

# Signal Transduction

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# SIGNAL TRANSDUCTION

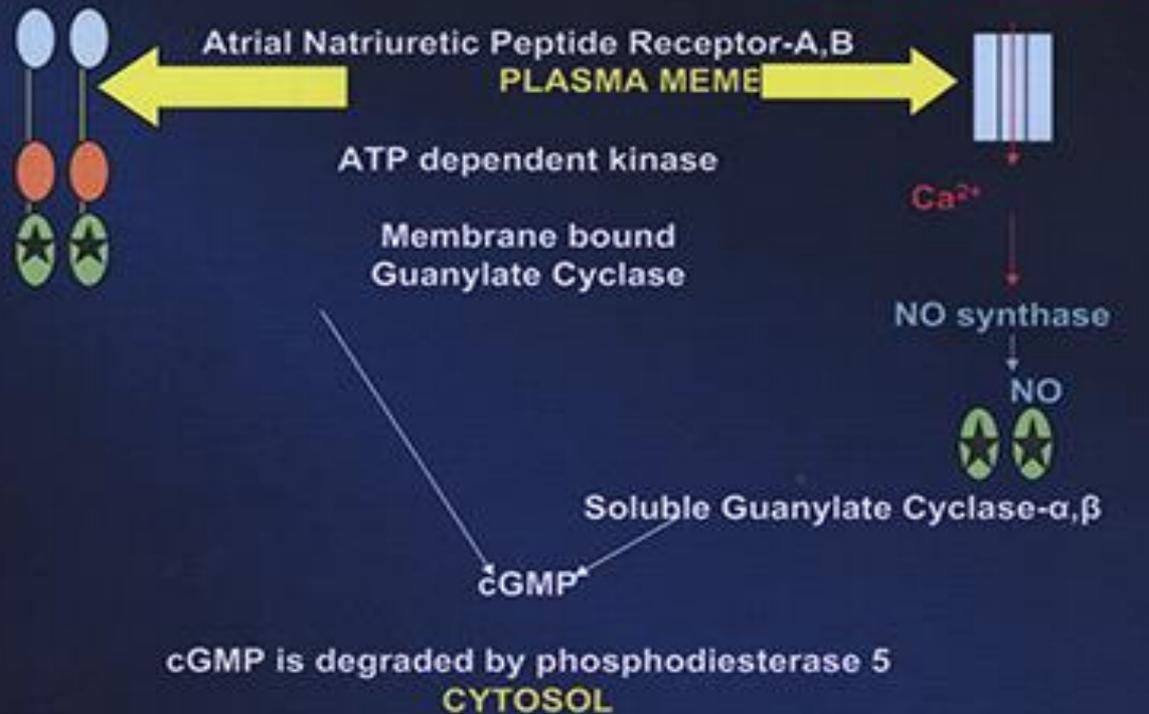
- **Low doses of RNS and ROS may stimulate proliferation of cancer cells.**
- **High doses of RNS and ROS may cause apoptosis of cancer cells.**

**Elevated cytosolic  $\text{Ca}^{2+}$  activates nitric oxide synthase (NOS) leading to cGMP.**

- **NO synthase uses arginine as a substrate to make the products NO and citrulline.**
- **Soluble guanylyl cyclase uses GTP as a substrate to make the product cGMP.**

# Cyclic GMP production

## CYCLIC GMP PRODUCTION



# Atrial Natriuretic Peptide Receptor

	<u>A</u>	<u>B</u>
# amino acids	1061	1047
Molecular weight	118,918	117,021
Signal sequence	1-32	1-22
Extracellular	33-473	23-458
Transmembrane	474-494	459-478
Kinase	528-805	513-786
Guanylate cyclase	876-1006	861-991

# Soluble guanylate cyclase

	<u><math>\alpha 2</math></u>	<u><math>\beta 1</math></u>
# amino acids	732	619
Molecular weight	81,749	70,514
Guanylate cyclase	521-648	421-554

**In soluble guanylate cyclase, the Fe is nitrosylated by NO. This increases enzymatic catalysis 400-fold**

- **Soluble guanylate cyclase has 3 domains, a heme binding domain, a dimerization domain and a catalytic domain**

# Elevated cGMP has 4 protein targets

- cGMP dependent protein kinase (PKGI) a 76 kDa serine/threonine kinase which ultimately leads to vasodilation
- PKGII which phosphorylates the cystic fibrosis transmembrane conductance regulator
- Cyclic nucleotide gated channel which translate visual signals to nerve impulses
- Phosphodiesterases (PDE). Viagra selectively inhibits PDE 5

# The NO delivery agent SPER/NO increases cGMP and ERK activation.

- SPER/NO increases cGMP 30 min. after addition to cells.
- Increased P44/P42 MAPK (ERK) tyrosine phosphorylation is observed after 30 min.
- *Thomas et al., PNAS 101:8894 (2004).*

# **Low doses of NO lead to:**

- **Increased cGMP**
- **Increased ERK tyrosine phosphorylation**
- **Increase proliferation**

# **High NO causes apoptosis of cancer cells.**

- **NO can induce stress proteins, disrupt mitochondria, release cytochrome c and activate caspases.**

## **SPER/NO causes phosphorylation of p53.**

- **The phosphorylated p53 results in less G1 to S transitions in the cell cycle, leading to increased apoptosis.**

## **NO and apoptosis.**

- **The NO donors S-nitroso-N-acetylpenicillamine (SNAP) and sodium nitroprusside (SNP) cause apoptosis of lung cancer cells.**

# Table I. NO inhibits lung cancer cellular proliferation.

Addition	% Proliferation	Nitrite, uM
None	100	3
SNAP, 0.4 mM	70	35
SNAP, 0.8 mM	60	55
SNP, 1 mM	80	35
SNP, 2 mM	55	45

SNAP and SNP were added to NCI-H1299 cells for 24 hr. *Chao et al., JBC, 20267 (2004).*

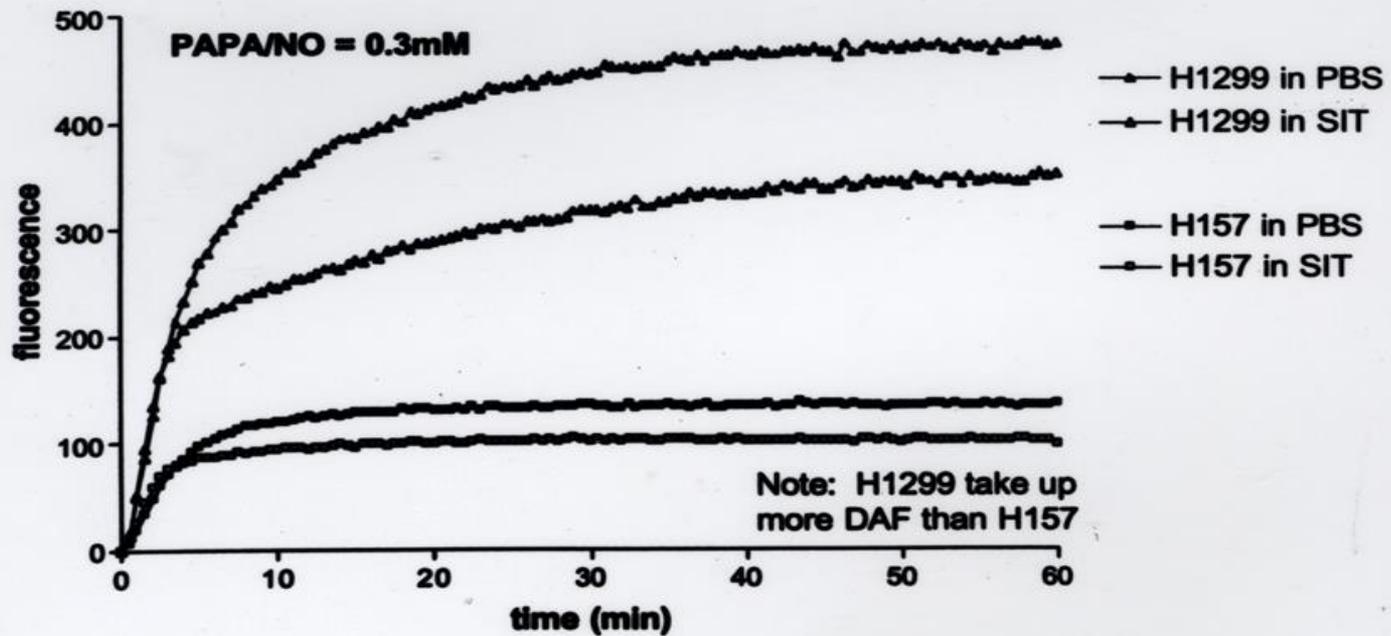
**NO delivery agents inhibit lung cancer cellular proliferation using the MTT assay.**

**Addition Absorbance at 540 nm**

<b>None</b>	<b>.332 <math>\pm</math> .057</b>
<b>DEA/NO</b>	<b>.201 <math>\pm</math> .021*</b>
<b>PAPA/NO</b>	<b>.193 <math>\pm</math> .025*</b>

**The mean absorbance  $\pm$  S.D. of 8 determinations is indicated using NCI-H1299 cells;  $p < 0.05$ , \*.**

# DAF reactive chemicals form in cells within minutes after the addition of PAPA/NO



# **PAPA/NO inhibits lung cancer colony formation**

# Macrophages inhibit colony formation.

Addition	Colony number
None	929 $\pm$ 72
Macrophages, 0.5 M	756 $\pm$ 98
Macrophages, 1 M	586 $\pm$ 117
Macrophages, 2 M	474 $\pm$ 58*
Macrophages, 5 M	456 $\pm$ 37*

The mean number  $\pm$  S.D. of 3 determinations is indicated;  $p < 0.05$ , \*.

