Studies on Amphotericin B

Current Formulations, the liposome concept and toxicity

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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

<table>
<thead>
<tr>
<th>Organism</th>
<th>Clinical syndrome</th>
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</thead>
<tbody>
<tr>
<td><em>Candida albicans</em></td>
<td>Thrush, vaginal candidiasis, esophageal candidiasis</td>
</tr>
<tr>
<td><em>Cryptococcus neoformans</em></td>
<td>Meningitis, pneumonia</td>
</tr>
<tr>
<td><em>Penicillium marneffei</em></td>
<td>Fever alone or with pulmonary infiltrates, lymphadenopathy, or cutaneous lesions</td>
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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 (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.
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?

Cholesterol: humans
Ergosterol: fungi

Nystatin

Binds weakly to AmB
Binds strongly to AmB

Amphotericin B
Ergosterol: fungi

Amphotericin B binds strongly to AmB, whereas Nystatin binds weakly to AmB.
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...104° 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.
- “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

- The drug

Cationic

Conventional

Stealth™

Immunoliposomes
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

Back to Amphotericin...
### TABLE 2. FDA Approved AmB pharmaceutical preparations

<table>
<thead>
<tr>
<th>AmB Preparation</th>
<th>composition</th>
<th>physical state, shape</th>
<th>Cost</th>
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<tr>
<td></td>
<td>mole ratio</td>
<td>diameter, (µM)</td>
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<tr>
<td></td>
<td>net charge</td>
<td></td>
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<tr>
<td>Fungizone</td>
<td>DOC/AmB</td>
<td>micelles</td>
<td>Dirt</td>
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<tr>
<td></td>
<td>7:3</td>
<td>&lt;0.4</td>
<td>Cheap</td>
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<tr>
<td></td>
<td>negative</td>
<td></td>
<td>$5-10</td>
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<tr>
<td>AmB-Lipid complex</td>
<td>DMPC/DMPG/AmB</td>
<td>sheets</td>
<td>$$$$$$$</td>
</tr>
<tr>
<td>(Ablecet)</td>
<td>7:3:3</td>
<td>1.6-11</td>
<td>$125-150</td>
</tr>
<tr>
<td>AmBisome</td>
<td>HSPC/Chol/DSPG/AmB</td>
<td>small unilamellar vesicle</td>
<td>$$$$$$$</td>
</tr>
<tr>
<td></td>
<td>2:1:0.8:0.4</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>AmB-Colloidal Dispersion (Amphocil)</td>
<td>CS/AmB</td>
<td>discs</td>
<td>$$$$$$$</td>
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<tr>
<td></td>
<td>1:1</td>
<td>0.12</td>
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**Ampho gets Liposomes**

*Not a Liposome, but not encapsulated*

*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
Where Have We Been?

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Cholesterol channels ≠ Ergosterol channels
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Where Have We Been?


TNF-α = fever, anorexia, hypermetabolism and wasting
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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? $\text{Ca}^{2+}$ or other ion leaks, Toll-like Receptors (TLR), membrane potential?
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

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<th></th>
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<th>b</th>
<th>c</th>
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<tr>
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<td>IL-15</td>
<td>IFN-γ</td>
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</tr>
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Here’s how it works:

Samples

Incubation of Sample with protein array membrane

Incubation with Biotin-Ab

Incubation with HRP-Streptavidin

ECL

Different signals corresponding to differentially expressed proteins
**Searchlight Antibody Array**

**Proteomics-Quantitative**

**Assay Procedure Summary**

1. **Step 1.** Add 50 μl of Standards and samples in duplicate.
2. **Step 2.** Incubate the covered plate at room temperature (20-25°C) for 1 hour with shaking at 200 rpm.
3. **Step 3.** Wash the plate THREE times.
4. **Step 4.** Add 50 μl of prepared Biotinylated Antibody Reagent to each well.
5. **Step 5.** Incubate the covered plate at room temperature (20-25°C) for 30 minutes with shaking at 200 rpm.
6. **Step 6.** Wash the plate THREE times.
7. **Step 7.** Add 50 μl of Streptavidin-HRP Reagent to each well.
8. **Step 8.** Incubate the covered plate at room temperature (20-25°C) for 30 minutes with shaking at 200 rpm.
9. **Step 9.** Wash the plate THREE times.
10. **Step 10.** Prepare SuperSignal® Substrate. Add 50 μl of substrate to each well. **Read within 1-10 minutes.**
11. **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/full/278/40/38105)
- [http://www.jbc.org/cgi/content/abstract/278/39/37561](http://www.jbc.org/cgi/content/abstract/278/39/37561)
- [http://jac.oxfordjournals.org/cgi/content/abstract/55/2/214](http://jac.oxfordjournals.org/cgi/content/abstract/55/2/214)
Toll-like receptors??
An “innate” immune system
TOLL mediated
Pro-inflammatory cascade
Engulfing and slow release from macrophages & reduced cytokine response

Toll-like receptors?

AmB channel w/ cholesterol or no sterol
AmB/sterol channel w/ ergosterol

Fungizone

Drug Delivery Systems

active AmB oligomer
AmB monomer

active AmB oligomer
AmB monomer


Some Important Points:
- Fungizone and Amphotec cause secretion of pro-inflammatory 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!