

# Ruthenium-containing Linear Helicates and Mesocates with Tuneable p53 Selective Cytotoxicity in Colorectal Cancer Cells.

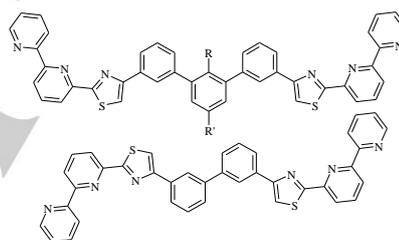
Simon J. Allison, David Cooke, Francesca S. Davidson, Paul I. P. Elliott, Robert A. Faulkner, Hollie B. S. Griffiths, Owen J. Harper, Omar Hussain, P. Jane Owen-Lynch, Roger M. Phillips,\* Craig R. Rice,\* Samantha L. Shepherd and Richard T. Wheelhouse.

**Abstract:** The ligands  $L^1$  and  $L^2$  both form separable dinuclear double stranded helicate and mesocate complexes with Ru(II). In contrast to clinically approved platinates the helicate isomer of  $[Ru_2(L^1)_2]^{4+}$  was preferentially cytotoxic to isogenic cells (HCT116 p53<sup>-/-</sup>) which lack the critical tumour suppressor gene. The mesocate isomer shows the reverse selectivity with the achiral isomer being preferentially cytotoxic towards HCT116 p53<sup>+/+</sup>. Other structurally similar Ru(II)-containing dinuclear complexes showed very little cytotoxic activity. This study demonstrates that alterations in ligand or isomer can have profound effects on cytotoxicity towards cancer cells of different p53 status and suggests that selectivity can be 'tuned' to either genotype. In the search for compounds that can target difficult to treat tumours that lack the p53 tumour suppressor gene,  $[Ru_2(L^1)_2]^{4+}$  is a promising compound for further development.

The transition metal helicate is one of the simplest architectures found in supramolecular chemistry.<sup>1</sup> This species is formed by the use of a ligand which can partition into two separate binding sites, each of which coordinates a different metal ion. The cation's coordination sphere is completed by another ligand which wraps around both metal ions giving (in the simplest form) a dinuclear double helicate  $[M_2L_2]^{n+}$ . The varieties of linear transition metal helicates can be diverse with examples containing 2, 3 and 4 ligands and between 2 – 5 metal ions reported.<sup>2-7</sup> To produce a "true" helicate assembly the ligand must adopt an S-type arrangement where each of the metal binding domains coordinates a different metal ion but the ligand twists in the centre generating the homochiral ( $\Delta\Delta$  or  $\Lambda\Lambda$ ) helicate. If the ligand coordinates two different metal ions but the ligand strand doesn't twist (referred to as a C-type arrangement) then this "side-by-side" complex is referred to as the achiral ( $\Delta\Delta$  or  $\Lambda\Lambda$ ) meso-helicate (or mesocate).<sup>8-14</sup>

Previously, it has been demonstrated that the formation of mesocate and helicates can be controlled by the steric interactions between ligand strands. For example, the ligand  $L^1$ ,

(Fig. 1) forms dinuclear self-assembled complexes with divalent transition metal ions e.g.  $[M_2(L^1)_2]^{4+}$ . In these species there is a substantial twist about the ligand strand and it adopts an S-type arrangement, resulting in the formation of a dinuclear double helicate. Reaction of divalent metal ions with  $L^3$ , which contains a methoxy substituent on the central phenyl unit, again produces a dinuclear species e.g.  $[M_2(L^3)_2]^{4+}$  but in these self-assembled architectures there is no twist around the ligand chain and it adopts a C-type arrangement giving a dinuclear double mesocate. The difference in structures is attributed to *intra*-ligand steric interactions which governs the formation of either helicate or mesocate.<sup>15</sup>



