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Duplex Healing of Selectively Thiolated Guanosine Mismatches through a Cd^{2+} Chemical Stimulus

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Abstract: The on-column selective conversion of guanosine to thioguanosine (**tG**) yields modified oligomers which exhibit destabilisation over the fully complementary duplex. Restoration to a stabilised duplex is induced through thio-directed Cd^{2+} coordination, a route for healing DNA damage. Short oligomers are G-specifically thiolated through a modified on-column protocol without the need for costly thioguanosine phosphoramidites. Addition of Cd^{2+} ions to a duplex bearing a highly disrupted **tG** central mismatch sequence, 3'- $\text{A}_6\text{tG}_4\text{T}_6$ -5', suggests a (**tG**)₈ Cd_2 central coordination regime, resulting in increased base-stacking and duplex stability. Equilibrium molecular dynamic calculations support the hypothesis of metal induced healing of the thiolated duplex. The 2 nm displacement of the central **tG** mismatched region is dramatically reduced after addition of a chemical stimuli, Cd^{2+} ions, returning to a minimized fluctuational state comparable to the unmodified fully complementary oligomer.

DNA has moved beyond its biological function into the arena of nanotechnology where it has been re-purposed as a building material for the construction of functional nano-architectures.^[1] Artificial oligonucleotides incorporating modified nucleosides at predetermined sites are of increasing interest due to their potential applications in conducting nanowires,^[2] functional DNA origami structures^[3] and drug delivery systems.^[4] Of particular interest is the modified purine, 6-thio-2-deoxyguanosine (**tG**) due to potential applications in therapeutics^[5] and the ability to coordinate metal ions forming coordination polymers.^[6] Thiol-bearing artificial nucleobases are typically exploited as soft ligands to bind soft transition metals, e.g. gold nanoparticles^[7] and silver ions.^[8] Here we firstly report a facile and cost effective synthetic route for incorporating **tG** into oligonucleotides. Secondly, duplexes containing multiple mis-matched **tG** sites show considerable de-stabilisation of the helix. Finally, dramatic re-stabilisation is achieved via metal-mediated healing of the **tG** : **tG** mismatches through the addition of Cd^{2+} ions. Scheme 1 summarises these three concepts. Understanding the stabilising effects of metal ions on **tG** bearing duplexes would be of benefit in the directed assembly of DNA nanomaterials through a chemical stimulus.

Scheme 1. Schematic overview highlighting the on-column conversion of **G** to **tG**, the formation of a **tG**:**tG** mismatched duplex and the subsequent binding of Cd^{2+} to the **tG** region to re-stabilise the helix.

Reported syntheses of DNA containing **tG** include incorporation by chemical^[9] or enzymatic^[10] approaches. Enzymatically can prove problematic due to the polymerases limit on structure toleration.^[11] Chemically, incorporation of protected **tG** bases can be achieved^[12] however, the synthesis can involve complex organic chemistry and is often prohibitively expensive. A synthetic route for the facile conversion of guanosine (**G**) to **tG** that is compatible with the standard phosphoramidite protecting chemistries is therefore attractive. Here, simple homobasic oligonucleotides (consisting of only guanine bases), **G_n**, where $n = 2, 3, 4$ and 5 , were synthesized on controlled porous glass (CPG) beads. The surface immobilised oligomers were subjected to thiolation conditions using a procedure developed by Jones,^[13] and were then cleaved from the solid support,^[14] see SI for full synthesis and characterisation. Thus we demonstrate that **G_n** oligonucleotides can be converted to **tG_n** oligonucleotides whilst still bound to the standard CPG column, greatly aiding in purification and isolation and without the need to purchase costly pre-thiolated phosphoramidite reagents.

To study the effect of the replacement of **G** with **tG** on the overall stability of duplex formation, the oligomer (**T₆G₄A₆**), **oligo-1**, and its complement, (**T₆C₄A₆**), **oligo-2** were synthesized. **Oligo-1**, upon thiolation yielded **oligo-3**, (**T₆tG₄A₆**), for full synthesis details see SI. The sequence details of these three 16-base oligomers and the perfectly matched **G-C**, (**duplex-1**), the mismatched **G-G** (**duplex-2**), the mismatched **tG-tG**, (**duplex-3**) and the partially matched **tG-C** (**duplex-4**), formed from them are given in Table 1.

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Table 1. Base sequences of the oligomers and duplexes formed to study the effect of the central 4-base sequence on thermal stability.

oligo-1	5'- TTT TTT GGGG AAA AAA-3'
oligo-2	5'- TTT TTT CCCC AAA AAA-3'
oligo-3	5'- TTT TTT tGtGtGtG AAA AAA-3'
duplex-1	5'-TTT TTT GGGG AAA AAA-3' 3'- AAA AAA CCCC TTT TTT-5'
duplex-2	5'-TTT TTT GGGG AAA AAA-3' 3'-AAA AAA GGGG TTT TTT-5'
duplex-3	5'-TTT TTT tGtGtGtG AAA AAA-3' 3'-AAA AAA tGtGtGtG TTT TTT-5'
duplex-4	5'-TTT TTT tGtGtGtG AAA AAA-3' 3'-AAA AAA C C C C TTT TTT-5'

