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ORIGINAL RESEARCH

Analysis of the cytotoxic effects of ruthenium–ketoconazole and ruthenium–clotrimazole complexes on cancer cells

**Elisa Robles-Escajeda · Alberto Martínez · Armando Varela-Ramirez ·
Roberto A. Sánchez-Delgado · Renato J. Aguilera**

Abstract Ruthenium-based compounds have intriguing anti-cancer properties, and some of these novel compounds are currently in clinical trials. To continue the development of new metal-based drug combinations, we coupled ruthenium (Ru) with the azole compounds ketoconazole (KTZ) and clotrimazole (CTZ), which are well-known antifungal agents that also display anticancer properties. We report the activity of a series of 12 Ru–KTZ and Ru–CTZ compounds against three prostate tumor cell lines with different androgen sensitivity, as well as cervical cancer and lymphoblastic lymphoma cell lines. In addition, human cell lines were used to evaluate the toxicity against non-transformed cells and to establish selectivity indexes. Our results indicate that the combination of ruthenium and KTZ/CTZ in a single molecule results in complexes that are more cytotoxic than the individual components alone, displaying in some cases low micromolar CC_{50} values and high selectivity indexes. Additionally, all compounds are more cytotoxic against prostate cell lines with lower cytotoxicity against non-transformed epidermal cell lines. Some of the compounds were found to primarily induce cell death via apoptosis yet weakly interact with DNA. Our studies also demonstrate that the cytotoxicity induced by our Ru-based compounds is not directly related to their ability to interact with DNA.

Keywords Ruthenium · Ketoconazole · Clotrimazole · Apoptosis · Prostate cancer cell lines

Introduction

The increasing understanding of the biochemistry of cancer has resulted in the generation of a wide variety of compounds with potential antitumor properties. Many of those compounds are often used in combination chemotherapy, as each component may elicit a different mechanism of action by reaching diverse biological targets. In line with this, the combination of a number of organic drugs with well-known metal-based antitumor agents, notably cisplatin and carboplatin, has been widely explored (Lippert 2006; Sweetman 2010). Significant examples include combinations of 5-fluorouracil with carboplatin, cisplatin, and oxaliplatin in the treatment of gastrointestinal, head and neck, colorectal, and pancreatic cancer (Lorch et al. 2011; Posner et al. 2007; Van et al. 2006). Combination therapies, however, may present pharmacokinetic issues related to

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the difficulty in reaching multiple targets or achieving peak blood concentrations of each component drug at similar times; in that sense, hybrid compounds containing multiple pharmacophores in a single molecule might present an advantage.

For some time, we have been designing potential chemotherapeutics based on the metal–drug synergism (Martinez et al. 2010, 2012; Rademaker-Lakhai et al. 2004; Rajapakse et al. 2009; Strasberg et al. 2004), which may be achieved by combining a drug of known therapeutic value with a biologically relevant metal in a single molecule (Sanchez-Delgado et al. 2004). Such combinations can lead to new chemical entities with enhanced activity, decreased toxicity, and more controlled pharmacokinetic properties than each separate component, or than the mere physical combination of two drugs. Because of the high toxicity of platinum and the common occurrence of resistance, other metals are receiving increasing attention in cancer research, and ruthenium appears as a particularly interesting element in terms of anticancer activity and low toxicity. The compound NAMI-A, ImH[*trans*-RuCl$_4$(DMSO)Im] (Im=imidazole), has shown very promising properties in preclinical and phase I clinical trials against lung metastases (Bergamo et al. 1999; Morbidelli et al. 2003; Rademaker-Lakhai et al. 2004; Sava et al. 1998, 2002) while KP1019, IndH[*trans*-RuCl$_4$(Ind)$_2$] (Ind= indazole), is effective in ovarian and colon carcinomas (Berger et al. 1989; Bratsos et al. 2007b; Garzon et al. 1987; Hartinger et al. 2008; Jakupec et al. 2008; Kapitza et al. 2005; Seelig et al. 1992). In addition, three octahedral Ru (II) complexes were found to induce apoptosis when tested on human hepatoma (BEL-7402 and HepG-2), ovarian (HeLa), and osteosarcoma (MG-63) cancer cell lines (Huang et al. 2011). Most recently, two symmetric ruthenium(II) complexes were found to exert apoptosis on the BEL-7402, HeLa, MG-63, and SKBR (breast) cancer cell lines (Xie et al. 2013). In addition, novel Ru-letrozole compounds were found to exhibit cytotoxocity against a breast cancer cell line (MCF-7) and glioblastoma (U251N) cells, and one of these compounds was implicated in the induction of autophagy-induced cell death (Castonguay et al. 2012). Ru-quinolone adducts were also found to have anti-cancer potential as monitored on the HeLa cancer cell line (Kljun, et al. 2013). Arene-ruthenium "semi-sandwich" compounds like [Ru(η^6-arene)(X)(Y–Z)] (where Y–Z is a chelating ligand, and X is monoanionic ligand), and related derivatives incorporating the 1,3,5-triaza-7-phosphaadamantane (PTA) ligand (Aird et al. 2002; Dougan et al. 2006; Morbidelli et al. 2003; Morris et al. 2001; Scolaro et al. 2005; Wang et al. 2005) also display very interesting antitumor activities. In our search for metal–drug synergy, we synthesized a series of arene–Ru–chloroquine complexes that proved to be active against Jurkat human T lymphocyte leukemia and SUP-T1 lymphoma cell lines; we also demonstrated that induction of apoptosis is the main path for cell death in these cases (Martinez et al. 2010). The complex [Ru(η^6-arene)Cl$_2$(CQ)], also proved to be active in vitro against dedifferentiated liposarcoma (LS141), a type of tumor for which no chemotherapy is available (Rajapakse et al. 2009).

