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# Role of RONS in Plasma Medicine: Lessons from Nature

David B. Graves

PPPL, Princeton University and UC Berkeley

June 23, 2020

# Acknowledgements

My debts to others in this field are too numerous to properly list. The people listed below are very important for this talk but there are many others as well from whom I have learned a great deal.

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- Dominika Sersenová, Division of Environmental Physics, Faculty of Mathematics, Physics and Informatics, Comenius University, Bratislava, Slovakia

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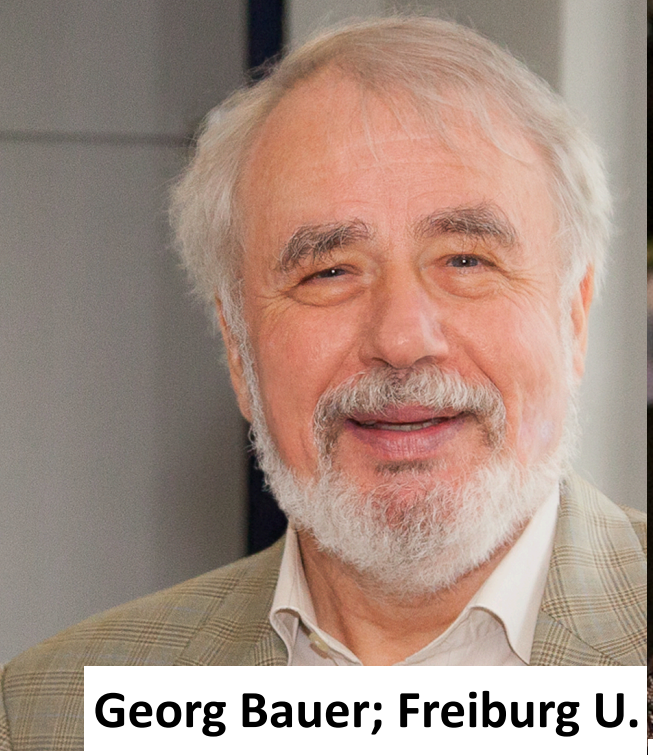

Georg Bauer; Freiburg U.

Dominika Sersenová; Comenius U.

ko Machala; Comenius U.

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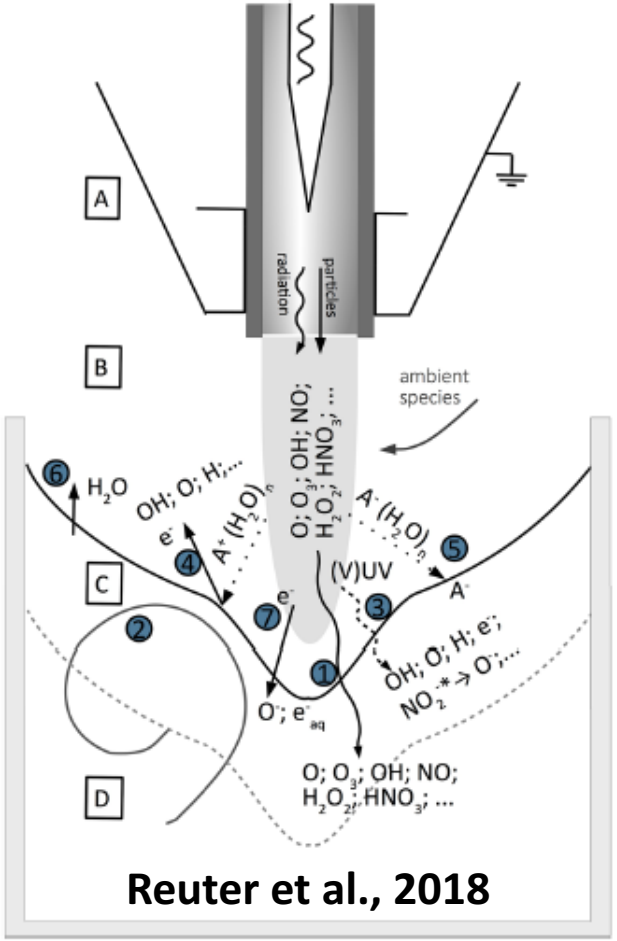
# Major Plasma-Generated RONS

| | |
|---|---|
| $^{\bullet}O$ | Oxygen ra |
| $^{\bullet}O_2^-/^{\bullet}O_2H$ | Superoxid |
| $H_2O_2$ | Hydrogen |
| $^{\bullet}OH$ | Hydroxyl |
| $^1O_2$ | Singlet (d |
| $O_3$ | Ozone |
| $^{\bullet}N$ | Nitrogen |
| $^{\bullet}NO$ | Nitric oxi |
| $^{\bullet}NO_2$ | Nitrogen |
| $NO_2^-/HNO_2$ | Nitrite/ni |
| $NO_3^-/HNO_3$ | Nitrate/n |
| $ONOO^-/ONOOH$ | Peroxynit |
| $OCl^-/HOCl$ | Hypochlo |
| $O_2NOO^-/O_2NOOH$ | Peroxynit |




Reuter et al., 2018

**Species generated mainly via electron-impact and/or Penning dissociation reactions and subsequent reactions in plasma with $N_2$, $O_2$, $H_2O$ (i.e. humid air).**

**Liquid phase species, e.g. $NO_2^-$, $ONOO^-$, $O_2NOO^-$, $OCl^-$, include liquid precursors such as $Cl^-$.**

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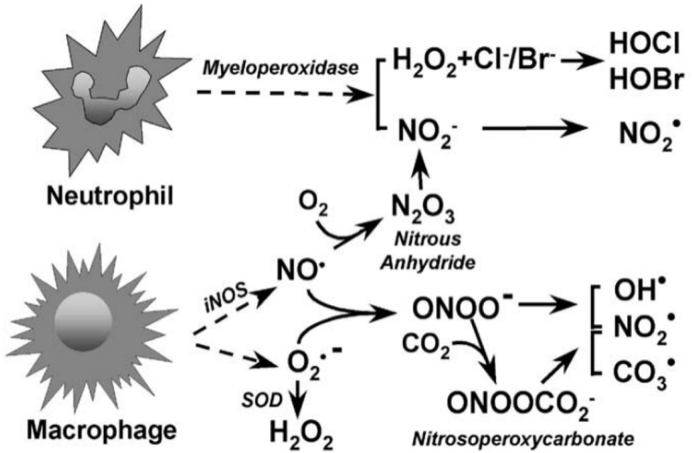
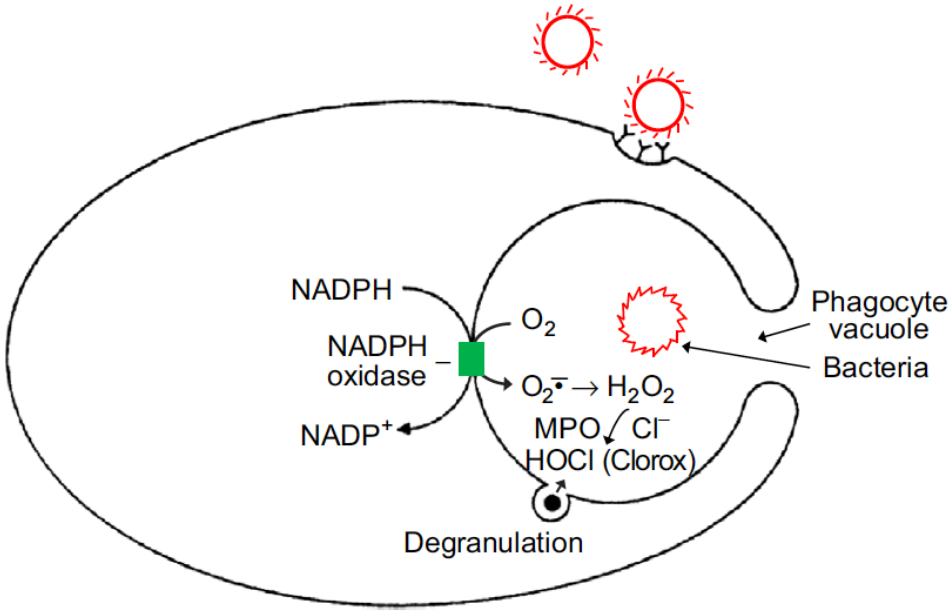
# Biological RONS in Innate Immunity

## Innate Immune System Chemistry

Dedon and Tannenbaum, 2004




## *Phagocytic Degradation of Microbes via ROS*




Redox Biology 1 (2013) 244–257

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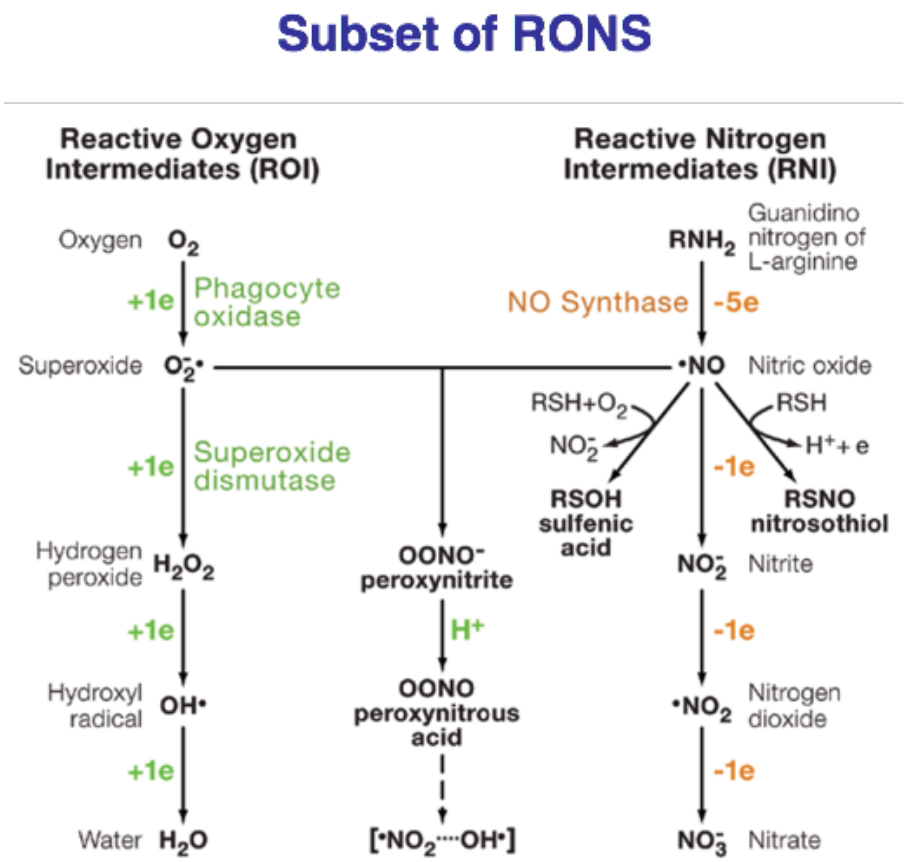
# RONS: Oxidation-Reduction (Redox) Chemistry



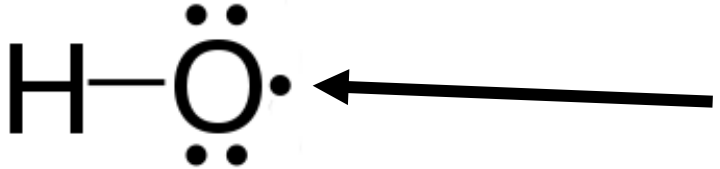

(Nathan and Ding, 2010)

RONS are formed by, and react in the context of, so-called oxidation-reduction (redox) reactions that can be thought of as involving the exchange of electrons.

