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Synthesis, biological activities and bioavailability of moschamine, a safflomide-type phenylpropenoic acid amide found in *Centaurea cyanus*

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Moschamine is a phenylpropenoic acid amide found in plants. In this article, the synthesis and two biological activities (serotoninergic and cyclooxygenase (COX) inhibitory activities) and bioavailability of moschamine were described. Moschamine was synthesised and confirmed using NMR spectroscopic methods. Using the moschamine synthesised, serotoninergic and COX inhibitory activities were investigated. At the concentration of $10\,\mu mol\,L^{-1}$, moschamine was able to inhibit forskolin-stimulated cAMP formation by 25% ($p < 0.015$), *via* inhibiting serotonin receptors in the OK cells. The inhibition was repressed by two 5-HT1 antagonists (Nan-190 and spiperone), suggesting that moschamine may suppress cAMP formation *via* binding to 5-HT1 receptors in the cells. Also, moschamine was a very potent compound that is able to inhibit COX-I by 58% ($p < 0.012$) and COX-II by 54% ($p < 0.014$), at the concentration of $0.1\,\mu mol\,L^{-1}$. The oral bioavailability of moschamine was also determined in mice.

Keywords: moschamine synthesis; *Centaurea cyanus*; NMR; serotoninergic and cyclooxygenase (COX) inhibitory activities; HPLC; bioavailability

1. Introduction

Moschamine (syn; *N*-feruloylserotonin) is a phytochemical belonging to the group of safflomide-type phenylpropanoid acid amides, originally isolated from the seeds of an Asteraceae family plant, *Centaurea cyanus* (cornflower) (Sarker, Laird, Nahar, Kumarasamy, & Jaspars, 2001; Sarker, Savchenko, Whiting, Šik, & Dinan, 1997). For the last 10 years, several safflomide-type phenylpropanoid acid amides have been isolated from several plants including *Coffea canephora*, *Theobroma cacao* and *Carthamus tinctorius* (Jenett-Siems, Weigl, Kaloga, Schulz, & Eich, 2003; Niwa, Etoh, A. Shimizu, & Y. Shimizu, 2000; Sarker et al., 2001; Stark, Justus, & Hofmann, 2006; E. Tanaka, C. Tanaka, Mori, Kuwahara, & Tsuda, 2003). In plants, safflomide-type phenylpropanoid acid amides are synthesised via forming an amide bond between the carboxyl group of phenylpropanoid acids and the amine group of serotonin

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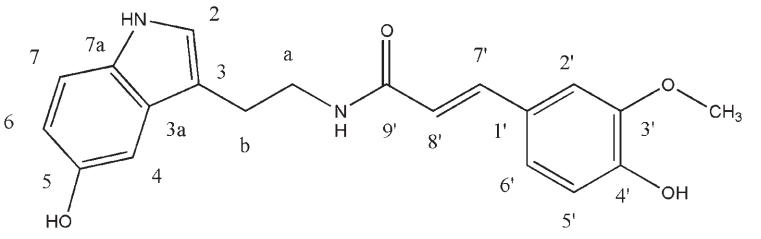

Figure 1. Chemical structure of moschamine.

derivatives by their respective transferases (Andrianaivoravelona, Terreaux, Sahpaz, Rasolondramanitra, & Hostettmann, 1999; Jang, Ishihara, & Back, 2004; Kang et al., 2006; Niwa et al., 2000; Sarker et al., 2001). Several studies indicate that these amides may possess anti-oxidant, anti-inflammatory and other activities, potentially providing beneficial effects on human chronic diseases such as inflammation and cardiovascular diseases (Koyama et al., 2006; Ohnishi, Morishita, Toda, Yase, & Kido, 1998; Takii et al., 2003). Interestingly, a recent study suggests that serotomide, a safflomide-type phenylpropanoid acid amide, is likely to act as a serotonergic agent binding to serotonin receptor type-1 (5-HT1R) in the cells (Park, 2008).

