Evolution of Long-Term Adjuvant Anti-hormone Therapy: Consequences and Opportunities. The St. Gallen Prize Lecture

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Abstract

The successful translation of the scientific principles of targeting the breast tumour oestrogen receptor (ER) with the nonsteroidal anti-oestrogen tamoxifen and using extended durations (at least 5-years) of adjuvant therapy, dramatically increased patient survivorship and significantly enhanced a drop in national mortality rates from breast cancer. The principles are the same for the validation of aromatase inhibitors to treat post-menopausal patients but tamoxifen remains a cheap, life-saving medicine for the pre-menopausal patient. Results from the Oxford Overview Analysis illustrate the scientific principle of "longer is better" for adjuvant therapy in pre-menopausal patients. One-year of adjuvant therapy is ineffective at preventing disease recurrence or reducing mortality, whereas five-years of adjuvant tamoxifen reduces recurrence by 50% which is maintained for a further ten-years after treatment stops. Mortality is reduced but the magnitude continues to increase to 30% over a 15-year period. With this clinical database, it is now possible to implement simple solutions to enhance survivorship. Compliance with long-term anti-hormone adjuvant therapy is critical. In this regard, the use of selective serotonin reuptake inhibitors (SSRIs) to reduce severe menopausal side effects may be inappropriate. It is known that SSRIs block the CYP2D6 enzyme that metabolically activates tamoxifen to its potent anti-oestrogenic metabolite, endoxifen. The selective nor-epinephrine reuptake inhibitor, venlafaxine, does not block CYP2D6, and may be a better choice. Nevertheless, even with perfect compliance, the relentless drive of the breast cancer cell to acquire resistance to therapy persists. The clinical application of long-term anti-hormonal therapy for the early treatment and prevention of breast cancer, focused laboratory research on the discovery of mechanisms involved in acquired anti-hormone resistance. Decades of laboratory study to reproduce clinical experience described not only the unique mechanism of SERM-stimulated breast cancer growth, but also a new apoptotic biology of oestradiol action in breast cancer, following 5-years of anti-hormonal treatment. Oestradiol-induced apoptotic therapy is currently shown to be successful for the short-term treatment of metastatic ER positive breast cancer following exhaustive treatment with anti-hormones. The "oestrogen purge" concept is now being integrated into trials of long-term adjuvant anti-hormone therapy. The Study of Letrozole Extension (SOLE) trial employs "anti-hormonal drug holidays" so that a woman's own oestrogen may periodically purge and kill the nascent

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sensitized breast cancer cells that are developing. This is the translation of an idea first proposed at the 1992 St. Gallen Conference. Although tamoxifen is the first successful targeted therapy in cancer, the pioneering medicine is more than that. A study of the pharmacology of tamoxifen opened the door for a pioneering application in cancer chemoprevention and created a new drug group: the Selective ER Modulators (SERMs) with group members (raloxifene and lasofoxifene) approved for the treatment and prevention of osteoporosis with a simultaneous reduction of breast cancer risk. Thus, the combined strategies of long-term anti-hormone adjuvant therapy, targeted to the breast tumour ER, coupled with the expanding use of SERMs to prevent osteoporosis and prevent breast cancer as a beneficial side effect have advanced patient survivorship significantly and promises to reduce breast cancer incidence.

Keywords

tamoxifen; selective oestrogen receptor modulators (SERMs); raloxifene; apoptosis; oestrogen; acquired drug resistance; chemoprevention

INTRODUCTION

Professor Hans-Joerg Senn asked me to cast light on future opportunities for improving adjuvant anti-hormone therapy that can be implemented or tested in clinical trial. This I will do, but first I will preface my remarks with a quote from Patrick Henry, the first elected Governor of Virginia, who said it best: "I have but one lamp by which my feet are guided, and that is the lamp of experience. I know no way of judging of the future, but by the past." In 1969, when I started my research on the pharmacology of non-steroidal anti-oestrogen, there was no tamoxifen (Fig 1), only ICI 46,474, an effective anti-fertility agent in rats[1]. The compound had anti-oestrogenic properties, so I proposed[2] to enhance its clinical application from an orphaned drug, with modest efficacy in metastatic breast cancer, to a targeted anti-cancer agent for adjuvant therapy and chemoprevention. Tamoxifen became my lamp, and subsequent laboratory research results shed light on the future of successful and safe adjuvant anti-hormone therapy, a new drug group of selected estrogen receptor modulation (SERMs)[3], a lead compound in the SERMs raloxifene for clinical applications, the promise of multi-functional medicines, the unique qualities of acquired anti-hormone drug resistance and a new apoptotic biology of oestrogen in breast cancer (Fig 1, Table 1)[4]. Tamoxifen, a failed contraceptive in women, is now a pioneering medicine in oncology [1] and is listed as an essential medicine by the World Health Organization.

The clinical validation[5, 6] of the laboratory principles of targeting the breast tumour oestrogen-receptor (ER)[7] with long-term adjuvant antihormonal therapy (tamoxifen and oestrogen withdrawal)[8, 9] using a long acting anti-oestrogen, metabolically activated to potent hydroxylated metabolites[9-12], established a treatment strategy that continues to enhance the survivorship of millions of women world-wide. The key to success was the application of the first effective medicine to target the tumour through blocking oestrogen-stimulated growth at the ER, but coupled with the application of the counter-intuitive laboratory finding, that long-term adjuvant therapy would be superior to short-term therapy to control recurrence. The strategy succeeded, despite initial clinical findings that the tumour response to tamoxifen was not strongly correlated to ER status[13, 14] and the legitimate concern that long-term therapy would precipitate early drug resistance. This concern was based on the fact that tamoxifen was only an effective treatment in unselected metastatic disease for about a year or two[15], so why would extended or indefinite adjuvant tamoxifen treatment be effective at preventing recurrence in the adjuvant setting?

Clinical trials finally demonstrated that the laboratory principle of "longer was more effective at controlling recurrence" was correct[5, 6]. The subsequent development of the aromatase (AIs)[16] expanded post-menopausal patient treatment options and reduced "oestrogen-like" side effects associated with tamoxifen, such as endometrial cancer and thromboembolic disorders[17]. There was also a modest improvement of disease-free survival compared with tamoxifen. The widespread acceptance of long-term antihormonal therapy as the standard of care and the intense and exhaustive examination of patient population databases, now permit questions to be addressed to improve patient survivorship. At a time of shrinking resources for biomedical research but expanding menus of purported targeted drugs to close one pathway or another, it is time to apply simple, basic rules that will make an impact immediately on enhancing survivorship. Only then, is it prudent to fine tune the results from a position of strength, by interrogating the tumour biology with blockers of survival pathways.

SIMPLE SOLUTIONS TO ENHANCE SURVIVAL

It seems obvious but it must be stated. The past 30-years of successful translational research is without value if an infrastructure does not exist to ensure that a patient's treatment is maintained when the medicine has proven value to aid survival from breast cancer. A medical team is available to support a patient's needs but there must be a refocus of the team to relearn basic principles: chronic therapy that requires years to provide benefit is worthless if the patients will not follow the regimen. This act will **dramatically** reduce their potential for survival. The fashion over the past four decades, for evidence based medicine, requires effective delivery. Significantly, delivery is a minor commitment compared to the effort behind discovering and proving the efficiency of a medicine in prospective clinical trials.

