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Synthesis physicochemical profile and PAMPA study of new NO-donor edaravone co-drugs

Barbara Rolando [a], Andrea Filieri [a], Konstantin Chegaev [a], Loretta Lazzarato [a], Marta Giorgis [a], Claudio De Nardi [b], Roberta Fruttero [a,*], Sophie Martel [c], Pierre-Alain Carrupt [c], Alberto Gasco [a]

[a] Dipartimento di Scienza e Tecnologia del Farmaco, Università degli Studi di Torino, Via Pietro Giuria 9, 10125 Torino, Italy
[b] Nicox Reasearch Institute, Via Ludovico Ariosto 21, 20092 Bresso (Milano), Italy
[c] School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, 30 Quai Ernest-Ansermet, CH 1211 Geneva 4, Switzerland

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ABSTRACT

A new class of co-drugs were synthesised by joining antioxidant edaravone with a vasodilating substructure containing NO-donor nitrooxy functions, and characterised for their stability in different media, lipophilicity and permeability profile. The products display good stability in water/co-solvent at different pH. Conversely, they are rapidly metabolised into edaravone and NO-donor moieties when incubated in human serum or rat-liver homogenates. In the latter conditions time dependent production of nitrite/nitrate (NO_x) occurs. The compounds display wide-ranging lipophilicity. PAMPA studies predict good gastrointestinal absorption for a number of these compounds. The title products are potentially useful for treating ROS-related conditions accompanied by decreased NO availability.

1. Introduction

Edaravone (MC-186) (**1**), is an acid pyrazoline drug that can exist in three tautomeric forms **a**, **b**, **c** (Fig. 1). It was developed in Japan for treating of patients in the acute stage of cerebral thrombosis or embolism, and has been reported to be effective also in the acute stage of stroke. Edaravone displays potent antioxidant properties, and consequently it might be useful in managing other pathological processes involving oxidative stress.[1] Generally, these processes are complex diseases which must be treated with a cocktail of drugs. An alternative could be the use of polyvalent drugs, namely single products active at more than one target, and consequently exercising more than one action simultaneously.[2] The design of a polyvalent drug is achieved by fusing, or joining through appropriate linkers, generally two drugs, or crucial parts of them. The linker can be susceptible to metabolic cleavage (co-drug), or can be a hard linker (dual drug). Another possibility is to overlap substructures which are common to the two leads. We recently proposed a new class of polyvalent drugs: the nitric oxide (NO)-donor antioxidants.[3–5] These compounds are obtained by combining known antioxidants with moieties able to release NO. All products display both antioxidant and NO-dependent activities. They are potentially useful in treating reactive oxygen species (ROS)-related diseases that are accompanied by decreased NO availability. Typical examples of such conditions are atherosclerosis and related diseases. Among the hybrid products we designed, there is also a series of derivatives obtained by combining edaravone with nitrooxy (ONO_2) or furoxan (1,2,5-oxadiazole 2-oxide) NO-donor moieties through a hard linker.[4]

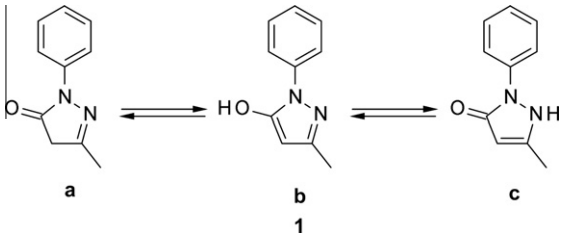

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Figure 1. Edaravone and its tautomeric forms.

As extension of this work, we describe here a new class of co-drugs obtained by joining edaravone through a vulnerable carbonate (**4a–c**) or ester (**7a–i**) linker with alcohols (**2a–c**) or carboxylic acids (**5a–i**) containing ONO_2 functions (Schemes 1 and 2). In contrast with the compounds described above, the antioxidant activity is linked to the edaravone part, recovered after hydrolysis of the vulnerable linker. This difference may lead to different in vivo pharmacokinetic and pharmacodynamics profiles. The synthesis of these products, their stability in different media, their lipophilicity as well as the prediction of their human gastrointestinal permeation (PAMPA assay) are described. Vasodilator

* Corresponding author.
E-mail address: roberta.fruttero@unito.it (R. Fruttero).

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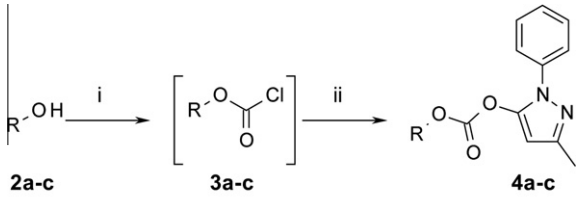
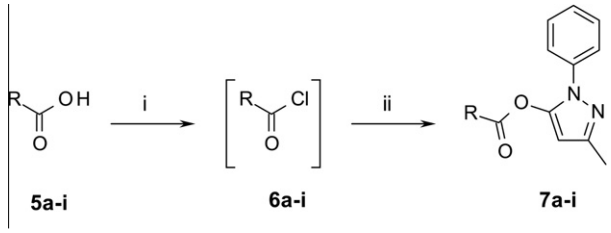


a R = $(CH_2)_3ONO_2$
b R = $(CH_2)_6ONO_2$
c R = $(CH_2)_4CH(ONO_2)CH_2ONO_2$

Scheme 1. Carbonates synthesis. Reagents and conditions: (i) $(Cl_3CO)_2CO$, Py, CH_2Cl_2, 0 °C to rt; (ii) edaravone, Et_3N, CH_2Cl_2, 0 °C to rt.



a R = $(CH_2)_5ONO_2$
b R = $(CH_2)_3CH(ONO_2)CH_2ONO_2$
c R = $C(CH_3)_2CH_2ONO_2$
d R = $C(CH_3)_2(CH_2)_4ONO_2$
e R = $C(CH_3)_2(CH_2)_2CH(ONO_2)CH_2ONO_2$
f R = *p*-$C_6H_4(CH_2)_3ONO_2$
g R = *p*-$C_6H_4O(CH_2)_3ONO_2$
h R = *p*-$C_6H_4CH_2CH(ONO_2)CH_2ONO_2$
i R = *p*-$C_6H_4OCH_2CH(ONO_2)CH_2ONO_2$

Scheme 2. Esters synthesis. Reagents and conditions: (i) $SOCl_2$, toluene, DMF, rt; (ii) edaravone, Et_3N, CH_2Cl_2, 0 °C to rt.

properties of the NO-donor related alcohols and acids used to build the co-drugs, and of selected co-drugs, are also reported.

2. Results and discussion

2.1. Chemistry

The carbonates **4a–c** were obtained by the procedure reported in Scheme 1. The already-known NO donor alcohols **2a–c** (see Section 4) were transformed into the corresponding chloroformates **3a–c**, by action of triphosgene in dichloromethane, in the presence of pyridine. Crude compounds thus obtained were immediately treated with edaravone in dichloromethane solution, in the presence of triethylamine, to give the expected final carbonates as principal reaction products. The assigned pyrazole structures were confirmed by NMR spectroscopy: ^{13}C NMR and ^{1}H NMR spectra of the compounds show a signal occurring at δ 95.6–95.8, attributable to the 4-C aromatic carbon, and a signal occurring at δ 6.09 or 6.10 attributable to 4-CH methyne proton. Additional evidence of the assigned structures derives from the capacity of the products to undergo hydrolysis, yielding edaravone. Synthesis of the final esters **7a–i** required preliminary preparation of the NO-donor acids **5a–i** (Scheme 2); a number of these, compounds **5a–c** and **5f,g**, are known products (see Section 4), while acids **5d,e** were obtained via the pathway reported in Scheme 3. Methylisobutyrate (**8**) was alkylated by action of 4-bromobut-1-ene in the presence of lithium diisopropylamide, prepared in situ. The resulting methyl 2,2-dimethylhex-5-enoate (**9**) was treated with $AgNO_3$ and I_2 in CH_3CN to give the dinitrooxy substituted ester **10**. Finally, acid hydrolysis of this ester afforded the corresponding acid **5e**. Hydroboration of the unsaturated ester **9** by action of diisoamylborane, prepared in situ, followed by oxidation with hydrogen peroxide, gave **11**. Basic hydrolysis of this intermediate, followed by the action of sulfonitric mixture in dichloromethane, afforded the corresponding final nitrooxy substituted acid **5d**.

