Original article

Interaction between ticlopidine or warfarin or cardioaspirin with a highly standardized deterpened Ginkgo biloba extract (VR456) in rat and human

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Abstract. Ginkgo biloba is available in Europe as an over-the-counter drug and it is reported to cause hemorrhage when co-administered with other anti-platelet agents. We set out to study the interactions of ticlopidine with Ginkgo biloba extract or VR456, a new highly standardized deterpened extract from Ginkgo biloba leaves. Male Wistar rats were used to study the effects of ticlopidine (50-100 mg/kg/day), given alone and in combination for 5 days with Ginkgo biloba extract (50 mg/kg/day) or VR456 (50 mg/kg/day), on bleeding time and ex vivo ADP-induced platelet aggregation measurements. In addition, human studies were performed with the compounds under investigation. Combined treatment of ticlopidine and undeterpened Ginkgo biloba extract increased anti-platelet effect and prolonged the bleeding time in the rat. On the contrary, the combination treatment of ticlopidine and VR456 increased anti-platelet effect but did not prolong bleeding time. Moreover, daily administration of 360 mg of VR456 for 14 days to ticlopidine-treated humans did not highlight any unwanted effect and did not alter PT/INR and PTT parameters. Same results have been also obtained in warfarin or in cardioaspirin-treated patients. These data point out the clear role played by the terpenoid, PAF-antagonist fraction of Ginkgo biloba extract in affecting bleeding risk in anticoagulant-treated subjects and suggest VR456 as a possible option treatment in geriatric people subjected to anticoagulant treatment where the use of standard Ginkgo biloba extracts are discouraged. (www.actabiomedica.it)

Key words: VR456, Ginkgo biloba, Ticlopidine, Warfarin, Cardioaspirin, Rat, Human

Introduction

Ticlopidine is one of the most worldwide used anti-platelets drugs. Being considered superior to aspirin as anti-platelet agent, it is widely used in transient ischemic cerebral infarction, stroke and ischemic myocardial infarction. It is described to block the binding of fibrinogen determining platelet aggregation inhibition (1,2).

A highly standardized Ginkgo biloba leaves extract, titred as 24% ginkgoflavonglucoside, 6% terpenes and < 5 ppm in ginkgolic acids is currently used to treat and prevent peripheral and central vascular disorders mainly affecting the elderly (3). The same extract has been the issue of some case reports and studies underlining its possible risk in inducing bleeding episodes when associated with anti-coagulant drugs like non-steroidal anti-inflammatory drugs, warfarin, ticlopidine and analogues. Nevertheless Authors still debate about the consistency of this effect, since a possible risk cannot be excluded (4,5). Ginkgo biloba extract is known to prolong bleeding time, when associated to anticoagulant drugs, and this could be linked to its terpenic fraction, because it is mainly constituted by a mixture of ginkgolide A, B, C, J and M described all to be endowed with a strong platelet

activating factor (PAF)-antagonist action (6). Since it cannot be excluded that the neuroprotective effect of the extract is potentially due to the ginkgoflavonglucoside fraction and not to the terpenoid one (7), we have developed a deterpened (less than 0.1%) form of Ginkgo biloba extract (VR456) according to the method shown in EPO 800222. The purpose of our study was to verify if terpenes absence reduces the bleeding effect when the product is associated to anticoagulant drugs, in order to later verify if the product still maintains its clinical neural protective action mainly observed in the elderly.

Materials and Methods

Animal

Male Wistar rats (Charles River Laboratories, Calco, Lecco, Italy) weighing 250-275 g were used. The animals were housed in a conditioned environment (22 ± 1 °C, 55 ± 5% relative humidity, 12-h light/dark cycles) and were fed standard laboratory chow and water. This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised in 1996).

Materials

A standardised Ginkgo biloba leaves extract was prepared applying the procedure described in patent EP 0 360556 B1. The extract (batch n° 8047, provided by Indena S.p.A. – Milan, Italy) was analysed according to Ph. Eur. for ginkgoflavonglucosides content, 25.1% w/w, and ginkgoterpenes content, 8.9 % w/w (as sum of bilobalide, ginkgolide A, ginkgolide B, ginkgolide C and ginkgolide J).

