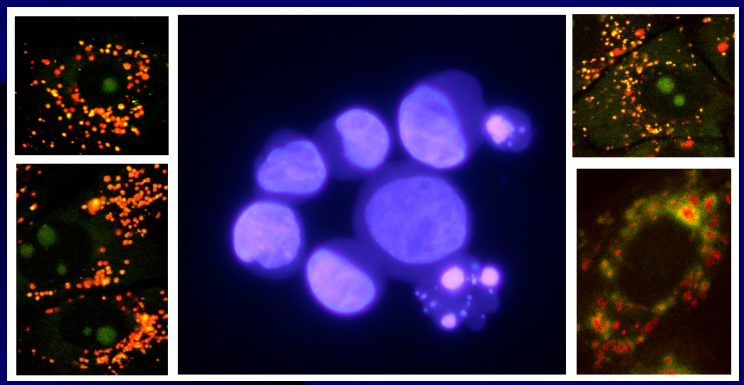
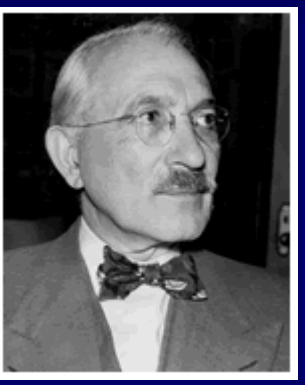
Lysosomes, weapons or shield in gentamicin-induced apoptosis.



Hélène Servais
Unité de Pharmacologie cellulaire et moléculaire
Promoteur: Prof. M-P Mingeot-Leclercq
Co-Promoteur: Prof. P.M. Tulkens

19th April 2006 - Public thesis

1. INTRODUCTION : Aminoglycosides (AG) history….



Dr Waksman

Streptomycin

Kanamycin

Dibekacin

Amikacin

Isepamicin

« New aminoglycosides »

1940 1950 1960 1970 1980 1990 2000

Neomycin

Paromomycin

Gentamicin isolated from *Micromonospora purpurea*

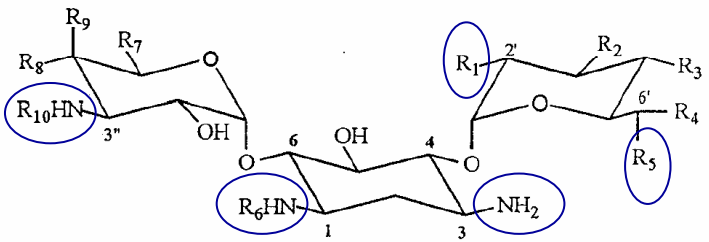
Tobramycin

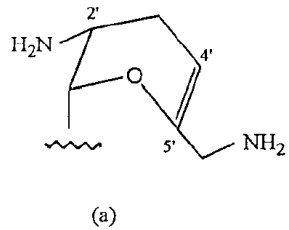
Netilmicin

Based on the identification of the exact interactions between AG with the bacterial ribosome

1. INTRODUCTION : Aminoglycosides structure

4,6-DISUBSTITUTED DEOXYSTREPTAMINE



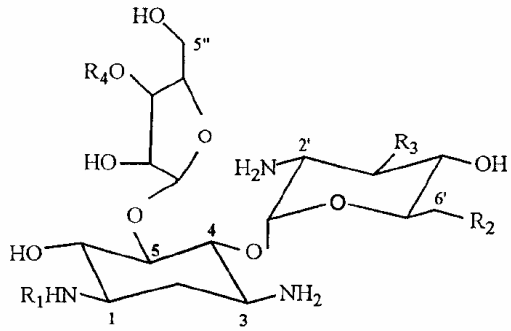


(a)

Aminoglycoside	R_1	R_2	R_3	R_4	R_5	R_6*	R_7	R_8	R_9	R_{10}
Kanamycin A	OH	OH	OH	H	NH_2	H	CH_2OH	OH	H	H
Kanamycin B	NH_2	OH	OH	H	NH_2	H	CH_2OH	OH	H	H
Kanamycin C	NH_2	OH	OH	H	OH	H	CH_2OH	OH	H	H
Amikacin	OH	OH	OH	H	NH_2	COR'	CH_2OH	OH	H	H
Tobramycin	NH_2	H	OH	H	NH_2	H	CH_2OH	OH	H	H
Dibekacin	NH_2	H	H	H	NH_2	H	CH_2OH	OH	H	H
Arbekacin	NH_2	H	H	H	NH_2	COR'	CH_2OH	OH	H	H
Gentamicin C_1	NH_2	H	H	CH_3	$NHCH_3$	H	H	CH_3	OH	CH_3
Gentamicin C_{1a}	NH_2	H	H	H	NH_2	H	H	CH_3	OH	CH_3
Gentamicin C_2	NH_2	H	H	CH_3	NH_2	H	H	CH_3	OH	CH_3
Gentamicin C_{2b}	NH_2	H	H	H	$NHCH_3$	H	H	CH_3	OH	CH_3
Gentamicin B	OH	OH	OH	H	NH_2	H	H	CH_3	OH	CH_3
Isepamicin	OH	OH	OH	H	NH_2	COR	H	CH_3	OH	CH_3
Sisomicin	---	---	---	---	---	H	H	CH_3	OH	CH_3
Netilmicin	---	---	---	---	---	CR''	H	CH_3	OH	CH_3

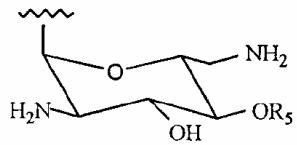
* $R = CHOHCH_2NH_2$; $R' = CHOH(CH_2)_2NH_2$; $R'' = CH_2CH_3$ (a) = primed sugar for sisomicin and netilmicin

4,5-DISUBSTITUTED DEOXYSTREPTAMINE

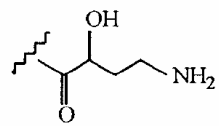


Aminoglycoside	R_1	R_2	R_3	R_4	R_5
Neomycin B	H	NH_2	OH	X	H
Paromomycin I	H	OH	OH	X	H
Lividomycin A	H	OH	H	X	Mannose
Ribostamycin	H	NH_2	OH	H	
Butirosin B	Y	NH_2	OH	H	

X =



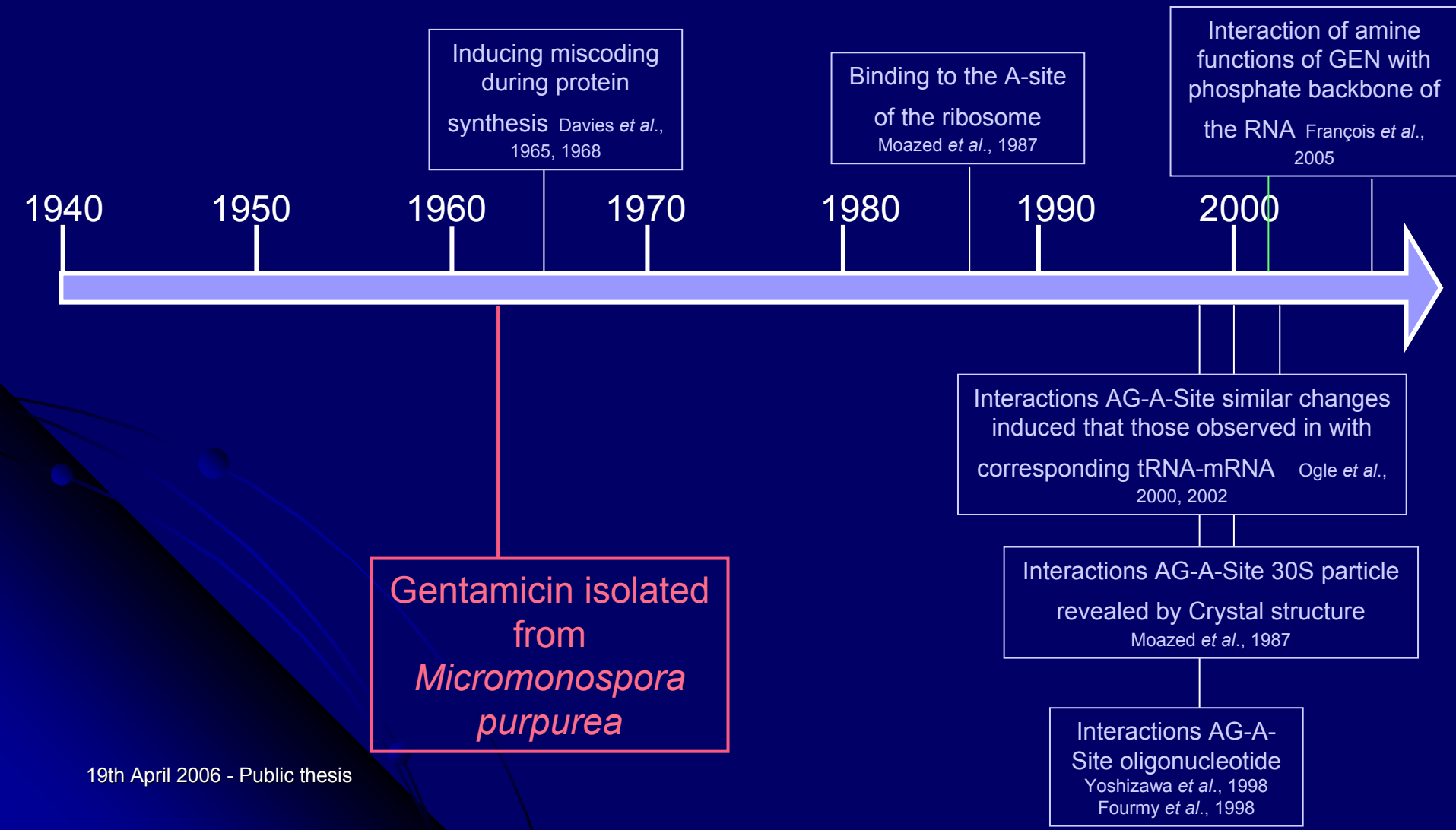
Y =



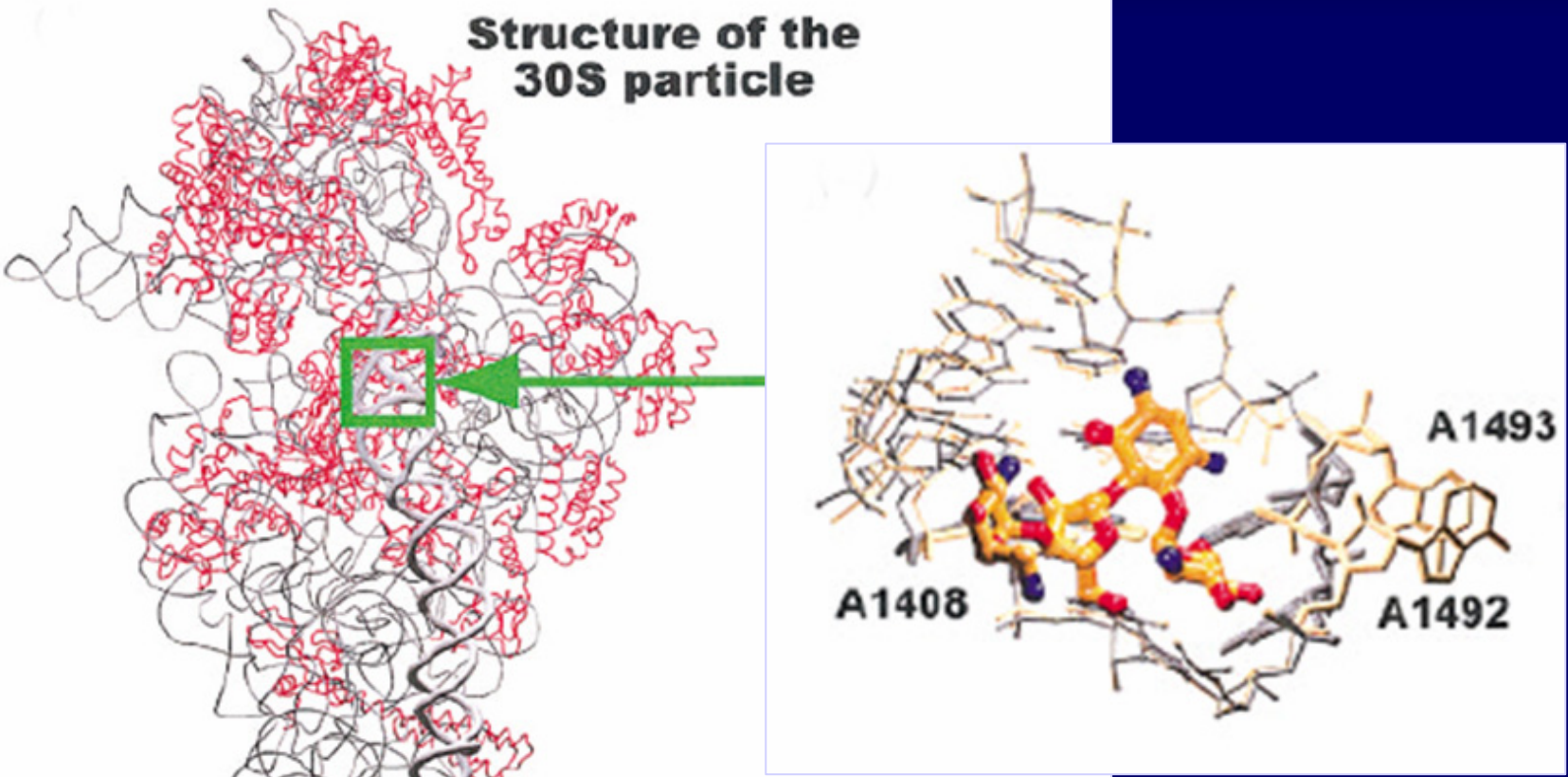
Mingeot-Leclercq and Tulkens 1999 *Antimicrob Agents Chemother* 43:727-737.

1. INTRODUCTION: Aminoglycosides mechanism of action

« New aminoglycosides »

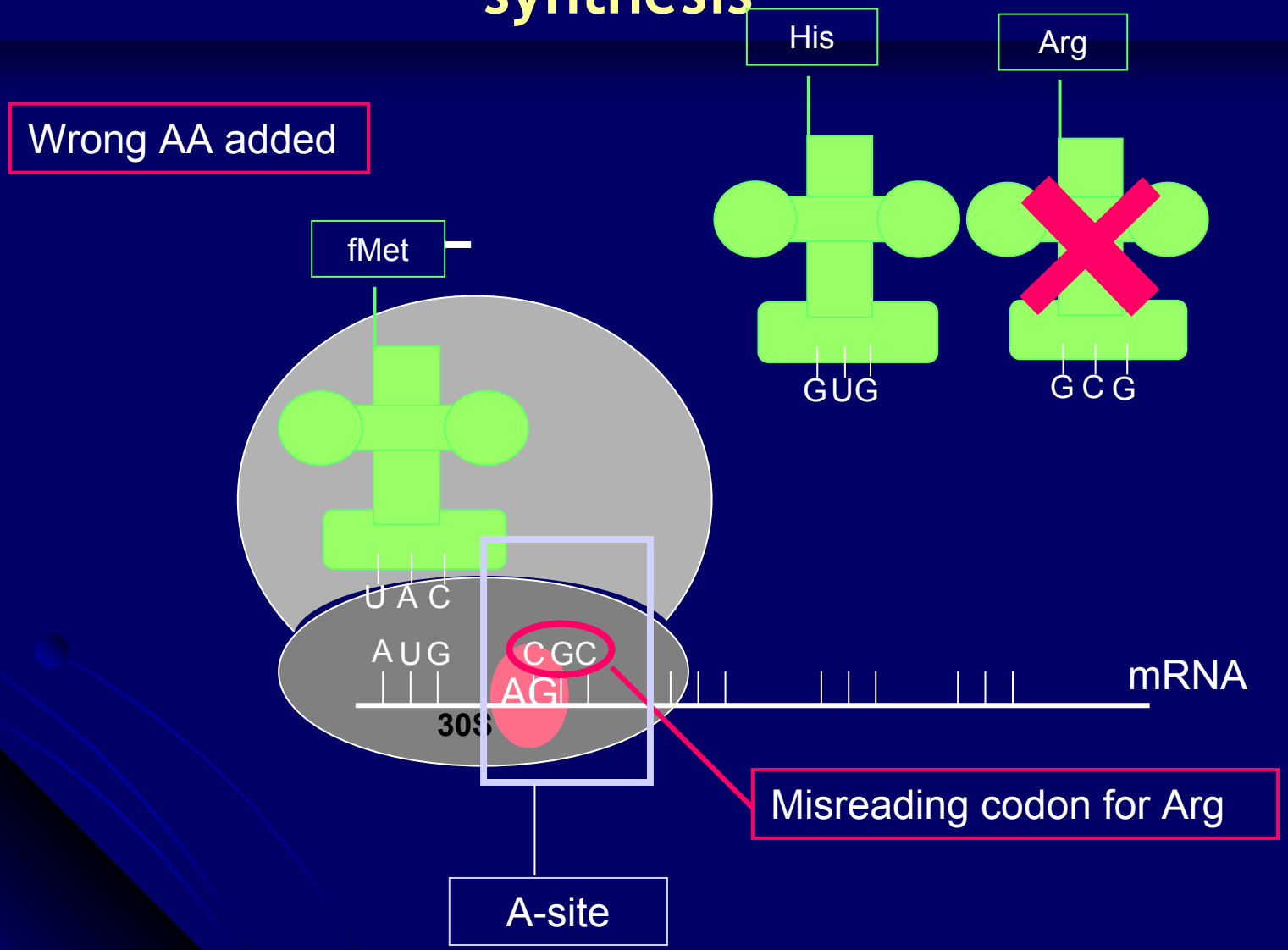


1. INTRODUCTION: Aminoglycosides insertion inside the 16S rRNA A-site



Vicens and Westhof 2003 *biopolymers* 70: 42-57

1. INTRODUCTION: Aminoglycosides disturb protein synthesis



Wimberly *et al.*, 2002 *Nature* 407:327-339, Ogle *et al.*, 2001 *Science* 292:897-902 , Ogle *et al.*, 2002 *Cell* 111:721-732
Vicens and Westhof., 2003 *Biopolymers* 70:42-57

1. INTRODUCTION: Clinical indications of aminoglycosides









- Serious, life-threatening gram-negative infection
- Complicated skin, bone or soft tissue infection
- Complicated urinary tract infection
- Septicemia
- Peritonitis and other severe intra-abdominal infections
- Severe pelvic inflammatory disease
- Endocarditis
- Mycobacterium infection
- Neonatal sepsis

1. INTRODUCTION: Aminoglycosides are interesting drugs…but…

- Bactericidal
- Post-antibiotic effect
- Synergism with cell wall active antibacterials

