Original Article
DOI: 10.4274/tjps.galenos.2021.85530

Advantages and disadvantages of two in vitro assays in evaluating aromatase activity: “a cell-based and a cell-free assay”

Aromataz aktivitesini değerlendirmede iki farklı in vitro yöntemin avantajları ve dezavantajları: “bir hücre temelli ve bir hücreden ari yöntem”

Elif İnce Ergüç[1], Senem Özcan Sezer[2], HANDE GURER-ORHAN[2]
[1]Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Izmir Katip Celebi University, Izmir, Turkey
[2]Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Ege University, Izmir, Turkey

Corresponding Author Information
Elif İnce Ergüç
elif.ince@ikcu.edu.tr
02323293535
https://orcid.org/0000-0003-0764-7694
16.09.2021
15.11.2021

Abstract
INTRODUCTION: Aromatase is an enzyme catalyzes conversion of androgens to estrogens. While inhibition of aromatase is useful approach in the treatment of breast cancer, it may also have toxicological consequences due to its endocrine disrupting/modulating effect. In the present study, sensitivity and performance of two in vitro assays-a cell free and a cell-based - for evaluating aromatase activity were investigated by testing known aromatase inhibitors and partial validation of the methods was performed. Advantages and disadvantages of the methods were also discussed.
METHODS: Aromatase activity was evaluated via two in vitro models; direct measurement with a cell-free assay using a fluorescence substrate and recombinant human enzyme and indirect evaluation with a cell-based assay where cell proliferation was determined in estrogen receptor positive (ER+) human breast cancer cells (MCF-7 BUS) in the absence of estrogen and the presence of testosterone.
RESULTS: In the cell free direct measurement assay reference compounds ketoconazole and aminoglutethimide has been shown to inhibit the aromatase enzyme with IC50 values concordant with literature. In cell-based indirect measurement assay only ketoconazole inhibited cell proliferation in a dose-dependent manner with 3,47.10-7 M IC50. Inter -assay and intra-assay reproducibility of both methods were found to be in the acceptable deviation levels.
DISCUSSION AND CONCLUSION: Both methods can be applied successfully. However, to evaluate the potential aromatase activity of the novel compounds in vitro it seems better to perform both the cell-based and the cell-free assays which allows low-moderate biotransformation and eliminate cytotoxicity potential respectively.

Keywords: aromatase inhibition, cell-based assay, cell-free assay, in vitro
İngilizce Kısa Başlık: in vitro assays for aromatase activity

Öz
GİRİŞ ve AMAÇ: Aromataz, androjenlerin östrojenlere dönüşümünü katalize eden bir enzimdir. Aromataz inhibisyonu meme kanserinin tedavisinde faydalı bir yaklaşım iken bu enzimin inhibisyonunun endokrin bozucu/modülatör etki nedeniyle bazı toksikolojik sonuçları da olabilmektedir. Bu çalışmada, hücre temelli ve hücreden ari olmak üzere iki in vitro yöntemin aromataz aktivitesini ölçmedeki hassasiyeti ve başarısı bilinen aromataz inhibitörü bileşikler kullanılarak araştırılmış ve yöntemlerin kısmi validasyonları gerçekleştirilmiştir. Ayrıca kullanılan yöntemlerin avantajları ve dezavantajları da tartışılmıştır.
YÖNTEM ve GEREÇLER: Aromataz aktivitesi iki in vitro modelde araştırılmıştır; hücreden ari yöntem floresans bir substrat ve rekombinant insan enziminin kullanıldığı doğrudan aktivite ölçüm yöntemi iken hücre temelli yöntem östrojensiz ama testosteron içeren besi ortamında östrojen reseptör pozitif insan meme kanser hücrelerinin (MCF-7 BUS) proliferasyonunun belirlendiği dolaylı bir ölçüm yöntemidir.
BULGULAR: Hücreden ari doğrudan ölçüm yönteminde referans bileşikler olan ketokonazol ve aminoglutetimitin aromataz enzimini inhibe ettiği ve elde edilen IC50 değerlerinin literatür ile uyumlu olduğu bulunmuştur. Hücre temelli indirekt ölçüm yönteminde sadece ketokonazol 3,47.10-7 M IC50 değeri ile hücre proliferasyonunu doza bağlı şekilde inhibe etmiştir. Her iki yöntemin de gün içi ve günler arası tekrarlanabilirlik değerleri kabul edilebilir sapma düzeylerinde olduğu bulunmuştur.
TARTIŞMA ve SONUÇ: Her iki yöntem de başarılı bir şekilde uygulanabilmektedir. Ancak sonuçların daha gerçekçi yorumlanabilmesi adına yeni bileşiklerin aromataz aktivitesi üzerine etkilerini değerlendirmede hücre temelli ve hücreden ari iki testin birlikte yapılması gerektiği düşünülmektedir.
Anahtar Kelimeler: aromataz inhibisyonu, hücre temelli yöntem, hücreden ari yöntem, in vitro
Türkçe Kısa Başlık: aromataz aktivitesinde in vitro yöntemler

