Cellular Pharmacokinetic/Pharmacodynamic Relationship of Platinum Cytostatics in Head and Neck Squamous Cell Carcinoma Evaluated by Liquid Chromatography Coupled to Tandem Mass Spectrometry

Dirk Theile, Jan-Christoph Detering, Christel Herold-Mende, Gerhard Dyckhoff, Walter E. Haefeli, Johanna Weiss, and Jürgen Burhenne

Department of Clinical Pharmacology and Pharmacoepidemiology (D.T., J.-C.D., W.E.H., J.W., J.B.), Division of Neurosurgical Research, Department of Neurosurgery (C.H.-M.), and Section of Molecular Cell Biology Group, Department of Otorhinolaryngology, Head and Neck Surgery (C.H.-M., G.D.), University of Heidelberg, Heidelberg, Germany

Received November 1, 2011; accepted December 28, 2011

ABSTRACT

Cisplatin (diaminodichloroplatinum) is the favored platinum (Pt) drug for the treatment of head and neck squamous cell carcinoma (HNSCC). However, Pt drug alternatives such as carboplatin (diaminoplatinum-cyclobutan-1,1-dicarboxylate) or oxaliplatin [oxalato[(1R,2R)-cyclohexanediylamino]platinum] have not been comprehensively investigated in HNSCC. Moreover, little data reveal the decisive efficacy determinant and whether Pt drug efficacy is truly concentration-dependent. Using five human HNSCC cell lines, we determined the concentrations of cisplatin, carboplatin, and oxaliplatin leading to 50% inhibition of cell proliferation (IC50). Concurrently we quantified cellular drug uptake by liquid chromatography coupled to tandem mass spectrometry and evaluated mRNA expression of drug transporters involved in Pt drug uptake by quantitative real-time polymerase chain reaction. Mean IC50 among the five cell lines was 6.2 \( \pm \) 1.9 \( \mu \)M for cisplatin and 11.6 \( \pm \) 4.2 \( \mu \)M for oxaliplatin, whereas carboplatin showed significantly lower proliferation inhibition (IC50 107.5 \( \pm \) 21.2 \( \mu \)M). In agreement with this finding carboplatin poorly accumulated in HNSCC cells, compared with cisplatin and oxaliplatin. HNSCC cell lines expressed Pt drug transporters. Taken together, the results demonstrate: 1) carboplatin was less effective and was poorly taken up; 2) a high individuality among cell lines was found concerning the accumulation of cisplatin and oxaliplatin despite similar in vitro efficacy; and 3) distinct expression of SLC22A2 and ABCC2 accompanies strong uptake and cytotoxicity of Pt drugs. In conclusion, we demonstrate that in vitro efficacy of cisplatin and oxaliplatin in HNSCC is concentration-independent because they exhibited different uptake characteristics but similar efficacies, suggesting oxaliplatin as a promising alternative against HNSCC that needs further evaluation in clinical trials.

Introduction

Eighty to 90% of all tumors arising from the upper aerodigestive tract are derived from squamous epithelial cells and are called head and neck squamous cell carcinoma (HNSCC) (Hoffman et al., 1998). In addition to surgery and radiation, systemic chemotherapy remains a cornerstone in the curative or palliative treatment of HNSCC (Panucci and Khuri, 2004; Colevas, 2006). Randomized clinical trials revealed that cisplatin (diaminodichloroplatinum) plus 5-fluorouracil (5-FU) was more effective than either compound alone or carboplatin (diaminoplatinum-cyclobutan-1,1-dicarboxylate) plus 5-FU, thus establishing cisplatin plus 5-FU as the reference regimen against HNSCC (Jacobs et al., 1992; Forastiere et al., 1998). However, the contribution of individual agents to overall efficacy is uncertain and therefore must be evaluated in single-agent clinical trials as performed earlier. In these trials, many antineoplastic agents including platinum (Pt)-containing cytostatics have been investigated with no evident superiority of a particular drug (Colevas, 2006). Above all, data are sparse or contradictory, which questions cisplatin as the standard Pt drug: cisplatin has been demon-
stratified to cause response rates of 15 to 17% (Jacobs et al., 1992; Clavel et al., 1994), whereas carboplatin caused higher response rates of 26% in earlier trials (Eisenberger et al., 1986). For oxaliplatin [oxalato[(1R,2R)-cyclohexanediaminoniclonium]platinum] the only single-agent clinical trial suggests efficacy with response rates of 10 to 17% (Degardin et al., 1996).

