Research Thesis

Designing distribution of adjuvants: synthesis of lipidated nucleic acid adjuvant compounds

by

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Abstract

Peptide vaccines can generate antigen-specific immune responses against tumours. However, peptides on their own are not usually immunogenic and require an adjuvant to ensure antigen-presenting cells are appropriately activated. Adjuvant localisation is essential for its activity; targeting an immunomodulatory compound to the lymph nodes appropriately positions it among a high density of immune cells, where immune responses are coordinated. Furthermore, systemic distribution of a potent immune modulator can lead to severe systemic toxicities. Lymph node targeting reduces systemic exposure with simultaneous reduction of side effects. Where a compound distributes *in vivo* is determined by its pharmacokinetic properties and its route of administration. Once the route has been defined, a drug's pharmacokinetic properties can be modified by structural changes. To this end, we modified existing adjuvants to distribute into the lymphatics preferentially. One method was to increase the hydrophilicity and size of α -galactosylceramide to favour lymphatic uptake. The second was to exploit albumin hitchhiking to access the lymph nodes. Here, α -galactosylceramide was chemically linked via an enzyme-labile linker to CpG ODN 1826, a TLR-9 agonist. The properties of each adjuvant mutually alter those of the other: to the CpG, α -galactosylceramide acts as an albumin binding domain; to the α -galactosylceramide, the CpG serves as a large hydrophilic group creating an amphiphile. *In vivo*, this should activate a strong, multilineage T cell response through the synergy of the two adjuvants. Furthermore, this should reduce the toxicity and side effects of the adjuvant by limiting its systemic distribution. This adjuvant may find further use in vaccines for diseases requiring a Th1 response for effective clearance.

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Declaration and Copyright

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The work in this thesis was completed by the author at Victoria University of Wellington, New Zealand. It has not been submitted, in part or whole, for any higher degree at this or any other University.

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Table of acronyms and abbreviations

°C	degrees Celsius
5-A-RU	5-amino-6-(D-ribitylamino)uracil
Ac	acetyl
APC	antigen-presenting cell
Ar	aryl
atm	atmosphere(s)
BCN	bicyclononane
Bn	benzyl
Boc	<i>tert</i> -butoxycarbonyl
bp	boiling point, base pair
br	broad (spectral)
Bu, <i>n</i> -Bu	primary butyl
CAD	charged aerosol detector
calcd	calculated
cat	catalytic
CD	cluster of differentiation
CFA	complete Freund's adjuvant
cm	centimeter(s)
COSY	correlation spectroscopy
CpG	cytosine-phosphate-guanosine
CTL	cytotoxic lymphocyte
d	doublet (spectral)
DC	dendritic cell
DCM	dichloromethane
delta	chemical shifts in parts per million downfield from tetramethylsilane

DEPT	distortionless enhancement by polarization transfer
DMAP	4-(<i>N,N</i> -dimethylamino)pyridine
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
equiv	equivalent
ESI	electrospray ionization
Et	ethyl
Fmoc	9-fluorenylmethoxycarbonyl
FT	Fourier transfer
g	gram(s)
GTP	guanosine 5'-triphosphate
h	hour(s)
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
HMBC	heteronuclear multiple bond correlation
HPLC	high-performance liquid chromatography
HRMS	high-resolution mass spectrometry
HSA	human serum albumin
HSQC	heteronuclear single quantum correlation
Hz	hertz
IFA	incomplete Freund's adjuvant
IFN	interferon
IL	interleukin
iNKT cell	invariant natural killer T cell
<i>i</i> Pr	isopropyl
<i>J</i>	coupling constant (in NMR spectrometry)
k	kilo
L	liter(s)

lit.	literature value
Log P	logarithm of partition coefficient
m	multiplet (spectral); meter(s); milli
M	molar (moles per liter); mega
<i>m/z</i>	mass-to-charge ratio
M+	parent molecular ion
MAIT	mucosal-associated invariant T cell
Me	methyl
MHC	major histocompatibility complex
MHz	megahertz
min	minute(s)
mM	millimolar (millimoles per liter)
mol	mole(s)
mp	melting point
MS	mass spectrometry
MW	molecular weight
NHS	N-hydroxysuccinimide
nm	nanometer(s)
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
Nu	nucleophile
ODN	oligodeoxynucleotide
PABCV	para-aminobenzoyl-citrulline-valine
PE	petroleum ether
PEG	polyethylene glycol
Ph	phenyl
ppm	part(s) per million

Pr	propyl
q	quartet (spectral)
rel	relative
<i>R_f</i>	retention factor (in chromatography)
rt	room temperature
s	singlet (spectral); second(s)
s-Bu	sec-butyl
SPDP	succinimidyl 3-(2-pyridyldithio)propionate
SPPS	solid-phase peptide synthesis
STD	saturation transfer difference, NMR method
t	triplet (spectral)
<i>t</i> -Bu	<i>tert</i> -butyl
TFA	trifluoroacetic acid
TFP	2,3,5,6-tetrafluorophenyl ester
THF	tetrahydrofuran
TLC	thin-layer chromatography
TLR	Toll-like receptor
TNF	tumour-necrosis factor
TOF	time-of-flight
<i>t_R</i>	retention time (in chromatography)
UV	ultraviolet
v/v	volume per unit volume (volume-to-volume ration)
vis	visible
vol	volume
w/w	weight per unit weight (weight-to-weight ratio)
α-GalCer	α-galactosylceramide
μ	micro

1 Introduction

Cancer is a major health problem in New Zealand. It is the most common cause of death accounting for nearly a third of mortality. Between 2004 and 2013 the number of cancer deaths increased 14.5% and will continue to grow as the population ages. Meanwhile, rates of cancer and death from cancer are consistently higher among Māori than non-Māori¹.

Cancer is typically treated in three ways: with surgery, chemotherapy, or radiation therapy. These methods can be highly toxic and invasive, killing both healthy and malignant tissue, and are often not successful in treating advanced or metastasized tumours^{2,3}. Exploiting the immune system to kill malignant cells is an attractive prospect for cancer therapy. The goal of immunotherapy is to retrain the immune system to recognise cancerous tissue as dangerous. In this way, components of the immune system will specifically target malignant cells with minimal impact on healthy tissue. Immune cells have access to almost all areas of the body and can attack tumours that may be unreachable by surgery. Furthermore, immunotherapy has the potential to target the less rapidly proliferating cancer stem cells that aren't targeted by current chemotherapeutics. Finally, it can develop a memory phenotype which can prevent the recurrence of cancer⁴.

1.1 Therapeutic cancer vaccines.

Three signals are required to generate an antigen-specific cytotoxic T cell response. Firstly, the target antigen presented in the context of antigen-presenting molecules, such as major histocompatibility complex (MHC) class I and II. Secondly, the T cell must be correctly activated through costimulation by interactions such as CD80/86:CD28. Thirdly,

soluble factors guide the maturation and polarisation of T cells. These signals can all be supplied by appropriately activated dendritic cells⁵.

Dendritic cells (DCs) are a specialised type of immune cell that is especially good at antigen presentation. Among antigen-presenting cells (APCs), DCs are considered to be the most potent and are often referred to as professional antigen-presenting cells. They are distributed widely throughout the body, primarily at the external barriers of the body particularly the skin and mucosa in an immature form⁶. DCs continuously sample their environment for antigen. Upon encountering antigen, their phenotype will change to reduce their ability to pick up antigen and to favour migration to the draining lymph node and ability to activate T cells. Upregulation of molecules like CCR7 allows for homing to the draining lymph node, where they present processed antigen to naive T cells⁶. This process requires the presence of both antigen and a danger signal to appropriately activate the DC for presentation.

Therapeutic cancer vaccines harness the power of DCs as professional APCs to induce a T cell response to tumour cells against tumour specific epitopes. They expand the population of T cells specific for this tumour epitope, and may also reactivate dormant or anergic cells. Both antigen and adjuvant are needed to stimulate APCs for optimal T cell activation⁷. The antigen provides T cell specificity while the adjuvant is necessary for generating an adequate innate immune response which in turn drives an adaptive - both humoral and cellular - response. The antigen alone usually does not carry a danger signal for appropriate activation. While many peptide antigens have been identified for use in therapeutic cancer vaccines, the adjuvants to improve immunogenicity are lacking. A successful therapeutic cancer vaccine needs to “achieve sufficient antigen concentration in DCs...utilize an effective route of administration...[and] activate DCs with adjuvants”⁸.

1.2 A type 1 response is key to tumour regression.

The third signal provides the polarisation of the immune response. Secreted factors determine the types of effector T cells that are formed and shape the response generated.

The polarisation of the immune response is critical for proper control of a disease. For example, only a type 1 immune response can effectively clear Leishmaniasis⁹.

Secretion of IFN- γ and IL-12 generates a Th1 phenotype. Activated Th1 cells are necessary for the killing of intracellular pathogens. They secrete effector molecules such as IFN- γ to activate macrophages, and IL-2 to promote T cell proliferation. Th2 cells are favoured by the release of IL-4 and subsequently release IL-4 and IL-5. They are involved in the immune response to parasites and allergic responses⁹.

For tumour eradication, several studies have demonstrated the need for a type 1 response for effective clearance of the tumour^{10,11}. IL-12 producing DCs stimulate Th1 CD4 T cell responses, secreting IFN- γ . This cytokine helps to expand the population of CD8 cytotoxic T lymphocytes which kill cancer cells through perforin and granzyme. DCs producing IL-6 and TNF- α promote Th2 CD4 differentiation. These cytokines stimulate the production of IL-4 and IL-13 which does not result in effective tumour rejection¹². Toll-like receptor (TLR) signalling generates IL-12 producing DCs. For example, a *Toxoplasma gondii* infection in mice typically generates a strong Th1 response. In mice lacking adapter protein MyD88, a key component of the TLR signalling pathway, cannot generate this response and the infection cannot be cleared¹³.

A successful therapeutic vaccine is possible if designed correctly to achieve a powerful mixed T cell response. Kenter et al¹⁴ present a good example: a synthetic long peptide vaccine adjuvanted with Montanide ISA-51, a potent stimulator of Th1 immunity. They reported a complete regression of grade 3 vulvar intraepithelial neoplasia in 47% of cases, and the effect correlates with induction of antigen-specific immunity. However, the failure of many therapeutic cancer vaccines is primarily due to the inability to generate CD4 and CD8 responses of sufficient size and avidity against the antigen. Furthermore, the common use of adjuvants which skew toward Th2 or humoral immunity does not lead to effective tumour elimination¹⁵.

1.3 Current adjuvants for cancer vaccines are lacking

Figure 1-1 Main adjuvant types and their mechanism of action. ISCOMs, immunostimulating complexes; O/W, oil-in-water emulsion; PRR, pattern-recognition receptor; TLR, Toll-like receptor; W/O, water-in-oil emulsion. Reproduced from Guy, 2007¹⁶, with permission.

Broadly, adjuvants augment the immune response to a vaccine – defined as “any substance that can enhance the immunogenicity of substances mixed with it”⁹. More closely, they have several roles and may serve to improve the strength and duration of an antigen-specific immune response, alter the polarisation of the immune response, help to overcome the immunosuppressive environment of the tumour, reduce the dose of antigen required, and even reverse immune tolerance^{5, 17}. Importantly, the adjuvant activity does not depend on tissue type in the same way as antigen recognition. Adjuvants are classed either as immunostimulatory agents or carriers that promote antigen uptake (Figure 1-1).

Many adjuvants exploit the innate immune system. The innate immune system can react rapidly to danger signals, mediated by pattern recognition receptors like the TLRs. These receptors recognise pathogen-associated molecular patterns (PAMPs) - conserved structures across a range of pathogens. Some examples include lipopolysaccharide from bacterial cell walls recognised by TLR-4, or oligonucleotides with CpG repeats from bacterial and viral DNA recognised by TLR-9. This type of recognition provides an early and broad immune response, limiting the spread of infection before the adaptive immune system is recruited¹⁸.

Historically, adjuvants have been like “alchemy”, chosen solely on the phenotype of generating inflammation with little knowledge of mechanism. Despite research and progress in the field, few discoveries have been translated into the clinic. Currently, only

two adjuvants have approval for universal use in humans: alum, and MF59¹⁹. Aluminium salts, or alum, have been used as an adjuvant in many vaccine formulations for over 80 years, and are currently included in vaccines against hepatitis A, human papillomavirus, and tetanus vaccines. Alum stimulates humoral and Th2-type responses²⁰. It is thought to act through a depot effect where the antigen is adsorbed to the aluminium and slowly released. This effect is enhanced by causing mild inflammation and affecting antigen-presenting cells²¹. MF59 is a squalene-based oil-in-water-emulsion which also generates a Th2 and enhanced humoral response. It is a proprietary adjuvant by Novartis and is used in flu vaccines^{22, 23}. A range of vaccines have been formulated as oil-in-water or water-in-oil emulsions for this adjuvant effect, including Montanide formulations. The exact mechanism of MF59 has not been confirmed but is thought to enhance antigen uptake into antigen-presenting cells and may aid recruitment of immune cells to the injection site²³.

Adjuvant safety has been a limiting factor in the development of new compounds. Many classical adjuvants showed high immunogenicity acting through unknown mechanisms. Proposed adjuvants have previously failed because of toxicity and side effects, poor stability, or bioavailability issues^{24, 25}. Unfortunately, highly potent adjuvants tend to have increased toxicity, such as Freund's complete adjuvant – highly potent and highly toxic¹⁷. Adverse reactions may be local or systemic, such as swelling at the site of injection, and as severe as anaphylaxis²⁴.

In a cancer vaccine, the adjuvant requires several key features. Firstly, the adjuvant must be able to activate DCs⁸. Second, the adjuvant must be potent enough to overcome the challenge of both an immunosuppressive environment and a poorly immunogenic antigen.

“Accumulated evidence obtained from various clinical trials and animal studies suggested that cancer vaccines need better adjuvants than those that are currently licensed, which include the most commonly used alum and incomplete Freund’s adjuvant, because of either a lack of potent anti-tumor immunity or the induction of undesired immunity”²⁶

1.4 CpG triggers both innate and adaptive immunity.

Figure 1-2 Action of CpG oligodeoxynucleotides as an adjuvant. CTL, cytotoxic T lymphocyte; IFN-γ, interferon-γ; IL, interleukin; TNF, tumour-necrosis factor. Reproduced from Klinman, 2004²⁷, with permission.

TLR-9 detects unmethylated CpG dinucleotides which occur far more commonly in bacterial and viral genomes. TLR-9 is located endosomally and is primarily expressed by plasmacytoid dendritic cells and B cells²⁸. Activation by CpG oligodeoxynucleotides (CpG ODN) leads to secretion of type 1 interferon, increased expression of costimulatory molecules, upregulation of CCR7, and secretion of Th1-promoting chemokines and cytokines (Figure 1-2)^{27, 29, 30, 31}. Natural killer cells, monocytes, and neutrophils, as well as Th1 type T cell responses, are generated. B cells activated by TLR-9 are more sensitive to antigen and are therefore more likely to develop into plasma cells³².

Three classes of CpG ODN exist, each with a different set of immunological properties. Class B (also known as class K) CpG ODNs contain a full phosphorothioate backbone. Class B ODNs strongly stimulate B cells, and plasmacytoid dendritic cells, while mildly inducing IFN- α and IL-6 secretion. They have previously been used as potent Th1 adjuvants, and demonstrate antitumour properties³³.

CpG ODNs have favourable properties for use as an adjuvant. CpG ODNs have been shown to be extremely potent activators of a Th1 response, even more so than the prototypical Th1 adjuvant, complete Freund's adjuvant (CFA)³⁰. The combination of cellular and humoral response generated is likely to be beneficial. Furthermore, many ODNs have been tested in humans and shown to be safe, with their ADMET properties well characterised³⁴. Vaccines against hepatitis B³⁵, influenza (Fluarix)³⁶, and peptide-based tumour vaccines³⁷ use CpG ODNs as adjuvants. Notably, in the hepatitis B vaccine, the CpG adjuvant induced protection more quickly and after fewer doses than the standard vaccine.

A common modification of therapeutic ODNs is a phosphorothioate backbone, where a non-bridging oxygen of the phosphodiester backbone is replaced with a sulfur. This modification makes the compound more resistant to degradation by nucleases with only minor changes in immunological properties compared to phosphodiester ODNs. This substitution creates a new phosphorous stereocentre between each nucleotide, creating 2ⁿ diastereomers for n phosphorothioates in the oligonucleotide³⁸. TLR-9 agonists can be chemically modified at the 3' end without loss of immunostimulatory activity. Modifications of the 5' end, however, will cause loss of activity³⁹.

1.5 α -galactosylceramide exploits the link between the innate and adaptive immune system

α -galactosylceramide (α -GalCer) represents a novel "cellular" adjuvant exerting its effect through a distinctly different mechanism, via invariant natural killer T (iNKT) cells⁴⁰. iNKT cells are considered "innate-like", acting as a link between adaptive and innate

immunity. α -GalCer is presented on DCs by the lipid antigen presentation molecule CD1d - like how MHC presents peptides. The invariant T cell receptor of iNKT cells can recognise glycolipids bound to this complex. The CD1d-ligand:TCR interaction activates the iNKT cell which, in turn, activates the APC through the provision of CD40L. This interaction causes the release of a range of both Th1 and Th2 cytokines. The DC is then licensed to stimulate a peptide-specific T cell response and improves the quality of the response^{41, 42}. Its use has been limited due to the development of anergy upon repeated injection.

Performing an N $\rightarrow$ O acyl migration in acidic conditions forms a prodrug of α -GalCer, migrated α -GalCer. The amine can be capped to “lock” this isomer. Uncapping can allow for the reverse to occur, reforming the active adjuvant (Figure 1-3)⁴¹.

Figure 1-3 N $\rightarrow$ O acyl migration to form a prodrug of α -GalCer.

A range of analogues of α -GalCer have previously been examined, with modifications to the lipid chains, glycosidic linkage, and substitutions on the galactosyl moiety. These have allowed for structure-activity relationships to be determined with respect to its immunological activity⁴³. Most notably, the six position of the galactosyl moiety can be

modified without loss of activity, while other galactosyl modifications may significantly reduce activity^{44, 45}.

