Phospholipidosis and its Assessment of Pharmaceuticals

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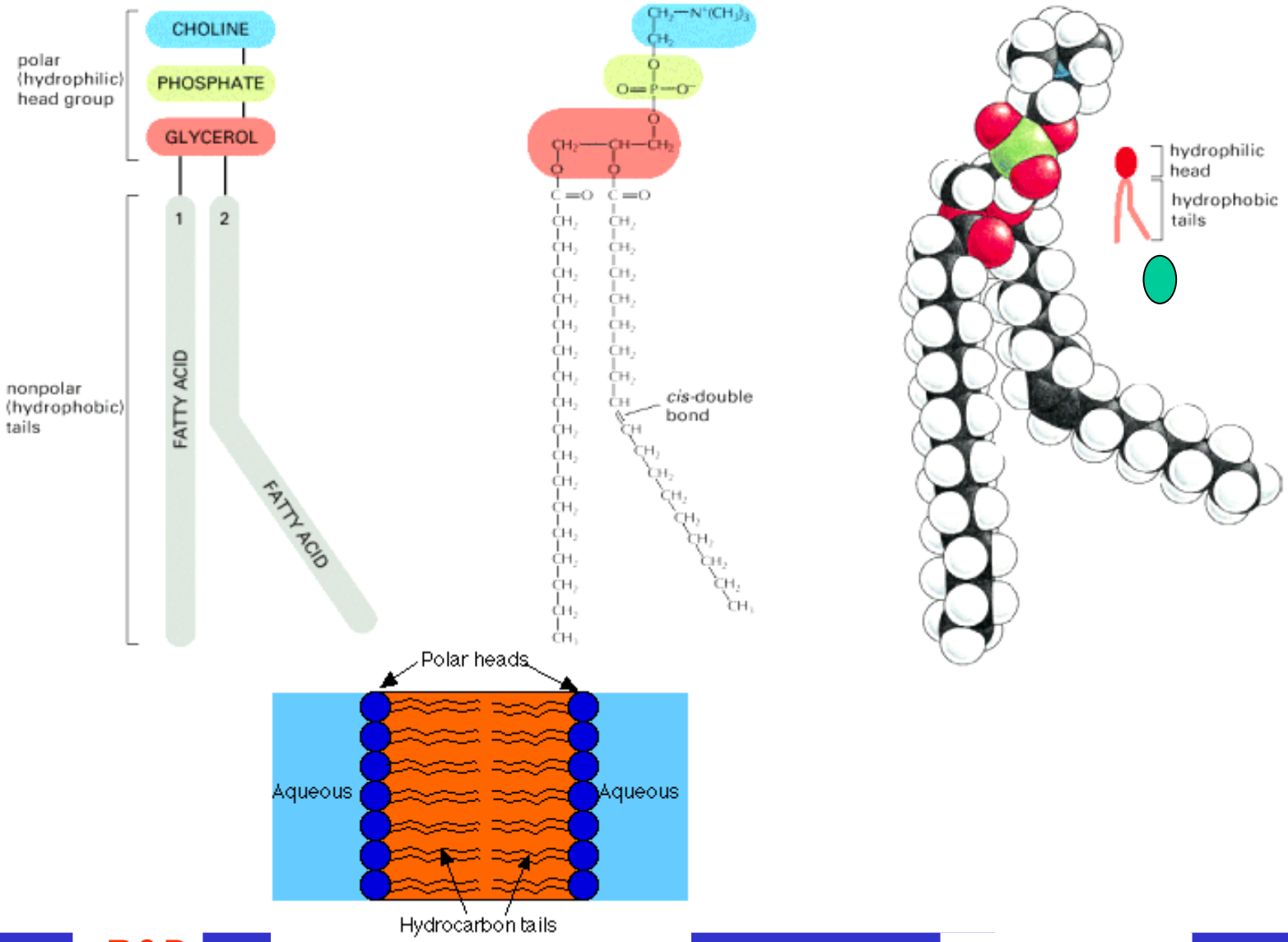
- Phospholipid & cell membrane
- Phospholipidosis and CADs
- Diagnostic hallmark
- Target organs
- Mechanisms
- Reversibility
- Pathological and functional effects
- Prediction & biomarkers
- Risk assessment & regulatory status
- Summary

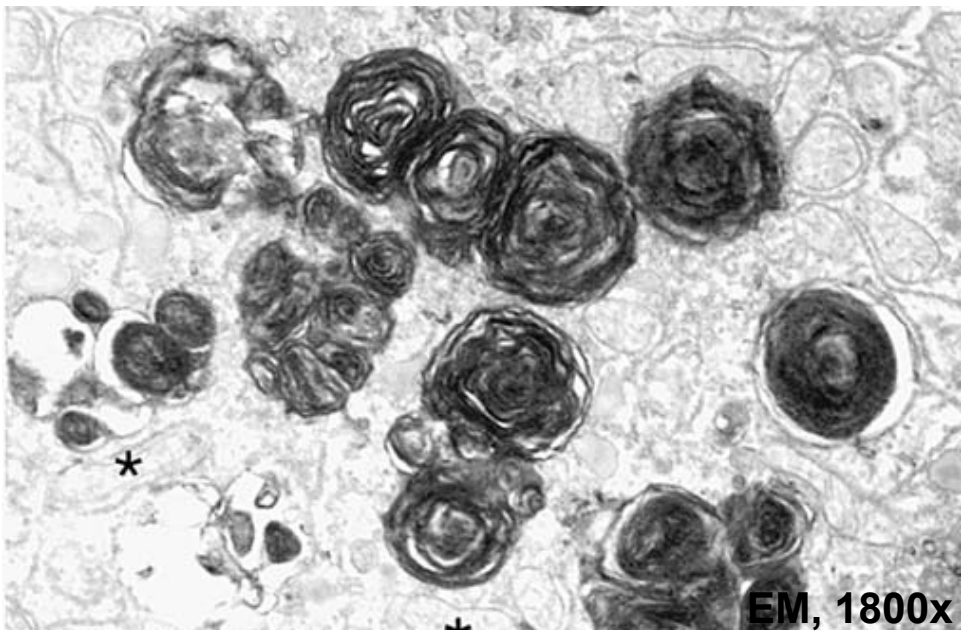
Phospholipids and Cell Membrane

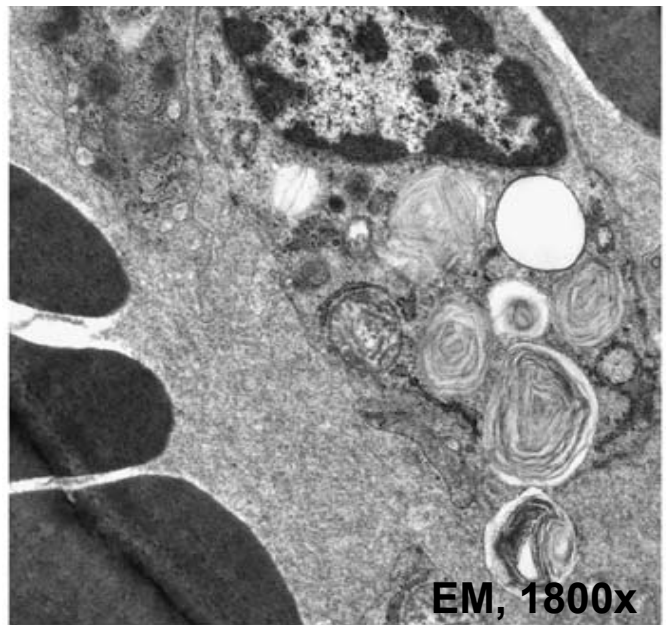


Phospholipidosis (PLDsis)

- Reversible accumulation of polar phospholipids with the development of unicentric or multicentric lamellated bodies/content within cells, most likely from an impaired PLD metabolism of the lysosomes.

- Cationic amphiphilic drugs (CAD) – lysosomotropic behaviour, weak basic property, mostly cross BBB



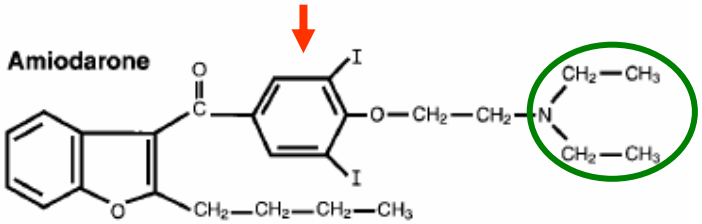


Cationic Amphiphilic Drugs - PLDsis

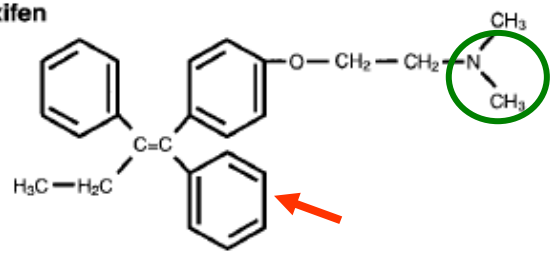
Antidepressants	Fluoxetine, Impipramine, Citalopram, Sertraline, Zimelidine, Fluvoxamine, Iprindole, Chlomipramine
Antibacterial	Gentamicin, Azithromycin, Erythromycin, Amikacin, Tobramycin, Trospectomycin, Netimicin
Antimalarial	Chloroquine, Mepacrine
Anorectic	Chlorophenetermine, Cloforex, Fenfluramine
Antiarrhytmic	Amiodarone
Antianginal	Perhexiline
Antipsychotic	Clozapine
Antiviral	Tilarone
MMP	ABT-770
Antiestrogen	Tamoxifen
Antihistaminic	Chlorcyclizine, Meclizine, Norchlorcyclizine, Hydroxyzine
Secretolytic	Bromhexine, Ambroxol

