Inhibition of Thrombus Formation In Vivo by Novel Antiplatelet Agent

Ulla M. Marzec, Andrew B. Kelly, Stephen R. Hanson, Andrew Lasslo, and Laurence A. Harker

The antithrombotic and antihemostatic effects of the novel antiplatelet compound, α,α′-bis[3-([N,N-diethylcarbamoyl]piperidine]-p-xylene dihydrobromide (GT-12), were investigated in a baboon model of platelet-dependent thrombosis under high flow conditions. In this model, segments of collagen-coated tubing and Dacron vascular graft were placed as thrombus-inducing extension devices in exteriorized femoral arteriovenous shunts. The deposition of 111In-labeled platelets was measured for each thrombogenic segment throughout 1 hour by using gamma camera imaging. In addition, the 111In fibrin retained in the forming thrombus was measured. Intravenous infusion of GT-12 (100 μmol/kg, 63.3 mg/kg) over a 15-minute period before the insertion of the test segments prolonged the bleeding time from a baseline value of 4.4 ± 0.4 min to 7.6 ± 1.0 min (p = 0.036) and inhibited platelet aggregation ex vivo induced by adenosine diphosphate (ED50 4.7 ± 0.9 to 10.3 ± 2.2 μM; p < 0.02) and collagen (ED50 2.0 ± 0.4 to 8.0 ± 2.4 μg/ml; p < 0.05). Deposition of platelets and fibrin was decreased in concert by 30% (p < 0.05) for vascular grafts and possibly collagen segments at the end of the 60-minute observation period. We conclude that GT-12 is antithrombotic for Dacron graft-induced thrombus formation in vivo. (Arteriosclerosis 10:367–371, May/June 1990)

Platelets participate in the formation of thrombus under high flow conditions after vascular injury or exposure of blood to artificial surfaces.1,2 Activation of platelets on these abnormal surfaces induces platelet recruitment of ambient platelets into forming thrombus by several different pathways, including: 1) adenosine diphosphate (ADP) released from platelet dense granules, 2) thromboxane A2 (TXA2) synthesized by platelets through metabolism of arachidonic acid, and 3) thrombin generated on the platelet surface.

The development of the antiplatelet agent, GT-12,3–10 provided the opportunity to investigate its effect on thrombosis in a well-characterized baboon model.11,12 In this model, segments of collagen-coated tubing and Dacron vascular graft are inserted as thrombogenic devices in an exteriorized arteriovenous (A-V) shunt circuit. Covalently bonded collagen-coated tubing and Dacron vascular grafts were selected as thrombogenic surfaces because of their clinical relevance, readily controllable blood flow, segment geometry, and highly reproducible thrombus formation and occlusion. GT-12 is thought to stabilize membrane complexes of the dense tubular system and other storage sites sequestering calcium, thereby blocking the mobilization of additional Ca++ into the platelet cytosol.3–10

Methods

Normal male baboons (Papio anubis/cynocephalus) were used in these studies. All procedures were approved by the Institutional Animal Care and Use Committee in accordance with federal guidelines (Guide for the Care and Use of Laboratory Animals, 1986). The animals weighed 8 to 12 kg each and had been observed to be disease-free for at least 6 weeks before use. Each animal had a chronic A-V shunt surgically implanted between the femoral artery and vein. This chronic shunt did not detectably activate platelets or coagulation proteins.12 For surgical procedures, the animals received atropine (0.4 mg/kg intramuscularly) as the preanesthesia and ketamine (10 mg/kg intramuscularly) as the inducing agent. Halothane was used as the anesthetic agent, and butorphanol (0.1 mg/kg twice daily for 2 days) was used for postoperative analgesia.

Segments of Dacron vascular grafts (U.S. Catheter, Billerica, MA; 4.0 mm i.d.) were rendered impervious to blood leakage by an external wrapping of Parafilm (American Can, Greenwich, CT) and were placed inside a 5 cm length of "heat-shrink" Teflon tubing as previously described.11,12 These test segments were interposed between the arms of the permanent A-V shunt, and the blood flow rates were measured continuously with a Doppler ultrasound flowmeter (L&M Electronics, Daly City, CA). In all studies, the initial blood flow rates ranged between 160 and 240 ml/min, with a calculated wall shear rate of 424 to 637 sec⁻¹. In these studies, collagen-coated silicone rubber tubing was prepared in 2 cm
lengths as previously described\textsuperscript{19} and was incorporated
into the exteriorized A-V shunts distal to the segments of
Dacron vascular grafts; the shear rates were calculated to
be 844 to 1267 sec\textsuperscript{-1} for the collagen segments.

