The red clover (Trifolium pratense) isoflavone biochanin A inhibits aromatase activity and expression

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Biochanin A is an isoflavone isolated from red clover (Trifolium pratense), and is a commercially available nutraceutical for women suffering from postmenopausal symptoms. Isoflavones resemble the structure of oestrogen, and display agonistic and antagonistic interactions with the oestrogen receptor. Overexposure of oestrogen is a major contributing factor in the development of breast cancer, and cytochrome P450 (CYP) 19 enzyme, or aromatase, catalyses the reaction converting androgen to oestrogen. In the present study the effect of biochanin A on the gene regulation and enzyme activity of aromatase was investigated. By assaying MCF-7 cells stably transfected with CYP19, biochanin A inhibited aromatase activity and hampered cell growth attributing to the enzyme activity. In addition, 25 μM-biochanin A significantly reduced CYP19 mRNA abundance in the oestrogen-receptor-negative breast cancer cells SK-BR-3. The transcriptional control of the CYP19 gene is exon-specific, and promoter regions I.3 and II have been shown to be responsible for CYP19 expression in SK-BR-3 cells. Luciferase reporter gene assays also revealed that biochanin A could repress the transcriptional control dictated by the promoter regulation. Interestingly, genistein did not inhibit aromatase but it might down regulate promoter I.3 and II transactivation. Since genistein is a major metabolite of biochanin A, it might contribute to biochanin A’s suppressive effect on CYP19 expression. The present study illustrated that biochanin A inhibited CYP19 activity and gene expression.

Biochanin A: Aromatase: Breast cancer cells

Epidemiological studies have shown that the use of exogenous oestrogen¹² or an augmented endogenous oestrogen concentration³⁴ is associated with increased breast cancer risk. In both cell and animal models a causal relationship between oestrogen exposure and breast cancer has also been established⁵.

The cancer-inducing mechanisms of oestrogen in the breast can be multifaceted, and may participate in either the initiation or promotion stage. Oestrogen can be metabolised into various catechol oestrogens, and oestrogen-2-hydroxylase and oestrogen-4-hydroxylase are cytochrome P450 (CYP) enzymes that hydroxylate oestrogen at the C-2 and C-4 positions, respectively⁶. These hydroxylated metabolites can further be converted into quinone and semiquinone structures, which have been shown to be carcinogenic in animal models⁷,⁸. In addition, free radicals generated by some of these metabolites may cause oxidative DNA damage⁹. These genotoxic effects of oestrogen have been demonstrated in MCF-7 cells¹⁰ and rat mammary tissues¹¹. The notion that oestrogen promotes breast cancer is reinforced in a transgenic mouse model that develops spontaneous mammary tumours. Treatment with oestrogen accelerates the development of neoplastic lesions and carcinomas in these mice¹². Oestrogen-induced cell proliferation has been a major focus in breast cancer research. The retained mechanisms lie in the regulation of cell-cycle¹³,¹⁴ Bel-2 family protein expression¹⁵, and the interaction with plasma membrane receptors¹⁶. Oestrogen is synthesised from cholesterol in several steps, and CYP19 (aromatase) catalyses the final rate-limiting reaction. Aromatase is encoded by a single-copy gene¹⁷,¹⁸. The promoter utilisation for CYP19 regulation varies in different tissues, which provides the basis for tissue-specific expression¹⁹. Polymorphisms in the CYP19 gene have been associated with breast cancer risk²⁰. Many aromatase inhibitors have recently been developed, and some of them are promising agents for breast cancer prevention and therapy²¹.

Some flavones have been documented to be aromatase inhibitors. The A and C rings of flavones may compete with the C and D rings of the androgen structure for binding to the active site²². Isoflavones are another class of flavonoids whose chemical structures highly resemble that of flavones. Nevertheless, biochanin A is the only aromatase-inhibitory isoflavone with reported 50 % inhibitory concentration (IC₅₀).

Abbreviations: CYP, cytochrome P450; IC₅₀, 50 % inhibitory concentration.
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values varying from about 10 µM to 113 µM. We would like to examine and clarify the CYP19 inhibitory potential of the isoflavone and its effect on mRNA expression specifically driven by promoters I.3 and II in the present study. Since promoters I.3 and II are typically employed in breast cancerous tissues, suppression on these promoters would halt oestrogen supply for their growth and development.

Materials and methods

Chemicals

Biochanin A was obtained from Sigma Chemicals (St Louis, MO, USA). All chemicals, if not stated, were purchased from Sigma Chemicals.

