Cellular pharmacokinetic/pharmacodynamic relationship of platinum cytostatics in head and neck squamous cell carcinoma evaluated by liquid chromatography coupled to tandem mass spectrometry

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Nonstandard abbreviations: Cisplatin, diaminodichloroplatinum; oxaliplatin, oxalato[(1R,2R)-cyclohexanediamino]platinum; carboplatin, diaminoplatinum-cyclobutan-1,1-dicarboxylate; 5-FU, 5-fluorouracil; HNSCC, head and neck squamous cell carcinoma; DDTC, iethyldithiocarbamate; SLC, solute carrier; OCT, organic cation transporter; MRP, multidrug resistance-associated proteins; ATP7A/ATP7B, copper-transporting ATPases.

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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)-cyclohexanediaminocyclohexanetetra-1,2-dionate]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 ± 1.9 μM for cisplatin and 11.6 ± 4.2 μM for oxaliplatin, whereas carboplatin showed significantly lower proliferation inhibition (IC50 107.5 ± 21.2 μM). In agreement with this finding carboplatin poorly accumulated in HNSCC cells, compared to cisplatin and oxaliplatin. HNSCC cell lines distinguishly expressed Pt-drug transporters. Taken together, the results demonstrate (1) carboplatin to be less effective and poorly taken up and (2) a high individuality among cell lines concerning accumulation of cisplatin and oxaliplatin despite (3) similar in vitro efficacy. (4) 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 as 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). Besides surgery and radiation, systemic chemotherapy remains a cornerstone in the curative or palliative treatment of HNSCC (Fanucchi et al., 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 (Forastiere et al., 1992; Jacobs et al., 1992). However, 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 questioning cisplatin as the standard Pt-drug: cisplatin has been demonstrated to cause response rates of 15 – 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)-cyclohexanediamino)platinum), the only single-agent clinical trial suggests efficacy with response rates of 10 – 17% (Degardin et al. 1996).

Based on the assumption that 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 studies (Fisch et al., 1996). 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 (hCTR1/ solute-carrier (SLC) SLC31A1), copper-transporting ATPases ATP7A (Menkes disease protein) and ATP7B (Wilson disease protein)) physiologically involved in copper homeostasis (Rabik et al., 2007). Additionally, multidrug resistance-associated protein 2 (MRP2/ABCC2) and organic cation transporter 2 (OCT2/SLC22A2) were 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(DDTC)_2 and Pt(DDTC)_3 chelate complexes (Drummer et al., 1984; Raghavan et al. 2000; Verschraagen 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, to evaluate the relationship between antiproliferative responses and cellular Pt concentration, and to assess its modulation by drug transporters.
MATERIALS AND METHODS

Materials

Culture media, medium supplements, antibiotics, trypsin, ethylenediaminetetraacetate solution, sodium diethyldithiocarbamate, palladium acetate, ammonium acetate and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Fetal calf serum (FCS) was purchased from PAA (Cölbe, Austria). Crystal violet was from AppliChem (Darmstadt, Germany). Methanol and acetonitrile used for LC/MS/MS analysis was highest grade from Merck (Darmstadt, Germany) and Carl Roth GmbH (Karlsruhe, Germany). 96-well (700 µl) collection plates were from Eppendorf (Hamburg, Germany). 96-well 0.66 mm glas-fiber filter plates were purchased from Corning Inc. (New York, USA). 96-well (300 µl) microtiter plates for cell culture were from Nunc (Wiesbaden, Germany) and cell culturing bottles were from Greiner (Frickenhausen, Germany). Deionized water was obtained from a HP 6 UV/UF TKA ultra-filtration system (TKA GmbH, Niederelbert, Germany). The Pt containing cytostatics cisplatin, oxaliplatin, and carboplatin were obtained from the University Hospital’s 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 TNM stages, but all lack chemotherapeutic approaches to minimize bias from antineoplastic agents potentially altering drug transporter expression or drug resistance, respectively.
Adequateness of cell lines for representation of HNSCC was recently confirmed by comprehensive tumor cell biological characterization (Freier et al., 2010). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin sulphate.