Continuing our search for new metal-based drug combinations, we have turned our attention to azole compounds like ketoconazole (KTZ) and clotrimazole (CTZ) (Fig. 1), two well-known antifungal agents that also display anticancer properties. KTZ is a cytochrome P-450 inhibitor that blocks both testicular and adrenal androgen biosynthesis (Eichenberger et al. 1989a) and causes apoptosis through p53-dependent and other pathways in specific cancerous cell lines, although the molecular mechanisms are still unclear (Ho et al. 1998). Because of its ability to compete with testosterone and dihydrotestosterone in binding androgen receptors, KTZ is also used as a treatment for hormone-refractory (Van Veldhuizen et al. 2003) and metastatic prostate cancer (Bok and Small 1999). When KTZ is administered in combination with vinblastine or etoposide, synergistic suppression of in vitro growth on human androgen-independent prostate cancer cells was observed (Eichenberger et al. 1989a, b). The effect of KTZ has also been investigated on the glucocorticoid receptor (GR) transcriptional activity on HeLa and HepG2 (hepatic) cancer cells (Duret et al. 2006). In addition to P450 enzymes, KTZ inhibits the GR transcriptional activity and competes with dexamethasone for human GR binding (Duret et al. 2006).

CTZ, also a cytochrome P-450 inhibitor, is in turn able to suppress tumor growth by acting as a calmodulin antagonist (Hegemann et al. 1993; Mac et al. 1993). The increase in mitochondrially bound hexokinase, a process associated to the high rate of glycolysis in cancer cells (Bratsos et al. 2007a), is induced by insulin and Ca^{2+}- and Ca^{2+}-mobilizing hormones, and it could be prevented by calmodulin antagonists (Penso and Beitner 1998). In addition, CTZ induces Ca^{2+} store-mediated inhibition of translation initiation (Cao et al.

Fig. 1 Chemical structures of (**a**) KTZ and (**b**) CTZ
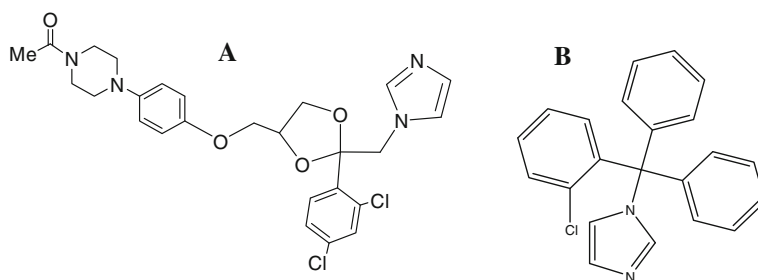
2004) and blocks calcium-activated potassium channel (IKCa1) on human T lymphocytes (Wulff et al. 2000).

Metal complexes of KTZ and CTZ have begun to receive attention as potential drugs. Some of us reported that Ru(KTZ)$_2$Cl$_2$ induces cytotoxicity and apoptosis-associated caspase-3 activation in several cancer cell lines including C8161 melanoma and HT-29 colon carcinoma, irrespective of p53 status, with IC$_{50}$ values around 25 μM; this complex targets the mitochondria and is more effective than KTZ, CTZ, Ru(CTZ)$_2$Cl$_2$, or cisplatin at inducing PARP fragmentation and proapoptotic Bak expression (Strasberg et al. 2004). Pt(CTZ)$_2$Cl$_2$ was found to be active against a panel of human tumor cell lines, including prostate, pancreas, breast, and colon, while the Pd analogue was inactive (Navarro et al. 2006, 2009). We recently reported that the arene-Ru–CTZ and arene-Ru–KTZ compounds 1–12 (Fig. 2) display high activity against *Leishmania major* and *Trypanosoma cruzi* with very low toxicity to normal mammalian cells (Iniguez et al. 2013; Martinez et al. 2012). In the present paper, we discuss the cytotoxicity of the Ru–KTZ and Ru–CTZ complexes 1–12 against a panel of tumor and non-tumor cell lines, the mechanism of cell death induced, and their ability to interact with DNA.

Materials and methods

Reagents

Calf thymus (CT) DNA, buffers, and solvents were purchased from Sigma-Aldrich. Solvents were purified by use of a PureSolv purification unit from Innovative Technology, Inc.; all other chemicals were used as received. Thermal denaturation experiments were performed on an Agilent 8453 diode-array spectrophotometer equipped with a HP 89090 Peltier temperature control accessory. Circular dichroism (CD) spectra were

taken in a Chirascan CD spectrometer also equipped with a thermostated cuvette holder. Complexes 1–12 were synthesized as previously described by us (Iniguez et al. 2013; Martinez et al. 2012).