Cationic Amphiphilic Drugs

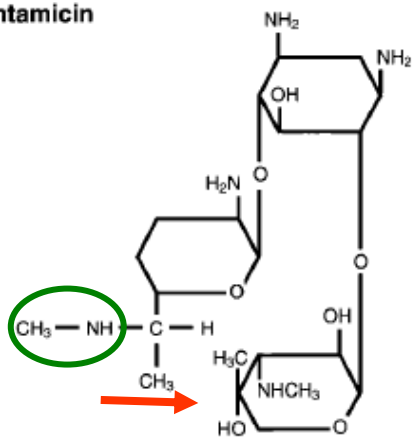
Amiodarone



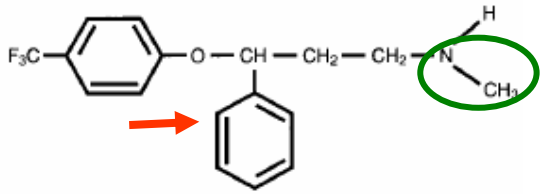
Tamoxifen



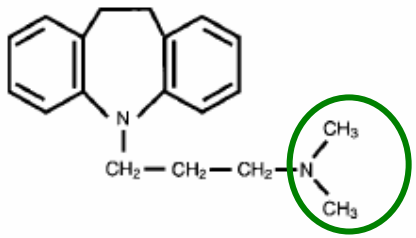
Gentamicin



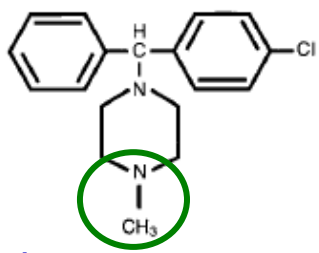
Fluoxetine



Imipramine



Chlorcyclizine



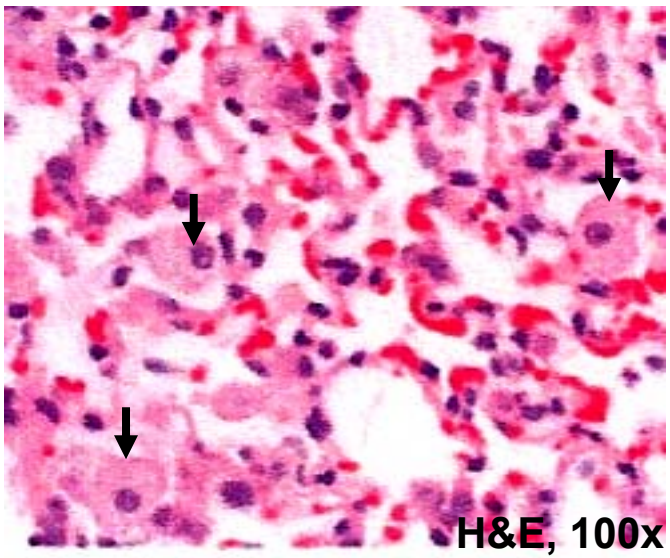
Hydrophilic region with amine gp., positively charged at physiological pH.

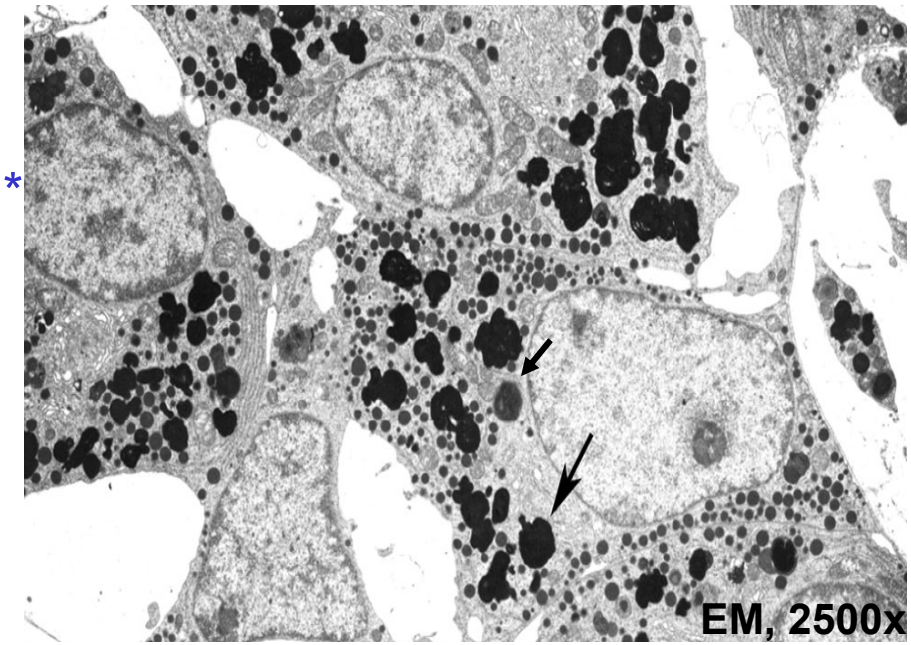
Hydrophobic region with aromatic ring str.

Diagnostic Hallmarks

- EM: Concentric lamellar bodies/cytoplasmic inclusions/myelin like bodies.

- Microscopy
  - Enlarged pale cells/vacuolated*

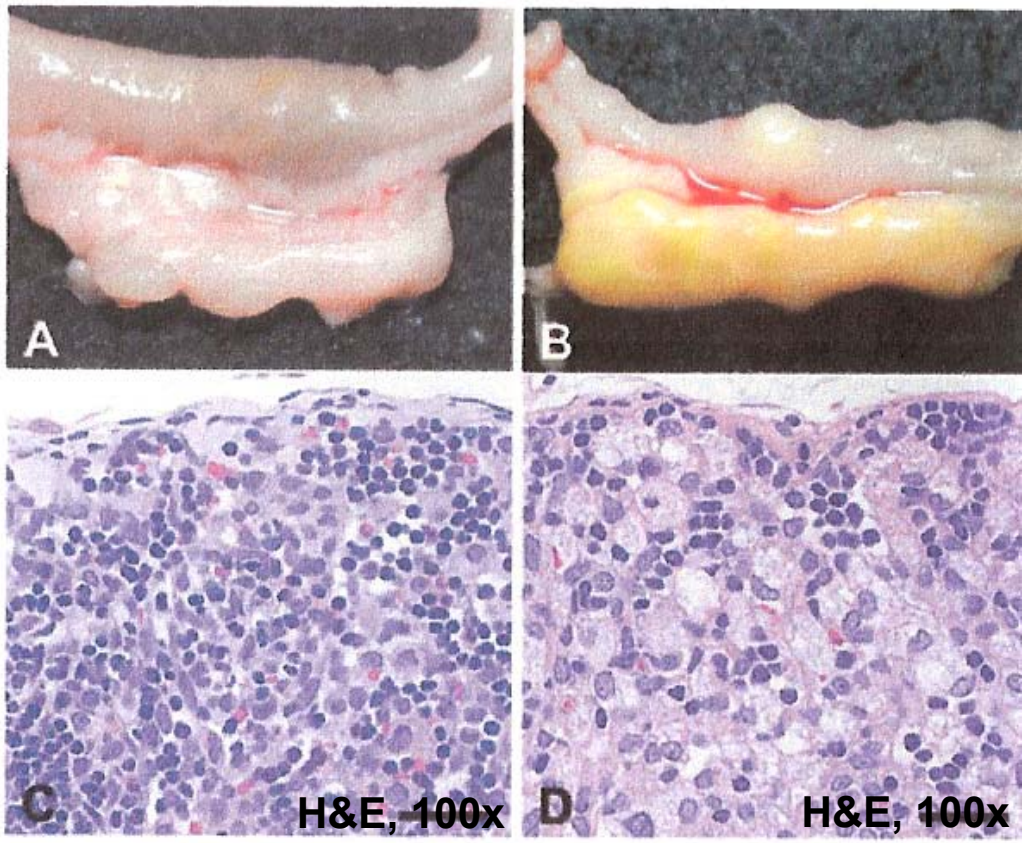




* Not always PLDsis. Nile blue stain for differentiation.

Diagnostic Hallmarks

- In some cases, organs show similar color to the drug



Mesentric LN:

A. Normal

B. Treated with GPCR antagonist

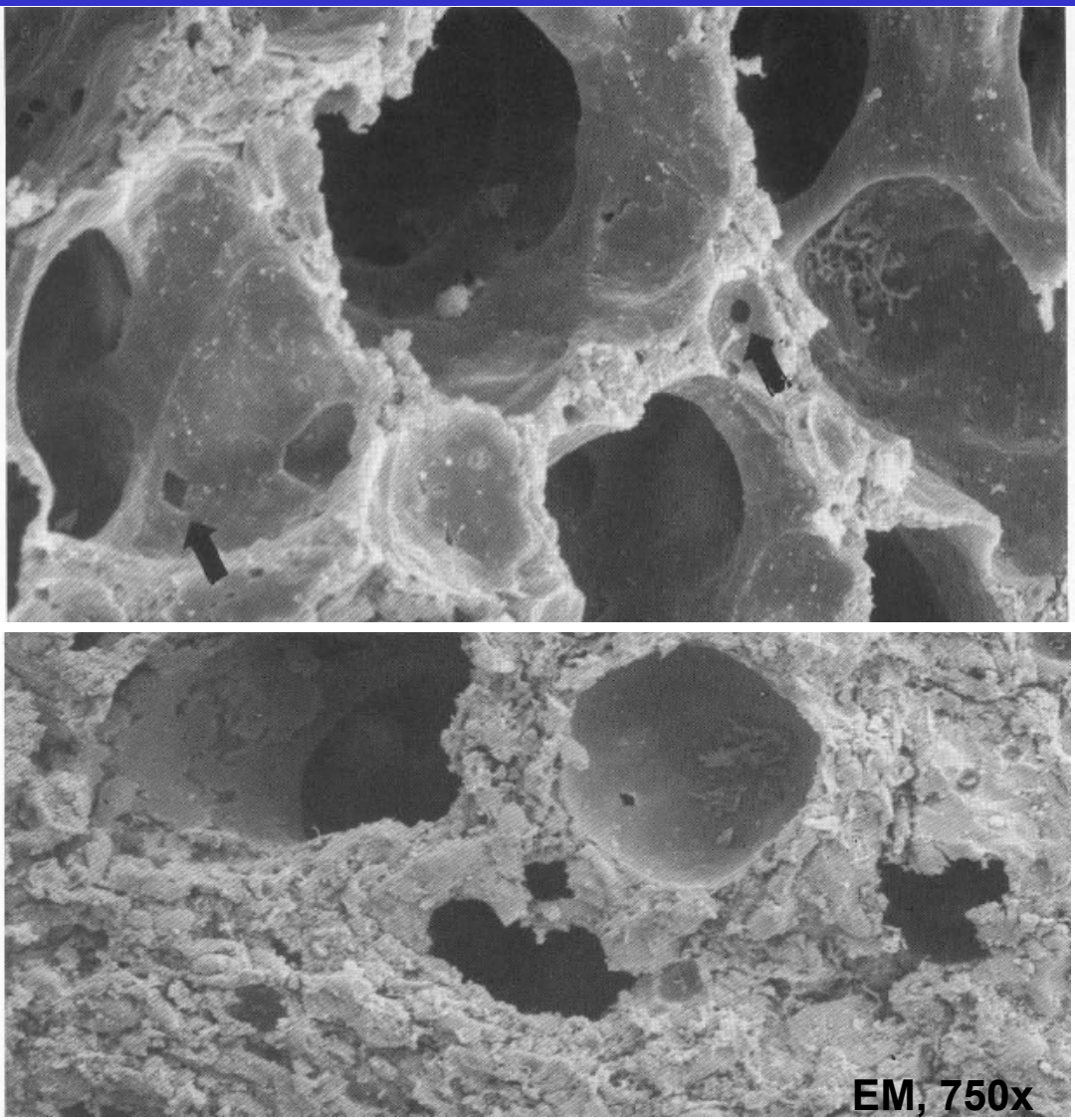
C. Normal

D. Macrophage showing foamy appearance

Nonoyama and Fukuda, 2008

- Cytoplasmic vacuolation of the lymphocytes
- In severe cases, increased content of phospholipid in cell or tissue

Diagnostic Hallmarks



Scanning EM:
Normal alveolar walls & lumen

Lumens filled with cellular & fibrous material

Robison et al., 1985

Diagnostic Hallmarks

Distribution of lesions:

1. Macrophage dominant
   - Infiltration of foamy macrophages in lung/LN.
   - Foamy macrophages lining beneath the pleural alveolar spaces than angular parts of pulmonary lobes
   - In advances cases, macrophages diffusely infiltrate, occupy the alveolar spaces with eosinophilic flocculent/granular materials in the air space.
   - Increased number of lamellar bodies. Lung type II cells and alveolar macrophages (secondary) contain lamellar bodies under normal condition. Lamellar bodies are involved in surfactant storage and secretion and represent about 20 % (maximal 30%) of the volume of type II cells.

