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Dual Inhibition of Platelet-activating Factor and Arachidonic Acid Metabolism by Ajmaline and Effect on Carrageenan-induced Rat Paw Oedema

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Abstract—The effects of ajmaline on human platelet aggregation, arachidonate metabolism and platelet activating factor (PAF)-induced lethality in rabbits were examined. Platelet aggregation induced by several stimuli (ADP, collagen, and PAF) was inhibited by increasing concentrations of ajmaline. The potency of ajmaline was higher when PAF was employed as stimulating agent in comparison with other agonists (IC50 70, 270 and 380 μM for PAF, ADP and collagen, respectively) whereas ajmaline had no effect against arachidonic acid-induced aggregation. In contrast however, ajmaline inhibited arachidonate metabolism by platelet homogenates. The formation of both thromboxane A_2 and 12-hydroxy-eicosatetraenoic acid was inhibited by ajmaline with comparable potencies. Pretreatment of rabbits with ajmaline (50 mg kg^{-1}) completely abolished the lethal effects of PAF (11 μg kg^{-1}) given intravenously ($P < 0.001$). In addition, ajmaline at doses ranging from 50 to 100 mg kg^{-1} inhibited carrageenan-induced rat paw oedema ($P < 0.001$). In this test ajmaline was three times more potent than aspirin. In the light of these results we conclude that ajmaline, a known anti-arrhythmic agent is a PAF antagonist and a dual inhibitor of platelet cyclo-oxygenase and lipoxygenase enzymes with anti-inflammatory properties.

Ajmaline, a rauwolfia alkaloid, belonging to the large group of indole alkaloids was first isolated and described chemically by Siddiqui & Siddiqui (1931). Subsequently, it was found to be effective against experimental and clinical atrial and ventricular arrhythmias (Arora & Madan 1956; Dick & McCawley 1963; Wellens et al 1980; Sethi et al 1984). These studies have established the use of this drug as a class I anti-arrhythmic agent with actions similar to those of quinidine. More recently, we have demonstrated that anti-arrhythmic drugs, such as quinidine, inhibit platelet-activating factor (PAF)-induced human platelet aggregation in-vitro (Gilani & Saeed 1989).

It has also been demonstrated that platelets play an important role in acute inflammation (Nachman & Weksler 1972; Vincent et al 1978). They accumulate and respond to injury by releasing important mediators such as 5-hydroxytryptamine, prostanoids, PAF and hydrolases (Holmsen et al 1977; Page et al 1984) which contribute to the inflammatory process. Furthermore, thromboxane A_2 (TXA_2), an arachidonic acid (AA) product formed by platelets has been reported to be a potent constrictor of blood vessels and an aggregator of platelets (Hamberg et al 1975; Lefer 1982). Recent evidence suggests that TXA_2 is involved in the pathogenesis of circulatory shock and cardiac ischaemia associated with arrhythmias (Lefer 1985). In addition, PAF has also been implicated in the pathogenesis of ischaemia and reperfusion-induced arrhythmias by activating platelets (Wainwright et al 1989).

This background prompted us to investigate the effects of ajmaline on platelet aggregation and arachidonic acid metabolism. The effects of ajmaline on carrageenan-induced rat paw oedema and PAF-induced shock, and platelet aggregation leading to death in rabbits have also been investigated.

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Materials and Methods

Materials

Ajmaline, acetylsalicylic acid, arachidonic acid (Grade 1, 99% pure), ADP, collagen, indomethacin, PAF, reduced glutathione (GSH), nordihydroguaiaretic acid (NDGA) and carrageenan were purchased from Sigma Chemical Co. (St Louis, MO, USA). [1-^{14}C]AA (sp. act. 58 mCi $mmol^{-1}$), [^{3}H]thromboxane B_2 (> 120 Ci $mmol^{-1}$) and 12-(*S*)-hydroxy-[^{3}H]eicosatetraenoic acid (12-HETE) (100 Ci $mmol^{-1}$) were obtained from Amersham International plc, UK. All other chemicals used were of the highest purity grade available.

Animals

New Zealand White male rabbits, 3–3·5 kg, and Sprague-Dawley male rats, 150–200 g, were obtained from the Laboratory Animal Unit, H.E.J. Research Institute of Chemistry. They were fed a standard diet for a minimum period of 7 days and food was withheld the night before an experiment. Water was freely available throughout.

