



Induction of caspase 8 and reactive oxygen species by ruthenium-derived anticancer compounds with improved water solubility and cytotoxicity

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ABSTRACT

Organometallic compounds which contain metals, such as ruthenium or gold, have been investigated as a replacement for platinum-derived anticancer drugs. They often show good antitumor effects, but the identification of their precise mode of action or their pharmacological optimization is still challenging. We have previously described a class of ruthenium(II) compounds with interesting anticancer properties. In comparison to cisplatin, these molecules have lower side effects, a reduced ability to interact with DNA, and they induce cell death in absence of p53 through CHOP/DDIT3. We have now optimized these molecules by improving their cytotoxicity and their water solubility. In this article, we demonstrate that by changing the ligands around the ruthenium we modify the ability of the compounds to interact with DNA. We show that these optimized molecules reduce tumor growth in different mouse models and retain their ability to induce CHOP/DDIT3. However, they are more potent inducers of cancer cell death and trigger the production of reactive oxygen species and the activation of caspase 8. More importantly, we show that blocking reactive oxygen species production or caspase 8 activity reduces significantly the activity of the compounds. Altogether our data suggest that water-soluble ruthenium(II)-derived compounds represent an interesting class of molecules that, depending on their structures, can target several pro-apoptotic signaling pathways leading to reactive oxygen species production and caspase 8 activation.

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1. Introduction

Cancer remains one of the first causes of death in industrialized countries. Nevertheless, few examples of successful chemotherapy are still driving the search for more potent, more selective, less prone to resistance and better tolerated drugs. One of these examples is cisplatin, a drug showing a significant role in the management of a number of tumors [1–3]. However, cisplatin

likewise platinum-derived drugs display an often-severe toxicity and a relatively frequent inactivity due to natural- or induced-resistance [4–6]. More recently, a significant interest was addressed to ruthenium-based drugs, because of some favorable properties that make them a suitable basis for the development of antitumor drugs, such as their metal oxidation state (Ru(II) or Ru(III)), the fairly facile ligand exchange and the binding to biologically relevant proteins. Compared to many organic compounds, ruthenium-based drugs also offer the advantage of a relatively low cost for their synthesis and purification.

Various ruthenium complexes were shown to present cytotoxicity against cancer cells, ligand-exchange abilities similar to those of platinum complexes, no cross-resistance with cisplatin and a reduced toxicity against healthy tissues at least in part explained by the selective transportation to cancer cells by the iron transport system [1–3,7]. Following the pioneering work with ruthenium red

Abbreviations: CHOP, C/EBP homologous protein; DDIT3, DNA damage inducible transcript 3; FACS, fluorescence activated cell sorter; FRET, Förster resonance energy transfer; NAC, n-acetylcysteine.

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[8] a number of ruthenium-based drugs were shown to endow antitumor potential. Indeed, several teams have synthesized and characterized compounds containing ruthenium in oxidative state (II) or (III), showing their anticancer activity [9–16]. Two, namely NAMI-A and KP1019, have successfully passed some initial phases of clinical trials [17,18]. Besides these partial successes, the emergence of new ruthenium-based therapies have been slowed down by limitations, such as a relatively poor level of water solubility, and/or stability in aqueous solutions, a not impressive cytotoxicity (IC₅₀ between 20 and 100 μ M), and an uncertainty on the molecular mechanisms of action responsible for the antitumor effect.

The mechanism of action and the direct targets of ruthenium-based drugs are still a matter of debate. Indeed, depending on the drug, several modes of action have been proposed, such as interaction with DNA and activation of DNA damage pathways [19–24], inhibition of kinases [25], or other enzymatic activities [26,27], including extracellular metallo-proteases [28]. The differences observed may be due to variations in their structure. Even if most of the ruthenium-containing compounds have ligands that are relatively weakly bound to the metal via a heteroatom (N, O, S), there are differences in the types of ligands attached.

In order to improve the stability of ruthenium complexes and possibly to enhance their cytotoxicity and their pharmacokinetics, we have previously generated several ruthenium-based complexes in which the ligand is bound to the metal via strong covalent bonds such as a C–M σ bond [22,29]. Beside the stability, these compounds present differences in their Ru(II)/Ru(III) redox potential and a new variety of ligands. We have called these molecules RDCs (Ruthenium-Derived Compounds) and we have previously shown that some RDCs are cytotoxic for several cancer cell lines resistant to cisplatin [22]. One of them, RDC11, showed a good antitumor activity both *in vitro* and *in vivo* [30] with an IC₅₀ often between 2 and 5 μ M, and anticancer properties on models of ovarian cancer, melanomas and gliomas. Importantly, they showed, compared to cisplatin a reduced neurotoxicity. As we previously showed that cisplatin exerts its neurotoxic effect in part through induction of the p53 pathway [31,32], we analyzed the ability of RDC11 to interact with DNA and induce p53 proteins. We demonstrated that RDC11 exerts its antitumor effect via DNA-dependent and DNA-independent modes of action. We also identified one of the DNA-independent signaling pathways by showing an activation of the endoplasmic reticulum stress pathway, and in particular the transcription factor CHOP/DDIT3. However, the silencing of CHOP/DDIT3 by siRNA was not able to completely abolish RDC11 cytotoxicity, strongly suggesting that other signaling pathways are also involved [30,33,34].

In the present study, we developed RDC11 variants designed for improving cytotoxicity and water solubility. The biological properties of these novel ruthenium-based organometallics are studied *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cell culture, MTT test, flow cytometry analysis

B16F10, U87, A172, 3LL, A2780, HCT116, SW480 cells were obtained from ATCC (Manassas, VA). Cells were maintained in DMEM with 10% FBS and incubated in presence of 5% CO₂/95% air at 37 °C. MTT tests were performed with cells cultured in 96-well culture dishes as previously described [35]. Hypodiploid DNA was measured as described [36] using propidium iodide (Sigma, MO). The fluorescence of 10,000 cells was analyzed using a FACScan flow cytometer and CellQuest software (Becton Dickinson, San José, CA).

2.2. Statistical analysis

In MTT ($n = 8$), RT-qPCR ($n = 3$), ROS ($n = 8$), caspase 8 activity ($n = 4$), FACS analysis ($n = 3$), bars are mean \pm SD and asterisks indicate a statistically significant difference ($p < 0.01$) compared to control, as calculated by a one-way ANOVA test followed by a Tukey post-test.

