Original Research Article

In vivo evaluation of the aromatase inhibition by 4-((1H-imidazol-1-yl) methyl)-2-(4-fluorophenyl)-8-phenylquinoline

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Abstract

Objective: Some of aromatase inhibitors (AIs) are Food and Drug Administration-approved agents which are used as first-line therapy in the treatment of endocrine-responsive breast cancer. In this study, we aimed to develop new quinoline derivative with higher specificity and potency.

Materials and Methods: The *in vivo* aromatase inhibition of these compounds was evaluated by measuring the inhibition of the androstenedione-induced uterine hypertrophy. The selectivity of aromatase inhibition has been investigated by the inhibition of adrenocorticotropic hormone stimulation on the plasma concentrations of aldosterone and cortisol.

Results: Letrozole (10 μg/kg) could significantly inhibit uterine hypertrophy in positive control group that received androstenedione 30 mg/kg/day. Furthermore, quinoline derivative could decrease the androstenedione-induced uterine hypertrophy in a dose-dependent manner. Interestingly, there was no significant difference between inhibitory potency of letrozole and quinoline derivatives. High dose of letrozole could significantly decrease the serum concentration of aldosterone and cortisol as compared to control group. On the other hand, the same doses of quinoline derivative were administered; it did not show any significant effects on the serum concentration of either aldosterone or cortisol.

Conclusion: This report introduced a new compound that can be considered as new lead for further investigation to explore the more potent and more selective AIs.

Keywords: Aromatase inhibitors, breast cancer, estrogen, hypertrophy, quinoline

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INTRODUCTION

The most common malignancy in women is breast cancer that is the second cause of cancer death in woman after lung cancer.[1] The conventional treatment modalities for breast cancer are surgery, radiation therapy, hormone therapy, chemotherapy, and/or a combination of them.[2]

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About 75% of breast cancers are estrogen receptor or progesterone receptor positive or both of them. These tumors are dependent on estrogen stimulation for their development and continued growth.[3] Hence, scientists expected that deprivation of estrogenic signaling can be effective in prevention of the appearance of these cancers and regression of established tumors.[4]

Estrogen biosynthesis differs according to menopausal status, in which the ovaries are the major source of estrogen in premenopausal women, while peripheral tissues are more important in postmenopausal patients.[5] Aromatase enzyme is responsible for the conversion of androgen to estrogen that is the last and rate-limiting step in the estrogen biosynthesis in peripheral tissues.[6,7] It has been showed that aromatase inhibitors (AIs) can decrease estrogen production by > 90% in postmenopausal women, so they have generated enormous attention in treating endocrine-responsive breast cancer in women without functioning ovaries.[8]

AIs are classified as Type I or Type II inhibitors based on the reversibility of their inhibitory function. Type I inhibitors, steroidal inhibitors, regularly have an androgen structure and covalently bind to substrate-binding site of aromatase enzyme and cause an irreversible inhibition of aromatase enzyme, thus earning the name "suicidal inhibitors." In contrast, majority of Type II inhibitors, nonsteroidal inhibitors, are azoles and bind reversibly to the cytochrome P450 moiety of aromatase enzyme, resulting in competitive inhibition. Both types of AIs are subclassified as first, second, or third generation according to their specificity and potency for the enzyme inhibition.[9,10] The third generation of Type II AIs (i.e., anastrozole, letrozole, and vorozole) is the most potent, most selective, and least toxic AIs, and they can decrease serum estradiol by >95%. Anastrozole and letrozole are Food and Drug Administration-approved agents which are used as first-line therapy in the treatment of estrogen-responsive cancer in postmenopausal women.[11,12]

Researchers are interested in developing the new nonsteroidal AIs with higher specificity and potency and lower side effects. The structure of nonsteroidal AIs contains two main parts. One part which is needed to interact with the heme iron atom of the cytochrome P450 of aromatase is the azole part with a nitrogen atom and the other part which mimics the steroid ring of the substrate is the bulky aryl part.[13,14] Synthesis of nonsteroidal AIs almost needs several chemical reactions and/or difficult purification process.