Cell culture

All cell lines were obtained from American Tissue Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured at 37 °C in a 5 % CO$_2$ humidified atmosphere, by incubating them in a regular water-jacketed incubator or in a temperature controlled chamber within the bioimaging system. Only cell cultures demonstrating cell viabilities of 90 % or higher were used for the cytotoxicity experiments (Lema et al. 2011). Human non-transformed keratinocyte HaCaT cells (Boukamp et al. 1988), mammary epithelial MCF-10A cells (Soule and McGrath 1986), and human cervical HeLa cancer cells were cultured in Dulbecco's modified Eagle medium, supplemented with 10 % heat-inactivated newborn calf serum (Hyclone, Logan, UT, USA) and antibiotics; 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B (Invitrogen, Carlsbad, CA, USA). Wild-type mouse embryonic fibroblasts (MEFs) were grown as above with the addition of 2 mM L-glutamine (Glutamax; Life Technologies) as previously described (Song et al. 2006). DU145 human prostate carcinoma cells (Stone et al. 1978) were grown in the previously described medium in which newborn calf serum was substituted with fetal bovine serum. Human prostate cancer lines 22Rv1 (Sramkoski et al. 1999) and LNCaP (Horoszewicz et al. 1983) and the YT NK-like lymphoblastic lymphoma (Yodoi et al. 1985) cell lines were cultivated in Rockwell Park Memorial Institute 1640 (RPMI-1640) culture medium, supplemented with 10 % heat-inactivated fetal bovine serum (Hyclone) and the same three antibiotics as described above.
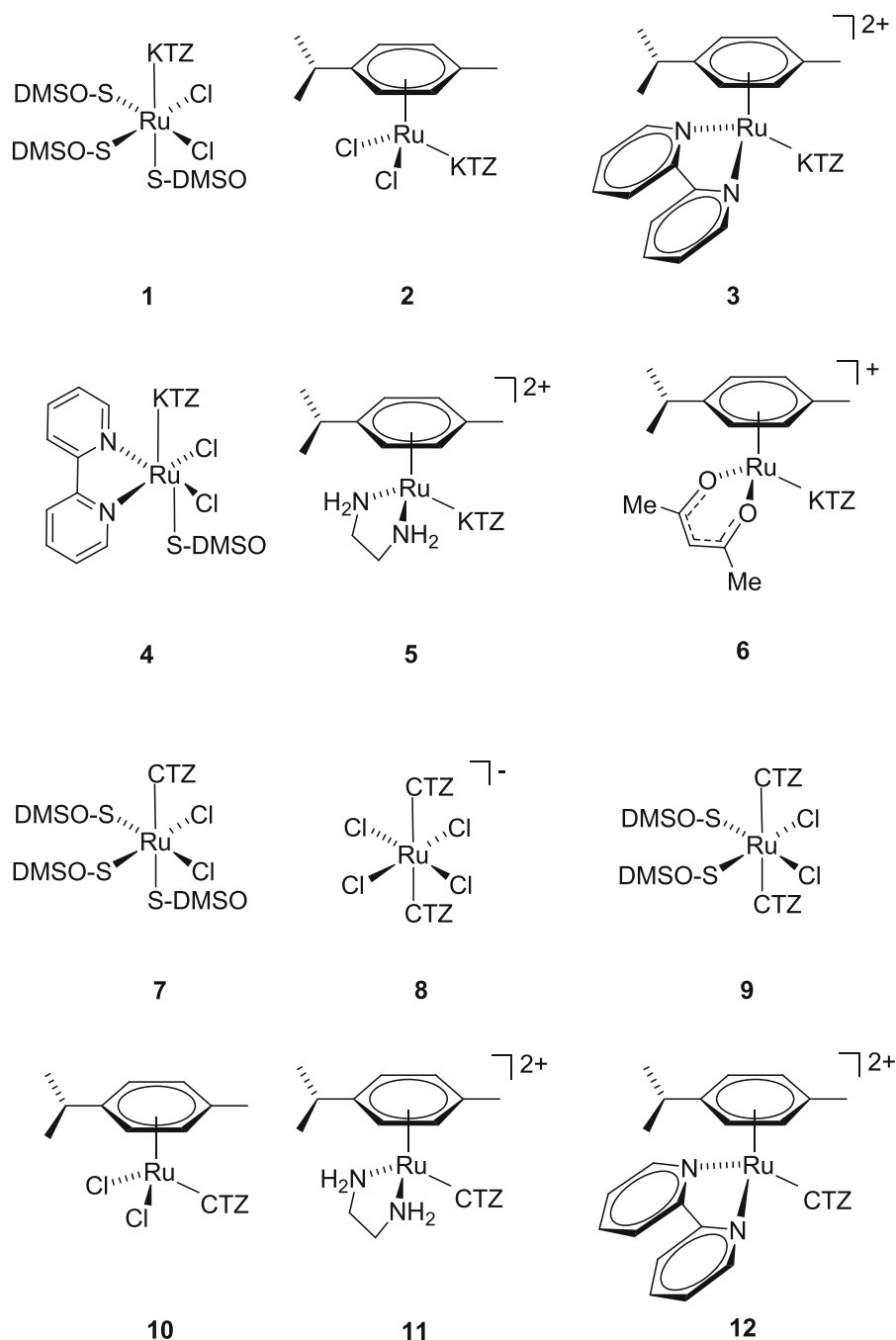
Fig. 2 Chemical structures of the compounds investigated. Synthesized as previously described (Iniguez et al. 2013; Martinez et al. 2012)

Drug screening assays

The cytotoxic effect of KTZ, CTZ, and complexes 1–12 was measured by using the differential nuclear staining (DNS) assay essentially as previously described (Lema et al. 2011). Cell lines plated on 96-well microplates were seeded at a density of 10,000 cells/200 μl media per well and incubated overnight to promote cell attachment. Subsequently, cells were incubated for a total of 20 h in the presence of the compounds. One hour prior to image capture in a live-cell mode, a mixture of Hoechst 33342 (Invitrogen, Eugene, OR) and

propidium iodide (PI; MP Biomedicals, Solon, OH) was added to a final concentration of 1 μg/ml each fluorophore. In this assay, Hoechst was utilized to label the total number of cells, while PI was used to obtain the number of dead cells. Dimethyl sulfoxide (DMSO), which was the compound diluent, was used as the solvent control at a final concentration of 0.5 %v/v. Hydrogen peroxide (H$_2$O$_2$; 300 μM) was used as the positive control for cytotoxicity while untreated cells were included to estimate the background of dead cells generated by the plating process. The fluorescent signal of stained cells was captured in a series of microscopic images utilizing a Pathway 855 Bioimager system (BD Biosciences Rockville, MD), and data analysis was conducted as previously described (Lema et al. 2011). To obtain adequate numbers of regions of interest (cells), montages (3×3) from nine contiguous image fields were captured per well utilizing a 20× objective. Every experimental point, as well as all controls, was evaluated in triplicate. The 50 % cytotoxic concentration (CC$_{50}$) values were determined as previously described (Varela-Ramirez et al. 2011). Briefly, the average percentage from triplicates of the two compound concentrations closest to the 50 % cytotoxicity value was plotted against the compound concentration in a xy (scatter) chart function (Microsoft Excel). The best-fit regression line and its equation was used to calculate the concentration of chemical compound required to damage the plasma membrane by 50 % of the cell population, as compared with solvent-treated cells (DMSO). Standard deviations of the mean are not shown since the CC$_{50}$ values were determined by linear extrapolation.