Based on the published evidence, several general principles are emerging about compliance. A recent analysis of anti-hormone therapy conducted in patients enrolled in the Kaiser Permanente of Northern California health system[18], revealed that approximately 30% of all patients discontinued either AI or tamoxifen early but of those who did continue, 70% were fully adherent for up to 5-years. Thus, only 49% overall are adherent for the full course of adjuvant anti-hormonal therapy. Predictors of non-adherence were African-American race, lumpectomy, unknown tumour site, lymph node involvement and other co-morbidities. Adherence was associated with Asian/Pacific Island ethnicity, married, earlier years of diagnosis (tamoxifen era), prior chemotherapy, radiation therapy and longer prescription refills. These and similar findings[19, 20] describe the extent of the problem but noncompliance with effective therapeutic agents also increases recurrence and mortality[21-23].

Another significant finding of the Hershman study[18] was that young women under 40-years old were more likely to discontinue anti-hormone therapy. This group would be prescribed tamoxifen but reasons for stopping could be because the women chose to start a family or the menopausal side effects were too severe. In regard to the latter, many women have been routinely prescribed selective serotonin reuptake inhibitors (SSRIs) over the past decade to reduce menopausal side effects. Members of this drug group block the CYP2D6 enzyme that metabolically activates tamoxifen to the potent anti-oestrogen endoxifen thereby (Fig. 2) impairing full drug benefit (Fig. 3)[24]. However, it must be stressed that not all SSRIs have the same ability to block tamoxifen metabolism and as a result, studies that group all SSRIs together are not uniformly consistent with the hypothesis[25, 26]. Nevertheless, the recent Canadian study of co-prescription of various SSRIs and the selective nor-epinephrine reuptake inhibitor (SNRI) venlafaxine does implicate paroxetine as increasing mortality during tamoxifen treatment and venlafaxine decreases mortality[27]. Overall, enhancing compliance and avoiding SSRIs that block CYP26D will significantly increase the chances

of patient survival. That being said, the next issue to address is anti-hormone drug resistance.

Anti-hormonal drug resistance can be manifest in two forms for the ER positive tumour: intrinsic resistance where the tumour does not respond at all to anti-hormone therapy, despite being ER positive and acquired anti-hormone therapy where the tumour initially responds to anti-hormone therapy but then grows despite the continuing treatment. Much effort has focused on an understanding of the molecular mechanism of intrinsic anti-hormone resistance and it seems that cross-talk between growth factor receptors and the low levels of ER have essentially made the ER irrelevant for cell survival. No scientific advance has yet reversed intrinsic resistance and aided patients. In contrast, there have been significant advances in understanding acquired anti-hormone resistance in the laboratory and these emerging data have been translated to clinical practice.

THE CHALLENGE: ACQUIRED DRUG RESISTANCE

Clinical experience with the successful application of long-term tamoxifen as an adjuvant therapy produced a clear survival advantage for patients[28]. Unselected patients treated for 5-years with adjuvant tamoxifen lived longer than patients in the non-treatment (placebo) arm but who were treated with tamoxifen at first recurrence as they had metastatic breast cancer. The clinical results with successful adjuvant tamoxifen therapy demonstrated[28] that our understanding of the development of drug resistance to tamoxifen treatment in ER positive disease were incorrect on July 25, 1987 (the publication date of the Scottish MRC trial), but supported the principle of early treatment of micrometastatic disease. Also, it highlights the fact that resistance to tamoxifen for the treatment of metastatic disease occurs rapidly within 2-years, and this biology did not apply to an adjuvant application of tamoxifen. Despite the fact that the rat mammary carcinoma model demonstrates that earlier, longer treatment with an anti-oestrogen was a suitable clinical strategy[8], there was no model of human diseases to test this hypothesis. However, in the mid-1980s, this was about to change. The ER positive breast cancer cell line MCF-7[29] exhibits oestradiol-stimulated tumor growth when transplanted into ovariectomized athymic mice. Tamoxifen blocks oestradiol-stimulated tumor growth but cannot maintain growth inhibition as ER positive tumors eventually grow despite tamoxifen treatment[30]. However, it seems that SERM and antihormonal resistance in breast cancer evolves and exposes a vulnerability in breast cancer that can be exploited in the clinic[31].

The first transplantable model of tamoxifen resistance in breast cancer demonstrated that drug resistance to tamoxifen was unique[32]. Although tamoxifen can initially block oestradiol-stimulated growth of MCF-7 cells, resistant ER positive tumors can use either oestradiol or tamoxifen to *stimulate* tumor growth (Fig. 4). Tumours do not grow unless treated with tamoxifen or oestradiol so in the ovariectomized mouse, this is equivalent to the "non-oestrogen state" created by aromatase inhibitors. Tumours also do not grow if treated with the pure anti-oestrogen fulvestrant that destroys the ER[33, 34]. This laboratory model replicates clinical experience with drug resistance to tamoxifen in metastatic breast cancer and explains why aromatase inhibitors or fulvestrant are effective second line treatments[35, 36]. So, how does a study of the drug resistance to tamoxifen in the laboratory explain the effectiveness of 5-years of adjuvant tamoxifen to reduce recurrence rates in ER positive breast cancer to tamoxifen by fifty-percent and continue to reduce mortality a decade after tamoxifen treatment is stopped? The answer is the evolution and reconfiguration of cell survival pathways that occurs in micrometastatic breast cancer during years of treatment.

Continuous retransplantation of successive generations of tamoxifen-stimulated MCF-7 tumor lines into athymic mice for more than 5-years results in a derived tumor line that does not respond to physiologic oestradiol with growth but rapid tumor regression through apoptotic cell death (Fig. 5)[37, 38]. These data were first presented at the St. Gallen meeting in 1992[37]. The concept offered at the time was that the ultimate and long lasting value of adjuvant tamoxifen therapy derives from stopping adjuvant tamoxifen when the woman's own oestrogen can now destroy the micrometastases that have been sensitized to oestrogen-induced apoptosis. The initial laboratory observations on low dose oestradiol-induced tumour regression were subsequently confirmed[38], expanded[39-42] and translated successfully to clinical trial[43, 44]. As a result, it is now possible to define the evolution of acquired anti-hormone therapy into a Treatment Phase where the anti-hormone blocks oestradiol stimulated tumour growth, Phase I when a SERM or oestradiol stimulates growth (or an aromatase inhibitor creates oestrogens independent growth) and Phase II when a SERM stimulates growth but physiological oestrogen provokes apoptosis either after stopping a SERM or after stopping an aromatase inhibitor (Fig. 6).

Thus, over the past four decades, general scientific principles have emerged and translated to clinical care for patients. The application of these principles of endocrine adjuvant therapy have benefited, and continued to benefit, millions of women worldwide, through a simple and cheap therapeutic intervention. We will now consider how emerging laboratory knowledge may reverse or at least hold Phase II resistance to enhance the longevity of the patient. We will, however then, revisit the clinical reality that increased tumour burden is a poor indicator of patient survival, so that the founding principles of our initial work, i.e. early treatment targeting the ER with long-term therapy[2] must be embraced by the clinical community.