**Figure 1.** Ligands  $L^1$  R = R' = H.  $L^2$  R = Me, R' = H.  $L^3$  R = OMe, R' = Me (top) and  $L^4$  (bottom).

Whilst initially the transition metal helicate was purely of academic curiosity the similarity of the shape of the helicate to an  $\alpha$ -helix (which is a common motif in the secondary structure of proteins) has fuelled interest in the potential biological applications resulting in the discovery of some interesting properties.<sup>16</sup> For example, Hannon and co-workers have shown that an Fe(II)-containing dinuclear triple helicate (e.g.  $[Fe_2L_3]^{4+}$ ) interacts strongly with duplex DNA, binding in the major groove,<sup>17</sup> and displays both anti-cancer<sup>18</sup> and anti-bacterial properties.<sup>19</sup> Other Fe(II)-containing examples include Scott and co-workers "head-to-head-to-tail" helicates which show *in vitro* cytotoxic activity against a range of cancer cell lines with IC<sub>50</sub> values lower than *cis*-platin against HCT116 p53<sup>+/+</sup> cancer cells.<sup>20</sup>

Work has also focused upon the synthesis of Ru(II)-containing helicates and the study of their cytotoxic activity.<sup>21</sup> However, whilst the formation of helicates using labile first-row transition metal ions is well established the formation of the corresponding Ru(II)-containing species is more challenging. This is a consequence of the reversibility of the bonding between labile metal ions and the ligand strands, which allows a number of molecular permutations to be accessed until the thermodynamic product is achieved. However, products arising from reaction with inert metal ions and ligand strands tend to produce kinetic products requiring the desired complexes to be separated, often from polymeric materials.<sup>22</sup>

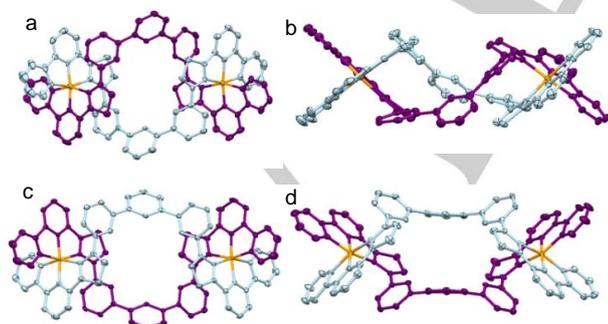
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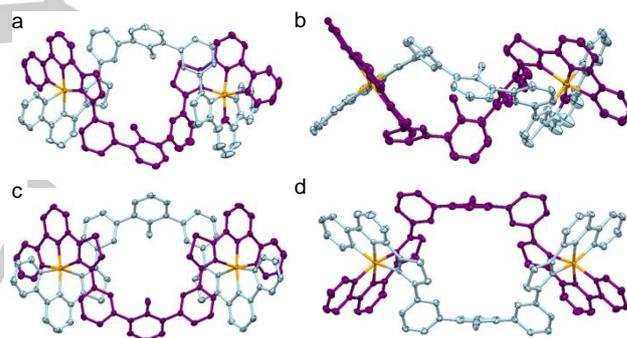
However, despite the synthetic challenge, Ru(II) compounds are attractive as they have been shown to possess interesting photophysical, redox and cytotoxic properties.<sup>23</sup> For example, Hannon and co-workers show a dinuclear triple helicate formed from a bis-pyridylimine and Ru(II) binds and distorts the structure of DNA resulting in cytotoxicity against breast cancer cell lines.<sup>22</sup> A similar bis-bidentate ligand containing two azopyridine donor units forms an unsaturated dinuclear double helicate with Ru(II) (e.g.  $[(RuCl_2)_2L_2]$ ) of which both the *cis/trans* and the *trans/trans* show activity against HBL100 breast-cancer cell lines but the latter isomer exhibits 30 fold more potent cytotoxicity.<sup>24</sup> To date the majority of this work has been limited to dinuclear triple helicates and saturated dinuclear double helicates have remained largely unexamined. Furthermore, biological activity of the helicate's achiral twin, the mesocate, has not been previously reported. Herein this paper discusses the formation of Ru(II)-containing double helicates and gives the first reported examples of ruthenium mesocates. The paper also reports the first example of the selectivity of these compounds towards a cancer genotype, namely p53.

