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## Accepted Article

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# Evaluation of the toxicity of two electron-deficient half-sandwich complexes against human lymphocytes from healthy individuals

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**Abstract:** Electron-deficient half-sandwich complexes are a class of under-studied organometallics with demonstrated potential as metallodrug candidates. The present study investigates the effect of two 16-electron organoruthenium complexes ( $[(p\text{-cym})\text{Ru}(\text{benzene-1,2-dithiolato})]$  (**1**) and  $[(p\text{-cym})\text{Ru}(\text{maleonitriledithiolate})]$  (**2**)) on the cell viability of non-immortalised human lymphocytes from healthy individuals. The genotoxic effects of **1** and **2** in lymphocytes using the Comet and cytokinesis-block micronucleus assays is also investigated. Gene expression studies were carried out on a panel of genes involved in apoptosis and DNA damage repair response. Results show that the two 16-electron complexes do not have significant effect on the cell viability of human lymphocytes from healthy individuals. However, an increase in DNA damage is induced by both compounds, presumably through oxidative stress production.

## Introduction

Reactive oxygen species (ROS) are oxygen-containing molecules which are constantly produced as byproducts from various cell functions such as cellular respiration and production of energy. Additionally, ROS can be generated as a result of exposure to irradiation, air pollutants, and toxic substances. Among the most important ROS within living organisms are species such as the superoxide ( $\text{O}_2^{\cdot-}$ ) and hydroxyl ( $\text{HO}^{\cdot}$ ) free radicals, and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ).<sup>[1]</sup> In order to survive oxidative stress, living organisms need to have a well-coordinated system which copes with stressors and high levels of generated ROS. Alterations in oxidative stress have been shown to be particularly effective against cancer cells,<sup>[2]</sup> which, owing to their active metabolism, are under constant oxidative stress.<sup>[3]</sup>

Nonetheless, the role of ROS in cancer is debatable and there has been an ongoing discussion whether or when ROS have a tumour-promoting or tumour-suppressive effect. Through pro-tumourigenic signaling, ROS promote cancer cell proliferation, survival, and adaptation to hypoxia. On the other hand, through anti-tumourigenic signaling these radicals induce oxidative stress followed by cell death. The two contradicting functions of ROS depend on the nature of the free radicals, their concentration, and their location. For instance, when the intracellular level of ROS is modest, this can contribute to tumour promotion.<sup>[4]</sup> However, when the level of ROS is high, this can lead to cellular damage and tumour suppression.<sup>[5]</sup> Additionally, location is a very important factor for determining ROS function as mitochondrial ROS have been shown to induce cell death, while NOX-generated ROS have been linked to cell proliferation and migration (NOX

enzymes being ROS-producing NADPH oxidases, found in most eukaryotic organisms).<sup>[6]</sup> The role of antioxidants in cancer treatment is also complicated as it can involve either protection of normal cells from toxic radicals, or stimulation of tumour growth. In normal cells, antioxidants prevent the formation of malignancies,<sup>[7]</sup> while in developed tumours antioxidants contribute to cell growth, enhance resistance mechanisms, and interfere with ROS-dependent anticancer therapies.<sup>[8]</sup> Current strategies for the treatment of cancer through modulation of the redox balance include ROS-depleting therapy with the use of antioxidants, or ROS-elevating therapy which involves the increase of intracellular ROS levels either directly or through the inhibition of antioxidant systems.

We recently reported the highly-promising *in vitro* anticancer properties of two electron-deficient half-sandwich complexes ( $[(p\text{-cym})\text{Ru}(\text{benzene-1,2-dithiolato})]$  (**1**) and  $[(p\text{-cym})\text{Ru}(\text{maleonitriledithiolate})]$  (**2**); Figure 1).<sup>[9]</sup> These two complexes are air- and moisture-stable and unreactive towards N-, S-, or O-donor ligands. Complex **1** exhibits significantly high cytotoxicity against colorectal cancer cell lines (12 to 34 × more potent than cisplatin,  $\text{IC}_{50}$  values in the nanomolar range), and high *in vitro* selectivity (>50-fold) towards the cancer cells tested, compared to PNT2 normal cells. *In vitro* complex **2** was found to be highly cytotoxic with  $\text{IC}_{50}$  values in the nanomolar range: 5 to 60 times more potent than cisplatin towards some ovarian, colon, and lung cancer cell lines. It showed no cross-resistance and, unlike cisplatin, the remarkable *in vitro* antiproliferative activity of this compound appears to be *p53*-independent. *In vivo* evaluation with the hollow-fibre assay across a panel of cancer cell types and subcutaneous H460 non-small cell lung cancer xenograft model hinted at the activity of the complex in mice. The ability of complexes **1** and **2** to generate ROS production and oxidative stress in cancer cells was demonstrated by co-incubation with the antioxidant molecule *N*-acetylcysteine and by using the fluorescent DCFH<sub>2</sub>-DA assay and flow cytometry.<sup>[9]</sup>

