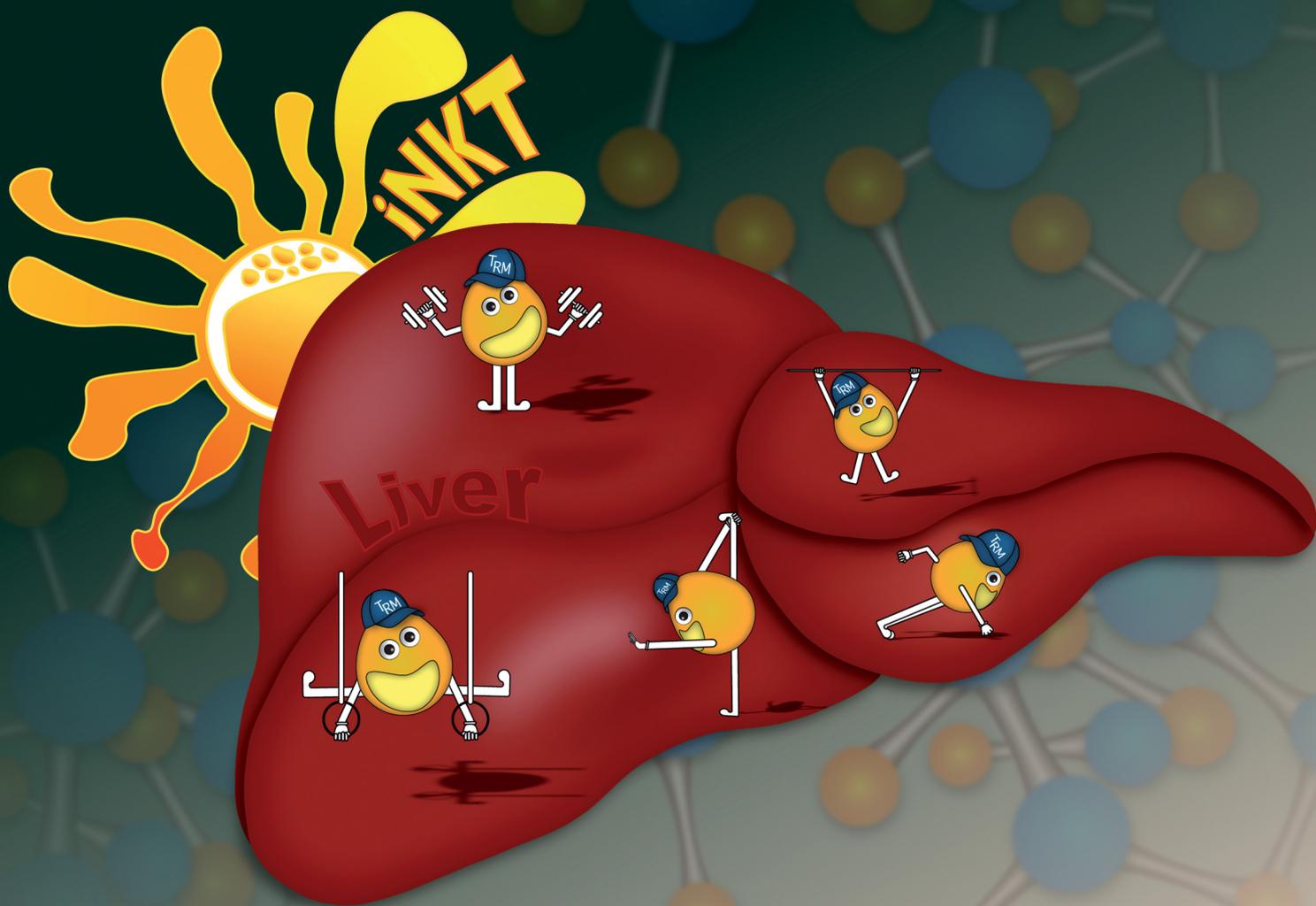


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## PAPER

Gavin F. Painter, Benjamin J. Compton *et al.*  
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## 6''-Modified $\alpha$ -GalCer-peptide conjugate vaccine candidates protect against liver-stage malaria†

Michael A. Meijlink,<sup>‡a</sup> Yu Cheng Chua,<sup>ib ‡b</sup> Susanna T. S. Chan,<sup>a</sup>  
Regan J. Anderson,<sup>ib a</sup> Matthew W. Rosenberg,<sup>ib a</sup> Anton Cozijnsen,<sup>c</sup>  
Vanessa Mollard,<sup>c</sup> Geoffrey I. McFadden,<sup>c</sup> Sarah L. Draper,<sup>ib a</sup> Lauren E. Holz,<sup>b</sup>  
Ian F. Hermans,<sup>de</sup> William R. Heath,<sup>ib §b</sup> Gavin F. Painter<sup>ib §\*ae</sup> and  
Benjamin J. Compton<sup>ib §\*a</sup>

Self-adjuvanting vaccines consisting of peptide epitopes conjugated to immune adjuvants are a powerful way of generating antigen-specific immune responses. We previously showed that a *Plasmodium*-derived peptide conjugated to a rearranged form of  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer) could stimulate liver-resident memory T ( $T_{RM}$ ) cells that were effective killers of liver-stage *Plasmodium berghei* ANKA (Pba)-infected cells. To investigate if similar or even superior  $T_{RM}$  responses can be induced by modifying the  $\alpha$ -GalCer adjuvant, we created new conjugate vaccine candidates by attaching an immunogenic *Plasmodium*-derived peptide antigen to 6''-substituted  $\alpha$ -GalCer analogues. Vaccine synthesis involved developing an efficient route to  $\alpha$ -galactosylphosphingosine ( $\alpha$ -GalPhs), from which the prototypical iNKT cell agonist,  $\alpha$ -GalCer, and its 6''-deoxy-6''-thio and -amino analogues were derived. Attaching a cathepsin B-cleavable linker to the 6''-modified  $\alpha$ -GalCer created pro-adjuvants bearing a pendant ketone group available for peptide conjugation. Optimized reaction conditions were developed that allow for the efficient conjugation of peptide antigens to the pro-adjuvants via oxime ligation to create new glycolipid-peptide (GLP) conjugate vaccines. A single dose of the vaccine candidates induced acute NKT and *Plasmodium*-specific CD8<sup>+</sup> T cell responses that generated potent hepatic  $T_{RM}$  responses in mice. Our findings demonstrate that attaching antigenic peptides to 6''-modified  $\alpha$ -GalCer generates powerful self-adjuvanting conjugate vaccine candidates that could potentially control hepatotropic infections such as liver-stage malaria.

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## Introduction

Immunomodulatory glycolipids play important roles in immunosurveillance and pathogen clearance. Through their agonist activity on cellular receptors, these compounds stimulate the release of signalling molecules that have a multitude of downstream effects including immune cell recruitment, cellular differentiation and proliferation, maturation and activation. As such, harnessing the immunostimulatory properties of glycolipids is widely recognised as holding tremendous potential

for developing vaccines to both treat and prevent communicable and non-communicable diseases.

A particularly potent and well-studied example of an immune-activating glycolipid is  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer, KRN7000), a synthetic analogue of a natural product isolated from the marine sponge *Agelas mauritianus*.<sup>1</sup> Since its first report in 1995,<sup>2</sup>  $\alpha$ -GalCer has been used in over 30 clinical trials<sup>3</sup> and has been the focus of an extensive list of publications that describe an important chemical aspect or biological function of the parent compound. Whereas most immune-activating (glyco)lipid classes function by directly binding specific pattern recognition receptors within or on antigen presenting cells (*i.e.* toll- and Nod-like receptors, C-type lectins),  $\alpha$ -GalCer derives its adjuvant activity by functioning as an agonist for type I (or “invariant”) natural killer T (iNKT) cells. Unlike conventional T cells, which exhibit considerable diversity in their antigen receptors and require several days of proliferation to generate sufficient effector function, iNKT cells express largely invariant receptors, are found in large numbers, and respond to antigen within hours of stimulation. Given this capacity to mobilise a strong and rapid response, iNKT cells straddle the

<sup>a</sup> Ferrier Research Institute, Victoria University of Wellington, Lower Hutt, New Zealand. E-mail: benji.compton@vuw.ac.nz, gavin.painter@vuw.ac.nz

<sup>b</sup> Department of Microbiology and Immunology, Doherty Institute for Infection and Immunity, University of Melbourne, Melbourne, VIC, Australia

<sup>c</sup> School of BioSciences, University of Melbourne, Parkville, VIC, Australia

<sup>d</sup> Malaghan Institute of Medical Research, Wellington, New Zealand

<sup>e</sup> Avalia Immunotherapies Limited, Lower Hutt, New Zealand

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‡ These authors contributed equally to this work as co-first authors.

