Antagonism of oestrogen action in human breast and endometrial cells in vitro: potential novel antitumour agents

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Abstract Purpose: There is a need to find novel oestrogen receptor (ER) ligands that antagonize oestrogen action in the reproductive tissues and would therefore have therapeutic potential in oestrogen-dependent tumours. We tested novel ER ligands in both breast and endometrial cells to profile agonism/antagonism in these oestrogen target reproductive tissues. Methods: Novel analogues of the ER antagonist ICI 182,780 were synthesized and tested for their ability to inhibit gene expression dependent on oestrogen response elements (ERE) in human breast (MCF-7) and endometrial (Ishikawa) cell lines. This activity was correlated with inhibition of oestrogen-induced cell proliferation and ER binding. Results: The sulphide analogue (compound 1) and sulphone analogue (compound 2) had no intrinsic ERE-dependent agonism in either breast cancer or endometrial cells in culture. All three compounds dose-dependently inhibited ERE-mediated oestrogen agonism. Moreover, these ER ligands inhibited oestrogen-stimulated proliferation of breast cancer and endometrial cells. ICI 182,780, compound 1 and compound 2 were all able to bind both isoforms of the ER (ERα and ERβ). In endometrial cells, the relative binding to ERβ correlated with the ERE-dependent anti-oestrogenic effect of these ligands, suggesting that in this tissue this receptor is the predominant isoform that determines antioestrogenic activity. Conclusions: The ability of these analogues of ICI 182,780 to inhibit oestrogen-stimulated transcriptional activity and cell proliferation suggests that these agents, in particular the sulphone analogue, have therapeutic potential in the treatment of breast cancer without exhibiting the unwanted oestrogenic effects in the endometrium.

Key words Oestrogen · Steroid · Breast · Antioestrogen · Hormone

Introduction

Although oestrogen is clinically useful in preventing bone loss, it exhibits tissue-specific side effects including endometrial hyperplasia which results in uterine cancer, and proliferative effects in mammary tissue which result in an increased risk of breast cancer [1, 3, 4]. The ideal postmenopausal ‘oestrogen’ would reproduce the beneficial effects of oestrogen on vasomotor symptoms, skeletal tissue and the cardiovascular system without producing the adverse effects of oestrogen on reproductive tissues. This idea has led to the development of selective oestrogen receptor (ER) modulators (SERMs), which are defined as compounds that have oestrogen agonism in one or more of the desired target tissues, such as bone or liver, and antagonism and/or minimal agonism (i.e. clinically insignificant) in reproductive tissue such as the breast or uterus [14, 16, 32]. The first of these, tamoxifen, is the only antioestrogen used widely for the treatment of breast cancer in women and behaves as a mixed agonist/antagonist of oestrogen action, thus potentially limiting its therapeutic potential for the treatment of breast cancer [16]. Attempts to improve on the pharmacological profile of tamoxifen have resulted in compounds which differ in their oestrogen agonist/antagonist characteristics [14, 23, 29].

The next generation of antioestrogens include the oestrogen antagonist ICI 182,780 [17, 28, 29]. ICI
182,780 is a C7-substituted analogue of oestrogen that has been shown to fully antagonize the trophic effects of endogenous oestrogen in the uterus in intact rats and to prevent bone loss following ovariectomy [26, 29]. In an effort to investigate the comparative antagonism of ICI 182,780, as well as other potential SERMs, we synthesized analogues of this pure oestrogen antagonist [28] and tested them for their activity in vitro. We rationalized that chemical modification of the sulphide group may enhance the antioestrogenic effect of ICI 182,780 in vitro cell models, and this was indeed the case with enhanced ER binding correlating with antioestrogenic activity. We profile the oestrogen antagonism of two of these potentially novel SERMs in both breast and endometrial cells. The in vitro data reported here suggest that these analogues, particularly the sulphide derivative of ICI 182,780, may have therapeutic potential in the treatment of breast cancer.

Materials and methods

Cell culture and materials

All chemicals were purchased from Sigma (St. Louis, Mo.) unless otherwise stated. ICI 182,780 and analogues 1 and 2 were prepared according to published procedures [2]. Endometrial Ishikawa cells (kindly provided by Dr. V.C. Jordan, Northwestern, Chicago) [10], human breast cancer cells (MCF-7, ATCC, HTB-22) and human cervical carcinoma cells (Hela; ATCC, CCL-2) were grown routinely in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, Utah), penicillin (10 U/ml) and streptomycin (50 ng/ml) (Gibco BRL Technologies, Grand Island, N.Y.). All experiments were performed in phenol red-free Eagle’s modified minimal essential medium (EMEM) containing 10% heat-inactivated charcoal dextran-stripped fetal bovine serum (HyClone) and supplemented as above. Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO₂. At confluence, cells were subcultured after exposure to trypsin-EDTA (Gibco).

