Distinct Effects of Dinuclear Ruthenium(III) Complexes on Cell Proliferation and on Cell Cycle Regulation in Human and Murine Tumor Cell Lines

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ABSTRACT

We have examined the biological and antitumor activity of a series of dinuclear ruthenium complexes. The aim of this study was to compare the in vitro effects of these new compounds on cell proliferation, cell distribution among cell cycle phases, and the expression of some proteins involved in cell cycle regulation. Results obtained show a mild cytotoxic activity against human and murine cell lines, more evident after prolonged exposure of cell challenge. Two of the eight dinuclear complexes [namely, compounds D3 (Na2[RuCl4(dmso-S)(μ-py)] and D7 (NH4)[RuCl3(dmso-S)(μ-py)] modify cell cycle distribution similarly to imidazolium trans-imidazolylidemethylsulfoxidotetrachlororuthenate (NAMI-A), whereas the others have a low or negligible effect on this parameter. If we correlate the induction of cell cycle modifications with ruthenium uptake by tumor cells and with the modulation of proteins regulating cell cycle, we may stress that the induction of G2-M cell cycle arrest is related to the achievement of a threshold concentration of ruthenium inside the cells, which is dependent on the cell line being used, and that only cyclin B, among cell cycle regulating proteins examined by immunoblotting assays, appears to be significantly modified. This in vitro study shows that dinuclear ruthenium complexes may have a behavior similar to that of the monomer NAMI-A. These results encourage the future experimentation of their pharmacological properties in vivo models.

The study of the pharmacological properties of ruthenium compounds led to the identification of the potent antitumor activity of compounds with ammine, heterocyclic, and sulfoxide ligands (Keppler and Rupp, 1986; Keppler et al., 1987; Clarke et al., 1988; Clarke, 1989; Sava et al., 1992, 1998, 1999). Among these latter, NAMI-A (ImH[trans-RuCl4(dmso-S)Im]) and NAMI-A. These results encourage the future experimentation of their pharmacological properties in vivo models. Among platinum compounds, a significant therapeutic advancement is given by multinuclear compounds that highlight the possibility of overcoming the problem of resistance, since they increase the interchain DNA binding, which is more refractory to cell repair systems (Farrell et al., 1999).

Although the activity of NAMI-A and related compounds on DNA and/or other related molecules still has not been clarified, we thought it worthwhile to test the pharmacological properties of a new series of ruthenium complexes characterized by two ruthenium centers, i.e., dinuclear ruthenium compounds (see Fig. 1). Seven of the eight Ru(III)
species that we investigated are dianions of general formula $X_2[(RuCl_4(dmso-S))_2(μ-L)]$ ($X = Na$ or $NH_4$), in which $L$ is a bridging aromatic N-ligand; each half of these dinuclear species maintains essentially the same coordination environment that characterizes NAMI-A (i.e., four trans-chlorides, one S-bonded DMSO, and one heterocyclic N-ligand). We also investigated a mono-anionic unsymmetrical compound (D7) of formula $[NH_4][(RuCl_4(dmso-S))(μ-pyz)\{RuCl_3(dmso-S)(dmso-O)\}]$, which bears a neutral fragment. The bridging ligands, which may be either rigid or flexible, allowed us to vary parameters such as the relative disposition of the two Ru(III) centers, their distance, and the electronic conjugation between them. The study focuses on in vitro cytotoxicity in human and murine cell lines on ruthenium uptake by tumor cells and on cell cycle modification that results from flow cytometry analysis and Western blotting.

**Materials and Methods**

**Compounds and Treatment.** Dimeric ruthenium complexes named D1 ($Na_2[(RuCl_4(dmso-S))_2(μ-pyz)]$), D2 ($Na_2[(RuCl_4(dmso-S))_2(μ-pym)]$), D3 ($Na_2[(RuCl_4(dmso-S))(μ-bipy)]$), D4 ($Na_2[(RuCl_4(dmso-S))(μ-ethbipy)]$), D5 ($Na_2[(RuCl_4(dmso-S))(μ-ethylbipy)]$), D6 ($Na_2[(RuCl_4(dmso-S))(μ-probipy)]$), D7 ($[NH_4][(RuCl_4(dmso-S))(μ-pyz)\{RuCl_3(dmso-S)(dmso-O)\}]$), and D8 ($[NH_4][(RuCl_4(dmso-S))(μ-pyz)]$) were synthesized according to already reported procedures (Lengo et al., 1999; Serli et al., 2001).

