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An integrated view of aromatase and its inhibition☆

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Abstract

Aromatase inhibition has become a major treatment strategy for postmenopausal women with oestrogen-dependent breast cancer. Its optimal application is, however, dependent upon (i) the accurate identification of cancers which are ultimately dependent upon the activity of the aromatase enzyme, (ii) the use of the best method/inhibitor by which to blockade aromatase activity.

The single best predictor of response to aromatase inhibitors is the presence of tumour oestrogen receptors; receptor-negative cancers rarely respond whereas those with high levels seem particularly likely to benefit. However, there is a need for additional discriminatory markers. The use of microarray technology coupled with neoadjuvant therapy is likely to yield promising candidate genes. The finding that, amongst peripheral tissues, the tumour itself may have high activity has led to the suggestion that the tumour aromatase measurements may be predictive; however, in situ studies and the lack of robust assays for tumour aromatase suggest that tumour aromatase may not be an influential marker.

Whilst drugs such as anastrozole, exemestane, formestane and letrozole are all effective and specific inhibitors of aromatase, they differ in structure, potency and mechanism of action. Thus, differential sensitivity of tissues/tumours and non-cross resistance mean inhibitors are not equivalent and individual agents may have differing roles according to the setting in which they will be used. Aromatase inhibitors have evolved as key endocrine agents in the treatment of breast cancer. They offer the promise of rational treatment management based on the accurate identification of individual cohorts of tumours responsive to specific drugs.

Keywords: Aromatase; Inhibition; Neoadjuvant therapy

1. Introduction

The first international symposium on aromatase was held in 1982 [1]. At that time, it was unforeseen that 20 years later the subject area would still be the centre of interest. This is because in the intervening years the central role of oestrogen in the development of both normal and abnormal tissues has been confirmed. Most particularly the involvement of oestrogen in the natural history of breast cancer has identified aromatase as a target for prevention and treatment of the disease [2]. Furthermore, the past 5 years has seen the clinical introduction and evaluation of therapeutic agents which block the aromatase enzyme with immense potency and exquisite specificity [3–9].

The aim of the current paper is to integrate several threads of research in an attempt to address two key issues, viz. (i) which breast cancers are most likely to respond to therapeutic intervention and (ii) which aromatase inhibitor should be used as treatment.

Many of the data presented in this review are derived from patients given neoadjuvant treatment during which aromatase inhibitors are given with the primary tumour within the breast. This form of therapy may provide clinical benefits for the patient. Thus, patients with large tumours may have these down-staged following successful therapy. As a consequence, inoperable tumours may become operable and women who may have required a mastectomy before therapy may be satisfactorily treated with breast-conserving surgery. Furthermore, those unfit for surgery may avoid such procedures. Additional clinical benefit includes the knowledge of response or otherwise of the primary tumour to a specific therapy which may be offered or avoided in the adjuvant setting.

However, there are major advantages of using neoadjuvant protocols in the research setting. Because the primary tumour is available for measurement, accurate assessment of response is possible. These measurements may be correlated with putative biological markers in a pre-treatment biopsy

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of the same tumour. Additionally, since many patients come to definitive surgery, effects of treatment may be monitored by serial samples of individual tumours.

2. Materials and methods

2.1. Patients for neoadjuvant therapy

Postmenopausal patients with large (>3 cm) oestrogen receptor (ER) positive (>20 fmol/mg cytosol protein or 80 histoscore) [10] primary breast cancers (staged as T_2, T_3, T_{4b}, N_0 or N_1, M_0) were entered into the study. None had received prior treatment with hormonal agents for breast cancer or were taking hormone preparations at the time of study. Tumour size was monitored clinically (by calipers) and by breast ultrasound before and at monthly intervals during treatment. All patients received primary endocrine therapy comprising either letrozole (2.5 mg daily, $n = 12$ or 10 mg daily, $n = 12$), anastrozole (1 mg daily, $n = 12$ or 10 mg daily, $n = 11$), exemestane (25 mg daily, $n = 12$) or tamoxifen (20 mg daily, $n = 24$).

2.2. Clinical response

Clinical response was based on change in tumour volume taken at monthly intervals over the treatment period. Ultrasound measurement of three orthogonal tumour diameters produced an estimate of tumour volume [11]. Reductions in tumour volume >25% were regarded as evidence of tumour response; those >50% were categorised as major response.