It is proposed that **duplex-3** could direct metal ion coordination to the central **tG₄** region, much like the artificial bases employed by Shionoya et al.^[15] Cd^{2+} has a well-known binding affinity for **tG**,^[16] and we hypothesise it could induce metal-ion mediated duplex stabilisation. Upon addition of an increasing concentration of Cd^{2+} to **duplex-3** the absorbance at 341 nm gradually decreased and exhibited a blue shift to 319 nm, see Figure 1. We attribute this change in absorbance to the complexation of Cd^{2+} ions through the metal coordinating thione $\text{C6}=\text{S}$ which undergoes binding induced deprotonation to yield the deprotonated thiol, see SI for full UV studies into the mode of deprotonation.^[17] The Job plot of this titration indicates a clear 1:4, Cd^{2+} to **tG** binding ratio, (Figure 1 inset) suggesting a total of two metal ions coordinated within the duplex to give **duplex-3** Cd_2 .

Figure 1. UV-Vis plot showing the change in absorbance of **duplex-3** at increasing concentrations of CdNO_3 (from red to purple). Inset: the Job plot of the mole fraction (χ) of thiobase multiplied by the change in absorbance at 341 nm against the mole fraction

Figure 2. Thermal denaturation curves for i) **duplex-1** (green line), **duplex-2** (blue line), and **duplex-3** (red line) all in the absence of metal ions and ii) **duplex-3** (red line) and **duplex-3** Cd_2 (yellow line). Inset: The first derivative for each melting curve highlighting the T_m is only pronounced for **duplex-1** and **duplex-3** Cd_2 .

We hypothesise that each Cd^{2+} coordinates to the **tG** bases on both strands through a combination of the C6 -sulphur and N7 donor groups to form the stabilized **duplex-3** Cd_2 . An analysis of crystal structures in the Cambridge Structural Database which contain sulphur and nitrogen ligands coordinated to Cd^{2+} , indicates that the most commonly reported structures consist of Cd^{2+} coordinated to four sulphurs and two nitrogens which corresponds with the 1:4, Cd^{2+} to **tG** binding ratio, see Table T1 in SI for full details.^[5, 16] This hypothesis is further supported from a low temperature NMR study of **tG** which upon addition of Cd^{2+} exhibits a downfield shift of the N1 proton from 12.55 ppm to 14.19 ppm, as previously reported for mercaptopurine.^[16]

The thermal stability of **duplex-1**, **-2** and **-3** were compared, see Figure 2. **Duplex-1** showed the expected UV-absorption denaturation curve with a T_m of 32.2 °C, whereas **duplex-3** does not, Figure 2i. Rather the steady increase in absorption with increasing temperature indicates extensive unwinding of the duplex due to increased destabilisation caused by the thiol groups on both strands. We estimated the T_m to be 22.5 °C which indicates a destabilisation of c.a 10 °C, comparable to a previous report by Somerville et al.^[18] We hypothesise that the 12 (2 x 6) complementary terminal A : T base-pairs do not fully overcome the destabilising central **tG₄ : tG₄** region.^[19] **Duplex-2** shows an intermediate denaturation curve indicating partial destabilisation of the duplex through the four **G : G** mismatches. **Duplex-3** upon complexation of Cd^{2+} , **duplex-3** Cd_2 , exhibits a more typical denaturation curve with a T_m of 30.0 °C which indicates c.a. 7.5 °C stabilisation over **duplex-3**, Figure 2ii.

Figure 3. Snapshots representative of the highest populated clusters in conformational ensembles of the DNA duplexes under investigation. Cadmium ions (green spheres) found interacting with the sulphur atoms of the four central **tG** bases (small yellow spheres) induced stabilisation upon the flexible **duplex-3**.

Equilibrium molecular dynamics (MD) simulations were carried out in order to assess the stability of the duplexes at the atomistic detail. All duplexes were subjected to 50 ns of unrestrained MD simulations, see SI, and obtained trajectories were analysed by monitoring system energetics, stability in terms of deviations from the native DNA geometry (**duplex-1**), and flexibility in terms of per-residue root-mean-square fluctuations (RMSF), see Figure 3. Compared to the fully complementary **duplex-1**, and to **duplex-2**, **duplex-3**, retains a B-type helix despite the introduction of the four **tG** residues into each strand, see MD simulation videos in SI for full details. The modelling images show that upon incorporation of the **tG** bases within the duplex, the stacking of the nucleobases in the central region becomes distorted but the duplex maintains a right-handed helical structure.^[20] Hydrogen-bond base-pairing between two **tG** bases is not expected and hence the **tG** bases are directed outwards from the centre of the helix, Figure 3, **duplex-3**.^[18] This lack of complementary hydrogen bonding between the two sets of four **tG** bases in the central region results in increased fluctuational movement of all bases along the duplex. The individual mobility of each of the 16-nucleobase residues within both strands of **duplex-3** was quantified in terms of per-residue RMSF, (red markers in Figure 4). In the fully complementary, **duplex-1**, (green markers) the RMSF of the central four base-pairs is c.a.0.2 nm, whereas for **duplex-3**, it is up to 2 nm, a tenfold increase in movement corresponding to a partial unwinding of the double helix.

