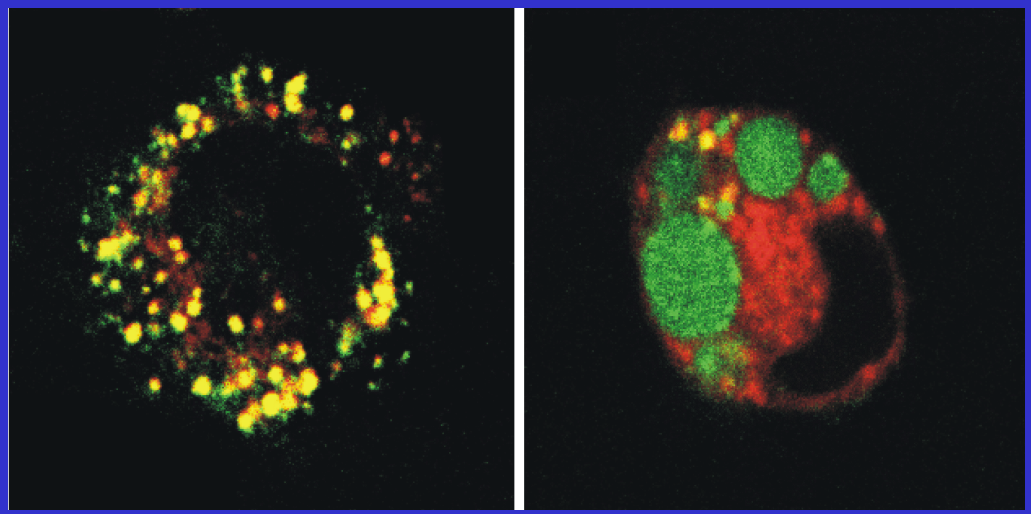
Azithromycin, a pharmacological agent which selectively inhibits some pathways of endocytosis: characterization, interests and mechanism of action.



Donatienne Tyteca, Pharm.

Thesis submitted for the dregree of Doctor in Pharmaceutical Sciences (PhD)

Promotor: Prof. M.P. Mingeot-Leclercq

Copromotors: Profs P.J. Courtoy & P.M. Tulkens

Brussels, December 4th, 2001

Endocytosis

➥ **mammalian cells take up extracellular material by a variety of mechanisms collectively termed endocytosis**

➥ **implications of endocytosis in physiology:**

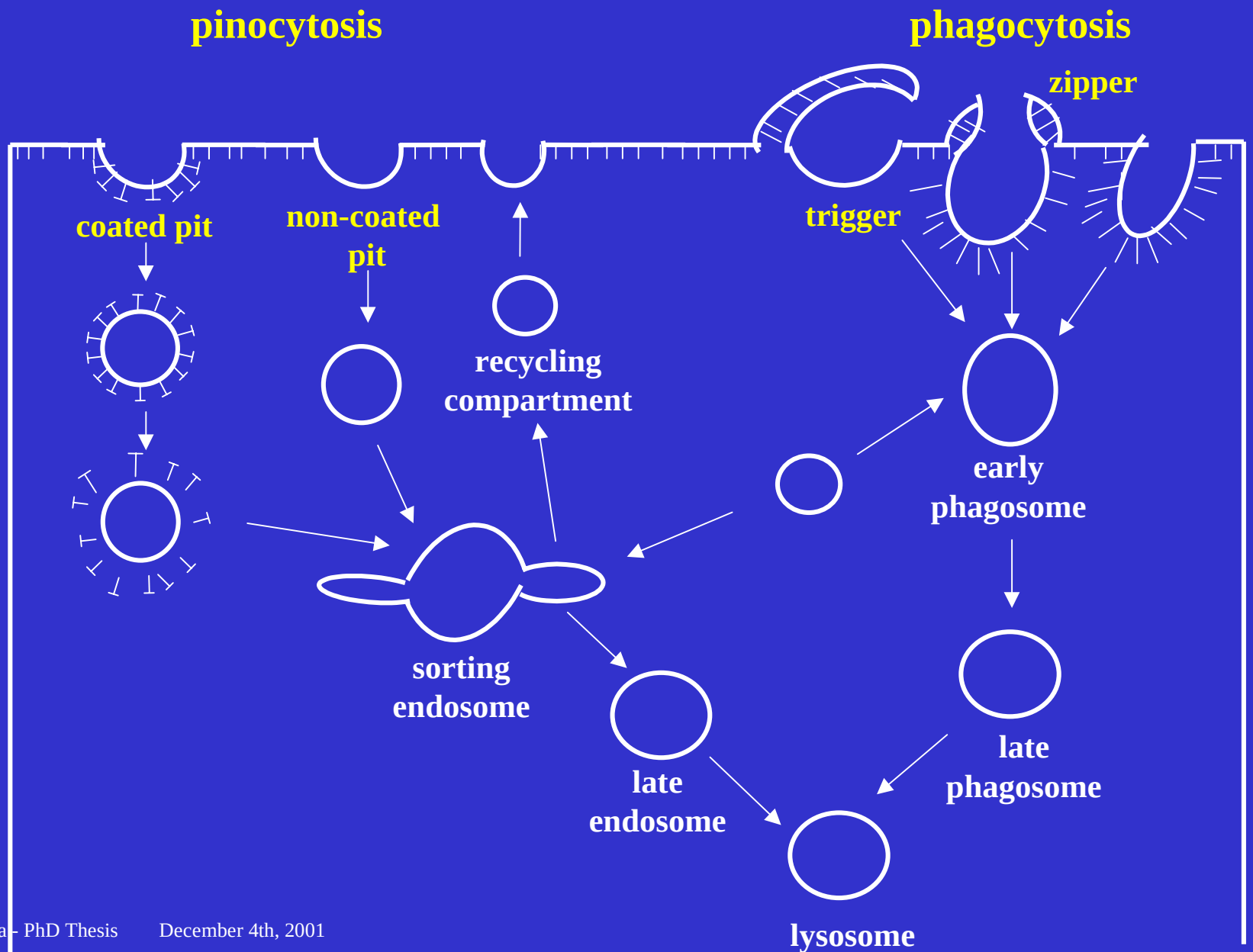
- **uptake of extracellular nutrients,**
- **cellular cholesterol homeostasis,**
- **regulation of hormonal response,**
- **maintenance of cell polarity,**
- **antigen presentation,**
- **....**

➥ **implications of endocytosis in pathology:**

- **atherosclerosis,**
- **entry of pathogens and toxins,**
- **neurogenerative diseases (Alzheimer, prion),**
- **....**

(Mukherjee et al, 1997)

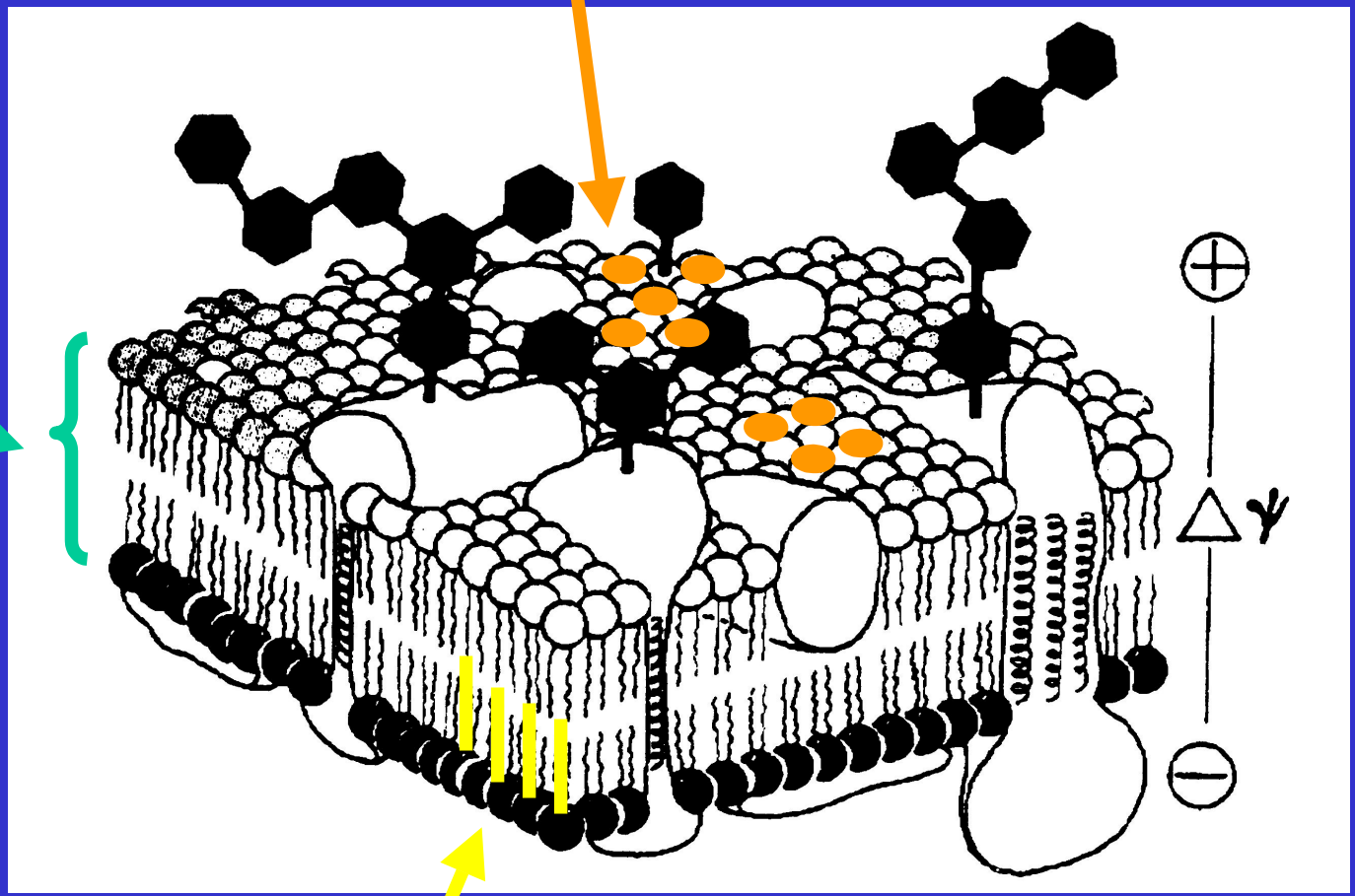
Pathways of endocytosis studied in this thesis



Membrane lipids are implicated at various stages of the endocytic process

membrane organization in domains

membrane asymmetry



membrane fluidity

Molecular machineries of endocytic pathways

budding

coat proteins:
clathrin, APs
COPs

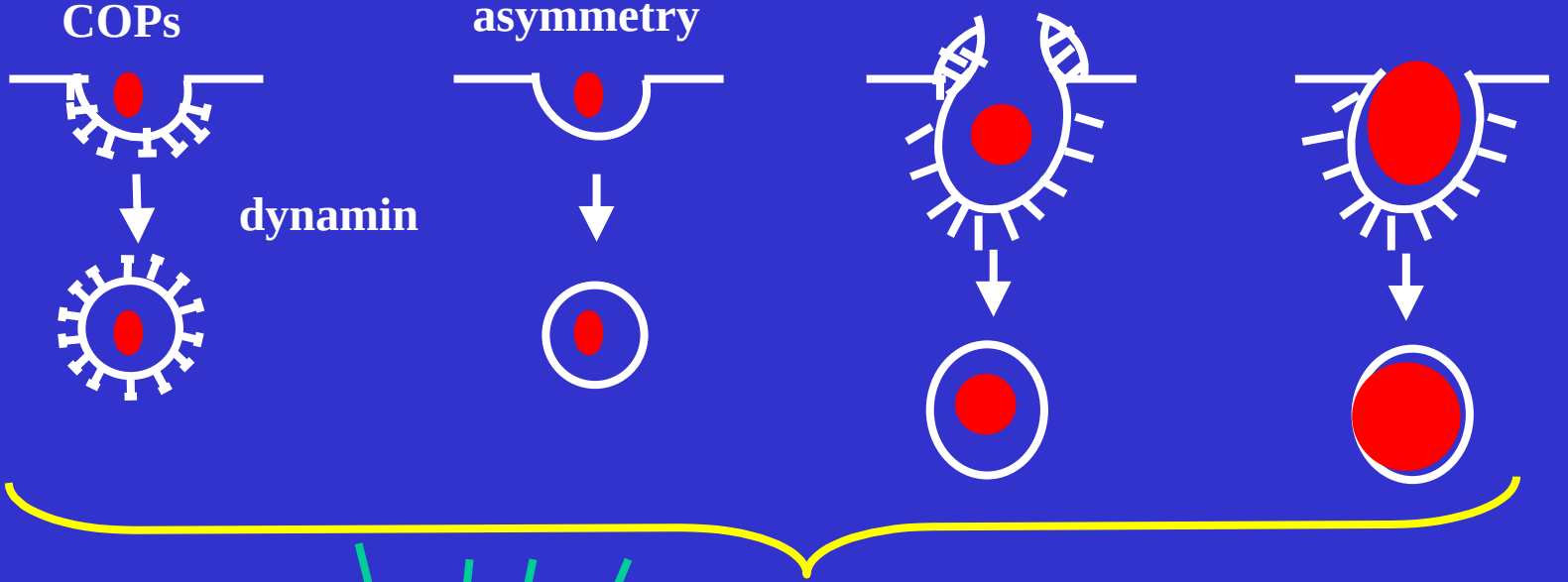
membrane
tension and
asymmetry

cytoskeleton

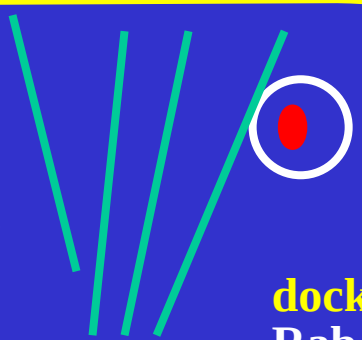
adhesion to a
curved particle

fission

dynamin



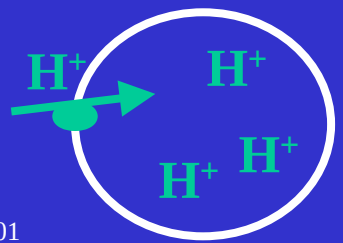
transport
actin
microtubules

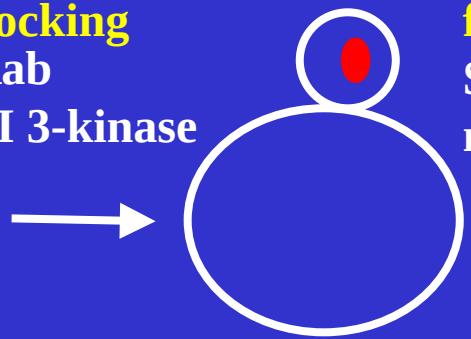


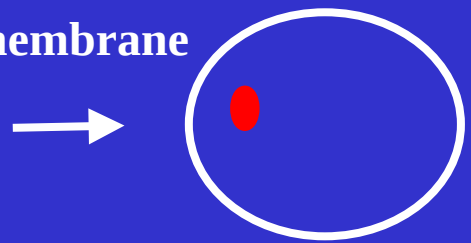
docking
Rab
PI 3-kinase

fusion
SNARE/SNAP
membrane

acidification
vacuolar
H^+-ATPase







What is the place of pharmacological inhibitors to dissect the endocytic apparatus ?

