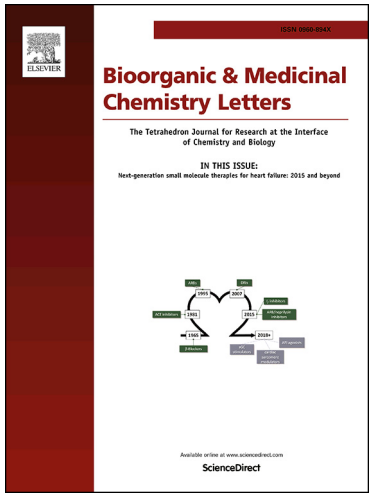
Accepted Manuscript



Synthesis of Novel Mono and Bis Nitric Oxide Donors with High Cytocompatibility and Release Activity

Tanya Sahyoun, Caroline Gaucher, Yi Zhou, Naïm Ouaini, Raphaël Schneider, Axelle Arrault

PII: S0960-894X(18)30740-6
DOI: https://doi.org/10.1016/j.bmcl.2018.09.009
Reference: BMCL 26030

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 9 July 2018
Revised Date: 4 September 2018
Accepted Date: 7 September 2018

Please cite this article as: Sahyoun, T., Gaucher, C., Zhou, Y., Ouaini, N., Schneider, R., Arrault, A., Synthesis of Novel Mono and Bis Nitric Oxide Donors with High Cytocompatibility and Release Activity, *Bioorganic & Medicinal Chemistry Letters* (2018), doi: https://doi.org/10.1016/j.bmcl.2018.09.009

Synthesis of Novel Mono and Bis Nitric Oxide Donors with High Cytocompatibility and Release Activity

Tanya Sahyoun[a,c], Caroline Gaucher[b], Yi Zhou[b], Naïm Ouaini[c], Raphaël Schneider[d*], Axelle Arrault[a*]

[a] *Laboratoire de Chimie Physique Macromoléculaire, Université de Lorraine, CNRS, LCPM, F-54000 Nancy, France*

[b] *Université de Lorraine, CITHEFOR, F-54000 Nancy, France*

[c] *Faculty of Sciences, Holy Spirit University of Kaslik, BP446, Jounieh, Mount Lebanon, Lebanon*

[d] *Laboratoire Réactions et Génie des Procédés, Université de Lorraine, CNRS, LRGP, F-54000 Nancy, France*

Abstract

Four compounds bearing amidoxime functions were synthetized: (1) **2a-b** bearing an aromatic amidoxime function, (2) **2c** bearing an aliphatic amidoxime function, and (3) **2d** bearing aromatic and aliphatic amidoximes functions. The ability of these compounds to release NO was evaluated *in vitro* using the oxidative metabolism of cytochrome P450 from rat liver microsomes. Results obtained demonstrate that all amidoximes were able to release NO with a highest amount of NO produced by the **2a** aromatic amidoxime. Moreover, all amidoximes exhibit cytocompatibility with human aorta smooth muscle cells. Using intracellular *S*-nitrosothiol formation as a marker of NO bioavailability, compounds **2a-c** were demonstrated to deliver a higher amount of NO in the intracellular environment than the reference. Considering that the concentration of the bis-amidoxime **2d** was two times lower that than of **2a** and **2b**, we can assume that **2d** is the most potent molecule among the tested compounds for NO release.

Keywords: Amidoximes; Nitric oxide; Cardiovascular disease; Double donor; Oxidation

Gasotransmitters are endogenously synthesized molecules presenting biological properties. Until today, three gasotransmitters have been identified.[1] The first described and studied gasotransmitter is nitric oxide (NO).[2] NO plays a key role in various physiological processes. In the nervous system, NO controls the neurotransmission, in the immune system, it also acts as a defense against bacteria, parasites, and tumor cells, and in the cardiovascular system, it controls vascular homeostasis including blood pressure.[3,4] The second gasotransmitter is carbon monoxide (CO) which plays a role in the cardiovascular system since it relaxes the vascular vessels and therefor lowers the blood pressure. CO was also identified as a