Cell culture

The breast cancer cell line SK-BR-3 was a generous gift from Dr Richard K. W. Choy (Obstetrics and Gynaecology Department, the Chinese University of Hong Kong, Kowloon, Hong Kong) and MCF-7 cells were obtained from the American Tissue Culture Collection (Rockville, MD, USA). MCF-7 cells stably transfected with human CYP19 (MCF-7aro) were prepared as previously described.

The stably transfected MCF-7 cells were maintained in Eagle's minimum essential medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Invitrogen Life Technology, Rockville, MD, USA) and the selective antibiotic G418 (500 µg/ml; USB, Cleveland, OH, USA). SK-BR-3 cells were cultured in McCoy's 5A medium (Sigma Chemicals) with 10% fetal bovine serum. Cells were incubated at 37°C and 5% carbon dioxide, and were routinely sub-cultured when reaching 80% of confluency. Biochanin A was administered in the solvent vehicle dimethyl sulfoxide, and the concentration was limited to 0.1% (v/v). Cells were seeded uniformly at a density of 5 x 10^4 cells/mm² in all experiments.

‘In-cell’ aromatase assays

The assays were performed as previously described. In brief, MCF-7aro cells were seeded and allowed 1d for attachment. Assays were started by replacing the culture medium with serum-free medium containing [1β-3H]androstenedione and biochanin A. The final concentration of androstenedione was controlled at 25 nM, and the reaction was incubated at 37°C for 1h. A sample of the medium was then mixed with an equal volume of chloroform, followed by a 10 000g centrifugation at 4°C for 10 min. The aqueous phase was removed into a new tube containing 500µl of 5% activated charcoal suspension. After 30 min incubation, a sample of the supernatant fraction was taken out for scintillation counting. The protein content of the cells, on the other hand, was determined by using a BCA kit (Sigma Chemicals) after dissolving the cells in 0.5 M NaOH.

A similar protocol was applied to assays performed on SK-BR-3 cells, except that the assays were designed to determine the level of expression as described previously.

In brief, the cells were seeded in twelve-well plates at a density of 2 x 10^5 per well. Biochanin A was administered in the cell cultures and incubated for 24h before adding the substrate [1β-3H(N)]androst-4-ene-3, 17-dione. The reaction was further incubated at 37°C for 3h before the assay was performed.

For the enzyme inhibition assays performed on recombinant protein, 2 pmol Supersomes was incubated with biochanin A and the substrate-containing assay buffer (25 mM-[1β-3H(N)] androst-4-ene-3, 17-dione, 3.3 mM-MgCl₂, 100 mM-KH₂PO₄ (pH 7.4)). The reaction was initiated by the addition of 1.3 mM-NADPH and incubated at 37°C for 15 min.

Quantitative real-time polymerase chain reaction assay

In order to quantify the suppression of mRNA abundance, a cell line with reasonable amount of aromatase expression had to be used. Because aromatase mRNA was barely detectable in wild-type MCF-7 cells, we employed an aromatase-expressing cell line (SK-BR-3) for this assay. The real-time quantitative PCR was carried out as previously described by our laboratory. In brief, CYP19 and β-actin cDNA fragments were amplified and cloned into pGEMT-Easy vector (Promega Corp., Madison, WI, USA) as templates for quantifying the absolute amount of mRNA expression. Plasmids containing the respective amplicon – pGEMT-CYP19 and pGEMT-β-actin – were sequenced and stored at −20°C until use. SK-BR-3 cells were cultured and treated as described earlier. After 24h of treatment, total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and purity of the isolated RNA were determined by the absorbance reading observed at 260 and 280 nm. Total RNA (3 µg), oligo-dT, and MLV Reverse Transcriptase (USB Corporation, Cleveland, OH, USA) were used for first strand synthesis. Target fragments were quantified by real-time PCR and an Opticon™ 2 system (MJ Research, Waltham, MA, USA). CYP19 copy number was determined by absolute quantification. A standard curve was constructed by 10-fold serial dilutions from 10 to 10⁷ copies amplified from pGEMT-CYP19 or pGEMT-β-actin. Sample copy number was read from the standard curve. A SYBR green PCR Master Mix Reagent kit was obtained from Applied Biosystems and PCR reactions were set up as described in the manual. A typical reaction contained 200 nmol/l of forward and reverse primer, 2 µl cDNA and the final reaction volume was 20 µl. The reaction was initiated by preheating at 50°C for 2 min, followed by 95°C for 10min. Subsequently, forty-five amplification cycles were then carried out with 15s denaturation at 95°C and 1min annealing and extension at 58°C. Copies of β-actin RNA were also determined and used for normalisation. The forward and reverse primers designed for CYP19 were 5'-ATC TCT GGA GAC ACA TTC ATTA-3' and 5'-CTG ACA GAC CCT TCA TAA AGA AGGG-3'; the forward and reverse primers for β-actin were 5'-CAC CAA CTC GGA CGA CAT-3' and 5'-AGG CTT ACA GGG ATA GCA-3'. Dissociation curve and gel image analysis did not review non-specific amplifications generated from these primers.
Measurement of cell viability