Apoptosis assays

After treatment of cells with the novel KTZ– and CTZ–ruthenium complexes, all subsequent operations were carried out on ice to slow down or arrest cell deterioration. Cells from each individual well were then collected and processed as previously described (Varela-Ramirez et al. 2011). Briefly, cells were simultaneously stained with annexin V–fluorescein isothiocyanate (FITC) and PI following the manufacture's specification and immediately analyzed via flow cytometry using a Cytomics FC500 flow cytometer (Beckman Coulter, Miami, FL). Approximately 10,000 events were acquired for each sample and the recorded data analyzed using CXP software (Beckman Coulter). In each experiment, the same controls used in the DNS assays were used which

included apoptosis, solvent, and untreated controls. The total percentage of apoptosis was calculated by summing the total annexin V–FITC positive signal that included early and late apoptosis (Shaik et al. 2009).

Circular dichroism (CD) and thermal denaturation (Tm) experiments

For CD experiments, stock solutions (1.5 mM) of each complex were freshly prepared in water prior to use. An appropriate volume of each solution was added to 3 ml samples of a freshly prepared solution of CT DNA (60 μM) in Tris/HCl buffer (5 mM Tris/HCl, 50 mM NaClO$_4$, pH=7.39) to achieve molar ratios ranging from 0.5 to 2.0 drug/DNA. The samples were incubated at 37 °C for 20 h. All CD spectra of DNA and DNA-drug adducts were recorded at 25 °C over a 220–420 nm range and finally corrected with blank and noise reduction. The final data are expressed in molar ellipticity (millidegrees). Melting curves were recorded in 5 mM Tris/HCl, 50 mM NaClO$_4$, pH 7.39. The absorbance at 260 nm was monitored for solutions of CT DNA (35 μM) before and after incubation with a solution of the drug under study (at a final 0.5 drug/DNA molar ratio) for periods of 3 and 20 h at room temperature. The temperature was increased by 0.5 °C/min in the range 65–82 °C and by 3 °C/min in the ranges 25–65 °C and 82–97 °C.

Results and discussion

Ru complexes are cytotoxic to cancer cell lines

The cytotoxicity of compounds 1–12 (chemical structures shown in Fig. 2) was measured by DNS against three prostate tumor cell lines (DU145, 22Rv1, LNCaP), a cervical cancer (HeLa), a lymphoblastic lymphoma (YT NK, Natural Killer-like), and non-tumorigenic human cell lines (immortalized keratinocyte, HaCaT, breast epithelial MCF-10A, and MEFs). In order to ascertain the effectiveness of the metal–drug synergy, the activity of KTZ and CTZ, and of metal compounds with molecular structures similar to complexes 1–12 but lacking the organic drug, viz. Na[*trans*-RuCl$_4$(DMSO)$_2$] (C1), *cis*-[RuCl$_2$(DMSO)$_4$] (C2), and [Ru(*p*-cymene)Cl$_2$]$_2$ (C3) was also measured. The results of these assays are shown in Table 1. We note that both KTZ and CTZ display moderate

Table 1 CC_{50} values, relative activity (R.A.), and selectivity index (S.I.) of Ru–KTZ and Ru–CTZ complexes

Cell line	DU145	22Rv1	LNCaP	YT	HeLa	HaCaT	MCF-10A	MEFs
C1	[b]	113.7[a]	46.5	[b]	[b]	[b]	N.D.	N.D.
C2	[b]	[b]	232.4	438.6	[b]	[b]	N.D.	N.D.
C3	217.6	140.9	29	164.2	[b]	[b]	N.D.	N.D.
KTZ R.A./S.I.[d]	28.6 1/>11	24.2 1/>13	27.9 1/>11	4.9 1/>64	66.7 1/>4	[b]	37	31.3
1 R.A./S.I.	[b]	49.4 0.5/1.6	55.1 0.5/1.4	56.3 0.1/1.4	[b]	76.8	62 [a]	55.8
2 R.A./S.I.	8.1 3.5/>39	21.4 1.1/>15	4.3 6.6/>74	32.5 0.2/>10	47.8 1.4/>7	[b]	6.7	5.2
3 R.A./S.I.	74.4 0.4/0.8	7.4 3.3/8.3	36.4 0.8/1.7	8.1 0.6/7.7	61.4 1.1/1	61.6	66.4	25.3
4 R.A./S.I.	[b]	132.3 0.2/>2.4	7.4 3.8/>43	<4[c] >1.2/>79	[b]	[b]	[b]	212.5
5 R.A./S.I.	34.3 0.8/9.1	14.7 1.6/21.3	6.6 >3/>31	30.0 0.2/10.4	254.1 0.3/1.2	312.6[e]	171	39.2
6 R.A.-S.I	94.8 0.3/1.2	128 0.2/0.9	6.8 >2.8/>11	30.6 0.2/3.7	33.3 2.0/3.4	113.1	12.3	37.3
CTZ R.A./S.I.	43.6 1/2.2	19.2 1/5	15.4 1/6.2	16.7 1/5.7	94.3 1/1	95.3	79.4	35.3
7 R.A./S.I.	[b]	[b]	23.2 0.7/>14	12.7 1.3/>25	[b]	[b]	31.6	31.8
8 R.A./S.I.	[b]	3.6 5.4/>87	2.4 6.3/>128	3.6 4.7/>87	38.2 2.5/>8	[b]	19.5	4.8
9 R.A./S.I.	[b]	24.2 0.8/>13	5.0 3.1/>63	4.3 3.9/>72	[b]	[b]	33.5	4.5
10 R.A./S.I.	67.1 0.7/0.4	20.4 0.9/1.3	5.3 2.9/5	5.2 2.9/5	36.9 2.6/0.7	26.1	42.9	5.6
11 R.A./S.I.	[b]	36.1 0.5/>9	14.7 1.1/>21	11.2 1.5/>28	126.7 0.7/>2	[b]	[b]	12.8
12 R.A./S.I.	546.97 0.1/0.2	[b]	25.38 0.6/3.8	42.18 0.4/2.3	359.3 0.3/0.3	97.1	79.1	64.7