# **SNP causes phosphorylation of p38 MAPK.**

- **P38 MAPK is a mediator of NO induced caspase-3 associated apoptosis.**
- **The p38 MAPK inhibitor SB202190 protects cells from NO-mediated cell death.**

# **SNP and SNAP decrease survivin and Bcl-2 levels.**

- **Survivin is critical for cell cycle progression.**
- **Bcl-2 is critical for cellular survival.**

# High NO leads to:

Decreased BCL-2 and increased p38-MAPK

Cytochrome c plus ATP plus Apaf1

Increased caspase-9 and decreased IAP

Increased caspase-3 activity

Cell death and apoptosis

# **The MAPK/AP-1 and NF- $\kappa$ B pathways are activated by oxidants**

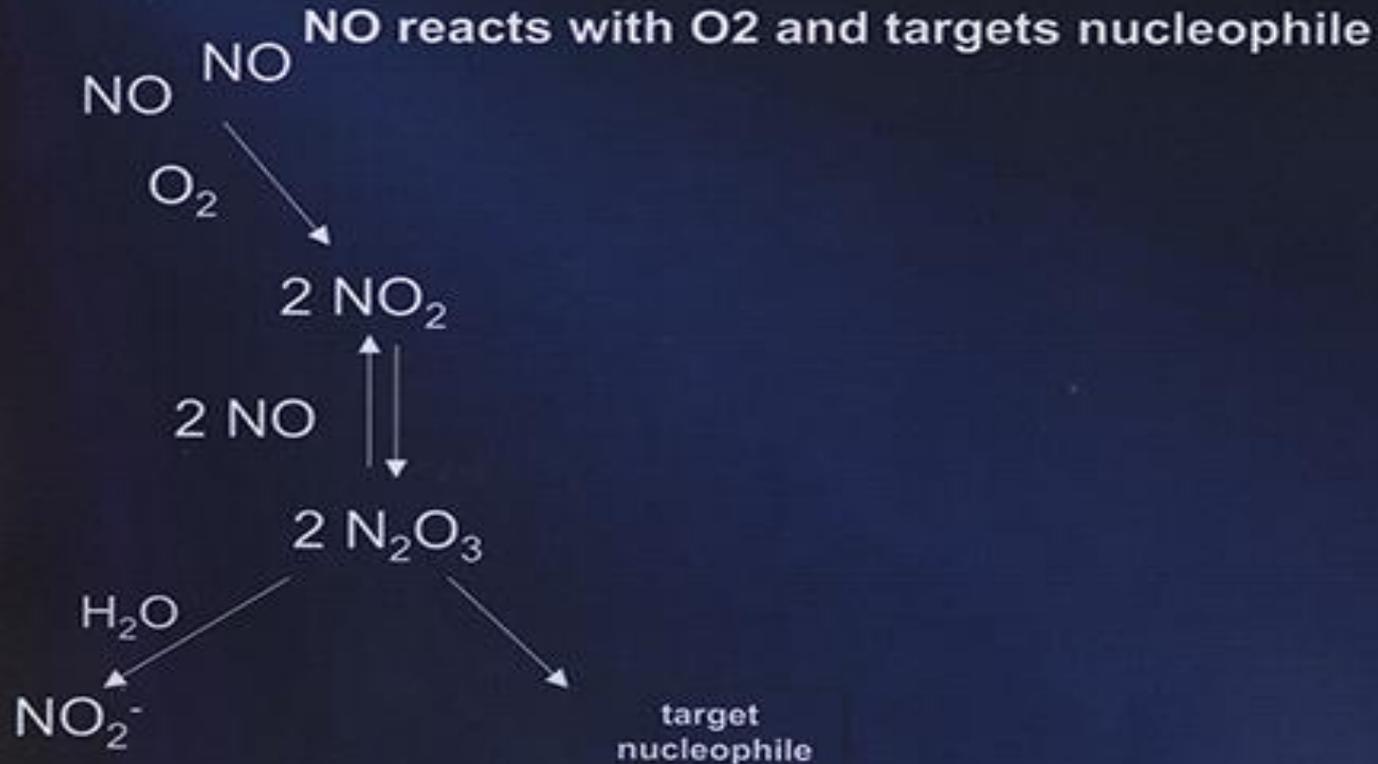
- **Activator Protein-1 leads to proliferation or apoptosis**
- **Nuclear Factors lead to inflammation and survival**

# **Growth factors (ROS/RNS) and Cellular Stress (ROS) lead to:**

- **Raf, MEK1/2, ERK 1/2 activation and proliferation or**
- **MEKK, MEK3, p38MAPK activation and**
- **MEKK, MEK4, JNK 1/2 activation leading to stress responses**

# NO oxidation

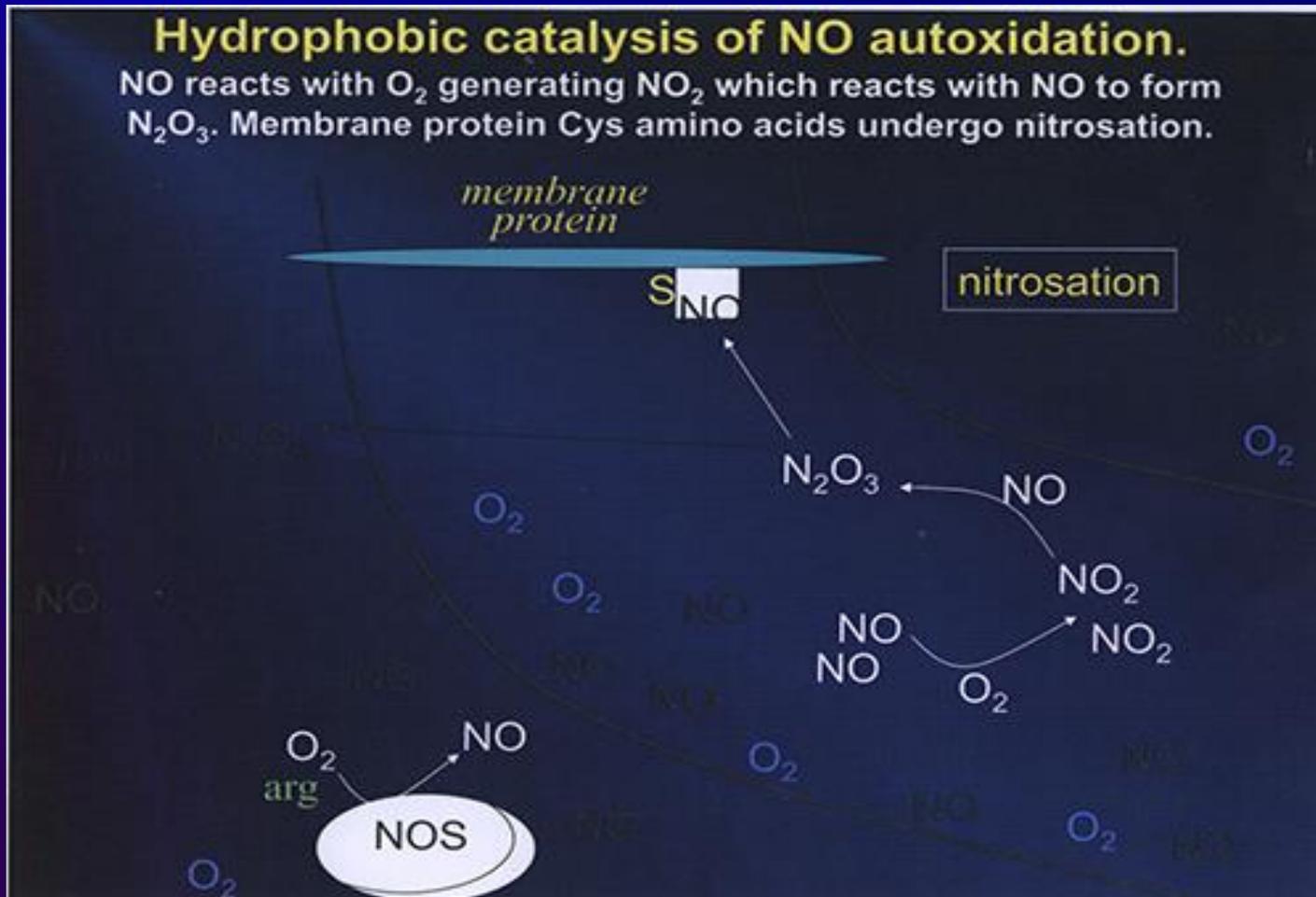
**NO autoxidation results in protein nitrosylation.**



# Protein nitrosation

## Hydrophobic catalysis of NO autoxidation.

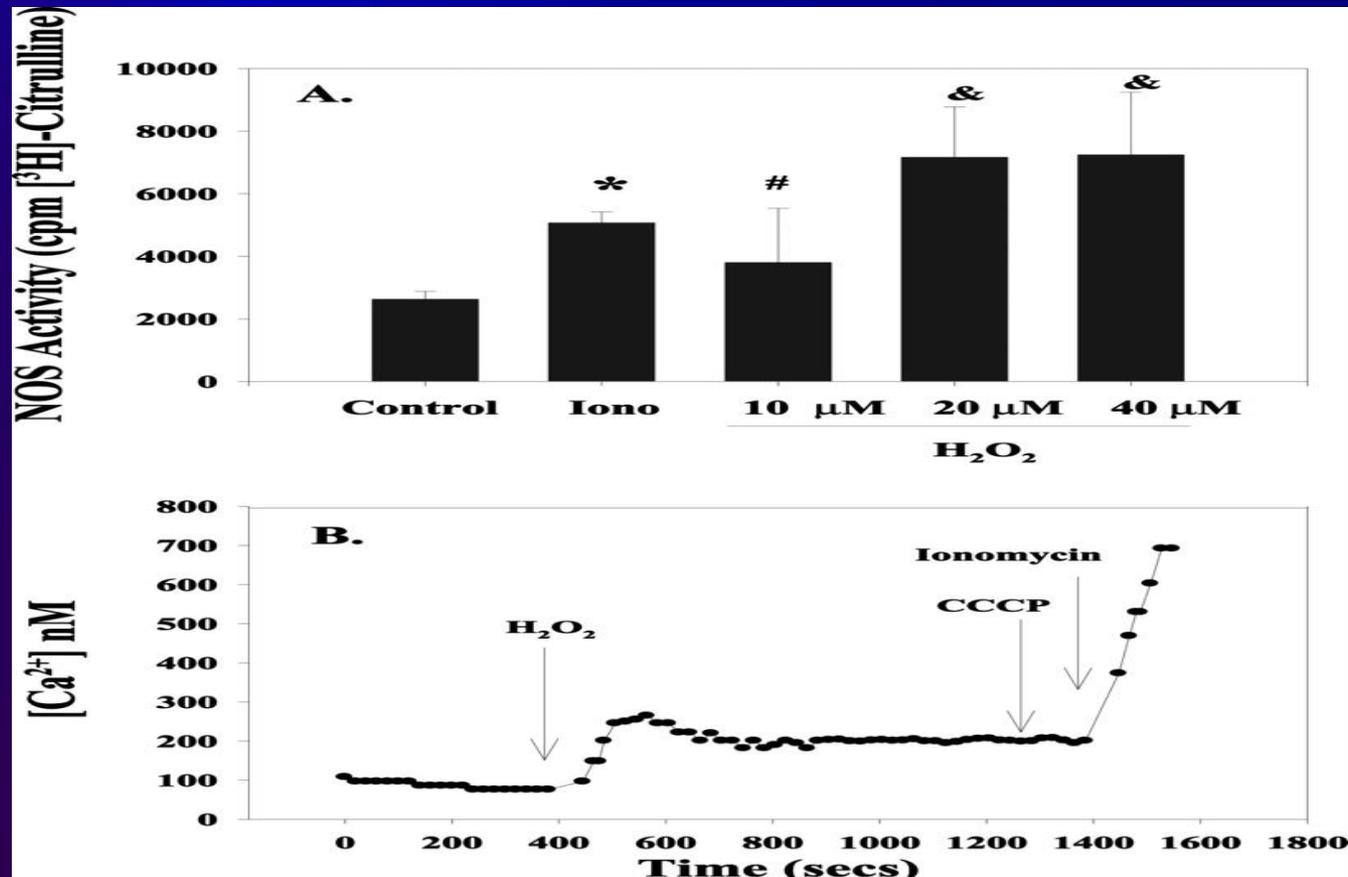
NO reacts with  $O_2$  generating  $NO_2$  which reacts with NO to form  $N_2O_3$ . Membrane protein Cys amino acids undergo nitrosation.