Plasmid construction

The ERα and ERβ mammalian expression constructs were prepared by PCR cloning cDNAs encoding human ERα or ERβ open reading frames into pCR3.1 (Invitrogen, San Diego, Calif.). The template for the ERα cDNA was pRST7-ERα, a generous gift from Donald McDonnell (Duke University) [30]. This cDNA contained a point mutation changing valine 400 to glycine, which was corrected by site-directed mutagenesis prior to cloning in pCR3.1. The ERβ cDNA was generated by RT-PCR from brain tissue and corresponds to the sequence in GenBank accession number X99101 [20].

Transient transfections

Cells were seeded either in six-well plates at 1.5 × 10⁵ cells/well or in 24-well plates at 1.5 × 10⁴ cells/well in phenol red-free medium. DNA was introduced into the breast cancer MCF-7 cell line by the lipofectin method (Life Technologies, Gaithersburg, Md.) and into the endometrial Ishikawa cell line by the calcium phosphate method (Invitrogen, San Diego, Calif.). Briefly, cells were cotransfected with 1 μg per well in six-well plates and 833 ng per well in 24-well plates of either MMTV-ERE(5)-Luc or C3-ERE(3)-Luc and 25 ng of the control renilla-luciferase vector (pRL-CMV). MMTV-ERE(5)-Luc is a DNA reporter construct comprising the mouse mammary tumour virus promoter, in which the glucocorticoid response elements have been replaced with five copies of a 33-bp vitellogenin ERE cloned upstream of the luciferase reporter gene (provided by D. McDonnell, Duke University) [30]. C3-ERE(3)-Luc is the natural complement 3 containing three nonconsensus ERs cloned upstream of the luciferase reporter gene (provided by D. McDonnell) [22]. Transfection efficiency was corrected by cotransfection with a renilla-luciferase vector (Promega, Madison Wis.). Cells were incubated overnight. The transfection medium was then removed and cells were incubated for 48 h with or without hormones as indicated in the figure legends.

Cell lysates were prepared as described in the manufacturer’s protocol for the dual luciferase reporter assay to assess transfection efficiency (Promega). Briefly, cells were washed in PBS and then lysed with 1 × passive lysis buffer (500 μl/well for six-well plates or 100 μl/well for 24-well plates) for 15 min while rocking the sample on a rocking platform. Lysates were centrifuged for 30 s at 14,000 g and the clear lysate was transferred to a tube prior to reporter enzyme analysis. Samples (20 μl) were transferred to a 96-well luminescence detection plate and reacted with 100 μl of each assay reagent (Promega). Each assay reagent was injected by a microtiter LB960P luminometer (Wallac, Gaithersburg, Md.), which measured luciferase activity. The results are expressed as relative light units (RLU).

Cell proliferation

MCF-7 or Ishikawa cells were seeded in 48-well plates at 5×10⁴ cells/well in phenol red-free medium containing 10% heat-inactivated charcoal-stripped fetal bovine serum, and left overnight. The following day, the cells were washed with PBS and the medium was changed to phenol red-free medium containing 1% serum, and incubated for an additional 24 h. Cells were then treated with ligands for 48 h. Following treatment, 1 μCi ³H-thymidine was added to each well of the 48-well plate and the cells were incubated for 6 h. The cells were then washed with ice-cold PBS and cell lysates were prepared by adding 100 μl 0.1 N NaOH for 15 min. The samples were then transferred to scintillation vials containing 10 ml scintillation cocktail for analysis.

ER competition binding assays

Hela cells were used for oestrogen binding studies based on the fact that they do not express either isomer of the receptor endogenously. Hela cells were transfected with pCR3.1 ERα or pCR3.1 ERβ using Lipofectamine Plus (Life Technologies) according to the manufacturer’s instructions. After 24 h the cells were removed from the plate with 200 mM EDTA and washed three times in PBS. Cytoplasmic extracts were prepared as described previously [18], except that the cells were resuspended in buffer A (25 mM Tris-HCl, 1.5 mM EDTA, 10 mM z-n-monomethylglycine, 10% glycerol, 10 mM sodium molybdate, pH 7.4) containing 0.2 U/ml aprotinin, 1 mM PMSF, and 10 μg/ml leupeptin at a concentration of 1 ml per 100 mg wet cell pellet [18]. Lysis was performed by dounce instead of polytron homogenization.

Unlabelled competitor diluted in 25 μl buffer A with 0.1% charcoal-stripped bovine serum albumin (BSA) was combined with 25 μl 24 nM [2,4,6,7-³H]Oestriol (Amersham-Pharmacia) in 96-well U-bottomed microtitre dishes. Cytoplasmic extract (50 μl at 0.7 mg/ml) from Hela cell transfecants was added, and the plates were incubated at 4°C for 18 h. The incubation was removed by adding 100 μl 0.5% dextran-coated charcoal (Sigma) in buffer A with 0.1% BSA, agitating for 15 min at 4°C, and centrifuging at 500 g for 5 min. A 100-μl aliquot of the supernatant was counted in 10 ml Ready Safe scintillation fluid (Beckman) in a Beckman Model LSS801 scintillation counter.

Specific binding was determined by subtracting the counts per minute bound in the presence of a 100-fold molar excess of dieth