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ABSTRACT

A number of new nitric oxide (NO)-precursors were synthesized by grafting nitrate-containing moieties on the structures of the benzyloxy isonipecotanilide derivatives **1** and **2** already reported as moderately potent antiplatelet agents. Various nitrooxy (ONO_2)-alkyl side chains were covalently linked to the piperidine nitrogen of the parent compounds through carbamate and amide linkage, and the synthesis of a benzyl nitrate analog (**15**) of compound **1** was also achieved. The in vitro vasodilatory activities, as well as platelet anti-aggregatory effects, of the newly synthesized organic nitrates were assessed. The (ONO_2)methyl carbamate-based derivative **5a** and the benzyl nitrate analog **15**, which on the other hand retain activity as inhibitors of ADP-induced platelet aggregation, exhibited strong NO-mediated vasodilatory effects on pre-contracted rat aorta strips, with EC_{50} values in the low nanomolar range (13 and 29 nM, respectively). Experiments carried out with the selectively inhibited soluble guanylate cyclase (*s*GC), which is the key enzyme of the NO-mediated pathway leading to vascular smooth muscle relaxation, confirmed the involvement of NO in the observed vasodilation. The nitrate derivatives proved to be stable in acidic aqueous solution and at pH 7.4. In human serum, unlike **5a**, which showed not to undergo enzyme-catalyzed decomposition, the other tested (ONO_2)-alkyl carbamate-based compounds (**5b** and **5e**) and benzyl nitrate **15** underwent a faster degradation. However, their decomposition rates in serum were quite slow ($t_{½}$ > 2.6 h), which suggests that nitrate moiety is poorly metabolized in blood plasma and that much of the in vitro anti-platelet activity has to be attributed to the intact (ONO_2)-containing molecules.

Keywords: Isonipecotamides; Nitrooxy alkyl carbamate derivatives; Nitric oxide-donors; Vasodilation; Antiplatelet activity.

Abbreviations: AChE, acetylcholinesterase; ADP, adenosine 5'-diphosphate; BuChE, butyrylcholinesterase; cGMP, cyclic guanosine monophosphate; CHF, congestive heart failure; CINOD, cyclooxygenase-inhibiting nitric oxide donator; GSH, glutathione; GST, glutathione *S*-transferase; GTN, glyceryl trinitrate; ISDN, isosorbide dinitrate; MLC, myosin light chain; MLCK, MLC kinase; NO, nitric oxide; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinaxolin-1-one; PON1, paraoxonase; PRP, platelet rich plasma; RP-HPLC, reversed phase-high performance liquid chromatography; *s*GC, soluble guanylated cyclase.

1. Introduction

The organic nitrates are known for over a century as coronary artery medications, since the use of glyceryl trinitrate (GTN) as anti-anginal drug (Münzel et al., 2014). Administered through transdermal and sublingual routes, GTN is an essential medication in angina, acute myocardial infarction, severe hypertension, and coronary artery spasm. Isosorbide dinitrate (ISDN, Fig. 1) is another long-acting nitrate reported in the World Health Organization's List of Essential Medicines (WHO, 2013), used for heart-related chest pain and congestive heart failure (CHF) as adjunct to other drugs.

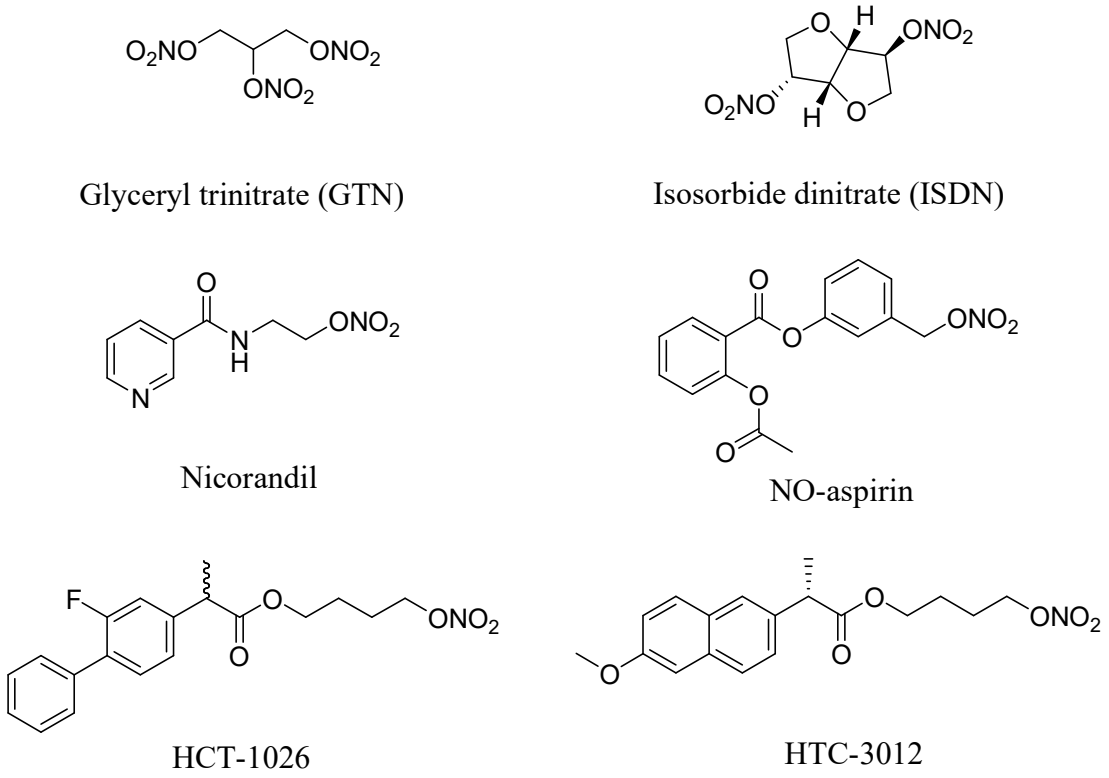


Fig. 1. Organic nitrates used in clinics and some representative experimental NO-donor drugs.

GTN and ISDN, which undergo denitration in vivo with production of the active metabolite nitric oxide (NO), act primarily via vascular smooth muscle relaxation, with decrease of cardiac output and

improvement of myocardial oxygen supply-to-demand ratio without affecting the heart's contractions.

The mechanism by which nitrovasodilators liberate NO in the body has not been completely elucidated. Nonenzymatic pathways involving endogenous sulfhydryl-containing molecules (Harrison, 2005) and several enzymes, such as the cytosolic glutathione *S*-tranferase (GST), xanthine oxidoreductase (XO), the mitochondrial aldehyde dehydrogenase, or the microsomal cytochrome P450 (CYP), have been proposed as mediators of bioactivation of organic nitrates (Chen et al., 2002; DiFabio et al., 2003; Keen et al., 1976; Kollau et al., 2005; Kurz et al., 1993; McDonald and Bennett, 1990; Schroder, 1992; Servent et al., 1989; Taylor et al., 1989). The released NO activates the soluble guanylate cyclase (*s*GC) (Follmann et al., 2013), thereby increasing the formation of cyclic guanosine monophoshate (cGMP), which, in turn, activates the myosin light chain kinase (MLCK), the enzyme phosphorylating MLC in the presence of ATP, ultimately preventing the phosphorylation of myosin and resulting in vascular muscle relaxation (Lucas et al., 2000; Murad, 2006; Sogo et al., 2000).

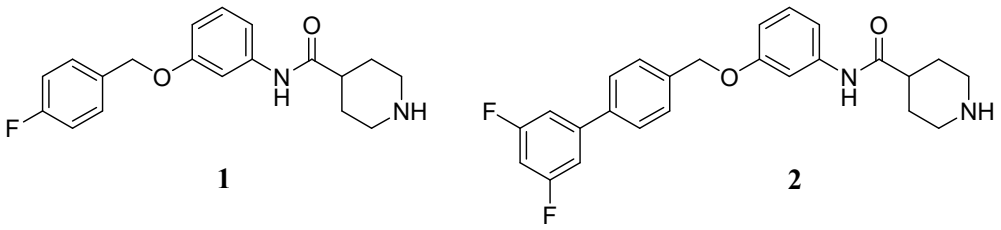
Endogenous NO, generated from L-arginine by the nitric oxide synthase (NOS) enzymes, has several different physiological actions targeted at kidney, reproductive apparatus, immunity system, inflammation, and neurotransmission (Gasco et al., 2005; Scatena et al., 2010). In the cardiovascular system NO predominates in large conduits, which supports its primarily anti-atherothrombotic effects (Miller et al., 2008, 2000; Miller and Megson, 2007; Schade et al., 2010). NO contributes to control the vascular endothelium smooth muscle cells tone and platelets' adhesion and aggregation (Miller and Megson, 2007; Moncada et al., 1991; Murad, 2006; Scatena et al., 2010; Schade et al., 2010).

Besides GTN and ISDN, nicorandil (Fig. 1) has been marketed in several countries as a vasodilatory medication for the treatment of angina pectoris and CHF. As a hybrid between organic nitrates and K^+-ATP channel agonists, it acts through dual mechanism of action, combining the vasodilatory property of both nitrates and nicotinamide with its ability to increase K^+ conductance (Edwards and Weston, 1990; Horinaka, 2011).

In the last decades a lot of hybrid nitrates as in vivo NO-donors have been studied for their potential use in the treatment of a variety of diseases, including pain and inflammation, thrombosis and restenosis, neurodegenerative diseases, cancer, liver disease, impotence, bronchial asthma and osteoporosis (Keeble and Moore, 2002). Since some disagreement about whether nitrates really generate NO at all, some authors prefer to use the term NO-mimetics (Thatcher et al., 2005). However, among the various pharmacologically relevant families of nitrate-containing agents (Fig. 1), NO-aspirin showed pharmacological effects in cardiovascular, cancer and inflammation models, and when tested in clinical trials showed little or no gastric toxicity (Cena et al., 2003; Keeble and Moore, 2002), due to gastro-protective effects of NO (Lazzarato et al., 2009). A new class of cyclooxygenase-inhibiting nitric oxide donors (CINODs) has been developed with the aim of achieving greater safety than the existing non-steroidal anti-inflammatory drug (NSAIDs) (Boschi et al., 2010, 2009). Two promising CINODs are HCT-1026 and HTC-3012 (Fig. 1), i.e., 4-(ONO_2)butyl esters of flurbiprofen and naproxen, respectively. HCT-1026 has been under study for its therapeutic use in a variety of conditions, including neurodegeneration and inflammation (Keeble and Moore, 2002; Scatena et al., 2005; Gasparini et al., 2005; Prosperi et al., 2004; Wenk et al., 2004, 2002; Ronchetti et al., 2009; Idris et al., 2004). HTC-3012, as single (*S*)-enantiomer, has been tested in clinical trials for the treatment of osteoarthritis (Geusens, 2009; Zhang et al., 2011). Other typical examples of NO-donor hybrids of existing drugs have been reported, which include ACE-inhibitors, statins, calcium antagonists, and phosphodiesterase inhibitors (Martelli et al., 2006; Napoli and Ignarro, 2003; Serafim et al., 2012).

Some years ago, we have reported a number of moderately potent isonipecotamide-based inhibitors of adenosine 5'-diphosphate (ADP)-induced human platelet aggregation (de Candia et al., 2003). Among them, *N*-(3-(4-fluorobenzyloxy)phenyl)piperidine-4-carboxamide **1** (Fig. 2), with half maximal inhibitory concentration (IC_{50}) of 68 μM, and the *N*-(3-[(3',5'-difluoro-1,1'-biphenyl-4-yl)methoxy]phenyl) analog **2** (IC_{50} = 27 μM), which proved to be an antiplatelet agent about two-fold more potent than **1** (de Candia et al., 2009), and a potent factor Xa (fXa)-selective inhibitor (K_i = 130

nM) as well, were chosen for further optimization through hybridization with the organic nitrate moiety, the first aim being to possibly strengthen the in vivo antiplatelet activity of the parent compounds **1** and **2**, conferring to them additional NO-mediated vasorelaxing properties.



1 **2**

Fig. 2. Fluorinated benzyloxyphenyl piperidine-4-carboxamide derivatives endowed with anti-thrombotic properties (de Candia et al., 2009). Both compounds proved to inhibit ADP-induced platelet aggregation (IC_{50} equals 68 and 27 μM for **1** and **2**, respectively), whereas **2** showed additional nanomolar inhibition potency against blood coagulation factor Xa (K_i = 135 nM).

In this work, we grafted nitrate moieties on the structures of antiplatelet compounds **1** and **2**, by covalently linking various nitrooxy (ONO_2)-alkyl side chains to the piperidine nitrogen via carbamate and amide linkages. A benzyl nitrate analog of compound **1** was also synthesized. The in vitro vasodilatory and antiplatelet activities of the newly synthesized compounds were evaluated, and the stability in aqueous solutions and human serum of the most potent compounds was assessed.

2. Materials and methods

Triethylamine (TEA), dichloromethane (DCM), chloroform, ethanol (EtOH), methanol (MeOH), acetone (Me_2CO), ethyl acetate (EtOAc), *n*-hexane (Hex), acetonitrile (ACN), *N*,*N*-dimethylformamide (DMF), tetrahydrofuran (THF), trifluoroacetic acid (TFA), sodium sulphate (Na_2SO_4), sodium bicarbonate ($NaHCO_3$), potassium carbonate (K_2CO_3), silver nitrate ($AgNO_3$),

deuterated dimethyl sulfoxide (DMSO-d_6) and deuterated chloroform ($CDCl_3$) and all other chemicals and reagents were purchased from Sigma-Aldrich (Milan, Italy). Unless otherwise stated, chemicals and reagents were of analytical grade and were used without further purification.

