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INHIBITION OF HUMAN PLATELET CYCLIC AMP PHOSPHODIESTERASE AND OF PLATELET AGGREGATION BY A HEMISYNTHETIC FLAVONOID, AMENTOFLAVONE HEXAACETATE

ALAIN BERETZ,* FRANÇOISE BRIANÇON-SCHEID,† ANITA STIERLÉ,* GILLES CORRE,* ROBERT ANTON† and JEAN-PIERRE CAZENAVE*‡

* Laboratoire de Biologie et Pharmacologie des Interactions du Sang avec les Vaisseaux et les Biomatériaux, Centre Régional de Transfusion Sanguine, 10 rue Spielmann, 67085 Strasbourg Cédex, France; and † Laboratoire de Pharmacognosie, Faculté de Pharmacie, Université Louis Pasteur, B.P. 10, 67048 Strasbourg Cédex, France

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Abstract—Amentoflavone hexaacetate (AmAc) was synthesized from natural amentoflavone (Am), a biflavonoid extracted from *Viburnum lantana* L. Am does not inhibit aggregation of intact platelets up to a concentration of 100 μM but inhibits human platelet cAMP phosphodiesterase (IC_{50} = 22.0 μM). AmAc is a potent inhibitor of the aggregation of washed human platelets induced by ADP (IC_{50} = 2.3 μM) or collagen (IC_{50} = 4.7 μM). AmAc inhibits crude (IC_{50} = 8.6 μM) or partially purified (IC_{50} = 42.2 μM) human platelet cAMP phosphodiesterase. In the presence of prostaglandin E_1, AmAc (10 μM) induces a 3.7-fold increase in total platelet cAMP. The characteristics of this action suggest a role for cAMP in the mechanism of action of AmAc. The incubation of AmAc with intact platelets for 5 min is necessary for its activity.

Flavonoids are potent inhibitors of cyclic AMP phosphodiesterase (EC 3.1.4.17) in many human and animal tissues [1–6]. Inhibition of cyclic AMP phosphodiesterase (cAMP-PDE)§ has been implied in some pharmacological actions of flavonoids, such as the inhibition of blood platelet aggregation and secretion [7–9]. The biflavonoids, other natural compounds produced by the coupling of two flavonoid molecules, are also PDE inhibitors; among them, amentoflavone (Fig. 1) inhibits a crude preparation of beef heart PDE with a I_{50} of 0.12 μM [10]. Thus, it could be expected that amentoflavone would modify the function of intact cells through mechanisms related to PDE inhibition. In this study, we report the effects of amentoflavone (Am) and especially of the hemisynthetic derivative, amentoflavone hexaacetate (AmAc) (Fig. 1) on the function of human blood platelets. The characteristics of the action of Am and AmAc suggest that the inhibition of platelet activation is mediated by an increase in cAMP.

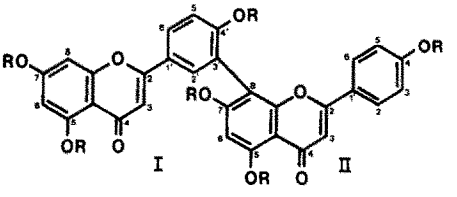

Fig. 1. Chemical structure of amentoflavone (R = H−) and amentoflavone hexaacetate (R = CH_3CO−).

MATERIALS AND METHODS

Preparation of amentoflavone. Amentoflavone was extracted from *Viburnum lantana* L. leaves, where its presence was described by Godeau *et al.* [11]. The ethyl ether soluble fraction (213 g) of a defatted methanolic extract obtained from 25 kg of dry powdered leaves was chromatographed over a column of Kieselgel 60 H (15 μm) (Merck, Darmstadt, F.R.G.) using a mixture of chloroform/methanol (95:5, v/v) as eluant (Chromatospac Prep-10 apparatus (Jobin-Yvon, Longjumeau, France)). When the flavonoid rich fractions were collected and concentrated, Am precipitated. The separated material was purified by several precipitations from methanol and obtained as a yellow amorphous powder. The isolated product (M.W. = 528) had an R_f value (Silica gel—chloroform/methanol, 90:10, v/v—R_f = 0.20) identical with that of an authentic sample of Am kindly provided by Prof. Dr. H. Wagner (Munich, F.R.G.). The purity of Am was

‡ Address reprint requests to: Dr J.-P. Cazenave, Centre Régional de Transfusion Sanguine, 10, rue Spielmann, 67085 Strasbourg Cédex, France.