**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 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 using a Multiskan RC photometer (Thermo Scientific, Langenselbold, Germany) with 555 nm excitation. Proliferation was expressed as proliferation index by calculating crystal violet absorption intensity as percentage relative to baseline (absorption intensity of wells with medium only (set to 0%)) and native proliferation (absorption intensity of untreated cells (set to 100%)). Each concentration was tested in octuplet and each assay was performed thrice.

**Concentration-dependent uptake of Pt-drugs**

Cells were seeded onto 96-well microtiter plates and preincubated for 24 h. After addition of blank medium into two columns and test compounds into four columns (5 µM, 10 µM, 50 µM, 100 µM, respectively), cells were incubated for 4 h, a duration found devoid cytotoxic effects in preliminary tests. Pt-drug uptake after 4h-exposure was evaluated by determining intracellular Pt-drug amount of six wells normalized to 10,000 cells evaluated using two wells by CASY cell counter methodology (Schärfe System, Reutlingen, Germany). Subsequent intracellular Pt-concentrations following exposure of cells with 5 µM, 10 µM, 50 µM, or 100 µM were
determined. Figure 1 explains the analytical approach: The corresponding area under the curve characterizing this interrelation was determined for every single HNSCC cell line to express uptake characteristics of the particular drug or cell lines, respectively (Figure 1).

**LC/MS/MS conditions**

For quantification of the Pt complex an LC/MS/MS (Thermo Fisher, Dreieich, Germany) system consisting of a HTC 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 A; 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 runtime 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, capillary heater temperature 350°C. No additional voltage for in-source collision induced dissociation (CID) was used. The multiplier voltage was set at 1.6 kV. The LC/MS/MS system was tuned by the 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)₂, m/z 639 → m/z 492 at 33V for Pt(DDTC)₃, and m/z 403 → m/z 116 at 25V for the internal standard palladium complex (Pd(DDTC)₂).

**Calibration, QC, and validation procedures**
Due to 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 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 FDA guideline (Food and Drug Administration Guidance) by analyzing three analytical batches, each containing calibration samples (with two calibration curves) and QC samples (sixfold for each concentration). The quality control (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 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 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 50 g for 5 min. Subsequently, 200 µl of methanol was added onto the filter plate and the plates were centrifuged again at 50 g for 5 min. The collection plate was sealed with rubber caps and analyzed by injecting 25 µl sample volume into the LC/MS/MS system.

Quantification of mRNA expressions by quantitative real time reverse transcription polymerase chain reaction (qRT-PCR)

RNA was isolated using the RNeasy Mini-Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. cDNA was synthesized with the RevertAid™ 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 qRT-PCR with a LightCycler® 480 (Roche Applied Science, Mannheim, Germany). PCR amplification was carried out in 20 µl reaction volume containing 5 µl 1:10 diluted cDNA using the SYBR Green format with the Absolute QPCR SYBR Green Mix (Abgene, Hamburg, Germany). Primer sequences were published previously (Theile et al., 2009, 2010). The following genes were quantified: ABCC2 (encoding for MRP2), SLC22A1 (encoding for OCT1), SLC22A2 (encoding for OCT2), SLC31A1 (encoding for hCTR1), 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 $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001). All samples were amplified in duplicate.
Calculations and statistical analysis

The amount of Pt-drug in treated wells was calculated using LCQuan Software Version 1.2 and linear calibration. The mean value per incubation concentration (sixfold determination) together with the corresponding mean cell number obtained by the CASY cell counter was normalized to 10,000 cells using Microsoft Excel 2002. Batch to batch accuracy was expressed as the deviation of the mean recovery from the nominal value in percent. Batch to batch precision was calculated as coefficient of variation by dividing standard deviation and nominal value and was expressed in percent. Intercomplex accuracy was expressed as the mean difference between the Pt(DDTC)$_2$ and Pt(DDTC)$_3$ values divided by the nominal value in percent. Intercomplex precision was calculated as the mean of the differences between the two Pt complexes value per sample in percent.

