PHYTOL DERIVED IMMUNOADJUVANTS AS OIL-IN-WATER MICRO-EMULSIONS FOR USE IN VACCINES

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ABSTRACT

The main objective of this study is to evaluate an oil-in-water micro-emulsion of phytanol which we have named PHIS59 with multiple antigens: ovalbumin, a protein and microorganism that include a reference laboratory strain of methicillin susceptible *Staphylococcus aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA). PHIS59 was compared with the previously described crude oil-in-water emulsion PHIS-01 and the similarly formulated squalene as an oil-in-water micro-emulsion based on the commercial product MF59 referred to here as NV59. Safety and efficacy of PHIS59 and NV59 was evaluated in a mouse and rabbit vaccine models. Determinations of safety were reported as the range of *in vivo* mouse LD50s for each compound. Vaccine efficacy was assessed in terms of antibody response titers and isotype profiles in sera of vaccinated animals. Vaccines consisted of ovalbumin or heat killed *S. aureus* adjuvanted with either PHIS59, NV59 or PHIS-01. Control animals received unadjuvanted controls. Currently, there is no approved MRSA vaccine. PHIS-01 has shown promise in preventing MRSA-associated mortality in a mouse model; this study provides cross species validation.

PHIS59 is easier to handle than PHIS-01 because it does not bind the syringe plunger, can be preloaded into syringes and is a stable emulsion which allows consistent dose delivery. PHIS59 has a lower LD50 but is effective at lower doses giving a similar therapeutic index. This

could be explained by increased bioavailability due to the micro-emulsion formulation. This is an improvement over PHIS-01 in that it is effective at lower doses.

Finally, we tested a novel water soluble phytol derivative, sodium phytanyl sulfate and characterized a safe working dose range. Based on the doses determined through this study, sodium phytanyl sulfate (PHIS-SO4) is currently being studied as an adjuvant with an effective mouse dose of less than 1mg. Additionally, because it is soluble in water, it requires no emulsification for formulation in vaccines.

PREFACE

Phytol derived adjuvants have been documented to boost the immune response to tumor antigens, bacterial antigens and whole bacteria vaccines. They are an attractive class of compounds for vaccine drug development as they appear to possess many of the qualities needed for an ideal adjuvant. Importantly, they seem to increase antibody production and isotype switching when administered with albumin protein or *S. aureus* vaccines. The next logical step is to document the efficacy against clinically important pathogens and continue the development of these compounds for use in vaccinations by refinement of the formulations (so that they are safe and easy to prepare and administer.)

Previous work with these phytol derived adjuvants revealed potential therapeutic value in *Staphylococcus aureus* vaccine preparations. The greatest value of a *S. aureus* vaccine would be the ability to prevent and treat antibiotic resistant infections. For this reason, the phytol-derived immunoadjuvants could be evaluated using clinically important strains of methicillin resistant *S. aureus* (MRSA). By employing vaccination strategies, concerns about creating additional drug resistance through treatment are ameliorated.

Influenza vaccines are a highly studied area of vaccinology. The high prevalence and seasonal appearance of novel strains of influenza combine to keep influenza vaccines a relevant topic. As currently available influenza vaccines are only between 40% and 90% effective new strategies to improve their efficacy (including the use of novel adjuvants,) are constantly being developed.

Finally, formulation refinements to allow easier storage, injection and handling of the phytol-derived adjuvants will allow for greater use in research and potentially a more viable commercial product. The current formulation involves adding the hydrophobic phytol compound to an aqueous antigen in physiological buffer. The resulting vaccine must be mixed by drawing it up in a needle several times immediately prior to inoculation. This leads to difficulty in preparing consistent batches as each inoculum must be mixed separately. Additionally, it precludes the use of preloaded syringes. This study aims to address these concerns by formulating phytanol as a submicron-emulsion.

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INTRODUCTION

Phytol-Derived Immunoadjuvants

Phytol is a diterpene alcohol that is produced during the metabolism of chlorophyll (Figure 1C.) It has structural similarity to the triterpene squalene (Figure 1A.) which is used in several commercially available vaccine formulations[1,2] but, phytol is a xenobiotic while squalene is endogenous[3]. Adjuvants are compounds that are not immunogenic themselves but increase the immune response when administered with certain antigens.[4,5] Squalene is contained in several commercially available oil-in-water emulsion adjuvants.[5,6] Phytol initially was considered as a possible adjuvant because of its similar structure and the lack of endogenous phytol in animals.[7]

As an adjuvant, phytol proved effective but had some undesired side effects such as toxicity at higher doses.[8] Later studies used a reduced from of the substance, phytanol, and the efficacy and safety of phytanol as an adjuvant have been demonstrated in several studies.[5,8-17] The use of phytanol adjuvants to boost the immune response to a variety of antigens has been demonstrated in numerous other studies. Phytanol has been demonstrated to be safe and effective in mouse models against simple antigens and bacterial lysates.[10,12,16] When used to boost vaccine efficacy, it provides increased survival in the mouse model against challenges with certain strains of *Escherichia coli* and *Staphylococcus aureus*. [16-18]

There are shortcomings of the formulation method previously used for the phytanol adjuvant that were observed while preparing injections. Notably, most previous studies with vaccines containing phytanol have relied on emulsification by drawing the solution into a syringe

and expelling it through a needle a number of times (typically 10) prior to vaccination.[8,14] This creates a crude emulsion of the hydrophobic phytanol in an aqueous physiological buffer with antigen. The particle size of the phytanol is unstable and if the syringe is allowed to sit for any length of time, it coalesces and the phases separate. Because of this, the dosage of phytanol can vary dependent on the degree of phase separation and whether it preferentially remains in the residual volume of the syringe.

Furthermore, during the mixing process, an interaction between phytanol and the rubber in the syringe plunger causes the plunger to stick. While alternatives such as glass or plastic syringes could overcome this problem,[19] this is an issue that would need to be addressed prior to clinical trials. In animal vaccine studies, we observed that this interaction between phytanol and the rubber plunger reduces the ability of the person injecting the vaccine to do so in a slow and controlled manor. This problem seems to be exacerbated when the prepared syringe is not immediately used for injection or when there is increased mixing of vaccine in the syringe. These problems can cause distress in experimental animals when the syringe unexpectedly sticks and unsticks leading to a quicker than intended injection. In the worst case scenario, with small syringes, the plunger can lock up during the injection and the remaining vaccine volume must be injected with a new syringe.

Phytol-Derived *Staphylococcus aureus* Vaccines

Phytanol and its derivative phytanyl chloride have been shown to be effective in the reduction of mortality in live *S. aureus* challenge after vaccination with Heat killed *S. aureus* in a mouse model.[14,17] This model has been tested with a laboratory reference strain of *S. aureus* and with the methicillin resistant strain MW2. Validating these results with clinically important strains of *S. aureus* is an important step in producing a vaccine candidate. By testing the

phytanol adjuvants with clinical isolates of these types, we can begin to establish the efficacy of the phytanol adjuvant.

Research Objectives

There are two primary objectives of this study: 1) to examine the use of phytol-derived adjuvants in oil-in-water micro-emulsion formulations, and 2) to use the formulations to expand research with phytol-derived adjuvants in *S. aureus* vaccines in a mouse model.

Several concentrations of phytanol formulated with the same buffers and emulsifiers as MF59 were made using double ended syringe homogenizers, an ultrasonic homogenizer and a vortex mixer. A control emulsion with squalene replacing phytanol was made at the same time. These were compared to phytanol emulsified with a needle immediately prior to injection as had been described previously for Freund's adjuvants.[8] Only the ultrasonic homogenization is able to produce an emulsion that is similar to the published descriptions of MF59.[6] While the strategy of using an ultrasonic homogenizer instead of a fixed geometry fluid processor may yield an emulsion that does not meet the particle size specifications for commercially produced MF59, it does yield a stable micro-emulsion. A formulation modeled after Novartis' MF59 formulation which we refer to as NV59 contains the same squalene and emulsifiers as MF59. NV59 differes from MF59 in that it is prepared by ultrasonic homogenization instead of in a micro-fluidizer. NV59 was manufactured at the same time and using the same methods as PHIS59, and was used as a control in all experiments.

MATERIALS AND METHODS

Formulation of Micro-Emulsion Adjuvants for Vaccine Studies

NV59 was made based on the published formulation of MF59.[6] Breifly, we dissolved squalene in one part to ten of sorbitan trioleate (Span85) (w/w). Separately, polysorbate 80 (Tween80) is dissolved in 10 mM citric acid/sodium citrate pH 6.0 buffer to make a 0.5% solution. The squalene solution and citrate buffer solutions were combined to create a 5% squalene (w/v), 0.5% sorbitan trioleate (w/v), 0.5% Tween 80 in 10 mM citric acid/sodium citrate pH 6.0. To make a micro-emulsion, up to 10 ml of this solution is processed for 5 minutes in a Branson Sonifier 250 ultrasonic homogenizer at 100% power (based on micro-tip output settings) with a 50% duty cycle using a titanium microtip. The resulting emulsion was placed in the refrigerator overnight to allow air trapped in the sample to leave the solution. This differs from the production method of commercially made MF59 as ultrasonic homogenization was used in place of a micro-fluidizer. We also developed a NV59 variation that contained one part of squalene to five parts of Span 85 combined with the citrate buffer solution to create a 10% squalene (w/v), 0.5% sorbitan trioleate (w/v), 0.5% Tween 80 in 10 mM citric acid/sodium citrate pH 6.0pH 6.0 which is referred to as NV59-10. To complete the vaccine formulation, 200µl of NV59 or NV59-10 was mixed with 200µl of antigen (either OVA or HKSA as specified) providing a 3400mg/kg or 6800mg/kg dose (for a 25g mouse.)

