The ruthenium complexes *cis*-(dichloro)tetramineruthenium(III) chloride and *cis*-tetraammine(oxalato)ruthenium(III) dithionate overcome resistance inducing apoptosis on human lung carcinoma cells (A549)

Cesar Augusto Sam Tiago Vilanova-Costa · Hellen Karine Paes Porto ·
Flávia de Castro Pereira · Aliny Pereira de Lima · Wagner Batista dos Santos ·
Elisângela de Paula Silveira-Lacerda

Abstract Lung cancer is one of the leading causes of death in the world, and non-small cell lung carcinoma accounts for approximately 75–85 % of all lung cancers. In the present work, we studied the antitumor activity of the compound *cis*-(dichloro)tetramineruthenium(III) chloride {*cis*-[RuCl$_2$(NH$_3$)$_4$]Cl} against human lung carcinoma tumor cell line A549. The present study aimed to investigate the relationship between the expression of MDR1 and CYP450 genes in human lung carcinoma cell lines A549 treated with *cis*CarboPt, *cis*CRu(III) and *cis*DRu(III). The ruthenium-based coordinated complexes presented low cytotoxic and antiproliferative activities, with high IC$_{50}$ values, 196 (±15.49), 472 (±20.29) and 175 (±1.41) for *cis*CarboPt, *cis*CRu(III) and *cis*DRu(III), respectively. The tested compounds induced apoptosis in A549 tumor cells as evidenced by caspase 3 activation, but only at high concentrations. Results also revealed that the amplification of P-gp gene is greater in A549 cells exposed to *cis*CarboPt and *cis*CRu(III) than *cis*DRu(III). Taken together all these results strongly demonstrate that MDR-1 over-expression in A549 cells could be associated to a MDR phenotype of these cells and moreover, it is also contributing to the platinum, and structurally-related compound, resistance in these cells. The identification and characterization of novel mechanisms of drug resistance will enable the development of a new generation of anti-cancer drugs that increase cancer sensitivity and/or represent more effective chemotherapeutic agents.

Keywords Carboplatin · *cis*-(Dichloro)tetramineruthenium(III) chloride · *cis*-Tetraammine(oxalato)ruthenium(III) dithionate · A549 · MDR-1 · CYPs

C. A. S. T. Vilanova-Costa · H. K. P. Porto ·
F. C. Pereira · A. P. de Lima · E. P. Silveira-Lacerda (✉)
Laboratório de Genética Molecular e Citogenética,
Instituto de Ciências Biológicas, Universidade Federal de
Goiás - UFG, Campus Samambaia (Campus II), Cx.
Postal: 131, Goiânia, GO 74001-970, Brazil
e-mail: silveiralacerda@gmail.com

C. A. S. T. Vilanova-Costa
e-mail: vilanovacosta@gmail.com

H. K. P. Porto
e-mail: paesporto@gmail.com

F. C. Pereira
e-mail: pereirafc@gmail.com

A. P. de Lima
e-mail: alinypereiralima@gmail.com

W. B. dos Santos
Instituto de Ciências Exatas e da Terra, Universidade
Federal de Mato Grosso - UFMT, Barra do Garças,
MT 78698-000, Brazil
e-mail: wbsantos32@yahoo.com.br

Introduction

Lung cancer is one of the leading causes of death in the world, and non-small cell lung carcinoma

(NSCLC) accounts for approximately 75–85 % of all lung cancers. At the time of diagnosis, 40 % of NSCLC cases are often at an advanced. Only 15 % of lung cancers are diagnosed at a localized stage, for which the 5-year survival rate is 52 %. The 5-year survival for NSCLC is 18 % (American Cancer Society 2013).

First-line treatment for patients with advanced NSCLC includes platinum compounds such cisplatin, carboplatin and oxaliplatin. The platinum-based chemotherapy drugs are among the most active and widely used agents for the treatment of malignancies, including testicular, head and neck, ovarian, lung, colorectal, and bladder cancers (Kelland 2007; Varbanov et al. 2012). Nevertheless, the clinical utility of these drugs has been proven limited due to the development of acquired drug resistance (Karki et al. 2007; Wagner and Karnitz 2009).

The clinical treatment of cancer with chemotherapeutic drugs is frequently hindered by either intrinsic or acquired resistance of the tumor cells (Kelland 2007). When tumor cells acquire resistance against a single chemotherapeutic drug, they often show cross-resistance to a variety of antitumor drugs, a state termed multidrug resistance (MDR). According to statistical data, the failure of treatment in over 90 % of patients with metastatic cancer is caused by MDR (Liu et al. 2006). Although MDR can develop by several mechanisms, a common cause is believed to be overexpression of an energy-dependent drug efflux pump, P-glycoprotein (P-gp). P-gp lowers the intracellular concentration of cytotoxic agents by pumping them outside of the tumor cells (Tang et al. 2013; Liu et al. 2006).

The side effects of chemotherapy hamper many normal activities of patients undergoing treatment (Kim et al. 2002). Ruthenium complexes have shown potential utility in chemotherapy with lower toxicities compared to platinum-based chemoterapeutics attributed to their specific accumulation in cancer tissues. Ruthenium complexes have shown potential utility in chemotherapy with lower toxicities compared to platinum-based compounds attributed to their specific accumulation in cancer (Silveira-Lacerda et al. 2010a).

Among the ruthenium complexes studied for anticancer application, the *cis*-(dichloro)tetramminer-uthenium(III) chloride and *cis*-tetraammine(oxalato)ruthenium(III) dithionate complexes have shown

promising results on tumour cells in humans and mice (Menezes et al. 2007; Pereira et al. 2009; Silveira-Lacerda et al. 2010a, b; Lima et al. 2012).

Although platinating agents are among the most widely used chemotherapy agents, and ruthenates presents a potential role as new anticancer drugs, the molecular mechanism of multidrug resistance to chemotherapy remained largely unknown, especially in lung tumor cells. Since previous studies show that the resistance to chemotherapy exhibited in multidrug-resistant human lung tumor cells was mediated by P-gp and others CYP450 proteins, the present study aimed to investigate the relationship between the expression of MDR-1 and CYP450 genes as mediators of metallocomplexes cytotoxicity in human lung carcinoma cells (A549) treated with Carboplatin and two ruthenium(III) compounds.

