

## Electronic Supplementary Information

### **Metallohelices with Activity Against Cisplatin-Resistant Cancer Cells; does the mechanism involve DNA binding?**

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#### **Contents**

<b>Chemicals</b>	<b>2</b>
<b>AFM imaging</b>	<b>3</b>
<b>DNA binding studies using spectroscopic methods</b>	<b>4</b>
<i>CD Spectroscopy</i>	<b>5</b>
<i>LD Spectroscopy</i>	<b>6</b>
<b>DNase I footprinting</b>	<b>10</b>
<b>UV melting experiments, Gel electrophoresis of DNA 3WJs</b>	<b>11</b>
<b>MTT Assay – Determination of Anticancer Activity</b>	<b>12</b>
<b>H2AX &amp; Cell Cycle Analysis FACS Assays</b>	<b>14</b>
<b>References</b>	<b>16</b>

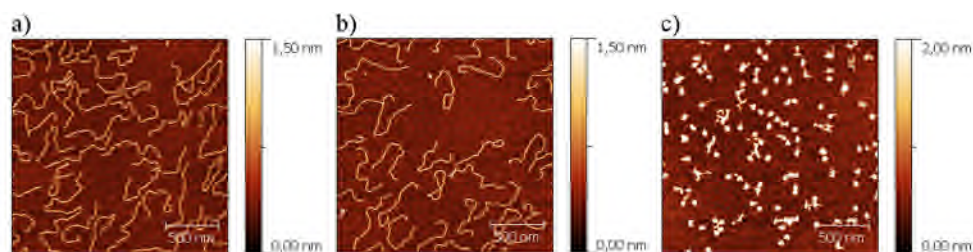
## Chemicals

The iron(II) flexicates  $[\text{Fe}_2\text{L}^n_3]\text{Cl}_4$  were synthesised as previously described.<sup>1</sup> Plasmid pSP73 (2464 bp) was isolated according to standard procedures. The synthetic oligodeoxyribonucleotides used in this work were purchased from VBC-genomics (Vienna, Austria). The purity of oligonucleotides was verified by fast protein liquid chromatography (FPLC) or gel electrophoresis. The Klenow fragment from DNA polymerase I (exonuclease minus, mutated to remove the 3'→5' proofreading domain) (KF<sup>-</sup>), T4 polynucleotide kinase, *NdeI* and *HindIII* restriction endonucleases were purchased from New England Biolabs (Beverly, MA). Deoxyribonucleoside 5'-triphosphates were from Roche Diagnostics, GmbH (Mannheim, Germany). Acrylamide, bis(acrylamide) and urea were from Merck KGaA (Darmstadt, Germany). Dimethyl sulfate (DMS),  $\text{MgCl}_2$  and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) was from Sigma (Prague, Czech Republic).  $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$  was from MP Biomedicals, LLC (Irvine, CA). ATP was from Boehringer (Mannheim, Germany). Wizard SV and PCR Clean-Up System used to extract and purify the 158-bp DNA fragment (*vide infra*) was purchased from Promega. Ethidium bromide (EtBr) was from Merck KGaA and deoxyribonuclease I (DNaseI) was from Roche (Mannheim, Germany).

### AFM imaging

The plasmid pSP73 (2464 bp) was linearized by digestion with *NdeI* restriction endonuclease and purified using the Promega Wizard SV Gel clean-up system. The flexicates were dissolved in Milli-Q water (Millipore System, Billerica, MA), mixed with DNA (20 ng) in 10  $\mu\text{L}$  at the required DNA base:flexicate molar ratio in the buffer containing 4 mM HEPES (pH 7.4), 5 mM KCl and 5 mM  $\text{MgCl}_2$  and incubated for 10 min at room temperature. A droplet (4  $\mu\text{L}$ ) of the sample was spotted directly onto freshly cleaved mica (SPI, West Chester, PA). After 2 min incubation, the mica was gently rinsed with 1 mL of MilliQ water and immediately blown dry with compressed air. Imaging was performed using a MultiMode 8 atomic force microscope (Bruker, Santa Barbara, CA). Scanning was carried out in the tapping mode using 125  $\mu\text{m}$  silicon cantilevers with resonance frequencies of  $\sim 300$  kHz (NT-MDT, Zelenograd, Moscow, Russia).

