Direct and irreversible inhibition of cyclooxygenase-1 (COX-1) by nitroaspirin (NCX 4016).

Teresa Corazzi, Mario Leone, Raffaella Maucci, Lanfranco Corazzi, Paolo Gresele

Division of Internal and Cardiovascular Medicine (T.C., M.L., P.G.), Section of Biochemistry (L.C.), Department of Internal Medicine, University of Perugia, Perugia, Italy, and NicOx Research Institute, Milan, Italy (R.M.).
a) Running title page: Inhibition of COX-1 by nitroaspirin

b) Corresponding authors: Paolo Gresele, MD, PhD, Dept. of Internal Medicine, Division of Internal and Cardiovascular Medicine, University of Perugia, Via Enrico dal Pozzo, 06126, Perugia, Italy. Phone: 0039/075-5783989; Fax: 0039/075-5716083; e-mail: grespa@unipg.it

c) Text pages: 27
Tables: 3
Figures: 6
References: 39
Words in abstract: 250
Words in introduction: 719
Words in discussion: 1207

d) Abbreviations: NSAIDs, non steroidal anti-inflammatory drugs; COX, cyclooxygenase; PGE₂, prostaglandin E₂; TxB₂, thromboxane B₂.

e) Section: Cardiovascular
Abstract

NCX 4016 (benzoic acid, 2-(acetyl-oxy)-3-[(nitrooxy)methyl]phenyl ester), 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 in order to inhibit COX-1, or if it can block it as a whole molecule. Aim of our study was to evaluate the effects of NCX 4016 and its analogue or metabolites on platelet COX-1 and whole blood COX-2 and on purified ovine COX-1 (oCOX-1) and COX-2 (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, while 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 prior to NCX 4016 prevent the irreversible inhibition of oCOX-1 by NCX4016 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 slower the kinetics of COX-1 inhibition by NCX 4016, as compared with aspirin.
Introduction

Cyclooxygenase (COX) catalyses the committed step in the conversion of arachidonic acid to 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 non-steroidal anti-inflammatory drugs (NSAIDs) (Marnett et al., 1999, Smith et al., 1997).

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 (Ser$^{529}$ of COX-1 and Ser$^{516}$ of COX-2), thus irreversibly inactivating it and preventing the generation of prostaglandins and thromboxane A$_2$ (TXA$_2$) (Loll et al., 1995). Ser$^{529}$ 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 or 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 thus preventing aspirin from reaching its target (Ouellet et al., 2001; Catella-Lawson 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 TXA$_2$-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, ulceration), resulting from the inhibition of vasodilator and cytoprotective prostanoids in the gastric mucosa, which limits the use of these compounds (Wallace, 1997).

In the last few years, nitric oxide (NO)-releasing NSAIDs 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-α-nitrate, NCX 4015) and the resulting molecule has a molecular mass 1.8 fold greater than aspirin (Del Soldato et al., 1999).

Nitroaspirin, besides its better gastric tolerability (Fiorucci et al., 2003), appears to be superior to aspirin in terms of platelet inhibition (Lechi et al., 1996; Mezzasoma et al., 1999) and antithrombotic activity (Momi et al., 2000; Momi et al., 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, while NCX 4016 inhibits COX-1 with a potency similar to 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 if 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 unravelling 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 analogues/metabolites inhibit directly COXs activities, 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 tested on platelet COX-1 activity were studied by adding the different compounds, at increasing concentrations, to non-anticoagulated human whole blood samples that were then immediately transferred into glass tubes and allowed to clot at 37°C for 60 minutes (Patrono et al., 1980; Gresele et al., 1987). Serum was separated by centrifugation (10 min, 2000 x g) and frozen at –80°C for later assay of TxB₂ (Gresele et al., 1988).

