Biochimica et Biophysica Acta, 777 (1984) 37–40
Elsevier

BBA 72260

THE EFFECT OF α,α′-BIS[3-(*N*,*N*-DIETHYLCARBAMOYL)PIPERIDINO]-*p*-XYLENE ON HUMAN BLOOD PLATELET STRUCTURAL PHYSIOLOGY

ANDREW LASSLO [a,*] and JAMES G. WHITE [b]

[a] *Department of Medicinal Chemistry, College of Pharmacy, University of Tennessee Center for the Health Sciences, Memphis, TN 38163 and* [b] *Departments of Pediatrics, and Laboratory Medicine and Pathology, College of Medicine, University of Minnesota Health Sciences Center, Minneapolis, MN 55455 (U.S.A.)*

(Received April 4th, 1984)

Key words: Piperidine derivative; Platelet aggregation; Membrane stabilization; Electron microscopy

α,α′-Bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-*p*-xylene enhances human blood platelet membrane integrity by exerting a stabilizing action at the level of the dense tubular system in surface membrane complexes known to sequester platelet calcium.

Introduction

In a comprehensive study, undertaken to develop agents enhancing human blood platelet membrane integrity during biodynamic impacts capable of triggering adhesion-release-aggregation chain reactions, structural features of synthetic entities were related to their effect on human blood platelet function [1–11]. The several series of compounds served, in effect, as molecular probes. Systematic alteration in the structure permitted interpretation of platelet-response patterns in terms of the most recently evolved chemical parameters, and allowed actual 'fine-tuning' of structural features to enhance the sought effects.

Here, we report the effect of one of the potent carbamoylpiperidine congeners [1], α,α′-bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-*p*-xylene, on human blood platelet structural physiology. In this compound, the distance between the two platelet aggregation-inhibitory specific functions (tertiary amine ring nitrogens), computed with Godfrey space-filling models, ranges 7.8–8.3 Å with a flexibility variance of only 0.5 Å; consequently, the functions within the rigid xylylene analog are locked into an intramolecular distance deemed optimal for inhibiting ADP-induced human blood platelet aggregation [1,2,5].

Materials and Methods

Inhibitor. α,α′-Bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-*p*-xylene dihydrobromide, m.p. 284.2–284.8 (dec.), is one of the analogs prepared and characterized by Quintana et al. [12]; they were, along with other carbamoylpiperidine congeners synthesized earlier by Lasslo et al. [13] and Lasslo and Waller [14], discussed extensively in previous articles [1–11]. Paralleling preceding studies [1,15], the compound was introduced in 0.5 μl redistilled 95% ethanol into citrated platelet-rich plasma to yield 10 and 100 μM final concentrations and, after 4-min incubation at 37 °C, the aggregation-inducing agent was added.

Aggregation. After informed consent, blood was obtained from normal adult donors tested repeatedly in our laboratory. Following venipuncture, the sample was mixed immediately with citrate-citric acid-dextrose (93 mM sodium

* To whom correspondence should be addressed.

citrate/7 mM citric acid/140 mM dextrose) (pH 6.5) in a ratio of nine parts blood to one part anticoagulant [16]. Citrated platelet-rich plasma was separated from whole blood by centrifugation at room temperature for 20 min at $100 \times g$. Platelet aggregation studies were performed using a Payton (Buffalo, NY) dual-channel aggregometer with platelet-rich and platelet-poor plasma. Agents used routinely in the evaluation of platelet aggregation were made up in stock solutions such as phosphate-buffered saline or Hank's balanced salt solution and maintained in the frozen state between experiments, unless fresh preparation was required [17]. Aggregants added to citrated platelet-rich plasma included ADP at 2.5 μM, acid-soluble collagen (Worthington, Freehold, NJ) at 30 μg/ml, epinephrine at 5.5 μM, bovine thrombin (Parke-Davis, Detroit, MI) at 0.2 U/ml, and the sodium salt of arachidonic acid (greater than 99% pure, NuChek Prep, Elysian, MN) at 0.8 mM; each was tested first on an untreated sample of citrated platelet-rich plasma on the aggregometer, and all responses were recorded on moving graph paper [18].

