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TETRAFIBRICIN HAS A HIGH SELECTIVITY FOR GPIIB/IIIA: COMPARISON OF THE EFFECTS OF TETRAFIBRICIN AND RGDS ON GPIIB/IIIA AND THE VITRONECTIN RECEPTOR

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SUMMARY: The specificity of tetrafibricin was examined by comparing its activities on GPIIb/IIIa and on the vitronectin receptor ($\alpha_v\beta_3$) with those of Arg-Gly-Asp-Ser (RGDS) on the same receptors. Tetrafibricin, which inhibited fibrinogen-GPIIb/IIIa binding 10 times more potently than RGDS, was three orders of magnitude less potent compared to RGDS on the inhibition of fibrinogen binding to $\alpha_v\beta_3$. Furthermore, tetrafibricin potently inhibited platelet adhesion to both fibrinogen and von Willebrand factor. Whereas, there was no significant inhibition observed in the GPIIb/IIIa-independent cellular adhesions. These results suggest that tetrafibricin is highly selective for GPIIb/IIIa.

Fibrinogen binding to its receptor, glycoprotein (GP) IIb/IIIa, on the platelet surface can result in the formation of platelet aggregates. A number of studies have clearly demonstrated that fibrinogen interacts with GPIIb/IIIa through two distinct amino acid sequences, Arg-Gly-Asp (RGD) present in the α chain and His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val(HHLGGAKQAGDV, γ400-411) at the carboxy terminus of the γ chain [1,2]. The RGD sequence was used as basis for the synthesis of much more potent GPIIb/IIIa antagonists such as cyclic peptides and peptidomimetics [3, 4, 5, 6].

Tetrafibricin is a novel fibrinogen receptor antagonist recently discovered in the cultural broth of *Streptomyces neyagawaensis*. In previous studies we could demonstrate that tetrafibricin exhibited potent inhibition on platelet aggregation through its blockage of the GPIIb/IIIa receptor on the platelet surface [7, 8]. Compared to the other fibrinogen receptor antagonists [3, 4, 5, 6], tetrafibricin is extremely unique in the respect of having no peptidic sequence in its molecule (Fig. 1). Besides platelet GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$), several other integrins (e.g. $\alpha_5\beta_1$, $\alpha_3\beta_1$ and $\alpha_v\beta_3$) are recognized by RGD-containing peptides [9].

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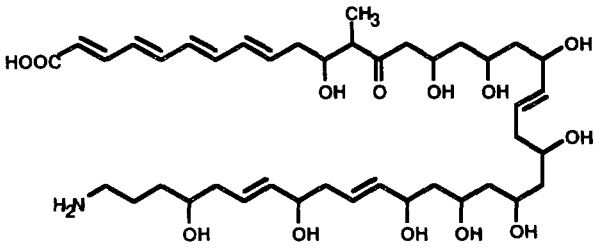

Fig. 1. **Structure of tetrafibricin.**

Therefore, the potential drawbacks of these peptides is their poor selectivity among integrin receptors.

In the present study we examined the selectivity of tetrafibricin for GPIIb/IIIa by comparing its effects on GPIIb/IIIa and $\alpha_v\beta_3$, the most closely related integrin, with those of RGDS. Tetrafibricin was three orders of magnitude less potent than RGDS in inhibiting fibrinogen binding to purified $\alpha_v\beta_3$. This was in contrast to the fact that tetrafibricin was 10 times more potent than RGDS in inhibiting the fibrinogen-GPIIb/IIIa binding. Furthermore, tetrafibricin showed no inhibition in the adhesion of endothelial cells to proteins containing RGD, whereas, the adhesion of platelets to these proteins was abolished in the presence of tetrafibricin.

These results indicate the selectivity of tetrafibricin towards GPIIb/IIIa versus $\alpha_v\beta_3$ and represent the possibility to design novel nonpeptidic and selective inhibitor of GPIIb/IIIa.

