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NOVEL INHIBITORY ACTIONS ON PLATELET THROMBOXANE AND INOSITOLPHOSPHATE FORMATION BY XANTHONES AND THEIR GLYCOSIDES

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Abstract—Xanthones and their glycosides were tested for their antiplatelet activities in washed rabbit platelets. Tripteroside acetate and norathyriol acetate were the most potent inhibitors. Tripteroside acetate inhibited platelet aggregation and ATP release induced by ADP, arachidonic acid, platelet-activating factor (PAF), collagen, ionophore A23187 and thrombin. The IC_{50} values of tripteroside acetate toward arachidonic acid- (100 μM) and collagen- (10 μg/ml) induced platelet aggregation were 10 and 30 μg/ml respectively. It inhibited thromboxane B_2 formation of washed platelets caused by arachidonic acid, collagen, thrombin and ionophore A23187 and also that caused by the incubation of lysed platelet homogenate with arachidonic acid. Tripteroside acetate decreased the formation of inositolphosphate caused by thrombin, collagen and PAF, whereas it had no direct effect on fibrinogen–platelet interaction. It is concluded that xanthone derivatives inhibited platelet aggregation and release reaction by diminishing thromboxane formation and phosphoinositide breakdown.

Chinese herbs have been widely used as important remedies in oriental medicine. In recent decades, many biologically active constituents have been isolated and their pharmacological actions investigated. Xanthones and their glycosides are potential therapeutic agents isolated from the *Gentianaceae*, especially *Tripterospermum taiwanense* and *Tripterospermum lanceolatum* [1–3]. Xanthone derivatives have been reported to be effective as anti-allergics or bronchodilators and may be useful for asthma, while their glycosides have been claimed to have a remarkable central stimulant effect in mice and a depressant effect in rats [4–6]. In this study, we compared the antiplatelet activities of xanthone derivatives and also investigated their mechanisms of action.

MATERIALS AND METHODS

Materials. Tripteroside (I) was isolated from *T. taiwanense* [2] and lancerin (V), mangiferin and methyllanceolin from *T. lanceolatum* [1, 3]. Norathyriol (III) and 1,3,7-trihydroxyxanthone (VII) are aglycones of mangiferin and lancerin respectively [1, 2]. 1-Hydroxy-3,4,7,8-tetramethoxyxanthone is the O-methylated product of methyllanceolin. The chemical structures of these compounds and their acetylated derivatives (II, IV, VI, VIII, IX, X) are shown in Fig. 1. Collagen (Type 1, bovine achilles tendon), obtained from the Sigma Chemical Co. (St. Louis, MO) was homogenized in 25 mM acetic acid and then stored at −70°. Arachidonic acid, ADP, bovine serum albumin (BSA), indomethacin, EDTA (disodium salt), sodium citrate, luciferase-luciferin, Dowex-1 (100–200 mesh: ×8, chloride) resin and *myo*-inositol also were purchased from the Sigma Chemical Co. Thrombin (bovine) was obtained from the Parke Davis Co. (Detroit, MI), and dissolved in 50% (v/v) glycerol to give a stock solution of 100 NIH units/ml. Ionophore A23187 and platelet-activating factor (PAF) were obtained from Calbiochem-Behring (San Diego, CA). *Myo*[2-^{3}H]Inositol was purchased from Amersham (Amersham, U.K.). Thromboxane B_2 RIA kits were obtained from New England Nuclear (Boston, MA).

Platelet aggregation and ATP release reaction. Platelet suspension was obtained from EDTA-anticoagulated platelet-rich plasma (PRP) according to washing procedures described previously [7]. Platelet numbers were counted by a Coulter Counter (model ZM) and adjusted to 4.5×10^8 platelets/ml. The platelet pellets were finally suspended in Tyrode's solution containing (mM): NaCl (136.8), KCl (2.8), $NaHCO_3$ (11.9), $MgCl_2$ (2.1), NaH_2PO_4 (0.33), $CaCl_2$ (1.0) and glucose (11.2) with bovine serum albumin (0.35%). Aggregation was measured by the turbidimetric method [8] and assigned the absorbance of platelet suspension as 0% aggregation and the absorbance of platelet-free Tyrode's solution as 100% aggregation. ATP released from platelets was detected by the bioluminescence method as described by DeLuca and McElory [9]. Both the aggregation and release of ATP were measured simultaneously by a Lumi-aggregometer (Chrono-Log Co., U.S.A.) connected to two dual channel recorders. The platelet suspension was stirred at 1200 rpm. To eliminate the effect of the solvent on the aggregation, the final concentration of dimethyl sulfoxide (DMSO) was fixed at 0.5%.

