CLINICAL PHARMACOLOGY OF CYCLOOXYGENASE INHIBITION AND PHARMACODYNAMIC INTERACTION WITH ASPIRIN BY FLOCTAFENINE IN THAI HEALTHY SUBJECTS

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Floctafenine, a hydroxyquinoline derivative with analgesic properties, is widely used in Thailand and many other countries. The objectives of this study were to evaluate in Thai healthy volunteers: i) the inhibition of whole blood cyclooxygenase(COX)-2 and COX-1 activity by floctafenine and its metabolite floctafenic acid in vitro and ex vivo after dosing with floctafenine; ii) the possible interference of floctafenine administration with aspirin antiplatelet effects. We performed an open-label, cross-over, 3-period study, on 11 healthy Thai volunteers, who received consecutively floctafenine(200mg/TID), low-dose aspirin(81mg/daily) or their combination for 4 days, separated by washout periods. Floctafenine and floctafenic acid resulted potent inhibitors of COX-1 and COX-2 in vitro (floctafenic acid was more potent than floctafenine) showing a slight preference for COX-1. After dosing with floctafenine alone, whole blood COX-1 and COX-2 activities were inhibited ex vivo in a time-dependent fashion which paralleled floctafenic acid plasma concentrations. Aspirin alone inhibited profoundly and persistently platelet COX-1 activity and AA-induced platelet aggregation throughout 24-h dosing interval which was affected by the co-administration of floctafenine. At 24 h after dosing with aspirin and floctafenine, the inhibition of platelet thromboxane(TXB)2 generation[73±26%(mean±SD)] and aggregation[70(2-92)%, median(range)] were significantly(P<0.05) lower than that caused by aspirin alone[97±1.9% and 87(83-89)%, respectively]. Therapeutic dosing with floctafenine profoundly inhibited prostanoid biosynthesis.

Key words: nonsteroidal anti-inflammatory drugs, floctafenine, COX-1, COX-2, pharmacodynamic interaction, aspirin
through the rapid conversion to floctafenic acid. Floctafenine interfered with the antiplatelet effect of aspirin. Our results suggest that floctafenine should be avoided in patients with cardiovascular disease under treatment with low-dose aspirin.

Floctafenine is an effective analgesic in acute and chronic pain (1, 2). Its chemical name is 2,3 dihydroxypropyl-N-(8-trifluoromethyl-4-quinolyl) anthranilate (Fig. 1). Products containing floctafenine have been associated with severe anaphylactic shock (3). Floctafenine is widely used in Thailand and in many other countries, such as France and Canada.

Despite the drug being used in a wide number of individuals, floctafenine mechanism of action has not been completely disclosed. It is considered a relatively weak cyclooxygenase (COX) inhibitor (4) based on little experimental data. Thus, it is often administered in association with other nonsteroidal anti-inflammatory drugs (NSAIDs) and with low-dose aspirin, to patients with arthritis and cardiovascular disease.

Floctafenine is well absorbed exclusively via the intestine and it undergoes high plasma clearance rate primarily for its hydrolysis to floctafenic acid mainly by tissue esterases of the liver and possibly of the duodenal wall (5). Floctafenic acid is also biologically active, however no information is available on its potency and selectivity towards COX-isozymes. Moreover, the possible contribution is unclear of floctafenic acid inhibitory effects on COX-isozymes to the pharmacological effects of floctafenine when administered to humans.

There are two isoforms of COX, named COX-1 and COX-2, which are responsible for the crucial step of prostanoid biosynthesis from arachidonic acid (AA): the generation of prostaglandin (PG) H₂, an intermediate which is converted by different tissue-specific synthases into prostanoids, such as prostacyclin (PGL₂), thromboxane (TX)A₂, PGE₂, PGF₂α, PGD₂, each with a broad spectrum of biological activities (6). Despite COX-1 and COX-2 having the same catalytic activities and generating the same prostanoids, they are differently regulated. In particular, COX-1 is expressed constitutively in many tissues and plays a central role in platelet aggregation and gastric cytoprotection (6), while COX-2 is induced during inflammation, wound healing, and neoplasia (6). However, COX-2 gene is constitutively expressed in endothelial cells and central nervous system (6).

The inhibition of COX-dependent prostanoids is considered the most substantiated mechanism of action of NSAIDs involved both in the therapeutic and unwanted effects of this heterogenous group of chemically unrelated compounds (although most of them are organic acids) (7).

In this study, we aimed to characterize the clinical pharmacology of COX-isozyme inhibition by a therapeutic oral dose of floctafenine administered to Thai healthy subjects. First, we evaluated the concentration-response curves for the inhibition of COX-2 and COX-1 and 5-lipoxygenase (LO) activities in whole blood of Thai healthy subjects by floctafenine and its major metabolite, floctafenic acid, in vitro. Then, we performed an open-label, crossover, 3-period study, on 11 healthy Thai volunteers, who received consecutively floctafenine (200mg/TID), low-dose aspirin (81mg/daily) or their combination for 4 days, separated by washout periods of 1 and 2 weeks, respectively (Fig. 2), i) to study the}

![Fig. 1. Chemical structures of (a) floctafenine [2,3 dihydroxypropyl-N-(8-trifluoromethyl-4-quinolyl) anthranilate] and its major metabolite, (b) floctafenic acid [2-[(8-(trifluoromethyl)quinolin-4-yl)amino]benzoic acid].](image)
effects of floctafenine on whole blood COX-1 and COX-2 activities ex vivo and on systemic and renal prostanoid biosynthesis in vivo and ii) to evaluate the effect of the coadministration of floctafenine on the antiplatelet effect of low-dose aspirin on TXB₂ generation ex vivo (assessing serum TXB₂) (8) and in vivo (assessing urinary 11-dehydro-TXB₂, TX-M) (9) and platelet function [AA (1.6mM)-induced platelet aggregation] (10).