MATERIALS AND METHODS

Materials

The purification of tetrafibricin from the microbial broth of *Streptomyces neyagawaensis* NR0577 has been described previously [10]. Rabbit anti-β_3 antibody was from Chemicon Int. Inc. (Temecula, CA). Rabbit anti-human fibrinogen antibody was from Dakopatts (Copenhagen, Denmark). Anti-LFA-1 (BCA-1) was from British Technology (UK) and anti-ELAM-1 was a gift from Barry. Wolitzky (Dept. Molecular Genetics, Hoffmann La Roche Inc. Nutley N.J.). All other materials were of reagent grade unless specified otherwise.

Purification of GPIIb/IIIa and vitronectin receptor ($\alpha_v\beta_3$)

The active GPIIb/IIIa was purified from the Triton X-100 lysate of outdated human platelets as described previously [10]. Glycoproteins retained by concanavalin A were eluted and then applied to an aminoethyl-glycyl(Aeg-)-RGDS-Sepharose column. Bound GPIIb/IIIa was eluted by RGDS (3 mM). Human $\alpha_v\beta_3$ was purified from placenta as described previously [11] using a concanavalin A-Sepharose 4B and an Aeg-RGDS affinity column.

Fibrinogen binding to immobilized GPIIb/IIIa and $\alpha_v\beta_3$

The binding of fibrinogen to immobilized active GPIIb/IIIa or to $\alpha_v\beta_3$ was performed according to the method described previously [11]. Briefly, fibrinogen (0.5 μg/ml for GPIIb/IIIa and 1.5 μg/ml for $\alpha_v\beta_3$) was added to the wells coated with purified active GPIIb/IIIa or $\alpha_v\beta_3$ at 0.5 μg/ml and incubated overnight at room temperature. Bound fibrinogen was detected by enzyme-linked immunosorbent assay (ELISA) using antihuman fibrinogen antibodies and antirabbit Ig antibodies.

Platelet preparation

Platelets were isolated by gel-filtration according to the procedure described [7] and then suspended at 1×10^8 cells/ml Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.5 mM glucose, 3 mM NaH_2PO_4 , 0.35% BSA and 3.5 mM HEPES, pH 7.2).

Cell adhesion assays

For the platelet adhesion, the plastic wells were coated with fibrinogen (0-20 μg/ml) or von Willebrand factor (vWF) (0-10 μg/ml). Platelets stimulated with ADP were prepared by incubating the platelet suspension (1×10^8/ml) with 20 μM ADP for 2 min at room temperature. Untreated or stimulated platelets (1×10^7 cells/well) were then allowed to adhere to protein-coated wells at room temperature in the presence of a test compound without shaking. After a 20 min incubation, the wells were washed three times and fixed with 1% paraformaldehyde for 1 hour. The extent of platelet adhesion was determined with an ELISA system by incubating the wells containing adherent platelets with polyclonal anti-β_3 antibodies followed by anti-rabbit Ig as described for the fibrinogen-GPIIb/IIIa binding assay.

Soluble form of ICAM-1 (sICAM-1) and E-selectin containing lec/EGF/CR6 domains (ELAM) were prepared and generously gifted from Drs. B. Wolitzky, D. Presky (Dept. Molecular Genetics, Hoffmann-La Roche Inc., Nutley, N.J.) and J. Merritt (Dept. Inflammation, Roche Products Ltd., Welwyn Garden City, U.K.). Before used for the adhesion assays, cells were fluorescently labeled. Briefly, HL-60 cells (5×10^6 cells/ml) were labeled for 30 min at 37°C with 40 μg/ml of 6 carboxy fluorescein diacetate (6-CFDA) (Calbiochem, C.A.). JY cells (2×10^6 cells/ml) were labeled for 1 hr at 37°C with 2 μg/ml of 1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethylindo carbocyanine perchlorate (DiI) (Molecular Probe Inc., O.R.). Adhesions of fluorescently labeled JY cells or HL-60 cells to the protein-coated wells were conducted as described in [12]. Non adherent cells were removed by aspiration and 2 gentle washings with Tyrode's buffer and the fluorescence associated with adherent cells was determined in the cytofluor ™ 2300 (Millipore M.A.).

