The Breast (1996) **5**, 186–191

ESO TASK FORCE ARTICLE

Models of new antioestrogen action in vivo: primary tumours

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SUMMARY. We have conducted a clinical trial of the novel pure antioestrogen ICI 182780 to assess its short-term biological effects in women with primary breast cancer. The results were compared against those obtained from a similar study of tamoxifen. In both studies, the drugs were administered for a short period of time in the interval between first clinic attendance and operation. Samples of tumour tissue were obtained both before and after treatment with the antioestrogens.

Seven days treatment with ICI 182780 caused a significant decrease in tumour proliferation as indicated by the Ki67 labelling index (Ki67LI). Tamoxifen caused a similar reduction in the Ki67LI after a median of 21 days treatment. In oestrogen receptor (ER) positive breast tumours, ICI 182780 caused a profound decrease in the level of receptor protein that could be detected immunocytochemically whereas tamoxifen was without effect. This reduction in ER-protein content was not reflected in a similar decrease in the mRNA for the receptor. ICI 182780 significantly reduced the expression of two oestrogen-regulated genes (the progesterone receptor and pS2) whereas tamoxifen was without effect. Finally, although ICI 182780 reduced ER expression to almost undetectable levels in some tumours, no other changes suggestive of an endocrine insensitive phenotype were apparent.

In conclusion, ICI 182780 produces demonstrable antioestrogenic effects on human primary breast tumours in vivo and is without any oestrogen agonist effects. The novel mechanism of action of the new pure antioestrogen, as determined in vitro, is reflected in its effects on human primary breast tumours in vivo.

INTRODUCTION

In 1991, the first phase I study of the new specific antioestrogen ICI 182780 in primary breast cancer patients was initiated. This trial followed several years developing new agents that would be more efficacious than conventional antioestrogens and with fewer side-effects.

Conventional triphenylethylene-based antioestrogens, typified by tamoxifen, compete with oestradiol (E_2) for binding to the ER but they also form a complex with the receptor that retains some transcriptional activity.[1] Consequently, tamoxifen exhibits a full range of biological activity from full oestrogen antagonism to full agonism depending upon the species, the target tissue and the target gene response being studied. Although some of the agonist effects of tamoxifen such as reduction in serum cholesterol levels and maintenance of bone mineral density are beneficial, others may be detrimental to patients receiving long-term therapy. Tamoxifen stimulates endometrial growth in animals and adjuvant use in women with breast cancer is associated with an increased risk of endometrial cancer in some, but not all, studies.[2–4] Moreover, evidence from animal studies and from observations of withdrawal responses suggests that the agonist properties of tamoxifen may stimulate tumour cell growth and is the cause of some treatment failures.[5,6] It is possible that the efficacy of tamoxifen and the other conventional non-steroidal antioestrogens may be compromised compared to that which might be achieved by complete oestrogen antagonism.

The new class of steroidal antioestrogens, exemplified by ICI 164384 and ICI 182780, differ significantly from conventional agents in both their chemical structure and their molecular pharmacology. Both ICI 164384 and ICI 182780 are derived from E_2 substituted at the 7α position with an alkylamide or an alkylsulfinyl moiety respectively.[7] Both ICI 164384 and ICI 182780 bind to the ER with high affinity and are complete antagonists in the rat uterus oestrogen bioassay. Co-administration of either of the new compounds completely inhibits the uterotrophic effects of tamoxifen in a dose-dependent manner.[7] However, the compound designated ICI 182780 has been chosen for further

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development because of its greater potency and bioavailability.[8] ICI 182780 shows a lack of agonist activity in several animal model systems including carcinogen-induced rat mammary tumours and human breast cancer cells grown as xenografts in athymic nude mice. Further studies have indicated that both ICI 182780 and ICI 164384 are more potent and effective than tamoxifen in promoting remissions in several models of human breast cancer. Importantly, the specific antioestrogens have growth inhibitory effects on tumour cells that are resistant to tamoxifen or whose growth is stimulated by the non-steroidal antioestrogen.[8,9]

