Potent Inhibition of Human Phosphodiesterase-5 by Icariin Derivatives

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Plant extracts traditionally used for male impotence (Tribulus terrestris, Ferula hermonis, Epimedium brevicornum, Cinnamomum cassia), and the individual compounds cinnamaldehyde, ferutinin, and icariin, were screened against phosphodiesterase-5A1 (PDE5A1) activity. Human recombinant PDE5A1 was used as the enzyme source. Only E. brevicornum extract (80% inhibition at 50 μg/mL) and its active principle icariin (1) (IC₅₀ 5.9 μM) were active. To improve its inhibitory activity, 1 was subjected to various structural modifications. Thus, 3,7-bis(2-hydroxyethyl)icaritin (5), where both sugars in 1 were replaced with hydroxyethyl residues, potently inhibited PDE5A1 with an IC₅₀ very close to that of sildenafil (IC₅₀ 75 vs 74 nM). Thus, 5 was 80 times more potent than 1, and its selectivity versus phosphodiesterase-6 (PDE6) and cyclic adenosine monophosphate-phosphodiesterase (cAMP-PDE) was much higher in comparison with sildenafil. The improved pharmacodynamic profile and lack of cytotoxicity on human fibroblasts make compound 5 a promising candidate for further development.

The inability to achieve or maintain an erection sufficient for satisfactory sexual function is an increasing problem with a considerable impact on interpersonal relationships and quality of life for men.¹ During erection, nitric oxide is released from the axon terminals of the parasympathetic nerves and diffuses into smooth muscle cells of the arterial walls of the corpus cavernosum. The consequent activation of guanylyl cyclase, converting guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP), causes smooth muscle relaxation, leading to dilation and increased influx of blood into the penile tissue. The trapping of blood in the penis results in an erection.² Selective inhibitors of cGMP-phosphodiesterase-5 (PDE5) such as sildenafil (Viagra), tadalafl, and vardenafil are currently used for erectile dysfunction (ED). However, several adverse effects have been recorded in clinical trials, including priapism and visual disturbances.³ Furthermore, therapy with PDE5 inhibitors is cost-effective. Thus, the search for new compounds of this type for drug development could be worthwhile. A variety of natural plant products, including berberine, forskolin, papaverine, and yohimbine, are claimed to be useful for improving sexual performance. Extracts from Lepidium meyenii Walp. (maca), Panax ginseng C.A. Meyer, Ginkgo biloba L., Ferula hermonis Boiss., and many other herbal remedies, alone or in combination, have been promoted for the treatment of sexual problems.⁴⁻⁵ With the aim of looking for new leads for selective PDE5 inhibitors, plant extracts and their putative active principles were selected for screening against human PDE5 activity in vitro. Our attention focused on Tribulus terrestris L., Ferula hermonis, Epimedium brevicornum Maxim., and Cinnamomum cassia L., since these extracts are claimed traditionally to improve sexual performance. T. terrestris caused vasodilating and antihypertensive effects in rats⁶ and a pro-erectile effect on the rabbit corpus cavernosum;⁷ F. hermonis has been studied for its effects on sexual behavior in male and female rats;⁸,⁹ C. cassia and “Epimedi Herba” are components of Chinese herbal products patented for the treatment of sexual dysfunction.¹⁰⁻¹⁴ “Epimedi herba” is the common name for the dried aerial parts of E. brevicornum, E. sagittatum Maxim., or E. koreanum Nakai, collected in the summer.¹⁵,¹⁶ Among the extracts, only “Epimedi Herba” was active against PDE5A1, for which the presence of icariin (1), the major pharmacologically active constituent,¹⁷⁻¹⁹ was considered a lead compound for chemical modifications in order to improve inhibitory activity. Modifications applied at the hydroxyl groups at C-3, C-7, and C-8 included partial or complete removal of the sugar moieties, partial or complete sugar replacement with a hydroxyethyl residue, and cyclization of the prenyl group (Scheme 1). All compounds produced (1—6) were tested for PDE5A1 activity. Also, selectivity versus human retina PDE6C and human platelet cAMP-PDE, and cytotoxicity on human fibroblasts were investigated.

Results and Discussion

The activity of plant extracts and individual compounds against human recombinant PDE5A1 is shown in Figure S1 (Supporting Information). Cinnamaldehyde, icariin (1), and ferutinin were considered as the putative active principles of C. cassia, E. brevicornum, and F. hermonis, respectively, since the compounds represent the most abundant secondary metabolites of those species. Only E. brevicornum and icariin (1) strongly inhibited PDE5A1 (~80% and ~72%, respectively), whereas the other test materials were much less active (~15 to ~23%). Inhibition by cinnamaldehyde (~16%) and ferutinin (~7%) was not significant. The medicinal plants tested in the present study had a reputation for aphrodisiac effects and therefore represented the start of a screening program to search for compounds to be developed as a new natural drug alternative to sildenafil. The observation that only E. brevicornum and its active principle 1 inhibited PDE5 in a significant manner, in agreement with previous results,¹⁷⁻²⁰ suggests that the other plant extracts may interfere with erectile function through mechanisms other than PDE5 inhibition.

