Resistance to Targeted Anti-Cancer Therapeutics 8
Series Editor: Benjamin Bonavida

Alexey Larionov *Editor*

Resistance to Aromatase Inhibitors in Breast Cancer

Springer

Resistance to Targeted Anti-Cancer Therapeutics

Volume 8

Series editor

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For several decades, treatment of cancer consisted of chemotherapeutic drugs, radiation, and hormonal therapies. Those were not tumor specific and exhibited severe toxicities in many cases. But during the last several years, targeted cancer therapies have been developed. Targeted cancer therapies—sometimes called "molecularly targeted drugs—are drugs or other agents (e.g., anti-bodies) that block the growth and spread of cancer by interfering with specific gene products that regulate tumor cell growth and progression".

We have witnessed in the last decade a significant explosion in the development of targeted cancer therapies developed against various specific cancers. These include drugs/antibodies that interfere with cell growth signaling or tumor blood vessel development, promote the cell death of cancer cells, stimulate the immune system to destroy specific cancer cells, and deliver toxic drugs to cancer cells. One of the major problems that arises following treatment with both conventional therapies and targeted cancer therapies is the development of resistance, preexisting in a subset of cancer cells or cancer stem cells and/or induced by the treatments. Tumor cell resistance to therapies remains a major problem and several strategies are being considered to reverse the resistance to various manipulations. *Resistance to Targeted Anti-Cancer Therapeutics* focuses on the basic and translational research behind the molecular mechanisms of resistance found in many kinds of anti-cancer therapeutics.

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ISSN 2196-5501 ISSN 2196-551X (electronic)
Resistance to Targeted Anti-Cancer Therapeutics
ISBN 978-3-319-17971-1 ISBN 978-3-319-17972-8 (eBook)
DOI 10.1007/978-3-319-17972-8

Library of Congress Control Number: 2015936686

Springer Cham Heidelberg New York Dordrecht London

Printed on acid-free paper

Springer International Publishing AG Switzerland is part of Springer Science+Business Media
(www.springer.com)

Preface

Breast cancer is the most common female cancer, affecting up to 10 % of women in the developed world through their lifetime. Aromatase inhibitors (AIs) are indicated to treat postmenopausal estrogen receptor positive (ER+ve) tumors, which constitute the majority of breast cancer patients. AIs significantly improve treatment outcomes compared to previously used endocrine treatments. However, 10–15 % of patients relapse within 5 years of adjuvant treatment, about 25–50 % of the patients do not respond to AIs in a neo-adjuvant or metastatic setting, and the majority of metastatic patients who initially respond develop resistance within 3 years. Thus, there is a need to understand the mechanisms and to develop methods of preventing or overcoming the resistance to AIs.

While some of the mechanisms of AI resistance may be in common with other endocrine treatments, such as Tamoxifen, there is no absolute cross-resistance in different endocrine treatments. This book reviews current experimental and clinical data specifically focused on AIs, including (i) genetic regulation and protein structure of aromatase, (ii) molecular mechanisms and markers of AI resistance, and (iii) data from clinical trials combining AIs with novel-targeted treatments. The goal was to bring together the current knowledge from different areas, ultimately putting the biological and experimental facts into the clinical context.

While each chapter has its own focus, they have been written to talk about different aspects of the same story, rather than as a collection of isolated stories. The book starts and ends with clinical chapters, which frame the central core focused on the biology of aromatization and on different mechanisms of resistance. In Chap. 1, Prof. David Cameron provides a concise introduction to the history and current role of aromatase inhibitors in breast cancer clinics. Then, Prof. Nobuhiro Harada gives a comprehensive review of structure, regulation, and polymorphisms of the aromatase gene, with particular focus on the alternative tissue-specific promoters and genetic regulatory elements. In Chap. 3, Prof. Debashis Ghosh and coauthors describe structural studies of the aromatase protein. They review the overall crystal structure, positioning in the membrane, and the possibility of oligomerization, as well as motion and flexibility within the aromatase molecule. This chapter also illustrates how new knowledge about the enzyme's active site lays the foundation

for the development of new aromatase inhibitors. Further, the book advances to a chapter on experimental models which have been devised to study aromatase inhibition in breast cancer, comparing a variety of cell lines and xenografts resistant to aromatase inhibitors, as reviewed by Gauri Sabnis and Angela Brodie. Then, Prof. Per Lonning addresses an apparently simple question of how can we measure the efficiency of aromatase inhibition in clinic. Plasma estrogen levels are low in postmenopausal women, in particular when on aromatase inhibitor therapy. Professor Per Lonning reviews methodical challenges of applying radio-immunoassays to measure estrogen levels in blood and tissues of breast cancer patients. In fact, because of the expertise required for such measurements, until very recently, the data on estrogen levels in AI treatments were limited by a relatively small number of studies with small numbers of enrolled patients. These studies reviewed by Per Lonning indicated the exquisite potency of AIs, which led him to the conclusion that inefficiency of inhibition is an unlikely cause for resistance (at least in a carefully controlled research setting). However, in a dramatic turn, just after the completion of this chapter, a new study was published, which implements mass spectrometry for simultaneous measurements of estrogens, AIs, and their metabolites in a large multicenter study with several hundreds of patients [1]. This study suggests that in a real-life clinical environment, there is possibility of inefficient inhibition in 8 % of patients. In some isolated cases, no drug was detectable in blood and the estrogen concentration was increasing during treatment. This study is discussed in a later chapter by Alexey Larionov and William Miller, who speculate that such variation in efficiency of AIs may be linked to differences in drug metabolism as well as to issues with treatment adherence or patient selection.

To characterize mechanisms of AI resistance in one word, a suitable term would be “diversity.” This is fully reflected in the following four chapters that focus on the mechanisms of AI resistance in cases when the aromatase inhibition had been efficient. Elizabeth Sweeney with Craig Jordan highlighted that not only can estrogens stimulate growth, but they can also cause apoptosis of breast cancer cells. The balance between these apoptotic and growth-stimulating aspects of estrogens is changed during estrogen deprivation. The authors review the biology of estrogen-induced apoptosis and relate it to the new concept of using “breaks” in aromatase inhibitor therapy (as tested in the SOLE clinical trial). The role of ligand-independent ER signaling in AI resistance is reviewed by Jean McBryan and Leonie Young, who discuss various sites of ER phosphorylation, role of ER cofactors, and involvement of the cross talk between ER and growth factor pathways into hypersensitivity of ER to low concentrations of estrogens. Epigenetic determinants of resistance to aromatase inhibitors are reviewed by Raffaella Maria Gadaleta and Luca Magnani. Starting with the epigenetic regulation of the aromatase gene, they then discuss the role of histone modifications and pioneering factors in facilitating ER-mediated transcription, specifically focusing on the recent studies relating genome-wide ER-binding patterns to AI response. This chapter also discusses epigenetic regulation of ER itself, and describes the current state of epigenetic-based medicine in the context of endocrine therapies. The section on diversity of molecular mechanisms of AI resistance is concluded by Abdul Aziz Bin Aiderus and

Anita Dunbier, who describe experimental aspects of resistance via non-endocrine signaling pathways (including PI3K/mTOR, IGF, GDNF, and Myc pathways) as well as the role of tumor microenvironment (including inflammatory immune cells and adipocytes) in AI resistance. A series of recent studies highlighted role of activating mutations in ligand-binding domain of ER, which might be detected in 20–50 % of breast cancers, acquired endocrine resistance [2, 3]. Interestingly, these mutations are not present in primary breast cancers [4]. A chapter was commissioned about the role of ER mutations in AI resistance. However, circumstances prevented completion of this chapter. Readers interested in this mechanism of resistance are advised to read recent papers of Robinson et al. [2] and Toy et al. [3] as well as earlier studies and comprehensive reviews of Prof. Fuqua [4, 5].

The final section of this book brings the reader back into the clinical realm. It includes three chapters, which (i) discuss prediction of response to aromatase inhibitors, (ii) review clinical trials aimed to prevent or overcome AI resistance, and (iii) describe clinical use of aromatase inhibitors beyond breast cancer. Accurate prediction of response is needed to select an effective treatment and to avoid unnecessary side effects in patients who are unlikely to respond to AIs. Numerous studies have evaluated the utility of routine biomarkers (ER, PgR, HER2, and Ki67), multigene signatures (e.g. Intrinsic subtypes, Oncotype Dx, SET, Endopredict, and others), and multi-component clinical indices (e.g. PEPI and Adjuvant! online). These studies and markers are reviewed by Alexey Larionov with William Miller; they also discuss the technologies of biomarker development and some future markers, which could be used for the patients' selection and monitoring. Numerous clinical trials attempted combining AIs with novel-targeted agents (including HER2, EGFR, mTOR, PI3K, Akt, CDK4/6, FGFR, HDAC, IGF-1, Src, Proteosome-, and angiogenic-targeted agents). These trials are reviewed by Hazel Lote and Stephen Johnston. A number of the combinations have not yet fulfilled expectations (e.g. the combination with anti-angiogenic agents). On the other hand, the first examples of success are the combinations of AIs with mTOR and with CDK4/6 inhibitors. An important aspect of the combined treatments is that the new agents need companion biomarkers, to personalize the treatment selection (consistent with the experimental data about diversity of AI resistance mechanisms). Finally, in the last chapter of this book, Prof. Lev Berstein summarizes AIs use outside of the treatment of breast cancer, including other malignancies (e.g. endometrial cancer and endometrial uterine sarcoma) and some non-oncological indications (e.g. endometriosis, fertility treatment and abortion).

Many of the chapters provide extensive historical overviews that show the inner logic of the field and connect the historical studies to the present state of the art. Overall, the book brings together current knowledge from different relevant areas, including molecular and clinical aspects of AIs resistance, and is directed at scientists developing new treatments for ER+ve breast cancer and at medics treating breast cancer patients with aromatase inhibitors.

Cambridge, UK
March 2015

Alexey Larionov

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Acknowledgments

I am grateful to my wife Elena Perelman and to my brother Sergey Larionov for their constant support of my work in this volume. I wish to thank Fiona Sarne, Joy Bramble, and Benjamin Bonavida for their friendly help and encouragement.

Volume Editor Alexey Larionov

I wish to acknowledge the assistance of Kathy Nguyen, Leah Moyal, Melanie Miller, and Ailina Heng for their valuable effort in the editing and formatting of the various contributions in this volume. Also, I acknowledge the valuable cooperation and coordination with Joy Evangeline Bramble, Publishing Editor, Cancer Research, Springer Science+Business Media.

Series Editor Benjamin Bonavida

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Chapter 1
Clinical Use of Aromatase Inhibitors in Breast Cancer: History and Present

David Cameron

Abstract The mainstay of endocrine therapy in breast cancer has been to deprive breast cancer cells of oestrogen. Traditionally done by surgical means, for post-menopausal women the treatment is now much simpler with the advent of highly specific and effective aromatase inhibitors. This chapter will summarise the history of endocrine deprivation therapy in post-menopausal women with breast cancer, and outline where these drugs are used today, what is known about their efficacy and potential causes of resistance. It will set the scene for the rest of the book and draw lessons from history to interpret contemporary clinical practices and research questions.

Abbreviations

AIs Aromatase inhibitors
ER Oestrogen receptor
PgR Progesterone receptor

Introduction

Endocrine therapy of breast cancer has arguably cured more women than chemotherapy. Its efficacy was evidenced before its mechanism of action was properly understood, and many important lessons in the systemic therapy of cancer were first learnt in this setting, and often not without controversy: rejection of a "one

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A. Larionov (ed.), *Resistance to Aromatase Inhibitors in Breast Cancer*,
Resistance to Targeted Anti-Cancer Therapeutics 8,
DOI 10.1007/978-3-319-17972-8_1

drug suits all" with the concept of targeting a therapy to cancers defined by their biology; relevance of pharmacogenomics, drug-drug interactions; the idea that two drugs are not always better than one; questions of duration of therapy, long term rarer toxicities etc.

From Beatson to Aromatase Inhibitors

The use of endocrine deprivation therapy to treat breast cancer was the first effective systemic therapy in breast cancer. Beginning with the work of Beatson in Glasgow in 1896 [1], oophorectomy became a management standard in pre-menopausal women, in part because of the important series of patients reported by Boyd in 1900 [2]. This approach had no efficacy in post-menopausal women, though at the time this was not fully understood. Further effective endocrine manipulations were achieved by hypophysectomy and adrenalectomy, which were active in post-menopausal women since they inhibited or removed (respectively) the source of the androgen precursors of oestrogens in post-menopausal women. This led to pharmacological approaches replacing surgery and in steps to today's use of potent, well-tolerated third generation aromatase inhibitors (AIs).

Such surgical approaches, pioneered by Huggins and Bergenstal in 1951 [3], were of course not without their surgical and endocrine morbidity. Operative mortalities of the order of a few percent were accepted, though it was noted that the presence of pulmonary and/or pleural disease increased this risk. This is a stark comparison to the mortality risks of today's endocrine therapies! Responses were seen in around 40 % of patients, though fewer in younger women, and of reasonable duration—one series reported that almost a third of women were still alive 5 years later [4]. These data are not substantially different from today's endocrine therapies, and in some ways the major developments since, such as the pharmacological approaches which began in the late 1960s, were more about tolerability and increasing the range of therapeutic options than about major improvements in efficacy.

Aminoglutethimide was developed as an inhibitor of the final enzymatic step in the synthesis of oestrogens from androgens, but it also inhibits the earlier conversion of pregnenolone from cholesterol, thus inhibiting the synthesis of all hormonally active steroids, so that when used in the full effective dose of 1000 mg daily, even with the required supplementary hydrocortisone, there were endocrine toxicities such as hypothyroidism and ACTH stimulation. Importantly, in a 96-patient randomised trial, it was demonstrated to be at least as effective as surgical adrenolectomy [5]. Subsequently it was also found that the lower dose of 250 mg daily was essentially both biochemically and clinically as effective as the standard 1000 mg daily [6, 7] but with less toxicity.

Tamoxifen had of course been developed somewhat in parallel, initially as a contraceptive, and had evidence of its efficacy established back in the 1960s [8], so that once medical approaches had supplanted surgical adrenalectomy, the

question arose as to which of the drugs, aminoglutethimide or tamoxifen was the superior therapy for women with advanced breast cancer. One very informative study comparing these drugs was a phase II randomised cross-over trial conducted in the UK [9]. No significant differences in efficacy were found, though what was informative was that there was a differential cross-resistance. More patients responded to aminoglutethimide after tamoxifen than with the opposite sequence of drugs, and unsurprisingly, tamoxifen was generally better tolerated. Thus tamoxifen remained first-line therapy: though the study was too small to exclude small differences in efficacy. This, and other data, established that when more potent aromatase inhibitors were developed, they started in the later lines of therapy and only once on the market were compared directly with tamoxifen. This phase II study is also noteworthy for the observation, confirmed with a later follow-up [10], that there was no benefit in combining tamoxifen and aminoglutethimide: a lesson perhaps forgotten in the design of the ATAC trial which again confirmed the lack of any benefit in the combination of tamoxifen and a later generation non-steroidal aromatase inhibitor [11].

One of the major developments in the endocrine therapy of women with advanced breast cancer was the ability to identify those tumours very unlikely to respond, namely those without nuclear expression of the oestrogen receptor (ER) and/or progesterone (PgR) receptor. Older studies did not select for ER and/or PgR positive breast cancers, but gradually it has become the norm to test for expression in the histopathological diagnosis of breast cancer, preferably in laboratories that have some form of certification (e.g. CLIA in North America, NEQAS in the UK) that their assays are robust and reproducible. There remains debate as to just how frequently woman have ER-negative PgR-positive (ER-ve/PgR+ve) breast cancer, but that notwithstanding, it is fair to say that the only indication for the use of an endocrine therapy is in patients with breast cancers that are ER and/or PgR positive. Those cancers that express both receptors are on the whole more likely to be endocrine sensitive, and have a lower rate of proliferation and as such are mostly Luminal A; those which only express the ER tend to have higher rates of proliferation, earlier relapses and fit into the category of Luminal B. The importance of proliferation in breast cancer biology led to extensive studies of Ki67, which is now reaching the status of an ancillary biomarker to separate Luminal A and B subtypes within ER+ve tumours [12].

Further research has been conducted to explore the drivers of endocrine resistance, both as a class of agents and specifically to aromatase inhibitors. There is a very extensive literature on this, with many markers all having some evidence that they are relevant, but none yet has been able to enter clinical practice as having sufficient analytical validity and strong enough positive and negative predictive values to allow identification of ER+ve patients whose cancer will not respond to endocrine therapy, over and above the presence of the oestrogen and/or progesterone receptor. One reason why this extensive research has yet to identify a better marker is perhaps illustrated in a study conducted using mRNA from ER+breast cancers treated in the neo-adjuvant setting [13] in which a heat map of similarity of gene expression shows that responders are more similar than non-responders,

such that accurate prediction of non-response before therapy is challenging. More recent data suggest that the use of the primary tumour to predict sensitivity of metastatic disease may not be ideal: many years of work looked for mutations in the *ESR1* gene (which codes for the ERα protein) in the historic primary tumour as a cause of resistance and failed to confirm its relevance; more recent studies have looked at biopsies taken from the metastases and reported that up to 20 % of cases have mutations in the *ESR* gene [14] and these are associated with resistance to endocrine therapy including aromatase inhibitors [15].

Thus whilst there remain controversies over the exact optimal selection tools for the endocrine sensitivity, the absence of nuclear steroid receptor expression means that endocrine therapy is not appropriate, and older studies were often unable to exclude such patients and so often reported slightly lower overall beneficial effects. The study by Murray and Pitt of 53 women treated with aminoglutethimide is important as it laid down three seminal observations which have not really been disproved in the subsequent 30+ years [16]. Firstly, the median duration of benefit in advanced breast cancer was about 1 year but with some patients having much longer disease control; and secondly, that sensitivity to prior endocrine therapy increased the chance of response to an AI with approximately twice as many women who had responded to prior tamoxifen responded to the AI (69 % vs. 35 %). It also confirmed that once a breast cancer has developed overt clinical metastases, resistance to sequential lines of therapy almost inevitably develops, though this can happen after months or years of any particular therapeutic approach to oestrogen deprivation.

Current Situation

Today there is widespread use of the three clinically available aromatase inhibitors. Letrozole and anastrazole, both non-steroidal compounds are the two more dominant in the market, and the third agent, exemestane, is often referred to as an aromatase inactivator as being a steroidal compound it irreversibly inhibits the enzyme. A series of trials have confirmed modest superiority of AIs over the previous standards of care, whether megestrol acetate in the second line therapy of advanced disease after tamoxifen, or against tamoxifen itself, both in the adjuvant setting and also in advanced breast cancer. In the adjuvant setting, the meta-analysis of 18,871 women randomised across several large studies confirms an overall small, but significant survival advantage for the sequence of an AI after a few years' tamoxifen [17]. Such confirmed survival advantages have not been robustly seen in advanced breast cancer, but either non-inferiority and/or some clinical superiority have been shown in large phase III trials, and on the basis of those data and their good tolerability, they have become the standard first line endocrine therapy in ER+ breast cancer for post-menopausal women.

Neo-Adjuvant Setting

Although acceptance of validity for the use of systemic therapy before definitive surgery has not yet been achieved universally, there is a growing body of data that this is justified in terms of improved surgical outcomes without any evidence of poorer long-term disease control. Initially it had been chemotherapy that has been the mainstay of neo-adjuvant therapy, except in much older women where tamoxifen (and more recently aromatase inhibitors) has been used often instead of surgery.

Early non-randomised studies in Edinburgh did use both aminoglethimide and the steroidal AI 4-hydroxy-androstendione for 3 months in post-menopausal ER+ve localised breast cancer, with evident clinical responses [18]. However, the most important neo-adjuvant study was probably the 024 trial conducted by Novartis which randomised 324 patients whose disease did not permit breast conservation at initial presentation between either tamoxifen or letrozole for 4 months and demonstrated significantly higher response rates to letrozole (both clinical and on imaging), and importantly, a 10 % increase in the rate of breast conservation (45 % vs. 35 %) at the time of definitive surgery [19]. Since then further studies in Edinburgh, Holland and Italy [20, 21] have demonstrated that in some women longer duration of therapy increases the response rate and AIs can offer surgical advantages, and similar benefits over tamoxifen have been reported with anastrozole and exemestane [22]. Thus, in many units it has become standard of care to offer some post-menopausal women with ER-rich breast cancers neo-adjuvant aromatase inhibition therapy, though whether there is an optimal group of tumours for this approach, or duration and/or regimen remains unclear. However, it is clear that this approach is not offered to patients as universally as the use of post-operative aromatase inhibition, with some conservatism either because immediate surgery is preferred, or the use of chemotherapy is the standard approach except where medically inappropriate.

Adjuvant Setting

Whilst it is clear that the use of aromatase inhibitors offers superior benefits as compared to tamoxifen, there are different toxicities and so for some women with good prognosis, the choice between AIs and tamoxifen may be driven by toxicity questions more than benefits. Some local guidelines will use tamoxifen as the first choice for good prognosis patients (e.g. those post-menopausal women with node negative smaller and/or lower grade tumours) and without history of deep vein thrombosis or pulmonary embolism.

A number of studies addressed the question of optimum choice and sequencing of adjuvant endocrine therapy. Based on the large phase III ATAC and BIG 1-98 trials [11, 23], today many women are offered 5 years of either anastrozole

(ATAC) or letrozole (BIG 1-98) upfront. However, the data from several studies that sequenced tamoxifen and AIs [24, 25] have also shown a benefit for the use of a 5 year sequence of tamoxifen followed by an AI. In many centres this is offered to women at slightly lower risk, though it is probably not used as widely as the upfront 5 years of an AI, since there is a slightly higher recurrence rate in the first 2–3 years when women are on the tamoxifen.

More recently, the question of duration of endocrine therapy has become less clear. In the 1990s 5 years' therapy tamoxifen was becoming a standard practice in many clinics, but in 2003 the MA17 trial, which randomised women to 5 years of letrozole after 4.5–6 years' tamoxifen, suggested that a longer duration may provide additional benefits [26]; this was supported by the ATLAS and ATTOM trials of longer versus shorter duration tamoxifen (with most women effectively to 10 vs. 5 years' tamoxifen) [27]. But, there are not data on superiority or tolerability of more than 5 years of aromatase inhibitors, though the studies are underway and there is clinical support for this on the basis of extrapolation from the MA17, ATLAS and ATTOM studies.

As to which AI to use—this often depends on local protocols and experience, but it is clear that some patients find one AI preferable to another, and so there are a modest number of patients who switch from one drug to another for reasons of tolerability rather than inefficacy.

In premenopausal women AI use has been negligible, with tamoxifen (with or without ovarian ablation) being the standard of care. However, the very recent SOFT/TEXT trial results which tested the combination of an AI (exemestane) plus ovarian ablation have challenged this, reporting superior benefits for the combination of the AI exemestane and ovarian suppression as compared to tamoxifen and ovarian suppression, and this may lead to much more widespread use of "total oestrogen blockade" by the combination of ovarian suppression and aromatase inhibition in pre-menopausal women [28].

Advanced Disease

The overall superiority of aromatase inhibitors has led to them being the first line choice of endocrine agent for a post-menopausal woman with advanced breast cancer that is likely to be amenable to an endocrine approach. However, there is no uniform approach for a number of reasons.

Firstly, increasingly patients will have been exposed to aromatase inhibition in the adjuvant setting. Unlike the patients in the trials that established aromatase inhibitors as first line therapy in metastatic disease, their disease has already developed some degree of resistance to AIs. Thus, one cannot be sure that the same degree of superiority still exists for the AIs in the majority of post-menopausal women who have had prior therapy with AIs. Nevertheless, the practice remains in many centres to use AIs as first line therapy, with a tendency to use a different class if the recurrence developed during, or shortly after, the use of adjuvant

aromatase inhibition. Thus, many patients will get exemestane if they had a non-steroidal aromatase inhibitor as their most recent adjuvant endocrine therapy. However, if patients relapse some time (usually more than 1 year) after last taking an AI, clinicians may often choose to restart the same class of AI, or even, if well-tolerated, the same actual drug that was last used. This approach is clinically effective with patients able to have both responses and stable disease with further AI therapy. There are, however, no robust randomised data to support these various approaches. Furthermore, because the mechanism by which disease can recur after therapy with an AI, yet still show residual sensitivity to this approach, is not known, optimal strategies, if they exist, are not defined.

Secondly, whilst it is clear that further aromatase inhibition can be effective for those women whose metastatic disease progresses during aromatase inhibition therapy, the probability of confirmed response is small. The best data exist for exemestane. Consistent across non-randomised series and in the use of exemestane monotherapy in the control arm of the BOLERO2 trial, it is clear that only around 25 % of patients will have stable disease with less than 10 % (and in BOLERO2 < 1 %) patients manifesting a confirmed response [29].

However, this situation is being further confused by the new studies that demonstrate that superior efficacy can be obtained by adding a non-endocrine agent to an aromatase inhibitor. The combination with tamoxifen was of no benefit, and similar lack of benefit was seen with the combination of an aromatase inhibitor and the ER-downregulator fulvestrant [30]. But building on the science of the pathways involved in endocrine resistance, the combination of the mTOR inhibitor everolimus and exemestane was found to be more active (BOLERO2 trial) and gained marketing approval around the world. Very recently, exciting phase II data have emerged on the activity of the combination of Letrozole and the CDK4/6 inhibitor pablociclib and [31] a series of pivotal phase III trials are under way to determine if this should become a new standard of care.

Resistance to Aromatase Inhibition

In all the situations in which aromatase inhibitors are used in the treatment of breast cancer, resistance can and does occur. For example, any patient whose tumour relapses during or after the use of adjuvant AIs can be considered to have resistant disease, and those women who are cured might be thought to had disease that was sensitive to the therapy. However, non-relapse after adjuvant use of AIs does not equate to sensitivity, since we know that some patients would have been cured following surgery alone, and thus separating out the biological predictors of drug sensitivity from those that are associated with a good prognosis irrespective of therapy is a further complexity.

For those women whose disease does relapse, depending on the time to relapse it may be classified as primary or secondary resistance. Thus early relapse [32] (usually defined as within 2 years of starting AI therapy) may suggest primary

resistance, while late relapses (after several years therapy, or even after adjuvant therapy has been completed) may be considered as evidence of initial response and only later development of secondary resistance. At the present time, data only exist from the pivotal adjuvant AI trials to about 10 years' from diagnosis. Over this time period those studies suggest that the rate of relapse is relatively constant after the first couple of years (TLO ATAC 100 month data), such that only a minority of patients whose disease relapses will meet the definition of primary resistance. This is consistent with the neo-adjuvant use where most studies report primarily refractive disease (i.e. it continues to grow despite the use of AI) in only a small percentage of patients.

The most intensive biological analyses of resistance have been made in the neo-adjuvant and to some extent advanced disease settings where the only "variable" determining outcome is the behaviour of the cancer under aromatase inhibition. On the other hand, identifying predictors of primary or secondary resistance in these settings is more difficult as there is more obviously a continuum of both the degree and/or duration of response. In the neo-adjuvant setting, the rate of resistance depends on the selection of patients (age and degree of ER expression, measurement techniques (calliper, mammography, ultrasound or MRI) and duration of therapy [20, 33]. Objective response rates reported in large randomised neo-adjuvant AI trials range between 40 and 58 % (PO24, IMPACT and PROACT trials) [19, 34, 35]. If one takes lack of response as the definition of resistance, then the rate varies accordingly. In contrast, in the setting of advanced disease, resistance eventually develops in most of the patients. But it is not clear what time on therapy for advanced disease equates to the definitions of primary resistance in the neo-adjuvant or adjuvant setting. Thus, some clinical trials interpret progression within 6 months as primary resistance, and progression after 6 months as acquired/secondary resistance [36].

Given these discrepancies in response assessment and resistance criteria between the clinical settings, the study of the mechanisms of resistance in the three different settings may appear to give conflicting data without the underlying biology necessarily being any different.

Conclusion

The use of the currently available aromatase inhibitors has offered women with post-menopausal breast cancer a very useful, and generally well-tolerated, oral therapy to treat advanced ER+ breast cancer, and in the adjuvant setting, to significantly reduce the risk of recurrence and subsequent death from breast cancer.

However, whilst these agents offer considerable advantages over the earlier surgical and pharmacological approaches to endocrine deprivation therapy in post-menopausal patients with breast cancer, the gains in efficacy have been

more modest, and they have not changed four fundamental characteristics of this disease, namely:

- once metastatic, resistance to this approach almost always develops;
- whilst response to prior endocrine therapy increases the chance of benefit, subsequent therapies are rarely more effective than the prior ones;
- defining an optimal regimen/sequence remains a challenge;
- and the mechanisms underpinning resistance to aromatase inhibition remain inadequately resolved for many patients.

The rest of this book will therefore address these questions to get to the heart of the challenge of overcoming resistance to aromatase inhibition therapy for women with breast cancer.

Conflict of Interest No potential conflicts of interest were disclosed.

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Chapter 2
Structure, Regulation and Polymorphisms of the Aromatase Gene

Nobuhiro Harada

Abstract Human aromatase is widely expressed in various tissues and shows complicated regulation by both inductive and suppressive factors. The aromatase gene has the unique characteristic of having multiple exons available for use as exon 1, which are flanked with unique promoters. Tissue-specific expression of aromatase is regulated by alternative use of these exons. The exon 1 termed exon I.4 (1b) is the one that is mainly used in breast tissues. However, during cancer development it is often switched from exon I.4 (1b) to exon I.3 (1c) or exon PII (1d), which causes enhancement of aromatase expression in cancer-associated adipocytes and fibroblasts. The aromatase gene is further regulated at both the transcriptional and post-transcriptional levels through PKA-, PKC-, and tyrosine kinase receptor-mediated signaling pathways that employ prostaglandin E_2 and class 1 cytokines. Epigenetic modifications of the aromatase gene and microRNA-mediated aromatase regulation may play a critical role in breast cancer progression. Several genetic polymorphisms in the aromatase gene may be prognostic factors of disease and may influence response to aromatase inhibitors.

Abbreviations

AP-1	Acivator protein-1
ATF-2	Activating transcription factor-2
CAA	Carcinoma-associated adipocytes
CAF	Carcinoma-associated fibroblasts
C/EBP	CCAAT/enhancer binding protein
CRE	cAMP-responsive element

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A. Larionov (ed.), *Resistance to Aromatase Inhibitors in Breast Cancer*, Resistance to Targeted Anti-Cancer Therapeutics 8,
DOI 10.1007/978-3-319-17972-8_2

CREB	CRE binding protein
E2	17β-estradiol
ERK	Extracellular signal-regulated kinase
GAS	γ-interferon activation site
GPER	G protein-coupled estrogen receptor
GRE	Glucocorticoid responsive element
IHC	Immunohistochemical staining
LRH-1	Liver receptor homologue-1
MAPK	Mitogen-activated protein kinase
PGE_2	Prostaglandin E_2
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
RT-PCR	Reverse transcriptase-polymerase chain reaction
SF-1	Steroidogenic factor-1
SNP	Single nucleotide polymorphism
Sp1	Specificity protein 1

Introduction: Tissue-Specific Expression of Human Aromatase

Aromatase (estrogen synthase, cyp19a gene product) is a unique member of the cytochrome P450 superfamily. This enzyme is a terminal component of the electron transport system in the endoplasmic reticulum, accepts electrons from NADPH via NADPH-cytochrome P450 reductase, and catalyzes aromatization of androgens to estrogens by three successive hydroxylations and elimination of the carbon atom in position 19 of androgens, which is a rate-limiting step in estrogen biosynthesis [1]. Initially, high aromatase enzyme activity was reported to be localized in ovary and placenta, and to participate in female reproductive functions through the production of estrogens. However, later new sensitive assays using high-performance liquid chromatography with tandem mass spectrometry (LC/MS/MS), reverse transcriptase-polymerase chain reaction (RT-PCR), and immunocytochemical staining have shown that aromatase is expressed not only in female gonadal tissues but also in male gonadal tissues such as the testis and epididymis, as well as in extra-gonadal tissues such as the prostate, brain, liver, skin, adrenal gland, hair follicles, and adipose, bone, and vascular tissues [2–5]. While it is well known that aromatase expression can be found in estrogen-dependent breast cancer tissues, its expression has also been observed in endometrial carcinoma and in liver, gastric, pancreatic, colorectal, lung, ovarian, and prostatic cancers [6, 7].

There is a complicated regulation of aromatase in a tissue-specific manner by both inductive and suppressive factors. Thus, the expression of aromatase in placenta-derived cells is induced by phorbol esters [8] and suppressed by insulin [9], whereas its expression in the ovary is antithetically induced by insulin [10] and suppressed by phorbol ester [11]. Similarly, dexamethasone is an inducer of aromatase in the skin or adipose tissue [12], whereas it is a suppressor of aromatase in the ovary [13]. Aromatase is induced by the gonadotropin (e.g., FSH, LH, or hCG)-initiated cyclic AMP (cAMP)-protein kinase A (PKA) intracellular signaling system in many tissues except for the brain, in which aromatase is known to be mainly induced by androgens [14]. These observations indicate that aromatase expression is strictly regulated by tissue-specific regulatory factors, supporting the concept of intracrinology through local production of estrogens by aromatase in multiple tissues.

Structure of the Human Aromatase Gene

Analysis of aromatase transcripts in various human tissues indicated the presence of tissue-specific unique nucleotide sequences at the 5′-ends of mRNA, following which there is a common translated nucleotide sequence that encodes 503 amino acids, indicating that aromatase proteins have the same amino acid sequence in all tissues. Interestingly, aromatase transcripts are transcribed from different positions on the human aromatase gene in a tissue-dependent manner [2]. Aromatase gene clones were subsequently isolated from a human genomic DNA library. The human aromatase gene (*cyp19a1*) is present in the haploid genome as a single copy and spans about 123 kb at the 15q21.1 region of chromosome 15 [15]. Analysis of the exon-intron organization of the gene showed that all of the tissue transcripts are composed of 10 exons (Fig. 2.1). The unique sequences that are observed in the 5′-ends of the transcripts are scattered over approximately 100 kb

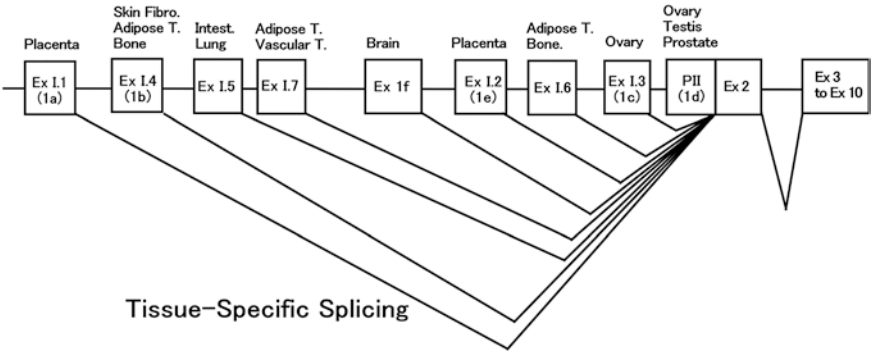

Fig. 2.1 Gene structure of the human aromatase. Multiple exons 1, encoding the only 5′-untranslational region of the human aromatase gene are tissue-specifically spliced and connected to exon (Ex) 2

upstream of exon 2, whereas the translated coding sequence was identified as exons 2-10 that lie within about 35 kb of the 3′-end of the gene [1, 2]. This gene organization suggests that the human aromatase gene includes multiple promoters and that the aromatase transcript is tissue-specifically spliced from the multiple alternative exons available for exon 1 (Fig. 2.1).

To date, nine unique alternative versions for exon 1 have been isolated (Fig. 2.1). Each exon 1 is used in a tissue-specific manner; I.1 (1a) and I.2 (1e) in the placenta, I.3 (1c) and PII (1d) in the ovary and testis, I.4 (1b) in adipose tissue, I.5 in the fetal lung and intestine, I.6 in adipose and bone tissues, I.7 in adipose and vascular endothelial tissues, and 1f in the brain [1, 16]. This selective utilization of these exons is possible due to tissue-specific promoters that flank each exon 1. The promoter structures of the exons that are predominantly used as exon 1 in the major estrogen-producing tissues are shown in Fig. 2.2 [17]. The 5′-upstream promoter regions of these exons have binding sites for diverse tissue-specific regulatory factors such as glial cell missing 1a (GCM 1a), activator protein-2γ (AP-2γ), LIM homeodomain box-2 (Lhx-2), and apolipoprotein regulatory protein-1 (ARP-1)/chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII). These promoter regions also contain regulatory elements such as the glucocorticoid responsive element (GRE), γ-interferon activation site (GAS), activator protein-1 (AP-1) and cAMP-responsive element (CRE), steroidogenic factor-1 (SF-1) binding sites, as well as basic transcription elements such as a TATA box, a CAAT box, and a GC box. Exons I.1 (1a) and 1f contain

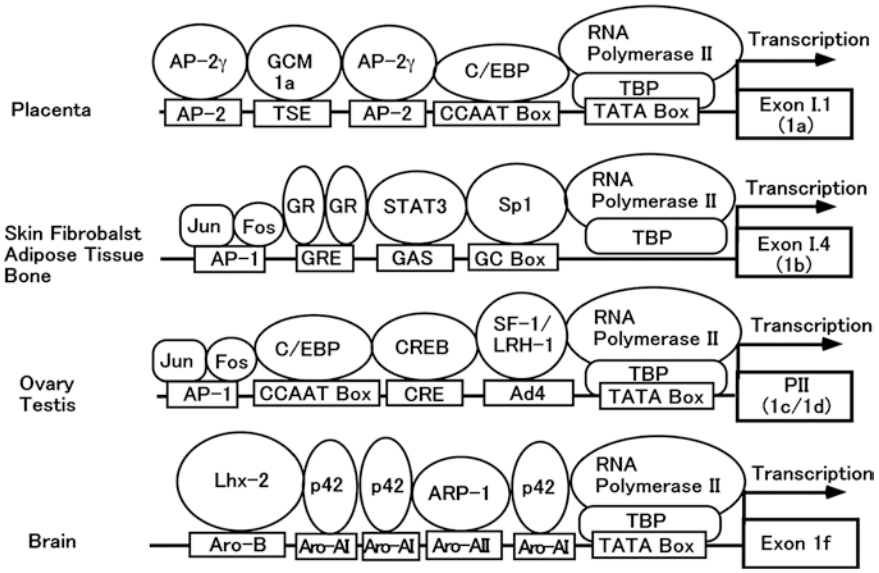

Fig. 2.2 Schematic promoter structures of exons I.1 (1a), I.4 (1b), PII (1c/1d), and 1f of the human aromatase gene. Factors are shown as *ovals* and cis-acting DNA elements as *boxes*. Details are in the text

binding sites for the trophoblast (placenta)-specific transcription factors GCM 1a and AP-2γ [18] and for the neuron-specific and/or developmental stage-specific transcription factors Lhx-2 and ARP-1/COUP-TFII [19], respectively, which illustrate remarkable differences in tissue-specific transcription regulation in the placenta and brain, respectively. Most of the exons that encode an exon 1, except for exons I.4 (1b) and I.7, have a TATA box in the proximal promoter region and form stable transcription pre-initiation complexes mediated by binding of TBP (TATA binding protein) to the TATA box. However, instead of a TATA box in the proximal promoter regions, exon I.4 (1b) and exon I.7 have a GC box, which is a specificity protein 1 (Sp1) binding site and is often found in housekeeping genes, and a GATA box, which is the binding site of a hematopoietic transcription factor, respectively [20]. Transcription from exon I.4 (1b) seems to be somewhat unstable, judging from the several transcriptional initiation sites observed in the transcripts. Switching of exon I.4 (1b) that is used in these transcripts to exon I.2 (1c)/PII (1d) is frequently observed when the tissues are exposed to cancerous or inflammatory conditions [2, 21]. This switching may be possibly explained by the relative instability of the transcription initiation complex on exon I.4 (1b). Of note, exon I.2 (1c)/PII (1d), which is predominantly used in gonadal tissues, has both CRE and AP-1 sites in the proximal promoter region for the binding of CRE binding protein (CREB)/activating transcription factor-2 (ATF-2) and c-Fos/c-Jun, respectively, suggesting that the aromatase in gonadal tissues is transcriptionally induced by cAMP/PKA and diacylglycerol (DAG)/protein kinase C (PKC) intracellular signals derived from hormones such as FSH and LH. This regulation may also explain the dynamic changes in aromatase expression in the ovary that are a response to the reproductive cycle. Transcriptional factors interacting with alternative promoters of aromatase gene in breast cancer will be discussed in more details this chapter.

Expression of Aromatase in Breast Cancer Stroma

Healthy breast tissues express low baseline levels of aromatase transcripts from exon I.4 (1b) under non-stimulated conditions. However, once the breast tissues tend towards carcinogenesis, in many cases transcription switches from using exon I.4 (1b) to using exon I.2 (1c)/PII (1d) [21], and levels of aromatase mRNA and catalytic activity are significantly increased in adjacent adipose tissue [22].

Aromatase has been reported to be localized in stromal spindle cells as well as in tumor epithelial cells of breast cancer tissues based on immunohistochemical staining (IHC) [6, 23]. High levels of aromatase mRNA expression have also been detected by a combination of RT-PCR analysis and laser capture microdissection of stromal cells, which supports previous IHC studies that stromal cells display the highest positivity for aromatase in many breast cancer cases [24]. Because cancer cells at the early stage of cancer development are usually surrounded by a large number of stromal cells, estrogen production by aromatase in

the stromal cells could play an important role in proliferation of the cancer cells. It has been suggested that the interaction between malignant epithelial cells and the stromal cells or inflammatory cells in breast cancer is important for tumor proliferation and progression [25]. In particular, invasive malignant tumor cells interact with surrounding adipocytes (carcinoma-associated adipocytes; CAA), fibroblasts (carcinoma-associated fibroblasts; CAF), and inflammatory cells, and induce a desmoplastic reaction in the surrounding stroma that is accompanied by breast cancer progression [26]. These dense fibrous or connective tissues that are formed in the desmoplastic reaction secrete various kinds of cytokines or bioactive substances, leading to induction of aromatase and consequently progression of the breast cancer by enhanced production of local estrogens [27]. Indeed, high-frequency switching from exon I.4 (1b) to exon I.2 (1c)/PII (1d) and enhanced expression of aromatase mRNA are often observed in invasive scirrhous cancers [21]. Fibroblast-like mesenchymal preadipocytes have been reported to be able to express high amounts of aromatase and this ability disappears upon differentiation into mature adipocytes [28]. In the desmoplastic reaction, cytokines such as TNFα and IL-11 that are secreted from the cancer cells promote the formation of fibrous cells thereby suppressing adipocyte differentiation in the stroma and causing elevation of aromatase expression in CAFs (Fig. 2.3) [29]. The suppressive effect of TNFα and IL-11 is believed to be mediated by selective down-regulation of the CCAAT/enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ), which are essential factors for adipocyte differentiation. Consequently the large quantities of estrogens produced by the elevated aromatase

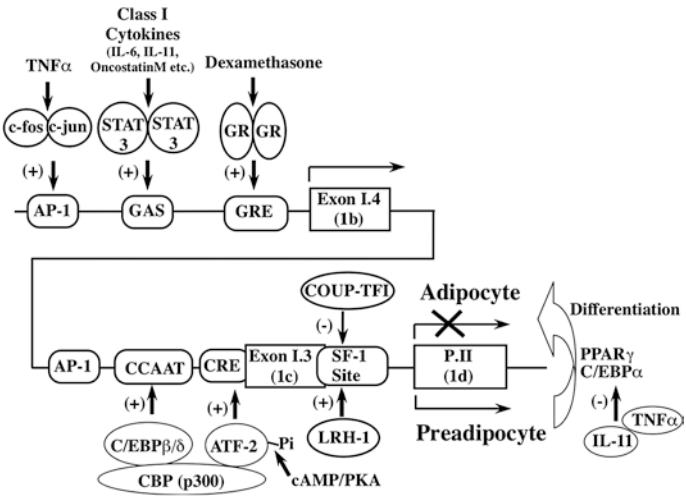

Fig. 2.3 Proposed regulation of aromatase gene expression in breast cancer tissue. Stimulatory (+) and inhibitory (−) transcription factors are differentially bound to promoter regions of exons I.4 (1b), I.3 (1c) and promoter II (1d) of the human aromatase gene in breast cancer tissue. Anti-adipogenic cytokines TNFα and IL-11 play an important role in E2 production through inhibitory effects on the differentiation of adipose tissue

in the CAFs promote the proliferation of estrogen-dependent cancer cells and, at the same time, act on the cancer cells to induce transcriptional activation of the *IL-11* gene, which ultimately forms a positive feedback and acts on CAFs and pre-adipocytes to increase TNFα receptor (TNFR1) mRNA [29].

It was previously suggested that the G protein-coupled receptor 30 (GRP30) acts as a membrane-bound mediator to interpret rapid non-genomic actions of estrogens. This receptor, also called the G protein-coupled estrogen receptor (GPER), was shown to be localized in the endoplasmic reticulum of CAFs by IHC and to increase expression of the CAF aromatase through the EGF receptor (EGFR)/extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway that is activated by estrogens, tamoxifen and the GPER agonist G1, thereby promoting CAF proliferation and cell-cycle progression (Fig. 2.4) [30]. These findings suggest that GPER/EGFR/ERK signaling may be involved in the progression of endocrine-resistant breast cancer cells by elevation of aromatase expression and estrogen production.

Aromatase expression is locally increased in stromal cells proximal to breast cancer cells by their switching of transcription from exon I.4 (1b) to one of the alternative exons I.2 (1c)/PII (1d), whereas aromatase is expressed at normal levels in distal stroma, which cells display a low switching frequency [21]. This finding may indicate that cytokines or bioactive substances that are secreted into the region proximal to the breast cancer as a result of cancer–stroma interactions contribute to exon 1 switching. Indeed, low levels of aromatase transcripts from exon I.4 (1b) were observed in isolated cultured breast stromal cells, whereas increased levels of aromatase transcripts from exon I.2 (1c)/PII (1d) were often observed in co-culture of stromal cells with cancer cells [31]. Because this increase was reproduced by replacing the cancer cells with their culture supernatant, the participation

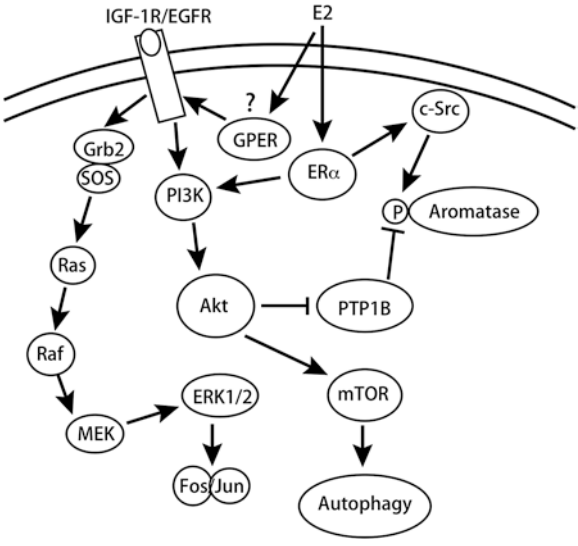

Fig. 2.4 Proposed model of potential signaling pathways from IGF-1 receptor/EGF receptor and E2 which modulate cell proliferation and aromatase activity in breast cancer cells

of humoral factor(s) secreted from the cancer cells in this exon switching and aromatase transcriptional activation was suggested. Simpson et al. reported that stromal aromatase in breast cancer tissues is induced by TNFα, ceramide, prostaglandin E_2 (PGE_2), and class 1 cytokines such as IL-6, IL-11, Leukemia inhibitory factor (LIF) and Oncostatin M (Fig. 2.3) [32, 33]. In other experiments, Yamaguchi et al. [25] evaluated the transactivation of an estrogen-estrogen receptor (ER) complex resulting from cancer-stromal cell interactions in the cancer microenvironment by using a reporter with an estrogen response element that drove expression of the green fluorescent protein gene (ERE-GFP reporter). When human breast cancer-derived MCF-7 cells that were stably transformed with this ERE-GFP reporter were co-cultured with stromal cells isolated from 67 different breast cancer patients, transactivation of the reporter gene in MCF-7 cells was observed to varying extents, depending on the case. All of these observations are consistent with the concept of a positive feedback loop of aromatase induction in stromal cells by cancer cell-derived humoral factors and of estrogen-dependent proliferation in cancer cells by cancer-stromal interaction.

Transcriptional Regulation of the Aromatase Gene in Breast Cancer Tissues

In breast cancer tissues, of the multiple exons that are available for exon 1, the aromatase gene is mainly transcribed from exons I.3 (1c), I.4 (1b), I.7, and PII (1d), and this transcription is controlled by transcription factors that interact with regulatory elements on the exon 1 flanking promoter regions (Fig. 2.2) [34]. The GRE, GAS, and AP-1 sites on the proximal promoter region of exon I.4 (1b) are predominantly used in non-malignant breast tissues. Zhao et al. [35] reported the induction of transcriptional activation from exon I.4 (1b) in breast adipose stromal cells by class 1 cytokines that were secreted by breast cancer cells or by lymphoid cells/macrophages (Fig. 2.3). These cytokines bound to gp130 cytokine receptors on the stromal cells resulting in the phosphorylation of Signal Transducer and Activator of Transcription 3 (STAT3) through activation of tyrosine kinases (JAK1/JAK2/TYK2) of the Janus activation kinase (JAK) family. STAT 3 subsequently dimerized and caused transcriptional activation by binding to the GAS site on the promoter. The aromatase in adipose stromal cells has also been reported to be induced by TNFα and its intracellular downstream factor, ceramide. Since TNFα is secreted by mature adipocytes, this finding indicates the participation of a type of adipokine in the induction of aromatase in breast tissues [33]. Aromatase induction by TNFα is considered to involve the mitogen-activated protein kinase (MAPK) signalling pathway, because it is inhibited by the p38 MAPK inhibitor, SB203580. As there is an incomplete AP-1 site in the promoter upstream of GAS, TNFα is considered to promote the binding of c-Fos and c-Jun to this AP-1 site through activation of MAPK signaling.

Frequent switching of the aromatase exon 1 that is used in breast cancer tissues from the dominant exon I.4 (1b) to exon I.3 (1c)/PII (1d) has been observed, concomitant with increased expression of aromatase [21]. Because the promoter regions of exon I.2 (1c) and PII (1d) partially overlap, there is similar transcriptional regulation of these two exons. They both share an AP-1 and a CRE that is present on the common region. For this reason, transcription from both of these exons is commonly regulated by various inducible factors in gonadal tissues. As shown in Fig. 2.3, and proposed by Simpson et al., transcription from exon I.3 (1c)/PII (1d) is suppressed in normal breast tissues by binding of the transcriptional suppressor, COUP-TFI, to an SF-1 binding site on the promoter, whereas surprisingly, transcription from this exon is activated with the progression of breast cancer [36]. This conversion from inhibition to activation is enabled by decreased expression of COUP-TFI and increased expression of liver receptor homolog-1 (LRH-1), a transcriptional activator with high affinity for SF-1 binding sites, and is accompanied by cancer progression [28]. Additionally, progressive cancer cells and inflammatory cells frequently secrete PGE_2 or cytokines, which activate the transcription factors ATF-2 and c-Fos/c-Jun in adipose stromal cells through PKA- and PKC-activating intracellular signals, respectively [37]. Consequently, transcription from exon I.3 (1c)/PII (1d) is enhanced by binding of activated ATF-2 and c-Fos/c-Jun to the CRE and AP-1 sites, respectively, on the promoter region. In addition, the adipocyte differentiation factors C/EBPα and PPARγ were reported to cause transcriptional suppression of the aromatase gene by lowering LRH-1 expression, whereas C/EBPβ, a preadipocyte differentiation factor, was suggested to participate in transcriptional activation by forming a stable transcription complex with the transcriptional coactivator, p300/CREB binding protein (CBP) [38], and AFT-2 which is activated by cAMP-PKA signaling from PGE_2 (Fig. 2.3) [39]. While the conditioned medium of cultured breast cancer cells is known to increase aromatase mRNA levels in cultured adipose stromal cells, it also induces expression of C/EBPβ and the formation of a stable transcription complex consisting of C/EBPβ, AFT-2 and p300/CBP, resulting in induction of aromatase [40]. As this induction was not completely suppressed by inhibitors of cyclooxygenase-2 (COX-2) and adenylate cyclase, the conditioned medium may contain other unknown aromatase-inducible factors in addition to PGE_2 that might synergistically induce aromatase. PGE_2 in breast cancer tissues was shown to increase the binding affinity of LRH-1 for the SF-1 binding site on the promoter of PII (1d) and to simultaneously induce aromatase in adipose stromal cells [28]. Therefore, non-steroidal anti-inflammatory drugs (NSAIDs) may be expected to suppress aromatase gene expression trough promoter PII and proliferation of breast cancer cells by inhibition of COX-2, a rate-limiting enzyme in PGE_2 synthesis. It was shown that the CREB-regulated co-activator CRTC2 binds directly to the PII (1d) promoter of the aromatase gene in preadipocytes and activates expression of the aromatase gene through mechanisms involving LKB1-AMP kinase (AMPK) that is regulated in response to PGE_2 [41, 42]. Tamoxifen-resistant breast cancer cells also displayed increased expression of aromatase

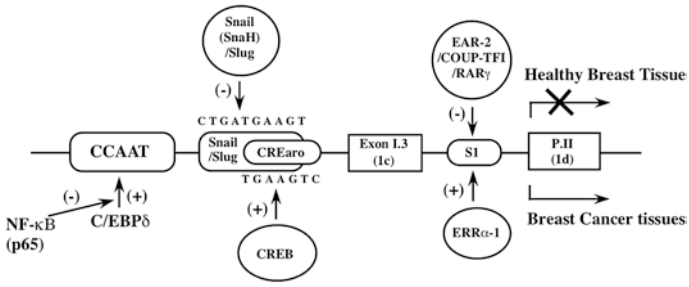

Fig. 2.5 Proposed mechanism of aromatase gene expression in healthy and malignant breast tissues. Stimulatory (+) and inhibitory (−) transcription factors are differentially bound to promoter regions of exon I.3 (1c) and promoter II (1d) of the human aromatase gene

together with phosphorylation of Akt, ERK and the p38 kinase and the resulting phosphoinositide 3-kinase (PI3K)/Akt-dependent CREB activation induced the expression of aromatase [43].

Chen et al. [44] identified an S1 site (silencer element), a CREaro site, and a Snail/Slug site on the promoter region of exon I.3 (1c)/PII (1d) using a yeast one-hybrid screening system and a promoter assay (Fig. 2.5). The S1 site, which is just upstream of PII (1d), includes the SF-1 binding site within it. Transcriptional repressors of COUP-TFI, V-erbA related protein 2 (EAR-2), and retinoic acid receptor γ (RARγ) bind to this S1 site in healthy breast tissues and suppress expression of aromatase [45]. The expression levels of these repressors decrease with cancer progression and in their stead ERRα-1, a transcriptional activator, binds to the S1 site, leading to an increase in transcription from PII (1d) [45]. That study also indicated that two types of CREB, CREB-1 and CREB-related factor, bind to CREaro, an incomplete CRE site (5′-TGAAGTCA-3′) that is just upstream of exon I.3 (1c), and induce transcriptional activation of the aromatase gene [46]. Two transcription factors with zinc-finger motifs, Snail (a human homologue of SnaH) and Slug, were implicated to regulation of this promoter by yeast one-hybrid screening [47]. Their DNA binding sequence (5′-CTGATGAAGT-3′) largely overlaps with that of CREaro. Because the expression of aromatase in breast cancer tissues showed an inverse correlation with that of SnaH, it was suggested that SnaH is a transcriptional repressor that binds to the Snail/Slug site. Indeed, the expression of aromatase is suppressed due to overexpression of SnaH in healthy breast tissues [47]. However, in breast cancer tissues, in which PGE_2 is secreted from cancer cells or inflammatory cells and activates cAMP-PKA signals in the stromal cells, CREB family factors are activated and bind to the CREaro site. Because there is a 6-base pair overlap between the DNA sequences of the CREaro and the Snail/Slug sites, binding of the CREB factors to the CREaro site hinders binding of SnaH to the Snail/Slug site, resulting in release of aromatase expression from SnaH suppression and the induction of aromatase [44].

Epigenetic Regulation of Aromatase

The above-described transcriptional regulation of aromatase has been studied since 1980s. More recently the epigenetic modification has also attracted attention as an aromatase regulatory mechanism and was shown to play an important role in the regulation of aromatase in physiological processes such as in temperature-dependent sex determination [48], placental development [49], and the ovarian sexual cycle [50]. Since epigenetic mechanisms regulate aromatase in human breast adipose fibroblasts, it was suggested that DNA methylation in the promoter region of the aromatase gene might contribute to this regulation [51]. In contrast to the well-studied role of epigenetic DNA methylation, little is known to date regarding the potential role of histone modifications as regulatory mechanisms of the human aromatase gene. Epigenetic histone modifications make it possible to regulate the expression of diverse genes through the regulation of DNA-chromatin interactions by using combinations of histone methylation, acetylation, phosphorylation, glycosylation, ubiquitination, and ADP-ribosylation. Generally, trimethylation of histone 3 lysine 4 (H3K4me3) and lysine 36 (H3K36me3) or acetylation of histone 3 lysine 9 (H3K9ac), lysine 14 (H3K14ac), and lysine 27 (H3K27ac) are observed in activated genes, whereas trimethylation of histone 3 lysine 9 (H3K9me3) and lysine 27 (H3K27me3) are observed in repressed genes. Lee et al. [52] recently showed that a decreased level of H3K4me3 and an increased level of H3K27me3 were found at the promoter region of the rat aromatase gene in ovary granulosa cells, concomitant with a decrease in aromatase gene expression. Methodical investigation will be required to evaluate the exact contribution of epigenetic regulation of the aromatase gene in breast cancer tissues in relationship to local estrogen supply and breast cancer progression.

Genetic Polymorphisms of the Human Aromatase Gene Associated with AI Response and Susceptibility to Breast Cancer

As estrogens are considered to be an important risk factor for the incidence and development of breast cancer, the transcriptional regulation of aromatase has been intensively studied as described above in order to elucidate its etiological role in this disease. In addition to transcriptional regulation, genetic variants of the aromatase gene including short tandem repeat polymorphisms and single nucleotide polymorphisms (SNPs) have also been shown to be associated with aromatase activity and are potential prognostic factors for breast cancer susceptibility. Indeed, several genetic polymorphisms of the aromatase gene have been reported to potentially affect aromatase gene expression, clinicopathological factors, prognostic factors, refractory factors, and cancer susceptibility.

Kristensen et al. [53] observed five different short tandem repeat polymorphisms of $(TTTA)_n$ in the intron 5 of the aromatase gene and showed that the $(TTTA)_{12}$ repeat allele is more frequent in Caucasian white women with breast cancer than in healthy controls. In contrast, Probst-Hensch et al. [54] reported the opposite result, showing no significant association of breast cancer susceptibility with any alleles of $(TTTA)_n$ repeats in African-American, Japanese, Latin, and non-Latin white populations. Since the allele distribution of the $(TTTA)_n$ repeat polymorphism varied depending on ethnic differences, the discrepancy between the results of the groups might reflect ethnic differences.

Ma et al. [55, 56] identified 88 SNP alleles of the human aromatase gene by exone re-sequencing in 240 subjects representing different ethnical groups with follow-up analysis on 10,592 cases and 11,720 controls. These studies firmly established functional associations between aromatase variation and function, with potential clinical implications for estrogen-dependent conditions, including breast cancer. A considerable number of other authors have also reported correlations of SNP alleles with levels of aromatase mRNA and activity, clinical factors, and susceptibility to breast cancer as well as to other cancers has been found to date (Table 2.1). A typical example is the genotype distribution of an SNP that is found in the 3′-untranslated region of exon 10 [57]. The TT genotype of this SNP was found at significantly higher frequency in breast cancer patients than in controls. Higher frequency was especially notable in patients with stage III and IV cancers and with tumors larger than 5 cm. The TT alleles also showed significant associations with the expression levels of aromatase mRNA and with switching from the adipose tissue-preferred exon I.4 (1b) to the gonad-tissue-preferred

Table 2.1 SNPs in the human aromatase gene

Cancer type	SNP	Risk	Reference number
Breast cancer	rs4646	Time to progression	Colomer et al. [73]
	rs6493497	AI-effect, E2 production	Wang et al. [58]
	rs10046	CS, E2 production, OS	Zins et al. [74]
	rs700518	Recurrence, bone loss (AI)	Napoli et al. [61]
	rs727479	Recurrence, OS	Miron et al. [75]
	rs934635	MASE (AI), VMS (AI)	Fontein et al. [60]
	rs1694189	VMS (AI)	Fontein et al. [60]
	rs7176005	VMS (AI)	Fontein et al. [60]
Ovary cancer	rs727479	E2 production	Haiman et al. [76]
	rs749292	E2 production, CS	Goodman et al. [77]
Prostate cancer	rs 2470152	E2 production, CS	Kanda et al. [78]
	rs10459592	E2 production, CS	Kanda et al. [78]
	rs4775936	E2 production, CS	Kanda et al. [78]
Lung cancer	rs3764221	E2 production, CS	Kohno et al. [79]
Colorectal cancer	rs1902584	CS	Lin et al. [80]

AI aromatase inhibitor, *CS* cancer susceptibility, *MASE* musculoskeletal adverse event, *OS* overall survival, *VMS* vasomotor symptom, *E2* 17β-estradiol

exon I.3 (1c) in breast cancer tissues. Wang et al. [58] reported two SNP alleles (rs6493497/rs7176005) on the promoter region of exon I.1 (1a), which were associated with elevation of aromatase enzymatic activity, with plasma 17β-estradiol (E2) level and with the potency of aromatase inhibitors for breast cancers; however some of these findings have not been confirmed in a different patients' cohort [59]. Some SNP alleles were potentially associated with increased risk of musculoskeletal adverse events (rers934635), vasomotor symptoms (rs 934635, rs1694189, rs7176005), or bone loss (rs700518) that accompany breast cancer treatment with aromatase inhibitors [60–62]. Of note, several SNP alleles were also reported to be associated with E2 production and ovary, prostate, lung and colorectal cancer susceptibility (Table 2.1). Since genetic polymorphisms are widely distributed throughout the human genome, not all of the SNP alleles on the aromatase gene are necessarily in the translated coding region or in the promoter region and some of these alleles exist in introns or untranslated regions. Therefore, SNP alleles that display association with clinically valuable factors may not directly affect aromatase expression but may rather be in linkage disequilibrium with another genetic variant.

Post-transcriptional Regulation of Aromatase

MicroRNA (miRNA) has recently been shown to be involved in the translational regulation of various genes through control of the translational rate or of transcript stability. Estrogen production in the ovary was shown to be regulated by miR-378, a microRNA that targets aromatase [63]. Aromatase activity is also controlled by post-translational modifications such as glycosylation and phosphorylation/dephosphorylation. Post-translational glycosylation of placental aromatase increased aromatase activity by 35–40 % [64]. On the other hand, aromatase activity was down-regulated by phosphorylation and was restored by dephosphorylation in various cultured cells [65]. Furthermore, aromatase protein levels are irreversibly decreased when aromatase is in a chronically phosphorylated state [66]. Conversely, the growth factors TGFα, EGF, and FGF, whose receptors are linked to a tyrosine kinase, enhanced aromatase activity in breast cancer cells [67]. Moreover, insulin-like growth factor-1 (IGF-1) was also found to increase aromatase activity in breast cancer cells by phosphorylation that was mediated through both the PI3K/Akt and the MAPK intracellular signaling pathways [68]. Barone et al. [69] suggested a critical role for estrogens in the regulation of aromatase in breast cancer cells. Interestingly, Zhang et al. [70] reported that IGF-1 enhanced both aromatase protein levels and its activity by inhibiting autophagy through activation of mTOR. As shown in Fig. 2.4, E2 may rapidly enhance aromatase activity through tyrosine phosphorylation of aromatase by the E2-activated c-Src kinase and through the suppression of protein tyrosine phosphatase 1B (PTP1B), which can dephosphorylate tyrosine-phosphorylated aromatase, by E2-activated PI3K/Akt kinases [69].

Other Possible Factors that Affect Aromatase

Non-steroidal aromatase inhibitors were reported to increase aromatase protein levels in cultured cells, probably through the formation of a stable aromatase protein-inhibitor complex that prevents its proteolytic degradation [71]. Aromatase catalyzes the aromatization reaction of androgens by supplying electrons from the NADPH-cytochrome P450 reductase. Therefore, estrogen-synthesizing activity is also dependent on the level of NADPH-cytochrome P450 reductase in the electron transport system of the endoplasmic reticulum. However, there have been few reports regarding changes in the expression level of NADPH-cytochrome P450 reductase in breast cancer tissues. In malignant liver tumors, the level of this reductase was observed to decrease at tumor sites compared with its level at distal sites, whereas the aromatase level increased locally at tumor proximal sites [7]. A number of polymorphisms have been reported for HADPH cytochrome reductase [72]. Given the important role of this cytochrome reductase in aromatization, mutations in this gene may have implications for AI response and resistance in breast cancer. More investigation is needed to evaluate the contribution of NADPH-cytochrome P450 reductase to estrogen production and to breast cancer progression.

Conclusion

The aromatase gene has an exceptionally complex organization. It consists of 9 coding exons and multiple alternative 1st exons, controlled by different promoters. A body of studies has been accumulated over the last 30 years about the interplay of multiple transcriptional modulators, post-transcriptional modifications and genetic variations for the human aromatase gene. Knowledge of tissue-specific regulation of the aromatase gene is essential for understanding AI resistance mechanisms and for minimizing side effects of AIs in breast cancer.

Acknowledgments This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan.

Conflict of Interest No potential conflicts of interest were disclosed.