Diagnostic Hallmarks

Distribution of lesions:

2. Parenchymal cell dominant

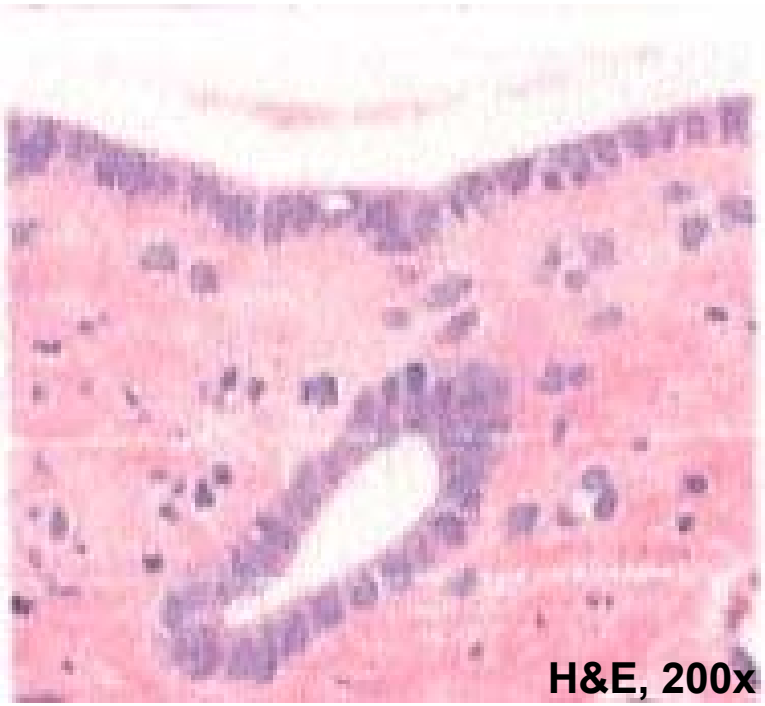
- Hepatocytes, renal tubular epithelial cells, bile ductal cells, endocrine cells, striated and smooth muscle cells, endothelial cells, nerve cells in CNS & PNS may be affected.

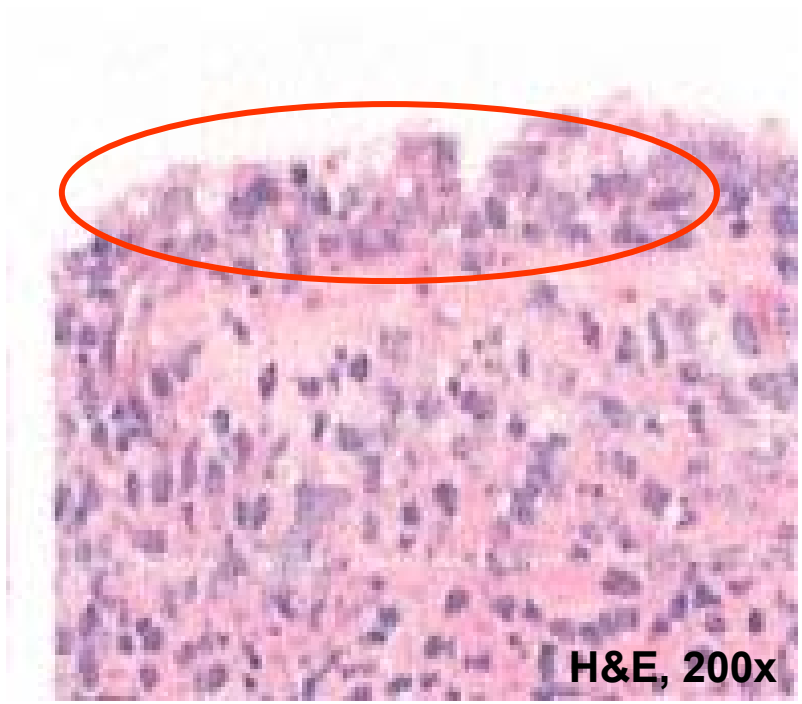
3. Localized PLDsis

- Vacuoles on the apical portion of cytoplasm in the bile ductal cells.
- Higher exposure of drug
- Similarly renal and nerve cells in the brain

Mark Reasor, 2006

Diagnostic Hallmarks – Bile Duct Change



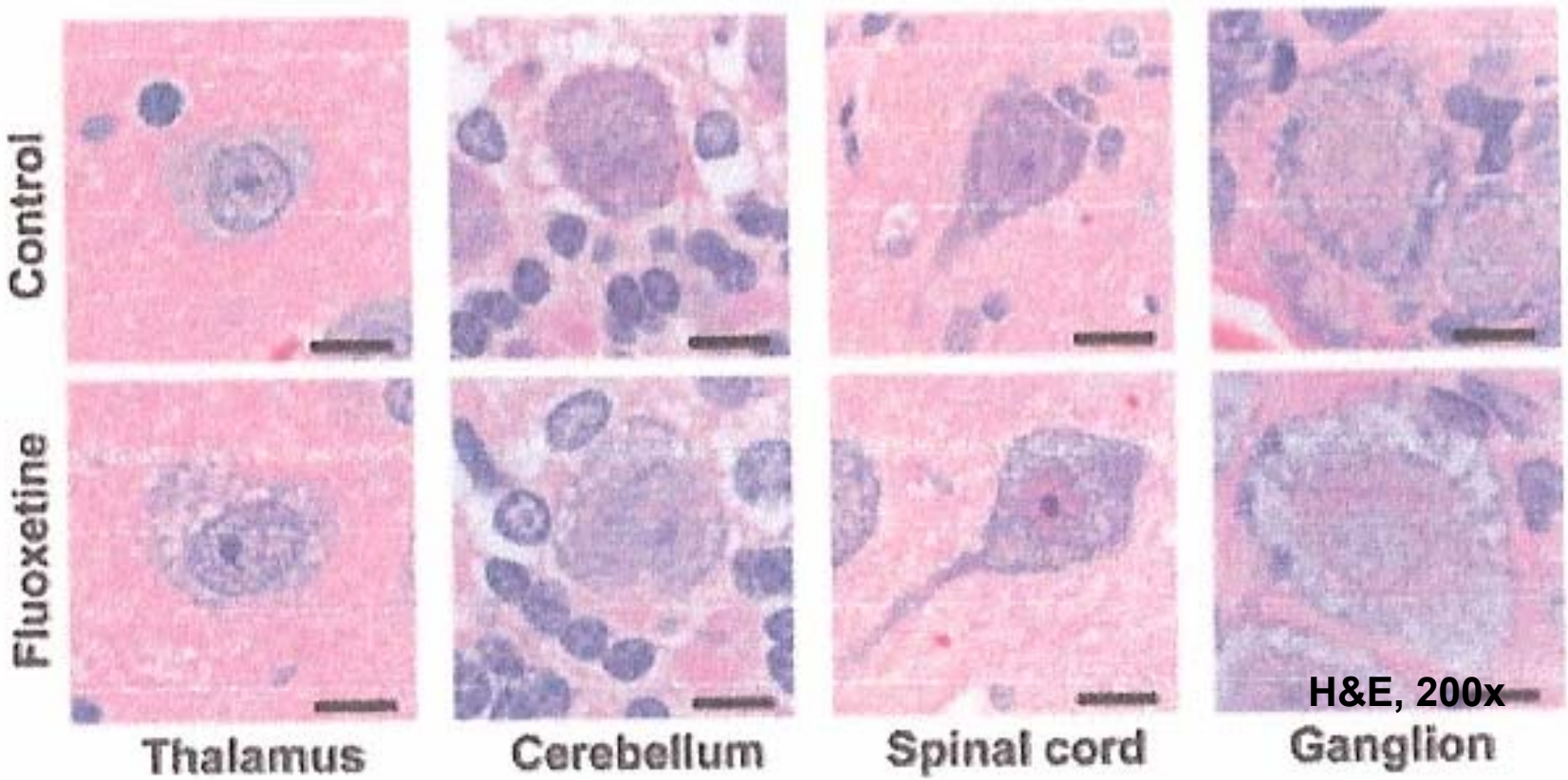


Bile duct:

a) **Control**

b) **Treated with anti-rheumatic agent showing vacuolation and necrosis of epithelium**

Diagnostic Hallmarks – Nervous System



Nervous system:

a) Upper plane – Normal appearance

b) Lower plane – Intracytoplasmic vacuoles

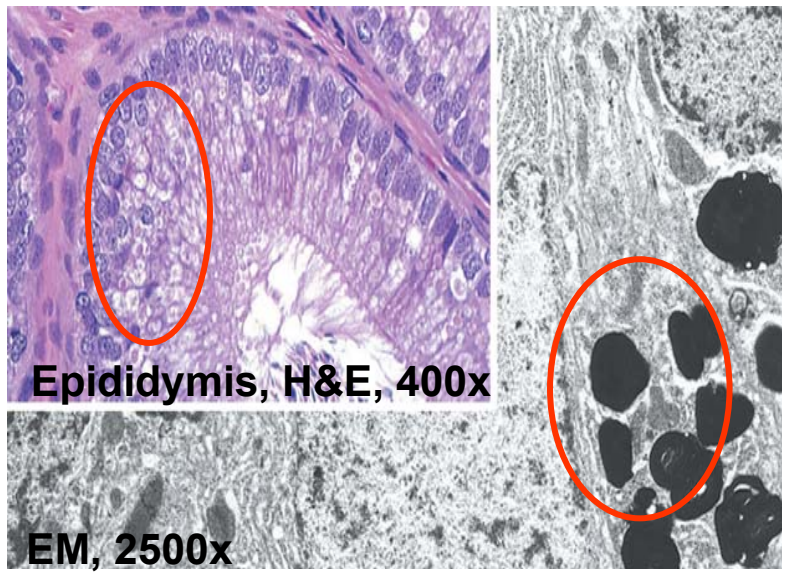
Nonoyama and Fukuda, 2008

Target Organs for PLDsis

- Virtually any tissue
- Lung, liver, brain, cornea, gall bladder, lymph nodes, kidneys, adrenal gland, epididymis, peripheral lymphocytes, pituitary and skeletal muscle.
- The process/induction depends of the affinity of CADs, species, strain age, dose and drug specific PK and PD property.

