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European Journal of Pharmacology 449 (2002) 23–28

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Antiplatelet aggregation activity of diterpene alkaloids from *Spiraea japonica*

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Received 12 March 2002; received in revised form 12 April 2002; accepted 16 April 2002

Abstract

Six diterpene alkaloids with an atisine-type C_{20}-skeleton isolated from the Chinese herbal medicines *Spiraea japonica* var. *acuta* and *S. japonica* var. *ovalifolia*, as well as eight derivatives of spiramine C and spiradine F were evaluated for the ability to inhibit aggregation of rabbit platelets induced by arachidonic acid, ADP, and platelet-activating factor (PAF) in vitro. The results showed that 12 of the 14 atisine-type diterpene alkaloids significantly inhibited PAF-induced platelet aggregation in a concentration-dependent manner, but had no effect on ADP- or arachidonic acid-induced aggregation, exhibiting a selective inhibition. It is the first report that C_{20}-diterpene alkaloids inhibit PAF-induced platelet aggregation. However, spiramine C1 concentration-dependently inhibited platelet aggregation induced by PAF, ADP and arachidonic acid with IC_{50} values of 30.5 ± 2.7, 56.8 ± 8.4 and 29.9 ± 9.9 μM, respectively, suggesting a non-selective antiplatelet aggregation action. The inhibitory effect of spiramine C1 on arachidonic acid was as potent as that of aspirin. Primary studies of the structure–activity relationships for inhibition of PAF-induced aggregation showed that the oxygen substitution at the C-15 position and the presence of an oxazolidine ring in spiramine alkaloids were essential to their antiplatelet aggregation effects. These results suggest that the atisine-type alkaloids isolated from *S. japonica* are a class of novel antiplatelet aggregation agents.

Keywords: Atisine-type diterpene alkaloid; Platelet aggregation; Arachidonic acid; Adenosine-5′ -diphosphate; PAF (platelet-activating factor); Structure–activity relationship

1. Introduction

Diterpenoid alkaloids including C_{19}- and C_{20}- diterpenoid are of great interest of pharmacy because of their diverse chemical structures and significant pharmacological activities (Pelletier and Page, 1986). Pharmacologically, the diterpenoid alkaloids with C_{19} skeleton have been extensively studied and reviewed for their anti-inflammation, analgesia, anti-arrhythmia and antifungal actions (Benn and Jacyno, 1983; Pelletier and Page, 1986; Chiao et al, 1995; Heubach and Schule, 1998; Atta-ur-Rahman et al., 1997). In contrast, there is little information about the pharmacological activities of the C_{20} diterpenoid alkaloids.

Since 1987, a number of new atisine-type diterpenoid alkaloids with a C_{20} skeleton have been obtained from the roots of *Spiraea japonica* (Rosaceae) (Hao et al., 1987; Hao et al., 1995; Hao and Nie, 1998; Wang et al., 2000a; He et al., 2001), a Chinese herbal medicine widespread in Yunnan Province which has long been used for anti-inflammation and analgesia in folk and ethnic traditions (Zhang and Wang, 1993). Previously, we found that the ethanol extract of this plant and the crude alkaloids inhibited rabbit platelet aggregation induced by platelet-activating factor (PAF) in vitro (data not shown). Recently, we reported that spiramine Q (Fig. 1, the structure of spiramine Q has been corrected to the present form, Wang et al., 2000b), a novel atisine-type diterpene alkaloid isolated from *S. japonica* var. *incisa*, was found to selectively inhibit rabbit platelet aggregation induced by arachidonic acid in vitro and ex vivo and that the inhibition was much stronger than that of aspirin (Shen et al., 2000). In the present study, the antiplatelet aggrega-

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PII: S0014-2999(02)01627-8

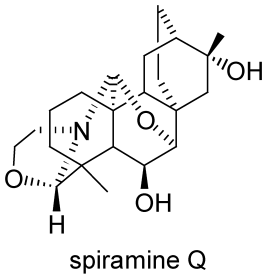

Fig. 1. The structure of spiramine Q.

tion activities of some natural atisine-type alkaloids isolated from *S. japonica* var. *acuta* and *S. japonica* var. *ovalifolia* (Fig. 2), and the semi-synthetic derivatives of spiramine C and spiradine F (Fig. 3) were investigated. Their structure–activity relationships are discussed as well.

