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Effects of fibrinogen receptor antagonist GR144053F and aurintricarboxylic acid on platelet activation and degranulation

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Abstract

Activated blood platelets play crucial role in restenosis due to their fundamental significance in thrombus formation. Therefore, platelets are attractive targets for the inhibition with a variety of antagonists. In this study, we present direct evidence that GR144053F [non-peptide antagonist of glycoprotein IIb-IIIa complex (GPIIb-IIIa)] inhibits activation and degranulation of human platelets, and opposes the action of aurintricarboxylic acid (ATA), the antagonist of von Willebrand factor, which augments platelet secretion. The effects of both drugs on platelet function were monitored by using various instrumental methods. Platelet-rich plasma and whole-blood aggregation was measured by using ADP and collagen as agonists. Platelet degranulation was assessed based on the expression of surface membrane activation markers: P-selectin, glycoprotein Ib, and activated GPIIb-IIIa complex. Measurements of closure time with platelet function analyzer PFA-100™ enabled us to reason on primary hemostatic capacity and reflected both aggregability and adhesiveness. GR144053F markedly reduced primary hemostatic platelet response (IC_{50} = 114.0 ± 9.6 nM) under conditions that closely mimicked natural blood flow in circulation, and inhibited aggregation in platelet-rich plasma (IC_{50} = 17.7 ± 7.0 nM). It was equally potent inhibitor of platelet activation, degranulation, fibrinogen binding, platelet consumption, and aggregate formation. Also, ATA was efficient in inhibition of platelet aggregation and adhesion (by up to 50% at 100 μM), but the combined action of both drugs on primary haemostatic capacity was not additive. GR144053F suppressed the activating effects of ATA on platelet degranulation and secretion. Overall, our data indicate that GR144053F is not only the efficient blocker of fibrinogen binding to GPIIb-IIIa, but also hampers platelet degranulation and may attenuate the activating effects of ATA.

Keywords: GPIIb-IIIa antagonist; Fibrinogen receptor antagonist (GR144053F); von Willebrand factor antagonist; GPIb; Aurintricarboxylic acid (ATA); Platelet activation

1. Introduction

Because blood platelets are known to participate in a plethora of clinical life-threatening disorders, including cardiovascular and cerebrovascular diseases, platelet membrane receptors have become targets for a variety of antagonists tested in both the *in vitro* studies and clinical trials. Among various receptors, which underlie physiological response of blood platelets to stimuli, the heterodimers GPIIb-IIIa ($\alpha_{IIb}\beta_3$) and GPIb-IX-V seem to be of a special significance.

General action of the GPIIb-IIIa antagonists is to block the receptor on virtue of their stereochemical structure, which leads to the inability of activated platelets to bind fibrinogen and other adhesive ligands, and further prevents platelet aggregation and impedes thrombus formation [1]. GR144053F, a non-peptide mimetic of the RGD-sequence, has been shown to be a potent inhibitor of GPIIb-IIIa receptor in thrombosis models in animals [2–4], as well as the efficient blocker of GPIIb-IIIa-dependent platelet aggregation in model systems [5], although it was not accepted for therapy in humans.

Among a variety of compounds tested as the inhibitors of

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Abbreviations: ACD, citric acid/trisodium citrate/glucose (recipe A), blood anticoagulant; ATA, aurintricarboxylic acid; CT_{CADP}, closure (occlusion) time determined with the use of collagen/ADP cassettes in PFA-100; GPIb-IX-V, a complex of glycoproteins Ib, IX, and V, a vWF receptor; GPIIb-IIIa, a complex of glycoproteins IIb and IIIa, a fibrinogen receptor; IC_{50}, the concentration of an inhibitor, at which 50% of the maximal estimated inhibition occurred; the extent of maximal inhibition was calculated based on the mathematical resolving of the equation describing the inhibition of platelet aggregation; PE, R-phycoerythrin; PFA-100™, platelet function analyzer; PRP, platelet-rich plasma; RGD, Arg-Gly-Asp; PBS, phosphate-buffered saline; TRAP, thrombin receptor activating peptide, SFLLRNPNDKYEPF; and vWF, von Willebrand factor.

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GPIb-mediated platelet activation, ATA has been shown to inhibit ristocetin-vWF-induced platelet aggregation in a dose-dependent manner [6, 7], and has been proven to be advantageous in the animal models of induced stenosis and vessel injury [8–15].

Very little is known about the impact of GR144053F and ATA on the initial processes of platelet activation, such as platelet degranulation and granule secretion. Therefore, our aim in this study was to define the roles of both drugs in the intraplatelet α-granule secretion and platelet release, as well as their effects on a platelet-derived primary hemostatic capacity of whole blood.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

All chemicals were from Sigma unless stated otherwise. Tubes for blood collection containing 0.105 M sodium citrate (Vacutainer tubes) were from Becton Dickinson. Bovine thrombin for flow cytometry was from Dade Behring. Adenosine 5′-diphosphate, collagen fibrils (type I) from equine tendons and arachidonic acid for whole-blood aggregometry were from Chrono-Log. Collagen/ADP cartridges for PFA-100™ closure time measurements were from Dade Behring. GR144053F (4-[4-[4-(aminoiminomethyl]-1-piperazinyl]-1-piperidineactetic acid, hydrochloride trihydrate) was a kind gift from Glaxo Wellcome. Mouse monoclonal antibodies anti-human platelet GPIIIa (anti-CD61, fluorolabeled with fluorescein isothiocyanate, CD61/FITC), mouse monoclonal antibodies anti-human platelet P-selectin (anti-CD62, fluorolabeled with phycoerythrin, CD62/PE), mouse isotype IgG_1 (fluorolabeled with R-phycoerythrin (PE)), FACSFlow (optimized PBS for cell preparation and washing), and CellFix (phosphate buffered fixative containing 10% v/v formaldehyde and 1% w/v sodium azide) were from Becton Dickinson. Mouse monoclonal antibodies anti-human platelet GPIb (anti-CD42b, fluorolabeled with phycoerythrin, CD42b/PE) were from Dako. Human fibrinogen labeled with Oregon Green was from Molecular Probes. Water used for solution preparation and glassware washing was passed through an Easy Pure UF water purification unit (Thermolyne Barnstead).

2.1.2. Subjects

Totally, 38 healthy volunteers (19 men and 19 women) aged 22–53 yr (34 ± 10 yr) were studied. None of the selected individuals had a history suggesting any underlying hemostatic disorder, and within 10 days prior to the study none of them reported to have taken aspirin or any other drug that is known to affect platelet function. Blood was collected under the guidelines of the Helsinki Declaration for human research and the study was approved by the committee on the Ethics of Research in Human Experimentation at Medical University of Lodz.

2.2. Blood collection

Blood from each of the enrolled subjects was collected by dripping from 18-gauge needle placed in a peripheral vein into a tube containing either 0.105 M sodium citrate (the final citrate:blood ratio 1:9 v/v) or ACD (for the flow cytometry experiments with PAC-1 antibody; the final citric acid/trisodium citrate/glucose (recipe A), blood anticoagulant (ACD):blood ratio was 1:7 v/v). Blood was withdrawn with a special caution to avoid undesirable artefactual platelet activation: donors rested for 20–30 min prior to blood withdrawal, the first 0.5 mL was discarded before the collection of 5 mL of blood used for further platelet function assays. All the measurements of platelet reactivity were performed within 1 hr after blood withdrawal.

2.3. GR144053F and ATA preparation and handling

The 100 μM stock solution of GR144053F was prepared in 0.85% w/v saline, portioned and stored frozen in small aliquots. The working solutions 1–10 μM were prepared in 0.85% w/v saline immediately before the experiment and used within 15 min. ATA working solution (unfractionated aurintricarboxylic acid, triammonium salt) was prepared in 0.85% w/v saline immediately before experiments and used at the final concentrations of 100, 200, and 500 μM.

