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COMMUNICATION

Agonistic or antagonistic mucosal-associated invariant T (MAIT) cell activity is determined by the 6-alkylamino substituent on uracil MR1 ligands

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Mucosal-associated invariant T (MAIT) are a subset of innate-like T cells that are activated by uracil ligands presented by MR1. For the first time, we demonstrate that changes to the 6-aminoalkyl chain on uracil agonist 5-OP-RU can determine agonistic or antagonistic MAIT cell activity. Insomuch, a simplified agonist with a functional profile similar to 5-OP-RU, and a new structural class of antagonist that exhibits similar activity to known MAIT cell antagonist Ac-6-FP, were identified.

Mucosal-associated invariant T (MAIT) cells are a subset of recently identified innate-like T lymphocytes bearing a T cell receptor (TCR) with a highly conserved Vα19Jα33 α-chain in mice or Vα7.2-Jα33 in humans, which pair with a limited array of TCR β-chains.[1,2] MAIT cells are abundant in human tissue and blood,[3] and are activated by ligands bound to the major histocompatibility complex (MHC) class I-like protein MR1 on antigen presenting cells.[4,5] Upon activation, MAIT cells migrate to sites of infection, rapidly proliferate, and secrete pro-inflammatory cytokines or cytotoxic molecules.[3,6] MAIT cells have been implicated in a number of diseases such as tuberculosis, HIV, diabetes, and cancer,[3] and through the manipulation of MAIT cell function, via the development of agonists and antagonists, it might be possible to develop better therapeutics for such diseases.[7] This is a pertinent goal given the highly conserved nature of both the manipulation of MAIT cell function, via the development of MAIT cell agonists or antagonists, it might be possible to MAIT TCR α-chain and MR1,[1,4] which allows for the targeting of MAIT cells in all individuals.

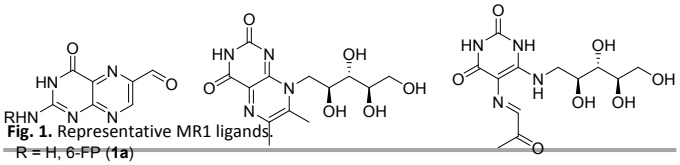

Fig. 1. Representative MR1 ligands.

6-Formylpterin (6-FP, **1a**, Fig. 1), a metabolite of vitamin B9 (folic acid), and its synthetic derivative, acetyl-6-formylpterin (Ac-6-FP, **1b**) are MAIT cell antagonists,[5,8] with the inhibitory activity of these compounds being attributed to their ability to covalently bind MR1 through formation of a Schiff base with Lys43 of MR1, leading to MR1 upregulation.[5,8,9] In contrast, the MAIT cell agonists, the ribityl lumazines [*e.g.*, 6,7-dimethyl-8-D-ribityllumazine (RL-6,7-diMe, **2**)], which are products of vitamin B2 (riboflavin) metabolism,[5] are unable to form a Schiff base with Lys43, although the ribityl tail of these ligands directly contacts Tyr95 of the MAIT TCR, thereby allowing for moderate levels of MAIT cell activity.[5,9] However, the most potent MAIT cell agonist identified is the unstable intermediate 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU, **3a**),[10] and its activity has been attributed to the combination of Schiff base formation with Lys43 of MR1, as well as the presence of the ribityl tail, which interacts directly with the MAIT TCR.

Although studies have focussed on developing more stable analogues of 5-OP-RU (**3a**),[11] much still remains unknown about the effects of the ribityl side chain on MAIT TCR binding and activity. However, we recently demonstrated that the incorporation of different sugar motifs at the 6-position of 5-OP-RU (**3a**) can affect MAIT cell activity,[12] and that removal of the 2'-hydroxy group of the ribityl tail significantly decreased

