Selective Small Molecule Inhibitors of NOX2

Jaideep Saha, Ph.D

Wipf Group Topic Seminar
17th November, 2012

Contents

- ✓ Reactive Oxygen Species (ROS)
- ✓ Structure and Function of NOX Enzyme Family
- ✓ Prior Art of NOX Inhibitor Development
- ✓ Our Approach to Find a Selective NOX2 Inhibitor
- ✓ Conclusions and Outlook

Reactive Oxygen Species (ROS)

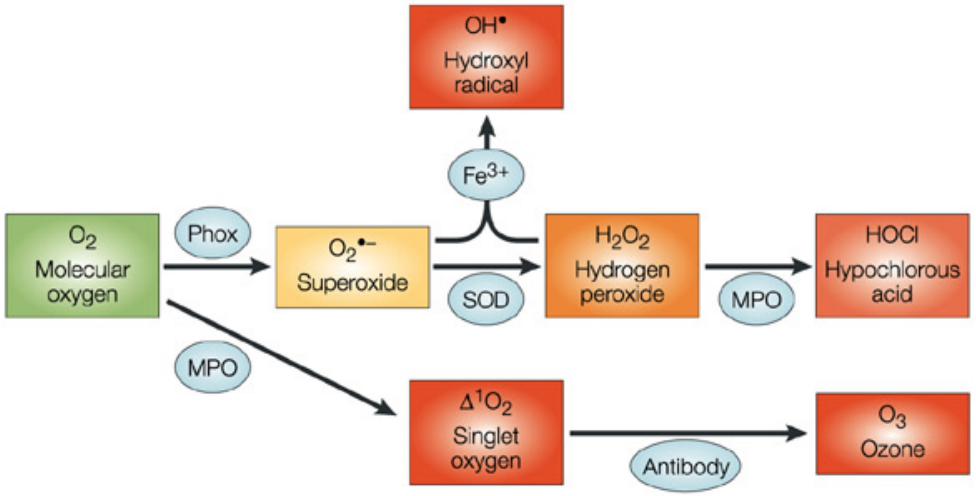
Radicals	
$O_2^{\bullet -}$	Superoxide
$HO^{\bullet}$	Hydroxyl
$ROO^{\bullet}$	Peroxyl
$RO^{\bullet}$	Alkoxyl
$HOO^{\bullet}$	Hydroperoxyl

Non-Radicals	
H_2O_2	Hydrogen Peroxide
HOCl	Hypochlorous Acid
$^1O_2$	Singlet Oxygen
O_3	Ozone
$ONOO^-$	Peroxynitrite

ROS are oxidants generated form metabolism of molecular oxygen.

Toxicology. **2000**, 149, 43-50.

Reactive Oxygen Species (ROS)



Equations:

$2O_2^{\bullet -} + 2\,H^+ \longrightarrow H_2O_2 + O_2$

$Fe^{3+} + O_2^{\bullet -} \longrightarrow Fe^{2+} + O_2$

$Fe^{2+} + H_2O_2 \xrightarrow{\text{(Fenton Reaction)}} Fe^{3+} + OH^- + \dot{O}H$

Facts:

- ✓ *Superoxide anion radical is the precursor of most ROS in the cell*
- ✓ *Removal of H2O2: It's a substrate for catalase and glutathione (GSH) peroxidase and converted to O_2 and H_2O. Its converted by myeloperoxidase (MPO) in neutrophils to HOCl; a strong oxidant that act as a bactericidal agent in phagocytic cell.*
- ✓ *H_2O_2 catalyzed by Fe^{2}+ to produce $OH^{\cdot}$ Which react with any biological molecule RH.*

Nat Rev Immunol. 2004, **4**, 181

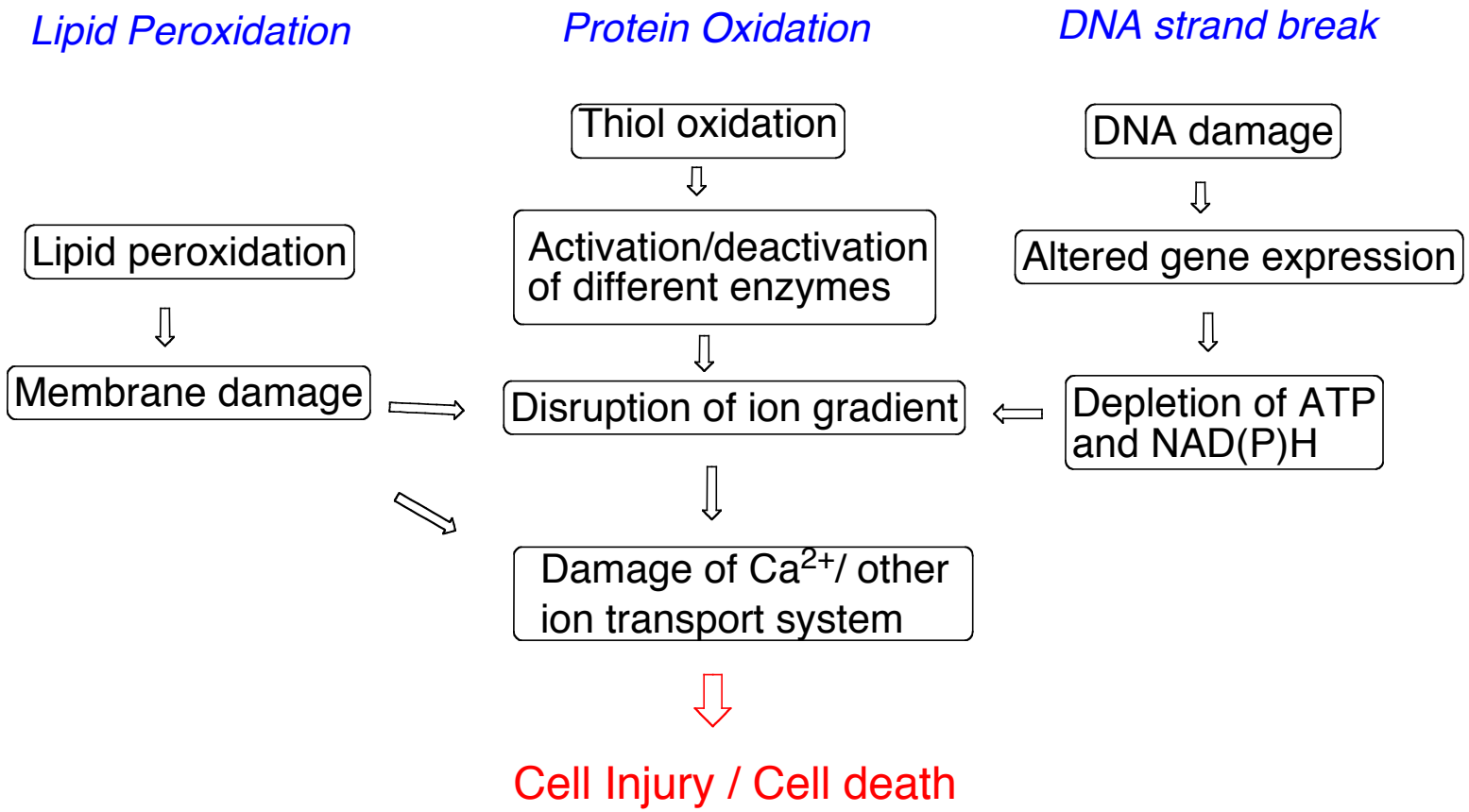
Half life of ROS

Hydroxyl radical	⇨	*nanoseconds*
Superoxide anion		
Singlet Oxygen	⇨	*microseconds*
Alkoxyl radical		
Peroxynitrite	⇨	*miliseconds*
Peroxyl radicals	⇨	*seconds*
Hydrogen peroxide		
Organic Hydroperoxides	⇨	*minutes*
Hypochlorous acid		

Toxicology. **2000**, 149, 43-50.

ROS mediated damages

ROS



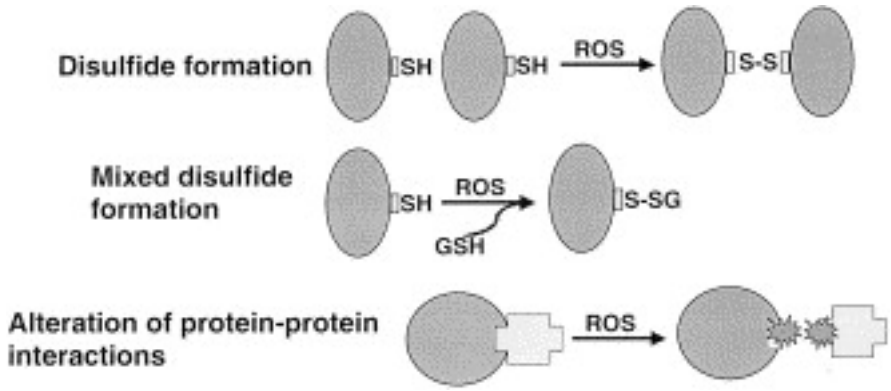
Crit Rev. Toxicol. 1993, **23**, 21-48

Consequences of thiol oxidation

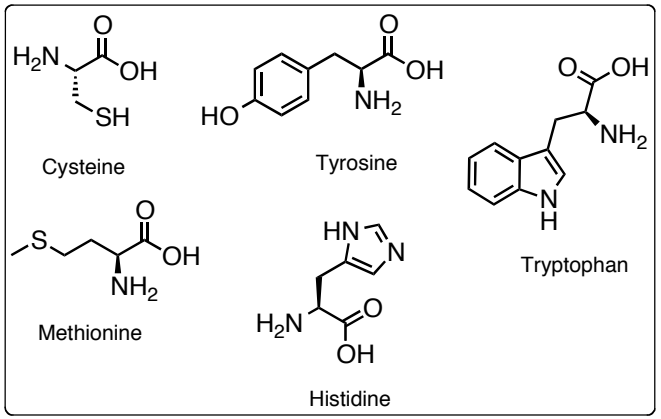
Formation of mixed sulfide bonds

Protein-protein linkages (RS-SR)

Protein-GSH linkages (RS-SG)



Protein targets for ROS



Alteration in 2° and 3° structure

Increased susceptibility to proteolysis

Toxicology. **2000**, 149, 43-50.