For the phytanol based oil-in-water adjuvant PHIS59, the same protocol as NV59 is followed except varying concentrations of phytanol, 10%, 5%, 2.5%, were dissolved in the Span85. This created a series of adjuvants that have named as follows: PHIS59-10 containing 40 mg of PHIS-01 per dose, PHIS59-5 containing 20 mg of PHIS-01 per dose, and PHIS59-2.5 containing 10 mg of PHIS-01 per dose. All form a stable emulsion that appeared similar to emulsion seen for NV59. Visual analysis by light microscopy confirms a sub-micron particle size (data not shown). High speed centrifugation (17,000 xg) for greater than 10 minutes caused the particles of the emulsifier to aggregate and formed a white solid on top of the aqueous phase with the oil phase at the top of the tube. After several weeks, slight separation (figure 3A) could be remedied by brief vortex mixing (figure 3B) which returned the solution to the homogenous micro-emulsion state. This was notable as vortex mixing alone for a minimum of 1 minute at full speed with a Fisher vortex genie 2 was insufficient to create a stable emulsion. Additionally, thorough mixing by 10 passages through a 22 gauge by 2-7/8 inch double ended micro-emulsifying needle syringe was not sufficient to create a stable emulsion.

Additionally, PHIS-59-10 was tested at a half dose where 100 µl of adjuvant was mixed with 100 µl of antigen solution. We refer to this formulation as PHIS59-10/2. PHIS-59 was tested in a double strength dose where 400 µl of PHIS-59-05 was mixed with 400 µl of antigen solution. We refer to this formulation as PHIS59-5x2.

The ultrasonically homogenized emulsions of PHIS59-05, NV59 and NV59-10 were visually examined and found stable for greater than 10 months at 4 °C storage (Figure 3.) A comparison of the components of PHIS-01, PHIS59 and NV59 are presented as table 1. Mass spectrometry was used to verify that the ultra-sonic homogenization of the phytanol did not change its chemical nature.

Table 1. Basic composition of PHIS59 and NV59 made in the laboratory for use as adjuvants.

Formulation Designation	Isoterpene	Buffer	Tween 80	Span 85
PHIS59-10	Phytanol 40mg/400µl dose	10 mM Citrate pH 6.0	0.5% (w/v)	0.5% (w/v)
PHIS59-5	Phytanol 20mg/400µl dose	10 mM Citrate pH 6.0	0.5% (w/v)	0.5% (w/v)
PHIS59-2.5	Phytanol 10mg/400µl dose	10 mM Citrate pH 6.0	0.5% (w/v)	0.5% (w/v)
PHIS-01	Phytanol 40mg/400µl dose	PBS pH 7.2	none	none
NV59	Squalene 20mg/400µl dose	10 mM Citrate pH 6.0	0.5% (w/v)	0.5% (w/v)
NV59-10	Squalene 40mg/400µl dose	10 mM Citrate pH 6.0	0.5% (w/v)	0.5% (w/v)
PHIS59-10/2	20mg/200µl dose	10 mM Citrate pH 6.0	0.5% (w/v)	0.5% (w/v)
PHIS59-5x2	40mg/800µl dose	10 mM Citrate pH 6.0	0.5% (w/v)	0.5% (w/v)

Formulation of Sodium Phytanyl Sulfate Adjuvant

Sodium phytanyl sulfate (PHIS-SO4) (figure 1D) was dissolved as a homogenous solution in phosphate buffered saline (PBS) at a concentration of 10 mg/ml and further diluted with additional PBS to obtain the desired dose. Originally, autoclaving was attempted but this resulted in dissociation of the sulfate group as evidenced by a drop of over one unit of pH. Instead, samples were sterile filtered. A sample was removed from each batch to test the pH remained neutral.

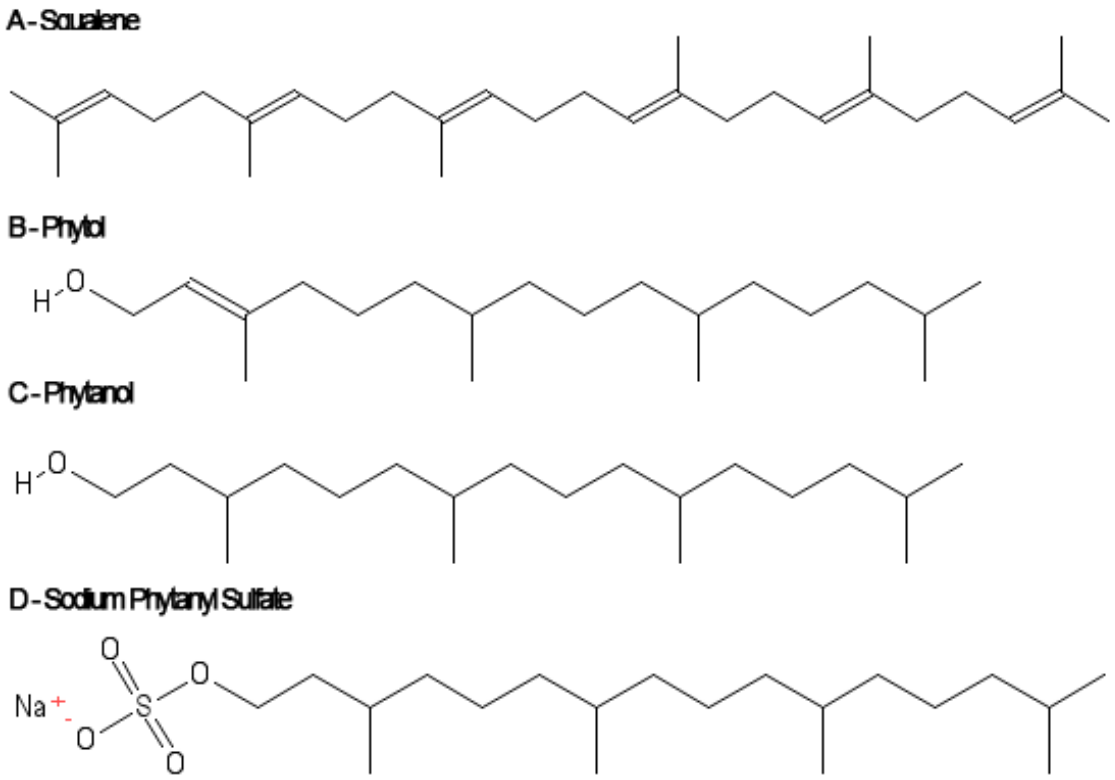

Figure 1. Molecular structures of (A) squalene, (B) Phytol, (C) Phytanol and (D) sodium phytanyl phosphate.

Vaccine Adjuvant Controls and General Manufacturing Process

All vaccine formulations were handled with best aseptic practices including sterile filtration through a 0.22 µm pore size cellulose acetate membrane syringe filter (Ahlstrom Munktell, Sweden.) Single dose syringes of all adjuvants except for PHIS-01 were made in the lab prior to being transported to the animal facility. PHIS-01 was emulsified in Freund's adjuvant-like manner (by vigorously expelling it through a 27 guage syringe 10 times immediately prior to injection) as previously described.[8]

Commercially produced aluminum hydroxide gel as Alhydrogel™ 2% (InvivoGen Cat # vac-alu-250) was used at a dose of 0.75 mg per dose for mice and 5.63 mg per

dose for rabbits. This dose was based on previous work and the recommended FDA allowed doses for humans.[20,21]

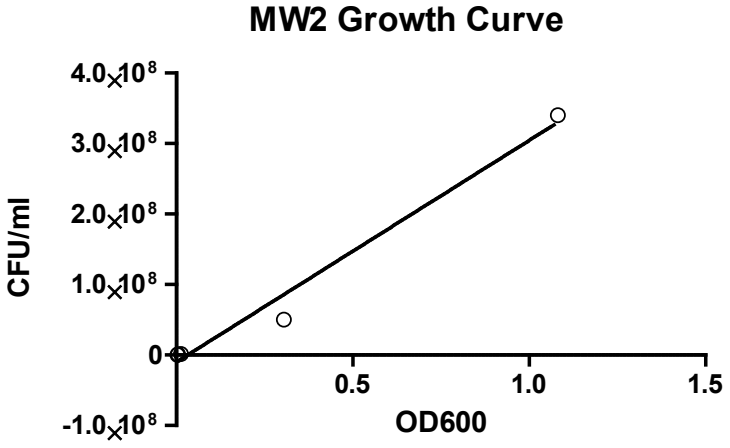

Figure 2. Linear portion of the growth curve of *S. aureus*, R2 = 0.98

Antigen Solutions for Vaccine Formulations

Three antigen solutions were used to complete the vaccines. Ovalbumin (OVA) purified from hen egg whites was purchased from Sigma Aldrich (Cat # A7641) as lyophilized powder and mixed with PBS at a concentration of 5 mg/ml. This solution was filter-sterilized and 20 µl/dose was mixed with autoclaved reverse osmosis water to make up an antigen solution containing 100 µg OVA/dose.