Data are presented as means ± SD and were analyzed using GraphPad Prism$^\text{®}$ Version 5.0 and InStat Version 3.05 (GraphPad Software, San Diego, CA, USA). Differences in in vitro efficacy of Pt-drugs (IC$_{50}$ values) and uptake characteristics (AUC values) were tested using one-way ANOVA with Bonferroni’s post-hoc test comparing Pt-drugs among each other. Different uptake of cisplatin or oxaliplatin in HNO211 was evaluated 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 respective single genes were tested using one-way ANOVA with Bonferroni’s post-hoc test comparing all cell lines among each other. A p-value < 0.05 was considered significant and was depicted by brackets connecting the respective pair of cell lines.
RESULTS

Performance of the analytical methods

Derivatization of the Pt-drugs with DDTC resulted in two- and threefold 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 Figure 2. The mass transitions for derivatized platinum compounds (Pt(DDTC)\(_2\), m/z 492 → m/z 116; Pt(DDTC)\(_3\), m/z 639 → m/z 492) and palladium compound (internal standard Pd(DDTC)\(_2\): m/z 403 → 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 5 - 6 times more sensitive than Pt(DDTC)\(_2\). Retention times for Pt- and Pd-complexes were 2.9 and 4.2 min (Figure 3). Both analyzed mass transitions for the Pt-complexes resulted in peaks at identical retention times.

Pt-complex concentrations were measured 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 using only the Pt(DDTC)\(_3\) mass transition. The derivatization and extraction efficacy (recovery) was 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 FDA with batch-to-batch accuracy (precision) ranging from 0.1 to 10.5% (2.0 to 12.4%). The mean ± SD 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 IC₅₀ of cisplatin, oxaliplatin, and carboplatin ranged from 3.50 µM to 8.13 µM, 7.91 µM to 18.11 µM, and 71.85 µM 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 (Figure 4A).

Concentration-dependent uptake of Pt-drugs.

HNSCC cell lines were exposed to 5 µM, 10 µM, 50 µM, or 100 µM of the respective Pt-drug for 4 h. Subsequent intracellular Pt-concentrations following exposure were determined. The corresponding area under the curve characterizing this interrelation (AUC) was determined to express uptake characteristics of the particular drug or cell lines, respectively. 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 (Figure 4B).

Cellular pharmacokinetic/pharmacodynamic relation

When proliferation inhibition was related to uptake characteristics of Pt-drugs in different HNSCC cell lines (Figure 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 (Figure 7). 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* (Figure 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 and 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) or oxaliplatin in colorectal cancer (Goodwin et al., 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 et al., 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 while cytotoxicity was similar thus questioning the relevance of DNA platination for cytotoxicity (Goodisman et al., 2005). Hence the effects of Pt-drugs appear 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 IC\textsubscript{50} 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; third, 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 using a LC/MS/MS method that can uniformly be used to quantify whole cell concentrations of both
cisplatin, carboplatin, and oxaliplatin. This allowed objectively comparing Pt uptake characteristics (expressed as AUC values) with cytotoxicity.

The developed LC/MS/MS method proofed 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 complexation 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 about 2,000 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(DDTC)_3 complex with the same retention time as the known Pt(DDTC)_2 complex. The scan around the mass 639 revealed an isotope pattern as expected for Pt(DDTC)_3. However, further analysis revealed, that this Pt(DDTC)_3 consists of a Pt(IV) ion which cannot originate from the Pt-drugs because they exclusively contain Pt(II) ions. While contamination can largely be excluded, it remained unclear how this Pt(IV)(DDTC)_3 complex was formed. Most probably it originated from an oxidizing ESI source effect, because both Pt(DDTC)_2 and Pt(DDTC)_3 co-elute 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 et al., 2007). Although platinum has a higher ionization potential than the iron of the steel capillary (Lloyd et al., 2009) it was shown that dithiocarbamates promote unusual oxidation states (Bond et al., 1984). This might explain the observed oxidation of the platinum atom. All samples were therefore quantified using both Pt(DDTC)_2 and Pt(DDTC)_3. These two calibration
curves and their results were equivalent markers for QC. Using only the Pt(DDTC)$_3$ complex it would be possible to further decrease the limit of quantification by factor 5-10.

