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DNA-based aptamer fails as a simultaneous cancer targeting agent and drug delivery vehicle for a phenanthroline-based platinum(II) complex

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Abstract

The sgc8c aptamer is a 41-base DNA oligonucleotide that binds to leukaemia cells with high affinity and specificity. In this work we examined the utility of this aptamer as both a delivery vehicle and an active targeting agent for an inert platinum complex [(1,10-phenathroline)(ethylenediamine)platinum(II)]$^{2+}$. The aptamer forms a stem-and-loop confirmation as determined by circular dichroism. This conformation is adopted in both water and phosphate buffered saline solutions. The metal complex binds to the aptamer through intercalation into the aptamer's double helical stem with a binding constant of approximately 4.3×10^4 M^{-1}. Binding of the metal complex to the aptamer had a significant effect on the aptamer's global conformation, and increased its melting temperature by 28 ^{o}C possibly through lengthening and stiffening of the aptamer stem. The effect of the aptamer on the metal complex's cytotoxicity and cellular uptake was determined using in vitro assays with the target leukaemia cell line CCRF-CEM and the off-target ovarian cancer cell lines A2780 and A2780cp70. The aptamer has little inherent cytotoxicity and when used to deliver the metal complex results in a significant decrease in the metal complex's cytotoxicity and uptake. The reason(s) for the poor uptake and activity may be due to the change in aptamer conformation which affects its ability to recognise leukaemia cells.

1. Introduction

Platinum-based chemotherapy agents represent one of the most important classes of drug used to combat a range of human cancers [1, 2]. Whilst effective, the six drugs currently on the market display poor selectivity for cancers compared with other rapidly dividing cells within the human body. As a result there has been enormous interest in the development of delivery vehicles for platinum drugs, which are able to either passively or actively target solid tumours, whilst sparing normal tissue from damage [3-5].

Aptamers are oligonucleotide-based recognition molecules which can be short single stranded DNA, RNA, or modified nucleic acid fragments (i.e. locked nucleic acid) [6]. These molecules are able to recognise proteins and peptides expressed on the surface of cells in a manner similar to antibodies. Aptamers with appropriate selectivity can be rapidly generated through an *in vitro* process known as Systematic Evolution of Ligand by EXponential enrichment (SELEX) and offer reduced immunogenicity, good tumour penetration, rapid uptake and clearance, as active targeting groups for drug delivery [7]. In particular, they have the potential to act as cancer targeting molecules as they are able to recognise and bind proteins that are over-expressed on the surface of cancerous cells [8]. Aptamers can be better targeting agents compared with essential small molecules, like folate or oestrogen, because of their high cell selectivity, are simpler to synthesise and produce than antibodies, have good stability in storage, and have little or no immunogenicity.

To date aptamers have been used successfully as active targeting agents on the outside of liposome- and micelle-like vehicles containing platinum drugs. Because the platinum drugs are sequestered within the delivery vehicle they do not interact or irreversibly bind to the guanosine and adenosine bases of the aptamer [9-12]. The need for both a separate delivery vehicle and a conjugated targeting aptamer is complicated to construct and control, with respect to particle size and consistent drug loading. We were therefore interested to see if it was possible to discard the delivery vehicle component of the system and instead use the aptamer as both the cancer targeting agent and the delivery vehicle simultaneously. An aptamer of particular potential for this application is the sgc8c aptamer [13]. This DNA-based aptamer consists of 41 bases and is able to recognise and bind T-cell acute lymphoblastic leukaemia cells with a binding constant of 0.78 nM [13].

Unfortunately, “normal” platinum drugs, like cisplatin and carboplatin, which form coordination bonds with DNA after aquation of their labile ligands, cannot be used in this manner. These drugs can form irreversible bonds with N7 atom of guanosine and adenosine bases in the aptamer, and therefore not go on to bind cellular DNA. Instead, platinum anticancer complexes which are coordinately saturated with non-labile amine groups are needed. In the last decade several families of platinum complexes that fit this description have been developed and which are able to reversibly bind to double helical DNA [14-16]. These platinum(II) complexes have displayed very high cytotoxicity, and are able to overcome drug-resistance in a variety of different cancer cell lines [15]. An example is a trinuclear platinum complex with bridging 1,6-diaminohexane ligands developed by Farrell and co-workers. With its 8^{+} charge it is highly water soluble and binds strongly, but reversibly, to double stranded DNA [17]. Another inert platinum complex which has

displayed potential is (5,6-dimethyl-1,10-phenanthroline)(1*S*,2*S*-diaminocyclohexane)platinum(II) (56MESS) which was developed by Aldrich-Wright [18-20]. The highly aromatic phenanthroline-based complex is able to intercalate into double stranded DNA, although its mechanism of action is not thought to be related to its DNA binding [21].

In this paper we report the first attempted use of an aptamer to act simultaneously as both a delivery vehicle and as an active cancer targeting agent. We examine the aptamer's tertiary structure by circular dichroism and ^{1}H NMR, the binding of the metal complex [(1,10-phenanthroline)(ethylenediamine)platinum(II)]$^{2+}$ (PHENEN) to the aptamer, and the effect of the aptamer on the on the metal complex's cytotoxicity and uptake in the on-target CCRF-CRM leukaemia cell line and the off-target A2780 and A2780/cp70 ovarian carcinoma cell lines.

2. Experimental

2.1 Materials

PHENEN was made as previously reported [20]. Potassium tetrachloroplatinate, ethylenediamine, 1,10-phenanthroline, monosodium phosphate, disodium phosphate, sodium chloride, dimethylsulfoxide, methanol, diethyl ether, ethanol and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich. The sgc8c aptamer was purchased from Alpha DNA, Canada. The sequence of the

sgc8c as reported by Shangguan et al is: 5'-ATC TAA CTG CTG CGC CGC CGG GAA ATT ACT GTA CGG TTA GA [13]. Prior to use the aptamer was annealed by dissolving it in water and heating to 90 ^{o}C in a water bath. It was then allowed to slowly cool to room temperature over a period of several hours, whilst still in the water bath. RMPI 1640 medium, phosphate buffered saline (PBS), trypsin 2.5%, fetal bovine serum, glutamine were purchased from Invitrogen. Glycine buffer chemicals were purchased from Fischer Scientific. The ovarian cell lines A2780 and cisplatin resistant derivative a2780/cp70 were obtained from Fox and Chase Cancer Centre, Philadelphia and the leukaemia cell line CCRF-CEM was obtained from the American Type Culture Collection. RPMI medium was modified by addition of 1% (5 mL) glutamine and 10% (50 mL) fetal bovine serum. All solutions were prepared using water obtained from a Millipore Direct-Q water purification system.

