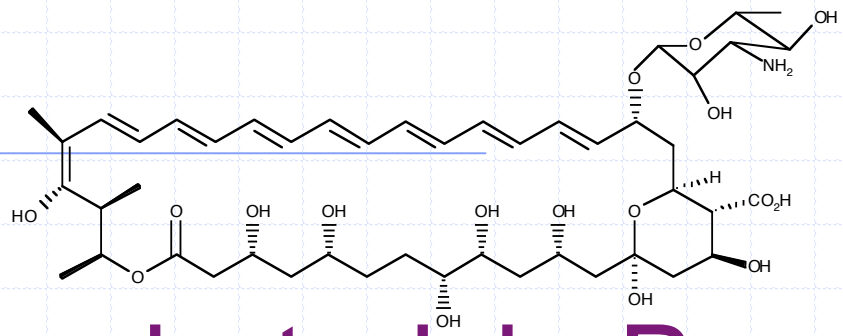


Studies on Amphotericin B

Current Formulations, the liposome concept and toxicity

Scott C. Hartsel

Chemistry Department

University of Wisconsin-Eau Claire

Overview

- An ominous threat
- What is Amphotericin B?
- The problem with Amphotericin B
- What are liposomes and what good are they?
- Where Have We Done in This lab?
- Where are we going?

An Ominous Threat

- Using a number of different models, researchers have concluded that the current incidence of both suspected and confirmed fungal infections in immunosuppressed AIDS, cancer, and organ transplant patients is nearly 400,000. -*International Association of Physicians in AIDS Care*,1996

- Ninety percent of people with AIDS develop at least one fungal infection over the course of the disease....10-20% of the systemic infections prove fatal -(Benedict S, Colagreco J. Fungal infections associated with malignancies, treatments and AIDS. *Cancer Nurs* 17:411-7, 1994.)

Some Fungal Pathogens in AIDS

Organism	Clinical syndrome
Candida albicans	Thrush, vaginal candidiasis, esophageal candidiasis
Cryptococcus neoformans	Meningitis,pneumonia
Penicillium marneffei	Fever alone or with pulmonary infiltrates, lymphadenopathy, or cutaneous lesions

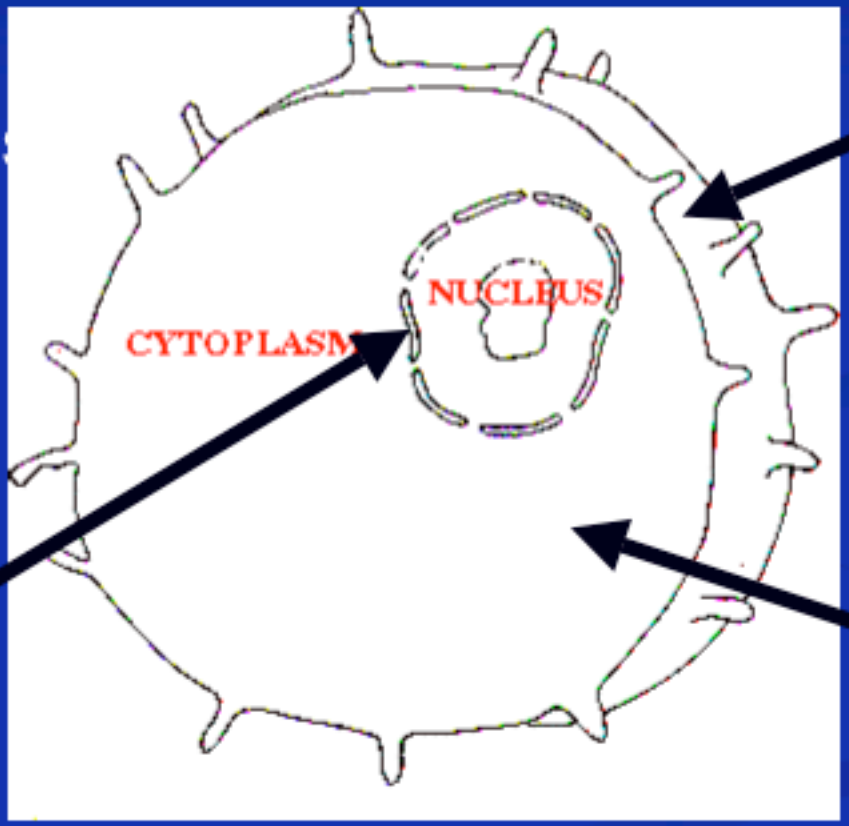
Drug therapy: ketoconazole, fluconazole
Amphotericin B

Current anti-fungal drugs

- Different classes of drugs target the plasma membrane, sterol biosynthesis, DNA biosynthesis, and β-glucan biosynthesis
- Fungal membranes and sterol biosynthetic enzymes are different enough from ours that these agents can kill fungi but not us
- Fungi make β-glucan, we don't, so drugs that target β-glucan biosynthesis have low side-effects

Mechanism of action (I)

Cell wall biosynthesis

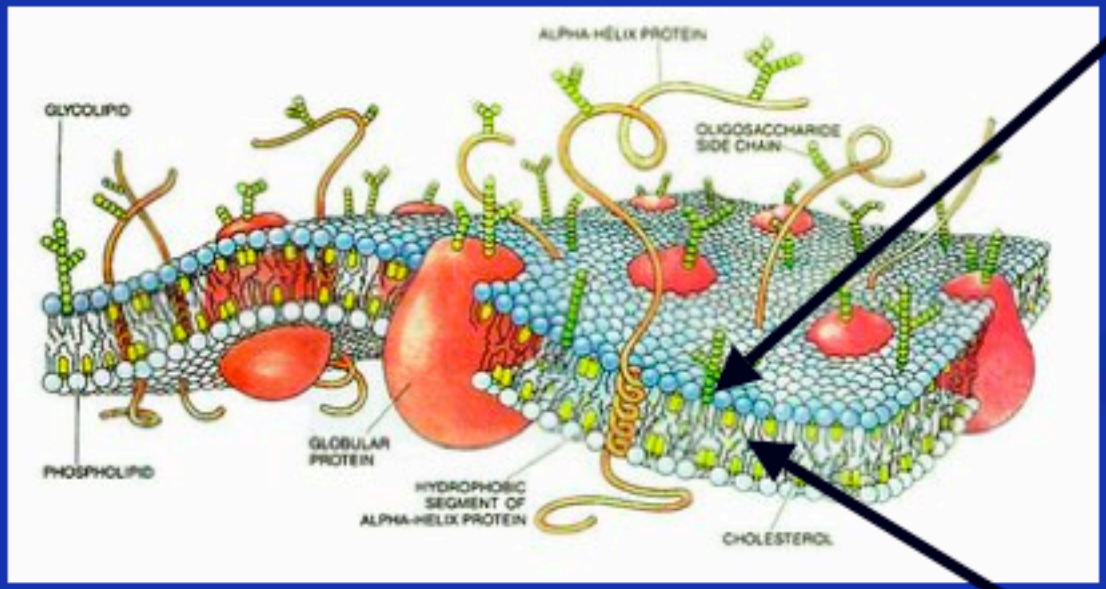


Cell Membrane

DNA Synthesis

Sterol biosynthesis

Mechanism of action (II)

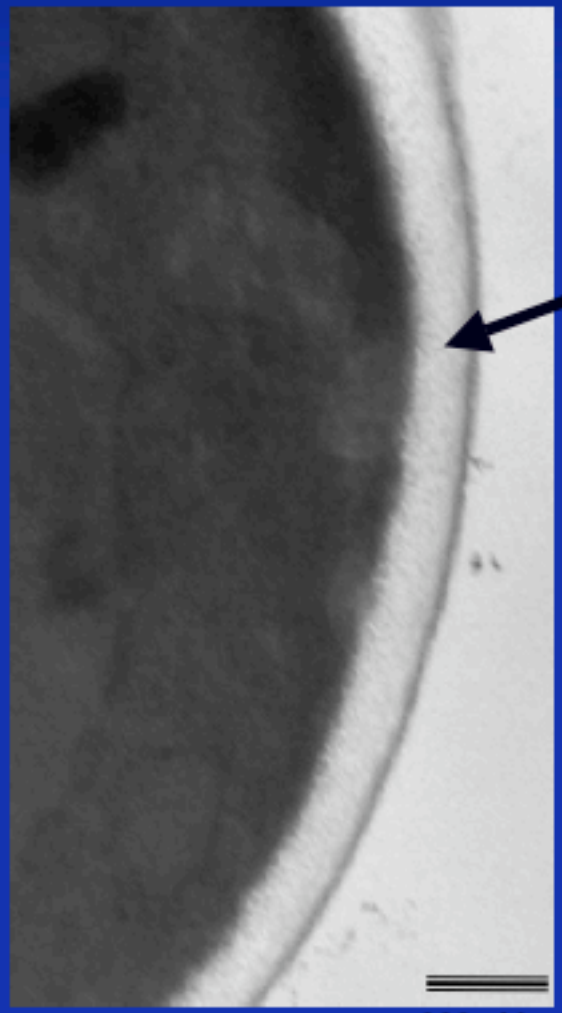


Azole drugs target the fungal-specific synthesis of membrane lipids

Amphotericin inserts preferentially into fungal membranes and disrupts their function

