Direct and Irreversible Inhibition of Cyclooxygenase-1 by Nitroaspirin (NCX 4016)

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ABSTRACT

Benzoic acid, 2-(acetyl-oxy)-3-[(nitrooxy)methyl]phenyl ester (NCX 4016), a new drug made by an aspirin molecule linked, through a spacer, to a nitric oxide (NO)-donating moiety, is now under clinical testing for the treatment of atherothrombotic conditions. Aspirin exerts its antithrombotic activity by irreversibly inactivating platelet cyclooxygenase (COX)-1. NCX 4016 in vivo undergoes metabolism into deacetylated and/or denitrated metabolites, and it is not known whether NCX 4016 needs to liberate aspirin to inhibit COX-1, or whether it can block it as a whole molecule. The aim of our study was to evaluate the effects of NCX 4016 and its analog or metabolites on platelet COX-1 and whole blood COX-2 and on purified ovine COX (oCOX)-1 and oCOX-2. In particular, we have compared the mechanism by which NCX 4016 inhibits purified oCOX enzymes with that of aspirin using a spectrophotometric assay. All the NCX 4016 derivatives containing acetylsalicylic acid inhibited the activity of oCOX-1 and oCOX-2, whereas the deacetylated metabolites and the nitric oxide-donating moiety were inactive. Dialysis experiments showed that oCOX-1 inhibition by NCX 4016, similar to aspirin, is irreversible. Reversible COX inhibitors (indomethacin) or salicylic acid incubated with the enzyme before NCX 4016 prevent the irreversible inhibition of oCOX-1 by NCX 4016 as well as by aspirin. In conclusion, our data show that NCX 4016 acts as a direct and irreversible inhibitor of COX-1 and that the presence of a spacer and NO-donating moiety in the molecule slows the kinetics of COX-1 inhibition by NCX 4016, compared with aspirin.

Cyclooxygenase (COX) catalyzes the committed step in the conversion of arachidonic acid into prostaglandins and thromboxanes (Smith et al., 2000). Two isoforms of the enzyme have been identified, COX-1 and COX-2, that are the product of two distinct genes (Appleby et al., 1994), and both isoforms are inhibited by nonsteroidal anti-inflammatory drugs (NSAIDs) (Smith et al., 1997; Marnett et al., 1999). Aspirin is unique among NSAIDs because it covalently modifies COX by acetylating a serine residue positioned in the arachidonic acid-binding channel of the enzyme (Ser529 of COX-1 and Ser516 of COX-2), thus irreversibly inactivating it and preventing the generation of prostaglandins and thromboxane A2 (Loll et al., 1995). Ser529 is not directly involved in catalysis; in fact, mutation of this residue has no effect on the enzyme activity, but it is strategically located, and its acetylation prevents the access of the substrate to the catalytic site of the enzyme (Loll et al., 1995). Other NSAIDs, such as ibuprofen and indomethacin, inhibit competitively and reversibly COX and prevent the irreversible acetylation of COX-1 by aspirin by occupying the active site channel of the enzyme, thereby preventing aspirin from reaching its target (Catella-Lawson et al., 2001; Ouellet et al., 2001). Mature normal human platelets express only COX-1, and because they are anucleate cells unable to synthesize enzyme de novo, the effect of aspirin on them is permanent and cumulative (Patrono, 1994). Thus, the cardioprotective effect of aspirin is exerted through the irreversible and permanent impairment of thromboxane A2-dependent platelet function, which reduces the development of acute arterial thrombosis (Patrono, 1994). One of the limitations of NSAIDs is the occurrence of side effects in the gastrointestinal tract (e.g., gastritis and ulceration), resulting from the inhibition of vasodilator and cytoprotective prostanooids in the gastric mucosa, which limits the use of these compounds (Wallace, 1997).

