Selective Inhibition of Aromatase by a Dihydroisocoumarin from *Xyris pterygoblephara*

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Aromatase is a well-established target for the chemoprevention of breast cancer. The dihydroisocoumarin (3*R*,4*R*)-(−)-6-methoxy-1-oxo-3-pentyl-3,4-dihydro-1*H*-isochromen-4-yl acetate (**1**) (IC_{50} = 1.6 ± 0.1 μM), isolated from aerial parts of *Xyris pterygoblephara*, showed aromatase inhibitory activity. The specificity of **1** was evaluated by inhibition assays with cytochrome P450 enzymes. CYP1A1 was inhibited modestly (IC_{50} = 38.0 ± 2.0 μM), while CYP2C8 and CYP3A4 enzymes were not affected. Dihydroisocoumarin **1** showed weak antiproliferative activity against MCF-7 (IC_{50} = 66.9 ± 2.3 μM) and LNCaP (IC_{50} = 57.5 ± 2.0 μM) cells and was inactive against LU-1 and HepG2 cells in culture. These results demonstrate the potential of dihydroisocoumarin **1** to serve as a selective aromatase inhibitor.

On a global scale, breast cancer continues to represent a major cause of death among women.[1] A large portion of breast tumors are potentially endocrine-responsive; 75% of cases occur in the postmenopausal period, while 50% are found in premenopausal women expressing estrogen receptor.[1,2] Endocrine therapy, employed to decrease estrogen production, is a useful approach for treating such tumors.[1,2] Aromatase (CYP19) is a key cytochrome P450 (CYP) enzyme, which catalyzes the rate-limiting aromatization step for the conversion of androgens (testosterone and androstenedione) to estrogens (estradiol and estrone).[3,4] The regulation and inhibition of aromatase activity have received considerable attention because of the crucial role in regulating estrogen synthesis in postmenopausal women.[1,2]

A third-generation of nonsteroidal aromatase inhibitors (AIs), including anastrozole and letrozole, have been developed based on quantitative structure−activity relationship (QSAR) studies, employing antimycotic drugs as templates.[1,2] Aromatase inhibitors have shown improved efficacy and reduced side effects against advanced and early stage breast cancer in comparison with the estrogen antagonist tamoxifen.[5–8] On the basis of these findings, approval has been obtained by the FDA for the therapy of breast carcinoma.[5,6] However, AIs in current use may possibly inhibit other P450 enzymes, resulting in emergence of resistance during long-term treatments.[5,6] In addition, reduced efficacy for the treatment of tumors in more advanced stages has been reported.[5,6] These factors stimulate the continuous search for new aromatase inhibitors, hopefully with better pharmacological and toxicological profiles. In this context, natural products represent a promising source for the discovery of new drugs and templates for developing innovative AIs.[9]

As part of our research for cancer chemopreventive agents of natural origin, we evaluated 38 extracts from Brazilian plants with an *in vitro* aromatase inhibition assay. Of these test materials, only an ethanol extract from the aerial parts of *Xyris pterygoblephara* Steud. (Xyridaceae) was found to be active, with an IC_{50} value of 3.1 ± 0.2 μg/mL.

Xyris plants are small shrubs, popularly named "sempre-vivas" (everlasting plants), being collected in Brazil for ornamental purposes and medicinal uses, mainly to treat eczemas and dermatitis.[10] In our previous work, the antifungal activity of the endemic species *X. pterygoblephara* was evaluated and its fractionation resulted in the new dihydroisocoumarin (3*R*,4*R*)-(−)-6-methoxy-1-oxo-3-pentyl-3,4-dihydro-1*H*-isochromen-4-yl acetate (**1**), which showed potent *in vitro* activity against clinical isolates of dermatophyte fungi.[11]

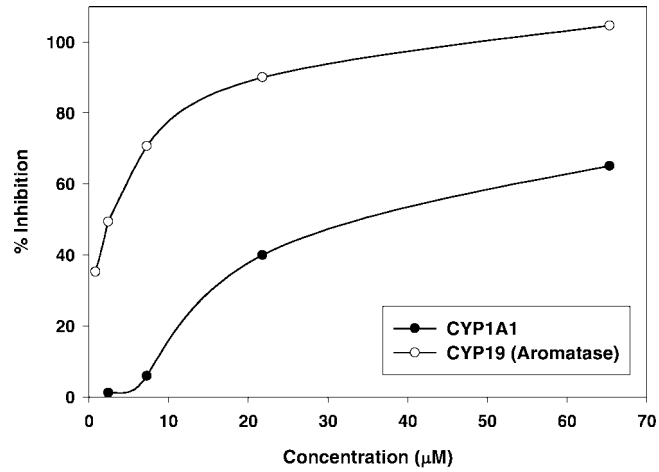
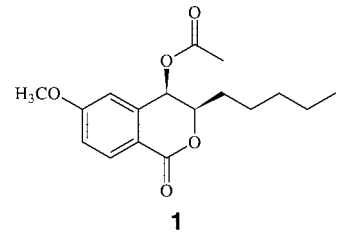


Figure 1. Dose−response studies of the inhibition of compound **1** on aromatase (CYP19) and CYP1A1. Results are the means of three assays, each one carried out in triplicate.



