Abstract. Background: The in vitro cytotoxic activity of two new platinum(II) complexes (3P-SK and PtAMP) in comparison with cisplatin (CDDP), oxaliplatin (OXA) and carboplatin (CARBO) was determined in four different human tumour cell lines. The in vivo efficiencies of CDDP and 3P-SK in MCA mammary carcinoma tumours induced in CBA mice were compared. Materials and Methods: The in vitro cytotoxicity of the platinum (II) complexes was determined by the colorimetric MTT assay. The tumours were treated with different doses of platinum compounds, alone or combined with the local application of electric pulses to the tumour (electrochemotherapy). The antitumour effectiveness was determined by tumour growth inhibition. Results: CDDP and OXA were the most cytotoxic in all cells tested. 3P-SK was more cytotoxic when compared to CARBO in all cells tested, except in MCF7. Intratumoral injection of 3P-SK alone or combined with electroporation (electrochemotherapy) induced significant tumour growth delay and tumour cure. Conclusion: 3P-SK was found to be less cytotoxic to human tumour cell lines than CDDP and OXA, but displayed a higher cytotoxicity compared to CARBO. In the experimental tumour model of mammary carcinoma, treatment with 3P-SK, alone or in combination with electroporation, was less effective compared to CDDP, but nevertheless resulted in tumour growth inhibition after a single application.

Platinum-based drugs are the most active anticancer agents and have been widely used in the treatment of a variety of human tumours. Cisplatin (CDDP; cis-[PtCl2(NH3)2]) and carboplatin (CARBO; [Pt(cbdc)(NH3)2] (cbdc=1,1-cyclobutanedicarboxylate)), as a CDDP analogue, have been in widespread use for many years to treat testicular, ovarian, cervical, head and neck and non-small cell lung cancer (1, 2). However, the treatment is limited by side-effects, including nephrotoxicity, emetogenesis and neuretoxicity (1, 2). The cellular processes by which CDDP enters and attacks cells include uptake and transport, formation of DNA adducts and their recognition by damage-response proteins and signal transduction leading to cell cycle arrest, repair and/or cell death (1, 3). However, cells posses several resistance mechanisms to avoid the damage caused by CDDP. Glutathione has been implicated in resistance since it was found to react with drugs to form inactive platinum thiol species, thus reducing drug accumulation. The major mechanisms are limitation of drug levels inside the cells by reduced uptake and/or increased efflux, increased cellular thiol levels, enhanced DNA repair and/or increased damage tolerance and failure of the cell-death pathway (1, 4, 5).

To overcome cellular resistance and/or reduce the toxicity of both first- (e.g., CDDP) and second-generation (e.g., CARBO) platinum drugs, a large number of platinum analogues have been synthesised and screened for anticancer activity, however only one, namely oxaliplatin (OXA), has been approved for treatment of colorectal cancer in clinical practice (6, 7). OXA has demonstrated antitumour activity in cell lines with acquired CDDP resistance and appeared to be active in tumour types that are intrinsically resistant to CDDP and CARBO. The sterically hindered platinum (II) compound AMD473 (cis-[PtCl2(2-pic)2] (2-pic = 2-methylpyridine) was designed based on an improved understanding of the mechanisms of platinum drug resistance and possesses a reduced susceptibility to binding thiols compared to CDDP (8-10). The large body of evidence on the cellular processing of platinum complexes, and an understanding of the induced DNA damage and how damage signals are transduced, provide an important basis for the design of new platinum-based compounds.
Another approach to increasing the antitumour effectiveness of platinum complexes and lowering their side-effects is the use of various drug delivery systems. One of these systems, already proven to be effective in clinical trials, is electroporation (11, 12). The local application of electric pulses to tumours causes the cells in the tumour tissue to become permeable, thus allowing up to two times higher CDDP concentrations in the cells (13). Therapy combining either systemically or locally administered CDDP and the subsequent application of electric pulses to tumours is termed electrochemotherapy. Electrochemotherapy is effective only with drugs whose transport into the cells is hampered, such as CDDP complexes and also bleomycin (11-13). The results of the preclinical studies and clinical trials, predominantly on subcutaneous metastases of malignant melanoma, have demonstrated that electrochemotherapy induced long-lasting local tumour control. Clinical studies demonstrated 60-80% complete responses. Furthermore, as increased uptake of CDDP is obtained by electroporation, the dose of CDDP used in electrochemotherapy is very low and, therefore, without systemic side-effects (11, 14).