Platelet counts and hematocrit determinations were
performed on whole blood collected in 2 mg/ml disodium
ethylene diaminetetraacetic acid (EDTA) with a J.T. Baker
(Allentown, PA) Model 810 whole-blood analyzer. Mean
whole-blood platelet counts were 405 000±52 000 plate-
lets/\mu l; hematocrits averaged 34.6%. Bleeding time mea-
surements were performed in duplicate on the shaved
volar surface of the forearm by using the standard tem-
plate method as previously described for studies in
baboons.\textsuperscript{14, 15}

Autologous baboon blood platelets were labeled with
\textsuperscript{111}In-oxine as previously described.\textsuperscript{11} In brief, whole
blood (100 ml) was collected directly into plastic bags
(TA-3, Fenwal Labs, Deerfield, IL) containing 20 ml of
acid-citrate-dextrose anticoagulant (NIH formula A). The
blood was centrifuged in the bag at 300 g for 10 minutes.
The supernatant platelet-rich plasma (PRP) was trans-
ferred to a second bag, and the pH was adjusted to 6.5
by the addition of 0.15 M citric acid (0.1 ml/10 ml PRP).
The red blood cell fraction was returned to the donor
animal. The platelets were formed into a pellet by cen-
trifugation of the PRP at 13 000 g for 15 minutes. The
supernatant platelet-poor plasma (PPP) was completely
decanted and discarded. To remove the residual plasma
proteins, the bag containing the platelet pellet was care-
fully washed once by overlaying with 30 ml of Ringers
citrate dextrose (RCD, pH 6.5), which was decanted and
discarded. The pellet was then gently resuspended in
5.0 ml of RCD and was incubated for 30 minutes with 800
to 1000 \muCi of \textsuperscript{111}In-oxine (Amersham, Arlington Heights,
IL). Contaminating red cells were removed by a final slow
centrifugation at 200 g for 5 minutes. The labeling effi-
ciencies averaged >90%. All the labeled platelets were
re-infused, together with the previously separated red
cells, for a total circulating platelet activity of 800 to
950 \muCi.

Images of the Dacron grafts, including proximal and
distal silicone rubber segments, were acquired with a
Searle PhoGamma V scintillation camera and were stored
on and analyzed by a Medical Data Systems A\textsuperscript{2}
computer (Medtronic, Ann Arbor, MI) interfaced with the
camera. Immediately before imaging the vascular grafts, images
were also acquired of 4.0 mm i.d. silicone rubber tubing
filled with autologous blood and having the same luminal
volume as the graft segment (blood standard). The
activities of the standard and 5 cm graft segments were
counted in the same region of interest as defined by
image analysis software routines. Images were acquired at
5 minute intervals. Deposited \textsuperscript{111}In-platelet activity
calculated by subtracting the blood standard activity from
all dynamic study images increased monotonically over
the exposure period. The total number of platelets depos-
ited after 1 hour (labeled plus unlabeled cells) was
calculated by dividing the deposited platelet activity by
the blood standard platelet activity, and multiplying by the
volume of the blood standard and the circulating platelet
count (platelets/ml).\textsuperscript{11, 16} The results were expressed as
platelets deposited/cm graft.

The concentration of fibrinogen was estimated spec-
trrophotometrically by a modification of Jacobssen's
method,\textsuperscript{16} in which the optical density of thrombin-
clottable protein was determined after collection on a
glass rod and subsequent solution in 1% sodium dodecyl
sulphate (SDS).

For quantifying the formation of fibrin on the collagen
and vascular graft segments, we injected homologous
\textsuperscript{125}I-fibrinogen (approximately 5 \muCi) intravenously 15
minutes before incorporation of the thrombogenic segments
into the A-V shunts. Homologous fibrinogen was purified
by \beta-alanine precipitation\textsuperscript{17} and was labeled with \textsuperscript{125}I
by using the iodine monochloride method.\textsuperscript{18, 19} This prepa-
ration was >95% clottable in vitro. Additionally 95% of the
\textsuperscript{125}I activity was associated with the clot, thereby document-
ing that it was functionally equivalent to unlabeled fibrino-
gen. At the time of segment exposure, blood was collected
in EDTA for the determination of both \textsuperscript{125}I-fibrinogen activity
and fibrinogen concentration, from which the specific activ-
ity was calculated. After a 1 hour exposure to flowing
blood, the collagen and vascular graft segments were
removed from the A-V shunt, were flushed with isotoninc
buffer, were placed in 10% buffered formalin fixative, and
were stored at 4°C. After approximately 30 days, when the
\textsuperscript{111}In-activity had largely decayed, the \textsuperscript{125}I-activity in
each segment was counted and related to the standard and
plasma \textsuperscript{125}I-activity and plasma fibrinogen level at that
time, using the formula:

deposited fibrin (mg) =
\textsuperscript{125}I-activity in the segment (cpm)
\times \frac{\text{clottable plasma } \textsuperscript{125}I-activity (cpm/ml)}{\text{plasma fibrinogen} (mg/ml)}