Ginkgo biloba terpenes-free extract, VR456, was obtained according to patent EPO 800222, having a ginkgoflavonglucosides (Fig. 1) content of 23.3 % w/w and a total ginkgoterpenes (Fig. 2) content of 0.03% w/w (batch n° 757/20/D – Indena S.p.A.)

GinkASA, the finished product containing 120 mg of VR456 per tablet, notified to the Italian Minister of Health as nutritional supplements in 2008 and used for the pilot trial in human, was manufactured by S.I.I.T. (Trezzano S/N, Milan, Italy)

Others drugs and products used during the study were: ticlopidine hydrochloride (purity > 99%, batch N° 017K1282), ADP sodium salt (Sigma-Aldrich, Milan, Italy), thiopentone sodium (Pentothal®; Abbott (Campoverde, Latina, Italy), Ticlopidina Dorom (Teva, Italy), Coumadin (Cipla, Italy) and Cardioaspirin (Bayer, Italy).

Experimental design in rats

Forty-two rats were randomly assigned to seven groups (n = 6 per group) according to the following protocol: group 1, vehicle (1% carboxymethylcellulose); groups 2 and 3, ticlopidine (50 and 100 mg/kg); groups 4, Ginkgo biloba extract (50 mg/kg); groups 5, VR456 (50 mg/kg); group 6, ticlopidine (50 mg/kg) plus Ginkgo biloba extract (50 mg/kg); group 7, ticlopidine (50 mg/kg) plus VR456 (50 mg/kg). Vehicle, ticlopidine alone, ticlopidine plus Ginkgo biloba or ticlopidine plus VR456 were orally administered by gavage (2 mL/kg) to rats once a day for 5 consecutive days. All experiments were performed 3.5-4 h after the final administration.

Indirect systolic blood pressure (BP) and heart rate (HR) measurements in rats

Systolic BP were recorded immediately before (0-day) and after a 5-day (5-day) oral treatment with the compounds under investigation. Systolic BP was indirectly measured by tail-cuff plethysmography (mod 8006; Ugo Basile, Comerio, Varese, Italy) in unanesthetized rats that had been placed in a warm cupboard (30 °C) for 20 min. Systolic BP values for individual rats were obtained from the average of 3 consecutive measurements and were considered valid only when these readings did not differ by more than 5 mmHg. At the same time, HR was measured from the arterial pulse wave.

Determination of bleeding time in rats

Bleeding time was measured according to the procedure described by Stella et al. (8). Rats were

