Ruthenium (II)-Derived Organometallic Compounds Induce Cytostatic and Cytotoxic Effects on Mammalian Cancer Cell Lines through p53-Dependent and p53-Independent Mechanisms

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ABSTRACT

The metallic compound cisplatin has been used for many years to treat various human cancers. Here, we describe the cytostatic and cytotoxic properties of a new class of organometallic compounds that contain a ruthenium (II) atom covalently linked to carbon and nitrogen atoms. We found that several ruthenium-derived compounds (RDCs) led to G1 arrest and induced apoptosis in tumor cell lines derived from glioblastomas, neuroblastomas, and lymphoid tumors at least as efficiently as cisplatin. We further analyzed the signaling pathways underlying these effects, and we showed that both RDCs and cisplatin induced p53 and p73 protein levels but with different intensities and kinetics. This accumulation of p53 and p73 proteins correlated with an increase in p21 and Bax expression, two p53 target genes linked to cell growth arrest and apoptosis. However, in contrast to cisplatin-induced apoptosis, overexpression of ΔNp73, a p53 and p73 dominant-negative isoform, only partly reduced RDC-induced apoptosis, suggesting p53-dependent and p53-independent modes of action. This observation was further confirmed by the ability of RDC to induce apoptosis in p53−/− cells. Altogether, this study highlights key cellular and molecular features of RDCs and suggests that further development of this new class of compounds may contribute to improve future chemotherapeutic protocols.

Cisplatin is one of the three most widely used antitumor drugs in the world. Cisplatin is particularly effective against ovarian and testicular cancers and contributes to the treatment of many other cancers. Cisplatin induces formation of intrastrand DNA adducts, primarily ascribed to its interaction with nucleophilic N7 sites of purine bases in DNA (Eastman, 1987). Formation of these DNA adducts correlates with cytotoxicity (Fraval and Roberts, 1979). Indeed, subsequent to DNA damage, stimulation of multiple damage recognition and repair mechanisms (hMSH2) leads to the activation of the p53 family of transcription factors (namely, p53 and p73 isoforms), which mediate cell growth arrest and apoptosis (Prives and Hall, 1999; White and Prives, 1999).

One of the upstream events linking p53 and p73 to DNA damage is the activation of kinases such as ATM, ATR, Chk1 and 2, or c-Abl. Cisplatin preferentially activates ATR (ATM and Rad-3-related kinase) (Damia et al., 2001), which can directly activate p53 by phosphorylating it at serine 15 or activate other kinases such as Chk-1, which results in the phosphorylation of p53 at serine 20 (Shieh et al., 2000; Zhao and Piwnica-Worms, 2001). Chk-1 also phosphorylates p73 at position 49 and induces its activity (Gonzalez et al., 2003). However, the most accepted pathway that leads to p73 activation after DNA damages involves tyrosine phosphorylation of p73 by c-Abl (Agami et al., 1999; Gong et al., 1999; Yuan et al., 1999). Once activated, p53 and p73 stimulate the transcription of several genes involved in cell growth arrest (p21 and p57), DNA repair (GADD45a), or apoptosis (Bax). The

ABBREVIATIONS: ATM, ataxia telangiectasia, mutated; ATR, ataxia telangiectasia, mutated and Rad-3-related; RDC, ruthenium-derived compound; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; FACS, fluorescence-activated cell sorting.
regulation of these genes accounts for the p53-dependent cell growth arrest and apoptosis induced by cisplatin treatment. Various anticancer drugs use these pathways that are initiated by DNA damages and lead to cell growth arrest or apoptosis through activation of kinases and the p53 family. However, depending on the drug, there exists several differences in which pathways are activated and the kinetics observed.

Despite its major contribution to actual anticancer therapies, cisplatin presents two disadvantages: 1) severe toxicity such as nephrotoxicity, neurotoxicity, and emetogenesis; and 2) limited applicability to a narrow range of tumors. Indeed, some tumors exhibit natural resistance, whereas others develop resistance after initial treatment (Wong and Giandomenico, 1999). One mechanism of resistance is the deletion or inactivating mutation of antiproliferative or proapoptotic genes such as p53 (El-Deiry, 2003). For p53, some of the mutants are able to abrogate the functions of other p53 family members (Marin et al., 2000; Gaiddon et al., 2001). In addition, the onset of resistance creates a further therapeutic complication because tumors failing to respond to cisplatin exhibit cross-resistance to diverse unrelated antitumor drugs (Ozols, 1992). Therefore, efficient therapy that circumvents resistance should be based on a combination of antitumor drugs, which would stimulate multiple and various DNA damage signaling pathways.

