CURRENT STATUS OF SYNTHETIC HEMOZOIN ADJUVANT: A PRELIMINARY SAFETY EVALUATION

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ABSTRACT

Although adjuvants are a “must-have” component of successful vaccines, there are very few adjuvants licensed for use in humans, there is therefore an urgent need to develop new and safer adjuvants. Synthetic hemozoin (sHZ), a chemical analog of hemozoin which is produced by the malaria parasite, exhibits a potent adjuvant effect which enhances antigen-specific immune responses to vaccines. The potency of sHZ adjuvant activity is not limited to malaria specific vaccines, it has also been demonstrated to be effective in influenza and dog allergy models. While the synthesis of uniformly sized sHZ with consistent characteristics has proven difficult, we have recently successfully **optimized the manufacture of sHZ product with** an optimal adjuvant effect. Here, we summarize recent developments on the adjuvant properties of **optimized** sHZ adjuvant, including its **good laboratory practice (GLP) non-clinical** safety profile in animals. These studies ensure the safety of **optimized sHZ product** to be readily used as vaccine adjuvant beforehand in veterinary medicine.

Keywords: Synthetic hemozoin, Adjuvant, Safety, **Non-clinical toxicity**

Introduction

It is now widely accepted that, if rationally designed, adjuvants improve vaccine formulations for better and controllable immune responses [1,2]. Despite of this fact, however, aluminum hydroxide, oil-in-water emulsions MF59 and ASO3, and alum with monophosphoryl lipid A (ASO4) are still the very few adjuvants that have been licensed for humans, suggesting that there is a great need to increase the numbers of “rational adjuvants” [3,4]. On the other side, although alum has been considered as a safe adjuvant in humans, the local reactions and the increase of IgE antibody responses have been observed [5–7]. Therefore, greater efforts are needed to further understand the mode of action of adjuvants in order to overcome the limitations of adjuvants, in terms of safety issues and/or choosing the suitable adjuvant depending on the desirable vaccination outcome. In the current review, we focus on the production and vaccine adjuvant properties of a new adjuvant, synthetic hemozoin, a synthetic analog of *Plasmodium*-produced hemozoin, and its preliminary GLP non-clinical safety and toxicology studies in several animals and infection models.

Hemozoin

Hemozoin (HZ) is produced during the erythrocytic life cycle of *Plasmodium* parasites as a by-product of their hemoglobin metabolism. While hemoglobin catabolism is required for protein and energy resource of parasites, catabolized free heme, which is toxic to parasites, is immediately detoxified into hemozoin crystals at the inner surface of the parasite digestive vacuole in an oriented manner [8–10]. The crystallization procedure occurs initially by the binding of free monomeric heme molecules to each other via iron-carboxylate bonds that form reciprocal cyclic dimers. Then, these dimers establish a stable, triclinic structure via hydrogen

66 bonds which results in insoluble ~500 nm sized brick-shaped crystals [11]. The presence of
67 hemozoin crystals is readily noticed as dark-brown pigment in the red blood cells of malaria
68 infected patients/animals and in the midgut of malaria transmitting mosquitoes [12].

69 Malarial hemozoin exhibits various properties that have been a matter of interest in the
70 biology, chemistry and immunology fields. For example, the detoxification process of heme by
71 parasites has been utilized as antimalarial drug target [13,14]. The well-known anti-malarial drug
72 chloroquine acts by binding to free heme and preventing hemozoin formation; a similar
73 mechanism has been proposed for the current frontline antimalarial, artemisinin [15,16].
74 Although hemozoin formation has been considered essential for most *Plasmodium* parasites,
75 recent studies have implied additional amino acid resources for *Plasmodium* parasites other than
76 hemoglobin, suggesting multiple facets of hemozoin biology for *Plasmodium* parasites [17]. Of
77 note, in the absence/or reduced conditions of hemozoin formation in *Plasmodium* parasites, the
78 susceptibility to chloroquine is altered, whereas no alterations have been observed for
79 artemisinin.

80 The unique optical birefringent properties of hemozoin have been exploited as an early
81 diagnostic marker for malaria via several novel detection modalities [18–23]. These new
82 approaches and techniques may help to develop early diagnosis and treatment of malaria.

