Supplementary Information: Rotaxanes as cages to control DNA binding, cytotoxicity and cellular uptake of a small molecule

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S1 General experimental procedures

$^1$H NMR spectra were recorded on either a Bruker Avance 400 MHz or 500 MHz Ultrashield NMR spectrometer and chemical shifts are reported in parts per million (ppm). Assignments were carried out where possible and for the cases in which assignments were not possible, the peaks were labelled to indicate which interlocked component they were from. Mass spectrometric analysis was performed on a LCT Premier mass spectrophotometer. All chemicals were purchased from Sigma–Aldrich, Fluorochem or VWR and used without further purification. Flash chromatography was performed using a Teledyne ISCO RF 200 Combiflash system with Redisep Rf Silica Gel flash columns. LCMS was carried out using a Waters ACQUITY UPLC system using a gradient of 5-95% MeCN in water with 0.1% formic acid using a C$_{18}$ column.
S2 Synthesis

The following compounds were prepared according to literature procedures; S1, S4, S7 and 3.

Supplementary Figure S1: Reaction scheme for the compounds synthesised in this work.
S2.1 Compound S2

Compound S1 (630 mg, 2.07 mmol), 3,4-diaminobenzoic acid (150 mg, 0.986 mmol), and Zn(OAc)$_2$·2H$_2$O (238 mg, 1.08 mmol) were dissolved DMSO (5 mL). The reaction was stirred at 80 °C for 2 hours before PtCl$_2$ (288 mg, 1.08 mmol) was added. The reaction mixture was stirred at 80 °C for 3 days before being allowed to cool to room temperature. Methanol (150 mL) was added and the resulting orange precipitate was filtered and washed with methanol (50 mL) and DCM (50 mL) to afford S2 in 82% yield as an orange solid (742 mg, 0.808 mmol). $^1$H NMR (400 MHz, DMSO-d$_6$) δ 9.37 – 9.32 (m, 2H, D), 8.78 (s, 1H, C), 8.39 – 8.33 (m, 1H, B), 7.88 (d, J = 8.8 Hz, 2H, F), 7.76 (d, J = 9.1 Hz, 1H, A), 6.63 (s, 2H, G), 6.49 (t, J = 6.7 Hz, 2H, E), 4.56 (s, 4H, H), 3.87 – 3.77 (m, 4H, I), 3.20 (s, 19H, J). $^{13}$C NMR (101 MHz, DMSO-d$_6$) δ 166.54, 166.30, 163.56, 163.37, 150.19, 144.02, 137.35, 137.09, 128.04, 117.17, 108.16, 107.74, 103.17, 63.89, 61.73, 53.16, 48.62. TOF MS (ES$^+$) calculated for [C$_{31}$H$_{38}$N$_4$O$_6$Pt]$^{2+}$ 378.6221, found 378.6215. HPLC-MS(ESI) calculated for [M]$^{2+}$ 378.6, found 378.6.

Supplementary Figure S2: Left, HPLC chromatogram of S2; right, mass profile (ESI) of S2.
Supplementary Figure S3: $^1$H NMR spectrum S2.