Compound 1 was a good PDE5 inhibitor (IC₅₀ of 5.9 μM), but required improvement in order to have equivalent potency to sildenafil, which gave an IC₅₀ of 75 nM. The inhibitory effects of icariin derivatives 2–6 on PDE5A1 is shown in Table 1. Since aglycons might be expected to possess higher activity than the corresponding glycosides, the first general structural modification to 1 was the removal of one or both of the sugar moieties at the hydroxyl groups at positions C-3 and C-7 of the flavone scaffold. Enzymatic hydrolysis of 1 with cellulase and naringinase allowed the partial or total removal of the sugar moieties, respectively,

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affording the known compounds 2 and 3. Indeed, the removal of the glucose at the hydroxyl group in C-7, thus furnishing icariside II (2), improved drastically the enzyme inhibition, attaining an IC$_{50}$ value on the nanomolar order (IC$_{50}$ 156 nM). Conversely, icaritin (3), where both sugars were removed, was only around 3-fold more potent than 1 (IC$_{50}$ 2.2 µM).

To investigate if the prenyl moiety is essential for enzyme inhibition activity, β-anhydroicaritin (6) was tested. The cyclization
led to a dramatic drop in inhibitory activity. The IC\textsubscript{50} value for 6 was 45.5 \mu M, indicating that a free prenyl group at position C-8 is important for enzyme inhibition. To confirm that the prenyl group is required for enzyme inhibition, the 8-prenyl derivatives of naringenin (8-PN), quercetin (8-PQ), and apigenin (8-PA) were tested and their activity compared to that of the corresponding free flavonoid. As shown in Figure S2 (Supporting Information), all prenyllflavonoids inhibited PDE5A1 with the following order of potency: 8-PQ (IC\textsubscript{50} 0.70 \pm 0.10 \mu M) > 8-PA (IC\textsubscript{50} 1.29 \pm 0.11 \mu M) > 8-PN (IC\textsubscript{50} 16.23 \pm 1.16 \mu M). Quercetin, apigenin, and naringenin (10 \mu M) showed 23\%, 12\%, and 6\% inhibition, respectively, much lower than the corresponding prenyl derivatives. Data from the literature confirm the importance of the prenyl group: sophoravescenol, a prenylated flavonol from Sophora flavescens Ait. (Leguminosae), and osthole, a prenyl coumarin from Angelica pubescens Maxim., are two additional examples of PDE-5 inhibitors in the class of prenylated phenolic compounds.\textsuperscript{21,22}

The last modification to 1 was the replacement of one or both sugar moieties with the hydroxyethyl side chain, representing a µ with respect to that of sildenafil. Compound 5 for PDE5 was improved with a crude drug standard and/or authoritative literature source by Tabulae Medicinae. The IC\textsubscript{50} values for 5 and 367 for sildenafil. These results were as follows: elution gradient 0.01% trifluoroacetic acid in CH\textsubscript{3}CN (A) and 0.01% trifluoroacetic acid (v/v) in water (B) eluting in gradient mode starting from 10\% (A) up to 60\% (A) in 40 min at a flow rate of 1.0 mL/min.

HPLC-UV analysis was carried out with a Kontron 325 pump/system controller equipped with a Merck-Hitachi UV–vis detector set to 278 nm. The analyses were performed on Phenomenex Luna RP C\textsubscript{18} (3 \mu m, 4.6 mm \times 1.5 cm) columns. Analytical conditions were as follows: elution gradient CH\textsubscript{3}CN (A) and 0.01% trifluoroacetic acid (v/v) in water (B) according to the following profile: 0–60 min, 15–100\% A, 85–0\% B; flow rate 1.0 mL/min.

All solvents and reagents were obtained from commercial sources and used without further purification unless otherwise noted. Reaction course and product mixtures were routinely monitored by TLC and HPLC. TLC was carried out on precoated silica gel F\textsubscript{254} (Merck) plates or on silica gel 60 (Merck) plates (visualizing developed chromatograms by spraying with plates with 10% (v/v) phosphomolybdic acid in H\textsubscript{2}SO\textsubscript{4} at 100 °C for 3 min). Column chromatography was carried out with silica gel (Kieselgel 40, 0.040–0.063 mm; Merck) using the flash technique. For the semisynthetic derivatives, yields are reported after chromatographic purification and crystallization.