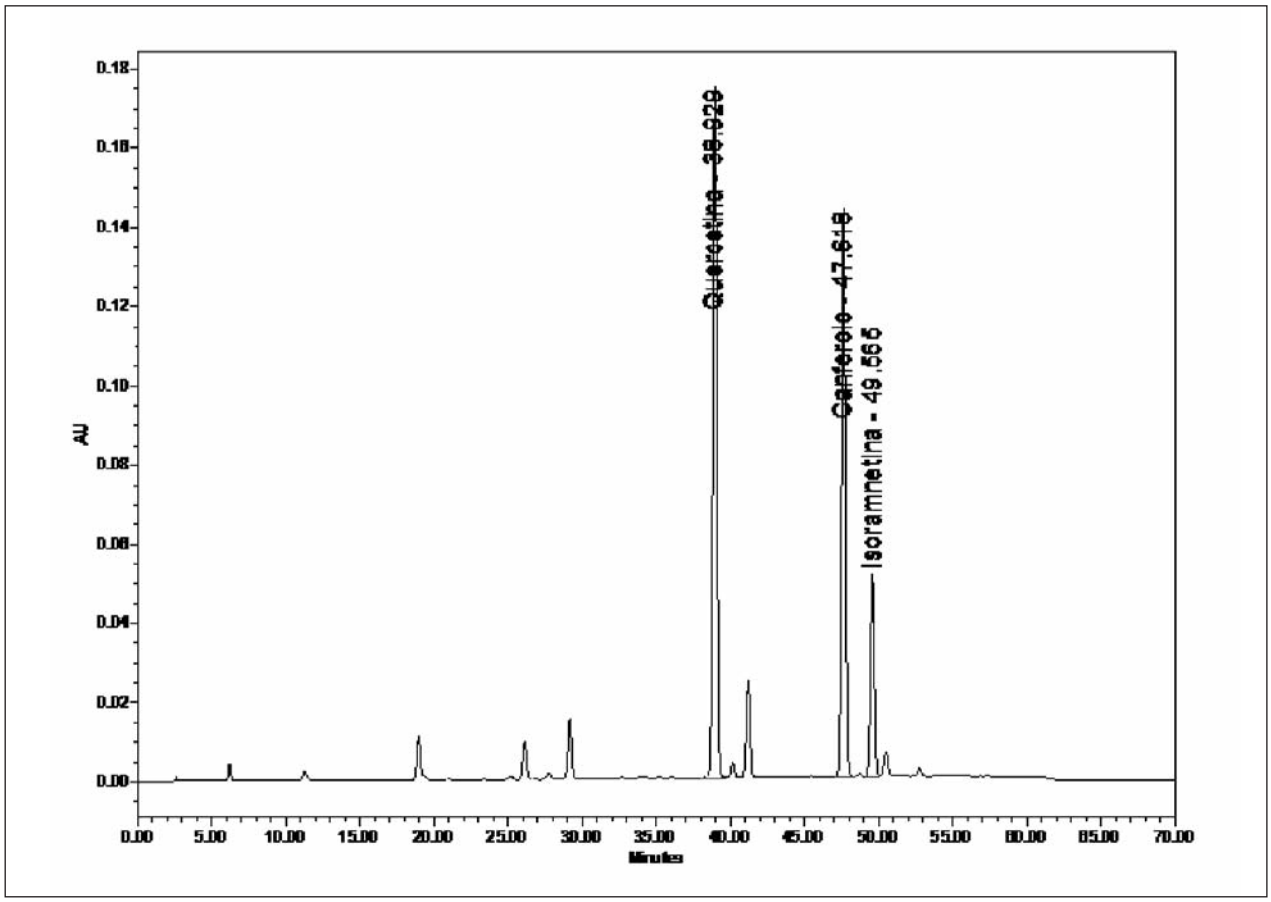

Figure 1. Ginkgoflavonglucosides HPLC profile of VR456, after acidic hydrolysis – method adapted from Ph. Eur. 6.2

anesthetized with 50 mg/kg i.p. thiopentone sodium (Pentothal®), and fixed in supine position on a temperature-controlled (37 °C) heating-table. To assess bleeding time, the template device was longitudinally applied between the median and lower dorsal portion of the tail (between 4-5 cm from the end of the tail), avoiding the artery and large vein. Blood from the wound was carefully collected onto filter paper every 30 sec. Bleeding times were recorded as the interval between incision and bleeding arrest. The time until bleeding stops is determined within a maximum observation time of 30 min.

Measurement of "ex vivo" platelet aggregation in rats

At the end of the bleeding time measurements, and exactly 4 h after the last treatment with the compounds under investigation, 5 ml of peripheral venous blood was collected from the vena cava into a plastic (polystyrene) syringe containing 3.8% (w/v) trisodium citrate (1:9 volume of blood) as an anticoagulant. Platelet-rich plasma (PRP) was prepared through centrifugation at 150 x g for 15 min at room temperature. Platelet-poor plasma (PPP) was obtained through centrifugation of the remaining blood at 1800 x g for 20 min at 20 °C. Platelet counts in PRP were adjusted to 3 x 108 platelets/ml by adding PPP. Duplicate samples of 450 µL PRP from drug-treated and vehicle control rats were incubated at 37 °C for 3 min in the aggregometer with continuous stirring at 1000 r.p.m. (Born turbidimetric technique), and then stimulated with 50 µL of 10 µM adenosine diphosphate (ADP) to induce platelet aggregation. Changes in light transmission were recorded for 7 min after stim-