Ghodsi *et al.* have synthesized the quinoline derivatives as nonsteroidal AIs, and *in vitro* biological evaluation was also studied. The compound 4-((1H-imidazol-1-yl) methyl)-2-(4-fluorophenyl)-8-phenylquinoline (8b) [Figure 1] as one of the derivatives of nonsteroidal AIs inhibits aromatase enzyme activity more than reference drug letrozole.[15]

The main objective of this study is to investigate the anti-aromatase activity and anticancer activity of 4-((1H-imidazol-1-yl) methyl)-2-(4-fluorophenyl)-8-phenylquinoline using *in vivo* studies. The *in vivo* aromatase inhibitory activity was determined using a standard assay based on the inhibition of uterine hypertrophy occasioning after treatment of sexually immature female rats with a standard dose of androstenedione.

MATERIALS AND METHODS

Materials

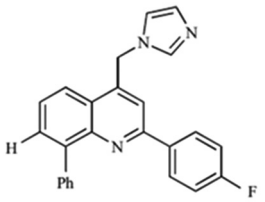
All commercial reagents and solvents used in this study were purchased from Merck KGaA and Sigma-Aldrich Chemical.

Apparatus

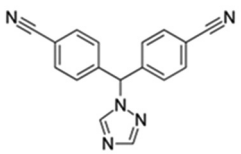
Melting points were determined with a Stuart- F 500MA apparatus. Infrared spectra were acquired using a Unicam SP 1100 spectrometer. ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra were collected to confirm the structure. ^{1}H NMR was performed in $CDCl_3$ and dimethyl sulfoxide (DMSO) using an NMR instrument (Bruker AC-80) at 300 MHz at room temperature through tetramethylsilane as an internal standard.

Procedure for preparation of 4-((1H-imidazol-1-yl) methyl)-2-(4-fluorophenyl)-8-phenylquinoline

To a solution of the appropriate alcohol (1.59 mmol) in 15 ml N-methyl-2-pyrrolidone, carbonyl 1, 1-diimidazole (1.29 g, 7.9 mmol) was added. Then, the solution was



4-((1H-Imidazol-1-yl) methyl)-2-(4-fluorophenyl)-8-phenylquinoline



Letrozole

Figure 1: Scheme of 4-((1H-imidazol-1-yl) methyl)-2-(4-fluorophenyl)-8-phenylquinoline and letrozole

refluxed for 20 h at 170°C. Then, it is cooled to room temperature, and water (50 ml) was added and extracted with ethyl acetate. The obtained organic phases were washed with brine and water, dried over Na_2SO_4, and evaporated. Then, the obtained product was purified by flash chromatography on silica gel (dichloromethane/methanol, 90:10 v/v) (yield: 13%–62%).

Yield: 21%; cream crystalline powder; mp = 157–158°C; ^{1}H NMR (CDCl3): δ (ppm) 5.74 (s, 2H, CH2), 7.07 (s, 1H, imidazole H5), 7.12 (t, 2H, 4-fluorophenyl H3 and H5, J = 8.62 Hz), 7.27 (s, 1H, imidazole H2), 7.32 (s, 1H, imidazole H4), 7.48 (t, 1H, phenyl H4, J = 7.35 Hz), 7.55 (t, 2H, phenyl H3 and H5, J = 7.54 Hz), 7.68 (t, 1H, quinoline H6, J = 7.79 Hz), 7.76 (s, 1H, quinoline H3), 7.81 (d, 2H, phenyl H2 and H6, J = 7.46 Hz), 7.86 (d, 1H, quinoline H5, J = 6.94 Hz), 7.92 (d, 1H, quinoline H7, J = 8.26 Hz), and 8.02 (t, 2H, 4-fluorophenyl H2 and H6, J = 7.04 Hz); liquid chromatography-mass spectrometry (electrospray ionization): 380.9 (M + 1) +100; and Anal. Calcd. for C25H18N3F: C, 79.14; H, 4.78; N, 11.07. Found: C, 79.38; H, 4.92; N, 11.22.[15]