Redox reactions pervade the earth's environment, are key to terrestrial biogeochemistry, as well as to aerobic biology.

*OIL RIG*: Oxidation Is Loss (of electrons); Reduction Is Gain (of electrons)

# What is a ‘Radical’ or ‘Free Radical’?



**Unpaired electron on hydroxyl radical**

***OH radical is very reactive!***

**The unpaired electron often causes this kind of chemical species to be more reactive than normal molecules.**

**Because of this, biologists were originally inclined to believe that radicals could not be important in living matter. This proved to be incorrect…**

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# Radicals in Chemical Biology: circa 2008

## Biologically Relevant Small Radicals

Chimia 62 (2008) 704–712

Georg Bauer[a], Chryssostomos Chatgilialoglu[b], Jerzy L. Gebicki[c], Lidia Gebicka[c], Georg Gescheidt[d], Bernard T. Golding[e], Sara Goldstein[*f], Jozsef Kaizer[g], Gabor Merenyi[h], Gabor Speier[g], and Peter Wardman[i]

**"Free-radical chemistry has moved from being an esoteric curiosity, mainly limited to applications in nuclear reactor chemistry and related specialties, to being a core component of numerous biological processes central to both normal and pathological conditions.**

**While we cannot yet weigh out superoxide and make solutions as we would sodium chloride, the realization that this radical and its chemistry is commonplace and important has been an important step in chemical biology.**

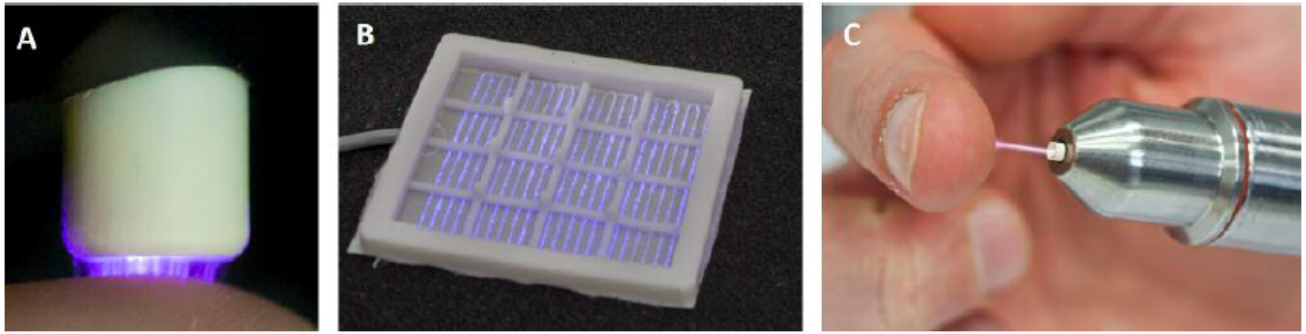
**The same is true of nitric oxide, and as this short article has demonstrated, these two simple molecules initiate a rich chemistry."**

# Review of Plasma-Generated RONS

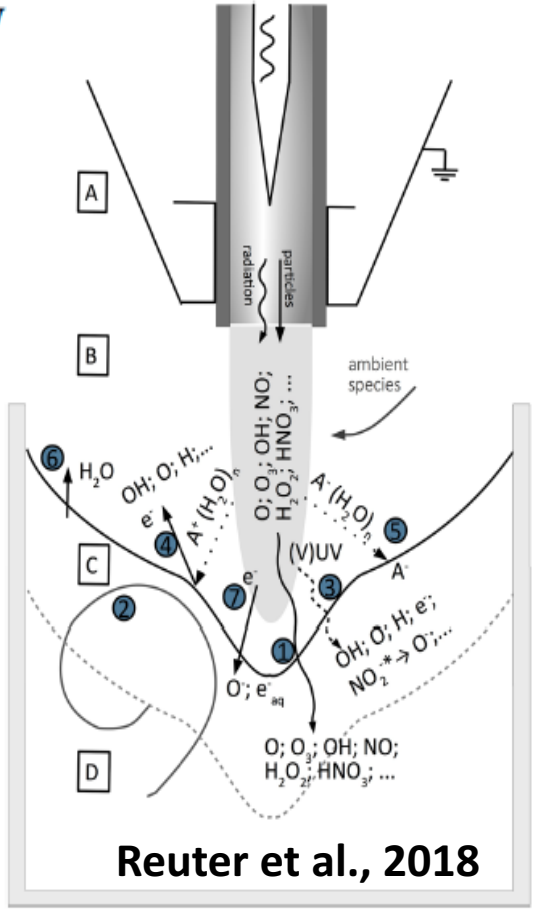
## Plasma Medicine: A Field of Applied Redox Biology

THOMAS VON WOEDTKE[1,2], ANKE SCHMIDT[1], SANDER BEKESCHUS[3], KRISTIAN WENDE[3] and KLAUS-DIETER WELTMANN[1]

**in vivo** *33*: 1011-1026 (2019)
doi:10.21873/invivo.11570




**An excellent recent review associating plasma biomedicine with the large field of 'redox biology.'**




Reuter et al., 2018

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# Cold Atmospheric Plasma: Selective Anti-Cancer Treatment

www.impactjournals.com/oncotarget/ Oncotarget, 2017, Vol. 8, (No. 9), pp: 15977-15995

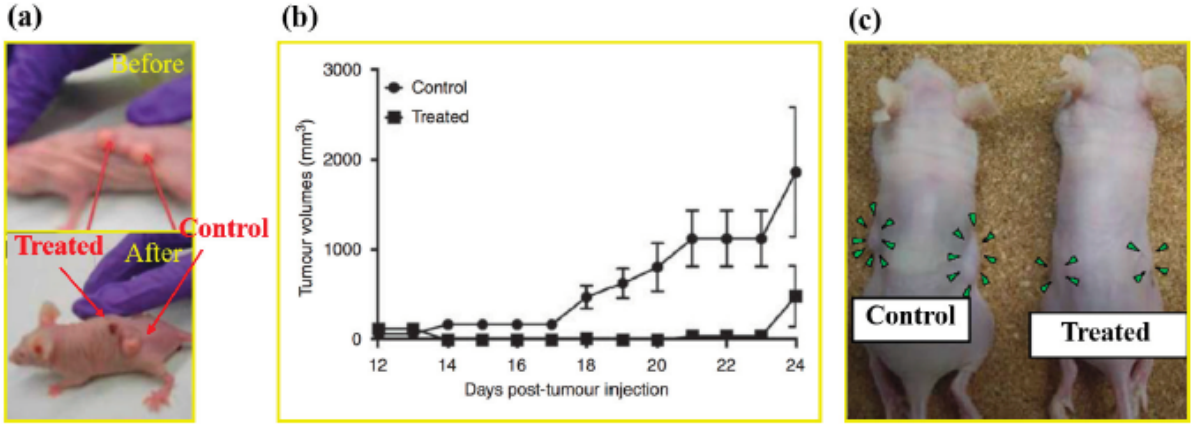
Review

## Cold atmospheric plasma, a novel promising anti-cancer treatment modality

**Dayun Yan[1], Jonathan H. Sherman[2] and Michael Keidar[1]**

[1] Department of Mechanical and Aerospace Engineering, The George Washington University, NW, Washington, DC, USA

[2] Neurological Surgery, The George Washington University, NW, Washington, DC, USA




Plasma treatment of sub-cutaneous tumors shown by multiple groups

What is basis for selectivity?

How does plasma act (through skin) to shrink relatively large tumors?

*What mechanisms control these effects??*

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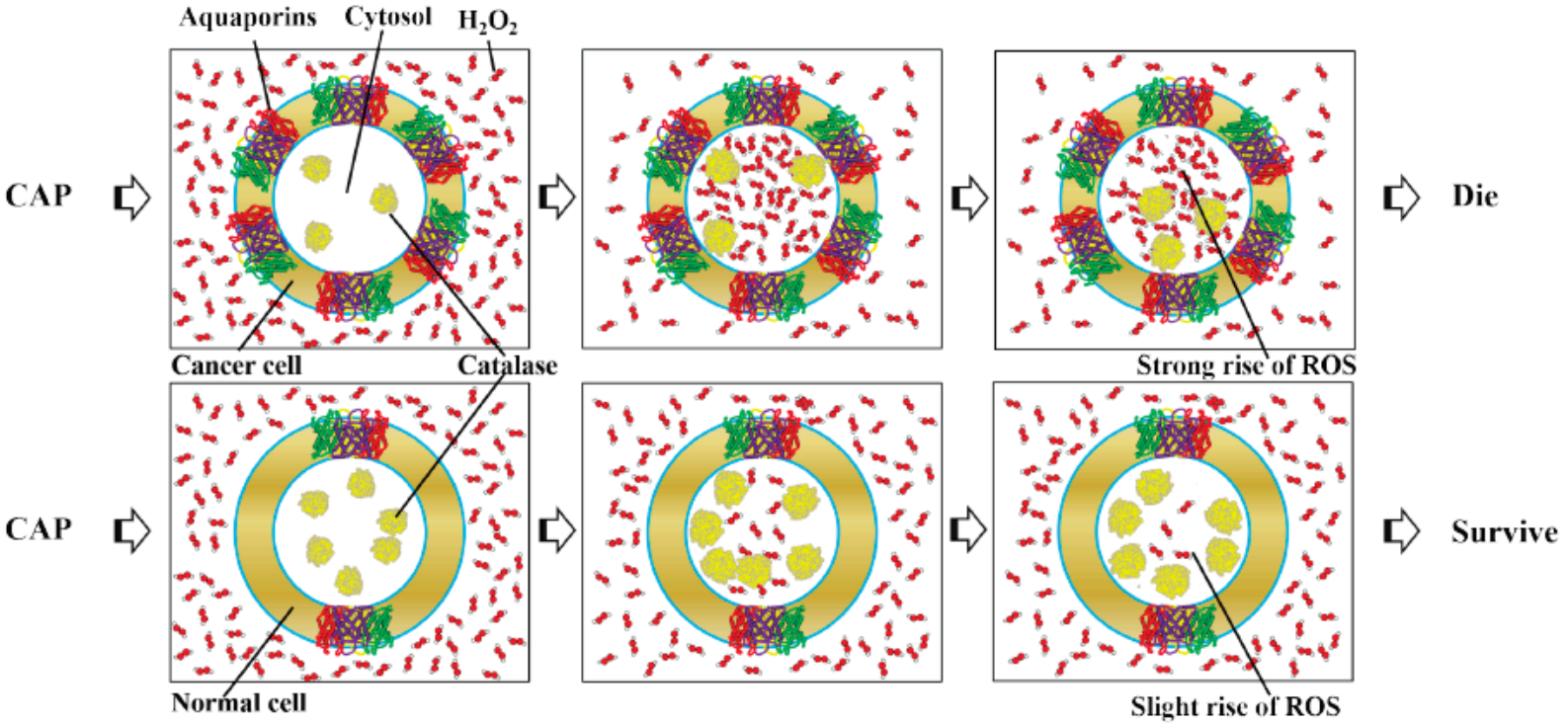
# Cold Atmospheric Plasma: Selective Anti-Cancer Treatment