Materials and methods

Ruthenium(III) compound synthesis, carboplatin and chemicals

The ruthenium(III) complexes *cis*-(dichloro)tetrammineruthenium(III) chloride {*cis*-[RuCl$_2$(NH$_3$)$_4$]Cl} and *cis*-tetraammine(oxalato)ruthenium(III) dithionate {*cis*-[Ru(C$_2$O$_2$)(NH$_3$)$_4$]$_2$(S$_2$O$_6$)} were synthesized at the Chemistry Institute of Universidade Federal de Mato Grosso by the methods described in detail previously (Pavanin et al. 1985; Pereira et al. 2009; Silveira-Lacerda et al. 2010a). Carboplatin [*cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II)] was obtained from Sigma-Aldrich (Sigma-Aldrich Corp, St. Louis, MO, USA).

The stock solutions of Ru(III) complexes and Carboplatin were freshly prepared before use in DMSO (Sigma-Aldrich Corp, St. Louis, MO, USA). The final concentration of DMSO in cell culture medium did not exceed 0.25 % (v/v).

Cell culture

The human lung carcinoma cells (A549 ATCC®# CCL-185™) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Tumor cells were cultured in Dulbecco's modified Eagle medium (DMEM) (pH 7.2–7.4) at 37 °C, under a 5 % CO$_2$

humidified atmosphere. Media were supplemented with 100 U mL^{-1} penicillin G, 100 µg mL^{-1} streptomycin, 2 mM L-glutamine, 1.5 g L^{-1} sodium bicarbonate, 4.5 g L^{-1} glucose, 10 mM HEPES, 1.0 mM sodium pyruvate and 10 % fetal calf serum (FCS) (w/v) (all reagents were obtained from Gibco®, Invitrogen, Carlsbad, CA, USA) and subcultured 2 times a week at an appropriate plating density.

Cell viability determination by MTT assay

The cytotoxic activity of ruthenium(III) compounds was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described (Mosman 1983). Carboplatin and Ru(III) metallodrugs were added to final concentrations from 0 to 500 µM. The logarithmically growing A549 cells were washed twice with supplemented DMEM medium and centrifuged at (1,000 rpm) $300 \times g$/15 min/10 °C. Cells were plated at a density of 5×10^4 cells/well into flat-bottomed 96-well microplates (Techno Plastic Products, Trasadingem, Switzerland) and cultured for 24 h.

Cell death was evaluated after tumor cells were exposed to the complexes for 48 h; then, after removal of medium with the complexes, a fresh medium was added, and cells were incubated for 24 h of recovery time. Then, 10 µL MTT (5 mg mL^{-1}) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated for an additional 6 h. The microplates were then centrifuged ($300 \times g$/15 min/10 °C) and the culture media were discarded. Then, 200 µL of PBS/20 % of SDS was added to each well and plates were kept in the dark overnight.

Absorbance was measured at 545 nm (OD$_{545nm}$) using an automated microplate reader (Stat Fax 2100, Awareness Technology Inc, Palm City, FL, USA). The viability rate was calculated as follows: Viability (%) = (absorbance of the treated wells)/(absorbance of the control wells) × 100 %. The IC$_{50}$ values (compound concentrations that produce 50 % of cell growth inhibition) were calculated from curves constructed by plotting cell survival (%) versus drug concentration (µM) using GraphPad Prism 4.02 for Windows (GraphPad Software, San Diego, CA, USA). All experiments were made in triplicate.

Cell toxicity measurement with lactate dehydrogenase (LDH) assay

LDH is a cytoplasmic enzyme, and LDH reduction is often associated with cell membrane damage and cell death (Lobner 2000). The activity of LDH was measured spectrophotometrically by assaying reduced nicotinamide adenine dinucleotide (NAD) oxidation at 510 nm during LDH-catalyzed reduction of pyruvate to lactate. Briefly, cells were cultured at a density of 5×10^4 cells/well into flat-bottomed 96-well microplates (Techno Plastic Products, Trasadingem, Switzerland) and incubated for 24 h with Cisplatin and ruthenium(III) complexes at IC$_{50}$ concentrations. After 24 h of incubation, the cell culture supernatant was removed and centrifuged to eliminate nonadherent particles and cell debris. Samples of supernatant for each drug tested were then analyzed for DHL activity using a DHL colorimetric kit (Doles LTDA, Goiânia, GO, Brazil). All experiments were made in triplicate.

Reverse transcription and Real time quantitative PCR

The human lung carcinoma cells A549 were treated with different concentrations of Ru(III) complexes and Carboplatin for different periods. Total RNA was extracted with Tri Reagent® (Sigma-Aldrich Co., St. Louis, MO, USA) following the manufacturer's protocol. Total RNA (2.0 µg) was used to produce cDNA with ABgene one-step Verso™ RT-PCR Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) in a total of 25 µL reaction mixture according to the manufacturer's protocol. Real-time PCR reactions were then carried out by a LineGene K fluorescence quantitative PCR detection system (Hangzhou BIOER Tech Co., Tokyo, Japan). *Homo sapiens* apoptosis-related cysteine peptidases (Caspases) 3, 8 and 9 mRNAs expression were quantified for apoptosis cell profiling using specific primers (Table 1).

Futhermore, MDR-1 mRNA [*H. sapiens* ATP-binding cassette, sub-family B (MDR/TAP), member 1 (ABCB1)]; CYP3A4 mRNA (*H. sapiens* cytochrome P450, family 3, subfamily A, polypeptide 4); CYP2C9 mRNA (*Homo sapiens* cytochrome P450, family 2, subfamily C, polypeptide 9) and CYP2C19 mRNA (*H. sapiens* cytochrome P450, family 2, subfamily C, polypeptide 19) were detected in order

Table 1 Primers sequences used in reverse transcription real time quantitative polymerase chain reactions