§ These authors contributed equally to this work as co-senior authors.



boundary between innate and adaptive immunity. The antigen receptors expressed by iNKT cells recognise glycolipids (such as  $\alpha$ -GalCer) displayed by the cell surface presenting molecule CD1d. Upon activation, iNKT cells rapidly release potent cytokines and direct molecular signals that activate antigen presenting cells leading to their improved capacity to stimulate T cells and drive antibody production. Because CD1d is non-polymorphic and iNKT cells are found in all humans,  $\alpha$ -GalCer is an appealing adjuvant for the development of new vaccines.

Investigations in animal models have shown that  $\alpha$ -GalCer boosts immune responses when co-administered with antigen, either as peptide,<sup>4</sup> protein<sup>5,6</sup> or through mRNA<sup>7–9</sup> translation. Studies where  $\alpha$ -GalCer is covalently bound to antigen consistently demonstrate superior T cell and antibody responses compared to admixture comparators.<sup>10,11</sup> This can be attributed to the close temporal release of both components inside the same antigen presenting cell, thereby focusing iNKT-derived stimulatory signals on the cells engaged in driving adaptive responses.

However, the possible points for attaching peptide antigens to  $\alpha$ -GalCer appear limited as the galactosyl and ceramide hydroxyls function as important H-bond donors when bound within the CD1d-T cell receptor (TCR) complex,<sup>12–14</sup> perhaps with the exception of the 6''-position, which appears solvent exposed in X-ray crystal structures. An elegant way to overcome this limitation was through migration of the ceramide acyl chain of  $\alpha$ -GalCer to the neighboring 4-OH allowing the phytosphingosine nitrogen atom to be further modified with an immolative linker capable of undergoing chemoselective peptide conjugation.<sup>10</sup> Upon intracellular processing by antigen presenting cells, such as dendritic cells, the linker underwent enzymatic cleavage followed by facile O  $\rightarrow$  N acyl migration co-releasing the active constituents (*i.e.*  $\alpha$ -GalCer and peptide antigen).<sup>10</sup> Conjugates of this nature (Fig. 1) have been shown to induce potent antigen-specific T cell responses with therapeutic potential for treating cancer<sup>15,16</sup> and infectious diseases<sup>17</sup> including malaria.<sup>18</sup>

In this work, we explore an alternative vaccine design that takes advantage of the galactose-6-OH (Fig. 1), which is considered non-essential for binding of the CD1d: $\alpha$ -GalCer complex to the TCR.<sup>14</sup> Previously reported 6''-modified  $\alpha$ -GalCer analogues include the amino,<sup>19</sup> azido,<sup>20</sup> thio,<sup>21</sup> hydro (deoxy),<sup>22</sup> and uronic acid<sup>23</sup> derivatives, which have been further modified to produce a range of ether,<sup>19</sup> ester,<sup>24</sup> amido,<sup>25–29</sup> carbamoyl,<sup>26</sup> carbamido,<sup>27</sup> triazole,<sup>20,30–32</sup> disulfide<sup>21</sup> and thioether<sup>21</sup> analogues, many of which display potent immunostimulatory activities that are biased for either a Th1<sup>19,20,22,23,26,27,30,32</sup> or Th2<sup>19,20,25</sup> response compared to  $\alpha$ -GalCer. Notable among these 6''-modified  $\alpha$ -GalCer analogues are ABX196 and PBS-57, both of which are 6''-NHAc derivatives with disparate *N*-acyl ceramide chains. ABX196 has been advanced into clinical trials for hepatocellular carcinoma as a combination therapy with the checkpoint inhibitor nivolumab<sup>33</sup> while PBS-57 is used for the creation of CD1d monomer or tetramer reagents for the detection of NKT cells.<sup>34</sup> In light of these successes, we viewed the simple 6''-deoxy-6''-thio and -amino  $\alpha$ -GalCer analogues as

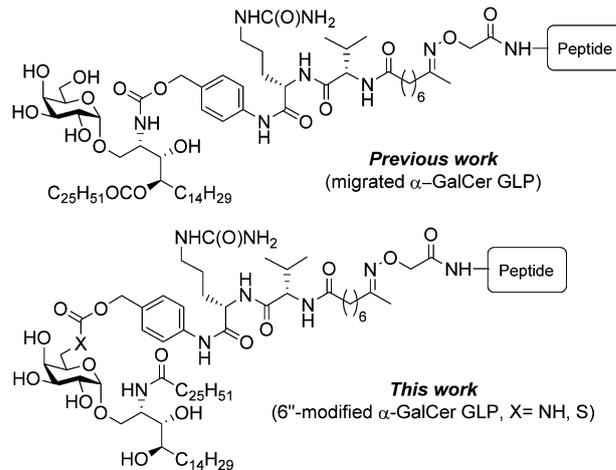


Fig. 1 Self-adjuvanting peptide-conjugate vaccine designs based on  $\alpha$ -GalCer illustrating the 'migration' approach (previous work) and the 6''-modified approach (this work).

attractive adjuvants for glycolipid-peptide (GLP)-based vaccine candidates due to their structural similarity to  $\alpha$ -GalCer and the fact that these heteroatoms offer a chemically orthogonal site for peptide antigen attachment. Herein, we report the synthesis of these 6''-modified derivatives, the appendage of an enzymatically labile self-immolative linker to the *N*- and *S*-atoms, and their subsequent conjugation to a peptide antigen. Head-to-head *in vivo* testing of the two vaccines against the original migrated design enabled us to probe the immunological implications of attaching peptide antigen *via* the phytosphingosine portion of the molecule (*i.e.* the 'migration' approach) *versus* appending it at the sugar head group (*i.e.* the '6''-modified' approach), in particular, the ability of the vaccine candidates to generate antigen-specific memory T cell responses in the liver and their ability to protect against *Plasmodium* sporozoite challenge.