2.3. Pathological response

Histological sections from the initial biopsy and the final surgical excision were assessed for decrease in cancer cellularity and increase in fibrosis. Where such changes occurred, the tumour was classified as having a clear subjective pathological response and where clear changes in cellularity and/or fibrosis were not apparent the tumour was graded as no pathological response.

2.4. Immunohistochemical studies

Measurement of oestrogen receptor and progesterone receptor (PgR) status was assessed after microwave antigen retrieval (ID5 Dako, and PG88 Biogenex, respectively) and scored according to Allred et al. [12] for intensity (four levels) and proportion (five levels); the values are summed into a category score (range 0–8).

2.5. Aromatase activity

2.5.1. In situ estimates

In situ aromatase activity within the breast was based on measuring radioactivity in purified oestrogen fractions extracted from breast tissue after infusion with radioactively labelled steroids. To determine the effect of aromatase inhibitors on such activity, tumour material (and where possible adjacent non-malignant tissue) was taken by biopsy (before treatment) or by wide local excision of the tumour (at the end of treatment) and blood was obtained by veni-puncture at the same time-points.

Infusion with radio-labelled steroids (20 MBq 1,2,6,7-^{3}H-androstenedione (85 Ci/mmol, Amersham, Little Chalfont, UK) and 1 MBq (4-^{14}C) oestrone (56 m Ci/mmol, Amersham) was performed immediately preceding biopsy and prior to excision of the tumour after treatment as previously described [13].

2.5.2. In vitro estimates

This methodology was based on identifying and characterising radio-labelled oestradiol following incubation of homogenates of breast cancer with 7-^{3}H testosterone as previously described [14,15].

Fibroblast cultures were derived from breast adipose tissue as described by Miller and Dixon [13]. Before assaying for aromatase activity, cells were incubated with dexamethasone (1 nM) for 18 h. The aromatase assay was based on the release of [^{3}H] water after incubation with [1β-^{3}H]androstenedione as previously described [13].

2.6. Endogenous oestrogens

Oestrogens were extracted as described by Thijssen et al. [16] and the oestrone and oestradiol purified in Sephadex LH-20 columns before being measured by radioimmunoassay [13].

2.7. Oligonucleotide arrays

RNA was extracted from tumour biopsies, amplified and subjected to micro-array analysis on Affymetrix chips. Data were analysed and dendrograms derived. All samples were clustered using hierarchical clustering and Euclidean distances. In order to reduce noise from non-expressed genes, pair-wise differences between samples were derived based on only the present genes (according to Affymetrix' absolute call values). For each individual gene, relative expression was compared in pre-treatment and 10–14-day biopsies in an additional eight patients offered neoadjuvant therapy with letrozole (2.5 mg daily).

3. Results

3.1. Prediction of response to aromatase inhibitors

3.1.1. Steroid receptors

In early studies of neoadjuvant therapy, treatment was given to postmenopausal women irrespective of steroid

Table 1
Oestrogen receptor status and response to aromatase inhibitors

ER status	Response	Non-response
Rich	6	8
Poor	0	8

$P = 0.05$ (by Fisher's exact test).

Table 2
Progesterone receptor status and response to aromatase inhibitors

PgR status	Response (%)	Non-response
Positive	46 (88)	6
Negative	4 (57)	3

$P = 0.06$ (by Fisher's exact test).

receptor status. The relationship between oestrogen receptor status as measured by ligand-binding assay and response to either aminoglutethimide/hydrocortisone or 4-hydroxyandrostenedione is shown in Table 1. Thus, no tumour which was ER-poor (<20 fmol/mg cytosol protein) responded to treatment. Whilst the responders were all amongst the ER-rich cohort, the presence of receptor did not guarantee clinical benefits (the response rate amongst ER-rich tumours being 50%). Because of these results, management practice changed and aromatase inhibitors were reserved for patients with ER-rich tumours. However, further predictor parameters were required to subdivide ER-rich tumours into responding and non-responding cancers.

As the progesterone receptor is induced as a result of oestrogen signalling through a viable ER, PgR has been used as an additional predictive parameter. The relationship between PgR status (as measured immunohistochemically) with response to neoadjuvant treatment with letrozole, anastrozole or exemestane in postmenopausal patients with ER-rich tumours is summarised in Table 2. The incidence of PgR positivity (50 of 59) was high presumably on account of high levels of ER. Whilst the response rate was higher in PgR positive tumours (88%) compared with those which were PgR negative (57%), the clinical utility of PgR was limited. Thus, some PgR positive tumours failed to respond whereas other PgR negative cancers regressed on therapy (and responses were observed with any level of receptor). However, change in PgR expression could be used as evidence of the anti-oestrogenic mechanism of action of aromatase inhibitors. Thus, as is shown in Table 3, 46 of 50 tumours had a reduction in staining on treatment (in many cases PgR became undetectable after 3 months' therapy). This should be contrasted with the effects of tamoxifen in which the most consistent phenotypic change was an increase in PgR in a series of 50 tumours studied concurrently with the aromatase inhibitor cohort.

