B-539

Oral Encochleated Amikacin Demonstrates Activity *In Vitro* and *In Vivo* In Biofilm Models of *Mycobacterium avium*

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ABSTRACT

Background: ***M. avium*** **causes disseminated disease in immunocompromised patients and lung infection in patients with chronic lung diseases.** ***M. avium*** **has been shown to form biofilm** ***in vitro*** **and** ***in vivo*****, which appears to be associated with lung infections. The infections are frequently recurrent and often resistant to standard antibiotics. Amikacin is effective against the bacterium, but is limited by intra-venous administration and toxicity. Encochleated Amikacin is a lipid-crystal, nano-particle formulation designed for targeted oral delivery of amikacin to infected tissue without the toxicity. Previously, we demonstrated Encochleated Amikacin had activity in a mouse model of disseminated** ***M. avium*** **infection.**

Methods: ***In Vitro*** **- Polarized A549 alveolar epithelial cells were cultured for 6-days and** ***M. avium*** **104 (10^5 bacteria) were seeded on the apical surface of the cells and given 7 days for biofilm formation. Monolayers were treated daily for 7days on the baso-lateral surface, before lysing and plating to determine the CFUs.**

In Vivo **- C57 BL/6 mice were infected with 8.3 x 10^6 of** ***M. avium*** **104 intranasal and the infection was allowed to establish for 7 days. Baseline bacterial load was determined and mice treatment was initiated, daily for 4 weeks (orally or with intraperitonal injection of amikacin). Three days after the end of treatment, mice were harvested and bacterial load in the lungs determined.**

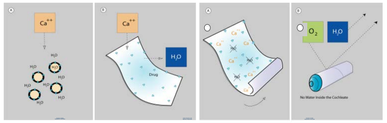
Results: Encochleated amikacin dosed orally (20 and 100 mcg/ml) was significantly more active than empty cochleates and as active as free amikacin IP. The results of the ***in vivo*** **experiment are shown.**

Conclusions: Encochleated amikacin showed significant activity against ***M. avium*** **in the in vitro biofilm model and the** ***in vivo*** **respiratory biofilm mouse model. Further studies will have to be conducted to evaluate the effects of Encochleated amikacin in humans.**

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 API 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 API is trapped in or between the layers protecting the API from harmful environmental elements

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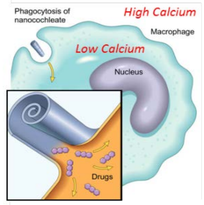
Cell-Targeted Delivery

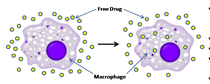
- Macrophage readily engulf cochleates and their cargo
- Once inside the macrophage, the low level of calcium in the cytoplasm causes the cochleate to open, releasing the cargo molecule



Divalent cation concentrations *in vivo* 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 API.

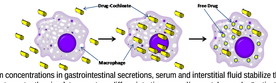
Cochleates Can Change the Pharmacokinetics and Biodistribution of Drugs

Traditional Model of Drug Delivery



- 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.

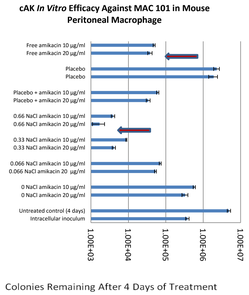
Model of Drug Delivery - The "Trojan Horse" Hypothesis



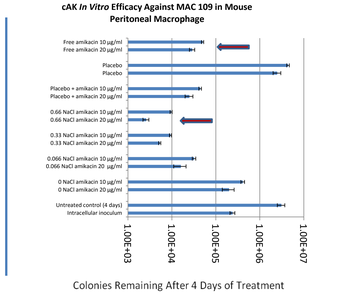
- High calcium concentrations in gastrointestinal secretions, serum and interstitial fluid stabilize the drug-cochleate crystal.
- Drug cochleates enter the circulatory system, diffuse into tissues and/or are taken up by "activated" and/or infected cells.
- Intracellular levels of drug-cochleates increase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

AMIKACIN COCHLEATE *IN VITRO* ACTIVITY IN MACROPHAGE CULTURE

The efficacy of cochleate-amikacin (cAK) against intracellular *Mycobacterium avium* (MAC) infections was evaluated *in vitro* using mouse peritoneal macrophage infected with *M. avium* strains MAC 101 or MAC 109. Mouse peritoneal macrophages (Mφ) Raw 264.7 cells were seeded at 10^5 cells/well. Mφ monolayers were infected at ratio 1:10 for 1 h and extracellular bacteria removed. Monolayers were treated with free amikacin and/or cochleate preparations for 4 days and the number of intracellular bacteria determined. Assays were repeated three times.