neurotransmitter. The third and recently studied gasotransmitter is hydrogen sulfide (H_2S) which was discovered to play an important role in neuromodulation and neurotransmission.[2, 5] In this study, we focus on the firstly discovered gasotransmitter, NO. *In vivo*, NO is produced by oxidation of the guanidine function of *L*-arginine. In the cardiovascular system, this oxidation is catalyzed by the endothelial nitric oxide enzyme (eNOS) and occurs in two steps.[6] The first one is the *N*-hydroxylation of one of guanidino nitrogen atoms of arginine consuming one mole of NADPH and one mole of dioxygen. The second step is the oxidation of the intermediate *N*-hydroxy-*L*-arginine (NOHA) allowing the breaking of the C=N-OH bond and producing NO and citrulline (Fig. 1).[7] This step consumes 0.5 mole of NADPH and 1 mole of dioxygen. The *L*-arginine oxidation resembles a classical P450 dependent monooxygenation.[8] This reaction has been the subject of extensive studies and it has been demonstrated that the oxidation of NOHA, but also of other arginine-mimetics like amidoximes, can be performed by cytochromes P450.[9]

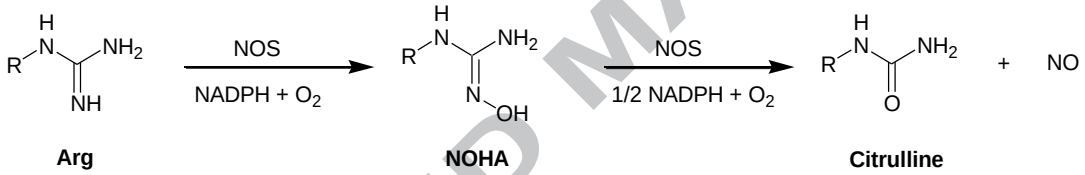


Fig. 1. *In vivo* oxidation of *L*-Arginine by NO Synthases.

Jousserandot *et al.* were the first to describe the oxidation of some amidoximes by cytochromes P450, using rat liver microsomes in the presence of NADPH and dioxygen.[10,11] Their work focused on the quantification of nitrite ions released by aromatic amidoximes substituted by para electron-donating substituents, such as Cl, that were demonstrated to exhibit the highest NO release rates.[11] Other studies were devoted to the synthesis of prodrugs bearing an amidoxime function and on their effects on many diseases like cardiovascular disease. These reports showed the capacity of seventeen different amidoxime prodrugs to inhibit platelet aggregation, thrombus formation and to decrease blood pressure in spontaneously hypertensive rats.[12,13] Some aliphatic bis-amidoximes showed the highest inhibition of thrombus formation while the aromatic mono-amidoxime bearing a p-CF_3 group was demonstrated to be of high efficiency for the decrease of the blood pressure.[13] Furthermore, the capacity of some amidoxime functions to induce vasorelaxation of rat aortic rings after the NO release was also highlighted.[14, 15]

In this work, the synthesis of mono-amidoximes **2a-c** substituted by various electron-donating groups in the para position and of a bis-amidoxime **2d**, bearing one aromatic and one aliphatic amidoxime function, is described from the corresponding nitriles (Fig. 2).

