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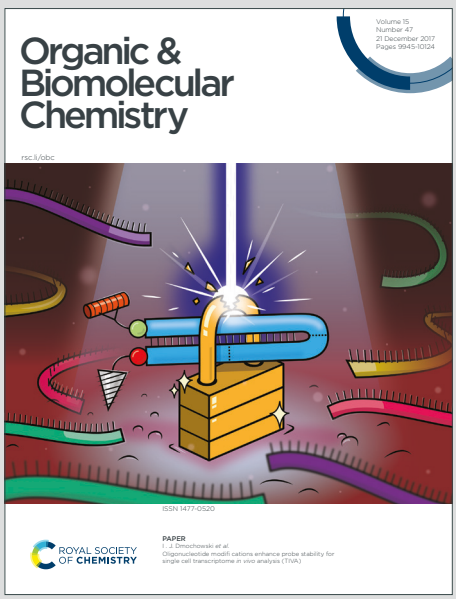

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The effect of MR1 ligand glyco-analogues on mucosal-associated invariant T (MAIT) cell activation

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Abstract

Mucosal-associated invariant T (MAIT) cells are a subset of recently identified innate-like T lymphocytes that appear to play an important role in many pathologies ranging from viral and bacterial infection, to autoimmune disorders and cancer. MAIT cells are activated via the presentation of ligands by MR1 on antigen presenting cells to the MAIT T cell receptor (TCR), however few studies have explored the effects of systematic changes to the ligand structure on MR1 binding and MAIT cell activation. Herein, we report on the first study into the effects of changes to the sugar motif in the known MAIT cell agonists 7-hydroxy-6-methyl-8-D-ribityllumazine (RL-6-Me-7-OH) and 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU). Tetramer staining of MAIT cells revealed that the absence of the 2'-hydroxy group on the sugar backbone of lumazines improved MR1–MAIT TCR binding, which could be rationalised

using computational docking studies. Although none of the lumazines activated MAIT cells, all 5-OP-RU analogues showed significant MAIT cell activation, with several analogues exhibiting comparable activity to 5-OP-RU. Docking studies with the 5-OP-RU analogues revealed different interactions between the sugar backbone and MR1 and the MAIT TCR compared to those observed for the lumazines and confirmed the importance of the 2'-hydroxy group for ligand binding and activity. Taken together, this information will assist in the development of future potent agonists and antagonists of MAIT cells.

Introduction

Mucosal-associated invariant T (MAIT) cells are a subset of innate-like T lymphocytes that comprise 1-10% of peripheral blood T lymphocytes and up to 50% of liver T cells in humans.[1,2] These cells bear a T cell receptor (TCR) with a highly conserved α chain (Vα7.2-Jα33 in humans, Vα19-Jα33 in mice),[3,4] and are activated by antigens bound to the major histocompatibility complex (MHC) class I-like molecule MR1 on antigen presenting cells (APCs) (Figure 1). Despite their high prevalence in humans, much remains unknown about the exact function of MAIT cells, though they are considered to have key roles in immunity, particularly as MR1 is highly conserved in mammalian species.[5,6] MAIT cells are absent in germ-free mice, highlighting the requirement of microbiota for their expansion,[3, 7] and have been found to migrate to inflammatory sites in diseases such as tuberculosis,[8,9] multiple sclerosis,[10,11] HIV,[12,13] and type 1[14] and type 2 diabetes.[15] Once activated via ligand presentation to their TCRs, MAIT cells develop an effector phenotype and secrete pro-inflammatory cytokines, proliferate, and can kill intracellular bacteria[16,17] and/or modulate the development of autoimmune diseases.[18]

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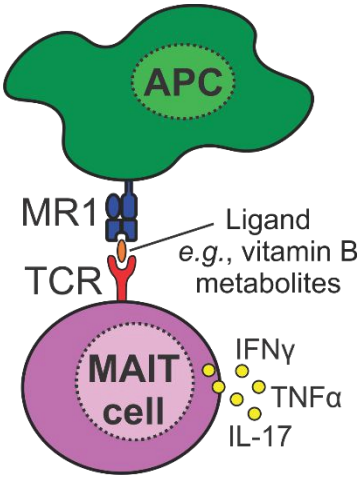

Figure 1. Ligand presentation by MR1 to MAIT cells

The ability of MAIT cells to respond to a variety of microbes in an MR1-restricted manner[19] has led to much recent interest in identifying MAIT cell ligands and in understanding how ligand structure influences the ensuing immunological response. The first ligand for MR1 was identified by Kjer-Nielsen *et al.* in 2012 and determined to be 6-formylpterin (6-FP, **1a**, Figure 2), a degradation product of folic acid (vitamin B9).[20] This ligand and subsequently others (*e.g.*, acetyl-6-formylpterin [Ac-6-FP], **1b**) were found to be potent antagonists of MAIT cells, for while such ligands do not interact with the MAIT cell TCR, they can form a Schiff base with the Lys43 residue of MR1.[16] The first series of MR1 agonists, also identified by Kjer-Nielsen *et al*.,[20] were the ribityl lumazines 6,7-dimethyl-8-D-ribityllumazine (RL-6,7-diMe, **2**), 7-hydroxy-6-methyl-8-D-ribityllumazine (RL-6-Me-7-OH, **3a**) and 6-hydroxymethyl-8-D-ribityllumazine (RL-6-CH_2OH, **4**), which are products of riboflavin (vitamin B2) metabolism.[20] These ligands bind MR1 through a variety of aromatic and non-covalent interactions analogous to those seen with 6-FP (**1a**), with the key difference being no Schiff-base formation but rather an interaction of the ribityl moiety with the MAIT TCR through hydrogen-bonding.[20,21] Subsequently, 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU, **5a**) and related derivatives were identified as highly potent MAIT cell ligands due to their ability to form a Schiff base with Lys43 on MR1 and interact with the MAIT TCR,[17] with recent studies being undertaken to develop more stable analogues of these compounds.[22,23] A variety of drugs and drug-like molecules were also recently screened as ligands for MR1, with direct contact of the ligand with the MAIT TCR being required for agonist activity.[24]