Mechanisms of action (III)

Echinocandins target synthesis of β-glucan, a fungal-specific cell wall molecule



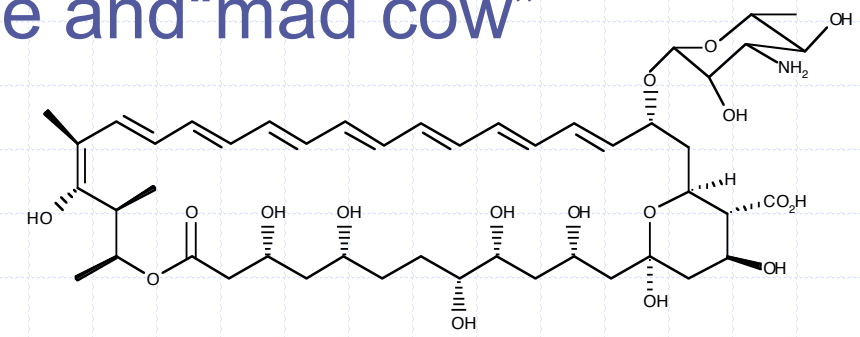
Candida albicans on human epithelium Source: Holland/Özel, Robert Koch-Institut Ber

What's missing in antifungal therapy?

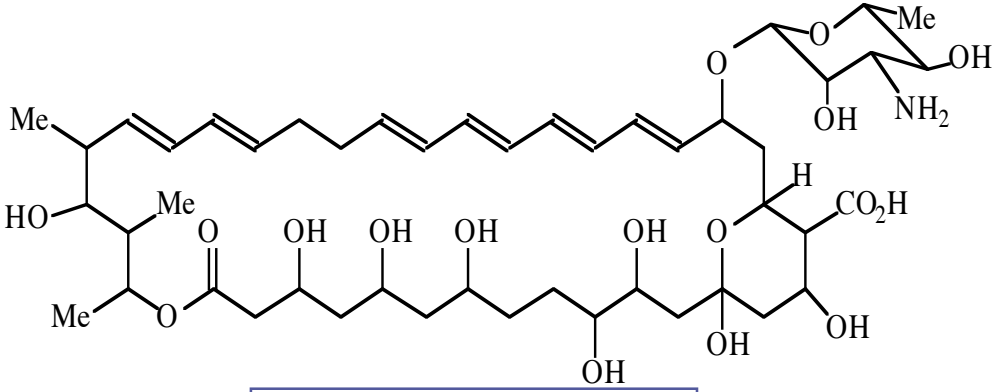
- Specificity (no toxicity)
  - Activity throughout the body
- Broad spectrum
  - Kill microbes, not just prevent growth
- No drug-drug interactions
  - Low cost

What is Amphotericin B?

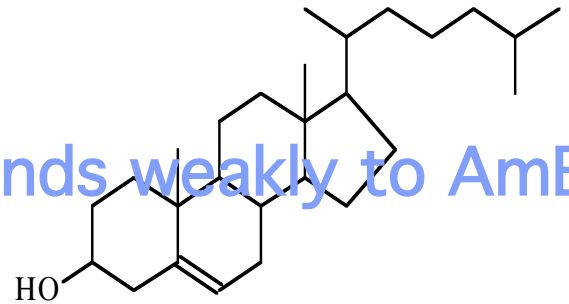
- The "Swiss Army Knife" of drugs
  - Antifungal (1st line for serious fungal infections)
  - Antiparasitic (*Leishmania*)
  - Antiviral (HIV)
  - Antimicrobial (indirect?)
  - Antiprion (*e.g.* scrapie and"mad cow" disease !!)
  - Anticancer?



What is Amphotericin B?

