Supplementary Material

DNA-binding properties of cytotoxic naphtindolizinedione-carboxamides acting as type II Topoisomerase inhibitors. A combined *in silico* and experimental study

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Figure S2. UV-Vis spectra used for the calculation of molar extinction coefficient, recorded in 240-500 nm range, for buffer solutions (Tris-HCl 10mM, NaCl 20mM, pH= 7.5) of compounds. (a) **1**, (b) **2**, (c) **3**.

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Table S2. Inhibition of human topoisomerase IIα decatenation assay IC_{50} values for **1**-**3** and mitoxantrone.

1. General Experimental Methods

Deoxyribonucleic acid sodium salt from *Calf thymus* (CG = 41.9%) and dihydrochloride mitoxantrone were from Sigma. Synthetic CGCGAATTCGCG oligonucleotide (GC = 66.7%) was bought from Thermo Fisher Scientific. Reagents and solvents were used without purification. All evaporations were carried out at room temperature at reduced pressure. UV-Visible spectra were recorded with a double beam spectrophotometer UVIKON 923. The DNA melting point has been obtained by JASCO V−550 UV/Vis Spectrophotometer equipped with a thermostatic unit. CD spectra were recorded with a Jasco J−710 spectrophotometer. pH Measurements were obtained by pH-meter Delta OHM H2256.

2. Preparation and use of $(CGCGAATTCGCG)_2$ oligonucleotide

Single strand oligonucleotide, dissolved in DNase/RNase free 10mM Tris, was heated at 85°C for 5 minutes and slowly cooled during 12 hours till room temperature. It was used in UV analysis of the complex with compound **2** at a fixed concentration (18.3 μM) and at increasing oligonucleotide amounts (from 1.4 to 5.6 μM).

3. Spectroscopic Analyses

3.1. Binding constants evaluation by UV/Vis spectroscopy

A stock solution of *Calf thymus* DNA was diluted in 10 mM Tris (pH 5.8) and the exact concentration was determined by UV/vis spectroscopic analysis (λ= 260 nm, ε= 13200 M^{-1} cm^{-1} established relatively to base pairs). The same procedure has been applied for protonated salts of compounds **1** (λ= 331 nm, ε= 8791 M^{-1} cm^{-1}), **2** (λ= 323 nm, ε= 12208 M^{-1} cm^{-1}), **3** (λ= 339 nm, ε= 8710 M^{-1} cm^{-1}) and mitoxantrone (λ= 660 nm, ε= 19684 M^{-1} cm^{-1}). DNA solution (in the range 1,6 ÷ 16 μM) was incubated individually with compound **1**, **2**, **3** or mitoxantrone at 20 μM for 90 minutes at room temperature in the dark. The complexes and the controls were subjected to spectroscopic analysis by a double beam spectrophotometer UVIKON 923 equipped with quartz cuvettes. The calculation of binding constants was achieved following two different approaches reported in literature[1,2], obtaining comparable results (Figure S2).

3.2. Thermal denaturation analysis

DNA melting experiments were carried out by monitoring the absorbance (260 nm) of *Calf thymus* DNA (14 μM base-pairs) at different temperatures, ranged between 40 and 90 °C, in the absence and presence of the complex, in a 1:1 DNA/complex ratio. Measurements were performed by a JASCO V−550 UV/Vis spectrophotometer equipped with a thermostatic unit. The solution containing the complex and *Calf thymus* DNA in 10 mM Tris buffer (pH 5.8) was heated with a rate of temperature increase of 1°C/ min and reading the absorbance at ΔT=0.25 °C- (Figure S3).

3.3. Circular dichroism

It was performed using a Jasco J−710 spectrophotometer. The spectra of *Calf thymus* DNA (60 μM) or synthetic oligonucleotide in 10 mM Tris pH 5.8 and in the absence and presence of various concentrations of each compound **1**-**3** and mitoxantrone was recorded in the range of 220– 305 nm with a spectral resolution of 1 nm. The scan speed was 20 nm/min and the response time was 0.3330 sec with a band width of 1 nm. Quartz cell with a path length of 10 mm was used and all measurements were carried out at 25°C. Results are expressed as molar ellipticity [θ], in deg × cm^2 × $dmol^{-1}$ (Figure S4).

