Cytotoxic Activity of Platinum(II) and Palladium(II) Complexes of N-3-Pyridinylmethanesulfonamide: the Influence of Electroporation

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The series of complexes: cis-[Pd(PMSA)X2], cis-[Pt(PMSA)X2], trans-[Pt(PMSA)I2] and [Pt(PMSA)4]Cl2 (PMSA = N-3-pyridinylmethanesulfonamide; X = Cl, Br, I), previously synthesized and characterized by us, as well as the free ligand PMSA, were tested for their cytotoxic activity without electroporation – against murine leukemia F4N and human SKW-3 and MDA-MB-231 tumour cell lines – and with electroporation – against the latter two cell lines. The majority of the complexes exhibited cytotoxic effects (IC50 < 100 μmol/l) under the conditions of electroporation. Both cis- and trans-[Pt(PMSA)I2] had pronounced cytotoxic effects (29–61 μmol/l against MDA-MB-231 cells).

Key words: Platinum Complexes, Sulfonamides, Cytostatic Agents, Electroporation

Introduction

cis-Diamminedichloroplatinum (cis-[Pt(NH3)2Cl2], cisplatin) is the first and still the most important metal-based antineoplastic drug, which has been widely applied in cancer chemotherapy for the treatment of lung, head and neck, ovarian, bladder and testicular cancers (Boulikas and Vougiouka, 2003; Cohen and Lippard, 2001). The drug cisplatin has, however, numerous disadvantages, e.g. nephro- and neurotoxicity, as well as other severe side-effects and low aqueous solubility (Boulikas and Vougiouka, 2003; Kostova, 2006a). The search for metal cytostatics with improved therapeutic characteristics has led to the synthesis and screening of a vast number of new platinum and other metal complexes (Bose, 2002; Kostova, 2006b; Reedijk, 2003; Stordal et al., 2007). A significant disadvantage of the “classical” (conventional) cisplatin analogues is their inefficacy against cisplatin-resistant tumours. For this reason, a topical trend is the design of “non-classical” platinum complexes which differ not only in their structure from cisplatin, but display distinct mechanisms of action and hence are quite promising for treating cisplatin-resistant tumours (Kostova, 2006a; van Zutphen et al., 2006). After Farrell et al. (1989) and Van Beusichem and Farrell (1992) established the cytostatic activity of Pt(II) pyridine complexes with trans configuration, the interest in platinum coordination compounds with planar N-heterocyclic ligands considerably increased and now trans-platinum complexes with such ligands acquire the significance of a new class of non-conventional platinum cytostatic agents, that retain their activity against cisplatin-resistant cells (Jakupec et al., 2003; Kostova, 2006a; Coluccia and Natile, 2007).

On the other hand, the antitumour activity of sulfonamide derivatives and its relation to the processes of enzyme inhibition have attracted much attention in the last years (Scozzafava et al., 2003; Supuran et al., 2004). Numerous metal complexes with sulfonamide ligands act as carbonic anhydrase inhibitors (Supuran et al., 1994, 1996), and some of them exhibit cytotoxic activity (Chohan et al., 2005). The molecule of N-3-pyridinylmethanesulfonamide (PMSA) combines both structural fragments, a N-heterocycle and a sulfonamide group. We have performed an ab initio quantum chemical and IR spectroscopic study on PMSA (Dodoff, 2000) and have firstly synthesized and structurally characterized Pd(II) and Van Beusichem and Farrell (1992) established the cytostatic activity of Pt(II) pyridine complexes with trans configuration, the interest in platinum coordination compounds with planar N-heterocyclic ligands considerably increased and now trans-platinum complexes with such ligands acquire the significance of a new class of non-conventional platinum cytostatic agents, that retain their activity against cisplatin-resistant cells (Jakupec et al., 2003; Kostova, 2006a; Coluccia and Natile, 2007).

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and Pt(II) complexes of this ligand (Dodoff, 2001; Dodoff et al., 2004).

The application of high electric field pulses (rectangular or exponential) leads to a transient permeabilisation of the cellular membrane, and this phenomenon is known as electroporation (Gehl, 2003). Electroporation permits exogenous membrane proteins or DNA to be inserted into the cells (Neumann et al., 1996; Tsoneva et al., 2005). Electroporation, combined with the chemotherapeutic drugs bleomycin and cisplatin (electrochemotherapy), finds advanced application for the enhanced delivery of drugs into solid tumours (Serša et al., 2000; Spugnini and Porrello, 2003; Fujimoto et al., 2005; Spugnini et al., 2008). A valuable advantage of this modern drug delivery method is the selective application of the drug onto the tumour (e.g. melanoma) while preserving the normal tissues (Serša et al., 2000; Peycheva and Daskalov, 2004).