Primer	Sequence	Amplicon length (pb)
Homo sapiens Caspase 3	Forward 5′-CCTCTTCCCCCATTCTCATT-3′	122
	Reverse 5′-CCAGAGTCCATTGATTCGCT3′	
Homo sapiens Caspase 8	Forward 5′-GGAGTTAGGCAGGTTAGGGG-3′	188
	Reverse 5′-GCTTCCTTTGCGGAATGTAG-3′	
Homo sapiens Caspase 9	Forward 5′-AACAGGCAAGCAGCAAAGTT-3′	198
	Reverse 5′-CATGCTCAGGATGTAAGCCA-3′	
Homo sapiens MDR-1	Forward 5′-CTATGCTGGATGTTTCCGGT-3′	147
	Reverse 5′-GCTTTGGCATAGTCAGGAGC-3′	
Homo sapiens CYP3A4	Forward 5′-GAAACACAGATCCCCCTGAA-3′	250
	Reverse 5′-CATGTACAGAATCCCCGGTT-3′	
Homo sapiens CYP2C9	Forward 5′-TCTGGTTGACCCAAAGAACC-3′	110
	Reverse 5′-AGGCCATCTGCTCTTCTTCA-3′	
Homo sapiens CYP2C19	Forward 5′-GCTGCATGGATATGAAGTGG-3′	219
	Reverse 5′-GCTTCCTCTTGAACACGGTC-3′	
Homo sapiens GAPDH	Forward 5′-ACAGTCAGCCGCATCTTCTT-3′	127
	Reverse 5′-GTTAAAAGCAGCCCTGGTGA-3′	
Homo sapiens B2M	Forward 5′-GTGCTCGCGCTACTCTCTCT-3′	143
	Reverse 5′-TCAATGTCGGATGGATGAAA-3′	

to evaluate the expression profile of these genes in cells exposed to the cisplatin and ruthenium(III) complexes (Table 1).

A total of 25 μL reaction mixture: 2 μL of cDNA, 10 μL of SYBR Green PCR Master Mix (LGC Biotecnology, Middlesex, UK), 2.5 μL of each forward and reverse primer (100 nM μL^{-1}). The PCR program was initiated at 15 min at 95 °C before 40 thermal cycles, each of 30 s at 95 °C and 30 s at 55 °C and 30 s at 72 °C. Data were analyzed according to relative quantification method. Each sample was normalized by the expression of following endogenous genes: GAPDH (*H, sapiens* glyceraldehyde-3-phosphate dehydrogenase) and B2 M (*H, sapiens* beta-2-microglobulin) (Table 1). Checking of the products was performed by melting curve analysis and agarose gel eletroforesis of qPCR products.

Statistical analysis

Statistical analysis of the results was performed using one-way ANOVA and Tukey's post test for multiple comparisons with controls. All statistical analyses were performed using the statistical software GraphPad Prism 4 (GraphPad Software Inc., La Jolla, CA, USA). A probability of 0.05 or less was deemed statistically significant. The following notation was used throughout: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, relative to control.

Results

The *cis*CarboPt, *cis*CRu(III) and *cis*DRu(III) cytotoxicity on A549 tumor cells

The anticancer drug *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II) [*cis*CarboPt] and the two ruthenium(III) complexes, *cis*-(dichloro)tetramminerutheni um(III) chloride [*cis*CRu(III)] and *cis*-tetraammine (oxalato)ruthenium(III) dithionate [*cis*DRu(III)] (Fig. 1), were evaluated in a comparative in vitro MTT cell viability assay with human lung carcinoma cells A549.

Cell viability was detected after the cells were treated with *cis*CarboPt, *cis*CRu(III) and *cis*DRu(III) at concentrations from 0.2 to 1,000 μM for 24 h.

Table 2 lists the IC$_{50}$ values obtained with the tested compounds *cis*CarboPt, *cis*CRu(III) and *cis*-CRu(III). The inhibitory effect of the test drugs on cell viability was measured by the MTT colorimetric method as described previously.
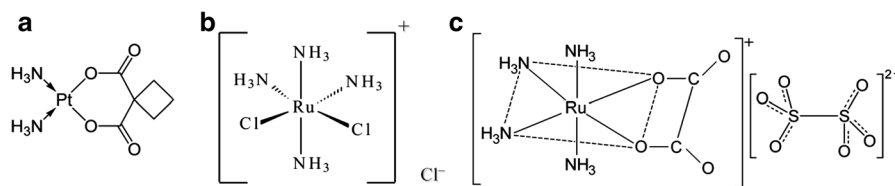
Fig. 1 Chemical structure of *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II) (**a**), *cis*-(dichloro)tetramineruthenium(III) chloride (**b**) and *cis*-tetraammine(oxalato)ruthenium(III) dithionate (**c**)

Table 2 IC$_{50}$ values of compounds *cis*CarboPt, *cis*CRu(III) and *cis*DRu(III) on A549 lug carcinoma cell line after 48 h of incubation, determined using the MTT assay

Compound	IC$_{50}$ A549 (µM)
*cis*CarboPt	196 (±15.49)
*cis*CRu(III)	472 (±20.29)
*cis*DRu(III)	175 (±1.41)

Results are expressed as means (±standard deviation) of at least three independent experiments
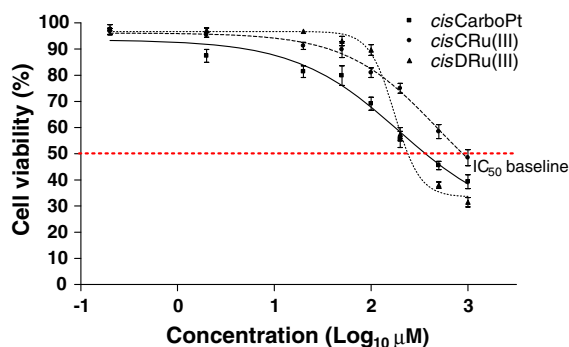
Fig. 2 Effect of *cisCarboPt*, *cis*CRu(III) and *cis*DRu(III) (0.2–1,000 µM) upon cell viability, determined by MTT assay in human A549 tumor cells. Data are mean ± SD ($n = 3$)

Fig. 3 Effect of *cis*Carbo*Pt*, *cis*CRu(III) and *cis*DRu(III) (IC$_{50}$ concentration) upon A549 cellular membrane integrity determined by LDH activity (U.I./L), in human A549 tumor cells. Data are mean ± SD (n = 3). Significant differences from the untreated control are indicated by *$p < 0.05$. Means among *bars* without a common *letter* are statistically different (Tukey's test, $p < 0.05$)

The data indicate that *cis*CRu(III) proved be the most cytotoxic compound of three on tested cell line, presenting the value of IC$_{50}$ 472 (±20.29), followed by *cis*CarboPt and *cis*CRu(III) (Fig. 2).