**Figure S1.** AFM images of nonmodified linear plasmid pSP73 (2464 bp) (a, b) and plasmid DNA mixed with  $\text{M-}[\text{Fe}_2(\text{I})_3]^{4+}$  helicate at 5:1 DNA base:helicate ratio (c).



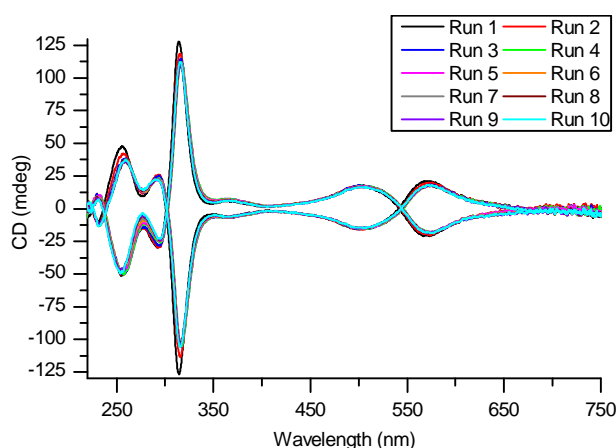
### DNA binding studies using spectroscopic methods

For the DNA binding spectroscopic studies, CD and LD spectra were recorded using a Jasco J815 spectrometer adapted for LD spectroscopy. The instrument was calibrated conventionally using 0.060% ACS for intensity and a holmium filter for wavelength, and also against our recently introduced Na[Co(EDDS)] system.<sup>2, 3</sup> CD measurements were collected in a 1 cm path-length quartz cuvette. The LD cell used is a large volume (1 mL) Couette flow cell built by Crystal Precision Optics based on the design by A. Rodger<sup>4</sup> and is now available from Dioptica Scientific Ltd. The parameters used were: bandwidth 1 nm, response time 1 sec, wavelength scan range 200 – 800 nm, data pitch 0.2 nm, scanning speed 200 nm/min and accumulation 1.

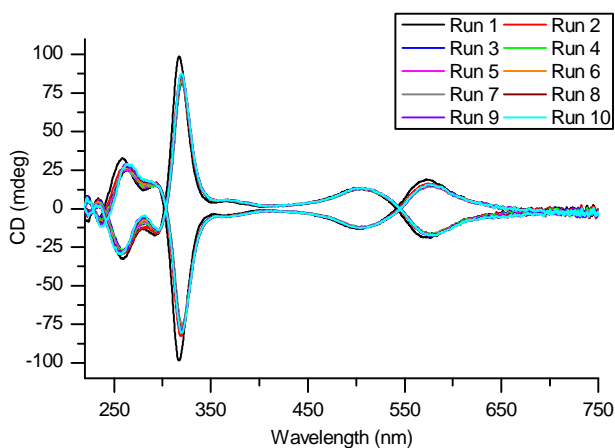
**Table S1.** Ratios of ct-DNA base:flexicate used in all titration experiments.

<b>Run</b>	<b>Concentration of ct-DNA (<math>\mu\text{M}</math>)</b>	<b>DNA Base / Flexicate Ratio</b>
1	0.00	0.00
2	16.19	1.08
3	30.91	2.06
4	44.35	2.96
5	56.67	3.78
6	68.00	4.53
7	78.46	5.23
8	88.15	5.88
9	97.14	6.48
10	105.52	7.03

