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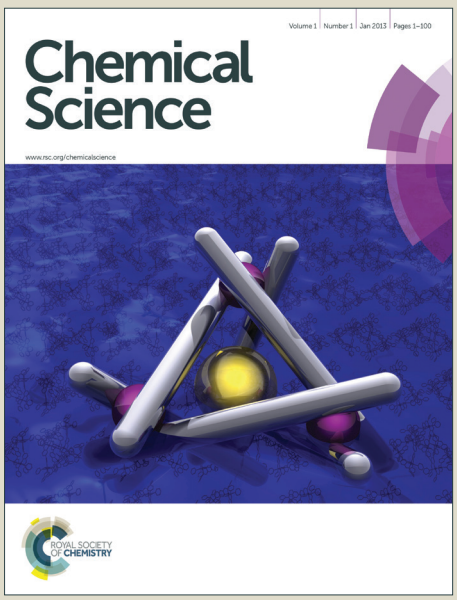

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Glycopolymers with Secondary Binding Motifs Mimic Glycan Branching and Display Bacterial Lectin Selectivity in Addition to Affinity.

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The application of synthetic glycopolymers to anti-adhesive therapies has so far been limited by their lack of lectin specificity. Here we employ a macromolecular engineering approach to mimic glycan architecture. A new, 3-step tandem post-polymerisation methodology was developed which afforded precise control over both chain length and carbohydrate (galactose)-polymer backbone linker distance. This route also allowed a secondary binding (branched) motif to be introduced onto the linker, increasing specificity and affinity towards bacterial toxins without the need for extensive carbohydrate or organic chemistry. Sequential variation of this motif was found to dramatically alter both the affinity and the specificity of the glycopolymers towards two lectins, CTx and PNA, by up to 20-fold either via direct binding, or increased steric constraints. Using this method, a glycopolymer that showed increased specificity towards CTx was identified.

Introduction

Protein-carbohydrate interactions dictate the outcomes of a large and varied number of cellular recognition processes, controlling immune responses, tumour metastasis, gamete fertilisation and many more.[1] The structure, function or even identity of many glycans remains unknown. It is estimated that 50 % of human proteins are glycosylated and there remains significant analytical challenges associated with the isolation and characterisation of complex glycans.[2] Proteins which recognise and process the signals associated with carbohydrates are termed lectins: carbohydrate binding proteins which are neither antibodies nor enzymes and they are widely distributed in Nature.[3] Despite their role in normal physiology, lectins/glycans can also act as a potential site for infection, which can be exploited by pathogenic organisms to interface with their host. For example, pathogenic *Escherichia coli*, *E. coli,* expresses the FimH adhesin, which can bind mannose residues in the urinary tract, influenza has sialic acid binding lectin (heamaglutinins) for adhesion to erythrocytes and *Vibrio cholerae* secretes a toxin which binds to intestinal epithelial cells.[1, 4-6] Conversely, HIV expresses high-mannose structures on its capsid that enables it to bind to DC-SIGN lectin on the surface of dendritic cells in the human immune system.[7] With the widespread emergence of antibiotic resistance, new interventions to prevent and detect infectious disease are urgently required.[8, 9] Anti-adhesion therapy, which seeks to use compounds that have higher affinity than the pathogen for the target binding site, thus preventing the adhesion step and hence reducing the infectivity, has emerged as a promising potential treatment.[4, 10-14] As this process does not involve killing the pathogen, there should be no evolutionary stress, hence reducing resistance development and could be administered prophylactically.

The binding affinity of a carbohydrate to its target lectin is typically very weak ($K_d = 10^3 – 10^6$ M^{-1}), limiting their use in anti-adhesion therapy. The sugars' weak affinity is overcome in Nature by the presentation of multiple copies on cell surfaces which gives rise to an increase in affinity which is greater than the linear sum of the individual sugars, the so-called cluster glycoside effect.[15-19] Lee *et al.* first demonstrated that multivalent *N*-acetyllactosamine with one to four carbohydrates showed progressively increasing binding affinities, over several orders of magnitude, towards rabbit hepatocytes.[15] Kiessling and coworkers have elegantly shown that polymer architecture (linear, branched, dendritic) has profound effects on lectin binding affinity, with particular focus on their ability to cluster receptors.[20] Ambrosi *et al.* found that galacto-functional polymers have a 100-fold increase in binding affinity compared to free galactose.[21] STARFISH-based monodisperse glycodendrimers were shown by Kitov *et al.* to neutralise Shiga-like toxins, with a measured affinity over 10^6-fold greater than the monovalent carbohydrate.[22] Exploitation of the high affinity of glycopolymer-lectin interactions also has applications in biosensing.[23, 24]