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Taking into account the significant aromatase inhibitory activity exhibited by *X. pterygoblephara* extract, dihydroisocoumarin **1** was evaluated in the assay, yielding an IC_{50} value of 1.6 ± 0.1 μM (Figure 1). Compound **1** can be considered a potent aromatase inhibitor in view of data previously reported for natural products derivatives. For example, the synthetic prenylated flavonone abyssinone II, evaluated as a racemic mixture, showed IC_{50} values of 0.6 μM (radiometric method), 62 μM (fluorimetric high throughput method),[12] and 40.95 ± 11.31 μM (fluorimetric method).[3] Furthermore, the potency exhibited by **1** was approximately 3-fold greater than the flavonoid naringin (IC_{50} = 5.0 μM), one of the positive controls employed in our study. Coumarin derivatives have been also reported to mediate considerable

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aromatase inhibitory activity, showing IC_{50} values in the range of 0.08 to 50 μM, when assayed by the radiometric method.[13]

As a class, coumarins have been extensively evaluated for aromatase inhibitory activity, including studies of structure−affinity relationships (SAFIR).[14] Some compounds have been designed to present different lipophilic aromatic cores, responsible for extended and strong hydrophobic (or $\pi-\pi$) interactions at the enzyme binding site, among other features. On the basis of these findings, it is feasible to suppose that the *n*-pentyl group of compound **1** may confer the required lipophilic function necessary for strong enzymatic interaction.

Aromatase bears an iron-containing porphyrin system at the active site which shows planar conformation due to the extended aromatic π-delocalization.[15] Several studies have shown that CH/π interactions play an important role in protein−ligand interactions.[15−17] Therefore, we can assume that compound **1** may interrelate with the porphyrin system through CH/π interactions, thus altering the conformation of the active site. To the best of our knowledge, this is the first report of aromatase inhibition by a dihydroisocoumarin. Only a few dihydroisocoumarins substituted at either C-3 or C-4 have been described, including compound **1**. Therefore, derivatives will be obtained in the future to investigate the structural features required for enzyme inhibition and to evaluate their potential as leads for new aromatase inhibitors.

The specificity of aromatase inhibition by compound **1** was evaluated by assays carried out with xenobiotic-metabolizing cytochrome P450 enzymes CYP1A1, CYP2C8, and CYP3A4. They are key enzymes in drug metabolism and thus potential sites for drug interactions.[18−25] CYP3A4, the most abundant P450 isoform in human liver, accepts as substrate roughly 50% of all drugs in clinical use. Therefore, oxidation modulation catalyzed by this enzyme is a major concern in terms of drug interactions.[19]

Dihydroisocoumarin **1** moderately inhibited CYP1A1, with an IC_{50} value of 38.0 ± 2.0 μM (Figure 1). Several studies have demonstrated previously an association between CYP1A1 expression and high-risk estrogen-receptor (ER) breast cancer, prostate cancer, and lung cancer.[21−25] Therefore, inhibition of CYP1A1 by compound **1** may be beneficial in the chemoprevention of different types of cancer. Compound **1** did not show inhibitory activity with CYP2C8 and CYP3A4, yielding IC_{50} values above 65 μM, which is regarded as inactive.[26]

To further assess the potential use of compound **1** as a selective aromatase inhibitor, toxicity was determined against four cell lines (HepG2, LU-1, LNCaP, and MCF-7), at concentrations up to 65 μM. Proliferation suppression with LU-1 and HepG2 cells was insignificant, whereas a weak antiproliferative effect against MCF-7 and LNCaP cell lines was observed, with IC_{50} values of 66.9 ± 2.3 and 57.5 ± 2.0 μM, respectively. The MCF-7 antiproliferative activity exhibited by compound **1** could be related to CYP19 inhibition, as this cell line is known to present aromatase activity and to be estrogen-receptor positive.[27,28]

Taken together, the results reported here demonstrate that dihydroisocoumarin **1**, isolated from *X. pterygoblephara* aerial parts, mediates potent *in vitro* aromatase inhibitory activity with some selectivity. This compound can be employed as a prototype for future development, which may result in a new chemopreventive agent directed toward the prevention or treatment of breast cancer.