The second vaccine antigen, heat killed *Staphlococcus aureus* strain MW2 (HKSA-MW2) contained methicillin resistant *S. aureus (ATCC® BAA1707™)* obtained from American Type Culture Collection was prepared based on the procedure described previously [14] with the several modifications. A standard growth curve was generated by seeding *S. aureus* strain MW2 at low density in Luria-Bertani broth at 37 °C and taking samples at fixed time points. For each sample, the absorbance at 600 nm was measured using a Beckman DU640 spectrophotometer. Additionally, 10 µl was plated at various dilutions on blood-agar plates to determine the colony

forming units (CFU) per ml of culture. A linear regression of the data from the linear growth phase of the bacteria was generated (figure 2) and used to find the CFU/ml for vaccine preparations.

Bacterial cultures were grown for approximately 7 hours and the CFU/ml were found by comparison to the standard curve. The bacteria were centrifuged at 1500 x g for 10 minutes in a Beckman TJ6 centrifuge and washed twice with PBS before being resuspended in PBS at a concentration of $5x10^6$ CFU/dose for heat killing. The first trial at 60 °C for 1 hour showed significant growth from a 10µl of the suspension plated on blood-agar and grown overnight to confirm killing. Therefore, for the second trial, we heat-killed the bacteria at 90 °C for 5 minutes instead of the previous 60 °C for 1 hour. This resulted in no bacterial growth after a week when 10µl of the suspension was plated grown on blood-agar.

A laboratory reference strain of methicillin susceptible *Staphylococcus aureus subspecies aureus* (ATCC® 25923™) , a generous gift from Dr. H. K. Dannelly to Dr. R. Roy Chowdhury, was revived from glycerin stocks stored at -80 °C. After recovery, an antigen solution of the heat killed *S. aureus* lab strain (HKSA-LS) was prepared in an identical method to HKSA-MW2 with 90 °C heat killing. A consolidated summary of the completed vaccine formulations along with data comparing the overall survival of mice injected with the formulation is table 2.

Table 2. Survival of mice vaccinated with PHIS-01, PHIS59 or NV59 at varying doses.

	Isoterpene (mg/dose)	Twee80 and Span85	Antigen	Dose	Emulsification Method	Mice Treated	Mice Surviving
PHIS-01	Phytanol (40 mg)	No	OVA, HKSA	400 µl	Freund-Like	31	31
NV59	Squalene (20 mg)	Yes	OVA HKSA	400 µl	Ultrasonic Homogenization	27	27
NV59-10	Squalene (40 mg)	Yes	OVA	400 µl	Ultrasonic Homogenization	2	2
PHIS59-5	Phytanol (40 mg)	Yes	OVA	400 µl	Ultrasonic Homogenization	6	6
PHIS59-10/2	Phytanol (20 mg)	Yes	OVA	200 µl	Ultrasonic Homogenization	2	2
PHIS59-05	Phytanol (20 mg)	Yes	OVA	400 µl	Ultrasonic Homogenization	11	11
PHIS59-05	Phytanol (20 mg)	Yes	HKSA	400 µl	Ultrasonic Homogenization	6	4 (1*)
PHIS59-05x2	Phytanol (40 mg)	Yes	OVA	800 µl	Ultrasonic Homogenization	2	0
PHIS59-2.5	Phytanol (40 mg)	Yes	OVA, HKSA	400 µl	Ultrasonic Homogenization	15	15
PHIS59-10	Phytanyl Sulfate (10 mg)	Yes	OVA	400 µl	N/A (soluble)	4	4

*Overall survival rate with 3 doses over a month long period.

Endotoxin Testing of PHIS59 and NV59

An endotoxin assay was conducted using the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific Inc., Il, USA) according to the manufacturer's instructions to ensure that the PHIS59 and NV59 manufactured in the lab were non-pyrogenic. A representative sample of PHIS59-5 and NV59 without antigen were tested and were found to have less than 1 endotoxin unit (EU) per ml (figure 7.)

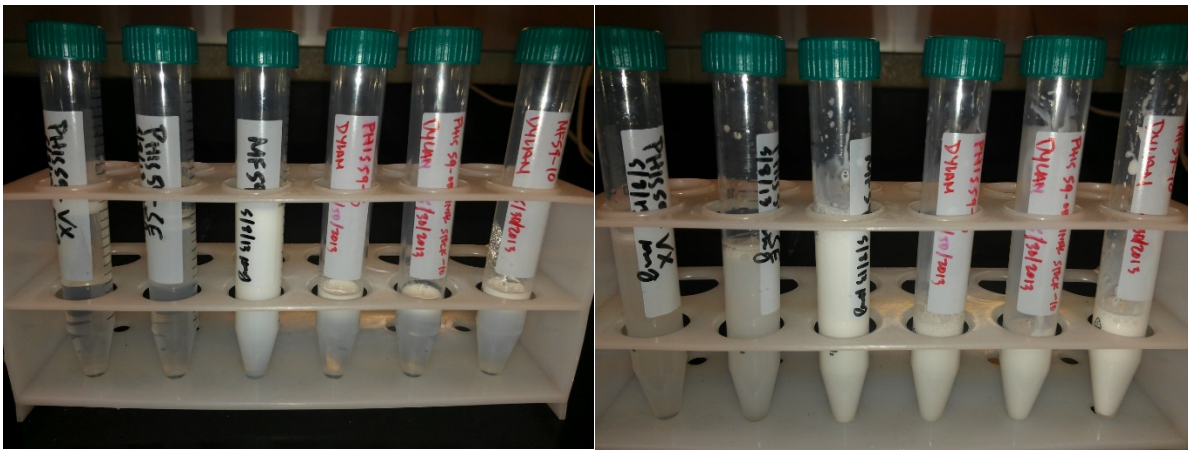

Figure 3A (left). Adjuvants from refrigerator storage >5 months. From left to right: 1) PHIS59-10 processed with a vortex mixer only 2) PHIS59-10 processed with a double ended syringe emulsifier, 3) NV59 processed with a ultrasonic homogenizer, 4) PHIS59-5 processed with a ultrasonic homogenizer, 5) PHIS59-10 processed with a ultrasonic homogenizer, 6) NV59 with 10% Squalene processed with a ultrasonic homogenizer. Figure 3B (right). Adjuvants after brief inversion mixing from refrigerator storage >5 months. From left to right: 1) PHIS59-10 processed with a vortex mixer only 2) PHIS59-10 processed with a double ended syringe emulsifier, 3) NV59 processed with a ultrasonic homogenizer, 4) PHIS59-5 processed with a ultrasonic homogenizer, 5) PHIS59-10 processed with a ultrasonic homogenizer, 6) NV59 with 10% Squalene processed with a ultrasonic homogenizer. Macroscopic appearance is identical to freshly prepared for homogenized adjuvants. The appearance of the adjuvants was unchanged after 10 months of storage. All adjuvants were filter-sterilized and loaded into sterile 15 ml tubes for storage without antigen added to the formulation.

Mouse and Rabbit Study Approvals and General Methods

All animal studies were conducted with the approval of the Indiana State University Institutional Animal Care and Use Committee (IACUC) under protocols 01-03-11-RRC/SKG for all HKSA-MW2 formulations, 404519-1 for all OVA formulations including all PHIS-SO4 vaccinations, and 450531-1 for all HKSA-LS vaccinations including all rabbit vaccinations.

All mouse studies were conducted in female Balb/c mice aged 6-18 weeks except for the third trial of the phytanyl sulfate vaccine which utilized male mice. All vaccinations were given intra-peritoneal (IP) into the lower right quadrant using a 27-gauge needle. The injection site was cleaned before and after injections with sterile alcohol prep pads. Blood collection from mice was performed by retro-orbital bleeding of isoflurane anesthetized mice. Serum was separated from whole blood by centrifugation at 3000 x g for 15 minutes and pooled from the individual mice in each experimental group.

All rabbit studies were conducted in female New Zealand White rabbits at approximately one year of age. All vaccinations were formulated as 7.5 times the equivalent dose for a mouse, split between two subcutaneous injection sites bilaterally over the rear hind quarter. Blood collection from rabbits was performed by venipuncture of the marginal ear vein. Serum was separated from whole blood by centrifugation at 1840 x g for 15 minutes at 4 °C.

Vaccine Trials of PHIS59 with Ovalbumin

The first trial of PHIS59 with OVA compared NV59, PHIS59-10, PHIS-01 and citrate buffer with emulsifiers. Four mice were vaccinated from each group with the adjuvants as previously described injected in 400 µl total volume. The trial had three planned doses but was terminated after the first dose caused the mortality of all mice in the PHIS59-10 group 4 days after injection.

A second trial used a single dose of adjuvant with OVA antigen to assess whether the mortality was caused by contamination of the PHIS59-10 formulation or was a result of the ultrasonic homogenization. To test if it was a result of the ultrasonic homogenization, we used PHIS59-10 which had been emulsified by 10 passages through a 22 guage 2 7/8 inch micro-emulsifying double ended syringe instead of ultrasonic homogenization and PHIS59-10 which had been vortexted to mix but not emulsified. Additionally, a new batch of PHIS-59-10 and PHIS59-5 were used. PHIS-01, NV59, and citrate buffer alone were injected as controls. All vaccinations were given in 400 µl total volume into groups of 2 mice each.