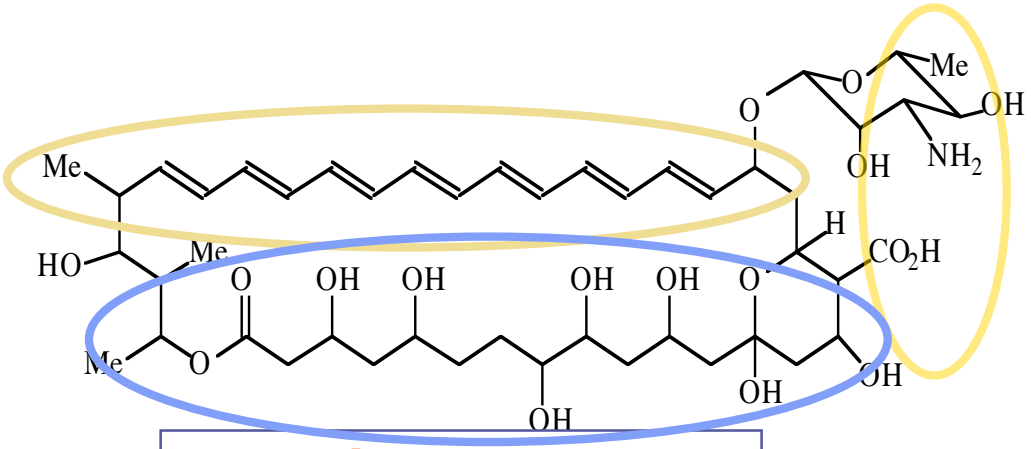


Nystatin

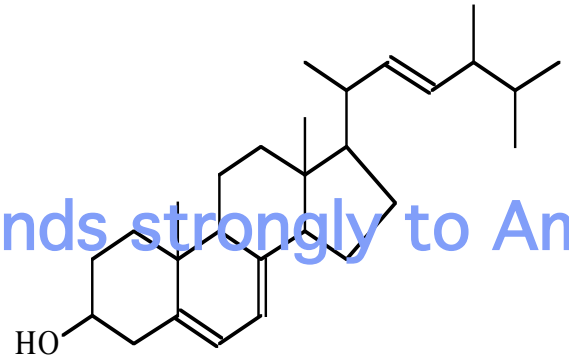


Binds weakly to AmB

Cholesterol: humans



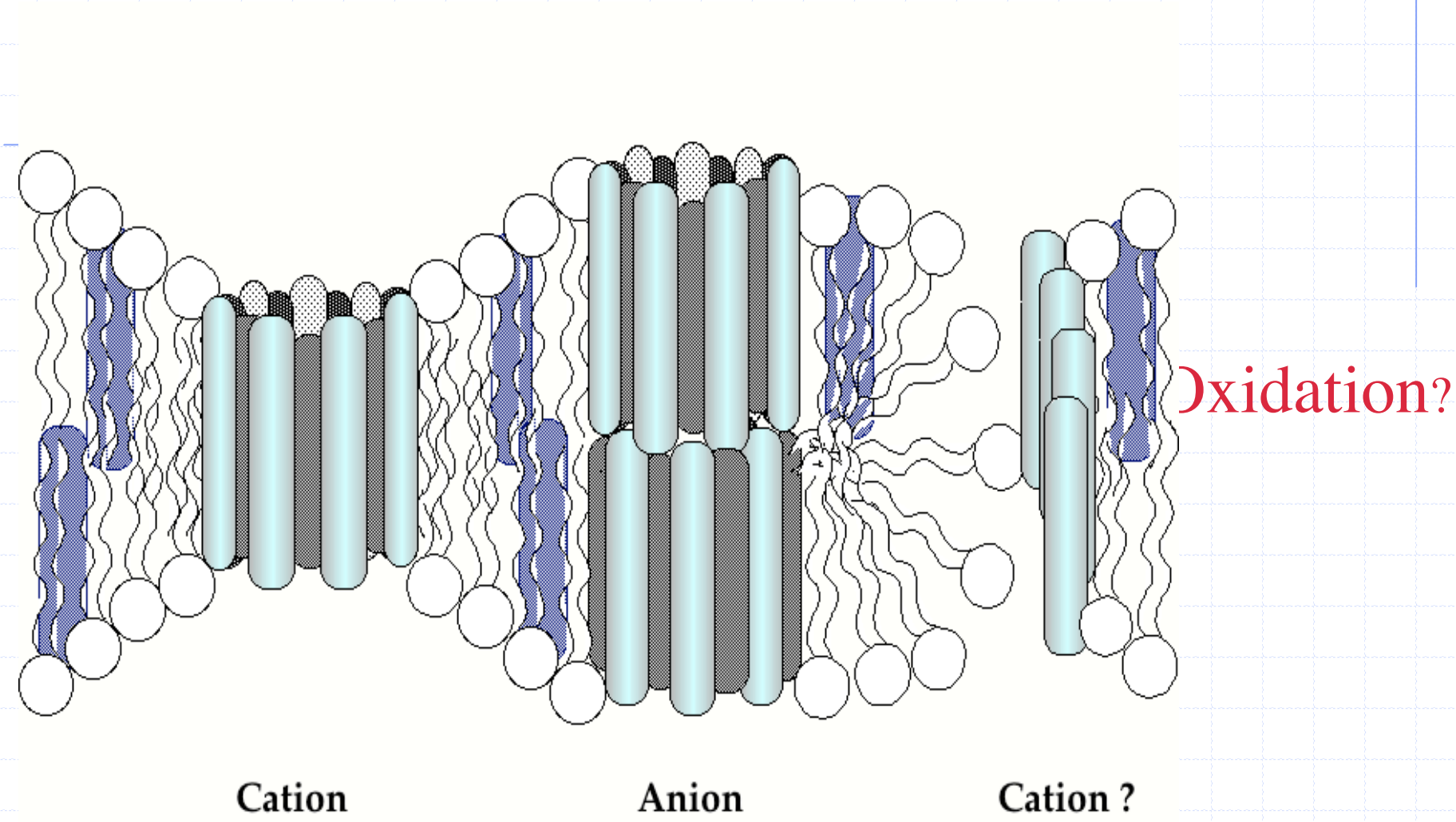
Amphotericin B



Binds strongly to AmB

Ergosterol: fungi

AmB Mode of Action



- **The bottom line:** Amphotericin preferentially forms pores in fungal membranes, causing death or inhibition

The Problem with Amphotericin B-
Side Effects of Fungizone (7:3 AmB/deoxycholate, a bile detergent)

- *high* fever, chills, nausea, phlebitis, aches
- permanent kidney damage
- long course-6 months ~2x/week
- I.V. delivery *only*/not absorbed orally
- called “Shake n’ bake” “amphoterrible” in medical slang
- terribly toxic *but* terribly effective

Sarah, a patient with histoplasmosis, on Amphotericin B treatment:

- “the drug made me the sickest I have ever been-it was worse than the disease”
- “I lost 30 pounds and had to quit school for 6 months”
- “I hurt everywhere…nausea…dry heaves…104º fever…my mouth tasted metallic”
- “my insurance wouldn’t pay for the better stuff”

Amphotericin's Three Modes of Action

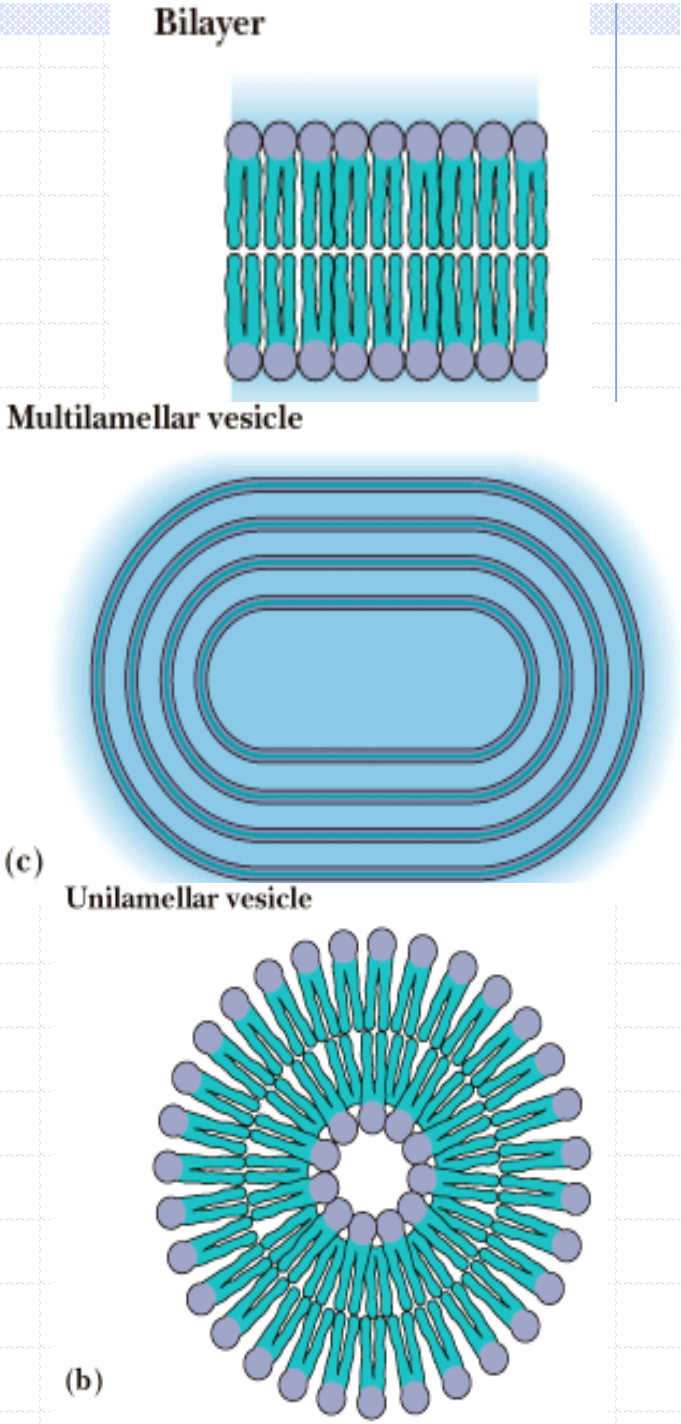
- The Three Facets
  - Intrinsic activity of different supramolecular forms
  - Contextual activity-e.g. in serum with proteins, lipoproteins, macrophages, etc.
  - Immune modulation/cytokine gene expression patterns (pro and con)

- Question: What effect do the various liposome systems have on these facets?

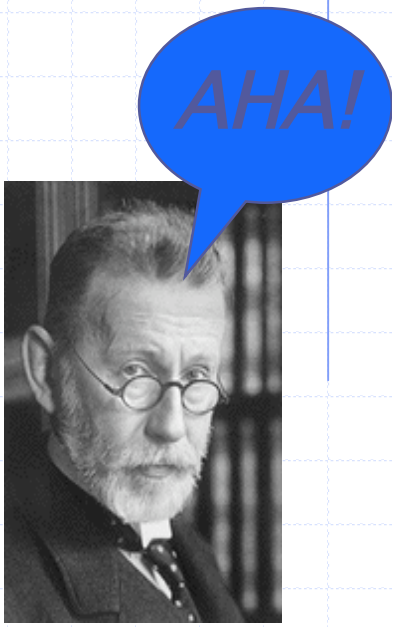
WAIT...
What Are Liposomes?
And...
What good are they?

Liposomes

- Phospholipids will spontaneously form bilayers in aqueous solutions
- Sir Alec Bangham coins term "liposome"(Bangham AD, et al."Diffusion of univalent ions across the lamellae of swollen phospholipids. J Mol Biol. 1965 Aug;13(1):238-52.).
- "Bangosomes", multilamellar vesicles, were good models of biological membranes and had an enclosed aqueous space like cells.
- Papahadopoulos, Szoka, Gregoriadis (1970's)- Maybe single-layered liposomes could be used as mini drug capsules!

