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Pharmacological characterization of 4-methoxy-N[1]-(4-*trans*-nitrooxycyclohexyl)-N[3]-(3-pyridinylmethyl)-1,3-benzenedicarboxamide (2NTX-99), a potential antiatherothrombotic agent with anti-thromboxane and NO-donor activity in platelet and vascular preparations

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Thromboxane A_2, TXA_2; prostacyclin, PGI_2; nitric oxide, NO; cyclooxygenase-1, COX-1; cyclooxygenase-2, COX-2; 4-methoxy-N^1-(4-*trans*-nitrooxycyclohexyl)-N^3-(3-pyridinylmethyl)-1,3-benzenedicarboxamide, 2NTX-99; norepinephrine, NE; arachidonic acid, AA; 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one, ODQ; thromboxane A_2 receptor isophorm alpha, TPα; inositol phosphate, IPs; isosorbide mononitrate, ISMN; glyceryl trinitrate, GTN

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Abstract

Thromboxane (TXA_2), prostacyclin (PGI_2) and nitric oxide (NO), regulate platelet function and interaction with the vessel wall. Inhibition of TXA_2, implemented synthesis of PGI_2, and supply of exogenous NO, may afford therapeutic benefit. 2NTX-99, 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 (EC_{50} 7.9, 17.1 μM, respectively), an effect abolished by 10 μM ODQ. 2NTX-99 inhibited arachidonic acid (AA) induced washed platelet aggregation (EC_{50} 9.8 μM) and TXB_2 formation (-71% at 10 μM) and its potency increased in the presence of aortic rings (EC_{50} 1.4 μM). In whole rabbit aorta incubated with homologous platelets, AA caused contraction and TXA_2 formation, reduced by 2NTX-99 (10-40 μM): contraction -28, and -47 %, TXA_2 formation -37 and –75.4 % respectively, with concomitant increase in PGI_2. 2NTX-99 (20-40 μM) inhibited U46619-induced aggregation in rabbit PRP (-74±6.7, -96±2.4% respectively), and inhibited collagen-induced aggregation in human PRP (-48.2±10 and -79.2±6%), while ozagrel was ineffective. In HEK293 cells, transfected with the TPα receptor, 2NTX-99 did not compete with the ligand (^{3}H-SQ29,548) nor prevented IP accumulation. After oral administration (50-250 mg/Kg), 2NTX-99 inhibited TXA_2 production in rat clotting blood (-71 and -91 %); at 250 mg/Kg an $AUC_{0\text{-}16\ hours}$ of 149.5 h•μg/ml and a $t_{1/2}$ of 6 h were calculated, with a C_{max} value of 31.8±8.2 μg/ml. An excellent correlation between plasma concentrations and TXA_2 inhibition occurs. 2NTX-99 controls platelet function and vessel wall interaction by multifactorial mechanisms and possesses therapeutic potential.

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Introduction

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

Novel approaches to the modulation of platelet function by preventing either 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. Up to 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 $P2Y_{12}$ receptor.

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 (Cure Study Investigators), (Matha and 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. (Maclouf 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 towards an alternative pathway, e.g., in platelet-endothelial cell coincubates PGI_2 synthesis is enhanced when thromboxane synthase is inhibited (Nowak and FitzGerald, 1989).

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The search for TXA_2 inhibitors has targeted thromboxane synthase or thromboxane receptors, but has also developed "dual" inhibitors which 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-$PGF_{2\alpha}$ (Dogne et al., 2000). In addition, shunting of PGH_2 towards 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 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, while preserving its antithromboxane activity, allowed the insertion of an NO-donor moiety, leading to the synthesis of compound 2NTX-99 (4-methoxy-N^1-(4-*trans*-nitrooxycyclohexyl)-N^3-(3-pyridinylmethyl)-1,3-benzenedicarboxamide, Fig.1).

2NTX-99 is a new molecular entity that targets three powerful regulators of platelet and vascular function, i.e., TXA_2, prostacyclin (PGI_2), and nitric oxide (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; Maclouf et al., 1998). In this paper 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.

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Methods

1. Synthesis of 4-methoxy-N^1-(4-*trans*-nitrooxycyclohexyl)-N^3-(3-pyridinylmethyl)-1,3-benzenedicarboxamide (2NTX-99, Fig 1)

The synthesis (US Patent 6,525,078) started from the regioselective mono-amination of the dimethyl ester of 4-methoxy-1,2-benzenedicarboxylic acid with 3-pyridynilmethylamine, 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. 153-154 °C; its structure was confirmed by ^{1}H-NMR in DMSO-d_6.

2. Pharmacology

2.1 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 2-2.5 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 2-3 mm wide transverse rings, while a 3-4 cm thoracic-abdominal segment was isolated for contractility studies.

2.2 Platelet preparation and aggregation studies

Rabbit blood was anti-coagulated with EDTA, to obtain platelet-rich plasma (PRP) and further centrifuged at 1800 *g* 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, 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

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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 µl) 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 AA (1.5-3.0 µM). Six min after challenge, aggregation was stopped by adding 10 µM indomethacin and 7.6 mM EDTA. The platelet suspension was then centrifuged at 11,600 *g* for 5 min at room temperature. The supernatant was divided in 2 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 (mg) of uniform density.

Platelet aggregation induced by U46619 (9,11-dideoxy-9α,11α-methanoepoxy-prosta-5Z,13E-dien-1-oic acid) was determined in rabbit PRP, obtained by centrifugation of citrated blood (trisodium citrate final concentration 0.3% w/v, 150 *g*, 15 min). Threshold aggregating concentration of U46619 was used (1-3 µM), 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 (Tremoli et al., 1984). For each subject, a collagen concentration (0.5-1.0 µg/ml) that induced a 50%-60% decrease of optical density within 5 min was selected to test the effect of the drugs. Data are expressed as % inhibition of platelet aggregation.