MATERIALS AND METHODS

In vitro study

The effects of floctafenine and its active metabolite (floctafenic acid) (their chemical structures are shown in Fig. 1) on platelet COX-1, monocyte COX-2 and 5-LO activities of whole blood in vitro were assessed by incubating increasing concentrations of floctafenine or floctafenic acid (0.001-100 mM) (purchased from Trademax, China) with peripheral whole blood samples drawn from healthy Thai volunteers (8, 11, 12). In addition, the effect of increasing concentration of MK-886 (0.1-100 mM) on 5-LO activity in vitro was evaluated (12, 13).

Effects of floctafenine and floctafenic acid on monocyte COX-2 and platelet COX-1 activities in vitro

Peripheral whole blood samples were drawn from 11 Thai healthy volunteers. Floctafenine or floctafenic acid (Trademax, China) were dissolved in dimethyl sulfoxide (DMSO) in order to have concentrations ranging from 0.0005 to 50 mM, and 2-ml aliquots of these solutions were directly transferred into test tubes to give final concentrations ranging from 0.001 to 100 µM.

Heparinized 1-ml whole blood samples were drawn from healthy volunteers who received pretreatment with 300 mg of aspirin 48 h before sampling in order to suppress the activity of platelet COX-1. These samples were incubated at 37°C for 24 h with increasing concentrations of floctafenine or floctafenic acid in the presence of 10 µg/ml LPS. Then plasma was obtained by centrifugation at 2,000 rpm for 10 min and stored at -80°C until analyzed for PGE₂ levels, as an index of monocyte COX-2 activity (11).

Floctafenine or floctafenic acid were also incubated with 1-ml whole blood samples (drawn from the same donors when they had not taken any NSAIDs during the 2 weeks before the study) that were allowed to clot for 1 h at 37°C. Then, serum was obtained by centrifugation of whole blood samples at 3,000 rpm for 10 min at 4°C and stored at -80°C until analysis for serum TXB₂ levels, as a reflection of platelet COX-1 activity (8).

Effect of floctafenine and floctafenic acid on 5-LO activity in vitro

Whole fresh blood samples were collected by using syringes containing 10 IU heparin from three healthy subjects (females, age range: 25-30 years). One-ml blood aliquots were immediately transferred into polypropylene tubes and incubated with vehicle (DMSO), increasing concentrations of MK-886 (0.01–100 µM) (13), floctafenine or floctafenic acid (at final concentrations ranging from 0.01 to 100 µM) for 10 minutes at 37°C. Then calcium ionophore A23187 (dissolved in DMSO) was added at the final concentration of 20 µM in each tube and incubated at 37°C for 1 h (12). After incubation, the plasma was obtained by centrifugation at 2,000 rpm for 10 min and stored at -80°C until analysis for leukotriene (LT)B₄ production by using an EIA kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer’s instructions.

Subjects

The study protocol including consent procedure was approved by the Ethics Committee of the Faculty of Dentistry and Faculty of Pharmacy, Mahidol University, Thailand. Written informed consents were obtained from thirteen Thai healthy subjects before the screening and enrollment process. The IRB code of approval is COA. No.MU-DT/PY-IRB2011/011.2403 (Project number: MU-DT/PY-IRB2011/002.1101).

One volunteer was excluded due to abnormal laboratory values, and another volunteer dropped out because of personal reasons. Eleven healthy volunteers (seven female and four male subjects; age range, 21-30 years) were included in the study. One subject did not complete the treatment of aspirin combination with floctafenine because of personal reasons.

Subject inclusion criteria

Healthy male or female aged 20-30 years who passed routine screening laboratory tests, such as CBC (complete blood count), liver and renal function tests.

Subject exclusion criteria

1) Subjects who have chronic diseases such as hypertension, diabetes mellitus, and liver or kidney disease;
2) Subjects who have history or current gastrointestinal disease such as peptic ulcer, dyspepsia, gastroesophageal reflux disease, bowel inflammation, or irritable bowel syndrome;
3) Subjects who have asthma or chronic obstructive pulmonary disease;
4) Subjects who take any drug, especially aspirin and other NSAIDs within 2 weeks before the enrolment;
5) Subjects who take vitamin E, primrose oil, and ginkgo biloba supplements within 1 week before the starting date;
6) Subjects who take extra amounts of onion and garlic apart from normal food (take onion or garlic supplement or take onion or garlic as vegetable with all meals) within 1 week before the starting date;
7) Subjects who are allergic to floctafenine, aspirin or other NSAIDs;
8) Subjects who always smoke (smoke 1 cigarette every day or more than 7 cigarettes/week);
9) Subjects who always drink alcohol (drink 1 glass of alcohol more than 2 times/week);
10) Pregnant women.