Paradigm of understanding the role of ROS

Early assumptions…..

- ✓ Due to early discovery of enzymes (SOD, catalase and peroxidase) over past three decades, a general assumption was ROS are produced accidentally In the cell.

- ✓ ROS were considered as harmful but unavoidable consequences of aerobic cellular process.
- ✓ SOD and other catabolic enzymes are evolved to protect cell from accidental production of ROS.
- ✓ Major Source: Mitochondria and NOX of phagocytes during oxidative burst.

Current understanding…..

- ✓ Enzymes with sole biochemical function of producing ROS have discovered recently. It indicates that catabolic enzymes are only to regulate ROS level in cells.
- ✓ more elaborate role for regulated level of ROS has been attributed to intracellular signaling pathways

Nat Rev Immunol. 2004, **4**, 181

How cell regulates ROS level?

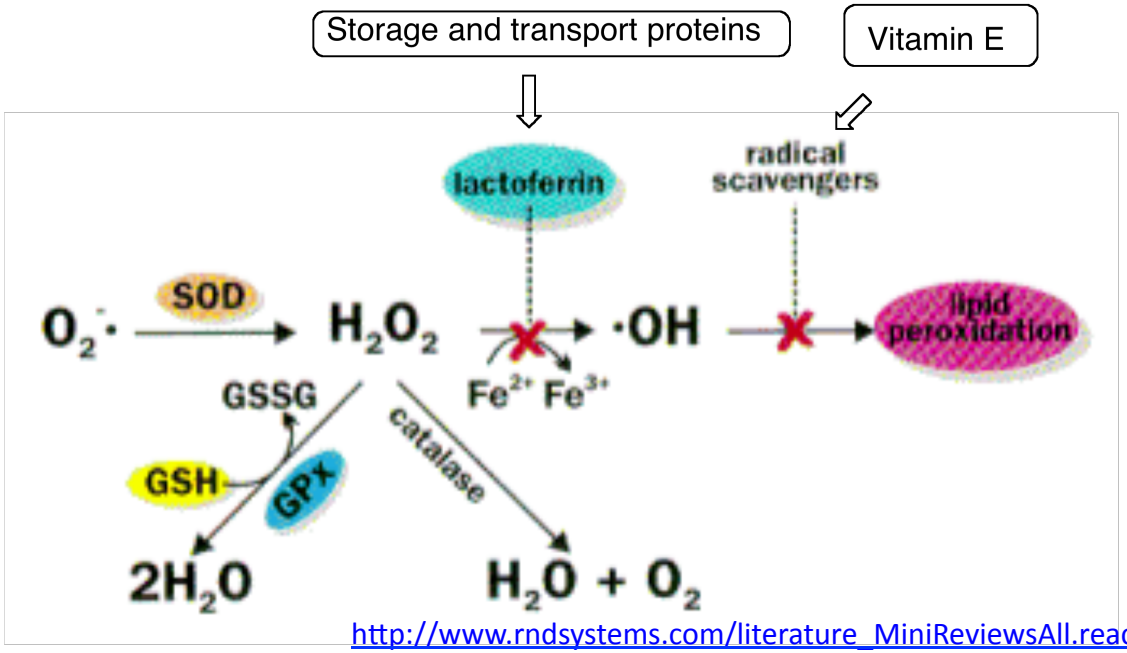
Cellular Antioxidants:

Small molecules.

- ✓ Water soluble: Glutathione, uric acid, ascorbate (Vit. C)
- ✓ Lipid soluble : α-tocopherol (Vit. E), β-carotene, coenzyme Q

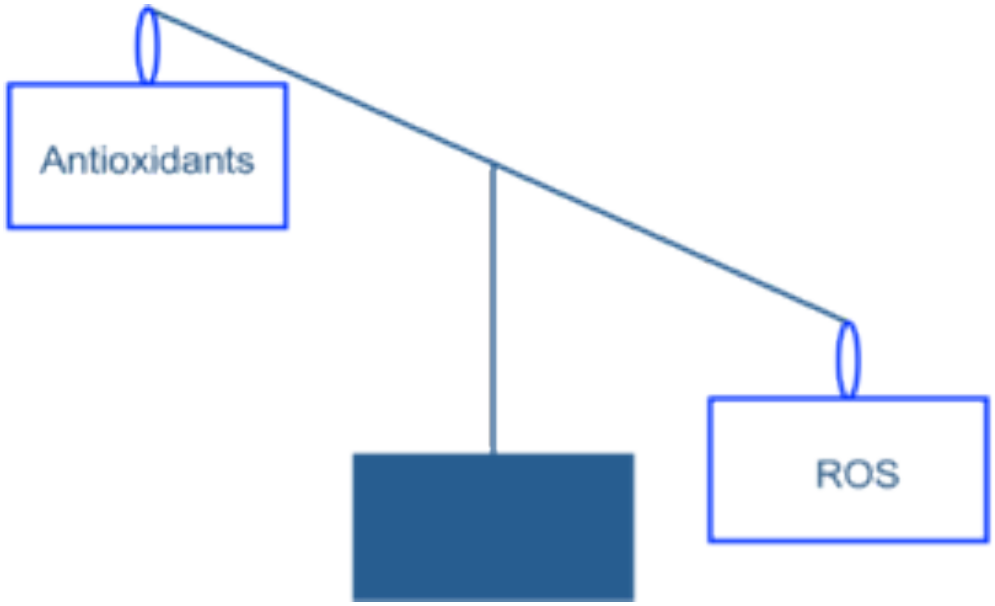
Proteins.

- ✓ Intracellular: SOD (I & II), glutathione peroxidase, catalase
- ✓ Cell membrane : SOD (III), plasma proteins (eg, albumins)
- ✓ Extracellular : phospholipid hydroperoxide GPx (PHGPx)