Based on the assumption that the efficacy of Pt drugs is closely linked to DNA platination, studies investigated the relationship between pharmacodynamic biomarkers and clinical efficacy. For instance, the extent of DNA platination in leukocytes correlated with clinical outcome in some (Fichtinger-Schepman et al., 1990; Schellens et al., 1996), but not all (Fisch et al., 1996), studies. The causes for the observed highly variable efficacies of Pt drugs and the underlying molecular mechanisms remain unknown for HNSCC.

In other cancer entities, Pt drug efficacy was modulated by increased repair of Pt-caused DNA damage, inactivation of Pt by Pt-binding metallothioneins, or action of Pt drug transporters [human copper transporter 1/solute carrier (SLC; SLC31A1), and copper-transporting ATPases ATP7A (Menkes disease protein) and ATP7B (Wilson disease protein)], which are physiologically involved in copper homeostasis (Rabik and Dolan, 2007). In addition, multidrug resistance-associated protein 2 (MRP2/ABCC2) and organic cation transporter 2 (OCT2/SLC22A2) have been implicated in Pt drug transport and resistance (Guminski et al., 2006; Zhang et al., 2006; Burger et al., 2010). Taken together, these remaining uncertainties raise the question of whether Pt drug efficacy in HNSCC is truly concentration-dependent and whether and which of these mechanisms is the decisive determinant of individual drug efficacy.

For the quantification of intracellular Pt concentration gas chromatography–mass spectrometry (Aggarwal et al., 1993), flameless atomic absorption spectrometry (Drummer et al., 1984), and liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) have been successfully used (Minakata et al., 2006). After complexation with diethyldithiocarbamate (DDTC) all three analyzed Pt drugs result in the same Pt(DTDC)2 and Pt(DTDC)3 chelate complexes (Drummer et al., 1984; Raghavan et al., 2000; Verschaeren et al., 2003; Minakata et al., 2006) that can be separated under reversed-phase LC conditions, ionized by electrospray ionization (ESI), and sensitively detected.

The aim of this in vitro study was to develop an LC/MS/MS method to quantify Pt disposition in human HNSCC cell lines, evaluate the relationship between antiproliferative responses and cellular Pt concentration, and assess its modulation by drug transporters.

### Materials and Methods

**Materials.** Culture media, medium supplements, antibiotics, trypsin, EDTA solution, sodium diethylthiocarbamate, palladium acetate, ammonium acetate, and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Fetal calf serum was purchased from PAA Laboratories GmbH (Colbe, Austria). Crystal violet was from AppliChem (Darmstadt, Germany). Methanol and acetonitrile used for LC/MS/MS analysis were of the highest grade from Merck (Darmstadt, Germany) and Carl Roth GmbH (Karlsruhe, Germany), respectively.

Ninety-six-well (700 μl) collection plates were from Eppendorf AG (Hamburg, Germany); 96-well, 0.66-mm glass-fiber filter plates were purchased from Corning Glassworks (Corning, NY); 96-well (300 μl) microtiter plates for cell culture were from NUNC GmbH & Co. KG (Wiesbaden, Germany); and cell culturing bottles were from Greiner Bio-One GmbH (Frickenhausen, Germany). Deionized water was obtained from a HP 6 UV/UF TKA ultra-filtration system (TKA GmbH, Niedereberg, Germany). The Pt-containing cytostatics cisplatin, oxaliplatin, and carboplatin were obtained from the University of Heidelberg’s hospital pharmacy.

