



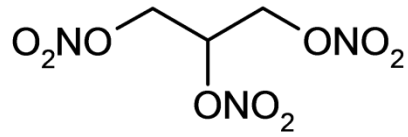
4th International Webinar on Chemistry and Pharmaceutical Chemistry

Overcome nitrate tolerance: new perspectives with multitarget antioxidant NO-donor nitrates

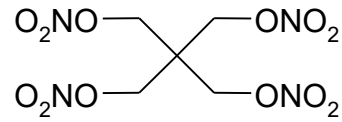
Elisabetta Marini

Webinar
March 11-12, 2022

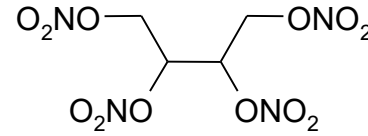
Organic Nitrates



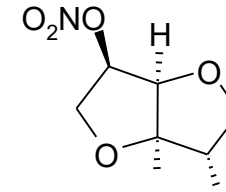
Glyceryl trinitrate (GTN)



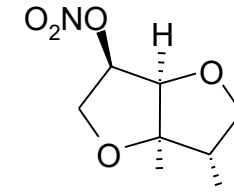
Pentaerythritol tetranitrate (PETN)



Erythrityl tetranitrate

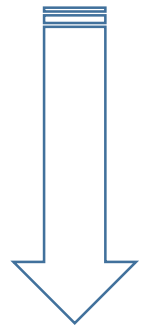


Isosorbide dinitrate (ISDN)



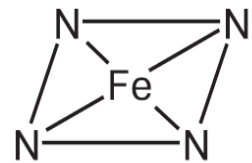
Isosorbide mononitrate (ISMN)

➤ The most commonly adopted vasodilators in coronary artery diseases

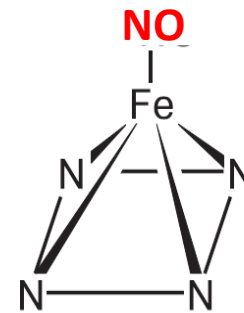


smooth muscle cell

Ferroprotoporphyrin IX



Nonactivated GC



Activated GC



Kinase protein

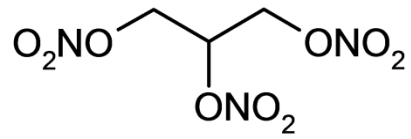


Myosin ~~×~~ P

↓ [Ca²⁺]_i

relaxation

Organic Nitrates: Bioactivation



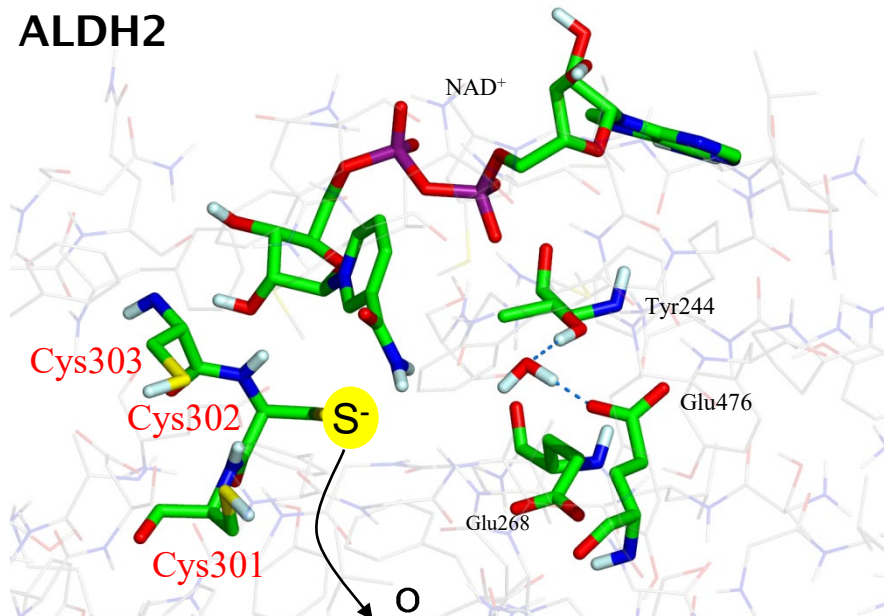
Glyceryl trinitrate
(GTN)



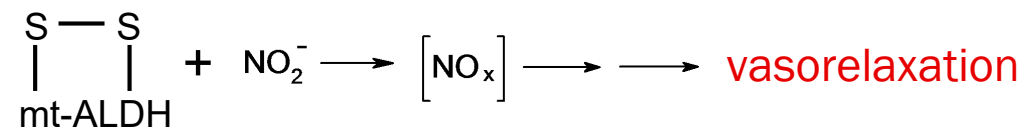
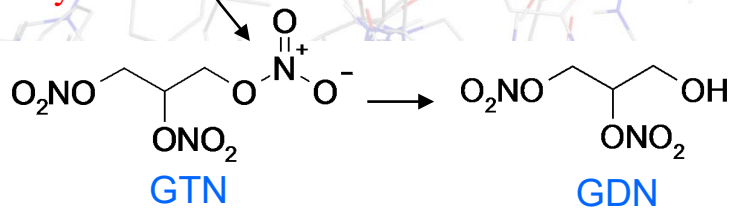
Enzymatic bioactivation :

- Glutathione S-transferases
- Cytochrome P450
- Xanthine oxidoreductase
- Glyceraldehyde-3-phosphate dehydrogenase
- Aldehyde dehydrogenase (ALDH) 2

