Nitroaspirin plus clopidogrel versus aspirin plus clopidogrel against platelet thromboembolism and intimal thickening in mice

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Summary
Clopidogrel plus aspirin is the treatment of choice for patients undergoing percutaneous, coronary interventions with stenting, but it does not prevent restenosis. NCX-4016, a nitric oxide-releasing aspirin (nitroaspirin), exerts a wider range of antiplatelet actions compared to aspirin, superior antithrombotic activity and reduces restenosis after arterial injury in animals. The aim of the present study was to compare the combination of nitroaspirin plus clopidogrel with aspirin plus clopidogrel in a model of platelet pulmonary thromboembolism, bleeding and intimal thickening in mice. Drugs were administered orally for 5 days; the antithrombotic effects were evaluated against collagen plus epinephrine-induced pulmonary thromboembolism, the haemorrhagic effects by tail transection bleeding time and the effects on neointima proliferation by histomorphology of photochemically injured femoral arteries. Lung platelet emboli were reduced significantly and more effectively by nitroaspirin plus clopidogrel (-56%, p<0.05 vs control) than by aspirin plus clopidogrel (-26%, p<0.05 vs control). Ex vivo platelet aggregation was inhibited maximally by nitroaspirin plus clopidogrel. Aspirin plus clopidogrel strikingly prolonged the bleeding time while nitroaspirin plus clopidogrel induced a lesser prolongation. Nitroaspirin plus clopidogrel significantly reduced intimal thickening of the femoral artery while aspirin plus clopidogrel was ineffective. Nitroaspirin plus clopidogrel may represent a new regimen to be tested in patients undergoing coronary revascularization procedures.

Keywords
Nitric oxide, platelet aggregation, pulmonary thromboembolism, restenosis, thromboxane

Thromb Haemost 2005; 93: 535-43

Introduction

The rupture of an atherosclerotic plaque with the exposure of subendothelial thrombogenic substances and the subsequent formation of an arterial thrombus are the main initiating mechanisms of acute coronary syndromes. Platelets are the pivotal blood element in arterial thrombosis and antiplatelet agents represent the gold therapeutic standard in the antithrombotic treatment of acute coronary syndromes (1, 2). Platelet aggregation on a ruptured plaque is a complex process involving multiple stimuli and disturbed flow conditions, thus multiple therapeutic agents are required to block its redundant pathways (3–5). In particular, percutaneous coronary interventions (PCI), with the damage inflicted to diseased, atheromatous vessels, represent a particularly strong thrombotic stimulus and several observations suggest that platelet activation started by intracoronary revascularization maneuvers is the initial trigger for the accelerated atherogenesis that leads to restenotic phenomena.

Indeed, the development of restenosis due to neointimal hyperplasia is the main long-term complication after percutaneous transluminal coronary angioplasty (PTCA) (6, 7) affecting up to 33% of patients 6 months after the procedure (8), especially those with risk factors, such as diabetes (9). Antithrombotic treatment following elective PCI with stenting involves the use
of aspirin plus clopidogrel, a combination that has shown benefit both in short- (10) and long-term trials (11), as well as in acute coronary syndromes (12). However, no evidence has been provided that this antiplatelet combination has any effect on the main long-term complication of PCI plus stenting, i.e. restenosis, especially in high-risk patients, such as diabetics (9).

Moreover, the risk of bleeding, and especially of gastrointestinal haemorrhage, remains one possible limitation to the chronic use of this antiplatelet combination therapy (11–13) and ongoing long-term clinical trials (CHARISMA) are exploring the risk/benefit ratio of this drug combination in the chronic treatment of ischemic cardiovascular disease in patients at high risk. Also the adjunctive antiplatelet treatment with intravenous GPIIb/IIIa antagonists has definitely reduced acute ischemic events and improved survival in patients undergoing percutaneous coronary interventions (14), but no evidence of prevention of long-term restenosis has been obtained (15). NCX-4016 (NicOx Research Institute, Milan, Italy), a novel nitric oxide-donating aspirin (nitroaspirin), has been shown to exert a wider range of antiplatelet actions and an antithrombotic effect superior to that of aspirin in different animal models (16, 17). Moreover, nitroaspirin does not induce gastric damage, either in animals (18) or in humans (19). Recently it was shown that nitroaspirin reduces restenosis after balloon angioplasty in hypercholesterolemic mice and in aged rats (20–22) while aspirin is ineffective.