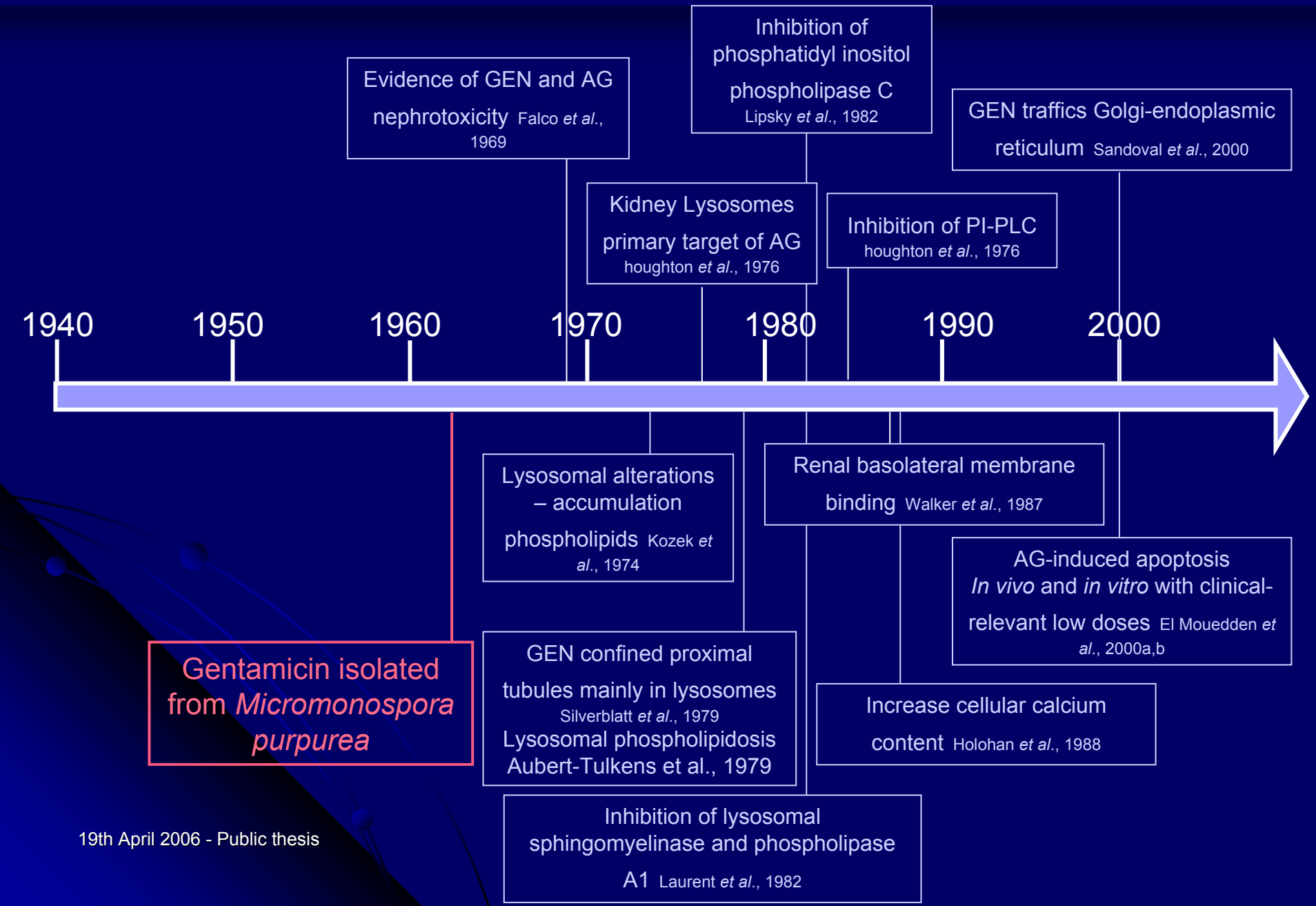
  (penicillin, cephalosporin, monobactam, carbapenem and glycopeptide)

- But toxicity limits their clinical use
  - Choclear and vestibular toxicity
  - Nephrotoxicity: 5-25% (0-50%)
    * Risk factors nephrotoxicity:
    * Clinical features:

      Nonoliguric renal failure

      Slow rise in creatinine

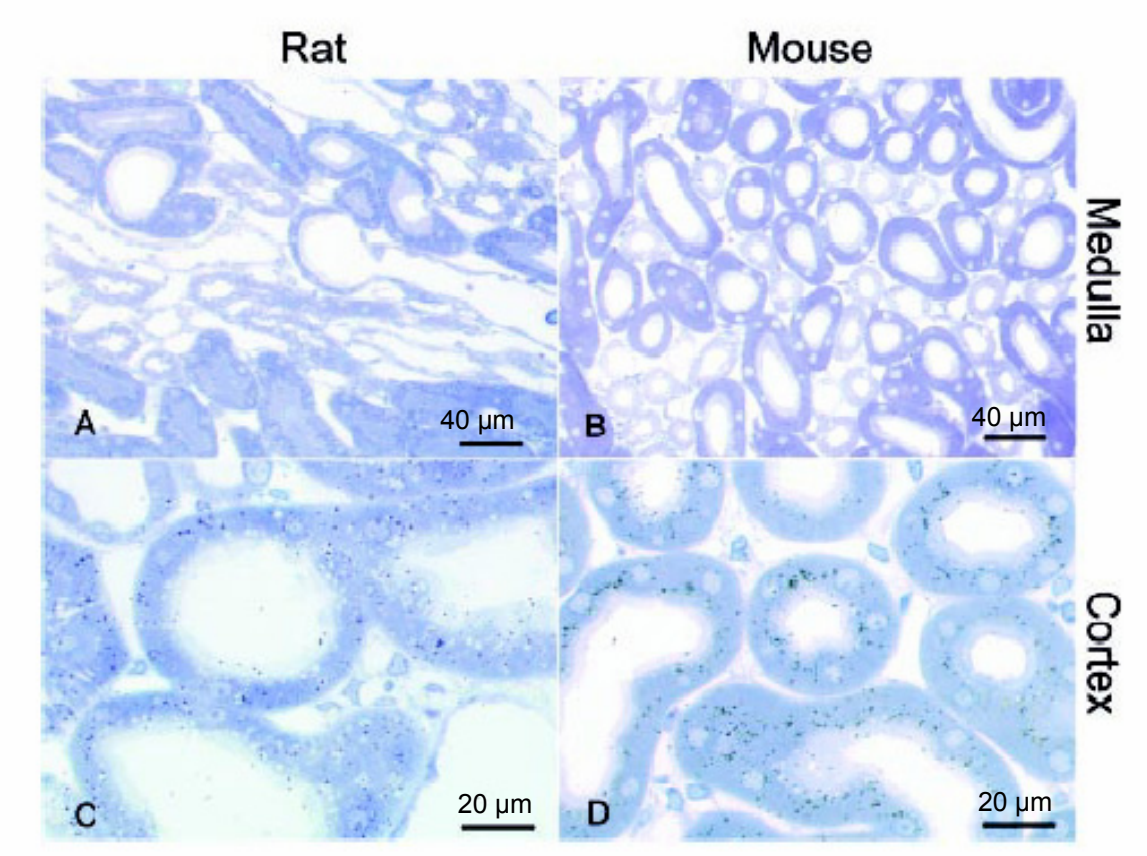
1. INTRODUCTION: Aminoglycosides nephrotoxicity



1. INTRODUCTION: Gentamicin accumulation in renal cortex

- Accumulated in the renal cortex – in proximal tubular epithelial cells

^{3}H-gentamicin – collection of tissue 24hour after injection



Schmitz *et al.*, 2002 J Biol Chem 277: 618-622

1. INTRODUCTION: Gentamicin uptake by kidney cells - accumulation in lysosomes

Gentamicin = **95%** excreted glomerumar filtration

GEN

Lumen- urine

Apical membrane

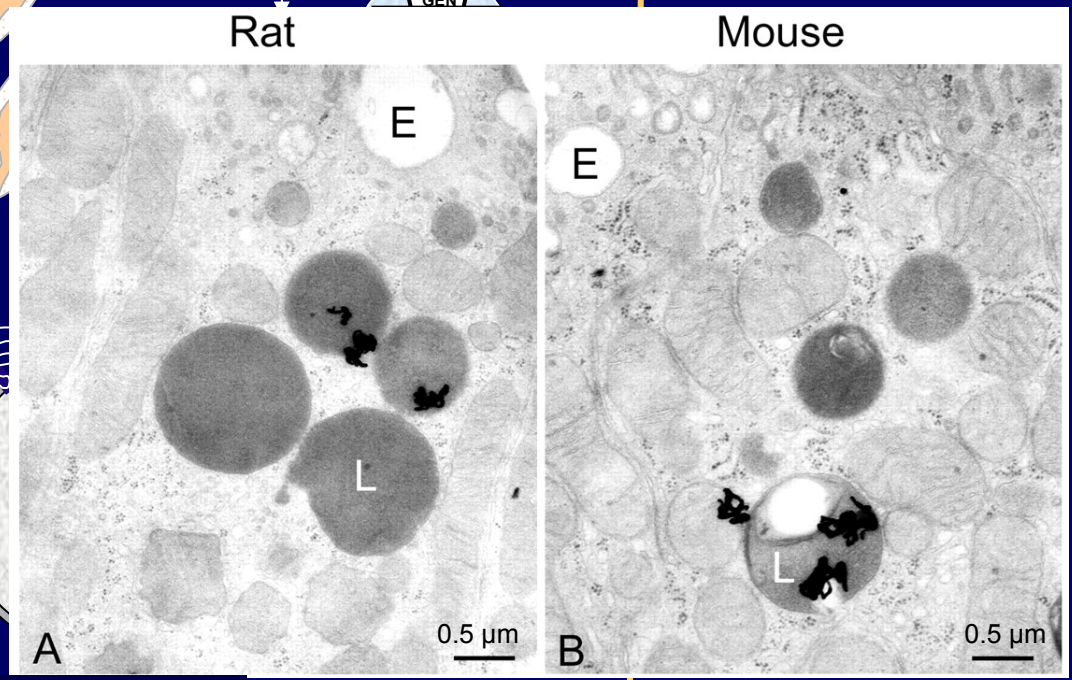
GEN

Endocytic Route

Proximal tubular epithelial cell

Mitochondria

Endoplasmic reticulum



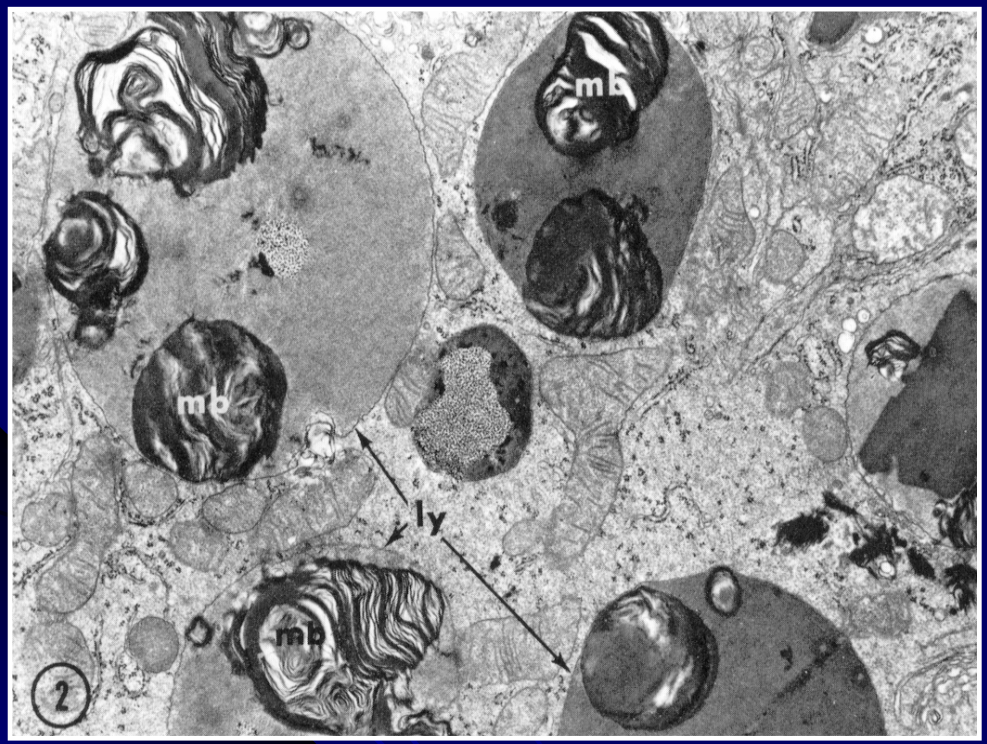
Basolateral membrane

Blood

Schmitz *et al.*, 2002 *J Biol Chem* 277: 618-622

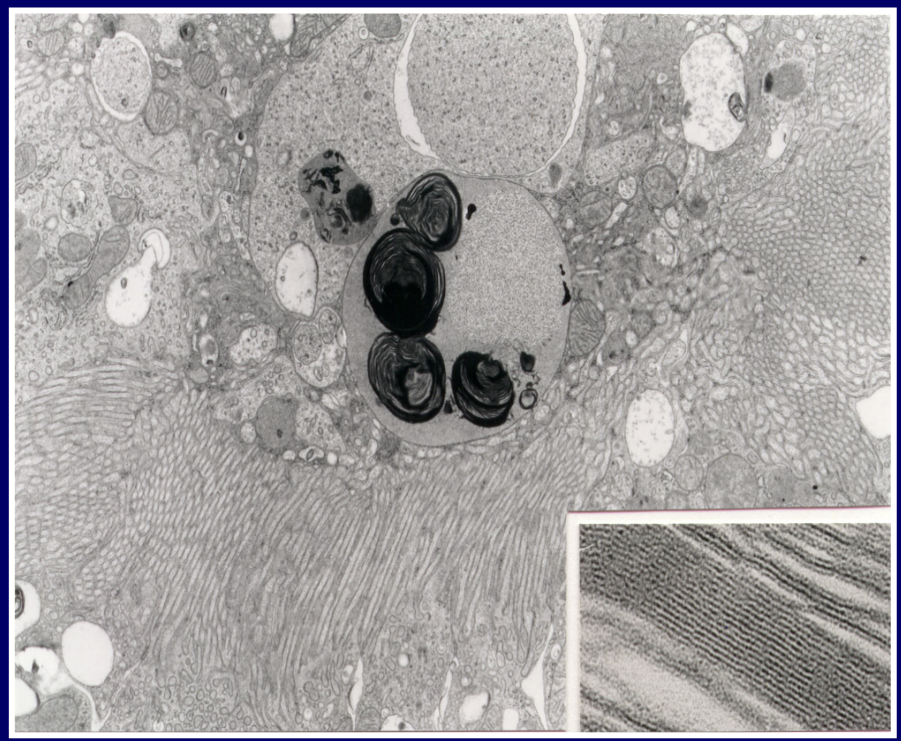
1. INTRODUCTION: Lysosomal perturbations induced by gentamicin

Proximal tubular cell - rat treated with 10 mg/kg.day gentamicin for 7 days



Kosek *et al* 1974 *Lab. Invest* 30: 48-57

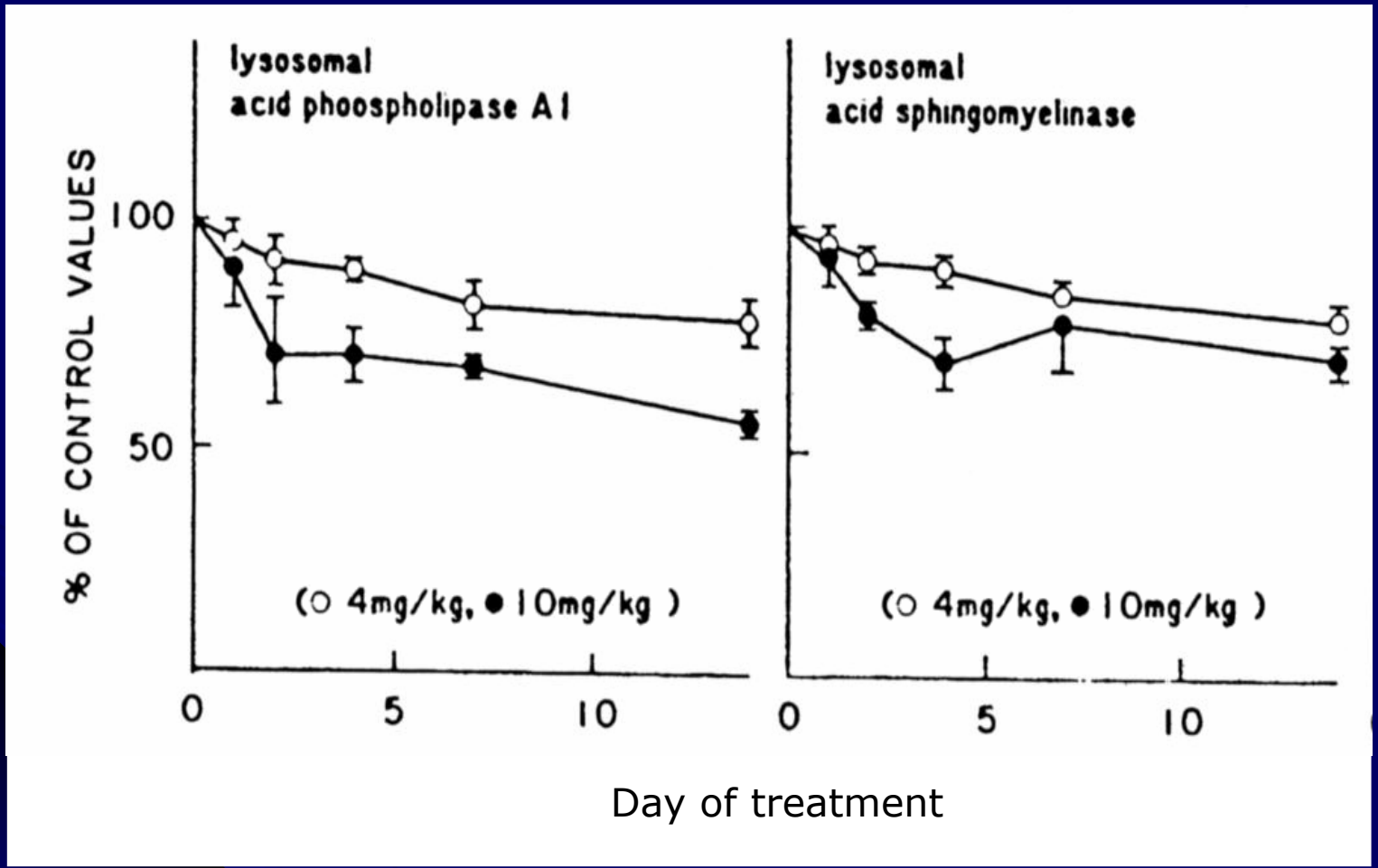
Proximal tubular cell - rat treated with 4 mg/kg of gentamicin for 4 days



Pictures from P.M. Tulkens & M.B. Carlier, adaptated from Tulkens 1986 *Am J Med* 80 Suppl 6B:105-114
Magnifications: X 75,000 (& 200,000, insert)