Melting points were determined by using the capillary method on a Stuart Scientific SMP3 electrothermal apparatus and are not corrected. IR spectra were recorded using KBr disks on a Perkin-Elmer Spectrum One FT-IR spectrophotometer (Perkin-Elmer Ltd., Buckinghamshire, UK), and the most significant absorption bands expressed in cm^{-1} are listed. ^1H NMR spectra were recorded at 300MHz on a Varian Mercury 300 instrument. Chemical shift values are expressed in δ and the coupling constants J in Hz. Abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet, dd, doublet of doublets; m, multiplet; br, broad. Signals due to NH and OH protons were located by deuterium exchange with D_2O. Mass spectra were recorded on Agilent GC-MS 689-973. Chromatographic separations were performed on silica gel 60 for column chromatography (Merck 70-230 mesh, or alternatively 15-40 mesh for flash chromatography). Purity (≥ 95%) of the pharmacologically tested compounds was established by HPLC and elemental analysis. Elemental analyses (C, H, N) were performed on Euro EA3000 analyzer (Eurovector, Milan, Italy) in the Analytical Laboratory Service of the Dipartimento di Farmacia - Scienze del Farmaco, University of Bari, and the results agreed to within ± 0.40% of the theoretical values.

2.1 Synthesis

*2.1.1. General procedure for preparation of chloroalkyl carbamate derivatives **3a**, **3b** and **3e***

A solution of the appropriate chloroalkyl chloroformate (1.50 mmol) in dry DCM (3 ml) was added dropwise to a stirred solution of 1.37 mmol of compound **1**·HCl (de Candia et al., 2009) and TEA (2.74 mmol) in dry DCM (10 ml). The mixture was stirred at room temperature overnight. Then, the precipitate was filtered, and the filtrate was evaporated in vacuum. The residue was dissolved in 50 ml of chloroform, and the solution was sequentially washed with 3×20 ml of saturated aqueous $NaHCO_3$, 1N HCl and brine, dried over anhydrous Na_2SO_4, filtered and evaporated in vacuum. The

crude product was purified by crystallization or by silica gel flash chromatography to afford the desired compound.

*2.1.1.1 Chloromethyl 4-[({3-[(4-fluorobenzyl)oxy]phenyl}amino)carbonyl]piperidine-1-carboxylate, **3a**.* Compound **3a** was prepared according to the general procedure from compound **1**·HCl (500 mg, 1.37 mmol) and chloromethyl chloroformate (0.13 ml, 1.50 mmol). The crude product was purified by crystallization from EtOH in 54% yield (310 mg) as a pale brown solid; mp 137-138 °C. IR (cm^{-1}): 3264, 1662, 1612, 1445, 1208, 1088, 838, 699. ^1H NMR ($CDCl_3$) δ 7.45 (s, br, 1H), 7.41-7.37 (m, 2H), 7.23 (d, J = 8.0, 1H), 7.18 (s, 1H), 7.06 (t, J = 9.0, 2H), 6.91 (d, J = 7.5, 1H), 6.72 (dd, J = 8.0 and 2.0, 1H), 5.80 (d, J = 13, 2H), 5.01 (s, 2H), 4.28 (d, J = 13, 1H), 4.18 (d, J = 13, 1H), 2.99 (d, J = 13, 1H), 2.90 (d, J = 13, 1H), 2.50-2.35 (m, 1H), 2.00-1.90 (m, 2H), 1.85-1.70 (m, 2H).

*2.1.1.2. 2-Chloroethyl 4-[({3-[(4-fluorobenzyl)oxy]phenyl}amino)carbonyl]piperidine-1-carboxylate, **3b**.* Compound **3b** was prepared according to the general procedure from compound **1**·HCl (500 mg, 1.37 mmol) and 2-chloroethyl chloroformate (0.15 ml, 1.56 mmol). The crude product was purified by crystallization from EtOH in 78% yield (466 mg) as a brown solid ; mp 132-134 °C. IR (cm^{-1}): 3230, 1660, 1605, 1445, 1205, 1078, 838, 696. ^1H NMR ($CDCl_3$) δ 7.46 (t, J = 2.0, 1H), 7.43-7.36 (m, 2H), 7.25-7.18 (m, 2H), 7.10-7.02 (m, 2H), 6.92 (d, J = 8.0, 1H), 6.72 (dd, J = 8.0 and 1.5, 1H), 5.01 (s, 2H), 4.34 (t, J = 6, 2H), 4.30-4.15 (m, 2H), 3.69 (t, J = 6.0, 2H), 3.00-2.85 (m, 2H), 2.45-3.35 (m, 1H), 2.00-1.85 (m, 2H), 1.75-1.20 (m, 2H).

*2.1.1.3. Chloromethyl 4-[({3-[(3',5'-difluoro-1,1'-biphenyl-4-yl)methoxy]phenyl}amino)carbonyl] piperidine-1-carboxylate, **3e**.* Compound **3e** was prepared was prepared according to the general procedure from compound **2**·HCl (175 mg, 0.38 mmol) and chloromethyl chloroformate (0.1 ml, 1.14 mmol). The crude product was purified by silica gel flash chromatography (eluent: Hex/EtOAc, 7:3

v/v) in 89% yield (175 mg) as an orange oil. IR (cm^{-1}): 3333, 2928, 1728, 1660, 1610, 1442, 1206, 1119, 1086, 989, 847, 783, 689. ^{1}H NMR (DMSO-d_6) δ 9.93 (s, 1H), 7.75 (d, J = 8.0, 2H), 7.52 (d, J = 8.0, 2H), 7.50-7.40 (m, 3H), 7.30-7.05 (m, 3H), 6.68 (dd, J = 8.0 and 1.5, 1H), 5.88 (d, J = 3.5, 1H), 5.11 (s, 2H), 4.10-3.90 (m, 2H), 2.95 (t, J = 12, 2H), 2.89 (t, J = 12, 2H), 2.60-2.45 (m, 1H), 1.90-1.70 (m, 2H), 1.60-1.40 (m, 2H).

*2.1.2. General procedure for preparation of halogenoalkyl carbamate derivatives **3c** and **3d***

4-Nitrophenyl chloroformate (3.60 mmol) was added portionwise to a 0 °C cooled solution of the appropriate halogenoalkyl alcohol (3.60 mmol) and TEA (5.40 mmol) in dry THF (10 ml), and the mixture was stirred 3 h at 0 °C. Then, compound **1**·HCl (3.60 mmol) and TEA (3.60 mmol) were added, and the mixture was stirred at room temperature overnight. The formed precipitate was filtered, and the filtrate evaporated in vacuum. The residue was dissolved in 50 ml of chloroform, and the solution was sequentially washed with 3×20 ml of saturated aqueous $NaHCO_3$, 1N HCl and brine, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The crude oil product was purified by silica gel flash chromatography to afford the desired compound.

*2.1.2.1. 3-Bromopropyl 4-[({3-[(4-fluorobenzyl)oxy]phenyl}amino)carbonyl]piperidine-1-carboxylate, **3c***. Compound **3c** was prepared according to the general procedure from compound **1**·HCl (1.31 g, 3.60 mmol) and 3-bromo-1-propanol (0.32 ml, 3.60 mmol). The crude product was purified by silica gel flash chromatography (eluent: Hex/EtOAc, 4:6 v/v) in 50% yield (0.890 g) as a brown oil. IR (cm^{-1}): 3230, 1656, 1600, 1448, 1210, 1068, 840, 700. ^{1}H NMR ($CDCl_3$) δ 7.48 (s, 1H), 7.46-7.36 (m, 2H), 7.23-7.17 (m, 2H), 7.06 (t, J = 9.0, 2H), 6.91 (d, J = 7.0, 1H), 6.72 (dd, J = 8.0 and 2.5, 1H), 5.02 (s, 2H), 4.23 (t, J = 6, 2H), 4.30-4.20 (m, 2H), 3.47 (t, J = 6.5, 2H), 2.95-2.80 (m, 2H), 2.50-2.35 (m, 1H), 2.20 (t, J = 6.5, 2H), 2.00-1.87 (m, 2H), 1.85-1.70 (m, 2H).

2.1.2.2. 4-Chlorobutyl 4-[({3-[(4-fluorobenzyl)oxy]phenyl}amino)carbonyl]piperidine-1-carboxylate, ***3d***. Compound **3d** was prepared according to the general procedure from compound **1**·HCl (850 mg, 2.34 mmol) and 4-chloro-1-butanol (0.23 ml, 2.34 mmol). The crude product was purified by silica gel flash chromatography (eluent: Hex/EtOAc, 7:3 v/v) in 55% yield (570 mg) as a brown oil. IR (cm^{-1}): 3227, 1650, 1600, 1445, 1208, 1080, 837, 696. ^{1}H NMR ($CDCl_3$) δ 7.45 (s, 1H), 7.42-7.36 (m, 2H), 7.32-7.20 (m, 2H), 7.08 (dd, J = 8.5 and 2.0, 1H), 7.03 (d, J = 2.0, 1H), 6.91 (d, J = 7.0, 1H), 6.72 (d, J = 8.0, 1H), 5.01 (s, 2H), 4.40-4.00 (m, 4H), 3.80 (t, J = 6.0, 2H), 3.58 (t, J = 5.5, 2H), 2.95-2.85 (m, 2H), 2.55-2.45 (m, 1H), 1.98-1.70 (m, 6H).

2.1.3. General procedure for preparation of halogenoalkyl amide derivatives ***4a*** *and* ***4b***

A solution of the appropriate bromoacyl halide (2.10 mmol) in dry THF (2 ml) was added dropwise to a 0 °C cooled solution of compound **1**·HCl (1.37 mmol) and TEA (2.74 mmol) in dry THF (20 ml). The mixture was stirred at 0 °C for 1 h and at room temperature overnight. The precipitate was filtered, and the filtrate evaporated in vacuum. The residue was diluted with EtOAc (50 ml) and the organic phase was sequentially washed with 3×20 ml of saturated aqueous $NaHCO_3$, 1N HCl and brine, dried over anhydrous Na_2SO_4, filtered and evaporated in vacuum. The crude product was purified by silica gel flash chromatography to afford the desired compound.

2.1.3.1. 1-(Bromoacetyl)-N-{3-[(4-fluorobenzyl)oxy]phenyl}piperidine-4-carboxamide, ***4a***. Compound **4a** was prepared according to the general procedure from compound **1**·HCl (500 mg, 1.37 mmol) and bromoacetyl bromide (0.18 ml, 2.10 mmol). The crude product was purified by silica gel flash chromatography (eluent: Hex/EtOAc, 1:9 v/v) in 55% yield (340 mg) as a brown oil. IR (cm^{-1}): 3233, 1652, 1605, 1443, 1198, 1080, 838, 696. ^{1}H NMR ($CDCl_3$) δ 7.43 (t, J = 2.0, 1H), 7.45-7.32 (m, 2H), 7.25-7.18 (m, 2H), 7.08-7.00 (m, 2H), 6.90 (d, J = 8.0, 1H), 6.75 (dd, J = 8.0 and 1.5, 1H), 5.00 (s, 2H), 4.30-4.15 (m, 2H), 3.20 (t, J = 6.0, 2H), 3.00-2.85 (m, 2H), 2.45-3.35 (m, 1H), 2.00-1.85 (m, 2H), 1.75-1.20 (m, 2H).

2.1.3.2. 1-(Bromopropanoyl)-N-{3-[(4-fluorobenzyl)oxy]phenyl}piperidine-4-carboxamide, ***4b***. Compound **4b** was prepared according to the general procedure from compound **1**·HCl (500 mg, 1.37 mmol) and 3-bromopropionyl chloride (0.20 ml, 2.10 mmol) in DCM (15 ml). The crude product was purified by silica gel flash chromatography (eluent: Hex/EtOAc, 1:9 v/v) in 64% yield (400 mg) as a brown solid; mp 164-165 °C. IR (cm^{-1}): 3275, 1653, 1638, 1433, 1206, 1022, 838, 690. ^{1}H NMR (CDCl3) δ 7.57 (s, br, 1H), 7.44 (s, 1H), 7.41-7.35 (m, 2H), 7.20 (t, J = 8.0, 1H), 7.05 (t, J = 8.0, 2H), 6.93 (t, J = 8.0, 1H), 6.71 (dd, J = 8.0 and 2.0,1H), 5.00 (s, 2H), 4.60 (d, J = 13, 2H), 3.86 (d, J = 13, 2H), 3.64 (t, J = 7.0, 2H), 3.12 (d, J = 12, 1H), 2.92 (t, J = 7.0, 2H), 2.73 (t, J = 13, 1H), 2.55-2.45 (m, 1H), 2.00-1.90 (m, 2H), 1.85-1.65 (m, 2H).

2.1.4. General procedure for preparation of nitric ester derivatives ***5a-e*** *and* ***6a-b***

A suspension of the appropriate alkyl halide intermediates (1.0 mmol) and $AgNO_3$ (2.0 mmol) in dry ACN (10 ml) was heated at reflux until reaction completion (TLC monitoring). After cooling, the suspension was filtered on Celite, and pad was washed with ACN (50 ml). The combined filtrates were evaporated in vacuum and the residue was purified by silica gel flash chromatography and/or crystallization.