In this study, the cytotoxicity of two newly synthesised platinum complexes was examined in four human tumour cell lines and their cytotoxicity was compared to that of CDDP, CARBO and OXA. Complex 3P-SK ([Pt(skv)(3py)2] (skv=3,4-dioxocyclobut-1-ene-1,2-diolate, 3py=3-hydroxymethylpyridine)) was the most potent of the newly synthesised complexes. In three cell lines, T24, IGROV1 and IGROV1/RDDP, 3P-SK was even more effective than CARBO. Therefore, the antitumour effectiveness of intratumoral injection of 3P-SK, alone or combined with electroporation, was tested in vivo in MCA mammary carcinoma in mice and was compared to the effectiveness of CDDP.

Materials and Methods

**Drugs.** Five platinum(II) complexes were used in the study: CDDP (Platamine, Pharmacia & Upjohn S.p.A., Milan, Italy), CARBO (Paraplatin, Bristol-Myers Squibb, Sermoneta, Italy), OXA ([Pt(oxalato) (trans-R,R-dach)] (dach=cyclohexane-1,2-diamine)), Elonaite, Sanofi-Synthelabo, Paris, France) and two new platinum(II) complexes which were synthesised at the University of Ljubljana, Faculty of Chemistry and Chemical Technology, Ljubljana, Slovenia: 3P-SK and PtAMP ([Pt(AMP)(AMPo)] (AMP=2-amino-2-methylprop-1-ol, AMPo=2-amino-2-methylprop-1-olate)). The synthesis of the platinum(II) complexes will be described in detail in a separate publication. Stock solution of the compounds, except CARBO, were prepared in distilled water and kept at −20 °C. Further dilutions were made in serum-free medium and were freshly prepared for each experiment. CARBO, which had been dissolved in distilled water at 10 mg/ml, was kept at 4 °C. For in vivo experiments, different doses of the compounds were prepared in 0.9% NaCl.

**Cell lines.** Four different human tumour cell lines were used in the experiments. Bladder carcinoma T24 (HBT-4, ATTC, Manassas, USA) and mammary carcinoma MCF7 (HBT-22, ATCC) cells were grown in Eagle’s minimum essential medium (EMEM; Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated foetal calf serum (FCS; Sigma). The ovarian carcinoma IGROV1 and ovarian carcinoma IGROV1/RDDP cell lines resistant to CDDP, generously provided by Dr. J. Benard, Institut Gustave Roussy, Villejuif, France, were grown in RPMI 1640 (Sigma) supplemented with 15% FCS. The cells were maintained in a humidified atmosphere containing 5% CO2 at 37 °C.

**Treatment protocol in vitro.** The cells were plated into 96-well microtitre plates (CorningCostar, Acton, USA) at a density of 3000 or 5000 cells/well in 50 μl medium. After allowing time for the cells to attach (~ 30 min), 50 μl of different concentrations of the platinum complexes were added to the wells (eight wells/drug concentration). The cells were incubated with the platinum complexes for 3 or 5 days at 37 °C in a humidified atmosphere containing 5% CO2. The cytotoxicity of the compounds was determined by the colorimetric MTT assay. At the end of the incubation period, 20 μl of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) were added to each well and the microtitre plates were further incubated for 4 h at 37 °C. The solution was then carefully removed and the formed formazan crystals were dissolved in 100 μl of dimethyl sulphoxide (Sigma). The microtitre plates were shaken for 60 sec to ensure adequate solubilisation before the absorbance of the resulting solution was measured at 570 nm using a microplate reader (Anthos Labtec, Salzburg, Austria). The survival of cells treated with different compounds is presented as a percentage of the absorbency obtained from the control untreated cells. The inhibitory concentration of each compound that reduced the survival of cells to 50% (IC50) was determined for all the cell lines graphically. The experiments were performed at least twice for each compound in all the cell lines.

**Animals and tumours.** The animals used in the experiments were female CBA mice obtained from the Institute of Pathology, Medical Faculty, University of Ljubljana (Ljubljana, Slovenia). The mice were kept in a conventional animal colony at 22 °C and in a natural day/night cycle. The tumour used was MCA mammary carcinoma (15). Single cell suspensions for the induction of subcutaneous tumours were obtained by mincing of the tumour from the donor mouse. Tumours were induced in the right flank of the mice by inoculation of 1x10⁶ viable MCA cells subcutaneously. The treatment started 6-8 days post-inoculation when the tumours had reached approximately 45 mm³ in volume. Mice subjected to the specific experimental protocol were randomly divided into experimental groups consisting of six to eight mice.