Yan et al., 2017

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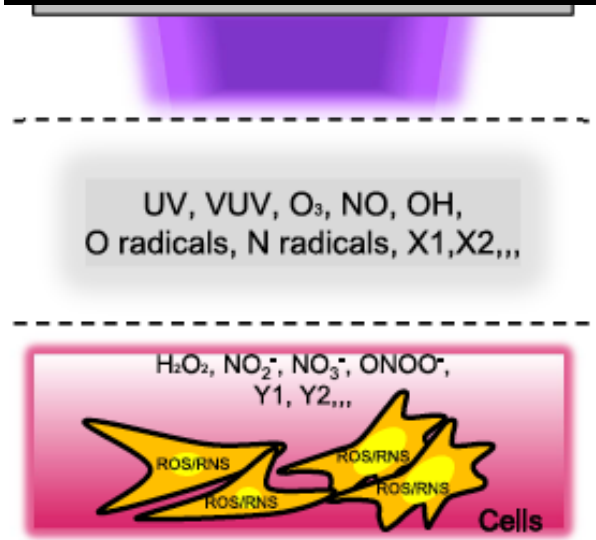
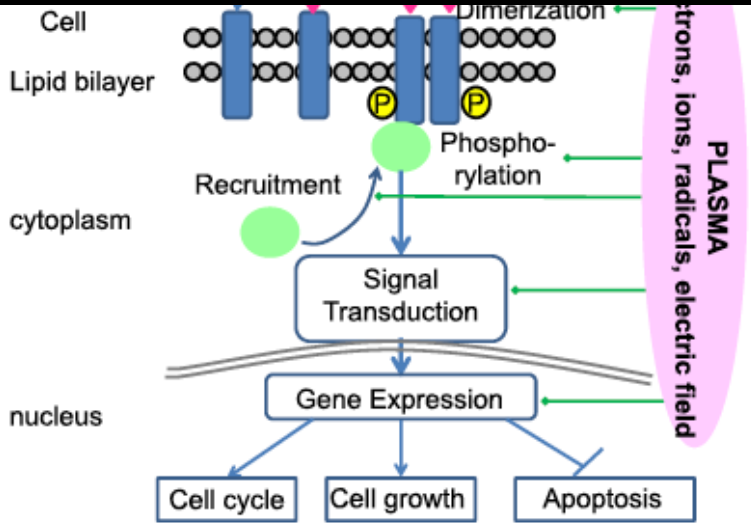
# RONS and Cancer: Mechanistic Investigations

Plasma Medical Science for Cancer Therapy:
Toward Cancer Therapy Using Nonthermal
Atmospheric Pressure Plasma

"It is challenging to figure out where plasma or plasma-generated reactive species would act on the signaling networks to induce apoptosis…A p53-mediated DNA damage response Is one signaling pathway to explain the apoptosis by plasma treatments."






IEEE TRANSACTIONS ON PLASMA SCIENCE, VOL. 42, NO. 12, DECEMBER 2014

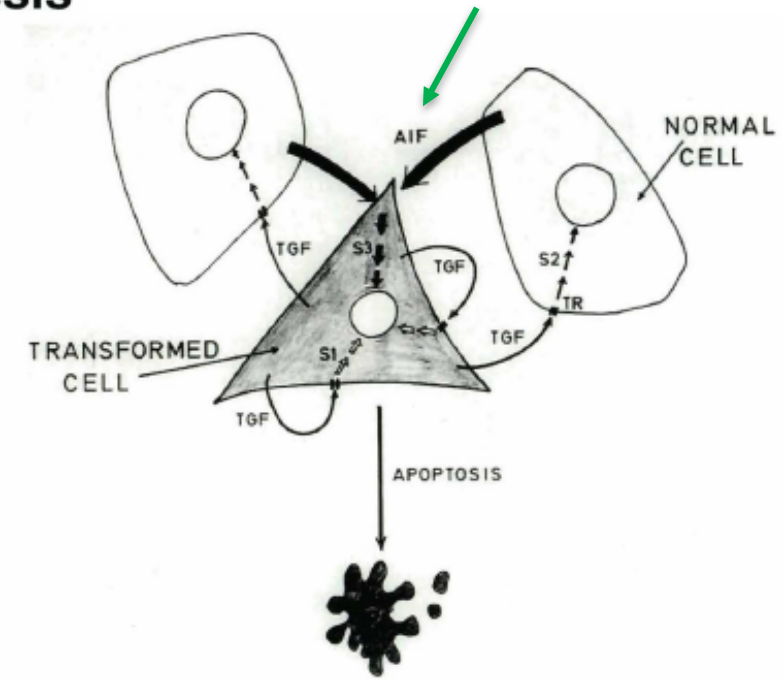
# Natural RONS Signaling: Context for CAP Biomedicine

*Invited Review* Histol Histopathol (1996) 11: 237-255

## Elimination of transformed cells by normal cells: a novel concept for the control of carcinogenesis

**G. Bauer**
Abteilung Virologie, Institut für Medizinische Mikrobiologie und Hygiene der Universität Freiburg, FRG

**AIF: 'apoptosis inducing factor'**




**"Inhibition of transformed cells by neighbouring normal cells has been known for as along as transformation studies have been performed in vitro."**

**Berwald and Sachs (1963)**
**Stoker (1964, 1967)**
**Stoker et al. (1966)**

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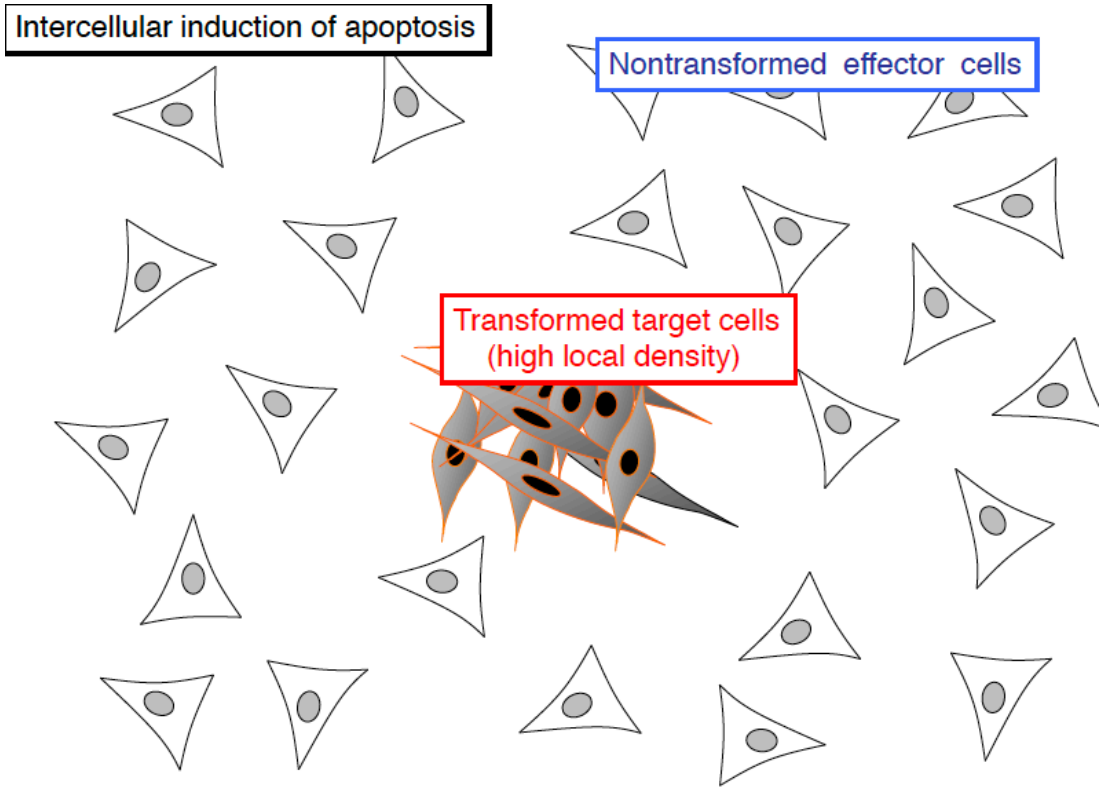
# Intercellular Inhibition of Transformed Cells




**First observed in 1960s that nontransformed 'effector' cells can induce a decrease in number of malignant cells or cause them to disappear.**

**Later work showed malignant cells at low density surrounding malignant cells at high density can do the same job as the nonmalignant cells. (NO/POD)**

***Decades of subsequent research elucidated key roles played by RONS, including $O_2^-$, $H_2O_2$, OH, HOCl, NO and $ONOO^-$.***

**Courtesy: Professor G. Bauer**

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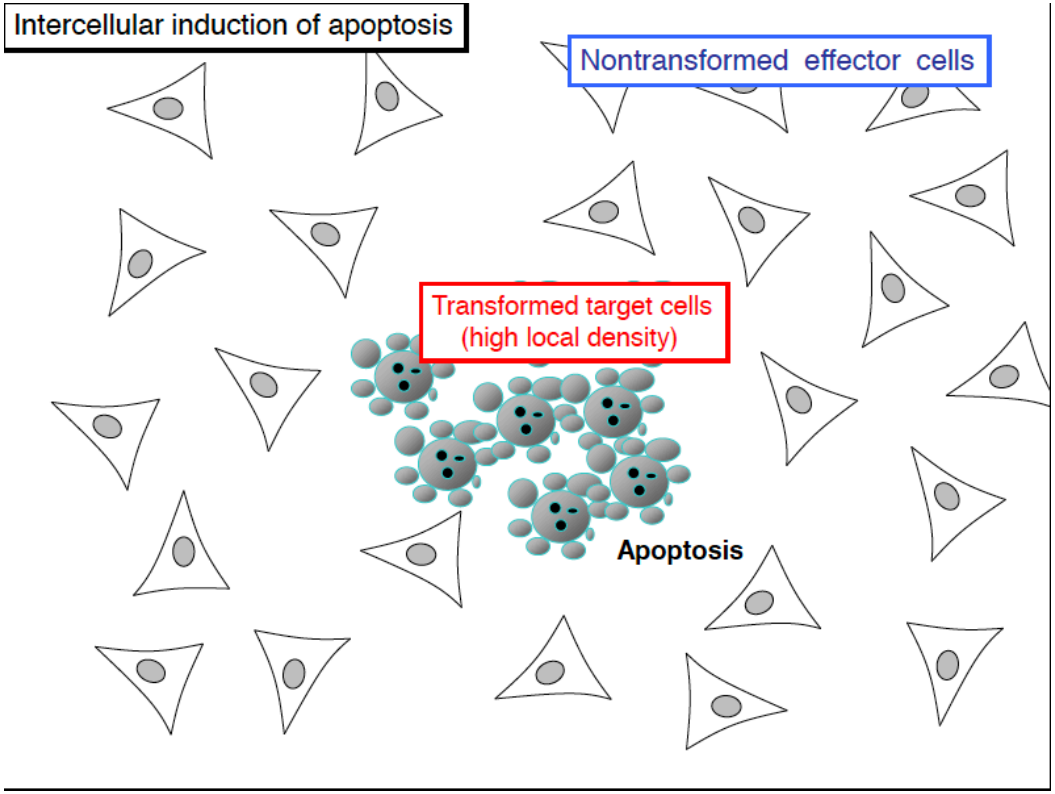
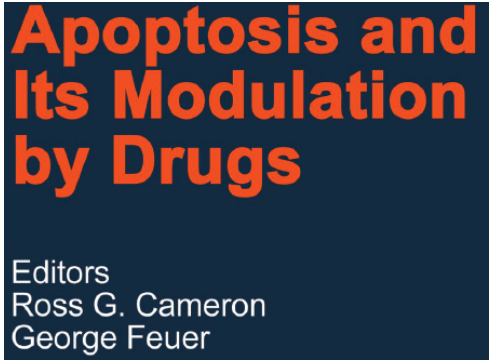
# RONS Play a Natural Anti-Tumor Role