The aim of our study was to compare the antithrombotic effect of nitroaspirin plus clopidogrel versus aspirin plus clopidogrel in a platelet pulmonary thromboembolism model in mice, to evaluate their effects on bleeding and to assess their comparative effects in preventing restenosis in a model of photochemically induced localized arterial damage in mice.

Materials and methods

Reagents

The sources of the reagents used were as follows: U46619 (9, 11-dideoxy-11α, 9α epoxymethano-prostaglandin F2α), ADP, Rose Bengal, polyethylene glycole 400, arachidonic acid (Sigma Chemicals, St. Louis, MO); epinephrine bitartrate, a 5 mM solution in Tris buffer (Mascia Brunelli, Milan, Italy); equine tendon collagen in suspension (Hormon Chemie, Munich, Germany); acetyl salicylic acid (Flectador, Carlo Erba, Milan, Italy); non enzymatic NOx method (Oxford Biomedical Research, Michigan, USA); cGMP EIA kit (Amersham Biosciences, Freiburg, Germany); NCX-4016 (2 acetoxybenzoate-2-[1-nitroxy-methyl]-phenyl ester) (NicOx Research Institute, Milan, Italy).

All drugs were dissolved in 0.9% saline; nitroaspirin was dissolved in polyethylene glycole.

In vivo thrombosis model in the mouse

Animals

6 to 8 week-old male CD1 mice (20–25 g) were from Charles River (Lecco, Italy). Animals were randomly distributed into seven treatment groups: 1- nitroaspirin (60 mg/kg); 2- aspirin (30 mg/kg); 3- clopidogrel (0.5 mg/kg); 4- aspirin+clopidogrel (30 and 0.5 mg/kg, respectively); 5- nitroaspirin+clopidogrel (60 and 0.5 mg/kg, respectively); 6- aspirin+nitroaspirin+clopidogrel (30, 60 and 0.5 mg/kg, respectively); 7- and vehicle (PEG 400, 100 μl). Drugs were orally administered in a fixed volume of 100 μl, once a day for five days. Animals were weighted before the first and one hour after the last drug administration (n=5 animals per treatment regimen). Haematological parameters (blood cells counts and haematocrit) were assessed by an automatic cell analyzer (Genius, Seac, Florence, Italy) 1 hour after the last drug administration (n=5 animals per treatment group).

Pulmonary thromboembolism

Pulmonary platelet thromboembolism was induced by a method described previously (23, 24). At least 5 animals per treatment group were studied in each experimental session, and at least two experimental sessions for each treatment were carried out. One hour after the last drug administration a thrombotic challenge was induced by the rapid i.v. injection of 100 μl of a mixture of collagen (250 μg/ml; 12.5 μg/mouse) and epinephrine (1.5 μg/ml; 0.075 μg/mouse) (16, 23, 24).

Lung histology

Two minutes after the i.v. injection of collagen+epinephrine, the lower right-lung lobe, prepared as described (23, 24), was collected and embedded in paraffin. Several sections 5–6 μm thick, were cut and stained with haematoxylin and eosin, staining evidencing platelet thrombi. The total number of lung vessels per high magnification microscopic field was counted and the percentage of them occluded by platelet thrombi was annotated (at least 20 fields for each animal tested) (24).

Platelet count

Platelet counts were carried out on blood samples collected 2 min after the thrombotic challenge in saline-pretreated (controls) or drug-pretreated animals, as previously described (23, 24).

Bleeding time

Bleeding time was assessed by a tail transection method, as previously reported (25). Bleeding was recorded for a maximum of 900 sec. and the end point was the arrest of bleeding; if bleeding restarted within 30 sec, bleeding time recording continued until a new arrest lasting for more than 30 sec had occurred.

All animal experiments were approved by the Committee on Ethics of Animal Experiments of the University of Perugia and by the Italian Ministry of Public Health (Authorization n° 21/2000-B).