### CD Spectroscopy



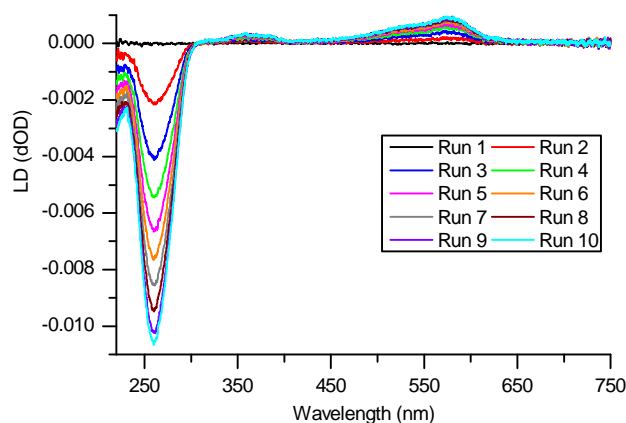
**Figure S2.** CD titration series for the  $\Delta_{\text{Fe}}/\Lambda_{\text{Fe}}\text{-[Fe}_2\text{L}^{2\text{a}}\text{]Cl}_4$  flexicates at constant flexicate concentrations (15  $\mu\text{M}$ ) and increasing ct-DNA concentrations. TRIZMA<sup>®</sup> base buffer (1 mM, pH 7.2). Path length 1.0 cm.



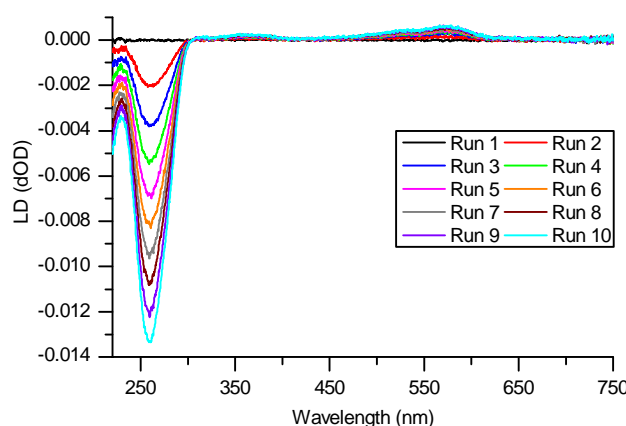
**Figure S3.** CD titration series for the  $\Delta_{\text{Fe}}/\Lambda_{\text{Fe}}\text{-[Fe}_2\text{L}^{2\text{b}}\text{]Cl}_4$  flexicates at constant flexicate concentrations (15  $\mu\text{M}$ ) and increasing ct-DNA concentrations. TRIZMA<sup>®</sup> base buffer (1 mM, pH 7.2). Path length 1.0 cm.

### LD Spectroscopy

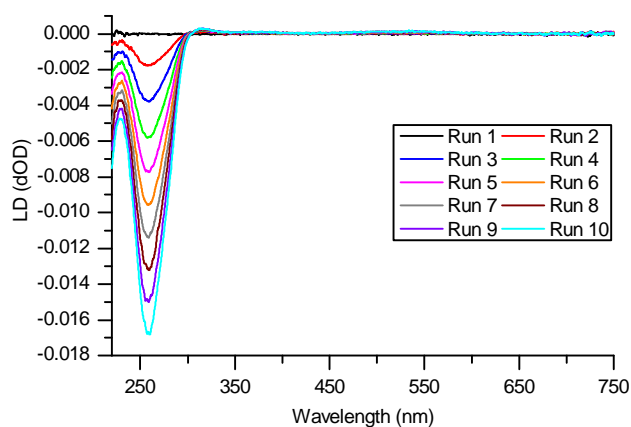
LD spectroscopy measures the difference in absorption of light polarised parallel and perpendicular to the orientation axis of the molecule [ $LD = A_{\parallel} - A_{\perp}$ ]. Laminar flow is used to orient molecules in one direction and this was achieved using a large volume (1 mL) Couette flow cell. In general, small molecules do not orient in flow and therefore exhibit no LD signal, whereas molecules which are very long and thin (*e.g.* natural DNA) orient very easily in flow and display strong LD signals. The helical axis orients along the flow axis, with the base pairs orienting approximately perpendicular to this axis.<sup>5</sup> The  $\pi$ - $\pi^*$  transitions in the DNA base pairs therefore result in a negative LD signal at 260 nm. Data for  $[\text{Fe}_2\text{L}^{\text{1a}}_3]^{4+}$  is presented elsewhere.<sup>1</sup>