Addition of Cd^{2+} ions stabilised **duplex-3** through direct interactions between the Cd^{2+} ions and the sulphur atoms of the central **tG** residues (Cd-S distances of 2.60 – 2.75 Å), resulting in increased stacking of the **tG** bases which were previously protruding from the duplex. The restorative effect of the Cd^{2+} on the distorted **duplex-3** is considerable, resulting in reduced residue fluctuations from end-to-end of **duplex-3Cd₂**, (yellow markers in Figure 4). The induced local ordering of the structure and the decreased overall flexibility of the system, support the observed Cd^{2+} mediated stabilisation of the T_m for **duplex-3Cd₂**.

It is conceivable that **oligo-3** could form an intrastrand hairpin structure due to the terminal self-complementary A:T sequences. In this structure the four **tG** bases would lie within a

loop section which could coordinate a single Cd^{2+} , thus giving a 1:4 Cd^{2+} / nucleobase ratio. To test this possibility, **oligo-3** was annealed with **oligo-2** to form **duplex-4**, where the central section contains four **tG** bases on one single strand opposite four “complementary” C bases. The T_m measurement of **duplex-4** indicated an intermediate thermal stability between **duplex-1** and **duplex-3**, as expected. However the binding titration of **duplex-4** with Cd^{2+} , to yield **duplex-4Cd₂**, indicated a 1:2, Cd^{2+} to **tG** binding ratio, (see Figure S20) which suggests that the Cd is associated between two adjacent bases in the linear duplex. In addition, there was no noticeable difference in the thermal stability between **duplex-4** and **duplex-4Cd₂** (see Figure S23), supporting the proposal that the Cd^{2+} ions are critical in the stabilisation of the mis-matched **tG:tG** region only in **duplex-3**. Furthermore, molecular dynamic simulations of the possible intrastrand hairpin structures formed from **oligo-1** and **oligo-3** indicated that the overall structures were flexible and had minimum energy values of -4240 kJ/mol and -927kJ/mol for **hairpin-1** and **hairpin-2** respectively, see SI for full details. This suggests that hairpin formation within the **tG** modified **oligo-3** is unlikely due to its higher energy conformation. Energy calculations after the inclusion of Cd^{2+} ions showed some stabilisation of the hairpin-2 structure but the MD simulations showed no intimate interactions of Cd^{2+} with the **tG** nucleobases, see Figure S17. We therefore conclude that the predominant structure of the **tG** modified **oligo-3** after the addition of Cd^{2+} is the **duplex-3Cd₂** rather than the formation of a hairpin.

Figure 4. Graphical representation of the RMS fluctuations of each of the 16 nucleobase residues within a single strand of **duplex-1**, **duplex-2**, **duplex-3** and **duplex-3Cd₂**. Data for the second strand of each duplex is provided in the SI.

To investigate further the structures of **duplexes-1**, **-2** and **-3** predicted from the MD simulations, circular dichroism spectra of each duplex were recorded, Figure 5. In all cases the exciton splitting centred around 260 nm is consistent with a B-form DNA spectrum.^[21] In **duplex-3**, the conversion from **G** to **tG** can be observed by the additional absorption band at 344 nm. However, as no peak splitting is observed the **tG** bases are not stacked concertedly. On addition of Cd^{2+} , the **tG** CD band does now exhibit an exciton splitting centred at 320 nm, which is indicative of the Cd^{2+} binding to the DNA through the **tG** sites. Therefore,

the Cd^{2+} induces some stacking of the previously displaced **tG** bases and hence increases the stability of the overall duplex.

Figure 5. Circular dichroism spectra of duplex-1, -2, -3 and duplex-3 Cd_2 .

We conclude that **G** can be selectively converted to **tG** within oligonucleotides which are still immobilised on a CPG-solid support. The **tG** bases are shown to destabilise the duplex as seen by increased nucleobase fluctuations in the central region and evidenced by the loss of base-stacking order in the CD spectrum. Upon coordination of Cd^{2+} ions to the sulphur atoms of **tG**, induced base stacking and a reduction of base fluctuations re-stabilises the DNA duplex. Here, the Cd^{2+} induced stabilisation of multiple single-atom base mutations within a DNA duplex, illustrate the potential for the metal mediated healing of DNA damage. Future work could exploit this approach to develop a route for the design of functional DNA hybrid nanomaterials from highly artificial and potentially unstable oligomeric building blocks, which in response to chemical stimuli can be re-assembled.

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COMMUNICATION

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**Duplex Healing of Selectively
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Duplex Healing of Selectively Thiolated Guanosine Mismatches through a Cd²⁺ Chemical Stimulus

Supporting information

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Experimental Materials and Methods

DNA Synthesis. DNA synthesis was performed using an EXPEDITE™ Nucleic Acid System. All reagents used to synthesise DNA oligonucleotides were purchased from Glen Research or Sigma Aldrich, and used as received unless stated otherwise. Oligomers were purchased from Eurofins (Ebersburg, Germany) whilst still bound to the CPG solid support.