Experiment: Treatment of *M. avium* 101-infected macrophages with cochleate preparations
(1) $p < 0.05$ compared with the intracellular inoculum
(2) $p < 0.05$ compared with untreated control (4 days)
(3) $p < 0.05$ compared with the amikacin/cochleate placebo or free amikacin



Experiment: Treatment of M. avium 109-infected macrophages with cochleate preparations
(1) $p < 0.05$ compared with the intracellular inoculum
(2) $p < 0.05$ compared with untreated control (4 days)
(3) $p < 0.05$ compared with the amikacin/cochleate placebo or free amikacin

Results: Untreated control MAC strains grew within Mφ from 3.8×10^5 to 4.9×10^6. MAC within Mφ treated with free amikacin (10 and 20 μg/ml) were killed to 6.1 and 3.4×10^4 bacteria, respectively. Optimized cAK (10 and 20 μg/ml) demonstrated **greater than 10-fold enhanced efficacy**, reducing bacterial count to 3.9 and 1.7×10^3 bacteria within Mφ ($p<0.05$ compared with free amikacin).

AMIKACIN COCHLEATE TREATMENT OF MICE WITH *M. AVIUM* DISSEMINATED INFECTION

To evaluate the activity of the cochleate-amikacin (cAK) preparation, C57 BL/6 mice were infected with *M. avium* 104 (clinical isolate) I.V. and one week later some mice where harvested to establish the bacterial load before treatment. Then, oral therapy was initiated with cAK (either 25 mg/kg or 100 mg/kg), empty cochleate, and free amikacin (100 mg/kg). As an additional control, mice were also treated with I.P. AK (100 mg/kg). Treatment was delivered for 4 weeks and after the mice were harvested, the number of bacteria CFU/liver and spleen quantified and histopathological examination of tissues was also performed.

Table 1: Organisms (CFU) in organs of C67BL/6 mice (4 wks)

	Liver	Spleen
Baseline	$6.83 \pm 0.5 \times 10^5$	3.67×10^6
Water (4 weeks)	$2.9 \pm 0.5 \times 10^6$	$9.17 \pm 0.4 \times 10^6$
Oral amikacin	$2.32 \pm 0.4 \times 10^6$	$1.81 \pm 0.5 \times 10^7$
15* mg/kg cochleates	$6.02 \pm 0.4 \times 10^5$	$5.64 \pm 0.4 \times 10^6$
60* mg/kg cochleates	$6.88 \pm 0.3 \times 10^5$	$1.22 \pm 0.6 \times 10^6$
Empty cochleates	$8.15 \pm 0.6 \times 10^6$	$2.07 \pm 0.4 \times 10^7$
IP Amikacin	$4.51 \pm 0.4 \times 10^4$	$6.83 \pm 0.4 \times 10^5$

Figure 1: CAMK *In Vivo* Efficacy – SPLEEN

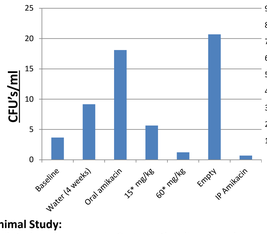
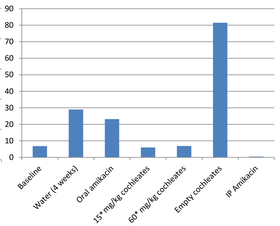
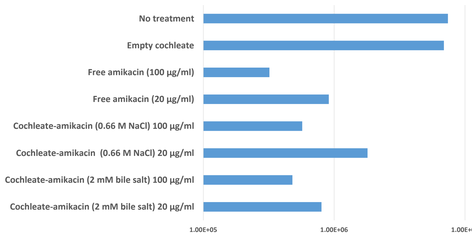