Tissue	Dog	Rat
Adrenal gland	-	+
Alveolar Macrophages	+	++++
Skeletal muscle	-	+
Epididymis	++	+++

E.g., Dopamine selective antagonist, PNU-177864, tissue distribution of PLDsis in dog and rat



Rudman et al., 2004

Target Organs for PLDsis

Species differences in tissue distribution of PLDsis with Dopamine selective antagonist, PNU-177864

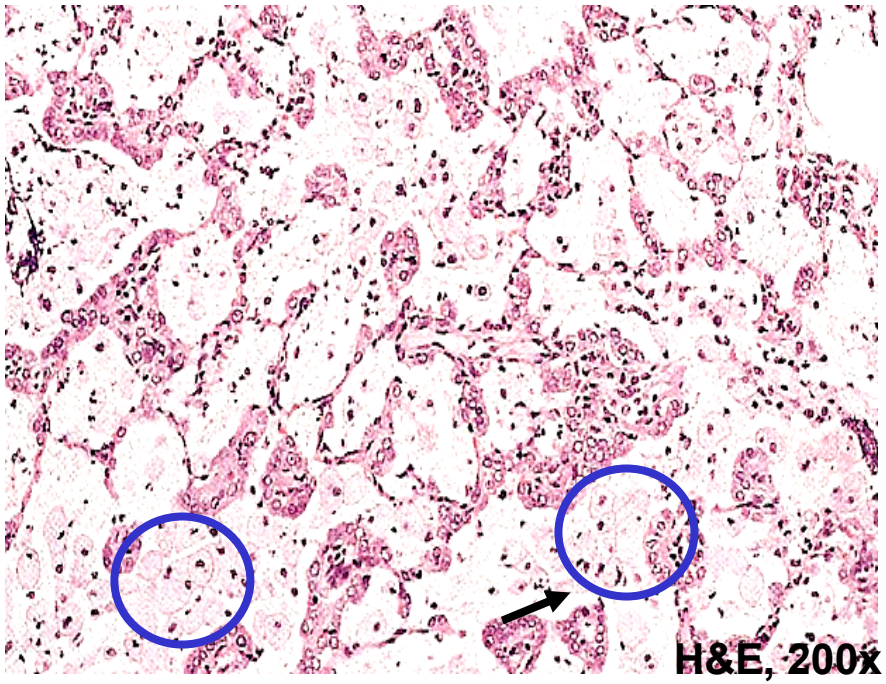
Tissue	Dog	Rat
Adrenal gland	-	+
Alveolar Macrophages	+	++++
Skeletal muscle	-	+
Epididymis	++	+++

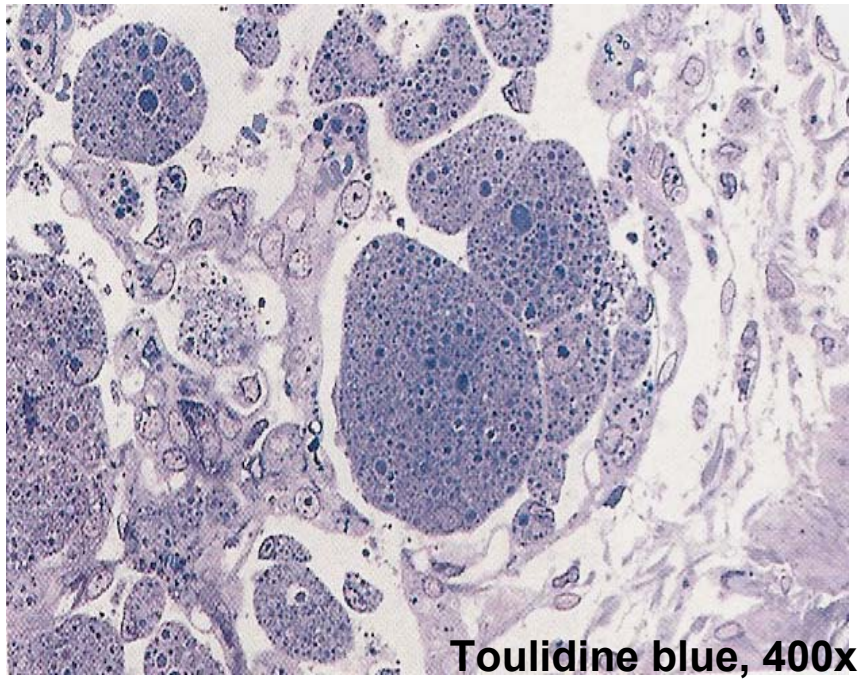
Target organ specificity with various drugs in inducing PLDsis in rats

Tissue	Control	Amiodarone	Azithromycin	Gentamicin
Liver	-	+	-	+++
Lung	-	+++	++	++
Kidney	-	+++	++	+++
Lymph node	-	+	_	+

- Not observed, + very few, ++ mild, +++ moderate

Target Organs for PLDsis


H&E, 200x

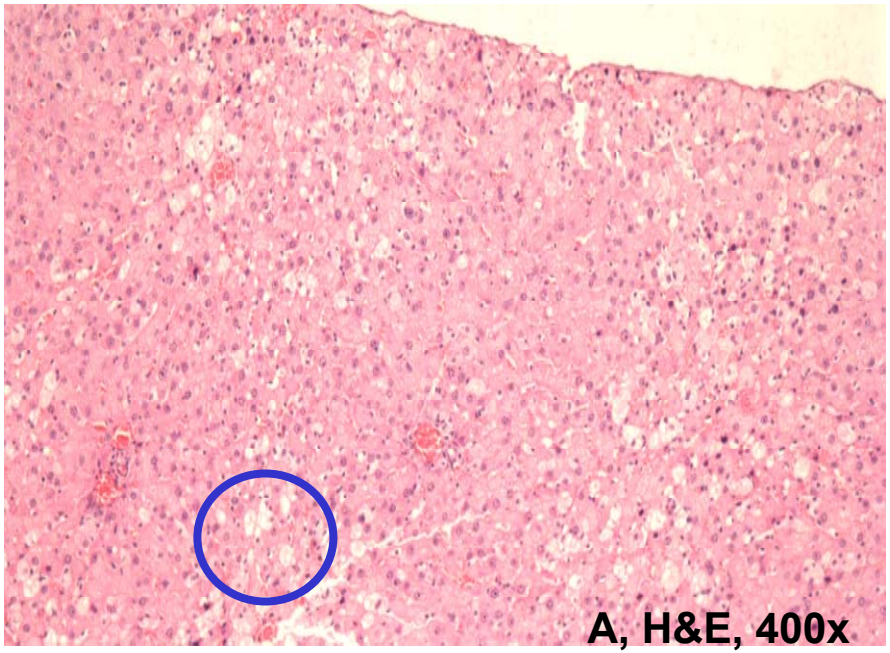

Toulidine blue, 400x

Swollen alveolar macrophage with a foamy appearance. Also see for thickened alveolar wall.

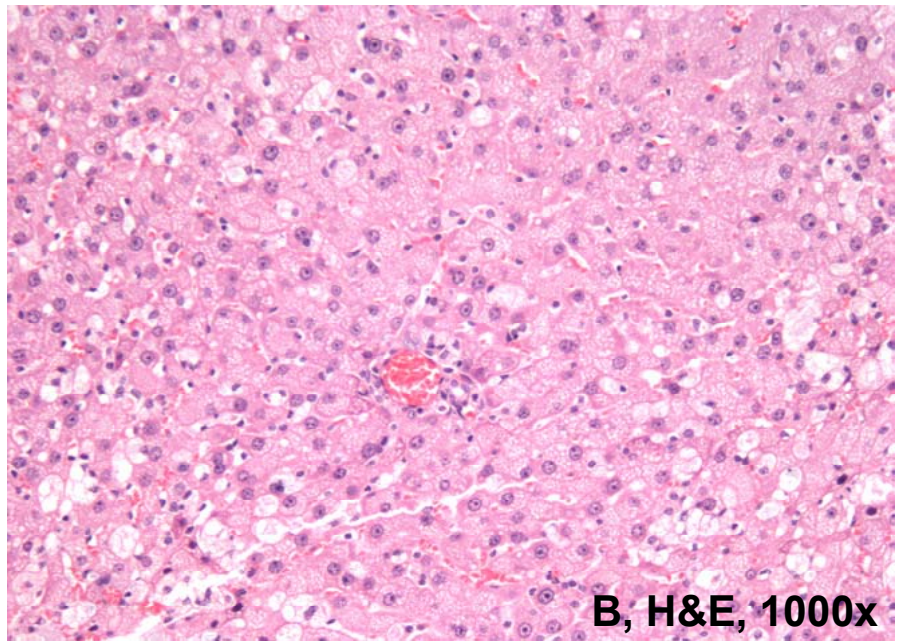
Alveolar macrophage with a distended, packed with round inclusions.

