### Potent Fluoro-oligosaccharide Probes of Adhesion in Toxoplasmosis


Unnatural, NMR- and MRI-active fluorinated sugar probes, designed and synthesised to bind to the pathogenic protein TgMIC1 from *Toxoplasma gondii*, were found to display binding potency equal to and above that of the natural ligand. Dissection of the binding mechanism and modes, including the first X-ray crystal structures of a fluoro-oligosaccharide bound to a lectin, demonstrate that it is possible to create effective fluorinated probe ligands for the study of, and perhaps intervention in, sugar–protein binding events.

### Introduction

It is estimated that the protozoan parasite *Toxoplasma gondii* (Tg) infects up to a third of the world’s human population[1] and causes diseases that can prove lethal, ranging from those that affect immunocompromised patients to birth defects when mothers are exposed during pregnancy.[2] Unusually, this parasite forces entry into host cells by a process that is initiated by so-called micronemal proteins (MICs) that mediate this first-phase adhesion not only by direct binding to host cell surface markers, but also through mutual interaction. Of these, TgMIC1, the first discovered, is essential for parasite entry.[3] absence of the *mic1* gene reduces invasion and virulence in vivo.[4] The identity of the host cell surface markers that act as binding points for TgMIC1 has been the subject of some speculation,[5–7] but the broad range of hosts that *Tg* can infect suggests a generic ligand. Recent studies have identified natural sialylated glycans, such as the trisaccharide sialyl-lactosamine (1), as putative ligands and shown that these are recognised by the microneme adhesive repeat (MAR) domains of TgMIC1 (TgMIC1-MAR).[7] In the heart of such glycans the natural disaccharide N-acetyllactosamine (Galβ(1,4)GlcNAc) is a common motif. However, during the design of probes for TgMIC1 we speculated that the wide host glycans recognition might reflect ligand tolerance and a flexibility that might valuably allow the design of alternative and unnatural ligands with higher TgMIC1 specificity and/or incorporating reporter groups that might allow direct assessment of binding. Such unnatural molecules might also provide leads for small molecule inhibitors of binding of parasite infection that would, by virtue of unnatural constitution, be resistant to degradation (unlike the natural sialyllactosamine structures) by endogenous host enzymes. We describe here the creation of a small panel of TgMIC1 trisaccharide ligands including fluorinated oligosaccharide probes 2 and 4 that, despite unnatural constitution and substituents, display better binding than natural ligand 1.

We chose two key features for probe design (Figure 1): 1) incorporation of fluorine at C-2' of the central α-galactose (Gal) residue; 2) alteration of the dominant Gal-to-GlcNAc connectiv- ity from β(1,4) to β(1,3).[8–10] The fluorine substituent is a powerful probe substituent. 19F is NMR/magnetic resonance imaging (MRI) active with no background signals in biological systems,[11,12] and the scalar coupling across 19F hydrogen bonds is unusually strong.[13] 18F is positron emission tomography (PET) active with potential, therefore, for noninvasive in vivo imaging. Furthermore, elegant “F scanning” strategies[14] for noncarbohyd ral ligands have identified the potential value of additional interactions with proteins (e.g., C–F–HC–C–O or C–F–C–O) and with some[11] H-bond donors, such as amide N–H, or by creating "pockets" in proteins for additional bound waters.[16] This latter strategy has been particularly neglected in examining and exploiting carbohydrate-binding protein interactions despite the fact that monosaccharide ligands suggest that replacement of C–OH by C–F bonds might be successful.[11,12] Fluorinated sugars have proven to be powerful enzyme inhibitors that might also provide leads for small molecule inhibitors of *Toxoplasma* infection.

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inhibitors,\textsuperscript{[18–26]} yet fluorosugars have proven to be usually weaker ligands,\textsuperscript{[18, 27, 28]} and therefore poorer inhibitors of carbohydrate-binding proteins (lectins, adhesins). We reveal here the first structures of fluoro-oligosaccharides complexed to nonenzymatic carbohydrate-binding proteins.

