Use of the furoxan (1,2,5-oxadiazole 2-oxide) system in the design of new NO-donor antioxidant hybrids

Clara Cena*, Massimo Bertinaria, Donatella Boschi, Marta Giorgis, and Alberto Gasco

Dipartimento di Scienza e Tecnologia del Farmaco, via P.Giuria, 9, 10125 Torino, Italy
E-mail: clara.cena@unito.it

Abstract
The design, the synthesis and the study of the antioxidant and vasodilating properties of new hybrids obtained by linking different antioxidant phenolic moieties to the furoxan substructure present in CHF2363, which can release nitric oxide, are described. All the final NO-phenols were endowed with both antioxidant and vasodilating properties. The antioxidant activities were assessed on the ferrous salt/ascorbate induced autooxidation of lipids present in microsomial membranes of rat hepatocytes. The vasodilating activities were assessed on rat aorta strips precontracted with phenylephrine, in the presence and absence of ODQ. The antioxidant potencies (IC_{50} values) and the vasodilating potencies (EC_{50} values) were widely modulated into the series. Further in vivo studies should clarify whether these products may become preclinical candidates for the treatment of atherosclerosis and related disorders.

Keywords: Nitric oxide, furoxans, NO-donors, phenol antioxidants, NO-donor phenol antioxidants

---

Contents

1. Introduction
   1.1 Furoxan, a heterocycle able to release NO
   1.2 Atherosclerosis: a new therapeutic strategy with NO-Donor Antioxidants
   1.3 The design of a new series of NO-Donor Antioxidants
2. Chemistry
   2.1 Synthesis
3. Biological characterization
   3.1 Antioxidant activity
   3.2 Vasodilating activity
4. Results and discussion

1. Introduction

1.1 Furoxan, a heterocycle able to release NO

Furoxan, is an old heterocycle known to chemists thanks to an argument over its structure and its intriguing chemistry (see Chart 1). NMR spectroscopy and X-ray crystallography resolved the problem of its structure and showed that it is the 1,2,5-oxadiazole 2-oxide, **1.**[1] Furoxan derivatives can consequently exist as a pair of position isomers when the substituents at the ring are different. The controversy over its structure, its chemistry, and the reactivity of its side-chain functional groups, have been exhaustively reviewed[2-5] and are also the subject of a very complete two-volume monograph.[6,7]

1

Chart 1

The capacity of activating soluble guanylate cyclase (sGC) by furoxans was showed by our group working with phenylsulphonylfuroxan derivatives.[8] It was hypothesised that these products released nitric oxide (NO) with consequent sGC activation. Simultaneously Feelish found that a similar activation was triggered by furoxancarboxamides and proved its dependence on NO release.[9] He showed that these derivatives produce NO when dissolved in physiological solution in the presence of thiols. Among the reaction products, they isolated nitrite and, in lesser amounts, nitrate, which are the final oxidation products of nitric oxide in aerobic water solution. They also evidenced that *S*-nitrosothiols as intermediates could be produced following a marked pH-dependence and that when using L-Cysteine the rate of NO formation increased as the L-Cys/furoxan ratio increased and became constant for ratios above 50:1. A mechanism to justify their findings was proposed which implies the attack of the thiolate anion on the 3- or/and the 4-position of the ring.

When the reaction between 4-phenyl-3-furoxancarbonitrile and an excess of thiophenol in water solution at physiological pH was closely investigated by our group,[10] a mechanism was suggested in which the attack of the thiolate anion occurred principally on the 3-position of the furoxan ring. Nitrosothiol intermediates formation, which can decompose giving NO, could be explained as well as the formation of other reaction products by this hypothesis.

Interestingly, some furoxans can also produce NO, detected as nitrite, spontaneously without the assistance of thiols.[11] In conclusion, either thiol-induced or spontaneous NO-release from furoxans, depending on the substituent at the ring, can occur at physiological pH values through complex mechanisms still in need of thorough investigation. In addition, both the rate

and the amount of nitric oxide generated can be modulated by introducing appropriate substituents at the ring.

