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In vitro evaluation of the cyto-genotoxic potential of Ruthenium(II) SCAR complexes: a promising class of antituberculosis agents

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ABSTRACT

Tuberculosis is a top infectious disease killer worldwide, caused by the bacteria *Mycobacterium tuberculosis*. Increasing incidences of multiple drug-resistance (MDR) strains are emerging as one of the major public health threats. However, the drugs in use are still incapable of controlling the appalling upsurge of MDR. In recent years a marked number of research groups have devoted their attention toward the development of specific and cost-effective antimicrobial agents against targeted MDR-Tuberculosis. In previous studies, ruthenium(II) complexes (SCAR) have shown a promising activity against MDR-Tuberculosis although few studies have indeed considered ruthenium toxicity. Therefore, within the preclinical requirements, we have sought to determine the cyto-genotoxicity of three SCAR complexes in this present study. The treatment with the SCARs induced a concentration-dependent decrease in cell viability in CHO-K1 and HepG2 cells. Based on the clonogenic survival, SCAR 5 was found to be more cytotoxic while SCAR 6 exhibited selectivity action on tumor cells. Although SCAR 4 and 5 did not indicate any mutagenic activity as evidenced by the Ames and Cytokinesis block micronucleus cytome assays, the complex SCAR 6 was found to engender a frameshift mutation detected by *Salmonella typhimurium* in the presence of S9. Similarly, we observed a chromosomal damage in HepG2 cells with significant increases of micronuclei and nucleoplasmic bridges. These data indicate that SCAR 4 and 5 complexes did not show genotoxicity in our models while SCAR 6 was considered mutagenic. This study presented a comprehensive genotoxic evaluation of SCAR complexes were shown to be genotoxic *in vitro*. All in all, further studies are required to fully elucidate how the properties can affect human health.

1. Introduction

Tuberculosis (TB) is a major global health problem, ranks alongside the human immunodeficiency virus (HIV) as a leading cause of death from infectious disease worldwide. The latest estimate of the World Health Organization (WHO) recorded 9.6 million new cases in 2014, and has claimed the lives of over 1.5 million people. Out of that number, 26% of the patients infected by TB had co-infection with HIV [1].

In spite of the fact that the standard therapy for TB is still active, more effective treatments have become essentially necessary if we are to reduce the burden imposed by infectious processes [2]. In addition, it is noteworthy to point out that there has been a rise in the number of new cases involving multi-drug resistant TB (MDR-TB), where the *Mycobacterium tuberculosis* strains are found to be resistant to at least rifampicin and isoniazid and other drugs other than a fluoroquinolone, and extensively-drug resistant TB (XDR-TB) defined by the resistance to rifampicin, isoniazid, any fluoroquinolone and at least one of the second line drugs, in other words rendering us (the patients infected) practically without any pharmacological alternative [1,3].

The protocols for MDR-TB involve months of treatment alongside a combination of several drugs. These regimes are associated with significant adverse effects and psychological comorbidity, as a result of social isolation [4]. After 50 years, bedaquiline and delamanid have now been allowed into the market with rational use though they are still under clinical trials, given the lack of new and more effective drugs against TB-MDR [5,6]. Although new

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molecules are in clinical development phase, fully effective drugs against resistant strains are yet out of sight.

In the search for new safer and more effective molecules, the ruthenium complexes have exhibited promising results, with minimum inhibitory concentrations (MIC), which are less than or equal to first-line drugs [7]. Ruthenium likewise most metals can form electron deficient cations, which tend to interact with molecules that have electron pairs available to be shared [8]. Ruthenium stands out among the transition metals thanks to its ability to assume different oxidation states [9] and it is this variety of states that gives ruthenium a very diversified chemistry [10]. In particular importance, the ruthenium(II) complexes containing ligands such as phosphine/diimine/picolinate (SCAR), synthesized by the Inorganic Chemistry group of the Federal University of São Carlos (UFSCar, Brazil), showed promising activity against the MDR strains of *Mycobacterium tuberculosis* [11,12].

Given the important activity of the ruthenium(II) complexes SCAR as far as MDR-TB strains are concerned, this study investigated the genetic toxicology profile of three ruthenium(II) complexes, SCAR 4, 5 and 6 (Fig. 1) as part of the recommended pre-clinical studies with the aim of giving subsidies and ensuring their safe development as possible candidates in anti-TB therapy.

The mutagenicity was investigated by Ames test with and without exogenous activation (S9) in different strains of *Salmonella typhimurium* (TA1535, TA98, TA100, TA97a and TA102) capable of identifying agents that cause gene mutations. To assess the mutagenicity in mammalian cells, cytokinesis-block micronucleus cytome assay (CBMN-cyt) was carried out in HepG2 (human hepatocellular carcinoma) and CHO-K1 (chinese hamster ovary) cells, where the effect of *in vitro* metabolism of the complexes was compared. For cytotoxicity detection and the determination of a suitable concentration range for the CBMN-cyt test, the clonogenic survival assay was used to determine the viability of cell lines after 48 h of incubation.

2. Material and methods

The ruthenium complexes [Ru(pic)(dppb)(phen)]PF_6 (SCAR 4), *cis*-[Ru(pic)(dppe)$_2$]PF_6 (SCAR 5) and *cis*-[$RuCl_2$(dppb)(bipy)] (SCAR 6) were synthesized at Federal University of São Carlos, São Carlos, São Paulo, Brazil. The synthesis procedures and characterization tests are described in [13] and [11,12].