In the past few years, nitric oxide (NO)-releasing NSAIDs

ABBREVIATIONS: COX, cyclooxygenase; NSAID, nonsteroidal anti-inflammatory drug; NO, nitric oxide; TxB2, thromboxane B2; LPS, lipopolysaccharide; PG, prostaglandin; oCOX, ovine cyclooxygenase; TMPD, N,N,N’,N’-tetramethyl-p-phenylenediamine; HPLC, high-performance liquid chromatography; NCX 4016, benzoic acid, 2-(acetyl-oxy)-3-[(nitrooxy)methyl]phenyl ester; NCX 4019, 2-(hydroxy)-3-(hydroxymethyl)phenyl ester; NCX 4215, acetylsalicylic acid 4-(nitroxy)butyl ester; NCX 4015, benzenemethano-3-hydroxy-α-nitrate.
have been developed (Burgaud et al., 2002) with the aim of reducing gastrointestinal toxicity by exploiting the protective activity of locally released NO on gastric mucosa (Wallace, 1997; Lanas et al., 2000). They are synthesized by the ester linkage of a NO-releasing moiety to conventional NSAIDs, such as aspirin (NCX 4016), flurbiprofen (HCT 1026), or naproxen (HCT 3012). For NCX 4016, the acetylsalicylic acid molecule is linked to a chemical spacer (hydroxybenzylalcohol) bearing the NO-donating moiety benzenemethanol-3-hydroxy-o-nitrate (NCX 4015), and the resulting molecule has a molecular mass 1.8-fold greater than that of aspirin (Del Soldato et al., 1999).

Nitroaspirin, besides its better gastric tolerability (Fiorucci et al., 2003), seems to be superior to aspirin in terms of platelet inhibition (Lechi et al., 1996; Mezzasoma et al., 1999) and antithrombotic activity (Momoi et al., 2000, 2005), and this is probably due to a combination of the antiplatelet effects of aspirin and the broad-range cardiovascular activities of NO (Mezzasoma et al., 1999; Gresele et al., 2003). Moreover, differently from aspirin, NCX 4016 inhibits the activity of COX-2 of stimulated monocytes in human whole blood through a NO-mediated mechanism (Corazzi et al., 2003).

However, although NCX 4016 inhibits COX-1 with a potency similar to that of aspirin (Lechi et al., 1996; Mezzasoma et al., 1999; Corazzi et al., 2003), it is not clear whether the inhibitory activity is exerted directly by the whole molecule or whether it is the consequence of its enzymatic transformation, in whole blood or in other organs, into acetylsalicylic acid. It is indeed conceivable that the steric hindrance created by the spacer and the NO-releasing moiety of NCX 4016 may hamper it from entering the COX-1 enzymatic channel and thus from irreversibly blocking the enzyme. In fact, some reports have questioned the effectiveness of nitroaspirin in blocking platelet COX-1 (Wallace et al., 1999; Wainwright et al., 2002), and it has been hypothesized that NCX 4016 may either block only reversibly COX-1 or it may act as a reservoir of aspirin that is then slowly released in blood to exert its pharmacologic effects.

The understanding of the mechanism of COX blockade by NCX 4016 is relevant to the complete unraveling of the pharmacokinetic/pharmacodynamic profile of the drug required for further clinical studies. Aims of the present study were to test whether NCX 4016 and/or its analog/metabolites inhibit directly the activities of COXs, to assess whether the inhibition of COX is reversible, and to evaluate whether other NSAIDs may compete with this inhibition.

Materials and Methods

**COX-1 Activity in Human Whole Blood.** The effects of the drugs under study on whole blood monocyte COX-2 activity were assessed by incubating each compound, or its vehicle, with 1-ml aliquots of heparinized (10 IU/ml) human whole blood for 15 min before the addition of 10 μg/ml LPS (derived from *Escherichia coli* 026:B6; Sigma-Aldrich, St. Louis, MO). Samples were incubated for 24 h at 37°C in a 5% CO₂ atmosphere, and then plasma was separated by centrifugation (10 min; 2000g at room temperature) and immediately frozen at −80°C for later assay of PGE₂ (Patrignani et al., 1994; Corazzi et al., 2003). Given that the above-mentioned assay, although useful for the testing of the effects of NSAIDs and cyclooxygenase-2 inhibitors (Patrignani et al., 1994; Chan et al., 1999), is largely insensitive to aspirin due to rapid deacetylation of the molecule by plasmonic esterases, we also used a modified whole blood assay for the measurement of monocyte COX-2 activity in which aspirin was previously proven to be effective (Warner et al., 1999). Human whole blood monocytes were isolated from buffy coat, as described previously (Corazzi et al., 2003), seeded into plastic Petri dishes, and exposed to LPS (10 μg/ml) for 24 h to induce COX-2 expression. The medium was then removed, and human heparin-anticoagulated whole blood (100 μl) was added together with the test agents or vehicle. Sixty minutes later, A23187 (calcimycin; 50 μM) was added, and the samples were further incubated for 30 min. The culture plates were then centrifuged, and supernatant plasma was removed for later assay of PGE₂ as a measure of the activity of COX-2 in human monocytes.

