FULL PAPER

# Preclinical Anticancer Activity of an Electron-Deficient Organoruthenium(II) Complex

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**Abstract:** Ruthenium compounds have shown to be promising alternatives to platinum(II) drugs. However, their clinical success depends on achieving mechanisms of action that overcome Pt-resistance mechanisms. Electron-deficient organoruthenium complexes are an understudied class of compounds which exhibit unusual reactivity in solution and may offer novel anticancer mechanisms of action. Here, we evaluate the *in vitro* and *in vivo* anticancer properties of the electron-deficient organoruthenium complex [(*p*-cymene)Ru(maleonitriledithiolate)]. This compound is found to be highly cytotoxic: 5 to 60 times more potent than cisplatin towards ovarian (A2780 and A2780cisR), colon (HCT116 *p53*+/+ and HCT116 *p53*-/-), and non-small lung H460 cancer cell lines. It shows no cross-resistance and is equally cytotoxic to both A2780 and A2780cisR cell lines. Furthermore, unlike cisplatin, the remarkable *in vitro* antiproliferative activity of this compound appears to be *p53*-independent. *In vivo* evaluation in the hollow fibre assay across a panel of cancer cell types and subcutaneous H460 non-small cell lung cancer xenograft model hints at activity of the complex. Although the impressive *in vitro* data are not fully corroborated by the *in vivo* follow-up, this work is the first preclinical study of electron-deficient half-sandwich complexes and highlights their promises as anticancer drug candidates.

## Introduction

There is an urgent need to find molecules with different anticancer mechanisms of action (MoA) than platinum(II) drugs, particularly for patients who relapse after having been initially treated with a platinum-containing chemotherapy regimen (*ca*. 50% of all anticancer chemotherapy treatments[1]). In this context, ruthenium-based drugs are promising[2] with several complexes exhibiting their anticancer properties *via* MoA different than nuclear DNA binding. For example, half-sandwich ruthenium complexes have shown great potential as catalytic metallodrug candidates,[3] some inert polypyridyl Ru(II) complexes target mitochondria and induce apoptosis[4] while other inert octahedral Ru(II) complexes can act as highly potent and selective inhibitors of kinases.[5]

A class of under-explored ruthenium compounds is the family of electron-deficient half-sandwich complexes. Electron-deficient organometallics are involved in a number of catalytic processes as generally unstable intermediates, thus rendering their use as metallodrug candidates difficult.[6] Nonetheless, the groups of Suzuki, Koelle, Tilley among others reported some stable coordinatively unsaturated 16-electron (16-e) organometallics.[7] We have recently developed a strong interest in elucidating the chemistry of 16-e dithiolate-half-sandwich complexes of precious metals (Ru, Os, Rh, Ir). Their unusual reactivity in solution (*e.g.* thermochromism,[8] lack of reactivity with σ-donor ligands and/or σ-donor/π-acceptor ligands,[9] carbon monoxide capture/release,[10] behavior under irradiation[11]) makes them fascinating molecules to study. We also investigated their anticancer[12] and anti-inflammatory properties,[13] as well as their potential for boron neutron capture therapy.[14]

In our efforts to confirm/infirm the anticancer potential of such electron-deficient organometallics, the antiproliferative activity of the 16-e complex [(*p*-cymene)Ru(maleonitriledithiolate)] (**1**) towards ovarian (A2780 and A2780cisR), colon (HCT116 *p53*+/+ and HCT116 *p53*-/-), and lung (H460) cancer cells is reported herein. The stability in solution and reactivity with potential (bio)ligands is investigated, while the MoA of this metal complex is studied *via* gene expression studies, cell cycle analysis, and *N*-acetylcysteine (NAC) co-incubation assay. Finally, the complex is progressed *in vivo* to assess toxicity and efficacy. The maximum tolerated dose is determined, along with the effects of **1** on human tumor cell lines grown in hollow fibers implanted subcutaneously or intraperitoneally in mice (hollow fiber assay; HFA), and in a human H460 non-small cell lung cancer (NSCLC) subcutaneous tumor xenograft model.