2. Materials and methods

2.1. Plant materials

The roots of *S. japonica* var. *acuta* and *S. japonica* var. *ovalifilia* (Rosaceae) were collected in Lijiang and Songming counties, Yunnan Province, P. R. China, respectively. The plant materials were identified by Prof. H. Sun at Kunming Institute of Botany, Chinese Academy of Sciences, and the voucher specimens are deposited in the Herbarium in Kunming Institute of Botany.

2.2. Extraction and isolation

The powdered air-dried roots (5000 g) of *S. japonica* var. *acuta* were extracted three times with 95% ethanol under reflux. The removal of solvents by evaporation under vacuum produced 300 g of extracts that were dissolved in 1700 ml of 3% aqueous hydrocaloric acid. The aqueous hydrochloric acid solution was extracted three times with petroleum ether–benzene (1:1, vol/vol) and then made basic with 5% aqueous sodium hydroxide to pH 11, followed by exhaustive extraction with trichloromethane to yield 35 g of basic portion. This basic extract was subjected to repeated flash column chromatography over silica gel eluted with a gradient mixture of petroleum ether–acetone–diethylamine to give spiramine A, and spiradine F (Fig. 2) (Hao et al., 1987). The preparation of the derivatives, spiramines C1–4 and spiradines F1–4 (Fig. 3), of spiramine C and spiradine F will be reported in a forthcoming paper. The isolation of spiramine Z-2, deacetylspiramine F and spiramine V from *S. japonica* var. *ovalifolia* (Fig. 2) was described previously (Zuo et al., 2001). All drugs were dissolved in 0.1 M aqueous hydrochloric acid. These solutions were adjusted to pH 6.5–7.0 with 0.1 M aqueous sodium hydrogen carbonate.

2.3. Reagents

Arachidonic acid and PAF were all purchased from Sigma, and ADP was the product of Amresco. Arachidonic acid, ADP and PAF were dissolved in 100 mM aqueous sodium hydrogen carbonate, 100 mM (pH = 7.2) phosphate buffer solution and 10 mM (pH = 7.0) Tris–NaCl buffer containing 0.25% bovine serum albumin, respectively.

2.4. Platelet aggregation assay in vitro

Healthy Japanese rabbits of either sex weighing 1.8–2.5 kg were obtained from Experimental Animal Center,

