189 Efficacy of Oral Encochleated Amphotericin B (CAMB) in a Mouse Model of Cryptococcal Meningoencephalitis

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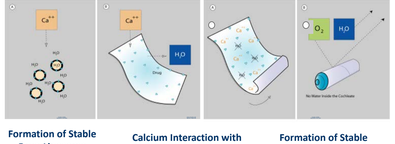
ABSTRACT

BACKGROUND: Cryptococcal meningoencephalitis (CM) is an important infection in HIV/AIDS, responsible for an estimated half million deaths annually. Amphotericin B deoxycholate is a broad-spectrum fungicidal drug that is the standard treatment for cryptococcal disease; however, its use is limited by toxicities and intravenous administration. To help mitigate these limitations a novel orally available lipid-crystal nano-particle, cochleate, formulation of amphotericin B has been developed (CAMB) that has a favorable tolerability profile. In the present study the efficacy of oral CAMB was evaluated in an intravenous mouse model of CM.
METHODS: Groups of 5 mice each were inoculated with 10^4 of *C. neoformans* (strain H99/ATCC 208821) intravenously in 100 µl. Therapy was delayed 72 hours and then daily treatment commenced with Fungizone + flucytosine (5-FC), CAMB, CAMB + 5FC, 5FC, CAMB + fluconazole, or fluconazole for 28 days and mice were followed for up to 150 days and sacrificed when moribund. In addition, to study cochleate delivery to the brain, three mice were infected as above, 5 days later 2 were treated once daily x 3 d by oral gavage with a Rh-CAMB fluorescent cochleate preparation equivalent to 10mg/kg/d of CAMB. An equivalent group of 3 mice remained uninfected. Mice were then sacrificed at 7 d and brain material recovered and observed for fluorescence.
RESULTS: Mortality studies: Median mouse mortality was as follows: vehicle control: 19 d; CAMB 25 mg/kg/d PO: 49 d; CAMB 25 mg/kg/d PO + 5-FC 250 mg/kg/d PO: 102 d; CAMB 25 mg/kg/d PO + fluconazole 25 mg/kg/d PO: 56 d; 5-FC: 250 mg/kg/d PO: 47 d; fluconazole 25 mg/kg/d PO: 53 d. The CAMB formulation led to a significantly increased survival over untreated, infected mice (19 v 49 d; p = 0.0025, Log Rank, Mantel-Cox). Combinations with 5FC prolonged survival over CAMB alone (102 d vs. 49 d; p = 0.007) combination with fluconazole did not prolong survival of mice (56 vs. 49 d; p = 0.28). Equivalent survival was observed between CAMB + 5FC and the gold standard Fungizone IP + 5FC (102 d vs. 80d; p = 0.44). Cochleate delivery to CNS: Fluorescent imaging demonstrated numerous fluorescent particles in the brains of mice treated with CAMB oral preparations with increased delivery evident in brains of infected vs. uninfected mice.
CONCLUSION: CAMB is an effective oral anti-fungal agent equivalent to systemic fungizone + 5FC in an intravenous mouse model of *Cryptococcus neoformans* brain infections and delivery of CAMB was evident by imaging of CAMB fluorescently labeled particles.

COCHLEATE TECHNOLOGY

How Cochleates Encapsulate Drugs

Cochleate delivery vehicles have been shown to mediate **oral bioavailability for injectable drugs, reduce toxicity**, and significantly **enhance intracellular drug delivery**. Cochleates are stable, lipid-crystal, nano-particles composed of simple, naturally occurring materials: phosphatidylserine and calcium. They have a unique multilayered structure consisting of a large, continuous, solid, lipid bilayer sheet rolled up in a spiral or as stacked sheets, with no internal aqueous space. This unique structure provides protection from degradation for "encochleated" molecules. Components within the interior of the cochleate remain intact, even though the outer layers of the cochleate may be exposed to harsh environmental conditions or enzymes.