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 to generally exhibit minor uptake properties. In contrast, cisplatin and oxaliplatin demonstrated higher potency to penetrate at least some of the cell lines (Figure 4B). These drugs also exhibited most potent proliferation inhibition (Figure 4A). Cisplatin and oxaliplatin had quite similar mean IC$_{50}$ values. Surprisingly, carboplatin’s efficacy to inhibit proliferation was one order of magnitude lower.

Merging uptake characteristics and proliferation inhibition into a pharmacokinetic/pharmacodynamic relation model (Figure 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 to play 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 µM vs. 8.05 µM) (Figure 4C). Additionally, 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 (Figure 4C). Moreover, with respect to individual cell lines, cisplatin and oxaliplatin appeared to be of similar type: HNO199, HNO41, and HNO150 showed the same rank order for cisplatin or oxaliplatin uptake (Figure 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 et al., 2007).

Because Pt-drug transporters are believed to play a major role for Pt-drug safety, efficacy, and drug resistance (Rabik et al., 2007; Safaei et al., 2005; Zhang et al., 2006; Burger et al., 2010; Guminski et al., 2006), we also evaluated per se mRNA expression levels of ABCC2, SLC22A2, SLC31A1, ATP7A, and ATP7B. No particular Pt-drug transporter was clearly demonstrated to correlate with magnitude of uptake or cytotoxicity among all 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 (Figures 4B and 5). In particular, HNO210 with the highest expression of SLC22A2 (encoding OCT2) concurrently demonstrated profound accumulation of both cisplatin and oxaliplatin underlining recent findings demonstrating OCT2 to be a major determinant of uptake and cytotoxicity of these drugs (Burger et al., 2010). HNO210 also had the highest expression of ABCC2 (encoding MRP2) that is involved in efflux of Pt-drugs thus lowering intracellular Pt concentrations and mediating drug resistance (Cui et al., 1999; Guminski et al., 2006; Taniguchi et al., 1996). But on the other hand high ABCC2/MRP2 expression levels were also associated with 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 glutathione 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 and that (2) there is a high variability between cell lines concerning drug accumulation of cisplatin and oxaliplatin despite similar in vitro efficacy (3). (4) Profound expressional differences were observed for ABCC2 and SLC22A2, the later associating with high drug uptake characteristics and efficacy of cisplatin and/or oxaliplatin.
In conclusion, we newly developed and validated an LC/MS/MS methodology to quantify intracellular Pt-drugs and demonstrated that *in vitro* efficacy of cisplatin and oxaliplatin in HNSCC is concentration-independent as they exhibited different uptake characteristics but similar efficacies suggesting oxaliplatin as a promising alternative against HNSCC that should be further evaluated in clinical trials.
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Authorship contributions:

Participated in research design: Theile, Detering

Conducted experiments: Theile, Detering

Contributed new reagents or analytical tools: Burhenne, Weiss

Performed data analysis: Theile, Detering, Burhenne, Weiss

Wrote or contributed to the writing of the manuscript: Theile, Detering, Weiss, Burhenne, Haefeli, Herold-Mende, Dyckhoff
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Footnotes:

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FIGURE LEGENDS:

Figure 1: Graphical explanation describing interrelation between exposure concentration of Pt-drugs and resulting intracellular Pt-drug concentration. Area under the curve (AUC) was determined to express and compare uptake characteristics of the respective Pt-drugs.