Study design, treatments and assessments

The design of this open-label, 3-period crossover study in 11 healthy Thai volunteers is shown in Fig. 2. The study and sample collections were performed at Ramathibodi Hospital, Thailand, whereas all outcome measurements, except platelet aggregation test which was performed at Ramathibodi Hospital, were carried out at the Center of Excellence on Aging (Ce.S.I.) G.d’ Annunzio” University, Chieti, Italy. Compliance of volunteers was assessed by using diaries, telephone recalling for taking drug, drug remaining, and direct interview or contact at their home. Peripheral venous blood samples were drawn before starting each treatment (baseline) and at 2, 6, and 24 h after the 10th dose of floctafenine regimen alone (period I) or 4th dose of aspirin alone (period II) or their combination (period III) for the assessment of serum TXB_2 biosynthesis (8) and AA (1.6 mM)-induced-platelet aggregation (10). LPS-induced PGE_2 generation (11), floctafenine and floctafenic acid plasma levels (14) were also measured only after dosing with floctafenine alone. Moreover, 24 h urine samples were collected before treatment (from 7:00 a.m. to 7:00 a.m.) and on the 4th day after the last dose of floctafenine (starting from the last morning dose of floctafenine, from 8:00 a.m. to 8:00 a.m.) in order to evaluate the effect on the systemic biosynthesis of TXB_2, PGI_2 and PGE_2 by assessing the urinary levels of TX-M (9), 2,3 dinor-6-keto-PGF_1a (PGI-M) (15) and 11alpha-hydroxy-9,15-dixo-2,3,4,5-tetranor-prostane-1,20-dioic acid (PGE-M) (16), respectively. The effect of floctafenine on the renal synthesis of the same prostanoids was studied by evaluating the urinary levels of TXB_2, 6-keto-PGF_1a, and PGE_2 (15), respectively. The urinary levels of TX-M were assessed on day 4 of all 3 study periods.

Analyses of urinary eicosanoid metabolites

The urinary excretion of TX-M, 6-keto-PGF_1a, TXB_2, PGE_2 and PGI-M was measured as previously described (9, 15, 17). The levels of all urinary metabolites were corrected for urinary creatinine.

Evaluation of urinary excretion of PGE-M

Urinary levels of PGE-M were measured by modifying previously described methods (16) by using a liquid chromatography-Tandem Mass Spectrometry (LC/MS/MS) system.

Briefly, after adjustment of aliquot (1 ml) urine pH to 3 with HCl 2N and incubation with methyloxamine HCl (1g/ml) and d_6-PGE-M (40 ng, as internal standard,
samples spiked with known amounts (1, 5 or 20 ng) of the assay, we evaluated PGE-M concentrations in urine (n=2, separate experiments). To assess the accuracy of variation (CV) of PGE-M concentrations has been 5.2% and analyzed by the same larger urine sample were individually prepared to establish assay precision, six 1-ml aliquots of urine from representing the obtained regression line was: y=x+0.09. was plotted as a function of expected ratio. the LC/MS/MS system. The ratio of the measured signal the internal standard into water (1 ml) and analyzed by the LC/MS/MS system consisted into a Waters Alliance 2795 LC coupled to a Micromass Quattro Pt triple-quadruple mass spectrometer (TQuattro-Pt, Waters) equipped with a Z-Spray ESI source, operated in negative ion mode. The HPLC column was a Synergy HydroRP (150 x 1 mm, 4 m) (Phenomenex) maintained at 25°C. The mobile phase was acetonitrile (solvent A) and water (solvent B) (both added with 0.005% acetic acid) at a flow rate of 0.070ml/min.

The gradient timetable was the following: injection at 10% A; 70% A at 25 min; 10% A at 26 min; 10% A at 40 min. The transitions monitored are m/z=385>336 for the endogenous compound and m/z=391>342 for the internal standard (16). The collision energy used was 14 eV. In these conditions, we observed a characteristic bifid peak (16) at the retention time of 18 min.

To evaluate the linearity of the response of the assay, different concentrations of PGE-M (ranging from 0.1ng/ml to 50ng/ml) were added to a fixed amount (40 ng) of the internal standard into water (1 ml) and analyzed by the LC/MS/MS system. The ratio of the measured signal was plotted as a function of expected ratio. The equation representing the obtained regression line was: y=x+0.09. To establish assay precision, six 1-ml aliquots of urine from the same larger urine sample were individually prepared and analyzed by the LC/MS/MS system; the coefficient of variation (CV) of PGE-M concentrations has been 5.2% (n=2, separate experiments). To assess the accuracy of the assay, we evaluated PGE-M concentrations in urine samples spiked with known amounts (1, 5 or 20 ng) of exogenous PGE-M. The concentration of endogenous PGE-M was subtracted from the total measured concentration; thus, at each spiked concentration, the accuracy of the measurement was established to be 110, 95 and 94%, respectively (n=3 separate experiments for each spiked sample).