Platelet aggregation studies

Platelet aggregation ex vivo was performed in platelet rich plasma (PRP) prepared by centrifuging citrated blood at 150 x g for 15 min and then adjusting platelet count to 250,000/μl with platelet poor plasma (PPP), as described (26). Blood was collected by cardiac puncture 1 hour after the last drug administration on day 5 and U46619 (4 μM)-, (a stable TxA2 analogue), arachidonic acid (0.2 mM)- and ADP(0.8 μM)-induced platelet aggregation was performed.

Measurement of thromboxane

For serum TxB2 measurement, blood was collected by cardiac puncture from anesthetized mice into non-anticoagulated glass
tubes and placed in a water bath at 37°C for 1 h for serum preparation.

Immunoreactive TxB₂ was measured in unextracted serum samples by a highly specific radioimmunoassay, as described (27, 28).

The urinary excretion of 11-dehydro-TXB₂ (urine collections from groups of 5 mice treated orally once a day for 5 days) was measured by a previously described and validated radioimmunoassay, after extraction and purification on Sep-Pak C18 cartridges (Waters Associates) and silicic acid chromatography (29), on urines collected for 24 h before the first and for 24 h after the last drug administration.

Plasmatic nitrite and nitrate
Nitrite and nitrate (NOx) were determined in platelet poor plasma (PPP), prepared from blood collected by cardiac puncture in sodium citrate 4% (1:10), by a colorimetric, non enzymatic method (Oxford Biomedical Research, Michigan, USA)(30).

Intraplatelet cGMP
In order to investigate if the effects of nitroaspirin are mediated by in vivo release of NO, intraplatelet levels of cGMP were measured by a commercially available enzyme immunoassay kit. Blood samples were taken by cardiac puncture one hour after the last drug administration. PRP was obtained by centrifugation at 150 xg for 10 min and then centrifuged again at higher speed to obtain a platelet pellet that was extracted with the lysis reagent provided by the manufacturer and stored at –80°C.

Intraplatelet cGMP was expressed as pmol/10⁵ platelets.

Photochemically-induced localized vessel wall injury in mice
Mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and a butterfly 25 G needle was inserted in one of the tail veins for Rose Bengal infusion (31). The right femoral artery was surgically exposed and transilluminated with green light (wavelength 540 nm), by a xenon lamp with a heat-absorbing filter, through an optic fiber positioned 5 mm away from the arterial segment (Hamamatsu Photonics, Shizuoka, Japan). Green light irradiation was protracted for 25 min; the infusion of Rose Bengal (20 mg/kg) was started 5 min after the beginning of irradiation and lasted for 5 min. The wound was then closed and animals returned to their cage. 21 days after endothelial injury mice were anesthetized, the chest and abdominal cavities were opened and a catheter was inserted into the left ventricle. The circulatory system was initially washed with saline and then perfused with a solution of 2% glutaraldehyde and 1% paraformaldehyde in 0.1 mM PBS, pH 7.4, at physiologic pressure (90 to 100 mmHg) for 10 minutes. The femoral artery was then removed and fixed overnight in the same fixative. Femoral artery segments were embedded in paraffin and cut consecutively in 5 μm thick sections. The sections for morphometric analysis, taken at intervals of 500 μm, were stained with hematoxylin and eosin. The area of media and intima were measured using a computerized apparatus (NIH Scion Image, Maryland, USA). Measurements were made in a blinded manner as regards to treatment. Some sections were stained with an α-actin monoclonal antibody (Sigma, USA).

Statistical analysis
Given that the minimum effect size considered to be of relevance was an increase of the proportion of surviving animals in the treated groups up to 50%, choosing a significance level of 0.05 and a power of 80%, the number required in each group was estimated to be of 14 animals (Graphpad 4 software Stat-Mate, S.Diego, CA, USA). When in the first two experimental sessions the proportion of surviving animals approximated the efficacy threshold, additional experimental sessions were carried out in order to fulfill the required sample size. The Chi-square test was applied to the studies on mortality; the Bonferroni’s correction to all the studies comparing different treatments (statistically significant p<0.05/number of comparisons). One-way analysis of variance (ANOVA), followed by the Bonferroni’s multiple comparisons test, was used for all the other studies. Data are expressed as means ± SEM (n).

