*J. Enzyme Inhibition*, 1993, Vol. 7, pp. 203-212
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# SCREENING AND DETERMINATION OF KINETIC PARAMETERS OF AROMATASE INHIBITORS USING HUMAN GENITAL SKIN FIBROBLASTS

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*(Received 5 April 1993)*

The screening of aromatase inhibitors using human genital skin fibroblasts (GSF) in culture is described. Using this system the selectivity in detecting inhibition compares well with that for human full-term placenta as a source of enzyme. $IC_{50}$ and $K_i$ values may be determined using GSF and the ranking of a group of known inhibitors tested compares with that using placental enzyme. The advantages of this method are that the tissue is readily available and the long term freezing of viable cells allows a ready supply of material for repeated experiments.

KEY WORDS: Aromatase inhibitors, placental aromatase, genital skin fibroblasts, aromatase screening.

## INTRODUCTION

Oestrogens are considered to stimulate breast cancer growth[1]. In postmenopausal women with breast cancer, removal of the tumour mass is followed by radiotherapy and anti-endocrine chemotherapy to prevent further tumour growth in the breast and metastases. The anti-oestrogen tamoxifen and aromatase inhibitors are used in this connection[2].

Aromatase inhibitors are commonly screened *in vitro* using full-term human placental tissue or rat ovarian tissue. Aromatase activity is measured by the release of $^3H_2O$ for both tissues using either [1$\beta$, 2$\beta$-$^3$H]-androstenedione (or testosterone) or [1$\beta$–$^3$H]-androstenedione as substrate for placenta and [1$\beta$–$^3$H]-androstenedione as substrate for rat ovary[3].

Human genital skin fibroblasts (GSF) in culture express a wide range of enzymes including aromatase[4,5]. There are distinct advantages in using such a cell culture system to study enzyme inhibition. Skin tissue is readily accessible for sampling in humans and establishes well in primary culture. The environment (ie culture medium) of the cells

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can be changed under controlled conditions and the effects measured. Furthermore the efficient long term storage of viable cells frozen at −70°C allows a ready supply of cell strains for repeated experiments. We describe its use as a means to screen aromatase inhibitors, amenable to large scale studies. A range of known inhibitors of aromatase and some chemically-related but inactive compounds, previously screened using placental tissue, have been examined to confirm the sensitivity and accuracy of the GSF system for determining inhibitory potency.

## MATERIALS AND METHODS

[1$\beta$-$^3$H]-Androstenedione (25–30Ci/mmol) was obtained from Dupont (UK) Ltd., Stevenage, Herts. $^3H_2O$ (90$\mu$Ci/mmol) was purchased from Amersham International plc, Bucks. All other materials were obtained as previously described[5,6].

Aminoglutethimide (AG), glutethimide, nitroglutethimide, 4-hydroxyandrostenedione and CGS16949A (fadrozole hydrochloride) were gifts from Ciba-Geigy, Basle. R76713 and rogletimide (E) were gifts from Janssen Research Foundation, Beerse, Belgium and Professor M. Jarman, the Institute for Cancer Research, Sutton respectively. Econazole and miconazole were purchased from Sigma Chemical Co. Ltd., Poole, Dorset. Most of the compounds have been synthesized in our laboratories and reported elsewhere: A, B, C, D, WSP-1 and WSP-3[1,7]; F and G[8]; H[9]; K[10]; N-acetylaminoglutethimide[11].