Advances in controlled (radical) polymerisation methods[11, 25, 26] together with the development of highly efficient and orthogonal 'click'[27] reactions has facilitated the synthesis of glycopolymers by pre- and post-polymerisation modification thus widening the chemical and architecture diversity of glycopolymers.[25, 28] Despite

the interest in developing synthetic glycopolymers with high affinity for their respective lectins, there has been significantly less focus placed on their *specificity*.[6] What is often not studied is the relative affinity of the glycopolymer for various lectins with similar specificities, which is essential to avoid unwanted therapeutic side effects or to enable precise diagnostics. An important target for multivalent anti-adhesion therapies/diagnostics is the toxin CTx, secreted by *Vibrio cholerae*, the causative agent of cholera infection which is estimated to cause over 100,000 deaths per year, and infects more than 3 million people.[29] β-Galactose functional inhibitors have been shown to have high affinity to CTx, but there is a need to avoid unwanted interactions with other mammalian lectins, such as the galectins, which also bind β-galactose and could lead to immune responses such as cytokine production.[30] Lectin targets for which selective anti-adhesive therapies have been studied include DC-SIGN/Langerhin in HIV therapy, or BmbL/DC-SIGN to treat *Burkholderia ambifaria*.[31-34] The relative affinity of a series of bivalent galactosides towards chicken galectins has also been studied.[35] Moreover, selectivity presents a challenge when identifying biological warfare agents based on lectins such as ricin.[36] Oligosaccharide-mimetic agents have also been developed with high specificity based on tuning their 3-D structure to fit the lectin binding site but without the need for total oligosaccharide synthesis.[37]

Examples of synthetic polymers that have demonstrated lectin selectivity are rare, despite the obvious benefits of their multivalent nature.[38] We have demonstrated that galactose-functional polymers can be engineered to have increased selectivity for cholera toxin B-subunit (CTxB).[39] This was achieved by modulating the distance between backbone and carbohydrate to match the relatively deep cleft of the CTx binding domain, compared to other galactose-binding lectins with shallower domains.[40] Selectivity is required here to discriminate between other pathogenic lectins (or bacteria) that bind galactose including ricin[36] or indeed dietary lectins which can reduce the inhibitor's potency as this would need to function in the intestinal tract.

In this work, we present a macromolecular engineering approach to introduce specificity into glycopolymers, inspired by glycan branching and guided by structural biology information. In particular, we were motivated to achieve selectivity without resorting to multi-step total glycan synthesis, which is non-trivial. Using a new, 3 step-tandem post-polymerisation process, secondary binding (branched) motifs are introduced to the polymer side chain, to increase specificity and affinity towards bacterial toxins.

Results and Discussion

As the first step, microarray data were extracted from the Consortium for Functional Glycomics database to enable a short bioinformatics study to probe lectin specificity/affinity.[41] The relative affinity of CTx and a model galactose-binding non-pathogenic lectin, PNA (peanut agglutinin), to several oligosaccharides was measured and the most relevant results are shown in Figure 1A (full analysis in ESI). PNA was found to bind the disaccharide Gal-β-GalNAc 100-fold more than CTx. However, changing this to a branched oligosaccharide (GM1), which retains the Gal-β-GalNAc unit but also introduces a neuraminic acid branch results in the CTx affinity increasing approximately 100-fold, but with no change in PNA affinity. The increased binding affinity of CTx to the branched saccharide is attributable to allosteric interactions of the neuraminic acid with a secondary binding pocket within CTx, which is not present in PNA.[42] We therefore reasoned that if a secondary-binding motif could be installed on the linker between galactose and backbone on a polymer it would be possible to attenuate the binding affinity of the polymer to CTx as shown by Tran *et al.*,[43] but also selectivity towards the CTx over PNA. Figure 1B shows the crystal structure of CTx binding to the branched glycan unit from GM1 showing both the primary and secondary binding pockets, which is simplified in Figure 1C using standard glycan notation. Figure 1D shows the proposed polymer, with a sufficiently long linker to penetrate the cleft in CTx and a secondary motif to target the allosteric neuraminic acid site.