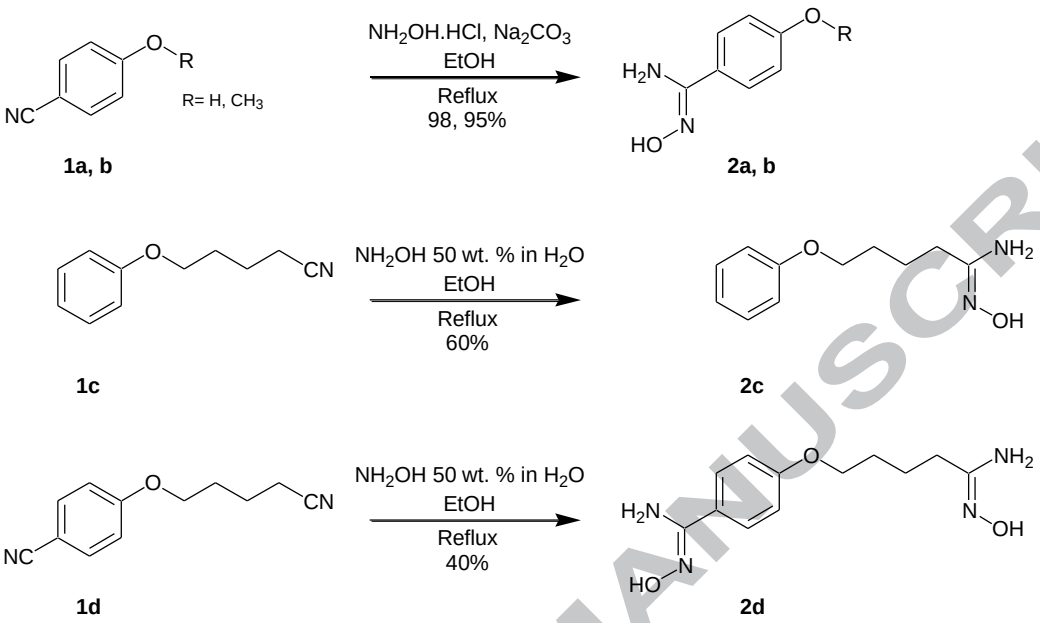


Fig. 2. Synthesis of amidoximes

Nitriles **1a** and **1b** are commercially available, while **1c** and **1d** were prepared from the corresponding bromides by nucleophilic substitution using KCN in DMF. The synthesis of amidoximes **2** from nitriles **1** was performed either in ethanol using hydroxylamine hydrochloride associated to Na_2CO_3 or using an aqueous solution of hydroxylamine and a protocol adapted from Ovdiichuk *et al.*[16-18] The synthetic conditions are listed in Table 1 in the Supporting Information. Aromatic nitriles **1a and 1b** could easily be converted into amidoximes **2a**[19,20] and **2b**[19,21] using hydroxylamine hydrochloride and Na_2CO_3. Due to the lower reactivity of aliphatic nitriles compared to aromatic ones, compound **2c**[19] could only be prepared from **1c** with a modest yield of 37% under these synthetic conditions. With aliphatic nitriles, better conversions were obtained using a solution of hydroxylamine in water and **2c**[22,23] was isolated with 60% yield after column chromatography. Under these reaction conditions, bis-amidoxime **2d**[22, 24] was obtained in 40% yield.

The ability of compounds **2** to release NO by oxidation was first evaluated using rat liver microsomes containing cytochromes P450 in the presence of NADPH and dioxygen (see Supporting Information). 4-Chlorobenzamidoxime (Fig. 3), which is known to release high amounts of NO in the presence of cytochrome P450,[11] was used as a reference.

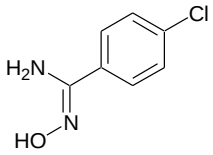


Fig. 3. Structure of 4-chlorobenzamidoxime.

After 10 min of incubation with rat liver microsomes and NADPH, supernatants were collected for nitrite ions quantification using the Griess assay (see Supporting Information). Four control samples were prepared for each molecule, namely without microsomes, without amidoximes, without NADPH and finally using nitriles **1** instead of amidoximes **2**. The amount of nitrite ions measured in these controls was subtracted from the values measured with amidoximes **2**. As can be seen from Fig. 4, all compounds **2** generate nitrite ions, indicating their ability to be oxidized by rat liver microsomes and NADPH. The nitrite ions release was the highest for compounds **2a** and **2b** (2.4 and 1.5 nmol NO_2^-/mg P450 for 10 min, respectively). Noteworthy is that these values are higher than that obtained for the reference (1.3 nmol NO_2^-/mg P450 for 10 min). The modest capacity of compounds **2c** and **2d** to produce nitrite ions may originate from their inadequate size/shape that does not allow their fast oxidation by the enzyme and/or to the lower reactivity of aliphatic amidoximes in the oxidation reaction. Compound **2a** is of high interest due to its high capacity of release NO compared to the reference.