To improve anticancer therapies, new platinum-based but also nonplatinum antitumor drugs such as metalloccenes, titan IV complexes, gold I complexes, and gallium III salts (Clarke et al., 1999; Guo and Sadler, 1999) are under development. In this respect, ruthenium-based agents appear especially attractive because they exhibit three important properties suitable to biological applications: 1) these metal complexes have similar ligand exchange kinetics to those of platinum II complexes; 2) different oxidation states are accessible under physiological conditions; and 3) ruthenium mimics iron in binding to albumin and transferrin, two iron-carriers that reduce toxicity of ruthenium (Allardyce and Dyson, 2001; Clarke, 2003). Several ruthenium (II) compounds have already been described for their antitumoral activity (Morris et al., 2001; Aird et al., 2002). However, these compounds displayed an activity 10 times lower than cisplatin in vitro (Morris et al., 2001). Furthermore, the molecular mechanisms and the signaling pathways activated by ruthenium-derived molecules were not examined.

Based on these observations, we decided to test several cycloruthenated arene complexes (Fernandez et al., 1999) that present structural analogy with recently published antitumour ruthenium II arene complexes. We tested the biological activity of these molecules on various cancer cell lines and compared their effects with cisplatin. We found that several ruthenium-derived compounds efficiently induced cell growth arrest and apoptosis. We further characterized the contribution of the p53 signaling pathway into the biological activity of ruthenium-derived compounds.

**Materials and Methods**

**Chemicals.** The dicationic compound RDC-2 (Bennett and Smith, 1974), the chloro complex RDC-3 (Abbenhuis et al., 1993), and the monocationic complexes RDC-8 and RDC-10 (Fernandez, 1999) have been prepared according to published methods. RDC-13 was a gift from Dr. R. Le Lagadec (Instituto de Quimica, Universidad Nacional Autonoma de Mexico, Ciudad Universitaria, Mexico City, Mexico). RDC-6 and RDC-9 were obtained by phosphane exchange starting, respectively, from RDC-3 and RDC-8. RDC-11 and RDC-12 were prepared from RDC-8 by diimine exchange.

**Cell Culture.** A172, HS683, N2A, SHY55, HCT116, TK6 (p53+/+), and 293 cells were obtained from American Type Culture Collection (Manassas, VA). NH32 cells, the TK6 p53 knockout derivatives, were generously provided by Dr. H. Liber (University of Washington, Seattle, WA). RDM4 was obtained from Dr. D. Oth (Institut Curie, Frappier, Laval-des-Rapides, QC, Canada). Dr. Stephen B. Howell (Department of Medicine, University of California, San Diego, La Jolla, CA) kindly provided the 2008 stable cell lines (control and ATTP7B). RDM4, TK6, and NH32 cells were maintained in RPMI 1640 Glutamax medium (Invitrogen, Cergy-Pontoise, France) supplemented with 1 mM sodium pyruvate, 1 µM nonessential amino acids, and 50 µM gentamicin. The other cell lines were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, and all cell lines were incubated in presence of 5% CO2/95% air at 37°C.

**Preparation of Whole Cell Extracts and Immunoblotting Analysis.** Cells were grown in six-well plates, and for each condition, two wells were treated. Cells were lysed in 150 µl of lysis buffer [TEGN; 20 mM Tris-HCl (pH 8), 1 mM EDTA, 0.5% NP40, 150 mM NaCl, 1 mM dithiothreitol, 10% glycerol, protease inhibitors (Sigma-Aldrich, St. Louis, MO)], and the extracts from two wells were mixed and centrifuged at 13,000 rpm for 12 min. Protein concentrations were determined using a colorimetric assay (Bio-Rad, Hercules, CA). Sample buffer (Silhavy et al., 1984) was added to 75 µg of proteins, and samples were heated to 95°C for 3 min followed by electrophoresis through a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (Whatman Schleicher and Schuell, Keene, NH). For p20 (the active fragment of caspase 3) detection, a polyclonal antibody (R&D Systems, Minneapolis, MN) was used at 1/1000. Histone H3 phosphorylation at serine 10 was observed using a phospho-specific antibody (1/2000; Upstate Biotechnology, Lake Placid, NY). p53, p73, p21, Bax, and actin were detected, respectively, anti-p53 (pAb 1801), anti-p73 (Ab2; Oncogene Science, Cambridge, MA), anti-p21 (Oncogene), anti-Bax (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-actin antibodies (Dr. D. Aunis, U575, INSERM, Strasbourg, France). Proteins were visualized by enhanced chemiluminescence detection.