In our efforts to evaluate the anticancer potential of such electron-deficient half-sandwich complexes, the toxicity of complexes **1** and **2** against human lymphocytes from healthy individuals is reported herein; the aim of this study being to confirm the surprising selectivity we previously observed for these complexes between cancer and normal, immortalised, cells. In this work, we used non-immortalised healthy lymphocytes isolated from freshly-taken blood to assess the toxicity of these complexes. The DNA damage induced by these compounds using the Comet and cytokinesis-block micronucleus assays was also investigated, in order to reinforce our hypothesis that oxidative stress is an important parameter in the anticancer mechanism of action of

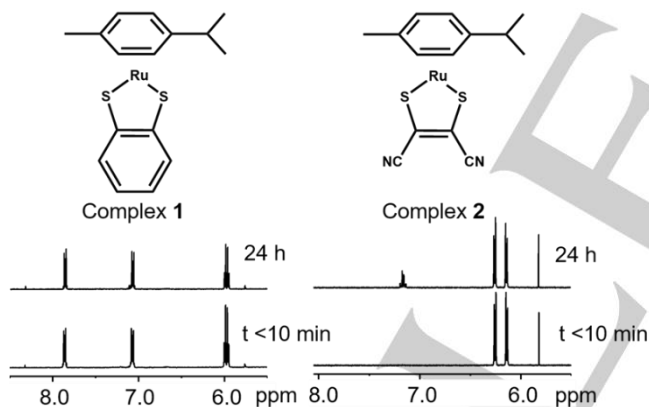
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such complexes. In general, DNA responses to damage could be variable depending on the types of cells examined. However, we believe that primary human cells freshly isolated *ex-vivo/in-vitro* are the best surrogate model to examine responses in human, owing to their intact metabolic system.

## Results and Discussion

## Chemosensitivity assay

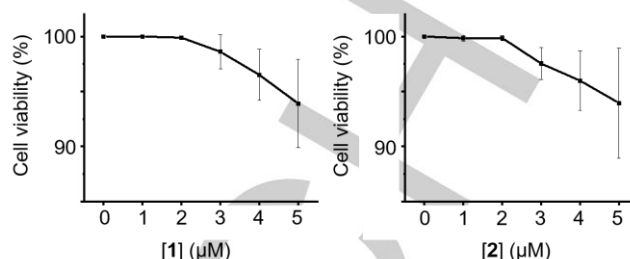
Complexes **1** and **2** were synthesised according to previously-reported methods with slight variations (see Experimental Section).<sup>[9]</sup> Their stability in the media necessary for treatment of lymphocytes was first tested. Owing to their poor water solubility at millimolar concentrations, the complexes were dissolved in pure deuterated dimethylsulfoxide (DMSO- $d_6$ ) (1 mM concentration) and  $^1\text{H}$  NMR spectra at  $t = 0$  h and 24 h were recorded. The complexes are 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 (Figure 1). Nonetheless, both complexes are stable in pure DMSO at millimolar concentration and they 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). Both compounds are stable in a mixture RPMI drug-media/DMSO (1/1; v/v) at ambient temperature over a minimum of 72 hours, as determined by UV-visible spectroscopy (Figure S1).



**Figure 1.** Molecular structures and stability studies in DMSO- $d_6$  of complexes **1** and **2**.

Chemosensitivity studies were then undertaken using a Cell Counting Kit-8 (CCK-8; see Experimental Section). Cell viability was determined against isolated human lymphocytes from healthy individuals exposed for 24 h to either complex **1** or **2** at concentrations of 1, 2, 3, 4 and 5  $\mu\text{M}$  (higher concentrations than the  $\text{IC}_{50}$  values determined for both complexes against cancer cells, in the nanomolar range). Both compounds were shown to have no significant or little effect on the cell viability of human lymphocytes (Figure 2). This result is not only in accordance with our preliminary investigations on the cytotoxicity of **1** and **2** against normal PNT2 cells, but also demonstrates the absence of cytotoxicity towards healthy, non-immortalised, isolated

lymphocytes from blood samples taken by venipuncture from healthy control individuals within the University of Bradford.