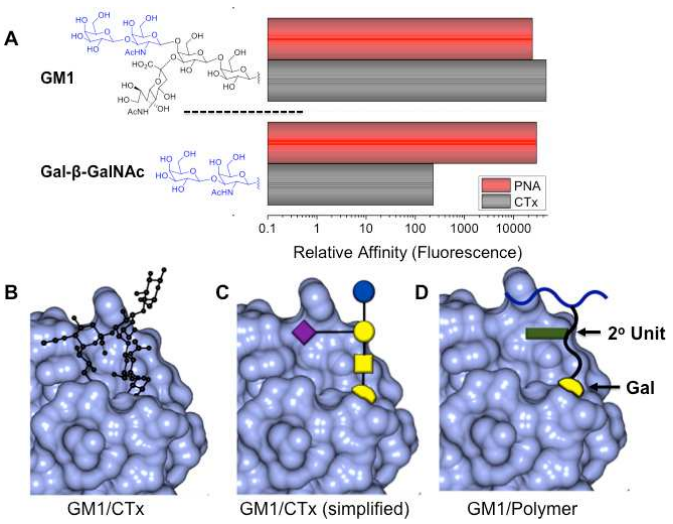

Figure 1. A) Glycan microarray analysis showing relative affinity of CTx and PNA to two related glycans; B) Crystal structure of CTx (blue) binding to the oligosaccharide portion of GM1; C) CTx crystal structure with glycan drawn in standard ball/stick notation. (ESI for key); D) Synthetic polymer design concept with idealised polymer shown (schematic, not simulation).

To enable installation of the branched motifs in a semi-combinatorial manner, we have developed a new synthetic methodology based on three tandem post-polymerisation modifications.[19, 44] This introduces large chemical diversity that is not normally found on glycans, but ensures chain length (and hence valency) homogeneity across all samples overcoming a common challenge in functional polymers, Figure 2.

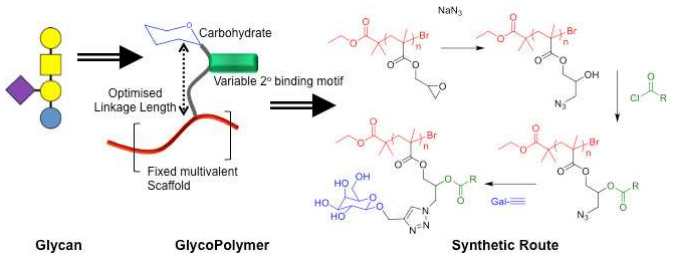

Figure 2. Glycopolymer design principle and the newly developed synthetic route. Glycan structure is shown in standard notation (ESI).

The synthetic method fulfilled the following criteria: i) sufficient separation between backbone and carbohydrate to enable penetration into the CTx binding site; ii) an azide group for subsequent glycosylation with β-D-propargyl galactose; iii) esterification of the hydroxyl group, generated during epoxide ring-opening. Poly(glycidyl methacrylate) was synthesised by Cu(I)-mediated polymerisation to produce a well-defined polymer with a degree of polymerisation ~ 100 and M_w/M_n = 1.2. This molecular weight was targeted as our previous results have shown that above a DP of ~30, no further increase in avidity towards CTx was observed.[39] The polymer was produced by controlled radical polymerization to ensure a lack of low molecular weight tail which would confuse the interpretation of the activity measurements (*vide infra*). Installation of the azide was achieved by addition of sodium azide in DMF at 50 °C, which simultaneously, and quantitatively, installed the necessary orthogonal handle and produced a secondary alcohol as confirmed by infrared spectroscopy (IR), Figure 3. In the second step a range of acyl chlorides were reacted with the alcohol to install secondary motifs as confirmed by the disappearance of the OH stretch at 3400 cm^{-1} and the addition of a second carbonyl stretching frequency. The acyl chlorides were chosen based on evidence that aromatic groups can bind the sialic acid site. [43] In the final modification reaction, β-D-propargyl galactose was installed by Cu(I)-catalysed cycloaddition, which could be monitored by the reduction in the azide vibration at 2100 cm^{-1}. Table 1 summarises the polymer library obtained, the side chains installed and the calculated LogP values of a single repeat unit of the polymer (*vide infra*). LogP values are included as an estimate of the relative hydrophobicity of the binding units.