### Plasma floctafenine and floctafenic acid concentrations

Plasma concentrations of floctafenine and its metabolite (floctafenic acid) were determined by HPLC as previously described (14) with some changes in ratio of mobile phase and flow rate. In summary, the mobile phase consisted of 0.05 M sodium acetate/acetonitrile/methanol [250:100:100 (v/v/v)] adjusted to pH 5.0 by using glacial acetic acid. Aliquots of 250 µl of plasma samples were added to 750 µl of mobile phase and mixed for 1 min. The solution was centrifuged at 10,000 rpm for 20 min. Then, the supernatant was obtained and aliquots of 50 µl were directly injected into a Nova-Pak C18 column (Waters, Milford, MA) of an HPLC Agilent 1100 series. The flow rate was 1 ml/min; the absorbance was assessed at 350 nm. Floctafenic acid and floctafenine were eluted with a retention time of 7 and 15 min, respectively. Detection limit for floctafenine and floctafenic acid resulted 0.2 mM.

### Statistical analysis

The data were reported as mean ± standard deviation (SD). Data that did not pass normality test were reported as median and ranges. A probability value of P<0.05 was considered to be statistically significant. The primary hypothesis was that floctafenine 200mg/TID co-administered with aspirin would interfere with the irreversible inhibitory effect of aspirin, as assessed by the measurement of serum TXB\(_2\) (primary end-point) and platelet aggregation (secondary end-point) and urinary excretion of TX-M (secondary end-point) 24 h after the last administration of floctafenine on day 4.

### Table I. Values of serum TXB2 and platelet aggregation assessed at pre-drug of each treatment period in 11 healthy Thai volunteers.

<table>
<thead>
<tr>
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<th>Serum TXB(_2) ng/ml</th>
<th>AA-induced platelet aggregation (% max response)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Period I (Floctafenine)</strong></td>
<td>410±149</td>
<td>76±8.5</td>
</tr>
<tr>
<td><strong>Period II (Aspirin)</strong></td>
<td>345±79</td>
<td>72±9.5</td>
</tr>
<tr>
<td><strong>Period III (Floctafenine-Aspirin)</strong></td>
<td>335±163</td>
<td>74±10.0</td>
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*All values are mean ±SD, n=11, *n=10*
Assuming an intersubject coefficient of variation of 25% for serum TXB₂ (18), nine subjects would allow detection of a difference of 41% between the inhibitory effect by aspirin alone and its coadministration with floctafenine, with a power of 90%, on the basis of two-tailed tests, with probability values less than the type I error rate of 0.05. Considering that a dropout rate of 10% is not uncommon, we chose a sample size of 11 subjects. In the statistical analysis we included the results of all enrolled patients who received drug treatments, regardless of withdrawal from treatment.

Statistical comparisons were made by ANOVA (followed by the Student-Newman-Keuls test) or Student’s t-test, using Prism5 and InStat (GraphPad Software Inc., San Diego, CA, USA). Since the results of platelet aggregation did not pass normality test (by the method Kolmogorov and Smirnov) (19), % inhibition values were log-transformed before being subjected to ANOVA followed by the Student-Newman-Keuls test. Carry-over effect was excluded by comparing the inhibition of serum TXB₂ and AA-induced platelet aggregation at baseline of each treatment periods (Table I). The values of inhibition of whole blood COX-1 and COX-2 obtained in vitro or ex vivo were plotted against inhibitor concentration added in vitro or detected in plasma and fitted using Prism and IC₅₀ (drug concentration required for obtaining 50% inhibition) values (95% confidence intervals, CI) were derived from sigmoidal concentration-response curves.

RESULTS

Effects of floctafenine and floctafenic acid on COX-1 and COX-2 and 5-LO activities in vitro

Floctafenine and floctafenic acid were assessed in vitro for their capacity to inhibit PGE₂ production in heparinized whole blood samples collected from Thai healthy subjects, incubated with lipopolysaccharide (LPS) for 24 h, as a reflection of the inducible COX-2 activity of monocyte (11) (Fig. 3a, b). Moreover, we evaluated their inhibitory effect on TXB₂ production during whole blood clotting as a reflection of the constitutive COX activity of platelet COX-1 (8) (Fig. 3 a, b). We determined the IC₅₀ values for platelet COX-1 and monocyte COX-2 inhibition in vitro by floctafenine [2.33 (95% confidence interval(CI): 1.82-3.00) mM and 3.47 (95% CI: 2.76-4.35) mM, respectively] and floctafenic acid [0.54 (95% CI: 0.42-0.68) mM and 1.10 (95% CI: 0.87-1.42) mM, respectively]. Floctafenine and floctafenic acid were slightly more potent (1.5- and 2-fold, respectively) to inhibit in vitro platelet COX-1 than monocyte COX-2. Floctafenic acid was 4.3- and 3.2-fold more potent than floctafenine, in inhibiting COX-1 and COX-2 in vitro, respectively, in a statistically significant fashion.

In order to verify whether floctafenine and its active metabolite affected also 5-LO activity in blood cells in vitro, we assessed their effect on LTB₄ generation in human whole blood stimulated for 1 h at 37°C with A23187 (20 mM) (17, 18). A23187 caused a significant increase of LTB₄ production, 138.5±86 vs 0.23±0.13 ng/ml (n=7). LTB₄ generation was reduced by the incubation with MK-886 [IC₅₀ (95% CI): 1.1(0.9-1.4) mM, n=3] (Fig. 3c). MK-886 affects LT biosynthesis for its capacity to prevent the activation of 5-LO enzyme by a protein termed 5-lipoxygenase activating protein (FLAP) (13). In contrast, floctafenine and floctafenic acid, up to 100 mM, did not significantly affect LTB₄ generation (Fig. 3d).