2.1. Cell culture

CHO-K1 and HepG2 cells were grown in Dulbecco's Modified Eagle Medium (DMEM, GIBCO) containing 10% of fetal bovine serum (FBS, GIBCO), 100 IU/mL penicillin and 100 streptomycin μg/mL (Sigma–Aldrich) in a humidified atmosphere with 5% CO_2 at 37 °C. The highest DMSO (Sigma–Aldrich) concentration used was 1.0%.

2.2. Clonogenic survival assay

To determine the cytotoxicity, the Clonogenic Survival Assay was performed in accordance with the guidelines of Ballal et al [14]. with modifications. 1 × 106 cells of CHO-K1 and HepG2 were seeded in cell culture bottles of 25 cm^2 in 5 mL of complete culture medium and incubated at 37 °C in CO_2. Following 24 h of cultivation, the treatment was proceeded with 1.5, 3.1, 6.2, 12.5, 25, 50 and 100 μM of SCAR complexes at stock solutions (500 μg/mL). 10% of DMSO (v/v) was used as positive control (PC) (Sigma-Aldrich) and 1.0% of DMSO (v/v) was used as negative control (NC) in the culture medium. All the treatments were performed for 48 h.

At the end of the treatments, the cells were washed and trypsinized, and replated into 6-well plates (Corning) at a concentration of 150 cells per well for each treatment in triplicate.

After 7 days of culture, the cells were washed and fixed with methanol: acetic acid: distilled water (1:1:8) for 30 min. The colonies were stained with 2.5 mL of Giemsa for 20 min. After staining, the cells were washed with distilled water, and the colonies were subsequently counted.

The average of colonies in the NC was regarded as 100%. Based on that, the calculations of survival fractions were performed (SF) for each treatment:

$$SF = \frac{\text{Number of colonies counted in each treatment}}{\text{Number of colonies counted in NC}} \times 100$$

The SF percentage of each treatment of three independent experiments was subjected to analysis of variance (ANOVA) followed by Tukey's pair-wise comparison and by Dunnett's multiple comparisons. All the tests were carried out taking into account a significance level of 5% ($p < 0.05$).

2.3. Cytokinesis block micronucleus cytome (CBMN-cyt) assay

The CBMN-cyt was performed in HepG2 and CHO-K1 cells previously described by Fenech [15], with modifications. A total of 5.0×10^5 cells were incubated in 25 cm^2 culture flasks for 24 h before being treated with 3 varying concentrations of the SCAR complexes, which were selected from the clonogenic survival assays. In the treatment period, CHO-K1 cells received 5.1, 10.2 and 20.5 μM of SCAR 4, 0.64, 1.3 and 2.6 μM of SCAR 5 and 25.0, 50.0 and 100.0 μM of SCAR 6, while the HepG2 cells received 3.8, 7.6 and 15.5 μM of SCAR 4, 0.32, 0.64 and 1.3 μM of SCAR 5 and 7.5, 15.0 and 30.0 μM of SCAR 6. Doxorubicin chloride (Sigma–Aldrich) at a concentration of 0.05 μM was used as an inducing agent (PC). Another PC was composed of 5 μM of aflatoxin B_1 (AFB_1) (Sigma–Aldrich), which was used as a model of indirect mutagen. DMSO (1.0%) as negative control and the control medium (MC) was composed of DMEM, without the action of any treatment. After 24 h of treatment, the cells were washed with PBS, the culture medium was changed, and the cells were incubated with cytochalasin B (3 μg/mL in culture medium - Sigma–Aldrich).

The cells were then incubated for 24 h, harvested using 0.25% of trypsin-EDTA (GIBCO), treated with 1% (v/v) cold potassium chloride (Sigma–Aldrich) and fixed with formaldehyde and methanol:acetic acid (3:1) solution for 5 min. Immediately prior to the analysis, the slides were stained using 40 μg/mL of Acridine Orange (Sigma–Aldrich), and the frequencies of binucleated cells with micronuclei (MNi) were determined at 1000x magnification.

The frequency of nucleoplasmic bridges (NPBs, biomarkers of dicentric chromosomes resulting from telomere end fusions or DNA mis-repair) and nuclear buds (NBUDs, biomarkers for gene amplification and altered gene dosage events) were also evaluated according to the methods described by [15]. In addition, the cytostatic effects were assessed using the Nuclear Division Index (NDI). A total of 500 viable cells per experimental point were scored to determine the percentage of cells with one, two, three and four nuclei, and the NDI was calculated in accordance with [16]: NDI = [M1 + 2 (M2) + 3 (M3) + 4 (M4)]/N, where M1–M4 represent the numbers of cells with 1–4 nuclei, respectively, and N representing the total number of cells scored.

This index provides a measure of the proliferative status of viable cells. Thus, the smallest possible value of NDI is 1.0, which occurs when all the cells are neither divided nor have had the cytokinesis blocked and thus are all mononuclear. If all the cells are able to complete a division cycle and are therefore by their entirety binucleated, then the NDI is 2.0.

For each treatment the mean and standard deviations were calculated. The MNi, NPB and NBUD frequencies were evaluated in a total of 1000 binucleated cells. The NDI calculation was measured in 500 cells per treatment. Three independent experiments were per-