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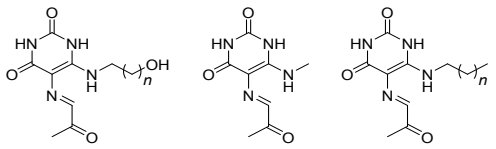
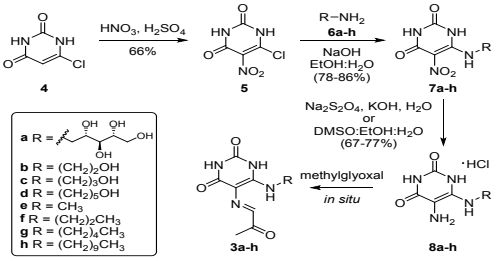


Fig. 2. Proposed 6-alkylamino analogues of 5-OP-RU (**3a**).

MAIT cell activation. In this study, we sought to investigate the effects of gross structural changes to the 6-alkylamino side chain on MAIT cell activation through the synthesis and subsequent biological evaluation of 5-OP-RU analogues hydroxyethylamine **3b**,[13] hydroxypropylamine **3c,** hydroxypentylamine **3d**, methylamine **3e**, propylamine **3f**, pentylamine **3g**, and decylamine **3h** (Fig. 2). From this work, we were able to demonstrate how changes to the 6-aminoalkyl chain can switch the function of the ligands from agonist to antagonist.

To synthesise 5-OP-RU (**3a**) and analogues **3b**-**h**, 6-chlorouracil (**4**) was first nitrated under the agency of fuming HNO_3 and H_2SO_4 to produce 6-chloro-5-nitrouracil (**5**) (Scheme 2).[12] Substitution of the chloride in **4** was then undertaken under basic conditions[12] using 1-amino-1-deoxy-D-ribitol (**6a**), 2-aminoethanol (**6b**), 3-amino-1-propanol (**6c**), 5-amino-1-pentanol (**6d**), methylamine (**6e**), 1-aminopropane (**6f**), 1-aminopentane (**6g**) and 1-aminodecane (**6h**) to give the corresponding nitrouracils **7a**-**h** in good yields (74-86%). Next, reduction of the nitro groups was performed to give the desired diaminouracil products ready for condensation with methylglyoxal. Here, reductions of hydroxyalkyl nitrouracils **7a**, **7c** and **7d** were carried out under the agency of KOH and $Na_2S_2O_4$ in H_2O,[12,14] and upon completion of the reactions, as determined by high resolution mass spectrometry (HRMS) analyses, the reaction mixtures were directly loaded onto C18 reversed-phase columns. Elution with water, and acidification of the product fractions with 1M HCl, followed by lyophilization, then gave diaminouracils **8a**, **8c** and **8d** in good yields (71-75%) as HCl salts, in order to improve their stability.[14] The poor aqueous solubility of hydroxyalkylated nitrouracil **7b** and alkylated nitrouracils **7e**-**h** required reductions to be performed in DMSO:EtOH:H_2O (1:1:1). After completion of these reactions, the mixtures were acidified with 1M HCl, dried using a flow of argon to remove EtOH, followed by lyophilisation to remove DMSO and H_2O. Purification of the residue by C18 reversed-phase chromatography and acidifycation of the product fractions gave the desired diaminouracils **8b**, **8e**-**h** as HCl salts in good yields of 67-77%.

With the diaminouracil analogues **8a**-**h** in hand, we then sought to assess their ability to activate MAIT cells. To this end, analogues **8a**-**h** (10 µM) were combined with methylglyoxal *in situ* to obtain 5-OP-RU analogues **3a**-**h**,[12,14] immediately before incubation with mouse MR1-overexpressing NiH.cl9 cells[12] and the mouse MAIT cell line 6C2.[2,12] The expression of MAIT cell activation marker CD137[12] was then measured after 24 hours via flow cytometry to determine ligand activity. Here, hydroxyethyl analogue **3b** led to the highest activity from the



Scheme 1. Synthesis of hydroxyalkyl and alkyl analogues.