Oestradiol-Induced Apoptosis under Laboratory Conditions

The administrations of physiologic oestradiol to athymic mice implanted with phase II SERM (tamoxifen or raloxifene) resistant ER positive MCF-7 tumours[38, 40, 41, 45] causes tumours to stop growing and/or rapidly regress. Similarly, the long-term oestrogen deprived clinical cell line MCF-7:5C[42, 46] rapidly undergoes oestrogen-induced apoptosis both *in vitro* and *in vivo*. These laboratory observations are reminiscent of the pioneering studies of Sir Alexander Haddow FRS with his application of the first Chemical Therapy to successfully treat any cancer – high dose synthetic oestrogens to treat metastatic breast cancer[47, 48]. He observed a 25% response rate but these were short-lasting[47]. The observation was made that no responses were observed close to menopausal but often dramatic responses occurred in women in their late 60s and 70s. By 1970, during the presentation of the Inaugural Karnofsky Award Lecture at the American Society of Clinical Oncology (ASCO)[48] (incidentally, when I was starting my PhD in Pharmacology at Leeds University) he stated: "…the extraordinary extent of tumour regression observed in perhaps 1% of post-menopausal cases (with oestrogen) has always been regarded as of major theoretical importance, and it is a matter for some disappointment that so much of the underlying mechanisms continues to elude us…"

Now we know that the responses Haddow observed occur because of oestrogen deprivation following the menopause. Longer oestrogen withdrawal after menopause was more effective at creating Phase II resistance in select patients, but high dose oestrogen therapy was necessary. Based on laboratory studies and clinical correlations, anti-hormone therapy does a better job in driving the rapid evolution to Phase II resistance and as a result, only physiological oestrogen is necessary to trigger apoptosis. Haddow's paradox that stood for 40-years now has clarity and we can start to offer treatment options to exploit the concept further.

Cell culture models provide a vehicle to examine, over time, oestrogen-induced apoptosis with the aim of pharmacologic modulation and the discovery of mechanisms that may have relevance for patient care. Through a knowledge of mechanisms, the elegant oestrogen trigger for naturally initiating tumour cell death may subsequently be exploited to other treatment scenarios. If we can decipher the process of ER-induced apoptosis from its current obscurity, this knowledge could be applied with the discovery of new drugs to trigger the mechanism without the involvement of ER. The ER is our current guide and light to find a new drug group.

We have undertaken an extensive examination of the actions of oestradiol on the growth (MCF-7), immediate apoptosis (MCF-7:5C) and delayed apoptosis (MCF-7:2A)[49] of our model cells using a 2-week time course of gene activity documented through mRNA analysis, creation of cDNA libraries and competitive hybridization with a cDNA library from no treatment controls using Agilent Gene Arrays. These studies were conducted in collaboration with Dr. Eric Ariazi and Dr. Heather Cunliffe. We extensively analyzed the gene time course, and completed gene segregation based on hierarchical pathway analysis. We found that MCF-7 and MCF-7:2A, our control cells remained quiescent during the initial few days of oestradiol treatment (1nM) whereas the pre-apoptotic MCF-7:5C cells responded with a massive rise in the activation of inflammatory genes. Analysis of the sequence of events during the first few days of gene activation, we propose that apoptosis occurs in MCF-7:5C cells by the exploitation of the non-canonical pathway for NF-κB signal transduction (Fig. 7). Furthermore, we have mapped out the time-course activation of each caspase (except caspase 3 that is absent in MCF-7) and determined that caspase 4 is the first and controlling executioner to provoke programmed cell death. We have interrogated the apoptotic process with purported inhibitors of individual activated caspases to confirm our conclusion of the role of caspase 4. Blockade of caspase 4, blocks oestrogen-induced apoptosis.

Most importantly, the activation of inflammatory genes suggests that oestradiol-induced apoptosis could be inhibited or at least modulated by glucocorticoids. We have subsequently established that dexamethazone inhibits oestrogen-induced apoptosis in a concentration related manner. This novel observation may have important implications for the application of oestradiol-induced apoptosis for individualized patient care. It is possible that the inadvertent administration of glucocorticoids during patient care could block oestrogen-induced apoptosis or that a patient's own glucocorticoids may also inhibit apoptosis, if patients are challenged with oestrogen following exhaustive anti-hormone therapy? The anti-glucocorticoid mifipristone (RU486) could potentially be used with oestrogen to block glucocorticoid action temporarily for a few weeks during low dose oestrogen administration to enhance apoptosis.

Examination of the Agilent gene array data confirmed our previous work[49] that elevated synthesis of glutathione, is protecting MCF-7:2A cells from immediate apoptosis in response to oestrogen. Apoptosis appears to be retarded in MCF-7:2A cells but an activation of autophagy heralds an enhanced transcription of caspase 4 and then triggers oestrogen-induced apoptosis during the second week of oestradiol treatment. We have previously successfully used pharmacological inhibitors to test our hypothesis. Buthionine sulfoximine (BSO), an inhibitor of glutathione synthesis[49] enhances oestradiol-induced apoptosis from a slow event lasting 2-weeks to an immediate event. Unfortunately, BSO, though used extensively in clinical trial a decade or more ago, is no longer available to examine whether it is possible to enhance oestrogen-induced apoptosis in patients with select tumours.

Thus far, our studies have described what happens, but the real question is how does the oestradiol/ER complex triggers apoptosis? Are there clues about the actual shape or

structure of the oestrogen ER complex that can be modulated and investigated further? The MCF-7:5C cells depend on a functioning ER for oestradiol-induced apoptosis. The pure anti-oestrogen fulvestrant binds to the ER and causes the rapid destruction of the protein complex. As a result, fulvestrant blocks oestradiol-induced apoptosis in a concentration related manner. Interestingly enough, the tamoxifen metabolites 4-hydroxytamoxifen (4OHTam) and endoxifen do not block or affect the autonomous growth of MCF-7:5C cells but do block the initiation of oestradiol-induced apoptosis. Herein lies a clue to the mechanism that triggers oestradiol-induced apoptosis (Fig. 8). X-Ray crystallographic studies of the ER ligand binding domain and the oestrogens, oestradiol and diethylstilboestrol (DES) and the SERMs 4OHTam[50] and raloxifene[51] provide a fascinating insight into oestrogen and anti-oestrogen action. The solution of the crystal structures demonstrate that the planar oestrogens are sealed within the ligand binding domain by helix 12 which then allows co-activators to bind to the activating function (AF)-2 site on the complex. This event amplifies oestrogen action through gene transcription. In contrast, the bulky side chain of the triphenylethylene 4OHTam and the benzothiophene raloxifene prevent helix 12 from sealing the hydrophobic ligand binding domain which prevents coactivator binding to AF-2. The promiscuous oestrogen-like activity of 4OHTam is explained by the inability of the anti-oestrogenic side chain to neutralize and shield the exposed aspartate at position 351 at the surface of the ligand binding domain. This exposed carboxylic acid communicates with AF-1 to induced oestrogen-like actions. Raloxifene completely blocks and neutralizes the aspartate at 351 and the raloxifene ER complex does not activate AF-1. This hypothesis has been successfully interrogated with changes in the ligand and the aspartate at 351 to modulate the activation of a model oestrogen target gene Transforming Growth Factor α[52-55]. Overall, we concluded that activation of AF-1 by an exposed surface aspartate 351 confirms that helix 12 is not sealing the ligand binding domain so it can, therefore, communicate a signal to AFI to induce oestrogen-like gene activation. If aspartate 351 is masked under helix 12 with a planar oestrogen than AF-2 is activated and the communication between AF-1 and aspartate 351 is mute. These data and conclusions subsequently resulted in a reclassification of oestrogens into class 1 (planar) and class 2 (non-planar)[56] using a simple assay to determine whether helix 12 was locking the ligand into the hydrophobic ligand binding domain or not. However, the biological significance of this molecular insight was not apparent until recently.

