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

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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),
- ....
Pathways of endocytosis studied in this thesis

pinocytosis

coated pit

non-coated pit

recycling compartment

sorting endosome

late endosome

lysosome

phagocytosis

zipper

trigger

early phagosome

late phagosome
Membrane lipids are implicated at various stages of the endocytic process.

membrane organization in domains

membrane fluidity

membrane asymmetry
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

**acidification**
- vacuolar H⁺-ATPase

**docking**
- Rab
- PI 3-kinase

**fusion**
- SNARE/SNAP
- membrane
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)
budding cytoskeleton adhesion to a curved particle
membrane tension and asymmetry

agents:
chlorpromazine
benzyl alcohol
cytochalasins
nacodazole
batilomycins

coop proteins: clathrin, AP COP
membrane docking
Rab PI 3-kinase

acidiification
H+-ATPase

adhesion to a curved particle

fission

transport actin microtubules

wortmannin
gentamicin

fusioin SNARE/SNAP membrane
phospholipid dysis

H+ H+ H+ chloroquine

H+ H+ H+ acidification

H+-ATPase

coat proteins: clathrin, AP COP

dynamyn
cytoskeleton

coat proteins:
cation, AP COP

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 ???
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)

diagram showing molecular interactions

perturbs the fusion of lysosomes with horseradish peroxidase (HRP)-containing endosomes (unpublished observation of Van Bambeke)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

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

- Experimental test
  Fluid-phase endocytosis
Fluid-phase endocytosis

\( \rightarrow \) horseradish peroxidase (HRP)

(a) coated pit
(b) non-coated pit

recycling compartment

trigger

zipper

Coated pit

Non-coated pit

Early phagosome

Late phagosome

Sorting endosome

Late endosome

Lysosome

Fluid-phase endocytosis

\( \rightarrow \) horseradish peroxidase (HRP)

(Cupers et al, 1994)

(Cupers et al, 1994)
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
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

Is AZ specific to fluid-phase endocytosis?
Fluid-phase endocytosis

- HRP and lucifer yellow (LY)

(Cupers et al, 1994; Swanson et al, 1987)

Fluid-phase endocytosis involves the uptake of fluid and particles into cells, such as HRP and lucifer yellow (LY). The process can be divided into two main stages:

(a) Coated pit:
- HRP and LY are taken up into the cell through coated pits.
- They are then transported to the non-coated pit.
- From the non-coated pit, they are recycled to the cell membrane or move to the recycling compartment.

(b) Sorting endosome:
- Particles are sorted into early phagosomes, late phagosomes, and lysosomes.
- The early phagosome can fuse with late phagosomes or lysosomes.

Early phagosomes and late phagosomes are involved in the uptake of large particles, while lysosomes are responsible for the degradation of internalized materials.
AZ inhibits fluid-phase endocytosis and this inhibition is reversible.
Bulk-membrane endocytosis

\[ \Rightarrow \ N\text{-}rhodamine\text{-}phosphatidylethanolamine (N-Rh-PE) \]

(a) aggregates of N-Rh-PE

(Kok et al, 1990)
AZ slows down bulk-membrane endocytosis
Receptor-mediated endocytosis

125I-labelled transferrin

(a) Tf

(b) TfRY

(c) Y Y Y Y Y Y Y Y Y

(early phagosome)

(late phagosome)

(sorting endosome)

(recycling compartment)

(coated pit)

(non-coated pit)

(early endosome)

(late endosome)

(late phagosome)

(lysosome)

(zipper)

(trigger)

(Cupers et al, 1994)
AZ strongly decreases the surface-pool of transferrin receptors

![Graph](image-url)
AZ delays sequestration of ligand/receptor in endocytic pits and recycling vesicles
Receptor-mediated endocytosis

PAP immune complexes

(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

(Pratten and Lloyd, 1986)
AZ does not affect phagocytosis
AZ impairs accessibility of HRP and PAP, but not of latex beads, to swollen endosomes/lysosomes
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 ?
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)

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

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

- pH 5.4
- pH 6.0
- pH 7.0

Chemical shift anisotropy (ppm) vs. temperature (°C)
AZ interacts with lipids and perturbs the organization of DPPC: cholesterol Langmuir-Blodgett monolayers

membrane organisation in domains

atomic force microscopy (AFM)
AZ reduces incorporation in the plasma membrane of three membrane tracers

insertion of membrane probes in the plasma membrane of J774

extracellular tracer concentration (μM)

 нескольque insertion

monomers; external leaflet of the PM

monomers; deep in the PM
plasma membrane fluidity of J774

fluorescence anisotropy of TMA-DPH

\[
p = \frac{l_{\text{par}} - l_{\text{per}}}{l_{\text{par}} + l_{\text{per}}}
\]

\[
r = \frac{2p}{3 - p}
\]
AZ decreases J774 plasma membrane fluidity
**General conclusion**

- **Coated pit**
- **Non-coated pit**
- **Receptor-mediated endocytosis**
- **Fluid-phase endocytosis**
- **Phagocytosis**

**Processes**:
- **Fluid-phase endocytosis**
- **Rh-PE**
- **Tf-PAP**
- **HPR**
- **LY**
- **Beads**

**Components**:
- **Trigger**
- **Recycling compartment**
- **Sorting endosome**
- **Late endosome**
- **Early phagosome**
- **Late phagosome**
- **Lysosome**

**Conclusion**

*General conclusion*
AZ decreases membrane fluidity
(J774 plasma membrane)

AZ perturbs membrane organization in domains (monolayers)

AZ decreases incorporation of:
N-Rh-PE,
C6-NBD-SM
TMA-DPH
(J774 plasma membrane)

AZ interacts with phospholipid headgroups (liposomes)
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 pharmacological agent, azithromycin

a physiological process, endocytosis

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