N.D. not determined

[a] Concentration of chemical compounds is in micromolars

[b] Experimental chemical compound with cytotoxicity below 25 % at the maximum compound concentration tested

[c] When this compound was tested at 4 μM, the percentage of dead cells observed was 82.48

[d] R.A.=CC_{50} of compound/CC_{50} of KTZ or CTZ; S.I.=CC_{50} in HaCat cells/CC_{50} in tumor cells

[e] For those complexes where the toxicity on HaCaT could not be established, the selectivity indexes were calculated using the highest value reported for HaCaT toxicity (312.58 μM), corresponding to compound 5

cytotoxicity against all the cancer cells lines studied, while the control metal compounds C1–C3 not containing KTZ or CTZ were essentially inactive. The Ru–KTZ and Ru–CTZ complexes, in turn, display activity against all cancer cell lines, in some cases, considerably higher than the uncomplexed KTZ or CTZ, validating our synergistic approach. Importantly, the new complexes display low to very low toxicity to non-cancerous HaCaT cells. In addition to the HaCaT cell line, the experimental compounds were also tested on the MCF-10A human breast epithelium cell line and MEFs. As shown on Table 1, half of the compounds tested (1, 3, 4,CTZ, 10–12) exhibited similar toxicity levels when tested on HaCaT and the MCF-10A cell line while only two compounds (1 and 12) had similar effects on the three control cell lines. Control compounds C1–C3 were not evaluated on the MCF-10A and MEF cell lines as these proved to have little if any inherent cytotoxicity.

It is interesting to highlight some observations concerning the three prostate cancer cell lines employed in this study: First, our Ru-KTZ and Ru-CTZ complexes are more active on 22Rv1 and LNCaP than on DU145. DU145 cells are androgen–receptor-negative, while 22Rv1 cells are androgen–receptor-positive and weakly stimulated by dihydroxytestosterone (DHT), and LNCaP cells express high levels of androgen receptors and are highly sensitive to DHT (Horoszewicz et al. 1983). KTZ has been used as treatment for androgen-dependent prostate cancer (De et al. 1996) on the basis that its cytochrome 450 inhibition properties may result in inhibition of testicular and adrenal biosynthesis of androgen by competing with testosterone and DHT in the binding of androgen receptors (Borlak and Thum 2001; Thum and Borlak 2000). The ability of CTZ to inhibit cytochrome P450, on the other hand, has been linked to the modulation of Ca^{2+} channels

and consequently, to its cytostatic antitumor properties (Xiao et al. 1998).

The higher cytotoxicity observed for the Ru–KTZ and Ru–CTZ complexes to the androgen receptor positive lines might thus be related to: (1) the ability of Ru–KTZ compounds to act as androgen receptor antagonists, and/or (2) changes in Ca^{2+} levels induced by the Ru–CTZ drugs, as reported in the case of apoptotic prostatic cell death of LNCaP (Gong et al. 1995). Interestingly, the same trend is not observed for the parent compounds KTZ or CTZ, indicating that new mechanisms of action are triggered when the azole molecules are linked to the metal fragment. As a result, some of the Ru compounds exhibit CC_{50} values in the low micromolar range, an enhanced activity relative to KTZ or CTZ, together with good selectivity in relation to non-cancerous HaCaT cells. For instance, compounds 2 and 8 are particularly active against the LNCaP line, over six times more active than the parental drugs KTZ or CTZ, in line with our metal–drug synergy hypothesis. They also display the highest selectivity indexes, more than 74 and 128 times less toxic to HaCaT than to LNCaP cells.

In the case of lymphoblastic lymphoma YT NK, we note that the Ru–KTZ complexes are less active than KTZ alone, while Ru–CTZ compounds show significantly higher activity than CTZ, with CC_{50} values in the low micromolar range. These results suggest that Ca^{2+} homeostasis could be related to the apoptotic process in some lymphomas, as previously described (Ito et al. 2002). For HeLa cells, complex 2 is again the most active of the Ru–KTZ series, and complex 8 is the best of the Ru–CTZ series in terms of relative activity and selectivity index. Other Ru complexes, including KP1019 analogues with a chemical structure similar to 8, have been reported to be active against HeLa cells with IC_{50} values in the micromolar range, comparable to our present data (Frasca et al. 1996; Qian et al. 2013).

