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Inhibitory Effects of Furoquinoline Alkaloids from *Melicope confusa* and *Dictamnus albus* against Human Phosphodiesterase 5 (hPDE5A) *In Vitro*

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Eight furoquinoline alkaloids were purified from two plants belonging to the Rutaceae family. Kokusaginine, skimmianine, evolitrine, and confusameline were purified from *Melicope confusa*, and haplopine, robustine, dictamine, and γ-fagarine from *Dictamnus albus*. In this study, the eight furoquinoline alkaloids were examined for inhibitory potency against human phosphodiesterase 5 (hPDE5A) *in vitro*. DNA encoding the catalytic domain of human PDE5A was amplified from the mRNA of T24 cells by RT-PCR and was fused to GST in an expression vector. GST-tagged PDE5A was then purified by glutathione affinity chromatography and used in inhibition assays. Of the eight alkaloids, γ-fagarine was the most potent inhibitor of PDE5A, and its single methoxy group at the C-8 position was shown to be critical for inhibitory activity. These results clearly illustrate the relationship between PDE5A inhibition and the methoxy group position in furoquinoline alkaloids.

Key words: Furoquinoline alkaloids, cGMP, Smooth muscle, hPDE5A

INTRODUCTION

Cyclic nucleotides regulate many functions in various tissues, and their levels are altered by signals that modulate the activities of adenylate and guanylate cyclases or phosphodiesterases (PDEs) (Lin *et al.*, 2002; Kim *et al.*, 2001; Uckert *et al.*, 2001). Cyclic nucleotide PDEs are classified into 11 families based of their structural, kinetic, and regulatory characteristics. PDEs hydrolyze cyclic nucleotides and control the duration and amplification of nucleotide signals (Sausbier *et al.*, 2000; Mehats *et al.*, 2002; Lacas *et al.*, 2000). Phosphodiesterase 5 (PDE5) is a cyclic GMP (cGMP)-specific hydrolytic enzyme that regulates the intracellular levels of cGMP in many tissues and influences vascular smooth muscle tone (Rybalkin *et al.*, 2003; Funayama *et al.*, 2001; Wyatt *et al.*, 1998). In smooth muscle, NO activates soluble guanylate cyclase, which leads to the production of cGMP (Chen *et al.*, 2002). The degradation of cGMP is controlled by cyclic PDEs, and PDE5A is the most highly expressed cGMP-hydrolyzing PDE in smooth muscle cells. Numerous pharmacological compounds have been developed that target specific PDEs in order to regulate cellular cAMP and cGMP concentrations as a means of treating ailments such as allergic diseases and heart failure (Crocker *et al.*, 1999; Schmit *et al.*, 1999; Chen *et al.*, 2003). PDE5A inhibitors increase the intracellular levels of cGMP in many tissues (Blount *et al.*, 2004; Tejada 2004). The PDE5A inhibitor zaprinast has been found to promote the induction of cerebellar long-term depression in rats (Wang *et al.*, 2004; White *et al.*, 1989). A potent PDE5A-specific inhibitor, sildenafil, is effective in treating male erectile dysfunction by increasing cGMP in the smooth muscle of the corpus cavernosa (Turko *et al.*, 1999; Boolell *et al.*, 1996).

It has been reported that the quinoline alkaloids japonine and eduline, isolated from the extract of the leaves of *Orixa japonica*, show similar potency in the relaxation of rat small intestine smooth muscle (Funayama, *et al.*, 2001). Our interest in naturally occurring biologically active compounds led us to purify eight furoquinoline alkaloids from *Melicope confusa* and *Dictamnus albus* and to study their inhibitory activities against PDE5A.

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PDE5As share a common structure, with a conserved catalytic domain flanked by regulatory domains (Mehats *et al.*, 2002; Natochin *et al.*, 1996; Francis *et al.*, 2002). There are no differences between the inhibitory profiles of the PDE5A holoenzyme and its catalytic fragment, indicating that the monomeric catalytic domain of PDE5 has a sufficiently high affinity for its inhibitors in the absence of the regulatory domain interaction (Fink *et al.*, 1999; Turbo *et al.*, 1996).