In order to assess the effect of the prolongation of preincubation time on the COX-1 suppressing activity, the tested compounds were incubated at room temperature, for 1, 4 and 24 hours, with one millilitre aliquots of citrated (3.8%, 1/10 v/v) whole blood;
0.2 mM CaCl₂ and 1 U/ml thrombin were then added and the samples were allowed to clot at 37°C for 60 minutes and serum was then separated, as above described, for later assay of TxB₂.

**COX-2 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 one millilitre aliquots of heparinized (10 I.U./ml) human whole blood for 15 min before the addition of 10 µg/ml LPS (derived from Escherichia Coli 026:B6; Sigma-Aldrich Co, St.Louis, MO, U.S.A). Samples were then incubated for 24 hours at 37°C in a 5% CO₂ atmosphere and then plasma was separated by centrifugation (10 min, 2000 x g 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 assay, while useful for the testing of the effects of NSAIDs and Coxibs (Patrignani et al, 1994; Chan et al., 1999), is largely insensitive to aspirin due to rapid deacetylation of the molecule by plasmatic 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 previously described (Corazzi et al, 2003), seeded into plastic Petri dishes and exposed to LPS (10 µg/ml) for 24 hours 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 (50 µM) was added and the samples further incubated for 30 minutes. 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 previously described (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, which comprised ≈4000 cpm of radiolabelled [3H]TxB2 (sp. Act. 104 Ci/mM; Amersham International, Buckinghamshire, England) and a highly specific rabbit anti-TxB2 antiserum diluted 1:35,000 in assay buffer, was 200 µl.

IC50 for unlabelled TxB2 was 170 pg/ml and the least detectable amount was 25 pg/ml of TxB2. Crossreactivities of the antiserum are: PGD2=1.6%; PGF2α=0.04%; PGE2 < 0.01%; PGF1α < 0.01%; PGE1 < 0.01%; 6-keto-PGF1α < 0.01%; arachidonic acid < 0.01%.

Radioimmunoassay of PGE2 was performed on non-extracted, highly diluted serum samples using a highly specific antiserum and tritiated tracers as previously described (Vezza et al., 1993).

Purified ovine COX-1 and COX-2 (oCOX-1; oCOX-2) assay. COX activity was determined by a spectrophotometric assay (O.D. 610 nm) using 200 units oCOX-1 or oCOX-2 (Cayman Chemicals, Ann Arbor, MI, U.S.A.) in 600 µl of enzyme reaction buffer (100 mM sodium phosphate, pH 6.5, 0.5 µM hematin, 1 mg/ml gelatine), and 0.6 µl of the tested compound dissolved in DMSO.

The enzymatic reaction was initiated by adding N,N,N′,N′-tetramethyl-p-phenylenediamine 100 µM (TMPD; Sigma-Aldrich, Milan, Italy) and arachidonic acid 100 µM (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 percent 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 previously described (Ouellet et al., 2001). In our experimental conditions one unit of enzyme activity produced an increase in TMPD absorbance of 0.001 per minute, as also reported by Copeland (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 inhibitors in a volume of 1 ml of enzyme reaction buffer for 15 minutes at 4°C. After incubation the samples were dialyzed against 500 ml of inhibitor-free reaction buffer for 5 hr. After dialysis the remaining enzymatic activity, assessed as described above, was measured in comparison with samples of oCOX-1 incubated with vehicle prior to dialysis.

Competition experiments were carried out by adding the potential competing inhibitor 15 minutes prior to the addition of the tested inhibitor (aspirin or NCX 4016). After an additional 15 minutes, the reaction mixture was extensively dialyzed for four hours 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, in order to exclude degradation in vitro of NCX 4016. The samples (0.2 ml aliquots) were extracted with ice-cooled CH₃CN (2 vol) containing 1% orto-phosphoric acid (50%), centrifuged at 20,000 x g at 4°C for 10 minutes, and supernatants immediately submitted to Liquid Chromatography (LC).