Courtesy: Professor G. Bauer




CHAPTER 11

**Reactive Oxygen Species and Apoptosis**

G. Bauer, S. Dormann, I. Engelmann, A. Schulz, and M. Saran

**Volume 142 of Handbook of Experimental Pharmacology (2000).**

***Explicit evidence accumulated by 2000: RONS "..are involved in triggering and mediating apoptosis under physiological and pathophysiological conditions."***

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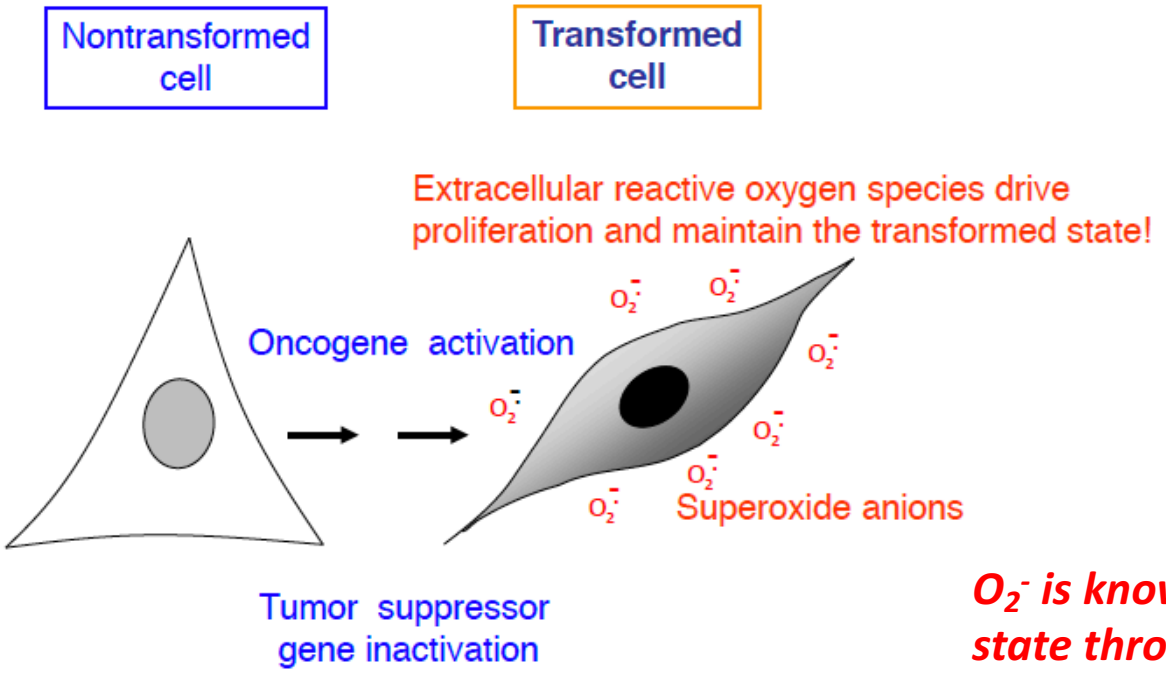
# $O_2^-$: A Two-Edged Sword in Cancer




**Normal cells become transformed (precancerous) cells due to oncogene activation and tumor suppressor gene inactivation.**

**One key feature of transformed cells is high concentration of extracellular superoxide anions.**

**Courtesy: Professor G. Bauer**

***$O_2^-$ is known to initiate the transformed state through mutagenesis (among others), drive transformed cell proliferation and maintain the transformed state. It is also key to apoptotic signaling.***

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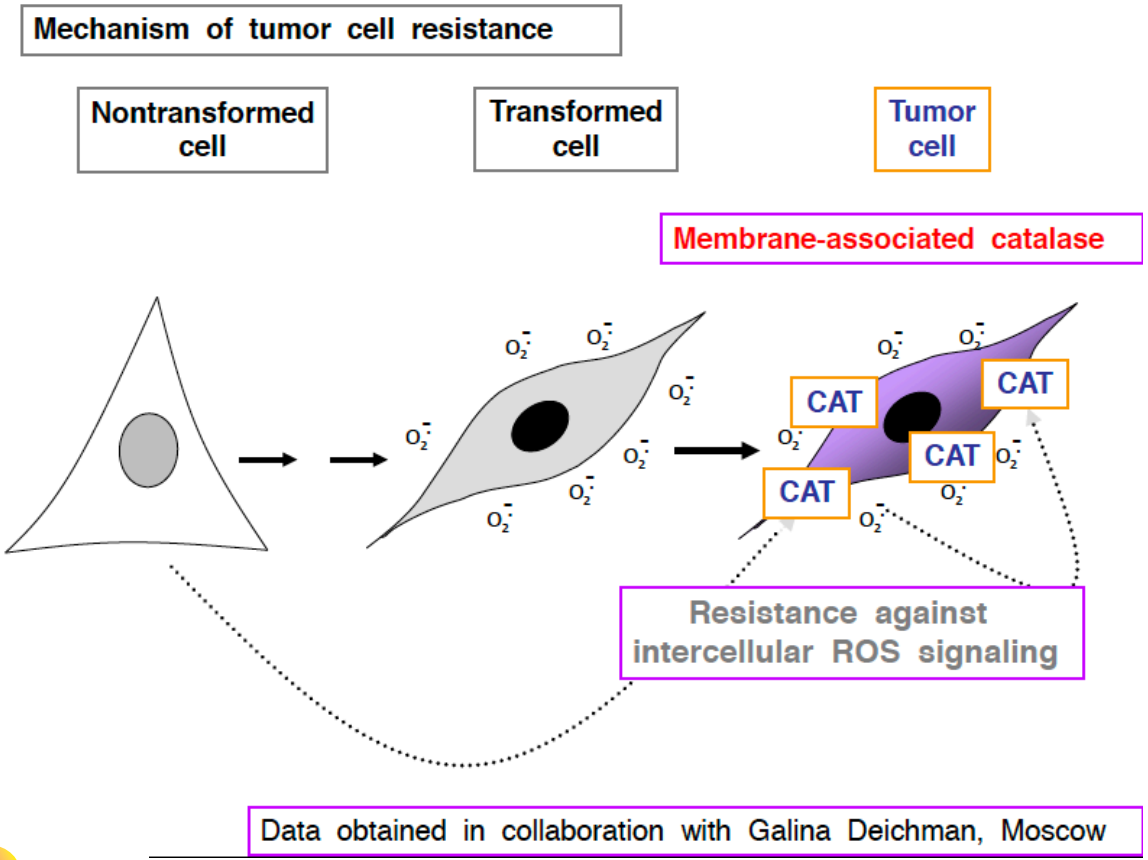
# Tumor Cells *Resist* Intercellular Apoptotic Signaling




Another key step was recognition of protective effect of membrane associated catalase (CAT), allowing transformed cells to become 'bona fide' tumor cells.

CAT protects the tumor cell from attack via intercellular RONS signaling.

***Selectively inactivating CAT is central to tumor cell induction of apoptosis.***

**Courtesy: Professor G. Bauer**

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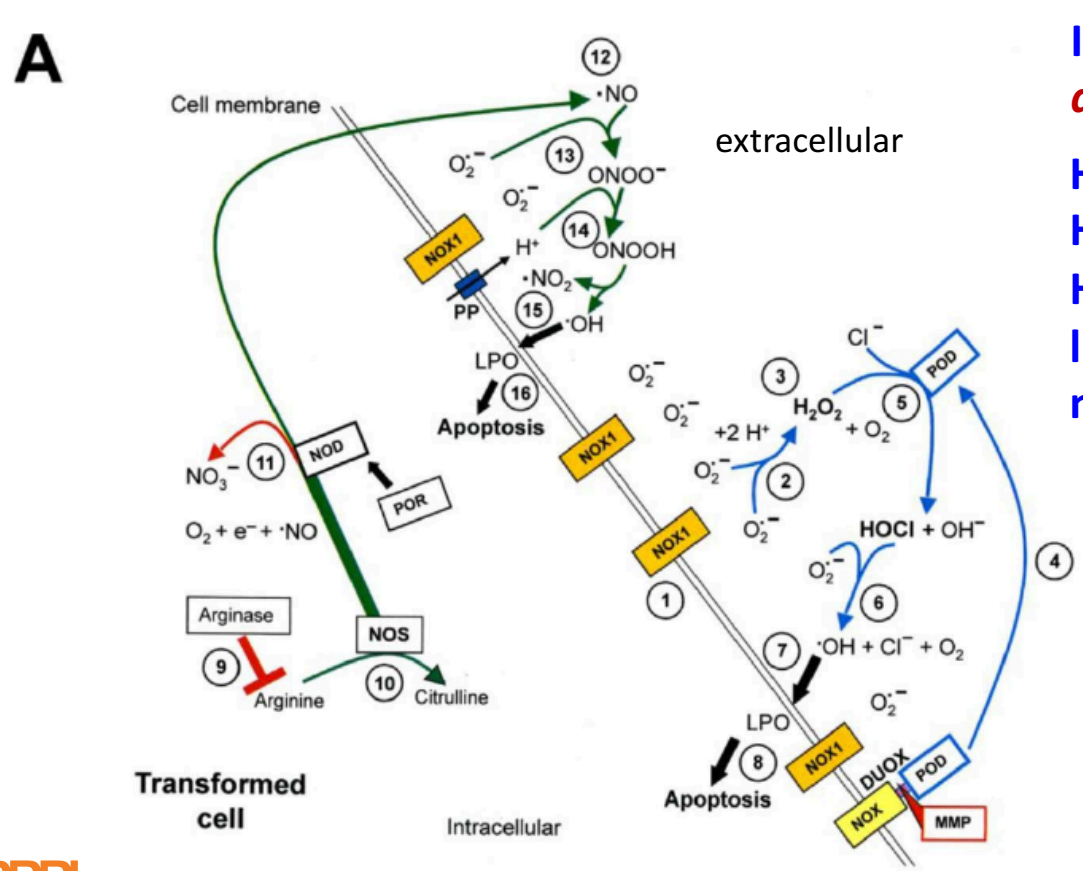
# Intercellular Signaling via $ONOO^-$ and HOCl Pathways




Mechanisms of Ageing and Development 172 (2018) 59–77

**Intercellular signaling *induces apoptosis in transformed cells*.**

**HOCl: $O_2^-$ from NOX1 dismutates to $H_2O_2$. POD creates HOCl from $Cl^-$ and $H_2O_2$. HOCl + $O_2^-$ creates OH, results in lipid peroxidation (LPO). Triggers mitochondrial apoptosis pathway.**

**$ONOO^-$: NOS makes NO, some consumed by NO dioxygenase (NOD). NO leaves cell and reacts with $O_2^-$ to form $ONOO^-$. $H^+$ from proton pumps (PP) create ONOOH that decomposes to $NO_2$ and OH. LPO and apoptosis results.**