1.6 Potential for adjuvant synergy

The combination of multiple innate stimuli can be extremely powerful. In an actual infection or vaccination with live or attenuated pathogens, a range of danger signals, as well as antigen, are present to initiate a powerful, multilineage immune response^{46, 47, 48}. Induction of powerful immunity occurs after few doses; these types of vaccines have been extremely successful in preventing and even eradicating diseases. A peptide vaccine, however, lacks the same immunogenicity and will require a carefully designed combination of adjuvants to generate a similar response.

The combination of adjuvants may have synergistic effects to either promote an immune response or generate tolerance. For example, Bagchi et al.⁴⁹ showed that TLR agonists acting through different pathways (MyD88-dependent vs. MyD88-independent) synergised and generated a response greater than either agonist alone. However, agonists acting through the same pathway induced tolerance. Moreover, others have demonstrated the potential for synergism between TLR and CD1d agonists. For example, upon administration of α -GalCer and monophosphoryl lipid A (MPL), a TLR-4 agonist, in a DNA vaccine, lymphocyte numbers and CTL activity were higher with the combined adjuvants than with either adjuvant alone⁵⁰. In a study by Semmling et al.⁵¹, it was demonstrated that stimulation by α -glycuronosylceramide and CpG were both able to initiate cross-priming of DCs, showing the potential for functional synergism between CD4⁺ T cells and NKT cells in the licensing of DCs. Furthermore, this study demonstrates that the combination of adjuvants would be able to stimulate to different populations of CTL – CCR4⁺ and CCR5⁺ positive subsets – which may induce a different quality of immunity and have the potential for improving vaccination. Finally, the administration of α -GalCer-loaded tumour cells and a CpG ODN adjuvant was shown to redirect a Th2 response towards a Th1 response and synergy between the two adjuvants⁵².

1.7 MAIT cell biology.

Mucosal-associated invariant T cells (MAIT cells) are a population of innate-like T cells, comprising up to 10% of peripheral blood T cells in humans. As their name suggests, they are found in mucosal sites such as the gastrointestinal tract, as well as mesenteric lymph nodes and comprise up to 50% of liver T cells. MAIT cells may present a better clinical target than other innate-like cells given their higher abundance, compared to NKT cells which only make up less than 0.1% of peripheral blood T cells⁵³.

MAIT cells express a semi-invariant $\alpha\beta$ T cell receptor which binds to MHC class I-like molecule, MR1. They express invariant T cell receptor α -chain V α 7.2Ja in combination with either TCR-V β 2 or V β 13. Vitamin B metabolites, found in some yeast and bacteria, activate MAIT cells. 5-amino-6-D-ribitylaminouracil (5-A-RU) is an intermediate of the riboflavin biosynthetic pathway. It does not directly bind to MR1 but forms potent ligands by reacting with simple aldehydes such as glyoxal and methylglyoxal to form the pyrimidine “neo-antigen” (Figure 1-4). These unstable intermediates spontaneously dehydrate to form lumazines but can be captured by MR1 by forming a reversible Schiff base complex with Lys43⁵⁴.

MAIT cells have many parallels with other innate-like T cells. Both MAIT and iNKT cells rapidly release a range of cytokines on activation, both express a semi-invariant TCR and are restricted by MHC-like receptors⁵⁵. Cells like iNKT cells can be harnessed for their adjuvant-like activity to modulate the immune system. Unpublished work by Hermans et al. suggests that MAIT cells can be harnessed in a similar manner.

MAIT cell ligands can be harnessed for therapeutic use by creating a prodrug in a similar fashion to α -GalCer. A prodrug is a compound which undergoes a transformation following

Figure 1-4 Example reaction of 5-A-RU with methylglyoxal. The intermediate forms a Schiff base with Lys43 of MR1. Adapted from⁵⁴

administration to release the active drug⁵⁶. 5-A-RU can be harnessed as an adjuvant by forming a prodrug which breaks down in the right context to allow the formation of the lumazine neo-antigen.

1.8 Lymph node targeting.

The lymphatic system is a network of nodes and vessels which plays a vital role in fluid homeostasis, lipid distribution, and immune control. Lymphocytes circulate through the lymph vessels which provide structure for antigen presentation and immune activation⁵⁷.

Targeting an immunomodulatory compound to the lymphoid organs allows it to be present in the tissues where immune responses are coordinated. The vaccine may access the lymph node by being transported from the site of injection into the draining lymph node, or by being carried by APCs into the lymph node⁵⁸. Lymph node targeting allows for reduced systemic dosing with simultaneous reduction of off-target side effects.

It has previously been shown that targeting vaccine components to the lymph node has therapeutic benefits. In its most basic form, this was achieved using intranodal injection⁵⁹. However, this is a difficult procedure which requires special equipment. It would be easier to use a discrete molecular entity that targets to the lymph node that does not need a complicated administration method.

Access to the lymphatics is dependent on molecular weight or particle size. While the blood clears up to ten times more interstitial fluid than the lymphatics, the basement membrane and tight junctions of the endothelium limits large molecules entering the

blood. Conversely, the lymphatics lack a basement membrane and are highly fenestrated. Molecules with a molecular weight less than 1000 are almost entirely absorbed by the bloodstream, while molecules above 16,000 preferentially distribute into the lymphatics⁶⁰. This effect can be exploited to target compounds to the lymphatics – for example associating small molecules with macromolecular carriers like nanoparticles or liposomes, or linking to endogenous macromolecules⁶¹.

Sentinel lymph node tracers are administered to cancer patients to identify lymph nodes closest to the tumour where metastases may have spread. These compounds are injected subcutaneously at the site of the tumour and drain into the nearest lymph node^{62, 63}. Many of these dyes bind with high affinity to human serum albumin present in the serum and interstitial fluid. Human serum albumin is a plasma fatty acid carrier, able to bind up to seven equivalents of C14-C18 fatty acids (Figure 1-5)^{64, 65}. Albumin is present in interstitial fluid, blood and lymph⁶⁶. It circulates from the interstitial fluid back into systemic circulation through the lymphatics. Therefore, it would be expected that compounds which bind to albumin would preferentially accumulate in the lymphatic system^{61, 66}.

Inspired by sentinel lymph node tracers, Liu et al.⁶⁷ chemically linked a CpG vaccine to a diacyl lipid to exploit the role of albumin as a fatty acid carrier and target their treatment to the lymph nodes. They reported that this increased the amount of the compound in the lymph nodes and overall increased the potency. Importantly, their vaccine displayed reduced inflammatory signals systemically, and in the spleen which indicates a safer vaccine as these effects are usually toxic. They attributed this effect to reduced systemic

distribution due to preferential accumulation in the lymph nodes. This method may be applied more broadly to other drug molecules.

Figure 1-5 Serum albumin, with bound fatty acids in white and red.⁶⁸

This paper presents two interesting ideas relevant to the pharmacokinetics of adjuvants. Firstly, that a discrete chemical structure can exploit the role of albumin as a fatty acid carrier to target a compound to the lymph nodes. Secondly, that these compounds must be amphiphilic to prevent non-specific insertion into membranes. The albumin binding domain presented in this paper structurally resembles the adjuvant α -GalCer, the adjuvant used in the synthetic vaccines produced by our group (Figure 1-6). To this end, we hypothesised that α -GalCer could act as an albumin binding domain when attached to adjuvant compounds. Attaching this glycolipid to water soluble adjuvants would create a similar amphiphilic vaccine compound, additionally incorporating a bioactive albumin binding domain, and thereby creating a bifunctional, lymph node targeting adjuvant.

m-aGalCer

albumin binding domain

Figure 1-6 Structural similarity between α -GalCer and the diacyl lipid albumin binding domain

1.9 Aims and hypothesis

We, therefore, hypothesise that the properties of a glycolipid vaccine adjuvant can be improved by controlling its distribution. Specifically, targeting the adjuvant to the lymph nodes by altering its physicochemical properties will enhance its potency and safety. Targeting will be achieved using a prodrug strategy by attaching groups to modify pharmacokinetic properties through a cleavable linker.

We aim to:

1. Design and synthesise a series of chemically modified vaccine adjuvants over a spectrum of hydrophilicity.
2. Design and synthesise a bifunctional adjuvant.
3. Chemically synthesise a lymph node targeting peptide antigen.
4. Investigate the albumin binding properties of the compounds *in vitro*.
5. Test the efficacy and safety of the vaccine *in vivo* (performed independently at the Malaghan Institute of Medical Research).

Three sets of compounds will be generated. Firstly, derivatives of 5-A-RU designed to target the lymph node by attachment of the diacyl lipid moiety, and α -GalCer. Secondly, a set of PEGylated α -GalCer over a range of PEG lengths to improve hydrophilicity and hydrodynamic radius to target the lymph nodes. Finally, a highly potent, safe, adjuvant with dual activities by chemically linking α -GalCer with TLR-9 agonist CpG intended to target the lymph nodes by albumin hitchhiking.

In summary, we aim to develop a safe and potent compound to be used as an adjuvant. It will be altered such that it preferentially distributes to the lymph nodes rather than systemically to both induce potent immune responses and reduce the systemic distribution. This proposed adjuvant harnesses innate-like cells, which are present in all individuals irrespective of tissue type.

2 Synthetic route to MALT ligands

We wanted to explore the potential to create novel adjuvants based on 5-amino-6-(D-ribitylamino)uracil (5-A-RU) (**2.01**). This compound was only recently reported as an immunomodulatory compound, with the potential to be developed as an adjuvant. This water soluble compound was a good starting place to examine the albumin binding properties of the diacyl lipid chains and α -GalCer. We aimed to create two compounds: first, an albumin hitchhiking 5-A-RU using the identical diacyl lipid albumin binding domain; second, a dual adjuvant compound by chemically linking 5-A-RU to α -GalCer to examine whether the lipid chains of α -GalCer could exploit this same effect and target the compound to the lymph nodes. This product would be a bifunctional adjuvant targeting two populations of innate-like T cells. Unfortunately, neither of these compounds were synthesised as we changed our focus to different adjuvants and hydrophilic groups.

For this thesis, we still had the opportunity to improve the synthetic route to 5-A-RU. This compound is an intermediate in the biosynthesis of riboflavin. *In vivo*, riboflavin is produced through a cascade of enzymatic reactions from GTP. The synthetic route to 5-A-RU is already well established as a step in the synthesis of riboflavin^{69, 70, 71, 72, 73}.

(**2.01**)

A reductive amination of ribose will yield ribitylamine. This product can then be reacted via an addition-elimination reaction with a substituted uracil to give (2.06). Next, the 5-amine must be introduced. This can be done by installing an oxidized nitrogen group, followed by reduction leading to 5-A-RU.

Most recently, Kjer-Nielsen et al.⁷¹ performed this synthesis to examine the role of these compounds in the activation of MAIT cells (Scheme 2-1). The oxime of D-ribose (2.03) was formed using hydroxylamine in the presence of sodium methoxide in ethanol. Next, this was reduced using platinum oxide with acetic acid under pressurised hydrogen. Ribitylamine (2.04) was then reacted with 6-chlorouracil in water with heating to form 6-ribitylaminouracil (2.06). A nitroso group was then installed at the five position to give (2.07). This was subsequently reduced using sodium dithionite to form 5-A-RU (2.01). In total, this is a seven-step synthesis with the installation of the nitroso group being particularly low-yielding. We aimed to improve the synthesis by reducing the number of steps and improving the yields of the poorly yielding steps. To do this, we firstly wanted to telescope products into subsequent reactions, and secondly, we wanted to use a more reactive substituted uracil.

Scheme 2-1 Synthetic route to 5-A-RU (2.01), as described in the literature and has been used in our group.

2.1 Purification of ribitylamine: formation of a Schiff base.

Dr R J Anderson had previously synthesised ribitylamine through the oxime reduction with platinum oxide and acetic acid. However, this reaction had been determined to contain up to 30% of ribitylamine dimer (2.10) with $[M+H] = 286$. Other impurities included N-acylated products as detected by NMR. Kagan et al.⁷⁴ reported a method to purify a similar mixture of compounds for ribitylgalactamine (Scheme 2-2). They proposed the formation of an aromatic Schiff base to separate the desired product from side products which could then be extracted and hydrolyzed by steam distillation to yield ribitylamine (2.04) as a pure product.

Scheme 2-2 - Overview of the mechanism of purification of ribitylamine mixture.

The mixture of (2.04) and (2.10) was treated, as described, with either benzaldehyde or the more reactive salicylaldehyde - but with little success in our hands. Kagan⁷⁴ reports a yellow solid precipitating after as little as ten minutes of stirring. Even with refluxing, the addition of base, and cooling on ice, no precipitate formed, nor was product detected by NMR or TLC. It was noted that the aldehydes remained as oil droplets on the surface, even with vigorous mixing. Reactions were attempted in mixtures of water and methanol or ethanol to encourage aldehyde solubility. These reactions were still unsuccessful. The presence of acetic acid in the reaction mixture may have had some influence on the failures of the reaction. It may have been necessary to neutralise the acetic acid prior to these reactions as it may be affected by pH.

While these methods use aqueous conditions to conduct the reaction, it is likely that the exclusion of water, and precipitation of the imine, would favour the equilibrium towards the product. In these reactions, there was no precipitate indicating that the reaction is not being driven forward. There is some conflict in the literature about water in reactions for the formation of imines – normally water should be excluded, to the extent that drying agents or molecular sieves have been included in the reaction mixture, while others use water as the solvent. Saggiomo et al.⁷⁵ investigated the formation of imines in aqueous conditions. From their studies, they suggest that the reaction does not occur in water and that concentrating the reagents in the work up causes the formation of the imine. When they conducted their experiments neat, and under reduced pressure, a solid would form with the release of heat in 10 minutes. The reaction mixtures from the various conditions trialled were concentrated under vacuum overnight. Even following this treatment no product was detected.

2.2 Formation of D-ribityl benzyl amine

An alternative route to ribitylamine is via a reductive amination of ribose with benzylamine, followed by cleavage of the benzyl group (Scheme 2-3). Ribitylbenzylamine (2.02) was prepared from D-ribose as reported by Davoll et al⁷⁶. This method forms an imine with benzylamine which is subsequently reduced by platinum oxide in a one pot, two step reaction. This method was particularly more convenient as the product is crystalline and could be purified by recrystallization from ethyl acetate.

Scheme 2-3 Synthesis of ribitylbenzylamine (2.02)

Hydrogenolysis of ribitylbenzylamine (2.02) had previously been attempted in our group and found to stall before complete conversion. Benzyl amine (2.02) was therefore subjected to a range of hydrogenolysis conditions (Scheme 2-4). The reactions were monitored by ¹H NMR and TLC, but the products were not isolated. Percentage conversion is estimated from integrations of the proton spectrum (Figure 2-1). The integration was taken from the peak at 3.89 ppm (ribitylbenzylamine, 1H) which would reduce over the course of the reaction, relative to the integration of the doublet of doublets at 2.90 ppm (ribitylbenzylamine + ribitylamine, 1H). Therefore the amount of starting material remaining could be calculated using Equation 2-1. Results of these experiments are summarised in Table 2-1.

$$\% \text{ conversion} = 100 - \left(\left(\frac{SM}{SM + TM} \right) * 100 \right)$$

Equation 2-1

Scheme 2-4 Hydrogenolysis of ribitylbenzylamine to ribitylamine.

Figure 2-1 Representative crude spectra of the hydrogenolysis reaction.

Ribitylbenzylamine was hydrogenolyzed using 10% palladium on carbon in methanol as described⁷⁶. This experiment was used as a benchmark to establish how well other conditions worked in comparison – achieving approximately 60% conversion of starting material. The addition of, or use of, acetic acid as the solvent was recommended for hydrogenolysis of N-benzyl groups. It has been posited that protonation of the amine improves the reaction⁷⁷. However, no real difference was noted between hydrogenolysis in methanol alone, or with the addition of acetic acid.

Transfer hydrogenolysis is the reduction by addition of hydrogen from a source other than gaseous hydrogen. The setup of a transfer hydrogenolysis may be more desirable as it does not involve handling gaseous hydrogen, and is effective without the use of

pressurised equipment. Adger et al.⁷⁸ suggest the use of ammonium formate or hydrazine hydrate for the deprotection of N-benzyls, refluxing the substrate in an alcoholic solvent with catalytic amounts of 10% palladium on carbon. We investigated three reagents for transfer hydrogenolysis: formic acid, ammonium formate, and hydrazine hydrate. Of all these additives, the ammonium formate had the best conversion. Qualitatively, the ammonium formate reaction generated far cleaner spectra.

The best method, however, used ammonia and palladium hydroxide under an atmosphere of hydrogen to selectively remove N-benzyl groups^{79,80}. This reaction proceeded a lot more cleanly and with high conversion. Palladium hydroxide appeared to be a more active catalyst and could be used at lower loading.

Catalyst	Additive	Percent conversion
10% palladium on carbon	-	59%
	Acetic acid	59%
	Formic acid*	67%
	Hydrazine hydrate*	69%
	Ammonium formate*	87%
10% palladium hydroxide on carbon	NH ₃	91%

Table 2-1 a summary of the reaction conditions explored for deprotection of N-benzyl. * indicates transfer hydrogenolysis reagent, reaction not conducted under an atmosphere of hydrogen.

Analysis of these compounds was made difficult by the lack of protons on the uracil heterocycle. Furthermore, negligible change in the chemical shift of the ribityl moiety was observed. Previous literature lacks spectroscopic data, only reporting melting points. By TLC, even polar mobile phases such as 50:50:13:2 DCM:MeOH:water:ammonia barely moved these compounds off the baseline

2.3 Synthesis of ribitylaminouracil

Scheme 2-5 Summary of attempted reactions to synthesis ribityluracil and derivatives.