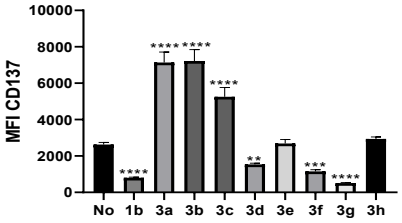


Fig. 3. MAIT cell activation assay for 5-OP-RU analogues **3b**-**h**. The MAIT cell line 6C2 was cultured for 24 h on NiH.cl9 cells overexpressing mouse MR1 in the presence of **3b**-**h** (10 µM) and the mean fluorescence intensity (MFI) of CD137 expression was measured after gating on viable TCRβ$^+$ cells. Ac-6-FP (**1b**, 100 µM) and 5-OP-RU (**3a**, 10 µM) were used as negative and positive controls, respectively. Data represents two independent experiments (n = 3). (**) P ≤ 0.01; (***) P ≤ 0.001; (****) P ≤ 0.0001 compared to unstimulated cells.

series of synthesised analogues (Fig. 3), with this activity being comparable to that elicited by agonist 5-OP-RU (**3a**). The activity of the hydroxyalkyl analogues decreased as chain length increased, with hydroxypentyl analogue **3d** being unable to activate MAIT cells. Conversely, removal of the terminal hydroxy group abolished all activity, as no MAIT cell activation was observed for the alkylated analogues **3f**-**h**. In this assay, **3d**, **3f** and **3g** behaved in a similar manner to Ac-6-FP (**1b**) as they led to significant downregulation of CD137 when compared to the no-ligand control. This data indicated that **3d**, **3f** and **3g** represent potential MAIT cell antagonists.

To understand more about the ability of the compounds to bind MR1, we then examined the effect of these ligands on MR1 surface expression. Here, compounds were incubated at two concentrations (10 µM and 1 µM) with NiH.cl9 cells for 4 hours before the surface expression of MR1[8] was assessed using flow cytometry. Overall, antagonist Ac-6-FP (**1b**) induced the highest upregulation of MR1 surface expression at both concentrations (Fig. 4A). Consistent with the MAIT cell activation assay, hydroxyethyl analogue **3b** showed similar activity in this assay to that observed for 5-OP-RU (**3a**). Hydroxypentyl analogue **3d** induced slightly higher MR1 expression at 10 µM when compared to **3a**, whereas the propyl (**3f**) and pentyl (**3g**) analogues led to lower MR1 expression levels. Altogether, these results confirmed that the agonistic hydroxyethyl analogue **3b** binds MR1.

Further evidence for MR1 binding and MAIT cell interaction was provided by the ability of the compounds to bind MR1

tetramers and stain MAIT cells. To this end, compounds were loaded onto empty MR1 tetramers[12] and the resulting antigen-

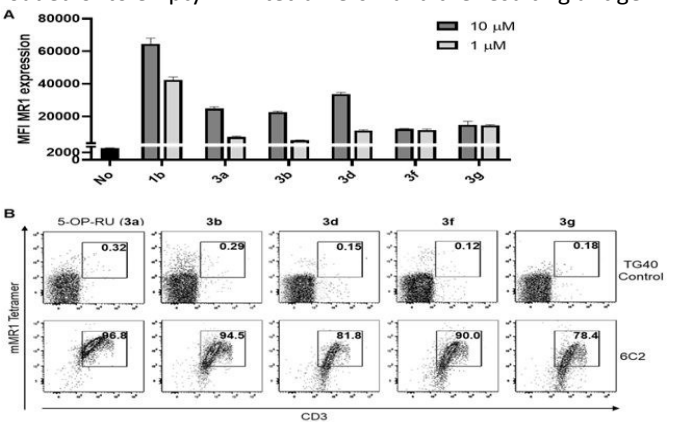


Fig. 4. A. MR1 surface expression assay. NiH.cl9 cells overexpressing mouse MR1 were stimulated with compounds **1b**, **3a**, **3b**, **3d**, **3f** and **3g** at 10 μM and 1 μM for 4 h, after which time the MFI of MR1 expression was measured. Data represents two independent experiments (n = 2). B. MR1–analogue tetramer staining of MAIT cells. Empty MR1 tetramers were loaded with **1b**, **3a**, **3b**, **3d**, **3f** and **3g** and assessed for their ability to stain the MAIT cell line 6C2 and the parental cell line TG40 (negative for TCR). Data represents two independent experiments.

