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# In-vitro and Ex-vivo Inhibition of Blood Platelet Aggregation by Naftazone

P. DURAND, C. BLOY*, F. PELTIER-PUJOL* AND D. BLACHE

*Laboratoire de Biochimie des Lipoprotéines, Inserm CJF 93-10, Université de Bourgogne, Dijon, and *Laboratoires Cassenne, Osny, France*

## Abstract

Because of the considerable interest in the role of platelets and antiplatelet therapy in cardiovascular disease, including the aggregation of platelets to each other during arterial thrombosis and atherogenesis, we have studied the effect of naftazone (Etioven), an original vasculotropic drug on platelet aggregation.

Rat and human platelets were prepared and incubated in-vitro with different concentrations of naftazone. We found that naftazone inhibited both platelet secretion and aggregation in platelet-rich plasma (PRP) and washed platelets after stimulation with thrombin or ADP. Rats were also treated intraperitoneally for five days with various naftazone doses (0·125–10 mg $kg^{-1}$) and ex-vivo platelet aggregation compared, at various times after the last injection, with that of control animals. Inhibition by naftazone was dose-dependent in both PRP and isolated platelets. The inhibition was transient, a maximum value ($\sim 50\%$) being obtained about 3–6 h after the last injection, with a return to near-control values after 24 h. Naftazone also facilitated platelet deaggregation after in-vitro stimulation with thrombin or ADP. In another series of experiments, rats were treated intraperitoneally for five days with 10 mg $kg^{-1}$ of aspirin, ticlopidine, dipyridamole or naftazone. Platelets were prepared and tested for aggregation 90 min after the last injection. Thrombin-induced aggregation in PRP and washed platelets was significantly reduced after in-vivo treatment with ticlopidine and naftazone. Except for dipyridamole, all the drugs inhibited ex-vivo ADP-induced aggregation in PRP. In isolated platelet preparation, only naftazone induced a significant inhibition of ADP- or thrombin-stimulated aggregation.

We conclude that naftazone inhibits platelet aggregation in-vitro and ex-vivo.

Blood platelets are involved in haemostasis. They can adhere to blood vessel walls, release bioactive compounds and aggregate to each other. The well-established increase in these properties during arterial thrombosis and atherogenesis (Meade et al 1985; Sussman 1985; Ross 1986) provided the rationale for many drugs which inhibit platelet function (Ciavatti et al 1982; Blache & Ciavatti 1989; Antiplatelet Trialists ± Collaboration 1994) and also explains the considerable interest in the role of platelets and antiplatelet therapy in cardiovascular disease.

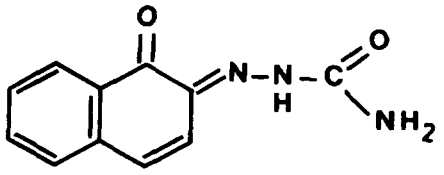
Aggregation is the final event of a multiple step process. Several agonists, such as ADP, adrenaline, thrombin and collagen induce the release of arachidonic acid after phospholipase activation through calcium mobilization (Hallam & Rink 1985; Blache 1992; Hashizume et al 1994). This fatty acid is metabolized by cyclo-oxygenase to endoperoxide and subsequently to thromboxane $A_2$ ($TXA_2$). After interaction with its receptor, $TXA_2$ induces an increase in free cytoplasmic calcium concentration. Mobilized calcium stimulates the release of the secretory granule contents including ADP, 5-hydroxytryptamine and other bioactive compounds (growth factors). Cyclic AMP plays an important role in the activation process, as prostacyclin is a potent inhibitor of platelet aggregation possibly through resequestration of cytoplasmic calcium (Feinstein et al 1985; Blache et al 1987**b**).

Several drugs have been developed to block the different steps in these activation pathways. Inhibition of platelet function by aspirin has been described by many investigators (De Gaetano et al 1985; Patrono 1994). Although other mechanisms have been proposed (Buchanan et al 1982), aspirin is thought to acetylate platelet and endothelial cyclo-oxygenase irreversibly, and blocks both thromboxane and prostacyclin production at high concentrations (FitzGerald et al 1988; Rao & White 1994).

New antithrombotic compounds are required to overcome the side-effects of existing agents, as well as population variability (Beving et al 1994; Pappas et al 1994; Roth & Calverley 1994). Thromboxane synthase inhibitors should not have major side-effects as they allow prostacyclin biosynthesis vessel in the wall. $TXA_2$/endoperoxide receptor antagonists can block these agents at the receptor level (Hoet et al 1993; Modesti et al 1994). Another step of the pathway can be blocked by monoclonal antibodies against glycoprotein IIb/IIIa receptors or peptides bearing the arginine-glycine-aspartic acid sequence (Coller 1992; FitzGerald & Meagher 1994; Kiss et al 1994).

Naftazone (Etioven, 1,2-naphthoquinone-2-semicarbazone; Scheme 1) is used in man for the treatment of vascular conditions such as peripheral vascular or veinous insufficiency (Berson 1977; Winand 1977). In view of the unexpected beneficial effects of naftazone and more recent results dealing with endothelial cell proliferation (Klein-Soyer et al 1991), specific studies have been carried out to determine whether naftazone affects platelet function. In this paper, we present the results of in-vitro and ex-vivo studies indicating that naftazone inhibits platelet aggregation.

Correspondence: D. Blache, Inserm CJF 93-10, Laboratoire de Biochimie des Lipoprotéines, Université de Bourgogne, 7 bd Jeanne d'Arc, 21033 Dijon Cedex, France.



SCHEME 1. Chemical structure of naftazone. Molecular weight 215.