2.3. Western blot

Cells were treated in triplicates and Western blots were performed as previously described [37]. Immunoprobings were performed with anti-phospho 137-H2AX antibody (1/3000, Millipore, Molsheim, France), anti-CHOP (1/1000, Santa Cruz Biotechnology, CA), anti-human p53 (421, supernatant 1/3, C. Prives, Columbia University NYC), or actin (Dr. Aunis, Strasbourg) antibodies. Membranes were probed with a secondary horseradish-peroxidase-conjugated antibody (anti-rabbit, -goat or -mouse, 1/2000, Thermo Fisher, IL), and revealed with ECL (Thermo Fisher, IL).

2.4. Quantitative real-time PCR

RNA was extracted using RNeasy Nucleospin (Macherey-Nagel, Strasbourg, France). Reverse transcription was performed with 1 μ g RNA (iScript kit, Bio-Rad), followed by qPCR in Bio-Rad iCycler thermal cycler using iQ SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA). Starting quantities of genes of interest were reported to those of housekeeping genes (TBP, 18s). Specificity of the amplification was controlled by a melting curve [31]. Probes: for noxa, 5'-ggagatgctgggaagaag-3'; 5'-cctgagttagtagcacactcg-3'; for fas, 5'-atggccaattctgccaataag-3', 5'-tgactgtgcagtccttagctt-3'; ttp, 5'-cgctgtttaacttcgcttc-3', 5'-cacacgccaagaacagtga-3'.

2.5. Evaluation of mitochondrial membrane potential

The changes in $\Delta\Psi_m$ were assessed using the membrane potential sensitive dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide, Life technology, France). Cells were grown on cover glasses and the staining procedure was the following: DMEM was removed and 10 μ g/ml JC-1 solution (in PBS) was added for 15 min [38]. The staining solution was removed and cultures were rinsed with PBS. Subsequently, cells were fixed in 4% PFA for 15 min and then covered with Vectashield mounting medium (Vector labs, CA) on glass slides. Fluorescence was measured at 488 nm excitation/510 nm emission.

2.6. Assay for caspase 8 activity

Caspase 8 activity was assayed by measuring the light intensity using a kit (Caspase-Glo[®] 8 Assay, Promega) and a luminometer (Perkin Elmer HTS 7000, France). Briefly, cells were cultured in 96-well plates in a final volume of 200 μ l. Then 50 μ l caspase-8 reaction buffer was added and incubated at room temperature for 1 h before measurement.

2.7. FRET

FRET measurements are performed on a home build setup as previously described [34].

2.8. Intracellular reactive oxygen species (ROS) measurement

5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) (Life Technology, France) was used to detect intracellular ROS according to the manufacturer's instructions. For

ROS quantification, cells were seeded in 96-well black plates (Greiner Bio-One, France) and treated with RDCs at the indicated concentrations and times. Afterwards, cells were washed with PBS and incubated with 10 μM carboxy-H2DCFDA in DPBS for an hour. Cells were then washed and fluorescence was measured by a plate reader (Perkin Elmer, France) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

2.9. Chemical synthesis

RDC11 [39], RDC34,37,40,41,44 [40] were synthesized according to published procedures. All complexes were purified over chromatography columns carried out on Merk aluminum oxide 90 standardized. Complexes were used with a purity >98%, as demonstrated by several protocols [40]. ES-MS spectra and elemental analyses were carried out by the corresponding facilities at the Institut de chimie, Université de Strasbourg and at the Service Central d'Analyse du CNRS, Vernaison.

3. Results

3.1. Generating ruthenium compounds with an IC50 in the nanomolar range

On the basis of previously structure–function studies showing an improved cytotoxicity of RDCs having a phenanthroline ligand [22], we substituted the two acetonitrile ligands of RDC11 by a second phenanthroline, naming the new molecule RDC34 (Fig. 1). An equivalent of RDC34 with another counter-ion (PF_6^-) was also synthesized (RDC37). Previous works have established the ability of RDCs to modulate the activity of cellular enzymes through their redox potential [39]. We therefore modified the redox potential of RDC34 by adding either a NO_2 (electron withdrawing) or a NH_2 unit (electron releasing) on the phenylpyridine ligand, leading to RDC40 and RDC41 respectively. Finally, in order to increase the water solubility of RDC34, we added a spermine unit to the phenylpyridine ligand, leading to RDC44 (water solution of up to 25 mM was obtained) [40].

As colon cancers are one of the indications for platinum-derived treatments, the cytotoxicity of the RDC was first tested on a human colon cancer cells (HCT116). Interestingly, RDC34, RDC37, and RDC40, characterized by the presence of the NO_2 group, showed an increased cytotoxicity ($\text{IC}_{50} < 2 \mu\text{M}$) (Fig. 1) compared to RDC11

and cisplatin. However, the addition of the NH_2 group decreased the cytotoxic activity ($\text{IC}_{50} = 2\text{--}4 \mu\text{M}$, RDC41) and that of the spermine moiety had an even worse effect with rising the IC_{50} to over 16 μM . Additional experiments gave IC_{50} s for RDC34, RDC40 and RDC41 of respectively 0.25 μM , 0.75 μM and 2–4 μM (supplementary data 1).

3.2. Effects of the hydrophilic spermine substituent chain on cytotoxicity, cell uptake and DNA binding

The addition of the spermine chain (leading to RDC44) significantly decreased the cytotoxicity (Fig. 1). In order to understand the reason for this behavior, we tested RDCs' cellular uptake. The two phenanthrolines confer to RDC34 and RDC44 luminescent properties allowing us to follow their entry into living cells (Fig. 2A and B). The maximum of accumulation of RDC34 inside the cells was reached 1 h after the compound was added. Cell uptake of RDC44 was lower. In PBS, the emitted intensity of RDC44 is 20% lower than the emission of RDC34, whereas in DMEM, RDC44 cell uptake is strongly diminished (Fig. 2C). However, the increased cell uptake of RDC44 in PBS did not lead to an increased cytotoxicity of this compound as the IC_{50} s were closed to those observed when cells were treated in culture medium (Fig. 2D).