The lack of agonist activity of the new specific antioestrogens may be related to their mechanism of action which is completely different to that of tamoxifen. The major effect of ICI 164384 and ICI 182780 is to reduce substantially the cellular content of the ER by decreasing the half-life of the protein.[10,11] This reduction in half-life has been attributed to inhibition of the energy-dependent nucleocytoplasmic shuttle of the ER between the cytoplasm and the nucleus which blocks re-entry of the receptor into the nucleus and promotes its degradation.[11] However, dimerization of the ER and consequent binding to the ER element (ERE) may also be destabilized which further enhances receptor degradation.[12]

As administration of ICI 182780 in animal studies was associated with little or no toxicity and as the compound appeared to be highly active in several experimental systems, a phase I study in primary breast cancer patients was carried out in three centres (Manchester, Nottingham and London). The aims of the study were three-fold: 1. to determine the pharmacokinetics and short-term tolerability of ICI 182780; 2. to discover whether there was evidence of biological activity in human primary breast tumours in vivo and; 3. to compare the effects of the new compound with those of tamoxifen administered in a similar short-term protocol. The clinical aspects of this study are reported elsewhere.[13] The aim of this report is to summarize the biological actions of ICI 182780 on human primary breast tumours with particular reference as to whether ICI 182780 exerts any agonist effects and whether the drug's novel mechanism of action is reflected in its effects in vivo.

THE EFFECTS OF ICI 182780 ON PRIMARY BREAST TUMOURS

Fifty-six postmenopausal women with primary breast cancer satisfied the entry criteria [see 13 for details] and participated in the study. After giving informed consent, the patients were randomized to either a control group (n = 19) who received no pre-operative treatment, a low dose treatment group (n = 21) who received 6 mg ICI 182780 per day and a high dose treatment group (n = 16) who were treated with 18 mg ICI 182780 per day. Both the low and the high dose treatment groups were given daily i.m. injections of ICI 182780 for 7 days prior to surgery. Wherever possible, pre-treatment tumour samples were obtained by multiple needle core biopsies. Post-treatment samples were obtained from the operative specimen at the time of surgery. The tumour samples were divided and portions were snap frozen in liquid nitrogen for steroid receptor measurement, assay of the proliferation-associated antigen Ki67 and mRNA extraction. The other portions were fixed in formalin and embedded in paraffin wax for subsequent histological examination and assay of oestrogen-regulated genes such as pS2. All the immunocytochemical assays were carried out as previously detailed[13] and were scored blinded as to whether the samples came from treated or control patients.

The tamoxifen study against which the effects of ICI 182780 were compared, was carried out on 103 patients who presented at the University Hospital of South Manchester with primary breast cancer.[14] After giving informed consent, the patients were randomized to receive either placebo (n = 44) or tamoxifen (n = 59) at a loading dose of 4 × 40 mg for 1 day and then 20 mg per day thereafter in the interval between clinic attendance and surgery (median time = 21 days; range = 6–65 days). Pre- and post-treatment tumour samples were obtained and processed as described above for the ICI 182780 study. The tamoxifen protocol differed from that used for the study of ICI 182780 in that the patients received the drug for a variable period of time. In addition, tumour ER and PR content in the tamoxifen study was calculated as the percentage of tumour cells stained positively for the receptor whereas, in the ICI 182780 study, receptor measurements were presented as an index which combined the percentage of positively stained cells with the intensity of staining. The proportion of receptor positive tumours identified by each of these methods was not significantly different. Moreover, the changes in the percentage of receptor positive cells after antioestrogen treatment reflected those of the receptor indices.