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. For in vitro studies the compounds were dissolved in PBS or in complete medium with 5% fetal bovine serum and sterilized by filtration with a 0.2-μm filter. Compounds were tested at doses ranging from 1 to 100 μM.

**Tumor Cell Lines.** The B16-F10 murine melanoma cell line was obtained from the American Type Culture Collection (Manassas, VA; catalog number CR7964). Cells were cultured in minimal essential medium with Hanks’ salts (Euroclone Ltd. UK, Wetherby, Yorkshire, UK) adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 1 mM sodium pyruvate (Euroclone Ltd. UK), and 10% fetal bovine serum (FBS) (Invitrogen Italia, Milan, Italy), 2 mM l-glutamine (Euroclone Ltd. UK), 100 IU/ml penicillin, and 100 μg/ml streptomycin solution (Euroclone Ltd. UK), 1% nonessential amino acids (Euroclone Ltd. UK). The KB human oral carcinoma cell line was obtained from the European Collection of Animal Cell Cultures (Porton Down, UK; catalog number 86100304). Cells were cultured in minimal essential medium with Hanks’ salts (Euroclone Ltd. UK), adjusted to contain 1.5 g/l sodium bicarbonate, and 10% FBS (Invitrogen Italia), 2 mM l-glutamine (Euroclone Ltd. UK), 100 IU/ml penicillin, and 100 μg/ml streptomycin solution (Euroclone Ltd. UK), 1 mM sodium pyruvate (Euroclone Ltd. UK), 1% nonessential amino acids (Euroclone Ltd. UK), and 1 mM Heps solution.

**In Vitro Cytotoxicity Evaluation.** Cell growth was determined by the MTT assay (Mosmann, 1983). Cells were grown on 96-well plates (Corning Costar Italia, Milan, Italy) and, 24 h after sowing, were incubated for 24 or 72 h with concentrations from 1 to 100 μM of each compound dissolved in the appropriate medium containing 5% fetal bovine serum. Analyses of cell cytotoxicity were performed at the end of the incubation time. Briefly, MTT, dissolved in PBS at 5 mg/ml, was added (10 μl per 100 μl of medium) to all wells, and plates were incubated at 37°C for 4 h. At the end of incubation, the medium was discarded and 100 μl of acidified isopropanol (0.2 ml of 0.04 N HCl in 10 ml of isopropanol) was added to each well according to the modified protocol proposed by Galeano et al. (1992). Optical density was measured at 570 nm on a SpectraCount (PerkinElmer Life Sciences, Boston, MA).

**Cell Cycle Analysis.** Viable cells (0.5 × 10^6) of a single cell suspension were fixed in 70% ethanol at 4°C for at least 1 h. Before analysis, ethanol was removed by centrifugation, and cells were washed twice with PBS. Cells were resuspended in PBS containing 1 mg/ml RNase, kept at 37°C for 30 min, and further stained with propidium iodide (40 μg/ml) for at least 30 min at room temperature in the dark (modified from Crissman and Steinkamp, 1973). Red fluorescence (610 nm) was analyzed, using a peak fluorescence gate...
to discriminate aggregates. Each analysis consisted of 10,000 events counted. The flow cytometry analyses were done with an EPICS XL flow cytometer (Beckman Coulter, Inc., Miami, FL). Cell distribution among cell cycle phases was determined by analysis with Multicycle software (Phoenix Flow Systems Inc., San Diego, CA).