A third trial used 3 doses of PHIS59-5, PHIS59-2.5, PHIS-01, NV59, PHIS-SO4-10 mg/dose or citrate buffer alone given every 14 days. Three mice were assigned to each group of adjuvant and two mice to the unadjuvanted citrate buffer group. All PHIS-SO4-10 mice were found dead the day after the first vaccination. Blood was collected 7 days after the second and third vaccinations from the remaining groups to determine the antibody titer and isotype by ELISA.

A fourth trial with 6 mice per group assigned to either PHIS59-5, NV59, PHIS-01 or unadjuvanted citrate buffer given three doses 14 day intervals. Blood was collected 7 days after the last vaccination from the remaining groups to determine the antibody titer and isotype by ELISA.

Vaccine Trials of PHIS59 with HKSA-MW2

Two trials of HKSA-MW2 vaccinations were run. Both used groups of 6 mice vaccinated at 14 day intervals with blood collected 7 days after the last injection. The first trial had 4 groups: NV59, PHIS59-5, PHIS-01 and unadjuvanted buffer. The second also had 4 groups although PHIS59-2.5 was used in place of PHIS59-5.

Vaccine Trials of PHIS59 with HKSA-LS

A single trial of HKSA-LS on groups of 5 or 6 mice each assigned to the following groups: PHIS-01, NV59, Alum, PHIS59-2.5 or unadjuvanted buffer. Vaccines were administered at 14 day intervals. Blood was collected 7 days after the first two doses and 10 days after the third dose. Additionally, a trial HKSA-LS was run in rabbits with a single rabbit assigned to each group.

Vaccine Trials of PHIS-SO4 with OVA

After establishing the LD50 of PHIS-SO4 was less than 10 mg per mouse, three additional vaccine trials with OVA as the antigen were run to find a safe dose and establish the potential adjuvanticity of PHIS-SO4. In the first trial, 2 mice were assigned to each of the following groups: PHIS-SO4-1, PHIS-SO4-250µg, a 200 µl half-dose of PHIS-59-10 (PHIS59-10/2), a 800 µl double-dose of PHIS59-5 (PHIS59*2), NV59 or unadjuvanted vaccine.

The second and third vaccination trials both employed the strategy of a series of 3 vaccinations at 14 day intervals with blood collection 7 days after each vaccination. In the second vaccine trial, five mice per group were vaccinated with PHIS-SO4-3, PHIS-01 or unadjuvanted vaccine. For the third vaccination trial, 4 mice per group were vaccinated with PHIS-SO4-100µg, PHIS-SO4-50µg, PHIS-SO4-25µg, PHIS-01 or unadjuvanted vaccine. A summary of all vaccination trials is presented as Table 3.

Table 3. Summary of vaccination trials.

Vaccine Trial	Experimental Adjuvant(s)	Major Finding
OVA-PHIS59-1st	PHIS59-10	PHIS59-10 causes mortality in all mice 4 days after injection
OVA-PHIS59-2nd	Various emulsion methods for PHIS59-10	PHIS59-10 mortality is only seen with ultrasonic homogenization
OVA-PHIS59-3rd	PHIS59-5, PHIS59-2.5, PHIS-SO4-10	PHIS-SO4-10 causes mortality the first day (Figure 4); PHIS59 is comparable to NV59 (Figure 8)
OVA-PHIS59-4th	PHIS59-5	Figure 9
HKSA-MW2 1st	PHIS59-5	Figure 5, Figure 9
HKSA-MW2 2nd	PHIS59-2.5	Figure 9
HKSA-LS-1st Trial	PHIS59-2.5	Data not shown
HKSA-LS-Rabbit 1st	PHIS59-2.5	Figure 11
OVA-PHIS-SO4-1st	PHIS-SO4-1, PHIS-SO4-250μg, PHIS59-10/2, PHIS59-5*2	The LD50 of PHIS-SO4 is above 1 mg/dose; the toxicity of PHIS59 is not related to dose volume. (Figure 6)
OVA-PHIS-SO4-2nd	PHIS-SO4-3	The LD50 of PHIS-SO4 decreased for multiple vaccinations in a two week period and is less than 3mg/dose in this case (Figure 6)
OVA-PHIS-SO4-3rd	PHIS-SO4-100μg, PHIS-SO4-50μg, PHIS-SO4-250μg	Multiple doses of ≤100μg of PHIS-SO4 are well tolerated (Figure 6).

ELISA Determination of Antibody Titer and Isotypes Induced by Vaccination

Lysates of HKSA-MW2 and HKSA-LS were made by growing bacteria overnight followed by centrifugation as described above. Our pellet was suspended in approximately 1.5 ml of PBS for each 100 ml of initial culture. The sample was placed in a 90 °C water bath for 5 minutes. The bacteria was then subjected to 3 freeze thaw cycles followed by sonication in a Branson Sonifier 250 with the macro tip on ice for 3 periods of 6 minutes (power setting 3, 20% duty cycle, approximately 35% output.) A sample was plated on blood agar as above to check for growth. Lysates and an OVA solution were tested by ELISA at various dilutions against serum pooled from the third bleed serum of the NV59 group from the 2nd trial of HKSA-MW2 vaccinations or the HKSA-LS vaccination trial. It was determined that optimal binding was achieved by a 1:6000 dilution of the lysed HKSA-MW2, a 1:1000 dilution of the lysed HKSA-LS and a 10 µg/ml solution of OVA.

For all ELISAs, antigen was coated to 96 well flat bottom polystyrene plates (Corning Cat # 2595) by adding 100 µl of optimally binding antigen concentration in carbonate (0.01M)-bicarbonate (0.35M) buffer pH 9.6 with 0.05% (w/v) sodium azide overnight at 4 °C. The plates were then washed with ELISA wash buffer containing 0.05% (v/v) Triton X-100 in PBS. The residual binding capacity of the plate was blocked with ELISA blocking buffer containing 1% (w/v) bovine serum albumin with 0.05% (v/v) Triton X-100 and 0.05% (w/v) sodium azide in PBS. The plates were stored with blocking buffer in plastic wrap at 4 °C. ELISA blocking buffer was used for the dilution of all serum. ELISA blocking buffer without sodium azide was used for dilution of all horse radish peroxidase (HRP) linked antibodies to prevent diminished activity due to the HRP.

Antibody titer dilution: Blocking buffer was removed from ELISA plates prepared as described above by washing 3 times with ELISA wash buffer. Next, 100 µl of various dilutions of serum were then added to the plates. The plates were incubated at 37 °C for 1-2 hours and washed 3 times with ELISA wash buffer. Secondary rabbit anti-mouse-HRP was diluted 1:1000 and 100 µl was added to each well. The plates were incubated at 37 °C for 1 hour and washed 6 times with ELISA wash buffer. Ortho-phenylenediamine (OPD) solution containing 1 mg/ml OPD with 2 µl of 50% hydrogen peroxide per ml of pH 5.0 citrate buffer was freshly prepared and 100 µl added to each well. After 5 minutes, the colorimetric development of OPD was stopped by the addition of 100 µl of 10% sulfuric acid and the plates were read in a Bio-Tek EL808 ultra micro-plate reader at 492 nm. Titration of rabbit serum was essentially the same as for mouse serum with the exception that goat anti-rabbit-HRP (Bio-rad Cat # 170-6515) was used in place of rabbit anti-mouse-HRP.

Antibody Isotyping: blocking buffer was removed from ELISA plates prepared as described above by washing 3 times with ELISA wash buffer and 50 µl of a 1:50 dilution of serum were added to the plates. The plates were incubated at 37 °C for 1-2 hours and washed 3 times with ELISA wash buffer. Rabbit-anti-isotype antibodies (50 µl) from a Mouse Typer Isotyping Kit (BioRad Cat # 172-2051) were added by row to the plates followed by a 1 hour incubation at 37 °C. The plates were then washed 3 times with ELISA was buffer. Secondary goat anti-rabbit-HRP (Bio-rad Cat # 170-6515) was diluted 1:1000 and 50 µl was added to each well. The plates were incubated at 37 °C for 30 minutes to 1 hour and washed 6 times with ELISA wash buffer. OPD measurement was performed as described above.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA). Linear regression was performed for the MW2 growth curve and endotoxin test. ELISA data was analyzed with a 2-way analysis of variance (ANOVA) with a Bonferroni post hoc test using serum from mice with unadjuvanted vaccines as the control group. For titers, the independent variables tested were bleed number and adjuvant group. For isotypes, the independent variables tested were isotype and adjuvant group. All 2-way ANOVAs had a significant interaction at $p < 0.05$ except for the first two isotype ELISAs of the HKSA-LS mouse trial. Bonferroni post hoc tests are specified in figures with an asterisk (*) above columns that are significantly different from unadjuvanted controls. Error bars on all figures represent the mean plus or minus the standard error of the means.

RESULTS

General Tolerability of Phytol-Derived Vaccine Formulations

The lethal dose for half of the mice receiving (LD50) of PHIS-01 has been established to be greater than 40 mg per mouse (average weight 25 gg) or 1.6 mg/kg [8,10] When formulated with a simple antigen such as OVA, the LD50 values for PHIS59 vaccine preparations appears to be between 20-40 mg per mouse or greater than 800 mg/kg but less than 1600 mg/kg for the PHIS59-10 and PHIS59-5 formulation. The required injection volume required to test PHIS59-2.5 precluded determining an LD50 value. Results of individual trials with formulations of OVA antigens are presented in figure 4. When the antigen is replaced with the more complex HKSA-MW2, the LD50 appears to continue to fall to between 10-20 mg per mouse (400 mg/kg - 800 mg/kg, figure 5.)