## Results and Discussion

### Stability studies

Complex **1** was synthesized according to a previously reported method.[15] Its stability in the presence of different solvents or reactants was first evaluated. Owing to poor water-solubility at millimolar concentration, complex **1** was dissolved in pure deuterated dimethylsulfoxide (DMSO-$d_6$) (1.1 mM concentration; Figure 1(A)) and spectra at t = 0 h, 24 h, 48 h were recorded. The complex is stable under these conditions, although a slight loss of *para*-cymene can be observed (free *p*-cym signals at *ca*. 7.2 ppm) after 24 hours. Nonetheless, complex **1** does not significantly decompose in pure DMSO at millimolar concentration and the complex is 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).

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The reactivity of complex **1** was then evaluated in the presence of 1 mol. equiv. glutathione (GSH) by $^1$H NMR spectroscopy (298K, $CD_3CN:D_2O$, 1:1 v/v; 1.1 mM). Glutathione is involved in cisplatin resistance mechanisms. Indeed, the redox-regulating capacity of GSH is involved in detoxifying cisplatin while GSH also regulates the intracellular copper pool that affects cisplatin uptake.[16] Furthermore, reactivity between complex **1** and GSH could lead to facile excretion of the 18-e GSH-adduct from the cells. As can be seen in Figure 1(B), no reaction occurs between complex **1** and 1 mol. equiv. of GSH, which indicates that the electron-deficient complex does not accept electrons from the electron-donor ligand.

Nucleobase binding studies were then carried out with 2 mol. equiv. of 9-ethylguanine or 9-methyladenine using $^1$H NMR spectroscopy (298K, $CD_3CN:D_2O$, 1:1 v/v; 1.1 mM). The spectra show that complex **1** does not react with 9-ethylguanine (Figure 1(C)), nor with of 9-methyladenine (Figure 1(D)). The remarkable inertness of electron-deficient complex **1** is further demonstrated by the absence of reactivity with 4-dimethylaminopyridine, a strong σ-donor ligand (Figure S1). However, in the presence of the σ-donor/π-acceptor triphenylphosphine ligand, the 18-e adduct [(*p*-cymene)Ru(maleonitriledithiolate)$PPh_3$] can be synthesized (Figure S2).

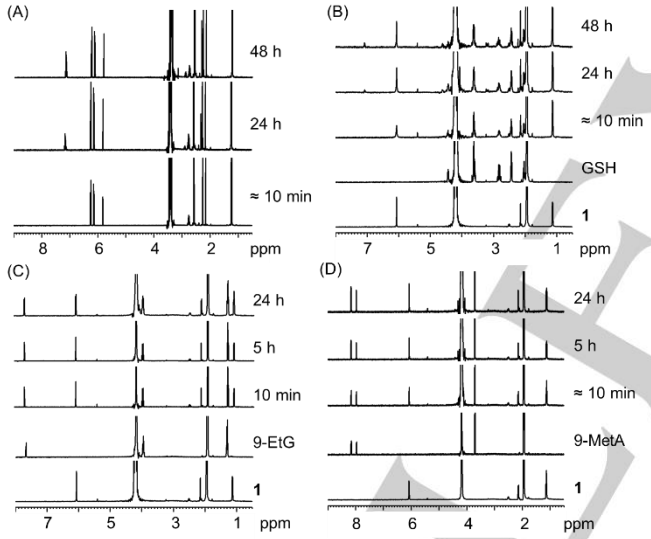


Figure 1. Stability studies for complex **1**. (A) $^1$H NMR spectra of complex **1** in DMSO-$d_6$ over a period of 0 – 48 h (298K, 1.1 mM); (B) $^1$H NMR spectra of complex **1** + 1 mol. equiv. GSH in a mixture $CD_3CN:D_2O$, 1:1 v/v (298K, 1.1 mM) over a period of 0 – 48 h; (C) $^1$H NMR spectra of complex **1** + 2 mol. equiv. 9-ethylguanine in a mixture $CD_3CN:D_2O$, 1:1 v/v (298K, [**1**] = 1.1 mM) over a period of 0 – 48 h; (D) $^1$H NMR spectra of complex **1** + 2 mol. equiv. 9-methyladenine in a mixture $CD_3CN:D_2O$, 1:1 v/v (298K, [**1**] = 1.1 mM) over a period of 0 – 48 h.