**Immunoblot Analysis.** PCNA, cdk1, cdk2, p27, and cyclin B were detected by Western blot analysis. SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970). Cells were harvested by scraping into ice-cold phosphate-buffered saline. Protein concentration was measured on an aliquot of cells by Bradford’s method (Bradford, 1976). Cells were then lysed by an SDS-containing buffer (125 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromphenol, 1.8% β-mercaptoethanol). Lysates were heated for 5 min in boiling water and then centrifuged for 10 min at 10,000g. For the detection of PCNA, cdk1, cdk2, and p27, a volume of lysate containing a quantity of protein between 25 and 40 μg was loaded onto Laemmli 15% polyacrylamide gels and electrophoresed; for the electrophoresis of cyclin B, 10% polyacrylamide gel was used. The proteins were then transferred electrophoretically onto a 0.45-μm pore size Trans-Blot nitrocellulose membrane (Bio-Rad, Hercules CA). To assess protein loading and transfer quality, protein bands were stained using Ponceau S solution, followed by destaining with deionized water. Blots were blocked overnight at 4°C with 4% nonfat powdered milk in TBS buffer (10 mM Tris, pH 7.4, 0.1 M sodium chloride). The membranes, washed three times in Tween TBS (0.1% Tween 20, 10 mM Tris, pH 7.4, 0.1 M sodium chloride), were incubated for 1 h at 37°C with the specific monoclonal antibody at the appropriate concentrations (PCNA, 1:500; cyclin B, 1:1000; cdk2, 1:1000; p27, 1:500; all obtained from Transduction Laboratories, Lexington, KY; cdk1, 1:1000, Chemicon International, Temecula, CA). The antibodies were diluted in TBS, containing 0.05% Tween 20 and 0.1% nonfat powdered milk. After the end of the incubation, membranes were again washed three times in TBS at room temperature. Alkaline phosphatase goat-anti-mouse IgG (Sigma-Aldrich) was used as a secondary antibody, for another incubation of 1 h at 37°C, in the same buffer used for the primary antibodies. The membranes were washed again for 1 h. Proteins were then detected by adding phosphatase substrate solution (100 mM Tris, pH 9.5, 100 mM sodium chloride, 5 mM magnesium chloride) containing 0.3 mg/ml precipitating agent BCIP (5-bromo-4-chloro-3-indolyl phosphate) and 0.6 mg/ml NBT (2,2′-diphenyl-1-p-sulfophenyl-5′-5″-diphenyl-3′-3″-dimethoxy-4′-4″-diphenylene-ditrazoil chloride).

**DNA Extraction.** Cells incubated with ruthenium dimeric solutions or medium alone of 100 μM concentrations, or control cells were washed four times with PBS, removed from the monolayer by scraping, transferred into a 15-ml polypropylene tube, and pelleted by centrifugation (300g for 7 min). DNA extraction (Miller et al., 1988) was performed by adding 3 ml of cell lysis buffer (10 mM Tris-HCl, 400 mM NaCl, 2 mM EDTA, pH 8.2), 0.2 ml of SDS 10%, and 0.5 ml of proteinase K solution (2 mg/ml proteinase K, 1% SDS, and 2 mM EDTA) on cell pellets, and tubes were placed at 37°C overnight. At the end of digestion, cell lysates were added with 1 ml of a saturated NaCl solution (6 M) and centrifuged at 4500g for 10 min. Supernatants were transferred in another 15-ml polypropylene tube, added with 2 volumes of absolute ethanol, and gently inverted until precipitation of filamentous DNA. After another centrifugation at 4500g for 10 min, the liquid supernatant was removed. Recovered DNA was resuspended in 150 μl of TE buffer (10 mM Tris-HCl, 2 mM EDTA, pH 7.5) and incubated for 1 h at 37°C with 300 U of DNase-free RNase. Concentration and purity of the DNA sample were determined by UV spectrometry.

**Atomic Absorption Spectroscopy.** Cells grown in multiwell plates and treated with dinuclear ruthenium compounds were extensively washed and harvested with a solution of trypsin-EDTA. The cell specimens, counted by trypan blue exclusion test, and DNA samples were dried overnight at 80°C and then at 105°C in Nalgene cryovials. Cell decomposition was facilitated by the addition of an aliquot of tetramethylammonium hydroxide (25% in water) (Aldrich Chimica, Gallarate, Milan, Italy) and Milli-Q water at a ratio of 1:1 directly in each vial at room temperature and under shaking (modified from Tamura and Arai, 1992). Final volumes were adjusted to 1 ml with Milli-Q water. Ruthenium concentration was measured using a graphite furnace atomic absorption spectrometer, model SpectrAA-220Z, supplied with GTA 110Z power and a specific ruthenium emission lamp (hollow cathode lamp P/N 56-101447-00) (Varian, Mulgrave, VIC, Australia). To correct for possible deterioration of the graphite furnace during a daily working session, a reslope standard was measured every six samples and a full recalibration was done every 12 samples. Changes in the readings of this standard are included in the calculation of dimeric compound concentration of the test samples. The graphite furnace was replaced when the values of two subsequent reslope readings deviated by more than 20%. The lower and higher limits of quantitation were set at the levels corresponding to the lower (20 ng of ruthenium per milliliter) and higher (100 ng of ruthenium per milliliter) standard concentrations, respectively. The limit of detection 10 ng of ruthenium per milliliter was estimated according to the EURACHEM guide (www.eurachem.ul.pt/guides/valid.pdf 1998). The quantification of ruthenium was carried out in 10-μl samples at 349.9 nm with an atomizing temperature of 2500°C, using argon as the carrier gas at a flow rate of 3.0 l/min. Before each daily analysis session, a five-point calibration curve was obtained using ruthenium custom-grade standard, 998 μg/ml (Inorganic Ventures Inc., Lakewood, NJ).