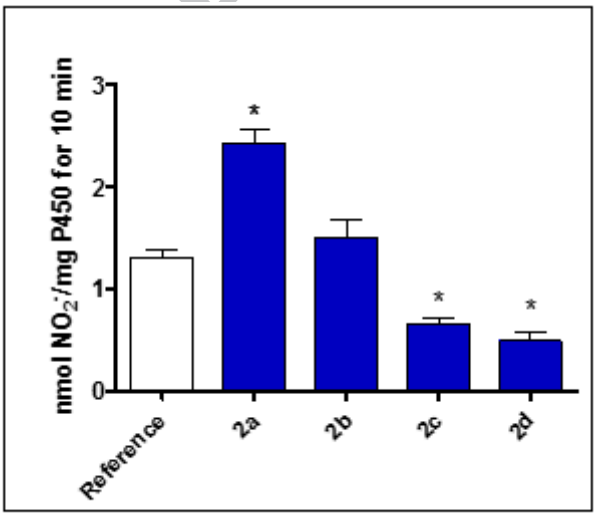


Fig. 4. Concentration of released nitrite ions from compounds **2** and the reference after a 10 min incubation with microsomes. Results are presented as mean ± SEM of three to five independent experiments and compared with a One-way ANOVA; * $p<0.05$ *versus* reference

We further evaluated the capacity of compounds **2** to release NO in the presence of human vascular smooth muscle cells (HVSMC). In preliminary experiments, the cytocompatibility of amidoximes **2** toward the HVSMC was evaluated (see Supporting Information). As shown in Fig. 5, except **2a** which induced the lowest cell viability at 100 µM (74.67% ± 2.03), HVSMC cells remained alive and active in the presence of amidoximes even used at high concentration. Further experiments were conducted at the 100 µM concentration to reach NO releases higher than the limit of detection of the fluorometric method used (*vide infra*). Noteworthy is also that the cytotoxicity observed at 100 µM was detected after 24 h-incubation while the release test requires only a 1 h-incubation.

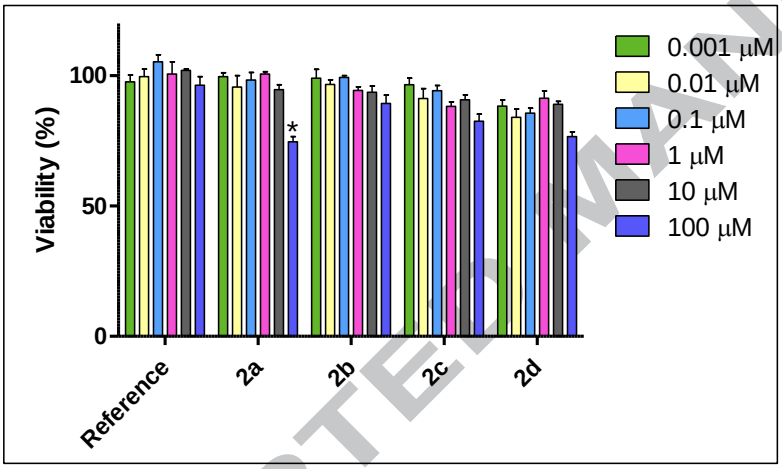


Fig. 5. Viability of the smooth muscle cells after 24 h incubation at concentrations ranging from 0.001 to 100 µM. Results are presented as mean ± SEM of three to four tests and compared with a two-way ANOVA for compound 2a; * $p<0.001$ 100 µM *versus* all the concentrations.

For NO release experiments using amidoximes **2**, *S*-nitrosoglutathione (GSNO),[25] a well-known NO donor, able to deliver bioavailable NO for smooth muscle cells and blood vessels,[26-28] was prepared and used as a reference. HVSMC cells were seeded in 6-wells plates 48 h before incubation with compounds **2** for 1 h at 37°C. Amidoximes **2a-c** and GSNO were used at 100 µM while compound **2d** bearing two amidoxime functions was used at 50 µM. After washing and lysis of the cells, *S*-nitrosothiols (RSNO) and nitrite ions were immediately quantified using a fluorometric method based on the reaction of N_2O_3 with 2,3-diaminonaphthlene producing naphthotriazole that emits at 415 nm after excitation at 375 nm