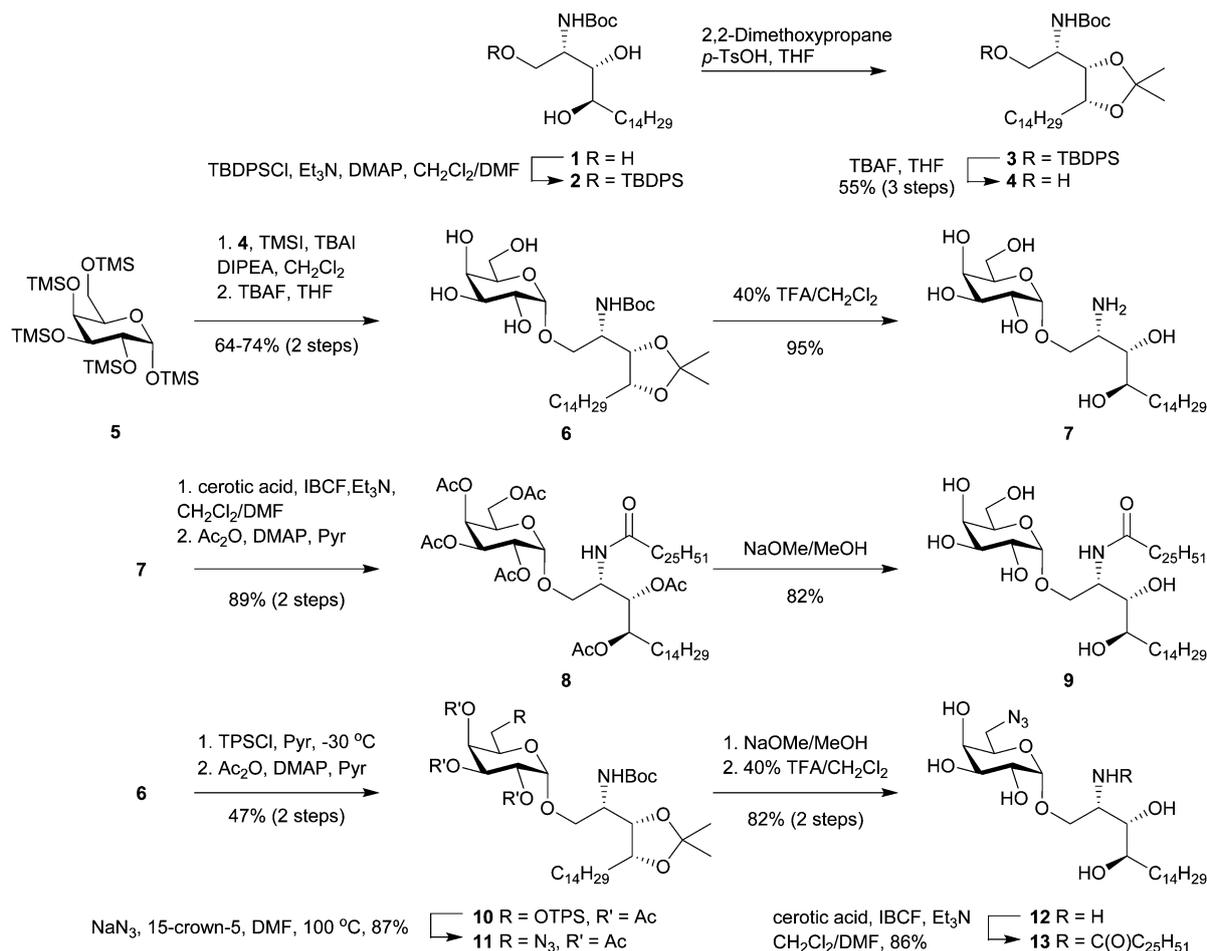
## Results and discussion

### Vaccine synthesis

Of the many reported syntheses of  $\alpha$ -GalCer (reviewed in Banchet-Cadeddu *et al.*<sup>35</sup>), Gervay-Hague's galactosyl iodide mediated glycosylation<sup>36</sup> offers an elegant approach that is both high yielding and stereoselective. The high  $\alpha$ -selectivity obtained from this reaction is due to the *in situ* formation of a  $\beta$ -galactosyl iodide and its subsequent displacement by a phytosphingosine acceptor. Because the donor can be easily prepared from D-galactose in one step which does not require chromatographic purification, this synthetic approach appears amenable to scale-up, an important consideration for GMP manufacture.

The synthesis of  $\alpha$ -GalCer and its 6''-modified analogues commenced from commercially available *N*-Boc-protected phytosphingosine **1** which was converted into the acetonide-containing acceptor (**4**) in three steps *via* temporary protection of the primary hydroxy group as a silyl ether (Scheme 1). This route provides the glycosyl acceptor in 55% overall yield





Scheme 1 Synthetic route to  $\alpha$ -GalCer (9) and its 6''-deoxy-6''-azido analogue (13).

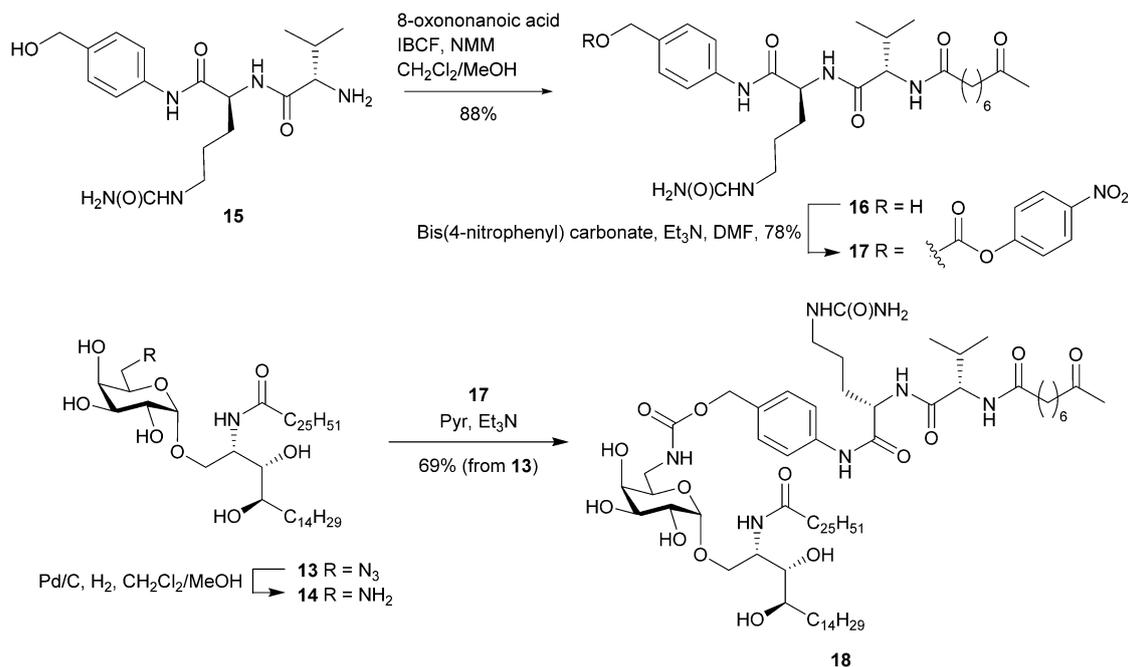
(>95% pure) and, importantly, requires only one simple chromatography purification step. Glycosylation with excess per-TMS-protected galactose 5 in the presence of iodotrimethylsilane (TMSI), tetrabutylammonium iodide (TBAI) and *N,N*-diisopropylethylamine (DIPEA) converted 4 into the desired  $\alpha$ -linked product after 48 h at ambient temperature. Monitoring the glycosylation reaction by HPLC-MS, we observed that freshly prepared TMSI was superior to a commercially acquired solution of TMSI in dichloromethane whereby the former reagent effected the glycosylation in 87% compared to 76% for the latter. Treating the crude product with tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF) gave 6 in 74% isolated yield (based on a 2.2 mmol reaction of 4). The feasibility of scaling up this reaction was demonstrated by performing the reaction on 11 mmol scale to afford multi-gram quantities of 6 in 64% yield. Removal of the acid-labile protecting groups with 40% trifluoroacetic acid in dichloromethane gave  $\alpha$ -galactosylphytosphingosine ( $\alpha$ -GalPhs, 7). *N*-Acylation of 7 with cerotic acid, pre-activated as a mixed carbonic anhydride, followed by per-*O*-acetylation afforded a protected form of  $\alpha$ -GalCer (8) that is easily purified from low level contaminants on scale by silica gel chromatography. Zemplén deacetylation followed by precipitation from hot

ethanol afforded gram quantities  $\alpha$ -GalCer (9) in good overall yield.

Access to the *O*-6'' position was achieved by selective sulfonation of the primary hydroxyl group in 6 using the sterically hindered 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCI).<sup>21</sup> Subsequent per-*O*-acetylation was performed to improve the separation of this compound from small amounts of contaminating bis-sulfonated materials. Azide displacement at *C*-6'' followed by removal of the protecting groups afforded 12 which was *N*-acylated to give 6''-azido-6''-deoxy- $\alpha$ -GalCer (13). Palladium-mediated reduction of 13 to 6''-amino-6''-deoxy- $\alpha$ -GalCer (14) gave a product that was poorly soluble in most common organic solvents and mixtures thereof with the apparent exception being hexafluoroisopropanol (HFIP). To avoid the difficulties surrounding its handling, 13 was reduced using Pd/C and H<sub>2</sub>, and the resulting amine was carried forward in the synthesis without purification. Here, filtration of the catalyst and swapping of the solvents from dichloromethane/methanol to pyridine provided a suspension of crude 14 which, upon reaction with the pre-activated dipeptide-ketone linker 17,<sup>37</sup> afforded the pro-adjuvant 18 in readiness for peptide conjugation (Scheme 2).