Bovine aortic endothelial cells (BAEC) were donated from Barry Wolitzky and maintained as described previously [8]. The adhesion of BAEC (1×10^6/ml) to the wells coated with fibrinogen (15 μg/ml), vWF (15 μg/ml) or vitronectin (0.23 μg/ml) was measured by a modification of the method described by Ruoslahti et al. [13]. The adherent cells were fixed with 3.7% formaldehyde for 30 min and stained with 0.5% crystal violet solution. The dye was solubilized with the addition of 50% ethylene glycol. The number of adhered cells was quantified by reading the absorbance at 595 nm.

RESULTS

Tetrafibricin selectively inhibits the fibrinogen binding to GPIIb/IIIa

In Fig. 2, the potency of tetrafibricin was compared to that of RGDS in two solid phase assay systems, fibrinogen bindings to purified active GPIIb/IIIa (α_{IIb}/β_3) and to purified vitronectin receptor ($\alpha_v\beta_3$). Even though tetrafibricin was 15-fold more potent than RGDS in inhibiting fibrinogen binding to GPIIb/IIIa (IC_{50} = 0.2 and 2.9 μM, for tetrafibricin and RGDS, respectively) (Fig.2-A), this compound was 3 orders of magnitude less potent than RGDS in inhibiting fibrinogen binding to the immobilized $\alpha_v\beta_3$ receptor, with an IC_{50} of 15 μM, as compared with an IC_{50} of 0.008 μM for RGDS (Fig. 2-B). These results indicate that tetrafibricin is highly selective for GPIIb/IIIa versus $\alpha_v\beta_3$ in a purified system.

Tetrafibricin inhibits platelet adhesion to proteins containing RGD

Platelet adhesion to proteins containing RGD is reported to be mediated by the binding between GPIIb/IIIa and the RGD motif in the adhesive proteins. In order to further

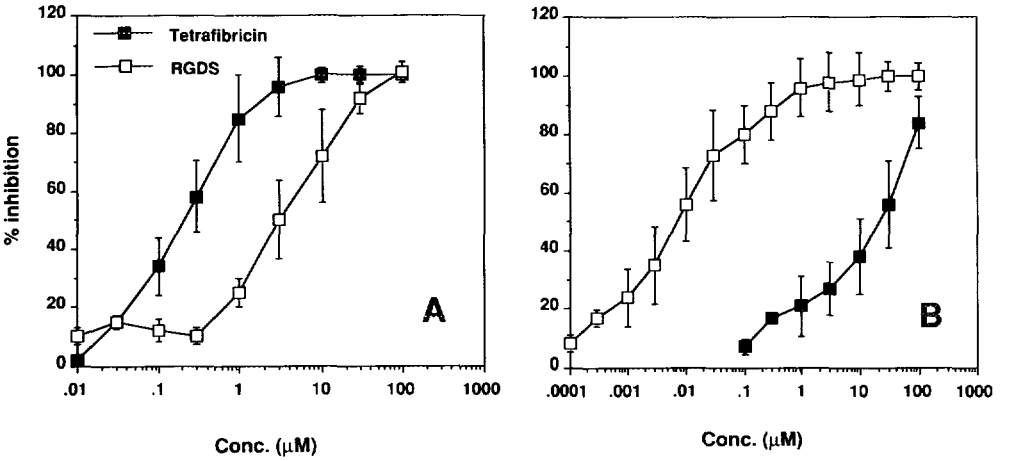

Fig. 2. **Inhibition of fibrinogen binding to immobilized GPIIb/IIIa (A) and vitronectin receptor (B).**