## Materials and Methods

*Subjects*

After informed consent, all subjects were asked not to take any drug that might interfere with platelet function for 10 days before blood sampling. They were fasted for 12–14 h. After discarding the first 2 mL, blood was drawn as in previous studies (Blache et al 1992; Blache 1995) with plastic syringes containing either 1 vol sodium citrate anticoagulant (129 mM adjusted to pH 7·4 with 10% citric acid) for 9 vols blood for studies on platelet-rich plasma (PRP) or 3 vols acid citrate dextrose for 7 vols blood for studies of washed platelets.

*Animals*

Male Sprague-Dawley rats (200–250 g, Charles River, Cléon, France) were maintained in stainless steel cages in a well-ventilated room at 23–24°C on a 12-h light-dark cycle. They were maintained in compliance with the Inserm policy on animal care and use (similar to the National Research Council Guidelines; NRC 1985). Blood was removed as described elsewhere (Blache et al 1986, 1987a), from the jugular vein of animals fasted overnight, into plastic syringes containing either 1 vol sodium citrate anticoagulant (as for humans) for 9 vols blood for studies on PRP, or 1 vol acid citrate dextrose for 3 vols blood for washed platelet studies.

*Platelet preparations and aggregations*

Platelet aggregation was studied with PRP or platelets re-suspended in Tyrode buffer containing NaCl (149 mM), KCl (2·6 mM), $NaHCO_3$ (9·5 mM), glucose (5·5 mM), $NaH_2PO_4$ (0·5 mM), $MgCl_2$ (0·6 mM) and gelatine (Merck; 0·25%) and adjusted to pH 7·4 with 0·25 M HCl. PRP was obtained by centrifuging blood (120 *g*, 15 min). For aggregation in PRP, the platelet count was adjusted to $0{\cdot}5 \times 10^9$ mL with platelet-poor plasma (PPP). Two inducers (Sigma) were used, human thrombin and ADP at final concentrations of 1 int. units $mL^{-1}$ and 3 $\mu$M, respectively.

Platelet aggregation was recorded using Born's method (Born 1962) either with 0·5 mL of the platelet suspension in disposable polystyrene cuvettes placed in the turbidimetric coagulo-aggregometer (Renaud-Rubel) and stirred at 1100 rev $min^{-1}$ at 37°C or with a computerized four-channel laser-light aggregometer (Servibio, France). In the case of thrombin-induced aggregation, the platelet suspension contained 20% calcium-free Tyrode buffer (pH 7.4) to avoid coagulation (Blache et al 1992). Results represent the aggregation amplitude measured in mm or light transmission, and are usually expressed as percentage of control values.

Platelets were also isolated from PRP by centrifugation (1000 *g*; 18 min) and re-suspended in Tyrode buffer pH 7·4. Final inducer concentrations were 0·1 int. units $mL^{-1}$ and 1 $\mu$M for thrombin and ADP.

For in-vitro testing of the effect of naftazone on platelet aggregation, the drug, supplied by Laboratoires Cassenne (Puteaux, France), was solubilized in dimethylsulphoxide and added to the platelet suspension at different concentrations for 1 min at 37°C before treatment with the stimulating agents (Blache & Ojeda 1992).

*Platelet secretion*

Platelet 5-hydroxytryptamine (5-HT) was monitored by means of an electrochemical procedure developed in our laboratory and described elsewhere (Marcenac & Blache 1985). Using a pyrolytic carbon fibre microelectrode (MFC-1, Taccussel, Villeurbanne, France) electrochemically treated, 5-HT release was measured 2 min after aggregation, in supernatant obtained after quick centrifugation (9800 g, 10 s) of thrombin-stimulated platelets. Measurements were made by differential pulse voltammetry (polarograph PRG5, Taccussel, Villeurbanne, France) in the presence of 0·2 mM L-ascorbic acid as anti-oxidant. Results (means ± s.d.) were expressed as nmol 5-HT $10^{-9}$ platelets.

*Ex-vivo experiments*

*Experiment A.* This work combined a dose–response study and a time-course study of the effects of naftazone on ex-vivo platelet aggregation. A total of 128 male rats were used for these studies. Each animal received naftazone, solubilized in 1% methylcellulose, (Sigma) in 0·9% NaCl at doses of 0·125, 0·25, 0·5, 1·0, 5·0 and 10 mg $kg^{-1}$ (i.p.). Injections were made in the morning on five consecutive days (between 0700 and 0900 h); control animals received the vehicle (1% methylcellulose) only. Blood sampling was performed 1, 2, 3, 6 or 24 h after the last injection. Platelets were prepared and thrombin- and ADP-induced aggregation was analysed as indicated above.

*Experiment B.* Sixty rats were divided into five equal groups and were treated intraperitoneally on five consecutive days at 0700–0900 h, as follows: group A, vehicle only (1% methylcellulose); group B, 10 mg $kg^{-1}$ aspirin; group C, 10 mg $kg^{-1}$ ticlopidine; group D, 10 mg $kg^{-1}$ dipyridamole; group E, 10 mg $kg^{-1}$ naftazone. Blood samples were withdrawn 90 min after the last injection. Platelet-induced aggregation was analysed with PRP and isolated platelets, as indicated above.

*Deaggregation.* Deaggregation was studied after 600 s, when the plateau of the aggregation curve had been reached following addition of the platelet inducers. Results are expressed as a percentage of total aggregation amplitude.

*Statistical analysis*

Means were compared by using Student's *t*-test for unpaired samples. For multiple comparisons, we used one-way analysis of variance followed by the Newman-Keuls test, on PCSM Software (Meylan, France).

