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Anticancer activity of electron-deficient metal complexes against colorectal cancer *in vitro* models

Maria Azmanova, Dr. Joan Soldevila-Barreda, Hira Bani Hani, Dr. Rianne M. Lord, Dr. Anaïs Pitto-Barry, Dr. Steven M. Picksley, Dr. Nicolas P. E. Barry*

Abstract: An evaluation of the *in vitro* cytotoxicity of nine electron-deficient half-sandwich metal complexes towards two colorectal cancer cell lines (HCT116 *p53*^{+/+}, HCT116 *p53*^{-/-}) and one normal prostate cell line (PNT2) is presented herein. Three complexes were found to be equally cytotoxic towards both colorectal cancer cell lines, suggesting a *p53*-independent mechanism of action. These complexes are 12 to 34 × more potent than cisplatin against HCT116 *p53*^{+/+} and HCT116 *p53*^{-/-} cells. Furthermore, they were found to exhibit little or no cytotoxicity towards PNT2 normal cells, with selectivity ratios greater than 50. To gain an insight into the potential mechanisms of action of the most active compounds, their effects on the expression levels of a panel of genes were measured using qRT-PCR against treated HCT116 *p53*^{+/+} and HCT116 *p53*^{-/-} cells, and cell cycle analysis was carried out.

Introduction

Colorectal cancer is the third most common cancer, and is responsible for more than 600,000 deaths every year, worldwide.¹ A current limitation in the treatment of colorectal cancer is the reduced cytotoxicity of clinically used chemotherapeutics towards cancer cells which lack the tumour suppressor gene *p53*.² The *p53* transcription factor is considered to be the most important tumour suppressor,³ commonly named as the “Guardian of the genome”,⁴ and protects cells from acquiring genetic damage on a daily basis. However the *p53* tumour suppressor is frequently itself inactivated or mutated during the development of colorectal cancer. The activation of *p53* involves a number of phosphorylation events and post-translational modifications which in turn affect the expression of various *p53*-target genes related to DNA repair, growth arrest, apoptosis, senescence, angiogenesis, and autophagy.⁵ The cellular levels of *p53* protein are controlled by its two negative regulators, MDM2 and MDMX, leading to degradation or inactivation by mutation of the tumour suppressor.⁶ The investigation of the impact of *p53* on the cytotoxic activity of anticancer drug candidates is therefore of great importance. In recent years, half-sandwich complexes of ruthenium, osmium, and iridium have attracted much attention as metallodrug candidates with potential novel mechanisms of action (MoA).⁷⁻¹⁶ In this broad context, we have developed a strong interest in air- and water-stable electron-deficient half-sandwich complexes based on a carborane ligand, and have investigated their applications in materials science¹⁷⁻²³ and in biology.²⁴⁻²⁶ More

recently, we synthesised a family of electron-deficient metal complexes based on the benzene-1,2-dithiolato ligand, a more readily available and easier-to-functionalise ligand than carboranes.²⁷

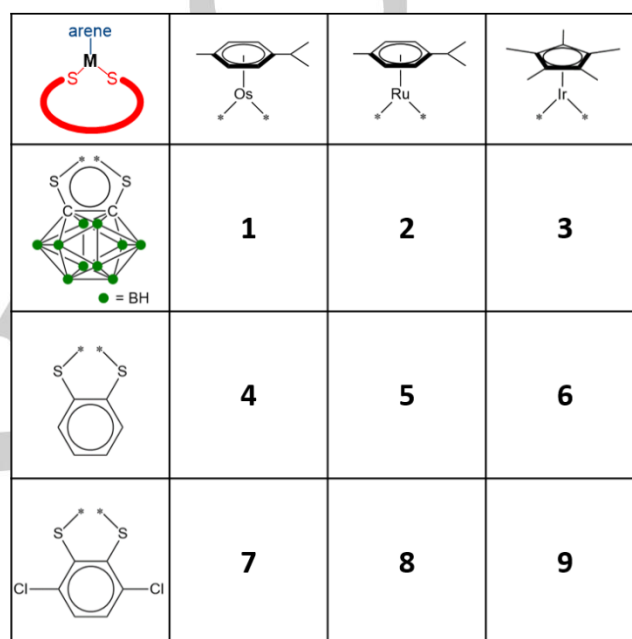


Figure 1. Molecular structures of the electron-deficient half-sandwich metal complexes **1 – 9** studied in this work.