**Cell Proliferation Assays.** Cells were grown in 96-well plates and treated at 30% confluence. After 48 h, medium was removed, and a mixture of Dulbecco’s modified Eagle’s medium with MTT (0.5 mg/ml) was added for 1 h. Then, medium was removed, and a solution of 0.04% HCl in isopropanol was added. Differences in coloration were quantified by an enzyme-linked immunosorbent assay plate reader (Mettechrif Inc., Taipei, Taiwan) at 490 to 650 nm.

**Immunocytochemistry.** A172 cells were grown on cover slips until 50% confluence. After 24 h of treatment, cells were fixed in platelet-activating factor (4%), permeabilized in PBS containing 0.1% Triton, and blocked in PBS containing 3% BSA for 1 h. Cells were then incubated overnight in PBS containing 3% BSA and an anti-p20 antibody (1/1000; R&D Systems). After three washes with PBS, cells were incubated for 1 h in PBS containing 3% BSA and an anti-rabbit secondary antibody link to Cy3 (1/2000; Jackson Immunoresearch Laboratories Inc., West Grove, PA). After two washes with PBS, cells were incubated 10 min in PBS containing the Hoechst colorant (1 µg/ml; Calbiochem, San Diego, CA). After one wash in PBS, cover slips were mounted in Moviol and observed under a fluorescent microscope (Nikon, Melville, NY) linked to a digital camera (Bio-Rad).

**Cell Cycle Analysis and Apoptosis Assays.** Hypodiploid DNA was measured as described according to Nicoletti et al. (1991). Briefly, 10^6 cells were centrifuged and fixed in 1 ml of ice-cold 70% ethanol at 4°C for 1 h, washed once in PBS, 2 mM EDTA, and resuspended in 1 ml of PBS containing 0.25 mg of RNase A, 2 mM H9262/H11001/
EDTA, and 0.1 mg of propidium iodide. After incubation at 37°C for 30 min, cells were analyzed. The fluorescence of 10,000 cells was analyzed using a FACScan flow cytometer and CellQuest software (BD Biosciences, San Jose, CA).

Phosphatidylserine externalization was measured with the Annexin-V-FLUOS staining kit (BD Biosciences Clontech, Palo Alto, CA). Cells were washed in RPMI 1640, and for each template, 10⁶ cells were incubated in 100 μl of reaction buffer (100 μl of HEPES buffer and 2 μl of Annexin-V-FLUOS; Roche Diagnostics, Indianapolis, IN). Templates were incubated 15 min in the dark at room temperature. Next, they were analyzed with a flow cytometer FACScan, and data were measured with the CellQuest software (BD Biosciences).

Results

Ruthenium-Derived Compounds Inhibit Cell Growth in Vitro. To evaluate the antitumor potential of the various RDCs (represented in Fig. 1A), we first analyzed their effect on cell proliferation. A172 cells, derived from a glioblastoma, were treated with different doses of each RDC or cisplatin, and the number of cells was determined after 48 h by measuring a specific cellular enzymatic activity. We observed that several RDCs (6, 9, 11, and 12) exhibited in vitro antiproliferative activity on A172 cells, whereas others (i.e., RDC-2) did not have a significant effect (Fig. 1, B–D). Interestingly, the four most effective RDCs had an antiproliferative effect equivalent to cisplatin or even higher. We next determined the effect of RDCs on other cell lines derived from glioblastoma (HS683), neuroblastoma (N2A, SH5Y), lymphoblastoma (RDM4, TK6), and adenocarcinoma (HCT116) and a commonly used cell line (293) and evaluated an approximate IC₅₀ value. Equivalent results were obtained with these cell lines and were summarized in Table 1. The only exception was the RDM4 cells, which were particularly less sensitive compared with the other cell lines tested. Based on these results, we decided to focus our study on RDC-9, one of the most efficient RDCs.