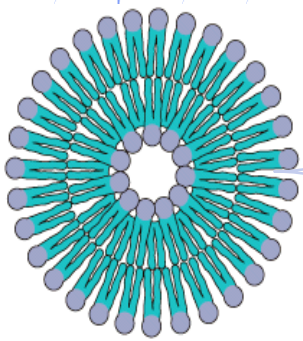


Hypothesis: Liposomes could be used to deliver drugs



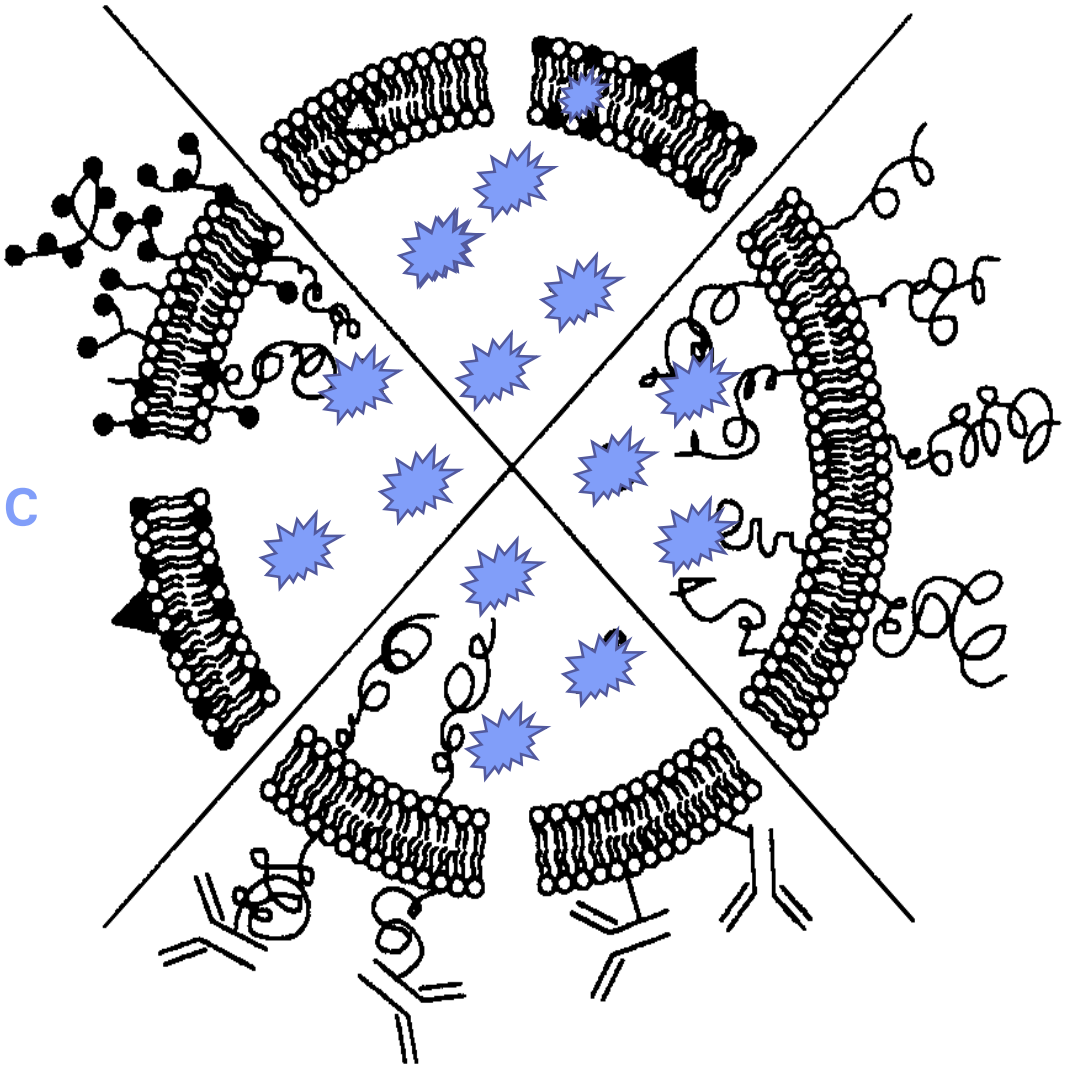
- Liposomes could encapsulate drugs for a long period without leakage .
- Encapsulated toxic drugs could safely circulate for a long time without harm to the host.
- The liposome “capsules” could be specifically targeted only to diseased tissue, tumors or pathogens *(Like Paul Erlich’s magic bullet model of 1907 !)*

Using Liposomes as Magic Bullets



Cationic

-The drug



Conventional

Stealth™

Immunoliposomes

Back to Amphotericin...

Methods for reducing AmB toxicity

- Chemical derivatives (*e.g.* AME, MS 8209, oligo-ethylene glycol conjugates)
- AmB (Fungizone)+ 10% Intralipid
- Liposomal / lipid associated formulations
- Something new?-Hot-Zone

Ampho gets Liposomes

TABLE 2. FDA Approved AmB pharmaceutical preparations

AmB Preparation	composition mole ratio net charge	physical state, shape diameter, (μM)	Cost
Fungizone	DOC/AmB 7:3 negative	micelles <0.4	Dirt Cheap $5-10
AmB-Lipid complex (Ablecet)	DMPC/DMPG/AmB 7:3:3 negative	sheets 1.6-11	$$$$$$ $125-150
AmBisome	HSPC/Chol/DSPG/AmB 2:1:0.8:0.4 negative	small unilamellar vesicle 0.06	$$$$$
AmB Colloidal Dispersion (Amphocil)	DS/AmB 1:1 negative	discs 0.12	$$$$

Not a Liposome

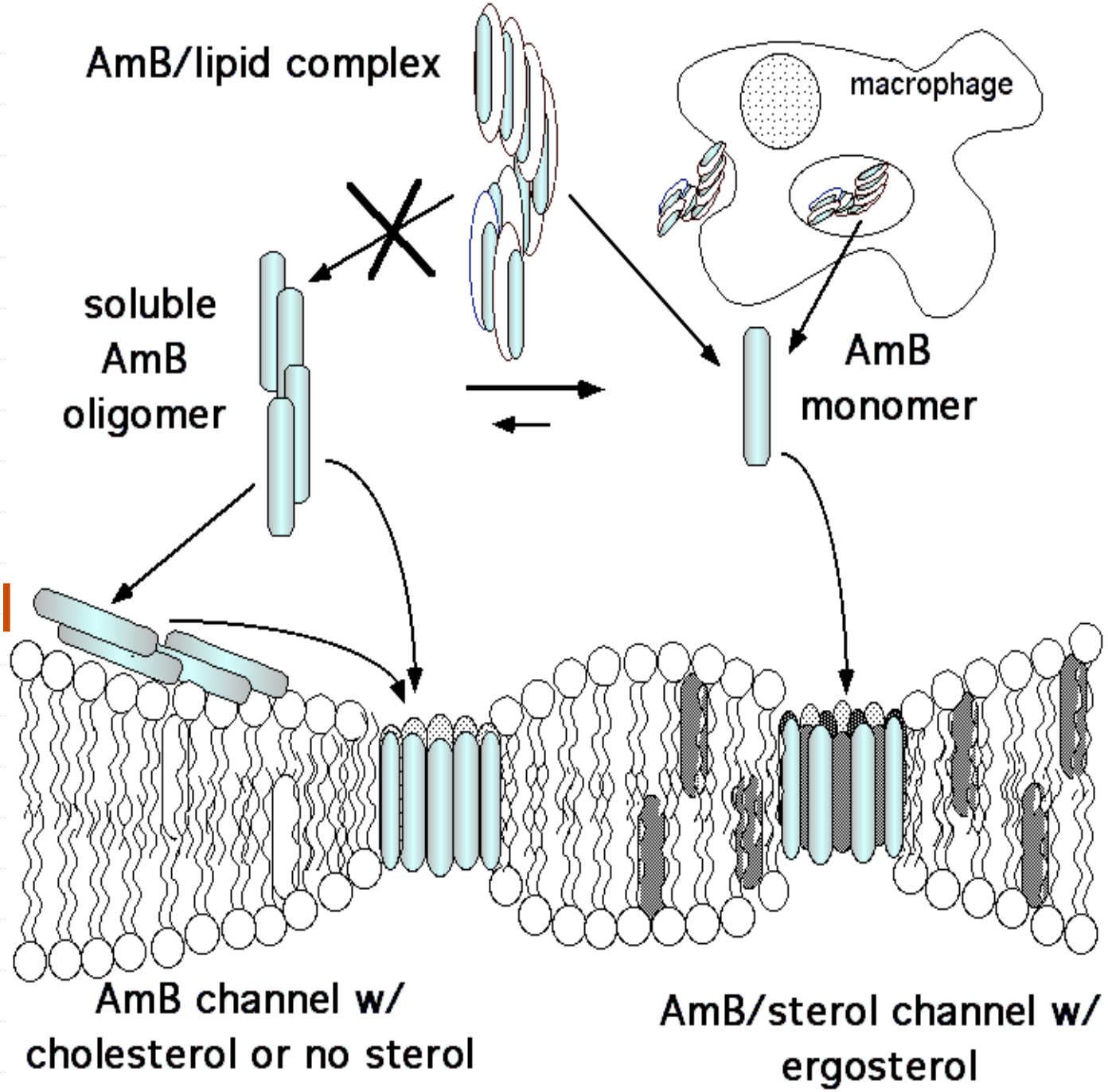
A Liposome, but not encapsulated

Not a Liposome

How do liposomes reduce toxicity?

Bolard's model

- Hence, reducing effective chemical potential of AmB by "tying up" or by macrophage consumption is key.



Where Have We Done in This lab?