§ Abbreviations used: cAMP, adenosine 3′,5′-cyclic monophosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; PDE, cyclic nucleotide phosphodiesterase (EC 3.1.4.17); Am, amentoflavone: I-4′, II-4′, I-5, II-5, I-7, II-7 hexahydroxy I-3′, II-8 biflavone; AcAm, amentoflavone hexaacetate; ADP, adenosine-5′-diphosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PGE_1, prostaglandin E_1; DMSO, dimethylsulfoxide; DTT, 1,4-dithio-DL-threitol.

checked by analytical HPLC [12]. The spectral analysis was in agreement with that described in the literature [13–17].

Preparation of amentoflavone hexaacetate. Two hundred milligrams of Am were acetylated [15] with pyridine and acetic anhydride, at room temperature overnight. The hexaacetate was separated from the obtained acetate mixture by preparative TLC (Silica gel—chloroform/methanol, 90:10, v/v—$R_f = 0.90$) and then purified by crystallization from chloroform/methanol as thin white needles (M.W. = 790). The spectral analysis of AmAc was in agreement with the literature [15, 16].

Chemicals. Adenosine-5′-diphosphate, sodium salt (ADP), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and *Crotalus atrox* snake venom were obtained from Sigma Chemical Co. (St Louis, MO). PGE_1 was from Upjohn (Kalamazoo, MI). Acid-soluble collagen was prepared as described previously [18]. Human fibrinogen (grade L) (Kabi, Stockholm, Sweden) was treated with diisopropylfluorophosphate (Sigma Chemical Co., St. Louis, MO) as described previously [19]. [8-3H] adenosine 3′,5′-cyclic monophosphate (18.3 Ci/mmol) was purchased from Amersham, U.K., and further purified by thin-layer chromatography [20]. cAMP was obtained from Boehringer (Mannheim, F.R.G.). All other chemicals were of analytical grade.

Preparation of washed human platelet. Blood was collected from a forearm vein of healthy human volunteers, who had not taken any medication for 2 weeks. Six volumes of blood were collected into 1 vol. of acid–citrate–dextrose anticoagulant [21]. Twice-washed platelet suspensions were prepared exactly as described [22]. The final resuspending medium (pH 7.35) was Tyrode solution (which contained 2 mM Ca^{2+} and 1 mM Mg^{2+}) with 0.35% human albumin (Centre Régional de Transfusion Sanguine, Strasbourg, France), 5 mM HEPES buffer and apyrase. The platelets were stored at 37° throughout the experiments. Platelet count was adjusted in the final suspension to 300,000/mm^3 using a Baker 810 platelet counter (Baker Instruments, Allentown, PA).

Platelet aggregation studies. Platelet aggregation was studied at 37° using a turbidimetric device (Payton aggregometer, Scarborough, Ontario, Canada) [22]. One microlitre of Am or AmAc or the solvent (DMSO) was added with a micro-pipette to 0.5 ml platelet suspension and stirred at 1100 rpm in the aggregometer for 0.5 to 10 min before addition of the aggregating agent. The final concentration of the aggregating agent was 2.5 μg/ml collagen or 5 μM ADP in the presence of 0.8 μg/ml of human fibrinogen treated with diisopropylfluorophosphate [22]. The extent of aggregation was estimated quantitatively by measuring the maximum height, in millimeters, above baseline level for aggregation induced by ADP and by measuring the height of the curve reached above baseline level after 3 min for aggregation by collagen. Control aggregation curves were obtained in the presence of 1 μl DMSO and were compared with the curves obtained in the presence of the inhibitor. The results were used to calculate the percentage inhibition.

Cyclic AMP assay. One microliter of Am or AmAc solubilized in DMSO was added in the aggregometer cuvette (37°, 1100 rpm) containing 900 μl of washed human platelets and incubated for 0.5 to 10 min. Then 100 μl of PGE_1 (or buffer) was added; incubation was terminated 30 sec after the addition of PGE_1 by transfering 400 μl of the suspension in 100 μl of cold 3.5 N perchloric acid. The protein precipitate was eliminated by centrifugation; the supernatant was neutralized to pH 6–7 by 9 N KOH and succinylated [23]. Cyclic AMP was assayed using a commercial radioimmunoassay kit (Immunotech, Luminy, France).