Platelet aggregation

Blood was taken by venepuncture from normal volunteers reported to be free of medication for 7 days. Blood samples were mixed with 3·8% (w/v) sodium citrate solution (9:1) and centrifuged at 260 *g* for 15 min at 20°C to obtain platelet-rich plasma (PRP). The remaining blood sample was centrifuged at 1200 *g* for 10 min to obtain platelet poor plasma (PPP). Platelet count was determined by phase contrast microscopy and all aggregation studies were carried out at 37°C with PRP having platelet counts between 2·5 and 3.0×10^8 mL^{-1} of plasma. Aggregation was monitored with a

four-channel NKK Hema Tracer aggregometer (Model PAT 4M, Niko Bioscience Co., Japan) using 225 μL samples of PRP. The PRP was preincubated with an appropriate amount of the test compound for 1 min before challenge with the aggregating agent. The light transmission was adjusted to 0 and 100% with PRP and PPP, respectively. Aggregation was induced by ADP (2·2 μM), AA (0·8 mM), collagen (20 μg mL^{-1}) or PAF (0·8 μM). The resulting aggregation was recorded and expressed as percentage inhibition compared with control at 4 min after challenge. Test compounds were tested at 3 or 4 concentrations in duplicate. Differences between control and test measurements were assessed by Student's *t*-test.

Arachidonic acid metabolism by platelets

Human blood platelets were routinely obtained in plastic bags containing 30–40 mL concentrated PRP from The Aga Khan University Hospital laboratory. The PRP was centrifuged at 1200 *g* for 20 min and the sedimented platelets were washed twice with an ice-cold phosphate buffer (50 mM, pH 7·4), containing NaCl (0·15 M) and EDTA (0·2 mM). After centrifugation platelets were resuspended in the same buffer without EDTA at the initial PRP cell concentration. The PRP suspension was homogenized at 4°C using a polytron homogenizer for 15 s and the homogenate centrifuged at 1200 *g* for 20 min. Supernatant (300 μL containing 0·4 mg protein) was incubated with 10 μg unlabelled AA and 0·1 μCi [1-^{14}C]AA in the presence and absence of test compound. After 15 min with gentle shaking in air at 37°C the reaction was stopped by adding 0·4 mL citric acid (0·4 M) and ethyl acetate (7·0 mL). After mixing and centrifuging at 600 *g* for 5 min at 4°C, the organic layer was separated and evaporated to dryness under nitrogen. Residues were dissolved in 40 μL ethanol and 20 μL was applied to silica gel G thin layer chromatography (TLC) plates (Analtech, Delaware, USA). The AA, TXB_2 (a stable degradation product of TXA_2) and 12-HETE standards were spotted separately. The plates were developed in ether/petroleum ether [boiling range 40–60°C]/acetic acid (50 : 50 : 1, v/v) to a distance of 17 cm. By use of this solvent system the various lipoxygenase products (HETEs) are separated with TXB_2 and prostaglandins remaining at the origin. The solvent system used for the separation of various prostaglandins and TXB_2 in dried organic extracts of platelet incubates as above, was ethylacetate/isooctane/water/acetic acid (11 : 5 : 10 : 2, v/v, upper phase). Radioactive zones were located and quantified by use of a Berthold TLC linear analyser and chromatography data system (Model LKB 511, Berthold, Germany). Protein concentration was determined by the method of Lowry et al (1951) using human serum albumin as standard.