http://www.rndsystems.com/literature_MiniReviewsAll.reactive oxygen species

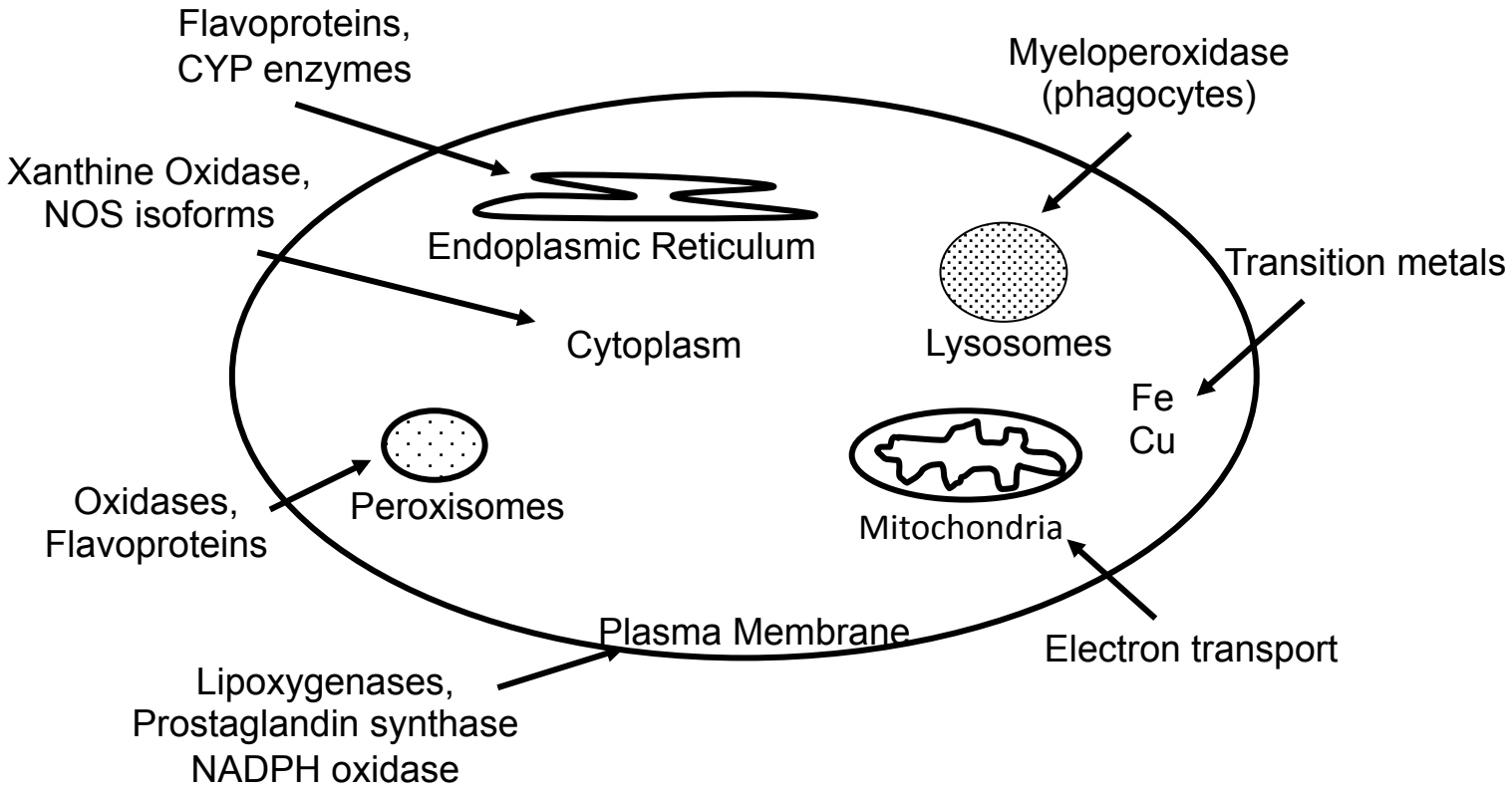
Oxidative Stress



An Imbalance favoring ROS generated to the antioxidants present in the cell

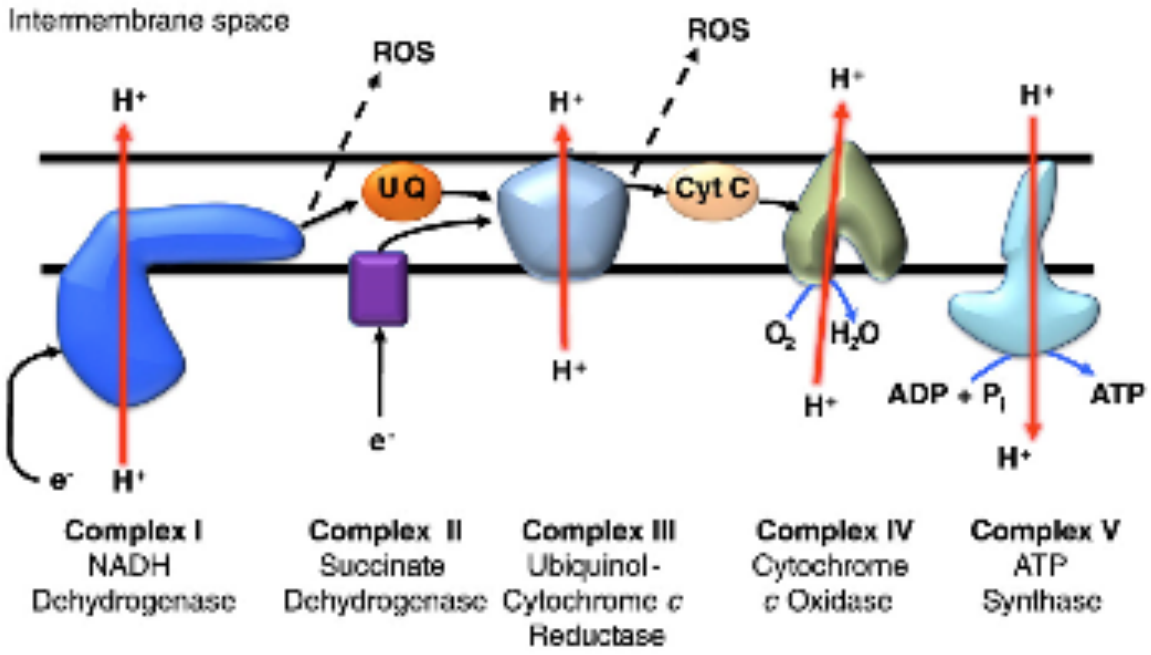
Experimental Physiology. 1997, **82**, 291-295

Endogenous sources of ROS



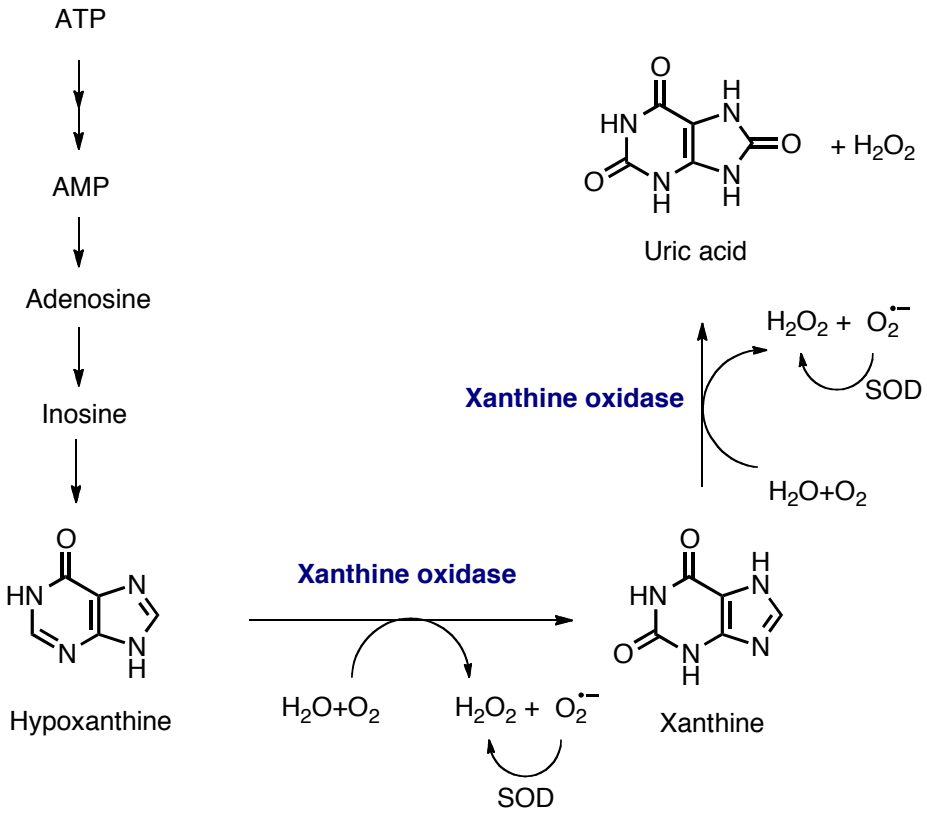
www.unc.edu/courses/2008fall/envr/430/001/Rusyn3**ROS**

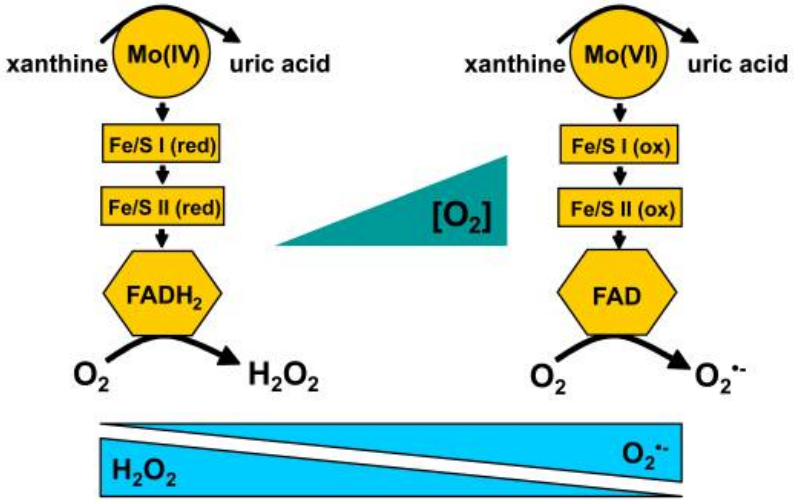
ROS generation in mitochondria



Free Radic. Biol. Med. 2011, **51**, 1271

Cytoplasmic sources of ROS: Xanthine oxidase





Free Radic. Biol. Med. 2010, **48**, 493

NADPH oxidases (NOX)

- ✓ NADPH oxidase activity was first described in liver microsomal isolates and bacteria In 1963.

- ✓ Until 1990, the phagocyte NADPH oxidase was only known for the "deliberate" generation of ROS (*non-mitochondrial*) in mammaliun cells that catalyses the *respiratory burst*. The oxidase consist of the catalytic subunit gp91phox (otherwise known as NOX2).

- ✓ During 1990s, other sources of ROS was suspected to be a flavoproteins that is similar to gp91phox. In 1999, the first of the NOX homologues of gp91phox was describes as NOX1.

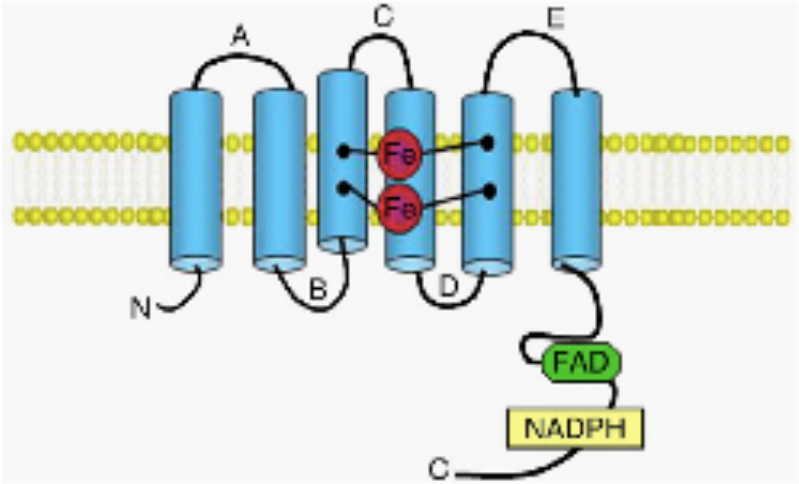
- ✓ Currently the total number of enzymes in this family is increased to seven; NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1 and DUOX2.

Nat Rev Immunol. 2004, **4**, 181

NADPH oxidases (NOX)

- ✓ NOX's are membrane integrated proteins. No crystal structure is available
- ✓ NOX isoforms differ in tissue distribution, subunit requirement, domain structure and mechanism of activation.