Figure 2: Mass spectra of the Pt and Pd complexes: A) MS (full scan) of Pt(DDTC)2 (m/z 492) and Pt(DDTC)3 (m/z 639). B) MS/MS spectra (daughter scan) of Pt(DDTC)2 (m/z 492). C) MS/MS spectra (daughter scan) of Pt(DDTC)3 (m/z 639). D) MS (full scan) of Pd(DDTC)2 (m/z 403).

Figure 3: LC/MS/MS chromatograms of A) a full blank sample, B) a blank sample (spiked with internal standard), C) a calibration sample at 0.5 ng/ml (LOQ), and D) cell sample at 9.6 ng/10,000 cells. Upper trace shows m/z 403 → m/z 116 at 25V for Pd(DDTC)2, middle trace shows m/z 492 → m/z 116 at 33V for Pt(DDTC)2, and lower trace shows m/z 639 → m/z 492 at 33V for Pt(DDTC)3.

Figure 4

A: Comparison of inhibitory concentrations leading to 50% of proliferation inhibition (IC50) of Pt-drugs in five different HNSCC cell lines. Each dot represents the mean of three independent replicates. Statistical significance was evaluated using one-way ANOVA with Bonferroni’s multiple comparison test. * P <0.05.

B: Pt-drug uptake characteristics in different HNSCC cell lines expressed as AUC values. Statistical significance was evaluated using one-way ANOVA with Bonferroni’s multiple comparison test.

C: Relationship between Pt uptake and inhibition of proliferation after exposure to cisplatin, oxaliplatin, or carboplatin.
Figure 5: mRNA expression profile of Pt-drug transporters in HNSCC cell lines. mRNA expression was normalized to RPL13. Data are presented as mean ± SD for n=3 experiments. Statistical significance was evaluated using one-way ANOVA with Bonferroni’s multiple comparison test and was depicted by brackets connecting the respective pair of cell lines. * P <0.05; *** P<0.001.
Table 1: Patient data

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<tr>
<th>Patient</th>
<th>Sex</th>
<th>Localization</th>
<th>Age</th>
<th>TNM</th>
<th>G</th>
<th>Therapy</th>
<th>PFS</th>
<th>OS</th>
<th>M</th>
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<td>HNO41</td>
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<td>Tons/Oropharynx</td>
<td>52</td>
<td>T2N2bM0</td>
<td>2</td>
<td>Op+Rad</td>
<td>10</td>
<td>14</td>
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<td>f</td>
<td>Larynx</td>
<td>47</td>
<td>T3N2bM0</td>
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</tbody>
</table>

Localization, localization of tumor; Tons, tonsils; Age, age in years at time of diagnosis; TNM, initial tumor staging according to UICC; G, histological grading; Op, operation; Rad, radiation; PFS, progression free survival in months; OS, overall survival in months after surgery; M, occurrence of distant metastases.
Table 2: Results of accuracy and precision testing

<table>
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<tr>
<th></th>
<th>Cisplatin</th>
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<th>Carboplatin</th>
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<td>Pt(DDTC)₃</td>
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<td>C 109.0</td>
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<tr>
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<tr>
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<tr>
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<td>10.8</td>
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</table>

All values in %
Figure 1

The graph illustrates the relationship between the exposure concentration of Pt-drug (in μM) and the subsequent intracellular concentration of Pt-drug (in pg/10,000 cells). The area under the curve (AUC) represents the cumulative amount of drug uptake over different exposure concentrations.
Figure 2
Figure 4

AUC (µM*pg/µl * 10^4 cells)

- Cisplatin
- Oxaliplatin
- Carboplatin
Figure 4

C

AUC (μM×pg / ml×10^5 cells)

IC_{50} (μM)

- Cisplatin
- Oxaliplatin
- Carboplatin
Figure 5

The graph shows the mRNA levels normalized to RPL13 for different genes (ATP7A, ATP7B, SLC22A2, SLC31A1, and ABCC2) across different conditions (HNO41, HNO150, HNO199, HNO210, and HNO211).

Significant differences are indicated by asterisks: * indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001.