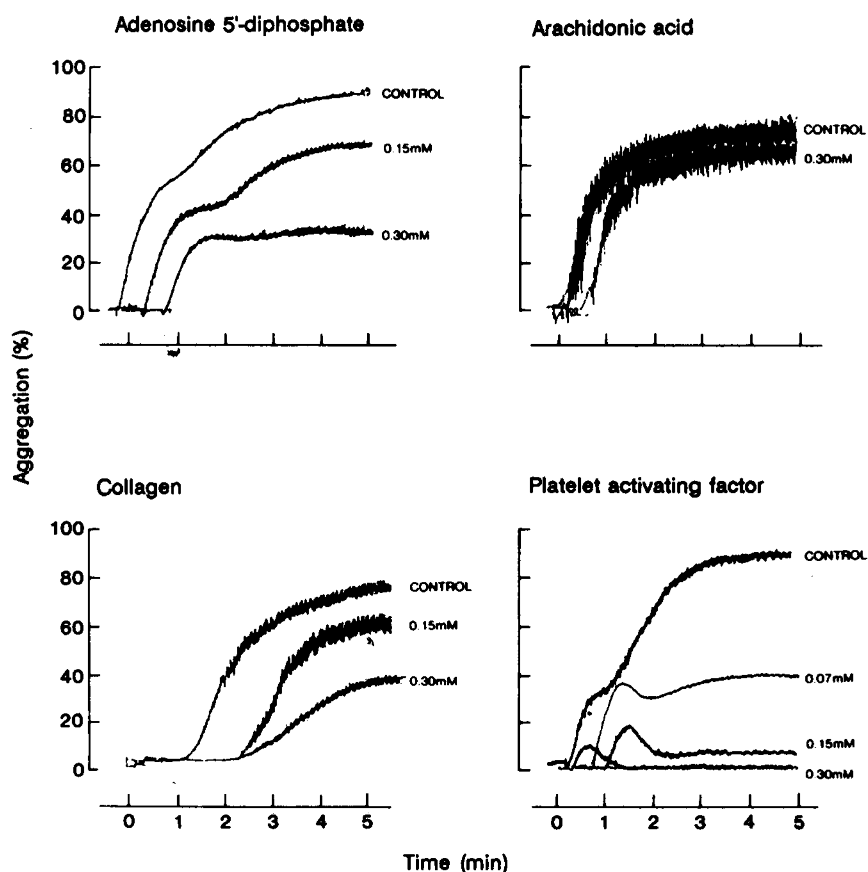

FIG. 1. Platelet aggregation tracings showing inhibition of human platelet aggregation by ajmaline. Ajmaline was dissolved in 0·9% NaCl and added 1 min before challenge with the aggregating agent as shown.

Carrageenan-induced paw oedema in rats

Paw oedema was induced by injecting 0·1 mL of freshly prepared 1% suspension of carrageenan in 0·9% NaCl (saline) into the subplanter region of the right hind paw. The plethysmographic method (Winter et al 1962) was used for recording volume changes in the rat paw. Sprague-Dawley male rats, 150–200 g, divided into five groups of 10 animals each, were used. Ajmaline at doses of 50 and 100 mg kg^{-1} was injected intraperitoneally into two groups of rats. The other two groups received the reference substance, aspirin, at a dose of 150 and 300 mg kg^{-1}, while the control group received distilled water. Carrageenan was injected after an interval of 30 min. The paw volumes were measured 30 min before and 5 h after injection of carrageenan. The difference in paw volumes before and after indicated the volume of inflammation. The anti-inflammatory activity was calculated as $A-B/A \times 100$, where A and B denote mean paw volume of control and drug-treated animals, respectively.

PAF-induced mortality in rabbits

Male rabbits (NZ White, 3–3·5 kg) were used to test the lethality of intravenous PAF (Saeed et al 1988, 1989). PAF was prepared before each injection by dissolving sufficient PAF in saline to give a final concentration of 22 μg mL^{-1}. The PAF was injected into the marginal ear vein of the rabbits over a period of approximately 1 min. In preliminary experiments, we established that an intravenous dose of 11 μg kg^{-1} PAF was required to induce consistently fatal pulmonary thrombosis in control animals (n = 6); this dose of PAF was used in subsequent experiments with ajmaline. Animals were pretreated by intraperitoneal injection of ajmaline (25 and 50 mg kg^{-1}) or aspirin (50 mg kg^{-1}) 2 h before the challenging dose of PAF. Each animal was used for only one experiment.

Results

The effects of ajmaline on platelet aggregation induced by PAF and other agents is shown in Fig. 1. When PRP was preincubated with ajmaline for 1 min before challenge, platelet aggregation induced by PAF was completely suppressed in a concentration-related manner, whereas higher concentrations (> 0·1 mM) of ajmaline were required to inhibit aggregation induced by other agents, such as ADP or collagen. The mean values (±s.e.m, n = 7) for inhibiting aggregation by 50% against different inducing agents were; ADP, 0·27±0·03; collagen, 0·38±0·05; and PAF, 0·07±0·01 mM, respectively. Ajmaline, however, had no effect on platelet aggregation induced by AA. These results demonstrate that ajmaline preferentially inhibited PAF-induced aggregation as compared with aggregation induced by other agents.