Table 3
The effects of 3 months' neoadjuvant treatment with either aromatase inhibitors (letrozole, anastrozole or exemestane) or tamoxifen on progesterone receptor expression

Treatment	Decrease	No change	Increase
Aromatase inhibitors	46	3	1
Tamoxifen	12	13	27

Table 4
c-erbB2 expression and response to neoadjuvant treatment with aromatase inhibitors (letrozole, anastrozole and exemestane)

c-erbB2 expression	Response	Non-response
Positive	8	1
Negative	50	11

P = NS (by Fisher's exact test).

3.1.2. c-erbB2 expression

Following the report of Ellis et al. [17] that the presence of c-erbB1/2 is associated with an increasing likelihood of response to neoadjuvant treatment with letrozole, a cohort of 70 tumours from patients treated neoadjuvantly with letrozole, anastrozole or exemestane was identified retrospectively and stained for c-erbB2. The results are shown in Table 4. The incidence of positivity was low (9 of 70) but eight of the nine staining tumours responded to treatment. This response rate (88.9%) was higher than that in the c-erbB2 negative group (82%) but the difference between the groups was not significant. c-erbB2 status therefore does not aid prediction of response to aromatase inhibitors.

3.1.3. Tumour aromatase

Many breast cancers possess aromatase activity which can be measured both in vitro and in situ. Thus, in a large study of 250 breast cancers incubated in vitro with 7-^{3}H androgen, radioactively labelled oestrogen could be identified in the medium in 181 (73%) tumours [18]. This potential has been confirmed as occurring in situ. Following administration of ^{3}H androstenedione to patients with breast cancer, labelled oestrogen was found in 46 of 60 (77%) cases [13]. Furthermore, as is shown in Fig. 1, tumours with the highest level of endogenous oestrogen invariably have evidence for in situ synthesis. It was therefore of interest to examine the relationship between in situ aromatase and response to aromatase inhibitors. These data have been published previously [13] and indicate that whilst the majority of responding tumours possess aromatase and most resistant tumours do not, the correlation is not absolute and not of practical utility. Nevertheless, as is shown in Fig. 2, there is a non-significant trend between degree of clinical response and level of in situ synthesis; tumours with very high aromatase activity were associated with significant shrinkage in volume on treatment with letrozole.

3.1.4. Oligonucleotide arrays

In order to discover novel candidate genes whose expression might be associated with response to aromatase inhibitors, we have undertaken studies using the strategy