Conditions, mutations and agents have been extensively used to dissect cellular mechanisms of endocytosis:

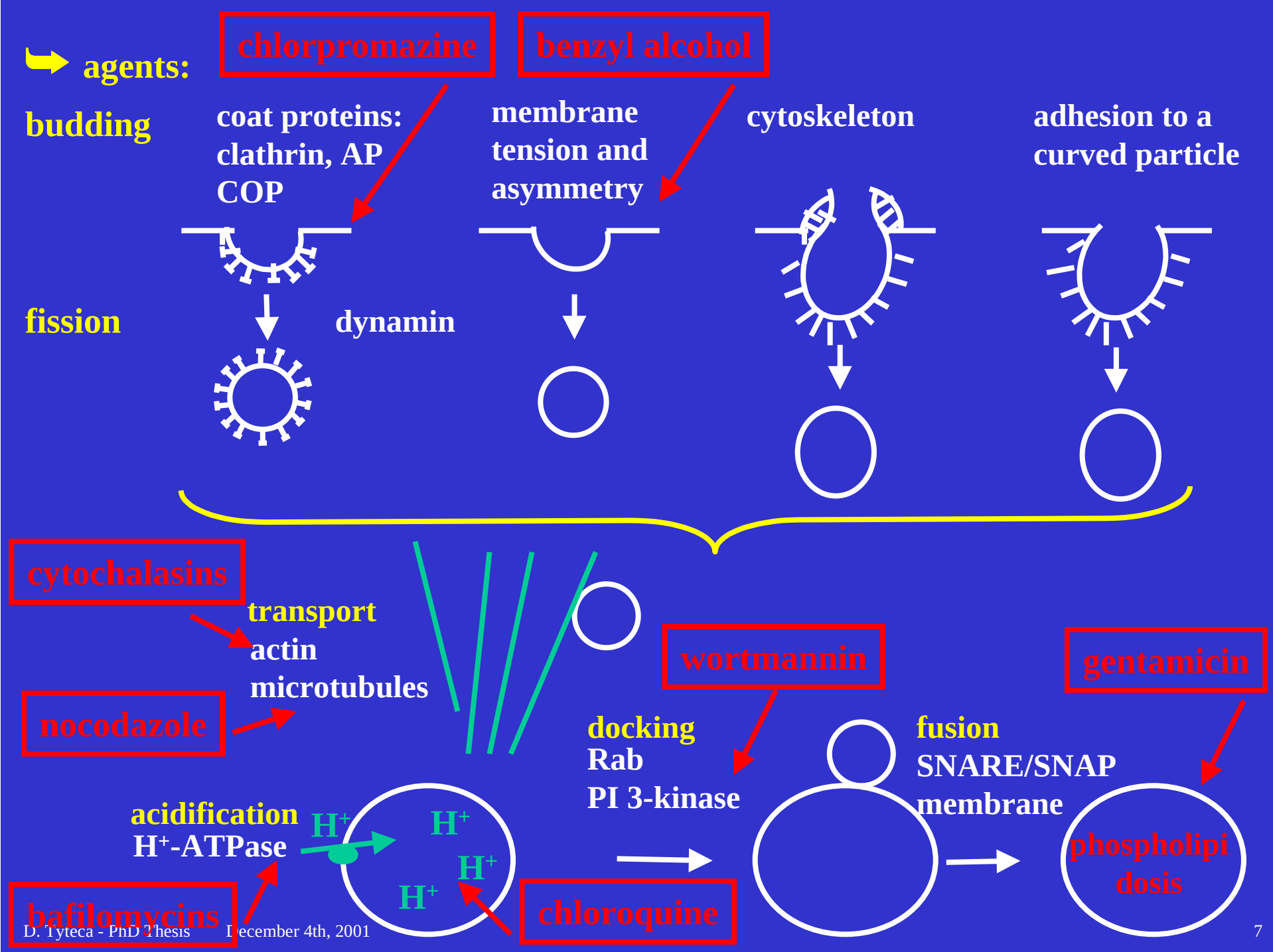
➥ **conditions:**

- **K+ depletion** (Cupers et al, 1994)
- **incubation in hypertonic medium** (Cupers et al, 1994; 1997)
- **cytosol acidification** (Sandvig et al, 1987)

➥ **mutants:**

- **clathrin, adaptor proteins and associated proteins**
- **COP**
- **dynamin**
- **Rabs**
- **....**

(for a review, see Dautry-Varsat, 2001)

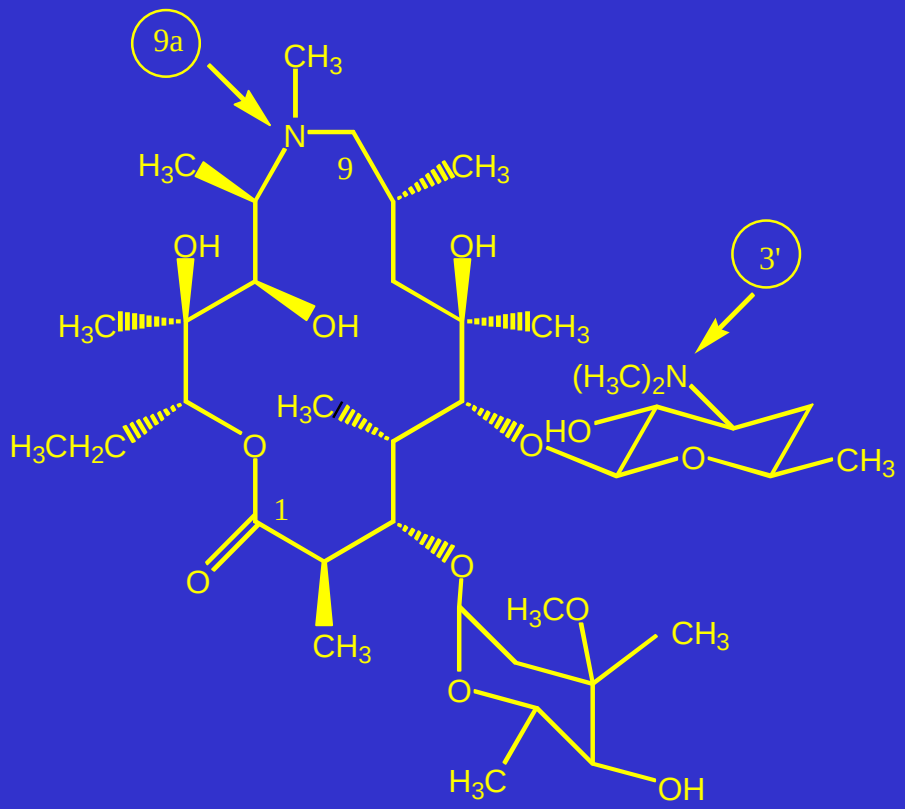

agents:
chlorpromazine
benzyl alcohol
budding
coat proteins:
clathrin, AP
COP
membrane
tension and
asymmetry
cytoskeleton
adhesion to a
curved particle
fission
dynamin
cytochalasins
transport
actin
microtubules
wortmannin
gentamicin
nocodazole
docking
Rab
PI 3-kinase
fusion
SNARE/SNAP
membrane
acidification
H+-ATPase
H+
H+
H+
H+
phospholipi
dosis
bafilomycins
chloroquine

limitations:

- ➥ **almost all are unspecific and show pleiotropic effects**
- ➥ **none inhibit the earliest steps of clathrin-independent pinocytosis**

and azithromycin ?

Azithromycin (AZ),
a dicationic amphiphile



Pharmacological properties of AZ

➥ **spectrum of activity**

- **Gram +**
- **some Gram -**

➥ **therapeutic use**

- **upper and lower respiratory tract infections**
- **skin infections**
- **sexually transmitted diseases**
- ***Mycobacterium avium* complex in AIDS patients**

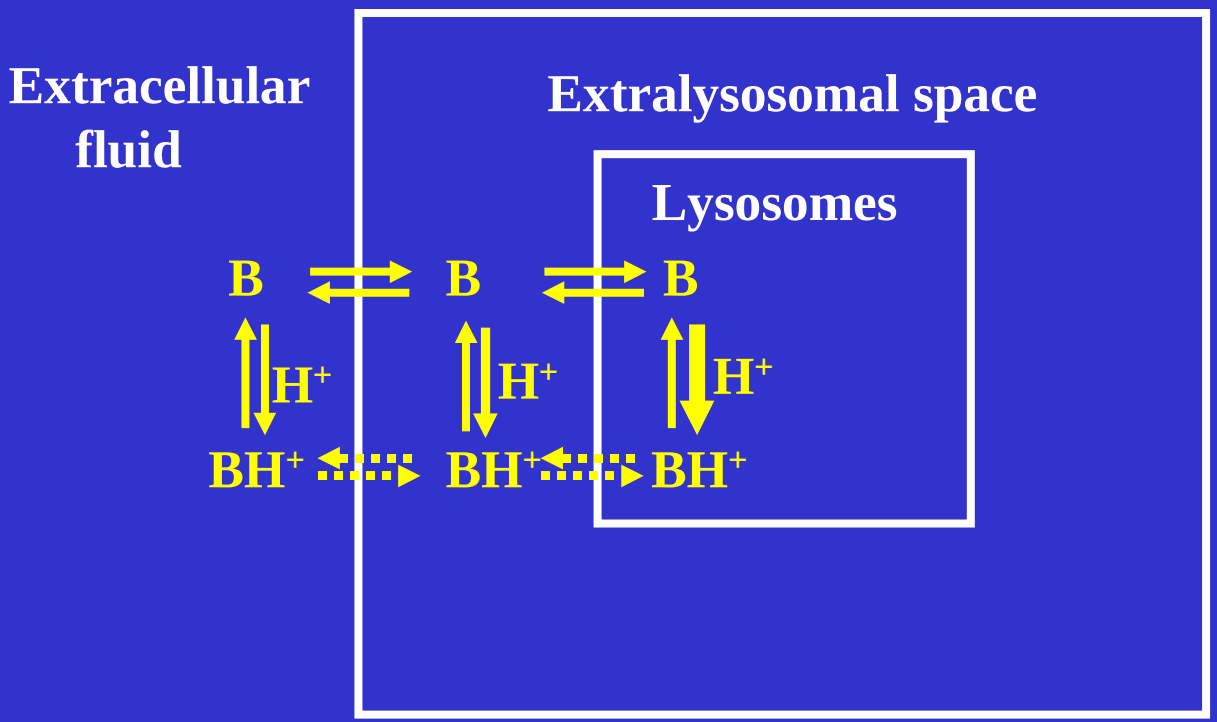
➥ **pharmacokinetic properties *in vivo***

- **exceptionally high and rapid accumulation in tissues, and slow release** (Foulds et al, 1990)
- **consequences**
  - ➥ **low serum concentrations**
  - ➥ **decrease of the length of treatment**
  - ➥ **toxicity ???**

Pharmacological properties of AZ

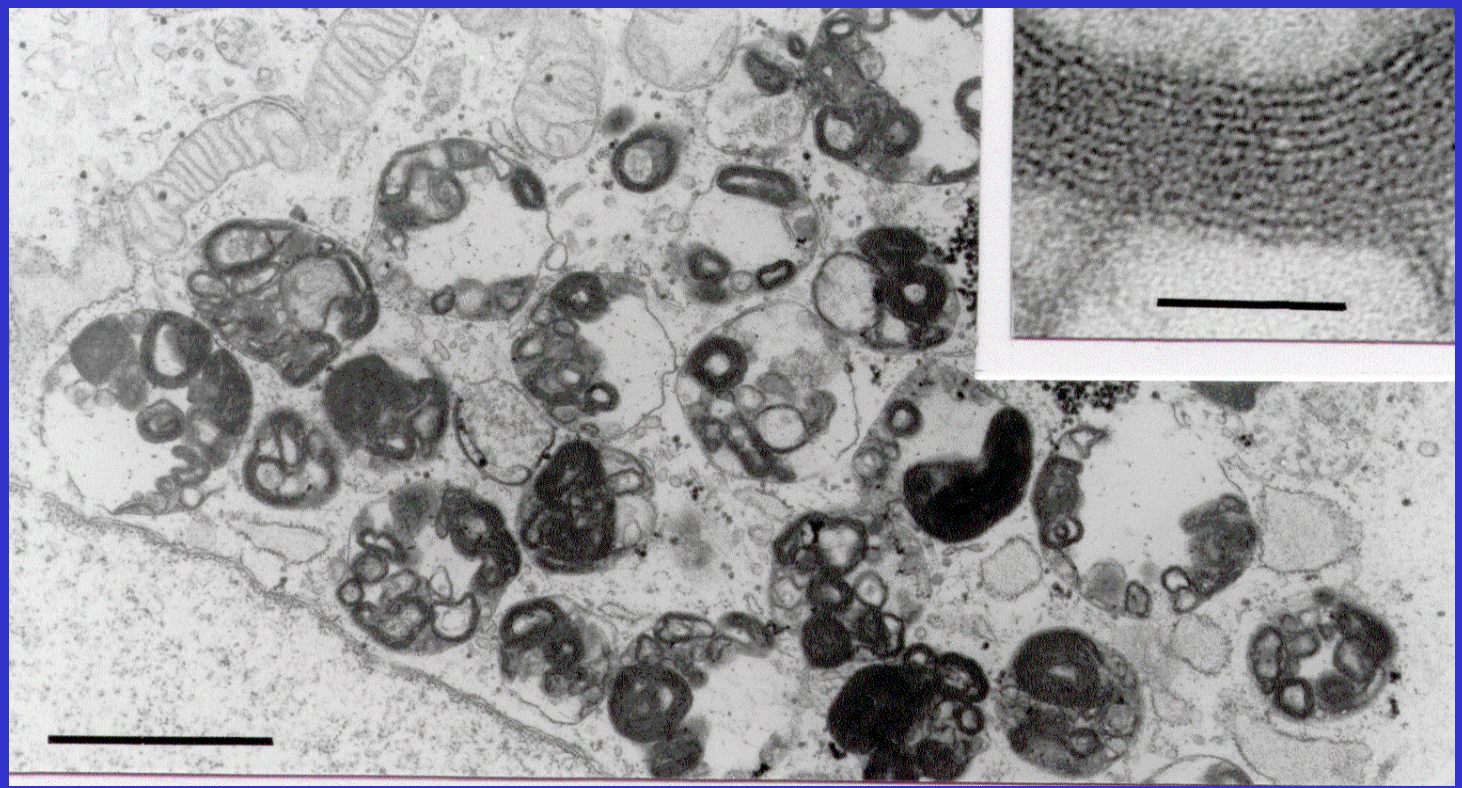
Cellular pharmacokinetic properties of AZ

- **accumulates in lysosomes of fibroblasts and macrophages** (Carlier et al, 1994)
- **acidotropic sequestration** (de Duve et al, 1974)



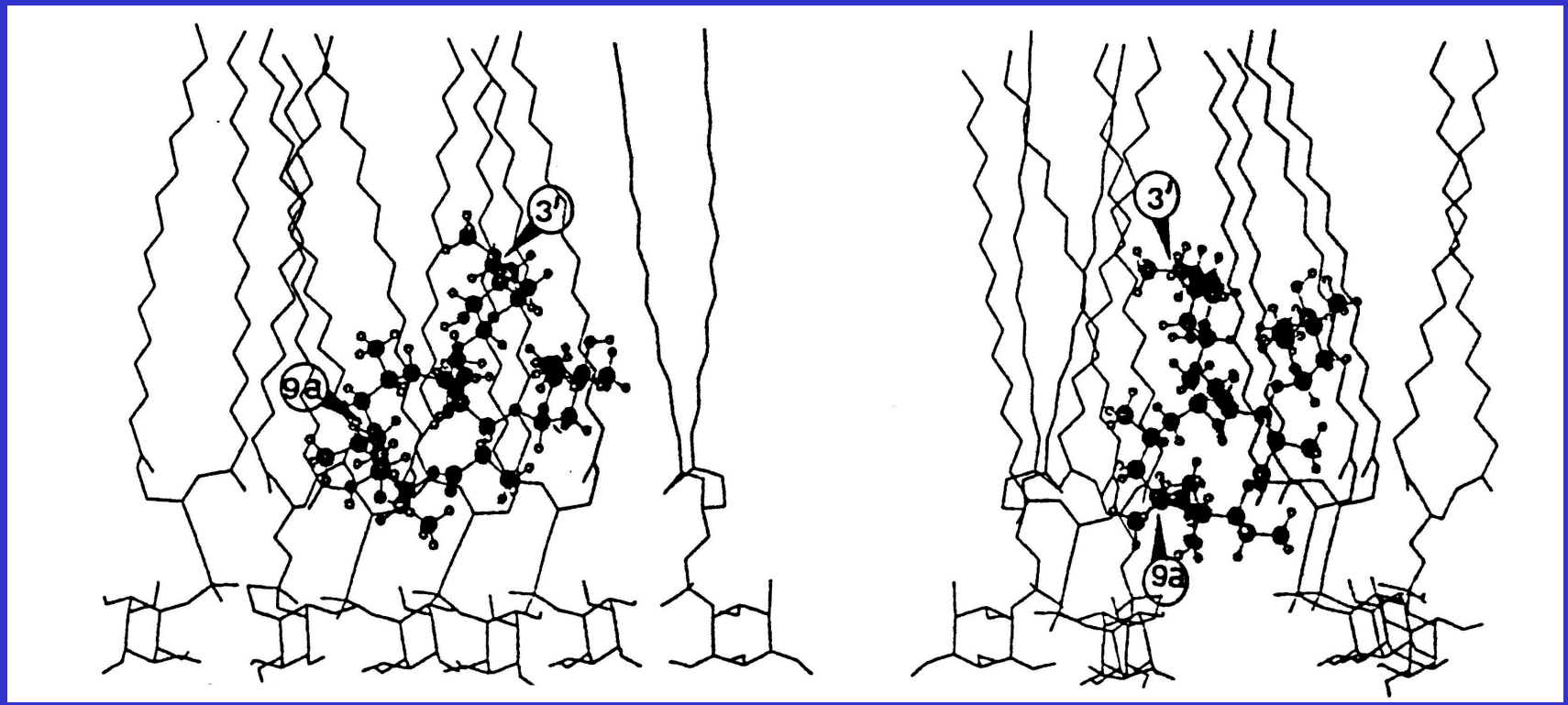
Cellular toxicological properties of AZ

➥ **induces a lysosomal phospholipidosis in fibroblasts** (Van Bambeke et al, 1996)



➥ **inhibits lysosomal phospholipase A1** (Montenez et al, 1996)

➥ **binds to negatively-charged bilayers at acidic pH** (Montenez et al, 1996)



➥ **perturbs the fusion of lysosomes with horseradish peroxidase (HRP)-containing endosomes** (unpublished observation of Van Bambeke)

① Could AZ affect earlier steps of the endocytic apparatus ?