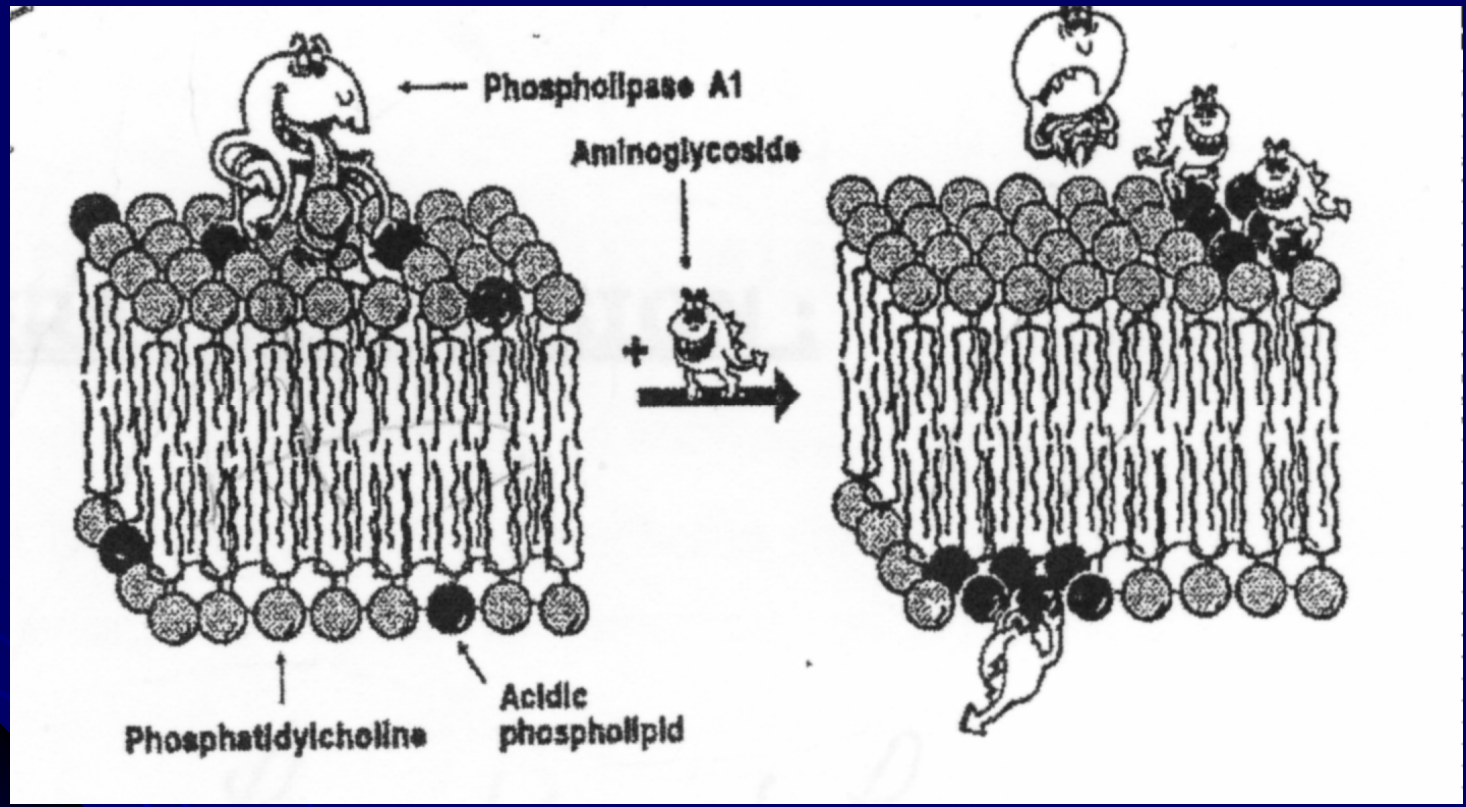
1. INTRODUCTION: Inhibition of lysosomal phospholipase and sphingomyelinase

Cortex of rat treated with gentamicin 4 or 10 mg/kg.day



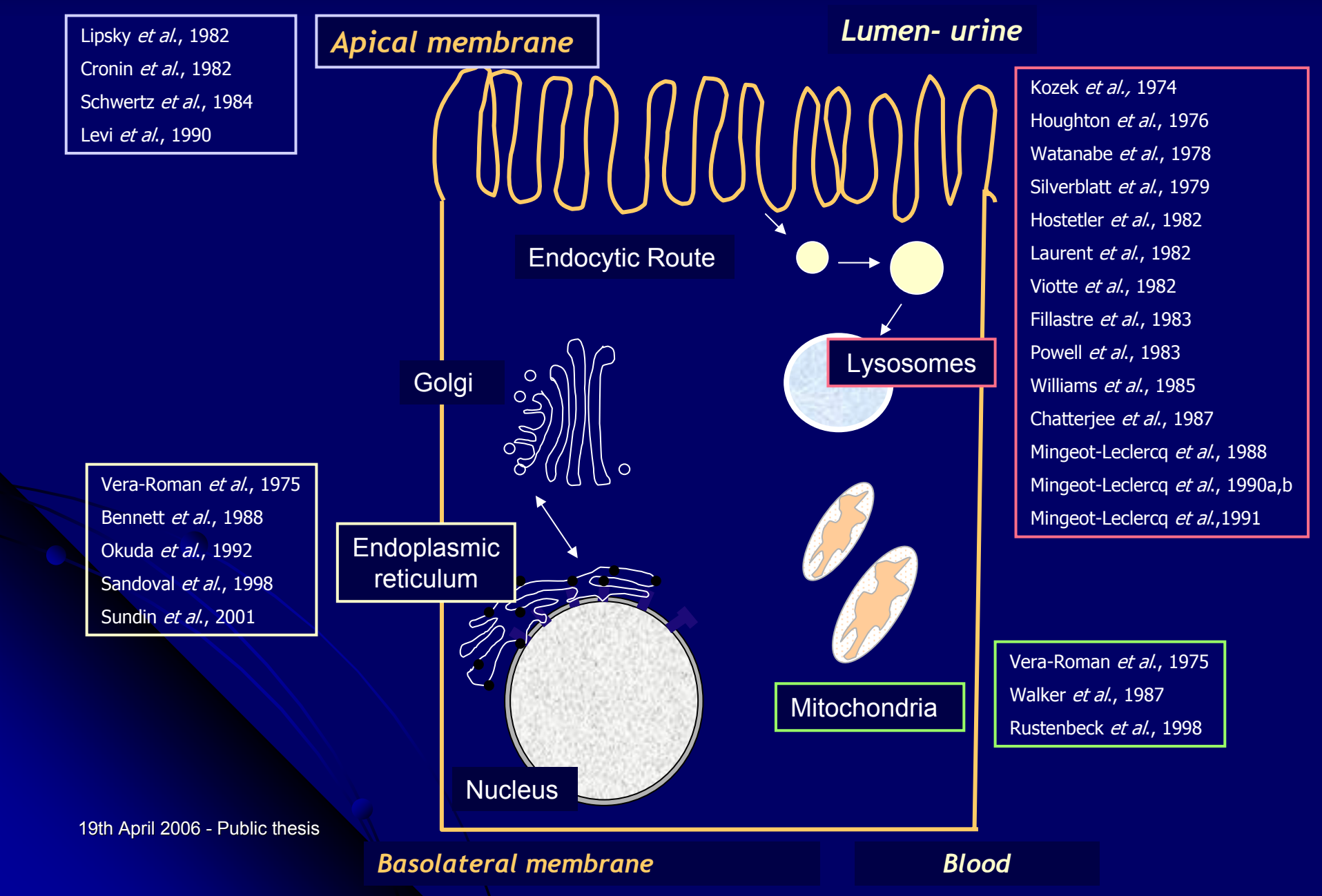
Carlier *et al* 1982 *Arch Toxicol Suppl*. 5:287-290

1. INTRODUCTION: Inhibition of lysosomal phospholipase and sphingomyelinase

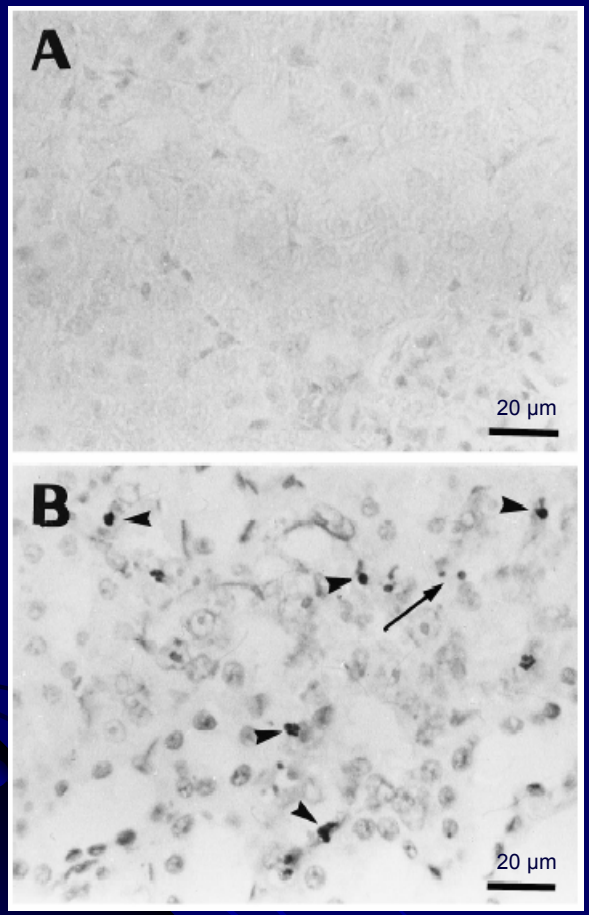


Adaptated from Mingeot-leclercd *et al* 1991 *Biochem (Life Sci Adv)*. 10:113-141

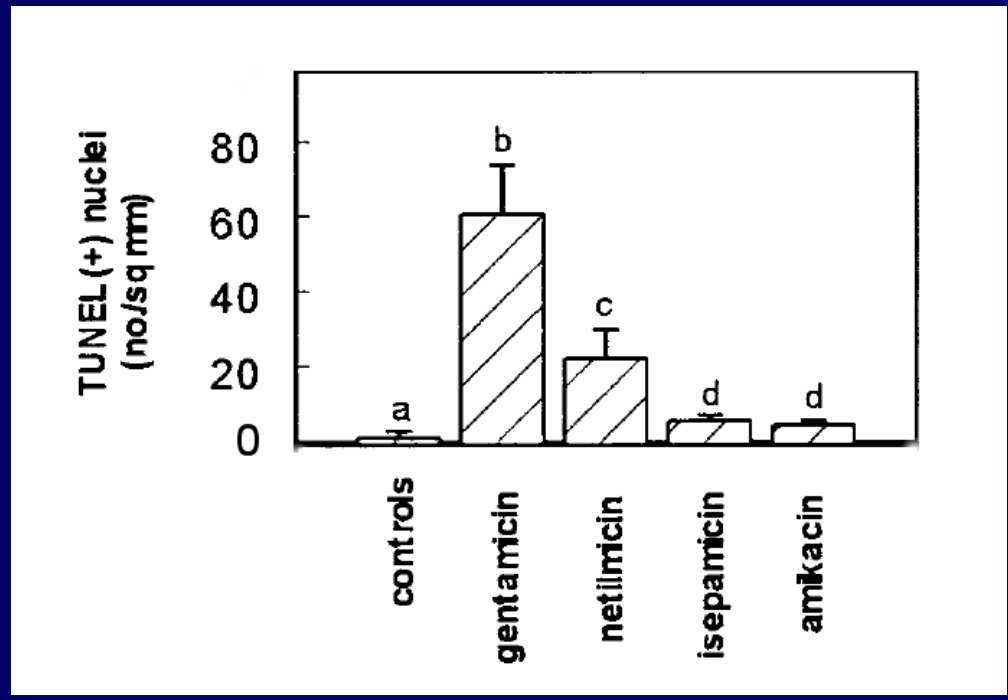
1. INTRODUCTION: Cellular alterations induced by GEN



1. INTRODUCTION: Gentamicin induces apoptosis *in vivo*



Rat kidney cortical specimens of controls (A) and animal treated for 10 days with 10mg/kg of gentamicin.



Rat treated for 10 days with saline (control), 10mg/kg of gentamicin and netilmicin; and 40mg/kg isepamicin and amikacin.

Apoptosis

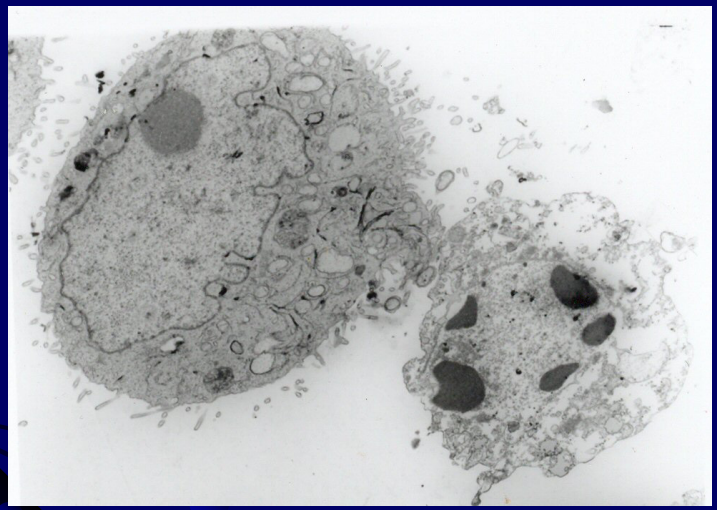
↕

Nephrotoxicity

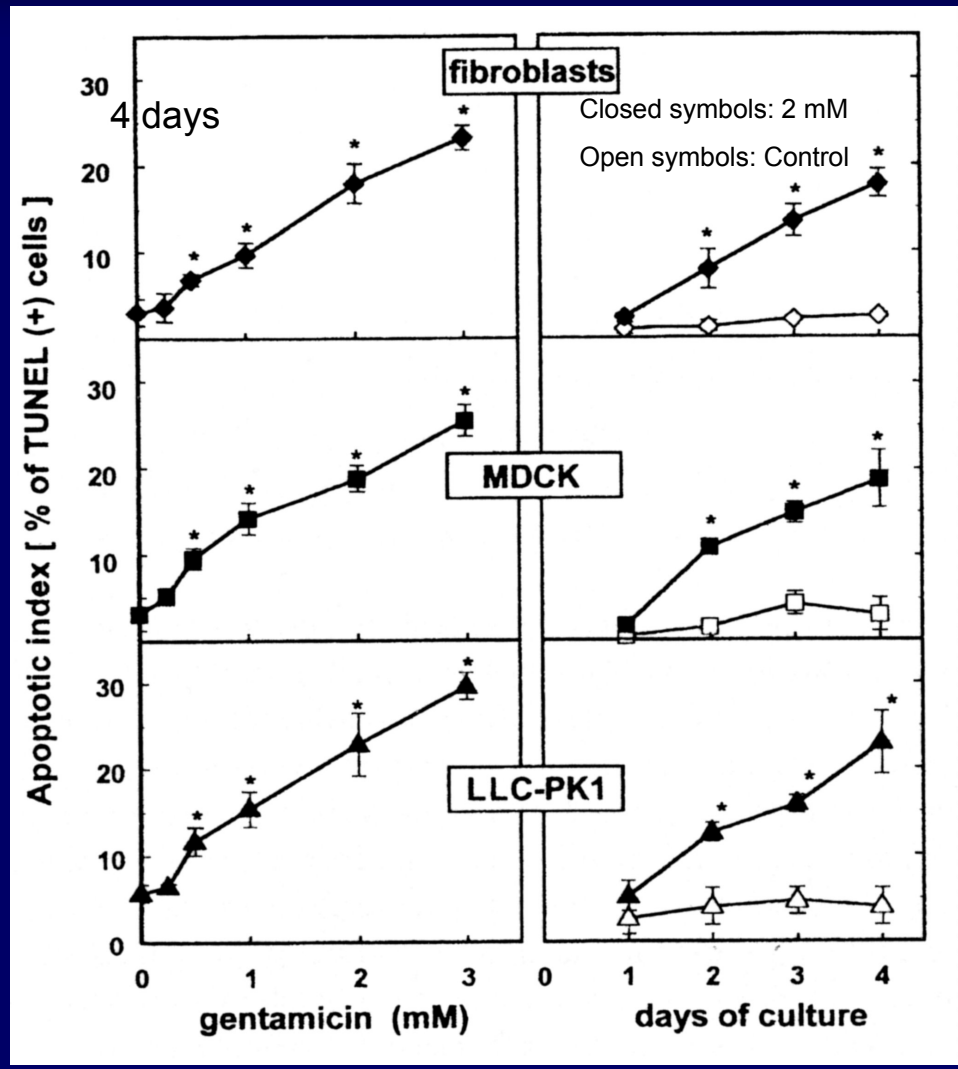
In vivo

El Mouedden *et al.*, 2000 *Antimicrobial Agents and Chemother* 44: 665-675

1. INTRODUCTION: Gentamicin induces apoptosis *in vitro*



LLC-PK1 treated 4 days with 2 mM of GEN



In vitro

El Moudden *et al.*, 2000 *Toxicological Sciences* 56: 229-239

1. INTRODUCTION: Apoptotis or programmed cell death

A. Apoptosis:

Apoptosis: Programmed cell death (PCD)