Ruthenium-Derived Compounds Induce G1 Arrest. To understand how RDCs affect cell growth, we directly examined their effect on cell cycle by FACS analysis. Figure 2 shows the results of A172 (Fig. 2A) and RDM4 cells (Fig. 2B) treated with different doses of cisplatin, RDC-9, or RDC-11, stained with propidium iodide, and analyzed by flow cytometry. In A172 cells (Fig. 2A) at 5 μM, cisplatin and RDC-9 induced an increase of the cells in G0/G1 and in sub-G₁. At 15 μM, RDC-9 induced a further increase in the percentage of cells in sub-G₁, which was 15% at 24 h and was up to 35% at 72 h. As observed in Fig. 1, A172 cells and RDM4 cells do not have the same sensitivity toward RDCs. Indeed, RDM4 cells are more resistant than A172 cells, with an IC₅₀ value 3 times higher. Therefore, we used different doses of RDCs to treat RDM4 cells (15 and 45 μM). Treatment with 15 μM of both RDCs led to a marked increase in the number of cells in G0/G1 in RDM4. On the other hand, treatment with 45 μM of both RDCs led to the formation of hypodiploid particles that created an important sub-G₁ phase. The number of cells accumulated in the sub-G₁ phase was lower than 10% at 24 h but exceeded 50% at 48 h and reached 60% at 72 h. These results showed that RDCs are able to both induce a G₁ cell cycle arrest and DNA fragmentation, which is characteristic of apoptosis.

Ruthenium-Derived Compounds Induce Apoptosis. To further analyze the characteristics of the death induced by
TABLE 1
An approximation of the IC50 values found for various cell lines.
Experiments have been done in the same conditions as in Fig. 1. Each IC50 value was evaluated on four independent experiments.

<table>
<thead>
<tr>
<th>A172</th>
<th>HS683</th>
<th>N2A</th>
<th>SH5Y</th>
<th>HCT116</th>
<th>293</th>
<th>RDM4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisp</td>
<td>3.9 ± 0.2</td>
<td>4.1 ± 0.1</td>
<td>3.3 ± 0.2</td>
<td>5.4 ± 1.1</td>
<td>1-5</td>
<td>1-5</td>
</tr>
<tr>
<td>RDC-2</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>RDC-3</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>RDC-6</td>
<td>4.8 ± 0.2</td>
<td>3.2 ± 0.3</td>
<td>1-5</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>RDC-8</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>RDC-9</td>
<td>1.7 ± 0.3</td>
<td>2.3 ± 0.2</td>
<td>1-5</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>RDC-10</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>RDC-11</td>
<td>1.9 ± 0.2</td>
<td>3.1 ± 0.3</td>
<td>1-5</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>RDC-12</td>
<td>12 ± 0.2</td>
<td>9.8 ± 1.1</td>
<td>15.1 ± 0.8</td>
<td>7.9 ± 0.2</td>
<td>5-15</td>
<td>5-15</td>
</tr>
</tbody>
</table>

Cisp, cisplatin.

RDCs, we performed immunocytochemistry experiments to assess the status of the nucleus and the possible activation of caspases, such as caspase 3, which are proteases induced by and involved in the apoptotic process (Hengartner, 2000). A172 cells were treated with RDCs for 24 or 48 h. After fixation, cells were labeled with an antibody that recognizes the active fragment of caspase 3 (p20, in red, Fig. 3A). The nuclei were stained by the Hoechst colorant (in blue, Fig. 3A). Cells treated with cisplatin showed nuclei alterations, such as fragmentation or condensation, and a strong increase in p20 labeling (Fig. 3A). Multiple examples of cells with fragmented or condensed nuclei and also stained by the anti-p20 antibody were observed. A quantification of the cells with nuclei fragmented or condensed showed that the most active RDCs induced a similar effect (Fig. 3B). Equivalent observations were realized with cells treated with RDC-9, RDC-6, and RDC-11 (Fig. 3, A and B). In contrast, RDC-2 and RDC-3, which had no effect on the MTT test, failed also to induce nuclear fragmentation and caspase 3. We also examined p20 production by Western blot and confirmed that RDC-9 and -6, but not RDC-2, were able to induce p20 as efficiently as cisplatin (Fig. 3C). The blot was reprobed with anti-actin antibody for controlling the loading of protein. These results showed that RDCs induced caspase 3 activity.