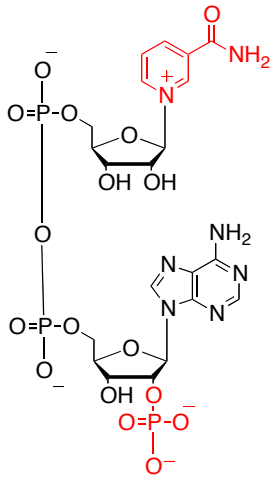
NOX1	**NOX5**	**DUOX1**
NOX2		**DUOX2**
NOX3		
NOX4		



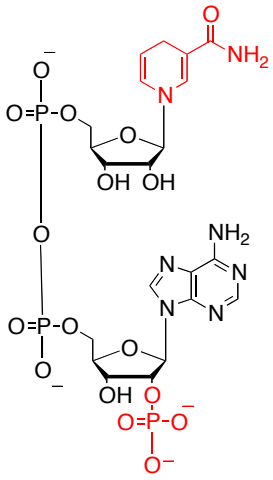
Schematic representation of common NOX features

Free Radic. Biol. Med. 2011, **51**, 1271

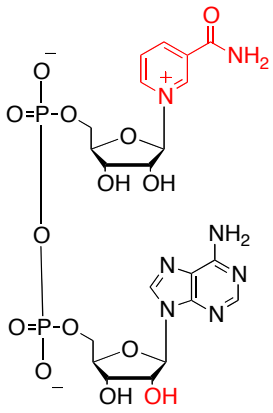
Structure of some related coenzymes



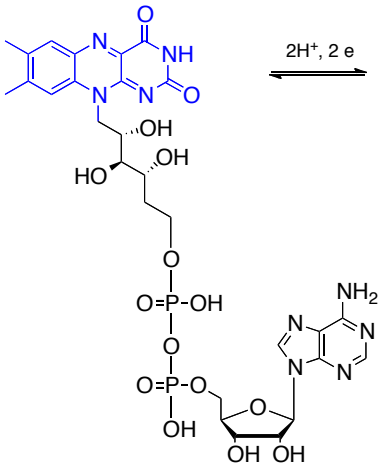
NADP+



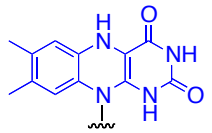
NADPH



NAD+

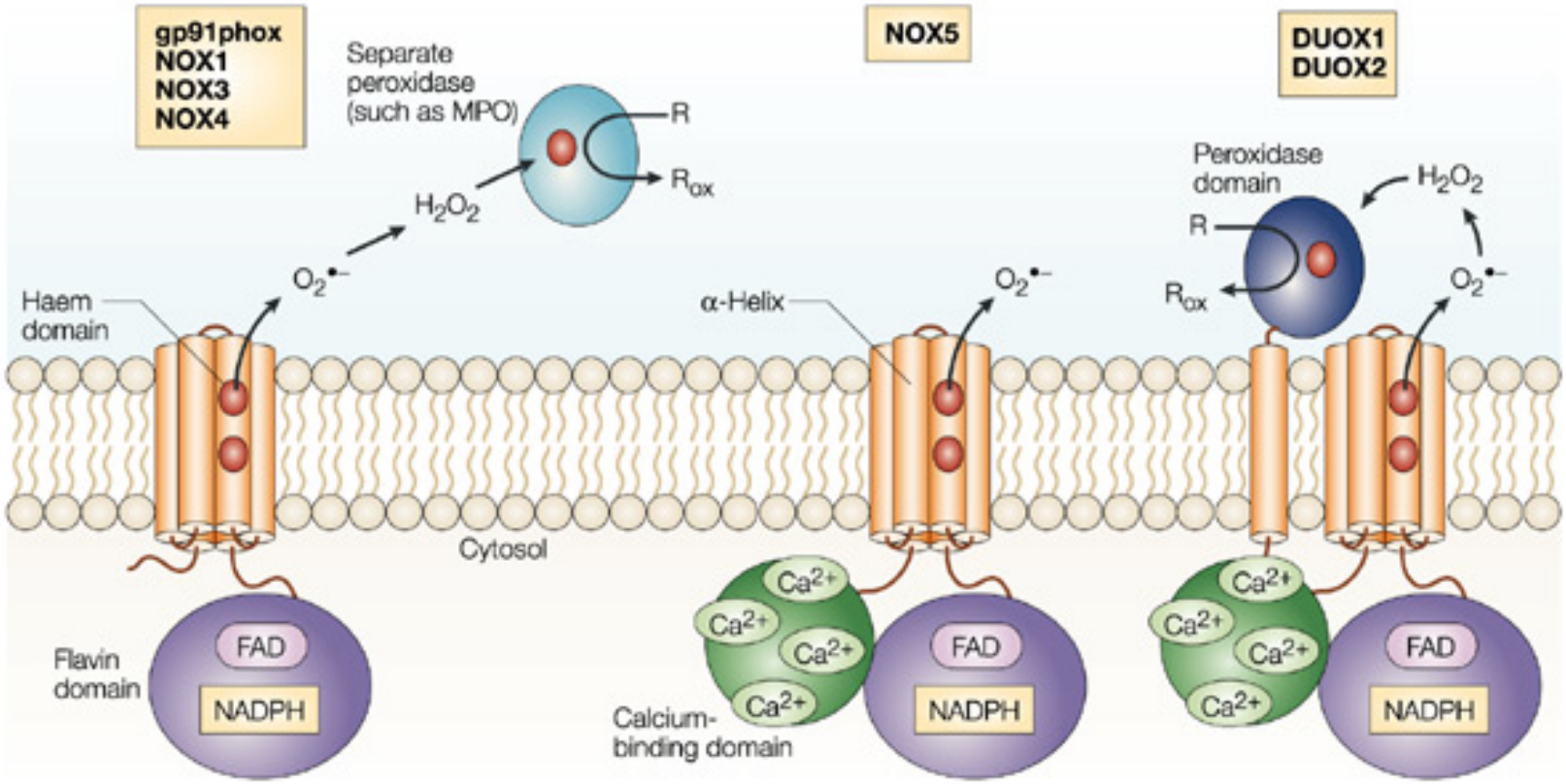


FAD



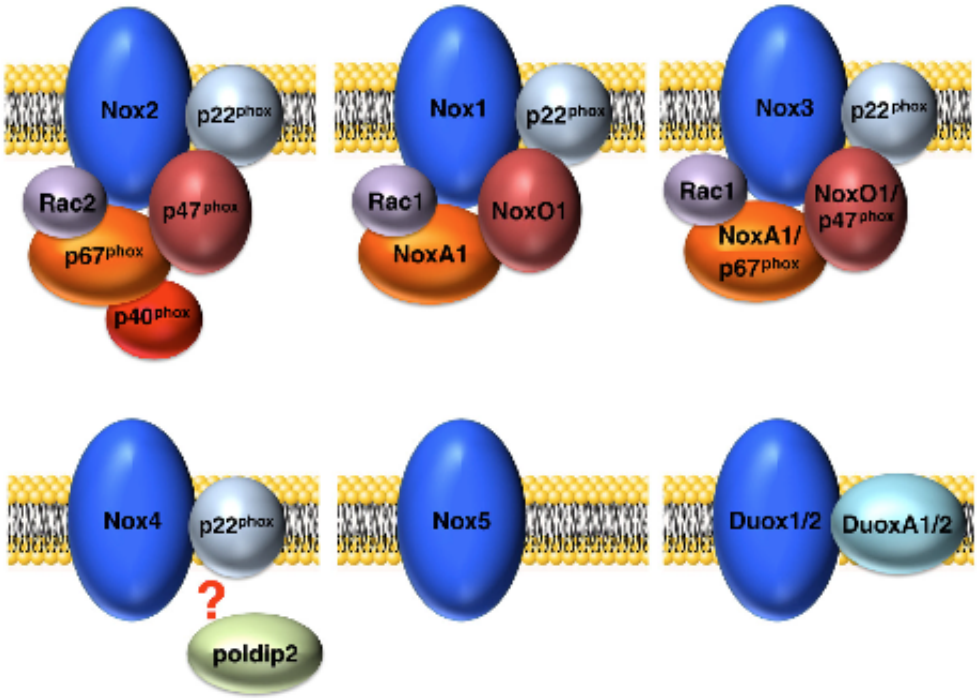
$FADH_2$

Transmembrane topology and domain structure of NOX & DUOX enzymes



Nat Rev Immunol. 2004, **4**, 181

Subunit requirements in the active NOX complexes

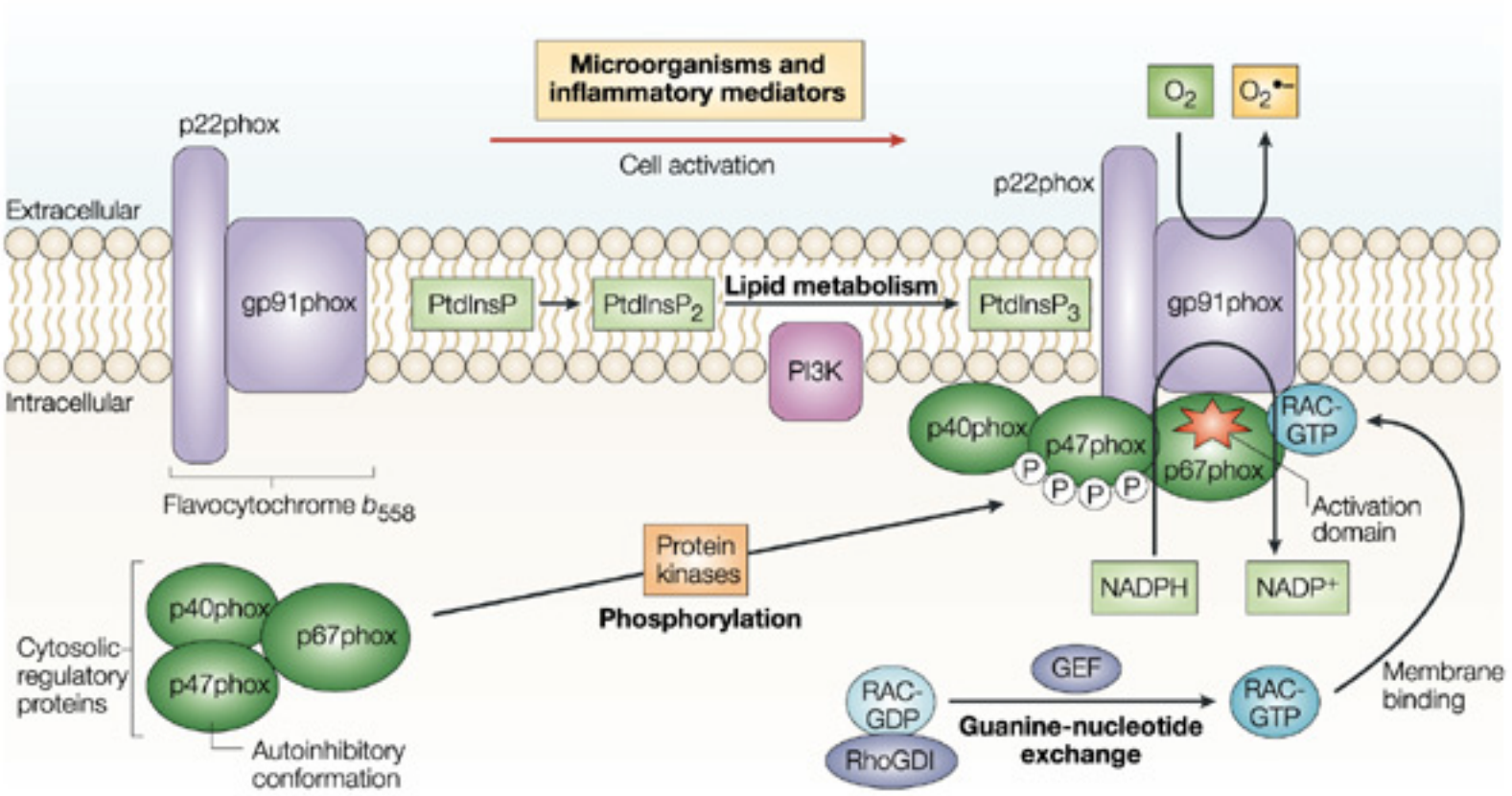


Three molecular triggers that initiate cell activation in presence of microorganism or inflammatory mediators:

a. *Protein phosphorylation*
b. *Lipid metabolism*
c. *Guanine nucleotide exchange on RAC.*

Free Radic. Biol. Med. 2011, **51**, 1271

ROS generation by assembly of Phox regulatory proteins in phagocytes



Nat Rev Immunol. 2004, **4**, 181

NOX Inhibitors

Overview:

Discovery of new homologues of NADPH oxidases has stimulated the interest to target this family of enzyme as potential therapeutic agent.

The majority of the inhibitors were indentified earlier were based on their ability to block the neutrophil oxidative burst mediated by phagocyte oxidase (ie, NOX2).

Early NOX inhibitors were limited by several factors;