Preparation of cyclic AMP phosphodiesterase from human platelets. Human platelets were washed as described above. The suspensions, examined by phase contrast microscopy, were contaminated by less than 0.1% erythrocytes and 0.01% leukocytes. The final suspending medium was 20 mM Tris/HCl buffer (pH 7.5) containing 2 mM $MgCl_2$, 1000 U/ml aprotinine (Choay, Paris, France). The platelet pellet was resuspended in 4 vol. of cold (4°) buffer and immediately sonicated at 115 V, 30 sec/ml (Sonimasse 150T, Ultrasons Annemasse, France). The sonication was done with 1 min pauses between each 10 sec stroke of sonication to avoid a rise in temperature. The homogenized solution was immediately centrifuged at 100,000 *g* for 60 min at 4°, yielding a crude platelet PDE preparation.

To purify human platelet PDE, 1 mM DTT and 5 mM EDTA were added to the final suspending medium. All manipulations were performed at 4°. The 100,000 *g* supernatant was applied to a DEAE Trisacryl (IBF, Villeneuve-la-Garenne, France) column (1.5 × 6 cm), previously equilibrated with 35 ml of 20 mM Tris/HCl, 2 mM $MgCl_2$, 1 mM DTT, 1000 U/ml aprotinine (pH 7.5). Elution was performed by a linear gradient from 0 to 0.25 M NaCl with a flow rate of 10 ml/hr and a total gradient volume of 150 ml. One ml fractions were collected and stored at 4° in the presence of 1 mg/ml human serum albumin. Two peaks of activity were obtained, one (peak I) eluted at 0.05 M NaCl hydrolysed essentially cGMP and the second (peak II) eluted at 0.17 M NaCl hydrolysed both cAMP and cGMP.

Platelet cyclic AMP phosphodiesterase assay. Activity of cAMP-PDE was determined by the method of Wells *et al.* [24]. The medium contained 48 mM Tris/HCl (pH 7.5), 2 mM $MgCl_2$, 10 μM $CaCl_2$, 1 mg/ml of human albumin, 0.2 mM EGTA. The inhibitors, prepared as stock solutions in DMSO, were further dissolved in 20 mM Tris/HCl, 2 mM $MgCl_2$ (pH 7.5) buffer. Fifty microlitres of the inhibitor solution was added to the incubation medium (final vol. 250 μl). Similar dilutions of DMSO did not influence cAMP-PDE activity. The medium was incubated at 37° after addition of the platelet PDE preparation and the substrate ([3H] cAMP, 1 μM). The reaction was stopped by the addition of 25 μl of an aqueous solution of 50 mM EDTA, 30 mM theophylline, 10 mM cyclic AMP, 10 mM cyclic GMP and 100 mM Tris/HCl (pH 7.5). Ten microlitres of *Crotalus atrox* venom (20 mg/ml) was then added and the medium was incubated for 20 min at 37°. The reaction mixture was then diluted to 1 ml with a solution containing 0.1 mM adenosine,

0.1 mM guanosine and 15 mM EDTA. Tritiated dephosphorylated products were separated with small disposable QAE-Sephadex (Pharmacia, Uppsala, Sweden) formate columns and mixed with 10 ml of ACS-II scintillation fluid (Amersham, U.K.). Radioactivity was counted in a liquid scintillation counter (LKB 1211 Minibeta, Turku, Finland). Product accumulation was linear with time and enzyme concentration under the conditions studied, where hydrolysed substrate was always less than 25%.

RESULTS

Effect of Am and AmAc on the aggregation of human platelets by ADP or collagen

AmAc inhibited platelet aggregation induced by ADP or collagen by less than 20% when added to the suspension 30 sec before the aggregating agent. When the preincubation time of AmAc was increased to 5 min, it inhibited strongly platelet aggregation induced by ADP ($IC_{50} = 2.3 \pm 0.4$ μM, mean ± S.E.M., N = 5) and by collagen ($IC_{50} = 4.7 \pm 2.5$ μM, N = 4). The inhibition of ADP-induced platelet aggregation was fully achieved when AmAc was incubated for at least 5 min with the platelet suspension (Fig. 2). Am, after 30 sec or 5 min incubation (data not shown), had no inhibitory activity up to a concentration of 100 μM, where it precipitated. The inhibition of ADP-induced aggregation by AmAc was potentiated by a low concentration (20 nM) of PGE_1 (Fig. 3).