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Chapter 3
Structure, Function and Inhibition of Aromatase

Debashis Ghosh, Jessica Lo and Chinaza Egbuta

Abstract Human cytochrome P450 aromatase catalyzes with high substrate specificity the synthesis of estrogens from androgens. The crystal structure of human aromatase has revealed an androgen-specific active site. The structural insights have been utilized in the investigation of its transmembrane integration, roles of critical residues, reaction mechanism, implications of motion and flexibility on its function, ligand-binding interactions, and oligomeric states. Some of these results provide glimpses into the enzyme function as a membrane-embedded molecule. The structural and chemical basis of steroid-protein interactions have been harnessed to rationally design novel steroidal inhibitors with exclusive aromatase specificity. Several of these compounds exhibit superior inhibitory properties in purified human aromatase when compared with the breast cancer drug exemestane. The antiproliferative potencies of some of these compounds assayed in an MCF-7 breast cancer cell line exceed that of exemestane. The X-ray structures reveal that these new inhibitors exploit previously unknown aromatase-specific interactions. The newly developed structural and chemical biology knowledge lay the foundation for understanding the mechanisms of modulation of enzyme activity upon estrogen-dependent phosphorylation, and novel non-genomic aromatase-estrogen signaling feed-back reported in malignant breast cells as well as in neuroendocrine systems. The structural insights also provide the molecular basis for discovery of next generation inhibitors.

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A. Larionov (ed.), *Resistance to Aromatase Inhibitors in Breast Cancer*,
Resistance to Targeted Anti-Cancer Therapeutics 8,
DOI 10.1007/978-3-319-17972-8_3

Abbreviations

A	Androstenedione
AI	AROM inhibitor
ANZ	Anastrozole
AROM	Cytochrome P450 aromatase
CPR	NADPH cytochrome reductase
E2	17β-estradiol
ER	Estrogen receptor
EXM	Exemestane
LTZ	Letrozole
mER	Plasma membrane associated estrogen receptor
NMA	Normal mode analysis
OR	Opioid receptor
T	Testosterone

Introduction

Cytochrome P450s are members of a superfamily of heme-containing enzymes present both in eukaryotes and prokaryotes [1]. There are 18 gene families, 44 subfamilies, and 57 sequenced proteins of cytochrome P450s in human. Cytochrome P450 aromatase (AROM) is the product of the *CYP19A1* gene on chromosome *15q21.1*, which has one family and one subfamily. P450s in general catalyze metabolism of a wide variety of endogenous and xenobiotic compounds, and drugs with low substrate specificities. AROM, on the contrary, uses with high specificity androstenedione (A), testosterone (T), and 16α-hydroxytestosterone (all with the same androgen backbone) as substrates converting them to estrone, 17β-estradiol (E2), and 17β, 16α-estriol (all with the same estrogen backbone), respectively (Fig. 3.1). It is the only known enzyme in vertebrates capable of

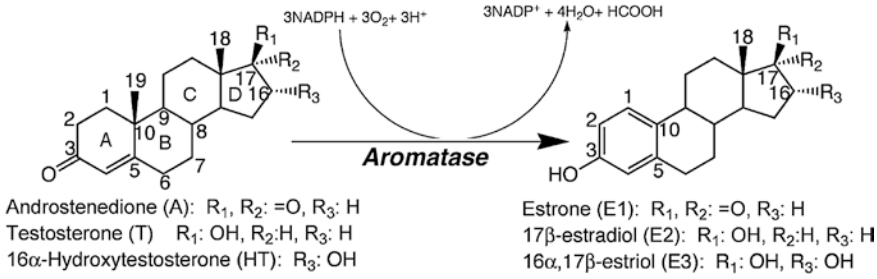

Fig. 3.1 Catalysis of androgens to estrogens by AROM. Androgens are converted to estrogens in a three-step reaction that requires 3 mol of O_2, 3 mol of NADPH, and coupling with CPR for the transfer electrons from NADPH to AROM

catalyzing the aromatization of a six-membered ring. Inhibition of estrogen biosynthesis by AROM inhibitors (AI) constitutes one of the foremost therapies for postmenopausal estrogen-dependent breast cancer today [2, 3].

The functional human enzyme is comprised of a heme group and a polypeptide chain of 503 amino acid residues. It is an integral membrane protein of the endoplasmic reticulum, anchored to the membrane by an amino (N)-terminal transmembrane domain [4, 5], in addition to other membrane-associated regions. Being a catalyst in the estrogen biosynthesis pathway for a unique hydroxylation reaction that involves a carbon-carbon bond cleavage and a ring aromatization, AROM has been the subject of intense biochemical and biophysical investigations for the past 50 years [6–8].

Nevertheless, many aspects of the AROM-catalyzed reaction, especially the third step of aromatization, remained poorly understood. Until 2009 [4] the absence of a crystal structure for human aromatase led to a number of homology models for the enzyme based on other experimental P450 structures and site-directed mutagenesis data [9–13]. Several androgen-binding scenarios at the active site, possible involvements of side chains in the catalytic process, as well as models for enzyme's mechanism of action were proposed based on these structural and functional analyses. Validation of all these results necessitated an experimental three-dimensional model of the enzyme showing the binding mode of the steroidal substrate and its interactions with active site amino acids. Details of the substrate and inhibitor binding interactions at the active site were crucial for the development of next generation AIs.

Despite concerted efforts in many laboratories, no experimental molecular structure of AROM emerged for a very long time. The major impediments to AROM crystallization were its strong hydrophobic character, and susceptibility to rapid denaturation in the absence of the protective lipid bilayer. Using term human placenta as a rich source of AROM and a purification technique that employs a highly specific monoclonal antibody-based affinity chromatography [14], we were able to purify large quantities of the enzyme in a pristine, active form that permitted the growth of diffraction-quality single crystals under suitable detergent conditions. This was the first and only microsomal P450 purified from a native source to date, and the first full length P450 to be crystallized [4, 15]. Employing the same crystallization protocols, a recombinant form of human AROM has more recently been crystallized [16].

Crystal Structure of Human Placental Aromatase

The crystal structure of the highly active human placental AROM in complex with the substrate A was originally determined at 2.90 Å resolution [4]. More recently, the resolution has been extended to 2.75 Å [17]. The tertiary structure of AROM follows the characteristic cytochrome P450 fold. There are 12 major α-helices (labeled A through L) and 10 β-strands (numbered 1 through 10) distributed into 1

major and 3 minor sheets (Fig. 3.2). In addition, the N-terminal transmembrane helix A_{NT}, (residues Thr14 to Trp39) built into the weak electron density [5], is shown. The N-terminal residues 47-50 make one backbone hydrogen bond with β1 and add an extra β-strand-like element to this sheet in AROM. The major β-sheet is a mixed 4-stranded sheet beginning near the N terminus (first two strands are β1:83-88 and β2:93-97) and ending in two strands from the carboxyl (C)-terminal half of the polypeptide chain (β3:373-376 and β6:393-396). Three minor sheets consist of two anti-parallel strands scattered over the polypeptide chain (sheet2: β4:381-383 and β5:386-388; sheet3: β8:473-475 and β9:479-481; sheet4: β7:458-461 and β10:491-494). The lengths, locations and orientations of the 12 major helices, namely I (293-324), F (210-227), G (242-267), H (278-287), C (138-152), D (155-174), E (187-205), J (326-341), K (354-366) and L (440-455) are similar to those found in most of the cytochrome P450s. Minor helices A' (57-68), A (69-80), B (100-109), B' (119-126), G' (232-236), H' (271-274), J' (346-349), K' (398-404), and K" (414-418) are 1 to 4 turns long and have more variability among P450s in terms of their locations, lengths and orientations. A comparison of AROM to human P450s 3A4 and 2D6 (~16–20 % sequence identity) [18, 19] shows that, the helix A' in AROM is longer than that of 3A4 by 2 turns and is absent in 2D6. Furthermore, the long continuous helix F in AROM is separated by a polypeptide stretch into two shorter helices in 3A4. This region of the secondary structure contributes significantly to the architecture of the catalytic cavity. Other notable differences in the secondary structures between AROM and 3A4 or 2D6 are as follows: the long continuous helix F in AROM, adjacent to the active site, is separated by a polypeptide stretch into two shorter helices in 3A4; the G-helix in AROM is at least one turn longer than the equivalents in 3A4 and 2D6; the helix F-loop-helix G region, in general, is different from other P450s in that the position of helix G' in the middle causes the loop to be

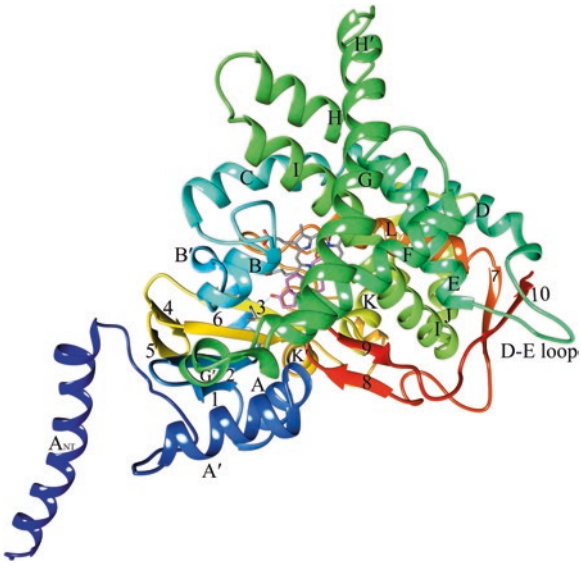

Fig. 3.2 A ribbon diagram showing the secondary and tertiary structures of AROM. The N terminus, beginning with Asn12, is *dark blue* and the C terminus ending at residue 496 is colored *red*. AROM consists of 12 major α-helices (labeled A through L) and 10 β-strands (numbered 1 through 10) distributed into 1 major and 3 minor sheets. The N-terminal transmembrane helix A_{NT} is also shown. The heme group and the bound androstenedione molecule at the active site are shown in *grey* and *magenta* respectively. (Adapted from Ref. [4, 5])

tighter in AROM than in either 3A4 or 2D6, both of which have longer intervening loops. More recently, the X-ray structures of six more human/mammalian microsomal P450s, namely 11A1 [20], 11B2 [21], 17A1 [22], 21A1 [23], 24A1 [24], and 51A1 [25], have been determined. The sequence identities of these P450s with AROM, again, range between 15 and 20 %, and all have subtle but characteristic differences with AROM in the overall tertiary structure organization.

A common feature of all cytochrome P450s is the heme ligation by arginine and tryptophan side chains through ionic and hydrogen bonding interactions to the propionate moieties of the heme. Arg115, Trp141, Arg145, Arg375, and Arg435 are involved in heme coordination. Despite the overall similarities of cytochrome P450 structures, homologs such as 3A4 and 2D6 are drug/xenobiotic-metabolizing enzymes with diverse substrate selectivity, while AROM, like 17A1 and 24A1, performs a unique catalytic role in steroid biosynthesis using a very specific substrate. This dichotomy between AROM and other less specific P450s is a manifestation of the differences in the detailed atomic architecture of the active site that result from these subtle but specific changes in the overall organization of the tertiary folds.

Architecture of the Active Site

The active site of AROM is located at the heme distal cavity, buried deep within the roughly spherical molecule near its geometrical center. Analysis of the refined AROM model revealed the well-defined position of the bound androstenedione (A), and permitted unequivocal modeling of the active site within the human placental aromatase model [4] (Fig. 3.3a). A binds with its β-face oriented towards the heme group and C19 of the methyl group positioned 4.0 Å from the Fe-atom. As expected for a high spin species, the refined Fe-position was displaced slightly from the heme plane towards the ligand Cys437.

The residues and polypeptide segments that comprise the catalytic cleft are Ile305, Ala306, Asp309 and Thr310 from I-helix, Phe221 and Trp224 from F-helix, Ile133 and Phe134 from the B-C loop, Val370, Leu372 and Val373 from the K-β3 loop, Met374 from β3, and Leu477 and Ser478 from the β8-β9 loop. The hydrophobic residues and porphyrin rings of heme surround and pack tightly against the steroid backbone to form a cavity that is complementary in shape to A. As shown in Fig. 3.3a and b, the side chains of residues Arg115, Ile133, Phe134, Phe221, Trp224, Ala306, Thr310, Val370, Val373, Met374 and Leu477 make direct van der Waals contacts with the bound A. Ile133, Phe134, Phe221, Trp224 and Leu477 approach the substrate from the α-face and follow contour and puckering of the steroid backbone, while Arg115, Ala306, and Met374 make contacts at its edge, and Thr310, Val370, and Val373 on the β-face of A. The combined surface creates a pocket of ~400 Å [3] that tightly encloses the bound A. This is considerably smaller than the volumes of the active site space in 3A4 [26], 2D6 [26, 27], 11A1 [20], 11B2 [21], 17A1 [22], 21A1 [23], 24A1 [24], and 51A1 [25], indicative of the specificity with which AROM selects its substrates.

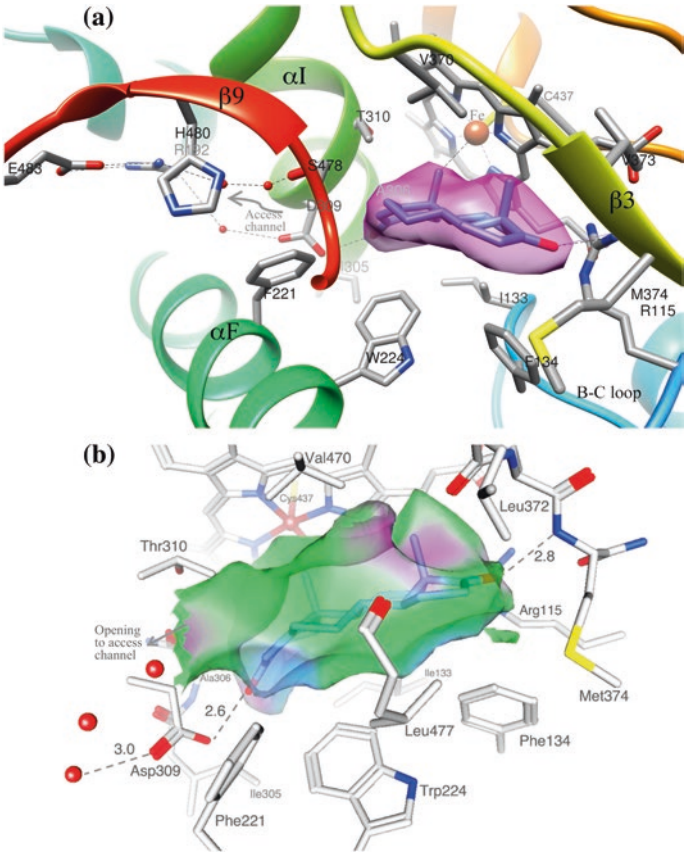

Fig. 3.3 Close-up view of the active site of AROM. **a** An unbiased difference electron density (contoured at 4.5σ) for the bound androstenedione prior to its inclusion in the model refinement, is colored purple. The backbone ribbon is *rainbow-colored*. Protonated Asp309 makes a hydrogen bond with the 3-keto group of androstenedione. Asp309 is also linked to Arg192 via water. Arg192 forms a salt bridge with Glu483. These two residues sit at the mouth of the active site access channel. **b** A van der Waals interaction surface formed by the protein and heme atoms at the active site. The semi-transparent surface, colored green for hydrophobic interactions and *magenta* for polar interactions, closely resembles the shape, size and puckering of the steroid backbone. *Red* spheres represent water molecules. (Adapted from Ref. [4])

The 17-keto oxygen of A is 2.8 Å from the amine backbone of Met374 to accept a proton and make a hydrogen bond. Additionally, NH1 of Arg115 is positioned at 3.4 Å from O2 (of 17-keto) of the bound A (Fig. 3.3a). Although NH1 and NH2 of Arg115 primarily interact with the two heme propionate moieties electrostatically, the existence of a weak hydrogen bond between the 17-keto oxygen of A and Arg115 is a distinct possibility. The 3-keto oxygen O1 at the other end is situated at 2.6 Å from the carboxylate Oδ2 of the Asp309 side chain, suggesting protonation of the carboxylate moiety and formation of a hydrogen bond.

A protonated Asp309 side chain at the crystallization pH 7.4 is probably a direct consequence of the presence of a water molecule, at a distance 3.0 Å from Oδ1, that donates a proton to the carboxylate, as shown in Fig. 3.3a, b. The geometries of these two hydrogen bonds are such that 3-keto O1 and the water oxygen atom lie roughly in the carboxylate plane, thereby ensuring strong hydrogen bonding interactions. The Asp309 side chain, involved in substrate-binding interactions as well as in catalysis requiring protonation and deprotonation of its carboxylate group, is thus coupled to a proton relay network of water molecules and polar side chains that may function as a proton source and a proton sink for the androgen to estrogen conversion reaction. An elongated electron density adjacent to the Ser478 side chain was modeled as two water molecules, hydrogen-bonded to each other and also to the Ser478 side chain OH, which in turn donates its proton to His480Nδ1 (Ser478OH—Nδ1His480: 2.9 Å), further away from the active site (Fig. 3.3a). Moreover, the Ser478 side chain is linked via these two water molecules to Arg192 by a weak hydrogen bond (3.4 Å).

The only significant hole to the binding pocket is where 3 water molecules are located, leading to a channel that opens to the exterior of the protein surface (Fig. 3.3b). The salt bridging Arg192-Glu478 side chain pair as well as those of Asp309 and Ser478 line the channel. It is conceivable that this channel that hosts the proton relay network is also the major transport route to and from the active site for water, oxygen and steroids molecules. This channel appears to be a confluence of what was previously described as channels 2a, 2ac and 2c for other P450's [28]. Although the channel at some points is narrow for steroidal passage, it is likely that flexibility in the tertiary structure, especially in the channel-bordering regions, permits the channel to "breathe" and swell when necessary allowing the passage of steroids.

Structural Perspective on the Mechanism of Action

AROM converts androgens to estrogens in a three-step catalytic process that requires 3 mol of O_2, 3 mol of NADPH, and CPR for transferring electrons from NADPH (Fig. 3.1). The active site of AROM provides validation for the salient features of a consensus model for reaction mechanism [29, 30] while shedding new lights and raising new questions. The residues that are directly involved in catalysis are Ala306, Asp309 and Thr310 (Fig. 3.4a). The location of Thr310 with respect to the heme iron is similar to the corresponding residue in other P450s, and hence is expected to perform a similar role in the first two steps of hydroxylation yielding C19-aldehyde derivative of A through the 19,19-*gem*-diol formation and retention of the *pro-S* hydrogen [31]. However, contrary to the proposed mechanisms where Asp309 was assumed to play critical roles in all three oxygenation steps, the AROM crystal structure shows that, because of its strong interaction with 3-keto of the substrate, the Asp309 side chain is not available for deprotonation of the Thr310 side chain and activation of the peroxo-ferric to the oxy-ferryl

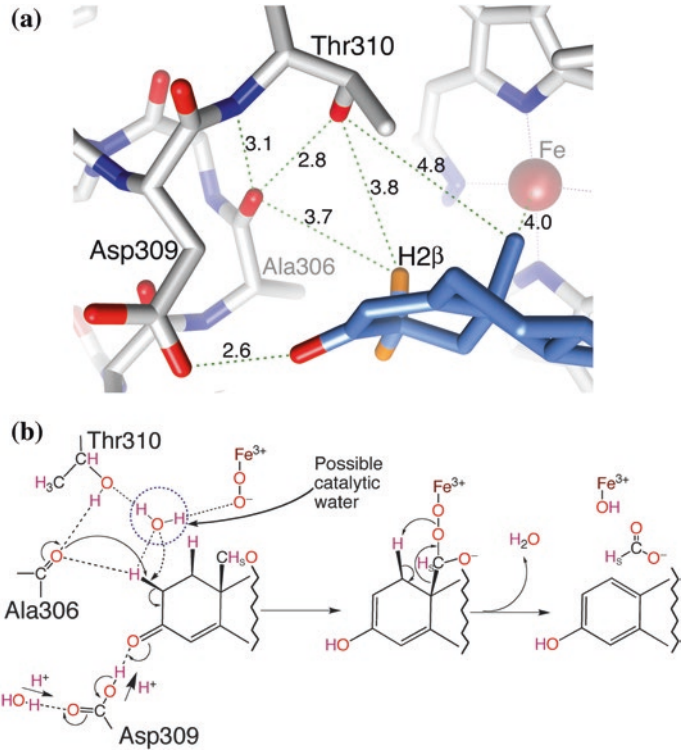

Fig. 3.4 Proposed aromatization mechanism. **a** Ala306CO·HOThr 310 pair that could function in the aromatization of the A-ring of androstenedione. Calculated hydrogen-atom positions of C2 of the bound androstenedione are shown. Distances are in ångströms. **b** A possible mechanism for H2β abstraction and 2, 3-enolization that could be initiated by a nucleophilic attack on C2-H2β by the Ala306CO·HOThr310 pair, along with an electrophilic attack on the C3 carbonyl by a protonated Asp309 side chain. The direction of proton flow from the proton relay network through Asp309 carboxylate to the substrate is indicated by *arrows*. Involvement of a catalytic water in H2β abstraction is a possibility. The backbone carbonyl of the Ala306CO·HOThr310 pair aided by a potential catalytic water molecule, or the water oxygen itself (as indicated by *dotted arrow*) could act as the nucleophile. H1β abstraction is drawn as proposed previously. (Adapted from Ref. [4])

intermediate (Fig. 3.4b). Moreover, Asp309 side chain is unable to rotate in order to approach the Thr310 side chain without causing steric conflicts with the bound substrate. Additionally, the relative juxtaposition of Asp309 and the steroid molecule is such that the 2β-hydrogen of C2 is not accessible to the side chain for extraction, unless the steroid molecule undergoes large rotational motion (> ~ 40°) about the O1-O2 axis, which would cause significant steric clashes with the hydrophobic envelope (Fig. 3.4a, b).

Interestingly, the structure reveals a specific interaction involving Thr310 side chain that not only restricts its mobility but also indicates its crucial role in all three steps of catalysis. As illustrated in Fig. 3.4a, b, -OγH of Thr310 makes a

strong hydrogen bond (2.8 Å) to the backbone C=O of Ala306. Although a similar interaction can be found in 3A4 and 2D6, the hydrogen bonds are either significantly longer or not geometrically favorable. In fact, the Ala306 carbonyl oxygen is involved in two bifurcated hydrogen bonds; the weaker one is the normal CO—HN bond to the Thr310 backbone amide (Fig. 3.4a). This is a direct consequence of distortion in helicity of the I-helix owing to Pro308 [4]. Thus, the deprotonation and protonation of Thr310 OγH for the activation of peroxo-ferric to the oxy-ferryl intermediate, previously postulated to be carried out by Asp309, is likely being conducted through the partial polarization of the Thr310 side chain by this interaction with C=O (Fig. 3.4b). Although the Ser478 side chain is too far away (>8 Å) to have a direct involvement, water molecules attached to it belong to a solvent channel network and could be involved in this proton transfer process (Fig. 3.3). More importantly, the presence of the Ala306C=O—HOγThr310 pair in close proximity to the steroid A-ring is probably what imparts to this P450 the unique ability to catalyze the aromatization reaction step, i.e., the removal of 2β hydrogen, enolization of 3-keto, and the removal of 1β hydrogen of 19-aldehyde (Fig. 3.4b). The crystal structure shows that the C=O oxygen of 306Ala and OγH of Thr310 are at 3.7 and 3.8 Å, respectively, from H2β (Figs. 3.3 and 3.4b). This finding prompts us to propose (Fig. 3.4b) that 306AlaC=O alone or in conjunction with a deprotonated Thr310Oγ^{-} acts as a nucleophile for the abstraction of H2β. This action at C2 in conjunction with a protonated Asp309 side chain acting as an electrophile and interacting strongly with the C3-keto oxygen could promote H2β abstraction and enolization of the 3-keto. The presence of a water molecule could facilitate the deprotonation of the Thr310 side chain by weakening the Ala306CO—HOγThr310 hydrogen bond and freeing C=O for the nucleophilic attack for the H2β abstraction. The 1β hydrogen, on the other hand, is too far from this carbonyl (6.2 Å) to be similarly abstracted. It points at and is closer to the heme Fe (4.2 Å), and is probably removed during the peroxo-ferric nucleophilic attack on 19-aldehyde (Fig. 3.4b) as previously postulated [31]. However, partial electron delocalization in the A-ring, caused by tautomerization, will yield a thermodynamically favorable situation for the removal of H1β. The deprotonated Asp309 side chain is quickly reprotonated by the proton relay network after the product leaves and before the next substrate molecule enters the catalytic cleft. The direct involvement of Asp309 in protonation and aromatization appear to be unique to AROM.

Theoretical calculations supports that peroxo-ferric is the active intermediate since proton transfer (for formation of the oxy-ferryl intermediate) is hindered in AROM compared to other P450s [32, 33]. Electron paramagnetic resonance studies have shown that the primary species formed after cryo-reduction of oxy-ferrous is peroxo-ferric [34]. Using resonance Raman spectroscopy, two separate groups report a shift in the Fe-CO stretching bands by ~5 cm^{-1} upon the binding of 19-aldehyde A [35, 36]. Kitagawa and colleagues concluded that the low frequency shift of the stretching band indicates reduced electron donation and hindered compound I formation; thus suggesting that the active species is peroxo-ferric [35]. On the other hand, Kincaid and colleagues concluded that a shift of

~5 cm^{-1} is an insignificant change; therefore, the same intermediate is involved in the first and third steps [36]. Kinetic solvent isotope effects for the first and third steps were shown to be ≥2.5, indicative of the involvement of proton transfer during these steps [37] and compound I as the active species.

We have also considered the possibility that the bound A molecule is already in the tautomeric form, i.e. enolized at the 3-position. Given the resolution of the X-ray structure and that X-ray photoelectrons could initiate the hydroxylation reaction within the crystal, this cannot be completely ruled out. However, the facts that C-19 fits the electron density best as a methyl group, and that the refined geometrical parameters are consistent with those of an idealized substrate A-ring, suggest that such a possibility is unlikely.

Membrane Integration

The organization of the tertiary structure along with hydrophobicity data for the polypeptide chain provide clues as to how the enzyme could integrate into the endoplasmic reticulum and/or golgi membrane and how lipophilic substrates like A and T could utilize the lipid bilayer integration to gain access to the active site. These factors, along with orientation of the putative active site access channel dictate that AROM sits on the bilayer with residues 43-48, 72-80, and 450-470 penetrating the membrane surface (Fig. 3.5). This model also allows the helix A_{NT} to span the membrane and position a putative glycosylation site at Asn12 in the luminal space, in agreement with a previous proposal [38]. Furthermore, the scenario correctly localizes the bulk of AROM having a cysteine ligand to the heme iron, 7 cysteines in the reduced form and no disulfide, in the reducing environment of cytoplasm. Observed weak electron density beyond residue 45 towards the N terminus is consistent with the likelihood that structure held together by lipid interaction could be rendered dynamically mobile when stripped off the lipid bilayer. The normal mode analysis (NMA) results showing highest fluctuations for the A_{NT} region provide strong support to this hypothesis [5]. Coincidentally, endoplasmic reticulum membrane-spanning 43 residues at the C terminus of human placental and recombinant estrogenic 17β-hydroxysteroid dehydrogenase were similarly found to be disordered in the crystal structure and could not be traced [39].

Based on the structural data, it is likely that the hydrophobic helix A' (residues 57-68) and at least partially helix A (residues 69-80), are embedded into the membrane, thereby positioning several arginine (Arg64, Arg79 and Arg86) and tryptophan (Trp67 and Trp88, as well as Trp239 from the F-G loop) residues at the lipid-protein interface, a telltale sign of lipid integration of proteins [40]. In addition, electron densities for at least 2 detergent molecules were identified in the proximity of helix A', near the Trp67 side chain. Thus, the lipid integration of AROM begins with these helices, as the N terminus traverses further into the bilayer towards the lumen side. When the model is inserted into the membrane (Fig. 3.5), the entrance to the active site access channel rests on the membrane

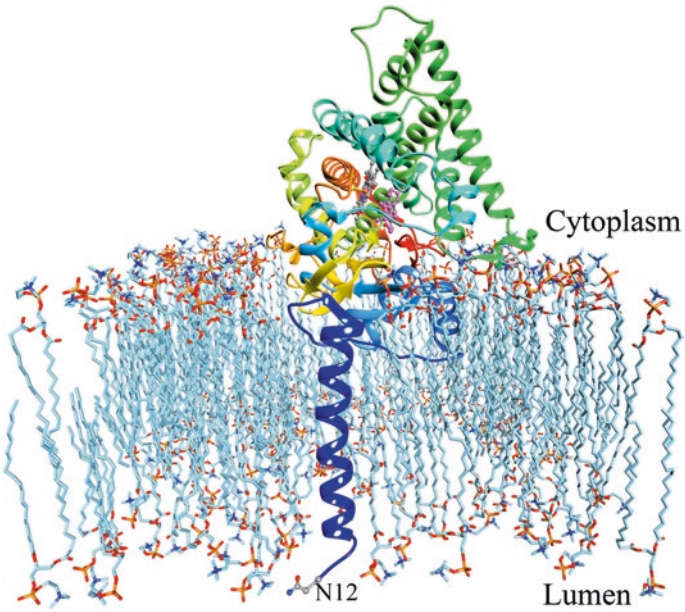

Fig. 3.5 A putative membrane integration model. In the proposed model, the opening to the active-site access channel rests on the lipid bilayer surface, allowing the steroids to enter the aromatase active site directly from within the bilayer. The model suggests lipid integration/association of the N terminus up to helix *A′*, and other loops near the C terminus. *Asn12* is shown as facing the lumen of the endoplasmic reticulum. Atom color scheme: oxygen in *red*; *nitrogen blue; phosphorous: orange*; *carbon magenta* (androstenedione), *gray* (heme and protein side chain) and *light blue* (lipid)

surface. Although other possible substrate/product entry/exit routes cannot be excluded, this arrangement allows the steroidal substrate to enter the AROM active site directly from within the membrane (Fig. 3.5). The channel to the active site should have enough flexibility, perhaps by breaking the salt bridge to open further allowing the steroidal substrate to pass through and possibly the product to exit as well. Furthermore, in this orientation of AROM on the lipid bilayer, the hydrophobic loop of residues 462-471 between β7 and β8 touches the interior of the membrane, while the amphipathic F-G loop including the G' helix sits on the membrane surface. This membrane integration model, in addition, conveniently orients the heme group in an upright plane not far from the membrane interface, thereby allowing a lipid-associating CPR molecule from the cytoplasmic side to bind and occupy the heme proximal site for the transfer of electrons [4]. Other recently published membrane insertion models [41, 42] of AROM have similar features. The structure of AROM, thus, provides a rationale for its crucial membrane integration as evidenced by the necessity for phospholipids reconstitution of the purified enzyme for demonstrable catalytic activities [4].

Interestingly, the N-terminal helices A_{NT} from AROM molecules align themselves about the 3_2 symmetry axis within the crystal in the space that constitutes

the largest void (a region of lowest electron density in the crystal), a channel filled with dynamically disordered detergent and solvent occupying a significant fraction of the crystal volume [16]. This intermingling of the N-terminal region with the disordered lipidic phase provides a rationale for the observed weak electron density of the region.

Oligomerization of Aromatase

In the AROM crystal, the head-to-tail intermolecular interaction between the D-E loop of one monomer and the proximal cavity of the other generates a polymeric AROM (Fig. 3.6) [5]. The proximal site is the cavity behind the cysteine thiolate ligand of the heme moiety, while the distal site comprises the substrate-binding pocket. The D-E loop consisting of residues Val178-Thr179-Asn180-Glu181-Ser182-Gly183-Tyr184-Val185-Asp186 between helices D and E inserts itself into the proximal cavity primarily made of residues 354-361 from the K helix, 418-432 from the K-L loop and 440-444 from the L helix (Fig. 3.6). Two polymer chains

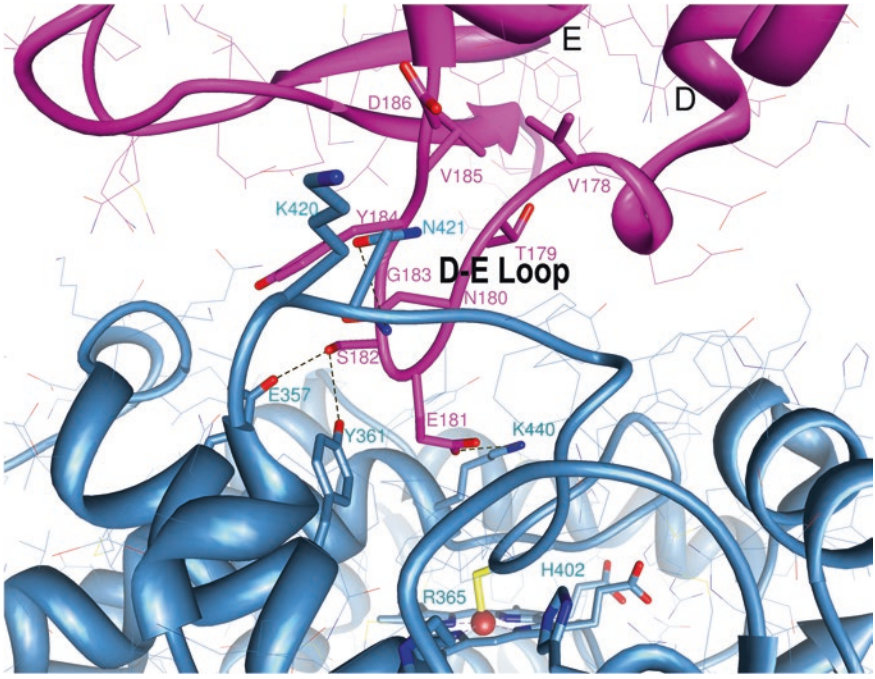

Fig. 3.6 Intermolecular interaction between neighboring AROM molecules. The D-E loop of one aromatase molecule is colored in *magenta* and the heme-proximal region of the neighboring molecule *blue*. Important resides involved in interfacial interactions and/or subjects of mutational analysis are shown. Amino acids have roles implicated in oligomer formation are labeled. (Adapted from Ref. [41])

pack about a 2-fold rotational symmetry axis normal to the screw axis, forming the $P3_221$ space group symmetry. The D-E loop makes several polar contacts, forming five hydrogen bonds and one salt bridge with residues within the cavity. The hydrogen bond forming contacts are backbone O of Tyr 184 to ND2 of Asn421, backbone N of Asn180 to ND2 of ASN 421, OD of Asn180 to backbone N of Val422, OG of Ser182 to OH of Tyr361, and OG of Ser182 to OE2 of Glu357 (Fig. 3.6). In addition, interactions also exist between two charged side chains OE1 of Glu181 and NZ of Lys440, and backbone O of Glu176 and backbone N of Arg425. Together, these provide additional stability to the loop-proximal cavity association. Furthermore, calculation of electrostatic potentials at these two interacting regions shows that electrostatic attraction between the two oppositely charged surfaces, and their shape complementarity could actually be responsible for driving the head-to-tail dimer formation (Fig. 3.7) [5]. The D-E loop is primarily negatively charged (Glu181, Asp186), whereas the heme-proximal site contains predominantly positively charged side chains (Lys108, Lys354, Lys420, Arg425, Lys440, Lys448). The formation of an open proximal cavity is made possible by a long K-L loop and two helices K and L that line the cavity optimally in depth to accommodate the D-E loop. The innermost point of the loop, the OE1 atom of Glu181, is 10.4 Å from the heme iron of the neighboring monomer (Figs. 3.6 and 3.7). Computational and mutational data probing the intermolecular association suggest that there is more involvement of the interfacial surfaces than just the D-E loop to proximal cavity interaction [5, 16]. Both the full length placental

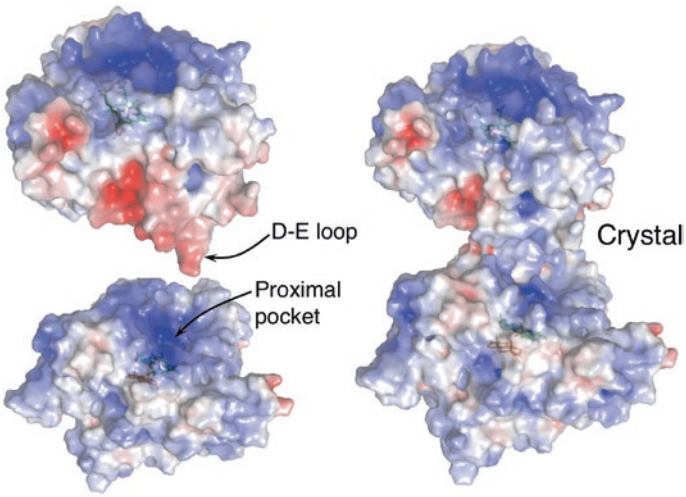

Fig. 3.7 Electrostatic potentials mapped on the van der Waals surface of a head-to-tail AROM dimer. Color scale red to *blue* represents a potential scale from -7kT/e to +7kT/e. The positive potential surface of the heme-proximal region couples to the negative potential surface of the D-E loop region. The head-to-tail oligomeric association is driven by both electrostatics and shape complementarity, not only of the D-E loop to proximal cavity, but also of the entire interaction surface of roughly 1500 $Å^2$. (Adapted from Ref. [5])

and the N-terminal modified recombinant AROM maintain exactly the same intermolecular and packing interactions in the crystalline state [16]. In solution the evidence suggests that AROM retains a concentration-dependent higher order oligomeric form [16]. Interestingly, the interface involved in aromatase oligomerization has also been implicated in the formation of electron transfer complexes of AROM and P450s with CPR [43, 44] and may be targeted by estrogen-dependent phosphorylation, as discussed later.

Roles of Critical Residues

Roles of specific amino acids in the aromatization reaction have been probed by site-directed mutagenesis for more than 30 years [45–47]. These residues were identified mainly by sequence alignment and homology with other P450s and site-directed mutagenesis [48–50]. One example is Thr310, which is fairly well conserved across the P450 enzyme family, and shown to be in the active sites of bacterial P450s and catalytically important [51]. However, the roles of neighboring residues such as Asp309 were much less clear although by homology it appeared to be in or around the active site and demonstrably crucial for AROM function [50]. The X-ray structure provided the molecular basis for interpreting the old mutational data in the new light, as well as for new mutations probing various aspects of the structure-function relationship.

Thus, the crystal structures suggest that D309N mutant is capable of hydrogen bonding to the 3-keto group of A. However, since asparagine has no dissociable proton, it is unable to participate in proton transfer, and hence the D309N mutant would be inactive, in agreement with the mutagenesis data [16]. The proposal that Asp309 is protonated, and participates in proton relay and enolization of the A-ring was first put forth on elucidation of the X-ray structure [4]. The residue R192, distant from the active site at the mouth of the active site access channel, is a participant in the proton relay network and was proposed to play critical roles in catalysis [4]. This “gatekeeper” residue along with Glu483 with which it forms a salt bridge at the lipid-protein interface, could also have some roles in steroidal substrate selectivity and guidance [4]. The R192Q mutant is virtually inactive [16], supporting its proposed role [4]. Mutations of R192 and several other residues in the open reading frame of the human *CYP19A1* gene have been clinically identified, as well as their phenotypical properties. Summarized in Table 3.1, all of these “loss of function” mutations are predictable and easily explained by the crystal structure of AROM [52–61].

Mutational analysis at the intermolecular interface and solution studies suggest that the AROM oligomer observed in the crystals of both the full-length placental and the truncated recombinant enzymes could have functional implications. Proximal side mutations K440Q and Y361F are virtually inactive; whereas, the D-E loop mutants have activity comparable to the wild type enzyme [16].

Table 3.1 Summary of clinically observed mutations in the CYP19A1 gene

Mutations	Structural consequences	Functional implications	Phenotype
M85R [49]	Destabilizes helix A and interferes with membrane association	Loss of activity (predicted)	Ambiguous genitalia at birth
			Puberty absent
			Bone age delayed
			Virilization at pubertal age
M127R/ R375H [50]	M127R- located in hydrophobic area, destabilizes the B-C loop. R375H- loss of heme coordination	Loss of activity (predicted)	Tall stature
			Diffused bone pain
			Indeterminate sexual characteristics
R192H [52]	Loss of proton relay network necessary for aromatization	Greatly reduced activity	Virilization in 46XX
			Undervirilization in 46XY
			No maternal virilization
E210 K [53]	Loss of the hydrogen bond, destabilizes D helix	Fully active	Persistent linear growth
			Diffused bone pain
R365Q [54]	Loss of a charge, may affect CPR coupling	Inactive	Linear growth
			Infertility
			Moderate skeletal pain
V370 M [55]	Loss of van der Waals contacts with the bound androstenedione	Loss of activity (predicted)	Ambiguous genitalia at birth
			Hyperandrogenism
			Delay in bone age
R375C [56]	Loss of heme coordination	Inactive	Ambiguous genitalia at birth
			Puberty absent
			Ovarian cyst
			Virilizing signs at pubertal age
R375H [51]	Loss of heme coordination	Loss of activity (predicted)	Ambiguous genitalia at birth
			Puberty absent
			Ovarian cyst
			Virilizing signs at pubertal age
N411S [57]	Loss of hydrogen bond with R403 on membrane surface	Inactive	Maternal virilization
			Ambiguous genitalia at birth

(continued)

Table 3.1 (continued)

Mutations	Structural consequences	Functional implications	Phenotype
R435C/C437Y [58]	Loss of heme coordination/loss of heme Fe ligation	Inactive	Ambiguous genitalia at birth
			Puberty absent
			Delayed bone age
			Multicystic ovaries
			Virilizing signs at pubertal age
R457X (premature stop codon) [49]	Interference with membrane association and transport	Loss of activity (predicted)	Ambiguous genitalia at birth
			Hyperandrogenism
			Progressive delay in bone age

Motion and Flexibility of the Aromatase Molecule

Motion and flexibility of a protein molecule are directly related to its function. Normal mode analysis (NMA) of the X-ray structure revealed the intrinsic fluctuations of AROM, the internal modes in membrane-free and membrane-integrated monomers, as well as the intermolecular modes in oligomers [5]. The results confirmed that the rigid-core structure of AROM is intrinsic regardless of the changes in steroid binding interactions, and that AROM self-association does not deteriorate the rigidity of the catalytic cleft.

According to the NMA results, the N-terminal helix is the most mobile and flexible structural element identified, in agreement with the X-ray observation. The F-G loop is the next most flexible region of the AROM structure that is not significantly influenced by self-association and membrane integration. The F-G loop flexibility is one of the common features of P450s [28]. Evidence of the flexible loop undergoing an open/close motion, perhaps to allow steroids to enter into or leave from the active site through the access channel [4] was observed [5]. Furthermore, the NMA of a monomer revealed that the access channel could serve as a hinge for intramolecular bending (Fig. 3.8a) and an interface for twisting motions (Fig. 3.8b). These motions, together with the intrinsic flexibility of the access channel, are likely to contribute to channel "breathing", opening and closing of the channel mouth and the cavity, perceived necessary for entry and exit of steroids to and from the active site [4, 5].

The hinge bending and twisting motions at the access channel are also present in the lipid-embedded AROM, but at a higher frequency. The membrane penetrating areas, such as helices $A_{NT,}$ A' and A, have reduced amplitudes, owing perhaps to dampening of the oscillation by the surrounding lipid molecules (Fig. 3.8c, d). However, the twisting motion is similar to the membrane free molecule, which suggests that twisting could be more closely related to a functional AROM in vivo.

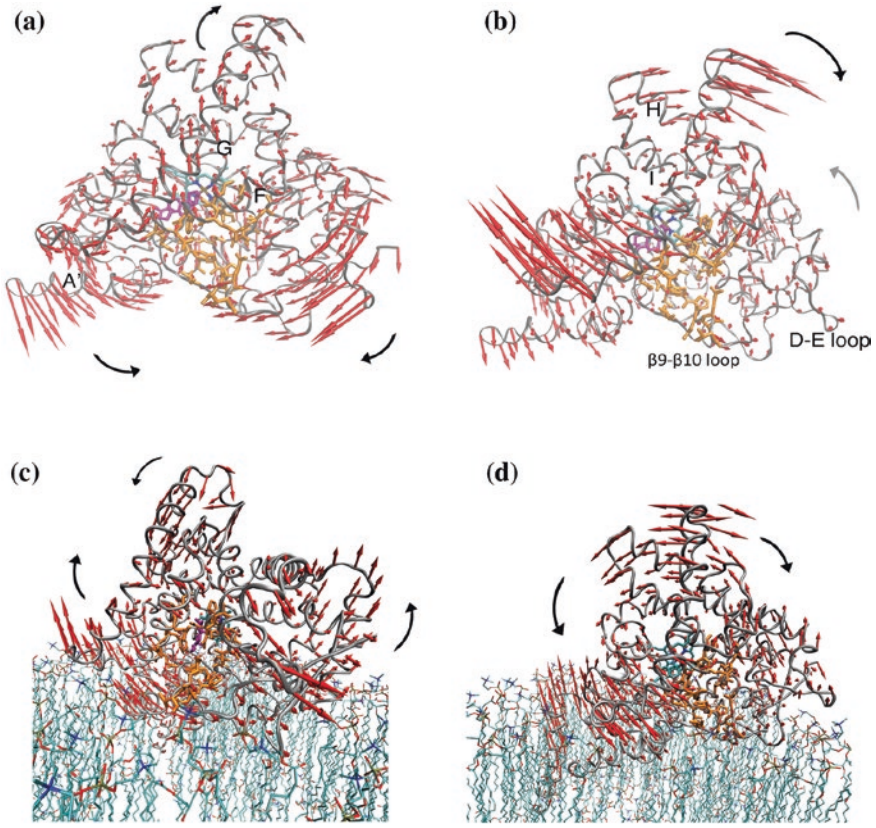

Fig. 3.8 Motion and Flexibility of AROM. **a** Three moving parts of a membrane-free monomer produce the hinge-bending motions with the hinge at the active site access channel. **b** Two moving parts contribute to a twisting motion with the access channel at the interface. **c** and **d** Two internal normal modes show the intramolecular bending and twisting motions for a membrane-integrated monomer. The eigenvector *arrows* represent the relative amplitudes and directions of motion of the associated **c**α atoms. The *black arrows* depict the directions of collective motion. The residues of the access channel are represented by sticks and rendered in *orange* color. The residues include Arg192, Ile217, Gln218, Phe221, Asp222, Ala225, Pro308, Asp309, Thr310, Ser312, Val313, Val369, Ile474, Ser478, Leu479, His480, Pro481, Asn482, Glu483 and Thr484. (Adapted from Ref. [5])

Interestingly, the N-terminal helix motion does not coordinate with either of these two movements; instead, it is associated with the rear half of the molecule, suggesting that membrane integration of the N-terminal helix may have roles different from "breathing" or steroid passage, perhaps in intramembrane stabilization or CPR coupling. One of the slowest modes of the membrane-embedded AROM suggests a periodic movement of the active site region deeper toward the lipid interior. Such a motion could be associated with the enzyme's substrate sequestration and/or product release phases of the catalytic cycle.

The collective intermolecular hinge bending and twisting modes provide the flexibility in the quaternary association necessary for membrane integration of the AROM oligomers. Two slowest modes at the interface of the head-to-tail association are intermolecular rigid-body hinge bending and twisting motions. They provide the flexibility for the AROM molecules to reorganize themselves retaining the interface in order to form an oligomeric structure. However, reorganization and reorientation are necessary to position the transmembrane helices on the same side of each monomer for the oligomer as a whole to penetrate the lipid bilayer. The heme-proximal electropositive site of AROM has been proposed to be critical for electron transfer by the FMN moiety from CPR [62]. The observed flexibility of the intermolecular interaction from this work suggests that the FMN moiety of CPR [39] could bind at the interface, either by flexing the head-to-tail organization for a three-way binding or by competitively replacing an AROM monomer.

The biological relevance of fluctuations of the active site, the access channel, and the heme-proximal cavity, and quaternary organization could be that these are essential components of a dynamically active functional AROM molecule in its role as an ER membrane-embedded steroidogenic enzyme. Others have shown that the dynamics of Trp224, located near the active site, are reduced in the presence of A or a non-steroidal AI (although to a greater extent by A) thus suggesting slower dynamics and altered flexibility in the presence of ligands [63]. Furthermore, direct simulation of protonated and deprotonated Asp309 reveals that the active site titration affects ligand positioning, dynamics of the access channel, and the aromatization process [42]. Computational data suggest influence of the lipid bilayer on the substrate accessibility of the catalytic site [41, 42].

Aromatase Inhibitors: Recent Developments

AROM has long been considered to be an ideal target for selective inhibition of estrogen biosynthesis for the treatment of estrogen-dependent breast cancer in post-menopausal women [2, 3]. The third generation AIs letrozole (LTZ), anastrozole (ANZ) (both non-steroidal), and exemestane (EXM) (steroidal) have been remarkably effective against breast cancer. AIs have also been used for the treatments of endometriosis [64, 65], ovarian [64, 66] and lung [67] cancers. However, these AIs were developed by combinatorial processes in 1980s [6], long before any structural data was available [4, 15]. These AIs have high affinities for AROM, but not necessarily the optimal specificity or selectivity. P450s such as CYP1A2, CYP2C9 and CYP3A4, are inhibited by ANZ, and CYP2A6 and CYP2C19 by LTZ [68–71]. CYP2A6 and CYP3A4 metabolize LTZ [72–74]. Despite high efficacy, some patients may fail to respond to AIs, which is known as AI resistance [72]. Furthermore, EXM is androgenic [75] and also has weak ERα agonistic activity [76]. Structure-guided approach towards the next generation AIs could minimize these non-specific and adverse effects [3]. Availability of the AROM crystal structure has prompted urgency for the incorporation of additional AROM specificity into the inhibitors and revitalized new AI discovery research. The

design that exploits the androgen-specific architecture of the active site and interactions exclusive to the substrate-binding pocket is likely to minimize cross-reactivities of the current AIs. The progress already made in designing novel steroidal AIs by this methodology is a proof of the principle [17].

Crystallographic and computational results have shown that AROM structure has a rigid core, and that the active site is small and compact, unlike many other P450s [4, 5, 15, 17]; therefore, ligands are accommodated by modest adjustments of the catalytic cavity [17]. Hydrophobic interactions via the EXM C6-methylidene group within the hydrophobic crevice surrounded by Thr310-Cγ, Val370-Cγ2 and Ser478-Cβ (Fig. 3.9) could add to its binding affinity. The crevice, acting like a "hydrophobic clamp", holds firmly C6-methylidene of EXM, which has a better overall shape complementarity to AROM than the substrate A [17]. Contrary to the published reports [77, 78], no covalent bond formation between the EXM molecule and catalytic active site residues was found. Indeed, the covalent bond formation would require coupling with CPR and steps through the catalytic cycle, which the crystallized AROM-EXM complex did not undergo. Structural data shows that, in the absence of CPR, EXM is held at the C6-methylidene by the "hydrophobic clamp", reducing the mobility of Thr310 critical for the

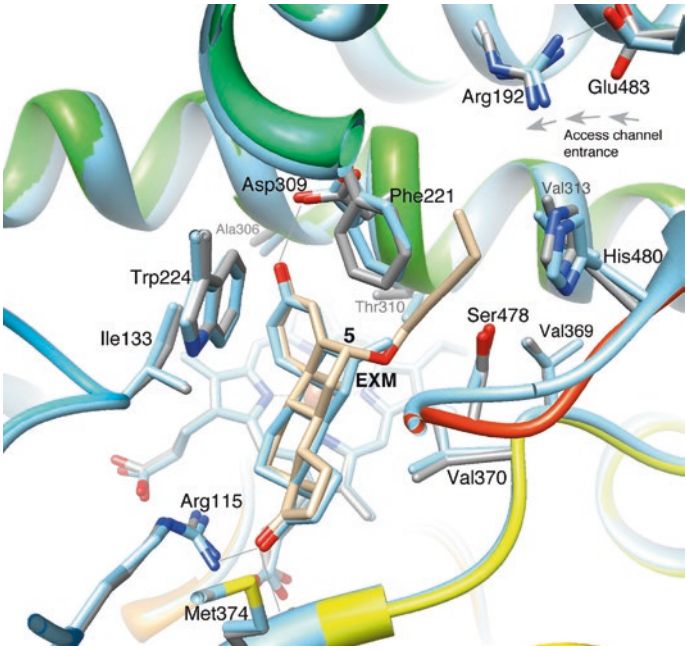

Fig. 3.9 Structure-guided design of novel inhibitors and validation of design by X-ray structure of the enzyme-inhibitor complex. Superposition of the structures of exemestane (EXM) and compound **5** complexes of AROM, illustrating that the orientation of the unsaturated C6-methylidene group in exemestane is different from that of the C6β-alkoxy group. Exemestane complex is shown in *light blue* carbon and backbone (PDB code: 3S7S). Compound **5** (PDB code: 4GL7) is in *beige* color. Side chain carbons are either in *gray*, and the backbone is in *rainbow*. (Adapted from Ref. [15])

hydroxylation reaction (Fig. 3.9) [4, 5]. Thus, a "tighter" binding and "immobilization" of the catalytic machinery could also be the means of inactivation by EXM.

Nine novel steroidal inhibitors designed as derivatives of androsta-1,4-diene-3,17-dione using structure-guided methodology have undergone complete in vitro characterization in a MCF-7 breast cancer cell-based antiproliferation system and cell-free purified enzyme inhibition assays [17]. Of these compounds, three C6β-alkoxy/alkynyloxy derivatives have been identified as most potent AROM inhibitors to date [17]. The IC_{50} and EC_{50} values of the most potent derivatives are comparable to or better than those of EXM. Table 3.2 summarizes the inhibitory and antiproliferative potencies of the three most potent compounds **4**, **5**, and **9**, in comparison to EXM. Compound **5**, the best in the series, has IC_{50} and EC_{50} roughly 4 and 187 folds, respectively, better than EXM (Table 3.2). The X-ray structures of compounds **4** and **5** reveal that the 2-alkynyloxy side chains fit tightly within the hydrophobic environment of the channel [4, 17]. The C6β-alkoxy group is suitably oriented to pierce through the "hydrophobic clamp" to bury the longer side chains within the access channel, thereby immobilizing the catalytic residues as well (Figs. 3.9 and 3.10) [17]. Both the structural and functional data are consistent in that **5** and **9** possess the right dimensions to traverse the access channel. The side group of **5** nearly extends to the polar residues Arg192 and Glu483 at the channel entrance, which is presumed to be at the membrane-protein interface (Figs. 3.9 and 3.10). Compound **6**, a C25 methyl derivative of **5**, has much reduced inhibitory and antiproliferative potencies. It is likely that the terminal methyl group of **6** interferes with the water molecules trapped in the channel by polar residues Ser478, Arg192 and Asp309 [4, 17]. These data suggest that an optimal size/nature of the side group lies between compounds **5/9** and **6**, leaving room for design improvement.

Several other groups have utilized the structure of AROM to design new inhibitors. However, most of the binding modes and/or mechanisms are hypothetical and can only be validated by experimental data. The inhibitors synthesized as derivatives of A [79] and T [80] are reported to have sub μM potencies in placental microsomes. Potencies of the active C7α testosterone derivatives ranged from 0.5 to 0.8 μM [80]. Non-steroidal 2-phenylpyrroloquinolinones have been shown to possess anti-AROM activity [81] with nM potency of AROM inhibition, and low cross-reactivity with P450 11B1 and 17A1. Dual AROM/steroid

Table 3.2 Summary of IC50 and EC50 of most potent C6-beta-alkoxy/2-alkynyloxy series of steroidal aromatase inhibitors and controls

Compounds	IC_{50} (nM)	95 % Confidence interval (nM)	Potency relative to EXM	EC_{50} (nM)	95 % Confidence interval (nM)	Potency relative to EXM
Exemestane (EXM)	50.1	40.9–61.4	–	5.6	2.7–6.5	–
4 (HDDG029)	112.3	78.2–161.3	0.5	1.7	1.2–2.2	3.3
5 (HDDG046)	11.8	9.3–14.9	4.2	0.03	0.02–0.06	187.0
9 (HDDG065)	20.0	18.1–22.0	2.5	0.3	0.2–0.4	18.7

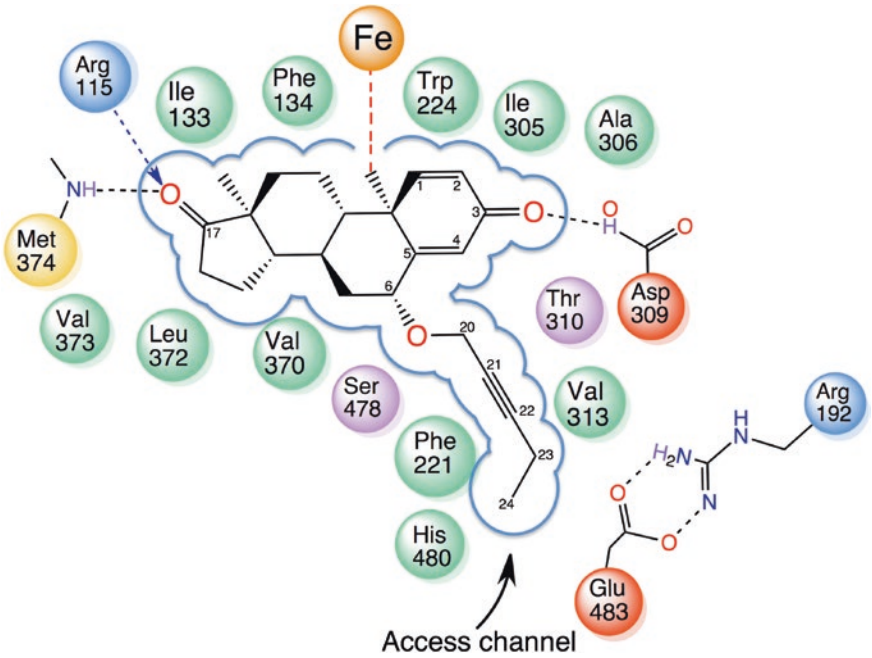

Fig. 3.10 Utilization of novel AROM-specific interactions in inhibitor design. Schematic diagram depicting the X-ray structure of the tight hydrophobic binding pocket for the designed steroidal inhibitor **5**, the proton donors at the 3- and 17-keto positions, and the 6β-alkoxy-substituted alkyne side group that nearly fills the access channel. The residues lining the binding pocket making hydrophobic and hydrogen-bonding contacts are shown (*hydrophobic*, *green*; *acidic*, *red*; *basic*, *blue*; *polar*, *purple*; *sulfur containing*, *yellow*). (Adapted from Ref. [15])

sulfatase inhibitors generated by introducing the AI pharmacophore into a steroid sulfatase inhibitor template were evaluated for antiproliferative effect on JEG cells and were shown to have nM half-maximum effect [82, 83]. However, the reported inhibition/antiproliferation potencies are not comparable in the absence of standardized assay systems.

No structural data is available yet for unequivocal elucidation of the binding modes of non-steroidal AIs LTZ and ANZ. Recent inhibition kinetics data on LTZ and other azole compounds are indicative of mixed inhibition modes [84].

Phosphorylation of Aromatase and Estrogen Signaling: The New Frontier

The heme proximal cavity plays key roles in enzyme function, not only during the transfer of electrons to heme [43], but also being at the intermolecular interface as discussed above in the oligomerization section. X-ray data suggest that the cavity

could serve as a binding scaffold for small molecules and that the cavity is considerably larger than other known P450s (Ghosh et al. unpublished). Could the presence of a small molecule at this site interfere with the transfer of electrons from CPR to AROM? It has been shown that the intermolecular contact at the heme-proximal cavity could be functionally and physiologically meaningful in the lipid bilayer. It is also known that the CPR to P450 coupling and electron transfer is driven by electrostatics via the proximal cavity. Targeting the proximal cavity for AROM inhibition is a new concept and could lead to the discovery of a novel class of inhibitors of estrogen biosynthesis.

Interestingly, phosphorylation of the proximal cavity residue Tyr361, involved in intermolecular interaction as discussed above (Fig. 3.6), has been reported to increase AROM activity in breast cancer cells [85]. Short exposure of estrogen-dependent MCF-7 and ZR75 breast cancer epithelial cells to E2 induces an increase of AROM enzymatic activity. Rapid E2-to-plasma membrane associated estrogen receptor α/β (mER)-induced enhancement of AROM activity in MCF-7 cells does not correlate with increase in AROM mRNA and protein content. Site-directed mutagenesis experiments reveal that phosphorylation of Tyr361 is crucial in the up-regulation of enzymatic activity after E2 treatment. E2 treatment enhances Tyr361 phosphorylation and activity of AROM by activating c-Src kinase or blocking the tyrosine phosphatase PTP1B. In the absence of E2, PTP1B reduces AROM activity by dephosphorylation of Tyr361 [86].

Rapid E2 synthesis by a phosphorylated AROM and non-genomic autocrine E2 loop signaling via the mER has long been proposed to be a mechanism by which estrogen performs neuroprotective and neurotransmitter roles in brain and CNS [87]. This positive feed-back loop involving phosphorylation of AROM and rapid E2 synthesis was first reported by neuroscientists in the brains of songbirds and other mammals [88–90]. AROM activity was found to be elevated in male zebra finches that were singing for 30 min as compared to non-singing males. This elevation occurred only within the cellular compartment that contains synaptic terminals. In the brain of male and female zebra finches, local E2 synthesis at the synaptic terminals was shown to increase dramatically under phosphorylating conditions [91, 92]. Other residues, such as Ser118, have also been proposed as likely sites of phosphorylation that cause reduction of enzyme activity in mammals [89, 93]. The residue Ser118 is located at the N terminus of the B-helix and phosphorylation at this site would result in steric clashes that could destabilize the B-helix as well as helix G′ and the F-G loop (Fig. 3.2), thereby explaining the observed reduction in activity. Activation of the motor pathway for song production was linked to local elevations in synaptic AROM activity within the forebrain of male zebra finches [92]. Regulation of E2 fluctuations occurred within the auditory cortex of song birds is sex-specific during the presentation of natural audio-visual stimuli in males, and the presentation of auditory stimuli only in females [94]. These rapid changes in local E2 levels were proposed to be mediate by a nonclassical, membrane-bound ER signaling. Furthermore, neuroprotective effects of E2 and neuroinflammation-induced glial AROM expression have also been reported [95].

Expression of AROM in other CNS tissues, such as spinal cord, is well-documented [96, 97]. Reports that estrogens act on the mER located in plasma membrane of neurons modulate nociception and antinociception have been published [98]. Involvement of opioid receptors in this process has also been postulated [99, 100]. The equilibrium between monomeric κ-opioid receptor (OR), hypothesized to mediate pro-nociception, and heterodimeric κ-OR/μ-OR, hypothesized to mediate anti-nociception, shifts the net effect of endogenous as well as exogenous dynorphin functionality from pro-nociception to anti-nociception. In this way, rapid E2 synthesis and signaling via spinal cord plasma membrane ER can dynamically regulate pain processing. These novel functions of AROM-estrogen signaling compliment the classical endocrine genomic actions of E2 and suggest that E2 signaling could play other significant roles in diverse physiological and pathological conditions. Interestingly, the AROM-E2-mER signaling system is present in the CNS of male as well as female but while there are considerable data supporting the functional significance of the AROM-E2-mER system in females, its implication in males remains very poorly understood [101].

Concluding Remarks

The exact molecular mechanism of conversion of androgens to estrogens by AROM is very complex and still is under intensive investigation. Recent advancement on structure-function studies amounts to significant progress towards that goal; however, much remain to be achieved in this regard. Capturing the reaction intermediates in situ within the AROM crystals and time-resolved crystallography of the aromatization reaction in the crystalline state are two difficult but worthy goals. Additionally, more comprehensive understanding of the aromatization process requires analysis of the structure of electron-transfer complex of AROM with CPR. This has been proven to be difficult as well for any P450.

Although crystallization of AROM in complex with steroidal inhibitors has been achieved, no experimental structural data on the mechanism of inhibition of the enzyme by non-steroidal AIs LTZ and ANZ are available yet. This constitutes a significant void in our knowledge on the molecular basis of AROM inhibition. Paradoxically, crystallization of these complexes with AROM has been unsuccessful thus far under the conditions similar to the steroidal complexes. It is likely that the AROM active site undergoes structural rearrangement induced by the non-steroidal agents, and/or the inhibition mechanism has mixed modes and is intrinsically different. The dearth of structural data on the complexes of AROM with non-steroidal LTZ/ANZ is an impediment to understanding of resistance mechanisms and to the development of next generation AIs.

Recent reports of phosphorylation of AROM at the proximal cavity residue Y361, elevation of its enzymatic activity and non-genomic estrogen signaling in MCF-7 breast cancer cells are reminiscent of rapid estrogen synthesis and non-genomic signaling by E2 in the brains of vertebrates proposed decades ago. Interestingly, a

similar mechanism of AROM-E2 signaling in CNS has also been put forth for pain perception. These newly discovered roles of E2 and issues still unresolved account for continued challenges to the investigation of this fascinating enzyme and its inhibition in breast cancer clinic.

Acknowledgment This work is supported in part by grant GM086893 from the National Institutes of Health.

No Conflict of Interest We have no potential conflict of interest to disclose.

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Chapter 4
In Vivo Models of AI Resistance

Gauri Sabnis and Angela Brodie

Abstract The goal of endocrine therapy is to deprive the breast tumor of estrogens, which are key to the growth and progression of the tumor. This can be accomplished by blocking the receptor action via antiestrogens or blocking the biosynthesis of estrogen using aromatase inhibitors. Model systems have been devised to study the effectiveness of hormonal therapy on breast tumor growth. These models proved essential in bringing endocrine agents to the forefront of breast cancer therapy. Now, these models are being exploited to develop strategies to overcome resistance to endocrine agents. Cell lines or tumor xenografts that are deprived of estrogen or treated extensively with aromatase inhibitors serve as models of breast cancers of patients that are receiving AIs in the clinical setting.