In this study, we examined eight furoquinoline alkaloids (kokusaginine, skimmianine, evolitrine, confusameline, haplopine, robustine, dictamine, and γ-fagarine) for inhibitory activity against the human PDE5A catalytic fragment, which was obtained by RT-PCR.

MATERIALS AND METHODS

Materials

Eight furoquinoline alkaloids (kokusaginine, skimmianine, evolitrine, and confusameline from *Melicope confusa*; haplopine, robustine, dictamnine, and γ-fagarine from *Dictamnus albus*) were used. Botanical specimens are stored at the herbarium of the Natural Products Research Institute (Seoul National University, South Korea). Bovine brain phosphodiesterase was obtained from Sigma-Aldrich (St. Louis, MO).

Cell culture

The T24 cell line, derived from a human urinary bladder carcinoma, was purchased from American Type Culture Collection (U.S.A.). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 in air. Confluent cultures were used in experiments.

Cloning of the human PDE5A catalytic fragment

Sense (5'-GGAATTCCAAGCAAATGGTCACATTGGAG-3') and antisense (5'-GGAATTCAGTTCCGCTTGGC-CTGGCC-3') primers flanking the catalytic region and containing EcoRI restriction sites were used. RT-PCR was performed on 1 μg of total RNA from T24 cells with the following cycle parameters: 1 cycle of 50°C/30 min, 90°C/2 min; 30 cycles of 94°C/30 s, 48°C/30 s, 70°C/180 s; and 1 cycle of 70°C/5 min. The PCR product was digested with EcoRI and ligated into a pGEX-4T3 GST fusion vector (Amersham Biosciences Korea Ltd., South Korea), and the resulting construct was confirmed by DNA sequence analysis.

Expression and purification of GST-PDE5A

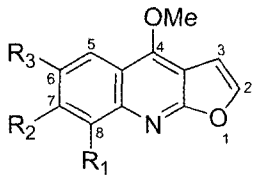
The pGEX4T3-PDE5A plasmid was used to transform BL21 (DE3) bacterial cells. GST-PDE5A fusion protein was highly expressed after the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubation at 37°C for 5 h. Cells were lysed by sonication, and the GST-tagged protein was purified from the lysate on glutathione Sepharose 4 Fast Flow beads for large-scale purification (Amersham Biosciences Korea Ltd., South Korea) The eluted GST-PDE5A protein was aliquoted in buffer containing 20% glycerol and stored at -70°C until use. The total protein concentration was determined according to the method of Bradford (Bradford, 1976). The molecular mass of GST-PDE5A was analyzed by SDS-PAGE. Gels (10% polyacrylamide) were stained with 0.25% Coomassie Blue in methanol:water:acetic acid (50:40:10) and destained in 30% methanol and 10% acetic acid.

Phosphodiesterase assay

The hPDE5A activity was measured using a modification of the [^{3}H]cGMP SPA assay system (Amersham). The incubation mixture (100 μL) contained 40 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7.5), 0.5 mM EGTA, 15 mM magnesium acetate, [^{3}H]cGMP (0.005 Ci), and 0.1 mg GST-PDE5A. The reaction was incubated for 10 min at 30°C. The reaction was stopped by the addition of 50 mL Yttrium silicate beads (20 mg/mL) and thorough mixing, followed by a 20-min incubation at room temperature. PDE5A activity was detected on microtiter plates in a beta counter (Wallac 1450 MicroBeta TM). The inhibitory activity of samples was also confirmed against bovine brain phosphodiesterase.

Statistical analysis

The results were analyzed as the mean ± S.D. of at least three separate experiments. The differences between the means of the groups were determined using Student's *t*-test and analysis of variance. The minimum level of significance was set at $p < 0.05$.