To further discriminate the anti-PAF activity of ajmaline, we tested the effect of indomethacin, a cyclo-oxygenase inhibitor, on platelet aggregation induced by AA, ADP, collagen and PAF. The mean values (±s.e.m., n = 4) for inhibiting aggregation by 50% against different inducing agents were; AA, 0·35±0·03; ADP, 0·57±0·02; and collagen, 11±1 μM, respectively. Indomethacin inhibited only the second wave of aggregation without affecting the first wave, induced by PAF. These results demonstrate that indomethacin selectively inhibited AA- and ADP-induced aggregation as compared with aggregation induced by other agents.

Table 1. Effect of ajmaline and aspirin on PAF-induced mortality in rabbits.

Compound	Dose (mg kg^{-1})	Route	Survival
PAF-control	0	i.v.	0/10
Ajmaline	25	i.p.	0/10
	50	i.p.	10/10**
Aspirin	50	i.p.	7/10*

* $P < 0·01$, ** $P < 0·001$ compared with control.

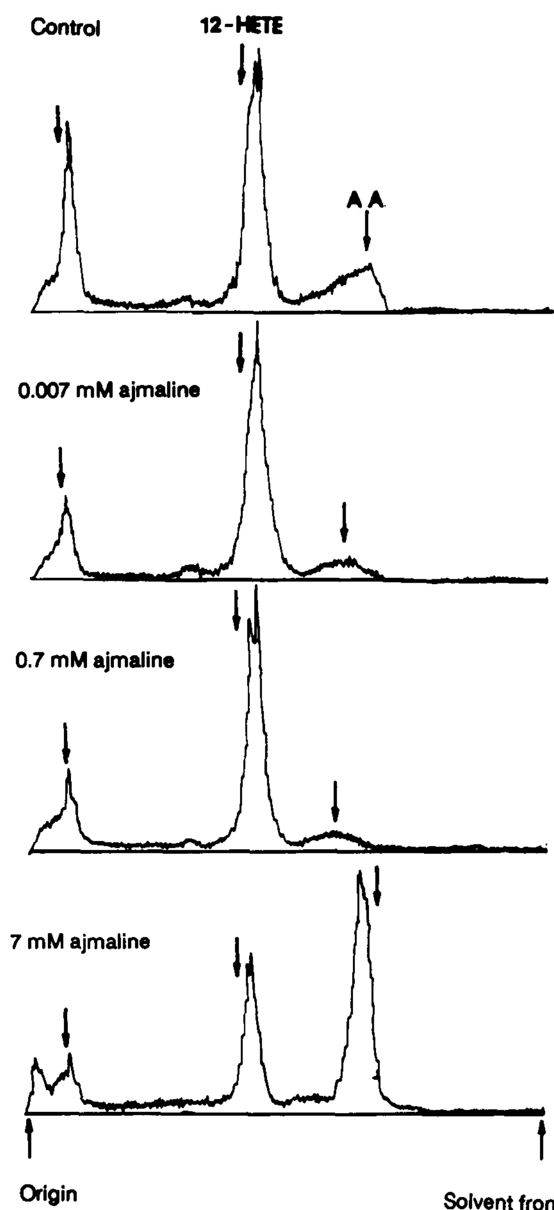

FIG. 2. Radiochromatogram scan showing the profile of arachidonate lipoxygenase metabolites produced by homogenate of human platelets incubated with [1-^{14}C]AA in the absence and presence of ajmaline.

These results strongly suggest that ajmaline acts as an antagonist of PAF rather than an antagonist of TXA_2 in platelet aggregation.

In view of our present finding that ajmaline more selectively inhibited PAF-induced platelet aggregation, we also investigated the effect of ajmaline on PAF-induced sudden death associated with platelet aggregation in the rabbit. We found that the survival rate after ajmaline pretreatment was significantly higher ($P < 0.001$, n = 10) than the survival rate found without pretreatment (control group). These results are presented in Table 1.