Thromboxane B_2 assay. After challenging platelets

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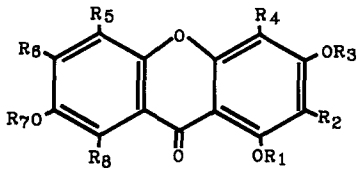

Ac: acetyl
Me: methyl
G : glucosyl
G*: glucosyl tetraacetate

		R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
I.	Tripteroside	H	H	H	H	H	OG	H	H
II.	Tripteroside acetate	Ac	H	Ac	H	H	OG*	Ac	H
III.	Norathyriol	H	H	H	H	H	OH	H	H
IV.	Norathyriol acetate	Ac	H	Ac	H	H	OAc	Ac	H
V.	Lancerin	H	H	H	CG	H	H	H	H
VI.	Lancerin acetate	Ac	H	Ac	CG*	H	H	Ac	H
VII.	1,3,7-Trihydroxyxanthone	H	H	H	H	H	H	H	H
VIII.	Mangiferin acetate	Ac	CG*	Ac	H	H	OAc	Ac	H
IX.	Methyllanceolin diacetate	Ac	H	Ac	OMe	H	H	Me	OMe
X.	1-Hydroxy-3,4,7,8-tetra-methoxyxanthone acetate	Ac	H	Me	OMe	H	H	Me	OMe

Fig. 1. Chemical structures of xanthones and xanthone glycosides.

with an aggregation inducer for 6 min, 2 mM EDTA and 50 μM indomethacin were added. Thromboxane B_2 was assayed by centrifugation in an Eppendorff centrifuge (model 5414) for 2 min, using the radioimmunoassay kit according to the procedure described by the manufacturer. In some cases, platelets (4.5×10^8/ml) were homogenized by ultrasonic disruption. The lysed platelet homogenate was incubated with arachidonic acid (100 μM) for 6 min, and thromboxane B_2 formation was determined by the same procedure described above.

Labeling of membrane phospholipids and measurement of the production of [^{3}H]*inositolphosphate.* The methods of Huang and Detwiler [10] and Neylon and Summers [11] were modified as follows. EDTA-PRP was centrifuged at 500 *g* for 10 min, and the platelet pellets were suspended in 700 μl of Ca^{2+}-free and BSA-free Tyrode's solution containing 75 μCi/ml of [^{3}H]inositol and 1 mM EDTA. After incubation for 2 hr at 37°, the platelets were collected by centrifugation (500 *g* for 4 min) and resuspended in Ca^{2+}-free Tyrode's solution. The reaction was carried out at 37° for 6 min with 1 ml of platelet suspension in a 3.5-ml cuvette with a stirring bar driven at 900 rpm. An equal volume of 10% (w/v) trichloroacetic acid was added to stop the reaction. After centrifugation at 1000 *g* for 10 min, 1 ml of supernatant fraction was pooled and trichloroacetic acid was removed by washing with 5×2 vol. of diethyl ether. The aqueous phase, containing the inositolphosphate, was adjusted to pH 7–8 and diluted to 4 ml with distilled water before application to a Dowex-1 ion-exchange column for separation of the inositolphosphates. All the experiments were carried out in the presence of 5 mM LiCl to inhibit inositolphosphate phosphatase. Because the levels of inositol bisphosphate and inositol trisphosphate were very low, we measured the inositolphosphate as an index of the total inositolphosphate formation.

RESULTS

The antiplatelet activities of xanthones and their glycosides were compared in collagen-induced aggregation of washed rabbit platelets. Among them,

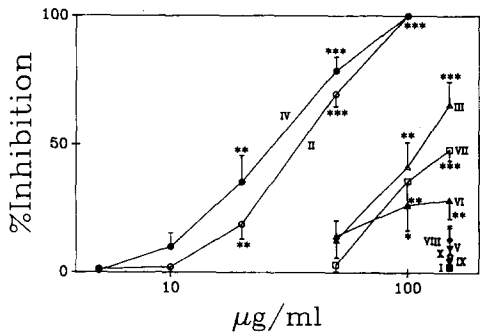

Fig. 2. Effects of xanthones and xanthone glycosides on the platelet aggregation induced by collagen. Washed rabbit platelets were incubated with various concentrations of each analogue for 3 min; then collagen (10 μg/ml) was added to trigger the aggregation. Percent inhibition is presented as means ±SE (N = 4). The numbers on the curves refer to the compounds listed in Fig. 1. Key: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ compared with the control value.