Role: Important in
*Tissue **Homeostasis**
*embryogenesis

Diseases

Toxicological involvement

Neurodegenerative disorders

AIDS

Excess of apoptotic process

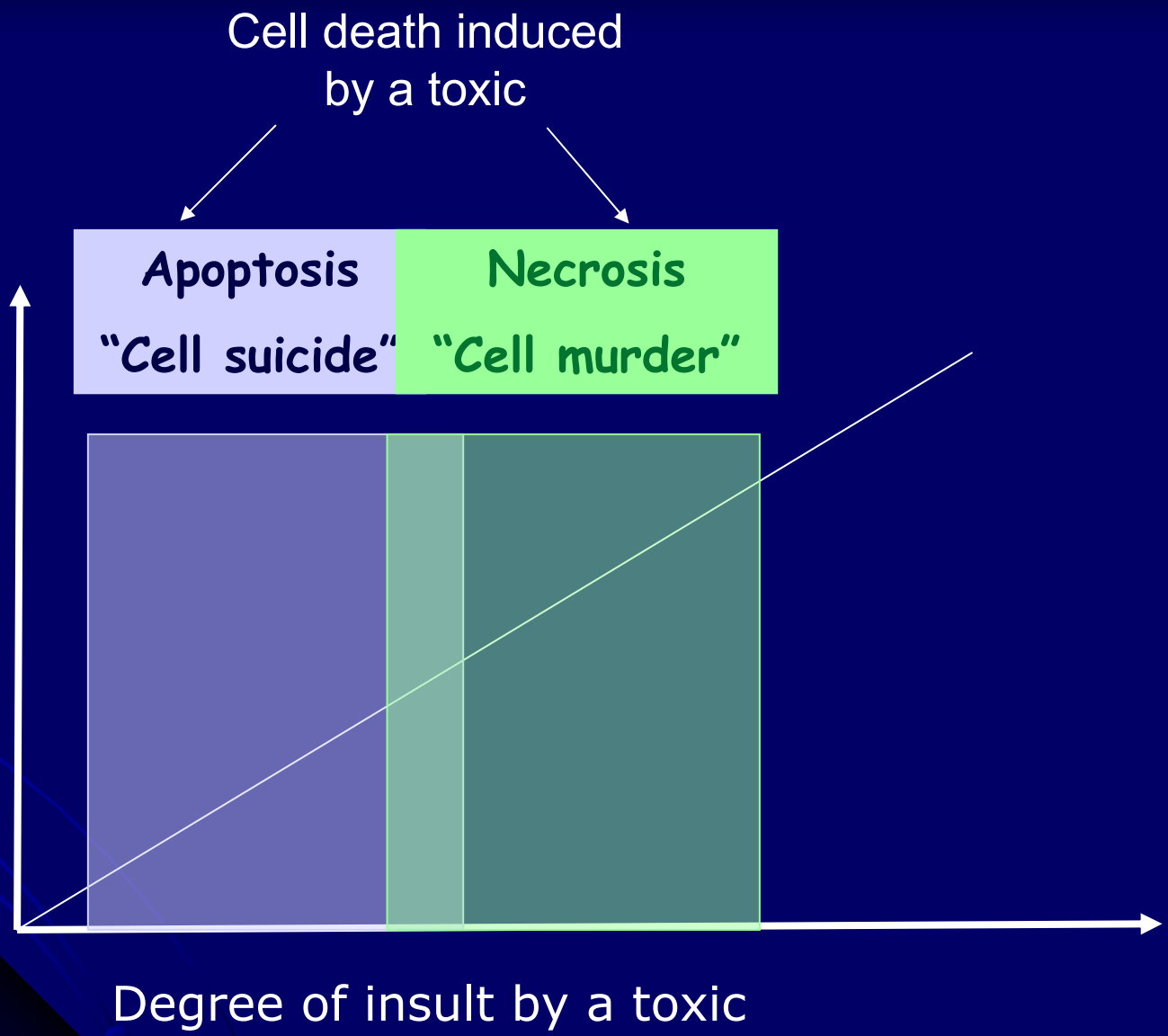
Cancer

Autoimmune disorders

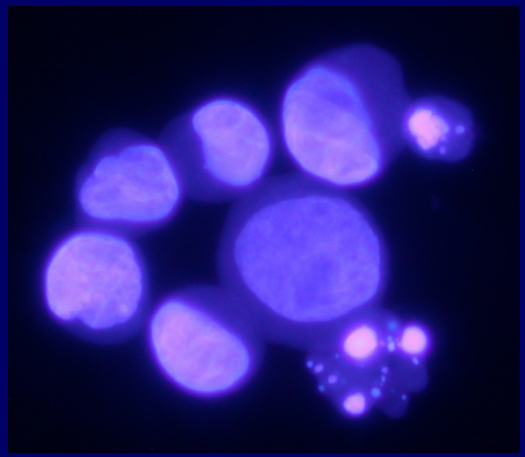
Decrease of apoptotic process

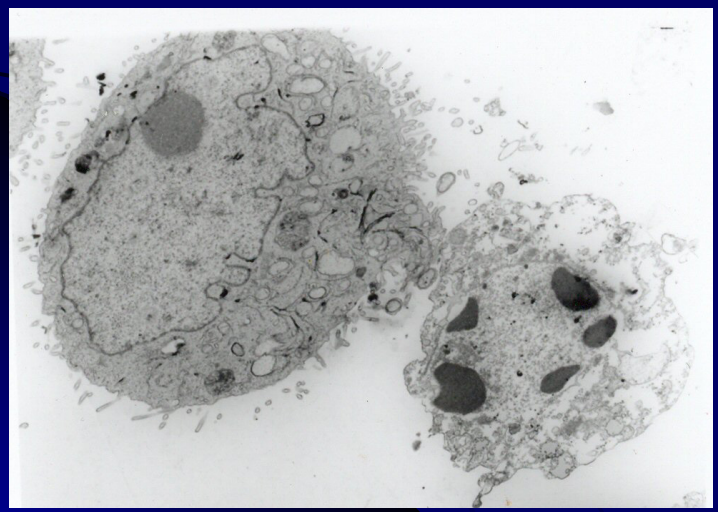
Liver
CNS
Kidney

1. INTRODUCTION: Apoptotis and necrosis



1. INTRODUCTION: Morphological appearance of apoptotic cell

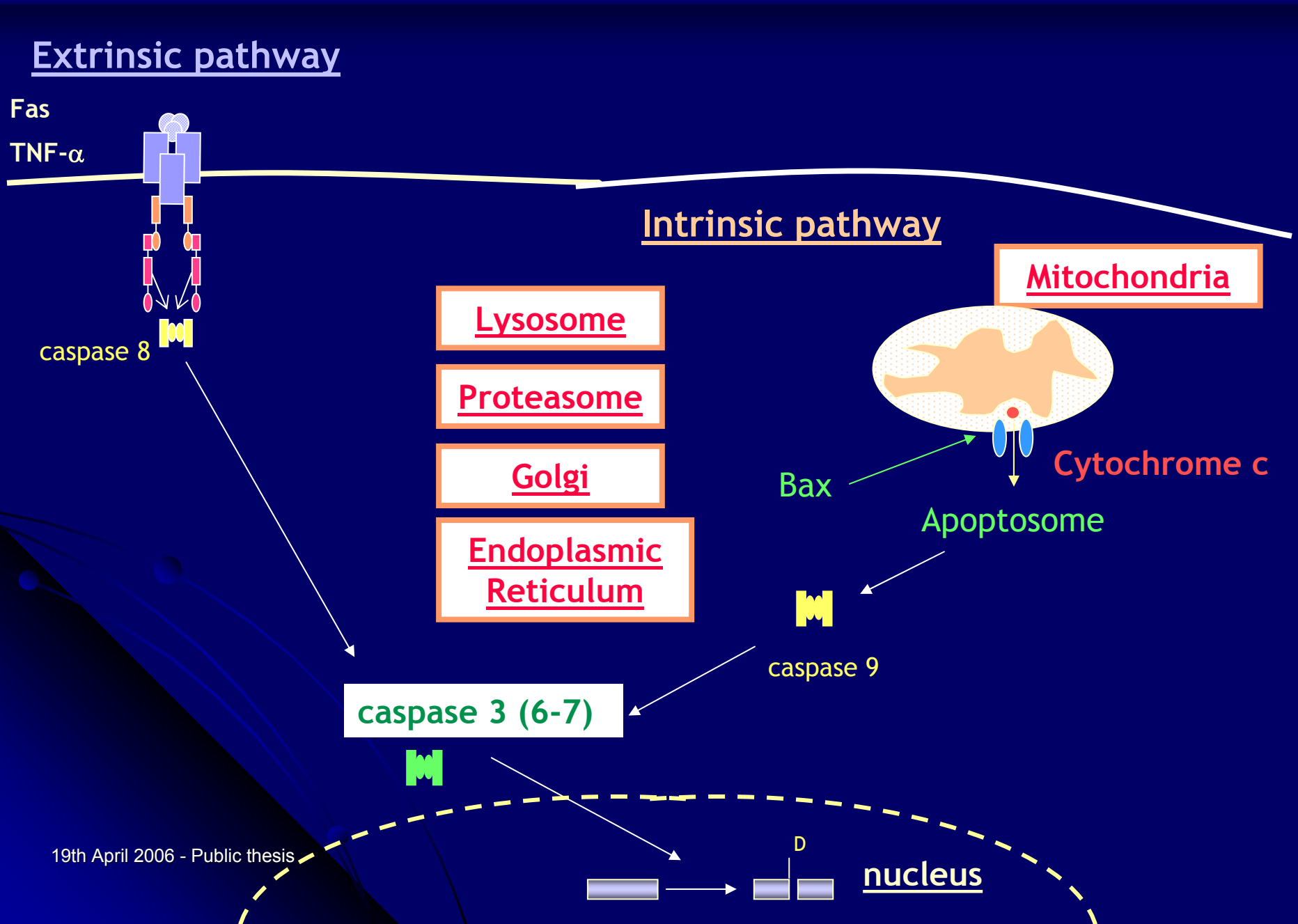




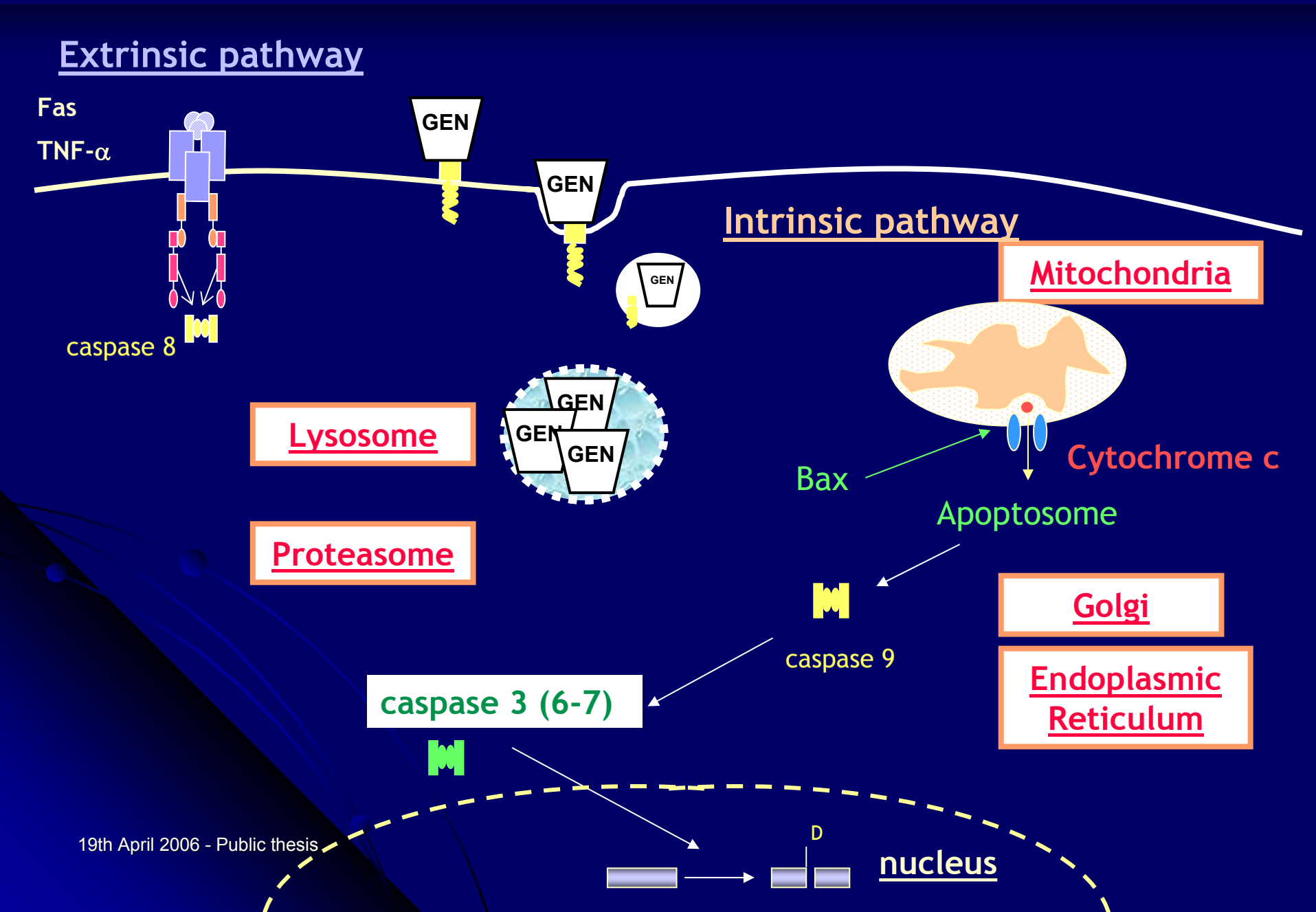
Morphological changes in apoptotic cells

- Cytoplasm shrink
- Nuclear condensation – margination – fragmentation
- Formation of apoptotic bodies
- Results from the activation of special enzyme: CASPASE

1. INTRODUCTION: Apoptotic Pathways

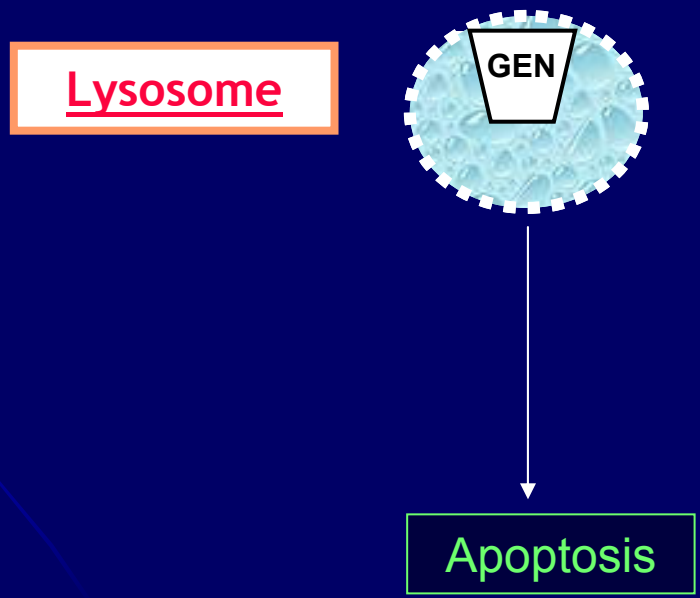


2. AIM of the study: HOW GEN INDUCES APOPTOSIS?



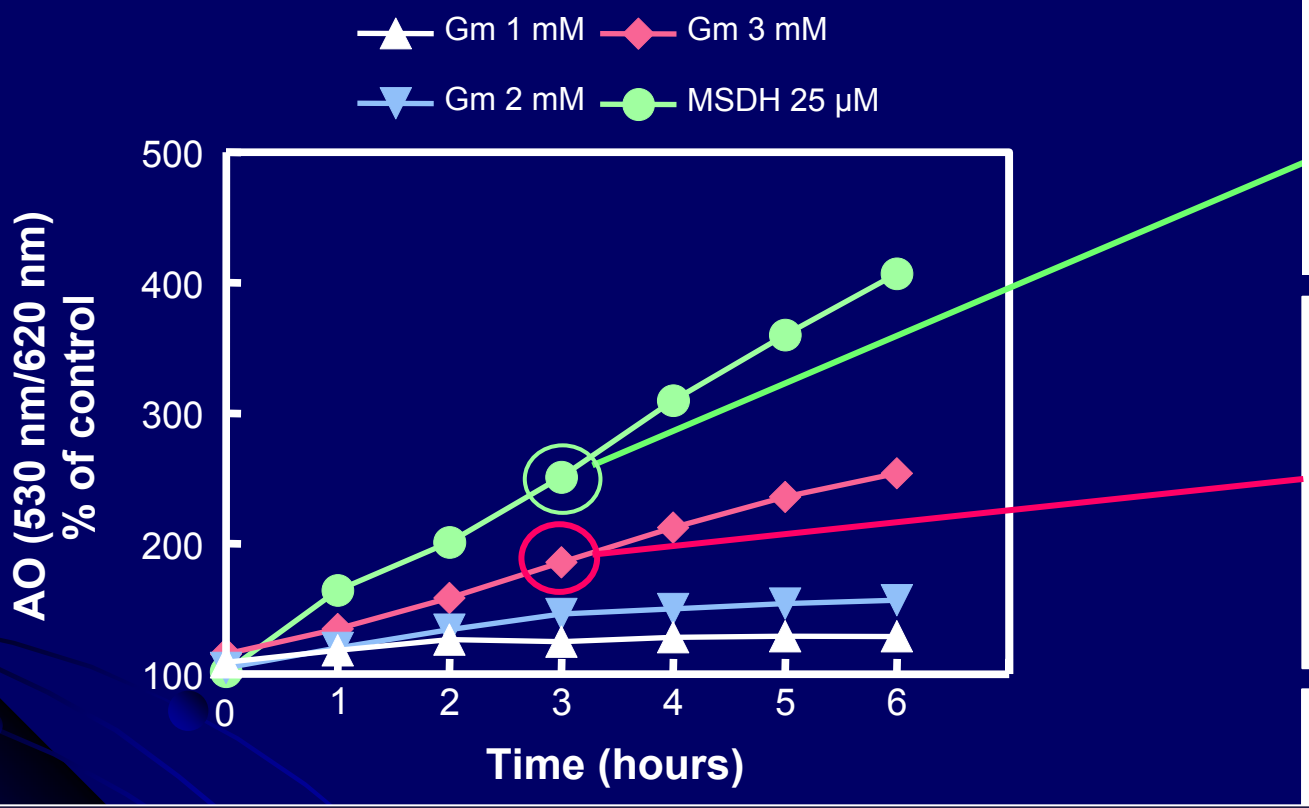
3. RESULTS

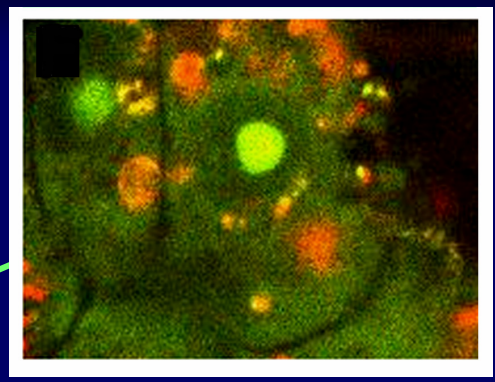
Part I: Are lysosomes involved in GEN-induced apoptosis?

