6782 Efficacious and Commercially Viable DNA Vaccines: Plasmids Formulated into Lipid-Crystal Nano-Particles for Oral and Systemic Immunization

Raphael J. Mannino and Ruying Lu - Matinas BioPharma, Inc.

ABSTRACT

Background: The use of DNA plasmids for protective and therapeutic vaccination continues to progress. One of the major growth areas for DNA vaccination is the development of an efficient and effective formulation and DNA plasmid delivery technology that is cost effective and commercially viable. Cochleates are a unique stable, multilayered, essentially anhydrous lipid-crystal nano-particle that, following either oral or systemic delivery, safely and efficaciously delivers drugs and oligonucleotides to target tissues. MAT2203, an orally-administered, encochleated formulation of amphotericin B, (CAMB)) has commenced a Phase 2a clinical study, funded and led by the NIAID. Relative to oligonucleotides, in early stage studies, cochleates successfully formulate and mediate *in vitro* and *in vivo* delivery and efficacy of potential oligonucleotide based therapies, including DNA plasmids.

Results: Oral Administration of HIV-1 DNA cochleates: Encochleated formulations of plasmid pCMV HIV-1 containing 3.5µg or 17µg of DNA were given to BALB/c mice by oral or intramuscular routes.

Cytotoxicity Oral administration of two 3.5µg or 17µg doses yielded strong splenocyte cytolytic responses (73 to 85% specific lysis at an E:T ratio of 100:1) analogous to intramuscular injection. Oral administration of a higher dose (50µg) of naked DNA, was inactive.

Proliferation Low doses (3.5µg) of orally administered encochleated DNA induced antigen specific splenocyte proliferation 8-11 fold above background, similar to intramuscular. Naked plasmid was negative.

Adjuvant Enhancement: HSV-2 DNA Cochleates plus IL-12 Cochleates: Plasmids - Mice were immunized intramuscularly with HSV-2 gD2 DNA, 25µg/dose, (pc DNA 3.1 vector backbone) and two IL12 plasmids (equal mixture of the p35 and p40 subunits), 35µg/dose.

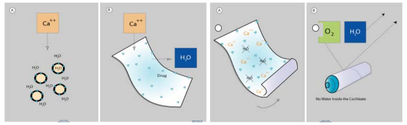
Results - Encochleation of gD and IL-12 plasmids induced 2X greater HSV-specific cytolytic T cell responses than Herpes infection, as well as enhancement of T helper 1 cellular responses and antibody. These studies affirmed that co-administration of cytokines can enhance the immunogenicity of a DNA-based vaccine. Naked DNA was inactive.

Conclusion: Plasmid-Cochleate formulations show promise as commercially viable oral and systemic vaccines.

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.

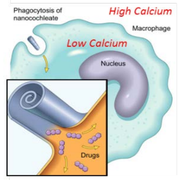


Formation of Stable Drug-Liposome Intermediate | Calcium Interaction with Negatively Charged Lipid | Formation of Stable Drug-Cochleate Nano-Crystal

- 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

Cell-Targeted Delivery

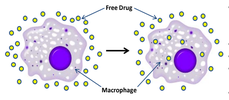
- Macrophage readily engulf cochleates and their cargo
- Once inside the macrophage, the low level of calcium in the cytoplasm causes the cochleate to open, releasing the cargo molecule



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.

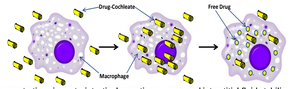
Cochleates Can Change the Pharmacokinetics and Biodistribution of Drugs

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

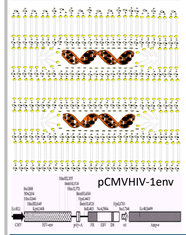


- High calcium concentrations in gastrointestinal secretions, serum and interstitial fluid stabilize the drug-cochleate crystal.
- Drug cochleates enter the circulatory system, diffuse into tissues and/or are taken up by "activated" and/or infected cells.
- Intracellular levels of drug-cochleates increase 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.

DNA COCHLEATES FORMULATED WITH PLASMIDS EXPRESSING HIV-1 PROTEINS– ORAL ADMINISTRATION

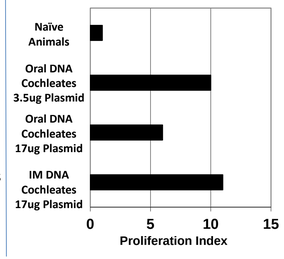
Experimental Design

DNA Cochleates



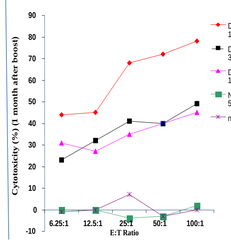
Plasmid pCMV HIV-1 expresses the HIV-1 proteins env (gp160), rev, and tat, in mammalian cells. DNA cochleates containing 3.5ug or 17ug of plasmid were given to BALB/c mice by oral or IM administration. A second dose was administered 4 weeks after the primary dose. Naïve control animals were given cochleates without DNA. Animals were sacrificed two weeks after the second immunization.