**Measurement of TxB₂ and Prostaglandin E₂.** Immunoreactive serum TxB₂ was measured by a specific radioimmunoassay, as described previously (Gresele et al., 1987; Vezza et al., 1995). Briefly, unextracted serum samples were diluted with the assay buffer (Tris-HCl-buffered isotonic saline; pH 7.5). The final volume of the assay mixture was 200 μl, and the mixture comprised ~4000 cpm of radiolabeled [³H]TxB₂ (specific activity 104 Ci/mM; GE Healthcare, Little Chalfont, Buckinghamshire, UK) and a highly specific rabbit antibody-TxB₂ antiserum diluted 1:35,000 in assay buffer.

The IC₅₀ for unlabelled TxB₂ was 170 pg/ml, and the least detectable amount was 25 pg/ml TxB₂. Cross-reactivities of the antiserum are as follows: PGD₂, 1.6%; PGF₂α, 0.04%; PGE₂, <0.01%; PGF₁α, <0.01%; PGE₁, <0.01%; 6-keto-PGF₁α, <0.01%; and arachidonic acid, <0.01%. Radioimmunoassay of PGE₂ was performed on nonextracted, highly diluted serum samples using a highly specific anti-serum and tritiated tracers as described previously (Vezza et al., 1995).

**Purified Ovine COX (oCOX)-1 and oCOX-2 Assay.** COX activity was determined by a spectrophotometric assay (optical density 610 nm) using 200 units of oCOX-1 or oCOX-2 (Cayman Chemical, Ann Arbor, MI) in 600 μl of enzyme reaction buffer (100 mM sodium phosphate, pH 6.5, 0.5 μM hematin, and 1 mg/ml gelatin), and 0.6 μl of the tested compound dissolved in dimethyl sulfoxide.

The enzymatic reaction was initiated by adding 100 μM TMPD (Sigma-Aldrich, Milan, Italy) and 100 μM arachidonic acid (saturating condition; Sigma-Aldrich) in assay buffer. Inhibitors were added to the incubation reaction at different time intervals before the addition of TMPD and arachidonic acid. The velocities observed in the presence of different inhibitor concentrations were divided by the velocity observed for enzyme samples preincubated with vehicle, and this ratio was multiplied by 100 to yield percentage of control activity. The enzyme activity was measured by the estimation of the initial velocity of TMPD oxidation as indicated by the increase in absorbance at 610 nm, as described previously (Ouellet et al., 2001).

In our experimental conditions, 1 unit of enzyme activity produced an increase in TMPD absorbance of 0.001 per minute, as also reported by Copeland et al. (1994).

The irreversibility of the inactivation of oCOX-1 by the tested inhibitors was assessed by repeating the COX activity assay after extensive dialysis. Enzyme (2000 units) was incubated with inhibi-
tors in a volume of 1 ml of enzyme reaction buffer for 15 min at 4°C. After incubation, the samples were dialyzed against 500 ml of inhibitor-free reaction buffer for 5 h. After dialysis, the remaining enzymatic activity, assessed as described above, was measured in comparison with samples of oCOX-1 incubated with vehicle before dialysis.

Competition experiments were carried out by adding the potential competing inhibitor 15 min before the addition of the tested inhibitor (aspirin or NCX 4016). After an additional 15 min, the reaction mixture was extensively dialyzed for 4 h, and then COX-1 activity was determined as described above.