## Results

*In-vitro effects of naftazone*

Various concentrations of naftazone incubated with rat PRP tended to inhibit ADP and thrombin-induced aggregation (Fig. 1) with a significant difference relative to the control value at final concentrations of $10^{-6}$, $10^{-5}$ and $10^{-4}$ (inhibition of 7 to 17% and 62 to 85% after ADP and thrombin stimulation, re-

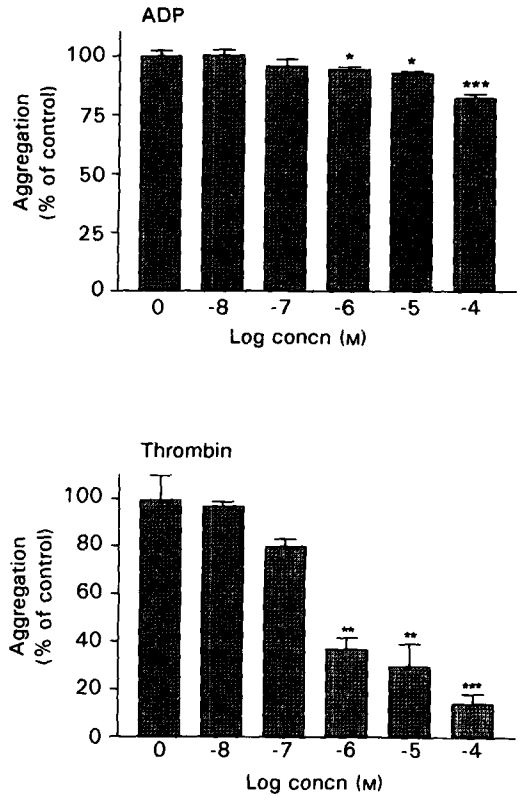



FIG. 1. Effect of naftazone on rat platelet aggregation in-vitro. Aggregation was studied with PRP after incubation for 1 min with naftazone and stimulation with ADP or thrombin. Transmittance values were expressed as a percentage of control values. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

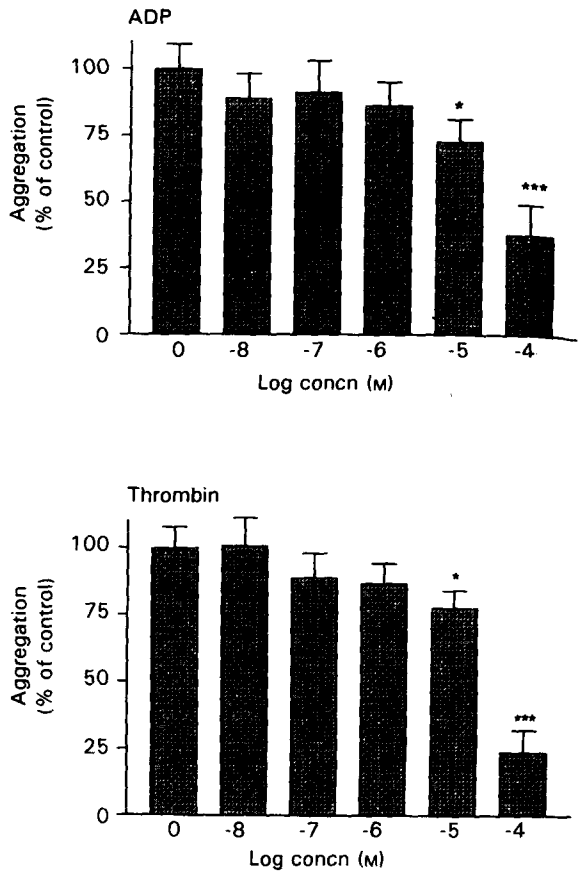



FIG. 2. Effect of naftazone on rat platelet aggregation in-vitro. Aggregation of washed platelets was studied after incubation for 1 min of naftazone and stimulation with ADP or thrombin. Transmittance values are expressed as a percentage of control values. $*P < 0.05$; $***P < 0.001$.

spectively). Similar results were obtained with rat platelets re-suspended in buffer, although statistical significance was only reached with $10^{-5}$ and $10^{-4}$ M naftazone (Fig. 2), possibly owing to the wider dispersion of values. Naftazone inhibited the thrombin-induced release of 5-HT from washed rat platelets at concentrations as low as $10^{-7}$ M ($P < 0.05$) (Fig. 3). Naftazone reduced both ADP- and thrombin-induced aggregation of human platelets (Figs 4, 5), but the effect was more marked for re-suspended platelets (Fig. 5) than for PRP (Fig. 4). For example, with $10^{-6}$ M naftazone, ADP- and thrombin aggregation of re-suspended platelets was inhibited by 45 and 34%, respectively, whereas inhibition was only 10 and 21% with PRP.

*Ex-vivo effects of naftazone*

Aggregation induced by ADP and thrombin was studied with PRP (Table 1) and washed platelets (Table 2) at different times after the last of five naftazone injections. Ex-vivo inhibition of platelet aggregation by naftazone relative to controls was both dose- and time-dependent.

One hour after the last injection of 0·5 mg $kg^{-1}$ naftazone, a dose close to that used in clinical practice, inhibition of thrombin-induced aggregation reached nearly 10% with PRP, compared with about 35% with 10 mg $kg^{-1}$ naftazone at the same time point. As shown in Table 1, marked inhibition was obtained between 1 and 3 h after the last injection; platelet reactivity returned close to baseline 24 h after the last injection.

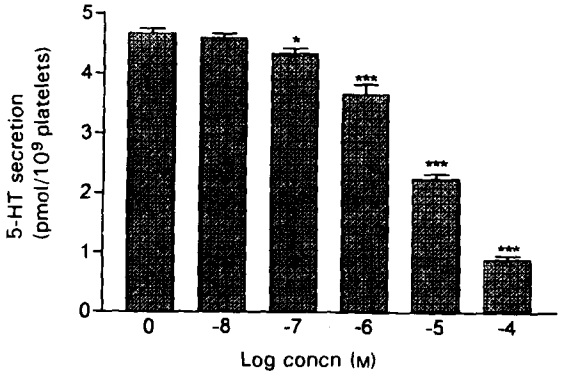



FIG. 3. Effect of naftazone on rat platelet 5-HT release in-vitro. Endogenous 5-HT release by washed platelets was studied after incubation for 1 min with naftazone and stimulation with thrombin. 5-HT was assayed by means of voltammetry, as described in Materials and Methods, using a carbon fibre microelectrode. $*P < 0.05$; $***P < 0.001$.