Here we report an evaluation of the cytotoxicity of nine electron-deficient metal complexes ([Os/Ru(*p*-cymene)(1,2-dicarba-*closo*-dodecarborane-1,2-dithiolato)] (**1/2**), [Ir(pentamethylcyclopentadiene)(1,2-dicarba-*closo*-dodecarborane-1,2-dithiolato)] (**3**), [Os/Ru(*p*-cymene)(benzene-1,2-dithiolato)] (**4/5**), [Ir(pentamethylcyclopentadiene)(benzene-1,2-dithiolato)] (**6**), [Os/Ru(*p*-cymene)(dichlorobenzene-1,2-dithiolato)] (**7/8**), [Ir(pentamethylcyclopentadiene)(dichlorobenzene-1,2-dithiolato)] (**9**); Figure 1) towards *p53* wild-type (HCT116 *p53*^{+/+}) and *p53*-null (HCT116 *p53*^{-/-}) isogenic cancer cell clones of the human colorectal cancer cell line HCT116, and towards one normal human prostate cell line (PNT2). HCT116 *p53*^{+/+} and HCT116 *p53*^{-/-} cell lines are routinely used as *in vitro* models to study *p53*-dependent anticancer modes of action of drug candidates.²⁸ Therefore, we have investigated the *p53*-dependence and gene regulation in HCT116 *p53*^{+/+} and HCT116 *p53*^{-/-} cells for the most promising compounds.

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Supporting information for this article is given via a link at the end of the document.

Results

Chemosensitivity assay

Owing to poor water-solubility at millimolar concentration, the stability of all complexes was assessed in pure deuterated dimethylsulfoxide (DMSO- d_6) (1 mM concentration; Figure S1) and NMR spectra at $t = 1$ h, and 24 h were recorded. All complexes are stable under these conditions and are expected to be stable at micromolar concentration in the drug-media solutions which are added to cells (the final DMSO concentrations being less than 0.5% (v/v) in all cases; a slight loss of arene can be noted (free p -cym signals at ca. 7.2 ppm) for complexes **1** and **2** after 24 hours, which is not surprising since DMSO is a strong coordinating solvent). Chemosensitivity studies were then undertaken using a 24-hour MTT assay, with a 72-hour recovery period. The IC_{50} values were determined against HCT116 $p53^{+/+}$ (human colorectal carcinoma, $p53$ -wt) and HCT116 $p53^{-/-}$ (human colorectal carcinoma, $p53$ -null) exposed to each of compounds **1** – **9** or cisplatin (Table 1, Figures S2-S5). Controls used were cisplatin (positive control), and untreated cells for 100% viability (negative control). The three iridium compounds **3**, **6**, and **9** were found to be inactive against all cell lines and have IC_{50} values $> 100 \mu M$. The osmium compounds **1**, **4**, and **7** exhibit a range of cytotoxicity: carborane-containing Os complex **1** is highly active towards both cell lines, whilst the benzene-containing Os complexes **4** and **7** are inactive, whilst the ruthenium complexes **2**, **5**, and **8** show significantly high cytotoxicity against both colorectal cell lines.

Selectivity ratio

One of the major limitations of existing anticancer drugs is their poor selectivity towards cancer cells. Comparing the response of the cancer cell lines to the normal PNT2 cells provides a preliminary indication towards the selectivity of the compounds. The IC_{50} values obtained for complexes **1** – **9** against PNT2 cells highlight compounds which have significant selectivity towards

cancerous cell types (Table 1 and Figure 2). Complexes **1**, **3**, **4**, **6**, **8**, and **9** were found to be non-cytotoxic against PNT2 normal cells, whilst cisplatin and complexes **5** and **7** were moderately cytotoxic. Complex **2** was found to be highly potent against PNT2 cells and lacks any selectivity towards a particular cell type, which could be explained by the ruthenium-carborane combination (ruthenium complexes in this series being the most cytotoxic compounds, and carborane leading to the most active complexes; see Discussion).

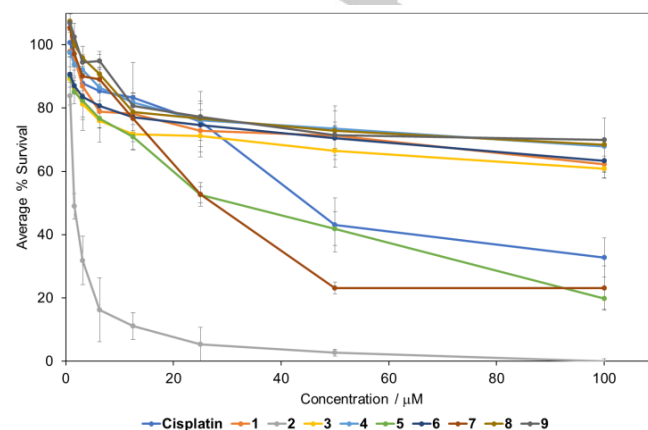


Figure 2. IC_{50} (\pm SD) graphs for complexes **1** – **9**, and cisplatin, against PNT2 normal cells.