For PHIS-SO4, the LD50 of a single dose is between 10 mg/mouse and 3 mg/mouse or between 400 mg/kg and 120 mg/kg. This is reduced for multiple doses to below 120 mg/kg although we have noted that 4 mg/kg is well tolerated over a series of 3 vaccinations. (Figure 6.) A summary of LD50s is provided as table 4.

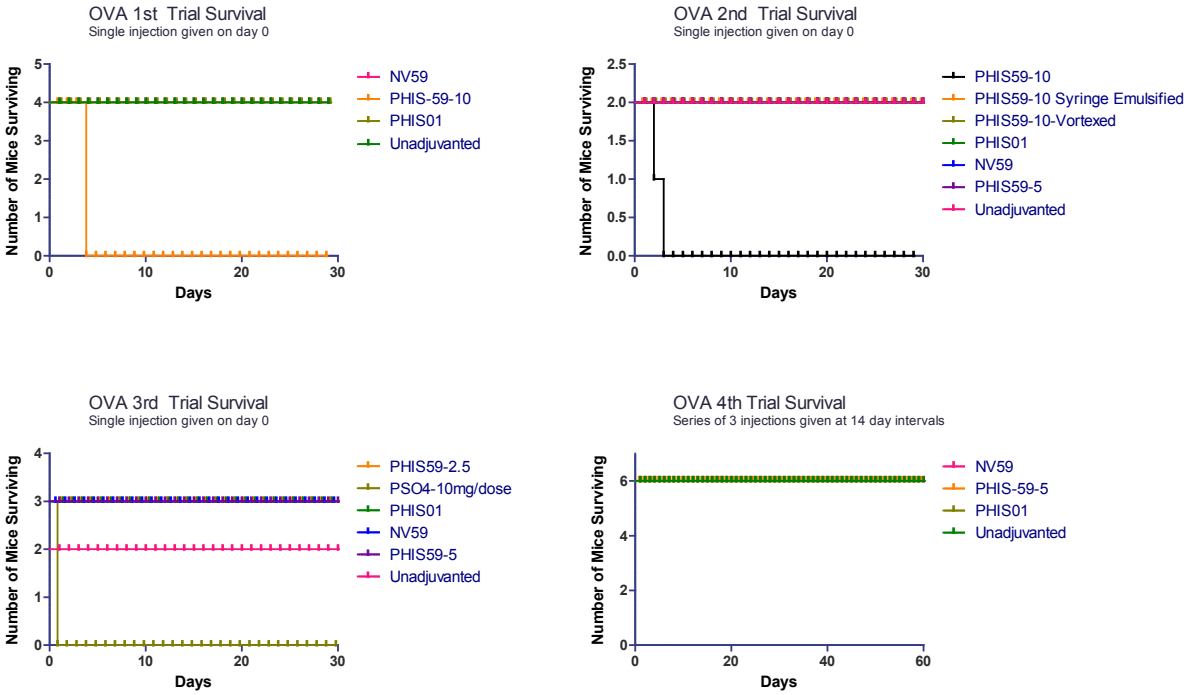

Figure 4. Survival curves of the mice from the four vaccine trials which contained the antigen OVA. PHIS-59-10 appears to be toxic to all mice receiving a 40 mg vaccine dose. PHIS-SO4-10 caused the mortality of all mice within the first day of injection.

Table 4. LD50 of novel phytol-derived immunoadjuvant formulations

Formulation	Isoterpene	Emulsification	Antigen	Range for LD50
PHIS-01	Phytanol	Freund's adjuvant like	Simple or Complex	>40 mg/dose >1.6 mg/kg
PHIS59	Phytanol	Ultrasonic Homogenization with Emulsifiers	Simple	20-40 mg/dose 800-1600 mg/kg
PHIS59	Phytanol	Ultrasonic Homogenization with Emulsifiers	Complex	10-20 mg/dose 400-800 mg/kg
NV59	Squalene	Ultrasonic Homogenization with Emulsifiers	Simple or Complex	>40 mg/dose >1.6 mg/kg
PHIS-SO4	Sodium Phytanyl Sulfate	Not needed, water soluble	Simple	3-10 mg/mouse 120-400 mg/kg (single dose) 0.1-3 mg/mouse 4 – 120 mg/kg (two doses at 14 day intervals)

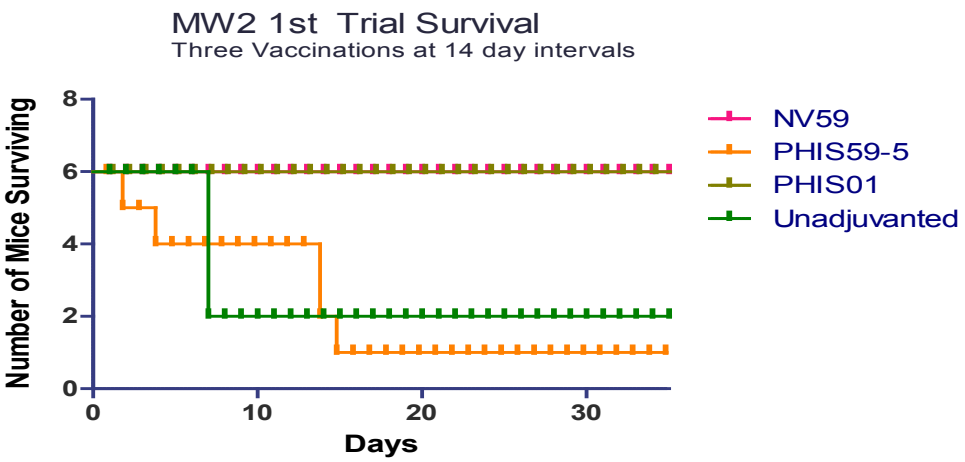

Figure 5. Survival curve from mice receiving vaccination formulations containing heat killed *S. aureus*. Mice in the control group that died from the first vaccination were inadvertently given active *S. aureus* infection. No mortality was observed in the second MW2 trial or the HKSA-LS trial which had the same treatment groups except PHIS59-5 was replaced with PHIS59-2.5.

All of the mice receiving PHIS59-05 during the course of this study appeared ill for the first four days following a dose of the vaccine formulation. This was observed through decreased movement and response to stimuli, ruffled fur, perianal inflammation with or without bloody stool and a hunched posture. Mice that received PHIS59-05 with OVA all recovered from this condition by the fifth day post vaccination. Mice who were given PHIS59-10 displayed similarly ill after vaccination but never recovered and died between the third and fifth day post vaccination. Necropsy did not display any gross anatomical changes, particularly to the spleen, liver or kidneys.

PHIS-SO4 vaccinations containing 1 mg/mouse or more of PHIS-SO4 appeared to cause injection site discomfort. This was observed through vocalization and hyperactivity during a 1-3 minute period following vaccination. After this, the mice appeared normal. Mice that received doses above 1 mg of PHIS-SO4 that were not fatally toxic displayed signs of distress including

hunched posture, ruffled fur and decreased response to stimuli for 1 or 2 days following vaccination.

Mice administered the PHIS59-2.5 vaccines with HKSA-MW2 or HKSA-LS consistently displayed hunched posture, ruffled fur and decreased response to stimuli for 3-5 days post vaccination and had recovered completely by day 6. A similar effect was not observed in mice who received PHIS59-2.5 with OVA. No physical or behavioral changes was observed in any of the vaccinated rabbits.

