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Anita K. Dunbier, Lesley-Ann Martin, Mitch Dowsett. 'New and translational perspectives of oestrogen deprivation in breast cancer'. *Molecular and Cellular Endocrinology*, 2011, 340 (2), pp.137. 10.1016/j.mce.2010.12.034 . hal-00717914

HAL Id: hal-00717914

<https://hal.science/hal-00717914>

Submitted on 14 Jul 2012

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Accepted Manuscript

Title: 'New and translational perspectives of oestrogen deprivation in breast cancer'

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PII: S0303-7207(11)00023-2
DOI: doi:10.1016/j.mce.2010.12.034
Reference: MCE 7741

To appear in: *Molecular and Cellular Endocrinology*

Received date: 10-8-2010
Revised date: 21-12-2010
Accepted date: 22-12-2010

Please cite this article as: Dunbier, A.K., Martin, L.-A., Dowsett, M., 'New and translational perspectives of oestrogen deprivation in breast cancer', *Molecular and Cellular Endocrinology* (2010), doi:10.1016/j.mce.2010.12.034

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'New and translational perspectives of oestrogen deprivation in breast cancer'

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Abstract

Over the last 20 years, aromatase inhibitors have been developed to become a highly effective treatment strategy for treatment of hormone receptor positive breast cancer. Despite their success, poor response and resistance limit the effectiveness of these agents in up to 50% of patients. In recent years, studies using highly sensitive hormone assays have provided insight into the source of oestrogen production for the stimulation of oestrogen receptor positive breast cancer growth, suggesting that uptake from the circulation is likely to make a significant contribution to intratumoural oestradiol. To obtain insight into how tumours become resistant to oestrogen after aromatase inhibition, long term oestrogen deprivation of cultured cells has been used to mimic acquired resistance to aromatase inhibitors. This work has aided the selection of agents to rationally combine with aromatase inhibitors to combat resistance. Molecular profiling using genome-wide approaches has shed new light on the heterogeneity of responses to oestrogen deprivation and predictors of resistance *in vivo*. Testing new agents and combinations in short-term pre-surgical studies using biomarkers such as Ki67 is critical for increasing the rate at which new rational combinations can be assessed for efficacy.

Introduction

Over the last 20 years the introduction of 3rd generation aromatase inhibitors has led to a highly effective treatment strategy for hormone-receptive positive breast cancer in post-menopausal women, and more recently has been demonstrated to have effectiveness in premenopausal women with ovarian suppression. This has led to these inhibitors being recommended to be included in the treatment of all cases of oestrogen receptive positive (ER+) primary breast cancer in postmenopausal women[1]. Nonetheless, challenges to control the disease in such patients remain, since poor response and acquired resistance continue to occur. Opportunities to address these challenges spring from our improved understanding of the mechanism of resistance to oestrogen deprivation, a plethora of novel agents to target the pathways of resistance and the harnessing of the presurgical or neoadjuvant treatment scenario for the validation of mechanisms of resistance identified *in vitro* and for rapid assessment of new agents in the clinical setting. This article aims to summarise some of the most recent developments that have affected our thinking and some of the ongoing approaches to meeting the continued challenge of ER+ breast cancer.

Systemic vs. intra-tumoural oestrogen synthesis as the stimulant for ER+ breast cancer

There has been much debate over the past three decades on the most important source of oestrogen production for the stimulation of ER+ve breast cancer growth. In postmenopausal women oestrogen is synthesised in a variety of peripheral tissues and creates plasma levels which are 10% or less those of premenopausal women, yet the intra-tumoural levels of oestradiol vary little between postmenopausal and

premenopausal women[2]. Thus, the concept has developed that the low, but measurable, level of aromatase activity in breast tumours results in the high concentrations seen in postmenopausal women[3]. This origin of the stimulatory oestradiol is significant for the development of therapies and potentially of resistance mechanisms. In the recent past we have conducted two studies which suggest that plasma oestradiol levels, and therefore the systemic conversion of androgen to oestrogen, are the more significant source of stimulation.