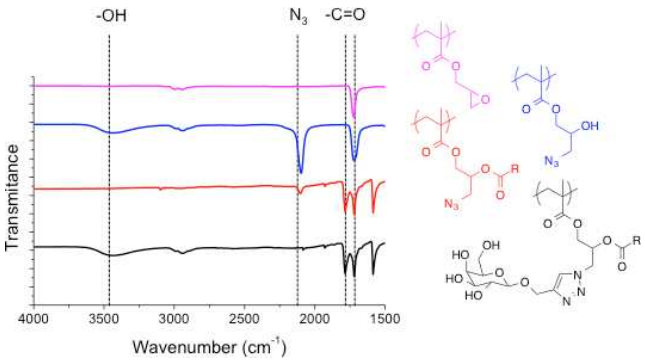

Figure 3. Infrared analysis of the 3-stage, glycan mimetic, tandem post-polymerisation strategy used here. IR analysis was of purified product.

With this panel of sequentially modified glycopolymers in hand, a sorbent assay was used to evaluate the affinity of the polymers towards each of the lectins.[24, 39, 45] Briefly, the glycopolymers were incubated at various concentrations with fluorescently labelled lectins. The solutions were then added to galactose-functionalised microtitre plates. The concentration of polymer required to inhibit 50 % of binding to the plates was reported as the MIC_{50} value. Upon initial testing it was found that several members of the library were insufficiently soluble in buffer to be used in the assays. It was possible to solubilise the polymers in 5 % (v/v) aq. DMSO, but we found this compromised the CTx-assay giving false positive results and hence only the polymers which could be directly dissolved into buffer were tested. The less soluble polymers are still shown in Table 1 to highlight the synthetic diversity achieved by this approach. As predicted, addition of the branched motifs had a dramatic influence on the inhibitory potential of the glycopolymers against both of the lectins, Figure 4.

Table 1. Side chains installed onto the polymers and LogP values.

Code	Structure	LogP	Code	Structure	LogP	Code	Structure	LogP
P1		-1.57 ± 0.61	P5		1.3 ± 0.7	P9		- 0.17 ± 0.6
P2		1.96 ± 0.68	P6		1.48 ± 0.75	P10		-0.36 ± 0.82
P3		1.70 ± 0.7	P7		0.81 ± 0.75	P11		0.73 ± 0.68
P4		3.08 ± 0.6	P8		0.22 ± 0.63			

LogP values are calculated based on a single repeat unit of the polymer, with methyl capping groups at each chain end.

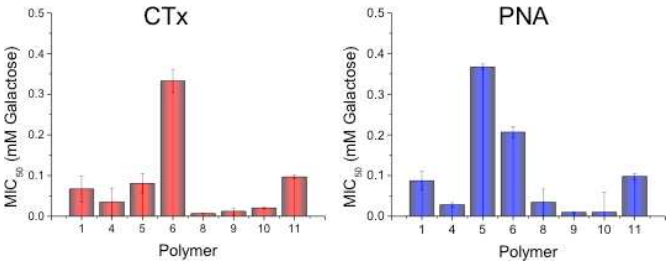

Figure 4. Inhibition of lectin binding by synthetic glycopolymers. Values shown are the average of at least 3 measurements, and errors are the standard deviation.