We looked first at the effects of the new antioestrogen on tumour cell proliferation as indicated by immunocytochemical detection of the Ki67 proliferation associated antigen. Paired samples were available for estimation of the Ki67 labelling index (% tumour cells positively stained with the Ki67 antibody) in 44 of the 56 subjects in the phase I study of ICI 182780. There were no significant differences in the pre-treatment Ki67 is between ICI 182780 treated and control tumours.[13] The pure antioestrogen had no effect on proliferative activity of ER-negative tumours but, as shown in Figure 1, it reduced significantly the Ki67LI of ER-positive tumours. There was evidence that this effect was dose dependent as the decrease in Ki67LI was significant only in those treated with the higher (18 mg) dose of ICI 182780. A similar reduction in Ki67LI was seen after

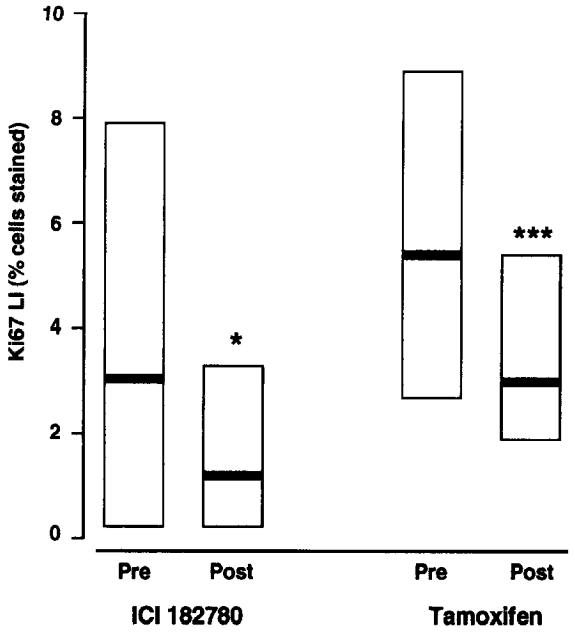

Fig. 1 Pre- and post-treatment Ki67LIs (% tumour cells showing positive immunostaining) in patients with ER-positive tumours treated with ICI 182780 (both doses have been combined) or all patients treated with tamoxifen. Columns = interquartile ranges; bars= medians; * = $P < 0.05$ and *** = $P < 0.001$ by Wilcoxon's matched-pair signed rank test.

tamoxifen treatment (see Fig. 1) except anti-proliferative effects were seen in both ER-positive and ER-negative tumours (as determined in the pre-treatment sample). This is the first demonstration of a reduction in proliferative activity of human breast tumours treated with ICI 182780 in vivo. The absence of a similar effect on ER-negative tumours treated with ICI 182780 or on untreated controls suggests that this reduction was drug related and ER dependent. In addition, the reduction in Ki67LI appeared to be dose dependent because a significant decrease could only be demonstrated in the patients treated with the higher (18 mg) dose. This may have been due, in part, to the small number of patients in the 6 mg dose group who had ER-positive tumours as previous studies of ICI 182780 have shown clear dose-dependent anti-proliferative effects on human breast cancer cell lines in vitro. The effects of ICI 182780 on proliferation were achieved with just 7 days' treatment and at serum levels of 5–25 ng/ml[13] which are much lower than the steady state levels reported for tamoxifen and its metabolites (250–450 ng/ml).[15] Thus, it appears that in vivo, as in vitro, the new pure antioestrogen is more potently anti-proliferative than tamoxifen.

Paired pre- and post-study steroid receptor measurements were carried out on 45 (80%) of the 56 participants in the ICI 182780 trial. In this study, steroid receptor expression was assessed semiquantitatively by determining the percentage of positively stained tumour cells and assessing the intensity of staining to produce an ER or PR index. In the control tumours, we noted that there was a significant tendency towards under-estimation of the ER level in the pre-treatment biopsy specimens compared to that seen in the operative sample. This difference is probably due to the difficulties in preserving the receptor content of small Tru-cut biopsy samples. Nevertheless, ICI 182780 treatment caused a profound decrease in the ER index of the tumours in a dose dependent manner (Fig. 2A & B). This decrease in receptor content was such that some, initially ER-positive, tumours appeared ER-negative after treatment with the pure antioestrogen. Although it had pronounced effects on ER-protein expression, ICI 182780 did not alter the amount of ER mRNA that could be extracted from tumours after treatment as measured by Northern analysis (Fig. 2C).[16] Tamoxifen treatment did not affect either receptor protein or mRNA expression in ER-positive breast tumours (Fig. 2). The lack of effect of ICI 182780 on ER mRNA expression is consistent with its proposed mechanism of action where destabilization of the ER dimer, enhanced ER degradation and a reduced half-life of the ER obliterate ER protein content without changing ER mRNA transcription.[10–12] Thus, it appears that at least some of the changes described after ICI 182780 treatment in vitro also occur in human primary breast tumours in vivo.