2.3 Rabbit aorta contractility

Four aortic rings were set up for isometric recording in oxygenated buffer (Krebs Henseleit: 5 mM KCl, 1 mM $MgSO_4x7H_20$, 119 mM NaCl, 1mM KH_2PO_4, 25 mM $NaHCO_3$, 5 mM glucose, 2.5 mM $CaCl_2$) at 37°C. Responses to an endothelium-dependent vasodilating agent such as acetylcholine (Ach, 1-3 µM) were tested following enhancement of vascular tone with a submaximal (1 µM) concentration of NE (or alternatively with the TXA_2 analog, compound U46619, 10 nM) in order to verify endothelium integrity. Vessels that gave a relaxation lower than 50% were not used. After

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wash out 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 μM) 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 μM, 3 hours), or appropriate DMSO blank.

A vascular segment with the endothelial lining exposed, was prepared for isotonic contraction recording as described (Buccellati et al., 2002). Following 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/μl), pretreated for 15 min with the drugs under test or with vehicle. AA (12 μM) 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 μl) were collected for EIA quantitation 15 min after AA-challenge.

2.4 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 enzyme-immunoassay (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, USA).

Nitrite (NO_2^-) was measured using the Griess reaction, that possesses significant sensitivity limitations but allows measurement of the cumulated amount of nitrite, a significant marker of NO release in the assay sample. 2-NTX99, 2-NTX101 and isosorbide mononitrate 120 μM, or GTN (40 μM), were incubated with rat aortic rings (20 mg wet weight) in 0.2 ml 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% naphtylethylendiammine dihydrochloride, 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 ng/ml NO_2^-.

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2.5 Thromboxane receptor studies

2.5.1. Culture and Transfection of HEK293 Cells

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 units/ml penicillin, 100 µg/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 TPα construct was performed as previously described (Capra et al., 2004).

2.5.2. Ligand Binding Assays

Receptor expression was monitored 48 hours after the transfection. A mixed type protocol together with heterologous competition were performed as described (Capra et al., 2003; Capra et al., 2004). Briefly, confluent adherent cells in 250 µl of serum-free DMEM, containing 0.2% (w/v) BSA were assayed in the presence of 0.1-3 nM of the specific receptor antagonist [^{3}H][1S-[1α,2β(5Z),3β,4α]]-7-[3-[[2-(Phenylamino)-carbonyl]hydrazino]methyl]-7-oxabicyclo[2,2,1]-hept-2-yl]-5-heptanoic acid (SQ29,548; 48 Ci/mmol), 0.01 to 10 µM of the homologous cold ligand, or 0.1 nM to 10 µM of the heterologous cold ligands (U46619, 2NTX-99). After 30 min incubation at 25°C, cells were washed with ice-cold PBS containing 0.2% (w/v) BSA and lysed in 0.5 N NaOH. Analysis of binding data was performed by means of the LIGAND program (Munson and Rodbard, 1980).

2.5.3. Total Inositol Phosphate Determination

The functional activity of receptor was assessed 48 hours after transfection by measuring total inositol phosphate (IPs) accumulation as previously described (Habib et al., 1997; Capra et al., 2004). HEK293 cells were labeled with 1 µCi of *myo*-[2-^{3}H]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 incubated for 30 min with either vehicle or 1 µM of the agonist U46619. Cells were then lysed and extracted with an anion exchange AG 1X-8 column (BioRad). Free inositol and glycerophosphoinositol were washed with

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40 mM ammonium formate/formic acid buffer, pH 5, and total IPs eluted with 4 ml of a 2 M ammonium formate/formic acid buffer, pH 5.

2.6 2 NTX-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 mg/kg or 250 mg/kg). Blood was collected 30 min after i.v. 2NTX-99 infusion, or 30 - 360 min and 18 hours after p.o. administration. Samples were incubated for 30 min at 37°C to obtain serum, or added with EDTA (10 µg/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, USA).

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 sec.. Bleeding times were recorded as the interval between incision and bleeding arrest starting at 30 min after i.v. treatment.

2.7 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 µm, RP-SELECT B, C8, 25 cm x 4 mm I.D. column (Merck) as well separated peaks at 5.3±0.5, 9.5±0.5 and 34±1.8 min, using solvent A (30% acetonitrile; 70% NaH_2PO_4 x H_2O 0.05 M, 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 alkalinization on C18 Bond Elut cartridges, followed by elution with ethyl acetate, evaporation and reconstitution in 300 µl HCl 0.6 M and 600 µl ethyl acetate (Fossati et al., 1992). The lower acidic aqueous phase was taken to dryness and redissolved in 40 µl solvent A before injection into the HPLC system.

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

3. Data analysis

The concentration-response curves of platelet aggregation were analysed and drawn by means of the computer programme 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 ANOVA or ANOVA repeated measure with one grouping factor, as indicated); $p<0.05$ was considered statistically significant.

Statistical analysis of ligand-binding data was 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 ± mean standard error of multiple independent experiments, each performed at least in duplicates. A statistical level of significance of $p< 0.05$ was accepted.

4. 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, USA). Thromboxane B_2 and 6-keto prostaglandin $F_{1\alpha}$ EIA kits, SQ 29,548 and U46619 were from Cayman Chemical. Gelatin powder and all inorganic salts were from Merck (Darmstadt, Germany). Ultrapure water (MilliQ) was from Millipore Co. (Bedford, MA, USA). 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. Cell culture media, serum, supplements, and molecular biology reagents were purchased from Gibco Invitrogen Co. (Carlsbad, CA, USA).

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Inositol-free-DMEM was from ICN Pharmaceuticals Inc. (Costa Mesa, CA, USA). HEK293 cells were obtained from ATCC (Rockville, MD, USA). Ultima Gold was from Packard Instruments (Meriden, CT, USA). [5,6-^{3}H]SQ29,548 and myo-[2-^{3}H]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 BioRad Laboratories (Hercules, CA, USA). All other reagents were of the highest purity available from Sigma Chemical Co. (St Louis, MO, USA).