2.2 Preparation of Buffers

PBS solutions (10 mM and 100 mM) were produced using a standard method. A 100 mM stock solution of NaCl was prepared in water. This stock solution was then used for the preparation of both 10 mM and 100 mM phosphate solutions, using a mixture of monosodium phosphate and disodium phosphate. For the 10 mM NaCl phosphate buffer, monosodium phosphate (1.8 g) in NaCl stock solution (150 mL) was combined slowly with disodium phosphate (2.1 g) in NaCl stock solution (150 mL) while the pH of the solution was measured until a pH of 7 was attained. This method was employed in the same manner for the preparation of the 100 mM buffer solution. Glycine buffer was prepared by the addition of glycine (0.1 M) to NaCl (0.1 M) with the pH adjusted to 10.5 using NaOH (0.1 M).

2.3 Instrumental techniques

2.3.1 UV-Vis spectroscopy

UV-visible spectrometry was carried out on a Cary 50 Bio spectrophotometer between 200-700 nm, with the scan speed set to medium and using a 1 cm quartz cuvette. All samples were analysed at room temperature.

2.3.2 NMR spectroscopy

^{1}H NMR spectra of the free sgc8c aptamer, the aptamer bound with PHENEN and 2D nuclear overhauser effect spectroscopy (NOESY) of the free aptamer were obtained on either a 400 MHz or 600 MHz Bruker Avance spectrometer in D_2O at 27 ^{o}C with 128-256 scans.

2.3.3 CD spectrophotometry

All CD spectra were measured in a 2 mm quartz cuvette using a Chirascan CD spectrometer at 25 ^{o}C. An air background was run followed by a blank sample of water with 2 repeats prior to sample analysis. All samples were prepared to a concentration of 3.4 µM, confirmed by UV, in water, 10 mM or 100 mM PBS solutions. Spectra were recorded from 200-350 nm with 5 repeats, at a step value of 0.5 nm and a time of 3 seconds per step. The N_2 carrier gas flow for the sample was 2 mL min^{-1}. The binding constant of PHENEN to the sgc8c aptamer was determined using equation 1, where E is the measured ellipticity at each

titration point, E_0 is the ellipticity when no PHENEN has been added, E_∞ is the maximum ellipticity, [PHENEN] is the concentration of the metal complex at each titration point and k is the binding constant:

Equation 1

$$E/E_0 = 1 + (E_\infty/E_0 - 1)\frac{[\text{PHENEN}]k}{1+[\text{PHENEN}]k}$$

2.3.4 Thermal UV-Vis Spectrometry

Thermal UV spectra were obtained using a Cary spectrophotometer. Spectra were recorded for 4 samples simultaneously using a moveable cuvette holder, at a fixed wavelength of 260 nm over the temperature range 10-80 ^{o}C. Samples were measured in 1 cm quartz cuvettes, with the melting temperature (T_m) recorded for each sample. Four aptamer samples were prepared to a concentration of 3.4 µM in 10 mM PBS solution with 0, 1, 2 and 3 equivalents of PHENEN being added, respectively. Four heating and 4 cooling cycles were run and the average of these results obtained for each PHENEN titration point.

2.4 In Vitro Testing

2.4.1 Cytotoxicity

The cytotoxicity of PHENEN and the sgc8c aptamer were measured using growth inhibition MTT assays and is reported as their IC_{50} concentration in micromolar (µM). Cytotoxicity was determined in the leukaemia cell line CCRF-CEM, and the ovarian cell lines A2780 and A2780/cp70 and compared with cisplatin. In all assays PHENEN was dissolved in water and diluted to the desired concentration (100 µM) using modified RPMI 1640 medium. Cisplatin was dissolved in 3% DMSO/97% RPMI medium and again diluted to the desired

concentration (100 μM) using modified RPMI 1640 medium. For assays carried out in the ovarian cell lines a cell density of 1 x 10^3 per well was selected for both the A2780 and A2780/cp70 lines and a cell density of 2 x 10^5 per well for the CCRF-CEM line was used. The ovarian cell lines were grown into flat 96 well plates and incubated for 48 h. Following this, the medium was removed and replaced with either PHENEN or cisplatin solutions at concentrations between 0 to 100 μM. The plates were further incubated for 24 h before the medium and drug were removed, replaced by fresh medium and incubated for a further 48 h. Plates were then fed with fresh medium and MTT solution (50 μL), wrapped in tinfoil and incubated for 4 h. The medium and MTT were removed from the wells leaving the purple MTT-formazan crystals. These were dissolved by addition of DMSO (200 μL) and glycine buffer (25 μL). Plates were then read by their absorbance at 570 nm with the resulting dose-response curve displaying absorbance (y-axis) with respect to drug concentration in μM (x-axis). The same procedure was employed for measurement of drug cytotoxicity in the leukaemia cell lines but as they were non-adherent, circular bottomed 96 well plates were used, with these being centrifuged at 1,000 g for 5 min to pellet cells prior to removal of medium. Removal of medium was carried out using a fine gauge hypodermic needle (24 G) to prevent removal of the cell pellet. All incubations were undertaken in a 5% CO_2 humidified atmosphere at 37 ^oC.

2.4.2 Cell Uptake

Selectivity and uptake of PHENEN and PHENEN bound sgc8c aptamer were measured in on-target leukaemia CCRF-CEM and the off-target ovarian A2780 and A2780/cp70 cell lines. For drug uptake in the adherent ovarian cell lines a cell density of $1x10^6$ cells/well was used with incubation overnight at 37 ^oC in a 10% CO_2 humidified atmosphere. Following incubation, the medium was replaced with either fresh medium or medium containing either cisplatin, PHENEN or PHENEN bound sgc8c aptamer, at a concentration of 10 μM, before the cells were incubated for another 2 or 4 h. The medium/drug solutions were then removed and the ovarian cells washed with ice cold PBS and lysed by adding 215 μL of nitric acid to each well. For the non-adherent CCRF-CEM leukaemia cell line a density of 2×10^6 cells in 0.5 mL was added into microfuge tubes. To this, 0.5 mL of medium or medium containing either cisplatin, PHENEN or PHENEN bound sgc8c aptamer, all at a concentration of 20 μM, was added and the tubes incubated at 37 ^oC for 2 or 4 h with constant mixing. The tubes were then centrifuged at 300 g for 4 min to pellet the cells allowing the medium to be removed. Cells were washed with ice cold PBS (1 mL) and centrifuged again at 300 rpm for 4 min before the PBS was removed and the cells were digested in 215 μL nitric acid.

The digested leukaemia and ovarian cells were then collected in a microfuge tube and incubated overnight at 65 ^oC on a heating block. The digest (50 μL) was then diluted 100-fold using water and 0.1% Triton-X100 anionic surfactant. The platinum content of each sample was then determined using inductively coupled plasma mass spectrometry (ICP-MS) and the uptake recorded as picomoles of platinum per 10^6 cells for the mean ± standard error of the mean (SEM) of three wells.