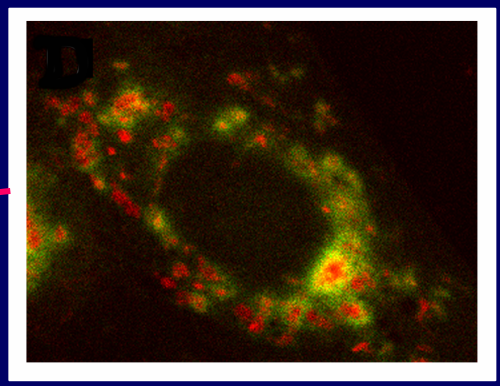


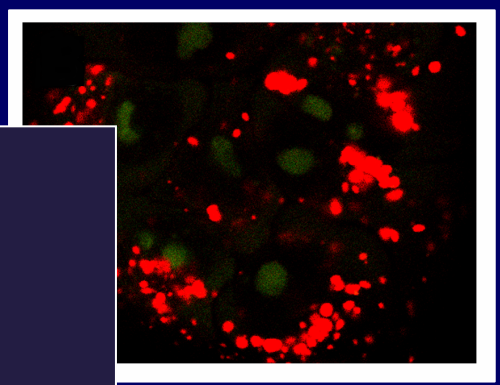
PI.1. Destabilization of lysosomal membrane by gentamicin:

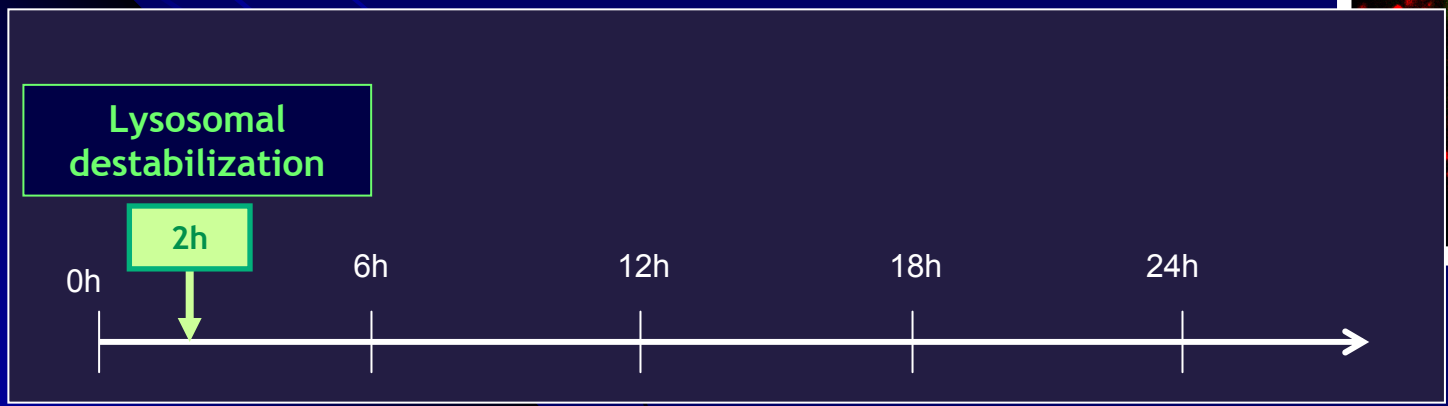
LLC-PK1cells: renal proximal tubular cells from pig



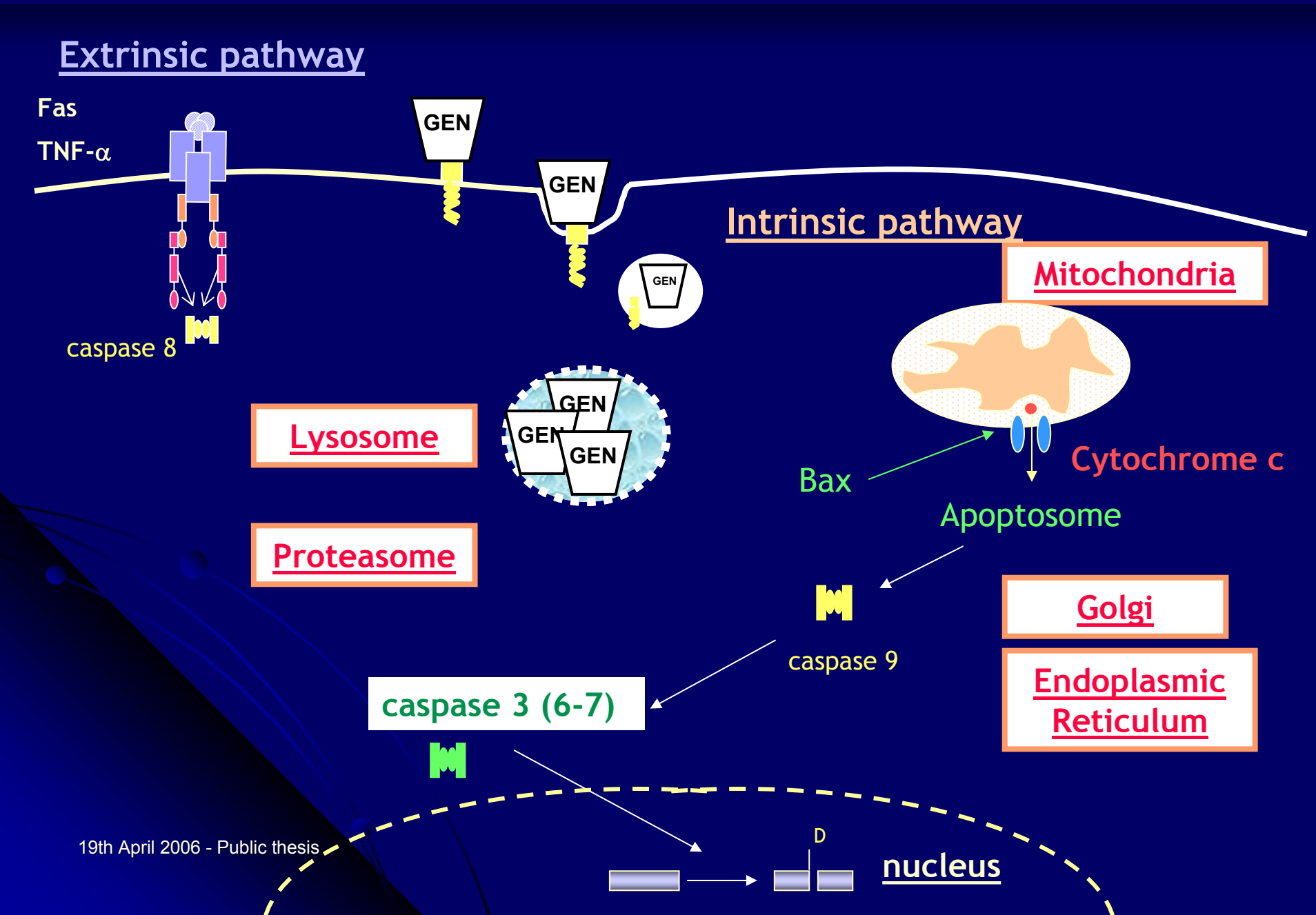




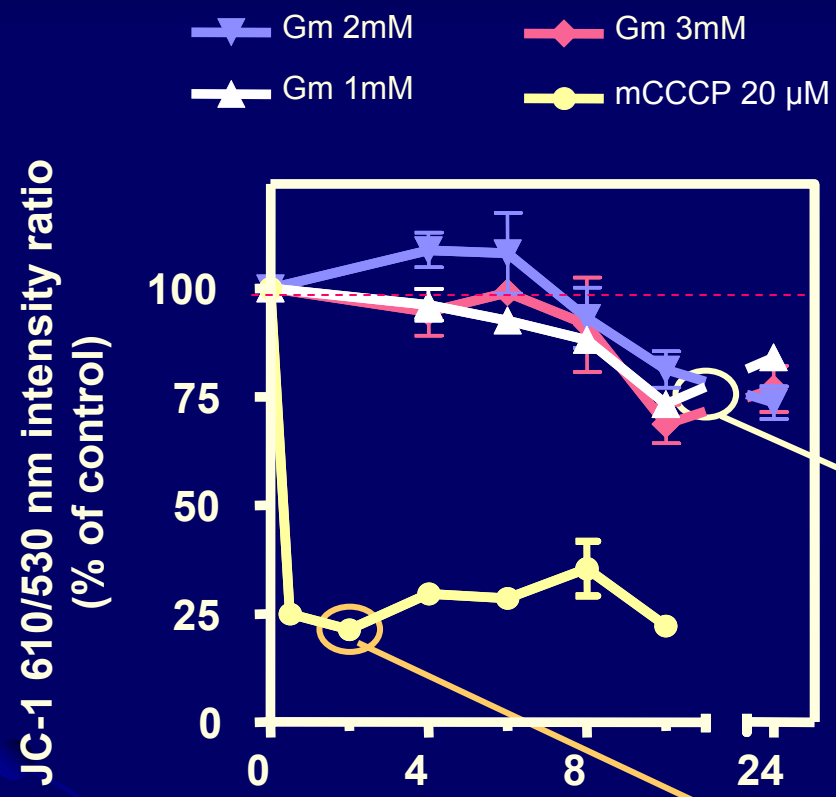


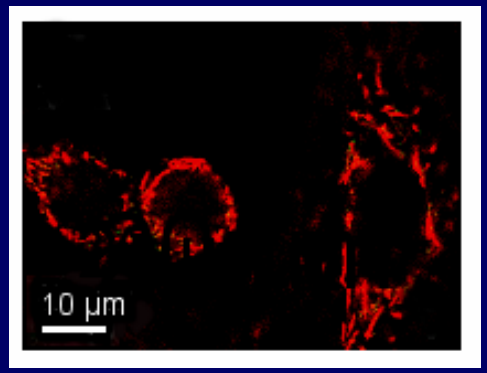


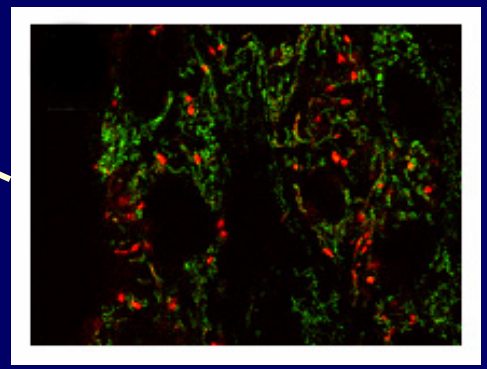
Apoptotic signalling:

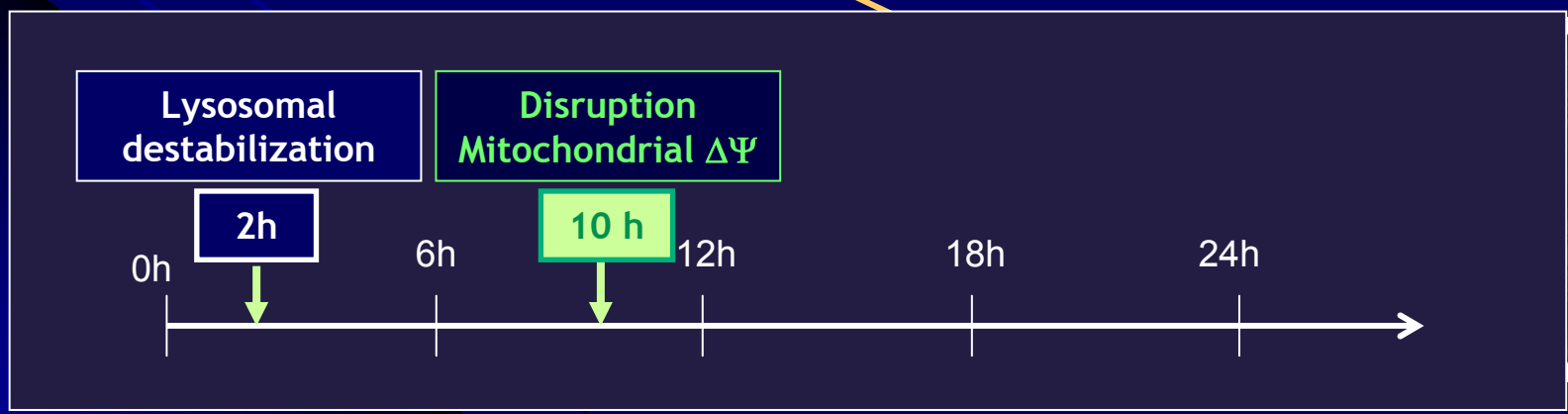


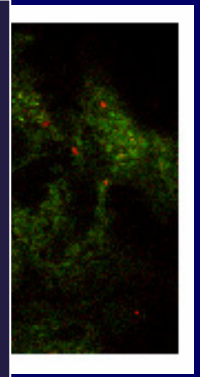
PI.2. Disruption of mitochondrial membrane by GEN



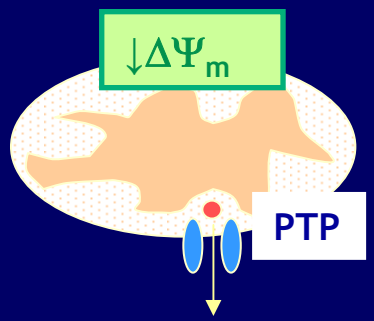






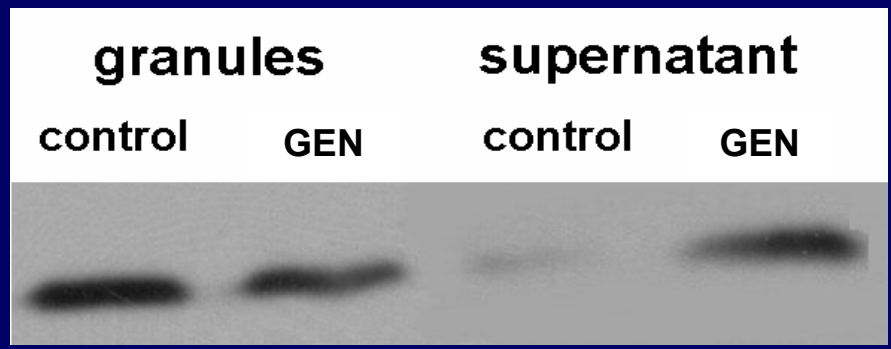


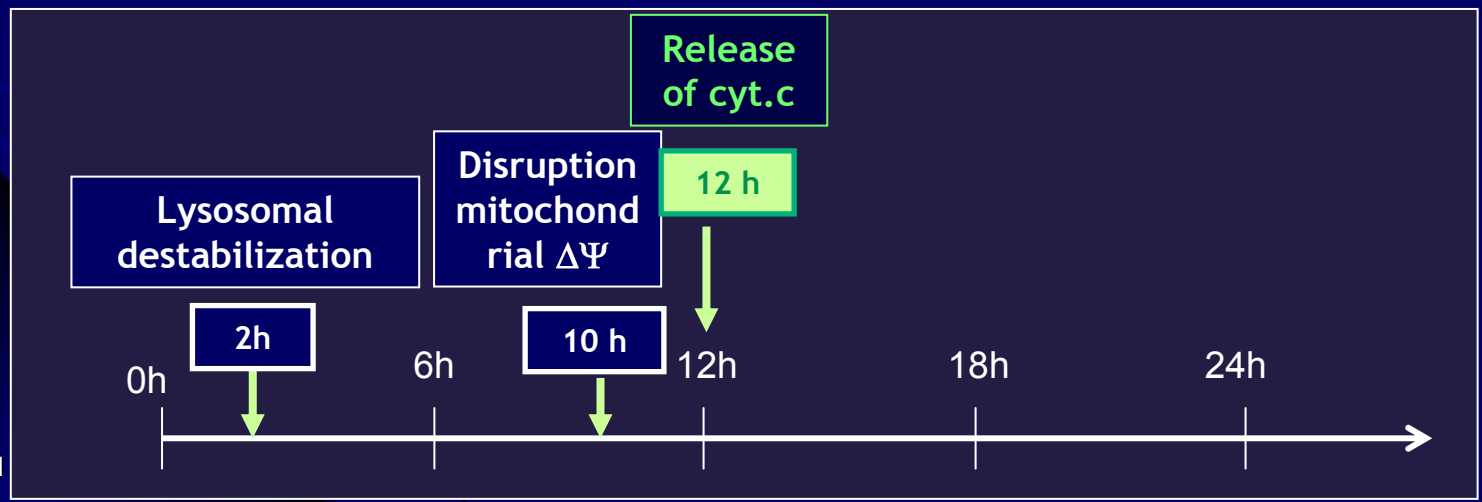
PI.3. Release of cytochrome c :