Cell number was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) staining as described by Mosmann. Briefly, MCF-7aro cells were seeded in ninety-six-well plates and maintained in Eagle’s minimal essential medium supplemented with 10% charcoal dextran-treated serum (HyClone, Logan, UT, USA). The cells were allowed 24 h for attachment and they were treated with testosterone and/or biochanin A for 48 h. At the end of the treatment, 50 µl of MTT (1 mg/ml) was added to the cells and incubated at 37°C for 4 h. Cell viability was assessed with respect to the absorbance at 544 nm.

Luciferase gene reporter assay

Construction of CYP19 promoter-driven reporter plasmid.

A human CYP19 gene fragment (~446/+118) upstream to exon II was amplified from genomic DNA isolated from SK-BR-3 cells. The promoters I.3 and II26 have been reported to be associated with CYP19 expression in breast cancer cells. Primers were designed with the incorporation of KpnII and XhoI restriction sites. The amplified products were then digested and subcloned into a firefly luciferase reporter vector pGL3 basic (Promega Corp.), and the sequences were verified.

Dual luciferase assays. SK-BR-3 cells were plated in twenty-four-well dishes. After 24 h, the cells were transiently transfected with 0.25 µg of the CYP19 reporter plasmid and 20 ng of renilla luciferase control vector pRL (Promega Corp.) in LipofectAmine reagent (Invitrogen Life Technologies). After 1 d, the medium was removed and the cells were treated with biochanin A for 24 h. The cells were lysed and the activities of the luciferases were determined using the Dual-Luciferase Assay Kit (Promega Corp.). The luciferase bioluminescence was quantified by using a FLUOstar plate reader (BMG Labtechnologies GmbH, Offenburg, Germany). The transcription activity of the CYP19 promoter represented by firefly luciferase light units were then normalised with that of renilla luciferase.

Western analysis

Cells were washed once by PBS (pH 7.4) and harvested into a 1.5 ml microtube with 0.5 ml lysis buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS). The lysis buffer contained protease inhibitors (phenylmethylsulfonyl fluoride (40 µg/ml), aprotinin (0.5 µg/ml), leupeptin (0.5 µg/ml), 1-1 mM EDTA and pepstatin (0.7 µg/ml)). The harvested cells were then lysed with a cell disruptor (Branson Ultrasonics Corp., Danbury, CT, USA) on ice for 30 s. The protein concentration of cell lysate was determined by the DC protein assay (BioRad, Danbury, CT, USA) on ice for 30 s. The protein concentration then lysed with a cell disruptor (Branson Ultrasonics Corp., Danbury, CT, USA) and the harvested cells were incubated at 37°C for 4 h. Cell viability was assessed with respect to the absorbance at 544 nm.

Biochanin A inhibited aromatase enzymatic activity

Enzyme inhibition assay performed on MCF-7aro cells and recombinant protein. A previous study31 has shown that MCF-7aro cells can be used for enzyme inhibition analysis. Biochanin A displayed an inhibitory effect with an IC50 value of about 8 µM in the MCF-7aro cells (Fig. 1 (A)). No significant drop in activity was observed in other isoflavones. The enzyme inhibition was further confirmed in the recombinant enzyme system (human CYP19 Supersomes®; BD Gentest, Woburn, MA, USA) and the IC50 value was determined to be 12.5 µM (Fig. 1 (B)).

Enzyme kinetic assay. Five concentrations, i.e. 0, 6.25, 12.5, 25 and 50 µM-biochanin A, were selected for kinetic analysis. A Lineweaver–Burk plot showed that biochanin A had a mixed type of inhibition on CYP19 with a Ki value of 10.8 µM in MCF-7aro cells (Fig. 2).

Specific inhibition on testosterone-induced proliferation in MCF-7aro cells. Biochanin A was able to reduce the testosterone-induced proliferation of MCF-7aro cells through the inhibition of aromatase (Fig. 3). The administration of 10 nM-testosterone increased the cell number by 67% as shown at 0 µM-biochanin A. At 12.5 µM, biochanin A could significantly (P < 0.05) reduce the cell proliferation. At 25 µM, biochanin A brought down the testosterone-induced cell growth to a level comparable with their testosterone-less counterparts.