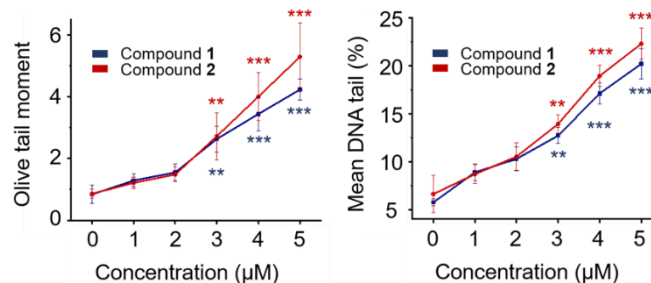


**Figure 2.** Cell viability of isolated human lymphocytes treated with either complex **1** or **2**. Data represent the means  $\pm$  SD obtained from three repetitions.

## DNA damage studies

The responses of lymphocytes from healthy individuals to **1** for the Comet assay parameters (Olive tail moment (OTM) and % tail DNA) are shown in Figures 3, S2, and Tables S1 and S2. The tail moment is defined as the product of the tail length and the fraction of total DNA present in the tail. This also includes measurements of smallest detectable size of migrating fragmented DNA and relaxed and broken pieces of DNA. Tail length is used to describe the distance of DNA migration from the body of the nuclear core region (the head of comet) and is used to evaluate the extent of DNA damage.

A significant increase from 0.84 (OTM) 5.74 (% tail DNA) to 2.63 (OTM) and 12.70 (% tail DNA) can be observed when increasing the complex concentration from 0 to 3  $\mu\text{M}$ . Further significant increase of both parameters can be observed with higher concentrations of **1** with a maximum of 4.23 (OTM) and 20.20 (% tail DNA) when using 5  $\mu\text{M}$ . Similarly, when cells are treated with complex **2**, an increase from 0.86 (OTM) and 6.61 (% tail DNA) to 2.72 (OTM) and 13.92 (% tail DNA) can be observed when raising the concentration from 0 to 3  $\mu\text{M}$ . Cells treated with 5  $\mu\text{M}$  showed an even greater increase to 5.19 (OTM) and 22.29 (% tail DNA). Both complexes **1** and **2** show a similar increase in DNA damage through the Comet assay.



**Figure 3.** DNA damage measured as mean OTM and % tail DNA after treatment of human lymphocytes from healthy individuals with **1** and **2** (0, 1, 2, 3, 4, 5  $\mu\text{M}$ ) for 30 minutes. Data represent the means  $\pm$  SE obtained from three repetitions.  $^{**}P < 0.01$ ,  $^{***}P < 0.001$  when compared with untreated lymphocytes.

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To confirm the results obtained by the Comet assay, chromosome damage induced by **1** and **2** was determined by assessing the frequencies of micronuclei (MNI). After treating the cells with 3  $\mu\text{M}$  of either complex **1** or **2**, a significant increase of the MNI can be observed, which indicates DNA damage. Increasing concentrations of either compound also correlates with an increase of MNI frequencies (Table 1). Thus, the extent of DNA damage by the Comet assay was reflected by the CBMN assay.

**Table 1.** Cytological scoring parameters, including cell mitotic status (BiNC, and MultiNC), NDI and chromosomal damage/instability parameters in the form of NPBs and NBUDs in lymphocytes following exposure to complexes **1** and **2**. Data represent the mean  $\pm$  SE obtained from healthy individuals

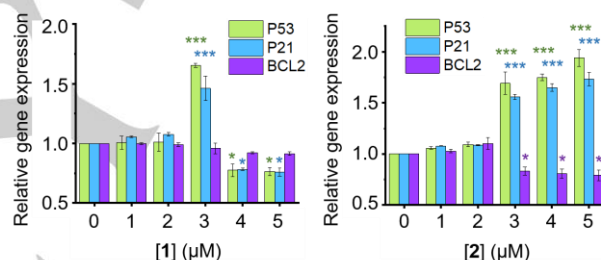
Treatment	NDI	Mean % BiNC	Mean % MultiNC	Mean			Mean of % MNI in MoNC
				BiMN	BiBuds	BiNPB	
Complex 1							
Untreated	1.8	56.6	12.1	1.66	0	0	0
1 $\mu\text{M}$	1.9	59.6	10.6	3.0	0	0	1.0
2 $\mu\text{M}$	1.8	47.3	11.2	4.3	0	0	1.0
3 $\mu\text{M}$	1.6	51.8	10.0	10.3***	0	0	4.0
4 $\mu\text{M}$	1.8	47.3	13.0	12.0***	1.0	0	4.3
5 $\mu\text{M}$	2.0	62.0	13.60	15.3***	2.1	1	11.6
Complex 2							
Untreated	1.8	62.0	9.1	2.0	0	0	1.0
1 $\mu\text{M}$	1.9	59.6	8.6	3.3	0	0	1.0
2 $\mu\text{M}$	1.9	60.0	14.1	4.0	0	0	2.1
3 $\mu\text{M}$	1.9	63.1	13.1	9.0***	0	0	6.6
4 $\mu\text{M}$	2.0	62.0	13.3	13.0***	1.0	0	8.0
5 $\mu\text{M}$	2.0	69.1	12.1	16.0***	2.5	2.1	14.6