Effect of ajmaline on platelet arachidonic acid metabolism. Incubation of [1-^{14}C]AA with homogenate (1200 *g* supernatant fraction) of human platelets resulted in the formation of two lipoxygenase products (Fig. 2). The mobility of one product (Rf 0·62) on a silica gel G chromatogram was similar to that of the authentic 12-HETE. The formation of a more polar lipoxygenase product (Rf 0·2) was also observed. Its absolute identification however, remains to be determined; it may be a tri-hydroxy-eicosatrienoic acid (THETE) as described previously by Bryant & Bailey (1979).

The synthesis of lipoxygenase products by human platelets was inhibited by ajmaline (7–700 μM) and NDGA (5–50 μM), an antioxidant and a known inhibitor of lipoxygenase (Bray et al 1981), in a concentration-related manner (n = 5). The mean values of ajmaline (±s.e.m., n = 5) for inhibiting formation of these products by 50% (IC50) were 375 ± 14 μM for the polar product and 700 ± 15 μM for 12-HETE; aspirin (7–700 μM) selectively inhibited the production of TXB_2 in a concentration-related manner ($P < 0.01$ for slopes of concentration-response curves). The mean values (±s.e.m., n = 5) for inhibition of TXB_2 by 50% were 590 ± 6 for ajmaline and 240 ± 11 μM for aspirin. These results show that ajmaline is a dual inhibitor of platelet cyclo-oxygenase and lipoxygenase enzymes.

Inhibition of carrageenan-induced rat paw oedema

Ajmaline showed a marked reduction in the carrageenan-induced oedema. The paw volume observed in the control animals (0·804 mL) was effectively reduced to 0·52 and 0·24 mL after treatment with ajmaline at doses of 50 and 100 mg kg^{-1}, respectively. The percent reduction of oedema in ajmaline-treated rats was dose-dependent (Table 2). The reduction induced by aspirin (a positive control) at 150 and 300 mg kg^{-1} was 28 and 71%, respectively (Table 2). This showed that ajmaline and aspirin produced a significant ($P < 0.001$) reduction of oedema, with ajmaline being three times more potent than aspirin.

Table 2. Anti-inflammatory effects of ajmaline and aspirin on carrageenan-induced rat-paw oedema.

Compound	Dose (mg kg^{-1})	Mean paw volume ±s.e.m. after 5 h (mL)	Inhibition Mean ± s.e.m.
Control	—	0·80 ± 0·04	—
Ajmaline	50	0·52 ± 0·11*	35 ± 2
	100	0·31 ± 0·1*	70 ± 5
Aspirin	150	0·58 ± 0·08*	28 ± 9
	300	0·23 ± 0·03*	71 ± 3

Each value represents the average value for 8–10 animals. * $P < 0.001$ compared with controls.

Discussion

In our experiments, ajmaline inhibited platelet aggregation induced by several aggregating agents tested. Some differences can be observed in the aggregation induced by different agonists. Ajmaline had no effect against AA-induced platelet aggregation but inhibited (at higher doses) AA metabolism by platelet homogenate (Figs 2, 3). The reason for this discrepancy is not understood. It is possible that the drug is more accessible to cyclo-oxygenase in a platelet homogenate preparation. On the other hand, ajmaline inhibited ADP and collagen-induced aggregation and their inhibitory potencies

