

Pharmacological Characterization of 2NTX-99 [4-Methoxy-*N*¹-(4-*trans*-nitrooxycyclohexyl)-*N*³-(3-pyridinylmethyl)-1,3-benzenedicarboxamide], a Potential Antiatherothrombotic Agent with Antithromboxane and Nitric Oxide Donor Activity in Platelet and Vascular Preparations

Carola Buccellati, Angelo Sala, Giuseppe Rossoni, Valérie Capra, G. Enrico Rovati, Antonio Di Gennaro, Giancarlo Folco, Susanna Colli, and Cesare Casagrande

Department of Pharmacological Sciences, School of Pharmacy, University of Milano, Milano, Italy

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ABSTRACT

Thromboxane (TX) A₂, prostacyclin (PGI₂), and nitric oxide (NO) regulate platelet function and interaction with the vessel wall. Inhibition of TXA₂, implemented synthesis of PGI₂, and supply of exogenous NO may afford therapeutic benefit. 2NTX-99 [4-methoxy-*N*¹-(4-*trans*-nitrooxycyclohexyl)-*N*³-(3-pyridinylmethyl)-1,3-benzenedicarboxamide], a new chemical entity related to picotamide, showed antithromboxane activity and NO donor properties. 2NTX-99 relaxed rabbit aortic rings precontracted with norepinephrine or U46619 (9,11-dideoxy-9 α ,11 α -methanoepoxy-prosta-5Z,13E-dien-1-oic acid; EC₅₀, 7.9 and 17.1 μ M, respectively), an effect abolished by 10 μ M 1*H*-(1,2,4)oxadiazolo(4,3-*a*)quinoxalin-1-one (ODQ). 2NTX-99 inhibited arachidonic acid (AA)-induced washed platelet aggregation (EC₅₀, 9.8 μ M) and TXB₂ formation (–71% at 10 μ M), and its potency increased in the presence of aortic rings (EC₅₀, 1.4 μ M). In whole rabbit aorta incubated with homologous platelets, AA caused contraction and TXA₂ formation, reduced by 2NTX-99 (10–40 μ M): contraction, –28 and –47%, TXA₂ formation, –37 and –75.4%, respectively, with concomitant

increase in PGI₂. 2NTX-99 (20–40 μ M) inhibited U46619-induced aggregation in rabbit platelet-rich plasma (PRP) (–74 \pm 6.7 and –96 \pm 2.4%, respectively) and inhibited collagen-induced aggregation in human PRP (–48.2 \pm 10 and –79.2 \pm 6%), whereas ozagrel was ineffective. In human embryonic kidney 293 cells transfected with the TXA₂ receptor isophorm α receptor, 2NTX-99 did not compete with the ligand, [³H]SQ29,548 ([³H][1*S*–[1 α ,2 β (5*Z*),3 β ,4 α]]-7-[3-[[2-(phenylamino)-carbonyl]hydrazino]methyl]-7-oxabicyclo[2,2,1]-hept-2-yl]-5-heptanoic acid), or prevent inositol phosphate accumulation. After oral administration (50–250 mg/kg), 2NTX-99 inhibited TXA₂ production in rat clotting blood (–71 and –91%); at 250 mg/kg, an area under the curve, 0 to 16 h, of 149.5 h/ μ g/ml and a *t*_{1/2} of 6 h were calculated, with a C_{max} value of 31.8 \pm 8.2 μ g/ml. An excellent correlation between plasma concentrations and TXA₂ inhibition occurs. 2NTX-99 controls platelet function and vessel wall interaction by multifactorial mechanisms and possesses therapeutic potential.

Aspirin, a nonreversible inhibitor of platelet cyclooxygenase (COX)-1, has been the mainstay of antiplatelet therapy for over 20 years (Antithrombotic Trialist Collaboration, 2002). Novel

approaches to the modulation of platelet function by preventing platelet secretion and/or aggregation, their adhesion to the vessel wall, or progression of thrombus development (Bhatt and Topol, 2003) have been addressed in experimental and clinical studies. Until now, the only novel drug suitable for chronic therapy, which in some trials appeared superior to aspirin in preventing cardiovascular accidents (CAPRIE, 1996), is clopidogrel, an inhibitor of platelet P2Y₁₂ receptor.

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ABBREVIATIONS: COX, cyclooxygenase; PG, prostacyclin; TX, thromboxane; NCX-4016, 2-acetoxy-benzoate 2-(2-nitroxymethyl)-phenyl ester; 2NTX-99, 4-methoxy-*N*¹-(4-*trans*-nitrooxycyclohexyl)-*N*³-(3-pyridinylmethyl)-1,3-benzenedicarboxamide; NO, nitric oxide; PRP, platelet-rich plasma; AA, arachidonic acid; OKY-046, ozagrel; EIA, enzyme immunoassay; U46619, 9,11-dideoxy-9 α ,11 α -methanoepoxy-prosta-5Z,13E-dien-1-oic acid; NE, norepinephrine; GTN, glyceryl trinitrate; DMEM, Dulbecco's modified Eagle's medium; TP α , thromboxane A₂ receptor isophorm α ; SQ29,548, [³H][1*S*–[1 α ,2 β (5*Z*),3 β ,4 α]]-7-[3-[[2-(phenylamino)-carbonyl]hydrazino]-methyl]-7-oxabicyclo[2,2,1]-hept-2-yl]-5-heptanoic acid; IP, inositol phosphate; HPLC, high-performance liquid chromatography; CV, coefficient(s) of variation; ISMN, isosorbide mononitrate; ODQ, 1*H*-(1,2,4)oxadiazolo(4,3-*a*)quinoxalin-1-one; PGH₂, prostaglandin H cyclic endoperoxide.

Significant advancement in our understanding of the role of platelets in the atherothrombotic process (Bhatt and Topol, 2003) supports the concept that therapeutic efficacy may be improved by a combined action on platelet activation and interaction with the vascular wall. Indeed, several studies have shown that combined treatment with clopidogrel and aspirin in acute coronary syndromes (Mehta et al., 2001) or with dipyridamole and aspirin in the prevention of stroke (Forbes, 1998), offer advantage over single treatment. However, concern about lack of response in subsets of patients and about development of resistance during chronic treatment (Gurbel and Bliden, 2003; Eikelboom and Hankey, 2004) stresses the need of novel therapeutic approaches (Bhatt and Topol, 2003).