NDI = Nuclear division index, BiNC = Binucleated cells, % BiNC, is % expressed out of all types of 500 cells scored; MonoNC = Mononucleated cells. MultiNC = Multinucleated cells % MultiNC, is % expressed out of all types of 500 cells scored. MNI = Micronuclei score/500 cells each of BiNC and MonoNC; NPBs = Nucleoplasmic bridges and NBUDs = Nuclear buds. \*\*\* $p < 0.001$  versus untreated cells.

Evaluations of DNA damage induced by half-sandwich ruthenium compounds using the Comet assay or the cytokinesis-blocked micronucleus assay have been previously reported.<sup>[10]</sup> In most cases, such complexes had the capacity to covalently bind to nucleobases and DNA, in a similar manner than cisplatin; DNA damage is therefore not unexpected.<sup>[10-11]</sup> In the present study, both complexes **1** and **2** induce DNA damage, whereas they were previously demonstrated to be not capable of binding DNA covalently due to their unique properties as stable pseudo 16-electron species.<sup>[9, 12]</sup> Other ruthenium(II) complexes such as [Ru(bipy)<sub>2</sub>(dppz)]PF<sub>6</sub> or [Ru(dmb)<sub>2</sub>(1-Ph- $\beta$ C)]<sup>+</sup>, where dppz = dipyrrodo[3,2-a:2',3'-c]phenazine, 1-Ph- $\beta$ C = 1-phenyl-9H-pyrrodo[3,4-b]indole, dmb = dimethylbipyridine, have also been shown to produce DNA damage despite binding to DNA non covalently or not binding due to low affinity.<sup>[13]</sup>

## Gene expression studies

Gene expression studies were carried out on a panel of genes involved in apoptosis and DNA damage repair response (Figure 4): *p53*, known as guardian of the genome, whose role is preserving the stability and preventing genome mutations; *p21*, associated with linking DNA damage and cell cycle arrest; and *BCL2* involved in regulation of apoptosis. Below 3  $\mu\text{M}$ , compounds **1** and **2** do not show any significant effect on gene expressions. Above 3  $\mu\text{M}$  ( $>> \text{IC}_{50}$  values on cancer cells), compound **2** shows a significant increase in *p53* and *p21* gene expressions, consistent with genome damage. Additionally, a downregulation of *BCL2* can also be observed indicating pro-activation of apoptosis. In contrast, at concentrations of 4 and 5  $\mu\text{M}$  of compound **1** in cells, a 1.3 x times reduction of the mRNA *p53* and *p21* levels was observed. This clear 3  $\mu\text{M}$  threshold indicates that healthy cells can recover well from the stress induced by the two compounds at concentrations much higher than the previously determined half-maximal inhibitory concentrations on cancer cells, but that above 3  $\mu\text{M}$  genotoxicity starts being observed. We believe that this observation supports, and certainly does not disprove, our hypothesis of oxidative stress induction as possible anticancer mechanism of action for these metal complexes.



**Figure 4.** Effect of **1** and **2** on the P53, P21 and BCL2 mRNA expression levels in human lymphocytes. Values were normalized using  $\beta$ -actin as an internal control. Data from three different healthy individuals ( $n=3$ ) in triplicate. \* $p < 0.05$  and \*\*\* $p < 0.001$  compared with untreated lymphocytes.