**Treatment protocol in vivo.** The animal studies were carried out according to the guidelines of the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (permission #: 323-02-170/2004) and in compliance with the EU directive 86/609/EEC. The MCA tumours were treated with CDDP or 3P-SK intratumorally and, 1 min thereafter, electric pulses were locally applied to the tumour. CDDP was injected at doses of 4, 8 and 12 mg/kg and 3P-SK at equimolar doses of 7, 14 and 21 mg/kg. The injection volume was 0.1 ml. Electroporation of the tumours was performed by application...
of eight square-wave electric pulses, delivered in two sets of four pulses in perpendicular directions, of 1040 V (1300 V/cm), with a pulse width of 100 µs and a repetition frequency of 1 Hz. The electric pulses were delivered to the tumours by two flat, parallel stainless-steel electrodes 8 mm apart (two stainless-steel strips: length 15 mm, width 7 mm, with rounded corners), which were placed percutaneously at the opposite margins of the tumour. Good contact between the electrodes and the skin was assured by means of ultrasonographic conductive gel. The electric pulses were generated by an electroporator Jouan GHT 1287 (Saint Herblaine, France). All the treatments were performed without anaesthesia and were well tolerated by the animals (16).

Tumour growth was followed by measuring three mutually orthogonal tumour diameters (e1, e2 and e3) with a vernier calliper three times per week. The tumour volumes were calculated by the formula \( V = \pi \times e_1 \times e_2 \times e_3 / 6 \). The arithmetic mean of the tumour volumes and the standard error of the mean (SE) were calculated for each experimental group for each day of measurement. The tumour doubling-time was determined for each individual tumour from the growth curves. The tumour growth delay was calculated for each individual tumour by subtracting the doubling-time of each tumour from the mean doubling-time of the control group and then averaging for each experimental group. Animals that were tumour-free for 100 days were declared cured.

**Statistical analysis.** Significance tests were carried out on the data groups using analysis of variance (ANOVA) followed by the Student's t-test for individual pair-wise comparisons, with values of \( p < 0.05 \) considered to be significant. The statistical analysis was carried out using SigmaStat statistical software (SPSS, Chicago, USA).

**Results**

**In vitro cytotoxicity.** The cytotoxicity of the platinum (II) complexes CDDP, OXA and CARBO, as well as the new platinum complexes, was determined in four human tumour cell lines, T24, MCF7, IGROV1 and IGROV1/RDDP, using the MTT assay. Among three clinically used platinum (II) compounds, CDDP was the most cytotoxic in all cell lines, except for T24 bladder carcinoma, where OXA was the most effective. Of the two newly synthesised platinum (II) compounds, 3P-SK was more cytotoxic than PtAMP. Its IC\(_{50}\) value was statistically significantly smaller than that of CARBO in three cell lines (T24, IGROV1 and IGROV1/RDDP). The other compound was only marginally toxic and the IC\(_{50}\) values were at the limit of solubility of the compound. However, IGROV1/RDDP, a cell line resistant to CDDP, was less resistant to these new compounds compared to CDDP, as well as to CARBO and OXA. The increase in the IC\(_{50}\) value in the IGROV/RDDP cells compared to the IC\(_{50}\) value in IGROV cells was 20-fold for CDDP, 32-fold for OXA, 16-fold for CARBO, but only 6- and 4-fold for 3P-SK and PtAMP, respectively (Figure 1, Table I).

**In vivo antitumour effectiveness.** From the two newly synthesised platinum (II) complexes, the 3P-SK complex was selected, due to its high cytotoxicity in vitro, for the in vivo evaluation of its antitumour effectiveness in comparison with equimolar doses of CDDP. In addition, electroporation, a physical drug delivery system, was used to potentiate their delivery to the cells in tumours.

3P-SK, injected intratumourally, induced pronounced antitumour effectiveness with an approximately 3-day tumour growth delay and even a cured tumour at the highest dose tested. However, 3P-SK was less effective than CDDP at all the doses tested. CDDP induced from 9-13 days' tumour growth delay and approximately 35% of the tumours were cured. The antitumour effectiveness of both the platinum (II) complexes was not dose-dependent.

When intratumoural injection of platinum (II) complexes was combined with the application of electric pulses to the tumour, the antitumour effectiveness of both complexes was potentiated by approximately 2-fold (Figure 2, Table II).

**Discussion**

The results of this study indicate that the newly synthesised platinum complexes, 3P-SK and PtAMP, could be potentially useful in the treatment of CDDP-resistant tumours. The CDDP-resistant ovarian carcinoma cell line, IGROV1/RDDP, was approximately 4-fold less resistant to 3P-SK and PtAMP compared to other clinically used platinum compounds.