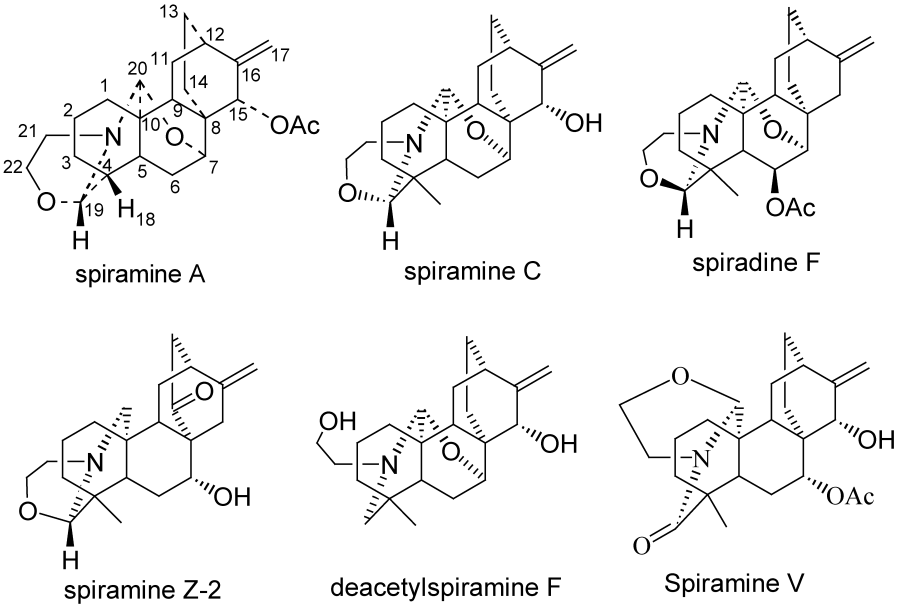

Fig. 2. The structures of natural diterpene alkaloids.

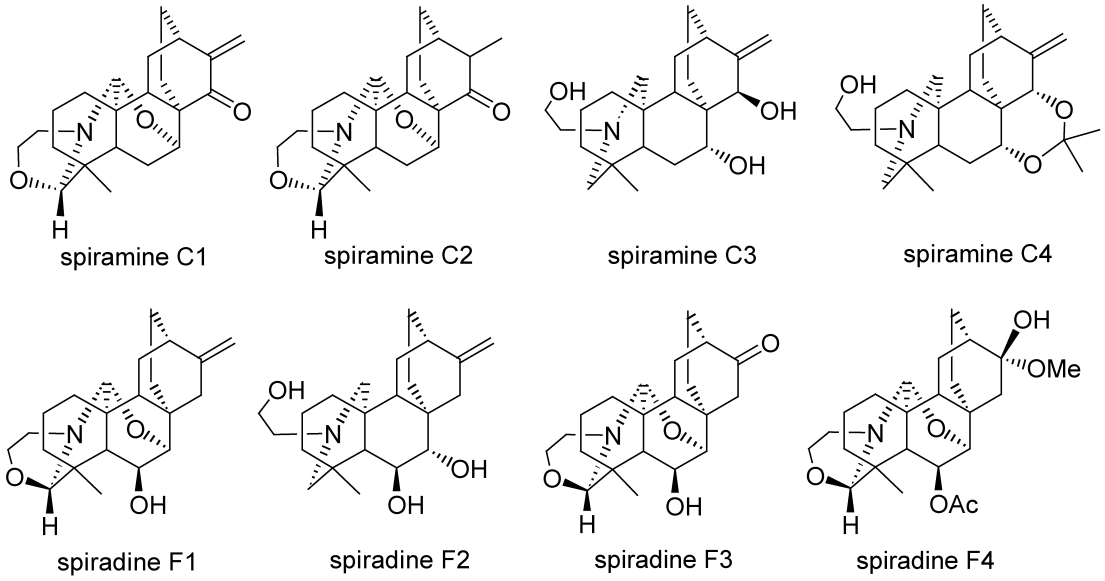

Fig. 3. The structures of the derivatives of spiramine C and spiradine F.

Kunming Medical College (Certificate number: 99003, the Administrative Commission of Medical Experimental Animals of Yunnan Public Health Bureau).

Blood from the rabbit carotid artery was anticoagulated with 3.8% sodium citrate solution (9:1, vol/vol). Platelet-rich plasma and platelet-poor plasma were obtained from the supernatant fraction of blood after centrifugation at room temperature for 10 min at 1000 and 3000 rpm, respectively. The final cell count in platelet-rich plasma was adjusted to 5×10^8 platelets/ml with platelet-poor plasma.

Platelet aggregation was monitored by Born's method (Born, 1962) with an aggregometer, model SH-93 (Shanghai Biochem Equipment) at 37 °C under stirring (900 rpm). The test drugs were pre-incubated with platelet-rich plasma for 10 min at 37 °C. After incubation, platelet aggragation was induced by the addition of arachidonic acid, ADP and PAF (final concentration: arachidonic acid 100 μM, ADP 5 μM, and PAF 4.5 nM), respectively. The maximal aggregation was recorded. The aggregation is expressed as % inhibition (X) calculated by using the following equation:

$$X(\%) = (A - B)/A \times 100$$

where A = maximal aggregation of the control, and B = maximal aggregation of drug-treated PRP.