- Dogma about polyene antibiotics *ca.* 1987
  - Amphotericin B and nystatin form cation selective ion channel barrels composed of stoichiometric amounts of drug alternating with sterols
  - The “barrels” are more stable with ergosterol but identical barrels also form with cholesterol
  - Therapeutic index can only be improved by liposomal encapsulation
  - Toxic side effects can be traced to ion channel formation in affected tissue
  - Efficacy against fungi is solely predicated upon ion leakage
  - All Amphotericin’s effects are channel-mediated

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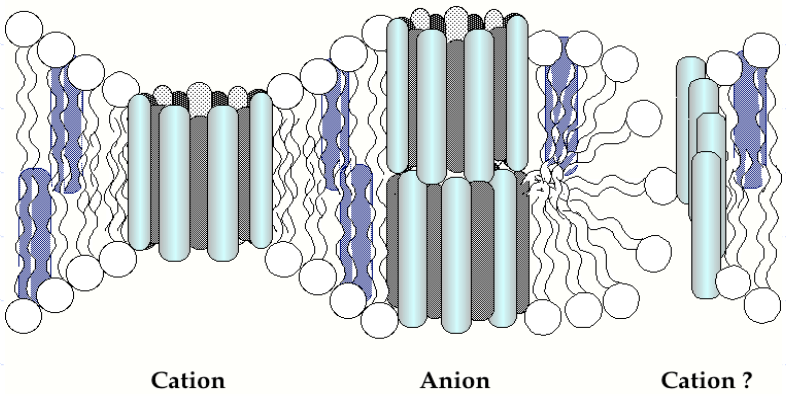
Where Have We Been?

- *Wolf, B.D., and Hartsel, S.C. " Osmotic Sensitizes Sterol-Free Phospholipid Bilayers to The Action of Amphotericin B" ***Biochimica et Biophysica Acta*., 1995,** 1238: 156-162**.**
- Hartsel, S. C.; *Benz, S. K.; *Peterson, R. P.; and *Whyte, B. S. "Potassium Selective Amphotericin B Channels are Predominant in Vesicles Regardless of Sidedness." ***Biochemistry* 1991**, 30: 77-82.
- *Whyte, B. S.; *Peterson, R. P. and Hartsel, S. C. "Amphotericin B and Nystatin Show Different Activities on Sterol-Free Vesicles:" ***Biochemical and Biophysical Research Communications* 1989**, 164: 609-614.



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- Hartsel, S.C., *Benz, S.K.,*Ayenew, W., and Bolard, J. " Na+, K+ and Cl- Selectivity of the Permeability Pathways Induced Through Sterol-containing Membrane Vesicles by Amphotericin B and other Polyene Antibiotics."***Eur. Biophysics Journal*, 1994** 23: 125-132
- *Lambing, H.E., *Wolf, B.D. and Hartsel, S.C. "Temperature Effects on the Aggregation State and Activity of Amphotericin B."***Biochimica et Biophysica Acta*., 1993** 1152: 185-188.

Cholesterol channels ≠ Ergosterol channels

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Where Have We Been?

- *Kwong, Evan H. , Ramaswamy, M., *Bauer, E.A., Hartsel, S.C. and K. M. Wasan " Heat Treatment of Amphotericin B modifies its Serum Pharmacokinetics, Tissue Distribution and Renal Toxicity Following a Sing[illegible]ous Dose to Rabbits." ***Antimicrob[illegible]rapy*, 2001**, 45: 2060-

- *Baas, B[illegible], A., *Scott, J., *Mikulecky, P, and Hart[illegible]Activity and Kinetics of Dissociation and Transfer[illegible]mphotericin B from a Novel Delivery Form" ***PharmSci* , 1999** Volume 1 Issue 4 October - December

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- Hartsel, S.C.,*Bauer, E.A., *Kwong, E. H. and K. M. Wasan " The Effect of Serum Albumin on Amphotericin B Aggregate Structure and Activity." ***Pharmaceutical Research*, 2001-Sep;18(9):1305-9.**

TNF-α= fever, anorexia, hypermetabolism and wasting

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Newer Stuff

Cytokine and other gene and protein expression profiles caused by AmB preps in immune cells (with L. Turtinen). Is this responsible for toxicity or efficacy?

- How do they correlate with antifungal activity?
- Human toxicity?
- What is the cause of the cytokine stimulation? Ca^{2+} or other ion leaks, Toll-like Receptors (TLR), membrane potential?

What are Cytokines?

- Cytokines are small proteins released from immune cells such as monocytes and they are responsible for a host of uncomfortable inflammatory responses in humans.
- These include pain, fever, nausea, wasting-the same symptoms that Amphotericin causes.
- Hence, part of Amphotericin's toxicity may be related to cytokine induction from monocytes.
- We first tested a model human monocyte line (THP-1) for stimulation of tumor necrosis factor-α (TNF) by Fungizone and found increased levels.
- What about other cytokines like IL-8?

Measuring Cytokines:

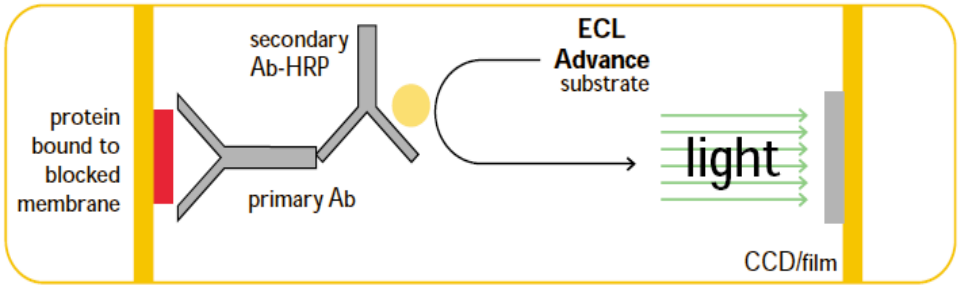
Antibody Array -Qualitative

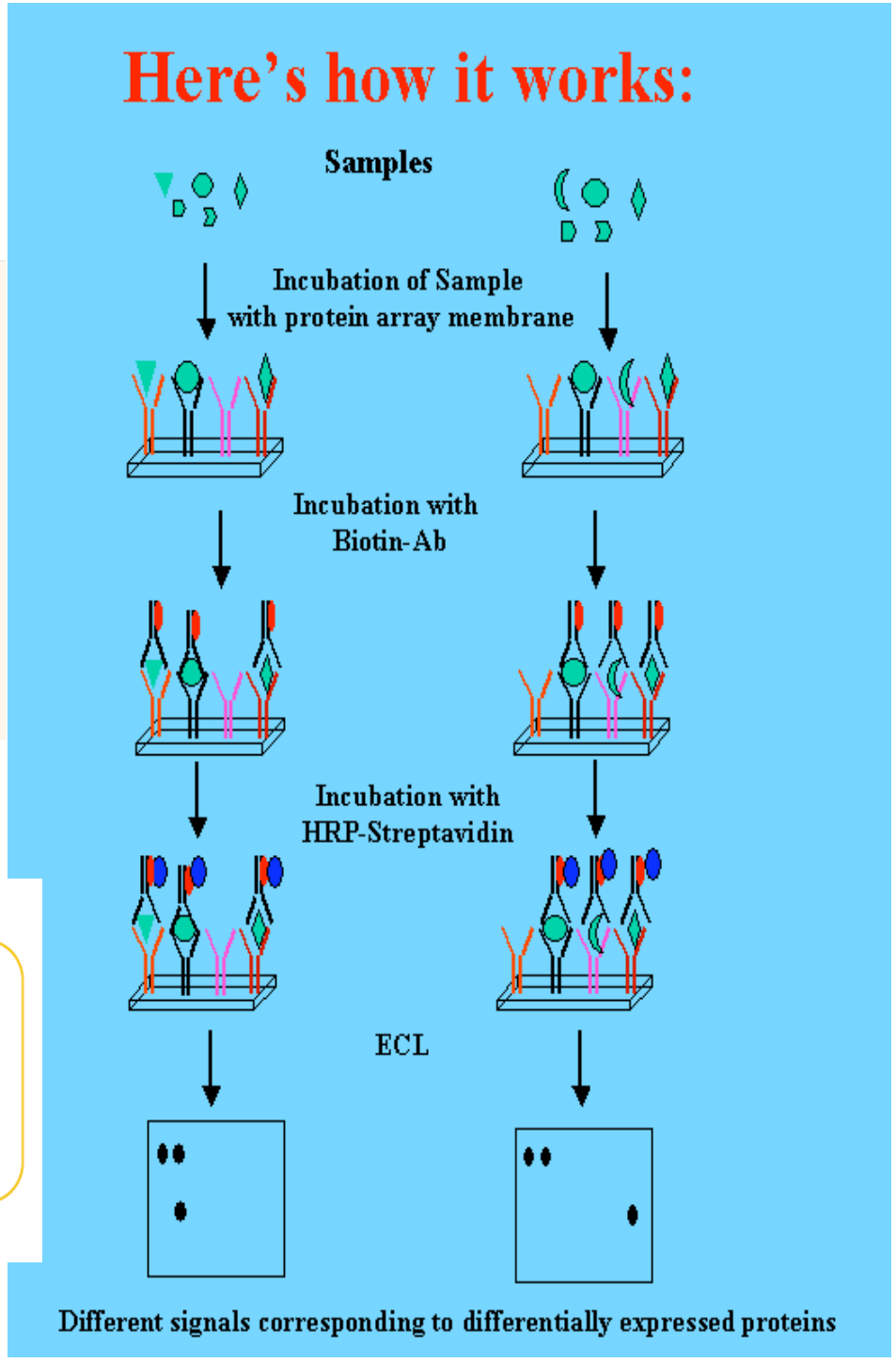
RayBio™ Human Cytokine Array I & 1.1 Map