Results and Discussion

Synthetic strategy

The creation of 2-fluoro-2-deoxy-oligosaccharides through the use of 2-fluoro-2-deoxyglycosyl donors is rare, in part, as a consequence of the destabilisation of putative glycosyl transfer transition states by the strongly electron withdrawing substituent. Indeed, there are only very few examples of such oligosaccharides.\textsuperscript{[27–33]} Target trisaccharides 1–4 were constructed through a chemoenzymatic strategy (Scheme 1) by using parallel routes (Schemes 2–5); four variant Gal–GlcNAc intermediate disaccharides 5–8 containing different substituents F/OH or different connectivities \( \beta(1,4)/\beta(1,3) \) were accessed through chemical glycosylation by using trichloroacetimidate (TCA) donors 9\textsuperscript{[34]} and 10\textsuperscript{[35]} (Scheme 4). These, in turn, were converted into target trisaccharides 1–4 by using regio- and stereoselective \( T. \) cruzi transsialidase (\( TcTS \))-catalysed glycosylation (Scheme 5). \( TcTS \) is known to be a promiscuous enzyme that will tolerate a variety of functional group modifications and linkage patterns.\textsuperscript{[36–38]} As a first stage, various reducing terminus monosaccharide acceptors were synthesised. Acceptee identity had a strong effect on stereoselectivity and reactivity (vide infra) necessitating a survey of acceptors 11–14 and conditions. GlcNAc glycosyl acceptors 11/12 and 13/14 were used to access \( \beta(1,4) \) and \( \beta(1,3) \) disaccharides, respectively (Schemes 2 and 3). Reducing terminus protection was chosen carefully: orthogonal substituents were chosen in both systems that could be retained after removal of all other protecting groups (to allow biocatalysis) that would be compatible with biocatalytic sialylation (accommodated by \( TcTS \)) and aid purification (readily separated from more polar protein components).

Building block synthesis

\( \beta(1,4) \) Disaccharide syntheses first utilised a rarely employed\textsuperscript{[39, 40]} chemical regioselective OH-4-over-OH-3 glycosylation strategy of diol GlcNAc acceptor 11. Compound 11 was readily constructed in three different ways (Scheme 2): highly
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Fluorodicarbohydrate and disaccharide syntheses

Disaccharides 5–8, the substrates for enzymatic glycosylation, were accessed through chemical glycosylation (Scheme 4) and subsequent partial deprotection (Scheme 5). Lactol precursors to chemical glycosyl donors Gal-TCA (9) and 2-fluoroGal-TCA (10) donors were accessed through global acetylation then regioslective OH-1 deprotection by using benzylamine[34] or Selectfluor™-mediated electrophilic fluorination[47] of triacetyl galactal.[35] Both TCA donors 9 and 10 were readily formed by using base and trichloroacetonitrile.[46] The different reactivities and selectivities of 9 and 10 were explored (Scheme 4): glycosylation reactions with 2-fluoroGal-TCA were found to require higher activation temperatures (typically 0 °C cf. −40 °C) than the corresponding 2-OAc analogue 10. As expected, participatory 2-OAc donor 9 gave excellent β selectivities, whereas fluoro-TCA 10, which lacks C-2 neighbouring group participation, showed a preference for α selectivity and led to marked acceptor decomposition due to prolonged reaction times.

β(1,4) Glycosylations were investigated, initially, through the use of regio- and stereoselective 1,4-over-1,3 methodology (Scheme 4). The elegance of regioselective glycosylation[45–58] is underexploited and exploring its utility here allowed access to the desired 1,4 product in reasonable yield (59%) with excellent regio- and stereoselectivity (> 15:1 1,4/1,3; > 98% β). However, although regioselective glycosylation of β(1,4) acceptor 11 was successful with 9, it gave poor results with 10 despite variation of solvent or conditions. 2-Fluoro-2-deoxy glycosyl donors have only been used successfully rarely (often with low yields) due to the strongly deactivating/disarming nature of the 2-F substituent and are typically dominated in their stereoselectivity by the formation of α anomers due to a lack of neighbouring group participation and enhanced anomeric effect.[28,29,48] Nonetheless, choice of an appropriate, alternative acceptor (12) proved viable. Thus, glycosylation of 12 by using fluoro-TCA 10 gave 2-deoxy-2-fluoroLacN 18 (18) with improved yield and unusually high stereoselectivity towards the desired β anomer (71% brsm, α/β 1:5).