Abbreviations

Δ4A	Androstenedione
AE	Antiestrogen
AEs	Antiestrogens
AIs	Aromatase inhibitors
Ans	Anastrozole
E_2	Estradiol
ER	Estrogen receptor
Exm	Exemestane
Let	Letrozole
Tam	Tamoxifen

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A. Larionov (ed.), *Resistance to Aromatase Inhibitors in Breast Cancer*,
Resistance to Targeted Anti-Cancer Therapeutics 8,
DOI 10.1007/978-3-319-17972-8_4

Introduction

Effectiveness of estrogen deprivation as a therapy for breast cancer was shown as early as the 19th century, when Sir Beatson indicated that ovariectomy can induce mammary tumor regression [3]. It is now widely accepted that estrogen signaling is of primary importance in the proliferation and progression of breast cancer. Two types of breast cancer treatment have been developed to target this signaling pathway. The antiestrogens, such as tamoxifen, target the estrogen receptor whereas the more recent aromatase inhibitors target the biosynthesis of estrogen by directly interacting with the enzyme aromatase (Fig. 4.1). Both antiestrogens and aromatase inhibitors (AIs) have the advantage that they are effective treatments that are well tolerated compared to cytotoxic chemotherapy. Furthermore, the benefits of hormone therapy are long lasting. Thus, tumors remain responsive for 5 years or even 10 years. Nevertheless, some patients may eventually relapse during treatment. Researchers have investigated the mechanisms involved as the tumor develops resistance to AIs, in order to gain a clearer understanding of how tumors adapt and survive the pressure of suppressive treatment. The ultimate goal of these studies is development of treatment strategies to overcome the resistance.

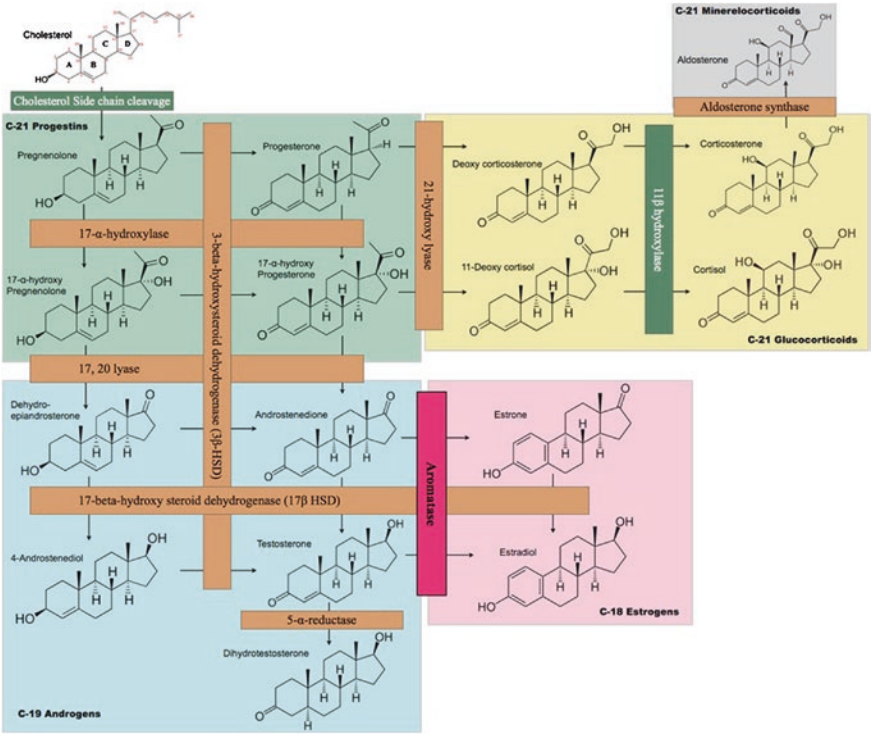

Fig. 4.1 Steroid biosynthesis pathway showing the target of aromatase inhibitors

Results to date indicate that over time tumors survive in a low estrogen environment by adapting to alternate signaling pathways allowing them to escape the antiproliferative effects of AIs.

Carcinogen Induced Syngeneic Tumor Model

The original rodent model of breast adenocarcinoma was introduced by Huggins et al. was a syngeneic rat tumor model [33]. In this model, rats (50–55 days of age) were given oral gavage of 20 mg of a carcinogen 7,12-dimethylbenzanthracene (DMBA) (Fig. 4.2) to induce multiple mammary tumors in about 90 % of the rats in 19 weeks [12, 28]. It was essential that these rats had mature ovaries (>21 days old). Thus, no tumors formed in immature rats or ovariectomized rats. As such, this model could be used to study inhibition of ovarian aromatase activity as well as antitumor effects of the AIs. Alternatively, *N*-nitrosomethylurea (NMU) was also used to induce mammary tumors in rats 50 days or older [31].

Using this model, Brodie lab showed that 4-hydroxy androst-3,17-dione (later known as formestane) was able to reduce the growth of carcinogen induced tumors significantly (Fig. 4.3) [10, 12]. Formestane was then tested in several clinical trials (Fig. 4.4) and showed to cause objective breast cancer regression and reduced serum estrogen levels [10, 22]. This led to the approval of formestane by the National Health Service in the United Kingdom, making formestane the first selective aromatase inhibitor to be used clinically [26].

However, these models represented pre-menopausal breast cancer whereas majority of hormone dependent breast cancers occur in post-menopausal patients. Furthermore, the use of this model is complicated by the feedback mechanisms via the pituitary gonadal axis, whereby gonadotropin secretion increases when ovarian function is suppressed. This can eventually lead to increased estrogen levels and override of the tumor inhibition.

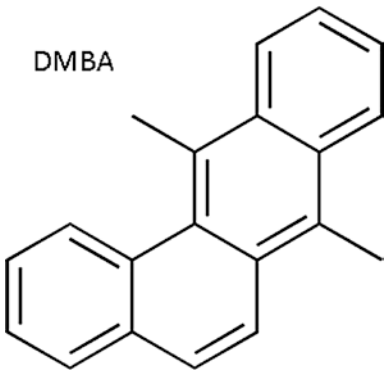

Fig. 4.2 Chemical structure of 7,12-Dimethylbenz(a) anthracene (DMBA)

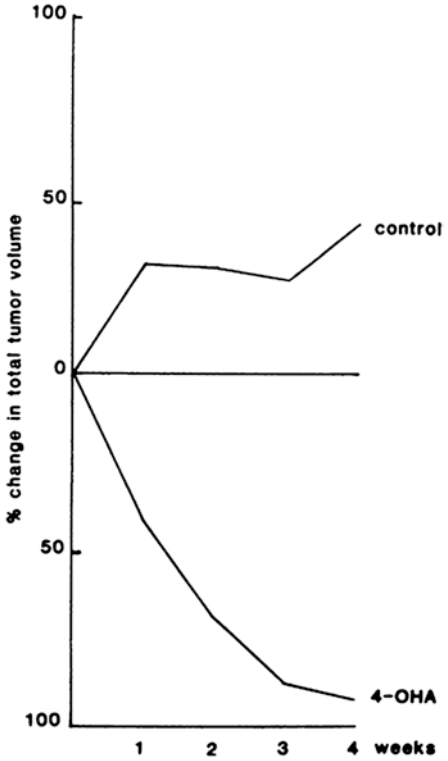

Fig. 4.3 Effect of 4-OHA on DMBA-induced mammary tumors of the rat. Seven rats with 29 tumors were treated with twice-daily sc injections of 4-OHA (50 mg/kg/day). Control animals received injections of the steroid-suspending vehicle at the same time

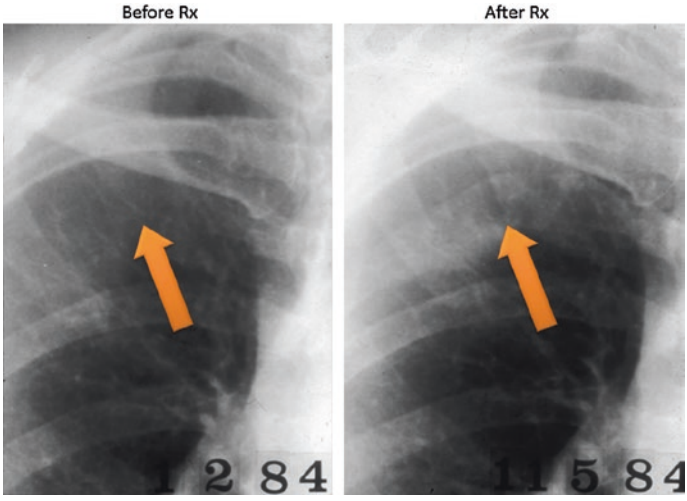

Fig. 4.4 Chest X-ray of patient treated with formestane. Rib metastatic lesion: re-ossification after treatment

Xenograft Model Using the Nude Mouse

Use of syngeneic rodent models was further complicated by the fact that these were not "human tumors". Immunocompromised mice were shown to support the growth of human tumors as early as 1969, when athymic nude mice were used to heterotransplant malignant human tumors [61]. However, breast and prostate cancer tumors were found to be more difficult to transplant. This could be attributed to the fact that the majority of the breast (and prostate) tumors are hormone dependent. Estrogen supplementation increased the tumor transplantation rate of breast cancers. Subsequently, the estrogen receptor positive MCF-7 breast cancer cell line was isolated from the pleural effusion of a patient with invasive ductal carcinoma [14, 75]. This cell line showed the hallmarks of hormone sensitive breast cancer and was also able to form tumors in immune compromised athymic nude mice when supplemented with estrogen [41, 55, 56, 71, 72]. Matrigel, a mixture of basement membrane proteins such as laminin, collagen IV, heparin sulphate, proteoglycan and entactin, further increased tumorigenicity of breast cancer cells such as MCF-7, in the nude mouse [27, 53].

This model has proved excellent in evaluating effectiveness of antiestrogens [38, 50]. However, AIs could not be effectively evaluated, since the mice were given estradiol, which could override the inhibition by AIs.

Intra-tumoral Aromatase Xenograft Model

Aromatase inhibitor screening in vitro was initially performed using purified preparations of human placental microsomes [60], rat [11] or hamster ovaries [32]. About 200 μg of placental microsomes were mixed with 1.25 IU/mL of NADPH generating system (NADP, glucose-6- phosphate and glucose-6-phosphate dehydrogenase) in 1 mL of 0.1 M phosphate buffer (pH 7.4). Samples are mixed with 0.5 nM androstenedione with 0.3 μCi of 1β-^{3}H androstenedione and incubated at 37 °C for 30 min. This assay depends on the findings by Brodie et al. that 1β hydrogen from the androgen substrate is lost during aromatization of the A ring of the steroid [13]. In this reaction, 1 molecule of androstenedione forms 1 molecule of estrone, while releasing 1 molecule of water. Using androstenedione labeled with ^{3}H at the 1-β position, ^{3}H from 1β position is lost and forms $^{3}H_2O$. Radioactive $^{3}H_2O$ is measured by scintillation counting after removing unconverted substrate with chloroform and charcoal extraction. Microsomal preparations all have a disadvantage of heterogeneity between tissues and in differences between tissues and species. A method for measuring aromatase activity in tumor cells for in vitro and in vivo studies was therefore developed. Although, aromatase is expressed in ovarian and choriocarcinoma cells, not all breast cancer cells

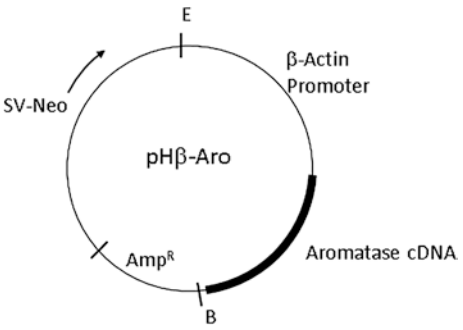

Fig. 4.5 Schematic of aromatase (pHβ-Aro) plasmid transfected in the MCF-7Ca cells

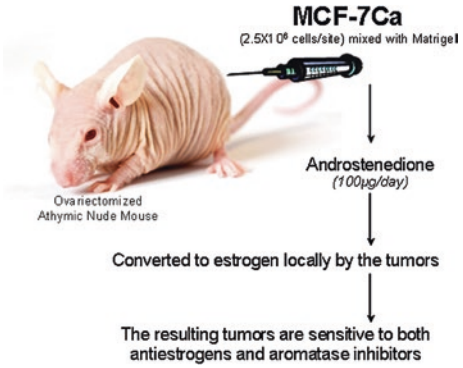

Fig. 4.6 Schematic representation of intra-tumoral aromatase MCF-7Ca xenograft model

express aromatase. MCF-7 breast cancer cells have low aromatase activity [16, 39]. Zhou et al. stably transfected MCF-7 breast cancer cells with human placental aromatase gene (MCF-7Ca or MCF-7_{Aro}) under the control of a constitutively active β-actin promoter (Fig. 4.5) [79]. This cell line was useful for studies of aromatase inhibitors. Brodie lab employed the cell line to develop a model for in vivo studies of aromatase inhibitors [77, 78]. The model simulates a post-menopausal breast cancer patient as it utilizes an ovariectomized mouse. In post-menopausal breast cancer patients, the source of estrogen is aromatase in the peripheral tissues and is not regulated by the gonadotropins. In the xenografts, the source of estrogen is aromatase expressed by the tumors, which is not regulated by gonadotropins. The MCF-7Ca based intra-tumoral aromatase xenograft model (Fig. 4.6) has been successfully used in studying aromatase inhibitors for the treatment of breast cancer [76]. The MCF-7 human breast cancer cells provide tumorigenicity in athymic nude mice and the presence of aromatase in the cells provides an endogenous non-ovarian source of estrogen that can support the tumor growth. However, unlike humans, athymic mice have a low adrenal androgen production [57]. The mice are therefore supplemented with androstenedione to enhance estrogen production by the tumor cells. As such, tumors develop in response to locally produced estrogen converted from androstenedione by the aromatase in the MCF-7Ca cells (Fig. 4.7). The cells, therefore, respond to both antiestrogens and aromatase

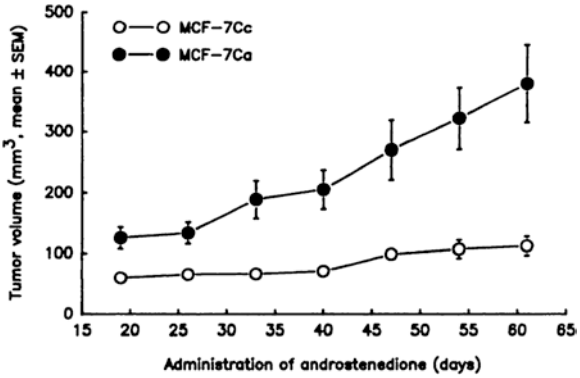

Fig. 4.7 Effect of androstenedione supplement on the growth of MCF-7Ca and MCF 7cc tumors in OVX mice. Four mice were given inoculations of MCF-7Ca cells and 5 mice given empty vector transfected MCF-7cc cells (2.3 × 106 cells/site, 2 sites/mouse). Administration of androstenedione (100 μg/mouse/day sc) began on the second day after inoculation and continued for 60 days. Tumor volumes were measured every week from Day 20 after inoculation

inhibitors. Tumor growth rate is determined by measuring the tumors using calipers and the volume is calculated using the formula $4/3\ \pi\ r_1^2 r_2$ (where $r_1 < r_2$).

Aromatase Inhibitors as First Line Agents

The initial studies with first-generation aromatase inhibitors such as formestane involved breast cancer patients who had relapsed after prior endocrine therapies such as ovariectomy, high-dose estrogens, and tamoxifen. In order to compare efficacy of the treatments in the xenograft model, mice that received tamoxifen first, were later switched to letrozole. Although efficacious, this strategy proved inferior to treatment with letrozole as first-line treatment (Figs. 4.8, 4.9) [35, 42, 43]. Furthermore, tamoxifen and the steroidal antiestrogen fulvestrant (Faslodex) administered alone were ineffective as second-line therapy after letrozole treatment (Fig. 4.10). These results are now confirmed by several large clinical trials, establishing AIs as superior to tamoxifen and as standard first line therapy for postmenopausal hormone sensitive breast cancer [1, 17, 20, 21, 25, 58]. Furthermore, this model showed that the combination of letrozole (or anastrozole) with the pure antiestrogen fulvestrant (Fig. 4.11) was better as first line treatment [34, 44]. This finding was also later confirmed in the clinical setting [51]. The combination of tamoxifen and AIs was also tested in this model but combination of letrozole and tamoxifen was not better at controlling tumor growth than letrozole alone [35]. These results have also been confirmed in the ATAC clinical trail [1, 25]. On the other hand, mice switched to receive letrozole after their tumors had progressed and doubled in volume on tamoxifen treatment, showed tumor regression [42]. Similar findings were also obtained in the MA.17 clinical trial [29, 30].

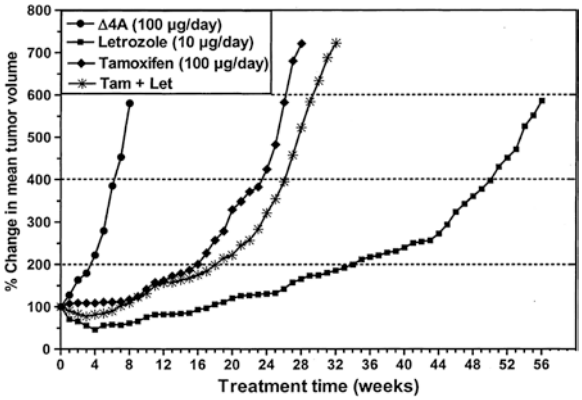

Fig. 4.8 Effects of letrozole (Let; 10 μg/day) and tamoxifen (Tam; 100 μg/day) and their combined or alternating treatment on the growth of MCF-7Ca breast tumor xenografts in female ovariectomized athymic nude mice. Mice were inoculated with MCF-7Ca human breast cancer cells at two sites per flank and were supplemented daily with androstenedione (Δ4A; 100 μg/day) until mean tumor volumes were approximately 300 mm^3. Mice were then divided into groups ($n = 20$ per group) and injected subcutaneously daily with letrozole (10 μg/day) and/or tamoxifen (100 μg/day) in addition to the Δ4A supplement. Tumor volumes were measured weekly and are expressed as the percent change relative to the initial tumor volume. Treatment with letrozole was statistically significantly better than the other treatments at 16 weeks. Tumor volumes were statistically significantly larger in the tamoxifen treatment group than in the letrozole treatment group at 28 weeks

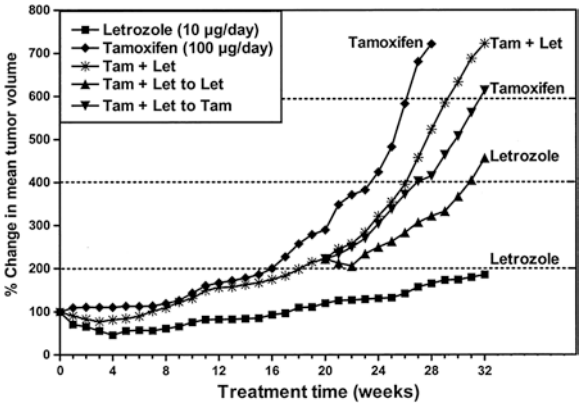

Fig. 4.9 The effect of second-line treatments with letrozole (Let) or tamoxifen (Tam) on the growth of MCF-7Ca breast cancer xenograft tumors progressing on treatment with the combination of tamoxifen plus letrozole. Tumors in the mice treated with the combination of tamoxifen (100 μg/day) plus letrozole (10 μg/day) ($n = 9$) doubled in volume after 18 weeks of treatment. After 20 weeks of treatment, the mice were divided into separate groups for continued treatment with the same combination ($n = 3$) or for second-line treatment with letrozole alone (10 μg/day) ($n = 3$) or tamoxifen alone (100 μg/day) ($n = 3$). Second-line treatment lasted for 12 weeks, and tumor volumes were measured weekly for a total of 32 weeks. Tumor volumes are expressed as the percent change relative to the initial tumor volume. Tumor weight in the tamoxifen plus letrozole group was higher than that in the letrozole-alone group. At week 32, tumor volumes in the group treated with letrozole alone were lower than those in all other treatment groups

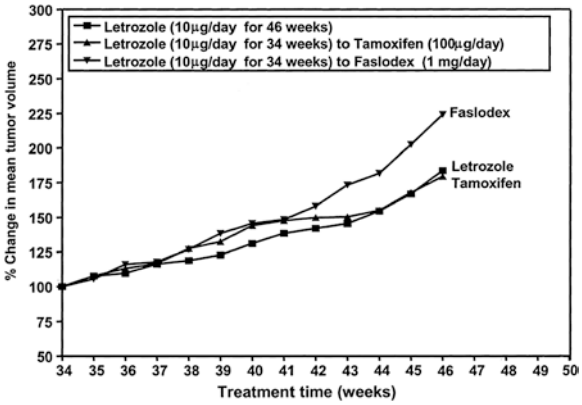

Fig. 4.10 The effect of second-line treatments with tamoxifen or faslodex on the growth of MCF-7Ca tumors progressing on treatment with letrozole. When tumors in the mice treated with letrozole (10 μg/day) doubled in volume (i.e., at 34 weeks), they were separated into smaller groups for continued treatment with letrozole (n = 3) or for second-line treatment with tamoxifen (100 μg/day; n = 5) or fulvestrant (1 mg/day; n = 3). Second-line treatment lasted 12 weeks, and tumor volumes were measured weekly for a total of 46 weeks. The mice in the tamoxifen-alone group and the fulvestrant group were killed at 46 weeks, and those in the letrozole-alone group were killed at 56 weeks. There was no statistically significant difference in tumor volume between any of the groups

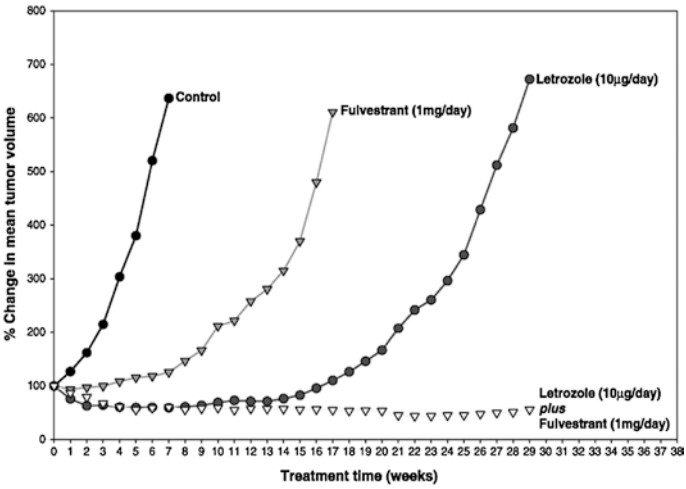

Fig. 4.11 The effect of letrozole (10 μg/d) and fulvestrant (1 mg/d) alone or in the combination on the growth of MCF-7Ca breast tumor xenografts in female ovariectomized athymic nude mice. When tumors reached ~300 mm^3, animals were divided into four groups and injected sc daily with vehicle (control; n = 6), fulvestrant (1 mg/d; n = 7), letrozole (10 μg/d; n = 18), or letrozole (10 μg/d) plus fulvestrant (1 mg/d; n = 5). Tumor volumes were measured weekly and expressed as the percent change in mean tumor volume relative to the initial size at week 0

AI Resistance Models

Despite long lasting growth inhibition seen in the MCF-7Ca xenograft model, the tumors eventually started growing again and had doubled in volume in approximately 16 weeks. This situation is also similar to patients who after years of responding to AIs may relapse and tumors become resistant. In order to understand the mechanism of resistance and to devise secondary treatment options several AI resistance models have been developed.

Estrogen Deprivation Based Models

LTED Model

In order to understand the effect of low levels of estrogen on breast cancer cells, a cell line was developed in vitro, named LTED (Long-term Estrogen Deprived) from MCF-7 cells [47]. To derive LTED cells, MCF-7 cells were cultured in the presence of phenol red free growth medium treated with activated charcoal (to remove steroids and growth factors) for 6 months to 2 years [47]. The LTED cells showed hypersensitivity to E_2 as three-log lower concentration of E_2 was able to stimulate the proliferation of cells compared to the parental MCF-7 cells whereas higher dose of E2 would kill the cells [69, 70]. The LTED cells also showed activation of growth factor pathways such as MAPK and Akt and dual inhibition of MAPK and Akt pathway shifted the E_2 sensitivity towards the same level as the MCF-7 cells [69]. These studies also suggested a role of mTOR, which is downstream of both MAPK and Akt, in resistance to AIs.

The LTED cells were then grown as xenografts in athymic nude mice [73]. These LTED xenografts grow to a greater extent than wild-type MCF-7 xenografts in response to very low concentrations of E_2. In contrast, higher E_2 concentrations cause greater growth of wild-type MCF-7 xenografts than LTED tumors [73]. However, these results were obtained in LTED cells that were still adapting to E_2 deprivation (LTED-H) [18, 46]. When the cells were cultured for over 80 weeks in steroid free conditions (LTED-I), they grew independent of E_2 but removal of insulin from the culture medium again made them hypersensitive. This suggested that the cells were growing more dependent on insulin mediated signaling with continued E_2 deprivation. The LTED-I cells however, still expressed ERα and activated ERα mediated transcription in response to E_2. The cells also upregulated total and phosphorylated forms of Her-2 and IGF-1R. Fluorescent in situ hybridization (FISH) test showed that Her-2 overexpression was not due to gene amplification (Chan et al. [18]. These data suggested that crosstalk between the ERα and growth factor receptor pathways is a critical mediator of resistance to estrogen deprivation.

UMB-1Ca Model

Long-term estrogen deprivation was also used as a model for AI resistance in MCF-7Ca cells. Early passage MCF-7Ca cells were transferred to steroid-depleted medium and examined twice weekly. For the first 2 weeks, cell growth was slower than that of parental MCF-7Ca cells. Cell growth then slowed considerably for 4 weeks before the cells entered a period of proliferative quiescence. This lasted for ~4 weeks, after which time the cells began to proliferate slowly. Six months after the cells were transferred to steroid-depleted medium, normal growth had resumed and the resulting cell line, designated UMB-1Ca, proliferated at a rate comparable with the parental MCF-7Ca [43]. Upon examination of protein expression profile, UMB-1Ca cells showed significant upregulation of ERα protein and no sensitivity to E_2 [43, 67]. The cells also exhibited resistance to androstenedione; letrozole and tamoxifen in vivo (Fig. 4.12) [43, 64]. However, unlike the LTED cells, UMB-1Ca cells did not exhibit upregulation of p- MAPK but PI3K-Akt pathway was pivotal in regulating resistance [64, 67]. The in vivo model of UMB-1Ca also showed that the cells retained their sensitivity to fulvestrant and the combination with an inhibitor of PI3 K-Akt pathway was able to reverse the resistance to tamoxifen [64]. A similar cell line model ($LTED_{aro}$) was also developed by Chen group in vitro, which similarly showed that ERα was still critical in AI resistant cell lines [48, 49].

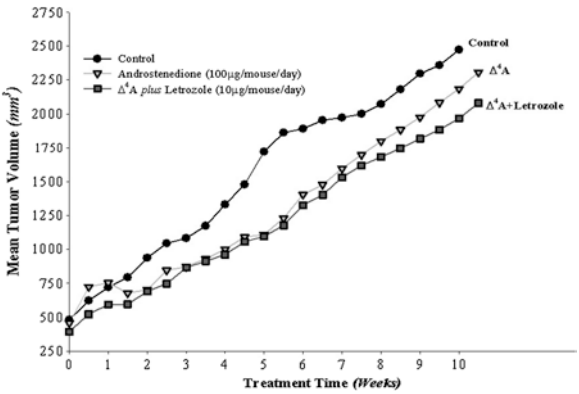

Fig. 4.12 Effects of Δ4A and Δ4A plus letrozole on the growth of UMB-1Ca xenografts. Each mouse received sc injections at one site on each flank with 100 µL of suspension of UMB-1Ca cells (2.5 × 107/mL). Mice were divided into three groups (n = 10) and injected sc daily with vehicle (n = 7), Δ4A 100 µg/day (n = 10), or Δ4A plus letrozole 10 µg/day (n = 10) from the day of inoculation. Measurements began when the tumors reached a measurable size (~300 mm^3) and tumor volumes were measured twice weekly

Letrozole Resistance Model

Although, LTED and UMB-1Ca models mimicked the estrogen deprivation that ensues after treatment with an AI, it did not mimic the actual pressure of the drug on the tumor. Genome wide analysis done by Chen and colleagues also showed that resistance to AIs was different from resistance to tamoxifen or long-term estrogen deprivation [48, 49]. To simulate actual drug induced resistance, the LTLT-Ca model was introduced by the Brodie group [36, 42, 43]. MCF-7Ca cells were inoculated into ovariectomized mice and grown as tumors under the influence of estrogen produced by aromatization of androstenedione. The mice were then treated with AI letrozole for an extended period of time (56 weeks) until tumor growth was no longer inhibited by the treatment and tumors were actively growing (Fig. 4.13). Despite, long lasting control over tumor growth by AIs such as letrozole, the tumors eventually began to proliferate in the presence of letrozole. To elucidate the mechanisms of this loss of sensitivity, two approaches were adopted. In the first approach, tumors were collected at various time points during continuous letrozole treatment. The tumors were collected on week 4 (when tumors were regressing), week 28 (when tumors had resumed growth) and week 56 (when tumors were rapidly growing in the presence of letrozole). These tumors were tested for expression of proteins in various growth factor receptor pathways and compared with parental MCF-7Ca tumors (control) (Fig. 4.14). Tumors actively growing in the presence of letrozole had significantly higher expression

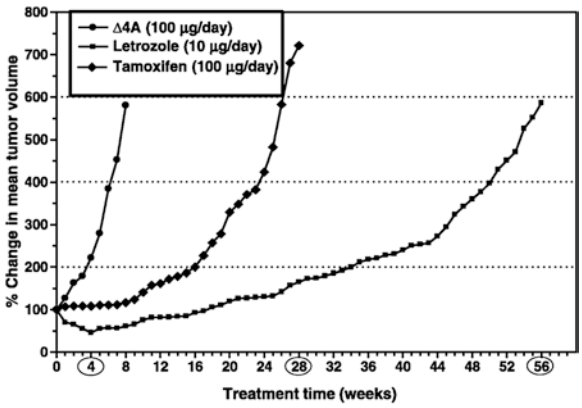

Fig. 4.13 Effect of letrozole and tamoxifen as a first-line treatment on the growth of MCF-7Ca xenografts. Animals were inoculated with MCF-7Ca cells at two sites on each flank and were supplemented with androstenedione (100 µg/d) for the duration of experiment. When the tumors reached a measurable size (~300 mm^3), animals were assigned to three groups (n = 20 per group) and injected sc daily with vehicle (control), or tamoxifen (100 µg/d), or letrozole (10 µg/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

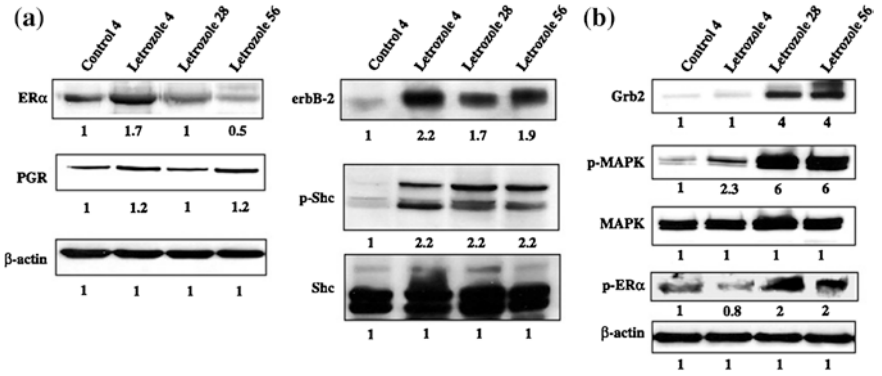

Fig. 4.14 Effect of letrozole treatment on ERα, p-ERα, PGR, Grb2, p-MAPK, Her-2, and p-Shc expression in MCF-7Ca tumor xenografts. Letrozole-treated tumors were collected at 4 weeks (when they were responding to letrozole), 28 and 56 weeks (when they were growing on letrozole), analyzed by Western immunoblotting, and were compared with vehicle-treated tumors collected at week 4 (control). Tumors were homogenized in lysis buffer, and equal amounts of protein (60 μg) were separated on a denaturating polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking nonspecific binding with 5 % nonfat milk in PBS-T, the membranes were incubated with respective primary antibodies, and specific binding was visualized by using species-specific immunoglobulin G followed by ECL detection (ECL kit) and exposure to ECL X-ray film. After exposure to X-ray film, the membranes were stripped and probed for β-actin to confirm that equal amount of proteins were loaded in each lane. Numbers below the blots represent fold change in protein expression compared with the control obtained by densitometric analysis

of growth factor receptor such as Her-2 and pathway proteins such as Grb-2 and phosphor-MAPK compared to control tumors. Furthermore, expression of ERα was downregulated, as the tumors got resistant (responding tumors had overexpression of ERα). Despite low levels of ERα, the expression of p-ERα was higher in tumors growing rapidly on letrozole. In addition, the expression of progesterone receptor (PgR) was unchanged. This suggested ligand independent activation of ERα in the tumors. Tumors of mice treated with letrozole had lower tumor and serum E2 concentration suggesting letrozole was still effective in inhibiting estrogen synthesis [43]. Next, cells were isolated from the resistant tumors and designated **L**ong **T**erm **L**etrozole **T**reated (LTLT-Ca) cells (Fig. 4.15). These cells were further maintained in medium containing 1 μM of letrozole.

This cell line was found to be useful for studying the mechanisms of letrozole resistance and potential methods for reversing the resistance. Similar to the tumors, LTLT-Ca cells showed increased expression of Her-2 and phosphor-MAPK as well as reduction in ERα protein (Fig. 4.16). Aromatase expression was also reduced in LTLT-Ca cells [36, 65]. These cells were resistant to letrozole in vitro. They exhibit a cross-resistance to other AEs such as tamoxifen, fulvestrant and AIs such as anastrozole and exemestane. The LTLT-Ca cells formed tumors in immunosuppressed, ovariectomized mice without estrogen stimulation and were unresponsive to AI treatment (Fig. 4.17).

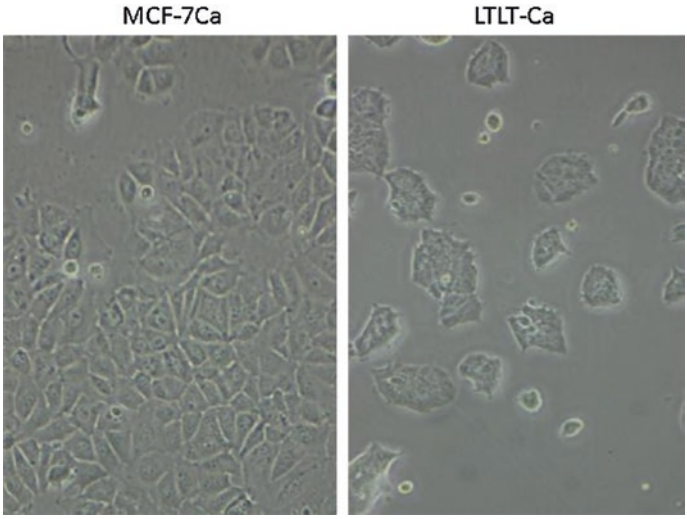

Fig. 4.15 Photographic image of MCF-7Ca and LTLT-Ca cells under 20X magnification using phase contrast microscope

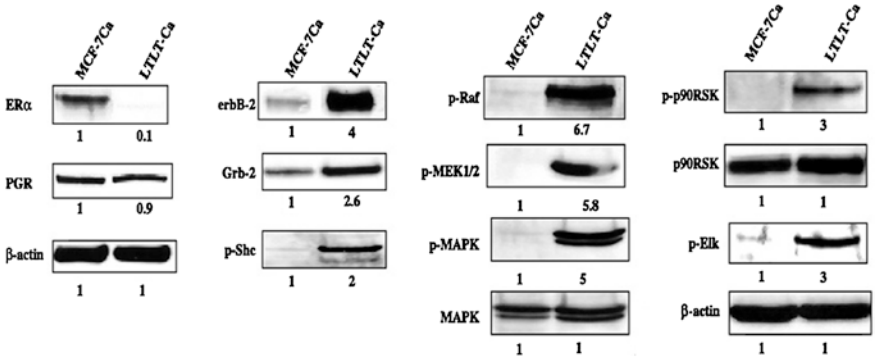

Fig. 4.16 Expression of steroidal receptors ER and PGR, growth factor receptor Her-2, and adapter proteins Grb2 and p-Shc and signaling proteins p-Raf, p-MEK1/2, p-MAPK, p-p90RSK, and p-Elk in MCF-7Ca and LTLT-Ca cells. Equal amounts of protein (60 μg) from whole cell lysates were separated on a denaturating polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking nonspecific binding with 5 % nonfat milk in PBS-T, the membranes were incubated with the respective primary antibodies, and specific binding was visualized by using species-specific immunoglobulin G followed by ECL detection (ECL kit) and exposure to ECL X-ray film. After exposure to X-ray film, the membranes were stripped and probed for β-actin to confirm that equal amount of proteins were loaded in each lane. Numbers below the blots represent fold change in protein expression compared with the control obtained by densitometric analysis

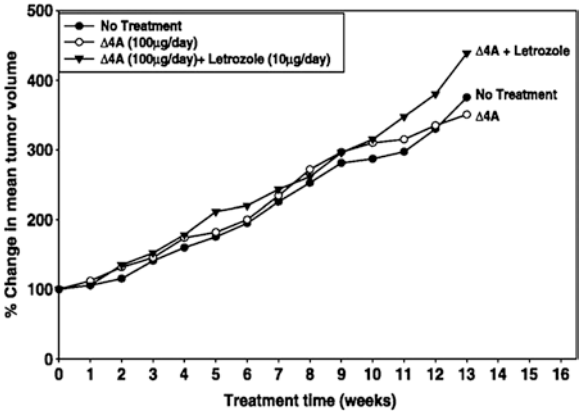

Fig. 4.17 Effect of Δ4A and letrozole on the growth of LTLT-Ca xenografts. Each mouse received sc injections at one site on each flank with 0.1 mL of suspension of LTLT-Ca cells (2×107 cells/mL). Mice were divided into three groups ($n = 5$ per group) and injected sc daily for 13 weeks with vehicle, androstenedione (Δ4A, 100 μg/d), or Δ4A plus letrozole (10 μg/d) from the day of inoculation. Tumor volumes were measured weekly and were expressed as the percent change relative to the initial tumor volume

Transplanted Tumors

Using the intra-tumoral xenograft model, the effect of second line endocrine therapy was examined. Tumors were treated with letrozole until they were resistant. One of the biggest tumors was then minced into small pieces and dissociated into a single cell suspension that was inoculated into new female ovariectomized athymic nude mice. The transplanted tumors grew equally well in the presence and absence of Δ4A (Fig. 4.18), indicating that estrogen supplementation was not required for tumor growth [43]. Although, the tumor growth slowed in response to letrozole, tumors did not regress and they were insensitive to other AIs. The tumors were however, still sensitive to fulvestrant, suggesting the importance of ERα is maintained in resistance.

Activation of Growth Factor Pathways

To study the mechanisms of resistance, the LTLT-Ca cell line was subjected to western blotting, RT-PCR and ChIP assays. The cells showed increased activation of growth factor receptor pathways mediated by Her-2 and MAPK, whereas ERα levels were markedly downregulated (Fig. 4.16) [36, 65]. When these pathways were inhibited (trastuzumab to inhibit Her-2), sensitivity to AIs was restored [65]. Similar results were also obtained in anastrozole resistant cells; inhibition of MAPK activation resulted in reversal of resistance (Fig. 4.19) [68]. These results were then corroborated by clinical trials showing the beneficial effect of combining lapatinib (small molecule inhibitor of Her-2) with letrozole [37].

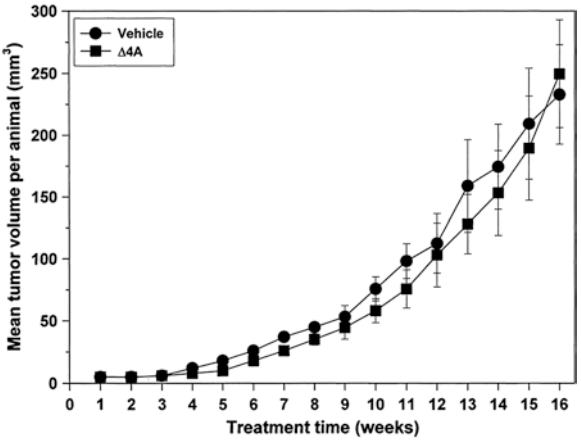

Fig. 4.18 The effect of vehicle and Δ4A (100 μg/day) on the growth of transplanted letrozole-treated tumors in female OVX athymic mice. One of the long-term letrozole-treated tumors was mixed to a single cell suspension, suspended in Matrigel, and inoculated into animals at one site per flank. From the day of inoculation, animals (n = 5) were treated daily with vehicle or Δ4A. Tumor volumes were measured weekly and are expressed as mean tumor volume per animal ±SEM

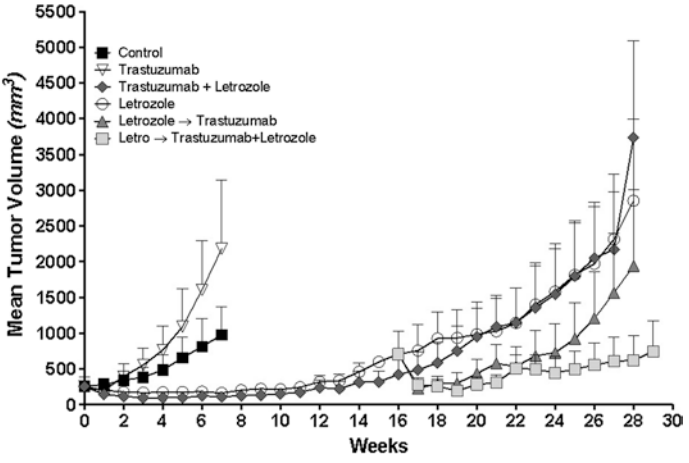

Fig. 4.19 Effect of trastuzumab alone or in combination with letrozole on the growth of MCF-7Ca xenografts. MCF-7Ca xenografts were grown in female ovariectomized athymic nude mice. When tumors reached measurable size ~300 mm^3, mice were assigned to four groups: (a) control (n = 5); (b) trastuzumab, 5 mg/kg/wk, divided in two doses (n = 5); (c) letrozole, 10 μg/d, plus trastuzumab, 5 mg/kg/wk, divided in two doses (n = 5); and (d) letrozole, 10 Ag/d (n = 30). The tumors were measured weekly. Trastuzumab alone did not inhibit the growth of MCF-7Ca tumors ($p = 0.86$). Combination of letrozole plus trastuzumab was effective in reducing the tumor growth rate. However, the combination was no more effective than letrozole as single agent. Letrozole alone inhibited the growth of these tumors for a prolonged period (13 weeks). Nevertheless, tumors ultimately began to grow on continued treatment and had doubled in volume by week 15. At this time, the mice were subdivided into three groups: (a) trastuzumab, 5 mg/kg/wk (two doses; n = 10); (b) letrozole, 10 μg/d, plus trastuzumab (n = 10); and (c) letrozole, 10 μg/d (n = 10). Tumors in mice switched to combination therapy following letrozole resistance responded better than combination treatment from week 0 ($P < 0.0001$)

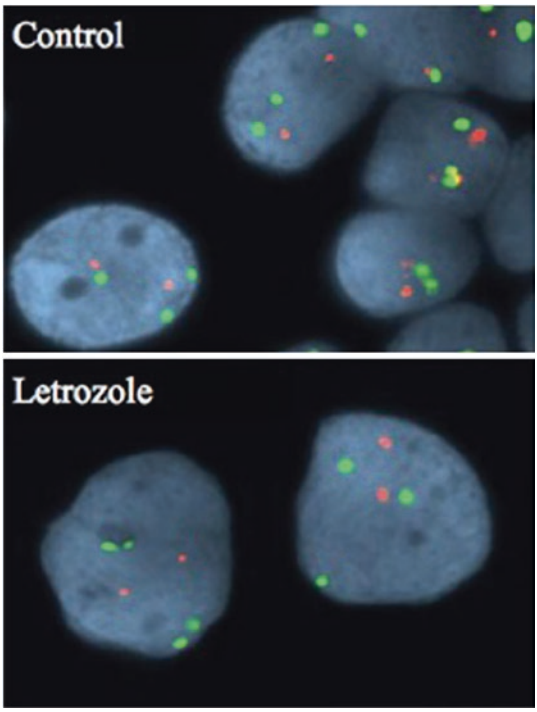

Fig. 4.20 FISH analysis of tumors of MCF-7Ca tumors. MCF-7Ca xenografts grown in ovariectomized athymic female nude mice and treated with Δ4A −100 μg/day (control at 7 weeks) or letrozole −10 μg/day (at week 33). Both tumors show no amplification of Her-2 gene (*red*)

In our model, parental MCF-7Ca cells were ERα/PR positive and Her-2 negative. However, as the tumors progressed on letrozole treatment, they gained Her-2 expression. This increase in Her-2 expression was not due to gene amplification (Fig. 4.20), but greater stability of Her-2 protein and mRNA [63, 66]. Hence, inhibition of stability of Her-2 protein and/or mRNA using HDAC inhibitor was also able to reduce Her-2 expression and thus reverse resistance [66]. However, inhibition of ERα mediated transcription using AIs was also required. ChIP studies showed that the ERα promoter was active in LTLT-Ca cells, suggesting ligand independent activation through crosstalk with growth factor pathways.

Other Models

To depict the role of Her-2 in modulating resistance to endocrine therapy, the group of Schiff and Osborne have used MCF-7 cells stably transfected with Her-2 [5, 19, 24]. This model (MCF-7/Her-2-18) exhibited resistance to tamoxifen, but retained sensitivity to estrogen deprivation. Other Her-2 overexpressing cell lines such as BT-474 or SKBr3 have also been employed as models of endocrine therapy resistance [59].

Variations Between the Models of AI Resistance

Resistance to AIs has been depicted in various models. However, these models differ in their method of creation; in vitro versus in vivo or using estrogen deprivation versus treating with an AI. Further, tumors or cell lines treated with different AIs have different molecular characteristics. Some retain or even upregulate ERα (UMB-1Ca) whereas some loose ERα (LTLT-Ca). Cells in different models were also subjected to selective pressures of the drug for varying length of time, which could also be a result of response to the drug or due to pharmacokinetic barriers. For example, tumors treated with anastrozole acquired resistance sooner than with letrozole, which could be attributed to the poor pharmacokinetic profile of anastrozole in mice. One common observation in all the models is that ERα was still shown to be important in driving the growth of the tumors. Secondly, all cell lines or tumor models showed activation of growth factor receptor pathway, mainly Her-2. Downstream targets of Her-2 such as MAPK, Akt or mTOR were differentially expressed in various models and inhibitors of any of these proteins were able to reverse resistance. As such, which pathway or which protein is activated in any patient is a key factor in determining which secondary therapy will reverse resistance.

Discrepancies Between Animal Models and Clinical Data

Clinical trials based on preclinical animal data have successfully launched aromatase inhibitors as standard of care for hormone sensitive post-menopausal breast cancer (or with gonadotropin analogs for premenopausal). However, the tumors eventually get resistant. Several of the compounds that showed promise in preclinical setting for treating the AI resistant tumors have only provided marginal improvement in human disease. These discrepancies between preclinical data and actual clinical observations could be due to many reasons. Several different pathways have been implicated in resistance to AIs in preclinical studies and which pathway is activated in a particular subset of patients is not understood. This underlines the importance of patient selection for the clinical trials. In addition to inter-tumor variability, there is also intra-tumor variability [45, 52], whereas cell line data is more homogenous. To circumvent this limitation of cell lines, patient derived xenografts (PDX) have been employed [40]. Here, the entire tumor is transplanted into a mouse and as such includes all the clones within the tumor. However, not all PDX tumors establish and it is harder to grow hormone sensitive tumors.

Conclusion

Estrogen is the major stimulus to breast cancer progression in both pre- and post-menopausal patients. The actions of estrogen on the tumor are mediated by the estrogen receptor alpha (ERα). Activation of ERα leads to binding of the receptor

to the DNA causing activation of gene transcription. These events lead to cell cycle entry and progression following expression of cell cycle regulating genes. Additionally, several genes associated with cell survival such as Bcl-2 are also upregulated in estradiol treated cells in vitro and ERα positive tissues in vivo with increased estrogen induced cell survival contributing significantly to breast cancer growth in response to estrogens. In young women, the main source of estrogen is the ovary. After menopause, ovarian production declines and extra-gonadal sites such as adipose tissue, which are not under the control of the pituitary, are the main source of circulating estrogen. However, tissue concentrations within the breast are comparable with those of premenopausal women as a result of local estrogen synthesis and uptake. About two thirds of the breast cancer patients are postmenopausal women with ER positive (ER+) tumors. In breast cancer patients, tumor ERα concentrations are higher after menopause, resulting in cancers that are sensitive to even low levels of estrogens. Estrogen signaling is of primary importance in the proliferation and progression of breast cancer and has led to two types of breast cancer treatment being developed to target this signaling pathway. The antiestrogens (AEs), such as tamoxifen, target the estrogen receptor alpha (ERα) whereas the more recent aromatase inhibitors target the biosynthesis of estrogen by directly interacting with the enzyme aromatase. Synthesis of estrogen is the last step in the steroid biosynthesis pathway and as such inhibition of aromatase does not affect the production of any other steroids. Aromatase inhibitors are now the gold standard for treating postmenopausal hormone sensitive breast cancer, as they are both efficacious and well tolerated. The tumors response may last for years with few side effects. However, not all patients respond and some of the ones who do respond may eventually relapse with a resistant cancer. To assess the mechanisms of resistance and develop strategies for reversing the resistance for second line therapy, several model systems have been employed. The intra-tumoral aromatase xenograft model of MCF-7Ca cells has predicted results of numerous clinical trials. Using this model, Brodie et al. showed that tumors adapted to the low estrogen environment by activating alternative signaling pathways [7–9, 62]. These pathways also interacted with ERα. Although there is no change in the ability of AIs to inhibit aromatase, there is ample evidence suggesting that ER mediated pathways still play a role in growth of the breast cancer cells and tumors, despite showing resistance to endocrine agents such as tamoxifen or the AIs. Some tumors respond to another agent (switching to steroidal AI after non-steroidal AI); tamoxifen resistant tumors may respond to AIs. Model systems developed by Santen et al. and Brodie et al. such as LTED, UMB-1Ca and LTLC exhibit upregulation of ER upon acquisition of resistance [4, 67, 69]. Whereas the LTLT-Ca model developed by Brodie et al. shows that ERα levels are down-regulated and Her-2/MAPK pathway upregulated [7, 36, 65]. When LTLT-Ca cells were treated with MAPK inhibitor (PD98059), ER expression was increased to levels in MCF-7Ca cells. This suggests that inhibiting the MAPK pathway may restore hormone sensitivity [34]. Similar results were also obtained with anti-Her-2 agent trastuzumab [65]. El-Ashry et al. have also shown that overexpression of activated MAPK results in loss of ERα and inhibiting MAPK activity reverses ERα

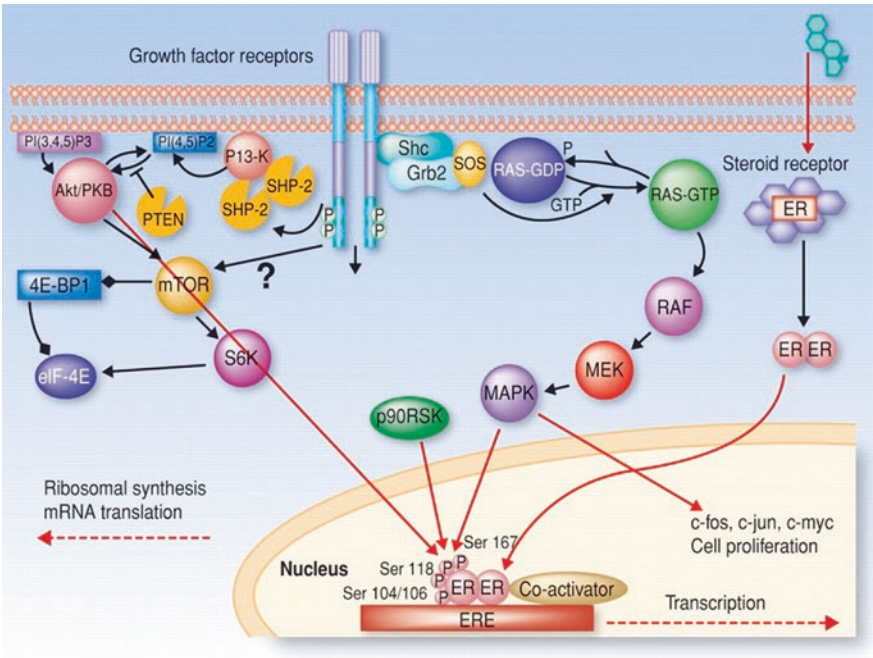

Fig. 4.21 Schematic of the crosstalk between ERα and growth factor pathways

downregulation [2, 6, 23, 54]. More recently, clinical studies have confirmed that Her-2 and ERα status changes in the secondary tumor or metastatic lesion compared to the primary tumor [15, 74]. As such, crosstalk between the growth factor pathways and ERα mediated transcription is a key target for future interventions. Figure 4.21 shows a schematic of this crosstalk.

Conflict of Interest There are no potential conflicts of interests.

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Chapter 5
Ineffective Inhibition of Aromatase: A Cause for AI Resistance?

Per E. Lønning

Abstract While treatment with aromatase inhibitors have improved outcome as compared to tamoxifen in advanced as well as in adjuvant breast cancer therapy, similar to what has been recorded with tamoxifen as well as for oophorectomy in premenopausal women, the reduction in risk for a relapse as well as breast cancer death in the adjuvant setting is less than 50 %. Thus, many patients reveal resistance to aromatase inhibitor therapy even in the early setting. As for metastatic disease, endocrine therapy with aromatase inhibitors, like other forms of endocrine treatment and chemotherapy, remains palliative. Looking at plasma estrogen levels as well as total body aromatization, patients treated with third-generation aromatase inhibitors (anastrozole, letrozole and exemestane) all seem to get profound plasma estrogen suppression as well as effective total body aromatase inhibition. As for studies assessing intratumour estrogen levels, they all point in the same direction; so far, there is no evidence indicating lack of tissue estrogen suppression among individual patients. Important, these findings do not exclude the possibility that in some patients intratumour aromatase to some degree may escape inhibition; however, due to rapid equilibrium with the plasma pool, such a phenomenon most likely should have limited effect on intratumoural estrogen levels.

Abbreviations

AIs	Aromatase Inhibitors
ER	Estrogen Receptor
E2	Estradiol E1, Estrone
E1S	Estrone Sulfate
HR	Hazards Ratio

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A. Larionov (ed.), *Resistance to Aromatase Inhibitors in Breast Cancer*,
Resistance to Targeted Anti-Cancer Therapeutics 8,
DOI 10.1007/978-3-319-17972-8_5

Introduction

While treatment with aromatase inhibitors has improved outcome as compared to tamoxifen in advanced [1] as well as in adjuvant [2] breast cancer therapy, similar to what has been recorded with tamoxifen [3] as well as for oophorectomy in premenopausal women [4] the reduction in risk for a relapse as well as breast cancer death (HR) in the adjuvant setting is less than 50 %. Thus, many patients reveal resistance to aromatase inhibitor therapy even in the early setting. As for metastatic disease, endocrine therapy with aromatase inhibitors, like other forms of endocrine treatment and chemotherapy, remains palliative.

Similar to what has been recorded with respect to other types of endocrine treatment, the degree of estrogen receptor (ER) expression predicts likelihood of successful response to aromatase inhibitor therapy [5]. Further, HER-2 overexpression is associated with a reduced chance of having a response to an aromatase inhibitor, resembling what has been recorded also for tamoxifen [5]. An unexpected observation was the fact that patients acquiring resistance to non-steroidal aromatase inhibitors may subsequently respond to treatment with a steroidal compound; this relates to the second-generation steroidal compound formestane as well as the third-generation compound exemestane [6]. Also, patients acquiring resistance toward aromatase inhibitors in some cases may benefit from additional endocrine therapy, including treatment with tamoxifen, faslodex or high-dose estrogen therapy [7]. While we currently lack explanations to these observations, one possibility could be a "pharmacological escape" or ineffective aromatase inhibition related to individual compounds. In this respect, aromatase inhibitors (and estrogen suppression with use of LH-RH analogues) differ from endocrine treatment with tamoxifen. While tamoxifen, similar to other antiestrogens like droloxifene, exerts effects on circulating levels of plasma parameters like SHBG, IGF-I and its binding protein-1 [8–12], alterations in these parameters in general reflect their influence on hepatic protein synthesis and do not reflect their effect at the tumor level. Thus, no direct surrogate parameter for anti-tumour efficacy exists. In contrast, aromatase inhibitors act by suppressing estrogen levels, and evidence from comparing different aromatase inhibitors with a different potency points to a dose-response effect [13]. Thus, to address whether ineffective aromatase inhibition may explain treatment failure among individual patients, it is necessary to examine current knowledge with respect to estrogen disposition in postmenopausal women and data from studies examining estrogen disposition in response to treatment with aromatase inhibitors.

The Aromatase Enzyme and Estrogen Disposition in Postmenopausal Women

Considering estrogen disposition in postmenopausal women, ovarian estrogen production ceases at the menopause. Postmenopausal estrogens are synthesized from circulating androgens, mainly androstenedione, which is converted into estrone; in

addition, a minor pathway includes aromatization of circulating testosterone into estradiol [14]. Plasma (and tissue) estradiol seems to have a dual origin; some arising from direct aromatization of testosterone, with the rest is synthesized by reduction of estrone [14]. While the adrenal gland is the main contributor of circulating androgens, there has been some controversy over potential ovarian contribution. While most recent evidence indicates ovarian contribution to circulating androgens to be of minor importance [15, 16], notably use of LH-RH analogues in postmenopausal women has been found to cause reduction in plasma testosterone in concert with minor suppression of plasma estradiol levels [17].

An interesting observation for more than two decades is the finding of elevated tissue, in particular breast cancer tissue, estradiol levels as compared to plasma [18–23]. This in general has been attributed to local expression of the aromatase enzyme [24, 25]. While the aromatase enzyme has been studied for decades, its crystallographic structure was first reported in 2009 [26]. Importantly, while the human genome harbours one aromatase gene only, the gene is subject to alternative exon I transcription in different tissues due to use of alternative promoters located along exon I into the start of exon II [27]. Thus, the gene harbours at least 11 different promoters [27], subject to different ligand stimulations in different tissue compartments [28–31]. In breast cancer, aromatase expression is mainly regulated by promoters PII but also 1.3 and 1.7 [27]. Thus, if local synthesis is a main contributor to local estrogen levels, this opens for the possibility to generate promoter-specific targeting therapy.

However, there may be alternative explanations to the observation of elevated estrogen levels. In a recent study, we found the ratio between normal breast tissue and plasma estrogen levels to be similar among pre-and post-menopausal women [32]. As plasma levels of estradiol may vary 10–100 fold between pre- and postmenopausal individuals (pending on time of the menstrual cycle), this finding argues strongly against a significant contribution from local synthesis. As for breast cancer tissue (Fig. 5.1), we recorded elevated tissue concentrations of estradiol but reduced levels of estrone in breast tumours as compared to the

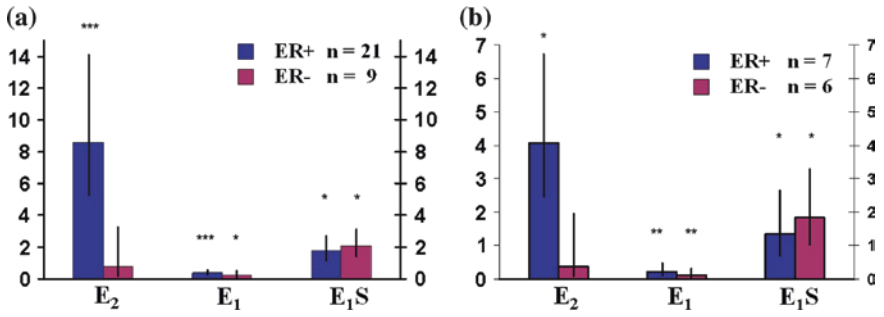

Fig. 5.1 Ratio between intratumour and benign breast tissue estrogen levels related to estrogen receptor expression and menopausal status. Blue columns = ER+, red columns = ER−. **a** Postmenopausal women. **b** Premenopausal women. Reproduced with permission from Ref. [32]. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

surrounding normal breast tissue [32]. Moreover, elevated tumour estradiol concentrations were seen among ER positive tumours only [32]. In addition, we found a strong correlation between intra-tumour estradiol levels on the one hand and plasma estradiol and intra-tumoural ER but also dehydrogenase 2, 7 and 12 as well as ER expression levels on the other [33]. Further, circulating estrogen levels have been found to correlate to subsequent breast cancer risk [34], to time to relapse [35] and, not at least, to expression of estrogen-stimulated genes in tumour tissue [36] in hormone-sensitive breast cancer.

A detailed discussion of these observations may be found elsewhere [37]. While local (normal breast and breast cancer tissues) aromatisation may occur, such local synthesis may have limited influence on local estrogen levels due to rapid equilibrium with the plasma pool. The fact that the ratio between tissue estradiol and tissue estrone correlated to expression levels of certain dehydrogenases in the breast cancer tissue may indicate a somewhat delayed equilibrium. This may be consistent with a positive correlation between intratumour estradiol levels and ER mRNA expression, indicating the bulk of intratumoural estradiol to be receptor-bound [33] and, thus, a delayed turnover rate. As mentioned above, these findings may have significant implications to future therapeutic strategies; while the fact that intratumoural aromatase expression is regulated by distinct promoters (as I.3 and PII) may suggest specific promoter targeting as a therapeutic strategy, this is unlikely to become successful due to rapid equilibration with the plasma pool [37].

Plasma Estrogen Measurements in Relation to Treatment with Aromatase Inhibitors

Plasma estrogen levels are low in postmenopausal women; thus, there is a need for highly sensitive assays to determine estrogen levels, in particular while on aromatase inhibitor therapy [38]. Taking into account plasma levels of estradiol (E_2), E_1 and estrone sulfate (E_1S) to be in the 15–20, 70–80 and 4–500 pM ranges, respectively [39], the assays need sensitivity limits of a few pM to detect potential suppression >98 %, which is what may be expected for many patients during treatment with potent third-generation aromatase inhibitors. Developing highly sensitive radioimmunoassays with a detection limit of about 1 pg/ml (3.7 pM), the Herhsey group revealed significant differences with respect to plasma estrogen suppression between the second-generation aromatase inhibitor CGS16949A [40] and letrozole [41]. Notably, as for both studies the difference in plasma estrogen suppression was corroborated by similar findings with respect to suppression of urinary estrogen secretion.

A problem related to use of ^{3}H-labelled standards for radioimmunoassays relates to limited specific activity (in the 50–160 mCi/mmol range). A higher specific activity may be achieved with use of ^{125}I-labelled compounds (specific activity in the 2000 Ci/mmol range). The first sensitive ^{125}I-based RIA for

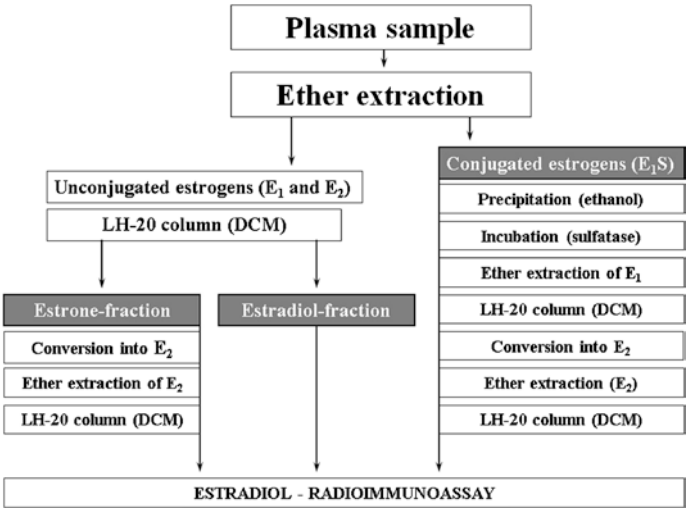

Fig. 5.2 Flow diagram depicting pre-purification steps required prior to determination of plasma estrogens with radioimmunoassay. Reproduced with permission from Ref. [50]

E_2 measurement in patients treated with aromatase inhibitors was developed by Professor Mitch Dowsett at the Royal Marsden Hospital [42] and subsequently used to measure plasma E_2 suppression with different aromatase inhibitors [43–48]. Some years later, we extended this assay to plasma E_1S measurement. The procedure involved taking the samples through multiple purification steps (Fig. 5.2), hydrolysis and finally conversion of unconjugated E_1 into E_2 [49].

Taking this approach further, we improved our assay, allowing E_1 as well as E_1S to be converted into E_2, each steroid to be measured with the same ^{125}I-E_2 assay (Fig. 5.2) [50] Applying this assay to patients on treatment with anastrozole versus letrozole [51], we recorded a mean suppression of plasma E_2 of 92.8 % versus 95.2 %, for E_1 96.3 % versus 98.8 %, and for E_1S 95.3 % versus 98.9 %, respectively [52]. It should be noted however that, even with this sensitive assay, 5 out of 12 patients had plasma levels of E_2 below detection limit during anastrozole treatment; corresponding figures for letrozole were as high as 11 out of 12 [52].

A particular problem relates to plasma estrogen measurement for patients on steroidal aromatase inhibitors, such as exemestane. Due to potential interacting metabolites (Fig. 5.3), samples collected from patients on treatment with such compounds need pre-purification with use of HPLC before radio immunoassaying [53].