Cell death was further characterized by quantification of anionic phospholipids externalization, which is an earlier apoptosis marker. RDM4 cells were stained with annexin V after a treatment of 24, 48, and 72 h with RDC-9 and RDC-11. Figure 4 shows that a treatment with 15 μM of either RDCs did not induce apoptosis. Indeed, even after 72 h of treatment, the apoptosis rate was still as low as in control cells. In contrast, with 45 μM of either RDCs, this rate was close to 70% at 24 h and exceeded 99% after 48 h, indicating that all cells have undergone apoptosis. Taken together, these results clearly showed that RDCs are able to induce apoptosis in several tumor cell lines.

Ruthenium-Derived Compounds Induce p53 and p73 Proteins. Since we had demonstrated that RDCs induced cell growth arrest and apoptosis, we investigated the molecular mechanisms that may account for these cellular processes. p53 and p73 activities have been reported to be induced by several anticancer drugs (Prives and Hall, 1999). Therefore, we examined the effect of RDCs on p53 and p73 protein levels in A172 cells. We treated the cells at a dose close to the IC50 (2 μM) and a dose 5 times higher (10 μM) that induces moderately apoptosis in A172 cells. We observed that p53 and p73 protein levels were already higher after 6 h of cisplatin treatment and increased further after 24 h of treatment (Fig. 5A). Interestingly, p53 and p73 protein levels were strongly induced by RDC-9 after 6 h of treatment in A172 cells but not anymore at 24 h of treatment. The lack of induction after 24 h was not due to a decrease of cell quantity,
as actin protein levels remained unchanged under these conditions. Note also that even with a lower dose of RDC-9, we did not see any increase of p53 protein level at 24 h. Thus,

Fig. 3. Ruthenium-derived compounds induce apoptosis. A, A172 cells were grown on cover slips. At 50% confluence, cells were treated for 24 h with cisplatin (15 μM), RDC-2 (25 μM), or RDC-6 (15 μM). Cells were fixed, permeabilized, labeled with an anti-caspase 3 active fragment antibody (p20, in red), and the nuclei were stained with Hoechst (in blue). B, the graphic represents a quantification of fragmented or condensed nuclei observed as indicated in A. Bars are means of duplicates from one experiment out of three independent experiments. C, in parallel, protein extracts were prepared from A172 cells treated with the indicated drugs. Proteins were separated on a 10% SDS-PAGE gel, transferred onto nitrocellulose, and immunoblotted with the anti-caspase 3 active fragment antibody. The blot was reprobed to detect actin as a control for protein loading.

Fig. 4. Ruthenium-derived compounds induce phosphatidylserine externalization. Apoptosis measurement by annexin V staining on RDM4 cells treated with complexes 9 and 11 at 15 and 45 μM. Phosphatidylserine externalization is measured after treatment with ruthenium complexes. Apoptosis is expressed by the rate of annexin V-positive cells.
although the accumulation of p53 and p73 proteins by the two drugs was equivalent in intensities, there was a clear difference in kinetics.

To confirm whether RDCs treatment leads to p53 and p73 activation, we analyzed the expression of two p53 target genes, p21 and Bax, that can account for cell growth arrest and apoptosis (Xiong et al., 1993; Brady and Gil-Gomez, 1998). As previously described, p21 and Bax were induced by cisplatin treatment (Fan et al., 1994; Simonian et al., 1996). RDC-9 and -11 (24-h treatment) also induced Bax and p21, whereas RDC-2 did not have any significant effect (Fig. 5B).

We also verified whether these observations could be extended to cell lines derived from other origins. Upon treatment with cisplatin and RDC-9, we also observed an induction of p53 in HCT116 cells (Fig. 5C). Indeed, RDCs and cisplatin induced p53 protein levels at 6 and 24 h. However, in HCT116 cells, cisplatin was more potent to induce p53 protein levels than RDC, especially at 6 h. Interestingly, the two drugs induced a similar amount of p21 proteins and decreased similarly the phosphorylation of histone 3 (a marker of cell proliferation).

These results showed that the most active RDCs regulate and activate p53 and p73 proteins. However, we also observed several differences when compared with cisplatin suggesting that the mode of action of RDCs might be in part different from cisplatin.