Microtiter wells were coated with purified GPIIb/IIIa (A) or $\alpha_v\beta_3$ (B) at 0.5 μg/ml and blocked with 1% BSA. Fibrinogen (0.5 μg/ml for GPIIb/IIIa (A) or 1.5 μg/ml for $\alpha_v\beta_3$ (B)) was bound to the immobilized receptor at room temperature overnight in the presence of increasing concentrations of the test compound. Bound fibrinogen was detected by an ELISA system as described in Materials and Methods. Results show the mean values from 5-7 experiments.

demonstrate the inhibitory properties of tetrafibricin, we next tested tetrafibricin on the adhesion of platelets to fibrinogen and vWF. GPIIb/IIIa recognizes the soluble form of fibrinogen only after activation of the receptor, whereas, no activation is required for platelet adhesion to fibrinogen immobilized onto a plastic plate [14].

The adhesion of non-stimulated platelets to immobilized fibrinogen (0 to 20 μg/ml) was dependent on the coating concentration of fibrinogen (Fig. 3-A). Two to threefold more platelet adhesion was observed when GPIIb/IIIa on the platelet surface was activated by 20 μM ADP. In contrast, the platelet adhesion to vWF was entirely dependent on the activation of GPIIb/IIIa, since no significant adhesion was observed when the resting platelets were added to wells coated with vWF (Fig. 3-B).

As shown in Table 1, the platelet adhesion to these two proteins (fibrinogen or vWF), either with or without stimulation, was inhibited in the presence of tetrafibricin (IC_{50}s of 24-105 μM). In this assay, tetrafibricin was 14- to 20-times more potent than RGDS. These results agree well with the above results that tetrafibricin was approximately 10 times more potent than RGDS on fibrinogen binding to purified GPIIb/IIIa (Fig. 2-A). Compared to the adhesion of resting platelets, slightly more amount of tetrafibricin or RGDS is required to inhibit the adhesion of ADP-stimulated platelets (Table 1).

Tetrafibricin showed little inhibition on the vitronectin receptor-dependent adhesion

In order to test the specificity of tetrafibricin for GPIIb/IIIa on the cellular level, we selected three cellular adhesion systems, endothelial cell adhesion to RGD-containing

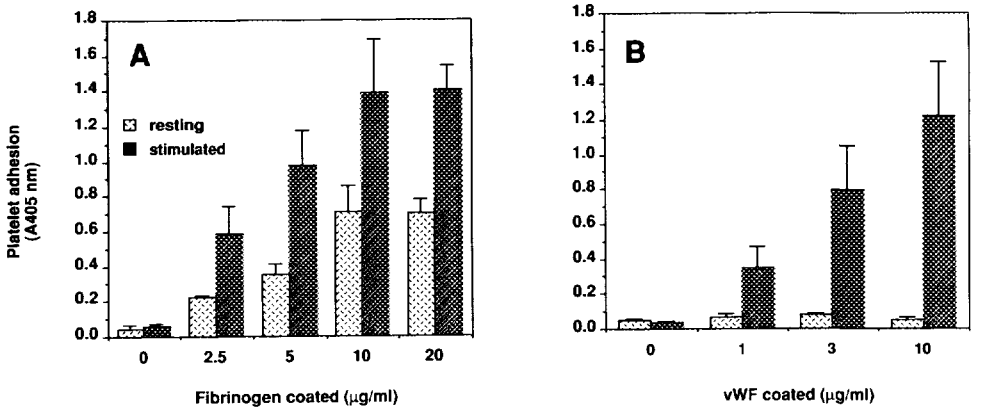

Fig. 3. **Adhesion of resting- or stimulated-platelets to immobilized fibrinogen (A) or von Willebrand factor (B).**