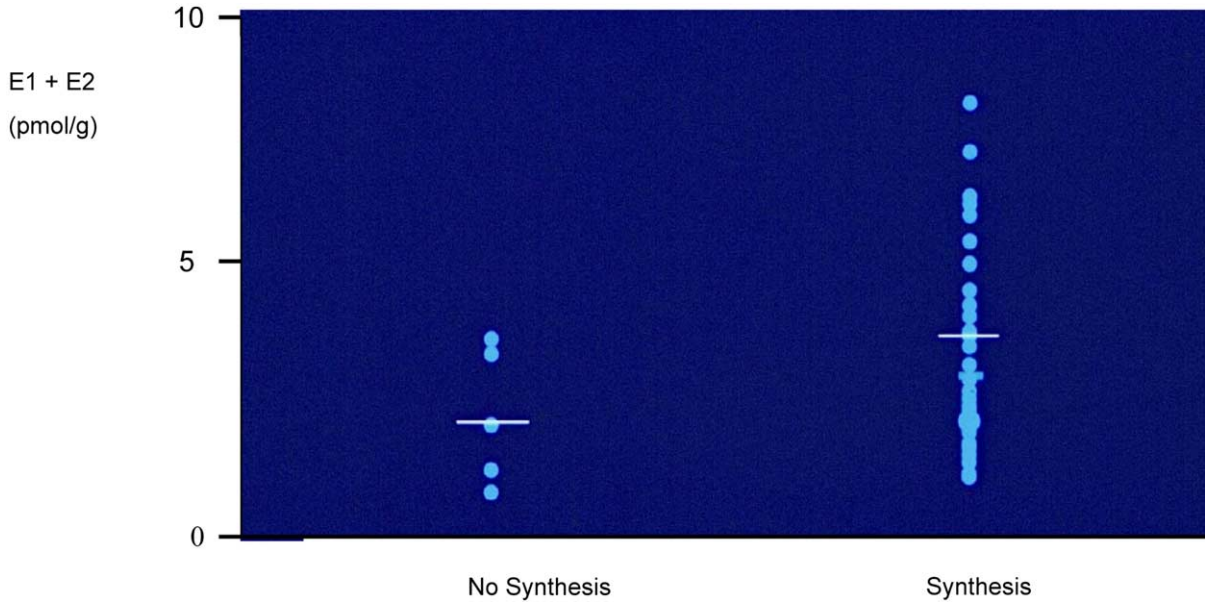

Fig. 1. Levels of oestrogens (oestrone plus oestradiol) in tumours with and without evidence of in situ synthesis (lines are median values).

outlined in Fig. 3. In this protocol tumour biopsies are sequentially taken before and after 10–14 days and 3 months of treatment. These are extracted and the mRNA converted to cDNA and amplified before being subjected to micro-analysis on Affymetrix chips. In a pilot study to determine the feasibility of the approach, 32 biopsies from 11 different patients were arrayed and their phenotype analysed to produce a dendritic tree. As is shown in Fig. 4, the analysis produced a pattern in which multiple biopsies from the same patient tended to cluster together. Perhaps most informative was patient ML from which six tumour cores were obtained. The analysis was able to link the duplicate biopsies taken at the same time and separate the pre-treatment, 10–14-day and 3 months time-points. This provides evidence of reproducibility of measurement whilst offering hope that the effects of treatment might be greater than those due to tumour heterogeneity. A further facet of these pilot studies has been to compare micro-array analysis of pairs of tumour cores taken before and after 10–14 days of letrozole treatment in eight patients. Part of the computer generated display for this comparison is shown in Fig. 5, left panel. It shows that genes may be identified which tend to be down-regulated, up-regulated or remain unchanged in the same tumour following treatment and that the pattern of change may be different between individual patients. Effects of treatment on two particular genes are shown in Fig. 5, middle and right panels. For gene A (U73328. Cluster Incl U73328:Human DLX7 (Dlx7) mRNA, complete cds/cds = (246, 749)/gb = *U*73328/gi = 1657866/ug = Hs.172648/len = 1393), substantial increase in expression was seen in tumours 2, 4, 5 and 7 whereas the remaining cancers displayed no change. In contrast, gene B (X62534. Cluster Incl X62534:H.sapiens HMG-2 mRNA/cds = (214, 843)/gb = *X*62534/gi = 32332/ug = Hs.80684/len = 1288), substantial decrease in expression was seen in most tumours but no change or an increase in tumours 3 and 8. Interestingly, tumours 3 and 8 rarely showed changes in expression of other genes.

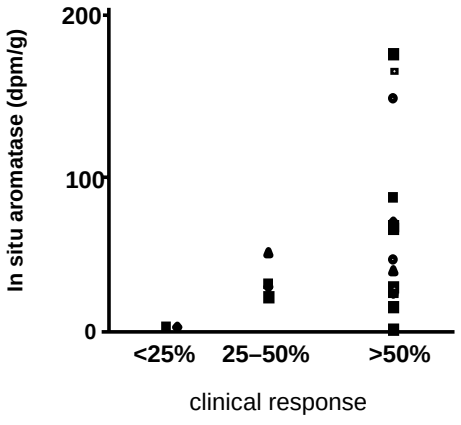

Fig. 2. Levels of in situ synthesis in breast cancers subdivided according to clinical response to neoadjuvant letrozole (clinical response expressed as tumour volume shrinkage).

3.2. Comparative effects of different aromatase inhibitors

Prototype aromatase inhibitors such as aminoglutethimide were used to treat breast cancer in the 1980s without the realisation that their major effects were on oestrogen biosynthesis [19]. Since that time, there have been programmes of rational drug design which have developed second generation inhibitors such as formestane and fadrozole and then third generation agents such as anastrozole, letrozole and

