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Debio 0507 primarily forms diaminocyclohexane-Pt-d(GpG) and -d(ApG) DNA adducts in HCT116 cells

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Abstract

Purpose—To characterize the cellular action mechanism of Debio 0507, we compared the major DNA adducts formed by Debio 0507- and oxaliplatin-treated HCT116 human colon carcinoma cells by a combination of inductively coupled plasma mass spectrometry (ICP-MS) and ultra-performance liquid chromatography mass spectrometry (UPLC-MS/MS).

Methods—HCT116 cells were treated with IC₅₀ doses of Debio 0507 or oxaliplatin for 3 days. Total cellular Pt–DNA adducts were determined by ICP-MS. The DNA was digested, and the

major Pt–DNA adducts formed by both drugs were characterized by UPLC/MS/MS essentially as described previously for cisplatin (Baskerville-Abraham et al. in Chem Res Toxicol 22:905–912, 2009).

Results—The Pt level/deoxynucleotide was $7.4/10^4$ for DNA from Debio 0507-treated cells and $5.5/10^4$ for oxaliplatin-treated cells following a 3-day treatment at the IC_{50} for each drug. UPLC-MS/MS in the positive ion mode confirmed the major Pt–DNA adducts formed by both drugs were dach-Pt-d(GpG) (904.2 m/z 610 m/z and 904.2 m/z 459 m/z) and dach-Pt-d(ApG) (888.2 m/z 594 m/z and 888.2 m/z 459 m/z).

Conclusions—These data show that the major DNA adducts formed by Debio 0507 are the dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts and at equitoxic doses Debio 0507 and oxaliplatin form similar levels of dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts. This suggests that the action mechanisms of Debio 0507 and oxaliplatin are similar at a cellular level.

Keywords

Debio 0507; Oxaliplatin; DNA adducts; LC/MS; UPLC/MS/MS

Introduction

Platinum compounds with the (*trans-R,R*) diaminocyclohexane (dach) carrier ligand are often effective in the treatment of cisplatin-resistant tumors. One of these compounds, oxaliplatin (*trans-R,R*-diaminocyclohexaneoxalatoplatinum(II)), is widely used in the treatment of colon cancer and other cisplatin-resistant tumors. However, oxaliplatin suffers from a number of drawbacks, including a rapid onset cold-sensitive neuropathy and cumulative neurotoxicity [1]. Debio 0507 is a nanoparticulate, micellar dach-Pt compound in which the dach-Pt moiety is complexed to a polyglutaminated polyethylene glycol polymer (which will be referred to as PEG-pGlu). It has a number of properties that suggest it may be an effective anticancer agent. For example, (1) it has very slow clearance and excellent stability in plasma [2, 3]; (2) it is rapidly taken up by cancer cells [3, 4]; (3) it appears to have very low toxicity [3]; and (4) it has demonstrated anti-tumor activity in several animal models [3, 4].

However, a number of important questions remain. For example, it is not known how Debio 0507 is activated once it is inside the cell. If one knew the mechanism of activation, it might suggest strategies for increasing the efficacy and/or further decreasing the toxicity of Debio 0507. In addition, it is not known whether the Pt–DNA adducts formed by Debio 0507 in the tumor cell are similar to, or different from, those formed by oxaliplatin. There is an abundant literature on the chemistry and biology of the dach-Pt–DNA adducts formed by oxaliplatin [5–10]. There is also an extensive literature on the selective recognition of cisplatin- and oxaliplatin-DNA adducts by cellular proteins [11–17] and the relation between that differential adduct recognition and the efficacy of oxaliplatin in cisplatin-resistant tumors [5, 18–22]. Thus, if the adducts formed by Debio 0507 are similar to those formed by oxaliplatin, it would go a long way toward defining the cellular action mechanism of Debio 0507. On the other hand, if the Pt–DNA adducts formed by Debio 0507 are fundamentally different from those formed by oxaliplatin, it would suggest that Debio 0507 might be effective in the treatment of both cisplatin- and oxaliplatin-resistant tumors.

Formation of Pt–DNA adducts by most Pt(II) compounds is a two-step process [23–25]. The rate-limiting steps of the reaction sequence are the aquation reactions prior to the formation of Pt–DNA monoadducts and prior to the conversion of Pt–DNA monoadducts to Pt–DNA diadducts. Because the rate of reaction of the aquated intermediates with DNA are so rapid, the aquation rates become the *de facto* rates for monoadduct formation and monoadduct to