### In vitro antiproliferative activity

Chemosensitivity studies were undertaken using a 24-hour MTT assay, with a 72-hour recovery period. The $IC_{50}$ values (which correspond to inhibitions of cancer-cell growth at the 50% levels) were determined against HCT116 *p53*+/+ (human colorectal carcinoma, *p53*-wt), HCT116 *p53*-/- (human colorectal carcinoma, *p53*-null), A2780 (ovarian adenocarcinoma), A2780cisR (cisplatin-resistant variant of A2780), and H460 (NSCLC) exposed to compound **1** or cisplatin (Table 1, Figure S3).

Complex **1** is highly active towards all cell lines (5 to 70 × more active than cisplatin), with $IC_{50}$ values in the nanomolar range against ovarian and lung cancer cells, and in the low micromolar range against colon cancer cells. This metal compound ranks among the most active half-sandwich complexes ever reported, and compares well with the highly potent thiolato-bridged arene ruthenium complexes reported by Süss-Fink, Therrien and co-workers in 2012.[17]

Table 1. $IC_{50}$ values / μM ± SD (triplicates of triplicates) for cisplatin and compound **1** against HCT116 *p53*+/+, HCT116 *p53*-/-, A2780, A2780cisR, and H460 cell lines.

| *Complex* | *$IC_{50}$ / μM ± SD* | | | | |
|---|---|---|---|---|---|
| | HCT116 *p53*+/+ | HCT116 *p53*-/- | A2780 | A2780cisR | H460 |
| **1** | 1.14±0.29 | 1.10±0.16 | 0.5±0.04 | 0.32±0.15 | 0.8±0.1 |
| cisplatin | 51±17 | 67±22 | 8±1 | 18±1 | 4.0±0.7 |

Furthermore, compound **1** is highly cytotoxic against the difficult-to-treat colorectal HCT116 *p53*-/- cells. A current limitation in the treatment of colorectal cancer is the reduced cytotoxicity of clinically used chemotherapeutics towards cancer cells which lack the tumor suppressor *p53*.[18] Identifying molecules that are active towards both *p53* wild-type and *p53*-null isogenic cancer cell clones of the human cancer cell line HCT116 is therefore of great interest.[19]

Another important result is the submicromolar activity of compound **1** against A2780cisR. Ovarian cancers are commonly treated with platinum-containing regimens,[20] and acquired and intrinsic resistance mechanisms limit the efficacy of Pt-chemotherapy. The ability of complex **1** to circumvent Pt resistance mechanisms is therefore of interest.

### Gene expression studies

To gain an insight into the possible anticancer MoA of complex **1**, gene expression studies were then carried out in HCT116 *p53*+/+ and HCT116 *p53*-/- cells on a panel of genes involved in apoptosis and DNA damage repair response (Figure 2 and Table S1 for the names, primers sequences and roles of the genes used in this study). Complex **1** induces significant upregulation of some key genes associated to apoptosis, including *BAX* and *CDKN1A* genes (600% and 900% increase, respectively). Furthermore, complex **1** only induces moderate DNA damage response: *ALKBH2* is upregulated, a gene involved in the protection against methylating agents which induces repair of DNA lesions.[21] Moreover, the expression level of *PARP1* is moderately increased,

which indicates a repair of modified bases,[22] as does the upregulation of *BRCA1* which facilitates homologous recombination to maintain genomic stability.[23] These results suggest that complex **1** might have a different MoA than cisplatin (as the chemosensitivity studies on A2780cisR already suggested), which has been shown to significantly upregulate genes involved in DNA damage response and repair (*e.g., PARP1*, *BRCA1, ALKBH3*, *RAD51*; which is consistent with the DNA alkylating MoA of cisplatin).[24]

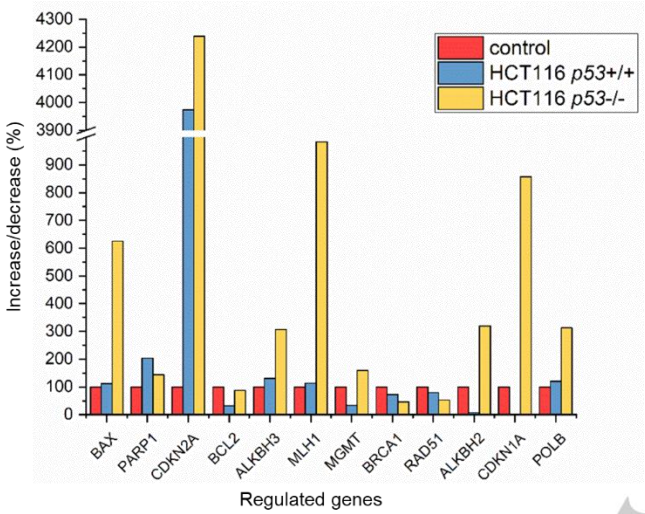


Figure 2. Percentage of increase/decrease of genes expressed in HCT116 *p53*+/+ and HCT116 *p53*-/- cells treated with complex **1**.