Based on the fact that 4OHTam blocks oestradiol induced apoptosis at the ER and the statement that the "bulky side chain" of 4OHTam altered the conformation of the ER preventing helix 12 from sealing the ligand binding domain[50], we advanced the hypothesis that the "bulky side chain" of 4OHTam was the phenyl ring of the oestrogenic triphenylbut-l-ene not just the *para*-dimethylaminoethoxy group traditionally associated with anti-oestrogen action. Perhaps the phenyl ring of the triphenylbut-l-ene anti-oestrogen was stopping helix 12 from sealing the binding site? A series of triphenylethylenes (TPEs), previously known to be classified exclusively as oestrogens in rodent uterine weight and vagina cornification assays, was used to establish oestrogenic activity in MCF-7 breast cancer cells. All compounds were found to be full oestrogens in growth assays compared with oestradiol and DES and fully-activate an ERE luciferase report ER gene system in MCF-7 cells[57]. In contrast, while oestradiol and DES will trigger apoptosis and cell death in MCF-7:5C cells within a week, the synthetic TPE "oestrogens" do not provoke massive apoptosis and indeed block oestradiol-induced apoptosis. Studies using the CHIP assay at the ERE site in the promoter region of the oestrogen responsive pS_2 gene demonstrate that whereas oestradiol E_2ER complex is recruited with the co-activator SRC3 in AF-2 neither 4OHTam nor the TPE ER complexes are recruited to the promoter[58].

Overall, these data demonstrate that oestrogen-induced apoptosis is governed and programmed by the shape of the ER complex. As a consequence, shape governs coactivator

binding at AF-2 and these events subsequently trigger apoptosis. A recent study by[59] advances our initial oestrogen reclassification paper[56] and confirmed, using a phage display library, that the shape of the ligand programs the external shaped of the ER complex. A precise evaluation of the immediate early genes involved in the apoptotic response will describe the mechanism of the oestrogenic trigger for cell death. Exploitation of this knowledge may find applications in other disease states.

OESTROGEN TREATMENT: CURRENT CLINICAL FINDINGS AND TRANSLATION TO ADJUVANT THERAPY

The laboratory finding[37-39] that acquired resistance to anti-hormone therapy evolves and exposes a vulnerability of breast cancer cells to the apoptotic actions of physiological oestrogen, provides an important insight into potential therapeutic applications. As previously noted in this paper, the anti-tumour effect of physiological oestrogen is reminiscent of the early therapeutic use of high dose oestrogen therapy for the treatment of metastatic breast cancer in post-menopausal women[47]. It was noted that the further from menopause patients were, the more likely there was to be a tumour response, but these responses never exceeded 30% in any given population.

It is now clear that the acute oestrogen deprivation caused by anti-hormones speeds up the molecular adaptation and reconfiguration of vulnerable survival pathways. The surviving populations of susceptible breast cancer cells also have increased sensitivity to oestrogen-induced apoptosis. Low dose oestrogen therapy now becomes a clinically viable strategy with the prospect of reducing oestrogen associated side effects.

The laboratory data generated and published in the 1990s proposed the clinical strategy of using low-dose oestrogen therapy to "purge" breast cancer cells with Phase II–acquired anti-hormone resistance, but then the re-introduction of anti-hormone therapy would control oestradiol-stimulated tumour growth[37, 38]. Nevertheless, a European trial lead by Dr. Per Lonning[43] recruited patients with metastatic breast cancer following exhaustive anti-hormone therapy were treated with standard high-dose DES (5mg tid). Results are summarized in Table 2a. Select patients responded well with one patient subsequently reported[60] being disease-free more than 10-years after first initiating a high dose oestrogen "purge" therapy. "One of the patients (AO) who achieved a complete response of a 16×16 mm cytological confirmed chest wall relapse, received DES treatment for five years, where after she been subject to regular follow-up without active treatment. To this day, she remains disease-free 10 years and six months after commencing DES treatment."

In a follow-up study, Ellis[44] compared and contrasted high dose (10mg tid) and low dose (1mg tid) oestradiol therapy in patients who relapsed during adjuvant aromatase inhibitor therapy. Results are summarized in Table 2b. Results were not as impressive as the Lonning study probably because patients did not receive "exhaustive" endocrine therapy prior to an oestrogen "purge". Nevertheless, the clinical trial confirms that low dose oestrogen can produce similar clinical benefit when compared with high-dose oestrogen treatment but with fewer, serious side effects.

Finally, there is further clinical evidence from the Women's Health Initiative (WHI) that oestrogen replacement therapy (ERT) alone causes a decrease rather than increase in the incidence of breast cancer[61] and a recent report from the Million Women Study in the UK demonstrates that oestrogen alone increases breast cancer incidence immediately following the menopause but if ERT is used more than 5-years after oestrogen exposure, oestrogen replacement therapy does not cause a rise in breast cancer incidences[62]. An overarching explanation for these apparently confusing clinical observations is clarified by our evolving

molecular model to exploiting the role of oestradiol in the life and death of breast cancer cells[63, 64]. We interpret these clinical findings based on the evolution of anti-hormone resistance as follows: breast cancer cells in an environment of oestrogen only grow in response to exogenous oestrogen, but following long-term oestrogen depriving surviving breast cancer cells either die or at least do not develop into tumours.

The clinical and laboratory database also provides continuing support for the ongoing adjuvant Study of Letrozole Extension (SOLE) trial (Fig. 9). Patients who have completed 5-years of adjuvant therapy with tamoxifen, an AI or any sequence are then randomized to an AI continuously for 5-years or an AI with a drug holiday for 3-months a year. The trial seeks to exploit the hypothesis, advanced at the 1992 St. Gallen Meeting, that a woman's own oestrogen may act as an anti-tumour agent after adjuvant anti-hormone therapy is stopped. The SOLE trial proposes a rigorous test of the hypothesis under controlled conditions that promises to create a practical advantage for patients following drug holidays. Results from this trial coupled with the expanding molecular database concerning the modulation of oestrogen-induced apoptosis may result in the proposition of regularly purging patients for a week or two with ERT if decades of anti-hormone therapy are to become common place in order that the disease is held in check and prevented from recurring. The question is now – at what point is oestrogen intervention too late?

FIGHTING OVERWHELMING CANCER CELL FLEXIBILITY

The enemy is us, Haddow[48] in the Inaugural Karnofsky Lecture reasoned that it would not be possible to develop a cancer specific therapy in the same way Ehrlich had for syphilis, as cancer was our own cells. What he did not know was that the situation is worse than that. The replicative fidelity of normal cells replace exactly what is lost, but in its own special place. Cytotoxic chemotherapy kills the patient by indiscriminately killing normal differentiated cells, and perhaps stem cells, so life saving repopulation for the host organism is impossible or too late. In contrast, human populations eventually adapt to external destructive forces such as fatal infectious diseases (plague, small pox, etc.) but individuals only survive through their preprogrammed nimble immunology. The survivors repopulate. And so it is with cancer at the cellular level within the body. However, immunology has not yet been proven to be of significance for breast cancer prevention. Haddow was right there – the enemy is us. The tumour at diagnosis has hundreds of mutations compared to the (purportedly) normal human genome[65, 66]. This and activated oncogenes, or loss of tumour suppression genes, provides the random survival flexibility within the cancer cell population to adapt and eventually thrive in a hostile (cytotoxic) environment within a few months. The principle is a microscopic adaptation of simple Darwinian evolution that has played out over the millennia by animals on earth. Random mutations create a preferred trait that permits survival, while the non-adaptive species or population dies out. The situation with cancer only becomes worse through adaptive survival responses preprogrammed in the cancer stem cell. These cellular "spores" seek to expand and prepare for massive repopulation in an enforced anoxic environment. The clinician is confronted with a perverted microcosm of the struggle for life by cancer cells programmed to create infinite candidates in the quest for survival. The patient is overwhelmed by sheer numbers in the wrong places. This is the challenge of targeted molecular therapeutics but how to build rationally on the advances in survivorship achieved over the past 40-years in breast cancer?