83 Hemozoin that is released into the blood stream is immediately phagocytosed by immune
84 cells, causing immune system activation. Several reports, from ourselves and others, have
85 suggested receptor mediated and/or non-mediated immune activation mechanisms by
86 *Plasmodium falciparum* hemozoin [24–27]. However, the details of the interaction of hemozoin

crystals with immune cells is still a matter of debate, with several detailed review articles addressing this subject [28–30].

Synthetic hemozoin production

Synthetic hemozoin (sHZ, also called β -hematin, Figure 1A) is a synthetic analog of *Plasmodium*-produced hemozoin (HZ, or natural hemozoin, Figure 1B). It is produced from hemin chloride by various acid-catalyzed methods and contains water insoluble black crystals with a molecular formula of $C_{68}H_{62}Fe_2N_8O_8$. It has been shown that HZ and sHZ are chemically and spectroscopically identical [11,18,31]. Recent studies also suggest that both structures share paramagnetic properties due to the transformation of the redox state of iron (from Fe^{2+} diamagnetic state to Fe^{3+} paramagnetic state) [32–34]. However, despite such similarities, differences between natural and synthetic hemozoin are evident (i.e. size and shape, Figure 1A and B). Importantly, it is relatively simple to produce sHZ crystals, however, different synthesis protocols (e.g. using different solvents and temperatures) produce diverse sizes of sHZ crystals, and very few protocols can produce sHZ crystals which are similar to *Plasmodium*-produced natural hemozoin crystals [28,35]. This is of concern, as it is reasonable to propose that immune cells may respond differently to different sizes of sHZ crystals [30,35]. Indeed, we previously demonstrated that very big crystals ($> 4 \mu m$) could not induce an adjuvant effect when with antigen immunization, as compared to sHZ crystals of around 200 nm (ranging from 50 nm to 1 μm) which had a potent vaccine adjuvant effect [31]. Synthetic hemozoin crystals are becoming widely used for immunological and vaccine studies, therefore, it has been crucially important to produce the optimal size of sHZ crystals, especially for adjuvant studies. However, our attempts to produce crystals around 200 nm faced reproducibility and scale-up problems (unpublished

observation). Nevertheless, years of evaluation with Zenoaq (a veterinary company certified with GLP production and evaluation facility, Fukushima, Japan) have led us to successfully produce optimized sHZ crystals (patents applied and/or pending, WO2011074711 and WO2014196657 A1).

Manufacturing process of optimized synthetic hemozin.

The controlled optimization strategies for sHZ production has been carried out at Zenoaq, a GLP/GMP certified laboratory. A GLP/GMP laboratory provides laboratory results with a proper guidance of planning, performing, recording and reporting to produce product in high purity, sterility and reproducibility. With this guidance, we have developed a method to increase the yield of sHZ crystals by incorporating an additional heating step (patent applied WO2014196657 A1). The production of optimized sHZ is carried out, first, by the usual acid-catalyzed step via dissolving hemin-chloride in sodium hydroxide (NaOH) solution. After brief addition of aqueous HCl to reduce the pH, acetic acid is added dropwise until pH is stabilized around pH 4.8. Subsequently, crystallization reaction takes place when temperature is raised up to around 80°C for 1-3 hrs. The crystals are subjected to subsequent washings, one time with disodium phosphate (Na_2HPO_4 , pH 9) solution, followed by 3-4 times with pure water. Finally, crystals in water are autoclaved at 120 °C for 20 min. and stored at 4 °C. The final product has been evaluated for its quantity, particle size, stability, purity and chemical composition by various assays and has been issued a certificate of analysis in a lab compliance with GLP. This new protocol has yielded homogenously distributed, non-aggregated, sterile and approximately 1 μm size sHZ crystals namely optimized sHZ, a “GLP grade” product (Figure 1A and C shows SEM and laser particle size analysis of optimized sHZ crystals). Notably, while this optimized sHZ is

131 mostly 1 μm with sharp edges, *P. falciparum*-produced hemozoin crystals are mostly brick-
132 shaped and rarely reach 1 μm .