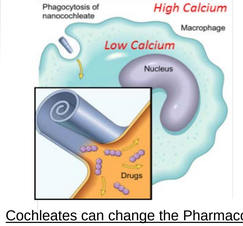


Formation of Stable Drug-Liposome Intermediate | Calcium Interaction with Negatively Charged Lipid | Formation of Stable Drug-Cochleate Nano-Crystal

- The drug product is associated with the negatively charged lipid.
- The addition of calcium creates a calcium-phospholipid anhydrous crystal.
- Nano-crystals are composed of layers of a lipid-calcium complex.
- The drug product is trapped in or between the layers protecting it from harmful environmental elements

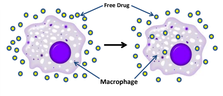
Cell-Targeted Delivery

- Macrophage readily engulf cochleates and their cargo
- Once inside the macrophage, the low level of calcium in the cytoplasm causes the cochleate to open, releasing the cargo molecule



Divalent cation concentrations in serum and mucosal secretions are such that the cochleate structure is maintained. Hence, the majority of cochleate associated molecules are present in the inner layers of a solid, stable, impermeable structure. Once within the interior of a cell, however, the low calcium concentration results in the opening of the cochleate crystal and release of the entrapped drug product.

Cochleates can change the Pharmacokinetics and Biodistribution of Drugs



- Free drug in the extracellular milieu must cross the cell membrane in order to be effective against intracellular microorganisms.
- High plasma and interstitial drug levels are needed
- A relatively low percentage of circulating drug enters the cell.
- Drugs with these properties have difficulty treating intracellular infections.
- High circulating drug levels can result in nonspecific toxicity.

Model of Drug Delivery – The "Trojan Horse" Hypothesis

- High calcium concentrations in GI-secretions, serum and interstitial fluid stabilize the cochleate crystal.
- Cochleates enter the circulatory system, diffuse into tissues and are taken up by "activated" or infected cells.
- Intracellular levels of cochleates increase and reach high levels.
- The low intracellular calcium concentration causes the cochleates to open releasing their cargo.
- Lower plasma levels are required to reach efficacious intracellular drug concentrations.
- These lower plasma levels may result in less systemic toxicity.

METHODS

ND4 mice were infected with an inoculum of 10^4 colony forming units of *C. neoformans* strain H99/ATCC208821. A 100 µL aliquot of the inoculating solution (10^4 H99 cells) was taken and plated on two YPD plates to validate that the mice were infected with an inoculum containing the correct number of cells

Mice were treated intraperitoneally (IP) with conventional Amphotericin B deoxycholate (Fungizone) or by gavage (PO) with CAMB for 28 days, beginning 3 days post-infection. Body weights were taken daily to calculate the correct dosage. For the indicated groups, 200 mg of 5-FC/200 mL of sterilized water was prepared every three days and aliquoted into clean, autoclaved water bottles and placed in the cages of the four groups of mice, estimated to deliver approximately 200-25 mg/kg/d of 5FC. Fluconazole, where indicated, was dosed either alone or in combination at 25 mg/kg/d in two doses by gavage.

Primary End Point: Mortality. Mice were sacrificed when moribund. Death was recorded in a survival study log using GraphPad Prism.

Secondary End Point: Colony Forming Units (CFU). When a death occurred during the study, the carcass of the mouse was saved and the brain was collected, weighed and recorded. The brain of each mouse was homogenized in 1 mL of sterilized water and serially diluted (brain homogenate diluted to 10,000X) using sterile Phosphate-buffered Saline (PBS) for serial dilutions. From the subsequent serial dilutions, 100 uL of the diluted homogenate solutions were plated on YPD plates and labeled with the group and animal number, date (plates were incubated for two days at 37°C), and labeled as "brain" on the plates. After two days of incubation, any colonies that grew from the mouse brain homogenates were counted. The number of colonies were divided by the weight of each organ (brain) in grams (g) and recorded to calculate the number of colonies per gram (CFU/g) of tissue.

RESULTS – Mortality Endpoint & CFU Endpoint

Figure 1. Efficacy of an oral CAMB in a delayed-therapy model of cryptococcal meningoencephalitis.