In vivo aromatase activity evaluation

Aromatase inhibitory activity assay in immature female rat

The *in vivo* aromatase inhibitory activity was determined using a standard assay based on the inhibition of uterine hypertrophy occasioning after treatment of sexually immature female rats with a standard dose of androstenedione.[16] Androstenedione was dissolved in olive oil for intraperitoneal (IP) injection with standard dose 30 mg/kg/day. Letrozole and 4-((1H-imidazol-1-yl) methyl)-2-(4-fluorophenyl)-8-phenylquinoline (quinoline derivative) were dissolved in DMSO and diluted by distilled water to different concentrations (final DMSO concentration was < 1%). The immature female rats with the average weight of 35 ± 5 g were selected and randomly divided into eight experimental group. Group 1: negative control has been orally treated with olive oil, Group 2: positive control has received androstenedione 30 mg/kg/day (IP) with oral dose of olive oil, and other groups were treated with different doses of either quinoline derivative (1, 10, 100, and 200 μg/kg) or letrozole (10 μg/kg) orally as well as IP injection of standard dose of androstenedione (30 mg/kg/day). Treatments were administered once a day for 4 consecutive days and 2 h after the last treatment, and animals were sacrificed as shown in Figure 2. The uterus was dissected and weighted in a Mettler AT261 Delta Range balance (±0.1 mg), and body weights were measured before sacrifice by a Mettler PM4600 Delta Range balance (±10 mg). Results were expressed

Figure 2: Dissected uterus

as uterine relative weight as a ratio of (uterine wet weight [mg]/body weight [g]) treated sample/(uterine wet weight [mg]/body weight [g]) negative control.[17]

In vivo aromatase selectivity assay

It has been showed that adrenocorticotropic hormone (ACTH) significantly raises plasma concentrations of aldosterone and cortisol in male rats.[18] Eighteen male rats with the average weight of 225 ± 25 g were divided into three groups of six each and stored in single cages. On the first day, all animals received a subcutaneous injection of 10 mg/kg of ACTH (Synacthen®, Ciba-Geigy Ltd., Switzerland). Sixteen hours after the ACTH administration, control group of animals received normal saline (P.O.) and other groups were treated with 4 mg/kg of either compound 8b or letrozole (P.O.) dissolved in water. Two hours later, all animals were beheaded and whole blood was collected in a covered test tube. The blood was allowed to clot by leaving it undisturbed at room temperature for about 15–30 min. Then, the clot was removed by centrifuging at 3000 ×*g* for 10 min at 4°C, and the liquid component (serum) was transferred into a clean polypropylene tube and stored at 2–8°C for immediately hormone determination or stored at −20°C for later analyses. Aldosterone and cortisol were measured by ELISA kits.

Statistical analysis

Statistical analyses were performed using SPSS 16.00 software. Results (mean ± standard deviation) were reported as three independent occasions each performed in duplicate or triplicate. Statistically significant differences between values were calculated using multivariate analysis of variance with Tukey's *post hoc* or Student's *t*-test, and a value of $P < 0.05$ was considered statistically significant.

RESULTS

Aromatase inhibition *in vivo* has been studied by measuring the inhibition of the androstenedione-induced uterine hypertrophy in immature female rats. The results showed that letrozole (10 μg/kg) could significantly inhibit uterine hypertrophy in positive control group that received androstenedione 30 mg/kg/day. Furthermore, quinoline derivative could decrease the androstenedione-induced uterine hypertrophy in a dose-dependent manner. Interestingly, there was no significant difference between inhibitory potency of letrozole and quinoline derivative [Figure 3]. Letrozole inhibits the androstenedione-induced uterine hypertrophy in immature female rats with an IC_{50} of 1.5 μg/kg, and IC_{50} of quinoline derivative was 4.1 μg/kg as shown in Figure 4.

Effects of single oral doses of quinoline derivatives and letrozole on serum concentrations of cortisol and aldosterone as an *in vivo* aromatase selectivity assay are shown in Figure 5. Male rats received a subcutaneous injection of 10 mg/kg of ACTH, and 16 h later, they were treated with 4 mg/kg of either compound 8b or letrozole (control group received normal saline). After 2 h, all animals were beheaded, and their whole blood was collected. Serum concentration of aldosterone and cortisol was measured successful by ELISA assay. The results showed that high dose of letrozole could significantly decrease the serum concentration of aldosterone and cortisol as compared to control group.

