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Research Paper

Antiplatelet effects of piplartine, an alkamide isolated from *Piper tuberculatum*: possible involvement of cyclooxygenase blockade and antioxidant activity

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Abstract

Objectives Piplartine (piperlongumine; 5,6-dihydro-1-[1-oxo-3-(3,4,5-trimethoxyphenyl]-2(1H) pyridinone) is an alkaloid amide isolated from *Piper* species (Piperaceae). It has been reported to show multiple pharmacological activities *in vitro* and *in vivo*.
Methods We evaluated the in-vitro antiplatelet effect of piplartine isolated from the roots of *P. tuberculatum,* on human platelet aggregation induced in platelet-rich plasma by the agonists collagen, adenosine 5′-diphosphate (ADP), arachidonic acid (AA) and thrombin.
Key findings Piplartine (100 μg/ml) caused a 30% inhibition in platelet aggregation when collagen was the agonist. At 200 μg/ml, piplartine significantly inhibited the aggregation induced by arachidonic acid (100%), collagen (59%) or ADP (52%) but not that induced by thrombin. The highest concentration of piplartine (300 μg/ml) inhibited thrombin- (37%), ADP- (71%) and collagen- (98%) induced aggregation. The inhibitory effect of piplartine on ADP-induced platelet aggregation was not modified by pretreatment with pentoxifylline (a phosphodiesterase inhibitor), L-arginine (a substrate for nitric oxide synthase) or ticlopidine (a $P2Y_{12}$ purinoceptor antagonist). However, aspirin, a well-known inhibitor of cyclooxygenase, greatly increased the inhibitory effect of piplartine on arachidonic-acid-induced platelet aggregation.
Conclusions The mechanism underlying the piplartine antiplatelet action is not totally clarified. It could be related to the inhibition of cyclooxgenase activity and a decrease in thromboxane A_2 formation, similar to that occurring with aspirin. This and other possible mechanisms require further study.
Keywords antiplatelet effects; *Piper tuberculatum*; piplartine; platelet aggregation

Introduction

Cardiovascular and cerebrovascular diseases are still major causes of morbidity and mortality worldwide. The formation of platelet aggregates is an important pathogenetic factor in a variety of cardiovascular diseases. Platelets play a significant role not only in normal haemostasis but also in arterial thrombosis, particularly under conditions of high shear stress.[1] The high incidence of thromboembolic diseases[2] means that there is continuous research for new antithrombotic agents with limited adverse effects. Herbal medicines are being increasingly recognised as therapeutic alternatives, and are beginning to be considered by the pharmaceutical industry. A survey of the literature of the two last decades indicates growing interest in this field of research, as reflected by a number of studies concerning the antiplatelet and anticoagulant properties of natural products.[3,4]

Piper species (Piperaceae) are important plants in traditional medicine. In particular, they are useful against asthma, bronchitis, fever, haemorrhoids and rheumatism.[5,6] The Piperaceae family is largely found in tropical areas, and has five genera and 1499 species. *Piper* is the most representative genus, with 700 species.[7] In traditional Chinese medicine, *Piper* species are used for rheumatic diseases and ailments of the respiratory tract, and many of them have been shown to have considerable inhibitory activity against the key enzymes of arachidonic acid (AA) metabolism: 5-lipoxygenase (LOX) and cyclooxygenase (COX)-1 and -2, and particularly COX-1.[8] Analytical investigations of these *Piper* species resulted in the identification of 20 constituents, most of them amides. Amides constitute

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characteristic metabolites of the Piperaceae family, and chemical studies carried out on Brazilian species of the Piperaceae family have identified isobutyl, pyrrolidine, dihydropyridone and piperidine moieties.[9]

In some Brazilian Northeast communities, the fruit from *P. tuberculatum*, known as 'pimenta-longa' and 'pimenta d'arda' is used largely as a spice[10,11] and as a folk medicine with many properties.[12,13] Previous publications describe the presence of amides, such as piplartine and its dimer, in *P. tuberculatum*.[14]

In the last few years, *Piper* species have received considerable attention because of their reputation for producing lignans with antagonist activity against platelet activating factor (PAF)[15] and cytotoxic alkaloids.[16,17] A series of substances with high specific PAF receptor antagonist activity *in vitro* was isolated from *P. betle*.[18] Jatan and colleagues[19] investigated some species of Malaysian plants, and showed that *P. aduncum* is a potential source of PAF antagonists with potent inhibitory antiplatelet activity.