Similar results were obtained with washed platelets induced to aggregate by thrombin and ADP (Table 2). In particular, the inhibition of ex-vivo platelet aggregations was again dose- and time-dependent.

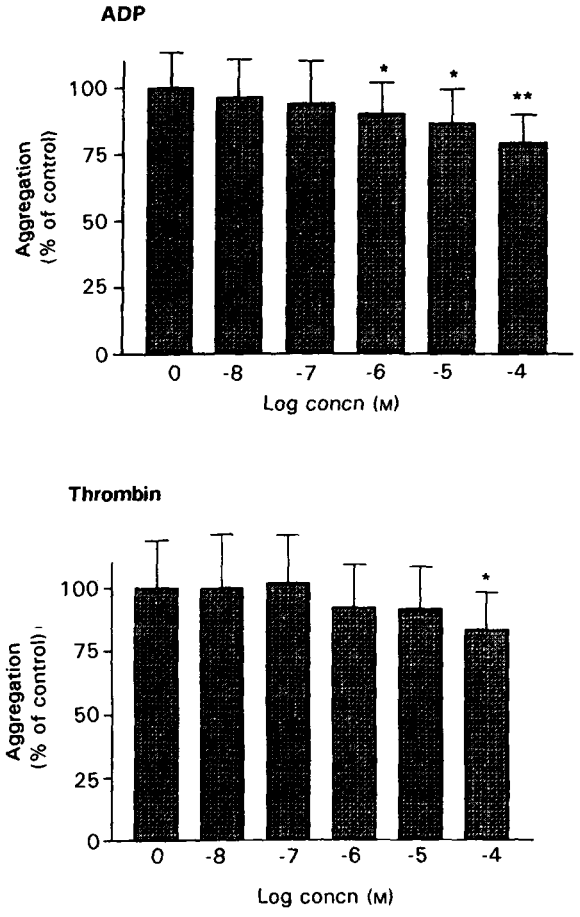



FIG. 4. Effect of naftazone on human platelet aggregation in-vitro. Aggregation was studied in PRP after incubation for 1 min with naftazone and stimulation with ADP or thrombin. Transmittance values are expressed as a percentage of control values. $*P < 0.15$; $**P < 0.01$

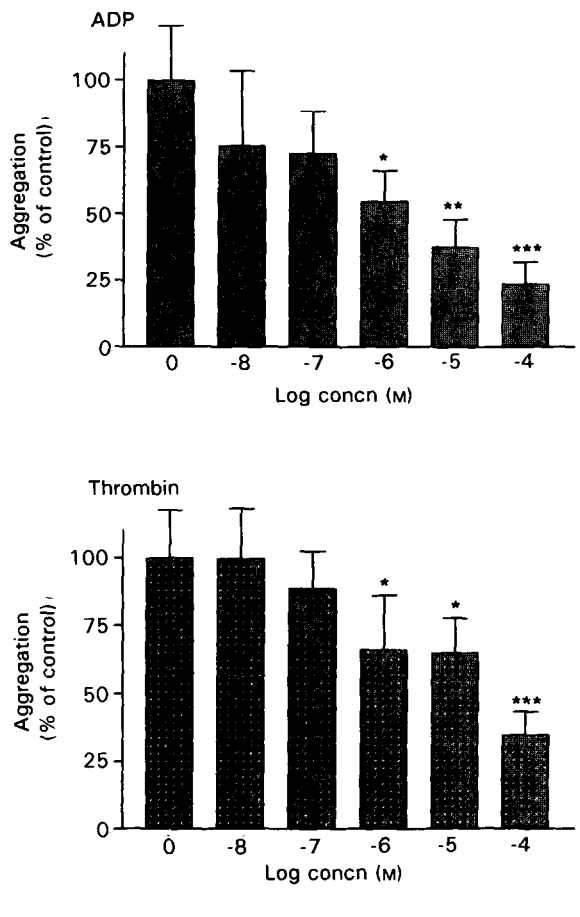



FIG. 5. Effect of naftazone on human platelet aggregation in-vitro. Aggregation was studied with washed platelets after incubation for 1 min with naftazone and stimulation with ADP or thrombin. Transmittance values are expressed as a percentage of control values. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Table 1. Effect of various naftazone doses on ex-vivo aggregation of platelets stimulated with ADP or thrombin in platelet-rich plasma.