133 **Optimized synthetic hemozoin is a potent vaccine adjuvant**

134 Synthetic hemozoin has been shown to exhibit a potent adjuvant effect in several animal
135 models with different antigens [31,36–38] (Table 1 summarizes up-to-date studies in various
136 animals, disease models and with various antigens, **modified from [39]**). Mouse studies using
137 different model antigens together with sHZ have suggested that sHZ may act as a type-2
138 adjuvant by leading mainly an increase in antigen-specific IgG1 antibody and spleen cell
139 initiated IL-5 and IL-13 cytokine responses (with no detectable IFN- γ or IL-17 production) [31],
140 although this seems to be dependent on the antigen (Table 1). For example, when inactivated
141 bacterial (*E.coli*) and viral (PRRSV) antigens were immunized with sHZ, the antigen-specific
142 antibody responses were mainly IgG2a isotype (**Table 1**, Zenoaq). This may be explained by
143 having another “built-in-adjuvant” in the inactivated microbial antigens which may interact with
144 a second adjuvant like sHZ.

145 The anti-allergen vaccine studies using a canine allergy model, on the other hand, additionally
146 suggested that not only the nature of antigen, but the host itself could be a determining factor for
147 the immune response [28,31]. Beagle dogs develop atopic dermatitis similar to that seen in
148 humans, which is associated with the production of high levels of IgE antibodies against the
149 house dust mite allergen, Derf2. Immunization of dogs with Derf2, together with alum and/or
150 sHZ, and subsequent sensitization by the allergen showed significantly elevated levels of IgG2,
151 but not IgG1, antibodies in the sHZ-treated group, resembling a Th1-like immune response [28].
152 Moreover, Derf2-specific IgE responses were remarkably reduced in the sHZ co-administered

dogs. Of note, the same antigen and adjuvant administration resulted in mainly IgG1 responses in mice (Table 1, unpublished observation, Zenoaq), suggesting that more research is needed to understand the contribution of antigen and host factors to adjuvants and vaccines.

Synthetic hemozoin was evaluated in non-human primate *cynomolgus monkey* model in comparison with other TLR9 ligand adjuvants, K3 CpG oligodeoxyribonucleotides (K3 CpG ODN) and D35 CpG ODN [40], in a formulation with SE36/AHG antigen (a candidate blood-stage malarial antigen produced mainly in late trophozoites and schizont stages [41]) for safety, immunogenicity and protective efficacy [36]. Synthetic hemozoin was an effective adjuvant somewhere between K3 ODN and D35 ODN adjuvants for the SE36/AHG vaccine, significantly elevating serum antigen-specific IgG levels in immunized monkeys and IFN- γ , IL-5, IL-13 and IL-17 responses from PBMCs. However, sustainability of the antibody titers was not as effective as in the K3 ODN administered group. In addition, due to limitations in performing real *Plasmodium* infections, immunized *cynomolgus* monkeys were administered with crude extract of *P. falciparum* to mimic the exposure to malaria infection, in order to determine if SE36-specific IgG can be boosted by natural antigen exposure. SE36-specific IgG titers were significantly and equally enhanced by crude extract administration in all groups, suggesting that this vaccine formulations including with sHZ adjuvant may form the basis of a safer and more effective malaria vaccine.

Synthetic hemozoin adjuvant was recently evaluated in influenza vaccination models in mice and ferrets. In mice, sHZ administration with inactivated whole influenza viruses (A/California/04/2009 (H1N1pdm09) or A/Vietnam/1203/2004 (H5N1)) elicited higher virus-specific antibody responses and provided better protection from lethal challenge, compared to