Dulbecco’s modified Eagle’s medium, trypsin, protease inhibitors, naringinase (from Penicillium decumbens, 596 units/g solid; \beta-glucosidase activity: 69 units/g solid), and all chemical reagents for cell culture were purchased from Sigma Aldrich (Milan, Italy). Cellulase (from Aspergillus niger) was from Fluka (Milan, Italy). Penicillin, streptomycin, and L-glutamine were from GibCO (Grand Island, NY); fetal calf serum was provided by Mascal Brunelli SpA (Milan, Italy). The COS-7 cell line was purchased from ATCC (Manassas, VA). Superfect reagent for transient transfections was obtained from Qiagen GmbH (Hilden, Germany). The expression plasmid pCDNA3 containing the full-length cDNA of PDE5A1 was a kind gift of Prof. C. S. Lin (Department of Urology, University of California, San Francisco, CA). Human recombinant PDE6C, cloned from the human retina and expressed in S. frugiperda insect cells using a baculovirus expression system, was purchased from Scottish Biomedical (Glasgow, UK). [\textsuperscript{3}H]-cGMP and [\textsuperscript{3}H]-cAMP were from Amersham Pharmacia Biotech (Amersham Place, Little Chalfont, Buckinghamshire, UK). DEAE-Sephadex A25 was from Pharmacia (Uppsala, Sweden), cGMP, cAMP, and 8-prenylated snake venom were purchased from Sigma Aldrich. Sildenafil was supplied by Sequoia Research Products (Oxford, UK). Cinnamaldehyde and ferutinin were supplied by Indena Spa (Milan, Italy). 8-Prenylnaringenin, 8-prenylquercetin, and 8-prenylapigenin (purity >98\%) were donated by Prof. Giovanni Appendino (Università del Piemonte Orientale, Italy).

Plant Material. T. terrestris L. dried extract (44\% furosantinolic sapogenins) was from Farmbio Ltd. (Sofia, Bulgaria); the ethanolic extract of the aerial parts of E. brevicornum Maxim. (20.9\% icariin) was from Chengdu Wagott Natural Products Co. Ltd., Xian City, People’s Republic of China. The root methanolic extract from F. hermonis Boiss. (26.3\% furanocoumarin) and C. cossyri L. oil extract (73.4\% cinnamaldehyde) were supplied by Indena Spa (Milan, Italy). Plant material was identified against a crude drug standard and/or authoritative literature source by a suitable qualified person. A voucher of each plant is kept at the botanical laboratory of the company. Extracts were quantified by HPLC, and the chromatographic profiles are shown in Figures S3–S6 (Supporting Information).

Extraction and Isolation of Icariin (1). A dried extract of E. sagittatum as a greenish-brown residue (4 g) was dissolved in a mixture of CH\textsubscript{3}OH/H\textsubscript{2}O (1:1) (200 mL). The solution was stirred for 20 min and then washed with CH\textsubscript{3}Cl\textsubscript{2} (3 \times 80 mL). Methanol was evaporated under vacuum, and the remaining aqueous solution was diluted with water to 400 mL. The solution was extracted with Et\textsubscript{2}O (5 \times 400 mL), and the organic phase was taken to dryness. The extract was resuspended with CH\textsubscript{3}Cl\textsubscript{2} (200 mL) and filtered under vacuum to yield 1.08 g of extract (A), from which icariin (1) was purified by precipitation with methanol (50 mL) as a yellow powder (purity 95.3\%) (530 mg; 13\% yield on the dry extract); mp 224–226 °C; \textsuperscript{1}H NMR (DMSO-\textsubscript{d}_6, 300 MHz, 30 °C) \delta 0.80 (3H, d, J = 5.4 Hz, rha Chls), 1.60 (3H, s, CH\textsubscript{3}–14), 1.70 (3H, s, CH\textsubscript{3}–15), 3.05–3.20 (4H, m, H-11 and sugar protons), 3.40–3.80 (7H, m, sugar protons), 3.87 (3H, s, OCH\textsubscript{3}), 4.00 (1H, m, sugar proton), 4.55–4.78 (3H, m, OH), 4.85–5.22 (6H, m, sugar protons and OH), 5.30 (1H, t, J = 6.9 Hz, H-12), 6.60 (1H, H-6), 7.15 (2H, d, J = 8.4 Hz, H-3, H-5); 7.85 (2H, d, J = 8.4 Hz).
Hz, H-2', H-6'), 12.60 (s, 1H, OH-5); ESIMS (positive-ion mode) m/z: 677 [M + H]+, 699 [M + Na]+.