# Numerous cellular proteins are nitrosylated

- Ras, the p21 monomeric GTPase is nitrosylated at Cys 118 resulting in activation of MAPK and PI-3-K.
- Denitrosylation of caspase-3 is essential for apoptosis

# Low concentration of H<sub>2</sub>O<sub>2</sub> increase transiently cytosolic free Ca<sup>2+</sup> and NOS activity



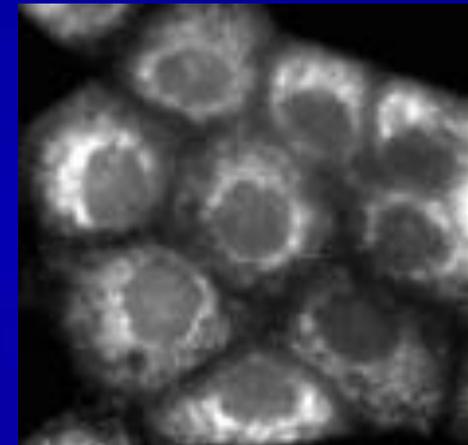
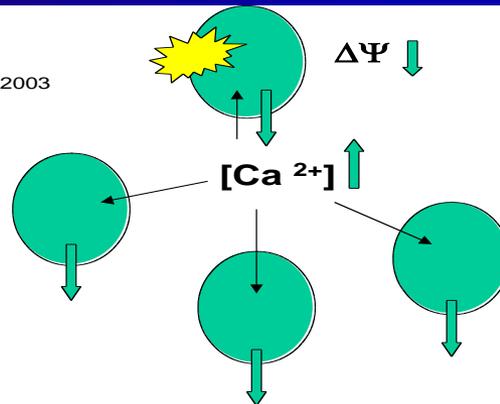
# How do cells sense and transduce a cytoplasmic oxidative event.

NO synthase activation leads to NO which forms metal nitrosyl complexes in cytochrome C oxidase and guanylate cyclase; Cys nitrosylation in PTP, caspase, Zn proteins and ATM; and RNS causing tyrosine nitration in NF- $\kappa$ B, Bcl, pte and Keap1/Nrf2w

## Propagating MPT:

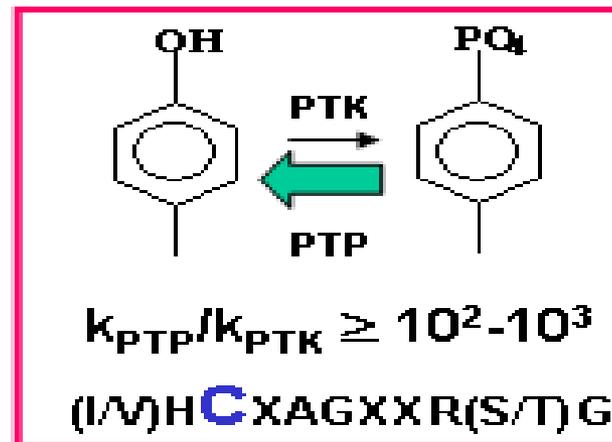
Leach *et al*, 2001, 2002; Mikkelsen and Wardman, 2003

- Localized increase in  $[Ca^{2+}]$ ; uptake by adjacent mitochondria
- Depolarization and release of  $Ca^{2+}$
- Transient and reversible: Self propagating  $\Delta\Psi$
- Mitochondria and ER are structurally and functionally linked



# Protein Tyrosine Phosphatases can be oxidized.

Protein-Cys, S-Nitrosylation, Tyr Phosphorylation and Cellular Redox Homeostasis in **Autocrine** Regulated Tumor Cells



Work by Tonks, Rhee on the oxidation of PTP Cys active site and Tyr kinase activity:



# Effects of PGE2 on cancer cells

- **NO activates COX2 increasing PGE2.**
- **PGE2 binds to EP2R increasing VEGF**
- **EP2R transactivates EGFR**

# **Lung cancer cells produce LTs and PGs**

- **Phospholipids are metabolized by PLA2 to arachidonic acid.**
- **Arachidonic acid is metabolized by LOX to leukotrienes and by Cox to prostablandins.**

**Arachidonic acid is slowly  
metabolized by the rate  
limiting enzyme COX**

# Two subtypes of COX are present, COX-1 and COX-2

● COX-1 is a constitutive house keeping enzyme expressed in the normal kidney, platelets and GI tract. COX-1 is inhibited by non-steroidal antiinflammatory drugs (NSAIDs).

● COX-2 is induced in inflammation and neoplasia by EGF, TGF $\beta$ , TNF $\alpha$ , hypoxia and uv B light. COX-2 is inhibited by NSAIDs and celecoxib.

# Cyclooxygenase (COX)

- **COX 2 has 604 amino acids, has a molecular weight of 68996 daltons, has a distal His at 193, has an iron binding site at 374 and aspirin acetylates Ser516.**
- **COX 1 has 599 amino acids, a molecular weight of 68,856 daltons, has a distal His at 206, has an Fe binding site at 387 and aspirin acetylates Ser 529.**

# **The A/J mouse represents an animal model for lung carcinogenesis**

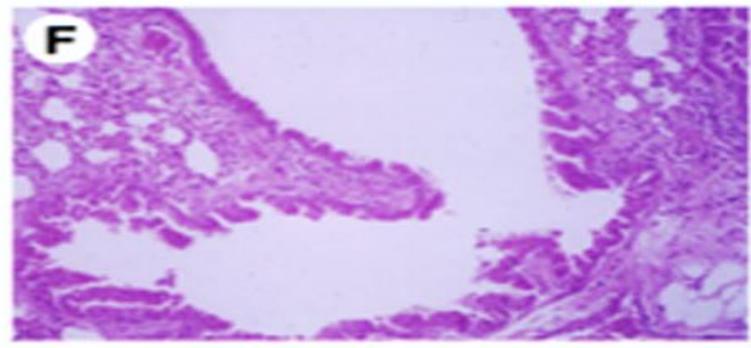
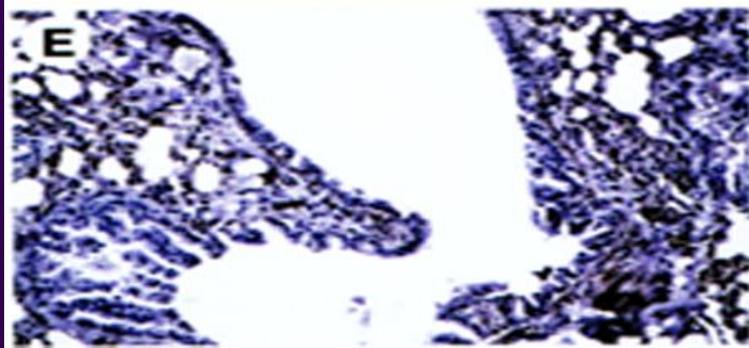
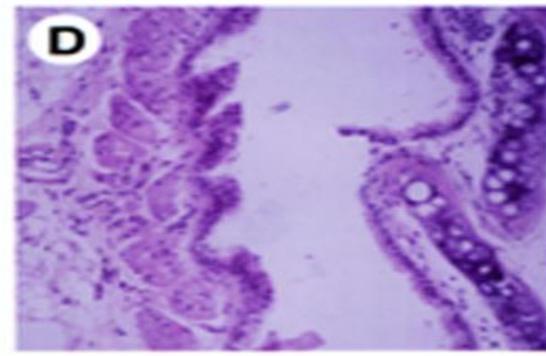
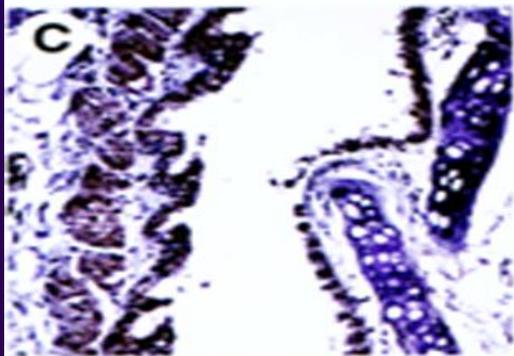
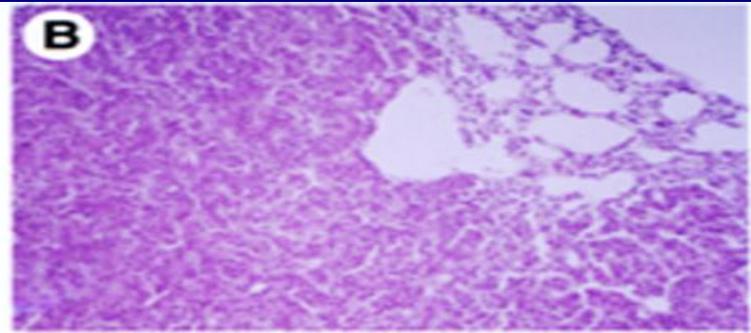
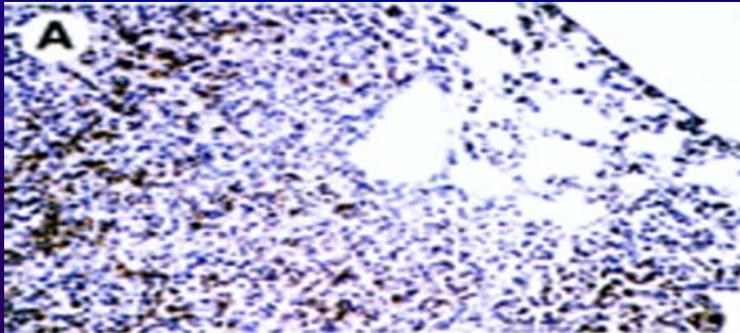
- **COX-2 is present in all lung compartments including the alveoli, bronchi and bronchioles**

# Lung adenomas

Lung adenomas develop 4 months after injections of carcinogen.



# COX-2 immunostaining in the A/J mouse lung.



**Celecoxib (CELEBREX) is approved by the FDA for arthritis and treatment of colorectal polyps in FAP patients.**

**Oral celecoxib inhibited corneal angiogenesis and PGE<sub>2</sub> levels by 79%**

**Oral celecoxib reduced endothelial cell proliferation by 2.5-fold and increased apoptosis 2.7 fold**

**In lung cancer patients treated with celecoxib paclitaxel and carboplatin, serum VEGF declined.**

# S-NSAIDs and a COX-2 inhibitor reduce PGE<sub>2</sub> in NSCLC cells.

Table I. S-NSAIDs and PGE<sub>2</sub>.