## Conclusion

In conclusion, the data presented herein demonstrate that the two electron-deficient half-sandwich complexes [(p-cym)Ru(benzene-1,2-dithiolato)] (**1**) and [(p-cym)Ru(maleonitriledithiolato)] (**2**) do not present a significant toxicity against healthy, non-immortalised, isolated lymphocytes from blood samples taken by venipuncture from healthy control individuals. Furthermore, although not able to covalently bind to nucleobases, these complexes induce a significant DNA damage response in such lymphocytes, as demonstrated by the Comet and the cytokinesis-blocked micronucleus assays. This study, combined with our previous results obtained on generation of ROS species and apoptosis, suggests that the cytotoxicity of complexes **1** and **2** against cancer cells may come from their ability to generate a high-enough oxidative stress in cells to cause apoptosis in cancer cells but a low-enough level to allow normal and healthy cells to recover. We believe that such results are valuable and encouraging for the future development of electron-deficient organometallics as anticancer drug candidates.

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## 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. Dichloromethane was dried over molecular sieves 4Å. All procedures were performed under nitrogen atmosphere and with pre-dried glassware, unless otherwise stated. All reagents used in the different tests were purchased from Sigma-Aldrich Company Ltd. (Sigma Chemical Ltd., Gillingham, UK).

## Synthesis

[(*p*-cym)Ru(benzene-1,2-dithiolato)] (**1**): Ruthenium dimer [(*p*-cym)RuCl<sub>2</sub>]<sub>2</sub> (100 mg, 0.16 mmol) was placed in a 50 mL 2-neck round bottom flask and dissolved in 20 mL of dry dichloromethane. Benzene-1,2-dithiolato (40 mg, 0.34 mmol) dissolved in dry dichloromethane (5 mL) was then added dropwise to the solution containing the ruthenium dimer. The dark red mixture was then left stirring under a nitrogen atmosphere at room temperature for 1 h. After removing the solvent under vacuum, a dark precipitate was obtained. The crude product was purified by chromatography (hexane/dichloromethane 1:2 v/v).

[(*p*-cym)Ru(maleonitriledithiolate)] (**2**): Ruthenium dimer [(*p*-cym)RuCl<sub>2</sub>]<sub>2</sub> (150 mg, 0.25 mmol) and disodium maleonitriledithiolate (100 mg, 0.50 mmol) were placed in a 50 mL 2-neck round bottom flask and dissolved in 20 mL of dry dichloromethane. The dark brown mixture was then left stirring under a nitrogen atmosphere at room temperature for 1 h. After removing the solvent under vacuum, a dark precipitate was obtained. The crude product was purified immediately by chromatography (dichloromethane).

## Stability studies

Complexes **1** and **2** were dissolved in DMSO-*d*<sub>6</sub> (1.1 mM) and <sup>1</sup>H NMR spectra (400 MHz, 298 K) were recorded over a period of 24 h. Complexes **1** and **2** were dissolved in DMSO-*d*<sub>6</sub> were dissolved in DMSO/RPMI (1/1; v/v) at ambient temperature (5 × 10<sup>-5</sup> M) and UV-visible spectra were recorded over a period of 72 h.

## Collection of blood samples

After informed consent, approximately 10 mL heparinised blood was taken by venepuncture from the healthy control individuals within the University of Bradford, UK (West Yorkshire, UK). Ethical permission was obtained from Leeds East Ethics Committee (Reference no: 12/YH/0464) and the University of Bradford's Sub-Committee for Ethics in Research involving Human Subjects (Reference no.: 0405/8).

## Isolation of lymphocytes

Three mL of whole blood were diluted 3:3 with 0.9% saline and carefully layered on top of three mL of Lymphoprep in 15 mL Falcon tubes. The tubes were centrifuged for 20 min at 800 × g. Lymphocytes were then harvested, washed with saline (10 mL) and centrifuged again for 15 min at 500 × g at room temperature. Lymphocytes were re-suspended in Roswell Park Memorial Institute (RPMI) 1640 Medium and used for the *in vitro* experiments.

## Chemosensitivity assay

The cytotoxicity assay using the Cell Counting Kit-8 (CCK-8) was performed to determine the effect of **1** and **2** on the cellular viability of isolated human lymphocytes. Isolated cells were plated in a 96-well plate at a concentration of 5000 cells per well. Cells were either treated with

different concentrations of **1** and **2** of 1, 2, 3, 4 and 5 μM or left untreated and considered as control for 24 h in a humidified incubator at 37 °C, 5% CO<sub>2</sub>. Ten microliters of CCK-8 solution were added to each well of the plate, followed by incubation at 37 °C for 4 h. Absorbance was measured at a wavelength of 450 nm using a Microplate reader MRX II (Dyner Technologies, Chantilly, USA). Viability was also measured by the trypan blue exclusion test indicating intact cell membranes.<sup>[14]</sup> Ten microliters of 0.05% trypan blue was added to 10 μL of cell suspension and the percentage of cells excluding the dye was estimated using an improved Neubauer haemocytometer.<sup>[15]</sup>