Experimental Section

General Experimental Procedures. Dihydroisocoumarin **1** was isolated in our laboratory from an EtOH extract of *X. pterygoblephara* aerial parts, as previously described.[11] All other chemicals, unless specified otherwise, were purchased from Sigma Chemical Co. or Aldrich. Cell culture media and supplements were obtained from Gibco. The substrate DBF (dibenzylfluorescein) was obtained from Gentest Corporation (Woburn, MA). All human recombinant cytochrome P450 enzymes were purchased from BD Biosciences, San Jose, CA.

Cell Lines. HepG2, human hepatoma, cells were supplied by Dr. Hong-Jie Zhang (University of Illinois at Chicago, Chicago, IL); MCF-7, human breast carcinoma (ATCC HTB-22), and LNCaP, hormone-dependent human prostate carcinoma (ATCC-CRL-1740), were purchased from American Type Culture Collection; LU1, human lung carcinoma, was supplied by the Department of Surgical Oncology, University of Illinois at Chicago, Chicago, IL. All media contained 10% fetal bovine serum (FBS) in the presence of 100 U/mL penicillin and 0.1 μg/mL streptomycin. LNCaP were cultured in the presence of testosterone (2 nM). Cells were incubated at 37 °C with 95% air and 5% CO_2. All cells were maintained below passage 20 and used in experiments during the linear phase of growth.

Cytochrome P450 Assays [CYP19 (Aromatase), CYP1A1, CYP2C8, and CYP3A4]. Aromatase inhibition was quantified by measuring the fluorescent intensity of fluorescein, the hydrolysis product of dibenzylfluorescein (DBF), by aromatase, as previously described.[3] For inhibition of CYP1A1, CYP2C8, and CYP3A4, experimental conditions were similar to the aromatase assay, with some modifications.[29,30] In brief, the test substance (10 μL) was preincubated with a NADPH regenerating system (90 μL of 2.6 mM $NADP^+$, 7.6 mM glucose 6-phosphate, 0.8 U/mL glucose 6-phosphate dehydrogenase, 13.9 mM $MgCl_2$, and 1 mg/mL albumin in 50 mM potassium phosphate, pH 7.4), for 10 min, at 37 °C, before 100 μL of the enzyme and substrate (E/S) mixture were added (4.0 pmol/well of CYP19/0.4 μM DBF; 5.0 pmol/well of CYP2C8/2.0 μM DBF; 5.0 pmol/well of CYP3A4/2.0 μM DBF; and 0.5 pmol/well of CYP1A1/2.0 μM DBF). The reaction mixtures were incubated for 30 min (except CYP1A1, 25 min) at 37 °C to allow the generation of product, quenched with 75 μL of 2 N NaOH, shaken for 5 min, and incubated for 2 h at 37 °C, to enhance the noise/background ratio. Finally, fluorescence was measured at 485 nm (excitation) and 530 nm (emission). Three independent experiments were performed, each one in triplicate, and the average values were used to construct dose−response curves. At least four concentrations of the test substance were used, and the IC_{50} values were calculated (Tablecurve 2D, AISN Software, EUA, 1996). Significant differences of inhibition values were determined by Student's *t*-test ($p < 0.05$). Naringenin and aminoglutethimide were used as positive controls, yielding IC_{50} values of 5.0 and 0.27 μM, respectively. Compound **1** was dissolved in dimethyl sulfoxide (DMSO) and diluted to final concentrations. An equivalent volume of DMSO was added to control wells, and this had no measurable effect on cultured cells or enzymes.

Cell Proliferation Assay. Cell density determinations were performed as described by Skehan et al.[31] This assay is based on the selective binding of sulforhodamine B with cellular protein.[31] The human cancer cell panel was comprised of LU-1, LNCaP, HepG2, and MCF-7 cells. Experiments were performed in 96-well plates. Cells were seeded (1×10^4 cells/well), test samples were added at various concentrations at day 1, and cell growth was estimated at day 4. After the incubation period, cell monolayers were fixed with 10% (wt/v) trichloroacetic acid and stained for 30 min. Excess dye was removed by washing repeatedly with 1% acetic acid, protein-bound dye was dissolved in 10 mM Tris base solution, and measurements were performed at 510 nm using a microplate reader. For each cell line, four concentrations were tested in triplicate.

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