Various linker strategies have been developed for  $\alpha$ -GalCer-based self-adjuvanting conjugate vaccine candidates that allow





Scheme 2 Synthetic route to the pro-adjuvant **18** from 6''-azido-6''-deoxy- $\alpha$ -GalCer and immolative dipeptide linker.

release of the glycolipid adjuvant and its antigen cargo inside the same immune cell. Examples include that of Seeberger and De Libero *et al.*<sup>11</sup> who attached a synthetic amine-equipped  $\alpha$ -GalCer derivative non-selectively to the capsular polysaccharide of *Streptococcus pneumoniae* through the action of cyanogen bromide. The resulting isourea, *N*-substituted imidocarbonate and *N*-substituted carbamate linkages were believed to degrade upon exposure to an acidic environment. Another approach has been to attach peptide antigen to 6''-deoxy-6''-thio- $\alpha$ -GalCer *via* maleimido and disulfide linkages that exploit glutathione-mediated release mechanisms.<sup>21</sup> For this work, our vaccine design employs the versatile *p*-aminobenzyl-citrulline-valine (PAB-Cit-Val) linker<sup>38</sup> which provides a substrate for proteolytic cathepsin enzymes (cysteine proteases which are found at high levels inside immune cells). Recognition and cleavage of the citrulline carboxylamide bond in **18** (or its peptide conjugate) would result in a facile 1,6-elimination cascade releasing a molecule of CO<sub>2</sub>, azaquinone methide and the adjuvant, 6''-amino-6''-deoxy- $\alpha$ -GalCer (**14**). Prior to the synthesising the full GLP-vaccine candidates, susceptibility of the PAB-Cit-Val linker to human liver-derived cathepsin B was investigated in a simplified system whereby the pro-adjuvant **18** was exposed to the enzyme for 24 h at 37 °C in a mixture of HFIP/DMSO in ammonium acetate buffer. Following the reaction by HPLC-MS revealed that after 1 h, 4 h and 24 h, approximately 44%, 17% and 4% (respectively) of **18** remained whilst the cleaved products 8-oxononanoyl-valine-citrulline (Cit-Val-Non) and 6''-amino-6''-deoxy- $\alpha$ -GalCer (**14**) were detected (ESI,† Fig. S1).

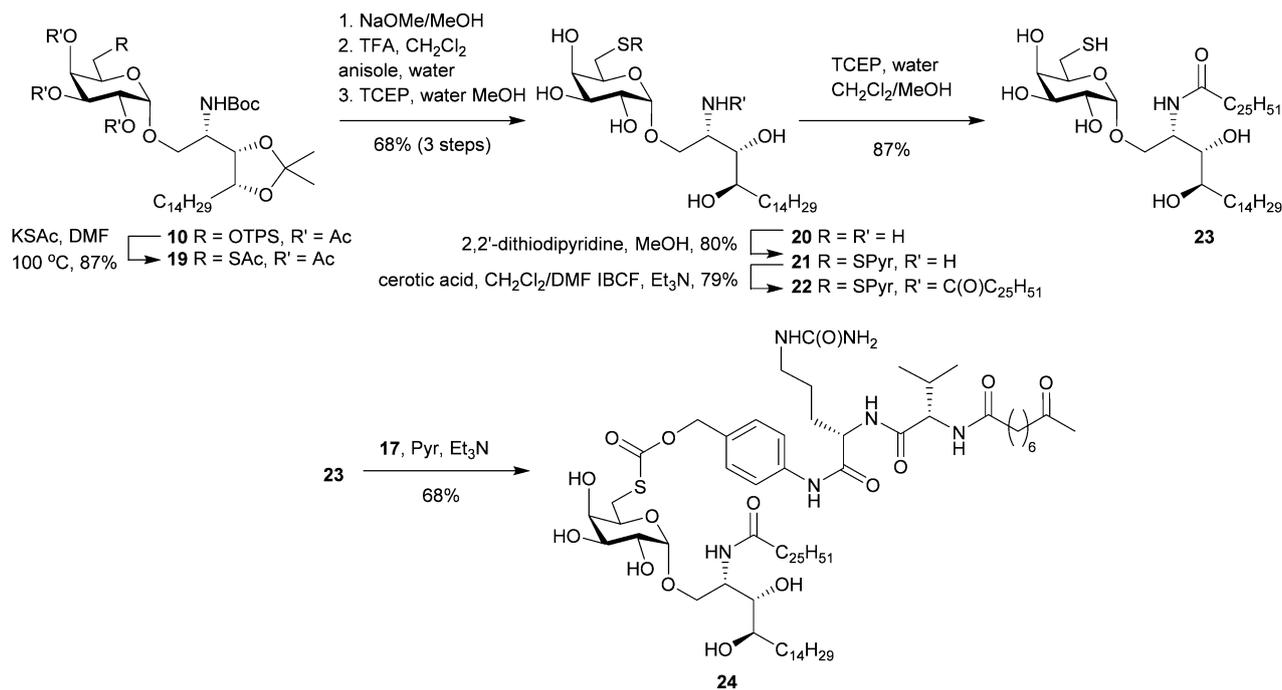
Having demonstrated the enzyme-mediated release of 6''-amino-6''-deoxy- $\alpha$ -GalCer from its pro-adjuvant form, we were interested to see if this same strategy could be applied to the potent iNKT cell agonist, 6''-deoxy-6''-thio- $\alpha$ -GalCer.<sup>21</sup>

To the best of our knowledge, this would be the first use of a self-immolative *p*-aminobenzyl linker to release a thiol-containing drug. Towards this, we embarked on the synthesis of 6''-deoxy-6''-thio- $\alpha$ -GalCer and its conversion to the corresponding thiocarbonate-containing pro-adjuvant.

Starting from the advanced intermediate **10**, thioacetate displacement of the triisopropylbenzenesulfonyl group was effected by heating in DMF with potassium thioacetate (KSAC) affording **19** in high yield (Scheme 3). Deacetylation was achieved by treating **19** with catalytic NaOMe in methanol to afford the crude product as an 85:15 mixture of thiol and disulfide. Subsequent treatment with 50% TFA/dichloromethane in the presence of water and anisole removed the acetone and *N*-Boc protecting groups without altering the thiol/disulfide ratio. Reduction of this mixture to the sulfhydryl product (**20**) was achieved by treating the mixture with TCEP in methanol/water and the resulting product was isolated using RP-C18 chromatography. Protection of the thiol as the pyridyl disulfide permitted *N*-acylation with cerotic acid to give **22**. TCEP-mediated reductive cleavage of the pyridyl disulfide group gave **23** which was subsequently reacted with the PAB-Cit-Val-ketone linker (**17**) under basic conditions to afford the thiocarbonate-containing pro-adjuvant **24**.

Incubating **24** with cathepsin B and following the reaction by HPLC-MS revealed the pro-adjuvant cleaved to its respective constituents (Cit-Val-Non and **23**) as intended with approximately 34% of **24** remaining after 24 h. (ESI,† Fig. S2). When cathepsin B was omitted, pro-adjuvant **24** remained intact demonstrating that thiocarbonate cleavage was enzyme mediated and not the result of direct hydrolysis. Although **24** appears more resistant to cathepsin B activity than **18** *in vitro*, work by Dubowchik and Firestone<sup>38</sup> suggests that other





Scheme 3 Synthetic route to 6''-deoxy-6''-thio- $\alpha$ -GalCer (**23**) and its pro-adjuvant form (**24**).

intracellular enzymes can also cause linker immolation *in vivo* thus we considered that the utility of this design was best examined in a functional assay.

With the 6''-modified pro-adjuvants **18** and **24** in hand, the next step was to conjugate these substrates with peptide antigen to prepare the GLP-conjugate vaccine candidates. The antigen selected for this work was the short peptide epitope NVDFDFNNL. Identified from the *Plasmodium berghei* ANKA ribosomal protein (PbRPL6), the NVDFDFNNL sequence (PbRPL6<sub>120–127</sub>) has previously been shown to be an excellent antigen for vaccination against liver-stage malaria.<sup>39</sup> To investigate if this epitope could also induce antigen-specific CD8<sup>+</sup> liver T<sub>RM</sub> cells when attached to our 6''-modified  $\alpha$ -GalCer pro-adjuvants, the epitope was modified by adopting a similar design to what has been previously shown to work for peptide-conjugate vaccines.<sup>18</sup> Here, the CD8 epitope was adorned with naturally flanking amino acid residues – ST- (*N*-terminus) and –S (*C*-terminus) from the protein antigen and the *N*-terminal –FFRK – proteasomal cleavage sequence, separated by a tri-alanine (–AAA–) spacer.

