Responses to Pure Antiestrogens (ICI 164384, ICI 182780) in Estrogen-Sensitive and -Resistant Experimental and Clinical Breast Cancer[a]

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INTRODUCTION

The last ten years has seen the emergence of a new class of pharmacological agents termed pure antiestrogens (reviewed in Refs. 1, 2). These compounds, which were originally discovered by ICI Pharmaceuticals Division (now Zeneca Pharmaceuticals) in the UK, have the unique property of binding to the estrogen receptor (ER),[3] producing a receptor complex which lacks estrogenic activity.[4,5] They are of use in two important areas of breast cancer research. Firstly, as clinical agents, where it is hoped that their ability to induce total estrogen deprivation will improve the effectiveness of endocrine therapy. Secondly, as pharmacological probes to investigate the cellular and molecular actions of estrogens and tamoxifen. Inherent in each of these areas of research are questions associated with the impact pure antiestrogens may have on the therapy of endocrine-resistant states and whether resistance develops as a consequence of incomplete estrogen withdrawal; with tumor cells more efficiently utilizing either a reduced estrogenic pool or the agonistic activity of an antiestrogen,

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or whether the resistant cells have completely circumvented the need for ER-mediated growth and hence sensitivity to pure antiestrogens.[2]

Since pure antiestrogens are now entering clinical development, the current paper seeks to outline some of their basic cellular and antitumor properties on estrogen-responsive (MCF-7) human breast cancer cells *in vitro,* primarily using the lead compound ICI 164384. This information will be briefly compared with the properties exhibited by pure antiestrogens in endocrine-resistant variants of human breast cancer cells (see refs. in Ref. 2) and phase I and II trials of ICI 182780 in primary[5,6] and advanced[7] breast cancer patients. Where possible examples will be given from immunohistochemical studies, since this technique is most readily applicable to clinical material and ultimately should facilitate an assessment of the degree to which pure antiestrogens are fulfilling their potential as complete antagonists of estrogen action in clinical breast cancer and thereby aid in defining the importance of estrogens in the regulation of breast cancer growth and development.

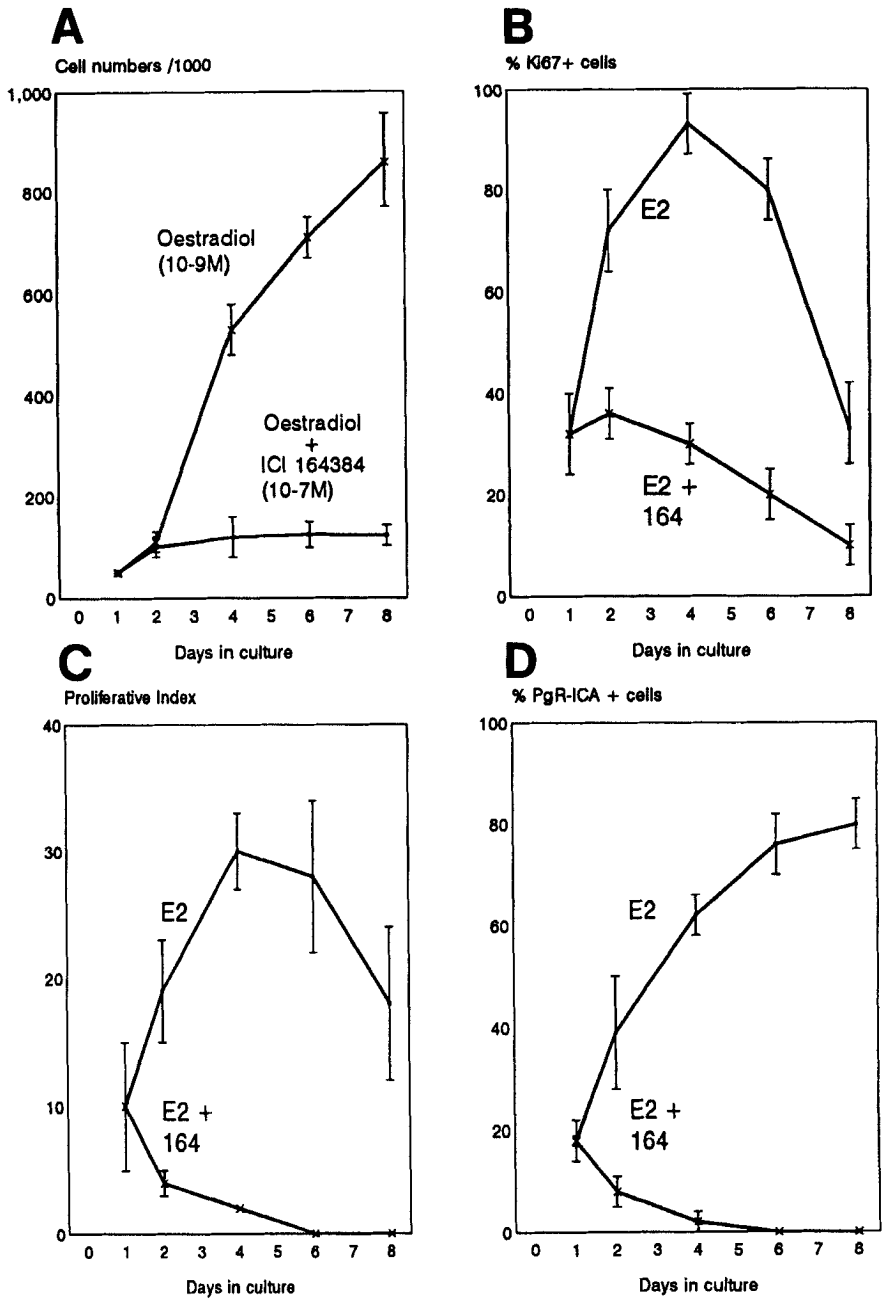
Experimental Studies with Pure Antiestrogens