In the first of these[4] we obtained genome-wide RNA expression profiles from pre-treatment core-cut biopsies from 104 post menopausal patients with primary ER+ breast cancer treated with neoadjuvant anastrozole, and found that the expression of many known oestrogen responsive genes and gene sets were highly significantly associated with plasma oestradiol levels before treatment: the average expression of 4 of these, termed the AvERG, showed a correlation of 0.51 ($p < 0.0001$) (Figure 1). The observations were validated in an independent set of 73 ER+ tumours. Of particular note, plasma oestradiol was significantly correlated with 2 week Ki67, a marker associated with clinical outcome[5].

In the second piece of work[6] we collaborated with Professor Lønning's group in assessing the expression of genes involved in oestrogen synthesis, metabolism and signalling in 34 matched samples of breast tumour and normal breast tissue. Intratumoural oestradiol levels showed a strong positive correlation with ER expression in all patients ($R = 0.55$; $P < 0.0001$), and was highest in the post-menopausal women ($R = 0.76$). Of particular note, expression of two of the enzymes which interconvert oestrone and oestradiol, HSD17B7 and HSD17B2, showed

significant positive and negative correlations respectively with intra-tumoural oestradiol. In contrast, *CYP19* (the gene encoding aromatase) showed no correlation with intratumoural oestradiol. Overall these data suggest that uptake due to binding to ER rather than intratumoural synthesis by aromatase, is the probable determinant of intratumoural oestradiol.

Long-term oestrogen deprived cells

There is a paucity of clinical and biological data on acquired resistance to AI. In order to address this several groups have assessed this in MCF7 cells that have escaped from the anti-proliferative effects of oestrogen deprivation. In our work and that of others, MCF7 cells during adaptation to long term oestrogen deprivation (LTED) appear to pass through two distinct phases: quiescent, followed by hypersensitivity where basal cell growth is maximally stimulated by concentration of oestradiol (E2) as low as 10^{-13} M. In contrast the wt-MCF7 cells required concentrations in excess of 10^{-10} M [7-10] (Figure 2). Clinical data supporting hypersensitivity as a means of resistance comes from a study of pre-menopausal patients who initially responded and then relapsed after oestrogen withdrawal by ovarian suppression, and subsequently responded to further suppression of oestrogen levels by the addition of an aromatase inhibitor at the time of subsequent relapse[11]. This indicates that the initial resistance was due to the acquisition of an increased sensitivity to residual postmenopausal levels of oestrogen, which can be overcome by further reducing circulating levels of oestrogen in these patients.

Of particular note in the LTED model system, concentrations of E2 which resulted in maximum proliferation in the wt MCF7 cells induced apoptosis in the LTED

setting[10]. It was subsequently shown that was mediated via a Fas/FasL driven mechanism and may have clinical implications for the use of high dose oestrogen for the treatment of patients who have replaced on AIs[12].

Hypersensitivity to oestradiol coincided with enhanced ER expression and function as evidenced by the 10-fold increase in ER-mediated transcription[9]. This was associated with increased cross-talk between the ERBB2 signalling pathway and ER at the point of resistance. We hypothesized that the ER was acting in a ligand-independent manner as evidenced by increased levels of ER phosphorylated at serine 118, the target for ERK1/2, together with an increase in levels of pp90RSK — one of the kinases involved in ER phosphorylation at ser167. This was further supported by the fact that ER-mediated gene transcription could be inhibited by several treatments that interrupt upstream signaling, including gefitinib and the MEK1/2 inhibitor UO126. However, assessment of the phosphorylation status of the ER showed that while inhibition of the signalling cascades inhibited ER-mediated proliferation and transcription it did not alter phosphorylation of ERser118. This suggested the LTED cells remained ligand-dependent and that the associated effects of the growth factor signalling pathways were most likely via activation of the ER coactivators influencing recruitment of the basal transcription machinery, providing a hypersensitive reception to residual E2. Treatment of the LTED cells with the ER downregulator ICI182780 (fulvestrant), which degrades the ER, inhibited both proliferation and ER-transactivation in the absence of exogenous E2[9]. These data suggested that ER remained an integral part of LTED phenotype and supported the design of the SoFEA study, which is layering fulvestrant with anastrozole in advanced breast cancer patients. By elucidating the signalling pathways that operate

in this process of adaptation, a logical combination of inhibitors targeting the various signal-transduction pathways could be developed for use in the endocrine resistant setting as discussed in the following section.