Here we report the cytotoxic effect of PMSA and its Pd(II) and Pt(II) complexes 1–6 (Fig. 1) under usual conditions, i.e. without applying an electric field and under the conditions of electroporation.

Material and Methods

Compounds

The ligand PMSA was prepared by a known procedure (Jones and Katritzki, 1961) and purified as described by us (Dodoff, 2000). The synthesis of the complexes tested, 1–6, was described in detail elsewhere (Dodoff, 2001). Cisplatin of the quality pro analysi and N,N-dimethylformamide (DMF), >99.8%, were purchased from Fluka. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and (3Z)-5-amino-3-[[4-[4-[(2Z)-2-(8-amino-1-oxo-3,6-disulphonaphthalen-2-ylidene)hydrazinyl]-3-methylphenyl]-2-methylphenyl]hydrazinylidene]-4-oxonaphthalene-2,7-disulfonic acid (trypan blue) were from Sigma.

Cell lines

Murine erythroleukemia cells, clone F4N (Dube et al., 1975), were cultured in Dulbecco’s modified Eagle medium (Gibco, Grand Island, NY, USA) supplemented with 10% calf serum, under 5% CO₂ atmosphere at 37 °C, and passaged every day at a concentration of 5 · 10⁵ cells/ml.

The human T-cell leukemia cell line SKW-3 was established from the peripheral blood of a man with T-cell chronic lymphocytic leukemia (Hirono et al., 1979). The human breast cancer cell line MDA-MB-231 was originally derived from a pleural effusion and grown adherently (Cailleau et al., 1974). Both cell lines were grown in RPMI-1640 medium (Lonza, Walkersville, USA), supplemented with 10% fetal calf serum (FCS) and 1% L-glutamine, at 37 °C in an incubator with humid atmosphere and 5% CO₂. The cells were passaged two or three times per week to keep them in the log phase.

Cytotoxicity assays and electroporation protocol

The compounds studied were dissolved immediately before use in DMF (cisplatin, PMSA and complexes 1–5) or sterile distilled water (complex 6) to obtain stock solutions, which were then diluted to obtain the desired final concentrations. Control samples (except those for 6) were incubated in the presence of 1% DMF.

F4N cells

Exponentially growing cells were incubated in culture medium with varying concentrations (100, 200 and 400 μmol/l) of the test compounds at 37 °C in an atmosphere containing 5% CO₂. After 24, 48 and 72 h of drug exposure the cells were counted hemocytometrically. The number of dead cells was determined by staining with trypan blue. The cell growth inhibition was expressed as the percentage of living cells in the treated samples with respect to that of the control. The mean of three determinations was taken.

SKW-3 and MDA-MB-231 cells

The cells were harvested, centrifuged and resuspended in electroporation medium (0.3 M mannitol solution in sterile distilled water, with 0.1 mM Mg²⁺ and 0.1 mM Ca²⁺ added) to give a final concentration of 5 · 10⁵ cells/ml (5 · 10⁵ cells in 100 μl of sample). To those 100-μl samples, the corresponding amounts of the drugs were added. The samples were subjected or not to electroporation (electroporated or non-electroporated samples). Immediately after the treatment all samples were additionally diluted with RPMI-1640 complete medium to give a total volume of 1 ml (5 · 10⁵ cells/ml), and then 100-μl fractions (5 · 10⁵ cells) from each sample were distributed.
into an 8-well column of a sterile 96-well plate and left for 72 h for incubation (37 °C, 5% CO₂). The final concentrations of the compounds were 1, 10 and 100 μmol/l.

The electroporation of the samples was performed in chambers equipped with flat parallel electrodes (BioRad Laboratories, Richmond, CA, USA) and an electrode distance of 0.4 cm. Each electroporated sample was treated with one rectangular pulse of 1000 V/cm for 5 ms. A detailed description of the electroporation equipment used can be found in the literature (Haenze et al., 1998). The controls of the electroporated set of samples were subjected to an electrical pulse in the absence of any drug. The controls of the non-electroporated set of samples were subjected neither to electrical nor to drug treatments.