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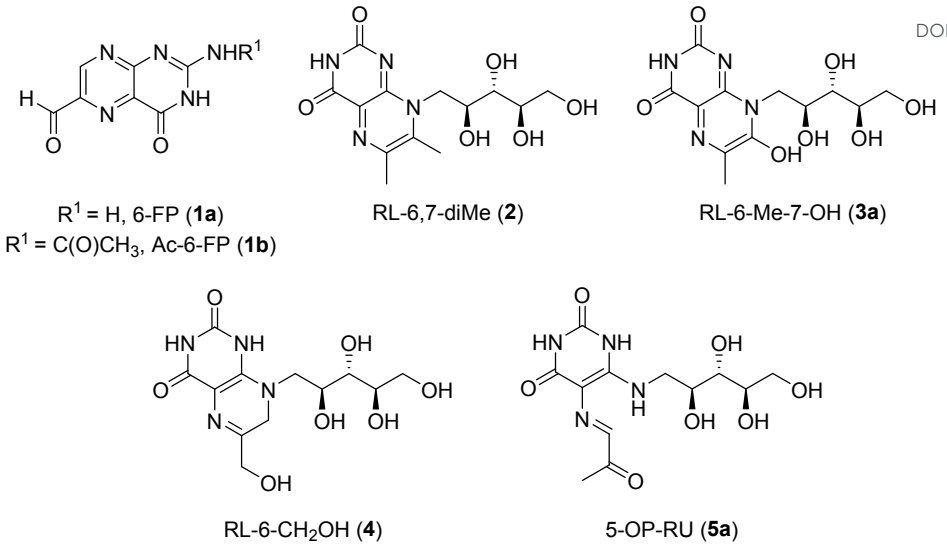

Figure 2. Representative MR1 ligands

Given the ability of the ribityl lumazines **2** and **3** to act as MAIT cell agonists, we became interested in exploring how the substitution pattern of the sugar-motif influences MAIT cell activation. This information, coupled with the knowledge about those structural motifs required for irreversible binding to MR1,[16-20,22,24] would aid in the design and synthesis of potent MAIT cell agonists. However, the current synthetic route to the sugar-substituted lumazines suffers from low overall yields (< 10%), and involves the synthesis of ribitylamine through D-ribose-oxime formation and high pressure catalytic hydrogenation, followed by condensation of the amine with 5-chloro-uracil, nitrosylation at the uracil 6-position and reduction to form the corresponding 6-amino-uracil. Condensation of the diamine with either diacetyl or pyruvic acid then forms RL-6,7-diMe (**2**) or RL-6-Me-7-OH (**3a**), respectively.[20] Thus, we first sought to develop an efficient three-step protecting-group-free synthesis of D-ribityl-substituted lumazines RL-6,7-diMe (**2**) and RL-6-Me-7-OH (**3a**). Glyco-analogues of the lumazine scaffold showing the greatest ability to bind MR1 would then be prepared using the established route, which in turn would allow the effects of the sugar moiety on MAIT cell activation to be explored. Given the recent interest in MAIT cells and the knowledge that their activation, expansion, or suppression can play a key role in numerous diseases,[11, 25-29] the studies reported herein will therefore assist with the development of MAIT cell-mediated therapeutics.

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Results and Discussion

The synthesis of RL-6,7-diMe (**2**) and RL-6-Me-7-OH (**3a**) began with the preparation of 1-amino-1-deoxy-D-ribitol (**7a**) from D-ribose (Scheme 1) through a protecting-group-free reductive amination reaction previously developed in our group.[30] Here, D-ribose (**6a**) was treated with $NaCNBH_3$, NH_4OAc (sat.) and NH_3 (30% aq. soln.) in EtOH at room temperature to smoothly afford the corresponding primary amine **7a** in 82% yield following purification by silica gel flash column chromatography. Nitration of 6-chlorouracil (**8**) with fuming HNO_3 in the presence of conc. H_2SO_4 afforded 6-chloro-5-nitrouracil (**9**) in 66% yield,[31] which was subsequently subjected to a substitution reaction with ribitylamine **7a** under basic conditions using aq. NaOH (pH 8) to give 5-nitro-6-ribitylaminouracil (**10a**) in a 74% yield.[32] Next, reduction of the nitro group in **10a** using Fe powder in 10% AcOH (aq.), followed by condensation with butane-2,3-dione under acidic conditions in a one-pot, two step procedure afforded both RL-6,7-diMe (**2**) and its reduced form (**11**) in 65% combined yield. Here, it should be noted that hydrolumazine **11** slowly reverted to the oxidised form when kept in aqueous solution at room temperature. The same methodology was then applied to the synthesis of RL-6-Me-7-OH (**3a**), whereby reduction of 5-nitro-6-ribitylaminouracil (**10a**) and subsequent condensation with sodium pyruvate afforded the target compound in 40% yield over the two-steps. Thus, we established a simple route to synthesise RL-6-Me-7-OH in 19% overall yield, which was a significant improvement on the reported yield of < 10%.[20]