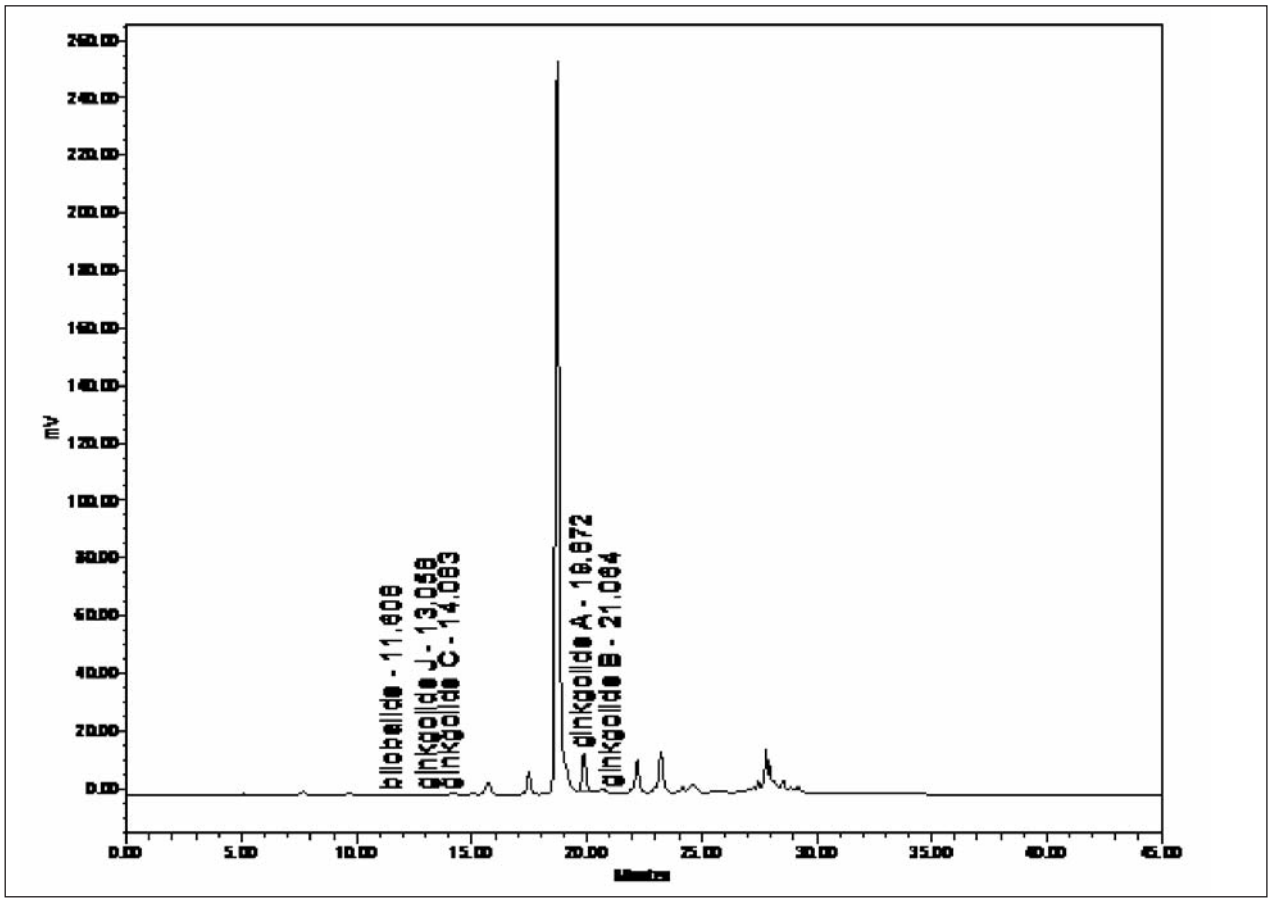

Figure 2. Terpenes HPLC profile of VR456,– method adapted from Ph. Eur. 6.2

ulation with ADP, and the degree of aggregation was monitored using a dual-channel Elvi 840 aggregometer (Elvi Logos, Milan, Italy) interfaced with a personal computer.