2.4. Turbidimeric aggregation assay

Platelet aggregation was monitored according to the microplate method [16] in platelet-rich plasma (PRP) obtained by blood centrifugation (190 *g*, 15 min at 37°). In selected samples platelet aggregation was also monitored by means of classical turbidimetric (optical) aggregometry (APACT dual-channel aggregometer). Briefly, each well of microtiter plate was loaded with 150 μL of PRP, supplemented with the appropriate amount of GR144053F or ATA solution, and the total volume was adjusted to 300 μL with 0.85% w/v saline. The plate was then incubated for 10 min at room temperature and next the content of each well was transferred to the fresh well containing a given platelet agonist to give the final agonist concentration of either 20 μM ADP (in the experiments with GR144053F) or 1.5 mg/mL ristocetin (when ATA was used). The rates of platelet aggregation inhibition at a given concentration of either antagonist were calculated on a virtue of the maximal drop of $A_{655\ nm}$ recorded for the control samples (no antagonist present), which was considered the 100% aggregation (and 0% inhibition). All the measurements were performed in triplicates (for GR144053F) or duplicates (for ATA) for each blood donor, and the readings for the samples with ATA were corrected for the light absorbance by ATA. Raw data gathered for each PRP sample incubated with the increasing GR144053F concentrations were used to generate a competition curve and to estimate the individual IC_{50} value based on the concentration dependence of GR144053F-mediated inhibition of platelet aggregation.

2.5. Whole-blood aggregometry

Platelet aggregation in whole-blood in the presence of collagen (2 μg/mL) was studied by the impedance method using Whole Blood Aggregometer Chrono-Log 592 [17]. Shortly, whole blood diluted 1:1 with 0.85% w/v saline was incubated with the respective amount of GR144053F for 6 min at 37°C, then supplemented with a given agonist, and the impedance value reflecting the extent of platelet aggregation was read after additional 6 min.

2.6. Flow cytometry studies

Basically, with both preparation and staining we referred to the modified [18,19] Becton Dickinson Procedure for Flow Cytometric Analysis for Platelets. In order to analyze surface membrane antigen expressions in circulating (resting) platelets, immediately after venipuncture 25-μL aliquots of whole blood were either supplemented with PAC-1 monoclonal antibodies (for the analysis of the expression of activated GPIIb-IIIa complex) or added to 0.25 mL of 10-fold diluted CellFix, mixed by gentle moving forth and back, and left at 4°C overnight (for the analysis of the expression of P-selectin and GPIb). In order to evaluate the ability of platelets to respond to activating agents, the samples of fresh whole blood were incubated with either 0.15 IU/mL thrombin (4 min), 8 μM TRAP (4 min) or 3 μM ADP (4 min). In parallel, the aliquots of fresh whole blood were incubated in polystyrene tubes at room temperature for up to 1 hr in order to evaluate the "static" or time-dependent model of platelet activation. Alternatively, 500-μL portions of a whole blood were placed in polystyrene 7-mL tubes immobilized in the rack, placed in a rotary stirrer, agitated (up to eight 360° rotations per min equivalent to 7–8 sec cycles) for up to 1 hr at room temperature in order to evaluate the "dynamic" platelet activation. Following platelet activation the samples were fixed in CellFix solution, left at 4°C overnight and further used for the staining with monoclonal antibodies [18,19].

Fibrinogen binding assay

Stock solution (1.5 mg/mL) of human fibrinogen labeled with Oregon Green was prepared in sodium biscarbonate buffer (0.1 M, pH 8.3). The aliquots of a fibrinogen stock solution was added to 100 μL of whole blood to the final concentration of 100 μg/mL and the mixture was incubated for 5 min at room temperature. Alternatively, GR144053F was added prior to the incubation with labeled fibrinogen (100 nM).

Flow cytometric assay was performed within 2 hr after staining on fixed blood cells with scatter and FL1 or FL3 fluorescence gates set on the platelet fraction. Fluorescence of 2000–5000 platelets was measured with FACSCalibur instrument (Becton Dickinson) with a laser excitation at 488 nm. All data were processed using Lysis II software (Becton Dickinson). Percentage of platelets positive with the specific fluorescence was obtained after the subtraction of non-specific mouse IgG_1 binding and referred to as the expression of a given platelet surface membrane antigen. Fibrinogen binding was assessed on the basis of percentage of the Oregon Green-positive platelets. The extent of a forward light scatter was employed to evaluate the fractions of platelet microparticles and platelet aggregates (range of channels 0–30 and 180–256 on the log 4 decade 256-channel scale, respectively) [18,19].

All flow cytometry measurements were fluorescence-compensated on a daily basis for each set of measured samples by using calibration beads (Becton Dickinson), to ensure that there was no considerable green, orange, and red fluorescence overlap. Flow cytometric measurements were performed at the Flow Cytometry Unit, 2nd Clinic of Children Diseases, Institute of Pediatrics, Medical University of Lodz.

2.7. PFA studies

Flow analyzer PFA-100™ (Dade Behring) allows studying a reactivity of blood platelets under conditions similar to those in the circulation. Blood samples are aspirated under steady-flow conditions through a small aperture cut in a membrane diaphragm coated with type I collagen and either epinephrine or ADP. Platelets, when becoming activated, attach to the area surrounding the aperture, form platelet plug, and occlude it, while the instrument records the time necessary for the occlusion (closure time), which is an indicative of platelet function and referred to as primary hemostatic capacity [20, 21]. In our study the respective amounts of GR144053F or/and ATA were added to 800 μL of undiluted citrated whole blood and the samples were incubated for 10 min.

2.8. Statistical analysis

Mean ± SD is given for all parameters. The extent of the percent inhibition of platelets to occlude (PFA-100™ CT_{CADP}) was expressed as:

$$100\% - [100\% \times (CT_{CADP\text{-}inh} - CT_{CADP\text{-}con})/(CT_{CADP\text{-}max} - CAT_{CADP\text{-}con})]$$

where $CT_{CADP\text{-}con}$ is the initial closure time, $CT_{CADP\text{-}inh}$ is the closure time in the presence of an inhibitor, and $CT_{CADP\text{-}max}$ is the maximum measurable closure time, which for PFA-100™ equals 300 sec.

Due to the relatively small sample size the distributions of some variables showed departure from normality (according to Shapiro–Wilk's test). Therefore, to estimate the significance of differences we employed Wilcoxon matched pair test, Mann–Whitney *U*-test and the non-parametric version of post-hoc Tukey test for multiple comparisons. We used Student *t*-test for comparing slopes to evaluate the additive effects of GR144053F and ATA. Non-linear estimation (quasi-Newton algorithm) was used to calculate the IC_{50} values for GR144053F [22].