norathyriol acetate (IV) and tripteroside acetate (II) were the most potent inhibitors with minimal effective concentrations around 10 μg/ml and maximal effective concentrations at 100 μg/ml. Norathyriol (III), 1,3,7-trihydroxyxanthone (VII) and lancerin acetate (VI) were effective at concentrations higher than 50 μg/ml. Tripteroside (I), methyllanceolin diacetate (IX), 1-hydroxy-3,4,7,8-tetramethoxyxanthone acetate (X), lancerin (V) and mangiferin acetate (VIII) did not have a significant antiplatelet effect even at 200 μg/ml (Fig. 2).

Tripteroside acetate was then used in the following experiments to study the properties and mechanisms of the antiplatelet actions of xanthone derivatives. Tripteroside acetate (50 μg/ml) almost completely inhibited aggregation of washed rabbit platelets stimulated by ADP (20 μM), arachidonic acid (100 μM) and PAF, (2 ng/ml). It also inhibited collagen- (100 μg/ml) and ionophore A23187-(2 μM) induced aggregation markedly but inhibited thrombin-induced aggregation only slightly (Table 1). All these effects were concentration-dependent with

Table 1. Effect of tripteroside acetate on the platelet aggregation induced by ADP, arachidonic acid, PAF, collagen, thrombin and ionophore A23187

Inducer	Aggregation (%)	
	Control	Tripteroside acetate
ADP (20 μM)	80.2 ± 2.3 (4)	8.8 ± 1.5* (4)
Arachidonic acid (100 μM)	86.6 ± 1.4 (10)	5.6 ± 4.3* (10)
PAF (2 ng/ml)	92.0 ± 1.6 (4)	0.6 ± 0.5* (4)
Collagen (10 μg/ml)	90.0 ± 0.8 (10)	22.7 ± 5.2* (10)
Thrombin (0.1 units/ml)	93.9 ± 1.0 (9)	89.2 ± 1.5† (9)
A23187 (2 μM)	73.1 ± 1.1 (3)	24.2 ± 11.9‡ (4)

Platelets were preincubated with tripteroside acetate (50 μg/ml) or DMSO (0.5%) (control) at 37° for 3 min; then the inducer was added. Values are means ± SE (N). *–‡ Significantly different compared to the respective control: *$P < 0.001$, † $P < 0.05$ and ‡ $P < 0.01$.

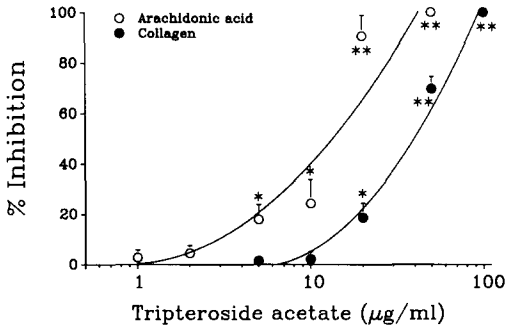

Fig. 3. Concentration–response curves of tripteroside acetate on the platelet aggregation induced by collagen and arachidonic acid. Washed rabbit platelets were incubated with various concentrations of tripteroside acetate for 3 min; then collagen (10 μg/ml, ●—●) or arachidonic acid (100 μM, ○—○) was added. Percent inhibition is presented as means±SE (N = 4). Key: (*) $P < 0.05$, and (**) $P < 0.001$ compared with the respective control value.

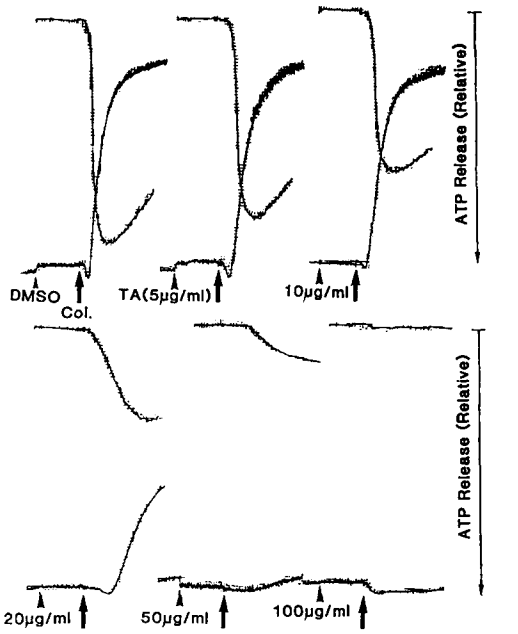

Fig. 4. Inhibitory effect of tripteroside acetate on platelet aggregation and ATP release induced by collagen. Washed rabbit platelets were incubated with various concentrations of tripteroside acetate (TA) or DMSO (0.5%) for 3 min; then collagen (10 μg/ml) was added to trigger the aggregation (upward tracings) and ATP release (downward tracings).