Human studies

Patients, after signing their approval for the proposed treatment, have been enrolled at the Clinic "Terme di Fontecchio" (Perugia, Italy) according to the following criteria. Inclusion criteria: age between 55 and 85, treatment with ticlopidine (250 mg; twice/day) or with warfarin (6-9 mg/die) or cardioaspirin (100 mg/die) since not less than 3 months (for ticlopidine) or since not less than 4 years (for warfarin and cardioaspirin), uncritical and stabilized myocardial ischemia, atheromatous carotid or chronic cerebral ischemia with transitory ischemic attack episodes and memory impairments (ticlopidine), cardiac valves substitution with mechanical prosthesis (warfarin), or coronary by-pass (cardioaspirin). Exclusion criteria: coagulation pathologies, recent events of ischemic myocardial attack, strokes, cerebral ictus, chronic kidney insufficiency in kidney dialysis, recent episodes of unexpected bleeding, active gastro and/or enteric ulcers, colon polyposis and diverticulosis with or rectal bleeding, concomitant treatment with antibiotics, non-steroidal anti-inflammatory drugs, cimetidine and analogues, barbiturates, oral contraceptives, hormonal replacement therapy, vitamin K. Patients have been instructed to avoid foods containing vitamin K or ingredients described to affect coagulation parameters such us: cow and pork liver, green tea, broccoli, soy, chick peas, cabbages, turnips. Without

stopping ticlopidine, warfarin or cardioaspirin, patients have been administered with VR456 at the dose of 360 mg/day (120 mg every 8 h) for 14 days and have been plasma monitored at 0, 7 and 14 days in terms of prothrombin time-international normalized ratio (PT-INR) and activated partial thromboplastin time (APTT), being the first parameter established by the World Health Organization to standardize results from all over the world as regards to PT (9) and the second parameter showing the measure of the functionality of both the intrinsic and common pathway of the coagulation cascade (10); or with VR456 at the dose of 120 mg/day for 6 months (still ongoing) with plasma monitoring every 4 weeks for PT-INR.

Statistical Analysis

Result are presented as mean ± SEM. The differences between the treatment groups were compared through the unpaired t test or one-way ANOVA, followed by the Student-Newman-Keuls post hoc test for multiple comparisons. $P<0.05$ was considered as statistically significant.

Results

As regards to the systolic BP and HR evaluation in conscious rats, oral administration of ticlopidine, Ginkgo biloba extract and VR456 given either alone or in combination for 5 consecutive days, was well tolerated by rats in all experimental groups. At the end of the treatment they appeared healthy, with no significant changes in systolic BP and HR (Table 1).

As regards to the bleeding time evaluation in rats, according to the critical assessment of this method, numerous variables which can influence rodent's bleeding time measurements are present (11): position of the tail (horizontal or vertical), the environment (air or saline), temperature, anesthesia, procedure of injury (simplate method, transection). All these variables are responsible for the different results reported in literature on compounds like aspirin and heparin under different assay condition (8, 12). Furthermore, it is impossible to exactly transect one blood vessel, because the transected tail region consists of a few major arteries and veins with mutual interaction between one another. Furthermore, in our experimental conditions, when 50 and 100 mg/kg ticlopidine were administered to rats for 5 consecutive days, the bleeding time was increased 1.4-fold and 2.2-fold ($P<0.001$), respectively (Fig. 3), as compared to that observed in vehicle-treated animals (481.8 ± 41.6 sec). The combination treatment of 50 mg/kg ticlopidine with 50 mg/kg Ginkgo biloba extract, but not with 50 mg/kg VR456, significantly ($P<0.001$) prolonged the bleeding time than 50 mg/kg ticlopidine alone (Fig. 3).