To reach (2.06) we attempted two reactions: the substitution of (2.04) with 6-chlorouracil, and the reaction of the, still benzyl protected, (2.02) with this uracil compound (Scheme 2-5). The first of these reactions has been conducted successfully in our lab. The second would be able to reduce the number of steps in the synthesis. Theoretically, the secondary, protected, amine will be more nucleophilic, however the steric bulk of the benzyl group may limit its reactivity. For both these reactions, in aqueous conditions, with the pH modified to pH 8 no reaction occurred. By ^1H NMR and TLC, only starting product was present.

One final approach to (2.06) was considered, which involved the reaction of 6-aminouracil (2.15) with ribose – either under reductive amination conditions or two separate steps, imine formation, followed by reduction (Scheme 2-6). 6-aminouracil is readily available. Similar imine formations have been reported with aryl aldehydes. However no examples of reducing sugars were found in the literature. We thought it would be worthwhile to attempt this approach. α -Picoline-borane has previously been

used for one-pot reductive aminations⁸¹. It was a particularly attractive candidate to try as it could be used in solvents like methanol and water, or neat. It is also a less toxic, greener, alternative to reagents such as sodium cyanoborohydride.

Figure 2-2

We, therefore, attempted the reductive amination of D-ribose with 6-aminouracil to form (2.05) with α-picoline borane.

Scheme 2-6 Reductive amination of D-ribose and 6-aminouracil using α-picoline-borane.

The aminouracil was poorly soluble in most solvents examined, including water, methanol, and ethanol. Furthermore, and despite previous reports of similar usage⁷⁵, the α-picoline borane formed snowflake-like crystals that did not appear to be soluble in these polar solvents. Nevertheless, the reaction was attempted in methanol with acetic acid, as well as in water, however without success.

We also attempted to synthesise an imine by mixing 6-aminouracil and D-ribose with HCl, followed by a subsequent reduction with 10% palladium on carbon under hydrogen. This reaction did not appear to proceed as monitored by TLC or ¹H NMR. While this reaction may look good on paper, the amine of 6-aminouracil is only weakly nucleophilic given the electron withdrawing carbonyls within the heterocycle. This may have limited the ability of this compound to react here.

2.4 Summary and future directions

Purification of the products from the oxime reduction was unsuccessful by the method described. This approach was not pursued any further as the other route was convenient. However, if it were to be tried again, more measures could be taken to exclude water from the reaction. The reaction could be carried out in a dry solvent in which the starting materials are adequately soluble, subjected to vacuum to remove water or use a Dean-Stark apparatus to extract water as the reaction progressed.

We successfully improved the reaction conditions for the hydrogenolysis of ribitylbenzylamine. The conditions were optimised to achieve greater than 90% conversion to the deprotected product, compared with earlier methods which stalled at around 60% conversion. For these deprotection reactions, a hydrogen atmosphere was introduced using hydrogen in a balloon. Many of these methods might have been more efficient if the reaction took place under pressurised conditions or with more advanced equipment such as an H-Cube continuous-flow hydrogenation reactor.

To improve the efficiency of the addition of (2.04) to a 6-substituted uracil, one idea was to use the more reactive 5-nitro-6-chloro-uracil (2.11). Following substitution, the nitro group could be reduced to the free amine, skipping a step to introduce the nitroso then perform a reduction. Furthermore, depending on the reactivity displayed by the nitro-compound, it could be reacted directly with the ribitylbenzylamine, removing two steps as the N-benzyl could be removed in the same reduction that would convert the nitro to an amine. This step is still the poorest yielding step in the pathway. A similar reaction has been reported with arabinitylamine and 6-chloro-5-nitrouracil with good yields⁸².

3 Designing a water soluble α -GalCer

3.1 α -GalCer pharmacokinetic properties

While α -GalCer is an effective adjuvant, it has less than optimal physicochemical and pharmacokinetic properties. α -GalCer is a difficult compound to work with, being completely insoluble in water and most alcoholic solvents, and is only soluble in DMSO above 60 °C. For *in vivo* use, α -GalCer must be solubilized with a matrix containing sucrose, L-histidine and Tween-20⁸³. These may not be ideal conditions for biological testing due to safety concerns or their own immunological properties. A water-soluble derivative of this adjuvant would, therefore, be useful.

The covalent attachment of polyethylene glycol (PEG) or “PEGylation” has previously been used to improve the pharmacokinetic properties of pharmaceuticals. PEGylation may be used to increase the water solubility of a compound and to enhance the stability and half-life of the compound. Biological drugs such as antibodies have been PEGylated to prevent proteolytic degradation. Furthermore, PEG is non-toxic and non-immunogenic^{84,85}. PEGylated compounds are well established, and many are available on the market currently, including Pegasys (a PEGylated interferon alpha for the treatment of chronic hepatitis B and C)⁸⁶, ADAGEN (PEGylated adenosine deaminase for treatment of severe combined immunodeficiency disease (SCID))⁸⁷, and Cimzia (PEGylated certolizumab, a monoclonal antibody for the treatment of rheumatoid arthritis)⁸⁵. A PEGylated α -GalCer has previously been reported⁸⁸, which demonstrated higher potency at lower doses than α -GalCer. PEG was attached to 6-amino- α -GalCer by forming an amide at the six position. The PEGylated compound, however, stimulated a Th2-biased response.

3.2 Methodology

We set out to create a set of water-soluble α -GalCer compounds spanning a range of hydrophilicity (Figure 3-1). To do so, we attached different lengths of PEG to 6-thio- α -GalCer (**3.01**). We chose to use 6-thio- α -GalCer, as the reactive thiol can be used as a selective reaction site with many orthogonal linker groups. Scheme 3-1 demonstrates the planned methodology using the succinimidyl 3-(2-pyridyldithio)propionate (SPDP)-PEG₂₄-N-hydroxysuccinimide (NHS) heterobifunctional linker. This can be added to 6-thio- α -GalCer through a thiol-disulfide exchange. The NHS intermediate can then be reacted with different PEG amine caps to form an amide. Linking the PEG groups to α -GalCer through a disulfide bond would allow for the formation of a cleavable linkage. *In vivo*, free thiols, such as glutathione, will reduce the disulfide releasing the active 6-thio- α -GalCer.

Alternatively, a 2,3,5,6-tetrafluorophenyl-ester (TFP)-PEG-maleimide heterobifunctional linker could be used to achieve these targets. However, the addition of a thiol to a maleimide creates a stable covalent bond that typically cannot be cleaved *in vivo*. This compound may still be able to bind to CD1d, which accommodates the lipid tails of α -GalCer, in two hydrophobic pockets, however, its immunological activity may be limited, especially with the largest PEG groups, preventing TCR binding of to the top galactosyl group and CD1d.

The dispersity of a polymer is a measure of the homogeneity of molecules in the mixture. Polymers typically exist over a range of molecular masses and are therefore polydisperse or non-uniform. A monodisperse mixture contains particles of homogeneous size. The heterobifunctional PEG linkers and PEG amines purchased from QuantaBioDesign Inc. are marketed as discrete PEGs, as opposed to monodisperse. They only contain a single length of PEG polymer rather than a range. However, this definition is used as they are not formed by polymerisation, but repeated organic synthesis steps.

In addition to creating a water soluble derivative, the increase in size thanks to the PEG groups would also be expected to passively target the adjuvant to the lymph nodes. The smallest of these compounds had a MW of 2200, while the largest was approaching 6000.

Figure 3-1 PEG- α -GalCer targets

Scheme 3-1 Reaction scheme for synthesis of PEG-α-GalCer via a disulfide linkage. From the NHS-intermediate a range of amine PEG caps can be introduced.

3.3 α -GalCer disulfide PEG conjugates

The initial goal was to attach the PEG groups through a cleavable disulfide. However, reactions using SPDP linker (3.02) required optimisation to improve the yield of product and reduce the production of by-products. These reactions produced a range of compounds – effectively a statistical mixture of disulfide compounds was present (Figure 3-3).

Firstly, we examined the effect of pH on this reaction as a way of controlling side reactions. In theory, the reaction should work best at a high pH as the reactive thiolate can form, whereas, in acidic conditions, the sulfur will be protonated and far less nucleophilic. For example, a thiol with a pKa of 10 will, at pH 7, have only 0.1% of the compound present as the thiolate, but, at pH 8, 1% will be in the anionic form. A thiolate will react with a disulfide, but a thiol and disulfide will not react. Thiolates are best generated by the addition of base in polar aprotic solvent, such as DMSO⁸⁹.

Four reactions were conducted side-by-side. Each reaction was conducted with the same amount of 6-thio- α -GalCer, and linker at the same concentration. To modify the pH, the reactions were carried out in 250 mM ammonium acetate buffer (pH 3), 250 mM ammonium formate buffer (pH 5), 200 mM phosphate buffered saline (pH 7), or neat DMSO. The reactions ran side-by-side at 40 °C for one hour. Following this, the reactions were quenched with N-ethylmaleimide and allowed to react for an hour, followed by addition of aminoethoxyethanol and left again for a further hour.

The results are shown in Figure 3-2. In the most acidic conditions, the least 6-thio- α -GalCer dimer was formed, however, these conditions also formed the least amount of product. As the pH increased, so did the amount of both 6-thio- α -GalCer dimer and product. In the neutral reaction mixture, however, nearly equal amounts of product and dimer were formed. In the unbuffered reaction mixture, little product is formed, but high levels of the dimer are formed. This appears to fit with the theory. At low pH, little product or dimer forms as the thiols remain protonated and unreactive. As the pH increased, more thiolates are available to react to form either product or dimer which

would be in equilibrium. In the unbuffered reaction, however, it is likely that an equilibrium develops where the 6-thio- α -GalCer starting material reacts with the disulfide product being formed to generate the dimer (Figure 3-4). Alternatively, oxidation may be leading to the formation of the dimer. Taking care to conduct these reactions under an inert atmosphere may be required to suppress the formation of the dimer.

N-ethylmaleimide is a Michael acceptor which creates a strong thioether through a Michael addition. This was used to quench any free thiols in the reaction which would continue to undergo thiol-disulfide interchange.

The peak at 1.1 mins in Figure 3-2 corresponds to the heterobifunctional linker, as determined by LC-MS in the same conditions. However, in some traces, a second peak is appearing at 1.3 mins with a mass corresponding to hydrolysis of the NHS ester to a carboxylic acid. Mass of SPDP-PEG₂₄-carboxylic acid calculated = 1342.67 mass found $[M+Na]^+ = 1366.6$.

Figure 3-2 - Representative chromatogram of the reaction mixture, post-quench with N-ethylmaleimide and addition of aminoethoxyethanol, CAD. From top to bottom: pH 3, pH 5, pH 7, DMSO.

3.4 α -GalCer maleimide PEG conjugates

Despite the improvements in the selectivity of the disulfide exchange reaction, parallel experiments using the maleimide-TFP linker appeared to be a more preferable route to our targets. This, however, is not normally cleaved *in vivo* though may be cleaved through a retro-Michael reaction. We used the same methodology as described above, with the MAL-PEG-TFP linker in place of the SPDP-PEG-NHS linker.

The first small scale reaction trialling the use of this linker appeared to produce a side product with a mass corresponding to the addition of two aminoethoxyethanol groups, $[M+H]^+ = 1489.8$. To counter this product forming, we trialled the reaction of the MAL-PEG-TFP linker with aminoethoxyethanol at different concentrations. The aminoethoxyethanol was added to the linker as a 50%, 25%, and 12.5% v/v solution in DMSO. This appeared to work best diluted to 12.5%, as well as reducing from 1.2 equivalents to 1:1 with the heterobifunctional linker.

With this adaptation, the reactions proceeded without issue, with none of the side products of the SPDP reaction, nor requiring a quench. Each reaction was first tested on a 1 mg scale, before scaling to 5 mg. A general procedure for these reactions is as follows. The MAL-dPEG₂₄-TFP linker was reacted with 1 equivalent of PEG-amine in DMSO, heated to 60 °C for 15 minutes. To this was added 0.5 equivalents of 6-thio- α -GalCer in DMSO, and again heated to 60 °C for 15 minutes. Reaction progress was monitored by HPLC.

Notably, with the MAL-PEG-TFP linker, we did not observe the formation of the 6-thio- α -GalCer dimer. It is likely that the thiol addition the maleimide is a much faster reaction, outcompeting any oxidation forming the dimer. Nor was hydrolysis of the MAL-PEG-TFP linker observed in any of these reactions.

These compounds were purified using preparative HPLC on C18 using mixtures of water, methanol, and isopropanol as the mobile phase. Fractions were examined for purity by

analytical HPLC. After combining they were concentrated then lyophilized. Results of these syntheses are outlined in Table 3-1.

Compound	Yield	Purity
3.07	8%	79.6
3.08	12%	90.5
3.09	33%	94.1

Table 3-1 Yields and purities of 6-thio- α -Gal-PEG conjugates.

3.5 Log P

We hypothesised that the covalent attachment of PEG groups would change the biodistribution of α -GalCer, as well as affect its size to access the lymph nodes. To gain an idea of how the compound may distribute *in vivo*, we attempted to determine the log P, or partition coefficient, of the compounds. Log P is the ratio of concentrations of a compound between two immiscible phases, usually octanol ($C_{n-octanol}$) and water (C_{water}), approximating the distribution between an aqueous cell cytoplasm and lipophilic cell membrane.

$$P_{ow} = \frac{C_{n-octanol}}{C_{water}}$$
$$\log P = \log_{10} \frac{C_{n-octanol}}{C_{water}}$$

Equation 3-1

The shake-flask method is the gold standard of experimentally measuring log P. However, the method is time- and labour-intensive. Alternative methods to calculate log P include HPLC retention or software prediction. HPLC methods typically analyse a range of reference compounds of known log P and extrapolate the log P of the analyte from its observed retention time. Many of these methods are limited to a log P between 0 and 6⁹⁰. Software methods calculate log P through a variety of methods, including additive methods of different atoms or groups present in the compound^{91, 92}. However different calculation methods can give entirely different results, as demonstrated in Table 3-2

Method	α -GalCer	6-thio- α -GalCer	(3.07)	(3.08)	(3.09)
clogP	17.06	-1.36			
ALogPs	6.21		3.96		
miLOGP	10.12	10.23	8.46	1.88	-5.59

Table 3-2 Software calculated values of log P. Blank spaces indicate that the software could not calculate the log P for that compounds due to the size of the molecule.

Given the values calculated for our compounds, it would not be suitable to determine the log P of our compounds using HPLC methods. Therefore we attempted to miniaturise the shake flask experiment using less than 1 mL of solvent in Eppendorf tubes. Analyte concentrations were measured using HPLC methods. Before carrying out the experiment using any α -GalCer derivatives, we attempted to validate this approach using compounds with log P values in the literature.

The method was adapted from an EPA product properties test guideline^{93,94}. 24 h before the experiment the solvents were shaken together and allowed to separate to allow mutual saturation. A stock solution of the compound in an aqueous solution was mixed with an equal volume of n-octanol. The tube containing the two phases was vortexed to mix the phases thoroughly. The tube was then centrifuged for 5 minutes at 13,400 rpm to separate the water and octanol phases efficiently. The concentration in each phase was determined using HPLC methods. A sample of each phase would be extracted, and the concentration determined based on the intensity of the UV or CAD signal against a calibration curve. To ensure no mixing of the phases upon extraction, the pipette tip, partially filled with air, would be inserted into the lower aqueous phase and the air expelled before taking a sample. The calibration curve and extracts from each phase were run in triplicate, and the experiment was run in duplicate. Alternatively, equal volumes of water and octanol were added to a vial. To this was added the analyte dissolved in DMSO, or dissolved in water.

The method is able to determine a log P up to 4.65, when the concentration of the solute in either phase is not more than 10 mM.

Initially, we tried to validate this method with caffeine, a compound with a log P close to zero (nearly equal concentrations in octanol and water), and a UV chromophore. Next, we planned to use cholesterol, a much more lipophilic compound, and then α -GalCer. The best results for caffeine are displayed in Table 3-3. This method used a stock solution of caffeine in water as the aqueous phase. It is likely that the alternative methods of adding caffeine in DMSO or water to the aqueous phase meant the concentration of caffeine was too low in either phase to get an accurate measurement. We observed high inter-run variability between trials, as shown in Table 3-4.

Replicate	C _{n-octanol}	C _{water}	log P
1	0.465	0.534	-0.0599
2	0.462	0.537	-0.0656
3	0.449	0.550	-0.0884
		mean	-0.0713
		literature	-0.07

Table 3-3 Representative log P determination for caffeine across side-by-side triplicate of the experiment. The concentration of caffeine determined from the absorbance at UV 254 nm, mg/mL.

Experiment	Calculated log P
DMSO calibration curve/added in DMSO	-0.015
DMSO calibration curve/added in DMSO	-0.10
Water calibration curve/added in DMSO	-0.19
Water calibration curve/added in water	-0.24
Water calibration curve/added in water	0.043
Water calibration curve/added in water	-0.75
Water calibration curve/added in water	-0.071
Literature	-0.07

Table 3-4 Inter run variability between results of log P experiments.

We created a calibration curve for α -Gal-PEG 25 to determine the limits of detection. This compound was predicted to have a log P as high as 8.46 by one software prediction. This would mean that the concentration of the analyte would be $10^{8.4}$ fold higher in octanol than water. This calculation does seem unlikely as the PEG should make the compound more hydrophilic not lipophilic. Using the value calculated for the PEG100 compound, an estimated log P of -5.7, there would be $10^{5.7}$ times more compound in the aqueous phase than the octanol. It would be expected that the PEG-25 compound would be somewhat less hydrophilic. By LC-MS, however, this compound was out of the linear range of response below 0.00001 mg/mL, therefore, be below the limits of detection in the octanol phase, without using a much larger quantity of it in the aqueous phase. The opposite would be true for α -GalCer or 6-thio- α -GalCer being so lipophilic.

The log P of α -GalCer derivatives was not determined. It is likely that these measurements would be inaccurate. Molecules such as α -GalCer may be surface active, with a polar head and lipid tail, which can cause emulsions between the aqueous and lipophilic phases leading to inaccurate concentrations measured in each phase. Given the extremely hydrophilic and hydrophobic nature of these compounds, it would be difficult to measure such low levels of compound present in the opposite nature phase.