Rational combination of drugs

Recent efforts to enhance the benefit of existing endocrine therapies have been directed towards using the improved understanding of the molecular basis for endocrine resistance that has resulted from studies such as those in the model systems described above. Important in application of these new treatments is a recognition that functional ER expression is maintained in most patients, relapsing after tamoxifen treatment. While the data are fewer for recurrence on aromatase inhibitors, early indications are that the proportions of patients with ER positivity are even higher in such patients[13]. There is some evidence that ER expression may be suppressed by enhanced peptide growth factor receptor signalling, such as that from HER2 or EGF receptor (EGFR). Pre-clinical studies with the dual EGFR/HER2 TKI lapatinib, including those from our own group, have shown that an inhibition of this growth factor activity in endocrine resistant cells *in vitro* is associated with an increase in ER expression and ER signalling[14]. Thus targeting these growth factor receptor pathways alongside aromatase inhibitors is a rational approach to impact on endocrine resistance.

Early trials with the TKIs gefitinib or erlotinib, either alone or in combination with endocrine therapy, were disappointing. Some data however from a recently reported study of anastrozole plus or minus gefitinib in advanced disease showed greater promise[15]. This study reported a prolongation of progression free survival from a

median of 8.2 months with anastrozole to 14.6 months with the combination, but this benefit was not observed in a second randomised phase 2 trial of the same combination[16]. Other somewhat encouraging results came from the randomised phase III TAnDEM trial in 207 patients with known ER+ HER2+ metastatic breast cancer, given that this doubled progression-free survival with the addition of trastuzumab to anastrozole[17]. However, the outcome for the combination patients was still poor with a median progression-free survival of only 4.8 months.

More recently, a large trial in advanced breast cancer patient with ER+ breast cancer not selected for HER2 positivity, reported on the comparison of letrozole vs. letrozole plus lapatinib in the HER2+ patients[18]. The addition of lapatinib to letrozole markedly reduced the risk of progression in this group (hazard ratio 0.71), improving the median progression free survival from 3.0 to 8.2 months. Overall, in the ER+ HER2- group there was no benefit of added lapatinib, although there was a trend to a potential benefit from the addition of lapatinib in the sub-set of patients that relapsed during adjuvant tamoxifen therapy.

Thus, while the targeting of HER2 in tumours which over-express HER2 and are ER+ de novo provides a clear benefit, the identification of patients that may benefit from targeting the *acquisition* of such growth factor signalling remains a major challenge. One of the reasons for this may be that during advanced disease, many secondary signaling pathways may be available for circumventing the block of just one pathway as practised to date. Combinations of therapies which are rationally targeted to characterised aberrations in the tumour, as well as known escape routes, may provide the way forward.

Pre-surgical studies

One of the greatest difficulties in developing a new agent for promising targets in endocrine resistance is the time that it takes to complete such studies in conventional phase III trials and the frequent absence of tissue from the index tumour that allows the linkage of benefit or not to molecular characteristics of the tumour. So far as studies of resistance are concerned, the pre-surgical or neoadjuvant scenario is proving particularly attractive, since biomarkers of clinical response in individual patients are easily determined in all patients, and tissue biopsies are available before, during and at the end of therapy.

Possibly the most important advance in taking this area forward in terms of endocrine therapy has been the validation of the proliferation marker Ki67 as an intermediate marker of treatment benefit. These data initially emerged from the IMPACT trial of anastrozole vs. tamoxifen vs. the combination[19,20]. The differences between these three arms in the change in tumour Ki67 at 2 weeks and 12 weeks was parallel to difference in recurrent survival seen with the same 3 arms in the ATAC adjuvant trial[21,22]. Importantly, the value of Ki67 at 2 weeks (and 12 weeks) in patients in IMPACT correlated with their recurrence-free survival with patients with higher Ki67 levels recurring significantly more rapidly[5](Figure 4). The clear implication from these data is that the 2-week value reflects in part the improved outcome that is associated with endocrine therapy in patients that benefit from it.