3.0 Results and discussion

3.1 Aptamer structure

How aptamers recognise and bind to proteins and peptides expressed on the surface of cancer cells is not fully known but is thought to be a function of both their primary sequence and their subsequent three dimensional shapes. The sgc8 aptamer, from which sgc8c is derived is known to be taken into cells via receptor mediated endocytosis [22]. Computer modelling. using the online program OligoCalc [23] [24], has predicted that the sgc8c aptamer most likely forms a stem-and-loop type conformation from 5 possible structures (ΔG values ranging from 0.46 to -0.31 Kcal mol) as well as a possible complete random coil state. The proposed stem and loop conformation would maximise Watson-Crick base pairing and be the most stable; although this has never been experimentally determined (Figure 1 and supplementary information). Therefore, before the metal complex [(1,10-phenanthroline)(ethylenediamine)platinum(II)]$^{2+}$ (PHENEN) was added to the aptamer, the aptamer's tertiary structure was examined in solution using circular dichroism and Watergate ^{1}H NMR spectroscopy.

It is known that calf-thymus DNA adopts a typical B-type DNA conformation in solution [25], with a characteristic positive peak centred around 275 nm, a negative peak centred around 240 nm and zero ellipticity around 258 nm, as shown in Figure 2. In comparison, the sgc8c

DNA aptamer displays a similar CD spectrum with a positive peak around 275 nm, which is indicative of the DNA aptamer strand possessing 10.4 bases per turn and adopting a B-type DNA conformation [25]. The characteristic negative peak at 240 nm of the B-type DNA is shown to be present but at a lower intensity and slightly higher wavelength, centred around 250 nm. In place of the small negative peak arising at 228 nm for B-type DNA, a strong positive peak is observed for the sgc8c aptamer. This positive peak may be due to the increased amount of expected random coil (non-base-paired) and single stranded bases within the aptamer compared with calf-thymus DNA..

The three-dimensional structure of the aptamer and the conformation adopted are likely key to its molecular recognition properties and use as a drug delivery vehicle. Due to the variety of different environments present within the body (high/low salt concentration and pH) it was of interest to determine whether the aptamer changed structure under different conditions. Of particular importance is its structure in both high and low salt concentrations which represent the conditions found in blood serum (Cl^- = 100 mM) and inside cells (Cl^- < 20 mM). The CD spectrum of the sgc8c aptamer was therefore examined in both pure water and PBS (Figure 3). Comparison of the spectra shows only a minimal change in conformation in each solution, which is important as any change could affect the performance of the aptamer.

A number of protons are involved in Watson-Crick base pairing. When DNA is in the random coil state resonances for these protons are not observed due to exchange with the

deuterated solvent. When the protons are involved in Watson-Crick base pairing however, because they are locked in place, they are seen in Watergate ^{1}H NMR in the region downfield of 10 ppm [16]. For the free sgc8c aptamer, a number of weak imino resonances are observed between 10 and 14 ppm (supplementary information). These resonances are weak because no resonances would be expected for the DNA bases in the loop region of the aptamer and also because the aptamer may be in dynamic equilibrium between a random coil state and the stem-and-loop conformation, with the equilibrium lying towards the random coil state.

3.2 Aptamer binding by PHENEN

The metal complex PHENEN (Figure 4) and other 1,10-phenanthroline containing platinum complexes have been known to intercalate into double helical DNA since the 1970s [26-29]. The X-ray crystal structure of PHENEN intercalation was first reported by Lippard in 1976 [30], and a molecular model showing a 3D representation of a 1,10-phenanthroline-based platinum(II) complex intercalating in DNA is given in reference [18]. As a well-studied DNA intercalator, with modest cytotoxicity, PHENEN was therefore chosen as a simple model of a coordinately saturated amine platinum complex for this study. The next step was to therefore determine whether PHENEN is able to intercalate into the stem of the sgc8c aptamer and what effect intercalation had on the aptamer's structure.

Upon intercalation into DNA, the aromatic proton resonances of phenanthroline-based platinum complexes are known to shift significantly upfield in one dimensional ^{1}H NMR spectra [18, 27, 28]. The ^{1}H NMR of free PHENEN has four aromatic resonances between 8.0

and 9.0, ppm (Figure 5). The ^{1}H NMR spectrum of the free aptamer is much more complex with considerable overlap of all the aromatic and H1' proton resonances, which excludes individual assignment.

The addition of one equivalent of PHENEN to the sgc8c aptamer has two effects. The PHENEN H_d and H_b resonances shift upfield by at least 0.65 ppm. The H_a and H_c resonances may also shift, but they are indistinguishable from the aptamer resonances and so their movement cannot be determined. As the concentration of phenanthroline complexes in solution increases, they are known to self-aggregate which has the effect of shifting the aromatic resonances upfield; although the degree of the shift is relatively small (about 0.3 ppm for concentrations up to 30 mM) [31]. The large shift of the PHENEN H_d and H_b resonances by more than 0.6 ppm is therefore indicative of the metal complex intercalating into the stem of the sgc8c aptamer [29] [18, 28]. In previous work, intercalation of PHENEN into sequence specific oligonucleotides resulted in shifts of the metal complex proton resonances of between 0.5 and 0.9 ppm, consistent with our results with the sgc8c aptamer [28]. The second effect is an exchange broadening of the aptamer resonances. Binding of the PHENEN complex to the aptamer can be either slow, intermediate or fast on the NMR time scale. For either slow or fast binding kinetics only sharp resonances for the aptamer would be observed. The general broadening of the aptamer resonances upon PHENEN addition is indicative of intermediate exchange kinetics and further evidence of PHENEN binding to the aptamer [27].

The binding of PHENEN to the sgc8c aptamer was also analysed by CD spectrophotometry. Aliquots of PHENEN were added to the sgc8c aptamer in 10 mM NaCl in 0.5 mole equivalent increments up to a ratio of 10-to-1 and the CD spectrum recorded at each step (Figure 6).

The resulting spectra show significant changes in the sgc8c aptamer's conformation upon the addition of PHENEN. A decrease in the peak intensity of both positive peaks and the negative peak is observed. As more PHENEN is added, further changes in the shape of the aptamer's curve are observed. This includes a hypsochromic shift of the positive peak shown from 220 nm to a shorter wavelength; a total shift of 2.5 nm is observed after addition of ten PHENEN equivalents. In addition, the intense negative peak at 250 nm splits, forming two less intense negative peaks at 255 nm and 230 nm. The primary stages of this peak formation can be seen after addition of three equivalents of PHENEN.

The observed reduction in peak intensity is consistent with binding of the PHENEN to the DNA, however, the overall change in the aptamers curve observed with increasing concentration of PEHENEN is indicative of a change to the secondary structure of the aptamer such as unwinding and lengthening of its stem region to accommodate the intercalation of multiple PHENEN molecules. As the CD curve of the aptamer also continues to change after the addition of more than one equivalent of PHENEN, then this is consistent with the occupancy of more than one binding site on the DNA by the platinum complex. Given the length of the stem and the nearest neighbour exclusion principle [29], it would be expected that the sgc8c aptamer stem could accommodate up to four PHENEN molecules at

a time. The continued changes in the aptamer's CD spectrum at high PHENEN-to-aptamer ratios therefore suggests that the binding of the metal complex to the aptamer is in equilibrium with free PHENEN, with a continual shift to more drug being bound at high PHENEN concentrations.