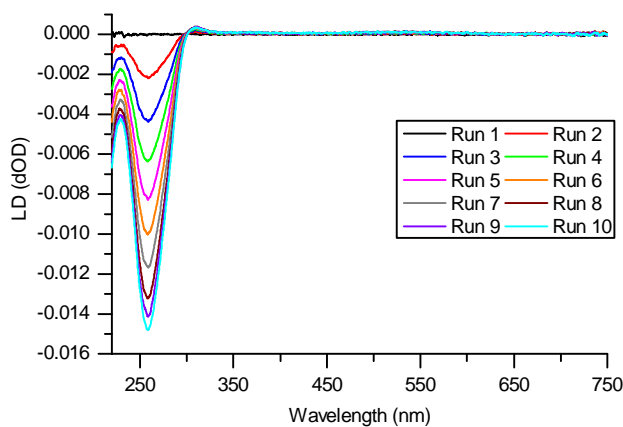
**Figure S4.** LD titration series for  $\Lambda_{\text{Fe,S-C}}\text{-}[\text{Fe}_2\text{L}^{\text{1a}}_3]\text{Cl}_4$  at constant flexicate concentration (15  $\mu\text{M}$ ) and increasing ct-DNA concentrations. TRIZMA<sup>®</sup> base buffer (1 mM, pH 7.2).



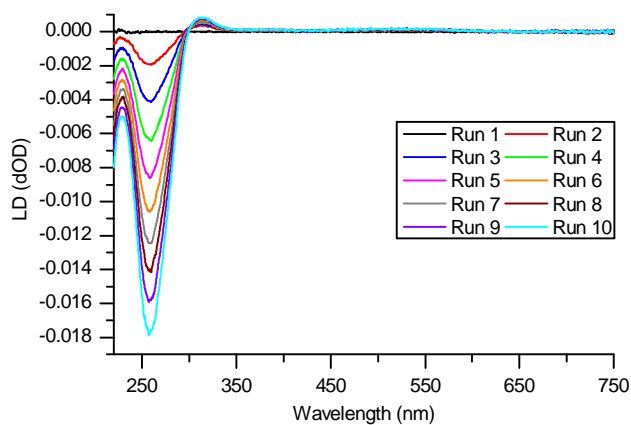
**Figure S5.** LD titration series for  $\Delta_{\text{Fe,R-C}}\text{-}[\text{Fe}_2\text{L}^{\text{1a}}_3]\text{Cl}_4$  at constant flexicate concentration (15  $\mu\text{M}$ ) and increasing ct-DNA concentrations. TRIZMA<sup>®</sup> base buffer (1 mM, pH 7.2).



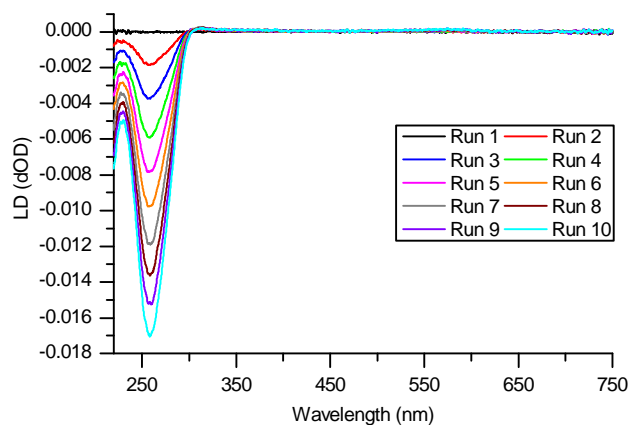
**Figure S6.** LD titration series for  $\Delta_{\text{Fe,S}_C}\text{-[Fe}_2\text{L}^{2a}_3\text{]Cl}_4$  at constant flexicate concentration (15  $\mu\text{M}$ ) and increasing ct-DNA concentrations. TRIZMA<sup>®</sup> base buffer (1 mM, pH 7.2).