Supplementary Figure S4: $^{13}$C NMR spectrum S2.
S2.2 Compound S3

Compound S2 (500 mg, 0.603 mmol), HOBt (123 mg, 0.905 mmol), EDC·HCl (174 mg, 0.905 mmol) and triethylamine (126 µL, 0.905 mmol) were dissolved in DMSO (5 mL). The reaction mixture was stirred under nitrogen for 30 mins before propargyl amine (67.6 µL, 0.905 mmol) was added. The reaction was stirred at 45 °C for 12 hours. The reaction mixture was diluted with water (100 mL) and a solution of sat. aq. NaPF₆ (15 mL) was added. The resulting orange precipitate was filtered and washed with water (20 mL) and dried under vacuum to obtain S3 in 95% yield as an orange solid (622 mg, 0.573 mmol). ¹H NMR (400 MHz, DMSO-d₆) δ 9.40 (m, 2H, D), 9.07 (t, J = 5.5 Hz, 1H, K), 8.80 (s, 1H, C), 8.44 (d, J = 9.1 Hz, 1H, B), 7.90 – 7.83 (m, 2H, F), 7.80 (d, J = 9.1 Hz, 1H, A), 6.70 (s, 2H, G), 6.59 – 6.47 (m, 2H, E), 4.57 (t, J = 4.8 Hz, 4H, H), 4.14 (dd, J = 5.5, 2.5 Hz, 2H, L), 3.82 (t, J = 4.8 Hz, 4H, I), 3.19 (s, 18H, J), 3.16 (s, 1H, M). ¹³C NMR (101 MHz, DMSO-d₆) δ 166.67, 166.48, 164.80, 163.72, 163.55, 150.70, 150.32, 144.15, 137.17, 132.28, 125.78, 117.18, 117.06, 116.05, 115.75, 108.33, 108.05, 103.27, 81.11, 73.25, 63.89, 61.73, 53.14, 30.69, 28.73. TOF MS (ES⁺) calculated for [C₃₄H₄₁N₅O₅Pt]²⁺ 397.1379, found 397.1386. HPLC-MS(ESI) calculated for [M]²⁺ 397.1, found 397.1.

Supplementary Figure S5: Left, HPLC chromatogram of S3; right, mass profile (ESI) of S2.
Supplementary Figure S6: $^1$H NMR spectrum S3.

Supplementary Figure S7: $^{13}$C NMR spectrum S3.
S2.3 Compound S5

A solution of EDC·HCl (387 mg, 2.02 mmol) and pivalic acid (228 mg, 2.24 mmol) in DCM (30 mL) was cooled to 0 °C for 30 mins, followed by the addition of compound S4 (200 mg, 0.896 mmol) and DMAP (246 mg, 2.02 mmol). The solution was allowed to warm to room temperature and stirred for 1.5 hours. Next, a solution of v/v 5% citric acid (aq.) (20 mL) was added and the organic layer extracted with DCM (3 x 20 mL). The organic layer was concentrated under reduced pressure, dried with MgSO₄ and purified via column chromatography (Silica, hexane:ethyl acetate, 50:50) to afford compound S5 as a pale yellow oil in 51% yield (157 mg, 0.457 mmol). ¹H NMR (400 MHz, Chloroform-d) δ 7.62 (d, J = 2.2 Hz, 2H, B), 7.10 (t, J = 2.2 Hz, 1H, A), 4.53 – 4.46 (m, 2H, C), 3.62 – 3.57 (m, 2H, D), 1.35 (s, 18H, E). ¹³C NMR (101 MHz, Chloroform-d) δ 176.50, 164.71, 151.51, 131.44, 120.64, 120.26, 64.12, 49.84, 39.17, 26.52. TOF MS (ES⁺) calculated for [C₁₉H₂₅N₃O₆Na]⁺ 414.1641, found 414.1635.

Supplementary Figure S8: ¹H NMR spectrum compound S5.
Supplementary Figure S9: $^{13}$C NMR spectrum compound S5.
S2.4 Compound S6

Compound S4 (200 mg, 0.896 mmol), 4,5-dimethoxy-2-nitrobenzyl bromide (520 mg, 1.88 mmol) and K$_2$CO$_3$ (310 mg, 2.24 mmol) were dissolved in DMF (10 mL). The reaction mixture was stirred at room temperature in the dark for two days. The precipitate formed was collected, washed with water and dried under vacuum to afford compound S6 as a white solid in 85% yield (467 mg, 0.762 mmol), which was used without further purification.

$^1$H NMR (400 MHz, Chloroform-d) $\delta$ 7.80 (s, 2H, E), 7.39 (d, J = 2.4 Hz, 2H, B), 7.32 (s, 2H, D), 6.92 (t, J = 2.3 Hz, 1H, A), 5.57 – 5.52 (m, 4H, C), 4.57 – 4.49 (m, 2H, F), 4.01 (d, J = 2.0 Hz, 12H, I, H), 3.65 – 3.58 (m, 2H, G).

$^{13}$C NMR (101 MHz, Chloroform-d) $\delta$ 165.56, 159.33, 153.95, 148.03, 139.18, 131.89, 128.39, 109.46, 109.32, 108.11, 107.42, 67.43, 64.31, 56.54, 56.43, 49.90. TOF MS (ES$^+$) calculated for [C$_{19}$H$_{25}$N$_3$O$_6$Na]$^+$ 414.1641, found 414.1635.