The change in the CD spectrum with increasing PHENEN concentration was then used to approximate the association constant of the metal complex binding to the aptamer. A plot of the ellipticity at 250 nm against PHENEN concentration (supplementary information) gives a typical binding curve which increases until approximately 7.5 equivalents of PHENEN has been added. From this, a binding constant (K_b) of 4.3 (± 0.5) x 10^4 M^{-1} was calculated. Previous studies have shown that PHENEN binds to double helical DNA with a K_b of ~10^4-10^6 M^{-1} [26, 28] so the value obtained for sgc8c binding is consistent with what would be expected.

To further confirm binding of PHENEN to the sgc8c aptamer via an intercalation mode, DNA melting experiments were performed using UV spectrophotometry [29]. The free sgc8c aptamer has a very low temperature of melting (Tm = 18.5 ^oC) which is consistent with the Watergate NMR experiments which suggested that the aptamer is in equilibrium between a random coil and stem-and-loop conformation, with the equilibrium shifted toward the random coil state.

Addition of one equivalent of PHENEN increases the melting temperature to 29.5 ^oC. Addition of more PHENEN results in further increases in the melting temperature with a Tm

of 36.5 ^{o}C for two equivalents of PHENEN and a Tm of 46.5 ^{o}C after three equivalents of PHENEN.

Normal double stranded DNA, such as short oligonucleotides and calf thymus DNA (ct-DNA) have melting points (without drug/complex) of around 60-70 ^{o}C [32-34]. A drug or metal complex that does not intercalate into DNA raises the DNA melting temperature by less than 5 ^{o}C [35]. An intercalating platinum(II) complex in comparison has been shown to increase the melting temperature anywhere between 6 and 20 ^{o}C depending on the nature of the intercalating ligand and the DNA:metal complex ratio [32, 34-36].

The very large change in Tm is consistent with an intercalation mode of binding although the magnitude of the increase in Tm was unexpected (2.5-fold higher). If PHENEN were binding to the sgc8c aptamer via simple electrostatic attraction or groove binding then only a modest increase in Tm would be expected (< 5 ^{o}C). The much larger increase in Tm for the aptamer could be a function of its structure compared with double stranded oligonucleotides and ct-DNA. For both the oligonucleotides and ct-DNA, the Watson-Crick base pairing is largely intact; in fact usually only the terminal and adjacent bases on the end of the strands are unpaired. The result is an already highly stable structure, so the addition of an intercalating ligand can only have a relatively small effect. In contrast, because much of the sgc8c aptamer is most likely in a random coil state, the structure is quite unstable and therefore the addition of an intercalating ligand would be expected to have a significantly bigger effect on its structural stability. An effect that is consistent with the observed increase in Tm for sgc8C.

3.3 Aptamer effect on PHENEN cytotoxicity and uptake

The effect of the sgc8c aptamer on the cytotoxicity of PHENEN was examined using the on-target CCRF-CEM leukaemia cell line and the off-target ovarian carcinoma cell line A2780 and its cisplatin-resistant derivative A2780/cp70 (Table 1). PHENEN has moderate activity in the CCRF-CEM and A2780/cp70 cell lines but is not able to overcome cisplatin resistance.

The sgc8C aptamer alone is not cytotoxic to CCRF-CEM cells and is poorly active in A2780/cp70, but appears to have some inherent cytotoxicity in the A2780 line. It is not clear why the aptamer has inherent cytotoxicity towards the non-target cells. It is possible that for these cells that it binds to different peptide/proteins compared with the CCRF-CEM target cells, and the binding of these peptides/proteins affects cell growth. Some aptamers have been shown to bind to specific cell receptors and in one case thioaptamers were developed that bind the primary receptor for hyaluronic acid [37]. As this receptor plays an important function in tumour growth, it is possible that sgc8c may also bind similar receptors on the ovarian cancer cells, which may be the cause of their inherent cytotoxicity.

The binding of PHENEN to the sgc8c aptamer decreases the activity of the platinum complex significantly ($P < 0.01$). Of importance, the sgc8c aptamer has little effect on the cytotoxicity of PHENEN in the off-target ovarian cell lines ($P = 0.076$), which is not unexpected, but also decreased the cytotoxicity of PHENEN in the on-target cell line.

The loss of PHENEN activity when bound to the aptamer could arise for three different reasons: (1) the conformational changes made to the aptamer upon PHENEN binding means the aptamer is no longer able to recognise and bind leukaemia cells, (2) the PHENEN bound sgc8c aptamer recognises and binds leukaemia cells but they are not internalised by the cell and/or (3) the PHENEN bound sgc8c aptamer recognises, binds and is internalised by the cells, but the PHENEN is not released inside the cell and therefore can't act to induce apoptosis.

To determine which of the three possible reasons is responsible for the loss of cytotoxicity, cellular uptake experiments were conducted using the same three cell lines using the sgc8c aptamer and another aptamer with the reverse sequence of sgc8c. This second aptamer was examined as it should display no selectivity for the CCRF-CEM cells and therefore the uptake results for this aptamer can be used to help differentiate between a lack of cell recognition and surface trapped PHENEN bound aptamer complex.

The cellular uptake result of PHENEN with the sgc8c aptamer demonstrates that in most cases the addition of the aptamer results in a decrease in the uptake of the metal complex. The only exception is at the 2 hour mark where the aptamer has no discernible ffect on PHENEN uptake in the A2780/cp70 line. Importantly, the decrease in cellular uptake increases over time such that after 4 hours the uptake of PHENEN in the on-target CCRF-CEM line has dropped 19.5% and has dropped in the off-target A2780 and A2780/cp70 lines 21.1 and 10.8%, respectively.

When PHENEN is added to an aptamer with the reverse sequence of sgc8c (sequence given in supplementary information) a similar effect on the metal complex's cytotoxicity and

uptake was observed. The reverse sequence aptamer decreases the cytotoxicity of PHENEN by 2.6-fold and the metal complex's cellular uptake by 56%.

4. Conclusions

In this work we examined the utility of the T-cell leukaemia targeting DNA sgc8c aptamer as a simultaneous delivery vehicle and active targeting agent for the delivery of an amine-coordinately saturated platinum anticancer complex. The sgc8c form a stem-and-loop type conformation although the equilibrium appears to be shifted more towards the random coil state. The metal complex PHENEN binds the sgc8c aptamer via an intercalative mode, which affects the sgc8c's conformation.

The sgc8c aptamer and the reverse sequence aptamer in vitro results taken together suggest that the decrease in PHENEN cytotoxicity in the on-target CCRF-CEM cell line is due to an inability of the sgc8c aptamer to recognise the leukaemia cell lines. If the PHENEN-aptamer complex was still able to recognise and bind to the cells but remained trapped on the surface of the cells, then it would be expected that the determined cellular platinum content would be higher compared with the free PHENEN uptake. Instead, the results show that the platinum content of the cells decreases, as it does for the reverse aptamer which cannot recognise CCRF-CEM cells.