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Results

1. Vascular response and modulation of AA metabolism by 2NTX-99

2NTX-99 (0.1-100 µM) caused a concentration dependent relaxation of aortic rings precontracted with NE (1 µM), with a potency approx. 300-fold lower than GTN (EC_{50} 7.9±0.4 µM and 0.03±0.002 µM, respectively) and twice as potent as ISMN (EC_{50} 15.9±0.9 µM). The relaxation curve of 2NTX-99 was shifted (approx. 12-fold) to the right, in a parallel way, by pretreatment with 10 µM 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 µM and no EC_{50} value could be calculated (Fig. 2A). The EC_{50} values of the compound varied moderately before (2.5±0.38 µM, n=4) and after (6.2±1.0 µM) exposure to 300 µM of 2NTX-99.

Compound 2NTX-99 (0.1-100 µM) caused a concentration dependent relaxation (EC_{50} 17±1.2 µM) of aortic rings precontracted with the stable thromboxane analogue U46619, at a concentration (10 nM) as efficaceous as 1µM NE. The effect of 2NTX-99 (30 µM) and of ISMN (60 µM) were prevented by pretreatment with ODQ (10 µM), a selective inhibitor of NO sensitive guanylate cyclase enzyme activity (Fig. 2B).

Incubation of rat aortic rings with compound 2NTX-99 (120 µM) triggered a time-dependent formation of nitrite (NO_2^-; 30 min: 103±42, 60 min: 179±32, 120: 213±31, 180 min: 353±97, 360 314±58 ng/ml; n=4), that plateaued after 180 min, while 2NTX-101 was ineffective. ISMN (120 µM) also led to nitrite formation (103±33 ng/ml, n=4) and, as expected, GTN (40 µM, n=3) led to a significant increase in NO_2^- (4.96±0.36 fold over 2NTX99).

The ability of compound 2NTX-99 to inhibit TXA_2 synthesis and action (and to stimulate PGI_2 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/µl) and challenged with 12 µM AA, a strong contraction occurred; pretreatment with 2NTX-99 (10 and 40 µM, 30 min) caused a significant reduction of AA-induced contraction (-24.5%, -47%, respectively) (Fig. 3A).

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The coincubation of washed platelets with aortic rings "per se" triggered significant synthesis of TXB_2 and 6-keto-$PGF_{1\alpha}$ which was markedly enhanced following challenge with AA (data not shown). Pretreatment with 2NTX-99 (10 and 40 μM) reduced TXB_2 synthesis and increased PGI_2 formation significantly (Fig. 3B).

2. 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/μl) stimulated with a sub-maximal aggregating concentration of AA (1.5-3 μM). 2NTX-99 inhibited platelet aggregation concentration-dependently (IC_{50} 9.83±1.1 μM); its potency was increased in the presence of aortic rings, (IC_{50} 1.45±0.15 μM) (Figure 4A). 2NTX-99 inhibited TXB_2 formation either in the absence or presence of aortic rings (-71 and -80% respectively, at 10 μM); conversely, the compound stimulated 6-keto-$PGF_{1\alpha}$ formation in a concentration-dependent way only when vascular rings were present (Fig. 4B).

2NTX-99 (20-40 μM) also prevented the aggregation induced by threshold U46619 concentrations (1-4 μM) in rabbit PRP (-74±6.7% and 96.4±2.4% respectively, n=8) thus sharing the behavior of picotamide (Gresele et al., 1989).

2NTX-99 (20-40 μM) inhibited (-48.2±10% and -79.2±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 μM) were ineffective. In this experimental setting, ozagrel (40 μM) inhibited TX formation by approx. 80% whereas equimolar concentrations of 2NTX-99 or 2NTX-101 reduced TX formation by 40% (data not shown).

3. Binding of 2NTX-99 to human TPα and total IPs determination.

Mixed type curves of [^{3}H]-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 K_d = 3.48 ± 36 %CV; U46619 K_i = 64.2 ±

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

4. Animal Studies

4.1 **Effect of 2NTX-99 on systemic blood pressure and bleeding time in the rat.**

Administration of 2NTX-99 to anesthetized rats (25 μg/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 mm Hg to 109.3±1.3 mm Hg (n=3); the decrease in blood pressure peaked 3-4 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.

4.2 Determination of 2NTX-99 in rat plasma and inhibition of thromboxane A_2 synthesis

Intravenous administration of 2NTX-99 to rats (25 mg/kg for 30 minutes) suppressed TXA_2 production in clotting blood (94% inhibition when compared with vehicle treated animals, n=5); plasma levels (n=2) were 25.67 and 27.44 μg/ml (mean concentration 61 μM).

After oral administration (250 mg/kg) plasma levels of 2NTX-99 and TXA_2-synthesis inhibition were assessed up to 16 hours post dosing, 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 hours; inhibition of TXA_2

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production peaked at 90 minutes (-71%) and plasma levels (n=2) were 9.29 and 6.95 µg/ml (mean concentration 19 µM). (Fig. 6A, 6B).

An AUC $_{0\text{-}16\ hours}$ of 149.5 h•µg/ml was calculated for the 250 mg/kg dose, with a C_{max} value of 31.8±8.2 µg/ml and an estimated half-life of 6 h, even if the spread (from 0.29 to 9.69 µg/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. AUC $_{0\text{-}3h}$ were respectively calculated as 66 and 18.3 h•µg/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 TXA_2 synthesis was observed (r^2 = 0.72, EC_{50} 11.8 µM).

4.1 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 to 6% of the concentrations of 2NTX-99

Incubation of 2NTX-99 (40 µM) 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±0.26 % (mean±S.D.) of the parent compound, whereas 2NTX-101 was not detected in incubation of 2NTX-99 in plasma alone.