Results
Effect of treatment on body weight and haematologic parameters
Drug administration was well tolerated by all the animals and no animal died or showed signs of discomfort in any of the treatment groups.

The administration of nitroaspirin (60 mg/kg), aspirin (30 mg/kg) and clopidogrel (0.5 mg/kg) once a day for five days, alone or in combination, did not modify body weight or the hematologic parameters (Table 1).

In vivo thrombosis model in the mouse
Pulmonary thromboembolism
The i.v. injection of collagen plus epinephrine led to 85–90% death in control animals.

Aspirin protected significantly animals from death only at a dose of 300 mg/kg, reducing mortality to 50% (p=0.0007 vs control), similarly to what was previously reported (22). Clopidogrel also exerted a protective effect, which was significant only at the dose of 75 mg/kg (50% mortality, p=0.0051). Nitroaspirin dose-dependently protected animals, with a significant reduction of mortality starting at the dose of 60 mg/kg and a reduction down to 28.5% mortality with the highest dose tested (120 mg/kg) (Table 2).

The doses for the drug combination studies were selected taking into account the effects of the single drugs both on mortality, choosing non protective doses, and on the bleeding time, choosing doses not prolonging maximally bleeding in order to permit the evaluation of possible additive effects; nitroaspirin was used at a dose equimolar to its parent compound (aspirin).

Clopidogrel prolonged the bleeding time above maximum starting from the dose of 10 mg/kg and aspirin starting from the dose of 200 mg/kg. Clopidogrel at the dose of 0.5 mg/kg, aspirin at 30 mg/kg and nitroaspirin at 60 mg/kg, prolonged the bleeding time significantly but not maximally, and to a similar extent (Table 2), and therefore these doses were chosen for subsequent drug combination studies.

The combination of aspirin (30 mg/kg) and clopidogrel (0.5 mg/kg), at doses not effective when used alone, significant-
Table 1: Body weight and hematological parameters.

<table>
<thead>
<tr>
<th>Drugs (mg/kg)</th>
<th>MORTALITY</th>
<th>BLEEDING TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dead / % mortality</td>
<td>P vs control</td>
</tr>
<tr>
<td>Control</td>
<td>36/44</td>
<td>81.8</td>
</tr>
<tr>
<td>Aspirin 15</td>
<td>n.d.a</td>
<td>n.d</td>
</tr>
<tr>
<td>Aspirin 30</td>
<td>12/16</td>
<td>75</td>
</tr>
<tr>
<td>Aspirin 60</td>
<td>18/24</td>
<td>75</td>
</tr>
<tr>
<td>Aspirin 100</td>
<td>7/10</td>
<td>70</td>
</tr>
<tr>
<td>Aspirin 200</td>
<td>18/30</td>
<td>60</td>
</tr>
<tr>
<td>Aspirin 300</td>
<td>35/70</td>
<td>50</td>
</tr>
<tr>
<td>NCX-4016 15</td>
<td>8/10</td>
<td>80</td>
</tr>
<tr>
<td>NCX-4016 30</td>
<td>11/18</td>
<td>61.1</td>
</tr>
<tr>
<td>NCX-4016 60</td>
<td>7/19</td>
<td>36.8</td>
</tr>
<tr>
<td>NCX-4016 120</td>
<td>4/14</td>
<td>28.5</td>
</tr>
<tr>
<td>Clopidogrel 0.25</td>
<td>22/26</td>
<td>84.6</td>
</tr>
<tr>
<td>Clopidogrel 0.5</td>
<td>22/28</td>
<td>78.6</td>
</tr>
<tr>
<td>Clopidogrel 1.0</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Clopidogrel 2.5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Clopidogrel 10</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Clopidogrel 18</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Clopidogrel 25</td>
<td>10/15</td>
<td>66.6</td>
</tr>
<tr>
<td>Clopidogrel 50</td>
<td>11/18</td>
<td>61.1</td>
</tr>
<tr>
<td>Clopidogrel 75</td>
<td>15/30</td>
<td>50</td>
</tr>
</tbody>
</table>

* = not done; † = not significant; §: test refers to Chi square; ‡ data refer to ANOVA followed by the Bonferroni’s multiple comparison test.