Apart from differentiating between first/second generation aromatase inhibitors on the one hand as compared to the highly potent third-generation compounds on the other side, plasma estrogen measurements have been able to discriminate also between highly potent third-generation compounds like anastrozole and letrozole. Thus, two independent studies, both applying a cross-over design, have confirmed letrozole to be a more portent plasma estrogen suppressor as compared to anastrozole [48, 52], consistent with tracer study findings [51]. Recently, data have been analyzed with respect

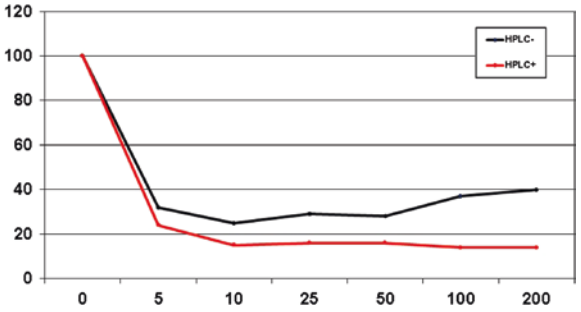

Fig. 5.3 Plasma estrogen levels in patients receiving escalating doses of exemestane during a phase II trial. Etsrogen levels measured by radioimmunoassay without pre-purification in black, following pre-purification in red. Data based on Ref. [53]

to body mass index (BMI). Conflicting data have challenged the efficacy of aromatase inhibition for overweight/obese patients [54–56]; analyzing plasma estrogen levels with these sensitive radioimmunoassay's [57, 58] revealed slightly higher plasma estrogen levels related to high BMI despite a similar degree of aromatase inhibition among overweight as compared to normal-weight individuals [58].

Tracer Studies

Following the seminal tracer study by Santenet al. [59] directly assessing in vivo aromatase inhibition in patients on treatment with aminoglutethimide, tracer injection studies have been considered the "gold standard" monitoring aromatase inhibitor efficacy in vivo. In collaboration with Professor Mitch Dowsett and his team, we initiated a program for in vivo assessment of aromatase inhibition. Using an HPLC method to separate estrogen metabolites [60], we developed an assay allowing assessment of in vivo aromatase inhibition with an average detection limit >99.1 % suppression [51, 61–67]. The results are depicted in Table 5.1; in brief, while most compounds (the so-called 1st and 2nd generation compound) caused in vivo aromatase inhibition <90 %, the three 3rd generation compounds; the steroidal inhibitor exemestane, as well as the non-steroidal compounds anastrozole and letrozole, each caused on average >98 % aromatase inhibition. Importantly, these tracer studies revealed no evidence suggesting suboptimal plasma estrogen suppression among any patients on treatment with either anastrozole, letrozole or exemestane [51, 61–67]. Most importantly, these endocrine results were paralleled by clinical findings; while the 1st and 2nd generation compounds in general revealed clinical efficacy similar to tamoxifen [68], the three 3rd generation compounds revealed superiority, also with respect to clinical efficacy [2].

Tissue Estrogen Levels

Much interest has focused on the issue of estrogen levels since van Landeghem [18] and others three decades ago reported breast cancer tissue E_2 levels a magnitude higher as compared to plasma levels in postmenopausal women. Thus, issues

Table 5.1 In vivo aromatase inhibition by different drugs evaluated in the clinical setting

Drug	Dose	Mean inhibition (%)	Reference
First/second generation compounds			
Aminoglutethimide (AG)	1000 mg daily	90.6	[63]
Roglethimide	400/800/1600 mg daily	50.6/63.5/73.8	[63]
Fadrozole	2 mg/4 mg daily	82.4/92.6	[61]
Formestane[a]	125 mg/250 mg daily	62.3/70.0[b]/57.3[c]	[64]
Formestane[a]	250 mg/500 mg i.m./2w	84.8/91.9	[62]
Formestane[a]	500 mg/w	91.3	[65]
Formestane + AG	500 mg/w/1000 mg daily	94.2	[65]
Third-generation compounds			
Exemestane	25 mg daily	97.9	[67]
Anastrozole	1 mg daily	97.3/96.7	[51, 66]
Anastrozole	10 mg daily	98.1	[66]
Letrozole	2.5 mg daily	>99.1	[51]

[a]Formestane = 4-Hydroxyandrostenedione
[b]Administered as 125 mg b.i.d
[c]Administered as 250 mg once daily

have been raised with respect to local estrogen synthesis by aromatization [69] as well as de-conjugation of E_1S [70]. The hypothesis; that circulating E_1S could be a main contributor to intratumour E_2 was based on previous studies applying direct immunoassays, revealing a high tissue concentration of E_1S. Recent studies by us, using sample purification prior to analysis have found tissue levels of E_1S to be quite low [39], consistent with theoretical assumptions related to physic-chemical properties of steroid conjugates [37].

Interestingly, Professor Miller and his team several years ago reported ineffective aromatase inhibition by formestane on a small fraction of tumour samples in vitro [71]. However, there is no evidence indicating somatic mutations to occur in the aromatase gene in breast cancer tissue, and so far studies by our own group as well as by professor Miller have not indicated local tissue "estrogen escape" during treatment with aromatase inhibitors (see below).

Using our sensitive radioimmunoassays on tissue samples following HPLC purification (Fig. 5.4), we were able to detect tissue levels of E_2 as well as E_1 and E_1S with high degree of sensitivity [72]. Studying tumor tissue samples collected before and during treatment with anastrozole or letrozole [22, 52], we confirmed effective tissue estrogen suppression with no evidence of "escape" for any single tumor. These findings are consistent with data from Professor Miller's group studying patients on treatment with letrozole [21]. Further, studying tissue estrogens across benign and malignant breast tissues [39], we confirmed elevated tissue to plasma E_2 as well as E_1 gradients; as for benign tissue, the tissue to plasma ratio for E_2 and E_1 averaged about 2 and 5, respectively. As for E_1S, however, we found a tissue to plasma gradient averaging 0.1 only, contrasting previous findings obtained by others with the use of direct radioimmunoassay [73]. Interestingly,

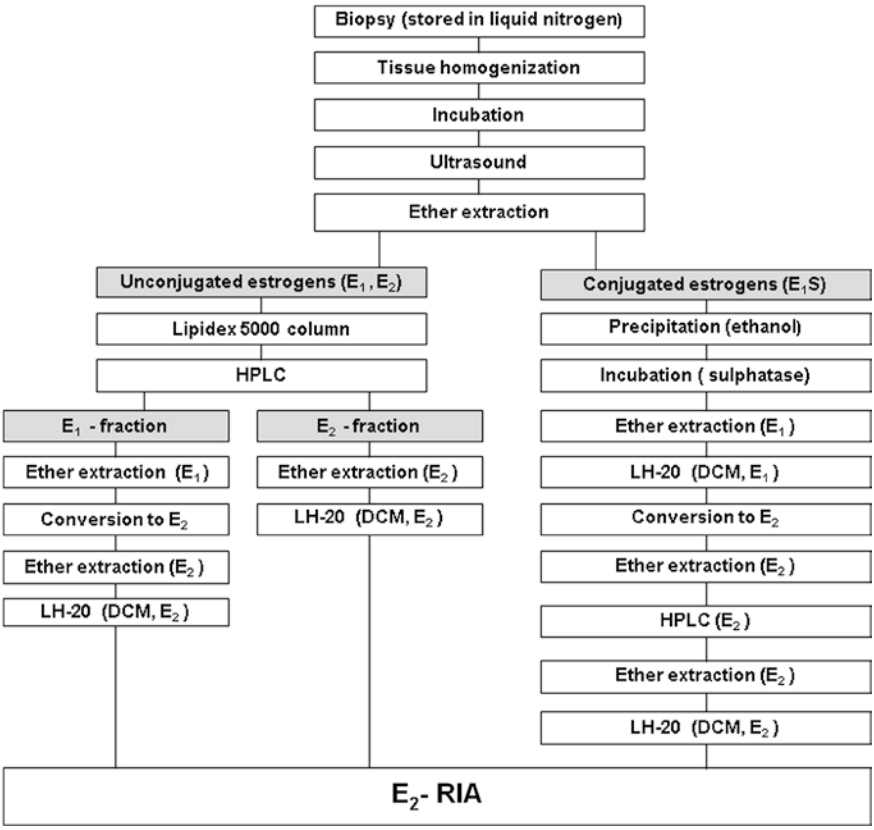

Fig. 5.4 Flow diagram depicting pre-purification steps required prior to determination of tissue estrogens with radioimmunoassay. Reproduced with permission from Ref. [72]

we confirmed elevated tumor E_2 levels in estrogen receptor positive but not in estrogen receptor negative tumors; these elevated levels were found positively correlated to transcriptional levels of the estrogen receptor as well as the reductive 17hydroxy steroid dehydrogenase B7, but negatively correlated to the oxidative B2 and B12 dehydrogenases [33]. However, tissue E_2 and E_1 levels correlated even stronger to their corresponding plasma estrogen concentrations [33, 39]. These findings are in agreement with the results from Professor Miller's group revealing the bulk of tumor tissue estrogens to have a plasma origin [74] and the findings of Dunbier et al. reporting a strong correlation between postmenopausal plasma E_2 levels and tumor tissue expression of estrogen-regulated genes [36].

Based on these findings, we proposed a new hypothesis, explaining tissue to plasma hormone gradients based on physical-chemical properties for each individual compound [37]. Considering the fact that E_2 and E_1 are unconjugated, these compounds are highly lipophilic, explaining a high tissue to plasma concentration gradient. In contrast, E_1S is a water-soluble conjugate. Our hypothesis does not

exclude local estrogen production; nor is it inconsistent with the finding that estrogen receptor expression as well as dehydrogenase activity to some degree may influence the ratio between E_2 and E_1 in breast cancer tissue. However, the finding of similar tissue to plasma estrogen gradients in pre- and postmenopausal women, despite substantial differences with respect to plasma estrogen levels between these groups, indicates tissue to plasma equilibrium to be a rapid event.

Conclusions

To this end, there is no evidence from in vivo studies arguing in favour of a "pharmacological escape" as a cause of resistance toward aromatase inhibitors. Looking at plasma estrogen levels as well as total body aromatization, patients treated with third-generation aromatase inhibitors (anastrozole, letrozole and exemestane) all seem to get profound plasma estrogen suppression as well as effective total body aromatase inhibition. As for studies assessing intratumour estrogen levels, they all point in the same direction; so far, there is no evidence indicating lack of tissue estrogen suppression among individual patients. Important, these findings do not exclude the possibility that in some patients intratumour aromatase to some degree may escape inhibition; however, due to rapid equilibrium with the plasma pool, such a phenomenon most likely should have limited effect on intratumoural estrogen levels.

Conflict of Interest No potential conflicts of interest were disclosed.

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Chapter 6
Understanding the New Biology of Estrogen-Induced Apoptosis and Its Application in Patient Care

Elizabeth E. Sweeney and V. Craig Jordan

Abstract Aromatase inhibitors (AIs) and selective estrogen receptor modulators (SERMs) are effectively used as treatments for breast cancer, but acquired resistance still occurs. Laboratory studies and clinical trials have demonstrated that after long-term estrogen deprivation, either by exhaustive anti-hormone therapy or years after menopause, estrogen is paradoxically able to kill breast cancer cells. Estrogen-induced apoptosis can be used as a potent strategy to treat anti-hormone-resistant breast cancer as well as prevent occult breast cancer from developing. This work outlines the background leading to the current understanding of estrogen-induced apoptosis and the clinical opportunities it presents both now and in the future.

Abbreviations

AI	Aromatase inhibitor
ATLAS	Adjuvant tamoxifen longer against shorter
CEE	Conjugated equine estrogen
EBCTCG	Early Breast Cancer Trialists' Collaborative Group
ER	Estrogen receptor
HRT	Hormone replacement therapy
LTED	Long term estrogen deprived
PR	Progesterone receptor
SERM	Selective estrogen receptor modulator
SOLE	Study of Letrozole Extension
WHI	Women's Health Initiative

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A. Larionov (ed.), *Resistance to Aromatase Inhibitors in Breast Cancer*,
Resistance to Targeted Anti-Cancer Therapeutics 8,
DOI 10.1007/978-3-319-17972-8_6

Introduction

During the early years of the 20th century, Professor Paul Ehrlich [1] conceived the idea of targeted therapy and selective toxicity. He created of a process of synthesizing a broad range of toxic chemicals based on arsenic, but he chose to use organic chemistry to carry the toxic chemical to the bacterial target to kill the disease selectively, and not the patient. Testing would occur in appropriate animal models of the human disease, and active but selectively safe drugs would be tested in clinical trials. In November 1908, Professor Paul Ehrlich learned he was to receive the Nobel Prize in Medicine for "performing enduring services to medical and biological work, notably in determining the potency of serum preparation." He used his lecture to map out his current findings and contributions with diphtheria toxin, his side chain theory, and receptors. He then turned to his new work on the development of resistance in trypanosomes to arsenicals. Salvarsan, an organic arsenical was to be his breakthrough success story as it was the first chemical therapy to cure a fatal disease, syphilis, in humans—by design.

Ehrlich next turned his attention to cancer chemical therapy. He initially spent much time creating an animal model but the year before he died on August 20th, 1915, he declared, "I have wasted 15 years of my life in experimental cancer research" [2]. So the situation was to remain for the next 25 years until the discovery of synthetic estrogens during the late 1930s [3, 4] became an intellectual link between estrogen and breast cancer growth regulation.

The first successful chemical therapy for cancer was reported by Sir Alexander Haddow in his preliminary paper describing the ability of estrogen to treat breast cancer [5, 6]. He observed the efficacy of high-dose synthetic estrogens as a treatment for metastatic breast cancer. However, he too noted the limitations of such therapy as well as our little understanding of how such treatments provide benefit. He laments in his 1970 Karnofsky lecture, "…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 continue to elude us…" [6].

It was not until 30 years later when tamoxifen, a selective estrogen receptor modulator (SERM), was developed for the treatment of breast cancer that the scientific community gained insight into the mechanism of estrogen-induced tumor regression and estrogen-induced apoptosis in breast cancer. Ironically, it was the study of acquired resistance to long-term tamoxifen therapy that began to reveal estrogen's apoptotic potential [7, 8]. We will provide a background of the past investigations of estrogen-induced apoptosis and then consider how it now applies to patient care.

Selective Estrogen Receptor Modulators (SERMs)

Non-steroidal anti-estrogens were discovered to be SERMs, which led to the idea that one drug could simultaneously treat osteoporosis and prevent breast cancer [9–11]. Tamoxifen was found to be an estrogen receptor (ER) agonist in bone, but an ER antagonist in the breast. Unfortunately, it was also found that tamoxifen increased endometrial cancer growth, which presented a difficulty for women seeking preventative medicine [12, 13]. Raloxifene is another SERM with a similar structure to tamoxifen which provides the benefits of tamoxifen (e.g. maintains bone density and prevents breast cancer growth) plus the additional advantage of not promoting endometrial cancer [4–17]. Raloxifene, however, unlike tamoxifen, must be taken indefinitely for benefit to continue.

SERMs are standard treatment for both therapy and prevention of breast cancer. In fact, The Early Breast Cancer Trialists' Collaborative Group (EBCTCG) found in their clinical trials' analyses the clear 15 year benefit tamoxifen offers to ER-positive breast cancer patients when taken for five years versus placebo [18]. Further, the adjuvant tamoxifen longer against shorter (ATLAS) trial found that women with ER-positive breast cancer gain more benefit if tamoxifen is taken for ten rather than five years, in terms of recurrence and mortality [19].

Contribution of SERM Resistance in Understanding Estrogen-Induced Apoptosis

To understand estrogen-induced apoptosis, acquired resistance to SERMs must first be addressed in an appropriate model of human disease. ER-positive MCF-7 cells were originally grown in vitro in media containing phenol red, a redox indicator that monitors the culture condition. Early studies showed an inconsistent effect occurred with these cells in that they could form tumors in animals when treated with estrogen [20], but added estrogen did not cause them to grow significantly in vitro [21]. It was eventually found in 1986 that phenolsulfonphthalein, a component of phenol red used in cell culture, was actually estrogenic [22, 23]. Therefore, the cells were already growing maximally without additional estrogen, so no significant difference could be found with the addition of further exogenous estrogen. Once phenol red was removed from MCF-7 media, the cells responded to estrogen treatment in vitro by growing and proliferating [22]. This important discovery allowed for a much more precise evaluation of the effect of estrogen on MCF-7 cells, and also allowed for extensive study of the direct effects of SERMs on breast cancer cells.

In the 1980s, laboratory in vivo studies documented that MCF-7 cells grow in an estrogen-dependent manner, either via endogenous estrogen produced from the ovaries or via exogenous estrogen given to ovariectomized athymic mice [20]. Tamoxifen was confirmed to block estrogen-stimulated growth in vivo by competitive inhibition [24], but becomes ineffective after four months of treatment [25], illustrating acquired resistance to tamoxifen therapy. Furthermore, when tamoxifen-resistant MCF-7 tumors were re-transplanted into other athymic mice, the tumors were able to grow because of either estrogen or tamoxifen, not despite tamoxifen treatment [26]. These and other data suggested that the tamoxifen-stimulated growth seen in tamoxifen-resistant breast cancer cells was due to tamoxifen's estrogenic activity intrinsic in its classification as a SERM [27]. These early in vivo data generated a platform upon which to study acquired resistance to SERM therapy during breast cancer treatment.

Laboratory studies in vitro and in vivo have explored SERM resistance that can now be divided into distinct phases. Phase I resistance is characterized by acquired resistance to SERMS; that is, SERMs (e.g. tamoxifen) are not able to inhibit tumor growth [25]. If tamoxifen treatment is extended, Phase II resistance eventually occurs, wherein tumors can regress in response to physiological estrogen [7, 8]. These laboratory data illustrate estrogen-induced apoptosis after long-term antihormone therapy or long-term estrogen deprivation, a concept that has since been well-studied.

Long-Term Estrogen Deprivation

Breast cancer cells that have the ability to grow in the absence of estrogen represent AI-resistant disease. This knowledge is important, as AIs are widely used to treat post-menopausal women with breast cancer. In fact, AIs have been proven to be more effective than tamoxifen in the adjuvant treatment of breast cancer with fewer side effects in this application [28].

Santen's [29] group made a breakthrough in the 1990s when they found that short-term estrogen-deprived breast cancer cells developed hypersensitivity to estrogen after deprivation; that is, the cells were able to grow with very low concentrations of estradiol. These data demonstrated the adaptive capacity of breast cancer cells to acclimate in response to varying levels of estrogen in their environment. However, these data revealed a different response after the development of a long-term estrogen-deprived (LTED) cell line, MCF-7:LTED. Unlike their short-term counterparts, MCF-7:LTED cells grow in the absence of estrogen, and eventually estradiol inhibits their growth [30]. More specifically, they found that estradiol induced Fas ligand-mediated apoptosis in this cell line [30].

Other cellular models have been established in the laboratory to model AI resistance and interrogate the signaling and mechanisms involved in estrogen-induced apoptosis. The ER-positive and progesterone receptor (PR)-positive MCF-7 cell line is the most frequently used cell line to study breast cancer in the laboratory,

and many sub-clones have been created to model various clinical circumstances. Especially applicable to anti-hormone resistance investigation are cell lines grown in the absence of estrogen which have the ability to survive after estrogen deprivation. This condition mimics clinical AI resistance of breast cancer cells that continue to grow during or after AI treatment. The MCF-7:5C [31, 32] and MCF-7:2A [33] sub-clones of parental MCF-7 cells are able to illustrate this phenomenon. Both cell lines were established through long-term estrogen deprivation, and were clonally selected. These cell lines exhibit the particularly interesting attribute in that they undergo apoptosis within 7 days (MCF-7:5C) or 14 days (MCF-7:2A) of estrogen treatment [34]. Interrogation of these cell lines has allowed for elucidation of molecular mechanisms involved in estrogen-induced apoptosis.

Molecular Mechanisms of Estrogen-Induced Apoptosis

Our laboratory has shown that primarily, estrogen triggers apoptosis in our estrogen-deprived breast cancer cells through pathways associated with oxidative stress, the unfolded protein response, and inflammatory response [34, 35]. Oxidative stress correlates with timing of estrogen-induced apoptosis in different estrogen-deprived cell lines, and is an integral characteristic [36]. These data begin to reveal estrogen's paradoxical role and function in breast cancer.

We have found that the initial site for triggering estrogen-induced apoptosis is the ER. An estrogenic ligand binds to the ER to trigger a stress response and generate apoptosis. Recent studies with triphenylethylenes have demonstrated that the biological response generated through the liganded ER complex depends on the conformation of the ligand binding and the cellular context. It was shown that triphenylethylenes can exhibit both agonist and antagonist functions at the ER; they can generate estrogen-induced apoptosis or estrogen-induced growth depending on the shape of the complex, the environment, and the duration of treatment [37–39].

Another interesting component of these studies involves the role of proto-oncogene tyrosine-protein kinase c-Src. Investigation of the interaction of c-Src with ER and estrogen-induced apoptosis has provided new mechanistic insight. c-Src is an established oncogene known to mediate breast cancer growth. It has been shown to be essential for ER-negative breast cancer; that is, inhibition of c-Src can block its growth. However, ER-positive disease is likely to exhibit c-Src inhibitor resistance [40], suggesting its growth may not require c-Src. Furthermore, c-Src is involved in estrogen-induced apoptosis in long-term estrogen-deprived breast cancer cells, as it can modulate the stresses caused by estrogen treatment that trigger apoptosis [35]. Recent data demonstrate that inhibiting c-Src has the ability to block estrogen-induced apoptosis and allow estrogen to generate cell growth in long-term estrogen-deprived breast cancer cells [35, 41]. These data illustrate that c-Src plays a critical role in estrogen-induced apoptosis, and that its inhibition could potentially block the beneficial effects of physiological estrogen seen in post-menopausal women or women who have undergone exhaustive anti-hormone therapy. It can mediate the stress pathway involved in triggering apoptosis.

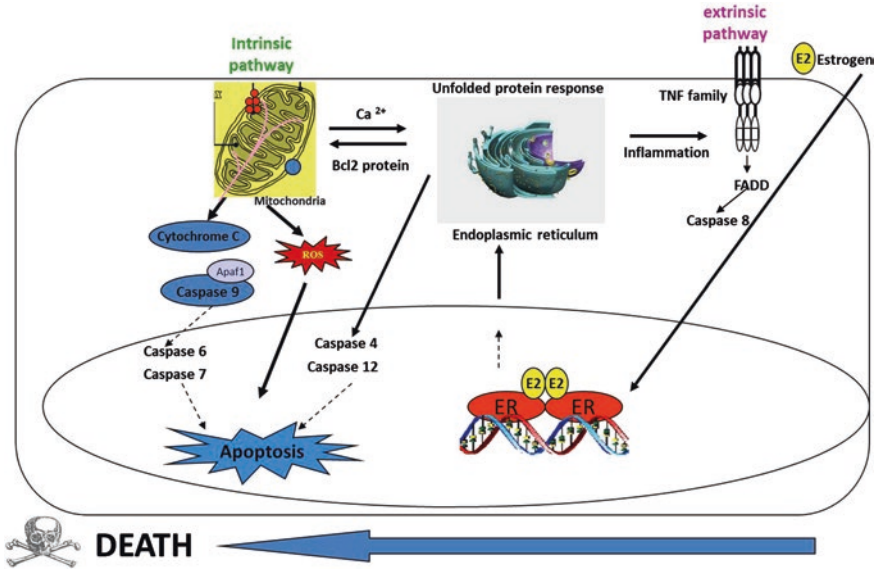

Fig. 6.1 Estrogen-induced apoptosis in aromatase inhibitor (AI)-resistant breast cancer cells. This schematic represents an estrogen-deprived AI-resistant breast cancer cell. Estrogen (estradiol, E2) binding to the estrogen receptor (ER) triggers oxidative stress and endoplasmic reticulum stress which manifests itself in the unfolded protein response (UPR). The UPR can stimulate the intrinsic pathway of apoptosis, triggering mitochondrial release of cytochrome C, a caspase cascade, and ultimately cell death. UPR can recruit and activate the inflammatory pathway through the tumor necrosis factor (TNF) family of receptors, resulting in the initiation of the extrinsic pathway of apoptosis. Reactive oxygen species (ROS) are produced during oxidative stress and can also promote cell death through apoptosis

It was also recently found that although both chemotherapy and estrogen can generate apoptosis in breast cancer cells, they do so using different mechanisms. Paclitaxel, a chemotherapeutic agent, induces rapid apoptosis whereas estrogen induces a slightly delayed apoptotic response [42]. This finding elucidates the unique activity of estrogen in this context, as well as its unique signaling pathway. Figure 6.1 diagrams a summary of the major mechanisms involved in estrogen-induced apoptosis in AI-resistant breast cancer cells, i.e. endoplasmic reticulum stress, inflammation, and both intrinsic and extrinsic pathways of apoptosis.

Estrogen-Induced Apoptosis: Clinical Translation Opportunities

Several situations pertain in medicinal oncology and gynecology where the role of estrogen-induced apoptosis can be implicated or explained. Laboratory evidence has been presented in the previous sections to prove in vitro the characteristic

biology and mechanisms involved in breast cancer. Clinical correlations of estrogen-induced apoptosis are evident in this disease as well.

First of all, the benefit of long-term adjuvant tamoxifen therapy extends after treatment has stopped. In fact, mortality decreases once the patient is no longer taking tamoxifen [18, 19]. Estrogen-induced apoptosis can be used to explain this phenomenon. Long-term tamoxifen therapy sufficiently deprives the women's breast cancer cells of estrogen. When the treatment is stopped, the small level of physiological endogenous estrogen that reappears in the women's body is able to trigger apoptosis in the nascent breast cancer cells, thereby decreasing incidence of and mortality from breast cancer. Lønning and colleagues [43] found that post-menopausal women with breast cancer who had been heavily treated with anti-hormone therapy responded well with high-dose estrogen salvage therapy. Ellis and collaborators [44] then established that low-dose estrogen therapy works too; both doses generated benefit for post-menopausal women who had undergone exhaustive AI therapy.

Epidemiological studies have also confirmed the beneficial ability of estrogen-induced apoptosis in breast cancer. Beral and collaborators [45] compared the timing of estrogen alone hormone replacement therapy (ERT) on breast cancer incidence in post-menopausal women. They found the relative risk for breast cancer to be higher if ERT was begun during or immediately following menopause versus beginning ERT later (five years after menopause). This suggests the importance of the estrogen withdrawal period after menopause. A "gap" after menopause of 5-10 years is sufficient to deplete the woman's nascent breast cancer cell of estrogen and prime them to undergo apoptosis when estrogen is re-introduced [46].

Haddow found in his early trials that women older than 60 were more likely to benefit from estrogen therapy than younger women, and that estrogen therapy may actually promote breast cancer in younger women [6]. His work suggested restricting the use of estrogen therapy to women who were at least five years past menopause. At the time, it was not established that this "gap" provided the necessary estrogen deprivation required for estrogen-induced apoptosis to occur, but it is more corroborative evidence suggesting the power of this strategy.

The Women's Health Initiative (WHI) drew similar conclusions when they illustrated in their randomized clinical trials that hysterectomized women taking conjugated equine estrogen (CEE) alone therapy exhibited a decrease in the risk of breast cancer and overall mortality [47]. However, women who were randomized to CEE plus a progestin (HRT, only women with intact uteri) exhibited an increase in their risk of breast cancer when compared to placebo-treated women [47]. There has yet to be a unified explanation for this increase in breast cancer when the progestin is added to estrogen; somehow the progestin is preventing the beneficial effects of estrogen-induced apoptosis in that particular setting. Designing a safer and effective HRT combination therapy that prevents osteoporosis and endometrial cancer while decreasing breast cancer risk is an important challenge facing scientists and physicians.

Recent unpublished data from our laboratory suggests that the glucocorticoid activity of particular synthetic progestins used in HRT may be able to block the

favorable effect of estrogen-induced apoptosis seen in women taking CEE. There have been recent advances showing promise in this field with the introduction of a new drug combining bazedoxifene with CEE for HRT. This drug combination has been approved by the Food and Drug Administration and is shown to be effective in reducing menopausal symptoms while potentially retaining estrogen's apoptotic ability [48].

We have shown that glucocorticoids can prevent estrogen-induced apoptosis from occurring in our estrogen-deprived cellular models [49]. Perhaps we can improve response rates in estrogen salvage therapy by combining the estrogen with an anti-glucocorticoid. Clinical trials are necessary to build on this hypothesis. Our data also show that glutathione exerts a protective mechanism in some long-term estrogen-deprived cells, delaying them from undergoing estrogen-induced apoptosis. If we block glutathione synthesis with an inhibitor, the cells succumb to apoptosis in response to estrogen [36, 50]. This strategy could potentially serve usefully in the clinic as well, by treating breast tumors with an anti-glutathione in combination with estrogen to sensitize the cells to apoptosis.

Another clinical opportunity to consider is improving the response rates to aromatase inhibition. The Study of Letrozole Extension (SOLE) clinical trial (Fig. 6.2) is ongoing and seeks to determine whether intermittent letrozole is more beneficial than continuous letrozole treatment [51]. The hypothesis here would

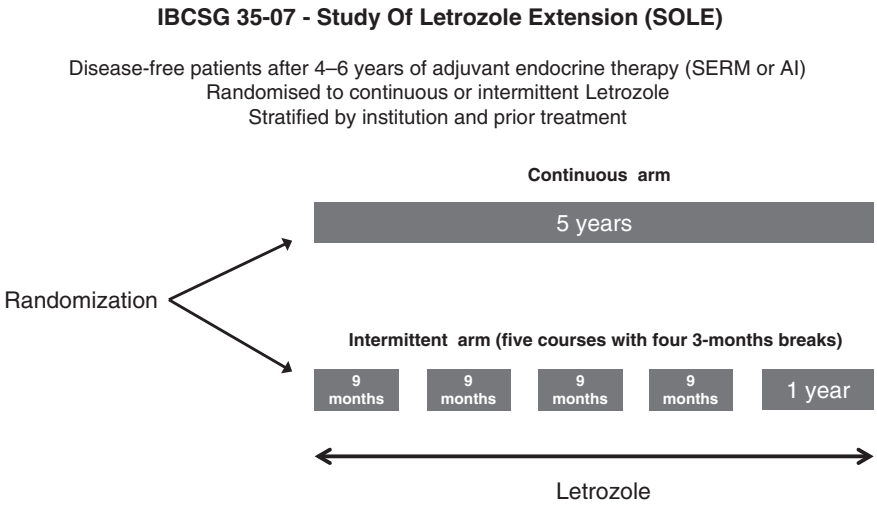

Fig. 6.2 Schema for the Study of Letrozole Extension (SOLE; IBCSG 35-07) conducted by the International Breast Cancer Study Group (IBCSG). Upon completing 4–6 years of prior adjuvant endocrine therapy with a SERM(s) and/or aromatase inhibitor(s) (AI), patients were randomly assigned to continuous or intermittent letrozole (3-month drug holidays per year) for 5 years. The rationale for this approach was that the woman's own estrogen in the intermittent arm would trigger apoptosis in long-term estrogen-deprived breast cancer and reduce recurrence rates. Adapted from International Breast Cancer Study Group—Study of Letrozole Extension (www.ibcsg.org). Reproduced with permission from Jordan, VC and Ford LG [77]

be that a break from letrozole treatment may allow endogenous estrogen to kill remaining nascent tumor cells. The SOLE trial is a clinical evaluation of the original hypothesis that long-term adjuvant tamoxifen creates selection pressure of micrometastases to be vulnerable to a woman's own estrogen to trigger apoptosis once tamoxifen is stopped [8]. There is laboratory [52] and anecdotal clinical support [53, 54] for the new concept of using "breaks" in aromatase inhibitor therapy. If correctly predicted, the results from this trial will provide yet another example of estrogen-induced apoptosis having important clinical application.

Perspectives and Conclusions

We have followed a path for progress in endocrine treatment of breast cancer that had twists and turns, as the fashions for research changed over the past century. This initially sounds quite alarming but it is a reality; Professor Paul Ehrlich conceived a new idea of how to conquer cancer but others demonstrated that success could be achieved. Sir Alexander Haddow was the first to achieve success with translational "chemical therapy" in metastatic breast cancer [5, 6]. However, the paradox of why estrogen could cause the regression of some breast cancers went unresolved. Dogma decreed that estrogen caused the formation and growth of breast cancer [55], so estrogen-induced tumor regression was an inconvenient observation. The clinical finding was to be ignored once the non-steroidal anti-estrogen tamoxifen (ICI 46,474) demonstrated anti-tumor action in metastatic breast cancer. This result followed dogma and tamoxifen had fewer side effects than high-dose estrogen therapy [56, 57].

However, the rise of rational mechanisms with a palliative anti-hormone therapy did not subvert the dogma of the 1970s that combination cytotoxic chemotherapy was going to cure breast cancer. The first results with adjuvant cytotoxic chemotherapy were showing promise [58, 59] so with patience and the practice of trial and error, eventually the correct combinations of new cytotoxic agents would be discovered to achieve cures. Success had been achieved with childhood leukemia and Hodgkin's disease so it was only a matter of time. Chemotherapy was the king!

By conceiving a novel and unconventional way of strategically using tamoxifen, a failed "morning after pill," an unanticipated advance had been made with an anti-estrogen used as the first targeted therapy for the treatment and prevention of breast cancer [60, 61]. This was neither an obvious advance nor a likely one, as the new drug group called non-steroidal anti-estrogens had uniformly failed to be developed by the pharmaceutical industry for good reasons—they were too toxic or if they had few side effects (like tamoxifen), there was no lucrative market to provide profits [62, 63]. Only the play of chance, and people in the right place at the right time [60, 64] proved to be the unlikely formula for success. Tamoxifen was an orphan drug placed on the market in the UK in 1973 for the palliative treatment of breast cancer, and the medicine would not be approved in the United States until December 31st, 1977, for the same palliative indication.

But a strategy had been conceived in the laboratory starting at the Worcester Foundation in Shrewsbury, Massachusetts, in 1974 [61, 65] and finishing at the University of Leeds in England by 1980, to prevent (chemoprevention) carcinogen-induced rat mammary cancer [66], and to use long-term adjuvant therapy to prevent recurrence of breast cancer [67] by targeting the ER [68]. Success was achieved by others to save perhaps millions of lives over the past 30 years, and will continue to save lives for the foreseeable future. But there is more with the tamoxifen tale and this has a touch of irony.

Tamoxifen gave medicine the concept of long-term administration to achieve decreases in mortality [69, 70], but it also gave medicine safer SERMs [71, 72]. Again, long-term therapy is necessary for SERMs to prevent breast cancer and prevent osteoporosis. However, it was the laboratory requirement to study acquired drug resistance to long-term treatment during the 1980s that was to close the circle of the paradox of estrogen action as a treatment for breast cancer.

The re-transplantation of MCF-7 tumors with acquired resistance to tamoxifen [26] into tamoxifen-treated athymic mice showed that the selection process of acquired resistance may produce a vulnerable population of cells that respond to physiological estrogen by tumor regression [7, 8]. This was an important milestone in deciphering estrogen-induced apoptosis [7, 8]. Subsequent studies in vivo confirmed and consolidated the process for SERMs [73–76].

It was, however, the ability to study mechanisms in well-characterized cell models of acquired resistance to AIs, i.e. estrogen withdrawal [27] that led to our current understanding of the new biology of estrogen-induced apoptosis. The new science translates to patient care [43, 44, 51] and is important to understand how [46] estrogen therapy alone causes a decrease in the incidence and mortality from breast cancer in post-menopausal women in their mid-sixties.

If this long journey with the new biology of estrogen-induced apoptosis sounds familiar, then perhaps you are correct. No one (or very few) would have given tamoxifen a chance of success in achieving significant advances in women's health in the 1970s. We anticipate that estrogen-induced apoptosis will evolve from curiosity to a general principle underlining new treatment strategies against resistance to aromatase inhibitors.

Acknowledgments This work was supported by the Department of Defense Breast Program (award number W81XWH-06-1-0590) Center of Excellence, the Susan G. Komen for the Cure Foundation (award number SAC100009), and the Lombardi Comprehensive Cancer Center Support Grant (core grant NIH P30 CA051008).

Conflict of interest The authors disclose no potential conflicts of interest.

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Chapter 7
Ligand-Independent Signalling Through Estrogen Receptor Pathways in Breast Cancer

Jean McBryan and Leonie S. Young

Abstract Aromatase inhibitors (AIs) target the production of estrogens with the intention of reducing estrogen receptor signalling in breast cancer. One mechanism by which tumour cells can evade AI therapy is to find alternative ways to activate the estrogen receptor in the absence of ligand. Here we discuss mechanisms of ligand-independent receptor activation including growth factor cross talk, kinase induced phosphorylation, the involvement of co-factors as well as hypersensitivity of the estrogen receptor. Understanding how cell signalling pathways regulate estrogen receptor activity is helping to identify biomarkers of AI resistance. The research discussed here has also led to the development of a number of new treatment strategies to help combat AI resistant disease.

Abbreviations

AI	Aromatase inhibitor(s)
ER	Estrogen receptor
DBD	DNA binding domain
LBD	Ligand binding domain
AF	Activation function
EREs	Estrogen response element(s)
PTMs	Posttranslational modification(s)
SERMs	Selective estrogen receptor modulator(s)
LTED	Long term estrogen deprived
FFPE	Formalin fixed paraffin embedded

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A. Larionov (ed.), *Resistance to Aromatase Inhibitors in Breast Cancer*,
Resistance to Targeted Anti-Cancer Therapeutics 8,
DOI 10.1007/978-3-319-17972-8_7

Introduction

The estrogen receptor is expressed in approximately 70 % of breast tumours and is one of the defining features in classifying tumour subtype and assigning treatment strategies in breast cancer. The functional importance of the estrogen receptor is evident in that the majority of breast cancer patients will be prescribed endocrine therapies aimed at preventing ligand binding and subsequent activation of the estrogen receptor. Indeed in recent years, a subset of healthy individuals deemed to be at high risk of developing breast cancer, have also been prescribed endocrine therapies as a preventative measure and clinical trials have shown clear benefits to this strategy [1].

The downstream signalling pathways of the estrogen receptor, which endocrine therapies aim to inhibit, are diverse. In the breast cancer context a central function of the estrogen receptor is in promoting cell proliferation with endocrine therapies certainly aiming to inhibit cell growth and proliferation of tumour cells. However, the intervention of blocking estrogen receptor signalling also has many other systemic effects, both desirable and undesirable [2]. With this in mind it is important to understand endocrine resistance to ensure that patients are receiving benefit from their treatment and not suffering unnecessary side effects.

Mechanisms of resistance to endocrine therapies, and more specifically to aromatase inhibitors (AIs) are varied. Much of our understanding of ligand-independent estrogen receptor activity comes from analysis of tamoxifen resistance although it must be stressed that while tamoxifen and AI resistance share many common features, their effects on estrogen receptor signalling are far from identical. This chapter focuses specifically on resistance mechanisms which involve continued activation of the estrogen receptor despite the absence, or reduced presence, of ligand as a result of aromatase inhibitor therapy.

Background: Classical Ligand-Dependent Estrogen Receptor Signalling

The estrogen receptor family consists of two receptors: ERalpha, encoded by the ESR1 gene on human chromosome 6 and ERbeta encoded by the ESR2 gene on chromosome 14 [3, 4]. Despite sharing a high degree of homology and both being expressed in the normal breast, the two receptors appear to play different roles in breast cancer. The function of ERbeta in breast cancer remains unclear and indeed only ERalpha expression is routinely analysed in the pathological diagnosis of breast cancer. This chapter will therefore focus only on signalling through ERalpha which from here on in will be designated simply as ER.

The structure of the ER has been divided into six domains (Fig. 7.1). Closest to the N-terminal, the A/B domain possesses an activation function (AF) and is often referred to as the AF-1 domain. Activation of ER transcriptional activity

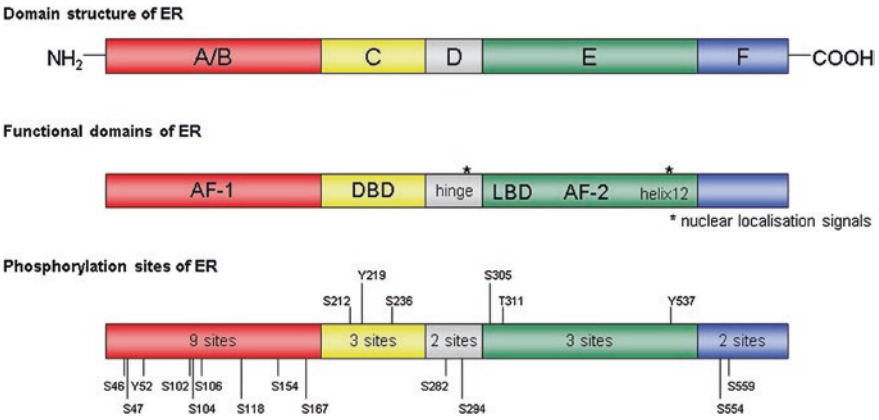

Fig. 7.1 Schematic representation of the functional domains and phosphorylation sites of ER. *AF* activation domain, *DBD* DNA binding domain, *LBD* ligand binding domain, *S* serine, *Y* tyrosine, *T* threonine, * nuclear localisation signals

through the AF-1 domain usually occurs in a ligand-independent manner [5]. Next to this is the C domain or DNA binding domain (DBD) which contains two zinc fingers and is the most highly conserved of the ER domains between species [6]. Classical transcriptional activity of the ER involves ER dimerisation followed by binding of the ER to estrogen response elements (EREs) on the DNA of target genes. The C domain of the ER is known to be involved in receptor dimerisation and is essential for recognition of and binding to the DNA [7]. Non-classical ER transcriptional activity involves ER binding indirectly to the DNA via other transcription factors. The C domain may not always be essential for this mechanism of ER activity although its involvement appears to depend on the specific ligand activation of ER as well as potentially the transcription factors involved [8]. Next to the highly conserved sequence of the C domain is the more variable D domain, often referred to as the hinge region. The function of this region is less defined although it is known to contain a nuclear localisation signal [9]. Moving towards the C-terminal end of the receptor is the E domain, also referred to as the ligand binding domain (LBD). The E domain contains another nuclear localisation signal, a 12 helix region involved in ligand binding and a second activation function (AF-2) which is responsible for ligand-dependent activation [10]. Finally the F domain at the C-terminal end is capable of modulating both AF-1 and AF-2 although it is not essential for transcriptional activity [11, 12].

Inactive ER is normally located in the cytosol of a cell associated with hsp90 chaperone proteins. Estrogen binding triggers a number of events resulting in transcriptional activation of the ER. Upon estrogen binding, heat shock proteins and other chaperone proteins dissociate from the ER permitting translocation to the nucleus and receptor dimerisation [13]. Estrogen binding also triggers a conformational change in the LBD of the ER allowing the receptor complex to bind to specific DNA sequences (EREs) and creating a binding site for nuclear co-activators

to assemble [14]. Co-activators, such as the p160 proteins, recognise agonist-bound nuclear receptors through a short LXXLL motif, where L is leucine and X is any amino acid [15]. Helix 12 of the LBD (the helix closest to the C-terminal end of the ER) has received much attention for its role in forming a specific binding site for co-activators. In the presence of estrogen, helix 12 is repositioned forming a recognition surface for co-activators but in the presence of tamoxifen, helix 12 is positioned in such a way that co-activators are prevented from binding [16].

Non-classical ER transcriptional activity also occurs whereby ligand activated ER translocates to the nucleus but binds to the DNA indirectly via other transcription factors such as AP-1. The DBD of the ER is not always required for this mechanism of action [17]. Finally, a non-genomic mechanism of ER signalling has also been identified. Stimulated by estrogen, membrane bound ER can signal in a manner that is too rapid to involve gene transcription and protein synthesis. Non-genomic ER action is mediated by the LBD of the ER and usually involves activation of various protein kinase cascades [18, 19].

ER Phosphorylation

The ER can undergo many posttranslational modifications (PTMs) which impact its activity and phosphorylation is an example of one such PTM. In classical ligand-dependent ER signalling, binding of estrogen results in phosphorylation of the ER, particularly at the serine 118 amino acid (Ser118). Several kinases have been shown to mediate this ligand-dependent phosphorylation of Ser118 including CDK7 [20], IKKα [21] and somewhat controversially the ERK1/2 MAPK pathway [22, 23]. At a functional level, phosphorylation of Ser118 has been shown to enhance protein stability of the ER [24], enhance binding of ER with co-activators [25], enhance transcriptional activity of the ER [20], and ultimately to enhance ligand-dependent cell growth [26].

Ligand-Independent Phosphorylation of Serine 118

Ligand-independent phosphorylation of ER at Ser118 has also been shown to occur and the ERK1/2 MAPK pathway certainly plays an important role in ligand-independent phosphorylation [27]. Several other protein kinases have also been implicated either directly or indirectly in phosphorylation of Ser118 including GSK3β [28], ILK [29], EGFR [30], IGF1R [30], DNA-PK [31] and RET [32] (reviewed by Murphy et al. [33]). Based on the known functional consequences of phosphorylation of Ser118, the fact that this amino acid can become phosphorylated in the absence of ligand offers a mechanism by which ER can become activated in the absence of ligand. This therefore represents a potential mechanism by which ER can evade AI therapy to remain functionally active in the absence of estrogen synthesis.

Evidence supporting a role for pSer118 in AI resistance is provided by expression studies of AI resistant cell lines. Relative to sensitive cell lines, pSer118 expression was found to be elevated in letrozole-resistant, anastrozole-resistant and long-term estrogen deprived cell lines [34, 35]. The cell line data supports the hypothesis that Ser118 is being phosphorylated in a ligand-independent manner to assist in the development of AI resistance. Evidence from clinical samples however is less clear. High expression of pSer118 is detected in approximately 50–60 % of ER positive patient tumour samples [36, 37]. pSer118 expression is regularly associated with good clinical outcome for patients who subsequently receive endocrine treatment with either tamoxifen [36, 37] or an AI [38]. Importantly this observation is specific to endocrine treated patients and expression of pSer118 did not correlate with disease free survival in ER positive patients who did not subsequently receive an endocrine therapy [37]. Based on these findings, pSer118 can be considered a marker of a functional ligand-dependent ER pathway and a predictive marker for good response to endocrine treatment (Fig. 7.2).

However, it should be noted that a large number of clinical studies have now examined pSer118 expression in patients who subsequently received tamoxifen treatment and not all have observed this correlation. Several studies observed no correlation between pSer118 and clinical outcome following tamoxifen [39–42]

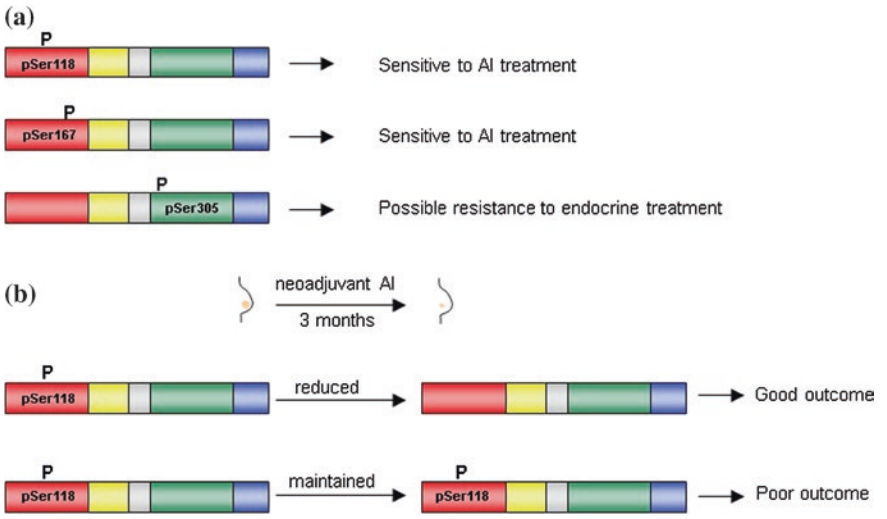

Fig. 7.2 Simplified schematic representation of the correlations observed between expression of phosphorylated ER and subsequent response to endocrine therapy. **a** Phosphorylation of both pSer118 and pSer167 have been correlated with improved sensitivity to AI therapy. By contrast, phosphorylation of Ser305 correlated with reduced response to endocrine therapy. These correlations were made by assessing ER phosphorylation status in the treatment-naïve primary tumour. **b** Changes in pSer118 levels following neoadjuvant AI treatment can also be predictive of AI sensitivity. A large reduction of pSer118 during 3 months of neoadjuvant AI treatment correlates with good response to endocrine therapy. By contrast, maintained expression of pSer118 may be indicative of a poor clinical response to AI therapy

and one observed a negative correlation between high pSer118 expression and good clinical outcome following tamoxifen therapy [43]. Possible explanations for the apparently contradictory observations include patient and tumour heterogeneity, differences in pSer118 cut offs and differences in the methodology of detecting pSer118. Interpreting the results from tamoxifen based studies is further complicated by the fact that tamoxifen is a partial agonist for the ER and therefore does more than just prevent ligand from binding. Phosphorylation of ER has been shown to reduce the affinity of the ER for tamoxifen binding, thus implying that pSer118 may lead to reduced antagonistic activity of tamoxifen by a mechanism which is not relevant to AI studies [44].

To date, the limited number of clinical studies of pSer118 in AI treated patients have been more straight forward and consistent. Generali and colleagues examined pSer118 expression in a cohort of 114 patients who were all subsequently treated with letrozole. A significant positive association was observed between nuclear pSer118 expression and sensitivity to endocrine treatment [38]. Zoubir and colleagues examined pSer118 expression in 80 patients who received neoadjuvant endocrine treatment, predominantly AI treatment. The same trend was observed for pSer118 expression to correlate with sensitivity to AI treatment although patient numbers were too low to reach statistical significance [45]. This study also looked at the changes in pSer118 expression by comparing matched tumour samples before and after neoadjuvant AI treatment. A significant decrease in pSer118 was observed following neoadjuvant AI treatment. Furthermore, the magnitude of pSer118 decrease was reflective of the responsiveness to AI, with refractory tumours displaying less of a decrease than sensitive tumours [45]. Combined, these data support a role for pSer118 as a marker of functional ligand-dependent ER signalling in the treatment-naïve tumour and therefore a marker of tumours which are likely to respond to AI therapy. However, following AI treatment, maintained expression of pSer118 may be indicative of ligand-independent ER signalling and the emergence of AI resistance (summarised in Fig. 7.2).

Ligand-Independent Phosphorylation of Other Sites

Ser118 is not the only amino acid within the ER that can become phosphorylated. At least 18 other amino acid residues within ER have been identified as potential targets for phosphorylation and many of these, like Ser118, are clustered within the AF-1 domain (Fig. 7.1). Not surprisingly given their location, a number of these other phosphorylation sites have also been linked to ligand-independent activation of ER. With regards to AI resistance, a study by Motomura and colleagues examined phosphorylation of Ser167 in primary tumours of 41 patients who subsequently developed metastatic disease despite receiving AI therapy. The authors observed a correlation between high pSer167 expression in the primary tumour and longer progression-free survival [46]. Thus pSer167, like pSer118 may be a predictive marker for sensitivity to endocrine treatment. A decrease in

pSer167 following neoadjuvant AI treatment has also been reported with no significant difference between responders and non responders although this was a very small (n = 16) population [47]. An association between high pSer167 in primary tumours and longer disease-free survival has also been reported for tamoxifen treated patients [43].

By contrast to Ser118 and Ser167, phosphorylation at Ser305 (located within the hinge region of ER) in the primary tumour may be an indicator of endocrine resistance (Fig. 7.2). Clinical studies have shown that expression of phosphorylated Ser305 in the primary tumour predicted poor response to tamoxifen in ER positive patients [48]. While pSer305 negative patients significantly benefited from tamoxifen treatment, pSer305 positive patients did not [49]. Experimental results however suggest that this finding may be specific to tamoxifen and not applicable to other endocrine therapies such as AIs or fulvestrant [50]. Mechanistic studies have shown that phosphorylation of Ser305 by protein kinase A induces a switch from tamoxifen being antagonistic to having an agonistic role, a mechanism which is not applicable to the AI setting [51]. On the other hand, support for a role of pSer305 in AI resistance comes from mutation experiments by Barone and colleagues. A cell line study examined aromatase expressing MCF7 cells with an ER mutation at amino acid 303. This lysine to arginine mutation conferred AI resistance on the cells but the resistance was shown to be dependent on phosphorylation of Ser305 and blocking phosphorylation was sufficient to restore sensitivity [52]. To date, no large scale clinical analysis of pSer305 has been completed for AI treated patients so a definitive role of pSer305 in AI resistance has yet to be established.

For the remaining phosphorylation sites in ER, the majority of work to date has been carried out in cell line models to assess the presence of phosphorylation, to identify the kinases responsible for phosphorylation, and through mutational studies to assess the functional significance of these phosphorylation sites (reviewed by Lannigan [53], de Leeuw et al. [54] and Murphy et al. [33]). Further studies of patient tumours will be required to assess the clinical relevance of these specific phosphorylation sites to the development of AI resistance.

ER Phosphorylation as a Marker of AI Resistance

Our current understanding of ER phosphorylation leads to a number of interesting avenues for clinical research. In addition to the potential of ER phosphorylation sites as predictors of response to AI therapy, expression of related kinases may also play a useful role in this regard. A wide range of kinases have been demonstrated or are predicted to play functional roles in the phosphorylation of ER (summarised in Table 7.1). Expression of active forms of several of these kinases has been associated with poor outcome in breast cancer. For example, kinases from the PI3K/AKT/mTOR pathway may phosphorylate ER at Ser118 and Ser167 (Table 7.1). A study of 165 invasive breast cancers (approximately half of which

Table 7.1 Kinases and growth factor pathways involved in ER phosphorylation

Kinase	Phosphorylation site	References	Potential drug intervention
MAPK/ERK signalling pathway			
ERK1/2	S104, S106, S118	[142]	
P38 MAPK	T311	[143]	
RET	S118, S167	[32]	
PI3K/AKT/mTOR signalling pathway			
AKT	S167	[144]	Everolimus (mTOR inhibitor)
DNA-PK	S118	[31]	
S6 K1	S167	[145]	
Wnt signalling pathway			
CK2	S167, S282, S559	[146, 147]	
GSK3B	S102, S104, S106, S118	[28]	
Growth factor pathways			
EGFR	S118	[148, 149]	Gefitinib (EGFR inhibitor) Lapatinib (dual EGFR/HER2 inhibitor) Trastuzumab (HER2 inhibitor)
IGF1R	S118	[148]	
Other signalling pathways			
cABL	Y52, Y219	[150]	Dasatinib (multi BCR/ABL/ Src inhibitor)
CDK2/Cyclin A2	S104, S106	[151]	
CDK7	S118	[20]	
c-Src	Y537	[152]	
IKK	S118, S167	[21, 153]	
LMTK3	Unknown	[154]	
PAK1	S305	[155]	
PKA	S236, S305	[51, 156]	

were ER positive), showed significant individual associations between high pAKT, high pmTOR and high p-4E-BP1 with worse overall disease free survival [55]. A subsequent study of 252 patients, including some AI treated patients, found that pAKT specifically correlated with poor disease free survival in patients who received postoperative endocrine therapy. These findings imply that pAKT may be a marker of poor response to endocrine therapy and suggest that targeting this pathway may help to improve responses to endocrine therapies [56]. It is worth noting that these are multifunctional kinases and their correlations with endocrine resistance may be due to more than simply their functional role in phosphorylation of ER. For example, cAbl tyrosine kinase is also well known to phosphorylate the co-activator AIB1 which can enhance activity of the ER signalling pathway [57]. Details of the involvement of co-activators and co-repressors in ligand-independent ER signalling will be discussed in the next section.

For many kinases with known functions in ER phosphorylation, clinical analyses have not yet been performed to specifically assess their association with

response to endocrine therapies, let alone to AI therapy. In fact, for a number of these kinases such as MAPK, cSrc and S6K1, more general studies have identified correlations between kinase expression and poor outcome in the general breast cancer population [58–60]. Again, this reflects the multifunctional nature of kinases, suggesting that they are playing a role (or perhaps multiple roles) in both ER positive and ER negative breast cancers, independent of endocrine therapy. This again emphasises that ER phosphorylation may not be the only mechanism leading to associations between kinases and response to endocrine therapy, such as that observed for pAKT.

Targeting ER Phosphorylation in Breast Cancer Treatment

In terms of clinical intervention, kinase inhibition offers the potential to prevent ligand-independent activation of ER and therefore prevent disease progression in the AI resistant setting. Toxicity is often an issue with inhibiting signalling pathways but so far there have been a number of success stories where benefits are deemed to outweigh the toxic side effects. The PI3K/AKT/mTOR pathway has so far been one of the most successfully targeted kinase pathways in endocrine resistance. Everolimus and temsirolimus are mTOR inhibitors which have undergone phase II and III clinical trials (summarised in Table 7.2). Everolimus is currently approved in both the USA and Europe for treatment of hormone receptor positive, postmenopausal breast cancer patients who have advanced disease after failure of a non steroidal AI. The initial phase II trial (TAMRAD, n = 111 patients) to support the approval of everolimus demonstrated extended time to progression and improved overall survival in patients treated with tamoxifen in combination with everolimus compared to those treated with tamoxifen alone [61]. The subsequent larger phase III trial (BOLERO-2, n = 724 patients), compared the AI exemestane in combination with everolimus to exemestane alone. All patients on this trial were ER positive, HER2 negative and refractory to either letrozole or anastrozole. The median time to progression was significantly extended from 4.1 to 10.6 months with the addition of everolimus [62]. Although everolimus has been shown to reduce ligand-independent phosphorylation of ER, this may not be the only function which contributes to its clinical benefit in the endocrine resistant setting [63]. Indeed, the multifunctionality of the mTOR signalling pathway is evident by the range of both toxic side effects and clinical benefits associated with everolimus which has also been approved for treatment of advanced kidney and pancreatic cancers. Clinical data for temsirolimus are similar but not as strong as those for everolimus. Despite promising phase II study results [64], a phase III study (HORIZON, n = 992 patients) comparing letrozole and temsirolimus to letrozole alone, showed no significant improvement in progression free survival with addition of the mTOR inhibitor [65]. Similar toxic side effects such as stomatitis were observed for temsirolimus as those reported for everolimus. Current research in this area focuses on the use of everolimus as an adjuvant therapy for breast cancer and there is ongoing debate

Table 7.2 Clinical trials of kinase inhibitors in combination with endocrine therapy

Kinase pathway	Drug	Trial	Disease setting	Companion biomarker	Brief result	References
mTOR	Everolimus	Phase II TAMRAD n = 111	Advanced	None	Extended time to disease progression Improved overall survival	[61]
	Everolimus	Phase III BOLERO-2 n = 724	Advanced	None	Extended time to disease progression	[62]
	Temsirolimus	Phase II n = 109	Advanced	None	Partial responses observed	[64]
	Temsirolimus	Phase III HORIZON n = 992	Advanced	None	No significant improvement	[65]
EGFR	Gefitinib	Phase II NCT00077025 n = 93	Advanced	None	Extended time to disease progression	[67]
	Gefitinib	Phase II NCT00229697 n = 206	Advanced	None	Extended time to disease progression	[68]
	Gefitinib	Phase II NCT00057941 n = 141	Advanced	None	No significant improvement	[69]
	Lapatinib	Phase III n = 219	Advanced	HER2	Extended time to disease progression	[71]
	Trastuzumab	Phase III TAnDEM n = 207	Advanced	HER2	Extended time to disease progression	[72]
IGF1R	AMG479	Phase II n = 156	Advanced	None	No significant improvement	[73]
Src Abl	Dasatinib	Phase II n = 120	Advanced	None	Extended time to disease progression	[74]

as to whether tumours need to acquire drug resistance before mTOR inhibitors can truly be beneficial or if mTOR inhibitors have a role in treatment of early stage disease. Development of new drugs which more comprehensively block the mTOR signalling pathway is also ongoing although still at the preclinical stage [66].

The area of growth factor inhibitors is another field which has seen much clinical activity. Several growth factors including EGFR and IGF1R have been implicated in ligand-independent phosphorylation of ER (see Table 7.1). Gefitinib, an EGFR inhibitor, has been used in several phase II clinical trials of advanced breast cancer with mixed results. In combination with anastrozole, gefitinib was found to extend progression free survival [67]. Similar results were found for gefitinib in combination with tamoxifen [68] although a separate phase II trial examining gefitinib with anastrozole or gefitinib with fulvestrant concluded that there was no clear benefit to the addition of gefitinib to either endocrine therapy [69]. Interpreting the results of these trials is complicated by the intricate treatment histories of metastatic breast cancer patients. Interestingly, preliminary data suggests that patients who have not previously received an endocrine therapy and particularly those who have not displayed signs of AI resistance may respond best to gefitinib [67, 68]. Further clinical studies, perhaps with selection of EGFR expressing tumours, will be required to assess if gefitinib can provide reliable benefit to AI resistant patients [70]. Lapatinib, a dual EGFR and HER2 inhibitor and trastuzumab, a monoclonal antibody targeting HER2 have also made good progress through clinical trials. Lapatinib in combination with letrozole has proven beneficial in a phase III trial of metastatic breast cancer patients with hormone receptor positive tumours, although only HER2 positive patients received benefit [71]. Trastuzumab in combination with anastrozole also produced successful phase III study results but only HER2 positive patients were included in this trial [72].

By contrast to the EGFR family inhibitors, drugs targeting IGF1R have had poor success in the clinic. A phase II clinical trial showed that AMG479, an IGF1R inhibitor, provided no benefit in terms of progression free survival when given in combination with exemestane/fulvestrant compared to patients treated with the AI alone [73]. At this point in time it seems that while growth factor receptor inhibitors have shown some very positive and promising results, further studies will be required, perhaps segregating patients based on previous treatment regimes, and maybe selecting patients based on expression of growth factor signalling markers, to truly assess the benefit of combining growth factor inhibitors with endocrine therapy for treatment of AI resistant breast cancer.

Similar conclusions have been reached based on clinical trials of the multikinase inhibitor dasatinib [74]. An inhibitor of both Src and ABL kinases, dasatinib is already approved for treatment of chronic myeloid leukemia and is relatively well tolerated in patients. Phase II clinical trial data were less convincing than anticipated but suggest that dasatinib may be able to inhibit the development of acquired AI resistance. The study results are once again complicated by the diversity of patients in terms of which treatments they have previously been exposed to. Dasatinib therapy may well benefit from a companion biomarker to identify patients who are likely to benefit based on activity of the relevant kinases [74].

In summary, ligand-independent phosphorylation and activity of ER is a well recognised mechanism of endocrine resistance. Extensive research has been performed to identify the detailed mechanisms of phosphorylation and the relevant kinases that are involved. Further research in this area would be beneficial to confirm the relevance of these kinases specifically to AI resistance. To date, everolimus, trastuzumab and lapatinib are approved drugs which may be given in combination with AI therapy. Amongst other functions, they may combat the ligand-independent phosphorylation of ER and re-sensitise tumour cells to AI therapy.

Role of Co-factors in Ligand-Independent ER Signalling

Transcriptional activity of ER is dependent on the presence of co-factor proteins. Co-activators induce transcriptional activation of the nuclear receptor, whereas co-repressors decrease transcriptional activity. Over 350 co-regulatory proteins have been identified for nuclear receptors, the most studied ER co-activator proteins are the SRC/p160 family (reviewed by Lonard and O'Malley [75]). There are three homologous family members SRC-1, SRC-2 (TIF2/GRIP1) and SRC-3 (AIB1/RAC3/ACTR). Each of the SRC family members has been implicated in the development and/or progression of ER positive-breast cancer [76]. Most notably, SRC-3 is amplified in approximately 10 % of breast cancers and transcript levels are found in the majority of primary breast tumours [77].

During normal transcriptional regulation, co-activators bind via their LXXLL motifs (NR-box) to the AF-2 domain of the nuclear receptor in the presence of an agonist ligand [78]. Co-activator proteins themselves have low intrinsic histone acetylation capacity and tend to recruit histone acetylating co-integrator proteins, including CBP/p300 and pCAF [79]. In the absence of ligand (or bound to an agonist/modulator) the ER interacts with co-repressor proteins such as nuclear receptor co-repressor (NCoR) and silencing mediator of steroid retinoid and thyroid hormone receptor (SMRT). These co-repressors bind via their LXXI/HIXXXI/L motifs (CoRNR box) to helix 3 and 5 of the receptor [80, 81], showing that surprisingly subtle differences in the recognition motifs may mediate the switch from co-repressors to co-activators upon ligand binding to receptors. In endocrine resistant breast cancer, however, enhanced growth factor receptor signalling induces receptor co-activator interactions in the absence of ligand or in the presence of SERMs such as tamoxifen. In this setting, co-activators such as the SRC/p160 family members, have been shown to bind to the AF-1 ligand-independent transactivator domain of the ER [82].

The development of resistance to endocrine therapy is due at least in part to cellular plasticity, leading to growth factor cross talk and a shift from steroid dependence to steroid independence/growth factor dependence. Enhanced growth factor signalling may result, not only in ligand-independent activation of ER, but also in inappropriate nuclear receptor co-activator interactions, leading to transcriptional

activations of genes important in disease progression. Aberrant expression of the p160 steroid receptor co-activators, in particular SRC-1 and SRC-3 (AIB1) in patients has been associated with resistance to endocrine therapies and the development of metastatic disease [83, 84]. Similar to the growth factors involved in regulation of ER phosphorylation, the co-repressors and co-activators of ER may also be involved in a complex interplay with other nuclear factors. For instance association of p160 with endocrine resistance may be mediated through cMyc [84].

Systemic and tumour levels of estrogen are suppressed in patients following treatment with aromatase inhibitors. Alterations in the steroid environment have an impact on growth factor signalling and expression levels of co-activator proteins, which together may have impact on ligand-independent activity of ER. In breast cancer patients treated with neoadjuvant aromatase inhibitors, elevations in transcript levels of tyrosine kinase receptor, HER2 and co-activators have been reported following neoadjuvant AI treatment [85]. Molecular studies reveal that HER2 overexpression can induce ligand-independent recruitment of AIB1 and CBP to ER target genes in models of AI treated breast cancer [86]. In the adjuvant setting, expression of AIB1 in primary breast tumours predicts poor response to AI therapy in ER positive breast cancer patients [34]. Moreover, SRC-1 was found to associate with reduced disease free survival in primary and/or resistant metastatic AI treated tumours [87]. These molecular and clinical studies suggest that enhanced growth factor signalling following steroid depletion may result in inappropriate co-activator ER interactions and subsequent poor response to AI therapy in patients.