Table 1. Systolic blood pressure (BP) and heart rate (HR) before (time 0) and after 5 days (5-day) oral administration in the rat of ticlopidine, Ginkgo biloba extract and VR456 given alone or in combination

Treatment		BP (mmHg)		HR (beats/min)	
		0-day	5-day	0-day	5-day
Vehicle		120 ± 9	125 ± 12	326 ± 21	330 ± 14
Ticlopidine	50 mg/kg (A)	122 ± 12	118 ± 10	340 ±14	324 ± 17
Ticlopidine	100 mg/kg	125 ± 8	115 ± 13	335 ± 24	312 ± 21
Ginkgo biloba	50 mg/kg (B)	124 ± 10	116 ± 13	336 ± 31	322 ± 28
VR456	50 mg/kg (C)	120 ± 13	122 ± 8	329 ± 18	334 ±23
A + B		123 ± 16	117 ± 10	332 ± 23	318 ± 16
A + C		120 ± 7	118 ± 12	341 ± 20	334 ± 15

Data are mean + SEM (n = 6 per group). Vehicle and compounds under investigations were orally administered by gavage once a day for 5 consecutive days.

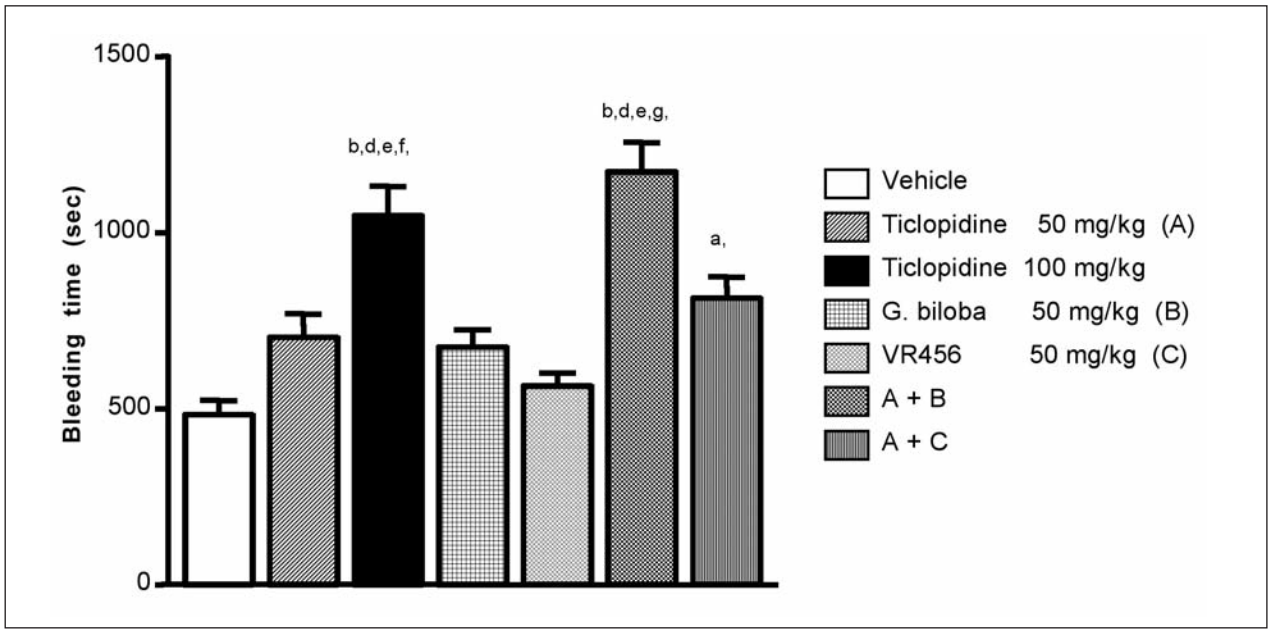

Figure 3. Bleeding time determined after 5 days oral administration of ticlopidine, Ginkgo biloba extract and VR456, given alone or in combination, in the rat. Each bar represents the mean ± SEM (n = 6 per group). a P<0.01 and b P<0.001 vs. vehicle; c P<0.05 and d P<0.001 vs. C; e P<0.001 vs. B; f P<0.01 and g P<0.001 vs. A; h P < 0.05 and ì P < 0.001 vs. A + C