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Discussion

In the present paper we describe the pharmacology of 2NTX-99, an orally active, innovative chemical entity with plural actions on TXA_2 synthesis, PGI_2 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 analogue of picotamide, a dual thromboxane synthase inhibitor/TXA_2 receptor antagonist (Gresele et al., 1989). 2NTX-99, while retaining the thromboxane synthase inhibitory activity did not bind to the TPα nor did affect TPα-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 (EC_{50} 7.9 μM) markedly lower than GTN (EC_{50} 0.03 μM), but higher than isosorbide 5-mononitrate (ISMN) (EC_{50} 15.9 μM). 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 (EC_{50} 17 μM), while 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 approx. 7-fold in the presence of aortic rings with intact endothelium. 2NXT-99 inhibited TXA_2 synthesis, either in the absence or presence of aortic rings (70-80% at 10 μM), and in the

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latter situation increased PGI_2 synthesis (about 3-fold at 10 μM), 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 A_2 receptor antagonist properties of 2NTX-99 we performed classical competition experiments in a recombinant system expressing the TPα receptor (it would have been the same utilizing the TPβ, as the two isoforms are identical for the first 328 residues, and differ only in C terminal tail of no relevance for ligand-binding). Receptor was labeled with the competitive antagonist [^{3}H]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 IPs 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 μg/ml) and reduced partially (-40%) TX formation. Ozagrel did not affect collagen-induced platelet aggregation in spite of a marked reduction of TX formation (80%), in line with the existence of a non-linear 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 a 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*

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vivo antiaggregatory effect in patients (De Caterina et al., 1990), comparable to those of ISDN (De Caterina et al., 1984). The action 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 three hours, 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 in order to equal its rate of release (7.9 and 15.9 µM respectively, vs. 30 nM of 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 generated 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

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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 hours. Importantly, an excellent correlation ($r^2 = 0.724$) between the plasma levels of 2NTX-99 and the inhibition of TXA_2 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 PGI_2 formation and suppress TXA_2 synthase activity; NO also activates guanylyl cyclase to increase cGMP and acts synergistically with PGI_2 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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Footnotes

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Legends for Figures

Figure 1.

Chemical structures of Picotamide, 2NTX-99 and 2NTX-101

Figure 2.

Panel A: dose-dependent relaxation by 2NTX-99, 2NTX-101, ISMN and GTN, on norepinephrine induced rabbit aortic rings contraction.

Panel B: dose-dependent relaxation induced by 2NTX-99 and ISMN on U46619 induced rabbit aortic rings contraction. Effect of the selective guanylate cyclase inhibitor ODQ.

Data represent mean values, bars represent the mean standard error of n replicates.

Figure 3.

Panel A: effect of 2NTX-99 on the contraction of isolated whole rabbit aorta, incubated with homologous platelets ($450x10^6$ /ml) and challenged with arachidonic acid (AA).

Panel B: effect of 2NTX-99 on TXA_2 and PGI_2 synthesis, in whole rabbit aorta incubated with homologous platelets and challenged with AA. TXA_2 and PGI_2 were detected as their stable metabolite, TXB_2 and 6keto-$PGF_{1\alpha}$ respectively.

Data represent mean values, bars represent the mean standard error of n replicates

Figure 4.

Panel A: dose-dependent inhibition by 2NTX-99 on arachidonic acid-induced aggregation (AA, 1.5-3 μM) of rabbit platelets ($450x10^6$/ml) in the presence or absence of an aortic ring.

Panel B: dose-dependent effect of 2NTX-99 on TXA_2 and PGI_2 synthesis, in rabbit platelets challenged by AA, in the absence or presence of an aortic ring. TXA_2 and PGI_2 were detected as their stable metabolite TXB_2 and 6keto-$PGF_{1\alpha}$ respectively.

Data represent mean values, bars represent the mean standard error of n replicates.

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Figure 5.

Panel A: binding of [^{3}H]SQ29,548 in HEK293 transiently expressing human TPα receptor.

For the sake of clarity, only curves from a representative experiment are shown.

Panel B: total inositol (IPs) formation in HEK293 transiently expressing human TPα 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 represent the mean standard error of n replicates.

Figure 6.

Time course of inhibition of rat serum TXB_2 (Panel A) and 2NTX-99 plasma levels (Panel 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 represent the mean standard error of n replicates.

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Figure 1.