INTRODUCTION

Endocrine disruptors are exogenous compounds which causes adverse effects via altering endocrine system functions (1). These compounds have several mechanisms of action, one is to modulate the cytochrome P450 (CYP450) enzymes involved in steroid hormone synthesis/metabolism (2).

Aromatase is a member of CYP450 enzyme superfamily which catalyzes conversion of androgens to estrogens at the last step of steroidogenesis (3). This conversion by aromatase is a rate-limiting step in estrogen synthesis and the enzyme is responsible for maintaining a homeostatic balance between androgens and estrogens. Aromatase is involved in numerous physiological functions such as reproduction, development, behavior as well as pathologies such as hormone dependent cancers. Especially in postmenopausal woman local estrogen synthesis via aromatization of androgens plays an crucial role in the development of estrogen-dependent breast cancer (3). Therefore, inhibition of aromatase is an useful approach to treat hormone- dependent breast cancer. On the other side inhibition of this enzyme may have toxicological consequences because of endocrine disruption/modulation.

In this paper aromatase activity was measured by two *in vitro* assays. The first one is a high throughput screening assay where a fluorescence substrate and recombinant human enzyme is used, and the enzyme activity is detected directly via the use of the substrate. In the second assay enzyme activity is indirectly evaluated via proliferation of the estrogen receptor positive human breast cancer cells, MCF-7 BUS, with the presence of testosterone in an estrogen-free

medium. The sensitivity and performance of both assays were evaluated by testing known aromatase inhibitors and partial validation of the methods was performed. Advantages and disadvantages of cell-based and cell-free assay were discussed.

MATERIALS AND METHODS

MCF-7 BUS cells were kindly provided by Prof. Ana Soto from Tufts Institute and maintained at 37 ˚C in 5% CO_2 atmosphere in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Reference compounds (ketoconazole and aminoglutethimide) and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Thermo Fisher Scientific. Aromatase activity assay kit was purchased from Corning Incorporated (New York USA).

Direct measurement of aromatase activity

Direct measurement of aromatase activity was evaluated by CYP19A/MFC screening kit from Corning Incorporated (New York USA). Reaction substrate 7-methoxy-4-trifluoromethyl coumarin (MFC) is converted to 7-hydroxytrifluoromethyl coumarin (HFC) by aromatase in the presence of NADPH generating system. So, reduction of the fluorescence intensity refers to aromatase inhibitor activity (4,5). Enzyme reactions were performed, according to the manufacturer's protocol as indicated in detailed previously (6). IC_{50} values of reference materials were obtained by using GraphPad Prism5 software.

Intra-assay reproducibility was determined via calculation of mean and standard deviation values of the enzyme activity which are measured in 5 different wells in the same day while inter assay reproducibility was determined via calculation of the values from 3 different days.

Indirect measurement of aromatase activity

If the estrogen dependent cells are seeded in estrogen-depleted media cell proliferation occurs via aromatization of androgens. Thus aromatase activity can be measured indirectly in MCF-7 cells by evaluating cell viability in the medium with testosterone/ without estrogen, according to the method (7) with minor modifications as previously described (6).

Briefly, MCF-7 BUS cells were plated in 96-well plates at a density of 6000cells/well, in DMEM supplemented with 10% FBS and incubated at 37 ˚C in a humid atmosphere containing 5% CO2. After 48 hour for attachment medium was replaced to DMEM without phenol red supplemented with 10 %charcoal stripped FBS, 1% sodium pyruvate and 1% non-essential amino acid solution containing either testosterone (10 µM) alone or testosterone and tested compounds together. A control group was also included, in which the cells are grown in estrogen-depleted media without any testosterone or test molecule. Following the 5 days incubation period cell viability was assessed via MTT assay. The medium was removed, cells were washed with phosphate buffered saline (PBS) and then incubated with MTT (1 mg/ml) for 4 h at 37 °C. MTT solution was removed, and formazan crystals were dissolved in DMSO. The absorbance was recorded at 550 nm on a microplate reader. The ratio of the absorbance of treated samples to the absorbance of control (taken as 100%) was expressed as % cell viability.