**Figure S7.** LD titration series for  $\Lambda_{\text{Fe,R}_C}\text{-[Fe}_2\text{L}^{2a}_3\text{]Cl}_4$  at constant flexicate concentration (15  $\mu\text{M}$ ) and increasing ct-DNA concentrations. TRIZMA<sup>®</sup> base buffer (1 mM, pH 7.2).



**Figure S8.** LD titration series for  $\Delta_{\text{Fe},R\text{C}}\text{-}[\text{Fe}_2\text{L}^{2b}_3]\text{Cl}_4$  at constant flexicate concentration (15  $\mu\text{M}$ ) and increasing ct-DNA concentrations. TRIZMA<sup>®</sup> base buffer (1 mM, pH 7.2).



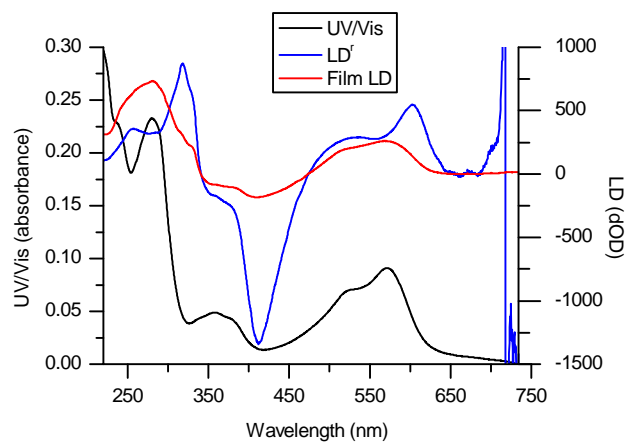
**Figure S9.** LD titration series for  $\Lambda_{\text{Fe},S\text{C}}\text{-}[\text{Fe}_2\text{L}^{2b}_3]\text{Cl}_4$  at constant flexicate concentration (15  $\mu\text{M}$ ) and increasing ct-DNA concentrations. TRIZMA<sup>®</sup> base buffer (1 mM, pH 7.2).

#### *Details of calculation of peaks intensity for the $[\text{Fe}_2\text{L}^{1a}_3]\text{Cl}_4$ flexicates*

As a result of absorption overlap we must subtract the spectrum of the complex flexicate in order to estimate the true reduction in the DNA peak intensity. Film LD spectra<sup>1</sup> reproduced in Fig. S10 were used to factor out orientation effects. The LD<sup>r</sup> spectrum of  $\Delta_{\text{Fe},R\text{C}}\text{-}[\text{Fe}_2\text{L}^{1a}_3]\text{Cl}_4$  (blue line) has three distinct horizontal flat regions in the spectrum which appear at the same wavelengths as the bands in the UV-Vis absorbance spectrum. The two flat regions, 255-290 nm and 515-580 nm, have positive LD<sup>r</sup> signals, and therefore correspond to transitions predominantly along the long (orientation) axis of the molecule. In contrast, the flat region 350-385 nm has a negative LD<sup>r</sup> suggesting this is predominantly a short axis polarisation transition. The relative intensities of the two transitions aligned predominantly along the long axis (ca 280 nm and ca 575 nm) are in a 2.8:1 ratio. Therefore, the positive LD signals around 280 nm when the complex binds to ct-DNA have estimated intensities of 0.0026 and 0.0017 dOD for the  $\Lambda_{\text{Fe}}$  and  $\Delta_{\text{Fe}}$  enantiomers respectively for Run 10 (i.e. height of peak at 575 nm multiplied by 2.8). Taking this into account, the difference between the LD peaks at 258.8 nm with and without the complex was calculated as a percentage.

$$[\text{LD}_{\text{DNA}} - \text{LD}_{\text{DNA+complex}} + \text{LD}_{\text{complex}}] / [\text{LD}_{\text{DNA}}]$$





**Figure S10.** UV-Vis absorbance, LD<sup>f</sup> and film LD spectra for  $\Delta_{Fe_c}R_C-[Fe_2L^1a_3]Cl_4$ .

### DNase I footprinting

Supercoiled pSP73 plasmid was digested with *Hind*III restriction endonuclease and 3'-end-labeled by treatment with Klenow *exo*- and [ $\alpha$ - $^{32}$ P]-deoxy-ATP. After radioactive labelling, the DNA first cleaved with *Hind*III was still digested with *Nde*I to yield a 158 and 2306 base pair fragments. The 158 bp fragment was purified by 1% agarose gel electrophoresis and isolated from the gel by Promega Wizard SV Gel clean-up system.