If this interpretation of the results is correct and the PHENEN bound sgc8C aptamer has lost its ability to recognise leukaemia cells, then it is important to determine why this is the case. From the structural analysis of the sgc8c aptamer conformation the results indicated that it formed and stem-and-loop structure, but this structure was dynamic with an equilibrium

shifted more towards the random coil state. Binding of PHENEN to the aptamer occurs via intercalation which is a process known to lengthen and stiffen helical DNA and prevent strand separation. From these results we therefore hypothesise that the sgc8c aptamer recognises and binds leukaemia cells not in its preferred stem-and-loop conformation, but either binds from a random coil state and adopts a defined conformation upon binding the cell, or it exists in the stem-and-loop conformation but must unwind to then bind the leukaemia cells. When PHENEN is bound to the aptamer it stops either of these processes from occurring leading to reduced cytotoxicity and uptake of the metal complex now that it is sequestered within the aptamer.

If this hypothesise is correct, then the results of this study can be used to design drug-aptamer complexes that can still transport drugs whilst simultaneously actively targeting cancer cells. To do this a drug-aptamer complex needs to be developed where the binding of the drug to the aptamer neither locks, nor changes, the aptamers conformation, but is still bound with sufficient strength so as not to disassociate upon injection into the blood stream. Such a drug-aptamer complex may be possible when minor groove DNA binding drugs are used. DNA minor groove binding drugs are known to have good binding strengths to double helical DNA (up to 10^5 M^{-1}), but do not significantly change or lock the DNAs conformation in the same way that intercalating drugs do.

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5. Abbreviations

56MESS	(5,6-dimethyl-1,10-phenanthroline)(1*S*,2*S*-diaminocyclohexane)platinum(II)
CD	circular dichroism
ct-DNA	calf-thymus DNA
ICP-MS	inductively coupled plasma mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NOESY	nuclear overhauser effect spectroscopy
PBS	phosphate buffered saline
PHENEN	(1,10-phenanthroline)(ethylenediamine)platinum(II) chloride
SEM	standard error of the mean

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Table 1. The in vitro cytotoxicity using growth inhibition assays of the sgc8c, PHENEN and PHENEN bound sgc8c aptamer complex after 24 hours of drug exposure, expressed as their inhibition concentration 50% (IC_{50}) values in the on-targeted CCRF-CEM leukaemia cell line and the off-target ovarian carcinoma cell lines A2780 and A2780/cp70.

Compound	IC_{50} (μM)		
	CCRF-CEM	A2780	A2780/cp70
cisplatin	0.57 ± 0.03	0.43 ± 0.05	5.60 ± 0.39
sgc8c aptamer	> 1000	6.59 ± 1.29	104.1 ± 48.0
PHENEN	7.77 ± 0.37	0.89 ± 0.47	12.36 ± 1.93
sgc8c + PHENEN	10.91 ± 0.51	2.26 ± 0.33	12.85 ± 3.98

Table 2. The in vitro cellular uptake of PHENEN and PHENEN bound sgc8c aptamer into on-target leukaemia CCRF-CEM cells and off-target ovarian carcinoma A2780 and A2780/cp70 cells as determined by inductively coupled plasma mass spectrometry.

Compound (10 μM)	Incubation time (h)	Uptake (pmol Pt/10^6 cells)		
		CCRF-CEM	A2780	A2780/cp70
cisplatin	4	14.7 ± 0.5	48.7 ± 1.9	35.5 ± 2.4
PHENEN	2	27.5 ± 1.8	165.3 ± 6.2	163.5 ± 2.0
sgc8c + PHENEN	2	20.7 ± 1.2	126.2 ± 3.0	166.1 ± 24.0
PHENEN	4	49.9 ± 1.0	265.6 ± 8.4	252.2 ± 5.3
sgc8c + PHENEN	4	40.2 ± 0.8	209.1 ± 3.8	225.9 ± 11.8

Table 3. The in vitro cytotoxicity and cellular uptake of PHENEN and PHENEN with a reverse sequence sgc8c aptamer into on-target leukaemia CCRF-CEM cells and off-target ovarian carcinoma A2780 and A2780/cp70 cells.

Compound	**Incubation Time (h)**	**IC_{50} (μM)**		
		CCRF-CEM	**A2780**	**A2780/cp70**
Cisplatin	24	0.57 ± 0.03	0.43 ± 0.05	5.60 ± 0.39
Aptamer	24	> 200	14.7 ± 4.1	55.0 ± 3.5
PHENEN	24	7.77 ± 0.37	0.89 ± 0.47	12.36 ± 1.93
PHENEN-reverse aptamer	24	5.78 ± 0.56	13.6 ± 2.1	> 200
		Uptake (pmol/10^6 cells)		
PHENEN	2	55.6 ± 4.8	77.1 ± 4.8	16.0 ± 2.2
PHENEN-reverse aptamer	2	27.0 ± 2.6	36.6 ± 2.3	6.38 ± 0.23
PHENEN	4	135.2 ± 9.6	141.7 ± 7.2	23.0 ± 1.5
PHENEN-reverse aptamer	4	59.3 ± 3.3	76.3 ± 3.7	9.07 ± 0.53

Figure(s)

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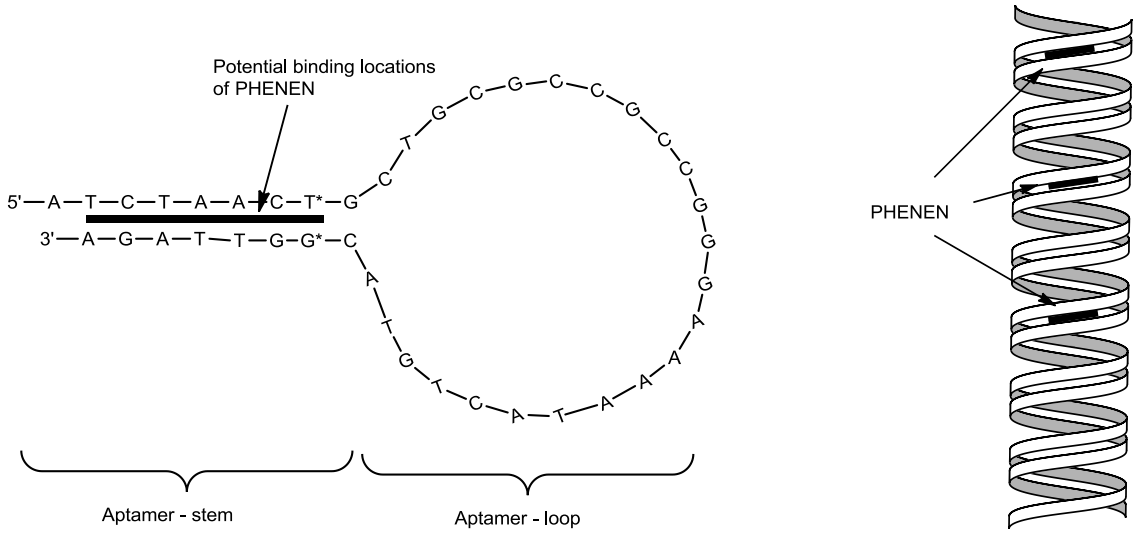

Figure 1. The predicted structure (left) of the T-cell leukaemia targeting sgc8c aptamer, determined from computer modelling and showing the potential binding area of PHENEN into the aptamer's double helical stem (the * indicates a base-pair mismatch) and a schematic diagram (right) showing the process of intercalation of PHENEN between base-pairs in double helical DNA.