Biological Consequences of Exposure of Breast Cancer Cells In Vitro *to Pure Antiestrogens*

Evidence from breast cancer cells grown in culture suggests that pure antiestrogens may be highly efficient in counteracting the stimulatory effects exhibited by estrogens both on cell proliferation and on steroid hormone-regulated gene expression.

One of the most important early observations arising from the functional disablement of ER signalling by pure antiestrogens in estrogen-sensitive human breast cancer cell lines was that, in contrast to the stimulatory activity of estradiol, treated cells became efficiently growth arrested (FIG. 1A).[8–10] This action is reflected in the growth dynamics of the tumor cells, with several groups showing that while estradiol increases the tumor cell growth fraction and acts to stimulate the passage of cells through the cell cycle, pure antiestrogens promote a highly effective restriction of the cell cycle approximately 5 hours into G1 and hence cause a reduction of the proportion of cells undergoing DNA synthesis.[8,9] On continuous exposure to pure antiestrogens there is almost a complete loss of those nuclear antigens which mark cell proliferation (FIG. 1B,C)[10] as a large proportion of the cells pass into a noncycling population.[9] Such cells show a reduced RNA/DNA ratio, characteristic of Go (Nicholson, Francis and Hoy, unpublished results). It is noteworthy that the growth-inhibitory activity of pure antiestrogens is not solely restricted to cytostatic activity; on continuous exposure there also appears to be a limited cytotoxic component.[5]

The growth-inhibitory activity of pure antiestrogens on human breast cancer cell lines is characteristically preceded by changes in the expression of several estrogen-regulated genes,[5,10–14] with, for example, the high levels of nuclear immunodetectable PR induced in estradiol-treated MCF-7 cells being rapidly reversed by a 100-fold molar excess of ICI 164384 (FIG. 1D).[5] Indeed, examination of the percentage of PR-positive cells throughout estradiol and ICI 164384 treatment shows that not only does the pure antiestrogen block estradiol-induced PR levels, but that it also obliterates all PR signalling after 4 days of culture. Such cells are as a consequence no longer responsive to progesterone.



Similarly, the substantial increase in cytoplasmic pS2 immunostaining (a protein of unknown function in the breast) that is induced by estradiol in human breast cancer cells is largely absent following ICI 164384 treatment.[5] Any residual pS2 staining tends to be present towards the outer cell membrane in small secretory vesicles. Once secreted, however, the cells remain negative, with no evidence of further pS2 production within the endoplasmic reticulum.

Predictably, the decrease in estrogen-regulated proteins often parallels a highly significant fall in their mRNA levels,[5,11,13,14] with, for example, pS2 mRNA levels being undetectable following 5 days of ICI 164384 treatment.[5] Indeed, even after reverse transcription PCR (30 cycles), the pS2 mRNA has been shown to be barely detectable in ICI 164384- and ICI 182780-treated cells,[5] indicating that pure antiestrogens can produce a rapid and complete shutdown of estrogen-regulated gene function. These actions contrast with the effect of both ICI 164384 and ICI 182780 on the estrogen-suppressed gene sequence pMGT-1, the expression of which is very significantly upregulated in their presence.[15]

