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ANALYTICAL METHODOLOGY

Assessing the intracellular concentration of platinum in medulloblastoma cell lines after Cisplatin incubation

Christoph A. Wehe[a,1], Georg Beyer[b,1], Michael Sperling[a,c], Giuliano Ciarimboli[b], Uwe Karst[a,*]

[a] University of Münster, Institute of Inorganic and Analytical Chemistry, Corrensstr. 28/30, 48149 Münster, Germany
[b] University of Münster, University Hospital, Medical Clinic D, Experimental Nephrology and Interdisciplinary Center for Clinical Research (IZKF), Albert-Schweitzer-Campus 1 – A14, 48149 Münster, Germany
[c] European Virtual Institute for Speciation Analysis (EVISA), Mendelstr. 11, 48149 Münster, Germany

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ABSTRACT

Two different analytical approaches, external calibration and isotope dilution analysis both using flow-injection inductively coupled plasma mass spectrometry, have been developed and applied to determine the intracellular platinum concentration after Cisplatin incubation of two different medulloblastoma cell lines (UW228 and DAOY). As the internal or isotopically enriched standard was already used for cell lysis, maximum accuracy of the results was obtained, whereas a new home-built and inert injection system dramatically lowered carry-over effects and analyte loss. With limits of the detection well below 0.4 $\mu g\,L^{-1}$ and typical relative standard deviations of 2%, a strong correlation between the cell viability in MTT assays and the incorporated amount of Pt could be shown, which was subsequently normalized to the protein content of the samples. DAOY cells did significantly ingest more Pt and showed a higher mortality, which supports the fact that transporter expression needs to be taken into account in order to obtain meaningful results.

Introduction

After their discovery in the 1960s and since first clinical tests in 1974, platinum based cytostatic agents are frequently and effectively used in the fight against different types of tumors [1–3]. Here, among others, Cisplatin, a neutral and planar complex with two cis-chlorido and two ammine ligands binding at the central platinum atom (cis-diamminedichloroplatinum(II), CDDP), has gained a lot of scientific interest [4–10]. This is not only due to the fact that CDDP was the first cytostatic agent utilizing Pt for crosslinking of DNA and hence apoptosis triggering, but also due to its serious known side effects, which include infertility, oto- and nephrotoxicity [11–13]. A variety of solid tumors are treated with CDDP, such as testicular and ovarian cancer, bladder and lung tumors as well as the high malignant brain tumor called medulloblastoma [14–21].

In vitro cell culture experiments are commonly applied to assess the effectiveness as well as toxicology of potent anti-cancer drugs. However, little is known about the diverse response of different cell lines toward the same amount of an incubated active substance. In this study, the cell lines DAOY and UW228, derived from two medulloblastoma patients, were examined concerning their concentration-dependent CDDP toxicity and the relation to the incorporated amount of Pt utilizing flow-injection analysis (FIA) on-line coupled to inductively coupled plasma mass spectrometry (ICP-MS), known inter alia for its high sensitivity and species-independent response.

The FIA concept was already introduced in the late 1970s [22] and has been successfully used for on-line chemical reactions, pre-concentration or isotope dilution analysis (IDA) [23–26]. Due to the low required sample volume it is ideally suited for cell culture studies where no further dilution is possible [27–30]. Inspired by the work of Björn et al. [6], who revealed the concentration of Pt in human subcellular (exosomes and DNA) microsamples, two different quantitation strategies were developed and thoroughly evaluated.

Materials and methods

Instrumentation

Two different ICP-MS systems were used during the studies in order to estimate the capabilities of both instruments. Whereas the experiments involving an external calibration approach were carried out on the double-focusing sector field instrument

* Corresponding author. Tel.: +49 251 83 33141; fax: +49 251 83 36013.
E-mail address: uk@uni-muenster.de (U. Karst).
[1] Both authors contributed equally to this work.

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ElementXR (Thermo Fisher Scientific, Bremen, Germany), all studies regarding the isotope dilution approach were conducted on an quadrupole mass analyzer system (iCAP Qc, Thermo Fisher Scientific) equipped with a flatapole collision/reaction cell (QCell). Both of the instruments were coupled to a liquid chromatography (LC) device, consisting of an AccelaPump (model 1000 or 1250) to deliver the carrier solution, and an AccelaAutosampler (both Thermo Fisher Scientific, San José, CA, USA), without any column mounted, as injection device for the FIA. All capillaries as well as the 20 μL sample loop used throughout all studies were made from polyether ether ketone (PEEK) with an inner diameter (ID) of 0.13 mm.

The sample introduction system for the sector field ICP-MS instrument consisted of a small inner volume cyclonic quartz glass spray chamber (Twinnabar, Glass Expansion, West Melbourne, Australia) for fast wash-out and a perfluoralkoxy polymer (PFA) μFlow ST nebulizer (Elemental Scientific, Omaha, NE, USA). The ICP system was equipped with a Ni sampler and skimmer (H geometry) and a quartz injector pipe with an ID of 1.75 mm. The data acquisition was started externally by the connected LC using a contact closure trigger. The ElementXR was operated with Element Software 3.1 (Thermo Scientific) and data were processed with OriginPro 8.5G (OriginLab, Northampton, MA, USA), Office Excel 2013 (Microsoft, Redmond, WA, USA) and Graph Pad Prism 5 (Graph Pad, La Jolla, CA, USA).