β(1,3) Galactosylation (Scheme 4) of 13 proceeded well and, as expected, with exclusive β selectivity to yield 19. This behaviour was in stark contrast to the poor reactivity of acceptor 14, which differs only in the substituent at C-2; 14 yielded only prepared from 13 by using benzylamine under Purdie–Irvine conditions,[40] followed by regioselective reductive benzylidene ring opening (Scheme 3).

α stereoselective ytterbium triflate catalysed glycosylation of BnOH either by using peracetylated β-GlcNAc[41] or peracetylated α/β-GlcNAc under microwave-accelerated conditions or protic acid-catalysed furanosyl oxazoline ring opening.[42] All allowed access to 15 in up to 77% yield on scales of 1–7 g; regioselective silylation of 15 gave acceptor 11 quantitatively. The presence of NHAc within acceptors and, in particular at C-2, has been suggested[43,44] to have a strong effect on reactivity and stereoselectivity, especially in β(1,4) glycosylation reactions, and so alternative β(1,4) azido acceptor 12[45] was also
the corresponding orthoester-linked disaccharide. To promote β selectivity in glycosylation of β(1,3) acceptor 13 with fluoro-TCA 10 a range of solvents were explored. In MeCN (TMSOTf, −18 to 0 °C) despite the use of 2 equiv donor, only a poor yield (40%) of disaccharide product 20 was obtained, albeit with desired selectivity (α/β 1:6). The yield in DCM was a much improved 85%, but gave disappointing anomeric selectivity (α/β 5:1). Compromise use of a 1:1 mixture of DCM/MeCN in combination with the use of 4 Å molecular sieves yielded 72% of 20 (80% brsm) and an anomeric selectivity in favor of β-galactoside (α/β 1:4). Thus, solvent optimisation in glycosylation of β(1,3) acceptor 13 with 10 allowed fine-tuning of selectivity (α/β) and yield (%): 5:1, 85%, DCM—1:6, 40%, MeCN—1:4, 72%, MeCN:DCM.

**Fluorotrisaccharide and trisaccharide syntheses**

The synthesis of trisaccharide probes 1–4 (Scheme 5) used β-linked disaccharides 5–8 carrying a protecting group at the reducing terminus. T. cruzi transsialidase (TcTS)[59] tolerates a variety of functional group modifications and linkage patterns.[36–38] After surveying a number of potential biocatalytic sialyl donors,[60–62] including para-nitrophenyl sialosides, the readily available and highly sialylated mammalian glycoprotein fetuin was selected as an appropriate enzymatic sialyl donor that could be easily removed during purification.

Although the TcTS enzyme used here carries a C-terminal His-tag and can be purified by nickel affinity chromatography,[63] the crude cell lysate is suitable for synthetic biotransformation[64] and was prepared by centrifugation, size-exclusion chromatography and resuspension in either phosphate buffered saline (PBS) (pH 7.0) or ammonium acetate buffer (pH 7.5). All synthetic β-linked disaccharides 5–8[65] carrying anomeric protecting groups were pleasingly found to be substrates for TcTS and could be sialylated in yields of up to 83% for β(1,4) sugars to lower yields for the unnatural β(1,3) sugars 7 (63%) and 8 (67% brsm). Anomeric deprotection of the resulting trisaccharides proceeded cleanly and in high yield by using either hydrogenolysis with Pearlman’s catalyst in methanol or hydrolysis with Dowex50WX8 acidic ion-exchange resin in water. These yielded 1–4 in final overall yields in 5–8 steps of 14–45%.