Supplementary Figure S10: $^1$H NMR spectrum S6.
Supplementary Figure S11: $^{13}$C NMR spectrum S6.
S2.5 Compound \(1^{\text{enz}}\)

Macrocycle 3 (23.0 mg, 0.05 mmol) was dissolved in DMSO (2 mL), followed by [Cu(CH$_3$CN)$_4$](PF$_6$) (16.5 mg, 0.04 mmol). To this reaction mixture was added salphen S3 (50 mg, 0.05 mmol) and stopper S5 (18.0 mg, 0.05 mmol) and the reaction stirred for 12 hours at room temperature. Subsequently, DCM (50 mL) and a solution of v/v 1:9 NH$_4$OH/H$_2$O with 0.1 M EDTA (50 mL) was added and stirred for 1 hour. The organic layer was separated, dried using MgSO$_4$ and the solvent removed under reduced pressure. The resulting solid was purified via preparative thin layer chromatography (Silica, 50% acetonitrile:DCM with 50 mM NaPF$_6$) to afford \(1^{\text{enz}}\) in 10% yield as an orange solid (9.10 mg, 0.005 mmol). $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 9.39 (s, 1H, D), 8.76 (s, 1H, K), 8.60 (s, 1H, A), 8.12 – 8.01 (m, 6H, a/b/c), 7.78 (s, 1H, Q), 7.63 – 7.55 (m, 3H, C, E, L), 7.42 (d, J = 2.2 Hz, 2H, T), 7.37 (d, J = 8.7 Hz, 1H, B), 7.32 (t, J = 2.2 Hz, 1H, U), 6.94 (d, J = 8.2 Hz, 4H, d), 6.67 (d, J = 2.4 Hz, 1H, G), 6.62 (d, J = 2.4 Hz, 1H, N), 6.46 (dd, J = 8.9, 2.4 Hz, 1H, F), 6.36 (s, 1H, O), 6.25 – 6.14 (m, 5H, M, e), 4.66 (t, J = 5.2 Hz, 2H, P), 4.59 – 4.46 (m, 6H, H, R), 3.96 – 3.87 (m, 2H, macrocycle), 3.83 (t, J = 3.4 Hz, 2H, S), 3.76 – 3.61 (m, 10H, macrocycle), 3.54 – 3.44 (m, 4H, macrocycle), 3.20 – 3.15 (m, 18H, J), 1.29 (s, 18H, V). $^{13}$C NMR (101 MHz, DMSO-d$_6$) $\delta$ 176.49, 166.54, 166.29, 164.46, 164.31, 163.72, 163.40, 160.41, 158.81, 156.76, 151.75, 145.37, 144.45, 143.05, 139.10, 133.42, 131.72, 129.03, 123.73, 121.65, 121.42, 120.44, 114.03, 108.44, 107.77, 103.94, 70.83, 70.46, 69.13, 67.26, 64.41, 62.11, 53.61, 29.47, 27.12.

TOF MS (ES$^+$) calculated for [C$_{83}$H$_{96}$N$_{10}$O$_{16}$Pt]$^{2+}$ 842.3338, found 842.3355. HPLC-MS(ESI) calculated for [M+H]$^{3+}$ 561.9, found 561.7.

Supplementary Figure S12: Left, HPLC chromatogram of \(1^{\text{enz}}\); right, mass profile (ESI) of \(1^{\text{enz}}\).
Supplementary Figure S13: $^1$H NMR spectrum 1$^{enz}$.