Ultrastructure. Citrated platelet-rich plasma, incubated with the inhibitor as described above, was prepared for study in the electron microscope according to methods reported in detail in previous publications [16,17,19–21]. Samples of citrated platelet-rich plasma stirred with aggregating agents, following the referenced incubation, were fixed 1 min after maximum aggregation or at the height of upward deflection of the recording pen if aggregation was inhibited. Briefly, the samples were combined with an equal volume of 0.1% glutaraldehyde in White's saline (pH 7.3) (a 10% solution of a 1 : 1 mixture of: (A) 2.4 M NaCl, 0.1 M KCl, 46 mM $MgSO_4$, 64 mM $Ca(NO_3)_2 \cdot 24\ H_2O$, and (B) 0.13 M $NaHCO_3$, 8.4 mM $Na_2HPO_4 \cdot 7\ H_2O$, 3.8 mM anhydrous KH_2PO_4, and 0.1 g/l Phenol red). After 15 min at 37 °C, the samples were sedimented to pellets, the supernatant was discarded and replaced with 3% glutaraldehyde in the same buffer. Fixation was continued at 4 °C for 60 min. The cells were then washed in buffer and combined with 1% osmic acid in veronal acetate (0.02 M HCl and a 20% solution of a stock buffer solution containing 0.14 M sodium barbital, 0.145 M sodium acetate $\cdot$ 3 H_2O, and a 6.8% solution of a stock salt solution containing 1.7 M NaCl, 54 mM KCl and 18 mM $CaCl_2$). After exposure to the second fixation for 1 h, the cells were dehydrated in a graded series of alcohol and embedded in Epon 812. Contrast of thin sections cut from plastic blocks on an ultramicrotome was enhanced with uranyl acetate and lead citrate. Observations were made in a Philips 301 electron microscope.

Results and Discussion

The results obtained in this investigation further corroborate earlier conclusions that certain carbamoylpiperidine derivatives, including α,α'-bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-*p*-xylene [1,5,7], exert a stabilizing action on platelets at the level of the dense tubular system in surface membrane complexes known to sequester platelet calcium [22]. For aggregometric studies on α,α'-bis[3-(*N*,*N*-diethylcarbamoyl)piperidino]-*p*-xylene, employing ADP and other platelet stimulants, the reader is referred to previous papers [1,3,6]. The compound's influence on platelet response effected by exogenously added and intracellularly released Ca^{2+} was also reported earlier [6]. Its striking interaction with anionic phospholipids, phosphatidylserine and phosphatidylinositol, and the lack of it with phoshatidylcholine and phosphatidylethanolamine, was described as well [7]. The rationale underlying the mechanism by means of which this class of compounds are envisioned to exert their action has been discussed in a recently published monograph [5] and, more concisely, in an equally current paper [4].

In evaluating the effect of this carbamoylpiperidine derivative on platelet morphology, the incubation of platelet-rich plasma with 100 μM concentrations of the compound, for 60 min, did not produce any alteration. In fact, the compound tended to preserve platelet discoid shape (Fig. 1).

The extent of the compound's influence in enhancing platelet membrane stability was relfected in its pattern of inhibitory responses to a series of aggregation-inducing agents. The compound completely inhibited collagen-induced aggregation and shape change at 100 μM concentrations (Fig. 1b). Such effect was not discernible, however, at 10 μM concentrations of the inhibitor; in fact, the electron photomicrograph taken in this instance il-