A 9  $\mu$ L solution containing  $1.11 \times$  TKMC buffer (10 mM Tris pH 7.9, 10 mM KCl, 10 mM MgCl<sub>2</sub>, and 5 mM CaCl<sub>2</sub>),  $4.5 \times 10^{-4}$  M DNA, and flexicate was incubated for 15 min at 25°C. Cleavage was initiated by the addition of 1  $\mu$ L of 50  $\mu$ g mL<sup>-1</sup> DNase I and allowed to react for 30 s at room temperature before quenching with 2.5  $\mu$ L of DNase stop solution (3 M NH<sub>4</sub>OAc and 0.25 M EDTA). This was then precipitated with ethanol, lyophilized and resuspended in a formamide loading buffer. DNA cleavage products were resolved by polyacrylamide (PAA) gel electrophoresis under denaturing conditions (8%/8 M urea PAA gel). The autoradiograms were visualized and quantified by using the bio-imaging analyzer. Assignment of the cleavage to a particular base has been made so that it corresponds to the cleavage of the phosphodiesteric bond on the 5' side of that base.

### UV melting experiments

Absorption spectra were measured with a Varian Cary 4000 UV–Vis spectrophotometer equipped with a thermoelectrically controlled cell holder and quartz cells with the pathlength 1 cm.

The stability of DNA in the presence of the flexicates was monitored by measuring the absorbance at 260 nm (1 nm bandwidth, average time: 10 s, heating rate 0.4°C/min) as a function of temperature. The experiment was run simultaneously on six masked 1 cm pathlength cuvettes of 1.2 mL volume using a Peltier controlled 6-sample cell-changer in a Varian Cary 4000 UV-Vis spectrophotometer.  $T_m$  was calculated within the thermal heating program by applying a first derivative calculation.

The DNA three-way and four-way junctions were measured at the concentration of  $3 \times 10^{-6}$  M (per strand) in a buffer containing 10 mM sodium phosphate (pH 7.0), 100 mM NaCl and 5 mM MgCl<sub>2</sub>. The melting of bulge oligonucleotides and other oligonucleotides was carried out in 10 mM sodium phosphate (pH 7.0) and 100 mM NaCl at the concentration of  $3 \times 10^{-6}$  M.

### Gel electrophoresis of DNA 3WJs

Stoichiometric amounts of oligonucleotides at the concentration of  $1.07 \times 10^{-5}$  M per strand were mixed together in the buffer to form a 3WJ. One of the strands was 5'-end labelled using T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP. Flexicates were then added to the mixture so that the final concentration of the 3WJs in the samples was  $7.5 \times 10^{-6}$  M. The samples were analyzed by electrophoresis on 15% polyacrylamide gels in buffered solutions consisting of 89 mM Tris(hydroxymethyl)amino methane, 89 mM borate (pH 8.3) and 10 mM NaCl run at room temperature.

### Other physical methods

Purification of oligonucleotides was carried out on a Pharmacia Biotech FPLC System with a MonoQ 5/50 GL column. The gels were visualized by using the BAS 2500 FUJIFILM bioimaging analyzer, and the radioactivities associated with bands were quantitated with the AIDA image analyzer software (Raytest, Germany).