(see Supporting Information).[29] The RSNO quantification corresponds to the released NO able to react with cell proteins thiol groups (intracellular *S*-nitrosation). [30] Control cells incubated with PBS containing 0.1% DMSO were under the limit of detection for both RSNO and nitrite ions. Fig. 6 shows that all compounds induced the formation of RSNO inside the cells. In all cases, the concentration of nitrite ions was under the limit of quantification of the method. GSNO, **2a**, **2b** and **2d** produced a significantly higher RSNO intracellular concentration (0.20 ± 0.01 µM, 0.21 ± 0.01 µM and 0.17 ± 0.02 µM, respectively) compared to the 4-chlorobenzamidoxime reference (0.03 ± 0.03 µM). The intracellular *S*-nitrosation process is less pronounced for **2c**, which contains a similar aliphatic amidoxime function than **2d**. Noteworthy is that compounds **2a**, **2b** and **2d** exhibit a higher potential than GSNO to generate intracellular RSNO.

Among all new synthesized amidoximes, **2a** and **2b** seem the most potent since they showed very high NO release compared to other mono-donors like the reference and GSNO. On the other hand, **2d** is also of high interest since its ability to release NO and thus to increase NO storage through RSNO formation inside the cells is similar to compounds **2a** and **2b** but at a twice lower concentration.

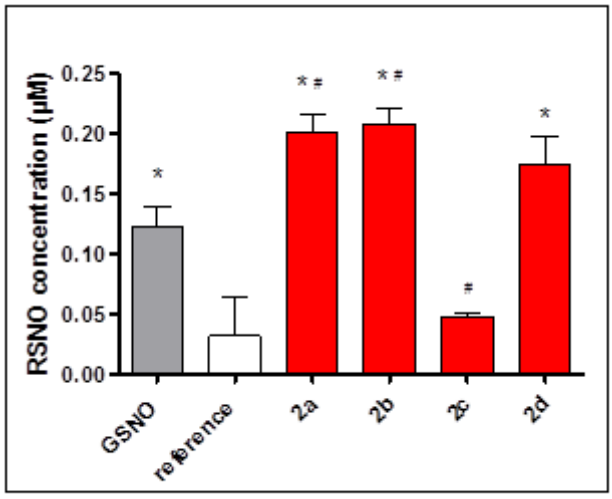


Fig. 6. RSNO intracellular concentration after a 1 h incubation of smooth muscle cells with 100 µM of each compound except **2d** that was used at 50 µM. Results are presented as mean ± SEM of three independent experiments and compared with a One-way ANOVA; * $p<0.05$ *versus* reference; # $p<0.05$ *versus* GSNO (Dunnett's multiple comparisons post-test).

In summary, a series of mono- and bis-amidoximes were synthesized and tested for their capacity to release NO. Using rat liver microsomes, only the aromatic mono-amidoximes **2a-b** showed high ability to release NO in the presence of cytochromes P450, while the aliphatic mono-amidoxime **2c** and the bis-amidoxime **2d** exhibit weaker NO liberation capacity. All of the studied amidoximes were demonstrated to be cytocompatible with human vascular smooth muscle cells and were further tested for their ability to deliver NO inside the cells. Amidoximes **2a**, **2b** and **2d** showed a high capacity to form intracellular RSNO indicating that these compounds were able to enter the cells and to be oxidized by the P450 of smooth muscle cells. The lowest delivery of NO inside the cells was observed with aliphatic amidoxime **2c**. Among the amidoximes able to form a high amount of intracellular RSNO, bis-amidoxime **2d** is of high potential since its concentration was two times lower than that of other amidoximes. This makes new bis-amidoxime **2d** of interest since it should allow to halve the drug dosage and thus to reduce the potential secondary effects. Results obtained with mono-amidoximes **2a-b** show their high capability of being used as NO donors and these compounds are among the best existing mono-NO-donors reported to date.

Acknowledgments

The authors thank the Agence Universitaire de la Francophonie (AUF) middle east for financial support of this work. The authors also thank Olivier Fabre for NMR analysis and Isabelle Fries for the cytocompatibility tests.