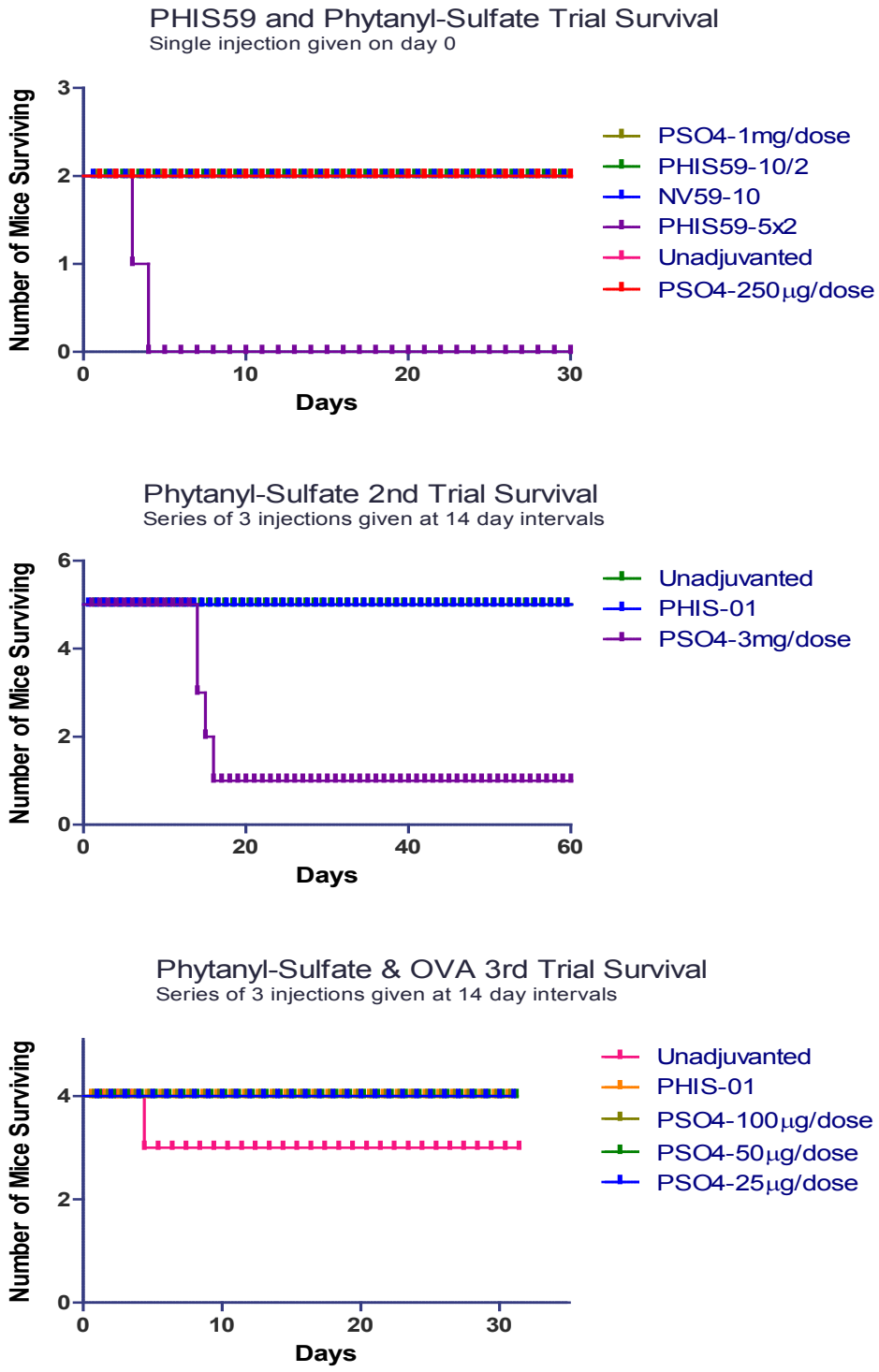

Figure 6. Survival curves of mice receiving PHIS-SO4. Single doses of 3 mg or multiple doses of 100 μg or less were tolerated well.

Endotoxin Testing

Human vaccines are regulated by the US Food and Drug Administration (FDA) to contain less than 5 EU/kg (Brito & Singh, 2011). This would convert to an estimated mouse equivalent dose of 62.5 EU/kg based on FDA published tables (Food & Administration, 2005). Considering the average mouse in our study weights approximately 25 grams, this would place the limit per dose at 1.56 EU/dose. As the doses contain 200 µl of adjuvant, corresponding to 0.14 EU/dose for NV59 and 0.11 EU/dose for PHIS59, both at least ten times less than the limit.

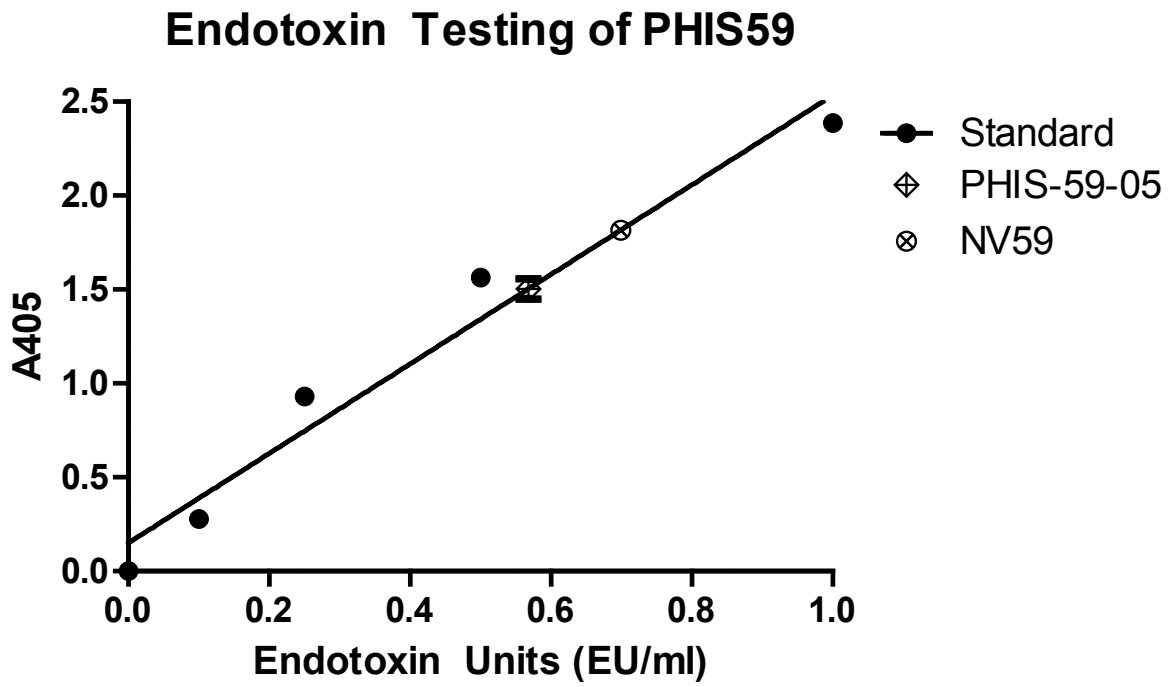

Figure 7. Endotoxin levels of NV59 (0.699 EU/ml) and PHIS59 (0.568 EU/ml) prepared for use as vaccines as based on the linear regression of 1 EU/ml, 0.5 EU/ml, 0.25 EU/ml, 0.10 EU/ml and 0 EU/ml standards. The standard curve was generated through linear regression. For the standard curve, R^2 = 0.96. Each data point presented is the mean of 3 replicate wells with error bars denoting SEM as appropriate.

Antibody Titer and Isotypes in Response to OVA Vaccines

The third and fourth trials of OVA antigenic vaccines produced ELISA data. From the third trial, PHIS59-2.5 is able to match the titer of NV59 after second and third doses are administered at a titer of 1:100 and 1:1000 respectively. PHIS-01 outperformed both NV59 and PHIS59-2.5 with tenfold greater titers at both blood collection time points. PHIS59-5 was able to match titer of PHIS59-2.5 and NV59 after the second dose but the titer did not increase after the third dose. Isotyping date from this trial suggested that NV59 was superior at inducing isotype changing towards IgG isotypes following the first booster. After the third dose, IgG1 remained elevated for NV59 and PHIS-01 displayed more IgG2b than the other adjuvants (Figure 8.)

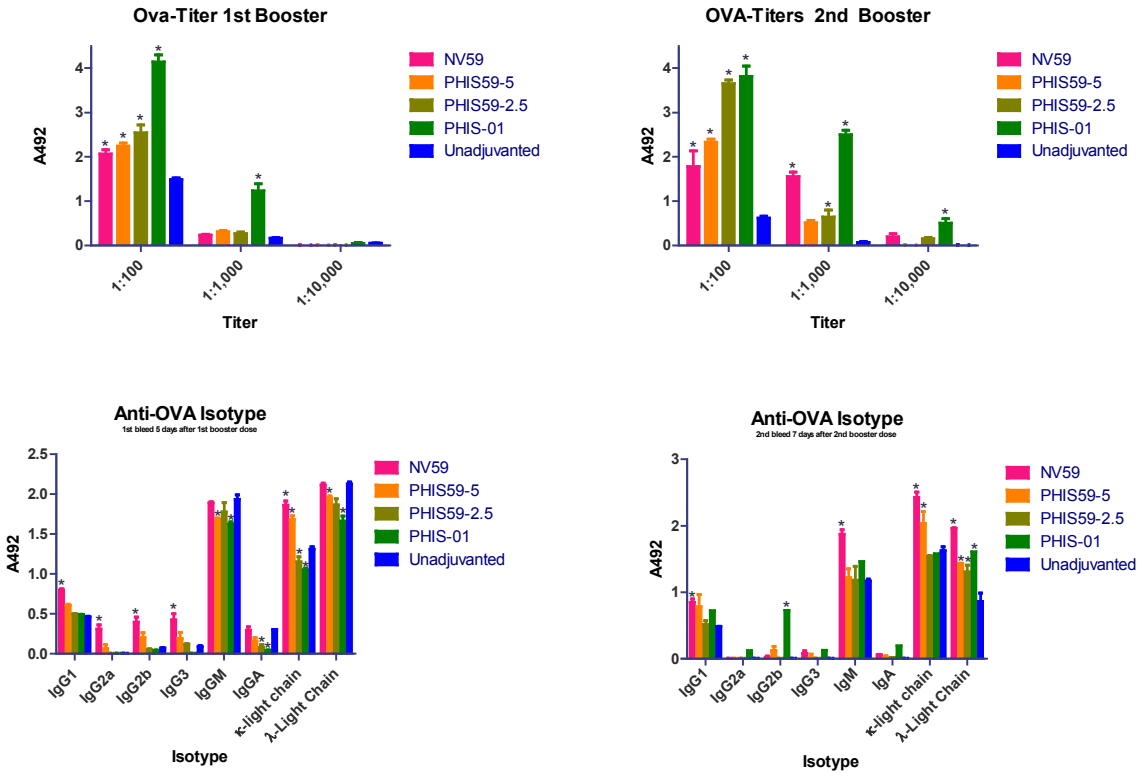

Figure 8. Titers and isotypes of antibodies after the second (first booster) and third (second booster) doses of OVA vaccinations from the third OVA vaccine trial.