To compare the results from colorectal cancer cell lines and PNT2 normal cell line, values have been expressed in terms of a selectivity ratio (SR), which is defined as the ratio of the mean IC_{50} for the normal PNT2 cells divided by the mean IC_{50} for each individual cancer cell line tested (Table 1, Figure S6). SR values > 1 indicate *in vitro* selectivity for cancer cells, and SR = 1 states equitoxicity. Some of these values cannot be stated as an absolute value due to the IC_{50} values being greater than the tested threshold concentration ($> 100 \mu M$).

Table 1. IC_{50} values/ $\mu M \pm SD$ for cisplatin and compounds **1** – **9** against HCT116 $p53^{+/+}$, HCT116 $p53^{-/-}$, and PNT2 cell lines, along with selectivity ratios (SRs; ratio of the mean IC_{50} for the normal PNT2 cells divided by the mean IC_{50} for each individual cancer cell line), selectivity factors (SFs: IC_{50} value of cisplatin for HCT116 $p53^{-/-}$ divided by the IC_{50} values of each compound for HCT116 $p53^{-/-}$), and $p53$ -selectivity (IC_{50} values of HCT116 $p53^{+/+}$ divided by IC_{50} values of HCT116 $p53^{-/-}$).

Complex	Metal	IC_{50} / $\mu M \pm SD$			Selectivity ratio			
		HCT116 $p53^{+/+}$	HCT116 $p53^{-/-}$	PNT2 normal cells	PNT2 / HCT116 $p53^{+/+}$	PNT2 / HCT116 $p53^{-/-}$	Selectivity factor	$p53$ -selectivity
1	Os	6.1 ± 1.6	5.9 ± 2.1	> 100	> 17	> 17	12	1
2	Ru	0.77 ± 0.01	1.05 ± 0.03	1.6 ± 0.3	2	1.5	66	0.7
3	Ir	> 100	> 100	> 100	NA	NA	< 0.7	NA
4	Os	> 100	> 100	> 100	NA	NA	< 0.7	NA
5	Ru	2.8 ± 0.1	2.7 ± 0.2	30 ± 2	11	11	26	1
6	Ir	> 100	> 100	> 100	NA	NA	< 0.7	NA
7	Os	> 100	> 100	27 ± 3	< 0.3	< 0.3	< 0.7	NA
8	Ru	2.2 ± 0.3	2.0 ± 0.3	> 100	> 50	> 50	34	1.1
9	Ir	> 100	> 100	> 100	NA	NA	< 0.7	NA
cisplatin	Pt	51 ± 13	69 ± 18	45 ± 3	0.88	0.65	1	0.74

The iridium compounds **3**, **6**, and **9** show no activity for either cancerous or normal cell lines, and therefore SR have not been determined. Compounds **2** and **7** show SR values which are only moderately >1 and <1 , respectively, and can be classified as non-selective for cancer, similarly to cisplatin (SR = 0.75). Compounds **1**, **5** and **8** demonstrated significant selectivity towards the HCT116 cell lines, with SR > 17 , 11, and 50, respectively. Importantly compounds **1** and **8** show no cytotoxicity towards the normal cell line PNT2, with IC_{50} values $> 100 \mu\text{M}$.

Selectivity factor and p53-selectivity

To obtain an indication on the efficiency of complexes **1** and **8** towards the difficult-to-treat colorectal cell lines, the selectivity factors (SF; defined as the ratio of the IC_{50} value of cisplatin for each cell line divided by the IC_{50} values of each compound for the same cell line) for all the complexes were determined using cisplatin as a reference drug (Table 1, Figure S7). Complex **2** is $66 \times$ more potent than cisplatin, but suffers from moderate selectivity against PNT2 cells. On the other hand, complex **5** is $26 \times$ more potent than cisplatin against HCT116 *p53*^{-/-} cells. Furthermore, complexes **1** and **8** (highly selective) are significantly more potent than cisplatin with SF = 12 and 34, respectively.

Interestingly, complexes **1** – **9** do not exhibit any significant *p53*-selectivity (Table 1, Figure S8), which indicates that their mechanism of action is *p53*-independent. This is of importance since the HCT116 *p53*^{-/-} line can be seen as a more advanced type of colorectal cancer model than the HCT116 *p53*^{+/+}, due to the loss of *p53* gene function that occurs in advanced colon cancer cells.²⁹

The two most active Os and Ru complexes (**1** and **2**, respectively) have been assessed using gene expression studies, and cell cycle analysis by flow cytometry against the cancer cell lines HCT116 *p53*^{+/+} and HCT116 *p53*^{-/-}.