**Ruthenium-Derived Compounds Induce Apoptosis through p53-Dependent and p53-Independent Mechanisms.** The induction of several p53 family members by RDCs suggested that these proteins might mediate the pro-apoptotic activity of RDCs, as previously described for cisplatin. We therefore overexpressed in A172 cells a dominant-negative isoform of p73, ΔNp73, which is able to inhibit p53 and p73 activity, and examined their response to cisplatin and RDCs treatments (Pozniak et al., 2002). Cells were also transfected with a p53 dominant-negative isoform, p53DD, which is a truncated variant of p53 that contains only the C terminus part and the oligomerization domain. To identify the ΔNp73- or p53DD-expressing cells, we cotransfected them with a GFP expression vector (Fig. 6, A and B). The apoptotic cells were characterized by the fragmentation or the condensation of their nuclei visualized by Hoechst staining. Most of the nontreated cells overexpressing ΔNp73 or control cells exhibited a normal, round and homogenous, light blue nucleus (Fig. 6A). When control cells were treated with RDCs or cisplatin, a significant proportion (10–15%) harbored a condensed or a fragmented nucleus (Fig. 6, B and C). Interestingly, ΔNp73 or p53DD overexpression only partly reduced the number of apoptotic nuclei observed after RDCs treatment and this, in striking contrast to cisplatin treatment where ΔNp73 or p53DD expression, almost completely abolished the apoptotic process (Fig. 6C).

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**Fig. 5.** Ruthenium-derived compounds induce p53 and p73 protein levels. A, A172 cells were treated at the indicated concentration (2 and 10 μM) of the drugs. Cells were lysed 6 or 24 h after treatments. Proteins were separated on a 10% SDS-PAGE and immunoblotted with anti-p53, anti-p73, or anti-actin antibodies. B, cells were lysed 24 h after treatments. Proteins were separated on a 10% SDS-PAGE and immunoblotted with anti-p21, anti-Bax, or anti-actin antibodies. C, ruthenium-derived compounds induce p53 in HCT116 cells. HCT116 cells were treated with cisplatin and RDC-9. Cells were lysed 6 or 24 h after treatments. Proteins were separated on a 10% SDS-PAGE and immunoblotted with anti-p53, anti-p21, anti-phospho S10 histone H3, or anti-actin antibodies.
Ruthenium-Derived Compounds Are Less Sensitive to ATP7B Expression Than Cisplatin. An important drawback of anticancer therapies is the development of a cellular resistance. One of the mechanisms of resistance against cisplatin has been identified. It involves the overexpression of ATP7B, a membrane transporter that can expulse cisplatin and its derivatives (carboplatin) out of the cell (Komatsu et al., 2000). To assess whether RDCs could be more advantageous than cisplatin, we tested the efficacy of RDCs on 2008 cells stably overexpressing ATP7B and compared the response with parental cells that do not overexpress it (Katano et al., 2003). The two 2008 cell lines were treated with increasing concentrations of cisplatin or RDC-9, and the number of cells was determined by a MTT test. As previously described, cells overexpressing ATP7B were less sensitive to cisplatin than control parental cells. In contrast, cells overexpressing ATP7B remained as sensitive as control parental cells to RDC-9 at an equivalent dose (Fig. 6A). The fact that cells developing any of the two resistance mechanisms against chemotherapies (p53 inactivation or ATP7B overexpression) were still sensitive toward RDCs suggested that RDCs might use different signaling pathways to induce apoptosis than cisplatin.

Discussion

The discovery of new anticancer drugs, which would be more efficient, with less toxicity and less sensitivity to resistance mechanisms, remains a fundamental challenge. One of the objectives of the pharmaceutical industry is the identification of new platinum drugs, presenting no cross-resistance with cisplatin and targeting a larger clinical spectrum in chemotherapy that cisplatin. However, in spite of the screening of hundreds of compounds, only two major cisplatin derivatives, carboplatin and oxaliplatin, are today clinically available as anticancer agents, even if they do cross-desensitize. Ruthenium-based compounds have been recently explored for their cytotoxic effects, and one of them is used in clinical trial to cure cancer. The molecular basis of their effects is still almost completely unknown.