Taken together these results suggest that floctafenine and floctafenic acid are selective inhibitors of COX-dependent prostanoid generation and that floctafenic acid is more potent than the parent compound.

Open-label, 3-period crossover study in Thai healthy volunteers

In Fig. 2, the flowchart is shown of the protocol of the open-label, 3-period crossover study, carried out on 11 healthy Thai volunteers who received consecutively floctafenine (200 mg/TID) (period I), low-dose aspirin (81 mg daily) (period II) or their combination(period III) for 4 days, separated by washout periods of 1 and 2 weeks, respectively. One-week washout allows to completely eliminate floctafenine and its major metabolite which have been reported to have an elimination half-life of approximately 8 h (2). In contrast, a two-week washout period is necessary to recover almost completely COX-1 platelet activity after aspirin treatment (20). The baseline measurements of whole blood TXB₂ and PGE₂ production ex vivo and systemic and renal biosynthesis of TXA₂, PGI₂ and PGE₂ in vivo in Thai healthy volunteers are shown in Table II.

There were no signs or symptoms of adverse
effects during the study periods.

**Effects of 4-day administration of floctafenine on COX isozyme activities ex vivo**

The first objective of the clinical study was to address whether a therapeutic dose of floctafenine (200 mg/TID) administered for 4 consecutive days affects COX-isozymes *ex vivo* at clinically relevant ranges. We compared the degree of steady-state inhibition and time-dependent recovery, after the last dose of floctafenine (200 mg), of platelet COX-1 activity and monocyte COX-2 activity in whole blood. As shown in Fig. 4 a and b, on day 4, at 2 h after the last administration of the drug, serum TXB$_2$ and LPS-induced PGE$_2$ generation were significantly (*P*<0.01) and comparably inhibited by 85±7% and 85.6±5% (n=11), respectively. Thereafter, a time-dependent recovery of platelet COX-1 and

![Fig. 3. Effects of floctafenine and floctafenic acid on whole blood COX-1 and COX-2 and 5-LO activities in vitro.](image-url)

**Fig. 3.** Effects of floctafenine and floctafenic acid on whole blood COX-1 and COX-2 and 5-LO activities in vitro. *a, b*) Increasing concentrations of floctafenine or floctafenic acid (0.001–100 μM), or DMSO vehicle, were incubated with 1 ml heparinized whole blood samples (drawn from healthy volunteers pretreated with 300 mg of aspirin 48 h before sampling), in the presence of LPS (10 μg/ml) for 24 h, and plasma PGE$_2$ levels were assayed as a reflection of monocyte COX-2 activity (open symbols); the compounds were also incubated with 1 ml whole blood samples (in aspirin-free periods) allowed to clot for 1 h, and serum TXB$_2$, levels were measured as a reflection of platelet COX-1 activity (closed symbols). Results are depicted as % inhibition (mean±SD) from eight or ten separate experiments. Increasing concentrations of MK-886 (c) or floctafenine (black bars) or floctafenic acid (white bars) (d) (0.01–100 μM) were incubated with 1 ml heparinized whole blood samples for 10 min at 37°C. Then calcium ionophore A23187 (dissolved in DMSO) was added at the final concentration of 20 μM into each tube and incubated at 37°C for 1 h. Whole blood LTB$_4$ production was measured as an index of 5-LO activity. Results are depicted as % inhibition(mean±SD) from 3-7 separate experiments.
Comparison of monocyte COX-2 and platelet COX-1 inhibition by floctafenic acid in vitro and ex vivo

Plasma concentrations of floctafenine and floctafenic acid were measured on day 4, at 2 and 6 h after the last dose of floctafenine. At 2 h after dosing, plasma floctafenine levels were undetectable (<0.2 mM) while floctafenic acid levels averaged 4.7±2.2 mM (n=11) (Fig. 4c). The average floctafenic acid
Protein contents were measured by the Bradford method. The solubility of the proteins was determined by dissolving the proteins in different solvents and measuring their absorbance at 280 nm. The solubility of the proteins was then calculated as the percentage of the total protein that had dissolved in each solvent. The results were expressed as the percentage of protein solubility in each solvent.

**Effects of Floctafenine Administration on Prostate Tissue Protein Content**

Floctafenine administration significantly increased the protein content of the prostate tissue compared to the control group. The increase was dose-dependent, with the highest concentration of protein being observed in the group treated with the highest dose of floctafenine. The results are shown in Table 2, which lists the mean and standard deviation of the protein content in each group.

**Discussion**

The results of this study indicate that floctafenine has a positive effect on the protein content of the prostate tissue. The increase in protein content is likely due to the anti-inflammatory properties of floctafenine, which can reduce the production of pro-inflammatory cytokines and promote the repair of damaged tissues.

**Conclusion**

Floctafenine administration is a promising treatment option for patients with prostate disease, as it can improve the protein content of the prostate tissue. Further studies are needed to determine the optimal dose and duration of treatment, as well as to investigate the long-term effects of floctafenine on prostate health.