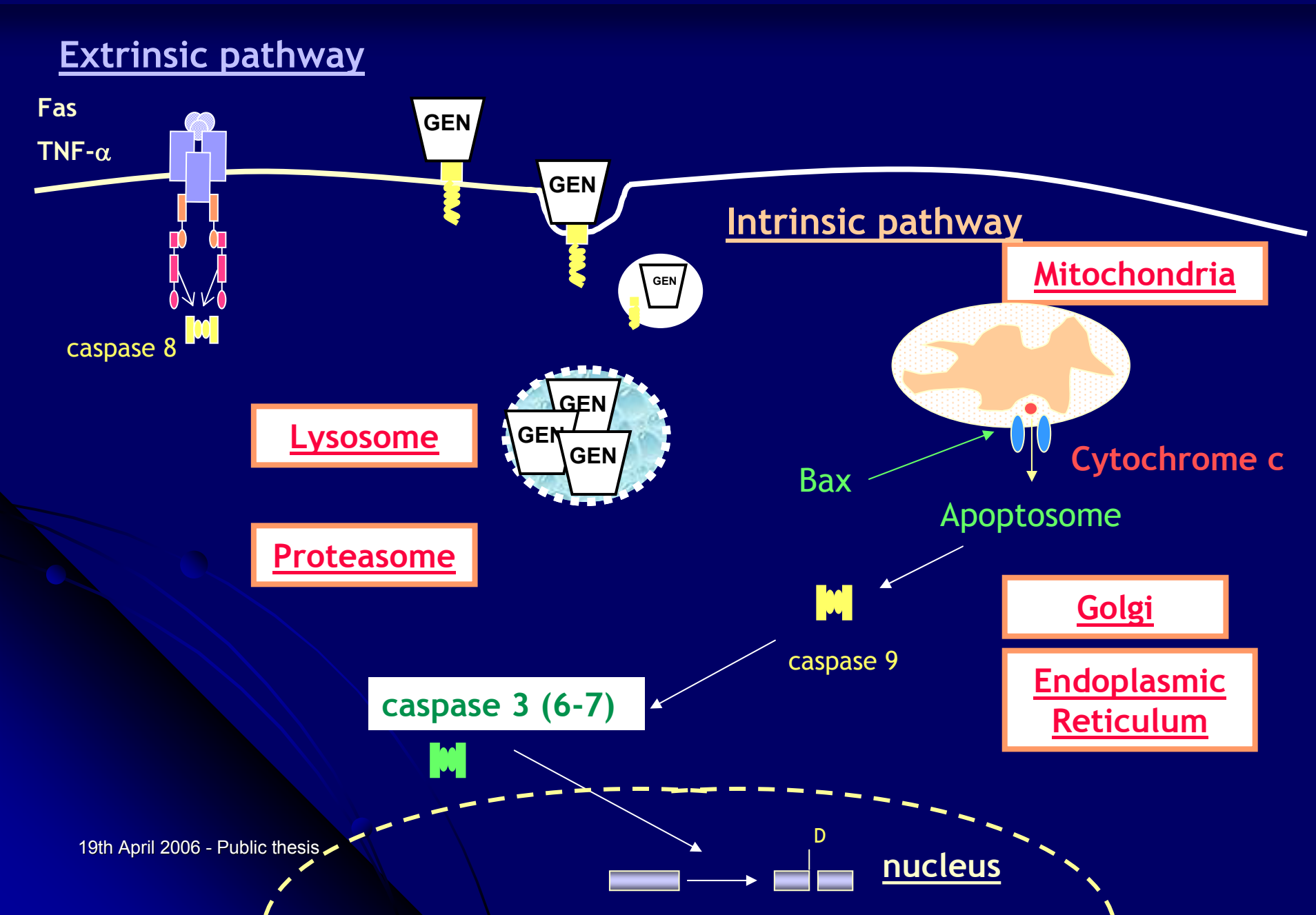
Cytochrome c

GEN 2 mM – 12h

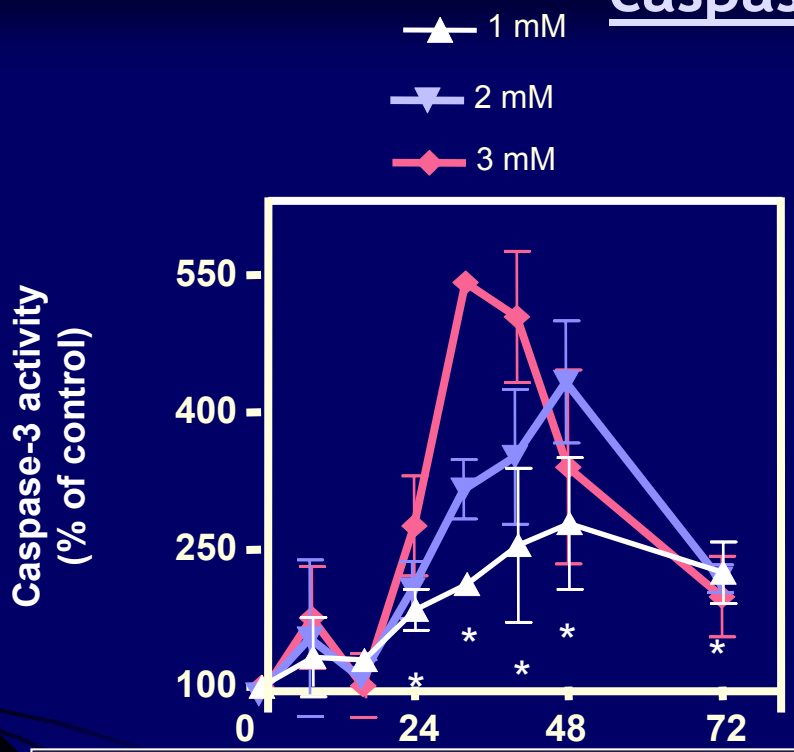


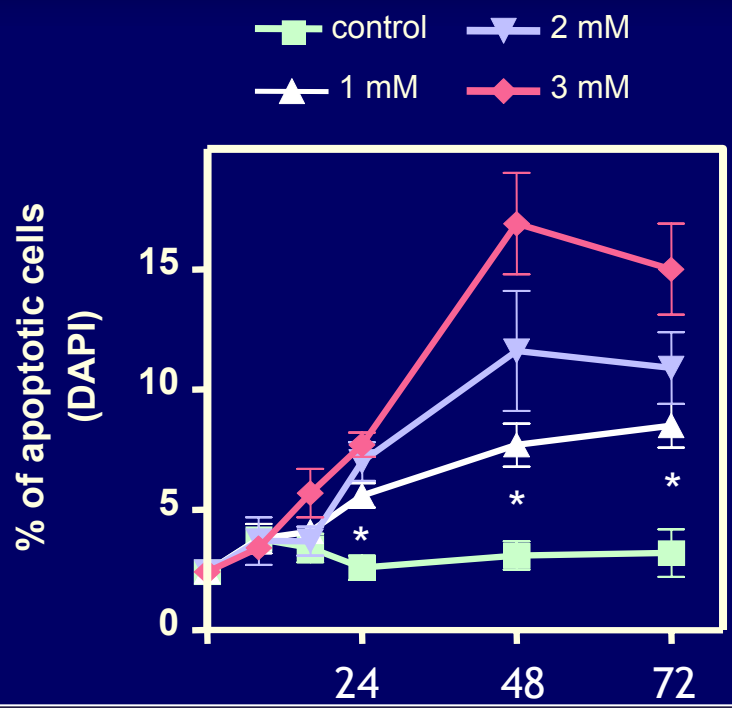


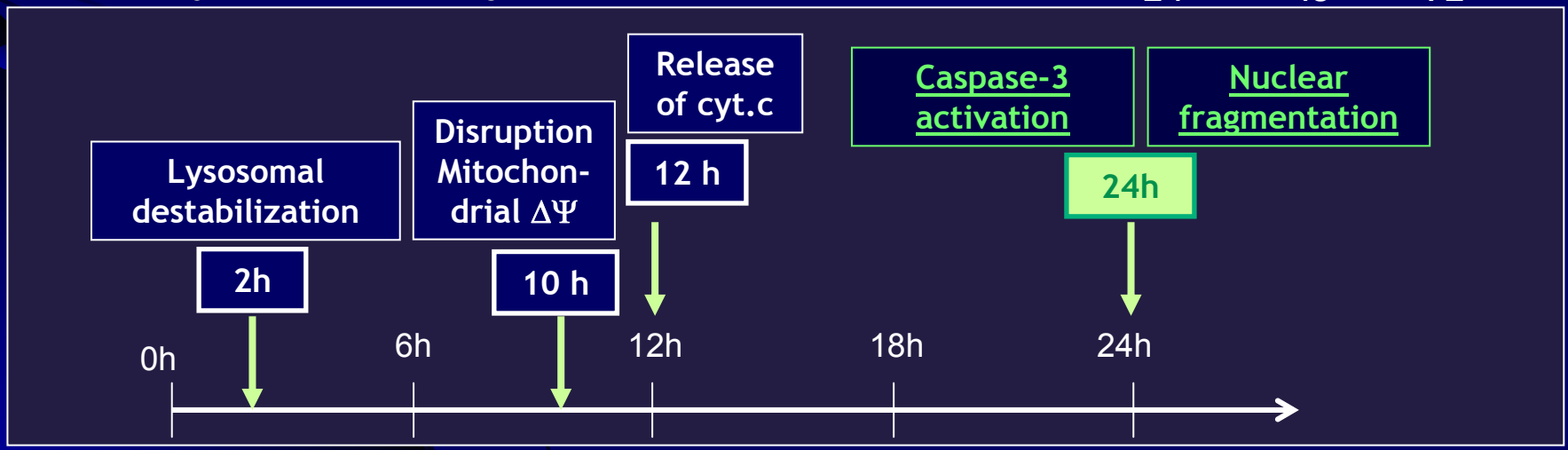
Apoptotic signalling:



PI.4. Time-sequence of the development of apoptosis and caspase-3 activity:







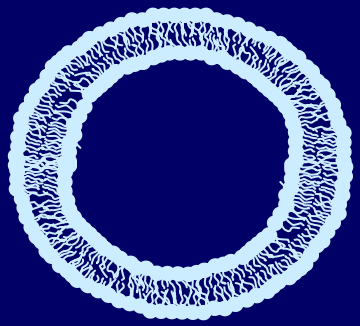
3. RESULTS : PART II

Is GEN able to destabilize membrane?

Mather and Rottenberg 2001 Biochem Biophys Acta 1503:357-368

Van Bambeke *et al.*, 1993 Eur J Pharmacol 247:155-168

PII. Mimicking lysosomes and mitochondria…:



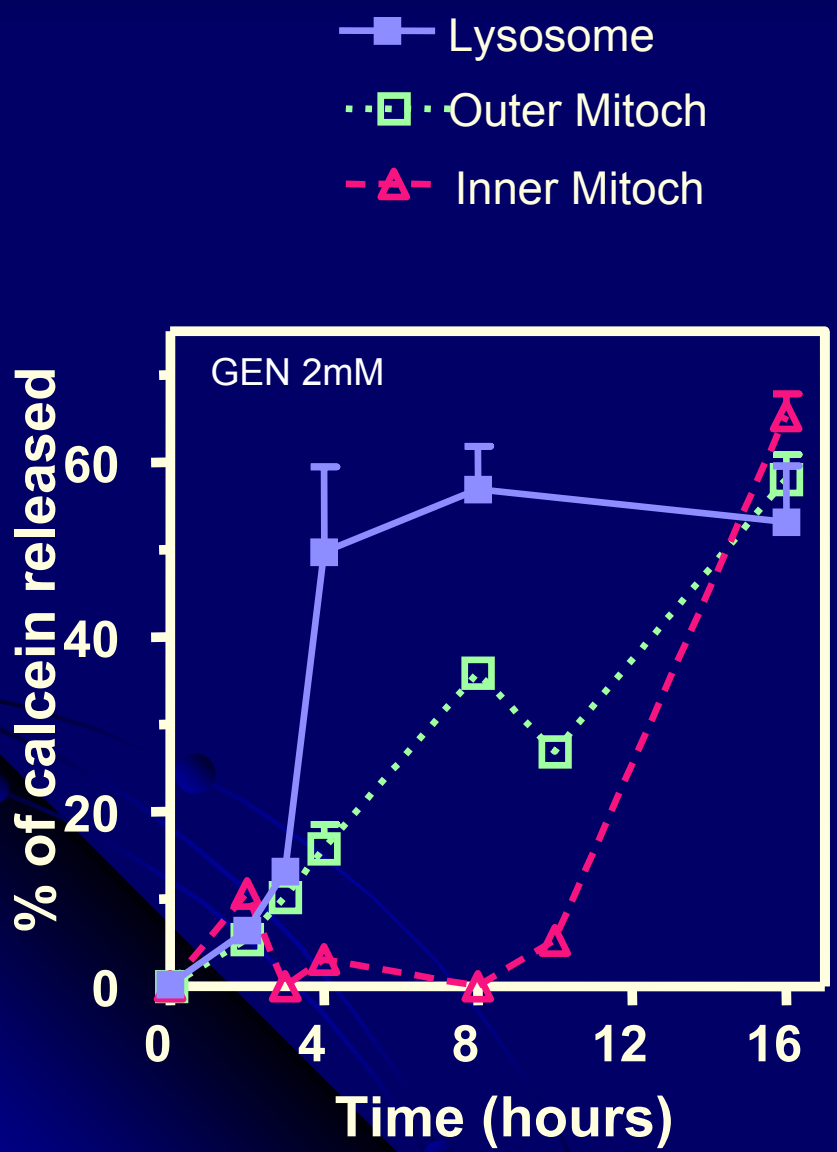
SUV: Small Unilamellar vesicles

Diameter: 50-80 nm

	CHO	PC	SM	PI	CL
LYSO **5.4** Mimic the lysosomal membrane	33.3 %	24.2 %	24.2 %	18.3 %	-
OUTER MITO **7.4** Mimic the outer mitochondrial membrane	33.3 %	24.2 %	24.2 %	18.3 %	-
INNER MITO **7.4** Mimic the inner mitochondrial membrane	33.3 %	24.2 %	24.2 %	-	**18.3 %**

CHO: Cholesterol; PC: Phosphatidylcholine; SM: Sphingomyelin; PI: Phosphatidylinositol; CL: Cardiolipin

PII. Ability of gentamicin to destabilize liposomes :



PI (Lysosomes) pH 5.4

>

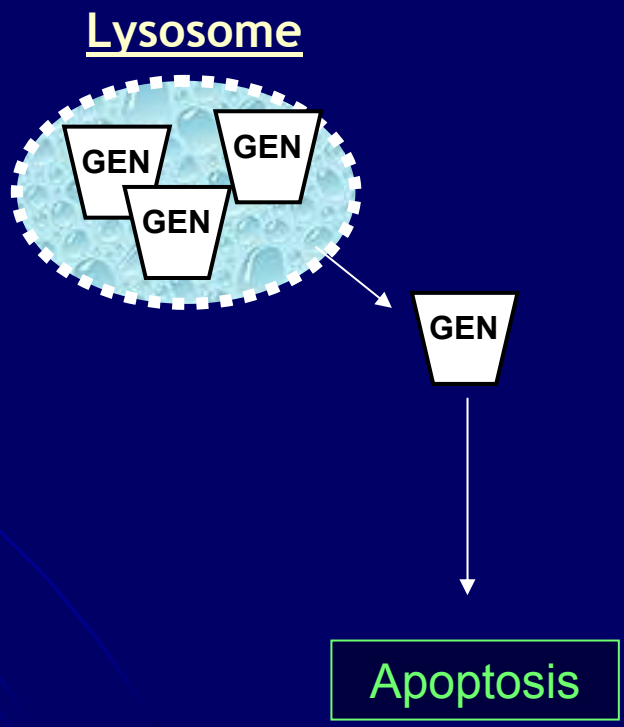
PI (outer mitochondria) pH 7.4

>

Cardiolipin (inner mitochondria) pH 7.4

3. RESULTS : PART III

Is GEN able to initiate apoptotic signalling if localized in the cytoplasm?



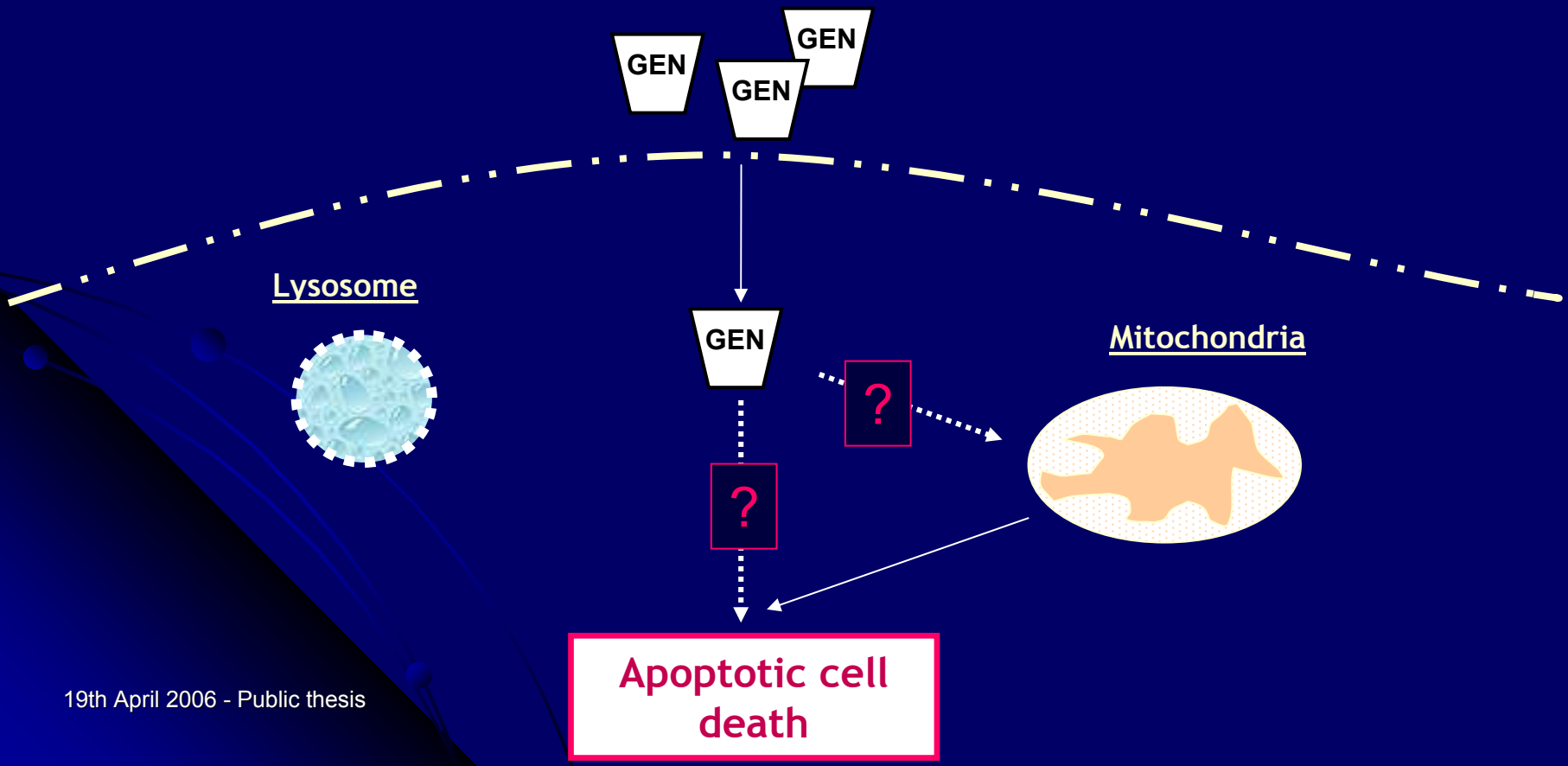
Delivery of GEN inside cellular cytosol

Definition of Electroporation (EP):