3.6 Summary and future work

A water-soluble derivative of α -GalCer was successfully achieved. These compounds dissolved well in water, forming clear solutions with no visible turbidity at concentrations up to 10 mg/mL. Their biological activity is yet to be tested. It will be interesting to see the immunological properties of these compounds with such drastically different physiochemical properties to α -GalCer.

An *in vitro* assay to measure whether these compounds are large enough to distribute preferentially in the lymph would also be interesting. *In vivo* studies for this purpose may also be useful, though detecting a small amount of compound in a large volume of distribution may be challenging.

The synthetic route to these PEG conjugates using the TFP-PEG-MAL heterobifunctional linker is straight forward, after optimisation. The reaction proceeds quickly with few side products, and the linker itself is not prone to hydrolysis. These methodologies could be adapted to introduce a fluorophore or other labels to make chemical probes of α -GalCer.

Use of the SPDP/NHS linker still has potential. Further optimisation is still required to minimise the formation of side products and maximise yields. Quenching with N-ethylmaleimide followed by acidification with formic acid was the best improvement in this synthetic route. More rigorous efforts to exclude oxygen from the reaction may aid in suppressing dimer formation. These efforts may include conducting the reaction under an inert atmosphere and degassing the solvents before use.

Further method development is needed to achieve reproducible determination of log P. It may be necessary to control the pH of the aqueous phase with a buffer especially for compounds with ionizable sites. More advanced methods to measure log P have been reported, such as micelle-based measurements, or generator columns, but were not attempted in this body of work. However, for these compounds with more extreme log P and other properties negatively affecting the determination of log P it may be necessary to

try different methods. Automation of this methodology to allow high throughput determination of log P for new compounds would also be useful.

4 Design and synthesis of CpG- α -GalCer conjugate

4.1 Vaccine design.

Our goal was to design an adjuvant that, when combined with appropriate peptide antigens, would generate a powerful cellular response against target epitopes for use in vaccine development. The compound would exploit the role of albumin as a fatty acid carrier to target it to the lymphoid tissues thereby reducing systemic side effects by controlling its distribution properties. To this end, we designed dual-adjuvant conjugates (4.01) and (4.02). Conjugates (4.01) and (4.02) are comprised of several features as depicted in Figure 4-1: CpG ODN 1826 (A), a short linker (B), a cleavable para-aminobenzoyl-citrulline-valine-bicyclononane linker (C), and a prodrug form of α -GalCer (D). Administered in combination with the adjuvant is antigen (4.03), consisting of the antigenic peptide (E), a PEG spacer (F), and albumin binding domain (G).

How an adjuvant distributes *in vivo* will have a significant effect on its activity. For compounds (4.01) and (4.02) chemically linking the two adjuvants mutually alters the biodistribution of each – the lipid tails of α -GalCer should target the CpG to the lymph nodes via albumin hitchhiking; the CpG will render the α -GalCer much more water soluble, creating an amphiphilic compound. The amphiphilic nature of the compound is designed to allow albumin binding while discouraging non-specific insertion into membranes.

Figure 4-1 Components of the vaccine, CpG- α -GalCer adjuvant (4.01), (4.02) and antigen (4.03).

A – CpG ODN 1826

CpG ODN 1826 (5'-tccatgacgttctctgacgtt-3') is a prototypical class B CpG ODN optimised for murine TLR-9. It was selected because it strongly activates plasmacytoid dendritic cells, B cells, and NK cells, causing secretion of IL-6 and moderate secretion of IFN- α . It has previously been used as a potent Th1 stimulating adjuvant. This compound was synthesised commercially by IDT Bio. with a 3'-amino modification and containing a full phosphorothioate backbone.

B – 5-azidopentanoic acid linker

This short linker was attached at the 3' terminus of the modified CpG to introduce an azide for chemoselective ligation.

C – para-aminobenzyl-citrulline-valine linker

The citrulline-valine dipeptide motif is a substrate for cathepsin B, a lysosomal cysteine protease abundant in APCs^{95, 96}. Upon cleavage, the linker collapses to release the α -GalCer intact (Figure 4-3). The bicyclononane portion of this linker introduces a site for strain promoted azide-alkyne cycloaddition (SPAAC) reactions, to cleanly and selectively attach the CpG.

Figure 4-2 Collapse mechanism of the cathepsin labile linker containing a strained cyclooctyne for SPAAC conjugation.

D – α -GalCer

α -GalCer is a potent activator of iNKT cells. Upon cleavage of the PAB-CV linker, the free amine of migrated α -GalCer will be released to allow an O $\rightarrow$ N acyl migration and reforming the active adjuvant (Figure 4-3).

Figure 4-3 Reformation of the active α -GalCer upon release of the free amine following cathepsin cleavage

To optimise albumin binding, we will also synthesise a truncated derivative of α -GalCer (4.02) bearing the optimal acyl chain length for lipid binding to albumin. Human serum albumin has only one binding site for fatty acids with a chain length of 26 carbons, but up to seven for fatty acids with a chain length up to 18 carbons⁹⁷. Liu et al. optimised the albumin binding domain in several assays with a range of lipid structures, arriving at the best being albumin binding domain x.

Figure 4-4 Structure of the optimal albumin binding domain compared to migrated a-GalCer and truncated a-GalCer

E – C-FFRK-SIINFEKL

The FFRK sequence is a linker sequence designed to contain cathepsin and other proteasomal enzyme cleavage sites to improve the processing and presentation of peptide antigens⁹⁸. OVA₂₅₇₋₂₆₄ (SIINFEKL) is a CD8 epitope of the model antigen ovalbumin⁹⁹. The cysteine at the N-terminus of the peptide allows for selective conjugation to maleimides.

This peptide sequence can be altered to incorporate, in theory, any desired antigen to elicit an antigen-specific T cell response.

F – PEG-2000

This PEG spacer is non-uniform, with an average molecular weight of 2000. Liu et al. used a similarly modified peptide in their vaccine and found that without the PEG block the peptide would insert into membranes non-specifically.

G – DPSE albumin binding domain

This diacyl lipid is the optimised albumin binding domain to allow the peptide to hitchhike to the lymph nodes concurrently with the adjuvant.

4.2 Synthesis of CpG- α -GalCer conjugate

Manual synthesis of oligonucleotides is a labour-intensive process, especially without automation for longer polymers. We purchased 100 mg of CpG ODN 1826 modified at the 3' terminus with an amine handle (**4.04**) from IDT Bio, Iowa. The amine modification was chosen as a range of reagents can be used to selectively react without modifying the nucleobases of the oligonucleotide. We wanted to use a strain promoted azide-alkyne cycloaddition (SPAAC) to attach the ODN to α -GalCer as this has reliably been used in our group to attach α -GalCer and other adjuvant compounds to peptides. SPAAC reactions occur rapidly and with few side products¹⁰⁰. The conjugates made in our group utilise a strained alkyne, bicyclononane, as a site for SPAAC chemistry, allowing for the attachment of azido-peptides.

Figure 4-5 - Amine modified CpG ODN purchased from IDT Bio

Firstly, an azide needed to be introduced to the oligodeoxynucleotide (**4.04**) to react with the strained alkyne (**4.07**) selectively. Two standard options are available to attach the azido tether, either using an activated ester or a carbodiimide coupling. The use of activated esters is common for labelling amines with fluorescent or pull-down tags. Carbodiimide couplings have been used with oligonucleotides, though to introduce different modifications at the 5' or 3' termini. Previous reports suggested that carbodiimides like EDC could potentially react with exocyclic amines of the bases, leading to undesired side products¹⁰¹.

5-Azidopentanoic acid was chosen as a short linker to introduce an azide. This was converted to NHS ester (**4.05**) using EDC and N-hydroxysuccinimide in DCM and purified by flash column chromatography. To prepare the CpG-3'-azide, CpG-3'-amine (**4.04**) was dissolved in water and sodium tetraborate buffer, pH 8.6. NHS-ester (**4.05**)

was dissolved in DMSO and added to the CpG-3'-amine (**4.04**) solution in two equal portions (Scheme 4-1). The reaction buffer must be basic to maintain the amine in its non-protonated, nucleophilic form. Tetraborate buffers (pH 8.5 – 9) are recommended for labelling of oligonucleotides, as opposed to the bicarbonate buffers commonly used for protein labelling reactions¹⁰². The reaction takes place at a concentration much lower than most standard organic reactions, with the concentration of the CpG-3'-amine (**4.04**) at only 200 μM ¹⁰². Despite significant excesses of the NHS ester, the reaction only went to completion, as determined by LC-MS (method development and conditions for analysis are discussed below), upon addition of a second portion of NHS ester. For example, after 20 hours of reaction only 50% of the starting material had been consumed, yet, only two hours after addition of the second portion less than 5% of starting material was remaining. The need for two portions may be due to hydrolysis of the NHS ester.

Scheme 4-1 Synthesis of CpG-3'-azide

The oligonucleotide (**4.06**) was precipitated at this stage by addition of 3 M NaCl and ice-cold ethanol. The supernatant was removed and (**4.06**) was telescoped into the next step without further purification. Methods were developed to determine if any residual azide remained as this would react with α -GalCer-PABCV-BCN (**4.07**) in the next step. Using HPLC with MS or charged aerosol detection (CAD), no peak nor mass corresponding to the NHS ester, or 5-azidopentanoic acid was detected. Nor could the NHS ester be detected by TLC; an authentic sample stains bright orange with ceric ammonium molybdate.

The C18 version of α -GalCer (**4.08**) was prepared from N-Boc protected glycolipid (**4.09**) (Scheme 4-2). Firstly, glycolipid (**4.09**) was treated with a 1:1 mixture of TFA:chloroform with thiophenol to remove the Boc group. Free amine (**4.10**) was purified on a reverse phase C18 cartridge (water/methanol). Several methods were attempted to install a C18 acyl chain. Firstly, an acylation using isobutyl chloroformate was performed, but this resulted in very low yield. Fractions eluting with 20% methanol, where this product would be expected, contained high levels of the triethylamine salt. After trituration with water, very little product was actually present. Better results were achieved using an HBTU coupling with stearic acid however the yield was still low. HBTU, or HOBT by-products, were not separated by column chromatography and were subsequently removed by trituration with water. Next, N $\rightarrow$ O migration of the stearyl group was performed under acidic conditions with heating to give the free amine (**4.12**) with approximately 60% conversion. It has previously been shown with α -GalCer that deglycosylated side products arise when this reaction is pushed to completion, negatively impacting the yield and purity of the product. The reaction forms two isomers – the acyl chain may migrate to either hydroxyl, with the major product shown in the scheme below, and the minor product with the acyl chain on the 3-OH. No more than 10% of the minor product was detected. The ester (**4.12**) was sufficiently stable to isolate and characterise. Then, the free amine was reacted with the p-NP-carbonate (**4.13**) in pyridine. This C18- α -GalCer - PABCV-BCN (**4.08**) was purified by flash column chromatography. The full C26 acyl chain α -GalCer-PABCV-BCN (**4.07**) was provided by Dr Regan J. Anderson, prepared in the same manner as (**4.08**), using cerotic acid in place of stearic acid.

Scheme 4-2 Synthesis of C18-a-GalCer-PABCV-BCN

To prepare the CpG- α -GalCer conjugate (Scheme 1-3), initially, the pellet of CpG-3'-azide (**4.06**) was redissolved in 30% DMSO/water v/v. These conditions were appropriate for dissolution of CpG-3'-azide, however, they were not suitable for α -GalCer-PABCV-BCN. Particles were visible as a fine suspension, though the C18- α -GalCer -PABCV-BCN was better dissolved than the C26 version. After concentrating the reaction mixture, the solution was redissolved in neat DMSO to a concentration of 10 mM – a more standard concentration for a SPAAC reaction. The reaction mixture was neon yellow in DMSO. A white solid was not soluble in the DMSO. This appeared to be the salts from the buffer and precipitation of the CpG-3'-azide. The solid was repeatedly washed with DMSO to rinse out any product. The salts did not appear to negatively affect the reaction, though their presence was not ideal given the small scale of reaction. The reaction proceeded quickly and was purified on reversed-phase by preparative HPLC.

Scheme 4-3 Synthesis of CpG- α -GalCer conjugate

4.2.1 TLC

A preparative TLC method has previously been reported¹⁰³ – this was used in an attempt to monitor the reaction attaching α -GalCer-PABCV-BCN to the CpG-3'-azide. A solvent system of 10% water, 35% ammonia, 55% n-propanol was used, and the oligonucleotides were visible under short-wave UV though did not stain using permanganate or ceric ammonium molybdate. Surprisingly, the compounds separated well (Figure 4-6). The unreacted CpG-3'-amine eluted with an R_f of 0.28, the α -GalCer-PABCV-BCN compounds eluted with an R_f of 0.7. A blur appeared with an R_f of 0.5. However, it could not be identified as the CpG-3'-azide or the CpG- α -GalCer conjugate, as no pure sample of the CpG-3'-azide was isolated. Ultimately, HPLC techniques were used as they were much more powerful for compound identification, though this method of analysis could be applied for quick reaction monitoring.

Figure 4-6 TLC plate visualised under short-wave UV. From left to right: α -GalCer-PABCV-BCN, cospot, reaction mixture, CpG-3'-amine.

4.2.2 Conjugate isolation – preparative HPLC.

For purification of this compound by preparative HPLC, methods were developed on a Luna 1 C18 column then scaled to a preparative Luna 2 C18 column. These columns were chosen based on availability and that they would be expected to perform in a similar manner to the Phenomenex Kinetex C18 column used for reaction monitoring (analytical method development, *vide infra*)

We first attempted purification with simple mobile phases, such as 100 mM ammonium acetate. The buffer concentration was ten-fold higher than their concentration in the analytical mobile phase. This is thought to be necessary to maintain ion-pairing efficiency on a larger scale and in the absence of HFIP. The column used has 20-fold the column volume of the 3.0 mm x 50 mm analytical Kinetex column, and much larger injections of were being injected. We wanted to avoid the use of triethylamine buffer systems, as they are less volatile than ammonium acetate, and would require ion-exchange following preparative HPLC. However, it became evident that triethylammonium as an ion-pairing agent was needed for good separation. We ended up reusing the same solvent system used for the analytical methods (8.3 mM triethylamine and 100 mM HFIP in water), with a gradient from 65% to 100% methanol over 15 minutes. In this method, the excess α -GalCer-PABCV-BCN could be detected eluting in the 100% methanol hold.

This method worked well for separation of the CpG- α -GalCer from remaining starting materials. However, the triethylammonium salt of the compound is not ideal for biological testing. During concentration of the preparative fractions before lyophilisation, the pH of the solution did not increase above pH 9, remaining around the same pH as the mobile phase. It would be expected that the HFIP would be removed easily under vacuum, quickly increasing the pH as the triethylamine remained in solution. However, even with concentrating, the solution smelt of HFIP and not triethylamine. It is possible that the pH did not increase as triethylamine may be removed under vacuum, and any remaining triethylamine will be paired with the phosphorothioates of the oligonucleotide. The compound was passed through ion exchange resin to exchange triethylammonium

cations for sodium. A large excess of resin was used to ensure that all anionic sites would exchange. The compound had some retention on the ion exchange resin, requiring washes of up to 20 mL to elute the small amount of compound loaded on to the column. Exocyclic amines in the nucleotide bases may be responsible for some retention.

The isolated product was a fluffy white powder. It was soluble to some extent in water, forming a pale white translucent solution when dissolved in less than 1 mL, but clearing as it became more dilute.

4.3 Albumin binding antigen

The second component of the vaccine is the antigen or peptide. The same albumin binding domain was attached to the peptide to target it to the lymph nodes via the same mechanism. C-FFRK-SIINF-EKL (4.15) was reacted with DPSE-PEG₂₀₀₀-MAL (4.14) in DMSO for two hours and purified by preparative HPLC almost quantitatively (93%) to yield (4.03) as a white powder (Scheme 4-4). The addition of the thiol to the maleimide occurred quickly and with minimal side products detected during the reaction. Some disulfide form of the peptide was present in the reaction mixture. This did not negatively affect the yield, though if it were forming in larger quantities, more care could be taken to exclude oxygen from the reaction.

This methodology can be broadly applied to any peptide antigen with an N-terminal cysteine.

Scheme 4-4 Synthesis of albumin binding peptide antigen.

4.4 *In vitro* measurements of albumin binding

Given the important consequences of drug binding to albumin, many *in vitro* methods exist to determine the extent of compound binding to albumin¹⁰⁴. We focused on two approaches, NMR and size exclusion chromatography.

4.4.1 NMR

A saturation-transfer difference experiment relies on the exchange of a ligand between the bound and unbound state. In an STD experiment, the sample is made up of a low concentration of the protein or receptor with a high excess of the ligand, or pool of ligands. Two spectra are then recorded: a 1D ¹H NMR – the reference, or “off-resonance” spectrum - and a second ¹H NMR where some resonances of the protein are selectively irradiated – the “on-resonance” spectrum”^{105, 106}. The STD spectrum is the difference between the “off-resonance” and “on-resonance” spectra, displaying the resonances that experienced saturation in the “on-resonance” spectrum.

The selective irradiation of the protein protons causes spread of magnetisation across the entire protein by the nuclear Overhauser effect (NOE). If a ligand is bound, the magnetisation will transfer to the ligand protons nearby. These ligands diffuse into solution where they are detected. Unbound ligands do not become saturated¹⁰⁷. In the “on-resonance” spectra, the signal intensity of bound molecules will decrease, therefore, in the difference spectra, their intensity will increase, while unbound molecules will have no change and are therefore removed from the difference spectra.

An STD experiment is not useful for ligands with high affinity for the protein. The saturated ligands are not effectively transferred into solution and are not observed. Conversely, very weak affinity ligands are less likely to be present in the binding site and may never get saturated³.

It is possible to measure the K_d of a ligand from an STD experiment. They may be limited in accuracy due to non-specific binding which will also contribute to the STD signal¹⁰⁵.

We first attempted to validate this experiment using 6-methyltryptophan as described by Viegas et al¹⁰⁵. We were unable to obtain a spectra showing a real difference signal.

