BRIEF COMMUNICATION

ANTIPLATELET EFFECT OF THE PYRIMIDO-PYRIMIDINIC DERIVATIVE RA-642

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INTRODUCTION

Amongst pyrimido-pyrimidine derivatives (p-p), dipyridamole (Dip) acts as a coronary vasodilator and platelet aggregation inhibitor (1-3), mopidamole (Mop) has been used in association with antimetastatic drugs (4,5), and RA-642 is endowed with vasodilating and hypertensive effects (6-8). We also reported that RA-642 inhibited lipid peroxidation induced by oxygen free radicals (9). We have found no references to RA-642 as a possible inhibitor of platelet aggregation. In the present study, we assessed the in vitro antiplatelet effect of RA-642 in comparison with Dip and Mop.

MATERIAL AND METHODS

Material

Dipyridamole (2,6-bis(di-ß-hydroxyethilamine)-4,8-di-(pyperidin-1)-pyrimido(5,5-d)-pyrimidine) was obtained from Boehringer Ingelheim S.A., Barcelona, Spain. RA-642 (2,2'-(4,8-bis(diethylamine)-pyrimido(5,4-d-pyrimidine-2,6-diyl)di-(2-metoxyethyl)imino)diethanol) and Mopidamole (2,6-bis(diethanolamine)-8-(N-py喋ridin)pyrimido(5,4-d)pyrimidine) were supplied by Dr Karl Thomae, Biberach an der Riss, Germany. ADP, adrenaline and collagen were obtained from Menarini Diagnostica, Barcelona, Spain. Arachidonic acid was obtained from Bio-data Corp, Hatboro PA, USA. Radioimmunoassay for thromboxane B2, and 8-14C-adenosine, were obtained from Amersham International plc, Amersham, UK. Other reagents were obtained from Sigma Chemical Co, St. Louis MO, USA.

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Blood samples
Blood samples were drawn from healthy male volunteers who had not taken any drug for at least 15 days prior to the study and mixed with 3.8% trisodium citrate in a proportion 1:10. All the tests were performed on at least six different samples.

Platelet aggregometry
The electric impedance method described by Cardinal and Flower (10) in whole blood (WB) and platelet-rich plasma (PRP) in a double channel Chrono-Log aggregometer was used. PRP was obtained from WB by centrifugation at 180 x g for 10 minutes. ADP (2.5 µM), adrenaline (25 µM), collagen (1 µg/ml), and arachidonic acid (AA) (0.4 mM) were used as inducers. Platelet aggregation was measured as the maximum change in impedance (ohms) 10 minutes after the addition of the aggregating agent.

8-14C-adenosine uptake
A modification of the method described by Roos and Peleger (11,12) was used to measure the uptake of adenosine into erythrocytes. The pellet obtained in the above-mentioned process of PRP preparation was removed and the red cells were washed twice with a solution of NaCl 153.8 µM, glucose 5 µM, and TRIS 5 µM, by centrifugation at 1250 x g for 20 min. To each tube 1 nmol/ml of 8-14C-adenosine was added and incubated at 37°C for 30 min. The final count of red blood cells was 203,000 ± 24,000/µL.

Lipid peroxidation assay
The production of malondialdehyde (MDA) after incubation with arachidonic acid (0.4 mM) for 3 minutes was determined by spectrophotometry, as described by Smith et al. (13).

Statistical analysis
Results are expressed as mean ± standard error. The statistical analysis of the results was carried out using the Epistat program. The Student's t test for unpaired data was applied to determine significant differences.

RESULTS
RA-642 showed a dose-related inhibitory effect on platelet aggregation both in WB and in PRP, except when AA was used as inducer (data not shown). The concentrations of RA-642, Dip, and Mop that produce 50% inhibition in platelet aggregation (IC-50) are shown in Table 1. Almost in all cases, IC-50's were significantly higher in PRP assays than in WB.
RA-642 showed a dose-related inhibition of platelet MDA production. IC-50's values were 139.9 ± 5.7 µM for RA-642, 140.5 ± 6.3 µM for Dip (p = 0.859 vs RA-642), and 91.4 ± 4.9 µM for Mop (p = 0.00024 vs RA-642 and Dip). Likewise, RA-642 showed a dose-related inhibition of 8-14C-adenosine uptake by erythrocytes. IC-50's values were 41.3 ± 6.5 µM for RA-642, 0.56 ± 0.17 µM for Dip, and 120.3 ± 10.8 µM for Mop (baseline uptake: 1120 ± 60 pmol/10^8 erythrocytes).
**TABLE 1**

IC-50 (µM) values for RA-642, dipyridamole and mopidamole for platelet aggregation in whole blood (WB) and platelet-rich plasma (PRP)