However, the effect of moschamine on 5-HT1R is currently unknown and the effects on cyclooxygenase (COX) I and II have not been investigated, even though some parts of *C. cyanus* have been traditionally used for treating inflammation-related diseases (e.g. abscess, ulcer, inflammation and cold) (Yesilada, Gürbüz, Bedir, Tatli, & Khan, 2004). Therefore, in this study, moschamine (1) was chemically synthesised and its potential effects of moschamine on 5-HT1R and COX (I and II) enzymes were investigated. Also, its bioavailability was determined in mice, related to its potential biological activities. This report suggests that moschamine can be absorbed as its intact form, able to inhibit COX I and II enzymes as well as to bind to 5-HT1 expressed on the renal epithelial (OK) cells.

2. Results and discussion

2.1. *Chemical synthesis and NMR analyses of moschamine*

Moschamine (*N*-feruloylserotonin; Figure 1) was chemically synthesised using the method previously described (Park, 2005, 2008). The synthesis was simple, and the yield of moschamine was greater than 40%. The synthesised product was purified by HPLC, and analysed by NMR spectroscopic methods as described in 'Materials and methods'. The structure of the synthesised product was determined by NMR as being N-[2-(6-Hydroxy-1H-indol-3-yl)-ethyl]-3-(4-hydroxy-3-methoxy-phenyl)-acryl-amide (*N*-feruloylserotonin).

2.2. *The effect of moschamine on forskolin-stimulated cAMP formation in the OK cells*

Forskolin is a cell-permeable diterpenoid possessing adenylyl cyclase activating properties (Pauwels & Palmier, 1994; Zgombick & Branchek, 1998). The activation of

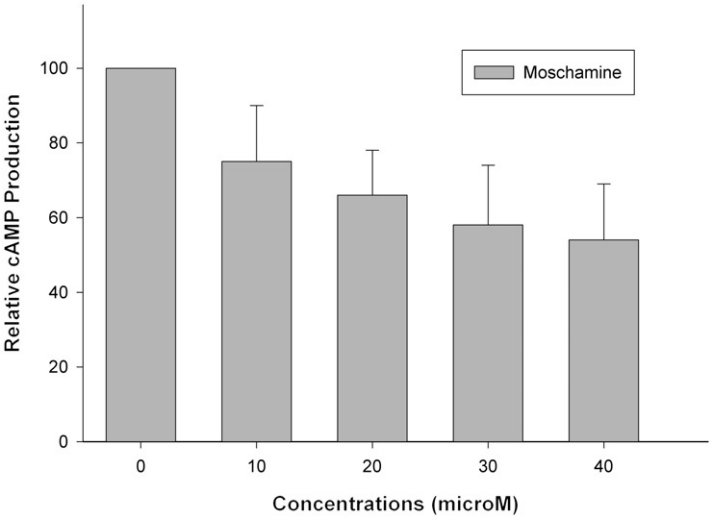

Figure 2. The effect of moschamine on forskolin-stimulated cAMP formation in the OK cells. Renal epithelial (OK) cells (1×10^6) were treated with moschamine for 10 min, prior to adding forskolin ($100\,\mu mol\,L^{-1}$) for 15 min. The cAMP production was measured as described in 'Materials and methods'. Data points in all figures represent the mean ± SD of more than five samples.

adenylyl cyclase by forskolin results in the increase of intracellular cAMP concentration (Majumdar, Peterson-Ford, & Uphouse, 2006; Zgombick & Branchek, 1998). In renal epithelial (OK) cells, 5-HT1 receptors are negatively coupled to adenylate cyclase, thus 5-HT1 receptor agonists can decrease cAMP formation stimulated by forskolin *via* inhibiting adenylate cyclase (Majumdar et al., 2006). At the concentration of $10\,\mu mol\,L^{-1}$, moschamine was able to inhibit forskolin-stimulated cAMP formation in the OK cells by 25% ($p < 0.015$). As shown in Figure 2, the inhibition was positively correlated to the concentrations of moschamine. These data suggest that moschamine may inhibit forskolin-stimulated cAMP formation via binding to 5-HT1 receptors in renal epithelial (OK) cells.

