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Report

Is ICI 182,780 an antiprogestin in addition to being an antiestrogen?

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Key words: estrogens, antiestrogens, antiprogestins, progestins

Summary

The pure antiestrogen ICI 182,780 has been shown to have antiprogestin activity in reporter gene constructs. Cell lines, naturally devoid of progesterone receptors (PR) were transfected with either the A or B forms of the human PR and a luciferase construct driven by a progesterone-response element (PRE). Because this system is an artificial one, our purpose was to determine whether these observations could be made in a human breast cancer cell line, naturally containing PR. We further evaluated the dose-response of ICI 182,780 and RU-486 (mifepristone) on PR and estrogen receptors (ER) in the presence of either progesterone, norgestrel or estradiol. These effects were measured using immunoassays for prostate-specific antigen (PSA) and human glandular kallikrein (hK2) and pS2. We found that ICI 182,780 blocked progesterone-stimulated PSA and hK2 production 100% at 10^{-5} M, which decreased significantly by 10^{-6} M. This inhibition did not occur when norgestrel was the progestin used. RU-486 showed 100% blockade for both progestins at all concentrations used. We concluded that the antiprogestin activity of ICI 182,780 exists for progesterone only. This weak antiprogestin activity may be unlikely to have significant clinical implications.

Abbreviations: ER: estrogen receptor; PR: progesterone receptor; GR: glucocorticoid receptor; PSA: prostate specific antigen; hK2: human glandular kallikrein: HRE: hormone response element; ERE: estrogen response element; PRE: progesterone response element; NSAE: non-steroidal antiestrogen; ELISA: enzyme linked immunosorbent assay

Introduction

ICI 182,780 (Faslodex) is a pure antiestrogen, used for the treatment of advanced breast cancer after failure of long-term adjuvant tamoxifen therapy [1, 2]. Like other antiestrogens, ICI 182,780 may eventually find applications in other aspects of breast cancer (e.g. prevention), as well as in other gynecological and non-malignant conditions. This compound is a derivative of estradiol, and as such, has high affinity for estrogen receptors (ER). However, because of its long side chain at the 7α position (Figure 1), ICI 182,780 stearically hinders receptor dimerization. In the absence of dimerization, binding of the ER to estrogen response elements (EREs) may be abolished or attenuated [3]. ICI 182,780 has also been shown to cause the destruction of ER [4, 5]. It has therefore been demonstrated that ICI 182,780, *in vitro* mediates virtually no transcription of ER [6].

Other compounds that interact with or affect ER function are also under investigation or are already used in breast cancer therapy. These include the non-steroidal antiestrogens (NSAE), of which tamoxifen is the classic example. Second generations NSAE, which have greater antagonistic and lesser agonistic activity than tamoxifen [7–12], include raloxifene and droloxifene. Progestins, such as R4020 and antiprogestins, such as RU-486 (mifepristone) are also

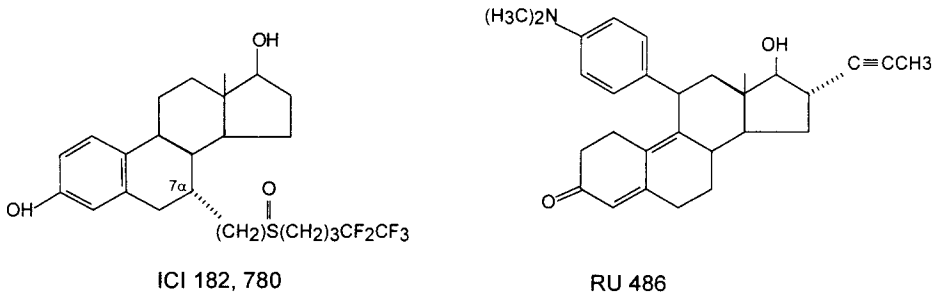

Figure 1. Structures of ICI 182,780 (faslodex) and RU 486 (mifepristone).

being examined. Progestins are currently the most commonly used second-line endocrine therapy for advanced breast cancer [6]. Antiprogestins have been in clinical trial for a few years [6]. However, the question remains as to their precise mechanism of inhibiting receptor-positive tumours.