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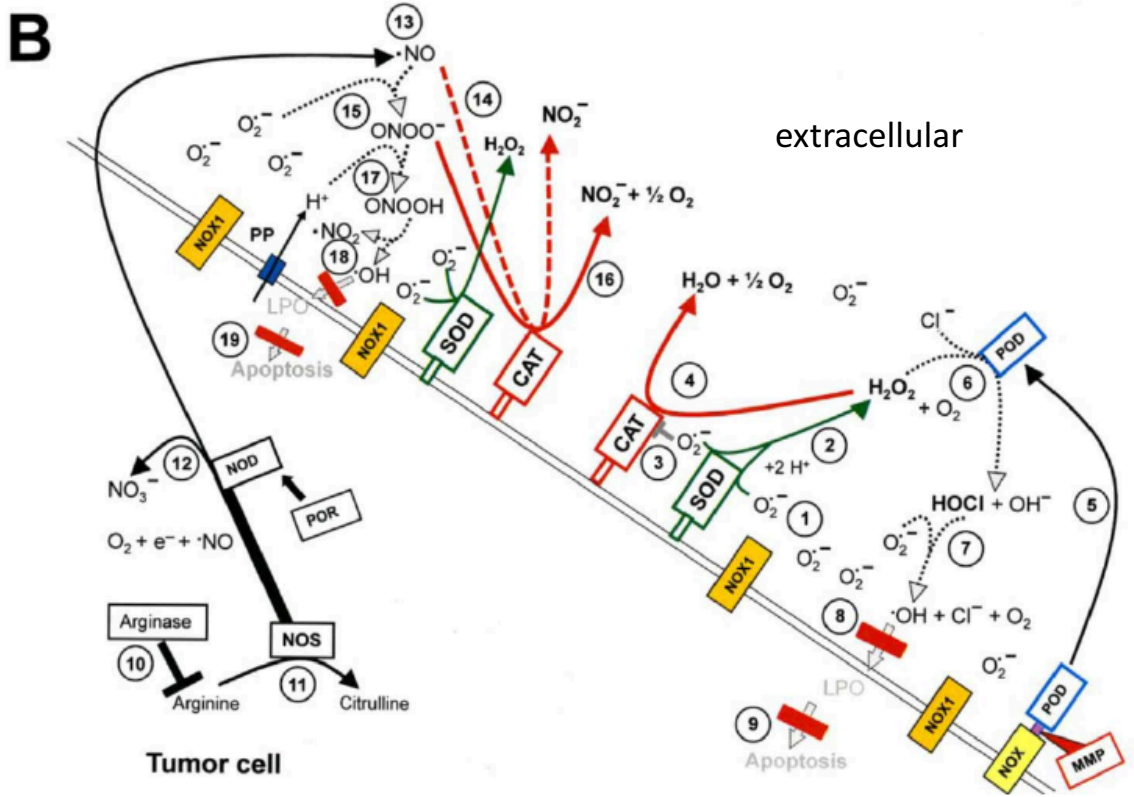
# Tumor Cells (via CAT, SOD) Stop Intercellular Signaling




**Membrane associated CAT and SOD: $O_2^-$ and $H_2O_2$ lost. POD-dependent HOCl synthesis and subsequent *apoptosis pathways eliminated.* Catalase oxidizes NO and decomposes $ONOO^-$.**

**A logical way to restore apoptotic RONS signaling *is to selectively inactivate CAT & SOD.***

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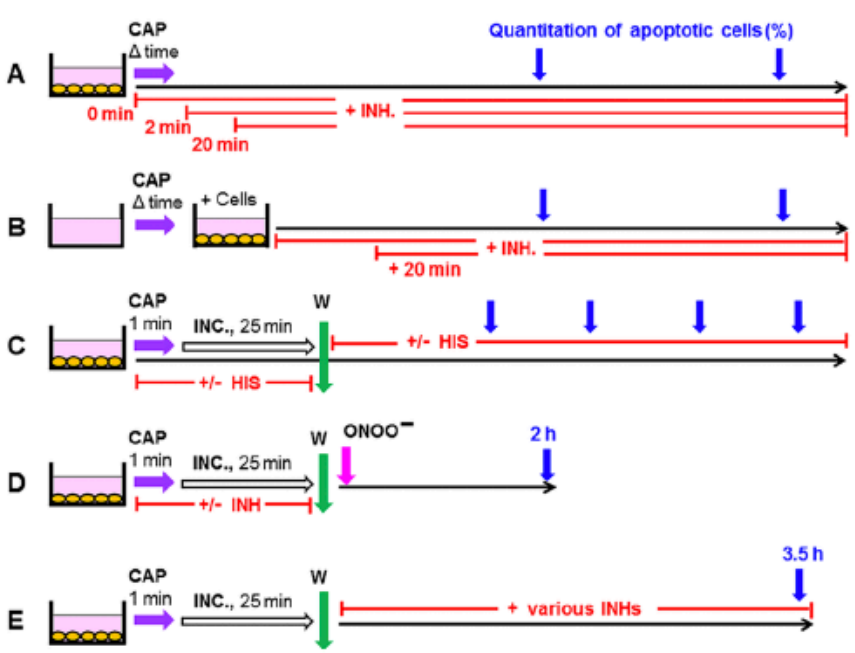
Mechanisms of Ageing and Development 172 (2018) 59–77

# Summary of Recent Study

SCIENTIFIC REPORTS | (2019) 9:14210 | https://doi.org/10.1038/s41598-019-50291-0

and
Trigger
poptosis

hala[3]




**Typical experiments: Air CAP treatment of medium & cells or PAM then applied to cells. Variable: Incubation (INC); Wash (W); Inhibitor (INH); etc. Observe % apoptosis.**

Human MKN-45 gastric carcinoma cells; human neuroblastoma cells; Ewing sarcoma and cervical carcinoma cells; normal dipoid fibroblasts

# Major Experimental Tools

**Table 1.** Summary of enzyme inhibitors, reactive species scavengers, reactive species donors, mimetics, and antibodies used in the present study to elucidate apoptotic and protective mechanisms.

| Purpose | Compound name | Compound abbreviation and standard working concentration |
|---|---|---|
| Singlet oxygen scavenger | Histidine | HIS 2 mM |
| Peroxynitrite decomposition catalyst | 5-, 10-, 15-, 20-Tetrakis(4-sulfonatophenyl)porphyrinato iron(III) chloride | FeTPPS 25 μM |
| NOX1 inhibitor | 4-(2-Aminoethyl) benzenesulfonyl fluoride | AEBSF 100 μM |
| HOCl scavenger | Taurine | TAU 50 mM |
| Aquaporin inhibitor | $AgNO_3$ | $Ag^+$ 5 μM |
| Catalase inhibitor | 3-aminotriazole | 3-AT 25 mM |
| Catalase donation (bovine liver catalase) | Catalase | CAT 10 - 1000 U/ml |
| glutathione synthesis inhibitor | Buthionine sulfoximine | BSO 10 - 50 μM |
| $^{\cdot}OH$ scavenger | Manitol | MANN 20 mM |
| $^{\cdot}OH$ scavenger | Dimethylthiourea | DMTU 20 mM |
| NO donor | Dieethylamine NONOate | DEA NONOate 0.5 mM |
| HOCl donor | Sodium oxychloride | NaOCl as indicated |
| Generation of $H_2O_2$ | Glucose oxidase | GOX as indicated |
| Nitric Oxide Synthase inhibitor | N-omega-nitro-L-arginine methylester hydrochloride | L-NAME 2.4 mM |
| Proton pump inhibitor | Omeprazole | |

# Major Experimental Tools

| | | |
|---|---|---|
| Peroxidase inhibitor | 4-Aminobenzoyl hydrazide | ABH 150 μM |
| Caspase-3 inhibitor | | Z-DEVD-FMK 50 μM |
| Caspase-8 inhibitor | | Z-IETD-FMK 25 μM |
| Caspase-9 inhibitor | | Z-LEHD-FMK 25 μM |
| SOD mimetics | Mn(III) 5,10,15,20-tetrakis(N-methylpyridinium-2-yl)porphyrin and Mn (III) meso-tetrakis(N-ethylpyridinium-2-yl)porphyrin | MnTM-2PyP and MnTE-2-PyP 20 μM |
| Mn-SOD donation (*E. coli*) | Manganese superoxide dismutase | Mn-SOD 100 U/ml |
| $ONOO^-$ decomposition catalyst and $O2^-$ scavenger | Fe(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachlorideporphyrin pentachloride | FeTMPyP 25 μM |
| Catalase mimetic | chloro([2,2′-[1,2-ethanediylbis[(nitrilo-κN)methylidyne]]bis[6-methoxyphenolato-κO]]]-manganese | EUK-134 20 μM |
| Antibody for human superoxide dismutase (SOD) | | cb 0989 (binding and neutralizing) cb 0987 (binding without neutralization) |

**Strategy: Reconstitution; inhibitors/scavengers; varying incubation and washing times; antibody studies; gene knockdown (SiRNA) to eliminate selected enzymes from cells**

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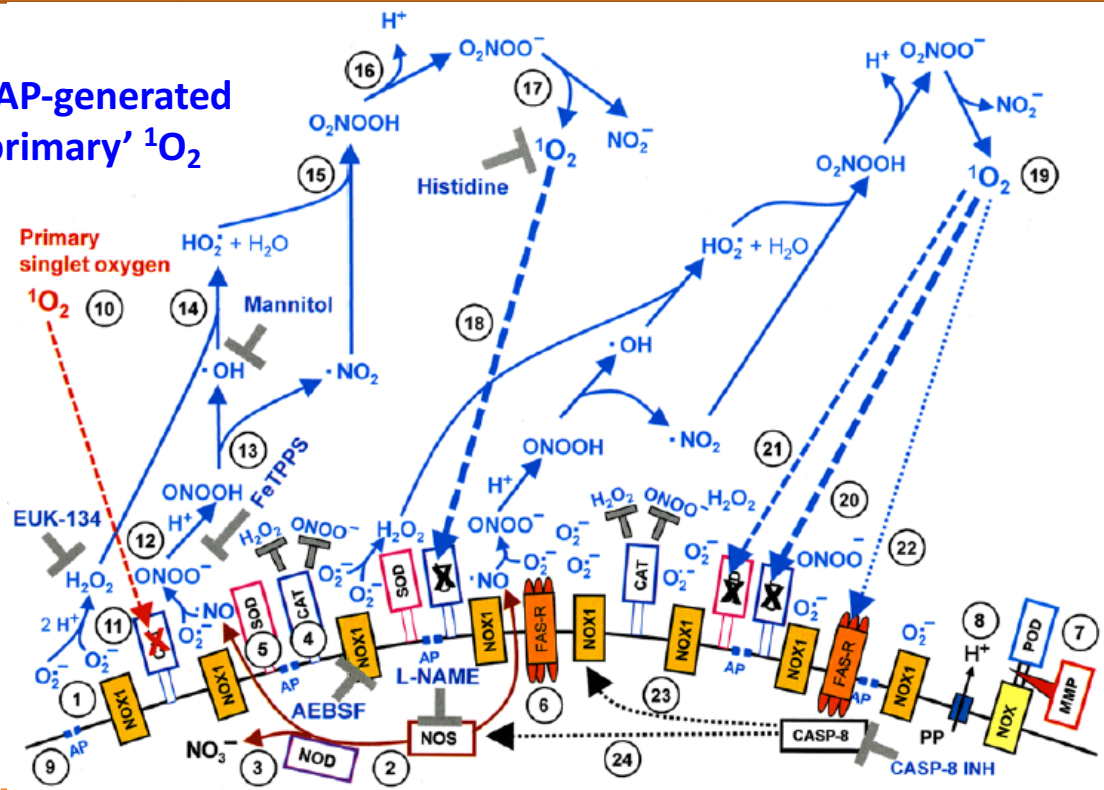
# $^1O_2$ Inactivates CAT: Key Trigger to Restore Intercellular Apoptosis




Primary $^1O_2$ inactivates a few CAT, then cell-derived $ONOO^-$ and $H_2O_2$ lead to additional, or secondary, $^1O_2$

Secondary $^1O_2$ initiate a chain reaction, inactivating adjacent CAT after CAT.