The literature has plenty of data on antiplatelet activities of *Piper* species.[18–22] For instance, *P. betle* is known to inhibit mainly AA- and collagen-induced platelet aggregation, as well as thromboxane (TX) B_2 and prostaglandin (PG) D_2 production, and to a lesser extent thrombin- and adenosine 5′-diphosphate (ADP)-induced platelet aggregation.[22] Another study showed that the *P. betle* aqueous extract is a potential reactive oxygen species scavenger, and may prevent platelet aggregation, possibly by scavenging reactive oxygen species or inhibiting TXB_2 production.[21] However, little is known about the properties of piplartine on platelet function. Piplartine isolated from the stems of *P. arborescens* has been reported to inhibit collagen-induced platelet aggregation *in vitro*.[20] Thus, the objective of the present work was to study the effects of piplartine, an alkaloid/amide isolated from the roots of *P. tuberculatum*, on human platelet aggregation induced by a variety of agonists, such as collagen, ADP, AA and thrombin. Comparisons of piplartine with several antiplatelet drugs with known mechanisms of action were also performed, in order to demonstrate their modulatory effects on piplartine action.

Materials and Methods

Plant material and isolation of piplartine

The roots of *P. tuberculatum* were harvested in September 2004 from a wild population on the Pici Campus of the Federal University of Ceará, Fortaleza, Brazil. A voucher specimen (#34736) was deposited at the Prisco Bezerra Herbarium, Department of Biology, Federal University of Ceará.

For the isolation of piplartine, a total of 420 g of ground roots of *P. tuberculatum* was macerated with a 1 : 1 mixture of petroleum ether and ethyl acetate (1.5 L) for 24 h (three times). The solvent mixture was rota-evaporated under reduced pressure to yield a yellowish solid (13.24 g) which, after crystallisation in methanol, provided a first yield of piplartine (4.35 g; Figure 1). The melting point was 122.2–122.6°C, and piplartine (synonyms piperlongumine, *N*-(3,4,5-trimethoxycinnamoyl)-Δ^3-piperidin-2-one, MW 317.33 g/mol, PubChem ID 442653) was characterised by one- and two-dimensional NMR analyses.[14,23]

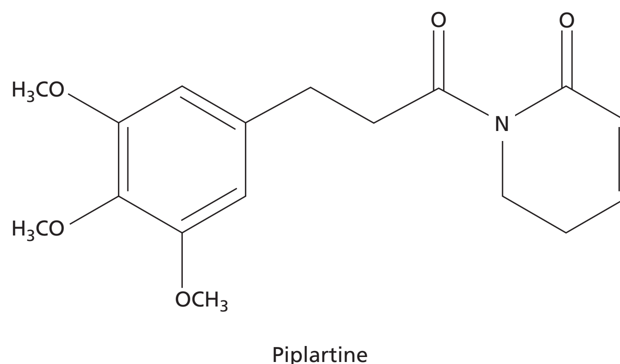

Figure 1 Chemical structure of piplartine (5,6-dihydro-1-[1-oxo-3-(3,4,5-trimethoxyphenyl)-2-propenyl]-2(1H) pyridinone)

Drugs

ADP, AA, aspirin (acetylsalicylic acid), collagen, L-arginine and ticlopidine were purchased from Sigma Chemical Co., (St Louis, MO, US). Bovine thrombin was from Roche (Rio de Janeiro, Brazil) and pentoxifylline from Hoechst (Novo Hamburgo, RS, Brazil). All other reagents were of analytical grade.

Platelet aggregation test

Preparation of platelet-rich and platelet-poor plasma

This study was approved by the Ethics Committee of the University Hospital, Federal University of Ceará. Blood from healthy volunteers (with their previous consent), who had not taken any drug for the last 15 days, was collected by venipuncture into siliconised glass flasks containing 3.8% sodium citrate (9 : 1 v/v). Platelet-rich plasma was prepared by centrifugation of blood at 1000 rpm for 10 min at room temperature. Platelet-poor plasma was obtained immediately afterwards by centrifugation of an aliquot of platelet-rich plasma at 3000 rpm for 10 min. The platelets were counted according to the method of Brecher and Cronkite,[24] and adjusted to a concentration of 300 000/mm^3, using platelet-poor plasma to dilute platelet-rich plasma.