LC analyses were carried out on an Agilent 1100 series system equipped with a binary pump, an UV/VIS Diode Array programmable detector operating at 230, 240,
280 and 300 nm wavelengths, an autosampler, an in line degasser and a Agilent Chemstation software. Separations were achieved by reverse phase elution with a Synergi Polar-RP 80A column (150 x 4.60 mm i.d., particle size 4 µm), equipped with a Polar-RP precolumn (4 x 3 mm i.d.) and maintained at 25°C. The mobile phase, KH2PO4 0.1 M 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 minutes. Under these conditions the retention times (minutes) were: NCX 4023=30.7, NCX 4016=29.5, NCX 4215=27.1, NCX 4019=22.3, NCX 4017=20.8, NCX 4015=19.0, SA (salicylic acid)=13.6, ASA (acetyl salicylic acid)=11.9 and 3-HBA=5.3 minutes.

**Statistical Analysis.** Results are expressed as means±SEM. Differences between controls and treatments were analysed by using the two ways ANOVA 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, analogue and metabolites on platelet COX-1 activity.

Several metabolites of NCX 4016, deriving from single or multiple hydrolytic cleavages of the ester functions of the molecule, have been identified or postulated (Fig.1) (Carini et al., 2002; Carini et al., 2004).

We tested the effect of all NCX 4016 derivatives and of the analogue NCX 4215 (kindly provided by NicOx Research Institute, 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 pre-incubation time produced a time-dependent increase in the potency of NCX 4016, with a reduction of the IC₅₀ of 25±1.6 fold (IC₅₀ for NCX 4016 at different incubation times: time zero = 10.22±0.05 μM; 1 h = 9.9±0.17 μM; 4 h = 0.553±0.08 μM; 24 h, 0.4±0.06 μM). On the contrary the prolongation of the pre-incubation time had no effect on the inhibitory potency of aspirin on platelet COX-1 (IC₅₀ for ASA: time zero = 0.25±0.02 μM; 1 h = 0.38±0.06 μM; 4 h = 0.35±0.07 μM; 24 h = 0.3±0.01 μM ) (Fig. 3).

Effect of NCX 4016, analogue and metabolites on monocyte COX-2 activity.

Incubation of heparinized whole blood with 10µg/ml LPS resulted in an approximately 20 fold increased production of PGE₂ at 24 hours as 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 (over 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 vs LPS stimulated control), while 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 non-acetylated NCX 4016 metabolites were instead almost inactive (NCX 4019 30% inhibition; NCX 4023 23% inhibition; NCX 4015 10% inhibition, p=ns) (Table 1). In this assay, which uses large doses of the calcium ionofore A23187 as a trigger of PGE₂ formation, NO-donors only marginally inhibit monocyte COX-2 activity (data not shown).

**Effect of NCX 4016, analogue and metabolites on isolated, purified oCOX-1 and oCOX-2.** Among NCX 4016 analogue and derivatives, only its acetyl-containing metabolites and its analogue NCX 4215 suppressed the activity of isolated, purified oCOX-1 and oCOX-2, while all the deacetylated derivatives were ineffective (Tab.2). NCX 4016 was somewhat less potent at inhibiting oCOX-1 as compared with aspirin, with IC₅₀s of 68 µM and 27 µM, respectively. Concerning the NCX 4016 analogue/metabolites, relative potency was, 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 metabolization (Tab. 3).

Concerning the time taken to inhibit isolated oCOX-1, aspirin inhibited enzyme activity already by 56.5±1.5% 30 seconds after the addition, while NCX 4016 at this time point gave almost no inhibition (0.86±0.1% inhibition). The prolongation of the pre-incubation 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 minutes vs 26.8±1.5 seconds, respectively; p<0.05) (Fig. 4).