| Group | n | | t+1 h | t+3 h | t+6 h | t+24 h |
|---|---|---|---|---|---|---|
| 0 | 8 | Thrombin | 86·13 ± 3·4 | 86·50 ± 4·4 | 87·12 ± 3·6 | 87·50 ± 3·7 |
| | 8 | ADP | 69·00 ± 4·9 | 69·25 ± 3·6 | 69·25 ± 4·3 | 67·50 ± 5·5 |
| 0·125 mg kg$^{-1}$ | 4 | Thrombin | 85·25 ± 4·1$^{b}$ | 76·25 ± 3·3$^{a,b}$ | 82·75 ± 7·9 | 82·25 ± 2·9 |
| | 4 | ADP | 70·00 ± 2·7 | 60·75 ± 2·2 | 65·25 ± 5·9 | 66·50 ± 4·2 |
| 0·25 mg kg$^{-1}$ | 4 | Thrombin | 83·70 ± 3·8 | 77·40 ± 1·8$^{a}$ | 82·50 ± 4·8 | 84·75 ± 3·0 |
| | 4 | ADP | 63·75 ± 5·2 | 62·90 ± 6·7 | 62·50 ± 2·6 | 67·00 ± 2·9 |
| 0·5 mg kg$^{-1}$ | 4 | Thrombin | 78·70 ± 2·1$^{a}$ | 73·50 ± 2·6$^{a}$ | 73·75 ± 5·9$^{a}$ | 75·25 ± 9·6$^{a}$ |
| | 4 | ADP | 63·80 ± 4·3 | 62·75 ± 5·6 | 58·00 ± 6·0$^{a}$ | 57·75 ± 3·9$^{a}$ |
| 1 mg kg$^{-1}$ | 4 | Thrombin | 67·75 ± 4·0$^{a,b,c}$ | 81·00 ± 2·6$^{a,b,d}$ | 72·50 ± 5·1$^{a,d}$ | 77·00 ± 4·7$^{a,c}$ |
| | 4 | ADP | 49·50 ± 3·1$^{a,b}$ | 67·50 ± 7·9$^{b,c,d}$ | 49·75 ± 6·7$^{a,c}$ | 50·75 ± 6·8$^{a,d}$ |
| 5 mg kg$^{-1}$ | 4 | Thrombin | 61·50 ± 4·4$^{a}$ | 67·25 ± 3·6$^{a}$ | 65·38 ± 7·0$^{a}$ | 67·75 ± 7·3$^{a}$ |
| | 4 | ADP | 41·13 ± 5·3$^{a,b}$ | 49·75 ± 5·6$^{a,c}$ | 46·18 ± 6·0$^{a,d}$ | 59·50 ± 2·9$^{b,c,d}$ |
| 10 mg kg$^{-1}$ | 4 | Thrombin | 56·50 ± 5·0$^{a,b,c}$ | 52·00 ± 2·9$^{a,d}$ | 47·50 ± 6·2$^{a,b,e}$ | 64·50 ± 3·3$^{a,c,d,e}$ |
| | 4 | ADP | 40·50 ± 2·4$^{a,b}$ | 38·25 ± 4·9$^{a,c,d}$ | 31·00 ± 4·2$^{a,b,c,e}$ | 54·00 ± 3·3$^{a,d,e}$ |

Results were expressed as transmittance (%). Statistical comparison between groups was performed using analysis of variance followed by the Newman-Keuls test: means indicated with superscript a are significantly different (at least $P < 0.05$) for a given time; means indicated with the same superscript letters (b, c, d or e) are significantly different from each other (at least $P < 0.05$) for a different time.

Table 2. Effect of various naftazone doses on ex-vivo aggregation of platelets stimulated with ADP or thrombin in isolated platelets.

| Group | n | | t+1 h | t+3 h | t+6 h | t+24 h |
|---|---|---|---|---|---|---|
| Control | 8 | Thrombin | 85·60 ± 4·6 | 87·30 ± 8·0 | 86·21 ± 8·2 | 89·40 ± 3·2 |
| | 8 | ADP | 66·80 ± 3·1 | 68·10 ± 5·1 | 66·50 ± 6·7 | 67·70 ± 3·3 |
| 0·125 mg kg$^{-1}$ | 4 | Thrombin | 91·10 ± 4·6 | 79·60 ± 3·8 | 84·50 ± 10·3 | 90·30 ± 2·2 |
| | 4 | ADP | 68·00 ± 7·8 | 65·30 ± 2·2 | 66·00 ± 1·6 | 72·00 ± 4·0 |
| 0·25 mg kg$^{-1}$ | 4 | Thrombin | 88·30 ± 8·3 | 76·80 ± 9·7 | 76·30 ± 14·0 | 89·10 ± 6·5 |
| | 4 | ADP | 61·80 ± 3·5 | 65·40 ± 4·4 | 61·10 ± 2·1 | 61·70 ± 2·5 |
| 0·5 mg kg$^{-1}$ | 4 | Thrombin | 79·30 ± 5·3$^{b}$ | 75·00 ± 5·4$^{b}$ | 61·00 ± 8·0$^{b}$ | 88·00 ± 4·7$^{b}$ |
| | 4 | ADP | 61·50 ± 5·8 | 66·50 ± 9·5 | 66·00 ± 8·1 | 61·00 ± 9·5 |
| 1 mg kg$^{-1}$ | 4 | Thrombin | 64·30 ± 5·0$^{a,b,c}$ | 84·00 ± 5·5$^{b,d}$ | 70·90 ± 6·0$^{d,e}$ | 82·00 ± 3·9$^{c,e}$ |
| | 4 | ADP | 46·00 ± 4·4$^{a,b,c}$ | 66·50 ± 6·8$^{b,d}$ | 46·00 ± 6·4$^{a,d,e}$ | 57·90 ± 4·4$^{a,c,e}$ |
| 5 mg kg$^{-1}$ | 4 | Thrombin | 61·30 ± 6·5$^{a,b}$ | 76·20 ± 4·8 | 66·80 ± 11·1 | 72·30 ± 5·1$^{a}$ |
| | 4 | ADP | 43·00 ± 2·9$^{a,b,c}$ | 62·30 ± 6·0$^{b,d}$ | 42·50 ± 8·7$^{a,d,e}$ | 55·40 ± 2·5$^{a,c,e}$ |
| 10 mg kg$^{-1}$ | 4 | Thrombin | 48·80 ± 8·2$^{a}$ | 56·00 ± 7·9$^{a}$ | 51·00 ± 23·5$^{a}$ | 65·80 ± 6·4$^{a}$ |
| | 4 | ADP | 38·00 ± 7·8$^{a,b}$ | 42·30 ± 4·7$^{a,c,e}$ | 34·90 ± 3·7$^{a,d}$ | 54·00 ± 3·2$^{a,b,c,d,e}$ |

Results were expressed as transmittance (%). Statistical comparison between groups was performed using analysis of variance followed by the Newman-Keuls test: means indicated with superscript a are significantly different (at least $P < 0·05$) for a given time; means indicated with the same superscript letters (b, c, d or e) are significantly different from each other (at least $P < 0·05$) for a different time.