**Statistical Analysis.** Experimental data were subjected to computer-assisted statistical analysis using analysis of variance (ANOVA) and Dunnett’s post test. Differences of p < 0.05 were considered to be significantly different from controls.

**Results**

**Effects on Cell Viability.** The effects of the dinuclear ruthenium complexes D1 to D8 on tumor cell proliferation were investigated on murine (TSA/Adenocarcinoma and B16-F10 melanoma) and human (KB oral carcinoma) cell lines. Table 1 reports data of the effects on viability of in vitro cultured TSA cells treated for 24 or 72 h at 1, 10, or 100 μM ruthenium complex concentrations. At the two lower (1 and 10 μM) concentrations, there was no significant reduction of cell viability with any of the compounds, at any time of analysis; significant results were measured only with the highest concentration tested, 100 μM, which showed reduction of cell proliferation after 24 h of exposure to compounds D1, D3, and D5. After a 72-h incubation, all the compounds significantly reduced cell viability with maximal inhibitions of about 50% of untreated controls (compound D5).

The effects on cell proliferation of the KB human carcinoma cell line (Table 2), in the same experimental conditions described for TSA cells, showed no inhibition of cell proliferation with any of the tested compounds, at any concentration and at any time of analysis. Also murine B16-F10 melanoma cells (Table 3) showed only a weak response for some of the tested ruthenium binuclear complexes, apparently not proportional to the concentration used or the length of treatment.

**Effects on Cell Cycle.** Cell distribution among cell cycle phases of in vitro cultured TSA cells was assessed after treatment for 24 h (Fig. 2, panel A) and 72 h (Fig. 2, panel B) with the dinuclear ruthenium compounds D1 to D8 (100 μM) in complete medium. After a 24-h challenge, control cells were 30% in G0/G1, 60% in synthesis, and 10% in G2-M; treatment with compounds D1, D3, D4, D5, D6, and D7 did not significantly modify this distribution (Fig. 2), whereas compound D2 caused a significant reduction in the percent-
TABLE 1
Effects of dimeric ruthenium compounds on proliferation of murine TS/A adenocarcinoma cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>24 h 1 μM</th>
<th>24 h 10 μM</th>
<th>24 h 100 μM</th>
<th>72 h 1 μM</th>
<th>72 h 10 μM</th>
<th>72 h 100 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>94 ± 9</td>
<td>100 ± 6</td>
<td>60 ± 11*</td>
<td>89 ± 2</td>
<td>93 ± 4</td>
<td>59 ± 1**</td>
</tr>
<tr>
<td>D2</td>
<td>107 ± 4</td>
<td>110 ± 6</td>
<td>92 ± 5</td>
<td>104 ± 8</td>
<td>94 ± 9</td>
<td>60 ± 4**</td>
</tr>
<tr>
<td>D3</td>
<td>88 ± 4</td>
<td>111 ± 12</td>
<td>60 ± 13**</td>
<td>94 ± 6</td>
<td>90 ± 2</td>
<td>68 ± 4**</td>
</tr>
<tr>
<td>D4</td>
<td>100 ± 9</td>
<td>82 ± 1</td>
<td>78 ± 4</td>
<td>88 ± 4</td>
<td>84 ± 5</td>
<td>76 ± 4*</td>
</tr>
<tr>
<td>D5</td>
<td>106 ± 4</td>
<td>97 ± 5</td>
<td>49 ± 11**</td>
<td>88 ± 9</td>
<td>89 ± 1</td>
<td>54 ± 2**</td>
</tr>
<tr>
<td>D6</td>
<td>102 ± 7</td>
<td>104 ± 9</td>
<td>70 ± 6</td>
<td>84 ± 1</td>
<td>91 ± 4</td>
<td>65 ± 5**</td>
</tr>
<tr>
<td>D7</td>
<td>84 ± 4</td>
<td>99 ± 9</td>
<td>101 ± 3</td>
<td>86 ± 7</td>
<td>84 ± 4</td>
<td>66 ± 2**</td>
</tr>
<tr>
<td>D8</td>
<td>97 ± 7</td>
<td>89 ± 4</td>
<td>76 ± 7</td>
<td>89 ± 3</td>
<td>90 ± 7</td>
<td>62 ± 1**</td>
</tr>
</tbody>
</table>