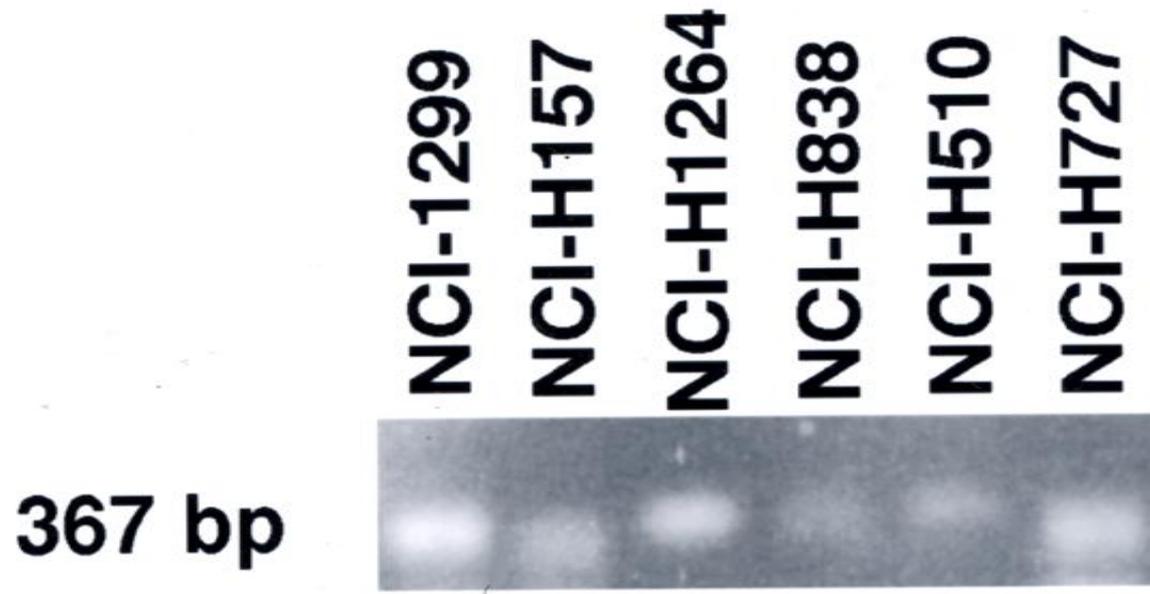
Addition	Relative PGE <sub>2</sub> , %	
	A549	H1299
None	100 ± 13	100 ± 8
NO-Asa, 1 ug/ml	18 ± 3**	23 ± 4**
S-Valproate, 1 ug/ml	26 ± 8**	33 ± 8**
S-Diclofenac, 1 ug/ml	18 ± 6**	25 ± 4**
S-Sulindac, 1 ug/ml	64 ± 9*	75 ± 10
DuP-697, 1 ug/ml	30 ± 7**	21 ± 4**

The mean value ± S.D. of 4 determinations is indicated; (p < 0.05, \*; p < 0.01, \*\* using student's t-test).

# EP2 receptor

- The EP2R has 358 amino acids, has a molecular weight of 39,760, has 7 transmembrane domains, has a 23 amino acid N-terminal extracellular domain, has a 34 amino acid C-terminal intracellular domain and is N-glycosylated at Asn 3,6,96 and 287.

PGE2 binds to the EP2R in lung cancer cells.



# **PGE<sub>1</sub>, PGE<sub>2</sub>, PGF<sub>2α</sub> and AH6809 bind with high affinity.**

<b>Compound</b>	<b>IC<sub>50</sub>, μM</b>
<b>Arachidonic acid</b>	<b>&gt;10</b>
<b>AH6809</b>	<b>5 ± 0.7</b>
<b>PGD<sub>2</sub></b>	<b>&gt;10</b>
<b>PGE<sub>1</sub></b>	<b>0.2 ± .03</b>
<b>PGE<sub>2</sub></b>	<b>0.04 ± .01</b>
<b>PGF<sub>2α</sub></b>	<b>2 ± 0.2</b>
<b>PGG<sub>2</sub></b>	<b>&gt;10</b>
<b>PGI<sub>2</sub></b>	<b>&gt;10</b>

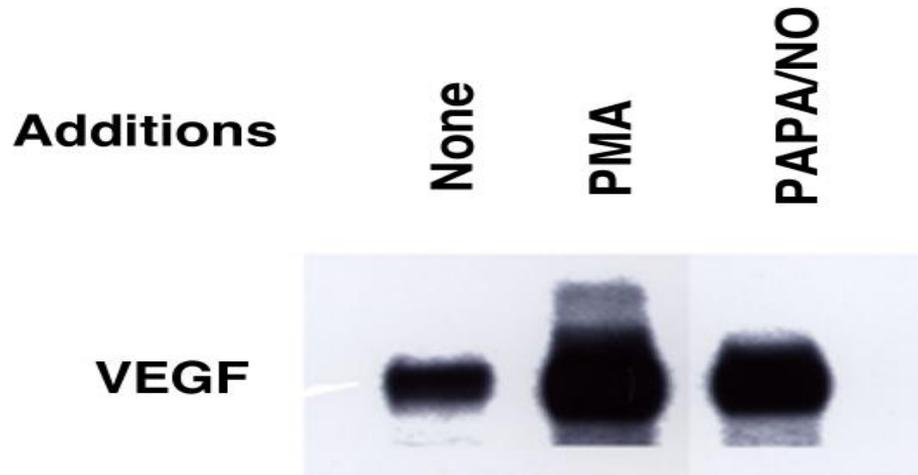
*Casibang, M. et al., Lung Cancer 2001; 31: 203*

## **The EP2R activates adenylyl cyclase**

- **PGE2 is an agonist which increases cAMP in lung cancer cells.**
- **AH6809 is an antagonists which reversibly blocks the receptor.**

**EP<sub>2</sub> receptor antagonists block the increase in cAMP caused by PGE<sub>2</sub>.**

# NO causes increased VEGF mRNA



# VEGF mRNA is increased by PGE2 in a PKA-dependent manner

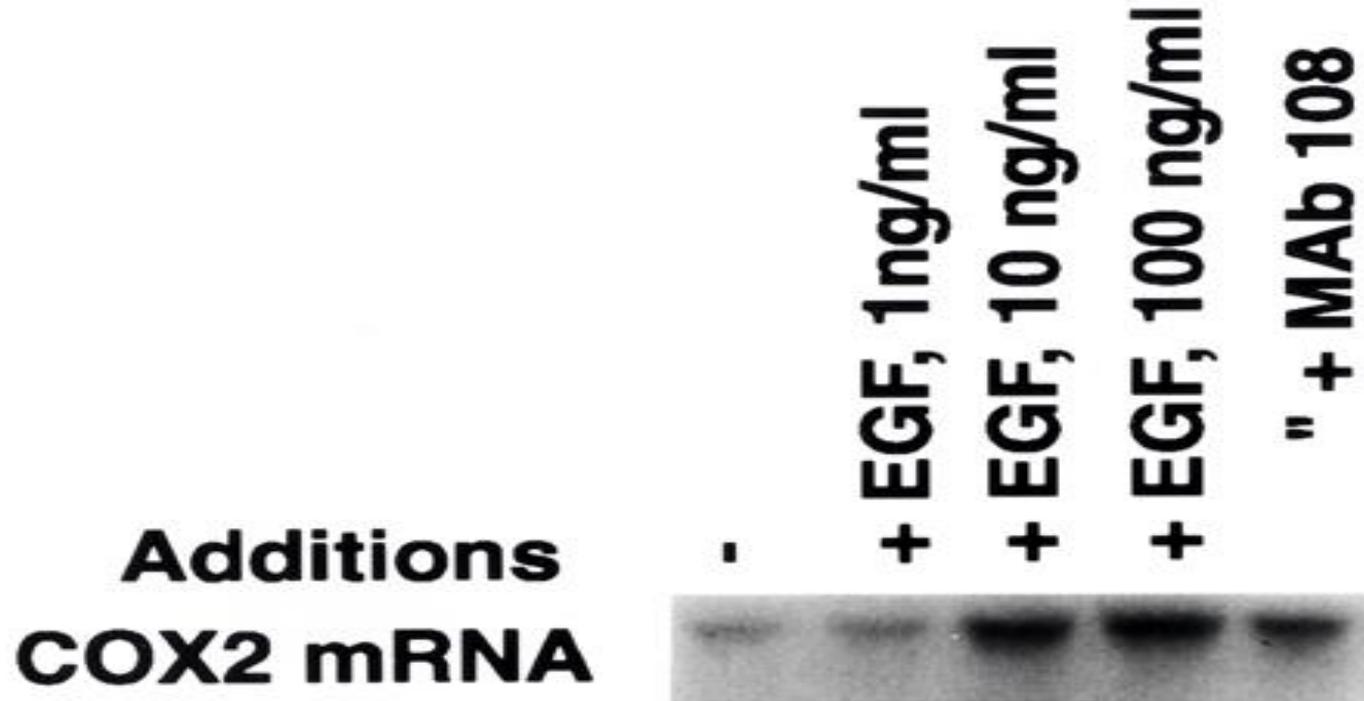
- Addition                      Relative VEGF mRNA
- None                       $100 \pm 5$
- PGE2                       $200 \pm 17^*$
- EGF                       $185 \pm 16^*$
- H89                       $104 \pm 3$
- PGE2 + H89               $110 \pm 6$
- The mean value  $\pm$  S.D. of 4 determinations is indicated;  $p < 0.05$ , \*.