MR1 complexes were tested for their ability to stain the 6C2 MAIT cell line and the TG40 cell line,[15] which was used as a negative control as it lacks TCR-α and β chains. Here, all compounds were capable of specifically staining MAIT cells, as indicated by the co-staining of cells bearing the T cell marker CD3ε (Fig. 4B). 5-OP-RU (**3a**)-loaded tetramers showed the highest level of staining, with 96.8% of cells co-staining with both the MR1-ligand complex and CD3ε. As expected, hydroxyethyl analogue **3b** showed a high level of MAIT cell staining (94.5%), which was comparable to 5-OP-RU (**3a**). Non-agonistic compounds **3d**, **3f** and **3g** were also capable of staining MAIT cells, albeit at slightly lower levels of 78-90%. This data suggests that analogues **3b**, **3d**, **3f** and **3g** bind MR1 and interact with MAIT cells in a similar manner to 5-OP-RU (**3a**).

To determine whether lead agonist hydroxyethyl analogue **3b** binds in the same binding site as Ac-6-FP (**1b**), a competitive inhibition experiment was carried out whereby NiH.cl9 cells were incubated with antagonist Ac-6-FP (**1b**) for 1 hour before the addition of 5-OP-RU (**3a**) or **3b** and 6C2 cells for 24 hours. The expression of CD137 was then determined by flow cytometry. Here, a significant reduction in MAIT cell activity for both **3a** and **3b** was observed when the cells were pre-incubated with antagonist **1b** (Fig. 5A), thereby indicating that analogue **3b** binds the known MR1-MAIT TCR binding site. Next, we sought to directly compare the agonistic profile of **3b** to 5-OP-RU (**3a**). A dose-response assay was undertaken where 6C2 cells were cultured for 24 hours on NiH.cl9 cells in the presence of **3a** or **3b** at titrated concentrations. Hydroxyethyl agonist **3b** was found to have a similar activation profile to **3a** (Fig. 5B), with similar EC_{50} values of 0.4 μM and 0.6 μM being observed for **3b** and **3a**, respectively. Taken together, these results demonstrate that hydroxyethyl analogue **3b** is a new, fully water soluble, MAIT cell agonist with potent activity and a functional profile similar to the current best known MAIT cell

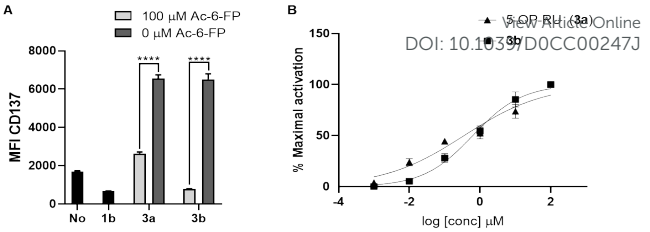


Fig. 5. A. MAIT cell competitive inhibition assay. NiH.cl9 cells were incubated with Ac-6-FP (**1b**, 100 μM or 0 μM) for 1 h before the addition of 10 μM 5-OP-RU (**3a**) or **3b** and 6C2 cells for 24 h. MFI of CD137 expression was measured after gating on viable TCRβ⁺ cells. Data represents two independent experiments (n = 3). (****) $P \leq 0.0001$. B. Dose-response assay for 5-OP-RU (**3a**) and **3b**. 6C2 cells were cultured for 24 h on NiH.cl9 cells with **3a** or **3b** at 100 μM, 10 μM, 1 μM, 0.1 μM, 0.01 μM and 0.001 μM and MFI of CD137 expression was measured after gating on viable TCRβ⁺ cells. Data represents % maximal activation of 6C2 cells from two independent experiments (n = 3).

agonist 5-OP-RU (**3a**). Moreover, analogue **3b** can be obtained from commercially available 2-aminoethanol via a three-step synthesis that is shorter than the current synthesis of 5-OP-RU (**3a**), which requires the generation of ribitylamine prior to substitution of the uracil moiety.