Figure 4 reveals some general trends between molecular structure and lectin affinity. Three of the secondary units, **P8**, **P9** and **P10** gave rise to large 10-fold decreases in the MIC_{50} towards both lectins, compared to **P1**. Whilst these groups were relatively diverse, the common theme was that they did not contain an aromatic group, but **P8** and **P10** did contain halogenated alkanes. **P8** and **P10** side chains are significantly larger than in **P9** which suggests that their affinity modulation was not entirely due to steric constraints and may indicate that branching at the side chain increases affinity to CTx. Polymers **P11**, **P5** and **P6**, led to either no changes, or

significantly increased the MIC_{50} values. Due to the size and rigidity of these functional groups, steric constraints might be crucial, preventing access of the polymer to both lectins, or limiting conformational flexibility. Polymer **P4**, which had a linear, but flexible, hexamethylene group gave modest improvements (lower MIC_{50}) in binding to both lectins. Our observations are in contrast to those of Bundle and coworkers who have observed that pendant aromatic units can enhance the binding to CTx *via* interactions with the neuraminic acid binding pocket, but they used polymers with a very low density of carbohydrate side chains and different length side chains on polydisperse scaffolds, making comparisons difficult.[43] Here we have densely packed side-chains that impose additional steric restraints. The high affinity of CTx to GM1 in Nature is attributable to the intrinsic rigidity of GM1, which has also been found to be important in small-molecule GM1 mimics and is probably contributing here.[46, 47] Comparison of the observed inhibitory values against the calculated partition coefficient did not reveal any obvious trend suggesting simple hydrophobic/hydrophilic interactions are not responsible (ESI).

As indicated in the introduction, the key aim of this study was to use glycan-mimetic branching to introduce *specificity/selectivity* as well as *affinity* into synthetic glycopolymers. Analysis of the data in Figure 4 revealed that **P1** and **P5** (chlorobenzyl) showed the most dramatic differences in terms of relative affinities for each lectin. Figure 5 shows the relative affinity (shown as $1/MIC_{50}$ for convenience), for **P1** and **P5** against the two lectins. **P1** shows similar affinity for both PNA and CTx indicating that it cannot discriminate/select between two different galactose-binding lectins. However, addition of 4-chlorobenzyl unit (**P5**) leads to dramatic differentiation in response to PNA and CTx, with a significant decrease in affinity towards PNA, but essentially no change in affinity to CTx. This demonstrates that **P5** displays lectin selectivity, using biomimetic macromolecular engineering, but without multistep carbohydrate chemistry. The exact mechanism of binding which leads to selectivity cannot be rationalised at this stage, but the additional bulk of the chlorobenzyl group may prevent access to the PNA binding site, but still be of correct dimensions to fit the neuraminic acid site in CTx. This will be the subject of future investigations. Furthermore, the influence of substituting the chloro- for bromo- group (**P5** – **P6**) cannot be explained. **P6** has vastly increased MIC_{50} values towards CTx relative to **P5**, but less effect on PNA binding. The additional steric bulk of a bromide might simply be too large for a good fit into the binding cleft in CTx. **P1**/**P5** also displayed similar affinities for RCA_{120}, another galactose-binding lectin indicating that the structural motifs added here only affect the target lectins (ESI.). This supports our hypothesis that the secondary motif is giving us the specificity based on interactions with the neuraminic acid binding site in CTx, which is not present in either RCA_{120} nor PNA, and hence the polymers' have decreased affinity to both of these lectins. These observations rule out non-specific hydrophobic association between polymer/lectins as this would be expected to give enhancements to all tested lectins.

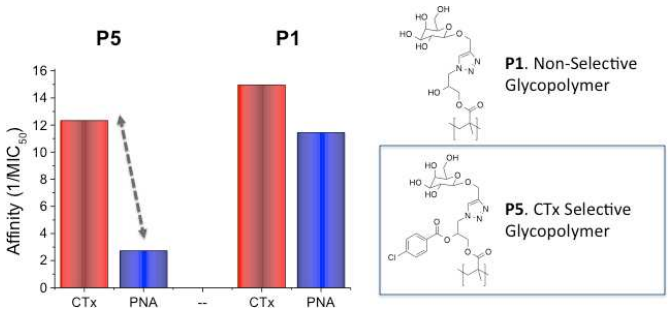

Figure 5. Relative affinity ($1/MIC_{50}$) of **P1** and **P5** for CTx and PNA.