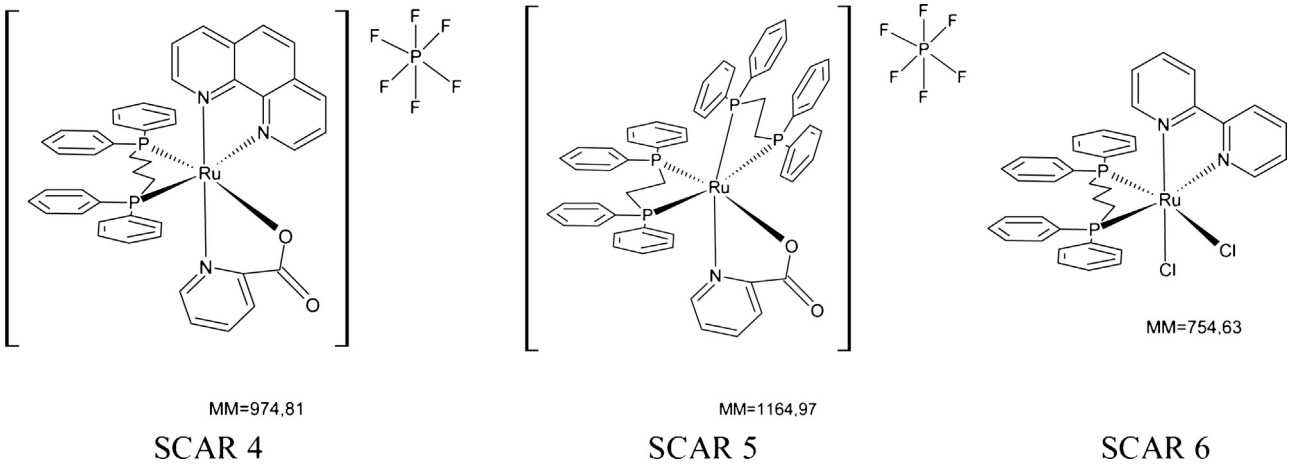

Fig. 1. SCARs structures.

formed. The results were subjected to ANOVA (assuming $p < 0.05$) followed by Dunnett's post-test for comparison with the negative control.

2.4. Ames test

The *S. typhimurium* tester strains TA1535, TA98, TA100, TA97a and TA102, were kindly provided upon request by Dr. B.N. Ames (Berkeley, CA, USA) and used in line with the preincubation methodology developed by Maron and Ames [17].

These strains were grown overnight from frozen cultures for 16 h in Oxoid Nutrient Broth No.2. The metabolic activation mixture (S9 fraction) prepared from the livers of Sprague Dawley rats treated with the polychlorinated biphenyl mixture Aroclor 1254 (500 mg/kg), was purchased from Molecular Toxicology Inc. (Boone, NC, USA) and freshly prepared prior to each test. The metabolic activation system consisted of 4% S9 fraction, 1% of 0.4 M $MgCl_2$, 1% of 1.65 M KCl, 0.5% of 1 M D-glucose-6-phosphate disodium and 4% of 0.1 M NADP, 50% of 0.2 M phosphate buffer and 39.5% of sterile distilled water.

For the mutagenic activity assay, varying concentrations of each complex (3.2–102.6 μM/plate for SCAR 4, 0.67–85.8 μM/plate for SCAR 5 and 4.14–265.3 μM/plate for SCAR 6) dissolved in DMSO were tested. The SCAR concentrations were selected on the basis of a preliminary toxicity test. In all the subsequent assays, the upper limit of the dose range tested was either the highest non-toxic dose or the lowest toxic dose determined in the preliminary assay. Toxicity was detected either as a reduction in the number of histidine revertants (His^+) or as a thinning of the auxotrophic background lawn.

The various amounts of the complexes to be tested, dissolved in DMSO, were added to 0.5 mL of 0.2 M phosphate buffer or 0.5 mL of 4% S9 mixture, plus 0.1 mL of bacterial culture and then incubated at 37 °C for 20 min. Thereafter, 2 mL of top agar were added, and the mixture was poured on to a plate containing minimal agar. The plates were incubated for 48 h at 37 °C and the His+ revertant colonies were counted with the aid of colonies counter Synbiosis Protocol.

The results were analyzed with the statistical software package Salanal 1.0 (U.S. Environmental Protection Agency, Monitoring Systems Laboratory, Las Vegas, NV, from Research Triangle Institute, RTP, NC, USA), adopting the [18] model. The data (revertants/plate) were assessed using the analysis of variance (ANOVA), followed by linear regression. The mutagenic index (MI) was also calculated for each concentration tested, this being the average number of revertants per test plate divided by the average number of revertants per negative (solvent) control plate. A sample was considered mutagenic when a dose–response relationship was detected and MI > 2, at one or more concentrations [19]. The standard mutagens used as positive controls in experiments without S9 mix were NPD (10 μg/plate) for TA98 and TA97a, SA (1.25 μg/plate) for TA1535 and TA100 and MMC (0.5 μg/plate) for TA102. In experiments with S9 activation, 2-AA (1.25 μg/plate) was used with TA1535, TA98, TA97a and TA100 and 2-AF (10 μg/plate) with TA102. DMSO served as the negative (solvent) control (100 μL/plate).

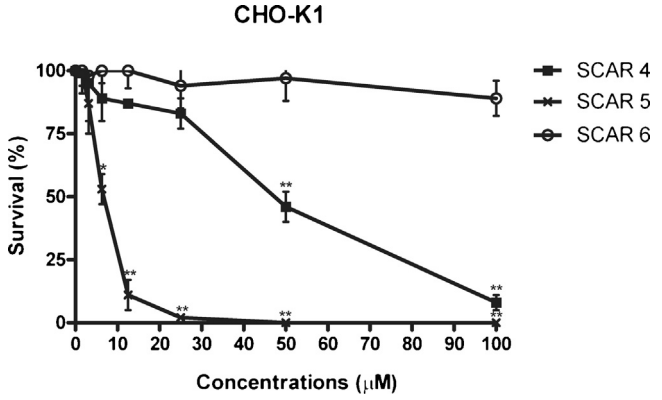

Fig. 2. Clonogenic survival assay of CHO-K1 cell lines after treatment with the complexes SCAR 4, 5 and 6 (1.5; 3.1; 6.2; 12.5; 25; 50 and 100 μM) for 48 h. Each value represents the mean derived from at least three individual experiments in triplicate (mean ± SD). (*)Statistically significant difference compared to vehicle control ($p < 0.05$); (**)Statistically significant difference compared to the control and between treatments ($p < 0.05$).