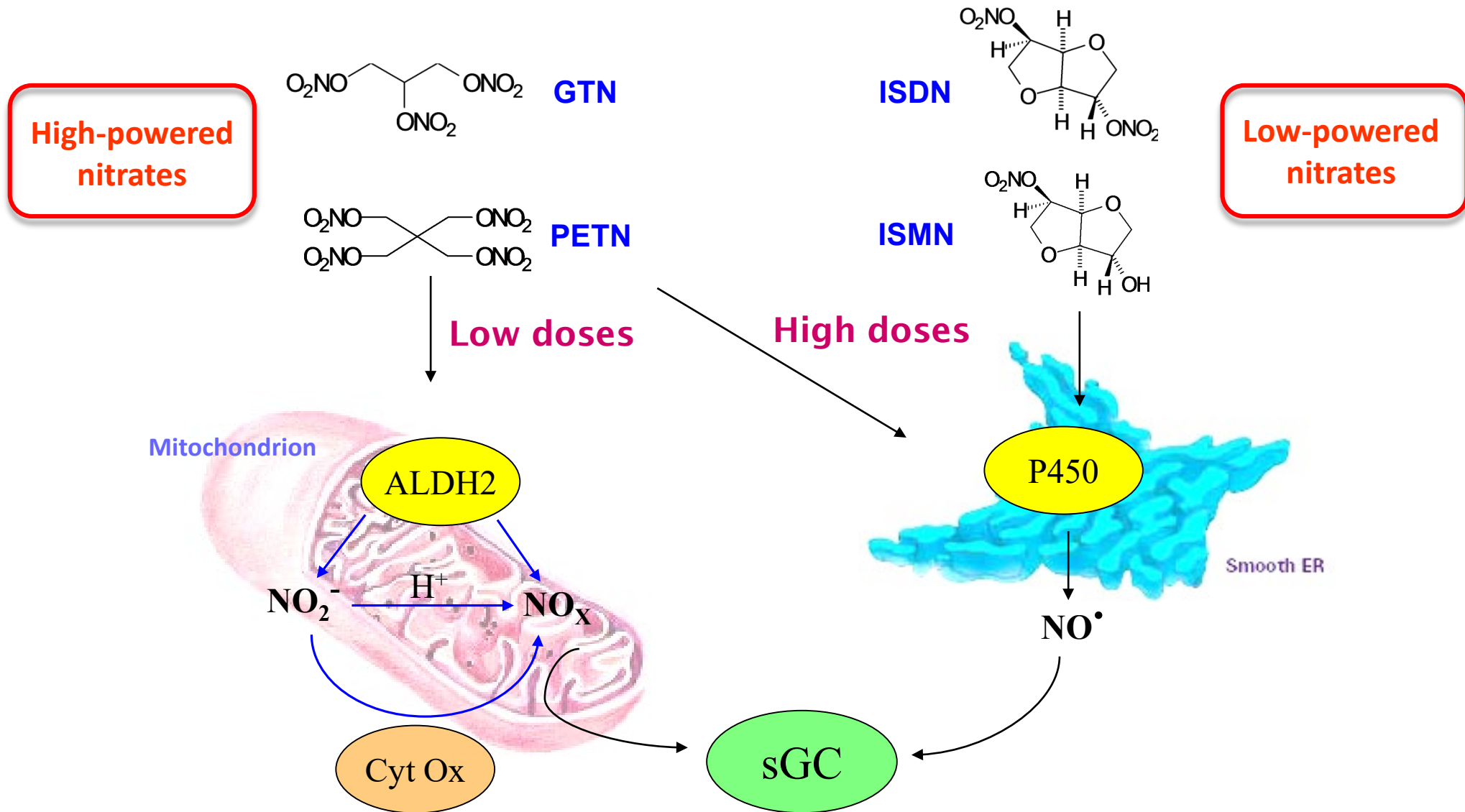
ALDH2



Not all organic nitrates are able to interact with ALDH2



Organic Nitrates: Bioactivation



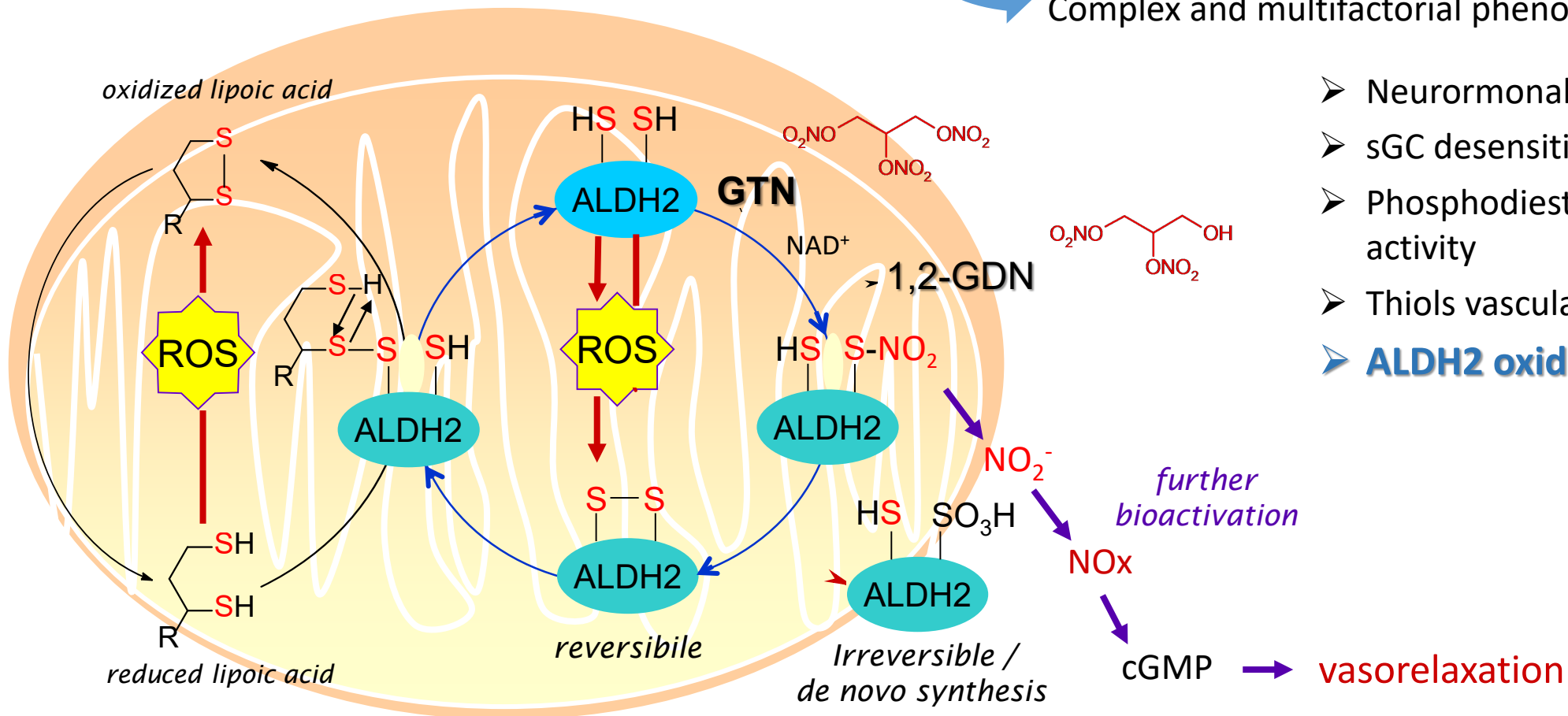
Organic Nitrates: Tolerance

The chronic efficacy of nitrates is blunted due to the development of:

- endothelial dysfunction
- nitrate tolerance and/or cross-tolerance to endothelium-dependent vasodilators

Complex and multifactorial phenomenon:

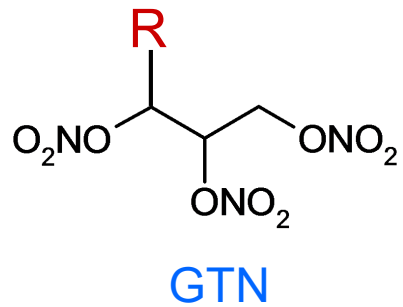
- Neuromonal counter-regulation
- sGC desensitization
- Phosphodiesterases increased activity
- Thiols vascular depletion
- **ALDH2 oxidative inactivation**



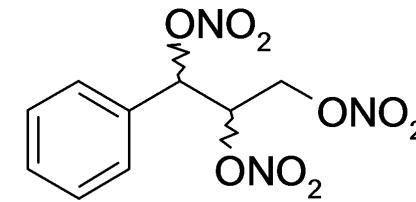
Organic Nitrates: in the search of novel NO-donors

Cardiovascular effects of GTN are well established from decades, but:

- the search for new NO-donors for clinical use is still actual
- the study of the mechanisms involved in tolerance development continues



- ✓ new compounds available as potential drugs
- ✓ new compounds available for use as probes to deepen the nitrates mechanism of action

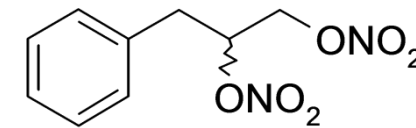


(+) **1a** erythro isomer

(-) **1a** erythro isomer

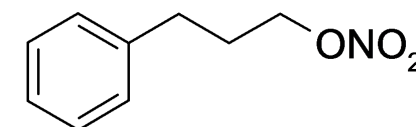
(+) **1b** threo isomer

(-) **1b** threo isomer



(+) **2**

(-) **2**

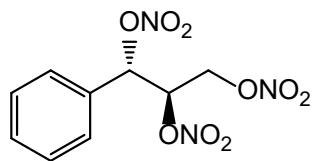


3

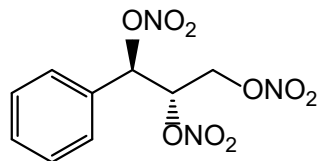
In vitro vasodilator profile

➤ Endothelium denuded rat aorta strips, precontracted with 1 μM L-phenylephrine

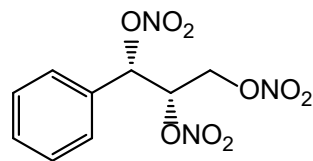
Compd	EC ₅₀ ± SE (μM)	+ Benomyl 1 μM	+ ODQ 1 μM
GTN	0.029 ± 0.004	0.42 ± 0.07	> 100
(+) 1a	0.29 ± 0.09	0.36 ± 0.07	21 ± 2
(-) 1a	0.21 ± 0.06	0.32 ± 0.08	18 ± 2
(+) 1b	0.28 ± 0.06	0.32 ± 0.06	30 ± 1
(-) 1b	0.22 ± 0.05	0.35 ± 0.08	21 ± 2
(+) 2	0.061 ± 0.010	0.60 ± 0.13	> 100
(-) 2	0.063 ± 0.010	0.63 ± 0.13	> 100
3	0.16 ± 0.03	1.5 ± 0.5	> 100



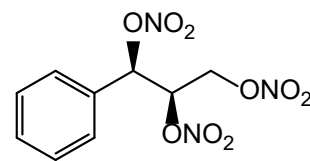
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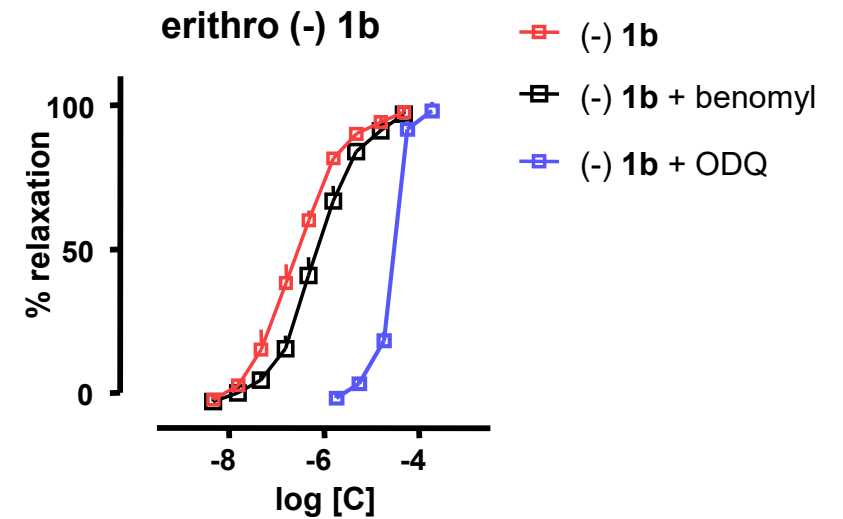
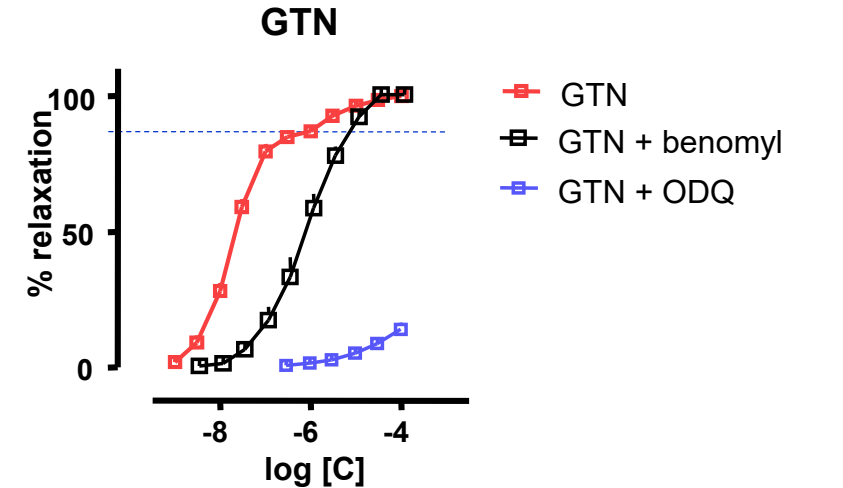
(-) **1a** erythro isomer



(+) **1b** threo isomer



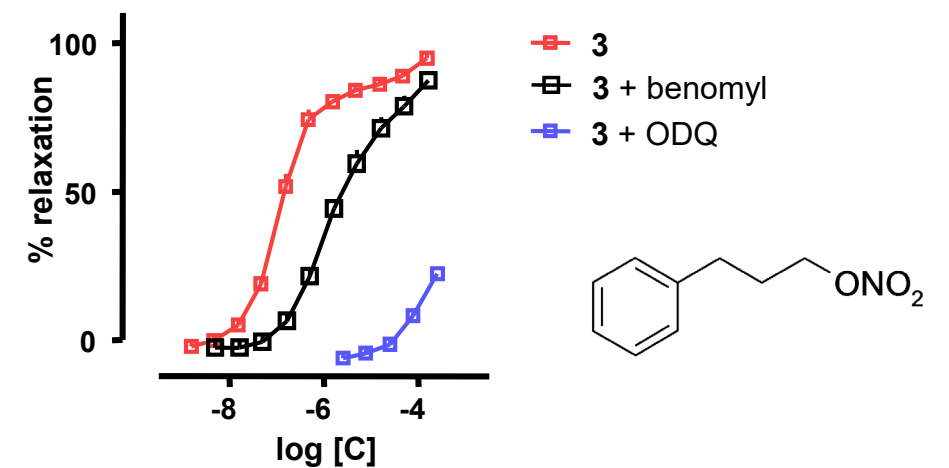
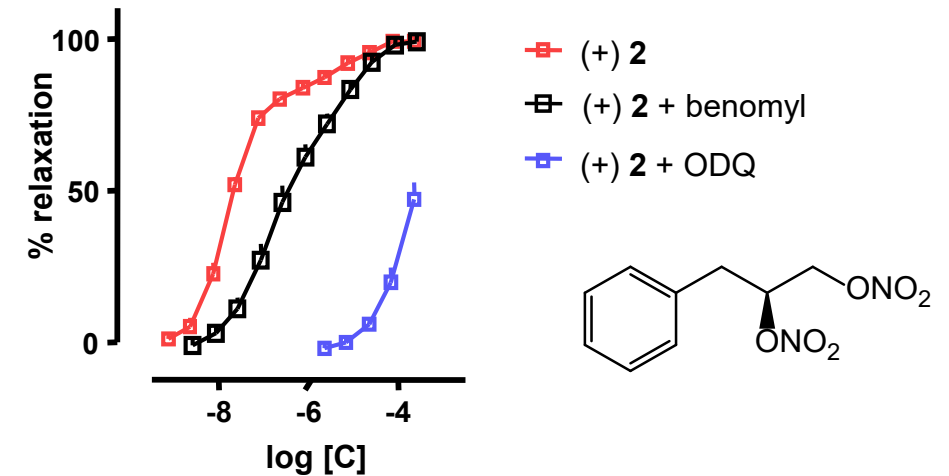
(-) **1b** threo isomer