Studies, using cells transfected with ER and progesterone receptor (PR) reporter genes, have indicated one mechanism of action of antiprogestins. They demostrate extensive inhibitory cross-reactivity between ER and PR. Both progestins (R5020) and antiprogestins (RU-486) have been shown to act as potent ligand-dependent repressors of ER activity when bound either isoform of PR [13]. Moreover, RU-486 has been shown to stimulate growth of MCF-7 cells at 10^{-6} M, which can be blocked by either 4OH-tamoxifen and ICI 164,384, suggesting weak estrogenic activity [14]. A recent study [15] has pointed to 'reverse' cross-reactivity between these receptors, i.e. blocking of progestin-induced transcription by the antiestrogen ICI 182,780. In this study, cell lines devoid of endogenous hormone receptors were transfected with PR expression plasmids, and a luciferase reporter gene driven by progesterone response elements (PREs).

Reconstituted hormone action in cells devoid of endogenous receptors has advantages and disadvantages. Among the advantages is the simplicity of the system and the avoidance of 'cross-talk' between various types of receptors. The incorporation of reporter genes with minimal promoter elements and hormone response elements (HREs) further facilitates quantification of semi-quantitative detection of the response. However, such systems may provide misleading or physiological non-relevant results, especially when multiple HREs are incorporated into the promoters of the reporter gene which then become 'too-sensitive' reporters [16]. Furthermore, physiologically relevant ratios of various hormone receptors, co-activators, and co-repressors and transcription factors are not present in cell lines naturally devoid of hormone receptors. It would be important to verify newly identified actions of hormonal or antihormonal agents in artificial systems by using cells containing natural receptors and endogenous genes which are regulated by such receptors. In this study we examine the recently identified antiprogestin action of ICI 182,780 by using the hormone receptor-positive breast carcinoma cell line BT-474 and the endogenously regulated genes pS2 (estrogen-regulated), prostate-specific antigen (PSA) (androgen-progestin-regulated) and human glandular kallikrein (hK2) (androgenprogestin-regulated).

Materials and methods

Materials

The BT-474 human breast carcinoma cell line was obtained from the American Type Culture Collection (ATCC), Rockville, MD. The levels of ER and PR are 29 and 389 fmol/mg, respectively. Although at present AR cannot be quantified, we know from Northern blot studies that this cell line contains AR [17]. ICI 182,780 was purchased from Tocris Cookson, Inc., Ballwin, MO, and RU-486 (mifepristone) was a gift from Roussell UCLAF, Paris, France. All steroids used were obtained from Sigma Chemical Co., St. Louis, MO, and stock solution were prepared at 10^{-3} M in anhydrous alcohol.

Methods

BT-474 cells were grown to confluency in phenol-free RPMI media (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum, 10 mg/ml insulin and 200 mM L-glutamine at 37°C, 5% CO_2. Once confluent, they were subcultured in 24-well microtiter

plates using the same media, but with substitution of charcoal-stripped fetal calf serum for the regular fetal calf serum. The cells were then stimulated with either a steroid (progesterone, norgestrel or estradiol) alone at a concentration of 10^{-8} M blocker (ICI 182,780 or RU-486) at 10^{-8}, 10^{-7}, 10^{-6} or 10^{-5} M, alone, or both blocker and steroid together. In wells with both blocker and steroid, blocker was added first, then incubated for 1 h prior to addition of steroid at the conditions specified above. The plates were then incubated for 7 days, at which time the supernatants were harvested. They were then analyzed quantitatively for pS2, PSA and human glandular kallikrein (hK2) as exemplified below.

Assays

pS2 Assay

We have used an ELISA-type competitive immunoassay for pS2 which was developed in-house. The details of this assay will be described elsewhere. In short, we coat microtiter wells with a monoclonal anti-pS2 antibody and perform a competition between a biotinylated 28-mer peptide derived from the *N*-terminal sequence of pS2 and endogenous pS2. After washing unreacted moieties, we detect bound biotinylated peptide with a streptavidin-alkaline phosphatase conjugate and time-resolved fluorometry, essentially as described elsewhere [18]. The detection limit of this assay is $\sim$ 20 ng/ml.

PSA Assay

Prostate specific antigen was quantified with an ELISA-type immunofluorometric procedure essentially as described elsewhere [19]. The detection limit of this assay is $\sim$ 1 ng/l.