**P < 0.01 and *P < 0.05 versus controls.

TABLE 2
Effects of dimeric ruthenium compounds on proliferation of human KB carcinoma cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>24 h 1 μM</th>
<th>24 h 10 μM</th>
<th>24 h 100 μM</th>
<th>72 h 1 μM</th>
<th>72 h 10 μM</th>
<th>72 h 100 μM</th>
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<tbody>
<tr>
<td>D1</td>
<td>112 ± 7</td>
<td>95 ± 5</td>
<td>108 ± 8</td>
<td>97 ± 2</td>
<td>100 ± 6</td>
<td>91 ± 9</td>
</tr>
<tr>
<td>D2</td>
<td>102 ± 20</td>
<td>103 ± 5</td>
<td>100 ± 8</td>
<td>94 ± 3</td>
<td>89 ± 1</td>
<td>103 ± 6</td>
</tr>
<tr>
<td>D3</td>
<td>117 ± 3</td>
<td>100 ± 3</td>
<td>97 ± 2</td>
<td>87 ± 3</td>
<td>103 ± 6</td>
<td>103 ± 11</td>
</tr>
<tr>
<td>D4</td>
<td>120 ± 5</td>
<td>102 ± 3</td>
<td>102 ± 3</td>
<td>108 ± 9</td>
<td>91 ± 6</td>
<td>114 ± 6</td>
</tr>
<tr>
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<td>124 ± 1</td>
<td>124 ± 17</td>
<td>95 ± 8</td>
<td>123 ± 3</td>
<td>114 ± 9</td>
<td>86 ± 3</td>
</tr>
<tr>
<td>D6</td>
<td>112 ± 5</td>
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<td>111 ± 9</td>
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<td>D7</td>
<td>100 ± 7</td>
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<td>100 ± 7</td>
<td>89 ± 3</td>
<td>123 ± 14</td>
<td>103 ± 6</td>
</tr>
<tr>
<td>D8</td>
<td>127 ± 8</td>
<td>105 ± 8</td>
<td>100 ± 12</td>
<td>120 ± 11</td>
<td>97 ± 9</td>
<td>109 ± 2</td>
</tr>
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</table>

**P < 0.01 and *P < 0.05 versus controls.

TABLE 3
Effects of dimeric ruthenium compounds on proliferation of murine B16-F10 melanoma cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>24 h 1 μM</th>
<th>24 h 10 μM</th>
<th>24 h 100 μM</th>
<th>72 h 1 μM</th>
<th>72 h 10 μM</th>
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<td>D1</td>
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<td>88 ± 3</td>
<td>91 ± 3</td>
<td>112 ± 7</td>
<td>109 ± 6</td>
<td>81 ± 1</td>
</tr>
<tr>
<td>D2</td>
<td>95 ± 1</td>
<td>84 ± 3</td>
<td>77 ± 1**</td>
<td>91 ± 2</td>
<td>90 ± 2</td>
<td>80 ± 2**</td>
</tr>
<tr>
<td>D3</td>
<td>81 ± 5*</td>
<td>93 ± 5</td>
<td>86 ± 9</td>
<td>106 ± 1</td>
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<td>88 ± 6</td>
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<tr>
<td>D4</td>
<td>83 ± 1</td>
<td>84 ± 3</td>
<td>107 ± 7</td>
<td>107 ± 1</td>
<td>98 ± 3</td>
<td>91 ± 5</td>
</tr>
<tr>
<td>D5</td>
<td>91 ± 3</td>
<td>84 ± 1</td>
<td>86 ± 3</td>
<td>100 ± 6</td>
<td>97 ± 1</td>
<td>105 ± 6</td>
</tr>
<tr>
<td>D6</td>
<td>115 ± 9</td>
<td>90 ± 3</td>
<td>88 ± 7</td>
<td>96 ± 3</td>
<td>83 ± 12</td>
<td>100 ± 2</td>
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<tr>
<td>D7</td>
<td>76 ± 2**</td>
<td>90 ± 1</td>
<td>86 ± 7</td>
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<td>74 ± 5**</td>
<td>84 ± 7</td>
<td>105 ± 6</td>
<td>95 ± 4</td>
<td>66 ± 5**</td>
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</tbody>
</table>