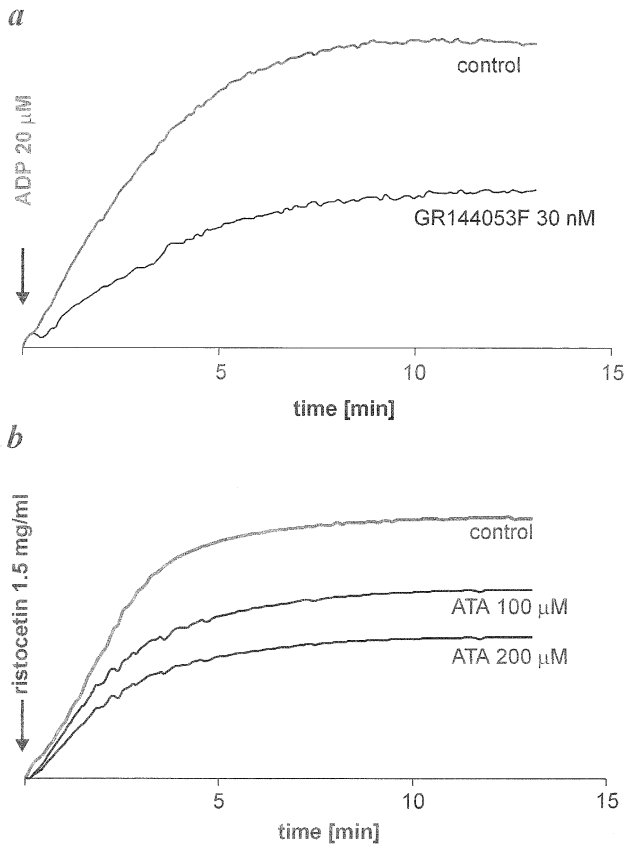

Fig. 1. Representative curves of ADP-(a) and ristocetin-induced (b) platelet aggregation monitored by a microplate method. Platelet-rich plasma was optionally supplemented with GR144053F or ATA and aggregation was triggered by adding the agonist. The extinction $A_{655\ nm}$ corresponding to maximal aggregation was 0.45 ± 0.09 for ADP and 0.53 ± 0.08 for ristocetin.

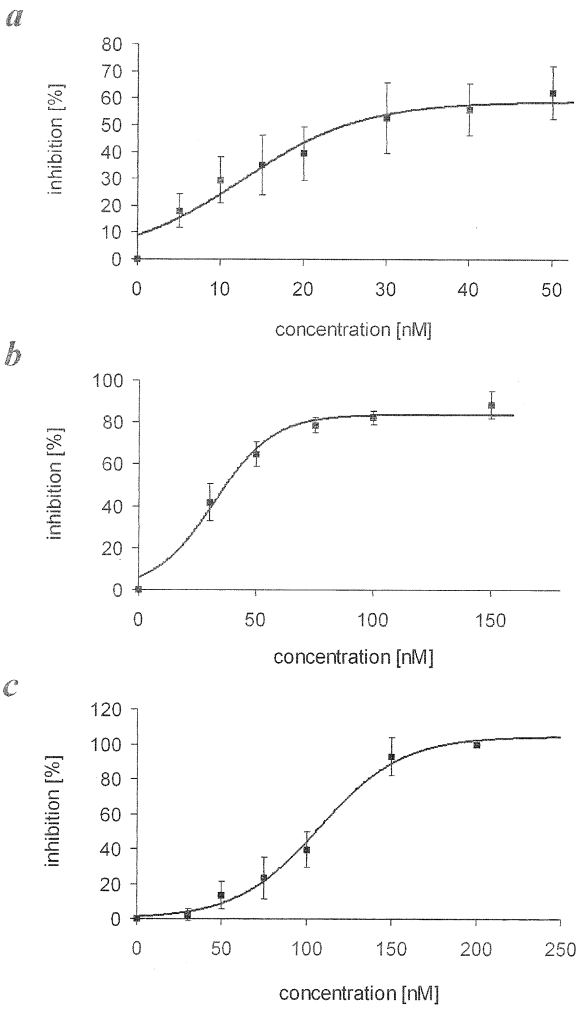

Fig. 2. Curves of the inhibition by GR144053F of: (a) the ADP-induced aggregation in platelet-rich plasma; (b) the collagen-induced platelet aggregation in a whole blood; and (c) primary hemostatic capacity in PFA-100™ CT_{CADP}. For every blood donor each GR144053 concentration was tested in 1–3 replicates. Points in the graph represent the mean inhibition values averaged for 15 individuals. Based on the experimental data, theoretical curves of inhibition were generated and plotted by using a nonlinear estimation (quasi-Newton algorithm) and IC_{50} (antagonist concentration for 50% of the maximal estimated inhibition) was calculated. The extinction $A_{655\ nm}$ corresponding to maximal aggregation was 0.45 ± 0.09 for ADP and 0.57 ± 0.06 for collagen. CT_{CADP} of control blood samples (no antagonist added) was 89 ± 9 sec.

3. Results

3.1. Influence of GR144053F and ATA on platelet aggregation

Platelet aggregation induced in PRP by 20 μM ADP (Fig. 1a) was hampered by GR144053F in a concentration-dependent manner. The maximal inhibition occurred at 40–50 nM (Fig. 2a) and did not deepen significantly with the increasing antagonist concentration (not shown). The estimated IC_{50} (antagonist concentration for 50% of the maximal estimated inhibition) was 17.7 ± 7.0 nM.

Also, in the presence of ATA the dose-dependent inhibition of the ristocetin-induced platelet aggregation in PRP was observed (Fig. 1b): 34.0 ± 18.8% inhibition at 100 μM ATA ($P < 0.02$) and 45.3 ± 18.9% at 200 μM ATA ($P < 0.04$ 100 μM vs. 200 μM ATA).

The inhibitory effect of 30–150 nM GR144053F on platelet aggregation induced by collagen (2 μg/mL) was also dose-dependent and the estimated averaged value of IC_{50} was 31.8 ± 4.8 nM (Fig. 2b).

3.2. Effect of GR144053F and ATA on primary hemostatic capacity monitored with PFA-100

The mean closure time in the cassettes coated with type I collagen and ADP (PFA-100™ CT_{CADP}) in control samples (no antagonist added) was 95 ± 22 sec. Upon the addition of GR144053F platelet reactivity became reduced ($P < 0.005$ or less), which resulted in longer closure times (Fig. 2c). GR144053F used at the concentration of 200 nM prolonged CT_{CADP} over 300 sec, which is above the upper measurement limit of PFA-100™. The relevant estimated value of IC_{50} for the prolongation of PFA-100™ CT_{CADP}

Table 1
Expression of P-selectin and GPIb and the fractions of platelet microparticles and aggregates in "static" and "dynamic" model of platelet activation

	Resting platelets	"Static" activation	"Dynamic" activation
P-selectin (%)	2.5 ± 2.3	15.2 ± 9.5*	24.8 ± 8.6*
GPIb (%)	93.7 ± 4.7	91.9 ± 5.9	69.7 ± 14.0*
Microparticles (%)	6.0 ± 6.2	7.0 ± 6.6	26.2 ± 18.7**
Aggregates (%)	2.2 ± 1.7	4.9 ± 4.4**	5.5 ± 2.2**

Means ± SD are given; $n = 15$. In "static" model of platelet activation the aliquots of freshly drawn whole blood were incubated in polystyrene tubes at room temperature for up to 1 hr. In "dynamic" model of platelet activation portions of whole blood were placed in polystyrene tubes and agitated for 1 hr in a rotary stirrer (for details see section 2). Significance of difference *vs*. resting circulating platelets, estimated by means of Wilcoxon's pair matched test, was: * $P < 0.0001$; ** $P < 0.01$.

Table 3
Effect of GR144053F on the activation of GPIIb-IIIa complex in blood platelets

Activation	(−) GR144053F (%)	(+) GR144052F (%)	Significance
"Static" activation	4.3 ± 4.5	3.5 ± 4.5	$P < 0.05$
"Dynamic" activation	5.7 ± 9.3	3.5 ± 5.8	$P < 0.01$
ADP 3 μM, 4 min	24.3 ± 10.3	17.6 ± 10.6	$P < 0.03$
TRAP 8 μM, 4 min	48.3 ± 18.6	39.7 ± 16.3	$P < 0.05$
Thrombin 0.15 U/mL, 4 min	9.0 ± 3.6	7.6 ± 3.3	NS

Means ± SD are given; $n = 15$. The binding of PAC-1 to the activated GPIIb-IIIa in resting circulating platelets was 1.0 ± 0.3%. Whole-blood platelets were activated by the incubation at room temperature, stirring, or in the presence of 0.15 IU/mL thrombin or 8 μM TRAP (4 min, RT) without or with 60 nM GR144053F. Significance was estimated by means of Wilcoxon's pair matched test; NS, not significant.

was 114.0 ± 9.6 nM. Also 100 μM ATA significantly prolonged the closure time up to 190 ± 71 sec ($P < 0.01$), which corresponded to the inhibition of 54 ± 30% ($P < 0.001$). None of the samples incubated with 100 μM ATA showed the closure time exceeding 300 sec.