2.3. *The effects of serotonin receptor antagonists on moschamine-suppressed cAMP formation in the OK cells*

As mentioned above, moschamine was able to suppress forskolin-stimulated cAMP formation. However, it is still unclear whether the inhibition of cAMP production ensues *via* 5-HT1 receptors, negatively coupled to adenylate cyclase in the cells (Albert & Tiberi, 2001; Della Rocca et al., 1999; Odagaki & Toyoshima, 2005). Typically, 5-HT1 receptor antagonists are capable of repressing the inhibition of adenylate cyclase by 5-HT1 receptor agonists (Della Rocca et al., 1999). Therefore, if the inhibition of forskolin-stimulated cAMP production by moschamine occurs *via* binding to 5-HT1 receptors in the OK cells, 5-HT1 antagonists are able to repress the inhibition. In this experiment, two 5-HT1 receptor antagonists (Nan-190 and spiperone) were used to demonstrate that moschamine is able to

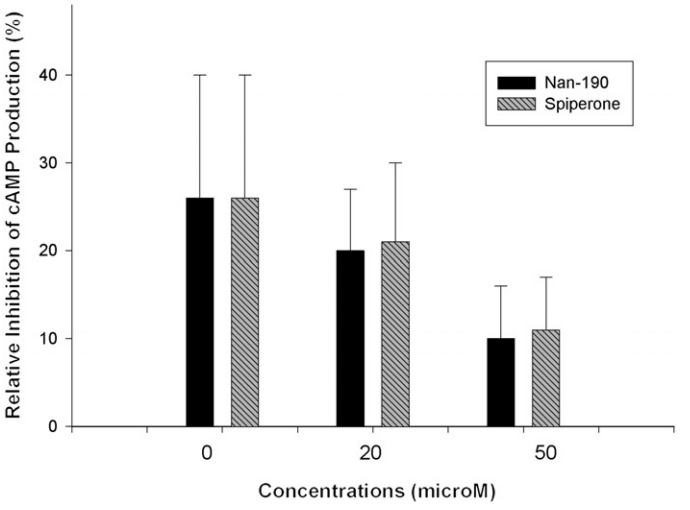

Figure 3. The effect of serotonin receptor antagonists on moschamine-suppressed cAMP formation in the OK cells. Renal epithelial (OK) cells (1×10^6) were treated with 5-HT antagonists, prior to the treatment of moschamine ($20\,\mu\,mol\,L^{-1}$). The cAMP production was induced by forskolin and measured as described in 'Materials and methods'. Data points in all figures represent the mean ± SD of four samples.

suppress forskolin-stimulated cAMP formation *via* 5-HT1 receptors in OK cells. As shown in Figure 3, at the concentrations of $20\,\mu\,mol\,L^{-1}$, both Nan-190 and spiperone repressed the inhibition of forskolin-stimulated cAMP production by moschamine in the OK cells by 24% ($p < 0.016$) and 20% ($p < 0.018$), respectively. The inhibition was further repressed upon the treatment with high concentrations ($\geq 40\,\mu\,mol\,L^{-1}$) of the two serotonin antagonists (Figure 3). These data show that moschamine is able to inhibit forskolin-stimulated cAMP production *via* binding to 5-HT1 receptors on the OK cells.

2.4. *The effects of moschamine on COX-I and II*

COX-I and II are expressed or induced in numerous cell types, deeply involved in prostaglandin homeostasis. By doing so, the enzymes are critically participated in various processes of inflammation and others (Smith, Meade, & DeWitt, 1994). In traditional medicines, some parts of *Centaurea* species (e.g. *C. cyanus, Centaurea drabifolia*, *Centaurea pulchella*, *Centaurea solstitialis*) have been mainly used for treating inflammation-related diseases (Yesilada et al., 2004). However, moschamine has not been investigated related to the inhibition of COX (I and II) enzymes. Therefore, effects of moschamine on COX-I and II were investigated in this article. As shown in Figure 4A, moschamine was very potent in inhibiting COX-I enzyme by 58% ($p < 0.012$) at the concentration of $0.1\,\mu\,mol\,L^{-1}$. On comparison with a well-known COX-I inhibitor (salicylic acid), moschamine was able to inhibit COX-I to a greater extent than salicylic acid. The effects of moschamine on COX-II were also investigated herein, because COX-II is also involved in many important physiological processes.