The sample introduction system of the quadrupole instrument consisted of a Peltier-cooled cyclonic quartz glass spray chamber operated at 2.7 °C, a μFlow ST nebulizer, a quartz injector pipe (ID = 1.8 mm) and Ni sampler and skimmer cones, the latter with a 2.8 mm insert. As collision/reaction gas inside the flatapole, a mixture of 8% (v/v) H_2 in He (purity 99.999%) was used for the applied high sensitivity kinetic energy discrimination (KEDS) mode. The instrument was operated using Qtegra 1.5 (Thermo Fisher Scientific) and tuned on a daily basis following the recommendations of the manufacturer. This software was also used to integrate the obtained peaks using the interactive chemical information system (ICIS) peak identification and integration algorithm. Detector's dead time [31] was determined by analyzing different concentrations of Lu.

Materials and chemicals

CDDP was ordered from TEVA Pharma (Radebeul, Germany). Hydrochloric acid (37% (w/w)) was obtained from Merck (Darmstadt, Germany) and nitric acid (65% (w/w)) from Fisher Scientific (Schwerte, Germany). Both were classified as suitable for "trace element" analysis. The elemental Pb and Pt standards (1000 mg L^{-1} each) were purchased from J. T. Baker/Avantor Performance Materials (Center Valley, PA, USA) or CPI International (Santa Rosa, CA, USA), respectively. All other elemental standards were ordered from Spetec (Erding, Germany). The metallic ^{194}Pt enriched material (certified enrichment grade = 96.45%) was a product of Isoflex (San Francisco, CA, USA; certification number 78-02-194-4221). After dissolution in *aqua regia*, the obtained solution was diluted and its concentration was verified using total reflection X-ray fluorescence analysis (S2 Picofox, Bruker AXS, Karlsruhe, Germany), utilizing Sr as internal standard and by reverse isotope dilution. Water was freshly purified for each experiment by an Aquatron Water Stills purification system model A4000D from Barloworld Scientific (Nemours Cedex, France). All chemicals were used in the highest quality available.

Cell culture

The cell line DAOY was established by Jacobsen [32] from the medulloblastoma cells of a 4 year old boy after biopsy. It was derived from ATCC-LGC (Promochem, Wesel, Germany). UW228 cells were obtained from M. Frühwald (University Children's Hospital Münster, Department of Pediatric Haematology and Oncology, Münster, Germany) with kind permission of J. Silber (Department of Neurological Surgery, University of Washington, Seattle, WA, USA).

The cells were cultivated in RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 1 mmol L^{-1} L-glutamine, 100 U mL^{-1} penicillin G, 100 μg mL^{-1} streptomycin, and 10% fetal calf serum in 25 cm^3 tissue culture flasks (Greiner Bio One) in a humidified atmosphere of 8% CO_2 at 37 °C. For MTT assays, 100 μL of cell suspension were dispensed per well on 96-well flat-bottom microtiter plates (Greiner Bio One) and incubated in a humidified atmosphere of 8% CO_2 at 37 °C for 24 h before experiments. For ICP-MS experiments, 2000 μL of the cell suspension were dispensed per well on 6-well flat-bottom microtiter plates (Greiner Bio One) and incubated in a humidified atmosphere of 8% CO_2 at 37 °C for 72 h. Typically, 80–90% of complete confluence was reached assuring a monolayer of cells. The DAOY cells were hereby sowed thinner in order to compensate for the different, faster growth rate compared to UW228 cells.

Cytotoxicity testing

A modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to test the chemosensitivity of DAOY and UW228 cells toward CDDP by determining the glycolysis rate via the reduction of the yellow tetrazolium salt MTT to a purple formazan dye [33–38]. After growth for 24 h in the incubator, 100 μL of CDDP containing cell culture medium was added to the present 100 μL cell suspension in each well. The incubation solutions contained twice the desired drug concentration to result in the final CDDP concentrations of 10, 50 and 100 μmol L^{-1}. Control cells were incubated with drug-free complete cell culture medium. All cells were incubated for 10 min with CDDP and afterwards, the medium was carefully removed and replaced by 200 μL of fresh complete cell culture medium. After incubation for another 48 h, 10 μL of MTT solution containing 5 mg mL^{-1} of the dye per well were added and cells were again incubated for 3 h. Afterwards, the medium was removed gently and 100 μL of lysis buffer containing 10% (w/v) sodium dodecyl sulfate and 40% (v/v) dimethylformamide per well were added. The plates were shaken for 10 min to destroy the cell structure and dissolve the blue formazan dye. Finally, the absorbance was measured at 590 nm using an automated microtiter plate reader (Infinite M200; Tecan, Männedorf, Switzerland). The percentage of viable cells in the untreated controls was compared to the different CDDP concentrations applied.

Treatment of cells and preparation of lysates for ICP-MS

After growth for 72 h in the incubator, the 2000 μL of used medium were gently removed from all wells and replaced by 2000 μL fresh complete cell culture medium containing 0, 50 and 100 μmol L^{-1} CDDP. For each concentration, two wells per cell line were treated and the test was repeated three times. In addition, one well per cell line was treated with 2000 μL complete cell culture medium containing 100 μmol L^{-1} CDDP each time in order to calculate the mass bias of the utilized ICP-MS device later on.

After adding the incubation solutions, the plates were incubated for 10 min. Immediately thereafter, the medium was removed and the cells were washed carefully three times with 2 mL ice cold phosphate-buffered saline (PBS, Biochrom) to remove all extracellular CDDP and to stop the transport processes via the cell membrane. After the PBS was removed, the cells were lysed with bidistilled water containing either about 4.2 μg L^{-1} of the isotopically enriched Pt standard or 100 μg L^{-1} of Pb with natural isotopic