Aggregation in platelet-rich plasma

Platelet aggregation was measured with an aggregometer (model 450, Chrono-Log Co., Havertown, PA, US) according to the method of Born and Cross.[25] Briefly, platelet aggregation was induced at 37°C in the aggregometer, with stirring at 1000 rpm, by addition of ADP (4 μM), AA (20 μM), thrombin (1.6 U/ml) or collagen (2 μg/ml) as agonists. Piplartine or 5% DMSO (vehicle) was added in increasing concentrations (50, 100, 150, 200 and 300 μg/ml) 10 min before the addition of agonist. The resulting aggregation, measured as the change in light transmission, was recorded for 8 min. Values are presented as percentage aggregation compared with the control (100%).

Statistical analysis

All values are expressed as mean ± SEM. Differences between treated samples and controls were subjected to analysis of

variance, followed by the Student–Newman–Keuls test for multiple comparisons. Significance was set at $P < 0.05$.

Results

The effects of piplartine on platelet aggregation induced in human platelet-rich plasma by the agonists ADP, AA, thrombin and collagen are shown in Table 1. Piplartine (100 μg/ml) inhibited platelet aggregation by 30% when the agonist was collagen ($P < 0.001$) but did not significantly inhibit ADP- or thrombin-induced aggregation. The curve for AA-induced platelet aggregation was shifted to the right at this concentration of piplartine (data not shown), and a concentration of 150 μg/ml produced 100% inhibition of platelet aggregation ($P < 0.001$). Significant inhibitory effects on platelet aggregation were seen with piplartine (200 μg/ml) when the inducer was ADP (52%) or collagen (59%) but no noticeable inhibitory effect on thrombin-induced platelet aggregation (14%) was seen at this concentration. Thrombin-induced aggregation was significantly inhibited only at a piplartine concentration of 300 μg/ml, (37%, $P < 0.001$), suggesting that piplartine probably does not interfere with thrombin receptors (a protease-activated receptor (PAR)-dependent phenomenon). At this concentration, piplartine inhibited ADP-induced platelet aggregation by 71% and collagen-induced aggregation by 98%.

The combination of a subthreshold dose of piplartine (50 μg/ml) with pentoxifylline (a known phosphodiesterase inhibitor; 120 μg/ml) caused no significant alteration of the antiplatelet effect of piplartine alone (17%) on ADP-induced platelet aggregation (Table 2). Inhibition of phosphodiesterase activity increases intracellular cAMP or cGMP levels, and regulates platelet function.[26] Thus, piplartine probably does not interfere with molecular phenomena associated with phosphodiesterase inhibition.

In order to verify whether the nitric oxide system interferes with the inhibitory action of piplartine, L-arginine (10 μg/ml), a substrate for nitric oxide synthase, was preincubated with piplartine (50 μg/ml) prior to ADP-induced platelet aggregation. The effect of piplartine alone on platelet aggregation (9%) was not modified in the presence of L-arginine (Table 2). In fact, the effect of L-arginine plus piplartine (26%) seemed to be only a sum of their effects. Thus, platelet-derived nitric oxide does not seem to modulate the antiplatelet activity of piplartine. In the same way, the combination of piplartine (50 μg/ml) with ticlopidine (500 μM), an antagonist of the $P2Y_{12}$ purinoceptor (ADP is its natural agonist), did not produce an inhibitory effect (26%) significantly different from the effect of piplartine (16%) or ticlopidine (17%) alone, on the platelet aggregation induced by ADP (Table 2).

Table 1 Effects of piplartine on aggregation of human platelets induced by adenosine 5′-diphosphate (ADP; 4 μM), thrombin (1.6 U/ml), collagen (2 μg/ml) and arachidonic acid (AA; 20 μM)

Piplartine concn (μg/ml)	ADP	Thrombin	Collagen	AA
50	99.4 ± 3.74	94.2 ± 5.35	87.8 ± 6.97	99.5 ± 4.08
100	93.5 ± 4.83	93.3 ± 4.93	70.7 ± 4.98a	94.5 ± 2.76
150	–	–	–	0
200	48.1 ± 11.22*	86.1 ± 4.83	40.8 ± 5.30*	0
300	29.3 ± 2.07*	62.7 ± 5.47*	2.1 ± 2.10*	–

Values are means ± SEM of aggregation percentages, relative to controls (100%), from 5–7 experiments. *$P < 0.01$ vs control.