The results were expressed as the total fibrin (mg/cm)
contained in the thrombus associated with each segment.
The plasma levels of the platelet-specific \alpha-granule
proteins, PF\textsubscript{4} and \beta\textsubscript{G}, and fibrinopeptide A (FPA), a
thrombin cleavage product of fibrinogen, were also deter-
mined by radioimmunoassay on blood samples collected
and processed as described previously.\textsuperscript{12}

Blood samples for platelet aggregation were collected
in 0.1 vol of 3.2% sodium citrate before the start of the
infusion, at the end of the infusion, and at the end of the
imaging. PRP was prepared by centrifugation at 180 g for
10 minutes, and the concentration was adjusted to
250 000 platelets/\mu l with PPP, which was prepared by
centrifugation at 13 000 g for 5 minutes. The maximal
aggregation response to doses of ADP (Sigma Chemical,
St. Louis, MO) and collagen (Hormon Chemie, Munich,
FRG) bracketing the full range of reactivity was measured
by using a dual-channel aggregometer (Chrono-Log,
Havertown, PA). The concentration of agonist required
to produce half-maximal aggregation of platelets, ED\textsubscript{50},
was calculated for each agonist.\textsuperscript{20}

GT-12 (100 \mu mol/kg [63.3 mg/kg]) was dissolved in
15 ml of sterile physiological saline, was then sterilized by
filtration through a 0.22 \mu m syringe filter (Acrodisc, Gel-
Table 1. Effect of GT-12 on Platelet Function

<table>
<thead>
<tr>
<th>Determination (n)</th>
<th>Baseline</th>
<th>15 min post GT-12 (100 μmol/kg)</th>
<th>60 min post GT-12 (100 μmol/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleeding time (7)</td>
<td>4.4±0.4</td>
<td>7.6±1.0</td>
<td>5.1±0.6</td>
</tr>
<tr>
<td>*p=0.036†</td>
<td></td>
<td>*p&gt;0.5†</td>
<td></td>
</tr>
<tr>
<td>Platelet aggregation (7)</td>
<td>ADP ED₅₀* (μM)</td>
<td>4.7±0.9</td>
<td>10.3±2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>*p&lt;0.02†</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*p&gt;0.5†</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Collagen ED₅₀* (7)</td>
<td>2.0±0.4</td>
<td>8.0±2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>*p&lt;0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*p&gt;0.5</td>
<td></td>
</tr>
</tbody>
</table>

*That dose of agonist inducing half-maximal aggregation. †Compared with baseline values.

Ex vivo platelet aggregation to ADP and collagen was significantly reduced after infusion of GT-12 (100 μmol/kg, 63.3 mg/kg). The ED₅₀ of ADP shifted from 4.7±0.9 μM to 10.3±2.2 μM (*p<0.02), and for collagen from 2.0±0.4 μg/ml to 8.0±2.4 μg/ml (p<0.05). Sixty minutes after the infusion of GT-12, the ED₅₀ had returned to baseline for both agonists (Table 1).

The deposition of platelets and fibrin on Dacron grafts in six animals treated with GT-12 was reduced in concert by 30% at 60 minutes compared to the matched control values (Table 2). Moreover, while one graft in the control group occluded at 50 minutes, all the grafts in the animals infused with GT-12 remained patent for the 60 minute observation period with no change in the blood flow. A time course for platelet deposition is displayed in Figure 1. A significant reduction in the number of deposited platelets was present at each time point. In two animals studied with collagen-coated segments, there was a reduction of fibrin and platelet deposition similar to results using the Dacron graft (Table 2).

The bleeding time was prolonged from a baseline of 4.4±0.4 minutes in the control group to 7.6±1.0 minutes by GT-12 at the end of the infusion period. There was no change in the fibrinogen concentrations or platelet counts from the control values of 2.86±0.38 mg/ml and 405 000±52 000 platelets/μl, respectively. The elevation of the platelet-specific α-granule proteins, PF4 and βTG, in plasma after the 1 hour exposure to the grafts, was less for the GT-12-treated animals (PF4 values decreased from 24.8±13.9 to 13.2±2.1, *p<0.04, Table 3). The plasma FPA levels were significantly less in GT-12-treated animals compared to untreated controls (reduced from 10.6±4.3 pmol/l to 5.5±0.9 pmol/l, *p<0.05).