HK2 Assay

An ELISA-type immunofluorometric assay was also used. The details of this assay have been described elsewhere [20]. The detection limit of this assay is $\sim$ 6 ng/l.

All measurements were performed in duplicate and all experiments described were performed at least twice.

Results

pS2 is an estrogen-regulated gene and the concentration of pS2 in the tissue culture supernatants reflects estrogenic activity. Estradiol stimulates pS2 production in a dose-dependent manner at concentrations from 10^{-5} M down to 10^{-11} M. Other steroids do not induce any pS2 upregulation (data not shown). Similarly, the concentration of PSA and hK2 in the tissue culture supernatants reflect progestational/androgenic activity. Both progesterone and norgestrel (and in addition testosterone and dihydrotestosterone, data not shown) stimulate PSA and hK2 production in a dose-dependent manner, from 10^{-5} M down to 10^{-11} M. Estrogens upregulate these two genes, but only at concentrations higher than 10^{-8} M (data not shown). All three endogenous reporter genes used (pS2, PSA, hK2) are secreted proteins and we found that 1 week post-single stimulation provide the best quantitative and reproducible data.

ICI 182,780 and mifepristone were tested at concentrations from 10^{-8} to 10^{-5} M for estrogenic (pS2) and progestational/androgenic activity (PSA and hK2). The data are shown in Table 1. ICI 182,780 did not show any agonistic activity at these concentrations for either receptor. This was as expected for a pure antiestrogen. RU-486 showed no estrogenic activity, but some progestational/androgenic activity was found (Table 1). This is in line with other reports of agonistic activity of this antiprogestin [21]. Our controls worked as expected, i.e. induction of pS2 by estradiol and of PSA and hK2 by progesterone and about 100-fold more potently by the synthetic progestin, norgestrel (Table 1).

Blocking experiments demonstrated that ICI 182,780, at concentrations of 10^{-5}– 10^{-7} M, showed 97–100% blocking of estradiol action. At 10^{-8} M, this blocking was minimal (only 16%). Interestingly,

Table 1. Production of pS2, PSA and hK2 by Blockers and Steroids

Compound	Concentration (M)	pS2 ng/ml	PSA ng/l	hK2 ng/l
ICI 182,780	10^{-5}	< 20	15	< 6
ICI 182,780	10^{-6}	< 20	21	6
ICI 182,780	10^{-7}	< 20	27	8
ICI 182,780	10^{-8}	< 20	23	11
RU 486	10^{-5}	< 20	617	1054
RU 486	10^{-6}	< 20	1050	4800
RU 486	10^{-7}	< 20	656	2428
RU 486	10^{-8}	< 20	434	600
Estradiol	10^{-8}	202	49	8
Progesterone	10^{-8}	< 20	799	1943
Norgestrel	10^{-8}	< 20	66253	162822