Supplementary Figure S14: $^{13}$C NMR spectrum 1$^{enz}$.
Supplementary Figure S15: COSY NMR spectrum $1^{\text{enz}}$. 
Supplementary Figure S16: ROESY NMR spectrum $1^{enz}$. 
Supplementary Figure S17: NOESY NMR spectrum $1^{\text{enz}}$. 
S2.6 Compound 1\textsuperscript{photo}

Macrocycle 5 (23.0 mg, 0.05 mmol) was dissolved in DMSO (2 mL), followed by [Cu(CH\textsubscript{3}CN)\textsubscript{4}]\textsubscript{(PF\textsubscript{6})} (16.5 mg, 0.04 mmol). To this reaction mixture was added salphen 1 (50 mg, 0.05 mmol) and stopper 3 (28.3 mg, 0.05 mmol) and the reaction stirred for 12 hours at room temperature. Subsequently, DCM (50 mL) and a solution of v/v 1:9 NH\textsubscript{4}OH/H\textsubscript{2}O with 0.1 M EDTA (50 mL) was added and stirred for 1 hour. The organic layer was separated, dried using MgSO\textsubscript{4} and the solvent removed under reduced pressure. The resulting solid was purified via preparative thin layer chromatography (Silica, 50% acetonitrile:DCM with 50 mM NaPF\textsubscript{6}) to afford rotaxane 1\textsuperscript{photo} in 9% yield as an orange solid (9.11 mg, 0.004 mmol). \textsuperscript{1}H NMR (500 MHz, DMSO-d\textsubscript{6}) \textdelta 9.40 (s, 1H, D), 8.78 (s, 1H, K), 8.62 (s, 1H, A), 8.16 – 8.01 (m, 6H, a, b, c), 7.69 (s, 1H, Q), 7.67 – 7.61 (m, 2H, E, L), 7.59 (d, J = 9.0 Hz, 1H, C), 7.39 (d, J = 9 Hz, 1H, B), 6.98 (d, J = 8.1 Hz, 4H, d), 6.94 (t, J = 1.6 Hz, 1H, V), 6.68 – 6.61 (m, 4H, G, N, U), 6.47 (dd, J = 9, 2.4 Hz, 1H, F), 6.40 (s, 1H, O), 6.22 (m, 5H, M, e), 4.56 – 4.47 (m, 4H, H), 4.38 (t, J = 7.1 Hz, 2H, R), 3.95 – 3.89 (m, 2H, macrocycle), 3.89 – 3.76 (m, 8H, I, T, macrocycle), 3.75 – 3.68 (m, 2H, macrocycle), 3.61 (s, 4H, macrocycle), 3.50 (s, 4H, macrocycle), 3.30 (s, 2H, macrocycle), 3.18 (m, 18H, J), 2.08 (q, J = 6.5 Hz, 2H, P), 1.22 (s, 18H, W).

TOF MS (ES\textsuperscript{+}) calculated for [C\textsubscript{91}H\textsubscript{98}N\textsubscript{12}O\textsubscript{22}Pt\textsuperscript{2+}] 953.3296, found 953.3330. HPLC-MS(ESI) calculated for [M+H]\textsuperscript{3+} 635.6, found 636.1.

Supplementary Figure S18: Left, HPLC chromatogram of 1\textsuperscript{photo}; right, mass profile (ESI) of 1\textsuperscript{photo}.
Supplementary Figure S19: $^1$H NMR spectrum $^{1}\text{photo}$. 
Supplementary Figure S20: COSY NMR spectrum $1^{\text{photo}}$. 
Supplementary Figure S21: ROESY NMR spectrum $I_{\text{photo}}$. 
Supplementary Figure S22: NOESY NMR spectrum $\text{1}^{\text{photo}}$. 
S2.7 Compound 1\textsuperscript{ctrl}

Macrocycle 5 (23.0 mg, 0.05 mmol) was dissolved in DMSO (2 mL), followed by [Cu(CH\textsubscript{3}CN)\textsubscript{4}](PF\textsubscript{6}) (16.5 mg, 0.04 mmol). To this reaction mixture was added salphen 1 (50 mg, 0.05 mmol) and stopper 4 (13.3 mg, 0.05 mmol) and the reaction stirred for 12 hours at room temperature. Subsequently, DCM (50 mL) and a solution of v/v 1:9 NH\textsubscript{4}OH/H\textsubscript{2}O with 0.1 M EDTA (50 mL) was added and stirred for 1 hour. The organic layer was separated, dried using MgSO\textsubscript{4} and the solvent removed under reduced pressure. The resulting solid was purified via preparative thin layer chromatography (Silica, 50% acetonitrile:DCM with 50 mM NaPF\textsubscript{6}) to afford 1\textsuperscript{ctrl} in 12% yield as an orange solid (12.2 mg, 0.005 mmol).