Reaction of  $L^1$  with  $Ru(dmsO)_4Cl_2$  in ethylene glycol at 200°C produces a dark red solution after 24 hrs. Column chromatography produced an orange crystalline material which gave an ion in the ESI MS at  $m/z$  2048 corresponding to a dinuclear species containing two metal ions and two ligand strands *i.e.*  $\{[Ru_2(L^1)_2](PF_6)_3\}^+$ . However, examination of the  $^1H$  NMR showed more than one species is present and further chromatography showed this initial fraction could be isolated as two species, both of which had almost identical ions in the ESI-MS but different signals were observed in the  $^1H$  NMR (see ESI). Analysis by X-ray crystallography showed that both fractions are dinuclear species containing two Ru(II) ions with the ligand partitioned into two tridentate thiazole-bipyridine domains separated by a triphenylene spacer unit. Each domain coordinates a different metal ion with the other ligand completing the Ru(II) coordination sphere. However, in one of the dinuclear complexes there is a substantial twist around the ligand axis giving a dinuclear double helicate **1a** (Fig 2 a and b). In the other fraction the ligands do not twist and a "side-by-side" complex is produced and the resulting species is a dinuclear double mesocate **1b** (Fig 2 c and d).



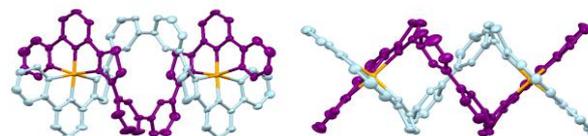
**Figure 2.** The dinuclear complexes of Ru(II) with  $L^1$ . a) and b) two views of the helicate **1a**  $[Ru_2(L^1)_2]^{4+}$  and c) and d) Two views of the dinuclear double mesocate **1b**  $[Ru_2(L^1)_2]^{4+}$ . Thermal ellipsoids shown at 50% probability level. Hydrogen atoms and counter ions omitted for clarity.

Ligand  $L^2$  is similar to  $L^1$  but contains a methyl substituent on the central phenyl ring. In an analogous fashion to  $L^1$ ,  $L^2$  reacts with  $Ru(dmsO)_4Cl_2$  and after initial purification an orange crystalline material was produced which gave ions in the ESI-MS at  $m/z$  2075 corresponding to  $\{[Ru_2(L^2)_2](PF_6)_3\}^+$  and 965 corresponding to  $\{[Ru_2(L^2)_2](PF_6)_2\}^{2+}$  (see ESI). After further chromatography, these could be separated into two species and analysis by X-ray crystallography confirmed that these two species are both dinuclear assemblies *i.e.*  $[Ru_2(L^2)_2]^{4+}$  but one is the helicate **2a** (Fig 3 a and b) and other the mesocate **2b** (Fig 3 c and d). We have previously shown that in these types of ligand systems the helicate assembly is favoured due to *inter-* and *intra*-ligand  $\pi$ -stacking interactions within the dinuclear assembly. However, in the Ru(II) system the helicate and mesocate are formed in similar amounts, although this can slightly vary from reaction to reaction. Molecular modelling shows that in both cases the mesocate is the more stable species; for  $[Ru_2(L^1)_2]^{4+}$  the mesocate **1b** is more stable by 11.21 kJmol<sup>-1</sup> whereas this is more pronounced for the methyl derivative ( $[Ru_2(L^2)_2]^{4+}$ ) with the mesocate **2b** 13.71 kJmol<sup>-1</sup> more stable than the helicate isomer **2a** as would be expected due to the steric bulk of the  $-CH_3$  unit on the central spacer. However, due to the kinetic inert nature of Ru(II) both the helicates **1/2a** and mesocates **1/2b** can be isolated as the Ru(II) ion allows access to both the kinetic and thermodynamic products.



**Figure 3.** The dinuclear complex form from reaction of Ru(II) with  $L^2$ . a) and b) two views of the helicate **2a**  $[Ru_2(L^2)_2]^{4+}$  and c) and d) Two views of the dinuclear double mesocate **2b**  $[Ru_2(L^2)_2]^{4+}$ . Thermal ellipsoids shown at 50% probability level. Hydrogen atoms and counter ions omitted for clarity.

Reaction of  $L^4$  with  $Ru(dmsO)_4Cl_2$  in an analogous fashion gives after purification the dinuclear species  $[Ru_2(L^4)_2]^{4+}$  (Fig 4). However, only the helicate isomer is obtained and no mesocate is observed. This can be attributed to the reduced flexibility of the diphenylene spacer and imparts a natural twist on the ligand strand preventing the formation of the mesocate.