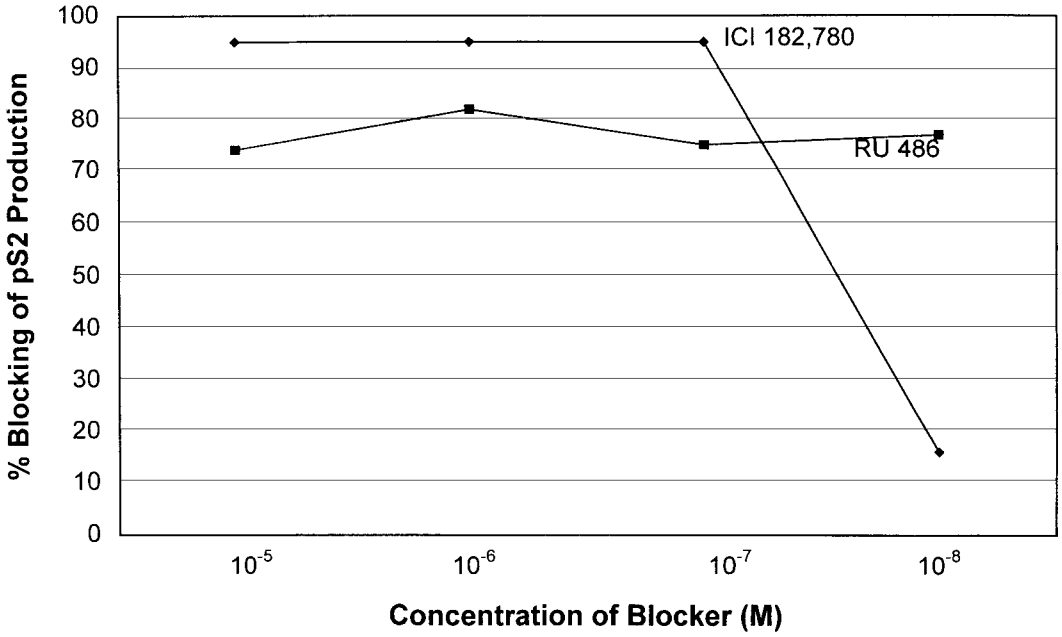

Figure 2. Percent blocking of pS2 production, by the anti-estrogen ICI 182,780 and anti-progestin RU 486. BT-474 cells were stimulated by 10^{-8} M estradiol.

RU-486 showed 74–82% blocking of ER at all concentrations used (Figure 2). There was no dose-response for this effect, suggesting that the phenomenon does not operate directly through the estrogen receptor but by an alternative pathway, likely involving a cross-talk with the progesterone receptor.

RU-486 showed complete blocking of PR, measured through both PSA and hK2, when either progesterone or norgestrel was used as the progestin. For hk2, however, mifepristone decreased blocking ability at 10^{-7} M and below (Figures 3 and 4). This was not seen for PSA, where blocking was 100% throughout (Figures 5 and 6).

Discussion

The mechanism of gene regulation by steroid hormones is quite complex and involves many molecules including the steroid hormone receptors, heat shock proteins, transcriptional factors, co-activators, co-repressors, protein kinases, etc. [22–24]. The final result, regarding specificity and potency of response, will depend on the availability and concentration ratios of all these factors and may be heavily dependent on tissue type or, in *in-vitro* experiments, on type of cell line used. Reconstituted steroid hormone action in cells that are devoid of hormone receptors is possible by exogenously transfecting plasmids containing the coding sequences of the receptors and plasmids containing minimal promoters with hormone response elements in front of indicator genes like CAT or luciferase. These simple systems have enjoyed widespread use because of their simplicity, ease of use and avoidance of complicating cross-talks between various receptor pathways. On the other hand, these systems should be approached with caution since they may produce results that have no relevance to the physiological situation. For example, a plasmid containing three or four EREs is progressively much more sensitive to estrogen stimulation than a plasmid containing only two EREs [16]. Moreover, Weaver et al. have reported, for example, that the antiestrogen ICI 164,384 had disparate actions on expression of the endogenous gene pS2 and on transfected reporters containing the pS2 ERE [25]. Thus, caution should be exercised in interpreting data of reconstituted hormone action experiments until these are verified in cells which possess the steroid hormone receptor system and utilize endogenous genes as reporter systems.

The study of Nawaz et al. [15] is important since they report for the first time progestin-induced inhibition of transcription of the pure antiestrogen ICI 182,780. Thus, they conclude that ICI 182,780 acts as an antiprogestin with potency presumably similar to that of the classical antiprogestin RU-486 (mife-