I. Dictamnine
R_1, R_2, R_3= H

II. Robustine
R_1= OH, R_2= H, R_3= H

III. γ–Fagarine
R_1= OMe, R_2= H, R_3= H

IV. Kokusaginine
R_1, R_2, R_3= OMe

V. Confusameline
R_1= H, R_2= OH, R_3= H

VI. Haplopine
R_1= OMe, R_2= OH, R_3= H

VII. Evolitrine
R_1= H, R_2= OMe, R_3= H

VIII. Skimmianine
R_1= OMe, R_2= OH, R_3= H

Fig. 1. Structures of the eight furoquinoline alkaloids.

RESULTS AND DISCUSSION

Schematics of the cloning steps used to create the GST-tagged hPDE5A and of the DNA sequence, including the conserved catalytic domain, are shown in Fig. 2A. The consensus PDE catalytic domain is located between amino acids 466_{Lys} and 823_{Asn} (Fink *et al.*, 1999).

Oligonucleotide primers flanking the catalytic domain of hPDE5A were synthesized and used to generate a 1.1-kb PCR product from the cDNA of T24 cells. The PCR product was digested with EcoRI and ligated into the EcoRI site of the pGEX-4T3 vector (Amersham Pharmacia Biotech), yielding pGEX4T3-CPDE5A. The insert sequence was identical to that of the human PDE5A catalytic domain (Fig. 2A). The GST-tagged construct, expressed in *E. coli* (BL21) and isolated by affinity chromatography, had an approximate molecular mass of 67 kDa (Fig. 2B) and was used in assays with the alkaloid PDE inhibitors.

Furoquinoline alkaloids were isolated from two plants, *Melicope confusa* and *Dictamnus albus*. The chemical structures of all eight alkaloids are presented in Fig. 1. A stock solution of each alkaloid was prepared in 100% dimethyl sulfoxide (DMSO), and the *in vitro* inhibitory activity was tested at 0.1 mM final concentration. Table I shows the results averaged from three independent experiments. The alkaloids evolitrine and γ-fagarine had

Table I. Inhibitory activity (%) of eight furoquinoline alkaloids (γ-fagarine, evolitrine, dictamnine, kokusaginine, robustine, skimmianine, haplopine, and confusameline) against hPDE5A.

Number	Compounds	Final concentration (mM)	% of inhibition (hPDE5A)
1	γ-fagarine	0.1	67.0+0.9
2	Evolitrine	0.1	52.7±0.6
3	Dictamnine	0.1	21.6±0.6
4	Kokusaginine	0.1	17.1+0.7
5	Robustine	0.1	17.1+0.7
6	Skimmianine	0.1	16.6+0.5
7	Haplopine	0.1	14.7±0.6
8	Confusameline	0.1	14.6±0.3