**Measurement of NCX 4016 and Its Derivatives by HPLC.**

After each oCOX-1 assay, the reaction mixture was collected and assessed by HPLC for NCX 4016, its metabolites, or aspirin to exclude degradation in vitro of NCX 4016. The samples (0.2-ml aliquots) were extracted with ice-cooled CH₃CN (2 volumes) containing 1% orthophosphoric acid (50%) and centrifuged at 20,000g at 4°C for 10 min. Supernatants were immediately submitted to liquid chromatography.

Liquid chromatography analyses were carried out on an Agilent 1100 series system (Agilent Technologies, Palo Alto, CA) equipped with a binary pump; a UV-visible diode array programmable detector operating at 230-, 240-, 280-, and 300-nm wavelengths; an autosampler; an in-line degasser; and Agilent Chemstation software. Separations were achieved by reverse-phase elution with a Synergi Polar-RP 80A column (Phenomenex, Torrance, CA) (150 × 4.60 mm i.d., particle size 4 μm), equipped with a Polar-RP precolumn (4 × 3 mm i.d.) and maintained at 25°C. The mobile phase, 0.1 M KH₂PO₄, pH 2/CH₃CN (9:1) (solvent A), and CH₃CN (solvent B), was delivered at a flow rate of 1 ml/min with a gradient elution: from 100% solvent A to 60% solvent B in 30 min. Under these conditions, the retention times (minutes) were as follows: NCX 4023, 30.7; NCX 4016, 29.5; NCX 4215, 27.1; NCX 4019, 22.3; NCX 4017, 20.8; NCX 4015, 19.0; salicylic acid, 13.6; acetylsalicylic acid, 11.9; and 3-hydroxybenzoic acid, 5.3 min.

**Statistical Analysis.** Results are expressed as means ± S.E.M. Differences between controls and treatments were analyzed by using the two-way analysis of variance followed by Tukey’s test for multiple comparisons or by the Student’s t test. A p value of less than 0.05 was considered as statistically significant.

**Results**

**Effect of NCX 4016, Analog, and Metabolites on Platelet COX-1 Activity.** Several metabolites of NCX 4016, derived from single or multiple hydrolytic cleavages of the ester functions of the molecule, have been identified or postulated (Fig. 1) (Carini et al., 2002, 2004).

We tested the effect of all NCX 4016 derivatives and of the analog NCX 4215 (kindly provided by NicOx Research Institute, Milan, Italy) on serum TXB₂ formation. In addition to NCX 4016 (Corazzi et al., 2003), only the acetylated compounds NCX 4017 and NCX 4215 strongly inhibited TXB₂ production. On the contrary, the deacetylated metabolites (NCX 4023 and NCX 4015) did not affect TXB₂ formation or only marginally inhibited it (NCX 4019) at the highest concentration tested (Fig. 2).

The prolongation of the preincubation time produced a time-dependent increase in the potency of NCX 4016, with a reduction in the IC₅₀ of 25 ± 1.6-fold (IC₅₀ for NCX 4016 at different incubation times: time 0, 10.22 ± 0.05 μM; 1 h, 9.9 ± 0.17 μM; 4 h, 0.553 ± 0.08 μM; and 24 h, 0.4 ± 0.06 μM). On the contrary, the prolongation of the preincubation time had no effect on the inhibitory potency of aspirin on platelet COX-1 (IC₅₀ for acetylsalicylic acid: time 0, 0.25 ± 0.02 μM; 1 h, 0.38 ± 0.06 μM; 4 h, 0.35 ± 0.07 μM; and 24 h, 0.3 ± 0.01 μM) (Fig. 3).

**Effect of NCX 4016, Analog, and Metabolites on Monocyte COX-2 Activity.** Incubation of heparinized whole blood with 10 μg/ml LPS resulted in an approximately 20-fold increased production of PGE₂ at 24 h compared with saline-incubated samples.