Table 2. Effects of GT-12 on Thrombus Formation

<table>
<thead>
<tr>
<th>Determination (n)</th>
<th>60 min post-placebo infusion</th>
<th>60 min post-GT-12 (100 μmol/kg)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet deposition (10⁸ plat/cm²)</td>
<td>Dacron graft (6)</td>
<td>2.9±0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Collagen (2)</td>
<td>1.81±1.33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fibrin deposition (mg/cm²)</td>
<td>Dacron graft (6)</td>
<td>1.47±0.22</td>
</tr>
<tr>
<td></td>
<td>Collagen (2)</td>
<td>0.13±0.90</td>
<td></td>
</tr>
</tbody>
</table>

Whereas baseline values were obtained before incorporation of the Dacron vascular grafts into the arteriovenous shunt, post-graft results were performed on samples drawn after the grafts had been in place for 60 minutes. *p values relate to the post-graft data in treated vs. untreated animals.

PF4=platelet factor 4, βTG=β-thromboglobulin, FPA=fibrinopeptide A.
Discussion

The results obtained in these experiments clearly show reduced platelet reactivity and, concomitantly, decreased thrombus formation on Dacron vascular grafts after infusion of the carbamoylpiperidine congener, GT-12, into baboons compared with the control results (<0.05 in all cases). The findings for collagen-coated segments appeared to be similar. These in vivo effects were relatively short-lived after bolus infusion. There was a significant prolongation of the bleeding time which, however, normalized within minutes after the bolus infusion of GT-12. ADP- and collagen-induced platelet aggregation also completely normalized at the end of the 60 minute observation period. These findings are concordant with previous in vitro observations of inhibition of platelet aggregation and PF4 release.22,23

The results obtained in the thrombosis model used in this study are quantitative and reproducible and were obtained under physiological conditions of clearance and regulation of activities. Earlier work in this model with aspirin, dipyradomale, sulodil, and heparin alone or in combination with aspirin or dipyradomale has demonstrated no detectable effect in the platelet deposition or thrombus formation.11-12,20,24,25 On the other hand, ticlopidine, a potent global inhibitor of platelet function,12,26 produces a thrombathenic-like impairment in platelet hemostatic function with an intermediate effect on platelet deposition, similar to the effects of GT-12. More complete inhibition of platelet deposition on grafts has been reported with murine monoclonal antibodies directed against platelet receptor GPIIb/IIIa, which blocks the binding of fibrinogen,15 and the synthetic covalent anti-thrombin III-phenylalanine-L-prolyl-L-arginyl chloromethyl ketone (FPRCH₂Cl).20

Previous observations indicate that placement of the collagen segment distal to the Dacron vascular graft segment did not affect platelet or fibrin deposition on the collagen surface.13 For example, in the present study, platelet deposition onto collagen in control studies was 1.81 and 1.33 x 10⁹ plat/cm compared with 1.23 ± 0.21 plat/cm for controls without associated segments of Dacron vascular graft.13 Similarly, the fibrin deposition onto collagen segments in the present study was compared to previous experience without proximal segments of vascular graft.13 Thus, the data suggest that proximal positioning of the segment of vascular graft did not affect the thrombogenicity of the collagen segment.

The compound, GT-12, or α,α'-bis[3-(M,N-diethylicarbamoyl) piperidin]-p-xylene dihydrobromide, is one of a series of carbamoylpiperidine and nipecoctyopiperazine congeners because of its low acute toxicity in vivo (i.p.) in male IRC mice (LD₅₀=837 μM/kg) and its potent inhibition of ADP-induced human platelet aggregation in vitro (IC₅₀, 11.4 μM).10 Also, the compound blocks thrombus growth in human whole blood induced by collagen-coated glass under controlled flow (platelets per thrombus reduced by 86.7% at 50 μM concentrations).8

Dogs receiving GT-12 intravenously (150 μmol/kg, 94.9 mg/kg) showed substantial inhibition of ADP-induced platelet aggregation ex vivo.6 The dose chosen for the present study was based on these preliminary data in dogs in which 50, 100, or 150 μmol/kg of the compound was injected intravenously over 3 to 6 minute period; side effects were noted with the highest dose consisting of salivation, perspiration, and muscle tremors. The infusion of 100 μM/kg in the baboons produced a detectable decrease in blood pressure of about 15 mm Hg in the awake animal lasting about 15 minutes, with no noticeable effect on the heart rate. Higher doses in two baboons were not well tolerated, and lesser doses in two other animals were judged to be ineffective.

The complex mechanisms involved in the formation of thrombus entail several different pathways of activation that interact to a variable extent. We have shown that the infusion of GT-12 in vivo inhibits several pathways of platelet activation and significantly reduces the deposition of platelets and fibrin during the formation of thrombus onto Dacron graft. It is our conclusion that GT-12, an inhibitor of platelet activation, affects platelet-dependent processes and has antithrombotic effects in vivo.

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References


34. Rink TJ. Cytosolic calcium in platelet activation. Experientia 1988;44:97–100


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