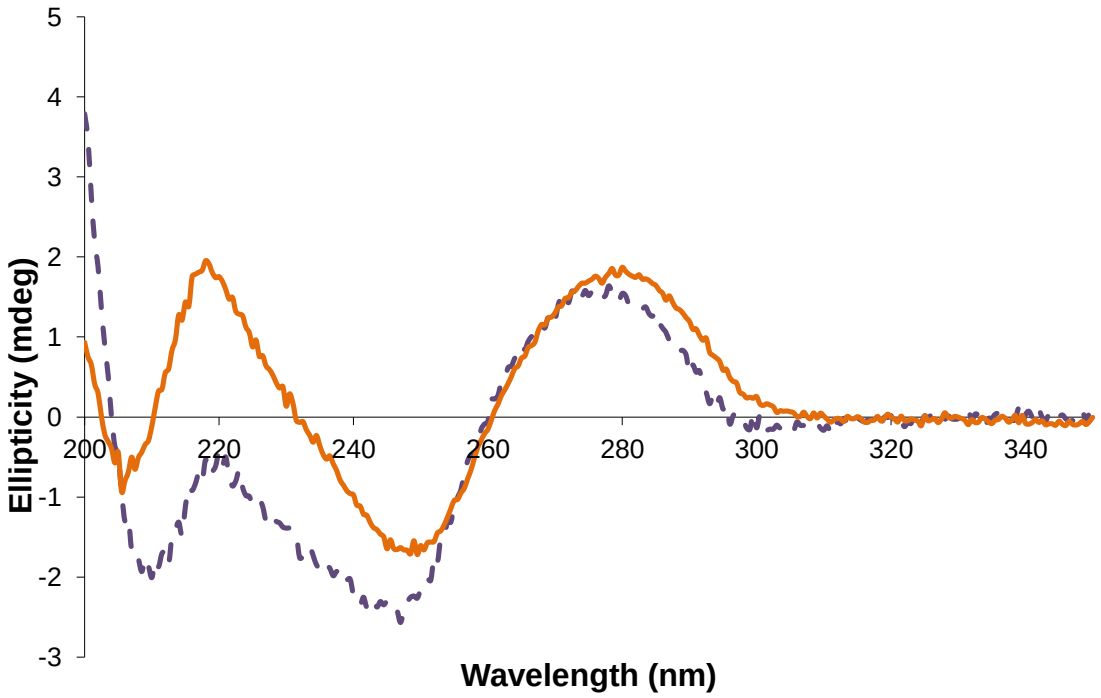

Figure 2. A comparison of the measured circular dichroism spectra of free sgc8c DNA aptamer in water (orange, solid line) and calf-thymus DNA (purple, dashed line). Calf-thymus DNA is known to form a typical B-type DNA conformation and the similarity of the aptamer's CD spectra is consistent with the formation of a B-type DNA conformation within a part of the aptamer.

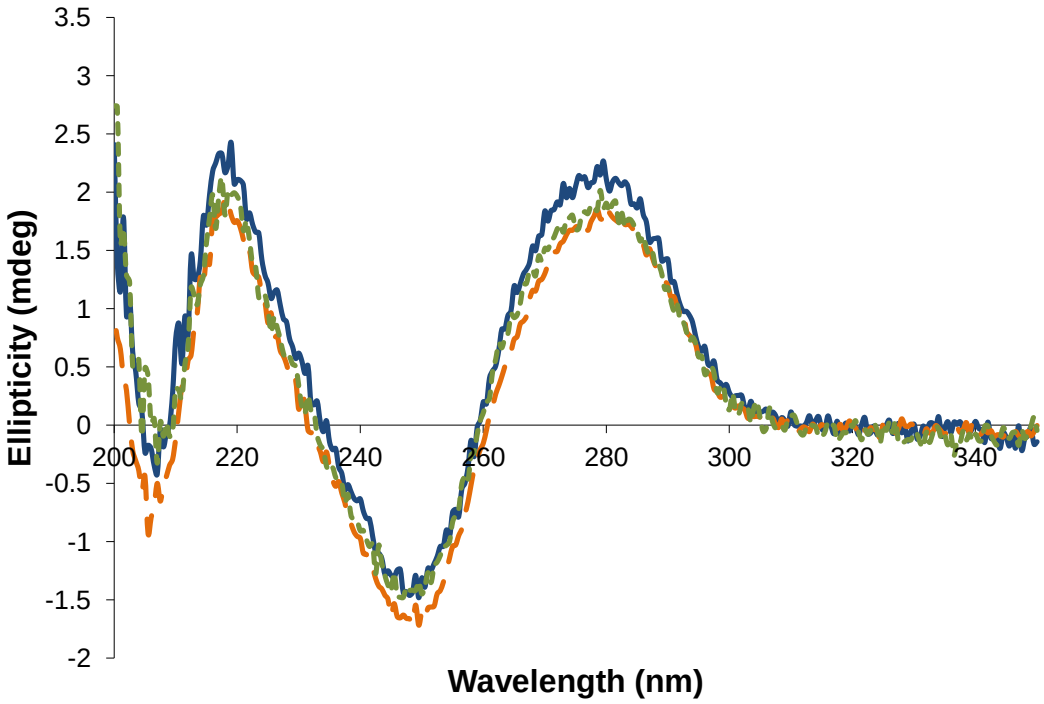

Figure 3. The CD spectra of free sgc8c aptamer in water (orange, dashed line), 10 mM (blue, solid line) and 100 mM (green, dotted line) phosphate buffered saline showing that the aptamer retains its structure in all three environments.

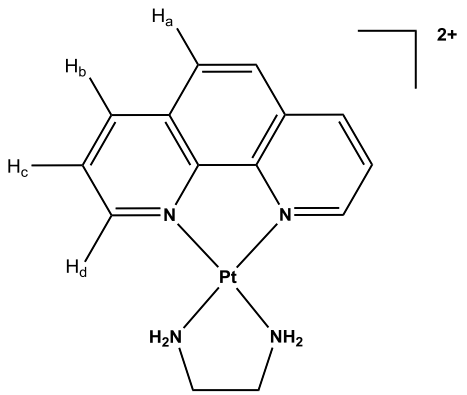

Figure 4. The chemical structure of [(1,10-phenanthroline)(ethylenediamine)platinum(II)]$^{2+}$ (PHENEN) showing the proton naming scheme used. Counter ions are omitted, but the general synthetic method produces a chloride salt of the complex.