Given the size of the final compound (MW > 6000), it will tumble slowly in solution and not experience efficient magnetisation. Experiments could be conducted to determine whether α -GalCer binds to albumin.

4.4.2 Size exclusion chromatography

Liu et al. demonstrate binding of their CpG vaccine to albumin through a range of methods. One key method they used to demonstrate binding to albumin is size exclusion chromatography. Size exclusion chromatograph separates compounds based on molecular weight. Large molecules pass easily through the column as they are not retained in the pores, while small molecules must travel a longer path through the pores of the column, getting retained¹⁰⁸. Human serum albumin and the analyte will have different retention times based on the difference in molecular weight. Binding of the analyte to human serum albumin would allow it to pass through the column with the same retention time as albumin. Therefore, binding can be measured by running the protein and analyte each alone, and a mixture where the two have been incubated together. A reduction in the intensity of the peak corresponding to the analyte and an increase in intensity at the retention time of human serum albumin would indicate binding¹⁰⁹.

Liu et al. described several other experiments to validate binding of the analyte to albumin such as SDS-PAGE after protein pull down, biolayer interferometry and binding to albumin-conjugated agarose. We do not have the capabilities for some of these methods, and furthermore, they require further chemical modification of the compound.

Due to difficulties purifying the compound, we did not have time to replicate these experiments.

4.5 Analysis and purification of oligonucleotide compounds

LC-MS is the gold-standard of analysis for oligodeoxynucleotides, however, it comes with several caveats. The polyanionic backbone of the oligonucleotide predominantly dictates the chromatographic and mass spectrometry behaviour of the compounds. LC-MS of oligonucleotides is a delicate balance of several factors to achieve retention and good signal to noise ratio in ESI-MS. Factors affecting chromatographic properties are not independent of factors that influence ESI-MS behaviour.

ESI requires the analyte to exist as ions in solution. Therefore the mobile phase will greatly affect the ESI-MS signal obtained¹¹⁰. ESI-MS detection performs best when there is low analyte flow with low viscosity, low surface tension, high volatility and low ionic strength. Yet for optimal separation on reversed-phase, the mobile phase must be low pH, and highly aqueous; the properties of such a solvent system will have unfavourable properties for ESI-MS. For an oligonucleotide, an envelope of multiple charge states will be generated from the loss of a number of protons. H^+ -alkali⁺ exchange ions will be detected from alkali metals in solution. Both these will reduce the mass spectrometer detection sensitivity. The degree of this problem increases with the length of the oligonucleotide, and even more so with phosphorothioate backbone ODNs.

A phosphorothioate backbone in the oligonucleotide can make analysis even more challenging as the replacement of a non-bridging oxygen with sulfur forms stereocentres at every linkage. For CpG ODN 1826 there are therefore 2^{20} possible diastereomers, therefore peaks tend to be broad.

4.5.1 Chromatographic conditions

Ion-pair RP-HPLC has become the best method for analysis of oligonucleotides. An organic base, such as triethylamine, forms an ion pair with the acidic phosphate/phosphorothioate backbone of the oligonucleotide. This improves the retention of the oligonucleotide as the compound is effectively neutrally charged to the column, and the hydrophobic counterions will interact more with the column than the

anionic backbone. This demonstrates one of the key compromises that must be accepted: increasing the concentration of triethylamine/triethylammonium ion-pairing reagents will provide improved separation of compounds, however, suppress the intensity of the MS signal. The extent of signal suppression depends on the affinity of the ion-pairing reagent to the analyte in the gas phase¹¹¹.

A key development for LC-MS analysis of oligonucleotides was the addition of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to the mobile phase. Appfel et al.¹¹² propose the use of 16.3 mM triethylamine and 400 mM HFIP in methanol as the optimal solvent system for LC-MS analysis of oligonucleotides. Compared to a triethylammonium acetate buffer system, HFIP is much more volatile – a boiling point of 57 °C compared to 118 °C of acetic acid – and remains predominantly uncharged at the pH of the system allowing for easy desolvation. Despite the low concentration of triethylamine in the eluent, the resolution is maintained or improved as it is suggested that the HFIP enhances the hydrophobicity of the triethylamine. The MS signal is also improved, as the HFIP can overcome suppression by triethylamine and reduces the cation adduct formation. It is proposed that “the behaviour of this system is based on the dynamic adjustment of the pH in the electrospray droplet as a function of the preferential removal of anionic counterion from the droplet by evaporation”¹¹². Essentially, as the HFIP evaporates from the surface of the droplet, the pH continues to rise and favours dissociation of the ODN-TEA ion pair, forming an ODN anion in the gas phase which is then detected.

Two columns were examined for analytical method development: a Phenomenex Kinetex® 2.6 µm C18 50 x 3 mm column and an Agilent Zorbax SB-C18 1.8 µm column. These columns were chosen based on availability, and previous use with glycolipid conjugates. The Zorbax column was investigated as it can tolerate higher temperatures which may be useful for disrupting secondary which could potentially form. The reduced particle size may also improve resolution. However, the analyses on this column were not as good as those on the Kinetex column, and the column was prone to blockage. Method

development on the Zorbax was not pursued, all further analytical work was conducted on the Kinetex column.

With respect to the mobile phase, initially, we examined the use of ammonium acetate and triethylammonium acetate buffers for the eluent. These gave good retention on the column, with reasonable peak shape. However, in these conditions, only a very weak MS signal was detected. From the analyses conducted, eluent containing HFIP increased the MS signal over 80-fold. For these analyses, the mobile phase was aqueous triethylamine and HFIP and unmodified methanol. We used a lower concentration of triethylamine and HFIP (8.3 mM and 100 mM respectively) than what Appfel described without issue. In addition to a good MS signal, the peaks were also much narrower, with a peak width at half height of 3.1 compared to 13.3 seconds, and, with less tailing. This system worked well for the detection and reaction monitoring of the CpG-3'-amine and CpG-3'-azide (Fig 4-7).

After conjugation of the CpG-3'-azide to α -GalCer, HFIP remained an important component of the eluent. This compound was eluting at 90% MeOH, therefore the concentration of HFIP was as low as 1% in the mobile phase, as the aqueous triethylamine-HFIP buffer was the only eluent containing HFIP. Following addition of 100 mM HFIP into the methanol, a mass for the CpG- α -GalCer conjugate could be detected as the mobile phase had a constant concentration of HFIP across the entire gradient. This again emphasises the necessity of HFIP for a good MS signal.

Developing analytical LCMS methods was essential for reaction monitoring and compound identification. We were unable to obtain high-resolution MS for these compounds, and ^1H NMR analysis was not feasible on this scale of synthesis, nor with the molecular weight of the compounds.

Figure 4-7 - Chromatography method development, CpG-3'-amine (4.04), UV 260 nm. All gradients were 10-90% Methanol Top to bottom: aqueous mobile phase containing 10 mM ammonium acetate pH 5.5, 10 mM triethylammonium acetate pH 6.3, 100mM HFIP + 8.3 mM TEA pH 8.6.

Solvent system	Signal to noise ratio
10 mM ammonium acetate & methanol	-
10 mM triethylammonium acetate & methanol	1.6
100 mM HFIP + 8.3 mM TEA & methanol	155.9

Table 4-1 Table 1 - Signal to noise ratio for MS signal, TIC.

4.5.2 Mass spectrometer settings

A range of settings can be adjusted on the mass spectrometer to optimise the signal obtained. During the analytical method development for these oligonucleotide compounds, we experimented with a range of detector variables.

Firstly, the negative mode was found to be significantly better than the positive mode.

This is because the mass spectrometer will be detecting the anionic oligonucleotide after dissociation from the ODN-TEA ion pair. Secondly, narrowing the scan range of the mass spectrometer can increase the sensitivity of detection. Reducing the scan window to 900 –

1700 m/z allowed an improvement of s/n by three-fold. Single ion monitoring allows for specific detection of certain masses and offers greater sensitivity to a full scan. Thirdly, altering the fragmentor voltage offered an only slight advantage when buffers with high triethylamine concentrations were used. A three-fold increase in fragmentor voltage afforded only three-fold increase in signal to noise ratio, with the total ion count still below 1000. In the HFIP buffer system, the signal was actually improved slightly by decreasing the fragmentor voltage. While some advantage was gained from adjusting these settings, by far the biggest improvements in MS detection were obtained by optimising the mobile phase components as previously described.

4.6 Summary and future directions

In summary, we were able to synthesise two bifunctional CpG- α -GalCer vaccine adjuvant. Methods were developed for the synthesis, analysis, and purification of these compounds, the likes of which had not been previously handled in our group. Methods for analysis and purification were particularly challenging and time consuming given the nature of the oligonucleotide compounds' behaviour on reversed phase columns. The methods used were successful for the preparation of reasonable quantities of pure compound.

CpG-3'-5-azidopentanamide-BCN-PABCV-C18- α -GalCer (4.02)

Figure 4-8 LC-MS trace of (4.02), top trace UV 260 nm, bottom, mass spectrum extracted from highlighted peak.

CpG-3'-5-azidopentanamide-BCN-PABCV-C26- α -GalCer (4.01)

Figure 4-9 LC-MS trace of (4.01), top trace UV 260 nm, bottom, mass spectrum extracted from highlighted peak.

In vitro measurements of albumin binding were not obtained. More work is needed to confirm the albumin binding properties of the compound, and whether this correlates with lymph node accumulation *in vivo*. Furthermore, whether lymph node accumulation actually generates a better response.

While biological testing did not occur during the body of this work, it will be conducted in the future now that the compound has been prepared and characterised. *In vitro* and *in vivo* studies will be carried out to measure the stimulatory ability of the bifunctional

adjuvants. Human peripheral blood mononuclear cells will be used to measure the stimulatory ability of the adjuvant with respect to cytokine production and the APC-stimulating capacity of the compounds. The activation of dendritic cells and bias of the response generated will be measured. Further assays may include *in vivo* cytotoxicity assays, like the VITAL assay¹¹³, assessment of *in vivo* dendritic cell activation, assessment of the T cell response, and testing in a therapeutic tumour model, depending on the activity demonstrated *in vitro*. We expect that the bifunctional adjuvant would generate a stronger response than CpG or α -GalCer alone, and than the admixture of CpG and α -GalCer, with a strong bias to a Th1 phenotype. While the adjuvants may synergise, more complex factors such as the timing of different signals and cellular uptake and localisation of the compound may mean it does not perform as well as we would expect.

The synthesis of this compound still has room for improvement. The method for synthesis of CpG-3'-azide works well. It could be improved by the addition of a third portion of NHS-ester to ensure complete conversion of CpG-3'-amine starting material. Ideally, serial dilution to determine the limit of detection of remaining azide would be conducted to ensure it was not present after precipitation. While precipitation was remarkably effective, a better way to purify this compound would be the use of a PD-10 size exclusion column. This disposable gel column traps compounds with a molecular weight below 5,000 in the matrix. This may be more efficient than precipitation with ethanol and NaCl as it would ensure removal of any small molecule linker regardless of its solubility in ethanol. The column would remove any buffer components and avoid carrying high concentrations of salt through to the following reaction. With respect to the final purification step, ideally, a preparative HPLC method would avoid the use of triethylamine to remove the need for an ion exchange column as the last step. As an alternative to preparative HPLC, size exclusion chromatography could be an option for purification based on size, especially if a peptide were to be incorporated into one compound.

For future immunological studies, it will be necessary to create negative controls. The structure of this would be similar to the CpG- α -GalCer conjugate synthesised here, but lacking immunological activity. Two compounds would be synthesised: an inactive glycolipid conjugated to an active CpG, and the active glycolipid conjugated to a scrambled CpG. This would be able to decouple immunological activity from distribution properties as they would have similar physicochemical properties but distinct immunological activities. These compounds may use β -galactosylceramide or β -mannosylceramide, both far less potent activators of iNKT cells. A scrambled ODN would have all the same bases as present in the active ODN but reordered such that CpG motifs are not present and therefore are not recognised by TLR-9. For example, a known negative control of a TLR-9 agonist is 5'-tcctgagcttgaagt-3' which does not demonstrate agonistic nor antagonistic activity¹¹⁴.

5 Summary and Discussion

In the first chapter, steps in the synthetic methodology for the MAIT ligand compounds were improved. The method used for hydrogenolysis to produce ribitylamine (**2.04**) was improved, achieving conversions of over 90%. The previous methodology had only reached 60% conversion. Current methodologies still require optimisation, and new methodologies may need to be explored for optimal yields and purities of these compounds. No bifunctional adjuvant came about from this work, though the proposed bifunctional adjuvant targeting two different subsets of innate-like T cells would be an interesting target.

In the second chapter, synthetic methodology for the use of PEG heterobifunctional linkers was also improved. These linkers and methods can find use in a range of settings. Whether to improve the physicochemical properties of other compounds or for other purposes such as labelling to create a chemical probe. We were able to prepare three derivatives of α -GalCer across a range of hydrophilicity, which were shown to be water soluble.

In the third and final chapter, we successfully prepared and characterised two bifunctional, CpG- α -GalCer compounds. While biological testing of these compounds was not conducted during this thesis, it will be in the future. The outcome of these tests will be particularly interesting.

Methods were developed for the synthesis, analysis and purification of these compounds. These compounds required different handling methods to the glycolipids that are normally handled within our group. Each adjuvant had disparate physicochemical properties, α -GalCer, a comparatively low molecular weight glycolipid, compared to the

CpG, a large, polyanionic biomolecule. This created challenges for the handling, analysis, and conjugation of these compounds. Developing analytical HPLC methods was essential for the synthesis of these compounds. Without these methods, reaction monitoring and compound identification would not have been possible as ^1H NMR analysis was not feasible on this scale of synthesis, nor with the molecular weight of the compounds. For conducting reactions, it was fortunate that both dissolved reasonably well in DMSO, as more complex solvent systems may have been needed to account for their differing solubility properties. These methods will continue to find use as different work with these adjuvants is conducted.

We can speculate that the biodistribution properties of each adjuvant were mutually altered. From the handling of this compound, we saw that it dissolved to some extent in water alone, which α -GalCer does not. The C18-version (**4.02**) appeared to be more soluble than the C26-version (**4.01**) by visual inspection. Furthermore, the retention time on reversed-phase of the conjugate was greater than that of the CpG-3'-amine, and less than the α -GalCer. Similarly, (**4.02**) was retained less on reversed-phase than the (**4.01**). This suggests that the compound has amphiphilic properties – the CpG is hydrophilic enough to bring α -GalCer into solution. The distribution of this bifunctional adjuvant will be significantly different to the distribution of either adjuvant alone. The albumin binding and lymph node targeting properties of the compounds synthesised in this work remain to be tested. If these claims hold, both strategies could be applied more broadly to other immunomodulatory compounds to improve their potency and safety and improve the strategies used in vaccine development. They may also find use in other applications such as for targeted drug delivery for cancer lymph node metastases, or imaging purposes.

In this work, peptide antigens were not chemically linked to the bifunctional adjuvant. This allows for the use of the adjuvant compound in combination with any desired antigen or in different vaccine formulations. Previous studies have combined, or chemically linked, CpG adjuvants with peptides to improve the response¹⁵. Future work in

our group may focus on the chemical attachment of antigens to this new adjuvant. A third biorthogonal handle will need to be installed to chemically link the peptide in a way which will release it after administration.

While adjuvant development has previously been “alchemy”, this will not suffice for future vaccine development. Synthetic vaccines hold promise for treatment of chronic viral infections and cancer and will be more likely to succeed if they are well designed. A rationally designed vaccine will consider the suitable formulation, ideal antigens, and custom adjuvants. Advances in immunological principles will help to guide the development of these vaccines.

The most successful vaccines, such as the polio vaccine, contain a range of danger signals and antigens. It is clear that a single adjuvant alone will not generate an immune response of the same magnitude or quality as a combination. Synergy displayed through chemical linking of CpG and α -GalCer will demonstrate the utility of chemically linking adjuvants working through different pathways, and different cell types, and encourage research into other bifunctional, or polyvalent adjuvants for use in and outside of the therapeutic cancer vaccine space.

6 Experimental

6.1 General experimental details

Anhydrous solvents were purchased commercially and used without further treatment.

Air-sensitive reactions were carried out under argon. 1,4-Dioxane was distilled from acidic 2,4-dinitrophenylhydrazine.

NH₂-dPEG₄-(m-dPEG₂₄)₃, NH₂-dPEG₄-(m-dPEG₈)₃, and heterobifunctional dPEG linkers, SPDP-dPEG₂₅-NHS and TFP-dPEG₂₅-MAL were purchased from Quanta Biodesign Ltd. DPSE-PEG₂₀₀₀-MAL was purchased from Laysan Bio Inc. CpG-3'-NH₂ was purchased from IDT Bio.

Thin-layer chromatography was performed on aluminium sheets coated with 60 F₂₅₄ silica (Merck). Plates were visualised using an ultraviolet lamp (shortwave – 254 nm, longwave 366 nm), ceric ammonium molybdate, or potassium permanganate staining. Flash column chromatography was performed on Reveleris silica cartridges (38.6 µm) or SiliCycle silica gel (40 – 63 µm).

NMR spectra were recorded on a Bruker 500 MHz spectrometer. ¹H NMR spectra were referenced to tetramethylsilane at 0 ppm as an internal standard or to the residual solvent peak. ¹³C NMR spectra were referenced to tetramethylsilane at 0 ppm as an internal standard or to the residual solvent peak. CDCl₃-CD₃OD mixtures were always referenced to the methanol peak at 3.31 ppm.

High-resolution electrospray ionisation (ESI) mass spectra analyses were undertaken on a Waters Q-TOF Premier™ Tandem Mass spectrometer fitted with a Waters 2795 HPLC.

Analytical HPLC data was obtained on an Agilent 1260 HPLC with an Agilent 6130 single quadrupole mass spectroscopic detector using ESI, or Dionex Corona Ultra RS charged aerosol detector (CAD) as required. Preparative HPLC was performed on an Agilent 1260 Infinity system equipped with a quaternary and preparative pump with DAD and ELSD detectors.