Ru complexes induce cytotoxicity via apoptosis

After a cytotoxic treatment, cells can undergo two main death pathways: necrosis or programmed cell death (apoptosis). Necrosis is typically initiated at the cell surface and is the consequence of a direct injury; it does not require metabolic energy and evolves through a traumatic cellular lysis. On the other hand, apoptosis is a much slower metabolic energy-dependent structured program that requires RNA and protein synthesis,

leading to a series of concerted biochemical events that culminate in cellular suicide (Fink and Cookson 2005; Kanduc et al. 2002). Both processes have different biochemical implications, and, consequently, discerning the mode of death mediated by any drug is of evident relevance. In line with this, we have quantified the percentage of apoptotic cells after treatment with our Ru complexes by flow cytometry. The results obtained for KTZ, CTZ, and the Ru complexes 1–12 on YT NK-like cells after 18 h exposure are shown in Fig. 3. One of the significant biochemical features in the early stages of apoptosis is loss of plasma membrane phospholipid asymmetry because of translocation of phosphatidylserine (PS) from the cytoplasmic to the extracellular side. This characteristic allows detection of externalized PS by the specific binding of annexin V (FITC-conjugated). Initiation of cell death will eventually result in the permeabilization of the cell membrane, allowing propidium iodide (PI) to stain DNA within the nucleus. Cells identified as annexin V–FITC-positive were considered as early apoptotic. Both annexin V–FITC and PI staining is typical at late stages of apoptosis, whereas cells that were stained only with PI due to the loss of plasma membrane integrity were considered necrotic cells (black bars in Fig. 3). The total percentage of apoptotic cell populations is expressed as the sum of percentages of both early and late stages of apoptosis (white bars in Fig. 3).

Our results show different trends when YT NK-like (YT) cells are treated with Ru–KTZ and Ru–CTZ complexes and their parent compounds, KTZ and CTZ. On one hand, cells treated with 15 and 10 μM concentrations of KTZ and CTZ, respectively, undergo death preferentially through apoptosis. This behavior agrees with previously reported results in which the treatment of primary bone marrow cell cultures from patients suffering of acute lymphoblastic leukemia with 10 μM clotrimazole depleted intracellular Ca^{2+} stores, provoking cytotoxicity via apoptosis, without affecting mature T lymphocytes and bone marrow stromal cells (Ito et al. 2002). Regarding the Ru–KTZ metal compounds, the presence of increasing concentrations (ranging 20–60 μM) of complexes 3, 4, and 5 induce YT cells to undergo cell death primarily by apoptosis, especially in the case of 4 (~52 % apoptosis vs. ~8 % necrosis) and 5 (~41 % apoptosis vs. ~4 % necrosis), in agreement with what was observed for other Ru compounds (Martinez et al. 2010; Strasberg et al. 2004). Complex 4 was
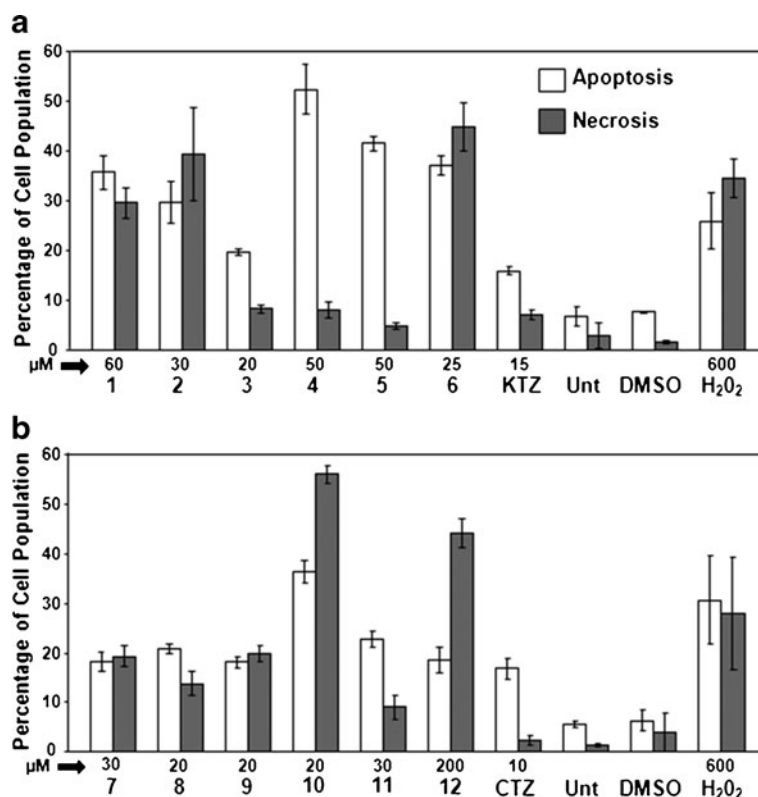
Fig. 3 Evaluation of cell death via flow cytometry. YT cells were exposed to the various Ru-based compounds for 18 h and stained with annexin V–FITC and PI. Cells treated with Ru–KTZ (**a**) or Ru–CTZ (**b**) complexes were compared with cells treated with uncomplexed KTZ and CTZ. Cells treated with the solvent control (0.3 %v/v DMSO) and cells exposed to 600 μM hydrogen peroxide (H_2O_2) were included as negative and positive cytotoxicity controls, respectively. Untreated (*Unt*) cells were also included to determine the viability of cells during the course of the experiments. Cells identified as annexin V–FITC positive were considered as apoptotic, and the percentage of these cells is expressed as the sum of percentages of both early and late stages of apoptosis (*white bars*). Cells that were only stained with PI (but not with annexin V–FITC) due to the loss of plasma membrane integrity are considered necrotic cells (*grey bars*). The concentration (in micromolars), as well as the name of each experimental compound, is shown below of the *x*-axis. Each *bar* represents the mean of three independent measurements with their respective standard deviations

significantly more active against YT ($CC_{50}<4$ μM) and the prostate cancer cell line LNCaP ($CC_{50}=7.37$ μM) while 5 had similar activity to LNCaP ($CC_{50}=6.64$ μM) and was more cytotoxic to the other prostate cancer cell lines used (CC_{50} between 16 and 34 μM; see Table 1). Interestingly, only two of the Ru–CTZ complexes 8 and 11 elicited slightly (no more than twofold) more apoptosis than necrosis while the rest of the CTZ-based compounds were more necrotic. It is important to note that CTZ-alone induced more (≥3-fold) apoptosis than any of the Ru–CTZ complexes. Taken together, our results indicate that Ru–KTZ complexes are more cytotoxic than the Ru–CTZ ones and that KTZ-based complexes act via distinct cytotoxic mechanisms (compare 4 and 6 for example, Fig. 3a).