tripteroside acetate. Typical concentration–inhibition curves are shown in Fig. 3. The IC_{50} values of tripteroside acetate on arachidonic acid- and collagen-induced platelet aggregation were about 10 and 30 μg/ml respectively.

Meanwhile, tripteroside acetate also dose-dependently inhibited ATP release of the activated platelets, in parallel with its inhibitory effect on aggregation (Fig. 4). Shape change of platelets caused by inducers (e.g. collagen in Fig. 4), however, was not affected by tripteroside acetate even when the aggregation and release reaction of platelets were suppressed completely. A longer incubation of tripteroside acetate with washed platelets (for 10 min, instead of 3 min) did not cause more inhibition. The antiplatelet effect of tripteroside acetate could be washed out and the aggregability of platelets restored.

Thromboxane B_2 formation in washed rabbit platelets was measured 6 min after the aggregation inducers were added. Thromboxane B_2 formations caused by arachidonic acid, collagen, thrombin and ionophore A23187 were inhibited profoundly by tripteroside acetate (Table 2). ADP or PAF increased thromboxane B_2 in rabbit platelets only slightly; thus, the thromboxane B_2 formation in ADP- or PAF-treated platelets was not studied. Tripteroside acetate also inhibited thromboxane B_2 formation caused by the incubation of the lysed platelet homogenate and arachidonic acid. As shown in Table 3, tripteroside acetate (50 μg/ml) inhibited thromboxane B_2 formation by 65%, and indomethacin (20 μM) showed 86% inhibition on thromboxane B_2 formation under this condition.

Phosphoinositide breakdown is observed in platelets activated by many agonists [12, 13]. In this study, we found that thrombin (0.1 units/ml), collagen (10 μg/ml) and PAF (2 ng/ml) increased inositolphosphate formation 6.7 ± 0.2, 3.0 ± 0.1 and 3.1 ± 0.4 fold respectively (compared with resting

Table 2. Effect of tripteroside acetate on thromboxane B_2 formation induced by arachidonic acid, collagen, thrombin and ionophore A23187 in washed rabbit platelets

Inducer	Thromboxane B_2 (ng/ml)	
	Control	Tripteroside acetate
Arachidonic acid (100 μM)	646.1 ± 89.5	22.4 ± 1.8*
Collagen (10 μg/ml)	248.1 ± 23.9	8.1 ± 3.9*
Thrombin (0.1 units/ml)	160.4 ± 30.5	4.7 ± 1.7*
A23187 (5 μM)	219.9 ± 29.2	15.2 ± 5.3*

Tripteroside acetate (50 μg/ml) or DMSO (0.5%) (control) was preincubated with platelets at 37° for 3 min; then the inducer was added. Aggregation and thromboxane formation were terminated by EDTA (2 mM) and indomethacin (50 μM) 6 min after the addition of the inducer. Values are means ±SE, N = 4.

* $P < 0.001$ compared with the respective control.

Table 3. Inhibitory effect of tripteroside acetate on thromboxane B_2 formation caused by arachidonic acid in lysed platelet homogenate

	Thromboxane B_2 (ng/ml)
Resting	2.2 ± 0.3
DMSO + AA	30.4 ± 2.1
Tripteroside acetate + AA	11.0 ± 0.9*
Indomethacin + AA	4.3 ± 0.8*

Lysed platelet homogenate was preincubated with tripteroside acetate (50 μg/ml), indomethacin (20 μM) or DMSO (0.5%) for 1 min; then arachidonic acid (AA, 100 μM) was added and the mixture was incubated for 6 min. EDTA (2 mM) and indomethacin (50 μM) were used to terminate the reaction. Values are means ±SE, N = 4.

*$P < 0.001$ compared with the control (DMSO).