A number of the changes in gene expression may directly contribute to the mechanism of action of the drugs, with ICI 182780 promoting decreases in immunodetectable TGFα, an estrogen-inducable mitogenic growth factor,[16] and the bcl-2 protein, a factor which has been implicated in the protection of cells against programmed cell death.[17] In each instance, while these proteins are readily detectable in a high proportion of cells treated by estradiol, their levels are lowered by estrogen withdrawal and further reduced by the pure antiestrogen.[5] This is especially evident for the bcl-2 protein, with estradiol-related immunostaining being largely abolished by ICI 182780. Indeed, bcl-2 positivity is a relatively rare event following the administration of pure antiestrogens and, in line with its role in cell survival, its absence is often associated with the presence of pyknotic tumor cell nuclei.[5]

Comparison with Antiestrogens Exhibiting Partial Estrogen-like Activity In Vitro

The inhibitory actions of pure antiestrogens, initially on estrogen-induced transcriptional events and subsequently on cell proliferation and survival, consistently exceed those effects which may be achieved by established antiestrogens with partial estrogen-like activity.

A comparison of the potency and efficacy of tamoxifen, ICI 164384 and ICI 182780 as inhibitors of the growth of MCF-7 cells showed that the pure antiestrogens are up to two orders of magnitude more potent,[12,21,22] reflecting, in part, their higher

FIGURE 1. Growth and immunohistochemical characterization of MCF-7 cells. The cells were grown in multiwell dishes in white RPMI tissue culture medium with 5% DCC stripped FCS (medium A) containing estradiol ± ICI 164384. **(A)** Cell numbers were assessed using a Coulter Counter; **(B,C)** Ki67 and **(D)** PR assays were performed according to the methods of Bouzubar *et al.*[18] and Press & Greene,[19] respectively. The Ki67 proliferative index (c) was calculated as the proportion of cells showing intense nucleoplasmic and nucleolar staining patterns.[20] The results are shown as the mean ± SD of six replicates.

affinity for estrogen receptors.[3,22] More significantly, flow cytometric analysis of the growth dynamics of the cultured cells showed that, although both classes of agent share the ability to block cell division in the G1 phase of the cell cycle, both ICI 164384 and ICI 182780 were more effective than tamoxifen[9,22] or hydroxyclomiphene[8] in reducing the proportion of cells which remain able to synthesize DNA after prolonged exposure. These activities, which are specific for estrogen receptor signalling,[10] are reflected in the tumor cell growth fraction[5,10] with pure antiestrogens abrogating growth responses to tamoxifen.[9]

These differences observed between pure and partial antiestrogens and the control of tumor cell growth have been ascribed to their interactions with other signalling pathways, with the partial agonistic activity of tamoxifen being amplified by the presence of growth factors.[9] This appears particularly evident for the interaction of tamoxifen and insulin/IGF-1, where a modest growth response to the antiestrogen is considerably increased by the presence of these factors.[9,23] Such activity is much weaker for ICI 164384, with the compound being more effective than tamoxifen in inhibiting the stimulatory activities of IGF-1 and TGFα.

A further feature of the cellular actions of pure antiestrogens which may relate to their improved antitumor activity has recently been revealed by studying their effects on the expression of estrogen receptors.[5,10,24] It has been observed that they are associated with a rapid loss of the receptor protein in estrogen receptor-positive cells, producing after relatively short periods of time cellular estrogen-receptor negativity.[5,10,24,25] This property contrasts with the increases in ER levels that are seen on either estrogen withdrawal or tamoxifen treatment.[5,10] Recent studies by Fawell *et al.*[26] with ICI 164384 have shown that dimerization of the receptor is impaired by the pure antagonist and this may result in the pure antiestrogen receptor complex becoming more fragile and perhaps more sensitive to the normal processes involved in receptor degradation. Certainly, the half-life of the ICI 164384 receptor complex appears substantially shorter[25] than the half-lives of the estrogen receptor and tamoxifen receptor complexes.[26,27]

Comparison with Estrogen Withdrawal In Vitro

Encouragingly and somewhat surprisingly, the effects of pure antiestrogens on the growth of estrogen receptor-positive breast cancer cells substantially surpass the effects of estrogen withdrawal.[10,21] This property has been demonstrated by several groups, with, for example, ICI 164384 severely impairing the growth of MCF-7 cells in phenol red-free medium where the 5% fetal calf serum has been extensively stripped of its endogenous estrogens by charcoal absorption, a procedure which reduces the level of endogenous estradiol to below 10^{-12} M.[5,10] Once again changes in cell numbers correspond to their recorded growth dynamics, with pure antiestrogens decreasing tumor cell S-phase fraction and increasing the proportion of cells in Go/G1 relative to estrogen withdrawn cells.[22]

The local production of hormones by breast cancer cells may play an important role in promoting some cell growth and gene expression in estrogen-withdrawn cells. The cells would potentially be highly sensitive to these, since estrogen receptor levels are elevated following oestrogen withdrawal.[5,10] However, the actions of locally

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