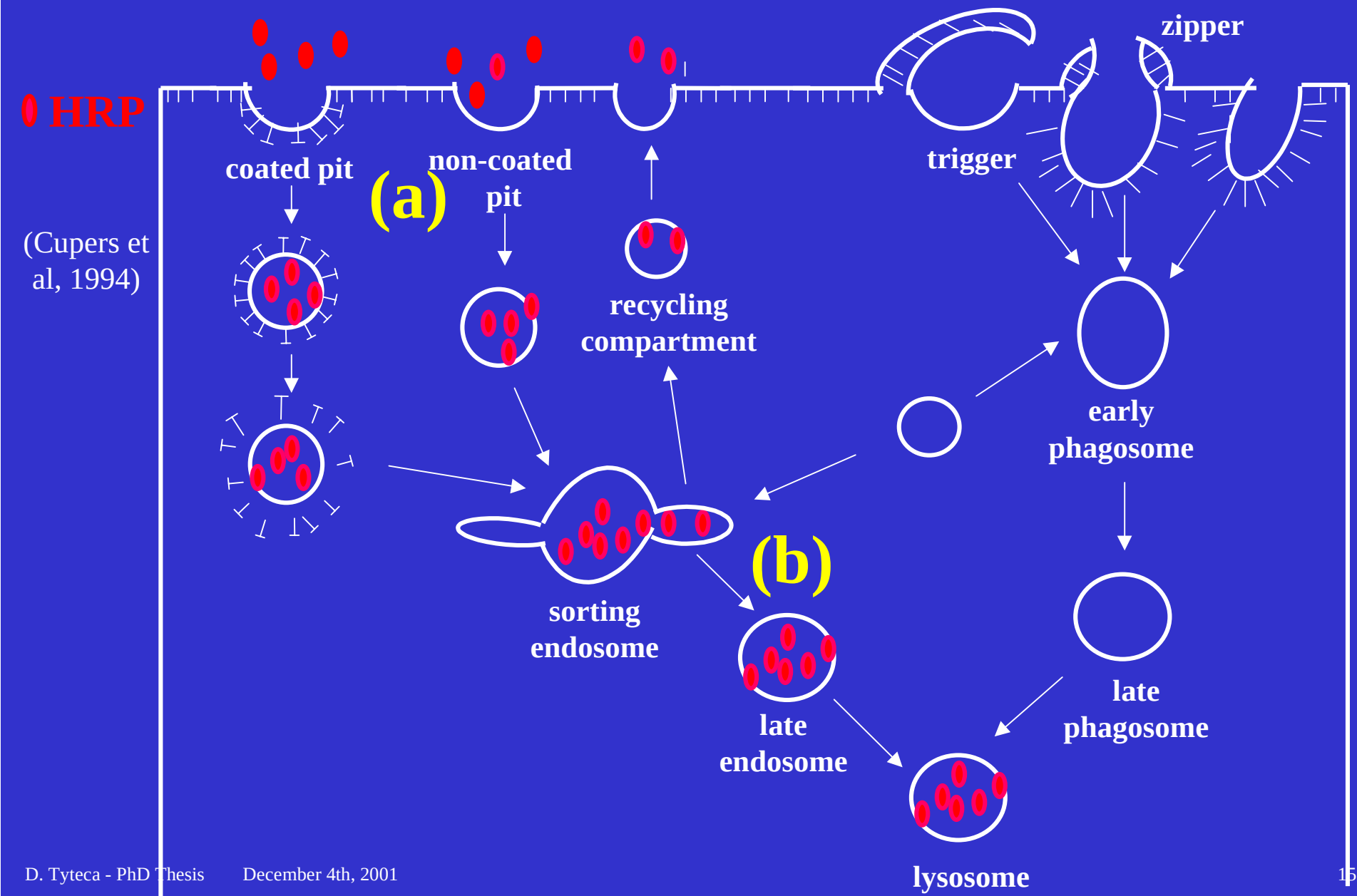
Azithromycin, a lysosomotropic antibiotic, impairs fluid-phase endocytosis in cultured fibroblasts
D. Tyteca, P. Van Der Smissen, F. Van Bambeke, K. Leys, P.M. Tulkens, P.J. Courtoy & M.-P. Mingeot-Leclercq
Eur. J. Cell Biol. 80: 466-478 (2001)

- **Selection of experimental system**
  Rat foetal fibroblasts
  - ➥ **avidly accumulate azithromycin**
  - ➥ **develop lysosomal phospholipidosis**
  - ➥ **extensively characterized system**

- **Experimental test**
  Fluid-phase endocytosis

Fluid-phase endocytosis

➥ horseradish peroxidase (HRP)



• General experimental protocol

confluent cells

↓

pretreatment with AZ (from 0 to 3 days)

↓

incubation with the endocytic tracer (from 0 to 4 h)

↓

washing, recovering and sonication

↓

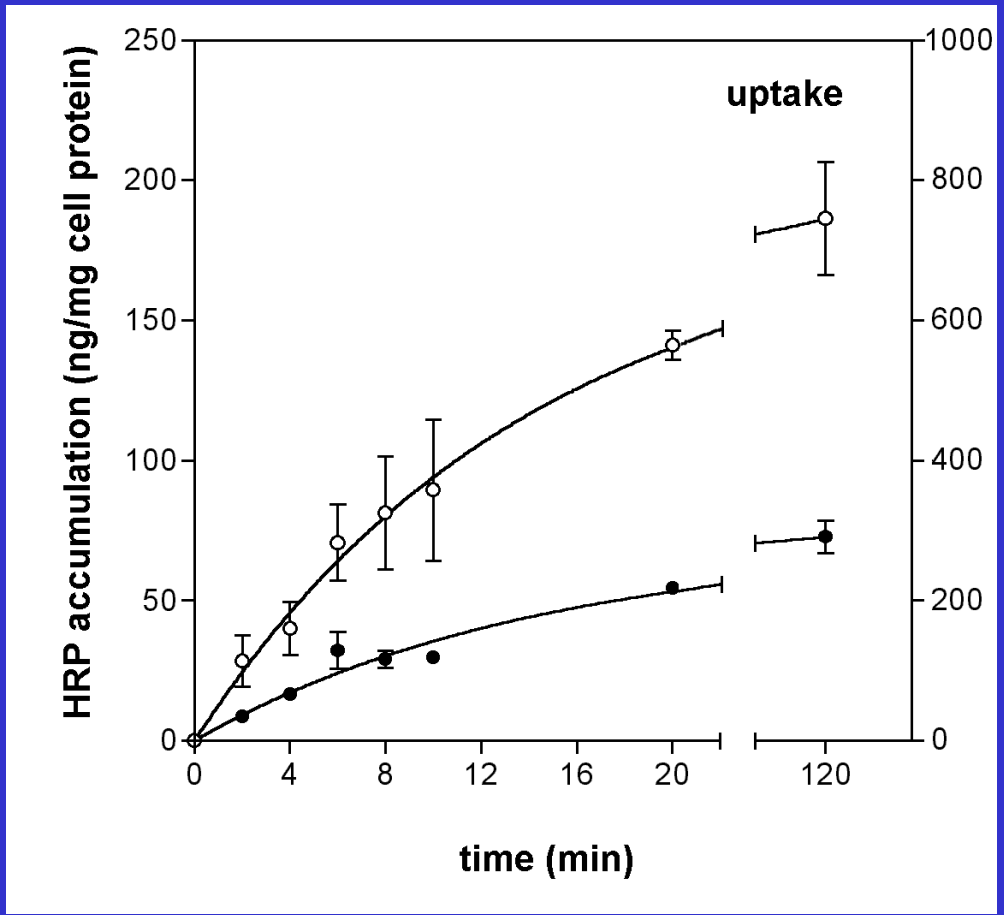
assays:

- ➥ **endocytic tracer**
- ➥ **proteins**
- ➥ **AZ**
- ➥ **phospholipids**

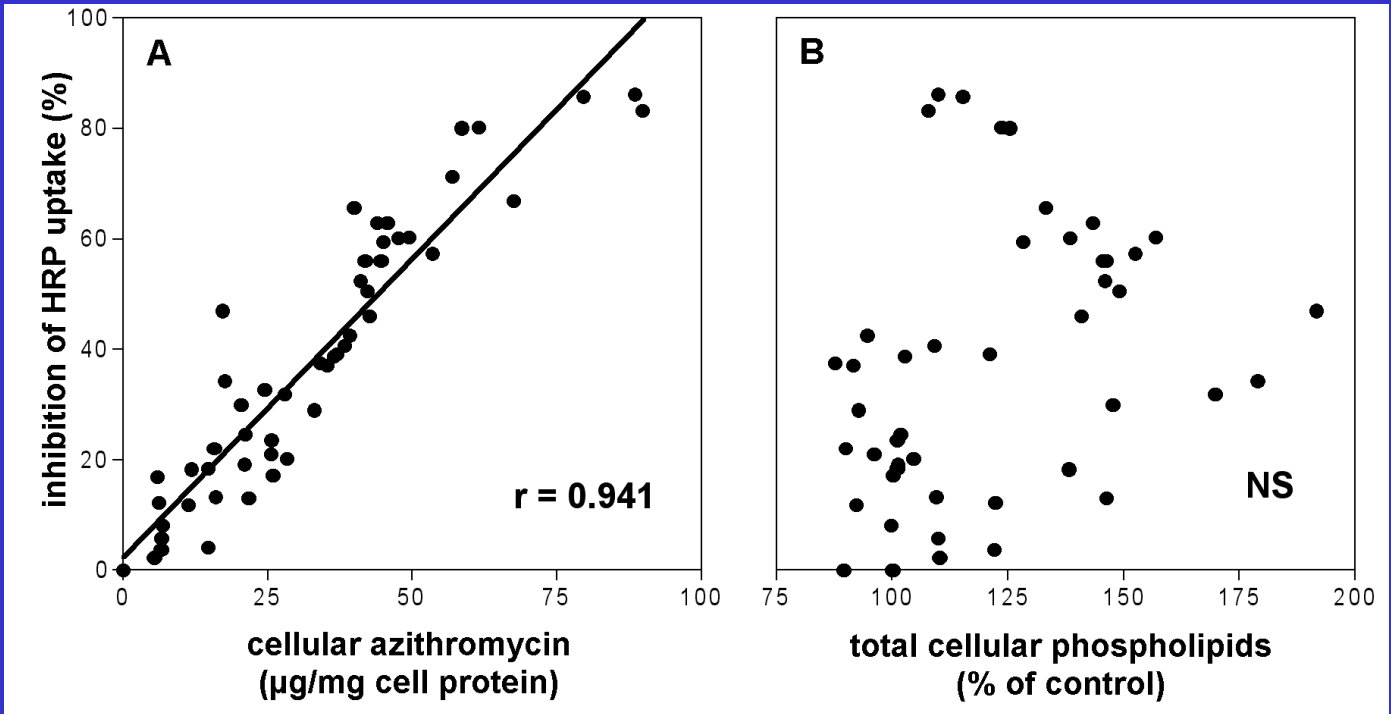
AZ slows down fluid-phase endocytosis

control cells

cells pretreated with AZ



Inhibition of fluid-phase endocytosis correlates with AZ content but is independent of phospholipidosis



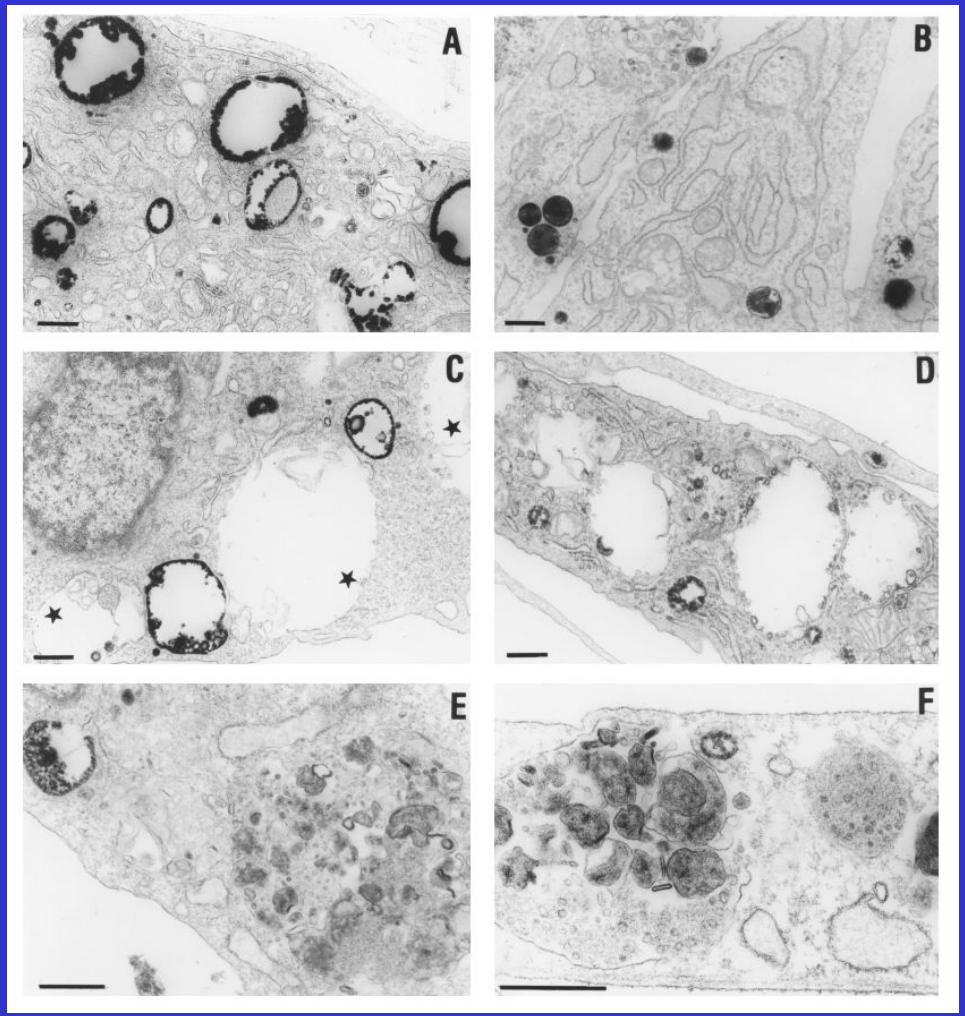
AZ causes a major reduction of the number of endosomes and lysosomes and impairs accessibility of HRP to swollen and overloaded endosomes/lysosomes

5 min HRP **2 h HRP**

CT

3 h AZ

3 days AZ



② Is AZ specific to fluid-phase endocytosis ?