**Fluorotrisaccharides and trisaccharides as probes of protein–carbohydrate interaction**

The binding of these putative TgMIC1 probes 1–4 was assessed and dissected in four complementary ways (Figures 2 and 3): 19F NMR spectroscopy, isothermal calorimetry (ITC), X-ray crystallography, and surface-arrayed binding (glycoarray).

Multiple binding sites and cooperativity in MIC proteins has led to a suggested multiplicity in the binding of glycans.[2] This was probed first by using clustered, oxime-linked neoglyco-lipids[66] forms of 1–4 arrayed on nitrocellulose-coated glass slides (Figure 2C). This method involves the formation of an oxime at the reducing terminus of the oligosaccharide; the equilibrium between ring-closed and ring-open forms of such adducts is condition dependent.[64] Two neutral disaccharides, Galβ(1,4)GlcNAc and Galβ(1,3)GlcNAc, as well as a natural glycolipid haematoside, Sia4α(2,3)Galβ(1,4)Glcβ(1) ceramide, were also included, respectively, as negative and positive controls.

The binding of TgMIC1-MAR was examined at five different concentrations (1–40 μM). Excitingly, the results revealed excellent binding of the two 2-fluoro analogues 2 and 4 (Figure 2C); at all the protein levels tested their binding intensities in clustered format were greater than those of the nonfluorinated analogues 1 and 3, and the naturally occurring ligand haematoside. ITC revealed that in solution, fluorinated and nonfluorinated compounds displayed similar overall apparent dissociation constants (Kd ~ 10–15 μM).[67] These observations appear consistent with the presence of multiple binding sites on TgMIC1 and the high density (clustered format) of immobi-
lised glycans in the array. It is also plausible that the array format might additionally allow dynamic “rafting” that effectively mimics the natural presentation of multiple copies of glycan ligands projecting from the surface of a host cell membrane.

Good binding of 4 to TgMIC1-MAR was also detected through $^{19}$F NMR titration. The interaction was in the slow exchange limit on the NMR timescale suggesting an upper limit for dissociation constant of $50 \text{ mM}$, which is consistent with published values for the natural carbohydrate.\(^7\) In NMR spectroscopy of biological systems, $^{19}$F provides a highly sensitive probe with no background signal. Line broadening and chemical shift are highly sensitive to changes in solvent, environment and conformation.\(^12\) Indeed, TgMIC1-bound or -unbound forms of 4 were readily distinguished by distinct line widths and chemical shifts (Figure 2A). Moreover, probe 4 also showed distinct resonances in $^{19}$F-MRI “phantoms”, thus highlighting its potential utility even in in vivo imaging (Figure 2B). Although detailed pharmacokinetics have not yet been performed on 4 or the other putative sugar probes described here, we have previously achieved local concentrations of up to $\sim 50 \text{ mM}$ in vivo through selective lectin-mediated sequestration of oligosaccharidic conjugates.\(^6\) Such concentrations are consistent with those used in “phantoms” here, and suggest that the TgMIC1-MAR-mediated probe binding characterised here might allow spatial resolutions suitable for reasonable images.

Finally, by virtue of the affinity of 2 and 4 for TgMIC1 we were able to successfully co-crystallise and determine by X-ray crystallography structures of ligands 2 and 4 complexed with TgMIC1; these first X-ray structures of fluorinated oligosaccharide ligands bound to a lectin/adhesin revealed the molecular basis for probe interaction.\(^6\) The conformation adopted by

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**Figure 2.** Fluorosugars as multifaceted probes. A) $^{19}$F NMR spectrum of 4 in the absence and presence of TgMIC1-MAR showing diagnostic bound resonance for 4 and stages of titration. B) Gradient echo MRI image of 4 in a 5 mm diameter cylinder phantom in red in (ii) by using a single turn $^{19}$F surface coil at 282.45 MHz; $T_1$, $\sim 150 \text{ ms}$. C) Microarray analyses of TgMIC1-MAR with seven lipid-linked oligosaccharide probes including 1–4. The lipid-linked oligosaccharide probes were printed in duplicate at 7 fmol per spot on nitrocellulose coated glass slides and the binding of His-tagged TgMIC1-MAR at different concentrations (1–40 $\mu\text{g mL}^{-1}$) was assayed.