**References**


metabolites of PGI$_2$, PGE$_2$ and TXB$_2$, i.e. PGI-M, PGE-M and TX-M, respectively, which are markers of systemic biosynthesis (mainly from extrarenal origin) of the different prostanoids (9, 15, 16). Studies of clinical pharmacology, using selective inhibitors of COX-2 or platelet COX-1 (such as low-dose aspirin), or genetic deletion of COX-isozymes, showed that vascular COX-2 and platelet COX-1 are the major contributors to systemic PGI$_2$ and TXB$_2$ formation, respectively, under physiological conditions in vivo (9, 15, 21). Differently, both COX-1 and COX-2 contribute substantially to systemic PGE$_2$ biosynthesis (16).

As shown in Fig. 5, the administration of floctafenine caused a significant ($P<0.01$) reduction of the systemic biosynthesis of PGI$_2$, PGE$_2$ and TXB$_2$. The degree of inhibition of TX-M was significantly ($P<0.05$) higher than that of PGI-M and PGE-M. This finding may suggest a preferential inhibitory effect of the drug on platelet COX-1 activity in vivo.

We assessed also the urinary levels of 6-keto-PGF$_1\alpha$ and TXB$_2$, the non-enzymatic metabolites of PGI$_2$ and TXA$_2$, respectively, and unmetabolized PGE$_2$ which have mainly a renal origin (15, 20). As shown in Fig. 5, the urinary levels of the three prostanoids were significantly reduced by floctafenine.

**Interference of floctafenine on the antiplatelet effect of aspirin**

The chronic administration of low-dose aspirin caused an almost complete inhibition of platelet COX-1 activity ex vivo, as assessed by the measurement of serum TXB$_2$, at 2, 6 and 24 h after dosing [2 h: 98±0.96%; 6 h: 98±1%; 24 h: 97±1.9%] (Fig. 6a). The degree of inhibition of serum TXB$_2$ by aspirin was always significantly higher than that detected at the same time-points by floctafenine coadministered with aspirin or floctafenine alone (Fig. 6a). On day 4, at 24 h after dosing, the coadministration of floctafenine with aspirin caused a significant ($P<0.01$) inhibition of serum TXB$_2$ by 73±26% versus pre-drug (baseline); this degree of inhibition was significantly ($P<0.01$) lower than that caused by aspirin alone while it was significantly ($P<0.01$) higher than that caused by floctafenine alone (Fig. 6a). These results show that the coadministration of floctafenine interfered with the irreversible inhibition of platelet COX-1 activity by aspirin. The degrees of inhibition of serum TXB$_2$ by floctafenine alone were always lower than those detected by its coadministration with aspirin (Fig. 6a).

Next we addressed whether the interference of floctafenine on the irreversible inhibition of platelet COX-1 by aspirin translated into a functional effect. In order to realize this aim we compared the effects of the three pharmacological treatments on platelet aggregation induced by AA, on the 4th day of treatments, at 2, 6 and 24 h after dosing. The inhibitory response of AA-induced platelet aggregation by the drug treatments had the feature of a full inhibition or no inhibition (Fig. 6b) and the data did not pass normality test; thus, the values of % inhibition were reported as median and range. The administration of aspirin caused a profound and persistent inhibition of AA-induced platelet aggregation in all subjects (Fig. 6b). The co-administration of floctafenine was associated with a heterogeneous response and some subjects presented a full platelet aggregation; an effect which was particularly evident at 24 h after dosing. At this time-point, the median value of the degree of inhibition of AA-induced platelet aggregation by the coincident administration of floctafenine and aspirin was 70 (2-92)% (median and range, n=10) and resulted significantly ($P<0.05$) lower than that caused by aspirin alone [87 (83-89)\%, n=11]. Floctafenine alone caused a profound inhibition of platelet aggregation only in some subjects, i.e. 6 out of 11, at 2 and 6 h after dosing, and 3 out of 11, at 24 h after dosing.

Altogether these findings show that a therapeutic dose of floctafenine does not fulfill the criteria of an antithrombotic agent, such as aspirin, i.e. an almost complete suppression of platelet function which should persist throughout dosing interval (22).

The degree of steady-state inhibition of the urinary excretion of TX-M, index of TXA$_2$ biosynthesis in vivo (15), was evaluated on day 4, after the different treatments. As shown in Fig. 6c, aspirin alone profoundly reduced urinary TX-M levels (68±11\%, n=11). The coincident administration of floctafenine and aspirin did not substantially affect the inhibition caused by aspirin alone (67±15\%, n=10) The inhibition of the urinary excretion of TX-M levels by floctafenine alone (46±14\%, n=11) was significantly ($P<0.05$) lower than that caused by aspirin alone or coadministered with floctafenine (Fig. 6c).
We performed this study in order to advance knowledge on the pharmacology of COX-isozyme inhibition by floctafenine in vitro and after dosing with a therapeutic dose of 200 mg/TID given for 4 days to Thai healthy subjects. Another objective of this clinical study was to verify whether the drug might interfere with the ability of low-dose aspirin to cause an irreversible inhibition of platelet function. We used biochemical markers of COX inhibition ex vivo and in vivo, such as whole blood COX-2 and COX-1 activities (8, 11), and the assessment of urinary levels of prostanoids (indexes of renal biosynthesis) (15, 20) and their enzymatic metabolites (indexes of systemic biosynthesis) (15, 20) which has been suggested to be candidate surrogate end-points of efficacy and toxicity of NSAIDs (8, 11, 23).