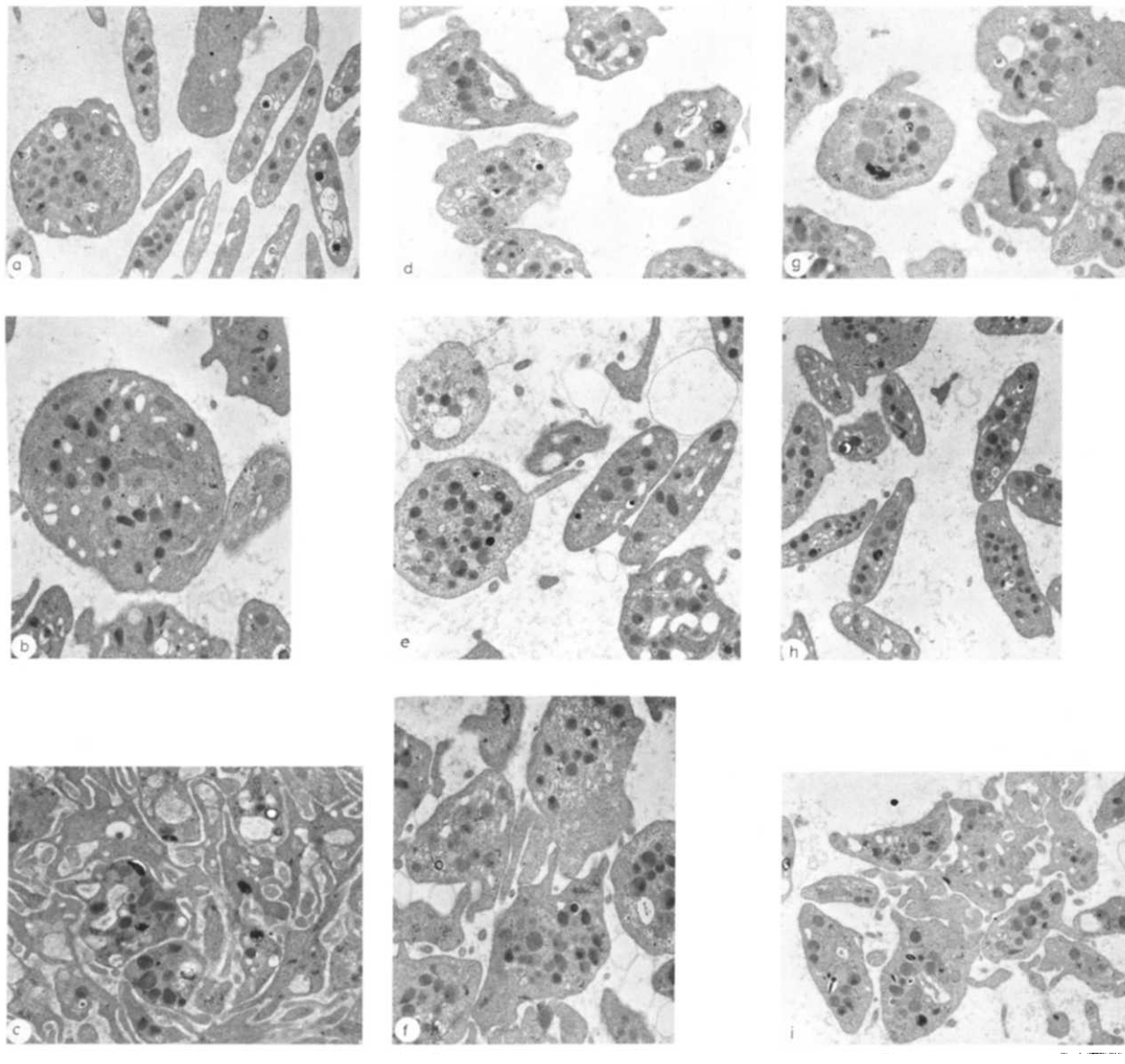

Fig. 1. Effect of the inhibitor, the carbamoylpiperidine derivative, on platelet morphology. (a) Platelets incubated for 60 min with the compound, at 100 μM concentration ($\times$6600). (b) and (c) The inhibitor's effect at 100 (b) and 10 μM (c) concentrations, respectively, on collagen-induced aggregation ($\times$8400). (d) The effect of the compound on thrombin-induced aggregation at 10 μM concentration ($\times$8400). (e) and (f). Inhibition of ADP-induced aggregation at 100 (e) and 10 μM (f) concentrations, respectively ($\times$8400). (g) Inhibition of arachidonic acid-induced aggregation at 100 μM concentrations of the compound ($\times$9600). (h) and (i) The inhibitor's effect at 100 (h) and 10 μM (i) concentrations, respectively, on epinephrine-induced aggregation ($\times$6600).

lustrates a typical aggregate of degranulated platelets from a collagen-induced aggregation (Fig. 1c). Lower concentrations (10 μM) of the compound remained effective, however, in impeding thrombin-induced aggregation, even though some shape change was evident (Fig. 1d). ADP-induced aggregation was inhibited at 100 μM concentrations, but the agent did not prevent some degree of shape change (Fig. 1e); this became more pronounced at the agent's lower concentration (10

μM) at which indications of adhesiveness also seemed to surface (Fig. 1f). Higher concentrations (100 μM) of the compound inhibited arachidonic acid-induced aggregation, but did not avert some shape change (Fig. 1g). Epinephrine-induced aggregation was blocked by the compound at 100 μM concentrations (Fig. 1h); however, at lower concentrations (10 μM), the inhibitor was not entirely effective in blocking adhesiveness and a few small aggregates could be observed (Fig. 1i).