Although the cytotoxicity of *cis*CRu(III) on A549 presented be lower than others two metallocomplexes, the results show that A549 tumor cells treated with different concentration of *cis*Carbo*Pt* and ruthenium complexes *cis*CRu(III) and *cis*DRu(III), ruthenium-based coordinated complexes, presented low cytotoxic and antiproliferative activities.
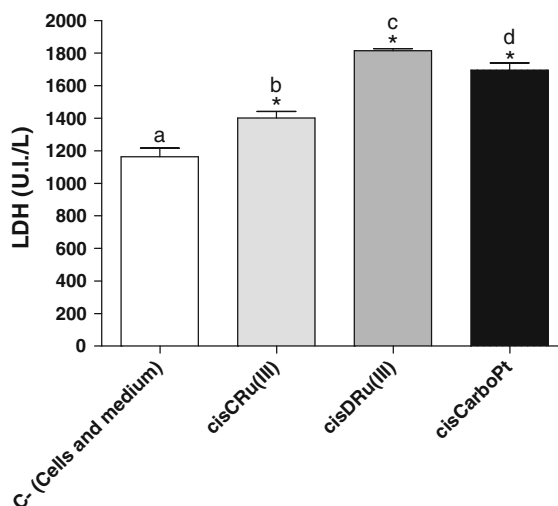
The three metallocomplexes showed low cytotoxic effects, where *cis*DRu(III) (IC$_{50}$ 175 µM) and *cis*CarboPt (IC$_{50}$ 196 µM) were more active than *cis*CRu(III) (IC$_{50}$ 472 µM) against the A549 cancer cells (Fig. 2).

Increased cytomembrane damage of A549 cells induced by cisCarboPt and ruthenium(III) complexes

The platinum and ruthenium compounds were also evaluated for cytotoxic potential via plasma membrane damage of A549 tumour cells assessed by the colorimetric quantification of LDH based on LDH release experiments (Fig. 3).

Using A549 IC$_{50}$ concentrations of *cis*Carbo*Pt*, *cis*CRu(III) and *cis*DRu(III) showed loss of cell vitality which corroborates results observed on MTT assays. The compounds *cis*DRu(III) and *cis*Carbo*Pt* caused A549 cytomembrane damage on a scale higher than *cis*CRu(III). The ruthenium complex *cis*DRu(III) caused the release of 650 U.I./L of DHL, followed by platinum complex *cis*Carbo*Pt* (530 U.I./L), whilst *cis*CRu(III) caused and increase of 235 U.I./L of DHL leaking, when compared to negative control.

Induction of caspase activity and apoptotic cell death on A549 tumor cell line by *cis*CarboPt, *cis*CRu(III) and *cis*DRu(III)

In one of our recent studies, we have shown that the expression of caspase 3 increases in the A549 cells treated with *cis*CRu(III) on a ratio of 3.8 fold, which is associated to apoptosis cell death (Lima et al. 2012). In present study we tested *cis*Carbo*Pt*, *cis*DRu(III), and *cis*CRu(III) as well, against A549 lung cancer cells for mRNA expression of caspase 3, caspase 8 and caspase 9, using Real-Time Quantitative PCR after 12 h of treatment (Fig. 3).

In our in vitro model system, we observed that A549 tumor cells overexpressed caspase 3, 8 and 9 in different levels when exposed to metallocomplexes
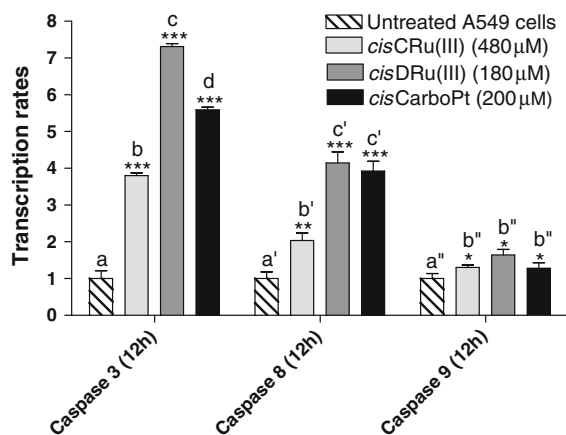
We found that *cis*DRu(III) induced overexpression of caspase 3, 8 and 9 by 7.3, 4.1 and 1.6 fold, respectively. The anticancer drug *cis*Carbo*Pt* induced overexpression of 5.5, 3.9 and 1.2; while *cis*CRu(III) was more modest showing 3.8, 2.1 and 1.3 on expression gain levels of caspase 3, 8 and 9, respectively (Fig. 4).

The *cis*CarboPt, *cis*CRu(III) and *cis*DRu(III) induced different profiles of MDR and CYPs expression in A549 tumor cell line

The expression levels P-gp production on A549 tumor cell were measured by real time reverse transcription PCR using specific primers for MDR-1, CYP2C9, CYP2C19 and CYP3A4 after 24 and 48 h of treatment with near IC$_{50}$ doses of *cis*Carbo*Pt*, *cis*CRu(III) and *cis*DRu(III). As shown in Fig. 5, *cis*Carbo*Pt* and *cis*CRu(III) increased MDR-1 expression by approximately 8 fold and ninefold, respectively, relative to the control group; while *cis*DRu(III) induced lower MDR-1 expression levels, nearly 2.5 fold that of the untreated cells group (Fig. 6).

The metallocomplexes *cis*Carbo*Pt*, *cis*CRu(III) and *cis*DRu(III) exert a significant effect on CYP3A4
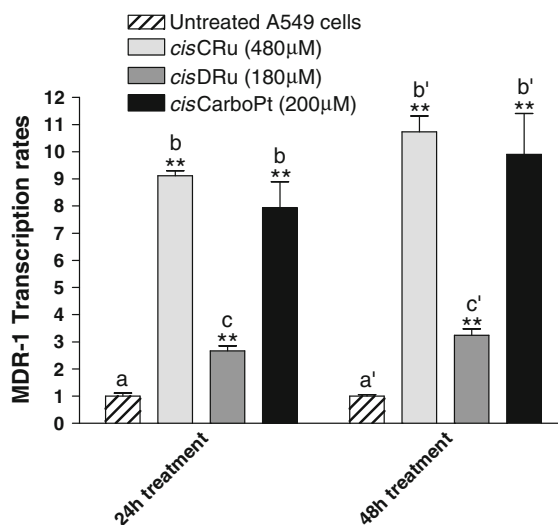

Fig. 4 Alterations of Caspases expression after treatment with *cis*Carbo*Pt*, *cis*CRu(III) and *cis*DRu(III), for 6 h. Samples are in relation to the untreated control cell line with a transcription rate set up to a value of one. The data are expressed as mean ± SEM of $n = 3$. Significant differences from the untreated control are indicated by *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Means among *bars* without a common *letter* are statistically different (Tukey's test, $p < 0.05$)

