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Tuning selectivity of triplex DNA receptors†

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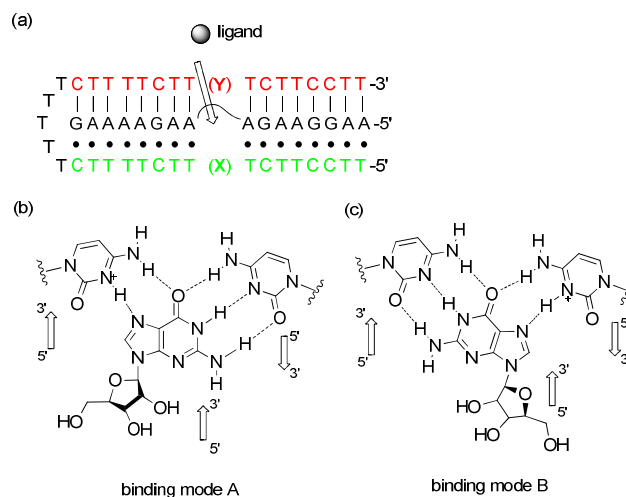
5 Effects of hydrogen bonding clamps on the selectivity of triplex DNA receptors were studied. Incorporation of a 5-methyl-2-thiocytosine base to the parallel homopyrimidine region of a triplex receptor enabled selective molecular recognition of an inosine ligand.

10 Synthetic receptors and protein receptors share similar binding principles.¹ However, the specificity of synthetic receptors is far less prominent than their natural counterparts. A major challenge in creating selectivity in synthetic receptors is to precisely position the binding groups on preorganized scaffolds. This challenge arises from the fact that molecular architectures are restrained by chemical bond lengths and angles. Therefore it is not always feasible to install the desired binding groups at the desired locations. In addition, many synthetic receptor scaffolds, particularly those of molecular containers, are highly symmetric.

20 Introduction of different binding groups on those scaffolds would break the symmetry, resulting in difficulties in organic synthesis. On a completely different track, researchers have been inspired by natural evolution and invented fast laboratory evolution for the development of artificial protein, peptide, and nucleic acid receptors.² However, the structural diversity of evolution-generated receptors is restricted by the limited number of building blocks. For example, many boron, sulfur, and halogen-containing building blocks are excluded from selection due to the lack of efficient orthogonal biochemical processing systems.

30 A middle ground of these two approaches might be a strategy to rationally design a scaffold on biooligomer receptors. Synthesis is less challenging here due to the well-developed automated biooligomer synthesizers, although prediction of the optimal locations to position binding groups is still difficult. For specific recognition of nucleobase and nucleoside targets, we and other groups have developed cavity-containing nucleic acid receptors.³⁻⁸ The binding occurs through the interactions that would normally be found in an intact nucleic acid helix and thus additional binding groups are not required. Receptors for adenosine, guanosine, 8-oxoguanine, and a few adenosine-containing cofactors have been successfully created. In many examples, triplex DNA was used to form a hydrogen bonding clamp (XLY) to fix the ligand in the binding cavity (Scheme 1a).⁴⁻⁵ Remarkably high affinities and selectivity were reported that had not been observed with the rival aptamers developed through SELEX.

As part of the efforts to expand the targets of triplex DNA receptors, we sought to design a selective receptor for inosine. In



50 **Scheme 1** (a) Binding of a ligand to the cavity of a triplex DNA receptor. The parallel and antiparallel homopyrimidine regions are shown in green and red respectively. (b) Guanosine binding to the cavity in a “normal” mode. (c) Guanosine binding to the cavity in a flipped mode

extracellular fluids, inosine alone was shown to activate the A1 and A3 receptors but considered ineffective *in vivo* as the endogenous adenosine was more potent.⁹ However, studies also showed that inosine levels in interstitial fluid of hypoxic and postischemic hearts were much higher than adenosine levels. It is inosine, not adenosine, that initiates glycolaldehydes degradation through the binding of A3 receptors.¹⁰ Therefore specific quantifications of adenosine and inosine concentrations are equally important. We previously developed an adenosine triplex sensor that can selectively detect adenosine in the presence of guanosine and inosine.^{4a} In principle, the same strategy can be used to design a selective inosine receptor.