3. Results

3.1. Cytotoxicity

This experiment was conducted to assess the cell viability after 48 h of treatment with SCAR 4, 5 and 6 in CHO-K1 and HepG2 cell lines.

In this test, the colonies were counted, to determine cell reproductive death after treatment with the complexes. Fig. 2 shows the SF of CHO-K1 cells, which was calculated [SF = number of colonies counted in the treatment/(number colonies counted in negative control) × 100], and the mean values obtained ranging from 99.0% to 7.7% in the treatment with SCAR 4, 99.3% to 0% when treated with the SCAR 5 and average values ranging between 99.8% and 89.5% in the treatment with SCAR 6.

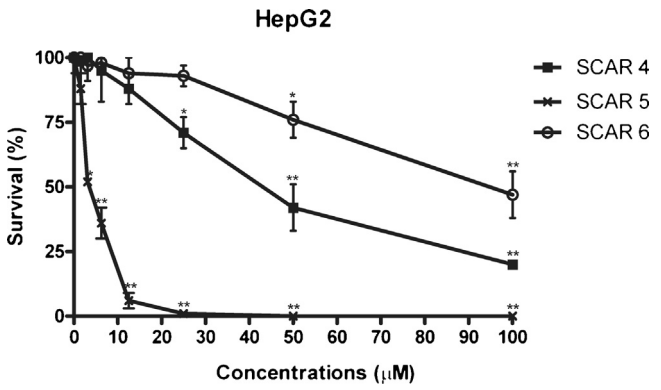

Fig. 3. Clonogenic survival assay of HepG2 cell lines after treatment with the complexes SCAR 4, 5 and 6 (1.5; 3.1; 6.2; 12.5; 25; 50 and 100 μM) for 48 h. Each value represents the mean derived from at least three individual experiments in triplicate (mean ± SD). (*)Statistically significant difference relative to the vehicle control ($p < 0.05$); (**)Statistically significant difference relative to the control and between treatments ($p < 0.05$).

Similarly, the SF was calculated for HepG2 cells treated with the complexes (Fig. 3). The mean values ranged from 99.1% to 20.4% in the treatment with SCAR 4, 88,4,3% to 0% when treated with SCAR 5 and 99.8% to 46.5% in the treatment with SCAR 6.

Statistically significant differences were observed ($p < 0.05$) in the treatment with SCAR 4 relative to the control for concentrations of 50 and 100 μM, and 6.2; 12.5; 25; 50 and 100 for SCAR 5 in the culture with CHO-K1 cells.

In HepG2 cells, statistically significant differences were observed ($p < 0.05$) at concentrations 25, 50 and 100 μM of SCAR 4, 3.1; 6.2; 12.5; 25; 50 and 100 μM in the treatment with SCAR 5 and concentrations of 50 and 100 μM for SCAR 6.

3.2. CBMN-cyt assay

The CBMN-cyt assay was performed in triplicate using CHO-K1 and HepG2 cell lines where the following parameters were analyzed: micronuclei frequency (MNi), nucleoplasmic bridges (NPBs) and nuclear buds formation (NBUDs) in 1000 binucleated cells. We also calculated the proportion of mono-, bi-, tri or multinucleated cells, which was used to calculate the NDI. The maximum concentrations were based on cytotoxicity previously selected from clonogenic survival assay. In this study, no statistically significant difference was found in the proliferative state of CHO-K1 cells and HepG2 cultures treated with the complexes as compared to the negative control.

Table 1 shows the MNi, NPBs and NBUDs induced by the treatment with the complexes SCAR 4, 5 and 6 in CHO-K1 cells culture. The experiments were performed in triplicate and the mean and standard deviation of the results are presented in Table 1.

In the CHO-K1 cells, the complexes did not lead to a statistically significant increase in the MNi, NPBs and NBUDs frequencies as compared to the vehicle control ($p < 0.05$), demonstrating the absence of mutagenic activity under the experimental conditions used in this study. On the other hand, the results showed a significant increase of MNs and NPBs frequencies in HepG2 cells after treatment with SCAR 6 ($p < 0.05$) (Table 2).

3.3. Ames test

Tables 3–5 show the mean number of revertants/plate (M), the standard deviation (SD) and the mutagenic index (MI) following the treatments with SCAR 4, 5 and 6 complexes, observed in *S. typhimurium* strains TA1535, TA98, TA100, TA102 and TA97a, in the presence (+S9) and absence (−S9) of metabolic activation.

Table 1
Assessment of mutagenic effects os SCAR 4, 5 and 6 on CHO-K1 cells using the cytokinesis-block micronucleus cytome assay (CBMN-cyt).