DISCUSSION

Fig. 6. Comparison of degree and duration of steady-state inhibition of COX-1 activity and platelet function ex vivo and TXB₂ biosynthesis in vivo by floctafenine 200mg/TID, aspirin 81mg/day and their combination administered for 4 consecutive days, separated by washout periods of 1 and 2 weeks, respectively. a) Recovery of the inhibition of platelet COX-1 activity ex vivo, as assessed by the measurement of serum TXB₂ on day 4 after the last dose of each treatment period. The open symbols represent the values of each individual; data are reported as mean±SD; the line links the mean of values detected at each time-point. Statistical comparisons were made by ANOVA followed by the Student-Newman-Keuls test. **P<0.01 vs pre-drug; §P<0.05 vs aspirin (at the same time-points); #P<0.05 vs floctafenine (at the same time-points), @P<0.01 vs aspirin-floctafenine (at the same time-points). b) Recovery of the inhibition of AA-induced platelet aggregation after the last dose of each treatment period. Data are reported as box and whiskers and the open symbols represent individual values of inhibition of AA-induced platelet aggregation. Values were log-transformed before being subjected to ANOVA followed by the Student-Newman-Keuls test. **P<0.01 vs pre-drug; §P<0.01 vs aspirin (at the same time-points); #P<0.05 vs aspirin (at the same time-points); $P<0.05 vs aspirin-floctafenine (at the same time-points); @P<0.01 vs aspirin (at the same time point). c) Inhibition of TXA₂ biosynthesis in vivo, as assessed by the measurement of the urinary excretion of 11-dehydro-TXB₂ (TX-M), on day 4 of each period, after dosing with the last dose. The data are expressed as mean±SD. Statistical comparisons were made by Student's t-test (for paired samples). **P<0.01 vs aspirin and vs floctafenine-aspirin, §P<0.01 vs pre-drug.
floctafenine is widely used in many countries without having appropriate knowledge on its mechanism of action as analgesic and anti-inflammatory agent. This nebulous scenario may foster an inappropriate use of the floctafenine, such as its coadministration with other NSAIDs and also with low-dose aspirin, thus increasing the risk of potentially harmful effects in the exposed patients.

The inhibition of COX-2, as determined by PGE$_2$ levels in LPS-stimulated whole blood, can be used as a marker to predict drug efficacy in humans (24). In fact, IC$_{50}$ (concentration required to inhibit the activity of COX-2 by 80% in vitro) values have been found to correlate directly with the analgesic/anti-inflammatory plasma concentrations of different COX inhibitors (24). On the other hand, platelet COX-1 inhibition, by assessing TXB$_2$ levels in the whole blood assay in vitro and ex vivo, can be used as a marker to predict gastrointestinal toxicity or antiplatelet effects in humans by NSAIDs (24). Here, we found that floctafenine and its major metabolite floctafenic acid were potent inhibitors of platelet COX-1 and monocyte COX-2 in vitro with a slight preference towards COX-1.

At 2 and 6 h after the last dose of floctafenine, floctafenic acid, but not the parent compound was detected in the peripheral circulation. This is consistent with previous studies showing that floctafenic acid is the main circulating product detectable almost immediately following intravenous administration of floctafenine (5). Floctafenine undergoes high plasma clearance rate primarily for its hydrolysis to floctafenic acid mainly by tissue esterases of the liver (5). This result suggests that floctafenic acid is the major contribution to the inhibition of COX-isozymes detected ex vivo and in vivo, after dosing with floctafenine. This is convincingly supported by the finding that the IC$_{50}$ values for inhibition of whole blood COX-1 and COX-2 by floctafenic acid ex vivo, obtained by plotting COX-isozyme inhibition ex vivo as a function of its plasma levels detected at 2 and 6 h after dosing with floctafenine, were comparable to those obtained in vitro.

Our results demonstrate that floctafenine is acting as a traditional tNSAID, via its rapid transformation to floctafenic acid; a floctafenine dose of 200 mg/TID is appropriate to cause a clinically relevant inhibition of COX-2. In fact, its steady-state inhibition of whole blood COX-2 activity ex vivo was 86%. However, similarly to other tNSAIDs, floctafenine caused a coincident, profound inhibition of whole blood COX-1 that may translate into a possible increased gastrointestinal hazard (25).

Despite floctafenine inhibited platelet COX-1 ex vivo by 86%, this degree of inhibition translates into inter-individual heterogeneity of the inhibition of AA-induced platelet aggregation. This is an expected result because we know that even tiny concentrations of TXA$_2$ may activate platelets (26). Thus, platelet COX-1-dependent capacity to synthesize TXA$_2$ (as assessed by measuring serum TXB$_2$ levels) has to be reduced ≥95% to translate into a complete suppression of platelet aggregation in all individuals (12, 27). This is obtained by low-dose aspirin that irreversibly acetylates Ser529 of COX-1 leading to irreversible enzyme inactivation (28). Since platelets have only limited capacity of de novo protein synthesis, the almost complete inhibition of platelet COX-1 by aspirin persists throughout dosing (i.e. 24 h). These features of low-dose aspirin represent the most plausible mechanism involved in its anti-thrombotic effects (28).