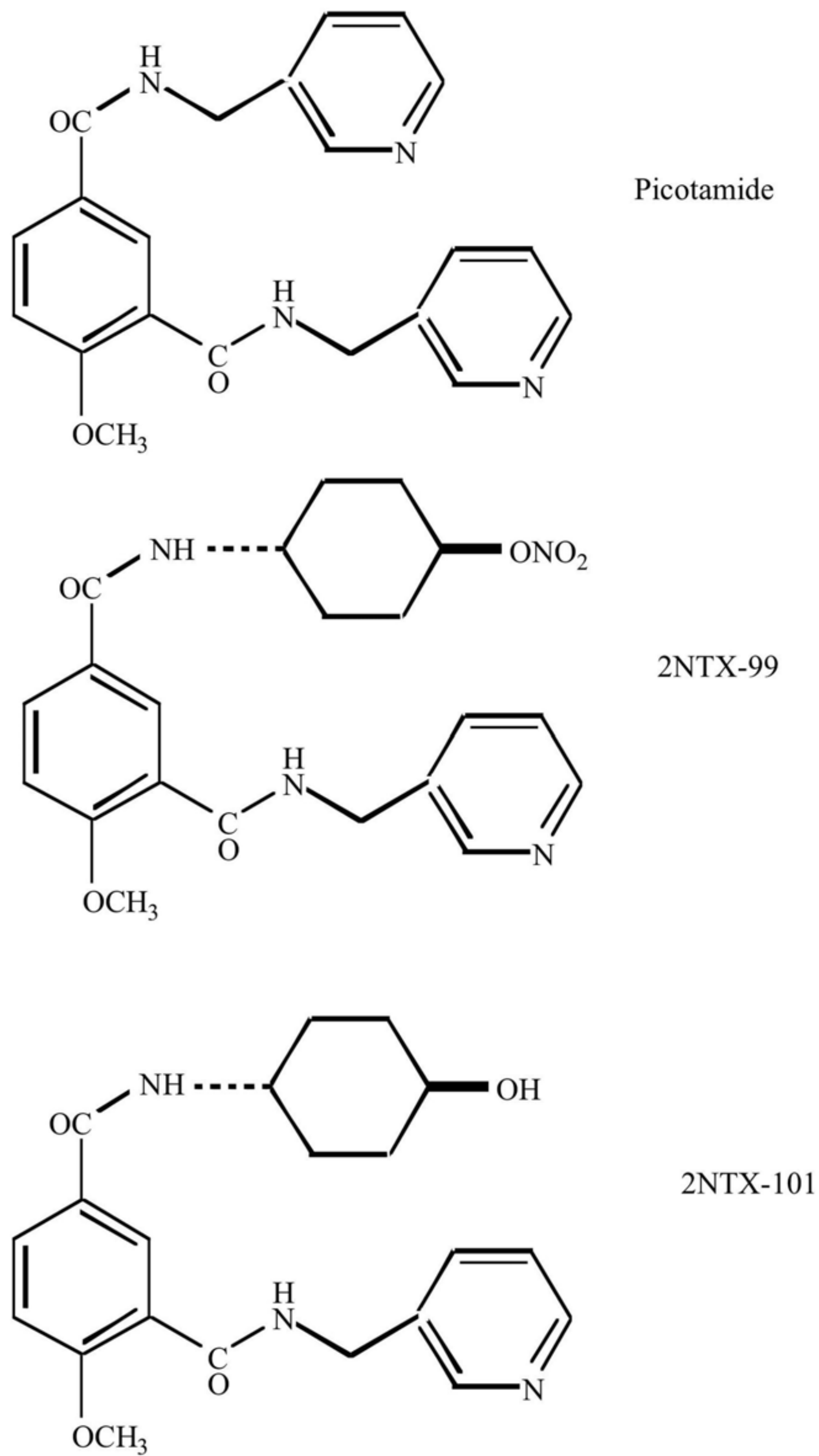

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Figure 2.

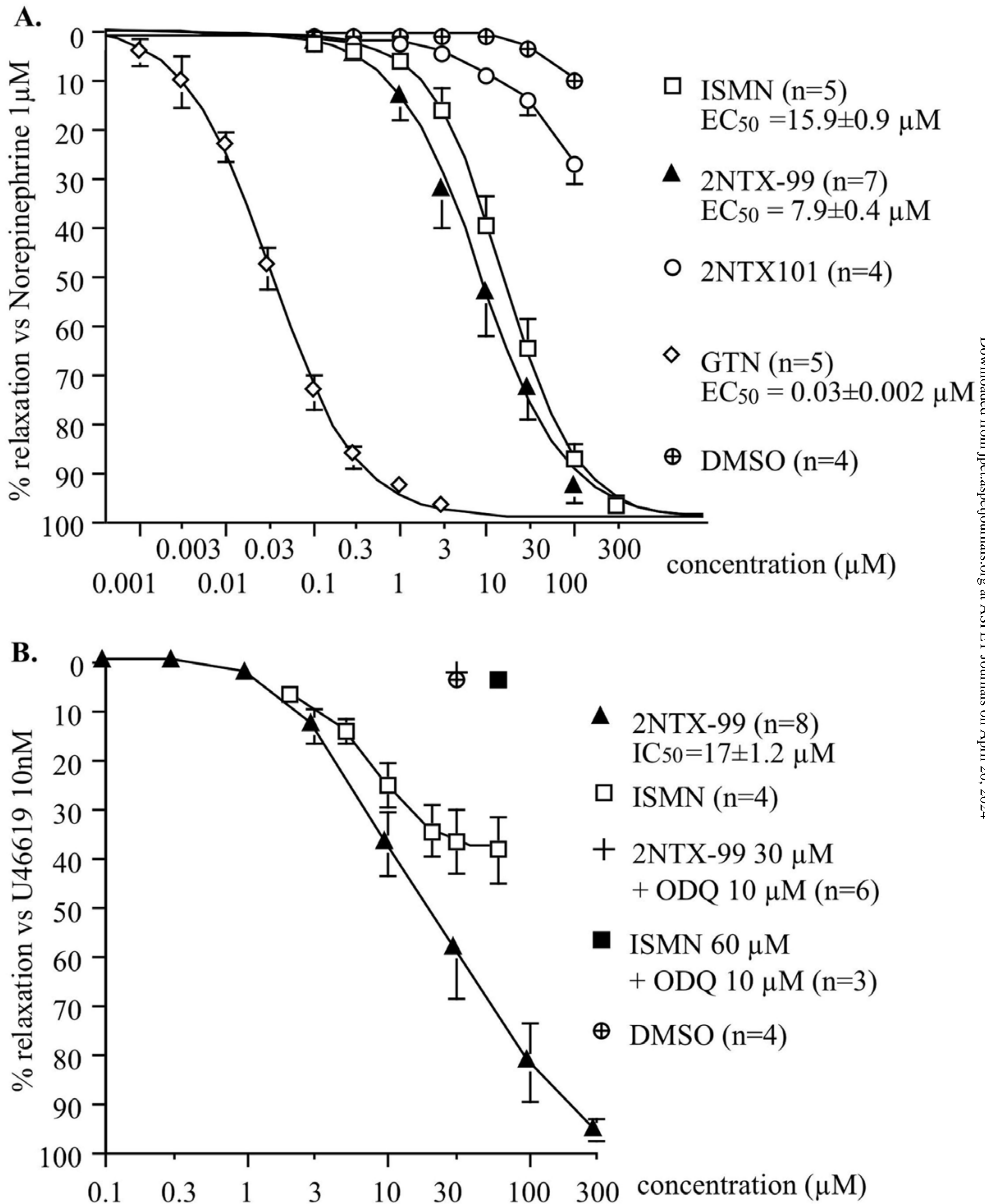

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Figure 3.