Fig. 5 Alterations of MDR1-mRNA expression using RT-qPCR after treatment with *cis*Carbo*Pt*, *cis*CRu(III) and *cis*DRu(III), respectively, for 24 and 48 h. Samples are in relation to the untreated control cell line with a transcription rate set up to a value of one. The data are expressed as mean ± SD of $n = 3$. Significant differences from the untreated control are indicated by **$p < 0.01$. Means among *bars* without a common letter are statistically different (Tukey's test, $p < 0.05$)
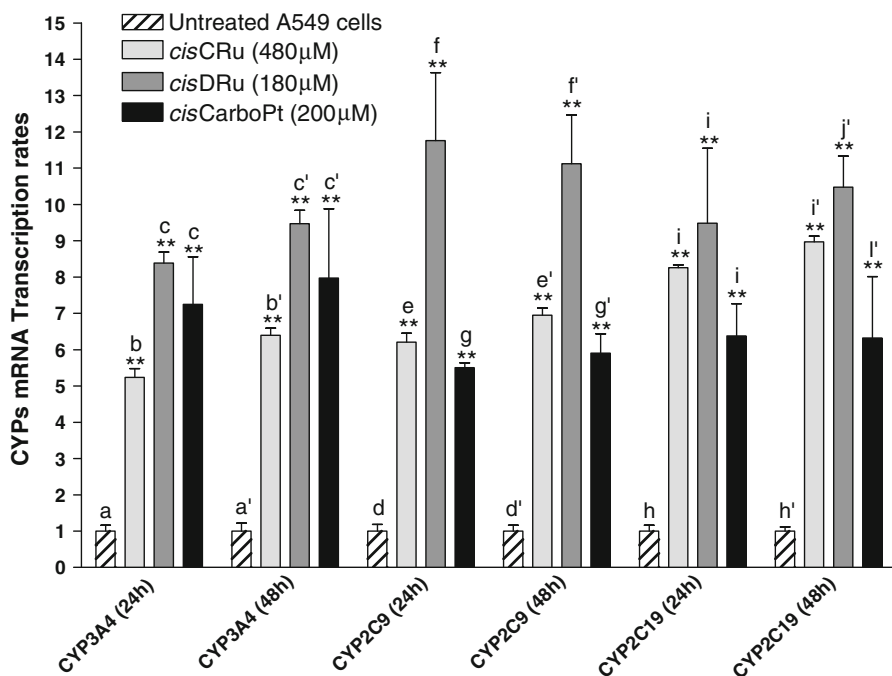
Fig. 6 Alterations of CYPs mRNAs expression using RT-qPCR after treatment with *cisCarboPt*, *cis*CRu(III) and *cis*DRu(III), respectively, for 24 and 48 h. Samples are in relation to the untreated control cell line with a transcription rate set up to a value of one. The data are expressed as mean ± SD of $n = 3$. Significant differences from the untreated control are indicated by **$p < 0.01$. Means among bars without a common letter are statistically different (Tukey's test, $p < 0.05$)

expression profile, inducing a overpression of this gene by 7.2–7.9, 5.2–6.3 and 8.8–9.5 for 24 and 48 h exposition time, respectively on A549 tumor cells. The compounds *cisCarboPt*, *cis*CRu(III) and *cis*DRu(III) also enhanced CYP2C9 and CYP2C19 mRNAs expression levels; *cis*DRu(III) caused a 11-fold CYP2C9 increase, while *cisCarboPt* and *cis*CRu(III) were shown to increase the expression of CYP2C9 by five and sixfold, and six and sevenfold on CYP2C19 by *cisCarboPt* and *cis*CRu(III) respectively, after 48 h or treatment.

Discussion

Chemotherapy is one of the primary treatments for lung cancer; however, primary or secondary drug-resistance is a significant reason why chemotherapy is often ineffective against NSCLC. This presents a major obstacle to successfully treatment of this disease, since NSCLC is extremely difficult to treat because of its low therapeutic and long-term survival rates. Thus, it is crucial to develop better therapeutic strategies for the management of lung carcinomas (Kim et al. 2002; Wu et al. 2010; Lima et al. 2012).

The chemotherapeutic drug Carboplatin is one of the most frequently used agents in the treatment of lung cancer. Still, the therapeutic effects of platinum-based drugs are weakened by its severe toxicity and the chemoresistance of most NSCLC (Rosell et al. 2002; Socinski 2004; Kelland 2007). It is thought that clinical multidrug resistance to chemotherapeutic agents is a key obstacle to curative treatment for advanced NSCLC (Clarke 2003).

Studies have shown ruthenium complexes to be active against certain tumors both in vivo and in vitro, with fewer side effects than cisplatin (Bergamo et al. 1999; Scolaro et al. 2005b). In addition, two ruthenium-based drugs, ImH[*trans*-RuCl₄(DMSO)Im] (NAMI-A) and indazolium *trans*-[tetrachlorobis (1*H*-indazole)ruthenate(III)] (KP1019), were the first ruthenium-based anticancer drugs transferred into to enter clinical trials (Rademaker-Lakhai et al. 2004; Hartinger et al. 2006). Many other compounds that include ruthenium centers are being developed and tested (Lima et al. 2012). Among these, the ruthenium(III)

complex *cis*-(dichloro)tetramineruthenium(III) chloride (*cis*-[RuCl$_2$(NH$_3$)$_4$]Cl) is particularly promising among non-resistant cells. It is being characterized by very low toxicity and has potential as an antitumor drug. Recently, the antitumor activity of *cis*CRu(III) was evaluated in vivo using Sarcoma 180 (S180) murine ascitic tumor cells and a number of in vitro murine (S180, A-20) and human (Jurkat, SKBR-3) tumor cell lines. In all cases *cis*CRu(III) demonstrated a very encouraging activity profile (Silveira-Lacerda et al. 2010b).

In the present study, we examined the citotoxic activities and multidrug resistance induction of two ruthenium(III) complexes, *cis*-(dichloro)tetramineruthenium(III) chloride and *cis*-tetraammine(oxalato)ruthenium(III) dithionate; and a common clinic used metallodrug, the *cis*-diamine(1,1-cyclobutanedicarboxylato)platinum(II), on NSCLC human lung carcinoma cell line A549.