**Cell Lines.** The HNSCC cell lines have been derived from intraoperatively obtained samples, established, and characterized as reported previously (Ninck et al., 2003) (Table 1). The five cell lines used represent tumors of different sexes (two females, three males), different localizations, and different tumor stages, but all lack chemotherapeutic approaches to minimize bias from antineoplastic agents potentially altering drug transporter expression or drug resistance.

**Proliferation Assay.** Cell proliferation was quantified by crystal violet staining (Peters et al., 2006). Cells were seeded onto 96-well microtiter plates and preincubated for 24 h. After the addition of test compounds, cells were incubated for another 48 h. Cells were then washed with PBS and stained with crystal violet (0.5%) as described previously (Peters et al., 2006). Absorption was measured by using a Multiskan RC photometer (Thermo Fisher Scientific GmbH, Langenselbold, Germany) with 555-nm excitation. Proliferation was expressed as proliferation index by calculating crystal violet absorption intensity as a percentage relative to baseline (absorption intensity of untreated cells; set to 100%). Each concentration was tested eight times, and each assay was performed three times.

**Concentration-Dependent Uptake of Pt Drugs.** Cells were seeded onto 96-well microtiter plates and preincubated for 24 h. After the addition of blank medium into two columns and test compounds into four columns (5, 10, 50, and 100 μM, respectively), cells were incubated for another 48 h. Cells were then washed with PBS and stained with crystal violet (0.5%) as described previously (Peters et al., 2006). Absorption was measured by using a Multiskan RC photometer (Thermo Fisher Scientific GmbH, Langenselbold, Germany) with 555-nm excitation. Proliferation was expressed as proliferation index by calculating crystal violet absorption intensity as a percentage relative to baseline (absorption intensity of cells with medium only; set to 0%) and native proliferation (absorption intensity of untreated cells; set to 100%). Each concentration was tested eight times, and each assay was performed three times.

### Table 1: Patient data

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Localization of Tumor</th>
<th>Age at Diagnosis</th>
<th>TNM</th>
<th>Histological Grading</th>
<th>Therapy</th>
<th>PFS</th>
<th>OS</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNO41</td>
<td>Male</td>
<td>Tonsils/oropharynx</td>
<td>52 years 5 months</td>
<td>T2N2bM0</td>
<td>2</td>
<td>Op + Rad</td>
<td>10</td>
<td>14</td>
<td>+</td>
</tr>
<tr>
<td>HNO150</td>
<td>Female</td>
<td>Larynx</td>
<td>47 years</td>
<td>T3N2bM0</td>
<td>2</td>
<td>Op + Rad</td>
<td>15</td>
<td>23</td>
<td>+</td>
</tr>
<tr>
<td>HNO199</td>
<td>Female</td>
<td>Oral cavity</td>
<td>69 years</td>
<td>T2N2cM0</td>
<td>3</td>
<td>Op + Rad</td>
<td>9</td>
<td>9</td>
<td>+</td>
</tr>
<tr>
<td>HNO210</td>
<td>Male</td>
<td>Larynx</td>
<td>69 years</td>
<td>T3N3Mx</td>
<td>3</td>
<td>Op + Rad</td>
<td>2</td>
<td>17</td>
<td>–</td>
</tr>
<tr>
<td>HNO211</td>
<td>Male</td>
<td>Tonsils/oropharynx</td>
<td>60 years</td>
<td>T4N2cM0</td>
<td>3</td>
<td>Op + Rad</td>
<td>10</td>
<td>10</td>
<td>–</td>
</tr>
</tbody>
</table>

TNM, initial tumor staging according to the Union for International Cancer Control; Op, operation; Rad, radiation; PFS, progression free survival; OS, overall survival after surgery; M, occurrence of distant metastases.
normalized to 10,000 cells evaluated in two wells by CASY cell counter methodology (Schärfe System, Reutlingen, Germany). Subsequent intracellular Pt concentrations after the exposure of cells with 5, 10, 50, or 100 μM compound were determined. Figure 1 shows the analytical approach. The corresponding area under the curve (AUC) characterizing this interrelation was determined for every single HNSCC cell line to express the uptake characteristics of the particular drug or cell line (Fig. 1).