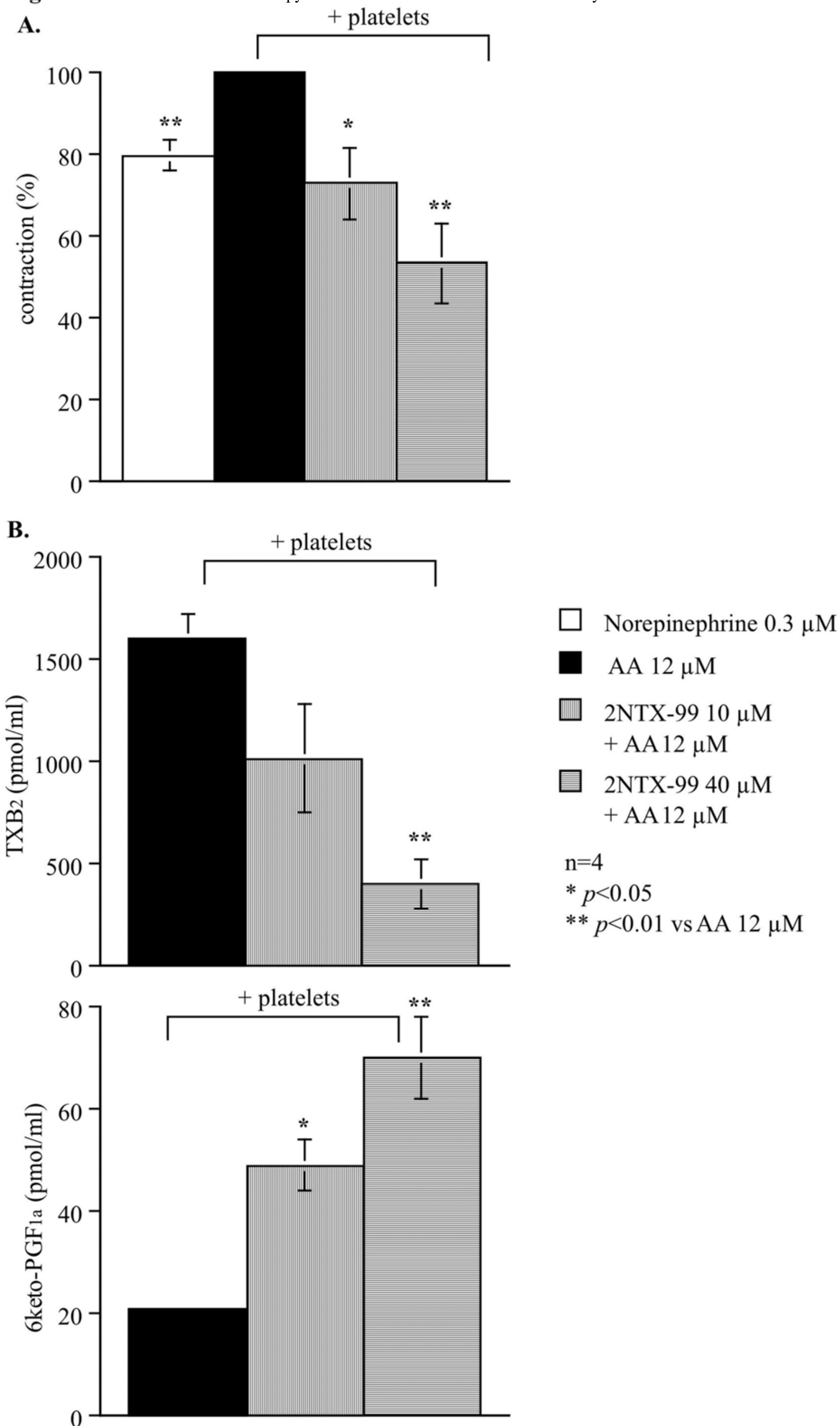

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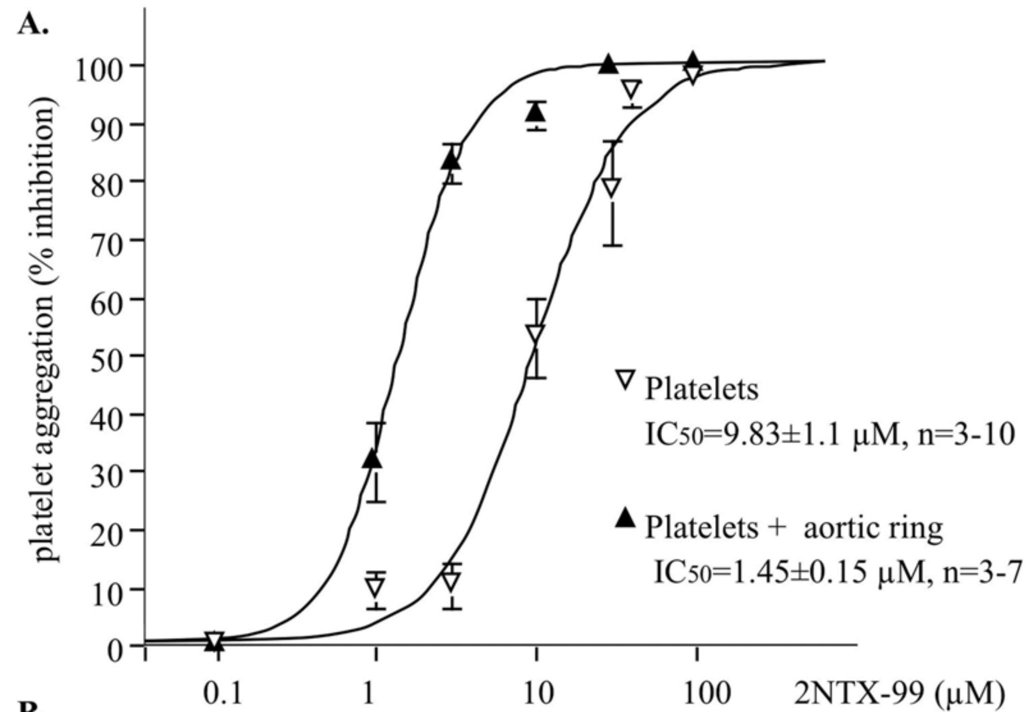
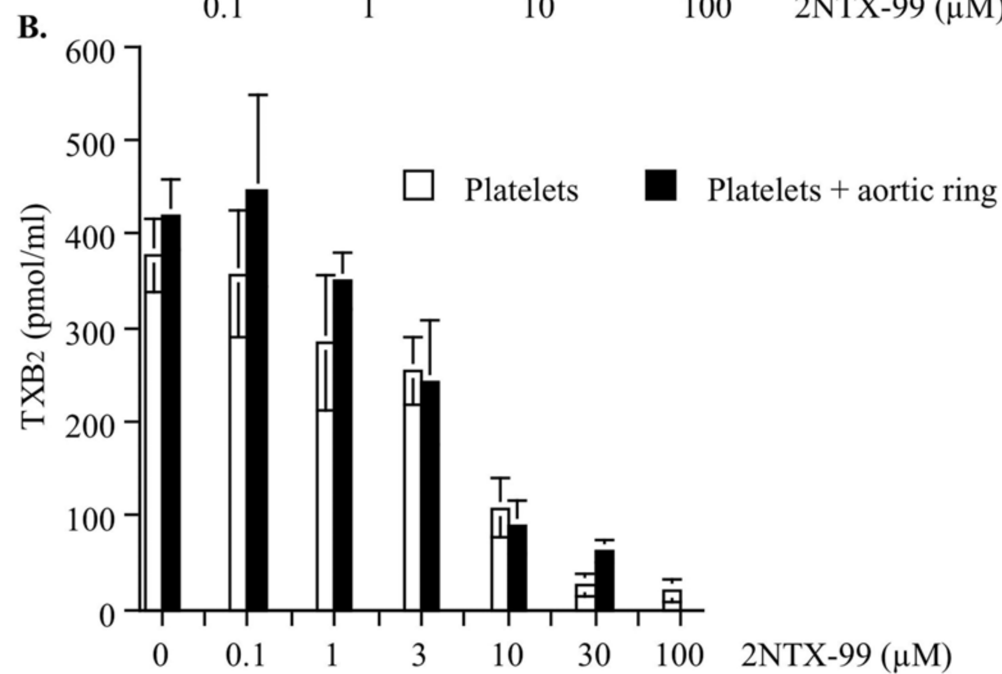
Figure 4