Table 2: Dose-response curves for aspirin, nitroaspirin and clopidogrel on collagen+epinephrine-induced mortality and bleeding time.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Red blood cells (x10^6/μL)</th>
<th>Platelets (x10^3/μL)</th>
<th>Hematocrit %</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (PEG 400)</td>
<td>7.6±0.25</td>
<td>1055.3±80</td>
<td>41.4±0.94</td>
<td>24.8±0.87</td>
</tr>
<tr>
<td>Aspirin (30mg/kg)</td>
<td>7.45±0.23</td>
<td>1185±116</td>
<td>40.6±1.36</td>
<td>25.9±0.33</td>
</tr>
<tr>
<td>Nitroaspirin (60 mg/kg)</td>
<td>7.36±0.15</td>
<td>1135.6±185</td>
<td>39.6±0.3</td>
<td>25.2±1.15</td>
</tr>
<tr>
<td>Clopidogrel (0.5 mg/kg)</td>
<td>7.67±0.21</td>
<td>1191.2±111</td>
<td>39.9±0.55</td>
<td>25.5±0.42</td>
</tr>
<tr>
<td>Aspirin (30 mg/kg)+ Clopidogrel (0.5 mg/kg)</td>
<td>7.6±0.11</td>
<td>1139±112.6</td>
<td>40±0.4</td>
<td>26.9±0.41</td>
</tr>
<tr>
<td>Nitroaspirin (60 mg/kg)+ Clopidogrel (0.5 mg/kg)</td>
<td>7.12±0.21</td>
<td>1068±109</td>
<td>41.6±0.67</td>
<td>24.7±0.45</td>
</tr>
<tr>
<td>Nitroaspirin (60 mg/kg)+ Aspirin (30 mg/kg)+ Clopidogrel (0.5 mg/kg)</td>
<td>7.33±0.18</td>
<td>1133±89</td>
<td>38.6±2.2</td>
<td>25.6±0.53</td>
</tr>
</tbody>
</table>

All drugs were administered orally once a day for five days; all the parameters were measured one hour after the last administration.
telet emboli. Aspirin, at 30 mg/kg, did not prevent platelet thromboembolic occlusion of lung vessels (96.6±3.3%, n=20) and clopidogrel (0.5 mg/kg) reduced only slightly, and not significantly, the number of pulmonary emboli (80.6±2.92%, n=62), while the combination reduced significantly the percentage of occluded lung vessels (69.1±4.8%, n=33, p<0.05 vs control), confirming a potentiation of platelet inhibition. Nitroaspirin (60 mg/kg) reduced significantly the number of occluded vessels (40.8±4.9%, n=20, p<0.001 vs control). The addition of aspirin to the combination nitroaspirin plus clopidogrel did not further reduce lung vessels occlusion (49.2±8.0%, p<0.001 vs control, n=20) (Fig. 1B). The direct comparison between aspirin plus clopidogrel versus nitroaspirin plus clopidogrel showed a superior effect of the latter (p<0.05).

In vivo platelet consumption
Intravenous collagen plus epinephrine reduced circulating platelets by 89.4% (111.0±12 x 10^9/L; n=25, p<0.001 vs basal). Aspirin (30 mg/kg) was ineffective in preventing agonist-induced platelet drop (121±13 x 10^9/L, n=11, p=NS vs collagen+epinephrine) and clopidogrel (0.5 mg/kg) reduced it only slightly, though significantly (173.7±13 x 10^9/L, n=7, p<0.05). The combination of clopidogrel and aspirin was significantly more effective in preventing the platelet drop than aspirin (210±14.7 x 10^9/L, n=7, p<0.05 vs collagen plus epinephrine) further confirming enhanced antiplatelet effect. Nitroaspirin (60 mg/kg) reduced significantly the drop of circulating platelets (239±52 x 10^9/L, n=7, p<0.001) and this effect was significantly greater when it was combined with clopidogrel (324±41 x 10^9/L, n=7, p<0.001) and with clopidogrel plus aspirin (333±29 x 10^9/L, n=7, p<0.001) (Fig. 1C). The direct comparison aspirin plus clopidogrel versus nitroaspirin plus clopidogrel showed no difference.