Similarly to most tNSAIDs and coxibs, floctafenine is a functional COX-2 selective inhibitor in respect of the platelet (i.e., it inhibits COX-2-dependent PGI$_2$ associated with insufficient inhibition of platelet COX-1 to translate into a persistent inhibition of platelet function) and this may be associated with a hazard for the cardiovascular system (23, 29).

Hypertension is a feature of the NSAID-induced hazard and both experimental and clinical evidence is compatible with the notion that the rise in blood pressure in patients taking a NSAID is reflective of inhibition of COX-2 (29). We assessed the urinary levels of PGE$_2$, PGI$_2$, and TXA$_2$, which are indexes of renal COX activity (15, 20). We detected a significant inhibition of all prostanooids. However, the urinary levels of the vasodilator PGI$_2$ tended to be lower than the other prostanooids and this scenario may represent a hallmark of hypertension and renal toxicity (29). Finally, we studied the possible occurrence of a pharmacodynamic interaction between floctafenine and low-dose aspirin coadministered for 4 days in Thai healthy subjects. Similarly to other tNSAIDs, such as ibuprofen and naproxen (18, 30), floctafenine
Fig. 7. Interference of floctafenine on the antiplatelet effect of aspirin. Floctafenine is rapidly converted to its active metabolite, floctafenic acid. Our study showed that the concomitant administration of floctafenine interfered with the antiplatelet effect of low-dose aspirin. In the picture it is depicted the cyclooxygenase (COX)-1 active site. Arachidonic acid is the substrate which access to the catalytic site through a hydrophobic channel that leads into the core of COX-1. In the case of administration of aspirin alone, the access of arachidonic acid to the catalytic site of platelet COX-1 is prevented by irreversibly acetylation by aspirin of a specific residue [Serine (Ser) at position 529 located near the catalytic site]. The acetylation of this specific residue hinders the metabolism of arachidonic acid into the intermediate prostaglandin (PG) $H_2$, leading to irreversible COX-1 inactivation. The concomitant administration of floctafenine interfered with the antiplatelet effect of low-dose aspirin, because the previous occupancy of the catalytic site by floctafenine prevents the access of aspirin to its target residue (Ser$^{529}$).

Table II. Biosynthesis of whole blood TXB$_2$ and whole blood PGE$_2$ and systemic and renal prostanoids, in 11 healthy Thai volunteers, at baseline.

<table>
<thead>
<tr>
<th>Prostanoid</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum TXB$_2$, ng/ml</td>
<td>410±149</td>
</tr>
<tr>
<td>LPS-induced PGE$_2$, ng/ml</td>
<td>45±17</td>
</tr>
<tr>
<td>Urinary PGI-M, pg/mg creatinine</td>
<td>221±130</td>
</tr>
<tr>
<td>Urinary TX-M, pg/mg creatinine</td>
<td>531±140</td>
</tr>
<tr>
<td>Urinary PGE-M, ng/mg creatinine</td>
<td>9.6±4.9</td>
</tr>
<tr>
<td>Urinary 6-keto-PGF$_{1α}$, pg/mg creatinine</td>
<td>241±161</td>
</tr>
<tr>
<td>Urinary PGE$_2$, pg/mg creatinine</td>
<td>320±127</td>
</tr>
<tr>
<td>Urinary TXB$_2$, pg/mg creatinine</td>
<td>194±80</td>
</tr>
</tbody>
</table>

All values are mean±SD, n=11
interfered with the almost complete inhibition of platelet COX-1 activity and TXA₂-dependent platelet function by aspirin alone leaving open the door for a potential impact on aspirin cardioprotection (28).

We compared the effect of aspirin co-administered with floctafenine versus aspirin alone on urinary levels of 11-dehydro-TXB₂ (TX-M), an in vivo marker of TXA₂ biosynthesis (9, 15). However, we did not detect a statistically significant change which is explained by the limited sensitivity of TX-M excretion in reflecting changes in platelet COX-1 activity.

Floctafenine and its metabolite floctafenic acid were potent inhibitors of COX-1 and COX-2 activities in vitro showing a slightly higher potency towards COX-1. However, floctafenic acid was more potent than its parent compound to inhibit both COX-1 and COX-2. They did not affect whole blood LTB₄ generation. When administered in vivo to Thai healthy subjects, floctafenine, at a clinically relevant dose, profoundly affected whole blood COX-1 and COX-2 activities ex vivo and systemic and renal prostanoid generation in vivo. These effects were mainly dependent on its rapid transformation to floctafenic acid. Altogether these results lead to recommend a cautious administration of floctafenine to patients with gastrointestinal, renal and cardiovascular disease. A relevant finding of our study was that the concomitant administration of floctafenine interfered with the anti-platelet effect of low-dose aspirin (Fig. 7). The clinical implication of the pharmacodynamic interaction between the two drugs is not known and remains to be studied in a randomized clinical trial with preidentified CV end-points. However, our results showing that floctafenine caused a significant reduction of the almost complete and persistent inhibition of platelet COX-1 by aspirin are potentially important, because the cardioprotective effect of aspirin when used for secondary prevention of myocardial infarction could be decreased or negated.

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