6.2 Buffer preparations

0.2M sodium tetraborate pH 8.5 labelling buffer

Sodium tetraborate decahydrate (19g , 94.4 mmol) was dissolved in 500 mL of distilled water. The pH was adjusted to 8.5 using HCl.

8.6 mM triethylamine – 100 mM HFIP HPLC solvent

HFIP (10.5 mL, 99.7 mmol) was dissolved in HPLC grade water (950 mL). To this was added HPLC grade triethylamine (1.2 mL, 8.61 mmol) while stirring vigorously. The solution was made up to 1 L with water. The pH was measured to be 8.5 and was not adjusted.

0.1 M Ammonium acetate

Ammonium acetate (7.7g, 99.9 mmol) was dissolved in 1 L of water. The solution was filtered through a 0.2 micron filter before use.

0.1 M Triethylammonium acetate pH 7

Glacial acetic acid (5.6 mL, 97.8 mmol) was dissolved in water (950 mL). To this was added triethylamine (13.86 mL, 99.4 mmol) with vigorous stirring. The pH was adjusted to approximately 7 using glacial acetic acid and triethylamine as needed, then the volume was made up to 1 L.

Thanks to other members of the Painter lab for synthesis of these compounds used during the course of this thesis:

Ribitylamine (**2.04**) was prepared by Dr. Regan J. Anderson through literature methods⁷³.

Glycolipid (**4.09**) was prepared by Dr. Benjamin Compton by literature methods.

6-thio- α -GalCer (**3.01**) was prepared by Dr Regan Anderson as described by Compton et al.¹¹⁵

(4.07)

α -GalCer-PABCV-BCN (4.07) was prepared by Dr. Regan J. Anderson by procedures currently unpublished (manuscript in preparation)

5-azidopentanoic acid was prepared from 5-bromopentanoic acid by Taylor Cooney.

C-FFRK-SIINKFEKL (4.15) peptide was prepared by Candice Gu using automated Fmoc SPPS.

(2*R*,3*S*,4*S*)-5-(((*Z*)-benzylidene)amino)pentane-1,2,3,4-tetraol (2.08)

To an ice-cold aqueous solution of ribitylamine (**2.04**) (590 mg, 3.90 mmol) in water (3 mL) was added benzaldehyde (350 μ L, 3.45 mmol). The mixture was stirred for 30 minutes on ice. No product formed. 5 mL of ethanol was added to the solution and was refluxed at 100 °C for 30 minutes. No product detected by NMR.

(2*R*,3*S*,4*S*)-5-(((*Z*)-2-hydroxybenzylidene)amino)pentane-1,2,3,4-tetraol (2.09)

To a stirred solution of ribitylamine (**(2.04)**) (532 mg, 3.52 mmol) in water (3 mL) was added 2-hydroxybenzaldehyde. The reaction mixture was stirred on ice for 10 minutes. No reaction occurred. Sodium bicarbonate (202 mg, 2.4 mmol) was added to the solution. The reaction was refluxed for 30 minutes at 100 °C. No precipitate formed, nor was product detected by NMR.

(2*R*,3*S*,4*S*)-5-(benzylamino)pentane-1,2,3,4-tetraol (2.02)

To a stirred solution of D-ribose (10.073 g, 67.095 mmol) in methanol (70mL) was added benzylamine (7 mL, 64.1 mmol). The reaction stirred for 20 h. After 24 h, PtO₂ (323mg, 1.14 mmol) was added to the solution and placed under an atmosphere of H₂ overnight. After 24 h of stirring, the reaction mixture was filtered through celite, concentrated to half the volume, and precipitated from ethyl acetate. The precipitate was filtered and washed with ethyl acetate. After drying under vacuum, the product was recrystallised from ethyl acetate and methanol to afford ribitylbenzylamine as yellow crystals (8.165 g, 53%).

¹H NMR (500MHz, CD₃OD) 7.35 -7.29 (m, 4H), 7.26 - 7.19 (m, 1H), 3.87 (ddd, *J* = 6.9, 5.6, 4.4 Hz, 1H), 3.80 (d, *J* = 13.0 Hz, 1H), 3.77 - 3.70 (m, 2H), 3.70 - 3.60 (m, 2H), 3.60 - 3.54 (t, *J* = 5.8, 1H), 3.31 (q, *J* = 1.55 Hz, 1H), 2.90 (dd, *J* = 12.0, 4.1 Hz, 1H), 2.74 (dd, *J* = 12.2, 6.7 Hz, 1H).

(2*R*,3*S*,4*S*)-5-aminopentane-1,2,3,4-tetraol (2.04)

Ribitylbenzylamine (**2.02**) (508 mg, 2.1054 mmol) was dissolved in MeOH (5 mL). 10% palladium on carbon (22 mg, 0.21 mmol) was added to the solution and placed under an atmosphere of hydrogen using a balloon. The reaction was left at room temperature, stirring under hydrogen for 20 h.

Hydrogenolysis with acetic acid

Ribitylbenzylamine (**2.02**) (501 mg, 2.0764 mmol) was dissolved in methanol (5 mL) with glacial acetic acid (0.2 mL). To this was added 10% palladium on carbon (22 mg, 0.21 mmol) and placed under an atmosphere of hydrogen using a balloon. The reaction was left at room temperature, stirring under hydrogen for 20 h.

Transfer hydrogenolysis with formic acid

Ribitylbenzylamine (**2.02**) (515 mg, 2.1344 mmol) was dissolved in methanol (4.5 mL) and formic acid (0.5 mL, 10 mmol, 88 mass%). To this was added 10% palladium on carbon (22 mg, 0.21 mmol) and the reaction was refluxed at 80 °C for 20 h.

Transfer hydrogenolysis with ammonium formate

Ribitylbenzylamine (**2.02**) (515 mg, 2.1344 mmol) was dissolved in methanol (4.5 mL) and ammonium formate (522 mg, 8.26 mmol). To this was added 10% palladium on carbon (22 mg, 0.21 mmol) and the reaction was refluxed at 80 °C for 20 h.

Transfer hydrogenolysis with hydrazine

Ribitylbenzylamine (**2.02**) (515 mg, 2.1344 mmol) was dissolved in methanol (5 mL). To this was added hydrazine hydrate (1.1 mL, 16.5 mmol, 80% aqueous) and 10% palladium on carbon (22 mg, 0.21 mmol) and was refluxed at 80 °C for 20 h.

Ribitylbenzylamine (**2.02**) (504 mg, 2.0889 mmol) was dissolved in methanol (5 mL) and ammonium hydroxide (0.3 mL, 2 mmol, 28 mass%). To this was added 10% palladium hydroxide on carbon (24.3 mg, 1.71 mmol). The reaction was placed under an atmosphere of hydrogen using a balloon. The reaction was left at room temperature, stirring under hydrogen for 20 h.

¹H NMR (500MHz, CD₃OD) 3.79 (dd, *J* = 10.0, 3.3, 1H), 3.74 – 3.68 (m, 2H), 3.65 (dd, *J* = 11.1, 5.8 Hz, 1H), 3.56 (t, *J* = 6.2 Hz, 1H), 3.36 (br s, 1H), 2.92 (dd, *J* = 13.1, 3.5 Hz, 1H), 2.76 (dd, *J* = 12.7, 7.5 Hz, 1H).

6-(((2*S*,3*S*,4*R*)-2,3,4,5-tetrahydroxypentyl)amino)pyrimidine-2,4(1*H*,3*H*)-dione
(2.06)

To a stirred solution of D-ribose (55 mg, 0.37 mmol) in ethanol (2 mL) was added 6-aminouracil (2.15) (63 mg, 0.50 mmol) and concentrated hydrochloric acid (0.2 mL). No product formed.

To a 10:1 mixture of methanol to acetic acid (1.1 mL) was added 6-aminouracil (64 mg, 0.504 mmol), D-ribose (75 mg, 0.50 mmol) and borane-2-picoline complex (59 mg, 0.469 mmol). The reaction stirred at room temperature for two hours. No product formed.

α -GalCer-PEG-25 conjugate (3.07)

(3.07)

A solution of 2-(2-aminoethoxy)ethanol (182 μ L, 0.0126 mmol, 12.5%) and MAL-dPEG₂₄-TFP (140 μ L, 0.0114 mmol) in DMSO was heated to 60 °C for 15 minutes. To this was added 6-thio- α -GalCer (0.00572 mmol) in DMSO (500 μ L). The reaction was purified by preparative HPLC (1.05 mg, 8%, 79.6% pure by HPLC)

HRMS-ESI calculated for C₁₁₂H₂₁₆N₄O₃₈S [M+Na]⁺ 2280.4711, found 2280.4897

α -GalCer-PEG-52 conjugate (3.08)

(3.08)

A solution of $\text{NH}_2\text{-dPEG}_4\text{-(m-dPEG}_8\text{)}_3$ (180.3 μL , 0.00816 mmol) and MAL-dPEG₂₄-TFP in DMSO was heated to 60 °C for 15 minutes in a water bath. To this was added 6-thio- α -GalCer (0.00572 mmol) in DMSO (500 μL). The reaction was purified by preparative HPLC. A white sticky film was obtained (2.9 mg, 12%, 90.5% pure by HPLC)

HRMS-ESI calculated for $\text{C}_{183}\text{H}_{354}\text{N}_8\text{O}_{71}\text{S}$ $[\text{M}+2\text{Na}]^{2+}$ 1939.69428, found 1940.2017

α -GalCer-PEG-100 conjugate (3.09)

(3.09)

A solution of NH₂-dPEG₄-(m-dPEG₂₄)₃ (411 μ L, 0.0108 mmol) and MAL-dPEG₂₄-TFP (140 μ L, 0.00969 mmol) in DMSO was heated to 60 °C in a water bath for 15 minutes. After 15 minutes, 6-thio- α -GalCer (0.00572 mmol) in DMSO (500 μ L) was added. The reaction mixture was heated for a further 15 minutes at 60 °C. The reaction was purified by preparative HPLC. Dried to afford a sticky white film (11.2 mg, 33%, 94.1% pure by HPLC)

HRMS-ESI calculated for C₂₇₉H₅₄₆N₈O₁₁₉S [M+3Na]³⁺ 2005.54667, found 2005.5393

DPSE-PEG₂₀₀₀-MAL-C-FFRK-SIINFELK (4.03)

C-FFRK-SIINFELK (3.45 mg, 0.00147 mmol) was dissolved in DMF (100 μ L) and sonicated to aid dissolution. The peptide solution was added to a solution of DPSE-PEG₂₀₀₀-MAL (2.18 mg, 0.00741 mmol) in DMF (100 μ L). The mixture was maintained at 25 °C in a water bath for two hours. The reaction was monitored by HPLC. After 2.5 h the reaction was determined to be complete. The product was purified using preparative HPLC (water, methanol, TFA). To 5 mL of t-BuOH were added the fractions containing the product. The mixture was concentrated to approximately 5 mL. To this was added 15 mL of water for injection. The solution was frozen and lyophilized to yield a white sticky fluff (3.2 mg, 95%).

MS-ESI: m/z calculated for C₂₁₇H₃₈₈N₂₂O₇₅PS

(2*S*,3*R*,4*S*,5*R*,6*R*)-2-(((2*S*,3*S*,4*R*)-2-amino-3,4-dihydroxyoctadecyl)oxy)-6-(hydroxymethyl)tetrahydro-2*H*-pyran-3,4,5-triol (**4.10**)

Boc-protected glycolipid (**4.09**) (175 mg, 0.302 mmol) was dissolved in 2:1 CHCl₃:MeOH (3 mL) to which was added TFA (1 mL, 13.5 mmol) and thiophenol (30 μ L, 0.292 mmol). The mixture was left stirring overnight under argon at room temperature. After 20 h the reaction was not complete. The solvent was removed under vacuum, and TFA (1 mL) was added neat. After the reaction was determined to be complete, the compound was purified by reversed-phase chromatography (water, methanol, TFA, 75%). This afforded a white film (118 mg, 66%).

¹H NMR (500 MHz, 2:1 CDCl₃:CD₃OD) δ 4.88 (d, J = 3.8 Hz, 1H), 4.09 (dd, J = 10.4, 2.8 Hz, 1H), 3.92 (br d, J = 3.7 Hz, 1H), 3.82 (td, J = 10.0, 3.9, 2H), 3.79 – 3.74 (m, 3H), 3.72 (dd, J = 10.3, 4.0, 2H), 3.67 – 3.62 (m, 1H), 3.60 – 3.52 (m, 1H) 3.39 (br s, 3H), 1.80 (br t, J = 10.3, 1H), 1.50 (br, 1H), 1.26 (br, 26H), 0.89 (t, J = 7.0, 3H)

N-((2*S*,3*S*,4*R*)-3,4-dihydroxy-1-(((2*S*,3*R*,4*S*,5*R*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)octadecan-2-yl)stearamide (4.11)

To a stirred solution of glycolipid **(4.10)** (50 mg, 0.104 mmol) in DMF (15 mL) was added stearic acid (52 mg, 0.174 mmol), followed by DIPEA (82 μ L, 0.470 mmol), and finally HBTU (67 mg, 0.173 mmol). The reaction was stirred for 72 h. Methanol (3 mL) was added to quench the reaction. The mixture was concentrated under vacuum and redissolved in 2:1 CHCl₃:MeOH (6 mL) to dry load on to silica. The product was purified using flash column chromatography (chloroform, methanol, 17%). HBTU was still present in the NMR of the product, therefore the solid was triturated with water. Drying under vacuum afforded a white film (14 mg, 22%).

¹H NMR (500 MHz, 2:1 CDCl₃:CD₃OD) δ 4.90 (d, J = 3.8 Hz, 1H), 4.20 (m, 1H), 3.9 (d, J = 2.7 Hz, 1H), 3.89 (dd, J = 11.4, 5.0, 1H), 3.83 – 3.67 (m, 6H), 3.56 (m, 2H), 2.21 (t, J = 7.7 Hz, 2H), 1.61 (m, 4H), 1.27 (br s, 54H), 0.89 (t, J = 6.9 Hz, 6H).

¹³C NMR (126 MHz, 2:1 CDCl₃:CD₃OD) δ 174.5, 99.7, 74.7, 71.9, 70.8, 70.2, 69.7, 68.9, 67.3, 61.7, 50.4, 36.4, 32.4, 31.8, 29.7, 29.6, 29.6, 29.5, 29.4, 29.3, 29.3, 29.2, 25.8, 25.7, 22.5, 13.7. Consistent with literature^{116, 117}.

HRMS-ESI calculated for C₄₂H₈₃NO₉ [M+H]⁺ 745.6068, found 746.6141

(2*S*,3*S*,4*R*)-2-amino-3-hydroxy-1-(((2*S*,3*R*,4*S*,5*R*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)octadecan-4-yl stearate (4.12)

1,4-dioxane was freshly twice-distilled twice from acidified 2,4-dinitrophenolhydrazine. Glycolipid (4.11) (10 mg, 0.01340 mmol) was dissolved in 1,4-dioxane (3mL) under Ar with heating to 60 °C. To this solution was added conc HCl (10 μ L, 37%). The mixture was heated at 70 °C for 20 minutes, then cooled and lyophilised. ^1H NMR analysis indicated approximately 60% conversion to the migrated product. The product was purified by flash chromatography on silica (chloroform, methanol, 25%) to yield a white film (10.8 mg, 54%).

^1H NMR (500 MHz, 2:1 CDCl_3 : CD_3OD) δ 4.92 (td, J = 17.5, 8.7, 3.0, 1H) 4.87 (d, J = 3.8 Hz, 1H), 4.12 (dd, J = 10.7, 2.8, 1H) 3.96 (br d, J = 2.9 Hz, 1H), 3.88 – 3.82 (m, 6H), 3.79 (d, J = 6.4, 2H), 3.77 – 3.67 (m, 3H), 3.54 (t, J = 3.5, 1H), 2.37 (t, J = 7.5 Hz, 2H), 1.61 (m, 4H), 1.27 (br s, 54H), 0.89 (t, J = 6.9 Hz, 6H).

(2*S*,3*S*,4*R*)-2-((((4-((*S*)-2-((*S*)-2-((((1*R*,8*S*,9*R*)-bicyclo[6.1.0]non-4-yn-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)-5-ureidopentanamido)benzyl)oxy)carbonyl)amino)-3-hydroxy-1-(((2*S*,3*R*,4*S*,5*R*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)octadecan-4-yl stearate (**4.08**)

To a flask containing solid migrated-C18- α -GalCer (**4.12**) (10.8 mg, 0.0145 mmol) was added PABCV-pNP-carbonate (**4.13**) (20.8 mg, 0.0289 mmol) which was left under vacuum for two hours. To the solid mixture was added pyridine (180 μ L) followed by triethylamine (2.1 μ L, 0.0290 mmol). After two hours, a second portion of triethylamine (2.1 μ L, 0.0290 mmol) was added. The reaction stirred for 72 h. The solution was concentrated under vacuum, then dissolved in 5% Methanol/DCM to be purified by column chromatography (chloroform, methanol, 35%) to yield a white film (6.8 mg, 33%).

^1H NMR (500 MHz, 2:3 CDCl_3 : CD_3OD). δ 7.58 (d, J = 8.1 Hz, 2H), 7.32 (d, J = 8.7, 2H), 5.14 – 4.95 (m, 3H), 4.84 (d, J = 3.8 Hz, 1H), 4.59 – 4.52 (m, 1H), 4.33 (s, 1H), 4.04 – 3.93 (m, 3H), 3.91 – 3.82 (m, 2H), 3.82 – 3.64 (m, 8H), 3.27 – 3.19 (m, 1H), 3.16 – 3.087 (m, 1H), 2.45 – 2.31 (m, 4H), 2.31 – 2.18 (m, 3H), 2.16 – 2.06 (m, 3H), 1.98 – 1.87 (m, 1H), 1.80 – 1.51 (m, 8H), 1.28 (br s, 54H), 0.98 (dd, J = 18.3, 6.7, 7H), 0.879 (t, J = 7.2 Hz, 7H), 0.79 – 0.66 (m, 4H).