Besides NCX 4016 (Corazzi et al., 2003), only the derivatives containing the NO-donating moiety (NCX 4023 and NCX 4215) inhibited human monocyte COX-2 in whole blood (more than 70% inhibition at 300 μM for both); the NO-donor moiety itself (NCX 4015; 300 μM) slightly but significantly inhibited PGE₂ production (48.8 ± 2.3% inhibition; p < 0.05 versus LPS-stimulated control), whereas the NCX 4016 derivatives devoid of the nitrooxy group (NCX 4017) and of the acetyl and nitrooxy group (NCX 4019) were totally inactive (data not shown).
When similar experiments were carried out in a modified human whole blood COX-2 assay, which does not involve extensive aspirin deacetylation, we found that 300 μM aspirin strongly inhibited LPS-induced COX-2 activity (98% of inhibition; p < 0.01) as did NCX 4016 (87% inhibition; p < 0.05) and the acetylated derivatives NCX 4017 and NCX 4215 (60 and 70% inhibition, respectively; p < 0.05). The nonacetylated NCX 4016 metabolites were instead almost inactive (NCX 4019, 30% inhibition; NCX 4023, 23% inhibition; NCX 4015, 10% inhibition; p = N.S.) (Table 1). In this assay, which uses large doses of the calcium ionophore A23187 as a trigger of PGE_2 formation, NO-donors only marginally inhibit monocyte COX-2 activity (data not shown).

Effect of NCX 4016, Analog, and Metabolites on Isolated, Purified oCOX-1 and oCOX-2. Among NCX 4016 analog and derivatives, only its acetyl-containing metabolites and its analog NCX 4215 suppressed the activity of isolated, purified oCOX-1 and oCOX-2, whereas all of the deacetylated derivatives were ineffective (Table 2). NCX 4016 was somewhat less potent at inhibiting oCOX-1 compared with aspirin, with IC_{50} values of 68 and 27 μM, respectively. Concerning the NCX 4016 analog/metabolites, relative potency was as follows: for oCOX-1, NCX 4016 > NCX 4215 > NCX 4017; and for oCOX-2, NCX 4016 > NCX 4017 > NCX 4215. HPLC analysis of the incubation mixtures collected after each reaction showed that NCX 4016 acts directly on oCOXs without undergoing further metabolism (Table 3).

Concerning the time taken to inhibit isolated oCOX-1, aspirin inhibited enzyme activity already by 56.5 ± 1.5% 30 s after the addition, whereas NCX 4016 at this time point gave almost no inhibition (0.86 ± 0.1% inhibition). The prolongation of the preincubation time produced a progressive increase of the degree of inhibition of oCOX-1 with both aspirin and NCX 4016; however, NCX 4016 took longer to reach its maximal effect, with a time to half-maximal inhibition significantly longer than that of aspirin (9 ± 1.05 min versus 26.8 ± 1.5 s, respectively; p < 0.05) (Fig. 4).

Reversibility and Competition Experiments. Similar to aspirin, the inhibitory effect of NCX 4016 on COX-1 was irreversible, whereas, as expected, that of indomethacin was largely reversible (Fig. 5).

In the study of the kinetics of inhibition by nitroaspirin, we also tested the competition with the reversible inhibitor indomethacin and with salicylic acid, the product of aspirin metabolism. Preincubation with indomethacin before dialysis prevented the irreversible inhibition of oCOX1 by both aspirin (inhibition was 84.6 ± 1.2 and 19.3 ± 1.4% in the absence or presence of indomethacin, respectively; p < 0.01) and NCX 4016 (inhibition was 58.3 ± 1.2 and 10 ± 2.5% in the absence or presence of indomethacin, respectively; p < 0.01) (Fig. 6).

When salicylic acid was preincubated with oCOX-1 before the addition of aspirin or NCX 4016, in a dose corresponding to its C_{max} when aspirin was assumed at 200 mg b.i.d. and nitroaspirin at 400 mg b.i.d. (Fiorucci et al., 2003), it prevented the inhibition of the enzyme by both aspirin and NCX 4016. In fact, salicylic acid (10.8 μM) preincubated for 15 min before the addition of aspirin (22.2 μM) produced a decrease in the inhibition observed with aspirin alone (35 ± 0.89% inhibition without salicylic acid; 16 ± 0.57% inhibition with salicylic acid). The same was observed by preincubating salicylic acid (25.3 μM) for 15 min before the addition of nitroaspirin (1.66 μM) (16 ± 0.7% inhibition without salicylic acid).