# **COX-2 and VEGF expression are intimately linked.**

**In Apc/COX-2 double knockout mice, VEGF protein is reduced by 94%.**

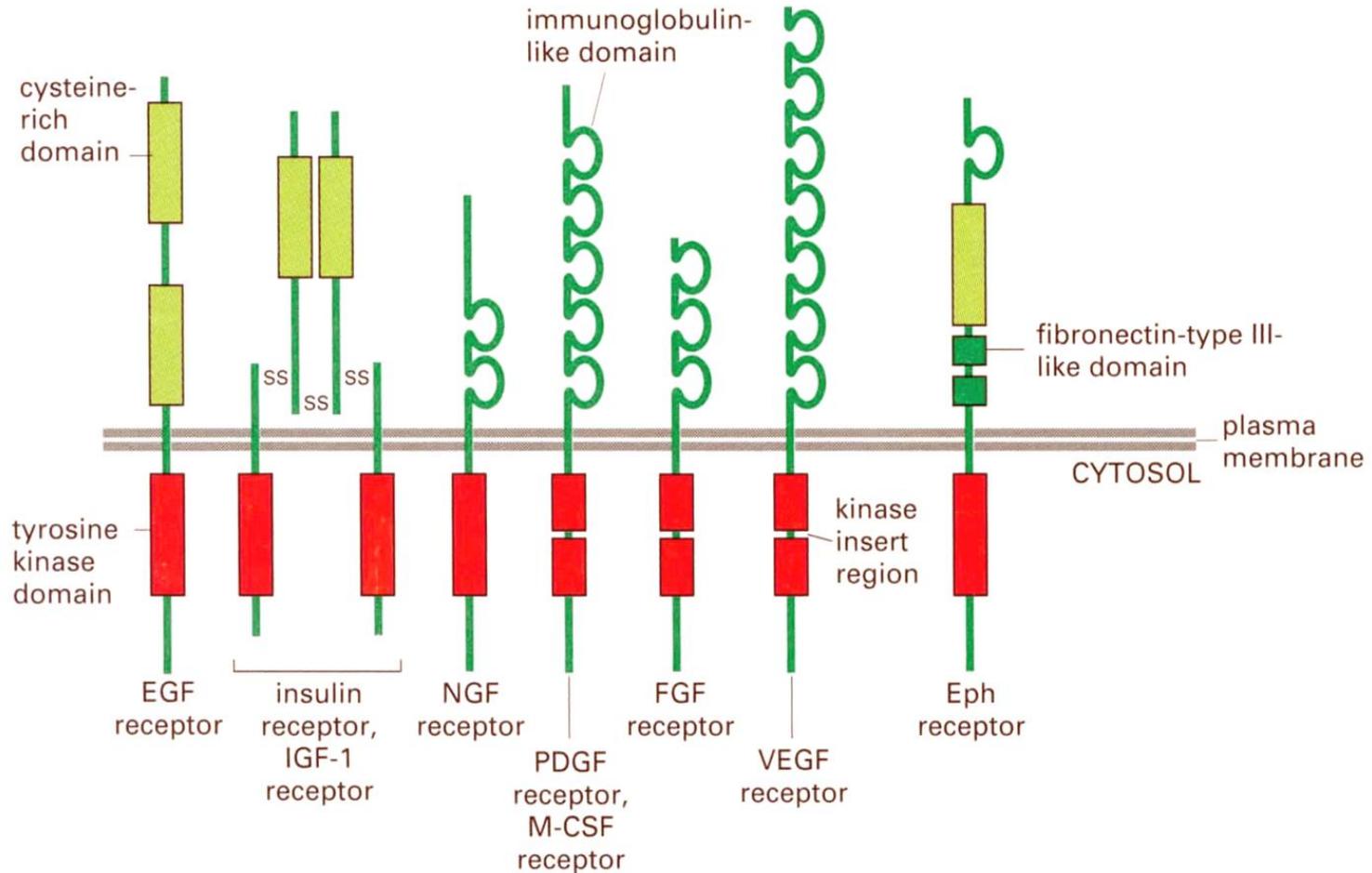
**In NSCLC patients, COX-2 mRNA expression correlates with VEGF mRNA, increased microvessel density, decreased patient survival and early relapse.**

# EGF causes increased COX-2 expression in NSCLC cells.



# Tyrosine kinase receptors

*Molecular Biology of the Cell, Alberts et al., 2001.*

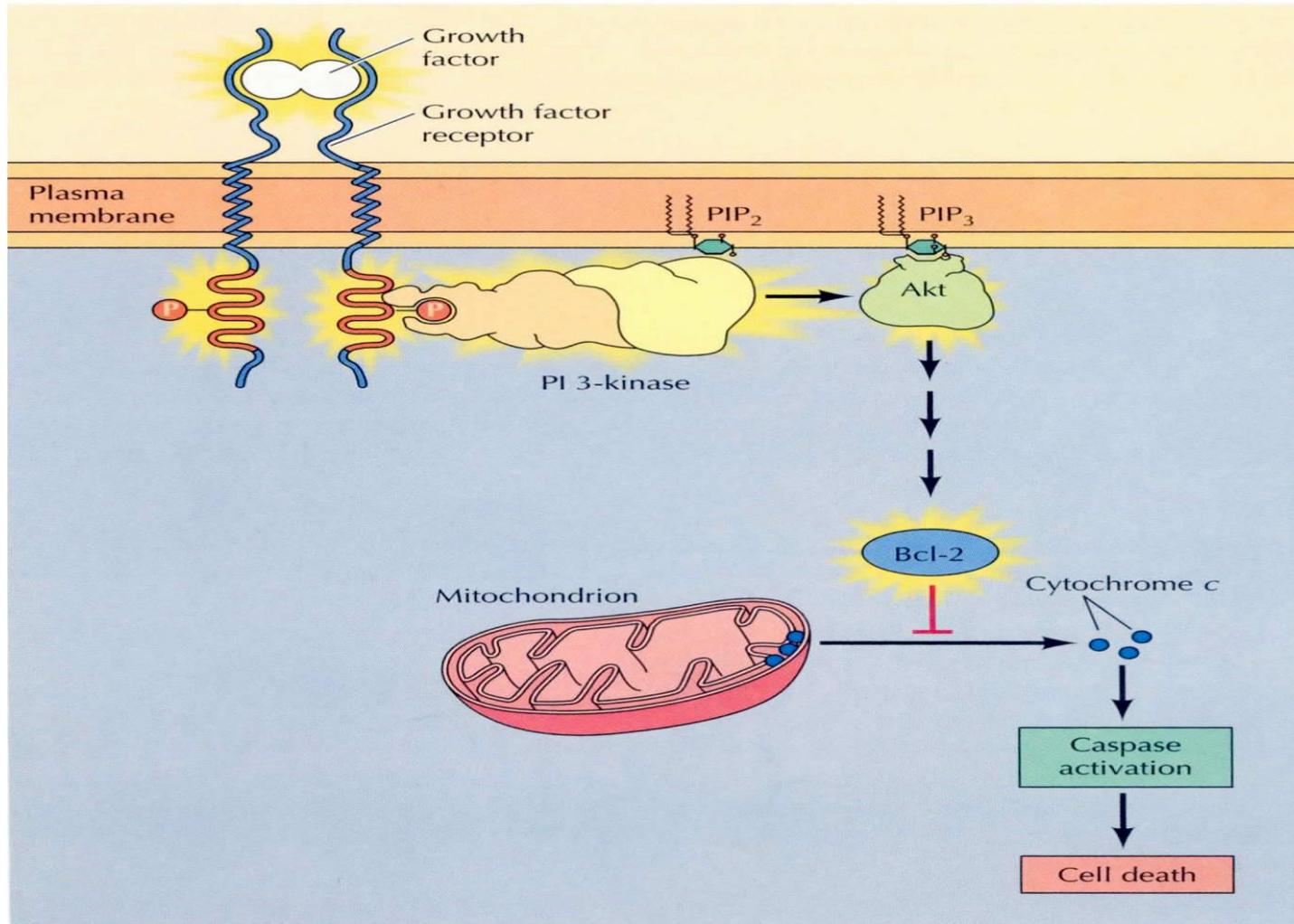


# **The EGFR is an 1186 amino acid integral membrane protein.**

- **The 621 amino acid extracellular domain binds EGF with high affinity.**
- **The 23 amino acid transmembrane domain anchors the receptor into the membrane and transduces signals.**
- **The 542 amino acid intracellular domain contains tyrosine kinase activity.**
- **Lys<sup>721</sup> binds ATP and Tyr<sup>1068</sup>, Tyr<sup>1086</sup>, Tyr<sup>1148</sup> and Tyr<sup>1173</sup> are subsequently phosphorylated.**

# Tyrosine kinase receptors cause increased cell survival.

*Molecular biology of the cell; Alberts et al. 2001*

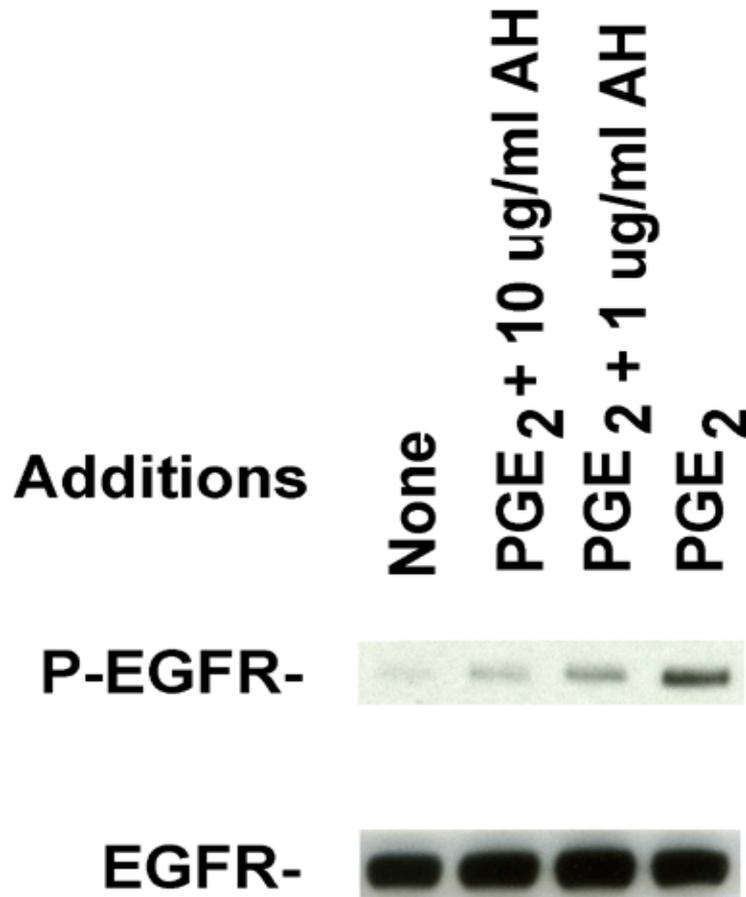


# **EGFR activation results in H<sub>2</sub>O<sub>2</sub> production which inactivates PTEN**

- Addition of EGF to cells, causes production of phosphatidylinositol 3,4,5-trisphosphate (PIP3) by activation of PI-3-kinase.
- PIP3 production results in AKT activation
- In cells overexpressing NADPH oxidase I, EGF or PDGF causes H<sub>2</sub>O<sub>2</sub> production
- H<sub>2</sub>O<sub>2</sub> causes reversible oxidation of PTEN resulting in formation of a Cys<sup>71</sup>-Cys<sup>141</sup> disulfide
- The disulfide is reversed by addition of thioredoxin