**LC/MS/MS Conditions.** For quantification of the Pt complex an LC/MS/MS system (Thermo Fisher Scientific, Dreieich, Germany) consisting of a HTE PAL autosampler, SpectraSystem P4000 HPLC System with ERC 310SP degasser, and a triple stage quadrupole mass spectrometer TSQ 7000 with API-2 ion source was used. Chromatographic separation was done on a Luna column [C18(2), 100 Å; 150 × 2.0 mm, 3 μm; Phenomenex, Aschaffenburg, Germany] with guard column at a temperature of 50°C. The isocratic eluent consisted of 80% methanol and 20% 5 mM ammonium acetate buffer with 0.1% acetonitrile. The flow rate was 0.25 ml/min, the run time was 5.5 min, and the first 2 min were diverted into the waste. ESI interface parameters were as follows: middle position, spray voltage 4.5 kV, sheath gas (N2) 90 psi, aux gas (N2) 20 scales, and capillary heater temperature 350°C. No additional voltage for in-source collision-induced dissociation was used. The multiplier voltage was set at 1.6 kV. The LC/MS/MS system was tuned by using Xcalibur system software version 1.2 to the Pt(DDTC)2 and Pt(DDTC)3 complexes using a stock solution and direct infusion via a syringe pump in the selected reaction monitoring mode. MS/MS transitions monitored in positive ion mode were m/z 492 → m/z 116 at 33V for Pt(DDTC)3, m/z 639 → m/z 492 at 33V for Pt(DDTC)2, and m/z 403 → m/z 116 at 25V for the internal standard palladium complex [Pd(DDTC)2].

**Calibration, Quality Control, and Validation Procedures.** Because of similar chemical properties palladium was chosen as internal standard. The stock solution was obtained by dissolving palladium-acetate in water. For calibration of the three Pt drugs standard stock solutions with a concentration of 1 mg/ml were diluted in water in volumetric flasks to yield eight standards per Pt drug with Pt complex target concentrations of 0.5, 3.0, 5.0, 12.5, 25.0, 50.0, 75.0, and 100.0 ng/ml (corresponding to 0.2, 1.0, 1.8, 4.9, 10.0, 20.3, 30.5, 40.7, and 81.4 pmol) in 200-μl samples. Three quality-control (QC) solutions were identically prepared from separate Pt drug stock solutions with target concentrations of 3.6, 22.5, and 67.5 ng/ml in 200-μl samples. The method was validated according to a Food and Drug Administration guideline (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf) by analyzing three analytical batches, each containing calibration samples (with two calibration curves) and QC samples (6-fold for each concentration). The QC samples were used for recovery determination including derivatization and extraction efficacy, for batch-to-batch accuracy, precision, and intercomplex accuracy and precision testing.

**Quantification of Intracellular Pt Concentration.** After 4-h exposure of the cells, the Pt drug-containing medium was discarded and the cells were washed twice with 200 μl of PBS for 10 min on a shaker at 200 rpm. Two wells of each Pt drug concentration were used for cell counting using CASY cell counter methodology. Ten wells with untreated cells were used to prepare calibration, and six were used for QC samples. For this purpose 50 μl of the respective calibration or QC solution was transferred into the respective wells, and 50 μl of water was added to the wells with Pt-treated cells for volume correction. Subsequently, 50 μl of internal standard was pipetted into all wells, and 100 μl of 0.002% DDTC in methanol was added. The wells were sealed with rubber caps, and the microtiter plate was placed into an ultrasonic bath for 1 min and incubated at room temperature for 1 h on a shaker at 1800 rpm. Samples were then transferred onto a Corning 96-well glass fiber filter plate, placed on a 700-μl Eppendorf collection plate, and centrifuged at 50g for 5 min. Subsequently, 200 μl of methanol was added onto the filter plate, and the plates were centrifuged again at 50g for 5 min. The collection plate was sealed with rubber caps and analyzed by injecting 25 μl of sample volume into the LC/MS/MS system.