High-Performance Liquid Chromatography (HPLC). HPLC was performed on a Waters 2487 machine using Picolog software, and a Varian Pro Star using a C18 reverse phase column for both systems. Buffer A was 0.1 M triethyl ammonium acetate at pH 6.5 with 5 % acetonitrile, and buffer B was 0.1 M triethyl ammonium acetate at pH 6.5 with 65 % acetonitrile at the gradient 0-30 % B over a period of 25 minutes (increasing 10 % buffer B at 5 minute intervals. The gradient was returned to 0% B after 25 minutes) at a rate of 1ml/min. Absorbance was monitored at two wavelengths: 254 nm and 340 nm.

Ultraviolet-Visible Spectroscopy (UV-Vis) and Titrations. UV-Vis spectra were recorded on a Varian Cary100 Bio UV-visible spectrophotometer with a Varian Temperature controller within the range of 200 nm to 600 nm. The spectrometer was blanked using nanopure water. The DNA concentrations were calculated using the absorbance at 260 nm and the Beer-Lambert law; $A = c\ell\epsilon$.

Extinction Coefficient calculation:

Using **oligo-3**:

At 21°C, $A_{260} = 0.479$, $A_{340} = 0.140$

At 95°C, $A_{260} = 0.597$, $A_{340} = 0.148$

Calculating ϵ_{oligo} for **oligo-3** = $0.9 \times ((6 \times 15400) + (4 \times 8800) + (6 \times 8700))$ nucleobase extinction coefficients taken from *Sigma and Jena biosciences*

$$= 161820 \text{ M}^{-1}\text{cm}^{-1}$$

Concentration of oligo = ss $A_{260} / \epsilon_{\text{oligo}}$

$$= 3.69 \text{ uM}$$

Therefore, concentration of dtG = $4 \times 3.69 = 14.76 \text{ uM}$

$$\text{ss tdG } \epsilon = 0.148 / 14.76 \times 10^{-6}$$

$$= 10027 \text{ M}^{-1}\text{cm}^{-1}$$

$$\text{ds tdG } \epsilon = 0.140 / 14.76 \times 10^{-6}$$

$$= 9485 \text{ M}^{-1}\text{cm}^{-1}$$

Compared to free tdGTP $\epsilon = 24800 \text{ M}^{-1}\text{cm}^{-1}$

Environment	$\epsilon \text{ M}^{-1}\text{cm}^{-1}$
tdGTP	24800
ss tdG	10027
ds tdG	9485

The thiobase concentration for the binding titration was 12 μM and the Cd^{2+} concentration was 200 μM . For the Job plot, the thiobase concentration was 12 μM and the Cd^{2+} concentration was 12 μM .

Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOF MS).

MALDI-TOF MS measurements were recorded on a Microflex MALDI-TOF MS instrument using a MSP Anchorchip 600-96. All reagents used for MALDI-TOF MS were purchased from Bruker Daltonics or Sigma Aldrich.

Preparation of the MALDI anchorchip target: 3- Hydroxypicolinic acid (3-HPA) (50 mg) and diammonium hydrogen citrate (5 mg) were dissolved in nanopure water (5 ml). 1 μL of the 3-HPA matrix solution was deposited onto the target position and allowed to dry. 1 μL of the sample solution was deposited onto the matrix spot and allowed to dry.

High resolution mass spectrometry (HRMS). HRMS spectra were recorded on a Waters Micromass LCT premier TOF system using Masslink software (version 4.1). Positive mode was used, with a desolvation temperature of 250 $^{\circ}\text{C}$ and the samples were injected in nanopure water.

^1H NMR and ^{13}C NMR. NMR spectra were recorded on a Bruker Avance 111 300 spectrometer using Topspin software (version 3.2). The spectra acquired was then processed using MestReNova.

For low temperature studies, NMR spectra were recorded on a 700 spectrometer at 214 K. NMR samples (2-5 mg) were dissolved in DMF-d_7 .

Infrared Spectroscopy (IR). FTIR analysis was performed on a Varian 800 FTIR Scimitar series AC 300 spectrometer and a Shimadzu IRAffinity-1S using Labsolutions IR software. For the analysis of oligomers, 1.5 μL of sample was dried directly onto the diamond plate and the spectra recorded at a resolution of 16 for 32 scans.

DNA Digestion. Snake venom phosphodiesterase (0.2 mg), bacterial alkaline phosphatase (100 units) in potassium phosphate buffer (10mM), magnesium chloride (10 mM, pH 7) was added to ds DNA (2 absorbance units at 260 nm) and incubated at 37 °C for 16-18 hours. 50 µL of digestion product was then analysed by reverse phase HPLC on an Spherisorb ODS C18 5µm 250 mm column with 0.1 M triethylammonium acetate, pH 6.5 with 5 % acetonitrile.