The path to progress in drug development has not changed significantly during this time, despite our new knowledge of the disease. The administrative plan for drug evaluation is in place to protect citizens and provide safe and therapeutically proven medicines for clinical care. To market a new drug to treat breast cancer, a precise system must be followed to obtain government approval. Phase I clinical trials must offer the hope of potentially

effective treatment to patients who have received all possible therapeutic options. The goal is to document dose limiting toxicities and at this stage of the disease, responses are a major bonus. Phase II trials focus on a cancer type of interest based on reasonable data from preclinical studies or an unanticipated response in Phase I trials. If a candidate is successful in Phase II trials, the drug is evaluated against or with the current standard of care. It should be emphasized that therapeutic results from Phase II trials with tamoxifen were not very dramatic, but Phase I data on toxicity for the patient was excellent compared with other therapies available. Only by targeting the ER in the tumour and applying long-term adjuvant therapy did patient survivorship increase. A discarded contraceptive became the "gold standard" for breast cancer therapy over a 30-year journey[1].

With this background, how do we build on success? Today there are dozens of good potential targets and dozens of plausible candidates for each target. However, unlike the ER which was, it is turned out, the principle messenger to stimulate breast tumour growth in about 30% of tumours, other candidate targets are proving to be not the star but part of the chorus. In late stage disease, one pathway is blocked but others now compensate. Pathways to preserve cellular life can be essential in all cells, but a cancer cell specific pathway is the only key to success in cancer therapeutics.

Based on our current work investigating oestradiol-induced apoptosis of breast cancer cells with long-term acquired resistance, we purposed a hypothesis: can we block breast cancer cell survival mechanisms and enhance the chances that the cell must undergo apoptosis in response to oestradiol?

c-Src was the first identified oncogene in cancer and is said to be present in more than 70% of breast cancer[67]. It controls AKT and MAPK phosphorylation cascades as the intermediary from growth factor receptor activation. It would appear to be an ideal target to subvert cell survival; almost as good as the ER! We posed the question, that if we blocked c-Src in breast cancer cells resistant to aromatase inhibitors would we then enhance apoptosis? In other words, would we generate value for the cancer patient by increasing cell kill as we have previously found that c-Src inhibitors were completely ineffective in affecting growth of oestrogen stimulated MCF-7 cells, but had significant efficiency in blocking the growth of ER-negative MDA-MB-231 and oestrogen stimulated ER-positive T47D cells. More importantly, long-term oestrogen-deprived MCF-7 cells have elevated pSrc. As most ER-positive cancers are exhaustively treated with antihormones before Phase I/II testing and we were building on a known efficacy of estrogen therapy, the proposition appeared sound. Our model cell, MCF-7:5C, had elevated phospho c-Src and are targeted inhibitor PP2 completely blocked phosphorylation. However, a 2 month course of treatment of MCF-7:5C cells with physiological oestrogen levels (1nM) that would be present in a postmenopausal patient plus the c-Src inhibitor (5 microMolar), resulted in the blockade of oestrogen-induced apoptosis and the reversion of the cell population to Phase I drug resistance (Fig. 10), i.e. estrogen or SERM-stimulated for growth. Within 2 months, the flexibility of cell populations had created no real advance that could realistically aid the patient.

Thus, as an illustration of the challenge, we face for the application of logical targeted therapy, one could conclude the following: an expanding menu of targeted medicines is available for testing, but only select populations will respond. Testing a c-Src inhibitor in the incorrect stage of antihormone resistance or patient populations cannot be successful. This is the problem: the testing populations for registration may be inappropriate for a drug candidate that is magnificent in a neoadjuvant therapy naïve disease study. However, does this enhance registration? Unfortunately not.

We need practical strategies to aid communities to hold the development and death from breast cancer while we attempt to decipher the enormous complexity of pathways and permutations of targeted therapies. This conclusion brings us back to the second piece of translational research started in our laboratory in the 1970's – chemoprevention. Remarkably, the lamp of tamoxifen shed light on an alternative strategy to reduce cancer incidence and preempt the aforementioned Gordian Knot. Unfortunately, the initial strategy for the clinical application of chemoprevention requires the identification of high-risk populations to be treated with the pioneer tamoxifen. This approach is flawed. However, a public health strategy for an aging population that creates wellness for as long as possible is a laudable goal now within our grasp.

TAMOXIFEN IS ALSO ABOUT PROGRESS IN CHEMOPREVENTION

An extensive study of the pharmacology of tamoxifen[68] identified its ability to modulate oestrogen target tissues around the body; tamoxifen is anti-oestrogenic in the breast and oestrogen-like in bone and lowers circulating cholesterol[69-72]. Translational research also first identified the potential of tamoxifen to increase the risk of developing endometrial cancer during extended treatment schedules[73-75]. Tamoxifen blocks breast tumour growth and development but enhances endometrial cancer growth. As a result, new procedures were introduced for the gynecological monitoring of post-menopausal patients receiving long-term tamoxifen therapy. New agents, without endometrial problems, were needed for investigation. Knowledge of selective ER modulation by tamoxifen and also the pharmacology of the structurally-related failed breast cancer drug, raloxifene, led to the creation of a new drug group, the Selective ER Modulators (SERMs)[76], with the potential to treat and prevent multiple diseases in women ***and*** prevent breast cancer at the same time. The fact that raloxifene was less oestrogen-like than tamoxifen in the rodent uterus and less likely to increase the incidence of the endometrial cancer in patients[77, 78] meant that safer compounds could be identified as chemopreventives for breast cancer but a new strategy to achieve the goal was essential. Benefits for a tiny, unidentifiable minority is unacceptable if the vast majority of women in a high risk population have side effects, some life-threatening. The road map for the pharmaceutical industry was clearly stated in 1990[79]. Is this the end of the possible applications for anti-oestrogens? Certainly not! We have obtained valuable clinical information about this group of drugs that can be applied in other disease states. Research does not travel in straight lines and observations in one field of science often become major discoveries in another. Important clues have been garnered about the effects of tamoxifen on bone and lipids so it is possible that derivatives could find targeted applications to retard osteoporosis or atherosclerosis. The ubiquitous application of novel compounds to prevent diseases associated with the progressive changes after menopause may, as a side effect, significantly retard the development of breast cancer. The target population would be postmenopausal women in general, thereby avoiding the requirement to select a high risk group to prevent breast cancer.

Raloxifene pioneered the concept in the clinic confirming the prediction that the prevention of breast cancer would occur during the treatment and prevention of osteoporosis in high risk post-menopausal women[80] with no increase in endometrial cancer. Today, the prediction that SERMs could control multiple diseases in women following the menopause is poised to become a reality. Lasofoxifene (Fig. 1) is approved in the European Union for the prevention and treatment of osteoporosis which simultaneously decreases the incidence of breast cancer, strokes and myocardial infarction, but without increasing endometrial cancer risk[81]. Lasofoxifene is more than one hundred times more potent than raloxifene and the aforementioned strategy[79] to improve women's health in aging populations is the new face of chemoprevention in breast cancer - treat the majority of women for major diseases like osteoporosis and coronary heart disease and prevent breast cancer as a beneficial side

effect. The saving in health care costs by **not** paying for the treatment of breast cancer in tens of thousands of women **without** breast cancer will be considerable, but admittedly hard to quantitate.