Gopinath et al, 1987

Target Organs for PLDsis

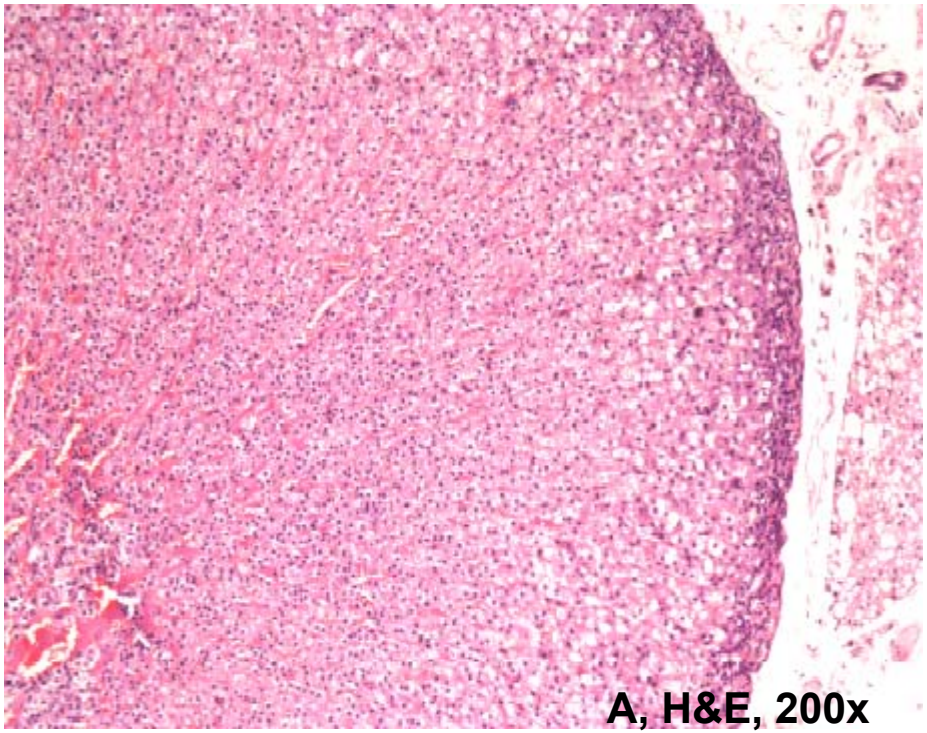


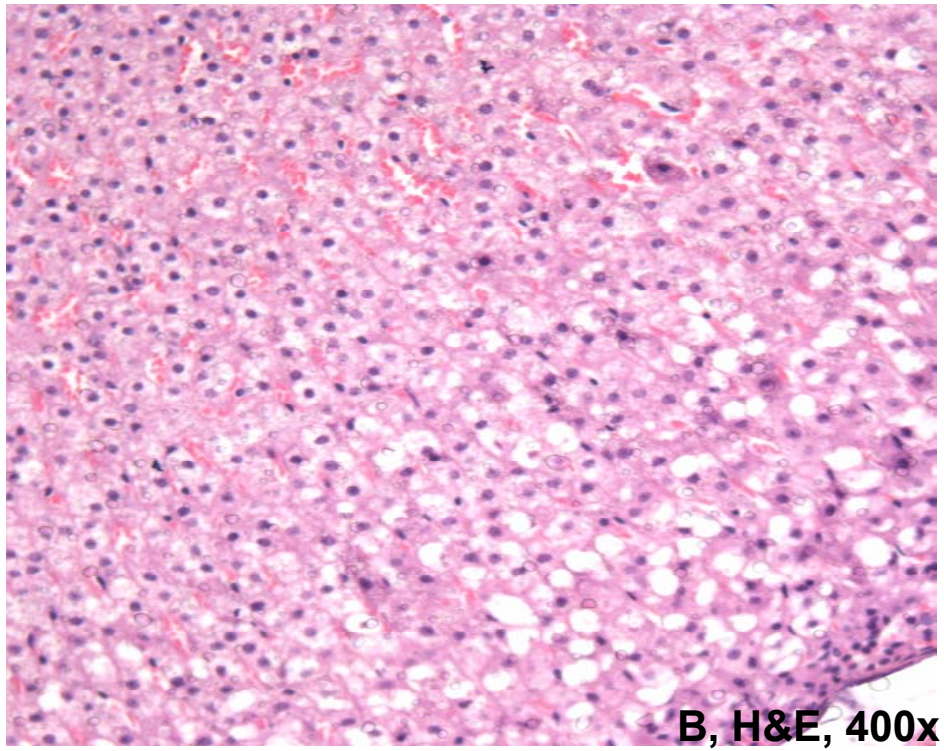
Hepatic parenchyma showing swollen hepatocytes & vacuolation, a typical feature in Chloroquine induced PLDsis



Same as A, higher magnification view.

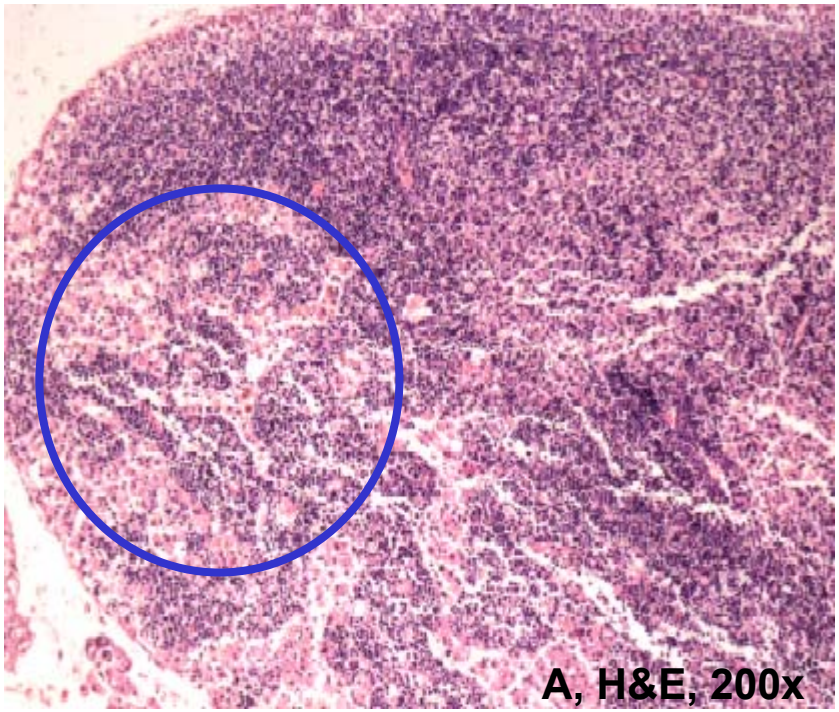
Target Organs for PLDsis

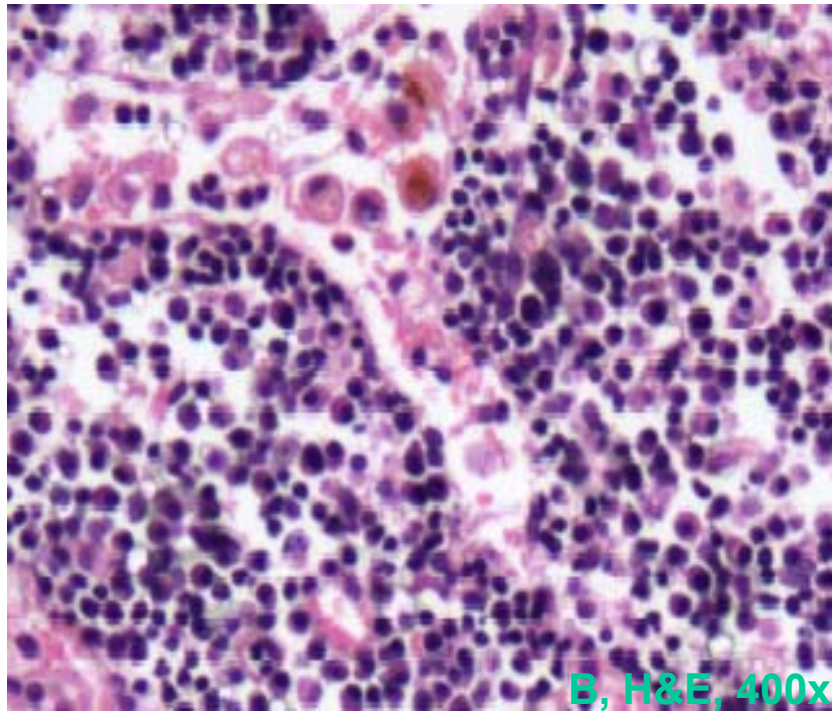




A & B (higher magnification) Adrenal gland – Cortical vacuolation in Chloroquine induced PLDsis

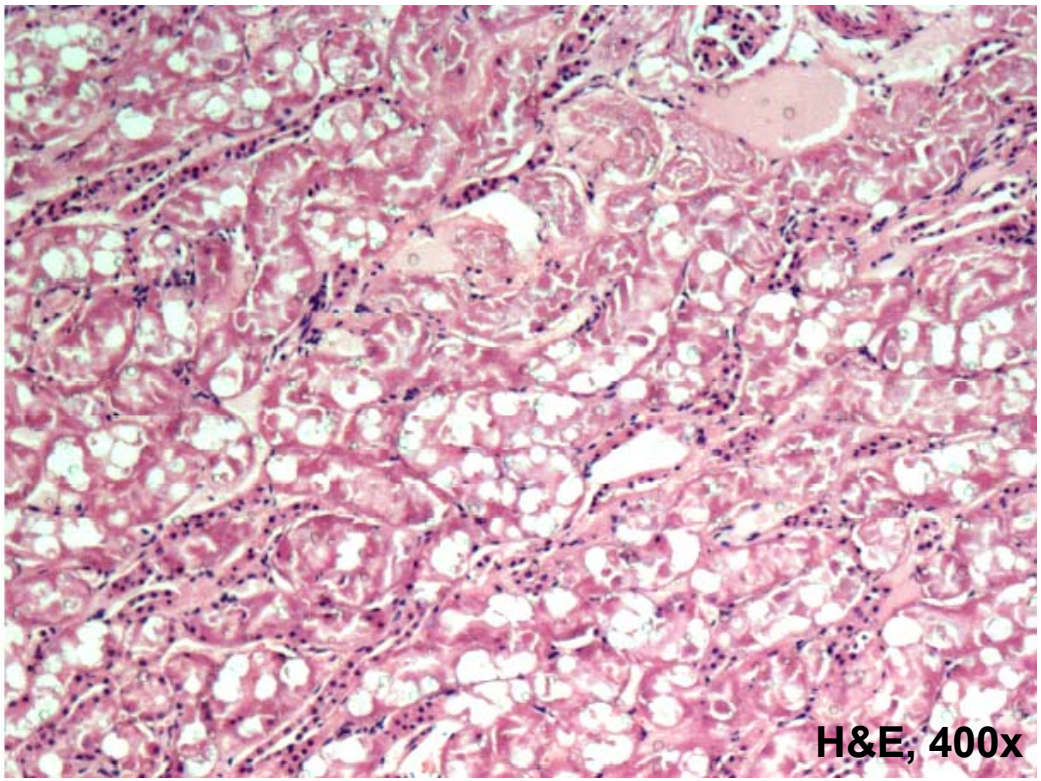
Target Organs for PLDsis





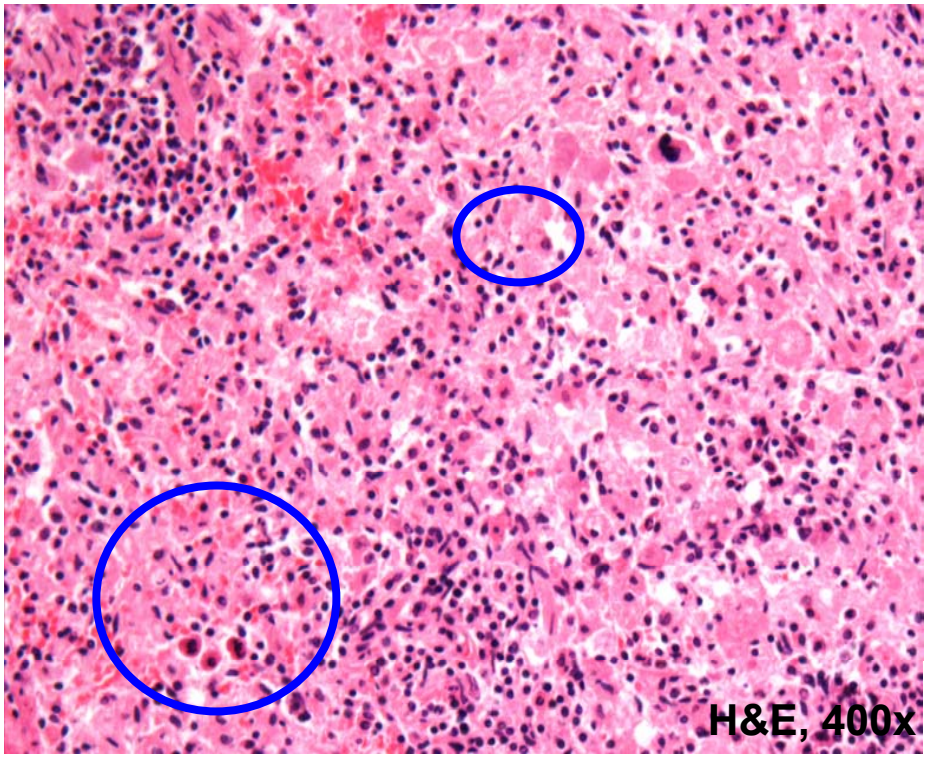
A & B (higher magnification) Lymph node - Large number of histiocytes in Chloroquine induced PLDsis