**Reversibility and competition experiments.** Similar to aspirin, the inhibitory effect of NCX 4016 on oCOX-1 was irreversible, while, 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 metabolization. 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 pre-incubated with oCOX-1 before the addition of aspirin or NCX 4016, in a dose corresponding to its $C_{\text{max}}$ when aspirin was assumed at 200 mg bid and nitroaspirin at 400 mg bid (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 minutes prior the addition of aspirin (22.2 µM) produced a
decrease in the inhibition observed with aspirin alone (35±0.89% inhibition without salicylic aid; 16±0.57% inhibition with salicylic acid). The same was observed preincubating salicylic acid (25.3 µM) for 15 minutes prior the addition of nitroaspirin (1.66 µM) (16±0.7% inhibition without salicylic aid; 7.5±0.8% inhibition with salicylic acid). Similarly, also the deacetylated NCX 4016 metabolites (NCX 4023, NCX 4019 and NCX 4015) competed with NCX 4016 or with aspirin for oCOX-1 inhibition (data not shown).
Discussion

Acetylation of cyclooxygenase and the consequent irreversible inactivation of the enzyme is a unique pharmacologic feature of aspirin, which makes 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, which include 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 anti-thrombotic effects through multiple mechanisms, extending beyond the COX-1 pathway.

Our data show for the first time that NCX 4016 is able to directly and irreversibly inhibit COX-1, without a requirement for metabolic breakdown. NCX 4016, which is stable in buffer, is rapidly enzymatically degraded in a series of metabolites in plasma or in whole blood (Carini et al., 2004), and so far it was still unknown whether the inhibition of COX-1 by NCX 4016 is exerted by the whole molecule or if it requires metabolism with release of acetylsalicylic acid.

We have worked in an in vitro system, with isolated, purified ovine COX-1 resuspended in a reaction buffer not containing plasma or cell fractions, thus excluding degradation of the drug by other enzymatic systems (glutathione transferase, aldehyde dehydrogenase) which could have accounted for the COX-
inhibitory activity previously detected *in vitro* or *in vivo* in whole blood or in platelet suspensions (Lechi et al., 1996; Mezzasoma et al., 1999; Momi et al., 2000; Corazzi et al., 2003). In such a condition, it has previously been demonstrated that NCX 4016 is stable, without any degradation into aspirin and/or de-acetylated or de-nitrated fragments (NicOx 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 previously reported (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 also the observation that among NCX 4016 analogue/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 appears 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, fifty % inhibition of the enzyme activity was reached only after 9 minutes of incubation with NCX 4016 as opposed to 26 seconds 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 as 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 pre-absorptionsal deacetylation of the drug, with only relatively little NCX 4016 absorbed as such, of the longer time taken to 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 (Fiorucci et al., 2003; NicOx, data on file).

Indeed, the potential competition exerted by *in vivo* generated salicylic acid, or by de-acetylated 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 de-acetylated NCX 4016 derivatives compete with NCX 4016 or aspirin, preventing in part the inhibition of COX-1. While 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 drug’s effect 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 appears 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 employed (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 pre-hepatic 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 \textit{in vitro} on COX-1 as a direct and irreversible inhibitor, similarly to aspirin, and simultaneously it displays a range of other actions related to NO.
Acknowledgements

Highly specific rabbit antisera against TxB\(_2\) and PGE\(_2\) were kindly provided by Dr. J. Beetens, Janssen Res Foundation, Beerse, Belgium. We thank E. Ongini for critical reading of the manuscript.
References


Footnotes

This work was supported in part by a grant from NicOx, Milan (Italy).
Legends to the Figures

Figure 1. Chemical structures of NCX 4016 (nitroaspirin) and its analogue/metabolites. NCX 4016: benzoic acid, 2-(acetyl-oxy)-3-[(nitrooxy)methyl]phenyl ester; NCX 4019: 2-(hydorxy)-3-(hydroxymethyl)phenyl ester; NCX 4017: 2-(acetyloxy)-3-(hydroxymethyl) phenyl ester; NCX 4023: 2-(hydroxy)-3-[(nitrooxy)methyl]phenyl ester; NCX 4215: acetyl salicylic acid 4-(nitroxy)butyl ester; NCX 4015: benzenemethano-3 hydroxy-α-nitrate. As a comparison the structure of aspirin: acetyl salicylic acid, is also shown.