Without protective CAT, cells undergo apoptosis

This leads to a form of 'bystander effect,' a key to the proposed mechanism

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# Extracellular $^1O_2$: Key Role in Selectively Inactivating CAT

Redox Biology 6 (2015) 157–168

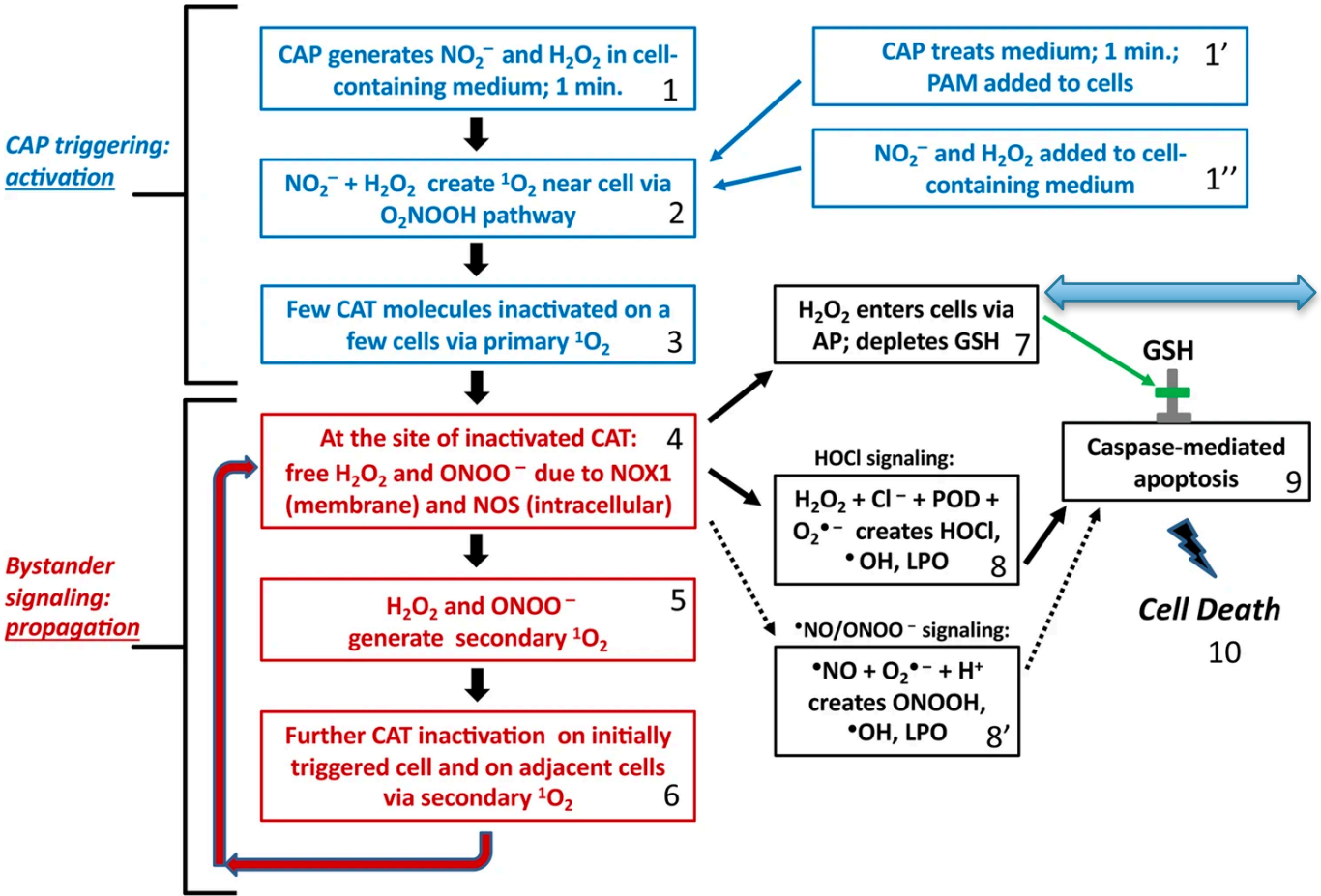
## Singlet oxygen treatment of tumor cells triggers extracellular singlet oxygen generation, catalase inactivation and reactivation of intercellular apoptosis-inducing signaling ☆

Michaela Riethmüller [1,3], Nils Burger [2,3], Georg Bauer *

*Institute of Virology, Department of Medical Microbiology and Hygiene, University Medical Center, Freiburg, Germany*

**"Model experiments using the singlet oxygen generating photosensitizer photofrin have shown that exogenous (*i.e. outside cell*) singlet oxygen triggers a well-defined biochemical cascade that leads to the generation of cell-derived extracellular singlet oxygen in an impressive auto-amplificatory process." (Bauer and Graves, 2016)**

# Summary of Proposed Mechanisms: Flowchart




**Recall Keidar et al. model involving AP and $H_2O_2$**

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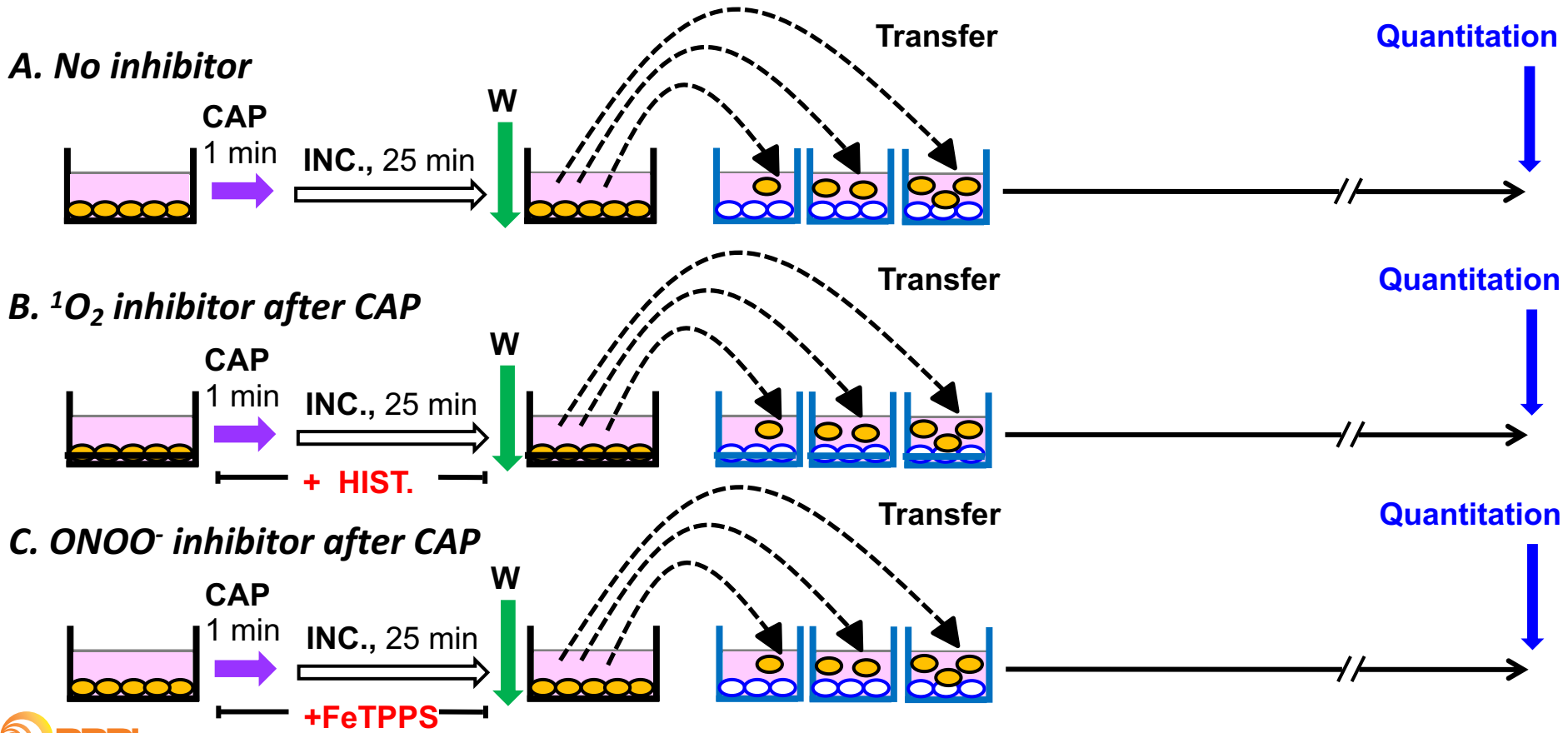
# Test of Bystander Mechanism: Cell Transfer Experiments

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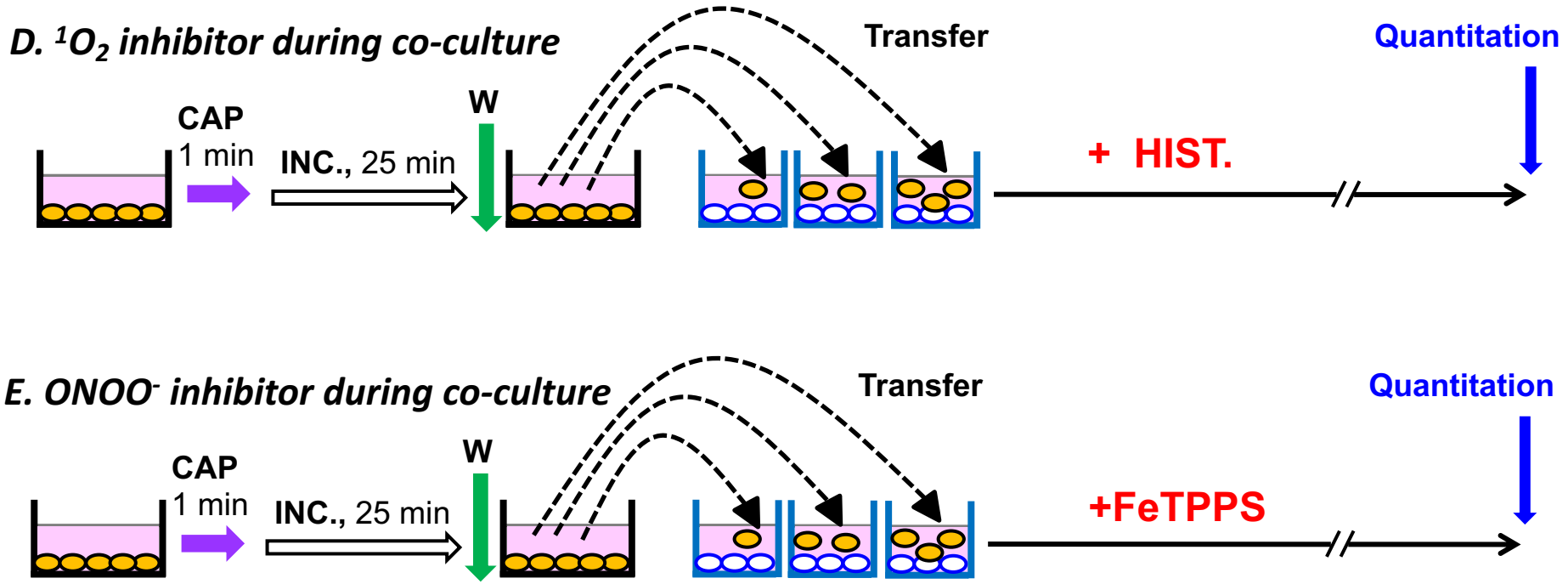
# Test of Bystander Mechanism