Comparison of the changes in oestrogen-regulated gene expression caused by ICI 182780 with those induced by tamoxifen suggests that the new drug exerts a greater anti-oestrogenic effect in vivo. In the patients treated with ICI 182780, there was a significant reduction in the median PR index from 0.5 before treatment to 0.01 afterwards ($P < 0.05$; Fig. 3A). There was no evidence of a dose-dependent effect as separate analysis of the two doses of ICI 182780 did not reach statistical significance. In contrast, tamoxifen increased the PR content of some ER-positive tumours although, for the group as a whole, this increase was not significant (Fig. 3A). The expression of another oestrogen regulated gene, pS2, was also reduced by ICI 182780 at both the protein and the mRNA levels (Fig. 3B & C). As in the case of PR, tamoxifen treatment increased pS2 expression in some primary breast tumours although overall this alteration was not significant. The effects of ICI 182780 on a third oestrogen-regulated gene pLIV-1 were rather less pronounced than its effects on PR or pS2 although there was a trend for pLIV-1 mRNA expression to be reduced after treatment with the pure antioestrogen (data not shown).

As both the PR and pS2 genes are regulated by oestrogen,[17,18] the levels of their respective protein products would be expected to fall in the presence of oestrogen antagonism. This is, indeed, the case in the present study of ICI 182780 where the decrease in the levels of PR and pS2 expression is consistent with the formation of a