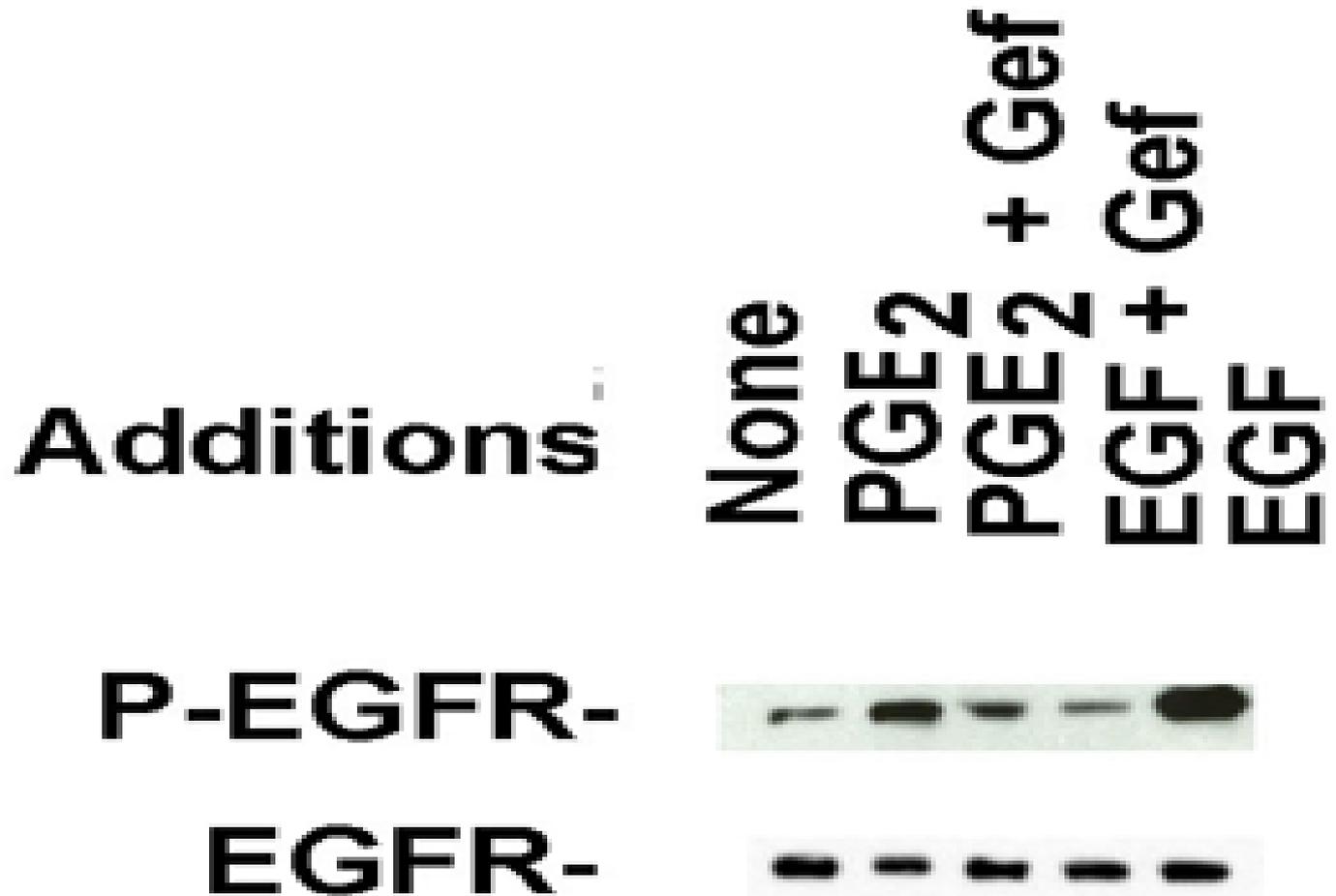
# AH6809 blocks EGFR transactivation caused by PGE<sub>2</sub>.

EPR transactivation of EGFR  
is inhibited by AH6809



IB Ab: anti-PY  
IP Ab: anti-EGFR  
PY1068

# Gefitinib is an EGFR tyrosine kinase inhibitor.



# Some NSCLC patients, who have failed chemotherapy, respond to tyrosine kinase inhibitors

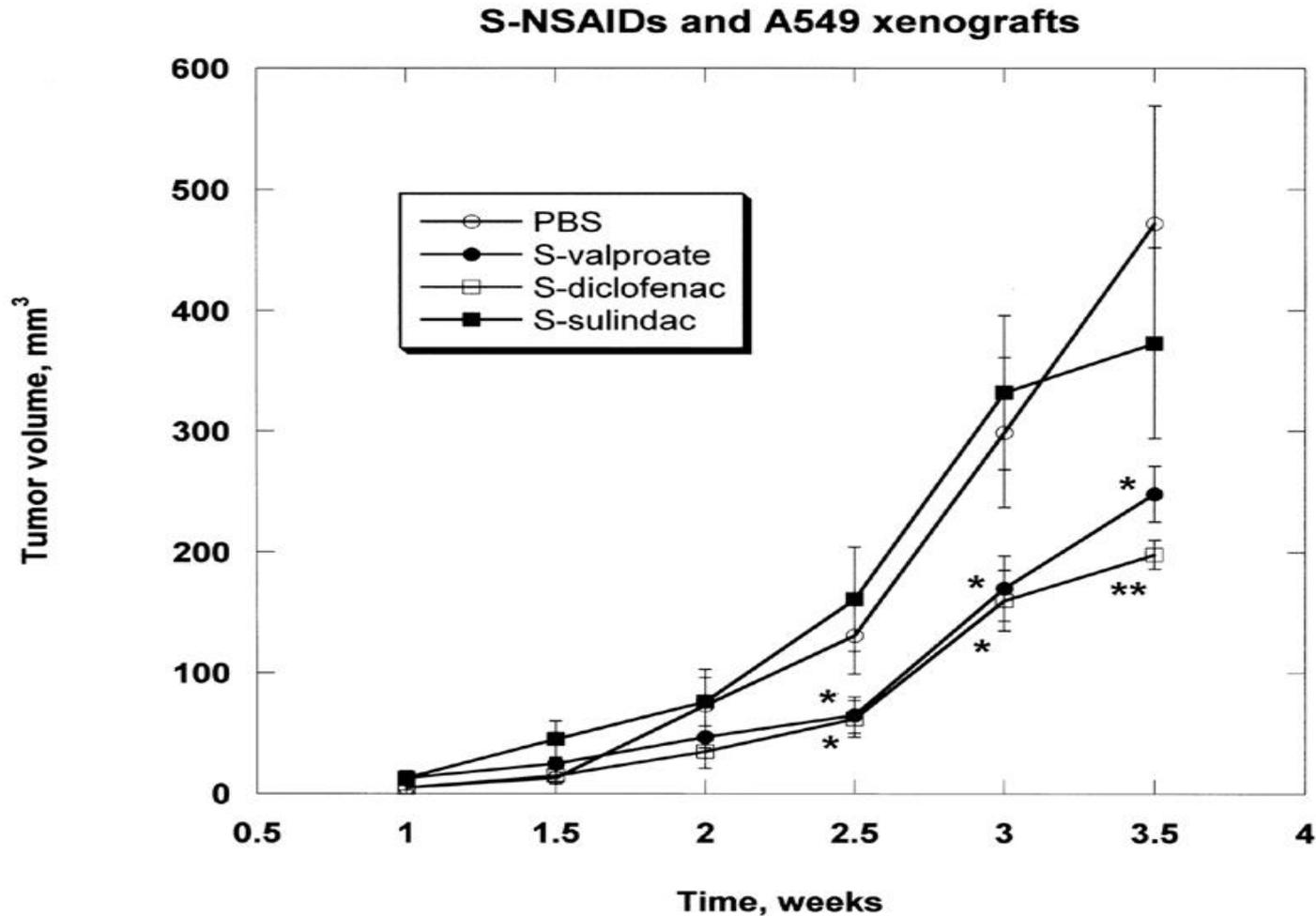
- In the IDEAL-1 and IDEAL-2 clinical trials, 250 mg of gefitinib caused an objective response in approximately 50% of the patients.
- Tumor responsiveness was not associated with EGFR expression but rather EGFR genetic mutations.
- EGFR mutations occurred in exons 18 through 21 of the tyrosine kinase domain, such as G719S or L858R.

# Signal Transduction

- **PGE2 binds to the EP2R causing Src activation and MMP activation. TGFa is released which binds to the EGFR.**
- **The EGFR increases COX2 expression leading to increased cAMP, PKA activation, CREB phosphorylation and increased VEGF expression.**

# S-Valproate or S-Diclofenac but not S-Sulindac reduce A549 xenograft growth in nude mice

Fig. 2



# S-valproate but not S-sulindac reduces NCI-H1299 xenograft growth *in vivo*

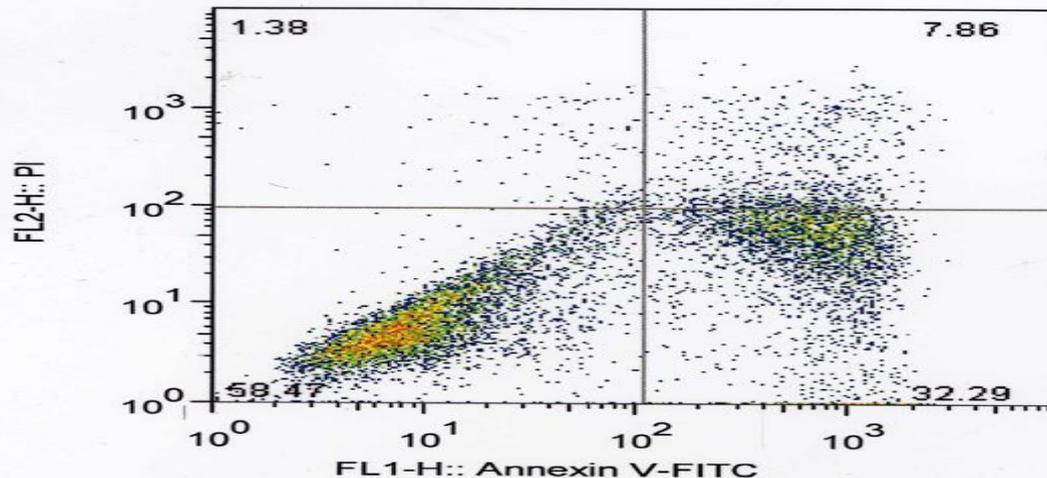
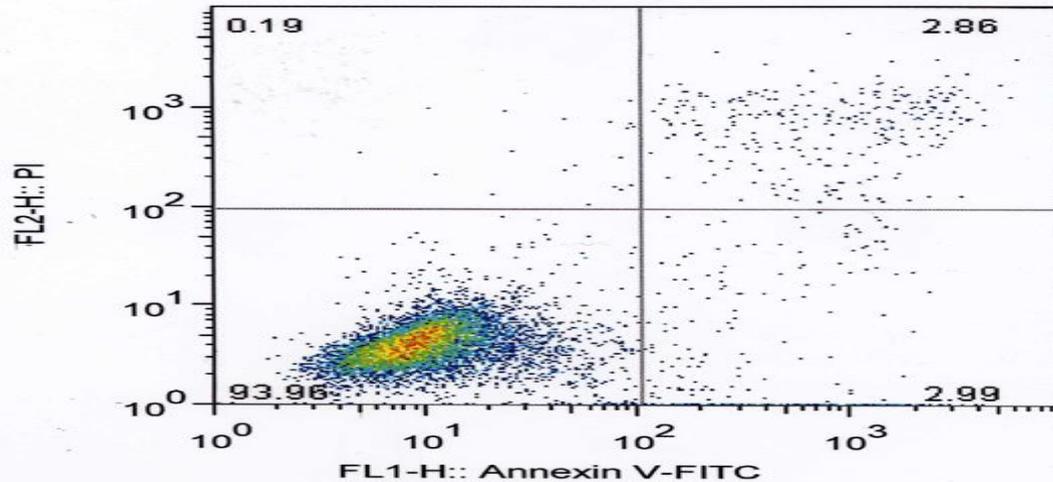
Table I. S-Valproate significantly inhibits NCI-H1299 lung cancer xenograft proliferation.