Table 3. Effect of various naftazone doses on ex-vivo platelet deaggregation after stimulation with thrombin or ADP.

| Group | n | Inducer | t+1 h | Inducer | t+3 h |
|---|---|---|---|---|---|
| 0 | 8 | Thrombin | 3·30 ± 2·5 | ADP | 21·40 ± 3·9 |
| 0·125 mg kg$^{-1}$ | 4 | Thrombin | 2·00 ± 0·8 | ADP | 21·90 ± 4·5 |
| 0·25 mg kg$^{-1}$ | 4 | Thrombin | 1·40 ± 1·2 | ADP | 26·70 ± 5·3 |
| 0·5 mg kg$^{-1}$ | 4 | Thrombin | 3·60 ± 5·0 | ADP | 32·90 ± 12·9 |
| 1 mg kg$^{-1}$ | 4 | Thrombin | 16·35 ± 6·6$^{a}$ | ADP | 34·30 ± 5·0$^{a}$ |
| 5 mg kg$^{-1}$ | 4 | Thrombin | 19·80 ± 6·4$^{a}$ | ADP | 38·30 ± 5·3$^{a}$ |
| 10 mg kg$^{-1}$ | 4 | Thrombin | 23·30 ± 5·8$^{a}$ | ADP | 43·80 ± 2·0$^{a}$ |

Platelet deaggregation was measured after thrombin- or ADP-induced aggregation for 600 s. It was expressed as percent of maximum aggregation amplitude. Statistical comparison between groups was performed using analysis of variance followed by the Newman-Keuls test: means indicated with superscript a are significantly different (at least $P < 0·05$) for a given time.

Table 4. Effects of various antiplatelet agents on ex-vivo rat platelet aggregation.

| | Group | n | PRP-Thrombin | PRP-ADP | Thrombin | ADP |
|---|---|---|---|---|---|---|
| A | Control | 9 | 62·4 ± 11·9$^{a,b}$ | 37·7 ± 11·5$^{a,b,c}$ | 70·5 ± 5·3$^{a}$ | 42·5 + 7·8$^{a}$ |
| B | Aspirin | 6 | 45·6 ± 6·5 | 24·4 ± 5·0$^{a}$ | 61·4 ± 12·3$^{b}$ | 43·5 + 12·6$^{b}$ |
| C | Ticlopidine | 6 | 33·3 ± 26·9$^{a}$ | 23·5 ± 9·9$^{b}$ | 63·9 ± 4·7$^{c}$ | 39·6 ± 9·2$^{c}$ |
| D | Dipyridamole | 6 | 54·5 ± 14·6 | 35·6 ± 11·8$^{d}$ | 67·4 ± 9·2$^{d}$ | 45·4 ± 3·8$^{d}$ |
| E | Naftazone | 6 | 32·9 ± 7·7$^{b,d}$ | 20·6 ± 5·6$^{c}$ | 24·3 ± 18·8$^{a,b,c}$ | 20·7 ± 19·2$^{a,b,c,d}$ |

Results were expressed as transmittance (%). PRP, platelet-rich plasma Statistical comparison between groups was performed using analysis of variance followed by the Newman-Keuls test: means indicated with the same superscript letter in the same column are significantly different (at least $P < 0·05$).

After thrombin-induced aggregation, ex-vivo platelet deaggregation was markedly enhanced by naftazone treatment (Table 3). This effect correlated with the dose when measured after 1 and 3 h of stimulation with thrombin and ADP, respectively.

*Comparative studies*

Ex-vivo platelet aggregation was compared after five daily intraperitoneal injections of the platelet-active compounds aspirin (group B), ticlopidine (group C), dipyridamole (group D) and naftazone (group E). Control animals received the vehicle alone. Platelets were prepared from blood taken 90 min after the last injection. Results of aggregation studies with PRP stimulated with thrombin and ADP are summarized in Table 4. Aspirin had a non-significant inhibitory tendency, whereas significant inhibition of thrombin-stimulated aggregation was obtained with ticlopidine (C) and naftazone (E). Except for dipyridamole (D), all the other compounds inhibited ADP-induced aggregation in PRP ex-vivo.

The effects of the different compounds on washed platelet aggregation stimulated with thrombin or ADP were not significantly different except for naftazone which resulted in ~50% inhibition. Again, inhibition was more marked with washed platelets than with PRP.

## Discussion

These in-vitro and ex-vivo experiments show that naftazone inhibits rat and human platelet aggregation. Although inhibition of ADP- and thrombin- induced aggregation was observed at a naftazone concentration of $10^{-7}$ M, significant differences relative to controls only occurred above $10^{-6}$ M. The inhibitory effect appeared to be weaker with rat platelets than with human platelets, and with PRP compared with washed platelets. It may be that, as for other drugs (Blache & Ojeda 1992), the high lipophilicity of naftazone contributes to its inhibitory potency, although we did not specifically determine naftazone binding to plasma proteins or lipoproteins. This hypothesis is in keeping with the greater inhibition of thrombin-induced aggregation with PRP than with platelets re-suspended in plasma diluted 1:5 with buffer. Binding of naftazone to platelet membranes, which could explain the interference of this compound in the platelet aggregation process, could be suggested; we are awaiting the results of further specific studies. In addition, naftazone appears to act on early events of platelet biochemistry, as the thrombin-induced release of 5-HT was markedly inhibited by concentrations as low as $10^{-7}$ M and fell by more than 50% in the presence of native $10^{-5}$ M naftazone. This could be related to the enhancement of platelet deaggregation ex-vivo as 5-HT participates in the autocrine platelet stimulation process (see below).