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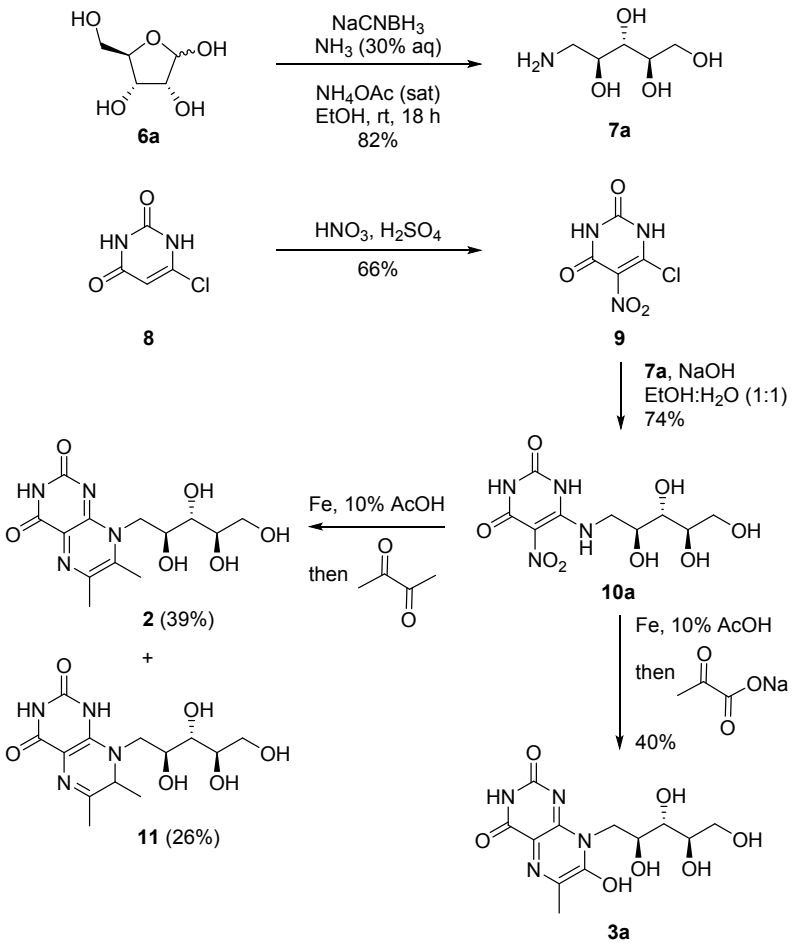

Scheme 1. Synthesis of lumazines **2**, **3a** and **11**

Having demonstrated proof-of-concept and an efficient 3-step synthesis of the ribityl-substituted lumazines, these ligands were then assessed for their MAIT cell activity. Compounds were loaded onto empty MR1 tetramers[33,34] and the resulting antigen-MR1 complexes tested for their ability to stain the 6C2 MAIT cell line[5] and the TG40 cell line,[35] which was used as a negative control as it lacks TCR-α and -β chains. The ligand 5-OP-RU (**5a**) was used as a positive control for MR1-tetramer staining. Here, lumazines **2**, **3a** and **11** were able to bind MR1 tetramers and stain the MAIT cells, as indicated by the co-staining of cells bearing the T cell marker CD3ε (Figure 3). Of these, RL-6-Me-7-OH (**3a**) possessed the strongest activity, with 96.4% of cells co-staining for both MR1-ligand complex and CD3ε. This lumazine scaffold was then used to explore the effects of changes in the sugar moieties on MAIT cell activation.