### Cell cycle analysis

Apoptosis induced by **1** in HCT116 *p53*+/+ and A2780 cells was confirmed by flow cytometry and cell cycle analysis. Flow cytometry allows a precise analysis of the impact of various functional modulators on the cell cycle,[25] and apoptosis can be detected from the loss of DNA from permeabilized cells. The permeabilization 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 G2M will be obtained with apoptotic cells being represented by a subG population. Figure 3 shows that compound **1** induces a significant increase of subG populations (43% in HCT116 *p53*+/+ and 68% in A2780), which thus confirms the strong apoptotic nature of this compound.

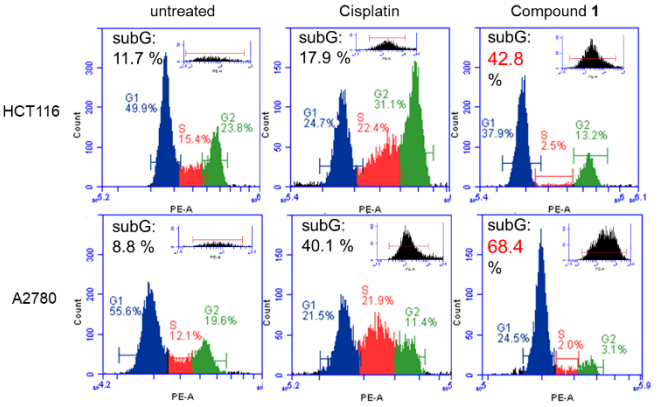


Figure 3. Cell cycle analysis of HCT116 *p53*+/+ (A) and A2780 (B) cells treated with cisplatin or compound **1**.

### Cell viability with *N*-acetylcysteine

The DNA damage response suggested by the gene expression study is not in accordance with the inertness of compound **1** towards biomolecules, and nucleobases in particular. We therefore hypothesized that complex **1** could lead to apoptosis *via* generation of reactive oxygen species (ROS). To test this hypothesis, HCT116 *p53*+/+ and HCT116 *p53*-/- cells were co-incubated with a large excess of *N*-acetylcysteine (NAC; 5 and 10 mM) 30 minutes prior to the treatment with complex **1** (at the $IC_{50}$ values). NAC is a known reductant which is used to protect the cells from ROS and oxidative stress. Pre-treatment with NAC at high concentrations inhibits the cytotoxic activity of complex **1** for both cell lines (Figure 4), which suggests that NAC protects the cells from the effect of complex **1** (indeed, **1** and NAC do not react together, which therefore rules out a deactivation of the metal complex by the ROS scavenger; Figure S4).

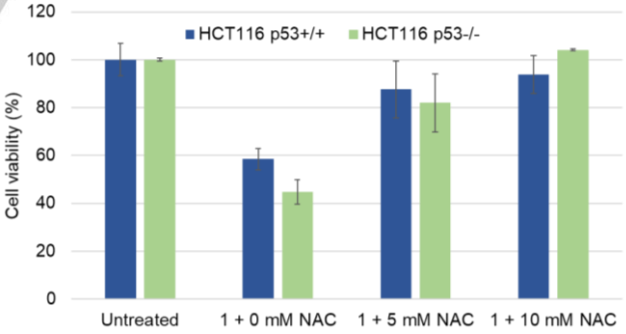


Figure 4. Cell viability of HCT116 *p53*+/+ and HCT116 *p53*-/- cells at the $IC_{50}$ concentration of complex **1** in the presence of a large excess of NAC.