**Quantification of mRNA Expression by Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction.** RNA was isolated by using the RNeasy Mini-Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions. cDNA was synthesized with the ReverTra A H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer’s instructions. Expression of mRNA of the respective genes was quantified by quantitative real-time reverse transcription polymerase chain reaction with a LightCycler 480 (Roche Applied Science, Mannheim, Germany). Polymerase chain reaction amplification was carried out in 20 μl of reaction volume containing 5 μl of 1:10 diluted cDNA by using the SYBR Green format with the Absolute QPCR SYBR Green Mix (Abgene, Hamburg, Germany). Primer sequences were published previously (Theile et al., 2009, 2011). The following genes were quantified: ABC2 (encoding for MRP2), SLC22A1 (encoding for OCT1), SLC22A2 (encoding for OCT2), SLC31A1 (encoding for human copper transporter 1), ATP7A, and ATP7B. Using LightCycler 480 software data were evaluated by calibrator-normalized relative quantification with efficiency correction as published previously (Albermann et al., 2005). The software calculated the relative amount of the target gene and the reference gene (ribosomal protein L13, RPL13) based on the crossing point. Results were expressed as the target/reference ratio divided by the target/reference ratio of the calibrator. Because expression data could not be normalized to untreated controls they were quantified by the 2−ΔΔCt method (Livak and Schmittgen, 2001). All samples were amplified in duplicate.

**Calculations and Statistical Analysis.** The amount of Pt drug in treated wells was calculated by using LCQuan Software version 1.2 and linear calibration. The mean value per incubation concentration (6-fold determination) together with the corresponding mean cell number obtained by the CASY cell counter was normalized to 10,000 cells by using Excel 2002 (Microsoft, Redmond, WA). Batch-to-batch accuracy was expressed as the deviation of the mean recovery from the nominal value in percentages. Batch-to-batch precision was calculated as the coefficient of variation by dividing the standard deviation and nominal value and was expressed in percentages. Intercomplex accuracy expressed as the mean difference between the Pt(DDTC)2 and Pt(DDTC)3 values divided by the nominal value in percentages. Intercomplex precision was calculated as the mean of the differences between the two Pt complexes value per sample in percentages.

Data are presented as means ± S.D. and were analyzed by using Prism version 5.0 and InStat version 3.05 (GraphPad Software Inc., San Diego, CA) by performing Student’s t-test.
San Diego, CA). Differences in in vitro efficacy of Pt drugs (IC_{50} values) and uptake characteristics AUC values were tested by using one-way ANOVA with Bonferroni’s post hoc test comparing Pt drugs. Different uptake of cisplatin or oxaliplatin in HNO211 was evaluated by using Student’s unpaired, two-tailed t test. A p value <0.05 was considered significant. Differences of cell lines concerning mRNA expression of the single genes were tested by using one-way ANOVA with Bonferroni’s post hoc test comparing all cell lines. A p value <0.05 was considered significant.

Results

Performance of the Analytical Methods. Derivatization of the Pt drugs with DDTC resulted in 2- and 3-fold complexed platinum derivatives. The expected natural abundance patterns for the five most frequent platinum isotopes in Pt(DDTC)_{2} of m/z 490 (23.7%), m/z 491 (27.8%), m/z 492 (26.3%), m/z 493 (7.9%), m/z 494 (9.4%), and Pt(DDTC)_{3} isotopes m/z 638 (20.3%), m/z 639 (25.4%), m/z 640 (26.2%), m/z 641 (10.8%), and m/z 642 (10.9%) were found, and the spectra are presented in Fig. 2. The mass transitions for derivatized platinum compounds [Pt(DDTC)_{2}, m/z 492 \rightarrow m/z 116; Pt(DDTC)_{3}, m/z 639 \rightarrow m/z 492] and palladium compound [internal standard Pd(DDTC)_{2}; m/z 403 \rightarrow m/z 116] were used for selective detection and sensitive quantification under reversed-phase liquid chromatography conditions. The mass transition of Pt(DDTC)_{3} was nearly five to six times more sensitive than Pt(DDTC)_{2}. Retention times for Pt and Pd complexes were 2.9 and 4.2 min, respectively (Fig. 3). Both analyzed mass transitions for the Pt complexes resulted in peaks at identical retention times.