Therapeutic approaches aimed at disrupting co-activator nuclear receptor interactions have been limited. However, recently two small molecule inhibitors have demonstrated efficacy in inhibiting SRC proteins. Gossypol has been shown to inhibit both SRC-1 and SRC-3 in vitro although early clinical trials showed negligible antitumour activity in a small cohort of refractory metastatic breast cancer patients [88, 89]. More recently, the cardiac glycoside bufalin was identified by a high throughput screening process to identify more effective SRC small molecule inhibitors. Bufalin has been shown to reduce SRC-1 and SRC-3 protein expression and retard cancer cell growth both in vitro and in vivo [90]. Furthermore, bufalin in combination with an AKT inhibitor was shown to be more effective at inhibiting cell proliferation than either agent alone [90]. While large scale clinical trials are still awaited, these small molecule inhibitors may prove powerful new agents to use in a combinatorial strategy with AIs to treat patients who have failed first-line endocrine therapy.

Hyper-sensitivity to Low Concentrations of Ligand

Although not strictly a “ligand-independent” mechanism, the concept of adaptive hypersensitivity to low levels of estrogen is well established and plays an important role in explaining endocrine resistance. The ability of tumour cells to adapt to

reduced levels of estrogen is evident from the clinic: premenopausal women often display tumour regression following ovariectomy and yet relapse can occur despite the substantially reduced estrogen levels. Furthermore, secondary tumour regressions can be observed following administration of an AI to further deplete the estrogen supply [91]. An in vitro study from the 1990s demonstrated that MCF7 cells, deprived of estrogen for up to 6 months, became hypersensitive to estrogen, displaying similar growth responses to 10,000 fold less estrogen than matched cells which had not been estrogen deprived [92]. In vitro, it is difficult to completely remove all traces of estrogens due to residual estrogens being leaked from plasticware and even from non-phenol derivatives [93, 94]. Research has suggested that the exact mechanisms of the hypersensitivity may depend on the levels of residual estrogen which are present [95]. In the clinical setting, it is quite likely that estrogen is not entirely depleted and it is certainly hypothesised that low levels of estrogen may remain despite endocrine therapy. A recent study, measuring plasma hormone levels in anastrozole treated patients, demonstrated large variation from one patient to the next, suggesting that the standard dose of 1 mg anastrozole daily may not be optimal for estrogen depletion in all patients [96]. This, combined with issues of adherence to treatment regimes, certainly suggests that many AI treated breast cancer patients may have residual low levels of estrogen which would be available to activate a hypersensitive receptor.

The mechanism of action of a hypersensitive ER, activated by low levels of estrogen, may not be identical to the mechanism of action of an ER which is in a plentiful supply of estrogen. Santen and colleagues are central to this area of research and have reported that classical ER-mediated gene transcription does not seem to be the primary mechanism by which the hypersensitive ER signals [94]. Indeed, interactions with signalling pathways such as IGF1R, EGFR, MAPK and PI3K, together with non-genomic roles of the ER at or near the cell membrane seem to play important roles [30]. The implications of these findings are that the hypersensitive actions of the ER could potentially be inhibited by disrupting some of these signalling pathways which may be a more achievable goal than trying to remove all residual levels of estrogen. As seen in Table 7.1, a number of these pathways are already the focus of clinical trials in AI resistant breast cancer and our understanding of ER hypersensitivity adds an additional mechanism by which these drugs may be producing their beneficial effects. Similarly, co-factors have also been implicated in ER hypersensitivity. Overexpression of the ER co-regulator Ciz1 confers estrogen hypersensitivity on breast cancer cells and so monitoring and combatting co-factor expression may be an appropriate way to combat ER hypersensitivity [97].

The original study by Masamura et al. [92], which illustrated ER hypersensitivity in cells which had been deprived of estrogen for a number of months, also demonstrated that by returning cells to estrogen, normal sensitivity could be regained. There is some logic to applying this same approach to the clinic. If AI resistance is developing through the mechanism of ER hypersensitivity, then withdrawing the patient from the AI would restore normal estrogen levels and may reverse the hypersensitivity. There is data to support withdrawal of tamoxifen as a

strategy [98] and multiple case studies report positive response upon withdrawal of AIs in advanced breast cancer patients who have previously progressed on endocrine therapy [99–103]. A single-arm phase II clinical trial with 24 patients demonstrated the benefit of AI withdrawal as a mechanism to combat AI resistance in selected metastatic breast cancer patients [104] but so far these treatment strategies have not become widely used in the clinic.

Ironically, treating AI resistance with additional estrogen is a more commonly used treatment strategy. High doses of estrogen are known to inhibit cell growth and can induce apoptosis [92]. Again, this strategy works particularly well to target the ER hypersensitivity mechanism of AI resistance because a hypersensitive receptor is likely to respond to lower levels of estrogen and therefore the steroid treatment can be given at low doses in these circumstances to prevent or limit adverse side effects. The therapeutic benefits of high dose estrogens were demonstrated in a phase II trial of diethylstilbesterol in advanced endocrine resistant breast cancer patients [105]. Subsequently, a phase II clinical trial showed that 6 mg of daily estradiol produced similar benefits to 30 mg in advanced breast cancer patients who had previously progressed on AI therapy [106]. The lower dose of estradiol produced substantially less toxicity than previously tested doses of estrogenic compounds.

Amplification and Overexpression of ER

Importantly, when considering the persistent ER activity which can be present in the AI resistant setting, it should be noted that expression levels of the ER are often considerably altered in AI resistant tumours relative to AI sensitive tumours. It has long been established that there is a direct correlation between the level of ER expression and the likelihood of clinical response to hormonal therapies. Thus patients whose primary tumours express high levels of ER are most likely to respond to AI therapy (reviewed by Brouckaert et al. [107]). There is now evidence for the existence of amplification of the ESR1 gene in some breast cancers although the frequency of this, the impact on ER protein expression and the clinical significance of amplification remain under debate. A study published in 2007 screened more than 2000 breast cancer tumours and detected ESR1 gene amplification in approximately 20 % of cases. Gene amplification correlated with strong protein expression of ER in this study. Furthermore, patients with ESR1 amplification displayed significantly longer survival, suggesting that ESR1 amplification is beneficial or at least predicts the likelihood of responding to endocrine therapy [108]. However, results from this study were strongly contested the following year by four other groups (summarised by Albertson [109]). They opened up debate about methodologies used to detect amplifications and contested the reported high frequency of ESR1 amplification suggesting that 5 % might be a more representative number than 20 %. Since then one further study supports the original 2007 paper and confirms the correlation between ESR1 amplification

and favourable prognosis [110]. However, two subsequent studies have reported correlations between ESR1 amplification and poor survival, in particular a poor response to endocrine therapy [111, 112]. In support of these latter studies, in vitro work using a cell line model of AI resistance, has observed ESR1 amplification during the development of long term estrogen deprived (LTED) cells. Thus the hypothesis certainly exists that amplification of ESR1, resulting in increased expression of ER, could contribute to the development of AI resistance although this hypothesis deserves more attention as the jury remains out as to whether this is the case or not. If a definitive link between ESR1 amplification and AI resistance is substantiated in the future, a reproducible method for detecting the amplification will be required.

Distinct from monitoring ER expression levels or activity before treatment as a predictor of response, a number of groups have researched changes in ER following AI treatment. Cell models have clearly shown that following estrogen deprivation, expression levels of ER can increase by four to tenfold which contributes to the hypersensitivity of the ER and the development of AI resistance [94]. While suggesting an attractive theoretical model, it should be noted that increased ER expression during AI treatment has not been confirmed in clinical samples [113]. Separately, a number of groups in recent years have detected the occurrence of single nucleotide mutations in ESR1 which develop following endocrine therapy and are likely to contribute to the mechanisms of AI resistance. These mutations, particularly those at amino acids 537 and 538 which are located at the start of helix 12 in the LBD, render the ER constitutively active, independent of the presence or absence of ligand [114–117]. The existence of ESR1 mutations is covered in detail in a separate chapter of this book but it is worth pointing out that such mutations certainly offer a mechanism for ligand-independent activity of the ER. Furthermore, assessing the presence of mutations in recurrent tissue is proposed as a biomarker for detecting persistent ER signalling and as such as a marker of AI resistance.

Activation of ER by Alternative Ligands

A classic difference between AI resistance and tamoxifen resistance is that tamoxifen, which binds directly to the ER and prevents estrogen from binding, has the potential to act itself as an agonist and modulate ER signalling. In contrast, aromatase enzyme inhibitors which prevent estrogen synthesis, do not have the potential for direct modulation of ER function. Therefore, AIs can not prevent interaction of ER with alternative ligands capable of such interaction. Recent in vitro studies demonstrated that androgen metabolites are capable of binding to and activating the ER under extreme estrogen-depleted conditions [118]. Preliminary clinical studies have also observed a correlation between elevated expression of androgen metabolising enzymes and ER activity in patient breast tumours, particularly those from postmenopausal women [119]. Furthermore, Takagi et al.

[120] demonstrated that intratumoral concentrations of the androgen DHT were higher in breast carcinoma tissues of women who had been treated with exemestane compared to levels in untreated women. Recently, androstenedione levels have been shown to be higher in patients receiving second line AI therapy (patients who already demonstrated resistance to one or more AIs) compared to adjuvant AI treated patients (who have not yet displayed signs of AI resistance) [121]. The consequences of higher androgen levels with regard to AI resistance remain unclear. The ability of androgens or their metabolites to activate ER is likely to be strongly influenced by the availability of AR, for which they have much higher affinity than ER [118]. As the majority of ER positive breast cancer patients also express AR, it remains unclear if androgens play a meaningful role in direct binding to and activation of ER. Accumulating evidence does suggest that androgens, through activation of the AR, may compliment ER signalling but the role of the AR in AI resistance is beyond the scope of this chapter [122]. Needless to say, it is important to recognise that, in contrast to tamoxifen, AIs have no role against exogenous and environmental estrogens that may have biological effects on breast cancer [123].

Challenges in Detecting Persistent ER Signalling

Having established that ligand-independent ER signalling plays a role in AI resistance, the challenge now is to detect, as early as possible, when inappropriate ER signalling is taking place and ideally to try to intervene. As we have seen, ligand-independent ER signalling can occur through a number of mechanisms and therefore the most comprehensive way to monitor it would be by examining the active phosphorylation status of the ER itself. As described above, monitoring the expression of pSer118 [38, 45] or pSer167 [46, 47] have potential value in terms of stratifying patients into those likely to respond to AI therapy and those less likely to respond. The development of reliable antibodies to specifically detect the individual sites of ER phosphorylation will be the key to bringing these biomarkers into routine clinical testing. This area is also complicated by questions over the stability of phosphorylation status in formalin fixed paraffin embedded (FFPE) tissue and so extensive testing is required to bring a phosphorylation antibody into routine, reliable clinical use.

Studying ligand-independent ER binding to chromatin and ligand-independent target genes is another area to potentially identify biomarkers of AI resistance. The advances in next generation sequencing technology have meant that much progress has been made in identifying the genome-wide targets of ER in endocrine resistance. ChIPseq has been performed for ER in a number of steroid depleted breast cancer cell models including MCF7 cells [124]; long term estrogen deprived, LTED, cells [125]; letrozole resistant, LetR, cells (McBryan, unpublished data) and tamoxifen resistant, TamR, cells [126]. The findings to date demonstrate that ligand-independent ER target genes are a subset of

ligand-dependent ER target genes and so a comprehensive understanding of these targets will be necessary in order to identify suitable signatures with which to monitor AI resistant ER activity. Recently, ER ChIPseq has also been completed in several patient breast tumours following endocrine treatment [125, 127]. This data confirms that ER-chromatin occupancy is still detected in vivo following AI therapy and following the development of drug resistance although insufficient patients have been profiled so far to suggest clinically relevant biomarkers.

Of course the major challenge with AI resistance is to identify patients who have "persistent" ER signalling. The word "persistent" implies that you need to look at a minimum of two time points such as before and during treatment to detect if signalling is maintained despite the treatment intervention. The neoadjuvant setting lends itself well to this. In addition to persistent pSer118 expression [45] (Fig. 7.2b), persistent expression of a number of ER target genes have been associated with poor clinical outcome following neoadjuvant AI therapy [128]. Identifying a gene signature to detect persistent ER signalling is additionally complicated by the dynamic nature of ER and the ability of tumour cells to adapt. A small number of genes, including several ER target genes, appear to respond to AI treatment within 2 weeks of treatment but subsequently adapt and become re-expressed by 3 months [129] (McBryan, unpublished data). Thus, it will be important not only to define a gene signature but also a specific time point or duration of AI treatment in which to monitor this signature.

While detecting phosphorylated ER or ER target genes might be the best way to identify persistent ER signalling, it may not be the most beneficial or informative to select a treatment strategy as it does not give information about the mechanism involved in resistance. Bearing in mind the current targeted treatment strategies that we have available, it would be more useful to identify which kinase or which growth factor signalling pathway is involved rather than simply whether ER activity is still maintained. Thus identifying biomarkers to act as companion diagnostics for each drug may help to identify the specific mechanisms of resistance and should lead to the development of personalised medicine for breast cancer patients.

Approaches to Combat Ligand-Independent ER Signalling

As mentioned throughout this chapter, there are a number of specific approaches to combat ligand-independent ER signalling. For example, small molecule inhibitors can combat the steroid receptor co-activators; growth factor and kinase inhibitors can combat ligand-independent phosphorylation; re-exposure to estrogen can combat ER hypersensitivity. Ultimately however, the research points to a continued role for ER signalling despite the intervention of AI therapy. A logical way to combat this would be to directly inhibit or remove the ER itself, in addition to removing its ligand.

Fulvestrant is an ER antagonist which, unlike tamoxifen, has no ER agonist functions. Fulvestrant is a competitive, reversible antagonist which works by inhibiting ER dimerisation and DNA binding as well as increasing ER turnover degradation and inhibiting nuclear uptake of the receptor [130–132]. Preclinical data shows that fulvestrant is effective, to a greater extent than tamoxifen, at suppressing the growth of ER positive tumours [133]. Successful phase II clinical trials have since led to the approval of fulvestrant as a treatment for metastatic hormone receptor positive breast cancer. Moreover, experimental studies suggest that combining fulvestrant with anastrozole may be more beneficial than using either agent alone [134]. Phase III trials however which combine fulvestrant with an AI are inconclusive. The SWOG S0226 trial reported better progression free survival and clinical improvement in patients treated with an AI and fulvestrant combination compared to those treated with AI alone, whereas the EFECT and FACT trials suggest there was no significant difference between treatment arms [135–137]. Once again, interpretation of these results is difficult due to the varied pre-trial treatment regimes that these metastatic patients had received. The SWOG trial contained a larger number of endocrine naïve patients and it has certainly been suggested that fulvestrant may prove more beneficial if placed earlier in the sequence of endocrine therapies for metastatic patients [138]. An additional confounding aspect is the issue of fulvestrant dosage. Initially approved for monthly delivery with a dose of 250 mg, it has since been shown that a 500 mg dose is superior to this and that patients can benefit by an additional loading dose within the first month [139]. The suboptimal 250 mg dose was predominantly used in the phase III trials listed above which may have contributed to the lack of clinical benefit observed. More recently a phase II study, FIRST, using the 500 mg dose has provided promising results [140] and a phase III study using the higher dose is ongoing (FALCON) with results anticipated in 2015.

Hopefully by adjusting dosage regimes and carefully selecting patients the efficacy of fulvestrant in the AI resistant setting can be improved. However, overall results from clinical trials have been a little disappointing for this drug, given our understanding of persistent ER signalling in AI resistance. Alternative ER antagonists have been developed such as EM-800 for which some positive preclinical and early clinical trial data has been gathered although no large scale clinical studies have been completed as of yet [141]. Our understanding of persistent ER signalling, occurring in a ligand-independent manner, certainly warrants further research to try to develop more clinically active antiestrogens for the treatment of AI resistant breast cancer patients.

Conclusions

Overall the aromatase inhibitors currently used in the clinic are extremely effective at minimising both circulating and tumour levels of estrogen. However, it is now clear that this does not always produce the anticipated consequence of removing

all ER signalling. Tumour cells seem adept at adapting to their changing environment and many survive by developing alternative mechanisms to activate the ER in a ligand-independent manner. The heterogeneity of each tumour and each tumour microenvironment make it challenging to find suitable treatments for this form of AI resistance. What is clear is that AI treated tumour cells are adapting to the lack of ligand and a key aspect to treating AI resistance is to sample recurrent tumour cells and monitor the signalling pathways that have become active rather than relying purely on the prognostic information available from the treatment naïve primary tumour. Extensive progress has been made to understand individual pathways involved, identify suitable biomarkers and develop drugs to target these pathways. This progress has led to expansion of the drug arsenal available to treat AI resistant patients. The options and drug combinations now available to tackle metastatic breast cancer have become far more advanced and are directed by the underlying molecular biology. With the exception of a pure antiestrogen, it is unlikely, based on disease heterogeneity, that any one blockbuster drug will be able to overcome AI resistance associated with ligand-independent ER signalling. However, the cross over of signalling pathways between ligand-independent ER signalling pathways and HER2 positive or triple negative pathways is substantial, thus newly designed drugs are likely to have a large impact across an array of breast and indeed other cancers.

Conflict of Interest The authors declare there is no conflict of interest.

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Chapter 8
Chromatin and Epigenetic Determinants of Resistance to Aromatase Inhibitors

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Abstract Resistance to aromatase inhibitors (AI) involves extensive transcriptional reprogramming to overcome drug-mediated cell cycle arrest. Chromatin accessibility and epigenetic modifications play a crucial role in regulating gene expression in normal development and contribute to aberrant transcription commonly found in malignant cells. In this chapter we will discuss the latest research findings linking AI resistance to epigenetics. Initially we will examine the epigenetic regulation of the aromatase gene. The estrogen receptor activity is significantly influenced by the chromatin template and we will present evidence on how the estrogen receptor-chromatin crosstalk can contribute to the development of resistance. Next we will consider alternative survival pathways and how they are epigenetically regulated in response to AI treatment. Afterward, we will review the current state of epigenetic-based medicine in the context of endocrine therapies. Finally, we will offer some insights on future challenges and possible breakthroughs.

Abbreviations

AI	Aromatase inhibitor(s)
BCa	Breast cancer
ERα	Estrogen receptor α
TFs	Transcription factors
ERE	Estrogen response elements
Pol II	RNA polymerase II
SERMs	Estrogen receptor modulators
HGP	Human genome project

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A. Larionov (ed.), *Resistance to Aromatase Inhibitors in Breast Cancer*,
Resistance to Targeted Anti-Cancer Therapeutics 8,
DOI 10.1007/978-3-319-17972-8_8

ChIP-seq	Chromatin immunoprecipitation followed by next generation sequencing
ArKO	Aromatase knockout
TSS	Transcriptional start site
REs	Transcription factors response elements
NRs	Nuclear receptors
LTED	Long-term estrogen deprived cells
CSCs	Cancer stem (like) cells
SERD	ERα downregulators
DNMT	DNA methyltransferases
ZFPs	Zinc fingers proteins
TADs	Topological associated domains
PDX	Patient's derived xenograft (PDX)

Introduction

Estrogens are steroid hormones involved in a number of physiological processes including the development and maintenance of the female reproductive systems, several neuro-endocrine and metabolic functions. Also, these hormones have crucial roles in certain disease states, particularly in mammary carcinomas. Cancer is the leading cause of death among women between the ages of 30 and 54, with breast cancer (BCa) comprising 28 % of all cancers in females per year [1]. Estrogens act by binding to a nuclear receptor called Estrogen Receptor α (ERα). Nuclear receptors are a large family of ligand-dependent transcription factors (TFs), involved in several physiological processes including differentiation, proliferation and metabolism [2, 3]. After the binding to estrogen, ERα forms homodimers and interact with the DNA at sequence-specific estrogen response elements (ERE) localized in regulatory regions (see next section) near ERα target genes. ERα binding to the DNA along with interaction with various co-factors, facilitate RNA Polymerase II (Pol II) recruitment and initiation of transcription at hundreds genomic loci (including coding and non coding RNAs) [4, 5]. ERα targets encompass enzymes, receptors, and secreted factors that orchestrate the steroid hormonal response regulating cell function, growth, and differentiation. It is well established that estrogens enhance growth and proliferation of ERα positive breast epithelial cells and estrogen-dependent mammary carcinoma cells with over 70 % of all breast cancers carrying ERα. ERα positive BCa exhibits highly variable prognosis, histological growth patterns and treatment outcomes [6]. Different approaches have been developed to reduce estrogen-dependent growth in BCa (endocrine therapies) [6]. Endocrine therapies abolish estrogenic signaling either through preventing estrogen synthesis or by inhibiting transcriptional activity of ERα. Antiestrogens compete for binding to the ERα and reduce the number of receptors available for binding to endogenous estrogens. This approach has proven effective as an anticancer strategy and has led to the development of agents such as Tamoxifen (and other estrogen receptor modulators-SERMs). However, Tamoxifen

present with several severe side effects and between 25 and 30 % of patients develop Tamoxifen resistance within the first 10 years. Importantly, Tamoxifen is not purely an ERα antagonist but can also act as an agonist in certain tissues. For all these reasons, the field has developed other modalities of endocrine therapies including aromatase inhibitors (AIs). These compounds can efficiently block peripheral production of estrogens without introducing any agonistic activity.

Aromatase is the enzyme that converts androgens (testosterone and androstenedione) to estrogens (estradiol and estrone, respectively). Aromatase inhibitors block the enzymatic activity of the aromatase protein (encoded by the CYP19A1 locus). In the last 10 years, third-generation AIs (Exemestane, Letrozole, Anastrozole) have largely replaced tamoxifen as adjuvant treatment for early and metastatic ERα-positive breast cancer in postmenopausal women [7, 8]. Both anastrozole and letrozole are more successful than tamoxifen as first-line therapy in postmenopausal women with advanced BCa [9, 10]. Exemestane has also shown enhanced efficacy over tamoxifen as first-line therapy in postmenopausal women with advanced breast cancer [7, 8, 11]. Despite being generally more effective than Tamoxifen, AIs treatment still results in the development or resistant BCa in a significant proportion of patients. In addition, estrogen deprivation does not completely arrest ERα activity, and transactivation of the unliganded receptor may continue through crosstalk with growth factor pathways. In this chapter we will introduce several concepts related to epigenetics and chromatin modifications and discuss their role in the context of aromatase inhibitor resistance. Starting from epigenetic regulation of the CYP19A1 locus, we will then explore how alternative pathways are epigenetically activated in response to drug treatment. Next, we will consider the potential of using chromatin marks as predictive and prognostic biomarkers. Finally, we will examine the current state of the art for epigenetic interventions to fight resistant disease.

On Epigenetics, Chromatin and Regulatory Elements

The human genome project (HGP) was completed in 2001 [12], and one of the most astonishing finding was that only about 5 % of the genome is actually coding for proteins. The cost of the HGP was around 1 billion dollars and it took 13 years, giving scientists an unprecedented understanding of the role of the genetic sequence. This also led to a dramatic improvement of our understanding of cancer, since tumours were substantially considered as a genetic driven disease. In the next few years a tremendous amount of resources have been devoted to analyze several cancer genomes, leading to important advance in the clinic. Nonetheless, it was noted that most of genetic variability occurs outside gene bodies and these results were not easily understood in the context of what has been for a long time a gene-centric field. Indeed, gene mutations cannot always explain cancer-related phenotype, including resistance to therapy.

Cancer cells are characterized by aberrant transcription and we had known for a long time that difference in gene expression does not impinge solely on the DNA

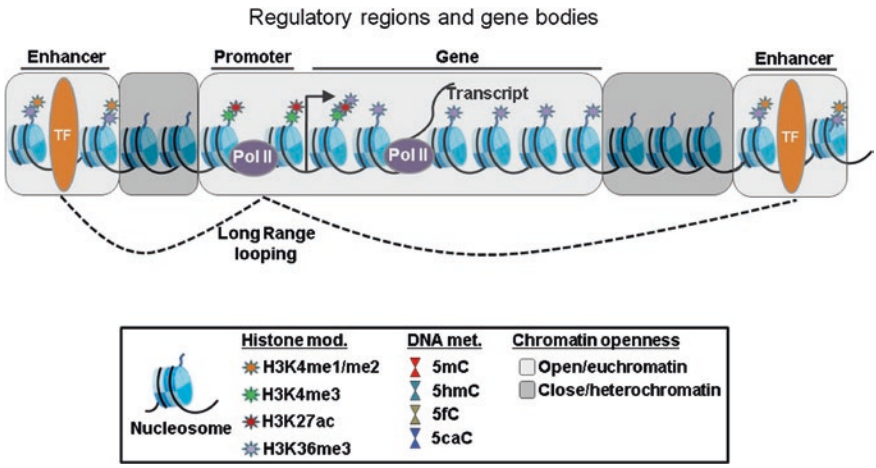

Fig. 8.1 Schematic representation of a prototypical gene with its associated regulatory regions. The transcriptional start site is represented as an *arrow*

sequence. Indeed, the tremendous amount of phenotypic difference caused by cell type specific gene expression during development is not driven by genetic variability at all. Slowly but steadily, in a process driven by the constant decrease of technical and economic barriers, the focus have shifted on the so-called "junk DNA", the dark matter of our genome. The ENCODE project [13, 14] was developed to understand the net contribution of the remaining 95 % of our genome with the underlying hypothesis that a significant proportion of non-coding DNA regions would be involved in regulating gene expression. With the development of novel biochemical and bioinformatic tools it emerged that an extensive collection of regulatory regions exists embedded within our genome. These regulatory loci are engraved with transcription factors binding motifs, and are frequently found in well-defined chromatin configurations [14]. Regulatory regions can broadly be categorized by their relative distance to genes and include promoters [15] (proximal to genes), enhancers [16] (distal and active in both orientations) and insulators [17] (Fig. 8.1).

Epigenetics is broadly defined as transmission of gene expression information through mitosis without changes within the DNA sequence. Development is a striking example of epigenetics where sequential epigenetic changes progressively shape cell lineages and cell types. Epigenetic information is stored in several chemical modifications occurring on the DNA template (DNA methylation) or on the minimal chromatin unit (the nucleosome). Nucleosomes are multi-subunit particles composed by 4 couples of histones (H2A, H2B, H3 and H4) [18]. The DNA is wrapped around the nucleosome in 147 bp segments in what is known as the "beads on a string model" [19]. The N-terminus tail of histone proteins can be modified by a variety of histone-modifying enzymes to include covalent chemical modifications such as lysine and arginine methylation, lysine acetylation, serine phosphorylation, etc. [20]. Interestingly, several of these chromatin modifying

enzymes are frequently mutated in cancer [21]. The collection of these modifications is at the base of the histone code theory [22].

Regulatory elements can be identified in a genome wide fashion by using these histone modifications. The histone code theory postulates that regulatory elements are defined by a combinatorial chemical alphabet written on histones. A technique called ChIP-seq (Chromatin Immunoprecipitation followed by next generation sequencing) has been extensively used to quickly and accurately map epigenetic modifications and TFs binding sites on a genome-wide manner [23]. For examples, active enhancers correlate with the presence of H3 lysine 27 acetylation mark (H3K27ac) and the H3K4monomethylation mark (H3K4me1) [24]. On the other hand, enhancers associated with repressed transcripts are enriched for H3K27me3 [24]. Therefore, TFs binding occurs mostly within epigenetically defined regulatory regions. It is thought that distal regulatory element (e.g. enhancers) interact with promoters via long-distance chromatin looping to bring TFs and RNA-Polymerase II close to the gene transcription starting sites [25].

The field is now quickly moving toward data integration between histone modifications and TFs binding maps, chromatin accessibility genome wide scans and other approaches in order to catalogue the entire repertoire of human enhancers while linking them to coding regions. Considering the central role of epigenetic events in cancer and several other diseases, it is not surprising that epigenetic factors may also contribute to the development of AI resistant breast cancer. In this chapter we will discuss how chromatin and epigenetics contributes to de novo AI resistance and how epigenetic reprogramming can partly explain the development of acquired AI resistance.

Genomics, Epigenetics and Transcriptional Regulation of Aromatase Gene

Aromatase inhibitors target the enzyme activity in peripheral tissues, such as adipose, and in certain estrogen-dependent tumours including breast cancer, endometrial cancer, endometriosis, and uterine fibroids. Although the reproductive effects of ovarian estrogen in females have been well understood for decades, only with the development of knockout mouse models the field has had a breakthrough in understanding the functions of non-ovarian estrogens in both males and females. The aromatase knockout (ArKO) mouse bear targeted disruptions of the Cyp19a1 gene, causing almost a complete abolition of estrogen production in homozygous animals [26, 27]. Fetuses of these mice display impaired gonadal development and defective mammary glands formation in females. Aberrations such as accumulation of excess adipose tissue, as well as reproductive and bone abnormalities were seen in both male and female ArKO mice [26, 27]. Mutations resulting in aromatase deficiency, accordingly, result in sexual infantilism and pubertal failure, primary amenorrhea, ambiguous external genitalia at birth, and polycystic ovaries [26, 28]. Genetic alteration resulting in overexpressed and overactivated aromatase

has also been clinically reported. In this conditions males present with gynecomastia [29–31] and females undergo premature onset of puberty, display gigantomastia, enlarged uterus and irregular menstrual cycle. These phenotypes results from excessive conversion of androgens into estrogens, and patients may be successfully treated with AI [32, 33].

Intratumoral aromatase expression can be detected in a large proportion of invasive ductal carcinoma [34, 35], and is retained during metastasis [36], suggesting the fundamental role of this enzyme in the survival of estrogen-dependent breast carcinoma cells. Tumours may also obtain estrogens by active uptake from circulation. The balance between uptake and synthesis may vary significantly between patients [37]. It has been suggested that local estrogens production in breast cancer can occur in fibroblasts surrounding cancer cells [38, 39] as well as in the cancer cells themselves [40, 41]. In fact, estrogen concentrations within the local breast tumour surroundings are up to ten times higher than circulating levels [42]. Altogether, endogenous aromatase activity in BCa appears to be one of the important mechanisms leading to increased local estrogen concentration.

Breast tumours are characterized by a heterogeneous population of cells that generate cell type-specific and cell type-codependent survival signals, which collectively contribute to malignancy [43]. Scientific reports have shown that a number of genomic rearrangements [44] and epigenetic changes [45] in BCa appear to contribute to this heterogeneity. Aromatase is encoded by the CYP19A1 gene, located on chromosome 15, band q21 of the human genome. The majority of studied vertebrates, with some exceptions, have a single Cyp19a1 gene copy [46, 47]. *CYP19A1* transcription is tissue-specific and tightly regulated [48]. Besides some subtypes of BCa and gonads, aromatase is widely expressed in a large variety of tissues such as the bones, skin, adrenals, adipose tissue, fetal liver and placenta [49]. In addition, aromatase is also well expressed in the central nervous system (e.g. in the hypothalamus, cerebral cortex, hippocampus, cerebellum) of all vertebrates [49]. Ten tissue-specific promoters have been identified within the 93-kilobase promoter region upstream of the Aromatase coding sequence [49]. Such a long distance between some of the alternative promoters and the coding sequence suggests an important role of enhancers and chromatin loops in the tissue-specific regulation of Aromatase gene, which is yet to be studied. Despite differences in the transcriptional start site (TSS), the aromatase protein is identical in all tissues in which it is expressed. In normal breast tissue, aromatase expression is mainly controlled through activation of promoter I.4 [49]. However, in BCa 3 additional promoters are used to initiate transcription [50], I.7, I.3, and II. Despite a switch to the preferential use of PII and PI.3 in the tumour [51], PI.4 transcripts are also upregulated and contribute a significant portion of total aromatase expression in BCa. The use and switch of different aromatase promoters result in 3–4 fold increase in aromatase transcripts in the tumour-bearing breast [52]. Therefore, local epigenetic control of CYP19A1 transcription may significantly contribute to AI resistance.

Several studies have investigated the effects of genetic polymorphisms within the aromatase gene. In contrast, little is known regarding its epigenetic regulation. Interestingly, recent data have suggested epigenetic regulation via differential DNA

methylation of PI.4. Typically, DNA methylation is associated with inhibition of gene expression, while genes activation has been linked to the demethylation of critical CpG loci at promoter regions [53]. However, intragenic DNA methylation seems to be strongly associated with increased gene expression [54]. Human ex vivo breast adipose fibroblasts and breast cell lines were used to test the association of DNA methylation within the I.4 and PI.3/II promoters and gene expression [55]. The authors found an inverse association between DNA methylation within the aromatase I.4 promoter region and aromatase gene expression in human breast adipose fibroblasts stimulated by dexamethasone and TNF-alpha [55]. In contrast, DNA methylation of specific CpG sites across the I.4 promoter region in human ex vivo omental and subcutaneous adipocytes is variable and exhibits both positive and negative correlations with aromatase expression depending on the tissue [56]. Importantly, this potential correlation of DNA methylation and aromatase expression may represent a complication to epigenetic therapies based on DNA methylation inhibitors (e.g. 5-Azacytidine) currently tested in ERα-negative BCa. In this case, it is thought that blocking DNA methylation would aid ERα expression ([57] see next sections). However, it may also favor aromatase re-expression.

Silencing of a gene by methylation involves the generation of an inactive chromatin structure characterized by deacetylated histones, resulting in chromatin condensation and transcriptional repression [58]. Historically, HDACs have been associated with formation of heterochromatin and gene repression in order to achieve growth control or tumour genes suppression. However, more recent reports are demonstrating that deacetylation of TFs and DNA binding proteins can be just as important in the activation of gene expression [59]. Indeed, the deacetylase sirtuin-1 (SIRT-1) is a positive regulator of PII/PI.3 activity [60]. SIRT1 is a nicotinamide adenine dinucleotide-dependent deacetylase; it has been shown to be up-regulated in a variety of cancers and influences multiple hallmarks of cancer [61] by regulating the function of histone and non-histone proteins [57, 62]. SIRT1 had also been implicated in the regulation of aromatase transcription on some level [60]. In fact, recently, SIRT1 was shown to bind directly to the PII/I.3 and PI.4 promoters in breast cancer cells, thereby, directly regulating expression of aromatase and likely contributing to its overexpression in BCa [60]. It was also reported that multiple small molecule inhibitors of SIRT1/2 and SIRT1 decrease the mRNA and protein levels of aromatase [60], thus, suggesting an alternative point of entry to modulate aromatase activity.

PI.4 contains several transcription factors response elements (REs) proximal to the TSS and also within the transcribed region. These REs are used by several cytokines, inflammatory mediators and nuclear receptors (e.g. IFNγ activation site, glucocorticoid, Sp1 and activating protein-1 binding site [63, 64]. Nuclear receptors appear to be key mediators in aromatase expression throughout the body, with many involved in PII regulation, including the retinoic acid receptor related orphan [65], thyroid hormone receptor β [66], peroxisome proliferator-activated receptor γ [67], dosage-sensitive sex reversal 1 [68] and others [69]. Additionally, it has been shown that approximately 40 % of BCa express the orphan nuclear receptor liver receptor homologue-1 (LRH-1), which binds to and activates PII via a nuclear receptor half-site [70]. The ability to therapeutically target nuclear receptors, in particular the

'orphan' nuclear receptors, pinpointed at the opportunity of using alternative agents other than classical AI to inhibit CYP19A1 expression in the breast.

Further work to clarify the epigenetics and transcriptional networks involved in aromatase regulation would be crucial to determine which additional factors are involved and whether they may be targeted as part of a consolidated approach to aromatase inhibition in BCa.

The Chromatin Landscape and Its Interaction with the Estrogen Receptor α, in the Context of Aromatase Inhibitor Resistance

Aromatase inhibitors specifically target the conversion of androstenedione to estrone with the consequence of lowering estrogens levels in the blood of post-menopausal women or ovariectomized pre-menopausal women [6, 8]. Estrone is a potent physiological ERα ligand and its binding amplifies the transcriptional response by inducing genome wide DNA binding [71]. Several studies using ERα positive BCa cell lines have demonstrated that estrogen deprivation (also known as estrogen starvation) significantly reduces ERα binding to the chromatin in a genome wide fashion [72, 73]. Two to four days in estrogen-free conditions are sufficient to inhibit almost all ERα binding while adding estradiol back in the media promptly induces ERα chromatin binding through a well-defined cyclical pattern [74–76].

Estrogen receptor binding is mediated by several other factors [77]. As most nuclear receptors, ERα dimerizes upon activation and recognizes specific DNA sequences throughout the genome (known as estrogen responsive elements, ERE) [78]. Yet, the human genome is interspersed with a >700,000 [78] of EREs and only a subset (10–60,000, depending on cells/clone/lab) of these is effectively bound by ERα. Genomic studies performed using tumour samples suggest that the loci bound by ERα are extremely heterogeneous and can be correlated with prognosis in endocrine treated patients [79]. During the last few years it has been discovered that ERα binding is mediated by additional chromatin and protein components and many of these are implicated in determining response to AI therapy [77].

Previous mechanistic models based on few promoter-biased studies were abandoned when ChIP-seq analysis demonstrated that ERα binding occurs primarily at distal regulatory elements [71, 80]. Indeed ERα has the strongest bias towards enhancer binding among all NRs [80]. ERα-bound elements are strongly enriched for ERE-DNA signature but they also contain other interesting features. For example, while nearly 50 % of ERα binding induces transcriptional repression [71], ERα binding is invariably associated with histone modifications associated with active enhancers, such as mono and di-methylation of lysine 4 on histone 3 (H3K4me1 and me2) [73, 81, 82]. This is not surprising considering that H3K4me1 and -me2 are strongly associated with cell type specific transcription [24]. Hence, ERα activity seems to be regulated at the chromatin level by epigenetic bookmarking of regulatory regions [73] (Fig. 8.2). It is still unclear if these regulatory regions are

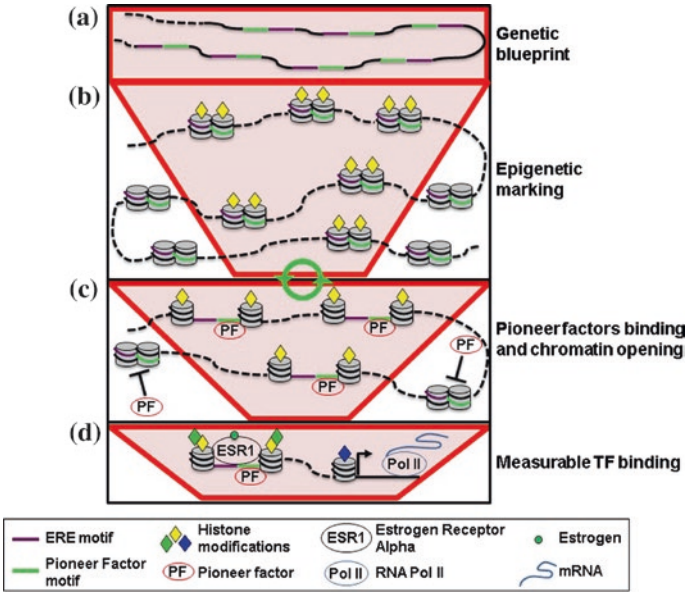

Fig. 8.2 Chromatin recruitment of the estrogen receptor is mediated via several hierarchical mechanisms. Genetic information (transcription factors specific motifs) can be found in accessible or inaccessible chromatin conformations. Secondly, pioneer factors occupancy is required for functional receptor binding. In addition, functional regions are epigenetically bookmarked via specific nucleosome modifications. It is likely that epigenetic bookmarking and pioneer factors occupancy are established through cross-talk mechanisms

established during normal breast development or if they may be altered during the early stages of malignancy similarly to what happen in colon cancers [83].

Epigenetic bookmarking may have implication in resistance to AI as recently suggested by Jansen and colleagues [84]. Mapping the genomic location of two promoter specific modifications (e.g. H3K4me3, commonly associated with active promoters and H3K27me3, a repressive chromatin hallmark [84]) was successfully used to cluster good and poor outcome patients in two separate groups, prior to AI treatment. These results were particularly convincing for H3K27me3 [84]. Epigenetically bookmarked promoters were then associated to their respective genes to create a transcriptional signature [84]. These signatures were capable of predicting outcome in an independent set of AI treated patients profiled using microarray analysis [84]. Similar data were obtained in a longitudinal study using ERα positive MCF-7 cell lines. This time around, H3K4me2 and FAIRE (chromatin accessibility) [85] were used to map distal regulatory regions in AI sensitive and estrogen independent cells [86]. These genomic data were then transformed into a transcriptional signature and tested in publically available datasets [86]. Again, differential expression of genes identified using chromatin biomarkers could discriminate ERα positive patients with good or poor prognosis. Of note, EREs were found to be highly enriched in regulatory regions of estrogen dependent cells [86]. On the other hand, EREs were found

at a much lower frequency within the regulatory regions of estrogen independent cells suggesting that ERα binding has been somewhat reprogrammed [86].

Similarly to other TFs, ERα binds ERE within nucleosome-depleted chromatin [87]. More specifically, ERα prefers to bind between epigenetically bookmarked nucleosomes rather than to ERE located on the DNA sequence wrapped around the histone octamer [77]. A class of TFs called "pioneer factors" (for a detailed review see [88, 89]) are involved in keeping the chromatin template accessible to ERα binding even in the absence of activated ERα. There is a strong connection between pioneer factor binding and histone bookmarking [77] (Fig. 8.2), suggesting that the interaction between the epigenome and ERα may be partly mediated by pioneer factors. Of note, PBX1, the second TF considered as a pioneer factor, controls the expression of ERα target genes associated with endocrine therapy resistance [82]. PBX1 is amplified in several luminal breast cancers (Magnani, unpublished). Thus, it is tempting to speculate that increased PBX1 expression may facilitate ERα binding near genes involved with AI resistance. These data are consistent with previous results that demonstrate that pioneer factor reprogramming is sufficient to relocate ERα binding [90]. Indeed, ERα cannot bind to the DNA in the absence of sufficient pioneering activity [91]. In summary, these data suggest that histone modifications and pioneer factors contribute to the development of AI resistance, partly modulating ERα binding toward very specific regions near genes actively involved in AI resistance (Fig. 8.3).

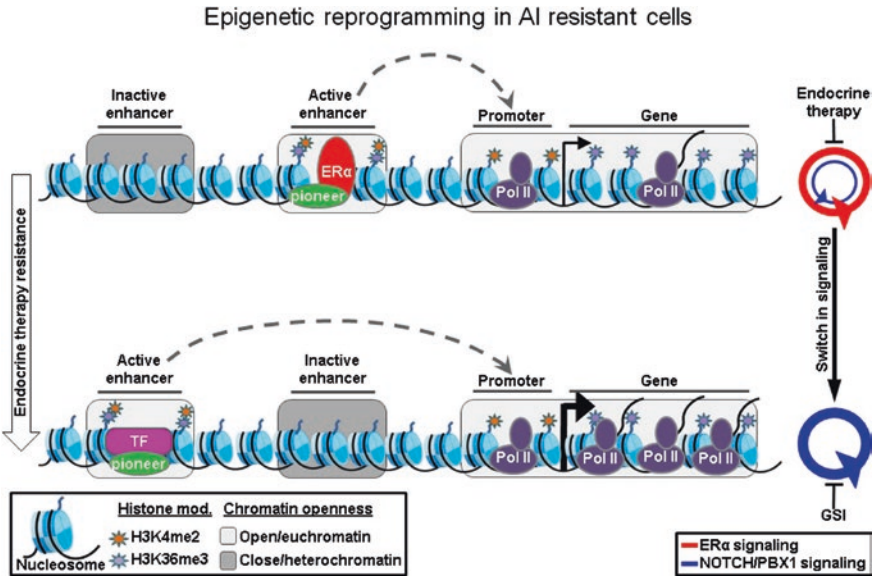

Fig. 8.3 Working model representing potential mechanisms involved in epigenetic reprogramming during the evolution of endocrine therapy resistance. Selective pressure from chronic treatment might impinge on regulatory element selection and leads to reprogrammed transcriptional output. An example of this is the switch from estrogen receptor signaling to notch signaling in cell lines that adapt to estrogen-deprived conditions

Alternative Ligands and Ligand-Independent ER-Activation: Role in Reprogramming of ERα Binding to the Chromatin Template

ERα binding can be also influenced by differential activation via alternative ligands. Although estradiol is certainly the most studied and physiologically relevant ERα ligand, several other compounds exhibit agonist activity, including Tamoxifen, chemicals and cholesterol derivatives [92–95]. Alternative ERα activation then could bypass the need of estradiol signaling, especially in AI resistant patients. Examples of additional ligands are oxysterols such as 25 and 27 hydroxycholesterol [92]. Recent work has shown that MCF-7 cells implanted in ovareoctimized mice (deprived of circulating estradiol) can grow in the presence of circulating 27-hydroxycholesterol. More importantly, MMTV-PyMT mice [96] with high levels of 27 hydroxycholesterol incur in a significant increase of metastatic lesions [97]. Of note, these alternative ligands may induce ERα binding at different chromosomal loci compared to estradiol. In fact, recent evidence point to the fact that AI resistant metastatic BCa patients do indeed have altered ERα binding profiles [79].

In addition to cholesterol intermediates, ERα can be also activated by growth factors. Epidermal growth factor (EGF) binding to its receptor activates a well defined signaling cascade responsible of ERα phosphorylation at Serine 118 [98]. The binding profile of phosphorylated ERα is significantly different from the one induced by estradiol stimulation, with only 20 % of loci bound in response to both treatments [99]. In this case, ERα binding seems not to rely on strong ERE motif [99]. Further experiments demonstrated that phosphorylated ERα piggybacks on the transcription factor AP1 to get access on the chromatin template and promote the expression of genes commonly found in AI resistant tumours [99]. These data suggest that, in tumours expressing both ERα and the orphan epidermal receptor Errb2 (aka HER2), amplified Erbb2 may contribute to drive proliferation via ERα phosphorylation and reprogrammed ERα binding [100]. Serine 118 may not be the only ERα phospho-residue capable of influencing ERα chromatin interactions. For example, serine 305 is targeted by the PKA kinase [101] and it has been shown that S305-P leads to alternative ERα chromatin binding while promoting the activation of a set of genes with strong prognostic significance [101].

In addition to various alternative ligands, recent evidence has also identified a role for un-liganded ERα in promoting gene expression, at least in BCa cell lines [102]. Un-liganded binding persisted for at least 14 days in the absence of estrogen (or serum deprivation). Un-liganded ERα seems to control basal expression of defined subset set of estrogen-dependent genes. Curiously, this subset of genes seems to contain only transcripts normally activated by estrogen while being void of estrogen repressed transcripts [102]. Silencing ERα via siRNA induced significant transcriptional repression of genes near un-liganded ERα binding sites, suggesting that the binding is indeed functional. These data indicate that AI resistance could be mediated, at least in part, by basal ERα-driven expression.

Overall, these data describe how the interplay between the chromatin template and ERα is occasionally involved in an escape route from AI treatment. Silencing

ERα can alter histone modifications levels at various enhancer elements [102]. A similar observation was also done for FoxA1, one of the most important ERα pioneer factors [103]. It is then possible that reprogrammed ERα binding plays an active role in shaping and reinforcing the selection of a defined set of regulatory elements [77]. More importantly, newly engaged regulatory elements may contain binding sites for other TFs including alternative nuclear receptors (Fig. 8.3). Activation of these alternative TFs may, in turn, contribute to AI resistance. In the next section these possibilities will be discussed in more details.

Epigenetic Modulation of ER Expression and Alternative Survival Pathways in AI Resistant Models

AI treatment is designed to significantly lower circulating estrogens to inhibit proliferation of ERα driven tumours. AI targets the aromatase enzyme contained in peripheral tissues such as fat and tumour itself. Several cell line models have been developed to study AI treatment including oestrogen withdrawal combined with different agents, and genetically modified breast cancer cells, overexpressing the aromatase gene [104–106]. Another well established model system for AI resistance is represented by long-term estrogen-deprived cells (LTED) [107]. LTED are derived from estrogen dependent MCF-7 cells cultured for a prolonged time in the absence of hormones. Using this system has allowed the discovery of a number of alternative pathways contributing to cell survival. Interestingly, recent evidence suggested that long term estrogen deprivation reprograms the entire set of regulatory regions and activates enhancers near Notch target genes [86] (Fig. 8.3) and Notch signaling has been associated with endocrine therapy resistance by several other groups [108–112]. The extensive epigenetic reprogramming observed during the development of AI resistance in vitro could be the result of active chromatin remodelling or underline the emergence and proliferation of a particular epigenetic subclone. ChIP-seq and other genomic approaches have technical limitations since they rely on millions of cells. Hence, epigenomics data then represent an average of all subpopulations present in the culture dish. Chromatin changes associate with Notch signaling, thus, can reflect the progressive enrichment of an AI resistant subpopulation of cells following estrogen deprivation. Interestingly, it has been proposed that cancer stem (like) cells (CSCs) are inherently resistant to AI and other anti-estrogen therapies (reviewed in [113]). In addition, accumulating evidence supports a role for Notch signaling in maintaining the CSC niche both in vivo and in vitro [114–116]. Thus, it may be possible that AI inhibitors target specifically more differentiated cells while sparing Notch driven CSC.

Epigenetic activation of other developmental pathways is likely to contribute to AI resistance as well. HOX TFs factors play a central role in embryonic development and HOX transcripts have been previously linked to BCa progression [117]. Interestingly, a genome-wide DNA methylation screen has identified HOXC10 to be epigenetically silenced in LTED cells via DNA methylation [118]. HOXC10

acts as a tumour suppressor and AI treatment is initially responsible for its upregulation in MCF7 cells. While HOXC10 is initially repressed by estrogen treatment, long term deprivation results also in silencing this gene through *enhancer of zeste 2* (EZH2) binding and DNA methylation at HOXC10 regulatory regions [118]. In agreement, the authors also observed a drastic increase of the repressive H3K27me3 chromatin mark, the histone modification catalyzed by EZH2. Permanent loss of HOXC10 in LTED cells is thought to contribute to estrogen-independent growth and acquired cellular motility. Interestingly, DNA methylation did not impact ERα binding itself [118], but estrogen depletion eventually led to DNA methylation and epigenetic repression of a large panel of regulatory elements. These observations are consistent with previous results originated by long term silencing of ERα [102, 119].

Another emerging observation from cell line work is that AI resistant cells may be less dependent on ERα signaling itself. LTED cells generally express higher levels of ERα protein [86, 120, 121] but at the same time are relatively more resistant to ERα downregulators (SERD) compared to parental cells [86, 120, 121]. In contrast, a small but consistent proportion of patients develops resistance to AI while losing ERα, probably via epigenetic silencing [122, 123]. Promoter DNA methylation has been long implicated with transcriptional silencing and a large body of evidence has accumulated through the years describing the link between loss of ERα transcription and promoter methylation. For example, triple negative cancer, defined by the absence of ERα, progesterone receptor and HER2, are commonly associated with strong promoter methylation at the ESR1 promoter [124]. DNA methylation is catalyzed by a set of specialized enzymes (DNA methyltransferases, DNMT). DNMT relocation onto the ESR1 promoter was observed in triple negative breast cancer cells (MDAMB231) [125]. In this case, the expression of a particular TF, called TWIST is directly associated with DNMT3B binding to the ESR1 promoter and stable DNA methylation [125]. In agreement, TWIST overexpression was sufficient to reproduce this phenotype in ERα positive cells. On the other hand, TWIST silencing in MDAMB231 led to partial ERα re-expression [125].

Although being an attractive mechanism, loss of ERα may accompany AI resistance in a relatively small proportion of cases. Importantly, decreased reliance on ERα has to be compensated by the activation of alternative growth-supporting pathways. For instance, an analysis of nuclear receptors network in breast cancer [80] suggests that disengagement of ERα with the chromatin template (for lack of ligand mediated binding or total loss of the receptor) could free regulatory elements to other receptors. Recent evidence suggests that the liver receptor homologue 1 (LRH1, NR5A2) contributes to endocrine therapy resistance. Depletion of LRH1 mRNA resulted in significant growth defect in MCF7 cells and to a larger extent in endocrine therapy resistant cells (LCC2 and LCC) [126]. LRH1 binds at several loci containing ERE elements and seems to cooperate with ERα itself at the chromatin level. Indeed LHR1 depletion results in decreased ERα binding while LRH1 overexpression increases ERα occupancy, a result in agreement with the assisted loading model [127]. Concurrently, another group also described the

role of LRH1 in regulating ERα target genes using MCF7 cells [128]. These data suggest that AI resistant cells may rely on alternative growth pathways activated through epigenetic reprogramming and remodeling of chromatin accessibility and implemented via alternative nuclear receptors.

Genetic-Epigenetic Crosstalk in AI Resistance

While epigenetic defects do not directly impinge on the genetic code, somatic mutations can have a direct impact on the epigenome. In the last few years several groups have published whole-exome sequencing data regarding many cancer types including ERα-positive BCa patients [129–132]. An interesting finding was that one of the genes most frequently mutated in luminal patients is the *histone methyltransferase MLL3*. MLL3 catalyzes the deposition of methyl groups on lysine residues including H3K4 [20]. Currently, it is not clear what the net effect of carrying mutated MLL3 is but it is tempting to speculate that it may facilitate the re-arrangement of the entire epigenetic landscape. Moreover, although identified at a much lower frequency, a whole set of chromatin modifiers was found mutated in a whole genome sequencing analysis of 46 AI resistant patients (histone methyltransferases: MLL2, MML3 MLL4 and MLL5; histone demethyltransferases KDM6A, KDM4A, KDM5B and KDM5C; acetyltransferases MYST1, MYST3 and MYST4 [132]). The idea that mutated chromatin modifiers could play a role in oncogenesis and tumour evolution is supported by many other genomic clues indicating an extensive crosstalk between the genome and the epigenome. For example, another commonly mutated gene in ERα positive breast cancer is *GATA3*, a TF commonly found at ERα binding sites [133]. GATA3 is thought to facilitate ERα binding and is considered as another pioneer factor [88, 133]. Similarly to MLL3, it is not completely clear what are the functional implications of these mutations, however, it appears they may regulate response to estrogen signaling by stabilizing GATA3 protein interaction with the chromatin [134]. On the other hand, GATA3 mutations can also preclude GATA3 binding altogether [135] thus interfering with ERα access to the chromatin [136].

While epigenetic bookmarking does not directly impact the genetic sequence, it can still modulate the effect of somatic mutations, especially the one falling within regulatory regions. There is now an extensive body of literature demonstrating how somatic mutations can interfere with TF binding by altering the DNA binding recognition sequence. Genetic variants at enhancers may influence gene expression by changing affinity to TFs [137]. Of importance, genetic variants that carry biological significance for ERα breast cancer development (often called risk-SNP) [138] are preferentially found near ERα binding sites [139]. Additionally, breast cancer risk-SNPs are also significantly associated with the ERα pioneer factor FOXA1 [139] suggesting that genetic variation is in fact filtered through the epigenetic landscape. In the future it will become important to evaluate if a genetic variability at regulatory regions plays a role in favoring AI resistance.

Epigenetic Intervention in the Context of Aromatase Inhibitor Resistance

In the previous sections we have described several examples of how epigenetic reprogramming directly or indirectly contributes to AIs resistance. Epigenetics is extremely attractive from a therapeutic stand-point since chromatin modifications (histone and DNA) are reversible by nature. This characteristic is likely due to the innate plasticity required through cell development. Indeed, epigenetic drugs are likely establishing a new era in the field, with some already in clinical development (see [140]). There is an increasing interest in the role of epigenetic contribution to diseases including tumour initiation and progression as well as tumour heterogeneity.

One long-standing problem linked to epigenetic intervention is that epigenetic drugs are virtually unselective and target the entire genome rather than concentrating on single defective loci. In addition, these agents are generally promiscuous and may hit several chromatin modifiers at the same time (see histone acetyltransferases inhibitors, HDAC). Epigenetic agents of the new generation have increased target specificity but they still lack locus specificity and do not discriminate between "oncogenic epigenetic drivers" and the normal epigenetic setting of cancer cells. It is not surprising that a large amount of work is currently carried on in trying to force ERα expression in tumours that do not express it via DNA methylation inhibitors (5-Aza) coupled with HDAC inhibitors (see [141, 142]). Treatments with these compounds have been associated with relative success. In some cases, ERα re-expression has not been achieved [143]. On the other hand, genome-wide hypomethylation is a common trait of tumours and interfering with DNA methylation in normal tissues may lead to the development of accessory malignancies. These data warrant for careful evaluation of these epigenetic strategies in the treatment of aromatase inhibitor resistant cancers.

The epigenetic field is gradually developing increasingly specific molecules designed to interfere with specific loci. Using epigenetic editing (aka epigenetic engineering) [144], several groups have demonstrated that gene specific transcriptional control is possible. Zinc Fingers proteins (ZFPs) can be engineered to target specific DNA sequence across the genome [145]. We have previously mentioned that HER2 transcription can contribute to AI resistance by phosphorylating ERα [146]. HER2 transcription can be modulated at the chromatin level by generating repressive histone marks at the HER2 promoter. By designing ZFP for the HER promoter fused with G9a, a histone methyltransferase inducing the repressive H3K9me3 mark, Falahi and colleagues successfully repressed HER2 transcription in several breast cancer cell lines [147]. By using a similar approach (ZFP fused with DNA demethylases), scientists now hope to re-activate tumour suppressors genes in breast cancer. For aromatase inhibitor resistant tumours, the hope is to improve specificity when reactivating ERα without the complications of deregulating other transcripts. Recent studies also speculated about the possibility that CYP19A1 expression itself may be lost in aromatase resistant tumours. Nevertheless, CYP19A1 is regulated via tissue specific promoters (10 different promoters [49]) and it may be difficult to specifically target them in resistant breast cancers.

Consideration on Epigenomics Studies in Cell Lines and Tumour Heterogeneity

One caveat of chromatin based assays such as ChIP-seq is that they discard potential information about population heterogeneity. For example, EGF or PKA reprogrammed ERα binding (which may be associated with resistance) is consistently weaker than standard recruitment of ERα [99, 101]. One possible explanation for this is that the reprogrammed ERα binding occurred only in a subpopulation of cells. The assumption that all cells within a tumour respond to stimuli in the same coherent fashion is frequently implied, while in fact it is possible that the response is either stochastic [148] or diverse, when only a subset of epigenetically defined cells gains the ability to sustain estrogen deprivation [149]. Hence, tumour heterogeneity may strongly contribute to response to AI and each epigenetic/genetic clone could activate very specific escape pathways rather than elude drug intervention as a coherent unit. If this is true, the best option would then become to identify the most prevalent epi/genetic clone(s) during a sequential therapy approach. Nonetheless, finding alternative ways to treat resistant tumours remains imperative and the study of the chromatin landscape in these cases holds tremendous potential.

Future Perspective

Cancer research has pioneered the exciting -omics era. We are finally approaching several aspects of cancer biology using system biology tools and considering the whole genome rather than forming hypothesis centered on few genes. Epigenomics is at the forefront of the -omics revolution and is quickly renewing our understanding of how cancer develops and progresses. Aromatase inhibitors have replaced Tamoxifen as first line endocrine therapy in post-menopausal ER-positive breast cancer [6]. The vast majority of research in the field of endocrine therapy resistance has dealt with Tamoxifen but it is conceivable that the mechanisms described in this chapter may not be shared across all endocrine treatments. A number of large clinical trials cohorts, such as BIG 1-98, ATAC, TEAM and the International Exemestane Study [9–11], have provided samples for analysis using various -omics approaches. This paves the way to understand which of the observations done in in vitro models will be still relevant in vivo. At any rate, several new discoveries lie ahead and will broaden our understanding of AI resistance.

For example, AI resistance is generally discussed as de novo or acquired. Are the mechanisms shared? Could it be that acquired simply represent clonal evolution of a pre-existing subpopulation? Or can it be that breast cancer cells acquire resistance to AI by epigenetic reprogramming and activation of alternative survival strategies in response to chronic drug exposure? We expect that several of the concepts and tools developed in the epigenetic fields will help solving these questions. Regulatory regions are not all equals, and studying super-enhancers (cluster of

enhancer with particular link to transcription) has already open another door in the treatment of leukemia [150]. Similarly, very recently we have come to understand how the genome is spatially organized (domains) and what the impact of this on epigenetic states is. Topological associated domains (TADs) seem to be regulated in a coherent fashion and we may gain further understanding of how AI resistance arise by studying how TAD changes between sensitive and resistant tumours [151].

In addition to using cell line models the field has now developed an entire array of new models including patient's derived xenografts (PDX). Moreover, it is now possible to access tumour cells and test drug sensitivity longitudinally by collecting circulating tumour cells [152, 153]. These samples, in addition to increasing diffusion of re-biopsing relapsing tumours and the expansion of tissue-banking, will potentially unlock better therapeutic agents, more reliable biomarkers and ultimately improve outcome for patients developing AI resistance.

Conflict of Interest The authors do not have conflicts of interest to disclosed.

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Chapter 9
Aromatase Inhibitor Resistance via Non-endocrine Signalling Pathways

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Abstract Mechanisms of resistance to aromatase inhibitor treatment have been examined in vitro, in mouse models and in tumours in human patients. These varying approaches have yielded a range of potential routes through which breast cancer cells may survive and continue to proliferate during treatment with aromatase inhibitors. Many of these mechanisms are not directly linked to endocrine signalling pathways, and include alterations in other growth factor pathways and the tumour microenvironment. In general, approaches focussing on long-term oestrogen deprivation of cultured cells have identified cell-intrinsic mechanisms of resistance while utilising whole tumour specimens has detected the contribution of stromal elements. Many mechanisms have been validated in more than one setting. In this chapter, we discuss the role of key growth factor pathways such as PI3K/mTOR, IGF, GDNF and Myc as well as the contribution of inflammatory immune cells and adipocytes. Advances in genomic analysis and greater understanding of the role of tumour heterogeneity look likely to provide further insight into the mechanisms through which cells withstand treatment with aromatase inhibitors and help improve therapy in the future.

Abbreviations

3D	Three-dimensional
AI	Aromatase inhibitor
AnaR	Anastrozole-resistant
AR	Androgen receptor
BCRP	Breast cancer resistance protein
CCND1	Cyclin D1

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A. Larionov (ed.), *Resistance to Aromatase Inhibitors in Breast Cancer*, Resistance to Targeted Anti-Cancer Therapeutics 8,
DOI 10.1007/978-3-319-17972-8_9

CDK4	Cyclin-dependent kinase 4
ChIP	Chromatin immunoprecipitation
CXCL	C-X-C motif ligand
ER	Oestrogen receptor
FGF1R	Fibroblast growth factor 1 receptor
GDNF	Glial-derived neurotrophic factor
HIF1α	Non-hypoxic hypoxia inducible factor 1 alpha
HRE	Hypoxia response element
IGF-1R	Insulin-like growth factor 1 receptor
InsR	Insulin receptor
LetR	Letrozole resistant
LTED	Long-term oestrogen deprived
MMP9	Matrix metalloproteinase 9
PGR	Progesterone receptor
PI3K	Phosphatidylinositol 3-kinase
qPCR	Quantitative real-time PCR
RET	Rearranged during Transfection
shRNA	Small hairpin RNA
SRC1	Steroid receptor coactivator 1
TFF1	Trefoil factor 1

Introduction

Even though the majority of patients respond well to endocrine therapy, a substantial population of patients become refractory to treatment and relapse with the disease. While many resistance mechanisms have been reported for tamoxifen particularly through in vitro studies, it is only recently that avenues of aromatase inhibitor (AI) resistance have been reported in the literature.

Whereas disturbances to oestrogen receptor function and signalling represent a direct mechanism of resistance, alterations in other growth factor pathways and the tumour microenvironment have also been shown to drive resistance to aromatase inhibitor treatment.

In Vitro Models of AI Resistance

The overarching finding of many clinical studies conducted to understand the phenomena of endocrine therapy resistance is that activation of alternative growth factor signalling pathways can mediate patients being refractory to endocrine agents [1]. A substantial body of knowledge regarding endocrine treatment resistance have been derived from studies performed using breast cancer cell lines. In order to better understand the biology of this phenomenon, in vitro

Table 9.1 Alterations in ER and signalling pathways in selected LTED and AI model systems

ER expression	ER activity	ER phosphorylation	Growth factor receptor/s altered	Kinases involved	Reference
Increased	Increased	Not available	IGF1R	MAPK, PI3K/AKT	Santen [51]
Increased	Increased	S118 increased	IGF1R, HER2	MAPKs	Martin [52]
Increased	Not available	S167 increased	HER2	PI3K/AKT	Sabnis [53]
Increased	Increased	S118 increased	Slight decrease in IGF1R	PI3K/AKT	Staka [54]
Increased	Not available	S167 increased	HER2	MAPK, PI3K/AKT	Jelovac [55]

models of AI resistance have been generated and studied in many laboratories. To model AI-mediated depletion of systemic residual oestrogen concentration, ER-positive breast cancer cell lines are grown in media containing oestrogen-depleted serum over a period of time, until the proliferation rates of the long-term oestrogen deprived (LTED) cells reach similar or higher levels than the parental line.

The LTED system, however, is limited by the fact that it is not a true representation of AI resistance per se [2]. In other words, different AIs may induce different resistance mechanisms, which may not be captured from an LTED model alone. This is instructive from a small trial that found patients with disease progression while on exemestane as a first line therapy do respond to either anastrozole or letrozole as a second line treatment [3, 4]. Based on this, another commonly utilised system to mimic AI resistance is the overexpression of aromatase in MCF7 cells and culturing these cells in the presence of testosterone, with and without an AI. Nonetheless, it is worthwhile mentioning that most of the oestrogen synthesis in postmenopausal patients occur peripherally in other tissues such as subcutaneous fat, and the rapid exchange between the plasma oestradiol and the tumour is likely to be more relevant in terms of determining the concentration of oestrogen within a breast tumour [5, 6]. Therefore, a system that overexpresses aromatase in a cell line may also not necessarily fully mirror the adaptive mechanism/s cancer cells acquire en route to treatment resistance. Key findings from some studies that have generated LTED and AI resistance systems are summarised in Table 9.1.

Cell Intrinsic and Extrinsic Mechanisms of Resistance

Potential mechanisms of AI resistance can be classified into two groups—(i) cell intrinsic pathways and (ii) external influences of the tumour stroma that provide alternative survival cues to as a means to evade oestrogen depletion.