	a	b	c	d	e	f	g	h
1	Pos	Pos	Neg	Neg	GCSF	GM-CSF	GRO	GRO-a
2	Pos	Pos	Neg	Neg	GCSF	GM-CSF	GRO	GRO-a
3	IL-1a	IL-2	IL-3	IL-5	IL-6	IL-7	IL-8	IL-10
4	IL-1a	IL-2	IL-3	IL-5	IL-6	IL-7	IL-8	IL-10
5	IL-13	IL-15	IFN-y	MCP-1	MCP-2	MCP-3	MIG	RANTES
6	IL-13	IL-15	IFN-y	MCP-1	MCP-2	MCP-3	MIG	RANTES
7	TGF-ß1	TNF-a	TNF-ß	Blank	Blank	Blank	Blank	Pos
8	TGF-ß1	TNF-a	TNF-ß	Blank	Blank	Blank	Blank	Pos

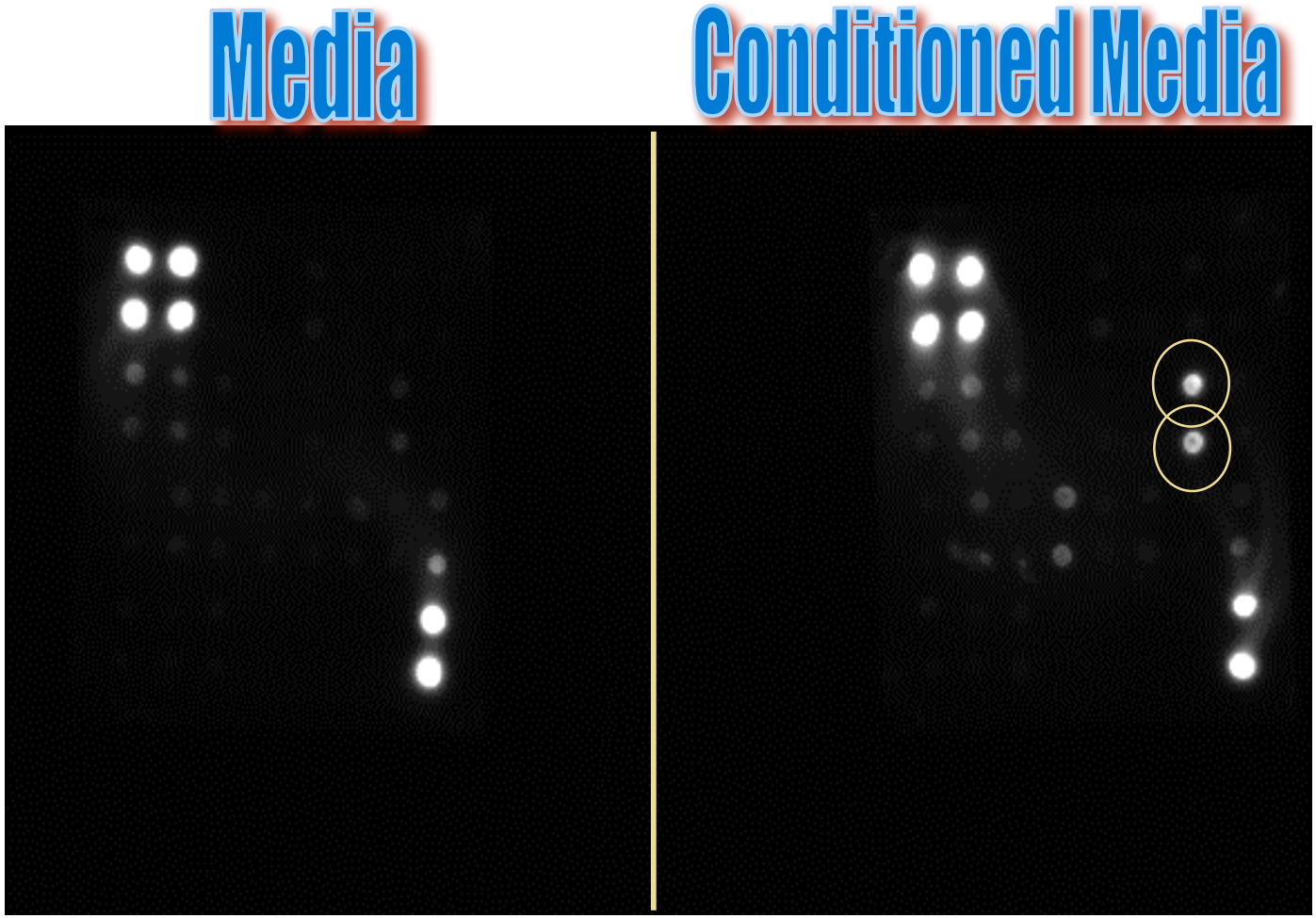
Back to Top

PRINCIPLE OF ECL ADVANCE CHEMILUMINESCENT DETECTION



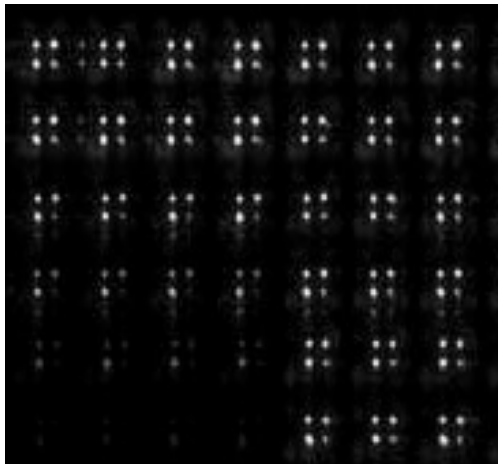


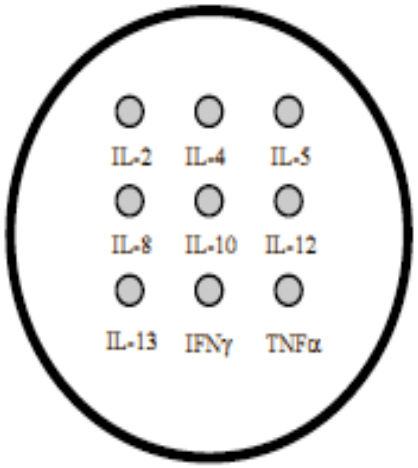
Antibody Array
Proteomics-Qualitative



IL-8

Searchlight Antibody Array Proteomics-Quantitative





Assay Procedure Summary

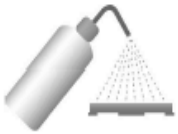
Step 1. Add 50 µl of Standards and samples in duplicate.

Step 2. Incubate the covered plate at room temperature (20-25°C) for 1 hour with shaking at 200 rpm.

Step 3. Wash the plate THREE times.

Step 4. Add 50 µl of prepared Biotinylated Antibody Reagent to each well.

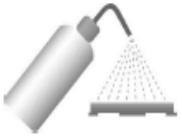
Step 5. Incubate the covered plate at room temperature (20-25°C) for 30 minutes with shaking at 200 rpm.



Step 6. Wash the plate THREE times.

Step 7. Add 50 µl of Streptavidin-HRP Reagent to each well.

Step 8. Incubate the covered plate at room temperature (20-25°C) for 30 minutes with shaking at 200 rpm.