NO-donor acids **5a–i** were transformed into the corresponding acyl chlorides **6a–i** by action of thionyl chloride in toluene, in the presence of a catalytic amount of DMF (Scheme 2). These intermediates were not purified or characterised, but immediately transformed into the desired products **7a–i** by reaction with edaravone, in the presence of triethylamine. The pyrazole structures assigned to these final compounds were confirmed by NMR spectroscopy: ^{13}C NMR and ^{1}H NMR spectra of the products show a signal occurring in the range δ 95.6÷95.8, attributable to the 4-C aromatic carbon, and a signal occurring in the range δ 6.06÷6.28 attributable to 4-CH methyne proton. Also for this class of products, additional evidence of the assigned structures derives from their capacity to undergo hydrolysis yielding edaravone.

2.2. Stability and metabolic studies

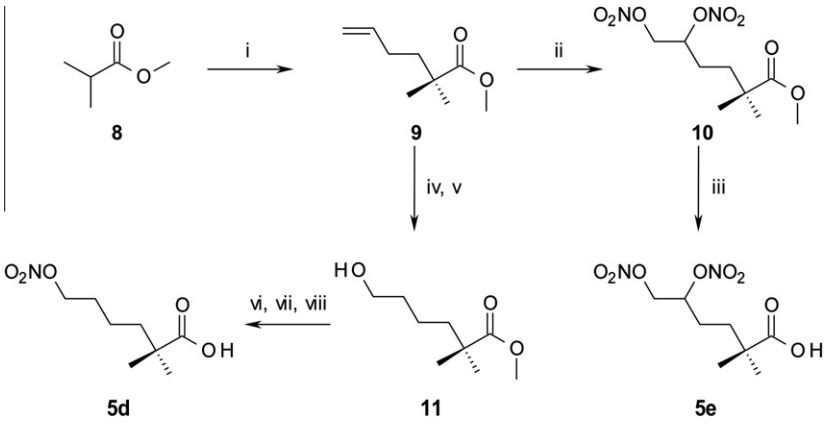
The stabilities of the co-drugs reported here were assessed by high-performance liquid chromatography (HPLC), in aqueous media, in human serum and in rat-liver homogenate.

2.2.1. Stability in aqueous media

The stability of all the compounds was assessed in water/DMSO 5% (v/v) (the experimental conditions of PAMPA permeability studies), and in acidic medium (0.1 M HCl/MeOH 60/40) to mimic the gastric environment. In both media, the products underwent hydrolysis following pseudo-first-order kinetics, affording edaravone and the related nitrooxy substituted alcohol and acid moieties. The observed pseudo-first-order rate constants (k_{obs}) and half-lives ($t_{1/2}$, Table 1) were determined by fitting the data with one-phase exponential decay equation (Graph Pad, Prism software version 5). Table 1 shows that half-lives are quite different in the two media used. The highest stabilities are in water/DMSO 5% (v/v). In this medium $t_{1/2}$ values of the three carbonates tested lie in the range 25–6 h and rank in the order **4b** > **4a** > **4c**, indicating that the stability of the products increases on moving from the propyl to the hexyl chain bearing at the ω-position the nitrooxy function, and decreases when two of these groups are present on the hexyl chain at ω- and ω-1 positions. The two esters **7a**,**b** are slowly hydrolyzed, and again the dinitrooxy compound **7b** is less stable than its mono-substituted analogue **7a**. The introduction of two methyl groups at α-position of the ester function gives rise to products which display high $t_{1/2}$ values in the order **7c** > **7d** > **7e**. Their low water solubility prevented us from studying the aromatic esters **7f–i**. In acidic medium, the compounds are less stable than in water, with the sole exception of **7d**. Also in this medium, dinitrooxy compounds are less stable than their mono-substituted analogues (compare **4b**/**4c**, **7a**/**7b**, **7d**/**7e**, **7g**/**7i**).

2.2.2. Stability in human serum and in rat-liver homogenate

When incubated in human serum or in rat-liver homogenates, all products were rapidly hydrolyzed, giving edaravone and the NO-donor moieties. In these conditions, hydrolysis is catalysed by carboxylesterases; these enzymes are ubiquitous and display broad substrate specificity. Frequently, an ester can be hydrolyzed by more than one of these enzymes, and as yet a precise classification has not been proposed.[6] Humans express carboxylesterases in several compartments, including liver, plasma, small intestine, brain, stomach, colon macrophages, and monocytes.[7] Table 1 shows that carbonates and unbranched aliphatic esters display $t_{1/2}$ values <1 min, while values for branched aliphatic esters and aromatic esters lie in the



Scheme 3. Synthesis of NO-donor acids **5d**,**e**. Reagents and conditions: (i) BuLi, DPA, 4-bromobut-1-ene, HMPA, THF dry, −78 °C; (ii) $AgNO_3$, I_2, CH_3CN, rt, then △; (iii) HCl 6 M, dioxane, △; (iv) $NaBH_4$, $BF_3{\cdot}Et_2O$, 2-methylbut-1-en, THF dry, 0 °C rt; (v) NaOH, H_2O_2, 40 °C; (vi) NaOH, MeOH, △; (vii) HCl; (viii) HNO_3, H_2SO_4, CH_2Cl_2, 0 °C.

Table 1
Stability in aqueous media, in human serum and in rat-liver homogenate

Compound	$t_{1/2}$			
	Water (5% DMSO, 0.1 M KCl)[a]	HCl 0.1 M/MeOH (60/40, v/v)[c]	Human serum[d]	Rat-liver homogenate[d]
Edaravone	Stable	Stable	Stable	n.d.
4a	13 h	1.4 h	<1 min	<1 min
4b	25 h	4.0 h	<1 min	<1 min
4c	6 h	3.1 h	<1 min	<1 min
7a	31 h	1.0 h	<1 min	<1 min
7b	10 h	0.7 h	<1 min	<1 min
7c	36 h	1.9 h	1.9 min	<1 min
7d	22 h	24 h	13.3 min	2 min
7e	16 h	10 h	16.2 min	3 min
7f	[b]	13 h	4.0 min	<1 min
7g	[b]	35 h	5.0 min	<1 min
7h	[b]	8 h	7.1 min	<1 min
7i	[b]	25 h	7.8 min	<1 min

[a] Room temperature; $n = 3$, SD <1.
[b] Not determined due to low solubility in the experimental conditions.
[c] $t = 37$ °C; $n = 3$, SD <0.1.
[d] $t = 37$ °C; $n = 3$, SD <0.3.

range 1.9–16.2 min in human serum. For all compounds, hydrolysis is almost immediate in rat-liver homogenate.

In liver homogenate, production of nitrite (NO_2^-) and nitrate (NO_3^-), the oxidised metabolites of NO derived from biotransformation of the organic nitrates, was time-dependent. Figure 2A and B reports, as an example, the time course of nitrite and nitrate generated from **7d** and **7e** over the incubation time. Figure 2C shows NO_x (nitrite + nitrate) produced by all compounds under study after 5 h incubation. As expected, the highest levels of NO_x were obtained from the dinitrooxy substituted products **4c**, **7e**, **7h**, **7i**.

2.2.3. In vivo study

Release of nitrite was studied preliminarily in vivo for compounds **4a** and **7d**, following oral administration of 10 mg/kg in mice (imwitor742/tween80 50/50, vehicle). Production of nitrite over 24 h was monitored. Nitrite AUC_{0-24h} was 964 μM min and 1790 μM min, respectively. Under the same conditions, AUC_{0-24h} for isosorbide 5-mononitrate (ISMN) was 2352 μM min.

2.3. Lipophilicity studies

The lipophilicity ($\log P_{oct}$) of the new products (Table 2) was determined by a RP-HPLC method (see Section 4 for details). The method was calibrated (Eq. 1) by plotting $\log P_{oct}$ values of 63 reference compounds against the corresponding extrapolated retention $\log k_w$ factors.[5] This equation was used for the evaluation of $\log P$ of edaravone and edaravone derivatives from their $\log k_w$ factors, measured under the same conditions as the reference compounds.

$$\log P_{oct} = 1.098(\pm 0.022)\log k_w + 0.335(\pm 0.047) \quad (1)$$

$N = 63$, $r^2 = 0.98$, $s = 0.15$, $F = 2531$.

The experimental $\log P_s$ of edaravone derivatives vary widely across the series (from 3.35 to 5.14) and are higher than that of parent compound (edaravone, $\log P_{oct} = 1.44$). Most of them are in accordance with calculated values (CLOGP, Bio-Loom for Windows v. 1.5, BioByte Corp., Claremont, CA, USA) within ±0.4, while others only slightly exceed this range.