The treated samples and the controls were seeded in 96-well plates (flat tissue bottom; Becton Dickinson, Heidelberg, Germany) at a density of 5 · 10⁴ cells/100 μl well. The plates were placed for 72 h at 37 °C in an incubator with humidified atmosphere and 5% CO₂. For each treatment, at least 8 wells were used. The cell viability fraction was determined by the Mosmann MTT dye-reduction assay (Mosmann, 1983) with some modifications (Konstantinov et al., 1998). In brief, after the incubation period of the samples, MTT solution (10 mg/ml) in phosphate buffer saline (PBS) was added (10 μl/well). The plates were further incubated for 4 h at 37 °C and the formazan crystals formed were dissolved by adding 110 μl acidified (0.04 M HCl) 2-propanol per well. Absorption was measured by an automated microtiter plate spectrophotometer (Anthos) at 540 nm, reference filter at 690 nm. A mixture of complete medium (100 μl), MTT stock (10 μl) and 110 μl acidified (0.04 M HCl) 2-propanol was used as blank solution. The cell growth inhibition was expressed as the percentage of the absorption of the treated sample with respect to that of the control. The mean of at least three determinations was taken.

The 50%-inhibitory concentration (IC₅₀) is defined as the drug concentration that reduces twice the number of living cells (trypan blue assay) or the absorption (MTT assay) as compared to the control. The IC₅₀ values were obtained by linear regression using Origin 6.0 software.

**Flow cytometry (FACS) analysis**

The MDA-MB-231 cell line was used for these experiments. The samples were subjected to cisplatin or PMSA and its complexes at different concentrations (10 and 100 μmol/l) in the presence or absence of electrical pulses and then further incubated for 72 h (37 °C, 5% CO₂). After that, FACS analysis of each sample was performed according to Watson and Erta (1992). Briefly, controls or treated samples were pelleted, washed with cold PBS and re-suspended in 100 μl PBS and 300 μl of 96% ethanol. The cells were kept at −20 °C. On the day of FACS measurements, the samples were centrifuged and re-suspended in 500 μl PBS containing 20 μg/ml RNase and 20 μg/ml propidium iodide at room temperature. 1–2 · 10⁴ Cells/sample were analyzed with a FACS Calibur cell sorter (Becton Dickinson), equipped with a 488 nm air-cooled argon laser, using CellQuest software.

**Results and Discussion**

**Cytotoxic effect**

*F4N cells*

The compounds were at first tested against this cell line without electroporation. PMSA and its complexes did not exhibit important cytotoxic effects: the most active was complex 1 [IC₅₀ = (248 ± 8) μmol/l (72 h of drug exposure)], whereas the IC₅₀ values for the free ligand and the other complexes were above 400 μmol/l.

*SKW-3 and MDA-MB-231 cells*

The cytotoxic effect of the free ligand and its complexes (Fig. 1) was examined with and without electroporation. The results of cell growth inhibition assays are presented in Figs. 2 and 3, and the IC₅₀ values are collected in Table I.

If we accept an IC₅₀ value below 100 μmol/l as criterion for interesting activity, only the cis-diiodo complex 4 exhibited this degree of cytotoxic activity against SKW-3 cells without electroporation [IC₅₀ = (69 ± 13) μmol/l], whereas its trans-analogue 5 showed marginal activity [IC₅₀ = (102 ± 8) μmol/l]. The electroporation, however, considerably increased the cytotoxicity of all compounds tested against SKW-3 cells. Complexes 3–6 exhibited IC₅₀ values lower than 100 μmol/l. The most active was again the cis-diiodo complex 4, showing a significant effect [IC₅₀ = (20 ± 2)
μmol/l], followed by its trans-isomer, which was three times less active. In this cell line the use of electroporation caused a 3.5-fold increase in the cytotoxicity of 4, whereas for cisplatin the effect was six-fold, in comparison with the result without electroporation.