\textbf{1H NMR (500 MHz, DMSO-d}\textsubscript{6}) \(\delta\) 9.37 (s, 1H, D), 8.73 (s, 1H, K), 8.57 (s, 1H, A), 8.03 (m, 6H, a, b, c), 7.78 (s, 1H, Q), 7.70 (s, 2H, X), 7.56 (s, 3H, C, E, L), 7.33 (d, \(J = 9\) Hz, 2H, B), 7.30 (s, 2H, W) 7.10 (d, \(J = 2.2\) Hz, 2H, T), 6.95 (s, 1H, U), 6.90 (d, \(J = 8.1\) Hz, 4H, e), 6.66 (s, 1H, G), 6.60 (s, 1H, N), 6.46 (d, \(J = 8.8\) Hz, 1H, F), 6.21 (d, \(J = 8.6\) Hz, 1H, M), 6.14 (d, \(J = 8.1\) Hz, 4H, e), 5.39 (s, 4H, V), 4.66 (s, 2H, R), 4.52 (d, \(J = 22.8\) Hz, 6H, P, H), 3.95 – 3.75 (m, 20H, macrocycle, Y), 3.70 (d, \(J = 10.0\) Hz, 2H, macrocycle), 3.58 (m, 4H, macrocycle), 3.50 (s, 4H, macrocycle), 3.17 (m, 18H, J), \textbf{13C NMR (101 MHz, DMSO-d}\textsubscript{6}) \(\delta\) 166.55, 166.30, 164.42, 163.73, 163.44, 160.36, 158.86, 158.39, 156.70, 152.54, 152.10, 150.50, 145.38, 144.33, 143.08, 139.19, 136.86, 135.65, 133.37, 131.07, 129.07, 125.90, 123.32, 121.48, 120.51, 177.79, 117.52, 117.03, 116.45, 114.85, 114.07, 109.11, 108.15, 107.74, 103.87, 79.42, 79.09, 70.81, 70.45, 70.25, 69.13, 67.28, 64.45, 62.12, 53.60, 46.80, 35.03, 34.60, 31.65, 30.19, 31.65, 30.19, 29.47.

TOF MS (ES\textsuperscript{+}) calculated for [C\textsubscript{81}H\textsubscript{98}N\textsubscript{10}O\textsubscript{11}Pt\textsuperscript{2+}] \(790.8532\), found 790.8668. HPLC-MS(ESI) calculated for [M+H]\textsuperscript{3+} 527.6, found 527.7.

Supplementary Figure S23: Left, HPLC chromatogram of 1\textsuperscript{ctrl}; right, mass profile (ESI) of 1\textsuperscript{ctrl}.
Supplementary Figure S24: $^1$H NMR spectrum $^1$ctrl.

Supplementary Figure S25: $^{13}$C NMR spectrum $^1$ctrl.
Supplementary Figure S26: COSY NMR spectrum 1<sup>ctrl</sup>. 
Supplementary Figure S27: ROESY NMR spectrum $1^{\text{ctrl}}$. 
Supplementary Figure S28: NOESY NMR spectrum 1\textsuperscript{ctrl}.
S2.8 Compound 2