diadduct conversion. Intermediate formation of a DNA monoadduct has been seen both in vitro and in cells for every Pt(II) complex investigated to date (e.g. cisplatin (*cis*-diamminedichloroplatinum(II)), Pt(en)Cl₂ (ethylenediaminedichloroplatinum(II)), Pt(dach)Cl₂ (*trans*-*R,R*-diaminocyclohexanedichloroplatinum(II)), carboplatin (*cis*-diammine(1,1-cyclobutanedicarboxylato) platinum(II)), Pt(dach)(mal) (*trans*-*R,R*-diaminocyclohexanemal-onatoplatinum(II)), and oxaliplatin) [23–25]. As might be expected, the rate of aquation, and hence the rate of formation of the DNA monoadduct, is strongly dependent on the nature of the leaving ligand. For cisplatin and Pt(dach)Cl₂, the half-life for aquation in vitro is around 2 h [23–25], while for carboplatin, Pt(dach)(mal) and oxaliplatin, it is several days [26, 27]. The rate of monoadduct to diadduct conversion is dependent on the nature of the leaving ligand, but it is also influenced by the nature of the carrier ligand. For example, the $t_{1/2}$ of monoadduct to diadduct conversion for cisplatin, Pt(en)Cl₂ and Pt(dach)Cl₂ is 144, 110 and 264 min, respectively [6].

The mechanism of intracellular activation of Pt(II) compounds with carboxylate leaving ligands, especially those with *bis*-bidentate carboxylate leaving ligands such as carboplatin, oxaliplatin and Debio 0507, is unknown. Obviously, the rate of intracellular activation and Pt–DNA adduct formation is greater than would have been anticipated from the in vitro rate of aquation. While chloride ions are fairly effective at displacing carboxylate ligands at extracellular chloride concentrations, they are much less effective at intracellular chloride concentrations. We [28–31] and others [32] have shown that HCO₃[−] and H₂PO₄[−] can facilitate the activation of Pt(II) complexes with carboxylate leaving ligands. This has led us to propose the activation model for Debio 0507 shown in Fig. 1. This model is modified from the one originally proposed for Pt(dach)(mal) and oxaliplatin [28, 29] because of data showing that Debio 0507 appears to be much more stable than oxaliplatin in plasma [2, 3].

In this model, HCO₃[−] and/or H₂PO₄[−] facilitate the monoaquation of the Pt(II) complex. The equilibrium is strongly in favor of the parent compound because of the proximity effect of the adjacent carboxylate groups on the leaving ligand. However, the monoaquated intermediate that does form is very reactive and can form monoadducts on the DNA, as well as presumably inactive complexes with amino acids and intracellular proteins. Because of the overlap of orbitals of stacked purine bases, the Pt–DNA monoadduct is most frequently formed on the N7 of the 3' guanine of GG (and less frequently AG) sequences in the DNA [33]. The N7 of guanine is more nucleophilic than the carboxylate moiety of the leaving ligand and has an even stronger proximity effect, so the monoadducts are fairly rapidly converted to diadducts [24, 25, 33]. HCO₃[−] and/or H₂PO₄[−] can also facilitate the formation of the diaquated Pt(II) complex, which could form diadducts on the DNA directly [28, 30]. However, the equilibrium under intracellular conditions strongly favors the intermediate formation of the monoaquated Pt(II) complex, probably by at least an order of magnitude [24, 29, 31].

This model suggests that Pt(II) complexes with *bis*-bidentate carboxylate leaving ligands are likely to form intracellular DNA adducts via a pathway involving the intermediate formation of Pt–DNA monoadducts with the leaving ligand still attached to one reactive center of the platinum. This appears to be a reasonable model for the reaction of carboplatin and oxaliplatin with cellular DNA and is supported by recent studies using accelerator mass spectrometry to demonstrate the intermediate formation of DNA monoadducts containing the 1,1-cyclobutanedicarboxylate leaving ligand when human bladder cancer cells were treated with carboplatin [34] and the oxalate ligand when cells were treated with oxaliplatin [35, 36]. While the geometry of the glutamate residues in Debio 0507 is not constrained to the same extent as the carboxylate groups of the 1,1-cyclobutanedicarboxylate (carboplatin) or oxalate (oxaliplatin) ligands, it is reasonable to expect that the reaction pathway for Debio 0507 is similar to that of carboplatin and oxaliplatin.

If it is a monoaquated Pt(II) species containing the leaving ligand that approaches the DNA to form the mono-adduct, it is reasonable to ask whether the presence of the leaving ligand can influence the site and type of adduct formation. Certainly, the leaving ligand could pose steric constraints, as well as affecting the charge distribution and lipophilicity of the platinum complex. There have been reports, for example, that the pattern of DNA adduct formation by carboplatin is not identical to that of cisplatin [37, 38], but those observations have not been reproduced using other techniques.

In the case of oxaliplatin, the data are not entirely clear-cut. Early in vitro studies used Pt(dach)Cl₂ [6], Pt(dach)(SO₄) [6] or Pt(dach)(NO₃)₂ [39] to speed the rate of Pt adduct formation. These Pt-dach complexes formed the same DNA adducts as cisplatin on the same sites on the DNA in vitro. More recently, Saris et al. [40] reported that the ratio of Pt-d(GpG) to Pt-d(ApG) adducts formed by cisplatin and oxaliplatin was very similar both in vitro and in cell culture. However, the ³²P-postlabeling methodology that they used was only semi-quantitative and did not take into account monoadduct to diadduct conversion during sample workup. When Woynarowski et al. [41] incubated DNA with cisplatin and oxaliplatin in vitro, they found that the sites of adduct formation were qualitatively similar but quantitatively different, suggesting that the oxalate leaving ligand might exert subtle effects on the site of adduct formation.