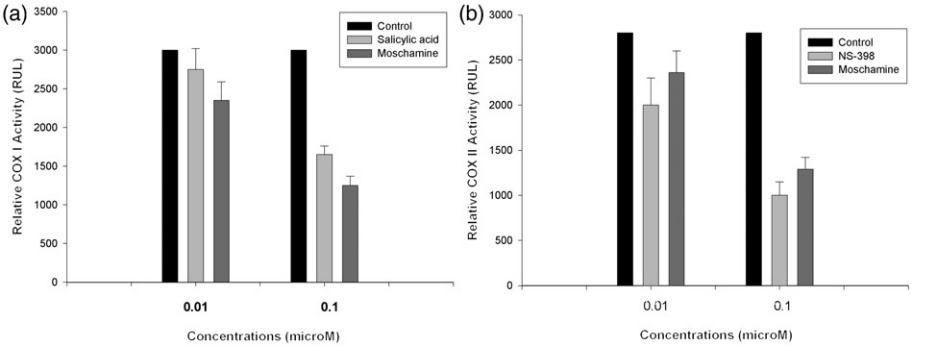

Figure 4. The inhibitory effect of moschamine on COX-I and II. Moschamine and COX inhibitors ($0.01\,\mu mol\,L^{-1}$ and $0.1\,\mu mol\,L^{-1}$) were added to the reaction mixtures, and the incubation was followed at room temperature (in the dark) for 10 min. Following the incubation, COX-I (A) and II (B) activities were measured according to the kit's protocol.

Like the COX-I inhibition, moschamine was also very effective in inhibiting COX-II enzyme by 54% ($p < 0.014$) at the concentration of $0.1\,\mu mol\,L^{-1}$. Compared to NS-398 (a COX-II specific inhibitor), moschamine was a bit less potent than NS-398 in inhibiting COX-II (Figure 4B), but the data overall suggest that moschamine may be a potent compound that is able to inhibit both COX-I and II enzymes.

2.5. *Bioavailability of moschamine following oral administrations in mice*

A number of studies indicate that moschamine analogues contain several biological activities, critically dependent on its plasma concentrations (Koyama et al., 2006; Park 2008; Takii et al., 2003). Therefore, in this study, plasma concentrations of moschamine were measured following two oral administrations (2 and 4 mg per 30 g body weight) to mice. The HPLC method described in 'Materials and methods' was used to measure moschamine in plasma samples prepared from two oral dosing groups ($n = 5$). The average concentrations of moschamine in the plasma collected at 0, 5, 10, 15, 20, 25, 30, 35 and 40 min after two oral administrations were 0, 0, 0, 25, 100, 40, 0, 0 and $0\,\mu mol\,L^{-1}$, and 0, 0, 8, 60, 210, 90, 10, 0 and $0\,\mu mol\,L^{-1}$, respectively. Moschamine appeared around 10 min after the high-dose oral administration (4 mg), and the highest amount was detected at around 20 min. After 20 min, plasma concentrations began to decrease rapidly, suggesting that moschamine may be metabolised quickly after that. The low-dose oral administration (2 mg) also produced similar patterns; the detection at later than 10 min, highest at around 20 min, and decreasing after that.

3. Experimental

3.1. *Materials*

Serotonin, ferulic acid, COX I and II enzymes, and others were purchased from Sigma Chemical Co. (St. Louis, MO). COX and cyclic AMP (cAMP) assay kits were purchased from Assay Designs Inc. (Ann Arbor, MI).

3.2. *Chemical synthesis*

Synthesis was performed using the method described previously (Park, 2005). Briefly, ferulic acid was dissolved in dichloromethane and converted to the symmetrical anhydride with 1,3-diisopropylcarbodiimide. Serotonin was added to the reaction mixture and incubated with a gentle stirring for 12 h. The following are the NMR data for moschamine. ^{1}H-NMR: (DMSO-d_6, 400 MHz) δ 7.38 (1H, d, $J = 15.6$ Hz, H-7′), 7.20 (1H, s, H-2), 6.88 (1H, dd, $J = 8.2; 1.4$ Hz, H-6′), 6.41 (1H, d, $J = 15.6$ Hz, H-8′), 7.58 (1H, s, H-4), 7.00 (1H, d, $J = 7.3$ Hz, H-6), 6.80 (1H, d, $J = 8.2$ Hz, H-5′), 7.04 (1H, br s, H-2′), 7.37 (1H, d, $J = 7.4$ Hz, H-7), 3.87 (3H, s, H-10′), 3.53 (2H, td, $J = 6.9$; 6.0 Hz, H-a) and 2.94 (2H, t, $J = 6.9$ Hz, H-b). ^{13}C-NMR; (DMSO-d_6, 100 MHz) δ 165.7 (C, C-9′), 151.4 (C, C-5), 149.5 (C, C-3′), 142.7 (C, C-4′), 141.3 (CH, C-7′), 132.4 (C, C-7a), 128.7 (C, C-3a), 127.6 (C, C-1′), 122.8 (CH, C-2), 120.6 (CH, C-6′), 118.8 (CH, C-8′), 108.4 (CH, C-4), 107.1 (CH, C-6), 116.0 (CH, C-5′), 114.0 (CH, C-2′), 112.0 (C, C-3), 111.5 (CH, C-7), 58.3 (CH3, C-10′), 45.8 (CH2, C-a) and 30.5 (CH2, C-b). The synthesised products were recovered and purified by HPLC (Waters, Milford, MA), as described previously (Park, 2008).