Different prostanoids originate from the common endoperoxide precursor PGH_2 , which is further metabolized by specific enzymes according to a strict cellular specificity, yielding, e.g., mostly TXA_2 in platelets, PGI_2 in endothelial cells, etc. (MacIouf et al., 1998). However, PGH_2 can be also made available extracellularly for further paracrine conversion to bioactive eicosanoids. The inhibition of a given enzymatic pathway within a defined cell facilitates an intercellular shunt of PGH_2 toward an alternative pathway, e.g., in platelet-endothelial cell cocultures, PGI_2 synthesis is enhanced when thromboxane synthase is inhibited (Nowak and FitzGerald, 1989).

The search for TXA_2 inhibitors has targeted thromboxane synthase or thromboxane receptors but has also developed "dual" inhibitors that combine inhibition of TXA_2 formation with antagonism of the receptor-mediated actions of PGH_2 , of any residual TXA_2 , and of the isoprostanoid 8-epi- $\text{PGF}_{2\alpha}$ (Dogne et al., 2000). In addition, shunting of PGH_2 toward PGI_2 may take place when, e.g., platelets interact with the vascular wall. Among dual inhibitors, only picotamide (Fig. 1) (Gresele et al., 1989) has found clinical application in peripheral arterial disease in diabetic patients (Modesti, 1995; Coto et al., 1998; Neri Serneri et al., 2004).

An impaired production of nitric oxide (NO) by damaged endothelium is considered one of the key factors in the development of atherosclerosis and thrombotic events (Napoli and Ignarro, 2001; Walford and Loscalzo, 2003). Potential benefit of NO supply is suggested by the preventive action of the NO-releasing derivative of aspirin, NCX-4016 (Napoli et al., 2002), in murine models of atherosclerosis and restenosis, an effect not shared by aspirin alone.

Structural modifications of picotamide, although preserving its antithromboxane activity, allowed the insertion of an NO donor moiety, leading to the synthesis of compound 2NTX-99 (Fig. 1). 2NTX-99 is a new molecular entity that targets three powerful regulators of platelet and vascular function, i.e., TXA_2 , PGI_2 , and NO. TXA_2 promotes platelet activation and increases vascular tone and neointima proliferation, whereas both PGI_2 and NO, per se or in synergy, counteract the biological actions of TXA_2 (Moncada et al., 1991; MacIouf et al., 1998). In this article, we report the pharmacological profile of 2NTX-99 on platelet and vascular preparations, as well as results from a preliminary kinetic study following oral administration to rats.

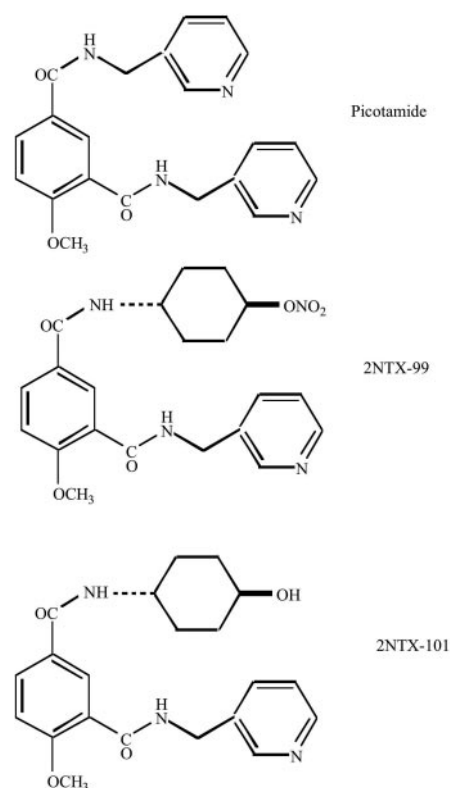


Fig. 1. Chemical structures of Picotamide, 2NTX-99, and 2NTX-101.

Materials and Methods

Synthesis of 2NTX-99 (Fig. 1)

The synthesis (U.S. Patent 6,525,078) started from the regioselective mono-amination of the dimethyl ester of 4-methoxy-1,2-benzenedicarboxylic acid with 3-pyridinylmethylamine, yielding the 3-*N*-pyridinylmethylamide 1-ester. The corresponding acid, obtained by alkaline hydrolysis, was activated with carbonyldiimidazole and allowed to react with *trans*-4-hydroxycyclohexylamine to give the isomerically pure N^1,N^3 -disubstituted amide 2NTX-101 (Fig. 1). Its esterification with nitric acid-acetic anhydride led to compound 2NTX-99, showing m.p. 153 to 154°C; its structure was confirmed by ^1H NMR in dimethyl sulfoxide- d_6 .

Pharmacology

Blood Collection and Aorta Isolation. The use of experimental animals adhered to the European Community guidelines. New Zealand male rabbits (Harlan Italy, Milan, Italy) weighing 2 to 2.5 kg were anesthetized (Zoletil 20, 1 ml/kg), the left carotid was isolated and cannulated for blood collection in 200 mM EDTA (1:40, v/v), and rabbits were sacrificed by complete bleeding. Part of the thoracic tract was cut into 2- to 3-mm-wide transverse rings, whereas a 3- to 4-cm thoracic-abdominal segment was isolated for contractility studies.

Platelet Preparation and Aggregation Studies. Rabbit blood was anticoagulated with EDTA to obtain platelet-rich plasma (PRP) and further centrifuged at 1800g for 20 min at 20°C to isolate platelets. The pellet was carefully resuspended in Tyrode-G (2.5 mM KCl, 1 mM MgCl_2 , 120 mM NaCl, 25 mM NaHCO_3 , 5 mM glucose, and 0.25% w/v gelatin, pH 6.5) with EGTA (0.2 mM) and centrifuged under the same conditions (Bossant et al., 1990); finally, the pellet was resuspended in Tyrode-G-Ca-HEPES (Tyrode G + 0.9 mM CaCl_2 and 4.2 mM HEPES, pH 7.4), and the platelet count was adjusted to 450,000 cells/ μL .

Platelet aggregation was studied using the Born turbidimetric technique in a dual-channel Elvi 840 aggregometer (Elvi Logos,

Milan, Italy). Before aggregation experiments, the aortic rings were preincubated with 1 mM acetylsalicylic acid, to inhibit endothelial cell cyclooxygenase activity, for 30 min at room temperature and then washed twice in Tyrode-G-Ca-HEPES.

Aliquots (250 μ l) of washed platelets, in the presence or absence of aortic rings, were preincubated for 2 min at 37°C under stirring and further preincubated for 3 min with drugs or their vehicles before challenge with arachidonic acid (AA) (1.5–3.0 μ M). Six minutes after challenge, aggregation was stopped by adding 10 μ M indomethacin and 7.6 mM EDTA. The platelet suspension was then centrifuged at 11,600g for 5 min at room temperature. The supernatant was divided in two parts and kept at –20°C until enzyme immunoassay (EIA) of AA metabolites. The extent of aggregation was quantified as the area under the aggregation curve from 0 to 6 min and expressed as weight of paper (milligrams) of uniform density.