The situation is even less clear for Debio 0507. In the case of Pt(dach)(mal) [28] and oxaliplatin [30, 42], there is significant conversion of the parent compound to Pt(dach)Cl₂ in plasma and tissue culture media. This is accompanied by the early intracellular appearance of Pt(dach)Cl₂ in both red blood cells and tissue culture cells [29, 30]. Since Pt(dach)Cl₂ is much more reactive than the parent compounds, it is likely that this Pt(dach)Cl₂ makes a significant contribution to intracellular Pt–DNA adduct formation; and since chloride concentrations are low in the cell, it is likely that most of the intracellular Pt(dach)Cl₂ arises from the extracellular reaction with chloride. In contrast, carboplatin is much more stable in plasma than either Pt(dach)(mal) or oxaliplatin, so the intracellular activation pathway is likely to be fundamentally different for carboplatin—which may explain why the ratio of Pt-d(GpG) to Pt-d(ApG) adducts appears to be different for carboplatin [37] compared to either cisplatin or oxaliplatin [40]. Since Debio 0507 is also extremely stable in plasma, its intracellular activation pathway may more closely resemble carboplatin than oxaliplatin.

Highly sensitive LC/MS/MS (liquid chromatography tandem mass spectrometry) methods have been described for the identification and quantification of the dach-Pt-d(GpG) and dach-Pt-d(ApG) DNA adducts formed by oxaliplatin [43–45], although only the dach-Pt-d(GpG) adduct has been quantified in tissue samples by these methods [43]. More recently, we have described a UPLC/MS/MS (ultraperformance liquid chromatography tandem mass spectrometry) method for identification and quantification of the intracellular CP-d(GpG) adducts formed by cisplatin [46]. In the present study, we have modified this method to identify the major intracellular Pt–DNA adducts formed by Debio 0507 and compare them to the Pt–DNA adducts formed by oxaliplatin in the same cell. Our working model was that non-enzymatic activation of Debio 0507 occurred, resulting in formation of dach-Pt intrastrand diadducts (primarily dach-Pt-d(GpG) and dach-Pt-d(ApG)) that would be indistinguishable from those formed by oxaliplatin.

Materials and methods

Drugs

Debio 0507 (Batch Number N0800263, 9.3 mg Pt/vial) was produced by Nanocarrier Co., Ltd, with the MediCelle Technology and was provided by Debiopharm. Oxaliplatin (Eloxatin), obtained from UNC hospital pharmacy, was manufactured by Ben Venue

Laboratories (Lot # 479738, 100 mg of oxaliplatin and 900 mg lactose monohydrate/ vial). Stock solutions of Debio 0507 (9.3 mg/ml) and oxaliplatin (5 mM, 2 mg/ml) were prepared by dissolving the drugs in water.

Cell culture

HCT116 human colon carcinoma cells were obtained from Dr. Jean Cook (Department of Biochemistry & Biophysics, University of North Carolina, Chapel Hill, NC) and were mycoplasma free. The cells were grown in McCoy's media, 5% FBS, non-essential amino acids and Penstrep.

Cell growth inhibition

Cytotoxicity was determined by the cell growth inhibition assay for Debio 0507 and oxaliplatin following a 3-day treatment by the following protocol: 2×10^5 cells were plated in 6-cm culture plates with 5 ml of McCoy's media, 5% FBS, non-essential amino acids and Penstrep. The cells were incubated overnight at 37°C to allow cells to become attached before treating with drug. Debio 0507 or oxaliplatin was then added at the indicated doses and incubation continued for 3 days. Cytotoxicity was determined by growth inhibition at the end of the 3-day incubation.

Preparation of dach-Pt-d(GpG) and dach-Pt-d(ApG) standards

(*trans-R,R*) Diaminocyclohexanedichloroplatinum(II) (Pt(dach)Cl₂) was converted to (*trans-R,R*) diaminocyclohexanediaquaplatinum(II) (Pt(dach)(H₂O)₂²⁺) by incubation with a 1:05-fold excess of AgNO₃ overnight at 30°C in the dark, and the AgCl₂ precipitant was subsequently removed by filtration. Aliquots of Pt(dach)(H₂O)₂²⁺ were stored at -20°C until use. 0.2 mM d(GpG) or d(ApG) was incubated with 0.8 mM Pt(dach)(H₂O)₂²⁺ in water for 16–20 h at 37°C in the dark. Samples of dach-Pt-d(GpG) and dach-Pt-d(ApG) prepared as described above were injected onto a Clarity oligo-RP 3u column (Phenomenex). Buffer A was 10 mM ammonium acetate, 0.1% acetic acid, pH 4 and buffer B was HPLC grade methanol. The gradient was 0–30% B over 20 min, followed by 30–70% B over 2 min and 70–0% B over 3 min. The flow rate was 1 ml/ min. The elution times for dach-Pt-d(GpG) and dach-Pt-d(ApG) were 8.76 and 9.73 min, respectively. The eluate between 8.5 and 8.9 min was collected for dach-Pt-d(GpG), and the eluate between 9.71 and 9.75 min was collected for dach-Pt-d(ApG). The samples were concentrated to dryness under vacuum using a Savant SVC100H SpeedVac Concentrator and resuspended in the appropriate buffer. The final concentration of the samples was determined by UV and confirmed by ICP-MS determination of platinum. It was 12 pmol/ul for the dach-Pt-d(GpG) standard and 9.2 pmol/ul for the dach-Pt-d(ApG) standard.