Salphen 1 (50 mg, 0.05 mmol) and azidoethanol (6.02 mg, 0.07 mmol) were dissolved in DMSO (2 mL). This was followed by the addition of [Cu(CH$_3$CN)$_4$](PF$_6$) (3.42 mg, 0.009 mmol). The reaction was stirred at room temperature for 12 hours before precipitation with DCM (20 mL). The solid was filtered and washed a further 3 times with DCM (10 mL) and purified by RP-HPLC to afford 2 in 60% yield as an orange solid (32.4 mg, 0.277 mmol). $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 9.41 (m, 2H, D), 9.19 (t, $J$ = 5.7 Hz, 1H, K), 8.83 (s, 1H, C), 8.44 (d, $J$ = 9.0 Hz, 1H, B), 7.98 (s, 1H, M), 7.90 (d, $J$ = 9 Hz, 1H, A), 7.88 – 7.78 (m, 2H, F), 6.73 – 6.67 (m, 2H, G), 6.58 – 6.52 (m, 2H, E), 5.04 (t, $J$ = 5.4 Hz, 1H, P), 4.65 – 4.50 (m, 6H, H, L), 4.39 (t, $J$ = 5.4 Hz, 2H, N), 3.83 (s, 4H, I), 3.77 (q, $J$ = 5.4 Hz, 2H, O), 3.19 (s, 18H, J). $^{13}$C NMR (101 MHz, DMSO-d$_6$) $\delta$ 166.64, 164.87, 164.67, 164.88, 163.74, 163.57, 150.70, 150.31, 146.39, 144.49, 144.14, 137.16, 132.60, 125.87, 123.54, 118.13, 117.19, 116.01, 115.72, 108.34, 108.07, 103.28, 63.89, 61.75, 59.87, 54.94, 53.14, 52.15, 48.62, 34.98, 30.73. TOF MS (ES$^+$) calculated for [C$_{36}$H$_{46}$N$_8$O$_6$Pt]$^{2+}$ 881.3188, found 881.3176. HPLC-MS(ESI) calculated for [M]$^{2+}$ 440.7, found 440.0.

Supplementary Figure S29: Left, HPLC chromatogram of 2 right, mass profile (ESI) of 2.
Supplementary Figure S30: $^1$H NMR spectrum 2.

Supplementary Figure S31: $^{13}$C NMR spectrum 2.
### S3 Photophysical properties

Absorption spectra were obtained using an Agilent Cary UV 60 spectrometer and a 1 cm quartz cuvette and emission spectra were obtained using an Agilent Cary Eclipse Fluorescence Spectrophotometer using a 1 cm quartz cuvette.

**Supplementary Figure S32:** Absorbance spectra of $1^{\text{enz}}$, $1^{\text{photo}}$, $1^{\text{ctrl}}$ and 2 in water at a concentration of 50 µM.
Supplementary Figure S33: Relative emission spectra of $1^{\text{enz}}$, $1^{\text{photo}}$, $1^{\text{ctrl}}$ and 2 in water at 50 µM. Excitation was at 440 nm.
S4 DNA binding experiments

S4.1 Oligonucleotide Preparation

c-Myc and HTelo oligonucleotides (see below for sequences) were purchased from Eurogentec (Belgium) and CT-DNA was purchased from Sigma Aldrich. Stock solutions were prepared via dilution in the appropriate buffers to concentrations of 300-400 µM determined by UV-Vis titrations using an Agilent Cary UV 60 spectrometer. The sequences used are in Table S1. Prior to use, the G4 DNA stock solutions were annealed by heating at 95 °C for 5 minutes and then cooling to room temperature overnight.

<table>
<thead>
<tr>
<th>DNA</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTelo</td>
<td>AGGGTTAGGTTAGGGTTAGGG</td>
</tr>
<tr>
<td>c-Myc</td>
<td>TGAGGGTGGGTAGGGTTAGGGTAA</td>
</tr>
</tbody>
</table>

Table S1: G4 forming sequences used in this paper.

S4.2 CD melting experiments

Experiments were carried out using a Jasco J-810 spectrophotometer using a 1 cm quartz cuvette. Temperature was controlled using a Peltier module. The signal at 295 nm was monitored as temperature was increased for 25 °C to 95 °C at a rate of 95 °C/min. CD melting experiments were carried out with HTelo G4 DNA (100 mM NaCl, 10 mM Li cacodylate, pH 7.3). Sample preparation was carried out by combining stock solutions of ligand and DNA in the appropriate buffer. The final G4 DNA concentration in the cuvette was 5 µM and the ligand concentration was 10 µM. Experiments were conducted in triplicate and melting temperatures were obtained by curve fitting in GraphPad Prism 8. The data was normalised and fitted to a variable slope Hill equation, and the melting temperature was defined at the temperature at which \( y = 0.5 \).
Supplementary Figure S34: CD spectra of HTelo (Na⁺) (5 µM) and HTelo (Na⁺) (5 µM + 1enz, 1photo, 1ctrl and 2 (all at 10 µM).
S4.3 Fluorescence titrations