Azithromycin inhibits clathrin-independent pinocytosis and slows down sequestration of ligand-receptor complexes into endocytic and recycling vesicles of J774 macrophages

D. Tyteca, P. Van Der Smissen, M. Mettlen, F. Van Bambeke, P.M. Tulkens, M.-P. Mingeot-Leclercq & P.J. Courtoy

Submitted for publication

- **Selection of experimental system**

  J774 mouse macrophages

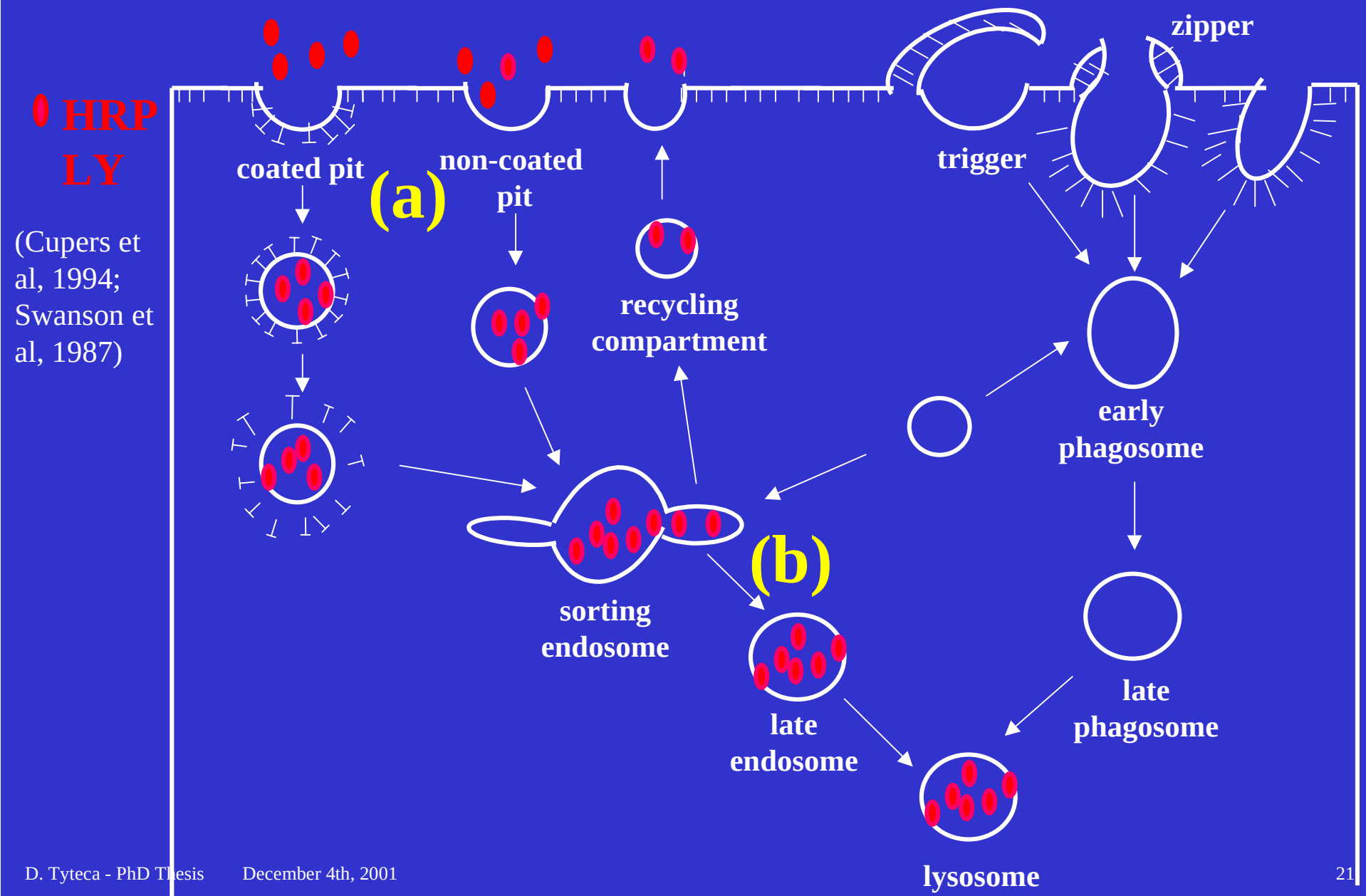
  - ➥ **homogeneous cell line**
  - ➥ **high endocytic activity**
  - ➥ **well-characterized system for pinocytosis and phagocytosis**

- **Experimental tests**

  - ➥ **fluid-phase endocytosis**
  - ➥ **bulk-membrane endocytosis**
  - ➥ **receptor-mediated endocytosis**
  - ➥ **phagocytosis**

Fluid-phase endocytosis

➥ HRP and lucifer yellow (LY)

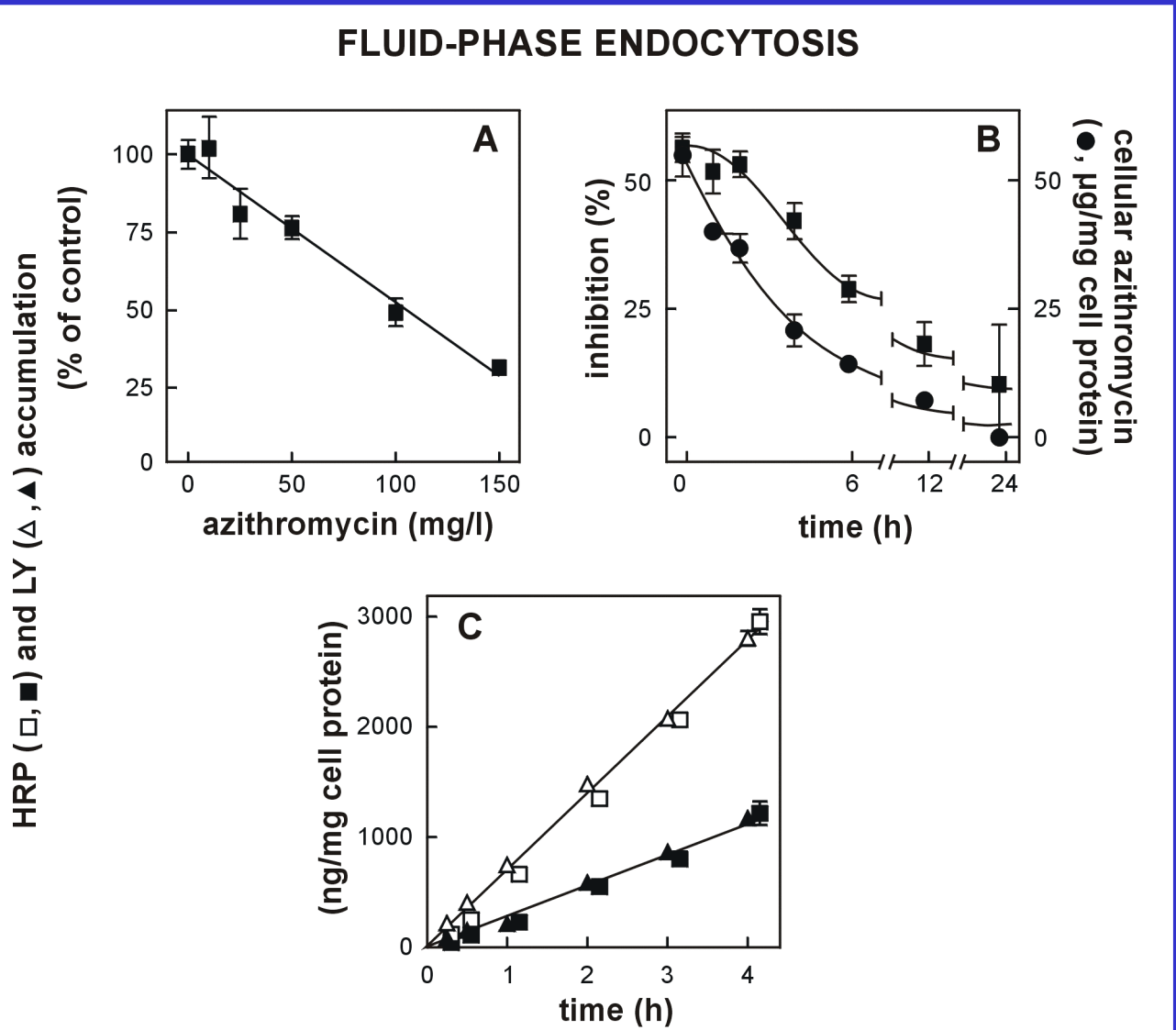


AZ inhibits fluid-phase endocytosis and this inhibition is reversible

AZ

HRP

LY

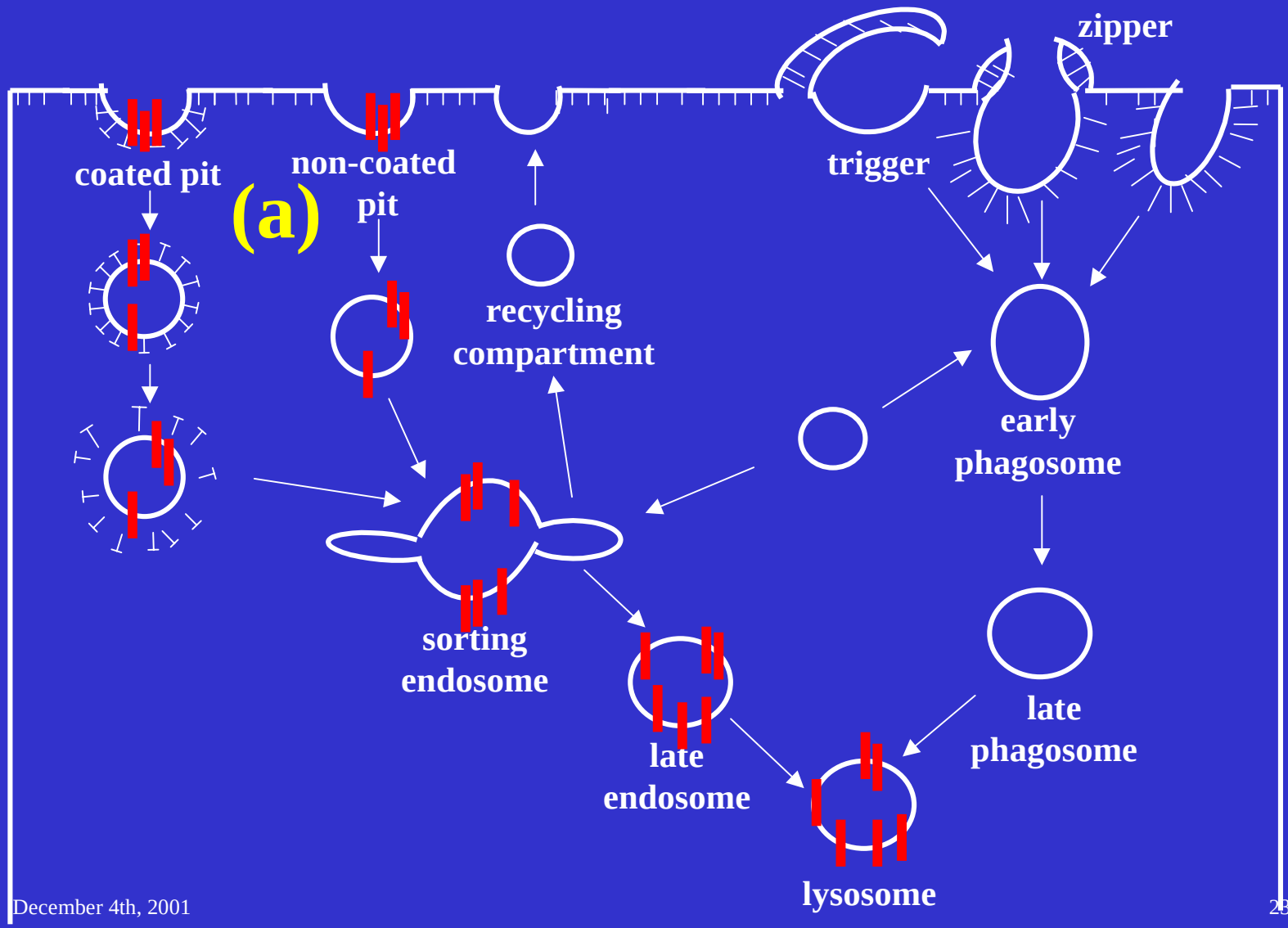


Bulk-membrane endocytosis

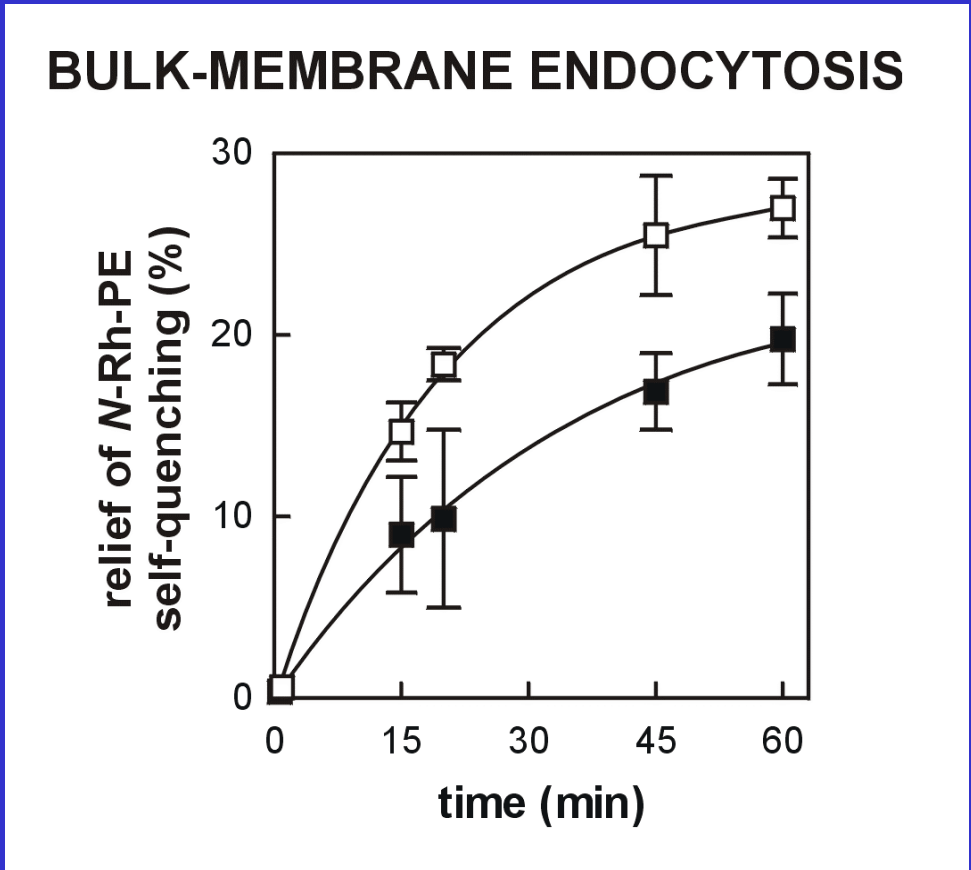
➥ *N*-rhodamine-phosphatidylethanolamine (*N*-Rh-PE)

aggregates of *N*-Rh-PE

(Kok et al, 1990)