Bleeding time
The combination aspirin plus clopidogrel strikingly prolonged the bleeding time (800±67.6 sec, n=9, p<0.001), while the combination nitroaspirin plus clopidogrel did not prolong it any more than the single drugs (484.6±90 sec, n=13, p=0.001). The addition of aspirin (30 mg/kg) to nitroaspirin plus clopidogrel, while
not further modifying protection against collagen+epinephrine-induced mortality (40% mortality), lead to a prolongation of the bleeding time to a level similar to the combination aspirin+clopidogrel (733±244 sec, n=8, p<0.001) (Fig. 1D). The direct comparison between aspirin plus clopidogrel and nitroaspirin plus clopidogrel showed lesser prolongation of the bleeding time with the latter (p<0.01).

**Ex vivo platelet aggregation**

ADP-induced platelet aggregation was inhibited in clopidogrel-pretreated mice, both when the drug was administered alone or in combination. Aspirin completely abolished arachidonic acid-induced aggregation; a similar effect was observed in platelets of animals pretreated with nitroaspirin, although not quite to the same extent. Nitroaspirin also inhibited U46619-induced aggregation, an effect characteristic of nitric oxide donating agents (32, 33), while this parameter was not affected by aspirin and only little affected by clopidogrel (Fig. 2). The combination of nitroaspirin and clopidogrel and this combination plus aspirin significantly inhibited platelet aggregation induced by all the agonists tested (Fig. 2). The direct comparison between aspirin plus clopidogrel versus nitroaspirin plus clopidogrel showed greater inhibition of platelet aggregation induced by ADP and by U46619 with the latter.

**Figure 2:** Effect of nitroaspirin (60 mg/kg), aspirin (30 mg/kg), clopidogrel (0.5 mg/kg) and their combinations on ex vivo platelet aggregation induced by U46619, arachidonic acid and ADP. *p 0<0.05, at least, vs control (by ANOVA followed by the Bonferroni's multiple comparisons test)(n=3).

**Figure 3:** Effect of nitroaspirin (60 mg/kg), aspirin (30 mg/kg), clopidogrel (0.5 mg/kg) and their combinations on: A) serum TxB2 formation; B) urinary excretion of 11-dehydro TxB2. Data are expressed as means±SEM (n=15, per treatment group). *p<0.05, at least, vs control (ANOVA followed by the Bonferroni's multiple comparisons test).
**Thromboxane measurements**

Serum TxB₂ in control mice was 118±18.9 ng/ml. Aspirin reduced strikingly TxB₂ formation, both alone (7.6±2.6 ng/ml, n=9, p<0.001) or in combination with clopidogrel (9.1±2.7 ng/ml, n=5, p<0.001). Nitroaspirin reduced serum TxB₂, although not quite to the same extent as equimolar aspirin (26.2±3.4 ng/ml, n=9, p<0.01 vs control), and the effect was maintained in association with clopidogrel (33.8±7.1 ng/ml, n=5, p<0.01 vs control). The combination of aspirin, nitroaspirin and clopidogrel strongly reduced TxB₂ formation (5.1±1.5 ng/ml, n=5, p<0.001) (Fig. 3A).

Aspirin reduced significantly urinary 11-dehydro-TxB₂ excretion (64.3% inhibition, p<0.01 vs control) (Fig. 3B) while clopidogrel was ineffective. Aspirin plus clopidogrel inhibited urinary 11-dehydro-TxB₂ excretion by 65.7% (p<0.05). Nitroaspirin also inhibited significantly urinary 11-dehydro-TxB₂ excretion (50.8% inhibition, p<0.05), and did so slightly more when administered in combination with clopidogrel (64.8% inhibition, p<0.05) and even more when associated with clopidogrel plus aspirin (65.9% inhibition) (Fig. 3B).