more importantly, The inhibitors often were not specific for NOX, either block upstream pathways or inhibit other targets along with NOX.

Specificity of action is a major challenge in the NOX field in general !

Antioxid Redox Signaling, 2009, **11**, 2535

Two-way Challenge

To identify the isoform-specific NOX inhibitor.

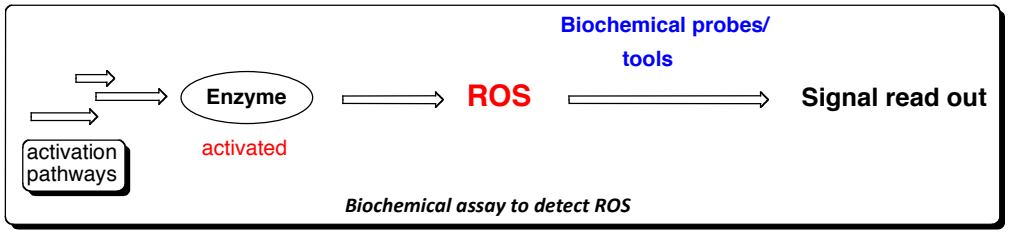
Isoforms share high percentages of residues
NOX1/ NOX2 – 60%
NOX2/ NOX4 – 39%

Enzymes are membrane-bound, no crystal structure available.

Existing antibodies are not useful

Proper measurement of the NOX activity using selective probes (colorometric, fluorescent or chemiluminescent) in the assay.

Challenges for precise measurement of NOX activity



Removal of ROS versus true enzyme activity

Many chemical probes detect only a specific ROS; decrease in the signal may reflect a decrease in the availability of that species through ROS scavenging or metabolism.

Inhibition through indirect pathways

Detectable NOX activity may be decreased through indirect or non-specific pathways; interfering with cell signaling pathways, physical disruption of the membrane or block pathways to NOX activation.

Non specificity or interference with the assay

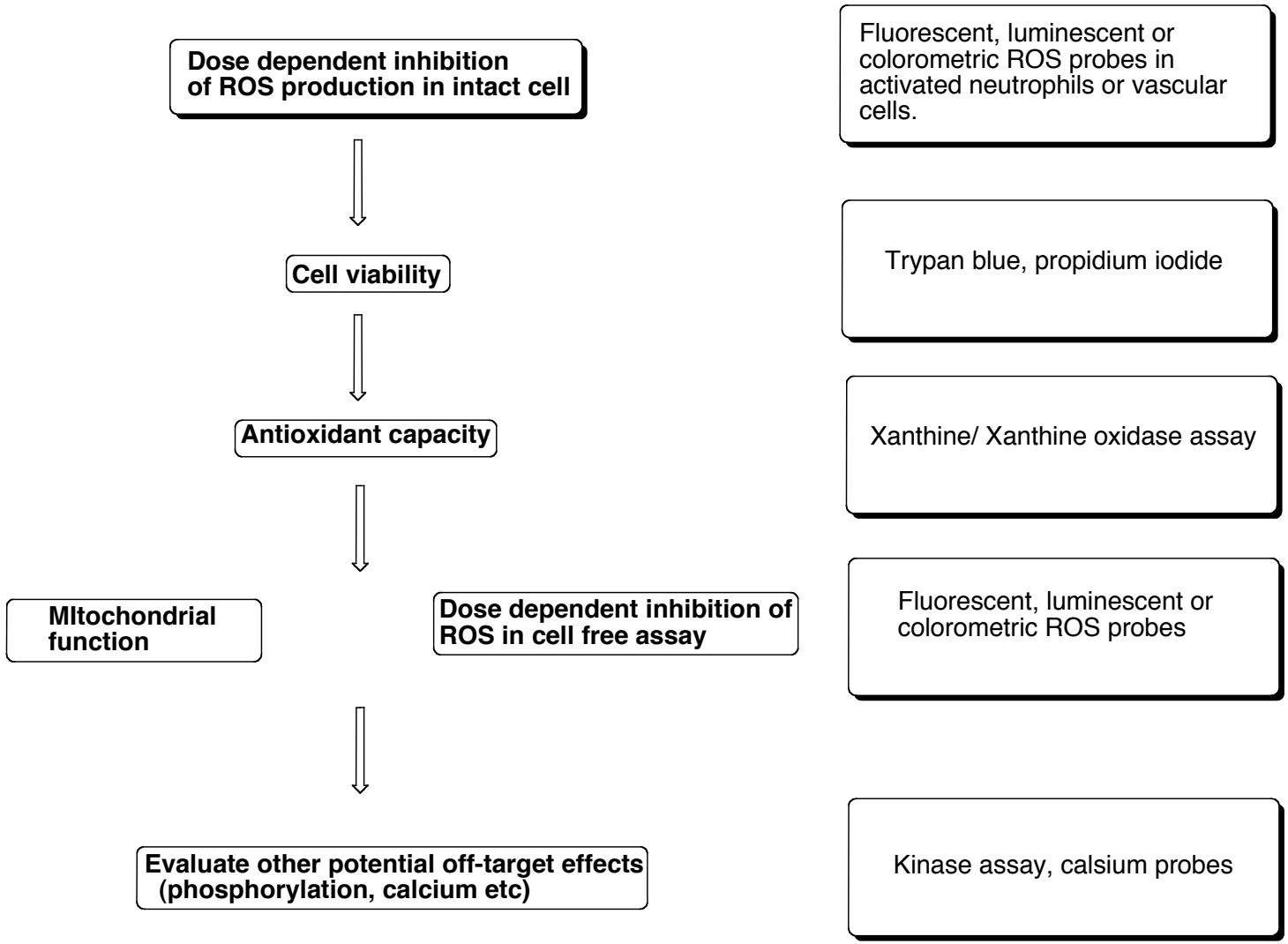
Many of the assay are based on detecting the free radical species. In some cases the ROS generated may not be detected because of the interfering reaction between the inhibitor and the detection probe.

Often two or more different methods are used to confirm the effectiveness and specificity of an inhibitor!