Target Organs for PLDsis



Kidney – diffuse tubular vacuolation in Chloroquine induced PLDsis

Target Organs for PLDsis



Spleen – Histiocytes in Chloroquine induced PLDsis

Mechanisms of CAD Induced PLDsis

i) Direct binding to phospholipids - indigestible drug-lipid complexes – accumulation. E.g., Amiodarone, Chloroquine

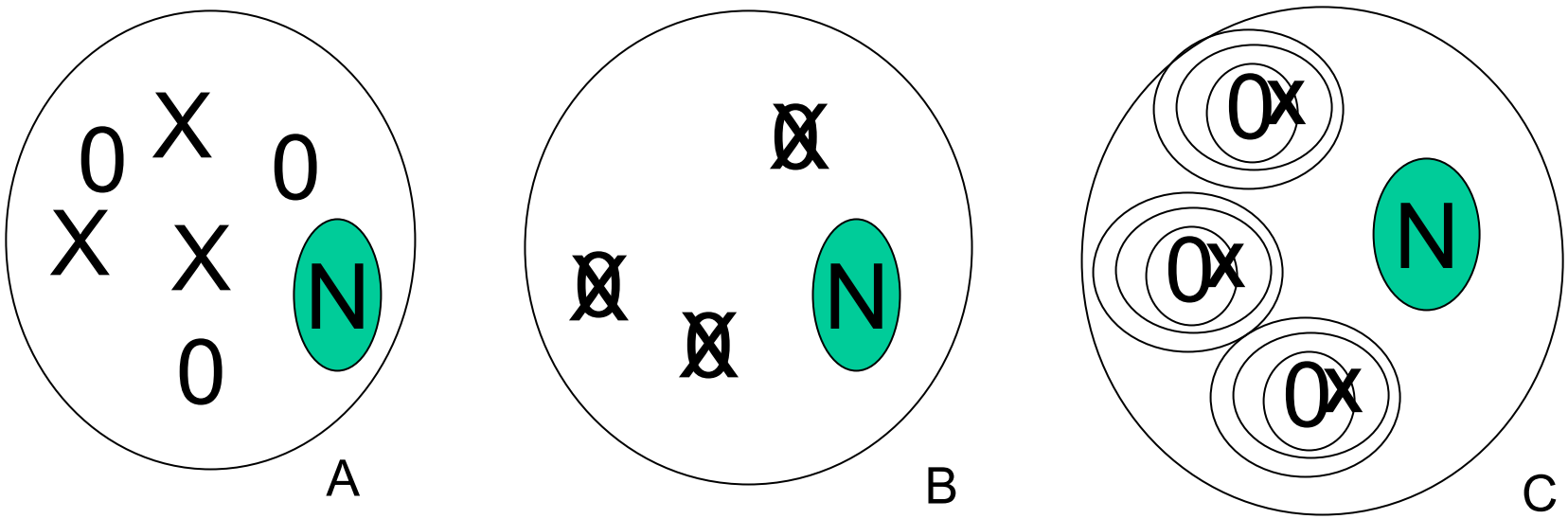
ii) Inhibition of the lysosomal phospholipases (PLA1, A2, C) & enzyme transport. E.g., Chlorpromazine, Amiodarone (IC50=16μM), Chloroquine, Gentamicin (decreased sphingomyelinase)

iii) PLA1 inhibition from charge neutralization of the lysosomal phospholipid bilayer. E.g., Erythromycin, Azithromycin

iv) Promotion of phospholipid and cholesterol synthesis

Mark Reasor, 2006

Mechanisms of CAD Induced PLDsis

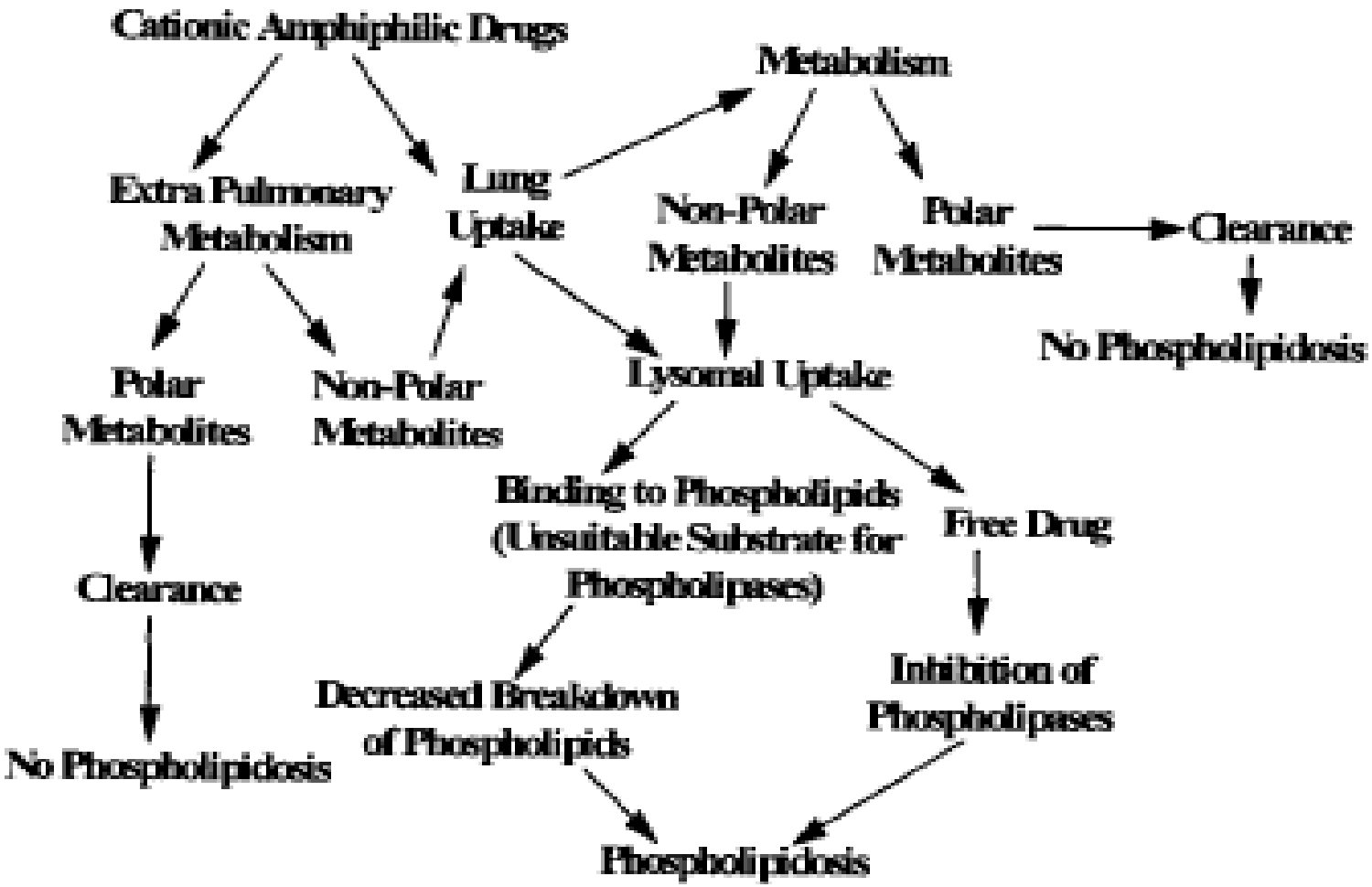


A. Cationic amphiphilic drugs (X) are take up by cells

B. Drugs (x) accumulates in lysosomes (0)

C. Drug inhibition of phospholipase activity results in deposition of phospholipids and development of lamellar bodies [ ]

Mechanisms of CAD Induced PLDsis



Joshi & Mehendale, 1989

Reversibility of PLDsis

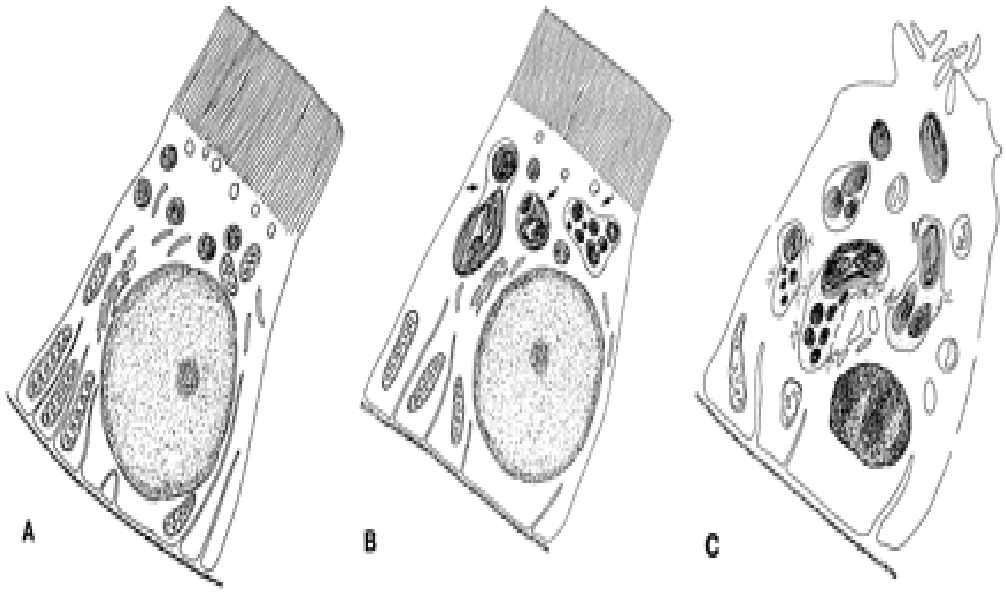
- Reversible upon termination of drug administration or exposure
- The time course of reversal is dependent on
  - the dissociation rate constant
  - elimination rate
- Duration - within weeks to a few months depending on these factors.
- Limited information available on the rate of reversal in humans. E.g., Amiodarone- Faster corneal lesion reversibility as compared to liver.

Functional & Pathological Effect of PLDsis

- Primarily an adaptive response to CAD exposure rather than a toxic response
  - Cells are protected from toxicity incase the drugs are confined to the lysosomes. E.g., Chlorphentermine offers protection against the lethal effects & pulmonary toxicity of nitrogen dioxide in mice.
  - Release of lamellar bodies via exocytosis, the cell could avoid excessive oxidation and the production of ROS – preventing stress.

Functional & Pathological Effect of PLDsis

- Rarely a toxic response (e.g., Gentamicin)



Sequence of events leading from aminoglycoside uptake in proximal tubular cells to tubular necrosis and kidney dysfunction.