As DNA is one of the direct targets of RDCs, we used the FRET approach based on a double-stranded oligonucleotide labeled at each end by two fluorophores to test RDC/DNA binding. RDC44 showed a higher affinity for DNA compared to RDC34, as demonstrated by the drop in FRET transfer energy that occurred at the lowest concentration of RDC44 (Fig. 2E). The affinity constants are obtained from the analysis of these data according to the McGhee and van Hippel model [41]: $K_a = 2.2 \times 10^3 \text{ M}^{-1}$ for RDC34 and $7.8 \times 10^5 \text{ M}^{-1}$ for RDC44. The number of base pairs occupied by RDC44 along the DNA chain is equal to 3.9, larger than the occupation site of RDC34 (2.3). It thus seems that the lack of significant cytotoxicity of RDC44 for HCT116 cells can be attributed neither to a default in cell entry and nor to the lack of interaction with DNA.

3.3. Modifications of RDC ligands modulate the selectivity between cancer cell lines

To further characterize the cellular effect of RDC34, we performed FACS analysis on HCT116 cells (Fig. 3A). RDC34, better than cisplatin, induced the accumulation of an elevated subG1

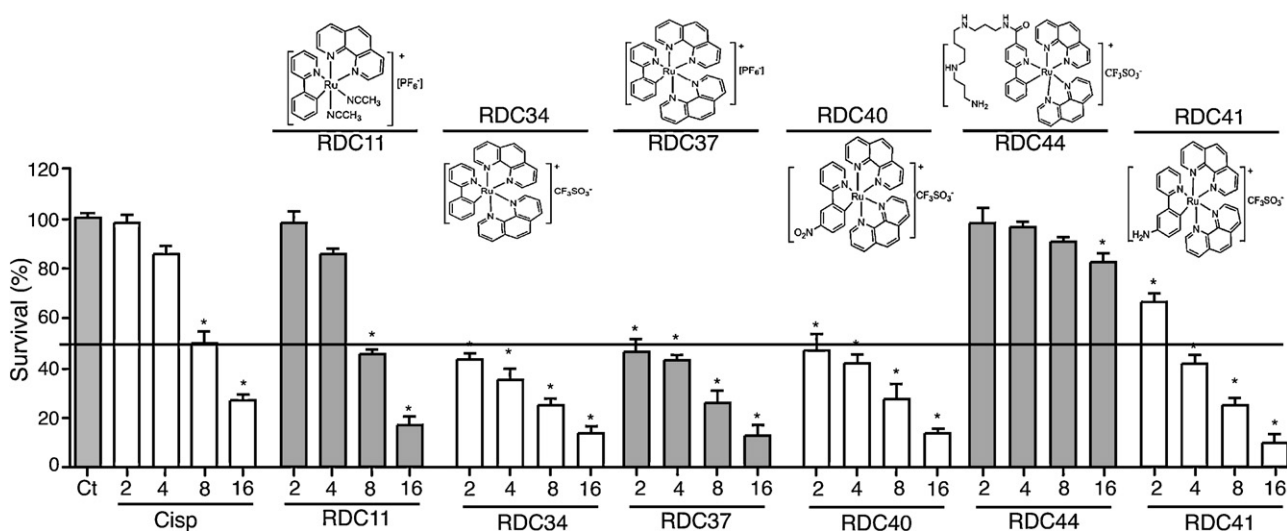


Fig. 1. Optimized RDC for water solubility and cytotoxicity reduce cell growth of HCT116 cells. HCT116 human colon cancer cells were treated for 48 h with cisplatin or RDC (μM). Viability was evaluated using a MTT test ($n = 8$). Insets: RDC structures. Data are representatives of 3 independent experiments. Statistical analyses are described in Section 2.

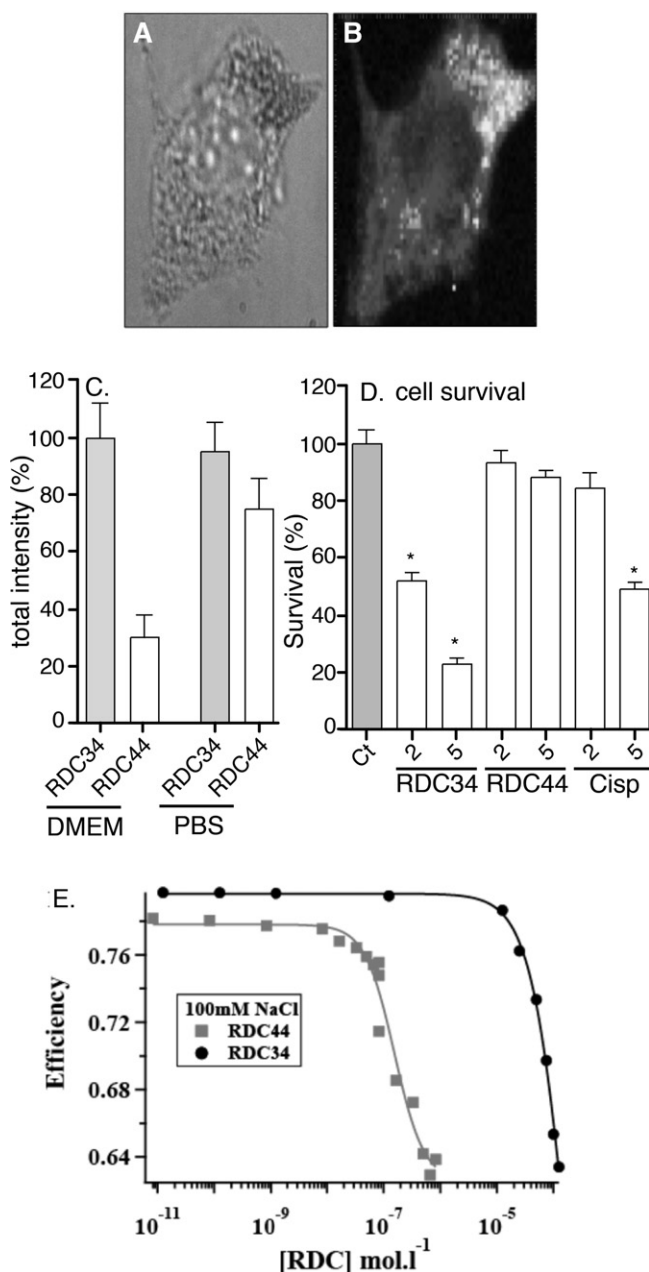


Fig. 2. Cell permeability and DNA interaction of optimized RDC. HCT116 cells incubated with RDC44 and visualized with white light (A) and fluorescence (at 750 nm, B) and quantification (C). Cells were incubated for 1 h with RDCs (5 μ M) in either DMEM or PBS. (D) HCT116 cells were treated for 2 h either in DMEM or PBS, medium was replaced by DMEM with serum for 48 h and MTT was performed. Data are representative of three independent experiments. (E) Efficiency of the Förster Resonance Energy Transfer (FRET) between the extremities of a 14 base pairs-long double-stranded DNA labeled with Alexa-488 and Alexa-568. The measurements were performed at the equilibrium of complexation of DNA with the metal complex. Solid line is a guide to the eye.