In vitro vasodilator profile

➤ Endothelium denuded rat aorta strips, precontracted with 1 μM L-phenylephrine

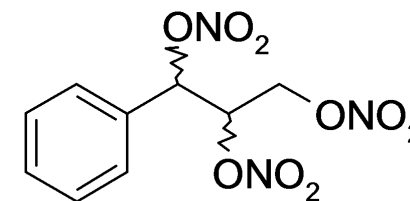
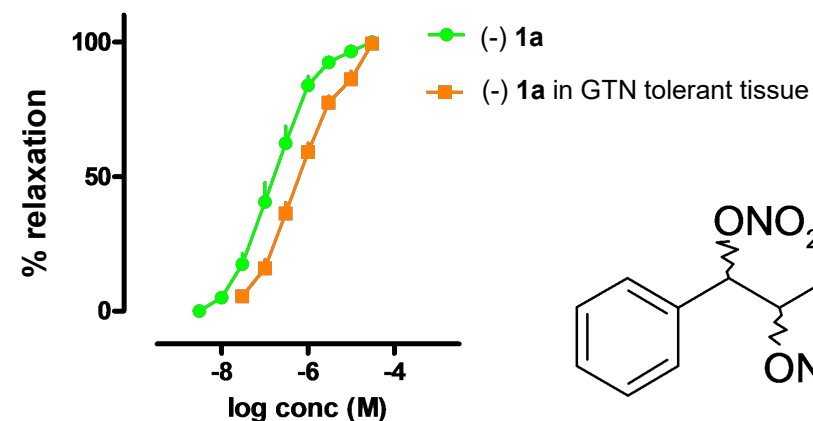
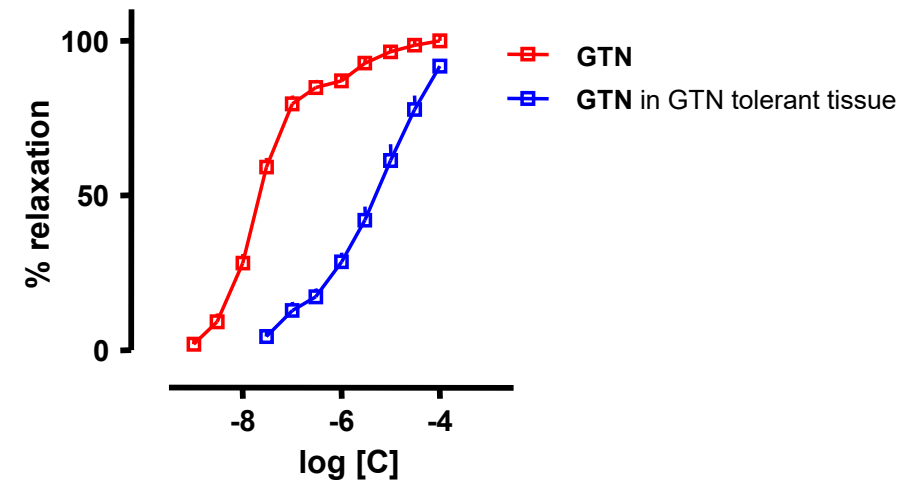
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(-) 1a	0.21 ± 0.06	0.32 ± 0.08	18 ± 2
(+) 1b	0.28 ± 0.06	0.32 ± 0.06	30 ± 1
(-) 1b	0.22 ± 0.05	0.35 ± 0.08	21 ± 2
(+) 2	0.061 ± 0.010	0.60 ± 0.13	> 100
(-) 2	0.063 ± 0.010	0.63 ± 0.13	> 100
3	0.16 ± 0.03	1.5 ± 0.5	> 100



In vitro GTN cross-tolerance study

- Endothelium denuded rat aorta strips, incubated with 0.55 mM GTN for 1 h, washout, precontracted with 1 μM L-phenylephrine

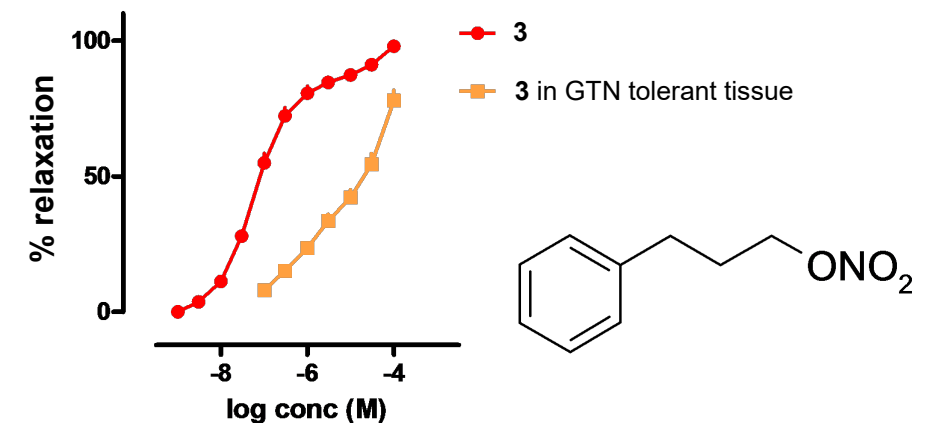
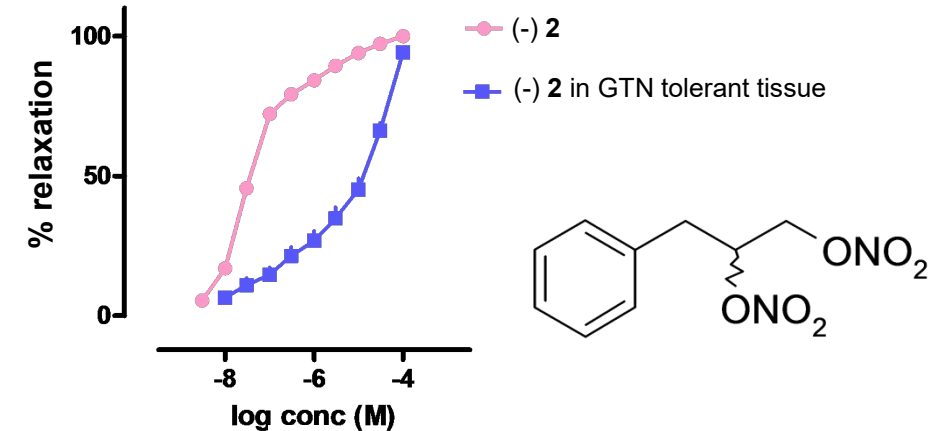
Compd	EC ₅₀ ± SE (μM)	Tolerant tissue	EC ₅₀ tol / EC ₅₀
GTN	0.029 ± 0.004	4.4 ± 1.3	152
(+) 1a	0.29 ± 0.09	0.78 ± 0.06	2.7
(-) 1a	0.21 ± 0.06	0.61 ± 0.10	2.9
(+) 1b	0.28 ± 0.06	1.0 ± 0.2	3.6
(-) 1b	0.22 ± 0.05	0.65 ± 0.05	3.0
(+) 2	0.061 ± 0.010	6.1 ± 1.0	100
(-) 2	0.063 ± 0.010	6.4 ± 1.1	102
3	0.16 ± 0.03	20 ± 4	125



In vitro GTN cross-tolerance study

- Endothelium denuded rat aorta strips, incubated with 0.55 mM GTN for 1 h, washout, precontracted with 1 μ M L-phenylephrine

Compd	EC ₅₀ \pm SE (μ M)	Tolerant tissue	EC ₅₀ tol / EC ₅₀
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(-) 1b	0.22 \pm 0.05	0.65 \pm 0.05	3.0
(+) 2	0.061 \pm 0.010	6.1 \pm 1.0	100
(-) 2	0.063 \pm 0.010	6.4 \pm 1.1	102
3	0.16 \pm 0.03	20 \pm 4	125



Organic Nitrates: in the search of novel NO-donors

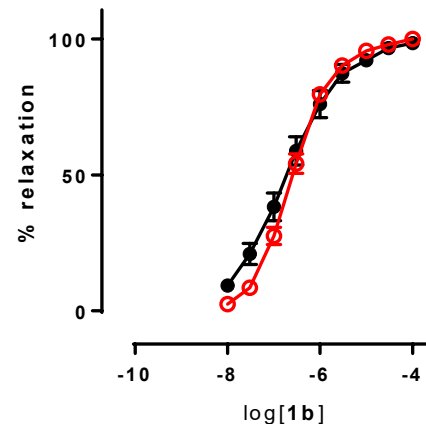
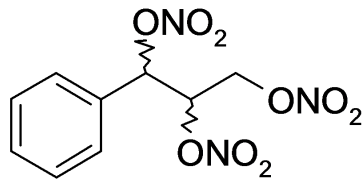
Ex vivo tolerance study

- Nitrate tolerance induced in rats by subcutaneous injection of 50 mg/kg/die GTN, or equimolar doses of nitrooxy derivatives, for 3 days consecutives
- Thoracic aortas removed and immediately used for functional studies
- Control animals treated with vehicle only, saline solution for GTN and DMSO for tested compounds

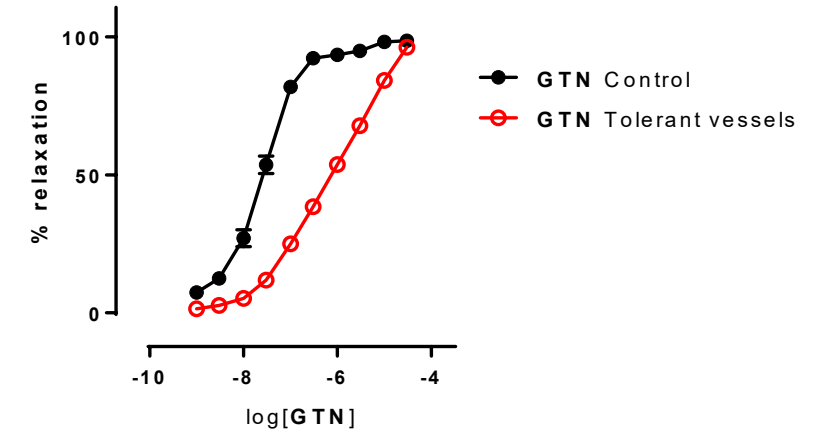
Compd	EC ₅₀ ± SE (μM)		Ex vivo tolerance	GTN cross-tolerance
	Control	Tolerant vessels	EC _{50tol} / EC ₅₀	EC _{50tol} / EC ₅₀
GTN	0.030 ± 0.004	0.80 ± 0.08	27	152
(±) 1a	0.12 ± 0.02	0.16 ± 0.03	1	2.8
(±) 1b	0.27 ± 0.07	0.36 ± 0.05	1	3.3
(±) 2	0.019 ± 0.004	0.26 ± 0.04 ^a	14	101
3	0.045 ± 0.004	0.43 ± 0.06 ^b	10	125

a *** P<0.0001 vs control (Student's t test);