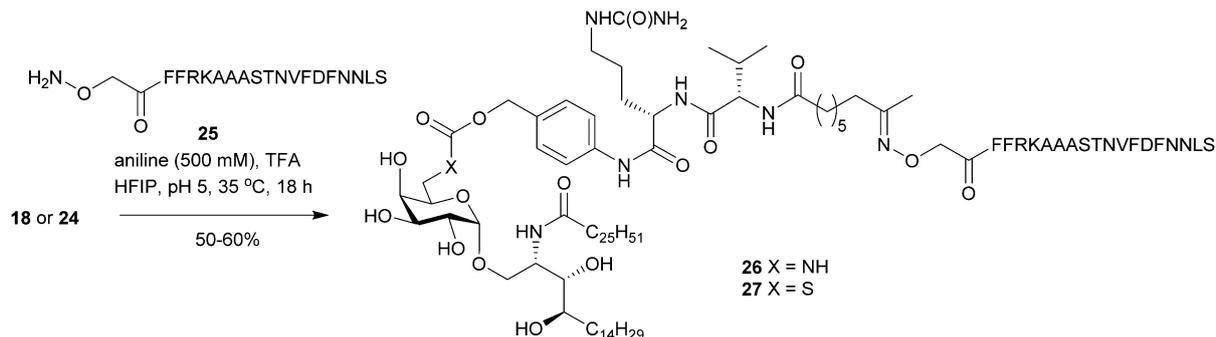
To create the GLP vaccine candidates, our strategy was to synthesise the modified peptide antigen with an *N*-terminal aminoxy acetyl residue (**25**) and attach it to the pro-adjuvants (**18** and **24**) *via* chemoselective oxime ligation. The oxime linkage was chosen for its relative hydrolytic stability<sup>40</sup> and its 'amide-like' small chemical footprint. Initial attempts to form the oxime bond employed conditions (4 : 2 : 3 THF/MeOH/300 mM aniline buffer at pH 4.1) which were successful for the synthesis of other GLP-vaccine candidates.<sup>18</sup> Unfortunately, **18** was sparingly soluble in these reaction conditions, even at temperatures approaching 50 °C, resulting in poor conversion

into the desired vaccine product. Because HFIP efficiently solvates **18** and has inherent acidity, we next evaluated this as a suitable solvent for oxime ligation. Pleasingly, HFIP efficiently dissolved both the pro-adjuvant (**18**) and modified peptide (**25**) while the addition of aniline (100 mM) catalysed the reaction such that it was complete within 48 h at ambient temperature. Swapping the aniline for the purportedly superior catalyst *m*-phenylenediamine<sup>41</sup> (100 mM in HFIP) failed to improve the reaction kinetics. Because HFIP readily dissolves arylamine catalysts of oxime ligation, a high concentration of these can be easily achieved using this system. Increasing the concentration of aniline from 100 to 500 mM in HFIP whilst maintaining the pH at ~5 by the addition of TFA, reliably afforded the desired oxime products with no obvious side reactions (as determined by HPLC-MS) within 18 h at 35 °C. Employing these optimized conditions, the pro-adjuvants **18** and **24** were reacted with peptide **25** (1.2–1.5 equiv.) to give conjugates **26** and **27** respectively (Scheme 4). The excess peptide and aniline catalyst were removed by preparative HPLC affording the GLP-vaccine products (> 98% purity by HPLC) for biological testing.

### Biological studies

The 6''-modified  $\alpha$ -GalCer GLP vaccine candidates (**26** and **27**) were assessed for their ability to induce memory T cell responses, particularly those capable of preventing malaria. These responses can involve several T cell subsets, including two that recirculate around the body termed central memory T (T<sub>CM</sub>) and effector memory T (T<sub>EM</sub>) cells and a subset that resides permanently within tissues, termed resident memory T (T<sub>RM</sub>) cells. While T<sub>RM</sub> cells can be found in a number of tissues, those localised in the liver are crucial for controlling liver-stage infection by





Scheme 4 Formation of self-adjuvanting antimalarial vaccine candidates **26** and **27**.

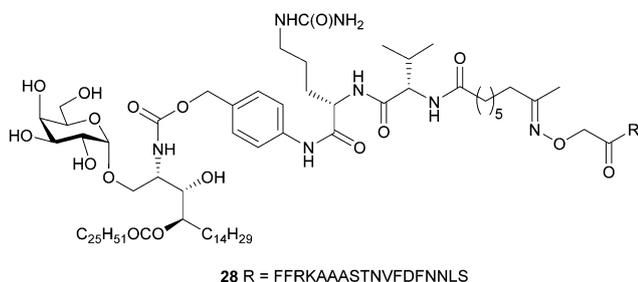


Fig. 2 Antimalarial GLP-vaccine (**28**) employing the 'migrated'  $\alpha$ -GalCer design.

*Plasmodium* parasites that cause malaria.<sup>42</sup> C57BL/6 (B6) mice were immunised intravenously with **26** or **27** or with our previously reported immunogenic vaccine candidate, **28** (Fig. 2), which is based on the migrated  $\alpha$ -GalCer design and known to efficiently generate T<sub>RM</sub> cell responses in the liver.<sup>18</sup> After 30 days, to allow formation of memory responses, antigen-specific T cells in the liver and spleen were enumerated by flow cytometry and assessed for key surface markers to determine their memory T cell subset composition and their antigen specificity. A H-2K<sup>b</sup> tetramer (tet) containing the NVDFNNL (NVF) peptide was used to identify T cells specific for the vaccine antigen (Fig. 3). This analysis revealed that both 6''-modified vaccine candidates efficiently generated NVF-specific (tet<sup>+</sup>) CD8<sup>+</sup> T<sub>RM</sub> cells in the liver (Fig. 3A, B), with vaccine candidate **26** inducing about twice as many as the control **28**. Significantly more NVF-specific memory T cells were also generated in the spleens of mice vaccinated with **26** compared to **28** (Fig. 3C), largely as a consequence of an increase in splenic T<sub>RM</sub> cell numbers. Overall, these results showed that both **26** and **27** were immunogenic and capable of inducing liver T<sub>RM</sub> cells, with the 6''-amino-6''-deoxy- $\alpha$ -GalCer conjugate (**26**), capable of generating the highest number of these cells.

To determine whether the greater ability of **26** to generate liver T<sub>RM</sub> cells was due to an enhanced ability to activate NKT cells, the NKT cell responses of mice vaccinated with **26**, **27** or **28** were assessed shortly after vaccination (day 7) (Fig. 4). This revealed that, relative to **28**, both **26** and **27** induced significantly more NKT cells in the liver and spleen (Fig. 4A, B and ESI<sup>†</sup> Fig. S3A, SB), most likely through proliferation. Phenotypic analysis showed that all three vaccine candidates stimulated

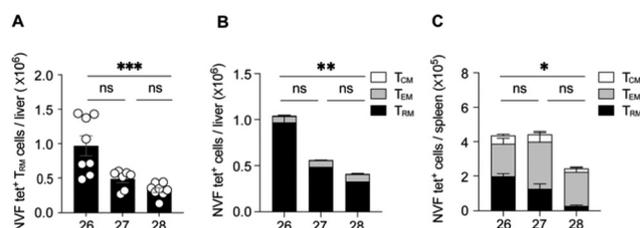
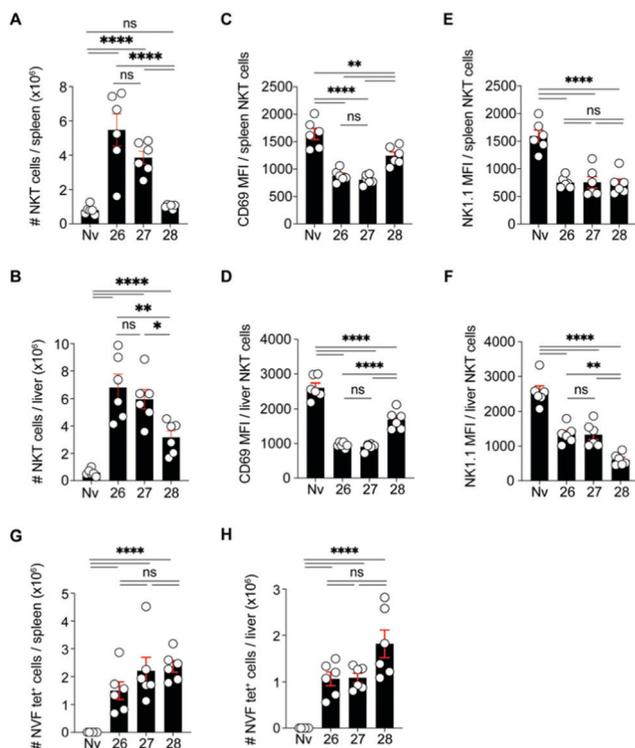