Results derived from cell viability MTT assay revealed that the ruthenium(III) compounds *cis*CRu(III) and *cis*DRu(III) and platinum compound *cis*CarboPt showed dissimilar concentration dependent reduction of cell viability in A549 tumor cell line, where *cis*DRu(III) and *cis*CarboPt presented an IC$_{50}$ concentration of 175 and 196 μM respectively, that are over a half of *cis*CRu(III) IC$_{50}$ = 472 μM. The LDH assay also showed that *cis*DRu(III) and *cis*CarboPt also induced more cell membrane injury and enzyme leakage on A549 tumor cells than *cis*CRu(III). Is important to note that *cis*CarboPt and *cis*DRu(III) are structurally similar, being broader and more complex molecules containing carbon and amines groups in their structures, differing from *cis*CRu(III), a structuraly cloride rich complex.

Sadler and Dyson studies, established that the mechanism of action of ruthenium chlorinated compounds, like *cis*CRu(III), has many of analogies to that of cisplatin. It first involves hydrolysis of the Ru–Cl bond of the prodrug to generate an active aquaruthenium species (Allardyce et al. 2003; Scolaro et al. 2005a). Detailed kinetic studies showed that the Ru-Cl bond hydrolysis can be strongly influenced by the nature of the coligands as well as the nature of the metal ion (Malina et al. 2001, Bacac et al. 2004). Importantly, this step could be suppressed in the blood because of the high chloride concentrations enabling to chloride rich ruthenium complexes cross the cell and nuclear membranes (Scolaro et al. 2005a). Inside the cell, the

hydrolysis of the chloro anion takes place because of the much lower chloride concentration (circa 25 times lower). It is then assumed that the aqua-ruthenium complex binds to nuclear DNA with a high affinity for the N7 position of guanine bases, damaging the cell DNA and leading to apoptosis (Nováková et al. 1995; Novakova et al. 2003; Gallori et al. 2000; Brabec and Nováková 2006; Silveira-Lacerda et al. 2010b).

Apoptosis is an important biological process in many systems and can be triggered by a variety of stimuli received by the cells. Caspase-family represents the key components of the apoptotic machinery within the cells and consists of at least 14 different cysteine proteases that act in concert in a cascade triggered by apoptosis signaling. The culmination of this cascade is the cleavage of a number of proteins in the cell, followed by cell disassembly, cell death, and, ultimately, the phagocytosis and removal of the cell debris (Krajewska et al. 2005; Galluzzi et al. 2012).

In the present study, exposure of A549 tumor cells to Platinum and Ruthenium compounds induced increased significant changes in the of RNAm expression of caspase 3 and caspase 8, although caspase 9 expression remains relatively unchanged. The *cis*DRu(III) complex induced overexpression of caspase 3 and 8 by 7.3 and 4.1 fold, respectively, *cis*CarboPt drug induced overexpression of 5.5 and 3.9 fold; whilst *cis*CRu(III) complex was more modest, showing 3.8 and 2.1 on expression gain levels of caspase 3 and 8, respectively. Those results corroborate recently studies reporting that ruthenium compound induced apoptosis in A549 cells as evidenced by sub-G1 peak observed in Flow cytometry assays and caspase-3 activation found by Lima et al. (2012).

Caspase-3 is the most prevalent caspase within cells, responsible for the majority of apoptotic effects. The activation of caspase-3 induces poly (ADPribose) polymerase (PARP) cleavage, chromosomal DNA breaks and finally lead to apoptosis (Cohen 1997). In some cell types, active caspase-8 directly catalyzes the proteolytic maturation of caspase-3, thereby triggering the executioner phase of caspase-dependent apoptosis in a mitochondrion-independent manner (Galluzzi et al. 2012). Since in present work, caspase-3 and caspase-8 showed be overexpressed by Ruthenium (III) complexes *cis*CRu(III) and *cis*DRu(III), we can infer that ruthenium(III) compounds-induced apoptosis by extrinsic pathway.

Although several studies have been performed regarding cytotoxic potential of ruthenium(III)

chloride and ditionate (Menezes et al. 2007, Pereira et al. 2009; Ribeiro et al. 2009; Silveira-Lacerda et al. 2010b; Lima et al. 2010a, b, Lima et al. 2012) no study has been performed regarding the potential multidrug resistance induction of these compounds on tumor cells.

A common mechanism of lung cancer chemoresistance is the overexpression of plasma membrane drug efflux proteins, such as P-gp. Herein we investigated if MRD1 expression profile on A549 lung cells is changed when tumor cells are treated with complexes containing mettalic ions such platinum and ruthenium. Results revealed that both Pt and Ru complexes upregulated MDR-1 expression, leading to tenfold overexpression pikes.

Importantly to point out that ruthenium complexes containing carbonic structures, like carboplatin and oxaliplatin, were found to be active against cisplatin-resistant cell lines, indicating that the detoxification mechanism is different from the one of cisplatin (Schluga et al. 2006). It was assumed that the carboxylato ligands would hydrolyze more slowly and in a more controllable way than the chloride ligands rich molecules, like cisplatin and *cisC*Ru(III) (Wang et al. 2003; Yan et al. 2005). All evidence taken together indicate that carboxylato rich compounds, like *cis*CarboPt and *cis*DRu(III), seem to operate by a different mode of action compared to cisplatin, Ru(II) arene ethylenediamine compounds, and most of the known anticancer compounds in general.

It was shown by mass spectrometry that carboxylato rich compounds form adducts with proteins and that their reactivity and cisplatin in the presence of proteins are much different. Other proteins have been proposed as the target for Ru organometallics (Scolaro et al. 2005a; Casini et al. 2009). P-Glycoprotein (Pgp) is a plasma membrane protein that is responsible for drug efflux from cells and is involved in MDR. Interestingly, for one of these ruthenium carboxylato derivatives, it was shown that the ruthenium coordination to the Pgp inhibitor derivative induced an even stronger protein inhibition (Vock et al. 2007). This can probably explain why MDR-1 was so overexpressed on *cisC*Ru(III) treated A549 cells when compared to *cis*DRu(III) treated A549 cells.

In a similar line of thought, other proteins can also be the target of cytotoxic ruthenium metallocomplexes, indicating that P-gp is not the major or only mechanism of chemoresistance (Bugarcic et al. 2009).

Cytochrome P450 superfamily represent key enzymes in drug metabolism, especially the CYP3A4. CYPs enzymes catalyze important bioactivations acting directly on oxidative stress and xenobiotics metabolism (Masubuchi and Horie 2007). Since CYPs are known to metabolize a number of drugs, and since their expression is modifiable by a number of drugs and agents, we investigated the expression of CYP3A4 expression in A549 tumor cells.