The compound is apparently capable of inhibiting aggregation without blocking platelet responses associated with shape change and internal transformation in most instances. The compound appears, therefore, to exert its effect by reinforcing platelet membrane surfaces interacting with the external environment, rather than by acting on critical sites in the interior of the platelet.

Acknowledgements

We wish to take this opportunity to express our gratitude for the continued fiscal support from the National Heart, Lung and Blood Institute (USPHS Grants HL-11880, HL-16833, HL-22236 and HL-25884), the National Cancer Institute (CA-21737), the Gustavus and Louise Pfeiffer Research Foundation and the March of Dimes.

References

1 Quintana, R.P., Lasslo, A., Dugdale, M. and Goodin, L.L. (1981) Thromb. Res. 22, 665–680
2 Quintana, R.P., Lasslo, A. and Dugdale, M. (1982) Biophys. J. 37, 130–133
3 Lasslo, A., Quintana, R.P., Dugdale, M., Johnson, R.W. and Naylor, J.L. (1983) Asaio J. 6, 47–59
4 Lasslo, A. (1984) Fed. Proc. 43, 1382–1389
5 Lasslo, A. and Quintana, R.P. (1984) in Blood Platelet Function and Medicinal Chemistry (Lasslo, A., ed.), pp. 229–315, Elsevier, New York
6 Lasslo, A., Quintana, R.P., Johnson, R.W., Naylor, J.L. and Dugdale, M. (1984) Biochim. Biophys. Acta 772, 84–92
7 Quintana, R.P., Lasslo, A. and Greer, L.T. (1981) Thromb. Res. 24, 379–395
8 Quintana, R.P., Lasslo, A. and Queen, G.S. (1982) Chem. Biol. Interactions 38, 135–144
9 Lasslo, A., Quintana, R.P., Crisan, D., Baier, R.E., Meyer, A.E. and Fornalik, M.S. (1984) Med. Prog. Technol. 6, 47–59
10 Lasslo, A., Quintana, R.P. and Dugdale, M. (to the Research Corporation, Tucson) (1982) U.S. Patent pending, Serial No. 347,037; Canadian Patent pending, Serial No. 433,890 (1983)
11 Lasslo, A., Quintana, R.P., Dugdale, M. and Johnson, R.W. (to the Research Corporation, Tucson) (1984) U.S. Patent 4, 443, 450; U.S. Patent pending, Serial No. 368,863 (1982); Canadian Patent pending, Serial No. 425,994 (1983); European Patent pending, Serial No. 83103664.5 (1983) (covering Belgium, Germany (West), France, Great Britain, Italy, Lichtenstein, Luxembourg, The Netherlands and Switzerland); Danish Patent pending, Serial No. 1670 (1983); Greek Patent pending, serial No. 71.072 (1983); Irish Patent pending, Serial No. 825 (1983); Spanish Patent pending, Serial No. 521.794 (1983); Japanese Patent pending, Serial No. 066818 (1983); Israeli Patent pending, Serial No. 68423 (1983); Australian Patent pending, Serial No. 13411 (1983); South African Patent pending, serial No. 2584 (1983)
12 Quintana, R.P., Smith, T.D. and Lorenzen, L.F. (1965) J. Pharm. Sci. 54, 785–787
13 Lasslo, A., Marine, W.M. and Waller, P.D. (1956) J. Org. Chem. 21, 958–960
14 Lasslo, A. and Waller, P.D. (1957) J. Org. Chem. 22, 837–839
15 Quintana, R.P., Lasslo, A., Dugdale, M., Goodin, L.L. and Burkhardt, E.F. (1980) Thromb. Res. 20, 405–415
16 White, J.G. (1968) Blood 31, 604–622
17 Gerrard, J.M., Phillips, D.R., Rao, G.H.R., Plow, E.F., Walz, D.A., Ross, R., Harker, L.A. and White J.G. (1980) J. Clin. Invest. 66, 102–109
18 Rao, G.H.R. and White, J.G. (1981) Am. J. Hematol. 11, 355–366
19 White, J.G. and Krivit, W. (1967) Blood 30, 625–635
20 White, J.G. (1968) Am. J. Pathol. 53, 281–291
21 White, J.G. (1969) Am. J. Pathol. 54, 467–478
22 White, J.G. (1984) in Blood Platelet Function and Medicinal Chemistry (Lasslo, A., ed.), pp. 15–59, Elsevier, New York