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/

References and notes

1. Steiger, A.K.; Zhao, Y.; Pluth, M.D. *Antioxid Redox Signal.* **2018**, *28*, 1516.
2. Fagone, P.; Mazzon, E.; Bramanti, P.; Bendtzen, K.; Nicoletti, F. *Eur. J. Pharmacol.* **2018**, *834*, 92.
3. Schröder, A.; Kotthaus, J.; Schade, D.; Clement, B.; Rehse, K. *Arch. Pharm.* **2010**, *343*, 9.
4. Snyder, S.H.; Bredt, D.S. *Sci. Am*. **1992**, *266*, 68.
5. Shefa, U.; Yeo, S.G.; Kim, M.S.; Song, I.O.; Jung, J.; Jeong, N.Y.; Huh, Y. *BioMed Res. Int.* **2017**, *2017*.
6. Sennequier, N.; Boucher, J.-L.; Battioni, P.; Mansuy, D. *Tetrahedron Lett*. **1995**, *36*, 6059.
7. Mansuy, D.; Boucher, J.-L. *Free Radical Biol. Med.* **2004**, *37*, 1105.
8. Mansuy, D.; Boucher, J.-L.; Clement, B. *Biochimie* **1995**, *77*, 661.
9. Fylaktakidou, K. ; Hadjipavlou-Litina, D.; Litinas, K.; Varella, E.; Nicolaides, D. *Curr. Pharm. Design* **2008**, *14*, 1001.
10. Jousserandot, A.; Boucher, J.-L.; Desseaux, C.; Delaforge, M.; Mansuy, D. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 423.
11. Jousserandot, A.; Boucher, J.-L.; Henry, Y.; Niklaus, B.; Clement, B.; Mansuy, D. *Biochemistry* **1998**, *37*, 17179.
12. Rehse, K.; Bade, S.; Harsdorf, A.; Clement, B. *Arch. Pharm.* **1997**, *330*, 392.
13. Rehse, K.; Brehme, F. *Arch. Pharm.* **1998**, *331*, 375.
14. Vetrovsky, P. *J. Pharmacol. Exp. Ther*. **2002**, *303*, 823.
15. Chalupsky, K.; Lobysheva, I.; Nepveu, F.; Gadea, I.; Beranova, P.; Entlicher, G.; Stoclet, J.-C.; Muller, B. *Biochem. Pharmacol.* **2004**, *67*, 1203.
16. Ovdiichuk, O.; Hordiyenko, O.; Medviediev, V.; Shishkin, O.; Arrault, A. *Synthesis* **2015**, *47*, 2285.
17. Ovdiichuk, O.; Hordiyenko, O.; Arrault, A. *Tetrahedron* **2016**, *72*, 3427.
18. Ovdiichuk, O.; Hordiyenko, O.; Fotou, E.; Gaucher, C.; Arrault, A.; Averlant-Petit, M.-C. *Struct. Chem.* **2017**, *28*, 813.
19. General protocol for the synthesis of mono amidoximes. To a stirred solution of nitrile **1** (4 mmol) in ethanol (40 mL), were added hydroxylamine hydrochloride and sodium carbonate (see Table 1 for details). The mixture was heated to reflux for 48 h. After cooling to room temperature, the solvent was evaporated and the residue was extracted with ethyl acetate (2 x 30 mL). The organic phase was washed with water (30 mL), dried over $MgSO_4$ and

finally concentrated under vacuum. Amidoximes **2** were purified by flash chromatography using methanol/dichloromethane (10/90) as eluent.

20. 4-Hydroxybenzamidoxime: Yellow-brown powder. $C_7H_8N_2O_2$. Yield: 596 mg (98%); mp 150.1°C; TLC: R_f = 0.15 (CH_2Cl_2/MeOH, 90:10) [silica gel]. ^{1}H NMR (300 MHz, DMSO-d_6, 25°C): δ = 9.57 (s, 1H, =N-OH), 9.33 (s, 1H, OH), 7.49-7.46 (m, 2H, H_{ar}), 6.74-6.72 (m, 2H, H_{ar}), 5.61 (s, 2H, NH_2). ^{13}C NMR (75 MHz, DMSO-d_6, 25°C): δ = 158.1, 150.8, 126.7, 124.1, 114.7. IR (neat): 3434 (br), 3319 (br), 3211 (br), 1645 (m), 1609 (m), 1509 (m), 1277 (m), 1247 (m). 1134 (m). HRMS (ESI, *m/z*): MH^+, found 153.0688. $C_7H_9N_2O_2$ requires 153.0659.