Pt complex concentrations were measured by using the three Pt drugs in five different cell lines in a concentration range of 0.5 to 100 ng/ml. Calibration was linear, and the correlation coefficient was always >0.99. The lower limit of quantification was 0.5 ng/ml, although much lower concentration could have been determined by using only the Pt(DDTC)_{3} mass transition. The derivatization and extraction efficacy (recovery) were equal for platinum and palladium compounds and ranged between 80 and 110% depending on concentration. Accuracy and precision testing (Table 2) fulfilled the requirements for bioanalytical method validation of the Food and Drug Administration with batch-to-batch accuracy (precision) ranging from 0.1 to 10.5% (2.0–12.4%). The mean ± S.D. cell numbers in all tests was 4645 ± 52.6% cells per well, rendering a normalized calibration range from 0.25 ng/10,000 cells to 60.01 ng/10,000 cells for all three Pt drugs.

Proliferation-Inhibiting Properties of Cisplatin, Oxaliplatin, and Carboplatin. Proliferation assays revealed high differences in proliferation-inhibiting potency among Pt
drugs tested. The IC_{50} of cisplatin, oxaliplatin, and carboplatin ranged from 3.50 to 8.13, 7.91 to 18.11, and 71.85 to 127.1 μM, respectively, indicating similar in vitro efficacy of cisplatin and oxaliplatin. Carboplatin showed significantly diminished potency to inhibit tumor cell proliferation in contrast to cisplatin or oxaliplatin (Fig. 4A).

**Concentration-Dependent Uptake of Pt Drugs.** HNSCC cell lines were exposed to 5, 10, 50, or 100 μM of the Pt drugs for
4 h. Subsequent intracellular Pt concentrations after exposure were determined. The corresponding AUC characterizing this interrelation was determined to express uptake characteristics of the particular drug or cell lines. In general, no statistically significant differences in AUC values of the three Pt drugs could be detected. However, a clear trend to accumulation efficiency of cisplatin was observed. In some distinct cases (HNO211), cisplatin accumulated 4-fold in contrast to oxaliplatin ($P < 0.0001$). Carboplatin was only marginally taken up into the cell lines (Fig. 4B).

**Cellular Pharmacokinetic/Pharmacodynamic Relation.** When proliferation inhibition was related to uptake characteristics of Pt drugs in different HNSCC cell lines (Fig. 4C), the limited uptake of carboplatin was accompanied with poor inhibition of tumor cell proliferation. In contrast, cisplatin and oxaliplatin exhibited equally potent cytostatic properties even though their accumulation in HNSCC differed.

**Drug Transporter mRNA Expression Levels of HNSCC Cell Lines.** The expression of Pt drug transporters in the HNSCC cell lines differed by more than an order of magnitude (Fig. 5). The highest expression levels were observed for ATP7A and ABCC2, whereas SLC22A2 was generally poorly expressed. The HNSCC cell line HNO210 stood out by significantly different expression of SLC22A2 and ABCC2 (Fig. 5).

**Discussion**

Although all Pt-containing cytostatics share the same principal mechanism of action, the response of certain cancer entities to individual Pt-containing drugs differs, thus certain Pt drugs are preferentially used in certain tumors. As an example carboplatin is preferred in gynecological tumors (Fung-Kee-Fung et al., 2007) and oxaliplatin is preferred in colorectal cancer (Goodwin and Asmis, 2009). The causes for different efficacies are not well understood but bear the potential to tailor therapies to individual tumor characteristics that might determine efficacy. Because DNA is considered to
be the preferential cytotoxic target (Rabik and Dolan, 2007), the relationship between DNA platination and in vitro cytotoxicity or growth inhibition has been studied. The few studies comparing cisplatin, oxaliplatin, or carboplatin with each other in the same tumor entity revealed surprising results. DNA adduct formation was not associated with cytotoxicity of oxaliplatin in colon cancer cells (Arnould et al., 2003), and oxaliplatin also caused considerably less DNA platination than cisplatin in Jurkat cells whereas cytotoxicity was similar, thus questioning the relevance of DNA platination for cytotoxicity (Goodisman et al., 2006). Hence the effects of Pt drugs seem to be linked to cellular actions beyond DNA platination. We therefore aimed to assess whole-cell Pt content rather than solely concentrating on Pt bound to DNA.