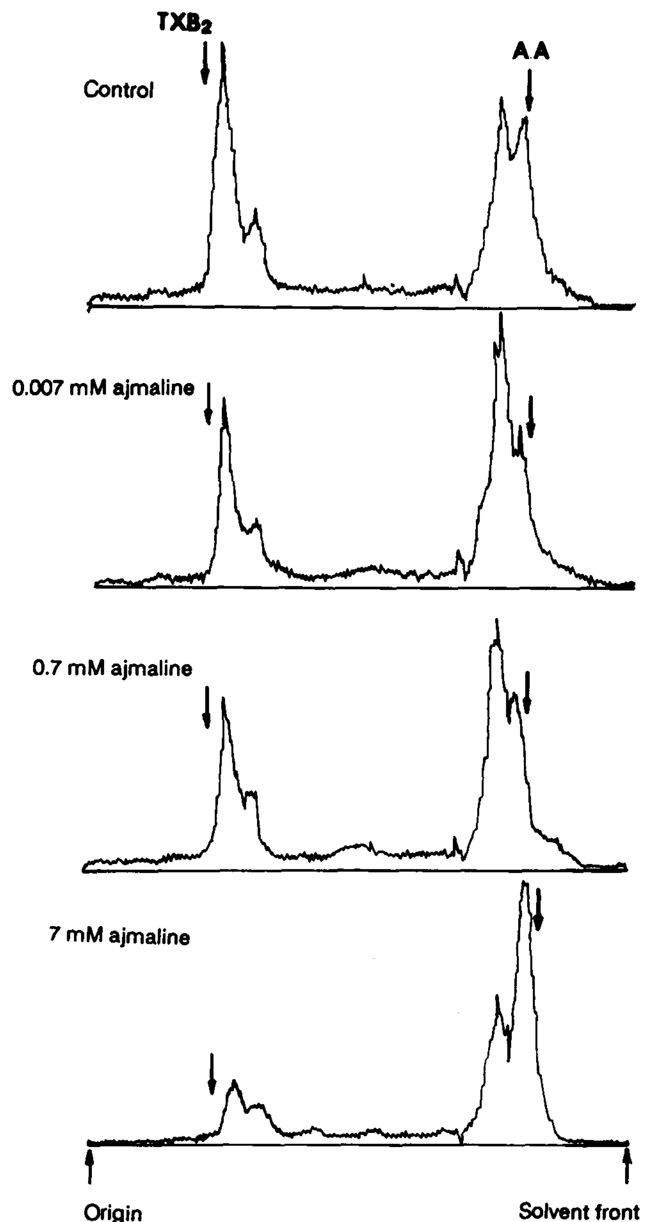

FIG. 3. Radiochromatograms of the products of cyclo-oxygenase metabolites of AA formed upon incubation of homogenate of human platelets with [1-^{14}C]AA in the absence and presence of ajmaline.

were comparable, whereas ajmaline was most active against PAF-stimulated platelets. This is in agreement with our previous results demonstrating that various anti-arrhythmic drugs selectively inhibited PAF-induced aggregation probably by a membrane stabilization effect (Gilani & Saeed 1989). In the present study we found that ajmaline (Table 1) also afforded protective effects in a severe model of PAF-induced sudden death in rabbits. While the precise sites of PAF-induced action in sudden death of rabbits remains to be clarified, some particular remarks can be drawn from PAF-involving experiments. PAF is a potent endogenous mediator of inflammation and cell-to-cell interactions. It activates human platelets, but the mechanism of this action is not well known. Evidence exists that intravenous injection of PAF induces platelet aggregation in several animal species through activation of PAF receptors (Martins et al 1988). Moreover, it has also been reported that the activation of AA metabolism increases cytosolic free calcium during PAF-induced platelet aggregation (Camussi et al 1983). Indeed it has been shown that PAF-induced aggregation is mediated, in part, by the formation of TXA_2 and is reduced by aspirin, an inhibitor of cyclo-oxygenase (McCulloch et al 1989). Since ajmaline, in common with aspirin also inhibited carrageenan-induced rat paw oedema (Table 2), the possibility exists that inhibition of AA metabolism by ajmaline may be involved in acute inflammation.

Thus, the actions of ajmaline on PAF, AA metabolism and acute inflammation must also be considered in concert with its beneficial properties in cardiac arrhythmias. It is noteworthy that release of PAF and TXA_2 by platelets has been implicated in the pathogenesis of shock, myocardial ischaemia and arrhythmias resulting in sudden death (Lefer 1985). There is evidence also supportive of the view that PAF may contribute to myocardial ischaemia and reperfusion-induced arrhythmias by activated platelets (Wainwright et al 1989; Koltai et al 1989). Ajmaline is beneficial against ventricular arrhythmias not controlled by other anti-arrhythmic drugs (Grenadier et al 1983) and is found to be effective against reperfusion-induced arrhythmia (Okumura et al 1988).

These results point to a new facet of pharmacological action of ajmaline, i.e. inhibition of PAF, AA metabolism and acute inflammation. Whether this is a general property of other anti-arrhythmic agents is not known at present. We believe that our present findings could be important in clarifying the mechanisms whereby ajmaline may exert anti-arrhythmic properties in conditions where PAF and AA metabolites have been implicated.

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