Microtiter wells were coated with fibrinogen (0-20 μg/ml) or vWF (0-10 μg/ml) and blocked with 1% BSA. Gel-filtered platelets (1×10^8 cells/ml) had adhered to the protein-coated wells during the 20 min period at room temperature. After fixation with 1% paraformaldehyde, the adhered platelets were quantified, based on the ELISA system using anti-β_3 antibodies followed by anti-rabbit Ig as described in Materials and Methods. Stimulated platelets were prepared by adding 20 μM ADP to the platelet suspension 2 min prior to the adhesion assay. Results show the mean values from 4-6 experiments.

proteins, JY cell adhesion to sICAM-1 and HL-60 cell adhesion to ELAM, and examined the effects of tetrafibricin and RGDS.

The adhesion of endothelial cells to proteins containing RGD appeared to be mediated predominantly via $\alpha_v\beta_3$ [15]. The fact that endothelial cells adhere to fibrinogen, vWF and vitronectin agrees well with the fact that $\alpha_v\beta_3$ recognizes all of these proteins as ligands [16, 17] and plays a predominant role in the observed adhesion. In addition to $\alpha_v\beta_3$, another RGD-dependent integrin, $\alpha_5\beta_1$ (fibronectin receptor) is reported to be expressed on the

Table 1 **Effects of tetrafibricin and RGDS on the adhesion of platelets to fibrinogen or von Willebrand factor**

Adhesion protein	stimulation	Tetrafibricin	RGDS
		(IC_{50} in μM)	
Fbg	resting	24 ± 5	480 ± 14
Fbg	+ADP	59 ± 11	1125 ± 25
vWF	+ADP	105 ± 9	1455 ± 25

Microtiter wells were coated with fibrinogen (Fbg, 15 μg/ml) or vWF (15 μg/ml) and blocked with 1% BSA. Gel-filtered platelets (1×10^8 cells/ml) were treated with 20 μM ADP (+ADP) or saline (resting) for 2 min prior to the addition to the protein-coated wells. Cell adhesion was performed at 37 °C for 20 min. The adhered cells were fixed with paraformaldehyde and quantified by an ELISA system. Each value represents the mean and SD of the IC_{50} (μM) from 4-7 experiments.

Table 2 **Effects of tetrafibricin and RGDS on vitronectin receptor-dependent endothelial cell adhesions**

Proteins coated	Tetrafibricin	RGDS
	(IC_{50} in μM)	
Fibrinogen	>3000	31 ± 6
von Willebrand factor	>3000	9 ± 3
Vitronectin	>3000	39 ± 8

Microtiter wells were coated with fibrinogen (Fbg, 1.5 μg/well), vWF (1.5 μg/well) or vitronectin (VN, 0.023 μg/well) and blocked with 1% BSA. Bovine endothelial cells were adhered to the protein-coated wells for 2 hours at 37°C. The adhered cells were fixed and quantified by staining with crystal violet. Each value represents the mean and SD of the IC_{50} (μM) from 2-4 experiments.

endothelial cell surface [15]. It seems, however, unlikely that $\alpha5\beta1$ plays a significant role in the endothelial cell adhesion observed here, because $\alpha5\beta1$ has a poor affinity for RGD-containing proteins other than fibronectin. Moreover, our preliminary experiments indicated that neither RGD-peptides nor tetrafibricin were effective in the adhesion of endothelial cells to fibronectin (unpublished observations). Tetrafibricin is a poor inhibitor of endothelial cell adhesion to the RGD-containing proteins tested. Because Tetrafibricin, up to 3 mM, exhibited no significant inhibition (less than 5% inhibition) on all the adhesions tested here. In contrast, the IC_{50} values of RGDS on endothelial cell adhesion were 9 to 39 μM (Table 2).