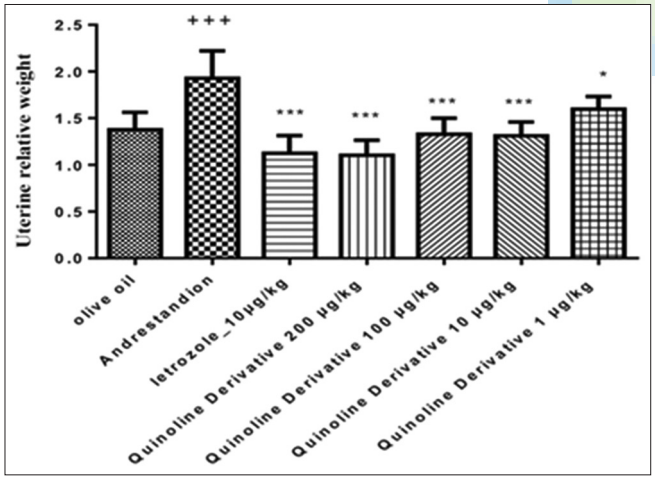


Figure 3: Inhibition of the androstenedione-induced uterine hypertrophy by quinoline derivatives and letrozole as an assay for the inhibition of aromatase *in vivo*. The immature female rats were treated with different doses of either quinoline derivatives or letrozole orally as well as IP injection of standard dose of androstenedione (30 mg/kg/day) for 4 consecutive days. Negative control group has been orally treated with olive oil, and positive control has received androstenedione. Results were expressed as uterine relative weight as a ratio of (uterine wet weight [mg]/body weight [g]) treated sample/(uterine wet weight [mg]/body weight [g]) negative control. The values are shown as mean ± standard error of the mean of determinations carried out of six rats in each group. +++$P \leq 0.001$ to negative control and ***$P \leq 0.001$ to positive control

DISCUSSION

Nowadays, aromatase competitive inhibitors are the first choice as adjuvant therapeutics for postmenopausal breast cancer patients. Researchers are interested in developing the new nonsteroidal aromatase competitive inhibitors with higher specificity and potency and lower side effects. The chemical structure of nonsteroidal AIs consists of two parts. One part is the azole part having nitrogen atom which binds to the heme iron atom of aromatase. The second part is usually the bulky aryl part that is hydrophobic and acts the same as the steroid ring of the substrate.[13] Ghodsi *et al.* designed and synthesized the quinoline derivatives (8a-g) as nonsteroidal AIs, and *in vitro* biological evaluation was also studied.[15] The ability of the quinoline derivatives 8a-g to inhibit aromatase enzyme activity was determined in subconfluent H295R cells by the titrated water release assay using [1-3H (N)]-androst-4-ene-3,17-dione, and letrozole (potent AI) at 5 μM concentration was used as positive control according to the previously reported method. Unexpectedly, most of the compounds were not able to inhibit aromatase enzyme activity at concentration ≤10 μM (in comparison with 5 μM letrozole). However, the compounds 8a, 8b, and 8c in this series display inhibition of aromatase enzyme activity at concentration ≤10 μM and 8b inhibited the enzyme activity strongly and was more potent than the reference drug letrozole. These results indicated that some of the lipophilic compounds, such as 8a, 8b, and 8c, apparently penetrate cells and inhibit aromatase activity. The significant inhibition of aromatase activity by compound 8b may be