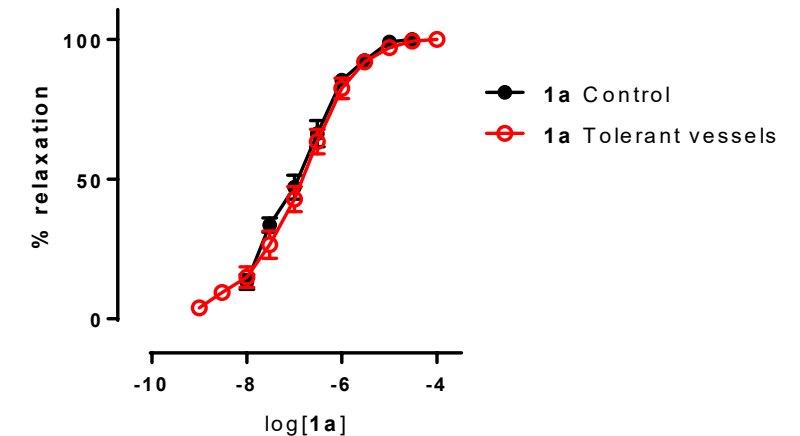
b *** P<0.0001 vs control (Student's t test)



● 1b Control
○ 1b Tolerant vessels



● GTN Control
○ GTN Tolerant vessels



● 1a Control
○ 1a Tolerant vessels

Organic Nitrates: in the search of novel NO-donors

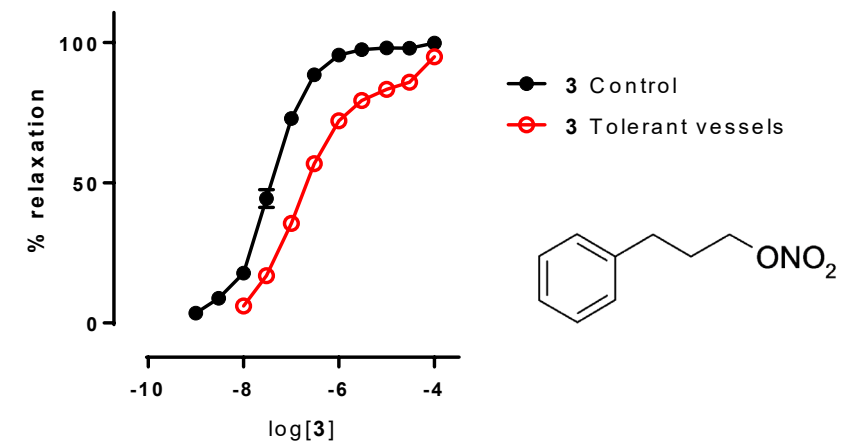
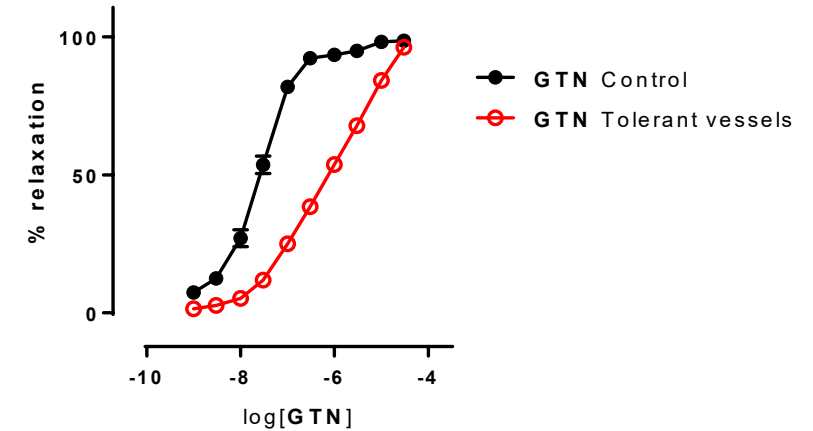
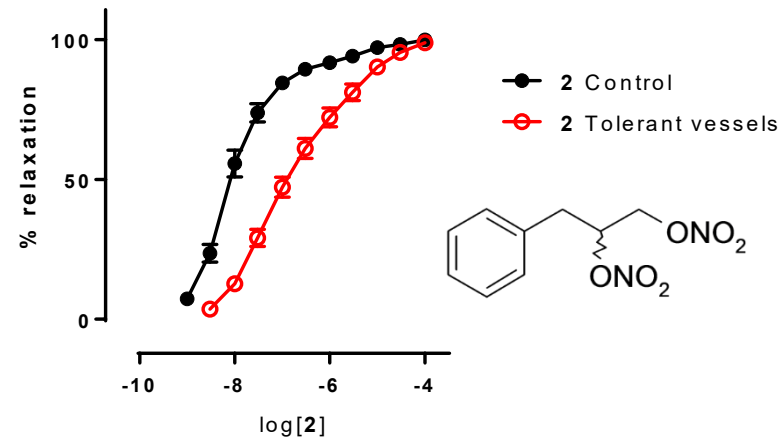
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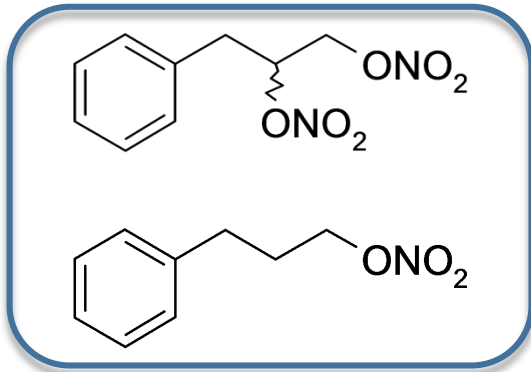
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b *** P<0.0001 vs control (Student's t test)

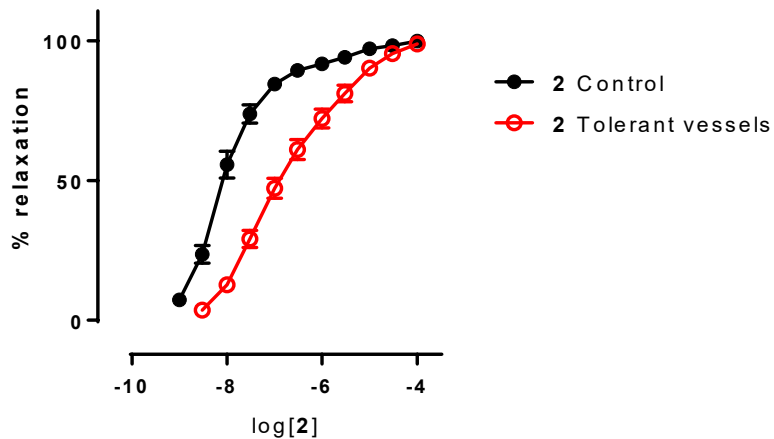


Organic Nitrates: in the search of novel NO-donors

GTN-like

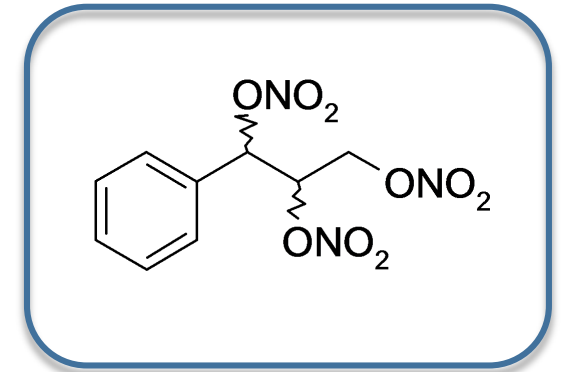


- Biphasic curve
- Sensitive to ALDH2 inhibitors:
bioactivation ALDH2 dependent
- GTN cross-tolerance
- **Direct tolerance**

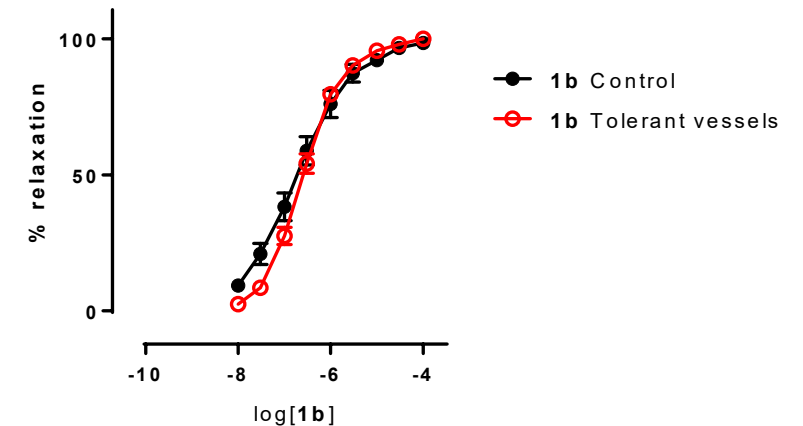


A nitrate, when
bioactivated by ALDH2,
can induce tolerance

GTN-unlike

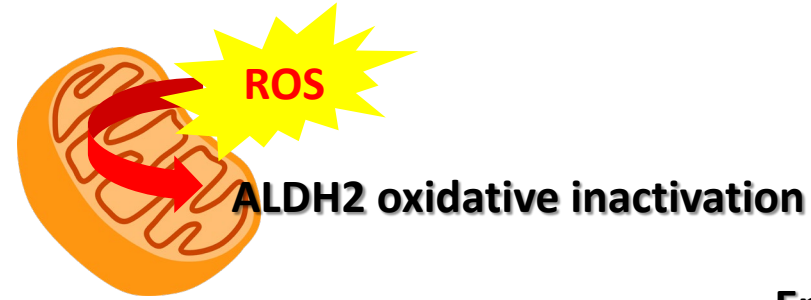


- Monophasic curve
- Not sensitive to ALDH2 inhibitors:
bioactivation ALDH2 independent
- No GTN cross-tolerance
- **No direct tolerance**

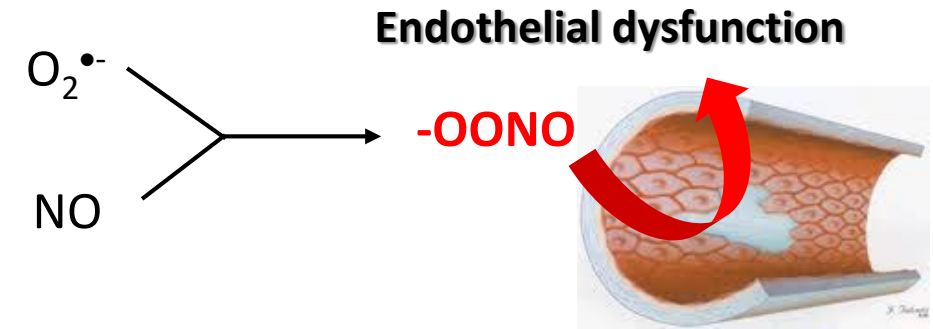


Organic Nitrates: new perspectives with multitarget antioxidant NO-donor nitrates