2.4. Permeability studies

The PAMPA assay is a recently-developed procedure for the rapid determination of passive transport permeability, which is widely accepted in pharmaceutical research circles. In this method, the donor compartment, containing a buffer solution of the tested compound, and the acceptor compartment, containing an initial fresh buffer solution, are separated by a 96-well filter plate coated with a liquid artificial membrane. To predict gastrointestinal absorption, phospholipids[8] or hexadecane[9] have been proposed as artificial membrane. Although the PAMPA-HDM (hexadecane membrane) does not contain phospholipids, the formation of a

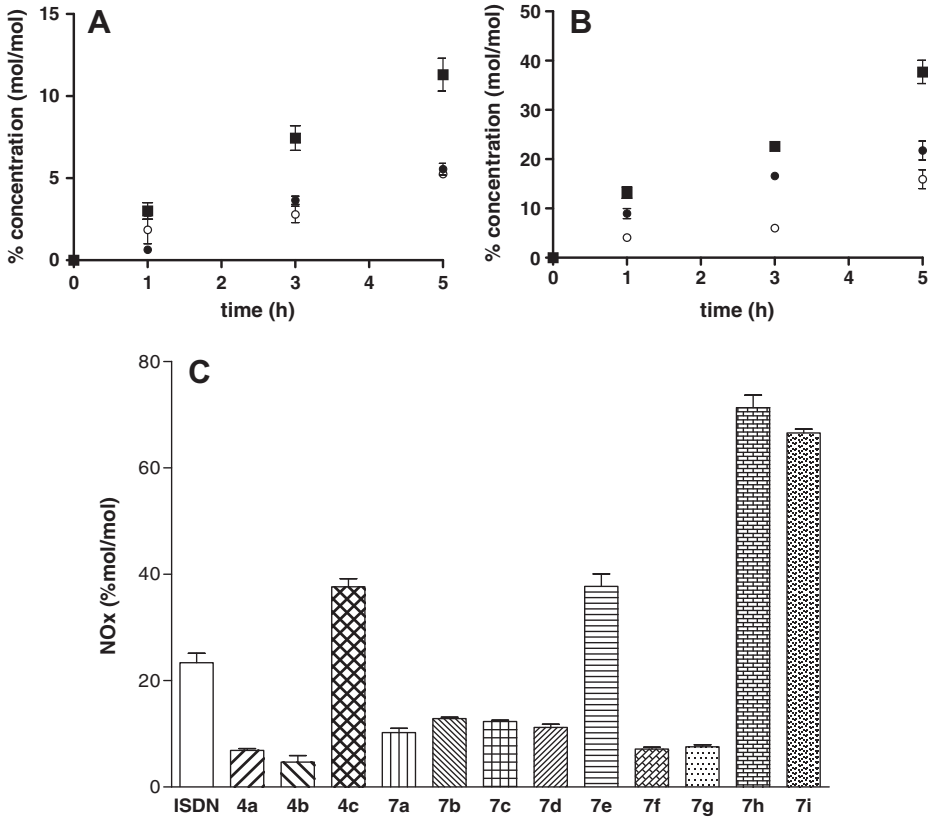


Figure 2. The time course of NO_x species generated from **7d** (A) and **7e** (B) over the incubation time in rat-liver homogenate. Data are expressed as %mol NO_x/mol compound: ■ NO_x (nitrite + nitrate); ● nitrite; ○ nitrate. (C) total NO_x (nitrite + nitrate) generated after 5 h of incubation of NO-donor edaravone co-drugs and ISDN (isosorbide dintrate, as reference compound), in rat-liver homogenate; data are expressed as %mol NO_x/mol compound. Values are presented as mean ± SD; number of determinations = 3.

Table 2
Calculated and measured $\log P_{oct}$, membrane retention (R) and effective permeability coefficient ($\log P_e$) obtained from PAMPA-HDM experiments

Compound	$C\log P_{oct}$[a]	$\log P_{oct}$[b]	PAMPA-HDM	
			R (±SD)	$\log P_e$ (±SD)
Edaravone	1.33	1.44	0.12 ± 0.00	−3.52 ± 0.01
4a	2.85	3.35	0.21 ± 0.03	−3.51 ± 0.05
4b	4.15	4.39	0.92 ± 0.04	−4.68 ± 0.24
4c	3.96	4.63	0.50 ± 0.01	−4.40 ± 0.08
7a	3.84	3.79	0.69 ± 0.08	−4.93 ± 0.10
7b	3.65	3.95	0.46 ± 0.01	−3.75 ± 0.08
7c	3.36	3.55	0.60 ± 0.05	−3.56 ± 0.05
7d	4.55	4.30	0.89 ± 0.02	−4.06 ± 0.12
7e	4.36	4.04	0.88 ± 0.04	−4.12 ± 0.15
7f	5.41	4.76	[c]	[c]
7g	5.17	4.74	[c]	[c]
7h	5.37	4.88	[c]	[c]
7i	5.26	5.14	[c]	[c]

[a] Bio-Loom for Windows v.1.5, Bio Byte Corp., Claremont, CA, USA.
[b] Determined by RP-HPLC (SE ⩽ 0.05).
[c] Not determined due to low solubility in the experimental conditions.

bilayer membrane has never been characterized in the phospholipid-based PAMPA assay. As a consequence, PAMPA-HDM was used as a simpler and more robust alternative to predict GI permeation.

Equations to calculate the effective permeability P_e can be deduced in several ways, depending on experimental conditions and on the design of the in vitro assay. When retention must not be neglected, the no-sink conditions in acceptor wells are fixed, and compounds degrade following a first-order kinetics in experimental conditions, thus the effective permeability coefficients P_e (cm/s) can be calculated using the following equation (see Supplementary data for details):

$$P_e = -\frac{2.303V_d}{(1+r_v)(t-\tau_{Lag})A}\log 10\left(1-\frac{(1+r_v^{-1})}{(1-R)e^{-kt}}\frac{C_a(t)}{C_d(0)}\right) \quad (2)$$

where $r_v = V_d/V_a$, A (cm^2) is the filter area, t is the incubation time (s), τ_{Lag} is the steady-state time (s), namely the time needed for the permeant's concentration gradient to became stabilised, V_a and V_d (cm^3) are the volumes in the acceptor and the donor wells, respectively, $C_a(t)$ is the concentration of the compound (mol cm^{-3}) in the acceptor well at time t, $C_d(0)$ is the concentration of the compound (mol cm^{-3}) in the donor well at time 0, and k is the pseudo-first-order rate constant of the hydrolysis. R is the retention factor defined as the mole fraction of compound that is lost in the membrane and in the microplates (filters and plate material) and calculated from Eq. 3.

$$R = 1 - \frac{C_d(t)}{C_d(0)e^{-kt}} - \frac{V_aC_a(t)}{V_dC_d(0)e^{-kt}} \quad (3)$$

where $C_a(t)/C_d(0)$ represents the fraction of compound that reaches the acceptor compartment after the incubation time t (for $V_a = V_d$).

PAMPA experiments were carried out on edaravone and its new derivatives, using filters impregnated with hexadecane to evaluate the gastrointestinal passive permeability of these compounds (PAMPA-HDM experiments). The $\log P_e$ and R values calculated from Eqs. 2 and 3 are in Table 2. The use of a simple solvent, namely hexadecane, as artificial membrane leads to a more robust model than using phospholipids. Indeed, for a set of 32 chemically-diverse drugs, Wohnsland et al. described quite a good sigmoidal correlation between their $\log P_e$ value, measured by PAMPA-HDM, and human absorption.[9] The original $\log P_e$ values of a set

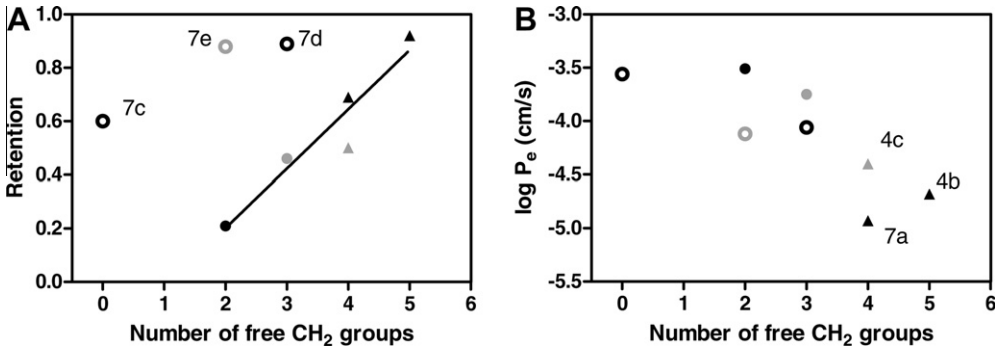


Figure 3. (A) Correlation between retention (R) and number of CH_2 groups. (B) Correlation between permeability coefficient ($\log P_e$) from PAMPA experiments and number of CH_2 groups. ● compounds with up to 3 free non substituted CH_2 groups; ▲ compounds with 4 or more free non substituted CH_2 groups; ○ compounds with substituted CH_2 groups. Black symbols correspond to compounds having a unique ONO_2 group, and grey symbols to compounds having 2 ONO_2 groups.