### ROS Analysis

To confirm the hypothesis of induction of oxidative stress by complex **1**, the intracellular production of ROS was investigated using the fluorescent $DCFH_2$-DA assay and flow cytometry with A2780 cells either left untreated, or exposed to **1** ($IC_{50}$ concentration), or to $H_2O_2$ as a positive control (200 µM), or to NAC as a negative control (5 mM). After 5 h drug incubation, it

can be observed that ROS levels in treated cells are significantly higher than in untreated cells (Figure 5). Furthermore, the ROS levels of treated cells are comparable to those of cells treated with 200 µM $H_2O_2$, which confirms intracellular ROS formation.

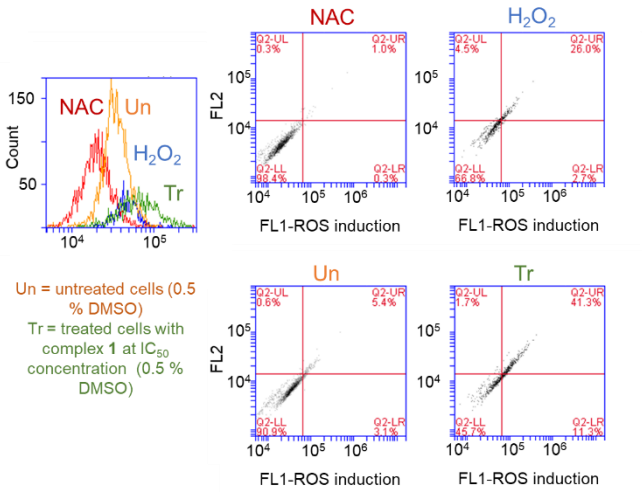


Figure 5. Induction of ROS in A2780 ovarian cancer cells using complex **1** ($IC_{50}$ concentration) and 5 h drug exposure. Controls with NAC (ROS inhibitor; 5mM) and $H_2O_2$ (200 µM) were also performed under the same conditions.

### In vivo toxicity studies

Since compound **1** showed great promise *in vitro*, preclinical studies were progressed *in vivo.* Initially the maximum tolerated dose (MTD) of complex **1** was established as 7.5 mg·kg$^{-1}$ when administered intraperitoneally (i.p.) daily for 4 days and monitored for a further 2 weeks. This is more than 3 times the MTD of cisplatin.

Following the establishment of the MTD for compound **1**, a preliminary *in vivo* efficacy screening was carried out using the *in vivo* hollow fibre assay (HFA). This assay was developed at the NCI, and bridges the gap between cell-based assays and *in vivo* xenograft studies in immunodeficient mice.[26] This intermediate assay, in a cell line panel, helps predict the activity a compound would have in a subsequent xenograft system. The method is based on propagating human cancer cells in inert hollow fibres with pores small enough to retain cancer cells but large enough to permit entry of potential chemotherapeutic drugs. Fibres which contain cancer cells are injected in the peritoneum (i.p.) or under the skin (s.c.). The host mice are then treated with the drug candidates through i.p. injection. The fibres are then retrieved for analysis of viable cell mass using a modified MTT assay. This assay has significant 3Rs benefits in terms of reducing the number of animals required, as well the refinement of a short assay time. The HFA was run with 5 cell lines: DLD-1 (colorectal adenocarcinoma cell line), H460, and MDA-MB-231 (breast cancer cell line), plus A2780 and A2780cisR in a second cohort. In the HFA the effects of a drug candidate on cell growth is determined in cells grown in hollow fibres implanted subcutaneously or intraperitoneally in mice on day 0. Using a standardised US National Cancer Institute protocol,[27] complex **1** (7.5mg/kg/dose) and cisplatin (2mg/kg/dose) were administered i.p. daily on days 3 to 6, with fibres retrieved for analysis on day 7. Significant reduction in growth ($p<0.1$) was seen in most cell lines for complex **1** for the i.p. implanted fibres, with less of an effect seen for the s.c. fibres. The fact that, for most cell lines, the compound lost activity when injected away from the fibres suggests issues with bioavailability of complex **1** and this will need to be investigated in a future study (Figure 6). However, in the cases of H460 and A2780cisR cells, such loss of activity between i.p. and s.c. implanted fibres was not observed, and we therefore chose to evaluate the efficacy of compound **1** in an H460 subcutaneous tumour xenograft model as it is more reliable in forming consistent xenografts compared to A2780cisR.