In the search of better anticancer compounds, we tested the cytostatic and the cytotoxic effects of a new class of organometallic ruthenium (II)-based compounds. We found that several of these RDCs matched or even exceeded cisplatin efficiency in cell growth inhibition and apoptosis induction. Importantly, RDCs might have an advantage over cisplatin, as they are less sensitive to some resistance mechanisms developed by cancer cells. We further identified several molecular mechanisms induced by RDCs, such as activation of p53 and p73, expression of p21 and Bax, induction of caspase-3, and nuclear fragmentation. Hence, our study represents the first thorough analysis of the cellular and molecular effects induced by RDCs and fully supports...
further development of this promising new class of compounds.

**Ruthenium-Derived Compounds Inhibit Cell Growth.**

Compound RDC-2 has been prepared as a reference arene ruthenium (II) complex: it presents an IC₅₀ higher than 50 μM. The chloro complex RDC-3 seemed particularly promising as it is isostructural to the published anticancer ruthenium (II) arene complexes (Morris et al., 2001; Aird et al., 2002), the only difference lying in the peculiar nature of the CN ligand. Surprisingly, RDC-3 had an IC₅₀ higher than 50 μM too (Fig. 1 and Table 1). At this stage, we formulated a different approach. Examples can be found in the literature of antitumoral cycloplatinated (Okada et al., 2001) or cyclopalladated (Rodrigues et al., 2003) compounds. In this respect, we tested cycloruthenated phenylpyridine RDC-8 and benzylpyridine RDC-10 with a lack of success (IC₅₀ > 50 μM). At the end, we came to the idea that it was necessary to mimic, in some manner, cisplatin itself. In consequence, we reduced the number of coordination sites on the metal by reaction with phosphane and diimine ligands to restore the two cis-exchangeable sites found in the platinum compound. This strategy paid as RDC-9, RDC-11, and RDC-12 inhibited significantly the proliferation of various cell lines. The efficiency of these complexes was at least equivalent to those of cisplatin tested in the same conditions, with an average IC₅₀ value of 5 μM. Among the cell lines tested, all responded similarly to RDCs and cisplatin, except RDM4 cells, which were much more less sensitive to cisplatin than RDCs.

**Ruthenium-Derived Compounds Induce G₁ Arrest.**

Anticancer drugs generally inhibit growth by blocking the cells in various phases of the cycle, G₁, S, or G₂, depending on the drugs and the cell type. The three RDCs (RDC-6, RDC-9, and RDC-11) that had the stronger effect on cell growth arrested the cells in G₁ phase in several cell lines tested, RDM4, TK6, and A172 (Fig. 2). We did not observe any significant block in other phases, but we cannot exclude that other cell lines would be differently affected. The ability of RDCs to arrest cells in G₁ is also supported by the induction of p21 protein levels, an inhibitor of the cell cycle that blocks CDK activity (Fig. 5B). We also observed a reduction of histone phosphorylation at serine 10, which is a marker of cell proliferation (Fig. 5C).

**Ruthenium-Derived Compounds Induce Apoptosis.**

Besides the block in G₁ phase, we showed that RDCs induce apoptosis in various cell lines, A172, TK6, and RDM4. Our demonstration that RDCs induce apoptosis is based both on morphological analysis (nuclear condensation and phosphatidyl serine inversion; Figs. 2, 3, and 4) and on the activation of several apoptosis effectors such as p53 and caspase 3 (Figs. 3 and 5). At a molecular level, RDCs stimulated p53 and Bax protein levels (Fig. 5B). p53, through Bax, has been extensively documented to induce apoptosis by the mitochondrial pathway (Xiong et al., 1993; Brady and Gil-Gomez, 1998). However, p53 activation has been also involved in other apoptotic pathways through up-regulation of Fas, the DR5 receptor, or even the reticulum stress pathway. The fact that there is up-regulation of Bax suggests that the mitochondrial pathway is induced by RDCs; however, we do not exclude that other pathways might be involved. In particular, the signaling pathway involving Fas and caspase 8 might be involved, as it has been described for cisplatin (Fulla et al., 1997).