2.5. Statistical analysis

Data are expressed as means ± S.D. Differences between drugs and control were analyzed using Student's t-test. Statistical significance was accepted at the level of $P < 0.05$.

3. Results

3.1. Effects of atisine-type alkaloids on platelet aggregation induced by PAF

In vitro, all test compounds significantly inhibited 4.5 nM PAF-induced rabbit platelet aggregation in a concentration-dependent manner (Fig. 4). Among the natural alkaloids, spiramine A was the most potent with an IC_{50} value of 6.7 ± 0.7 μM. The strongest semi-synthetic derivative was spiramine C1 with an IC_{50} value of 30.5 ± 2.7 μM. Deacetylspiramine F and spiradine F2 had modest activity with an inhibitory rate of 41.0 ± 3.0% and 37.7 ± 3.8% at a final concentration of 240 μM. The rank order of the potency of these compounds was spiramine A, spiramine C1, spiramine C, spiramine Z-2, spiramine C2, spiradine F1, spiramine V, spiradine F3, spiradine F4, spiradine F, spiramine C3, spiramine C4, and deacetylspiramine F according to the IC_{50} values (Table 1). The difference in inhibitory effects was significantly correlated with the chemical structure. The correlation coefficient was 0.9062 ($P < 0.01$).

3.2. Effects of atisine-type alkaloids on platelet aggregation induced by arachidonic acid and ADP

Spiramine C1 significantly inhibited 5 μM ADP- and 100 μM arachidonic acid-induced platelet aggregation in a concentration-dependent manner with an IC_{50} value of 56.8 ± 8.4 and 29.9 ± 9.9 μM, respectively (Fig. 5). Spiradine F1 showed a weak inhibitory effect on arachidonic acid-induced aggregation: 25.6 ± 2.5% at a final concentration of 240 μM. The natural atisine-type alkaloids and other semi-synthetic alkaloids did not inhibit aggregation