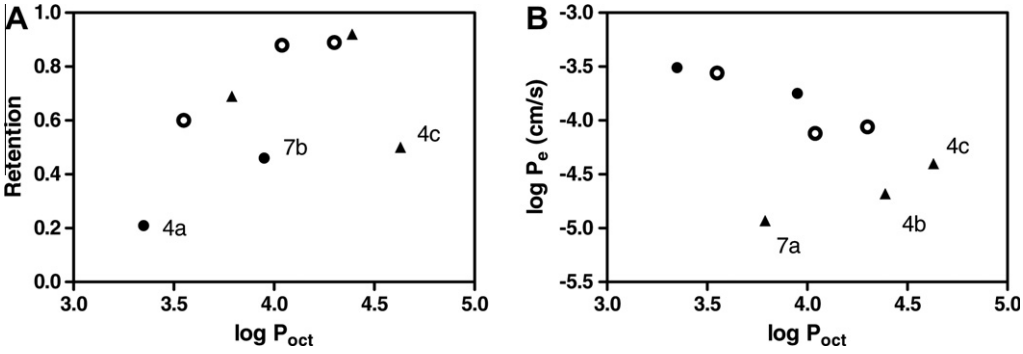


Figure 4. Correlation between octanol–water partition coefficients and retention (R) from PAMPA experiments (A) and permeability coefficients ($\log P_e$) (B). ● compounds with up to 3 free non-substituted CH_2 groups; ▲ compounds with 4 or more free non-substituted CH_2 groups; ○ compounds with substituted CH_2 groups.

of 9 reference compounds were first compared to $\log P_e$ values obtained in house. The correlation (data not shown) produced an r^2 of 0.83, a Y-intercept of 0.008 and a slope of 1.04. This means that values of $\log P_e$ for edaravone derivatives can be compared to those published in Wohnsland et al.[9] The $\log P_e$ values measured for these new edaravone NO-donor co-drugs (Table 2) vary across the series of compounds studied: around −3.5 for compounds **4a**, **7b**, and **7c**, in the range −4.06 to −4.40 for **4c**, **7d**, and **7e**, −4.68 for **4b**, and −4.93 for **7a**.

The retention factors R are quite high for most derivatives. The retention linearly correlates with the number of free CH_2 groups for most compounds, excepted for **7c, 7d** and **7e** (Fig. 3A). The higher retention of most compounds than edaravone may partly be due to adsorption processes on the plastic material from which the filter-microplates are made: it is known that molecules bearing nitrooxy groups can be adsorbed onto plastic materials.[10] However, the variation of retention factors is not correlated with the number of ONO_2 substituents, as shown in Figure 3A, in which black symbols represent compounds having a single ONO_2 group, and grey symbols to compounds having two ONO_2 groups. But the combination of fairly high lipophilicity (Fig. 4A) and the presence of ONO_2 substituents could explain significant adsorption onto plastic material.[10] However, since P_e is an 'effective' permeation value, it does not depend on the loss of compound through membrane retention or adsorption processes. The $\log P_e$ values obtained, ranging from −3.5 to −4.1 (excepted compounds **4b**, **4c** and **7a**), indicate that these edaravone derivatives can be classified as 'high permeant' compounds, compared to reference compounds. In the reference set, compounds having $\log P_e$ between −3.3 and −4.1 were those having intestinal absorption above 70%, whereas compounds with $\log P_e$ below −4.8 had 20% intestinal absorption or less.[9] Although membrane retention cannot be determined, since considerable adsorption onto the microplate probably occurred, and hypothesizing that the loss of compound observed in the PAMPA experiments is mainly due to this adsorption, edaravone derivatives (excepted for compounds **4b**, **4c** and **7a**) might possess high potential for passive penetration through the intestinal barrier. Permeability through artificial membrane appears to be lower for compounds **4b, 7a** and, to a lesser extent, for compound **4c**. It may therefore be assumed that intestinal absorption is limited by the increasing number of free CH_2 groups (Fig. 3B).

In addition, no clear relationship exists between these permeation parameters and the $\log P_{oct}$ value measured by HPLC (Fig. 4A and B). In general, permeability through an artificial membrane decreases, and membrane retention increases, with increased lipophilicity. However, a number of compounds deviate from this tendency, suggesting that permeability may be lowered by the presence of long linear hydrophobic CH_2 chains (compounds **4b**, **4c** and **7a**).

2.5. Vasodilator studies

The vasodilator activity of the NO-donor nitrooxy substituted alcohols and acids, which are rapidly generated when edaravone co-drugs are incubated in human serum, was evaluated on denuded rat aorta strips precontracted with phenylephrine. All compounds relaxed the contracted tissue in a concentration-dependent manner. Their potencies, expressed as EC_{50}, are in Table 3. The dinitrooxy substituted compounds **5b, 5h**, **5i**, **2c** were more potent than their mono-substituted analogues **5a, 5f, 5g** and **2b**. Alcohols **2b** and **2c** are better vasodilators than the structurally-related acids **5a** and **5b**, which might partly be due to their greater facility

Table 3
Vasodilator activity of nitrooxy-substituted alcohols **2a–c** and acids **5a–i** generated from the hydrolysis of edaravone co-drugs by esterases, and of **7d**,**7e**, the most stable compounds in human serum

Compound	EC_{50} (μM) ± SEM	EC_{50} (μM) ± SEM + 1 μM ODQ
2a	4.0 ± 0.6	>100
2b	1.5 ± 0.4	>100
2c	0.83 ± 0.17	>100
5a	8.3 ± 1.5[a]	>100
5b	5.8 ± 0.8[a]	>100
5c	13 ± 2	>100
5d	8.9 ± 2.2	>100
7d	11 ± 5	>100
5e	4.2 ± 1.2	>100
7e	2.5 ± 0.4	>100
5f	0.51 ± 0.08[a]	>100
5g	0.62 ± 0.07[a]	66 ± 12
5h	0.33 ± 0.06[a]	>100
5i	0.28 ± 0.04[a]	67 ± 6

[a] Taken from Ref.[12].

to penetrate into smooth muscle cells, consequent on their higher lipophilicity. When the experiments were repeated in the presence of 1 μM ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one), potencies decreased, in keeping with NO-induced activation of the sGC being the mechanism underlying the vasodilator effect. Vasodilator experiments were also carried out on the whole co-drugs **7d** and **7e**, the most stable versus serum esterases. As expected, their vasodilator activity is close to those of the individual related NO-donor moieties **5d** and **5e**.

3. Conclusion

In conclusion, we prepared new NO-donor edaravone co-drugs by joining edaravone, through a vulnerable carbonate or ester linker, with alcohols or carboxylic acids containing NO-donor nitrooxy functions. The resulting products display wide-ranging lipophilicity values, and for a number of these compounds PAMPA studies predict good gastrointestinal absorption. They display good stability in acidic medium; conversely, when incubated in human serum or in liver homogenate they undergo rapid hydrolysis by esterases, yielding edaravone and the nitrooxy functions (NO-donor alcohols and acids). In liver homogenate, all NO-donor moieties deriving from hydrolysis of the co-drugs produce inorganic nitrite and nitrate in a time-dependant manner. The NO-donor alcohols and acids relax rat aorta strips precontracted with phenylephrine in a concentration-dependent manner, through a cGMP dependent mechanism. The co-drugs described here are a new class of polyvalent agents, which combine edaravone-dependent activities with the NO-dependent activities of organic nitrates. For this reason they are potentially interesting for the treatment of ROS-related diseases accompanied by decreased NO availability, such as atherosclerosis and diabetes.