Melting Temperatures. Melting curves were obtained on a Varian Cary100 Bio UV-visible spectrophotometer with a Varian Temperature controller. The DNA solutions were preannealed. The temperature was increased from 5 °C to 95 °C at a rate of 1 °C/min and a data interval of 0.5°C. Normalized absorbance displayed in the figures was calculated as follows:

$$\text{Normalized } \Delta A_{260} = (A_{260}(t \text{ } ^\circ\text{C}) - A_{260}(5 \text{ } ^\circ\text{C})) / (A_{260}(95 \text{ } ^\circ\text{C}) - A_{260}(5 \text{ } ^\circ\text{C}))$$

Circular Dichroism (CD). Cd spectra were recorded on JASCO J-18 spectropolarimeter with 4 time accumulation and a path length of 1 cm at 20 °C.

CSD search. The Cambridge Structural Database was searched for discrete Cd complexes containing nitrogen and/or sulfur bearing ligands. The reported number of crystal structures that contained bonds between the Cd²⁺ centre and each of the two donor atoms was determined and tabulated, see below. Common geometries include; 4 sulfur and 2 nitrogen donors (80 structures), 4 nitrogen and 2 sulfur donors (60 structures), and 2 sulfur and 2 nitrogen donors (37 structures).

Number of structures		Nitrogen donors							
		1	2	3	4	5	6	7	8
SssSu	1	0	0	0	6	12	3	0	0
	2	0	37	10	60	0	0	0	
	3	4	16	4	0	0	0		
	4	28	80	0	0	0			
	5	1	0	0	0				
	6	0	0	0					
	7	0	0						
	8	0							

Table T1. Table representing the number of hits obtained from the CSD search of cadmium complexes bearing ligands containing both nitrogen and sulfur.

Molecular dynamics (MD) simulation protocol.

All simulations for the studied duplexes (**duplexes 1-4**) were carried out using GROMACS 5.1.2,¹ with Amber99SB-ILDN² force field for the duplexes and the TIP3P water model. Parameters for thioguanine were obtained by analogy to the native guanine and assigned using ACPYPE.³ Partial atomic charges on the thioguanine molecule were assigned using the RESP methodology⁴ and obtained with the Gaussian09 programme,⁵ using HF/6-31G* basis set.

The temperature was kept constant at $T = 298$ K by using velocity rescaling with a coupling time of 0.1 ps. The pressure was kept constant at 1 bar using an isotropic coupling to Parrinello-Rahman barostat with a coupling time of 0.1 ps.⁶ A cutoff of 1 nm was used for all nonbonded interactions. Long-range electrostatic interactions were treated with the particle-mesh Ewald⁷ method using a grid spacing of 0.1 nm with cubic interpolation. All bonds between hydrogens and heavy atoms were constrained using the LINCS algorithm.⁸

Each of the duplexes were immersed in a cubic TIP3P water box containing $\sim 50,000$ atoms. Simulation units were maintained neutral by adding calcium and chloride counterions (0.1 M concentration). In the simulations with cadmium, cadmium cations replaced calcium counterions. Initial conformations of all three systems were assumed to be helical.

Prior to MD simulations, the systems underwent 25000 steps of molecular mechanical energy minimization. This was followed by 100 ps MD simulations, during which position constraints were used on all duplex atoms. After the following unrestrained equilibration phase (1 ns) the production runs were carried for 50 ns, with an integration time step of 2 fs. The cutoff for non-bonded interactions was 0.1 nm. The coordinates were saved every 10 ps.

For the visual inspection of the results we used xmgrace and UCSF Chimera packages.^{9, 10}

The RMS fluctuations of the second strand of **duplex-3** is shown in the figure below, highlighting the increased movement of all nucleobases within the modified strand against **duplex-1**, **duplex-2**. In addition as reported in the main text, the addition of Cd^{2+} stabilizes the duplex.

Figure S1. Graphical representation of the second strand RMS fluctuations of each of the 16 nucleobase residues within a single strand of **duplex-1**, **duplex-2**, **duplex-3** and **duplex-3Cd₂**.

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Synthesis

Synthesis of *tG*: 2'-deoxyguanosine (0.20 g 0.75 mmol) was added to 15 ml of dry pyridine, which was cooled in an ice bath under a nitrogen atmosphere. Trifluoroacetic anhydride 0.9 ml (6.3 mmol) was added drop wise and stirred for 40 min. After that, a suspension of 1.3 g (23 mmol) of NaSH in 22.6 ml of anhydrous dimethylformamide was added and stirred. After 24 h, 38 ml of 0.16 M ammonium bicarbonate was poured into the reaction mixture with vigorous stirring. The mixture was concentrated to dryness then was dissolved in methanol and filtered. The filtrate was concentrated to dryness and 0.1 M of triethylammonium acetate (TEAA) was added. The product was crystallized from water and methanol to give yellow crystals (0.18 g, 85% yield). ¹H-NMR (399.78 MHz, DMSO-*d*₆) δ 11.89 (s, 1H, NH), 8.05 (s, 1H, H₈), 6.75 (br, 2H, NH₂), 6.04 (dd, 1H, J₁=6.33 Hz, J₂= 7.29 Hz H_{1'}), 5.23 (d, 1H, J=3.30 Hz, 3'-OH), 4.95 (t, 1H, J=4.94 Hz, 5'-OH), 4.27 (m, 1H, H_{3'}), 3.75 (m, 1H, H_{4'}), 3.46 (m, 2H, H_{5',5''}), 2.45 & 2.34 (m & m, 1H & 1H, H_{2'} & H_{2''}). ES-MS: m/z (positive mode) 306.063 (calcd for C₁₀H₁₃N₅O₃S (M+Na) 306.062).