21. 4-Methoxybenzamidoxime: White powder. $C_8H_{10}N_2O_2$. Yield: 631 mg (95%); mp 120.2°C; TLC: R_f = 0.15 (CH_2Cl_2/MeOH, 90:10) [silica gel]. ^{1}H NMR (300 MHz, DMSO-d_6, 25°C): δ = 9.42 (s, 1H, OH), 7.62-7.59 (m, 2H, H_{ar}), 6.93-6.90 (m, 2H, H_{ar}), 5.70 (s, 2H, NH_2), 3.77 (s, 3H, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6, 25°C): δ = 159.8, 150.5, 126.7, 125.7, 113.4, 55.1. IR (neat): 3443 (s), 3353 (s), 2968 (w), 2841 (w), 1646 (s), 1608 (m), 1518 (s), 1413 (m), 1386 (m), 1303 (m), 1243 (s), 1175 (m), 1033 (m), 925 (m), 835 (s). (ESI, *m/z*): MH^+, found 167.0836. $C_8H_{11}N_2O_2$ requires 167.0815.

22. General protocol of the bis-amidoximes and aliphatic amidoxime synthesis: To a stirred solution of nitrile **1** in ethanol (40 mL), was added an aqueous hydroxylamine solution (50 wt. % in H_2O). The reaction was heated to reflux for 24 h. After cooling to room temperature, the solvent was evaporated and the residue was extracted with ethyl acetate (2 x 30 mL). The organic phase was washed with water, dried over $MgSO_4$ and finally concentrated under vacuum. The product was purified by flash chromatography using methanol/dichloromethane (10/90) as eluent.

23. N-hydroxy-5-phenoxy-pentanamidine: Black purple powder. $C_{11}H_{16}N_2O_2$. Yield: 583 mg (70%); mp 96.5°C; TLC: R_f = 0.15 (CH_2Cl_2/MeOH, 90:10) [silica gel]. ^{1}H NMR (300 MHz, DMSO-d_6, 25°C): δ = 8.70 (s, 1H, OH), 7.30-7.24 (m, 2H, H_{ar}), 6.93-6.88 (m, 3H, H_{ar}), 5.32 (s, 2H, NH_2), 3.95 (t, *J* = 6.2 Hz, 2H, O-CH_2), 2.02 (t, *J* = 7.2 Hz, 2H, CH_2), 1.73-1.61 (m, 4H, $(CH_2)_2$). ^{13}C NMR (75 MHz, DMSO-d_6, 25°C): δ = 158.6, 152.6, 129.4, 120.3, 114.4, 67.0, 30.4, 28.2, 22.9. IR (neat): 3475 (br), 3361 (br), 3229 (br), 2926 (w), 2871 (w), 1681 (w), 1589 (w), 1498 (w), 1475 (w), 1296 (w), 1255 (m), 751 (w), 660 (m). (ESI, *m/z*): MH^+, found 209.1323. $C_{11}H_{17}N_2O_2$ requires 209.1285.

24. N-Hydroxy-4-[4-(N-hydroxycarbamimidoyl)-butoxy]-benzamidine: Green powder. $C_{12}H_{18}N_4O_3$. Yield: 330 mg (40%); mp 166.6°C; TLC: R_f = 0.15 (CH_2Cl_2/MeOH, 90:10) [silica gel]. ^{1}H NMR (300 MHz, DMSO-d_6, 25°C): δ = 9.42 (s, 1H, OH), 8.72 (s, 1H, OH),

7.60-7.57 (m, 2H, H_{ar}), 6.94-6.89 (m, 2H, H_{ar}), 5.69 (s, 2H, NH_2), 5.38 (s, 2H, NH_2), 3.98 (t, *J* = 6.0 Hz, 2H, O-CH_2), 2.02 (t, *J* = 7.1 Hz, 2H, CH_2), 1.73-1.61 (m, 4H, $(CH_2)_2$). ^{13}C NMR (75 MHz, DMSO-d_6, 25°C): δ = 159.2, 152.8, 150.6, 126.7, 125.6, 113.9, 67.2, 30.3, 28.1, 22.9. IR (neat): 3452 (br), 3303 (br), 2941 (w), 2869 (w), 1679 (s), 1627 (s), 1522 (m), 1393 (m), 1306 (w), 1260 (m), 1180 (m), 920 (w), 843 (w). (ESI, *m/z*): MNa^+, found 289.1291. $C_{12}H_{18}N_4NaO_3$ requires 289.1271.