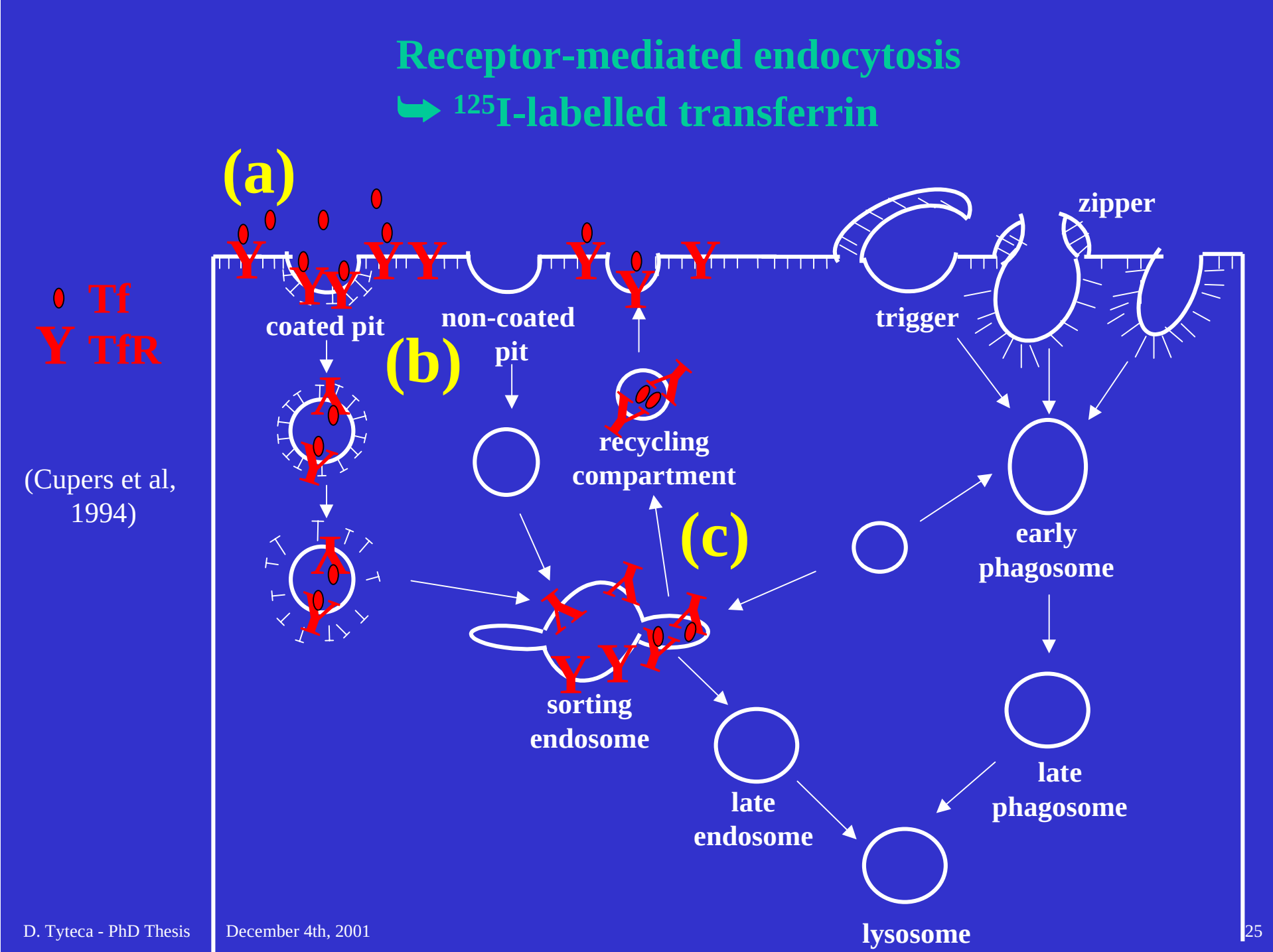


AZ slows down bulk-membrane endocytosis

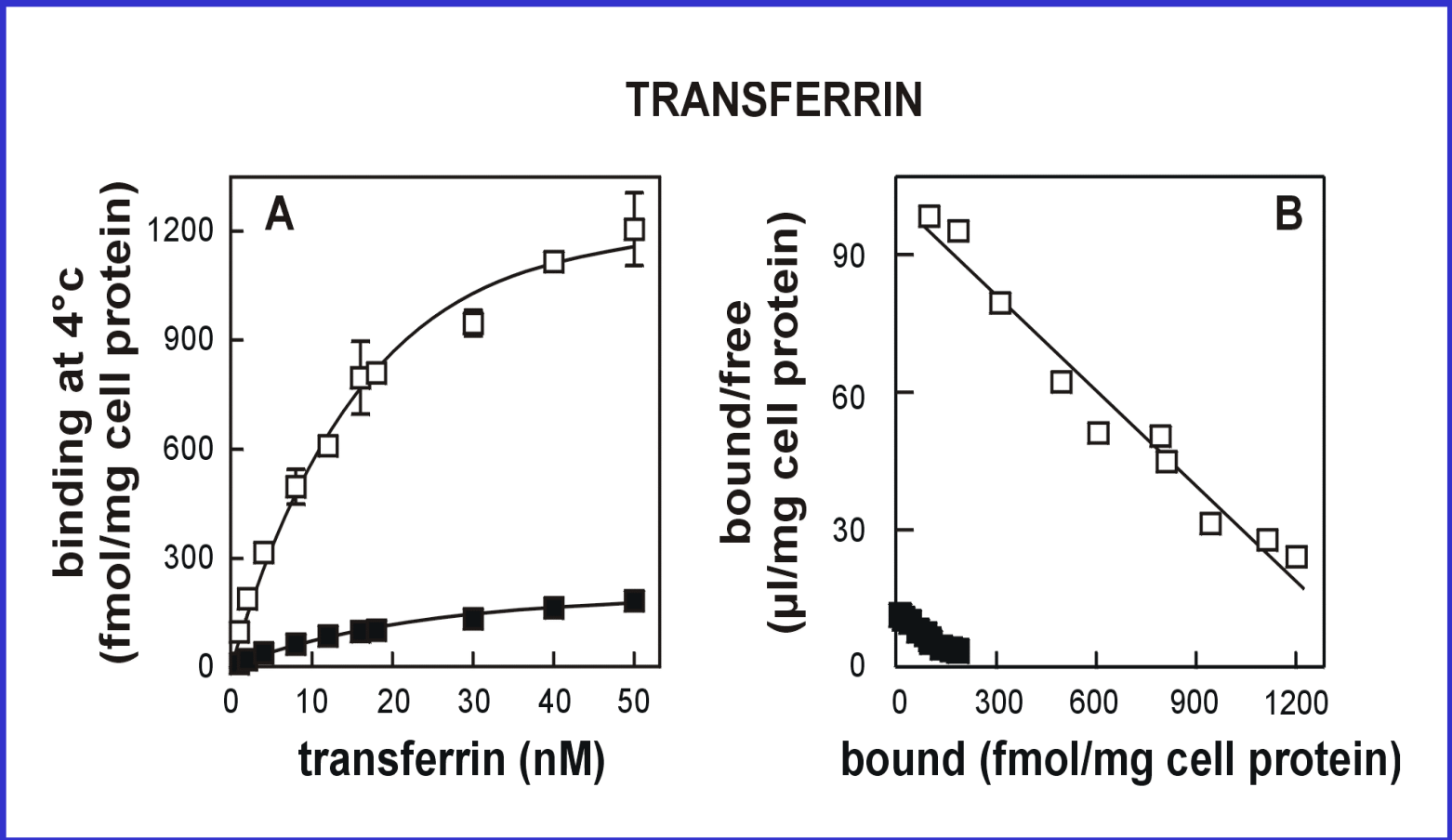


Receptor-mediated endocytosis

➥ ^{125}I-labelled transferrin

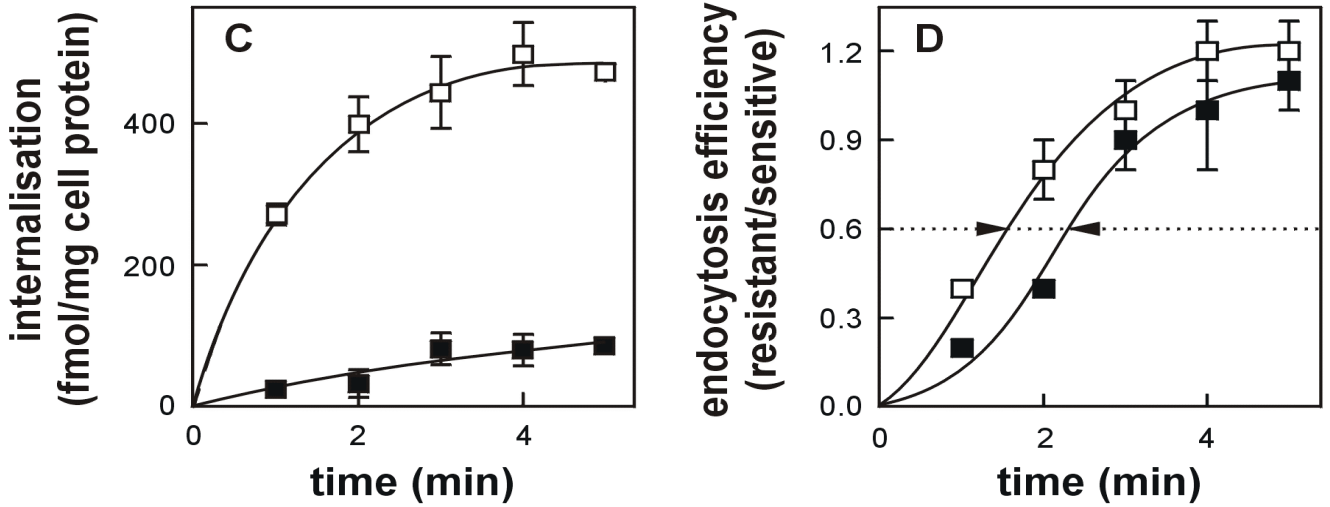


AZ strongly decreases the surface-pool of transferrin receptors

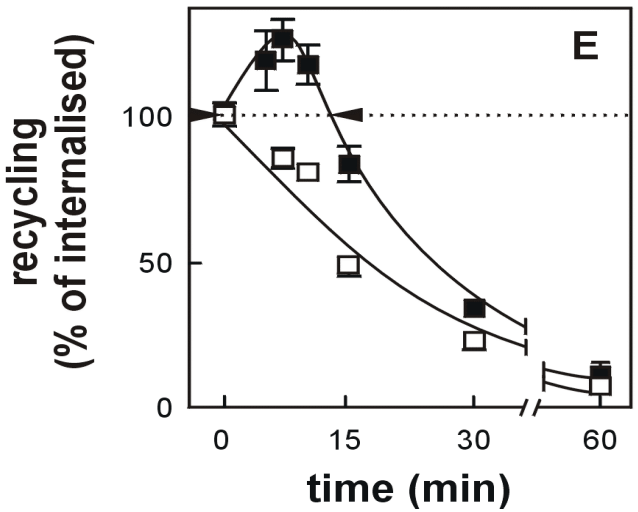


AZ delays sequestration of ligand/receptor in endocytic pits and recycling vesicles

(b)



(c)



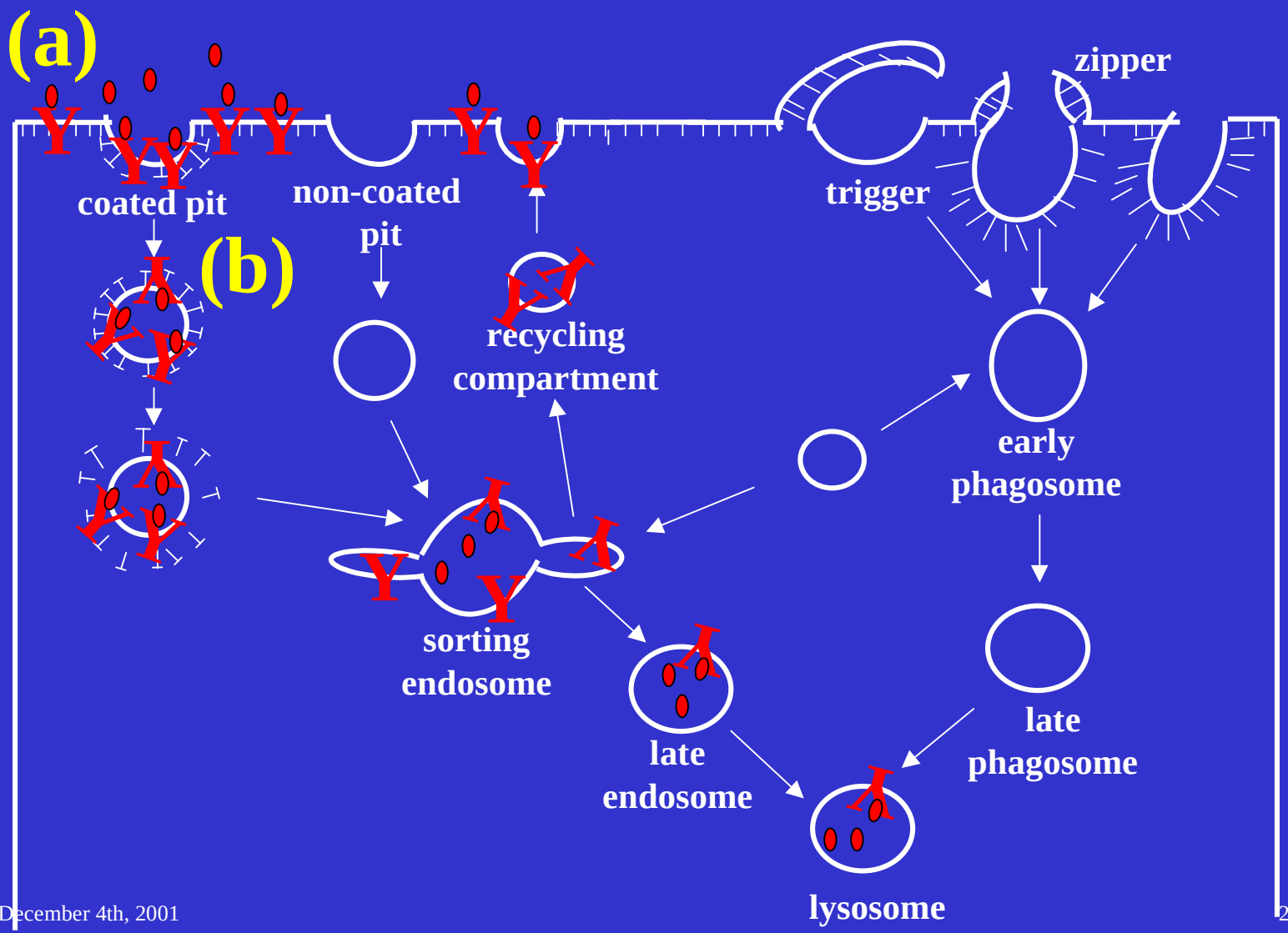
Receptor-mediated endocytosis

➥ PAP immune complexes

• PAP

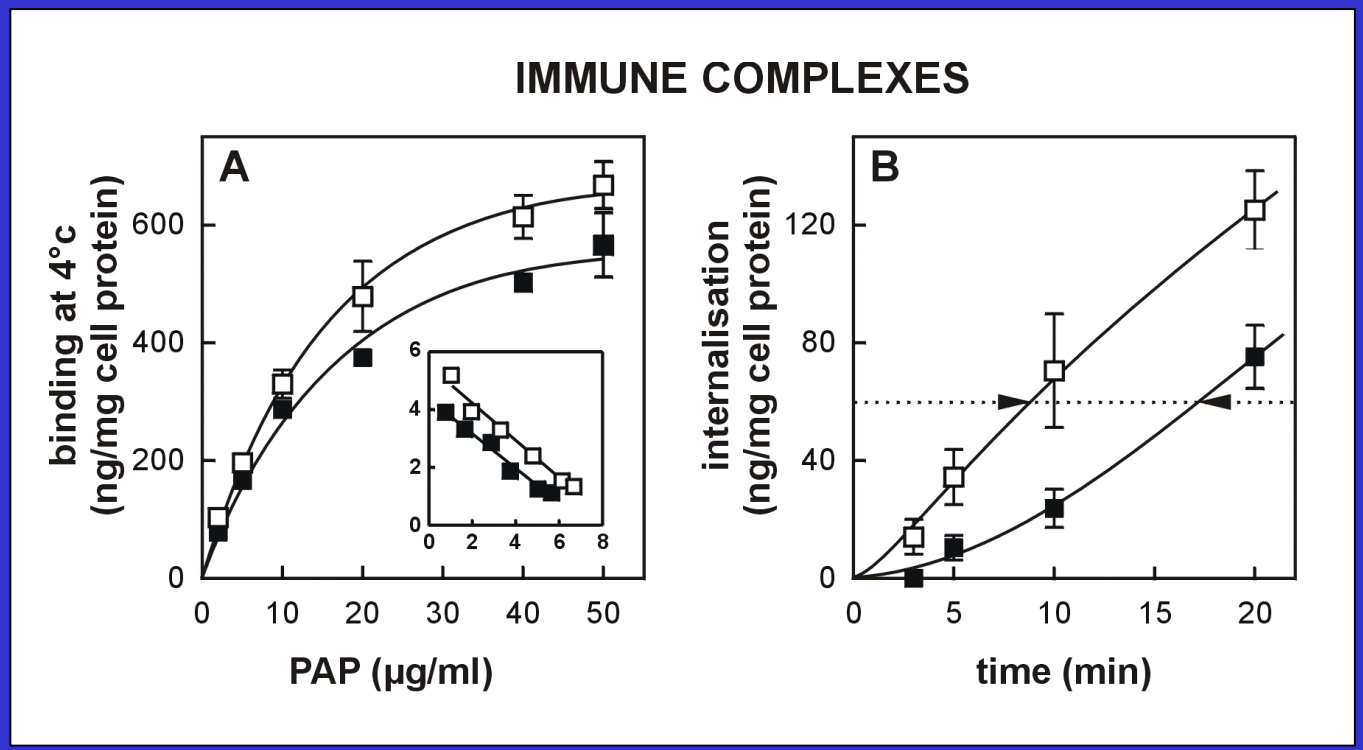
Y Fc?R

(Mellman et al, 1984; Kiss and Rohlich, 1984; 1987)