To evaluate performance and the sensitivity of the assay cells were incubated with 17-β-estradiol (1nM), and with testosterone (1 and 10 µM) for 5 days in the presence and absence of aromatase inhibitors.

Statistical analysis

Data were expressed as means ± SD. Statistical analysis was performed by using Student's t-test. Differences were considered significant $p<.05$. p Values are given in figure legends.

RESULTS and DISCUSSION

In the present study aromatase activity was measured by using two different *in vitro* assays; a cell free, direct measurement assay and a cell-based, indirect measurement assay. Performance and the sensitivity of the assays were compared via using reference compounds, ketoconazole, general CYP inhibitor and a well-known aromatase inhibitor aminoglutethimide.

In the cell free aromatase activity assay, human recombinant aromatase enzyme (CYP19) and a fluorescence substrate 7-methoxy-4-trifluoromethyl coumarin (MFC) was used. In NADPH generating system, fluorescence intensity is reduced as a result of demethylation of MFC by CYP19 and enzyme activity is calculated fluorometrically. Since this method is performed in 96 well plate format and allows high throughput screening, different groups have been previously used it in evaluating novel aromatase inhibitors (8).

Sensitivity and performance of the direct measurement assay in our laboratory conditions was evaluated via using a known aromatase inhibitor aminoglutethimide and a general CYP inhibitor ketoconazole. Ketoconazole and aminoglutethimide has been shown to inhibit the aromatase enzyme in the direct measurement assay with $2{,}3.10^{-6}$ M and $4{,}7.10^{-7}$ M IC_{50} values respectively (Table 1). Compared to the IC_{50} values from literature, our results were found to be concordant with the literature (Table 1).

Inter-assay and intra-assay reproducibility of the direct measurement assay was also evaluated by measuring enzyme activity in the presence of a fixed amount of recombinant enzyme and substrate (50 µM MFC). Intra –assay and inter-assay coefficient of variation values were % 2,8 and % 10 respectively (Table 2). According to these results, direct measurement assay is found to be in an acceptable reproducibility range.

Indirect measurement assay is performed in MCF-7 BUS cells by evaluating proliferation of the cells in estrogen deprived but testosterone added media. MCF-7 BUS is a well-established estrogen receptor positive cell line and is dependent to estrogen for proliferation. Cells are also known to possess aromatase activity (9). In the present study we also performed western blot analysis (data not shown) and confirmed the expression of aromatase in our cell line. Since cell proliferation is dependent on the presence of estrogens, in the absence of estrogen but presence of testosterone, cell proliferation depends on aromatization of testosterone to estrogen via aromatase enzyme (7) which is the principle of this indirect measurement assay.

Performance and the sensitivity of the indirect measurement assay was evaluated via using reference compounds (estradiol and testosterone) (Figure 1) in the presence and the absence of aromatase inhibitors. As expected, 17-β-estradiol significantly increased cell proliferation (approximately 2 fold) comparing to the control group. Testosterone also increased cell proliferation in a dose dependent manner. This effect was reduced by the aromatase inhibitors aminoglutethimide (100 µM) and ketoconazole (5 µM) indicating that cell proliferation was estrogen dependent and catalyzed with the aromatase activity of the cells (Figure 1).

Thereafter, cells were incubated with 10µM testosterone and varying concentrations of ketoconazole or aminoglutethimide for 5 days to obtain IC_{50} values in the indirect measurement assay. We found that ketoconazole (0,05-20 µM) inhibited cell proliferation as a result of aromatization of testosterone to estradiol (Figure 2A) with $3{,}47.10^{-7}$ M IC_{50} value. On the other hand aminoglutethimide did not inhibited cell proliferation in a dose dependent manner (data not shown). Therefore IC_{50} value of aminoglutethimide could not be calculated. Inter and intra assay reproducibility of the indirect measurement assay was evaluated. Intra-assay reproducibility was calculated using the results of the estradiol and testosterone obtained from four different wells on the same day. % coefficient variation values of

testosterone and estradiol were % 2,7 and 7,4 respectively (Table 3). For the inter-assay reproducibility, % coefficient variation values of testosterone and estradiol was calculated as % 2,5 and 2,6 respectively which were obtained from results of the experiments conducted in four different days. According to these results, it was concluded that the indirect measurement assay has high rate of successful replications and works successfully.