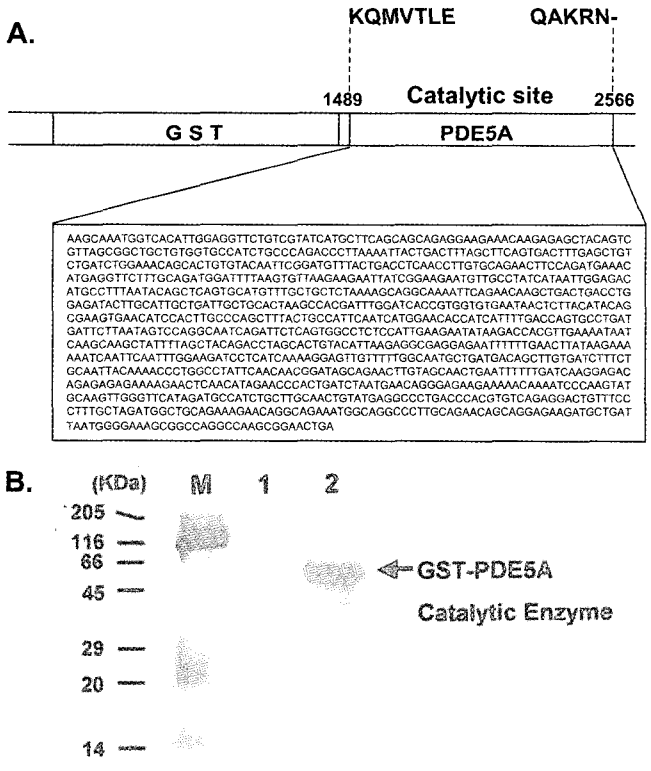


Fig. 2. A: Schematic showing the construction of the pGEX4T3-PDE5A plasmid and the DNA sequence of the PDE5A catalytic fragment (1.1 kb). B: Purified GST-PDE5A proteins were separated on 10% SDS-PAGE and visualized by staining with Coomassie Blue. Lane 1, Protein marker (M); Lane 2, GST-PDE5A (1 μg); and Lane 3, GST-PDE5A (20 μg). The molecular mass of the GST-PDE5A catalytic fragment is approximately 67 kDa.

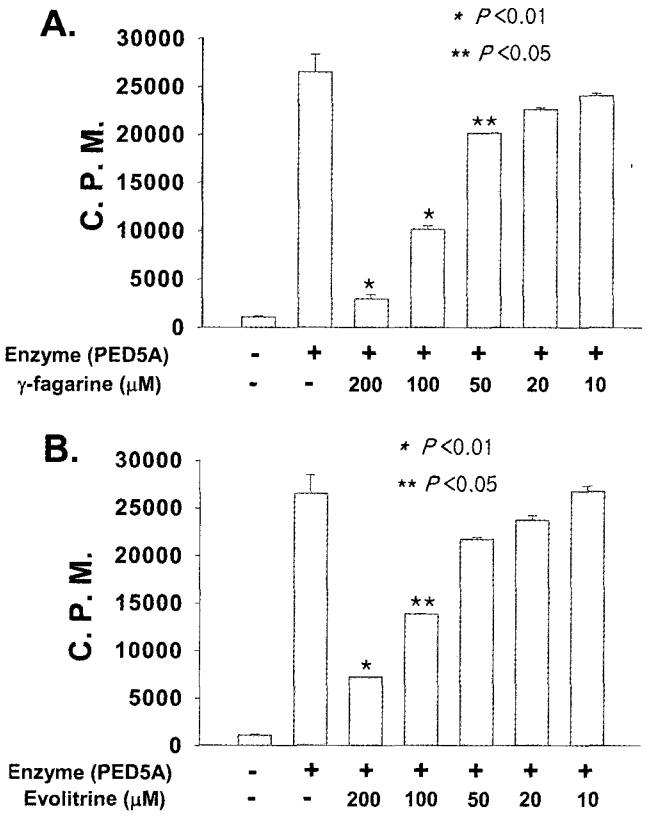


Fig. 3. Of the furoquinoline alkaloids, γ-fagarine and evolitrine were the most potent PDE5A inhibitors. A: γ-fagarine (200 μM to 10 μM) was tested for inhibitory activity against PDE5A. γ-fagarine inhibited PDE5A catalytic activity in a dose-dependent manner. B: Evolitrine (200 μM tc 10 μM) also significantly inhibited PDE5A. Data are presented as mean ± S.D. of three independent experiments. Statistical significance is represented as follows: * $P < 0.01$, ** $P < 0.05$.

IC_{50} values for hPDE5A of 98.2 μM and 60.0 μM, respectively. The six other alkaloids were less active against PDE5A (IC_{50} > 100 μg). The activities of the furoquinoline alkaloids differed according to the functional groups present and their positions (Table I and Fig. 1). Furoquinolines can have one to four methoxy groups, at the C-4, C-6, C-7, and C-8 positions. The furoquinolines with a single methoxy group at either C-7 or C-8 significantly inhibited PDE5A activity; the most potent had a methoxy at the C-8 position. Those with methoxy groups at both C-7 and C-8 or with a single hydroxy group or methoxy and hydroxy groups were inactive or less active inhibitors. These results were replicated in assays using purified bovine brain phosphodiesterase (data not shown).

Although a highly active compound was not identified in this series, two compounds, γ-fagarine and evolitrine, were the most potent inhibitors of PDE5A catalytic activity among the furoquinoline alkaloids. Modifications of these furoquinolines will be needed if they are to be of practical use in smooth muscle relaxation; further study of these alkaloids is currently underway.

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