### *Cell Culture*

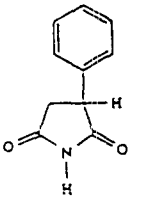
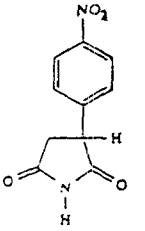
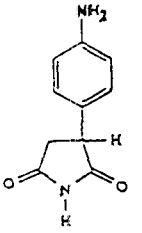
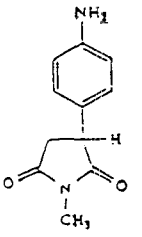
Normal genital skin fibroblasts (GSF) were established in culture using primary skin explants of foreskin samples established at the time of circumcision in prepubertal children. GSF were serially cultured from these explants as previously described[5]. All experiments were performed between cell passage numbers 5 and 20. Prior to assay, cells were seeded into 25cm$^2$ flasks (approximately $1 \times 10^6$ cells per flask) and kept in culture until very confluent (approximately two weeks) before assay in order to maximise aromatase activity.

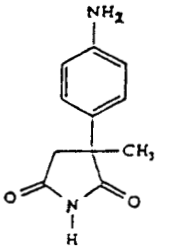
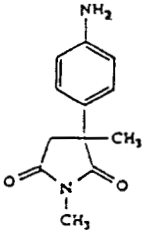
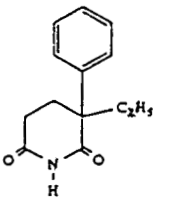
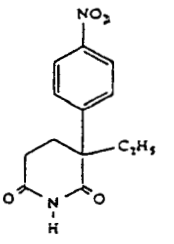
### *Aromatase Assay*

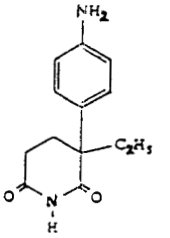
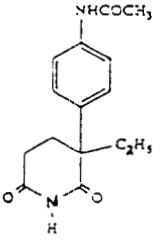
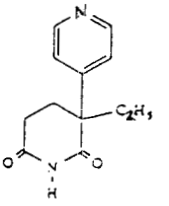
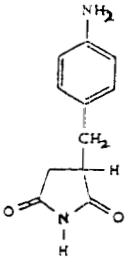
#### *GSF Cultures*

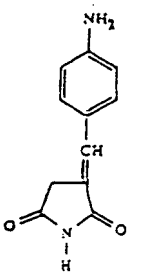
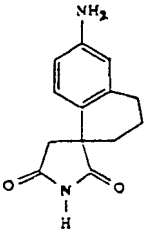
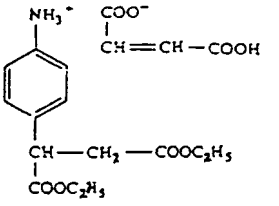
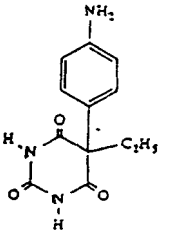
Confluent monolayers of GSF in 25 cm$^2$ flasks were preincubated (37°C; 72h) with 2 ml of culture medium containing 10% (v/v) newborn calf serum. The cells were then washed with 2 ml phosphate buffered saline and incubated with 2 ml of serum free medium[6] at pH7 containing [1$\beta$-$^3$H]-androstenedione and 5$\mu$M progesterone with or without inhibitor as described in the figure legends. Following incubation (37°C; 4h) aromatase activity was quantitated by the $^3H_2O$ release method as previously described[5,12]. Aromatase activity was expressed as fmol/mg protein/h and all results are presented as the mean of duplicate flasks of cells. The coefficient of variation for each point presented varied by less than 10%. All experiments were repeated with comparable results. Blank values were established in the absence of cells.

TABLE I
Screening for Aromatase Inhibitory Activity Using Cultured Genital Skin Fibroblasts and Placental Enzyme

| Compound | | % Inhibition | |
|---|---|---|---|
| | | Fibroblasts | Placental Enzyme |
| A |  | 3 | 0 |
| B |  | 0 | 3 |
| WSP-3 |  | 43 | 51 |
| C |  | 50 | 50 |

| Compound | % Inhibition | |
|---|---|---|
| | Fibroblasts | Placental Enzyme |
| WSP-1  | 53 | 73 |
| D  | 32 | 34 |
| Gluthethimide  | 0 | 5 |
| Nitroglutethimide  | 0 | 4 |

| Compound | % Inhibition | |
|---|---|---|
| | Fibroblasts | Placental Enzyme |
| AG  | 41 | 78 |
| Acetyl AG  | 0 | < 11 |
| E  | 0 | 36 |
| F  | 22 | 30 |

| Compound | % Inhibition | |
|---|---|---|
| | Fibroblasts | Placental Enzyme |
| G  | 2 | 0 |
| H  | 3 | 20 |
| J  | 70 | 63 |
| K  | 0 | 2 |