2.1.4.1. (Nitrooxy)methyl 4-[({3-[(4-fluorobenzyl)oxy]phenyl}amino)carbonyl]piperidine-1-carboxylate, ***5a***. Compound **5a** was prepared according to the general procedure from **3a** (400 mg, 0.95 mmol) and $AgNO_3$ (323 mg, 1.90 mmol). The crude product was purified by silica gel flash chromatography (eluent: Hex/EtOAc, 3:7 v/v) in 57% yield (240 mg) as a pale yellow solid, which was further purified by crystallization from EtOAc/Hex; mp 130-131 °C. IR (cm^{-1}): 3262, 1732, 1358, 1545, 1443, 1291, 1205, 1118, 951, 827. ^{1}H NMR (DMSO-d_6) δ 9.90 (s, 1H), 7.75 (d, J = 8.0, 2H), 7.52 (d, J = 8.0, 2H), 7.47-7.40 (m, 4H), 7.28-7.05 (m, 3H), 6.68 (d, J = 8.0, 1H), 6.07 (s, 2H), 5.11 (s, 2H), 3.98 (t, J = 13, 2H), 2.99-2.68 (m, 2H), 2.55-2.45 (m, 1H), 1.851.70 (m, 2H), 1.60-1.45 (m,

2H). MS (ESI) m/z 470 $[M + Na]^+$. Anal. calcd. for $C_{21}H_{22}FN_3O_7$: C, 56.37; H, 4.96; N, 9.39%; found: C, 56.55; H, 4.99; N, 9.43%.

2.1.4.2. 2-(Nitrooxy)ethyl 4-[({3-[(4-fluorobenzyl)oxy]phenyl}amino)carbonyl]piperidine-1-carboxylate, ***5b***. Compound **5b** was prepared according to the general procedure from **3b** (500 mg, 1.15 mmol) and $AgNO_3$ (390 mg, 2.30 mmol). The crude product was purified by crystallization from EtOAc in 75% yield (400 mg) as a brown solid; mp 89-91 °C. IR (cm^{-1}): 3430, 1635, 1442, 1384, 1280, 1204, 1040, 860. ^1H NMR (DMSO-d_6) δ 9.90 (s, 1H), 7.47 (t, J = 8.5, 2H), 7.39 (s, 1H), 7.25-7.00 (m, 4H), 6.66 (dd, J = 8.0 and 1.5, 1H), 5.02 (s, 2H), 4.76-4.70 (m, 2H), 4.32-4.25 (m, 2H), 4.05-3.90 (m, 2H), 2.95-2.75 (m, 2H), 2.55-2.40 (m, 1H), 1.85-1.70 (m, 2H), 1.60-1.40 (m, 2H). MS (ESI) m/z 484 $[M + Na]^+$. Anal. calcd. for $C_{22}H_{24}FN_3O_7 \times H_2O$: C, 55.11; H, 5.47; N, 8.76%; found: C, 55.41; H, 5.27; N, 8.74%.

2.1.4.3. 3-(Nitrooxy)propyl 4-[({3-[(4-fluorobenzyl)oxy]phenyl}amino)carbonyl]piperidine-1-carboxylate, ***5c***. Compound **5c** was prepared according to the general procedure from **3c** (845 mg, 1.71 mmol) and $AgNO_3$ (581 mg, 3.42 mmol). The crude product was purified by silica gel flash chromatography (eluent: Hex/EtOAc, 6:4 v/v) in 61% yield (500 mg) as a pale brown solid, which was further purified by crystallization from Hex/EtOAc; mp 91-93 °C. IR (cm^{-1}): 3432, 1702, 1622, 1436, 1279, 1207, 1037, 869. ^1H NMR (DMSO-d_6) δ 9.89 (s, 1H), 7.47 (t, J = 8.5, 2H), 7.38 (d, J = 2.0, 1H), 7.30-7.00 (m, 4H), 6.66 (dd, J = 7.5 and 1.5, 1H), 5.02 (s, 2H), 4.59 (t, J = 6.5, 2H), 4.07 (t, J = 6.5, 2H), 4.00 (t, J = 13, 2H), 2.90-2.75 (m, 2H), 2.55-2.45 (m, 1H), 1.99 (quintet, J = 6.5, 2H), 1.85-1.70 (m, 2H), 1.60-1.40 (m, 2H). MS (ESI) m/z 498 $[M + Na]^+$. Anal. calcd. for $C_{23}H_{26}FN_3O_7$: C, 58.10; H, 5.51; N, 8.84%; found: C, 58.34; H, 5.56; N, 8.58%.

2.1.4.4. 4-(Nitrooxy)butyl 4-[({3-[(4-fluorobenzyl)oxy]phenyl}amino)carbonyl]piperidine-1-carboxylate, ***5d***. Compound **5d** was synthesized according to the general procedure from **3d** (400 mg,

0.89 mmol) and $AgNO_3$ (303 mg, 1.78 mmol). The crude product was purified by silica gel flash chromatography (eluent: Hex/EtOAc, 7:3 v/v) in 71% yield (310 mg) as a brown solid, which was further purified by crystallization from Hex/EtOAc; mp 117-115 °C. IR (cm^{-1}): 3432, 2954, 1716, 1661, 1612, 1523, 1431, 1201, 1155, 1040, 865, 825, 781, 742, 689. ^1H NMR ($CDCl_3$) δ 7.45 (s, 1H), 7.42-7.36 (m, 2H), 7.24-7.15 (m, 2H), 7.11-7.00 (m, 2H), 6.92 (d, J = 7, 1H), 6.72 (dd, J = 8.0 and 2.5, 1H), 5.02 (s, 2H), 4.50-4.20 (m, 2H), 4.13 (t, J = 6.0, 2H), 3.58 (t, J = 6.0, 2H), 2.88 (d, J = 12, 1H), 2.84 (d, J = 12, 1H), 2.50-2.30 (m, 1H), 2.00-1.65 (m, 8H). MS (ESI) m/z 512 $[M + Na]^+$. Anal. calcd. for $C_{24}H_{28}FN_3O_7$: C, 58.89; H, 5.77; N, 8.58%; found: C, 59.02; H, 5.87; N, 8.58%.

2.1.4.5. (Nitrooxy)methyl 4-[({3-[(3',5'-difluoro-1,1'-biphenyl-4-yl)methoxy]phenyl}amino) carbonyl]piperidine-1-carboxylate, ***5e***. Compound **5e** was prepared according to the general procedure from **3e** (170 mg, 0.33 mmol) and $AgNO_3$ (70 mg, 0.39 mmol). The crude product was purified by silica gel flash chromatography (eluent: Hex/EtOAc, 7:3 v/v), in 65% yield (116 mg) as a pale yellow solid, which was further purified by crystallization from Hex/EtOAc; mp 130-131 °C. IR (cm^{-1}): 3200, 2930, 1732, 1658, 1598, 1545, 1443, 1384, 1291, 1205, 1118, 1086, 951, 827, 689. ^1H NMR (DMSO-d_6) δ 9.92 (s, 1H), 7.75 (d, J = 8.0, 2H), 7.52 (d, J = 8.0, 2H), 7.48-7.40 (m, 3H), 7.30-7.05 (m, 3H), 6.68 (dd, J = 8.0 and 1.5, 1H), 6.07 (s, 2H), 5.11 (s, 2H), 4.05-3.95 (m, 2H), 2.94 (t, J = 12, 1H), 2.87 (t, J = 12, 1H), 2.60-2.50 (m, 1H), 1.90-1.70 (m, 2H), 1.60-1.40 (m, 2H). Anal. calcd. for $C_{27}H_{25}F_2N_3O_7$: C, 59.89; H, 4.65; N, 7.76%; found: C, 60.02; H, 4.77; N, 7.90%.

2.1.4.6. 2-{4-[({3-[(4-Fluorobenzyl)oxy]phenyl}amino)carbonyl]piperidin-1-yl}-2-oxoethyl nitrate, ***6a***. Compound **6a** was prepared according to the general procedure from **4a** (230 mg, 0.5 mmol) and $AgNO_3$ (170 mg, 1.0 mmol). The crude product was purified by silica gel flash chromatography (eluent: MeOH/EtOAc, 5:95 v/v) in 56% yield (120 mg) as a white solid, which was further purified by crystallization from EtOAc; mp 135-136 °C. IR (cm^{-1}): 3306, 1651, 1552, 1446, 1376, 1291, 1207, 1037, 955, 856, 798, 785, 689. ^1H NMR (DMSO-d_6) δ 9.92 (s, 1H), 7.50-7.43 (m, 2H), 7.39 (s, 1H),

7.20 (t, J = 9.0, 2H), 7.18-7.05 (m, 2H), 6.66 (dd, J = 7.0 and 1.0, 1H), 5.39 (q, J = 13, 2H), 5.02 (s, 2H), 4.80 (s, 2H), 4.30 (d, J = 13, 1H), 3.73 (d, J = 13, 1H), 3.07 (t, J = 12, 1H), 2.69 (t, J = 12, 1H), 2.65-2.50 (m, 1H), 1.80 (d, J = 11, 2H), 1.75-1.55 (m, 1H), 1.55-1.35 (m, 1H). MS (ESI) m/z 478 [M + Na]$^+$. Anal. calcd. for $C_{22}H_{24}FN_3O_7 \times H_2O$: C, 55.11; H, 5.47; N, 8.76%; found: C, 55.41; H, 5.27; N, 8.74%.

2.1.4.7. 2-{4-[({3-[(4-Fluorobenzyl)oxy]phenyl}amino)carbonyl]piperidin-1-yl}-3-oxopropyl nitrate, ***6b***. Compound **6b** was prepared according to the general procedure from **4b** (500 mg, 1.10 mmol) and $AgNO_3$ (372 mg, 2.20 mmol). The crude product was purified by crystallization from EtOAc in 62% yield (300 mg) as a white solid; mp 154-156 °C. IR (cm^{-1}): 3330, 1683, 1633, 1551, 1381, 1285, 1200, 1159, 1041, 974, 857, 783, 690. ^{1}H NMR (DMSO-d_6) δ 9.86 (s, 1H), 7.50-7.45 (m, 2H), 7.38 (s, 1H), 7.19 (t, J = 7.0, 2H), 7.15-7.05 (m, 2H), 6.66 (dd, J = 8.0 and 1.5, 1H), 5.02 (s, 2H), 4.73 (t, J = 6.0, 2H), 4.38 (d, J = 13, 1H), 3.87 (d, J = 13, 1H), 3.03 (t, J = 12, 1H), 2.95-2.84 (m, 2H), 2.79-2.50 (m, 2H), 1.79 (d, J = 13, 2H), 1.70-1.55 (m, 1H), 1.55-1.35 (m, 1H). MS (ESI) m/z 468 [M + Na]$^+$. Anal. calcd. for $C_{22}H_{24}FN_3O_7 \times H_2O$: C, 55.11; H, 5.47; N, 8.76%; found: C, 55.41; H, 5.27; N, 8.74%.

2.1.5. Preparation of the nitric ester derivative ***9***

Compound **9** was synthesized through three main steps, starting with the preparation *t*-Boc-protected 2-(methylamino)ethanol, used in the first step, through the following procedure. A solution of Boc_2O (5.80 g, 26.6 mmol) in dry EtOAc (10 ml) was added dropwise to a 0 °C cooled solution of 2-(methylamino)ethanol (2.14 ml, 26.6 mmol) in dry EtOAc (9 ml). The mixture was stirred at room temperature for 2 h, and then concentrated under reduced pressure. The oil residue was partitioned between EtOAc (100 ml) and water (300 ml), and the collected organic phases dried over anhydrous Na_2SO_4, filtered and evaporated in vacuum to furnish *t*-butyl 2-hydroxyethyl(methyl)carbamate in 78% yield (3.66 g) of as a colorless oil. IR (liquid film, cm^{-1}): 3444, 2977, 1673, 1394, 1225, 1156,

1074, 877, 774. ^{1}H NMR ($CDCl_3$) δ 3.72 -3.80 (m 3H), 3.40 (t, J = 5.0, 2H), 2.92 (s, 3H), 1.47 (s, 9H).

2.1.5.1. 2-(Methyl)amino]ethyl 4-[({3-[(4-fluorobenzyl)oxy]phenyl}amino)carbonyl] piperidine-1-carboxylate, **7**. 4-Nitrophenyl chloroformate (498 mg, 2.47 mmol) was added portionwise to a 0 °C cooled solution of *t*-butyl 2-hydroxyethyl(methyl)carbamate (433 mg, 2.47 mmol) and TEA (0.86 ml, 6.18 mmol) in dry THF (10 ml), and the mixture was stirred at 0 °C for 3 h. Then, compound **1**·HCl (900 mg, 2.47 mmol) was added, and the mixture stirred overnight at room temperature. The formed precipitate was filtered, and the filtrate evaporated to dryness, leaving a solid residue which was dissolved in EtOAc (50 ml). The EtOAc solution was sequentially washed with 2×20 ml of saturated aqueous $NaHCO_3$, 1N HCl and brine, dried over anhydrous Na_2SO_4, filtered and evaporated in vacuum. The crude residue which was purified by silica gel flash chromatography (eluent: Hex/ethyl acetate, 4:6 v/v), providing *t*-Boc-protected compound **7** as a pale yellow oil in 50% yield (645 mg). IR (cm^{-1}): 3435, 2931, 1697, 1608, 1384, 1204, 1155, 864, 827, 770. ^{1}H NMR ($CDCl_3$) δ 7.45 (t, J = 2.0, 1H), 7.42-7.36 (m, 2H), 7.22-7.16 (m, 2H), 7.06 (dt, J = 8.5 and 2.0, 2H), 6.92 (dd, J = 8.0 and 1.5, 1H), 6.72 (dd, J = 8.0 and 2.0, 1H), 5.01 (s, 2H), 4.30-4.15 (m, 4H), 3.55-3.45 (m, 2H), 2.95-2.75 (m, 5H), 2.50-2.35 (m, 1H), 1.95-1.85 (m, 2H), 1.85-1.60 (m, 2H), 1.45 (s, 9H).