The biggest challenge in designing a selective receptor for inosine is to prevent guanosine from binding. It was reported that 5-methyl-2-thiocytosine (^{5m2S}C) specifically paired with hypoxanthine favorably over guanine in a duplex DNA.¹¹ However, a free guanosine, which as a ligand is not constrained by the phosphate backbone, may bind to the triplex cavity in a flipped mode (Scheme 1c). Therefore it is important to incorporate the ^{5m2S}C to the correct side where the 2-amino group of guanosine is predicted to face. If guanosine binds either side with similar affinities, two ^{5m2S}C bases need to be incorporated. Herein we report a binding study of triplex DNA receptors (Scheme 2) in which either or both cytosine bases surrounding

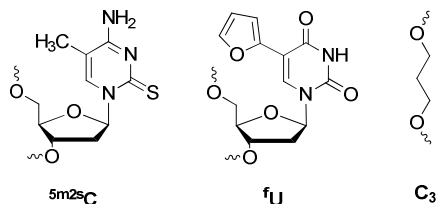
the cavity are replaced by ${}^5\text{m}2\text{sC}$.

5'-TTC CTT CTX TTC TTT TCT TTT TCT TTT CTT YTC TTC CTT-3'

- 1, X = C, Y = C
- 2, X = C, Y = ${}^5\text{m}2\text{sC}$
- 3, X = ${}^5\text{m}2\text{sC}$, Y = C
- 4, X = ${}^5\text{m}2\text{sC}$, Y = ${}^5\text{m}2\text{sC}$
- 5, X = T, Y = T
- 6, X = ${}^f\text{U}$, Y = ${}^f\text{U}$

5'-AAG GAA GA(C₃) AAG AAA AG-3'

7



Scheme 2 Oligonucleotides used in the study.

The binding study was carried out using a membrane filtration assay as previously reported.⁵ The membrane had a molecular weight cutoff of 3000 MW. During filtration, free triplex receptors and receptor-ligand complexes remained on the membrane, whereas unbound ligands passed through the membrane. The ligand concentrations were quantified by the measurement of UV absorbance at 260 nm. The dissociation constants were calculated according to the total ligand concentration, the total receptor concentration, and the unbound ligand concentration (ESI[†]). Receptors **1-4** were examined for the binding of guanosine and inosine (Table 1). The unmodified receptor (**1**) showed binding of both inosine and guanosine in the micromolar concentration range. The binding was weaker than previously observed for adenosine triplex receptors.^{4a,5b} The receptor was selective for guanosine over inosine ($K_{d,I} / K_{d,G} = 4.2$). Replacement of cytosine with ${}^5\text{m}2\text{sC}$ in the antiparallel homopyrimidine region (receptor **2**) increased the binding affinities of both inosine and guanosine. In contrast, replacement in the parallel homopyrimidine region (receptor **3**) showed significantly increased binding affinity toward inosine but decreased affinity toward guanosine. These results suggest that guanosine and inosine in fact bind to triplex receptors in a flipped mode as depicted in Scheme 1c. Substitution by ${}^5\text{m}2\text{sC}$ in the parallel homopyrimidine region disrupted normal Watson-Crick hydrogen bond formation for guanosine but not inosine. It is noteworthy to mention that stabilization effect of ${}^5\text{m}2\text{sC}$ on a Watson-Crick base pair with inosine was consistent with a previous report.¹² ${}^5\text{m}2\text{sC}$ is also known to increase triplex stability.^{11,13} Therefore substitution of cytosine with ${}^5\text{m}2\text{sC}$ on the antiparallel homopyrimidine region increased the binding affinities for guanosine and inosine. It was also revealed that replacement of both cytosine bases in the binding cavity with ${}^5\text{m}2\text{sC}$ (receptor **4**, $K_{d,I} / K_{d,G} = 0.32$) did not improve the selectivity for inosine compared to receptor **3** ($K_{d,I} / K_{d,G} = 0.18$). However, the binding of guanosine by **4** was stronger than **3**, indicating that although the Watson-Crick base pair of ${}^5\text{m}2\text{sC}:\text{G}$ was highly disfavored, guanosine was not forced to bind in mode A to avoid the clash.