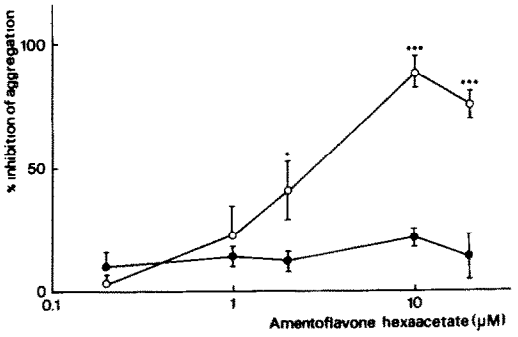

Fig. 2. Effect of AmAc on ADP-induced aggregation of washed human platelets. The drug was incubated for 30 sec (●) or 5 min (○) with the human platelet suspension before the addition of 5 μM ADP. Each point is the mean ± S.E.M. (vertical bars) of 3–5 experiments with platelets from different donors. Results were compared using Student's *t*-test: *P < 0.05, **P < 0.02, ***P < 0.001.

Effect of Am and AmAc on human platelet cAMP levels

Am or AmAc were added to the platelet suspension and incubated for various periods of time; PGE_1 (or buffer) was then added and the reaction stopped 30 sec after the addition of PGE_1 or control buffer. Under these conditions, there was no rise in total platelet cAMP levels over basal levels with AmAc or PGE_1 alone (Fig. 4). The combination of AmAc and PGE_1 resulted in a rise in cAMP (3.7-fold increase at 10 μM AmAc). This was dependent on the duration of incubation of AmAc and reached a plateau after 3 min (Fig. 4). The increase in total platelet cAMP was also a function of the concentration of AmAc (Fig. 5A). In contrast, Am had no effect on platelet cAMP levels when tested under the same conditions (Fig. 5B).

Effect of Am and AmAc on the activity of cAMP-PDE of human platelets

The effect of Am or AmAc on cAMP hydrolysis by a 100,000 *g* supernatant of fresh human platelets was studied (Fig. 6). When the drug was added to the medium immediately before the incubation of the enzyme with its substrate, AmAc was 2.6 times more active ($IC_{50} = 8.6 \pm 1.6$ μM, mean ± S.E.M., N = 3) than Am ($IC_{50} = 22.0 \pm 3.0$ μM). The crude 100,000 *g* platelet supernatant used in these experiments contained, in addition to cAMP-PDE, many

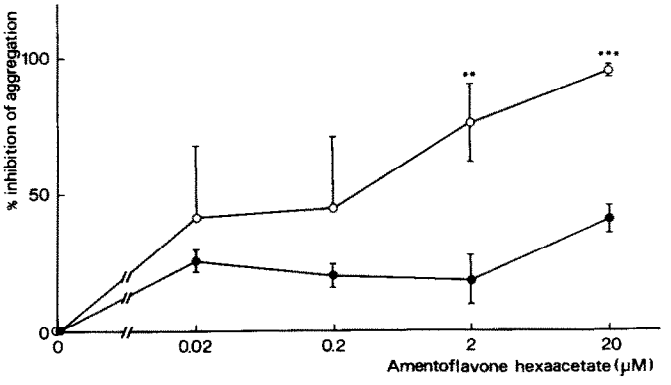

Fig. 3. Effect of PGE_1 on the inhibitory activity of AmAc on ADP-induced aggregation of washed human platelets. AmAc was preincubated 30 sec with the platelets; PGE_1 (○) or buffer (●) were then added 30 sec before addition of 5 μM ADP. The % of inhibition was calculated against a standard ADP-induced aggregation (closed symbols) or against the aggregation obtained in the presence of PGE_1 (open symbols). The dose of PGE_1 was chosen in order to produce approximately 25% of inhibition of the aggregation in each experiment (a mean dose of 10.8 ± 5.1 nM PGE_1 gave a mean 27.7 ± 2.5% of inhibition). Each point is the mean ± S.E.M. of 3 separate experiments using platelets from different donors. Results were compared using Student's *t*-test: **P < 0.02, ***P < 0.001.