^{13}C NMR (126 MHz, 2:3 CDCl_3 : CD_3OD) δ 175.8, 174.3, 172.1, 162.2, 159.1, 158.2, 139.3, 134.0, 129.8, 121.3, 101.4, 99.8, 76.0, 73.0, 72.2, 71.6, 71.1, 70.8, 70.4, 69.1, 67.6, 63.0, 62.1, 54.8, 53.8, 40.4, 35.8, 34.5, 33.2, 32.1, 31.0, 30.9, 30.9, 30.8, 30.6, 30.4, 29.8, 27.8, 26.6, 26.3, 25.0, 24.3, 23.9, 22.3, 20.2, 18.9, 14.9.

2,5-dioxopyrrolidin-1-yl 5-azidopentanoate (4.05)

To a stirred solution of 5-azidopentanoic acid (204.1 mg, 1.426 mmol) in dry dichloromethane (35 mL) under argon, was added 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (341mg, 2.153 mmol) followed by N-hydroxysuccinimide (302 mg, 2.624 mmol). The reaction stirred for 20 h at room temperature under argon. After 21 h, a further 53 mg of EDC and 51 mg of N-hydroxysuccinimide were added to the reaction mixture and left to stir for a further 20 h at room temperature. The mixture was then washed twice with 1% HCl, then once with distilled water and once with brine. The organic phase was dried with MgSO_4 , filtered and concentrated to obtain a cloudy colourless oil (241 mg, 70%). The yield was low, so the aqueous washes were re-extracted with DCM. All organic phases were concentrated. Proton NMR showed the presence of starting material. The compound was loaded onto silica from CHCl_3 and purified on silica (petroleum ether, ethyl acetate, 40%) to obtain a clear colourless oil, which was stored at -20 °C under argon (170 mg, 50%).

^1H NMR (500MHz, CDCl_3) δ 3.34 (t, J = 6.5 Hz, 2H), 2.84 (s, 4H), 2.66 (t, J = 7.4 Hz, 2H), 1.9 – 1.80 (m, 2H), 1.74 – 1.68 (m, 2H). ^{13}C NMR (126 MHz, CDCl_3) δ 169.0, 168.2, 50.8, 30.4, 27.9, 25.6, 21.9. Consistent with literature¹¹⁸.

CpG-3'-5-azidopentanamide (4.06)

To a solution of CpG-3'-NH₂ in water (1.3 mL) and sodium tetraborate buffer (7.5 mL) was added NHS-ester (11.5 mg, 0.0479 mmol) in DMSO (700 uL). The mixture was shaken overnight at room temperature. After 20 h, a second portion of NHS-ester (11.5 mg, 0.0479 mmol) in DMSO (700 uL) was added and shaken for a further 3 h. The reaction was determined to be complete by HPLC. To the reaction was added 3M NaCl (1 mL) and absolute ethanol (25 mL) instantly causing a white precipitate to form. The mixture was cooled to -20 °C for 30 minutes, then centrifuged at 5000 rpm for 45 minutes cooled to -20 °C. The supernatant was carefully removed and the pellet dried under vacuum. Without further purification, the product was used in the next step.

ESI-MS: m/z for C₂₀₅H₂₆₉N₇₁O₁₀₈P₂₀S₂₀ [M-5H]⁵⁻ calculated 1341.73, found 1342.04.

CpG-3'-5-azidopentanamide-BCN-PABCV-C18- α -GalCer (4.02)

CpG-3'-5-azidopentanamide (10 mg, 0.0015 mmol) was dissolved in DMSO (70 μ L). To this was added C18- α -GalCer-PABCV-BCN (1.26 mg, 0.018 mmol) in DMSO (70 μ L) to form a bright yellow solution. The mixture was heated to 30 $^{\circ}$ C for 20 h. When the reaction was determined to be complete by HPLC, it was purified on C18 by preparative HPLC. Following preparative HPLC, the fractions were combined and lyophilised to form off-white crystals. (1.1 mg, 9%, 95% pure by HPLC).

ESI calculated for C₂₇₇H₃₉₁N₇₇O₁₂₈P₂₀S₂₀ [M-8H]⁸⁻ 1004.067, found 1004.41

CpG-3'-5-azidopentanamide-BCN-PABCV-C26- α -GalCer (4.01)

CpG-3'-5-azidopentanamide (10 mg, 0.0015 mmol) was dissolved in DMSO (70 μ L). To this was added α -GalCer-PABCV-BCN (1.26 mg, 0.018 mmol) in DMSO (70 μ L) to form a bright yellow solution. The mixture was heated to 30 $^{\circ}$ C for 20 h. When the reaction was determined to be complete by HPLC, it was purified on C18 by preparative HPLC.

Following preparative HPLC, the fractions were combined and concentrated.

Lyophilisation afforded a white powder (3.4 mg, 28%, 96% pure by HPLC).

ESI calculated for C₂₈₅H₄₀₇N₇₇O₁₂₄P₂₀S₂₀ [M-9H]⁹⁻ 904.96, found 905.12

7 References

1. Ministry of Health. Cancer: New registrations and deaths 2013. Wellington: Ministry of Health,; 2016 9th November 2016.
2. Dougan M, Dranoff G. Immune therapy for cancer. *Annu Rev Immunol* 2009, **27**(1): 83-117.
3. Mellman I, Coukos G, Dranoff G. Cancer immunotherapy comes of age. *Nature* 2011, **480**(7378): 480-489.
4. Dimberu PM, Leonhardt RM. Cancer Immunotherapy Takes a Multi-Faceted Approach to Kick the Immune System into Gear. *The Yale Journal of Biology and Medicine* 2011, **84**(4): 371-380.
5. Schijns V, Tartour E, Michalek J, Stathopoulos A, Dobrovolskiene NT, Strioga MM. Immune adjuvants as critical guides directing immunity triggered by therapeutic cancer vaccines. *Cytotherapy* 2014, **16**(4): 427-439.
6. Cavanagh LL, Von Andrian UH. Travellers in many guises: the origins and destinations of dendritic cells. *Immunol Cell Biol* 2002, **80**(5): 448-462.
7. van der Burg SH, Arens R, Ossendorp F, van Hall T, Melief CJ. Vaccines for established cancer: overcoming the challenges posed by immune evasion. *Nat Rev Cancer* 2016, **16**(4): 219-233.
8. Melief CJ, van Hall T, Arens R, Ossendorp F, van der Burg SH. Therapeutic cancer vaccines. *J Clin Invest* 2015, **125**(9): 3401-3412.
9. Murphy K, Travers P, Walport M, Janeway C. *Janeway's immunobiology*. Garland Science: New York, 2012.
10. Winter H, Hu H-M, Poehlein CH, Huntzicker E, Osterholzer JJ, Bashy J, *et al*. Tumour-induced polarization of tumour vaccine-draining lymph node T cells to a type 1 cytokine profile predicts inherent strong immunogenicity of the tumour and correlates with therapeutic efficacy in adoptive transfer studies. *Immunology* 2003, **108**(3): 409-419.
11. Hu H-M, Urba WJ, Fox BA. Gene-Modified Tumor Vaccine with Therapeutic Potential Shifts Tumor-Specific T Cell Response from a Type 2 to a Type 1 Cytokine Profile. *The Journal of Immunology* 1998, **161**(6): 3033-3041.
12. Finn OJ. Cancer Immunology. *New England Journal of Medicine* 2008, **358**(25): 2704-2715.

13. Liu C-H, Fan Y-t, Dias A, Esper L, Corn RA, Bafica A, *et al.* Cutting Edge: Dendritic Cells Are Essential for In Vivo IL-12 Production and Development of Resistance against *Toxoplasma gondii* Infection in Mice. *The Journal of Immunology* 2006, **177**(1): 31-35.
14. Kenter GG, Welters MJP, Valentijn ARPM, Lowik MJG, Berends-van der Meer DMA, Vloon APG, *et al.* Vaccination against HPV-16 Oncoproteins for Vulvar Intraepithelial Neoplasia. *New England Journal of Medicine* 2009, **361**(19): 1838-1847.
15. van den Boorn JG, Barchet W, Hartmann G. Chapter 1 - Nucleic Acid Adjuvants: Toward an Educated Vaccine. In: Cornelis JMM (ed). *Advances in Immunology*, vol. Volume 114. Academic Press, 2012, pp 1-32.
16. Guy B. The perfect mix: recent progress in adjuvant research. *Nat Rev Micro* 2007, **5**(7): 505-517.
17. Coffman RL, Sher A, Seder RA. Vaccine adjuvants: putting innate immunity to work. *Immunity* 2010, **33**(4): 492-503.
18. Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol* 2003, **21**(1): 335-376.
19. O'Hagan DT, Valiante NM. Recent advances in the discovery and delivery of vaccine adjuvants. *Nat Rev Drug Discov* 2003, **2**(9): 727-735.
20. Lindblad EB. Aluminium compounds for use in vaccines. *Immunology and Cell Biology* 2004, **82**(5): 497-505.
21. Hem SL, HogenEsch H. Aluminum-Containing Adjuvants: Properties, Formulation, and Use. *Vaccine Adjuvants and Delivery Systems*. John Wiley & Sons, Inc., 2006, pp 81-114.
22. O'Hagan DT, Rappuoli R, De Gregorio E, Tsai T, Del Giudice G. MF59 adjuvant: the best insurance against influenza strain diversity. *Expert Review of Vaccines* 2011, **10**(4): 447-462.
23. O'Hagan DT, Singh M. MF59: A Safe and Potent Oil-in-Water Emulsion Adjuvant. *Vaccine Adjuvants and Delivery Systems*. John Wiley & Sons, Inc., 2006, pp 115-129.
24. Petrovsky N, Aguilar JC. Vaccine adjuvants: current state and future trends. *Immunol Cell Biol* 2004, **82**(5): 488-496.
25. Reed SG, Bertholet S, Coler RN, Friede M. New horizons in adjuvants for vaccine development. *Trends Immunol* 2009, **30**(1): 23-32.
26. Temizoz B, Kuroda E, Ishii KJ. Vaccine adjuvants as potential cancer immunotherapeutics. *International Immunology* 2016, **28**(7): 329-338.

27. Klinman DM. Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat Rev Immunol* 2004, **4**(4): 249-258.
28. Krieg AM. CPG MOTIFS IN BACTERIAL DNA AND THEIR IMMUNE EFFECTS. *Annual Review of Immunology* 2002, **20**(1): 709-760.
29. Sparwasser T, Vabulas RM, Villmow B, Lipford GB, Wagner H. Bacterial CpG-DNA activates dendritic cells in vivo: T helper cell-independent cytotoxic T cell responses to soluble proteins. *European Journal of Immunology* 2000, **30**(12): 3591-3597.
30. Chu RS, Targoni OS, Krieg AM, Lehmann PV, Harding CV. CpG Oligodeoxynucleotides Act as Adjuvants that Switch on T Helper 1 (Th1) Immunity. *The Journal of Experimental Medicine* 1997, **186**(10): 1623-1631.
31. Ballas ZK, Krieg AM, Warren T, Rasmussen W, Davis HL, Waldschmidt M, *et al.* Divergent Therapeutic and Immunologic Effects of Oligodeoxynucleotides with Distinct CpG Motifs. *The Journal of Immunology* 2001, **167**(9): 4878-4886.
32. Liu N, Ohnishi N, Ni L, Akira S, Bacon KB. CpG directly induces T-bet expression and inhibits IgG1 and IgE switching in B cells. *Nat Immunol* 2003, **4**(7): 687-693.
33. Gursel M, Verthelyi D, Gursel I, Ishii KJ, Klinman DM. Differential and competitive activation of human immune cells by distinct classes of CpG oligodeoxynucleotide. *J Leukoc Biol* 2002, **71**(5): 813-820.
34. Geary RS, Leeds JM, Fitchett J, Burckin T, Truong L, Spainhour C, *et al.* Pharmacokinetics and Metabolism in Mice of a Phosphorothioate Oligonucleotide Antisense Inhibitor of C-raf-1 Kinase Expression. *Drug Metabolism and Disposition* 1997, **25**(11): 1272-1281.
35. Halperin SA, Dobson S, McNeil S, Langley JM, Smith B, McCall-Sani R, *et al.* Comparison of the safety and immunogenicity of hepatitis B virus surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide and a licensed hepatitis B vaccine in healthy young adults. *Vaccine* 2006, **24**(1): 20-26.
36. Cooper CL, Davis HL, Morris ML, Efler SM, Krieg AM, Li Y, *et al.* Safety and immunogenicity of CPG 7909 injection as an adjuvant to Fluarix influenza vaccine. *Vaccine* 2004, **22**(23-24): 3136-3143.
37. Kim SK, Ragupathi G, Musselli C, Choi S-J, Park YS, Livingston PO. Comparison of the effect of different immunological adjuvants on the antibody and T-cell response to immunization with MUC1-KLH and GD3-KLH conjugate cancer vaccines. *Vaccine* 1999, **18**(7-8): 597-603.

38. Chan JHP, Lim S, Wong WSF. ANTISENSE OLIGONUCLEOTIDES: FROM DESIGN TO THERAPEUTIC APPLICATION. *Clinical and Experimental Pharmacology and Physiology* 2006, **33**(5-6): 533-540.
39. Putta MR, Zhu F-G, Wang D, Bhagat L, Dai M, Kandimalla ER, *et al.* Peptide Conjugation at the 5'-End of Oligodeoxynucleotides Abrogates Toll-Like Receptor 9-Mediated Immune Stimulatory Activity. *Bioconjugate Chemistry* 2010, **21**(1): 39-45.
40. Tefit JN, Serra V. Outlining novel cellular adjuvant products for therapeutic vaccines against cancer. *Expert Rev Vaccines* 2011, **10**(8): 1207-1220.
41. Anderson RJ, Compton BJ, Tang CW, Authier-Hall A, Hayman CM, Swinerd GW, *et al.* NKT cell-dependent glycolipid-peptide vaccines with potent anti-tumour activity. *Chemical Science* 2015, **6**(9): 5120-5127.
42. Cerundolo V, Silk JD, Masri SH, Salio M. Harnessing invariant NKT cells in vaccination strategies. *Nat Rev Immunol* 2009, **9**(1): 28-38.
43. Anderson BL, Teyton L, Bendelac A, Savage PB. Stimulation of Natural Killer T Cells by Glycolipids. *Molecules (Basel, Switzerland)* 2013, **18**(12): 15662-15688.
44. Zhou X-T, Forestier C, Goff RD, Li C, Teyton L, Bendelac A, *et al.* Synthesis and NKT Cell Stimulating Properties of Fluorophore- and Biotin-Appended 6'-Amino-6'-deoxy-galactosylceramides. *Organic Letters* 2002, **4**(8): 1267-1270.
45. Cheng JMH, Chee SH, Knight DA, Acha-Orbea H, Hermans IF, Timmer MSM, *et al.* An improved synthesis of dansylated α -galactosylceramide and its use as a fluorescent probe for the monitoring of glycolipid uptake by cells. *Carbohydrate Research* 2011, **346**(7): 914-926.
46. Querec T, Bennouna S, Alkan S, Laouar Y, Gorden K, Flavell R, *et al.* Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity. *J Exp Med* 2006, **203**(2): 413-424.
47. Trinchieri G, Sher A. Cooperation of Toll-like receptor signals in innate immune defence. *Nat Rev Immunol* 2007, **7**(3): 179-190.
48. Zom GGP, Khan S, Filippov DV, Ossendorp F. Chapter 7 - TLR Ligand–Peptide Conjugate Vaccines: Toward Clinical Application. In: Cornelis JMM (ed). *Advances in Immunology*, vol. Volume 114. Academic Press, 2012, pp 177-201.
49. Bagchi A, Herrup EA, Warren HS, Trigilio J, Shin H-S, Valentine C, *et al.* MyD88-Dependent and MyD88-Independent Pathways in Synergy, Priming, and Tolerance between TLR Agonists. *The Journal of Immunology* 2007, **178**(2): 1164-1171.

50. Gableh F, Saeidi M, Hemati S, Hamdi K, Soleimanjahi H, Gorji A, *et al.* Combination of the toll like receptor agonist and alpha-Galactosylceramide as an efficient adjuvant for cancer vaccine. *J Biomed Sci* 2016, **23**(1): 16.
51. Semmling V, Lukacs-Kornek V, Thaiss CA, Quast T, Hochheiser K, Panzer U, *et al.* Alternative cross-priming through CCL17-CCR4-mediated attraction of CTLs toward NKT cell-licensed DCs. *Nature Immunology* 2010, **11**(4): 313-U357.
52. Dong T, Yi T, Yang M, Lin S, Li W, Xu X, *et al.* Co-operation of α -galactosylceramide-loaded tumour cells and TLR9 agonists induce potent anti-tumour responses in a murine colon cancer model. *Biochemical Journal* 2016, **473**(1): 7-19.
53. Godfrey DI, Rossjohn J, McCluskey J. Fighting infection with your MAITs. *Nat Immunol* 2010, **11**(8): 693-695.
54. Corbett AJ, Eckle SB, Birkinshaw RW, Liu L, Patel O, Mahony J, *et al.* T-cell activation by transitory neo-antigens derived from distinct microbial pathways. *Nature* 2014, **509**(7500): 361-365.
55. Reantragoon R, Kjer-Nielsen L, Patel O, Chen Z, Illing PT, Bhati M, *et al.* Structural insight into MR1-mediated recognition of the mucosal associated invariant T cell receptor. *J Exp Med* 2012, **209**(4): 761-774.
56. Rautio J, Kumpulainen H, Heimbach T, Oliyai R, Oh D, Jarvinen T, *et al.* Prodrugs: design and clinical applications. *Nat Rev Drug Discov* 2008, **7**(3): 255-270.
57. Girard JP, Moussion C, Forster R. HEVs, lymphatics and homeostatic immune cell trafficking in lymph nodes. *Nat Rev Immunol* 2012, **12**(11): 762-773.
58. Liu H, Irvine DJ. Guiding principles in the design of molecular bioconjugates for vaccine applications. *Bioconjug Chem* 2015, **26**(5): 791-801.
59. Senti G, Prinz Vavricka BM, Erdmann I, Diaz MI, Markus R, McCormack SJ, *et al.* Intralymphatic allergen administration renders specific immunotherapy faster and safer: A randomized controlled trial. *Proceedings of the National Academy of Sciences* 2008, **105**(46): 17908-17912.
60. Supersaxo A, Hein WR, Steffen H. Effect of Molecular Weight on the Lymphatic Absorption of Water-Soluble Compounds Following Subcutaneous Administration. *Pharmaceutical Research* 1990, **7**(2): 167-169.
61. Trevaskis NL, Kaminskas LM, Porter CJ. From sewer to saviour - targeting the lymphatic system to promote drug exposure and activity. *Nat Rev Drug Discov* 2015, **14**(11): 781-803.