DNA isolation and digestion

DNA was isolated from the Debio 0507- or oxaliplatin-treated cells using the Gentra PureGene Cell Kit (Qiagen) according to the manufacturer's protocol. The DNA was then digested essentially as described by Eastman [47, 48]. The DNA was resuspended in the DNA Hydration Buffer provided with the Qiagen Gentra PureGene Kit, and the pH was brought to 7 with 190 mM sodium acetate with 11 mM MgCl₂ before addition of 0.24 KU DNase I. The reaction mixture was then incubated at 37°C for 4 h. Next, 24 µg nuclease P₁ was added and incubation was continued for 16 h. The pH was then brought to 9 with 10 mM Tris-HCl before addition of 5 U alkaline phosphatase, and the reaction mixture was incubated for an additional 4 h. Following digestion, the samples were purified with Microcon YM-3 columns to remove enzymes. DNA digests obtained from 10⁷ cells were concentrated to 500 µl under vacuum using a Savant SVC100H SpeedVac Concentrator.

ICP-MS determination of Pt–DNA adduct level

A Varian 820-MS inductively coupled plasma–mass spectrometer was used to determine Pt concentration. Samples were introduced via a concentric glass nebulizer with a free aspiration rate of 0.4 ml/min, a Peltier-cooled double-pass glass spray chamber and a quartz torch. A peristaltic pump carried samples from a SPS3 autosampler (Varian) to the nebulizer. All standards and samples were in 2% HNO₃, prepared with milliQ water. Prior to each experiment, optimization of the instrument operating parameters was performed using a tuning solution (Spectropure, Arlington, TX) diluted to 5 ppb each of Ba, Be, Ce, Co, In, Pb, Mg, Tl and Th. Ion optics and plasma parameters were optimized to maximize sensitivity while minimizing interferences. Pt standards were prepared by serial dilution of a solution containing 10 ppm Pt (Inorganic Ventures Inc., Lakewood, NJ). A four-point calibration curve was made over a concentration range of 0.5–8 ppb Pt. The two most abundant isotopes of Pt were monitored: ¹⁹⁴Pt (33.0% abundance) and ¹⁹⁵Pt (33.8% abundance). A 200 ppb dilution of the tuning solution was used for monitoring ¹¹⁵In as the internal standard. Data acquisition was done using peak hopping with a dwell time of 50 ms, one point per peak, 20 scans/replicate and five replicates per sample.

Identification and quantification of dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts by LC/MS

The protocol for the LC/MS experiments was as follows: LC was performed with Phenomenex Luna C18 100 mm × 2.1 mm; 3-μm column with a flow rate of 0.2 ml/min. Buffer A was water containing 10 mM ammonium acetate, 0.1% acetic acid, pH 4. Buffer B was methanol. The gradient was 0–30% B over first 20 min, followed by 30–70% B between 20 and 22 min, 70–0% B between 22 and 25 min and 30 min of re-equilibration at 0% B. The mass spectrometry was performed with a ThermoFinnigan LCQ Deca ion trap scanned from 150 to 1,500 m/z with ESI + electrospray ionization in the positive mode. The spray voltage was 5 kV, the sheath gas was 80 (arbitrary units), the auxiliary gas was 20 (arbitrary units) and the heated capillary temperature was 350°C.

Confirmation of dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts by UPLC/MS/MS

The protocol for the UPLC/MS/MS experiments was as follows: UPLC was performed with a Thermo Hypersil C18 100 mm × 2.1 mm; 3-μm column with a flow rate of 0.2 ml/min. Solvent A was 0.1% acetic acid. Solvent B was acetonitrile. The gradient was 1% B for 2 min, followed by 50% B for 23 min and 5 min of re-equilibration at 1% B. The MS/MS instrument was a ThermoFinnigan TSQ Quantum Ultra triple quadrupole with HESI + heat-assisted electrospray ionization in the positive mode. The probe temperature was 250°C. The spray voltage was 3 kV, the sheath gas was 35 (arbitrary units), the auxiliary gas was 30 (arbitrary units) and the heated capillary temperature was 285°C. The MRM (multiple reaction monitoring) scan method was used with a collision gas pressure of 1.5 mTorr. Dach-Pt-d(GpG) was monitored by 904.2–610 m/z with a collision energy of 25 V and 904.2 to 459 m/z with a collision energy of 37 V. Dach-Pt-d(ApG) was monitored by 888.2–594 m/z with a collision energy of 25 V and 888.2–459 m/z with a collision energy of 37 V.