Raloxifene is not only available in the United States of America for the treatment and prevention of osteoporosis but also for reduction of the incidence for breast cancer in post-menopausal, high-risk women[82]. However, the SERM must be given indefinitely to remain effective in both diseases[83]. In contrast, tamoxifen remains effective for decades after the limited treatment period of 5-years is stopped[84, 85]. As mentioned previously, the key to understanding this fact probably resides in the laboratory study of drug resistance to SERMs and aromatase inhibitors and the development of a cellular susceptibility to oestrogen-induced apoptosis. The fact that the same tumour responsiveness to raloxifene appears to be retarded in clinical practice suggests that the known poor pharmacokinetics and bioavailability of raloxifene is not able to rapidly produce an "anti-oestrogenic" state around the nascent tumour like tamoxifen. This may explain the reduced performance of raloxifene against tamoxifen in the STAR trial, following the cessation of 5-years of treatment[83].

SUMMARY AND CLOSING THOUGHTS

Over the past 40-years, we have witnessed, a dramatic improvement in the survivorship of the majority of patients with a diagnosis of ER positive breast cancer. The SERM tamoxifen pioneered the process. Translational research has added further cheap and effective targeted anti-hormonal therapies to the physician's armamentarium that are proven to be of benefit in randomized adjuvant clinical trials world-wide. Not only has therapy been improved substantially over the past 40-years, from the time in the early 1970s when there was stated to be little prospect of successful survival advances with "endocrine therapy", but also the parallel path of chemoprevention has been pioneered successfully with the same SERM tamoxifen. This SERM heralded a new era of general medicine where a family of SERMs would allow women to expect to reduce their risk of fractures but prevent breast cancer at the same time. This was only a laboratory concept 20-years ago[79, 86] but it seems obvious that with an aging population that seeks to remain active for as long as possible, that the SERMs will play their part in reducing the incidence of breast cancer if used wisely in the post-menopausal population.

The lessons learned with the lamp-light of the pioneer tamoxifen are now established principles in cancer therapeutics. The principles are: aim at the target (ER), start therapy as early as possible (i.e. as few lymph nodes as possible involved), long therapy is preferable to shorter therapy, compliance with the medicine is essential and drug interaction with SSRIs to stop menopausal side effect in a few should be avoided. Conforming to these principles aids patients' survival. The light from the lamp also taught us what we did not know. Firstly tamoxifen is a selective modulator of ER action around a woman's body and heralded a new drug group (the SERMs) that prevent osteoporosis and prevent breast cancer as a beneficial side effect. This avoids the need to find the exact women who would benefit from chemoprevention using the Gail Model[87]. Secondly, drug resistance evolves so that oestradiol becomes an apoptotic trigger. Further studies solved the concern expressed by Haddow in 1970 that the mechanism of oestrogen-induced apoptosis was a mystery. Now oestrogen therapy has a niche application in patient care.

During the past 40-years, the mosaic of endocrine adjuvant therapy and chemoprevention with SERMs has been clarified by effective translational research[4, 88]. The next challenge for a generation of "omic" scientists is to prioritize the opportunities in molecular therapeutics based on this solid start, so as to advance and individualize treatment.

Acknowledgments

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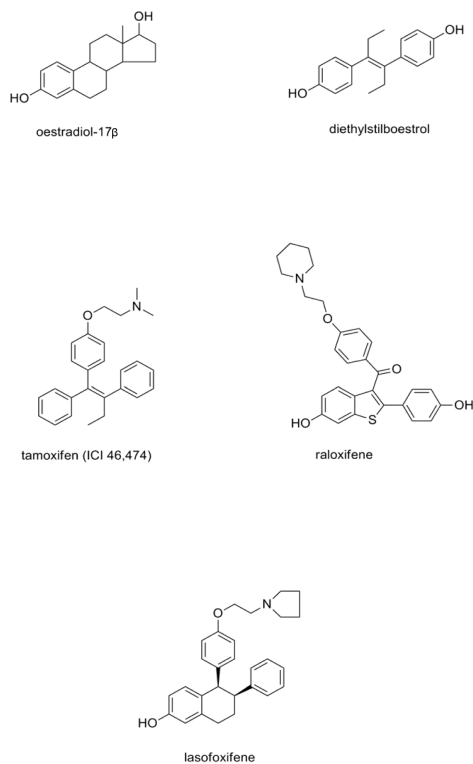
Fig. 1.
The structure of medicines and compounds mentioned in the text. Oestradiol and diethylstilboestrol are oestrogens, whereas all others are selective oestrogen receptor modulators (SERMs) used in medicine for the treatment and chemoprevention of breast cancer (tamoxifen), treatment and prevention of osteoporosis and the chemoprevention of breast cancer (raloxifene). The new SERM, lasofoxifene, is approved for the treatment and prevention of osteoporosis in the European Union.
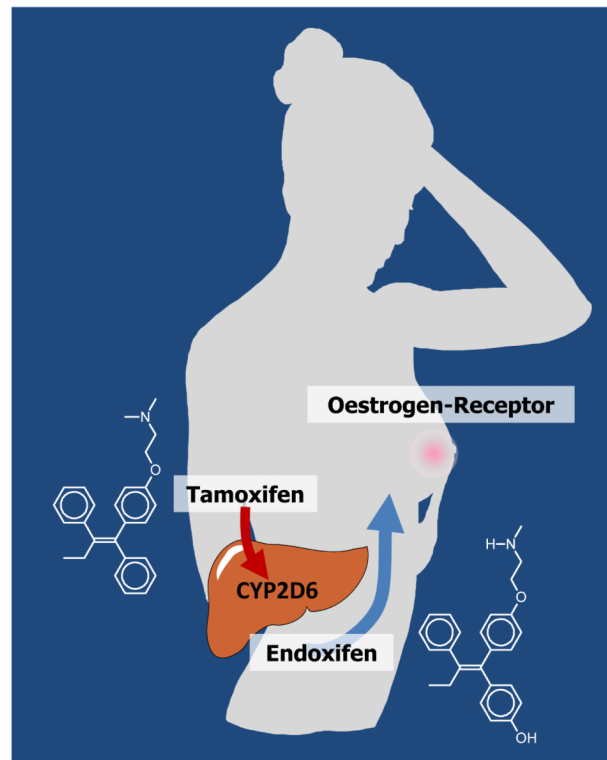
Fig. 2.
The metabolic activation of tamoxifen with a low affinity to the tumour oestrogen receptor by the P_{450} enzyme CYP2D6 enzyme to endoxifen with a high affinity for the tumour oestrogen receptor.
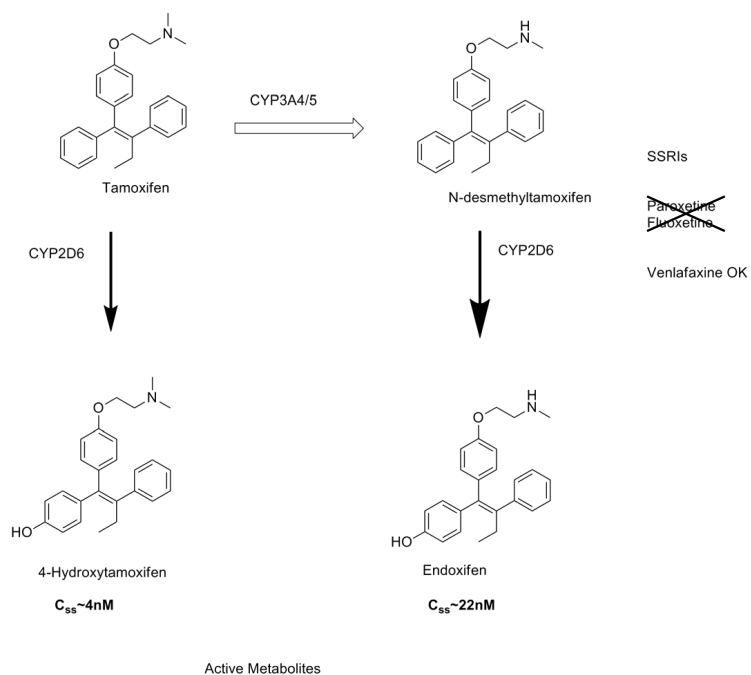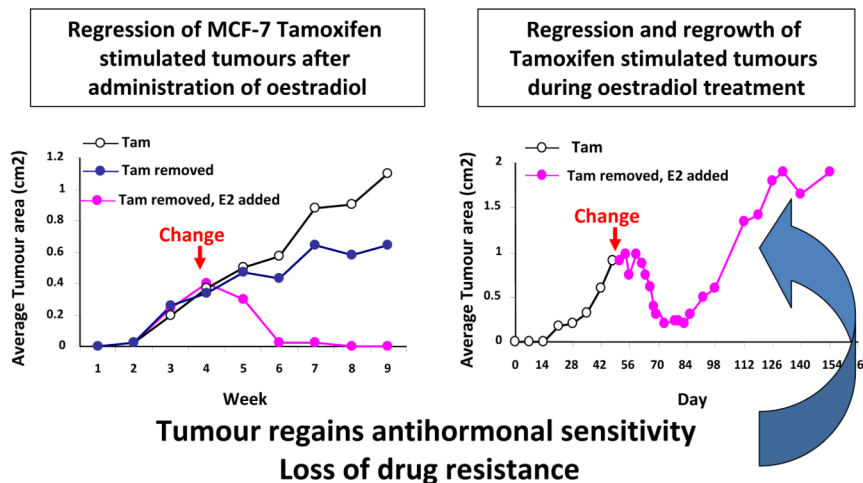
Fig. 3.
The metabolism of tamoxifen to 4-hydroxytamoxifen, a metabolite with a high affinity for the oestrogen receptor. Tamoxifen's major metabolite is N-desmethyltamoxifen that has a similar binding affinity to the oestrogen receptors as tamoxifen. However, N-desmethyltamoxifen is metabolically activated to endoxifen, with a high binding affinity for the oestrogen receptor. The selective serotonin re-uptake inhibitors (SSRIs), paroxetine and fluoxetine block the metabolic activation of tamoxifen by blocking CYP2D6. Venlafaxine, a selective norepinephrine re-uptake inhibitor (SNRI), does not affect tamoxifen's metabolic activation, and therefore is the preferred choice to treat menopausal symptoms experienced with tamoxifen.