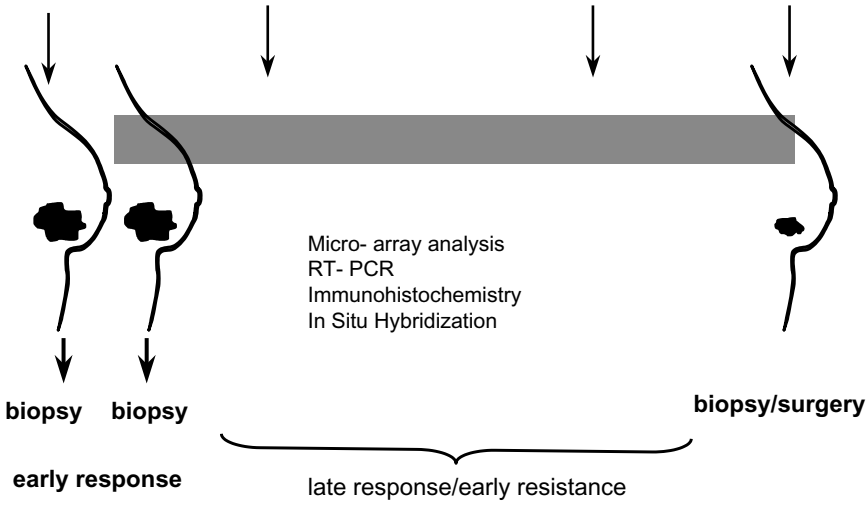

Fig. 3. Diagrammatic representation of neoadjuvant treatment protocol.

exemestane [3,4,8]. With each succeeding generation, the specificity and potency of the inhibitors toward the aromatase enzyme has increased. The drugs may be divided into two classes: Type I agents which compete for the substrate-binding site of the enzyme and are steroidal in structure; because some bind irreversibly and co-valently to the active site, they have been termed 'inactivators'. In contrast, Type II agents interact reversibly with the haem moeity of the cytochrome prosthetic group [20].

Differences in potency of aromatase inhibitors may be readily shown in vitro using model systems such as placental microsomes and homogenates of breast cancers. However, whole cell systems such as cultured fibroblasts from breast adipose tissue are more physiological and provide the opportunity to monitor drug uptake and cellular pharmokinetics. The inhibitory effects of aminoglutethimide, letrozole, anastrozole, exemestane and formestane are summarised in Table 5 and Fig. 6. All the agents are able to inhibit