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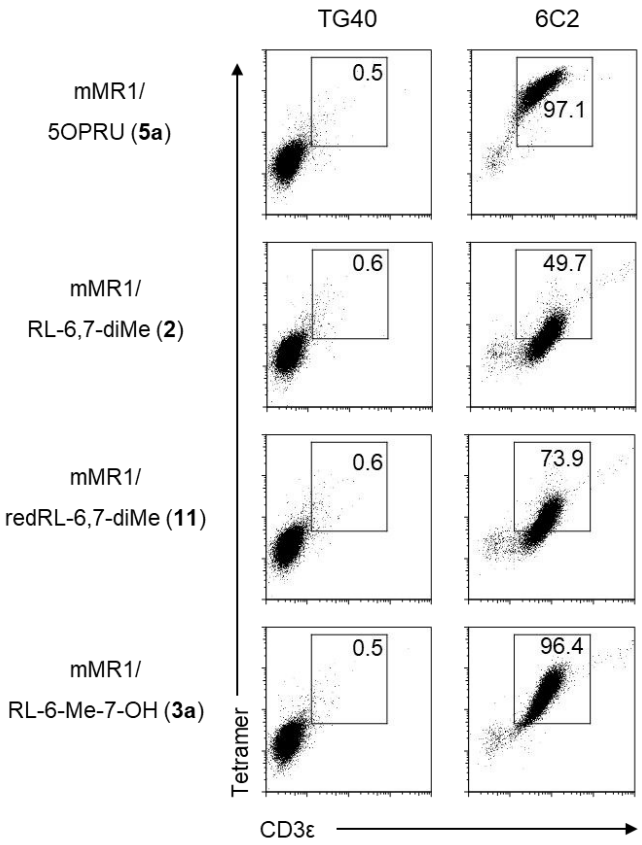
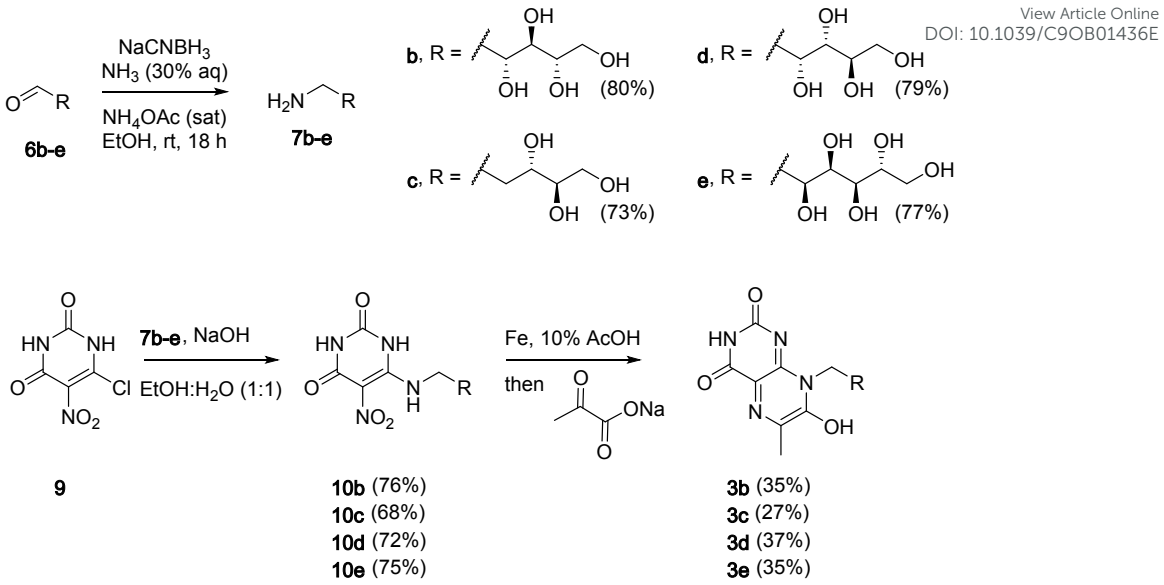


Figure 3. MR1-ligand tetramer staining of MAIT cells. Empty APC-conjugated MR1 tetramers were loaded with 5-OP-RU (**5a**) (positive control) and ligands **2**, **3a**, **11**, and the ability of the ligands to bind the tetramers determined using the MAIT cell line 6C2 and the parental cell line TG40 (negative for TCR), and PE-conjugated anti-mouse CD3 for the identification of T cells. Data is representative of three independent experiments.

With an efficient route for the synthesis of substituted lumazines in hand, the methodology was then extended to derivatives of RL-6-Me-7-OH (**3a**) containing L-ribityl- (**3b**), 2-deoxy-D-ribityl- (**3c**), D-arabinityl- (**3d**) and D-glucityl- (**3e**), rather than D-ribityl, sugar residues (Scheme 2). To this end, glycitylamines **7b-e** were prepared in good yields (73-80%) via reductive amination of the corresponding monosaccharides (**6b-e**), with subsequent substitution of the chloride in 6-chloro-5-nitrouracil (**9**) with the different glycitylamines, using the aforementioned methodology. Following *in situ* reduction of **10b-e** and condensation with sodium pyruvate, the target lumazines **3b-e** were obtained in 18-27% yield over the three steps.



Scheme 2. Glyco-analogues of RL-6-Me-7-OH (**3b-e**)

The lumazines **3a-e** were then assessed for their ability to bind MR1 and stain MAIT cells in the MR1 tetramer assay. All sugar analogues of RL-6-Me-7-OH (**3a**) were able to bind MR1 and stain 6C2 MAIT cells to some extent (Figure 4), with the configuration of the 2'-OH in RL-6-Me-7-OH (**3a**) proving unimportant for binding as the *arabino*-derivative **3d** complexed to MR1 was equably able to stain the cells. The 2-deoxy-D-ribityl analogue (dRL-6-Me-7-OH, **3c**) exhibited the highest level of staining, with 94.3% of cells co-staining for MR1-**3c** complex and CD3ε. This observation was initially surprising as it had been previously demonstrated that the ribityl 2'-OH of RL-6-Me-7-OH (**3a**) was involved in a hydrogen bond with the CDR3α Tyr95 residue in the crystal structure of an MR1–RL-6-Me-7-OH–MAIT TCR complex.[21] MAIT cell staining was largely abolished when using either L-ribityl analogue **3b** or D-glucityl analogue **3e** in the tetramer assay, indicating that the orientations of the 3'- and 4'-OH and the length of the glycityl chain are important for MR1-MAIT TCR binding.