**P < 0.01 and *P < 0.05 versus controls.

The effects of compounds D3 and D7 on KB cell distribution among cell cycle phases are shown in Fig. 4. Twenty-four hours after the end of the 1-h treatment, compound D3 did not induce any modification in the distribution of cells in cycle phases, whereas compound D7 caused a significant increase of the percentage of cells in G0/G1 and a corresponding decrease of cells in G2/M, which were completely abolished at 48 h from cell treatment.

Ruthenium Cell Uptake. Ruthenium uptake was studied for two compounds (D3 and D7) that showed the most interesting activity on TS/A and KB cells after 1 h of treatment (Table 4). Cell uptake depended on the tumor cell line being treated; ruthenium uptake was greater for compound D3 on murine TS/A cells, whereas on the human KB carcinoma cells, ruthenium uptake was greater for compound D3. Considering the amount of ruthenium bound to DNA, both compounds showed a similar DNA binding to TS/A cells.

The effects of dimeric ruthenium compounds on proliferation of human KB carcinoma cells

KB cells, sown 96 h before, were treated with the compounds at the indicated concentrations for 24 or 72 h in complete medium supplemented with 5% fetal calf serum. Cell viability was evaluated by MTT assay at the end of each treatment. Data are expressed as percentage of optical density of treated cells versus controls ± S.E. calculated on the average of two experiments performed in triplicate. Statistical analysis was done using ANOVA and Dunnett’s post test.

The average of two experiments performed in triplicate. Statistical analysis was done using ANOVA and Dunnett's post test.

age of cells in G0/G1, and compound D8 gave an increase of cells in G2/M and a corresponding decrease of cells in S phase.

After 72 h (Fig. 2, panel B), controls showed cells distributed by 70% in G0/G1, 20% in S, and 10% in G2/M. In these conditions, compound D1 caused a shift of cells from G0/G1 phase to S phase, compound D6 increased cells in G0/G1, and compound D7 increased cells in S phase. All other compounds were inactive.

When cell distribution in cell cycle phases is analyzed 24 h after a 1-h treatment with test compounds in PBS (Fig. 3, data represented as the percentage of treated groups value compared with the relevant control), only compounds D3 and D7 significantly increased the percentage of cells in G2/M phase (+70% and +100%, respectively); in the case of compound D3, the effect was still present 48 h after the end of treatment.
whereas a marked reduction of DNA binding, compared with compound D3, was measured on KB cells treated with compound D7.

**Effects on the Levels of Cell Cycle Regulating Proteins.** The study of the levels of some proteins involved in cell cycle regulation (PCNA, cyclin B, cdk1 and cdk2 kinases, and p27) measured by Western blot techniques was conducted in human carcinoma KB cells 24 and 48 h after treatment with compounds D3 and D7 in PBS at 100 μM concentration for 1 h (Fig. 5). PCNA levels, a marker of cell proliferation, did not change after treatment with D3 and D7, confirming the absence of effects measured with the MTT assay. Similarly, no change in the levels of kinases cdk1 and cdk2 and of the inhibitor p27 were recorded; the increased level of p27 48 h after the treatment was consistent with data showing an increase in the fraction of cells in the G0/G1 fraction. The intensity of the band of cyclin B increased in all the treated groups at 24 h after the treatment and returned to control values at 48 h.