Results

Preparation of dach-Pt-d(GpG) and dach-Pt-d(ApG) standards and characterization of those standards by LC/MS and UPLC/MS/MS

The dach-Pt-d(GpG) and dach-Pt-d(ApG) standards were prepared from Pt(dach)(H₂O)₂²⁺ and either d(GpG) or d(ApG) by a slight modification (“Materials and methods”) of the procedure that we have previously described for the synthesis of the CP-d(GpG) standard [46]. Positive ion electrospray LC/MS was then used to characterize the positive ion fragmentation patterns for the dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts. Again the

separation and detection conditions were a slight modification (“Materials and methods”) of those previously described for the CP-d(GpG) standard [46]. The results of the LC/MS analysis of the dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts are shown in Fig. 2.

For dach-Pt-d(GpG) (Fig. 2A) in addition to the parent compound at m/z of 904, the two major ion fragments observed were dach-Pt-(G)₂ at m/z of 610 and dach-Pt-G at m/z of 459. For dach-Pt-d(ApG) (Fig. 2B) in addition to the parent compound at m/z of 888, the two major ion fragments observed were dach-Pt-(A,G) at m/z of 594 and dach-Pt-G at m/z of 459. The experiments described above showed that the maximum sensitivity for LC/MS/MS detection of the dach-Pt-d(GpG) adduct in the positive ion mode was for a positive ion at 904 m/z with fragments at 610 and 459 m/z . This is identical to the electrospray positive ion fragmentation pattern observed by Kerr et al. [45]. Similarly, the maximum sensitivity for detection of dach-Pt-d(ApG) was for a positive ion at 888 m/z with fragments at 594 and 459 m/z . Similar experiments were performed for each of the deoxynucleosides (data not shown). In each case, the major positive ion fragment was the corresponding base. Thus, for the deoxynucleosides optimal sensitivity for detection in the positive ion mode was dA (252 136), dC (228 112), dG (268 152), dI (253 137) and dT (243 127).

These fragmentation patterns were used to guide the UPLC/MS/MS method for detection of the dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts in the presence of the de-oxynucleosides expected from a complete digestion of cellular DNA (Fig. 3). While the dach-Pt-d(GpG) peak shows some overlap with the dT peak, it is clear that the separation and identification procedure is sufficient for the separation and identification of dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts in a DNA digest.

Determination of the sensitivity of detection of dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts in the presence of excess undamaged deoxynucleosides by UPLC/MS/MS

In order to determine the assay sensitivity, the dach-Pt-d(GpG) and dach-Pt-d(ApG) standards were diluted with a DNA digest obtained from untreated calf thymus DNA at ratios ranging from 1:10 to 1:10,000 and analyzed by UPLC/MS/MS. This experiment showed that the LC/MS/MS methodology was capable of detecting both adducts in the presence of a 10,000-fold excess of undamaged DNA (Fig. 3).

Determination of Debio 0507 and oxaliplatin cytotoxicity in the HCT116 human colon carcinoma cell line

The cytotoxicity of Debio 0507 and oxaliplatin in the human colon carcinoma cell line HCT116 was determined by growth inhibition following a 3-day continuous incubation with drug as described in Materials and methods. The 3-day IC₅₀ values for Debio 0507 and oxaliplatin were 0.014 μ g Pt/ml and 0.003 μ M (0.0006 μ g Pt/ml), respectively.

Determination of Pt–DNA adduct levels in HCT116 human colon carcinoma cells treated with equitoxic doses of Debio 0507 and oxaliplatin by ICP-MS

Having determined the limits of sensitivity of the UPLC/MS/MS assay, we next determined what the Pt–DNA adduct concentrations were in DNA digests prepared from 1×10^7 HCT116 cells treated with IC₅₀ levels of Debio 0507 or oxaliplatin (using 3-day IC₅₀s and 3-day incubations). The cell culture conditions were the same as those described in Materials and Methods. DNA was isolated from the treated cells using the Gentra PureGene Cell Kit (Qiagen) according to the manufacturer’s protocol and was digested as described in “Materials and methods”. This digestion procedure has been shown by several laboratories to digest undamaged DNA to the deoxynucleoside level and quantitatively convert Pt–GpG and Pt–ApG intrastrand diadducts to free Pt-d(GpG) and Pt-d(ApG), respectively [43–48]. Following digestion, the samples were purified in Microcon YM-3 columns to remove

enzymes and concentrated to 500 μ l under vacuum using a Savant SVC100H SpeedVac Concentrator. Aliquots were diluted to 4 ml in 2% nitric acid for ICP/MS analysis as described in “Materials and methods”. The Pt levels were 5.5 Pt adducts/ 10^4 deoxynucleosides for the DNA digest obtained from HCT116 cells treated with an IC_{50} dose of oxaliplatin and 7.4 Pt adducts/ 10^4 deoxynucleosides for the DNA digest obtained from HCT116 cells treated with an IC_{50} dose of Debio 0507.