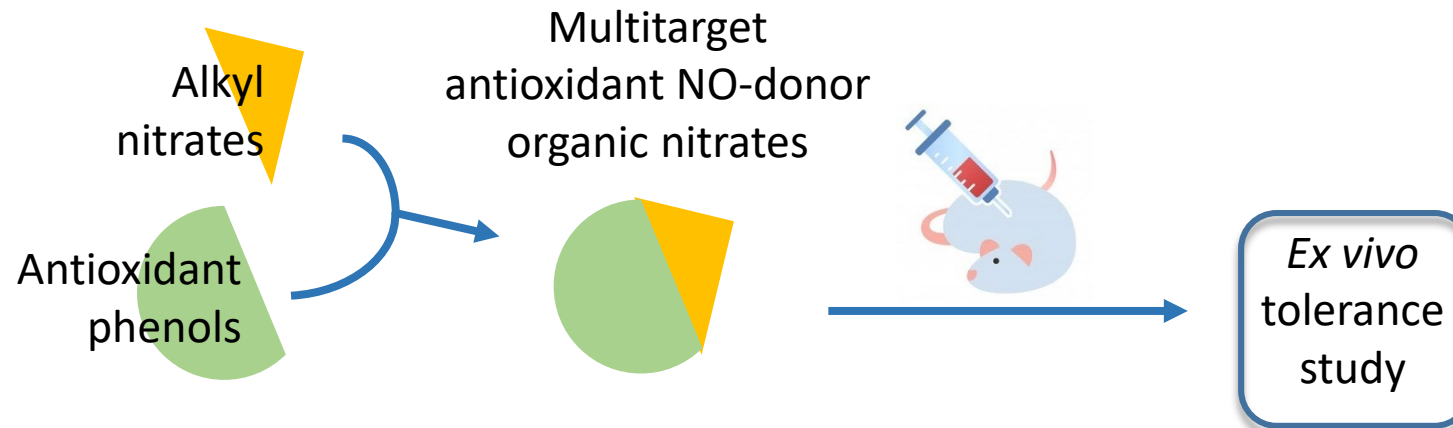
➤ Chronic nitrate treatment



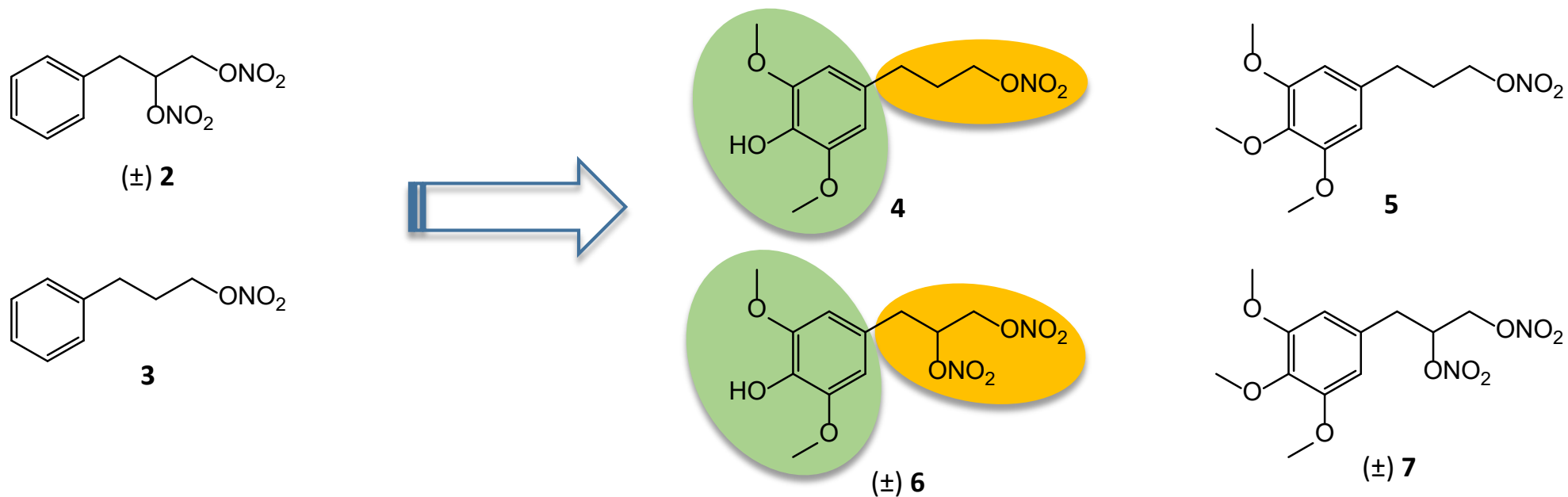
- **Antioxidants** preserved the sensitivity of the vasculature to organic nitrates in different animal models, but proved **ineffective** in clinical settings



INNOVATIVE APPROACH:



Organic Nitrates: new perspectives with multitarget antioxidant NO-donor nitrates



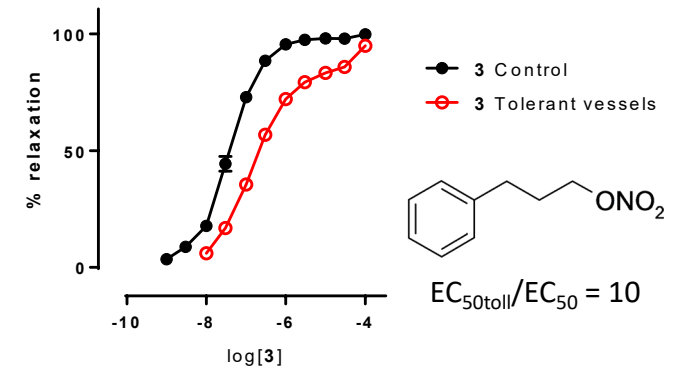
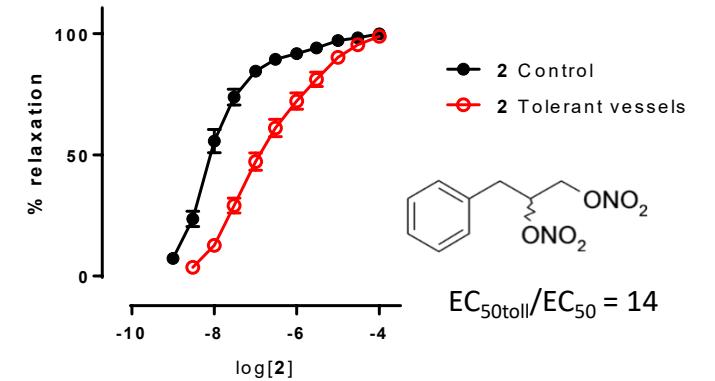
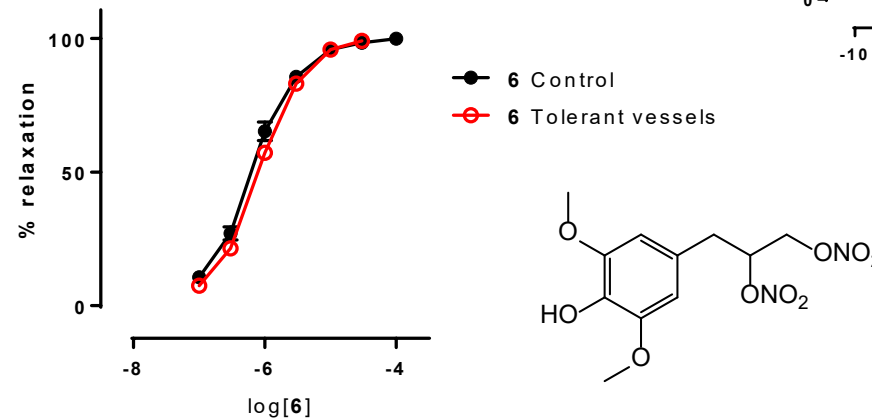
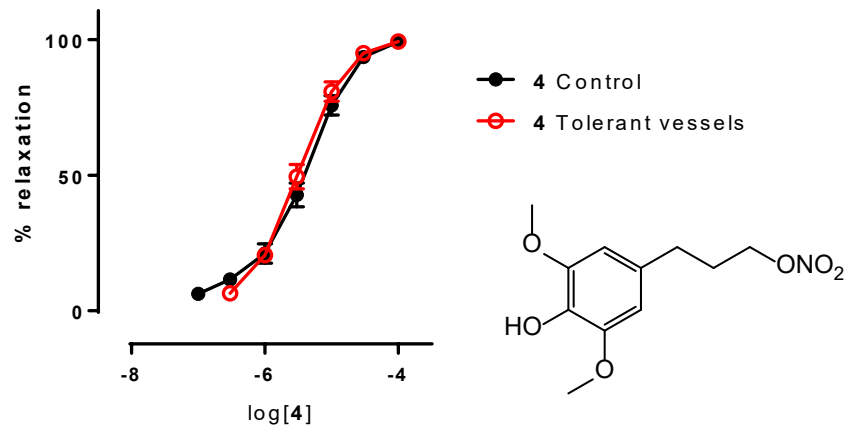
Compd	TBARS IC ₅₀ (CI 95%) μM	logZ
Ref. phenol	18 (17-20)	3.24
4	5.9 (5.5-6.4)	3.11
5	Not active at 1 mM	--
(±) 6	5.4 (5.1-5.8)	2.78
(±) 7	Not active at 1 mM	--

- TBARS: inhibition of lipid peroxidation induced by iron/ascorbate, in rat liver microsomes
- logZ: DPPH absorbance quencing in the first 15 s of reaction

Organic Nitrates: new perspectives with multitarget antioxidant NO-donor nitrates

Compd	EC ₅₀ ± SE (μM)	+ Benomyl 1 μM	EC ₅₀ ± SE (μM)		Ex vivo tolerance EC ₅₀ tol / EC ₅₀
			Control	Tolerant vessels	
GTN	0.029 ± 0.004	0.42 ± 0.07	0.030 ± 0.004	0.80 ± 0.08	27
4	3.5 ± 0.7	15 ± 2	3.9 ± 0.6	3.7 ± 0.4	1
5	3.2 ± 0.7	20 ± 3	3.0 ± 0.6	7.0 ± 0.8 ^a	2
(±) 6	0.64 ± 0.09	3.3 ± 0.6	0.74 ± 0.08	0.90 ± 0.05	1
(±) 7	0.28 ± 0.07	2.8 ± 0.5	0.32 ± 0.04	1.3 ± 1.1 ^b	4

