Comparative analysis of cytotoxic effects and intracellular accumulation of platinum(IV) nitroxyl complexes

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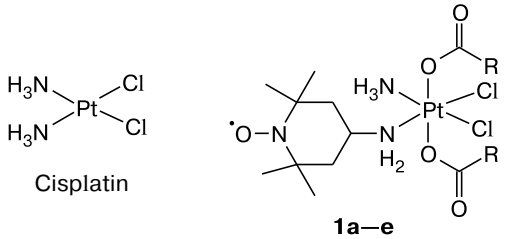
The relationship between the lipophilicity, cytotoxic effects, and intracellular accumulation for potential antitumor agents, *viz.*, amino complexes of platinum(IV) with the redox-active nitroxyl radicals *cis*,*trans*,*cis*-$Pt^{IV}(RNH_2)(NH_3)(OCOR')_2Cl_2$ (R is 2,2,6,6-tetramethyl-1-oxylpiperidin-4-yl, OCOR´ are *n*-alkylcarboxylates C_2—C_8), was studied in comparison with cisplatin. In a series of four platinum nitroxyl complexes (PNCs), an increase in the lipophilicity was found to lead to a considerable increase in the cytotoxicity and the efficiency of accumulation in HeLa cells. However, PNCs are characterized by the slower development of cytotoxic effects after a short time exposure compared with hydrophilic cisplatin. This can be attributed to the fact that Pt^{IV} complexes are prodrugs and need to be reduced to chemically more active Pt^{II} derivatives in order to exhibit cytotoxic effects.

Key words: platinum(IV) nitroxyl complexes, cytotoxicity, lipophilicity, intracellular accumulation.

Platinum group antitumor agents are among leading anticancer drugs. Cisplatin and its analogs (carboplatin and oxaliplatin) are used in almost half of the treatment regimens both for monotherapy and in combination with other antitumor drugs.[1,2] Substantial drawbacks of platinum complexes are high toxicity and serious side effects.[1] Yet another adverse factor for chemotherapy using cisplatin and its analogs is the rapid development of tumor resistance to these agents.[3] The development of resistance is associated with a decrease in the intracellular accumulation of the agents, an enhancement of their detoxification by glutathione and metallothioneins, and more efficient damage repair.[1,4,5] Therefore, efforts are being directed toward the development of new platinum compounds with improved characteristics, in particular, those having high efficiency of intracellular accumulation, which is required to overcome tumor cell resistance to chemotherapy. Investigations of platinum(IV) complexes with axial carboxylate ligands hold promise in this area of research.[6]

It is known that at near-millimolar concentrations, simple nitroxyl radicals have an effect on tumor cell viability through redox-mediated signals and induce cell death, which depends on numerous factors, including oxidative damage, the cessation of the cell cycle, and apoptosis.[7—10] At lower concentrations, nitroxyl radicals possess antioxidant properties[11] and can enhance chemotherapeutic properties of different classes of antitumor agents.[10,12]

In the present work, we investigated a series of platinum(IV) nitroxyl complexes (PNCs) containing aliphatic carboxylic acid (acetic, butyric, valeric, caproic, and caprylic) residues as axial ligands (compounds **1a**—**e**), which we have recently synthesized. The aim of this study is to determine the relationship between the lipophilicity, the accumulation of complexes in the cells, and the dynamics of the development of cytotoxic effects in comparison with the corresponding characteristics of cisplatin.



Cisplatin

1a—e

R = Me (**a**), $(CH_2)_2Me$ (**b**), $(CH_2)_3Me$ (**c**), $(CH_2)_4Me$ (**d**), $(CH_2)_6Me$ (**e**)

Experimental

Platinum(IV) nitroxyl complexes were synthesized by a procedure described earlier.[13,14]

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Experiments were performed in the HeLa cell culture. The cells were grown under a 5% CO_2 atmosphere in the DMEM cell culture medium supplemented with 10% fetal calf serum (PAA Laboratories).