4. Experimental section

4.1. Chemistry

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 300; abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; qi, quintet; m, multiplet; br, broad. Low resolution mass spectra were recorded with a Finnigan-Mat TSQ-700. Melting points were determined with a capillary apparatus (Büchi 540). Flash column chromatography was performed on silica gel (Merck Kieselgel 60, 230-400 mesh ASTM); PE stands for 40–60 petroleum ether. The progress of the reactions was followed by thin layer chromatography (TLC) on 5 × 20 cm plates with a layer thickness of 0.20 mm. Anhydrous magnesium sulphate was used as drying agent for the organic phases. Organic solvents were removed under vacuum at 30 °C. Preparative HPLC was performed on a LiChrospher® C_{18} column (250 × 25 mm, 10 μm) (Merck Darmstadt, Germany) with a Varian ProStar mod-210 with Varian UV detector mod-325. Elemental analyses (C, H, N) of the new compounds dried at 20 °C, pressure <10 mmHg for 24 h, were performed at the University of Geneva and the results are within ±0.4% of the theoretical values. Structures **2a**, **2b**,[11] **2c**,[12] **5a**,[13] **5b**,[14] **5c**,[15] **5f**, **5g**,[16] **5h** and **5i**[17] were synthesized by published methods. Methyl 2,2-dimethylhex-5-enoate (**9**) was obtained using a published procedure starting from methyl isobutyrate.[18] Tetrahydrofuran (THF) was distilled immediately before use from Na and benzophenone under a positive atmosphere of N_2.

4.1.1. General procedure for preparation of carbonate derivatives 4a–c

A solution of the appropriate alcohol (**2a–c**) (2.5 mmol) and anhydrous pyridine (0.20 mL; 2.5 mmol) in dry CH_2Cl_2 (10 mL) was added dropwise to a stirred solution of triphosgene (0.29 g; 1.0 mmol) in dry CH_2Cl_2 (5 mL) at 0 °C. The reaction mixture was stirred at 0 °C until reaction completion. Organic phase was washed with 1 M HCl (10 mL), brine and dried. The CH_2Cl_2 solution of the crude chloroformate (**3a–c**) thus obtained was added dropwise to a solution of **1** (0.45 g; 2.6 mmol) and Et_3N (0.36 mL; 2.6 mmol) in dry CH_2Cl_2 (20 mL) at 0 °C. The reaction mixture was stirred at rt for 1 h, then washed with H_2O (20 mL), 0.1 M HCl (50 mL), brine, dried, and the solvent evaporated. The product was purified by flash chromatography using the eluents indicated. Solid substances were further purified by crystallization.

4.1.1.1. 3-Methyl-1-phenyl-1*H*-pyrazol-5-yl 3-(nitrooxy)propyl carbonate (4a). Eluent PE/EtOAc 8/2 v/v; yellow oil which solidified in the freezer. The solid obtained was crystallized from *i*-Pr_2O to give the title compound as a white solid, mp = 42–43 °C; 51% yield. ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 2.11 (qi, 2H, CH_2), 2.33 (s, 3H, CH_3), 4.33 (t, 2H, CH_2), 4.51 (t, 2H, CH_2), 6.10 (s, 1H, C*H*), 7.30–7.35 (m, 1H), 7.42–7.47 (m, 2H), 7.54–7.57 ppm (m, 2H) (C_6H_5); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 14.5, 26.2, 65.5, 69.0, 95.4, 122.9, 127.3, 129.2, 137.8, 144.4, 149.0, 150.7 ppm. MS *m/z* 322 $(M+H)^+$. Anal. Calcd for $C_{14}H_{15}N_3O_6$: C, 52.34; H, 4.70; N, 13.08. Found: C, 52.36; H, 4.73; N, 13.12.

4.1.1.2. 3-Methyl-1-phenyl-1*H*-pyrazol-5-yl 6-(nitrooxy)hexyl carbonate (4b). Eluent PE/EtOAc 8/2 v/v; yellow oil which solidified in the freezer. The solid obtained was crystallized from hexane to give the title compound as a white solid, mp = 39–40 °C; 60% yield. ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 1.36–1.42 (m, 4H, 2CH_2), 1.66–1.74 (m, 4H, 2CH_2), 2.32 (s, 3H, CH_3), 4.22 (t, 2H, CH_2), 4.43 (t, 2H, CH_2), 6.09 (s, 1H, C*H*), 7.29–7.34 (m, 1H), 7.41–7.46 (m, 2H), 7.55–7.58 ppm (m, 2H) (C_6H_5); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 14.5, 25.1, 25.3, 26.6, 28.2, 69.5, 73.0, 95.4, 122.9, 127.2, 129.1, 137.9, 144.6, 148.9, 151.0 ppm. MS *m/z* 363 $(M+H)^+$. Anal. Calcd for $C_{17}H_{21}N_3O_6$: C, 56.19; H, 5.82; N, 11.56. Found: C, 56.23; H, 5.86; N, 11.58.

4.1.1.3. 5,6-Bis(nitrooxy)hexyl 3-methyl-1-phenyl-1*H*-pyrazol-5-yl carbonate (4c). Eluent PE/EtOAc 8/2 v/v; yellow oil; 64% yield. ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 1.43–1.56 (m, 2H, CH_2), 1.68–1.79 (m, 4H, 2CH_2), 2.32 (s, 3H, CH_3), 4.23 (t, 2H, CH_2), 4.44 (dd, 1H, C*H*H), 4.71 (dd, 1H, CH*H*), 5.23–5.27 (m, 1H, C*H*), 6.09 (s, 1H, C*H*), 7.29–7.35 (m, 1H), 7.41–7.47 (m, 2H), 7.54–7.58 ppm (m, 2H) (C_6H_5); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 14.5, 21.3, 28.0, 28.9, 68.9, 71.0, 78.7, 95.4, 122.9, 127.2, 129.1, 137.9, 144.5,

148.9, 150.9 ppm. MS *m/z* 424 $(M)^+$. Anal. Calcd for $C_{17}H_{20}N_4O_9$: C, 48.12; H, 4.75; N, 13.20. Found: C, 47.9; H, 4.80; N, 13.09.

4.1.2. General procedure for preparation of ester derivatives 7a–i

To a solution/suspension of appropriate acid (**5a–i**) (3.5 mmol) in dry toluene (5 mL) anhydrous DMF (two drops) was added, followed by $SOCl_2$ (0.3 mL; 4.2 mmol). The reaction mixture was stirred at rt until reaction completion (monitored by TLC). Solvent was evaporated and the acylchloride obtained (**6a–i**) was dissolved in dry CH_2Cl_2 (15 mL). The resulting solution was added dropwise to a solution of **1** (0.55 g; 3.2 mmol) and Et_3N (0.50 mL; 3.2 mmol) in dry CH_2Cl_2 (20 mL) at 0 °C. The reaction mixture was stirred at rt for 1 h, then washed with H_2O (20 mL), 0.1 M HCl (50 mL), brine, dried, and the solvent evaporated. The product was purified by flash chromatography with the eluent indicated. Solid substances were further purified by crystallization.

4.1.2.1. 3-Methyl-1-phenyl-1*H*-pyrazol-5-yl 6-(nitrooxy)hexanoate (7a). Eluent PE/EtOAc 9/1 v/v; yellow oil which solidified in the freezer. The solid obtained was crystallized from hexane to give the title compound as a white solid, mp = 39–40 °C; 45% yield. ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 1.34–1.44 (m, 2H, C*H*$_2$), 1.64–1.75 (m, 4H, 2C*H*$_2$), 2.32 (s, 3H, C*H*$_3$), 2.52 (t, 2H, C*H*$_2$), 4.39 (t, 2H, C*H*$_2$) 6.08 (s, 1H, *CH*), 7.29–7.34 (m, 1H), 7.40–7.46 (m, 2H), 7.50–7.53 ppm (m, 2H) (C_6H_5); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 14.5, 24.0, 25.0, 26.4, 33.7, 72.8, 95.8, 123.3, 127.2, 129.1, 138.0, 144.3, 149.0, 168.6 ppm. MS *m/z* 333 $(M)^+$. Anal. Calcd for $C_{16}H_{19}N_3O_5$: C, 57.65; H, 5.74; N, 12.60. Found: C, 57.56; H, 5.66; N, 12.56.

4.1.2.2. 3-Methyl-1-phenyl-1*H*-pyrazol-5-yl 5,6-bis(nitrooxy) hexanoate (7b). Eluent PE/EtOAc 8/2 v/v; yellow oil; 75% yield. ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 1.68–1.82 (m, 4H, 2C*H*$_2$), 2.32 (s, 3H, C*H*$_3$), 2.56–2.60 (m, 2H, C*H*$_2$), 4.40 (dd, 1H, C*H*H), 4.67 (dd, 1H, CH*H*), 5.21–5.22 (m, 1H, C*H*), 6.08 (s, 1H, C*H*), 7.35–7.36 (m, 1H), 7.41–7.52 ppm (m, 4H) (C_6H_5); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 14.5, 19.9, 28.4, 33.1, 70.9, 78.5, 95.8, 123.3, 127.4, 129.1, 138.0, 144.0, 149.0, 168.1 ppm. MS *m/z* 394 $(M)^+$. Anal. Calcd for $C_{16}H_{18}N_4O_8$: C, 48.73; H, 4.60; N, 14.21. Found: C, 48.74; H, 4.70; N, 14.16.