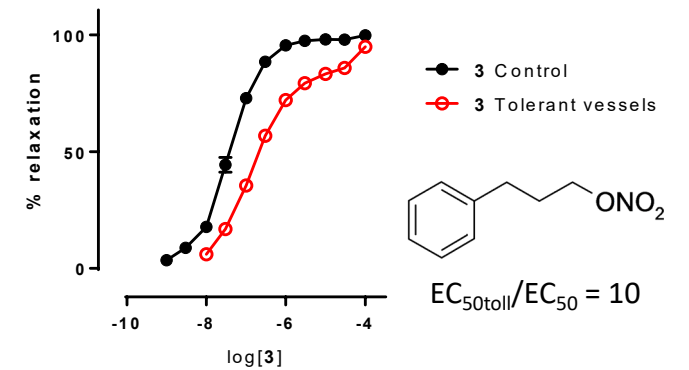
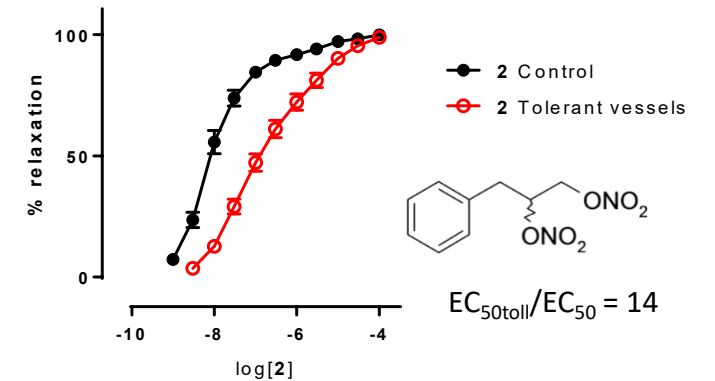
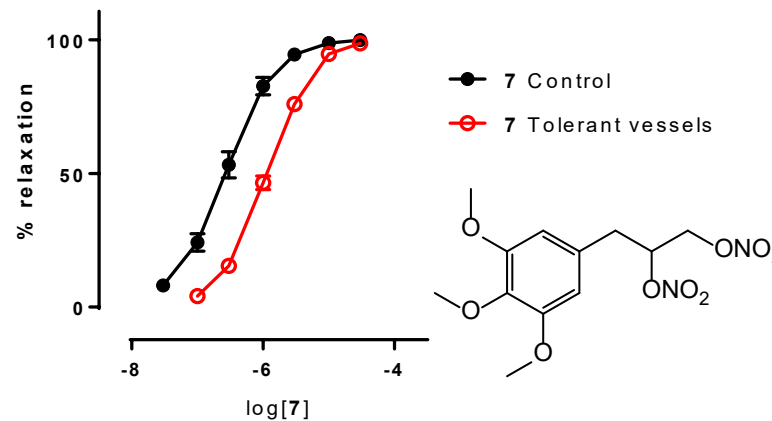
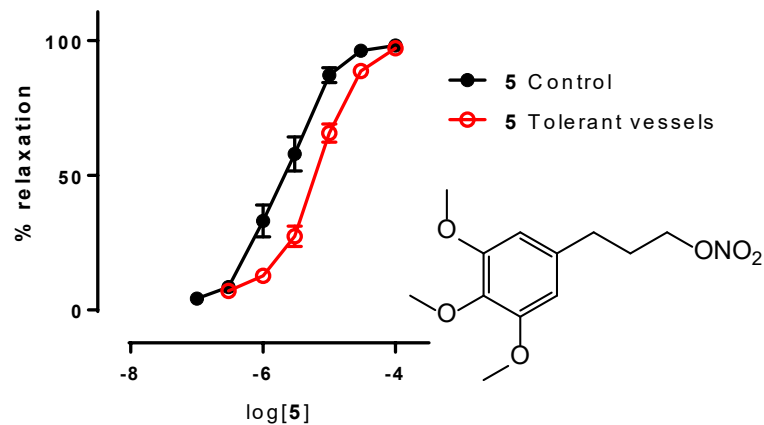
^a *** P<0.0003 vs control (Student's t test); ^b *** P<0.0001 vs control (Student's t test)



Organic Nitrates: new perspectives with multitarget antioxidant NO-donor nitrates

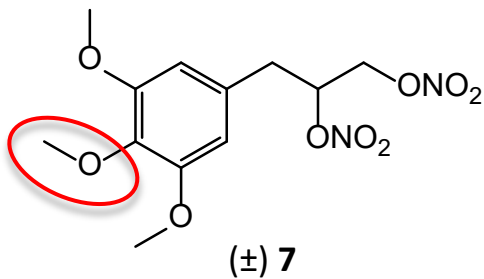
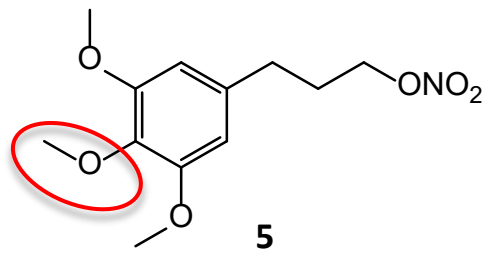
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^a *** P<0.0003 vs control (Student's t test); ^b *** P<0.0001 vs control (Student's t test)

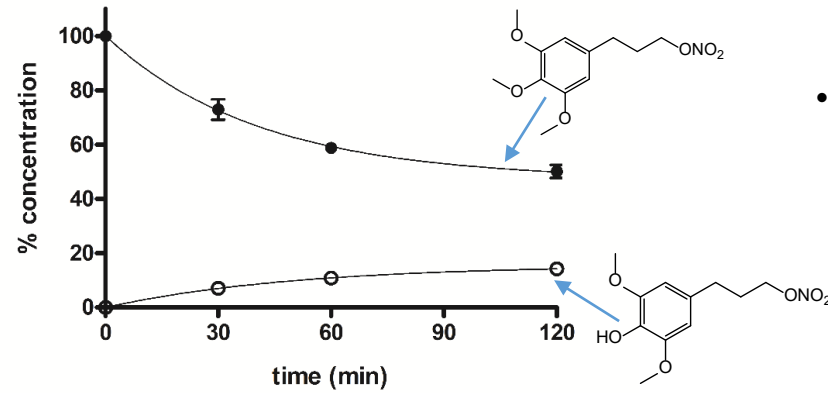


Weak rightward shift observed in tolerant vessels maybe due to metabolic transformations ?

Organic Nitrates: new perspectives with multitarget antioxidant NO-donor nitrates



In vitro study of phase I metabolism



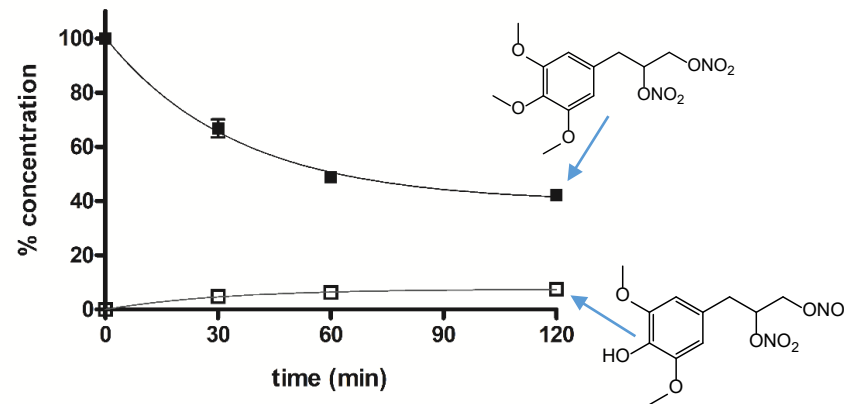
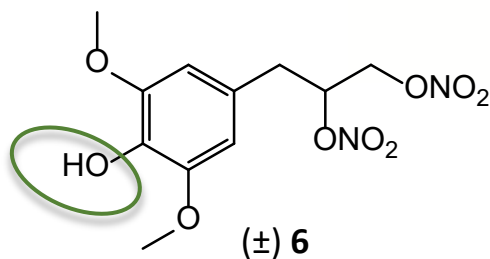
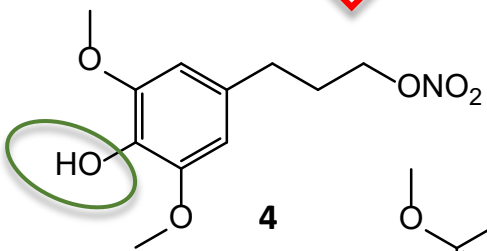
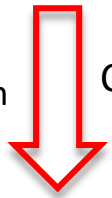
- Rat liver microsomes incubated with compounds (100 μ M; 37°C; 120 min) in the presence of a NADPH-generating system.
- At different time points samples analyzed by RP-HPLC

➤ Metabolites qualitative search

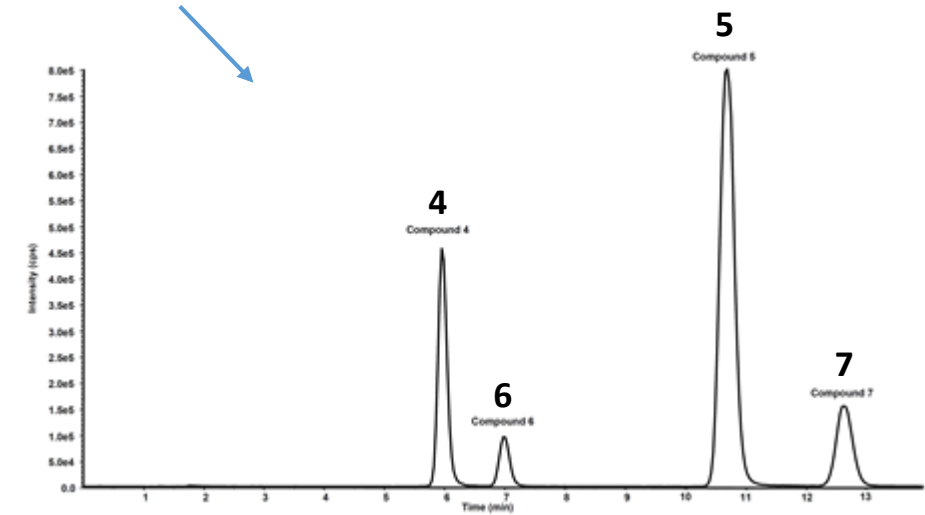
- HPLC-MS/MS analysis of the rat liver microsomal fraction, incubated with compound **5** and **7**

Oxidative demethylation

CYP450



Chromatographic profile in Total Ion Current of the transitions ion precursor-ion products of a standard solution of compounds **4**, **5**, **6** and **7**



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➤ Selected Reaction Monitoring (SRM) analysis

Standard solution of **4**
(10 µg/mL)