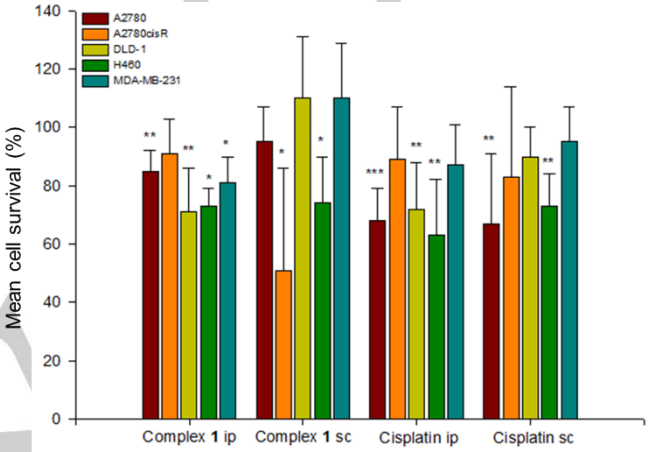


Figure 6. Activity of complex **1** against a panel of cancer cells *in vivo* in the HFA. Each cell line was implanted either intraperitoneally (i.p.) or subcutaneously (s.c.) in mice and treated with **1** (7.5 mg/kg) administered i.p. on days 3, 4, 5 and 6 after implantation.

The results from the more comprehensive evaluation of efficacy carried out in an H460 subcutaneous tumour xenograft model are shown in Figure 7. Complex **1** showed negligible signs of toxicity over the duration of the study, whereas some weight loss was observed with cisplatin (Figure S5). A reduction in tumour growth compared to the untreated and cisplatin control groups was evident over the first four days of treatment, with the growth delay being statistically significant on day 3. However the effect did not continue when further treatments were administered from day 7, which again suggests poor bioavailability of the complex.

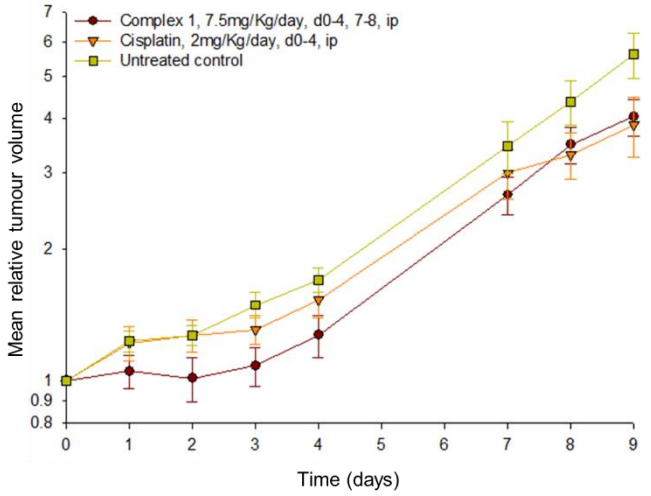


Figure 7. Xenograft study of the therapy of H460 tumours with complex **1** and cisplatin (mean relative tumour volume ± SEM against time).

## Conclusions

In conclusion, the data presented herein demonstrates that [(*p*-cymene)Ru(maleonitriledithiolate)], although electron-deficient at the metal center, is a chemically inert metal complex which does not interact with biomolecules acting as poisons for a number of organometallics. As a result, this complex shows remarkable antiproliferative and apoptotic properties *in vitro*, while being equally cytotoxic to cisplatin-sensitive and -resistant cell lines. Furthermore, the mechanism of action of this compound appears to be *p53*-independent. Whilst *in vivo* results were not as impressive as the *in vitro* data due to poor bioavailability, the slightly significant activity seen for the i.p.-implanted hollow fibers, and initial growth delay seen in the H460 xenograft model give encouragement for this class of molecules if suitable structural modifications can be carried out to improve bioavailability. Complex **1** is therefore a promising lead compound with a different MoA than cisplatin, being *p53*-independent, and resisting deactivation by sulfur- and nitrogen-containing biomolecules. We believe that these results highlight the potential of electron-deficient organometallics as anticancer drug candidates.

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**Keywords:** Electron-deficient • half-sandwich complexes • metallodrugs • hollow fibre assay • in vivo evaluation.

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**Table of Contents graphic and text.**

Anticancer evaluation (*in vitro* and *in vivo* in mice) of an electron-deficient half-sandwich ruthenium complex.