NO-release from furoxans in cells and tissues could also occur through enzymatic activation: this for example cannot be excluded for furoxans such as some furoxancarboxamide derivatives.[12]

Many furoxan derivatives display typical NO-donor dependent biological activities.[13,14] In particular, the presence at the 3-position of the furoxan ring of electron-withdrawing substituents such as NO_2, CN and $SO_2C_6H_5$ induced potent activating action of sGC, triggering both antiaggregatory on collagen-induced human platelet aggregation and vasodilating activity on rabbit aorta rings precontracted with noradrenaline. This was in keeping with the hypothesis that the thiol induced NO-release from furoxans in physiological solution is due to an initial nucleophilic attack on the ring by the thiolate anion.

In any case, these findings generated a renewed interest in the properties of this heterocycle, both by academia and industry.[1] In fact NO is a physiological messenger involved in a wide range of biological functions.[15] In particular NO exerts potent effects on vascular homeostasis, such as smooth muscle relaxation, inhibition of platelet adherence and aggregation, as well as attenuation of monocyte infiltration.

1.2. Atherosclerosis: a new therapeutic strategy with NO-Donor Antioxidants

Atherosclerosis is the main cause of morbidity and mortality in the western society. The main cause that triggers the atherosclerotic process is known to be “oxidative stress”. ROS (reactive oxygen species) are continuously produced in cellular metabolism through different pathways, but they are normally kept in physiological concentrations by the organism’s pool of antioxidant defences. After an abnormal production of ROS, this balance between the prooxidant/antioxidant status in the organism can be perturbed and a situation, called “oxidative stress” can arise. A prolonged situation of oxidative stress leads to cellular damage, due to alteration of lipids, enzymes, proteins and DNA.[16,17]

In atherosclerosis an abnormal production of superoxide anion ($O_2^{-\bullet}$) by the endothelium has been observed. Hydrogen peroxide is formed from this radical, under the action of the superoxide dismutase (SOD). Hydrogen peroxide is in turn a source of the very toxic hydroxyl radical (OH•) through Fenton and Haber-Weiss reactions. Under the action of this radical, low density lipoproteins (LDL), accumulated in the subendothelial space, can undergo oxidative modifications and this is the first step of a complex process which leads first to the formation of foam cells, then of the fatty streak and ultimately to atherosclerotic plaque.

In the mean time in the atherosclerotic vessel the endothelial dysfunction involves the attenuation of the effects mediated by endothelial nitric oxide (NO), such as vascular smooth muscle relaxation, inhibition of platelet adhesion and aggregation and of monocyte infiltration. This impairment of NO mediated bioactions could be derived from a reduced NO biosynthesis by endothelial NOS, at least in the advanced stages of atherosclerosis disease, or from increased inactivation of NO by the action of oxygen free radicals, or from a decreased responsiveness of

cellular targets to NO. Again the excess $O_2^{-\bullet}$ may be responsible for: 1) the trapping of nitric oxide to generate peroxynitrite (-OONO) which, in turn, can afford two very reactive and toxic radicals, the OH• and the nitrogen dioxide radical (NO_2•); 2) reaction with thiol residues of proteins. The consequent inactivation of these residues, normally involved in S-nitrosylation, prevents this reaction from occurring, thus resulting in the perturbation of this signalling mechanism with the consequent decrease of vessel responsiveness to NO•.[18-20]

By contrast there is experimental evidence that, at least in vitro, the responsiveness to the vasodilating action of exogenous NO released by NO-donors, such as glyceryl trinitrate and nitroprusside, is largely preserved and that the effects mediated by endogenous NO could be reconstituted/mimicked. This is most likely due to the relatively high doses of the compounds used in the experiments with respect to physiologically produced NO concentrations.[20]

1.3 The design of a new series of NO-Donor Antioxidants

From this picture the interest of joining an antioxidant moiety and the structure of a NO-donor in the same hybrid molecule clearly emerges.

With this rational in mind a project aimed at the realization of this class of new molecular hybrids was started. The choice of the reference models used in this chemical hybridisation approach was aimed at obtaining hybrids, characterised by extensively modulated antioxidant and vasodilating potencies.