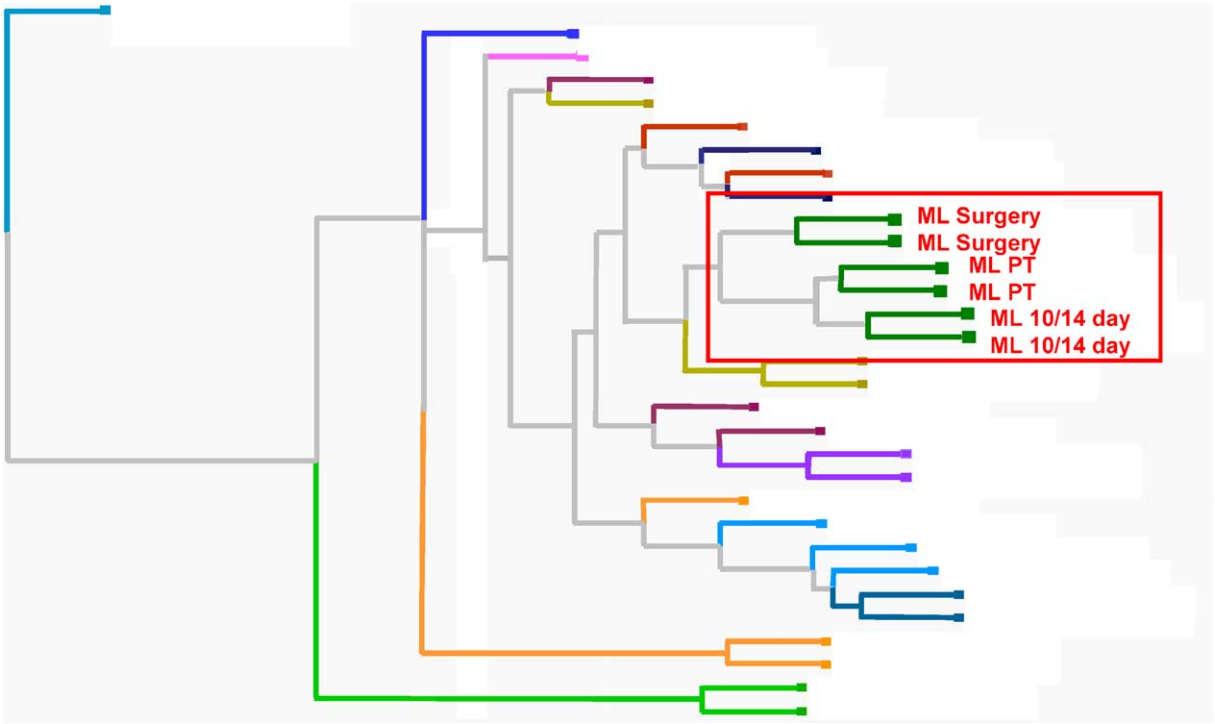

Fig. 4. Dendrogram illustrating clusters for micro-array analyses of breast biopsies, colour coded according to individual patient. Box identifies an undivided patient (ML) with duplicate biopsies taken before treatment (PT), after 14 days of treatment with letrozole (10–14 days) and after 3 months' treatment with letrozole (surgery). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

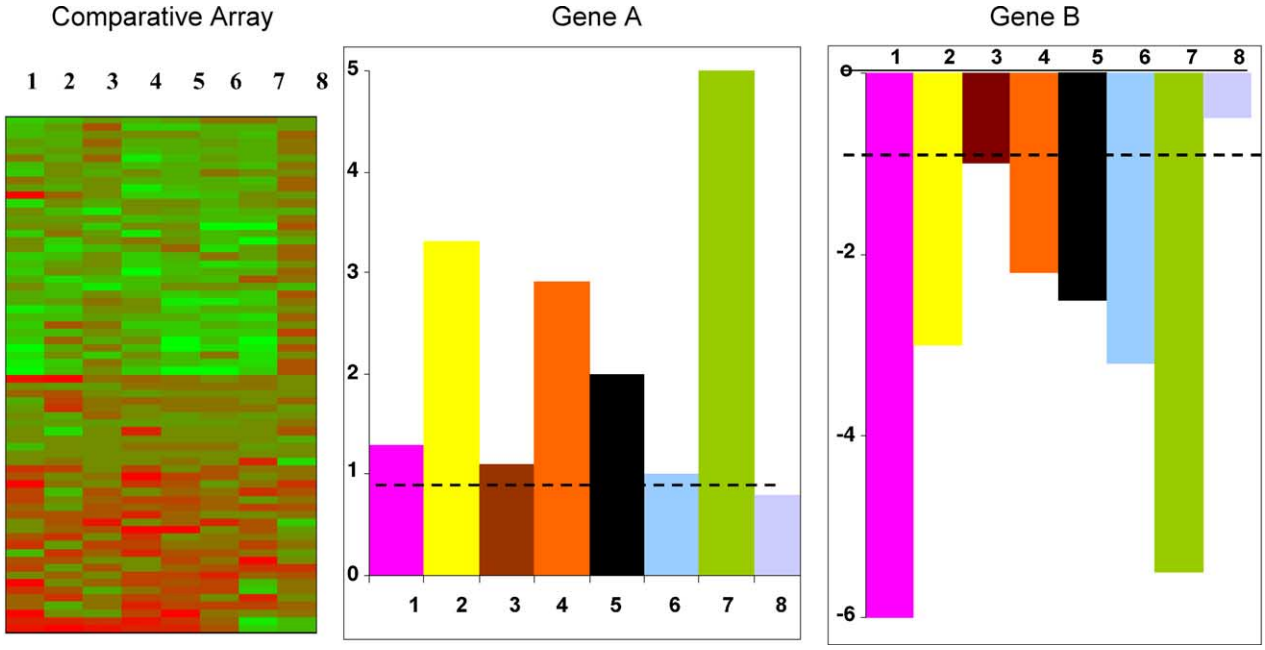

Fig. 5. Micro-array analysis of biopsies taken before and after 10–14-day neoadjuvant treatment with letrozole in eight different patients. Left panel—diagrammatic representation of changes in selected cohorts of genes (green represents reduction in expression and red increase in expression, degree of change is indicated by intensity of colour). Middle panel—change in expression of U73328. Cluster Incl U73328:Human DLX7 (Dlx7) mRNA, complete cds/cds = (246, 749)/gb = *U*73328/gi = 1657866/ug = Hs.172648/len = 1393. Right panel—change in expression of X62534. Cluster Incl X62534:H.sapiens HMG-2 mRNA/cds = (214, 843)/gb = *X*62534/gi = 32332/ug = Hs.80684/len = 1288 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