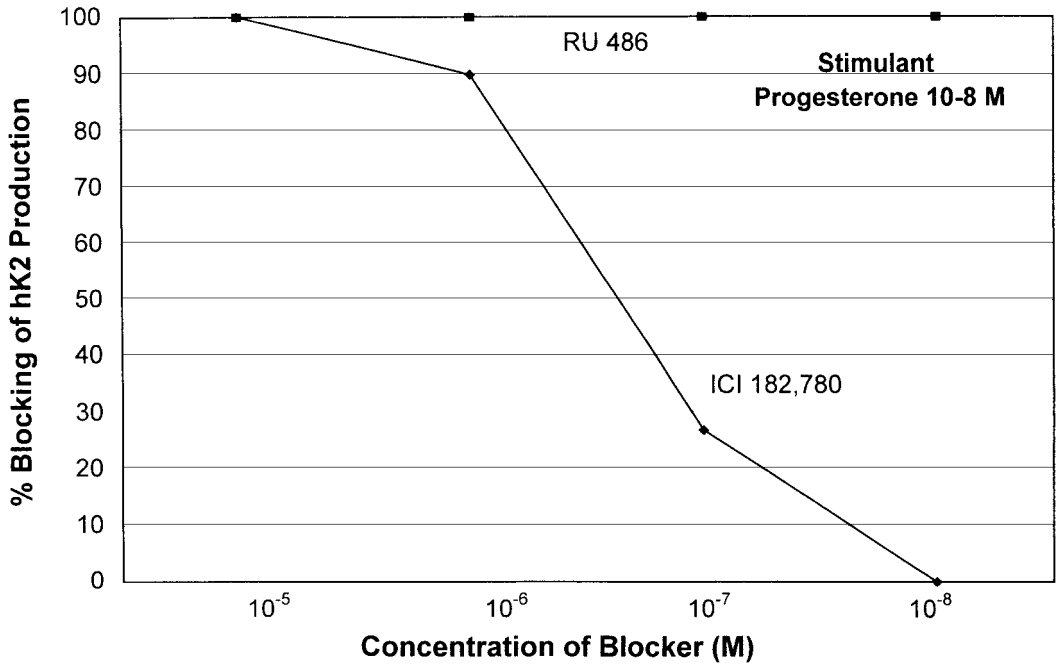

Figure 3. Percent blocking of human glandular kallikrein 2 (hK2) production by ICI 182,780 and RU 486. BT-474 cells were stimulated by 10^{-8} M progesterone.

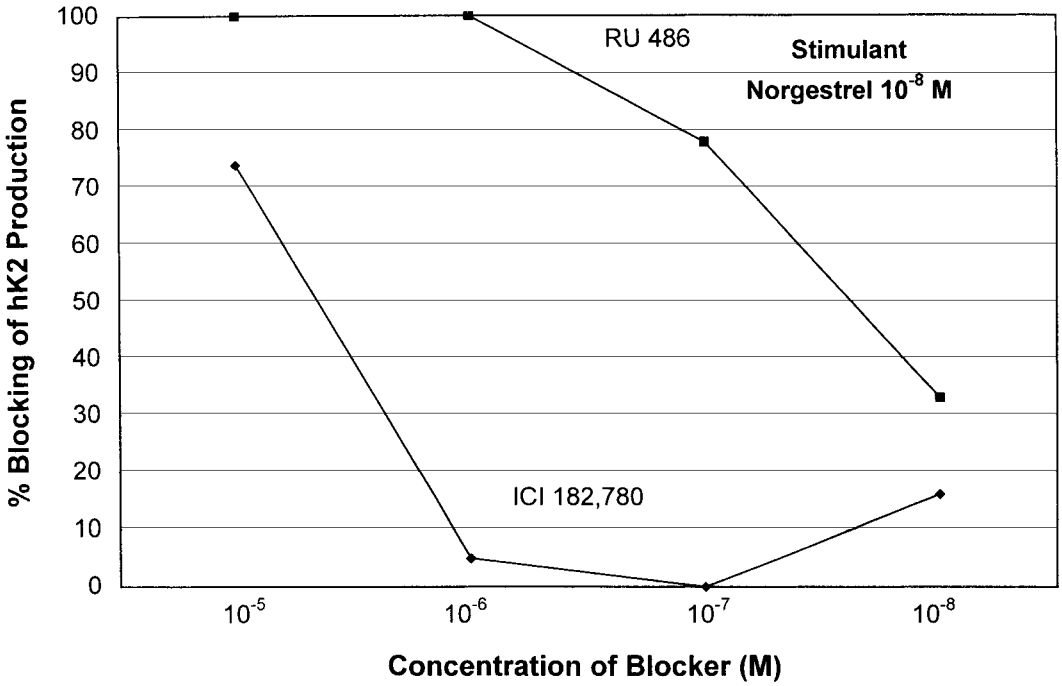

Figure 4. Percent blocking of hK2 production by ICI 182,780 and RU 486. BT-474 cells were stimulated by 10^{-8} M norgestrel.