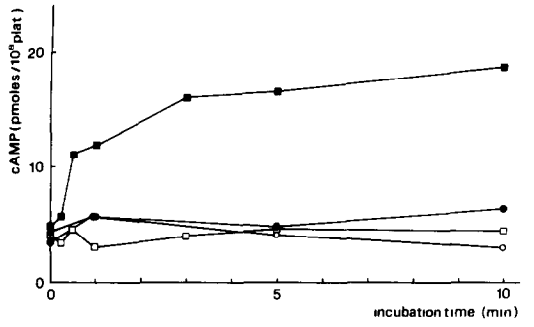

Fig. 4. Effect of 20 μM AmAc on total platelet cAMP levels. AmAc or DMSO was incubated for various times, then PGE_1 (or buffer) was added and incubated for 30 sec: ○, basal level of cAMP (DMSO + buffer); ●, PGE_1 20 nM; □, AmAc 20 μM; ■, AmAc 20 μM + PGE_1 20 nM. Each point is the mean of duplicate values from one experiment.

other cytosolic enzymes that may have induced chemical modifications of AmAc or Am. Thus the effect of Am or AmAc could be attributed to the drug itself or to some product(s) of its modification by platelet enzymes. This possibility was explored by incubating Am or AmAc with the crude platelet PDE preparation at 37° 15 min before adding cAMP. There was no significant change in the activity of either AmAc (IC_{50} = 7.4 ± 2.8 μM) or Am (IC_{50} = 24.2 ± 3.4 μM).

PDE from fresh human platelets was separated by DEAE-Trisacryl ion-exchange chromatography, in two active peaks. The first one (peak I) hydrolysed mainly cGMP, while the second (peak II) hydrolysed both cAMP and cGMP. Two apparent K_m for cAMP could be calculated (0.86 and 339 μM). The characteristics of peak II are comparable to those described by Hidaka *et al.* [25] and Grant *et al.* [26]. Am (IC_{50} = 41.0 ± 3.7 μM, N = 3) was 1.9 times less active on the partially purified peak II than on the crude platelet PDE preparation (Fig. 6). AmAc seemed much less active on this enzyme preparation, with an IC_{50} of 42.2 ± 18.1 μM, N = 3. However, Fig. 6 shows that the dose–action curve for AmAc tends to reach a plateau around 60% of inhibition for doses over 30 μM. At 10 μM, AmAc already inhibited by 36% the purified PDE whereas Am was inactive at this dose.

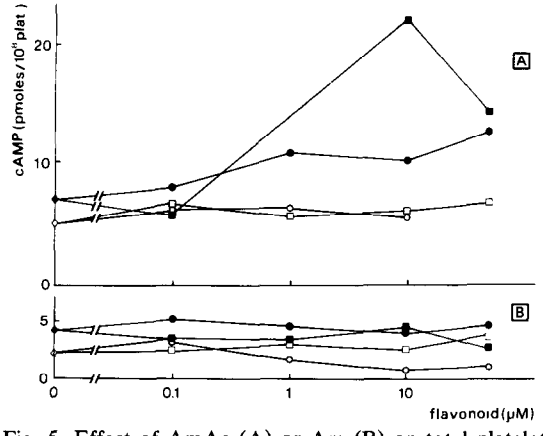

Fig. 5. Effect of AmAc (A) or Am (B) on total platelet cAMP levels. The flavonoids were incubated 30 sec before the addition of buffer (○) or 20 nM PGE_1 (●) and the incubations are stopped 30 sec after addition of PGE_1 or buffer. cAMP was also measured after incubation of the flavonoid 3 min before the addition of buffer (□) or 20 nM PGE_1 (■). Each point is the mean of duplicate values from one experiment.

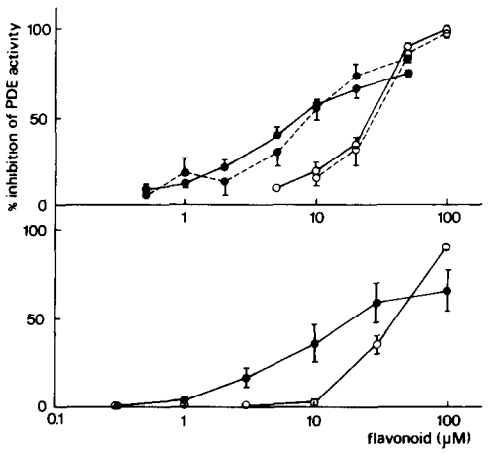

Fig. 6. Upper panel: Effect of Am (○) or AmAc (●) on the hydrolysis of 1 μM cAMP by a 100,000 *g* supernate of human platelets. The flavonoid was either added immediately before the incubation of the enzyme and its substrate (plain lines) or incubated with the enzyme 15 min at 37° before adding cAMP (dotted lines). Lower panel: Effect of Am (○) or AmAc (●) on the hydrolysis of 1 μM cAMP by a partially purified cAMP-PDE preparation (peak II) from human platelets. Each point is the mean ± S.E.M. of 3 separate experiments with each dose assayed in duplicate.