non-adjuvanted counterpart animals [38]. The ferret model is the most accurate animal model to test influenza vaccination efficacy. This model is also important in evaluating pyrogenicity of adjuvants which closely resembles vaccine-related fever in humans. Immunization of ferrets with HA split vaccine (SV) and sHZ was performed in comparison to Flud vaccine (a composition of influenza virus HA surface antigens from the three influenza strains and MF59 adjuvant). Synthetic HZ enhanced the immunogenicity of HA SV in ferrets, and was as effective as the Flud vaccine. Efficacy was dose-dependent against three HA antigens of A/California/7/2009 (H1N1), A/Victoria/210/2009 (H3N2), and B/Brisbane/60/2008, and there were no fever reactions [37]. Furthermore, the highest dose of sHZ adjuvant conferred sterile protection against infection with heterologous influenza virus without causing fever. Of note, MF59, an oil-in-water emulsion adjuvant included in the Flud vaccine, is the first adjuvant licensed in humans since the introduction of aluminum as an adjuvant [3]. Therefore, the equal potency and safety of sHZ adjuvant as compared to MF59 in ferret models suggests that sHZ could be used as a novel influenza vaccine adjuvant in humans.

Mode of action of synthetic hemozoin adjuvant

It is now widely accepted that vaccines and adjuvants are sensed by the innate immune system, which in turn regulates the magnitude, quality and persistence of adaptive immunity [42,43,7]. Several studies have suggested that hemozoin crystals, in either natural or synthetic form, have immunomodulatory properties effecting various immune cells, both in vitro and in vivo. They have been demonstrated to interact with various immune system molecules including TLR9 and NLRP3 [24–27,31,44]. Therefore, we've further investigated whether these innate sensors would affect sHZ-mediated adaptive immune responses. Increased antigen-specific

antibodies with sHZ adjuvant were significantly abrogated in MyD88-deficient mice, while TLR9- and ASC- or NLRP3-deficient mice showed comparable antibody titers [31]. The MyD88 adapter controls not only TLRs but also IL1/IL18-signaling, however, results from the immunization of IL1R-deficient mice suggest that IL1R-signaling is not involved in the adjuvanticity of sHZ [31]. Altogether, these studies suggest that distinct mechanisms exist for the adjuvant mode of sHZ, which culminate in MyD88-dependent signals, but that do not involve TLR9 or the inflammasome. Currently, the involvement of MyD88 in the mode of action of sHZ adjuvant is being intensely investigated in our laboratory.

GLP non-clinical safety, tolerability and toxicity profile of optimized synthetic hemozoin adjuvant

Vaccine development requires various pre-clinical testing before clinical safety evaluation. It is particularly important to evaluate potential adverse effects of adjuvants/vaccines by performing pre-clinical toxicology testing in animals [4]. Although sHZ adjuvant alone or in combination with various antigens has been safely administered to several strains of mice, ferrets, dogs and non-human primates for immunogenicity and efficacy testing, standard GLP non-clinical toxicology studies were recently completed in rats especially for the evaluation of single and repeated dose administration (Table 2). The GLP toxicology studies in rats (study design shown in Figure 1D) were performed three times, including the pre-evaluation and the evaluation of sub-acute repeated administration of various doses of sHZ adjuvant alone: i) repeated subcutaneous (s.c.) administration 3 times at doses of 8, 80, and 800 mg/kg/rat, ii) repeated s.c. administration 5 times at doses of 8, 80 and 800 mg/kg/rat, and iii) acute administration of high single dose (one time adjuvant alone at a dose of 910 mg/kg/rat) according to recommendation

by the Organization for Economic Co-operation and Development (OECD) principles of GLP. This complies with the requirement of the Ministry of Agriculture, Forestry and Fisheries Ordinance (No. 74, Series of 1997) which regulates GLP non-clinical studies on the safety of veterinary medicinal products in Japan [45]. Animal experiment guidelines are also approved by Japan Association for Laboratory Animal Science.

Throughout both acute (single dose) and sub-acute (repeated doses) studies in rats, even the repeated dose course of sHZ was well-tolerated and no death or behavioral abnormalities were observed as a sign of systemic toxicity (Table 3). Thickening of the treated site occurred following each injection of high dose sHZ, but subsided in less than a week. Pigmentation could be observed in subcutaneous tissue of the sites of injection as well as in lymph nodes, spleen, liver and lung after multiple administration of high dose of sHZ. Generally low doses of sHZ did not show any abnormalities in the hematological and biochemical blood test and tissue histology. Only after multiple administrations of high dose sHZ at 800 mg/kg, an increase in monocyte, neutrophil and platelet numbers in the blood, and slight decrease of albumin which could be associated with acute inflammation in the liver, was observed in rats. However, these effects were transient and disappeared 3 weeks after the last administration of sHZ.