Synthesis of *(tG)_n* oligomers: (**G**)_n (1 μ mol) was synthesized on a DNA synthesizer (EXPEDITE™ Nucleic Acid System) employing bases, ultra-mild reagents and following standard phosphoramidite procedures. The column then was removed from the machine and dried under vacuum for 24 h, after which it was used to synthesise (**tG**)_n-oligomer using Jones procedure. (**G**)_n (1 μ mol) column in dry pyridine (10 ml) was added to trifluoroacetic anhydride (1 ml). Sodium sulphide (1g) in anhydrous dimethylformamide (30 ml) was then added to give crude (**tG**)_n. After 24 hours the column was washed several times with water and ethanol and dried under vacuum for 24 hours. After drying, anhydrous CH₃NH₂ was passed through the column in order to deprotected and cleave (**tG**)_n. Finally, the (**tG**)_n yellow powder was dissolved in water and purified by HPLC. In this study, two HPLC machines were used; Gilson 715 HPLC and Varian Pro Star. After HPLC purification the pure (**tG**)_n oligomers were obtained, table 1.

Oligomer	Yield / %	Calculated m/z	Found Peak M ⁺
(tG) ₂	23	627.0958	627.0949
(tG) ₃	33	972.1254	972.1223
(tG) ₄	29	1317.1552	1317.2863
(tG) ₅	26	1662.1848	1662.3939

Synthesis of *oligo-3*: d(T₆G₄A₆) (1 μ mol) was synthesized on a DNA synthesiser (EXPEDITE™ Nucleic Acid System) applying phosphoramidite bases and standard reagents following standard phosphoramidite procedures. The column was then removed from the device and dried under a high

stream of N_2 , before the guanosine bases were converted to thio-guanosine bases using the Jones procedure. $d(T_6G_4A_6)$ ($1\ \mu\text{mol}$) CPG beads from inside the column were added to dry pyridine (10 ml). Trifluoroacetic anhydride (1 ml) was added dropwise and the resulting orange solution stirred for 40 min. A suspension of sodium sulphide (1 g) (either sodium sulfide or sodium hydrosulfide was used in this study, both producing the desired thio-guanosine base) in anhydrous DMF (30 ml) was added in portions. The resulting cloudy dark blue solution was stirred for 24 hours. The clear orange solution was filtered to obtain the CPG beads which were then washed several times with water and ethanol. After drying with N_2 , anhydrous CH_3NH_2 was passed through the column to deprotect and cleave the oligomer $d(T_6\text{thioG}_4\ A_6)$ from the CPG bead. The oligomer was dissolved in water and purified by HPLC. The purified sample was desalted using an illustra NAP-25 column following manufacturer's protocol.

Characterization of the monomer **tG**

The monomer 2-deoxy-6-thioguanosine (**tG**) was synthesised using the protocol in materials and methods. The conversion of guanosine to thio guanosine was characterised by ^1H NMR, ^{13}C NMR, IR, UV-Vis and mass spectrometry as shown in Figures S2-S5.

Figure S2. i) The comparison between the ^1H NMR shifts of the N(1), H(8) and NH₂ protons of **G** and **tG**. ii) The ^{13}C NMR spectra and the DEPT spectra for **tG**.

Figure S3. The comparison of the IR spectra (i) and UV spectrum (ii) for **G** and **tG**.

Figure S4. High resolution mass spectrum (positive mode) of **tG** (bottom) compared to the modelled spectrum (top).

Characterization of (tG)_n oligomers (2-5mers)

Synthesis of short oligomers (tG)_n (where n=2,3,4,5) was performed as written in the materials and methods. The yield after phosphoramidite synthesis, thiolation conversion and purification ranges from 25-35 %. The conversion was characterised by UV-Vis, HPLC and mass spectrometry in Figures S5-S8.

Figure S5. HPLC chromatogram of crude reaction mix from the synthesis of (tG)₂, peak at 8 min, exhibiting the truncated monomer (tG), peak at 4.5 min.

Figure S6. i) UV-Vis comparison of (G)₂ (red line) and (tG)₂ (black line). ii) HPLC spectrum of purified (tG)₂.

Figure S7. High resolution mass spectrum of $(tG)_2$ (bottom) compared to the modelled spectrum (top).

Figure S8. HPLC chromatograms of purified samples of a) $(tG)_3$, b) $(tG)_4$ and c) $(tG)_5$.

Characterization of standard base oligos (A_5 , T_5 and C_5)

Since most oligonucleotides are not homobasic but contain a mixture of all four nucleobases, the stability of adenine, thymine and cytosine bases to the thiolation reaction was investigated to ensure that the conditions for the conversion of **G** to **tG** leaves the other bases unmodified. The same thiolation reaction was attempted on penta-adenine, (A_5), penta-thymine (T_5), and penta-cytosine, (C_5) oligomers whilst still bound to their CPG supports. The cleaved products were characterised by UV-Vis, MALDI-TOF MS, IR, HPLC coinjection and DNA digestion. No changes in any spectroscopic data were observed when compared to the non-thiolated oligomers. This confirmed the selectivity of the modified Jones synthetic procedure for the thiolation of guanine over the three other standard nucleobases (Figure S9-S12).