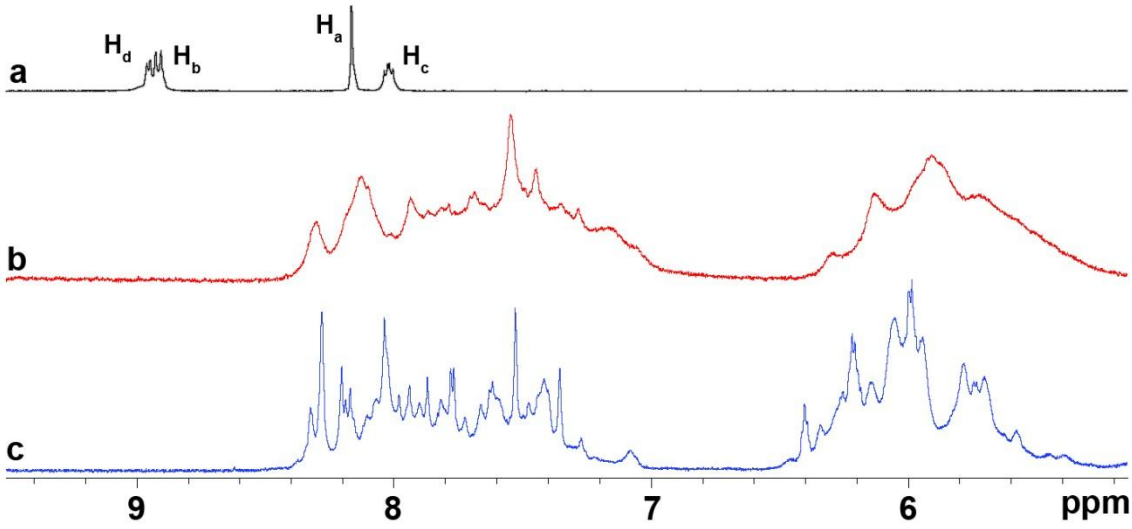

Figure 5. The ^{1}H NMR spectra (D_2O, 20 mM PBS, 400 MHz) of (a) PHENEN, (b) sgc8c aptamer with the addition of one equivalent of PHENEN and (c) the free sgc8c aptamer, showing the upfield shift of the PHENEN H_d and H_b proton resonances, consistent with DNA intercalation.

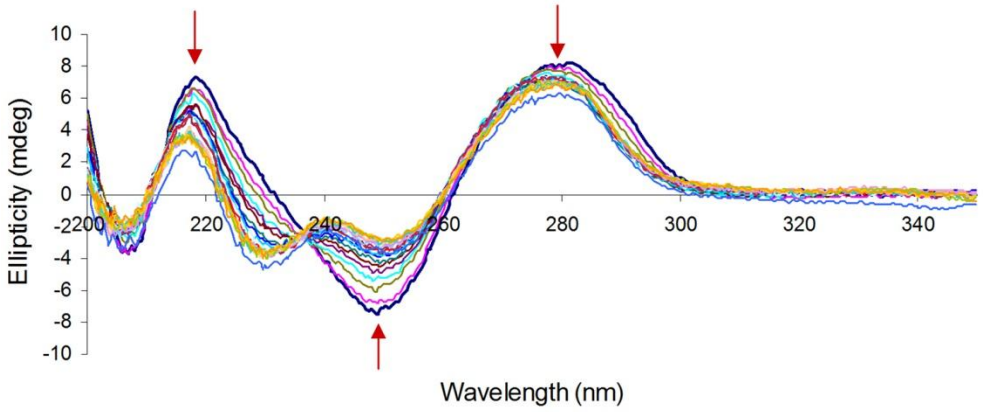

Figure 6. The circular dichroism spectra of the sgc8c aptamer in 20 mM PBS showing the free aptamer (dark blue) and its spectra upon the addition of PHENEN (0.5 to 10 mole equivalents). The arrows indicate the direction of change of the peaks.

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Supplementary information for

DNA-based aptamer fails as a simultaneous cancer targeting agent and drug delivery vehicle for a phenanthroline-based platinum(II) complex

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Keywords: platinum, phenanthroline, sgc8c, aptamer, DNA, drug delivery, active targeting, cancer.

Supplementary information

Reverse aptamer sequence: 5'- AGA TTG GCA TGT CAT TAA AGG GCC GCC GCG TCG TCA ATC TA-3'

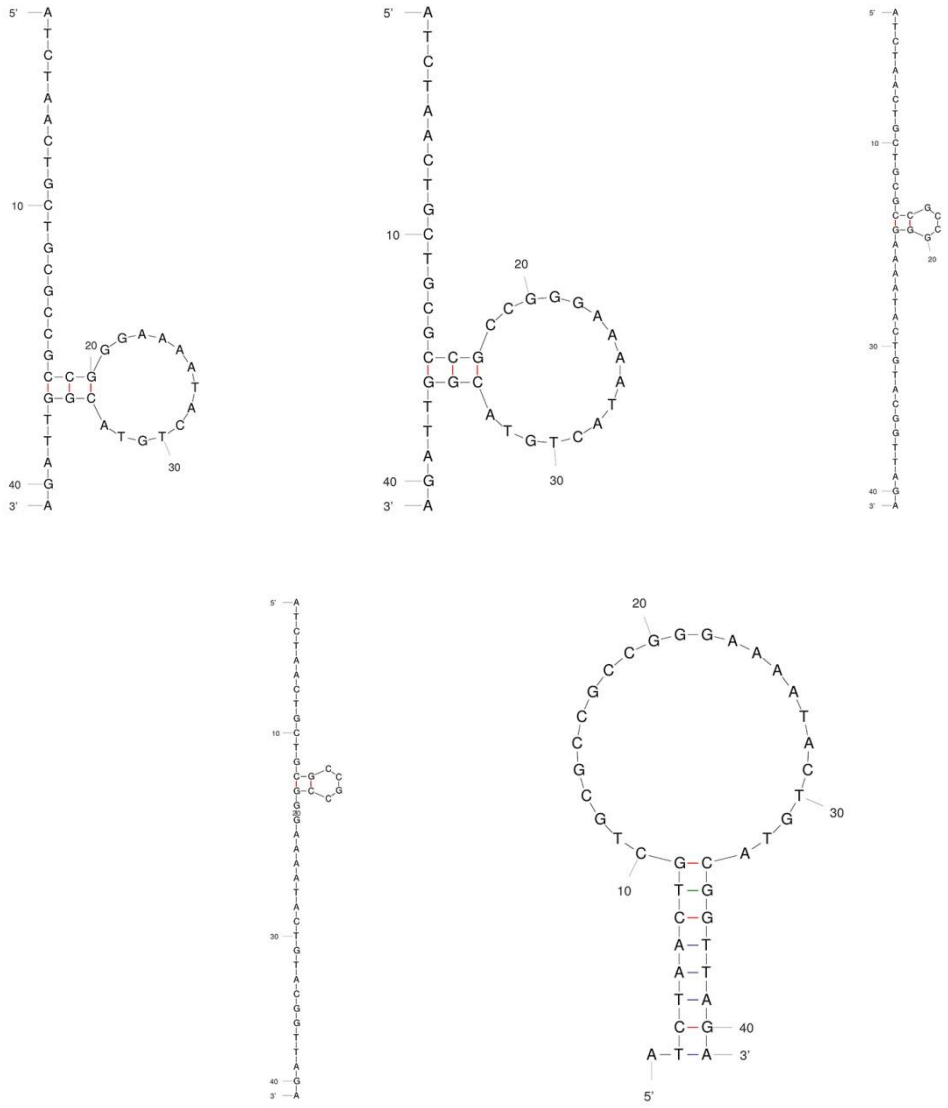

Figure S1. The predicted five possible conformations based on Watson-Crick pairing of the sgc8c DNA aptamer from computer modelling using OligoCalc [23]: available at www.basic.northwestern.edu/biotools/oligocalc.html. The ΔG values for each conformation going from left to right, top to bottom are: 0.46, 0.26, 0.20, -0.25 and -0.31.