Antioxid Redox Signaling, 2009, **11**, 2535

Criteria for selecting a NOX inhibitor In Vitro

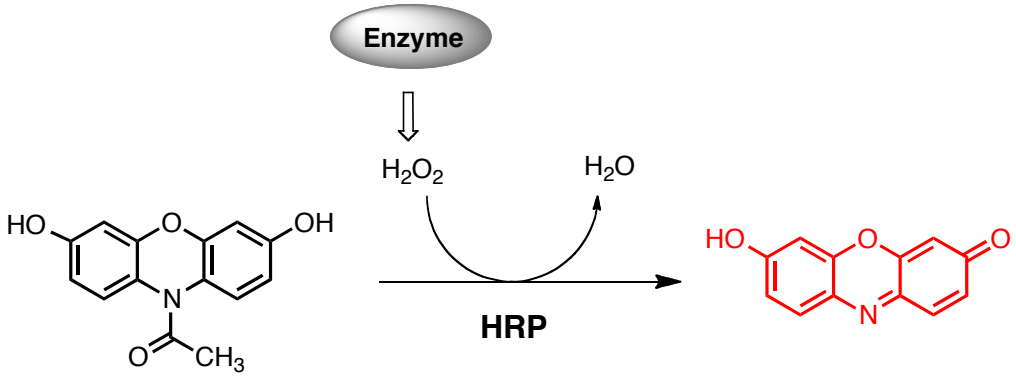
Flow Chart



Antioxid Redox Signaling, 2009, **11**, 2535

Chemical probes and tools in the assay for ROS detection/measurement

Fluorescence



Amplex Red

Resorufin
(Fluoroscent)

HRP: Horseredish peroxidase

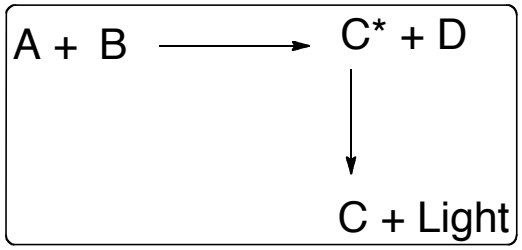
Chemical probes and tools in the assay for ROS detection/measurement

EPR: Electron Paramagnetic resonance

Advantages: This method enables not only detection but identification of the radical.

Disadvantages: The success of the detection depends on the steady state concentration of the reactive species, which in reality is often low.

Chemiluminescence:



Advantages

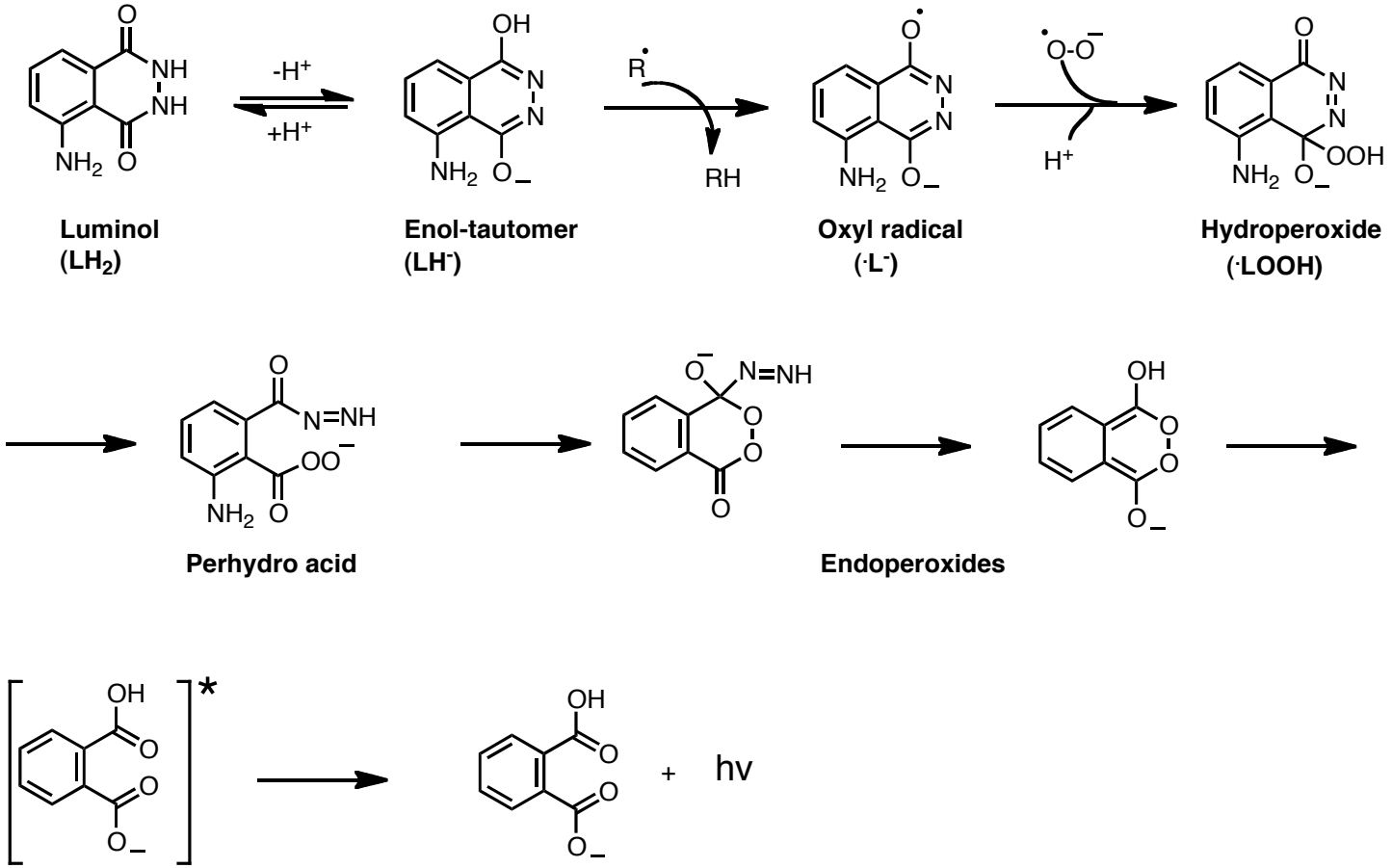
- ✓ Light emission is generated from a reaction, No source of excitation, hence excellent sensibility and detection limit (no noise or scatter).
- ✓ Simple, robust and inexpensive instrumentation
- ✓ suitable for both batch and flow analytical technique.

Disadvantages

- ✓ some chemiluminescent probes tends to generate ROS.

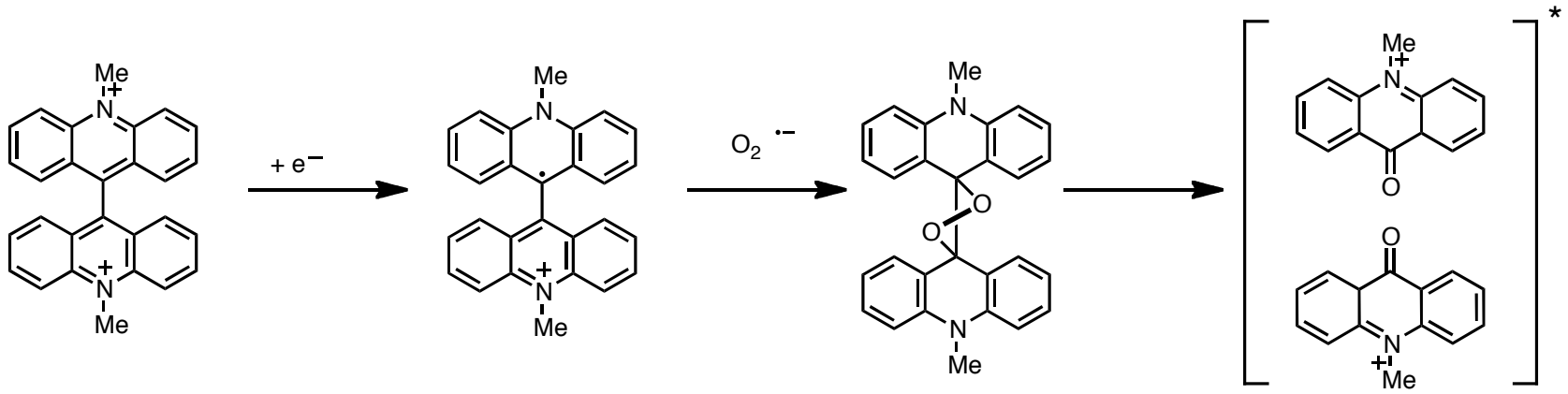
Chemical probes: Luminol, Lucigenin, L012

Mechanism for Luminol Luminnescence



Biochemistry (Moscow), 2009, **49**, 341-388

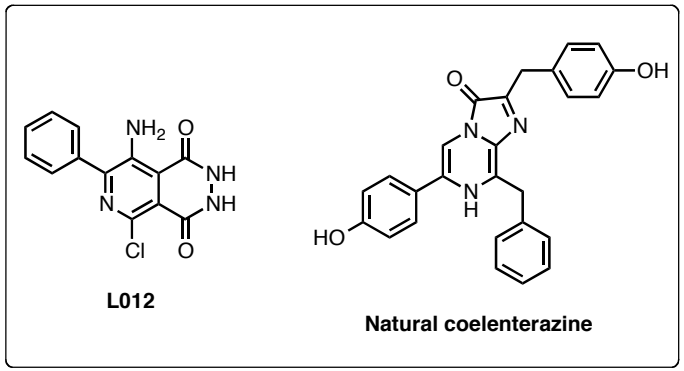
Lucigenic dependent chemiLuminescence



Lucigenin (Luc^{2+}) | **Lucigenin cation radical** | **Lucigenin dioxetane** | **N-Methylacridinium (NMA)**

NMA + hv

Other superoxide sensitive luminescence probes



L012 | **Natural coelenterazine**

Biochemistry (Moscow), 2009, **49**, 341-388

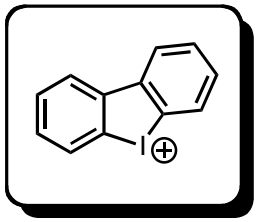
NOX Inhibitors

Direct inhibitors of the NOX catalytic unit

a. Measurement of ROS production
b. Oxygen consumption
c. Viability
d. Exclusion of a predominant scavenging agent
e. NOX inhibition in cell free assay

Indirect inhibitors of the NOX catalytic unit

Prior art:



Diphenyleneiodonium (DPI)

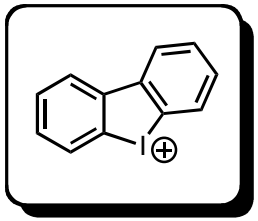
ROS Inhibition : IC_{50} = 0.90 µM for neutrophil NOX2.