4.1.2.3. 3-Methyl-1-phenyl-1*H*-pyrazol-5-yl 2,2-dimethyl-3-(nitrooxy)propanoate (7c). Eluent PE/EtOAc 9/1 v/v; yellow oil which solidified on standing. The resulting solid was crystallized from hexane to give the title compound as a white solid, mp = 35.5–36.0 °C; 92% yield. ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 1.33 (s, 6H, 2C*H*$_3$), 2.33 (s, 3H, C*H*$_3$), 4.49 (s, C*H*$_2$), 6.10 (s, 1H, C*H*), 7.34–7.48 ppm (m, 5H) (C_6H_5); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 14.5, 22.2, 42.5, 76.8, 95.7, 123.7, 127.7, 129.0, 137.7, 143.9, 149.0, 170.0 ppm. MS *m/z* 319 $(M)^+$. Anal. Calcd for $C_{15}H_{17}N_3O_5$: C, 56.42; H, 5.37; N, 13.16. Found: C, 56.40; H, 5.44; N, 12.99.

4.1.2.4. 3-Methyl-1-phenyl-1*H*-pyrazol-5-yl 2,2-dimethyl-6-(nitrooxy)hexanoate (7d). Eluent PE/EtOAc 9/1 v/v; yellow oil which solidified on standing. The resulting solid was crystallized from hexane to give the title compound as a white solid, mp = 51.0–51.5 °C; 90% yield. ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 1.17–1.28 (m, 8H, 2C*H*$_3$, C*H*$_2$), 1.54–1.63 (m, 4H, 2C*H*$_2$), 2.33 (s, 3H, C*H*$_3$), 4.32 (t, 2H, C*H*$_2$), 6.06 (s, 1H, C*H*), 7.30–7.52 ppm (m, 5H) (C_6H_5); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 14.5, 21.2, 24.9, 27.0, 39.8, 42.7, 72.8, 95.6, 123.8, 127.4, 128.9, 137.9, 144.5, 149.0, 173.0 ppm. MS *m/z* 361 $(M)^+$. Anal. Calcd for $C_{18}H_{23}N_3O_5$: C, 59.82; H, 6.41; N, 11.63. Found: C, 59.95; H, 6.41; N, 11.65.

4.1.2.5. 3-Methyl-1-phenyl-1*H*-pyrazol-5-yl 2,2-dimethyl-5,6-bis(nitrooxy)hexanoate (7e). Eluent PE/EtOAc 85/15 v/v. The oil obtained was further purified by reverse phase flash chromatography (RP-18, eluent MeCN/H_2O 6/4 v/v); yellow oil; 45% yield. ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 1.25 (s, 3H, C*H*$_3$), 1.27 (s, 3H, C*H*$_3$), 1.45–1.70 (m, 4H, 2C*H*$_2$), 2.33 (s, 3H, C*H*$_3$), 4.31 (dd, 1H, C*H*H), 4.58 (dd, 1H, CH*H*), 5.06–5.10 (m, 1H, C*H*), 6.07 (s, 1H, C*H*), 7.32–7.51 ppm (m, 5H) (C_6H_5); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 14.5, 24.5, 24.9, 25.3, 35.2, 42.4, 70.9, 78.8, 95.6, 124.0, 127.7, 129.0, 137.8, 144.3, 149.1, 172.5 ppm. MS *m/z* 422 $(M)^+$. Anal. Calcd for $C_{18}H_{22}N_4O_8$: C, 51.18; H, 5.25; N, 13.26. Found: C, 51.01; H, 5.24; N, 13.12.

4.1.2.6. 3-Methyl-1-phenyl-1*H*-pyrazol-5-yl 4-[3-(nitrooxy)propyl]benzoate (7f). Eluent PE/EtOAc 8/2 v/v. The oil obtained was further purified by preparative HPLC (eluent MeCN/H_2O 7/3 v/v); colourless oil which solidified in the freezer; 79% yield. The resulting solid was crystallized from hexane to give the title compound as a white solid, mp = 38.5–39.5 °C. ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 2.06–2.11(m, 2H, C*H*$_2$), 2.36 (s, 3H, C*H*$_3$), 2.82 (t, 2H, C*H*$_2$), 4.45 (t, 2H, C*H*$_2$), 6.27 (s, 1H, C*H*), 7.28–7.33 (m, 3H), 7.41–7.46 (m, 2H), 7.58–7.62 (m, 2H), 8.01–8.03 ppm (m, 2H) (C_6H_5 + C_6H_4); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 14.6, 27.9, 31.9, 71.9, 95.8, 123.2, 126.2, 127.2, 128.9, 129.1, 130.8, 138.2, 144.5, 147.3, 149.1, 161.7 ppm. MS *m/z* 381 $(M)^+$. Anal. Calcd for $C_{20}H_{19}N_3O_5$: C, 62.98; H, 5.02; N, 11.02. Found: C, 62.99; H, 5.02; N, 11.04.

4.1.2.7. 3-Methyl-1-phenyl-1*H*-pyrazol-5-yl 4-[3-(nitrooxy)propoxy]benzoate (7g). Eluent PE/EtOAc 8/2 v/v. The oil obtained was further purified by preparative HPLC (eluent MeCN/H_2O 6/4 v/v); colourless oil which solidified in the freezer; 79% yield. The resulting solid was crystallized from hexane to give the title compound as a white solid, mp = 61.5–62.5 °C. ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 2.25 (qi, 2H, *CH*$_2$), 2.36 (s, 3H, C*H*$_3$), 4.14 (t, 2H, C*H*$_2$), 4.67 (t, 2H, C*H*$_2$), 6.24 (s, 1H, C*H*), 6.94 (d, 2H), 7.30–7.32 (m, 1H), 7.40–7.45 (m, 2H), 7.58–7.61 (m, 2H), 8.03 ppm (d, 2H) (C_6H_5 + C_6H_4); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 14.6, 26.9, 63.9, 69.6, 95.8, 114.5, 120.6, 123.2, 127.1, 129.1, 132.6, 138.2, 144.6, 149.1, 161.5, 163.2 ppm. MS *m/z* 397 $(M)^+$. Anal. Calcd for $C_{20}H_{19}N_3O_6$: C, 60.45; H, 4.82; N, 10.57. Found: C, 60.42; H, 4.84; N, 10.57.

4.1.2.8. 3-Methyl-1-phenyl-1*H*-pyrazol-5-yl 4-[2,3-bis(nitrooxy)propyl]benzoate (7h). Eluent PE/EtOAc 8/2 v/v. The oil obtained was further purified by preparative HPLC (eluent MeCN/H_2O 7/3 v/v); colourless oil; 64% yield. ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 2.37 (s, 3H, *CH*$_3$), 3.11–3.15 (m, 2H, C*H*$_2$), 4.45 (dd, 1H, C*H*H), 4.73 (dd, 1H, CH*H*), 5.46–5.49 (m, 1H, C*H*), 6.28 (s, 1H, *CH*), 7.37–7.47 (m, 5H), 7.58–7.61 (m, 2H), 8.05–8.07 ppm (m, 2H) (C_6H_5 + C_6H_4); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 14.6, 35.7, 70.0, 78.7, 95.8, 123.3, 127.3, 127.6, 129.1, 129.8, 131.1, 138.1, 140.9, 144.3, 149.1, 161.3 ppm. MS *m/z* 442 $(M)^+$. Anal. Calcd for $C_{20}H_{18}N_4O_8$: C, 54.30; H, 4.10; N, 12.66. Found: C, 54.32; H, 4.06; N, 12.58.

4.1.2.9. 3-Methyl-1-phenyl-1*H*-pyrazol-5-yl 4-[2,3-bis(nitrooxy)propoxy]benzoate (7i). Eluent PE/EtOAc 8/2 v/v. The oil obtained was further purified by preparative HPLC (eluent MeCN/H_2O 7/3 v/v); colourless oil; 61% yield. ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 2.36 (s, 3H, C*H*$_3$), 4.31 (d, 2H, C*H*$_2$), 4.78 (dd, 1H, C*H*H), 4.92 (dd, 1H, CH*H*), 5.59–5.65 (m, 1H, C*H*), 6.25 (s, 1H, C*H*), 6.97 (d, 2H), 7.26–7.33 (m, H), 7.40–7.45 (m, 2H), 7.57–7.60 (m, 2H) 8.06 ppm (d, 2H) (C_6H_5 + C_6H_4); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 14.6, 64.8, 68.5, 76.3, 95.8, 114.6, 121.7, 123.2, 127.2, 129.1, 132.8, 138.2, 144.5, 149.1, 161.3, 162.0 ppm. MS *m/z* 458 $(M)^+$. Anal. Calcd for $C_{20}H_{18}N_4O_9$: C, 52.40; H, 3.96; N, 12.22. Found: C, 52.37; H, 4.02; N, 12.09.