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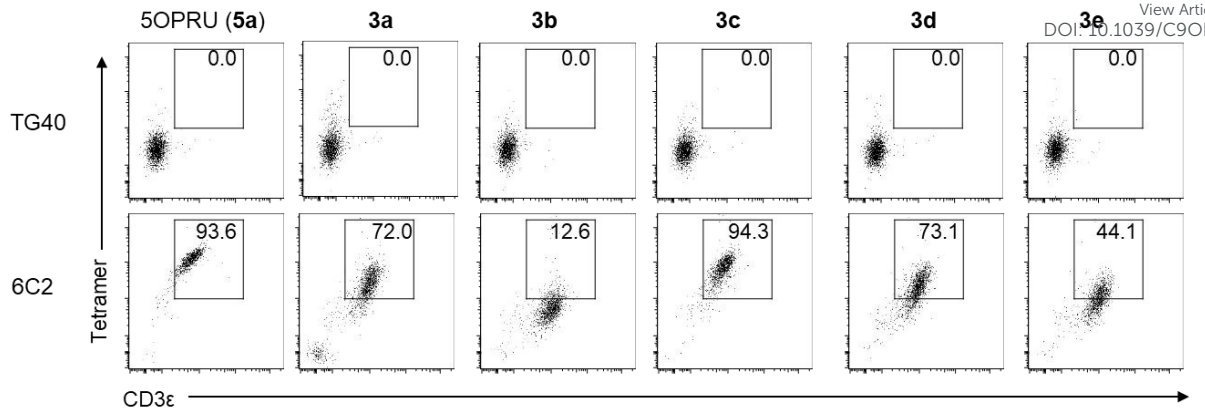

Figure 4. MR1-glyco-analogue tetramer staining of MAIT cells. Empty APC-conjugated MR1 tetramers were loaded with **3a-e** and assessed for their ability to stain the MAIT cell line 6C2 and the parental cell line TG40 (negative for TCR), and PE-conjugated anti-mouse CD3 for the identification of T cells. Data is representative of three independent experiments.

To better evaluate the importance of the 2'-OH in the binding of lumazine ligands to MR1 and the MAIT cell TCR, we docked dRL-6-Me-7-OH (**3c**) into the MR1-MAIT TCR complex. While the crystal structure of mouse MR1-MAIT TCR is unavailable, the analogous human complex is, and with high sequence identity with the mouse proteins (90% for the MR1-α1 and -α2 domains,[36] and 72% for the MAIT TCR[37]), the hMR1-MAIT TCR complex serves as a suitable substitute for our modelling studies. Moreover, it is also important to note that the CDR3α is highly conserved in humans and mice, with identical contact residues between MR1 and the MAIT TCR.[21] The CDR3β loop, however, is more diverse.[38]

As can be seen from the docked structure (Figure 5), the lumazine of dRL-6-Me-7-OH is held in the MR1 cleft by several hydrogen bonds and Van der Waals interactions, with overall bonding being very similar to that previously noted for RL-6-Me-7-OH (**3a**).[21] Notwithstanding, the 2'-deoxy-ribityl backbone and hydroxyls in **3c** are oriented in a slightly different conformation to that previously observed for **3a**, thereby facilitating hydrogen bonding to the Arg9, Arg94 and Tyr152 residues of MR1 (Figure 5A) as well as a hydrogen bond between the 5'-OH and the CDR3α Tyr95 residue (Figure 5B). In the crystal structure of

the RL-6-Me-7-OH (**3a**) complex, this hydrogen bond is present between the 2'-OH and Tyr95.[21] For dRL-6-Me-7-OH (**3c**), an additional hydrogen bond between the 5'-OH and CDR3β Gly98 was also observed (Figure 5B). Although sequence variability in the CDR3β chain means that not all TCRs will interact with MR1 in the same way, our observation that **3c** forms a hydrogen bond to the CDR3β backbone might indicate a measure of sequence independence for MR1-ligand recognition by MAIT TCRs. Overall, it seems that the lumazine **3c** can adopt an alternative orientation within the MR1 cleft so as to optimise binding to the MAIT TCR, and that the absence of a substituent at the 2-position may permit additional flexibility of the sugar moiety, allowing for better binding interactions.

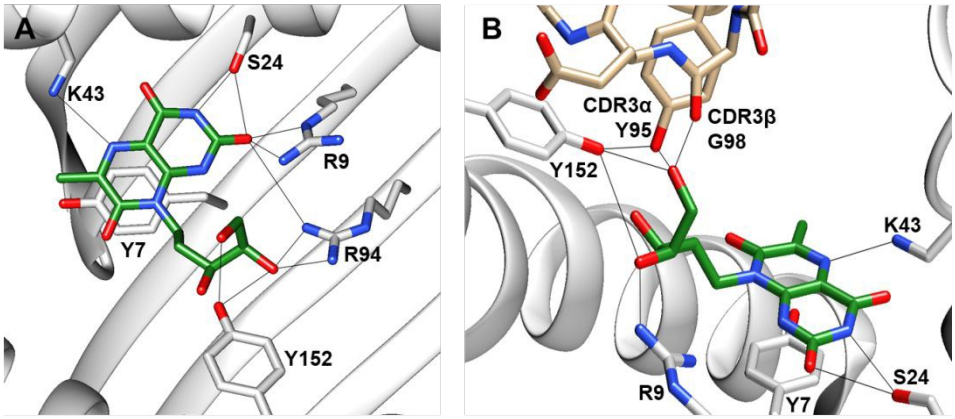

Figure 5. Docking of dRL-6-Me-7-OH (**3c**) into the hMR1-MAIT TCR complex (PDB: 4L4V). A. Contacts between **3c** and MR1. B. Contacts between **3c** and MAIT TCR and MR1. Hydrogen bonds are shown as black lines.