Viability : No cell toxicity at efficient concentration
Scavenging : Inhibits completely X/XO, but no scavenging activity.
Membrane assay : NOX2/NOX4

Others : All NOX enzymes are inhibited in submicromolar range
IC_{50} = 0.2 µM (NOX 4)
IC_{50} = 0.30 µM (NOX5).

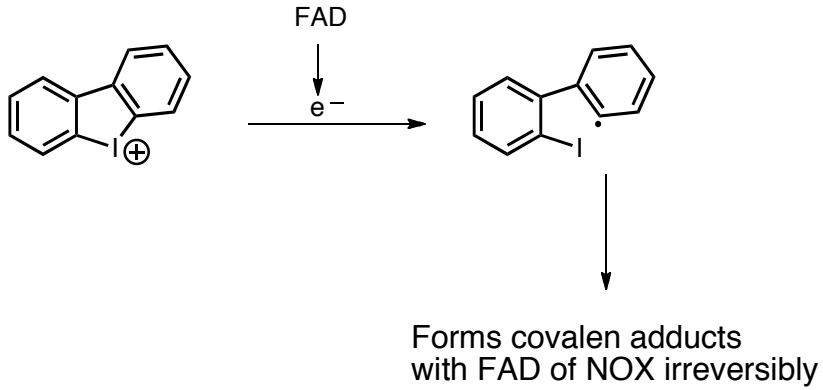
Inhibitors of all flavin-containing proteins and many other non specific activities.

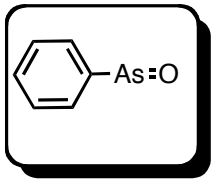
Antioxid Redox Signaling, 2009, **11**, 2535



Diphenyleneiodonium (DPI)

- ✓ Inhibit all NOX and DUOX isoforms in submicromolar concetration.
- ✓ Used widely to provide evidence of NOX activity in tissue.
- ✓ DPI is useful tools for studying NOX enzymes in vitro but not a drug candidate due to irreversible binding, off target effects, low solubility.
- ✓ Machanism of action:





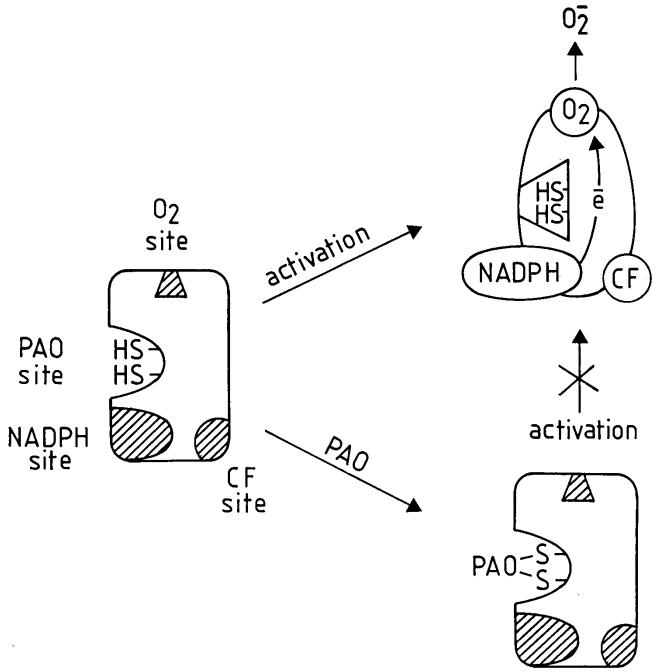
Phenylarsenic oxide (PAO)

ROS Inhibition : IC_{50} = 0.50 μM for PMA-activated neutrophils

Viability : No cell toxicity after 30 min incubation
Scavenging : ND
Membrane assay : NOX2, other NOX's not determined
Comments : No inhibition of protein kinases at concentration used

Antioxid Redox Signaling, 2009, **11**, 2535

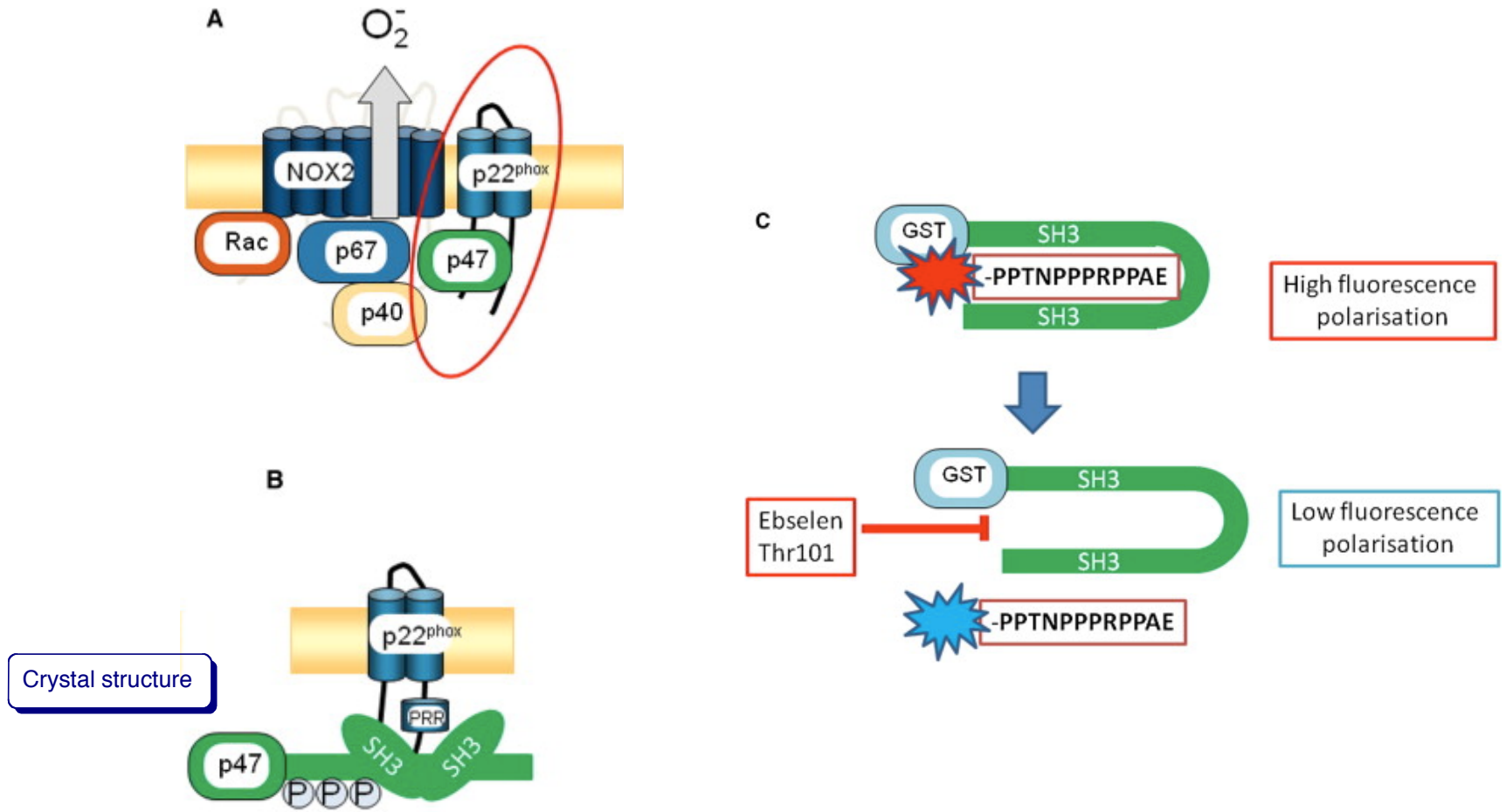
- ✓ PAO is ineffective once the oxidase complex is formed. It suggests that it blocks one active site/unit leading to the assembly
- ✓ PAO is known to interact with vicinal cysteine residue, which is only accessible in the resting set of the NOX subunit.
- ✓ A potential NOX2 specific inhibitor as this isoform contains two neighboring Cys residue.
- ✓ Inhibitory activity can be reversed by adding a vicinal dithiol competitor.



Eur. J. Biochem. 1998, **251**, 649-658

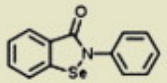
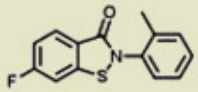
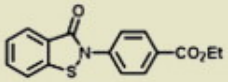
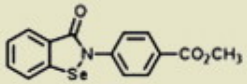
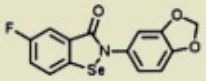
Cell-free screening for NOX inhibitors

This method is based on blockage of the cytosolic subunits required for Nox2 Activation.