Platelet aggregation induced by U46619 was determined in rabbit PRP, obtained by centrifugation of citrated blood (trisodium citrate final concentration 0.3% w/v, 150g, 15 min). Threshold aggregating concentration of U46619 was used (1–3 μ M), and percentage inhibition by different compounds was calculated by the reduction of the aggregation amplitude 5 min after challenge.

Collagen-induced platelet aggregation was determined in PRP from healthy donors as described previously (Tremoli et al., 1984). For each subject, a collagen concentration (0.5–1.0 μ g/ml) that induced a 50 to 60% decrease of optical density within 5 min was selected to test the effect of the drugs. Data are expressed as percent inhibition of platelet aggregation.

Rabbit Aorta Contractility. Four aortic rings were set up for isometric recording in oxygenated buffer (Krebs-Henseleit: 5 mM KCl, 1 mM $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 119 mM NaCl, 1 mM KH_2PO_4 , 25 mM NaHCO_3 , 5 mM glucose, and 2.5 mM CaCl_2) at 37°C. Responses to an endothelium-dependent vasodilating agent such as acetylcholine (1–3 μ M) were tested following enhancement of vascular tone with a submaximal (1 μ M) concentration of NE (or alternatively with the TXA_2 analog, compound U46619, 10 nM) to verify endothelium integrity. Vessels that gave a relaxation lower than 50% were not used. After wash of the preparations, the vascular tone was again increased with the submaximal concentration of NE, and a concentration-response curve of 2NTX-99 (0.1–100 μ M) was constructed. The capacity of 2NTX-99 to produce tachyphylaxis was investigated by exposing the aortic rings to a concentration of the compound that was the highest possible given its solubility profile (300 μ M, 3 h) or appropriate dimethyl sulfoxide blank.

A vascular segment with the endothelial lining exposed was prepared for isotonic contraction recording as described previously (Buccellati et al., 2002). After testing of the functionally intact endothelium as described above, the entire chamber volume was substituted with the washed platelet suspension (2.5 ml, 450,000 cells/ μ l), pretreated for 15 min with the drugs under test or with vehicle. AA (12 μ M) was added 15 min later to stimulate platelet TXA_2 formation and vascular contraction (30-min total platelet-drug incubation). Aliquots of the incubation suspension (200 μ l) were collected for EIA quantitation 15 min after AA challenge.

Assay of TXA_2 , PGI_2 , and NO. The stable metabolites of TXA_2 and PGI_2 (TXB_2 and 6-keto-PGF $_{1\alpha}$, respectively) were evaluated by selective EIA (Pradelles et al., 1985), carried out directly on aliquots of the incubation media, according to the manufacturer's instruction (Cayman Chemicals, Ann Arbor, MI).

Nitrite (NO_2^-) was measured using the Griess reaction, which possesses significant sensitivity limitations but allows measurement of the cumulated amount of nitrite, a significant marker of NO release in the assay sample. 2NTX-99, 2NTX-101, and 120 μ M isosorbide mononitrate or GTN (40 μ M) were incubated with rat aortic rings (20 mg wet weight) in 0.2 ml of Krebs-Henseleit buffer, pH 7.4, at 37°C for different times (30, 60, 120, 180, and 360 min). Supernatants were allowed to react (1:1, v/v) with the Griess reagent (0.5% sulfanilamide, 0.05% naphthylethylenediamine dihydrochloride, and 2.5% H_3PO_4) to form a chromophore absorbing at 546 nm.

Nitrite concentration was determined using sodium nitrite as standard. Results are expressed as nanograms per milliliter of NO_2^- .

Thromboxane Receptor Studies

Culture and Transfection of HEK293 Cells. HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin, 100 μ g/ml streptomycin, and 20 mM HEPES buffer, pH 7.4, at 37°C in a humidified atmosphere of 95% air and 5% CO_2 . Transfection with human $\text{TP}\alpha$ construct was performed as described previously (Capra et al., 2004).

Ligand Binding Assays. Receptor expression was monitored 48 h after the transfection. A mixed-type protocol together with heterologous competition were performed as described previously (Capra et al., 2003, 2004). In brief, confluent adherent cells in 250 μ l of serum-free DMEM, containing 0.2% (w/v) bovine serum albumin, were assayed in the presence of 0.1 to 3 nM of the specific receptor antagonist SQ29,548 (48 Ci/mmol), 0.01 to 10 μ M of the homologous unlabeled ligand, or 0.1 nM to 10 μ M of the heterologous unlabeled ligands (U46619, 2NTX-99). After 30 min of incubation at 25°C, cells were washed with ice-cold phosphate-buffered saline containing 0.2% (w/v) bovine serum albumin and lysed in 0.5 N NaOH. Analysis of binding data was performed by means of the LIGAND program (Munson and Rodbard, 1980).

Total Inositol Phosphate Determination. The functional activity of receptor was assessed 48 h after transfection by measuring total inositol phosphate (IP) accumulation as described previously (Habib et al., 1997; Capra et al., 2004). HEK293 cells were labeled with 1 μ Ci myo-[2- ^3H]inositol (17 Ci/mmol) for 24 h in serum-free, inositol-free DMEM, containing 20 mM HEPES buffer, pH 7.4, and 0.5% w/v Albumax I. Cells were washed and incubated with 25 mM LiCl for 10 min, pretreated with the indicated concentrations of study compounds (SQ29,548, 2NTX-99), and then were incubated for 30 min with either vehicle or 1 μ M of the agonist U46619. Cells were then lysed and extracted with an anion exchange AG 1X-8 column (Bio-Rad, Hercules, CA). Free inositol and glycerophosphoinositol were washed with 40 mM ammonium formate/formic acid buffer, pH 5, and total IP was eluted with 4 ml of a 2 M ammonium formate/formic acid buffer, pH 5.

2NTX-99 Oral and Intravenous Administration to Rats. 2NTX-99 was administered to anesthetized rats (thiopental sodium salt, 50 mg/kg i.p.) both i.v. (25 mg/kg) and p.o. (50 or 250 mg/kg). Blood was collected 30 min after i.v. 2NTX-99 infusion or 30 to 360 min and 18 h after p.o. administration. Samples were incubated for 30 min at 37°C to obtain serum or added with EDTA (10 μ g/ml) and Na-heparin (50 U/ml) to obtain plasma. Systemic blood pressure and heart rate were determined by insertion into a carotid artery of a PE-60 cannula connected to a pressure transducer (HP-1280; Hewlett-Packard, Waltham, MA).