Gene expression studies on compounds 1 and 2

Gene expression studies have been conducted for complexes **1** and **2**, using genes associated with apoptosis and DNA repair mechanisms (Figure 3; Table S2 for the biological roles of the genes studied). Indeed, DNA damage is one of the demonstrated potential MoAs for some half-sandwich complexes.³⁰ The gene expression results are consistent with the antiproliferative data, and show that both complexes **1** and **2** induce upregulation of some key genes associated to apoptosis, including *MGMT* and *CDKN* genes.

Furthermore, both complexes do not seem to induce high levels of DNA damage response: *ALKBH2* is marginally regulated, a gene involved in the protection against methylating agents, which induces repair of DNA lesions.³¹ Moreover, the expression level of *PARP1* is less than 2-fold downregulated, which indicates very low-level of repair of modified bases,³² whilst almost no changes are observed for *BRCA1* which facilitates homologous recombination to maintain genomic stability.³³ These results suggest that complexes **1** and **2** might have a different MoA than cisplatin, a drug which has been shown to significantly upregulate genes involved in DNA damage

response and repair (e.g. *PARP1*, *BRCA1*, *ALKBH3*, *RAD51*; consistent with the DNA alkylating MoA of cisplatin).³⁴

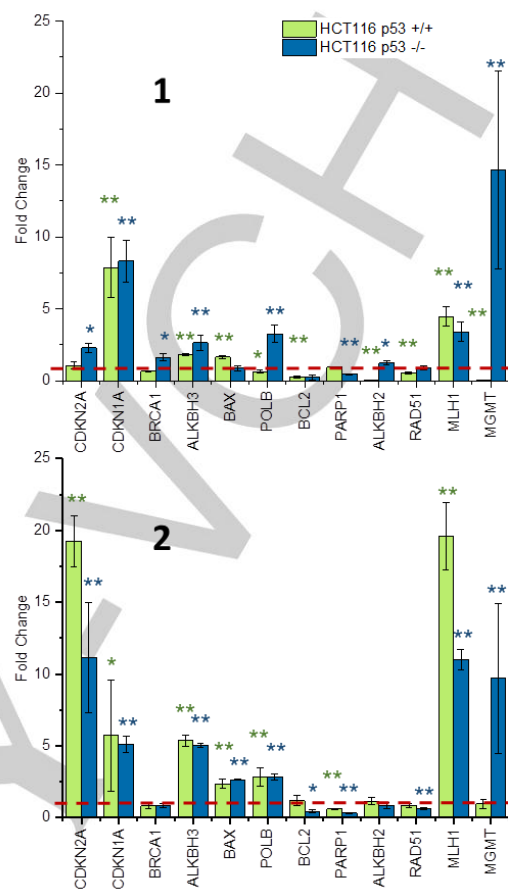


Figure 3. Percentage of increase/decrease \pm SD ($p < 0.01 = **$, $p < 0.05 = *$; 3 independent experiments) of genes expressed in HCT116 *p53*^{+/+} and HCT116 *p53*^{-/-} cells treated with complexes **1** and **2** ($1 \times IC_{50}$).

Cell cycle analysis on compounds 1 and 2

We then confirmed the apoptosis suggested by gene expression studies, by using cell cycle analysis *via* flow cytometry against both cell lines HCT116 *p53*^{+/+} and HCT116 *p53*^{-/-}. Flow cytometry allows a precise analysis of the impact of various functional modulators on the cell cycle,³⁵ and apoptosis can be detected from the loss of DNA from permeabilised cells. The permeabilisation leads to fragmented DNA multimers leaking out of the cells and therefore results in a population of cells with a reduced DNA content. The DNA profile representing cells in G1, S-phase and G2-M is obtained with apoptotic cells being represented by a subG population. As can be seen in Figure 4, compounds **1** and **2** induce a dramatic increase of subG populations (55% and 30%, respectively) thereby confirming the strong apoptotic nature of these complexes.