DISCUSSION

The characteristics of the pharmacological action of AmAc on human platelets are similar to those which have been described for the monomeric flavonoid quercetin [7, 8]. AmAc inhibits platelet cAMP-PDE and elevates total platelet cAMP in the presence of 20 nM PGE_1. However, as for quercetin [8, 9] and other PDE inhibitors [27–29], AmAc does not induce any measurable increase in total platelet cAMP at concentrations which have a pharmacological activity. It is possible that most of PDE inhibitors act only on a small pool of cAMP and these changes are not detected when measuring total platelet cAMP [30, 31].

Our results implicate cAMP in the mechanism of action of micromolar concentrations of AmAc on inhibition of intact human platelet function. In contrast, Am which is only 3.7-fold less active than AmAc on inhibition of partially purified cAMP-PDE, is not an inhibitor of platelet aggregation at concentrations at least 100 times greater. Am (50 μM) in combination with PGE_1 (20 nM) does not increase total cAMP levels in intact platelets, while micromolar AmAc in combination with 20 nM PGE_1 produces a clear increase in total cAMP in intact platelets (Fig. 5).

The lack of activity of Am on intact platelets can be due to the presence of free hydroxyl groups which could impair penetration of the drug into the platelet. It has been shown that structure-activity requirements for the platelet anti-aggregatory action of flavonoids (presence of C_2, C_3 double bond, absence of a carbohydrate substituent) [7] are also associated with increased lipophilic properties of molecule [32, 33]. Thus acetylation of Am to AmAc would have two effects. One is to improve slightly (3.7-fold increase) its inhibitory activity on soluble platelet cAMP-PDE, the second is probably to facilitate the transmembrane penetration of AmAc in intact platelets. In order to obtain the full activity of AmAc on inhibiting platelet aggregation (Fig. 2) or on elevating platelet cAMP (Fig. 4), it is necessary to incubate AmAc with the platelet suspension for 3–5 min. One possible explanation is that this is the time necessary for the drug to reach the target enzyme(s). The second explanation is that AmAc is metabolically transformed in the platelets into a more active molecule. However, prior incubation of AmAc with the crude PDE did not modify the IC_{50} (Fig. 6). It is also doubtful that AmAc is the prodrug of Am itself, because Am inhibited cAMP-PDE of human platelets at a concentration over 20 μM, while AmAc is active on intact platelets at a concentration of 2–5 μM.

The studies of Picq *et al.* [6] on the effects of pentasubstituted quercetin analogues on cAMP-PDE from rat brain have shown that pentaacetylquercetin was 4-fold less active than quercetin on cytosolic cAMP-PDE and 6-fold less active on the particulate enzyme. It seems that acetylation of the hydroxyl group of flavonoids can have opposite effects on cAMP-PDE inhibition when different types of flavonoids and/or PDEs extracted from different tissues are compared. We have shown previously that cyclic nucleotide PDEs from human platelets have different sensitivities to inhibitors compared to PDEs from vascular smooth muscle as demonstrated with a series of alkoxy-aryl-lactames [34]. This is also illustrated by our present results where Am is two orders of magnitude less active on platelet cAMP-PDE or bovine brain PDE [35] than on bovine heart [10], lung [4] or aorta [35] PDEs.

One of the best ways to inhibit platelet function is to raise platelet cAMP levels [36, 37]. Flavonoids inhibit platelet function, probably through this mechanism [7–9]. In addition, flavonoids could inhibit platelet function partly through other mechanisms such as inhibition of platelet cyclooxygenase [9, 38], inhibition of platelet lipoxygenase [39–41] or modifications of Ca^{2+} transport [33, 42–46]. In previous reports, the most active flavonoids inhibited platelet function at concentrations of about 10 μM. AmAc is the most potent human platelet aggregation inhibitor in the flavonoid group. AmAc is a parent compound of Am the most potent cAMP-PDE inhibitor in the flavonoid group [4, 10, 35]. Biflavonoids represent a source of potent platelet function inhibitors and simple chemical modifications, such as acetylation, can greatly improve their pharmacological activity.

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