Given the observations that hydroxypentyl analogue **3d**, propyl analogue **3f** and pentyl analogue **3g** induced MR1 surface expression and showed tetramer staining of MAIT cells, but not MAIT cell activation, we sought to further investigate the potential antagonistic activity of these compounds. Accordingly, the compounds were tested for their ability to inhibit 5-OP-RU-mediated MAIT cell activation, whereby NiH.cl9 cells were incubated with titrated amounts of compounds **3d**, **3f** or **3g** for 1 hour before the addition of 5-OP-RU (**3a**) and 6C2 cells for 24 hours. Flow cytometric analysis was then undertaken to determine CD137 expression levels and the known antagonist Ac-6-FP (**1b**) was used as a positive control. All three compounds showed concentration dependent inhibition of 5-OP-RU-mediated MAIT cell activation akin to **1b** (Fig. 6), with pentyl analogue **3g** inhibiting activity to the greatest extent at a concentration of 100 μM, although the antagonistic activity of this compound was less than **1b** at 10 μM. Altogether, these results suggest that we have identified a new class of MAIT cell antagonists with a structural scaffold that is different to known antagonists 6-FP (**1a**) and Ac-6-FP (**1b**), and that is instead structurally more similar to agonist 5-OP-RU (**3a**).

To better understand our observations that the hydroxyethyl (**3b**) and hydroxypropyl (**3c**) analogues are MAIT cell agonists, whereas the hydroxypentyl (**3d**), propyl (**3f**) and pentyl (**3g**) analogues act as antagonists, these structures were docked

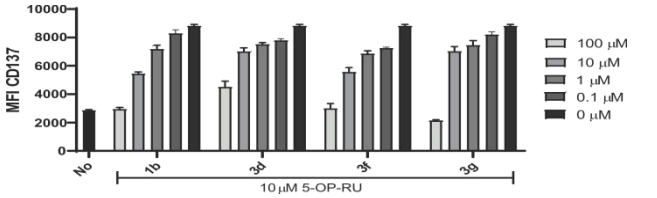


Fig. 6. MAIT cell inhibition assay for analogues **3d**, **3f** and **3g**. NiH.cl9 cells were incubated with Ac-6-FP (**1b**), **3d**, **3f** or **3g** at 100 μM, 10 μM, 1 μM, 0.1 μM and 0 μM for 1 h before the addition of 5-OP-RU (10 μM) and 6C2 cells for 24 h. MFI of CD137 expression was measured after gating on viable TCRβ⁺ cells. Data represents two independent experiments (n = 3).

into the MR1-MAIT TCR binding site and their binding interactions were compared to those observed in the crystal structure of MR1-5-OP-RU complexed with a human MAIT TCR (Fig. 7). For all compounds, the formation of a Schiff base between the ketone on the 5-position of the uracil ring and Lys43 of MR1 caused the uracil ring to sit in a slightly different orientation to 5-OP-RU (**3a**, yellow). The uracil ring of 5-OP-RU (**3a**) showed van der Waals interactions with Thr34, Ser24 and Arg9, and aromatic interactions with Tyr7 of MR1. These interactions were also observed for **3b**, **3d**, **3f** and **3g**, thereby allowing them to occupy the MR1-MAIT TCR binding site. Here, the 2-hydroxyethylamino (pink) and 3-hydroxypropylamino (blue) side chains of agonists **3b** and **3c**, respectively, were oriented towards, and formed interactions with, Tyr95 of the MAIT TCR (Fig. 7A). The hydroxypentyl side chain (green) of **3d** was oriented in the same direction as the ribityl tail of 5-OP-RU (**3a**, yellow), however, the hydroxy group was located further away from Tyr95 and could not interact with the MAIT TCR (Fig. 7B). Similarly, the lack of hydroxy groups in the propyl (**3f**, black) and pentyl (**3g**, orange) analogues prevented interactions with Tyr95, with the side chains oriented away from the MAIT TCR. Thus, in summary, although all the aminouracils bound MR1, antagonists **3d**, **3f** and **3g** are unable to interact with the MAIT TCR, whilst agonists 5-OP-RU (**3a**), **3b** and **3c** interact directly with Tyr95 of the MAIT TCR. Previously, we observed that for glyco-analogues of 5-OP-RU, ligand activity depended on the number of interactions between the sugar backbone and Tyr95,[12] and that an interaction between the 2'-hydroxy group on the sugar backbone enhanced MAIT cell activation. In this study, we show that an interaction between the 2'-OH of agonist **3b** and Tyr95 is favourable for MR1-MAIT TCR binding, and sufficient for potent agonistic activity.