*Inhibitor concentration = 5$\mu$M; androstenedione = 25nM.

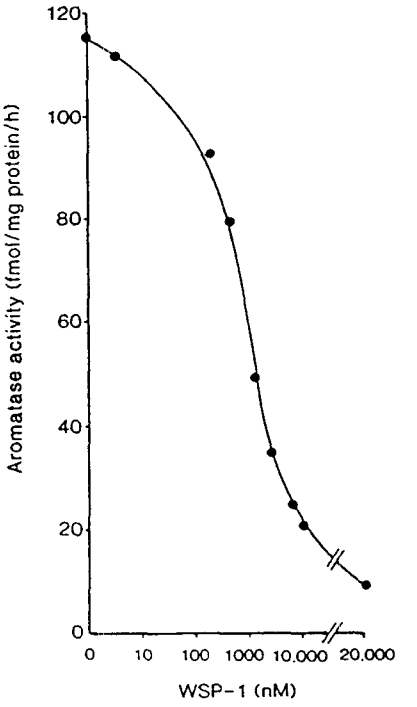



FIGURE 1 Determination of $IC_{50}$ for WSP-1. GSF were incubated at 37°C for 4h with [1$\beta$-$^3$H]-androstenedione (25nM), progesterone (5$\mu$M) and WSP-1 (0–20$\mu$M). Aromatase activity was quantitated by the $^3H_2O$ release method.

*Placental enzyme*

The activity of human term placental enzyme[13] was determined by measurement of $^3H_2O$ released from [1$\beta$, 2$\beta$-$^3$H]-androstenedione by the general method of Graves and Salhanick[14].

*Determination of Inhibitory Activity and Potency*

Normal GSF were incubated at 37°C for 4h with [1$\beta$-$^3$H]-androstendione (25 nM) and progesterone (5$\mu$M) in the presence and absence of inhibitor (5$\mu$M). The results are presented as percentage inhibition of the control value in Table I.

The $IC_{50}$ values were determined for some of the inhibitors from a plot of aromatase activity *vs* log [Inhibitor] (Figure 1 and Table II).

The $K_i$ values for the potent inhibitors R76713 (0.75nM) and CGS16949A (0.5nM) were determined from an Eadie-Hofstee Plot (Figure 2 and Table II) using [1$\beta$-$^3$H]-androstenedione (3–50nM) as substrate. The $K_i$ values for several other inhibitors were determined in a similar manner (Table II).