Cell Intrinsic Mechanisms

De Novo Resistance

Cell autonomous means of AI resistance can be further subdivided into two categories—de novo and acquired resistance. The former is defined as an inherent lack of response to initial endocrine treatment, while the latter refers to the development of refractory mechanisms after a period of treatment response.

1. *ER as key predictor of endocrine treatment response*
   By far, the main predictor of response to endocrine therapy is the expression of ER. This is evident, for instance, from the P024 neoadjuvant trial that compared the efficacy of tamoxifen against letrozole treatment and found ER-negative patients to have negligible response to either agents [7].

2. *Polymorphisms in CYP19 aromatase gene*
   Several genetic polymorphisms in the aromatase gene have been reported, with associated clinical relevance. Two single nucleotide polymorphisms—rs6493497 and rs7176005—significantly correlated with efficiency of the enzyme inhibition during AI treatment, and in a separate study, were also associated with higher plasma oestradiol levels pre- and post-AI treatment [8]. Conflicting findings, however, were made with regards to a 3′ untranslated region of the *CYP19* gene, where patients with the rs4646 variant allele had either a shorter or longer time to progression [9, 10]. More work is required to elucidate the significance of genetic variations in the *CYP19* gene, which may be of clinical significance in the management of patients with specific variants.
   Apart from the two factors mentioned above, very little else is known about de novo resistance mechanisms to AI. Given that this information would facilitate patient selection for endocrine therapy and early identification of poor responders, more effort should be channelled to identify and characterise the mechanisms associated with baseline resistant phenotype.

Alternative Signalling Pathways Associated with Aromatase Inhibitor Resistance

PI3K/MTOR and IGF1/IGF1-R Signalling Pathway

The phosphatidylinositol 3-kinase (PI3K) pathway is frequently altered in breast cancer and data from The Cancer Genome Atlas indicate that the catalytic subunit of PI3K—*PIK3CA* is the most common somatic aberration in ER+ve breast cancer, with approximately 30 % of ER+ve patients with mutated copy of the gene [11–13]. Alterations in other components of the pathway and other signalling cascades that converge on the PI3K pathway are summarised in Table 9.2 and Fig. 9.1. However, the clinical interpretation of PI3KCA mutations and PI3K signalling is still challenging [14–17].

Table 9.2 Genomic aberrations associated with PI3K pathway [11]

Gene	Type of aberration	Frequency	References
ERBB2	Amplification/ overexpression	~10 % of ER-positive tumours	Ellis [56], Arpino [57], De Laurentiis [58]
PTEN	Inactivating mutation	37–44 % of ER-positive tumours	Perez-Tenorio [59], Saal [60], Shoman [61]
PIK3CA	Activating mutation	28–47 % of ER-positive tumours	Perez-Tenorio [59], Baselga [14], Stemke-Hale [62], Ellis [63], Campbell [64]
PIK3CB	Amplification	5 % of total cases	Crowder [20]
IGFIR, INSR	Receptor activation	48 % of ER-positive tumours	Law [65]
FGF1R	Amplification	11.6 % of ER-positive tumours	Turner [66]
RPS6K1	Amplification	8.8–12.5 % of all tumours	Monni [67]
INPP4B	Decreased expression, deletion	8.4–37.7 % of all tumours	Gewinner [68], Fedele [69]
PIK3R1	Inactivating mutation	2 % of all tumours	Jaiswal [70]
AKT1	Activating mutation	2.6–3.8 % of all tumours	Stemke-Hale [62], Loi [71], Carpten [72]
AKT2	Amplification	2.8 % of all tumours	Bellacosa [73]
EGFR	Amplification	0.5 % of ER-positive tumours	Al-Kuraya [74]
PDK1	Amplification/ overexpression	21 % of total cases	Maurer [75]
KRAS	Activating mutation	4–6 % of total cases	Rochlitz [76], Di Nicolantonio [77]

In concordance with clinical observation of tumour heterogeneity, the phenotype of LTED cell lines may vary between and within laboratories. For instance, the expression of ER and response to a dose-dependent titration markedly varied between MCF7, ZR75-1, MDA-MB361, and HCC-1428 LTED cell lines [18]. While the MCF7 and HCC1428 cells upregulated ER expression, the expression of the hormone receptor was substantially decreased or lost from MDA-MB361 and ZR75-1, respectively. Furthermore, while LTED MCF7 cells have previously been shown to be hypersensitive to low doses of oestrogen, the LTED MCF7 cells derived by Miller et al. exhibited a similar dose-response profile to the parental line.

Protein array-based proteomic profiling of these LTED cell lines revealed a consistent activation of the mammalian target of rapamycin (mTOR) kinase, and the downstream activation of its substrates—p70S6, p85S6 and Akt. Pharmacological inhibition of PI3K and Akt led to cell death of the LTED cells, and also prevented cells from acquiring a hormone-independent phenotype. Analysis to identify upstream receptor tyrosine kinases that activate PI3K revealed an increase in activated insulin like growth factor 1 receptor (IGF-1R) and insulin

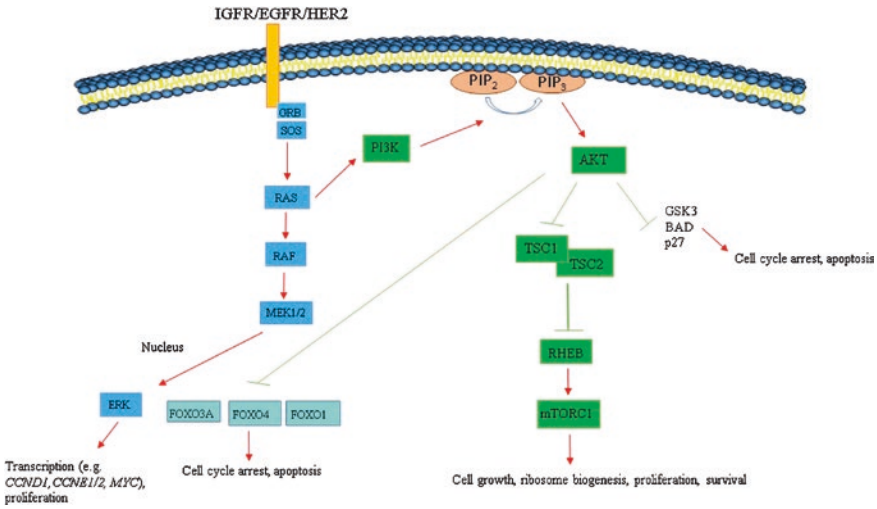

Fig. 9.1 Schematic of the IGFR/EGFR/HER2 pathway illustrating the crosstalk with the phosphatidyl inositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway. The pathway can be activated by receptor tyrosine kinases (RTKs) such as the insulin-like growth factor receptor (IGFR), the epidermal growth factor receptor (EGFR) and ERBB2 (also known as HER2). The pathway drives a number of critical cellular processes including cell growth, proliferation, and survival. Mammalian target of rapamycin (mTOR) is a signal transduction kinase in the PI3K pathway that exists in multiprotein complexes, mTOR complexes 1 and 2 (mTORC1 and mTORC2). mTORC1 consists of mTOR that is associated with raptor (regulatory-associated protein of mTOR). The mitogen activated protein kinase (MAPK) arm of the pathway leads to the ERK-mediated phosphorylation of effector proteins, such as transcription factors, and activation of subordinate kinases

receptor (InsR) in MCF7 and ZR75-1 LTED cells, while the epidermal growth factor receptor family—EGFR (HER1), HER2 and HER3—together with InsR, were upregulated in MDA-MB361 LTED cells. Indeed, treatment of the LTED cell line panel with an IGF-1R/InsR inhibitor AEW541 resulted in a decreased Akt activation, which corroborates the importance of this pathway in hormone-independent growth. Notably, ER-positive patients dichotomised based on the ratio of phosphorylated IGF-1Rβ to IGF-1Rβ were marginally significantly associated with poor endocrine therapy outcome (log-rank test $p = 0.053$).

As increased Akt is associated with an active PI3K signalling status, it is conceivable that inhibiting Akt may affect the viability of hormone-independent breast cancer cells. In an elegant study by Fox et al. [19], pharmacologic inhibition of Akt in the LTED cell line panel generated in the study mentioned above suppressed the growth of MCF7, ZR75-1 and MDA MB361 cell lines, but not HCC1428. It is possible that after a period of culture in oestrogen-depleted condition, the breast cancer cells may express ER that are ligand-independent. For instance, activation of PI3K in the absence of oestrogen can induce ER expression, and blocking PI3K activity in cells grown under hormone-depleted

conditions brings about a synthetic lethal effect [20]. Therefore, these findings are indicative of a crosstalk between PI3K and ER signalling. Inhibition of Akt led to an increased ER mRNA levels in the LTED lines, and treatment with the pan-PI3K inhibitor BKM120 upregulated the protein levels of the hormone receptor. Critically, treatment with fulvestrant, an ER destabiliser, further potentiated the effects of the Akt inhibitor in MCF7 LTED cells. Taken together, the data suggest that for some ER+ve tumours dual inhibition of Akt and ER is a more potent treatment combination compared to either target alone.

However, in the LTED cells, Akt inhibition was found to increase the expression levels of several receptor tyrosine kinases including InsR, HER3, and fibroblast growth factors 2–4, and increased activation of the Src kinase. Blocking InsR/IGF-1R or P13K activity pharmacologically resulted in the loss of Akt inhibition-induced Src activation, which suggests that PI3K and IGF-1R/InsR signalling are involved in the increased Src phosphorylated levels. The upregulation of the IGF-1R/InsR in response to Akt inhibition was driven by the FoxO3a transcription factor, and knockdown of *FOXO3A* in the presence of an Akt inhibitor led to the downregulation of IGF-1R and insulin-like growth factor (IGF) 1 and IGF2, but not InsR. Furthermore, treatment of MCF7 LTED cells with IGF binding protein 3 prevented IGF1 and IGF2-induced activation of IGF-1R/InsR and Akt. Importantly, siRNA knockdown or pharmacologic inhibition of IGF-1R and InsR further enhanced the inhibitory effects of an Akt inhibitor in MCF7 and MCF LTED cells, respectively. To summarise, the study by Fox et al. indicates that LTED cells are dependent on IGF1R/InsR-mediated activation of the Akt pathway for survival, and simultaneous inhibition of these pathways offers a potent treatment strategy in the management of patients who have developed AI resistance.

The findings of the study above is supported by Vilquin et al. [21] who observed the constitutive activation of the PI3K/mTOR/Akt pathway and deregulated expression of Erbb receptors in AI-resistant aromatase-overexpressing MCF7 cells. Additionally, control cells that were transfected with constitutively active Akt were found to be de novo resistant to anastrozole. Treatment with anastrozole together with either an Akt inhibitor or rapamycin increased the sensitivity of control cells to anastrozole and overcame resistance in the treatment refractory cells.

GDNF and RET Signalling

The expression of the Rearranged during Transfection (RET) receptor tyrosine kinase and its ligand glial-derived neurotrophic factor (GDNF) has been shown to be upregulated in a portion of ER-positive breast cancers [22, 23]. GDNF has also been reported to be upregulated by inflammatory cytokines and expressed on infiltrating fibroblasts of the tumour stroma [22].

Morandi et al. [24] reported increases in both RET and ER expression in LTED MCF7 cells compared to their parental line. RET expression was shown to be regulated by ER, and parental cells grown in normal serum and treated with the ER downregulator ICI182,780 resulted in decreased levels of RET. Furthermore, RET can

regulate ER activity in MCF7 LTED cells, as evidenced by phosphorylation of serine residues 118 and 167 of the hormone receptor, as well as upregulation of oestrogen-dependent genes *TFF1* and *PGR*. Pharmacologic and siRNA inhibition of RET in LTED cells treated with GDNF was observed to inhibit colony formation in two- and three-dimensional cultures. Additionally, in MCF7 cells expressing the aromatase gene and treated with androstenedione, GDNF administration markedly increased the concentration of letrozole required to eliminate half of the cell population (50 % survival fraction without GDNF = 1.71 nmol/L, with GDNF = 802 nmol/L).

Gene expression profiling of oestrogen-deprived MCF7 cells identified an 83-gene GDNF signature, which was functionally associated with immune system, cell death and response to various stimuli. Upon filtering out proliferation associated genes, the 67-gene GDNF signature was associated with a higher score in Luminal B than Luminal A subtypes, and patients dichotomized using the nearest centroid method were observed to have a lower relapse—or distant metastasis free survival in three independent datasets (dataset 1 log rank $p = 0.038$, hazard ratio (HR) 3.3; dataset 2 log rank $p = 0.006$, HR = 3.3; dataset 3 log rank $p = 0.015$, HR = 2.1). Notably, these datasets consist of patients that either did or did not receive systemic adjuvant treatment, which suggests that the GDNF signature may be of both prognostic and treatment predictive significance. Furthermore, gene expression profile of 52 patients before and after 2 weeks of neoadjuvant letrozole showed a significant decrease in the GDNF signature score only in responders. In an independent dataset, the signature score in the pretreatment samples had a weaker, albeit significant correlation with proportional 2 week change in Ki67, a molecular surrogate of response ($r_s = -0.24$, $p = 0.047$) [25].

Steroid Receptor Coactivator 1 Regulates Expression of Myc and Matrix Metalloproteinase 9 to Mediate AI Resistance

In their model of letrozole-resistant (LetR) MCF7 cells, McBryan et al. [26] showed that these cells had a slight increase in ER expression relative to letrozole-sensitive control cells. However, the LetR cells were more proliferative in response to epidermal growth factor than oestrogen treatment, indicative of hormone-independent growth. Migratory assays revealed that the LetR cells were 5 fold more motile relative to the sensitive control. Consistent with the migratory capacity of LetR cells, increased expression levels of matrix metalloproteinase 9 (MMP9) was observed in LetR cells compared to letrozole sensitive control cells. Three-dimensional culture of the two cell lines showed that the sensitive cells could form an organized, circular and hollow structures. In contrast, LetR cells displayed disorganized structures, with little indication of polarisation. Crucially, knockdown of SRC1, but not ER, significantly ablated the migration ability of the LetR cells. Taken together, the hormone-independence, in tandem with the high migratory ability and poor organization of the LetR model suggest that the system recapitulates a metastatic phenotype that would be expected of AI-resistant tumours.

ChIP-seq using antibody against steroid receptor coactivator 1 (SRC1) identified Myc as a likely target gene. Since the transcription factor Ets2 was previously shown to regulate Myc expression [27, 28], the authors assessed the protein expression levels of SRC1, Ets2, and Myc. In LetR cells, expression of Myc increased in the presence of androstenedione or oestrogen, which was not inhibited by co-treatment with letrozole. Furthermore, ChIP-seq analysis in LetR cells showed that SRC1 and Ets2 were recruited to the promoters of Myc and MMP9 independently of steroid treatment. Additionally, overexpression of either SRC1 or Ets2 in the sensitive cells resulted in increased transcript levels of MMP and Myc. Importantly, in Ets2 overexpressing LetR cells, siRNA knockdown of SRC1 abrogated the increased expression of target genes. Therefore, the data suggest that SRC1 interacts with Ets2 to activate expression of MMP and MYC, and may be a critical process in the development of AI-resistance in this cell model.

Evidence for the association of SRC1 with clinical outcome was less convincing. In a cohort of 150 patients; 84 of whom received tamoxifen, and 75 of whom received AI in an adjuvant setting, SRC1 was significantly associated with outcome only in the tamoxifen-treated ($p = 0.0326$), but not AI-treated cohort ($p = 0.6894$). Furthermore, SRC1 expression was only significantly correlated with tumour stage, but not tumour grade, progesterone receptor, HER2, and nodal status. Nonetheless, of 9 patients who relapsed on AI, while 3 primary tumours were SRC1-negative, all were SRC1-positive at relapse. Interestingly, when SRC1 status was taken into account at metastasis, the AI-treated cohort was significantly associated with disease-free survival, which suggests that while SRC1 is not predictive of treatment response per se, it plays a role in the progression of tumours acquiring AI resistance.

KRAS Mutation and FGF1R Gene Amplification Correlate with Distinct Outcome to Short-Term Letrozole Treatment

In an interesting case study reported by Balko et al. [29], a patient presented with bilateral ER-positive breast cancer and was treated with letrozole for 16 days prior to undergoing a bilateral mastectomy and sentinel lymph node biopsy. A mutation screen identified an activating mutation in *KRAS* (G12D) in the left breast tumour in both pretreatment biopsy and surgical sample, while no mutations were observed in the right breast tumour. Interestingly, the *KRAS* mutant tumour was observed to have a better response to letrozole, with a 93 % decrease in Ki67-positive cells post-treatment relative to pre-treatment, compared to a 43 % decrease post-treatment relative to pretreatment in the tumour with a wild type *KRAS*. ER scores were identical in both tumours which suggests that the differences in proliferation rate is not due to differential ER expression, and progesterone receptor expression was higher in the *KRAS* mutant tumours. Furthermore, an approximated Oncotype Dx recurrence score from the *KRAS* mutant classified it to have a ‘high’ recurrence risk, compared to ‘low’ risk for the wildtype counterpart.

Differential expression analysis revealed several chemokines, including CXCL9, 10, 11 and 13 to be upregulated in the mutant tumour, which might contribute to high proliferative capacity at baseline.

Intriguingly, the differential expression analysis also showed a high expression of the tyrosine kinase *FGFR1* in the wildtype tumour, and fluorescent in situ hybridisation revealed an amplification of the gene in both pre- and post-treatment samples. Since *FGF1R* has been shown to be correlated with endocrine treatment resistance, it may account for the weaker response of the wildtype *KRAS, FGF1R*-amplified tumour to letrozole. Taken together, this study, while only representing a single patient, highlights that a widely recognized phenomenon of heterogeneous genomic alterations within a patient, that may affect disease management. Additionally, signalling molecules (i.e., chemokines) of tumour or stromal origins can contribute to an aggressive tumour phenotype.

Androgen Receptor Interacts with ER to Facilitate Endocrine Treatment Resistance

The androgen receptor (AR) is frequently expressed in all subtypes of breast cancer, particularly in ER-positive tumours [30]. Recently, transgenic expression of AR in MCF7 cells expressing aromatase were shown confer resistance to anastrozole in this model [31]. In this study, the aromatase gene was cloned into the MCF7 cell line (MCF7-Aro), and a subclone of this line expressing AR (MCF-AR) was generated. Anastrozole treatment in the presence of androstenedione inhibited MCF-Aro, but not MCF7-AR cell growth, which suggests that androgenic signalling can drive anti-oestrogen treatment resistance. MCF7-AR cells were observed to have Akt activation, which was only slightly reduced by anastrozole, and treatment with an Akt1/2 inhibitor restored sensitivity of the cells to anastrozole. Interestingly, activation of HER2 was not observed in the MCF-AR model.

In the presence of both androstenedione and anastrozole, treatment with ER and AR modulators effectively suppressed cell growth. Proximity ligation assays showed that ER and AR were co-localised in one of the AR-overexpressing clones, while little interactions were observed in the aromatase-overexpressing only cell line. Using ChIP, both receptors were found to be recruited to the prostate specific antigen and pS2 promoter regions, further supporting the interaction between ER and AR to drive androgen- and oestrogen-responsive gene expression. Taken together, this study highlights for a role of AR in mediating AI resistance.

Non-hypoxic Hypoxia Inducible Factor 1 Alpha (HIF1α)

A hypoxic core environment is common in solid tumours due to the lack of blood vessel supply to innermost cells within a mass. HIF1α, a key protein that is post-translationally regulated by oxygen levels, has shown to be upregulated in hypoxic

regions of a tumour and activate genes in response to oxygen stress. Furthermore, HIF1α has been shown to be involved in tumour formation and metastasis, and is associated with increased disease-progression and decreased survival. In a recent study by Kazi et al. [32], the authors investigated the potential role of *HIF1α* in mediating AI resistance in breast cancer.

In letrozole-resistant and sensitive MCF7 cells, the protein expression of *HIF1α* was shown to be upregulated in the resistant cells compared to the sensitive parental control. Treatment of resistant cells with lapatinib (HER2 kinase inhibitor), UO126 (MEK inhibitor) or LY294002 (Akt inhibitor) led to a decrease in *HIF1α* expression, thereby implicating these pathways in regulation of *HIF1α* expression. Since the breast cancer resistance protein (BCRP) was previously shown by the authors to be upregulated in the resistant compared to sensitive MCF7 cells [33], they postulated that *HIF1α* regulates BRCP expression. ChIP analysis under non-hypoxic conditions revealed that *HIF1α* binds to the hypoxia response element (HRE) of the BCRP promoter region in resistant cells. Furthermore, stabilising HIF1α by cobalt chloride further increased its binding to the HRE, while lapatinib treatment prevented this event. Pharmacologic or siRNA inhibition of HIF1α led to the decreased in *BRCP* mRNA expression levels and decreased the viability of the resistant cells by 40 % after 24 h. Furthermore, antisense inhibition of *HIF1α* and *BRCP* transcripts decreased the formation of mammospheres relative to cells treated with non-silencing siRNA. Conversely, upregulating or stabilizing HIF1α in the letrozole-sensitive cells led to an increased viability in response to letrozole treatment. Taken together, data from this study suggest that in the studied MCF7 letrozole-resistant cell line model, upregulation of HER2 and associated signalling pathways activate HIF1α, which activates *BRCP* expression to mediate AI resistance.

Methylation of HOXC10 in During Transition to AI Resistance

Many studies so far have focused on identifying compensatory pathways that are altered in response to AI treatment. In performing a genome-wide methylation screen approach, Pathiraja et al. [34] found *HOXC10* to be hypermethylated in two independent LTED cell lines relative to wild type MCF7 control. Quantification of the *HOXC10* transcript levels showed that the methylation was associated with reduced expression of *HOXC10* in the LTED lines, and treatment with 5′-aza-deoxycytidine (DNA methylation inhibitor) and trichostatin A (histone deacetylase inhibitor) led to an increase in *HOXC10* mRNA expression in both LTED and wild type cells.

ChIP analysis revealed that oestrogen induced a strong recruitment of ER to a distal site of the ERE of *HOXC10*. The expression of *HOXC10* mRNA was silenced in MCF7 treated with oestrogen, while in LTED cells it was low independently of the oestrogen exposure. Similarly, in pre- and post-treatment clinical samples from 30 patients that received neoadjuvant exemestane, *HOXC10* expression was increased

in the latter sample, resembling the short-term oestrogen deprivation in cell line models. In contrast, the long-term oestrogen deprivation led to the recruitment of EZH2 and trimethylation of histone H3K27, resulting in a repressive modification at the distal promoter region of *HOXC10* in cell line models. Furthermore, reducing *HOXC10* expression by shRNA in MCF7 cells—with and without oestrogen—resulted in a better viability of cells compared to non-silencing controls. Under the long-term oestrogen-deprived conditions, silencing *HOXC10* expression in MCF7 cells significantly reduced apoptosis—reflected by a decrease in poly-ADP ribose polymerase—and exhibited enhanced migration compared to non-silencing control. Importantly, in xenograft mice that were oestrogen deprived and injected with MCF7 cells expressing the *HOXC10* shRNA, the resulting tumour continued to grow, while tumours with non-silencing shRNA shrunk under the same condition. Finally, in matched primary-recurrence pairs of patients treated with AI, *HOXC10* was found to be downregulated in 4 out of 5 recurrent samples. Taken together, the findings from this study suggest that *HOXC10* is downregulated in the tumours that relapsed during endocrine therapy, and the absence of *HOXC10* expression promotes treatment resistance by a mechanism that involves prevention of cell death.

Cell Cycle-Related Resistance Mechanisms

Amplification of CCND1 and Localisation of Its Expression

As mentioned above, genomic aberrations such as amplification and deletion occur at high frequencies, often with associated clinical significance [35]. The 11q13 loci, which harbours the gene encoding cyclin D_1—*CCND1* is commonly amplified in breast cancer [36]. Cyclin D_1 promotes the G1-S progression of the cell cycle, by forming a complex with cyclin-dependent kinase 4/6 to phosphorylate and inactivate the retinoblastoma protein.

A translational study performed on samples from the Arimidex, Tamoxifen, Alone or in Combination trial found that patients with *CCND1* amplification have an increased risk of recurrence in response to either anastrozole or tamoxifen (hazard ratio = 1.61, 95 % CI, 0.39 − 0.92), after adjusting for other clinicopathologic variables [37]. Interestingly, high nuclear expression of cyclin D_1, was associated with a decreased recurrence risk (hazard ratio = 0.6, 95 % CI, 0.39 − 0.92). Furthermore, in patients with no *CCND1* amplification, high cytoplasmic cyclin D_1 expression was correlated with a longer time to recurrence.

Low Molecular Weight Cyclin E

A second regulator of G_1-S phase progression is cyclin E. In tumour cells, the low-molecular weight (LMW) form of cyclin E and its associated kinase are

constitutively active through all phases of the cell cycle. In MCF7 cells transfected with the aromatase gene, overexpression of either LMW or full-length cyclin E was able to overcome letrozole-induced inhibition of cell growth in the presence of androstenedione [38]. However, flow cytometry analysis revealed that only the LMW cyclin E could overcome the S phase arrest by anastrozole. Furthermore, levels of phosphorylated CDK2, a cyclin E-dependent kinase, were increased by LMW but not full length cyclin E. Importantly, the authors showed that patients with high expression of LMW cyclin E and Cdk2 are more likely to relapse in response to AI treatment. Pharmacologic inhibition of CDK2 by roscovitine was able to restore sensitivity to AIs in cases of resistance associated with Cyclin E LMW.

ER-Dependent, CDK4/E2F-Mediated Growth in Hormone-Deprived Cells

AI resistance can be caused by a cross-talk of non-endocrine signalling pathways with ER-signalling. Thus, activated HER- and MAPK-signalling may lead to ER phosphorylation with subsequent activation of ER-regulated genes [39, 40]. Alternatively, membrane-associated ER may activate MAPK-signalling leading to hypersensitivity to residual oestrogens [41], which could still be found in blood of patients despite receiving the aromatase inhibitors [42]. However, the role of ER phosphorylation and ligand-independent ER signalling in development of AI resistance is discussed in more details elsewhere in this book.

In a comprehensive study by Miller et al. [43], the authors confirmed that hormone-deprived breast cancer cells can continue to utilise ER in a ligand-independent manner; however this study has also highlighted the role of CDK4 and E2F in cancer response to estrogen deprivation. Treatment of LTED cells with fulvestrant, an ER downregulator, effectively reduced the growth of cells in two out of four different LTED lines. ER ChIP-qPCR of both ER-dependent LTED cell lines and three ER-positive primary tumours from patients who received between 10 and 21 days of neoadjuvant anastrozole treatment revealed that two of three primary tumours had a similar ER binding profile to that of the ER-dependent LTED cell lines, which supports the notion of ligand-independent ER DNA-binding capacity under hormone-deprived conditions.

Ontology analysis of genes downregulated by ER-dependent LTED cells showed a strong enrichment of cell cycle and proliferation theme. Sixty percent of the top processes identified contained binding sites of the E2F transcription factor. Thirty-seven of 61 genes deregulated in LTED cells by fulvestrant treatment had an E2F motif, and were involved in the cell cycle. In neoadjuvant anastrozole-treated patients, the 61-gene signature was significantly correlated with Ki67, a proliferation marker used as a surrogate of response. Importantly, 24 genes in the signature that were not related to cell cycle were also correlated with changes in Ki67. A kinome siRNA screen identified CDK4 as a crucial regulator of

hormone-independent growth. Indeed, treatment of MCF7 and HCC1428 LTED cells with the CDK4/6 inhibitor PD-0332991 led to a marked decrease in cell growth. Notably, PD-0332991 also inhibited cell growth in a panel of six parental breast cancer cell lines, although the effect was more pronounced in the LTED lines. Taken together, the data suggest that under hormone-deprived conditions, CDK4 is activated in ER-positive breast cancer cells to inactivate Rb, thereby releasing the inhibition of E2F. The activated E2F then initiates related processes including cell division, thereby providing a mechanism of resistance to AI treatment. Importantly, these cell-line-based findings are consistent with the results of recent clinical trials, which showed that adding CDK4/6 inhibitors to AIs can significantly increase time to progression in an advanced setting [44].

Tumour Microenvironment as a Mediator of Endocrine Treatment Resistance

Much emphasis has been placed on intracellular mechanisms of resistance to endocrine therapy. However, there are accumulating evidence to suggest that the microenvironment plays an active role in contributing to the tumour phenotype. There are several components of the microenvironment that have been implicated in endocrine therapy resistance—the stroma which includes fibroblasts, endothelial and immune cells; the extracellular matrix; and soluble factors such as growth factors and cytokines.

Inflammatory Immune Profile Correlates with Poor Response to Neoadjuvant AI Treatment

Recently, Dunbier et al. [45] reported the differences in the molecular profile of 81 breast tumours between baseline and after 2 weeks of anastrozole treatment. Response was measured based on pre- and post-treatment changes in the proliferative marker Ki67. Expression of a baseline inflammatory gene signature was observed to be negatively correlated with response to anastrozole, which was validated in an independent dataset. In addition, histological analysis revealed that tumours with lymphocytic infiltration was significantly associated with poorer response to treatment. Using a PAM classification approach, the inflammatory signature was identified to be closely related to the profile of a dendritic cell.

The findings of Dunbier et al. are supported by a meta-analysis conducted by Gao et al. [16]. In this study, the associated published biologic gene modules were assessed for correlation with response to neoadjuvant AI at baseline and after 2 week treatment. At baseline, a STAT1-based immune signature was correlated with poor treatment response, as determined by Ki67 change. After removal of

proliferation-associated genes and p-value adjustment, the module was still significantly correlated with treatment response. Interestingly, after 2 weeks of AI treatment, the expression levels of immune module, together with a plasminogen activator module, are increased. Furthermore, in vitro studies using aromatase-expressing MCF7 and their anastrozole-resistant (AnaR) counterparts co-cultured with human cancer associated fibroblasts showed that the AnaR cells increased expression levels of interleukins 1 and 6 and their cognate receptors. Importantly, treatment of AnaR cells with the upregulated cytokines induced proliferation of these cells. Interestingly, when the two cell lines were cultured in conditioned medium from the stromal fibroblasts, anastrozole could inhibit the growth of wild type, but not AnaR cells. Blocking the activation of JAK2/STAT3 signalling axis inhibited condition medium-induced growth of AnaR cells. To conclude, cytokines of stromal origins may provide an alternative signalling cues to trigger cell division in breast cancer cells that have adapted to an oestrogen-deprived environment.

Taken together, data from these studies suggest that tumours with a strong immune-related gene expression profile and high levels of diffuse lymphocytic infiltration are correlated with resistance to neoadjuvant AI treatment. Indeed, patients with such tumours may benefit from an immune-modulating and oestrogen deprivation treatment strategy.

Adipocytes as a Driver of Oestrogen-Dependent Growth

Adipocytes are highly abundant in the tumour microenvironment and have been shown to release various factors that affect endocrine signalling, inflammation and angiogenesis. To study how adipocytes interact with a nascent tumour in vivo, Liu et al. [46] performed subcutaneous injection of murine pre-adipocytes and MCF7 cells into an immune-deficient mice. The control mice were injected with only MCF7 cells. No tumour growth was observed in the control mice, while mice coinjected with adipocytes showed gradual growth of the tumour 3 weeks post-injection. Immunoblotting of aromatase expression levels showed that the enzyme was expressed lower in the pre-differentiated adipocytes, compared to the differentiated counterparts. Furthermore, the expression levels of aromatase were increased when the adipocytes were treated with recombinant mouse leptin in vitro. Quantitative PCR performed on fat pads of mice treated with recombinant leptin showed that a six fold increase in aromatase mRNA expression relative to untreated control mice. Crucially, the authors showed that the aromatase mRNA expression level of mice placed on a high-fat diet was 45 fold higher than the low fat diet group. Also, obese mice with genetic leptin deficiency expressed lower levels of aromatase, compared to lean mice of similar genetic background, and leptin administration into the deficient mice increased the aromatase expression. Taken together, the findings from this study suggest that adipocytes within the immediate environment of a hormone-dependent breast tumour express aromatase via a

leptin-dependent mechanism, which likely results in the synthesis and release of oestrogen into adjacent tumour cells to spur their growth.

Future Directions

The management of breast cancer has been significantly improved by greater awareness in general, early detection from screenings, new treatments, and improved risk stratification of patients which aids clinical decisions. With increasing utility of high throughput—omics technologies, we are reaching a comprehensive appreciation of the molecular underpinnings of this disease. Importantly, knowledge from this effort has also generated more questions that require exploration.

Tumour heterogeneity is an important phenomenon that has only gained traction recently with the sequencing of multiple clonal populations within a tumour. The fact that different populations with different genetic aberrations coexist within a tumour would probably be critical in mediating treatment resistance [47–49]. The in vitro models of AI resistance provide a fairly homogenous population of cells at any one time, and therefore, limit the utility of this system to characterize heterogeneity at the molecular level. As a corollary to this, studies that perform gene expression profiling on such systems may only acquire information on that specific clone of cells. Since these clones are homogenous, any information on pathways correlated with resistance are likely to be 'dominant', which may preclude detection of the 'weaker', but biologically important signals that are lost from such methodology.

Another important research area that requires more effort is the role of the tumour microenvironment in treatment resistance [50]. Three-dimensional culture of cancer cells with known components of the stroma may to some extent model an in vivo tumour-stroma environment. Nevertheless, given the complexity of the stroma, such 3D models may only superficially capture the actual interactions that occur in an actual tumour.

No Conflict of Interest No potential conflicts of interest were disclosed.

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Chapter 10
Prediction of Response to Aromatase Inhibitors in Breast Cancer

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Abstract Aromatase inhibitors (AIs) are major treatment options for the management of patients with breast cancer. The drugs are effective and response rates can be high. However, resistance, either primary or acquired during treatment, may occur. Optimal clinical management requires accurate predictors of response to identify those tumours, which are most likely to respond (so sparing patients with resistant tumours needless side-effects of ineffective therapy). Currently, oestrogen receptor (ER) status is the only factor used routinely to select for treatment with aromatase inhibitors, but a substantial proportion of ER-positive tumours fails treatment. There is, therefore, an urgent need to identify additional markers by which accurately to predict clinical response on an individual basis. Whilst other markers (such as progesterone receptors or HER2) have some predictive powers, individually they have limited utility for routine use. The hope is that discovery strategies based on genome-wide searches will identify novel markers that can be used as predictive indices. Molecular phenotyping of individual tumours could then be used to decide not only which patients should be treated with AIs but whether AIs should be used alone or in combination or in sequence with other targeted agents in order that clinical benefits are maximized. This chapter will focus on utility of routinely assessed biomarkers, multi-component indices and gene signatures and outline the future perspectives in studies aimed to AI response prediction.

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A. Larionov (ed.), *Resistance to Aromatase Inhibitors in Breast Cancer*,
Resistance to Targeted Anti-Cancer Therapeutics 8,
DOI 10.1007/978-3-319-17972-8_10

Abbreviations

AIs	Aromatase inhibitors
ER	Oestrogen receptor
PgR	Progesterone receptor
ER+ve	Oestrogen receptor positive
ER−ve	Oestrogen receptor negative
pCR	Complete pathological response

Introduction

Endocrine therapy is a major treatment modality for hormone-dependent breast cancer. It has a relatively low morbidity and anti-hormone treatments have had a significant effect in reducing mortality for breast cancer [1]. The general principle of most endocrine treatments for breast cancer is to deprive tumours of oestrogen, which appears to be driving growth. This objective may be achieved in different ways but in recent years agents inhibiting the aromatase enzyme which catalyses the conversion of androgens to oestrogen have been increasingly used [2]. This is the outcome of rational drug development that has generated third generation inhibitors such as letrozole, anastrozole and exemestane which have exceptional potency and specificity [3]. When used clinically in postmenopausal women these agents can inhibit whole body aromatization of androgen by >99 % [4, 5] and often reduce circulating oestrogens to undetectable levels [6]. Consequently, AIs can reduce tumour proliferation [7, 8] and may cause tumour regression [9, 10]. Clinical benefits have been seen in advanced breast cancer [11] and in the adjuvant [12–14] and neo-adjuvant settings [15, 16]. Third generation AIs are now front-line treatments for breast cancer [2]. However, response rates range between 35 and 70 % in major neoadjuvant studies [17] and the benefits may be lower in advanced disease [18]. Acquired resistance after initial successful treatment is also common [19]. Optimal clinical management, therefore, urgently requires the identification of markers that predict accurately for response in individual tumours. In this chapter we will (i) consider factors which may be confounders in prediction of endocrine response in general and to aromatase inhibitors in specific (ii) review the current status of molecular markers such as ER, PgR and HER2 (iii) discuss the potential of approaches using multiple markers and multigene transcriptional signatures and (iv) assess the potential of new technologies and speculate on what the future holds.

Confounding Factors in Predicting Endocrine Response

Key factors to consider in reviewing predictive biomarkers for AI response include the confounding effect of different clinical settings, the diversity of ways to measure the response and various levels of evidence supporting different biomarkers.

Response Assessment in Different Clinical Settings

Endocrine treatment can be given in 3 major settings (i) as adjuvant therapy after surgery for patients with early breast cancer (ii) for treatment of advanced or metastatic disease and (iii) as neoadjuvant or pre-operative therapy for non-disseminated large tumours (Fig. 10.1).

Adjuvant Setting

Most women with breast cancer present with early stage disease. Adjuvant use of endocrine therapy after primary surgery, therefore, represents its major setting. The identification of predictive factors associated with clinical benefit to adjuvant therapy is, thus, particularly important. However, use of the adjuvant setting to investigate mechanisms and markers of endocrine response is beset with complicating factors. Treatment is given after breast surgery so that response of the primary tumour cannot be studied directly. Instead, time to disease recurrence and survival is usually monitored as surrogate markers of endocrine response. These long-term outcomes are, however, obscured in that they may be influenced not only by response to the therapy but also by the occult spread of disease and by the inherent aggressiveness of the cancer. Thus, patients may present with a good prognosis because they have a slow-growing, poorly metastasizing tumour rather than one which is particularly responsive to treatment. To account for such confounders it is important that adjuvant studies have a control arm so that comparisons can be made with patients not offered endocrine therapy. A further consideration is that adjuvant protocols potentially require many hundreds of patients to be studied over a relatively long time so that sufficient number of events

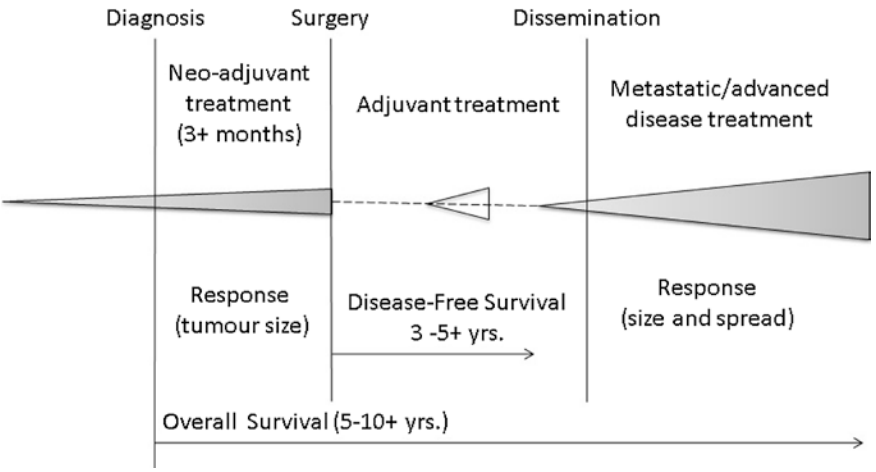

Fig. 10.1 Clinical history of breast cancer: clinical settings and modalities of treatment

accumulate for meaningful analysis. Additionally, markers are usually measured in excised primary tumours whereas the measured endpoints are largely dependent upon the behaviour of micro-metastatic disease. Most clinical trials comparing the adjuvant use of AIs versus tamoxifen have consistently shown clinical benefit in favour of AIs [12–14]. Effects on disease recurrence are usually statistically significant whereas those on survival are rarely so, unless trials are combined in a meta-analysis [20, 21]. These studies also suggest that benefits of AIs are not evident in all patients but more apparent in certain subgroups. Thus, the identification of predictive factors associated with clinical benefit in patients receiving adjuvant therapy is particularly important.

Neo-adjuvant and Advanced/Metastatic Settings

A more direct approach of studying endocrine response is to measure treatment effects in the tumour while it remains in the body. Historically, these studies have been performed in patients with metastatic disease [22]. Compared to adjuvant trials, studies in the metastatic setting required comparatively small numbers of cases followed up for a shorter time. However, it is not always possible to measure accurately response in metastatic sites. Consequently, there has been an increasing interest in the neoadjuvant setting in which therapy is given whilst the primary tumour is still within the breast, and surgery is usually delayed for several months [23, 24]. This has advantages in that the primary tumour is available for measurement of response and for biopsy (sometimes on multiple/sequential occasions) [25, 26].

There are, however, issues relating to duration of therapy and timing of tumour assessments. Historically in most studies of neoadjuvant endocrine therapy was given for 3–4 months [17]. Although somewhat arbitrary, length of treatment is based upon (i) allowing sufficient time for treatment to produce meaningful tumour shrinkage in responsive cases and (ii) not persisting with ineffective treatment in resistant tumours. However, recent studies showed that extended treatment may increase the response rates [27–30]. Moreover, since individual tumours are likely to respond in different time frames, the same time point of response assessment in all may lead to misclassification of response in some. Therefore, the current consensus is that neo-adjuvant endocrine treatment should last for at least 5–8 months, until sufficient response or until progression [31, 32].

The window between diagnosis and breast surgery also provides an opportunity to give preoperative endocrine therapy during which time, effects of treatment on the primary tumour may be monitored [33]. Furthermore, it was recently shown that molecular and proliferative response to short-term endocrine treatment may be prognostic for recurrence-free survival [34–36].

In this review, we will use term ‘response’ to designate tumour changes associated with therapy rather than clinical benefit following treatment; consequently the discussion of response types below is focused on neoadjuvant and advanced settings rather than adjuvant treatment.

Types of Response

Clinical Response

If undefined, response generally refers to clinical response in which tumour size or burden is monitored during treatment. Most studies routinely use clinical response as an endpoint. In the neoadjuvant setting, clinical response is usually assessed by measurements using callipers, mammography, MRI or ultrasound [17, 37, 38]. Most studies assessing the value of predictive markers employ change in tumour volume or size over a specified time period, although in patients offered extended neo-adjuvant therapy or in those with metastatic disease, time to progression (duration of response) or clinical benefit (objective response or durable stable disease for at least 6 months) may be used to measure clinical response. In the advanced setting, direct evaluation of tumour response may also be measured. Several approaches have been suggested including the WHO (World Health Organisation) [39] and RECIST systems (Response Evaluation Criteria in Solid Tumours) [40]. In the adjuvant setting, however, it is only possible to make indirect assessments of tumour response such as time to progression (duration of response).

Pathological Response

An alternative modality of response assessment is based on pathological changes caused by treatment. Thus, complete pathological response (pCR) to neoadjuvant chemotherapy has been shown to be a powerful determinant of long-term outcome [41, 42]. As a consequence, there has been pressure to use pCR as a surrogate endpoint in endocrine studies too—but there are complications. First, pCR is not always associated with long-term outcome, even for cytotoxic treatments [43]. Second, pCR is rare in endocrine treatment [44] and therefore it would be impractical to use, even if it was informative. Thus, pCR is not recommended for use in neo-adjuvant endocrine trials [42]. Nevertheless, neoadjuvant treatment with AIs can produce pathological changes (either reduction in histological grade or decrease in cellularity and increase in fibrosis) in 60–80 % of tumours by 3–4 months of treatment [9, 45]. However, quantitative measurements of partial pathological response can be subjective and, if such pathology is to be used routinely, robust criteria for assessment need to be agreed. Clinical response and pathological changes after treatment with AIs are significantly associated, but the concordance is not exact. About a fifth of tumours displays a discordant phenotype [46] in which tumours either (i) have a decrease in tumour volume without evidence of pathological changes or (ii) appear not to change in tumour size but display a decrease in cellularity.

Anti-proliferative and Molecular Response

Marked decrease in tumour proliferation in breast cancer is the most prominent pathological and molecular feature caused by AI treatment [25, 46]. Changes may be seen in about 80 % of cases after 3 months and in many cases as early

as 14 days. Different patterns of proliferation change with time of treatment can be observed. Most tumours display decreases in proliferation at 10–14 days, which are maintained or become more pronounced with treatment after 3 months. However, in a minority of tumours it is possible to observe other patterns in which (a) initial decrease in proliferation is followed by return to pre-treatment values, (b) tumours show delayed response in proliferation and (c) cases display little changes either at 14 days or 3 months [46]. Early changes in proliferation following AI treatment have been reported to relate to long-term outcomes [34, 36]. Proliferative responses are also positively and significantly correlated with both clinical and pathological response [46] but the types of response are not equivalent and discordant phenotypes are common. For example, clinical responses and cell cycle responses to letrozole in a major neoadjuvant trial were discordant in over one third of cases [38] and the size of proliferative response was not significantly different in cases clinically responding or not responding to anastrozole in another trial [7]. However, proliferative response appears to have a stronger association with pathological than with clinical response [46].

Molecular response to AI treatment will be considered in more detail later in the chapter. However, it should be noted that there is clear evidence of wide changes in molecular phenotype following treatment with AIs [25, 47]. For example, the progesterone receptor (PgR) which is classically regarded as a marker of oestrogenic activity is clearly reduced in 70–80 % of cases and in about one half of cases staining may completely disappear [46]. Early molecular changes on treatment may have predictive potential for clinical response and may be influential in determining subsequent tumour behaviour and patient outcome [48], suggesting that molecular responses may be used as putative surrogates for clinical or anti-proliferative outcomes of AI treatment.

Most molecular markers utilize a single measurement usually made in a tumour biopsy taken before treatment. Such markers may be considered truly ‘predictive’. However, a case can be made for starting treatment and screening for early changes in pathological or molecular events, which might occur in responsive but not resistant cases. Because of this, it may be more informative to biopsy tumour early into treatment (e.g. 10–14 days) [36, 49, 50]. The use of a dynamic approach comparing sequential biopsies taken before and early into treatment may be even more revealing. In this regard, it should be noted that using a single sampling early into treatment does not allow discrimination as to whether the level of marker at that time point occurs as a result of treatment or it is inherent to the tumour and was present before treatment. In contrast, the sequential sampling allows better understanding of the dynamics and interaction between treatment and tumour biology [25, 48].

Importantly, predictors of response to AIs may either (i) predict breast cancer response to endocrine therapy in general or (ii) be specific to AIs. In this respect, it may be pertinent that the mechanism of action of specific third generation AIs is more targeted than other forms of endocrine therapy such as ablative procedures, which may affect several classes of hormones, and drugs such as tamoxifen, which can have both anti-oestrogenic and oestrogenic effects. It is also possible to

envisage that predictive factors may vary between different inhibitors, particularly between the two major classes of AIs (type I steroidal and type II non-steroidal). In this respect, it is interesting that there is not a complete clinical cross-resistance between steroidal and non-steroidal AIs [51] and molecular response profiles may differ between inhibitors in experimental systems [52].

Biomarker's Development and Levels of Evidence

Clinical utility of prospective biomarkers depends on their phase of development and supporting evidence. Table 10.1 aligns the phases of biomarker development (as suggested by Pepe et al. [53]) with the framework for levels of evidence suggested by the American Society of Clinical Oncology [54]. Overall, a biomarker's utility depends on the analytical validity of clinical test, size and adequate design of the supporting studies. The higher levels of evidence require large prospective controlled randomized trials, which are costly and take long times for enrolment. Thus, the size of a randomized 2-group study is reciprocal to square of the expected effect size. Even using optimized study designs, it translates to millions of dollars/euros and years of enrolment (e.g. MINDACT study that evaluates the 70-gene classifier [55], and the TAILORx study evaluating the 21-gene classifier [56]). Such prospective studies are only feasible for already commercialized tests. For non-commercialized prospective markers the issue of sample size may be alleviated by using archival samples. To facilitate the use of archival specimens in biomarker research, Simon et al. [57] suggested a revision for the levels of evidence framework. Essentially they highlighted that differences between prospective and retrospective sample collection may become semantic when specimens are continuously collected by tissue banks under standard operating procedures.

Table 10.1 Development of biomarkers

Phases of development [53]	Levels of evidence [54]
1 Pre-clinical studies	NA
2 Clinical assay validation	5 Pilot exploratory studies to evaluate distribution of marker in relevant population
3 Retrospective validation	4 Small retrospective studies suggesting association of the marker with the clinical features in question
	3 Large retrospective study without pre-planned biomarker analysis at the time of samples collection
4 Prospective validation	2 Large prospective study, which included biomarker as a secondary objective, yet powered for different (e.g. therapeutic) primary endpoints
	1 Meta-analysis of the studies of 2–3 level or sufficiently powered prospective controlled study designed for the biomarker establishment as a primary endpoint
5 Impact on cancer control	NA

Therefore, consensus between two or more independent sufficiently powered and adequately designed studies may be sufficient to achieve the second or even first level of evidence, even if the studies utilize archival samples that had been physically deposited to tissue banks before commencing the trials.

Clinical role of biomarkers suggested for endocrine response prediction depends on the level of supporting evidence and on practical steps that could be implemented as a result of the test. For instance, it has been firmly established that HER2 amplification or lack of PgR expression are associated with reduced endocrine response rates in ER+ve breast cancers [58]. However, many of HER2+ve or PgR−ve tumours still respond to endocrine treatment [8]. Therefore, despite the solid evidence supporting association with endocrine response these markers have a low impact on clinical decision making, as discussed in more detail in the next section.

Routinely Measured Molecular Markers

To date expression of oestrogen and progesterone receptors, amplification of HER2 and expression of Ki67 have been the most commonly measured molecular markers in endocrine-treated breast cancer. They capture important aspects of tumour biology and have been intensively studied with regard to prediction of AI response.

Oestrogen Receptor

The proliferative effects of oestrogens in breast cancer cells are mediated through oestrogen receptors, which are ligand-dependent nuclear transcription factors, regulating expression of oestrogen-dependent genes [59]. Studies in a variety of clinical settings have demonstrated the clinical utility of oestrogen receptor as a molecular predictor for hormone response. Currently, therefore, tumour oestrogen receptors status is the main molecular marker routinely used to aid clinical decision-making with regard to hormone treatment. It has a strong negative predictive value: ER-negative tumours virtually never respond [60, 61]; in contrast, ER-positive tumours respond to endocrine treatment in 50–70 % of cases depending on clinical setting and response criteria [50, 60].

Most of the data linking ER expression to endocrine response was obtained using tamoxifen, before the introduction of the third generation AI inhibitors. This knowledge has deterred any randomized trial on AI for patients with ER−ve tumours. Therefore, there is little clinical information relating ER status with response to AI. However, early anastrozole and letrozole trials in the advanced setting whilst excluding ER−ve tumours included substantial numbers of patients with unknown ER status. The relationship between hormone

receptor status and treatment outcome in these trials has been reviewed by Buzdar et al. [62]. They found that positive ER status was important in determining an improved time to progression with the use of first-line treatment with AIs. However, such analysis has provided no evidence about AI responses in ER−ve disease. Anderson et al. [63] retrospectively identified 29 ER−ve patients with advanced breast cancer (ABC) treated with the third generation AIs in the Royal Marsden Hospital (London) from 1994 to 2008. These were compared with 146 ER+ve ABC patients treated in the same hospital over the same period. The clinical benefit was observed in only 6 of 29 ER−ve cases (21 %; 95 % CI 8.9–39.7) as compared to 88 of 146 ER+ve cases (95 % CI 51.9–68.3). The biological nature of response in some ER−ve patients is unclear. It could be explained by intra-tumoral heterogeneity [64] or by methodical issues with ER detection [65]. The small number of AI responses in ER-negative patients observed by Anderson with colleagues in advanced breast cancer is consistent with observations published for protocol violators in the neo-adjuvant setting. Thus, Ellis et al. [61] reported a study designed to compare Letrozole with Tamoxifen in a neo-adjuvant setting. The study was to include ER+ve patients only. However, central review re-classified 16 enrolled patients to ER−ve category. Only 3/16 (19 %) of ER−ve patients responded to letrozole; the response rate in ER+ve patients was significantly higher (72/120; 60 %). Similarly, a small number of ER−ve patients have been enrolled in the adjuvant AI trials as protocol violators, and indirect evidence from these trials suggests no benefit of adjuvant AIs in ER negative tumours [12].

Taken together (i) knowledge about AI's mechanism of action (ii) the results of endocrine trials with tamoxifen and (iii) the limited data from AI studies established tumour oestrogen receptors status as the main molecular marker used clinically to aid decision making, ER-negativity being used to exclude treatment with AIs. A dilemma surrounds the 20–40 % of ER-positive tumours apparently resistant to AIs. The challenge is now to discriminate accurately and on an individual basis, which ER-positive tumours respond to treatment and those that do not.

At this stage it is worth considering in more detail the methodology used to determine ER status. Initially ER expression in tumours was measured quantitatively using a radio-ligand method but this was superseded by immunohistochemical staining (IHC), which became the standard of practice [66]. A number of cut-offs have been suggested in order to define the ER "positivity" and "negativity" by IHC, varying from 0 to 20 % of the stained cells [67]. The most commonly used threshold is 1 %, as recommended by American Society of Clinical Oncology [65]. While the low cut-off setting is clinically justifiable to administer non-toxic treatment for a life-threatening disease, it also disposes for overtreatment. Indeed, the biology of tumours with low ER expression often resembles ER-negative tumours [68, 69] and numerous studies have confirmed that level of tumour ER expression is associated with the likelihood of endocrine responsiveness. In context of response to AIs, this was most clearly shown in the P024 trial which compared neo-adjuvant Letrozole and Tamoxifen [61]. The trial measured ER expression using semi-quantitative Allred score that splits tumours into 8 categories according to the proportion of stained cells and the intensity of staining.

Scores up to 2 correspond to ER−ve tumours, and a score of 8 means that 100 % of tumour cells show intensive ER staining [66]. A strong positive association was observed between ER score and clinical response for both Letrozole and Tamoxifen in the P024 trial. This is consistent with the data from adjuvant studies, which reported worse outcomes in ER-poor patients (e.g. the BIG 1-98 study) [70]. Importantly, the association between intensity of ER expression and response was not absolute: many of ER-low tumours still benefit from endocrine treatment, especially from aromatase inhibition [61].

Progesterone Receptor and HER2

One of the first attempts to explain the lack of endocrine response in some ER+ve tumours was made by Horwitz and Mc Guire in 1975, who hypothesized that mere presence of ER may not be sufficient for fully functional ER signaling. Thus, they suggested complementing ER measurements with measurement of oestrogen-induced proteins, such as progesterone receptor (PgR) [71]. Currently, PgR is routinely assessed to complement ER expression. A recent meta-analysis of 20 studies including 4111 patients suggests that PgR is virtually never expressed in ER−ve tumours [72]. At the same time, in agreement with the Horwitz and Mc Guire's hypothesis, expression of PgR reveals strong molecular diversity within ER+ve phenotype. Thus, meta-analyses of multiple trials showed that 43–75 % of ER+ve tumours expressed PgR, while 25–57 % did not [72, 73]. It has been shown that expression of PgR is associated with better prognosis [74, 75] and additional benefit from adjuvant endocrine therapy [73] in ER+ve tumours. At the same time, data about the predictive utility of PgR in AI trials are inconclusive. Thus, the neo-adjuvant IMPACT study reported a positive association of PgR expression with proliferative response [76] while P024 reported no linear association of PgR expression with clinical response [61]. Even assuming increased benefit for PgR+ve patients, many ER+ve PgR−ve patients respond to AI treatment. Therefore, PgR-negativity does not preclude treatment with AIs and baseline PgR assessment has a limited practical utility for administering AI treatment.

In the neoadjuvant setting, sequential measurements of PgR may be used to determine whether expression is reduced with AI treatment, using the change as evidence of reduced oestrogen stimulation. In this respect, PgR immunohistochemical staining is markedly reduced in most of the tumours after 2 weeks of treatment, being consistent with the antioestrogenic effects of AIs [46]. However, loss of PgR expression although marked and occurring early into treatment, may occur independently of pathological and clinical response [46].

Similarly, HER2 amplification indicates a possibility of oestrogen-independent tumour growth. In the adjuvant setting HER2 positivity is associated with poor prognosis [58]. Interestingly, neo-adjuvant studies show that following AI treatment many HER2-positive tumours still have high levels of proliferation even

when they show a clinical response [77, 78]. At the same time, despite the reduced anti-proliferative responses, neo-adjuvant studies have failed to demonstrate significant differences in clinical response rates between HER2+ve and −ve cancers [61, 79] and many HER2 positive tumours are responsive to AI treatment [80]. Despite some initial expectations [81] neither PgR nor HER2 could be used to detect additional benefit of AIs over tamoxifen [58, 82]. These observations, together with the fact that just 10 % of ER+ve tumours show HER2 amplification [83], suggest that HER2 has a limited utility as a marker of response or resistance to AIs. Instead, HER2 positivity is used as an indication for combining AIs with trastuzumab.

Ki67

Ki67 has been intensively studied as a predictor and surrogate of clinical response to endocrine treatment in breast cancer. Ki67 was discovered in 1983 as a nuclear antigen expressed in proliferating lymphocytes [84]. Later it has been found in other types of proliferating cells. Conveniently, Ki67 expression is limited to G1-S-G2-M phases of the cell cycle, not being detected in resting cells entering G0 [85–87]. The exact intracellular function of Ki67 is poorly understood [88–90]. It is a large protein (>350 Kda) interacting with chromatin [91, 92]. It can be phosphorylated [93]. It is associated with nucleoli, ribosome formation and protein biosynthesis [94, 95]. Several antibodies have been developed to detect Ki67 antigen; therefore in some studies it may be referred as MIB1 [96].

High pre-treatment Ki67 scores have been associated with poor long-term outcomes in ER+ve patients with breast cancer [97]. However, this does not necessarily relate to response to treatment: high proliferation might be expected to reduce the outcome irrespective of response. Moreover, the utility of Ki67 scores as a single predictive marker for response to AIs is questionable because pre-treatment Ki67 levels have not been found to be significant different in responding and non-responding tumours whether assessed clinically or pathologically [46]. This does not mean that Ki67 is totally without merit. As discussed above, early changes in Ki67 could be more informative than pre-treatment values [34], suggesting that if after treatment, Ki67 levels are still high, therapy has probably been ineffective. Conversely, it has been suggested that low residual proliferation after short-term letrozole therapy is an early predictive marker of response in high proliferative ER-positive breast cancer [36].

In summary, numerous studies attempted to analyze routinely measured molecular markers to guide endocrine treatment in general and AI treatment in particular. This established ER expression as the major marker of response to AIs. However, pre-treatment levels of PgR, HER2 or Ki67 only have a limited practical utility for AI response prediction. Therefore, a significant effort has been made to develop alternative molecular markers for guiding AI treatment within ER+ve patients.

Multigene Transcriptional Signatures and Multicomponent Clinical Indices

Making a Multi-gene Signature

Microarray technology developed in mid-1990th opened the door for simultaneous measurement of multiple gene expression [98], ultimately allowing capture of whole-genome expression patterns [99]. The diagnostic potential of the new tool was first demonstrated in haematological malignancies [100]; then it was promptly reproduced in breast cancer [101]. While microarray technology has progressed enormously since, the fundamental steps of designing a multi-gene transcriptional signature remain the same as outlined in the initial haematological studies [100]. These steps include (i) quality control and exploratory analyses (ii) selection of informative genes that differentiate between the clinical phenotypes in question and (iii) design of a classifier that utilizes a subset of the informative genes to classify new clinical specimens. Numerous specialized bioinformatics algorithms and tools have been developed to facilitate each of these steps. Thus, the common components of exploratory analysis include unsupervised class discovery and visualization procedures, such as clustering, principal component analyses, building heatmaps and dendrograms [102]. An important part of exploration may be focused on functional annotation of detected gene clusters (e.g. pathway fitting or GO enrichment analyses) [103].

The selection of informative differentially expressed genes is performed in a supervised manner. It is based on amplitude, frequency or statistical significance of gene expression differences between the known phenotypes in the discovery dataset [25, 48, 104]. Typically, different studies use different algorithms to derive the lists of differentially expressed genes. The statistical significance assigned for each candidate gene should be adjusted for multiple testing because many thousands of genes are tested at the same time in each microarray experiment. Most commonly used multiple testing adjustments include Bonferroni correction and FDR-based algorithms (FDR stands for a pre-specified False Discovery Rate) [105–107]. The former merely multiplies initial p-value by the number of simultaneous tests. This is a strict approach, which may lose many informative genes for the sake of avoiding any false-positives. In contrast, the FDR-methods explicitly allow for a certain rate of false-discovered genes for the sake of keeping all potentially informative ones. Again, the numerical values produced by the different correction procedures may drastically differ.

Finally, the last step in making a multi-gene signature includes (i) selecting a sub-set of informative genes to be measured in the new clinical samples and (ii) selecting and training an algorithm that interprets these measurements in terms of the clinical phenotypes. The exact sub-set of genes included into the final classifier may be manually restricted or expanded on a basis of the genes' informativeness, functional annotation or assay technicalities. For instance, additional reference genes are usually added to the test when a gene list is adapted for qPCR measurements (as in Oncotype DX or Endopredict tests) [108, 109]. Classification algorithms implemented in breast cancer multi-gene signatures tend to be based

on simple linear function of the genes expressions (e.g. Oncotype Dx) [108] or on a sum of distances from empirically detected centroids (e.g. PAM 50) [110]. Some studies benefit from more advanced classification algorithms (e.g. random forests or support vector machines) [111]. However, lack of transparency in such advanced algorithms may limit their acceptance by clinicians.

Selecting the optimal numerical values for the classifier's parameters is designated as "training" the model. This includes adjusting coefficients in the linear models and setting thresholds for classification calls. In some advanced classification algorithms training may also include selection of most informative genes within the larger list of candidates. Usually the models are trained on the same dataset, which have been used for exploratory analysis and for the candidate genes selection. Often, the number of genes in the microarray-based classifier may be higher than number of cases in the training dataset. If this is the case, special precautions should be made to avoid over-fitting during the training [112]. Over-fitting occurs when numerical parameters are tuned to the specific composition of the training set, rather than to the biology underlining the classification. Iterative training on the sub-sets of the entire training dataset reduces the possibility of over-fitting (e.g. the leave-one out procedure [113]). Finally, a signature intended for clinical use has to be validated on a completely new independent dataset(s), ideally collected in a separate medical centre(s) to achieve the required level of evidence for intended clinical use [54, 57, 114–116].

A large number of multi-gene signatures and multi-parameter indices have been developed over the last decade to aid clinical decision making in breast cancer. Some have been initially focused on endocrine treatment (e.g. SET, Endopredict and PEPI); others were developed for different purposes and later tested in context of endocrine response prediction (e.g. PAM50 or Oncotype Dx).

Intrinsic Subtypes

The first microarray-based classification of breast cancer was suggested in 2000 by Perou et al. [101, 117]. Analysing expression of 8102 genes in 65 tumours they showed that breast cancers could be separated into 5 major intrinsic molecular classes: Luminal A and B, Basal-, HER2-positive- and Normal-like subtypes. Except for the Normal-like cluster, this intrinsic subtypes classification appears robust and a recent large-scale analysis that involved samples from 825 patients analysed on multiple platforms (including genome-wide expression microarrays, DNA methylation chips, single nucleotide polymorphism arrays, miRNA and whole-exome sequencing and reverse-phase protein arrays) confirmed that absolute majority of the breast cancers can be classified to one of the intrinsic sub-types described by Perou and Sorlie in their initial study [118]. This intrinsic classification, however, may just provide a new way of identifying the previously known types of breast cancer, broadly corresponding to ER+ve (luminal), triple-negative (basal) and HER2+ve tumours. Nevertheless, the microarray-based classification has increased the molecular understanding of the known types and, most importantly in endocrine treatment context, highlighted the diversity of ER+ve tumours.

The intrinsic sub-types classification is implemented in the PAM50 test, which assigns tumours into intrinsic categories according to the expression of 50 genes characteristic for the different sub-types. The classification algorithm is based on similarity to the pre-defined centroids. PAM50 gene expression scores may be further translated to the risk of recurrence score (ROR), which is a linear function of individual sub-type scores with or without addition of the tumour size [110]. An alternative implementation of the intrinsic sub-type classification is based on the combined analysis of the routinely assessed IHC markers (ER, PgR and HER2) with addition of Ki67 score [119, 120]. In this case, luminal A and B subtypes are typically defined as ER and/or PR positive, HER2 negative tumours, with low (luminal A) or high (luminal B) expression of Ki67 (or low/high grade respectively).

The biological difference between the luminal A and B sub-types suggests a possibility of differences in their responses to AI. This hypothesis has been tested in two recent neo-adjuvant studies [121, 122]. Contrary to the expectations, these studies have not found significant differences in AI response rates between Luminal A and B subtypes. Despite the different pre-treatment Ki67 levels, similar proliferative responses were reported for luminal A and B tumours by Dunbier et al. [122] (75 % mean Ki67 suppression in both groups), which was consistent with the findings reported by the second group [121]. Surprisingly, Dunbier et al. reported a very high proportion of non-luminal phenotypes. Thus, of 104 enrolled ER+ve patients 36 % were classified as Luminal A, 19 % as Luminal B, 29 % as Normal, 12 % as HER2 and 5 % as Basal phenotype. As expected HER2 and basal phenotypes showed the lowest response rates (50 % and 15 % of Ki67 suppression respectively). Contra-intuitively, the Normal-like subtype showed higher response rates than luminal tumours (83 % vs. 75 % of Ki67 suppression respectively). These results suggest that some proportion of the ER+ve patients may have biological features similar to non-luminal phenotypes, which may influence clinical response to AI. Interestingly, the study of Ellis et al. [121] enrolled only ER-rich patients (Allred 6–8) and detected only a small proportion of non-luminal phenotypes in the study population. This suggests that the high proportion of non-luminal intrinsic subtypes observed by Dunbier et al. [122, 123] might be explained by the well-known differences between tumours with low- and high- expression of ER (as discussed in more details earlier in the oestrogen receptor section). If this is the case, then intrinsic sub-types testing may complement the binary ER assessment, especially when low threshold is set for ER-positivity [67, 68].