To assess bleeding time, a small incision was applied longitudinally between the median and lower dorsal portion of the tail (between 4 and 6 cm from the end of the tail), taking care to avoid the artery. Blood from the wound was collected onto a filter paper every 30 s. Bleeding times were recorded as the interval between incision and bleeding arrest starting at 30 min after i.v. treatment.

Determination of 2NTX-99 and 2NTX-101 in Rat Plasma by HPLC. The choice of the RP-HPLC and the extraction methods were investigated using reference standards of 2NTX-99 and 2NTX-101 (the primary, denitrated metabolite of 2NTX-99; Fig. 1), with picotamide as internal standard. Compounds eluted from a LiChrosorb 5- μ m, RP-SELECT B, C8, 25-cm \times 4-mm i.d. column (Merck, Darmstadt, Germany) as well separated peaks at 5.3 ± 0.5 , 9.5 ± 0.5 , and 34 ± 1.8 min, using solvent A (30% acetonitrile; 70% $\text{NaH}_2\text{PO}_4 \times 0.05 \text{ M H}_2\text{O}$, pH 6) as mobile phase; absorbance was monitored at 230 nm. Both 2NTX-99 and 2NTX-101 appeared to be pure, and only trace amounts of 2NTX-101 were present in 2NTX-99. Extraction from rat plasma was carried out after alkalization on C18 Bond Elut cartridges, followed by elution with ethyl acetate, evaporation,

and reconstitution in 300 μ l of 0.6 M HCl and 600 μ l of ethyl acetate (Fossati et al., 1992). The lower acidic aqueous phase was taken to dryness and redissolved in 40 μ l of solvent A before injection into the HPLC system.

Quantitation was performed using standard curves (200 ng–20 μ g of synthetic 2NTX-99 and 2NTX-101, together with the internal standard) prepared in rat plasma, and extracted, and analyzed as described. The curve was linear with a correlation coefficient of 0.99 for both compounds.

Data Analysis

The concentration-response curves of platelet aggregation were analyzed and drawn by means of the computer program ALLFIT, and evaluation of the statistical significance of the parameter difference was based on the *F* test for the extra sum of square principle (Draper and Smith, 1966). Statistical evaluation of the data was carried out by analysis of variance (one-way analysis of variance or repeated measure analysis of variance with one grouping factor, as indicated); *p* < 0.05 was considered statistically significant.

Statistical analysis of ligand-binding data were performed with the LIGAND program (Munson and Rodbard, 1980). Parameter errors are always expressed in percentage coefficient of variation (CV) and calculated by simultaneous analysis of at least two different independent experiments performed in duplicates or triplicates. Data are presented as means \pm mean S.E. of multiple independent experiments, each performed at least in duplicates. A statistical level of significance of *p* < 0.05 was accepted.

Materials

Zoletil 20 (tiletamine and zolazepam) was from Virbac (Milan, Italy); norepinephrine bitartrate salt, acetylcholine chloride, arachidonic acid sodium salt, and OKY-046 were from Sigma Chemical Co. (St. Louis, MO). Thromboxane B₂ and 6-keto prostaglandin F_{1 α} EIA kits, SQ 29,548, and U46619 were from Cayman Chemicals. Gelatin powder and all inorganic salts were from Merck. Ultrapure water (MilliQ) was from Millipore Co. (Bedford, MA). Isosorbide mononitrate (ISMN) was purchased from Chiesi Farmaceutici S.p.A. (Parma, Italy). Collagen was from Mascia Brunelli (Milano, Italy).

Transfection reagent ExGen 500 was from MBI Fermentas (Hanover, MD). Cell culture media, serum, supplements, and molecular biology reagents were purchased from Gibco Invitrogen Co. (Carlsbad, CA). Inositol-free-DMEM was from ICN Pharmaceuticals Inc. (Costa Mesa, CA). HEK293 cells were obtained from American Type Culture Collection (Rockville, MD). Ultima Gold was from Packard Instruments (Meriden, CT). [5,6-³H]SQ29,548 and myo-[2-³H]inositol were purchased from Perkin-Elmer (Boston, MA). Stock solution of these compounds were stored at –20°C. Anion exchange resin AG 1X-8 (formate form, 200–400 mesh) and Poly-Prep columns were from Bio-Rad. All other reagents were of the highest purity available from Sigma Chemical Co.

Results

Vascular Response and Modulation of AA Metabolism by 2NTX-99

2NTX-99 (0.1–100 μ M) caused a concentration-dependent relaxation of aortic rings precontracted with NE (1 μ M), with a potency approximately 300-fold lower than GTN (EC₅₀, 7.9 \pm 0.4 and 0.03 \pm 0.002 μ M, respectively) and twice as potent as ISMN (EC₅₀, 15.9 \pm 0.9 μ M). The relaxation curve of 2NTX-99 was shifted (approximately 12-fold) to the right, in a parallel way, by pretreatment with 10 μ M methylene blue, an inhibitor of guanylate cyclase (data not shown). Compound 2NTX-101, the primary denitrated metabolite of 2NTX-99, induced only a modest relaxation at 100 μ M, and no EC₅₀ value could be calculated (Fig. 2A). The

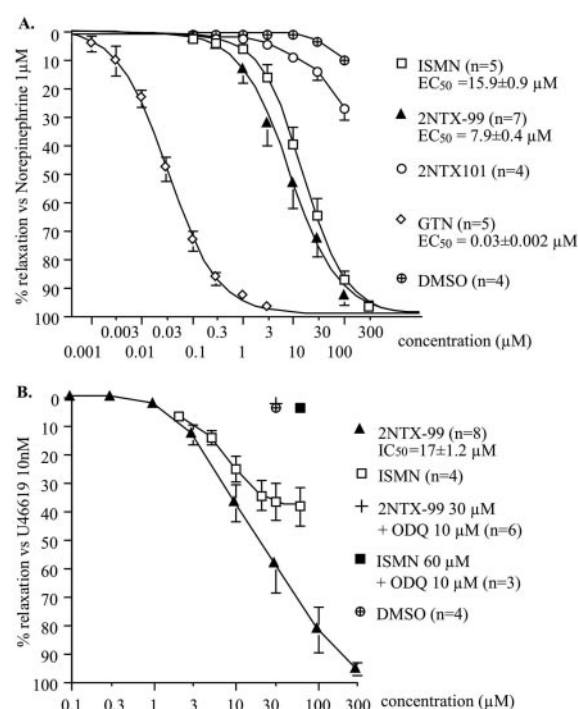


Fig. 2. A, dose-dependent relaxation by 2NTX-99, 2NTX-101, ISMN, and GTN on norepinephrine-induced rabbit aortic rings contraction. B, dose-dependent relaxation induced by 2NTX-99 and ISMN on U46619-induced rabbit aortic ring contraction. Effect of the selective guanylate cyclase inhibitor ODQ. Data represent mean values. Bars, mean \pm S.E. of *n* replicates.