Evaluation of cytotoxic effects of platinum complexes and their dependence on the time of incubation. The cytotoxic effects of the platinum complexes were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by a procedure described earlier.[15—17] The cells were seeded into wells of cell culture plates in a standard incubation medium at a density of ~60 cell/mm^2 of the surface area of the well bottom (15000 cells per well in 24-well plates or 2500 cells per well in 96-well plates). Before the application, cisplatin and complexes **1a**—**c** were dissolved in water; complexes **1d**,**e** that are poorly soluble in water, in DMSO. The compounds under consideration were added to the incubation medium at different concentrations 24 h after the seeding of the cells. Solutions of complexes **1d**,**e** in DMSO were added so that the final concentration of the latter in the incubation medium was 0.1%. It was found that this concentration of DMSO has no effect on the viability of HeLa cells.

In order to evaluate the cytotoxic effects, two series of experiments were performed. In one series, the cells were incubated at a constant exposure with the platinum complexes at different concentrations for 24 h, followed by MTT staining and the determination of the IC_{50} dose (the concentration of the compound at which MTT staining decreases by 50%). In another series, the dependence of the development of cytotoxic effects on the time of exposure of the compounds was studied using shorter exposures (from 2 to 12 h). In these experiments, the compounds under study were added to the incubation medium at the IC_{50} dose 24 h after the seeding of the cells. The medium was changed for a fresh portion, which did not contain the compounds under study, after 2, 4, 6, 8, 10, and 12 h. The compounds, which have not penetrated into the cells, were removed from the culture medium. Within 24 h after the addition of the platinum complexes, MTT staining of the cells was performed during 4 h at a dye concentration of 0.5 mg mL^{-1}. The crystals of MTT formazan that formed were dissolved in DMSO. The absorbance of the solutions was determined at a wavelength of 570 nm. The absorbance of the samples at a wavelength of 650 nm was set to zero. The intensity of MTT staining of the cell samples from the control wells (which were not treated with the platinum complexes) ($I = A^{570} - A^{650}$) corresponded to 100% cell viability.

Evaluation of lipophilicity. The lipophilicity of the complexes is characterized by the *n*-octanol—water partition coefficient ($\log P_{ow}$). The procedure is based on HPLC[18] and was accomplished on a Milikhrom-5 chromatograph (2×64 mm column, Separon C18 (5 μm), detection at 240 nm; MeCN—H_2O, 60 : 40, as the eluent). Anisole, toluene, ethylbenzene, biphenyl, and dibenzyl were used as reference compounds with known values of $\log P_{ow}$. The retention volumes (V_r) for the column used were determined by chromatography of the reference compounds, and the V_r—$\log P_{ow}$ calibration plot was constructed. The dependence is linear with a correlation coefficient of 0.98. Then the retention volumes of PNCs were determined and $\log P_{ow}$ for these compounds were found from the calibration curve. For the most hydrophilic complex **1a**, $\log P_{ow}$ was determined by EPR; for complex **1b**, by both methods. After the stirring of a solution of the complex in a mixture composed of equal volumes of the solvents for 0.5 h, the concentrations of the complexes in aqueous and *n*-octanol phases were determined by EPR. From these values, $\log P_{ow} = \log(C_o/C_w)$ were calculated, where C_o and C_w are the concentrations of the complexes in *n*-octanol and water, respectively.