population at 24 h and 48 h, indicative of cell death. RDC34 induced also a G2/M arrest at 24 h, while cisplatin blocked HCT116 cells in G1.

The cytotoxic potential of RDC11, RDC34 and RDC41 was submitted to the US National Cancer Institute (NCI) test on 60 cancer cell lines (Fig. 3B–D). RDC34 showed a strong cytotoxic effect (indicated by the % of cell survival compared to the initial number) on almost all cell lines (Fig. 3B–D, supplementary data #3). These results were similar to those we obtained on additional cell lines (N2A, U87, F10B16, SW480, supplementary data #2).

Kidney-derived cancer cell lines were mostly resistant toward RDC11 and RDC41. Globally, RDC34, unlike RDC11 and RDC41, was endowed with a broader range of sensitive cell isotypes. These tests also showed the negative effect of NH₂ function (RDC41), leading to a significant decrease of cytotoxicity in almost all the cell lines. However, RDC41 showed an activity better than RDC11 on EK VX cells, MDA-MB-435, UACC-257 and HS57BT (supplementary data #3). It is also interesting to stress the significant activity of RDC11 on lung-derived cancer cells, on which RDC41 is only marginally effective.

These data indicate the correlation between the modifications of the ligands around the ruthenium atom and the antitumor activity, and suggest a possibility to modulate the intensity of the cytotoxicity and the specificity for selected tumor types.

3.4. Modifications of RDC ligands change the mode of action: role of caspase 8 and ROS

In order to understand how each modification of the ligands affects RDCs cytotoxicity and specificity, we compared their DNA interactions. RDCs were incubated with double stranded supercoiled DNA and then samples were submitted to migration on an agarose gel (Fig. 4A). RDC34 showed an affinity for DNA slightly greater than that of RDC11, but inferior to RDC40 and RDC41. In particular, RDC40 formed a stable complex with DNA that remained in the loading pocket.

These data stress the lack of correlation between the DNA binding activity and the cytotoxicity. We therefore looked for another possible explanation for the higher cytotoxicity of RDC34. Previous observations indicated that redox potential allowed RDCs to modulate the oxido-reductase enzyme activity. We therefore hypothesized that such regulation might lead to the production of reactive oxygen species (ROS). Incubation of HCT116 cells with RDCs and a fluorescent ROS probe (Fig. 4B–G) showed that RDC34 was the most potent ROS inducer (Fig. 4G and H), suggesting a correlation between the induction of ROS and cytotoxicity, which was confirmed by the reduction of RDC34 cytotoxicity by NAC, a ROS inhibitor (Fig. 5I).

We therefore investigated in more detail the mode of action of RDC34, comparing how this ruthenium compound was regulating the expression of p53 and CHOP, two transcription factors previously shown to be regulated by RDC11 and ROS. We also followed the phosphorylation of H2AX at serine 137, which is a marker for DNA damages [30]. RDC34 was as efficient as RDC11 to induce CHOP, but was more potent to induce p53 protein levels (Fig. 5A, supplementary data 4). In these experimental conditions, cisplatin was unable to regulate CHOP, whereas both RDCs were less able to induce H2AX phosphorylation. Besides the significant induction of p53 protein levels, RDC34 increased the expression of pro-apoptotic p53 target genes involved in either the mitochondrial-dependent apoptotic pathway (noxa, bax, siva) or the mitochondria-independent pathway (fas, trail) (Fig. 5B and C).

A fluorescent probe (JC-1) was used to follow changes in the mitochondrial membrane potential, indicative of the involvement of the mitochondria in the apoptotic processes induced by RDC34 (Fig. 5E and G). The involvement of a mitochondria-independent pathway was conversely tested by following caspase 8 activity. Compared to RDC11, RDC34 was more potent in inducing caspase 8 (Fig. 5H). The concomitant use of ROS and caspase 8 inhibitors reduced the cytotoxicity of RDC34, suggesting their importance for the antitumor activity of this drug (Fig. 5I).

3.5. In vivo antitumor activity of optimized RDCs

To further examine the anticancer potential of the optimized compounds, we tested their activity on tumor growth. A