## Comet assay

Cell suspensions (1 mL, 10<sup>6</sup> cells/mL) were mixed with fresh RPMI 1640 medium (total volume 1 L). To each treatment tube, 100 mL of cell suspension, 890 mL of RPMI 1640 medium and 10 μL of **1** or **2** RPMI were added. Untreated lymphocytes from healthy individuals served as the negative control group. Cells were treated with different concentrations of 0, 1, 2, 3, 4, 5 μM of **1** and **2** for 30 min in a humidified incubator at 37 °C, 5% CO<sub>2</sub>. After treatment, the cells were used for the Comet assay to detect DNA damage (single and double-strand breaks in DNA, alkali-labile sites and oxidative base damage).

The Comet assay was processed with slight modifications according to the literature.<sup>[16]</sup> In brief, the lymphocytes were mixed with prewarmed 0.5 % low melting agarose (LMP) (Invitrogen, Paisley, UK: 15517-022). The cell suspension was then transferred to slides pre-coated with 1% normal melting point (NMP) agarose and covered with a coverslip. The slides were solidified on an ice block for 5 min. The coverslip was removed, and the slides incubated in cold lysing buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, with 1% Triton X-100 and 10% DMSO were added just before use) and kept overnight at 4 °C. The slides were placed on a horizontal gel electrophoresis platform to allow the DNA to unwind in cold fresh electrophoresis buffer (300 mM NaOH and 1 mM Na<sub>2</sub>EDTA, pH ~13.5) at 4°C, and electrophoresis was performed at 4°C for 30 min. The slides were neutralised with a 400 mM Tris (pH 7.5) buffer for 5 min. The slides were stained with ethidium bromide and covered with a coverslip. Slides were examined by a computerised image analysis system (Comet 6.0; Andor Technology, Belfast, UK). One hundred cells were scored per sample (50 cells from each slide); Olive tail moment (OTM) and % tail DNA were measured as DNA damage parameters.

## The cytokinesis-block micronucleus (CBMN) assay

CBMN was performed as described in the literature with modifications.<sup>[16b, 16c, 17]</sup> Five hundred microliters of whole blood were added to a T25 cm<sup>2</sup> Corning culture flask containing 4.5 mL RPMI 1640 medium supplemented with 1% of penicillin-streptomycin, 15% foetal bovine serum, 25 mM HEPES and L-glutamine with a final concentration of 15 and 1%, respectively, followed by 100 μL of phytohemagglutinin (PHA). In the next 24 h, 50 μL of excipient (original solution) was added to the negative control. Different concentrations of **1** and **2** of 1, 2, 3, 4, 5 μM were added to the rest of the flasks. Cultures were incubated at 37°C in the presence of 5% CO<sub>2</sub> for 44 h. After 44 h, cytochalasin-B (6 μg/mL, Sigma) was added and the cultures were incubated for another 28 h. The CBMN test preparations and slides scored were performed using the criteria, as recommended by Fenech *et al.* [18]. Micronuclei (MNi) were scored each from binucleated (BiNC) and mononucleated (MonoNC) cells. Other nuclear anomalies such as nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) were also evaluated as biomarkers of genotoxic events. The nuclear division index (NDI) was used as an indicator of the cytotoxicity and the following calculation was used to find the NDI: NDI = (M1 + 2 (M2) + 3 (M3) / N. Where: M1 = mononucleated cells, M2 = binucleated cells, M3 = multinucleated cells, N = the total number of viable cells scored.<sup>[17]</sup>

## Total RNA isolation

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Cell suspensions (1 mL,  $10^6$  cells/mL) were mixed with fresh RPMI medium (total volume 1 L). To each treatment tube, 100 mL of cell suspension, 890 mL RPMI medium and 10  $\mu$ L of **1** or **2** or RPMI were added. Untreated lymphocytes from healthy individuals served as the negative control group. Cells were treated with different concentrations of 0, 1, 2, 3, 4, 5  $\mu$ M of **1** and **2** for 24 h in a humidified incubator at 37 °C, 5% CO<sub>2</sub>.