4. Human topoisomerase IIα inhibition

The assay was performed according to the reported protocol [3] with minor modifications. The reaction was made in a final volume of 20μl; each sample was constituted by 200ng of kDNA, assay buffer (containing Tris HCl 50mM pH=7.5, NaCl 125mM, $MgCl_2$ 10mM, DTT 5mM and albumin 100μg/ml), ATP 10mM, 1U of human topoisomerase IIα (provided by Inspiralis Ltd, UK) and 2μl of compound solution, to have the desired final concentration. Samples were incubated for 60 min at 37°C and then stopped with 4μl of gel loading buffer made with 49% TE (10mM Tris, 1mM EDTA, 20mM NaCl, pH 7.4), 49% glycerol, 2% SDS, 0.025% bromophenol blue, and 0.025% xylene cyanol. Samples are resolved by electrophoresis on a 1% agarose gel running in 1X TBE

buffer (90mM Tris, 90mM boric acid, 20mM EDTA); the arrays for the electrophoresis are the Sub-Cell GT type provided by Bio-Rad. Gel was stained post run with an ethidium bromide solution 0.5μg/ml for 30 min. The bands were detected using the Geliance 600 Imaging System and densitometric analysis was performed using the software Gene tools from Perkin-Elmer. In each gel a blank control containing only kDNA, which correspond to 0% of activity, and a control containing kDNA end enzyme, corresponding to 100% of topoisomerase IIα activity, were added (Figures S5 and S6). The concentration of the inhibitor preventing 50% of the substrate (knotted DNA) from being converted into the reaction product (unknotted DNA) was determined from a standard sigmoid curve. By averaging at least three experiments, the IC_{50} values and the standard deviation were determined.

5. References

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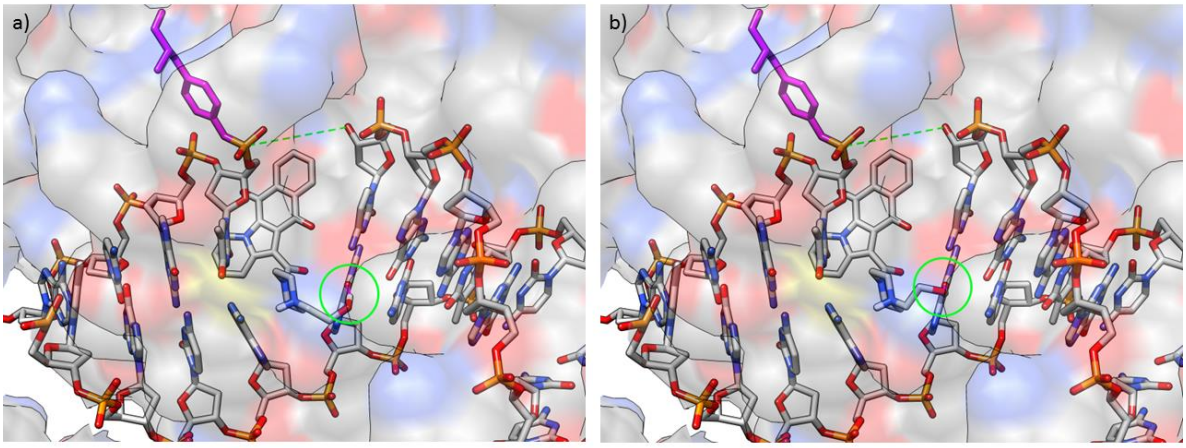

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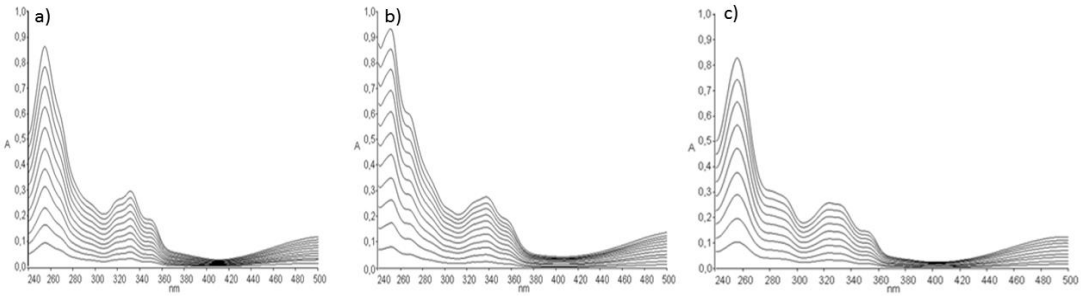

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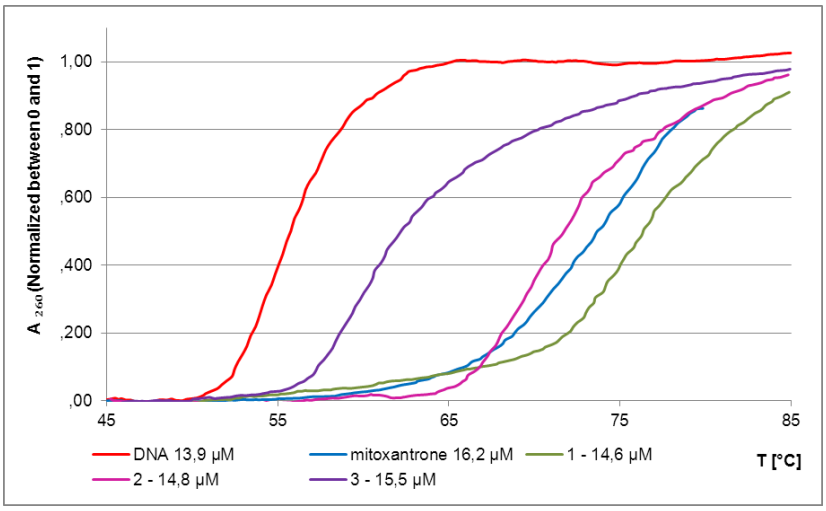