Figure 9. i) UV-Vis spectrum comparing A_5 and tA_5 . ii) A_5 MALDI-TOF: m/z (positive mode) 1503.67 (theoretical value for $C_{50}H_{57}N_{25}O_{23}P_4$ (M^+) 1503.07). tA_5 MALDI-TOF: m/z (positive mode) 1503.93 (theoretical value for $C_{50}H_{57}N_{25}O_{23}P_4S_5$ (M^+) 1660.36). iii) HPLC chromatogram for the coinjection of A_5 and tA_5 . iv) IR spectra comparing A_5 and tA_5 .

Figure S10. i) UV comparison between T_5 and tT_5 . ii) T_5 MALDI-TOF: m/z (positive mode) 1458.78 (theoretical value for $C_{50}H_{62}N_{10}O_{33}P_4$ (M^+) 1458.86). tT_5 MALDI-TOF: m/z (positive mode) 1459.57 (theoretical value for $C_{50}H_{62}N_{10}O_{33}P_4S_5$ (M^+) 1660.36). iii) HPLC spectra of a coinjection of T_5 and tT_5 . iv) Comparison of the IR spectra for T_5 and tT_5 . v) The MALDI-TOF spectra for 5tT extended to 1700 cm^{-1} highlighting the lack of peaks after 1600 cm^{-1} .

Figure S11. i) UV comparison between C_5 and tC_5 . ii) C_5 MALDI-TOF: m/z (positive mode) 1383.84 (theoretical value for $C_{45}H_{57}N_{15}O_{28}P_4$ (M^+) 1383.85). tC_5 MALDI-TOF: m/z (positive mode) 1383.85 (theoretical value for $C_{45}H_{57}N_{15}O_{28}P_4S_5$ (M^+) 1464.2). iii) HPLC spectra for the coinjection of C_5 and tC_5 . iv) Comparison of the IR spectra C_5 and tC_5 .

Figure S12: DNA digestion products analysed by HPLC.

Characterization of **oligo-3**

The synthesis of **oligo-3** was performed as in materials and methods. The oligomer was characterised by HPLC, IR, UV-Vis and DNA digestions, figures S13-S15. DNA digestion of the oligo into the mononucleoside constituents was performed as stated in the materials and methods. Analysis of the DNA digested product was performed by reverse phase HPLC which indicates that there is no **G** in **oligo-3** but only **tG** and that both the A and T components remain unmodified as previously demonstrated. Standards of the deoxynucleotides (Figure S14) were used to determine the HPLC elution times and can be compared to the digestion products.

Figure S13. i) UV comparison of **oligo-1** and **oligo-3** ii) The HPLC trace for the coinjection of **oligo-1** and **oligo-3**. iii) The comparison of the IR spectra for **oligo-1** and **oligo-3**.

Figure S14. HPLC trace of the standard deoxynucleotides for dA, dT, dG, dC and dtG.

Figure S15. HPLC traces of digestion products from i) **duplex-1** and ii) **duplex-3**.

Binding Titrations and Modelling of Hairpin Structures

A binding titration was performed as described in materials and methods on **duplex-3** and **duplex-4** with Cd^{2+} , Figure S16-17. It is conceivable that **oligo-3** could form an intra-strand hairpin structure due to the terminal self-complementary A : T sequences. In this structure the four **tG** bases would lie within a loop section which could coordinate a single Cd^{2+} , thus giving a 1 : 4 Cd^{2+} / nucleobase ratio, Scheme S1.

Scheme S1. Scheme illustrating the potential metal complexes of **duplex-3** and **duplex-4**.

The concentration dependence of **duplex-3** in the absence and presence of cadmium ions was investigated, Figure S16. In both the case of the hairpin and duplex conformations, the thermodynamics rely upon the AT complementary section; 12 bases for the duplex and 6 bases for the hairpin. For hairpin formation, the predicted T_m is 12 °C whereas duplex formation the predicted T_m is 24 °C. As both systems are relatively unstable, the T_m data relies upon the observation of small changes in the overall system stability. There is a concentration dependent difference in the absence of Cd^{2+} ions, although an exact T_m value is unobtainable due to the instability of the system. By comparing the low concentration T_m s from both situations indicates that the Cd(II) dissociation constant is probably greater than 1 μM so that it is weakly associated at the low concentration. The

difference in T_m of **duplex-3** with Cd(II) is marked indicating that the duplex is stabilised by the Cd(II) and the 7.5 °C increased stability is most likely due to the stabilisation of a duplex (°C) than from the more unstable hairpin (°C).

Figure S16. The T_m plots for **duplex-3** at differing concentrations (orange) and **duplex-4** + Cd₂ (yellow).

The plausible hairpin structures were also modelled, see figure S17, in the absence and presence of cadmium ions, and the overall energies of each system were calculated and compared to the energies for the duplex derivatives, Figure S18. It is clear that the hairpin structure is not able to direct the cadmium ions into a coordinated role, and hence the instability and overall energy of the system remains high.