Treatments	MNi	NPBs	NBUDs	NDI
Vehicle control	3.66 ± 1.52	4.00 ± 1.00	6.66 ± 4.16	1.85 ± 0.18
Positive control[a]	55.33 ± 7.55*	14.33 ± 4.58*	17.33 ± 6.02*	1.81 ± 0.05
Positive control[b]	4.66 ± 2.08	2.33 ± 2.51	6.66 ± 4.04	1.78 ± 0.83
SCAR 4				
5.1 μM	3.67 ± 0.89	1.33 ± 0.44	4.00 ± 0.67	1.82 ± 0.04
10.2 μM	3.33 ± 1.56	3.00 ± 0.67	3.67 ± 2.89	1.81 ± 0.02
20.5 μM	2.33 ± 1.11	3.33 ± 0.89	3.67 ± 2.22	1.85 ± 0.26
SCAR 5				
0.64 μM	1.33 ± 0.44	1.67 ± 1.11	2.00 ± 0.00	1.80 ± 0.09
1.3 μM	0.67 ± 0.89	1.33 ± 1.11	2.00 ± 0.67	1.80 ± 0.57
2.6 μM	2.00 ± 0.67	1.67 ± 0.44	3.00 ± 0.67	1.78 ± 0.45
SCAR 6				
25.0 μM	2.66 ± 1.52	3.00 ± 1.73	5.66 ± 2.08	1.78 ± 0.42
50.0 μM	4.33 ± 0.57	5.33 ± 2.08	5.33 ± 1.05	1.81 ± 0.04
100.0 μM	4.00 ± 1.73	6.66 ± 2.64	5.00 ± 3.46	1.82 ± 0.11

Values shown are the mean ± SD; BN: binucleated cell; MNi: micronuclei; NPBs: nucleoplasmic bridges; NBUDs: nuclear buds and NDI: nuclear division index. The data shown are based on three independent experiments. Vehicle control, 1.0% dimethylsulfoxide; Positive control[a], 0.05 μM doxorubicin; Positive control[b], 5 μM aflatoxin B1. *: Significantly different from the vehicle control ($p < 0.05$).

Table 2
Assessment of mutagenic effects of SCAR 4, 5 and 6 on HepG2 cells using the cytokinesis-block micronucleus cytome assay (CBMN-cyt).

Treatments	MNi	NPBs	NBUDs	NDI
Vehicle control	2.67 ± 1.53	1.00 ± 1.00	7.33 ± 1.53	1.84 ± 0.50
Positive control[a]	72.33 ± 6.66*	12.67 ± 2.08*	25.00 ± 14.93*	1.81 ± 0.17
Positive control[b]	41.14 ± 17.03*	16.67 ± 5.51*	19.33 ± 6.78*	1.78 ± 0.15
SCAR 4				
3.8 μM	1.67 ± 0.44	2.33 ± 0.89	3.00 ± 1.33	1.83 ± 0.20
7.6 μM	4.33 ± 1.11	2.33 ± 1.11	1.67 ± 0.89	1.75 ± 0.80
15.5 μM	2.00 ± 0.67	1.67 ± 0.44	1.67 ± 1.11	1.87 ± 0.20
SCAR 5				
0.32 μM	3.33 ± 1.11	2.33 ± 0.44	3.00 ± 1.33	1.77 ± 0.50
0.64 μM	6.00 ± 2.00	3.00 ± 1.11	2.67 ± 1.11	1.89 ± 0.70
1.3 μM	4.67 ± 1.78	2.00 ± 0.67	3.67 ± 1.11	1.85 ± 0.30
SCAR 6				
7.5 μM	9.60 ± 3.06	3.33 ± 1.53	12.67 ± 2.08	1.85 ± 0.21
15.0 μM	14.33 ± 3.06*	3.00 ± 1.00	11.00 ± 4.36	1.85 ± 0.18
30.0 μM	22.67 ± 8.33*	8.67 ± 4.04*	13.33 ± 4.93	1.88 ± 0.53

Values shown are the mean ± SD; BN, binucleated cell; MNi, micronuclei; NPBs, nucleoplasmic bridges; NBUDs, nuclear buds and NDI, nuclear division index. The data shown are based on three independent experiments. Vehicle control, 1.0% dimethylsulfoxide; Positive control[a], 0.05 μM doxorubicin; Positive control[b], 5 μM aflatoxin B1. *: Significantly different from the vehicle control ($p < 0.05$).

Table 3
Revertants/plate, standard deviation and mutagenicity index (in brackets) in the strains TA1535, of *S. typhimurium* after treatment with various doses of SCAR 4, with (+S9) and without (−S9) metabolic activation.

Treatments	Number of revertants (M ± SD)/plate and MI	
	TA 1535	
μM	−S9	+S9
0.00[a]	6 ± 1	7 ± 1
3.2	7 ± 2 (1.2)	6 ± 2 (0.8)
6.4	6 ± 2 (1.0)	7 ± 1 (1.0)
12.8	6 ± 1 (1.0)	7 ± 2 (1.0)
25.6	6 ± 1 (0.9)	8 ± 1 (1.1)
51.3	5 ± 2 (0.8)	6 ± 2 (0.8)
102.6	Toxic	Toxic
C+	140 ± 22[b]	234 ± 75[c]

C+: Positive control.
[a] Negative control: dimethylsulfoxide (100 μL/plate).
[b] sodium azide (1.25 μg/plate) in the absence of S9.
[c] 2-anthramine in the presence of S9.

Table 4
Revertants/plate, standard deviation and mutagenicity index (in brackets) in the strains TA1535, TA98, TA100, TA102 and TA97a of *S. typhimurium*after treatment with various doses of SCAR 5, with (+S9) and without (-S9) metabolic activation.