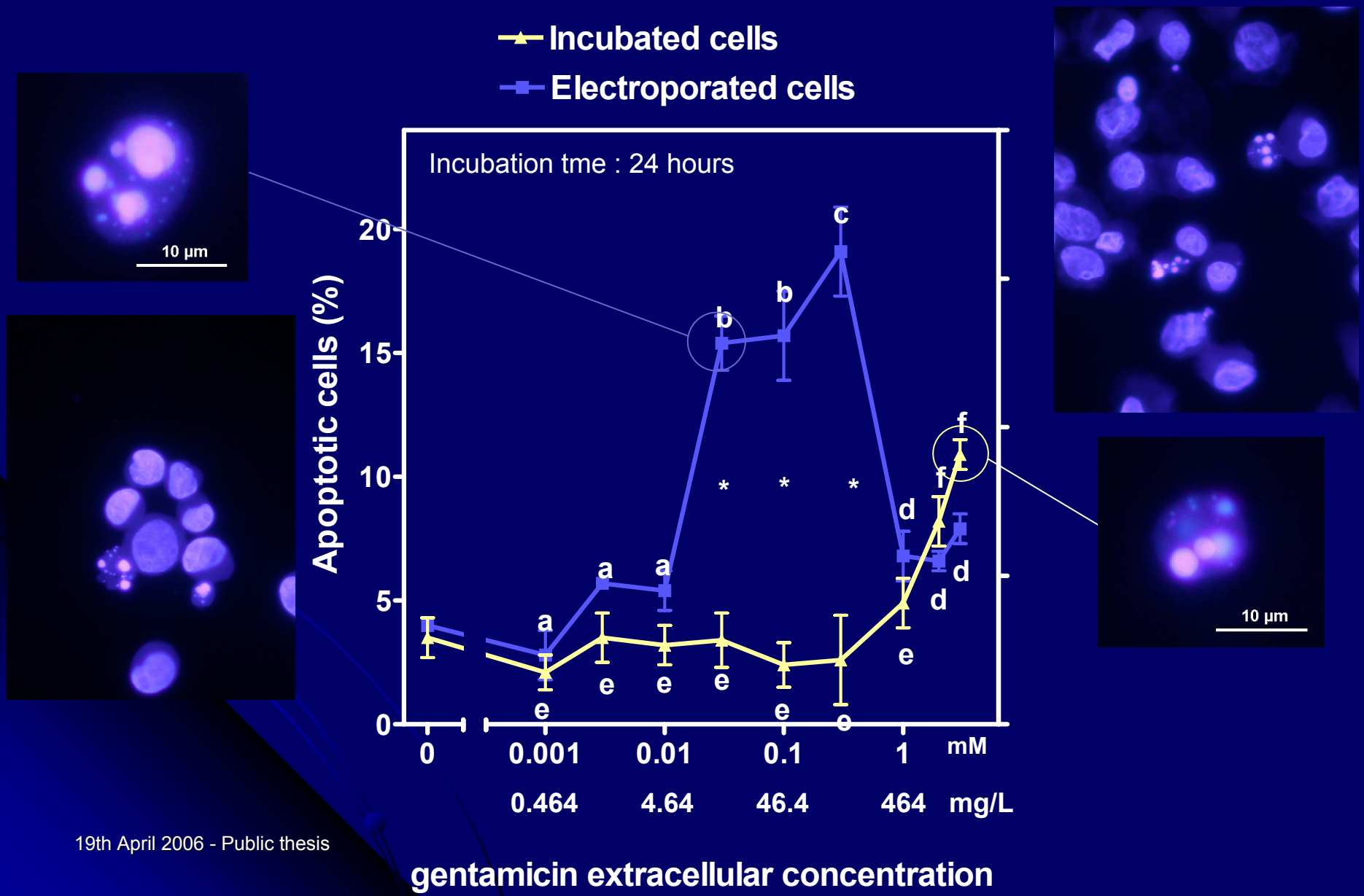
designates the use of SHORT HIGH VOLTAGE PULSE

to OVERCOME the BARRIER of the CELL MEMBRANE

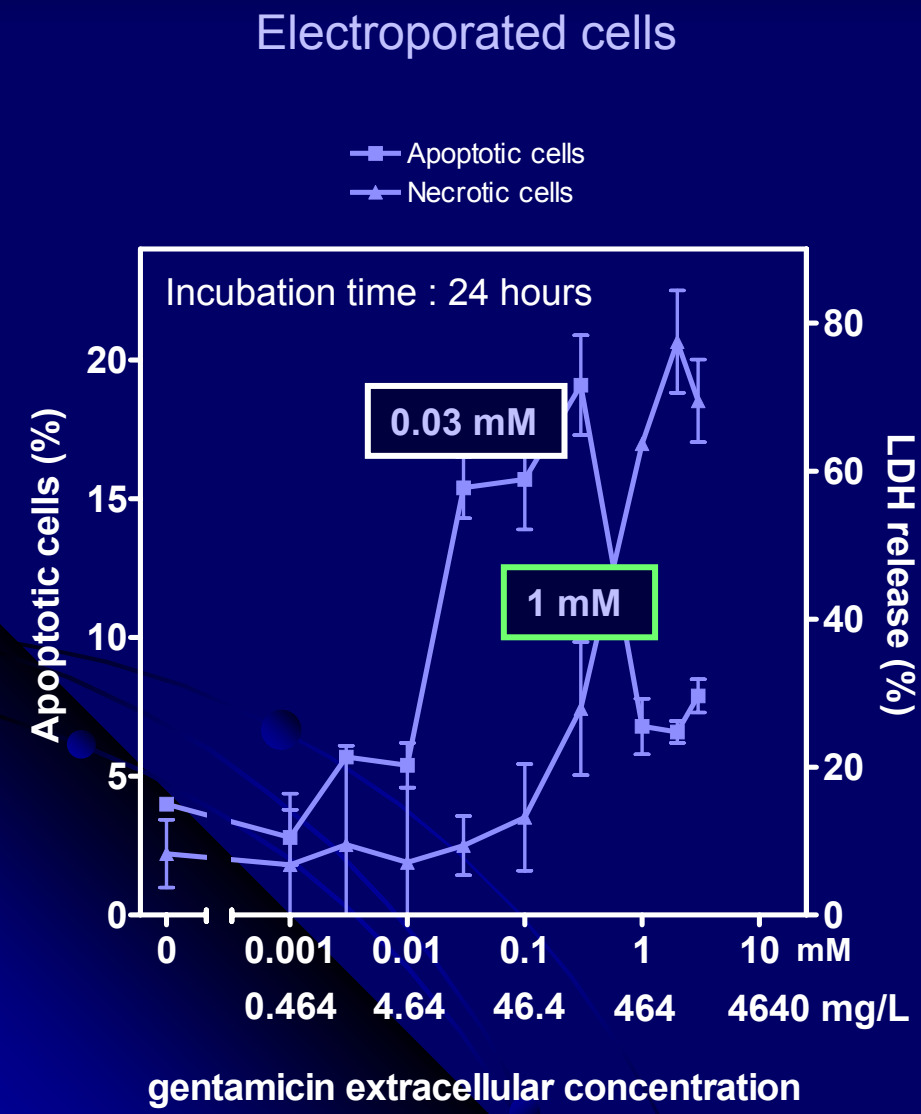
This rupture is TRANSIENT and REVERSIBLE

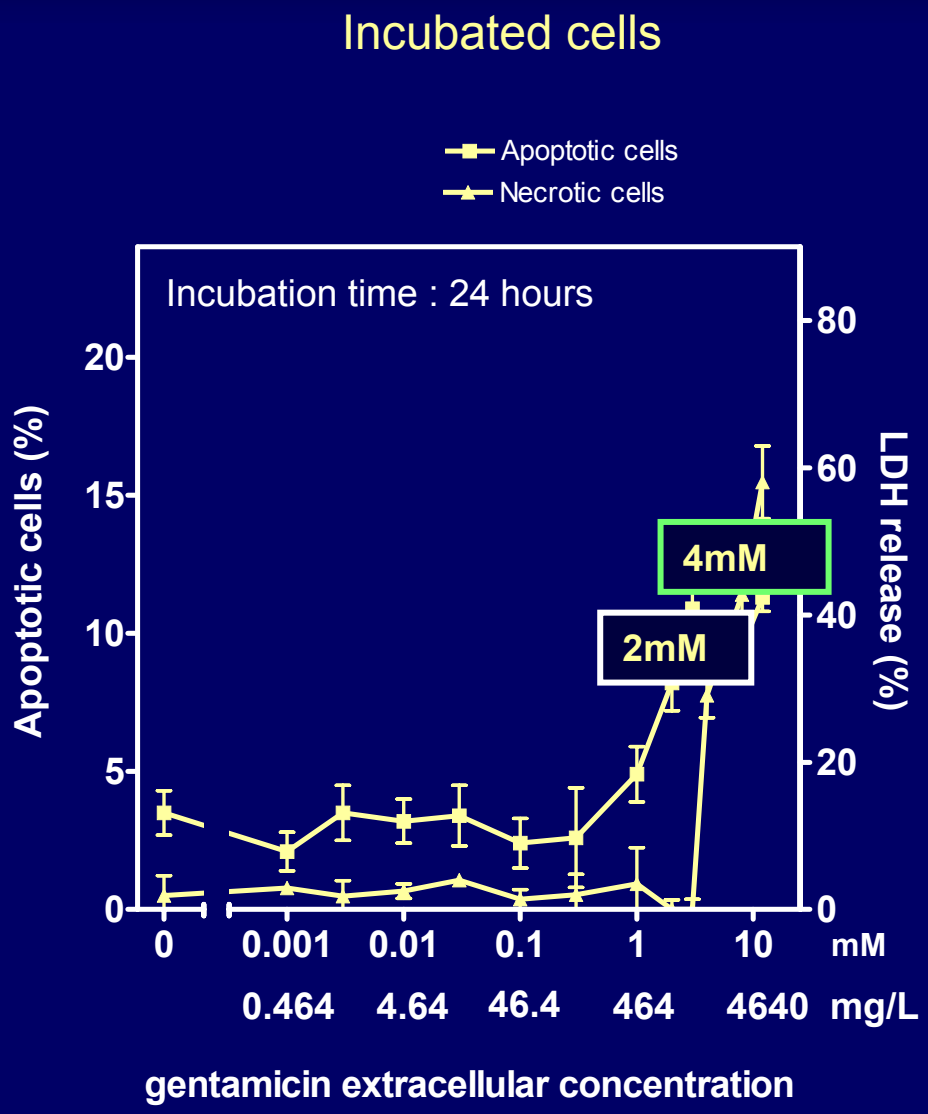


PIII.1. EP cells showed apoptosis with lower concentration of GEN

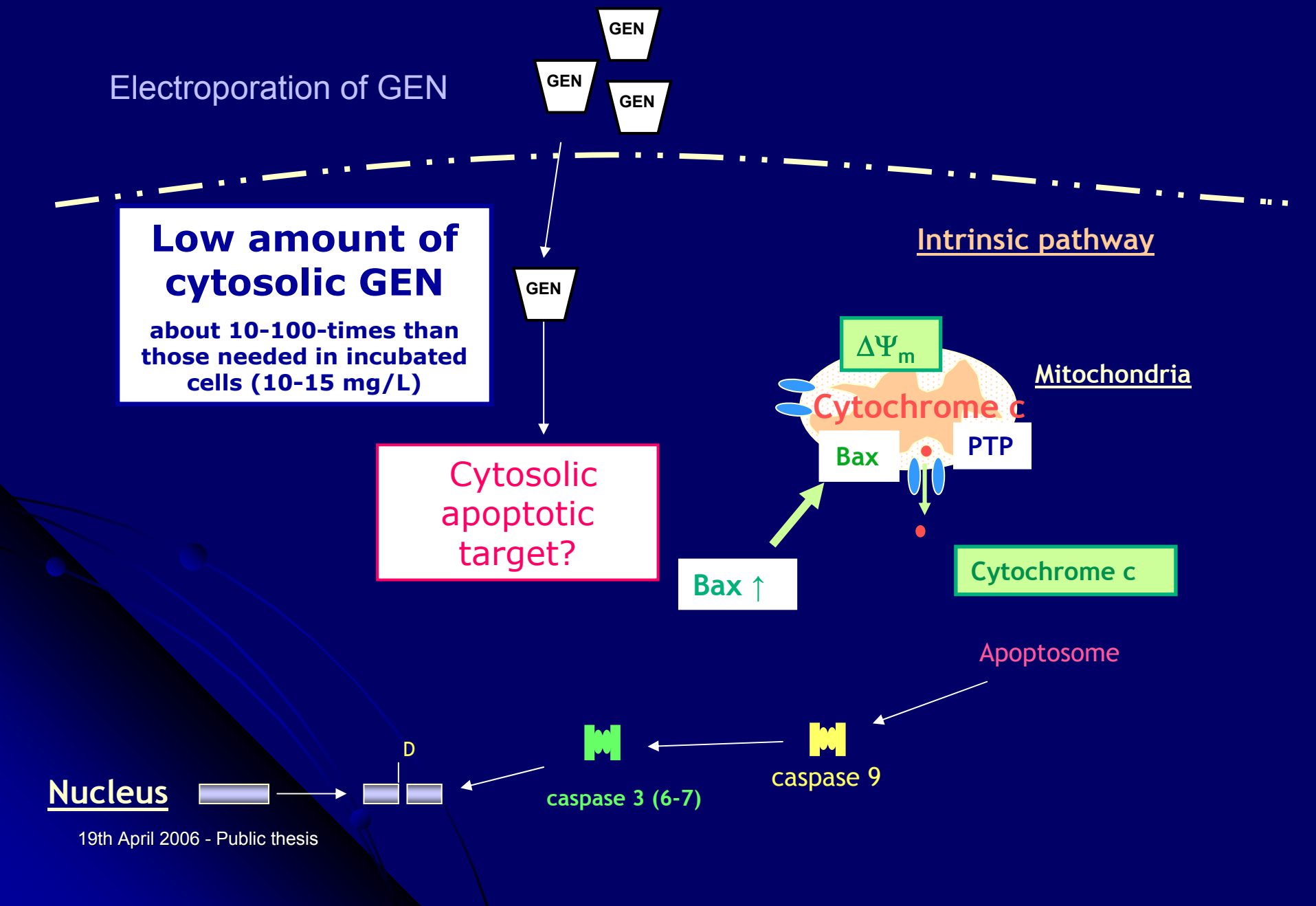


PIII.2. Apoptosis versus necrosis: a question of concentration in both cases (EP versus Inc)

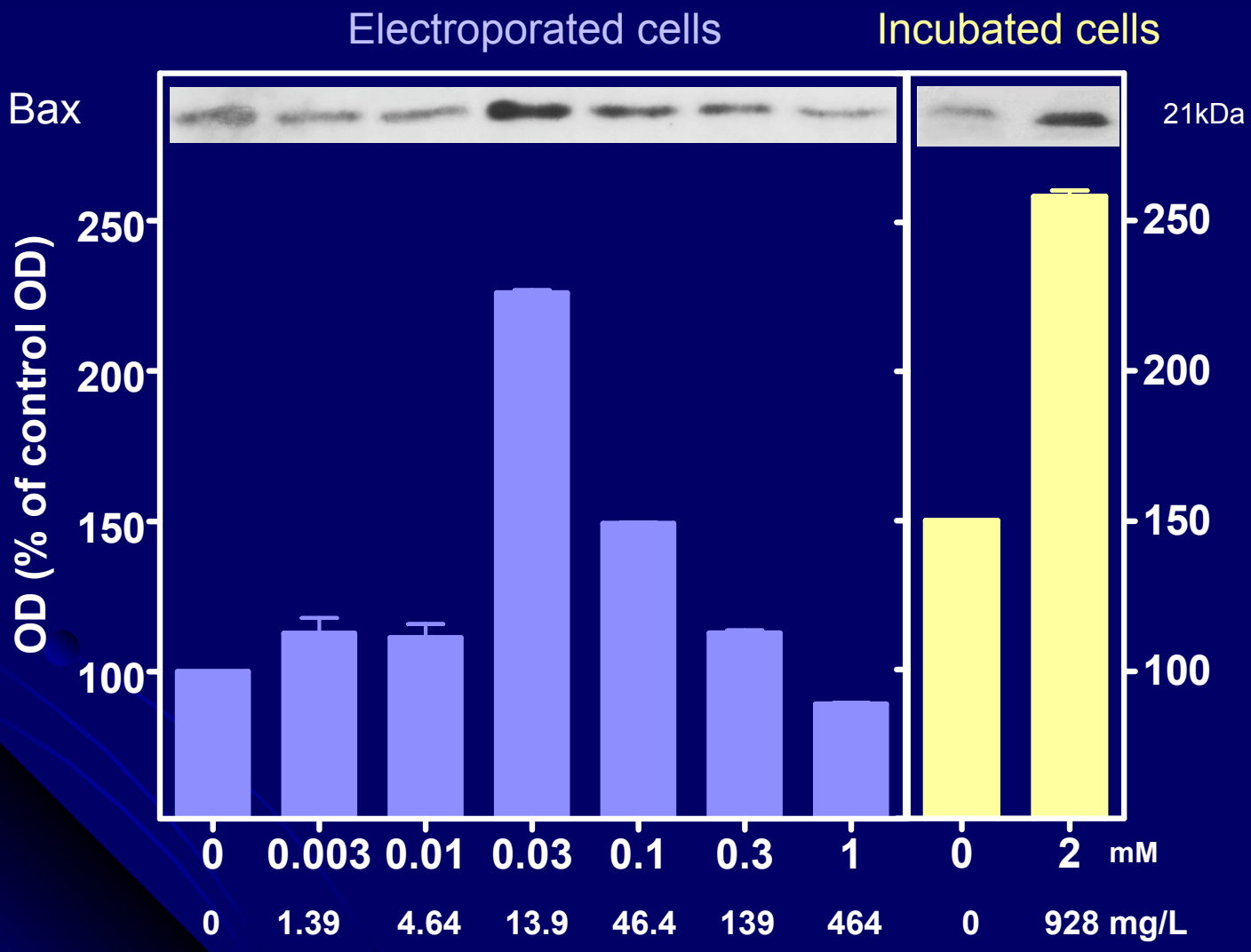




How cytosolic GEN induces apoptotic signalling?

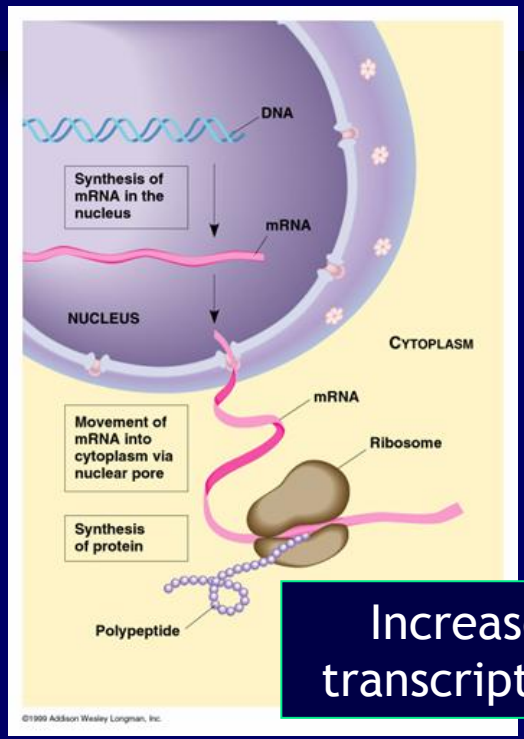


PIII.3 Increase in Bax content after incubation or after electroporation of GEN in LLC-PK1 cells



Incubation time : 8 hours

Increase in Bax cellular content



↑ Bax ⟷ Ubiquitinated Bax ↑

3. Decrease degradation

Ubiquitinated Bax ↓

Inhibitors proteasome

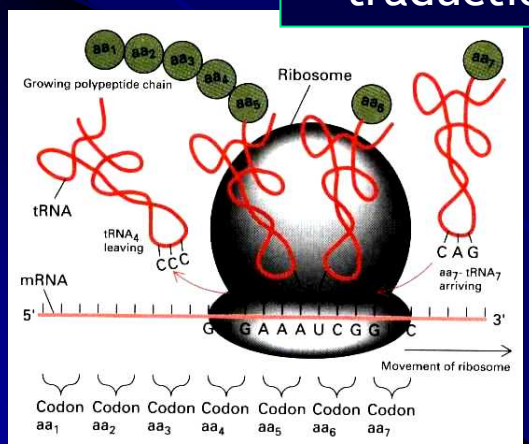
Proteasomal degradation

Increase transcription

Increase traduction

2.