Addition	Tumor volume, mm <sup>3</sup>	Weight, g
None	1941 $\pm$ 322	24.7 $\pm$ 0.7
S-Valproate, 3.6 mg/kg	1962 $\pm$ 510	24.1 $\pm$ 1.1
S-Valproate, 18 mg/kg	398 $\pm$ 64**	24.7 $\pm$ 0.7
PEG400	2134 $\pm$ 399	23.9 $\pm$ 0.4
S-Sulindac, 18 mg/kg	1154 $\pm$ 294	23.9 $\pm$ 1.0

S-Valproate was injected twice weekly i.p. in 100 ul of PEG400. The mean value  $\pm$  S.E. of 8 determinations is indicated;  $p < 0.01$  using Student's t-test, \*\*.

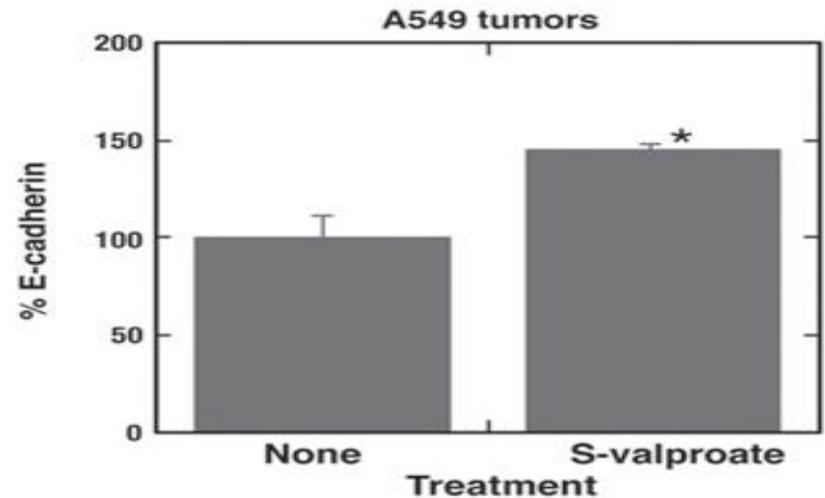
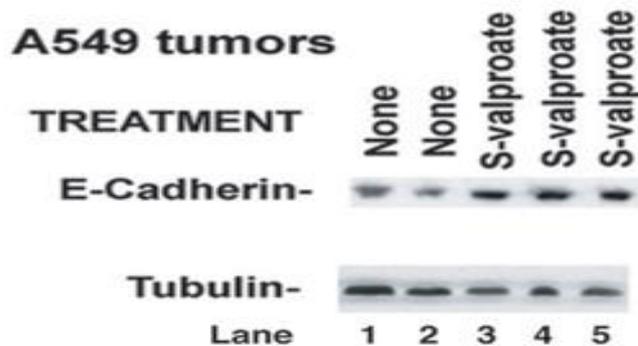
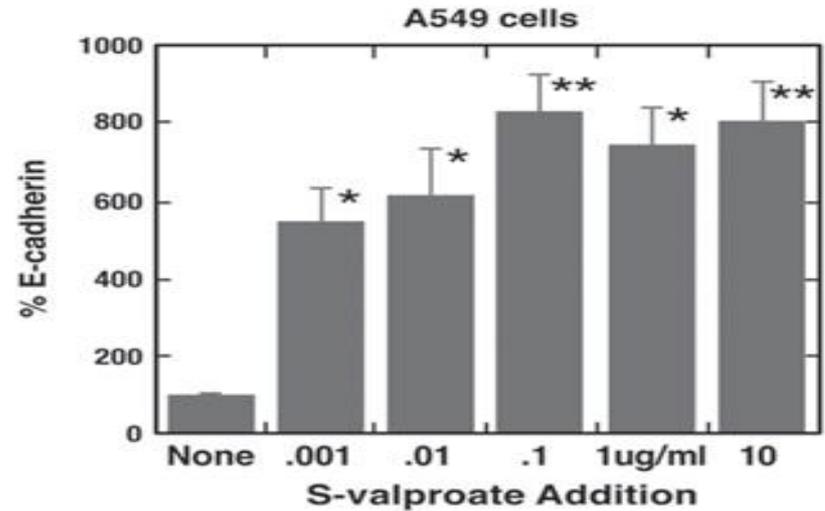
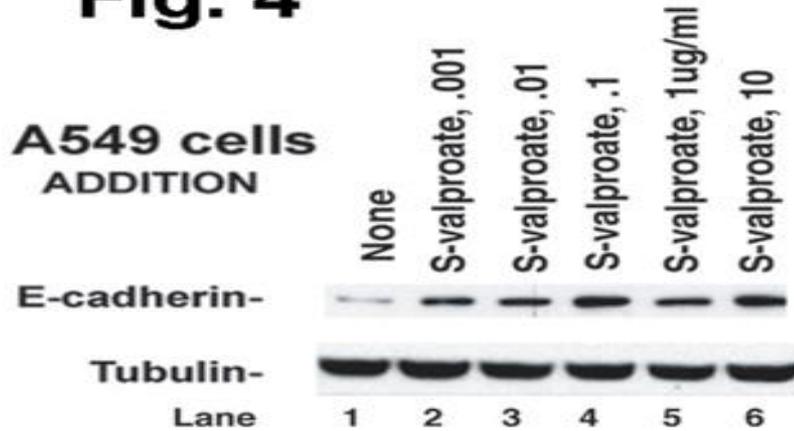
Moody, T.W. et al., *Lung Cancer* 2010:68:154