Determination of the types of Pt–DNA adducts formed in human HCT116 human colon carcinoma cells treated with equitoxic doses of Debio 0507 and oxaliplatin by LC–MS, UPLC-MS/MS and ICP-MS

HCT116 cells were treated with IC_{50} levels of Debio 0507 or oxaliplatin (using 3-day IC_{50} s and 3-day incubations). The cell culture conditions were the same as those described in Materials and Methods except that 2×10^7 cells were plated in 15-cm culture plates containing 25 ml of media. DNA was isolated from the treated cells using the Gentra PureGene Cell Kit (Qiagen) according to the manufacturer’s protocol. DNA was accumulated from a total of 1×10^8 Debio 0507- and oxaliplatin-treated cells and digested to the deoxynucleoside level as described in Materials and Methods.

The DNA digests were then pooled and fractions corresponding to dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts were HPLC purified as shown in Fig. 4. Briefly, at the beginning of each day HPLC elution profiles were obtained for DNA digest alone (Panel A) and DNA digest spiked with dach-Pt-d(GpG) and dach-Pt-d(ApG) standards (Panel B). On the basis of those two runs, a decision would be made with respect to which fractions would be collected to isolate the dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts, if they existed, from the DNA digests obtained from Debio-0507-treated cells (Panel D) and oxaliplatin-treated cells (Panel C). Because of the overlap of the dT and dach-Pt-d(GpG) peaks, only a subset of fractions corresponding to the location of the dach-Pt-d(GpG) peak could be collected.

Fractions corresponding to the elution positions of dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts were collected from the DNA digests obtained from 1×10^8 Debio 0507- and oxaliplatin-treated cells. These fractions were pooled and reduced in volume to 100 μ l under vacuum using a Savant SVC100H SpeedVac Concentrator. These pooled fractions were analyzed by LC/MS as described in Materials and Methods. The results are shown in Fig. 5. Dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts can clearly be identified in DNA digests from HCT116 cells treated with equitoxic doses of Debio 0507 and oxaliplatin. The dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts were identified based on their positive ion m/z (Fig. 5a–d) and characteristic platinum isotope distribution patterns (data not shown).

Their identity was further verified by UPLC/MS/MS performed as described in “Materials and methods”. These data are shown in Fig. 6. The UPLC/MS/MS analysis confirmed the identity of the dach-Pt-d(ApG) and dach-Pt-d(GpG) adducts isolated from DNA digests obtained from both Debio 0507- and oxaliplatin-treated cells on the basis of their characteristic fragmentation patterns (888.2 m/z 594 m/z and 888.2 m/z 459 m/z for the dach-Pt-d(ApG) adduct and 904.2 m/z 610 m/z and 904.2 m/z 459 m/z for the dach-Pt-d(GpG) adduct).

Finally, the amount of platinum in the HPLC fractions used in these LC/MS and UPLC/MS/MS analyses was determined by ICP-MS as described in “Materials and methods” (Table 1).

During the HPLC purification procedure, it was necessary to collect only a portion of the fractions corresponding to the dach-Pt-d(GpG) adduct because of overlap with the deoxythymidine peak. This probably explains the near-equivalence of dach-Pt-d(ApG) and

dach-Pt-d(GpG) and the relatively low overall recovery relative to the 90% recovery of cisplatin-d(GpG) reported by Baskerville-Abraham et al. [46].

The total recovery of dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts was similar from cells treated with Debio-0507 and oxaliplatin at IC₅₀ doses. However, it is important to keep in mind that the recoveries shown in Table 1 are only semi-quantitative estimates based on the total amount of platinum recovered from the DNA digests before and after HPLC purification. To determine an accurate recovery of each of the adducts, it would have been necessary to include an ¹⁵N-labeled internal standard as described by Baskerville-Abraham et al. [46].

Discussion

The major finding from this study is that levels of dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts are very similar in cells treated with Debio 0507 and oxaliplatin at equitoxic (IC₅₀) doses. While our determination of dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts is only semi-quantitative, this conclusion is strengthened by the observation that total Pt–DNA adducts are also very similar in cells treated with equitoxic doses of Debio 0507 and oxaliplatin. Taken together, these data suggest that the action mechanism of Debio 0507 and oxaliplatin may be similar at a cellular level.