A mixture of *t*-Boc-protected **7** (645 mg, 1.24 mmol) and redistilled TFA (0.55 ml, 6.18 mmol) in DCM (20 ml) was stirred at room temperature until reaction completion (TLC monitoring), to produce compound **7** as TFA salt in quantitative yield (535 mg) as a pale brown oil. IR (cm^{-1}): 3247, 2945, 1708, 1651, 1606, 1434, 1205, 1009, 837, 721. ^{1}H NMR (DMSO-d_6) δ 9.92 (s, 2H), 8.53 (s, br, 1H), 7.48-7.43 (m, 2H), 7.38 (s, 1H), 7.20 (t, J = 8.0, 1H), 7.12-7.00 (m, 2H), 6.67 (dd, J = 8.0 and 1.5, 2H), 5.02 (s, 2H), 4.19 (t, J = 5.0, 2H), 4.18-4.00 (m, 2H), 3.18 (t, J = 5.0, 2H), 2.90-2.70 (m, 3H), 2.60 (t, J = 5.0, 2H), 2.55-2.45 (m, 1H), 1.85-1.65 (m, 2H), 1.60-1.40 (m, 2H).

2.1.5.2. 2-[(Bromoacetyl)(methyl)amino]ethyl 4-[({3-[(4-fluorobenzyl)oxy]phenyl}amino)carbonyl] piperidine-1-carboxylate, ***8***. A solution of bromoacetyl bromide (0.17 ml, 1.93 mmol) in dry DCM (3 ml) was added dropwise to a 0 °C cooled suspension of **7**·TFA (750 mg, 1.75 mmol) and TEA (0.5 ml, 3.50 mmol) in dry DCM (8 ml). The mixture was stirred overnight at room temperature, then was filtered and the filtrate diluted with DCM (20 ml). The organic phase was washed with 2×20 ml of saturated aqueous $NaHCO_3$, 1N HCl and brine, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness, to give the crude product (407 mg, 48% yield), which was used without further purification in the subsequent reaction. MS (ESI) m/z: 550 $[M + H]^+$. IR (cm^{-1}): 3321, 2922, 1693, 1646, 1606, 1440, 1204, 1125, 1036, 873, 768.

2.1.5.3. 2-{Methyl[(nitrooxy)acetyl]amino}ethyl 4-[({3-[(4-fluorobenzyl)oxy]phenyl}amino) carbonyl]piperidine-1-carboxylate, ***9***. Compound **9** was prepared, according to the above described procedure from **8** (350 mg, 0.64 mmol) and $AgNO_3$ (162 mg, 0.95 mmol). The crude product was purified by crystallization from EtOAc in 55% yield (195 mg) as a brown solid; mp 120-121 °C. IR (cm^{-1}): 3433, 1650, 1541, 1439, 1384, 1286, 1045, 852. ^{1}H NMR (DMSO-d_6) δ 9.87 (s, 1H), 7.50-7.40 (m, 2H), 7.39 (s, 1H), 7.25-7.14 (m, 3H), 7.09 (d, J = 8.5, 1H), 6.66 (dd, J = 8.0 and 1.5, 1H), 5.34 (s, 2H), 4.22-4.10 (m, 2H), 4.07 (t, J = 5.0, 2H), 4.05-3.95 (m, 2H), 3.60-3.50 (m, 2H), 2.98 (s, 3H), 2.55-2.45 (m, 1H), 1.65-1.45 (m, 2H), 1.55-1.40 (m, 2H). MS (ESI) m/z 555 $[M + Na]^+$. Anal. calcd. for $C_{25}H_{29}FN_4O_8$: C, 56.39; H, 5.49; N, 10.52%; found: C, 56.58; H, 5.66; N, 10.68%.

2.1.6. General procedure for preparation of hydroxyalkyl carbamate derivatives ***12a*** *and* ***12b***

A solution of benzoyl chloride (1.7 ml, 14.7 mmol) in dry THF (10 ml) was added dropwise to a 0 °C cooled solution of ethylene glycol or 1,3-propanediol (16.1 mmol) for **10a** or **10b**, respectively, and TEA (4 ml, 29.4 mmol) in dry THF (20 ml). The mixture was stirred at room temperature overnight. The formed precipitate was filtered, the filtrate was evaporated to dryness and the solid residue was dissolved in EtOAc (50 ml). The organic phase was sequentially washed with 3×20 ml

of saturated aqueous $NaHCO_3$, 1N HCl and brine, dried over anhydrous Na_2SO_4, filtered and evaporated in vacuum, to provide a residue containing the desired crude products **10a** and **10b** (65% GC yield), which were used in the subsequent reactions without further purification.

A solution of 4-nitrophenyl chloroformate (950 mg, 4.70 mmol) in dry THF (5 ml) was added dropwise to a 0 °C cooled solution of crude product **10a** or **10b** (4.51 mmol) in dry THF (10 ml) and TEA (1.9 ml, 13.5 mmol), and the mixture was stirred at 0 °C for 2 h. Then, compound **1**·HCl (1.26 g, 3.86 mmol) was added, and the mixture was stirred at room temperature overnight. The formed precipitate was filtered, the solvent was evaporated in vacuum and the solid residue was dissolved in EtOAc (50 ml). The organic phase was sequentially washed with 2×20 ml of saturated aqueous $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The crude products **11a** and **11b** were purified by silica gel flash chromatography.

A mixture of **11a** or **11b** (1.20 mmol) in THF (10 ml), MeOH (2 ml) and aqueous 2N NaOH solution (3 ml) was stirred at room temperature for 6h, until reaction completion (TLC monitoring). The solution was then concentration under reduced pressure, and the oil residue was dissolved in EtOAc (50 ml) and partitioned with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The crude product was purified by crystallization or silica gel flash chromatography.

2.1.6.1. 2-(Benzoyloxy)ethyl 4-[({3-[(4-fluorobenzyl)oxy]phenyl}amino)carbonyl]piperidine-1-carboxylate, ***11a***. Compound **11a** was prepared was prepared according to the general procedure from crude 2-hydroxyethyl benzoate **10a** (750 mg, 4.51 mmol) and compound **1**·HCl (1.26 g, 3.86 mmol). The crude product was purified by silica gel flash chromatography (eluent: Hex/EtOAc, 1:1 v/v) in 45% yield (1.06 g) as a pale brown solid, which was crystallized from Hex/EtOAc; mp 142-144 °C. IR (cm^{-1}): 3269, 1729, 1698, 1647, 1605, 1276, 1208, 837, 707. ^1H NMR ($CDCl_3$) δ 8.08-8.02 (m, 2H), 7.56 (t, J = 7.5, 1H), 7.49-7.36 (m, 5H), 7.21 (t, J = 8.0, 1H), 7.16 (s, br, 1H), 7.06 (t, J = 9.0, 2H), 6.92 (dd, J = 8.0 and 1.5, 1H), 6.72 (dd, J = 8.0 and 1.5, 1H), 5.01 (s, 2H), 4.58-4.53 (m, 2H),

4.46-4.41 (m, 2H), 4.30-4.15 (m, 2H), 2.89 (d, J = 12, 1H), 2.85 (d, J = 12, 1H), 2.45-2.35 (m, 1H), 2.00-1.85 (m, 2H), 1.85-1.65 (m, 2H).

2.1.6.2. 3-(Benzoyloxy)propyl 4-[({3-[(4-fluorobenzyl)oxy]phenyl}amino)carbonyl]piperidine-1-carboxylate, ***11b***. Compound **11b** was prepared was prepared according to the general procedure from crude 3-hydroxypropyl benzoate (500 mg, 2.68 mmol) and compound **1**·HCl (750 mg, 2.28 mmol). The crude product was purified by silica gel flash chromatography (eluent: Hex/EtOAc, 1:1 v/v) in 55% yield (785 mg) as a white solid, which was crystallized from Hex/EtOAc; mp 135-137 °C. IR (cm^{-1}): 3290, 1715, 1703, 1661, 1278, 1202, 1122, 822, 709. ^{1}H NMR ($CDCl_3$) δ 8.03 (d, J = 7.0, 2H), 7.56 (t, J = 7.5, 1H), 7.48-7.35 (m, 5H), 7.23 (s, br, 1H), 7.19 (d, J = 8.0, 1H), 7.06 (t, J = 9.0, 2H), 6.93 (dd, J = 8.0 and 1.0, 1H), 6.71 (dd, J = 8.0 and 2.0, 1H), 5.00 (s, 2H), 4.43 (t, J = 6.0, 2H), 4.28 (t, J = 6.0, 2H), 4.27 (m, 2H), 2.85 (d, J = 12, 1H), 2.81 (d, J = 12, 1H), 2.45-2.30 (m, 1H), 2.14 (quintet, J = 6.0, 2H), 1.95-1.75 (m, 2H), 1.73-1.60 (m, 2H).

2.1.6.3. 2-Hydroxyethyl 4-[({3-[(4-fluorobenzyl)oxy]phenyl}amino)carbonyl]piperidine-1-carboxylate, ***12a***. Compound **12a** was prepared was prepared according to the general procedure from **11a** (635 mg, 1.22 mmol). The crude product was purified by crystallization from EtOH, with addition of few drops of water, in 55% yield (260 mg) as a pale yellow solid; mp 174-175 °C. IR (cm^{-1}): 3508, 3250, 2922, 2867, 1703, 1654, 1605, 1434, 1229, 1204, 1072, 839, 687. ^{1}H NMR (DMSO-d_6) δ 9.90 (s, 1H), 7.50-7.43 (m, 2H), 7.39 (t, J = 2.0, 1H), 7.25-7.05 (m, 4H), 6.66 (dd, J = 8.5 and 1.5, 1H), 5.02 (s, 2H), 4.77 (t, J = 5.0, 1H), 4.03 (d, J = 12.5, 2H), 3.98 (t, J = 5.0, 2H), 3.54 (q, J = 5.0, 2H), 2.95-2.85 (m, 2H), 2.50-2.40 (m, 1H), 1.80-1.75 (m, 2H), 1.55-1.40 (m, 2H). Anal. calcd. for $C_{22}H_{25}FN_2O_5$: C, 63.45; H, 6.05; N, 6.73%; found: C, 63.63; H, 6.12; N, 6.72%.

2.1.6.4. 3-Hydroxypropyl 4-[({3-[(4-fluorobenzyl)oxy]phenyl}amino)carbonyl]piperidine-1-carboxylate, ***12b***. Compound **12b** was prepared according to the general procedure from **11b** (365

mg, 0.68 mmol). The crude product was purified by silica gel flash chromatography (eluent: Hex/EtOAc, 3:7 v/v) in 92% yield (270 mg) as a yellow oil. IR (cm^{-1}): 3416, 3316, 2951, 2870, 1675, 1606, 1539, 1437, 1225, 1155, 1046, 955, 865, 826, 760. ^{1}H NMR ($CDCl_3$) δ 7.45 (s, 1H), 7.43-7.37 (m, 2H), 7.21 (t, J = 8.0, 1H), 7.17 (s, br, 1H), 7.06 (t, J = 9.0, 2H), 6.92 (d, J = 8.5, 1H), 6.72 (dd, J = 8.0 and 2.5, 1H), 5.02 (s, 2H), 4.28 (t, J = 6.0, 2H), 4.25-4.10 (m, 4H), 2.95-2.80 (m, 2H), 2.50-2.35 (m, 1H), 2.00-1.55 (m, 7H).

*2.1.7. Preparation of benzyl nitrate derivative **15***

Compound **15** was prepared through a two-step synthesis, starting from the already reported 1-*t*-Boc-protected *N*-(3-hydroxyphenyl)piperidine-4-carboxamide **13** (de Candia et al., 2009).

*2.1.7.1. Tert-butyl-4-{[(3-{[4-(bromomethyl)benzyl]oxy}phenyl)amino]carbonyl}piperidine-1-carboxylate, **14***. α,α'-Dibromo-*p*-xylene (298 mg, 1.13 mmol) and anhydrous K_2CO_3 (195 mg, 1.41 mmol) were added to a solution of compound **13** (300 mg, 0.94 mmol) in Me_2CO (15 ml). The mixture was refluxed until disappearance of **13** (TLC monitoring). After cooling and removal of the reaction solvent, the residue was partitioned between EtOAc (50 ml) and brine (50 ml). The organic phase was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The crude product was purified by silica gel flash chromatography (Hex/EtOAc, 1:1 v/v) in 60% yield (285 mg) as a brown oil. IR (cm^{-1}): 3300, 2973, 2929, 2851, 1694, 1651, 1584, 1421, 1286, 1213, 1163, 1041, 781. ^{1}H NMR ($CDCl_3$) δ 7.50-7.40 (m, 5H), 7.11 (s, 1H), 6.92 (d, J = 6.5, 1H), 6.72 (d, J = 6.5, 1H), 5.43 (s, 2H), 5.08 (s, 2H), 4.25-4.15 (m, 2H) 2.79 (t, J = 12, 2H), 2.45-2.30 (m, 1H), 1.95-1.85 (m, 2H), 1.80-1.60 (m, 2H), 1.46 (s, 9H).