Interaction of Ru complexes with DNA

The cytotoxicity of cisplatin, a widely used heavy metal-based anti-tumor agent, results mainly from the formation of bifunctional DNA cross-links, especially between consecutive guanines (Jamieson and Lippard 1999). This interaction induces important DNA structure modifications that trigger a cascade of biological processes, ultimately leading to inhibition of both DNA synthesis and replication, and resulting in death of cancer cells, primarily through apoptosis. It is also known that the anti-tumor activity of some ruthenium compounds like KP1019 (Kapitza et al. 2005) and [Ru(η⁶-arene)(X)(Y–Z)] (Aird et al. 2002; Dougan et al. 2006; Morris et al. 2001; Wang et al. 2005) is related to their

interactions with DNA. In contrast, the anti-metastasic NAMI and the arene–Ru–CQ complexes reported by us show some ability to interact with DNA, but their cytotoxic behavior appears to follow mechanisms different to DNA interactions. In view of the cytotoxic properties displayed by complexes 1–12, it was significant to determine whether they interact with DNA, and if so, whether that interaction contributes to the observed activity to a significant extent.

For this purpose, CD and thermal denaturation (Tm) experiments were carried out on selected complexes

(Fig. 4 and Table 2). The CD spectral technique is very sensitive for diagnosing changes in secondary structure of DNA resulting from drug–DNA interactions. A typical CD spectrum of calf thymus (CT) DNA shows a maximum at 275 nm due to the base stacking and a minimum at 248 nm attributed to the right-handed helicity, characteristic of the B conformation (Fig. 4) (Cutts et al. 1997). Thus, changes in CD signals can be assigned to corresponding changes in DNA structure. It is known that simple groove binding or electrostatic interaction of small molecules cause little or no
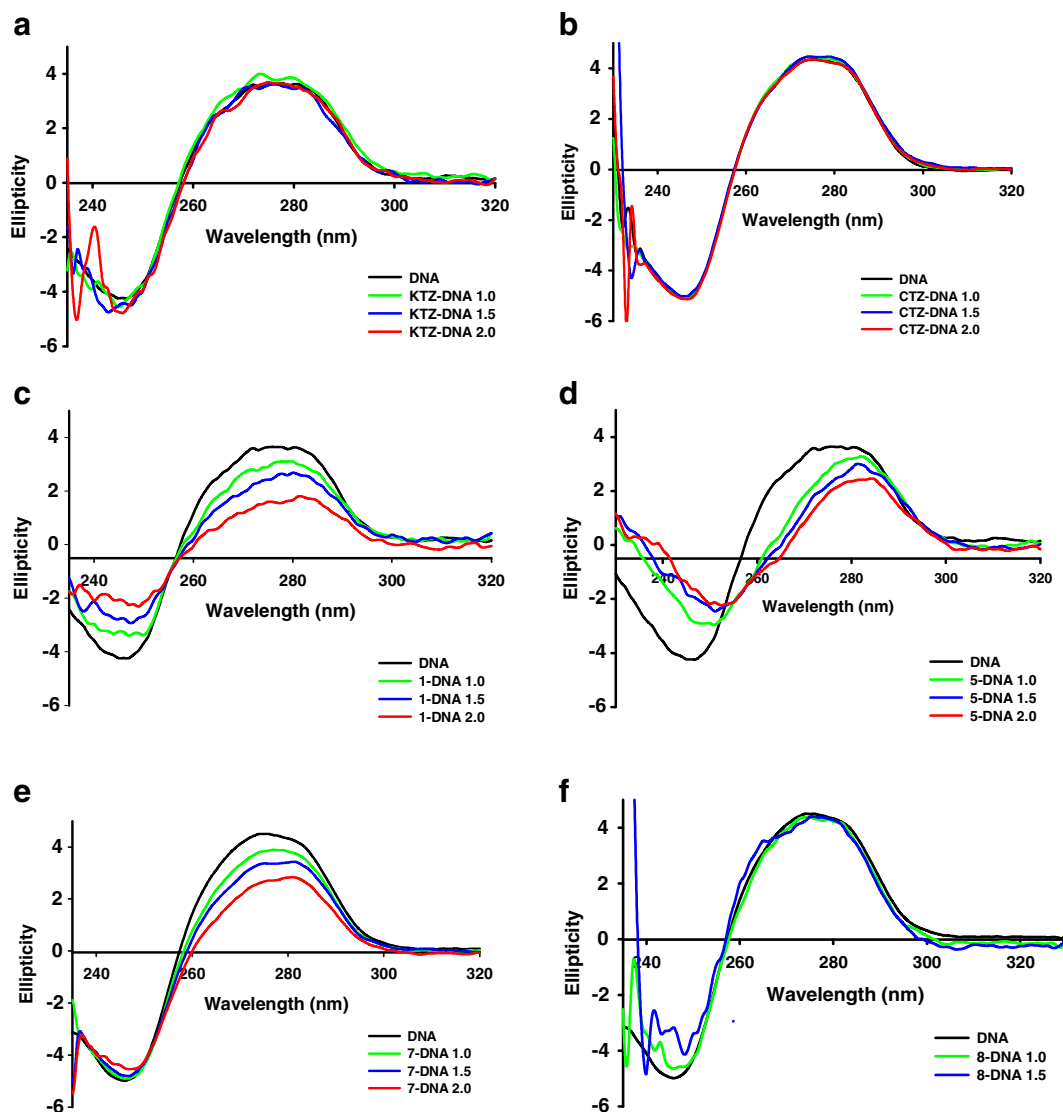