Determination of the platinum content in cells. The cells were seeded into culture flasks in a standard incubation medium and were grown to the culture density of 70—80%. The complexes were added to the medium at a concentration of 50 μmol L^{-1} and incubated for 4 h. Relatively high concentrations of the complexes were used because of the limited sensitivity of an AAS-3 spectrometer. The cells were removed from the support with trypsin and washed one time with a PBS solution (137 mmol L^{-1} NaCl, 2.68 mmol L^{-1} KCl, 4.29 mmol L^{-1} Na_2HPO_4, 1.47 mmol L^{-1} KH_2PO_4, pH 7.4). An aliquot of the cells was taken to measure the protein content; the other cells were used to determine the platinum content. The amount of the protein in the samples was determined by the Lowry method.[19]

In order to determine the platinum content, the samples were subjected to wet ashing by a method described earlier.[20—22] The cells were dissolved in concentrated (70%) nitric acid (0.55 mL) for 24 h at ~20 °C. The solution was refluxed for 10 min and cooled to ~20 °C. Then concentrated (30%) hydrogen peroxide (0.45 mL) was added to the solution, and the mixture was repeatedly refluxed for 10 min. The atomic absorption analysis was performed on an AAS-3 spectrometer (atomization in air—acetylene flame using the resonance line at 265.9 nm at a spectral slit width of 0.3 nm with deuterium background correction). The platinum concentration in the samples was calculated from the calibration curve, which was constructed using standard solutions. The platinum content in the cells was expressed as the ratio of the amount of platinum to the amount of the protein in the sample ((μg Pt) · (mg protein)$^{-1}$).

Results and Discussion

Earlier,[14] we have synthesized complexes containing axial carboxylate ligands of different structures, which can change the lipophilicity, and nitroxyl radicals, which can modulate the biological activity of the compounds. In the present work, we studied the relationship between the cytotoxic properties of PNCs and their lipophilicity and the intracellular accumulation in comparison with cisplatin. It is known that DNA is the major target for platinum complexes.[23,24] To be targeted to DNA, the agents should effectively penetrate into the cells. This ability of agents is well-characterized by the lipophilicity, which is expressed as the partition coefficient $\log P_{ow}$ in the two-phase octanol—water mixture.

The values of $\log P_{ow}$ determined by HPLC and/or EPR for the series of PNCs under consideration vary in a wide range from −0.41 to 4.8 (Table 1). The most hydrophilic complex in this series — compound **1a** — is much more lipophilic (based on the value of $\log P_{ow}$) than cisplatin[25] because it contains the bulky organic ligand (nitroxyl radical). The cytotoxic effect of complex **1a** is nearly an order of magnitude lower than that of cisplatin (see Fig. 1 and Table 1). In the series of complexes **1a**—**e**, which differ only by the length of the axial ligands, there is a correlation between the lipophilicity and cytotoxic effects (Fig. 1).

Table 1. Values of $\log P_{ow}$, 24-h IC_{50}, and intracellular accumulation of the platinum complexes in HeLa cells

Platinum complex	$\log P_{ow}$	Concentration IC_{50} /μmol L^{-1}	Platinum accumulation[a] /(μg Pt) (mg protein)$^{-1}$
Cisplatin	−2.53[25]	12.6±2.9	0.10±0.02
1a	−0.41[b]	≥120	0.06±0.02
1b	1.82[c]	4.2±0.8	4.0±0.7
	1.92[b]		
1c	2.10[c]	2.5±1.5	4.3±0.4
1d	2.55[c]	0.23±0.17	—
1e	4.80[c]	0.09±0.05	—

[a] Intracellular accumulation of the platinum complexes 4 h after the incubation of HeLa cells in a medium containing the complex at a concentration of 50 μmol L^{-1}.
[b] Determined by EPR.
[c] Determined by HPLC.

An elongation of the axial ligands in PNCs (complexes **1b—e**) leads to a substantial increase in their cytotoxicity and results in the formation of complexes, which have cytotoxicity higher than that of cisplatin. For example, IC_{50} of complexes **1b** and **1c** is 3—5 times smaller and IC_{50} of **1d** and **1e** is 50—100 times smaller than that of cisplatin.