EC₅₀ values of the compound varied moderately before (2.5 \pm 0.38 μ M, *n* = 4) and after (6.2 \pm 1.0 μ M) exposure to 300 μ M 2NTX-99.

Compound 2NTX-99 (0.1–100 μ M) caused a concentration-dependent relaxation (EC₅₀, 17 \pm 1.2 μ M) of aortic rings precontracted with the stable thromboxane analog U46619, at a concentration (10 nM) as efficacious as 1 μ M NE. The effects of 2NTX-99 (30 μ M) and ISMN (60 μ M) were prevented by pretreatment with ODQ (10 μ M), a selective inhibitor of NO-sensitive guanylate cyclase enzyme activity (Fig. 2B).

Incubation of rat aortic rings with compound 2NTX-99 (120 μ M) triggered a time-dependent formation of NO₂[–] (30 min, 103 \pm 42; 60 min, 179 \pm 32; 120 min, 213 \pm 31; 180 min, 353 \pm 97; 360 min, 314 \pm 58 ng/ml; *n* = 4) that plateaued after 180 min, whereas 2NTX-101 was ineffective. ISMN (120 μ M) also led to nitrite formation (103 \pm 33 ng/ml, *n* = 4), and, as expected, GTN (40 μ M, *n* = 3) led to a significant increase in NO₂[–] (4.96- \pm 0.36-fold over 2NTX-99).

The ability of compound 2NTX-99 to inhibit TXA₂ synthesis and action (and to stimulate PGI₂ formation) was investigated in preparations of rabbit aorta incubated with homologous platelets, as recently reported (Buccellati et al., 2002). When intact vessel specimens were incubated with a suspension of washed platelets (450,000/ μ l) and challenged with 12 μ M AA, a strong contraction occurred; pretreatment with 2NTX-99 (10 and 40 μ M, 30 min) caused a significant reduction of AA-induced contraction (–24.5 and –47%, respectively) (Fig. 3A).

The coincubation of washed platelets with aortic rings per se triggered significant synthesis of TXB₂ and 6-keto-PGF_{1 α} , which was markedly enhanced following challenge with AA

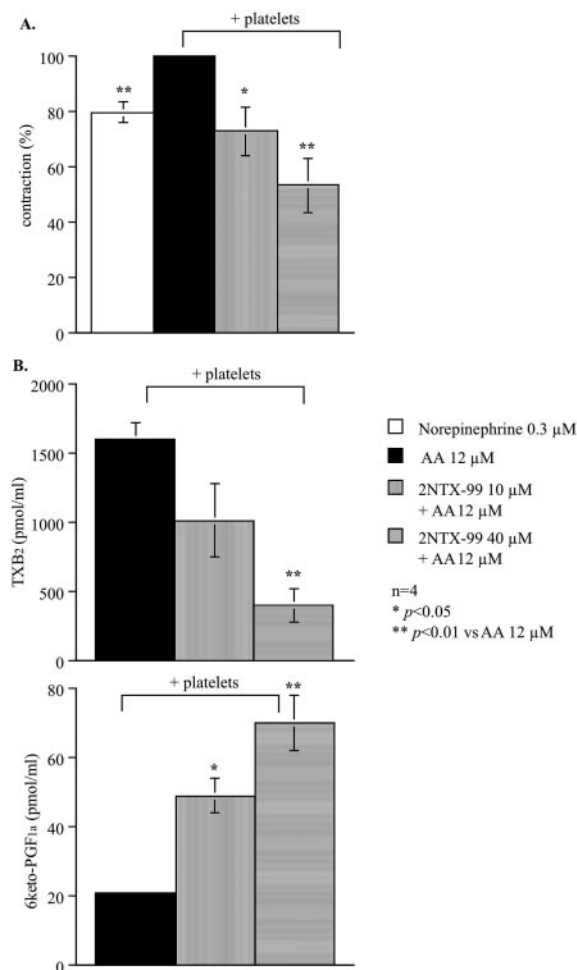


Fig. 3. A, effect of 2NTX-99 on the contraction of isolated whole-rabbit aorta incubated with homologous platelets ($450 \times 10^6/\text{ml}$) and challenged with AA. B, effect of 2NTX-99 on TXA₂ and PGI₂ synthesis in whole-rabbit aorta incubated with homologous platelets and challenged with AA. TXA₂ and PGI₂ were detected as their stable metabolite, TXB₂ and 6-keto-PGF_{1α}, respectively. Data represent mean values. Bars, mean \pm S.E. of *n* replicates.

(data not shown). Pretreatment with 2NTX-99 (10 and 40 μM) reduced TXB₂ synthesis and increased PGI₂ formation significantly (Fig. 3B).

Effect of 2NTX-99 on Platelet Aggregation

The effect of compound 2NTX-99 on platelet aggregation was evaluated in washed rabbit platelet suspensions ($450,000/\mu\text{l}$) stimulated with a submaximal aggregating concentration of AA ($1.5\text{--}3\text{ }\mu\text{M}$). 2NTX-99 inhibited platelet aggregation concentration-dependently (IC_{50} $9.83 \pm 1.1\text{ }\mu\text{M}$); its potency was increased in the presence of aortic rings (IC_{50} $1.45 \pm 0.15\text{ }\mu\text{M}$) (Fig. 4A). 2NTX-99 inhibited TXB₂ formation either in the absence or presence of aortic rings (-71 and -80% , respectively, at $10\text{ }\mu\text{M}$); conversely, the compound stimulated 6-keto-PGF_{1α} formation in a concentration-dependent way only when vascular rings were present (Fig. 4B). 2NTX-99 ($20\text{--}40\text{ }\mu\text{M}$) also prevented the aggregation induced by threshold U46619 concentrations ($1\text{--}4\text{ }\mu\text{M}$) in rabbit PRP ($-74 \pm 6.7\%$ and $96.4 \pm 2.4\%$, respectively; $n = 8$) thus sharing the behavior of picotamide (Gresele et al., 1989).