A) control B) changes at low doses- enlargement of lysosomes
C) changes at high doses - rupture of lysosomes, release of myeloid bodies in the cytosol

(*Mingeot-Leclercq & Tulkens, 1999*)

Functional & Pathological Effect of PLDsis

- No concrete proof on detrimental effect/carcinogenicity due to PLDsis to the organism. However,

Impaired phagocytic activity of alveolar macrophages	Chlorphenetermine
Increased zymosan stimulated release of superoxide anion & hydrogen peroxide	Iprindole, Amiodarone
Increased natural killer cell activity (pul lymphocytes) Decreased hepatocyte function preceding to death (cytotoxicity) due to high concentration Protection against silica induced pul toxicity	Amiodarone
Decreased mitochondrial volume density, LDH	Propranolol & Verapamil

Functional & Pathological Effect of PLDsis

Ion transport –inhibition of Mg+-ATPase and Na+, K-ATPase Inhibition of calmodulin stimulation of phosphodiesterases	Chlorphenetermine Chloroquine
Myopathy	Chloroquine, Perhexiline, Chlorphenetermine, macrolide, spinosad & dopamine D3 antagonist
Delayed hypersensitivity response, decreased ability to activate Ab-secreting cells	Chlorphenetermine

Functional & Pathological Effect of PLDsis

- Receptor mediated events
- Signal transduction pathways and other cell functions
- Inhibition of protein synthesis, PL and impairment of protein kinase C.

Prediction & Biomarkers of PLDsis

- *In silico* methods - SAR
- Pharmacogenomics/Toxicogenomics
- Physicochemical properties
  - Octanol/water partitioning (logP/logD) – Higher value-PLDsis
  - Ionization constant of the protonable amines (pKa) – basicity, higher value-PLDsis
- In vitro screening using human liver beads, HepG2 cells, lymphocytes, fibroblasts
- Phospholipase A2 knockout mice (deletion of exon 5)
- Ex vivo (BAL) and in vivo assays
  - 1-3 day tox, blood smear/HP/EM
  - Bronchoalveolar lavage

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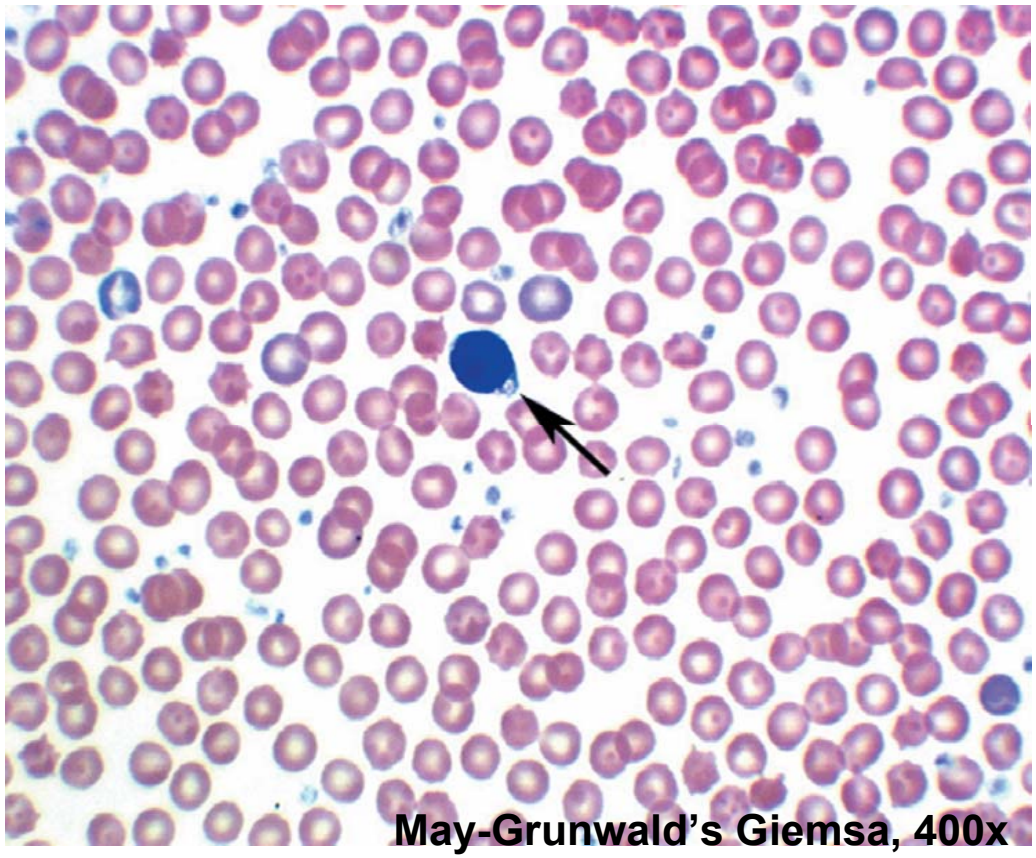
Prediction & Biomarkers of PLDsis

- Peripheral blood smear



May-Grunwald's Giemsa, 400x

Prediction & Biomarkers of PLDsis

- Measurement of urinary Lyso-bis-phosphatidic acid (Di-docosahexaenoyl phosphate, BMP), lysosomal metabolite or isomer of phosphatidylglycerol
  - Liquid chromatography coupled electro spray ionization-mass spectrometry/mass spectrometry method
- Fluorescence assay using 1-acyl-2-(12[(7-nitro-2-1-,3-benzoxadiazol-4-yl)amino]dodecanolyl (NBD) labeled phospholipids
- Uptake of Nile red fluorescence using flow cytometry
- Estimation of urinary and plasma phenylacetylglycine (PAG) using NMR technique (Metabonomics)
- Ratio of PAG/Hippuric acid determination through NMR technique

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Prediction & Biomarkers of PLDsis

- Toxicogenomics
  - Gene expression using DNA microarrays on human hepatoma HepG2 cells with 12 compounds.
    - Alterations of gene expression reflecting the inhibition of lysosomal phospholipase activity, lysosomal enzyme transport and induction of phospholipid and cholesterol biosynthesis.
    - 12 gene markers – significant concordance with the lamellar body formation

Toxicogenomics - PLDsis

Category/Function	Gene Title	Number of compounds that up-regulated	
		6 hours	24 hours
Lipid metabolism/phospholipid degeneration	N-acylsphingosine aminohydrolase (acid ceramidase) 1	0	7
	Sphingomyelin phosphodiesterase, acid-like 3A	3	6
	Hypothetical protein MGC4171	0	7
Lipid metabolism/cholesterol biosynthesis	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	10	4
	3-Hydroxy-3-methylglutaryl-Coenzyme A reductase	11	5
	Squalene epoxidase	7	3
	Lanosterol synthase	4	6
	7-Dehydrocholesterol reductase	4	6
Lipid metabolism/regulation of cholesterol metabolism	Nuclear receptor subfamily 0, group B, member 2	0	8
Lipid metabolism/cholesterol metabolism	Lipase A, lysosomalacid, cholesterol esterase	0	6
Lipid metabolism/fatty acid biosynthesis	ELOVL family member 6, elongation of long chain fatty acids Stearoyl-CoA desaturase	5	6
Lipid metabolism/fatty acid alpha-oxidation	Phytanoyl-CoA desaturase	8	7
Lipid metabolism/fatty acid transport	Fatty acid binding protein 1, liver	0	9
Lipid metabolism/lysosome enzyme	Ceroid-lipofuscinosis, neuronal 2, late infantile	0	6
Cell cycle, proliferation, death	Prostate differentiation factor	2	9
	Activin beta A	5	8
	p8 protein (candidate of metastasis 1)	7	7
	Chromosome 20 open reading frame 97	6	6
	connective tissue growth factor	0	6
	Granulin	0	6
	Insulin-like growth factorbinding protein 1	3	6
	Optineurin	0	6
	B-cell translocation gene 1, anti-proliferative	9	3
	Cyclin G2	8	3
Transport	Solute carrier family 7, (cationic amino acid transporter, y+ system) number 11	8	6
	Solute carrier organic anion transporter family, number 4C1	4	6
	Syntaxin 3A	1	7
	Transient receptor potential cation channel, subfamily V, number 2	1	6
	Transthyretin (prealbumin, amyloidosis type I)	0	8

Toxicogenomics - PLDsis

Proteolysis and peptidolysis	Calpain 3, (p94)	0	6
	Coagulation factor VII (serum prothrombin conversion accelerator)	0	7
	Hepsin (transmembrane protease, serine 1)	0	10
	Protease, serin, 8 (prostasin)	1	7
Endopeptidase inhibition	Pre-alpha (globulin) inhibitor, H3 polypeptide	0	7
	Serine (or cysteine) protease inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), number 3	0	8
	Serine protease inhibitor, Kazal type 1	0	7
Miscellaneous	Asparagine synthetase	0	8
	Argininosuccinate synthetase	0	7
	Yippee protein	2	7
	Deiodinase, iodothyronine, type I	4	6
	Interleukin 6 receptor	2	6
	J domain containing protein 1	3	6
	Lipin 1	8	4
	S100 calcium binding protein P	0	6
	Spondin 2, extracellular matrix protein	0	6
	Sulphotranspharase family, cytosolic, 2A, dehydroepiandrosterone (DHEA) – preferring, number 1	0	7
	Transmembrane 7 superfamily member 2	0	6

Nonoyama and Fukuda, 2008

Risk Assessment & Regulatory Views

- Concern on
  - tissue accumulation of drugs/metabolites coincident with PLDsis.
  - relationship between PLDsis dose and that inducing systemic organ toxicity
  - site or location involved – e.g. Neurons- disrupt cell to cell signaling.
- From regulatory perspective & task of determining drug safety, PLDsis has been considered an adverse finding.
- Niemann-Pick like syndrome – inborn errors of metabolism. Deficiency in sphingomyelin phosphdiesterase activity
- Recommend for perform macrophage function assay (immunotox)

Risk Assessment & Regulatory Views

- Go or No Go decision based on
  - SAR or other assay result
  - Pharmacological potential
  - ADME data
  - Chemical characteristics
  - Intended dosage, exposure duration, target organ and NOAEL
    - Renal/Hepatic PLDsis is manageable than CNS/pulmonary.