For the antioxidant action our choice was directed towards classical antioxidant molecules, which differ in terms of lipophilic properties, mechanisms of action, etc. such as phenols, vitamin C, melatonin, isoflavones, 1,4-dihydropyridines, while for the NO-donor the choice was directed both at nitrooxy functions and to the furoxan ring.

In this paper we discuss some preliminary results obtained from characterization of hybrids in which appropriately selected phenols, distinguished by their largely modulated lipophilicity[21] and -O-H bond dissociation energy (BDE),[22] are linked to a furoxan derivative to yield NO-donor antioxidants.

The dissociation energy of the O-H bond is considered to be one of the most important physico-chemical parameters involved in the definition of the antioxidant potency of phenolic derivatives. Literature data show a decrease for BDE of about 10 Kcal mol^{-1} from unsubstituted phenol to tocopherol, as a demonstration of the effect of the number and the nature of substituents present on the aromatic ring, in terms of sterical and electronic contributions. In particular for the series of phenolic reference compounds, energy values of bond omolitic dissociation (BDE) for O-H, either measured or calculated by different authors, are reported in literature.[22] In any case it is well-known that a number of other physico-chemical parameters are decisive, in particular the lipophilic-hydrophilic balance.

The NO-donating moiety linked to the antioxidant part is the 3-phenylsulfonylfuroxan-4-yloxy substructure present in the 4-ethoxy-3-phenylsulfonylfuroxan, a reference NO-donor developed by the Chiesi Company[23] (CHF 2363), characterized by a potent in vitro and in vivo oral activity as NO-dependent vasodilating agent, and by a powerful *in vitro* inhibition of platelet

aggregation induced by ADP, collagen and PAF. Evaluation in vivo of haemodynamic profile of CHF2636 in anesthetized rats showed a more marked fall of the arterial vascular tone after a continuous infusion of the product in comparison to an analogous treatment with isosorbide-5-mononitrate.Very interestingly crossed tolerance studies with GTN on isolated rat thoracic aorta precontracted with noradrenaline, characterize CHF2363 as a powerful vasodilatator compared to GTN with unmodified activity on vessels made tolerant to the GTN. These data should certainly be confirmed by chronic treatments on animals in vivo.

2. Chemistry

2.1 Synthesis

a)

HO R R H O 1 Prot-O R R H O 2 Prot-O R R OEt O 3

Prot-O R R OH 4 Prot-O R R O SO_2Ph N O N^+ O^- 5 HO R R O SO_2Ph N O N^+ O^-

Scheme 1

A general scheme of synthesis can be defined for the NO donor phenol hybrids (Scheme 1a). The common pathway starts with 1) the protection of the hydroxy group of the p-hydroxybenzaldehyde; 2) the elongation of the spacer carbon chain through a modified Wittig reaction affording the corresponding α,β-unsaturated esters; 3) the reduction of these products to obtain, first the saturated esters and then the related alcohols; 4) the selective displacement by the corresponding alcolates of the 4-phenylsulfonyl group of the 3,4-diphenylsulfonylfuroxan to yield the final protected hybrids; 5) the cleavage of the protection in acidic conditions to produce the expected final compounds.

The derivative bearing the chromanic substructure present in Vitamin E was synthetised starting from the ethyl ester of the commercially available carboxylic acid Trolox®. This ester was 1) protected on the phenol function, 2) reduced to the corresponding alcohol, 3) joined to the furoxan moiety, 4) deprotected to the final product (Scheme 1b).

b)

3. Biological characterization

All the phenol substituted furoxan hybrids as well as reference phenolic and furoxan compounds are assessed for antioxidant and vasodilating properties *in vitro*.