Total RNA was extracted from lymphocytes using the GenElute Mammalian Total RNA Purification kit (Sigma-Aldrich, UK). The RNA was treated with DNase I (Sigma-Aldrich, UK) to remove any DNA contaminants. The concentration and purity of total RNA were determined by measuring the absorbance at 260 and 280 nm ( $A_{260/280}$ ) ratios, using a NanoDrop™ Spectrophotometer.

## Real-time PCR

RNA was reverse transcribed using the iScript™ cDNA Synthesis Kit (Life Science Research, Bio-Rad). The reactions were performed using the StepOnePlus™ real-time PCR instrument (Applied Biosystems). The qPCR was used to measure the mRNA expression level of P53, P21 and BCL2 in lymphocytes. Each reaction was prepared in triplicate and consisted of 10  $\mu$ L of 10  $\times$  SYBR® Green PCR Master Mix (Applied Biosystems), 12.5 pmol each of forward and reverse primers, and 2  $\mu$ L of cDNA template, making up to a 20  $\mu$ L final volume per well. The qPCR was initially conducted at 50°C and 95°C for 2 and 20s, respectively, followed by amplification of the template for 40 cycles (each cycle involved 15 s at 95°C and 30s at 60°C). The data were analysed by StepOne™ Software v 2.2.2. The cycle threshold (Ct) mean value for the target gene was used to calculate the relative expression with the relative quantification (RQ) value and formula:  $RQ = 2^{-\Delta CT} \times 100$ , where  $\Delta CT = C_T$  of target gene -  $C_T$  of an endogenous housekeeping gene. Evaluation of  $2^{-\Delta CT}$  indicates the fold change in gene expression, normalized to the internal control ( $\beta$ -actin) which enables the comparison between differently treated cells.

## Statistical Analysis

All the experiments were performed in duplicate and repeated at least three times. Results are expressed as means  $\pm$  SEM of three experiments and data were analysed using one-way analysis of variance with Dunnett's post hoc test to determine significance relative to control; for all experiments, a *P* value of <0.05 was considered significant.

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**Keywords:** half-sandwich complexes • electron-deficient organometallics • bioinorganic chemistry • lymphocytes • anticancer metallodrugs