To examine the possible cooperative influence during the simultaneous action of both agents on primary hemostatic capacity we monitored platelet ability to occlude in the presence of 100 nM GR144053F and 100 μM ATA. Our results revealed that such simultaneous inhibitory effects were not additive: 40 ± 15% for 100 nM GR144053F, 39 ± 9% for 100 μM ATA, and 57 ± 8% for the combined action of 100 nM GR144053F and 100 μM ATA. Thus, we did not evidence the co-operative interaction of both agents in affecting PFA-100™ primary hemostatic capacity.

3.3. Influence of GR144053F and ATA on platelet activation and release reaction monitored by flow cytometry

3.3.1. The influence of GR144053F and ATA on the spontaneous platelet activation and the activation induced by blood agitation

In both "static" and "dynamic" model of platelet activation platelets acquired higher expression of surface membrane P-selectin (the recognized marker of platelet degranulation) and more GPIb became internalized, which points to platelet activation and degranulation of intraplatelet α-granules. These changes were particularly profound in the dynamic model of whole-blood platelet activation. There were more platelet aggregates in both models of activation and the fraction of platelet microparticles (the flow cytometric marker of platelet rupture/consumption) became vastly increased in agitated samples, thus reflecting the increased platelet clumping and rupture/consumption (Table 1).

The effects of both drugs were opposed to each other, and GR144053F suppressed the effects of ATA. GR144053F considerably reduced the release of platelet P-selectin, GPIb internalization, as well as the formation of platelet microparticles and aggregates in a concentration-dependent manner. Such a reduction occurred particularly in the dynamic model of platelet activation, as deduced from the values of IC_{50} and maximum inhibition (Table 2). In agitated samples (dynamic activation) GR144053F vastly reduced (up to 70−85%) the release of P-selectin from intraplatelet granules, the platelet consumption (decreased fraction of microparticles) and the formation of platelet aggregates, whereas the reduction of GPIb internalization was only moderate (Table 2). For the dynamic model the

Table 2
Inhibition of platelet activation, degranulation, consumption, and aggregation by GR144053F

	"Static" model of platelet activation		"Dynamic" model of platelet activation	
	IC_{50} [nM]	Maximum inhibition [%]	IC_{50} [nM]	Maximum inhibition [%]
Release of P-selectin	87.6 ± 22.9	24.8 ± 17.0	30.3 ± 6.6*	85.1 ± 7.4*
GPIb internalization	n.d.	n.d.	27.5 ± 12.0	20.7 ± 9.8
Formation of platelet microparticles	42.1 ± 15.4	31.8 ± 11.0	30.1 ± 12.3***	85.3 ± 6.7*
Formation of platelet aggregates	34.3 ± 8.6	50.4 ± 15.7	28.0 ± 15.0*	69.8 ± 8.1**

Data represent mean ± SD, $n = 11$; n.d., not determined. IC_{50} (antagonist concentration for 50% of the maximal estimated inhibition) and maximal estimated inhibition were estimated for the release of P-selectin, GPIb internalization, the formation of platelet microparticles and aggregates in a whole blood of each individual based on the inhibition curve generated by using a non-linear estimation (quasi-Newton algorithm). Significance of difference, estimated by means of Mann–Whitney *U*-test, was: * $P \ll 0.0001$ *vs*. "static" activation; ** $P < 0.002$ *vs*. "static" activation; *** $P < 0.06$ *vs*. "static" activation.