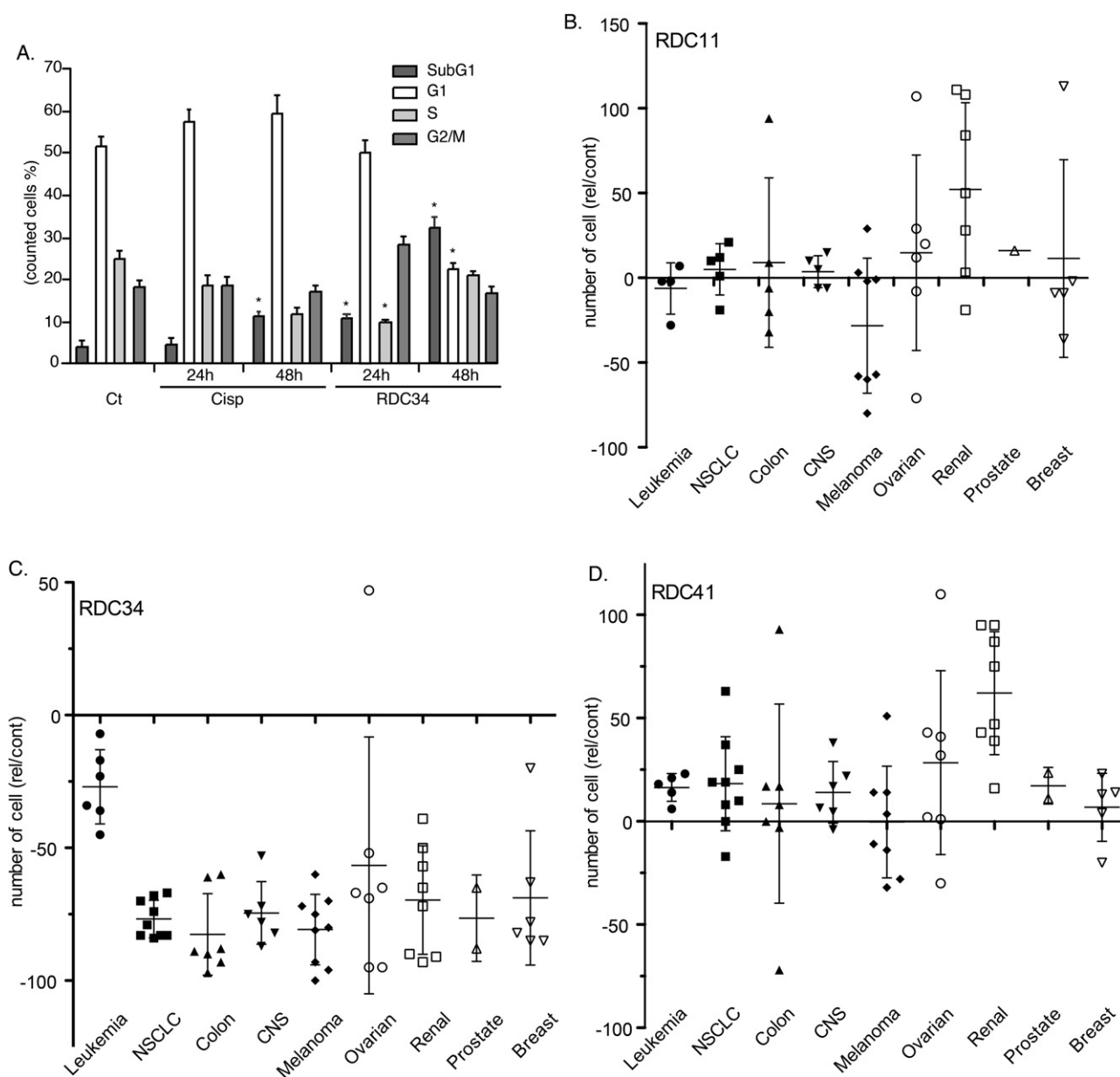


Fig. 3. Optimized RDC induced cytotoxicity on cancer cells of various origins. (A) Cell cycle profile analysis (FACS) of HCT116 cells treated with cisplatin or RDC34 (2.5 μ M) for 24 h or 48 h. (B–D) Graph indicating the cytostatic (above 0) and cytotoxic (below 0) response of 60 cancer cell lines of the specified origins (detailed in supplementary data #3). The test was performed by the NCI with the indicated RDC at a concentration of 5 μ M.

preliminary experiment to evaluate host toxicity after single dose and after chronic treatment (3 weeks, 2 injections a week) showed that repeated doses of RDC34 higher than 4 μ mol/kg were lethal (supplementary data #1). Similar results were obtained with RDC44, a compound with significant lower cytotoxicity in vitro (Fig. 1). RDC41 was the most tolerated compound and showed a DL50 of 30 μ mol/kg.

We first tested the compounds on a lung cancer as these cancers are commonly treated by cisplatin and we observed a good cytotoxicity of RDC34 on lung cancer cells, such as on 3LL cells (Figs. 3C and 6A). When tested at their maximum tolerated doses on the syngeneic model of 3LL cells implanted subcutaneously in B6 mice, RDC34 (4 μ mol/kg, 2 \times /week), RDC44 (4 μ mol/kg, 2 \times /week) and RDC41 (13 μ mol/kg, 2 \times /week) reduced tumor growth to approximately 40% (Fig. 6A). The anticancer activity of RDC34 on human ovarian cancer cells (A2780) implanted into nude mice (Fig. 6B) showed a significant reduction of tumor growth to

approximately 36% of the controls. In both models the activity of RDC34 is slightly higher compared to cisplatin.

4. Discussion

The development of novel anticancer compounds is a constant challenge. In the recent years, metal-based compounds have been the focus of a particular interest based on the proven relative efficiency of platinum-derived compounds and their intrinsic physico-chemical properties of transition metals, such as ruthenium. Especially, the vast possibilities of combination with organic ligands and small ions make ruthenium-based molecules particularly suitable to be modeled and adapted to a specific need. In particular, the wide range of redox states and the hexahedral structure of this metal allow endless metal/ligand combinations with variations in redox state, lipophilic/hydrophilic status, ligand exchange properties, stability, and geometry.