3.1 Antioxidant activity

The compounds are evaluated as inhibitors of ferrous salt/ascorbate induced lipidic peroxidation of membrane lipids of rat hepatocytes.[24] The progress of the peroxidation was followed by visible spectroscopy detection of 2-thiobarbituric acid reactive substances (TBARS) which are the final metabolites of the autoxidation. All the products are able to inhibit the lipid oxidation in a dose dependent manner. The potencies as antioxidants of these compounds (IC_{50}) are collected in Table 1 along with those of the parent phenols. The hybrid products show potencies near to those of the reference phenols, with only a partial exception of the unsubstituted phenolic derivative whose antioxidant activity is improved with respect to the reference phenol. It is noteworthy that the NO-donor furoxan CHF2363 also displays an antioxidant action.

3.2 Vasodilating activity

The assay employed is the dose-dependent vasodilation of endothelium-denuded rat aorta strips precontracted with 1μM phenylephrine. cGMP dependence of vasodilation is confirmed by decrease in the potency observed when repeating the experiments in the presence of 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), a well known inhibitor of the sGC. Vasodilating potencies are expressed as EC_{50} and reported in Table 1.

Table 1. Antioxidant and vasodilating activity of the NO-donor phenols, of the phenol parents and NO-donor CHF2363

A B C

Structure	R	R'	Antioxidant activity	Vasodilating activity		IC_{50} /EC_{50}
			IC_{50} (95% CL) μM	$EC_{50} \pm$ SE, μM	+1 μM ODQ	
A	H	CH_3	290 (260-324)	-	-	-
A	OCH_3	CH_3	18 (17-20)	-	-	-
A	*t*-Bu	CH_3	1.7 (1.6-1.9)	-	-	-
B	H	-	0.16 (0.16-0.17)	-	-	-
C	OEt	SO_2Ph	110 (98-122)	0.012 ± 0.002	1.2 ± 0.2	9167
A	H	O, SO_2Ph, N, O, N^+, O^-	47 (45-48)	0.012 ± 0.001	0.36 ± 0.09	3917
A	OCH_3	O, SO_2Ph, N, O, N^+, O^-	3.4 (3.2-3.5)	0.022 ± 0.003	0.50 ± 0.13	154
A	t-Bu	O, SO_2Ph, N, O, N^+, O^-	2.0 (1.9-2.0)	0.11 ± 0.03	4.8 ± 0.5	18
B	O, SO_2Ph, N, O, N^+, O^-	-	0.49 (0.48-0.50)	0.044 ± 0.004	0.67 ± 0.09	11

4. Results and Discussion

A comparison between the IC_{50} and EC_{50} values shows that in this series of products the vasodilating action prevails over the antioxidant activity. Work is in progress in order to obtain products with better balanced antioxidant and vasodilating activities. All the products reported in this preliminary paper could be of value as potential antiatherosclerotic agents and they are worthy of further *in vivo* investigations. From this screening a new interesting aspect of the

furoxan chemistry emerges, which is worthy of additional investigation: the potential antioxidant activity of furoxan derivatives. CHF 2363 in fact displays both a potent vasodilating activity and a 2-3 fold higher antioxidant action than *p*-cresol. This may be due to the product's ability to directly scavenge radicals and/or to small amounts of NO released by the product under the experimental conditions used for the evaluation of the antioxidant activity. It is known that low concentrations of NO display antioxidant actions.[25] Indeed we were able to detect, using a NO selective electrode (WPI, ISO-NO Mark II, equipped with ISONOP200) a release of NO from CHF 2363 when the product was incubated with microsomial membranes, ascorbate and ferrous salt. Further investigations are necessary to clarify this point.

Acknowledgements

This work was supported by a grant from MIUR Studi e Ricerche Finalizzate 40 % Roma and Regione Piemonte Ricerca Scientifica Applicata, 2003.