**Figure 3.** A) $2F_o - F_c$ electron density map (contoured 1.0 r.m.s) of 4 bound to TgMIC1-MAR; B) 4 bound to TgMIC1-MAR; C) overlay of 1 (blue\(^7\)), 2 (green) and 4 (orange).
the first two saccharidic residues in 2 and 4 together with the TgMIC1 binding site (Figure 3B) creates a pocket (7'-OH-Sia–6'-O-Sia–2'-F-Gal) for a key structural water (W170). Notably, 2'-F in both 2 and 4 is apparently able to maintain interaction with this water molecule, which would account for the excellent sensitivity of 4 as a probe in NMR-binding assays. The structure also highlights the excellent mimicry of the natural ligand 1[2] by unnatural probes 2 + W170 or 4 + W170 in binding to TgMIC1 despite altered connectivity and substituents (Figure 3C, both superimposed on 1). It should also be noted that the electron density does not allow entirely unambiguous interpretation of the conformation of the acetamide at C-4 in the sialic acid residue, an E conformation is consistent with local interactions (see the Supporting Information). The carbonyl of the acetamide engages[18] Arg217, which undergoes a dramatic side-chain alteration as compared with the apo structure. Analysis of a corresponding Z amide conformation reveals that the carbonyl oxygen is over 4.9 Å away from the entrapped water molecule, W170. The GlcNAc residue does not make any direct contacts with the protein and in both 1 and 2 with β(1,4) linkages the electron densities are not visible, suggesting that this group is disordered. Although the position of the GlcNAc is well resolved in the complex with 4, its conformation is stabilised exclusively by contacts from symmetry-related molecules in the crystal, which would suggest that this group is disordered in solution and might explain the excellent tolerance for the (1,3) linkage.

Conclusions

In summary, we have successfully constructed unnatural oligosaccharide ligands, and demonstrated through comprehensive analysis of their interaction with TgMIC1 that it is possible to create effective fluorinated sugar probes of an important pathogen-associated protein with binding equal to, or under clustered conditions in excess of, that of the natural ligand. Optimised sequential chemoenzymatic glycosylation methodology allowed the effective use of: 1) typically unreactive 2-F-glycolyl donors in construction of key glycosidic linkages with unusually good β stereoselectivity, and 2) highly regio- and stereo-selective enzymatic sialylation. Despite differing connectivity (β(1,3)) and an unnatural substituent (F), 4 proved to be a potent and, by virtue of its Z-fluoro group, a readily NMR-detectable probe. Replacement of C–OH by C–F in putative ligands can reduce binding affinity[16,27,28] by removing key interactions.[15] To our knowledge, this is the first fluorinated oligosaccharide ligand to show binding comparable (and even enhanced) to that of its natural counterpart. It is striking that in the only examples (here and with monosaccharide 6-F-Gal[17]) of fluorodeoxysugar analogues in complex with carbohydrate-binding proteins that have been characterised in molecular detail by X-ray crystallography, that the fluorine atom replacement of OH serves to create clear water "pockets"/binding sites. This is despite the often suggested belief that replacement of C–OH with C–F–H₂O would be too sterically congested; this is evidently not the case. Although the pharmacological effects of such fluorinated sugars has not yet been determined—and with the caveats of necessary local concentrations for MRI and late-stage introduction of F for PET noted above and below—we hope that the ability to incorporate a fluorine atom, as a small, powerful (NMR, MRI and possibly PET[73]) probe without loss of ligand potency might have useful implications for investigating sugar–protein interactions more generally. To this end, the in vivo interactions of such probes and the later stage introduction of F into sugars (including 18F) is a current key focus in our laboratory.

