Electronic Supplementary Material (ESI) for Dalton Transactions

Supporting Information

"Screening organometallic binuclear thiosemicarbazone ruthenium complexes as potential anti-tumour agents: cytotoxic activity and human serum albumin binding mechanism"

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Supporting Information includes the following Sections:

1. ***Cell Studies***

with figures

SI.1 – *In vitro* cytotoxic activity of ligands L1-L4 against A2780, MCF7 and PC3 cell lines;

SI.2 – In vitro cytotoxic activity of the thiosemicarbazone (TSC) ligands;

SI.3 – Dose-response profiles of cell viability in A2780, MCF7 and PC3 cell lines;

2. ***Time-dependent induced CD spectra for HSA binding of complexes 1 and 4***

with explaining text, and figures

SI.4 – Binding of complex 1 to HSA monitored by CD spectroscopy (time dependence and equilibrium conditions);

SI.5 – Time dependence of binding of complex 4 to HSA;

SI.6 – UV-Visible spectra on time dependence of complex 4 binding to HSA.

3. ***Mechanism of Förster Resonance Energy Transfer (FRET) for the HSA–complex 4 system***

SI.7 – Spectral overlap of the HSA fluorescence emission and complex 4 absorbtion.

Supporting Information

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1. Cell Studies

A total of 9 compounds – which comprise TSC ligands **L_1** to **L_4**, the $[Ru_2(p\text{-cym})_2Cl_4]$ precursor and $[Ru_2(p\text{-cym})_2(TSC)]X_2$ complexes **1** to **4** (see Scheme 1) – were screened for their cytotoxic activity against human tumour cell lines and compared against cisplatin (CDDP).