Fig. 3 Conjugate vaccines comprising 6''-modified  $\alpha$ -GalCer generate large numbers of liver T<sub>RM</sub> cells. B6 mice were vaccinated intravenously with vaccines **26**, **27** or **28** and examined for NVF-specific memory T cell responses >30 days later by flow cytometry after staining single cell suspensions with NVF-tetramers (NVF-tet) and various surface markers. (A) The number of NVF-tet<sup>+</sup> CD8<sup>+</sup> T<sub>RM</sub> (CD8<sup>+</sup>CD44<sup>+</sup>CD69<sup>+</sup>CD62L<sup>-</sup>) cells in the liver in individual mice (circles). Mean (black bar). (B and C) The number of NVF-tet<sup>+</sup> T<sub>RM</sub> (black), T<sub>EM</sub> (grey; CD8<sup>+</sup>CD44<sup>+</sup>CD69<sup>-</sup>CD62L<sup>-</sup>) and T<sub>CM</sub> (white, CD8<sup>+</sup>CD44<sup>+</sup>CD69<sup>-</sup>CD62L<sup>+</sup>) memory CD8<sup>+</sup> T cells in the liver (B) and spleen (C). Data are pooled from two independent experiments using 3–4 mice per group for each experiment. Error bars represent mean  $\pm$  SEM. Numbers of NVF-tet<sup>+</sup> CD8<sup>+</sup> T<sub>RM</sub> cells in (A) were log-transformed and compared using a Kruskal–Wallis test and Dunn's multiple comparisons test. Total numbers of NVF-tet<sup>+</sup> CD8<sup>+</sup> memory cells in (B and C) were log-transformed and compared using a Kruskal–Wallis test and Dunn's multiple comparisons test. ns, not significant, \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

both splenic and liver NKT cells to downregulate surface CD69 and NK1.1, indicating NKT cell activation, with greater down-regulation of CD69 after immunization with **26** and **27** relative to **28** (Fig. 4C–F and ESI<sup>†</sup> Fig. S3C, D). Examination of CD8<sup>+</sup> T cell responses at this early time point (day 7) showed similar numbers of NVF-specific effector T cells in the spleens and livers of mice vaccinated with all three compounds (Fig. 4G, H and ESI<sup>†</sup> Fig. S3E, F), indicating no obvious difference in priming at this early stage. Together, these data suggest that while vaccine candidate **26** induced about 2-fold more liver T<sub>RM</sub> cells than **28**, this difference could not be unequivocally attributed to better NKT cell activation as vaccine candidate **27** also showed improved NKT cell activation but did not generate significantly more liver T<sub>RM</sub> cells. Clearly, all three vaccine candidates efficiently activate NKT cells and are capable of excellent generation of liver T<sub>RM</sub> cells.

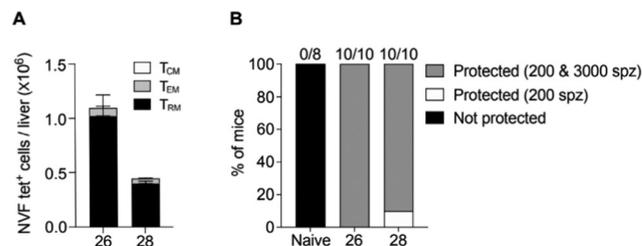
Although **26** induced superior numbers of memory T cells than the previous best vaccine candidate, **28**, it was unclear





**Fig. 4** Vaccine candidates **26** and **27** induce stronger NKT cell activation than their migrated  $\alpha$ -GalCer counterpart. B6 mice were immunised intravenously with **26**, **27** or **28** and then examined for acute NKT and NVF-specific T cell responses at day 7. (A and B) Enumeration of NKT cells in the spleen (A) and liver (B). (C and D) Expression of CD69 on NKT cells in the spleen (C) and (D) liver. (E and F) Expression of NK1.1 on NKT cells in the spleen (E) and liver (F). (G and H) The number of NVF-tet<sup>+</sup> CD44<sup>+</sup> CD8<sup>+</sup> T cells in the spleen (G) and liver (H). Data are pooled from two independent experiments using 3 mice per group for each experiment. Error bars represent mean  $\pm$  SEM. Groups in (A, B, G and H) were log-transformed and compared by one-way ANOVA with Tukey's multiple comparison post-test. Groups in (C-F) were compared by one-way ANOVA with Tukey's multiple comparison post-test. ns, not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\*\* $P < 0.0001$ . Nv, naive.

whether these cells were functional. To test function, we assessed whether liver T<sub>RM</sub> cells generated by **26**, like those of **28**, could mediate protection against malaria. B6 mice were immunised with **26** or **28** and then left for 30 days before assessing T cell numbers in the liver in some mice from each group and then challenging the remaining mice with 200 PbA sporozoites (Fig. 5). This confirmed that vaccine candidate **26** generated more liver T<sub>RM</sub> cells than **28** (Fig. 5A) and showed that all mice were protected from a 200 PbA sporozoite challenge (Fig. 5B and ESI,† Fig. S4A), indicating that T<sub>RM</sub> cells induced by **26** were indeed functional. To further assess function, surviving mice were rechallenged with a higher dose of 3000 PbA sporozoites, which showed that all mice immunized with **26** were protected from this higher dose (Fig. 5B and ESI,† Fig. S4B). Overall, these data indicated that vaccine **26** is highly effective at generating liver T<sub>RM</sub> cells and inducing protective immunity against malaria.



**Fig. 5** Compound **26** generates large numbers of liver T<sub>RM</sub> cells that protect mice from malaria. B6 mice were immunised intravenously with vaccines **26** or **28** and, after 30 days, were assessed by T cell enumeration or parasite challenge. (A) Livers of vaccinated mice were enumerated for NVF-specific memory T cell responses by flow cytometry after staining single cell suspensions with NVF-tet and various surface markers. Error bars represent mean  $\pm$  SEM. Total numbers of NVF-tet<sup>+</sup> CD8<sup>+</sup> memory cells were log-transformed and compared by one-way ANOVA with Tukey's multiple comparison post-test. \* $P < 0.05$ , \*\*\*\* $P < 0.0001$ . Note that the bar for naive (Nv) group shown in (A) is not visible due to low cell counts (<1600 cells). (B) Vaccinated mice were challenged intravenously with 200 PbA sporozoites and parasitemia measured up to day 12 post-infection. Mice showing no parasitemia were recorded as protected and were rechallenged intravenously with 3000 PbA sporozoites 21 days after the first challenge and monitored for parasitemia for 12 days. The percentage of mice that were protected from both 200 and 3000 sporozoites is shown in grey; the percentage only protected from 200 sporozoites shown in white; the percentage unprotected shown in black. Data are pooled from two independent experiments using 2–5 mice per group for each experiment. Numbers above bars indicate the number of mice that were protected against the initial 200 sporozoite challenge over total number of mice challenged.

## Conclusions

This work provides a succinct synthetic route to the potent iNKT cell agonist  $\alpha$ -GalCer and its 6''-deoxy-6''-thio and 6''-amino analogues. Attaching a traceless linker to the 6''-S or -N atoms of these glycolipids produced pro-adjuvants of 6''-substituted  $\alpha$ -GalCer that were readily cleaved by human cathepsin B *in vitro* with the self-immolating thiocarbonate version being the first example of its kind. Oxime ligation of peptide antigen to the pro-adjuvants was expedited by developing optimized HFIP-based reaction conditions. The resulting glycolipid-peptide conjugates were shown to co-stimulate NKT cells and peptide-specific CD8<sup>+</sup> T cells generating high levels of T<sub>RM</sub> cells in the liver with our lead 6''-amino-6''-deoxy- $\alpha$ -GalCer conjugate vaccine candidate inducing protective immunity against malaria in a *Plasmodium* sporozoite challenge model.

## Data availability

Full experimental details and compound characterisation are available in the ESI.†

## Author contributions

M. A. M., R. J. A., M. W. R., S. L. D., and B. J. C. synthesized the compounds. Y. C. C., S. T. S. C., and L. E. H. performed the experiments and analysed the data. A. C., V. M., and G. I. M. provided reagents. B. J. C., G. F. P., I. F. H., and W. R. H.



conceived the experiments. B. J. C., Y. C. C., and W. R. H. wrote the manuscript and all authors contributed to its reviewing and editing.