**Discussion**

Recent studies have shown that the in vivo antimitastatic activity of the ruthenium compound NAMI-A, and its in vitro lack of cytotoxicity for tumor cells, might be associated with a transient block of cell cycle at the G2-M level (Bergamo et al., 1999; Zorzet et al., 2000). Lack of in vitro cytotoxicity and marked metastasis inhibition were also shown by some NAMI-A analogs characterized by different N-donor ligands (Bergamo et al., 2002). In this study, we have extended the knowledge on the in vitro behavior of a series of dinuclear ruthenium compounds related to NAMI-A.

The enlargement of the chemical structure from the mononuclear species to dinuclear compounds does not modify the effects of ruthenium complexes on cell viability of in vitro tumor cell lines. The eight dinuclear complexes chosen show a low antiproliferative effect that is statistically significant at 100 μM, after 72 h of exposure, only on the TS/A adenocarcinoma cell line, and some of them, similar to NAMI-A, induce cell cycle arrest in the G2-M phase. Taken together, this activity and the lack of cell growth inhibition seem to characterize the biological behavior of ruthenium-DMSO complexes (Zorzet et al., 2000). Moreover, nitrogen ligand appears to be crucial for the concomitant occurrence of these biological effects in mononuclear compounds (Bergamo et al., 2002); in dinuclear complexes, it determines the distance between the two ruthenium atoms and the molecular flexibility, two parameters that might be relevant for interacting with biological substrates and “target receptors.”

Cisplatin, a cytotoxic drug that acts by binding to guanine DNA, was chosen as a control to establish the in vitro antimitastatic activity of the ruthenium complexes. Cisplatin was used at a concentration of 5 μM, that is, one order of magnitude lower than the IC50 values of the ruthenium compounds, to avoid a direct cytotoxic effect. As shown in Table 2, cisplatin was able to reduce the DNA binding of the complexes, but the reduction was much lower than that observed with compounds D3 and D7. This result suggests that the ruthenium compounds are not acting through a cytotoxic mechanism, at least at the concentrations used in this study.
Murine TS/A adenocarcinoma cells or human KB oral carcinoma cells were exposed for 1 h to 0.1 mM concentrations of compounds D3 and D7 in PBS. Ruthenium uptake by TS/A and KB tumor cells following 1-h exposure to compounds D3 and D7.

TABLE 4
Ruthenium uptake by TS/A and KB tumor cells following 1-h exposure to compounds D3 and D7

Murine TS/A adenocarcinoma cells or human KB oral carcinoma cells were exposed for 1 h to 0.1 mM concentrations of compounds D3 and D7 in PBS. Ruthenium concentration in cells and in DNA was determined immediately after the end of the cell challenge. Each value is the mean ± S.E. obtained in two separate experiments performed in triplicate and is expressed as nanograms of compound per million cells or per DNA extracted from one million cells.

It is known that among the cell cycle regulating mechanisms, arrest in G2/M and G2-M phases are events related to cellular damage and, in particular, to DNA damage. When damage occurs, DNA repair mechanisms are activated, to ensure the replication of an intact DNA. If the reparation is successful, then cells start again cycling; otherwise, apoptosis mechanisms are actuated. We may hypothesize that the arrest of the cell cycle observed in KB cells treated with the dinuclear ruthenium compounds D3 and D7 might be due DNA damage that cells are able to repair as shown by the reversibility of this event 48 h after the end of treatment. It should be noted that there is scarce structural correlation between D3 and D7, because the bridging ligand and the net charge are different [D7 is the only example of unsymmetrical dinuclear species among those reported here, because the pyrazine ligand bridges an anionic RuCl4(dmso-S) fragment and a neutral RuCl4(dmso-S)(dmso-O) fragment].