These experiments do not eliminate the possibility that Debio 0507 may form unique adducts that were not detected in these experiments. For example, in analogy with carboplatin [34] and oxaliplatin [35, 36], Debio 0507 might be expected to form detectable levels of Pt–G monoadducts at early times. These experiments were specifically designed to identify the Pt–DNA adducts that accumulated at 3 days of continuous incubation and did not address whether significant levels of Pt–DNA monoadducts might have been present at earlier time. In addition, the data do suggest that Debio 0507 may form small amounts of one or more Pt–DNA adducts not detected in these experiments. At IC₅₀ doses, the level of total platinum in DNA digests was 26% higher for DNA from Debio 0507-treated cells than for DNA from oxaliplatin-treated cells (7.4 Pt adducts/10⁴ deoxynucleosides for the DNA digest obtained from HCT116 cells treated with an IC₅₀ dose of Debio 0507 versus 5.5 Pt adducts/10⁴ deoxynucleosides for the DNA digest obtained from HCT116 cells treated with an IC₅₀ dose of oxaliplatin). Conversely, the recovery of dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts from the oxaliplatin-treated cells was about 30% greater than from Debio 0507-treated cells (Table 1). However, since the overall cytotoxicity is similar in Debio 0507- and oxaliplatin-treated cells at these adduct levels, it appears unlikely that the unidentified Pt–DNA adducts in the Debio 0507-treated cells make a significant contribution to cytotoxicity. Similarly, Debio 0507 may have interactions in the cell that do not involve the formation of Pt–DNA adducts, but by the same argument those interactions would also not appear to contribute significantly to the cytotoxicity of Debio 0507.

This study does not provide any information on the intracellular mechanism of activation for Debio 0507. Specifically, it did not address whether adduct formation proceeded via formation of a transient intermediate containing part of the polyglutaminated PEG polymer. While that could be an important avenue for future research, it is not immediately apparent how such an intermediate would be readily identified. LC–MS and LC–MS/MS identification of DNA adducts require that the precise chemical composition and ion fragmentation pattern of the adduct be known. Our experience with LC–MS analysis of the in vitro reaction between Debio 0507 and d(GpG) (data not shown) suggests that the early reaction products formed are heterogeneous and do not contain a simple ion fragmentation pattern that would be amenable to LC–MS analysis.

However, our data do suggest that the activation and formation of Pt–DNA adducts may occur more slowly for Debio 0507 than for oxaliplatin. For example, in our experiments the 3-day IC₅₀ for Debio 0507 in HCT116 human colon carcinoma cells was 0.04 µg Pt/ml compared to only 0.0006 µg Pt/ml for oxaliplatin (a 23-fold difference), yet the Pt–DNA adduct levels were almost identical. However, it is important to point out that both the cytotoxicity and the formation of Pt–DNA adducts were measured at 3 days in our experiments, while platinum has been detected in both the plasma and the tumor cells of Debio 0507-treated animals for up to 14 days [3]. Thus, the slower activation and Pt–DNA adduct formation of Debio 0507 is likely balanced by its greater persistence in the cell.

Even though the Pt–DNA adducts formed by Debio 0507 and oxaliplatin appear to be very similar at equitoxic doses, the pharmacokinetics, pharmacodynamics and intracellular biotransformations of Debio 0507 appear to be unique [2–4] and may result in a very different therapeutic profile. Previous studies have shown that Debio 0507 is eliminated from plasma very slowly and therefore accumulates to unusually high levels in both plasma and tumor tissue [2–4]. Preliminary animal model studies have also indicated that the tumor range of Debio 0507 may be different from that of oxaliplatin and that the toxicity of Debio 0507 may be less than that seen for oxaliplatin at therapeutic doses [3]. In conclusion, our studies have helped elucidate the cellular action mechanism of Debio 0507, a novel dach-Pt micellar formulation that holds significant promise as a chemotherapeutic agent.

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Fig. 1.

Proposed intracellular activation mechanism for Debio 0507 AA = amino acid, Prot = protein, Glu–Glu represents the PEG–pGlu leaving ligand of Debio 0507, N7G–N7G represents adjacent guanines on the DNA. This model is modified from the one originally proposed for Pt(dach)(mal) and oxaliplatin [28, 29] because of data showing that Debio 0507 appears to be stable in plasma [2, 3]

Fig. 2.

Positive ion LC/MS characterization of dach-Pt-d(GpG) and dach-Pt-d(ApG). **a** dach-Pt-d(GpG); **b** dach-Pt-d(ApG); *Panel A* UV elution profile at 260 m μ . *Panel B* Full spectrum positive LC scan of the elution profile (because of the instrument configuration, peaks elute ~0.1 min latter for MS compared to UV). *Panel C* Positive ion scan from 150 to 1,500 m/z for peak eluting at 9.9 min. *Panel D* Zoom of positive ion with m/z of 904 to show characteristic platinum isotope pattern. A Thermo LCQ Deca ion trap mass spectrometer was used for data acquisition, and Xcalibur software was used to construct graphs

Fig. 3.