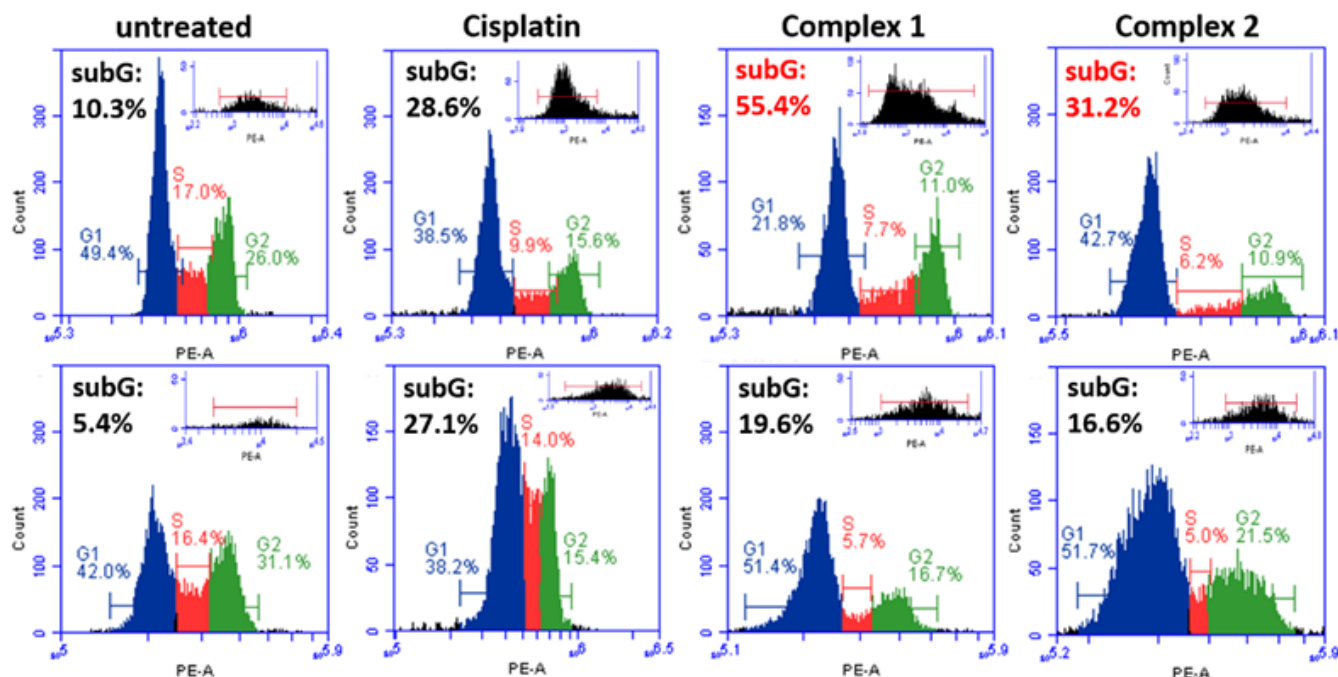


Figure 4. Cell cycle analysis of HCT116 *p53*^{+/+} (up) and HCT116 *p53*^{-/-} (bottom) cells treated with cisplatin, and compounds **1** and **2**.

Discussion

The Ru/Os/Ir complexes investigated in this work show distinct cytotoxic effects in specific cell lines, with complexes **1**, **5**, and **8** exhibiting increased cytotoxicity towards cancer cells than normal PNT2 cells. It is known that the inertness toward substitution reactions of low-spin d^6 half-sandwich complexes³⁶ depends both on the nature of the metal ion (for example, osmium complexes generally exhibit slower kinetics than their ruthenium analogues), and on the nature of the ligands, for example the extensive work of Meggers on rigid, globular, inert octahedral metal complexes of Ru^{II},³⁷ Os^{II},³⁸ Rh^{III},³⁹ and Ir^{III}⁴⁰ explains these effects.

In the case of 16-e organometallic complexes, the electron-deficient nature is believed to be crucial in terms of reactivity with biomolecules both extracellularly or intracellularly, and as such can affect their biological activity. In particular, the lack of toxicity of iridium complexes **3**, **6** and **9** may be due to their high reactivity in solution, as we recently reported that the binding constants between electron-deficient Ir complexes and σ -donor and σ -donor/ π -acceptor ligands are 10 to 100 \times greater than for their Ru and Os analogues.²⁷ As such, the Ir complexes **3**, **6**, **9** behave like “true” electron-deficient complexes (prone to make a more electronically stable 18-e adduct) and are more susceptible to reactions with S- or N-containing (bio)molecules than their “pseudo” electron-deficient Ru and Os analogues **1**, **2**, **4**, **5**, **7**, and **8**.²⁷ Within this work, we highlighted that the inertness of these Ru/Os complexes toward substitution reactions is related to the aromatic nature of the five-membered MS_2C_2 chelate ring (M = Ru/Os). One of the sulfur atoms acts as a 3-e donor to the metal centre, whilst the second sulfur atom is a 1-e donor. This gives the metal the more favorable pseudo

18e configuration, making the complexes unreactive towards nucleophiles and protecting them from poisons. It is our hypothesis that the “pseudo” electron-deficiency of the Ru and Os complexes makes them more inert and as such less prone to be deactivated in media or in cells than the Ir compounds.