Oncotype Dx

Oncotype Dx is one of the most validated multi-gene signatures and is commercially available [124]. It integrates expression of 21 genes into numerical index associated with time to relapse in adjuvant setting (RS, “Recurrence Score”). 16 of the genes are associated with breast cancer biology: proliferation (Ki67, STK15, Survivin, CCNB1, MYBL), ER signalling (ER, PgR, BCL2, SCUBE2), HER2 signalling (HER2 and GRB7), invasion (MMP11 and CTSL2) and other biological functions

(GSTM1, CD68, BAG1). The remaining 5 genes are used as references for normalisation in qPCR measurements (ACTB, GAPDH, RPLP0, GUS, TRFC) [108]. At present, the main utility of Oncotype DX is to identify patients with very good adjuvant prognosis, to avoid unnecessary cytotoxic chemotherapy [125, 126]. However, a recent study has explored Oncotype DX potential for response prediction for neoadjuvant treatment with exemestane [127]. The authors reported that response and conservation rates were significantly higher in low RS group (clinical responses: 59.4 % vs. 20.0 %, $p = 0.015$; conservation rates: 90.6 % vs. 46.7 %, $p = 0.028$; low vs. high RS groups respectively). These findings are consistent with biological significance of Oncotype Dx genes. However, because the genes are closely related to the routinely assessed markers, it has been questioned whether Oncotype Dx provides information beyond that available from assessment of ER and HER2 [128, 129].

Adjuvant Endocrine Signatures and Clinical Indices

SET

In contrast to Oncotype Dx, the genomic index of sensitivity to endocrine therapy (SET) was intentionally developed to aid decision making for endocrine treatment [130]. In essence, it is an alternative way to determine the functional activity of ER. However, to reduce the prognostic component associated with ER expression the authors excluded genes strongly associated with proliferation or stromal bias. The index integrates expression of 165 genes positively (106 genes) or negatively (59 genes) correlated with ER expression in primary breast cancers. To ascertain the predictive value in adjuvant setting the signature validation included two independent datasets without endocrine treatment. SET index was not associated with distant relapse rates in the non-treated patients, but there was a strong trend for such an association if patients received tamoxifen (none of endocrine-treated patients with high or intermediate SET developed distant relapse within 8 years). Unfortunately, only a minority of patients in the validation dataset achieved the high or intermediate SET values. Also, the practical utility of the SET index is limited by the procedure for index calculation: it is platform-specific and requires adjustments even when switching between different Affymetrix micro-array types. This complicates exploring this signature in AI datasets. However, SET index demonstrates that (i) predictive and prognostic components can be separated when analysing genes associated with ER-signalling and (ii) currently available datasets allow for design of predictive endocrine adjuvant signatures.

Endopredict

In contrast to SET index, Endopredict (EP) test was developed for direct clinical application [109, 131]. It includes 8 prognostic genes (BIRC5, UBE2C, DHCR7, RBBP8, IL6ST, AZGP1, MGP, and STC2) measured by qPCR and normalized

by 3 reference genes (CALM2, OAZ1, and RPL37A). Additionally, there is a version of EP score (EPclin) that incorporates tumour size and node status. To date, Endopredict is the only multigene breast cancer test, except for Oncotype Dx, which approximates to level 1 of evidence for prognosis in ER+ve tumours [124]. Gene selection was based on a training set, which excluded such confounders as HER2+ve tumours and concomitant cytotoxic chemotherapy. Similarly to SET index, the training set included only tamoxifen-treated patients; however, the validation sets included some anastrazole-treated patients too, suggesting that tamoxifen-derived signatures may maintain their relevance in AI context. EP has not yet been evaluated in patients without endocrine treatment, which precludes evaluation of its predictive value. Thus the clinical utility of EP is mainly limited to determining whether the ER+ve patient would benefit from addition of chemotherapy to endocrine treatment (as with Oncotype Dx in this respect) [132].

PEPI and Other Multicomponent Clinical Indices

A number of multicomponent clinical indices were developed to estimate expected outcome of adjuvant treatment by integration of a small number of key clinical parameters, such as patient's age, tumour size, lymph-node involvement, pathology and hormonal receptor status. Examples of such indices include NPI, Adjuvant!online, PREDICT and PEPI [133–136]. These indices are based on statistical associations between their components and clinical outcomes derived from retrospective analysis of large patient's cohorts. Historically NPI (Nottingham Prognostic Index) was the first of these indices [135]. It demonstrated that a mathematical model based on several parameters may be more informative than the same parameters considered separately. NPI was based on outcomes from 500 patients with non-disseminated breast cancer operated in Nottingham City Hospital in 1970th. The initial model utilized 9 clinical factors including age, reproductive status, tumour size, lymph node involvement, grade, oestrogen receptors, presence of adjuvant therapy and 2 additional pathological features that were of interest at the time. Only 3 of these factors were found to be significant: tumour size, lymph node stage and tumour grade. These were included into the final model, which outperformed any of the factors alone. While strongly influencing the later development of prognostic and predictive indices, NPI is purely prognostic and has no direct relevance to prediction of adjuvant response to AIs which were not widely used at the time of the index development.

Adjuvant!online and PREDICT [133, 134] have taken the NPI approach further, explicitly including the type of adjuvant treatment into the model. These tools compute a personalized prognosis that shows what proportion of "similar" patients benefited from a specific adjuvant treatment in the past. In principle, these models are being updated according to accumulated evidence and include some data for patients treated with AIs [137]. However, in practice the current breast cancer version of Adjuvant!online (version 8.0) is dated by 2005, which is before publication of the long-term outcomes of the major adjuvant AI trials [12–14]. At the

same time, the Adjvuant!online web site says that an updated breast cancer version is undergoing checking and will be released in future (version 9.0). To a degree Adjuvant!online and PREDICT aim to provide personalized outlook on prospective outcome of a specific adjuvant treatment. However, at present their estimates are limited by knowledge predating the molecular mechanisms of resistance. The authors of Adjuvant!online are working on inclusion of molecular indices into their model. However, this is a work in progress and the only index being considered for inclusion so far is Oncotype Dx.

The preoperative endocrine prognostic index (PEPI) is also based on a regression model that includes tumor size, node status, ER status, Ki67 proliferation index and histological grade [136]. However, the key difference from the previously discussed indices is that PEPI considers these parameters after pre-operative endocrine treatment and adds the neo-adjuvant response to the equation. Importantly, PEPI index was based on datasets, which included large proportion of patients treated with aromatase inhibitors (PO24 and IMPACT trials) [15, 138].

To a degree, PEPI index extrapolates neo-adjuvant response to the adjuvant setting. However, neo-adjuvant response is entirely dictated by the biology of the primary tumour. In contrast, outcome of adjuvant treatment is a composite of surgical aspects (such as clear margins) and biology of the occult micro-metastatic disease. Therefore, there is no direct link between pre-operative and adjuvant responses [139]. On the other hand, some studies reported an association between these responses (for both endocrine and cytotoxic treatments) [140, 141]. Consequently, the results of preoperative treatment have been considered as a valid approach for personalized prediction of adjuvant response to endocrine treatment, including treatment with aromatase inhibitors [50].

Signatures to Predict Neo-adjuvant Response to AIs

Development of response predictors in the adjuvant setting is confounded by surgical factors and prognostic aspects of the markers. Studies of response in advanced disease are often complicated by multiple previous treatments. In contrast, the neo-adjuvant setting provides an ideal opportunity directly to evaluate response in primary tumours.

Most recently, genome-wide searches have been performed in tumour material taken before, during and after neoadjuvant therapy with AIs. For example, Miller et al. [142] studied RNA expression in sequential biopsies taken before and after two weeks of neoadjuvant treatment with letrozole. The effects of 2-weeks preoperative treatment with either anastrozole or letrozole have also been examined [35, 122, 143]. Molecular responses detected in the studies were consistent. Genes most consistently up-regulated were associated with “stromal” signatures, for example specific types of collagens (COL3A1, COL14A1, COL1A2), members of a small leucine-rich proteoglycan family (DCN, LUM and ASPN), genes linked with cell adhesion and intercellular matrix turnover (MMP2, CD36,

CDH11, ITGB2, SRPX, SPON1, DPT) and immune response-associated genes (COLEC12, IL1R1, C1R, TNFSF10). As expected, letrozole decreased expression of classical estrogen-dependent and proliferation-related genes, such as TFF1, TFF3, KIAA0101, SERPINA3, IRS1, PDZK1, AGR2, ZWINT, CDC2, CCND1, CCNB1, NUSAP1, CKS2 (Fig. 10.2). The most prominent functional change, as detected by GO analysis, was a strong association of down-regulated genes with cell cycle and mitosis (Fig. 10.3a). These molecular changes were observed after 2 weeks of treatment and therefore preceded any evidence of tumour shrinkage or morphological changes. However, the neoadjuvant study of Miller et al. [48] continued treatment for 3 months so that clinical response could be assessed and molecular changes related to it. A 205-genes signature predictive for clinical response was derived based on gene expression (i) pre-treatment, (ii) profiles measured after 2 weeks of treatment and (iii) early changes in gene expressions between the time points. Generally, early changes on treatment were more informative than separate static assessments made either before or after 2 weeks of treatment. Ontology of genes associated with response was highly significantly related to structural constituents of ribosomes (Fig. 10.3b). Responsive tumours showed higher expression of ribosomal proteins before treatment and decreased expression after 2 weeks of letrozole therapy; in contrast, baseline expression of ribosomal proteins was low in resistant tumours and was not further reduced by treatment.

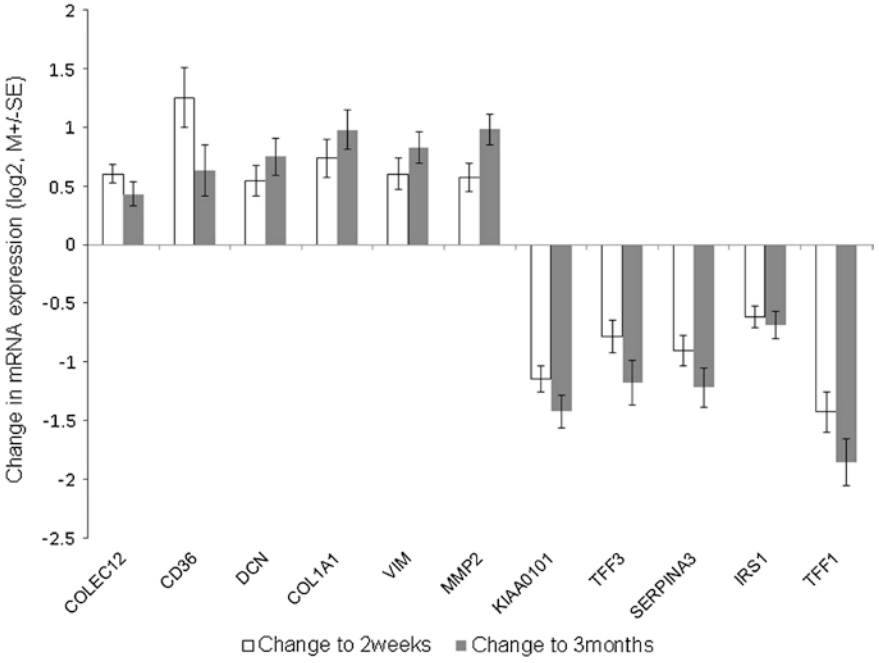

Fig. 10.2 Selected genes regulated by Letrozole. Gene expressions were measured by microarray in biopsies of breast cancer taken before treatment, after 2 weeks of neo-adjuvant letrozole and at surgery (after 3+ month of letrozole). Full data available in Miller et al. [25]

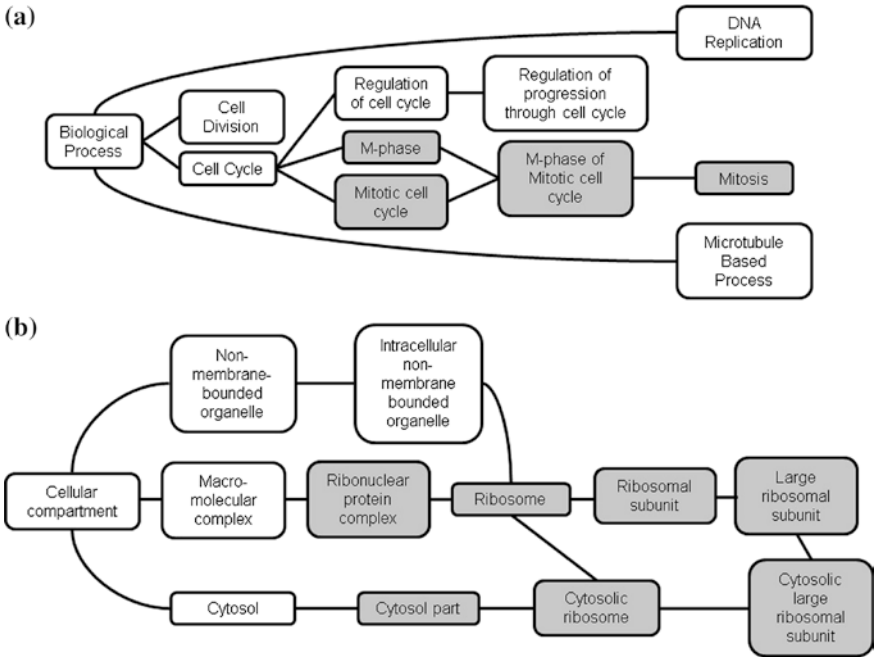

Fig. 10.3 Functional analysis of genes at 2 week of treatment with letrozole. Gene expressions were measured by microarray in biopsies of breast cancer taken before treatment, after 2 weeks of neo-adjuvant letrozole [25, 48]. **a** Shows GO terms enriched within genes most changed during treatment (biological process terms). **b** Shows biological processes enriched within the genes predictive for response (using cellular compartment terms). Most significant GO terms are highlighted in *grey*

Changes in proliferative and classic oestrogen-responsive genes had no strong association with clinical response—although more frequent in clinically responding tumours, fall of these genes could be detected in both clinical responders and non-responders [48, 144]. More recent interrogation of a similar dataset of letrozole-treated tumours has generated a 4-gene predictive index, which was validated on an independent neo-adjuvant anastrazole dataset [145]. Currently this 4-gene index is undergoing further validation.

In the study of MacKay et al. [35] the total number of genes significantly changing on treatment varied markedly between tumours and was related to changes in Ki67. The authors have integrated the gene changes into a global index of dependence on estrogen (GIDE), a measure of the number of genes with at least a 2-fold change on treatment. GIDE was found to be significantly associated with on-treatment changes in Ki67 and with pretreatment levels of HER2.

Recently Dunbier et al. [146] studied baseline and 2-week post treatment biopsies obtained from postmenopausal women receiving neoadjuvant anastrozole. Pathway analysis of gene expression data identified (i) the most prevalent changes in expression and (ii) the pretreatment genes/pathways most related to poor

Table 10.2 Utility of multi-gene signatures and clinical indices for AI response prediction

Test/signature	Phase of development	Relevance to predicting AI response
Intrinsic subtypes	Ready to use clinical tests (PAM50/ROR and IHC-based)	• Main utility is not directly relevant to AI response prediction [110]
		• Identifies distinctive biological subtypes within breast cancer [101]
		• Luminal A and B subtypes show similar response to AIs [121, 122]
		• Non-luminal phenotypes may be detected in ER+ve tumours, with possible implications to AI response [122]
Oncotype Dx	Ready to use clinical test	• Main utility is not directly relevant to AI response prediction [125, 126]
		• Designed to estimate prognosis for adjuvant tamoxifen treatment of ER+ve LN-ve tumours [108]
		• Associated with neo-adjuvant response rates to exemestane [127]
SET [130]	Exploratory signature	• Predicts adjuvant endocrine response
		• Prognostic component has been excluded (verified by a no-treatment dataset)
		• Designed and validated on tamoxifen-treated datasets, needs to be confirmed on an AI dataset
Endopredict [109, 131]	Ready to use clinical test	• Estimates prognosis for adjuvant endocrine treatment of ER+ve, HER−ve tumours
		• Validation dataset included AI-treated tumours
		• Predictive component has not yet been evaluated on an no-treatment dataset
PEPI [136]	Ready to use clinical index	• Estimates prognosis for adjuvant AI treatment basing on preoperative treatment outcomes
		• Predictive component has not been evaluated on a no-treatment dataset
205-genes Edinburgh signature [48]	Exploratory signature	• Predicts neo-adjuvant response to letrozole using baseline and early-on-treatment (2 weeks) molecular profiles
4-genes Edinburgh signature [145]	Clinical test at early stage of development	• Trained on letrozole dataset, validated on anastrozole dataset
		• Predicts neo-adjuvant response to AI using baseline and early-on-treatment (2 weeks) molecular profiles

anti-proliferative response. A total of 1327 genes were differentially expressed after 2-week treatment. Proliferation-associated genes and classical estrogen-dependent genes were strongly downregulated, whereas collagens and chemokines were upregulated. Pretreatment expression of an inflammatory signature correlated with antiproliferative response to anastrozole and this observation was validated in an independent series of cancers. Higher expression of immune-related genes such as SLAMF8 and TNF as well as lymphocytic infiltration were associated with poorer response and again validated in an independent cohort. In a similar study employing anastrozole pretreatment. Gao et al. [143] also found that multiple processes and pathways were affected by the AI treatment. Modules closely associated with ESR1 expression were predictive of good antiproliferative response to AIs, whereas modules representing immune activity and IGF-I/MAPK were predictive of poor Ki67 response, supporting their therapeutic targeting in combination with AIs. The added relevance of all these investigations is that early changes in proliferation in the neoadjuvant and pre-surgical settings may relate to likelihood of long-term benefit to adjuvant treatment as observed by comparing the IMPACT and ATAC trials [7].

Taken together the reviewed studies constitute a significant progress in development of multigene signatures and multi-component indices for endocrine response prediction. However, predicting response to aromatase inhibitors in ER+ve patients still remains a challenge (Table 10.2). Most of the adjuvant indices were developed using tamoxifen-treated cohorts; although tamoxifen-trained tests can be informative for AI patients too (e.g. in validation cohort for Endopredict test) [109]. Many signatures have not been developed beyond the exploratory phase (SET, 205- and 4-gene signatures) [35, 48, 130]. Practical utility of the tests that reached clinic (Endopredict, OncotypeDx and PAM50) is still limited to detection of the two extreme groups within ER+ve population. On one side, there is a relatively small group (up to ~20 %) of low risk patients with excellent prognosis, usually ER-rich, which do not need addition of cytotoxic therapy to adjuvant treatment [109, 125, 126, 132]. On the other side of the range, tests may identify a group of ER+ve tumours with low expression of ER (up to ~10 % of ER+ve tumours) [68, 69] and/or inconsistent with luminal phenotype [122]. Albeit these high-risk tumours are associated with poor response rates, some of these ER+ve tumours still may respond to AI treatment. Therefore, this high risk warrants addition of other treatments to AIs rather than exclusion of AIs. In the middle of the low- and high-risk phenotypes there is a significant number of ER+ve patients, whose response to AIs still cannot be predicted beyond the odds dictated by the ER-positivity.

Emerging Technologies and Markers

Arguably, until recently most studies addressing prediction of endocrine response were focused on multi-gene transcriptional signatures and/or routinely available clinical markers. However, the field is wider and is still expanding with the exploitation of new and alternative approaches, e.g. dynamic PET imaging [147],

proteomic signatures [148], genetic polymorphisms [149–154] and epigenetic changes (see Luca Magnani's chapter in this book). Reviewing of all the emerging directions would be beyond the scope of this review. Thus, below we focus on a few, including (i) pharmacogenetic markers relevant to AI treatment, (ii) monitoring of aromatase and oestrogen metabolites during AI treatment, (iii) pathway-focused analysis of tumours at time of relapse or progression on AI treatment and (iv) new findings and resources emerging from massive parallel sequencing technology in the post-genomic era.

Pharmacogenetic Markers

Aromatase gene (CYP19A1) has a peculiar structure and tissue-specific regulation is reviewed by Noburino Harada in another chapter of this book. Briefly: the CYP19A1 gene is large, spanning more than 120 kb (an average human gene is ~10–15 kb). The gene starts with 9 alternative versions of the 1st exon sparsely distributed over the initial ~90 kb of the gene. Each version of the 1st exon is controlled by its own promoter, which is used for tissue-specific regulation of the gene. This does not affect the coding part of the transcript because start of translation is located in exon 2; therefore the protein sequence is not affected by the tissue-specific alternative splicing [155]. In 2005 Ma et al. [156] sequenced the gene's exons and promoters in 240 individuals from 4 ethnic groups. The study identified 88 polymorphisms and reported 3 common non-synonymous SNPs (allele frequency 1–10 %) one of which significantly reduced activity and immune-reactivity of aromatase protein. Another rare genetic variant affected the aromatase interaction with inhibitors (exemestane and letrozole). Other authors reported polymorphisms in aromatase gene that can be associated with oestrogen production [151, 153, 157, 158], risk of breast cancer [159] and breast cancer survival [160]. A number of CYP19A1 polymorphisms were associated with AI response [151–154] and adverse effects during adjuvant AI treatment [161, 162].

Genetic variants may also affect the metabolism of aromatase inhibitors. Thus the Exemestane and Letrozole Pharmacogenomics (ELPH) trial studied letrozole concentration and letrozole metabolizing enzymes polymorphisms in 252 breast cancer patients received adjuvant letrozole [150]. Letrozole concentrations in blood varied more than 10 fold between patients and were strongly associated with CYP2A6 genotypes: the slow (n = 21) and intermediate (n = 40) CYP2A6 metabolizers had significantly higher concentrations of letrozole in blood than the normal (n = 200) metabolizers ($p < 0.0001$; median concentrations: 152, 112 and 81 ng/ml, numbers of patients: 21, 40 and 200 respectively). The authors concluded that hepatic CYP2A6 is a principal clearance mechanism for letrozole in humans and CYP2A6 genotyping may serve as a biomarker of the efficacy of letrozole or a predictor of adverse effects. Similarly, there are pre-clinical studies suggesting a possible role of UGT pharmacogenetics in metabolism of exemestane and anastrozole [163].

Monitoring of AIs Concentration and Estrogens in Blood

The observation of highly variable drug concentrations in blood of patients receiving letrozole [150] is consistent with a number of other AI studies [6, 164]. Surprisingly, such data have not been reported in the large adjuvant clinical AIs trials. Most likely this could be explained by historical and methodical reasons. Thus, small pilot studies with carefully selected patients did not show indications of between-patients variability in AI efficiency [165]. At the same time, challenges in measurements of ultra-low oestrogen concentrations in AI-treated post-menopausal patients (reviewed by Per Lonning in another chapter of this book) might complicate the inclusion of such measurements in the large trails. However, recent development of mass-spectrometry tools means that AIs and oestrogen metabolites can now be measured accurately and simultaneously in large cohorts of AI treated patients [6, 166]. The trial conducted by Ingle et al. [6] reported concentrations of oestrone, oestradiol, androstenedione, testosterone, anastrozole and its metabolites in 649 patients receiving adjuvant anastrozole [6]. While most of the patient demonstrated a strong decrease in oestrogen concentration after 4 weeks of treatment, detectable levels of oestrogens were still found in up to 30 % of patients. Moreover, in 8.9 % of patients the concentration of oestradiol did not fall during treatment and in some cases the oestradiol levels were of typical pre-menopausal range (up to 234 pg/mL of oestradiol on treatment, despite pre-menopausal patients not meeting trial inclusion criteria). There was ~2-fold interquartile range in anastrozole concentration in blood (Q1 23.5–Q3 44.8 ng/mL), with the drug not being detected in some samples. This suggests that periodic monitoring of AIs and steroids in blood might be considered in future during the long-term adjuvant treatment.

Multi-pathway Panels to Detect Mechanisms of Resistance

As reviewed earlier, most of the current multi-gene markers were derived either (i) from lists of genes differentially expressed in tumours with different outcomes on treatment or (ii) from lists of genes associated with expression and function of ER. Even when a signature is presented in a pathway-relevant way, the gene selection preceded the functional interpretation and the latter is done in very generic terms (e.g. Oncotype Dx) [108]. While such gene-lists may be informative for predicting resistance, they are not necessarily helpful in terms of predicting the resistance mechanisms. Because of the diversity of such mechanisms, mechanism-specific features may not be on the top of the differentially expressed genes, being overtaken by more generic features, such as proliferation, stromal response and protein biosynthesis [35, 48]. Therefore, the question of the mechanisms of resistance is better addressed by the pathway-focused analysis of clinical samples taken from resistant tumours. A number of transcriptomic signatures have been developed to

characterize activity of pathways potentially associated with alternative endocrine resistance mechanisms, such as DNA-repair pathways [167], PIK3A [168], MYC, E2F3, RAS, Beta-Catenin, SRC [169] and other signaling pathways. Simultaneous assessment and integrative interpretation of multiple pathways may potentially inform treatment strategies in individual patients, providing a basis for the next generation of breast cancer gene signatures [170]. Figure 10.4 illustrates such simultaneous application of 9 transcriptional signatures to 55 samples of breast tumours progressing or relapsing during AI treatment [171]. It suggests that oestrogen signalling remains active in 44 % (24/55) of the resistant tumours, despite

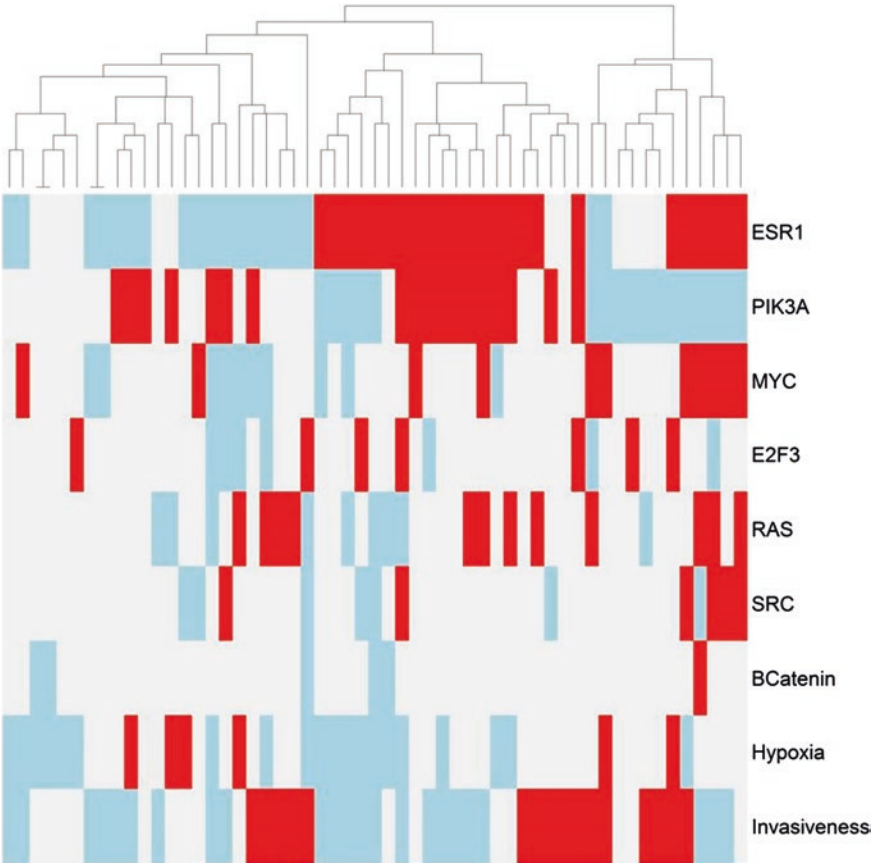

Fig. 10.4 Multi-pathway analysis of AI resistant tumours. Activity of signatures potentially associated with endocrine resistance was evaluated in AI resistant tumours from neo-adjuvant AI studies conducted in Edinburgh Breast Research Unit [25, 145, 181, 182]. *Red* shows high activity of signalling, *blue* shows low activity of signalling, *grey* shows inconclusive result for the given pathway. The activity of pathways was evaluated using Iterative Consensus PAM algorithm, cases clustered using Euclidean distances and complete linkage (see full details in [171])

the samples being collected during AI treatment. Pattern of activation of the other signatures may suggest particular pathways that could be targeted together with a switch of endocrine agents in individual patients.

New Molecular Methods and Bioinformatics Resources

Recent progress in massive parallel sequencing (NGS) technology provided a new powerful tool for molecular studies in endocrine resistance of breast cancer. In many cases, NGS studies are still focused on exploration rather than on biomarker development. Interpretation of NGS data is still limited by the enormous scale and complexity of data. However, there are already some examples, which illustrate the power of new sequencing techniques in predicting and explaining AI resistance in breast cancer.

Two independent studies have recently reported acquisition of activating mutations in the ligand-binding domain of ER in relapsing endocrine-resistant tumours [172, 173]. Robinson et al. [173] performed whole exome and transcriptome sequencing in 11 patients relapsed after multiple lines of endocrine treatment. Somatic mutations in ER were detected in 6 of 11 patients (55 %, all included AIs as one of the previous treatments). All mutations affected the same protein part: 536-538 Leu-Tyr-Asp section of ER. Three patients presented with Tyr537Ser, two patients with Asp538Gly and one with Leu536Gln. Functional studies in vitro showed that Tyr537Ser and Asp538Gly can lead to constitutive activation of ER causing resistance to oestrogen deprivation. Prevalence of ER mutations in primary breast cancers is ~1 % [174]. Therefore, it is likely that activating ER mutations may be acquired and harboured during tumour progression under selective pressure of endocrine treatment. The second paper reporting activating ER mutations in metastatic breast cancer [172] used massive parallel sequencing to study a panel of 230 genes in 80 metastatic ER-positive tumours. Activating mutations in ER were found in 14 of 80 cases (18 %). The mutations were recurrent and affected the same area of the protein (Tyr537Ser, Tyr537Asn, Asp538Gly). These studies suggest a mechanistic explanation to the finding of active ER signalling in a large proportion of resistant tumour despite the applied AI treatment (Fig. 10.4). Interestingly, one of these mutations (Tyr537Asn) had been reported in metastatic breast cancers 15 years ago [175].

A recent study correlated neo-adjuvant response to AIs to patterns of somatic mutations detected by next generation sequencing in 77 tumours [176]. The response was assessed by Ki67 index after treatment. Gene significance analysis based on background mutation rates identified 18 candidate genes (with FDR 26 %). Mutations in 8 of the genes had already been reported in breast cancer (PIK3CA, TP53, GATA3, CDH1, RB1, MLL3, MAP3K1 and CDKN1B); the others were not reported in breast cancer context earlier (TBX3, RUNX1, LDLRAP1, STNM2, MYH9, AGTR2, STMN2, SF3B1, CBFB and ENSG00000212670). A recurrence

screening for selected genes in 240 additional patients suggested that GATA3 mutations may be a positive predictive marker for AI response. TP53 mutations were associated with luminal B phenotype and higher proliferation both pre- and post-treatment. Mutations in MAP3K1 were more frequent in luminal A, low-grade and low-proliferation tumours [176]. Overall, the study highlighted the complexity of somatic mutation patterns and importance of algorithms for selecting the functionally important events out of the hundreds of somatic mutations detected in most tumours.

Finally, new important bioinformatics resources have been generated on the basis of the new molecular technologies. These resources contain vast information about thousands of clinical cancer specimens and already influencing studies in endocrine resistance. Cancer Genome Atlas project (TCGA) is one of the leading resources of the new type [118, 177]. It had been established by NIH to provide comprehensive multi-layer molecular data on clinical cancer specimens matched with normal tissues from the same patient. The data generated in dedicated large-scale sequencing centres include genome-wide somatic mutations, copy-number variation, mRNA and miRNA expression, methylation, proteomics data and clinical annotations of the specimens. Started in 2006 with lung, brain and ovarian cancers, in 2014 it provides public access to data about more than 11 thousand samples for 34 types of cancers, including more than a thousand breast cancer samples. Exploration of TCGA data confirmed the previously established genetic alterations associated with breast cancer. Thus, TP53 is the most commonly mutated gene in ER−ve cancers of TCGA dataset, while PIK3CA is the the most common in ER+ve tumours [119, 177, 178], suggesting a potential role of PIK3CA as a marker for endocrine resistance. This is in agreement with the finding of high activity of PIK3A in a significant proportion of endocrine-resistant tumours (Fig. 10.4). Additionally, a neo-adjuvant letrozole study reported poorer proliferative response to letrozole in patients with mutated exon 9 of PIK3CA [179]. However, another neo-adjuvant AI study reported positive association of PIK3CA mutations with favourable biomarkers and concluded that somatic mutations in PIK3CA do not preclude response to neo-adjuvant anastrozole [180]. Therefore the utility of PIK3CA mutations for predicting AI response is yet controversial.

Overall, the massive parallel sequencing studies have produced new interesting results and highlighted (i) the importance of bioinformatics algorithms to prioritise functionally significant genes out of the hundreds of somatic mutations detected in each tumour, (ii) the need for relevant clinical annotations in the molecular databases and (iii) importance of tumour evolution during growth under pressure of AI inhibitors.

Conclusion

Treatment with AIs is a major therapeutic regime in breast cancer. Currently, ER is the only factor used routinely to select for suitability for treatment, response rarely occurs in ER−ve tumours but a significant proportion of ER-positive tumours

fails therapy. Additional markers are, therefore, required which predict precisely response to aromatase inhibitors in ER-positive tumours. This review summarizes the progress and challenges in identifying predictive molecular signatures for aromatase inhibitors and suggests that, with rational study design and implementation of new technology, fundamental knowledge can be acquired about the nature of breast cancer response to aromatase inhibitors.

Conflicts of Interest No potential conflicts of interest were disclosed.

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Chapter 11
Clinical Trials Combining Aromatase Inhibitors with Other Targeted Treatments

Hazel Lote and Stephen Johnston

Abstract Aromatase inhibitors (AIs) play an important role in the treatment of both early and advanced hormone-receptor positive breast cancer. However, not all patients respond to first-line endocrine treatment due to primary de novo resistance, while others may initially respond but eventually progress with secondary *acquired* resistance. Strategies combining endocrine therapy with targeted inhibitors of growth factors or cell survival pathways to overcome AI resistance and enhance therapy are currently being investigated. This chapter will outline the clinical trials currently underway and will focus on whether AIs in combination with targeted therapies can translate into clinical benefit. Appropriate trial design and patient selection are important considerations for this approach to be successful. Enriching trial recruitment by molecular profiling of different ER+ subtypes will become increasingly important to maximize additional benefit that these new agents may bring to current AIs for breast cancer.

Abbreviations

AIs	Aromatase inhibitors
nsAI	Nonsteroidal AI
ER	Oestrogen receptors
ER+	Oestrogen receptor positive
E2	Estradiol
MBC	Metastatic breast cancer
TTP	Time to disease progression
CBR	Clinical benefit rate

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A. Larionov (ed.), *Resistance to Aromatase Inhibitors in Breast Cancer*,
Resistance to Targeted Anti-Cancer Therapeutics 8,
DOI 10.1007/978-3-319-17972-8_11

PFS	Progression-free survival
EGFR	Epidermal growth factor receptor
mTOR	Mammalian target of rapamycin
PI3K	Phosphoinositide-3-kinase (pathway)
CDK	Cyclin-dependent kinase
FGFR1	Fibroblast growth factor receptor-1
HDACI	Histone deacetylase inhibitors
VEGF	Vascular endothelial growth factor
PDGFR	Platelet-derived growth factor receptor
IGF-1	Insulin-like growth factor type 1
IGF-1R	Insulin-like growth factor receptor 1
IR-A	Insulin receptor-A
IR-B	Insulin receptor-B
NF-κB	Nuclear factor kappa B

Introduction

One of the main challenges facing oncologists today is the fact that most cancer therapies are unable to achieve durable responses. The oncological advances made over the past few decades have been enormous, and our understanding of aberrant cell signaling pathways and underlying genetic alterations has vastly expanded. However, a theme across cancer subtypes is that cancer cells can adapt and alter, ultimately developing resistance mechanisms.

In postmenopausal women, aromatase inhibitors (AIs) play an important role in the treatment of both early and advanced hormone-receptor (oestrogen and progesterone-receptor) positive breast cancer. As discussed elsewhere in this book, AIs are potent inhibitors of oestrogen biosynthesis by preventing conversion of androgens into oestrogen in the postmenopausal state. Given that approximately two-thirds of human breast cancer express oestrogen receptors (ER) and are dependent on oestrogen for their growth, AIs are used clinically as adjuvant therapy (to prevent the risk of disease relapse) and for treatment of advanced or metastatic disease. However, not all patients respond to first-line endocrine treatment due to *primary* de novo resistance, while others may initially respond but eventually progress with *secondary* acquired resistance [1]. Overcoming primary and secondary endocrine resistance remains critical to further enhancing the benefit of existing endocrine therapies [2].

The challenge of overcoming resistance to AIs has yielded success with the combination of AIs administered together with mTOR antagonists. Improvement of neoadjuvant responses may indicate that primary resistance can also be tackled by the combination of AIs and mTOR inhibitors [3]. However, the emergence of endocrine resistance during prolonged therapy is complex, and it is unlikely that any single mechanism is operative (Fig. 11.1). While the EGFR/HER2 and mTOR pathways have been studied extensively, numerous other signaling pathways may

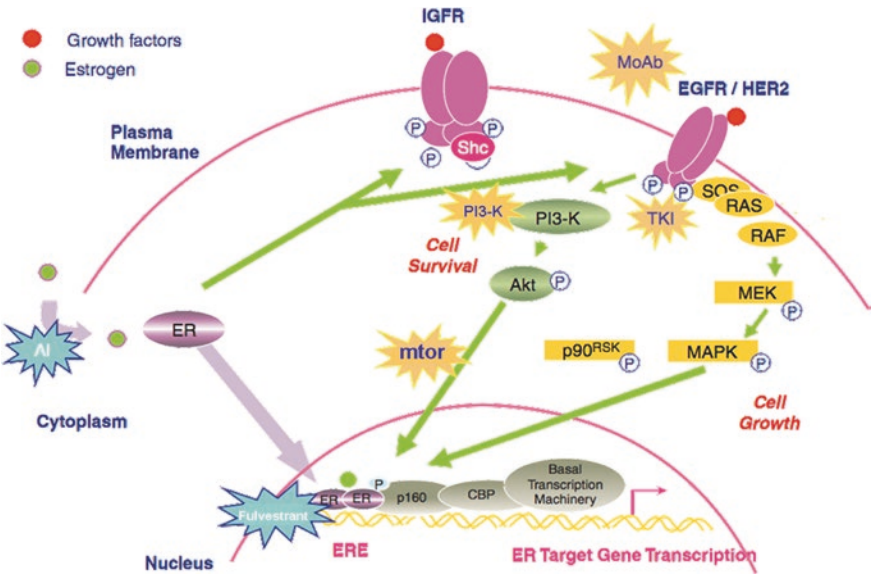

Fig. 11.1 Cross-talk between various growth factor receptor signaling pathways and ER at the time of relapse on long-term oestrogen deprivation (LTED), with ER becoming activated and super-sensitised by a number of different intracellular kinases, including mitogen-activated protein-kinases (MAPKs), epidermal growth factor receptor (EGFR) and HER2/HER3 signaling, and the insulin-like growth factor (IGF)/AKT pathway. In cells that become resistant to LTED (LTED-R), ER-mediated gene transcription is enhanced 10-fold, but can be abrogated by a number of different approaches to interrupt upstream signaling including phosphoinositide 3-kinase (PI3-K) inhibitors, the EGFR tyrosine kinase inhibitor (TKI) gefitinib, MEK inhibitors, and the ER down-regulator fulvestrant that degrades ER protein

also be implicated. Both pre-clinical and early phase clinical research are now trying to identify various other strategies to overcome endocrine resistance, based on the availability of targeted therapeutics that can be combined with endocrine therapy. Initial clinical trials suggested such combinations were less helpful in overcoming primary resistance; however, early results from recent clinical trials suggest that certain targeted therapies such as CDK 4/6 inhibitors may significantly prolong progression-free survival, and we eagerly await the formal results from these studies. As described in this chapter, there are numerous further clinical trials currently underway evaluating AIs in combination with targeted therapies including FGFR inhibitors, PI3K inhibitors, AKT inhibitors, HDAC, IGFR-1 and Src.

Earlier chapters have explored the mechanisms of resistance to AIs and the preclinical strategies to overcome this resistance. However, not all strategies that initially looked promising in experimental models have proved successful in the clinic. We will focus on whether AIs together with targeted therapies translate into clinical benefit, and whether there are associated biomarkers with which to predict response. We will examine clinical trial design issues that should be considered in order for this approach to be successful, and suggest areas to explore in future.

Enriching trial recruitment by molecular profiling of different ER+ subtypes will become increasingly important to maximize additional benefit that these new agents may bring to current endocrine therapies for breast cancer [2].

AIs—Background

From the mid 1990s, the potent third-generation oral aromatase inhibitors (AIs) become the standard first-line treatment option for postmenopausal patients with oestrogen receptor positive (ER+) advanced/metastatic breast cancer. Estrogens are normally synthesized in the ovary in premenopausal women; following menopause, mean plasma estradiol (E2) levels fall from about 400–600 pmol/L to around 25–50 pmol/L. These residual estrogens come solely from peripheral aromatase conversion particularly in subcutaneous fat, and plasma E2 levels correlate with body mass index in postmenopausal women [4]. The oral aromatase inhibitors anastrozole (ArimidexTM), letrozole (FemaraTM), and exemestane (AromasinTM) all reduce serum estrogen levels in post- menopausal women by preventing the conversion of adrenal androgens into oestrogens. Anastrozole and letrozole are third-generation nonsteroidal aromatase inhibitors that have similar pharmacokinetics with half-lives of approximately 48 h, allowing a once-daily schedule [5, 6]. Exemestane is a steroidal aromatase inactivator with a half-life of 27 h [7]. All three compounds are orally active, and were licensed and approved as first-line endocrine treatment for postmenopausal women with ER+ve advanced breast cancer.

For recent endocrine therapy trials, including those who are endocrine-therapy naïve, expected time to disease progression (TTP) for AIs as first-line therapy for metastatic disease are of the order between 10 and 15 months (Table 11.1). However, the influence of prior adjuvant endocrine therapy remains an important variable in the likelihood of success (Fig. 11.2). While resistance to aromatase inhibitors develops in many cases, it does not preclude further endocrine responses, and effective second-line endocrine options are indicated for these patients. On the basis of trials confirming benefit for AIs compared with tamoxifen in early breast cancer, AIs are the treatment of choice for adjuvant therapy in hormone-receptor positive breast cancer; they can also be used in the neo-adjuvant setting to shrink tumours prior to surgery.

AIs + Anti-HER2 Therapy

Co-targeting the type I epidermal growth factor receptor-2 (HER2) in ER+ breast cancer has been explored as a means of improving endocrine responsiveness, given the evidence that HER2 expression in breast cancer models is associated with primary resistance to endocrine therapy. Co-targeting HER2 may overcome endocrine therapy resistance through re-expression of silenced ER, as outlined in pre-clinical data [8]. Indeed, clinical evidence exists that trastuzumab can restore

Table 11.1 Main randomised clinical trials of different endocrine therapies as first-line treatment in metastatic breast cancer (MBC)

Study/arms	n	ORR (%)	CBR (%)	Median TTP or PFS (mo)	Median OS (mo)
AI versus tamoxifen					
Anastrozole versus tamoxifen [67]	171	21	59	11.1 (0.005)	33
	182	17	46	5.6	32
Anastrozole versus tamoxifen [68]	340	33	56	8.2	38
	328	33	55	8.3	42
Letrozole versus tamoxifen [69, 70]	453	32 (0.0002)	50 (0.0004)	9.4 (<0.0001)	34
	454	21	38	6.0	30
Exemestane versus tamoxifen [71]	182	46 (0.05)	–	9.9 (0.05)	37
	189	31	–	5.8	43
Fulvestrant versus tamoxifen or AI					
Fulvestrant 250 mg monthly versus tamoxifen [72]	313	31.6	54.3	6.8	36.9
	274	33.9	62	8.3	38.7
Fulvestrant 250 mg monthly versus anastrozole [73]	222	20.7	44.6	5.5	–
	229	15.7	45.0	5.1	–
Fulvestrant 250 mg monthly versus anastrazole [74]	206	17.5	42.2	5.4	–
	194	17.5	36.1	3.4	–
Fulvestrant LD + anastrozole versus anastrozole [75]	258	31.8	55.0	10.8	37.8
	256	33.6	55.1	10.2	38.2
Fulvestrant LD + anastrozole versus anastrozole [76]	355	–	–	15 (<0.007)	47.7 (0.049)
	352	–	–	13.5	41.3
Fulvestrant HD versus anatrozole [77]	102	36.0	72.5	23.4 (0.01)	–
	103	35.5	67.0	13.1	–

AI aromatase inhibitors, *CBR* clinical benefit rate, *HD* high dose (500 mg IM at day 0 + 500 mg IM at days 14 and 28, thereafter 500 mg IM monthly until progression), *LD* loading dose regimen (500 mg IM on day 0, 250 mg on days 14, 28 and 250 mg every 28 days thereafter), *mo* months; *n* number, *ORR* objective response rate, *OS* overall survival, *PFS* progression-free survival, *TTP* time to progression

both ER expression and endocrine responsiveness in a small series of patients with ER−ve HER2+ve advanced breast cancer who had serial biopsies during trastuzumab therapy [9].

Overcoming Primary Resistance Due to HER2 Over-Expression

Three randomised trials [10–12], have confirmed that co-targeting HER2 can treat primary endocrine resistance in known ER+ HER2+ advanced disease. A phase II

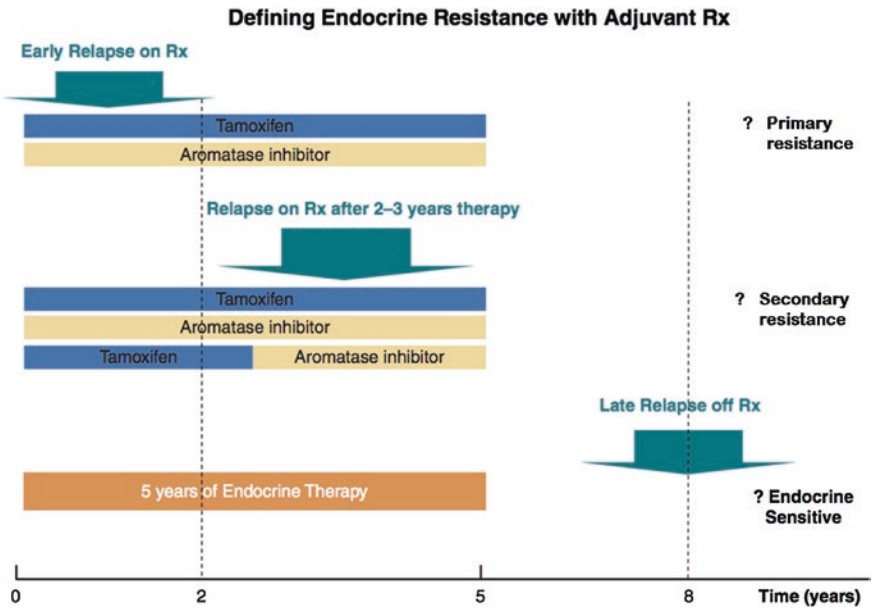

Fig. 11.2 Conventional definitions of endocrine sensitivity/resistance to adjuvant endocrine therapy. Both the time-point for relapse from diagnosis (disease-free interval) and also from prior adjuvant therapy (disease-free interval) might determine the response to endocrine therapy within the metastatic setting

clinical trial of letrozole and the monoclonal antibody trastuzumab in patients with ER+/HER2+ metastatic breast cancer revealed that the combination was well tolerated and had a clinical benefit rate (CBR) of 50 % [13]. Subsequently, a randomized phase II trial in 207 patients with known ER+/HER2+ MBC (TAnDEM) reported a doubling of PFS with the addition of trastuzumab over anastrozole alone (4.8 months vs. 2.4 months; $p = 0.0016$), although there was no significant impact on OS (Table 11.2) [10]. A small phase II study (eLEcTRA) showed a similar potential benefit for the addition of trastuzumab to letrozole as first-line treatment in ER+ HER2+ MBC [11].

Subsequently, lapatinib, a potent oral tyrosine kinase inhibitor of both EGFR and HER2, has been explored in combination with endocrine therapy based on in vitro data which demonstrated that addition of lapatinib significantly enhances the anti-proliferative effects of estrogen deprivation in HER2 negative breast cancer cell lines [14]. Likewise, preclinical evidence suggested that lapatinib could significantly enhance sensitivity to tamoxifen in cell lines with acquired tamoxifen resistance [15]. Results from a phase III trial of 1286 patients with metastatic ER+ breast cancer who were randomized to receive either letrozole alone or letrozole combined with lapatinib have been reported [12]. In patients with known ER+/HER2+ tumours (n = 219), the addition of lapatinib to letrozole significantly reduced the risk of progression (HR 0.71, 95 % CI, 0.53–0.96; $p = 0.019$),

Table 11.2 Main studies testing EFGF/HER2 inhibitors plus AIs to overcome endocrine resistance in MBC

Study	Phase	Arms	n	ORR%	Median TTP or PFS (mo)	Median OS (mo)
Cristofanilli et al. [17]	II RCT	Anastrozole + Placebo	50	12	8.4	nr
		Anastrozole + Gefitnib	43	2	14.7[a]	nr
TAnDEM [10]	III RCT	Anastrozole	104	6.8	2.4	23.9
		Anastrozole + Trastuzumab	103	20.3[a]	4.8[a]	28.5
EGF30008 [12]	III RCT	Letrozole + Placebo	108	15	3	32.3
		Letrozole + Lapatinib	111	28[a]	8.2[a]	33.3
eLEcTRA [11]	II RCT	Letrozole	31	13	3.3	nr
		Letrozole + Trastuzumab	26	27	14.1	nr

mo months, *nr* not reported, *ORR* objective response rate, *OS* overall survival, *PFS* progression-free survival, *RCT* randomized controlled trials, *TTP* time-to-progression

[a] Statistically significant difference

improving the median PFS from 3.0 months for letrozole to 8.2 months for the combination (Table 11.2). The clinical benefit was also significantly greater for the combination (48 % vs. 29 %; $p = 0.003$), and the combination became an approved treatment option in the United States and Europe from 2010 for ER+ HER2+ MBC in situations when chemotherapy was not indicated.

Delaying Secondary Resistance Due to HER2 Over-Expression

The large EGF30008 trial included an additional 952 patients with ER+ HER2-negative tumours. The hypothesis was that development of secondary resistance to letrozole due to adaptive EGFR or HER2 up-regulation could be prevented/delayed by dual targeting. However, there was no improvement in PFS for the combination in these patients, which implies that in ER+ve HER2-ve breast cancer specific co-targeting of HER2 together with ER from the outset does not delay resistance. Indeed, this result is consistent with experimental models which showed the failure of trastuzumab and letrozole combined together from the outset to delay endocrine resistance in hormone receptor-positive xenografts, in contrast to combined therapy that was very effective once resistance to letrozole had developed [16].

This important lesson needs to be borne in mind when designing other combined therapy trials: targeting a known resistance mechanism may simply allow the other resistance mechanisms to evolve over a similar time-frame.

AIs + EGFR Targeted Therapy

Expression of epidermal growth factor receptor (EGFR) in ER+ breast cancer has been shown to be enhanced in association with endocrine resistance in experimental models and various drugs have been developed to block this receptor (Table 11.2), including the tyrosine kinase inhibitor gefitinib. A randomised trial of gefitinib and anastrozole versus anastrazole alone was conducted in a first-line patient population of women with ER+ve advanced breast cancer, who had not received prior endocrine therapy for advanced disease, or who developed metastatic disease during or after adjuvant tamoxifen [17]. This trial reported a significant prolongation of progression free survival from a median of 8.2 months with anastrozole to 14.6 months with the combination of anatrozole + gefitinib (HR 0.55, 95 % CI 0.32–0.94) [17]. However, the number of patients in this study was only 93, and a subsequent combined analysis suggested that the benefit for the combination was seen exclusively in those patients that were endocrine therapy naïve, including no prior endocrine therapy in the adjuvant setting [2, 17].

On the basis of these results, a prospective multi-centre study (MINT, NCT 01151215) was conducted with a novel tyrosine kinase inhibitor AZD8931, a potent inhibitor of EGFR, HER2 and HER3, to test the hypothesis that combined therapy of growth factor blockade together with anastrozole could delay time to progression compared with anastrozole alone in endocrine therapy naïve metastatic breast cancer. Despite recruiting well (n = 359), this study failed to demonstrate any benefit for the addition of either 20 or 40 mg AZD8931 to anastrozole, with the mean PFS actually better for anastrozole alone (14.0 months vs. 10.9 months respectively) [18]. As such there is no evidence that co-targeting EGFR and/or HER2 can treat or prevent resistance to AI therapy.

AIs + mTOR Pathway Inhibitors

Preclinical data has suggested that the cell survival pathway regulated by the mammalian target of rapamycin (mTOR) protein is involved in causing resistance to oestrogen deprivation in ER+ breast cancer cells in vitro [19–21]. Inhibitors of mTOR have been shown to be synergistic with endocrine therapy in causing maximal inhibition of cell growth, suggesting that the combination may enhance endocrine responsiveness in the clinic.

Overcoming Secondary Resistance by mTOR Inhibition

There have been two important studies in the metastatic setting that have evaluated the addition of everolimus to endocrine therapy for postmenopausal women with ER+ve MBC who have already received prior endocrine therapy. Tamoxifen plus everolimus was compared with tamoxifen alone in patients with AI-resistant MBC in the small randomized phase II study TAMRAD (Tamoxifen plus Everolimus) [22, 23]. The combination therapy showed an improvement in TTP (8.6 months vs. 4.5 months), 6-month CBR (61 % vs. 42 %), and median overall survival compared with tamoxifen alone. Importantly, the trial design included stratification according to type of resistance to previous treatment with AIs, with primary resistance being defined as disease progression developing either during/within 6 months of completion of adjuvant AI therapy or within 6 months of starting AI therapy for MBC, while acquired secondary resistance was defined as those relapsing >6 months after stopping adjuvant AIs or responding for ≥6 months to AIs in the metastatic setting. An exploratory subgroup analysis showed that the greatest clinical benefit from the combination arm occurred in patients with acquired secondary resistance.

The clinical data from TAMRAD supported a hypothesis that tumours that initially respond and then develop resistance to AIs may utilize the PI3K/Akt/mTOR pathway, and that this combined approach should be most effective in those

patients with ER+ve advanced disease that progresses during or recurs after non-steroidal AI (nsAI) therapy [22]. This was confirmed in the Breast Cancer Trials of Everolimus-2 (BOLERO-2) study, a large randomised phase III trial that assigned 724 postmenopausal patients with ER-positive metastatic breast cancer in a 2:1 ratio to either exemestane alone or the combination of exemestane and everolimus [24]. All patients had progressed on a non-steroidal AI, and importantly 84 % of them had demonstrated prior hormone-sensitive disease defined as "at least 24 months of endocrine therapy before recurrence in the adjuvant setting, or a response or stabilisation for at least 24 weeks of endocrine therapy for advanced disease" [24]. In BOLERO-2 there was a statistically significant and clinically relevant improvement in PFS for the combination (median 7.8 vs. 3.2 months, HR = 0.45, $p < 0.0001$) [25]. The clinical benefit was primarily due to better control of the disease, although there was a significant improvement in tumour response rates from only 0.4 % in the exemestane-alone group to 9.5 % in the everolimus/exemestane group ($p = 0.001$) [24].

A key question remains as to whether the combination of an mTOR inhibitor with endocrine therapy will only be effective for secondary (acquired) endocrine-resistant breast cancer, or whether this is a new option for endocrine-sensitive MBC in the first-line setting that could delay or prevent endocrine resistance developing. A large first-line phase III study (HORIZON) recently reported the efficacy for the oral mTOR antagonist temsirolimus (30 mg orally for 5 days every 2 weeks) in combination with letrozole versus letrozole/placebo in 1112 patients with AI-naïve ER+ advanced breast cancer [26]. In contrast to BOLERO-2, the population in this larger study was mainly totally endocrine therapy naïve (approximately 60 %), and had received no prior AI therapy for locally advanced/metastatic disease. In HORIZON there was no improvement in PFS overall (median 9 months, HR = 0.90, $p = 0.25$), or in the 40 % patient subset that had received prior adjuvant endocrine therapy. These data suggest that as first-line therapy the combination may not be any better than an AI alone. However, it is possible that some tumours with primary resistance could still benefit from the addition of mTOR inhibitors to an AI, as shown by improved anti-proliferative responses in the neoadjuvant setting [3], although the challenge in primary breast cancer remains how best to identify these tumours using biomarker profiles.

Biological Feedback in the PI3K/Akt/mTOR Pathway

Despite the encouraging results reported for the combination of everolimus plus exemestane in the BOLERO-2 trial, at a molecular level two key regulatory loops may limit the effectiveness of current mTOR inhibitors. A negative feedback loop exists downstream in the PI3K/Akt/mTOR pathway whereby the mTOR activated kinase S6K1 phosphorylates and destabilises the IRS1 and IRS2 proteins in insulin like growth factor (IGF) responsive cells [27]. In these cells, mTOR inhibition leads to a reduction in S6K1 activity, which in turn allows IRS1/2 expression to

be increased with associated enhanced activation of IGFR-1 dependent Akt activity [28, 29]. Clinically phosphorylated Akt is upregulated in both tumour and skin biopsies of patients treated with everolimus [30], and as such this loss of negative feedback may counteract the anti-tumour effectiveness of mTOR blockade. In addition, a positive regulatory loop exists involving the mTORC2 complex which can be activated more directly by growth factors and activated phosphorylated Akt. This inability of some rapamycin derivatives to block mTORC2 could result in increased Akt signaling that result in ER phosphorylation on Serine 167 [31], negating the effect of combined aromatase inhibition [32, 33].

While these two mechanisms may limit the benefit of the current generation of mTOR inhibitors in combination with endocrine therapy, several other drugs that target the PI3K/AKT pathway upstream of mTOR are currently being tested in phase I/II trials in patients with advanced ER+ breast cancer in the hope that may prove more specific and effective than current mTOR inhibitors. These include pan- or isoform-specific PI3K inhibitors, dual PI3K/mTOR inhibitors, and AKT inhibitors (see Table 11.3).

Table 11.3 Current clinical trials investigating PI3K/Akt/mTOR pathway blockade in combination with AIs or fulvestrant

Study name	Stage and study number	Arms	Estimated enrollment (n pts)
MBC			
PI3K inhibition			
A phase III randomized, double blind placebo controlled study of BKM120 with fulvestrant, in postmenopausal women with hormone receptor-positive HER2-negative locally advanced or metastatic breast cancer which progressed on or after aromatase inhibitor treatment	Phase III (NCT01610284)	BKM120 (buparlisib, pan-PI3K inhibitor) plus fulvestrant versus placebo plus fulvestrant	1060
A phase III randomized, double blind, placebo controlled study of BKM120 with fulvestrant, in postmenopausal women with hormone receptor-positive HER2-negative AI treated, locally advanced or metastatic breast cancer who progressed on or after mTOR inhibitor based treatment	Phase III (NCT01633060)	BKM120 (buparlisib, pan-PI3K inhibitor) plus fulvestrant versus placebo plus fulvestrant	420

(continued)

Table 11.3 (continued)

Study name	Stage and study number	Arms	Estimated enrollment (n pts)
PI3K inhibition/mTOR dual blockade			
Phase 1/2 dose-escalation study of XL147 (SAR245408) or XL765 (SAR245409) in combination with letrozole in subjects with hormone receptor-positive and HER2-negative breast cancer refractory to a nonsteroidal aromatase inhibitor	Phase I/II (NCT01082068)	XL147 (inhibitor of PI3K) or XL765 (dual inhibitor of PI3K and mTOR) plus letrozole	99
A phase II, double-blind, placebo controlled, randomized study of GDC-0941 or GDC-0980 with fulvestrant versus fulvestrant in advanced or metastatic breast cancer in patients resistant to aromatase inhibitor therapy (FERGI) [36]	Phase II (NCT01437566)	GDC-0941 (pan PI3K inhibitor pictilisib) + fulvestrant or GDC-0980 (dual inhibitor of PI3K and mTOR) + fulvestrant or placebo + fulvestrant	270
Akt inhibition			
A phase 1 trial of MK-2206 in combination with anastrozole, fulvestrant, or anastrozole plus fulvestrant in postmenopausal women with estrogen receptor positive metastatic breast cancer	Phase I (NCT01344031)	MK-2206 (Akt inhibitor) plus anastrozole (Arm B), or fulvestrant (Arm C) or anastozole + fulvestrant (Arm D)	31
A Phase 1b/2 randomised placebo controlled trial of Fulvestrant ± AZD5363 in postmenopausal women with advanced breast cancer previously treated with a third generation aromatase inhibitor	Phase Ib/II (NCT01992952)	AZD5363 (Akt inhibitor) plus fulvestrant versus placebo plus fulvestrant	150
Early breast cancer			
Akt inhibition			
A phase II trial of neoadjuvant MK-2206 in combination with either anastrozole if postmenopausal or anastrozole and goserelin if premenopausal in women with clinical stage 2 or 3 PIK3CA mutant estrogen receptor positive and HER2 negative invasive breast cancer	Phase II (NCT01776008)	MK-2206 (Akt inhibitor) plus either anastrozole (if postmenopausal) or anastrozole plus goserelin (if premenopausal)	87

AIs + PI3K Inhibitors

BKM120 (buparlisib) is a potent oral pan-PI3K inhibitor that when given either continuously or intermittently in combination with letrozole in a phase I study has been demonstrated to be safe, with evidence of anti-tumour efficacy as assessed by FDG-PET scans [34, 35]. Clinical activity was seen regardless of phosphoinositide-3-kinase pathway mutation analysis (PIK3CA) mutation status [35]. The combination of BKM120 with fulvestrant has also been investigated, and a randomised phase III study of BKM120 with fulvestrant in patients with HR+/HER2-negative locally advanced/metastatic breast cancer who have progressed after prior AI therapy (BELLE-2, NCT01610284) is recruiting a second-line patient population very similar to that in the BOLERO-2 trial. Given the increased use of everolimus in combination with exemestane in the second-line setting, a further trial is currently underway, to assess the role of BKM-120 with fulvestrant who have progressed on or after mTOR inhibitors (BELLE-3, NCT01633060).

AIs + PI3K/mTOR Combined Blockade

Another approach is to develop drugs that target PI3K and mTOR together, and two pharmaceutical companies have set up studies comparing these dual inhibitors with pan-inhibitors of PI3K, both in combination with endocrine therapy versus endocrine therapy alone. For example, either XL147 (inhibitor of PI3K) or XL765 (dual inhibitor of PI3K and mTOR) have been combined with letrozole in a Phase I/II trial (NCT01082068) in ER+ advanced breast cancer. FERGI is a multi-centre, international, randomized, double-blinded, placebo-controlled phase II trial that recruited patients with advanced or MBC who had previously received treatment with an AI, randomised to receive either the pan PI3K inhibitor GDC-0941 (pictilisib) + fulvestrant or GDC-0980 (dual inhibitor of PI3K and mTOR) + fulvestrant, or placebo + fulvestrant (NCT01437566). Whether the dual targeted drugs are more effective than pan-isoform PI3K inhibitors remains to be seen, together with early assessments of toxicities which sometimes can be greater for drugs with broader target specificities. Preliminary data from the first part of the trial suggested that the addition of pictilisib improved progression-free survival in a subset of those patients with ER and PgR positive tumours from 3.7 to 7.4 months, regardless of PI3KCA mutation status [36].

AIs + Akt Inhibitors

Early phase trials have been set up investigating Akt inhibition in combination with AIs. MK-2206 is an Akt inhibitor currently being evaluated in phase I and II trials. In the metastatic setting, a multi-arm phase I trial is evaluating whether

administering an AI in combination with an Akt inhibitor can delay or prevent secondary endocrine resistance (NCT 01344031). This trial will also explore whether AI plus fulvestrant plus Akt inhibition is superior to an AI plus Akt inhibition alone. The same agent (MK-2206) is also being investigated in the neoadjuvant setting in combination with a neoadjuvant AI (NCT01776008). The primary outcome for this trial is pathological complete response.

AZD5363 is another Akt inhibitor being investigated in the established secondary resistance setting with a randomised placebo-controlled two-arm trial of either AZD5363 plus fulvestrant versus fulvestrant alone, following progression after a third-generation AI (NCT01992952).

AIs in Combination with Inhibition of Cyclin-Dependent Kinase (CDK) 4/6

Modulating the cell cycle has always been an attractive therapeutic target in cancer, and previously published data have suggested that cyclin-dependent kinase (CDK) 4/6 inhibition may play a key role in the treatment of subsets of breast cancers [37–39]. Palbociclib (previously known as PD 0332991) is a novel oral selective inhibitor of CDK 4/6, which prevents cellular DNA synthesis by blocking cell cycle progression from the G1 to the S phase. Synergy in combination with tamoxifen has been shown in ER+ cell lines, indicating that this combination could be effective in the clinical setting.

Recently, it was reported that the combination of palbociclib and letrozole given first line significantly improved median PFS in a randomised phase II study in patients with advanced ER-positive breast cancer, including those with identified cyclin D1 amplification and/or p16 loss in whom CDK 4/6 inhibition is expected to be most effective [38]. In the first part of this two-part phase II study (known as the PALOMA-1 or TRIO-18 study), 66 postmenopausal women with ER+ MBC were randomly assigned to either the combination of Palbociclib and letrozole or to letrozole alone 1st line. The second part of the study involved 99 patients with ER+ cancers possessing certain genomic alterations, specifically cyclin D1 amplification and/or p16 loss.