2NTX-99 ($20\text{--}40\text{ }\mu\text{M}$) inhibited ($-48.2 \pm 10\%$ and $-79.2 \pm$

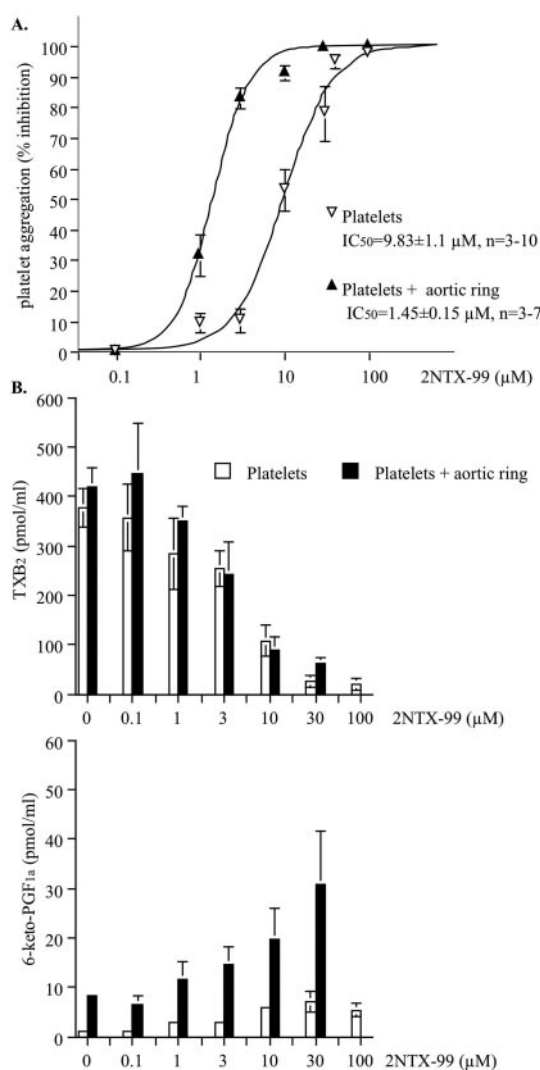


Fig. 4. A, dose-dependent inhibition by 2NTX-99 on AA-induced aggregation ($1.5\text{--}3\text{ }\mu\text{M}$) of rabbit platelets ($450 \times 10^6/\text{ml}$) in the presence or absence of an aortic ring. B, dose-dependent effect of 2NTX-99 on TXA₂ and PGI₂ synthesis in rabbit platelets challenged by AA in the absence or presence of an aortic ring. TXA₂ and PGI₂ were detected as their stable metabolite, TXB₂ and 6-keto-PGF_{1α}, respectively. Data represent mean values. Bars, mean \pm S.E. of *n* replicates.

6% , respectively; $n = 5$) platelet aggregation in human PRP challenged with threshold collagen concentrations. Compound 2NTX-101 (the primary denitrated metabolite of 2NTX-99) as well as the reference TX synthase inhibitor OKY-046 (ozagrel, $40\text{ }\mu\text{M}$) were ineffective. In this experimental setting, ozagrel ($40\text{ }\mu\text{M}$) inhibited TX formation by approximately 80% , whereas equimolar concentrations of 2NTX-99 or 2NTX-101 reduced TX formation by 40% (data not shown).

Binding of 2NTX-99 to Human TP α and Total IP Determination

Mixed-type curves of [³H]SQ29,548 and heterologous competition curves of the agonist U46619 clearly display monophasic binding curves fitting a single-site model by computerized analysis performed with the program LIGAND (Fig. 5A). The simultaneous analysis of three independent experiments indicated typical binding parameters (SQ29,548

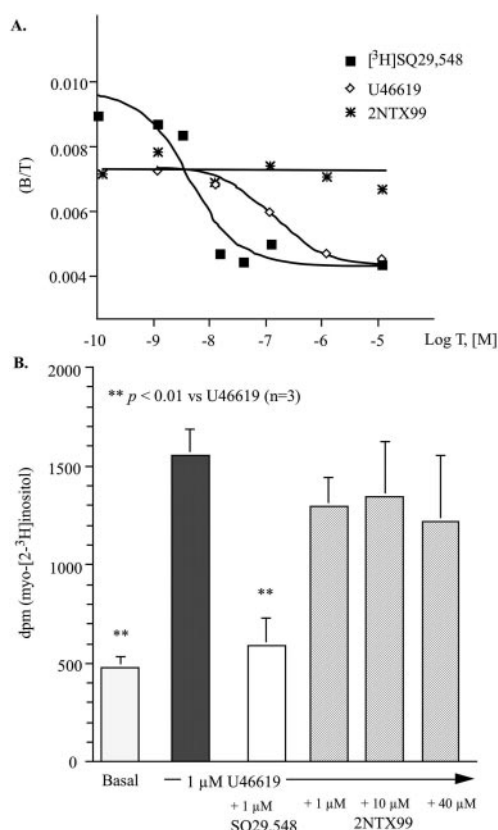


Fig. 5. A, binding of [³H]SQ29,548 in HEK293 transiently expressing human TP α receptor. For the sake of clarity, only curves from a representative experiment are shown. B, total IP formation in HEK293 transiently expressing human TP α receptor; accumulation was measured after incubation in the absence (basal) and presence of U46619 for 30 min. SQ29,548 and 2NTX-99 were added 30 min before U46619. Data represent mean values. Bars, mean \pm S.E. of *n* replicates.

$K_d = 3.48 \pm 36\%$ CV; U46619 $K_i = 64.2 \pm 83\%$ CV), as previously reported (Capra et al., 2004). On the contrary, compound 2NTX-99 did not compete for the labeled antagonist (Fig. 5A).

Signaling of TP α receptor was also investigated by measuring the capacity of 2NTX-99 to inhibit agonist-induced total IP production (Fig. 5B). HEK293 cells expressing the human TP α responded to 1 μ M U46619 stimulation with a marked elevation of total IPs (3.2-fold increase), an effect that was specifically and significantly ($p < 0.01$) prevented by 30-min pretreatment with 1 μ M SQ29,548. In contrast, 30-min pretreatment with 2NTX-99 up to 40 μ M was totally ineffective, suggesting that the compound does not inhibit TP α -induced phospholipase-C activation.