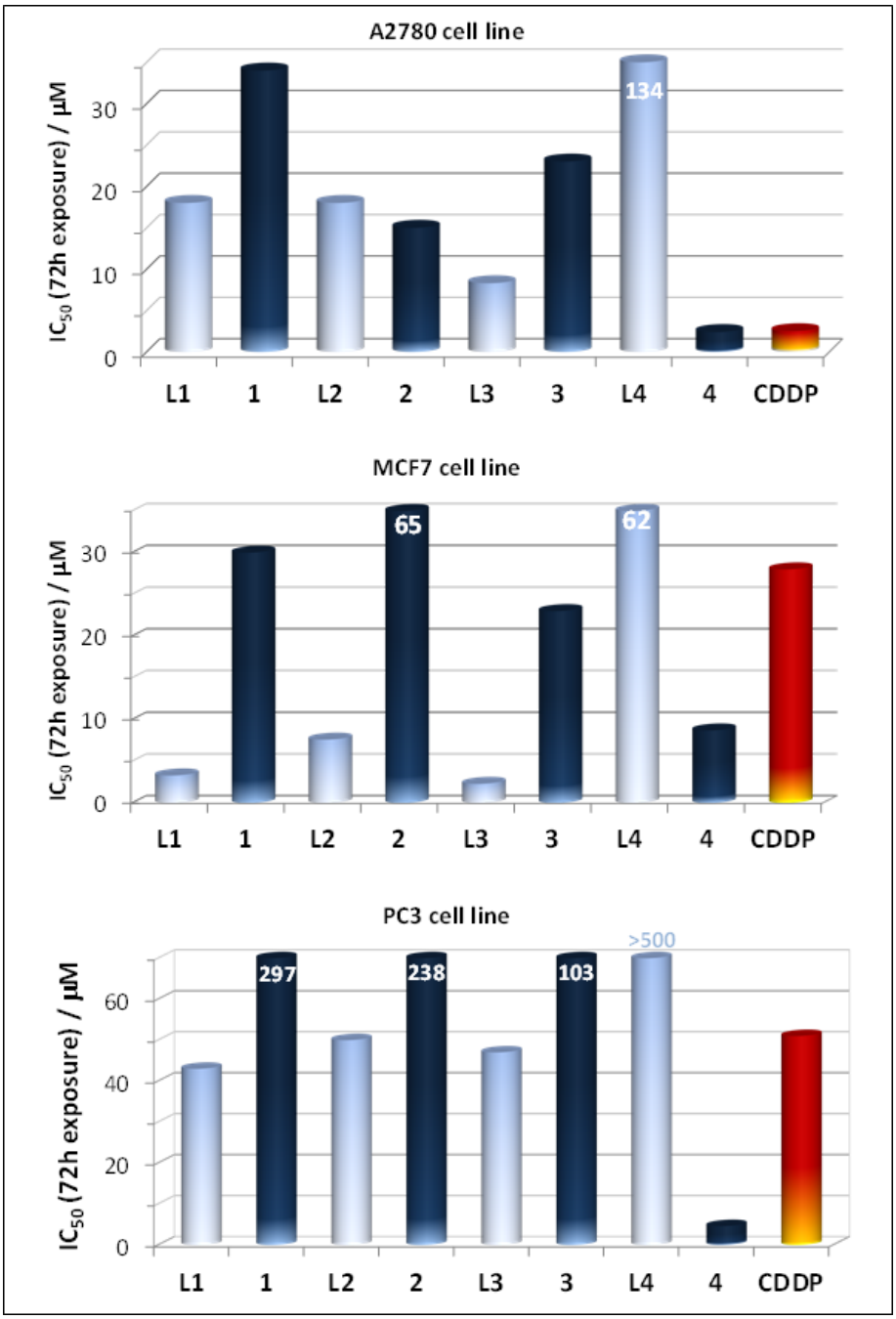

Figure SI.1. *In vitro* cytotoxic activity of all thiosemicarbazone compounds **1-4** and **L1-L4** against the human tumour cell lines A2780 (ovarian adenocarcinona), MCF-7 (breast adenocarcinoma) and PC-3 (grade IV prostate carcinoma). IC_{50} values are reported in µM for a 72h incubation period. CDDP is cisplatin incubated in the same conditions (IC_{50} values from[50] for MCF7 and PC3 cells obtained with the same experimental methodology).

Supporting Information

"Screening organometallic binuclear thiosemicarbazone ruthenium complexes as potential anti-tumour agents: cytotoxic activity and human serum albumin binding mechanism"

TSC ligands **L1, L2** and **L3** are much more active than cisplatin against the MCF7 cell line, and exhibit IC_{50} values close to those of CDDP in the very aggressive PC3 cell line as well (**Figure SI.2.**). The cytotoxicity of complexes **1** and **3** is comparable to that of CDDP in MCF7 and in PC3 cells. While **L4** is typically not cytotoxic, complex **4** is in contrast quite active against all tumorigenic cell lines tested.

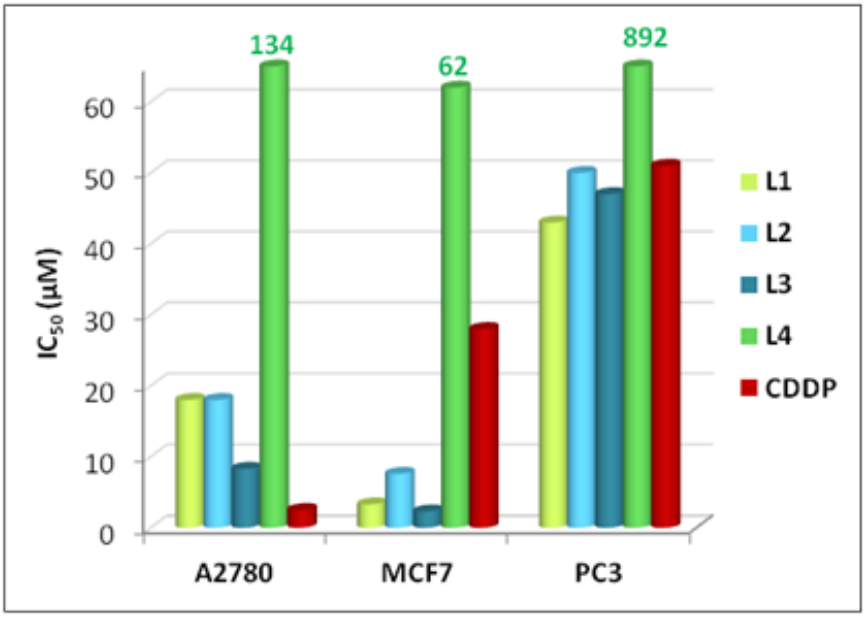

Figure SI.2. *In vitro* cytotoxic activity of the thiosemicarbazone (TSC) ligands **L1-L4** against A2780, MCF7 and PC3 cells. Cisplatin (CDDP) was included for comparison using the same experimental conditions (CDDP data from [47] for MCF7 and PC3 cell lines). IC_{50} values are reported in μM for a 72h incubation period.