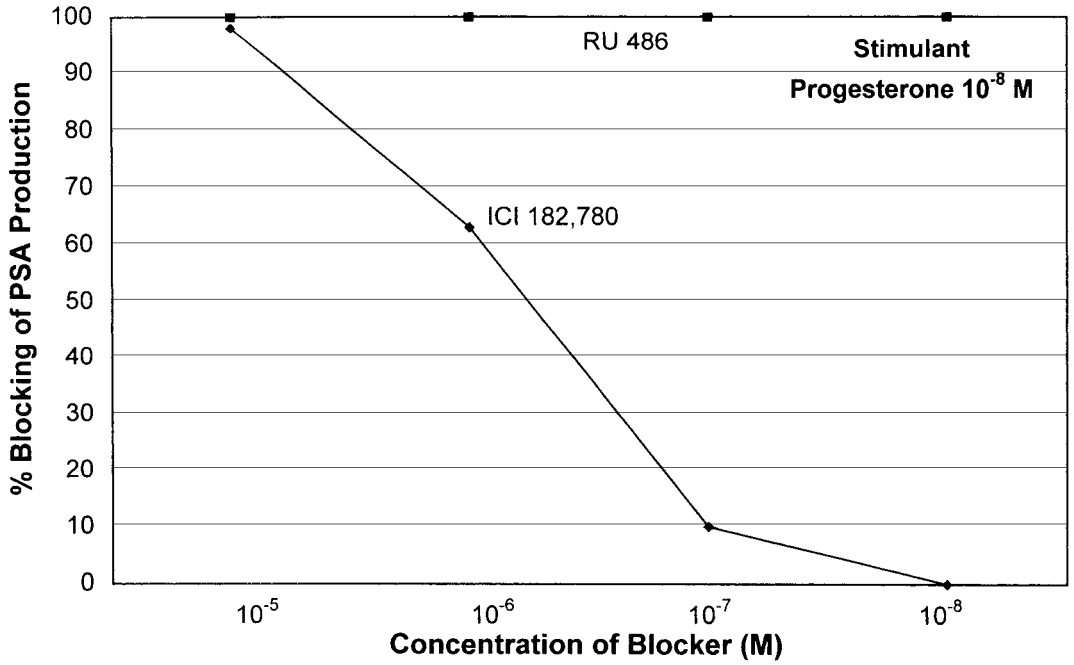

Figure 5. Percent blocking of prostate-specific antigen (PSA) production by ICI 182,780 and RU 486. BT-474 cells were stimulated by 10^{-8} M progesterone.