25. Parent, M.; Dahboul, F.; Schneider, R.; Clarot, I.; Maincent, P. *Curr. Pharm. Anal.* **2013**, *9*, 31.

26. Belcastro, E.; Wu, W.; Fries-Raeth, I.; Corti, A.; Pompella, A.; Leroy, P.; Lartaud, I.; Gaucher, C. *Nitric Oxide* **2017**, 69, 10.

27. Wu, W.; Perrin-Sarrado, C.; Ming, H.; Lartaud, I.; Maincent, P.; Hu, X.-M.; Sapin-Minet, A.; Gaucher, C. *Nanomedicine: Nanotechnology, Biology and Medicine* **2016**, *12*, 1795.

28. Dahboul, F.; Leroy, P.; Maguin Gate, K.; Boudier, A.; Gaucher, C.; Liminana, P.; Lartaud, I.; Pompella, A.; Perrin-Sarrado, C. *PLoS ONE* **2012**, *7*, e43190.

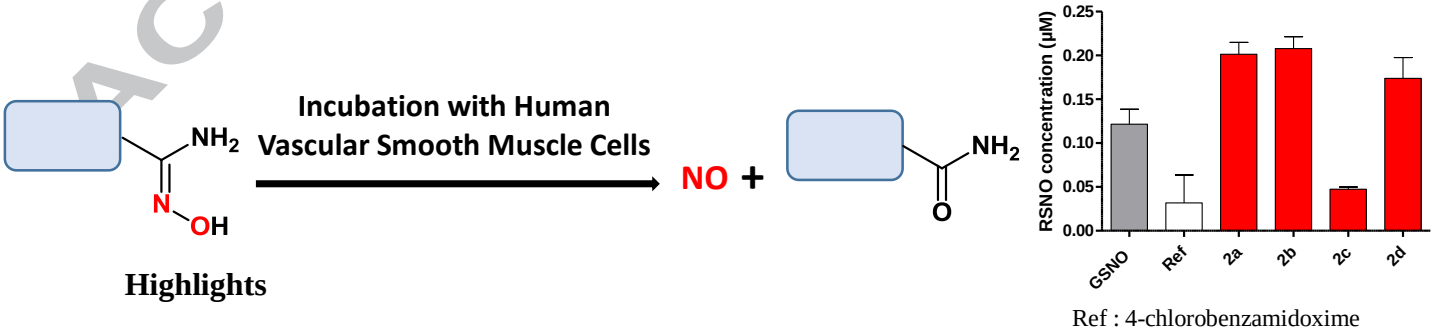
29. Wu, W. ; Gaucher, C.; Diab, R.; Fries, I.; Xiao, Y.-L.; Hu, X.-M.; Maincent, P.; Sapin-Minet, A. *Eur. J. Pharm. Biopharm.* **2015**, *89*, 1.

30. Nagababu, E.; Rifkind, J.M. *Cell Biochem. Biophys.* **2013**, *67*, 385.

Graphical Astract

Synthesis of Novel Mono and Bis Nitric Oxide Donors with High Cytocompatibility and Release Activity

Tanya Sahyoun, Caroline Gaucher, Yi Zhou, Naïm Ouaini, Raphaël Schneider, Axelle Arrault



Ref : 4-chlorobenzamidoxime

Highlights

- A series of aromatic and aliphatic mono-amidoximes and bis-amidoximes were synthesized.
- Amodoximes were tested for their NO release ability on rat liver microsomes and human vascular cells.

- Amidoximes are cytocompatible with human vascular smooth muscle cells.
- Mono-amidoximes **2a** and **2b** exhibit high NO release.
- Bis-amidoxime **2d** releases the same amount of NO than **2a** and **2b** at a twice lower concentration.