Herein, a series of hydroxyalkyl and alkyl analogues of 5-OP-RU were synthesised and their MAIT cell activity assessed. Hydroxyethyl analogue **3b** exhibited MAIT cell activation that was similar to the most potent agonist 5-OP-RU (**3a**). MR1 surface expression and dose-response assays revealed that **3b** also has a similar functional profile to 5-OP-RU (**3a**). However, an advantage of **3b** over **3a** is that it can be synthesised easily in three steps from commercially available 2-aminoethanol. Furthermore, the hydroxypentyl (**3d**), propyl (**3f**) and pentyl (**3g**) analogues of 5-OP-RU were found to exhibit antagonistic activity similar to Ac-6-FP, with competitive inhibition assays confirming that **3b**, **3d**, **3f** and **3g** bind the same site as 5-OP-RU and Ac-6-FP. Using computational docking studies, it was determined that the agonistic compounds contact the MAIT TCR directly, while the antagonistic analogues lack such interactions. Thus, for the first time, we have demonstrated that changing the 6-amino side chain of MR1 ligands can regulate MAIT cell agonistic or antagonistic activity. Additionally, we have identified a new class of MAIT cell antagonists which are structurally distinct from known antagonist Ac-6-FP. By providing further insight into the structural requirements for MR1-MAIT TCR binding, this work will undoubtedly aid in the development of therapeutics targeting the MR1-MAIT cell signalling axis.

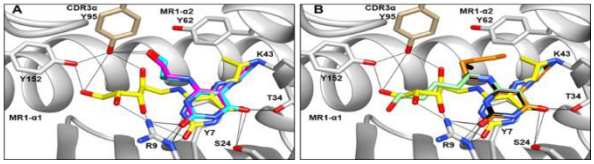


Fig. 7. Docking of analogues **3b**, **3c**, **3d**, **3f** and **3g** in the hMR1- MAIT TCR complex (PDB: 4PJ7). A. Contacts between agonistic ligands containing side chains: Hydroxyethyl (**3b**, pink) and hydroxypropyl (**3c**, blue) overlaid onto the crystal structure of 5-OP-RU (**3a**, yellow). B. Contacts between antagonistic ligands containing side chains: Hydroxypentyl (**3d**, green), propyl (**3f**, black) and pentyl (**3g**, orange) overlaid onto the crystal structure of 5-OP-RU (**3a**, yellow). Hydrogen bonds are shown as grey lines.

Conflicts of interest

There are no conflicts to declare.

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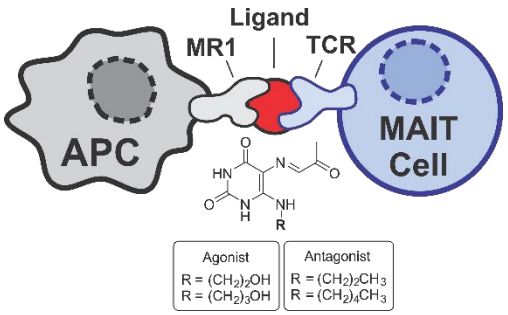

The 6-alkylamino side chain of aminouracil MR1 ligands controls MAIT cell agonistic or antagonistic activity.