Animal Studies

Effect of 2NTX-99 on Systemic Blood Pressure and Bleeding Time in the Rat. Administration of 2NTX-99 to anesthetized rats (25 μ g/kg i.v. over a 3-min period) led to a transient drop of systemic blood pressure from control values of 117.3 ± 1.3 to 109.3 ± 1.3 mm Hg ($n = 3$); the decrease in blood pressure peaked 3 to 4 min after administration and fully recovered thereafter, reaching control values (118.7 ± 1.3 mm Hg) between 30 and 60 min. No significant change in heart rate was observed. The effect of 2NTX-99 on bleeding time was also investigated 30 min after i.v. administration of 25 mg/kg, a level that fully inhibited platelet function (see

below); bleeding time was 4.3 ± 0.2 min ($n = 3$) in control conditions and was significantly prolonged (12.8 ± 0.6 min) by drug treatment.

Determination of 2NTX-99 in Rat Plasma and Inhibition of Thromboxane A₂ Synthesis. Intravenous administration of 2NTX-99 to rats (25 mg/kg for 30 min) suppressed TXA₂ production in clotting blood (94% inhibition compared with vehicle-treated animals, $n = 5$); plasma levels ($n = 2$) were 25.67 and 27.44 μ g/ml (mean concentration, 61 μ M).

After oral administration (250 mg/kg), plasma levels of 2NTX-99 and TXA₂-synthesis inhibition were assessed up to 16 h postdosing, showing sustained plasma concentrations and long-lasting pharmacological activity. In a few selected experiments, 2NTX-99 was administered at a lower dose (50 mg/kg), and plasma levels were followed up to 3 h; inhibition of TXA₂ production peaked at 90 min (-71%), and plasma levels ($n = 2$) were 9.29 and 6.95 μ g/ml (mean concentration 19 μ M) (Fig. 6, A and B).

An area under the curve, 0 to 16 h, of 149.5 h/ μ g/ml was calculated for the 250 mg/kg dose, with a C_{max} value of 31.8 ± 8.2 μ g/ml and an estimated half-life of 6 h, even if the spread (from 0.29–9.69 μ g/ml) of plasma values at 16 h was broad. The results of the 250 mg/kg dose at 16 h were compared with the more limited data of the 50 mg/kg dose. Areas under the curve, 0 to 3 h, were, respectively, calculated as 66 and 18.3 h/ μ g/ml, showing, when adjusted for the dose, a higher (+38%) value for the lower dose. The difference might be attributable to slower dissolution and absorption of the insoluble compound from the administered suspension of the higher dose. An excellent correlation between the plasma concentrations of 2NTX-99 and the inhibition of TXA₂ synthesis was observed ($r^2 = 0.72$; EC₅₀, 11.8 μ M).

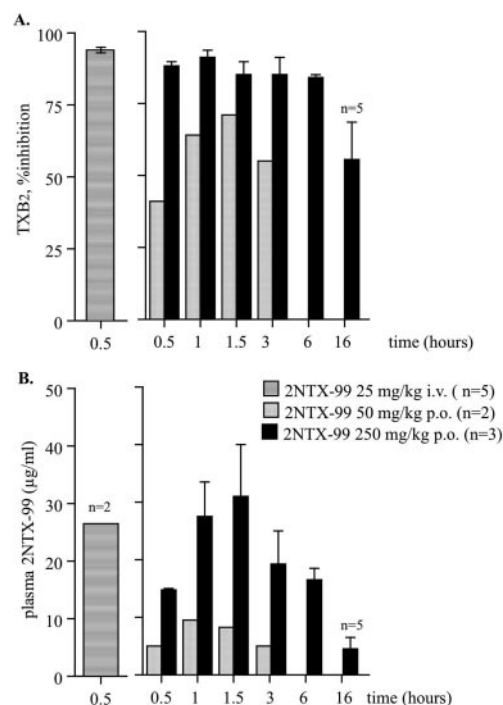


Fig. 6. Time course of inhibition of rat serum TXB₂ (A) and 2NTX-99 plasma levels (B) after oral (250 mg/kg) administration of 2NTX-99; comparison with i.v. (25 mg/kg, 0.5 h). In selected experiments (0.5–3 h) 2NTX-99 was administered p.o. at 50 mg/kg. Columns represent mean values. Bars, mean \pm S.E. of *n* replicates.

Formation of Denitrated Metabolite 2NTX-101. In all plasma samples from treated animals, a prominent chromatographic peak corresponding to intact 2NTX-99 was observed, together with less relevant peaks at the retention time of 2NTX-101, the primary, denitrated metabolite of 2NTX-99; quantitative analysis indicated amounts ranging between 3 and 6% of the concentrations of 2NTX-99. Incubation of 2NTX-99 (40 μ M) in rat plasma in the presence of aortic rings (at 37°C for 30 min) resulted in amounts of 2NTX-101 equal to $0.41 \pm 0.26\%$ (mean \pm S.D.) of the parent compound, whereas 2NTX-101 was not detected in incubation of 2NTX-99 in plasma alone.

Discussion

In the present article, we describe the pharmacology of 2NTX-99, an orally active, innovative chemical entity with plural actions on TXA₂ synthesis, PGI₂ formation, and NO availability, resulting in functional effects that span from vascular relaxation to inhibition of platelet aggregation and their interactions with the vessel wall. 2NTX-99 is a structural analog of picotamide, a dual thromboxane synthase inhibitor/TXA₂ receptor antagonist (Gresele et al., 1989). 2NTX-99, although retaining the thromboxane synthase inhibitory activity, did not bind to the TP α , nor did it affect TP α -induced signal transduction. Moreover, the contribution of the NO donor properties of 2NTX-99 has been well documented and confirmed by the lack of activity of the denitrated derivative 2NTX-101.

In NE-precontracted rings, 2NTX-99 showed NO-dependent vasorelaxant potency (EC₅₀, 7.9 μ M) markedly lower than GTN (EC₅₀, 0.03 μ M) but higher than ISMN (EC₅₀, 15.9 μ M). GTN is considered as an organic nitrate with high vasorelaxant potency, whereas our data clearly indicate that 2NTX-99 (and ISMN) belong to the group of organic nitrates with lower vasorelaxant potency but less prone to tolerance development (Daiber et al., 2004).

ISMN, the main metabolite of isosorbide dinitrate, is an orally active, clinically proven antianginal agent with sustained effects. ISMN is considered a more reliable reference for in vitro pharmacological comparison than its parent compound, whose pharmacokinetic and pharmacodynamic behavior is governed by biphasic NO release from two nitrate functions with very different rates of activation (Ahlner et al., 1991). 2NTX-99 fully relaxed aortic rings precontracted with U46619 (EC₅₀, 17 μ M), whereas ISMN caused only partial relaxation. The effect of 2NTX-99 was abolished by ODQ, an inhibitor of guanylate cyclase, and shifted to the right by the guanylate cyclase inhibitor methylene blue (data not shown).