While the IC_{50} value of ketoconazole was found to be 2.5 µM in the direct activity measurement method, it was about 10 times lower (0.35 µM) in the indirect aromatase activity. This difference is thought to be because of possible metabolites of ketoconazole. Nevertheless, in a study conducted in a primary culture system of rat hepatocytes, major metabolite of ketoconazole (N-deacetylated ketoconazole) has been shown to have more potent cytotoxic effect in MTT assay (10). Therefore, the reason for this difference may be due to potential cytotoxic, ER antagonist or of aromatase expression modulator effects of the possible metabolites. It was also demonstrated by Yan et.al. that ketoconazole downregulates aromatase gene expression in goldfish (11). So, lower IC_{50} value of ketoconazole in cell based indirect measurement assay may be the consequence of both inhibition of aromatase enzyme and downregulation of aromatase expression.

It should also be kept in mind that, substance concentration interacting with the active site of aromatase enzyme cannot be the same in the cell based and the cell free assay. There are lots of biological steps in cell-based assay such as passaging through the membranes, entering the cells and metabolism which can affect the results.

On the other hand, the cell-based method has a metabolic capacity compared to the direct measurement assay. Therefore, it is possible to evaluate potential effects of the active metabolites which makes it more advantageous method in reflecting the physiological state in a more realistic way.

CONCLUSION

In conclusion, partial validation results of the present study indicate that direct and indirect measurement assays can be used for evaluating aromatase activity, but both of them have some advantages and disadvantages indeed. Therefore, it seems better to perform these cell based and cell free assays together to evaluate potential aromatase activity of the novel compounds in order to comment on the results correctly. Additional tests like cytotoxicity, effect on enzyme expression levels should also be performed to prevent misinterpretation of the indirect measurement assay results.

ACKNOWLEDGEMENTS

This work was supported by The Scientific and Technological Research Council of Turkey (TUBITAK) Research and Development Grant 112S375, Ege University Research and Development Grant 13BIL009 and Grant 13ECZ008. Ege University FABAL facilities were used for biological activity assays.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Damstra T, Barlow S, Bergman A, Kavlock R, Van Der Kraak G. World Health Organization: Global assessment of the state-of-the-science of endocrine disruptors. WHO. 2002;
2. Sanderson JT. The Steroid Hormone Biosynthesis Pathway as a Target for Endocrine-Disrupting Chemicals. Toxicol Sci [Internet]. 2006 Nov [cited 2018 May 9];94(1):3–21. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16807284
3. Simpson E, Rubin G, Clyne C, Robertson K, O'Donnell L, Davis S, et al. Local estrogen biosynthesis in males and females. Endocr Relat Cancer [Internet]. 1999 Jun [cited 2018 Oct 6];6(2):131–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10731101
4. Maiti A, Reddy PVN, Sturdy M, Marler L, Pegan SD, Mesecar AD, et al. Synthesis of casimiroin and optimization of its quinone reductase 2 and aromatase inhibitory activities. J Med Chem [Internet]. 2009 Apr 9 [cited 2021 May 9];52(7):1873–84. Available from: https://pubmed.ncbi.nlm.nih.gov/19265439/
5. Stresser DM, Turner SD, McNamara J, Stocker P, Miller VP, Crespi CL, et al. A high-throughput screen to identify inhibitors of aromatase (CYP19). Anal Biochem [Internet]. 2000 Sep 10 [cited 2021 May 9];284(2):427–30. Available from: https://pubmed.ncbi.nlm.nih.gov/10964434/
6. Ozcan-Sezer S, Ince E, Akdemir A, Öztürk Ceylan Ö, Suzen S, Gurer-Orhan H. Aromatase Inhibition by 2-methyl indole hydrazone derivatives evaluated via molecular docking and in vitro activity studies. Xenobiotica. 2018 May 28;1–24.
7. Cos S, Martínez-Campa C, Mediavilla MD, Sánchez-Barceló EJ. Melatonin modulates aromatase activity in MCF-7 human breast cancer cells. J Pineal Res [Internet]. 2005 Mar [cited 2018 Feb 20];38(2):136–42. Available from: http://doi.wiley.com/10.1111/j.1600-079X.2004.00186.x
8. Bonfield K, Amato E, Bankemper T, Agard H, Steller J, Keeler JM, et al. Development of a new class of aromatase inhibitors: Design, synthesis and inhibitory activity of 3-phenylchroman-4-one (isoflavanone) derivatives. Bioorganic Med Chem [Internet]. 2012;20(8):2603–13. Available from: http://dx.doi.org/10.1016/j.bmc.2012.02.042
9. Zhou D, Wang J, Chen E, Murai J, Siiteri PK, Chen S. Aromatase gene is amplified in MCF-7 human breast cancer cells. J Steroid Biochem Mol Biol [Internet]. 1993 Aug [cited 2018 Oct 7];46(2):147–53. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8664162
10. Rodriguez RJ, Acosta D. N-Deacetyl ketoconazole-induced hepatotoxicity in a primary culture system of rat hepatocytes. Toxicology [Internet]. 1997 Feb 28 [cited 2021 May 10];117(2–3):123–31. Available from: https://pubmed.ncbi.nlm.nih.gov/9057891/
11. Yan Z, Lu G, Ye Q, Liu J. Modulation of 17β-estradiol induced estrogenic responses in male goldfish (Carassius auratus) by benzo[a]pyrene and ketoconazole.
12. Wouters W, De Coster R, Goeminne N, Beerens D, van Dun J. Aromatase inhibition by the antifungal ketoconazole. J Steroid Biochem [Internet]. 1988 [cited 2018 Jul 2];30(1–6):387–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3386265