**Ruthenium-Derived Compounds Cellular Effects Are p53-Dependent and p53-Independent.**

Our analysis of the signaling pathways that might be activated by RDCs showed that several members of the p53 family are induced. Indeed, both RDCs and cisplatin increased p53 and p73 protein levels (Fig. 5). However, the kinetics of p53 and p73 induction were different in A172 cells. In these cells, RDCs were quicker inducers of p53 protein levels than cisplatin, suggesting that the signaling pathways involved in the activation of p53 might be different. Early induction of p53 may also explain why RDCs cooperate better with other anticancer drugs that cisplatin if RDC induces early p53 signaling. Concomitantly to p53 and p73 accumulations, we observed an increase in p21 and Bax expression. Bax and p21 are two well-characterized targets of the p53 family members. This correlation between the increase in p53 proteins and the activation of their target genes suggests that RDCs activate p53 protein functions. Similar findings have been obtained for cisplatin (Prives and Hall, 1999). However, RDC-induced apoptosis was only partially dependent on p53 activity since inhibitors of p53 protein (ΔNp73 or p53DD) were not able to abolish this process (Fig. 6C). We cannot exclude that the expression of our dominant inhibitor was too low to be efficient. However, in the same conditions, the inhibitor was able to significantly reduce cisplatin-induced apoptosis. Furthermore, in a cell line where p53 had been deleted, RDCs were still able to induce cell growth arrest and apoptosis, strengthening the fact that RDCs may act through multiple pathways, dependent on p53 proteins and independent of p53 proteins. Our finding that RDCs exert their effect through alternate pathways different from p53 proteins is also supported by the cooperative activity of RDC with other anticancer treatments such as Taxol and ionizing radiation (data not shown). Such a cooperative effect unveils the existence of alternate pathways induced by the two treatments that converge to cancer cell growth inhibition.

DNA is the critical molecular target of alkylating agents. However, it must be kept in mind that only 1% of intracellular cisplatin reacts with nuclear DNA to form a variety of adducts (Gonzalez et al., 2001). Similarly, one may consider that, besides their binding to DNA, RDCs could also alter other intracellular structures. These damages may in turn contribute to the overall cytotoxicity of RDCs. However, whether the alternative platinum-binding sites are shared by RDCs or not is far from elucidated. We are currently prospecting the alternate pathways involved in RDCs biological effects. Since it has been described for other DNA damaging drugs, it might be possible that RDC activate Fas, caspase 8, or c-Jun NH₂-terminal kinase pathways (Fulda et al., 1997). Further studies on these proapoptotic mechanisms would bring new insights into the p53-independent pathway activated by RDC.

**Ruthenium-Derived Compounds Are Less Sensitive toward Resistance Mechanisms Than Cisplatin.**

One of the major drawbacks of anticancer drugs, including cisplatin, is the resistance developed by cancer cells to degrade or expulse the drugs or to inactivate several proapoptotic mechanisms. We found that RDCs are less sensitive to two resistance mechanisms developed by cancer cells. First, inactivation of p53 function does not reduce significantly RDC-induced apoptosis (Fig. 6, C and D). Second, overexpression of the ATP7B proteins, an event that accounts for cis-
Fig. 7. 2008 CMV and 2008 ATP7B cells were grown in 96-well plates. At 30% confluence, cells were treated for 48 h with cisplatin or various RDCs at the indicated concentrations (1, 5, 15, and 50 μM). The number of cells was assessed by a MTT test. Results are represented as percentage of the control. The graph shows means and standard deviation of eight wells from one representative experiment out of three independent experiments. The thick black line corresponds to the IC50, statistically significant differences tested by a one way analysis of variance followed by a Newman-Keuls test by pairs.

platin affects, less RDC biological activity (Fig. 7) (Komatsu et al., 2000). It is reasonable to think that other resistance mechanisms might reduce RDC efficiency. Indeed, long-term treatment (≥72 h) with submaximal doses of RDC-9, but not RDC-11, showed a reduction of the RDC-induced biological activities (Fig. 2; unpublished data). However, as RDCs are less sensitive to some of the resistance mechanisms affecting cisplatin activity, RDC may enlarge the panel of tools available to treat cancer.

Multiple aspects are now developed to increase our knowledge about RDCs and examine their potential uses in cancer treatments. First, we are testing in vitro the toxicity and the efficiency of the products that harbored interesting in vitro activity. We are also further investigating the molecular mechanisms involved in RDC biological activities. Another important aspect is the concomitant administration of radiation therapy and chemotherapy that represents a successful strategy to improve treatment outcome in some forms of cancer.

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References


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