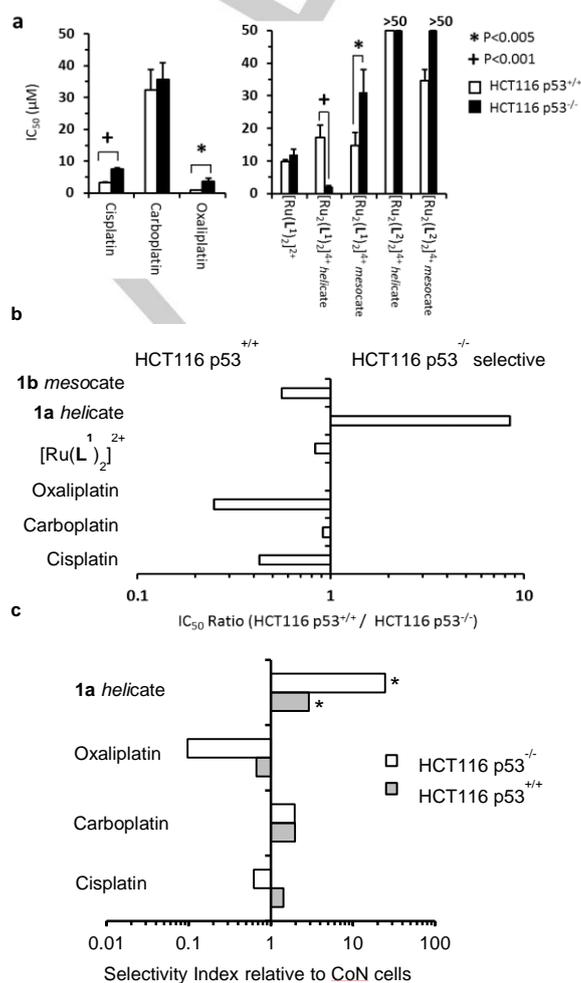


**Figure 4.** The dinuclear complex  $[Ru_2(L^4)_2]^{4+}$  formed from reaction of Ru(II) with  $L^2$ . Thermal ellipsoids shown at 50% probability level. Hydrogen atoms and counter ions omitted for clarity.

To investigate whether these new ruthenium helicates and mesocates have any cytotoxic activity against cancer cells *in vitro*, chemosensitivity studies were performed. One major limitation of many chemotherapeutic agents in clinical use is their reduced cytotoxicity towards cancer cells that lack the tumour suppressor p53.<sup>25-29</sup> To investigate the impact of p53 on any cytotoxic activity of these novel compounds, p53 wild-type and p53-null isogenic cancer cell clones of the human colorectal cancer cell line HCT116 were utilised.<sup>30</sup> These have been extensively used as *in vitro* cancer cell models to investigate and elicit p53-dependent effects.<sup>31-35</sup> Chemosensitivity assays revealed that neither the methyl substituted helicate **2a** or the diphenyl-containing helicate ( $[\text{Ru}_2(\text{L}^4)_2]^{4+}$ ) were active against either the p53<sup>+/+</sup> or p53<sup>-/-</sup> HCT116 cancer cells ( $\text{IC}_{50} > 50 \mu\text{M}$ ) whereas the mesocate isomer (**2b**) showed some, albeit modest, activity towards the p53 wild-type cancer cells that was comparable to the cytotoxicity of platinate carboplatin (Fig 5a). The mononuclear derivative  $[\text{Ru}(\text{L}^1)_2]^{2+}$  (see ESI), where two ligands are coordinated to one metal ion, showed a degree of potency that was comparable to cisplatin and oxaliplatin but lacked selectivity towards the p53<sup>+/+</sup> or p53<sup>-/-</sup> cancer cells (Fig 5b). The achiral mesocate **1b** was also active against both cell lines but it was ~2-fold more active against the p53<sup>+/+</sup> cells (Fig 5b). In terms of selectivity, this was similar to cisplatin and oxaliplatin which also showed selectivity towards the HCT116 p53<sup>+/+</sup> cancer cells. However, the unsubstituted helicate **1a** was substantially more cytotoxic towards the p53<sup>-/-</sup> cells (Fig 4a/b). This preferential cytotoxicity of the **1a** helicate towards the p53<sup>-/-</sup> cancer cells was independently confirmed by two different experimental approaches. First, the transient transfection of wild-type p53 into these p53<sup>-/-</sup> cancer cells and resulting expression of p53 reduced the activity of the **1a** helicate against the p53<sup>-/-</sup> cells such that the effects of 48h exposure to the **1a** helicate appeared similar to that of the vehicle control (Fig 6a). In the converse experiment, partial knockdown of p53 (~50% reduction in protein expression) in the HCT116 p53<sup>+/+</sup> cells using a previously validated siRNA against p53<sup>36</sup> led to a small but statistically significant increase in the potency of **1a** (Fig 6b). These results were reproduced in RKO and LoVo colorectal carcinoma cell lines (see ESI). Furthermore, initial studies demonstrate that knockdown of p53 in RKO cells is associated with increased apoptosis induced by **1a** (see ESI).