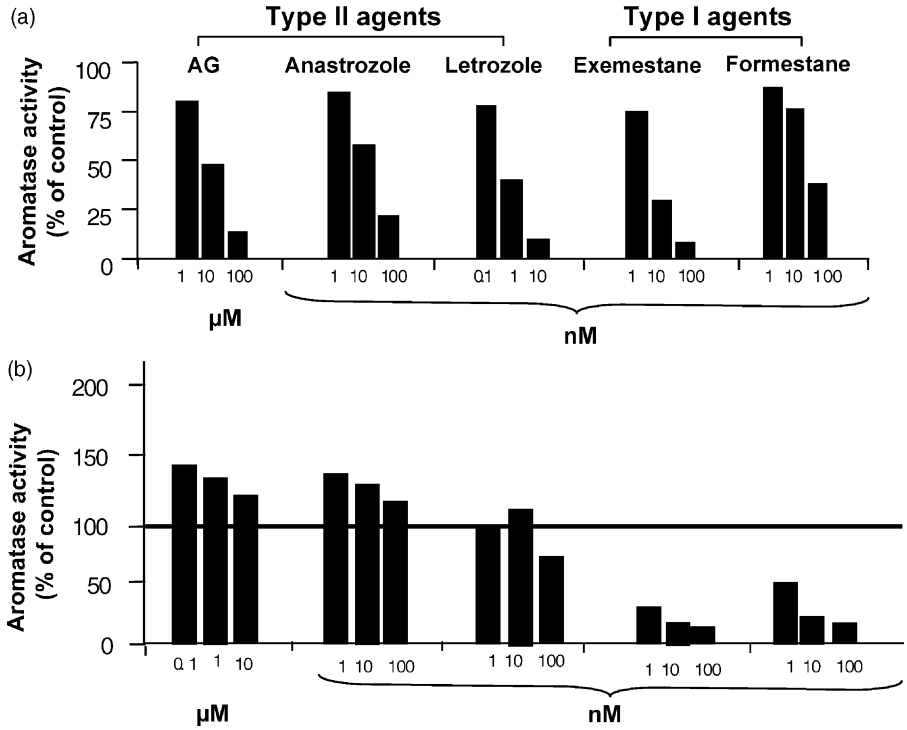

Fig. 6. The effect of inhibitors on aromatase measured in cultures of breast adipose tissue fibroblasts. Upper panel—aromatase activity induced by dexamethasone in the absence of inhibitors which are added during the assay phase. Lower panel—aromatase activity induced by dexamethasone in the presence of inhibitors which are removed during the assay for aromatase.

Table 5
Aromatase in cultured fibroblasts: sensitivity to inhibitors

	IC_{50} (nM)	Relative potency
Aminoglutethimide	8000	1
Formestane	45.0	180
Exemestane	5.0	1600
Anastrozole	14.0	570
Letrozole	0.8	10000

aromatase in a dose-related manner but the newer drugs are magnitudes of order more potent than aminoglutethimide. Amongst the Type II inhibitors letrozole is the most potent whereas exemestane is more potent than the other Type I agent formestane.

Cultures of fibroblasts can also be used to demonstrate differences in interaction between inhibitors and the aromatase enzyme, i.e. their (ir)reversibility of action. Thus fibroblasts may be preincubated with inhibitors but assayed in their absence as is shown in Fig. 6, lower panel. Under these conditions, Type I steroidal inhibitors continued to exert marked inhibitory properties (in keeping with their irreversible mechanism of action). In contrast, the Type II reversible agents, aminoglutethimide, letrozole and anastrozole, showed substantially less inhibition as compared with protocols when they are present during assay. Indeed, at least at one concentration, the drugs produced enhanced aromatase activity. This probably results from increased transcription of the aromatase gene and/or stabilisation of the aromatase protein [21,22].

These inductive effects are not an artefact of the culture system and can be seen when tumours from patients treated with reversible Type II inhibitors are assayed for aromatase ex vivo [4].

Ex vivo studies also show that aromatase in a subset of tumours appears resistant to the Type I inhibitor formestane (4-hydroxyandrostenedione) [4,23]. Interestingly, resistance can also be demonstrated in vitro. Thus Fig. 7 shows the results from in vitro studies in which tumour homogenates were incubated with increasing concentrations of 4-hydroxyandrostenedione and a Type II inhibitor (aminoglutethimide or CGP 16949). It can be seen that whilst certain tumours show classical sensitivity to both types of inhibitors, others displayed resistance to 4-hydroxyandrostenedione whilst remaining sensitive to the Type II inhibitor. Similar differential resistance/sensitivity has been shown in site mutagenesis studies of the aromatase enzyme [24] but the particular causative mutations have not been shown to be present in clinical specimens [25].