Table 2 Effects of pre-incubation with pentoxifylline (PTX), L-Arginine (L-Arg) and ticlopidine (TIC) on the inhibitory actions of piplartine (50 (μg/ml) against platelet aggregation induced by adenosine 5′-diphosphate (ADP), and with aspirin in arachidonic-acid-induced aggregation

	% Aggregation
ADP-induced aggregation	
Piplartine	96.1 ± 2.92
PTX (120 μg/ml)	84.1 ± 8.43
PTX + piplartine	79.6 ± 11.13
L-Arg (10 μg/ml)	91.8 ± 4.82
L-Arg + piplartine	74.2 ± 6.49*
TIC (500 μM)	82.9 ± 4.72
TIC + piplartine	75.5 ± 5.59
Arachidonic-acid-induced aggregation	
Piplartine	95.0 ± 3.63
Aspirin (7 μM)	90.0 ± 3.36
Aspirin + piplartine	0.0 ± 0.0†

Values are means ± SEM of aggregation percentages, relative to controls (100%), from six experiments. *$P < 0.05$ vs control. †$P < 0.001$ vs control, piplartine or aspirin groups.

Aspirin is an antithrombotic drug that produces antiplatelet effects by inhibiting COX activity, with a consequent decrease in TXA_2 and $PGF_{2\alpha}$ formation.[27] The addition of aspirin (4 μM) to a subthreshold concentration of piplartine (50 μg/ml) resulted in a shift to the right of the platelet aggregation curve induced by AA (data not shown), while the addition of 8 μM aspirin to the same concentration of piplartine resulted in a great potentiation of its effect (100% inhibition) on AA-induced platelet aggregation compared with the inhibitory effect of piplartine (5%) or aspirin (10%) alone. These results suggest a possible role of the COX system in the still-unknown antiplatelet action of piplartine.

Discussion

The present study reports the effects of piplartine (syn. piperlongumine), an alkaloid/amide constituent from *P. tuberculatum*, on human platelet aggregation *in vitro*, using the agonists ADP, collagen, AA and thrombin. We also investigated for the first time the relationship between the antiplatelet effect of piplartine and the effects of well-known inhibitors of platelet function.

Piplartine did not have a pronounced effect on thrombin-induced platelet aggregation, as only the highest concentration of piplartine had a significant effect. Thrombin plays a central role in normal and abnormal haemostatic processes. It is assumed that thrombin activates platelets by hydrolysing PAR-1.[28] Thus, it is unlikely that PAR-related molecular pathways are involved in piplartine-mediated inhibition of aggregation.

Piplartine inhibited both AA- and collagen-induced platelet aggregation more potently than that induced by

ADP or thrombin. Although ADP is a weak platelet activator, it plays a relevant role in platelet function. Platelets express three separate nucleotide receptors: the $P2X_1$ cation channels, which are activated by ATP, and two G-protein-coupled receptors, $P2Y_1$ and $P2Y_{12}$, both activated by ADP. Each of these receptors has a selective role during platelet activation.[29] The $P2Y_1$ receptor, coupled to $G\alpha q$, triggers calcium mobilisation from internal stores, resulting in a change in platelet shape and a weak and transient aggregation in response to ADP.[30,31] The human $P2Y_{12}$ receptor is coupled to G_{i2} and is responsible for the completion of platelet aggregation in response to ADP. It also plays a central role in the amplification of platelet aggregation induced by other agonists, including collagen, von Willebrand factor, thrombin, TXA_2, adrenaline and serotonin.[29,32,33] The $P2Y_{12}$ receptor is also involved in the potentiation of platelet secretion.[30,34] Thus, this receptor is pivotal in sustaining platelet aggregation.

The ADP receptor antagonists clopidogrel and ticlopidine inhibit ADP activity by preventing its binding to the platelet receptor. ADP stimulates the expression of GP IIb/IIIa receptors, and may mediate the release of other aggregation agonists and enhance platelet binding of the von Willebrand factor. Thus, the end result of ADP inhibition is the impairment of platelet aggregation and fibrinogen-mediated platelet crosslinking. GP IIb/IIIa receptor antagonists block the final common pathway in platelet activation. In the present study, we showed that piplartine (100 μg/ml) did not inhibit ADP-induced platelet aggregation. However, at this concentration, piplartine significantly inhibited collagen-induced platelet aggregation by 20%.