Preparation of Icariside II (2). A solution of 1 (500 mg) in DMSO (1 mL) was added dropwise for 48 h to a Na acetate-buffered hydroalcoholic solution at 37 °C (0.25 M, pH 5.0, in EtOH/H2O, 30:70) (50 mL) containing cellulase (210 mg). The suspension obtained was stirred at 37 °C for 4 days. Then, a further amount of cellulase (100 mg) was added, and the mixture was stirred under the same conditions for a further 4 days. EtOH was then removed under vacuum and the residue was diluted to 200 mL with H2O and extracted with EtOAc (3 × 200 mL). The organic layer was dried over anhydrous Na2SO4 and evaporated under reduced pressure to afford 2 (290 mg); yield 76%; mp 208–210 °C;H NMR (DMSO-d6, 300 MHz, 30 °C) δ -0.90 (3H, s, H-5), 1.82 (3H, s, CH3-14), 1.87 (3H, s, CH3-15), 3.05–3.60 (4H, m, rha protons, H-11), 3.85 (3H, s, OCH3), 4.22–4.24 (1H, m, rha proton), 4.55–4.80 (3H, m, sugar OH), 4.90 (1H, m, rha proton), 5.20 (1H, t, J = 6.8 Hz, H-12), 5.52 (1H, d, J = 1.5 Hz, rha proton), 6.37 (1H, s, H-6), 7.15 (1H, d, J = 8.4 Hz, H-3'), 7.83 (2H, d, J = 8.4 Hz, H-2', H-6'), 10.60 (1H, s, OH-7), 12.80 (1H, s, OH-5); ESIMS (positive-ion mode) m/z: 537 [M + Na]+.

Preparation of Icariside I (3). A solution of icaritin (1) (526 mg) in DMSO (1 mL) was added dropwise for 72 h to a Na acetate-buffered hydroalcoholic solution at 37 °C (0.25 M, pH 5.0, in EtOH/H2O, 30:70) (50 mL) containing naringin (207 mg). The obtained suspension was filtered and the mixture was stirred under the same conditions for a further day. EtOH was removed by evaporation and the aqueous suspension was filtered under vacuum and dried. The residue obtained was washed with H2O and dried to give icaritin (3, 290 mg; purity 95%) as a yellow powder. The mother liquors were diluted with H2O and extracted with EtOAc (2 × 200 mL). The organic phase was dried over anhydrous Na2SO4 and evaporated under reduced pressure to afford an additional amount of icaritin (3, 200 mg); purity 96.0%; mp 20.2% yield; mp 232 °C.

Preparation of 7-(2-Hydroxyethyl)-3-O-rhamnosylicariin (4). A stirred suspension of 2 (200 mg, 0.39 mmol), 2-bromoethanol (50 mg, 0.43 mmol), and anhydrous K2CO3 (240 mg, 1.7 mmol) in dry acetone (75 mL) was refluxed for 8 h. The hot reaction mixture was filtered, and the solvent was evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel (CH3Cl/acetone, 9:1) and crystallized from EtOH to give the desired compound as a yellow crystalline powder (70 mg, purity 96.0%); yield 20.2% yield; mp 233 °C; 1H NMR (CDCl3, 300 MHz, 30 °C) δ 1.77 (3H, s, CH3-14), 1.87 (3H, s, CH3-15), 2.70 (2H, t, J = 8.4 Hz, H-3'), 3.08–3.12 (4H, m, rha protons), 3.48 (2H, t, J = 8.4 Hz, H-11), 3.85 (3H, s, OCH3), 4.22–4.24 (1H, m, rha proton), 4.55–4.80 (3H, m, sugar OH), 4.90 (1H, m, rha proton), 5.20 (1H, t, J = 6.8 Hz, H-12), 5.52 (1H, d, J = 1.5 Hz, rha proton), 6.37 (1H, s, H-6), 7.15 (1H, d, J = 8.4 Hz, H-3'), 7.83 (2H, d, J = 8.4 Hz, H-2', H-6'), 10.60 (1H, s, OH-7), 12.80 (1H, s, OH-5); ESIMS (positive-ion mode) m/z: 369 [M + H]+.

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DMSO (9:1; 500 µL/well) for 15 min at 37 °C, and aliquots of 100 µL were read on a plate reader (Bio-Rad Laboratories) at 560 nm.

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Supporting Information Available: Figures showing the HPLC traces of the extracts under study and the effects of plant extracts and pure compounds on the inhibition of PDE5A1. This information is available free of charge via the Internet at http://pubs.acs.org.

References and Notes


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