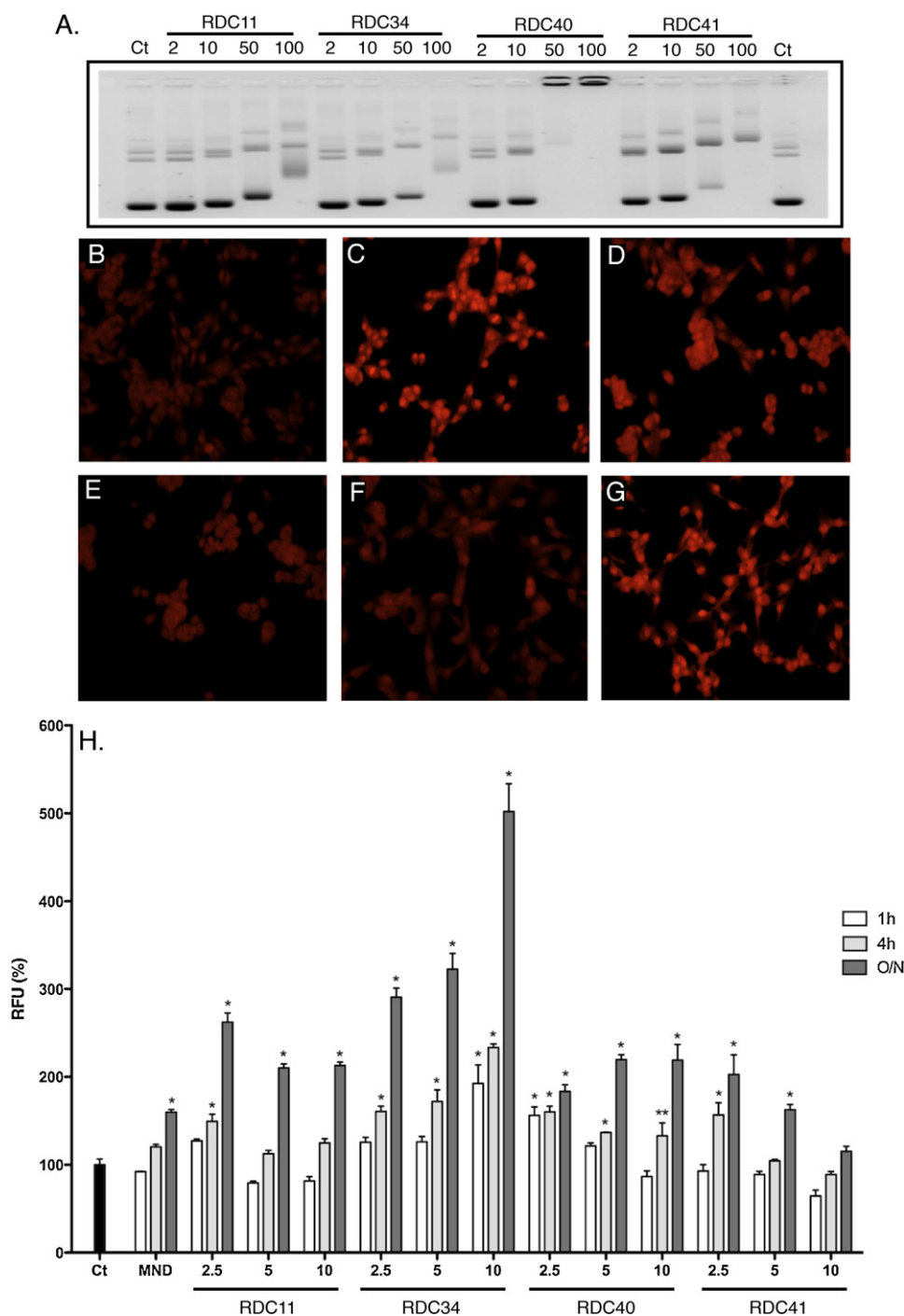


Fig. 4. Interaction of the optimized compounds with DNA and production of reactive oxygen species. (A) Circular double-stranded DNA was incubated with RDCs at the indicated ratio (DNA base pairs/molecule of drugs). Complexes were run on a 1% agarose gel, and then stained with ethidium bromide to observe DNA relaxation. Data are representative of 3 independent experiments. (B–G) HCT116 cells were treated with RDC (2.5 μ M) for 16 h and labeled with carboxy-H₂DCFDA for 1 h. (B) Control cells, (C) cells treated with the positive control, menadione, (D) cells treated with RDC11, (E) cells treated with RDC40, (F) cells treated with RDC41, (G) cells treated with RDC34. (H) HCT116 cells were treated with RDC as described in B–G, then fluorescence was quantified.

Recently, a ruthenium (II)-based organometallic compound, called RDC11, was shown endowed with anticancer properties. In order to optimize this compound, we have modified the ligands to gain in water solubility and in cytotoxicity.

As we hypothesized, the addition of a second phenanthroline significantly increased the cytotoxicity of the new compound RDC34 in comparison to RDC11, with an IC₅₀ often in the nanomolar range, confirming the important role of the phenanthroline residue in the cytotoxic properties of RDCs. Compared to

RDC11, RDC34 is also more toxic when given to mice [30]. However, at the maximum doses free of host toxicity, RDC34 significantly reduces tumor growth in two models. The increased toxicity of RDC34 could be related to its high lipophilicity [40], a property that might favor RDC34 cell uptake and distribution in the body after dosing. Although the addition of a second phenanthroline significantly increased the cytotoxicity, the modification of the phenylpyridine by addition of a NO₂, NH₂ or spermine moiety modify also significantly the cytotoxicity of the RDCs, highlighting