The cytotoxicity of the platinum complexes can be influenced by the difference in the level and/or the rate of their intracellular accumulation, as well as by other factors. In order to elucidate this question, we measured the intracellular accumulation of cisplatin and complexes **1a—c**, which are quite soluble in water (see Table 1). Although the lipophilicity of complex **1a** is higher than that of cisplatin, both compounds show comparable levels of accumulation in HeLa cells. This result could be attributed to the low rate of the transfer of complex **1a** from the solution into the cells. However, this is inconsistent with the fact that nitroxyl radicals of different structures easily penetrate through lipid membranes. The ease of their interphase transfer is evidenced by the nearly equal efficacy of the antioxidant action in the lipid and aqueous phases for radicals, the P_{ow} values of which differ by an order of magnitude.[26] Therefore, the efficiency of intracellular accumulation of complex **1a** is lower than that expected based on its higher lipophilicity compared with cisplatin.

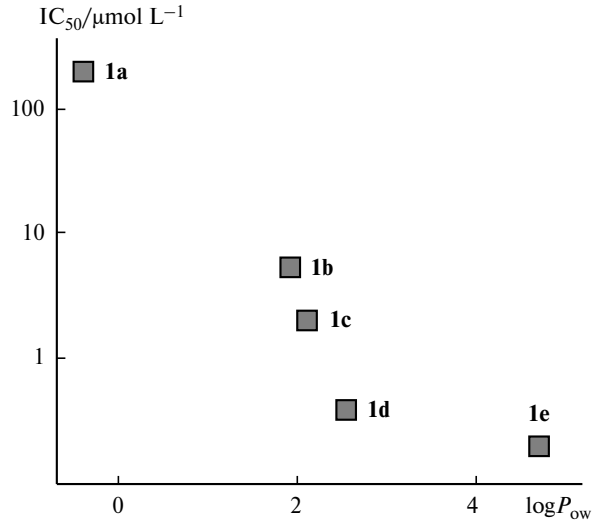

Fig. 1. Relationship between the lipophilicity ($\log P_{ow}$) and the cytotoxicity (IC_{50}) of complexes **1a—e**.

The efficiency of accumulation of complex **1a** is comparable with that of cisplatin, but its cytotoxicity is approximately an order of magnitude lower. The antioxidant action of the nitroxyl radical may be one of the factors responsible for the lower cytotoxicity of complex **1a** compared with cisplatin. The antioxidant action of the nitroxyl radical has been reported earlier.[6]

In order to study other factors that can influence the cytotoxic properties of the platinum complexes, we investigated the dependence of the cytotoxic effects on the time of incubation of HeLa cells with solutions of cisplatin and water-soluble PNCs (complexes **1a—c**). In this case, the cytotoxic effects are determined only by the amount of the complex accumulated in the cell during the incubation. As can be seen in Fig. 2, the level of cytotoxicity for cisplatin characteristic of 24-h incubation is achieved already within 6—8 h and the further incubation does not lead to the enhancement of cytotoxicity of cisplatin. This may indicate that the major part of cisplatin from the solution is accumulated in the cells and causes cytotoxic effects during the period of time ≥6 h.

Complex **1a**, which is less efficiently accumulated in the cells, does not exhibit noticeable cytotoxic effects up to 12-h incubation (see Fig. 2, *b*). Meanwhile, under the same conditions, complexes **1b** and **1c** are accumulated in the cells ~40 times more efficiently than cisplatin (see Table 1). Taking into account their efficient transfer from the solution into the cells, it would be expected that these complexes will cause a substantial decrease in the cell viability. However, the development of cytotoxic effects for complexes **1b** and **1c** after a short time exposure (see Fig. 2, *c*, *d*) occurs much more slowly compared with cisplatin than it was expected based on the difference in the efficiency of intracellular accumulation. Therefore, an additional time is required for complexes **1a—c** to exhibit cytotoxic effects. For complexes **1a** and **1b**, this time is >12 h; for complex **1c**, ~12 h.