Supporting Information

"Screening organometallic binuclear thiosemicarbazone ruthenium complexes as potential anti-tumour agents: cytotoxic activity and human serum albumin binding mechanism"

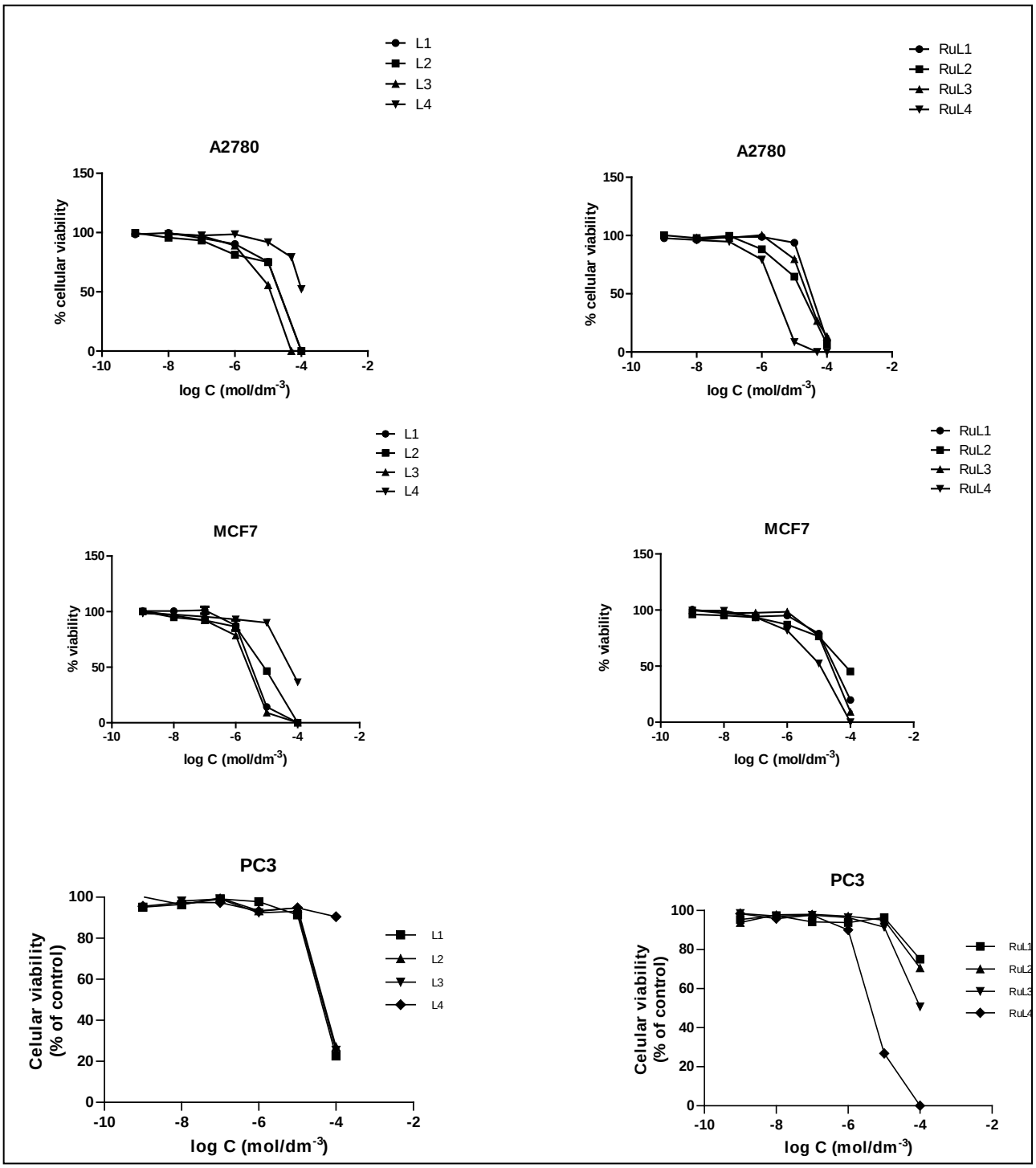

Figure SI.3. Dose-response profiles of cell viability in the human tumor cell lines obtained for all TSC ligands **L1-L4** and their corresponding complexes **1-4** after a 72 h incubation period (complex concentrations are indicated in M (mol.dm^{-3}). IC_{50} values obtained are reported in **Table 1**.

