

INTRODUCTION

Human aromatase (CYP19) converts C19 androgens to aromatic C18 estrogenic steroids (Thompson and Siiteri, 1974) and can also metabolize xenobiotics (Toma et al, 1996). Inhibitors of this enzyme are used to treat postmenopausal breast cancer and other estrogen-dependent diseases (Brodie et al, 1999). More recently, aromatase inhibitors have been identified as potential environmental toxins (so-called "endocrine disruptors" (Mak et al, 1999)). We have developed a high throughput screening method to detect aromatase inhibitors utilizing recombinant human aromatase and the fluorometric substrate dibenzylfluorescein (DBF). The methodology is similar to that developed previously for detecting drug candidates with metabolism based drug-drug interaction potential (Crespi et al, 1997).

Objective:

To develop a high throughput screen for aromatase inhibitors

METHODS & MATERIALS

Incubations with cDNA-expressed enzymes and liver microsomes

Assays were conducted in 96-well microplates. The substrate DBF was obtained from BD Gentest Corporation. Multiple test chemicals were evaluated and were obtained from Ultrafine Chemicals limited or Sigma-Aldrich except Org-30958 (19-ethylthio-4-ene-3,17-dione) and Org-30365 (19-mercaptopandro-4-ene-3,17-dione) which were kind gifts of Dr. Willem Schoonen of N. V. Organon, The Netherlands. Aromatase enzyme (baculovirus/insect cell-expressed) was obtained from BD Gentest Corporation. A 50% inhibitory concentration (IC₅₀) was determined utilizing 12 wells in each test. A cofactor/serial dilution (C/SD) buffer contained an NADPH-regenerating system and 0.1 mg/mL microsomal protein prepared from insect cells infected with wild type virus ("control protein"). To the first well in each row, 150 µl of the test compound (dissolved in acetonitrile) and C/SD buffer were added in a ratio of 6:144. The compound was diluted serially 1:3 through the 8th well. Wells 9 and 10 contained no inhibitor and wells 11 and 12 were used as controls for background fluorescence (enzyme and substrate were added after the reaction was terminated). The plate was then warmed to 37 °C and the reaction initiated by the addition of pre-warmed enzyme/substrate (E/S) mix. The E/S mix contained buffer, cDNA-expressed P450 (2 pmol/mL final), control protein (0.25 mg/mL final), substrate (0.2 µM final). Reactions were terminated after 30 min by addition of 75 µl 2 N NaOH. Fluorescence signal was measured (ex = 485, em = 538) using a FLUOstar model 403 fluorescence plate reader (BMG LabTechnologies, Inc., Durham, NC). The IC₅₀ values calculated by linear interpolation.

DISCUSSION

Assay Validation – Comparisons with the Literature

Several compounds were selected to evaluate the assay (Table 2) and the results compared to those found in the literature (Table 3). As shown, the assay detected the steroidal aromatase inhibitor 4-hydroxyandrostenedione (Brodie et al, 1981) and the non-steroidal inhibitor aminoglutethimide in the predicted rank order. The androstenedione analogs, Org 30365 and Org 30958, were the most potent of all compounds tested (Geelen et al, 1991). The IC₅₀ values found for the endogenous substrates testosterone and androstenedione are consistent with reported K_m values of 10-80 nM (Bullion et al, 1990; Kellis and Vickery, 1984; Ayub and Levell, 1988). Estradiol and estrone, the products of testosterone and androstenedione metabolism, respectively, were found to be relatively weak inhibitors, as expected. The flavonoid compounds alpha-naphthoflavone, chrysin, naringenin and quercetin were examined based on their reported activity as aromatase inhibitors (Kellis and Vickery, 1984). The rank order and potency was comparable to those found previously. The assay was also able to detect broad-spectrum cytochrome P450 inhibitors such as the antifungal agent ketoconazole and the mono-amine oxidase inhibitor tranylcypromine. By contrast, quinidine, sulfaphenazole and furafylline, selective inhibitors of the drug metabolizing cytochromes P450 isoforms, CYP2D6, CYP2C9 and CYP1A2, respectively, were without response at the concentrations tested.

Analysis of Assay Response and IC₅₀ values

Although absolute IC₅₀ values were, in some cases, quite different from specific reported values, they generally fall within the range of values reported. Such differences are likely attributable to differences in methodology, such as choice of substrate concentration in relationship to the K_m and use of cell-based systems versus cell-fraction systems. Reasonable comparisons between K_m and IC₅₀ values can be made when the substrate concentration is near the K_m. In the case of competitive inhibitors, they would differ by only a factor of two. Geelen et al (1991) used an androstenedione concentration of 3.5 µM, approximately 10-fold greater than the K_m usually reported for this substrate. This probably contributed to the large disparities between their results and those of others in Table 3. For screening purposes, it is the rank ordering of test compounds that is of concern. Our results were not consistent with the rank orders reported Ketoconazole and AG in two of the three reports where these two compounds were compared. Other than this exception, rank ordering was consistent with published data.

Investigation of Solvent Effects

Because most test compounds require organic solvent for delivery into the incubations, the effect of organic solvents on aromatase activity was examined (Figure 1). Final solvent concentration up to 2.0% acetonitrile and methanol or 0.5% ethanol and DMSO have minimal effect on the assay.