Results from the first part of the study showed a PFS of 26.1 months for the combination of palbociclib plus letrozole versus 5.7 months for letrozole alone ($p < 0.0001$). In part 2, PFS was 18.1 versus 11 months ($p = 0.0046$) [40]. Combined results from both parts of the study demonstrated a PFS of 20.2 months in patients who received palbociclib plus letrozole versus 10.2 months for those who received letrozole only (HR = 0.488; $p = 0.0004$) [40]. In patients with measurable disease, an improved response rate was seen (43 % vs. 33 %). Overall survival analysis (61 deaths) was 37.5 months versus 33.3 respectively (this was not statistically significant). The toxicity profile for the combination was favourable with the most common adverse events being (uncomplicated) neutropenia,

leukopenia, anemia and fatigue [37, 38, 40]. A potential biomarker for predicting the effectiveness of Palbociclib in combination with an AI is the possession of an intact Rb signaling pathway [40].

On the basis of these extremely promising results, a randomized, multi-centre, double-blind first-line study of Palbociclib plus letrozole versus letrozole/placebo in postmenopausal women with ER+ HER2− MBC who have not received any prior systemic anti-cancer treatment for advanced disease is ongoing (PALOMA-2 trial, NCT01740427) [40]. Similar studies are also underway with other CDK 4/6 inhibitors such as LEE011. The MONALEESA-2 trial is a randomized double-blind placebo-controlled study of LEE011 in combination with letrozole in the first-line metastatic setting (NCT01958021). Abemaciclib is also being studied in a series of phase III trials. The role of CDK 4/6 inhibition in the neoadjuvant and adjuvant setting in combination with an AI will also be investigated in phase 2 trials (NCT01919229, NCT01723774, NCT02040857).

The effectiveness of further endocrine therapy with fulvestrant in combination with CDK4/6 inhibition at overcoming acquired resistance to AI therapy is currently under investigation. The PALOMA-3 trial (NCT01942135) is a randomised (2:1), multi-centre, double blind Phase III study evaluating palbociclib in combination with fulvestrant versus fulvestrant plus placebo in women with hormone receptor-positive (HR+), HER2 negative metastatic breast cancer whose disease has progressed after prior endocrine therapy.

CDK 4/6 inhibition seems one of the more promising approaches to enhance endocrine response in ER+ endocrine sensitive breast cancer, and could potentially produce that quantum leap in response to first-line endocrine therapy that to date has eluded this area of clinical research in ER+ advanced breast cancer. Results from current trials evaluating the role of CDK 4/6 inhibition in overcoming resistance to AIs are eagerly awaited, but the sheer number of trials that are currently underway are testament to the promise that CDK 4/6 inhibitors hold (Table 11.4). However, appropriate clinical trial design, patient selection, and biomarker research remains crucial to enhancing the chance of success, and in particular finding the patient's own tumour profile that will determine most benefit from this approach.

Agents Targeting FGFR

Several studies have shown that the fibroblast growth factor receptor-1 gene (*FGFR1*) is amplified in approximately 10 % of all breast cancers, correlating with increased FGFR1 mRNA or protein expression [41]. Amplification of *FGFR1* is enriched in up to 20 % of ER-positive breast cancers. Amplification and over-expression of *FGFR1* may be a major contributor to poor prognosis in luminal-type B breast cancers, driving anchorage-independent proliferation and endocrine therapy resistance [41]. AZD4547 (Lucitanib) is a potent selective inhibitor

Table 11.4 Clinical trials investigating CDK 4/6 inhibitors plus endocrine agents

Study	Stage and study number	Arms	Estimated enrollment (n pts)
MBC			
Phase 1/2, open-label, randomized study of the safety, efficacy, and pharmacokinetics of letrozole plus PD 0332991 (oral CDK 4/6 inhibitor) and letrozole single agent for the first-line treatment of ER positive, HER2 negative advanced breast cancer in postmenopausal women (PALOMA-1) [40]	Phase II (NCT00721409)	Palbociclib + letrozole versus letrozole alone	165 (study completed)
A randomized, multicenter, double-blind phase 3 study of PD-0332991 (oral CDK 4/6 inhibitor) plus letrozole versus placebo plus letrozole for the treatment of postmenopausal women with ER (+), HER2 (−) breast cancer who have not received any prior systemic anti cancer treatment for advanced disease (PALOMA-2)	Phase III (NCT01740427)	Palbociclib + letrozole versus letrozole alone	650
Multicenter, randomized, double-blind, placebo-controlled, phase 3 trial of fulvestrant (Faslodex®) with or without PD-0332991 (Palbociclib) ± Goserelin in women with hormone receptor-positive, HER2-negative metastatic breast cancer whose disease progressed after prior endocrine therapy (PALOMA-3)	Phase III (NCT01942135)	Palbociclib + fulvestrant versus fulvestrant alone	417
A randomized, double-blind, placebo-controlled, phase 3 study of fulvestrant with or without LY2835219, a CDK4/6 inhibitor, for women with hormone receptor positive, HER2 negative locally advanced or metastatic breast cancer (MONARCH-2)	Phase III (NCT02107703)	Fulvestrant + LY2835219 versus fulvestrant alone	550

(continued)

Table 11.4 (continued)

Study	Stage and study number	Arms	Estimated enrollment (n pts)
A randomized double-blind, placebo-controlled study of LEE011 in combination with letrozole for the treatment of postmenopausal women with hormone receptor positive, HER2 negative, advanced breast cancer who received no prior therapy for advanced disease (MONALEESA-2)	Phase III (NCT01958021)	Letrozole + LEE011 versus letrozole alone	500
A phase Ib/II trial of LEE011 in combination with everolimus (RAD001) and exemestane in the treatment of postmenopausal women with estrogen receptor positive, Her2- locally advanced or metastatic breast cancer	Phase Ib/II (NCT01857193)	Everolimus + exemestane + LEE011 or exemestane +LEE011 versus everolimus + exemestane alone	185
Early breast cancer			
Adjuvant			
A phase 2 pilot feasibility study of palbociclib in combination with adjuvant endocrine therapy for hormone receptor positive invasive breast cancer	Phase II (NCT02040857)	Endocrine therapy (AI in postmenopausal women, tamoxifen in premenopausal) + palbociclib	120
Neoadjuvant			
A randomized pre-surgical pharmacodynamics study to assess the biological activity of LEE011 plus letrozole versus single agent letrozole in primary breast cancer (MONALEESA-1)	Phase II (NCT01919229)	Letrozole + LEE011 versus letrozole alone	120
A phase II trial of neoadjuvant PD 0332991, a cyclin-dependent kinase (Cdk) 4/6 inhibitor, in combination with anastrozole in women with clinical stage 2 or 3 estrogen receptor positive and HER2 negative breast cancer	Phase II (NCT01723774)	Letrozole (±goserelin) + palbociclib	29
A randomised phase 2 study of palbociclib with letrozole as neoadjuvant treatment for ER+ breast cancer (PALLET)	Phase II (awaiting NCT)	Letrozole ± palbociclib	300

of FGFR-1, 2 and 3 receptor tyrosine kinases (enzyme and cellular phosphorylation endpoints), and has a significantly lower potency for inhibition of IGF1R and KDR [42]. The co-administration of an FGFR inhibitor and exemestane has the potential to improve outcome for patients with aggressive disease or resistance to endocrine therapy. Therefore, GLOW is a randomised double-blind phase IIa study (with phase I combination safety run-in) designed to assess the safety and efficacy of AZD4547 in combination with exemestane versus exemestane alone in patients with ER-positive and *FGFR1* amplified (FISH $\geq$ 4) breast cancer who have failed treatment with one prior endocrine therapy (adjuvant or first-line metastatic) (NCT01202591).

In an interesting alternative trial design, the RADICAL trial is a phase IIa study investigating whether the addition of AZD4547 to the existing first-line AI (anastrozole or letrozole) following progression is superior to switching to second-line exemestane therapy (NCT 01791985). Likewise, FINESSE is a phase II study evaluating the efficacy of single-agent AZD4547 following development of resistance to endocrine therapy (NCT 02053636) (Table 11.5). It is hoped that these trials may evaluate whether targeting FGFR in endocrine resistant breast cancer is an

Table 11.5 Clinical trials investigating FGFR targeted agents in combination with AIs

Study name	Stage and study number	Arms	Estimated enrollment (n pts)
Safety and efficacy of AZD4547 in combination with fulvestrant versus fulvestrant alone in ER+ breast cancer patients (GLOW)	Phase IIa (NCT01202591)	AZD4547 + fulvestrant versus fulvestrant alone	120
A randomised piia study (with combination safety run-in) to assess the safety and efficacy of AZD4547 in combination with either anastrozole or letrozole versus exemestane alone in ER positive breast cancer patients who are progressing on current treatment with anastrozole or letrozole (RADICAL)	Phase IIa (NCT01791985)	AZD 4547 + either letrozole or anastrozole versus exemestane alone	99
A phase II trial testing oral administration of Lucitanib in patients with fibroblast growth factor receptor (FGFR)1-amplified or non-amplified estrogen receptor positive metastatic breast cancer (FINESSE)	Phase II (NCT02053636)	Lucitanib (AZD4547) only	123

appropriate strategy. Currently, there are no trials underway evaluating the FGFR in the first line endocrine naive setting.

AIs and Histone Deacetylase Inhibitors (HDACI)

Another possible approach to reverse hormone resistance is the use of histone deacetylase inhibitors (HDACI) to re-sensitize breast cancer cells to hormone manipulation [43, 44]. It has been shown that in some breast cancers, expression of ER can be repressed/lost by epigenetic modifications such as methylation and histone deacetylation, and this could be a mechanism for endocrine resistance. Entinostat is an HDACI that has been shown to increase expression of both ER and the enzyme aromatase in a dose-dependent manner both in vitro and in vivo, which then sensitized breast cancer cells to oestrogen and subsequent inhibition by the aromatase inhibitor letrozole [45]. Furthermore, in xenograft experiments the combination of letrozole plus entinostat was significantly more effective at inhibiting xenograft growth than either therapy alone. In a randomized phase II trial (ENCORE 301, NCT00676663), entinostat in combination with exemestane was compared to exemestane/placebo in patients who had received prior hormonal therapy [46]. This trial showed prolongation of median PFS (4.3 vs. 2.3 months) and extension of OS benefit (26.9 vs. 19.8 months). A randomized phase III trial is currently underway to investigate exemestane with or without entinostat in post-menopausal patients with recurrent hormone receptor-positive breast cancer that is locally advanced or metastatic (NCT 02115282). A phase II trial evaluating the addition of entinostat to continued AI upon progression has not yet released its results (NCT00828854) (Table 11.6).

Correlative studies from a phase II study testing vorinostat and tamoxifen suggest that HDAC2 expression could be a predictive biomarker, and that histone hyper-acetylation may be a valid pharmaco-dynamic marker for the efficacy of this combination [47]. The same may be true for HDACI in combination with AIs.

AIs and Anti-angiogenic Agents

Pre-clinical data [48] and retrospective clinical data [49] suggest that high vascular endothelial growth factor (VEGF) levels in breast tumours are associated with a decreased response to endocrine therapy. As several phase II studies had suggested the feasibility and activity of the combination of bevacizumab with endocrine agents [50, 51], a randomized phase III study (LEA) was conducted to test the hypothesis that anti-VEGF treatment with bevacizumab could prevent primary resistance to hormone therapy (either letrozole 2.5 mg/day or fulvestrant 250 mg/4 weeks) given as first-line therapy in endocrine responsive advanced

Table 11.6 Clinical trials evaluating AIs in combination with HDACI

Study name	Stage and study number	Arms	Estimated enrollment (n pts)
A phase 2, randomized, double-blind, multicenter study of exemestane ± entinostat in postmenopausal women with locally recurrent or metastatic ER+ breast cancer, progressing on treatment with a non-steroidal AI (ENCORE 301)	Phase II (NCT00676663)	Entinostat (SNDX-275) plus exemestane versus placebo plus exemestane	130 (completed with 125 patients recruited)
Exemestane with or without entinostat in treating postmenopausal patients with recurrent hormone receptor-positive breast cancer that is locally advanced or metastatic	Phase III (NCT02115282)	Entinostat (SNDX-275) plus exemestane versus placebo plus exemestane	600
A phase 2, randomized, double-blind, multicenter study of fulvestrant with and without entinostat in postmenopausal women with hormone receptor-positive advanced breast cancer	Phase II (NCT02115594)	Entinostat (SNDX-275) plus fulvestrant versus placebo plus fulvestrant	180
A phase 2, multicenter study of the effect of the addition of SNDX-275 to continued aromatase inhibitor (AI) therapy in postmenopausal women with ER+ breast cancer whose disease is progressing	Phase II (NCT00828854)	Entinostat (SNDX-275) plus continuation of current AI	25 (study completed, results not publicised)

breast cancer [52]. The PFS was better with the combination of bevacizumab plus endocrine therapy than with endocrine monotherapy (18.4 vs. 13.8 months), but this was not statistically significant. The combination had a significantly higher incidence of haematologic and non-haematologic toxicities, and does not appear to be a promising approach to enhance first-line therapy.

The absence of a robust positive effect in the LEA trial, together with negative data from the BEATRICE trial in women with triple-negative disease, questions the efficacy of first-line angiogenesis inhibition in breast cancer [53]. Results from a recently completed randomized first-line phase III trial of endocrine therapy

alone or endocrine therapy plus bevacizumab for women with hormone receptor-positive advanced breast cancer have not yet been released (NCT00601900).

Multi-target agents are being explored, which combine effects on angiogenesis with inhibition of other signaling pathways. Regulation of breast cancer cell proliferation depends on activation of MAPK, Ras and Raf [54]. Sorafenib is an oral multikinase inhibitor that inhibits tumour growth by acting on the tumour cells and tumour vasculature cells in preclinical models of human cancer, including breast cancer [55]. It targets the MAPK pathway at the level of Raf kinase, inducing tumour cell apoptosis, and potently inhibiting VEGFR-1, VEGFR-2, VEGFR-3, and the platelet-derived growth factor receptor (PDGFR)-β via tyrosine kinase autophosphorylation [56]. The role of sorafenib in the treatment of breast cancer which has developed acquired resistance to hormone therapy has therefore been investigated. Sorafenib in combination with endocrine therapy has been investigated with anastrozole and sorafenib in women with MBC: the combination demonstrated a 23 % CBR in 35 patients with hormone receptor positive, AI-resistant MBC. This may be attributable to the restoration of sensitivity to AIs [57].

Another multi-target inhibitor of angiogenesis is BMS-690514, which is a potent and selective inhibitor of epidermal growth factor receptor (EGFR), HER2, and HER4, as well as the VEGF receptor kinases. When BMS-690514 was tested in a panel of breast tumour cell lines, there was a clear demarcation between cell lines that were sensitive and those that were resistant. Overexpression of HER2 seemed to be sufficient to predispose breast tumour cell lines to inhibition by BMS-690514, again underscoring its intrinsic potency to that target [58]. An open-label randomized, parallel, two-arm phase II study comparing BMS-690514 plus letrozole with lapatinib + letrozole in recurrent/metastatic breast cancer patients who are hormone receptor positive despite HER2 status and who relapsed while receiving or after completing adjuvant anti-endocrine therapy has been completed and results are awaited (NCT01068704) (Table 11.7).

The absence of results from these trials and the lack of further trials into anti-angiogenesis agents suggests that this may not be a promising approach for preventing either primary or secondary resistance to endocrine therapy.

AIs in Combination with Agents Targeting Insulin-Like Growth Factor Type 1 (IGF-1)

The role of the insulin-like growth factor (IGF) system in endocrine resistant breast cancer has been studied, and inhibitors of this pathway are currently in clinical trials in ER+ve patients who have progressed on prior endocrine therapy. MEDI-573 is a dual-targeting human antibody which neutralizes IGF-I/-II ligands and inhibits insulin-like growth factor receptor 1 (IGF-1R) and insulin receptor-A (IR-A) signaling pathways which play a role in breast and other epithelial cancers. By sparing insulin receptor-B (IR-B) and its hybrid receptors, MEDI-573 is

Table 11.7 Recently completed anti-angiogenesis studies currently awaiting results

Study	Stage and study number	Arms	Estimated enrollment (n pts)
Endocrine therapy with or without anti-VEGF therapy: a randomized, phase III trial of endocrine therapy alone or endocrine therapy plus bevacizumab (NSC 704865) for women with hormone receptor-positive advanced breast cancer	Phase III (NCT00601900)	Bevacizumab plus tamoxifen or letrozole versus tamoxifen or letrozole alone	502
Multicenter, randomized study to evaluate the efficacy and safety of bevacizumab in combination with endocrine treatment compared to endocrine treatment alone, in postmenopausal women with advanced or metastatic cancer with indication of hormonotherapy as first-line treatment	Phase III (NCT00545077)	Bevacizumab plus letrozole or fulvestrant versus letrozole or fulvestrant alone	378
An open-label randomized, parallel, two-arm phase II study comparing BMS-690514 + letrozole with lapatinib + letrozole in recurrent and metastatic breast cancer patients who are hormone receptor positive despite HER2 status and who relapsed while receiving or after completing adjuvant antiendocrine therapy	Phase II (NCT01068704)	BMS-690514 (inhibitor of EGFR, HER2, and VEGF receptor kinases) plus letrozole versus lapatinib plus letrozole	140

expected to achieve anti-tumour activity without perturbing glucose homeostasis, and has showed acceptable safety and favorable PK profiles without significant changes in glucose levels [59]. A biomarker-rich phase Ib/II study of MEDI-573 with an aromatase inhibitor in patients who have developed acquired resistance to endocrine therapy and who have advanced ER+ve breast cancer is ongoing (NCT01446159) (Table 11.8).

Table 11.8 Current clinical trials investigating IGF inhibitors in combination with endocrine agents

Study	Stage and study number	Arms	Estimated enrollment (n pts)
A phase 1b/2 randomized study of MEDI-573 in combination with an aromatase inhibitor (AI) versus AI alone in women with metastatic breast cancer (MBC)	Phase Ib/II (NCT01446159)	MEDI-573 (dual IGF-I/II-neutralizing antibody) plus AI versus AI alone	193
A phase 2 study of BMS-754807 combined with letrozole or BMS-754807 alone in hormone receptor-positive breast cancer subjects with acquired resistance to non-steroidal aromatase inhibitors	Phase II (NCT01225172)	BMS-754807 plus letrozole versus BMS-754807 alone	59
A randomized double-blind phase 2 trial of exemestane ± MM-121 in postmenopausal women with locally advanced or metastatic estrogen receptor positive (ER+) and/or progesterone receptor positive (PR+) Her2 negative breast cancer	Phase II (NCT01151046)	MM-121 plus exemestane versus exemestane alone	130

Proteosome Inhibitors Targeting NF-κB

Several groups have now demonstrated that the PI3K/Akt pathway provides cancer cell survival signals, in part through activation of the nuclear factor kappa B (NF-κB) transcription factor, and that Akt activation of NF-κB may be an important mechanism in the development of tamoxifen- resistant breast cancer [60, 61]. Bortezomib is a proteasome inhibitor that blocks the NF-κB pathway. It was tested in a phase II study in combination with endocrine treatment [62]. Despite effective target inhibition that was demonstrated in peripheral blood mononuclear cells and tumour samples, no objective anti-tumour responses were observed. Addition of a proteasome inhibitor to anti-hormonal therapy resulted in 22 % CBR in a limited number of patients with endocrine resistant and progressive MBC. A randomized phase II study of fulvestrant versus fulvestrant in combination with bortezomib in women with ER+ MBC is ongoing (NCT01142401) (Table 11.9).

Agents Targeting Src Kinase

Results from preclinical studies showed that ER-Src kinase axis play an important role in promoting hormonal resistance by proto-oncogenes such as HER2, PELP1, and that blocking this axis prevents the development of hormonal independence in vivo [63]. Dasatinib is a potent, broad spectrum ATP-competitive inhibitor of Src tyrosine kinase. However, the addition of dasatinib to fulvestrant in a randomised phase II study in ER-positive post-menopausal MBC patients who had progressed after a NSAI did not improve PFS, CBR, or OS [64] Similarly, 157 patients were randomized in a double-blind Phase II trial (CA180-261) to receive dasatinib (100 mg daily) or matched placebo in combination with exemestane (25 mg daily). While the PFS difference was not significant in overall study population, a higher CBR in the dasatinib arm and higher PFS in patients with symptomatic bone metastasis (HR = 0.68) suggested that dasatinib may have efficacy in a subset of patients [65]. In contrast, a randomised phase II first-line study (NCT00696072) suggested in an exploratory analysis that the addition of dasatinib to letrozole may

Table 11.9 Clinical trials investigating AIs in combination with proteosome inhibitors

Study	Stage and study number	Arms	Estimated enrollment (n pts)
A randomized phase II study of fulvestrant versus fulvestrant in combination with bortezomib in women with ER positive metastatic breast cancer	Phase II (NCT01142401)	Bortezomib + fulvestrant versus fulvestrant alone	118

improve PFS (median 20.1 vs. 9.9 months, HR 0.69) [66]. It remains to be seen if Src is a viable target to enhance endocrine responsiveness.

Future Directions

As indicated above, there are numerous recently completed and ongoing clinical trials combining AIs with various different targeted signaling inhibitors. This combined approach is based upon sound preclinical evidence that each of the respective pathways has been implicated in causing either primary or secondary resistance to AIs. Despite this rationale, the level of clinical evidence to date for AIs in combination with targeted therapies remains low, and we need to gain further evidence by performing well-designed clinical trials.

From the evidence summarised in this chapter, AIs in combination with blockade of the mTOR pathway is one possible approach that appears to translate to clinical benefit, and this is already the only approved combination in the setting of secondary resistance. The other promising strategies based on early positive phase II randomised clinical trial data include both CDK4/6 inhibition in combination with AIs, and HDACI in combination with AIs. This is reflected by the large number of registration-based phase III clinical trials now evaluating these combinations, and results from these studies are expected in the next 2–3 years. The outcomes from these clinical trials will establish whether this early promise translates into confirmed clinical benefit.

The relatively disappointing results from targeting some specific growth factor receptors in combination with AIs resistance suggest that blocking any given single target as a means of preventing/delaying secondary endocrine may simply allow cancer cells to escape and bypass that particular signaling pathway. Finding a common pathway that determines endocrine response or resistance has proved elusive. What has become clear is that multiple pathways probably exist through which resistance to AIs can develop, which is the likely reason why clinical research to date has failed to establish the optimum approach to overcome resistance to AIs in daily clinical practice. There remain numerous unanswered questions that require further research in the future, including the following areas:

- Given that resistance to AIs can be either primary or secondary, should we aim to identify tumours with primary resistance before AI therapy starts, or should we focus on treating secondary resistance once it become established? Which of these strategic approaches would improve patient outcome the most?
- Should we put more effort into delaying the development of secondary resistance in endocrine sensitive MBC, and can we improve upon a median PFS of 12–15 months to AIs in the first-line setting?
- Should we explore sequential combinations of AIs with targeted treatments, or would combined blockade with triplet therapy (i.e. AIs with two different targeted treatments) be an effective strategy? Would toxicity for patients by this approach outweigh clinical benefit in this scenario?

- Might intermittent or pulsed therapy re-sensitise ER+ cells to AIs in combination with targeted therapy?
- What are the main drivers for resistance mechanisms? Is it more likely to be pathway driven in ER+ breast cancer, as opposed to specific driver mutations.
- Can we establish effective biomarkers to select the patients who will benefit from blockade of any specific signaling pathway? To date this has proved elusive with mTOR inhibitors in combination with AIs.
- If multiple mechanisms for AI resistance are operating contemporaneously, can effective biomarkers be developed to identify which of the resistance mechanisms are dominant in each patient? Ultimately, could we even predict primary or subsequent secondary resistance to AIs in ER+ breast cancer using molecular profiling of the primary tumour?

Conclusion

Endocrine therapy is the most important systemic treatment for ER+ breast cancer. While aromatase inhibitors are highly effective for postmenopausal women in both early and advanced stages of the disease, the resistance mechanisms limit their success. The substantial progress made in understanding the biology of ER+ breast cancer has yielded new approaches to treatment, some of which (i.e. mTOR inhibition) have now entered clinical practice in overcoming secondary resistance to AIs. There are other approaches now starting to show promise as discussed in this chapter. The ultimate goal will be to have accurate molecular profiling of patients with ER+ breast cancer that can personalise and refine treatment pathways, which in turn will enhance the effectiveness of AIs in the treatment of ER+ breast cancer.

Acknowledgments We acknowledge research support funding from the NIHR Biomedical Research Centre at The Royal Marsden NHS Foundation Trust.

Conflict of Interest Research funding support from Pfizer, GlaxoSmithKline: Speaker Honoraria from AstraZeneca, GlaxoSmithKline, Roche, Novartis.

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Chapter 12
Aromatase Inhibitors Beyond Breast Cancer: Endometrium Versus Breast Puzzle and Other Issues

Lev M. Berstein

Abstract Aromatase inhibitors were developed and intended for different purposes; however, in practice they are predominantly used to treat breast cancer. It is becoming increasingly clear that this approach proves to be useful. Unfortunately, not all patients show responsiveness to this class of drugs, and some lose it over time. The expansion of the attempts to use aromatase inhibitors beyond the mammary cancer field suggests that these drugs can be beneficial in some other cancers as well as noncancerous conditions. Some of the pathological states show different degrees of resistance to aromatase inhibitors. This phenomenon warrants further studies of its causes and ways to overcome it. In this regard, noteworthy are endometrial cancer on one hand and some variants of uterine sarcomas on the other. Endometrial cancer, so as breast cancer, is referred to estrogen-dependent conditions; therefore, the markedly low responsiveness of endometrial cancer patients to aromatase inhibitors is a puzzle calling for a solution. On the other hand, some cases of uterine sarcomas show significant responsiveness to aromatase inhibitors. The reaction of these tumors is higher than in other cancer and non cancer cases studied in this regard, except for breast cancer. Taken together, this makes another incentive to study the mechanisms of resistance to aromatase inhibitors and, due to this, to expand the latter usage beyond traditional targets.

Abbreviations

AI(s)	Aromatase inhibitor(s)
BC	Breast cancer
CR	Complete response

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A. Larionov (ed.), *Resistance to Aromatase Inhibitors in Breast Cancer*,
Resistance to Targeted Anti-Cancer Therapeutics 8,
DOI 10.1007/978-3-319-17972-8_12

EC	Endometrial cancer
EMA	European Medicines Agency
ER	Estrogen receptors
FDA	Food and Drug Administration (USA)
LC	Lung cancer
OC	Ovarian cancer
OS	Overall survival
PC	Prostate cancer
PFS	Progression free survival
PgR	Progesterone receptors
PR	Partial response
SD	Stable disease

Introduction

According to recommendations approved by FDA and European Medicines Agency (EMA), the modern aromatase inhibitors (AIs) letrozole, anastrozole and exemestane are indicated exclusively for breast cancer (Table 12.1). Nevertheless, attempts continue, often on empirical grounds, to use these drugs for other indications, sometimes successfully to varying extents. In essence, the issue of why the most common use of AIs is in breast cancer rather than in any other condition hinges on the main problem addressed in this volume: what are the causes of the natural resistance to AIs and why it develops eventually after a period of responsiveness to a treatment with an AI? Analyzing the areas of AIs applicability beyond breast cancer may not only clarify what other 'non-mammary' medical fields benefit or can potentially benefit from AIs but also may provide grounds to think about what can make AIs ineffective in breast cancer. The data presented below will be distributed in two sections, one related and the other unrelated, at least directly, to oncology. Wherever a specific disease/clinical situation will be considered, available data will be provided on the activity and/or expression of aromatase, the usability of AIs, and resistance to AIs and the attempts to overcome it.

AIs in Cancer

Let's Start from Breast Cancer…

By the time of preparing this Chapter (August 2014), PubMed yielded 9187 entries in response to the query "aromatase inhibitor", and 5400 to "aromatase inhibitor AND breast cancer"; that is, breast cancer (BC) occupies about 60 % of the entire area in question. On the whole, this looks as if AIs were designed mainly to treat BC, which seems true as follows from relevant evidence [1].

Table 12.1 Approved indications for using the main inhibitors of aromatase

Drug	FDA, 2013	EMA, 2011–2013
Letrozole	Letrozole (Femara) is indicated for the adjuvant treatment of postmenopausal women with hormone receptor positive early BC, for the extended adjuvant treatment of early BC in postmenopausal women who have received 5 years of adjuvant tamoxifen therapy, for first-line treatment of postmenopausal women with hormone receptor positive or hormone receptor unknown locally advanced or metastatic BC, and for the treatment of advanced BC in postmenopausal women with disease progression following antiestrogen therapy	In patients with advanced or metastatic BC, treatment with letrozole should continue until tumour progression is evident. In the adjuvant and extended adjuvant setting, treatment with letrozole should continue for 5 years or until tumour relapse occurs, whichever is first. In the adjuvant setting a sequential treatment schedule (letrozole 2 years followed by tamoxifen 3 years) could also be considered. In the neoadjuvant setting, treatment with letrozole could be continued for 4–8 months in order to establish optimal tumour volume reduction
Anastrozole	Anastrozole (Arimidex) is indicated for adjuvant treatment of postmenopausal women with hormone receptor-positive early BC, for the first-line treatment of postmenopausal women with hormone receptor-positive or hormone receptor unknown locally advanced or metastatic BC, and for the treatment of advanced BC in postmenopausal women with disease progression following tamoxifen therapy. Patients with ER-negative disease and patients who did not respond to previous tamoxifen therapy respond to anastrozole rarely	Anastrozole is indicated for the treatment of advanced BC in postmenopausal women, as adjuvant treatment of postmenopausal women with hormone receptor positive early invasive BC, and adjuvant treatment of early BC in hormone receptor positive postmenopausal women who have received 2–3 years of adjuvant tamoxifen. Co-administration of tamoxifen or estrogen-containing therapies with anastrozole should be avoided as this may diminish its pharmacological action
Exemestane	Exemestane (Aromazin) is indicated for adjuvant treatment of postmenopausal women with estrogen-receptor positive early BC who have received 2–3 years of tamoxifen and are switched to exemestane for completion of a total of five consecutive years of adjuvant hormonal therapy	Exemestane is licensed for BC treatment by a national health authorities and not the European Medicines Agency

At the same time, contemplating the reasons why this design proved to be a success may help to understand why AIs fail or only partly succeed in other conditions. Therefore, periodic recurrence of the BC theme in the subsequent discussion is warranted.

Without dwelling here on using AIs in female breast cancer patients, since this issue is covered by the previous chapters, it should be noted that EMA in its relatively recent statement asserted that “it was unaware of clinical trials or specific systematic investigation—as opposed to isolated case reports—on the use of letrozole in *male* breast cancer, and that neither efficacy nor safety data exist”

[2]. The initial part of this assertion seems true; however, papers published in 2013–2014 based on observational studies and inferences from earlier findings provide for some tentative conclusions. In particular, the well-known high rates (up to 90 %) of detecting of estrogen receptors in male mammary tumors is suggested to explain the beneficial effects of AIs in such patients in the curative and metastatic setting [3]. The additional use of GnRH analogues did not increase AIs efficacy estimated by a partial response and stabilisation of disease amounting to 26.1 and 56.5 %, respectively [4]; although, according to other authors, the combination of these two types of drugs looked attractive: 10.5 % of patients had complete response, 36.8 % experienced a partial response, and 36.8 % had stable disease lasting for not less than 6 months with overall disease control rate 84.2 % [5]. With all that, there are nuances, which should not be ignored. They include still prevalent use of tamoxifen, which interferes with the effects AIs, in male BC, the need to confirm the claim that mortality rate among males treated with AIs is higher than upon tamoxifen treatment [6], and doubts concerning the ability of AIs to efficiently prevent estrogen synthesis in the testes [7]. Taken together, this introduces some uncertainty in this male vs female aspect of the issue in question, which is reminiscent of confusing differences between AIs effects in breast cancer and endometrial cancer.

Endometrial Cancer (EC)

Extragonadal estrogen production in cancer may result from either (a) preexisting aromatase activity, which was significant in the parent tissue and could become quantitatively and often qualitatively, including the genomic level, altered in the neoplastic tissue (breast cancer is a typical case) or (b) de novo aromatase activity, which emerged in the course of neoplastic transformation. The second case is exemplified, in particular, with non-small cell lung cancer (which will be addressed below) and endometrial cancer [8, 9], which is significant in that in many countries it is the most common malignancy of the female genital tract.

Aromatase determined by radiometric or immunohistochemical methods is found in EC tissues in 55–80 % of cases [8, 10, 11]. This is not significantly different from findings in BC (60–70 %) [10, 12–14], although EC and BC markedly differ in their responsiveness to AIs (see below). There is no evidence of a clear-cut association between the presence of aromatase and steroid hormone receptors in either BC or EC, save that some findings suggest that this association may be inverse, which is not accepted unequivocally [12, 15, 16]. It cannot be ruled out that the final agreement on this topic is not achieved because it is still uncertain whether aromatase and steroid receptors are colocalised in the same cells, or their interactions are mediated in an autocrine or paracrine way. Nevertheless, the concomitant presence of both ER and PR remains the main indication for the use of AIs in breast cancer. Using aromatase activity as a marker for such indicative purposes still seems unreliable [12, 17], and the suitability of aromatase mRNA and

gene polymorphisms for the same purpose still needs further ascertaining [18–21]. This issue becomes even more complicated when it comes to endometrial cancer because it is less studied, in this respect, and because of the already mentioned low effectiveness of AIs in this disease.

In this regard, it is noteworthy that the history of studies of aromatase in the endometrium was not smooth at all. By early 1980-ies, it was concluded that there is no aromatase in the normal endometrium. Subsequently, this conclusion was doubted from time to time but finally confirmed when PCR showed no evidence of $P_{450arom(CYP19A1)}$ transcripts in endometrial tissues [22], although critique of this approach may still be encountered [11]. By contrast, in endometrial cancer tissue, aromatase is found with different methods including PCR, detection of the immediate products of androgen aromatisation, detection of 'hard water' released upon aromatisation of tritiated androgenic precursors, and immunohistochemical analysis [10, 11, 23].

The present author's opinion on a potential role of aromatase in endometrial cancer was formed based on original studies carried out in the beginning of this century [24, 25]. According to data obtained paradoxically at odds with observations on blood estrogens [26, 27], a higher intratumor aromatase activity [24, 28, 29] was featured by type II rather than type I pathogenetic variant of EC [27, 30, 31]. Moreover, in poorly differentiated tumors (G3) this increase was pronounced enough to suggest that aromatase is involved in the unfavorable clinical course of EC and thus may be used to predict such cases [24, 28]. Independent data either lend support to this observation [32, 33] or contradict it [11, 34] warranting further studies. Interestingly, in a study carried out in collaboration with the Laboratory of Molecular Oncology headed by Prof. Imyanitov, it was found that, among type II compared with type I EC patients, the bearers of the A6A6 allelic variant of aromatase (CYP19), which points at potentially higher activity, are detected more frequently [29]. It can be added that studied intronic TTTA(n) repeats of CYP19 vary in number from 1 to the ≥7, and bearing of genotypes with longer alleles (like A6A6 or A6A7), obviously, can lead to hyperestrogenization; this is confirmed also with higher lumbar spine bone mineral density and lower risk of spine fractures [35].

The polymorphisms of another steroidogenic enzyme, 17α-hydroxylase/17, 20-liase (CYP17), which is implicated in the synthesis of the androgenic precursors of estrogens, showed no difference in their occurence in type I and type II EC patients [29]. At the same time, although EC patients who bore different CYP17 polymorphic variants did not differ in their blood steroids, including estradiol, testosterone and dehydroepiandrosterone sulfate, the homozygous A2A2 bearers (the most rare variety) featured, as contrasted to A1A1 homozygotes and A1A2 heterozygotes, the lowest basal and reactive—i.e., after glucose load—blood insulin [36]. The latter observation can be put in association with the evidence that, although decreases in endometrial M-echo signal and increases in FSH and LH concentrations after neoadjuvant treatment with AIs were more pronounced in type I patients, decreases in tumor PgR content ($p = 0.04$) were more revealing in patients with type II EC. Besides, decreases in aromatase activity in tumor

tissue at the end of such treatment were found predominantly in patients with lower body weight (BMI < 27.5) [24], which can be associated with the aforementioned decreases in blood insulin [36]. Thus, although type II EC is often believed to be hormone-independent, the high rate of estrogen biosynthesis in such tumors may prompt a reconsideration of this belief [24], as also follows from the recent evidence that the risk factors of type I EC and type II EC are rather close [37]. Altogether, the above highlights such questions as which EC patients will benefit from taking AIs and what is currently known about the therapeutic efficiency of AIs in EC.

In reviewing any evidence relevant to this, one should bear in mind that, by contrast to BC, EC is a disease where adjuvant hormonal therapy (with progestins as the primary option) did not show any significant effect and, therefore, is virtually never used at present [38]; the factor of patients' selection probably needs to be studied additionally, though [39]. This seems also to be true with respect to AIs, although only a few relevant studies on small patient samples are available. For example, in a trial carried out at the Tom Baker Cancer Centre in Calgary (Canada) it was possible to assess the effectiveness of therapy with AIs, mainly nonsteroidal, in 7 patients only. Partial response was observed in 1 (14 %), stable disease in 5 (71 %), and progression in 1 (14 %) of the patients. Taking into account that in a larger sample where objective results were not available but subjective improvement was reported in 70 % of cases, the authors concluded that AIs can be used as a potential therapy in patients who have a contraindication to surgery or in whom therapy with progestins either have failed or cannot be used [40]. It is worthy to add, although this information is somewhat oblique, that gynecological abnormalities were assessed in the ATAC trial where BC patients received adjuvant tamoxifen, anastrozole (Arimidex) or a combination thereof. After 2 years of treatment, endometrial thickness remained within 5 mm (baseline: 3.0 mm) in patients treated with anastrozole, increased by 3.2–7.0 mm in patients treated with tamoxifen, and showed a similar trend in the combination group [41]. After 6-years follow-up, there were non-significantly fewer endometrial abnormalities with anastrozole than with tamoxifen (12.4 % vs. 20.2 %, odds ratio 0.52; $p = 0.17$); however, the effect of drug combination was not traced because this arm of the trial was discontinued [42]. On the whole, there are no grounds so far to claim a protective (antiestrogenic) effect of anastrozole on the endometrium.

Back to progestins already mentioned above, these are the drugs that should be rated as the most effective (responses were seen in 60 % of cases) neoadjuvant therapy for EC [43–45]. By now, only a few attempts to use AIs in this setting have been reported. In one such study, ten previously untreated postmenopausal patients (mean age 59 years) with endometrial cancer, predominantly stage I disease, received letrozole 2.5 mg/day for 14 days before surgery. The treatment was well-tolerated in all patients. In two patients, pain relief in the lower part of the abdomen and/or decrease in intensity of uterine discharge were reported. In three of the ten cases, substantial decreases in endometrial M-echo (ultrasound) signal, on average by 31.1 % versus baseline values, were noted [46]. Figure 12.1, which presents these and some other results of this work graphically, shows that,

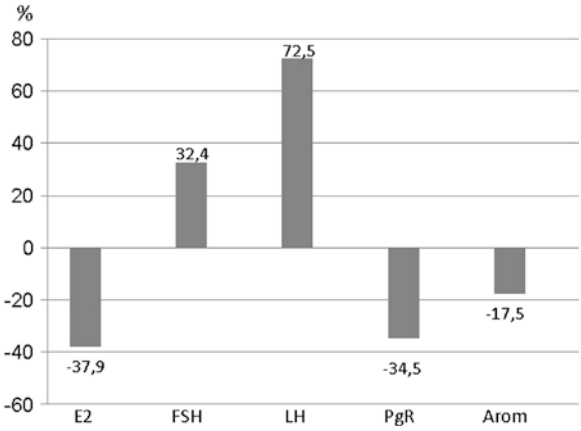

Fig. 12.1 Trends of the changes (%) in parameters studied before and after neoadjuvant therapy of endometrial cancer with the aromatase inhibitor letrozole (constructed from the data presented in [24, 25, 46]). E2, FSH, and LH: serum levels of estradiol, follicle-stimulating and luteinising hormone; PgR and AROM: tumor tissue progesterone receptor level and aromatase activity

during treatment, the mean blood estradiol decreased by 37.9 % and FSH and LH increased by 32.4 and 72.5 %, respectively, whereas the mean tumor tissue progesterone receptor (PgR) level decreased by 34.5 %, and aromatase activity only by 17.5 %, the latter decrease showing no correlation with changes in the endometrial M-echo signal [47].

As a follow-on of the above study, the effects of neoadjuvant letrozole where compared with those of anastrozole (n = 15, 1 mg/day, 28 days) and the non-steroid AI exemestane (n = 13, 25 mg/day, 14 day) [25]. Endometrial wall thickness (M-echo signal) decreased in 60 % of patients treated with anastrozole, in 58.3 % with exemestane, and in 30 % with letrozole. The differences might be attributed to treatment duration, which was longer with anastrozole, as well as to the steroid vs. nonsteroid nature of the drugs used. The latter possibility is consistent with that progesterone receptor downregulation in EC tissue (a marker of attenuated estrogenic stimulation) was most expressed with exemestane [25]. As an additional comment, the duration of the above neoadjuvant treatment with AIs was deliberately limited to one month, whereas the recommended duration of same therapy for breast cancer is 3–4 months, and proposals to increase in up to 7.5 months [47] have been repeatedly put forward.

To complete the consideration of neoadjuvant AIs in EC, the recent trial carried out at St. James University Hospital (Leeds, UK) [48] included 24 patients (mean age about 63 years) randomized into two groups: 16 patients received anastrozole (1 mg/day, 11–49 days, 20 days on average) and 8 patients received placebo (13–48 days, 23 days on average). Steroid receptors, Ki-67 antigen, and Bcl-2 protein were tested separately in endometrial glands and stroma. Anastrozole therapy resulted in a significant decrease in Ki-67, which was less pronounced in glands than in stroma, and significant decreases in ERα and androgen receptor in glands, whereas PgR (in contrast with [25]) and the apoptosis marker Bcl-2 were virtually unchanged. The authors acknowledged the importance of the decrease in the proliferation marker Ki-67 and provided no explanations to decreased ERα and unchanged PgR expression and no data about the EC course [48].

Clinical information proper may be found so far only in the results of using AIs in disseminated and recurrent/metastatic EC. The available evidence is summed up in Table 12.2, which presents data provided by three studies carried out 8–14 years ago [49–51] and in two recent publications: an original paper [52] and a review [53]. The evidence suggests that, despite of the good tolerability of these medicines, the overall response (CR + PR) in EC is 8.7–11.8 %, which is by 3.5–5-fold inferior to AIs effectiveness in BC in similar clinical settings [5, 54, 55].

Now, what is the cause of the relatively low responsiveness or high resistance of EC to AIs? What stands behind the resultant paradox based on the claims that estrogen dependence is repeatedly found in EC [27, 31]? With all the many approaches to answering these questions, the final solution is not yet known. Some tentative explanations should however be mentioned. Most importantly, no matter how prosaically it sounds, the mammary epithelium and the endometrial epithelium are two different epithelia. Differences between them encompass the discordant effects of progestins, tamoxifen and other hormone-associated factors, as summed up in Table 12.3 without citing extra literature, which is exceedingly vast. The possible causes of these differences may include the tissue-specific characteristic of the receptor apparatus and its coactivator and corepressor systems, signal transduction mechanisms, in particular peptide signalling, and the alternative promoters of aromatase gene expression [1, 8, 54, 56]. These factors are much more thoroughly studied in BC [21] thus delineating an enormous field of research related to EC and other potentially estrogen-related cancers as well as non-cancer pathologies [56, 57].

Ovarian Cancer

Whereas EC is the most frequently occurring tumor among gynecological malignancies, ovarian cancer (OC) is considered to be the most lethal, which explains so much effort devoted to searching for an effective therapy for this disease. Along with surgery, radiotherapy and chemotherapy, which remain the primary treatments for ovarian cancer, different endocrine therapeutic approaches were also tried for decades. Turning to AIs in these attempts may be explained by three considerations, at a minimum: the known roles of estrogens in OC pathogenesis, the discovery of steroid hormone receptors in the epithelial ovarian carcinomas, and the presence of aromatase in these tumors [58–62]. There is still no full consensus on the above, including possible therapeutic options. For example, aromatase activity in the tissues of normal ovaries, ovarian cysts and ovarian cancer was found to negatively correlate with ERα expression, which was the highest in the normal ovarian epithelium ($r = -0.34$, $P < 0.001$). At the same time, aromatase activity did not correlate with OC stage, grade and histological type and with patient survival [60]. Nevertheless, endometrioid ovarian carcinomas, which contain ER, are still believed to be the OC most likely to show beneficial effects upon therapy with AIs.

Table 12.2 Effectiveness of aromatase inhibitors in disseminated and recurrent endometrial cancer

Publication type	Drug	Regimen	Clinical effect	Reference number
Original study: 23 patients (9 cases with G3 tumor)	Anastrozole	1 mg/day, not more than 28 days	2 PR (8.7 %), 2 SD (8.7 %), mean PFS 6 mo	Rose et al. [49]
Original study: 32 patients (9 patients after progestin therapy); PgR and ER are found in 86 % of tumors	Letrozole	2.5 mg/day (until progression)	1 CR, 2 PR (9.4 %), 11 SD (34.3 %) mean PFS 8.8 mo	Ma et al. [50]
Original study: 28 patients (17 with ER + tumors)	Exemestane	25 mg/day (until progression)	In ER + tumor cases: 1 CR, 1 PR (11.8 %), 6 SD > 6 mo (35.3 %)	Nordstrom et al. [51]
Original study: 51 patients with advanced (FIGO stage III-IV) or relapsed EC (39 ER + tumor cases)	Exemestane	25 mg/day (until progression)	In the ER + patients: an overall response 10 %; a lack of progression after 6 mo in 35 % of the patients. No responses were registered in the ER-negative patients. In the ER + group OS was 13.3 mo, in the ER– group it was 6.1 mo	Lindemann et al. [52]
Review: advanced or recurrent EC	Third generation AIs		Response rates within 10 %	Lee et al. [53]

Notes CR complete response, *PR* partial response, *SD* stable disease; *PFS* progression-free survival; *OS* overall survival time

Table 12.3 Some hormone-associated distinctions between endometrial and breast cancer or respective normal tissues (according to available literature, references in the text; see also [130])

Feature	Endometrial cancer (endometrium)	Breast cancer (mammary epithelium)
Risk associated with estrogen replacement therapy in the menopause	Higher	Lower
Estrogen deficiency-associated femoral neck fracture rate in case histories	Lower	Higher
Mitotic index	Higher in the follicular phase of menstrual cycle	Higher in the luteal phase of menstrual cycle
Tamoxifen effect	Typically estrogenic	Antiestrogenic
Tissue estrogen level	Higher	Lower
Tobacco smoking effect on incidence ("antiestrogenic" effect)	More pronounced	Poorly pronounced
Diabetes mellitus	Risk factor in postmenopausal and reproductive period	Is found more often in post-menopausal period
Obesity	Prevalence in reproductive ages in not less than in post-menopausal females	Risk factor in postmenopausal variant of disease
Preventive effect of peroral steroid contraceptives	Pronounced	Not shown
Progestin use in endocrine therapy of disseminated disease	First-line therapy	Third- or fourth-line therapy (used rarely at present)
AIs use in therapy	AIs are still virtually unused	AIs use is prevalent and rather often effective

In practice, AIs have been used never as the first-line therapy for OC and, usually, supplement other chemotherapeutic drugs, such as platinum preparations and taxanes, are prescribed when other treatments for advanced or recurrent OC fail. The accumulated experience may be exemplified with several most recent publications, leaving aside the literature published since 1990-ies through the first decade of the present millenium. The most systematic review of recent findings is provided by Modugno et al. [63] who discuss the results presented in seven papers, which altogether cover the outcomes of treatment of 264 patients having persistent or recurrent OC, of whom 53 were treated with anastrozole, 22 with exemestane, and 189 with letrozole. Outcomes included only one CR case (0.3 %), 20 PR cases (7.6 %), and 81 SD cases (30.7 %). Having agreed with other authors in that the effectiveness of AIs is low in OC and even somewhat lower than in EC, Modugno et al. [63] also share the view that among OC patients there are always a few of those who can show more beneficial responses to AIs. Indeed, in their recent review van Meurs et al. recalled data on rather good responsiveness to AIs of granulosa cells ovarian tumors [64]. Therefore, what is needed is to select patients and

find predictive markers of responsiveness to AIs and/or the ways to escape resistance to them in OC, which is, naturally, a part of a broader agenda.

Here it is worthy to mention two more points that may, at least indirectly, be of relevance to OC proper. One of the points is a certain degree of similarity between the pathogenetic pathways of OC and endometriosis, which involve aromatase, sex steroid receptors, and some growth factors [65]. If confirmed, this might be important by providing some practical hints because the responsiveness of endometriosis foci to AIs is known to be higher than that of OC (see below). The second point is that many OC patients bear BCRA1 mutations [66], which are associated, similarly to decreased BCRA1 expression, with aromatase upregulation [67, 68]. Therefore, the possibility that OC patients who have BRCA1 mutations are most responsive to AIs cannot be ruled out and warrants special studies.

Lung Cancer

According to SEER data, more than 255 thousand newly detected lung cancer (LC) cases or about 14 % of all new cancer cases were expected to occur in the USA in 2013. In addition, LC-related death rate being twice as high as caused by all cancers and making 27.5 % of all cancer-related deaths, evidencing the high prevalence and severity of the disease [69]. Among all LC cases, 80–90 % are attributed to non-small cell carcinoma, including squamous cell carcinoma, which is often found in tobacco smokers, and adenocarcinoma, which is more prevalent in women. Although males are more vulnerable to LC than women are, females in many countries are gradually catching up, possibly because of changing smoking patterns and other factors, including endocrine ones.

Initially, the endocrine factors of LC development were generally thought to be limited to corticosteroids and their metabolites, although papers that suggest a potential role of estrogens in LC have been published since almost half a century ago [70]. Interest to this problem is on the rise since 1990-ies, particularly over the last 10–15 years, when the terms “aromatase” and “aromatase inhibitors” started to appear increasingly in publications relevant to LC. The idea emerged that estrogen replacement therapy during the menopause can influence LC risk and LC-related mortality in female smokers [71, 72] and that PgR and ER, especially ER-beta, found in lung cancer tissue may be involved [73, 74]. Noteworthy in this regard is that ER-beta in lung cancer tissue is often coexpressed with aromatase, and this combination is associated with a lower survival in male, but not female, LC patients, which suggests the feasibility of selective endocrine therapy, based on assessing these markers [75].

The ‘self-sufficient’ significance of aromatase activity in the lung tumor tissue, particularly in the non-small cell cancer, was assessed in a number of works. In some of them the association of a lower activity with a better survival was noted suggesting that aromatase activity can be among prognostic markers and that it is reasonable to try AIs as a therapy for LC [76, 77]. The latter suggestion is

supported by experiments showing that in nude mice with A549 lung tumor xenografts, administration of anastrozole for 21 days elicited pronounced inhibition of tumor growth in vivo [78]. No relevant clinical data are available by now; however, AIs are combined with estrogen receptors down-regulators in some ongoing Phase I-II clinical trials conducted among patients with advanced LC [77, 79]. Another therapeutic option in LC may be to combine an AI with an EGRF inhibitor as prompted by the experiments where the EGRF inhibitor gefitinib was given together with the pure antiestrogen fulvestrant [79, 80]; of note, though, no association between EGRF mutations and ER-beta expression was found in LC tumor tissue [81]. If mentioned approaches prove to be clinically beneficial, a certain similarity between LC and BC would be confirmed [82] promoting AIs expansion to therapies for cancers that feature unconventional hormone dependency patterns.

Other Tumors

It makes sense to begin this section with uterine sarcoma because, first of all, it is often reported to show beneficial responses to AIs. Sarcomas of the uterus are mesenchymal tumors with a poor prognosis and aggressive biology, although some of their forms are more differentiated and less aggressive. The recent review [83] contains reports about 7 cases (4 endometrial stromal sarcomas and 3 leiomyomas) treated by its authors with AIs. Besides, independently published papers are reviewed to cover 11 similar treatment reports and 6 retrospective studies. Taken together, this evidence suggests that the overall response rate of endometrial stromal sarcoma to AIs was 67 % [CR 7 % and PR 60 % (!)], and the partial response rate of leiomyosarcoma to AIs was 11 %, with no reported CR's [83]; however, in the ongoing Phase two clinical trial using letrozole to treat ER + or PgR + uterine leiomyosarcoma patients, somewhat more encouraging results are expected [84]. Since endometrial sarcoma responses to AIs are reported to be not inferior (if not superior) to responses to progestins [83], further studies are warranted to elucidate the causes of this fairly high responsiveness to AIs.

Prostate cancer endocrinology has been long centered 'around androgens'; however, due to studies carried out over the last decades, estrogens too are increasingly recognised as factors influencing prostate cancer development and progression [85]. With regard to a potential role of aromatase inhibitors, several findings and hypotheses deserve attention. In particular, aromatase is thought to be significant for balancing androgens and estrogens in prostate tissue as well as for mediating its diseases [86, 87]. More fundamental aspects of the prostate biology and carcinogenesis may relate to ER-containing stem/progenitor cells functioning [87], aromatase activation by prostaglandin E2 in the stromal cells [88], and the long standing idea advocated by Bosland that estrogens and androgens are synergetic in producing carcinogenic effects mediated by the catecholestogens' metabolites-DNA adducts in prostate [89]. Because AIs inhibit the synthesis of the classic estrogens and thus limit the generation of the progenotoxic metabolites of

estrogens, the above idea is interesting from the point of view of using AIs for PC prevention, which could be the objective of special investigation, at least in an experiment. In clinics, to the best of our knowledge, the use of modern AIs in PC has been limited (in spite of the above) to eliminating, albeit less efficient than with tamoxifen, of gynecomastia and breast pain in patients treated with antiandrogens [90] and to the old-established recommendation of aminoglutethimide combined with hydrocortisone for hormone-resistant PC [91]. In the latter case, remission based on laboratory findings (PSA level) was reported in 37 % of patients, median PFS in responders being 23 months [91]; however, this study had no continuation.

Of the other cancers, a high aromatase activity in melanoma tissue has once attracted attention [92]; however, aminoglutethimide proved to be inefficient in patients with this tumor [93]. There were no attempts so far to use AIs with the aim to treat patients having cervical cancer or tumors of the thyroid gland, colon or liver; however, the reasonability of such attempts deserves consideration in view of arguments presented in the number of papers [94–97]. In particular, there is some evidence that estrogens are involved in the promotion and, probably, even initiation of tumors in the liver and thyroid [98, 99].

Meanwhile, interests of researchers and clinicians expand to estrogens and to aromatase inhibitors for treatment of several non-cancer conditions which will be discussed further.

Non-cancer Conditions

Endometriosis

The prevalence of endometriosis in the general female population is 7–10 %, and may be up to 30–35 % in infertile women [100]. Clearly, to find successful therapies for this condition is an important task. In-depth studies of aromatase activity, expression and regulation in endometrioid lesions provided a large body of evidence suggesting an important role of estrogen synthetase in the pathogenesis of endometriosis and in the development of different variants of its clinical course and localisation, including the involvement of peritoneum and ovaries. In endometriosis, aromatase expression is primarly controlled by the proximally located promoter 1.3/II [8], which is regulated by a number of factors, such as prostaglandin E2 and peroxisome proliferator activated receptor-γ coactivator-1α (PGC-1α), assisted by auxiliary mediator [101, 102]. These and related findings make grounds for publications where AIs are proposed as therapeutic means for endometriosis, which can be no less potent than the conventionally used progestins, peroral steroid contraceptives etc.

In particular, it has been repeatedly observed that AIs prescribed to endometriosis patients of reproductive ages attenuate and, at times, eliminate for a while painful sensations associated with endometriosis as discussed in the comprehensive

reviews [103, 104]. In premenopause the effects of AIs have to be potentiated by other therapies [104], whereas in the postmenopausal endometriosis, AIs by themselves can be effective and even can reduce endometriotic lesions [105]. At the same time, there are publications where the reasonability of using AIs in therapies for endometriosis is disputed and the need for further studies is advocated [106, 107]. It is also noted that, in treating endometriosis with AIs, one should mind side effects, primarily a trend to decreased bone mineral density [104], which has been intensively studied in breast cancer field. It is also suggested to conduct more studies aimed at examining pregnancy rates and outcomes after AIs have been used to treat endometriosis [104], which seem important in view of the aforementioned association of endometriosis with infertility.

Infertility

Reproductive health problems occupy a special place in the attempts to use AIs outside the breast cancer area as will be relatively briefly reviewed in this and the subsequent sections.

Besides the above observations that the successful treatment of endometriosis with AIs can improve fertility in patients younger than 30–40 years, attempts were made in the recent years to use AIs to achieve the same result in other clinical situations, including unexplained infertility, infertility associated with the use of gonadotoxic therapy in cancer patients, and in male infertility. In women, AIs are used to induce ovulation in anovulatory states, including in vitro fertilisation (IVF) cycles, either independently or as an auxiliary to clomifen citrate and gonadotropin preparations as discussed in a recent Cochrane review and other publications [108–113]. Conclusions from the available evidence are sometimes unequivocal. Thus, it is not well known whether letrozole or other AIs can be used for this purpose independently (that is, as an only treatment). Also, the studies are in progress which will be helpful in understanding whether the total dose of gonadotropins should be modified upon their use in combination with AIs, whether there are differences in the use of letrozole in noncancer patients and after gonadotoxic therapy courses in cancer, and finally whether AIs or more conventional therapeutic modalities used to induce ovulation produce comparable results, including pregnancy rate etc. However, the consensus is that, in order to expand options available to treat infertility, it is reasonable to go on with trials including the use of combinations of AIs with other drugs, such as the antidiabetic biguanide metformin widely utilized in polycystic ovarian disease [108, 109, 112]. Among advantages of AIs relatively low cost and lower multiple pregnancy rates are mentioned, while limited data are presented on their potential teratogenic effects as well as on oocyte and embryo quality [113, 114].

There are reports on the attempts of using AIs to treat male infertility caused by impaired spermatogenesis. Anastrozole (1 mg/day) or letrozole (2.5 mg/day) administration has been reported to increase spermatozoid counts and blood

testosterone/estrogen ratio; however, it is still unclear whether oocyte fertilization is really improved in these cases [115].

Medical Abortion

In recent years AIs attract attention as a means for not only induction of ovulation but, on the contrary, for termination of pregnancy. This trend may be exemplified with a study where 20 women scheduled for abortion at 2 months of gestation received letrozole (10 mg/day) for 7 days and intravaginal misoprostol (prostaglandin E1) on the 7th day. Abortion was reported to be induced in 95 % women (95 %) at 7.5 h on average after misoprostol administration. Subsequent interviews showed that 17 women (88 %) would prefer this mode of abortion on a possible necessity in the future [116]. Importantly, letrozole used in such settings does not influence uterine contractility, and its abortion-inducing effect is mediated via estrogen production and metabolism [117].

The possible contraceptive effect of letrozole in women is contemplated tentatively because of its impact on luteal function [118].

Gynecomastia

Gynecomastia sometimes occurs in adolescents during normal maturation, but more often results from diseases associated with a disbalance between estrogens and androgens, such as upon liver cirrhosis, or from some medications. The latter may be categorised into two groups: cardiovascular drugs, including calcium channel blockers, angiotensin-converting enzyme inhibitors, spironolactone, etc., and drugs used to treat prostate cancer, including estrogens and antiandrogens. One of the causes of gynecomastia is the aromatase excess syndrome, a rare hereditary disorder manifested in the pre- or peripubertal period [119]. AIs have been used in a number of the above conditions, including excessive aromatase activity where AIs can be quite effective, liver cirrhosis-associated gynecomastia where AIs are most likely to be not inferior to tamoxifen, and antiandrogen-induced gynecomastia where AIs are less effective than tamoxifen is [1, 119, 120].

Other Conditions

A non-exhaustive list of additions to the above includes the attempts to use AIs in adolescents to prevent premature epiphysis closure in pubertas praecox and in other growth disorders [121]. It is still unclear, whether AIs can be used instead of testosterone substitution therapy for late-onset hypogonadism in elderly males

[122]. In children, AIs were tried in Peutz-Jeghers syndrome (autosomal dominant genetic disease characterized by the development of benign hamartomatous polyps in the gastrointestinal tract and pigmented macules on the lips and oral mucosa, sometimes associated with aromatase excess), McCune-Albright syndrome (a genetic disorder of bones manifested also in skin pigmentation and hormonal problems along with premature puberty), and in some forms of hyperandrogenism, including testotoxicosis and congenital adrenal hyperplasia [123].

As to women, noteworthy is the idea to use AIs to treat uterine myomas. In scarce reports about such attempts, more or less optimistic conclusions can be found. An optimistic publication reports about 30 premenopausal women aged 30–55 years having uterine myomas sized within 4 cm who received 2.5 mg of letrozole daily for 12 weeks. Myomatous nodes shrinked, on average by 1 cm in size and by twofold in volume, by the end of the 3rd month of the treatment. No changes in blood lipids and testosterone, FSH, LH and even estradiol were noticed (although the so-called rebound phenomenon might be expected in these cases); the most pronounced adverse effects being nausea and hot flushes [124]. On the other hand, in a Cochrane review on this subject [125] it was concluded that the trend to myoma shrinkage, although noticeable, is not always significant and that studies included only small samples of patients and were not blinded. Further studies are needed in this so as in several other cases discussed above.

Conclusions and Perspectives

The evidence discussed in the present chapter and generalized in Fig. 12.2 suggests that aromatase inhibitors can be used with broadly varying effectiveness for indications other than breast cancer, in conditions not limited to neoplasms, and in patients ranging from children to elders.

The causes of therapeutic failures with AIs are not always clear. They may relate to the tissue-specific features of the aromatase complex and its regulation as well as be disease-specific. Still poorly developed are approaches to escaping resistance to AIs, e.g. in EC, and to increasing responsiveness to AIs in conditions including non-cancer pathologies.

These are the problems to be tackled in the nearest future, in particular, by ascertaining which patients are most responsive to AIs, and by finding most appropriate combination of AIs with other drugs able to potentiate the effects of AIs in each specific indication. Such combinations may include cyclooxygenase inhibitors; however, their effects in experimental endometriosis were opposite to what was expected [126]. Combinations of AIs with the antidiabetic biguanide metformin, which is remarkable in its multisided effects, are already being tried in BC [127] and used in polycystic ovarian disease and other conditions associated with infertility [112].

The factors that limit the long-term use of AIs in cancer and non-cancer conditions include side effects, such as decreased bone mineral density, hyperlipidemia,

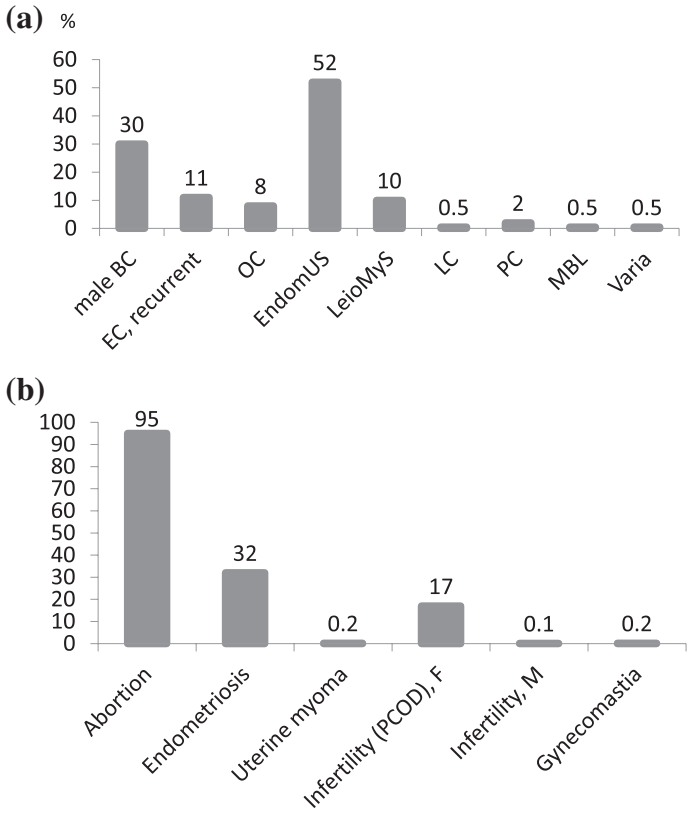

Fig. 12.2 The use and effectiveness of aromatase inhibitors in different medical fields (except female BC), contemporary situation (please see text for additional explanations, some data need confirmation). **a** Oncology: partial response rates (%) to AIs in various indications. *BC* male breast cancer; *EC* endometrial cancer, recurrent; *OC* ovarian cancer; *EndomUS* endometrial uterine sarcoma; *LeioMyS* leiomyosarcoma; *LC* lung cancer (no reliable clinical data with AIs so far); *PC* prostate cancer (AIs were used mainly for the alleviation of breast events in patients treated with antiandrogens); *MBL* melanoma (attempts to use AIs are rare and not successful); Varia: cervical, hepatocellular, thyroid, and colorectal cancer (only assumptions, no clinical or experimental data). **b** Non-cancer clinical conditions: success rates (%) for various indications. Medical abortion induction: of usage of AIs in combination with prostaglandin E1. Endometriosis: mainly alleviation of pain. Uterine myoma: solitary attempts to decrease myoma size were performed. Infertility: treatment attempts were made more often and were more successful in females (f.e. in polycystic ovarian disease, PCOD) than in males; randomized studies were carried out rarely so far. Gynecomastia: effects are disease type-dependent and so far most promising in cases of aromatase excess

and cardiovascular events, which must be taken seriously. Developing of means able to prevent these side effects may result in increasing the number of patients electable for being treated with AIs. At the same time, the endocrine side effects of AIs may be used as predictive factors of responsiveness to AIs in cancer patients [128, 129] and as such warrant confirmation and application beyond oncology field.

On the whole, with regard to a more general objective of this chapter, it could be summarised that estrogens, along with being potent mitogenic factors in breast, have a broader range of targets and effects in human physiology and pathophysiology. This warrants a persisting interest to the details of biosynthesis of these hormones and to means, including AIs, able to modify estrogen biosynthesis and (due to this) effects in cancer and in other diseases and clinical situations.

Acknowledgments Useful discussions with Dr. Alexey Golubev, St. Petersburg are highly appreciated.

No conflict statement "No potential conflicts of interest were disclosed".

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vDOI 10.1007/978-3-319-17972-8