4.1.3. Methyl 2,2-dimethyl-5,6-bis(nitrooxy)hexanoate (10)

To a vigorously stirred solution of **9** (1.0 g; 6.4 mmol) and $AgNO_3$ (3.3 g; 19.0 mmol) in MeCN (20 mL) I_2 (1.6 g; 6.4 mmol) was added in one portion. The reaction mixture was stirred at rt until all iodine dissolved and then refluxed for 6 h. The precipitate was filtered and the filtrate poured into H_2O (50 mL) and extracted with EtOAc (100 mL). The organic phase was washed with H_2O (50 mL), brine, dried, and the solvent evaporated. The resulting oil was purified by flash chromatography (eluent PE/EtOAc 9/1 v/v) to give the title compound as a colourless liquid; 74% yield. ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 1.21 (m, 6H, 2CH_3), 1.61–1.74 (m, 4H, 2CH_2), 3.69 (s, 3H, CH_3), 4.48 (dd, 1H, C*H*H), 4.75 (dd, 1H, CH*H*), 5.23–5.26 ppm (m, 1H, C*H*); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 24.8, 25.2, 25.5, 35.3, 41.9, 52.0, 71.1, 79.3, 177.5 ppm. MS *m/z* 281 $(M+H)^+$.

4.1.4. 2,2-Dimethyl-5,6-bis(nitrooxy)hexanoic acid (5e)

A mixture of **10** (1.3 g; 3.7 mmol) 1,4-dioxane (5 mL), 6 M HCl (5 mL) and DMSO (1 mL) was heated at reflux for 6 h. The reaction mixture was then cooled, poured into H_2O (20 mL) and extracted with EtOAc (25 mL). The organic phase was washed with brine, dried, and the solvent evaporated. The resulting oil was purified by flash chromatography (eluent CH_2Cl_2 + 0.1% HCOOH) to give the title compound as a colourless oil which solidified on standing; 36% yield. The resulting solid was crystallized from hexane/toluene mixture, mp = 87.5–88.5 °C. ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 1.24–1.25 (m, 6H, 2CH_3), 1.65–1.80 (m, 4H, 2CH_2), 4.49 (dd, 1H, C*H*H), 4.75 (dd, 1H, CH*H*), 5.24–5.26 ppm (m, 1H, C*H*); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 24.6, 25.1, 25.3, 35.1, 41.7, 71.0, 79.2, 174.7. MS *m/z* 267 $(M+H)^+$.

4.1.5. Methyl 6-hydroxy-2,2-dimethylhexanoate (11)

To a vigorously-stirred mixture of $NaBH_4$ (2.9 g; 76 mmol), 2-methylbut-1-ene (30 mL; 0.28 mol) and dry THF (30 mL) kept under positive N_2 pressure, $BF_3{\cdot}Et_2O$ (7.1 mL; 56 mmol) was added dropwise at −10 °C. The reaction mixture was stirred at rt for 5 h, cooled in an ice-bath, and a solution of **9** (0.84 g; 5.6 mmol) was added dropwise. The cooling bath was removed and the reaction mixture stirred at rt overnight. The following day, the reaction was cooled to −10 °C and, after the sequential addition of H_2O (36 mL), 3 M NaOH (36 mL) and H_2O_2 30% (54 mL), it was heated to 40 °C for 2 h. The organic phase was separated and the water phase extracted with Et_2O (2 × 75 mL). The combined organic extracts were washed with brine, dried, and the solvent evaporated. The resulting liquid was purified by flash chromatography (eluent PE/EtOAc 8/2 v/v) to give the title compound as a colourless liquid; 71% yield. ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 1.17 (s, 6H, 2CH_3), 1.26–1.34 (m, 2H, CH_2), 1.50–1.57 (m, 4H, 2CH_2), 1.79 (br s, 1H, O*H*), 3.62–3.66 ppm (m, 5H, CH_2 + CH_3); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 21.2, 25.2, 33.0, 40.4, 42.3, 51.7, 62.6, 178.6 ppm. MS *m/z* 175 $(M+H)^+$.

4.1.6. 2,2-Dimethyl-6-(nitrooxy)hexanoic acid (5d)

KOH (0.59 g; 11 mmol) was added to a solution of **11** (0.66 g, 3.8 mmol) in MeOH (15 mL) and H_2O (15 mL). The reaction mixture was heated at reflux for 6 h, after which it was cooled to rt, poured into H_2O (30 mL) and extracted with Et_2O (2 × 15 mL). The water phase was then acidified with 6 M HCl until pH 1 and extracted again with Et_2O (2 × 25 mL). The organic phases were washed with brine, dried, and the solvent evaporated. The resulting 6-hydroxy-2,2-dimethylhexanoic acid was used further without any purification. To vigorously-stirred fuming HNO_3 (2 mL), cooled in an ice bath, concd H_2SO_4 (2 mL) was added dropwise at t <15 °C. To the resulting sulfonitric mixture, CH_2Cl_2 (10 mL) was added, followed by the dropwise addition of a solution of the hydroxyl derivative in CH_2Cl_2 (5 mL) at t <10 °C. The ice bath was removed and the reaction mixture stirred at rt for 1 h. The organic phase was decanted, washed with H_2O (2 × 20 mL), 10% urea solution (15 mL), brine, dried, and the solvent was evaporated. The resulting oil was purified by flash chromatography (eluent CH_2Cl_2) to give the title compound as a colourless oil; 85% yield. ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 1.20 (s, 6H, 2CH_3), 1.35–1.46 (m, 2H, CH_2), 1.56–1.61 (m, 2H, CH_2), 1.68–1.77 (m, 2H, CH_2), 4.46 ppm (t, 2H, CH_2); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 21.3, 24.9, 27.2, 49.8, 42.1, 73.1, 184.7 ppm. MS *m/z* 206 $(M+H)^+$.

4.2. Stability studies

4.2.1. Stability in water solution

A solution of each compound (10 mM) in DMSO was added to water (0.1 M KCl) in order to obtain 5% DMSO (v/v) in the mixture. The suspension was filtered using 4 mm, 0.2 μm nylon filters (Titan), obtaining a solution useful for the experiments. The resulting solution was maintained at rt and, at appropriate time intervals, a 100 μL aliquot of reaction mixture was analyzed by RP-HPLC. The reverse-phase HPLC procedure allowed for the separation and quantitation of compounds and hydrolysis product. HPLC analyses were performed with a Hitachi Elite LaChrom equipped with a L-2130 pump, a L-2200 autosampler, a L-2400 UV detector, a L-7614 degasser and a Gynkotek STH585 oven. Data were analyzed using the EZChromElite V. 3.1.7. The analytical column was a Zorbax Extend C18 (150 × 4.6 mm, 5 μm particle size). The mobile phase consisted of methanol/water at 1.0 mL min^{-1} with gradient conditions: 70% methanol until 2.5 min, from 70% to 80% methanol between 2.5 and 5.0 min, 80% methanol for next 3 min and from 80% to 70% methanol between 8 and 12 min. The injection volume was 100 μL. The column effluent was monitored at 240 nm (for compounds) referenced against a 600 nm wavelength. Quantitation was done using calibration curves of compounds; the linearity of the calibration curves was determined in a concentration range of 1–200 μM (r^2 >0.99).

4.2.2. Stability in acidic medium

A 2 mL aliquot of 0.5 mM solution of each compound in methanol was diluted to 10 mL using a 0.1 M HCl/MeOH 60/40 (v/v) mixture. The resulting solution was maintained at 37 ± 0.5 °C and, at appropriate time intervals, a 20 μL aliquot of reaction solution was analysed by RP-HPLC. HPLC analyses were performed with a HP1100 chromatograph system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump (model G1311A), a membrane degasser (model G1379A), and a diode-array detector (DAD) (model G1315B). Data analysis was accomplished using a HP ChemStation system (Agilent Technologies). The analytical column was a Zorbax Eclipse C18 (150 × 4.6 mm, 5 μm particle size). The mobile phase consisted of acetonitrile/water at 1.2 mL min^{-1} with gradient conditions: 40% acetonitrile until 2.0 min, from 40% to 70% acetonitrile between 2.0 and 5.0 min, 70% acetonitrile for next 5 min and from 70% to 40% acetonitrile between 10 and 12 min. The injection volume was 20 μL (Rheodyne, Cotati, CA). The column effluent was monitored at 240 nm referenced against a 600 nm wavelength. Quantitation was done using calibration curves of compounds; the linearity of the calibration curves was determined in a concentration range of 1–200 μM (r^2 >0.99).