References

1. Gasco, A.; Schoenafinger, K. In *Nitric Oxide donors*; Wang P. G., Cai, T. B., Taniguchi, N., Eds.; Wiley-VCH: Weinheim, 2005; pp 131-175
2. Gasco, A., Boulton, A. J., *Adv. Heterocycl. Chem.* **1981**, *29*, 251
3. Boulton, A. J., Ghosh, P. B., *Adv. Heterocycl. Chem.* **1969**, *10*, 1
4. Friedrichsen, W.*, Methoden der organischen Chemie/ (Houben-Weyl);*Bd. E8c. Hetarene.-3; Thieme: Stuttgart and New York: Teil 3 **1994**; 716
5. Sheremetev, A. B., Makhova, N., Friedrichsen, W., *Adv. Heterocycl.Chem*. **2001**, *78*, 65
6. Khmelnitskii, L. I., Novikov, S. S., Godovikova, T. I., *Chemistry of Furoxans: Structure and Synthesis;* Nauka: Moscow, 1996 (in Russian)
7. 11 Khmelnitskii, L. I., Novikov, S. S., Godovikova, T. I., *Chemistry of Furoxans: Reactions and Applications;* Nauka: Moscow, 1996 (in Russian)
8. Calvino, R.; Fruttero, R.; Ghigo, D.; Bosia, A.; Pescarmona, G.P.; Gasco A. *J. Med. Chem.*, **1992**, *35*, 3296
9. Feelisch, M., Schoenafinger, K., Noack, E., *Biochem. Pharmacol*. **1992**, *44*, 1149
10. Medana, C., Ermondi, G., Fruttero, R., Di Stilo, A., Ferretti, C., Gasco, A., *J. Med. Chem.* **1994**, *37,* 4412
11. Sorba, G., Medana, C., Fruttero, R., Cena, C., Di Stilo, A., Galli, U., Gasco, A., *J. Med. Chem.* **1997**, *40*, 463; **1997**, *40*, 2288
12. Hecker, M., Vorhoff, W., Bara, A. T., Mordvintcev, P. I., Busse, R., *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1995**, *351*, 426

13. Ghigo, D., Heller, R., Calvino, R., Alessio, P., Fruttero, R., Gasco, A., Bosia, A., Pescarmona, G., *Biochem. Pharmacol.* **1992**, *43*, 1281
14. Ferioli, R., Folco, G. C., Ferretti, C., Gasco, A. M., Medana, C., Fruttero, R., Civelli, M., Gasco, A., *Br. J. Pharmacol*. **1994**, *114*, 816; (p. 820: 4-R should read 3-R and vice versa)
15. Kerwin, J. K. Jr; Heller, M. *Med. Res. Rev.* **1994**, *14*, 23.
16. Eberhardt, M.K. *Reactive Oxygen Metabolites;* CRC Press: BocaRaton, 2000.
17. Keaney, J.K.; Vita, J.A. *Prog. Cardiovasc. Dis*. 1995, 38, 129
18. Hare, J.M. *New Engl. J. Med.* **2004**, *351,* 2112.
19. Ogita, H.; Liao, J.K. *Endothelium* **2004,** *11,* 123.
20. Dillon, G. A.; Vita A. J. Nitric oxide and endothelial Dysfunction. In *Contemporary Cardiology, Vol.4: Nitric Oxide and the Cardiovascular system;* Loscalzo, J.; Vita, J.A., Eds.; Humana Press Inc.: Totowa, 2000; p 207.
21. Ancerewicz, J.; Migliavacca, E.; Carrupt, P.-A.; Testa, B.; Brée, F.; Zini, R., Tillement, J.-P.; Labidalle, S.; Guyot, D.; Chauvet-Monges, A.-M., Crevat, A.; Le Ridant, A. *Free Radical Biol. Med*. **1998**, *25*, 113.
22. Luccarini, M.; Pedrielli, P.; Pedulli, G.F.; Cabiddu, S.; Fattuoni, C. *J. Org. Chem*. **1996**, *61*, 9259.
23. Civelli, M.; Giossi, M.; Caruso, P.; Razzetti, R.; Bergamaschi, M.; Bongrani, S.; Gasco, A. Br. *J. Pharmacol*. **1996**, *118*, 923.
24. Mastrocola, R.; Aragno, M.; Betetto, S.; Brignardello, E.; Catalano, M.G.; Danni, O.; Boccuzzi, G. *Life Sci*. **2003**, *73*, 289.
25. Sharpe, M.A.; Robb, S.J.; Clark, J.B. *J. Neurochem*. **2003**, *87*, 386**.**