### MTT Assay – Determination of Anticancer Activity

The *in vitro* studies were performed at the Institute of Cancer Therapeutics, Bradford, on cell lines MCF7 (human breast adenocarcinoma), A2780 (human ovarian carcinoma) A2780cis (cisplatin resistant human ovarian carcinoma) and HCT116 p53<sup>+/+</sup> (human colon carcinoma). Cells were incubated in 96-well plates at a cell concentration of  $2.0 \times 10^4$  cells/mL. Complete cell media containing RPMI-1640, supplemented with 10% foetal calf serum, sodium pyruvate (1 mM) and L-glutamine (2 mM), was used to prepare the desired cell concentration and reference wells. Plates containing cells were incubated for 24 h at 37°C in an atmosphere of 5% CO<sub>2</sub>, prior to drug exposure. All compounds were dissolved in dimethylsulfoxide to give an initial concentration of 25 mM and diluted further with cell media to obtain concentrations ranging from 250 – 0.49 μM. A final dimethylsulfoxide concentration of 0.1% (v/v) was obtained, which is non-toxic to cells. Cell media (100 μL) was added to the reference cells and differing concentrations of drug solution (100 μL) were added to the remaining wells. The plates were incubated for a further 5 days at 37°C in an atmosphere of 5% CO<sub>2</sub>. 3-(4,5-Dimethylthiazol-1-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (20 μL, 5 mg/mL) was added to each well and incubated for a further 3 h at 37°C in an atmosphere of 5% CO<sub>2</sub>. Upon completion all solutions were removed from the wells *via* pipette, and dimethylsulfoxide (150 μL) was added to each well to dissolve the purple formazan crystals. A Thermo Scientific Multiskan EX microplate photometer was used to measure the absorbance at 540 nm. Lanes containing 100% cell media and 100% cell solution were used as a blank and 100% cell survival respectively. Cell survival was determined as the absorbance of treated cells minus the blank cell media, divided by the absorbance of the 100% cell solution; this value was expressed as a percentage. The IC<sub>50</sub> values were determined from a plot of percentage cell survival against drug concentration (μM). All IC<sub>50</sub> values were taken from an average of three different assays with good repeatability; assays were repeated if data did not correlate to within an approximate 10% error. The final IC<sub>50</sub> value was an average of the correlating data and errors were calculated as the standard deviation between each of the three IC<sub>50</sub> values.

### 24h Exposure MTT Assay

Assay was performed following the same procedure as 96h assay and HCT116 p53<sup>+/+</sup> cells, except after 24 h of drug treatment the supernatant was aspirated off, complete RPMI medium (200  $\mu$ L per well) was added and the cells were incubated for a further 72 h before being analysed.

**Table S2.** IC<sub>50</sub> values of 24h exposures of HCT116<sup>+/+</sup> cells to flexicates.

Complex	24 h IC <sub>50</sub> / $\mu$ M (esd)	% Cells killed by 10 $\mu$ M flexicate in 24 h (esd)
$\Delta$ -[Fe <sub>2</sub> L <sup>1a</sup> <sub>3</sub> ]Cl <sub>4</sub>	4.88 (1.36)	66.5 (18.5)
$\Lambda$ -[Fe <sub>2</sub> L <sup>2a</sup> <sub>3</sub> ]Cl <sub>4</sub>	4.11 (0.85)	54.5 (11.3)
$\Delta$ -[Fe <sub>2</sub> L <sup>2a</sup> <sub>3</sub> ]Cl <sub>4</sub>	4.52 (0.84)	71.9 (13.4)
$\Lambda$ -[Fe <sub>2</sub> L <sup>2b</sup> <sub>3</sub> ]Cl <sub>4</sub>	5.09 (1.36)	66.2 (17.7)
$\Delta$ -[Fe <sub>2</sub> L <sup>2b</sup> <sub>3</sub> ]Cl <sub>4</sub>	5.29 (0.35)	67.4 (4.5)

## **H2AX & Cell Cycle Analysis FACS Assays<sup>6</sup>**

### **Cell Preparation**

These studies were performed at Bradford Institute of Cancer Therapeutics. HCT116 p53<sup>+/+</sup> cells (5 X 10<sup>5</sup> cells/flask, 10 ml complete RPMI medium) were incubated for 18 h, then treated with 10µM flexicate (10 ml in complete RPMI medium) for 24 h. The supernatant, containing any dead cells, was collected and the cells were trypsinized with 1.5 ml trypsin. This single cell suspension in trypsin was added to the supernatant and centrifuged at 1500 rpm (300 g) for 5 min. The cells were washed twice with PBS, re-suspended in ice-cold methanol in PBS (90:10) and incubated in ice for 30 min, then stored at -20 °C until required for analysis.