*2.1.7.2. 4-({3-[(Piperidin-4-ylcarbonyl)amino]phenoxy}methyl)benzyl nitrate hydrochloride, **15***. Compound **15** was synthesized according to the general procedure, as described for **5a**, from compound **14** (350 mg, 0.70 mmol) and $AgNO_3$ (233 mg, 1.37 mmol). The crude *N*-Boc derivative

of compound **15** was purified by silica gel flash chromatography (Hex/EtOAc, 1:1 v/v) in 65% yield (220 mg) as a brown oil. IR (cm^{-1}): 3300, 2973, 2930, 2851, 1698, 1661, 1631, 1550, 1421, 1280, 1214, 1163, 1042, 957, 867, 856, 774, 690. ^1H NMR ($CDCl_3$) δ 7.48 (s, 1H), 7.46 (d, J = 8.0, 2H), 7.40 (d, J = 8.0, 2H), 7.21 (t, J = 8.0, 1H), 7.15 (s, br, 1H), 6.92 (d, J = 7.0, 1H), 6.71 (dd, J = 8.0 and 2.0, 1H), 5.43 (s, 2H), 5.08 (s, 2H), 4.25-4.10 (m, 2H), 2.78 (t, J = 12, 2H), 2.43-2.30 (m, 1H), 1.95-1.85 (m, 2H), 1.80-1.65 (m, 2H), 1.46 (s, 9H).

A 1.25 M HCl methanolic solution (2.0 ml, 2.25 mmol) was added to a solution of *N*-Boc-protected **15** (220 mg, 0.45 mmol) in MeOH (20 ml), and the mixture was stirred at room temperature until reaction completion (TLC monitoring). After solvent evaporation in vacuum and trituration of the residue with abs. EtOH, the hydrochloride salt of **15** was obtained in quantitative yield (175 mg) as a pale brown solid, which was crystallized from EtOAc/EtOH; mp 102-104 °C. IR (cm^{-1}): 3399, 2925, 2800, 2709, 1692, 1615, 1544, 1385, 1284, 1208, 1162, 1121, 892, 873. ^1H NMR ($CDCl_3$) δ 10.06 (s, 1H), 8.75 (s, br, 1H), 8.47 (s, br, 1H), 7.47 (s, 4H), 7.39 (d, J = 2.0, 1H), 7.18 (t, J = 8.0, 1H), 7.11 (d, J = 8.0, 1H), 6.68 (dd, J = 8.0 and 1.5, 1H), 5.56 (s, 2H), 5.08 (s, 2H), 3.97 (d, J = 12.6, 1H), 3.32 (d, J = 12.6, 1H), 2.88 (t, J = 12.6, 2H), 2.65-2.5 (m, 1H), 2.00-1.85 (m, 2H), 1.85-1.60 (m, 2H). MS (ESI) m/z 386 $[M + H]^+$. Anal. Calcd. for $C_{24}H_{28}FN_3O_7$: C, 58.89; H, 5.77; N, 8.58%; found: C, 59.02; H, 5.87; N, 8.58%.

2.3 Vasodilator activity

Male Wistar rats were obtained from Harlan Laboratories (San Pietro al Natisone, Italy) and individually housed at constant room temperature (25 ± 1 °C) and humidity (60 ± 5 %), with an artificial 12:12 h light/dark cycle. Thoracic aortas were isolated from rats weighing 180-200 g. As few animals as possible were used. The purposes and the protocols of our studies have been approved by Ministero della Salute (Rome, Italy). All the procedures were performed in accordance with the Ethical Animal Committee of the University of Turin (Italy).

The endothelium was removed and the vessels were helically cut: four to six strips were obtained from each aorta. The tissues were mounted under 1.0 g tension in organ baths containing 30 ml of Krebs-bicarbonate buffer with the following composition (mM): NaCl 111.2, KCl 5.0, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.0, $NaHCO_3$ 12.0, glucose 11.1, maintained at 37 °C and gassed with 95% O_2-5% CO_2 (pH 7.4). The aorta strips were allowed to equilibrate for 120 min and then contracted with 1 μM L-phenylephrine. When the response to the agonist reached a plateau, cumulative concentrations of the test compound were added. Results are expressed as EC_{50} values; data are the mean ± SEM of at least three experiments. The effects of 1 μM ODQ on relaxation were evaluated in separate series of experiments in which it was added to the organ bath 5 min before contraction. Responses were recorded by an isometric transducer connected to the MacLab System PowerLab. Addition of the drug vehicle (1% DMSO) had no appreciable effect on contraction level.

2.4 Inhibition of platelet aggregation

Human blood was obtained from healthy volunteers (25-45 years of age), who had not ingested any platelet inhibitory drug for at least one week prior to donation. All subjects provided informed consent, and were treated according to Helsinki protocol for biomedical experimentation.

Blood and blood products were handled in plastic ware, whereas siliconized glass cuvettes and stir bars were used in the aggregation assay. Platelet rich plasma (PRP) was prepared by centrifugation of citrated blood at 200×g for 20 min. The transmittance of PPP was taken as 100% aggregation. PRP (500 μl) was added into the aggregometer (Chrono-log 4902D) cuvettes and preincubated at 37 °C for 15 min with the tested compounds (final concentrations in the PRP solutions ranging from 10 μM to 300 μM) or with vehicle (to eliminate the effect of the solvent on the aggregation and release reaction of platelets; the final concentration of DMSO was fixed at 0.5%, v/v). Then, adenosine 5'-diphosphate (ADP, 5 μM final concentration), or collagen at submaximal concentration (0.8-1.5 μg/ml) were added to the incubated sample and aggregation was recorded as increased light

transmission under continuous stirring (1000 rpm) at 37 °C for 10 min after addition of the aggregation inducer. The antiaggregatory activity of the tested compounds is expressed as percent inhibition of platelet aggregation compared with vehicle control samples. For most of the active compounds, IC_{50} values (i.e., the concentration effecting 50% inhibition of aggregation), was calculated by nonlinear regression analysis ($r^2 > 0.80$); alternatively, percent inhibition at maximal concentration tested (300 μM) is reported. The number of experiments was 3-5 for IC_{50} values, and 3 for determination of % inhibition.

2.3 Hydrolysis studies

2.3.1 Hydrolysis in aqueous solutions. To 0.25 ml of a 10 mM stock solution of the compound in ACN, 1.75 ml of ACN and aqueous buffer solution (0.01 M HCl or 0.04 M phosphate buffer pH 7.4 in 0.15 M KCl) were added to give a final volume of 10 ml. The solution at the final concentration of 200 μM was thermostated at 37 ± 0.5 °C.

At appropriate time intervals, samples were withdrawn and analyzed by HPLC using 1260 Infinity Quaternary LC system (Agilent Technologies, Milan, Italy) equipped with autosampler, photodiode array detector and evaporative light scattering detector, and controlled by Lab Advisory software. A Phenomenex Kinetex C18 column 5 μm (150 × 3.0 mm i.d.) was used as the stationary phase; the analytes were eluted with a 23 min gradient from mobile phase A (65% v/v 20 mM ammonium formate aqueous solution in ACN) to mobile phase B (35% v/v 20 mM ammonium formate aqueous solution in ACN) at a constant flow rate of 0.5 ml/min; injection volume: 10 μl.

Pseudo-first-order rate constants (k_{obs}) for the hydrolysis of the compounds were calculated from the slopes of the linear plots of log (% remaining compound) against time. Each kinetic experiment was performed in triplicate.

2.3.2 Stability in human serum. To 1.47 ml of human serum (lyophilized and reconstituted with 4 ml of deionized water), preheated at 37 ± 0.5 °C, 30 μl of a 10 mM stock solution of each compound in

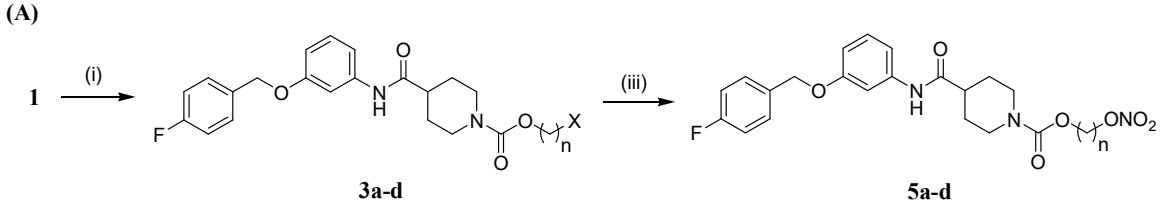
ACN were added and the solution was incubated at 37 ± 0.5 °C (final compound concentration 200 μM). Aliquots (100 μl) of the serum solution were taken at various times and deproteinized by mixing with 500 μl of cold MeOH. The suspension was vortexed 1 min, centrifuged 10 min at 4350 rpm, and 10 μl of the supernatant were analyzed by HPLC as described above. The % amounts of the remaining intact compound were plotted against the incubation time.

3. Results and discussion

3.1 Chemistry

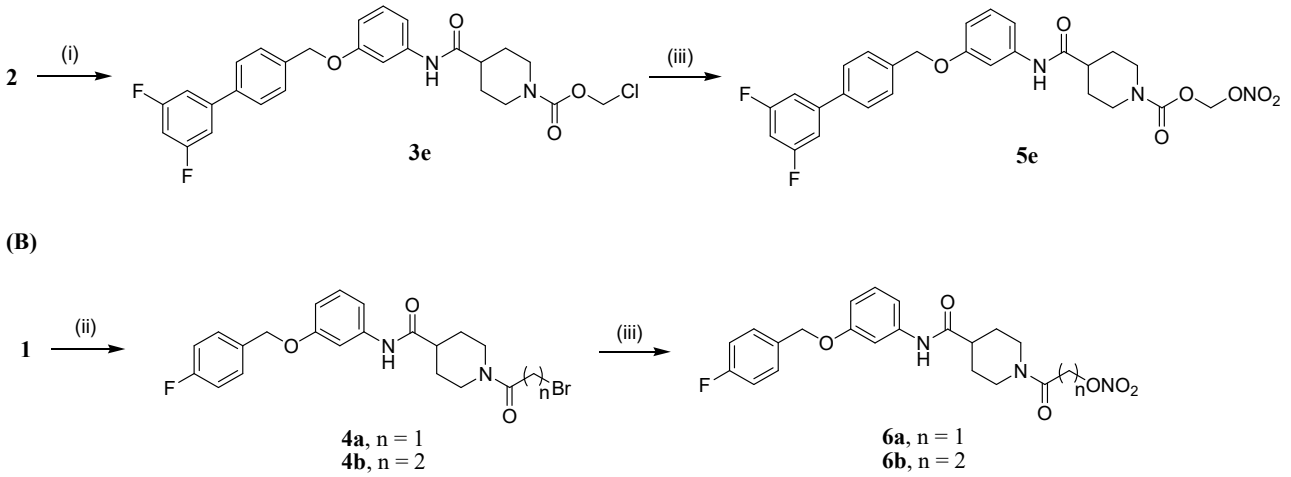
The nitrooxy (ONO_2)-containing compounds of compounds **1** and **2** were synthesized as outlined in Scheme 1. Compound **1**·HCl was reacted with chloromethyl- and 2-chloroethyl chloroformate, in the presence of triethylamine (TEA), to provide chloromethyl- and chloroethyl carbamate derivatives **3a** and **3b**, respectively, which were then transformed into the corresponding (ONO_2)methyl (**5a**) and (ONO_2)ethyl (**5b**) derivatives by treatment with $AgNO_3$ in acetonitrile (ACN) at reflux. Compound **5e**, i.e., the (ONO_2)methyl carbamate of **2**, was prepared using a similar procedure. Conversely, the synthesis of the intermediate bromopropyl (**3c**) and chlorobutyl (**3d**) carbamates was accomplished by activating 3-bromopropanol and 4-chlorobutanol as carbonates, by reaction with 4-nitrophenyl chloroformate, and subsequent aminolysis by **1**.