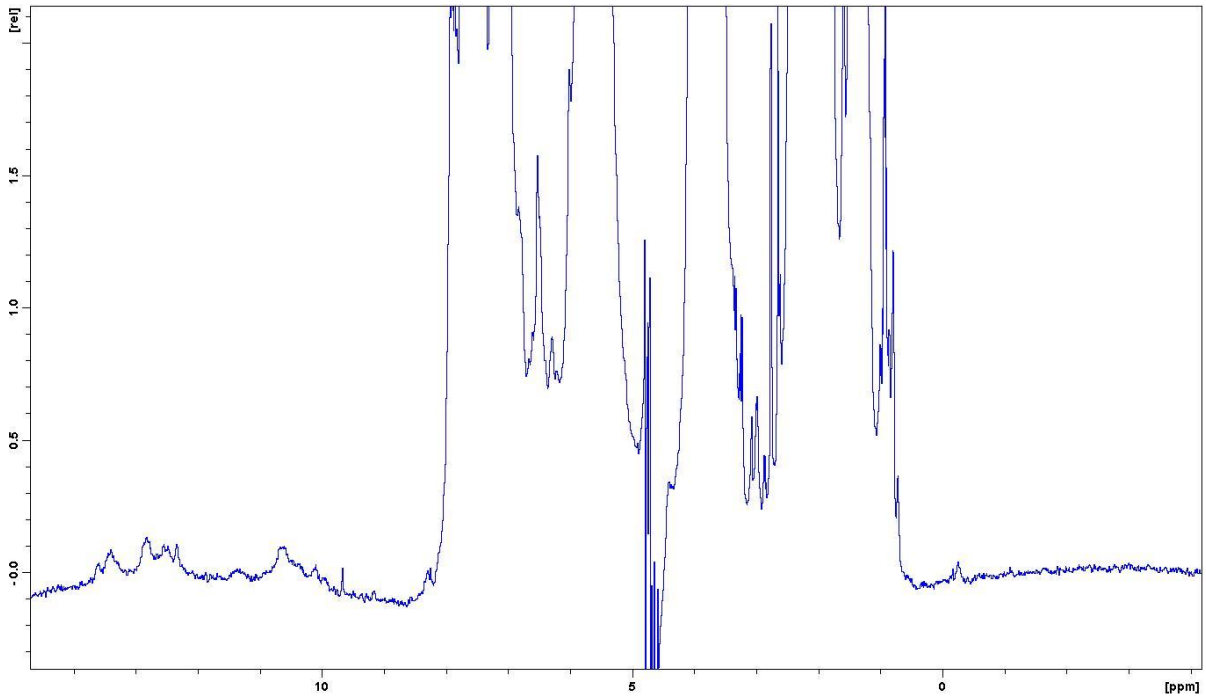

Figure S2. The ^{1}H Watergate NMR spectrum of the free sgc8c aptamer showing the weak imino protons which is indicative of Watson-Crick base pairing in the structure of the aptamer.

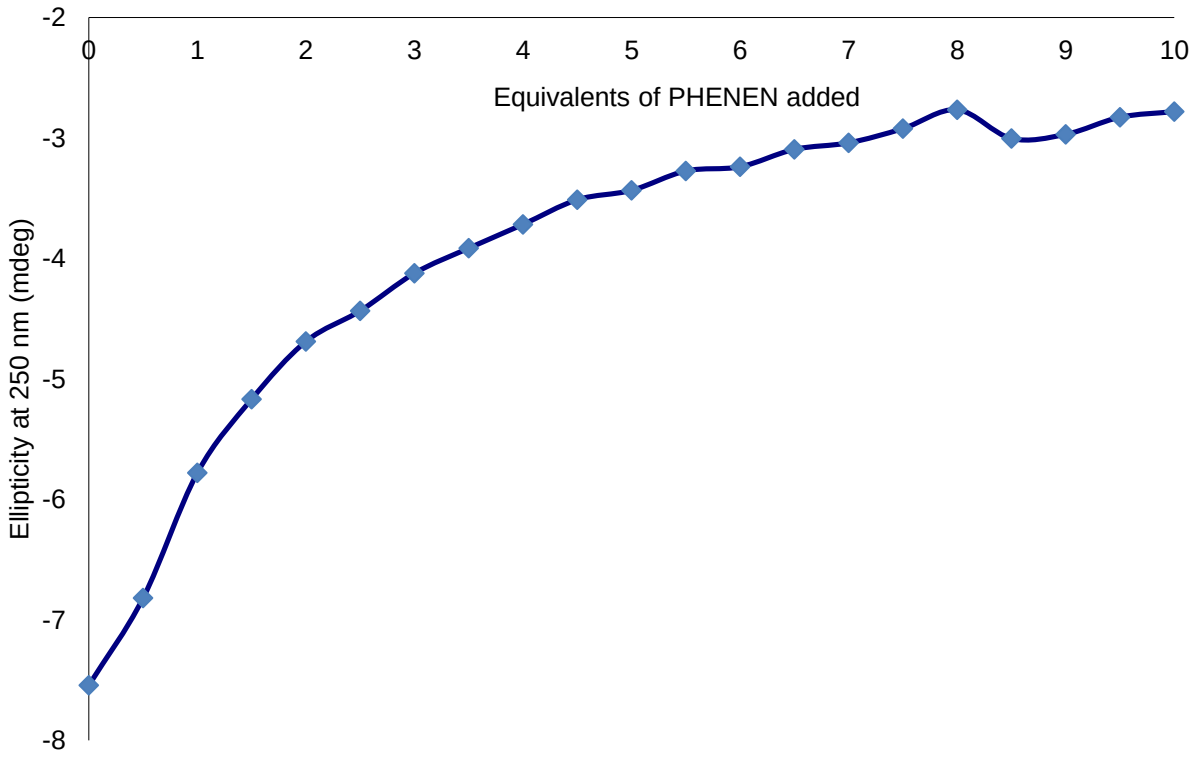

Figure S3. The binding curve of PHENEN to the sgc8c aptamer showing the change in ellipticity at 250 nm as determined by circular dichroism as 0 to 10 equivalents of the metal complex is added.