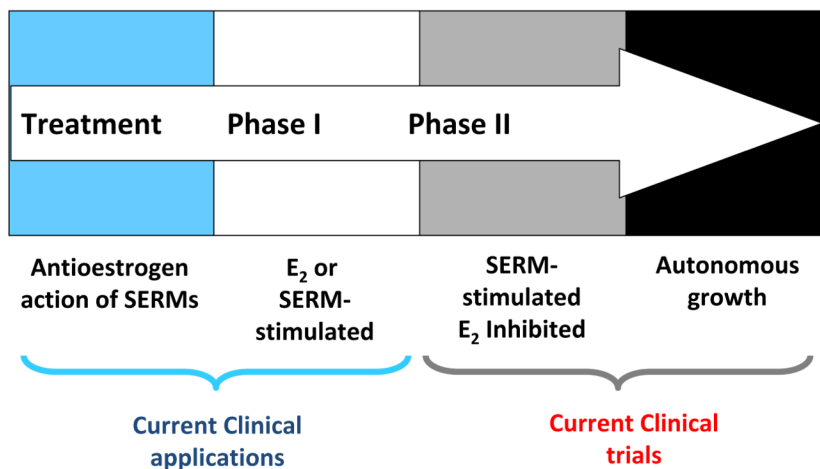# EVOLUTION OF SERM RESISTANCE
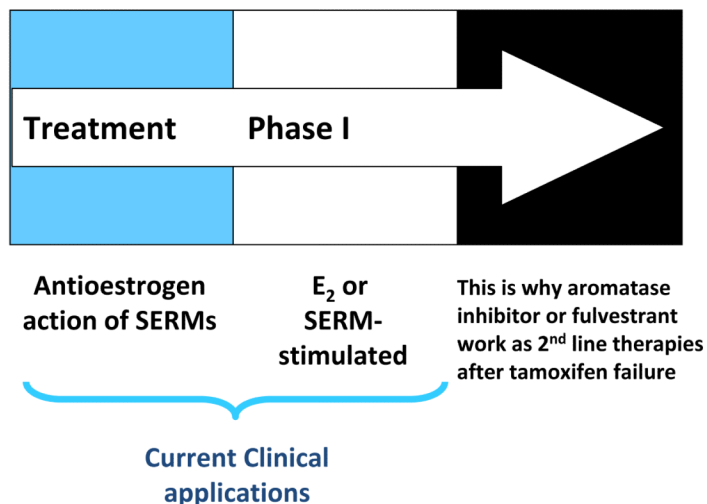
Fig. 4.

The development of acquired antihormone resistance to selective oestrogen receptor modulators (SERMs) (tamoxifen or raloxifene). The unique feature of Phase I antihormone resistance is that oestrogen receptor positive breast tumours grow in response to either physiological oestradiol or the SERM. In the clinical setting (and laboratory models), an aromatase inhibitor (no oestrogen) or the pure anti-oestrogen, fulvestrant, that destroys the oestrogen receptor, stops the growth of Phase I resistant tumours to tamoxifen.[31]

St. Gallen 1992

Regression of MCF-7 Tamoxifen stimulated tumours after administration of oestradiol

Regression and regrowth of Tamoxifen stimulated tumours during oestradiol treatment

**Tumour regains antihormonal sensitivity**
Loss of drug resistance

Fig. 5.
Diagrammatic representation of the actions of physiologic oestradiol (E2) on the growth of small phase II MCF-7 tamoxifen resistant tumors in ovariectomized athymic mice. A larger tumour will regress with oestradiol treatment but will eventually display oestrogen-stimulated growth. If tumours are re-transplanted into a new generation of ovariectomized athymic mice and treated with oestradiol, tamoxifen will block oestrogen-stimulated tumour growth.[38] First presented in St. Gallen, 1993.[37]

NEW CONCEPT
EVOLUTION OF SERM RESISTANCE

Fig. 6.
The evolution of drug resistance to SERMs. Acquired resistance occurs during long-term treatment with a SERM and is evidenced by SERM-stimulated breast tumour growth. Tumours also continue to exploit oestrogen for growth when the SERM is stopped, so a dual signal transduction process develops. The aromatase inhibitors prevent tumour growth in SERM-resistant disease and fulvestrant that destroys the ER is also effective. This phase of drug resistance is referred to as Phase I resistance. Continued exposure to a SERM results in continued SERM-stimulated growth (Phase II), but eventually autonomous growth occurs that is unresponsive to fulvestrant or aromatase inhibitors. The event that distinguishes Phase I from Phase II acquired resistance is a remarkable switching mechanism that now causes apoptosis, rather than growth, with physiologic levels of oestrogen. A similar evolution occurs with aromatase inhibitor resistance from oestrogen independent growth with a transition to oestrogen-induced apoptosis. These distinct phases of laboratory drug resistance have their clinical parallels and this new knowledge is being integrated into the treatment plan.