Figure 2: CAMK *In Vivo* Efficacy – LIVER



Results -Amikacin Cochleates (CAMK) Animal Study:
- AK administered orally at 100 mg/kg exhibited no decreases in CFU in either liver or spleen
- AK administered i.p. at 100 mg/kg exhibited decreases in CFU of 98.5% (liver) and 92.6% (spleen)
- cAMK administered orally at 15* mg/kg exhibited decreases in CFU of 79.3% (liver) and 38.5% (spleen). Indicates significant efficacy.
- cAMK administered orally at 60* mg/kg exhibited decrease in CFU of 76.3% (liver) and 86.7% (spleen). Indicates significant efficacy.
- **The significant efficacy of the 15* mg/kg and 60* mg/kg cAK administered orally has been demonstrated in mice. The pathology data for cAK is confirmatory of the *in vivo* efficacy data.**

AMIKACIN COCHLEATE TREATMENT OF *M. AVIUM* BIOFILM IN CULTURE



Cochleate – Biofilm

System: A549 alveolar epithelial cells were cultured in a transwell system. A549 cells became polarized after 6 days and integrity. Bacteria were seeded on the top (apical surface) of the cells. Seven days were allowed for biofilm formation.

Bacteria: MAC 104 (10^5 bacteria) infection inoculum.

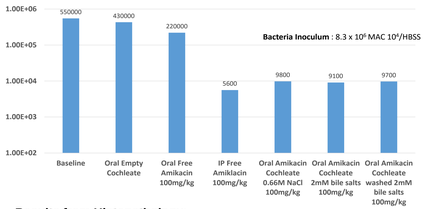
Treatment: 0.1 ml of the different treatments delivered to the bottom well daily. The basolateral surface (bottom) of the cells are immersed in the tissue culture medium present in the bottom well. Three replicas per experimental group in two different experiments.

Harvesting: Biofilm and epithelial cells were lysed and and lysed were diluted and plated onto 7H10.

Conclusions:

1. Biofilms of *M.avium* are encountered in lung infection.
2. Although empty cochleate has no activity against *M.avium* in biofilm, both preparations of cochleates (sodium and bile salt) showed significant activity in the model.
3. The anti-bacterial activity of the cochleates was similar to the activity of free amikacin, suggesting that in absence of infected cells (small percent of the total infection), both preparations achieve comparable effect.

AMIKACIN COCHLEATE TREATMENT OF MICE WITH *M. AVIUM* PULMINARY INFECTION



Cochleate Treatment in Mice with lung Disease Experimental model:

- C57 BL/6 mice were infected with 8.3 x 10^6 of *Mycobacterium avium subsp hominissuis* intranasal ant the infection was allowed to establish for 7 days,
- Then baseline bacterial load was determined in 10 mice and treatment protocols were initiated.
- Mice were treated daily for 4 weeks (orally or with intraperitonal injection of free amikacin.
- Mice were harvested and lung and spleens were removed, homogenized and plated to determine the bacterial load.
- Experimental groups had 12 mice each.

Results from Histopathology:

Infected control: Lung tissue showed many sites of focal inflammatory response, with non-cavitary granuloma formation in the majority of them. The areas have a few neutrophils, many lymphocytes and macrophages. In slides stained for acid-fast organisms one can see abundance of acid-fast bacilli indicative of *M.avium*.

Amikacin-Cochelates-treated mice: Lung parenchyma with a few small granulomatous areas, with a few of them with no visible acid-fast bacteria, and the majority with small numbers of acid-fast organisms. Lymphocytes and macrophages in reduced numbers are present in the lesions.

Empty cochleate-treated mice: Lung parenchyma with evidence of many granulomatous lesions with lymphocytes and macrophages. Acid-fast staining shows many *M. avium* organisms within the lesion limits.

Amikacin-treated mice: Lung tissues showing a few small granulomas with lymphocytes and macrophages. Acid-fast staining with few *M.avium* in some lesions, and some lesions without bacteria.

Conclusions

1. Free amikacin and cochleate amikacin preparations were effective for the treatment of lung infection by *M.avium*.
2. The effect observed was bactericidal for all the cochleates preparations and the free amikacin administered IP.
3. All three preparations of cochleates had comparable activity.
4. No toxicity was observed (including the histopathology of kidneys).

SUMMARY AND CONCLUSIONS

CAMK showed significant activity against *M. avium* in the *in vivo* respiratory biofilm mouse model and in the *in vitro* biofilm model. Further studies will have to be conducted to evaluate the effects of CAM in humans.