To assess the MAIT cell agonistic activity of lumazines **3a-e**, the MAIT cell line 6C2 was incubated with **3a-e** and a mouse MR1-overexpressing NiH.cl9 cell line. 5-OP-RU (**5a**) and antagonist Ac-6-FP (**1b**) were used as positive and negative controls, respectively (Figure 6A). The expression of activation marker CD137[39] was then assessed via flow cytometry. To our surprise, at the highest tested concentration of 100 µM, none of the lumazines were capable of activating MAIT cells. Instead, a significantly lower expression of CD137 was observed for lumazines **3a-c**, which indicated that these compounds may be acting as competitive inhibitors similar to Ac-6-FP. To investigate the effect of **3c** on 5-OP-RU activation, an

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inhibition assay was carried out whereby NiH.cl9 cells were incubated with Ac-6-FP (**1b**) or **3c** before stimulation with agonist 5-OP-RU and the addition of 6C2 cells (Figure 6B). A concentration-dependent reduction in 5-OP-RU-mediated CD137 expression was observed for both **3c** and Ac-6-FP, however **3c** was only capable of weakly inhibiting 5-OP-RU-mediated MAIT cell activity. This may be due to the ability of Ac-6-FP (**1b**) and 5-OP-RU (**5a**) to form Schiff bases with Lys43 of MR1, whereas lumazines **3a-e** are only capable of forming non-covalent bonds to MR1.[21] Thus, while the 2-deoxy-D-ribityl-analogue **3c** has the ability to interact with MR1 and the MAIT cell TCR, this interaction is not sufficient to lead to MAIT cell activation and instead leads to weak competitive inhibition of MR1. Hence, the generation of a potent MAIT cell agonist would greatly benefit from the presence of electrophilic motif capable of forming a covalent bond with MR1.[21]

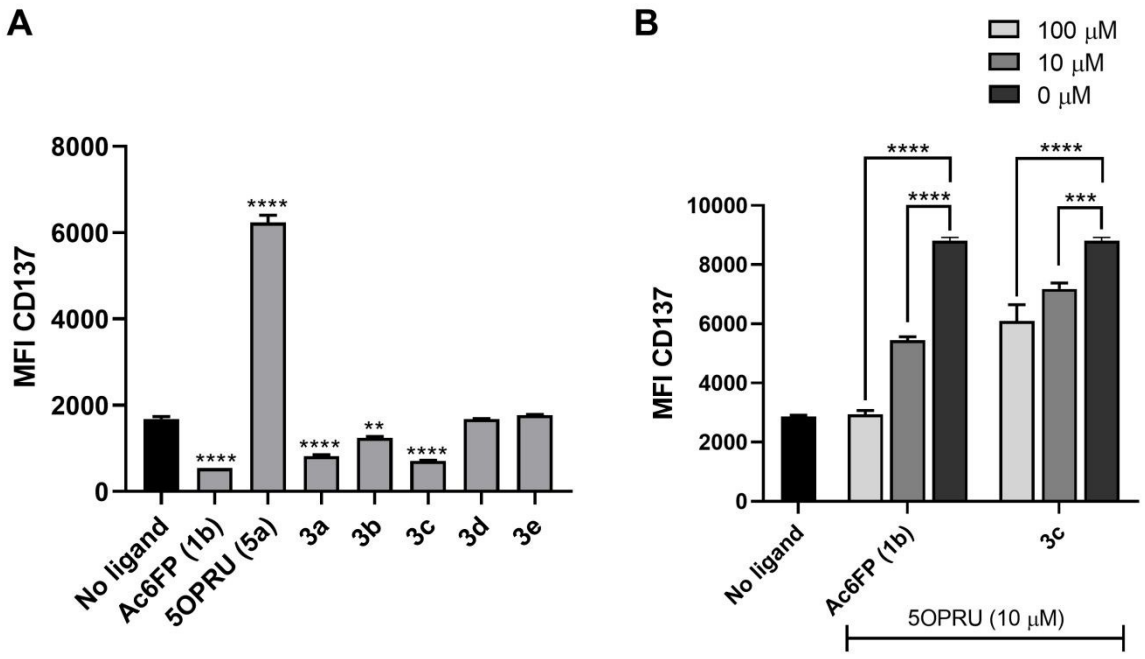

Figure 6. MAIT cell activation and inhibition assays. A. The MAIT cell line 6C2 was cultured for 24 h on NiH.cl9 cells overexpressing mouse MR1 in the presence of **3a-e** (100 µM) and the mean fluorescence intensity (MFI) of CD137 expression was measured after gating on viable TCRβ$^+$ cells. 5-OP-RU (10 µM) and Ac-6-FP (100 µM) were used as positive and negative controls, respectively. Data is representative of three independent experiments performed in triplicate. (**) $P \leq 0.01$; (***) $P \leq 0.001$; (****) $P \leq 0.0001$ compared to unstimulated cells. B.

NiH.cl9 cells were incubated with **3c** or Ac-6-FP (100 µM, 10 µM and 0 µM) for 1 h before the addition of **5a** (10 µM) and 6C2 cells for 24 h. MFI of CD137 expression was measured after gating on viable TCRβ$^+$ cells. Data is representative of two independent experiments performed in triplicate.

Accordingly, we sought to prepare glyco-analogues of the potent covalent agonist 5-OP-RU (**5a**) to investigate how changes to the sugar motif influence MAIT cell activation. Here, we included additional derivatives containing the D-xylityl- (**12f**) and L-lyxityl- (**12g**) residues to evaluate how systematic changes to the configuration of each hydroxyl group affects activity (Scheme 3). We also included the D-allityl- (**12h**) residue as it contains the same configuration as 5-OP-RU at the C2'-, C3'- and C4'-positions but contains an additional CH_2OH group, which may contribute to better activity via hydrogen bonding to residues in MR1 or the MAIT cell TCR. As 5-OP-RU (**5a**) can be prepared *in situ*, through condensation of its precursor molecule 5-amino-6-D-ribitylaminouracil (5-A-RU, **12a**) with methylglyoxal in the presence of MR1,[17, 22,23] 5-A-RU analogues **12a-h** were prepared *en route* to the synthesis of the corresponding 5-OP-RU analogues. To this end, the 5-nitro-6-glycitylaminouracils **10a-h** were reduced under the agency of sodium dithionite[23] to afford the aminouracil HCl salts **12a-h** in good yields of 58-71%.