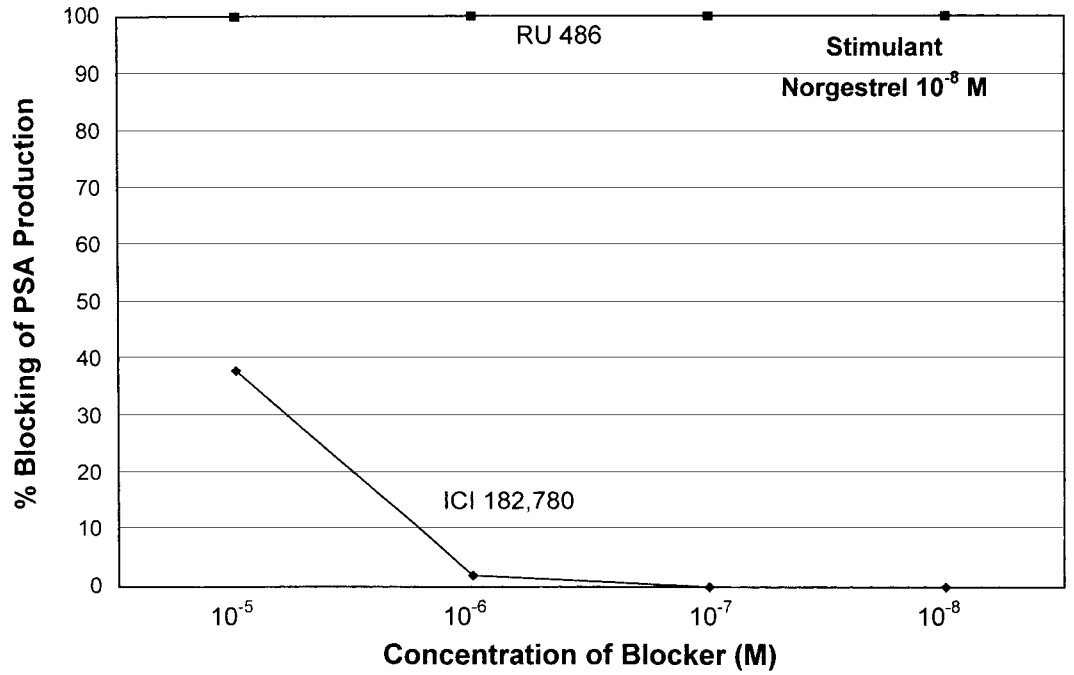

Figure 6. Percent blocking of PSA production by ICI 182,780 and RU 486. BT-474 cells were stimulated by 10^{-8} M norgestrel.

pristone). They found no intrinsic progestin activity of 182,780 and no antiandrogen or antiglucocorticoid activity. The IC_{50} of ICI 182,780 was around 2×10^{-7} M.

We set out to examine these data in a completely different experimental system. Our cell line (BT-474 breast carcinoma cell line) had endogenous hormone receptors. We also used endogenously regulated genes, i.e. pS2 (estrogen-regulated) and PSA and hK2 (androgen-progestin regulated). Quantitative analysis of the three secreted proteins in the tissue culture supernatants assures quantitative comparison of data.

The possible antiprogestational activity of ICI 182,780 and its comparison to that of RU-486 is important for three reasons: (a) This antiprogestational, in addition to its antiestrogenic activity of ICI 182,780 may be important in relation to its anti-cancer activity *in-vivo*; (b) This effect may allow us to better understand the clinical pharmacology of the drug and its possible therapeutic mechanisms, so that other compounds with similar activity are developed; (c) ICI 182,780 is used in *in-vitro* studies with cultured cells at concentrations around 10^{-6} M and the effects observed are attributed to its antiestrogenic activity. A possible antiprogestational activity of the drug at these levels will complicate the interpretation of data.

We have first verified that ICI 182,780 has no detectable estrogenic, androgenic or progestin activity even at concentrations as high as 10^{-5} M (Table 1). Further, we have shown that RU-486 has significant progestin agonist activity, as reported previously [18] and that norgestrel, a synthetic progestin, is about 100-fold more potent than progesterone, also in accordance with previous data regarding affinities of norgestrel and progesterone for the PR [18]. In addition, we verified the specific upregulation of the pS2 gene by estradiol (Table 1).

The potent antiestrogenic activity of ICI 182,780 is shown in Figure 2. At $\geq 10^{-7}$ M, ICI 182,780 inhibited $\geq$97% of estradiol's action, as expected (Figure 2). We have further observed a strong antiestrogenic activity of the antiprogestin RU-486 (approximately 70–80% inhibition of estradiol's activity) which was not dose-responsive (Figure 2). This activity does not seem to be directly related to the ability of RU-486 to block the ER, but rather, to be an indirect phenomenon associated with PRs liganded by RU-486. Such an effect has previously been published in the literature with progestins or antiprogestins demonstrating either inhibitory or stimulating estrogenic effects [13, 14].

In Table 1 we demonstrate, in agreement with the data of Nawaz et al., weak antiprogestational activity of ICI 182,780. The IC_{50} of ICI 182,780 for progesterone is between 10^{-6}–10^{-7} M, also in agreement with the previous report. However, the inhibition of the synthetic progestin norgestrel was only between 40–70% at an ICI 182,780 concentration of 10^{-5} M and not detectable at lower concentrations. Comparison of the inhibiting (antiprogestational) activity of ICI 182,780 to RU-486 clearly indicates that the latter is at least a 1000-fold more potent antiprogestin (Figures 3–6).

In conclusion, we have verified with a tissue culture system that contains endogenous receptors and with utilization of endogenous genes that indeed. ICI 182,780 has weak antiprogestational activity. However, this activity is far less than the activity of the synthetic antiprogestin RU-486. It seems unlikely that ICI 182,780 *in-vivo*, and at the concentrations used to treat breast cancer, will have any clinically significant antiprogestational activity in addition to its potent antiestrogenic activity. The weak antiprogestational activity of ICI 182,780 may complicate interpretations of tissue culture experiments in which the compound is used at concentrations $\geq 10^{-6}$ M and is considered a pure antiestrogen.

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