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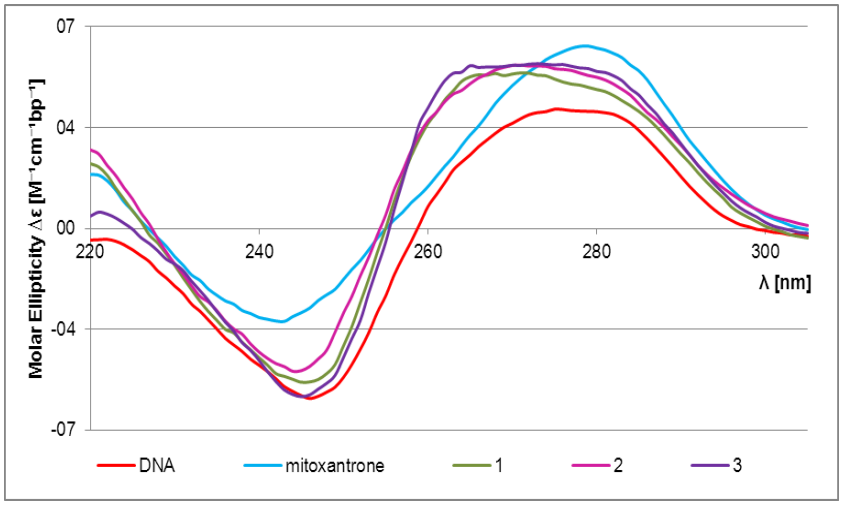

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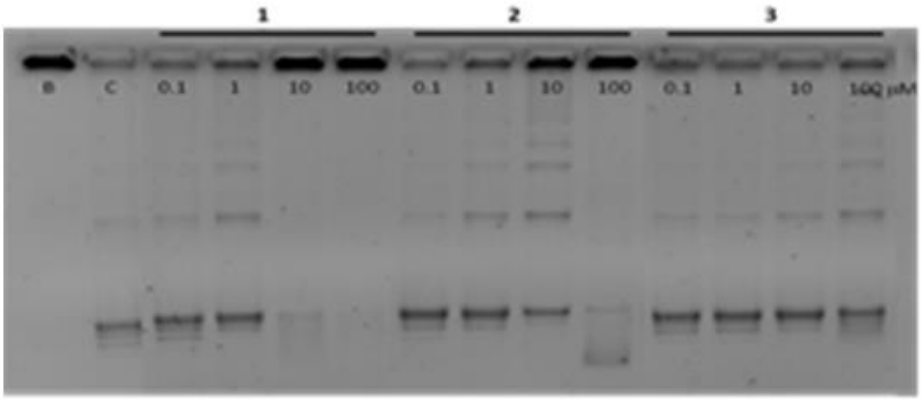

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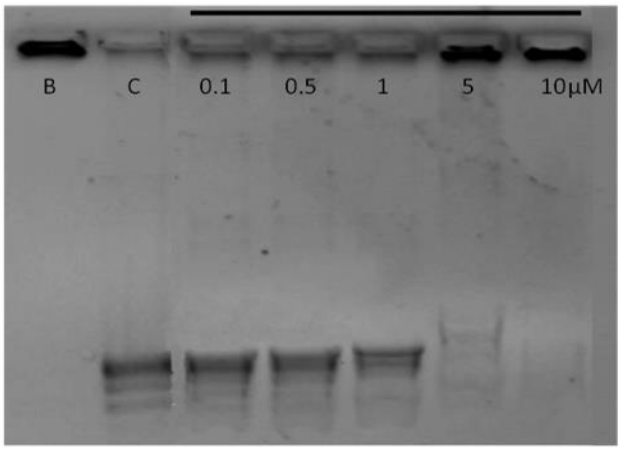

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System	**K** **[M^{-1}]**	**T_m** **[°C]**	**ΔT_m** **[°C]**
DNA	-	55.8±0.1	-
DNA/**1**	$(2,2 \pm 0,3)\cdot 10^6$	76.3±0.1	20.5±0.2
DNA/**2**	$(3,2 \pm 0,3)\cdot 10^6$	71.7±0.1	15.9±0.2
DNA/**3**	$(3,6 \pm 0,1)\cdot 10^5$	62.2±0.1	6.4±0.2
DNA/Mitoxantrone	$(2,3 \pm 0,1)\cdot 10^5$	73.6±0.1	17.8±0.2

Table S2. Inhibition of human topoisomerase IIα decatenation assay: IC_{50} values for **1**-**3** and mitoxantrone

Compound	**IC_{50} (μM)**
1	1.78±0.32
2	7.83±3.33
3	>100
Mitoxantrone	2.54±0.25