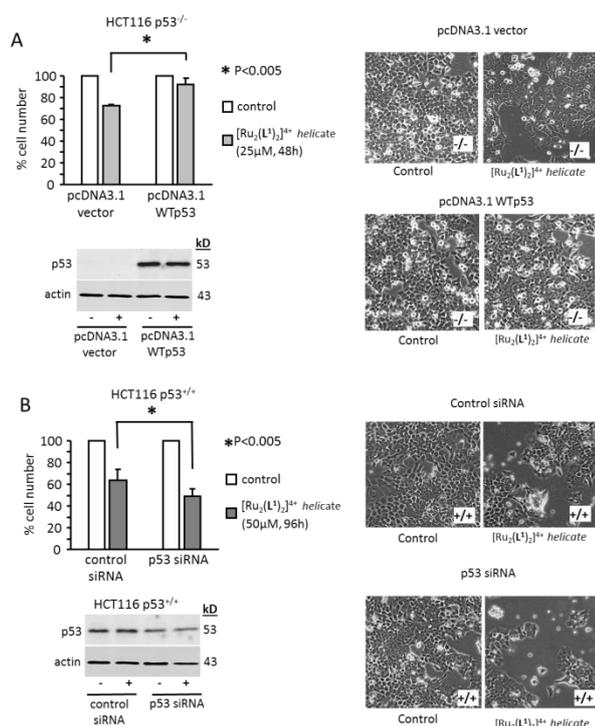
The observed preferential cytotoxicity of the **1a** helicate against the p53-null cancer cell clones is highly significant as mutations in the p53 gene leading to loss of p53 tumour suppressor function are very common in cancers and are typically associated with poor clinical outcome.<sup>37-38</sup> There is an urgent need for new chemotherapeutic agents that are effective against such cancers. The approach advocated here is to identify novel compounds that are active against cells that lack p53. Small molecule organometallic compounds including ruthenium (II) compounds have been shown to induce cell death via p53-dependent and independent mechanisms<sup>39</sup> but typically, the clinically approved platinum based complexes are less active against p53-deficient cells than wild-type cells (Fig 5).<sup>40</sup> The demonstration that the  $[\text{Ru}_2(\text{L}^1)_2]^{4+}$  helicate is significantly more potent against p53 null HCT116 cells is therefore a significant finding in the context of finding drugs that target hard to treat

p53-null tumours. In addition to selectivity towards HCT116 p53<sup>-/-</sup> cells, **1a** helicate is selectively toxic towards tumour cells compared to normal colon epithelia cells (Fig 5c). In contrast to the established platينات, selectivity for HCT116 cells as opposed to both normal colon epithelia CoN cells (Fig 5c) and non-cancer ARPE-19 cells (see ESI) was significantly higher.