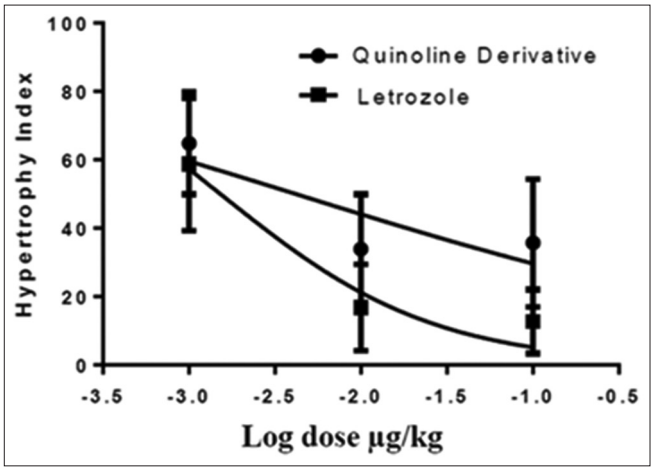


Figure 4: Uterine hypertrophy index of letrozole and quinoline derivative

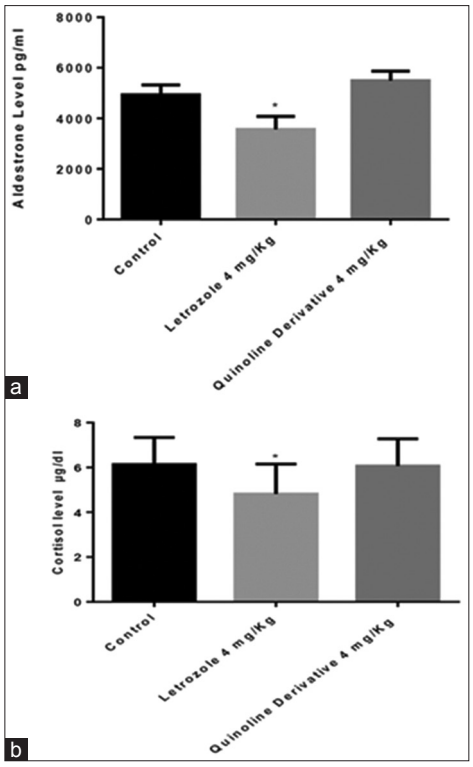


Figure 5: Effects of single oral doses of (a) quinoline derivatives and (b) letrozole on serum concentrations of cortisol and aldosterone as an *in vivo* aromatase selectivity assay. Male rats received a subcutaneous injection of 10 mg/kg of adrenocorticotropic hormone, and 16 h later, they were treated with 4 mg/kg of either compound 8b or letrozole (control group received normal saline). After 2 h, all animals were beheaded, and their whole blood was collected. Serum concentration of aldosterone and cortisol was measured by ELISA assay. The values are shown as mean ± standard error of the mean of determinations carried out of six rats in each group. ***$P \leq 0.001$

related to better interaction with amino acids present in the active site of aromatase. These quinolines 8a-g were more cytotoxic against MCF-7 cells in comparison with those of T47D. MCF-7 and T47D cell lines both are two estrogen-positive breast cancer cell lines and the amount of aromatase enzyme in MCF-7 cells is more than T47D cells, which may be due to their ability to inhibit aromatase or decrease aromatase activity. Furthermore, compound 8b that decreased the aromatase activity more than the others was not the most potent antiproliferative agent, suggesting other effects in addition to inhibition of aromatase activity.[15] Hence, the compound 8b (4-((1H-imidazol-1-yl) methyl)-2-(4-fluorophenyl)-8-phenylquinoline) among the other compounds (8a-g) was selected in this study for *in vivo* evaluation.

The *in vivo* effect of compound 8b has been studied by the measurement of the inhibition of androstenedione-induced uterine hypertrophy. In this method, androstenedione is aromatized to estrogen in the ovary that caused uterine hypertrophy. Therefore, inhibition of this uterine hypertrophy is considered as an index for aromatase inhibition *in vivo*. The results of this study showed that letrozole could significantly inhibit uterine hypertrophy that was the same as the previous reports that letrozole inhibits uterine hypertrophy with the ED_{50} (50% effective dose) 1–3 μg/kg.[16] Furthermore, these results indicated that the *in vivo* aromatase inhibitory potency of compound 8b was similar to letrozole that was in agreement with the results of *in vitro* studies. When the same doses of quinoline derivative 8b were administered, it did not show any significant effects on the serum concentration of either aldosterone or cortisol.

Previous studies showed that letrozole is highly selective for aromatase unlike first and second generation.[19] *In vivo* ACTH stimulation on the plasma concentrations of aldosterone and cortisol showed that compound 8b had no significant effect on either aldosterone or cortisol levels, even at a dose 400 times more than that required for *in vivo* inhibition of aromatase activity. Surprisingly, compound 8b showed more selective *in vivo* effects than letrozole in which letrozole (4 mg/kg) could significantly decrease the serum concentration of aldosterone and cortisol as compared to the control group.

CONCLUSION

In this study, we have described the *in vivo* evaluation of compound 8b as aromatase enzyme inhibitors. *In vivo* studies have shown that compound 8b is highly potent and selective AI which was similar to known AI letrozole and showed more selective *in vivo* effects than letrozole. Therefore, compound 8b can be considered as the new lead for further investigation to explore the more potent and more selective AIs with lower side effects compared to letrozole for hormone-dependent breast cancer.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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