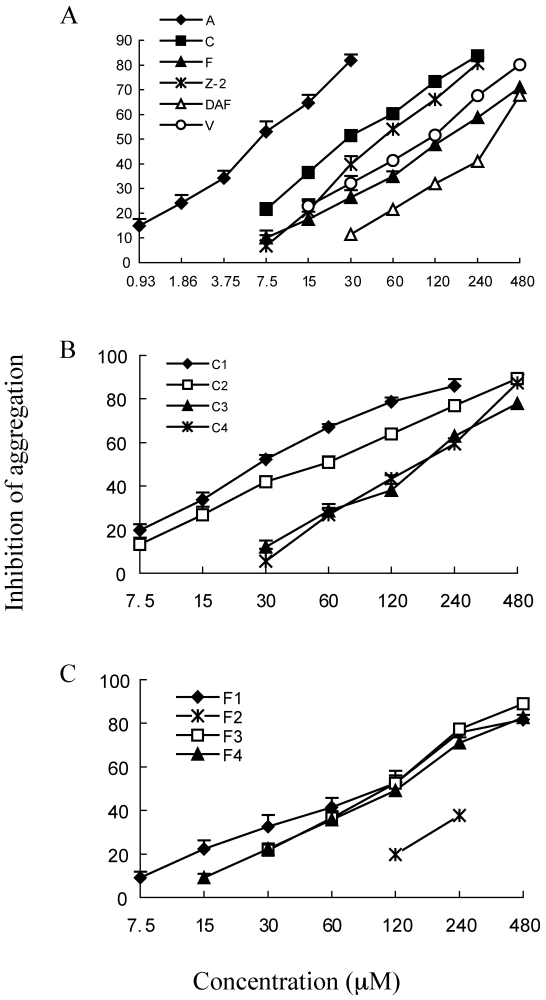

Fig. 4. Effects of natural atisine-type alkaloids (A), derivatives of spiramine C (B) and derivatives of spiradine F (C) on PAF-induced platelet aggregation in vitro. A, C, F, Z-2, DAF, V, C1–4 and F1–4 were the spiramines A, C, spiradine F, spiramine Z-2, deacetylspiramine F, spiramine V, spiramines C1–4 and spiradines F1–4, respectively. The platelet-rich plasma was incubated with vehicle or test compounds at 37 °C for 10 min and stimulated with PAF (4.5 nM) for 5 min. Data are expressed as means ± S.D., $n = 4$.

induced by ADP and arachidonic acid even at 240 μM in vitro (data not shown).

3.3. Comparison of in vitro antiplatelet activities of atisine-type alkaloids with those of ginkgolide B and aspirin

A comparison of the inhibitory effects of atisine-type alkaloids, ginkgolide B and aspirin on PAF, arachidonic acid and ADP-induced platelet aggregation is shown in Table 1. Of the 14 test compounds, 12 selectively inhibited platelet aggregation induced by PAF. The strongest one was spiramine A, which was over 40 times more potent than spiradine F2, but was less potent than ginkgolide B with an IC_{50} value of 0.43 ± 0.16 μM. Spiramine C1 markedly inhibited PAF, ADP and arachidonic acid-induced platelet aggregation. The potency of inhibition of arachidonic acid-induced aggregation was similar to that of aspirin with an IC_{50} value of 35.2 ± 8.6 μM.

Table 1
Comparison of the antiplatelet activity of atisine-type alkaloids

Compounds	PAF	IC_{50} (μM) arachidonic acid	ADP
Spiramine A	6.7 ± 0.7	–	–
Spiramine C	32.6 ± 3.3	–	–
Spiradine F	138.9 ± 10.0	–	–
Spiramine Z-2	58.4 ± 12.4	–	–
Deacetylspiramine F	309.3 ± 53.2	–	–
Spiramine V	87.1 ± 11.9	–	–
Spiramine C1	30.5 ± 2.7	29.9 ± 9.9	56.8 ± 8.4
Spiramine C2	53.3 ± 7.5	–	–
Spiramine C3	147.5 ± 18.1	–	–
Spiramine C4	172.6 ± 47.9	–	–
Spiradine F1	82.0 ± 15.3	>240	–
Spiradine F2	>240	–	–
Spiradine F3	92.9 ± 10.4	–	–
Spiradine F4	110.7 ± 11.2	–	–
Ginkgolide B	0.43 ± 0.16		
Aspirin		35.2 ± 8.6	

The IC_{50} values are expressed as the compound concentration required to produce 50% inhibition of agonist-induced platelet aggregation in comparison to vehicle treatment. Each value represents the mean ± S.D. for four experiments.

4. Discussion

Among the family of platelet activators, PAF, arachidonic acid, and ADP are three important platelet stimulants which induce platelet aggregation via different mechanisms. PAF is the most potent known platelet activator so far. As a phospholipid mediator, it is also involved in a variety of disorders, including arterial thrombosis, acute inflammation, endotoxic shock, acute allergic disease (Casals-Stenzel, 1987; Pretolani et al., 1987; Braquet et al., 1987). Recently, several PAF receptor antagonists were obtained from natural products including terpene, lignans, gliotoxin (Negro et al., 1997). In the present study, 12 of the 14 atisine-type