Ex-vivo platelet aggregation was also studied after intraperitoneal treatment of rats for five days with naftazone. The 5-day treatment schedule was necessary because of the short half-life of injected naftazone, probably owing to rapid metabolism (not shown). We found that the effect was both dose- and time-dependent. Indeed, ADP- and thrombin-induced aggregation were both inhibited by doses as low as 0·125 mg $kg^{-1}$ ($\sim$12% in PRP). This effect was, moreover, short-lived; it was observed 3 h after the last injection and had virtually disappeared after 24 h. Interestingly, significant inhibition was observed with the dose equivalent to that used in clinical practice (0·5 mg $kg^{-1}$), particularly, after thrombin stimulation.

Naftazone also significantly stimulated platelet deaggregation. Deaggregation can occur when platelet stimulation is low or when platelet membrane receptors are not activated or not fully expressed (Feinstein et al 1983). Deaggregation is more likely to occur when platelet secretion is inhibited. Indeed, platelet stimulation is a self-activating chain (Holmsen 1994). It is thus conceivable that naftazone, by inhibiting 5-HT secretion, facilitates deaggregation. The antiplatelet effect of naftazone may thus be mediated not only by inhibition of agonists of platelet aggregation, but also by inhibition of the stabilizing effect of released substances on thrombin-induced aggregates.

Dipyridamole was not active in our ex-vivo rat model with either PRP or washed platelets. This drug has been reported to inhibit platelet function by inhibiting phosphodiesterase activity and thereby contributing to an increase in platelet cycling AMP concentration. Aspirin was more active in experiments with PRP than isolated platelets: thrombin- and ADP-induced aggregation in PRP were inhibited by 27 and 35%, respectively. Aspirin is very effective in cardiovascular disease although the commonest side-effect of taking aspirin is its gastrotoxicity (FitzGerald & Meagher 1994). An alternative approach could be to use either the intravenous route or to use ticlopidine (Defreyn et al 1989; Haynes et al 1992). Ticlopidine was very effective under our conditions: respective inhibitions of 47 and 38% were obtained for thrombin- and ADP-induced aggregation in PRP. Ticlopidine was less active on isolated platelets. Naftazone was as active as ticlopidine in the case of thrombin-induced PRP aggregation ($\sim$47% inhibition) and more active in the case of ADP-induced aggregation ($\sim$46%). Naftazone was also active on isolated platelets, inhibiting aggregation stimulated by thrombin more effectively ($\sim$65%) than that induced by ADP ($\sim$22%). These data confirm the results obtained for dose-response studies. The shorter duration of the antiplatelet effect of naftazone relative to aspirin and ticlopidine may have therapeutic implications.

In conclusion, naftazone inhibited platelet aggregation induced by ADP or thrombin in-vitro together with thrombin stimulated 5-HT release. Ex-vivo studies revealed that aggregation was inhibited after intraperitoneal injection of rats with naftazone. This effect was dose- and time-dependent, and washed platelets from treated animals were also inhibited. Twenty four hours after the last injection, the platelet activity had, moreover, returned almost to baseline (about 80% of control values). Platelet deaggregation was facilitated by naftazone. Further work is required to identify the precise mechanism underlying the antiplatelet activity of naftazone.

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## References

Antiplatelet Trialists' Collaboration (1994) Collaborative overview of randomized trials of antiplatelet therapy I: prevention of death, myocardial infarction, and stroke by prolonged antiplatelet therapy in various categories of patients. Br. Med. J. 308: 81–106

Berson, I. (1977) Clinical study of a new venotropic drug: mediaven (DCI naftazone). Schweiz. Rundsch. Med. Prax. 66: 180–181

Beving, H., Eksborg, S., Malmgren, R. S., Nordlander, R., Rydén, L., Olsson, P. (1994) Inter-individual variations of the effect of low dose aspirin regime on platelet cyclooxygenase activity. Thromb. Res. 74: 39–51

Blache, D. (1992) Structure and function of blood platelets. Arch. Intern. Physiol. Biochem. Biophys. 100: A17–A24

Blache, D. (1995) Involvement of hydrogen and lipid peroxides in the acute tobacco smoking-induced platelet hyperactivity. Am. J. Physiol. 268: H679–H685

Blache, D., Ciavatti, M. (1989) Effects of organic and inorganic Ca antagonists on rat platelet arachidonic acid metabolism in the presence of $Ca^{2+}$, $Sr^{2+}$ and $Ba^{2+}$. Mol. Cell. Biochem. 85: 191–196

Blache, D., Ojeda, C. (1992) Comparative inhibitory effects of dihydropyridines on platelet aggregation, calcium uptake and cyclic AMP concentration. Pharmacology 45: 250–259

Blache, D., Nargeot, J., Nerbonne, J. M. (1986) Direct evidence for the inhibition of platelet aggregation and release by intracellular cyclic AMP produced with a new photoactivatable derivative. Thromb. Haemost. 55: 168–172

Blache, D., Ciavatti, M., Ojeda, C. (1987a) Platelet aggregation and endogenous 5-HT secretion in presence of $Ca^{2+}$, $Sr^{2+}$ and $Ba^{2+}$. Effects of calcium antagonists. Thromb. Res. 46: 779–791

Blache, D., Ciavatti, M., Ponsin, G., Nargeot, J. (1987b) Direct evidence for the modulation of human platelet cytosolic free $Ca^{2+}$ by intracellular cyclic AMP produced with a photoactivatable derivative. Biochem. Biophys. Res. Commun. 146: 321–331

Blache, D., Bouthillier, D., Davignon, J. (1992) Acute influence of smoking on platelet behaviour, endothelium and plasma lipids and normalization by aspirin. Atherosclerosis 93: 179–188

Born, G. V. R. (1962) Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 194: 927–929