Serial dilution of dach-Pt-d(GpG) and dach-Pt-d(ApG) standards with DNA digests obtained from undamaged DNA. Dach-Pt-d(GpG) and dach-Pt-d(ApG) standards prepared as described in Materials and Methods were diluted with deoxynucleosides obtained from a digested of undamaged DNA and analyzed by UPLC/MS/MS as described in “Materials and methods”. Each compound was detected based on their characteristic positive ion fragmentation pattern: dach-Pt-d(GpG) (904 m/z → 610 m/z and 459 m/z, dach-Pt-d(ApG) (888 m/z → 594 m/z and 459 m/z), dA (252 → 136), dC (228 → 112), dG (268 → 152), dI (253 → 137) and dT (243 → 127). **a** 1:10 dilution of dach-Pt-d(GpG) and dach-Pt-d(ApG) standards with DNA digest; **b** 1:100 dilution with DNA digest; **c** 1:1,000 dilution with DNA digest; **d** 1:10,000 dilution with DNA digest. A Thermo TSQ Quantum Ultra triple-quadrupole mass spectrometer was used for data acquisition, and Xcalibur software was used to construct graphs

Fig. 4.

HPLC purification of dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts from DNA digests obtained from Debio 0507- and oxaliplatin-treated cells. The HPLC separation conditions were as described in “Materials and methods” except that a Phenomenex Oligo-RP column was used. **a** UV elution profile of DNA digest obtained from oxaliplatin-treated cells; **b** UV elution profile of DNA digest obtained from oxaliplatin-treated cells spiked with dach-Pt-d(GpG) and dach-Pt-d(ApG) standards; **c** UV elution profile of DNA digest obtained from oxaliplatin-treated cells showing fractions collected for the dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts; **d** UV elution profile of DNA digest obtained from Debio 0507-treated cells showing fractions collected for the dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts. Xmgrace software was used to construct graphs

Fig. 5.

LC/MS analysis of dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts from DNA digests obtained from Debio 0507- and oxaliplatin-treated cells. The dach-Pt-d(GpG) and dach-Pt-d(ApG) HPLC fractions from DNA digests obtained from oxaliplatin- and Debio 0507-treated cells were analyzed by positive ion LC/MS as described in “Materials and methods”. **a** Positive ion LC/MS of HPLC fraction corresponding to the dach-Pt-d(ApG) adduct from oxaliplatin-treated cells; **b** Positive ion LC/MS of HPLC fraction corresponding to the dach-Pt-d(GpG) adduct from oxaliplatin-treated cells; **c** Positive Ion LC/MS of HPLC fraction corresponding to the dach-Pt-d(ApG) adduct from Debio 0507-treated cells; **d** Positive ion LC/MS of HPLC fraction corresponding to the dach-Pt-d(GpG) adduct from Debio 0507-treated cells. For each panel, the top graph is a positive ion scan at 888–891 m/z, the middle graph is a positive ion scan at 904–906 m/z, and the bottom figure is a UV trace of the elution profile. An Agilent 6520 Accurate Mass Q-TOF mass spectrometer was used for data acquisition, and Masshunter Qualitative Analysis software was used to construct graphs

Fig. 6.

UPLC/MS/MS analysis of dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts from DNA digests obtained from Debio 0507- and oxaliplatin-treated cells. The dach-Pt-d(GpG) and dach-Pt-d(ApG) HPLC fractions from DNA digests obtained from oxaliplatin- and Debio 0507-treated cells were analyzed by positive ion UPLC/MS/MS as described in “Materials and methods”. **a** Positive ion UPLC/MS/MS of HPLC fraction corresponding to the dach-Pt-d(ApG) adduct from oxaliplatin-treated cells; **b** Positive ion UPLC/MS/MS of HPLC fraction corresponding to the dach-Pt-d(GpG) adduct from oxaliplatin-treated cells; **c** Positive ion UPLC/MS/MS of HPLC fraction corresponding to the dach-Pt-d(ApG) adduct from Debio 0507-treated cells; **d** Positive ion UPLC/MS/MS of HPLC fraction corresponding to the dach-Pt-d(GpG) adduct from Debio 0507-treated cells. For each panel, the *top figure* is the positive ion scan for dach-Pt-d(ApG) (888.2 m/z → 594 m/z and 888.2 m/z → 459 m/z) and the *bottom figure* is the positive ion scan for dach-Pt-d(GpG) (904.2 m/z → 610 m/z and 904.2 m/z → 459 m/z). A Thermo TSQ Quantum Ultra triple-quadrupole mass spectrometer was used for data acquisition, and Xcalibur software was used to construct graphs

Table 1

Platinum content of HPLC fractions containing dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts

Drug	HPLC fractions tested	Platinum content (pmoles) ^a	Total for each drug (pmoles)	% Recovery (%) ^b
Debio 0507	dach-Pt-d(ApG)	32	63	29
Debio 0507	dach-Pt-d(GpG)	31		
Oxaliplatin	dach-Pt-d(ApG)	27	58	38
Oxaliplatin	dach-Pt-d(GpG)	31		

^aPlatinum content of the pooled, concentrated HPLC fractions (100 µl) from DNA digests obtained from 1×10^8 cells treated with IC₅₀ doses of each drug. Platinum content was determined by ICP-MS as described in “Materials and methods”

^bRecovery is based on the platinum content of DNA digests obtained from 1×10^7 cells prior to HPLC purification of the dach-Pt-d(GpG) and dach-Pt-d(ApG) adducts