Antibodies against hemozoin and/or heme or self-DNA

Given that adjuvants are designed to induce more robust immune responses to antigens in order to protect against real pathogens, safety concerns especially in the context of inducing autoimmune disorders have been raised [46,47]. Autoimmunity has been defined as the inappropriate self-recognition by one's own-immune system at the organ or systemic level which would lead to the production of autoantibodies which destroy self. Whether immune system

activation by adjuvants would induce reactivity of immune responses to self (i.e. injured tissue, released host DNA etc.), has been actively investigated in animal models. Therefore, concerning the possibility of inducing autoimmune responses, it has been crucial to address whether multiple injections of sHZ could raise antibodies against host DNA or the adjuvant itself. To address this, long term multiple intravenous (i.v.) administrations of sHZ were performed in mice: i) repeated i.v. administration every 5 days for total 6 times at a dose of 16 mg/kg/mouse, ii) repeated i.v. administration every 5 days for total 12 times at a dose of 16 mg/kg/mouse, and iii) repeated i.v. administration every 5 days for total 18 times at a dose of 40 mg/kg/mouse (Figure 1E). Local tolerability and potential adverse effects were assessed and found to be tolerable and safe. Neither IgM nor IgG against host DNA were detected following administration of sHZ, whereas natural *Plasmodium* infection (*P. yoelii* NL blood stage infection) caused substantial amount of anti-host DNA IgG and IgM responses (Figure 2A). Production of antibodies against sHZ or hemin, which is the subunit of hemozoin, were also not induced with any sHZ dose course, with only a subtle increase in the 18 times high dose injection group (Figure 2B). Importantly, hemin is a self-component of host, therefore, these results indicate that sHZ does not induce autoimmunity against this antigen. Of note, due to difficulties in coating plates with sHZ crystals for ELISAs, soluble hemin was used as a proxy for sHZ, as previously reported [31].

Conclusions

The importance of the development of better and safer adjuvants for human use is indisputable, however, it is difficult and laborious for new adjuvants to gain approval as a final product. In particular, controlling nanocrystal size and preventing crystal aggregation have been the major obstacles in delivering GMP grade nanoparticle adjuvants, including sHZ. We have

conducted a substantial amount of research into the optimization of “GLP grade” sHZ production, and have successfully produced a non-aggregated, sterile product. In addition, we completed first step GLP non-clinical tolerability and toxicology studies for sHZ, and performed several animal studies to assess its adjuvant ability and potency, including infection challenges. In conclusion, sHZ displays very low autoimmune properties, and is a potent adjuvant in many animal vaccines, including dogs and non-human primates. Our understanding of the mode of action of sHZ, as an adjuvant inducing adaptive immune responses to antigen via MyD88-dependent signaling pathways, also helps to evaluate its safety profile in vaccine formulations. Given that co-development of a vaccine for both veterinary and human health would be the optimal strategy [48], current status of optimized sHZ product shows that it is a very safe and potent vaccine adjuvant in animals including dogs and monkeys. Therefore, sHZ is readily attractive for use in animal vaccines as a cheap and easily produced vaccine adjuvant.

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Conflict of interest

Y. Igari and T. Tsukui are employees of Zenoaq, Koriyama, Fukushima, Japan. Y.I., T.T., C.C. and K.J.I applied patents for the usage of synthetic hemozoin as adjuvant.

FIGURE LEGENDS:

Figure 1. Optimized production of synthetic hemozoin (sHZ) in a Good Laboratory Practice (GLP) certified laboratory. A. An optimized “GLP grade” sHZ, B. *P. falciparum*-produced natural hemozoin by scanning electron microscope (SEM, by Hitachi 4800S). Scale bar 1 μ m. B is reproduced from Coban et al. Allergology International, 2010 [28]. C. Particle size distribution of sHZ by laser particle size analyzer (LPSA, LA-950, Horiba). Data in A and C are representative of similar results from at least 6 different optimized sHZ lots. D. Experimental design of GLP safety, tolerability and toxicology studies in rats. E. Non-GLP multiple administration of optimized sHZ into mice for the evaluation of autoimmunity. All animal experiments were performed under the appropriate laws and guidelines of Japan.