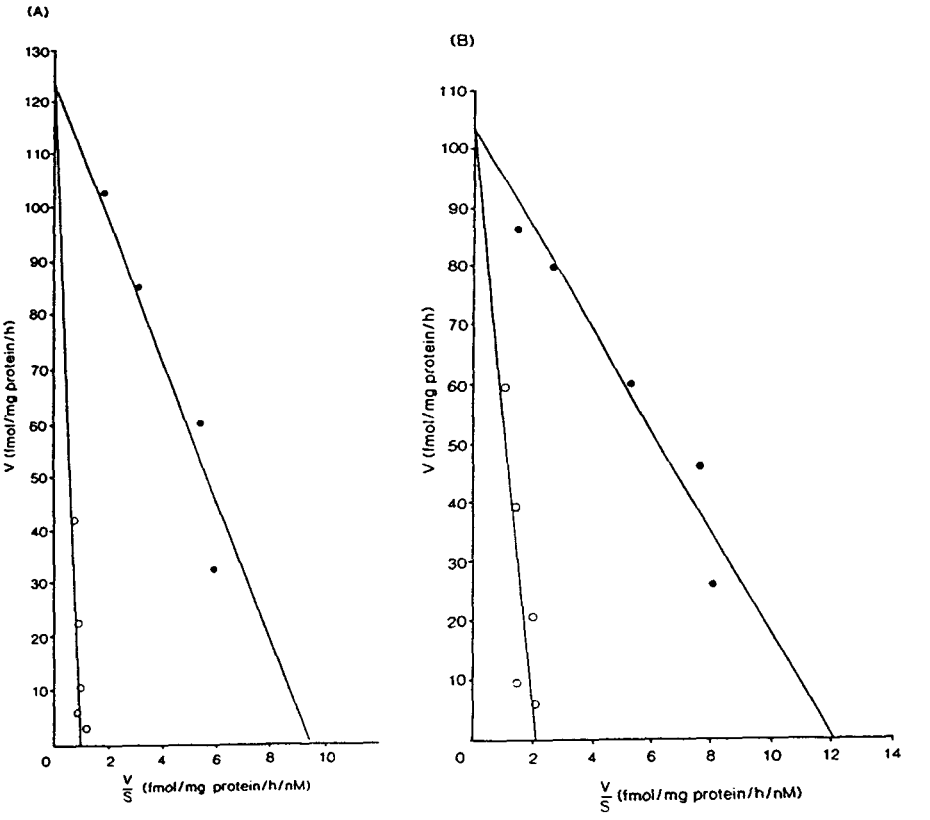



FIGURE 2 Eadie-Hofstee plot for the determination of $K_i$ values for; (a) R76713 (0.75nM), (b) CGS 16949A (0.5nM) in GSF using [1$\beta$-$^3$H]-androstenedione (3–50 nM) as substrate. Aromatase activity was quantitated by the $^3H_2O$ release method. Substrate alone (●); Substrate + inhibitor (○).

## RESULTS AND DISCUSSION

Using cultured human GSF as a source of aromatase we have found that a range of inhibitors of average to high potency give similar results to those previously obtained using human placental tissue (Table I). The only exception is rogletimide (compound E) which is a weak inhibitor using androstenedione as substrate (cf testosterone as substrate where it approaches AG in potency). Furthermore a range of compounds of low potency or lacking potency were similarly valued. Further examination of selected inhibitors showed that the system provided good plots of (aromatase activity) *vs* log [Inhibitors] for determination of $IC_{50}$ values and (aromatase activity) *vs* (aromatase activity)/[substrate] for determination of $K_i$ values. The values obtained were lower than those previously recorded using placental enzyme but showed a similar potency ranking for a group of inhibitors (Table II).

TABLE II

$IC_{50}$ and $K_i$ values for aromatase inhibitors using human GSF

| | $IC_{50}$* ($\mu$M) | $K_i$ (nM) |
|---|---|---|
| WSP-1 | 2 (10)[1] | 1013 |
| WSP-3 | 3 (10)[1] | N.D. |
| AG | 4 (8)[1] | 279 (6,500)[4] |
| 4-OHA | 0.001 (0.4)[2] | 0.27 (20)[3] |
| Econazole | 0.005 (.06)[3] | 2 (20)[3] |
| Miconazole | 0.2 (0.6)[7] | 35 (55)[7] |
| R76713 | ND | 0.1 (1.3)[5] |
| CGS16949A | ND | 0.1 (0.79)[6] |

*Androstenedione = 25nM. ND = not determined.

Values in parentheses are reported values for placental enzymes.

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In summary, the use of GSF provides a new, acceptable system for screening and determining the kinetic parameters of aromatase inhibitors. The long term freezing of viable cells allows a ready supply of material for repeated experiments.

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