(A)



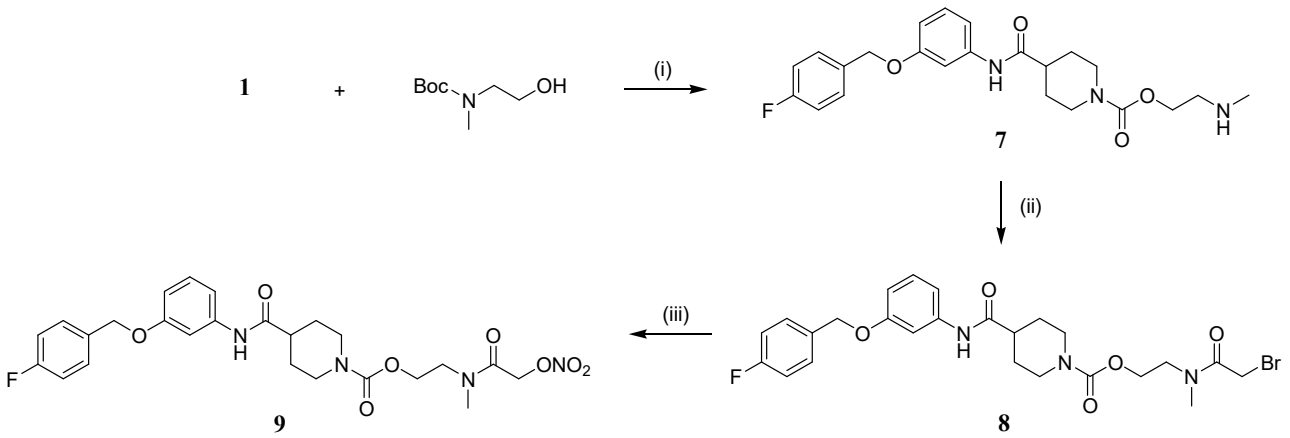
3a-d **5a-d**

	3a → 5a	**3b → 5b**	**3c → 5c**	**3d → 5d**
X	Cl	Cl	Br	Cl
n	1	2	3	4



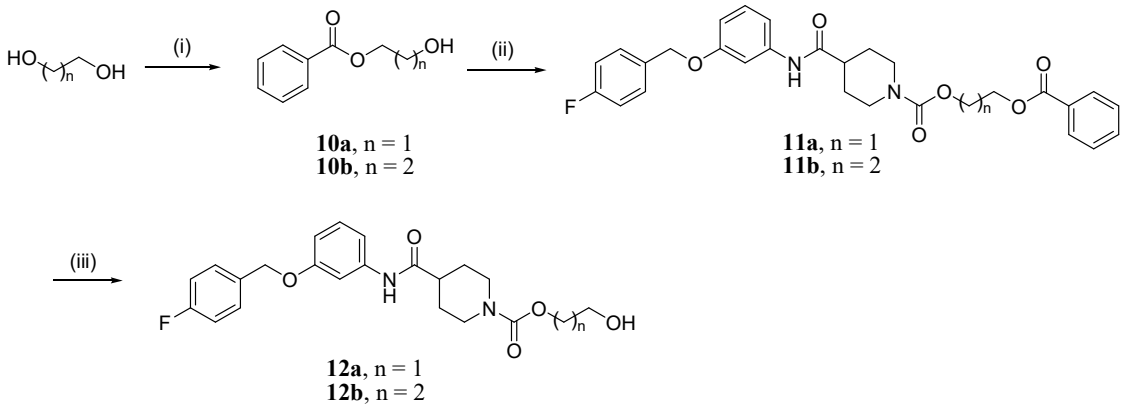
Scheme 1. Synthesis of (ONO_2)alkyl carbamate (A) and amide (B) derivatives of 3-substituted-benzyloxy isonipecotanilides **1** and **2**. Reagents and conditions: (i) a) $ClCOOCH_2Cl$ (for **3a** and **3e**), $ClCOOCH_2CH_2Cl$ (for **3b**), TEA, dry DCM, r.t., overnight, 54-78%; b) 4-nitrophenylchloroformate, TEA, $Br(CH_2)_3OH$ (for **3c**), $Cl(CH_2)_4OH$ (for **3d**), dry THF, 0 °C, 3 h, then **1**, r.t., overnight, 50-55%. (ii) $BrCH_2COBr$ (for **4a**), $BrCH_2CH_2COBr$ (for **4b**), TEA, dry THF, 0 °C, 1 h, then r.t., overnight, 55-64%. (iii) $AgNO_3$, CH_3CN, reflux, 16-20 h, 55-75%.

N-*t*-Boc-*N*'-methyl-2-aminoethanol was used to prepare the intermediate product **7**, which after *t*-Boc-deprotection and subsequent reaction with bromoacetyl bromide yielded compound **8**, which was finally refluxed with $AgNO_3$ in ACN to give the corresponding (ONO_2)-substituted compound **9**. The (ONO_2)-containing amide derivatives **6a**,**b** were synthesized by reacting compound **1** with the appropriate bromoacyl bromides and subsequent nitrooxylation of the intermediate bromides **4a,b** (Scheme 2).



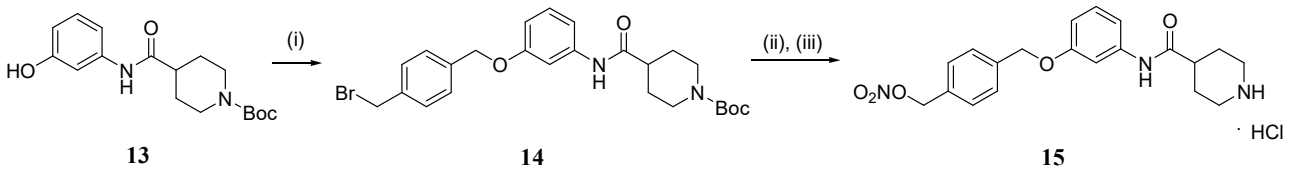
Scheme 2. Synthesis of the (ONO_2)-containing compound **9**. Reagents and conditions: (i) 4-nitrophenylchloroformate, TEA, dry THF, 0 °C, 3 h, then **1**, r.t., overnight; TFA, DCM, r.t., 50%. (ii) $BrCH_2COBr$, TEA, dry DCM, r.t., overnight, 48%. (iii) $AgNO_3$, CH_3CN, reflux, 16-20 h, 55-75%.

To prove the effectiveness of our (ONO_2)-containing derivatives as NO-releasing agents, the ethyl and propyl alcohols **12a** and **12b**, as hypothetical de-nitrated metabolites arising from biotransformation pathways involving hydrolytic cleavage of the nitric ester functions in **5b** and **5c**, respectively, were synthesized as outlined in Scheme 3. The mono benzoate esters **10a**-**b**, once activated as carbonates by reaction with 4-nitrophenyl chloroformate, were transformed through aminolysis by **1** into the ester products **11a**,**b**, which were then hydrolyzed in basic conditions to provide the respective hydroxyl derivatives **12a**,**b**.



Scheme 3. Synthesis of hydroxyalkyl carbamate derivatives of 4-fluorobenzyloxy isonipecotanilide **1**. Reagents and conditions: (i) Benzoyl chloride, TEA, dry THF, r.t., overnight, 55-65%; (ii) 4-nitrophenyl chloroformate, TEA, dry THF, r.t., 3 h; then **1**, r.t., overnight, 45-55%; (iii) 2N NaOH, THF/MeOH 5:1 v/v, r.t., 12-16 h, 55-92%.

Finally, the benzyl nitrate derivative **15** was synthesized as outlined in Scheme 4. Monobenzylation of the phenol OH in compound **13 (**de Candia et al., 2009) by reaction with *α,α'*-dibromo-*p*-xylene furnished the bromobenzyl derivative **14**, which was then transformed into the organic nitrate **15**.



Scheme 4. Synthesis of the 4-benzyl nitrate derivative **15**. Reagents and conditions: (i) α,α-Dibromo-*p*-xylene, K_2CO_3, dry acetone, reflux, 6 h, 60%; (ii) $AgNO_3$, ACN, reflux, 6 h, 65%; (iii) HCl gas, $CHCl_3$, quantitative yield.

3.2 Vasodilator and antiplatelet activities

All the newly synthesized nitric ester derivatives (**5a-e**, **6a-b**, **9** and **15**), along with the parent compound **1** were assayed to evaluate both the vasodilator and antiplatelet activities; vasorelaxing properties of the alcohol products **12a** and **12b**, hypothetically arising from the cleavage of the nitrate function correspondingly in **5b** and **5c**, were studied as well. The vasodilator activity was evaluated on isolated rat aortic strips, precontracted with L-phenylephrine. All the tested compounds showed concentration-dependent vasorelaxing effects, and their potencies, expressed as EC_{50} values, are summarized in Table 1.

The (ONO_2)-containing derivatives showed good vasodilatory activity with EC_{50} values ranging from 0.01 to 5 μM. The (ONO_2)-methyl carbamate **5a** and the benzyl nitrate derivative **15** proved to be the most potent compounds, with EC_{50} values falling in the low nanomolar range (13 and 29 nM, respectively). The carbamate derivatives **5a** and **5b** are several times more potent than the corresponding amide derivatives **6a** and **6b**. Within the small carbamate series (**5a-d**) the vasodilator potency is inversely correlated with the length of the alkyl spacer between the ONO_2 and >NCOO moieties (pEC_{50}/*n*CH_2 points lie on a straight line with *slope* = -0.84 and r^2 = 0.994), suggesting that the closer the secondary carbamate group, the greater its electron-withdrawing inductive effect on the vasorelaxing activity of the (ONO_2)alkyl piperidine-1-carbamate derivatives.

No substantial change in vasodilatory potency was observed when the NO-donor side-chain was elongated, as we did in compound **9**, via a link between the terminal 2-(ONO_2)acetamido fragment and the internal ethyl carbamate group.

Co-incubation of the aortic strips with the selective inhibitor of *s*GC, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinaxolin-1-one (ODQ), resulted in a rightward shift of the dose-response curves and higher EC_{50} values for all the tested nitrate compounds (Fig. 3). The observed decrease of potency provided support to NO-mediated activation of *s*GC, which is the key enzyme modulating smooth muscle relaxation (and platelet aggregation as well) through increase of cGMP concentration. Interestingly,

also the (ONO_2)-lacking compounds **1**, **12a** and **12b**, displayed vasodilatory effects, with EC_{50} values around 20 μM.

Table 1

In vitro vasodilator and anti-platelet activity of ONO_2-substituted compounds and some related alcohol derivatives of fluorinated benzyloxyphenyl piperidine-4-carboxamides.

	Vasodilator activity [a]	Platelet aggregation inhibition [b]	
Cmpd	EC_{50}, μM ± SEM [+1 μM ODQ]	ADP, 5 μM IC_{50}, μM (CL95%) [% inhib. ± SEM at 300 μM]	Collagen, 0.8-1.5 μg/mL IC_{50}, μM (CL95%) [% inhib. ± SEM at 300 μM]
1	17 ± 4	73 (68-79)	87 (82-92)
5a	0.013 ± 0.002 [1.3 ± 0.2]	117 (93-147)	[46 ± 6]
5b	0.11 ± 0.04 [4.5 ± 0.4]	188 (150-236)	[11 ± 7]
5c	0.50 ± 0.08 [3.1 ± 0.8]	180 (129-251)	[7.0 ± 4.0]
5d	5.0 ± 2 [> 100]	234 (198-277)	[14 ± 5]
5e	0.25 ± 0.06 [> 100]	108 (81-146)	[41 ± 11]
6a	0.42 ± 0.06 [17 ± 2]	[22 ± 11]	[12 ± 5]
6b	0.49 ± 0.10 [8.5 ± 0.6]	[4.0 ± 3.0]	[20 ± 13]
9	0.11 ± 0.02 [14 ± 1]	[16 ± 8]	[13 ± 4]
12a	24 ± 5		
12b	23 ± 1		
15	0.029 ± 0.006 [9.3 ± 1.0]	55 (43-70)	194 (182-205)

[a] Concentration of tested compound which reduces by 50% contraction of the rat thoracic aortic strips, previously treated with 1 μM L-phenylephrine, with and without addition of 1 μM ODQ, as *s*GC irreversible selective inhibitor; data are means ± SEM of at least three independent experiments.

[b] IC_{50} values or percent inhibition at the maximum concentration tested (300 μM); data are means of at least three independent experiments.

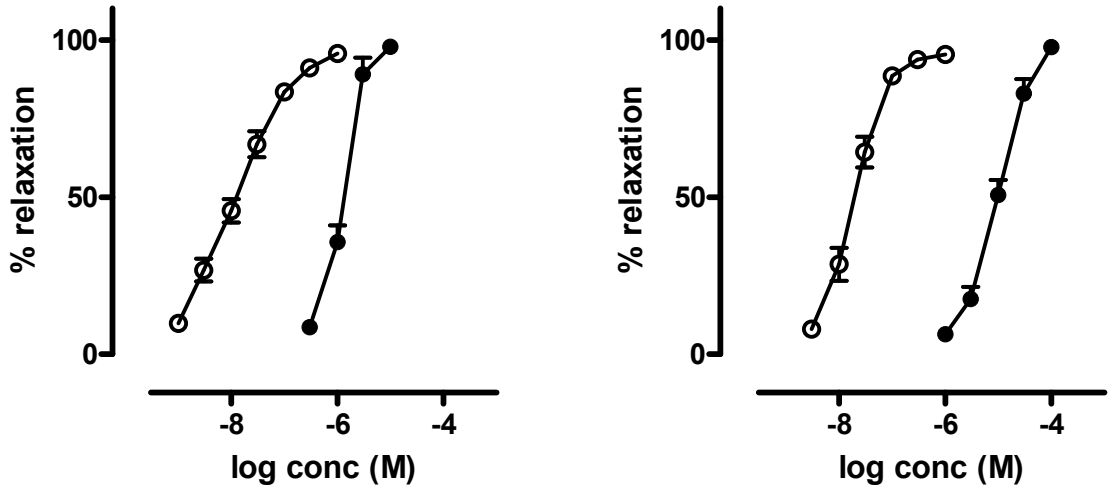


Fig. 3. Concentration-response curves of **5a** (left) and **15** (right), with (●) and without ODQ (○), in vasodilation assay on rat aortic strips precontracted with L-phenylephrine.

These data suggest, on one hand, that potent vasodilators, like the (ONO_2)-methyl carbamate **5a** and the benzyl nitrate **15**, attain smooth muscle relaxation also through mechanisms other than the NO-mediated prevention of myosin phosphorylation, whereas on the other hand compounds **1** or **12a**-**b**, as hypothetic metabolites of the nitrate derivatives **5b**-**c**, retain vasodilatory activity.