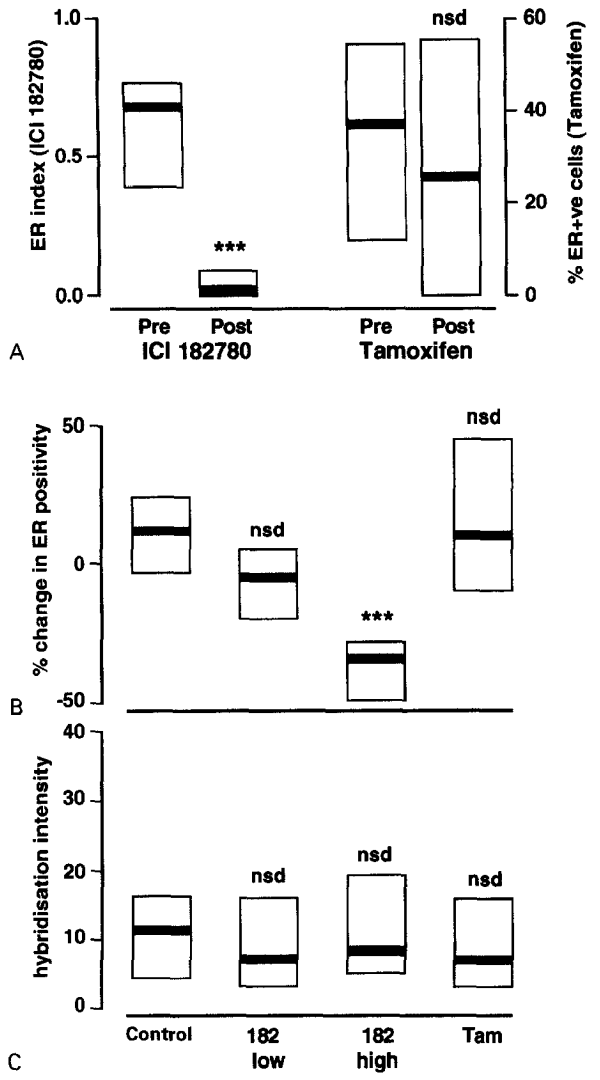

Fig. 2 (A) Pre- and post-treatment ER measurements in patients with ER-positive tumours treated with ICI 182780 or tamoxifen; *** = $P < 0.001$ and nsd= no significant difference by Wilcoxon's matched-pair signed-rank test; (B) percentage change in ER protein content following no treatment – control, low and high dose ICI 182780 (182 low and 182 high respectively) or tamoxifen (Tam) treatment; *** = $P < 0.001$ and nsd = no significant difference by Mann–Whitney U test; (C) levels of ER mRNA measured after treatment with ICI 182780 or tamoxifen; nsd = no significant difference by Mann–Whitney U test. Columns = interquartile ranges; bars = medians.

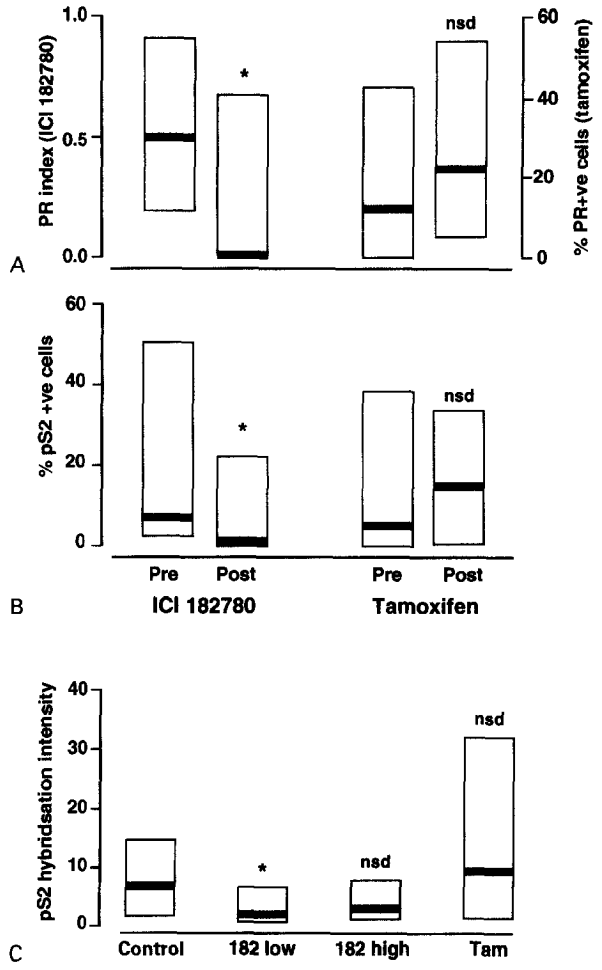

Fig. 3 (A) Pre- and post-treatment PR measurements in patients with ER-positive tumours treated with ICI 182780 or tamoxifen; * = $P < 0.05$ and nsd = no significant difference by Wilcoxon's matched-pair signed-rank test; (B) Pre- and post-treatment pS2 measurements in patients treated with ICI 182780 or tamoxifen; * = $P < 0.05$ and nsd = no significant difference by Wilcoxon's test; (C) levels of pS2 mRNA measured following no treatment – control, low and high dose ICI 182780 (182 low and 182 high respectively) or tamoxifen (Tam) treatment; * = $P < 0.05$ and nsd = no significant difference by Mann–Whitney U test. Columns = interquartile ranges; bars = medians.

transcriptionally inactive ICI 182780:ER complex and subsequent down-regulation of the ER protein.[10–12] We did find, however, that pS2 was expressed in some ER-negative tumours and that its expression was decreased in these tumours as well as in those that were ER-positive. This lack of association with ER positivity may be due to the fact that analysis of pS2 expression was carried out on a separate piece of tumour tissue from that used for estimation of steroid receptor expression and proliferation. The control of pLIV-1 gene expression is rather more complex than that of PR or pS2 and the rather small effects of ICI 182780 on expression of its mRNA are not surprising. Tamoxifen's effects on PR and pS2 expression are entirely consistent with its oestrogen agonist properties. This agonism appears to be due to the failure of tamoxifen to block all of the transcription activating functions of the ER. However, there is also evidence to suggest that the agonist activity of tamoxifen could be enhanced further by the interaction of other signal transduction pathways with the tamoxifen:ER complex.[19] As the pure antioestrogens obliterate cellular ER protein content, it has been suggested that opportunities for this type of interaction should not occur resulting in a more complete oestrogen antagonism.[20] Taken together, the differential actions of ICI 182780 and tamoxifen on

oestrogen-regulated gene expression in human primary breast tumours in vivo, suggest that the new drug is more effective and does not have the agonist effects associated with conventional antioestrogens.