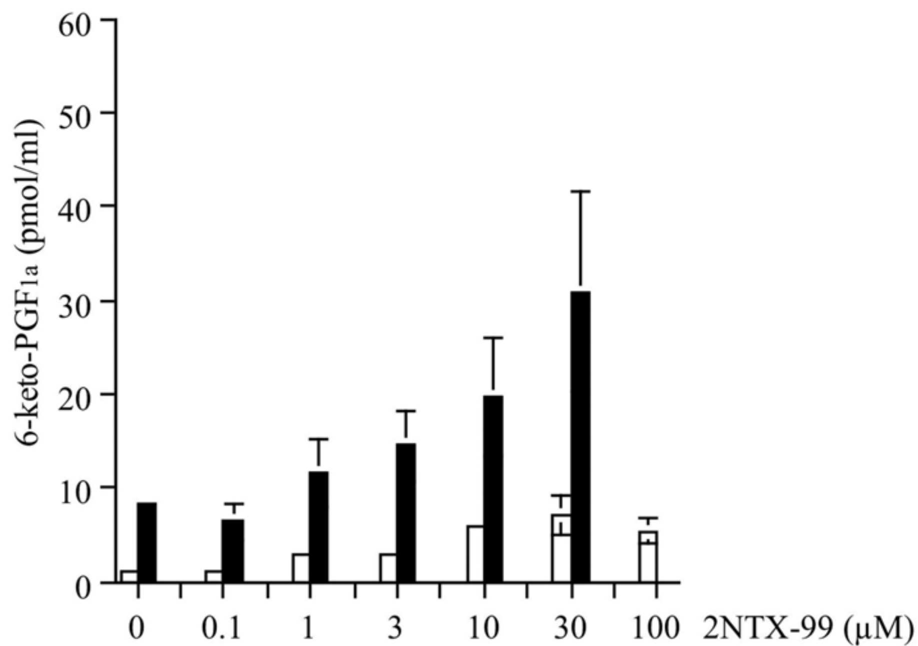


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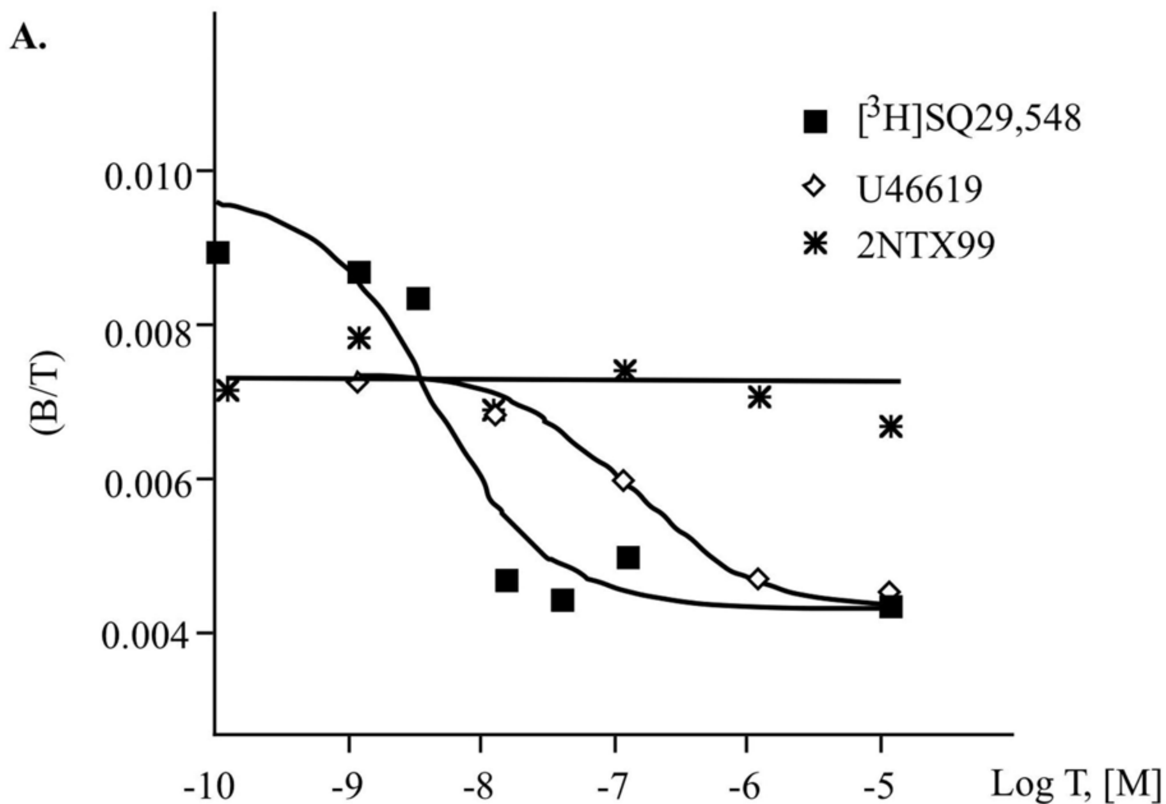
Figure 5.

A.



B.

2000
1500
1000
500
0

** $p < 0.01$ vs U46619 (n=3)

dpm (myo-[2-^{3}H]inositol)

**
**

Basal
— 1 μM U46619 →
+ 1 μM SQ29,548
+ 1 μM
+ 10 μM
+ 40 μM