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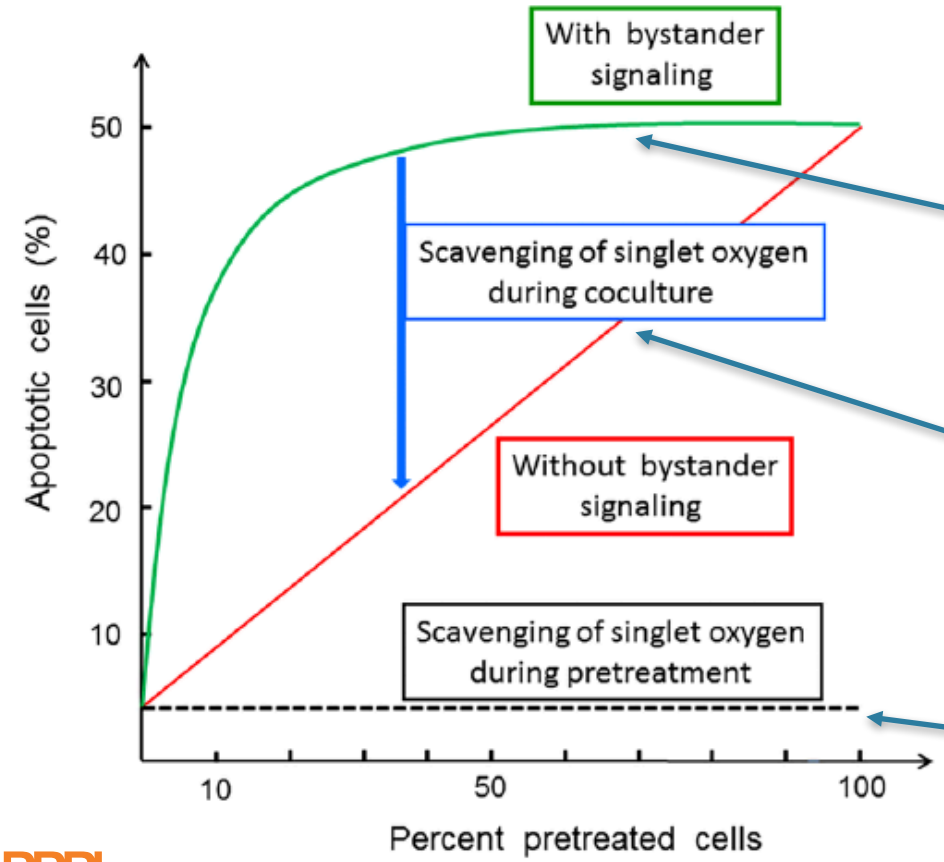
# Bystander Mechanism Prediction




Courtesy: Professor G. Bauer

Mixing varying percentages of pre-treated cells with untreated cells allows test of bystander mechanism

A. With no inhibitor, bystander signaling should result in *amplification* of apoptosis above original cell pretreatment percentage

D. & E. Scavenging $^1O_2$ during co-culture should result in *only pre-treated cell* apoptosis and no bystander signaling

B. & C. Scavenging $^1O_2$ during pre-treatment should eliminate 'activation' resulting in *minimal apoptotic cells*

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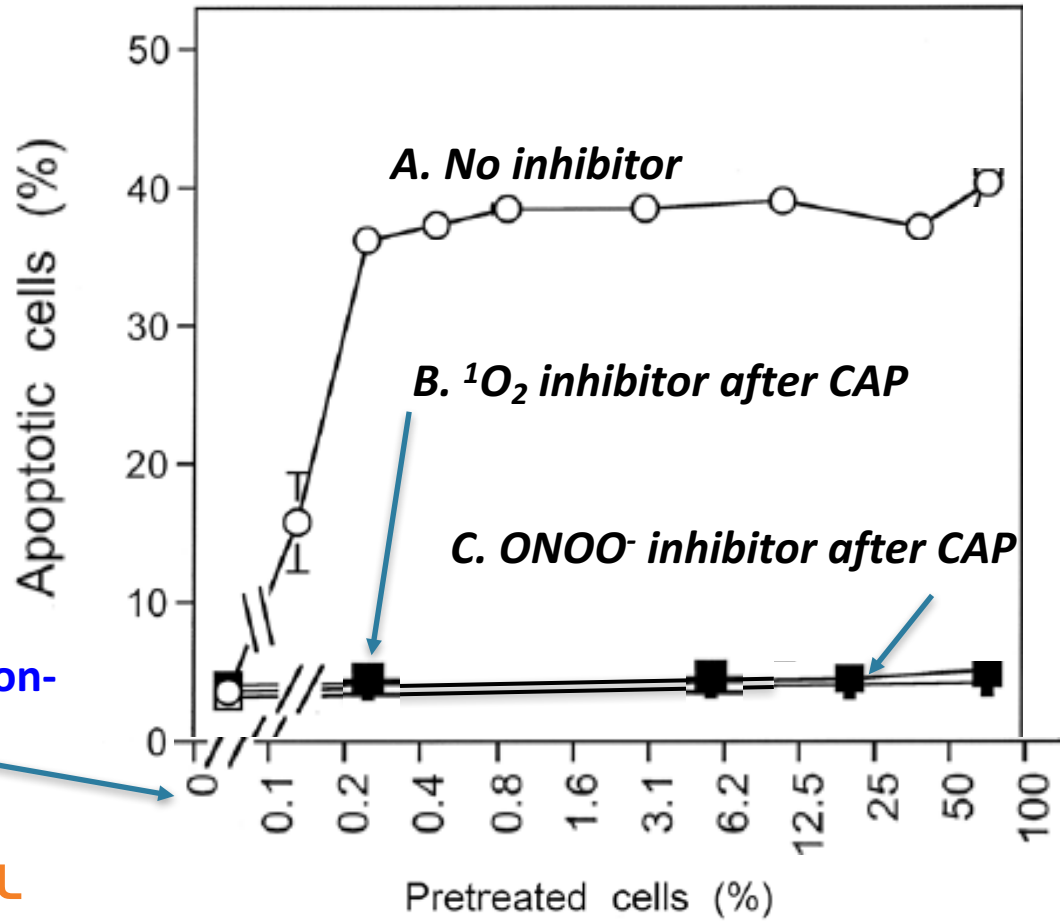
# Observation Confirms Bystander Mechanism




Note non-linear scale

Results follow predictions: no inhibitor followed by co-culture shows strong amplification: even 0.2% treated cells induces near maximal apoptosis in non-treated cells

Adding either $^1O_2$ or $ONOO^-$ inhibitors during pre-treatment/incubation eliminates any apoptosis

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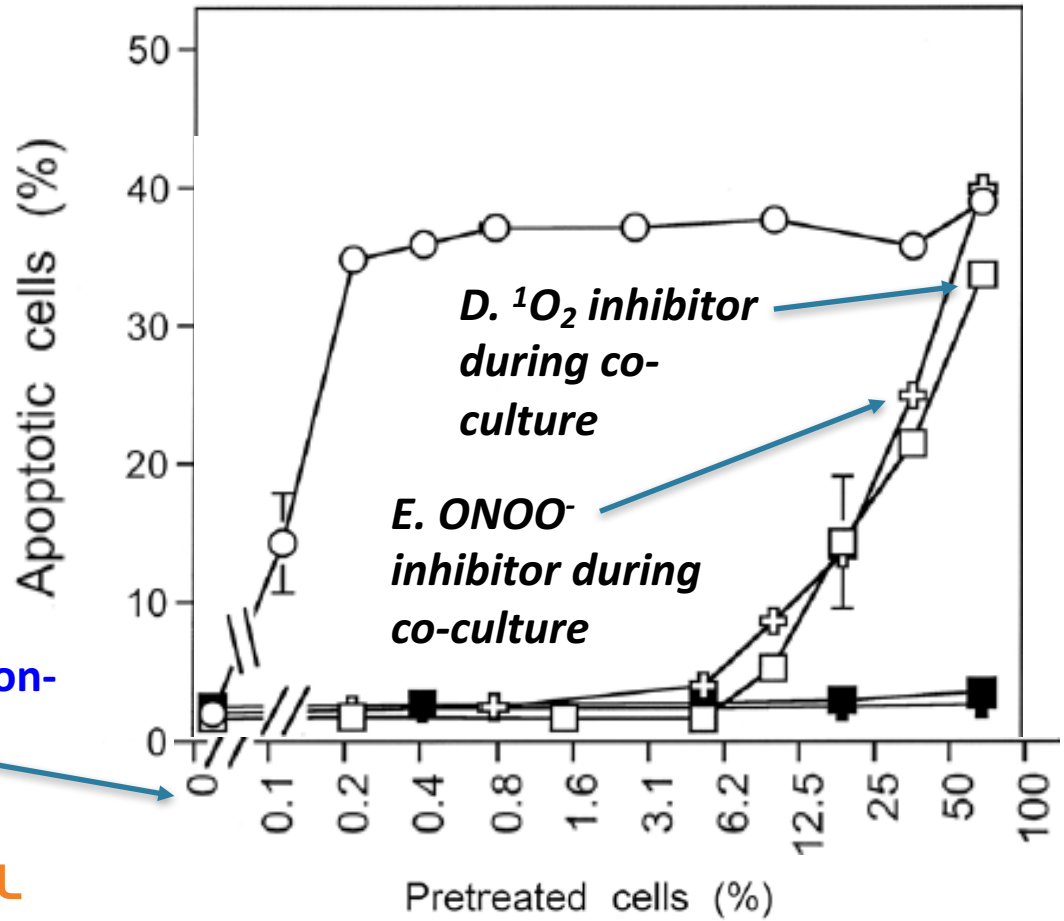
# Observation Confirms Bystander Mechanism




Note non-linear scale

Results again follow predictions: adding $^1O_2$ or $ONOO^-$ inhibitors during co-culture results on only pre-treated cells undergoing apoptosis

These results are consistent with the bystander mechanism proposed

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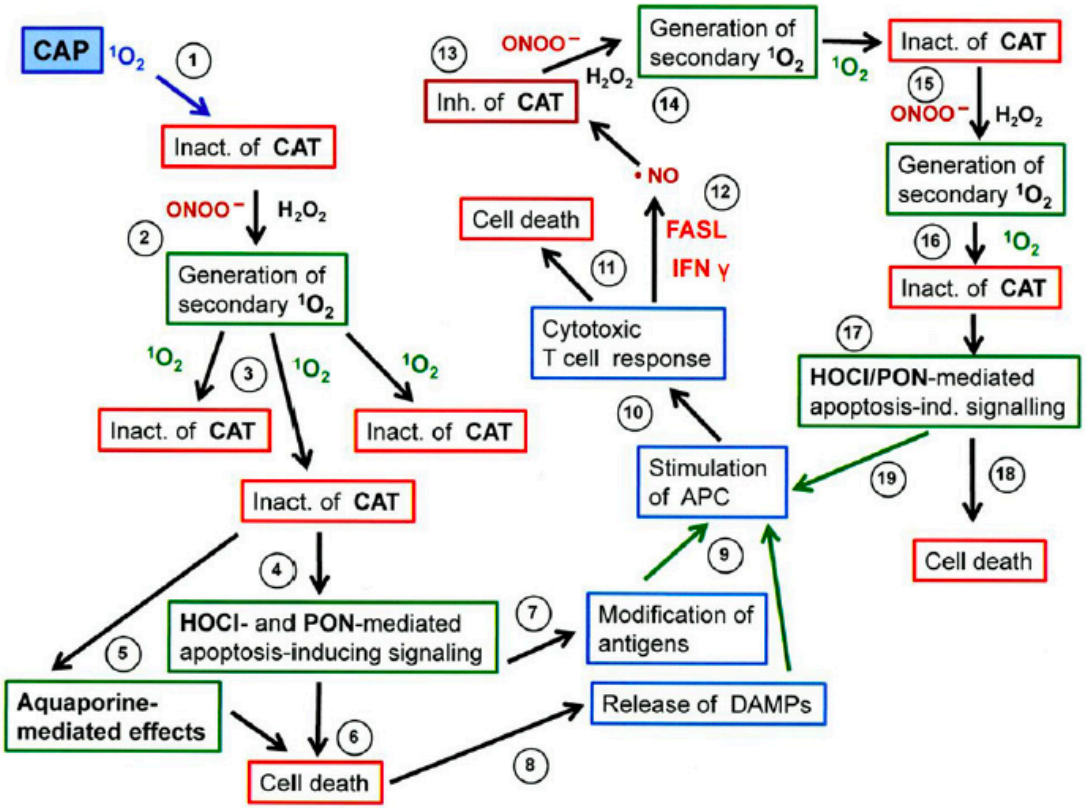
# *Proposed* Stimulation of Adaptive Immune Response




Bauer, IEEE TRPMS, 2, 2018

Apoptotic release of damage associated molecular pattern proteins (DAMPs) and modification of antigens by HOCl/PON signaling stimulates antigen presenting cells (APCs) and subsequent cytotoxic T cell response and cell death.