Treatments	Number of revertants (M ± SD)/plate and MI									
	TA 1535		TA 98		TA 100		TA 102		TA 97a	
μM	−S9	+S9	−S9	+S9	−S9	+ S9	−S9	+S9	−S9	+S9
0.00[a]	6 ± 0	5 ± 1	20 ± 6	15 ± 4	184 ± 1	180 ± 60	297 ± 73	333 ± 26	125 ± 4	121 ± 8
0.67	8 ± 5 (1.3)	8 ± 1 (1.3)	24 ± 1 (1.2)	21 ± 2 (1.4)	201 ± 39 (1.1)	178 ± 26 (1.0)	316 ± 8 (1.1)	389 ± 3 (1.2)	186 ± 26 (1.5)	126 ± 1 (1.0)
1.34	8 ± 1 (1.2)	6 ± 1 (1.2)	19 ± 4 (0.9)	20 ± 3 (1.3)	170 ± 7 (1.0)	200 ± 69 (1.1)	293 ± 40 (1.0)	359 ± 50 (1.1)	171 ± 8 (1.4)	122 ± 7 (1.0)
2.68	7 ± 4 (1.2)	7 ± 1 (1.4)	22 ± 4 (1.1)	20 ± 2 (1.3)	209 ± 26 (1.1)	176 ± 21 (1.0)	319 ± 15 (1.1)	351 ± 84 (1.0)	151 ± 40 (1.2)	128 ± 21 (1.0)
5.36	7 ± 1 (1.2)	7 ± 1 (1.4)	18 ± 5 (0.9)	15 ± 3 (1.0)	176 ± 10 (1.0)	196 ± 28 (1.1)	312 ± 22 (1.0)	424 ± 17 (1.2)	162 ± 27 (1.3)	119 ± 16 (1.0)
10.7	5 ± 3 (0.8)	6 ± 2 (1.2)	17 ± 7 (0.8)	13 ± 1 (0.9)	162 ± 6 (0.9)	162 ± 10 (0.9)	300 ± 22 (1.0)	414 ± 35 (1.2)	155 ± 27 (1.2)	124 ± 9 (1.0)
21.4	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic
42.9	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic
85.8	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic
C+	462 ± 22[c]	570 ± 33[e]	883 ± 71[b]	904 ± 80[e]	801 ± 38[c]	1020 ± 22[e]	970 ± 114[d]	1106 ± 51[f]	749 ± 71[b]	940 ± 115[e]

C+: Positive control.
[a] Negative control: dimethylsulfoxide (100 μL/plate).
[b] 4 -nitro-o-phenylenediamine (10.0 μg/plate – TA98, TA97a).
[c] sodium azide (1.25 μg/plate –TA100, TA1535).
[d] mitomycin (0.5 μg/plate – TA102), in the absence of S9.
[e] 2-anthramine (1.25 μg/plate - TA97a, TA98, TA100 and TA 1535).
[f] 2-aminofluorene (10.0 μg/plate – TA102), in the presence of S9.

Table 5
Revertants/plate, standard deviation and mutagenicity index (in brackets) in the strains TA1535, TA98, TA100, TA102 and TA97a of *S. typhimurium* after treatment with various doses of SCAR 6, with (+S9) and without (−S9) metabolic activation.

Treatments	Number of revertants (M ± SD)/plate and MI									
	TA 1535		TA 98		TA 100		TA 102		TA 97a	
μM	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
0.0[a]	6 ± 1	7 ± 3	23 ± 6	24 ± 4	85 ± 8	95 ± 12	398 ± 12	474± 26	102 ± 4	86 ± 10
4.14	8 ± 1.5 (1.3)	9 ± 2 (1.2)	26 ± 8 (1.1)	27 ± 6 (1.1)	100 ± 15 (1.2)	122 ± 13 (1.3)	402 ± 7 (1.0)	542 ± 39 (1.1)	119 ± 19 (1.2)	106 ± 22 (1.2)
8.29	7 ± 0 (1.2)	10 ± 3 (1.4)	27 ± 5 (1.1)	27 ± 3 (1.1)	99 ± 17 (1.2)	115 ± 20 (1.2)	400 ± 36 (1.0)	554 ± 32 (1.2)	120 ± 20 (1.2)	110 ± 14 (1.3)
15.5	7 ± 1 (1.1)	7 ± 0 (1.0)	27 ± 3 (1.1)	24 ± 1 (1.0)	101 ± 33 (1.2)	107 ± 22 (1.1)	403 ± 40 (1.0)	466 ± 25 (1.0)	119 ± 20 (1.2)	132 ± 9 (1.5)
33.1	6 ± 1 (1.0)	8 ± 0 (1.1)	26 ± 4 (1.1)	28 ± 2 (1.1)	105 ± 45 (1.2)	110 ± 5 (1.1)	400 ± 33 (1.0)	474 ± 12 (1.0)	121 ± 10 (1.2)	172 ± 11* (2.0)
66.3	6 ± 2 (1.0)	7 ± 3 (1.0)	26 ± 2 (1.1)	34 ± 12 (1.4)	83 ± 22 (1.0)	109 ± 9 (1.1)	326 ± 18 (0.8)	415 ± 12 (0.9)	124 ± 16 (1.2)	210 ± 16** (2.4)
132.6	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	156 ± 15 (1.5)	253 ± 15** (2.9)
199.0	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	54 ± 26 (1.5)	204 ± 19* (2.3)
265.3	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	84 ± 13 (0.8)	88 ± 15 (1.0)
C+	463 ± 48[c]	523 ± 107[e]	912 ± 43[b]	1010 ± 94[e]	1021 ± 38[c]	1200 ± 29[e]	1067 ± 74[d]	1126 ± 90[f]	824 ± 54[b]	904 ± 75[e]

Bernstein model *$p < 0.05$; **$p < 0.01$ (ANOVA); C+: Positive control.
[a] Negative control: dimethylsulfoxide (100 μL/plate).
[b] 4-nitro-o-phenylenediamine (10.0 μg/plate – TA98, TA97a).
[c] sodium azide (1.25 μg/plate –TA100, TA1535).
[d] mitomycin (0.5 μg/plate – TA102), in the absence of S9.
[e] 2-anthramine (1.25 μg/plate – TA97a, TA98, TA100 and TA 1535).
[f] 2-aminofluorene (10.0 μg/plate – TA102), in the presence of S9.