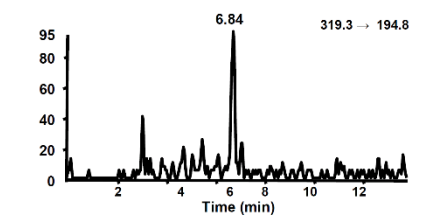
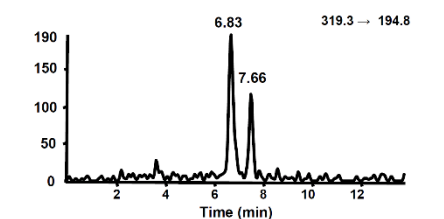
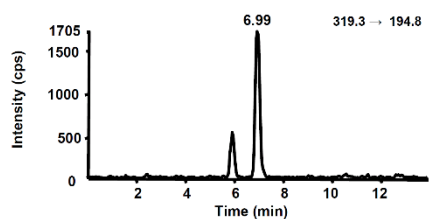
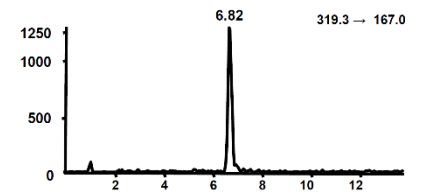
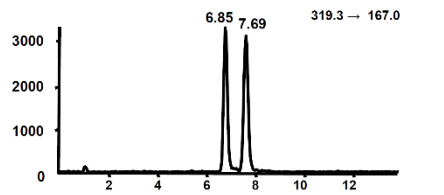
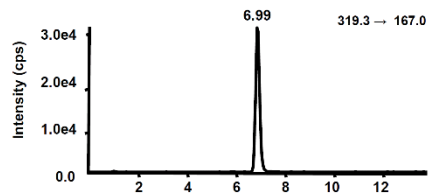
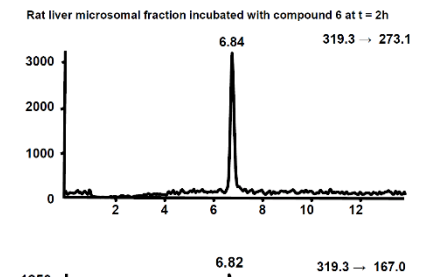
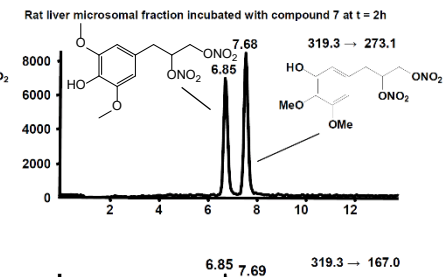
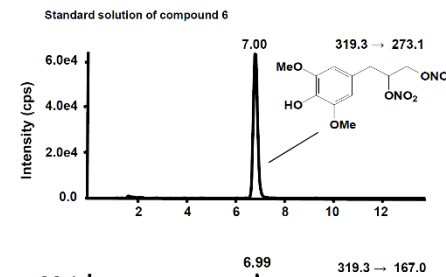
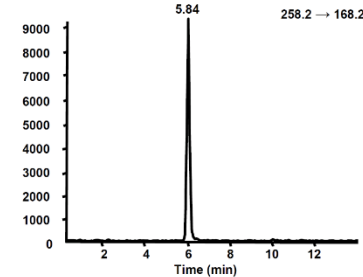
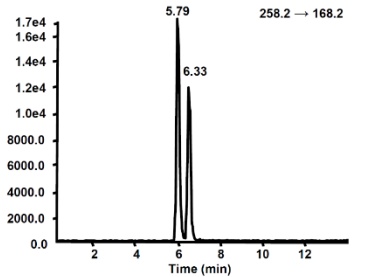
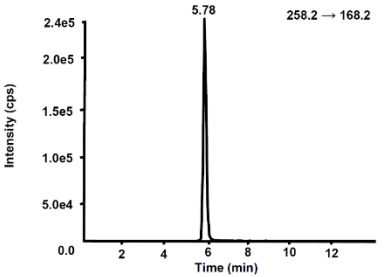
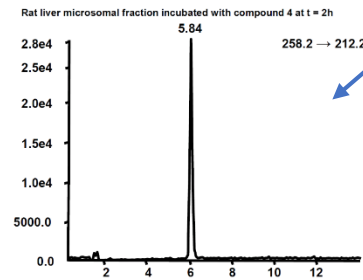
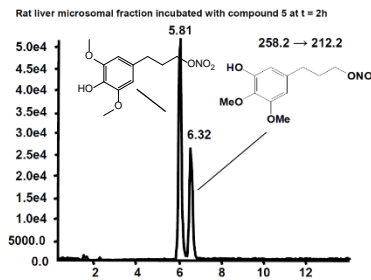
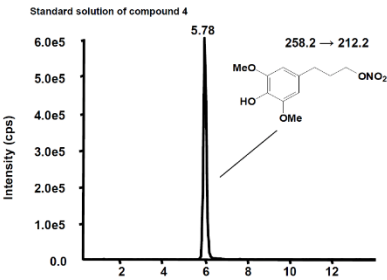
Rat liver microsomal fraction
after 2 h incubation with **5**

Rat liver microsomal fraction after
2 h incubation with **4**

Standard solution of **6**
(10 µg/mL)

Rat liver microsomal
fraction after 2 h
incubation with **7**

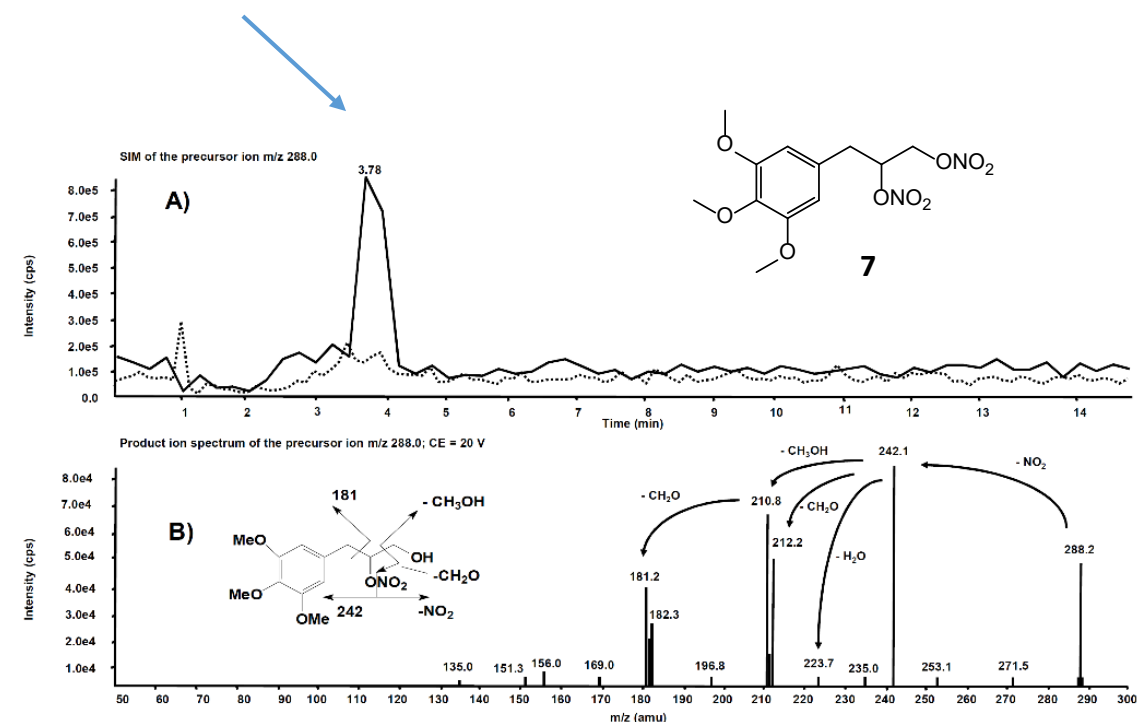
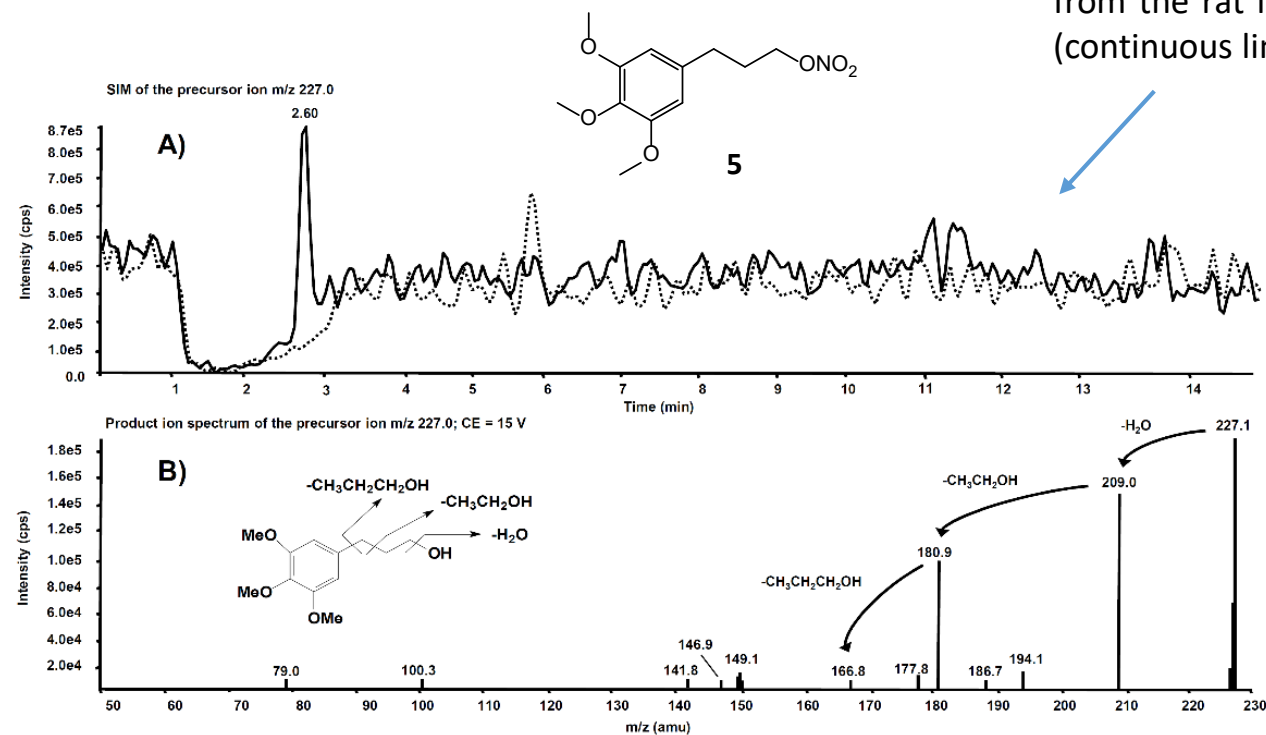
Rat liver microsomal
fraction after 2 h
incubation with **6**



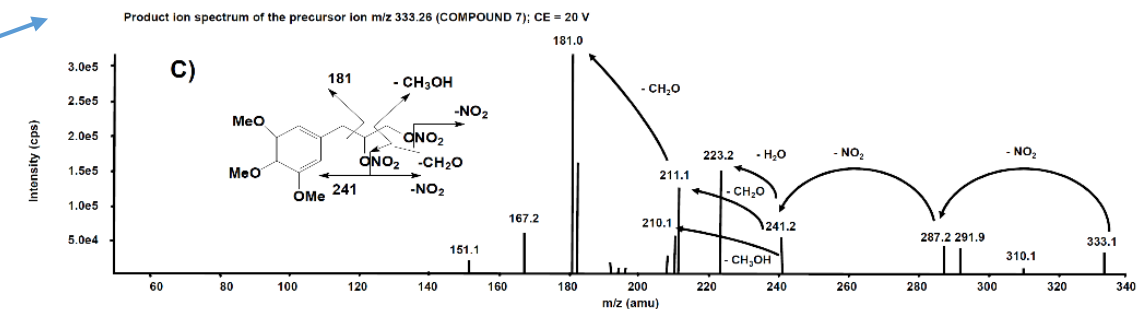
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➤ Product ion scan mode analysis