### **H2AX Assay**

The pre-treated cells were washed twice in incubation buffer (PBS containing 0.5 BSA) then re-suspended in 100 µl incubation buffer for 10 min at room temperature. 2 µl of primary rabbit anti-human phosphor Histon H2AX (Ser 139) antibody (final 1:50 concentration, cell signalling Technology) was added and incubated at RT and incubated for a further 1 h. This was then washed twice with incubation buffer, re-suspended in 100 µl incubation buffer containing Alexa Fluor conjugated antirabbit IgG secondary antibody (final 1:1000 concentration, cell signalling Technology) and incubated in the absence of light at room temperature for 30 min then the cells were stored at 0 °C until analysis using the FACS.

### **Cell Cycle Assay**

300 µl PBS containing PI 40µg/ml and RNase A 200µg/ml was added to the pre-treated cells and they were incubated in the absence of light at room temperature for 30 min. 200µl ice-cold PBS was added (final volume of 600 µl) and the cells were placed on ice until analysis using the FACS. Red fluorescence was observed at 488nm excitation by flow cytometry and data analysed using WinMDI2.9 and Cylchred software.

**Table S3.** Cell cycle FACS assay. Control is untreated cells.

Complex	% Sub G <sub>1</sub> (esd)	% G <sub>1</sub> (esd)	% S (esd)	% G <sub>2</sub> /M (esd)
Control	3.90 (1.44)	44.41 (3.79)	28.98 (4.80)	20.20 (2.24)
$\Lambda$ -[Fe <sub>2</sub> L <sup>1a</sup> <sub>3</sub> ]Cl <sub>4</sub>	31.31 (4.50)	13.72 (3.17)	9.49 (3.17)	38.77 (5.02)
$\Delta$ -[Fe <sub>2</sub> L <sup>1a</sup> <sub>3</sub> ]Cl <sub>4</sub>	18.83 (5.97)	37.49 (3.81)	8.09 (1.51)	30.81 (3.41)
$\Lambda$ -[Fe <sub>2</sub> L <sup>2a</sup> <sub>3</sub> ]Cl <sub>4</sub>	14.48 (7.56)	36.42 (5.46)	23.00 (1.99)	22.60 (3.02)
$\Delta$ -[Fe <sub>2</sub> L <sup>2a</sup> <sub>3</sub> ]Cl <sub>4</sub>	16.74 (3.58)	33.45 (8.27)	18.31 (4.91)	28.42 (1.08)
$\Lambda$ -[Fe <sub>2</sub> L <sup>2b</sup> <sub>3</sub> ]Cl <sub>4</sub>	17.05 (9.85)	30.49 (7.93)	25.37 (2.81)	23.94 (3.20)
$\Delta$ -[Fe <sub>2</sub> L <sup>2b</sup> <sub>3</sub> ]Cl <sub>4</sub>	13.07 (4.11)	32.58 (4.90)	27.00 (3.22)	24.13 (3.69)

## References

1. S. E. Howson, A. Bolhuis, V. Brabec, G. J. Clarkson, J. Malina, A. Rodger and P. Scott, *Nat. Chem.*, 2012, **4**, 31-36.
2. A. Damianoglou, E. J. Crust, M. R. Hicks, S. E. Howson, A. E. Knight, J. Ravi, P. Scott and A. Rodger, *Chirality*, 2008, **20**, 1029-1038.
3. N. P. Chmel, P. Scott and A. Rodger, *Chirality*, 2012, **24**, 699-705.
4. A. Rodger, in *Methods in Enzymology*, eds. F. R. James and L. V. Bert, Academic Press, 1993, vol. Volume 226, pp. 232-258.
5. P. J. Chou and W. C. Johnson, *J. Am. Chem. Soc.*, 1993, **115**, 1205-1214.
6. S. J. Allison, M. Jiang and J. Milner, *Aging*, 2009, **1**, 316-327.