The fourth trial with OVA based vaccinations seemed in line with the titer observations from the third trial. After three doses, PHIS59-5 and PHIS-01 displayed the highest titers at 1:10,000 with NV59 having increased activity over unadjuvanted controls at a tenfold reduced titer. All had similarly high isotype switching (Figure 9.)

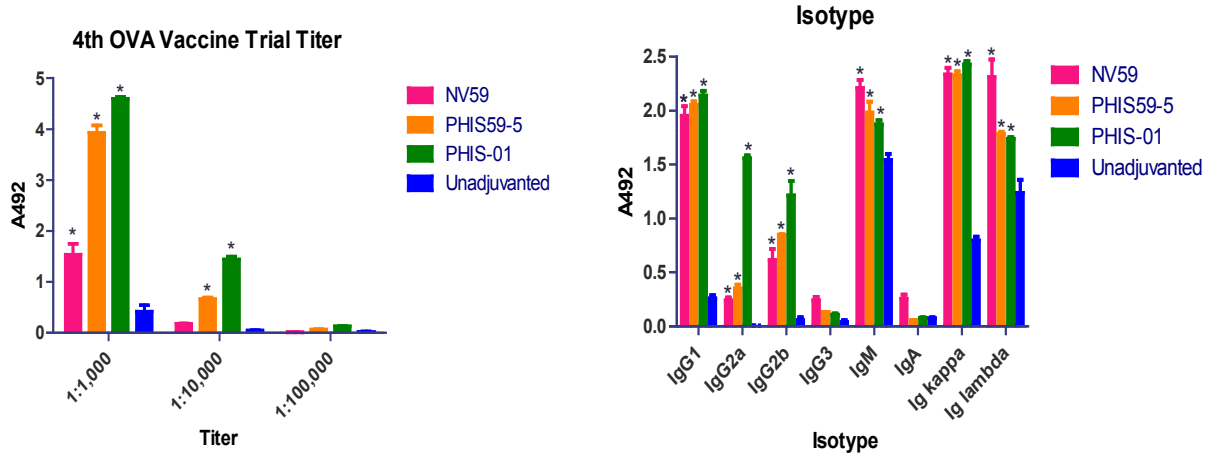

Figure 9. Titers and isotypes of antibodies third (second booster) doses of OVA vaccinations from the fourth OVA vaccine trial.

Antibody Titer and Isotypes in Response to *S. aureus* Vaccines

Titers from both trials of the HKSA-MW2 vaccines showed that PHIS59-5 and PHIS59-2.5 had efficacy over unadjuvanted controls at a hundredfold reduced titer compared to NV59. Similar increases in observed antibody were only observed at a tenfold titer reduction for PHIS59-2.5 compared to PHIS-01. PHIS59-5 displayed the same isotype switching as NV59 for the first MW2 trial. PHIS59-2.5 had significantly more IgG2a production than any other MW2 vaccine formulation. Aside from that, it displayed similar isotype switching to both PHIS-01 and NV59 (Figure 10).

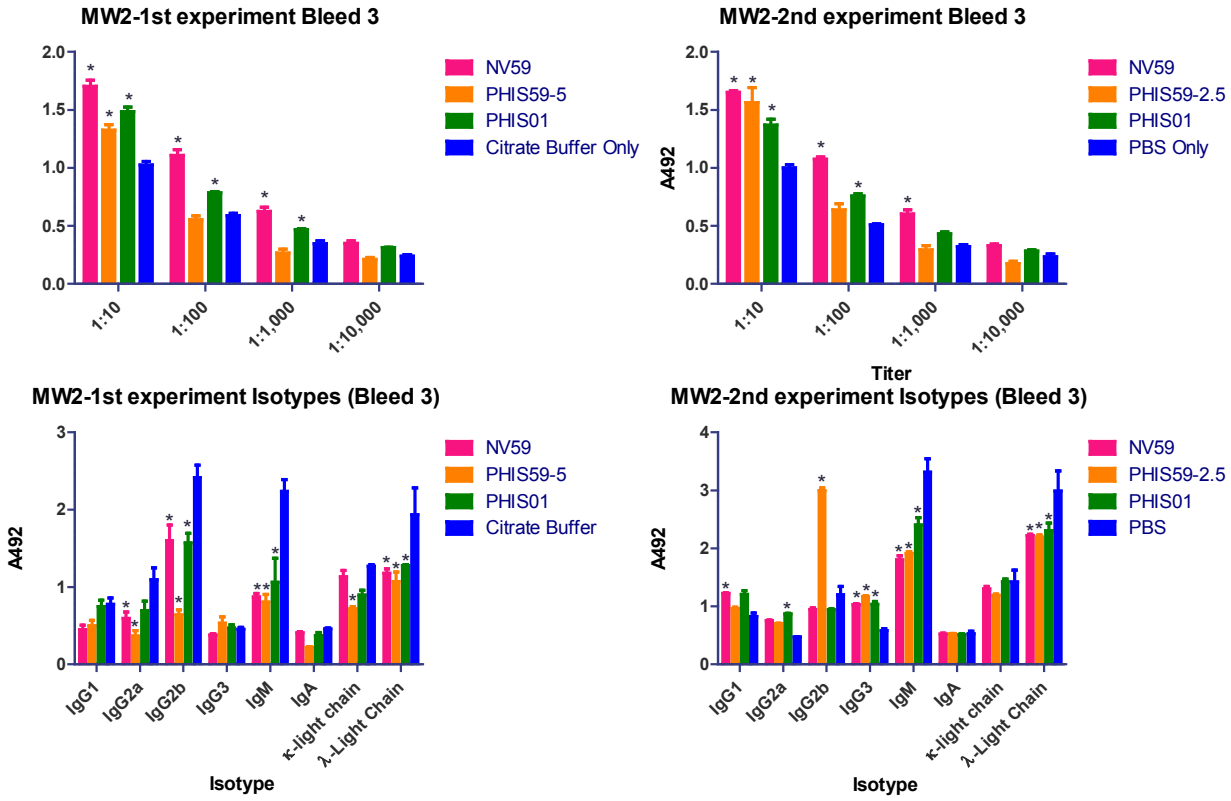

Figure 10. Titers and isotypes of antibodies generated in response to the third (second booster) doses of two separate MW2 vaccinations trials.

Blood was collected after each dose of the HKSA-LS vaccinations to observe changes in titer and isotypes as the vaccination series was administer. In the mice, for the first dose, no adjuvanted vaccine preformed significantly better than the unadjuvanted vaccine (data not shown.) For the second dose, while statistical significance was achieved, the effect size was so small that no real differences were observed between experimental groups. For the third dose, alum had significantly lower titers than any other vaccine group while PHIS-01 and PHIS59-2.5 appeared to have a similar effect. The isotypes from the first two doses did not show a significant interaction (data not shown) although the third showed NV59 had less IgG1, IgM and IgA than the unadjuvanted vaccine while PHIS59-2.5 had less IgM. PHIS-01 had equivalent

levels of IgG1 and IgG2a to the unadjuvanted with lower levels of all other isotypes while Alum had less of every isotype measured.

In the rabbits, no behavioral changes were observed after any vaccination; no adverse effects were observed. PHIS-01 and PHIS59-2.5 displayed similar titers in response to the first two doses which were both significantly elevated over NV59. By the third dose, the titer in response to the NV59 vaccination surpassed that of both PHIS-01 and PHIS59-2.5 (figure 11.)

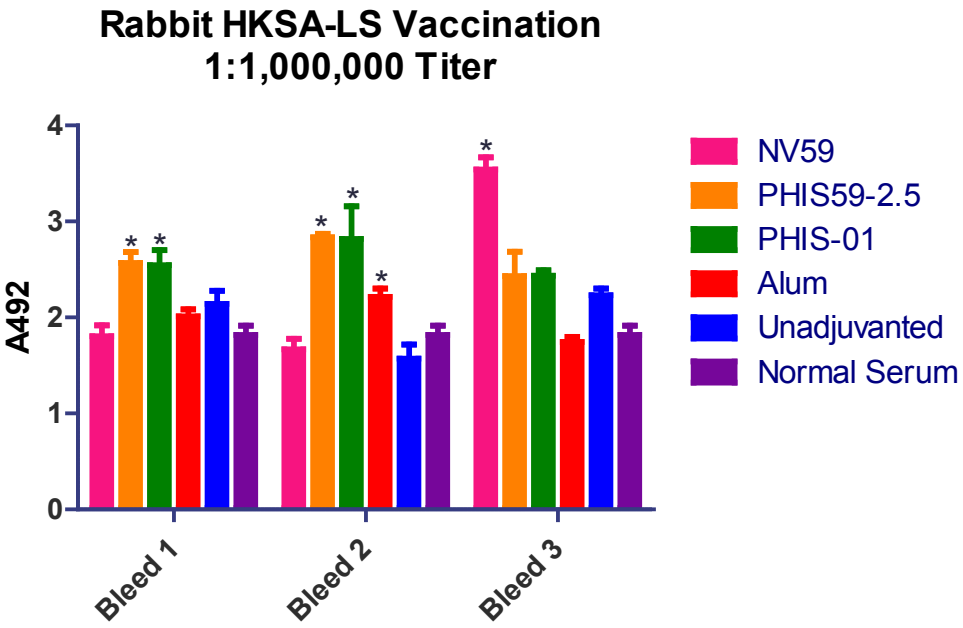

Figure 11. 1:1,000,000 titers of rabbit antibodies raised in response to vaccination with HKSA-LS vaccines. Rabbits received three doses at day 0, day 14 and day 28. Blood was collected on day 7 (bleed 1,) day 21 (bleed 2,) and day 31 (bleed 3.)