Encochleation of HIV-1 Plasmids Enhances HIV-1 T-Cell Prolifration



Proliferation. Proliferation was measured as uptake of 3H thymidine by splenocytes cultured with peptide p18. DNA cochleates mediated the induction of antigen specific splenocyte proliferation at a level 8-11 fold above background. The response to oral administration was similar to intramuscular. Very low doses of encochleated DNA were required to induce these cellular responses. Naked plasmid, even at a level of more than 30 fold greater than cochleate associated DNA, did not induce proliferation following oral administration

Encochleation of HIV-1 Plasmids Enhances HSV Specific CTL Responses



Cytotoxicity.Splenocytes were isolated and stimulated in vitro either with medium or peptide p18 (from gp160), and assayed for antigen specific cytotoxicity by measuring lysis of target cells expressing HIV gp160, (env). Oral administration of two 3.5ug or 17ug doses yielded strong splenocyte cytolytic responses (73 to 85% specific lysis at an E:T ratio of 100:1). These responses were essentiallythe same as those obtained by analogous intramuscular injection of 17ug DNA cochleates. Oral administration of a higher dose (50ug) of naked DNA, did not induce antigen specific cytolytic activity above background.

DNA COCHLEATES FORMULATED WITH PLASMIDS EXPRESSING HSV-2 PROTIENS AND PLASMIDS EXPRESSING IL-12 – INTRAMUSCULAR ADMINISTRATION

Experimental Design

Vaccine Formulation and Animal Inoculation: Encochleated plasmid preparations were formulated to allow for the comparison of encochleated or naked plasmids. HSV-2 gD2 DNA (made with the pc DNA 3.1 vector backbone) were mixed with encochleated or naked plasmid IL-12 DNA (made using the pED vector backbone). The IL-12 plasmid DNA consists of an equal mixture of two plasmid DNAs coding for the p35 and p40 subunits. The gD DNA or pc DNA 3.1 were administered at 25 mg/dose. IL-12 (each heterodimer plasmid) and pED plasmids were administered at 35 mg/dose. Mice were immunized via the i.m. route with either the naked or encochleated vaccine mixtures at weeks 0, 4 and 8. Four weeks post the last immunization the mice were sacrificed, and spleens and serum samples collected. Mice: Eight week old female BALB/c mice were purchased from Charles River (Wilmington, MA) and were maintained in microisolators. All animal protocols employed in this study met with established Institutional Animal Use and Care Committee guidelines.

Encochleation of IL12 Plasmid Enhances Th1 Antibody Responses

Immunization regimen		IgG1	IgG2a
Vaccine	Cytokine	GMT	GMT
gD Coch	IL-12 Coch	33,884	**42,658**
gD Coch	IL-12	26,485	26,195
gD Coch	pED Coch	45,708	25,293
pc3.1 Coch	pED Coch	25	25
pc3.1 Coch	pED	25	25
pc3.1 Coch	IL-12 Coch	25	25
gD	IL-12 Coch	35,481	**50,118**
gD	IL-12	29,512	37,757
gD	pED	22,542	36,057
pc3.1	pED	25	25
pc3.1	pED Coch	25	25
pc3.1	IL-12	25	25
HSV2		6,180	15,453
naïve		25	25

gD ELISA Wells of Immulon II were coated with gD, then washed and blocked prior to addition of serial two-fold dilutions of sera. After 1 hr at room temperature, plates were washed prior to the addition of biotinylated secondary antibody specific for either murine IgG1 or IgG2a. Following a 1 hr incubation at room temperature, the plates were washed and HRP-conjugated streptavidin was added to each well for a further 1 hour, then washed again prior to addition of the ABTS substrate. Resulting color was quantitated at 405 nm using a Biotek plate reader. Endpoint titers were defined as being 2 std deviations above the average OD of the sera obtained from naive mice.