Supporting Information

"Screening organometallic binuclear thiosemicarbazone ruthenium complexes as potential anti-tumour agents: cytotoxic activity and human serum albumin binding mechanism"

2. Time-dependent induced CD spectra for HSA binding of complexes 1 and 4

Time dependence for HSA binding of complexes **1** and **4** was monitored by CD spectroscopy since both complexes yield an induced CD signal upon binding to the protein.

Figure SI.4 shows a weak ICD signal (consisting of two negative bands at ~ 400 nm (396 nm, band 1) and ~500 nm (494 nm, band 2) detected after 1h and due to the binding of **1** to HSA. The fact that an ICD signal is observed after a short incubation time suggests that the complex does neither dissociates into monomeric species nor loses any of the coordinated ligands, and the binding of **1** to HSA is most likely to involve the original dimeric complex. This is supported by the UV-Visible spectrum of complex **1** after 1 h incubation with HSA, that shows in the lower energy absorption band a ~25 nm red shift from 415 nm (in the free complex) to 440 nm (in the HSA-bound form); further, the UV-Visible spectrum of **1** in the presence of the protein (for λ>350 nm) does not change during 24 h of contact time – data not shown.

The relative intensity of the two ICD bands 1 and 2 change with increasing incubation time (**Figure SI.4.A** and **SI.4.B**), with a concomitant blue shift (*ca.* 10 nm) in $\Delta\varepsilon_{max}$ for both. In equilibrium conditions the same two ICD bands are observed and their relative intensity seems to be concentration dependent (**Figure SI.4.C**): while band 1 is intensified by increasing the complex concentration, only modest changes are observed for band 2 after the 1:1 {complex **1**: protein} molar ratio. These observations can be tentatively explained supposing the existence of two different binding sites for complex **1** in HSA. One of the sites probably becomes saturated at a 1:1 molar ratio in equilibrium conditions. The time dependence of the relative intensity of the two bands suggests a kinetic preference for one of the binding sites (possibly more solvent accessible), yielding in due time a more stable thermodynamic interaction. Upon addition of a second mole equivalent of complex (**Figure 4.C**), the pattern of the CD signal changes: band 1 (at *ca.* 385 nm) increases intensity, while band 2 (at *ca.* 500 nm) shifts to the red. Therefore, it is clear that complex **1** binds to HSA, this binding occurring quite fast. If an excess of complex is added (not probable *in vivo*), a second weaker binding site (corresponding to a distinct CD signal) is also available.

This hypothesis is corroborated by the fluorescence measurements (see section 4.4.2). Steady-state and time resolved fluorescence data for the binding of **1** to HSA is consistent with two different interactions: one involving the loose binding of **1** to the protein (accounting for a dynamic quenching process observed) and a binding site within the van der Waals distance of Trp214 (originating the static quenching process).