The above-described results show that, apart from the lipophilicity and the efficiency of intracellular accumulation, the cytotoxic properties of PNCs are influenced by the dynamics of the processes. After the penetration into the cell, a small cisplatin molecule easily loses Cl ligands through the hydrolysis to form chemically active aqua complexes, which are capable of efficiently platinating

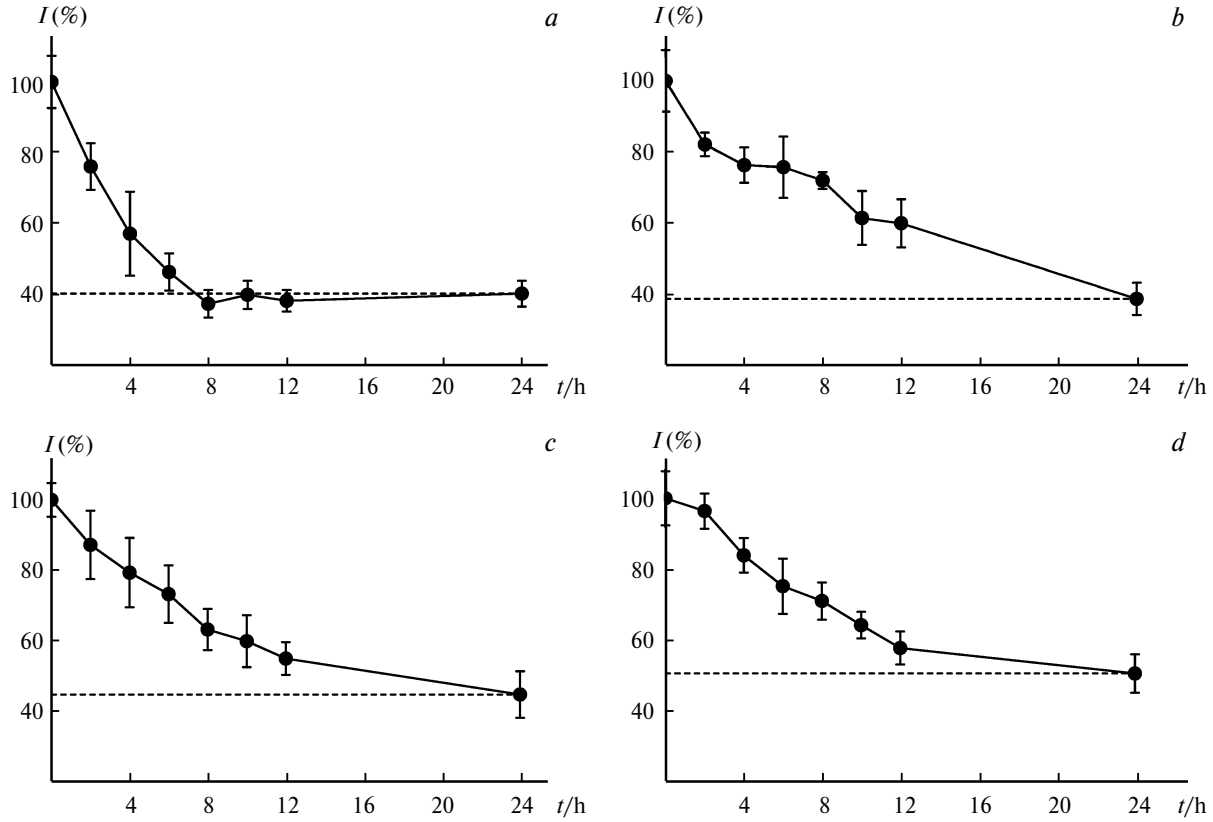

Fig. 2. Toxicity against HeLa cells of cisplatin (*a*) and water-soluble complexes **1a** (*b*), **1b** (*c*), and **1c** (*d*) depending on the time of incubation at the IC_{50} dose. The level of MTT staining of the cells after 24-h incubation of the cells in the presence of the compounds under study is indicated by a dashed line.

DNA[27] and inducing a chain of events, resulting in the cell death.