3.3. *NMR analyses*

For NMR experiments, the sample (20 mg) was prepared in DMSO-d_6 (0.75 mL). ^{1}H, ^{13}C, COSY and HMBC spectra were acquired at an ambient temperature on the JEOL BCX-400 NMR spectrometer operating 400 MHz for ^{1}H and 100 MHz for ^{13}C.

3.4. *cAMP measurement*

Forskolin (100 μ mol L^{-1}) was added to cultures containing renal epithelial (OK) cells (1×10^6) for 15 min, to induce cAMP production. Moschamine and/or serotonin receptor antagonists were added just prior to adding forskolin (Pauwels & Palmier, 1994). The cAMP production was determined in renal epithelial (OK) cells using Correlate-EIA™ Direct cyclic AMP kit (Assay Designs Inc., Ann Arbor, MI). All measurements were performed according to the kit's protocol. The measured optical density at 405 nm was used to calculate the concentrations of cAMP.

3.5. *COX-I and II assay*

COX-I and II activities were measured in a 96-well plate using a chemiluminescent COX kit (Assay Designs Inc., Ann Arbor, MI) according to the kit's protocol. Relative light units' output was measured to determine COX activity.

3.6. *Measurement of moschamine in plasma samples*

For determining plasma concentrations of moschamine, Swiss Webster mice, 20–25 weeks old (Charles River, Wilmington, MA.), were used for blood collections. All animal procedures were performed according to an animal protocol (BA06-031)

approved at Beltsville Agricultural Research Center. Mice in the experimental trial were divided into control, and two test groups ($n = 5$). For the two test groups, moschamine (2 and 4 mg per 30 g body weight) was administered into the animal using a dosing needle, because preliminary study showed that the oral administration of moschamine (1 mg per 30 g body) could provide C_{max} around 50 μ mol L^{-1}. Whole blood was collected and the plasma was prepared to extract moschamine. First, the samples were precipitated with methanol (40%) and centrifuged (14000 rpm) for 10 min. The supernatant was used for HPLC analysis. Ascentis RP-Amide (15 cm × 4.6 mm id., Sigma, St. Louis, MO) was used as the stationary phase to analyse moschamine, using a gradient condition (buffer A (50 m mol L^{-1} NaH_2PO_4, pH 4.3) for 0–5 min, a linear change from buffer A to buffer B (70% acetonitrile) for 5–20 min and buffer B for 5 min) at the flow rate of 0.8 mL min^{-1}. The detection was performed by CoulArray electrochemical detector with four electrode channels (ESA, Chelmsford, MA).

3.7. *Data analyses*

All statistical analyses were performed with the SigmaPlot 11.0 (Chicago, IL). The *p*-value was calculated using one-way ANOVA with Holm-Sidak method, and $p < 0.05$ was considered as statistically significant. Data points in all figures represent the mean ± SD of more than five samples.

4. Conclusion

In this study, moschamine was chemically synthesised and the synthesis was confirmed by NMR. Moschamine was able to inhibit COX I and II enzymes as well as to inhibit forskolin-stimulated cAMP formation *via* inhibiting 5-HT1 in the OK cells. Following oral administrations to mice, moschamine was found in the plasma in an intact form with COX inhibitory and serotonergic activities. To the best of our knowledge, this is the first report regarding the chemical synthesis, biological activities and bioavailability of moschamine found in the seeds of *C. cyanus* (cornflower).

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