Fig. 4 CD spectra of CT DNA and CT DNA incubated with 1.0, 1.5, and 2.0 equivalents of **a** KTZ, **b** CTZ, and complexes **c** 1, **d** 5, **e** 7, and **f** 8. See "Materials and methods" for assay details

Table 2 Change in the melting temperature (Tm) of DNA incubated with compounds

Compound[a]	ΔTm DNA (°C)
KTZ	NC
CTZ	NC
1	−1.1
2	[b]
3	NC
4	[c]
5	NM
6	NM
7	−2.3
8	NC
9	NC
10	NC
11	−0.3
12	[b]

NC no change in Tm beyond error is observed, *NM* Tm not-measured

[a] DNA was incubated with 0.5 equivalents of every compound for 3–24 h in 5 mM Tris/HCl buffer at pH=7.39

[b] Complex decomposes at high temperature

[c] Tm cannot be measured because of band overlap

alteration in any of the characteristic bands, whereas covalent interaction or intercalation between the base pairs will induce major perturbations in the CD spectrum. On the other hand, the thermal denaturation technique is an essential tool to detect the nature of drug–DNA interactions; destabilizing interactions (typically covalent) tend to decrease the thermal denaturation temperature of DNA, whereas a stabilizing interaction (electrostatic or intercalation) will increase it (Novakova et al. 2003)

CD spectra of CT DNA incubated for 20 h at 37 °C and pH 7.39 in Tris/HCl buffer with CTZ and KTZ up to r_i 2.0 (molar ratio drug/DNA) show no modifications with respect to untreated DNA (Fig. 4a and b), suggesting that none of the parent compounds interact with CT DNA to a significant extent or that the interaction does not induce any observable perturbation in the secondary structure. This is further supported by no changes being observed in the melting temperature (Tm) of CT DNA upon incubation with the azole molecules (Table 2). In contrast, the Ru–KTZ and Ru–CTZ complexes studied are able to modify the CD spectrum of CT DNA to a different extent, depending on the compound and the concentration. Complexes 1, 5, and 7 induce the largest alteration on the CT DNA secondary structure. A significant decrease in the intensity of the positive and/or the negative bands was observed, indicating a perturbation of the nucleobase pi-stacking and depletion of the B

conformation, similar to the effect induced by cisplatin (Brabec et al. 1990). Furthermore, complexes 1 and 7 decrease Tm of CT DNA by 1.1 and 2.3 °C, respectively (Table 2), suggesting that the strong interaction observed by CD is destabilizing DNA.

The amount of Ru covalently bound to DNA, determined by the Burton assay after 48 h of incubation with complex 7 was 585 nmol/mg DNA, which confirms that the changes observed in the CD spectra and Tm experiments are the result of a strong covalent interaction of the metal with the nucleobases of DNA (Anzelloti 2004). It is reasonable to hypothesize that the similar effect observed for complex 1 in CD spectral and Tm experiments is a consequence of the same type of interaction, given the structural analogy between both complexes. In the case of complex 5, the presence of ethylenediamine ligand and the overall positive charge likely allow this compound to form hydrogen bonds with the bases of DNA and establish electrostatic interactions with the phosphate backbone. The weak interaction observed for the other compounds (data not shown), reminiscent of that described for NAMI (Gallori et al. 2000), is likely due to electrostatic attraction/repulsion or minor groove binding, as previously reported for Pt–CTZ complexes (Navarro et al. 2009). This is further supported by results of the Burton Assay on complexes 8 and 9 (Anzelloti 2004), revealing 87 and 15 nmol/mg DNA of drug covalently bound to DNA after 48 h of incubation, respectively, much lower than was measured for complex 7. Importantly, our results show no correlation between the ability to interact with CT DNA and anti-tumor activity; complex 8 shows the best cytotoxic profile on 22Rv1, LNCaP, and YT NK but induces only slight changes in both the CD spectra (Fig. 4f) and the Tm of CT DNA, whereas complexes 1, 5, and 7 display a remarkably poor anti-tumor activity (Table 1) and strong interactions with DNA. This suggests that the enhancement in the anti-tumor properties of KTZ and CTZ when they are attached to ruthenium in a molecular complex is due to mechanisms/factors other than DNA interactions, as has been previously observed for other ruthenium complexes (Sanna et al. 2002). Importantly, the toxicity to non-tumorigenic cells (HaCaT) is not related to the ability of the complexes to interact with DNA, which could have important implications in drug safety.

Conclusions

We report the activity of a series of 12 Ru–KTZ and Ru–CTZ compounds against three prostate tumor cell lines with different androgen sensitivity, as well as cervical cancer and lymphoblastic lymphoma cell lines. A human epidermal cell line was used to evaluate the toxicity against non-transformed cells and to establish selectivity indexes. Our results show that the combination of ruthenium and KTZ/CTZ in a single molecule resulted in some of the complexes exhibiting more cytotoxic than any of the individual components separately. Additionally, some of the compounds are more cytotoxic against cancer cell lines with lower activity against non-transformed cell lines. Studies of DNA interactions reveal that neither the antitumor properties nor the toxicity against non-tumorigenic human cells for any of the metal compounds are related to their ability to interact with DNA. Some of the complexes display promising cytotoxic profiles among the different cell lines tested, including high activity accompanied by high selectivity indexes and cell death preferentially induced through apoptosis.

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