Unlike Pt^{II} complexes, Pt^{IV} complexes are prodrugs and need to be reduced to chemically more active Pt^{II} derivatives in order to display cytotoxic effects. It is known[28] that Pt^{IV} complexes are very rapidly reduced in the whole blood; the half-life time is 6.3 min. However, the data on the rate of their reduction in cell cultures are lacking. We believe that the low development of cytotoxic effects of PNCs can be attributed to the low rate of their reduction in HeLa cells and lower, compared with cisplatin, activity of the Pt^{II} metabolites of these complexes. Besides, the partial back diffusion from the cells cannot be excluded for lipophilic PNCs (**1b** and **1c**) after the replacement of the medium with a fresh portion, which does not contain the complex.

Therefore, the lipophilicity and the presence of polar moieties containing N and O atoms are the main structural factors responsible for the relationship between the structure and the cytotoxicity of platinum(IV) complexes.[29] The activity of the complexes depends on the efficiency of cellular accumulation, the rate of reduction of Pt^{IV} to Pt^{II}, and the structure of the corresponding Pt^{II} metabolites. Nitroxyl radicals[6] and other redox-active compounds[30] can modulate the biological activity of antitumor drugs and improve their chemotherapeutic properties: decrease the toxicity, alter the spectrum of antitumor activity, and reduce the rate of the development of resistance. The results of the present study, along with the available published data, suggest that the decrease in the cytotoxic activity of complex **1a** compared with cisplatin can be due to the antioxidant effect of the nitroxyl radical,[6,31] as well as due to the slow kinetics of the development of cytotoxic effects found for platinum(IV) complexes. A decrease in the cytotoxicity can be compensated by the enhancement of the lipophilicity and intracellular accumulation by varying axial RCOO ligands. Meanwhile, the development of cytotoxic effects even of lipophilic PNCs is slower compared with cisplatin. Therefore, the structure with the optimal activity and toxicity combination can be found in the series of PNCs (for example, compound **1c**). However, an additional time is needed for this compound to exhibit activity.