TABLE 1
Inhibition of PGE_2 production in a modified whole blood COX-2 assay

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>PGE_2 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>8 ± 0.2</td>
</tr>
<tr>
<td>Aspirin</td>
<td>136 ± 5</td>
</tr>
<tr>
<td>NCX 4016</td>
<td>3.7 ± 0.2*</td>
</tr>
<tr>
<td>NCX 4017</td>
<td>17 ± 1*</td>
</tr>
<tr>
<td>NCX 4019</td>
<td>54 ± 7*</td>
</tr>
<tr>
<td>NCX 4023</td>
<td>38 ± 6*</td>
</tr>
<tr>
<td>NCX 4015</td>
<td>95.3 ± 8</td>
</tr>
<tr>
<td>A23187-Stimulated (50 μM)</td>
<td></td>
</tr>
<tr>
<td>Solvent</td>
<td>104 ± 4</td>
</tr>
<tr>
<td>Aspirin</td>
<td>118 ± 4</td>
</tr>
</tbody>
</table>

*p < 0.05 vs. solvent.

TABLE 2
Inhibition of oCOX-1 and oCOX-2 enzymatic activities by aspirin, NCX 4016, and its analog/metabolites

<table>
<thead>
<tr>
<th>Drug</th>
<th>oCOX-1 % Inhibition</th>
<th>oCOX-2 % Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin 100 μM</td>
<td>94.1 ± 2.0</td>
<td>59.7 ± 1.3</td>
</tr>
<tr>
<td>Aspirin 300 μM</td>
<td>99.7 ± 1.5</td>
<td>62.6 ± 1.0</td>
</tr>
<tr>
<td>NCX 4016 100 μM</td>
<td>72.7 ± 0.8</td>
<td>96 ± 2.1</td>
</tr>
<tr>
<td>NCX 4016 300 μM</td>
<td>97.4 ± 1.2</td>
<td>99.8 ± 1.0</td>
</tr>
<tr>
<td>NCX 4017 100 μM</td>
<td>36.5 ± 1.0</td>
<td>49.5 ± 1.8</td>
</tr>
<tr>
<td>NCX 4017 300 μM</td>
<td>50.8 ± 0.4</td>
<td>73.7 ± 2.5</td>
</tr>
<tr>
<td>NCX 4215 100 μM</td>
<td>64.8 ± 1.5</td>
<td>421.1 ± 3.3</td>
</tr>
<tr>
<td>NCX 4215 300 μM</td>
<td>79.7 ± 1.9</td>
<td>73 ± 1.9</td>
</tr>
<tr>
<td>NCX 4019 100 μM</td>
<td>2.2 ± 0.4</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>NCX 4019 300 μM</td>
<td>3.8 ± 0.7</td>
<td>11.5 ± 1.6</td>
</tr>
<tr>
<td>NCX 4023 100 μM</td>
<td>4.4 ± 0.5</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>NCX 4023 300 μM</td>
<td>10.6 ± 0.8</td>
<td>16.8 ± 1.3</td>
</tr>
<tr>
<td>NCX 4015 100 μM</td>
<td>1.2 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>NCX 4015 300 μM</td>
<td>1.5 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of the preincubation time on the inhibitory effect of aspirin and NCX 4016 on thrombin-stimulated whole blood TxB2 formation. The prolongation of the preincubation time produced a time-dependent increase in the potency of NCX 4016 (•), whereas it had only a minor effect on the inhibitory potency of aspirin (○) (*p < 0.05 versus aspirin). Data represent mean ± S.E.M. (n = 5).
aid; 7.5 ± 0.8% inhibition with salicylic acid). Similarly, the deacetylated NCX 4016 metabolites (NCX 4019, NCX 4017, and NCX 4015) competed with NCX 4016 or with aspirin for COX-1 inhibition (data not shown).