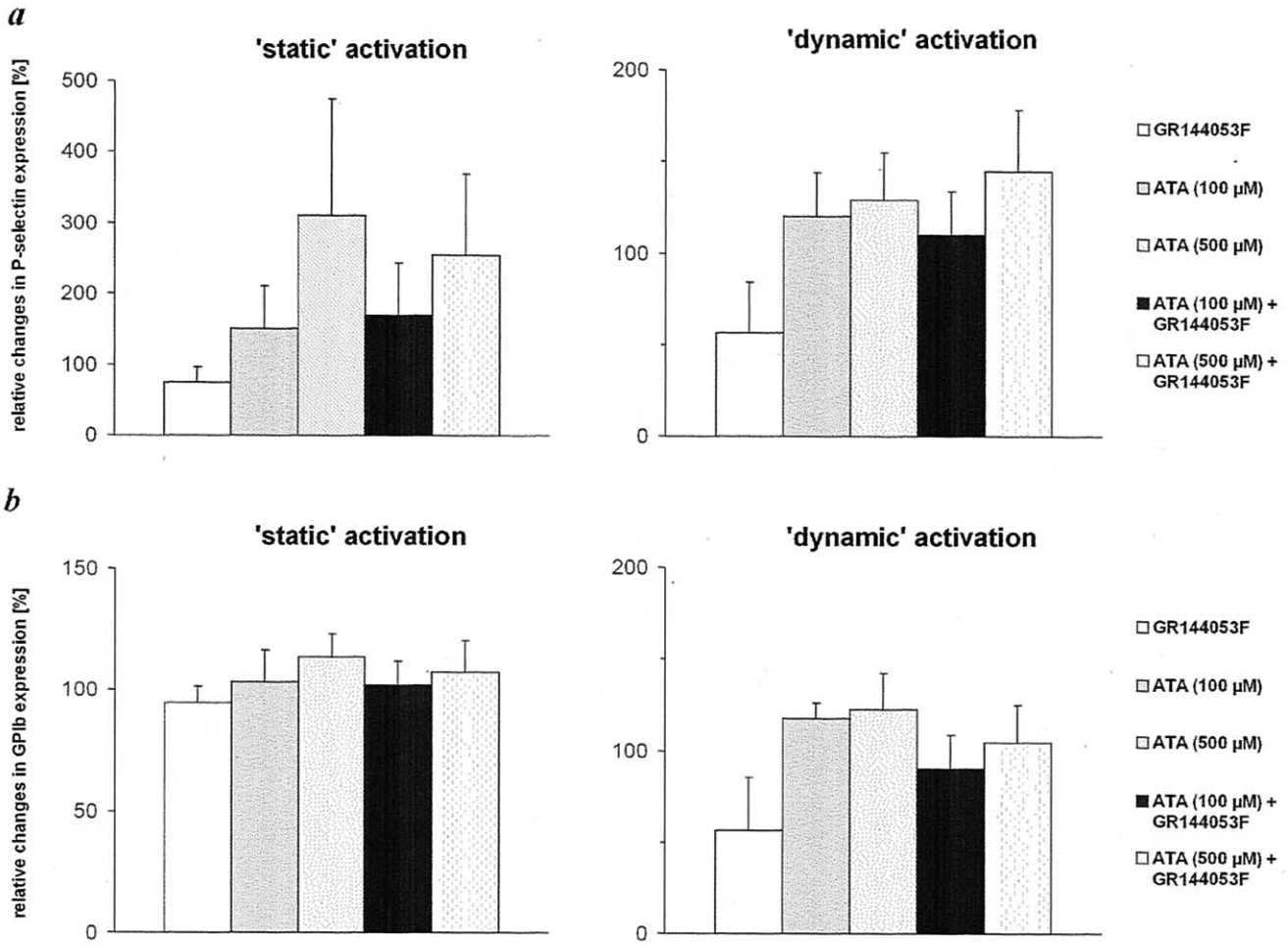

Fig. 3. Effect of GR144053F and ATA on the expressions of P-selectin (a) and GPIb (b) in "static" and "dynamic" model of platelet activation. The relative percent changes vs. control samples incubated without the antagonists (100%) are shown. The absolute values of the expression of P-selectin and GPIb are given in Table 1. Statistical significance was estimated by means of non-parametric version of *post-hoc* Tukey test for multiple comparisons. Statistically significant differences are: a) P-selectin, "static" model of platelet activation: $\mu_{\text{control}} \neq \mu_{\text{GR144053F}}$ $P < 0.01$, $\mu_{\text{GR144053F+ATA 500 }\mu\text{M}}$ $P < 0.01$: P-selectin, "dynamic" model of platelet activation: $\mu_{\text{control}} \neq \mu_{\text{GR144053F}}$ $P < 0.01$, $\mu_{\text{control}} \neq \mu_{\text{GR144053F+ATA 500 M}}$ $P < 0.01$; b) GPIb, "static" model of platelet activation: $\mu_{\text{control}} \neq \mu_{\text{GR144053F+ATA 500 }\mu\text{M}}$ $P < 0.01$, $\mu_{\text{ATA 500}\mu\text{M}} \neq \mu_{\text{GR144053F+ATA 500}\mu\text{M}}$ $P < 0.03$; GPIb, "dynamic" model of platelet activation: $\mu_{\text{control}} \neq \mu_{\text{GR144053F+ATA 100}\mu\text{M}}$ $P < 0.008$, $\mu_{\text{control}} \neq \mu_{\text{GR144053F+ATA 500 }\mu\text{M}}$ $P < 0.008$, $\mu_{\text{ATA 100 }\mu\text{M}} \neq \mu_{\text{GR144053F+ATA 100 }\mu\text{M}}$ $P < 0.008$, $\mu_{\text{ATA 500 }\mu\text{M}} \neq \mu_{\text{GR144053F+ATA 500 }\mu\text{M}}$ $P < 0.01$.

IC_{50} estimated for each of the above markers of platelet activation was about 30 nM, which corresponded to the value established for collagen-induced platelet aggregation in whole blood (32 ± 5 nM) (Table 2). Otherwise, the respective values calculated for the static model of platelet activation were significantly higher. These results clearly point that GR144053F was significantly more efficient inhibitor in the dynamic model of platelet activation.

GR144053F (60 nM) significantly attenuated the binding of PAC-1 to the activated complex GPIIb-IIIa in both the static ($P < 0.05$) and "dynamic" model ($P < 0.01$) of platelet activation (Table 3). The inhibitory effect of GR144053F on the activation of GPIIb-IIIa complex was paralleled by the diminished binding of plasma fibrinogen. When whole-blood platelets were treated with 100 nM GR144053F prior to their incubation for up to 5 min at room temperature, the binding of fibrinogen to GPIIb-IIIa receptor became reduced by 78.0 ± 11.7% (9.0 ± 3.9% in control vs. 1.8 ± 1.0% with GR144053F, $P < 0.001$).

Contrary to GR144053F, ATA at the concentration of either 100 or 500 μM significantly increased platelet degranulation in the static and dynamic model of platelet activation, as evidenced by the enhanced expression of surface membrane P-selectin (Fig. 3a). Also, the agitation of whole-blood samples with ATA resulted in higher GPIb internalization, thus pointing to the activating effect of ATA (Fig. 3b). These activating effects of ATA concerned also platelet consumption and the formation of platelet aggregates in both the static and dynamic model of platelet activation (Fig. 4). When 100 or 500 μM ATA was incubated simultaneously with 60–150 nM GR144053F, the latter antagonist significantly attenuated the ATA-induced platelet degranulation and GPIb trafficking, and lead to the reduced platelet consumption and