4. Discussion and conclusions

Neoadjuvant therapy has proved to be an invaluable setting in which to explore the endocrinology of breast cancer, the efficacy of aromatase inhibitors and the evaluation

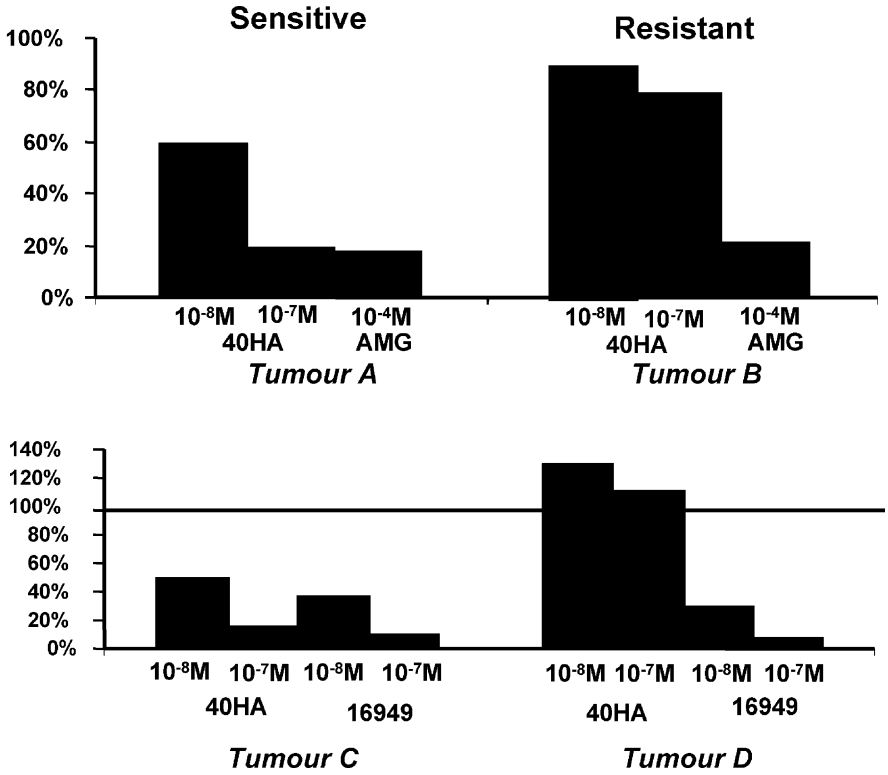

Fig. 7. In vitro tumour aromatase assayed in the absence and presence of aromatase inhibitors. Tumours A and C sensitive to both 4-hydroxyandrostenedione and Type II inhibitors. Tumours B and D resistant to 4-hydroxyandrostenedione but sensitive to Type II inhibitors.

of predictive indices of response. In terms of the latter, the oestrogen receptor is the single most influential parameter but it is clear that additional factors are required to distinguish between ER positive tumours which are truly responsive to aromatase inhibitors and those that are not. In this respect, markers may not be identical for other forms of endocrine therapy. Thus, the over-expression of c-erbB receptor proteins may indicate a poorer chance of response to anti-oestrogens such as tamoxifen but this has not been shown to be the case for aromatase inhibitors [17]. The use of micro-array technology has great potential in identifying novel genes which predict for response or are early markers of response but this has yet to make a major impact.

With regard to aromatase inhibitors themselves, the novel third generation drugs are extremely specific and potent agents. Clinical results also suggest that their anti-tumour potential is at least as great as tamoxifen [26–28]. It remains to determine whether amongst the group, an individual drug may transpire to be particularly effective. However, research has shown that the inhibitors should not be regarded as identical, there being important differences in terms of structure, potency and mechanism of action. Whilst it is clear that in the short- to medium-term Type II drugs are extremely effective, they do have the ability to induce aromatase [21] and it may be that in chronic situations, breakthrough synthesis of oestrogen could occur. Conversely, it appears that aromatase in a minority of breast cancers may show differential sensitivity to classes of inhibitors, particularly resistance to formestane [6,23]. The scenario can be painted that certain tumours should be managed with particular inhibitors. The last 20 years has seen the evolution of aromatase inhibitors as key endocrine agents in the treatment of breast cancer; the immediate future offers the promise of rational management based on accurate identification of tumours responsive to specific drugs.

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