Figure 2. Low levels of anti-DNA and anti-hemin antibodies after long time intravenous synthetic hemozoin exposure. Antibody responses to host DNA and hemin/hemozoin after multiple injections of sHZ in mice. The littermate female BALB/c mice were administered sHZ every 5 days for 6, 12 or 18 times via intravenous (i.v.) route. Mice were sacrificed after the last administration and sera were collected and subjected to serum ELISA. As control, naïve serum and *Plasmodium yoelii* NL-infected mice sera on day 30 after infection was used. Serum was

304 diluted 1/100. Data are depicted as mean $\pm$ SEM. To compare groups, one-way ANOVA analysis,
305 followed by Bonferroni's comparison test, was performed, ** p<0.01, *** p<0.001.

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Table 1. Up-to-date adjuvant properties of synthetic hemozoin (sHZ), extended from [39].

	IgG antibody (isotypes)				
	Mouse		Dog	Ferret	Cynomol. Monkey
	BALB/c	C57BL/6			
Model Antigens					
OVA	IgG1 IL5, IL13 ^[31]	IgG1>IgG2b IL5, IL13 ^[31]	-	-	-
HSA	IgG1>IgG2a≥IgG2b IL5, IL13 ^[31]	IgG1>IgG2b>IgG2c IL5, IL13 ^[31]	-	-	-
Infection antigens					
Inactivated <i>E. coli</i>	IgG2a	-	-	-	-
Inactivated <i>S. aureus</i> (Mucosal)	IgA in BALF IgG1 in serum	-	-	-	-
Inactivated PRRSV	IgG2a	-	-	-	-
Malarial antigens (SE36)	IgG1>IgG2a*	-	-	-	IgG IFN γ , IL5, IL13, IL17 ^[36]
Inactivated whole virion influenza	High HI titers, better protection ^[38]	-	-	-	-
Seasonal influenza SV HA antigen	-	-	-	High HI titers, sterile protection ^[37]	-
Allergen antigens					
Derf2	IgG1≥IgG2a	IgG1	High IgG2, Low IgG1, Low IgE ^[31]	-	-

Abbreviations: OVA; Ovalbumin, HSA; Human serum albumin, PRRSV; Porcine Reproductive and Respiratory Syndrome Virus, SE36; Antigen derived from serine repeat antigen 5 (SERA5) of *Plasmodium falciparum*, Derf2; *Dermatophagoides farinae* house-dust mite allergen, SV; split vaccine, HI titer; Hemagglutination inhibition antibody titers, Dog; Beagle dog, Cynomol. Monkey; Cynomolgus monkey (*Macaca fascicularis*), “-”; not applicable. *Prof. T. Horii and Dr. T. Tougan, personal communication.

Table 2. Procedures followed for Good Laboratory Practice (GLP) non-clinical safety tests for synthetic hemozoin adjuvant in rats.

GLP procedure	
Planning	√
Personnel and organization	√
Facilities and equipment (performance by an independent certified contract laboratory)	√
Standard operating procedures (SOPs)	√
Animal care	√
Toxicity (Single and repeated doses)*	√
Safety parameters	√
Analytical methods	√
Recording	√
Archiving	√
Reporting	√

*Details of experiments are given in Figure 1D.

Table 3. Preliminary GLP non-clinical toxicology test in rats for sHZ. Details of experiments are given in Figure 1D.

	Single dose	Repeated doses (5 times)
Mortality	None	None
Clinical signs	Temporary thickening of treated site	Temporary thickening of treated site with high dose
Food consumption	Normal	Normal
Body weight	No change	No change
Temperature	No change	No change
Clinical chemistry	None	Few temporary changes with high dose
Hematology	None	Few temporary changes with high dose
Gross pathology	None	None
Histopathology	None	Slight pigmentation in few organs with high doses

Figure 1

Figure 2

A

B