Summary

- Drug induced (CADs) PLDsis is observed frequently in preclinical toxicity studies in multiple organs.
- Unresolved issue due to unclear molecular causes. The morphological/ultrastructural changes are the hallmark feature.
- Sporadic relationship with adverse drug reactions
- Biological consequences is speculative
- No clear criteria or guidelines for morphological estimation of PLDsis exist.
- Screening for early biomarkers would be essential


Thank You

NBD Assay

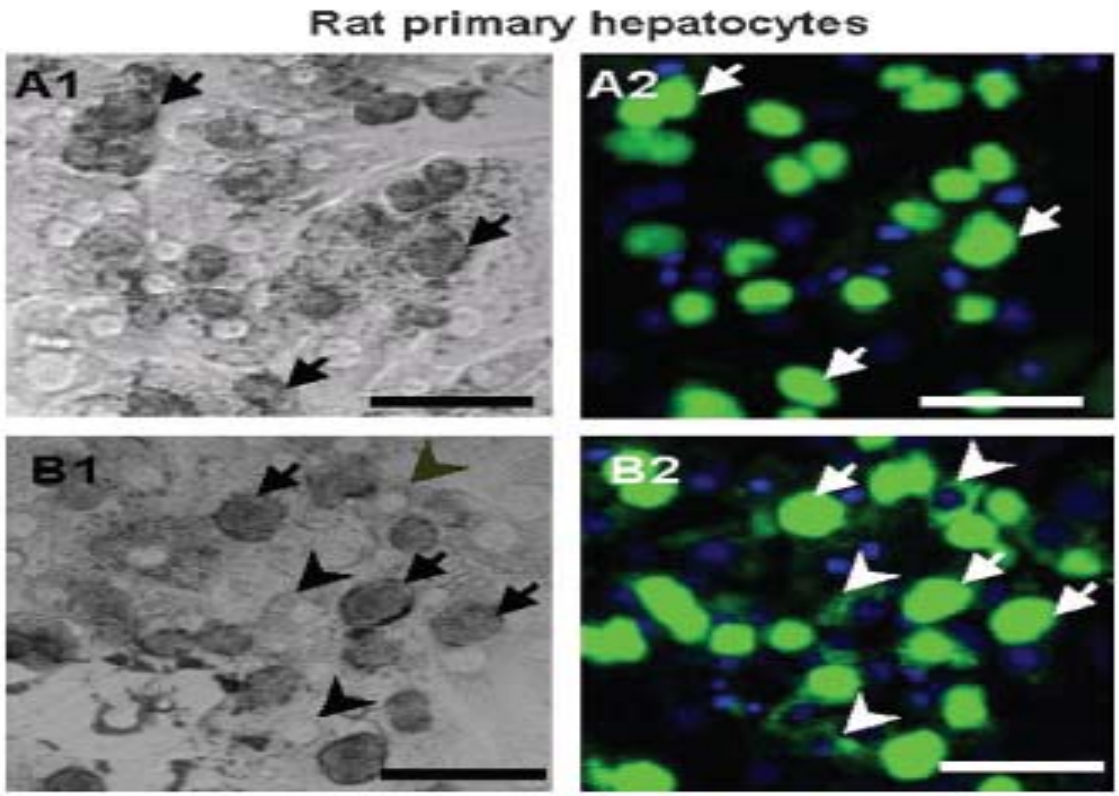

FIG. 3. Transmission laser and confocal laser scanning micrographs of rat primary hepatocytes treated with 10 μM amiodarone for 24 h. Photographs A1 and A2 show same microscopic field from the same cell, as do B1 and B2. A1 and A2 are controls, and B1 and B2 show cells treated with 10 μM amiodarone. A1 and B1 are transmission laser micrographs, and B1 and B2 are confocal laser scanning ones. Arrow and arrowhead indicate typical dead cells and living cells, respectively. Green fluorescence of NBD-PC was excited at 480 nm to detect accumulation of phospholipid, and blue fluorescence of Hoechst33342 was excited at 405 nm to identify the nucleus. Each bar indicates 50 μm.

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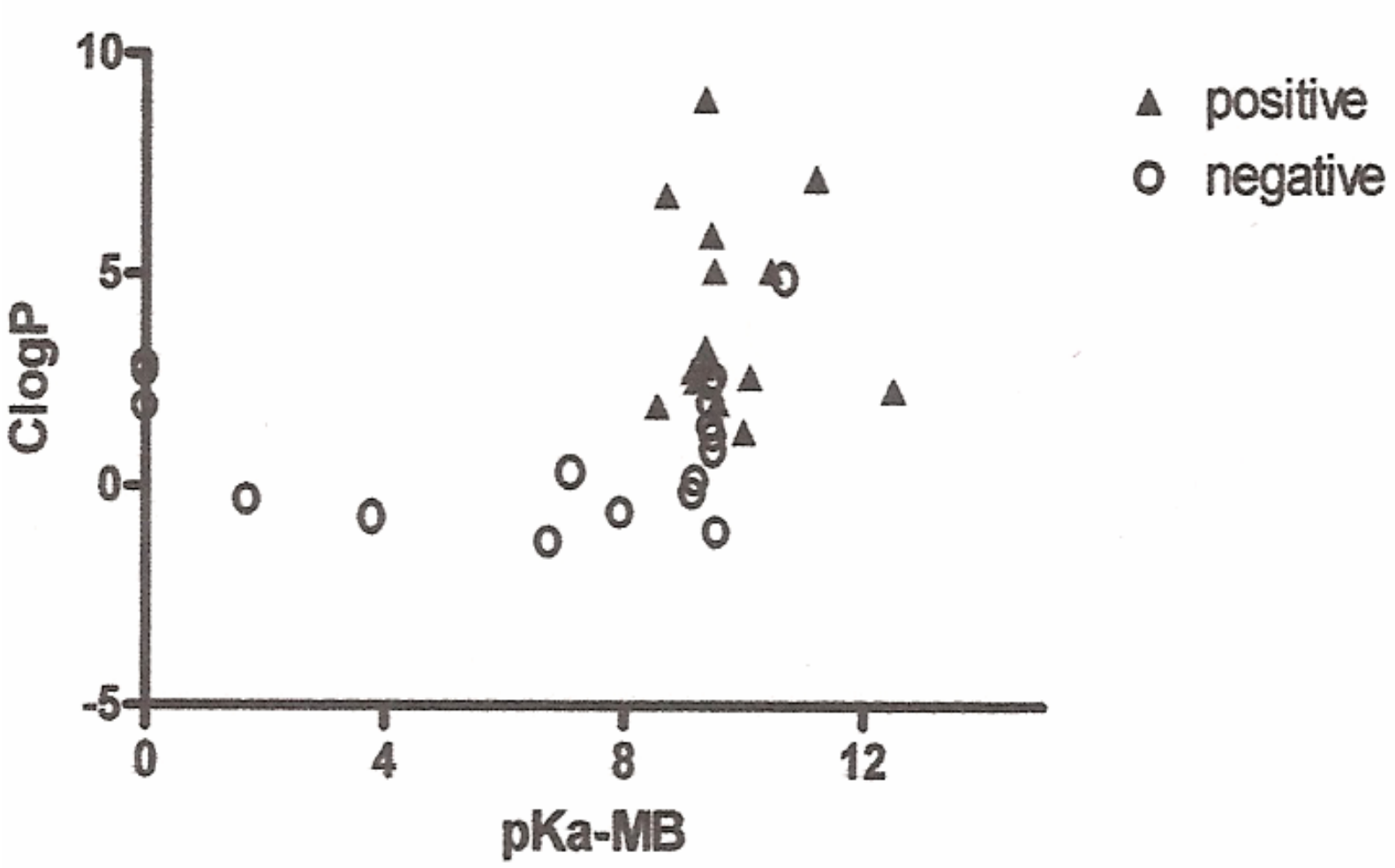
cLogP/pKa



Higher cLogP/pKa ratio for compounds positive for PLDsis.

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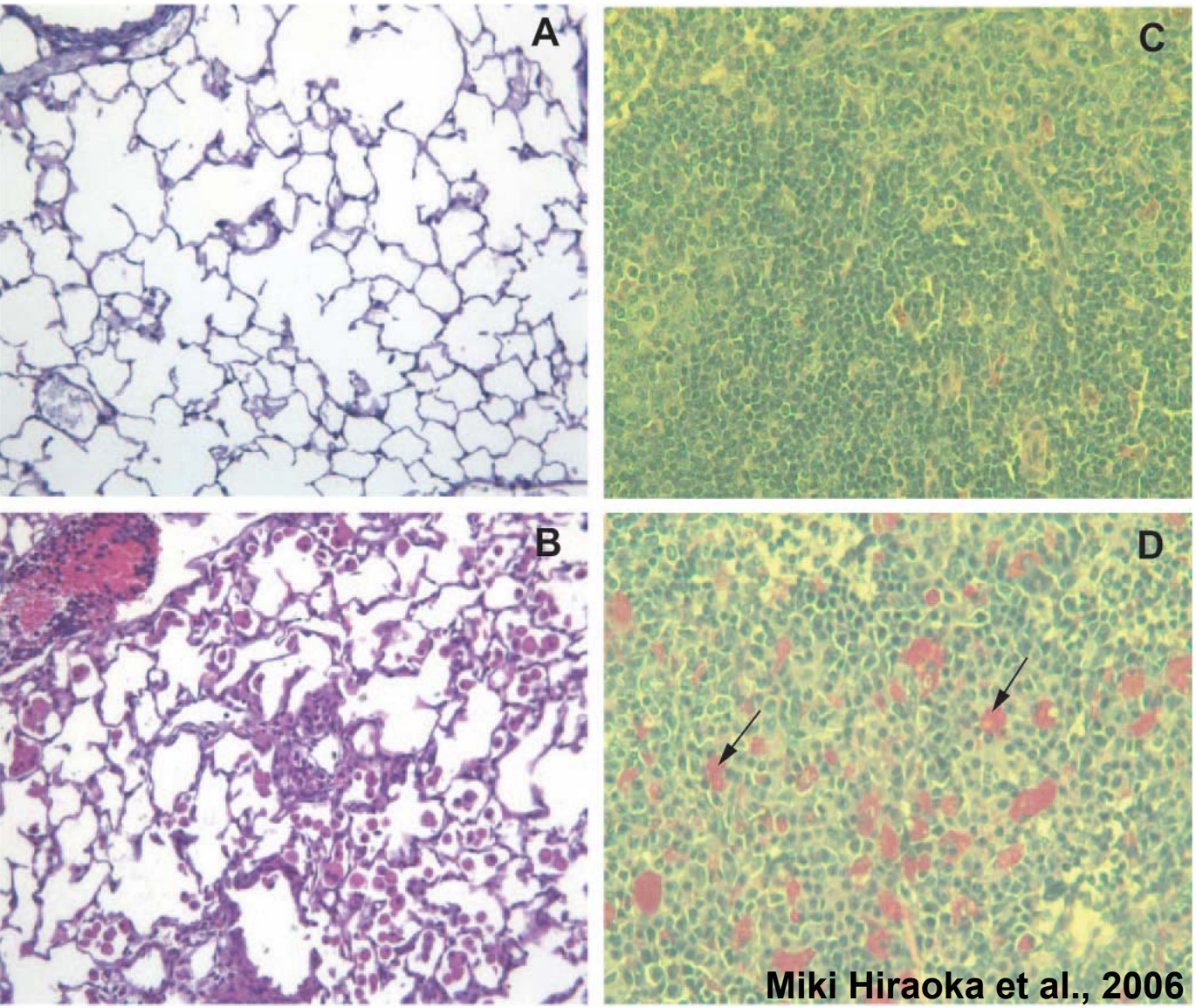
Prediction of PLDsis



A & C: Formalin fixed PAS stained normal lungs & spleen from wild type and wild-type mice.

B & D: Formalin fixed PAS positive macrophages in lungs & spleen from *Lpla2*$^{-/-}$ mice.

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