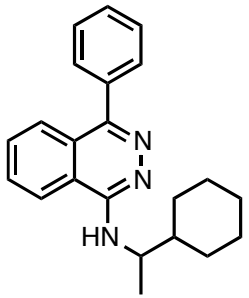
Chemistry & Biology. 2012,**19**, 752-763

Assay Results

Compound	Structure	EC_{50} (μM) for Inhibition of Nox Isoform Activity in Whole Cell Assays, and EC_{50} (μM) in Assay Controls					
		Nox1	Nox2	Nox4	Nox5	X/XO Activity	H_2O_2 Activity
Ebselen		0.15	0.5	ns	0.7	ns	ns
Thr101		3	0.3	8	8	ns	8
JM-87a		1.5	0.5	ns	4	ns	ns
TG4-193		0.3	0.3	ns	1.7	6	30
JM-77b		6.3	0.4	ns	17	5	ns

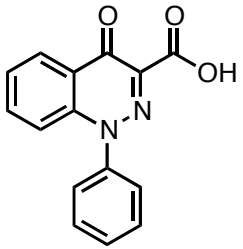
Chemistry & Biology. 2012,**19**, 752-763

Small molecule NOX inhibitors from pharmaceutical companies

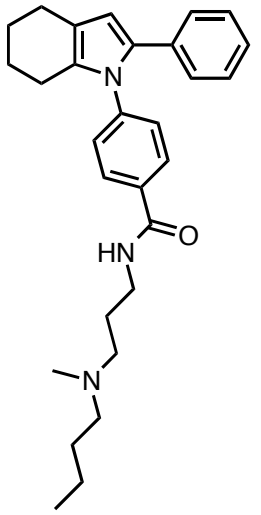


N-(1-cycloheylethyl)-4-phenylphtalazin-1-amine

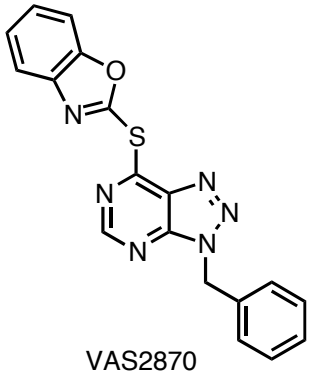
(Mitsubishi)



Compound C
(Mitsubishi)

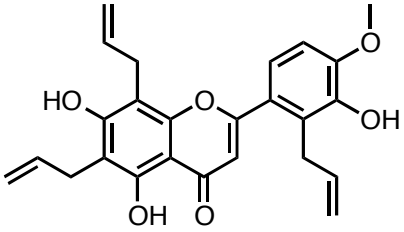


Tetrahydroindoles
(Genkyotex)

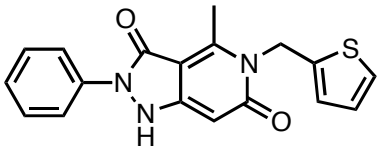


VAS2870

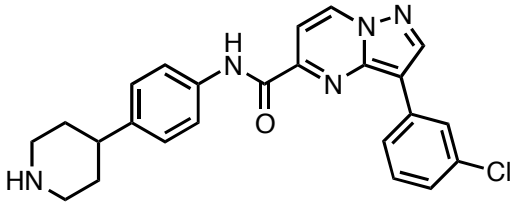
(Vasopharm)



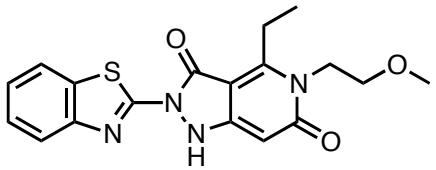
S17834
(Servier)



Pyrazolo pyridine
(Genkyotex)

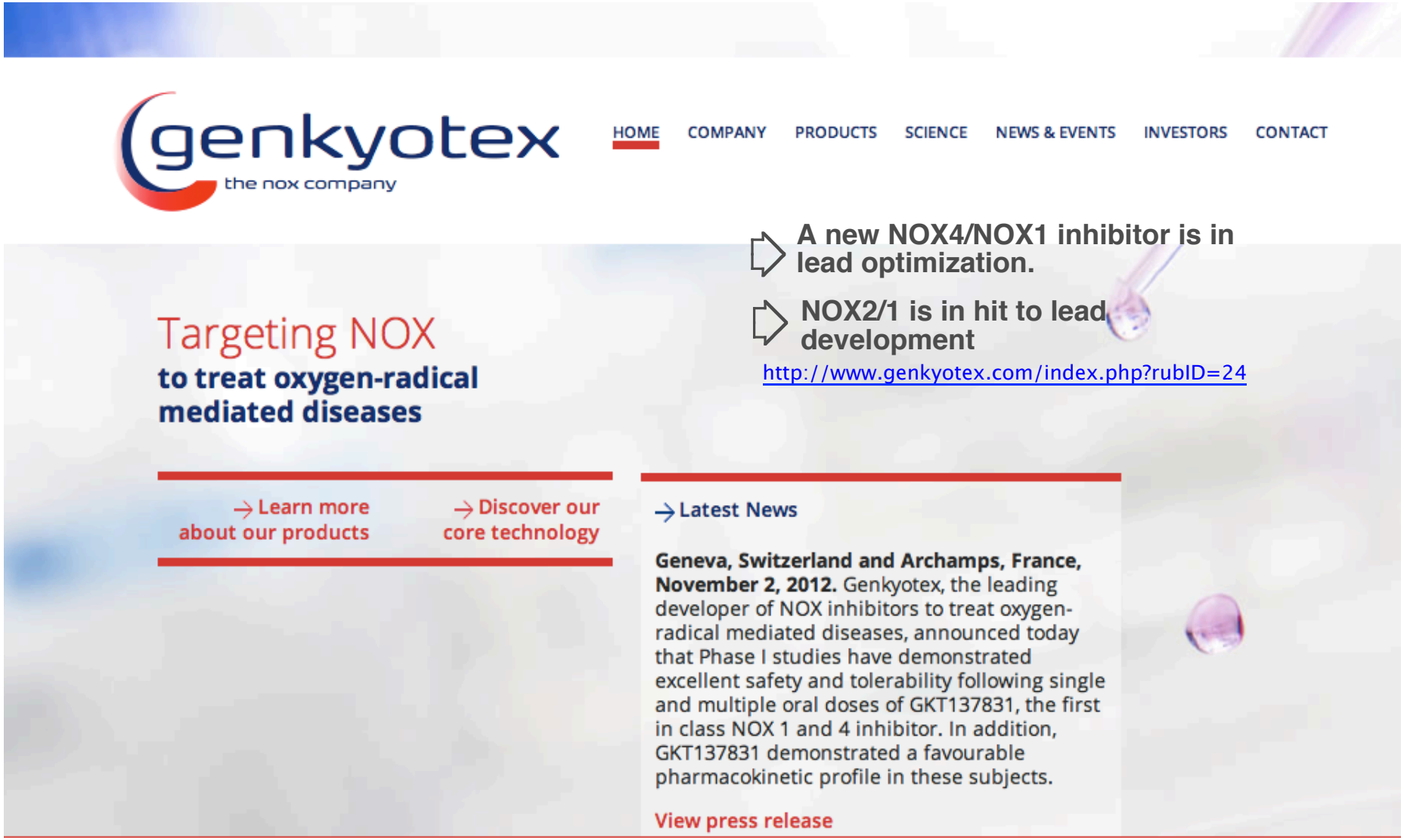


Pyrazolo pyrimidines
(Shionogi)

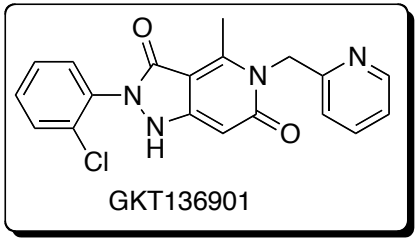


Pyrazolo pyridine
(Genkyotex)

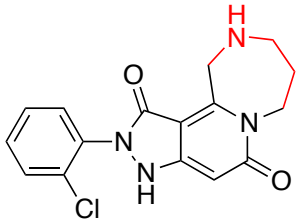
Genkyotex pipeline



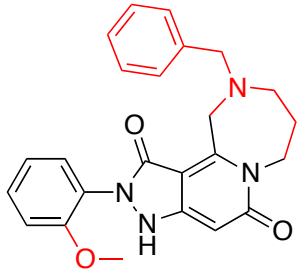
Genkyotex library for NOX4/NOX1 inhibitors



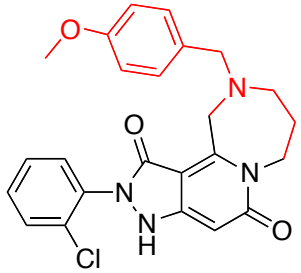
GKT136901



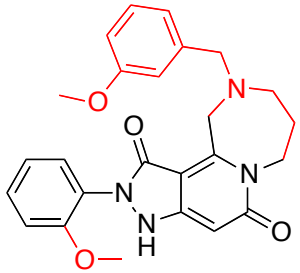
NOX1 K_i = 119 ± 20 nM
NOX2 K_i = 365 ± 5 nM
NOX4 K_i = 87 ± 8 nM
NOX5 K_i = 360 ± 74 nM



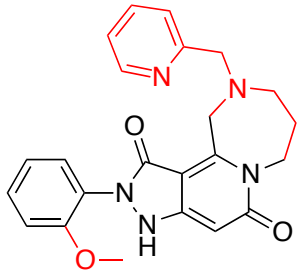
NOX1 K_i = 218 ± 39 nM
NOX2 K_i = 645 ± 75 nM
NOX4 K_i = 144 ± 22 nM
NOX5 K_i = 670 ± 30 nM



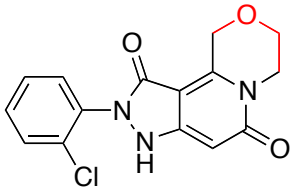
NOX1 K_i = 184 ± 24 nM
NOX2 K_i = 780 ± 170 nM
NOX4 K_i = 116 ± 9 nM
NOX5 K_i = 690 ± 10 nM



NOX1 K_i = 212 ± 51 nM
NOX2 K_i = 820 ± 150 nM
NOX4 K_i = 131 ± 6 nM
NOX5 K_i = 690 ± 60 nM



NOX1 K_i = 217 ± 18 nM
NOX2 K_i = 510 ± 20 nM
NOX4 K_i = 93 ± 11 nM
NOX5 K_i = 610 ± 40 nM



NOX1 K_i = 173 ± 14 nM
NOX2 K_i = 433 ± 19 nM
NOX4 K_i = 156 ± 11 nM
NOX5 K_i = 490 ± 67 nM

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