NF-κB Non-canonical Pathway
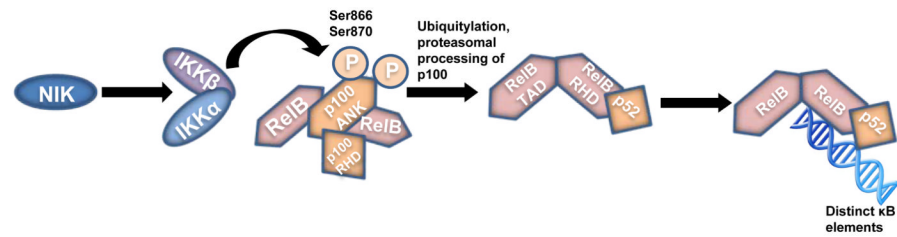
Fig. 7.
The non-canonical pathway results in the activation of IKKα by NIK and phosphorylation of the NF-κB subunit. This process results in the conversion of p100 to p52. It is the p52-RelB heterodimers that target distinct κB elements on DNA. ANK (ankyrin-repeat motifs). NIK (NF-κB kinase). RelB (NF-κB family member). RHD (Rel-homology domain). TAD (transcriptional activation domain).
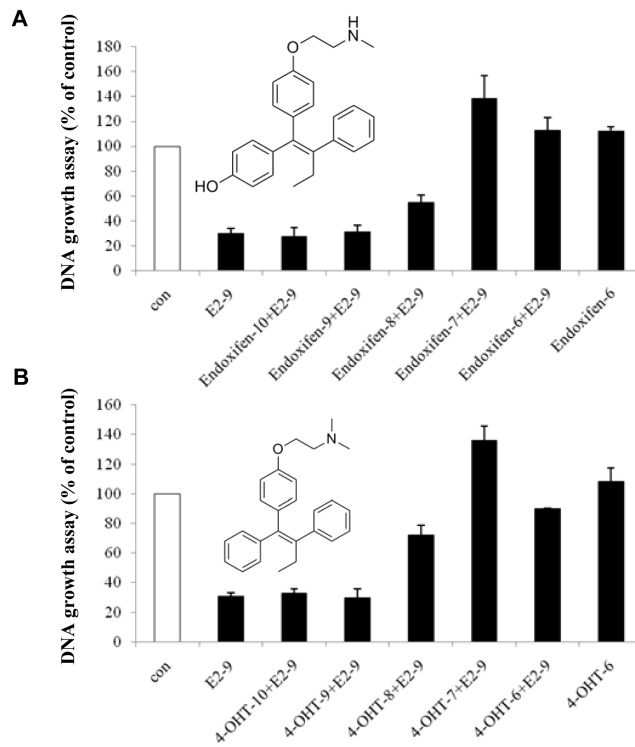
Fig. 8.
The reversal of oestradiol-induced apoptosis (1 nM) by increasing concentrations of 4-hydroxytamoxifen or endoxifen. This nonsteroidal antioestrogen effect highlights the ER dependence for oestradiol-induced apoptosis.

International Breast Cancer Study Group (IBCSG)

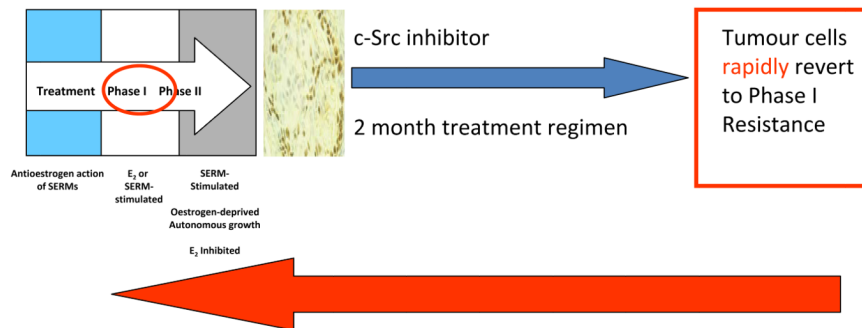### IBCSG 35-07 - Study Of Letrozole Extension (SOLE)

At completion of 4 to 6 years of prior adjuvant SERM/AI endocrine therapy, patients will be randomized to one of two treatment groups:
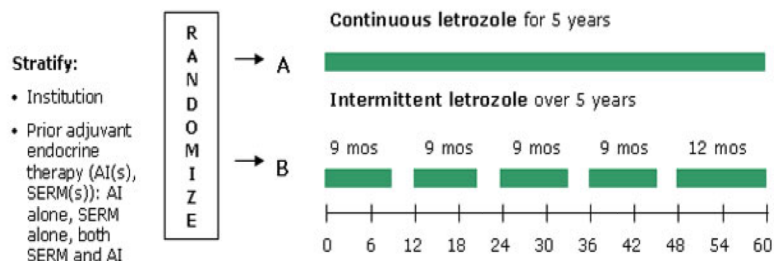
Fig. 9.
The Schema for the Study of Letrozole Extension (SOLE) conducted by the International Breast Cancer Study Group (IBCSG 35-07). Patients randomized following five years of adjuvant antihormone therapy to letrozole continuously or intermittent letrozole (3 month drug holidays per year for 5 years). The rationale is that the woman's own oestrogen in the intermittent arm will trigger apoptosis in aromatase inhibitor resistant cells and reduce recurrence rates.

Fig. 10.
The evolution of drug resistance and rapid alterations in cell populations if a c-Src inhibitor PP2 (5 μM) is incubated with MCF-7:5C cells in the presence of 1 nM oestradiol for two months to mimic a clinical scenario of a postmenopausal woman who fails an aromatase inhibitor to block growth. Apoptosis from oestrogen is blocked and the cells revert to Phase I resistance, i.e. oestrogen and SERM-stimulated growth.

Table 1

Decades of discovery. The development of scientific principles in the laboratory were translated to clinical trials ten years later and subsequently became the standards for clinic care for the treatment or chemoprevention of breast cancer, or in the case of the SERM, raloxifene, a treatment option for the treatment and prevention of osteoporosis with the prevention of breast carcinogenesis as a beneficial side effect.

Decades of Translational Discovery		
DECADE	**SCIENTIFIC PRINCIPLE**	**CLINICAL BENEFIT**
1970s	Long-term adjuvant tamoxifen therapy targeted to ER	---------------------
	Foundation of chemoprevention with tamoxifen	---------------------
1980s	Selective ER modulation	Survival benefits for long-term adjuvant tamoxifen
1990s	Evolution of drug resistance to hormones	Chemoprevention with SERMs, tamoxifen and raloxifene
	Anti-tumour actions of physiologic oestrogens	
2000s	Oestrogen-induced apoptosis	Clinical translation of oestrogen-induced apoptosis

Table 2

The proof of principle for a) high dose oestrogen (DES, 15 mg daily) triggering tumour responses in patients with metastatic breast cancer following exhaustive antihormone therapy[43] or b) a comparison of high dose oestrogen (oestradiol, 30 mg daily) or low dose oestrogen (oestradiol, 6 mg daily), producing similar clinical benefit rates following the failure of therapy with an aromatase inhibitor.[44]

A	Response	
COMPLETE	PARTIAL	SD
4[a]/32	6/32	2/32

B			
Dose	# patients	Response	Clinical benefit
6 mg	34	10/34	29%
30 mg	32	9/32	28%

[a]One patient remains disease-free 10 years and six months after commencing DES treatment."