## Conflicts of interest

The authors declare the following competing financial interest(s): G. F. P. and I. F. H. are the chief technical officer and chief scientific officer (respectively) of Avalia Immunotherapies Limited, and W. R. H. is a member of its Scientific Advisory Board. Avalia holds exclusive, worldwide license to patents related to aspects of the chemical design reported here. R. J. A., I. F. H., G. F. P., and B. J. C. are inventors on a granted patent (U.S. patent no. 10046046, granted 14 August 2018). B.J.C., R.J.A., I.F.H., G. F. P., L. E. H., and W. R. H. are named inventors on a PCT application (PCTNZ2020050048) submitted by Victoria University of Wellington, Malcorp Biodiscoveries Limited, and Victoria Link Limited that covers the production of tissue-resident memory T cells with glycolipid-peptide vaccines.

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## Notes and references

- 1 T. Natori, M. Morita, K. Akimoto and Y. Koezuka, Agelaphins, Novel Antitumor and Immunostimulatory Cerebroside from the Marine Sponge Agelas-Mauritianus, *Tetrahedron*, 1994, **50**(9), 2771–2784.
- 2 M. Morita, K. Motoki, K. Akimoto, T. Natori, T. Sakai, E. Sawa, K. Yamaji, Y. Koezuka, E. Kobayashi and H. Fukushima, Structure-Activity Relationship of Alpha-Galactosylceramides against B16-Bearing Mice, *J. Med. Chem.*, 1995, **38**(12), 2176–2187.
- 3 Y. T. Zhang, R. Springfield, S. Y. Chen, X. Li, X. T. Feng, R. Moshirian, R. R. Yang and W. M. Yuan, alpha-GalCer and iNKT Cell-Based Cancer Immunotherapy: Realizing the Therapeutic Potentials, *Front. Immunol.*, 2019, **10**, 1126.
- 4 A. N. Courtney, P. N. Nehete, B. P. Nehete, P. Thapa, D. Zhou and K. J. Sastry, Alpha-galactosylceramide is an effective mucosal adjuvant for repeated intranasal or oral delivery of HIV peptide antigens., *Vaccine*, 2009, **27**(25–26), 3335–3341.
- 5 S. Fujii, K. Shimizu, C. Smith, L. Bonifaz and R. M. Steinman, Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein, *J. Exp. Med.*, 2003, **198**(2), 267–279.
- 6 N. Singh, S. Hong, D. C. Scherer, I. Serizawa, N. Burdin, M. Kronenberg, Y. Koezuka and L. Van Kaer, Cutting edge: activation of NK T cells by CD1d and alpha-galactosylceramide directs conventional T cells to the acquisition of a Th2 phenotype, *J. Immunol.*, 1999, **163**(5), 2373–2377.
- 7 S. Fujii, A. Goto and K. Shimizu, Antigen mRNA-transfected, allogeneic fibroblasts loaded with NKT-cell ligand confer antitumor immunity, *Blood*, 2009, **113**(18), 4262–4272.
- 8 R. Verbeke, I. Lentacker, K. Breckpot, J. Janssens, S. Van Calenbergh, S. C. De Smedt and H. Dewitte, Broadening the Message: A Nanovaccine Co-loaded with Messenger RNA and alpha-GalCer Induces Antitumor Immunity through Conventional and Natural Killer T Cells, *ACS Nano*, 2019, **13**(2), 1655–1669.
- 9 M. L. Guevara, Z. Jilesen, D. Stojdl and S. Persano, Codelivery of mRNA with alpha-Galactosylceramide Using a New Lipopolyplex Formulation Induces a Strong Antitumor Response upon Intravenous Administration, *ACS Omega*, 2019, **4**(8), 13015–13026.
- 10 R. J. Anderson, C. W. Tang, N. J. Daniels, B. J. Compton, C. M. Hayman, K. A. Johnston, D. A. Knight, O. Gasser, H. C. Poyntz, P. M. Ferguson, D. S. Larsen, F. Ronchese, G. F. Painter and I. F. Hermans, A self-adjuncting vaccine induces cytotoxic T lymphocytes that suppress allergy, *Nat. Chem. Biol.*, 2014, **10**(11), 943–949.
- 11 M. Cavallari, P. Stallforth, A. Kalinichenko, D. C. K. Rathwell, T. M. A. Gronewold, A. Adibekian, L. Mori, R. Landmann, P. H. Seeberger and G. De Libero, A semi-synthetic carbohydrate-lipid vaccine that protects against S-pneumoniae in mice, *Nat. Chem. Biol.*, 2014, **10**(11), 950–956.
- 12 M. Koch, V. S. Stronge, D. Shepherd, S. D. Gadola, B. Mathew, G. Ritter, A. R. Fersht, G. S. Besra, R. R. Schmidt, E. Y. Jones and V. Cerundolo, The crystal structure of human CD1d with and without alpha-galactosylceramide, *Nat. Immunol.*, 2005, **6**(8), 819–826.
- 13 D. M. Zajonc, C. Cantu, J. Mattner, D. P. Zhou, P. B. Savage, A. Bendelac, I. A. Wilson and L. Teyton, Structure and function of a potent agonist for the semi-invariant natural killer T cell receptor, *Nat. Immunol.*, 2005, **6**(8), 810–818.
- 14 N. A. Borg, K. S. Wun, L. Kjer-Nielsen, M. C. J. Wilce, D. G. Pellicci, R. Koh, G. S. Besra, M. Bharadwaj, D. I. Godfrey, J. McCluskey and J. Rossjohn, CD1d-lipid-antigen recognition by the semi-invariant NKT T-cell receptor, *Nature*, 2007, **448**(7149), 44–49.
- 15 C. Grasso, C. S. Field, C. W. Tang, P. M. Ferguson, B. J. Compton, R. J. Anderson, G. F. Painter, R. Weinkove, I. F. Hermans and M. V. Berridge, Vaccines adjuvanted with an NKT cell agonist induce effective T-cell responses in models of CNS lymphoma, *Immunotherapy*, 2020, **12**(6), 395–406.
- 16 R. J. Anderson, B. J. Compton, C. W. Tang, A. Authier-Hall, C. M. Hayman, G. W. Swinerd, R. Kowalczyk, P. Harris, M. A. Brimble, D. S. Larsen, O. Gasser, R. Weinkove,