In HNSCC, cellular Pt amount after cisplatin exposure for 72 h slightly correlated with IC₅₀ values (Welters et al., 1997). However, some confounders were present: first, the correlation has been achieved by exclusion of a discordant data point; second, correlation was detected for only one single cisplatin exposure concentration; and third, the exposure period was 72 h, suggesting that results might have been observed in selected cells being intrinsically resistant.

In contrast, our study aimed at comprehensively comparing all three standard Pt cytostatics by using a LC/MS/MS method that can be uniformly used to quantify whole-cell concentrations of cisplatin, carboplatin, and oxaliplatin. This allowed the objective comparison of Pt uptake characteristics (expressed as AUC values) with cytotoxicity.

The developed LC/MS/MS method proved to be well suited for the analysis of cell culture samples. Using standard 96-well microtiter plates for cell culture, incubation with Pt drugs, and collection with one transfer step, our method is fast and minimizes sample loss. An eluent composition based on methanol was favorable because acetonitrile adducts with the Pt complex could not be dissociated with source fragmentation. The actual LC conditions allowed cycle times below 6 min, and by analyzing a minimum of approximately 2000 cells per well this high-throughput method was able to generate reliable data with many replicates of each Pt drug concentration while the handling effort for numerous samples was low.

During the development of this method we identified a Pt/DDTC₃ complex with the same retention time as the known Pt/DDTC₃ complex. The scan around the mass 639 revealed an isotope pattern as expected for Pt/DDTC₃. However, further analysis revealed that this Pt/DDTC₃ consists of a Pt(IV) ion that cannot originate from the Pt drugs because they exclusively contain Pt(II) ions. Although contamination can largely be excluded, it remained unclear how this Pt(IV)/DDTC₃ complex was formed. It probably originated from an oxidizing ESI source effect, because both Pt/DDTC₂ and Pt/DDTC₃ coelute from the LC column and calibration curves, and quantification of both complexes always results in the same Pt drug amount. During the sample preparation no oxidizing agent was present, but a known phenomenon that can occur in the electrospray ion source is electrochemical oxidation (Van Berkel and Kertesz, 2007). Although platinum has a higher ionization potential than the iron of the steel capillary (Lloyd and Hess, 2009) it has been shown that dithiocarbamates promote unusual oxidation states (Bond and Martin, 1984). This might explain the observed oxidation of the platinum atom. All samples were therefore quantified by using both Pt/DDTC₂ and Pt/DDTC₃. These two calibration curves and their results were equivalent markers for QC. Using only the Pt/DDTC₃ complex it would be possible to further decrease the limit of quantification by a factor of 5 to 10.

Using the described methodology, carboplatin was demonstrated to poorly accumulate into HNSCC cell lines. Little uptake differences among the five cell lines were observed for carboplatin, suggesting carboplatin generally exhibits minor uptake properties. In contrast, cisplatin and oxaliplatin demonstrated higher potency to penetrate at least some of the cell lines (Fig. 4B). These drugs also exhibited the most potent proliferation inhibition (Fig. 4A). Cisplatin and oxaliplatin had quite similar mean IC₅₀ values. We were surprised to find that carboplatin’s efficacy to inhibit proliferation was one order of magnitude lower.