4.2.3. Stability in human serum

A solution of each compound (10 mM) in MeOH was added to human serum (from human male AB plasma, Sigma) preheated at 37 °C. The final concentration of the compound was 250 μM. The resulting solution was incubated at 37 ± 0.5 °C and, at appropriate time intervals, a 300 μL aliquot of the reaction mixture was withdrawn and added to 450 μL of acetonitrile containing 0.1% trifluoroacetic acid, in order to deproteinize the serum. The

sample was sonicated, vortexed, and then centrifuged for 10 min at 2150*g*. The clear supernatant was filtered by 0.45 μm PTFE filters (Alltech) and analyzed by RP-HPLC using the HP1100 chromatograph system described in the 'acidic medium stability' experimental section.

4.2.4. Stability in rat-liver homogenate

Liver homogenate from male Wistar rats (200−250 g) was prepared by centrifuging (8000*g*, 20 min) a suspension of 20% tissue in a Tris–HCl/KCl (100 mM/150 mM, pH 7.4) and stored at −80 °C. Incubation was at 37 °C in a Tris–HCl/KCl (100 mM/150 mM, pH 7.4) containing a known amount of homogenate (6.4 mg prot/mL), 2 mM GSH, 1 mM NADPH; a solution of each compound (10 mM) in acetonitrile was added to incubation mixture preheated at 37 °C, the final concentration of the compound was 200 μM. The resulting solution was incubated at 37 ± 0.5 °C and, at fixed times, 700 μL of incubation mixture were removed and deproteinized with 700 μL of cold acetonitrile; the sample was sonicated, vortexed, and then centrifuged for 10 min at 2150*g*. The clear supernatant was filtered through 0.45 μm PTFE filters (Alltech). A 20 μL aliquot of filtrate was analyzed by RP-HPLC to determine the stability of the compound in the homogenate (analyses were performed using the RP-HPLC method described in the 'acidic medium stability' experimental section). An 800 μL aliquot of filtrate was analyzed by UV–vis spectroscopy (UV 2501-PC, Shimadzu) to determine nitrate and nitrite production. Nitrite and nitrate quantitation was performed by adapting a method described by Miranda et al.: nitrite and nitrite + nitrate (NO_x) concentrations were determined in parallel.[19] Briefly, for nitrite + nitrate (NO_x) analysis, a 400 μL aliquot of filtrate was added to 400 μL of VCl_3 saturated solution (400 mg in 50 mL HCl 1 M), rapidly followed by the addition of 400 μL Griess reagent (200 μL NEDD 0.1% w/v in water + 200 μL SULF 2% w/v in 5% HCl v/v); for nitrite analysis, a 400 μL aliquot of filtrate was added to 400 μL of HPLC-grade water and to 400 μL of the Griess reagent. The reaction mixtures were incubated at 37 °C for 45 min (NO_x detection) and for 10 min (nitrite detection) and in both cases the absorbance at 540 nm was measured. NO_x quantitation was done using calibration curves obtained for nitrite and nitrate standard solutions; the linearity of the calibration curves was determined in a concentration range of 1–100 μM (r^2 >0.99).

4.3. In vivo study of nitrite production

Compounds **4a**, **7d**, and ISMN as reference, were orally administered to mice (10 mg/kg in imwitor742/tween80 50/50) and nitrite blood level over 24 h was monitored. At fixed times blood was drawn, protein-precipitated using 3 volumes of acetonitrile and analyzed by adapting a method described by Misko et al.[20] 10 μL of 1 mM 2,3-diaminonaphtalene (DAN) were added to 200 μL of the deproteinized sample and left under gentle shake at room temperature to convert the nitrite to (1*H*)-naphtotriazole (NAT); after 20 min the reaction was stopped by adding 10 μL of 1 M NaOH. As the conversion of nitrite to NAT in deproteinized blood is not quantitative, a set of deproteinized blood samples spiked with $Na^{15}NO_2$ at four different concentrations (0, 0.1, 1.0 and 10 μM) were added to each batch prior to nitrite conversion. Samples and spikes were analyzed by LC–MS/MS instead of the usual fluorimetric detection against a calibration curve of NAT in the concentration range 0.01–10 μM. Nitrite basal level was obtained from the 0 μM spikes while the nitrite-to-naphtotriazole conversion factor was calculated from the ratio between experimental and nominal concentration of NAT in the 0.1, 1.0 and 10 μM spikes. This conversion factor was used to calculate the original nitrite concentration in the samples from the NAT experimental concentration.

4.4. Lipophilicity studies

The $\log P_{oct}$ of all compounds was determined by a RP-HPLC method using the HP1100 chromatograph system described in the 'acidic medium stability' experimental section. Retention times were measured on a Discovery RP-amide-C16 column (150 × 4.6 mm i.d., 5 mm; Supelco, Bellefonte, PA, USA) thermostated at 30 °C. The flow rate was 1.0 mL min^{-1}. The column effluent was monitored at 226 and 240 nm referenced against a 600 nm wavelength. Each analysis was performed isocratically using pH 7.0 phosphate buffer and methanol mixtures in proportions varying from 30 to 70% (v/v), with final ionic strength of 20 mM. Before use, the mobile phase was filtered under vacuum through a 0.45 μm HA Millipore filter (Millipore, Milford, MA, USA). Stock solutions (10 mM) of compounds were prepared in methanol and diluted (1–0.1 mM) in the mobile phase for injection (20 μL). All samples were injected at least three times for each mobile phase. Uracil was used as the non-retained compound. The logarithms of the capacity factor ($\log k$) for a minimum of four different methanol/buffer ratios were measured for each compound. $\log k_w$, namely the logarithm of the capacity factor corresponding to 0% methanol modifier, was obtained by linear extrapolation (r^2 >0.99, for all the compounds). $\log P_{oct}$ values of edaravone and derivatives were determined using the reference line obtained from the correlation between $\log P_{oct}$ and $\log k_w$ of 63 known structurally-diverse compounds.[5]

4.5. Permeability studies

Permeation experiments were carried out in 96-well microtiter polycarbonate filter plates obtained from Millipore AG (MPC4NTR10, Volketswil, Switzerland). Polycarbonate filter specifications were as follows: 0.45 μm pore size, 10 μm thickness, and 5–20% porosity. An average porosity value of 12.5% was used for permeability calculations. Each well of the filter plate was impregnated with 15 μL of 5% hexadecane dissolved in hexane and shaken for at least 10 min for complete evaporation of the hexane. Subsequently, the donor compartments were hydrated with 280 μL of solution (i.e., prepared as described in the 'water solution stability' experimental section) of test compound in water, containing 5% DMSO and 100 mM KCl, and placed upon a Teflon acceptor plate (MSSACCEPTOR, Millipore, Volketswil, Switzerland), which had been prefilled with water containing 5% DMSO and 100 mM KCl. The resulting sandwich was incubated at room temperature under constant light shaking (150 rpm). After appropriate time intervals (varying between 1 and 6 h), the sandwich was disassembled and a 100 μL aliquot of solutions in the acceptor and donor compartment were analyzed by RP-HPLC. Analyses were performed using the RP-HPLC method and the Hitachi EliteLaChrom system, described in the 'water solution stability' experimental section.

Membrane integrity was checked by electrical resistance measurements conducted on the filter plate at the end of the incubation time, using an electrometer system especially designed for PAMPA assays (EVOMX and MULTI96, World Precision Instruments, Sarasota FL).

4.6. Vasodilator studies

Thoracic aortas were isolated from male Wistar rats weighing 180–200 g. As few animals as possible were used. The purposes and protocols of these studies were approved by the Ministry for Health, Rome, Italy. The endothelium was removed, the vessels were cut helically, and three 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 1.5 h and then contracted with 1 μM L-phenylephrine. When the response to the agonist reached a plateau, cumulative concentrations of the vasodilator agent were added. Results are expressed as EC_{50} (±SEM) μM. The effects of 1 μM ODQ on relaxation were evaluated in a separate series of experiments in which it was added to the organ bath 5 min before contraction. In this protocol, the inhibitor is pre-incubated for at least 30 min before addition of the vasodilator compound. Responses were recorded by an isometric transducer connected to the MacLab System PowerLab. Addition of the drug vehicle, DMSO, had no appreciable effect on contraction. At least five experiments for each compound were performed.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.11.065.

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