The final issue that we wished to address in this phase I study of ICI 182780 was whether the decrease in tumour ER content was accompanied by other changes suggestive of the development of an endocrine insensitive phenotype. This question was raised by the finding that there is a strict inverse relationship between ER and expression of the epidermal growth factor receptor (EGFR) in clinical breast tumour samples.[21] Those tumours containing EGFR or its ligand transforming growth factor α (TGFα) are frequently unresponsive to endocrine therapy.[21,22] Furthermore, suppression of ER expression by other agents such as sodium butyrate or phorbol ester results in a concomitant rise in tumour cell EGFR content.[23,24] Accordingly, the expression of EGFR and TGFα was determined immunocytochemically on a subset of tumour samples taken before and after treatment with ICI 182780.[25] Figure 4 shows that neither dose of the pure antioestrogen changed the EGFR index even though ER expression was reduced in all cases. Likewise, no effects of ICI 182780 on expression of TGFα could be detected. Although the number of cases examined in each treatment group is small, it is reassuring to note the lack of effect of ICI 182780 on either EGFR or TGFα expression. These results suggest that, at least in the short term, treatment with the pure antioestrogen does not induce changes characteristic of resistance to endocrine therapy.

From the results of this first phase I study of ICI 182780 in human primary breast tumours in vivo it is evident that this new drug is more potently antioestrogenic than tamoxifen without any evidence of oestrogen agonism. Furthermore, the novel mechanism of ICI 182780 appears to be reflected in its effects on human breast tumour tissue in vivo.

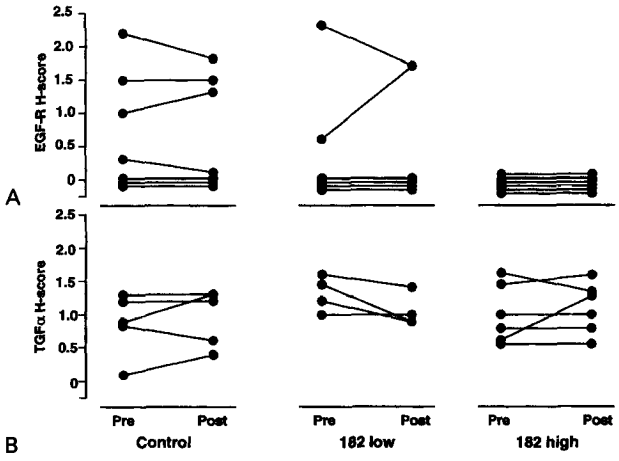

Fig. 4 (A) Pre and post-treatment measurements of EGFR content in control ER-positive tumours or those receiving the low (182 low) and high (182 high) doses of ICI 182780; (B) Pre- and post-treatment measurements of TGFα content in control ER-positive tumours or those receiving the low and high doses of ICI 182780. There were no significant differences between the pre- and post-treatment values for any of the treatment groups (Wilcoxon's matched-pair signed-rank test).

Acknowledgements

The above study was a collaboration between the Christie Hospital NHS Trust, the City Hospital, Nottingham and the Royal Marsden Hospital, London. In addition to the authors, the following people participated: David DeFriend, Ian Laidlaw, Robert Clarke, Richard MacClelland, Julia Gee, David Manning, Christobel Saunders, Robert Mansel, Michael Baum, Roger Blamey, John Robertson, Peter Walton and Alan Wakeling.

The European School of Oncology gratefully acknowledges an educational grant from Zeneca Pharmaceuticals which made the meeting possible.

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