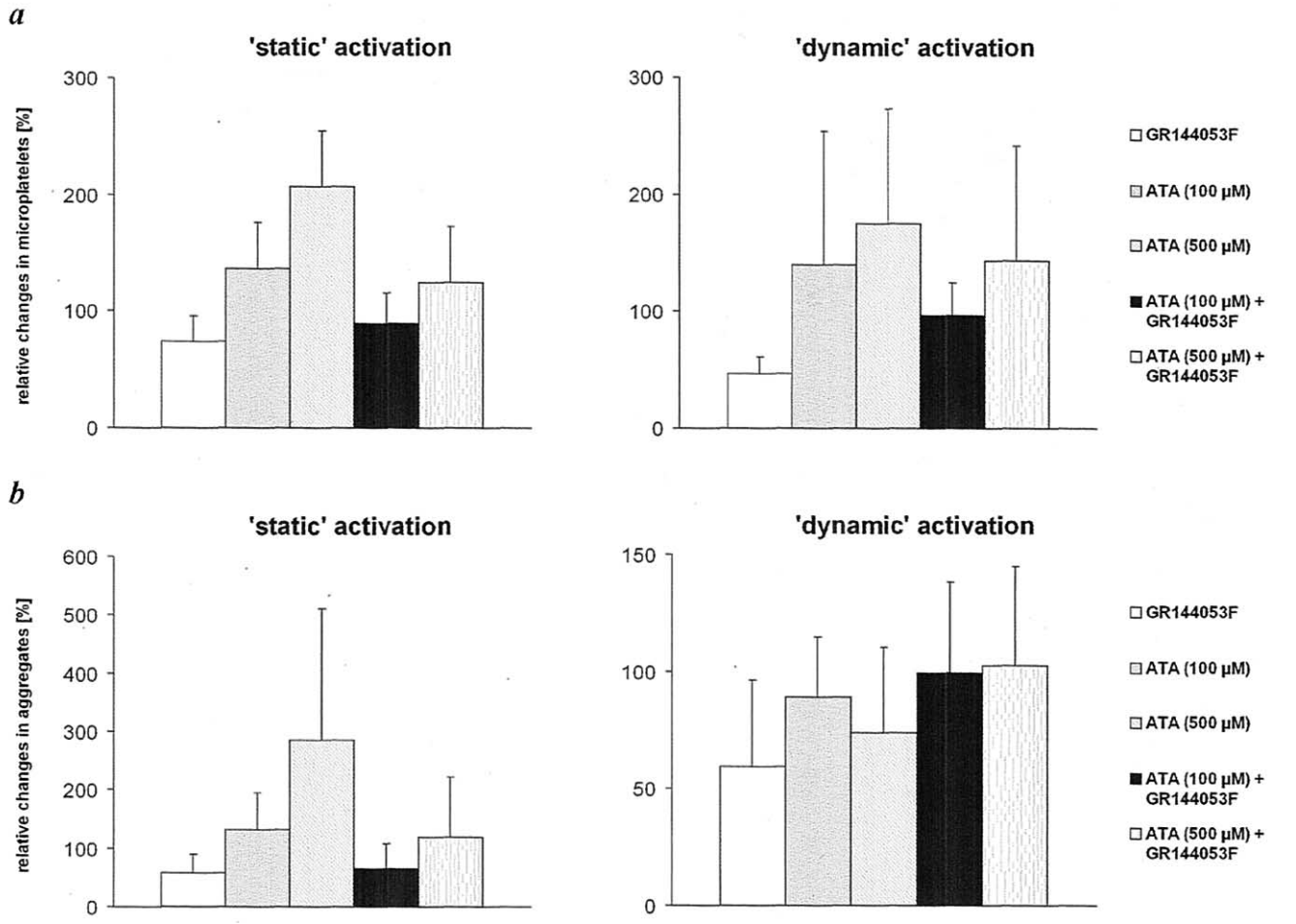

Fig. 4. Effect of GR144053F and ATA on the fractions of platelet microparticles (a) and aggregates (b) in "static" and "dynamic" model of platelet activation. The relative percent changes vs. control samples incubated without the antagonists (100%) are shown. The absolute values of the fractions of platelet microparticles and aggregates are given in Table 1. Statistical significance was estimated by means of non-parametric version of *post hoc* Tukey test for multiple comparisons. Statistically significant differences are shown below: a) microparticles, "static" model of platelet activation: $\mu_{control} \neq \mu_{GR144053F}$ $P < 0.01$, $\mu_{control} \neq \mu_{GR144053F+ATA\ 100\ \mu M}$ $P < 0.02$, $\mu_{control} \neq \mu_{GR144053F+ATA\ 500\ \mu M}$ $P < 0.008$, $\mu_{ATA\ 100\ \mu M} \neq \mu_{GR144053F+ATA\ 100\ \mu M}$ $P < 0.01$, $\mu_{ATA\ 500\ \mu M} \neq \mu_{GR144053F+ATA\ 500\ \mu M}$ $P < 0.02$; microparticles, "dynamic" model of platelet activation: $\mu_{control} \neq \mu_{GR144053F}$ $P < 0.008$, $\mu_{control} \neq \mu_{GR144053F+ATA\ 100\ \mu M}$ $P < 0.01$, $\mu_{control} \neq \mu_{GR144053F+ATA\ 500\ \mu M}$ $P < 0.008$, $\mu_{ATA\ 100\ \mu M} \neq \mu_{GR144053F+ATA\ 100\ \mu M}$ $P < 0.01$, $\mu_{ATA\ 500\ \mu M} \neq \mu_{GR144053F+ATA\ 500\ \mu M}$ $P < 0.008$; b) aggregates, "static" model of platelet activation: $\mu_{control} \neq \mu_{GR144053F}$ $P < 0.02$, $\mu_{control} \neq \mu_{GR144053F+ATA\ 500\ \mu M}$ $P < 0.03$, $\mu_{ATA\ 500\ \mu M} \neq \mu_{GR144053F+ATA\ 500\ \mu M}$ $P < 0.01$; aggregates, "dynamic" model of platelet activation: $\mu_{ATA\ 500\ \mu M} \neq \mu_{GR144053F+ATA\ 500\ \mu M}$ $P < 0.03$.

clumping (Figs. 3 and 4). Such a suppressive effect was observed already with 60 nM GR144053F, and did not deepen significantly with the increasing concentration of the drug.

3.3.2. Influence of GR144053F and ATA on the agonist-induced platelet activation

No inhibitory effect of 60 nM GR144053F was revealed with respect to platelet degranulation (the expression of P-selectin) and GPIb-IX-V internalization (GPIb expression). However, GR144053F was shown to markedly suppress the increased binding of PAC-1 in platelets stimulated by either TRAP or ADP (Table 3). In addition, the antagonist dramatically reduced the formation of platelet aggregates in the presence of thrombin (2.9 ± 1.4 vs. 13.5 ± 8.3%, $P < 0.01$) or TRAP (6.0 ± 3.0 vs. 14.5 ± 4.8%, $P < 0.003$) and significantly lowered platelet consumption in thrombin- (2.0 ± 1.2 vs. 5.2 ± 2.6%, $P < 0.01$) or TRAP-activated samples (2.2 ± 1.4 vs. 3.8 ± 1.9%, $P < 0.03$).

In series of experiments involving a group of seven healthy donors we also tested the impact of ATA (100 μM) and the combined effect of ATA and GR144053F (60 nM) on the binding of PAC-1 in either static or dynamic model of platelet activation, as well as in for platelets stimulated by 3 μM ADP or 8 μM TRAP. The combined action of both agents resulted in lower binding of PAC-1 (relevant to the reduced activation of the GPIIb-IIIa complex) in agitated platelets and platelets activated with ADP; these reductions, however, were beyond statistical significance.

4. Discussion

Antiplatelet therapy became the major challenge and clinical indication in the prevention of arterial thrombosis, because arterial thrombi are composed of predominantly platelets formed under conditions of elevated shear stress at sites of atherosclerotic vascular injury and disturbed blood flow [23–25]. Due to the considerable number of copies per platelet, two receptor systems that are present of platelet surface membrane deserve a special attention—the fibrinogen receptor (GPIIb-IIIa complex) and the receptor for vWF (GPIb-IX-V complex). Although there is a huge variability of the available antagonists for GPIIb-IIIa at present, and numerous are approved for clinical use, effective antagonists of GPIb-IX-V receptor are scarce. In addition to their ability to block the ligand binding, several other mechanisms have been suggested to contribute to the antiplatelet action of various GPIIb-IIIa antagonists, such as decreased thrombin generation, partial inhibition of the release reaction from dense and α-granules [26–28] and promotion of fibrinolysis [28]. Although none of the antagonists of GPIb-IX-V receptor complex has been approved hitherto to clinical use, some specific non-peptide inhibitors of the GPlb-vWF interaction, such as ATA, seemed once very promising [2,4,8,11,12,15,29,30]. Single reports pointed that the antithrombotic effect of ATA on platelet function may be not solely due to its interaction with vWF, but may also relate to the common pathway of the platelet aggregation induced by other agents, like fibrinogen [10,15,31].

In this work we evidence for the first time that GR144053F is a potent inhibitor of platelet degranulation, activation and aggregation under *in vitro* conditions. We investigated the efficiency of GR144053F and ATA acting alone, as well as the combined effects of both drugs on the inhibition of platelet function. For this purpose we employed different techniques, which enabled us to monitor various aspects of platelet activation and reactivity, i.e. their ability to aggregate, degranulation of intraplatelet granules, changes in the expression of surface platelet membrane receptors in response to platelet agonists, formation of microparticles, and platelet adhesion/clumping under conditions simulating a natural blood flow in vessels.

GR144053F was found to be a very efficient blocker of platelet functional responses to activation and we evidenced that in a triple way. First, we confirmed that GR144053F inhibited the ADP-induced platelet aggregation in nanomolar concentrations, which remains consistent with the respective figure reported for Chinese hamster platelets activated with ADP [29]. We further showed that GR144053F inhibited the collagen-induced aggregation of platelets in their natural milieu, i.e. in the presence of red and white blood cells, and revealed that the antagonist was twice less efficient compared to the inhibition of platelet aggregation in the presence of ADP. Despite of the fact that some collagen receptors (like $\alpha_2\beta_1$ or GP VI) are involved in the inside-out signaling leading to activation of GPIIb-IIIa [32], such a discrepancy may not be surprising, considering that the antagonist affects the influence of agonists acting on different platelet receptors. In addition, such a finding encourages us to reason that GR144053F may inhibit the final step of platelet transmembrane signalling. Hence, the final platelet response, i.e. platelet aggregation was blocked in the presence of GR144053F, regardless of the agonist used for platelet activation. Normally, when various agonists trigger platelet activation, the fibrinogen receptor becomes activated via the mechanism(s) of cellular inside-out signaling, binds fibrinogen and perpetuates platelet aggregation [1, 33]. Upon the action of GR144053F both the ligand binding and the activation of fibrinogen receptor may be hampered and platelet aggregation is reduced.

By using flow cytometry we showed that at the concentration of GR144053F, which was saturating for the inhibition of platelet aggregation, the release of P-selectin and the activation of GPIIb-IIIa complex became reduced, and so was the internalization of GPIb from platelet surface membranes to intraplatelet tubular system. Noteworthy, in the agitated blood samples the potency of GR144053F to prevent platelet degranulation and redistribution of GPIb was much higher compared to milder conditions, when platelets were incubated at room temperature in their natural milieu. In our opinion, this observation validates the conclusion that GPIIb-IIIa is more crucial for the mechanism of platelet activation in flowing blood. Our finding that GR144053F significantly reduced the activation of GPIIb-IIIa complex in platelets activated with ADP and to a much lesser extent with TRAP or thrombin, may be explained by a fact that thrombin and TRAP exert their effects via seven-domain transmembrane receptor that is apparently not under the influence of GR144053. To reasonably interpret the inhibitory effect of GR144053F on platelet consumption (reduced fraction of platelet microparticles), one should be aware that the ongoing platelet activation and release reaction usually results in a faster platelet consumption and the accelerated formation of platelet microparticles, which exhibit strong procoagulant properties. Obviously, more extensive platelet degranulation perpetuates faster consumption and, hence, the efficient inhibition of platelet activation by GR144053F might have resulted in an abating of platelet consumption.