Tetrafibricin showed little inhibition on the leukocyte adhesions

Furthermore, we found that tetrafibricin (up to 3 mM) exhibited no inhibition on leukocyte adhesions mediated via $\beta2$ integrin (LFA-1, CD11a/CD18) or E-selectin (ELAM-1). In the preliminary experiments, adhesions of JY cells and HL-60 cells were eliminated by adding

Table 3 **Effects of tetrafibricin on leukocyte cell adhesions**

compound	conc.	% inhibition on	
		JY cell adhesion to ICAM-1	HL-60 cell adhesion to E-selectin
Tetrafibricin	3 mM	7±4	1±5
BCA-1 (anti-LFA-1)	10 μg/ml	95±13	not tested
3B7 (anti-ELAM-1)	10 μg/ml	not tested	91±6

Microtiter wells were coated with sICAM-1 (0.25 μg/well) or ELAM (0.1 μg/well) and blocked with 1% BSA. Fluorescently labeled JY cells (2×10^5 cells/well) or HL-60 cells (5×10^5 cells/well) were added to sICAM-1 - or ELAM-1-coated wells, respectively, as described in Materials and Methods. For JY cell adhesion, 5 mM $MnCl_2$ was included throughout the incubation period. The adhered cells were quantified by measuring the fluorescence in each wells after removing the unbound cells with 2 gentle washings.

anti-LFA-1 (BCA-1) and anti-ELAM-1 (3B7) monoclonal antibodies, respectively (Table 3).

Taken together, these data can support the significant selectivity of tetrafibricin for GPIIb/IIIa.

DISCUSSION

Peptidic GPIIb/IIIa antagonists based on the RGD sequence have been designed as platelet inhibitors [3, 4, 5, 6]. Since the RGD peptide can be recognized by several integrins other than GPIIb/IIIa, the high selectivity of tetrafibricin for GPIIb/IIIa is an advantageous feature of this compounds.

Based on our previous results, tetrafibricin exhibited potent anti-platelet activities through blocking fibrinogen-GPIIb/IIIa interactions [8]. We have also identified that tetrafibricin could induce conformational changes in the inactive form of GPIIb/IIIa [18]. Taken together, these results suggest that tetrafibricin is able to interact with not only the active but also inactive forms of GPIIb/IIIa. The data presented in this study can be another evidence to support the ability of tetrafibricin to interact with inactive GPIIb/IIIa on the resting platelets, because adhesions of resting platelets, as well as that of stimulated platelets, were completely abolished in the presence of tetrafibricin. The reason why tetrafibricin was slightly less potent to inhibit the adhesion of stimulated platelets than to inhibit the adhesion of resting platelets is unclear at present.

Ultimately, the data in this study indicate a prominent difference between RGDS and tetrafibricin in their selectivity among GPIIb/IIIa and $\alpha_v\beta_3$. Among the members of integrin family, $\alpha_v\beta_3$ is closely related to GPIIb/IIIa. They share the same β subunit (β_3) and there is a significant homology between each α subunit [19]. In contrast to RGDS, tetrafibricin inhibits GPIIb/IIIa preferentially to $\alpha_v\beta_3$. One possible explanation for these results is that the mechanisms of tetrafibricin to interact with the integrin receptor might not be the same as that of RGDS. In the previous study [7], we observed that tetrafibricin, when combined with either RGDS or γ400-411, exhibited additive, not synergistic, effects. Recent several studies reported that the binding sites for RGD peptides are not identical to those for γ400-411 [20, 21, 22]. In order to account for the apparent additive character of two inhibitors, it is therefore most likely that the binding sites of two inhibitors are distinct but possibly inducing conformational change(s) in GPIIb/IIIa complex that preclude the binding of the other [23]. Judging from the additive effects observed, the site(s) recognized by tetrafibricin, RGDS and γ400-411 might not be identical to each other. At present we have no obvious explanation on whether the binding of tetrafibricin to GPIIb/IIIa differs from that of RGDS or not. Further analysis in light of the structural elements of tetrafibricin that account for its ability to interact with GPIIb/IIIa and/or the responsible regions of GPIIb/IIIa involved in the recognition of tetrafibricin remains to be investigated.

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