2NTX99

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JPET Fast Forward. Published on January 6, 2006 as DOI: 10.1124/jpet.105.097170
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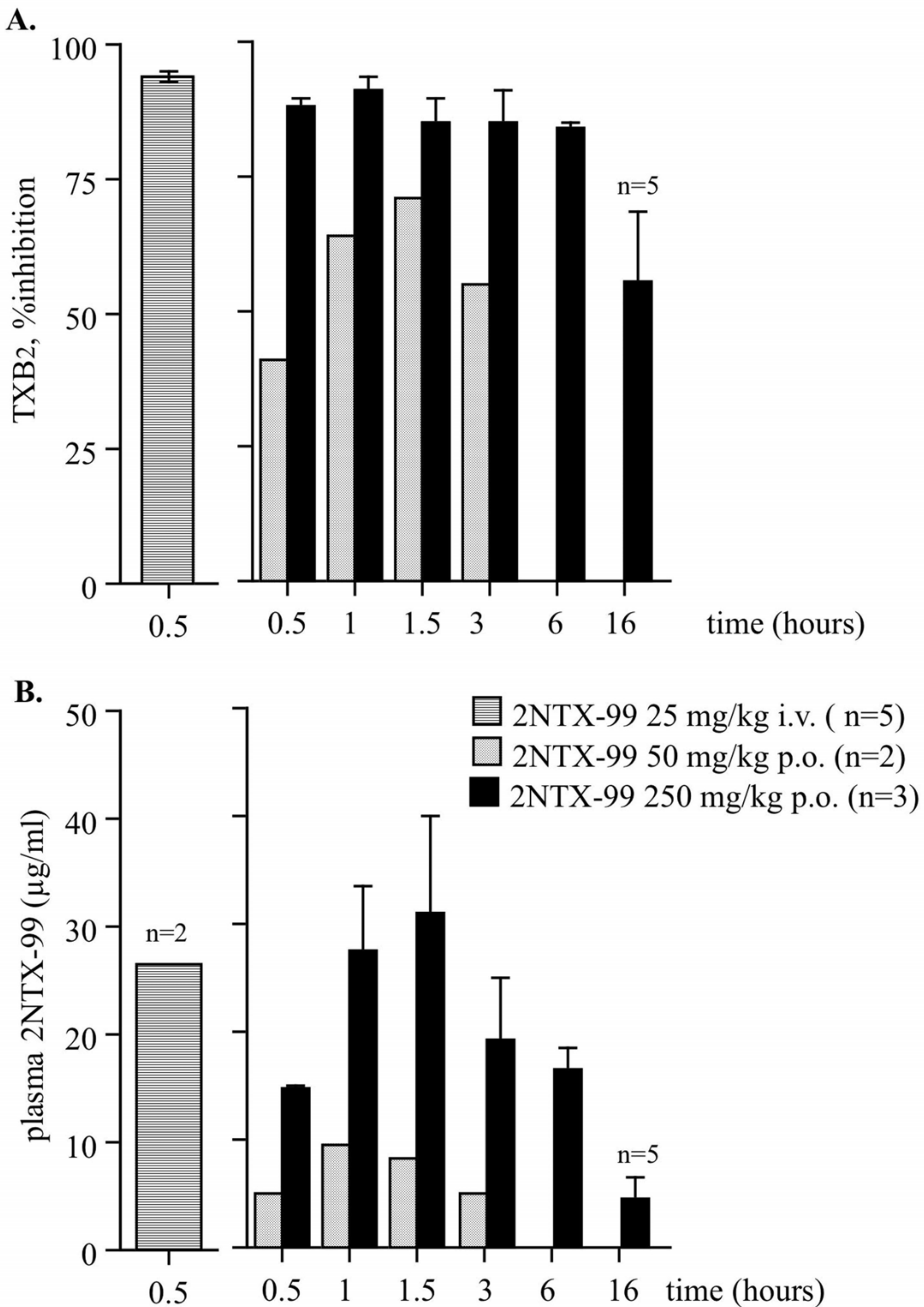
Figure 6.



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