Our results clearly demonstrate that the interaction of GR144053F with GPIIb-IIIa not only prevents fibrinogen binding but may also alter signaling properties of the heterodimer, and thus contribute to the attenuation of platelet activation and degranulation [26, 27]. There may be however, still other mechanisms—like the interaction of RGD-type ligands with $\alpha_2\beta_1$ integrin (collagen receptor) [34], which might possibly explain rather equated inhibition effects of GR144053F on platelet activation triggered by various agonists.

The effects exerted by ATA on platelet function cannot be uniformly classified. In micromolar concentrations ATA efficiently blocked the ristocetin-induced platelet aggregation in platelet-rich plasma in a dose-dependent manner,

which remains consistent with some literature data [7,35] and oppose others [15]. The efficient inhibition was observed within ATA concentration range of four orders of magnitude higher than those estimated for GR144053F. In our opinion, this may be consequence of the fact that such an inhibition results from the reversible binding of ATA to vWF but not platelets [14]. Despite the inhibitory action of ATA on ristocetin-induced and vWF-dependent platelet aggregation, the drug activated platelets and augmented their degranulation and consumption. This finding is consistent with literature data, which indicate that selected fractions of ATA may paradoxically trigger platelet activation by the mechanism most likely related to the direct activation of phospholipase C [36]. In an attempt to reconcile these opposing findings, one should remember that ATA solutions are heterogeneous mixtures of polyanionic, polyaromatic polymers of Mr 200 to greater than 6000. ATA polymers larger than 700 have been reported to inhibit ristocetin-induced and vWF-mediated platelet aggregation more effectively than smaller ATA polymers, whereas the shear-induced vWF-mediated platelet aggregation was optimally inhibited by ATA polymers of Mr $\geq$ 2500 [37]. It seems very likely that our solution of unfractionated ATA might contain predominantly smaller ATA polymers, which were effective in the inhibition of ristocetin-induced vWF-mediated platelet aggregation, but triggered platelet degranulation, as revealed by using flow cytometry. Further, we argue that the effect of ATA on GPIb-IX-V receptor is probably restrained only to the blockade of vWF and not to the complex internalization. It seems reasonable because platelet activation with thrombin, for which the GPIb-IX-V complex is the alternate receptor (with a higher thrombin binding affinity), was apparently unaffected even at high concentrations of ATA. The molecular mechanism(s) by which ATA might trigger platelet activation remain unexplained yet, however ATA may non-specifically activate platelet in at least two hypothetical ways. First, ATA may block the interaction of vWF and GPIb binding sites, while stimulating other domain(s) in the same GPIb-IX-V complex. Alternatively, ATA may bind to other platelet glycoprotein(s), induce their conformational changes and trigger platelet activation via different receptor system. Opposing to the above findings are the results pointing that ATA suppressed the activation of GPIIb-IIIa complex in the course of the ADP-induced platelet activation. Although the observed effect remains beyond statistical significance, the observation is worthy to be validated in further studies: it relates to the suggestion raised in the literature that ATA may interact with fibrinogen binding to GPIIb-IIIa [10].

Our finding that GR144053F suppressed the activation of blood platelets by ATA has two important implications. First, it validates our aforementioned conclusion on the direct inhibitory effect(s) of GR144053F on platelet activation. Second, it suggests that the action of GR144053F is more generalized and not restricted solely to its interaction with GPIIb-IIIa complex. It seems unlikely that GR144053F competes with ATA for the binding site on GPIb. More reasonably, GR144053F interferes with general mechanism(s) of platelet activation and degranulation, i.e. it acts beyond the receptor level.

Lastly, our data pointed to GR144053F and ATA as efficient inhibitors of the overall platelet haemostatic function, including both adhesion and aggregation, under conditions that closely mimic the natural blood flow in circulation. Noteworthy, GR144053F, which was very efficient blocker of platelet aggregation (IC_{50} ~20 nM), appeared much less active in attenuating the platelet-derived primary hemostatic capacity (IC_{50} >100 nM). Considering that both methods monitor different parameters of platelet function, it seems obvious that the estimated IC_{50} also vary: for PRP aggregometry IC_{50} reflects the efficiency to block platelet clumping, whereas for PFA-100 it reflects more the overall platelet ability to occlude vessels under particular conditions of blood flow. As far as it is believed that PFA-100 closure times are good approximation of platelet function in circulating blood, we might argue that IC_{50} evaluated for GR144053F with the use of PFA-100™ corresponds more to physiological values. This also indicates that GR144053F effectively blocks platelet aggregation, whereas it remains much less efficient in the modulation of platelet adhesion. These observations are consistent with other reports published in the literature using related methods [38]. They also relate to earlier papers reporting the decreased microaggregates fraction and the reduced flow retardation by the formed microthrombi in the presence of GR144053F in animal models of thrombosis [5].

In summary, the non-peptide antagonist of the platelet membrane fibrinogen receptor GR144053F affects a variety of blood platelet functional parameters, including aggregation and adhesion under conditions that closely mimic natural blood flow in circulation, activation of GPIIb-IIIa complex, and platelet degranulation. The molecular mechanisms of such an inhibition relate not only to the competitive binding of the drug to the RGD-sequence binding site, with the resultant prevention of the interaction with some physiological ligands, but also to the direct impairment(s) of platelet activation.

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References

[1] Nurden AT. New thoughts on strategies for modulating platelet function through the inhibition of surface receptors. Hemostasis 1996;26:78–88.

[2] Ito T, Matsuno H, Kozawa O, Niwa M, Sakai N, Uematsu T. Comparison of the antithrombotic effects and bleeding risk of fractionated