# NO-Asa but not S-valproate causes apoptosis of lung cancer cells



# S-Valproate increases E-cadherin in NSCLC cells and tumors.

**Fig. 4**

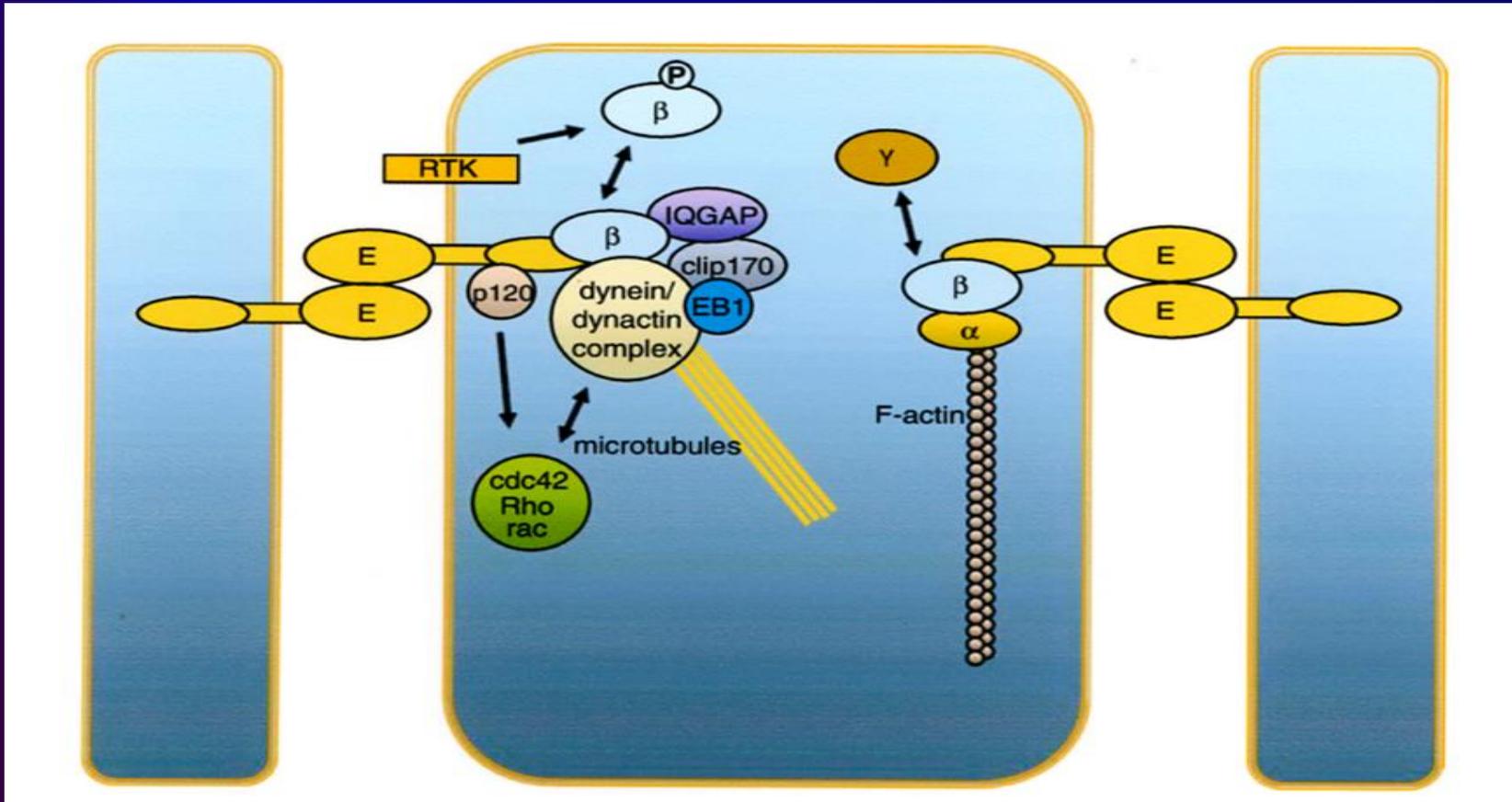


# E-Cadherin suppresses cancer cell metastasis

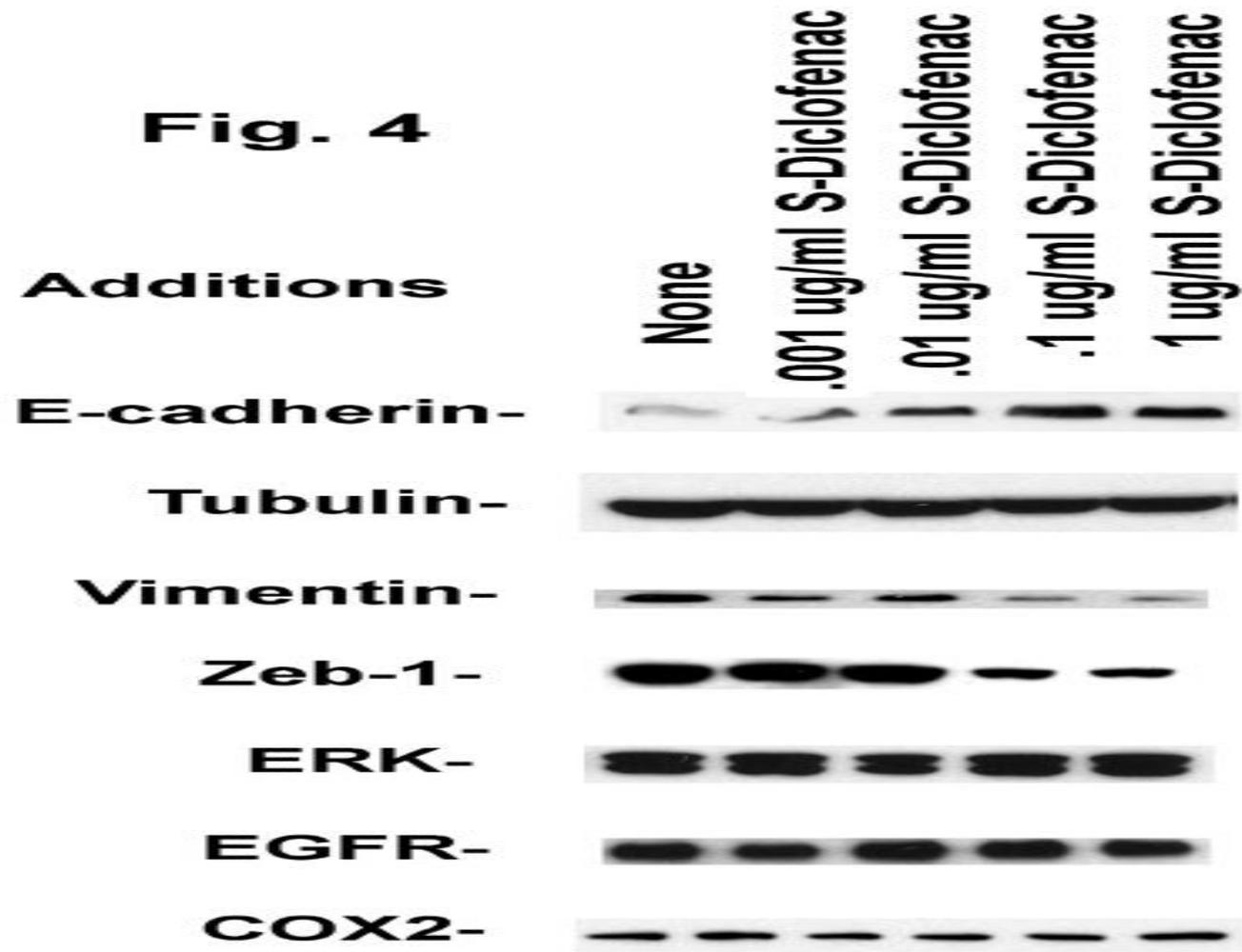
- Cadherins are cell surface transmembrane glycoproteins that play a major role in epithelial cell adhesion and connect the extracellular environment to the contractile cytoskeleton
- Loss of E-cadherin changes the cancer cell phenotype from epithelial to mesenchymal

*Brack, M.E. et al., Curr Top Microbiol Immunol 1996;213:123.*

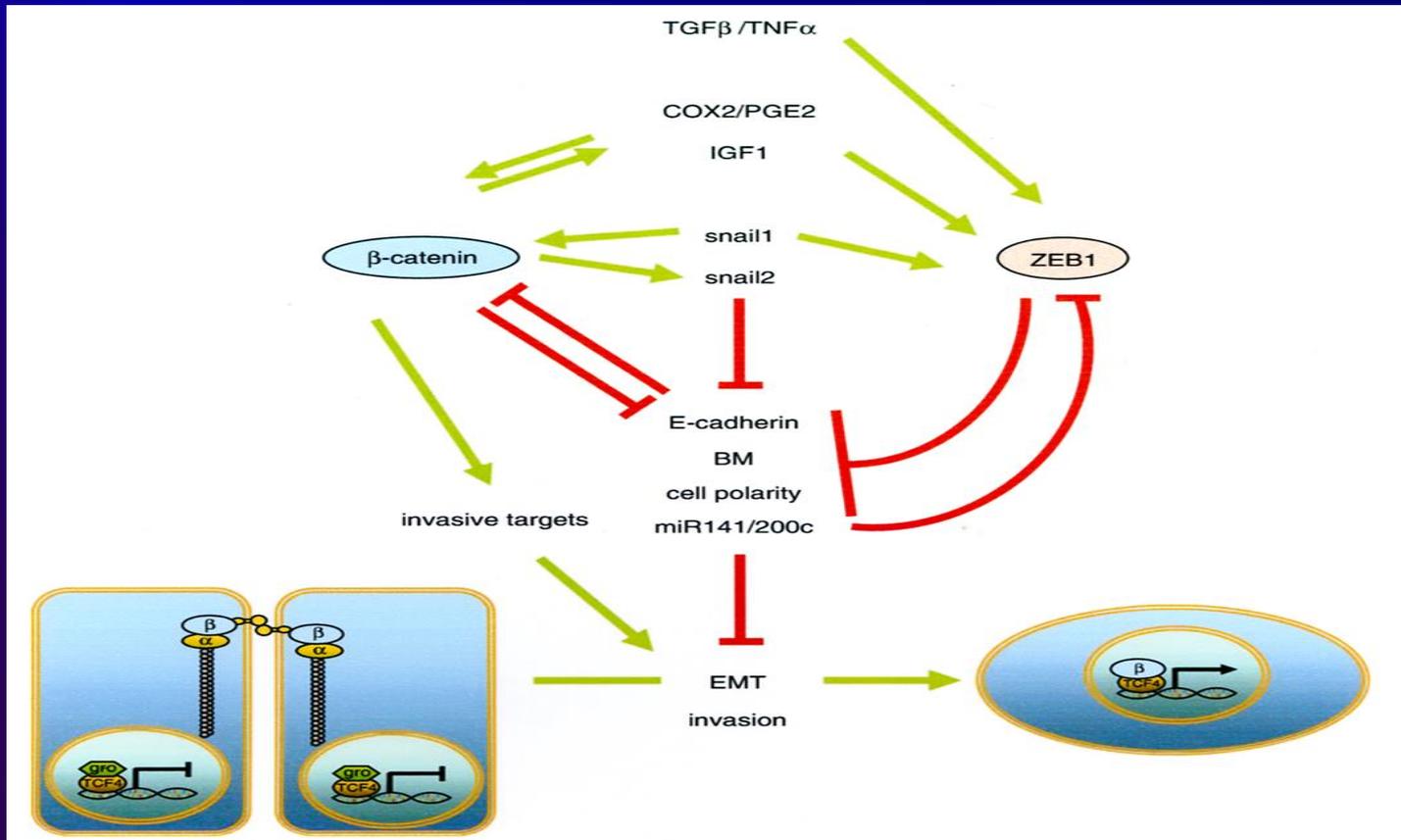
# E-Cadherin is linked to cytoskeletal proteins through $\beta$ -catenin.



# S-Valproate increases E-cadherin but decreases vimentin and ZEB1 in NSCLC cells.



# PGE<sub>2</sub> induces ZEB1 leading to loss of E-Cadherin.



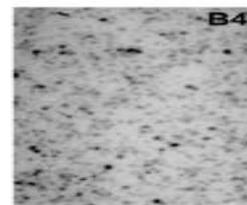
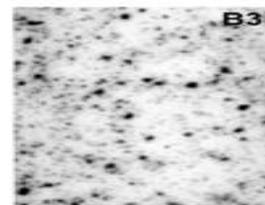
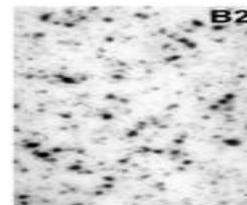
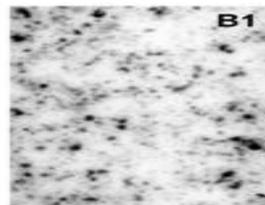
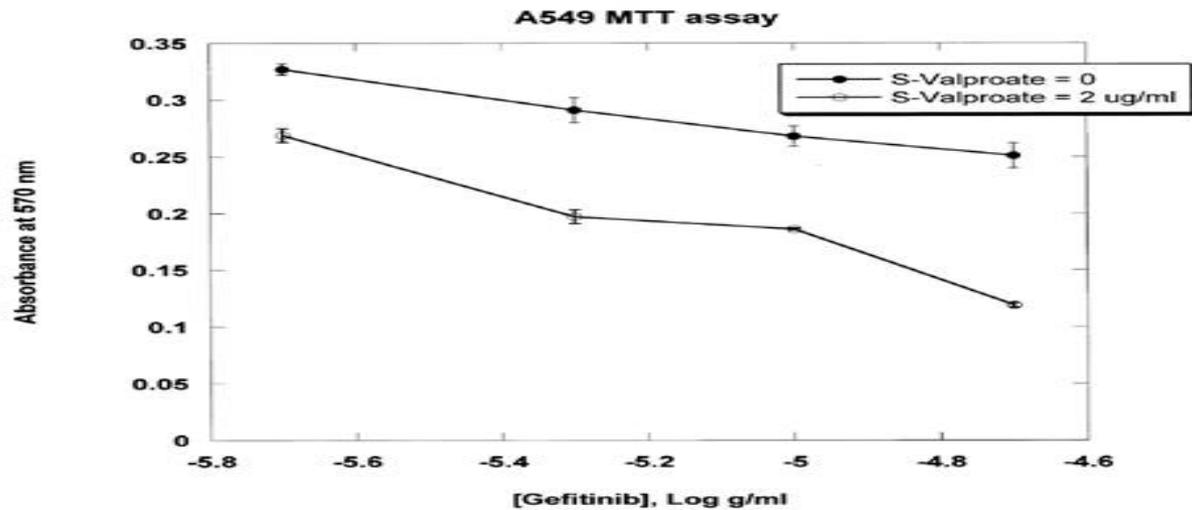
Schmalhofer, O. et al., *Cancer Metastasis Rev.* (2009) 28:151.

## **E-cadherin increases the sensitivity of NSCLC cells to EGFR tyrosine kinase inhibitors**

- **Gefitinib or erlotinib produce a 9-27% response rate in NSCLC patients**
- **E-cadherin transfection into a NSCLC cell line increases responsiveness to gefitinib**
- *Witta et al., Cancer Res 2006;66:944*

# S-Valproate increases Gefitinib potency in NSCLC cells.

Fig. 5



# Signal transduction pathways.

1. At low doses of NO, cGMP is increased leading to increased phosphorylation of p42 and p44 MAPK (ERK). At high doses of NO, p53 is phosphorylated, p38 MAPK is activated, bcl and survivin is reduced leading to cancer cell apoptosis.
2. At low doses of NO, COX-2 is activated leading to increased PGE<sub>2</sub> and activation of EP<sub>2</sub> receptors in cancer cells. PGE<sub>2</sub> increases VEGF expression in cancer cells. PGE<sub>2</sub> causes transactivation of the EGFR leading to increased proliferation of cancer cells.
3. S-Valproate and S-Diclofenac are cytostatic agents which inhibit COX-2, decrease the proliferation of NSCLC cells as well as tumors and increase E-cadherin expression. S-Valproate and S-Diclofenac increase the potency of gefitinib in NSCLC cells in vitro which have wild type EGFR.

# Acknowledgments

## NCI

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