**Discussion**

Acetylation of cyclooxygenase and the consequent irreversible inactivation of the enzyme are unique pharmacologic features of aspirin that make this drug ideal for chronic antithrombotic use (Patrono, 1994). So far, in spite of the ability of a large series of other compounds to inhibit COX-1, only aspirin has entered in the therapeutic armamentarium for thrombosis prevention.

Aspirin, however, has several limitations, including side effects at the gastrointestinal level, an antiplatelet activity limited to thromboxane-mediated activation, and little or no activity on atheroma formation. For these reasons, in the search of novel antithrombotic agents with wider activity, a NO-releasing aspirin (NCX 4016) has been developed by linking aspirin, through a spacer, to a NO-donating moiety (Del Soldato et al., 1999; Gresele et al., 2003). Nitroaspirin was found to exert anti-inflammatory and antithrombotic effects through multiple mechanisms, extending beyond the COX-1 pathway.

**TABLE 3**

Compounds recovered in the oCOX assay mixture after preincubation with aspirin or NCX 4016

<table>
<thead>
<tr>
<th>Compound Added</th>
<th>Aspirin</th>
<th>Salicylic Acid</th>
<th>NCX 4016</th>
<th>NCX 4019</th>
<th>NCX 4017</th>
<th>NCX 4023</th>
<th>NCX 4215</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>295.1 ± 6.3</td>
<td>4.9 ± 0.2</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>NCX 4016</td>
<td>N.D.</td>
<td>N.D.</td>
<td>263.4 ± 2.7</td>
<td>N.D.</td>
<td>N.D.</td>
<td>1.5 ± 0.1</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., compound not detected in the reaction mixture.

**Fig. 4.** Effect of time of preincubation on oCOX-1 inhibition by aspirin or NCX 4016. oCOX-1 (50 nM) was incubated with aspirin (300 μM; ○) or NCX 4016 (300 μM; ●). Increasing the preincubation time produced a progressive increase of the degree of inhibition of oCOX-1 with both aspirin and NCX 4016. However, the time to half-maximal inhibition of oCOX-1 was significantly longer with NCX 4016 than with aspirin (*, p < 0.05 versus NCX 4016). Data represent mean ± S.E.M. (n = 5).

**Fig. 5.** Reversibility of the inactivation of oCOX-1 by aspirin, NCX 4016, and indomethacin. oCOX-1 (50 nM) in 100 mM sodium phosphate was incubated with aspirin, NCX 4016, or indomethacin (300 μM for each) for 15 min. The reaction mixture was then dialyzed extensively against inhibitor-free buffer before testing its residual activity.

**Fig. 6.** Competition by indomethacin of the inhibitory effect of aspirin and nitroaspirin on oCOX-1. Preincubation with indomethacin (indo) (300 μM) for 15 min before the addition of aspirin (300 μM) or NCX 4016 (300 μM) prevented the irreversible inhibition of oCOX-1 by both drugs.
it has previously been demonstrated that NCX 4016 is stable, without any degradation into aspirin and/or deacetylated or denitrated fragments (NicOx Research Institute, data on file; Carini et al., 2002). A further confirmation that NCX 4016 acts as a whole molecule derives from HPLC assays of the reaction mixtures, which showed that NCX 4016 was active on isolated, purified COXs without undergoing metabolism.

The inhibition of isolated COX-1 that we observed is compatible with acetylation of the enzyme, as demonstrated by dialysis experiments in which oCOX-1 that had been incubated with aspirin or NCX 4016 was still largely inhibited after removal of the drug by dialysis, similarly to aspirin. On the contrary, when the purified enzyme was incubated with indomethacin, a reversible, competitive inhibitor of COX-1, the suppression of enzymatic activity was no longer evident after dialysis, indicating that there had been no covalent modification of COX by indomethacin, as reported previously (Kulmacz and Lands, 1985). Furthermore, we observed that the inhibitory effect of NCX 4016 on COX is to a large extent antagonized by the preincubation of the enzyme with a competitive inhibitor (indomethacin) or with salicylic acid. This result is consistent with competitive inhibition by indomethacin of the access of NCX 4016 to the acetylation site of platelet COX-1 (Loll et al., 1995; Catella-Lawson et al., 2001) and represents one further suggestion that nitroaspirin acts on COX-1 with the identical mechanism of aspirin.