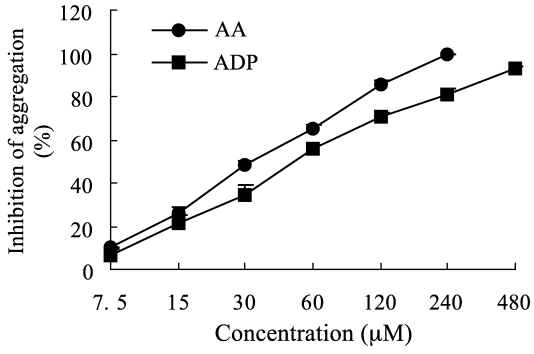

Fig. 5. Effects of spiramine C1 on ADP- and arachidonic acid-induced platelet aggregation in vitro. The platelet-rich plasma was incubated with vehicle or test compounds at 37 °C for 10 min and stimulated with ADP (5 μM) or AA (100 μM) for 5 min. Data are expressed as means ± S.D., $n = 4$.

alkaloids selectively inhibited PAF-induced platelet aggregation in vitro. The inhibitory effect was less potent than that of ginkgolide B, which is one of the well-known PAF receptor specific antagonists. However, these inhibitory effects were specific to PAF and had no effect on aggregation induced by arachidonic acid and ADP, like ginkgolide B. This suggested that atisine-type alkaloids might be a novel class of PAF receptor antagonists.

Inspection of the relationship between the activity and structure revealed that the antiplatelet aggregation activity induced by PAF was correlated with the oxazolidine ring. The compounds with the oxazolidine ring, such as spiramine C and spiradine F1, were much more potent than those with an open oxazolidine ring, such as spiramine C3, spiradine F2, spiramine C4 and deacetylspiramine F. Spiramine C was 9.4 times, 4.5 times and 5.3 times more potent than deacetylspiramine F, spiramine C3 and spiramine C4, respectively. This finding showed that the oxazolidine ring is important for the inhibitory effect on platelet aggregation induced by PAF, suggesting that the oxazolidine ring might be an active center.

Moreover, it was also found that the atisine-type alkaloids with oxygen substitution at C-15, such as spiramines A, C and C1, were active, while changing the position of oxygen substitution to C-6, as in spiradines F and F1, markedly decreased the inhibitory activity of these compounds ($P<0.01$). Spiramine A, with an acetyl group at C-15, was the most potent among these compounds, and was 20.7 times more potent than spiradine F. The results showed that the potency of these compounds was closely affected by substitution at C-15, suggesting that the group at the C-15 position might be another active center.

It is worth mentioning that when the acetyl group of spiramine A was oxided to the keto group to give spiramne C1, the compound was found to have a broad spectrum of antiplatelet aggregation activity. It concentration-dependently inhibited platelet aggregation induced not only by PAF, but also by ADP and arachidonic acid, showing a non-selective antiplatelet aggregation action. And the inhibition of arachidonic acid-induced platelet aggregation was as potent as that induced by aspirin, suggesting that the antiplatelet activity pattern of atisine-type alkaloids might be affected by changes in some functional groups.

In conclusion, 12 of 14 atisine-type diterpene alkaloids investigated in this study selectively inhibited rabbit platelet aggregation induced by PAF in a concentration-dependent manner. Their structure–activity relationships were characterized by the contribution of the oxygen substitution at the C-15 position and the presence of an oxazolidine ring. The results suggest that the atisine-type diterpene alkaloids isolated from *S. japonica* are a class of novel antiplatelet aggregation agents although they are less potent than ginkgolide B. However, more active derivatives might be obtained by structural modifications because a reasonable yield of crude alkaloids can be achieved and the plants are easy to cultivate. Moreover, spiramine C1 non-selectively inhibited aggregation induced by PAF, ADP and arachidonic acid, and the activity was as potent as that of aspirin in inhibiting arachidonic acid-induced aggregation. Therefore, a compound more potent than aspirin can be produced by structural modification.

Acknowledgements

This study was supported by the grants from National Natural Science Foundation of China (NSFC) for outstanding young scientists to X.-J. Hao contract No. 39525025 and Yunnan Provincial Natural Science Foundation contract No. 99C0079M.

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