Supporting Information

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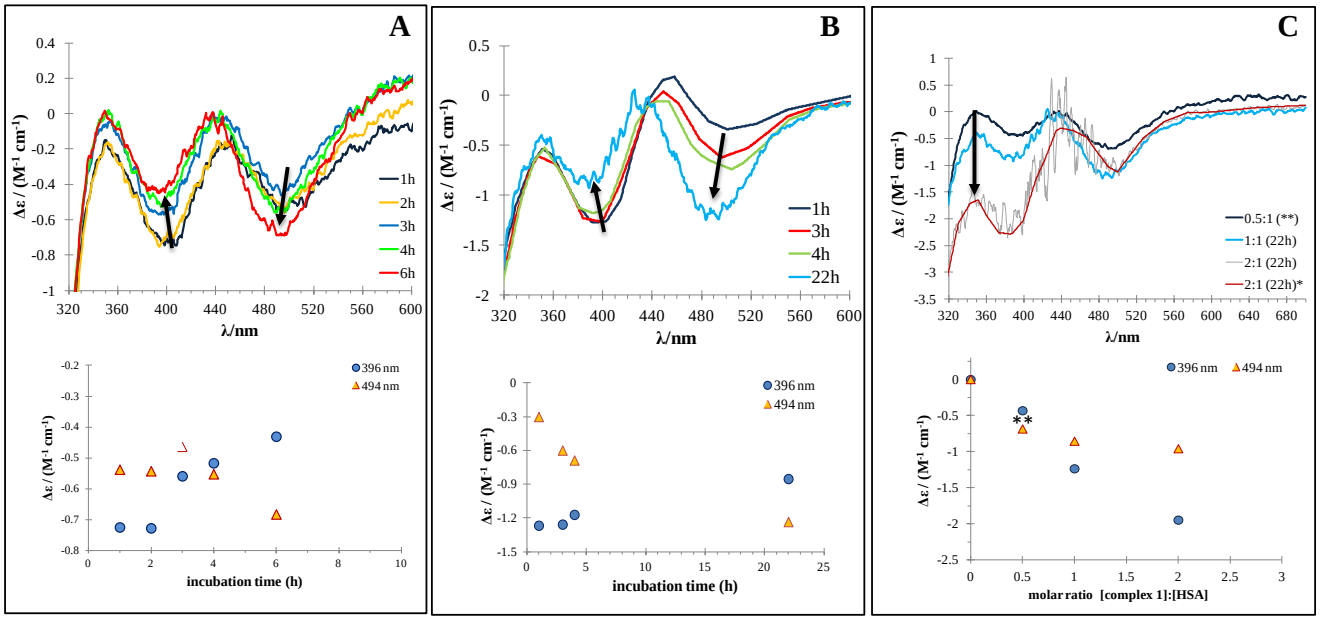

Figure SI.4. Binding of complex **1** to HSA monitored by CD spectroscopy: **A)** Time dependence: *top* - Induced CD (ICD) signal recorded for a solution of HSA (100 μM) with **1** at a 1:0.5 protein:complex molar ratio incubated with time at 37ºC (arrows indicate changes observed with increasing incubation time); *bottom* - corresponding evolution of the molar differential absorption values (Δε) for ICD band 1 (circles) and band 2 (triangles) with increasing incubation time; **B)** Time dependence till equilibrium conditions: *top* - ICD signal recorded for a solution of HSA (100 μM) with **1** at a 1:1 protein:complex molar ratio incubated with increasing time up to 22 h at 37ºC; *bottom* - corresponding evolution of Δε for band 1 (circles) and band 2 (triangles) with increasing incubation time; **C)** ICD spectra in equilibrium conditions. *top* - Changes in ICD signal with increasing concentration of **1** (*: noise-filtered spectrum for the 1:2 protein:complex molar ratio; **: ICD spectrum for 6 h incubation time): *bottom* - Changes in Δε observed for band 1 (circles) and band 2 (triangles) with increasing concentration of complex **1**.

ICD spectra observed in solutions containing HSA and **4** are far more intense than those recorded for **1**, exhibiting a quite different pattern that also changes with increasing incubation time – **Figure SI.5**. Strong ICD signals are recorded after as short as a 10 min incubation time (**Figure SI.5.A1**) showing several bands – an intense positive one at ~500 nm (503 nm), a less intense negative band at ~ 400 nm (416 nm) and a third negative band at *ca.* 340 nm (possibly another one hinted at lower wavelengths, also negative). With increasing incubation time the intensity of all ICD bands slightly increases with a concomitant blue shift of ca. 10 nm up to 1 h, after which the intensity of the positive ICD starts decreasing (**Figure SI.5.A2** and **SI.5.A3**). In equilibrium conditions (after 24 h) a completely different CD spectrum is observed with inversion of the signal of the two main ICD bands of lower energy (**Figure SI.5.B**).