It is known that the smooth muscle relaxation results from a prevention of myosin light chain (MLC) phosphorylation, thus maintaining the muscle in the relaxed state, through three main mechanisms: (i) increase of cAMP or cGMP leading to phosphorylation, and thereby inhibition, of MLC kinase (MLCK); (ii) reduction of intracellular Ca^{++} concentration and formation of the Ca^{++}-calmodulin complex which activates MLCK; (iii) phosphatase-activated MLC dephosphorylation. The pharmacological data (Table 1) suggest that the observed vasodilatory effects should mostly depend upon the release of NO which activates *s*GC, ultimately leading to prevention of muscle contraction. The significant, albeit poorer, activity shown by compounds **1**, **12a** and **12b** may be most likely

related to the capacity of benzyloxy isonipecotanilides of decreasing intracellular Ca^{++} concentration, which also plays a role in regulating smooth muscle contraction; the same mechanism could be involved in the residual activity observed for most of the (ONO_2)-containing derivatives tested in the presence of ODQ. To this purpose, we previously demonstrated that lipophilic nipecotanilides (De Marco et al., 2004), similarly to other lipophilic carbamoyl piperidine derivatives (Dillingham et al., 1989; Feng et al., 1992; Guo et al., 2000), are able to inhibit ADP-induced intraplatelet calcium mobilization. It could be reasonably assumed that the lipophilic benzyloxy isonipecotanilide derivatives investigated herewith may act by decreasing the intracellular Ca^{++} concentration also in vascular smooth muscle cells.

Most of the newly synthesized compounds were also evaluated in human platelet-rich plasma (PRP) as inhibitors of platelet aggregation induced by 5 μM ADP and collagen (0.8-1.5 μg/ml), using a turbidimetric method (Born, 1962). The platelet aggregation inhibition data (Table 1) are expressed as the half maximal inhibitory concentrations (IC_{50}, μM), calculated by nonlinear regression of the dose-response curves, or % inhibition at the maximum tested concentration (300 μM). Compound **1** has been re-assayed in this study as a positive control.

All the tested compounds attained 50% inhibitory activity of the collagen-induced platelet aggregation at concentrations significantly higher than those required for inhibiting the ADP-induced aggregation. At the tested agonists' concentrations, we should take into account that platelet aggregation triggered by collagen should depend upon the release of arachidonic acid and thromboxane A_2 (TXA_2) generation, while the ADP-induced aggregation should be less TXA_2-dependent. The anti-ADP data point out that the (ONO_2)-alkyl carbamate derivatives **5a**-**e** showed at least a 1.6-fold loss of antiplatelet potency over the respective parent compounds **1** and **2**, proving to be in vitro weak-to-moderate antiplatelet agents. Actually, the loss of antiaggregatory potency observed is not surprising, taking into account that in general alkyl nitrates decompose very slowly in plasma and buffer solution (Jones et al., 2009; Torfgard and Ahlner, 1994; Weber et al., 1993; Wendt, 1972). Whatever the rate of hydrolytic cleavage of the carbamates **5a**-**e** in plasma to

eventually yield the antiplatelet compounds **1** and **2**, and the (ONO_2)-substituted alcohol metabolites, the experimental results led us to rule out any significant contribution of NO release to the antiplatelet activity of the tested nitrates. This is consistent with the activities of non-nitrate compounds **1** and **2**, which are significantly higher than the corresponding nitrate derivatives, as well as by the generally reported failure of platelets to release substantial amounts of NO from organic nitrates in the absence of promoters of NO release, such as glutathione (GSH).

Within the examined series of organic nitrates, the benzyl nitrate derivative **15** and the (ONO_2)-methyl carbamates **5a** and **5e** are the most potent inhibitors of ADP-induced platelet aggregation. However, the antiplatelet IC_{50} values of the parent 1-unsubstituted isonipecotamides **1** (73 μM) and **2** (27 μM), significantly lower than those of any related 1-carbamoyl derivative, combined with that of **15** (55 μM), highlight the importance of the basicity of the piperidine nitrogen, protonated at physiological pH, in order to enhance the platelet aggregation inhibitory potency. Lipophilicity of the basic isonipecotamide derivatives should also affect the antiplatelet activity, taking into account that the antiplatelet potency of compounds **1**, **2** and **15** increase as the estimated log D values at pH 7.4 (by ACD/Labs software; values in parentheses) increase: **2** (2.22) > **15** (0.56) > **1** (0.47). These SAR trends are in reasonable agreement with the supposed mechanism of action of antiplatelet lipophilic (iso)nipecotamides (Dillingham et al., 1989; Feng et al., 1992; Guo et al., 2000), which involves interaction with anionic phospholipids of the platelet membrane as first step and decrease of intraplatelet Ca^{2+} concentration as final effect resulting in reduction of platelet activation. Indeed, it had been shown that, by virtue of their lipophilicity and surface activity, lipophilic nipecotamides can penetrate the platelet membranes and interact with anionic phospholipids (mainly phosphatidylinositol, PI, and phosphatidylserine, PS), thereby increasing their resistance to hydrolysis catalyzed by phospholipase-C to the second messengers inositol 1,4,5-triphosphate (IP_3) and *s*,*n*-1,2-diacylglycerol (DAG) and reducing the concentrations of IP_3 and cytosolic Ca^{2+} under the levels required for myosin phosphorylation and platelet activation. In a previous study, we ~~also~~ supported such a mechanism proving that a nipecotanilide derivative, almost isolipophilic with those

examined herein, inhibits the intraplatelet Ca^{2+} mobilization induced by ADP, this effect occurring at the initial phases of the signal transduction processes in platelets (De Marco et al., 2004).

3.3 Hydrolysis in aqueous media and human serum

The study of chemical stability focused on the most potent compounds combining good NO-dependent vasodilatory activities and moderate anti-platelet effects (**5a**, **5b**, **5e** and **15**) at 37 °C in acidic aqueous solution (0.01 M HCl), mimicking gastric environment, and in 0.04 M PBS at pH 7.4. In both aqueous media, all the examined compounds underwent pseudo-first-order hydrolysis kinetics, exhibiting good stability (half-lives ranging from 5 hours to 7 days). The observed rate constants (k_{obs}) and half-lives ($t_{½}$) are reported in Table 2.

Although the degradation products of **5a**, **5b** and **5e** were not fully characterized, RP-HPLC chromatograms recorded at regular intervals along the monitoring time between t_0 and $2\times t_{½}$ revealed disappearance of the carbamate derivatives and appearance of the parent 1-unsubstituted isonipecotamides **1** and **2**, which result from hydrolysis of the secondary carbamates and subsequent rapid CO_2 loss by the piperidine-1-carboxylate intermediates.

Table 2

Kinetic data for hydrolysis in aqueous media and in human serum at 37 °C. [a]

Cmpd	0.01 M HCl		pH 7.4 PBS		Human serum	
	$t_{½}$ (h)	k_{obs} (h^{-1})	$t_{½}$ (h)	k_{obs} (h^{-1})	$t_{½}$ (h)	k_{obs} (h^{-1})
5a	9.6	0.072	8.3	0.084	9.0	0.077
5b	110	0.0065	158	0.0045	11	0.063
5e	14	0.048	20	0.034	2.6	0.27
15	4.9	0.15	6.2	0.11	2.6	0.27

[a] Half-life ($t_{½}$) and pseudo-first-order rate constant (k_{obs}); values are means from three experiments (relative SD < 10%).

As a matter of fact, hydrolytic decomposition of **5a** and **5b** afforded, with significantly different k_{obs} values (0.072 and 0.0065 h^{-1}, respectively), predominantly compound **1**, whereas **5e** afforded predominantly the parent compound **2**, indicating that in both acidic and pH 7.4 aqueous solutions at 37 °C the carbamate group underwent hydrolysis faster than the nitric ester group. In case of **5b**, the related de-nitrated alcohol compound **12a**, which was synthesized and used in the stability study as analytical standard, was always below the limit of quantitation (LOQ, 3.8 nmol/ml) during the time of observation.

Compared to the (ONO_2)methyl carbamate **5a**, the rate of decomposition of the more lipophilic analog **5e** decreases by just a factor of 1.5 in 0.01 M HCl and 2.5 in 0.04 M PBS (pH 7.4), most likely due to restrictions on the hydration of its transition state. The rate of degradation of the (ONO_2)ethyl carbamate **5b** is ten-to-twenty slower than that of the lower homologue **5a**. As expected, the electron-withdrawing ONO_2 group accelerates the rate of hydrolysis of the secondary carbamates with an effect stronger on the α-carbon (**5a**) than on the β-carbon (**5b**).

The rate of decomposition of the benzyl nitrate derivative **15**, which revealed to be less stable than **5a** in both the aqueous media, is just 1.3-fold faster in acidic solution ($t_{½}$ = 4.9 h) than in solution buffered at pH 7.4 ($t_{½}$ = 6.2 h).

The stability of the (ONO_2)-containing compounds was also studied in human serum at 37 °C (Table 2). RP-HPLC revealed that also in human serum the main decomposition products of **5a**,**b** and **5e** are the 1-unsubstituted isonipecotamides **1** and **2**, respectively. With the exception of **5a**, which showed in human serum a stability similar to that measured in the aqueous media, the other carbamate derivatives **5b** and **5e** underwent a faster hydrolysis in serum. The rate of hydrolysis of **5e** to the main decomposition product **2** is some eight times faster in human serum than in PBS at physiological pH, in contrast to its less lipophilic analog **5a** which showed not to undergo enzyme-mediated reactions in serum (Fig. 4).

It has been established that the esterases present in human plasma are butyrylcholinesterase (BuChE), paraoxonase (PON1), albumin esterase, and acetylcholinesterase (AChE) in trace amounts, but not carboxylesterase; BuChE, PON1 and albumin contribute significantly to ester hydrolysis in human plasma (Li et al., 2005). Moreover, it is known that BuChE catalyzes carbamates' hydrolysis in human plasma, and more efficiently binds biaryl-containing substrates and inhibitors (Govoni et al., 2006; Lin et al., 2005). The stability data (Table 2) suggest that **5e** is a better substrate than **5a** for plasma esterases, which apparently prefer substrates with larger hydrophobic carbamoyl moieties.

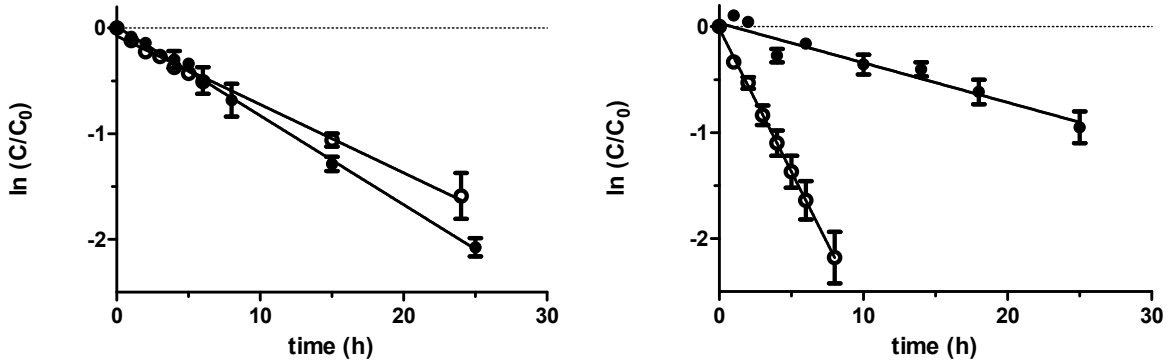


Fig. 4. Plots of first-order kinetics; disappearance of **5a** (left) and **5e** (right) in pH 7.4 PBS (●) and human serum (○) at 37 °C.

The times in which the compounds remain within 5% of the initial concentration ($t_{95\%}$), calculated from the apparent first-order rate constants in human serum, are equal to 40 and 11 min for **5a** and **5e**, respectively, suggesting that the compounds should remain almost intact during the PRP aggregation assay. On the other hand, **5a** and **5e**, which showed in vitro inhibition against the ADP-induced platelet aggregation with IC_{50}'s around 100 μM, decompose in serum, although slowly, to yield the more potent anti-platelet compounds **1** (IC_{50} 73 μM) and **2** (IC_{50} 27 μM).

The benzyl nitrate derivative **15** was also fairly stable in human serum ($t_{½}$ 2.6 h). It remains within 5% of the initial concentration ($t_{95\%}$) for 11 min, suggesting that most of the observed anti-platelet

activity (IC_{50} 55 μM) can be attributed to the intact (ONO_2)-containing molecule. Most likely benzyl nitrate **15**, similarly to GTN (Govoni et al., 2006), could be slowly hydrolyzed in blood plasma to yield the related de-nitrated metabolite.

4. Conclusion

We synthesized a number of organic nitrate derivatives of recently reported antiplatelet compounds **1** and **2**, built on the structure of benzyloxy isonipecotanilide. As a major outcome of this study, most of the investigated (ONO_2)-alkyl derivatives showed significant concentration-dependent vasodilation effects, as assessed through relaxation of precontracted rat aorta strips, while retaining appreciable activity as inhibitors of the ADP-induced platelet aggregation. The pharmacological data showed that the newly synthesized organic nitrates, which on the other hand proved to be very stable in water at acidic and neutral pH, and quite stable in pooled serum solution, exert their vasorelaxing action mainly stimulating the *s*GC/cGMP pathway, which is known to be damaged in patients with heart failure as a consequence of decrease in NO production and bioavailability. In particular, two (ONO_2)-containing compounds, namely the carbamate-based compound **5a** and the benzyl nitrate analog **15**, proved to be promising for further experimental investigation, including a thorough study of the bioactivation and metabolism pathways, as potential medications in the treatment of cardiovascular disease (e.g., acute coronary syndrome, angina pectoris, congestive heart failure), as they exhibited noteworthy vasodilatory potency in the low nanomolar range (EC_{50} of 13 and 29 nM, respectively) and good antiplatelet activity.

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References

Born, G.V.R., 1962. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 194, 927-929.

Boschi, D., Lazzarato, L., Rolando, B., Filieri, A., Cena, C., Di Stilo, A., Fruttero, R., Gasco, A., 2009. Nitrooxymethyl-substituted analogues of celecoxib: synthesis and pharmacological characterization. Chem. Biodivers. 6, 369-379.