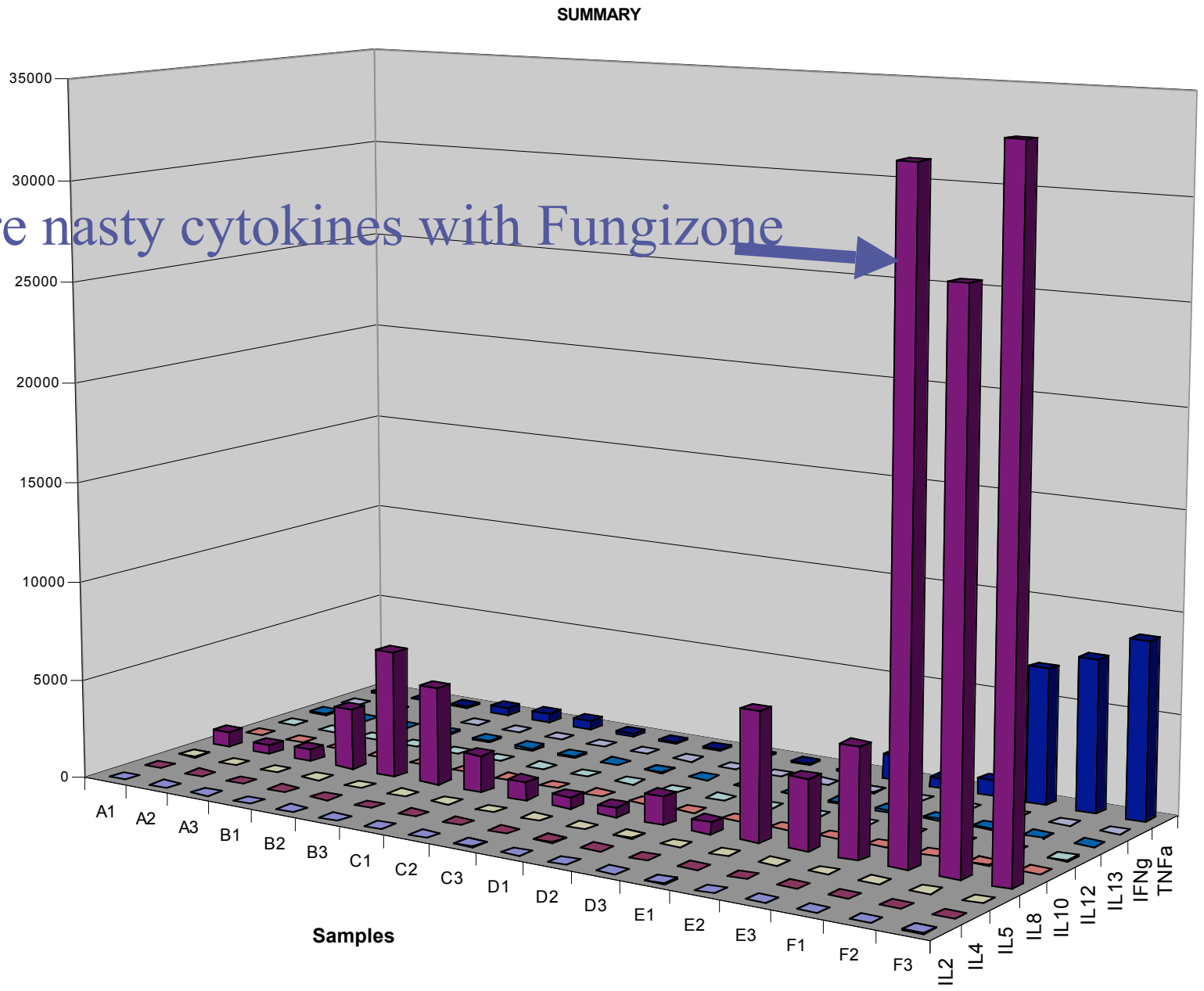
Step 9. Wash the plate THREE times.

Step 10. Prepare SuperSignal®Substrate. Add 50 µl of substrate to each well. **Read within 1-10 minutes.**

Step 11. Read the luminescence using a cooled CCD camera. Calculate results.

Searchlight Antibody Array Results

A LOT more nasty cytokines with Fungizone



Correlating Amphotericin B channel activity with cytokine response

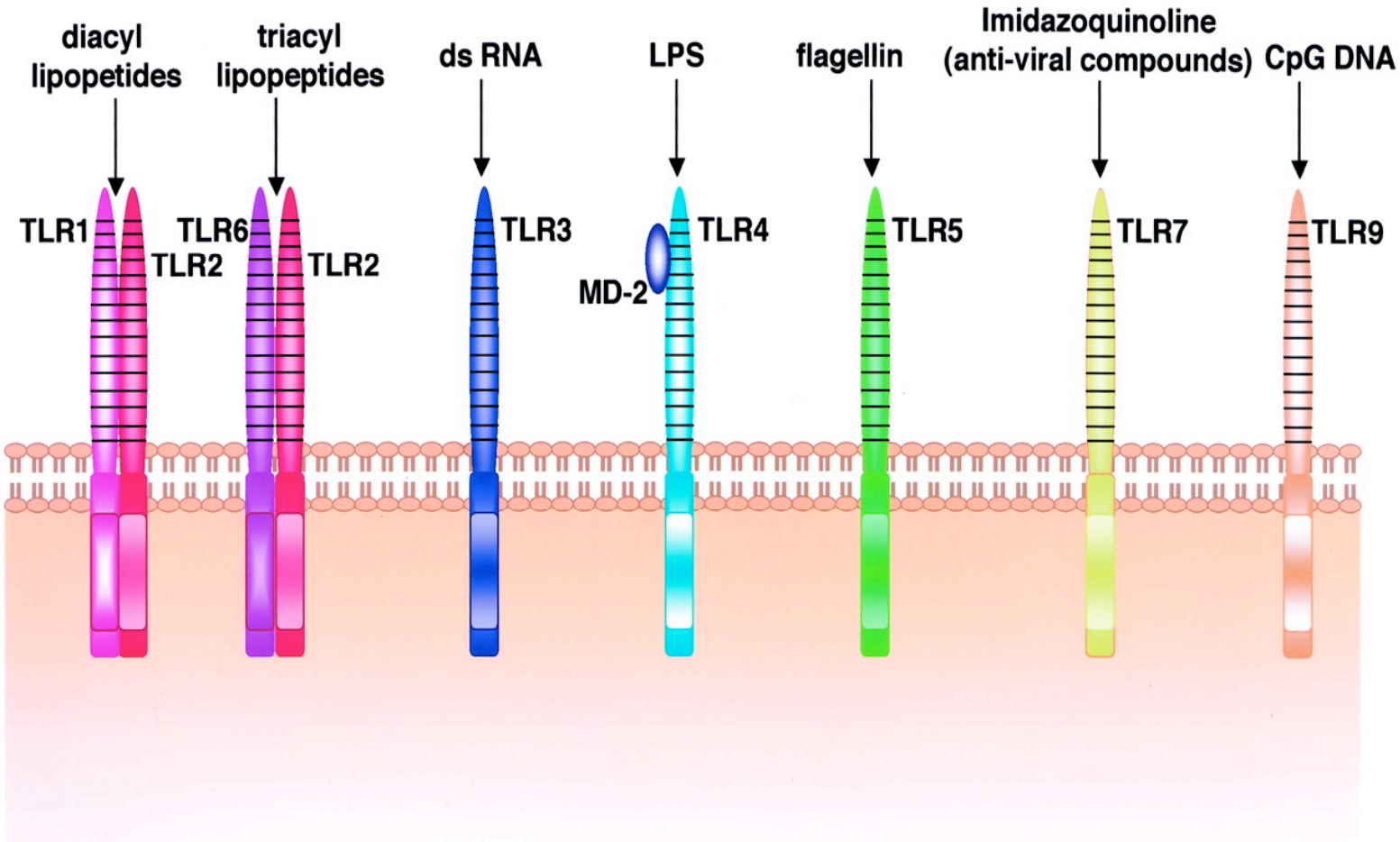
- K+ channel activity versus cholesterol was FZ>Amphotec(lag)>>Abelcet~AmBisome
- K+ channel activity vs. ergosterol-containing membranes was FZ>>Amphotec>Abelcet>AmBisome
- Side Effect: fever