Experimental Section

TcT5S reactions: Preparative reactions by using recombinant TcT5S preparation were followed effectively by TLC, and the proteins were cleared from an aliquot prior to using a membrane concentrator or chloroform/methanol precipitation. Trisaccharide compounds bearing the anomerical protecting group were purified by using flash chromatography: protein was first removed by filtration through a small plug of silica (eluent water/isopropanol/ethyl acetate 1:2:2), followed by flash chromatography (water/isopropanol/ethyl acetate 0.8:2:3); unreacted disaccharide compounds were recovered and recycled.

Crystallography: TgMIC1-MAR was expressed, purified and crystallised as described.[7] Diffraction data collection, processing and refinement were carried out in a similar fashion to those published[7] and will be described in more detail elsewhere.

ITC: Isothermal titration calorimetry measurements between protein and ligands were made by using a VP-ITC microcalorimeter (Microcal) at 27 °C. Ligand solution (0.5 mM in 20 mM Tris-HCl pH 7.0, 100 mM NaCl, 31 × 10⁻⁴ M) was added into a sample cell (volume 1.415 mL) containing protein (0.1 mM) over a period of 20 s with 5 min interval stirred at 300 rpm. After integration with respect to time, normalisation per mol of added ligand and subtraction of the heat of dilution of ligand into buffer, the parameters were calculated by using either a single or two-site model.

Microarray: Analyses were performed essentially as described[7] except that the concentration of TgMIC1-MAR was examined at different concentrations. A control experiment (protein concentration 0.1 µM in Figure 2C) was performed with the detection antibodies alone (50 µg/mL) in the absence of TgMIC1-MAR.

19F NMR titration: TgMIC1-MAR in the same buffer as used for ITC (pH 7.0) was introduced up to a twofold molar excess and 19F NMR spectra were recorded at 753 MHz at 300 K under identical experimental conditions.

MRI: A gradient echo image was acquired from a 5 mm diameter NMR tube containing a solution of 4 (20 mM) on a 7 Tesla magnet (Varian Inova console) by using a single turn 19F surface coil (i.d. 1.5 cm) operating at 282.45 MHz. Relaxation rate measurements indicated a T₁ for 4 of ~150 ms. Consequently, the following imaging parameters were used: t₁, 500 ms; t₂, 4 ms; pulse width, 20°; averages, 8; in-plane resolution, 300 × 300 mm; slice thickness, >1.5 cm (i.e., limited by diameter of coil). With these imaging parameters a signal/noise ratio of approximately 7.5 was achieved in an acquisition time of 4 min 16 s.
**Acknowledgements**

We thank the UK Basic Technology Initiative (S.A.A., H.H.J., Y.L., T.F., B.G.D., GR/S72968), the M.R.C. (J.G., E.L., S.M., G0400423/ G0800038) and the BBSRC (J.G., E.L., S.M., E02520X) for funding, the ESRF for generous beam time allocation and members of the UK Glycoarray Consortium for helpful discussions.

**Keywords:** fluorine · fluorosugars · lectins · oligosaccharides · toxoplasmosis

[8] Interestingly, the terminal sialylated Gal(1,3)GlcNAc “sialyl-neolactosamine” is in fact a rare but identified motif in human colon cancer.
[71] It should be made clear that the short half life of $^{18}$F would necessitate redesign of the synthesis of, for example, 4 presented here to allow late-stage incorporation compatible with the time scales for effective imaging.

Spiked candy: An unnatural fluorinated sugar probe (see figure), designed and synthesised to bind to the pathogenic protein TgMIC1, was found to display binding potency equal to and above that of the natural ligand. Dissection of its binding mechanism demonstrated that it is possible to create effective fluorinated probe ligands for the study of, and perhaps intervention in, sugar–protein binding events.