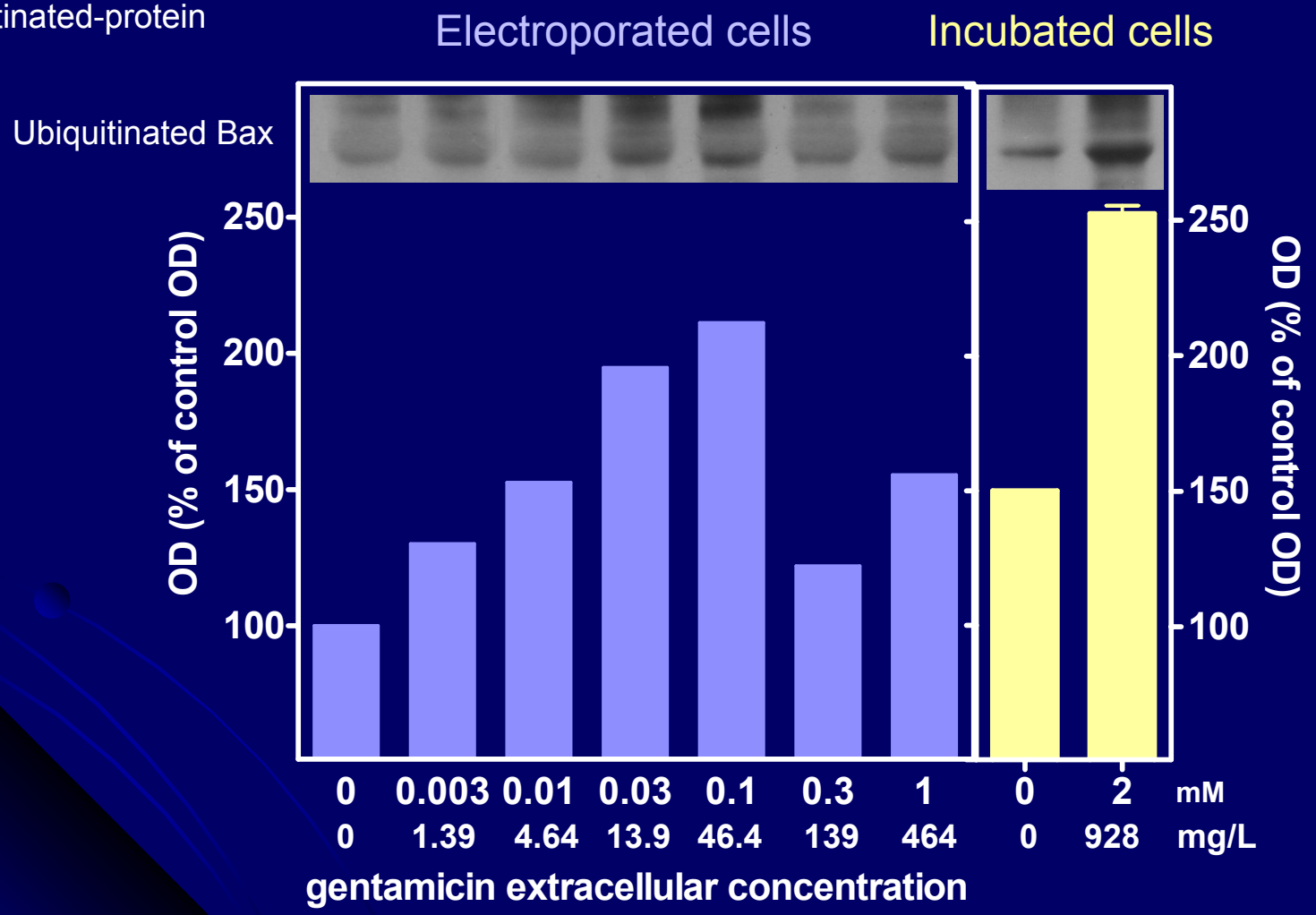
Bax ↑



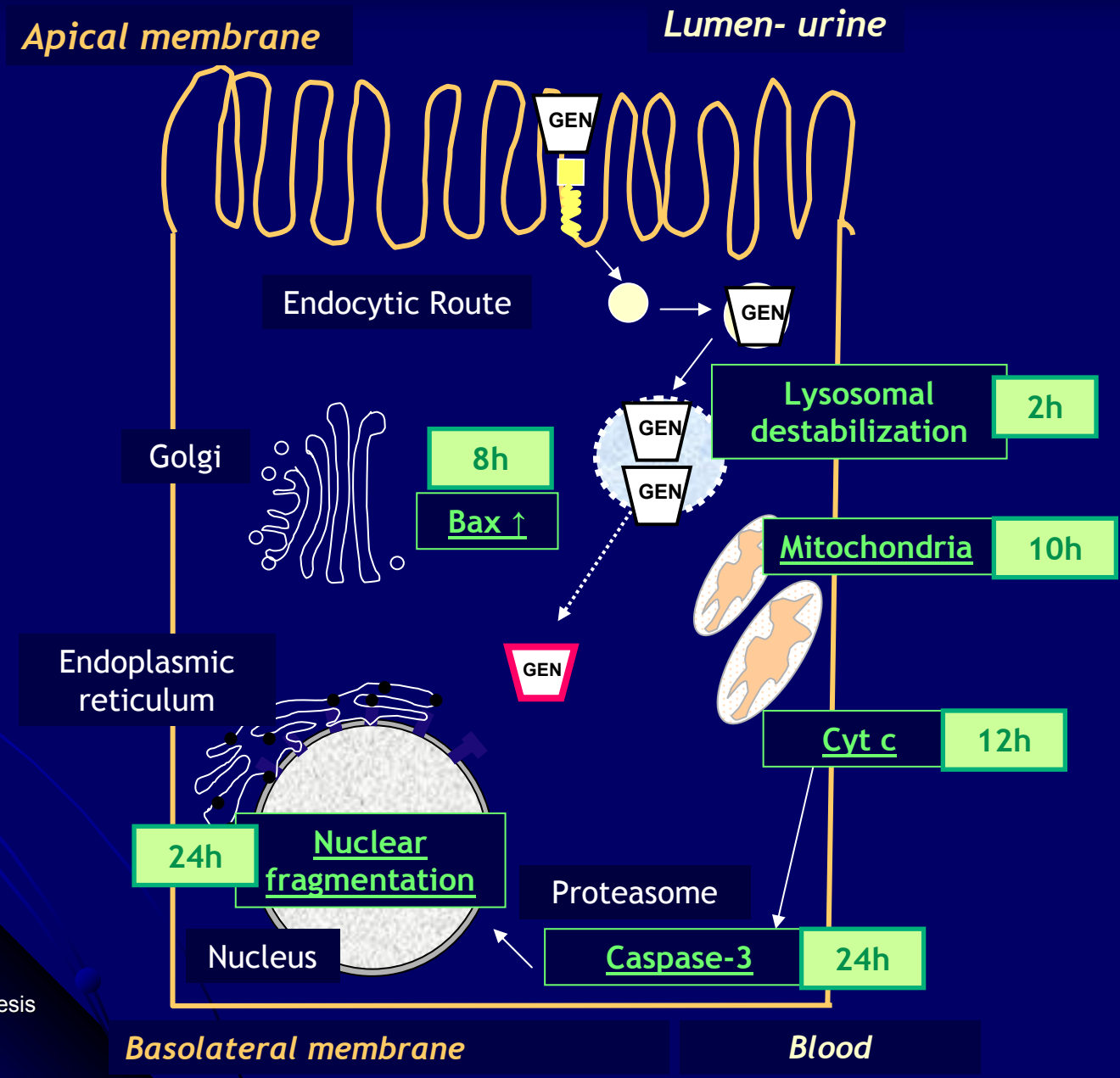
PIII.4. Increase of ubiquitinated Bax content after incubation or after electroporation of GEN in LLC-PK1 cells

IP: Bax

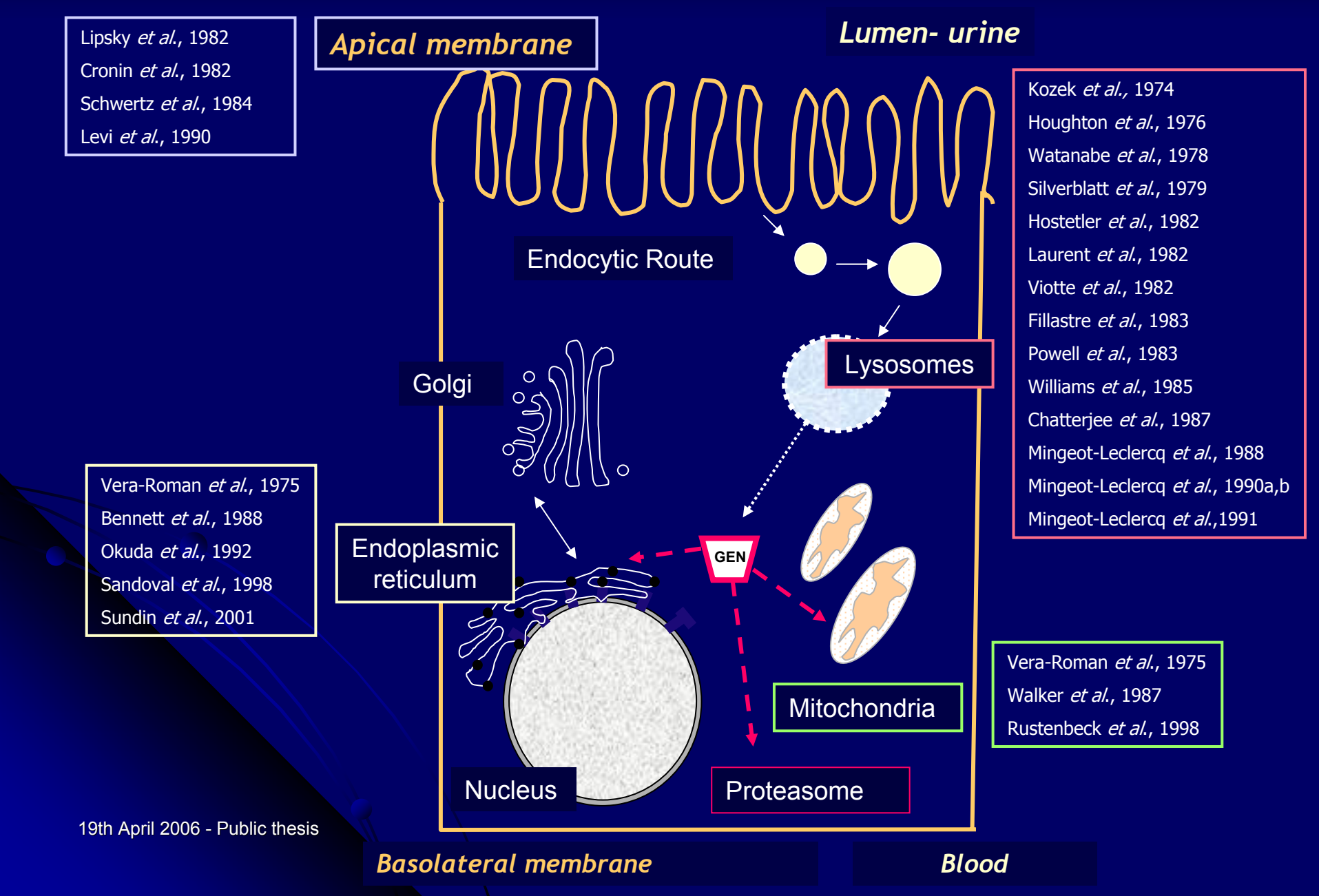
IB: Ubiquitinated-protein



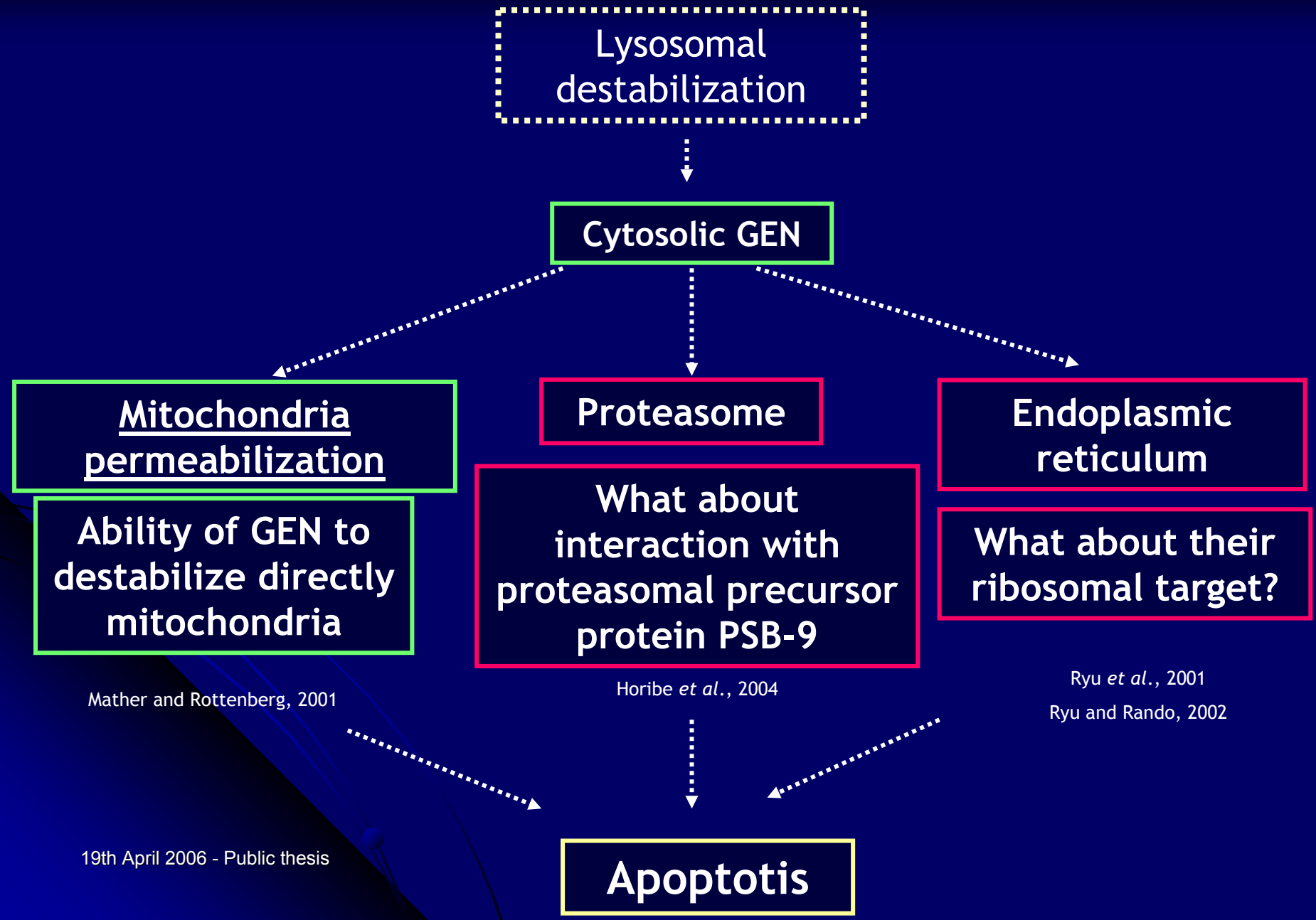
4.GENERAL CONCLUSION AND PERSPECTIVES (1)



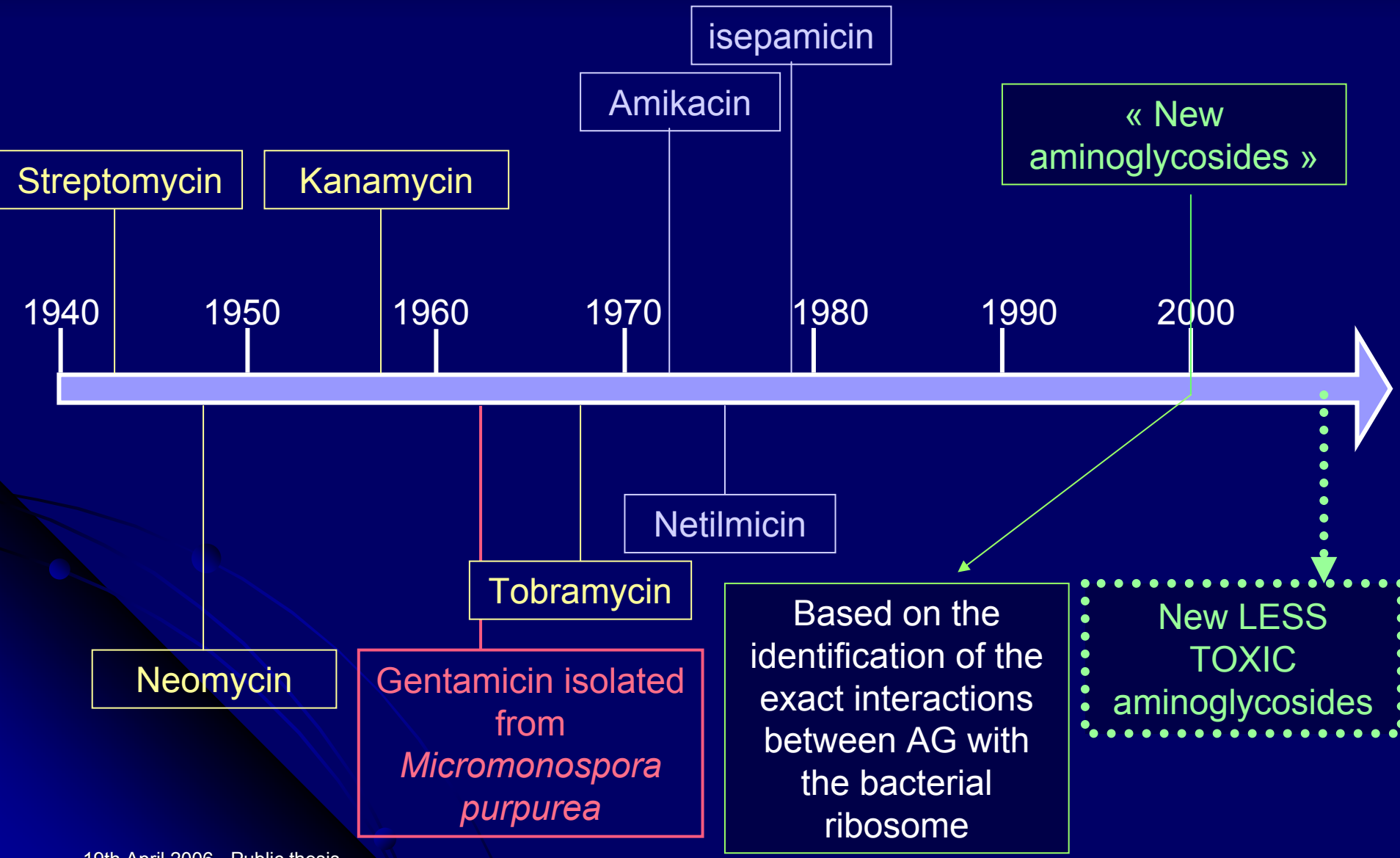
4. GENERAL CONCLUSION AND PERSPECTIVES (2)



4.1 Short term perspectives



4.2. Long term perspectives



And finally……

Lysosomes….

Shield?

Weapons?

….in gentamicin-induced apoptosis?

Acknowledgment

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- .....