aurin tricarboxylic acid and the GPIIb/IIIa antagonist GR144053 in a hamster model of stenosis. Thromb Res 1999;95:49–61.
[3] Matsuno H, Kozawa O, Ueshima S, Matsuo O, Collen D, Uematsu T. Lack of tPA significantly affects antithrombotic therapy by a GPIIb/IIIa antagonist, but not by a thrombin inhibitor in mice. Thromb Haemost 2000;83:605–9.
[4] Matsuno H, Kozawa O, Niwa M, Kaida T, Hayashi H, Uematsu T. GR144053, a fibrinogen receptor antagonist, enhances the suppression of neointima formation by losartan, an angiotensin II receptor antagonist, in the injured carotid artery of hamster. Br J Pharmacol 1997;122:1099–1104.
[5] Matsuno H, Kozawa O, Nagashima S, Kanamaru M, Uematsu T. Comparative antiplatelet effects of aspirin, vapiprost and GR144053, a GPIIb/IIIa antagonist, with a special reference to the role of platelet microaggregates. Br J Pharmacol 1999;127:1129–34.
[6] Girma JP, Fressinaud E, Christophe O, Rouault C, Obert B, Takahashi Y, Meyer D. Aurin tricarboxylic acid inhibits platelet adhesion to collagen by binding to the 509–695 disulphide loop of von Willebrand factor and competing with glycoprotein Ib. Thromb Haemost 1992;68:707–13.
[7] Phillips MD, Moake JL, Nolasco L, Turner N. Aurin tricarboxylic acid: a novel inhibitor of the association of von Willebrand factors and platelets. Blood 1988;72(Abstr):1898.
[8] Matsuno H, Kozawa O, Niwa M, Uematsu T. Inhibition of von Willebrand factor binding to platelet GP Ib by a fractionated aurintricarboxylic acid prevents restenosis after vascular injury in hamster carotid artery. Circulation 1997;96:1299–304.
[9] Takiguchi Y, Shimazawa M, Nakashima M. A comparative study of the antithrombotic effect of aurintricarboxylic acid on arterial thrombosis in rats, and guinea-pigs. Br J Pharmacol 1996;118:1633–8.
[10] Azzam K, Cisse–Thiam M, Drouet L, The antithrombotic effect of aurin tricarboxylic acid in the guinea pig is not solely due to its interaction with the von Willebrand factor-GPIb axis. Thromb Haemost 1996;75:203–10.
[11] Golino P, Ragni M, Cirillo P, Pascucci I, Ezekowitz MD, Pawashe A, Scognamiglio A, Pace L, Guarino A, Chiariello M. Aurintricarboxylic acid reduces platelet deposition in stenosed and endothelially injured rabbit carotid arteries more effectively than other antiplatelet interventions. Thromb Hemost 1995;74:974–9.
[12] Strony J, Song A, Rusterholtz L, Adelman B. Aurintricarboxylic acid prevents acute rethrombosis in a canine model of arterial thrombosis. Arterioscler Thromb Vasc Biol 1995;15:359–66.
[13] Bernat A, Lale A, Herbert JM. Aurin tricarboxylic acid inhibits experimental venous thrombosis [see comments]. Thromb Res 1994;74:617–27.
[14] Kawasaki T, Kaku S, Kohinata T, Sakai Y, Taniuchi Y, Kawamura K, Yano S, Takenaka T, Fujimura Y. Inhibition by aurintricarboxylic acid of von Willebrand factor binding to platelet GPIb, platelet retention, and thrombus formation in vivo. Am J Hematol 1994;47:6–15.
[15] Matsuno H, Kozawa O, Niwa M, Tanabe K, Ichimaru K, Takiguchi Y, Yokota M, Hayashi H, Uematsu T. Multiple inhibition of platelet activation by aurintricarboxylic acid prevents vascular stenosis after endothelial injury in hamster carotid artery. Thromb Haemost 1998;79:865–71.
[16] Walkowiak B, Kesy A, Michalec L. Microplate reader–a convenient tool in studies of blood coagulation. Thromb Res 1997;87:95–103.
[17] Ray MJ, Hawson GAT, Just SJE. Relationship of platelet aggregation to bleeding after cardiopulmonary bypass. Ann Thorac Surg 1994;57:981–6.
[18] Watala C, Boncler M, Golanski J, Koziolkiewicz W, Walkowiak B, Cierniewski CS. Release of calcium and P-selectin from intraplatelet granules is hampered by procaine. Thromb Res 1999;94:1–11.
[19] Watala C, Golanski J, Boncler MA, Pietrucha T, Gwozdzinski K. Membrane lipid fluidity of blood platelets: a common denominator underlying the opposite actions of various agents affecting platelet activation in whole blood. Platelets 1998;9:315–27.
[20] Bock M, de Haan J, Beck KH, Gutensohn K, Hertfelder HJ, Karger R, Heim MU, Beeser H, Weber D, Kretschmer V. Standardization of the PFA-100(R) platelet function test in 105 mmol/l buffered citrate: effect of gender, smoking, and oral contraceptives [in process citation]. Br J Haematol 1999;106:898–904.
[21] Sestito A, Sciahbasi A, Landolfi R, Maseri A, Lanza GA, Andreotti F. A simple assay for platelet-mediated hemostasis in flowing whole blood (PFA-100): reproducibility, and effects of sex, and age. Cardiologia 1999;44:661–5.
[22] Zar J. Biostatistical analysis. Upper Saddle River, NJ: Prentice-Hall, 1999.
[23] Lefkovits J, Plow EF, Topol EJ. Platelet Glycoprotein IIb/IIIa Receptor in Cardiovascular Medicine. N Engl J Med 1995;332:1553–9.
[24] Fuster V. Mechanisms of arterial thrombosis: foundation for therapy. Am Heart J 1998;135:S361–6.
[25] McGregor J, Berdeaux A, Bonnet J, Cambien F, Fitzgerald D, Lacolley P, Lu H, Le-Saux-Narjoz A, Miossec P, Netter P, Poston R, Laurent S. Proteines d'adhesion et applications pharmacologiques. Table Ronde No 3 de Giens XIII. (Cell adhesion molecules and pharmacologic applications. Round Table No 3 at Giens XIII). Therapie 1998;53:371–9 [in French].
[26] Lister–James J, Vallabhajosula S, Moyer BR, Pearson DA, Mcbride BJ, Derosch MA, Bush LR, Machac J, Dean RT. Pre-clinical evaluation of technetium-99m platelet receptor-binding peptide. J Nucl Med 1997;38:105–11.
[27] Reverter JC, Beguin S, Kessels H, Kumar R, Hemker HC, Coller BS. Inhibition of platelet-mediated, tissue factor-induced thrombin generation by the mouse/human chimeric 7E3 antibody—potential implications for the effect of c7E3 Fab treatment on acute thrombosis and "clinical restenosis." J Clin Invest 1996;98:863–74.
[28] Coller BS. GPIIb/IIIa antagonists, pathophysiologic, and therapeutic insights from studies of c7E3 Fab. Thromb Haemost 1997;78:730–5.
[29] Matsuno H, Kozawa O, Niwa M, Ito T, Tanabe K, Nishida M, Hayashi H, Uematsu T. Effect of GR144053, a fibrinogen-receptor antagonist, on thrombus formation and vascular patency after thrombolysis by tPA in the injured carotid artery of the hamster. J Cardiovasc Pharmacol 1998;32:191–7.
[30] Owens MR, Holme S. Aurin tricarboxylic acid inhibits adhesion of platelets to subendothelium. Thromb Res 1996;81:177–85.
[31] Kinlough-Rathbone RL, Packham MA. Unexpected effects of aurin tricarboxylic acid on human platelets. Thromb Haemost 1992;68:189–93.
[32] Nakamura T, Kambayashi J, Okuma M, Tandon NN. Activation of the GP IIb-IIIa complex induced by platelet adhesion to collagen is mediated by both $\alpha 2\beta 1$ integrin and GP VI. J Biol Chem 1999;274:11897–903.
[33] Hu DD, White CA, Panzer Knodle S, Page JD, Nicholson N, Smith JW. A new model of dual interacting ligand binding sites on integrin αIIbβ3. J Biol Chem 1999;274:4633–9.
[34] Cardarelli PM, Cobb RR, Nowlin DM, Scholz W, Gorcsan F, Moscinski M, Yasuhara M, Chiang SL, Lobl TJ. Cyclic RGD peptide inhibits $\alpha 4\beta 1$ interaction with connecting segment 1, and vascular cell adhesion molecule. J Biol Chem 1994;269:18668–73.
[35] Yamamoto H, Vreys I, Stassen JM, Yoshimoto R, Vermylen J, Hoylaerts MF. Antagonism of vWF inhibits both injury induced arterial and venous thrombosis in the hamster. Thromb Haemost 1998;79:202–10.
[36] Guo Z, Weinstein MJ, Phillips MD, Kroll MH. M(r) 6,400 aurin tricarboxylic acid directly activates platelets. Thromb Res 1993;71:77–88.
[37] Weinstein M, Vosburgh E, Phillips M, Turner N, Chute–Rose L, Moake J. Isolation from commercial aurintricarboxylic acid of the most effective polymeric inhibitors of von Willebrand factor interaction with platelet glycoprotein Ib. Comparison with other polyanionic and polyaromatic polymers. Blood 1991;78:2291–8.
[38] Li CK, Hoffmann TJ, Hsieh PY, Malik S, Watson WC. The Xylum clot signature analyzer: a dynamic flow system that simulates vascular injury. Thromb Res 1998;92:S67–77.