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New organic nitrate-containing benzyloxy isonipecotanilide derivatives with vasodilatory and anti-platelet activity

Modesto de Candia [a], Elisabetta Marini [b], Giorgia Zaetta [a], Saverio Cellamare [a], Antonella Di Stilo [b], Cosimo D. Altomare [a,*]

[a] Dipartimento di Farmacia-Scienze del Farmaco, Università degli Studi di Bari "Aldo Moro", Via E. Orabona 4, 70125 Bari, Italy
[b] Dipartimento di Scienza e Tecnologia del Farmaco, Università degli Studi di Torino, Via P. Giuria 9, 10125 Torino, Italy

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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.

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.

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*-transferase (GST), xanthine oxidoreductase (XO), the mitochondrial aldehyde dehydrogenase, or the microsomal cytochrome P450 (CYP), have been proposed as mediators of bioactivation of

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; PPP, platelet poor plasma; PRP, platelet rich plasma; RP-HPLC, reversed phase-high performance liquid chromatography; *s*GC, soluble guanylated cyclase.

* Corresponding author. Tel.: +39 080 5442781; fax: +39 080 5442230.
E-mail address: cosimodamiano.altomare@uniba.it (C.D. Altomare).

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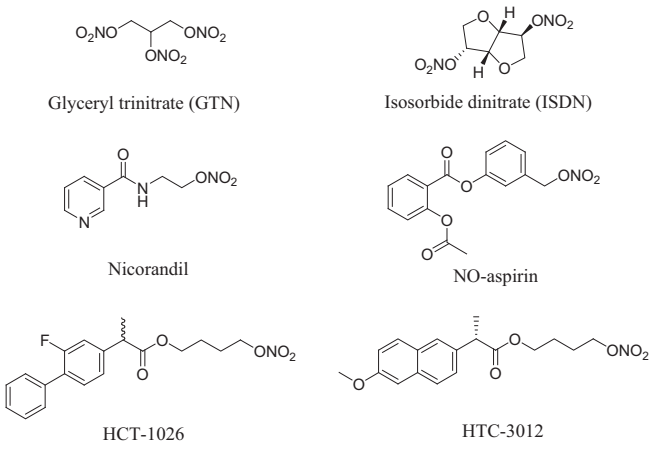

Fig. 1. Organic nitrates used in clinics and some representative experimental NO-donor drugs.

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 monophosphate (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, 2009; 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.

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 sulfate (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

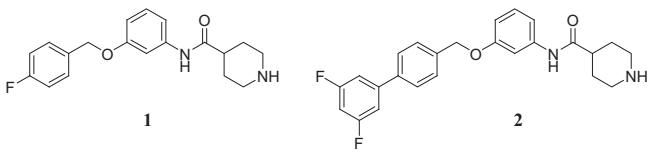

Fig. 2. Fluorinated benzyloxyphenyl piperidine-4-carboxamide derivatives endowed with anti-thrombotic properties (de Candia et al., 2009). Both compounds proved to inhibit ADP-induced platelet aggregation (IC_{50} equals 68 and 27 μM for **1** and **2**, respectively), whereas **2** showed additional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