Boschi, D., Cena, C., Di Stilo, A., Rolando, B., Manzini, P., Fruttero, R., Gasco, A., 2010. Nitrooxymethyl-substituted analogues of rofecoxib: Synthesis and pharmacological characterization. Chem. Biodivers. 7, 1173-1182.

Cena, C., Lolli, M.L., Lazzarato, L., Guaita, E., Morini, G., Coruzzi, G., McElroy, S.P., Megson, I.L., Fruttero, R., Gasco, A., 2003. Antiinflammatory, gastrosparing, and antiplatelet properties of new NO-donor esters of aspirin. J. Med. Chem. 46, 747-754.

Chen, Z., Zhang, J., Stamler, J.S., 2002. Identification of the enzymatic mechanism of nitroglycerin bioactivation. Proc. Natl. Acad. Sci. USA 99, 8306-8311.

de Candia, M., Summo, L., Carrieri, A., Altomare, C., Nardecchia, A., Cellamare, S., Carotti, A., 2003. Investigation of platelet aggregation inhibitory activity by phenyl amides and esters of piperidinecarboxylic acids. Bioorg. Med. Chem. 1, 1439-1450.

de Candia, M., Liantonio, F., Carotti, A., De Cristofaro, R., Altomare, C., 2009. Fluorinated benzyloxyphenyl piperidine-4-carboxamides with dual function against thrombosis: inhibitors of factor Xa and platelet aggregation. J. Med. Chem. 52, 1018-1028.

De Marco, A., de Candia, M., Carotti, A., Cellamare, S., De Candia, E., Altomare, C., 2004. Lipophilicity-related inhibition of blood platelet aggregation by nipecotic acid anilides. Eur. J. Pharm. Sci. 22, 153-164.

DiFabio, J., Ji, Y., Vasiliou, V., Thatcher, G.R., Bennett, B.M., 2003. Role of mitochondrial aldehyde dehydrogenase in nitrate tolerance. Mol. Pharmacol. 64,1109-1116.

Dillingham, E.O., Lasslo, A., Carter-Burks, G., Bond, S.E. Gollamudi, R. 1989. Relationships between chemical structure and inhibition of ADP-stimulated human thrombocyte release of

serotonin and platelet factor 4. Biochim. Biophys. Acta 990, 128-32.

Edwards, G., Weston, A.H., 1990. Structure-activity relationship of K^+ channel openers. Trends Pharmacol. Sci. 11, 417-422.

Feng, Z., Gollamudi, R., Dillingham, E. O., Bond, S. E., Lyman, B. A., Purcell, W. P., Hill, R. J., Korfmacher, W. A., 1992. Molecular determinants of the platelet aggregation inhibitory activity of carbamoylpiperidines. J. Med. Chem. 35, 2952-2958.

Follmann, M., Griebenov, N., Hahn, M.G., Hartung, I., Mais, F.J., Mittendorf, J., Schäfer, M., Schirok, H., Stasch, J.P., Stoll, F., Straub, A., 2013. The chemistry and biology of soluble guanilate cyclase stimulators and activators. Angew. Chem. Int. Ed. Engl. 52, 9442-9462.

Gasco, A., Fruttero, R., Rolando, B., 2005. Focus on recent approaches for the development of new NO-donors. Mini-Rev. Med. Chem. 5, 217-229.

Gasparini, L., Ongini, E., Wilcock, D., Morgan, D., 2005. Activity of flurbiprofen and chemically related anti-inflammatory drugs in models of Alzheimer's disease. Brain. Res. Brain. Res. Rev. 48, 400-408.

Geusens, P., 2009. Naproxcinod, a new cyclooxygenase-inhibiting nitric oxide donator (CINOD). Expert. Opin. Biol. Ther. 9, 649-657.

Govoni, M., Casagrande, S., Maucci, R., Chiroli, V., Tocchetti, P., 2006. J. Pharmacol. Exp. Ther. 317, 752-761.

Guo, Z., Zheng, X., Thompson, W., Dugdale, M., Gollamudi, R., 2000. New carbamoylpiperidines as human platelet aggregation inhibitors. Bioorg. Med. Chem. 8, 1041-1058

Harrison, R., 2005. Organic nitrates and nitrites, in: Wang, P.G., Cai, T. B., Taniguchi, N. (Eds.), Nitric Oxide Donors. Wiley-VCH, Weinheim, pp 33-54.

Horinaka, S., 2011. Use of nicorandil in cardiovascular disease and its optimization. Drugs 71, 1105-1119.

Idris, A.I., Del Soldato, P., Ralston, S.H., van't Hof, R J., 2004. The flurbiprofen derivatives HCT1026 and HCT1027 inhibit bone resorption by a mechanism independent of COX inhibition and nitric oxide production. Bone, 35, 636-643.

Jones, M., Inkielewicz, I., Medina, C., Santos-Martinez, M.J., Radomski, A., Radomski, M.W., Lally, M.N., Moriarty, L.M., Gaynor, J., Carolan, C.G., Khan, D., O'Byrne, P., Harmon, S., Holland, V., Clancy, J.M., Gilmer, J.F., 2009. Isosorbide-based aspirin prodrugs: integration of nitric oxide releasing groups. J. Med. Chem. 52, 6588-6598.

Keeble, J.E., Moore, P.K., 2002. Pharmacology and potential therapeutic applications of nitric oxide-releasing non-steroidal anti-inflammatory and related nitric oxide-donating drugs. Br. J. Pharmacol. 137, 295-310.

Keen, J.H., Habig, W.H., Jakoby, W.B., 1976. Mechanism for the several activities of the glutathione *S*-transferases. J. Biol. Chem. 251, 6183-6188.

Kollau, A., Hofer, A., Russwurm, M., Koesling, D., Keung, W.M., Schmidt, K., Brunner, F., Mayer, B., 2005. Contribution of aldehyde dehydrogenase to mitochondrial bioactivation of nitroglycerin: evidence for the activation of purified soluble guanylate cyclase through direct formation of nitric oxide. Biochem. J. 385, 769-777.

Kurz, M.A., Boyer, T.D., Whalen, R., Peterson, T.E., Harrison, D.G., 1993. Nitroglycerin metabolism in vascular tissue: role of glutathione *S*-transferases and relationship between $NO^{\cdot}$ and NO_2^- formation. Biochem. J. 292, 545-550.

Lazzarato, L., Donnola, M., Rolando, B., Chegaev, K., Marini, E., Cena, C., Di Stilo, A., Fruttero, R., Biondi, S., Ongini, E., Gasco, A., 2009. (Nitrooxyacyloxy)methyl esters of aspirin as novel nitric oxide releasing aspirins. J. Med. Chem. 52, 5058-5068.

Li, B., Sedlacek, M., Manoharan, I., Boopathy, R., Duysen, E. G., Masson, P., Lockridge, O., 2005. Butyrylcholinesterase, paraoxonase, and albumin esterase, but not carboxylesterase, are present in human plasma. Biochem. Pharmacol. 70, 1673-1684.

Lin, G., Chen, G., Lu, C., Yeh, S., 2005. QSARs for peripheral anionic site of butytylcholinesterase with inhibitions by 4-acyloxy-biphenyl-4'-*N*-butylcarbamates. QSAR Comb. Sci., 8, 943-952.

Lucas, K.A., Pitari, G.M., Kazerounian, S., Ruiz-Stewart, I., Park, J., Schulz, S., Chepenik, K.P., Waldman, S.A., 2000. Guanyl cyclases and signaling by cyclic GMP. Pharmacol. Rev. 52, 375-413.

Martelli, A., Rapposelli, S., Calderone, V., 2006. NO-releasing hybrids of cardiovascular drugs. Curr. Med. Chem. 13, 609-625.

McDonald, B.J., Bennett, B.M., 1990. Cytochrome P-450 mediated biotransformation of organic nitrates. Can. J. Physiol. Pharmacol. 68, 1552-1557.

Miller, M.R., Roseberry, M.J., Mazzei, F.A., Butler, A.R., Webb, D.J., Megson, I.L., 2000. Novel *S*-nitrosothiols do not engender vascular tolerance and remain effective in glyceryltrinitrate-tolerant rat femoral arteries. Eur. J. Pharmacol. 408, 335-343.

Miller, M.R., Megson, I.L., 2007. Recent developments in nitric oxide donor drugs. Br. J. Pharmacol. 151, 305-321.

Miller, M. R., Grant, S., Wadsworth, R.M., 2008. Selective arterial dilatation by glyceryl trinitrate is not associated with nitric oxide formation in vitro. J. Vasc. Res. 45, 375-385.

Moncada, S., Palmer, R.M.J., Higgs E.A., 1991. Nitric oxide: Physiology, pathophysiology, and pharmacology. Pharmacol. Rev. 43, 109-142.

Münzel, T., Steven, S., Daiber, A., 2014. Organic nitrates: Update on mechanisms underlying vasodilation, tolerance and endothelial dysfunction. Vascul. Pharmacol. 63, 105-113.

Murad, F., 2006. Nitric oxide and cyclic GMP in cell signaling and drug development. N. Engl. J. Med. 355, 2003-2011.

Napoli, C., Ignarro, L.J., 2009. Nitric oxide and pathogenic mechanisms involved in the development of vascular diseases. Arch. Pharm. Res. 32, 1103-1108.

Prosperi, C., Scali, C., Barba, M., Bellucci, A., Giovannini, M. G., Pepeu, G., Casamenti, F., 2004. Comparison between flurbiprofen and its nitric oxide-releasing derivatives HCT-1026 and

NCX-2216 on Abeta(1-42)-induced brain inflammation and neuronal damage in the rat. Int. J. Immunopath. Pharmacol. 17, 317-330.

Ronchetti, D., Borghi, V., Gaitan, G., Herrero, J. F., Impagnatiello, F., 2009. NCX 2057, a novel NO-releasing derivative of ferulic acid, suppresses inflammatory and nociceptive responses in *in vitro* and *in vivo* models. Br. J. Pharmacol. 158, 569-579

Scatena, R., Bottoni, P., Martorana, G. E., Giardina, B., 2005. Nitric oxide donor drugs: an update on pathophysiology and therapeutic potential. Expert. Opin. Investig. Drugs. 14, 835-46.

Scatena, R., Bottoni, P., Pontoglio, A., Giardina, B., 2010. Pharmacological modulation of nitric oxide release: new pharmacological perspectives, potential benefits and risks. Curr. Med. Chem. 17, 61-73.

Schade, D., Kotthaus, J., Clement, B., 2010. Modulating the NO generating system from a medicinal chemistry perspective: Current trends and therapeutic options in cardiovascular disease. Pharmacol. Ther. 126, 270-300.

Schroder, H., 1992. Cytochrome P-450 mediates bioactivation of organic nitrates. J. Pharmacol. Exp. Ther. 262, 298-302.

Serafim, R.A.M., Primi, M.C., Trossini, G.H.G., Ferreira, E.I., 2012. Nitric oxide: state of the art in drug design. Curr. Med. Chem. 19, 386-405.

Servent, D., Delaforge, M., Ducrocq, C., Mansuy, D., Lenfant, M., 1989. Nitric oxide formation during microsomal hepatic denitration of glyceryl trinitrate: involvement of cytochrome P-450. Biochem. Biophys. Res. Commun. 163, 1210-1216.

Sogo, N., Magid, K.S., Shaw, C.A., Webb, D.J., Megson, I.L., 2000. Inhibition of human platelet aggregation by nitric oxide donor drugs: relative contribution of *c*GMP-independent mechanisms. Biochem. Biophys. Res. Commun. 279, 412-419.

Thatcher, G.R., Bennett, B.M., Reynolds, J.N., 2005. Nitric oxide mimetic molecules as therapeutic agents in Alzheimer's disease. Curr. Alzheimer Res. 2, 171-182.

Taylor, I.W., Ioannides, C., Parke, D.V., 1989. Organic nitrate reductase: reassessment of its subcellular localization and tissue distribution and its relationship to the glutathione transferases. Int. J. Biochem. 21, 67-71.

Torfgard, K.E., Ahlner, J., 1994. Mechanisms of action of nitrates. Cardiovasc. Drugs Ther. 8, 701-717.

Weber, A.A.; Strobach, H; Schror, K., 1993. Direct inhibition of platelet function by organic nitrates via nitric oxide formation. Eur. J. Pharmacol. 247, 29-37.

Wendt, R.L., 1972. Systemic and coronary vascular effects of the 2- and 5-mononitrate esters of isosorbide dinitrate. J. Pharmacol. Exp. Ther. 180, 732-742.

Wenk, G.L., Rosi, S., McGann, K., Hauss-Wegrzyniak, B., 2002. A nitric oxidedonating flurbiprofen derivative reduces neuroinflammation without interacting with galantamine in the rat. Eur. J. Pharmacol. 453, 319-324.

Wenk, G.L., McGann-Gramling, K., Hauss-Wegrzyniak, B., Ronchetti, D., Maucci, R., Rosi, S., Gasparini, L., Ongini, E., 2004. Attenuation of chronic neuroinflammation by a nitric oxide-releasing derivative of the antioxidant ferulic acid. J. Neurochem. 89, 484-493.

World Health Organization, 2013. WHO Model List of Essential Medicines, 18th edition. Web site: http://www.who.int/medicines/publications/essentialmedicines/en/index.html.

Zhang, K., Xue, N., Yuan, Z., Li, L., Shi, X., Cao, L., Du, Y., 2011. Separation of the two enantiomers of naproxcinod by chiral normal phase liquid chromatography. J. Chromatogr. Sci. 49, 272-275.