The potential for studying mechanisms of de novo resistance in this scenario has already been reported in studies from our group and that of Miller[23-25]. Very

substantial changes in gene expression occur over a 2-week period with the aromatase inhibitors anastrozole and letrozole, but these vary widely between patients and seem likely to be determinants of the patient's outcome. These molecular changes may be dissected and disaggregated with the expectation that the important facets of molecular change with oestrogen deprivation for clinical response may be identified. The degree of heterogeneity between tumours, however, requires that very large numbers will require analysis to identify individual mechanics with confidence. In this regard a short-term pre-surgical study of aromatase inhibition vs. no aromatase inhibition (POETIC: Perioperative Endocrine Therapy for Individualising Care), currently underway in the UK, is highly encouraging: this is expected to recruit over 4,000 patients over the next 2-3 years, with recruitment rates already over 100 per month.

While such short-term studies of about 2 weeks duration are extremely helpful in conducting studies of de novo response/resistance, until recently studies of acquired resistance in the pre-surgical setting have not been possible since surgery is generally timed at a point to avoid clinical expansion of the tumour. Our recent data on the recovery of Ki67 in a proportion of patients between the 2-week and 12-week time-points strongly indicates that this early biological escape from control by oestrogen deprivation may be a valid measure of the acquisition of resistance since: recurrence-free survival in this group was similar to that in de novo Ki67 resistance and poorer than that in patients with persistent Ki67 response[26].

Clinical trials are therefore being constructed in which Ki67 is the primary endpoint and have a randomisation and design which allows the prevention of Ki67 recovery

to be specifically addressed. One such study has already been reported in which anastrozole was combined with gefitinib[27]. Unfortunately, in this instance the combination was no better than the aromatase inhibitor alone, on both measures of Ki67 and clinical parameters. Nonetheless, this observation contributed to the decision on not to move to a large phase III study of the combination in the adjuvant context.

Conclusions

There have been major advances in our understanding of mechanisms of resistance in model systems, and some of these show parallels with molecular evaluation of breast tumours using genome-wide approaches. To date, the advances in the clinic with agents targeted against individual resistance pathways have been modest. The application of molecular profiling alongside rationally targeted drugs in the presurgical setting, however, promises improved outcomes in the near future.

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Figure Legends

Figure 1: Plasma estradiol has recently been found to correlate with estrogen-responsive gene expression. In a set of 104 postmenopausal breast tumours, plasma estradiol correlates with the AvERG (**A**verage **E**strogen-**R**esponsive **G**enes; ie, mean of *TFF1*, *GREB1*, *PDZK1*, and *PGR*). This finding was also validated in an independent set of postmenopausal tumours[4]. Adapted from Dunbier et al., JCO, 28, 1161-7.

Figure 2. Summary of the molecular changes associated with adaptation to LTED. A. Proliferation assay showing the LTED cells are hypersensitive to E2 and proliferate maximally at E2 concentration in the range similar to those seen in a patient receiving an aromatase inhibitor. Of note E2 concentrations that result in maximum cell growth in the wt MCF7 reduce the proliferation of the LTED cells and is associated with Fas/FasL mediated apoptosis. B. The LTED cells showed increased expression of phosphorylated and total ERBB2 and blockade of ERBB2 with gefitinib an EGFR/ERBB2 inhibitor suppressed proliferation of the LTED cells in a concentration dependent manner showing enhanced sensitivity compared to the wt MCF7[8-10].

Figure 3. Signalling pathways associated with LTED and potential drug targets. Increased ERBB2 leads to activation of ERK1/2 and pp90RSK. Whilst in our setting ERK1/2 does not appear to phosphorylate the ER, previous studies have shown that ERK1/2 can activate the ER in a ligand independent manner via phosphorylation of ER serine 118[28] and indirectly via pp90rsk at ER serine 167[29]. ERK1/2 and p90rsk also phosphorylate and cause aberrant recruitment of ER- coactivators[30] and CBP potentiating ER-genomic activity allowing low levels of E2 to be perceived [31,32]. Agents targeting ER, the growth factor receptors or down stream kinases are currently under investigation.

Figure 4: Recurrence-free survival according to tertiles of tumor Ki67 expression at baseline (top) and after 2 wk of anastrozole treatment (bottom) in the IMPACT study[5]. The divisions refer to the natural logarithm of the percentage of Ki67-positive cells at baseline or 2 wk. Adapted from Dowsett et al., JNCI, 99, 67-70.

Figure 1