Results revealed that metallocomplexes cisCarboPt, cisCRu(III) and cisDRu(III) exert a significant effect on CYP3A4 expression profile, inducing a overpression of this gene by 7.9, 6.3 and 9.5, respectively, after 48 h or treatment. Our results suggest that oxidative stress may be affecting CYP expression in A549 tumor cells, which may alter the metabolism of drug cisCarboPt and metallocomplexes cisCRu(III) and cisDRu(III) thus, the toxicity of these compounds and that could be a promising approach to enhance the efficacy of chemotherapy treatment of multidrug-resistant cancer to cancer lung A549 cell.

In summary, the mechanisms by which ruthenium complexes induce cytotoxicity and apoptosis on A549 tumor cell line than *cis*CRu(III) may not only be by the consequence of direct DNA binding and damage, but also by the interactions with a myriad of proteins like Cytochrome P450, P-gp and caspases; via oxidative stress promotion, reduction of MDR-1 phenotype and apoptosis induction via extrinsic activation pathway.

Ethical Approval No studies involving humans or experimental animals were conducted in this work. The human lung carcinoma A549 cells were purchased from the American Type

Culture Collection (ATCC, Rockville, MD, USA) and cultured in vitro.

References

Allardyce CS, Dyson PJ, Ellis DJ, Salter PA, Scopelliti R (2003) Synthesis and characterisation of some water soluble Ruthenium(II)–arene complexes and an investigation of their antibiotic and antiviral properties. J Organomet Chem 668:35–42

American Cancer Society (2013) Cancer Facts & Figures. American Cancer Society, Atlanta

Bacac M, Hotze ACG, van der Schilden K, Haasnoot JG, Pacor S, Alessio E, Sava G, Reedijk J (2004) The hydrolysis of the anti-cancer ruthenium complexNAMI-Aaffects its-DNAbinding and antimetastatic activity: an NMR evaluation. J Inorg Biochem 98:402–412

Bergamo A, Gagliardi R, Scarcia V, Furlani A, Alessio E, Mestroni G, Sava G (1999) In vitro cell cycle arrest, in vivo action on solid metastasizing tumors and host toxicity of the antimetastatic drug NAMI-A and cisplatin. J Pharmacol Exp Ther 289:559–564

Brabec V, Nováková O (2006) DNA binding mode of ruthenium complexes and relationship to tumor cell toxicity. Drug Resist Updates 9:111–122

Bugarcic T, Habtemariam A, Deeth RJ, Fabbiani FPA, Parsons S, Sadler PJ (2009) Ruthenium(II) arene anticancer complexes with redox-active diamine ligands. Inorg Chem 48:9444–9453

Casini A, Gabbiani C, Michelucci E, Pieraccini G, Moneti G, Dyson PJ, Messori L (2009) Exploring metallodrug-protein interactions by mass spectrometry: comparisons between platinum coordination complexes and an organometallic ruthenium compound. J Biol Inorg Chem 14:761–770

Clarke MJ (2003) Ruthenium metallopharmaceuticals. Coord Chem Rev 236:209–233

Cohen GM (1997) Caspases: the executioners of apoptosis. Biochem J 326:1–16

Gallori E, Vettori C, Alessio E, Vilchez FG, Vilaplana R, Orioli P, Casini A, Messori L (2000) DNA as a possible target for antitumor ruthenium(III) complexes. A spectroscopic and molecular biology study of the interactions of two representative antineoplastic ruthenium(III) complexes with DNA. Arch Biochem Biophys 376:156–162

Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, Dawson TM, Dawson VL, El-Deiry WS, Fulda S, Gottlieb E, Green DR, Hengartner MO, Kepp O, Knight RA, Kumar S, Lipton SA, Lu X, Madeo F, Malorni W, Mehlen P, Nuñez G, Peter ME, Piacentini M, Rubinsztein DC, Shi Y, Simon H-U, Vandenabeele P, White E, Yuan J, Zhivotovsky B, Melino G, Kroemer G (2012) Molecular definitions of cell death subroutines: recommendations of lhe Nomenclature Commitee on Cell Death. Cell Differ 10:107–120

Hartinger CG, Zorbas-Seifried S, Jakupec MA, Kynast B, Zorbas H, Keppler BK (2006) From bench to beside- pre-clinical and early clinical development of the anticancer agent indazolium trans- [tetrachlorobis (1 H-indazazole)ruthenate (III)] KP1019 or FFC14A. J Inorg Biochem 100:891–904

Karki SS, Thota S, Darj SY, Balzarini J, Clercq E (2007) Synthesis, anticancer, and cytotoxic activities of some mononuclear Ru(II) compounds. Bioorg Med Chem 15:6632–6641

Kelland L (2007) The resurgence of platinum-based cancer chemotherapy. Nat Rev 7:573–584

Kim R, Tanabe K, Uchida Y, Emi M, Inoue H, Toge T (2002) Current status of the molecular mechanisms of anticancer drug-induced apoptosis e the contribution of molecular-level analysis to cancer chemotherapy. Cancer Chemother Pharmacol 50:343–352

Krajewska M, Kim H, Shin E et al (2005) Tumor-associated alterations in caspase-14 expression in epithelial malignancies. Clin Cancer Res 11:5462–5471

Lima AP, Pereira FC, Vilanova-Costa CAST, Mello FMS, Ribeiro ASBB, Benfica PL, Valadares MC, Pavanin LA, Santos WB, Silveira-Lacerda EP (2010a) The compound cis-(dichloro)tetramineruthenium(III) chloride induces caspase-mediated apoptosis in K562 cells. Toxicol In Vitro 24:1562–1568

Lima AP, Pereira FC, Vilanova-Costa CAST, Ribeiro ASBB, Pavanin LA, Santos WB, Silveira-Lacerda EP (2010b) The ruthenium complex cis-(dichloro)tetramineruthenium(III) chloride induces apoptosis and damages DNA in murine sarcoma 180 (S-180) cells. J Biosci 35:371–378

Lima AP, Pereira FC, Vilanova-Costa CAST, Soares JR, Pereira LCG, Porto HKP, Pavanin LA, Santos WB, Silveira-Lacerda EP (2012) Induction of cell cycle arrest and apoptosis by ruthenium complex cis-(dichloro)tetramineruthenium(III) Chloride in human lung carcinoma cells A549. Biol Trace Elem Res 147:8–15

Liu H-K, Wang F, Parkinson JA, Bella J, Sadler PJ (2006) Ruthenation of duplex and single-stranded d(CGGCCG) by organometallic anticancer complexes. Chem Eur J 12:6151–6165