Table 1 Apparent dissociation constants of receptor **1-4**.

Receptor	H bonding clamp	$K_{d,I}$ (μM)	$K_{d,G}$ (μM)	$K_{d,I} / K_{d,G}$
1	CLC	11.4 ± 3.2	2.7 ± 0.7	4.2
2	$\text{CL}{}^5\text{m}2\text{sC}$	6.4 ± 1.3	1.8 ± 0.5	3.6
3	${}^5\text{m}2\text{sC}\text{CLC}$	3.0 ± 0.9	17.1 ± 2.4	0.18
4	${}^5\text{m}2\text{sC}\text{CL}{}^5\text{m}2\text{sC}$	3.1 ± 0.7	9.7 ± 1.7	0.32

Table 2 Effects of ligand binding on the UV₂₆₀ melting temperatures of triplex receptors.^a $\Delta T_{m,A}$, $\Delta T_{m,G}$, and $\Delta T_{m,I}$ represent the increase of melting temperature when adenosine (30 μM), inosine (30 μM), and guanosine (30 μM) were added to the receptor respectively.

Receptor	H bonding clamp	T_m ($^\circ\text{C}$) ^c	$\Delta T_{m,I}$ ($^\circ\text{C}$) ^c	$\Delta T_{m,G}$ ($^\circ\text{C}$) ^c	$\Delta T_{m,A}$ ($^\circ\text{C}$) ^c
1	CLC	52.8	3.5	5.2	0.9
2	$\text{CL}{}^5\text{m}2\text{sC}$	53.0	3.5	6.2	ND ^b
3	${}^5\text{m}2\text{sC}\text{CLC}$	53.5	4.4	2.3	ND ^b
4	${}^5\text{m}2\text{sC}\text{CL}{}^5\text{m}2\text{sC}$	52.3	4.8	2.1	ND ^b
5	TLT	51.7	<0.5	<0.5	5.7
6	${}^f\text{UL}{}^f\text{U}$	54.5	<0.5	<0.5	3.2

^aAll measurements were performed in 60 mM PIPES (pH 5.5), 10 mM MgCl_2 , and 20 mM NaCl. The UV wavelength is 260 nm.

^bNot determined.

^cErrors < 0.5 $^\circ\text{C}$

To provide additional evidence to support the observation of binding, we examined the effects of ligands on the UV melting temperatures of receptors **1-6** (Table 2). These triplexes are “BiTrip” typed structures and thus each melting curve showed only one transition.¹⁴ The melting temperatures of the receptors in the absence of the ligands were all similar except for receptor **6**, which contained a pair of furanyl-uracil (${}^f\text{U}$), a fluorescent thymine analogue.¹⁵ In the absence of adenosine, the ${}^f\text{U}$ nucleosides are expected to adopt the *syn* conformation and insert the furan ring to the cavity, thus enhancing the stability of the abasic site. In the presence of purine nucleosides, the melting temperatures of all 6 triplexes more or less increased. The largest stabilization effect was observed when adenosine bound to the TLT triplet (+5.7 $^\circ\text{C}$). The stabilization effect of adenosine on the ${}^f\text{UL}{}^f\text{U}$ triplet was significantly smaller (+3.2 $^\circ\text{C}$), as it was likely that the ${}^f\text{UL}{}^f\text{U}$ triplet was already stabilized by the stacking of the furan rings in the cavity. The effect of guanosine imposed on the CLC triplet was also significant (+5.2 $^\circ\text{C}$). In contrast, the effect of inosine on the CLC triplet (+3.5 $^\circ\text{C}$) was smaller. The difference between the guanosine and inosine binding may be explained by the weaker stacking ability and fewer hydrogen bonding interactions associated with inosine compared to guanosine,¹⁶ and also agreed well with the dissociation constants shown in Table 1. When the cytosine surrounding the cavity was replaced with ${}^5\text{m}2\text{sC}$, the stabilization effects generated by the binding of ligands became different. The differences were more pronounced when the replacement occurred on the parallel homopyrimidine region: inosine showed enhanced stabilization effects (+4.4 $^\circ\text{C}$ and +4.8 $^\circ\text{C}$), whereas the effects of guanosine were much smaller (+2.3 $^\circ\text{C}$ and +2.1 $^\circ\text{C}$). These results further confirmed that inosine and guanosine bound in the flipped mode.