With the exception of the tetra-amine cationic complex 6, all the tested compounds exhibited interesting cytotoxic activity (marginal value for PMSA) against MDA-MB-231 cells, even without electroporation. The activity of 1, 4 and 5 was practically the same (IC\(_{50}\) ≈ 60 μmol/l), and it was only 1.3-fold smaller than that of cisplatin. This result is especially important taking into account that MDA-MB-231 is a cisplatin-resistant tumour cell line (Chu et al., 2005). Under the conditions of electroporation all the compounds, including the free ligand, showed increased cytotoxic activity against this cell line. As before, the cis-diiodo Pt(II) complex 4 was the most active [IC\(_{50}\) = (29 ± 6) μmol/l]. Practically the same activity exhibited its dichloro analogue 2. The trans-diiodo complex 5 also showed a considerable cytotoxicity [IC\(_{50}\) = (43 ± 9) μmol/l]. In the case of MDA-MB-231 cells, the electroporation caused a more than 20-fold increase in the cisplatin cytotoxicity [IC\(_{50}\) =

<table>
<thead>
<tr>
<th>Compound</th>
<th>SKW-3 cells</th>
<th>MDA-MB-231 cells</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>without electroporation</td>
<td>with electroporation</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>36 ± 2</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>PMSA 1</td>
<td>a</td>
<td>117 ± 8</td>
</tr>
<tr>
<td>2</td>
<td>a</td>
<td>134 ± 10</td>
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<tr>
<td>3</td>
<td>a</td>
<td>84 ± 6</td>
</tr>
<tr>
<td>4</td>
<td>69 ± 13</td>
<td>20 ± 2</td>
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<tr>
<td>5</td>
<td>102 ± 8</td>
<td>63 ± 5</td>
</tr>
<tr>
<td>6</td>
<td>a</td>
<td>94 ± 24</td>
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\(\text{IC}_{50}\) values are means of at least three independent experiments ± standard deviations (SD).

- a No inhibition of cell growth at concentrations up to 100 μmol/l.
- b Not more than 20% inhibition of cell growth at concentrations up to 100 μmol/l.
- c Not more than 30% inhibition of cell growth at concentrations up to 100 μmol/l.

**Fig. 1.** Chemical structures of PMSA and its complexes 1–6.
(2 ± 2) μmol/l]. For the dichloro Pt(II) complex 2, the activity-enhancing effect of electroporation was three-fold, whereas there was no effect in the case of its Pd(II) analogue.

**Cell cycle FACS analysis**

The cell cycle FACS measurements were used to determine whether the new compounds provoke an apoptotic response among the treated cell population and to compare this effect with that of cisplatin. The red fluorescence intensity of propidium iodide (with emission maximum at ca. 620 nm) was used for the quantitative determination of the distribution among the cell cycle phases in each sample. A representative set of results including the control, cisplatin and complex 3 are illustrated in Fig. 4. The ligand and the remaining complexes studied gave quantitatively the same pattern. The FACS analysis results showed that electroporation alone did not influence dramatically the apoptosis level of the sample, but treatment with 10 μmol/l cisplatin and electroporation increased the amount of apoptotic cells after 72 h of incubation. The induction of apoptosis by cisplatin is well known and was described previously (Henkels and Turchi, 1999; Park et al., 2002; Schwerdt et al., 2005). Unlike cisplatin, however, the treatment with PMSA and its complexes did not provoke a pronounced apoptotic response within the sample sets subjected to electroporation or not (Fig. 4), even at a concentration of 100 μmol/l. This finding implies that the mechanism of cytotoxic action of the compounds studied differs from that of cisplatin and, probably, does not involve apoptosis triggering. It should be pointed out, however, that some authors suggest a different mechanism of cell death induction for cisplatin as well (Gonzalez et al., 2001).

**Conclusion**

Most representatives of the Pt(II) and Pd(II) complexes with the sulfonamide substituted pyridine ligand PMSA exhibited cytotoxic activity against SKW-3 and MDA-MB-231 tumour cells
under the conditions of electroporation. Against the cisplatin-resistant cells MDA-MB-231, the non-ionic complexes were also active without electroporation. The most active complex was cis-[Pt(PMSA)_2I_2]; its trans-isomer showed also significant activity. Platinum complexes of PMSA with cis- and trans-configuration deserve further attention as potential cytostatic agents.

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Fig. 4. MDA-MB-231 cell cycle FACS analysis of: (A, B) control samples without and with electroporation; (C, D) samples treated with 10 μmol/l of cisplatin without and with electroporation; (E, F) samples treated with 10 μmol/l of 3 without and with electroporation. The different cell cycle phases are indicated by the markers: SubG1 phase (apoptotic cells), G1 phase (2n cells), S phase (synthetic), and M/G2 phase (mitotic). The exact values of the four markers are given below each marker as percentage of the total amount of cells in the sample.