  FZ~Amphotec>>Abelcet~AmBisome
- Some correlation…

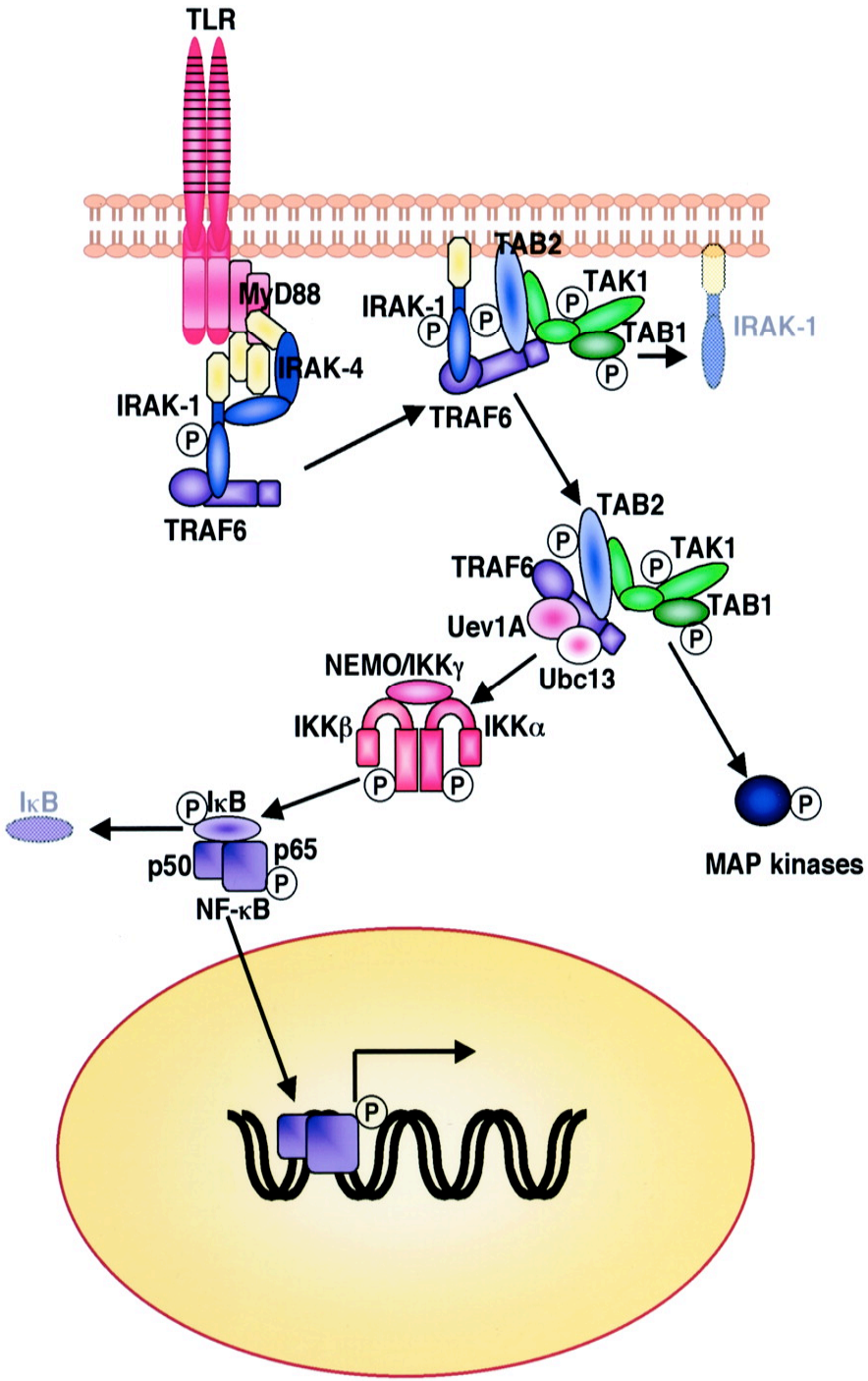
Correlating Amphotericin B channel activity with cytokine response

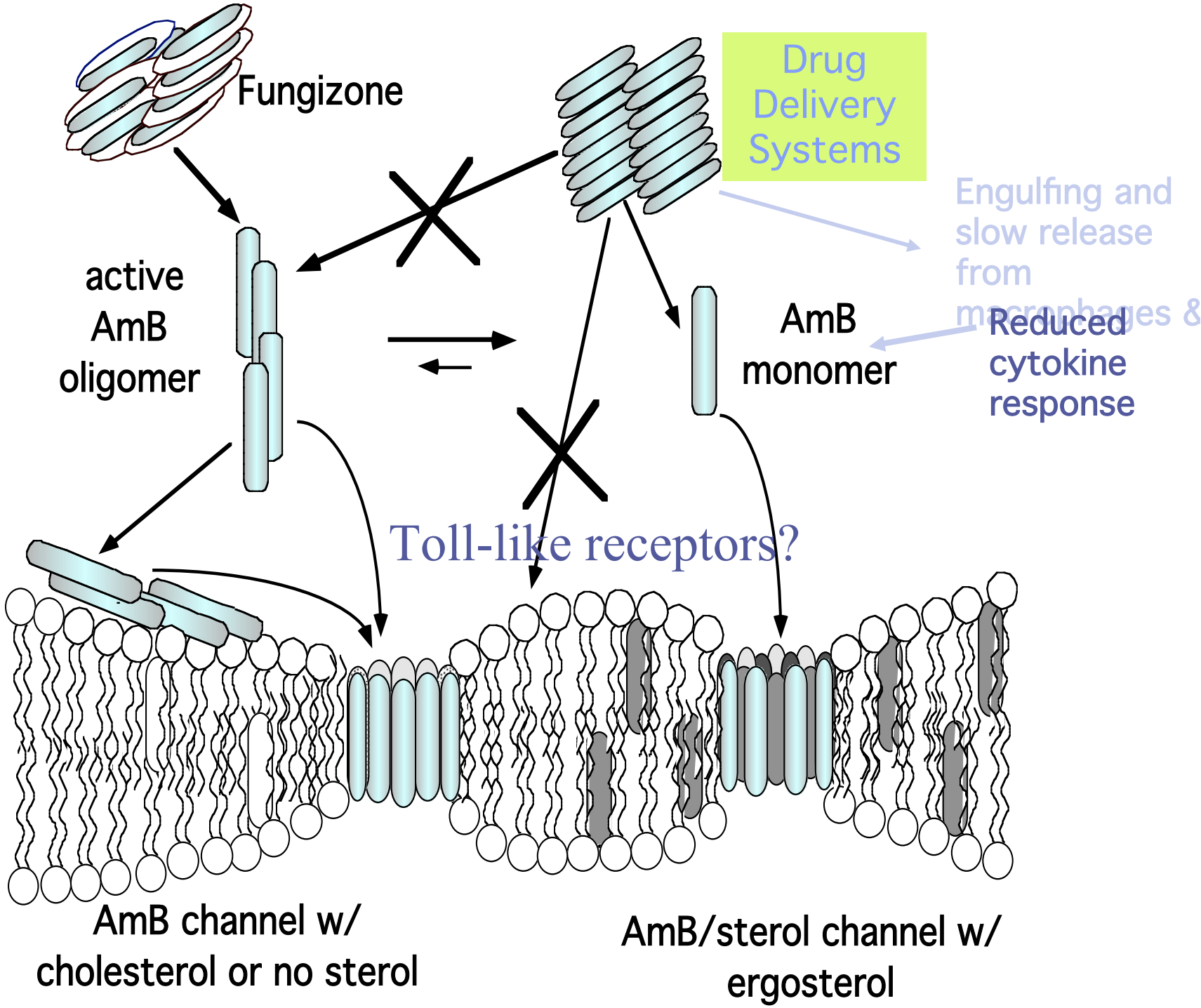
- But....are channels the actual CAUSE of IL-8 secretion or just a useful model system indicator ??????
- Could Toll-like receptors also (only?) involved??
  - http://www.jbc.org/cgi/content/full/278/40/38105
  - http://www.jbc.org/cgi/content/abstract/278/39/37561
  - http://jac.oxfordjournals.org/cgi/content/abstract/55/2/214
  - http://www.ncbi.nlm.nih.gov/pubmed/16625056

Toll-like receptors??
An “innate” immune system



TOLL mediated Pro-inflammatory cascade




Fungizone
Drug Delivery Systems
Engulfing and slow release from macrophages &
Reduced cytokine response
active AmB oligomer
AmB monomer
Toll-like receptors?
AmB channel w/ cholesterol or no sterol
AmB/sterol channel w/ ergosterol

Newer Stuff

- Lloyd W. Turtinen , David N. Prall* , Lindsay A. Bremer* , Rachel E. Nauss* and Scott C. Hartsel "*Antibody Array Generated Cytokine Release Profiles from THP-1 Monocytic Cells Exposed to Different Amphotericin B Formulations" Antimicrobial Agents and Chemotherapy*, 2004. Feb;48(2):396-403.
- Turtinen LW, Croswell A, Obr A. "Microarray analysis of amphotericin B-treated THP-1 monocytic cells identifies unique gene expression profiles among lipid and non-lipid drug formulations."J Chemother. 2008 Jun;20(3):327-35.

Some Important Points:

- Fungizone and Amphotec cause secretion of pro-imflammatory TNF-α,IL-8, and IL-6. Fungizone is worst
- But channel activity of AmB is not strong in a "real" serum situation
- These cytokines stimulate HIV replication
- AIDS patients should get Abelcet or Ambisome!