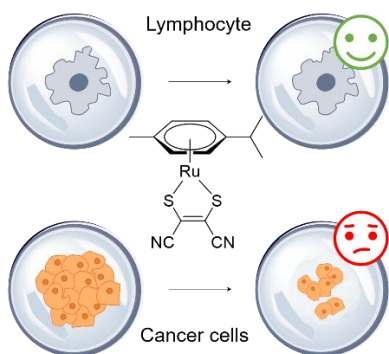
## References

- [1] M. Valko, C. J. Rhodes, J. Moncol, M. Izakovic, M. Mazur, *Chem.-Biol. Interact.* **2006**, *160*, 1-40.
- [2] a) J. J. Soldevila-Barreda, I. Romero-Canelón, A. Habtemariam, P. J. Sadler, *Nat. Commun.* **2015**, *6*, 6582; b) Z. Liu, I. Romero-Canelón, B. Qamar, J. M. Hearn, A. Habtemariam, N. P. E. Barry, A. M. Pizarro, G. J. Clarkson, P. J. Sadler, *Angew. Chem. Int. Ed.* **2014**, *53*, 3941-3946; c) J. P. C. Coverdale, I. Romero-Canelón, C. Sanchez-Cano, G. J. Clarkson, A. Habtemariam, M. Wills, P. J. Sadler, *Nat. Chem.* **2018**, *10*, 347-354.
- [3] E. O. Hileman, J. Liu, M. Albitar, M. J. Keating, P. Huang, *Cancer Chemoth. Pharm.* **2004**, *53*, 209-219.
- [4] V. Aggarwal, H. S. Tuli, A. Varol, F. Thakral, M. B. Yerer, K. Sak, M. Varol, A. Jain, M. A. Khan, G. Sethi, *Biomolecules* **2019**, *9*, 735.
- [5] C. Gorrini, I. S. Harris, T. W. Mak, *Nat. Rev. Drug Discov.* **2013**, *12*, 931.
- [6] E. C. Cheung, D. Athineos, P. Lee, R. A. Ridgway, W. Lambie, C. Nixon, D. Strathdee, K. Blyth, O. J. Sansom, K. H. Vousden, *Dev. Cell* **2013**, *25*, 463-477.
- [7] L. He, T. He, S. Farrar, L. Ji, T. Liu, X. Ma, *Cell. Physiol. Biochem.* **2017**, *44*, 532-553.
- [8] O. Firuzi, R. Miri, M. Tavakkoli, L. Saso, *Curr. Med. Chem.* **2011**, *18*, 3871-3888.
- [9] a) J. J. Soldevila-Barreda, M. Azmanova, A. Pitto-Barry, P. A. Cooper, S. D. Shnyder, N. P. E. Barry, *ChemMedChem* **2020**, *15*, 982-987; b) M. Azmanova, J. Soldevila-Barreda, H. Bani Hani, R. M. Lord, A. Pitto-Barry, S. M. Picksley, N. P. E. Barry, *ChemMedChem* **2019**, *14*, 1887-1893.
- [10] a) M. C. Ruiz, J. Kljun, I. Turel, A. L. Di Virgilio, I. E. León, *Metalomics* **2019**, *11*, 666-675; b) M. Kasim, M. Subarkhan, S. Sundar, R. Rengan, *Inorg. Chem. Front.* **2018**, *5*, 585-596; c) A. Skoczyńska, B. Pasternak, M. Malecka, U. Krajewska, M. Mirowski, A. Merez-Sadowska, B. T. Karwowski, J. Kusz, E. Budzisz, *Polyhedron* **2019**, *169*, 228-238; d) N. Mohan, M. Subarkhan, M. Kasim, R. Ramesh, *J. Organomet. Chem.* **2018**, *859*, 124-131; e) M. Ganeshpandian, R. Loganathan, E. Suresh, A. Riyasdeen, M. A. Akbarsha, M. Palaniandavar, *Dalton Trans.* **2014**, *43*, 1203-1219.
- [11] A. Rilak Simović, R. Masnikosa, I. Bratsos, E. Alessio, *Coord. Chem. Rev.* **2019**, *398*, 113011.
- [12] A. Pitto-Barry, A. Lupan, M. Zegke, T. Swift, A. A. Attia, R. M. Lord, N. P. E. Barry, *Dalton Trans.* **2017**, *46*, 15676-15683.
- [13] a) J. Chen, Y. Zhang, X. Jie, J. She, G. Dongye, Y. Zhong, Y. Deng, J. Wang, B. Guo, L. Chen, *J. Inorg. Biochem.* **2019**, *193*, 112-123; b) F. Mello-Andrade, C. Gomes Cardoso, C. Ribeiro e Silva, L. Chen-Chen, P. R. de Melo-Reis, A. Pereira de Lima, R. Oliveira, I. B. Machado Ferraz, C. Koppe Grisolia, M. A. Pinheiro Almeida, A. Azevedo Batista, E. de Paula Silveira-Lacerda, *Biomed. Pharmacother.* **2018**, *107*, 1082-1092; c) J. Chen, F. Peng, Y. Zhang, B. Li, J. She, X. Jie, Z. Zou, M. Chen, L. Chen, *Eur. J. Med. Chem.* **2017**, *140*, 104-117.
- [14] H. J. Phillips, in *Tissue Culture* (Eds.: P. F. Kruse, M. K. Patterson), Academic Press, **1973**, pp. 406-408.
- [15] B. L. Pool-Zobel, R. G. Klein, U. M. Liegibel, F. Kuchenmeister, S. Weber, P. Schmezer, *Clin. Investig.* **1992**, *70*, 299-306.
- [16] a) D. Anderson, T. Yu, D. B. McGregor, *Mutagenesis* **1998**, *13*, 539-555; b) R. R. Tice, E. Agurell, D. Anderson, B. Burlinson, A. Hartmann, H. Kobayashi, Y. Miyamae, E. Rojas, J.-C. Ryu, Y. F. Sasaki, *Environ. Mol. Mutagen.* **2000**, *35*, 206-221; c) OECD, *Test No. 487: In Vitro Mammalian Cell Micronucleus Test, Vol. Section 4*, OECD Publishing, Paris, **2016**; d) OECD, *Test No. 489: In Vivo Mammalian Alkaline Comet Assay, Vol. Section 4*, OECD Publishing, Paris, **2016**.
- [17] M. Fenech, *Nat. Protoc.* **2007**, *2*, 1084-1104.
- [18] a) M. Fenech, W. P. Chang, M. Kirsch-Volders, N. Holland, S. Bonassi, E. Zeiger, *Mutat Res-Gen Tox En* **2003**, *534*, 65-75; b) M. Fenech, *Nat Protoc* **2007**, *2*, 1084-1104; c) O. d. c. e. d. économiques, *Test No. 487: In Vitro Mammalian Cell Micronucleus Test*, OECD Publishing, **2016**.

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