Biochanin A suppressed CYP19 promoter I.3 and II-driven transactivation

Effect of biochanin A on promoter I.3 and II activity of CYP19 in SK-BR-3 and MCF-7 cells. As the enzyme activity of CYP19 was reduced by biochanin A, we subsequently determined the transcriptional activity driven by promoter regions I.3 and II. We employed the breast cancer cell line SK-BR-3, which had been demonstrated using promoters I.3 and II for CYP19 regulation26, for the assessment of promoter activity. At 50 µM, biochanin A was able to repress the promoter activity (Fig. 4 (A)) (P < 0.05). Similar suppression was observed in MCF-7 cells (Fig. 4 (B)), and the down regulation on CYP19 transactivity appeared to be universal for breast cells.

Biochanin A reduced aromatase mRNA and protein expression in SK-BR-3 cells. Quantitative RT-PCR indicated for horseradish peroxidase, and the targeted protein was visualised by autoradiography.

Statistical methods

A Prism® 3-0 (GraphPad Software, Inc., CA, USA) software package was utilised for statistical analysis. The results were analysed by one-way ANOVA followed by Bonferroni’s multiple comparison test if significant differences (P < 0.05) were observed. The t test was employed for comparison of the means between biochanin A-treated and control cultures. Another software package, SigmaPlot (SPSS Inc., Chicago, IL, USA), was used for graphing the Lineweaver–Burk plots.
that the mRNA abundance of aromatase was reduced by biochanin A. Cultures treated with 12.5, 25 and 100 μM-biochanin A revealed significant drops in aromatase expression, and 100 μM of the isoflavone could decrease the expression by more than 80% (Fig. 5 (A)). Western analysis also revealed a similar pattern (Fig. 5 (B)).

Aromatase activity in SK-BR-3 cells treated with biochanin A. Since the mRNA abundance could be suppressed by biochanin A, we measured the aromatase activity as an indicator for reduced expression. After 24 h of treatment the aromatase activity was found to be significantly reduced by 25 μM-biochanin A (Fig. 6).

A major metabolite of biochanin A – genistein – suppressed CYP19 promoter I.3 and II-driven transactivation

Effect of genistein on promoter I.3 and II activity of CYP19 in SK-BR-3 cells. Genistein is a major metabolite of biochanin A. Since biochanin A was shown to be active in suppressing CYP19 expression over a period of time, genistein might play some part at the transcriptional level. Genistein certainly suppressed promoter I.3 and II transactivity in SK-BR-3 cells, as depicted in Fig. 7 (A). Genistein treatment at 25 μM and above significantly suppressed the luciferase activity. The suppression was further supported by the respective enzyme activity (see Fig. 7 (B)).

Discussion

In the present study, we illustrated that biochanin A was the only aromatase inhibitor among the isoflavones tested. Enzyme kinetic analysis revealed that both competitive and non-competitive inhibitions were involved. Biochanin A could also suppress testosterone-induced MCF-7aro cell proliferation, which was attributed to the reduced aromatase activity. At the transcriptional level, the phytocompound also reduced the aromatase mRNA abundance in the breast cancer cell line SK-BR-3. The promoter utilisation of the human aromatase gene is tissue-specific and promoters I.3 and II have been identified to be responsible for the expression in breast cancer cells including SK-BR-3. We further demonstrated that the transactivation activity of the gene fragment containing promoters I.3 and II was deactivated by biochanin A, and this suppression could be extended to MCF-7 cells. Genistein, which is a major metabolite of biochanin...
A32,33, also blocked the transcriptional activity of promoters I.3 and II in SK-BR-3 cells. This implied that the metabolism of biochanin A could still be effective in suppressing CYP19 expression.

Fig. 5. Messenger RNA (A) and protein expression of aromatase (B) in SK-BR-3 cells treated with biochanin A. SK-BR-3 cells were seeded in six-well plates and maintained in McCoy’s 5A medium supplemented with 10% charcoal dextran-treated serum. Biochanin A was administered to the cultures for 24 h. (A) CYP19 expression result determined by real-time RT-PCR. Values are means (n 3), with their standard errors represented by vertical bars. * Mean value is significantly different from that of the control cultures with no biochanin A treatment (\( P < 0.05 \)). (B) Western analysis of aromatase. The image represents one of two blots with similar results. CYP, cytochrome P450.