Table 1. IC_{50} values of ketoconazole and aminoglutethimide that were obtained from literature and from direct aromatase activity assay in the present study (12).

Reference Compounds	Literature IC_{50}	Detected IC_{50}
Ketoconazole	$2{,}0.10^{-6}$ M	$2{,}3.10^{-6}$
Aminoglutethimide	$6{,}0.10^{-7}$ M	$4{,}7.10^{-7}$ M

Table 2. Inter-assay and intra-assay reproducibility values of direct aromatase activity assay. FI: Fluorescence intensity

	Intra-assay reproducibility	Inter-assay reproducibility
Mean of FI ± SD	2,33 ± 0,07 (n=5)	2,27 ± 0,23 (n=3)
% Coefficient of variation	2,8	10

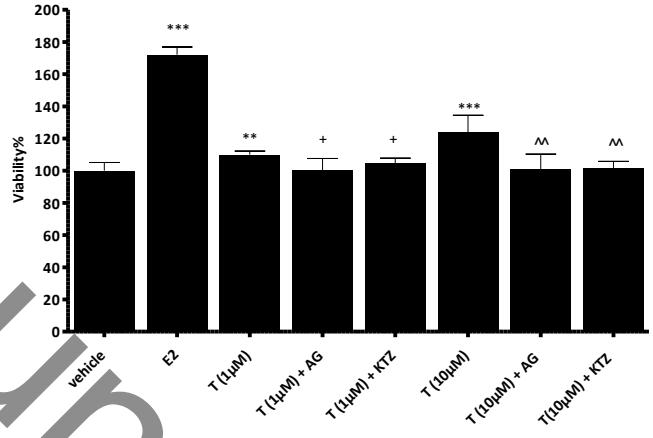
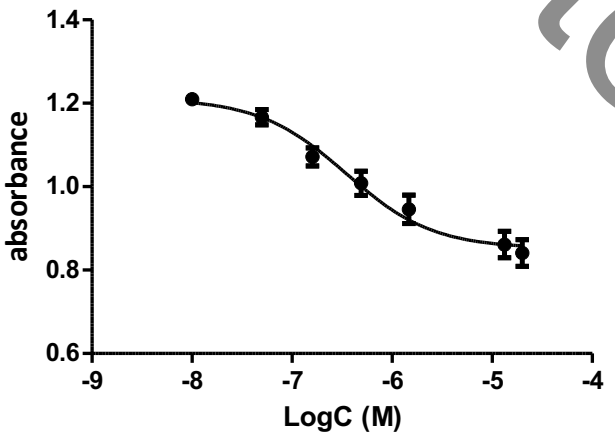


Figure 1. Effect of testosterone, aminoglutethimide (100μM) and ketoconazole (5μM) on MCF-7 BUS cell proliferation. Bars show percentage viability values compared to control group (mean ± SD). Statistical analysis was performed by using Student's t-test.
** $p<0.005$ versus vehicle; *** $p<0.001$ versus vehicle; + $p<0.05$ versus T(1μM); ^^ $p<0.005$ versus T(10 μM)



IC50	3.465e-007

Figure 2. Inhibitory effect of ketoconazole on indirect aromatase activity. Cells were incubated with testosterone (10μM) and ketoconazole for 5 days.

Table 3. Inter-assay and intra-assay reproducibility values for indirect aromatase activity measurement assay.

	Intra-assay reproducibility (n=4)		Inter-assay reproducibility (n=4)	
	Mean of % control ± SD	% Coefficient of variation	Mean of % control ± SD	% Coefficient of variation
Testosterone (10^{-5} M)	134 ± 4	2,7	134 ± 3	2,5
Estradiol (10^{-9} M)	202 ± 15	7,4	199 ± 5	2,6