- I. F. Hermans and G. F. Painter, NKT cell-dependent glycolipid-peptide vaccines with potent anti-tumour activity, *Chem. Sci.*, 2015, **6**(9), 5120–5127.
- 17 R. J. Anderson, J. Li, L. Kedzierski, B. J. Compton, C. M. Hayman, T. L. Osmond, C. W. Tang, K. J. Farrand, H. F. Koay, C. F. D. S. E. Almeida, L. R. Holz, G. M. Williams, M. A. Brimble, Z. F. Wang, M. Koutsakos, K. Kedzierska, D. I. Godfrey, I. F. Hermans, S. J. Turner and G. F. Painter, Augmenting Influenza-Specific T Cell Memory Generation with a Natural Killer T Cell-Dependent Glycolipid-Peptide Vaccine, *ACS Chem. Biol.*, 2017, **12**(11), 2898–2905.
- 18 L. E. Holz, Y. C. Chua, M. N. de Menezes, R. J. Anderson, S. L. Draper, B. J. Compton, S. T. S. Chan, J. Mathew, J. Li, L. Kedzierski, Z. F. Wang, L. Beattie, M. H. Enders, S. Ghilas, R. May, T. M. Steiner, J. Lange, D. Fernandez-Ruiz, A. M. Valencia-Hernandez, T. L. Osmond, K. J. Farrand, R. Seneviratna, C. F. Almeida, K. M. Tullett, P. Bertolino, D. G. Bowen, A. Cozijnsen, V. Mollard, G. I. McFadden, I. Caminschi, M. H. Lahoud, K. Kedzierska, S. J. Turner, D. I. Godfrey, I. F. Hermans, G. F. Painter and W. R. Heath, Glycolipid-peptide vaccination induces liver-resident memory CD8(+) T cells that protect against rodent malaria, *Sci. Immunol.*, 2020, **5**(48), eaaz8035.
- 19 J. T. Hung, R. C. Sawant, J. C. Chen, Y. F. Yen, W. S. Chen, A. L. Yu and S. Y. Luo, Design and synthesis of galactose-6-OH-modified alpha-galactosyl ceramide analogues with Th2-biased immune responses, *RSC Adv.*, 2014, **4**(88), 47341–47356.
- 20 P. J. Jervis, L. M. Graham, E. L. Foster, L. R. Cox, S. A. Porcelli and G. S. Besra, New CD1d agonists: Synthesis and biological activity of 6''-triazole-substituted alpha-galactosyl ceramides, *Bioorg. Med. Chem. Lett.*, 2012, **22**(13), 4348–4352.
- 21 B. J. Compton, C. W. Tang, K. A. Johnston, T. L. Osmond, C. M. Hayman, D. S. Larsen, I. F. Hermans and G. F. Painter, Synthesis and Activity of 6''-Deoxy-6''-thio-alpha-GalCer and Peptide Conjugates, *Org. Lett.*, 2015, **17**(24), 5954–5957.
- 22 T. Tashiro, R. Nakagawa, T. Shigeura, H. Watarai, M. Taniguchi and K. Mori, RCI-61 and related 6'-modified analogs of KRN7000: Their synthesis and bioactivity for mouse lymphocytes to produce interferon-gamma *in vivo*, *Bioorg. Med. Chem.*, 2013, **21**(11), 3066–3079.
- 23 Y. J. Chang, J. R. Huang, Y. C. Tsai, J. T. Hung, D. Wu, M. Fujio, C. H. Wong and A. L. Yu, Potent immunomodulating and anticancer effects of NKT cell stimulatory glycolipids, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**(25), 10299–10304.
- 24 D. Chennamadhavuni, N. A. Saavedra-Avila, L. J. Carreno, M. J. Guberman-Pfeffer, P. Arora, Y. Q. Tang, R. Pryce, H. F. Koay, D. I. Godfrey, S. Keshipeddy, S. K. Richardson, S. Sundararaj, J. H. Lo, X. S. Wen, J. A. Gascon, W. M. Yuan, J. Rossjohn, J. Le Nours, S. A. Porcelli and A. R. Howell, Dual Modifications of alpha-Galactosylceramide Synergize to Promote Activation of Human Invariant Natural Killer T Cells and Stimulate Anti-tumor Immunity, *Cell Chem. Biol.*, 2018, **25**(7), 571–584.
- 25 T. Ebensen, C. Link, P. Riese, K. Schulze, M. Morr and C. A. Guzman, A pegylated derivative of alpha-galactosylceramide exhibits improved biological properties, *J. Immunol.*, 2007, **179**(4), 2065–2073.
- 26 J. Guillaume, N. Pauwels, S. Aspeslagh, D. M. Zajonc, D. Elewaut and S. Van Calenbergh, Synthesis of C-5'' and C-6''-modified alpha-GalCer analogues as iNKT-cell agonists, *Bioorg. Med. Chem.*, 2015, **23**(13), 3175–3182.
- 27 M. Trappeniers, K. Van Beneden, T. Decruy, U. Hillaert, B. Linclau, D. Elewaut and S. Van Calenbergh, 6'-Derivatized alpha-GalCer Analogues Capable of Inducing Strong CD1d-Mediated Th1-Biased NKT Cell Responses in Mice, *J. Am. Chem. Soc.*, 2008, **130**(49), 16468–16469.
- 28 M. H. Hsieh, J. T. Hung, Y. W. Liw, Y. J. Lu, C. H. Wong, A. L. Yu and P. H. Liang, Synthesis and Evaluation of Acyl-Chain- and Galactose-6''-Modified Analogues of alpha-GalCer for NKT Cell Activation, *ChemBioChem*, 2012, **13**(11), 1689–1697.
- 29 X. T. Zhou, C. Forestier, R. D. Goff, C. H. Li, L. Teyton, A. Bendelac and P. B. Savage, Synthesis and NKT cell stimulating properties of fluorophore- and biotin-appended 6''-amino-6''-deoxy-galactosylceramides, *Org. Lett.*, 2002, **4**(8), 1267–1270.
- 30 W. W. Ma, J. J. Bi, C. F. Zhao, Z. G. Zhang, T. X. Liu and G. S. Zhang, Synthesis and biological activities of amino acids functionalized alpha-GalCer analogues, *Bioorg. Med. Chem.*, 2020, **28**(1), 115141.
- 31 P. J. Jervis, M. Moulis, J. P. Jukes, H. Ghadbane, L. R. Cox, V. Cerundolo and G. S. Besra, Towards multivalent CD1d ligands: synthesis and biological activity of homodimeric alpha-galactosyl ceramide analogues, *Carbohydr. Res.*, 2012, **356**, 152–162.
- 32 N. Pauwels, S. Aspeslagh, D. Elewaut and S. Van Calenbergh, Synthesis of 6''-triazole-substituted alpha-GalCer analogues as potent iNKT cell stimulating ligands, *Bioorg. Med. Chem.*, 2012, **20**(24), 7149–7154.
- 33 A Phase 1-2 Study of ABX196 in Combination With Nivolumab in Patients With Hepatocellular Carcinoma. <https://clinicaltrials.gov/ct2/show/NCT03897543>.
- 34 Y. Liu, R. D. Goff, D. P. Zhou, J. Mattner, B. A. Sullivan, A. Khurana, C. Cantu, E. V. Ravkov, C. C. Lbegbu, J. D. Altman, L. Teyton, A. Bendelac and P. B. Savage, A modified alpha-galactosyl ceramide for staining and stimulating natural killer T cells, *J. Immunol. Methods*, 2006, **312**(1–2), 34–39.
- 35 A. Banchet-Cadecdu, E. Henon, M. Dauchez, J. H. Renault, F. Monneaux and A. Haudrechy, The stimulating adventure of KRN 7000, *Org. Biomol. Chem.*, 2011, **9**(9), 3080–3104.
- 36 M. Schombs, F. E. Park, W. J. Du, S. S. Kulkarni and J. Gervay-Hague, One-Pot Syntheses of Immunostimulatory Glycolipids, *J. Org. Chem.*, 2010, **75**(15), 4891–4898.
- 37 R. J. Anderson; G. F. Painter; I. F. Hermans; O. S. Gasser and R. Weinkove, *MAIT Cell Agonists*, WO2019058289, 2018.
- 38 G. M. Dubowchik, R. A. Firestone, L. Padilla, D. Willner, S. J. Hofstead, K. Mosure, J. O. Knipe, S. J. Lasch and P. A. Trail, 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 Chem.*, 2002, **13**(4), 855–869.
- 39 A. M. Valencia-Hernandez, W. Y. Ng, N. Ghazanfari, S. Ghilas, M. N. de Menezes, L. E. Holz, C. Huang, K. English, M. Naung, P. S. Tan, K. M. Tullett, T. M. Steiner, M. H. Enders, L. Beattie, Y. C. Chua, C. M. Jones, A. Cozijnsen, V. Mollard, Y. P. Cai, D. G. Bowen, A. W. Purcell, N. L. La Gruta, J. A. Villadangos, T. de Koning-Ward, A. E. Barry, W. Barchet, I. A. Cockburn, G. I. McFadden, S. Gras, M. H. Lahoud, P. Bertolino, R. B. Schittenhelm, I. Caminschi, W. R. Heath and D. Fernandez-Ruiz, A Natural Peptide Antigen within the Plasmodium Ribosomal Protein RPL6 Confers Liver T-RM Cell-Mediated Immunity against Malaria in Mice, *Cell Host Microbe*, 2020, **27**(6), 950–962.
- 40 J. Kalia and R. T. Raines, Hydrolytic stability of hydrazones and oximes., *Angew. Chem., Int. Ed.*, 2008, **47**(39), 7523–7526.
- 41 M. Rashidian, M. M. Mahmoodi, R. Shah, J. K. Dozier, C. R. Wagner and M. D. Distefano, A Highly Efficient Catalyst for Oxime Ligation and Hydrazone-Oxime Exchange Suitable for Bioconjugation, *Bioconjugate Chem.*, 2013, **24**(3), 333–342.
- 42 D. Fernandez-Ruiz, W. Y. Ng, L. E. Holz, J. Z. Ma, A. Zaid, Y. C. Wong, L. S. Lau, V. Mollard, A. Cozijnsen, N. Collins, J. Li, G. M. Davey, Y. Kato, S. Devi, R. Skandari, M. Pauley, J. H. Manton, D. I. Godfrey, A. Braun, S. S. Tay, P. S. Tan, D. G. Bowen, F. Koch-Nolte, B. Rissiek, F. R. Carbone, B. S. Crabb, M. Lahoud, I. A. Cockburn, S. N. Mueller, P. Bertolino, G. I. McFadden, I. Caminschi and W. R. Heath, Liver-Resident Memory CD8(+) T Cells Form a Front-Line Defense against Malaria Liver-Stage Infection, *Immunity*, 2016, **45**(4), 889–902.