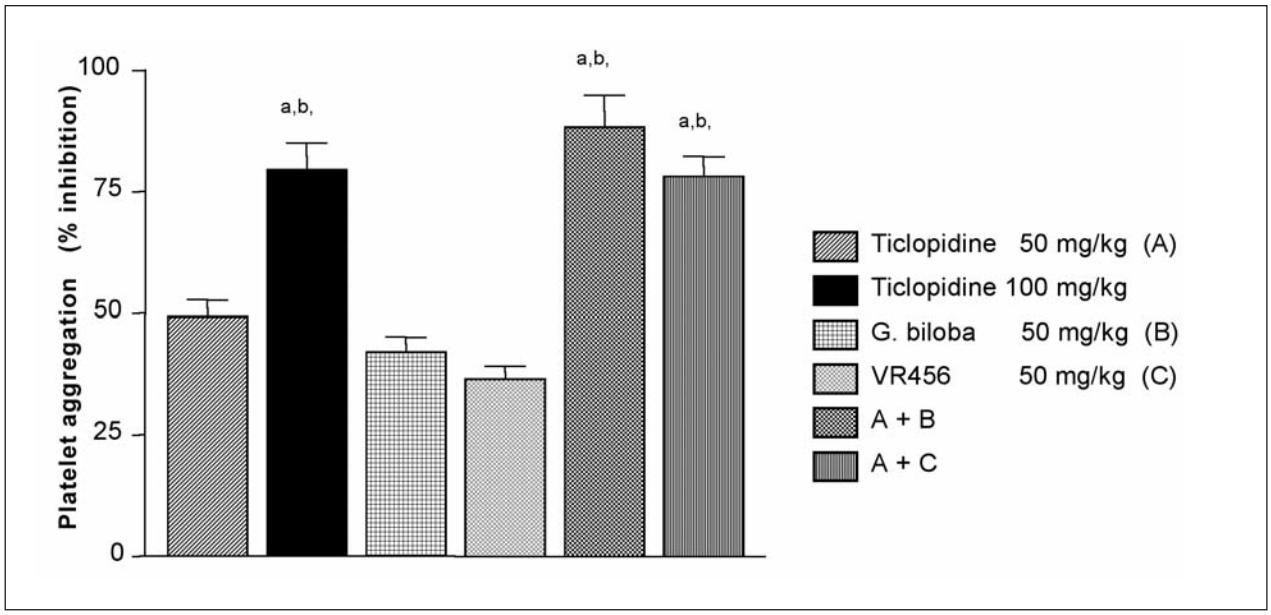

Figure 4. Percent inhibition of ex vivo adenosine diphosphate (10 μM)-induced platelet aggregation after 5 days oral administration of ticlopidine, Ginkgo biloba extract and VR456, given alone or in combination, in the rat. Each bar represents the mean ± SEM (n = 6 per group). a P < 0.001 vs. C; b P < 0.001 vs B; c P < 0.01 vs. A

As regards to platelet aggregation in rats, a dose-dependency of ticlopidine treatment on antiplatelet effects was observed. The oral administration of ticlopidine (50 and 100 mg/kg) for 5 days inhibited the ex vivo platelet aggregation by about 49.3% and 79.5%, respectively (Fig. 4). When 50

mg/kg ticlopidine and 50 mg/kg Ginkgo biloba extract were combined, the inhibitory effect (88.3%) was comparable to a single treatment of 100 mg/kg ticlopidine, and similar results were obtained when 50 mg/kg ticlopidine was combined with 50 mg/kg VR456 (78.2%) (Fig. 4).

As regards to PT-INR and APTT evaluation in humans, as shown in Table 2 nevertheless the concomitant administration of ticlopidine (500 mg/die) and VR456 (360 mg/die), PT-INR and APTT are not substantially modified and they keep constant along the 14 days of treatment showing no assumable coagulation differences due to the double therapy. Moreover, no visible bleeding occurred and no unwanted effects were registered along the study.

As shown in Table 3 and 4, not only the concomitant administration of ticlopidine and VR456 looks to be safe. As a matter of fact PT-INR does not significantly modify also both in patients taking warfarin (6-9 mg/die since 2005) and in patients taking cardioaspirin (100 mg/die since 2006). Evaluation lasted 6 months (and it is still ongoing in November 2009) and the patients were administered with the above reported drug along with VR456 at 120 mg/die.