The 3P-SK complex was more cytotoxic compared to PtAMP in vitro and its antitumour effectiveness was also tested in murine mammary carcinoma. The selection of the tumour model was based on the in vitro results, since mammary carcinoma cells are very resistant to 3P-SK and we wanted to test whether the high intrinsic resistance of mammary carcinoma to 3P-SK was also present in the in vivo system. The antitumour effect was pronounced and, when combined with electric pulses (electrochemotherapy), 37.5% of the tumours were cured after a single application. However, the antitumour effectiveness was less pronounced compared to CDDP, where at the highest dose tested 93% of the tumours were cured with electrochemotherapy. Therefore, in order to fully explore the potential of this new compound, a panel of different tumour models with different CDDP resistance should be tested.

Platinum compounds, especially CDDP, are widely used in the treatment of different cancers since they demonstrate a high effectiveness in chemotherapy protocols, as well as in combination with other therapies, such as radiotherapy (1, 17). The biggest obstacle to successful CDDP-based treatment protocols is that many tumours are intrinsically resistant or that some tumours acquire resistance during the treatment (1, 5). Therefore, the development of many new platinum compounds with different mechanisms of action to overcome resistance is
underway and the promising candidates are being tested for their antitumour effectiveness. In our study, two platinum (II) complexes, synthesised with the aim of overcoming CDDP resistance, were tested. Two novel Pt(II) complexes, 3P-SK and PtAMP, were prepared with the amine ligands 3-hydroxymethylpyridine and 2-amino-2-methylpropan-1-ol, respectively. Both ligands have a hydroxy group, which is a good hydrogen donor or acceptor and could additionally stabilise the interaction between the platinum (II) complex and DNA through the formation of hydrogen bonds. We previously prepared the platinum (II) squarato complex, which is very rare (18). The squarato anion as a leaving ligand usually engages two of its oxygen atoms in coordination with two platinum atoms (μ-1,2-bis(unidentate) coordination mode). The iodo analogues of cisplatin have not been studied to a great extent, mainly because of their low solubility in water. We prepared the platinum (II) iodo complex PtAMP with very good solubility in water.

The results of our previous study on the cytotoxicity of the platinum (II) complex with isopropylamine and squarato ligands showed that the survival of bladder carcinoma T24 cells was minimally reduced after exposure to these platinum compounds (18). Obviously, the replacement of

<table>
<thead>
<tr>
<th>Platinum(II) complexes</th>
<th>Cell lines</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>T24</td>
</tr>
<tr>
<td>CDDP</td>
<td>2.8±0.3</td>
</tr>
<tr>
<td>OXA</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>CARBO</td>
<td>72.2±4.8</td>
</tr>
<tr>
<td>3P-SK</td>
<td>28.8±3.5</td>
</tr>
<tr>
<td>PtAMP</td>
<td>241.4±14.7</td>
</tr>
</tbody>
</table>
isopropylamine as a carrier ligand by 3-hydroxy-methylpyridine generates a higher cytotoxic activity of the platinum (II) complex 3P-SK, as shown in the present study. Specifically, the 3P-SK complex was less cytotoxic than CDDP or OXA, but more than CARBO in all the cell lines tested, apart from MCF7. In addition, both compounds showed high cytotoxic action against a CDDP-resistant ovarian carcinoma cell line, although they were less cytotoxic in the parental cell line (IGROV1) compared to CDDP and OXA. Both of these clinically used compounds were cross-resistant with CDDP (resistance level ~20-fold) in CDDP-resistant IGROV1/RDDP cells, while 3P-SK and PtAMP circumvented the acquired CDDP resistance in the resistant subcloned cell line. The levels of resistance were only 4- to 6-fold. Many other platinum analogues also showed high efficiency against CDDP-resistant cells and tumours. Among them, AMD473, which can circumvent resistance to CDDP as well as to OXA, is already in clinical phase I and II studies (8-10).