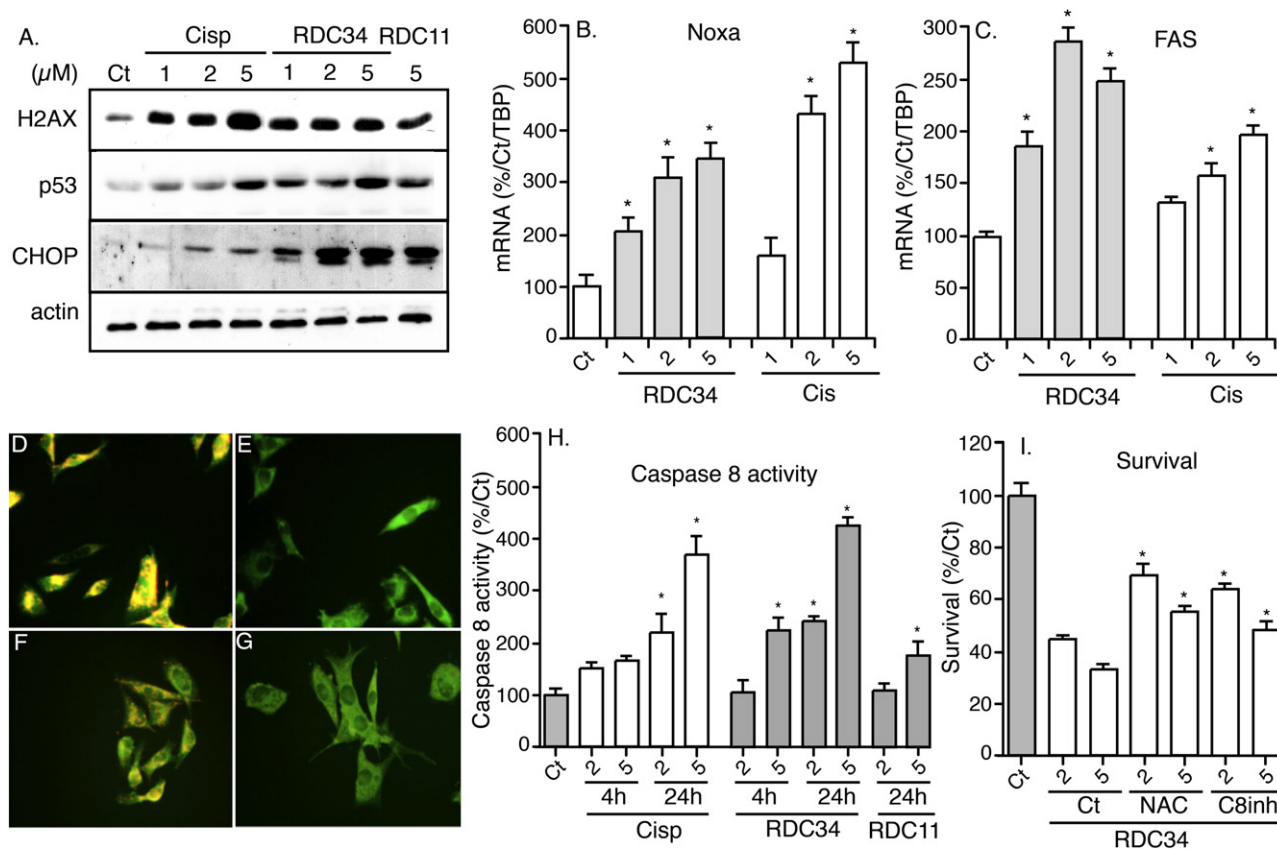


Fig. 5. Optimized RDCs induce multiple signaling pathways, including ROS and caspase 8. (A) Western blot analysis of cells treated for 24 h. Immunoblotting were performed with anti-p53, anti-actin, anti-phospho-H2AX, and anti-CHOP antibodies. Data are representative of 3 independent experiments. (B and C) HCT116 cells were treated with cisplatin, or RDC34 (in μM) for 24 h. RT-qPCR were performed using primers for noxa, and fas. Data are represented as fold inductions relative to untreated cells (Ct) and were normalized with both 18s and TBP levels. (D–G) Cells were incubated with the membrane potential sensitive dye JC-1. We used FCCP as positive control (E). Intracellular distribution of the dye was assessed by confocal microscopy at 488 nm excitation/510 nm emission. (H) Cells were incubated for the indicated time and concentration of drugs (in μM). (I) HCT116 were treated with the combination of drugs (NAC, 10 μM ; caspase 8 inhibitor, C8inh 10 μM) added to RDC34 at the indicated concentrations. Survival was tested 48 h after using MTT tests.

the functional interaction occurring between the ligands and their interdependence to produce a specific biological effect.

By adding the spermine chain (RDC44), the NH_2 function (RDC41), and in a lesser extend the NO_2 (RDC40) function, we purposely improve the water solubility of RDC34 as indicated by their LogP, which is 2.35 for RDC34, 2.05 for RDC40, 0.9 for RDC41 and inferior to 0 for RDC44. RDC44 is freely soluble in water up to a concentration of 25 mM, simplifying its in vivo dosing of the experimental animals. However, as observed with RDC44 and in a lesser extent with RDC41 and RDC40, increasing the water solubility decreased the cytotoxicity. Similar observation has been made previously for the ruthenium compound KP1019 and its water-soluble version KP1339 [42]. Although the low cytotoxicity of RDC44 was disappointing, the in vivo studies revealed that RDC44 displayed similar anticancer properties compared to RDC34. A possible explanation could be that the spermine moiety confers a favorable pharmacokinetics of distribution, or alternatively that the spermine is somehow removed after its injection in mice, suggesting that RDC44 is a water-soluble pro-drug of RDC34.

The lower cytotoxicity of RDC44 could be due to a diminished cellular uptake through the lipophilic membrane barrier. Indeed, in standard medium, RDC44 showed a diminished cellular accumulation. However, under PBS conditions both compounds (RDC34, RDC44) can enter the cells and the water-soluble compound RDC44 is still barely able to affect cell survival compared to the lipophilic RDC34. Moreover, RDC44 is still able to interact with DNA with an affinity similar to RDC34. This lack of correlation

between the cytotoxicity and the ability to interact with DNA is also supported by the results obtained with RDC40 and RDC41, which display a better affinity to DNA than RDC34, although they show a weaker cytotoxicity. Therefore, our data suggest that the improvement of RDC water solubility does not affect significantly RDC uptake and RDC-DNA interaction. We can therefore hypothesize that besides DNA, RDCs recruit additional direct intracellular targets that can account for a variation in their biological function depending on RDCs lipophilicity.

In favor of this hypothesis is the change of selectivity towards cancer cells of different origins observed in RDCs with variation in their ligands. Indeed, by modifying the ligand around the ruthenium center, we have observed that RDC34 displayed a strong cytotoxicity toward a broad range of cancer cell lines. RDC34 is particularly efficient on cancer cell lines of kidney origin that are poorly affected by RDC11. The introduction of the NH_2 function diminished the cytotoxicity of RDC41 at all levels comparable to that of RDC11. However, few exceptions exist in which RDC41 is more active than RDC11, such as EKVX cells, MDA-MB-435, UACC-257 and HS57BT. It is likely that the ability of the various RDCs to interact with cellular DNA does not explain their selectivity for different cell lines but rather their ability to interact with different intracellular targets and to induce different stress signaling.