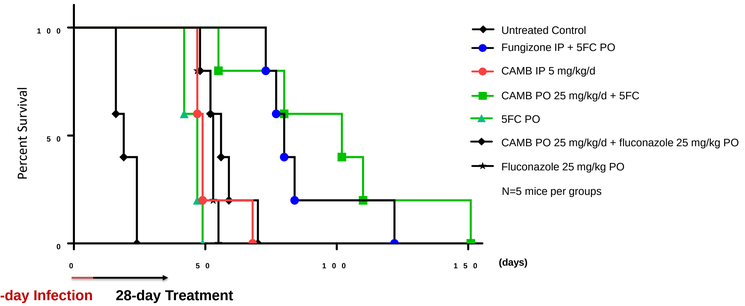
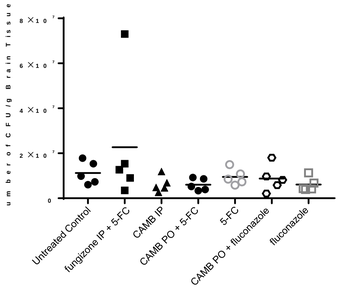


Table 1. Median Survival of Groups from Figure 1

Group	Route	Regimen	Mg/kg/d	Median Survival, days
Untreated control	PO	QD	-	19
Fungizone + flucytosine	IP, PO	QD	5	80*
CAMB	IP	QD	5	49*
CAMB + flucytosine	PO	QD	25, 250	102*
Flucytosine	PO	QD	250	47*
CAMB + fluconazole	PO, PO	QD, BID	25, 25	56*
Fluconazole	PO	BID	25	53*

N=5 mice per group; log-rank (Mantel-Cox, univariate)
*p=0.003 vs. Control
IP = intraperitoneal; PO = by mouth; QD = once daily; BID = twice daily

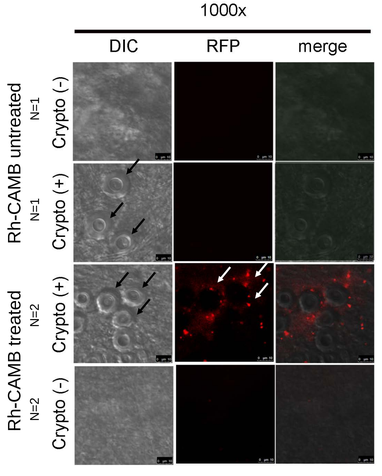
Figure 2. Colony Forming Units in brain tissue at death.



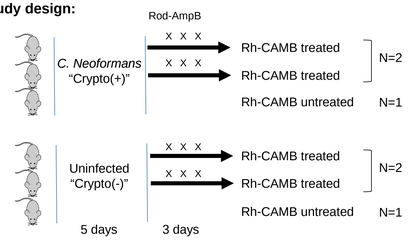
RESULTS – Delivery of CAMB to the Brain

Delivery of rhodamine-labeled CAMB to brains of mice: To study the delivery of CAMB to brain tissue, three mice were infected by tail vein with 10^4 *C. neoformans,* strain H99 and three remained uninfected. 5-days later 2 from each group were treated once daily for 3-days by oral gavage with rhodamine labeled CAMB (Rh-CAMB) equivalent at 10 mg/kg/d. Mice were sacrificed at day 7 and brain material was recovered, homogenized and subjected to microscopy using Differential interference contract (DIC), and observed for fluorescence (RFP).

Figure 3. Brain localization of fluorescent cochleates after oral dosing.
Three mice were infected by tail vein with 10^4 *Cn* and three remained uninfected. Five days later two from each group were treated daily for 3 days with fluorescent cochleate preparations (Rh-CAMB) by gavage and sacrificed. Brains were recovered and homogenized and subjected to microscopy using differential interference contrast (DIC), or red fluorescence (RFP) at the indicated magnifications. Black arrows indicate *C. neoformans* encapsulated organisms, white arrows indicate cochleate fluorescence. Bar = 10 mm



Study design:



SUMMARY AND FUTURE STUDIES

- Oral CAMB + 5FC exhibits equivalent efficacy as fungizone injection + oral flucytosine in a mouse model of cryptococcal meningoencephalitis
- Delivery of CAMB to the brain in mice infected with *C. neoformans* was demonstrated using fluorescently labeled CAMB particles
- Experiments using CAMB in a model of cryptococcal meningoencephalitis in an alternate species will be conducted
- Studies for evaluation of CAMB in human cryptococcal meningitis are warranted

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2. National Institute Allergy and Infectious Diseases/NIH, Bethesda, MD

*This research was supported by Matinas BioPharma and by the intramural research program of the NIAID.