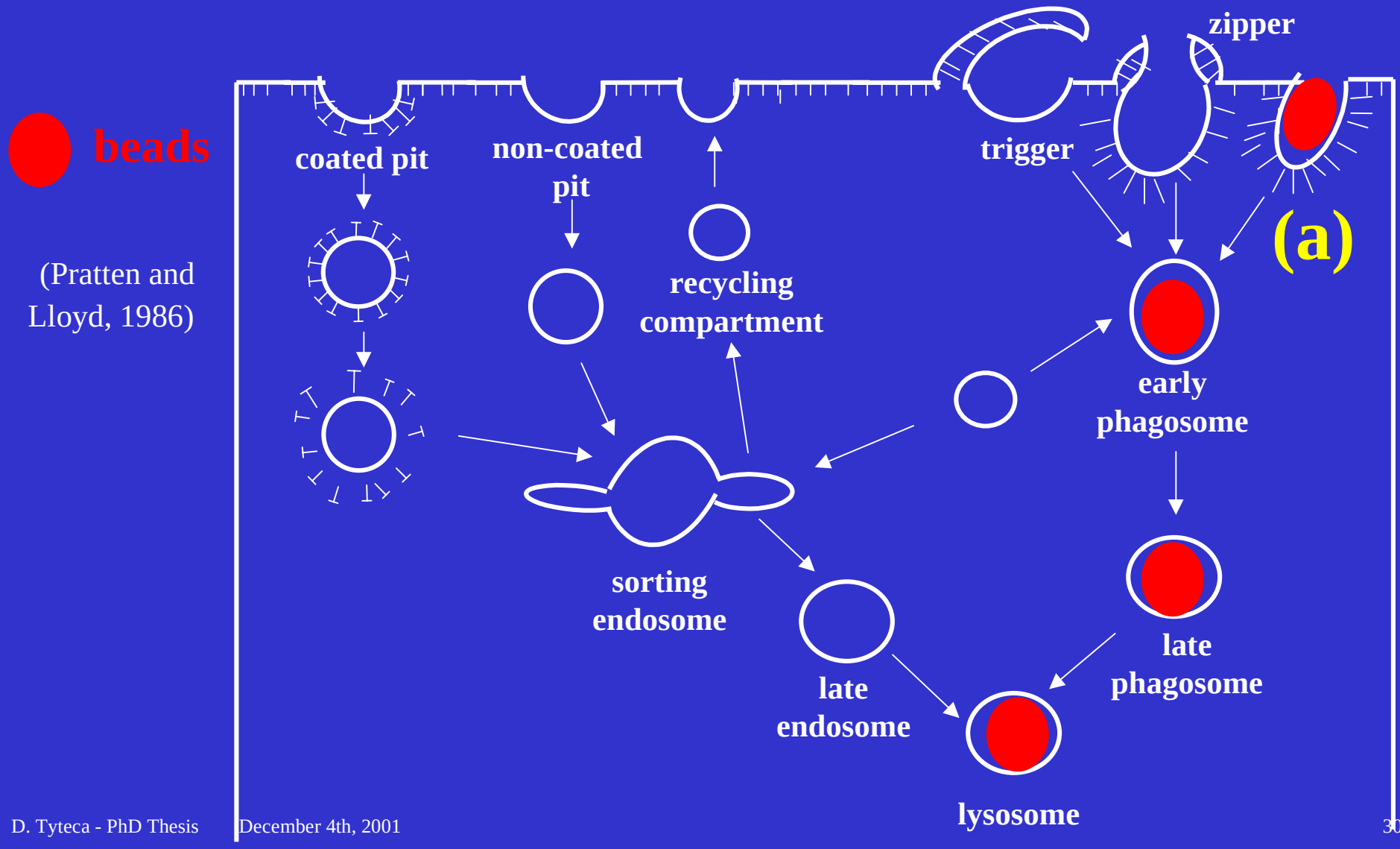
AZ marginally decreases the surface-pool of Fc? receptors and delays sequestration of ligand/receptor complexes into endocytic pits

(a) **(b)**

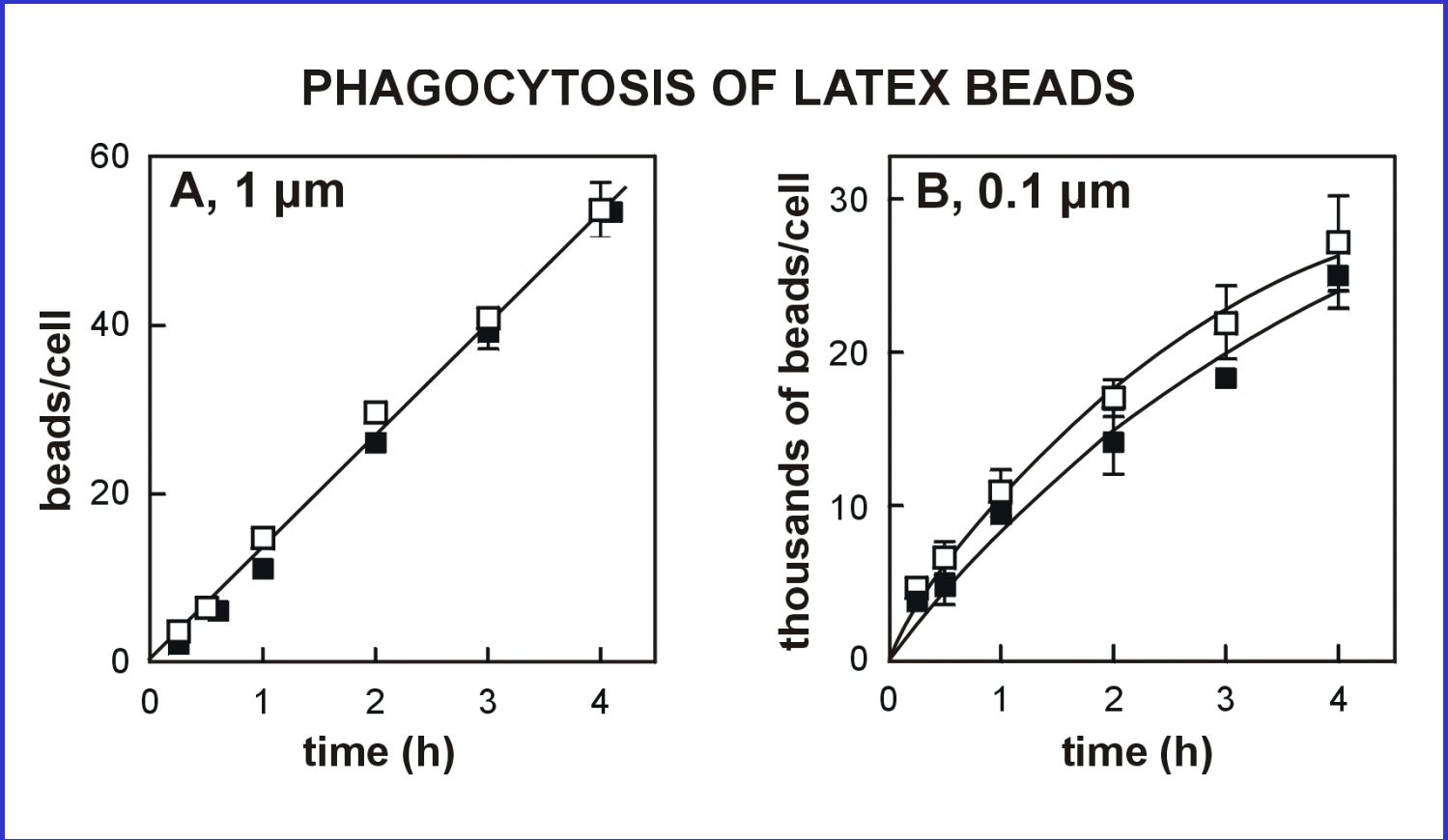


Phagocytosis

➥ latex beads of 1 and 0.1 µm



AZ does not affect phagocytosis

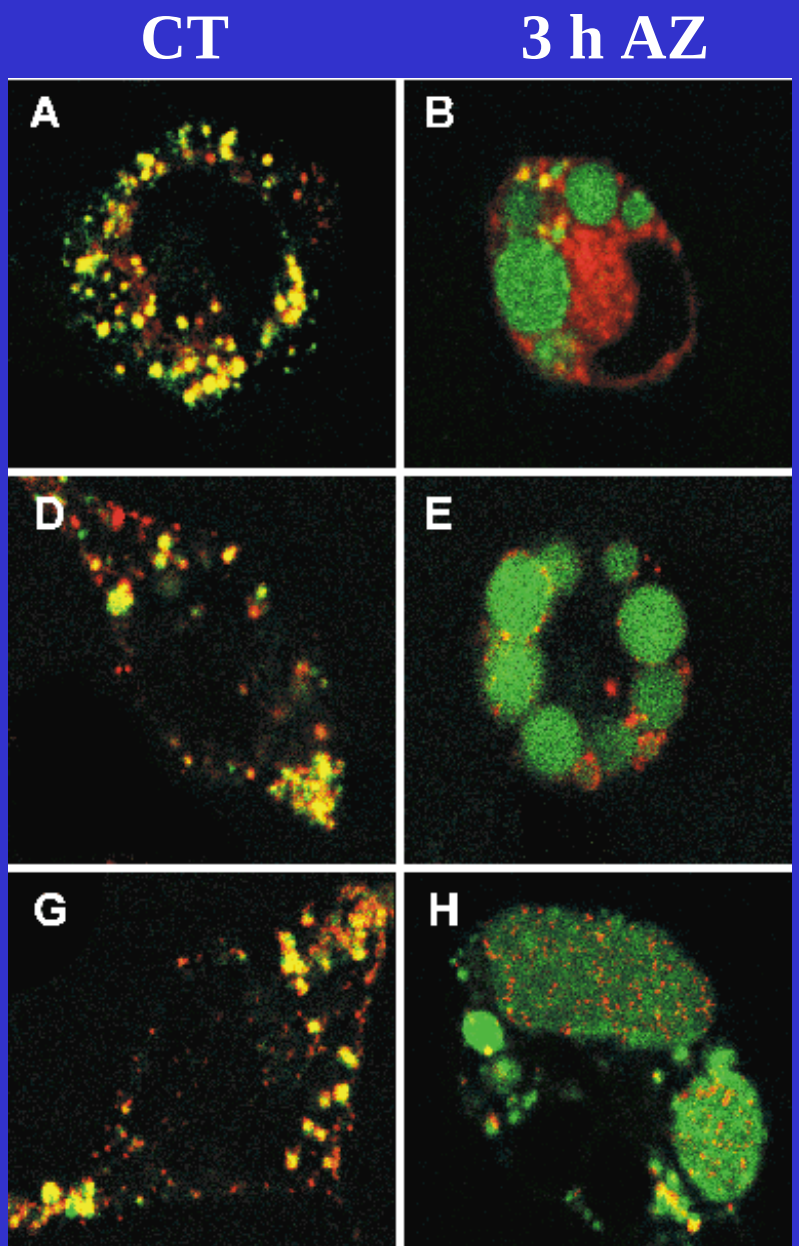


AZ impairs accessibility of HRP and PAP, but not of latex beads, to swollen endosomes/lysosomes

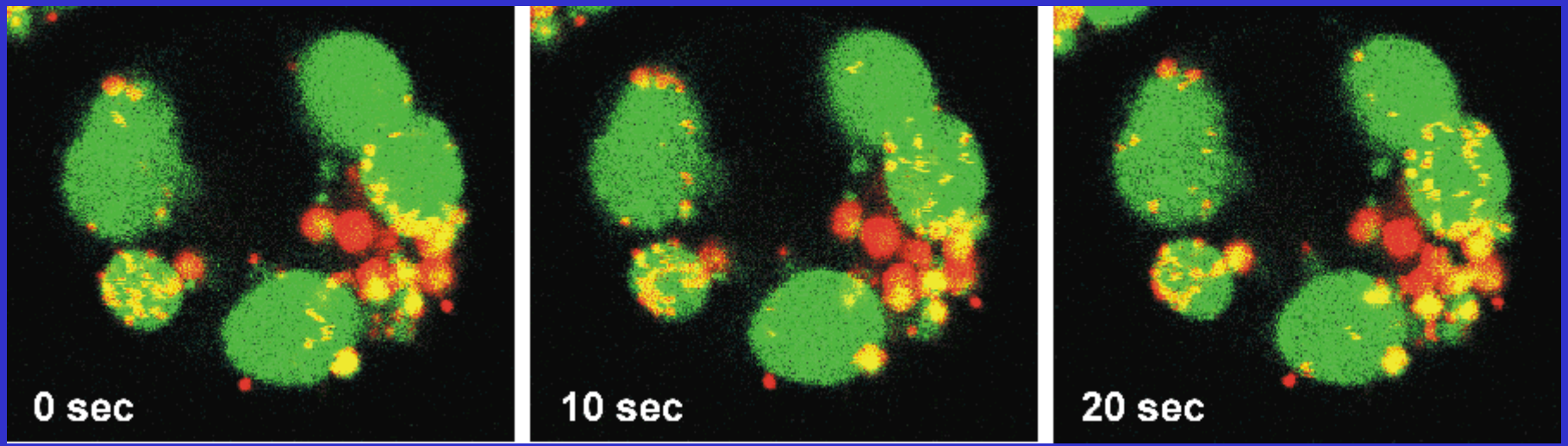
HRP
(fluid-phase endocytosis)

PAP
(receptor-mediated endocytosis)

latex beads
(phagocytosis)



Latex beads move within the structures vacuolated by AZ, demonstrating their presence in these structures



Interpretation

Does AZ perturb endocytosis by:

- a general toxic effect ? NO
- phospholipidosis ? NO
- AZ accumulation ? YES

AZ accumulation ...

- ➥ pH neutralization of endosome/lysosome ?
- ➥ swelling of endosome/lysosome ?
- ➥ membrane interaction ?

③ How does AZ inhibit pinocytosis ?

Azithromycin, a macrolide antibiotic that impairs endocytic trafficking, directly interacts with biomembranes and perturbs their organization and fluidity

D. Tyteca, A. Schanck, Y. F. Dufrêne, M. Deleu, P.J. Courtoy, P.M. Tulkens & M.-P. Mingeot-Leclercq

(to be submitted)

- **Selection of experimental system**
  - ➥ **liposomes**
  - ➥ **Langmuir-Blodgett monolayers**
  - ➥ **J774 mouse macrophages**

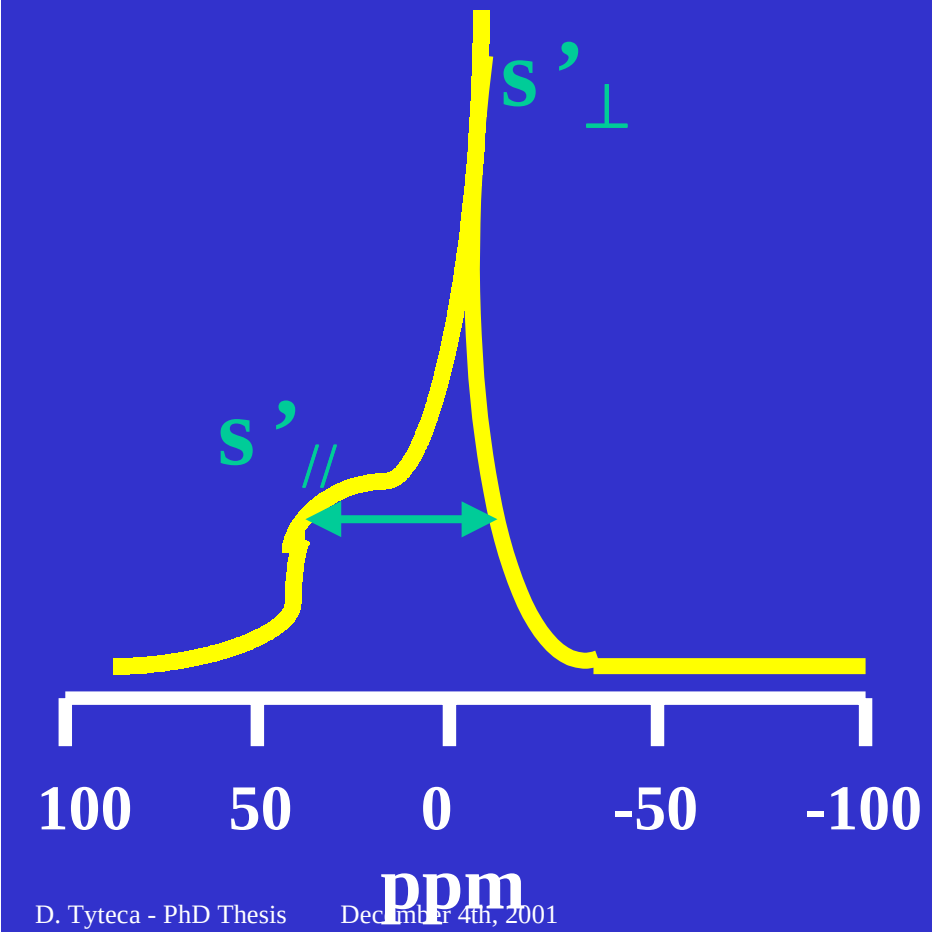
- **Experimental tests**
  - ➥ **interaction with membranes**
  - ➥ **membrane organization in domains**
  - ➥ **insertion of membrane probes in the plasma membrane**
  - ➥ **membrane fluidity**