As we have published for RDC11, the new generation of improved RDCs triggers at least two mechanisms: an interaction with DNA and an induction of CHOP expression. The interaction

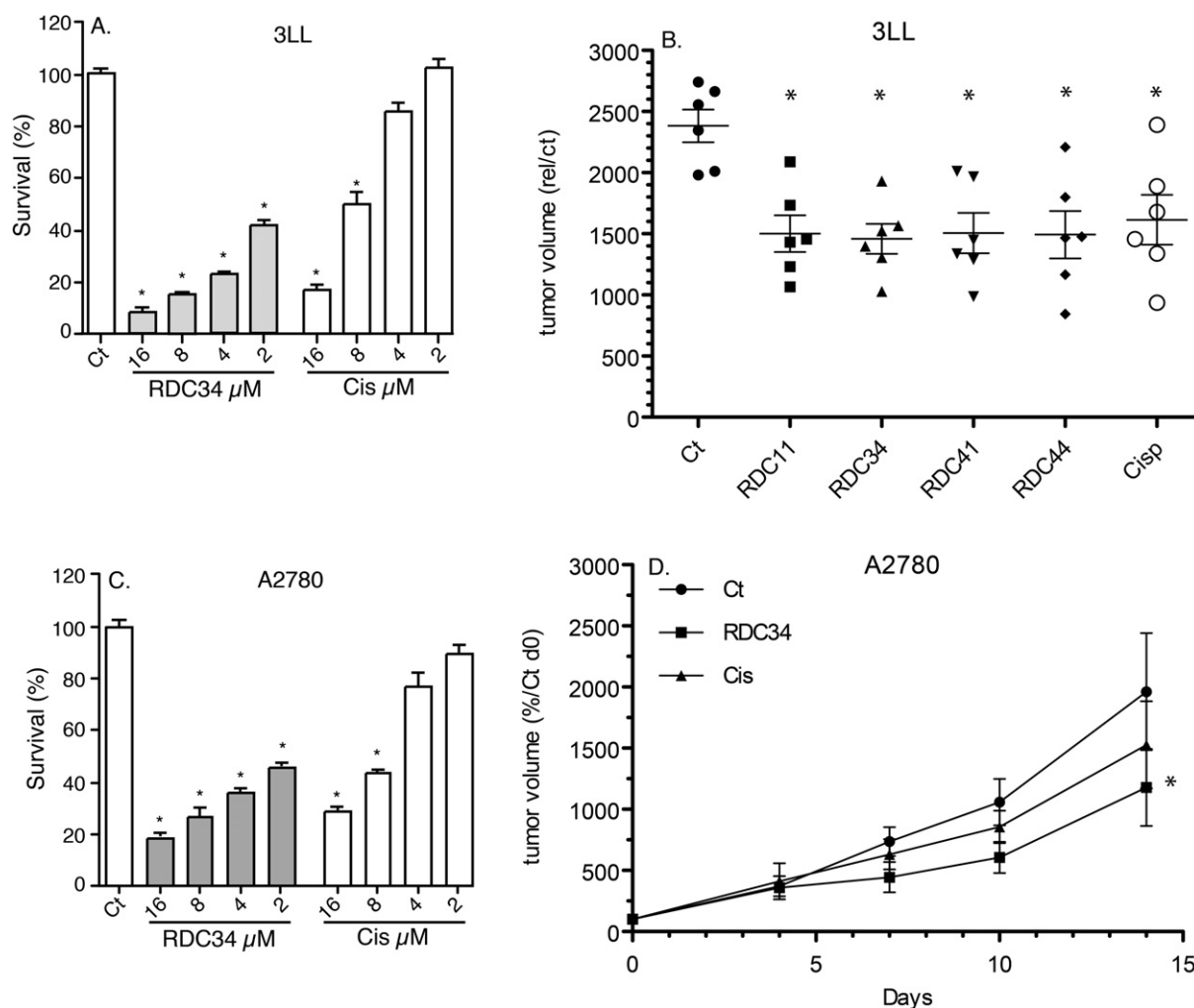


Fig. 6. Anticancer activity of optimized RDC. 3LL cells (A) or A2780 cells (C) were treated for 48 h with cisplatin or RDC (in μM). Viability was evaluated using a MTT test. (B) C57BL/6 mice (8-weeks old) were injected subcutaneously with 5×10^5 3LL cells. Injections of RDC (RDC34 and RDC44 at $4 \mu\text{mol/kg}$; RDC41, RDC11 at $13.3 \mu\text{mol/kg}$) or cisplatin ($13.3 \mu\text{mol/kg}$) started when tumors were palpable ($\pm 80 \text{ mm}^3$) and were performed twice a week. Solutions were prepared in PBS/5% Cremophore. (D) Nude mice (Swiss nu/nu, 8-weeks old) were injected subcutaneously with 5×10^6 A2780 cells. Injections of RDC34 ($4 \mu\text{mol/kg}$) or cisplatin ($13.3 \mu\text{mol/kg}$) were performed twice a week when tumors were palpable. Graph represents tumor volumes. Data are representative of two independent experiments ($n = 7$). Asterisks indicate statistically significant difference ($p < 0.01$) compared to control, as calculated by a one-way ANOVA test followed by a Tukey test.

with DNA does not seem to play a major role in their cytotoxicity, as there is not a good correlation between the cytotoxicity and their ability to interact with DNA. Interestingly RDC34 is a more potent inducer of the p53 protein levels and the p53 target genes compared to RDC11 even though its interaction with DNA is similar to RDC11. This stronger effect might involve the higher ability of RDC34 to induce ROS production, as ROS can induce p53. The role of ROS in the cytotoxicity of ruthenium compounds has been previously suggested [27,43] and is demonstrated by our present study as NAC significantly reduces RDC34 cytotoxicity. However, the correlation between production of ROS and RDC cytotoxicity is only partial, as there is no difference in ROS production between RDC40 and RDC11 even though RDC40 is more cytotoxic than RDC11. Previous studies indicated that RDCs modulate the activity of oxido-reductase enzymes [44], suggesting that the production of ROS might be triggered by alterations of the activity of enzymes committed to produce or remove ROS, although these targeted enzymes remain to be identified yet.

Anyhow, RDC34 is a strong inducer of p53 and p53 target genes connected to the extrinsic pro-apoptotic pathways involving caspase 8. The role of caspase 8 seems crucial to the pathway

leading to cell death after treatment with RDC34 since its inhibition reduced these cytotoxic effects.

Overall this study demonstrates the crucial role of modified ligand around the ruthenium center to optimize the molecules in order to improve the cytotoxicity and target cancers subtypes. The variation in cytotoxicity between the compounds tested in the present study might involve several factors: (1) the redox potential, (2) the lipophilic status, and (3) the geometry. These factors might influence the ability of the compound to enter the cells, interact physically with intracellular targets and modify their functions. These factors also certainly affect the pharmacokinetic and the tissues distribution of the compounds in vivo and might explain the differences in toxicity. Moreover, this study shows the possibility of producing a RDC pro-drugs that present fair water solubility without affecting significantly the in vivo anticancer activity of the pharmacophore.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bcp.2012.08.022>.

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