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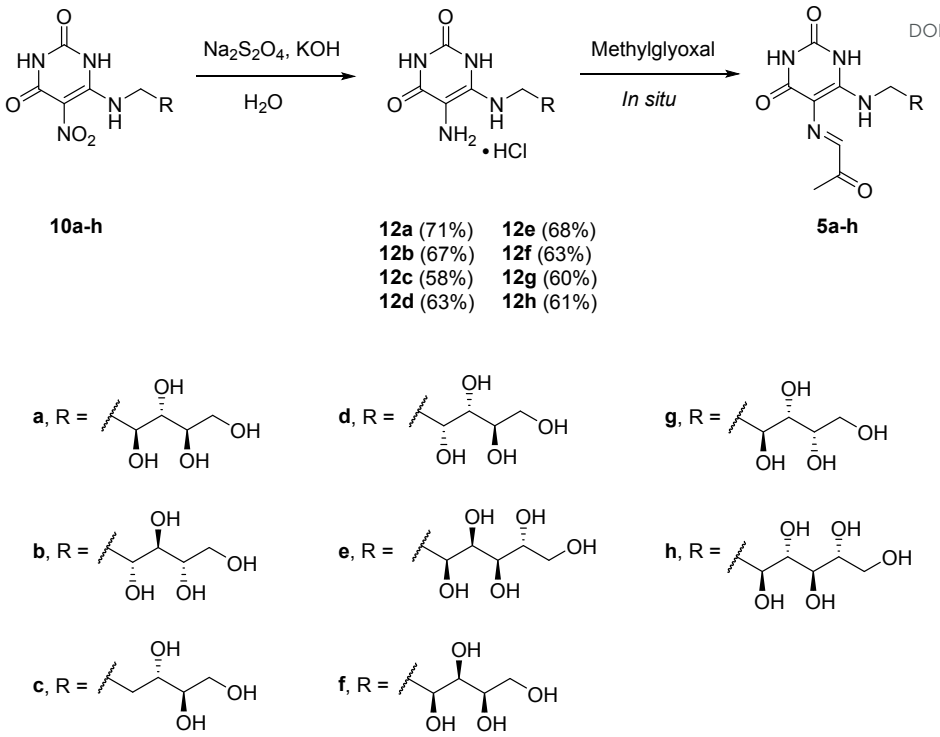

Scheme 3. Glyco-analogues of 5-OP-RU.

For biological assessment, the 5-A-RU analogues **12a-h** were converted to their 5-OP-RU counterparts **5a-h** via condensation with methylglyoxal immediately before testing against the MAIT cell line 6C2. At a concentration of 10 µM, the D-arabinityl (**5d**), D-xylityl (**5f**), L-lyixityl (**5g**) and D-allityl (**5h**) glyco-analogues exhibited significant levels of MAIT cell activity similar to that caused by 5-OP-RU (**5a**) (Figure 7). Glyco-analogues **5b**, **5c** and **5e** also caused significant MAIT cell activation, albeit at lower levels than **5a**. Here, the 2'-deoxy-D-ribityl analogue **5c** showed the lowest activity.

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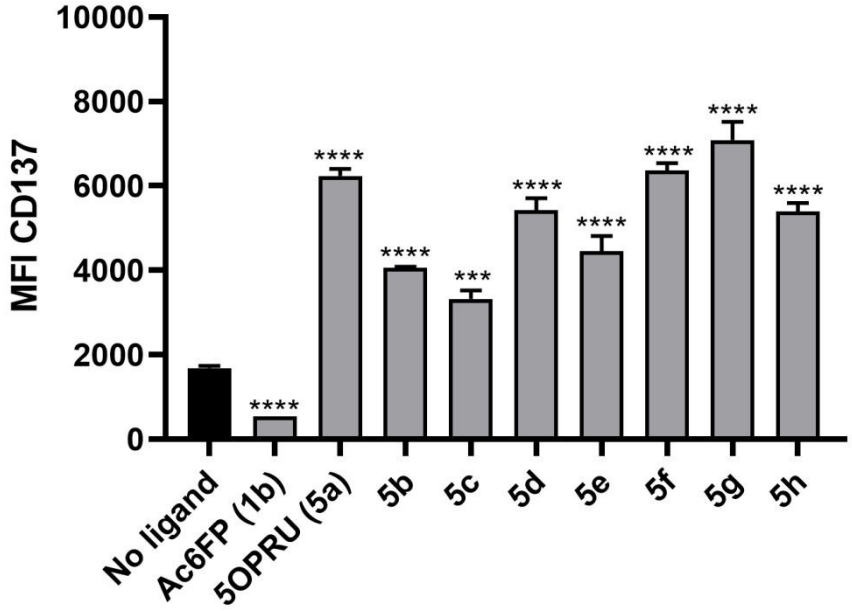

Figure 7. MAIT cell activation assay for 5-OP-RU analogues **5b-e**. The MAIT cell line 6C2 was cultured for 24 h on NiH.cl9 cells overexpressing mouse MR1 in the presence of **5b-e** (10 µM) and the mean fluorescence intensity (MFI) of CD137 expression was measured after gating on viable TCRβ$^+$ cells. 5-OP-RU (10 µM) and Ac-6-FP (100 µM) were used as positive and negative controls, respectively. Data is representative of three independent experiments performed in triplicate. (***) $P \leq 0.001$; (****) $P \leq 0.0001$ compared to unstimulated cells.