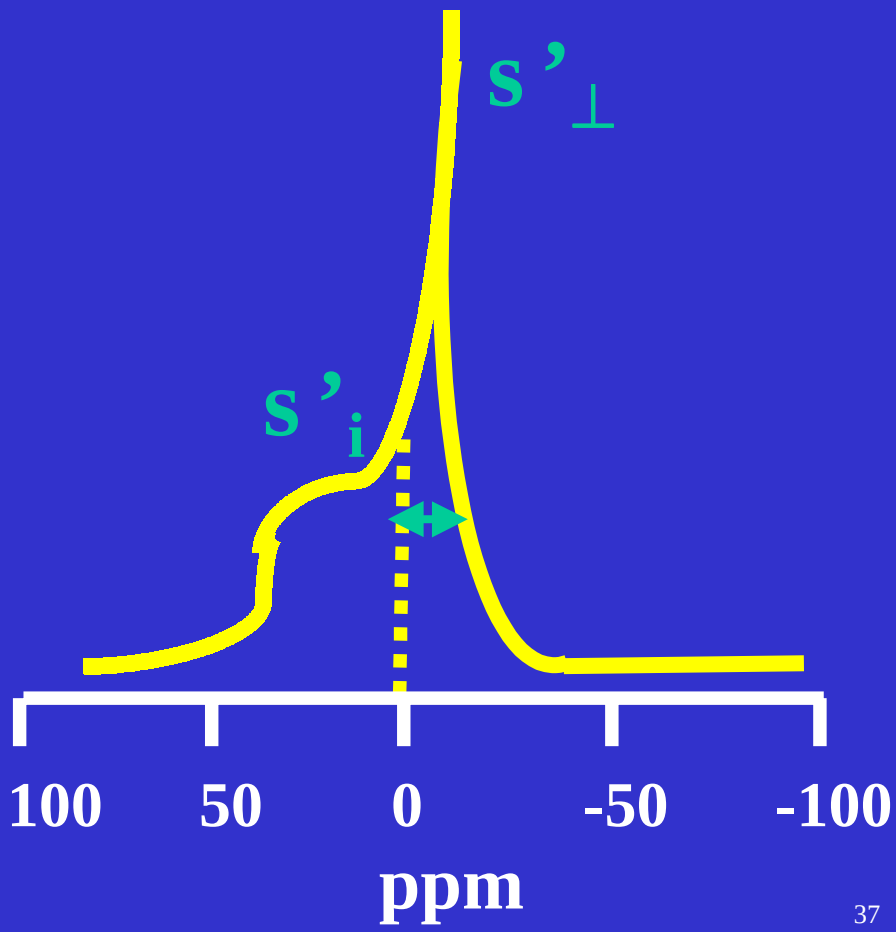
interaction of AZ with membranes

➥ ^{31}P nuclear magnetic resonance (NMR)

$\Delta' s = s'_{//} - s'_{\perp}$

$\Delta' s \text{ eff.} = s'_{i} - s'_{\perp}$



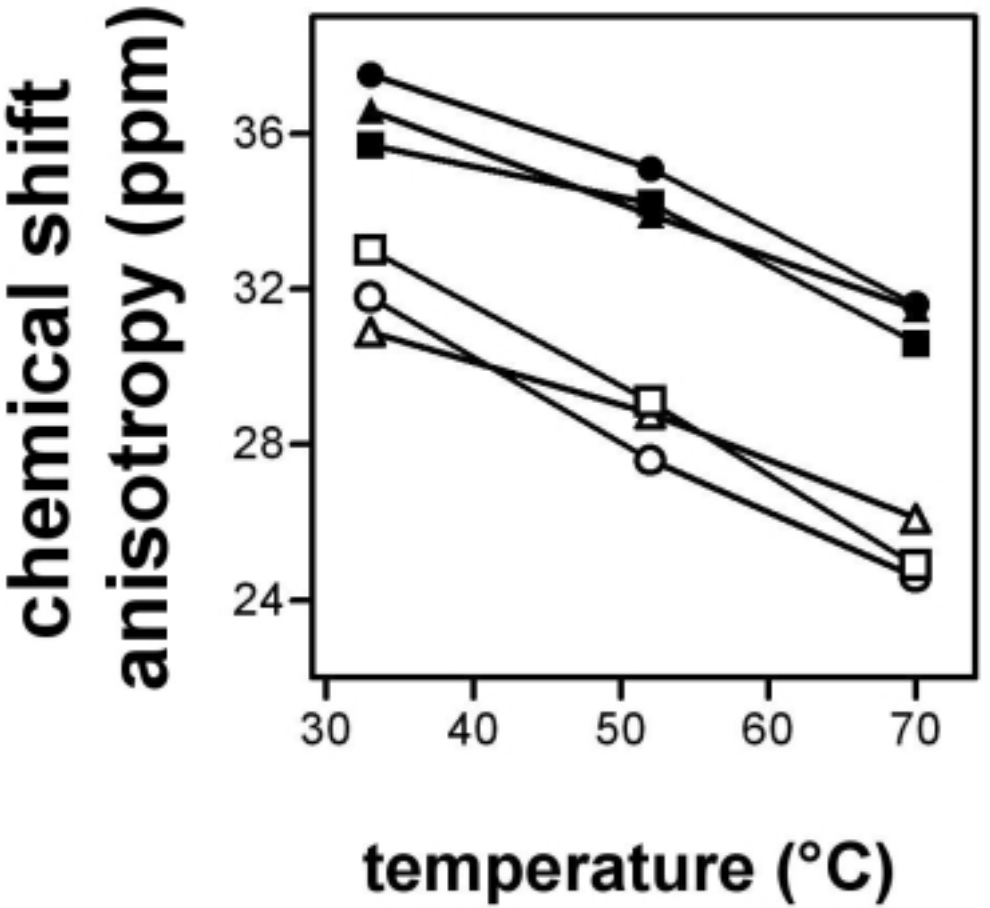


AZ interacts with lipids of liposomes made of cholesterol: PC: SM: PI in a pH-independent fashion

pH 5.4

pH 6.0

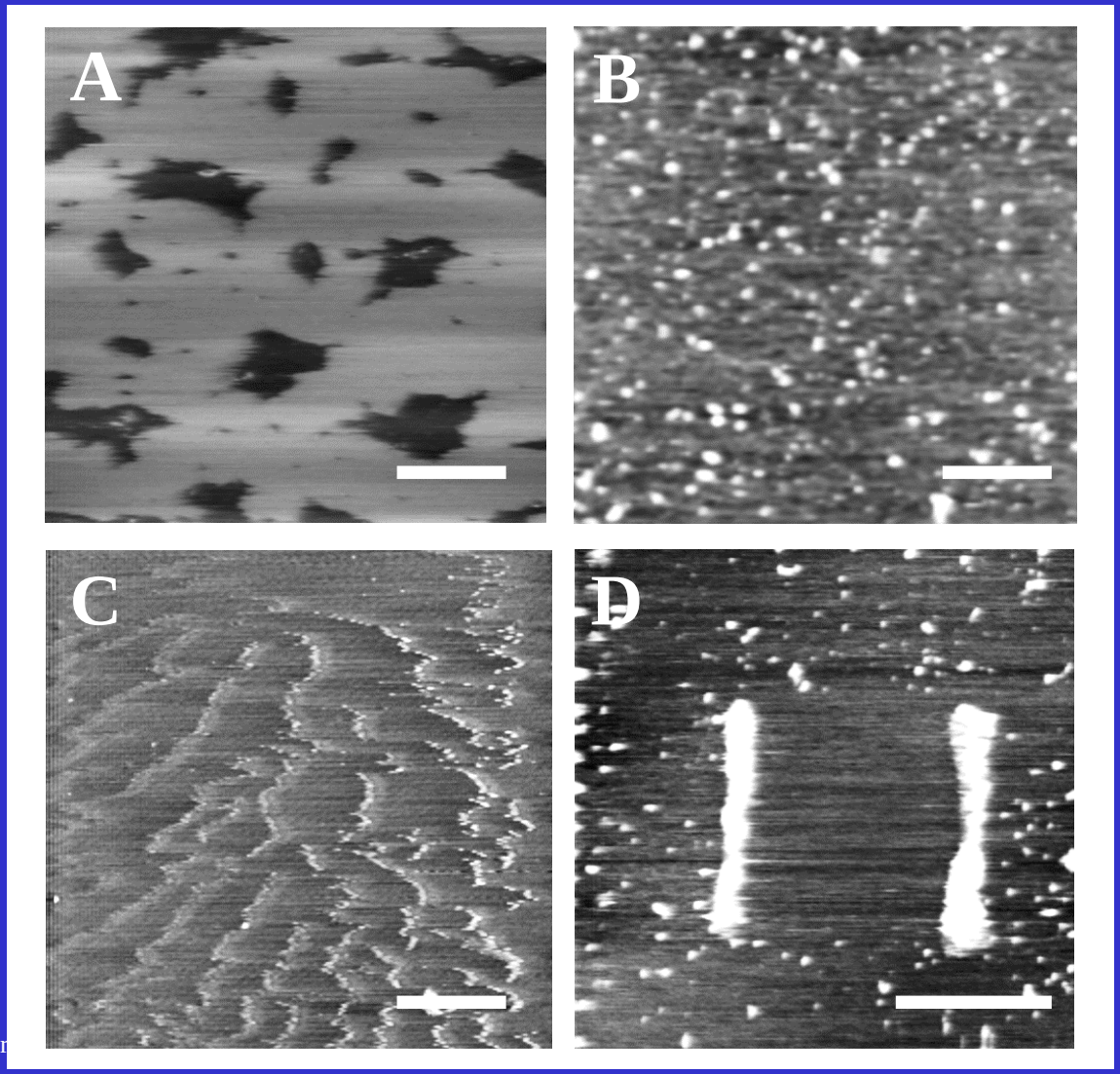
pH 7.0



membrane organisation in domains

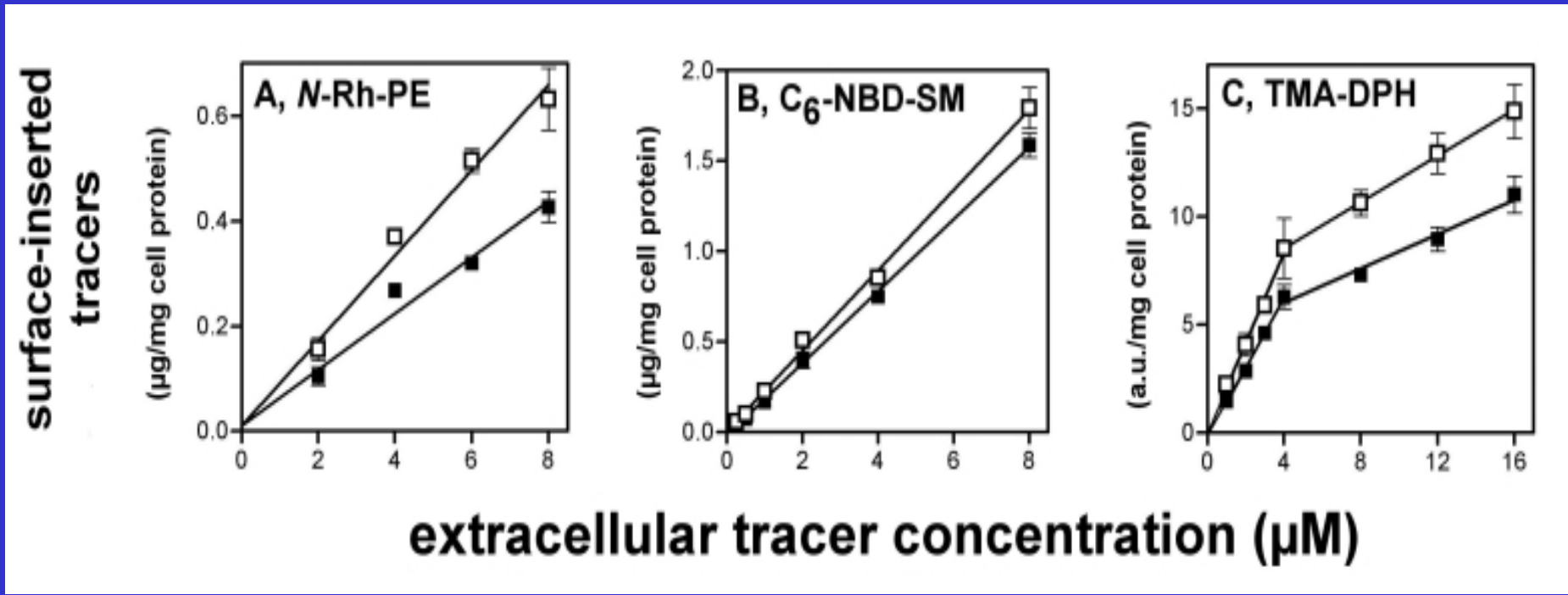
➥ atomic force microscopy (AFM)

AZ interacts with lipids and perturbs the organization of DPPC: cholesterol Langmuir-Blodgett monolayers