On the other hand, piplartine completely inhibited AA-induced platelet aggregation at a concentration of 150 μg/ml. In platelets, AA is metabolised to various compounds by COX isoforms. The metabolism of AA by COX-1 and COX-2 leads to the production of prostacyclin and TXA_2, respectively. TXA_2 stimulates vasoconstriction and platelet aggregation, whereas prostacyclin is a potent vasodilator and inhibitor of platelet aggregation.[35] In the present study, the anti-aggregant effect of piplartine is to some extent related to AA metabolism in platelets.

Previous studies have shown that peroxides are important stimuli for the activation of COX enzymes.[36] Therefore, hydrogen peroxide could amplify the platelet response to collagen by stimulating those enzymes. Alternatively, hydrogen peroxide could favour AA release by platelet membranes, as oxidant species have been shown to stimulate the phospholipase A_2 enzyme.[37] An earlier study also showed that collagen-induced platelet aggregation is associated with hydrogen peroxide release.[38]

Collagen is an important platelet agonist, thought to be involved in the early stages of platelet activation during both haemostasis and thrombosis. The activation of platelets by collagen leads to various events in signalling, generated by its interaction with the glycoprotein VI receptor. In fact, after an initial attachment to platelets through second-messenger pathways, collagen stimulates the release of TXA_2 and ADP, important agonists and platelet aggregators.[39] It has been shown that collagen-induced platelet aggregation is associated with a burst of hydrogen peroxide which, in turn, contributes to the activation of platelet function through calcium mobilisation and activation of the inositol pathway.[40,41] Moreover, the role of hydrogen peroxide on platelet function is closely related to collagen, as other agonists such as ADP and thrombin do not provoke platelet hydrogen peroxide formation.

Caccese and colleagues[41] showed that, among the platelet agonists ADP, thrombin and collagen, release of superoxide anions and hydroxyl radicals occurred mainly when platelets were stimulated by collagen. Such release was inhibited in platelets pre-treated with aspirin, suggesting that AA metabolism was the main source of these free radicals. The authors also demonstrated that in AA-stimulated platelet aggregation, superoxide anion and hydroxyl radical formation was dependent on the AA concentration. In addition, incubation of platelets with salicylic acid or ascorbic acid, which blunt hydroxyl radical and superoxide anion formation, respectively, inhibited both collagen-induced platelet aggregation and AA release. Furthermore, that study also demonstrated that the collagen-induced platelet aggregation was associated with free radical formation, which was dependent on AA release and metabolism.

A similar mechanism of action could possibly be implicated in the antiplatelet effect of piplartine, since the agonist AA, linked to activation of the COX pathway, was more sensitive to its inhibitory effect. A strong potentiation of the effect was seen when piplartine was associated with aspirin. Furthermore, our results demonstrated that piplartine, besides inhibiting aggregation induced by AA, also powerfully inhibited collagen-induced platelet aggregation. Similarly to aspirin, piplartine also shows anti-inflammatory effects, as assessed by carrageenan-induced paw oedema in mice, and protects mesencephalic cells against 6-OHDA, a neurotoxin whose cytotoxicity is associated with the production of free radicals (unpublished data).

Thus, our findings suggest that piplartine inhibits platelet function, and that this effect is dependent, at least in part, on the molecular pathways that mediate COX-related aggregation. Considering that previous data demonstrated that *Piper* species have considerable inhibitory activities against key enzymes of AA metabolism, such as 5-LOX, COX-1 and COX-2,[8] the inhibition of collagen-induced platelet aggregation by piplartine could be due to the inhibition of TXA_2 release by platelets. In addition, the PAF pathway could be another target for piplartine action. Further work is needed to elucidate the exact mechanism of action of piplartine as a platelet antiaggregant. Piperlongumine (synonym for piplartine) isolated from *P. longum* was recently shown to inhibit rabbit platelet aggregation induced by collagen, AA and PAF, without any inhibitory effect on thrombin-induced aggregation.[42] Since piperlongumine has been assumed to inhibit platelet aggregation as a TXA_2 receptor antagonist,[43] the same mechanism of action may be involved with piplartine.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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