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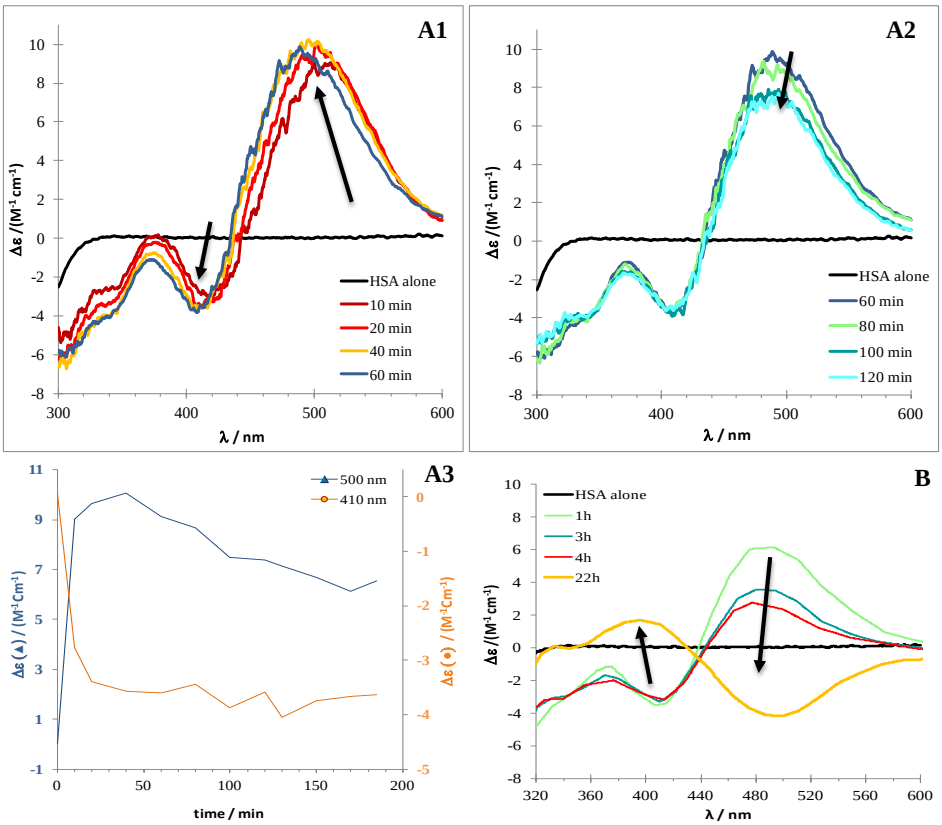

Figure SI.5. Time dependence of binding of complex **4** to HSA: **A1/A2)** Induced CD (ICD) signal for a 100 μM solution of HSA incubated with **4** at a 1:2 protein:complex molar ratio recorded at (32 ± 2) ºC up to 2h, and **A3)** corresponding evolution of the molar diferencial absorption values (Δε) for the positive 500 nm (triangles, *left Y axis*) and the negative 410 nm (circles, *right Y axis*) ICD bands with increasing incubation time; **B)** Evolution of the ICD signal for higher incubation times highlighting the inversion of the Cotton effect in equilibrium conditions (signal recorded at room temparature for a 100 μM HSA solution incubated with **4** at a constant 1:1 molar ratio kept with stirring at (37±0.5) ºC between measurements). Arrows highlight the changes observed with increasing time in all ICD spectra.

The strong ICD signal observed after an incubation time as short as 10 min suggests that the complex is binding to the protein as a whole entity. This is also supported by the UV-Visible spectra of **4** in the absence and in the presence of the protein (**Figure SI.6**). The low energy band shows a ~30 nm red shift after 10 min (at 32±2 ºC), followed by a small blue shift and an intensity loss with increasing incubation time up to 1h (~3 nm), after which the absorbance increases slightly until 2 h (**Figure SI-6.C**). After 22 h incubation, the spectrum of HSA-bound complex **4** shows λ_{max} at 455 nm (25 nm red shift) and a 23% hypocromism when compared to that of the free complex (see main text, section 4.4.1, for the conclusions drawn).