The results of the SCAR 4 complex obtained from the study conducted by our research group [7] showed no mutagenic activity in the strains TA98, TA100, TA102 and TA97 of *S. typhimurium*. However, to complement these results, the mutagenic activity in the TA1535 strain was also evaluated and no increase was observed in the number of revertant colonies in any concentrations of SCAR 4, in the absence and presence of metabolic activation (Table 3).

The SCAR 5 complex did not induce an increase in revertant colonies in any concentrations, in the absence and presence of metabolic activation (Table 4). These results lead to the conclusion that the SCAR 5 complex is not capable of causing genetic mutations, as evidenced by the Ames test.

In the absence of metabolization, SCAR 6 complex was not considered mutagenic in any concentration, and in none of the strains of *S. typhimurium* studied.

Nonetheless, in the experiments with metabolic activation (+S9), it was observed that the SCAR 6 complex after the metabolization process led to an increase in the number of revertant colonies, with mutagenic effect in the TA97a strain ($p < 0.05$). The mutagenicity index in the TA97a strain was equal/greater than 2.0 from the concentration of 33.1 μM to 199 μM/plate (Table 5). Thus, it can be inferred that after metabolic activation, the complex SCAR 6 induces frameshift mutations by adding C:G pairs, detectable through the TA97a strain of *S. typhimurium*. This outcome suggests that SCAR 6 reacts with DNA indirectly with bioactivation, and that bioactivation fosters DNA damage.

4. Discussion

The assessment that is required to be undertaken regarding the toxic effects of chemicals such as the candidates for new drugs, environmental and industrial compounds, among other agents, is an increasingly recurring challenge in our modern world. Contact with newly discovered molecules, even without knowledge of their biological activities, can cause harm to the human health in both short and long terms [20].

The absence of new drugs for TB treatment, despite the indisputably myriad efforts that have been made in this regard, is conceivably worrying, especially when dealing with MDR-TB [7]. Many research groups have been toiling in quest for new molecules

so as to enable them replace or supplement the standard therapy [21,22]. In the drug discovery field, for instance, the one can highlight the role of medicinal chemistry and inorganic metal complexes in the design of therapies that target different biochemical pathways [12].

Studies have brought into the limelight the vital role of the complex of gold, paladium, cobalt, copper and ruthenium with a promising activity against *M. tuberculosis*, with extra and intracellular targets, thus making thempotential candidates for future applications in therapy [23–26].

Encouraged by the promising results, our group has been seeking new candidates against MDR-TB. In previous studies, the complexes of ruthenium(II) SCAR 4, 5 and 6 showed MIC better or perhaps equivalent to the drugs used in first-line treatments, including activity against resistant clinical isolates of *M. tuberculosis* [7].

These promising results paved the way for the SCAR complexes to represent a new alternative therapy against TB, listed in the global pipeline of anti-TB drugs in research and stages of approval [27]

In this study, the clonogenic survival assay was conducted to determine the cell viability of HepG2 and CHO-K1 cells treated with seven different concentrations of SCAR 4, 5 and 6 complexes to set the concentrations to be used in subsequent *in vitro* assays.

Following 48 h of treatment and 7 days of subculture, SCAR 5 was found to have lower survival rates, presenting five statistically varying concentrations ($p < 0.05$) of control (1% DMSO), with SCAR 4 presenting only two cytotoxic concentrations. (Fig. 2).

Interestingly, we observed the same antiproliferative activity for the complexes in the tumor cell line HepG2, though with quite more pronounced effects. The SCAR 5 complex continued to have a major cytotoxic effect, followed by SCAR 4 and SCAR 6 that has also shown cytotoxic concentrations (50 and 100 μM) (Fig. 3).

The clonogenic survival assay is considered a more accurate test owing to the fact it takes into account the reproductive death, which is an important parameter to be analyzed and used in defining non-cytotoxic concentrations for *in vitro* assays [28].

The number of colony forming cells was reduced in a dependent manner as far as the complexes concentration is concerned. It is observed, moreover, that the number of HepG2 tumor cells colonies treated with the SCAR complexes was smaller compared tothe same concentrations in the normal cells (CHO-K1). It is worth considering the anti-proliferative effect of ruthenium complexes in tumor cells rather than in normal cells, suggesting a selectivity action mechanism and corroborating with recent studies that show a variety of ruthenium complexes with antitumor activity *in vitro* [29,30].

Our results showed that SCAR 6 complex in the highest concentration presents a SF of 46.5% in tumor cells, whereas in normal cells it is found to have a much less evident anti-proliferative, with 89.5% of survival cells in the same concentration.

These values reflect the selective nature of the complex, thus making them promising anticancer agents. Studies such as those published by [31] show comparative results among the antiproliferative action of ruthenium complexes in normal and tumor models, where in general, these complexes exhibit selectivity values that make them promising candidates for a safer therapy.

The mutagenic effect of complexes in a chromosomal level was evaluated using three selected concentractions for each complex that did not affect the viability of CHO-K1 and HepG2 cells that were considered adequate tools for the analysis of direct and indirect mutagens [32].

According to Fenech [33], in micronuclei analyses, cultures must present a percentage of binucleated cells above 35%. In this study, all the treatments as well as the controls the proportion of binucleated cells was greater than 65%, thereby rendering all the treatments feasible of being analyzed. The results obtained showed that none of theconcentrations of the SCAR 4 and 5 complexes led to a statistically significant increase in the total number of micronuclei, nucleoplasmic bridges and nuclear buds compared to the control (DMSO 1%), thus indicating that these ruthenium complexes are not capable of provoking chromosomal mutations in cellular systems assessed under the conditions used in this study (Table 1).