Lobner D (2000) Comparison of the LDH and MTT assays for quantifying cell death: validity for neuronal apopotosis. J Neurosci Meth 96:147–152

Malina J, Novakova O, Keppler BK, Alessio E, Brabec V (2001) Biophysical analysis of natural, double-helical DNA modified by anticancer heterocyclic complexes of ruthenium(III) in cell-free media. J Biol Inorg Chem 6:435–445

Masubuchi Y, Horie T (2007) Toxicological significance of mechanism-based inactivation of cytochrome p450 enzymes by drugs. Crit Rev Toxicol 37:389–412

Menezes C, Depaulacosta L, Ávila VMR, Ferreira MVC, Pavanin L, Homsi-Brandeburgo MI, Hamaguchi A, Silveira-Lacerda EP (2007) Analysis in vivo of antitumor activity, Cytotoxicity and Interaction between plasmid DNA and the cis-dichlorotetraammineruthenium(III) chloride. Chem Biol Interact 167:116–124

Mosman T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation an cytotoxicity assays. J Immunol Meth 16:55–63

Novakova O, Chen H, Vrana O, Rodger A, Sadler PJ, Brabec V (2003) DNA interactions of monofunctional organometallic ruthenium(II) antitumor complexes in cell-free media. Biochemistry 42:11544–11554

Nováková O, Vrana O, Kiseleva VI, Brabec V (1995) DNA interactions of antitumor platinum(IV) complexes. Eur J Biochem 228:616–624

Pavanin LA, Giesbrecht E, Tfouni E (1985) Synthesis and properties of the ruthenium(II) complexes *cis*-Ru(NH$_3$)$_4$(isn)L^{2+} spectra and reduction potentials. Inorg Chem 24:4444–4446

Pereira FC, Vilanova-Costa CAST, Lima AP, Ribeiro ASBB, Silva HD, Pavanin LA, Silveira-Lacerda EP (2009) Cytotoxic and genotoxic effects of *cis*-tetraammine(oxalato)ruthenium(III) dithionate on the root meristem cells of *Allium cepa*. Biol Trace Elem Res 128:258–268

Rademaker-Lakhai JM, van den Bongard D, Pluim D, Beijnen JH, Schellens JH (2004) A Phase I and pharmacological study with imidazolium-trans-DMSO-imidazole-tetrachlororuthenate, a novel ruthenium anticancer agent. Clin Cancer Res 10:3717–3727

Ribeiro ASBB, da Silva CC, Pereira FC, Lima AP, Vilanova-Costa CAST, Aguiar SS, Pavanin LA, da Cruz AD, Silveira-Lacerda EP (2009) Mutagenic and genotoxic effects of *cis*-(dichloro)tetraammineruthenium(III) chloride on human peripheral blood lymphocytes. Biol Trace Elem Res 130:249–261

Rosell R, Reginald VNL, Taron M, Reguart N (2002) DNA repair and cisplatin resistance in non-small-cell lung cancer. Lung Cancer 38:217–227

Schluga P, Hartinger CG, Egger A, Reisner E, Galanski M, Jakupec MA, Keppler BK (2006) Redox behavior of tumor inhibiting ruthenium(III) complexes and effects of physiological reductants on their binding to GMP. Dalton Trans. 14:1796–1802

Scolaro C, Bergamo A, Brescacin L, Delfino R, Cocchietto M, Laurenczy G, Geldbach TJ, Sava G, Dyson PJ (2005) In vitro e in vivo evaluation of ruthenium (II)–arene PTA complexes. J Med Chem 48:4161–4171

Silveira-Lacerda EP, Vilanova-Costa CAST, Pereira FC, Hamaguchi A, Pavanin LA, Goulart LR, Homsi-Brandeburgo MI, Soares AM, Santos WB, Nomizo A (2010a) The ruthenium complex *cis*-(dichloro)tetraammineruthenium(III) chloride presents immune stimulatory activity on human peripheral blood mononuclear cells. Biol Trace Elem Res 133:270–283

Silveira-Lacerda EP, Vilanova-Costa CAST, Pereira FC, Hamaguchi A, Pavanin LA, Goulart LR, Homsi-Brandeburgo MI, Soares AM, Santos WB, Nomizo A (2010b) The Ruthenium Complex *cis*-(dichloro)tetraammineruthenium(III) chloride presents selective cytotoxicity against murine B cell lymphoma (A-20), murine ascitic sarcoma 180 (S-180), human breast adenocarcinoma (SK-BR-3), and human T cell leukemia (Jurkat) tumor cell lines. Biol Trace Elem Res 135:98–111

Socinski MA (2004) Cytotoxic chemotherapy in advanced non-small cell lung cancer: a review of standard treatment paradigms. Clin Cancer Res 10:4210–4214

Tang J, Wang Y, Wang D, Wang Y, Xu Z, Racette K, Liu F (2013) Key structure of Brij for overcoming multidrug resistance in cancer. Biomacromolecules 14:424–430

Varbanov HP, Valiahdi SM, Kowol CR, Jakupec MA, Galanski M, Keppler BK (2012) Novel tetracarboxylatoplatinum(IV) complexes as carboplatin prodrugs. Dalton Trans 41(47):14404–14415

Vock CA, Ang WH, Scolaro C, Phillips AD, Lagopoulos L, Juillerat-Jeanneret L, Sava G, Scopelliti R, Dyson PJ (2007) Development of ruthenium antitumor drugs that overcome multidrug resistance mechanisms. J Med Chem 50:2166–2175

Wagner JM, Karnitz LM (2009) Cisplatin-induced DNA damage activates replication checkpoint signaling components that differentially affect tumor cell survival. Mol Pharmacol 76:208–214

Wang F, Chen H, Parsons S, Oswald IDH, Davidson JE, Sadler PJ (2003) Kinetics of aquation and anation of ruthenium(II) arene anticancer complexes, acidity and X-ray structures of aqua adducts. Chem Eur J 9:5810–5820

Wu J, Hu C, Gu Q, Li Y, Song M (2010) Trichostatin A sensitizes cisplatin-resistant A549 cells to apoptosis by up-regulating death-associated protein kinase. Acta Pharmacol Sin 31:93–101

Yan YK, Melchart M, Habtemariam A, Sadler PJ (2005) Organometallic chemistry, biology and medicine: ruthenium arene anticancer complexes. Chem Commun 38:4764–4776