Electrochemotherapy is used for the treatment of cutaneous and subcutaneous tumours of different histologies. The basis of this highly efficient local therapy is increased drug transport into the cells of accessible tumours due to electroporation of the tumour, which causes increased cell membrane permeability and thus increased drug uptake (12, 13). Currently, two drugs are employed in electrochemotherapy, including CDDP. Electrochemotherapy, using the intratumoral administration of CDDP, resulted in 88% long-term complete response in malignant melanoma cutaneous and subcutaneous metastases (11, 14). In the present study, the best conditions for electrochemotherapy with CDDP and electrochemotherapy with the potentially effective 3P-SK were compared. It was found that 3P-SK had a profound antitumour effectiveness against MCA murine mammary carcinoma, but was less effective than CDDP. It is worth mentioning that the application of electric pulses to a tumour potentiates the antitumour effectiveness of both compounds to approximately the same degree (2-fold). The transport mechanisms of CDDP and its mode of cytotoxic action have been the subject of many studies, but the exact mechanisms still remain unclear (1, 3).

In the view of the obtained data on antitumour effectiveness, it can be speculated that 3P-SK possesses a similar transport mechanism to CDDP, as well as a similar intracellular mode of action. However, taking into account the data from in vitro experiments, where the difference in cytotoxicity between

**Figure 2. Tumour growth delay (GD) and tumour cures (CR) of MCA tumours treated with CDDP or 3P-SK alone and combined with the application of electric pulses (electrochemotherapy; ECT). Data are mean±SE of at least 13 animals/group pooled from two independent experiments.**

**Table II. Tumour doubling-times (DT) and number of tumour cures (CR) of MCA tumours treated with 3P-SK or CDDP intratumorally alone or combined with the application of electric pulses (EP).**

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>no.</th>
<th>DT (days)</th>
<th>Cures no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14</td>
<td>2.1±0.2</td>
<td>0</td>
</tr>
<tr>
<td>EP</td>
<td>13</td>
<td>2.8±0.3</td>
<td>0</td>
</tr>
<tr>
<td>3P-SK 7 mg/kg</td>
<td>13</td>
<td>5.2±1.9</td>
<td>0</td>
</tr>
<tr>
<td>3P-SK 14 mg/kg</td>
<td>12</td>
<td>3.6±0.2</td>
<td>0</td>
</tr>
<tr>
<td>3P-SK 21 mg/kg</td>
<td>13</td>
<td>5.4±1.1</td>
<td>1 (7.7)</td>
</tr>
<tr>
<td>ECT+ 3P-SK 7 mg/kg</td>
<td>14</td>
<td>11.2±2.6</td>
<td>2 (14.3)</td>
</tr>
<tr>
<td>ECT 3P-SK 14 mg/kg</td>
<td>14</td>
<td>10.3±2.2</td>
<td>1 (7.1)</td>
</tr>
<tr>
<td>ECT 3P-SK 21 mg/kg</td>
<td>16</td>
<td>11.7±1.7</td>
<td>6 (37.5)</td>
</tr>
<tr>
<td>CDDP 4 mg/kg</td>
<td>15</td>
<td>11.2±2.9</td>
<td>5 (33.3)</td>
</tr>
<tr>
<td>CDDP 8 mg/kg</td>
<td>14</td>
<td>13.3±3.4</td>
<td>5 (35.7)</td>
</tr>
<tr>
<td>CDDP 12 mg/kg</td>
<td>14</td>
<td>14.8±3.7</td>
<td>5 (35.7)</td>
</tr>
<tr>
<td>ECT CDDP 4 mg/kg</td>
<td>14</td>
<td>29.7±4.0</td>
<td>11 (71.4)</td>
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<tr>
<td>ECT CDDP 8 mg/kg</td>
<td>14</td>
<td>28.0±7.1</td>
<td>12 (85.7)</td>
</tr>
<tr>
<td>ECT CDDP 12 mg/kg</td>
<td>14</td>
<td>35.5±0</td>
<td>13 (92.9)</td>
</tr>
</tbody>
</table>

*electrochemotherapy.
CDDP and 3P-SK was more that 2-fold (approximately 3 to 40 times, depending on the cell line), some other mechanism must be involved in the mode of action of 3P-SK in vivo. Currently, these mechanisms are not known, but they most probably involve either increased response of the immune system or an antivascular effect, or a combination of both.

In conclusion, the results of our study showed that 3P-SK, a new platinum (II) complex, was less cytotoxic to human tumour cell lines than CDDP and OXA, but possessed higher cytotoxicity compared to CARBO. Specifically, this compound was not cross-resistant to CDDP, as tested on a pair of ovarian carcinoma cell lines with acquired CDDP resistance. In an experimental tumour model of mammary carcinoma, treatment with 3P-SK, alone or in combination with electroporation, was less effective than CDDP, but, nevertheless, resulted in tumour cure after a single application. Further studies on other tumour models are foreseen to demonstrate antitumour effectiveness and to elucidate the mechanism involved in the response of tumours to 3P-SK.

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References


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