Figure S17. Model snapshots representing the highest populated clusters in the conformational ensembles of a) **hairpin-1**, b) **hairpin-2** and c) **hairpin-2** $\supset$ **Cd**. Cd²⁺ (green spheres) are not forming significant interactions with the sulphur atoms on the **tG**.

The lowest energy conformation obtained for **duplex-1** and **hairpin-1** are comparable, however after the conversion of **G** to **tG**, a noticeable difference between the energies is observed. **Hairpin-2** containing the **tG** base, drops to -927 kJ/mol indicating a substantial decrease in stability. The energy of **duplex-3** is also destabilised however only to -3315 kJ/mol which is then stabilised to -4135 kJ/mol upon cadmium binding. **Hairpin-2** is also stabilised in the presence of cadmium, however the energy calculated is -2460 kJ/mol which indicates that this structure is less stable compared to the destabilised **duplex-3**, even without cadmium ions. This suggests that although the natural hairpin containing **G** has a comparable energy to the fully complementary **duplex-1**, the hairpin conformation containing **tG** is profoundly destabilised with slight stabilisation upon cadmium addition however not as efficiently re-stabilised as when in duplex configuration.

Figure S18. A) a table displaying the sequences for potential hairpin conformations and duplex structures and the calculated energy of the systems. B) a graphical representation of the differences in energy of the oligomer conformations.

To test this possibility, **oligo-3** was annealed with **oligo-2** to form **duplex-4**, where the central section contains four **tG** bases on one single strand opposite four “complementary” C bases. The T_m measurement of **duplex-4** indicated an intermediate thermal stability between **duplex-1** and **duplex-3**, as expected. However the binding titration of **duplex-4** with Cd^{2+} , to yield **duplex-4 $\supset$ Cd₂**, indicated a 1 : 2, Cd^{2+} to **tG** binding ratio (Figure S20), which suggests that the Cd is associated between two adjacent bases in the linear duplex. In addition, there was no noticeable difference in the thermal stability between **duplex-4** and **duplex-4 $\supset$ Cd₂** (see Figure S23), supporting the proposal that the Cd^{2+} ions are critical in the stabilisation of the mis-matched **tG** : **tG** region in **duplex-3**.

Figure S19. Absorption titration spectrum of **duplex-3** with cadmium nitrate. Inset) Binding curve of the complex formed between **duplex-3** and Cd²⁺.

A standard fixed concentration binding titration was performed with **duplex-3**, Figure S19, which displays two isosbestic points at 357 nm and 325 nm, consistent with the presence of only two species in solution; free **tG**, or **tG** coordinated to Cd²⁺. The change in absorption intensity at 341 nm was plotted against the molar ratio of metal ions to thiobase, [Cd²⁺]/[**tG**], which also suggests a 1 : 4 metal to **tG** ratio supporting the result from the Job plot.

Figure S20. a) Absorption titration spectrum of **duplex-4** with Cd^{2+} . b) Binding curve of the absorbance at 345 nm against the molar ratio of Cd^{2+} to thiobase. c) Binding curve of the absorbance at 345 nm against the molar ratio of Cd^{2+} to thiobase, fitted with an exponential decay.

Binding studies of tG

The binding of Cd^{2+} to **tG** has not been documented, however it has been reported for mercaptopurine with Cd^{2+} .¹¹ The low temperature NMR comparison of mercaptopurine $\rightarrow$ Cd and **tG** $\rightarrow$ Cd indicated the same downfield shift of the NH(1) proton from 12.55 ppm to 14.19 ppm upon addition of Cd^{2+} , Figure S21. This observation allows us to conclude that in both cases the metal binding involves a bidentate chelation through N7 and the C6-thiol as seen in previous reports.^{12, 13}

Figure S21. ^1H NMR spectra (700 MHz, DMF- d_7) of a) mercaptopurine and b) **tG** with and without Cd^{2+} ions.

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Thione Thiol

The diagram illustrates the chemical equilibrium between two tautomeric forms of a nucleoside. On the left, the **Thione** form is shown, featuring a six-membered ring with two nitrogen atoms (one substituted with an R group) and a C=NH-S double bond. On the right, the **Thiol** form is shown, featuring a similar ring structure but with a C=N-S⁻ double bond. A double-headed equilibrium arrow connects the two structures.

Melting Temperature curves and CD analysis

DNA denaturation was performed as described in materials and methods. The first derivative was calculated from the change in absorbance with temperature against temperature.

Figure S23. Melting curve profiles for **duplex-1**, **-2** and **-4** with and without Cd^{2+} .

CD spectra were obtained as described in materials and methods.

Figure S24. CD spectra of **duplex-1**, **-2** and **-4** in the absence and presence of Cd^{2+} .

Figure S25. Snapshots representing the highest populated clusters in the conformational ensembles of **duplex-4** and **duplex-4**⊃**Cd₂**. Cd^{2+} (green spheres) forming interactions with the sulphur atoms on the **tG** however unlike in the case of duplex-3, the metal addition does not induce duplex stabilisation.