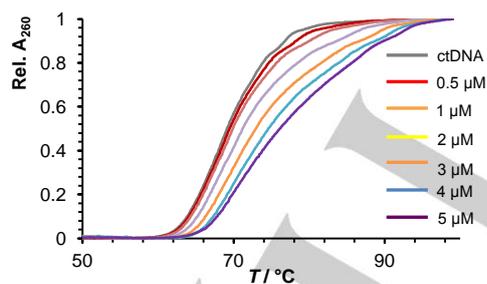


**Figure 5.** Potency and selectivity towards p53 wild-type and p53 null HCT116 colorectal carcinoma cells *in vitro*. a) The potency of  $\text{IC}_{50}$ . b) Differential selectivity of compounds towards either the p53<sup>+/+</sup> or the p53<sup>-/-</sup> HCT116 cancer cells. c) Selectivity index for HCT116 p53<sup>-/-</sup> and HCT116 p53<sup>+/+</sup> cells relative to normal colon epithelia CoN cells. The asterisk indicates that for **1a** helicate, true selectivity index values could not be determined as no  $\text{IC}_{50}$  could be obtained against CoN cells at the highest concentration (50  $\mu\text{M}$ ) tested.

UV thermal melting profiles for ctDNA in the absence and presence of **1a** are shown in Fig 7. These revealed a concentration dependent shift of the DNA melting temperature ( $T_m$ ) indicating that ruthenium helicate **1a** is able to stabilise genomic DNA. At ligand concentrations of 4  $\mu\text{M}$  and above, there was evidence of ligand redistribution and therefore the  $\Delta T_m$  analysis breaks down. At all ligand concentrations and the higher temperature region, the melting profile was disproportionately shifted to the right indicating a marked preference for stabilisation of GC- rather than AT-rich sequences (Fig 7).



**Figure 6.** Validation of the role of p53 in the response of cells to helicase **1a**. a) represents the transfection of wild-type p53 or vector control into HCT116 p53<sup>-/-</sup> cancer cells (left hand side) and modulation of p53 protein expression levels in these cells is indicated by immunoblot analyses. Representative images of vector control cells and transfected cells treated with or without **1a** is presented on the right-hand side. These results demonstrate that transfection of wild type p53 into p53 null cells significantly reduces the potency of **1a**. b) the effect of p53 knockdown in HCT116 p53<sup>+/+</sup> cells using siRNA on the potency of **1a**. siRNA knockdown partially reduced the expression of p53 as indicated in the immunoblot images and caused a small but statistically significant increase in the potency of **1a**. Representative images of cells treated with **1a** are presented on the right-hand side.

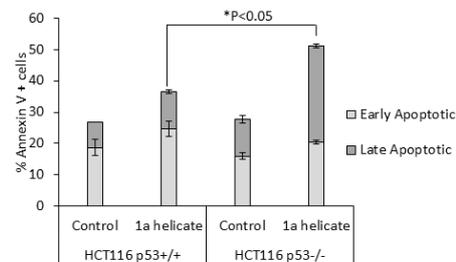


**Figure 7.** The interaction of helicase **1a** with DNA. Normalised thermal melting profiles of calf thymus DNA (50 μM) in the absence and presence of **1a** (from l to r, [1a] = 0, 0.5, 1, 2, 3, 4, 5 μM). All samples contained 0.25% DMSO.

The parameter  $\Delta T_m^{80}/\Delta T_m^{20}$  provides a semi-quantitative indication of the excess of GC over AT stabilisation<sup>41</sup> and whilst a  $\Delta T_m^{80}/\Delta T_m^{20} \approx 1$  indicates no sequence preferential effect on the melting curve, **1a** helicase generated a  $\Delta T_m^{80}/\Delta T_m^{20} \gg 1$  indicates a marked preference for stabilising GC-rich sequences (see ESI). ICP-MS studies demonstrated that **1a** helicase is taken up into the nucleus of cells (see ESI). The levels of **1a** helicase in both HCT116 p53<sup>+/+</sup> and HCT116 p53<sup>-/-</sup> cells are similar suggesting that differential drug uptake is unlikely to

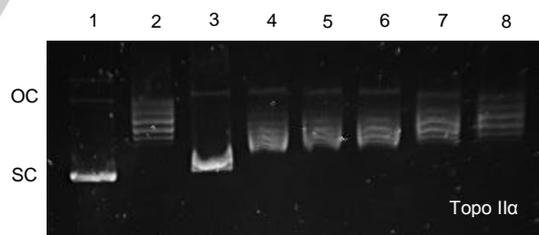
explain the increased sensitivity of HCT116 p53<sup>-/-</sup> cells to **1a** helicase.