If the nature of the metal ion plays an important role in the cytotoxicity of this family of half-sandwich complexes, the nature of the non-innocent ligand is also of importance. Carboranes in particular not only possess very unusual electronic properties (e.g. electron-deficient ligand, non-classical bonding interactions⁴¹) but also are highly hydrophobic. Carborane-containing complexes **1**, **2**, and **3** are therefore expected to be much more hydrophobic than their benzene or dichloro-benzene analogues. It is of note that only the carborane osmium complex **1** is found to be active in the osmium series.

Finally, there are no noticeable differences between the benzene and the dichloro-benzene complexes (both Ru complexes **5** and **8** are highly cytotoxic, both Os complexes **4** and **7** are not active). This suggests that, although halogens are very electronegative and electron withdrawing, therefore pulling the electron density away from the metal centre, the influence of the benzene derivative ligand is negligible compared to the influence of the nature of the metal ion on the antiproliferative activity of these compounds. This opens up interesting avenues for the functionalisation of the dithiol-benzene scaffold.

Conclusions

An evaluation of the cytotoxicity of nine electron-deficient metal complexes of ruthenium, osmium, and iridium (Figure 1; [Os/Ru(η^6 -*p*-cymene)(1,2-dicarba-*closo*-dodecarborane-1,2-dithiolato)] (**1/2**), [Ir(η^5 -pentamethylcyclopentadiene)(1,2-dicarba-*closo*-dodecarborane-1,2-dithiolato)] (**3**), [Os/Ru(η^6 -*p*-cymene)(benzene-1,2-dithiolato)] (**4/5**), [Ir(η^5 -pentamethylcyclopentadiene)(benzene-1,2-dithiolato)] (**6**),

[Os/Ru(η^6 -*p*-cymene)(dichlorobenzene-1,2-dithiolato)] (**7/8**), and [Ir(η^5 -pentamethylcyclopentadiene)(dichlorobenzene-1,2-dithiolato)] (**9**) has been reported, against two *in vitro* human colorectal carcinoma cell lines (HCT116 *p53*^{+/+}, HCT116 *p53*^{-/-}) and one normal prostate cell line (PNT2). Complexes **1**, **5**, and **8** exhibit significantly high cytotoxicity against both colorectal cell lines, and are 12 to 34 × more potent than cisplatin against HCT116 *p53*^{+/+} and HCT116 *p53*^{-/-} cells. Furthermore these three complexes exhibit high *in vitro* selectivity (>50-fold) towards the cancer cells tested, compared to PNT2 normal cells. Complexes **1**, **5**, and **8** have equitoxicity against both HCT116 *p53*^{-/-} and HCT116 *p53*^{+/+} cells, which suggests that they are good potential candidates for the treatment of advanced colorectal cancers and that their mode of action is *p53*-independent.

Finally, some complexes in the series are inactive towards all cell lines, whilst others (**2** and **7**) are toxic towards all cell lines. This result indicates that tailoring electron-deficient metal complexes, a biologically understudied class of half-sandwich complexes, with the right combination of ligands and metal ions, has the potential to open up exciting avenues for the development of anticancer metallodrug candidates.

Future work will focus on identifying the effect of these complexes on specific protein levels, and on understanding the origin of the observed selectivity of complexes **1**, **5**, and **8** towards cancer cells compared to normal cells.

Experimental Section

Materials and instrumentation.

Metals chloride hydrates were purchased from Precious Metals Online. All other reagents were obtained from commercial suppliers and used as received. THF was distilled over calcium hydride. Procedures were performed under nitrogen atmosphere and with pre-dried glassware, unless otherwise stated. Complexes **1** – **9** were prepared according to literature procedures.⁴²⁻⁴⁴

Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 medium, foetal bovine serum (FBS), penicillin and streptomycin, phosphate-buffered saline (PBS, pH 7.4), and other tissue culture reagents were purchased from Gibco (Thermo Fisher Scientific, UK). All other chemicals were purchased from Sigma-Aldrich (UK). DNA KiCqStart SYBR[®] Green validated oligomers were purchased from Sigma-Aldrich and diluted to a concentration of 3 μ M with diethylpyrocarbonate (DEPC)-treated water. Cell lines were provided by the Institute of Cancer Therapeutics, University of Bradford. Aurum[™] Total RNA MiniKit and iScript[™] cDNA Synthesis Kit were purchased from Bio-Rad.