Serum Neutralization Titers

Vaccine	Cytokine	Neut. GMT
gD Coch	IL-12 Coch	28
gD Coch	IL-12	38
gD Coch	pED Coch	37
pc3.1 Coch	pED Coch	< 5
pc3.1 Coch	pED	< 5
pc3.1 Coch	IL-12 Coch	< 5
gD	IL-12 Coch	75
gD	IL-12	133
gD	pED	23
pc3.1	pED	< 5
pc3.1	pED Coch	< 5
pc3.1	IL-12	< 5
HSV2		183
naïve		< 5

Virus Neutralization Assay: Sera were evaluated for HSV neutralizing titer using a microneutralization assay method. Test sera were heat inactivated for 30 min at 56°C, and then subjected to serial 2-fold dilution. An equal volume of HSV2 was added to each dilution. Guinea pig complement was also included. Mixtures were incubated for 1hr at 37°C (5% CO2) with gentle rocking, then inoculated directly onto confluent Vero cell monolayers in 96-well plates. Virus, medium and complement controls were included in each assay. Following incubation, cells were overlaid with 1% methylcellulose/MEM. Plates were incubated at 37°C (5% CO2) until approximately 50 plaques could be counted in virus control wells. Plaques were enumerated and titers were defined as the reciprocal dilution of the last serum dilution yielding a greater than 50% plaque reduction

Encochleation of gD and Il12 Plasmids Enhances Th1 Cytokine Responses

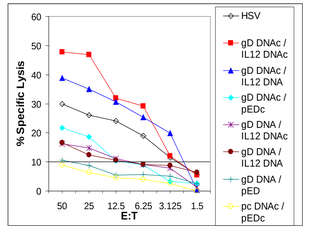
ELISPOT analysis of cytokine secretion: Spleen cells were restimulated in vitro with inactivated HSV for four days, then the viable cells plated in wells of nitrocellulose plates which had been coated with capture antibody specific for either murine IFNg or IL4. After a 20 hour incubation, cells were washed from the wells and the appropriate biotinylated anti-cytokine antibody added. Wells were washed after an overnight incubation and the peroxidase conjugated goat anti-biotin antibody added. Following a 3 hour incubation at 37°C, the AEC substrate was added. The reaction was stopped by washing the wells with tap water. Spots were counted using a dissecting microscope.

ELISpot analysis of cytokines

Immunization regimen		SFC/10^6 cells		
Vaccine	Cytokine	IFN-γ	IL-4	Th1/Th2
gD DNA Coch	IL-12 DNA Coch	520	15	**34.7**
gD DNA Coch	IL-12 DNA	407	18	**22.6**
gD DNA Coch	pED DNA Coch	333	33	**33**
gD DNA	IL-12 DNA Coch	390	55	7.1
gD DNA	IL-12 DNA	353	43	8.2
gD DNA	pED DNA	235	40	5.9
pc DNA 3.1 Coch	pED DNA Coch	168	53	3.2
pc DNA 3.1 Coch	pED DNA	148	58	2.6
pc DNA 3.1 Coch	IL-12 DNA Coch	278	87	3.2
pc DNA 3.1	pED DNA	170	47	3.6
pc DNA 3.1	pED DNA Coch	148	60	2.5
pc DNA 3.1	IL-12 DNA	242	62	3.9
HSV2		900	58	15.5
naïve		183	48	3.8

Encochleation of gD and IL12 Plasmids Enhances HSV Specific CTL Responses

CTL Assay: Spleen cell suspensions were restimulated with UV-irradiated, HSV infected autologous spleen cells. After 5 days of culture, the viable cell concentrations were adjusted to the cell concentration yielding the desired effector cell number and 100 ml aliquots added to wells of a round bottom 96-well plate. Each dilution was plated in triplicate. A20 targets cells, HSV-infected or uninfected, were labeled with 51Cr, then added to the wells in 100 ul aliquots. After a 4 hour incubation, 100 ml were harvested from wells and the gamma emissions counted. Specific release was determined by the following formula: Specific Release = (Experimental CPM-Spontaneous Release CPM) X 100 (Total CPM - Spontaneous Release)



Conclusions

Plasmid-Cochleates show promise as commercially viable effective oral and systemic vaccines.

- **Stable lipid-crystal nanoparticle formulations. Efficacious through oral and systemic delivery.**
- **Drives both mucosal and systemic immune responses.**
- **Induces both Th1 and Th2, antibody and cellular immunity.**
- **Antimicrobial cochleate formulations are currently in Phase I and Phase II human trials.**
- **Commercial scale manufacturing processes.**

These studies represent a collaboration among several investigators. The contributions and hard work of the following individuals is acknowledged and appreciated: Fan Zhang, Zheng Wand, and Susan Gould-Fogerite, Rutgers, New Jersey Medical School; Michael W. Pride, Robert J. Natuk, Laura York, David Giorgio, Jian She, Dongling Chen, John Eldridge, and Eric M. Mishkin, Wyeth-Lederle Vaccines and Pediatrics.