Experiments were carried out using a BMG Clariostar Microplate reader with Greiner Bio-One half volume (100 µL/well) plates. Excitation was carried out at 440 nm and recorded from 500-700 nm in addition to a matrix scan at 590 nm. Titrations were carried out using HTelo (Na⁺) G4 DNA (100 mM NaCl, 10 mM Li cacodylate, pH 7.3), HTelo (K⁺) G4 DNA (100 mM KCl, 10 mM Li cacodylate, pH 7.3), c-Myc G4 DNA (100 mM KCl, 10 mM Li cacodylate, pH 7.3) and CT-DNA (100 mM KCl, 10 mM Li cacodylate, pH 7.3). Ligand concentration was kept constant at 2 µM and the following DNA equivalents were tested: 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 1.5, 2, 3 and 5 (for CT-DNA 10x base pair equivalents (BPE) were used, ie 1 to 50 BPE). Sample preparation was carried out by preparing stock solutions of double concentration ligand (4 µM) and DNA in the appropriate buffer, before 50 µL of each was added to the appropriate well and mixed. Experiments were conducted in triplicate and binding constants were obtained by curve fitting in Graphpad Prism 8 using Equation 1.

\[
y = 0.5R\left[\frac{1}{K} + L + nx - \left(\left(\frac{1}{K} + L + nx\right)^2 - 4Lnx\right)^{0.5}\right]
\]  

Fluorescence response (y) was fitted against DNA concentration (x); \( R \) is the machine response, \( K \) is the association constant of ligand and DNA, \( L \) is the concentration of ligand and \( n \) is the number of binding sites per DNA (2 per G4 unit and 1 per CT-DNA BPE). The matrix scan results were used to plot the titration curves and 500-700 nm scan results were used to confirm that expected curve shapes and shifts were observed.
Supplementary Figure S35: Titration data for $1^{\text{enz}}$, $1^{\text{photo}}$, $1^{\text{ctrl}}$ and 2 with $HTelo$ (Na$^+$) and $HTelo$ (K$^+$) G4 DNA. In both cases the following equivalents of DNA were used: 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 1.5, 2, 3 and 5. Excitation at 440 nm.
Supplementary Figure S36: Titration data for $1^{\text{enz}}$, $1^{\text{photo}}$, $1^{\text{ctrl}}$ and 2. with c-Myc G4 DNA and CT-DNA. For c-Myc the following equivalents of DNA were used: 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 1.5, 2, 3 and 5. For CT-DNA 10 BPE equivalents were used. Excitation at 440 nm.
S5  Stability and activation experiments

S5.1  Buffer stability

A solution of the appropriate compound in PBS buffer was incubated at 37°C for 24 hours, after which analysis was carried out via HPLC.

Supplementary Figure S37: Stability of rotaxanes 1\textsuperscript{enz}, 1\textsuperscript{photo} and 1\textsuperscript{ctrl} in PBS buffer incubated at 37°C over 24 hours, followed by protein precipitation and HPLC analysis.

S5.2  Calculation of retention times

For compounds 2 and 3, a solution of the appropriate compound in acetonitrile was injected for HPLC analysis.

Supplementary Figure S38: Retention times for compounds 2 and 3.

In order to assign the intermediate product peaks a sample of 1\textsuperscript{photo} was irradiated using 365 nm light for 20 minutes. The sample was then injected for LCMS analysis and the peaks assigned to the intermediate products with one nitroveratyl group and both protecting group cleaved. The retention times and elution order were used to assign the analogous peaks in the experiment with 1\textsuperscript{enz}.
Supplementary Figure S39: Partially deprotected sample of 1\textsuperscript{photo} including MS data for the two intermediate peaks.

S5.3 Human serum stability

A solution of the appropriate compound in human serum was prepared and incubated at 37°C. After 24 h a sample was removed and diluted with 3x volume of ice-cold acetonitrile and kept on ice for 30 minutes for protein precipitation. After 30 minutes the sample was centrifuged, and the supernatant was removed and analysed via HPLC.
**Supplementary Figure S40:** Stability of rotaxanes $1^{\text{photo}}$ and $1^{\text{ctrl}}$ in human serum incubated at $37^\circ\text{C}$ over 24 hours, followed by protein precipitation and HPLC analysis.