Cells were incubated in a ThermoScientific HERAcCell 150 incubator, and observed under a Nikon ECLIPSE TS100 Microscope. Samples were centrifuged in a Sigma[®] 1-14 centrifuge. PCRs (Polymerase Chain Reaction) were performed in a Bio-Rad T100[™] Thermal Cycler. qPCR (quantitative Polymerase Chain Reaction) assays were performed in a PCR^{max} Eco[™] Illumina[®] Thermal Cycler and results were processed using PCR^{max} ECOstudy software.

Chemosensitivity Assays

In vitro chemosensitivity tests were performed against HCT116 (human colorectal carcinomas): HCT116 *p53*^{+/+} (*p53*-wild type) and HCT116 *p53*^{-/-} (*p53*-null). Cancer cell lines were routinely maintained as monolayer cultures in appropriate medium - RPMI 1640 or DMEM supplemented with 10% foetal calf serum, penicillin (100 I.U./ml) and

streptomycin (100 μ g/ml), sodium pyruvate (1 mM) and L-glutamine (2 mM). For chemosensitivity studies, cells were incubated in 96-well plates at a concentration of 1.0×10^4 cells per well and the plates incubated for 24 hours prior to drug exposure at 37 °C and 5% CO₂. Complexes or cisplatin were each dissolved in dimethylsulfoxide (DMSO) to provide stock solutions which were further diluted with media to provide a range of final concentrations. Drug-media solutions were added to cells (the final concentration of DMSO was 0.5% (v/v) in all cases) and incubated for 24 hours at 37 °C and 5% CO₂. The drug-media was removed from the wells and the cells were washed with PBS (100 μ L) twice, and fresh media (150 μ L) was added to each well. The plates were further incubated for 72 hours at 37 °C in an atmosphere of 5% CO₂ to allow for a period of recovery. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (20 μ L, 2.5 mg/mL) was added to each well and incubated for 2 hours at 37 °C and 5% CO₂. The liquid in each well was then removed and DMSO (100 μ L) was added to each well in order to dissolve the purple formazan crystals. A Thermo Scientific Multiskan EX microplate photometer was used to measure the absorbance in each well at 570 nm. Cell survival was determined as the absorbance of treated cells divided by the absorbance of controls and expressed as a percentage. The IC₅₀ values were determined from plots of % survival against drug concentration. Each experiment was repeated in triplicate of triplicates and a mean value was obtained and stated as IC₅₀ (μ M) \pm SD.

RNA extraction

Aurum[™] Total RNA Mini Kit was used for RNA extraction from treated and untreated HCT116 *p53*^{+/+} and HCT116 *p53*^{-/-} cells, and the concentrations of solutions are stated where provided by the manufacturer. A T-25 flask with cells was washed with PBS (137 mM NaCl, 2.7mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) twice, cells were scraped and transferred to a 2 mL Eppendorf tube, and centrifuged for 2 min at 1200 *g*. A small cell pellet was observed at the bottom of the tube, and the liquid was discarded. Lysis solution (supplemented with 1% β -mercaptoethanol) (350 μ L) and 70% (w/v) ethanol (350 μ L) were added, and the components mixed to reach the desired consistency. The lysate was transferred to an RNA binding column which was placed in a 2 mL wash tube, centrifuged for 30 s at 12000 *g*, and the remaining liquid in the wash tube was discarded. The binding column was then washed with low-stringency solution (700 μ L) and centrifuged for 30 s at 12000 *g*. Diluted DNase I was prepared by mixing reconstituted DNase I (5 μ L) with DNase dilution solution (75 μ L), and the resulting diluted DNase I (80 μ L) was added to the binding column, followed by incubation for 15 min at room temperature. The column was then washed with high-stringency solution (700 μ L) and centrifuged for 30 s at 12000 *g*. It was washed once more with low-stringency wash solution (700 μ L) and centrifuged for 1 min at 12000 *g*. The final RNA product was collected with elution solution (80 μ L) which was added to the binding column, followed by saturation of the membranes for 1 min and centrifuging for 2 min at 12000 *g*. The total eluted RNA samples were quantified by a Nanodrop spectrophotometer and stored at -80 °C.

Synthesis of complementary DNA (cDNA)

iScript[™] cDNA Synthesis Kit was used for the synthesis of cDNA from RNA samples for treated and untreated HCT116 *p53*^{+/+} and HCT116 *p53*^{-/-} cells. 5x iScript Reaction Mix (4 μ L), iScript Reverse Transcriptase (1 μ L), nuclease-free water (variable), and RNA template (variable, 1 μ g-1 pg) were mixed in a PCR tube, where the total volume of the reaction mix was 20 μ L. The complete reaction mix was incubated in a thermal cycler using the following protocol: priming for 5 min at 25 °C; reverse transcription for 20 min at 46 °C; RT inactivation for 1 min at 95 °C; hold at 4 °C. The generated cDNA samples were stored at -20 °C.