**Plasmonic nitrates and nitrates**

As expected, only animals receiving nitroaspirin, either alone or in association with clopidogrel, showed a significant enhancement of plasmatic NOx (control: 14.1±2.6 μM; nitroaspirin: 36.9±3.3 μM; nitroaspirin plus clopidogrel: 31.9±8.5 μM; nitroaspirin plus clopidogrel plus aspirin: 36.2±13.3 μM, p<0.05 vs control). This finding confirms that nitroaspirin was absorbed and metabolized after oral administration and the NO group liberated in the circulation.

**Intraplatelet cGMP**

A significant increase in intraplatelet cGMP was found after 5 days of oral treatment with nitroaspirin either alone or in combination (control: 0.13±0.04 pmol/10⁸ platelets; nitroaspirin: 1.27±0.56; nitroaspirin plus clopidogrel: 1.21±0.21; nitroaspirin plus clopidogrel plus aspirin: 1.22±0.22; p<0.05 vs control for all treatments) while aspirin (0.67±0.28 pmol/10⁸ platelets), clopidogrel (0.56±0.13 pmol/10⁸ platelets) or their combination (0.34±0.10 pmol/10⁸ platelets) did not significantly affect this parameter.

**Effects on intimal thickening**

Twenty-one days after vascular injury, an extensive neointima had formed at the injured arterial segment with a significant increase of the intima/media ratio as compared with sham-operated controls (Fig. 4A and B). Aspirin did not affect intimal thickening while clopidogrel slightly, but not significantly, reduced it. The combination of aspirin plus clopidogrel reduced the I/M ratio, although just not significantly. Nitroaspirin significantly reduced the I/M ratio, an even more visible effect when the

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**Figure 4: Effect of nitroaspirin (60 mg/kg), aspirin (30 mg/kg), clopidogrel (0.5 mg/kg) and their combinations on intimal thickening induced by photochemical damage in the femoral artery.** (A) Ratio between intima [I] and media [M] (bars represent mean±SEM, n=8); (B) Representative photomicrographs of hematoxylin and eosin staining (a=sham-operated, b=control, c=aspirin, d=clopidogrel, e=aspirin plus clopidogrel, f=nitroaspirin, g=nitroaspirin plus clopidogrel, h=nitroaspirin plus aspirin plus clopidogrel) or α-actin smooth muscle immunostaining (i=sham operated, m=control). Magnification is 600x. Bar=50 μm. Internal elastic lamina is indicated by arrowheads. At least 8 sections for treated animal were analyzed. *p<0.05, at least, vs control (by ANOVA followed by the Bonferroni’s multiple comparisons test).
drug was used in combination with clopidogrel or with clopi-
dogrel and aspirin (Fig. 4A). The reduction of intimal hyperplasia
observed with nitroaspirin plus clopidogrel was significantly
larger than that observed with aspirin plus clopidogrel (Fig. 4A)
(p < 0.01). Some sections from sham operated and control mice
were immunostained with an α-actin monoclonal antibody to re-
veal smooth muscle cells (Fig. 4B). At day 21, almost the entire
neointima was positive for α-actin confirming that photochemi-
cally-induced localized femoral artery injury produces smooth
muscle cell proliferation in mice.

Discussion

Our study shows that the combination of nitroaspirin with clopi-
dogrel inhibits platelet aggregation ex vivo, reduces platelet lung
emboli in vivo and displays antiproliferative effects in vivo in
mice more effectively than the aspirin plus clopidogrel com-
bination, but with a lesser prolongation of the bleeding time.

In our model, in which the drugs were administered orally
once a day for five days in order to mimic the clinical regimen,
a clear additive antiplatelet and antithrombotic effect was ob-
served by combining aspirin with clopidogrel, reproducing phar-
macodynamic (34, 35) and clinical data (10–12) in humans. The
bleeding time was also much more prolonged when the two
drugs were given in combination, and this too appears to repro-
duce human data (12, 35), suggesting that the results obtained in
this model may be of some clinical relevance.