It is worth pointing out, nevertheless, that SCAR 6 was capable of propelling a significant increase in the number of MN and NPBs in HepG2 cells (Table 2). The fact that mutagenic effect has only been observed in HepG2 cells may be related to the mechanism of action of the SCAR 6 molecule.

Its structure which is known to be similar to cisplatin has two chloride ligands complexed to the metal.

The action mechanism of cisplatin is undoubtedly well known, its chloride ligands are removed due to the hydrolysis that takes place inside the cell, and as such its hydrolyzed molecule generates a species that binds irreversibly to the DNA, usually the two adjacent guanine bases [10]. This interaction leads to the distortion of the molecule and a consequent break up of the DNA [34]. These mechanisms can lead to chromosomal aberrations as well as cell viability decrease in mammals [35].

As a result of the chemical similarity, because of square planar structure, that the SCAR 6 complex bears with cisplatin, it is suggested that it can interact with the DNA in a similar fashion, leading to the chromosomal breakage and a consequent formation of micronuclei in metabolizing cells.

The use of metabolizing cell lines, such as those derived from human liver, best reflects the sensitivity to detection of various classes of genotoxic chemical compounds [36]. HepG2 cells derived from human hepatocellular carcinoma, kept phase I and II enzymes active, as we know these enzymes play a crucial role in the activation of indirect mutagens and represent the *in vivo* metabolism in a more predictive way than experimental models with metabolically incompetent cells like CHO-K1. It is owing to the aforementioned reason that some chemicals may present negative results for mutagenicity in CHO-K1 cells, while exhibiting positive results in HepG2 cells under the same conditions [37,38].

In the CBMN-cyt assay, AFB_1 was additionally used as positive control, which once activated by CYP3A4, known to be not highly expressive in rats as in humans, can form various adducts in DNA [38]. Our results confirm the ability of our HepG2 cell lines to potentially metabolize genotoxic xenobiotics in the sense that the increase observed in MNs, NPBs and NBUDs were statistically significant in HepG2 cells (Table 2). Contrary to that, however, the mutagenic effect was not observed in CHO-K1 cells (Table 1).

These results can be compared with those found in the reverse mutation assay (Ames test), where the same complex, SCAR 6, showed mutagenic activity after undergoing metabolization. The Ames test was performed according to the OECD guideline N°. 471 [39] with 5 different strains of *S. typhimurium* (TA 1535, TA 98, TA 100, TA 102 and TA 97a).

No duplication was observed in the number of revertant colonies in any of the concentrations of SCAR 4 and 5 as compared to the negative control. Furthermore, there was not any dose-response relationship in any of these strains and as such, it can be concluded that the SCAR 4 and 5 do not have mutagenic effect.

In previous studies, [7] assessed, among other things, the mutagenic activity of some ruthenium complexes (SCAR) by the Ames test. Their results showed that the complexes studied did not show a statistically significant increase in the number of revertant colonies compared to the negative control, thereby indicating no mutagenic effect [7].

SCAR 6 complex was also not able to increase the number of revertants in the absence of metabolism experiments. However,

when S9 was added mutagenic effect was observed in the TA97a strain.

According to the strain involved, we can assume that the main genotoxic action mechanism of this compound is a frameshift mutation, a condition that occurs upon adding C:G pairs after undergoing metabolism [17].

The suggested mechanism of mutagenicity involves metabolic activation, causing the formation of metabolites that interact with bacterial DNA and leading to the reversion of mutations installed in *S. typhimurium strains* (TA97a). Thus, the results lead us to deduce that the SCAR 6 complex acts as an indirect mutagenic agent, in other words, the complex becomes mutagenic when metabolized.

This result confirms the effect observed in CBMN-cyt assay, suggesting that SCAR 6 complex may give rise to mutagenic products after being metabolized by cytochrome P-450 system.

In vitro studies have confirmed the ability of ruthenium complexes to bind to DNA [40]. The cytotoxicity in cell cultures and genotoxicity in *in vitro* and *in vivo* models indicate a direct correlation of this capacity [41–43].

It has evidently become clear the importance of the study of the mutagenic potential of new compounds to which organisms can be exposed. Obviously, a mutagenic event does not necessarily lead to the development of cancer, however, the detection of mutagenic activity is an important indicator for assessing the risk of the disease [44].

In summary, this study showed that two of the three ruthenium complexes do not have mutagenic activities *in vitro*. By some fortunate stroke of coincidence, these complexes are the ones that showed the most promising results against MDR *M. tuberculosis* strains in previous studies [7]. However, the SCAR 5 complex presented cytotoxicity in CHO-K1 and HepG2 cells at concentrations greater than 3.0 μM.

Cytotoxicity was investigated in this study with the aim of determining optimal concentrations for the CBMN-cyt assay. Oddly enough though, a possible selective cytotoxicity activity was observed for tumor cells. Further investigation into this issue is required using different models of human tumor and normal cells.

Our study makes a meaningful contribution in the sense that it helps to clarify a wide range of activities attributed to these complexes in biological systems providing valuable elucidations regarding the safety profile in the therapeutic use of these molecules.

It is noteworthy to point out that only one of the analyzed complexes (SCAR 6) was considered mutagenic in standard systems for the determination of *in vitro* mutagenicity. This effect, nonetheless, cannot be in the least bit considered discouraging owing to the fact that knowing the possible damage that chemical substances cause the DNA is indeed an essentially important information for the direction of appropriate therapy. This effect can be further explored given the known antitumor potential that ruthenium complexes have been known to have contributing to a novel and rational approach to developing a new metal-based drug. Other studies on the mechanisms of action of these complexes are underway and preliminary results have suggested mechanisms of interaction with DNA.

Conflict of interest

The authors declare no conflicts of interest.

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