T cell release of FAS ligand (FASL) and interferon gamma (IFN γ) induces further NO release from tumor and further inhibition of CAT, leading to subsequent apoptosis.

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# von Woedtke et al.: Question 1

## Plasma Medicine: A Field of Applied Redox Biology

THOMAS VON WOEDTKE[1,2], ANKE SCHMIDT[1], SANDER BEKESCHUS[3], KRISTIAN WENDE[3] and KLAUS-DIETER WELTMANN[1]

**in vivo** *33*: 1011-1026 (2019)
doi:10.21873/invivo.11570

***Are there single and specific RONS responsible for distinct biological effects or is it only a matter of the redox potential of the cellular target sites?***

**The mechanism we present identifies specific RONS – namely, CAP-generated $^1O_2$ attacks CAT on tumor cell membrane, triggering series of reactions and cell-cell communication, resulting in apoptosis of a collection of cells.**

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# von Woedtke et al.: Question 2

## Plasma Medicine: A Field of Applied Redox Biology

THOMAS VON WOEDTKE[1,2], ANKE SCHMIDT[1], SANDER BEKESCHUS[3], KRISTIAN WENDE[3] and KLAUS-DIETER WELTMANN[1]

**in vivo** *33*: 1011-1026 (2019)
doi:10.21873/invivo.11570

***How can we identify and analyze specific RONS at their site of action?***

**Reconstitution experiments; scavengers and inhibitors; vary incubation time and washing sequences; use siRNA to knock down gene expression of specific enzymes; test bystander effect by mixing varying amounts of pre-treated cells with untreated cells.**

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# von Woedtke et al.: Question 3

## Plasma Medicine: A Field of Applied Redox Biology

THOMAS VON WOEDTKE[1,2], ANKE SCHMIDT[1], SANDER BEKESCHUS[3], KRISTIAN WENDE[3] and KLAUS-DIETER WELTMANN[1]

**in vivo** *33*: 1011-1026 (2019)
doi:10.21873/invivo.11570

***Is it possible to find a measure for biological plasma effects that can serve as a kind of 'treatment dose'?***

**This can only be done with a firm understanding of CAP-cellular interaction mechanisms. For example, the bystander mechanism presented here requires a minimum density of cells. If a tumor is surgically excised, leaving only a few remnants of tumor cells, the bystander signaling described here might not be effective. Proper dose definition can only come through knowledge of mechanisms.**

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# Concluding Remarks

Cells are active generators and consumers of RONS and they are often key players in apoptosis. This fact must inform CAP biomedical studies of mechanisms and applications.

In experiments shown here, CAP acted (relatively briefly) to create $NO_2^-$ and $H_2O_2$ in medium. These species initiated or triggered complex signaling pathways that led to auto-amplification of apoptosis of tumor cells.

We *do not believe* that the only effect, in general, of CAP is generation of $NO_2^-$ /$H_2O_2$! In other experiments and certainly in vivo, many other effects are known and are undoubtedly important.

A key result from this work is strong evidence of cell-to-cell bystander communication.

# Concluding Remarks

**The most common forms of therapeutic intervention seek specific or selective action: e.g. eliminate invading pathogen or tumor with minimal negative effects on the host. Classic example: antibiotics.**

**But RONS tend to react *promiscuously* with everything! Nature solves this problem in part by using enzymes in precise locations to create precisely the species needed at the right concentration. Further, RONS reactivity is modulated through the use of enzymatic and non-enzymatic species (e.g. antioxidants). Finally, secondary reactions from relatively unreactive precursors (e.g. $NO_2^-$ and $H_2O_2$) react slowly to create more reactive intermediates (e.g. $^1O_2$).**

**The connection between RONS signaling, immunogenic cell death and adaptive immunity is important. Bystander signaling is relatively local; adaptive immunity-induced 'abscopal' signaling is fully systemic. CAP-stimulated adaptive immunity is probably the most promising direction for CAP biomedicine.**

***See Vandana Miller's talk in 2 weeks!***

# PPPL: My New Home!

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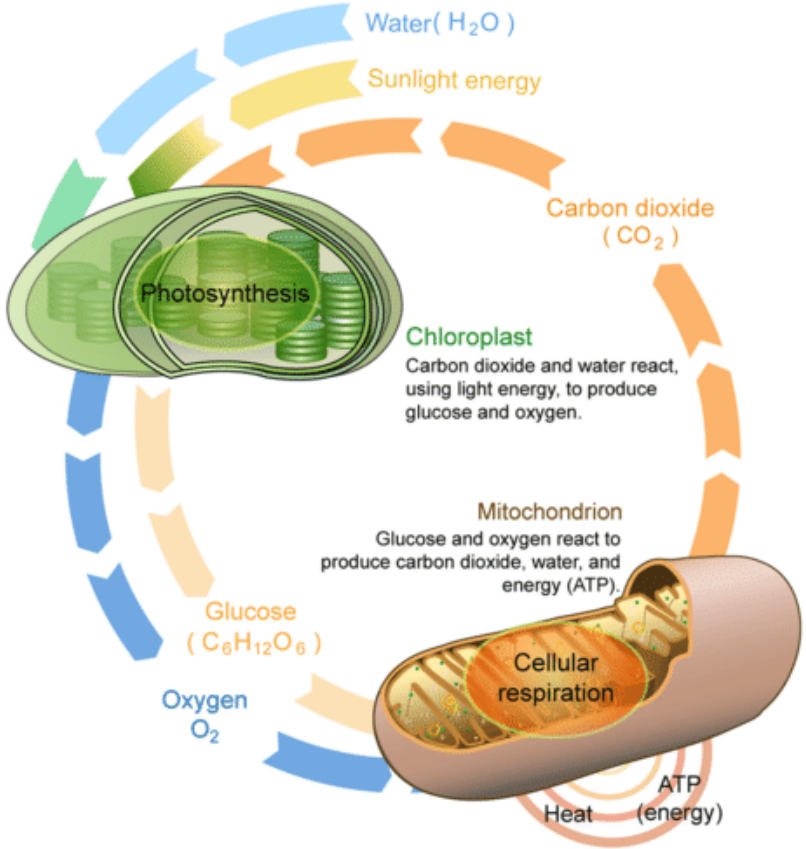
# Redox Biology is Based on Redox Chemsitry

**“Looking at life from the perspective of electron flow may be one of the most universal and fundamental approaches to Biology. This is because all known life forms depend on electrons that get stranded at the top of 'energy hills,' waiting to roll down the hill toward a low-energy resting place. This insight has been famously expressed in the words of Albert Szent-Gyorgyi: "Life is nothing but electrons looking for a place to rest." (Trefil et al., 2009).”**

Herrmann and Dick, Biol. Chem., 393, 2012

# Respiration and Photosynthesis: Classic Redox Chemistry




- **Cellular Respiration:** $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$
- **Photosynthesis:** $6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2$

(CK12.org)

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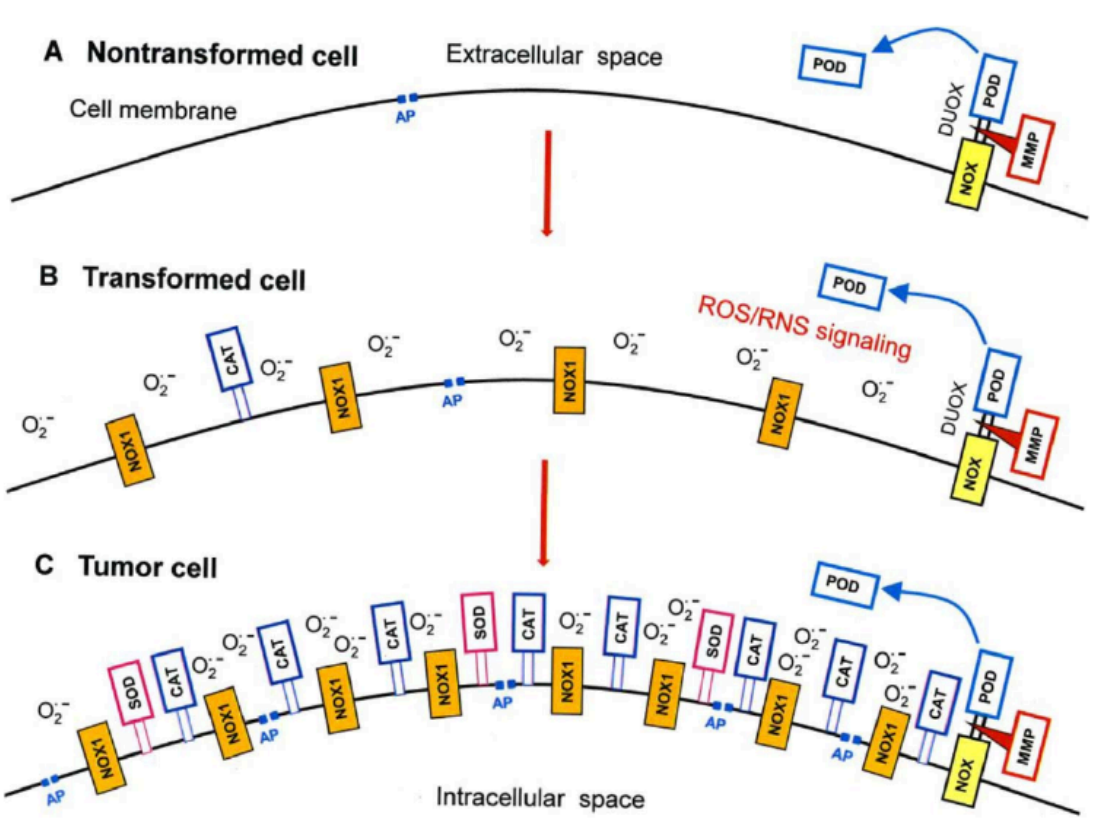
# Enzymes Associated with Cell Membranes




*Schematic picture of transition from nontransformed or 'normal' cell to transformed cell to 'bona fide' tumor cell.*

*Transformed cell: NOX1/$O_2^{\cdot-}$ and active RONS apoptotic signaling, including role of peroxidase (POD) enzyme, released by matrix metalloprotease (MMP).*

*Tumor cell: More NOX1/$O_2^{\cdot-}$, CAT and SOD. Inactive RONS apoptotic signaling.*

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Mechanisms of Ageing and Development 172 (2018) 59–77

Autoamplificatory singlet oxygen generation sensitizes tumor cells for intercellular apoptosis-inducing signaling

Georg Bauer[a,b,*]