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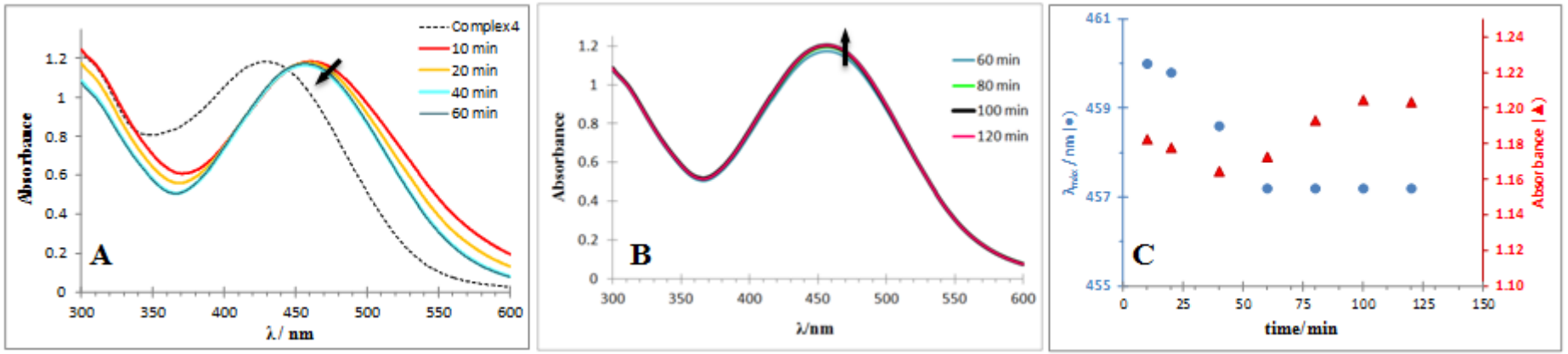

Figure SI.6. Time dependence of HSA binding for complex 4: **A+B)** UV-Visible spectra recorded for complex **4** (200 μM, dashed black line) and (colored full lines) for a solution of HSA:**4** at a 1:2 molar ratio (100 μM : 200 μM) recorded over time up to 2h at; **C)** Change in λ_{max} (blue closed circles, ●) and in the corresponding absorbance (red full triangles, ▲) in the spectra of **4** upon HSA binding over time (up to 2h). Samples in pH7.4 PBS/1%DMSO incubated at T = (32±2) º C; CD spectra were recorded at the same temperature.

Supporting Information

"Screening organometallic binuclear thiosemicarbazone ruthenium complexes as potential anti-tumour agents: cytotoxic activity and human serum albumin binding mechanism"

3. *Mechanism of Förster Resonance Energy Transfer (FRET) on the HSA–complex 4 system*

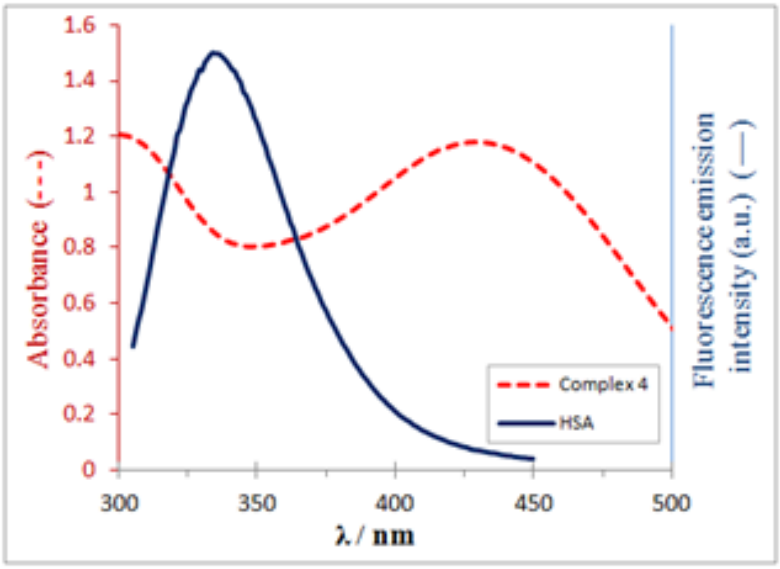

Figure SI.7. Spectral overlap in 2% DMSO/PBS of the HSA fluorescence emission and complex **4** absorption: (red dashed line, ---) Absorbance spectrum obtained for a 200 μM solution of complex 4 (0.5 cm path quartz cuvette); (blue full line, —) Trp214 emission of HSA (λ_{exc}=295 nm).