CONCLUSIONS

General Tolerability of Phytol-Derived Vaccine Formulations

Light microscopy confirmed that submicron particles were predominant in both the NV59 and PHIS59 vaccine formulations. It has been demonstrated before that the adjuvanticity of commercially available MF59 is due to the oil-in-water micro-emulsion [4] and it follows that since NV59 generally displayed good adjuvanticity in our study, a micro-emulsion similar to the commercial adjuvant may have been produced. This could be confirmed with particle size distribution analysis.[22]

Adjuvanticity in MF59, marked by enhanced antigen uptake and transport to regional lymph nodes is dependent on the micro-emulsion [4] It is possible that this increased uptake of vaccine material results in a higher bioavailability of phytanol from the PHIS59 formulation which may explain why it is toxic at lower doses. Phytanol, unlike squalene, is an adjuvant without significant emulsification.[5,11,15,16] Because of this, the increased bioavailability may lead to greater chemokine and cytokine signaling which in turn could increase the inflammatory reaction to the vaccine. Overall, PHIS59 holds promise as an adjuvant that increases the adjuvanticity of PHIS-01 while decreasing the effective dose. Additional studies should be focused on determining the minimal doses of PHIS59 which can induce a response similar to that of PHIS-01. In the course of these studies, it may be advisable to attempt smaller overall vaccine doses rather than reducing the phytanol content of PHIS59 as to deliver a smaller dose without losing the optimal oil in water emulsion properties. Further testing on the ratio of water to isoterpenoid is needed to determine the most immunogenic emulsion.

Although this study only provided the most basic toxicity data concerning PHIS-SO4, its development as an immunoadjuvant is already underway utilizing this data. Cell culture toxicity data suggested that a safe dose for mice should be in the range of 3-4 mg per dose (unpublished data, Johnson, Reeves and Ghosh) although we found that this was not tolerated in a multiple dose vaccination schedule. Ongoing work is focusing on characterizing the antibody response to sub-milligram doses of PHIS-SO4. Perhaps, the detergent nature of PHIS-SO4 is responsible for the observed toxicity. This could be tested using SDS as a positive control for detergent based toxicity.

By ensuring that trial vaccines meet the good manufacturing processes quality control standards (including sterile production and endotoxin testing,) it is easier to apply the results to future pre-clinical trials.[23] Emulsification of PHIS-01 prior to injection is similar to that used to prepare incomplete Freund's adjuvant (IFA).[8] In this method, the dose is drawn into and rapidly expelled from a small gauge (27 gauge was used here) needle 10 times immediately prior to injection. This has the drawback of creating a non-uniform emulsion that is not stable for any significant amount of time. Additionally, the rubber plunger of the syringe reacts with the oily PHIS-01 increasing the difficulty of injection (unpublished observation,) a phenomenon reported to occur with IFA.[24] While these drawbacks can easily be overcome by preparing the emulsion in a glass syringe immediately prior to injection, this would not be a desirable trait in a commercial vaccine preparation as it adds complexity to the vaccination procedure. PHIS59 can be prepared outside of the animal facility in under a laminar flow hood which reduced the chance of contamination by pathogens or endotoxin. This makes it an easier to deploy adjuvant than PHIS-01.

PHIS59 Adjuvants against OVA

Vaccine trials with OVA demonstrated that PHIS59 has comparable activity to PHIS-01 and NV59 with a reduced isoterpene dose. This is an important step in advancing the phytanol derived immune adjuvants for potential use in clinical trials. Currently, the mouse dose for PHIS-01 is approximately 1600 mg/kg [10,16] which translates to a human equivalent dose (HED) of approximately 128 mg/kg.[25] For a 50 kg person, this would translate to more than a 6 cc injection. With PHIS59-2.5, the HED is reduced to 32 mg/kg and the volume of the injection is also quartered.

PHIS59 Adjuvants for use in *S. aureus* Vaccines

Phytanol as PHIS-01 and its derivative phytanyl-chloride have been used in a similar HKSA-LS vaccine which demonstrated increased adjuvanticity in a vaccine against Staphylococcus aureus subsp. aureus (ATCC® 25923™) [17] *S. aureus* includes a variety of strains, many of them human commensals that are not commonly associated with disease. Drug resistant invasive forms often contain virulence factors and pathogenicity characteristics that distinguish them. Here we have extended the testing of phytanol derivatives to a methicillin resistant *S. aureus* (MRSA), strain MW2. Validation against human pathogens is an important step in developing phytanol-derivatives as adjuvants for MRSA vaccines.

Additionally, we have examined the use of phytol-derived adjuvants for use in HKSA vaccinations in a rabbits, a novel species for these vaccine formulations. Providing cross-species validation is critical in the preclinical development of vaccine candidates.[26] Our preliminary observations seem to show the safety and efficacy of PHIS-01 and PHIS59 in the rabbit HKSA-

LS vaccine as no adverse effects were evident. Furthermore, increased titers were raised with fewer vaccinations using PHIS-01 and PHIS59 compared to both NV59 and Alum.

Oil-In-Water Emulsion Adjuvants

Oil-in-water adjuvants are adjuvants in which liquid oil droplets are dispersed in an aqueous solution. [27-29] Freund's adjuvant, one of the first discovered and most heavily studied adjuvants, is a mineral oil-in-water emulsion.[30] MF59 is oil-in-water adjuvant with squalene, a lipid metabolite as the oil component.[6,31]

Oil-in-water adjuvants have been utilized to increase the efficacy of vaccines since mineral oil emulsions were approved for human use in the early 1950s.[32] Injection site reactions including abscess formation along with systemic inflammation required refinements to the emulsion formulation including the use of isoterpenes which were better tolerated. Several emulsion formulations using the triterpene squalene have been approved for use in humans and have been used extensively over the last three decades. [4,6,31]

Particle size is extremely important for oil-in-water vaccines. This is most easily demonstrated with squalene based oil-in-water emulsion adjuvants. Squalene alone is does not display adjuvanticity; only when it is emulsified in the MF59 formulation can it be used as an adjuvant.[4] MF59, a proprietary adjuvant marketed by Novartis, is a sub-micron emulsion of squalene in citrate buffer. A fixed geometry fluid processor is used to create the emulsion with sorbitan trioleate and polysorbate 80 used as stabilizers.[6] We hypothesized that given the similar structure, a sub-micron emulsion of phytanol could be prepared and that this formulation would lead to an easier to store and administer adjuvant.

Seasonal influenza vaccines have been shown to be better at preventing Influenza when adjuvanted with MF59.[33] MF59 has demonstrated safety for use in humans in multiple

studies [34,35] and is licensed in more than 20 countries.[1] Still, there have been concerns raised about adverse drug reactions [5] including injection site pain, erythema, induration, malaise, myalgia and headache.[2] Adjuvanted influenza vaccines are particularly important in populations that demonstrate a weaker immune response: children, the elderly and immunocompromised individuals. Phytanol is structurally similar to the main component of MF59, squalene, and phytanol-derived adjuvants have been promising in previous vaccine studies.

Commercially prepared oil-in-water emulsions are usually manufactured using a fixed geometry fluid processor. This type of homogenizer that is both expensive and geared more towards commercial scale applications, which led us to seek other means for creating a stable sub-micron emulsion.

Immunoadjuvants in Viral Vaccines

Oil-in-water emulsion adjuvants, aluminum hydroxide (alum) adjuvants and combinations of the two have been used in influenza vaccines. Additionally, both vaccines that provide neutralizing antibody titers as well as those that modulate T-cell associated responses have been effective. It has been hypothesized that phytol-derived adjuvants have the ability to balance humoral and cell-mediated immunity to effect protective immunity.[5] Based on this, we suggest that the inclusion of phytol-derived adjuvants may be especially useful in influenza vaccines.

MF59 has primarily been used to adjuvant influenza vaccines. Phytol-derived adjuvants have not been tested in viral vaccines. However, we theorize that because phytol-derived adjuvants are effective against other antigens and have similar structure to squalene that phytanol will also be effective against influenza A when formulated similarly to MF59. Additionally, MF59 has been associated with adverse drug reactions.[36,37] As phytanol has shown decreased

toxicity compared to trials with phytol, it may show decreased adverse reactions compared to MF59. We have laid here the groundwork for such testing by the establishment of tissue culture production of influenza virus and through testing of PHIS59 with the defined antigen ovalbumin (OVA).

Future Direction

A systematic dose response comparison of PHIS-01, PHIS-59, NV59 and MF59 could help to establish the therapeutic index for these adjuvants. Particle size analysis of micrographs or samples of PHIS59 and NV59 could be used to compare the emulsification efficiency of ultrasonic homogenization to that of a micro-fluidizer used to prepare commercially available MF59. Additionally, antibody titers for smaller doses of PHIS-01 and PHIS59 could be evaluated.

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