When merging uptake characteristics and proliferation inhibition into a pharmacokinetic/pharmacodynamic relation model (Fig. 4C) no interdependences were detectable. To our surprise, in vitro efficacy of cisplatin and oxaliplatin seems to be independent from measured AUC values, suggesting that intracellular concentration of Pt in HNSCC plays a minor role. For instance, oxaliplatin penetrated HNO211 less effectively in contrast to cisplatin (4-fold difference; P < 0.0001), but their cytostatic potencies were comparable (4.94 versus 8.05 μM) (Fig. 4C). In addition, in some cell lines (e.g., HNO199, HNO41) uptake of cisplatin or oxaliplatin was generally very low, whereas their impact on proliferation inhibition was also comparable and considerable (Fig. 4C). Moreover, with respect to individual cell lines, cisplatin and oxaliplatin seemed to be of a similar type: HNO199, HNO41, and HNO150 showed the same rank order for cisplatin or oxaliplatin uptake (Fig. 4C). In summary, this is why we consider oxaliplatin to be a worthwhile alternative to cisplatin in HNSCC. From a clinical point of view, this conclusion is comprehensible: Although oxaliplatin-based chemotherapy is regularly compromised by the development of a sensory neuropathy it has a clearly favorable toxicity profile, being markedly less nephrotoxic and myelosuppressive than cisplatin (Rabik and Dolan, 2007).

Because Pt drug transporters are believed to play a major role for Pt drug safety, efficacy, and drug resistance (Safaei and Howell, 2005; Guminiski et al., 2006; Zhang et al., 2006; Rabik and Dolan, 2007; Burger et al., 2010), we also evaluated the mRNA expression levels of ABC2, SLCC22A2, SLCC31A1, ATP7A, and ATP7B. No particular Pt drug transporter was clearly demonstrated to correlate with the magnitude of uptake or cytotoxicity among the cell lines tested. However, HNO210 that efficiently took up cisplatin and oxaliplatin additionally differed from other cell lines by a distinct mRNA expression profile of Pt drug transporters (Figs. 4B and 5). In particular, HNO210 with the highest expression of SLCC22A2 (encoding OCT2) concurrently demonstrated profound accumulation of both cisplatin and oxaliplatin, underlining recent findings demonstrating OCT2 to be a major determinant of the uptake and cytotoxicity of these drugs (Burger et al., 2010). HNO210 also had the highest expression of ABC2 (encoding MRP2) that is involved in the efflux of Pt drugs, thus lowering intracellular Pt concentrations and mediating drug resistance (Taniguchi et al., 1996; Cui et al., 1999; Guminiski et al., 2006). But, on the other hand, high ABC2/MRP2 expression levels were also associated with a favorable clinical outcome in HNSCC disease (van den Broek et al., 2009) and high in vitro efficacy of...
oxaliplatin (Theile et al., 2009). This might be related to MRP2-mediated deprivation of GSH levels increasing Pt drug efficacy (Theile et al., 2009). Taken together, the results demonstrate that: 1) carboplatin is less effective and poorly taken up into HNSCC cells; 2) there is a high variability between cell lines concerning drug accumulation of cisplatin and oxaliplatin despite similar in vitro efficacy; and 3) profound expression differences were observed for ABC22 and SLC22A2, the latter associated with high drug uptake characteristics and efficacy of cisplatin and/or oxaliplatin.

In conclusion, we developed and validated an LC/MS/MS methodology to quantify intracellular Pt drugs and demonstrated that the in vitro efficacy of cisplatin and oxaliplatin in HNSCC is concentration-independent because they exhibited different uptake characteristics but similar efficacies, suggesting oxaliplatin as a promising alternative against HNSCC that should be further evaluated in clinical trials.

Acknowledgments

We thank Corina Mueller for excellent technical help.

Authorship Contributions

Participated in research design: Theile and Detering. Conducted experiments: Theile and Detering. Contributed new reagents or analytic tools: Weiss and Burhenne. Performed data analysis: Theile, Detering, Weiss, and Burhenne. Wrote or contributed to the writing of the manuscript: Theile, Detering, Herold-Mende, Dyckhoff, Haefeli, Weiss, and Burhenne.

References