In agreement with this conclusion is the observation that among NCX 4016 analog/metabolites, only those containing the acetylsalicylic acid moiety suppressed the activity of platelet COX-1 and of monocyte COX-2, the latter under conditions preventing the degradation of the acetylated compounds by plasmatic esterases. The derivatives containing the NO-donating moiety also inhibited the activity of human monocyte COX-2 stimulated by LPS challenge of whole blood, an activity in part regulated by NO (Corazzi et al., 2003).

Therefore, it seems that in the NCX 4016 molecule, the hydroxybenzylalcohol spacer and the NO-donating moiety do not create a steric hindrance preventing NCX 4016 from reaching its target within the enzymatic channel of COX-1. However, some data suggest that they may delay it. In fact, in the experiments with isolated, purified oCOX-1, 50% inhibition of the enzyme activity was reached only after 9 min of incubation with NCX 4016 as opposed to 26 s with aspirin, suggesting a slower penetration of the molecule in the enzymatic channel of COX. Penetration of NCX 4016 through the cell membrane may to some extent be delayed, too, compared with aspirin, because experiments in whole blood showed an even longer time to maximal inhibition of platelet COX-1 with NCX 4016 than with aspirin.

In vivo studies in animals (Cuzzolin et al., 1996; Momi et al., 2005) as well as data in humans (Fiorucci et al., 2003) demonstrated that inhibition of serum TxB2 by NCX 4016 after oral administration is somewhat lower than with equimolar aspirin. This may be the consequence of a large preabsorptional deacetylation of the drug, with only relatively little NCX 4016 absorbed as such; of the longer time taken by NCX 4016 for platelet COX-1 inhibition; and of partial competition by larger amounts of deacetylated metabolites formed after NCX 4016 than of salicylate formed after aspirin administration (NicOx Research Institute, data on file; Fiorucci et al., 2003).

Indeed, the potential competition exerted by in vivo-generated salicylic acid or by deacetylated NCX 4016 derivatives (Carini et al., 2004) versus NCX 4016 for COX inhibition (de Gaetano et al., 1985) is a point worth considering. In our in vitro system, we observed that both salicylic acid and deacetylated NCX 4016 derivatives compete with NCX 4016 or aspirin, preventing, in part, the inhibition of COX-1. Although this competition may be of concern, it has previously been demonstrated that the combination of salicylate with aspirin, at therapeutically relevant dose ratios, may result in an almost complete dissociation of the effect of the drug on platelets and blood vessels by protecting primarily endothelial COX from aspirin inhibition (de Gaetano et al., 1985; Gambino et al., 1988). Considering that NCX 4016 seems to act on COX-1 with the identical mechanism of aspirin, it is conceivable that the effects of salicylate previously described with aspirin will be observed also with NCX 4016.

Although no unmetabolized NCX 4016 has been detected so far in peripheral blood after oral administration of the drug (Carini et al., 2004), the detection limits of the analytical methods used (approximately 1 μM) (Carini et al., 2004) are still compatible with the presence of amounts of NCX 4016 sufficient to inhibit COX-1 (Corazzi et al., 2003). Moreover, it is conceivable that some unmodified NCX 4016 may enter the mesenteric and preportal circulation and then undergo complete deacetylation in the liver. Thus, the demonstration that the whole NCX 4016 molecule affords irreversible inhibition of COX-1 is therapeutically relevant because the largest part of platelet COX-1 inhibition observed with low-dose aspirin takes place in the prehepatic circulation (Pedersen and Fitzgerald, 1984; Cerletti et al., 1985; Giuliano and Warner, 1999). Further studies with blood sampling in different circulatory districts after oral administration of NCX 4016 need to address whether the COX-1 inhibitory profile of NCX 4016 found with isolated cells or purified enzymes is reproduced in vivo in the portal or systemic circulation.

In conclusion, NCX 4016 may act in vitro on COX-1 as a direct and irreversible inhibitor, similarly to aspirin, and simultaneously display a range of other actions related to NO.

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References


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