62. Tsopelas C, Sutton R. Why certain dyes are useful for localizing the sentinel lymph node. *J Nucl Med* 2002, **43**(10): 1377-1382.
63. Wong JH, Cagle LA, Morton DL. Lymphatic drainage of skin to a sentinel lymph node in a feline model. *Ann Surg* 1991, **214**(5): 637-641.
64. Hamilton JA. NMR reveals molecular interactions and dynamics of fatty acid binding to albumin. *Biochimica et Biophysica Acta (BBA) - General Subjects* 2013, **1830**(12): 5418-5426.
65. Petitpas I, Grüne T, Bhattacharya AA, Curry S. Crystal structures of human serum albumin complexed with monounsaturated and polyunsaturated fatty acids1. *Journal of Molecular Biology* 2001, **314**(5): 955-960.
66. Szabo G, Magyar Z. Extravascular circulation of plasma proteins. *Acta physiologica Academiae Scientiarum Hungaricae* 1982, **60**(1-2): 65-74.
67. Liu H, Moynihan KD, Zheng Y, Szeto GL, Li AV, Huang B, *et al.* Structure-based programming of lymph-node targeting in molecular vaccines. *Nature* 2014, **507**(7493): 519-522.
68. Goodsell D. Serum albumin, with bound fatty acids in white and red. In: 37-SerumAlbumin-1e7i.tiff, editor. RSCB PDB: RSCB PDB; 2003.
69. Cresswell RM, Wood HCS. 928. The biosynthesis of pteridines. Part I. The synthesis of riboflavin. *Journal of the Chemical Society (Resumed)* 1960(0): 4768-4775.
70. Nielsen P, Bacher A. Biosynthesis of Riboflavin - a Simple Synthesis of the Substrate and Product of the Pyrimidine Deaminase and of Structural Analogs. *Z Naturforsch B* 1988, **43**(10): 1358-1364.
71. Kjer-Nielsen L, Patel O, Corbett AJ, Le Nours J, Meehan B, Liu L, *et al.* MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature* 2012, **491**(7426): 717-723.
72. Masuda T, Kishi T, Asai M, Kuwada S. Application of Chromatography. XXXVII. Total Synthesis of 6, 7-Dimethylribolumazine. *CHEMICAL & PHARMACEUTICAL BULLETIN* 1959, **7**(3): 361-365.
73. Maley GF, Plaut GWE. The Isolation, Synthesis, and Metabolic Properties of 6,7-Dimethyl-8-ribityllumazine. *Journal of Biological Chemistry* 1959, **234**(3): 641-647.
74. Kagan F, Rebenstorf MA, Heinzelman RV. The Preparation of Glycamines1. *Journal of the American Chemical Society* 1957, **79**(13): 3541-3544.

75. Saggiomo V, Lüning U. On the formation of imines in water—a comparison. *Tetrahedron Letters* 2009, **50**(32): 4663-4665.
76. Davoll J, Evans DD. 1976. The synthesis of 9-glycitylpurines, 3-glycetyl-[1,2,3]-triazolo[d]-pyrimidines, 8-glycetylpteridines, and 10-glycetylbenzo[g]pteridines, including riboflavin and riboflavin 2-imine. *Journal of the Chemical Society (Resumed)* 1960(0): 5041-5049.
77. Ji H, Jing Q, Huang J, Silverman RB. Acid-Facilitated Debenzylation of N-Boc, N-Benzyl Double Protected 2-Aminopyridinomethylpyrrolidine Derivatives. *Tetrahedron* 2012, **68**(5): 1359-1366.
78. Adger BM, O'Farrell C, Lewis NJ, Mitchell MB. Catalytic Transfer Hydrogenolysis of N-Benzyl Protecting Groups. *Synthesis* 1987, **1987**(01): 53-55.
79. Malle BM, Lundt I, Furneaux RH. A New Stereospecific Synthesis of 1,2,4-Trideoxy-1,4-Imino-D-Erythro-Pentitol. *Journal of Carbohydrate Chemistry* 2000, **19**(4-5): 573-583.
80. Painter GF, Falshaw A, Wong H. Conformation inversion of an inositol derivative by use of silyl ethers: a modified route to 3,6-di-O-substituted-l-ido-tetrahydroxyazepane derivatives. *Organic & Biomolecular Chemistry* 2004, **2**(7): 1007-1012.
81. Sato S, Sakamoto T, Miyazawa E, Kikugawa Y. One-pot reductive amination of aldehydes and ketones with α -picoline-borane in methanol, in water, and in neat conditions. *Tetrahedron* 2004, **60**(36): 7899-7906.
82. Schaefer K, Albers J, Sindhuwinata N, Peters T, Meyer B. A New Concept for Glycosyltransferase Inhibitors: Nonionic Mimics of the Nucleotide Donor of the Human Blood Group B Galactosyltransferase. *ChemBioChem* 2012, **13**(3): 443-450.
83. Giaccone G, Punt CJA, Ando Y, Ruijter R, Nishi N, Peters M, *et al.* A Phase I Study of the Natural Killer T-Cell Ligand α -Galactosylceramide (KRN7000) in Patients with Solid Tumors. *Clinical Cancer Research* 2002, **8**(12): 3702-3709.
84. Harris JM, Chess RB. Effect of pegylation on pharmaceuticals. *Nat Rev Drug Discov* 2003, **2**(3): 214-221.
85. Veronese FM, Mero A. The impact of PEGylation on biological therapies. *BioDrugs* 2008, **22**(5): 315-329.
86. Bailon P, Palleroni A, Schaffer CA, Spence CL, Fung W-J, Porter JE, *et al.* Rational Design of a Potent, Long-Lasting Form of Interferon: A 40 kDa Branched Polyethylene Glycol-Conjugated Interferon α -2a for the Treatment of Hepatitis C. *Bioconjugate Chemistry* 2001, **12**(2): 195-202.

87. Levy Y, Hershfield MS, Fernandez-Mejia C, Polmar SH, Scudieri D, Berger M, *et al.* Adenosine deaminase deficiency with late onset of recurrent infections: Response to treatment with polyethylene glycol-modified adenosine deaminase. *The Journal of Pediatrics* 1988, **113**(2): 312-317.
88. Ebensen T, Link C, Riese P, Schulze K, Morr M, Guzmán CA. A Pegylated Derivative of α -Galactosylceramide Exhibits Improved Biological Properties. *The Journal of Immunology* 2007, **179**(4): 2065-2073.
89. Singh R, Whitesides GM. Thiol—disulfide interchange. *Sulphur-Containing Functional Groups* (1993). John Wiley & Sons, Inc., 2010, pp 633-658.
90. OECD. *Test No. 117: Partition Coefficient (n-octanol/water), HPLC Method*. OECD Publishing.
91. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviews* 2012, **64**, **Supplement**: 4-17.
92. Mannhold R, Poda GI, Ostermann C, Tetko IV. Calculation of Molecular Lipophilicity: State-of-the-Art and Comparison of Log P Methods on more than 96,000 Compounds. *Journal of Pharmaceutical Sciences* 2009, **98**(3): 861-893.
93. OECD. *Test No. 107: Partition Coefficient (n-octanol/water): Shake Flask Method*. OECD Publishing.
94. Product properties test guidelines : OPPTS 830.7570 : partition coefficient (n-octanol/water), estimation by liquid chromatography.; 1996.
95. Dubowchik GM, Firestone RA. Cathepsin B-sensitive dipeptide prodrugs. 1. A model study of structural requirements for efficient release of doxorubicin. *Bioorganic & Medicinal Chemistry Letters* 1998, **8**(23): 3341-3346.
96. Dubowchik GM, Firestone RA, Padilla L, Willner D, Hofstead SJ, Mosure K, *et al.* Cathepsin B-Labile Dipeptide Linkers for Lysosomal Release of Doxorubicin from Internalizing Immunoconjugates: Model Studies of Enzymatic Drug Release and Antigen-Specific In Vitro Anticancer Activity. *Bioconjugate Chemistry* 2002, **13**(4): 855-869.
97. Choi J-K, Ho J, Curry S, Qin D, Bittman R, Hamilton JA. Interactions of very long-chain saturated fatty acids with serum albumin. *Journal of Lipid Research* 2002, **43**(7): 1000-1010.
98. Flechtner JB, Cohane KP, Mehta S, Slusarewicz P, Leonard AK, Barber BH, *et al.* High-Affinity Interactions between Peptides and Heat Shock Protein 70 Augment

- CD8⁺ T Lymphocyte Immune Responses. *The Journal of Immunology* 2006, **177**(2): 1017-1027.
99. Lipford GB, Hoffman M, Wagner H, Heeg K. Primary in vivo responses to ovalbumin. Probing the predictive value of the Kb binding motif. *The Journal of Immunology* 1993, **150**(4): 1212-1222.
 100. Agard NJ, Prescher JA, Bertozzi CR. A Strain-Promoted [3 + 2] Azide–Alkyne Cycloaddition for Covalent Modification of Biomolecules in Living Systems. *Journal of the American Chemical Society* 2004, **126**(46): 15046-15047.
 101. Hermanson GT. Chapter 23 - Nucleic Acid and Oligonucleotide Modification and Conjugation. *Bioconjugate Techniques (Third edition)*. Academic Press: Boston, 2013, pp 959-987.
 102. Thermo Fisher Scientific. Amine-Reactive Probe Labeling Protocol. Labeling Chemistry Protocols [Web Page] 2008 28 April 2008 [cited 2016 23/11/2016] Available from: <https://www.thermofisher.com/nz/en/home/references/protocols/cell-and-tissue-analysis/labeling-chemistry-protocols/amine-reactive-probe-labeling-protocol.html>
 103. Cohn WE. *Progress in Nucleic Acid Research and Molecular Biology*. Elsevier Science, 1981.
 104. Mashalidis EH, Śledź P, Lang S, Abell C. A three-stage biophysical screening cascade for fragment-based drug discovery. *Nat Protocols* 2013, **8**(11): 2309-2324.
 105. Viegas A, Manso J, Nobrega FL, Cabrita EJ. Saturation-Transfer Difference (STD) NMR: A Simple and Fast Method for Ligand Screening and Characterization of Protein Binding. *Journal of Chemical Education* 2011, **88**(7): 990-994.
 106. Angulo J, Nieto PM. STD-NMR: application to transient interactions between biomolecules—a quantitative approach. *European Biophysics Journal* 2011, **40**(12): 1357-1369.
 107. Mayer M, Meyer B. Characterization of Ligand Binding by Saturation Transfer Difference NMR Spectroscopy. *Angewandte Chemie International Edition* 1999, **38**(12): 1784-1788.
 108. GE Healthcare Life Sciences. Size Exclusion Chromatography Principles and Methods. 2014 [cited]139]. Available from: http://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1466158841539/litdoc18102218_20161012165335.pdf
 109. Wood GC, Cooper PF. The application of gel filtration to the study of protein-binding of small molecules. *Chromatographic Reviews* 1970, **12**(1): 88-107.

110. Niessen WMA, Falck D. Introduction to Mass Spectrometry, a Tutorial. *Analyzing Biomolecular Interactions by Mass Spectrometry*. Wiley-VCH Verlag GmbH & Co. KGaA, 2015, pp 1-54.
111. van Dongen WD, Niessen WMA. Bioanalytical LC–MS of therapeutic oligonucleotides. *Bioanalysis* 2011, **3**(5): 541-564.
112. Apffel A, Chakel JA, Fischer S, Lichtenwalter K, Hancock WS. Analysis of Oligonucleotides by HPLC–Electrospray Ionization Mass Spectrometry. *Analytical Chemistry* 1997, **69**(7): 1320-1325.
113. Hermans IF, Silk JD, Yang J, Palmowski MJ, Gileadi U, McCarthy C, *et al.* The VITAL assay: a versatile fluorometric technique for assessing CTL- and NKT-mediated cytotoxicity against multiple targets in vitro and in vivo. *Journal of Immunological Methods* 2004, **285**(1): 25-40.
114. Landrigan A, Wong MT, Utz PJ. CpG and Non-CpG Oligodeoxynucleotides Directly Costimulate Mouse and Human CD4⁺ T Cells through a TLR9- and MyD88-Independent Mechanism. *The Journal of Immunology* 2011, **187**(6): 3033-3043.
115. Compton BJ, Tang C-w, Johnston KA, Osmond TL, Hayman CM, Larsen DS, *et al.* Synthesis and Activity of 6''-Deoxy-6''-thio- α -GalCer and Peptide Conjugates. *Organic Letters* 2015, **17**(24): 5954-5957.
116. Schombs M, Park FE, Du W, Kulkarni SS, Gervay-Hague J. One-Pot Syntheses of Immunostimulatory Glycolipids. *The Journal of Organic Chemistry* 2010, **75**(15): 4891-4898.
117. Tashiro T, Shigeura T, Shiozaki M, Watarai H, Taniguchi M, Mori K. RCAI-133, an N-methylated analogue of KRN7000, activates mouse natural killer T cells to produce Th2-biased cytokines. *MedChemComm* 2013, **4**(6): 949-955.
118. Sachdeva A, Wang K, Elliott T, Chin JW. Concerted, Rapid, Quantitative, and Site-Specific Dual Labeling of Proteins. *Journal of the American Chemical Society* 2014, **136**(22): 7785-7788.

Appendix A – Additional compound characterisation data

Presented here are the HPLC traces of compounds. The top trace shows purity of the compound using UV at the stated wavelength or charged aerosol detection. Charged aerosol detection (CAD) is typically used to detect compounds without UV chromophores. MS data was obtained running the same sample in identical conditions attached to the mass spectrometer rather than CAD.

α -GalCer-PEG-25 conjugate (3.07)

HPLC/LCMS

Column: Phenomenex Kinetex® 2.6 μ m C18 100 Å, LC Column 50 x 3 mm

Mobile Phase A: 1:9 Water/Methanol + 0.1% formic acid

Mobile Phase B: Isopropanol + 0.1% formic acid

Gradient (A:B): T0 = 100:0, T7 = 20:80, T12 = 20:80; 0.5 mL/min, T=40°C

HPLC-CAD

LC-MS

Mass spectrum extracted from highlighted peak

α -GalCer-PEG-52 conjugate (3.08)

HPLC/LCMS

Column: Phenomenex Kinetex® 2.6 μ m C18 100 Å, LC Column 50 x 3 mm

Mobile Phase A: 1:9 Water/Methanol + 0.1% formic acid

Mobile Phase B: Isopropanol + 0.1% formic acid

Gradient (A:B): T0 = 100:0, T7 = 20:80, T12 = 20:80; 0.5 mL/min, T=40°C

HPLC-CAD

Direct infusion MS

α -GalCer-PEG-100 conjugate (3.09)

HPLC/LCMS

Column: Phenomenex Kinetex® 2.6 μm C18 100 Å, LC Column 50 x 3 mm

Mobile Phase A: 1:9 Water/Methanol + 0.1% formic acid

Mobile Phase B: Isopropanol + 0.1% formic acid

Gradient (A:B): T0 = 100:0, T7 = 20:80, T12 = 20:80; 0.5 mL/min, T=40°C

HPLC-CAD

Direct infusion MS

DPSE-PEG₂₀₀₀-MAL-C-FFRK-SIINFEKL (4.03)

HPLC/LCMS

Column: Phenomenex Kinetex® 2.6 µm C18 100 Å, LC Column 50 x 3 mm

Mobile Phase A: Water + 0.05% TFA

Mobile Phase B: Methanol + 0.05% TFA

Gradient (A:B): T0 = 70:30, T7 = 0:100, T9 = 0:100; 0.5 mL/min, T=40°C

HPLC-CAD

Mass spectrum extracted from highlighted peak

CpG-3'-5-azidopentanamide (4.06)

HPLC/LCMS

Column: Phenomenex Kinetex® 2.6 µm C18 100 Å, LC Column 50 x 3 mm

Mobile Phase A: 8.3 mM triethylamine + 100 mM HFIP, aqueous.

Mobile Phase B: Methanol

Gradient (A:B): T0 = 90:10, T7 = 10:90, T9 = 10:90; 0.4 mL/min, T=40°C

HPLC: UV 260nm

Mass spectrum extracted from highlighted peak

CpG-3'-5-azidopentanamide-BCN-PABCV-C18- α -GalCer (4.02)

HPLC/LCMS

Column: Phenomenex Kinetex® 2.6 μ m C18 100 Å, LC Column 50 x 3 mm

Mobile Phase A: 8.3 mM triethylamine + 100 mM HFIP, aqueous.

Mobile Phase B: Methanol + 100 mM HFIP

Gradient (A:B): T0 = 90:10, T7 = 0:100, T11 = 0:100; 0.4 mL/min, T=40°C

HPLC: UV 260 nm

Mass spectrum extracted from highlighted peak

CpG-3'-5-azidopentanamide-BCN-PABCV-C26- α -GalCer (4.01)

HPLC/LCMS

Column: Phenomenex Kinetex® 2.6 μ m C18 100 Å, LC Column 50 x 3 mm

Mobile Phase A: 8.3 mM triethylamine + 100 mM HFIP, aqueous.

Mobile Phase B: Methanol + 100 mM HFIP

Gradient (A:B): T0 = 90:10, T7 = 0:100, T11 = 0:100; 0.4 mL/min, T=40°C

HPLC: UV 260nm

Mass spectrum extracted from highlighted peak