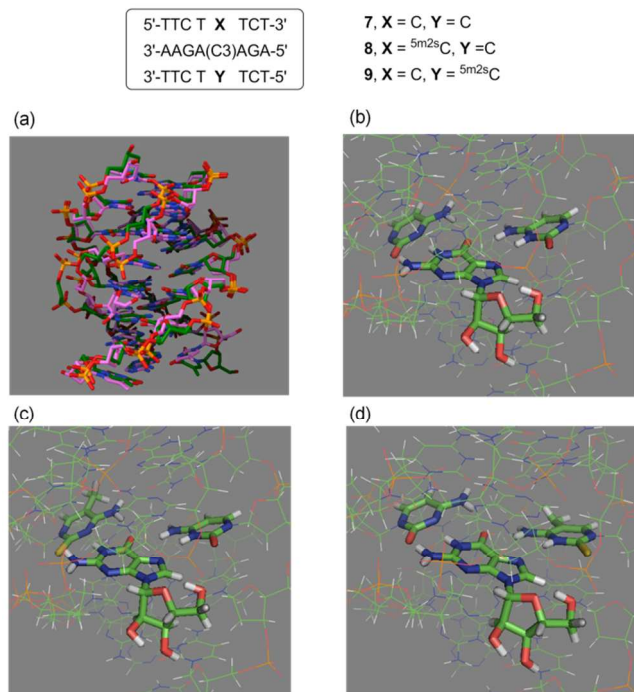


Fig. 1 Molecular modeling of binding between guanosine and triplex receptor 7-9. (a) Superimposition of the complexes between 7 and guanosine in two different binding modes. The image was obtained using Python Molecular Viewer 1.5.6.¹⁸ The carbon atoms of the structure in binding mode A and B were colored green and pink, respectively. (b) Binding of guanosine to 7 in mode B. (c) Binding of guanosine to 9 in mode B. (d) Binding of guanosine to 8 in mode B.

Finally, we examined the binding of guanosine to these receptors using molecular modeling. Triplex structures 7-9 were built in Hyperchem 8.1 starting from a known NMR structure.¹⁷ The complexes between the receptors (7-9) and guanosine were energetically minimized in the Amber Force Field (ESI†). Comparison of the structures of the complexes between triplex 7 and guanosine in two different binding modes suggests that the mode B would result in fewer disturbances to the triplex skeleton. The ribose moiety of guanosine in binding mode A would force the C3 spacer to bulge out into the solvent. In contrast, the C3 spacer in binding mode B was aligned well with rest of the phosphate backbone. Replacement of cytosine by ^{5m2s}C on the parallel homopyrimidine strand (triplex 9) caused slight distortions of a normal triplet: the N2-H of guanosine that formed a hydrogen bond with O2 of cytosine in triplex 7 now bent out-of-plane to avoid the clash with S2 of ^{5m2s}C, and the distance between the N1-H of guanosine and N3 of ^{5m2s}C increased to 2.2 Å, indicating a weaker hydrogen bond. In contrast, replacement of CH(+) with ^{5m2s}CH(+) on the antiparallel homopyrimidine strand (triplex 8) did not cause significant distortions. These models are consistent with the hypothesis that guanosine binds in a flipped mode and that ^{5m2s}C in the parallel homopyrimidine strand prevents guanosine from forming the normal triple Watson-Crick hydrogen bonds.

In summary, binding affinities between triplex receptors and purine nucleosides can be ranked in such an order: TLT-A > CLC-G > CLC-I. This trend is perhaps mainly dictated by the stacking and hydrogen bonding abilities of the purine nucleosides. However, chemical modifications can be employed

to reverse the binding preference between guanosine and inosine. Replacement of cytosine by ^{5m2s}C in the parallel homopyrimidine region of the triplex can produce a receptor selective for the inherently disfavored inosine ligand.

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Notes and references

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- † Electronic Supplementary Information (ESI) available: experimental procedures. See DOI: 10.1039/b000000x/
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