Fig. 6. Inhibitory effect of biochanin A on cytochrome P450 19 enzyme activity in SK-BR-3 cells. SK-BR-3 cells were seeded in six-well plates and maintained in McCoy’s 5A medium supplemented with 10% charcoal dextran-treated serum. Biochanin A was administered to the cultures for 24 h. The cultures were switched to serum-free medium upon assay. [1β-3H]androstenedione was administered and incubated for 1 h. Significant inhibition was seen at 25 \( \mu M \) and above. The 50% inhibitory concentration value was determined to be 40 \( \mu M \). Values are means (n 3), with their standard errors represented by vertical bars. * Mean value is significantly different from that of the control (\( P < 0.05 \)).
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subclass of flavonoid, display inhibitory actions on aromatase in human granulose-luteal cells. The differences in treatment are significant in inhibiting CYP19 at the enzyme and expression levels. The active ingredients in the extract could be procyanidin B dimers and resveratrol. Chalcones, which are a subclass of flavonoid, display inhibitory actions on aromatase. The biological relevance of biochanin A as a nutraceutical for preventing breast cancer has yet to be established. Like genistein, it exhibits biphasic effects on mammary cell proliferation. The phytochemical is growth inhibitory to human mammary epithelial cells and MCF-7 cells with IC50 values of about 20 μM after a 4 d incubation period, whereas it is growth stimulatory at a half maximal effective concentration (EC50) value of 9 nM after incubation for 6 d. In the present study, biochanin A had no significant effect on MCF-7aro cell proliferation in the testosterone-less treatment group after a 24 h incubation period.

In human subjects and the MCF-7 cell model, the phytochemical can be metabolised into genistein, biochanin A conjugates or hydroxy metabolites. Assuming the metabolites have an effect comparable with biochanin A, an oral dosage of 50 mg/kg could be able to sustain an aromatase-suppressing plasma concentration with respect to a pharmacokinetic study performed in rats.

In summary, the present study suggested that biochanin A inhibited the enzyme activity and suppressed the transcriptional control of CYP19 in breast cancer cells.

Biochanin A at 100 nm and 10 μM was found to be ineffective in inhibiting CYP19 at the enzyme and expression levels in human granulose-luteal cells. The differences in treatment concentration and cell type could separate this and the present study. Genistein, on the other hand, displays a similar suppressive effect on CYP19 in the former study. Other phytochemicals have also been reported to be aromatase inhibitors. Extract of red wine inhibits aromatase activity, and reduces mammary hyperplasia in transgenic mice over-expressing CYP19. The active ingredients in the extract could be procyanidin B dimers and resveratrol. Chalcones, which are a subclass of flavonoid, display inhibitory actions on aromatase in placental microsomes with IC50 values greater than or equal to 34-6 μM. Kao et al. have shown that the flavonone naringenin is a stronger inhibitor than the chalcones. In the present study biochanin A was the only isoflavone demonstrated to inhibit the enzyme activity. Given the structural resemblance between biochanin A and genistein, the methyl ether group substitute at the 4' C position may generate a significant steric hindrance in the active site of the enzyme.

At the transcriptional level, many factors have been described for the regulation of aromatase. Simpson et al. have reviewed that cyclic AMP, phorbol esters, dexamethasone, PG E2, transforming growth factor-β, and γ-interferon increase the transcriptional activity, whereas cyclo-oxygenase inhibitors suppress the mRNA expression. Kinoshita & Chen have previously reported that mitogen-activated protein kinase inhibitor may reduce CYP19 transcription in breast cells, and biochanin A may inhibit mitogen-activated protein kinase in a different cell system. This could also be a potential deactivating pathway in CYP19 transcription.

Many studies have documented biochanin A's chemopreventive effect on breast cancer. The isoflavone can protect against nitrosomethylurea-induced mammary carcinogenesis in rats, and mammary tumour virus-induced spontaneous breast cancer in mice. In the context of drug or xenobiotic metabolism, biochanin A also inhibits CYP145 and induces UDP-glucuronosyltransferase enzyme activities. The results of the present study provided a possible chemoprotective pathway for the isoflavone.

Fig. 7. Suppressive effect of genistein on cytochrome P450 19 in SK-BR-3 cells. SK-BR-3 cells were seeded in six-well plates and maintained in McCoy's 5A medium supplemented with 10% charcoal dextran-treated serum. Genistein was administered to the cultures for 24 h. (A) mRNA expression; (B) aromatase activity. Significant inhibition was seen in both mRNA expression and aromatase activity at 12.5 μM and above. Values are means (n=3), with their standard errors represented by vertical bars. *Mean value is significantly different from that of the control (P<0.05).

References

Effect of biochanin A on cytochrome P450 19