The molecular factors responsible for the G2-M arrest of in vitro cultured cells after treatment with different compounds have been related both to increase and to reduction of the cellular levels of cyclin B or cdk1 kinase, proteins that are essential for the start of mitosis (Ohi and Gould, 1999; Choi et al., 2000). NAMI-A, for example, significantly reduces cdk1 at doses active on cell proliferation (Pintus et al., 2002) in ECV304 endothelial cells. In this study, we extended the examination to cdk2 kinase, involved in the S phase (Reed, 1997), to p27, an inhibitor of cell progression among cell cycle phases (Polyak et al., 1994), and to nuclear proliferation antigen PCNA, whose expression levels are related and proportional to cell proliferation (Kurki et al., 1988). The mild effect of D3 and D7 ruthenium dinuclear complexes on KB cells that results from MTT and trypan blue exclusion tests has been confirmed at a molecular level by the quantity of PCNA protein that did not vary between control and treated groups. Even the levels of cdk1, cdk2, and p27 proteins were constant in control and treated groups, so none of these proteins might explain the cell cycle arrest occurring in our experimental conditions. Conversely, this study showed an increase of the intracellular level of cyclin B. The effect was evident in samples prepared 24 h after treatment, and in the case of compound D7, it was concomitant with cell cycle arrest. This effect disappeared on samples prepared 48 h after treatment, concomitant with the reversion of cell cycle arrest, confirming the temporal relationship between the two events. However, a cyclin B increase was detected also in KB cells treated with compound D3, a binuclear complex devoid

<table>
<thead>
<tr>
<th>Compound</th>
<th>Uptake/Cell</th>
<th>Uptake/DNA</th>
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<tr>
<td></td>
<td>In/Out</td>
<td>ng</td>
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<tr>
<td>TS/A murine adenocarcinoma cells</td>
<td>D3</td>
<td>283 ± 16</td>
</tr>
<tr>
<td></td>
<td>D7</td>
<td>345 ± 68</td>
</tr>
<tr>
<td>KB oral carcinoma cells</td>
<td>D3</td>
<td>466 ± 92</td>
</tr>
<tr>
<td></td>
<td>D7</td>
<td>158 ± 17</td>
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of effects on cell cycle arrest, as determined by flow cytometry.

These observations allow us to formulate the following considerations.

1. Cell cycle arrest needs an intracellular ruthenium concentration threshold that, in our experimental model and conditions, is reached with the binuclear ruthenium complexes D3 and D7 when used in PBS.

2. The lack of effects of compound D3 on cell cycle of KB cells may be due to the timing of analysis (24 and 48 h from the treatment), which is probably unfavorable to allow evidencing this event.

3. The increase of cyclin B protein, apparently in contrast to the effect on cell cycle arrest, is in agreement with studies of premitotic arrest related to the increase of this protein concentration (Suzuki et al., 1999; Vincent et al., 1999).

Cell cycle regulation is a complex phenomenon, comprising mechanisms more complex than simple variations in protein expression levels. In our experimental model, it will be useful to consider other events, for example intracellular localization of cyclin B during different cell cycle phases and the state of activation of cdk1 kinase. Indeed, a wrong localization of cyclin B, which increases in the cytosol but does not reach the nucleus, could explain the results of our analysis (Takizawa and Morgan, 2000). It is also possible that cdk1 kinase, if not properly phosphorylated (Morgan, 1995), could be a limiting factor in the start of mitosis in D3- and D7-treated cells, even when intracellular levels of this protein are the same in treated and control groups, as shown by our immunoblot analysis.

Moreover, cyclin B and cdk1 are just a little part of the very complex network of cell cycle regulating pathways. Thus, to explain the events revealed in our model, it will be important to consider the levels and the phosphorylation of other relevant regulating proteins, such as wee1 (Michael and Newport, 1998), myt1 (Liu et al., 1997), cdc25 (Ohl and Gould, 1999), and p21 (Sherr, 1994; Levine, 1997). Nevertheless, data of the present investigation show the interesting properties of some ruthenium dimeric compounds and the similarity of behavior of these compounds in vitro, as compared with NAMI-A, supporting the need for an appropriate test of their activity on in vivo tumor models.

References


Keppler BK and Rupp W (1988) Antitumor activity of imidazolium-bisimidazole- 

tetrachlororuthenate(III), in Cancer Res Clin Oncol 111:166–168.


Fig. 5. Effects on cell cycle proteins of human KB carcinoma cells. KB cells, sown 96 h before, were treated with the compounds at a 100 µM concentration for 1 h in PBS. At 24 and 48 h after the end of the treatment, cells were lysed to extract total protein content. Equal amounts of proteins were subjected to SDS-polyacrylamide gel electrophoresis, blotted onto a nitrocellulose membrane, and revealed immunoenzymatically.


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