2NTX-99 inhibited aggregation of washed rabbit platelets stimulated by AA, and its potency was increased approximately 7-fold in the presence of aortic rings with intact endothelium. 2NTX-99 inhibited TXA₂ synthesis, either in the absence or presence of aortic rings (70–80% at 10 μ M), and in the latter situation increased PGI₂ synthesis (approximately 3-fold at 10 μ M), as shown by other TX synthase inhibitors (Gresele et al., 1991; Buccellati et al., 2002). 2NTX-99 inhibited U46619-induced platelet aggregation, thus showing a profile similar to dual TX synthase inhibitors-TP antagonists (Gresele et al., 1989; Hanson et al., 2005). To assess the thromboxane A₂ receptor antagonist properties of 2NTX-99,

we performed classic competition experiments in a recombinant system expressing the TP α receptor (it would have been the same using the TP β because the two isoforms are identical for the first 328 residues and differ only in the C-terminal tail of no relevance for ligand binding). The receptor was labeled with the competitive antagonist [³H]SQ29,548 and competed with the unlabeled 2NTX-99. Furthermore, in the same system, we also demonstrated that 2NTX-99 does not antagonize the U46619-induced IP production, clearly demonstrating that 2NTX-99 is not a TP receptor antagonist. Rather, inhibition of U46619-induced platelet aggregation indicates that 2NTX-99 behaves as a functional antagonist, possibly with the contribution of the NO-releasing component of 2NTX-99. The exact molecular mechanisms behind this somehow unexpected finding are presently unclear and will be addressed specifically in separate investigations.

In addition, 2NTX-99 attenuated platelet aggregation in human PRP stimulated with threshold concentrations of collagen (0.5–1 μ g/ml) and reduced partially (–40%) TX formation. Ozagrel did not affect collagen-induced platelet aggregation despite a marked reduction of TX formation (80%), in line with the existence of a nonlinear relationship linking TX synthesis and platelet aggregation (Reilly and FitzGerald, 1987; Buccellati et al., 2002). These findings are of particular interest since exposed collagen represents a primer of platelet adhesion, activation, and release of inflammatory and prothrombotic mediators (Farndale et al., 2004). As expected, a similar degree of TX synthesis inhibition was shared by the denitrated derivative 2NTX-101, which failed to affect platelet aggregation. These results, altogether, are suggestive of an NO-mediated antiplatelet effect of 2NTX-99.

The in vitro antiaggregatory activity of organic nitrates is well recognized and shown to depend on structure and on steric orientation of the nitrate function (Weber et al., 1993); ISMN possesses ex vivo antiaggregatory effect in patients (De Caterina et al., 1990), comparable with those of ISDN (De Caterina et al., 1984). The actions of organic nitrates on vessels and platelets, at difference from spontaneous NO donors, are dependent upon enzymatic mechanisms for activation and release of NO (Ahlner et al., 1991). These mechanisms are generally more prominent in vascular cells than in platelets. 2NTX-99, combining the structural features for antithromboxane activity with the insertion of an NO donor moiety, i.e., a nitrate ester of secondary hydroxy group, equatorially oriented on a cyclohexane ring, was predicted to show a slow rate of activation. Indeed, this NO donor moiety afforded adequate stability and metabolic resistance, as needed for absorption and for sustained effect, at variance with other NO donors, mostly characterized by scarce stability, short duration of action, or lack of oral bioavailability (Megson, 2000). The cumulated release of NO from 2NTX-99 and ISMN following incubation with rat aortic rings at 3 h goes hand in hand with the potency of the compounds in relaxing rabbit aorta, i.e., 5.4 and 2.4% of the amount released by GTN, respectively, indicating for both compounds the requirement of concentrations over 200 times higher than GTN to equal its rate of release (7.9 and 15.9 μ M, respectively, versus 30 nM GTN), as well as the relaxing effect of nanomolar concentrations of the endothelial flow of endogenous NO (Moncada et al., 1991). 2NTX-99 did not spontaneously release NO in phosphate buffer nor generate detectable amounts of 2NTX-101 when incubated in rat plasma,

whereas 2NTX-101 formation was observed by incubation in rat plasma in the presence of aortic rings.

The i.v. administration of 2NTX-99 caused a moderate and transient drop in systemic blood pressure, without affecting heart rate, and significantly prolonged bleeding time. These findings were largely expected, given the capacity of the compound to cause vascular relaxation, and are in line with its ability to markedly inhibit platelet function and TX synthase enzyme activity. Moreover, the metabolic fate of 2NTX-99 was addressed in vivo in a preliminary study of oral administration in rats. Sustained plasma levels of the intact molecule were observed, along with low concentrations of the denitrated metabolite (3–6% with respect to the parent compound). These amounts may well represent the kinetic balance between the formation of the metabolite in vascular and other tissues and its elimination and appear compatible with the amount generated in vitro and the moderate, but pharmacologically significant, release of NO measured in arterial tissues.

The pharmacokinetic study, within the limits of the small number of animals and the degree of variability, allowed an estimate of the half-life of 2NTX-99 of 6 h. Importantly, an excellent correlation ($r^2 = 0.724$) between the plasma levels of 2NTX-99 and the inhibition of TXA₂ synthesis in clotting blood was observed. The in vivo experiments indicate that 2NTX-99 represents a novel chemical entity, not a prodrug or a mutual prodrug of two active molecules (Bolla et al., 2005), that is absorbed and exerts its sustained action in intact form. As a consequence, the multiplicity of its diverse effects, observed in vitro in a balanced fashion, can be elicited in vivo consistently with a sole pharmacokinetic pathway of absorption and distribution.

In conclusion, 2NTX-99 offers an innovative profile of plural actions on platelet activation and interaction with the vascular wall, inhibiting the synthesis of thromboxane, increasing that of prostacyclin, and providing a pharmacologically relevant supply of NO. NO, in turn, may stimulate PGI₂ formation and suppress TXA₂ synthase activity; NO also activates guanylyl cyclase to increase cGMP and acts synergistically with PGI₂ to increase cAMP levels in, e.g., platelets and vascular smooth muscle cells (Antman et al., 2005). Taken together, the net effect of these actions is to provide optimal control of platelet and vessel function in atherothrombosis.

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Address correspondence to: Dr. Giancarlo Folco, Department of Pharmacological Sciences, Center for Cardiopulmonary Pharmacology, School of Pharmacy, Via Balzaretti 9, 20133 Milano, Italy. E-mail: giancarlo.folco@unimi.it