RESULTS

TABLE 1

Kinetic properties of baculovirus/insect cell-expressed Aromatase (SUPERSOMES®) and assay parameters for inhibition analysis with DBF

| Substrate | Testosterone | |
|---|--------------------|------------------|
| | Estradiol | DBF ^b |
| Metabolite | Flourescein | |
| Apparent K _m (µM) | 0.043 ^a | 0.188 |
| Apparent V _{max} (min ⁻¹) | 8.4 | 0.32 |
| Substrate concentration for IC ₅₀ (µM) | NA | 0.20 |
| Pmol enzyme per well (200 µl vol.) | NA | 0.4 |
| Incubation Time (min) | NA | 30 |
| NA – Not applicable | | |

^a – Data from McNamara et al (1999).

^b – Apparent K_m and V_{max} values for DBF were determined with an enzyme concentration and incubation time as indicated using eight substrate concentrations ranging from 2 to 4440 nM. Kinetic parameters were calculated using nonlinear kinetics (SigmaPlot 4.0, SSPS, San Rafael, CA).

TABLE 2

Summary of IC₅₀ values for the test compounds^a

| Test compound | IC ₅₀ value (µM) | | | |
|----------------------|-----------------------------|--------|--------|------------|
| | Day 1 | Day 2 | Mean | Range/mean |
| Org 30365 | 0.0026 | 0.0016 | 0.0021 | 0.24 |
| Org 30958 | 0.0198 | 0.0079 | 0.0138 | 0.43 |
| 4-OHA | 0.028 | 0.034 | 0.031 | 0.10 |
| Androstenedione | 0.068 | 0.096 | 0.082 | 0.17 |
| Testosterone | 0.126 | 0.240 | 0.183 | 0.31 |
| Alpha-naphthoflavone | 0.149 | 0.207 | 0.178 | 0.16 |
| Chrysin | 0.60 | 0.79 | 0.70 | 0.13 |
| Aminoglutethimide | 0.61 | 0.93 | 0.77 | 0.21 |
| Ketoconazole | 0.64 | 1.17 | 0.90 | 0.30 |
| Tranylcypromine | 1.12 | 1.55 | 1.34 | 0.16 |
| (±)-Naringenin | 1.17 | 1.91 | 1.54 | 0.24 |
| Estrone | 1.17 | 2.72 | 1.95 | 0.39 |
| β-Estradiol | 6.99 | 9.40 | 8.20 | 0.15 |
| Quinidine | > 5 | > 5 | > 5 | - |
| Sulfaphenazole | > 10 | > 10 | > 10 | - |
| Quercetin | > 50 | > 50 | > 50 | - |
| Furafylline | > 100 | > 100 | > 100 | - |
| | | | Mean | 0.23 |

^a IC₅₀ values were determined in duplicate on separate days

HT assay values are consistent with literature values determined by traditional methods

TABLE 3

Comparison of IC₅₀ values for selected test compounds

| Test compound | Literature IC ₅₀ or K _i values with androstenedione as substrate (µM) | | | | | | | | |
|-----------------|---|---------------------------------|-------------------------------------|----------------------------------|--------------------------------------|----------------------------|------------------------------------|-----------------------------------|------------------------------------|
| | Present study | (Kao et al., 1998) ^a | (Bullion et al., 1990) ^b | (Ayub et al., 1998) ^c | (Campbell et al., 1993) ^d | (Kellis and Vickery, 1984) | (France et al., 1987) ^e | (White et al., 1999) ^f | (Geelen et al., 1991) ^g |
| Org 30365 | 0.0021 | - | - | - | - | - | - | - | 0.09 |
| Org 30958 | 0.0138 | - | - | - | - | - | - | - | - |
| 4-OHA | 0.031 | - | 0.015 | 0.18 | - | 0.05 | 0.04 | 0.039 | 1.4 |
| Androstenedione | 0.082 | - | - | - | - | - | - | - | - |
| Testosterone | 0.183 | - | - | - | - | - | - | - | - |
| ANF | 0.178 | 2.2 | - | - | 0.2 | 0.07 | - | - | - |
| Chrysin | 0.70 | 2.6 | - | - | 2.4 | 0.5 | - | - | - |
| AG | 0.77 | - | 0.62 | 30 | 2.4 | - | 55 | 6.53 | - |
| Ketoconazole | 0.90 | - | - | 7.3 | - | - | 65 | 6.04 | - |
| Tranylcypromine | 1.34 | - | - | - | - | - | - | - | - |
| (±)-Naringenin | 1.54 | 5.1 | - | - | - | - | - | - | - |
| Quercetin | > 50 | - | - | - | No effect | 12 | - | - | - |

^a K_i values, Chinese Hamster Ovary cells expressing human aromatase

^b K_i values, human placental microsomes

^c IC₅₀ values, human placental microsomes

^d K_i values, human preadipocytes

^e IC₅₀ values, human placental S9

Final solvent concentration up to 2.0% acetonitrile and methanol or 0.5% ethanol and DMSO have minimal effect on the assay.

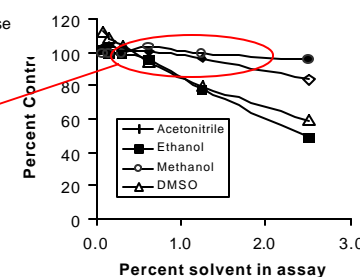


FIGURE 1

CONCLUSIONS

1. We have developed a high-throughput fluorometric screening method to detect inhibitors of human aromatase. The assay is useful for detecting novel drug candidates and/or environmental toxins (e.g. "endocrine disruptors").
2. The assay is reproducible [between day CV of 0.23] and efficient.
3. All materials are commercially available. No licensing fees are required.
4. Relative to traditional methodology, the assay is inexpensive and easy to perform
 - Materials costs are < \$ 0.5 per data point.
 - Mix and read

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