Helicase **1a** was found to induce cell death by apoptosis in both HCT116 p53<sup>+/+</sup> and p53<sup>-/-</sup> cancer cells. The proportion of cells in late stage apoptosis were higher in the p53<sup>-/-</sup> cancer cells than their p53<sup>+/+</sup> isogenic clones (Fig 8) correlating with the preferential cytotoxicity in chemosensitivity assays of **1a** towards p53<sup>-/-</sup> cells.



**Fig. 8.** Summary of the percentage of early and late apoptotic cells (annexin V-positive) in response to treatment of HCT116 p53<sup>+/+</sup> and p53<sup>-/-</sup> cells with **1a** helicase or solvent control.

In light of the observed binding of **1a** to DNA and its nuclear localization, its ability to inhibit topoisomerases I and II $\alpha$  was determined. **1a** induced a dose dependent, partial inhibition of topoisomerase II $\alpha$  (Fig. 9). The exact mechanism of inhibition is not known but is consistent with its ability to bind to DNA. In contrast, no inhibition of topoisomerase I was observed. Whilst p53 proficiency or deficiency does not affect cellular response to topoisomerase I inhibitors,<sup>42</sup> p53 deficiency is known to sensitize cells to topoisomerase II inhibitors.<sup>43</sup> It is possible therefore that the observed selectivity of **1a** helicase for p53 null cells is mediated through inhibition of topoisomerase II.



**Fig. 9.** Inhibition of purified human topoisomerase II  $\alpha$  by helicase **1a**. Lanes 1 and 2 represent control reactions with (lane 2) and without (lane 1) topoisomerase present. Lane 3 represents a control reaction without topoisomerase enzymes present but with **1a** at 10 μM. Lanes 4 to 8 represent reactions in the presence of **1a** at 10 μM (lane 4), 5 μM (lane 5), 2.5 μM (lane 6), 1.25 μM (lane 7) and 0.625 μM (lane 8). SC and OC denote the supercoiled and open circular forms of pBR322 DNA respectively.

This study gives valuable insight into the chemical composition and the shapes of the helicase system that are required to form species that are, a) selectively active against cancer cells as opposed to normal cells and, b) have preferential cytotoxicity towards cells either lacking or expressing the tumour suppressor p53. Compared with the mononuclear form, it is clear from the data presented that the dinuclear nature of the helicase is required to form a derivative that is preferentially selective towards cancer cells either with or without p53 (comparison of  $[\text{Ru}(\text{L}^1)_2]^{2+}$  vs  $[\text{Ru}_2(\text{L}^1)_2]^{4+}$ ). The data also

suggests that the type of twist present within the system (e.g. **1a** helicate vs **1b** mesocate) can switch the direction of p53 selectivity. However, subtle changes in the ligand strand can result in a significant reduction in the toxicity as very little activity was observed upon the introduction of a methyl unit (e.g. **2a** helicate and **2b** mesocate) or using a diphenyl spacer (e.g. helicate-[Ru<sub>2</sub>(L<sup>4</sup>)<sub>2</sub>]<sup>4+</sup>). These findings indicate that the helicate structure can be 'fine-tuned' with profound downstream effects both on toxicity and p53 selectivity. Given the frequent loss of p53 tumour suppressor function in cancers as well as p53 mutations that can result in oncogenic gain of function, this study demonstrates that the helicate system is worthy of future investigation as an emerging potential source of new anti-cancer drugs.

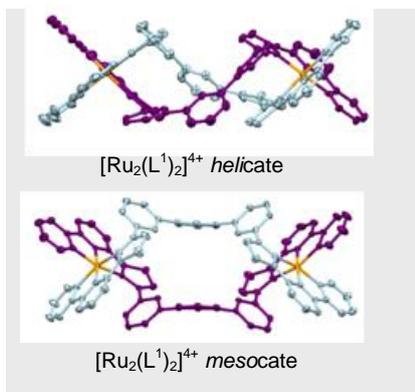
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Layout 1:

## COMMUNICATION

The bis-tridentate ligand  $L^1$  forms both the dinuclear double helicate and mesocate upon reaction with Ru(II). Cytotoxic studies show that the helicate is selective to HCT116 p53<sup>-/-</sup> cancer cells whereas the mesocate is selective to HCT116 p53<sup>+/+</sup>.

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Layout 2:

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