Genes

GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as a reference gene and twelve other genes have been used in this study. These are listed in Table S1, with names, primers sequence and their different roles.

Information for the genes have been retrieved from GeneCards⁴⁵ and OMIM⁴⁶.

qPCR assays

No-cDNA template controls for all PCR assays were prepared in order to verify that no contamination has occurred, and master mixes for replicate reactions were made so as to minimise statistical variation in experimental results. All samples were run in triplicates, and *GAPDH* was the housekeeping gene of choice, used as a reference point for the analysis of expression levels of other genes. DEPC-treated water (variable), forward primer (3 μ M, 1 μ L), reverse primer (3 μ M, 1 μ L), iQTM SYBR[®] Green supermix (SYBR[®] Green I dye, 50 U/mL iTaqTM DNA polymerase, dNTPs (0.4 mM each of dATP, dCTP, dGTP, and dTTP), 6 mM MgCl₂, 40 mM Tris-HCl, pH 8.4, 100 mM KCl, 20 nM fluorescein, and stabilizers; 5 μ L), and cDNA template (variable) were added to an EcoTM 48-well plate, with a total reaction volume of 10 μ L. The plate was covered with adhesive seal and placed in the qPCR instrument. The instrument was programmed with the following 2-step qPCR protocol: initial denaturation and enzyme activation for 3 min at 95 °C, 1 cycle; denaturing for 15 s at 95 °C and annealing extension for 30 sec at 60 °C for 40 cycles; melt curve for 10 s at 55-95 °C, 1 cycle.

Gene expression data analysis

The relative expression of twelve different genes, with *GAPDH* as the reference gene, was determined using the 2^{- $\Delta\Delta$ Cq} (Livak) method.⁴⁷ Δ Cq values between the reference and target genes were first calculated, followed by determination of $\Delta\Delta$ Cq values which were used to obtain the fold changes for the chosen genes.

Statistical analysis was performed as a 2 tailed T-test with equal variances using Δ Cq values for treated and untreated samples.

Cell cycle analysis

HCT116 *p53*^{+/+} and HCT116 *p53*^{-/-} cancer cells were seeded in T75 flasks and incubated for 24 hours at 37 °C, 5% CO₂ prior to drug exposure. Complex 1 or 2 was dissolved in DMSO and further diluted down with medium to reach a concentration corresponding to the IC₅₀ value for each cell line (the final concentration of DMSO was less than 0.5% (v/v) in all cases). Cells were washed with PBS twice, treated with the corresponding drug-medium solutions and incubated for 24 hours at 37 °C, 5% CO₂. Following the incubation period, cells were washed with PBS twice, trypsinised and suspended in fresh medium. A cell pellet was obtained by centrifugation at 200 g for 5 min. The medium was discarded; cells were washed with PBS, re-suspended in 70% EtOH and incubated for 30 min at 4 °C. Cells were washed with PBS, resuspended in PBS containing RNase (1 μ g/mL) and incubated for 30 min at 37 °C. Propidium iodide was added to the cells at a final concentration of 20 μ g/mL. Samples were run on a BD Accuri C6 Plus System Flow Cytometer and data was analysed using the BD Accuri C6 Plus software.

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Keywords: Electron-deficient • half-sandwich complexes • colorectal cancer • metallodrugs • gene expression

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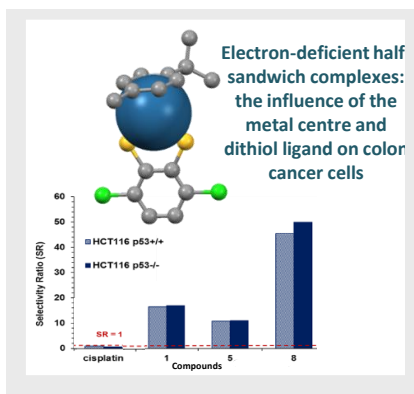
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Entry for the Table of Contents (Please choose one layout)

Layout 1:

FULL PAPER

The high potency of electron-deficient metal complexes against advanced colorectal *in vitro* models is reported. Some complexes exhibit very high selectivity for cancer cells, compared to normal cells, opening up new directions for the development of colorectal anticancer drug candidates.



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Anticancer activity of electron-deficient metal complexes against colorectal cancer *in vitro* models