References

1. N. J. Wheate, S. Walker, G. E. Craig, R. Oun, *J. Chem. Soc., Dalton Trans.*, 2010, **39**, 8113.

2. *Martindale: The Complete Drug Reference*, Ed. S. C. Sweetman, Pharmaceutical Press, London, 2007, 3322 p.
3. B. Koeberle, M. T. Tomicic, S. Usanova, B. Kaina, *Biochim. Biophys. Acta*, 2010, **1806**, 172.
4. F.-S. Liu, *Taiwan*, *J. Obstet. Gynecol.,* 2009, **48**, 239.
5. M. M. K. Shahzad, G. Lopez-Berestein, A. K. Sood, *Drug Resist. Updat.*, 2009, **12**, 148.
6. V. D. Sen´, A. A. Terentiev, N. P. Konovalova, *Nitroxides — Theory, Experiment and Applications*, Ed. A. I. Kokorin, InTech, Croatia, 2012, 385.
7. M. B. Gariboldi, S. Lucchi, C. Caserini, R. Supino, C. Oliva, E. Monti, *Free Rad. Biol. Med.*, 1998, **24**, 913.
8. M. B. Gariboldi, V. Rimoldi, R. Supino, E. Favini, E. Monti, *Free Rad. Biol. Med.*, 2000, **29**, 633.
9. M. B. Gariboldi, R. Ravizza, C. Petterino, M. Castagnaro, G. Finocchiaro, E. Monti, *Eur. J. Cancer*, 2003, **39**, 829.
10. S. Suy, J. B. Mitchell, A. Samuni, S. Mueller, U. Kasid, *Cancer*, 2005, **103**, 1302.
11. S. M. Hahn, Z. Tochner, C. M. Krishna, J. Glass, L. Wilson, A. Samuni, M. Sprague, D. Venzon, E. Glatstein, J. B. Mitchell, *Cancer Res.*, 1992, **52**, 1750.
12. N. M. Emanuel, N. P. Konovalova, in *Bioactive Spin Labels*, Ed. R. I. Zhdanov, Springer-Verlag, N.-Y, 1992, 439.
13. V. D. Sen´, V. A. Golubev, N. Yu. Lugovskaya, T. E. Sashenkova, N. P. Konovalova, *Russ. Chem. Bull.* (*Int. Ed.*), 2006, **55**, 62 [*Izv. Akad. Nauk, Ser. Khim.*, 2006, 60].
14. N. V. Komleva, G. V. Kostyuk, I. I. Parkhomenko, I. V. Balalaeva, V. A. Golubev, V. D. Sen´, A. A. Terent´ev, *Vestn. NNGU im. Lobachevskogo* [*Bull. N. I. Lobachevsky Nizhny Novgorod State Univ.*], 2011, No. 2, 82 (in Russian).
15. T. Mosmann, *J. Immunol. Methods*, 1983, **65**, 55.
16. F. Denizot, R. Lang, *J. Immunol. Methods*, 1986, **89**, 271.
17. J. Carmichael, W. G. DeGraff, A. F. Gazdar, J. D. Minna, J. B. Mitchell, *Cancer Res.*, 1987, **47**, 936.
18. C. V. Eadsforth, P. Moser, *Chemosphere*, 1983, **12**, 1459.
19. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.*, 1951, **193**, 265.
20. M. S. Clegg, C. L. Keen, B. Lönnerdal, L. S. Hurley, *Biol. Trace Elem. Res.*, 1981, **3**, 107.
21. K. M. Watling, J. F. Parr, L. Rintoul, C. L. Brown, L. A. Sullivan, *Spectrochim. Acta*, *Part A*, *Mol. Biomol. Spectrosc.*, 2011, **80**, 106.
22. T. Talarico, D. R. Phillips, G. B. Deacon, S. Rainone, L. K. Webster, *Invest. New Drugs*, 1999, **17**, 1.
23. S. Dhar, S. J. Lippard, in *Platinum and Other Heavy Metal Compounds in Cancer Chemotherapy. Cancer Drug Discovery and Development*, Eds A. Bonetti, R. Leone, F. Muggia, S. B. Howell, Humana Press, 2009, 135.
24. V. D. Sen´, A. V. Shugalii, A. V. Kulikov, *Russ. Chem. Bull.* (*Int. Ed.*), 2002, **51**, 1058 [*Izv. Akad. Nauk, Ser. Khim.*, 2002, 972].
25. D. Screnci, M. J. McKeage, P. Galettis, T. W. Hambley, B. D. Palmer, B. C. Baguley, *Br. J. Cancer*, 2000, **82**, 966.
26. A. M. Samuni, Y. Barenholz, *Free Rad. Biol. Med.*, 2003, **34**, 177.
27. A. V. Shugalii, A. V. Kulikov, M. V. Lichina, V. A. Golubev, V. D. Sen´, *J. Inorg. Biochem.*, 1998, **69**, 67.
28. J. L. Carr, M. D. Tingle, M. J. McKeage, *Cancer Chemother. Pharmacol.*, 2002, **50**, 9.
29. P. Gramatica, E. Papa, M. Luini, E. Monti, M. B. Gariboldi, M. Ravera, D. Osella, *J. Biol. Inorg. Chem.*, 2010, **15**, 1157.
30. G. T. Wondrak, *Antioxid. Redox Signal.,* 2009, **11**, 3013.
31. V. D. Sen´, N. A. Rukina, V. V. Tkachev, A. V. Pis´menskii, L. M. Volkova, S. A. Goncharova, T. A. Raevskaya, A. G. Tikhomirov, L. B. Gorbacheva, N. P. Konovalova, *Russ. Chem. Bull.* (*Int. Ed.*), 2000, **49**, 1613 [*Izv. Akad. Nauk*, *Ser. Khim.*, 2000, 1624].

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