Conclusions

We have demonstrated a new bio-inspired approach to mimicking glycan architecture by using macromolecular engineering, guided by structural biology, and without the need for multi-step oligosaccharide synthesis. Bioinformatics revealed that the addition of branched side chains to galacto-terminal carbohydrates could increase binding affinity to their corresponding lectins, relative to simple monosaccharides. To mimic this branched structure a new, 3 step, tandem post-polymerisation methodology was developed. This enabled precise control over not only chain length, but also carbohydrate-polymer backbone linker distance and the introduction of secondary binding (branched) motifs onto the linker. Sequential variation of this motif was found to dramatically (up to 20-fold) alter both the affinity and the selectivity of the glycopolymers towards two lectins; CTx and PNA. Using this method, a glycopolymer was identified which showed increased specificity towards CTx. Glycopolymers with high selectivity may feature in the development of sensitive and precise sensors or anti-adhesive therapies, which has so far limited the application of synthetic glycopolymers. These results show that combining structural biology tools with macromolecular chemistry enables the creation of synthetic glycans which can mimic, or outperform their natural counterparts and will find applications in anti-adhesive therapy and bimolecular sensors.

Experimental

Example Acylation Reaction using Benzoyl chloride. Poly(2-hydroxy-3-azidopropyl methacrylate) (200 mg, 8.73 μmol) was dissolved in anhydrous THF (50 mL), along with triethylamine (0.45 mL, 3.24 mmol – 3 eq. for each polymer repeat unit). Benzoyl chloride (0.46 g, 3.24 mmol - 3 eq. for each polymer repeat unit) was dissolved in 50 mL of anhydrous DCM and added dropwise to the solution over a period of 30 minutes. Following complete addition, the solution was left to stir at room temperature for 24 hours. A further portion of triethylamine (0.45 mL, 3.24 mmol) and benzoyl chloride (0.46 g, 3.24 mmol) were added to the solution and allowed to stir for a further 24 hours. The solution was then diluted with 100 mL of DCM and quenched with 100 mL of water. The organic layer was washed with water (2 × 50 mL), dried over anhydrous $MgSO_4$, filtered and the solvent removed. The crude polymer solution was then redissolved in 50 mL of THF and twice precipitated into a 1:1

mixture of diethyl ether/petroleum ether. The solids were isolated by centrifugation and dried under vacuum to yield the product as a off-white powder.

Example 1,3-Dipolar Cycloaddition Reaction of Benzoyl Chloride-Modified Polymer with Galactose Alkyne. Polymer (100 mg, 345.67 μmol), Cu(I)Br (4.9 mg, 34.16 μmol) and galactose alkyne (226 mg, 1.04 mmol) was dissolved in DMSO (8 mL) in a Schlenk tube. This solution was degassed by a minimum of 3 freeze-pump-thaw cycles and frozen with liquid nitrogen. The Schlenk tube was then opened, 2,2'-bipyridyl (10.8 mg, 69.15 μmol) was added and the tube re-sealed. The frozen solution was evacuated three times, back-filled with dry nitrogen and left to defrost. After stirring at ambient conditions for 4 days, the solution was diluted with distilled water and dialysed against water for 3 days. The resulting suspension was centrifuged and the supernatant was lyophilised to leave an off-white powder.

Example fluorescence-linked sorbent assay for inhibitory activity against cholera toxin B subunit (CTx) 96-well microtitre plates were incubated for 16 h with 150 μL of 100 $\mu g.mL^{-1}$ GCS (in 95% ethanol, 5% water and heated to 45 °C). Unbound GCS was removed by washing extensively with water. Polymer solutions were made up as serial dilutions (up to 10 dilutions per sample from either 1 $mg.mL^{-1}$ or 0.1 $mg.mL^{-1}$ in water). 10 μL of 100 $\mu g.mL^{-1}$ CTx-FITC in 10 mM Tris with 0.1 mM $CaCl_2$ and 0.5 mM NaCl (pH 8) was added to 90 μL of polymer solution to a final concentration of 11 $\mu g.mL^{-1}$. 100 μL of the PNA/polymer solutions were then added to GCS coated wells and incubated at 37 °C for 30 mins. After this the wells were extensively washed with water and fluorescence was measured at excitation/emission wavelengths of 485/528 nm. All experiments were carried out in triplicate. Percentage inhibition was compared to relative to controls of pure CTx-FITC (with no polymer).

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Notes and references

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Electronic Supplementary Information (ESI) available: Glycan notation and microarray analysis; full synthetic procedures and characterisation, additional inhibition assay details, and additional binding curve details. See DOI: 10.1039/b000000x/

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