The likely explanation of the lesser prolongation of the bleed-
ing time with NCX-4016 plus clopidogrel versus aspirin plus
clopidogrel is the lower degree of inhibition of in vivo thromb-
oxane biosynthesis. In this model, the degree of thromboxane
inhibition is critically involved in the prolongation of the bleeding
time, in fact aspirin starts to prolong it at dosages (15 mg/kg) that
suppress serum TxB2 production by ≥90% (unpublished data).
In agreement with this, when aspirin was added to nitroaspirin plus
clopidogrel, thus suppressing serum thromboxane by >90%, a
striking prolongation of the bleeding time was observed. A lower
degree of thromboxane inhibition by nitroaspirin as compared to
aspirin, previously reported both in animals (36) and in humans
(19), is probably due to an early, possibly pre-portal, deacety-
lation of a large fraction of the nitroaspirin absorbed by the gas-
trointestinal tract (36). Alternatively, lower absorption of the
whole molecule as compared with aspirin is another possibility.
On the contrary, in vitro, in human whole blood, nitroaspirin in-
hibits TxB2 formation by platelets at identical concentrations as
aspirin (37).

The lack of an excessive prolongation of the bleeding time
with the combination NCX-4016 plus clopidogrel, despite a
strong antithrombotic effect, seems a favourable finding, es-
pecially for patients that need to undergo urgent surgical pro-
cedures, such as coronary artery by-pass surgery (38).

Most interestingly, the combination of nitroaspirin and clopi-
dogrel, alone or in the presence of aspirin, inhibited the prolifer-
ation of arterial neointima triggered by oxygen radicals, a pro-
cess reproducing the phenomena seen after balloon angio-
plasty in humans (39), thus showing that the antiproliferative activity
of nitroaspirin (40) is maintained when the drug is used in com-
bination with other established antiplatelet agents. This action is
probably the consequence of the inhibitory effects of NO on
smooth muscle cells growth and of the anti-adhesive effects on
leukocytes and macrophages (40, 41). It is, however, interesting
that in our studies the drug combinations producing the most
striking inhibition of platelet aggregation (nitroaspirin plus
clopidogrel or nitroaspirin plus clopidogrel plus aspirin) gave the
most effective inhibition of intimal proliferation, suggesting that
a very profound suppression of platelet activation may con-
tribute to the inhibition of atherogenesis and that platelets may
play a role in vessel wall proliferation (42). In particular, nitroas-
pirin plus clopidogrel strongly inhibited both ADP- and TxA2
(U46619)-induced platelet aggregation, differently from aspirin
plus clopidogrel, and ADP and TxA2 are known to be powerful
stimulators of smooth muscle cell proliferation (42, 43).

The better antiplatelet and antithrombotic profile of the com-
bination of nitroaspirin with clopidogrel is probably to be as-
scribed largely to a NO-mediated effect on platelets, as docu-
mented by the enhancement of intraplatelet cGMP. Additional
findings supporting this view are the inhibition of U46619-in-
duced aggregation, an agonist insensitive or only partially sensi-
tive to aspirin or clopidogrel, and the increase of NO degradation
products in plasma of animals treated with the clopidogrel plus
nitroaspirin combination. Thanks to the peculiar slow and pro-
tracted release of NO in vivo by nitroaspirin (44), these NO-re-
lated effects take place without any significant hypotensive reac-
tion in mice (45) or humans (19).

In conclusion, the combination of nitroaspirin plus clopido-

grel exerts stronger antiplatelet effects, inhibition of platelet lung
emboli, and prevention of restenosis as compared with the com-
bination aspirin plus clopidogrel in mice.

If further testing of this drug combination in other animal
models confirms the advantages observed in our studies, it could
be hypothesized that nitroaspirin plus clopidogrel, or the addi-
tion of nitroaspirin to the gold standard aspirin plus clopidogrel,
may enhance clinical efficacy in patients undergoing coronary
revascularization procedures, by strengthening the antithrom-
botic effects and by reducing restenosis.

Acknowledgments

The help of Dr. Giuseppe Guglielmini, Dr. Roberta Caracchini and Dr.
Mario Leone with some parts of the analysis of the data is gratefully ac-
knowledged.

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