Figure 2. Effect of NCX 4016 and its analogue/metabolites on serum TxB₂ formation. All compounds were incubated with 1 ml of non-anticoagulated human whole blood that was then allowed to clot at 37°C for 1 hour. * = p<0.05 vs control (no drug). Data represent mean ± SEM (n=5).

Figure 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 pre-incubation time produced a time-dependent increase in the potency of NCX 4016 (-♦-), while it had only a minor effect on the inhibitory potency of aspirin (-•-) (* = p<0.05 vs aspirin). Data represent mean ±SEM. (n=5).

Figure 4. Effect of time of preincubation on oCOX-1 inhibition by aspirin or NCX 4016. 50 nM oCOX-1 was incubated with aspirin (300 µM; -•-) or NCX 4016 (300 µM; -♦-). Increasing the pre-incubation 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 vs NCX 4016). Data represent mean ±SEM (n=5).
Figure 5. Reversibility of the inactivation of oCOX-1 by aspirin, NCX 4016 and Indomethacin. 50 nM oCOX-1 in 100 mM sodium phosphate was incubated with aspirin, NCX 4016 or Indomethacin (300 µM for each) for 15 minutes; the reaction mixture was then dialyzed extensively, against inhibitor-free buffer, before testing its residual activity.

Figure 6. Competition by indomethacin of the aspirin’s and nitroaspirin’s inhibitory effect on oCOX-1. Preincubation with indomethacin (indo) (300 µM) for 15 minutes before the addition of aspirin (300 µM) or NCX 4016 (300 µM), prevented the irreversible inhibition of oCOX-1 by both drugs.
Table 1. Inhibition of PGE₂ production in a modified whole blood COX-2 assay.

<table>
<thead>
<tr>
<th></th>
<th>unstimulated</th>
<th>A23187-stimulated (50 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>solvent</td>
<td>aspirin (300 µM)</td>
</tr>
<tr>
<td>PGE₂ (ng/ml)</td>
<td>8±0.2</td>
<td>136±5</td>
</tr>
</tbody>
</table>

Isolated monocytes were stimulated with LPS for 24 hours to induce COX-2 expresion. The medium was then removed and whole blood was added with the vehicle or the tested agents (300 µM). PGE₂ production was triggered by adding the calcium inophore A23187. PGE₂ levels were measured by RIA in the supernatant plasma as an index of monocyte COX-2 activity (n=4;*p<0.05 vs solvent).
Table 2. Inhibition of oCOX-1 and oCOX-2 enzymatic activities by aspirin, NCX 4016 and its analogue/metabolites. Drugs were incubated with the purified enzymes for 15 minutes before starting the enzymatic reaction.

<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>42.1±1.3</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>1.3±0.1</td>
<td>1.1±0.1</td>
</tr>
</tbody>
</table>
Table 3. Compounds recovered in the oCOX assay mixture after preincubation with aspirin or NCX 4016.

<table>
<thead>
<tr>
<th>Compound added</th>
<th>Compound recovered</th>
<th>Aspirin (μM)</th>
<th>Salicylic acid (μM)</th>
<th>NCX 4016 (μM)</th>
<th>NCX 4019 (μM)</th>
<th>NCX 4017 (μM)</th>
<th>NCX 4023 (μM)</th>
<th>NCX 4215 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin (300 μM)</td>
<td>n.d.</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 (300 μM)</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>
<td>n.d.</td>
</tr>
</tbody>
</table>

After incubation of aspirin or NCX 4016 (300 μM) with oCOX-1 for 15 minutes, the reaction mixtures were collected and subjected to HPLC analysis (n.d.= compound not detected in the reaction mixture).