Discussion

The anti-platelet and antithrombotic effects were observed in thrombosis-induced as well as normal rats

Table 2. Prothrombin Time-International Normalized Ratio (PT-INR) and Activated Partial Thromboplastin Time (APTT) determined at different time in three patients concomitantly treated with ticlopidine (500 mg/die) and VR456 (360 mg/die).

Patients	PT-INR			APTT		
	Time (days)			Time (days)		
	0	7	14	0	7	14
P.M., male, 69 years	1.08	1.10	1.16	41.0	41.6	40.3
M.A., male, 70 years	1.15	1.25	1.30	43.1	42.1	46.1
C.M., female, 72 years	1.18	1.36	1.42	41.2	40.8	41.5

Table 3. Prothrombin Time-International Normalized Ratio (PT-INR) determined at different time (May-October 2009) in three patients concomitantly treated with warfarin (6-9 mg/die since 2005 due to cardiac valves substitution with mechanical prosthesis) along with VR456 (120 mg/die).

Patients	PT-INR					
	Time (weeks)					
	0	4	8	12	16	20
M.B., female, 72 years	3.72	3.83	3.25	3.34	3.79	3.42
A.G., male, 75 years	3.65	3.45	3.21	3.64	3.18	3.35
D.P., male, 69 years	3.75	3.65	3.86	3.24	3.52	3.37

Table 4. Prothrombin Time-International Normalized Ratio (PT-INR) determined at different time (May-October 2009) in three patients concomitantly treated with cardioaspirin (100 mg/die since 2006 due to coronary by-pass) along with VR456 (120 mg/die).

Patients	PT-INR					
	Time (weeks)					
	0	4	8	12	16	20
L.F., male, 63 years	2.50	2.23	2.52	2.54	2.62	2.51
M.S., male, 69 years	2.31	2.37	2.59	2.34	2.19	2.33
A.C., female, 62 years	2.75	2.69	2.54	2.49	2.42	2.38

by the oral administration of ticlopidine alone or the combination of ticlopidine with Ginkgo biloba extract or VR456. Our results showed that the combination treatment of ticlopidine and Ginkgo biloba extract increased anti-platelet effect and prolonged the bleeding time. On the contrary, the combination treatment of ticlopidine and VR456 increased antiplatelet effect but the bleeding time was in the same range obtained with ticlopidine or VR456 given alone. It is not surprising that nevertheless the total absence of the Ginkgo biloba PAF-antagonist fraction, VR456 demonstrates a clear anti-platelet activity. In fact, most (procyanidolic oligomers, anthocyanins, etc.) of the polyphenols show the same action (13) without anyway being reported to cause unexpected bleeding in humans when co-administered along with anticoagulant drugs. Removing from Ginkgo biloba extract the PAF-antagonist terpenes fraction does not mean to take off the ginkgoflavonglucoside one. Being this last constituted by natural polyphenols, VR456 keep on producing an in vitro anti-platelet activity.

Even if preliminary, due to the low number of patients enrolled (n = 9), and "uncontrolled", due to lacking of ethical consensus on behalf of the Hospital responsible for the study who denied the possibility to administer Ginkgo biloba extract to ticlopidine or warfarin or cardioaspirin treated-patients, the administration of 120 mg/die or 360 mg/die of VR456, being this last dose 3 times higher than the normal dose used for Ginkgo biloba extract, in people administered with ticlopidine (500 mg/die) or with warfarin (6-9 mg/die) or with cardioaspirin (100 mg/die) does not create any appreciable variation in terms of PT-INR and, when measured, APTT parameters. These data suggest a possible use of VR456, instead of Ginkgo biloba extract, in people affected by peripheral and central vascular disorders already in therapy with anticoagulant drugs.

In conclusion, larger numbers need to be evaluated in terms of bleeding risk to confirm what we have observed in order to start a trial to evaluate the clinical effectiveness of VR456 in people affected by vascular (peripheral and/or central) disorders like claudicatio intermittens, Raynoud's syndrome, dementia, and Alzheimer's.

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