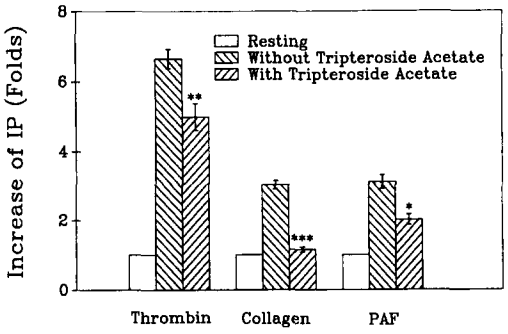

Fig. 5. Inhibitory effect of tripteroside acetate on the formation of inositolphosphate in washed rabbit platelets caused by thrombin, collagen and PAF. [^{3}H]Inositol-labeled platelets were incubated with thrombin (0.1 units/ml) in the absence of calcium or with collagen (10 μg/ml) or PAF (2 ng/ml) in the presence of calcium (1 mM) for 6 min. Multiple increases in inositolphosphate (IP) are presented as means ±SE (N = 4). Key: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ compared with the respective control (without tripteroside acetate).

platelets). The formation of inositolphosphate by these three agonists was decreased to 5.0 ± 0.4, 1.2 ± 0.1 and 2.0 ± 0.2 fold, respectively, by tripteroside acetate (50 μg/ml) (Fig. 5). PAF still induced a 2.7 ± 0.4 (N = 3) fold increase of inositolphosphate formation even in the presence of indomethacin (20 μM).

Tripteroside acetate did not affect fibrinogen-induced aggregation of elastase-treated human platelets (data not shown), indicating that it did not interfere with the fibrinogen–platelet interaction.

DISCUSSION

The hydrolysis of phosphoinositides (PI) by phospholipase C is proposed to be a very important step for signal transduction during the initial stimulation of platelet membrane by many agonists or aggregating agents [14, 15]. This process generates two active products, diacylglycerol and inositol trisphosphate. Diacylglycerol activates protein kinase C, leading to protein phosphorylation and release reaction. Inositol trisphosphate triggers calcium mobilization from intracellular compartments [16]. The rise of intracellular calcium is very important for both the release reaction and the activation of phospholipase A_2 [17], which is a rate-limiting enzyme for the generation of arachidonic acid.

The inositolphosphate formation caused by thrombin, collagen and PAF was inhibited by tripteroside acetate. This indicates that tripteroside acetate may suppress platelet aggregation, partly by inhibiting the breakdown of phosphoinositides, and also implies that the site of action of tripteroside acetate may be at a step after receptor activation. Although thromboxane A_2 could activate PI hydrolysis, the decrease of PI breakdown was not due to the inhibition of thromboxane formation because PAF does not cause thromboxane formation in rabbit platelets [7]. We also demonstrated the PAF-induced inositolphosphate formation in washed rabbit platelets

was not affected significantly by indomethacin. Furthermore, since inositolphosphate formation caused by thrombin, in this study, took place in the absence of exogenous calcium, the former inositolphosphate formation was decreased by tripteroside acetate independent of thromboxane generation.

Formation of thromboxane B_2, a stable metabolite of thromboxane A_2, induced by arachidonic acid, collagen, thrombin or ionophore A23187 was inhibited markedly by tripteroside acetate. This indicates that the antiplatelet effect of tripteroside acetate was due, at least partly, to the inhibition of thromboxane A_2 formation. Thromboxane A_2 is also important for mediating the release reaction of platelets [18]. This explains why the ATP release and aggregation were supressed in parallel. Although PI turnover is believed to be an earlier step before thromboxane synthesis, the inhibition of thromboxane formation caused by tripteroside acetate did not result entirely from the inhibition of PI hydrolysis. For example, thromboxane B_2 formation caused by the incubation of the lysed platelet homogenate and arachidonic acid was also inhibited by tripteroside acetate. Tripteroside acetate may exert its inhibitory action primarily on both thromboxane and inositolphosphate formations. Both actions are not necessarily directly linked.

Examining the structure–activity relationship, we found that a xanthone skeleton with three hydroxylations (1,3,7-trihydroxyxanthone, VII) was effective. Glucosyl substitution (e.g. tripteroside, I) decreased the antiplatelet activity. This could be due to the increase of the hydrophilic property of the molecule and the decrease of its penetration into the target sites of platelets. Thus, acetylation of xanthone (IV) or xanthone glycoside (II) greatly enhanced the inhibitory action on platelet aggregation. Therefore, the much more active antiplatelet agents from the derivatives of xanthones should be explored.

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