**S5.4 Fluorescence titrations**

Titrations were carried out using HTelo G4 DNA, c-Myc G4 DNA and CT-DNA (PBS buffer, pH 7.3 for all). Ligand concentration was kept constant at 2 µM and prepared via direct dilution of a solution of $1^{\text{enz}}$ incubated in buffer for 24 hours, $1^{\text{enz}}$ incubated for 24 hours with 50 U/µM of PLE and 2 also incubated with PLE for 24 hours. All other parameters were identical to previous titrations (Section S4.3).
Supplementary Figure S41: Titration data for $1_{\text{enz}}$, $1_{\text{enz}}$ + PLE and $2$ + PLE with HTelo and c-Myc G4 DNA. In both cases the following equivalents of DNA were used: 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 1.5, 2, 3 and 5. Excitation at 440 nm.
Supplementary Figure S42: Titration data for $1^{enz}$, $1^{enz} +$ PLE and $2 +$ PLE with CT-DNA. The following BPE of DNA were used: 0, 1, 2, 3, 4, 0.5, 0.75, 1, 15, 20, 30 and 50. Excitation at 440 nm.

S6  Cell experiments

S6.1  Cytotoxicity

Human Bone Osteosarcoma Epithelial Cells (U2OS, from ATCC) were grown in high glucose Dulbecco’s modified Eagle medium (DMEM) containing 10% foetal bovine serum at 37°C with 5% CO$_2$ in humidified air. U2OS cells were seeded ($5 \times 10^3$ cells, 200 µL, 32.2 mm$^2$) in a 96-well plate. After 24 hours a solution of an MTS/PMS mixture in fresh media was added, according to the Promega MTS assay protocol. After 4 h, absorbance at 490 nm (MTS) and 635 nm (background) was measured. Cell viability was calculated from the dose response curve of absorbance (MTS – background).
Supplementary Figure S43: Cell viability assays. Cells were seeded and incubated for 24 hours, followed by irradiation for either 10 or 20 minutes using 365 nm light. Cells were incubated for a further 24 hours, after which viability was measured using an MTS assay.

S6.2 Live cell imaging

Human Bone Osteosarcoma Epithelial Cells (U2OS, from ATCC) were grown in high glucose Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum at 37°C with 5% CO2 in humidified air. Cells were seeded on chambered coverglass (1.5 x 10^4 cells, 250 µL, 0.8 cm^2) for 24 h, before washing with PBS and adding fresh media containing the compounds under study (2 or 10 µM, 250 µL) for a further 24 h. The cells were co-stained with DAPI (50 µM, 1 hr) and LysoTracker green (200 nM, 1 hr). For all live cell imaging, cells were mounted in the microscope stage, heated by a thermostat (Lauda GmbH, E200) to 37°C, and kept under an atmosphere of 5% CO2 in air. Pt-Salphen emission (525-700 nm) was collected following 458 nm excitation. DAPI emission (400–450 nm) was collected following multi-photon excitation at 760 nm with 665 and 680 nm cut off filters. LysoTracker Green emission (492-525 nm) was collected following 488 nm excitation, and the same laser was used to collect the Pt-Salphen Emission (570-700 nm) simultaneously. A 100x (oil, NA = 1.4) objective was used to collect images at 512 x 512 pixel resolution.

Supplementary Figure S44: Live cell fluorescence imaging experiments with 1\textsuperscript{photo}, including co-staining with DAPI and LysoTracker Green. Pt-Salphen emission (525-700 nm) was collected following 458 nm excitation. DAPI emission (400–450 nm) was collected following multi-photon excitation at 760 nm with 665 and 680 nm cut off filters. LysoTracker Green emission (492-525 nm) was collected following 488 nm excitation, and the same laser was used to collect the Pt-Salphen Emission (570-700 nm) simultaneously.

S7 References

1. Rakers, V., Cadinu, P., Edel, J. B. Vilar, R. Development of microfluidic platforms for the synthesis of metal complexes and evaluation of their DNA affinity using online FRET melting assays. Chemical Science