<table>
<thead>
<tr>
<th>Inducer</th>
<th>RA-642</th>
<th>Dipyridamole</th>
<th>Mopidamole</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>WB 82.5 ± 8.7</td>
<td>13.0 ± 0.2</td>
<td>4.0 ± 0.7</td>
</tr>
<tr>
<td>2.5 µM</td>
<td>PRP 105.0 ± 9.2</td>
<td>200.8 ± 20.7</td>
<td>26.8 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>(p = 0.001)</td>
<td>(p = 0.0001)</td>
<td>(p = 0.0001)</td>
</tr>
<tr>
<td>ADRENALINE</td>
<td>WB 152.8 ± 6.7</td>
<td>148.8 ± 5.6</td>
<td>23.6 ± 0.9</td>
</tr>
<tr>
<td>25 µM</td>
<td>PRP 110.8 ± 13.5</td>
<td>&gt; 1000</td>
<td>59.8 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>(p = 0.00014)</td>
<td>(p = 0.0021)</td>
<td></td>
</tr>
<tr>
<td>COLLAGEN</td>
<td>WB 47.5 ± 4.6</td>
<td>57.8 ± 13.5</td>
<td>19.8 ± 4.3</td>
</tr>
<tr>
<td>1 µg/ml</td>
<td>PRP 121.4 ± 7.2</td>
<td>362.1 ± 8.4</td>
<td>17.8 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>(p = 0.00001)</td>
<td>(p = 0.00001)</td>
<td>(p = 0.297)</td>
</tr>
</tbody>
</table>

In brackets statistical differences between WB and PRP are shown. Each value is the mean of at least 6 independent samples.

**TABLE 2**

Concentrations (µM) of adenosine that doubled and theophylline that halved the antiaggregating effect of pyrimido-pyrimidinic compounds

<table>
<thead>
<tr>
<th>Adenosine</th>
<th>Teophylline</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRP</td>
<td>WB</td>
</tr>
<tr>
<td>RA-642</td>
<td>3.1 ± 0.5</td>
</tr>
<tr>
<td>Dipyridamole</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>Mopidamole</td>
<td>0.4 ± 0.03</td>
</tr>
</tbody>
</table>

In each case, the concentration of pyrimido-pyrimidinic compounds was that able to produce a 25±5% inhibition when incubated alone (RA-642: 40 µM in WB and 50 µM in PRP; Dip: 7.5 µM in WB and 100 µM in PRP; Mop: 2 µM in WB and 15 µM in PRP). Each value is the mean of 6 independent samples. Aggregations were induced by ADP (2.5 µM).

Adenosine and theophylline were able to enhance and to reduce, respectively, the inhibitory effects of RA-642, Dip and Mop on ADP-induced platelet aggregation, both in WB and in PRP. Table 2 shows the concentrations of adenosine that doubled and those of theophylline that halved the antiaggregating effect of the p-p compounds. Adenosine (10 µM) inhibits platelet aggregation 5.8 ± 1.05% in PRP and 11.3 ± 2.9% in WB; theophylline (200 µM) inhibits platelet aggregation 17.8 ± 1.5% in PRP.
DISCUSSION

This study indicates an antiplatelet activity of RA-642, a p-p derivative, at concentrations ranging from 10 to 200 μM. The present result cannot be compared with those of other authors, since we have found no references to the antiplatelet effect of RA-642 in the literature. The effect produced by RA-642 in WB was 30% greater than in PRP. Data from Dip and Mop are in agreement with those of Gresele et al. (3) and with previous results obtained by our group (12, 14). The antiplatelet action of Dip can be explained by its inhibitory effect on adenosine uptake by erythrocytes (3, 15). In WB, RA-642 shows an antiaggregating effect similar to that of Dip (except in ADP-induced aggregation), whereas in PRP the activity of RA-642 was intermediate between Mop and Dip. In addition, RA-642 exhibited an antiperoxidative effect quantitatively similar to the inhibition of platelet aggregation and adenosine uptake. We reported that Dip and RA-642 inhibited lipid peroxidation induced by oxygen free radicals in several tissues of the rat (9, 16); Iuliano et al. (17), demonstrated that the antiperoxidative effect of Dip may be explained by a scavenging action of superoxide radicals which, in turn, enhance platelet adhesion and aggregation (18).

Adenosine plays an important role in the mechanism of action of Dip (3, 15) and can influence its antiplatelet effect, as shown by Dawicki et al. (19) and Agarwall et al. (20). In the present study, adenosine enhanced the effects of p-p compounds in the following sequence: Mop > Dip = RA-642. The incubation with theophylline (adenosine receptor antagonist) inhibited the effects in the same order. These data are in agreement with the inhibition of cAMP phosphodiesterase caused by these compounds, whose Ki are 12 ± 2 μM for Dip (21), 0.55 ± 0.15 μM for Mop (22), and 15 μM for RA-642 (Weisemberger, personal communication). For this reason, inhibition of cAMP phosphodiesterase may play an important role in the antiaggregating effect of RA-642 in PRP.

Dawicki et al. (19) suggested that when adenosine is incubated with p-p compounds and ADP in WB, the possible inhibitory effect on adenosine uptake by erythrocytes is being studied, because 99% of adenosine is uptaken after 1 minute of incubation. In the present study, adenosine enhanced the antiaggregating effects of p-p derivatives in the following sequence: RA-642 > Dip > Mop (Table 2). This order was neither the same as that of the inhibition of adenosine uptake by erythrocytes (Dip > RA-642 > Mop)(see Results), nor that of the inhibition of cAMP phosphodiesterase (Mop > Dip = RA-642)(21,22, Weisemberger personal communication). RA-642 may therefore exert a complementary action at another level of the adenosine metabolism in WB.

In conclusion, RA-642 is a p-p derivative that exhibits antiplatelet properties and shares pharmacodynamic characteristics similar to Dip and Mop, although differences in the mechanism of action may provide further implications for new indications of RA-642 in clinical therapeutics.
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REFERENCES