To better understand these observations, we docked analogues **5b-h** into the MR1-MAIT TCR binding site and compared the binding interactions to those found in the crystal structure of MR1-5-OP-RU complexed with a human MAIT TCR (Figure 8A). For all analogues, the planar uracil ring adopted a very similar conformation to **5a** when a covalent Schiff base was formed with Lys43. However, the hydroxyl groups in each sugar analogue were oriented differently and formed different sets of contacts with residues of MR1 and the MAIT TCR (Figure 8B). The Tyr95 residue of the MAIT TCR showed interactions with the 2'-hydroxyl group of all the glyco-analogues, with the exception of the 2-deoxy-D-ribityl (**5c**) and D-glucityl (**5e**) analogues. Furthermore, the L-ribityl (**5b**) and 2-deoxy-D-ribityl (**5c**) analogues only made contacts with Tyr95 at one position of the sugar, which could also contribute to the lower activity observed with these analogues. These results indicate that for 5-OP-RU and its analogues, ligand activity

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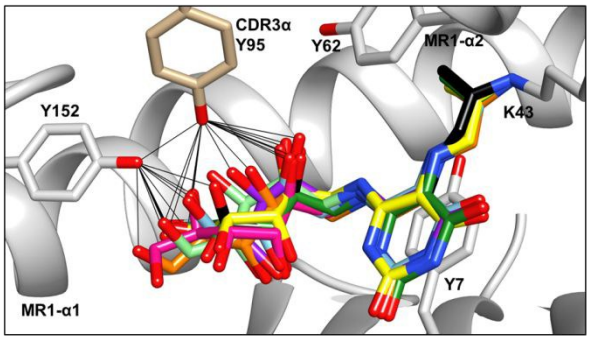
depends on the number of interactions with Tyr95 of the MAIT TCR, and that an interaction between the 2'-hydroxy group on the sugar backbone enhances MAIT cell activation. Taken together, our results demonstrate that the MR1-MAIT TCR binding pocket can accommodate changes to the sugar backbone, while still exhibiting ligand activity similar to 5-OP-RU. However, none of the sugar derivatives showed greater activity than 5-OP-RU, suggesting that the native D-ribityl chain in 5-OP-RU plays an important role in MR1-ligand-MAIT cell recognition.

A



B

Contacts between ligands and MR1-MAIT TCR

Sugar chain (colour)	Y95 (MAIT TCR CDR3α)	Y152 (MR1-α1)
5a D-ribityl (yellow)	2', 4'-OH	5'-OH
5b L-ribityl (light blue)	2'-OH	4', 5'-OH
5c 2-deoxy-D-ribityl (dark green)	5'-OH	5'-OH
5d D-arabinityl (light green)	2', 5'-OH	5'-OH
5e D-glucityl (orange)	3', 5'-OH	5', 6'-OH
5f D-xylityl (purple)	2', 3', 5'-OH	5'-OH
5g L-lyxityl (black)	2', 5'-OH	4', 5'-OH
5h D-allityl (magenta)	2', 5'-OH	5'-OH

Figure 8. Docking of 5-OP-RU analogues **5b-h** in the hMR1- MAIT TCR complex (PDB: 4PJ7). A. Contacts between docked ligands containing sugar chains: L-ribityl (**5b**, light blue), 2-deoxy-D-ribityl (**5c**, dark green), D-arabinityl (**5d**, light green), D-glucityl (**5e**, orange), D-xylityl (**5f**, purple), L-lyxityl (**5g**, black) and D-allityl (**5h**, magenta) overlaid onto the crystal structure of 5-OP-RU (**5a**, yellow) in the hMR1-TCR complex. Hydrogen bonds are shown as black lines. B. Table listing the depicted hydrogen bonds.

Conclusion

We have developed a highly efficient 3-step synthesis of MR1 ligands based on the lumazine scaffold. Key to this approach was the use of a protecting-group-free reductive amination protocol, which also allowed for ready incorporation of modified sugar residues in the target lumazines **3a-e** and 5-OP-RU analogues **5a-h**, and the first systematic analysis of the effect of the sugar motif on MAIT cell activation. The results of this study indicate that although the 2-deoxy-D-ribityl-lumazine **3c** can stain MAIT cells when complexed with MR1, **3c** and the other lumazines are unable to induce significant MAIT cell activation and instead act as weak

competitive inhibitors of MR1. In contrast, different pentose and hexose analogues of 5-OP-RU induced MAIT cell activation, albeit at lower or similar levels to 5-OP-RU (**5a**). Docking studies revealed the importance of the 2'-hydroxy group on the sugar backbone for MAIT cell activity, with the absence of the 2'-hydroxy enhancing MR1-MAIT TCR binding for the lumazines, while this hydroxy group enhanced MAIT cell activation by the 5-OP-RU derivatives. Taken together, these findings contribute towards an understanding of the structural requirements for an MR1 ligand to activate MAIT cells and suggest that while modifications to the sugar moiety can improve binding to MR1, a potent MAIT cell agonist greatly benefits from the presence of the native D-ribityl chain along with an electrophilic motif capable of forming a covalent bond with nucleophilic residues of MR1.

Conflicts of interest

There are no conflicts to declare.

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