Buchanan, M. R., Rischke, J. A., Hirsh, J. (1982) Aspirin inhibits platelet function independent of the acetylation of cyclooxygenase. Thromb. Res. 25: 363–372

Ciavatti, M., Davenas, E., Blache, D., Monnier, A., Renaud, S. (1982) Biosynthesis of platelet lipids in relation to aggregation in women using oral contraceptives. Contraception 25: 629–638

Coller, B. S. (1992) Inhibitors of the platelet glycoprotein IIb/IIIa receptor as conjunctive therapy for coronary artery thrombolysis. Coron. Artery. Dis. 3: 1016–1029

De Gaetano, G., Cerletti, D., De Jana, E., Latini, R. (1985) Pharmacology of platelet inhibition in human: implication of the salicylate-aspirin interaction. Circulation 72: 1185–1192

Defreyn, G., Bernat, A., Delebasse, D., Maffrand, J.-P. (1989) Pharmacology of ticlopidine: a review. Sem. Thromb. Haemost. 15: 159–166

Feinstein, M. B., Egan, J. J., Opas, E. F. (1983) Reversal of thrombin-induced myosin phosphorylation and the assembly of cytoskeletal structures in platelets by the adenylate cyclase stimulants $PGI_2$ and forskolin. J. Biol. Chem. 258: 1260–1267

Feinstein, M. B., Zavoico, G. B., Halenda, S. P. (1985) Calcium and cyclic AMP: antagonistic modulators of platelet function. In: Longenecker, G. L. (ed.) The Platelet: Physiology and Pharmacology. Academic Press, Orlando, pp 237–269

FitzGerald, G. A., Meagher, E. A. (1994) Antiplatelet drugs. Eur. J. Clin. Invest. 24 (Suppl. 1): 46–49

FitzGerald, G. A., Oates, J. A., Hawiger, J., Maas, R. L., Roberts, L. J., Lawson, J. A., Brash, A. R. (1988) Endogenous biosynthesis of prostacyclin and thromboxane and platelet function during chronic administration of aspirin in man. J. Clin. Invest. 71: 676–688

Hallam, T. J., Rink, T. J. (1985) Agonists stimulates divalent cation channels in the plasma membrane of human platelets. FEBS. Lett. 186: 175–179

Hashizume, T., Taniguchi, M., Sato, T., Fujii, T. (1994) Arachidonic acid liberation induced by phosphatidic acid endogenously generated from membrane phospholipids in rabbit platelets. Biochim. Biophys. Acta: Mol. Cell. Res. 1221: 179–184

Haynes, R. B., Sandler, R. S., Larson, E. B., Pater, J. L., Yatsu, F. M. (1992) A critical appraisal of ticlopidine, a new antiplatelet agent: effectiveness and clinical indications for prophylaxis of atherosclerotic events. Arch. Intern. Med. 152: 1376–1380

Hoet, B., Arnout, J., Deckmyn, H., Vermylen, J. (1993) Synergistic antiplatelet effect of ridogrel, a combined thromboxane receptor antagonist and thromboxane synthase inhibitor, and UDCG-212, a cAMP-phosphodiesterase inhibitor. Thromb. Haemost. 70: 822–825

Holmsen, H. (1994) Significance of testing platelet functions in vitro. Eur. J. Clin. Invest. 24 (Suppl. 1): 3–8

Kiss, R. G., Lu, H. R., Roskams, T., Jang, I.-K., Plow, E. F., Gold, H. K., Collen, D. (1994) Time course of the effects of a single bolus injection of F(ab')2 fragments of the antiplatelet GPIIb/IIIa antibody 7E3 on arterial eversion graft occlusion, platelet aggregation, and bleeding time in dogs. Arterioscler. Thromb. 14: 367–374

Klein-Soyer, C., Bloy, C., Archipoff, G., Beretz, A., Cazenave, J.-P. (1991) Naftazone accelerates human saphenous vein endothelial cell proliferation in vitro. J. Rheumatol. 18: 146–147

Marcenac, F., Blache, D. (1985) Endogenous platelet 5-hydroxytryptamine release monitored during aggregation by means of a new electrochemical technique. Biochim. Biophys. Acta 840: 377–382

Meade, T. W., Vickers, M. V., Thompson, S. G., Stirling, Y., Haines, A. P., Miller, G. J. (1985) Epidemiological characteristics of platelet aggregability. Br. Med. J. 290: 428–732

Modesti, P. A., Cecioni, I., Colella, A., Costoli, A., Paniccia, R., Serneri, G. G. N. (1994) Binding kinetics and antiplatelet activities of picotamide, a thromboxane A2 receptor antagonist. Br. J. Pharmacol. 112: 81–86

Pappas, J. M., Westengard, J. C., Bull, B. S. (1994) Population variability in the effect of aspirin on platelet function: implications for clinical trials and therapy. Arch. Pathol. Lab. Med. 118: 801–804

Patrono, C. (1994) Drug therapy: aspirin as an antiplatelet drug. N. Engl. J. Med. 330: 1287–1294

Rao, G. H. R., White, J. G. (1994) Influence of various doses of aspirin (in vivo) on platelet arachidonic acid metabolism (ex vivo) and function. Prostaglandins, Leukot. Essent.Fatty Acids 51: 63–67

Ross, R. (1986) The pathogenesis of atherosclerosis – an update. N. Engl. J. Med. 314: 488–500

Roth, G. J., Calverley, D. C. (1994) Aspirin, platelets, and thrombosis: theory and practice. Blood 83: 885–898

Sussman, I. I. (1985) The role of platelets in atherogenesis/atherosclerosis. In: Longenecker, G. L. (ed.) The Platelet: Physiology and Pharmacology. Academic Press, Orlando, pp 237–269

Winand, R. (1977) La mono-semicarbazone-2 de la naphthoquinone (DCI naftazone) dans la maladie variqueuse. Rev. Med. Liege 14: 429–431.