AZ reduces incorporation in the plasma membrane of three membrane tracers

insertion of membrane probes in the plasma membrane of J774



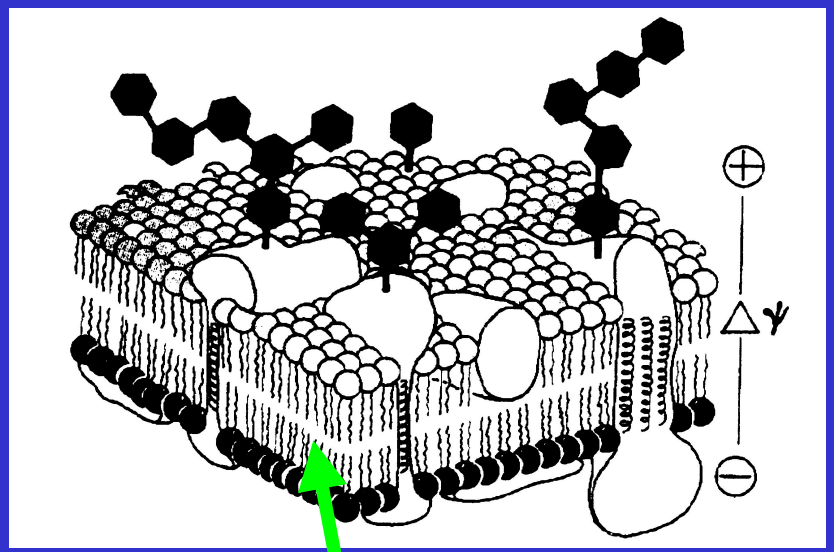
➥ aggregates

➥ monomers; external leaflet of the PM

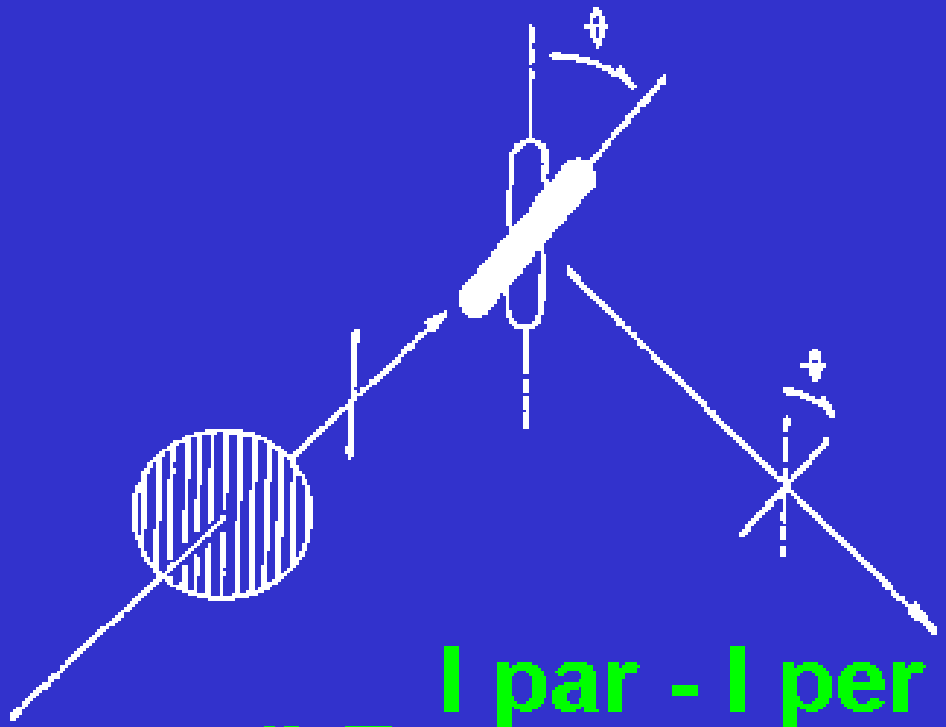
➥ monomers; deep in the PM

plasma membrane fluidity of J774

➥ fluorescence anisotropy of TMA-DPH



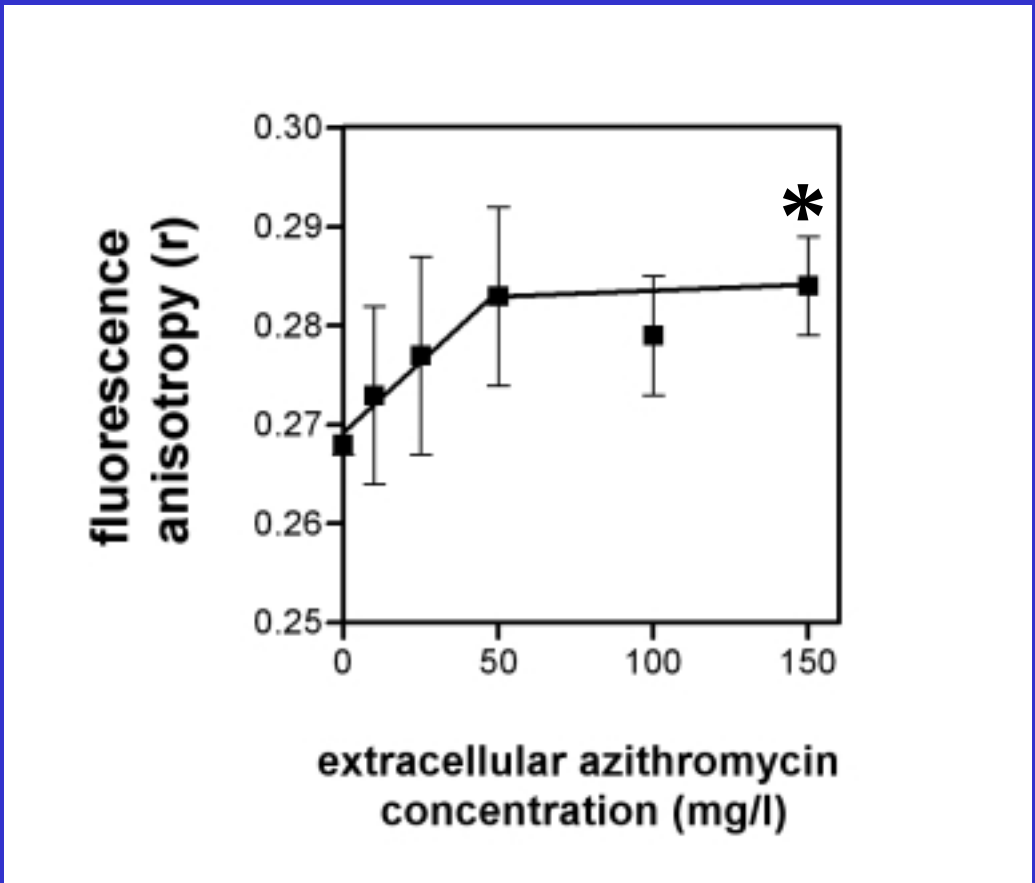
TMA-DPH



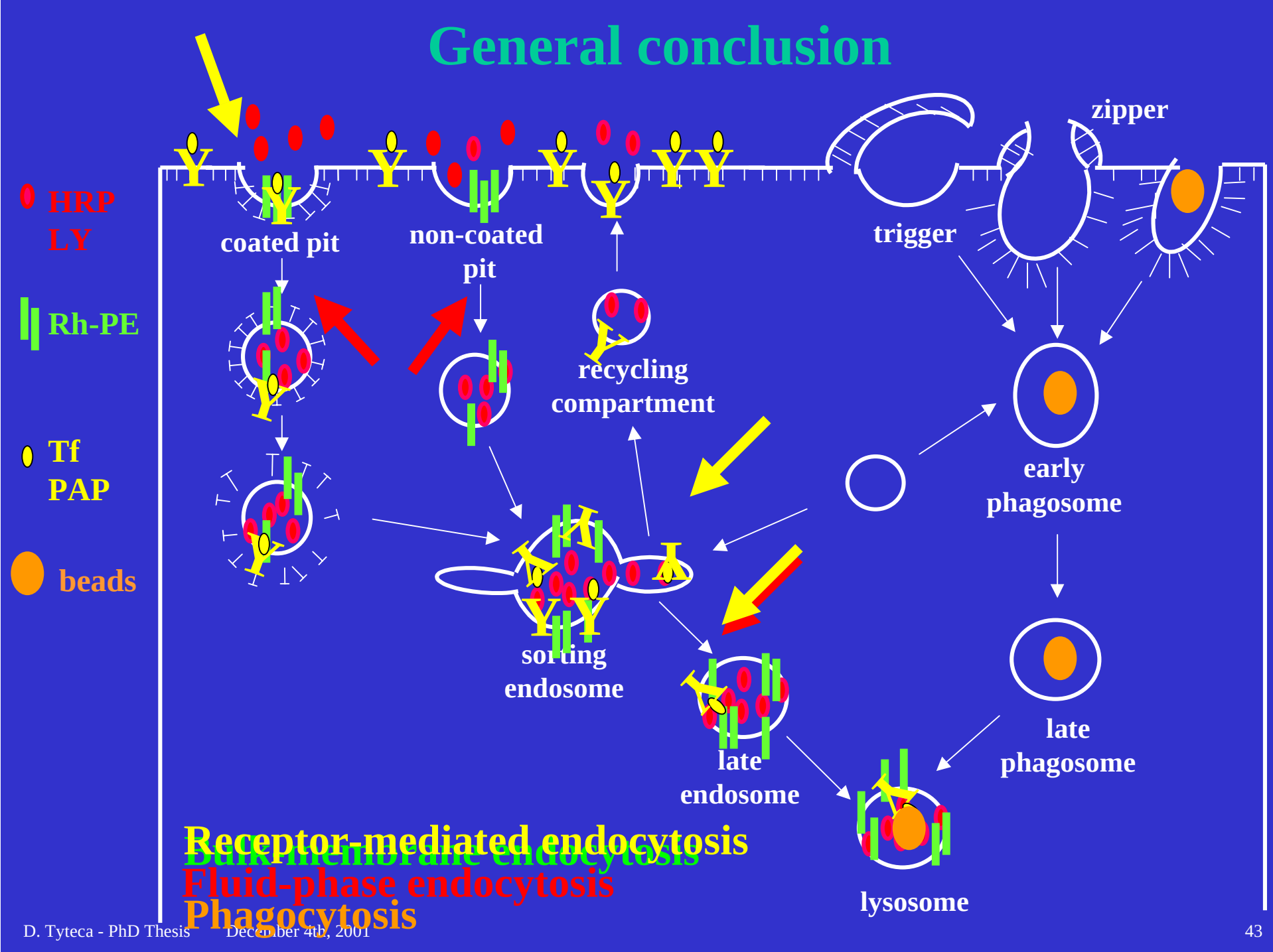
$$p = \frac{I_{par} - I_{per}}{I_{par} + I_{per}}$$

$$r = \frac{2p}{3 - p}$$

AZ decreases J774 plasma membrane fluidity

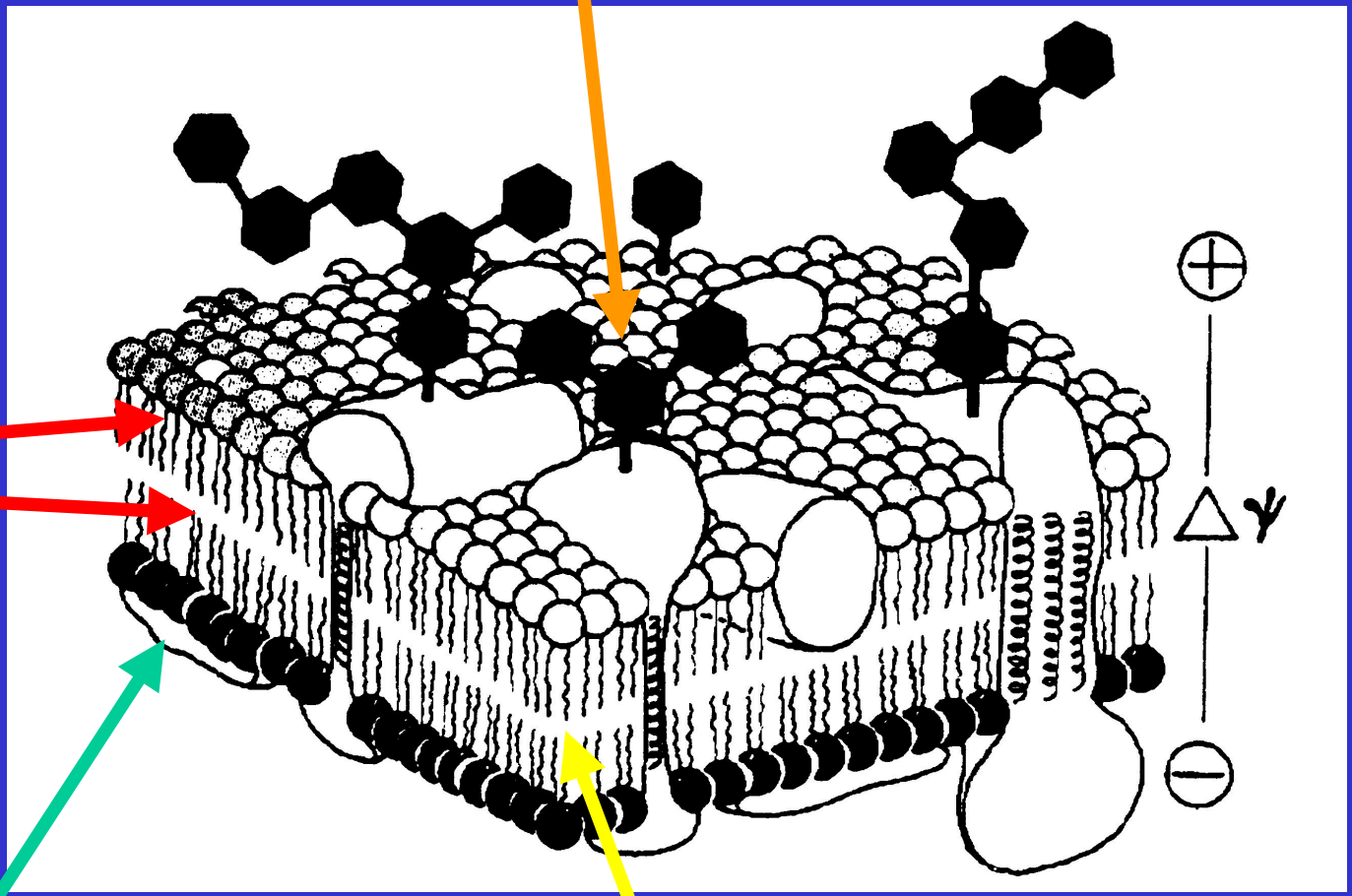


General conclusion



AZ perturbs membrane organization in domains (monolayers)

AZ decreases incorporation of: *N*-Rh-PE, C_6-NBD-SM } TMA-DPH (J774 plasma membrane)



AZ interacts with phospholipid headgroups (liposomes)

AZ decreases membrane fluidity (J774 plasma membrane)

Short-term perspectives

- **Mechanism of AZ action:**
  - **role of membrane properties**
    - **membrane fluidity, tension, transverse asymmetry and composition**
    - **receptor mobility in the plasma membrane and incorporation into coated pits**
  - **role of vacuolation**
  - **role of pH neutralization**

Long-term perspectives

➥ **Usefulness of AZ**

- **inhibition of selective endocytic modes/pathways/steps**

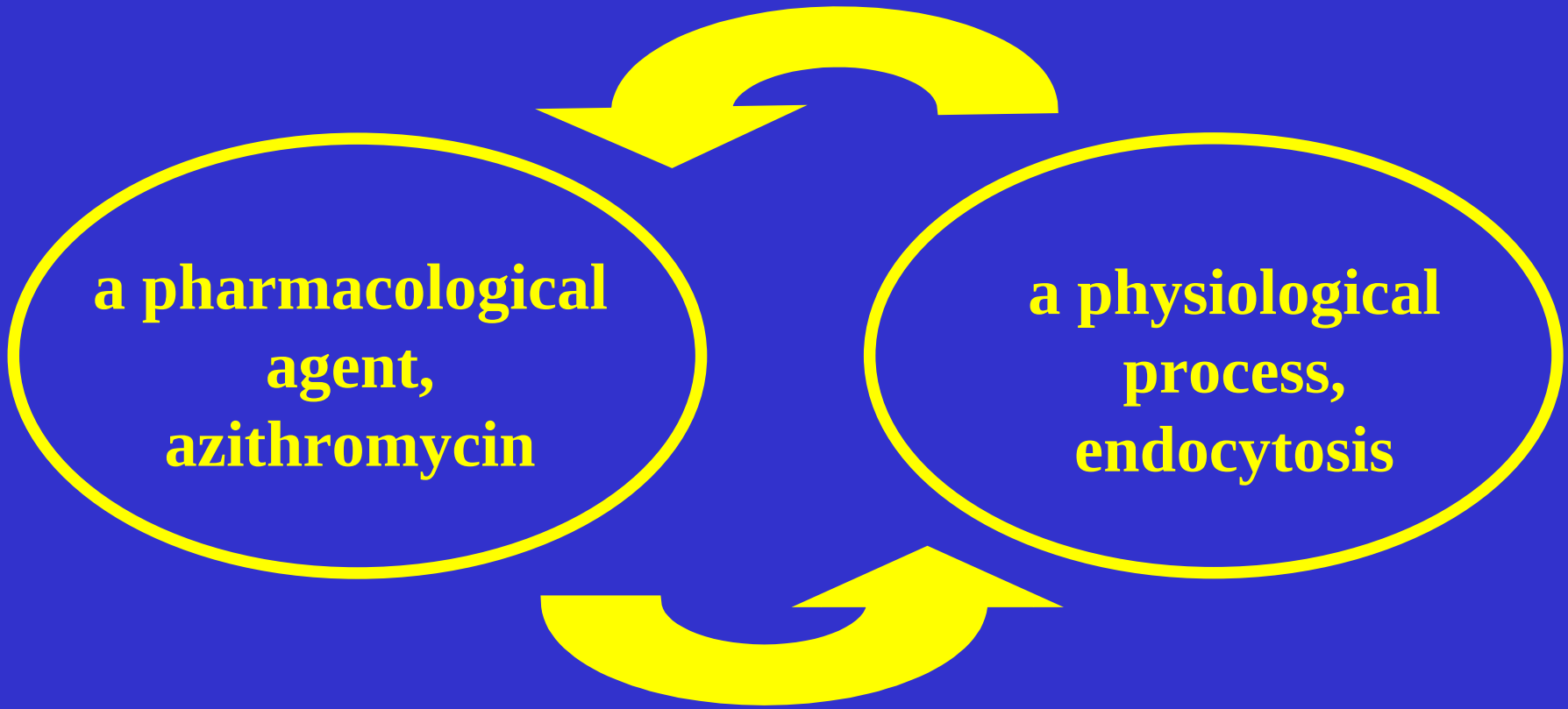
  study of a series of ligands, e.g. cholera toxin internalization

- **differential modulation by drug concentration**

➥ **Practical application: mechanism and function**

- **clathrin-independent endocytosis**
- **recycling pathway**
- **fusogenicity between endosomes and lysosomes**

Progress in cellular toxicology of azithromycin



a tool to better understand mechanisms and molecular machineries of endocytic modes/pathways/steps