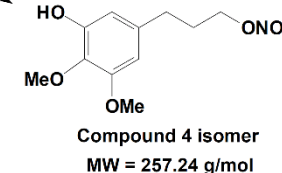
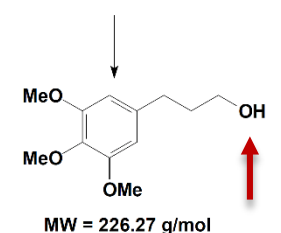
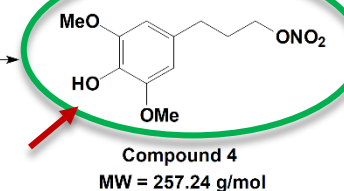
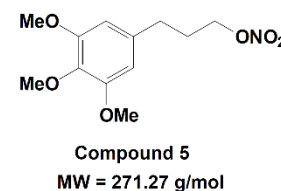
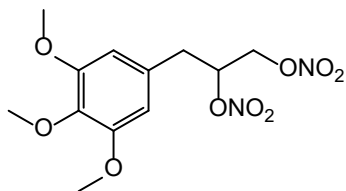
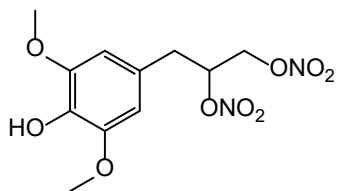
Superimposed mass chromatograms of the precursor ion, obtained from the rat liver microsomal fraction at t=0 (dotted line) and t=2h (continuous line) incubation with compound **5** and **7**



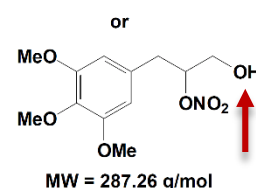
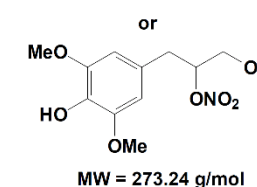
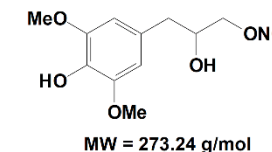
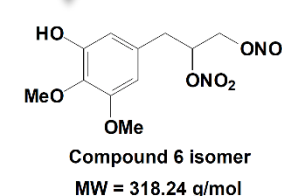
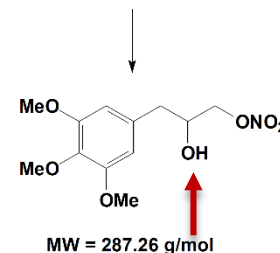
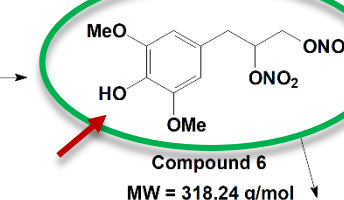
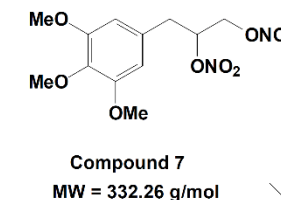
Product ion spectrum of the selected precursors of compound **5** and **7**



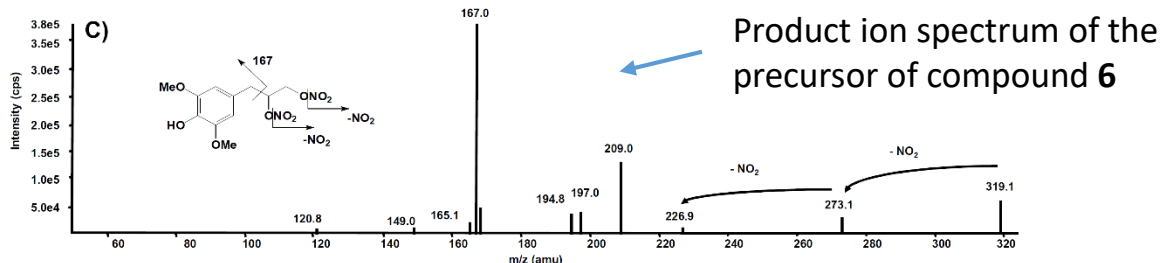
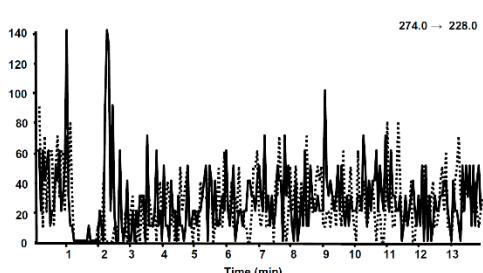
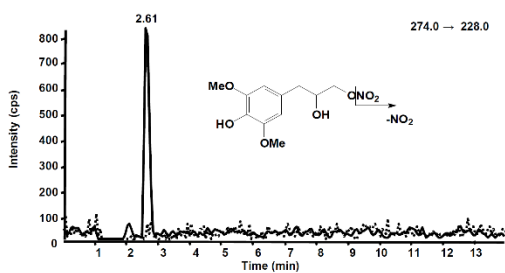
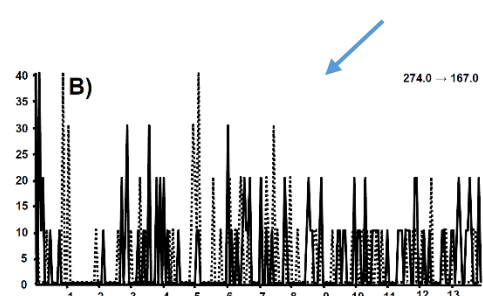
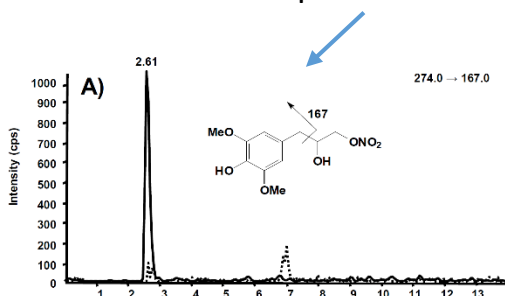
Organic Nitrates: new perspectives with multitarget antioxidant NO-donor nitrates



Metabolic pathway
hypothesized for
compounds 5 and 7

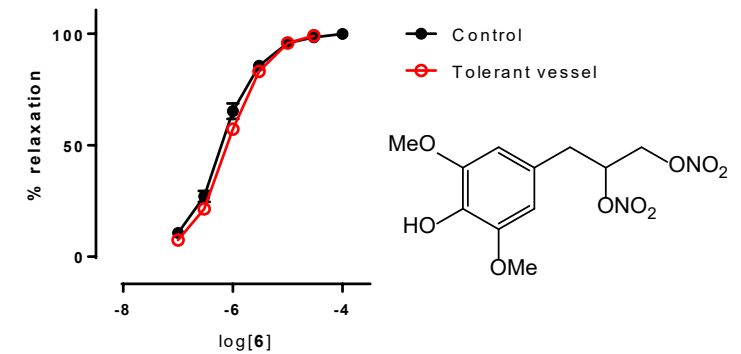
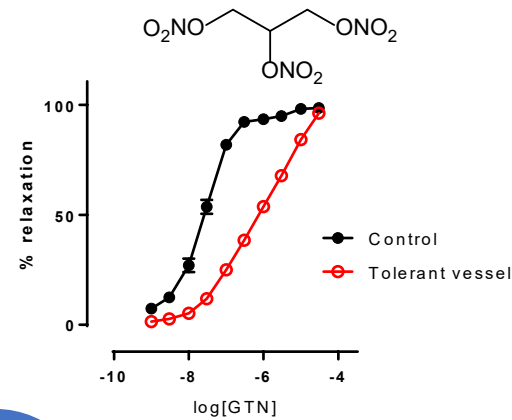
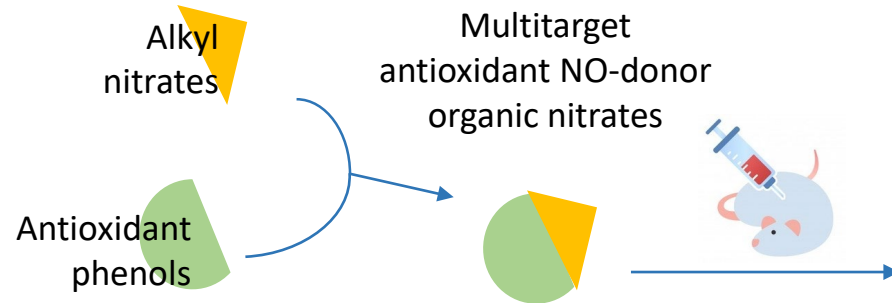


Rat liver microsomal fraction at t=0
(dotted line) and t=2h (continuous line)
of incubation with compound 6



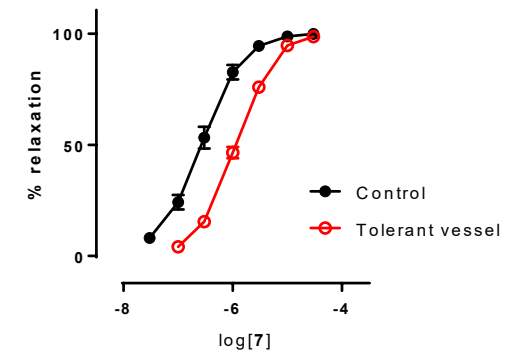
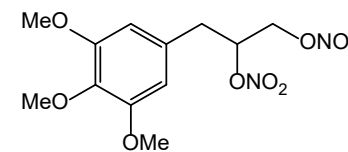
Organic Nitrates: new perspectives with multitarget antioxidant NO-donor nitrates

Multitarget antioxidant NO-donor organic nitrates: a novel approach to overcome nitrates tolerance



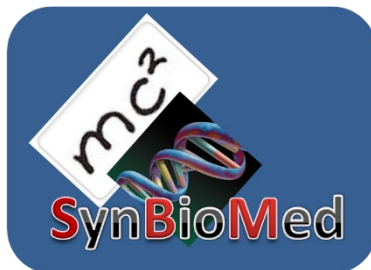
Conclusions

- Nitroxy derivatives endowed with antioxidant properties did not determine the onset of tolerance, even if bioactivated by ALDH-2.
- The behaviour of organic nitrates with antioxidant properties supports the hypothesis of the involvement of ROS in inactivating ALDH-2 and in the development of nitrate tolerance
- Potential role for multitarget drugs as a therapeutic tool in the prevention of the tolerance that accompanies the chronic use of GTN in patients.

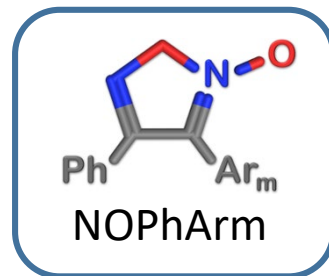




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