Reduction of *cis,trans,cis*-[$PtCl_2(OCOCH_3)_2(NH_3)_2$] by Aqueous Extracts of Cancer Cells

Alina Nemirovski, Yonit Kasherman, Yael Tzaraf, and Dan Gibson*,†

Department of Medicinal Chemistry and Natural Products, School of Pharmacy, The Hebrew University of Jerusalem, Jerusalem, 91120, Israel

Received June 24, 2007

Abstract: Pt(IV) complexes must be reduced to kill cancer cells. While reduction rates correlate with reduction potentials, we wanted to check if the rates of reduction depend on the cell line used. The reduction of *cis,trans,cis*-[$PtCl_2(OCOCH_3)_2(NH_3)_2$] by extracts of three cell lines was measured, and the rates follow the order A2780cisR > A2780 > HT-29. The reduction is not carried out by the low molecular weight (MW) antioxidants but primarily by cellular components with MW > 3000.

The *cis,trans,cis*-[$PtCl_2(OCOCH_3)_2(NH_3)$(Am)] complexes (where Am = cyclohexylamine and adamantylamine, Figure 1) are potent anticancer agents that are in advanced clinical trials.[1] There is evidence that inert octahedral Pt(IV) complexes must be reduced to the more reactive square-planar Pt(II) complexes before binding to DNA, their critical pharmacological target.[2] Pt(IV) complexes can be considered as prodrugs that are activated by biological reducing agents. If the reduction occurs outside the cancer cell, it results in inactivation of the drug, but if the reduction takes place primarily intracellularly, the prodrug is activated and can kill the cancer cell. Thus, the potency of Pt(IV) anticancer agents depends on where they might be reduced, which is a function of their reduction potentials. The axial and equatorial ligands affect the reduction potentials and the reduction rates of Pt(IV) complexes by low molecular weight biological reducing agents such as thiols and ascorbic acid.[3] The reduction potential is influenced primarily by the nature of the axial ligands where the most difficult to reduce are complexes with axial hydroxy ligands {*ctc*-[$PtCl_2(OH)_2(NH_3)$(Am)]}, the most easily reduced are complexes with axial chloride ligands {*ctc*-[$PtCl_2(Cl)_2(NH_3)$(Am)]}, and those with axial carboxylate ligands {*ctc*-[$PtCl_2(OCOCH_3)_2(NH_3)$(Am)]} have intermediate reduction potentials.[3] Choi et al. demonstrated that reduction rates correlate with reduction potentials.[3b] Attempts to correlate reduction potentials with cytotoxicity led the researchers to conclude that in general there is no correlation between cytotoxicity and reduction potentials.[1a] In the studies correlating reduction potentials with reduction rates, the reduction rates were measured in aqueous solutions having "physiological conditions" (37 °C, pH ∼7.2) with a single reducing agent. This approach does not consider the possibility that the intracellular reduction rates might depend on the unique intracellular composition of a given cancer cell line.

Our working hypothesis was that the reduction rates of a given Pt(IV) complex will vary from one cell line to another because each cell line has a unique combination of reducing agents resulting in different reducing capacities. Thus, we set out to compare the reduction rates of the same compound, *ctc*-[$PtCl_2(OCO^{13}CH_3)_2(NH_3)_2$], by aqueous extracts from different cancer cell lines by continuously monitoring the reduction process using [^{1}H,^{13}C]HSQC[a] 2D NMR spectroscopy.

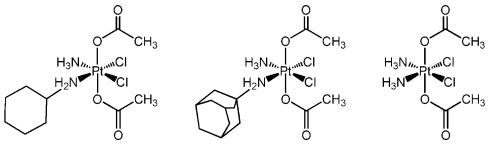


Figure 1. Pt(IV) anticancer agents: satraplatin (JM216) (left); the adamantylamine complex (LA-12) (center); ctc-[$PtCl_2(OCOCH_3)_2(NH_3)_2$] (compound **1**) (right).

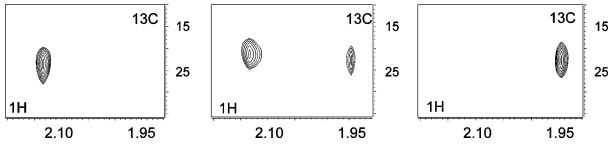
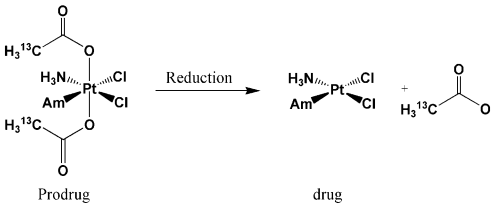


Figure 2. [^{1}H,^{13}C]HSQC spectra of the reaction of **1** with extracts of A2780 cells after 10 min (left), 110 min (center), and 160 min (right).

Scheme 1.[a]



[a] Reduction of the ^{13}C labeled *ctc*-[$PtCl_2(OCO^{13}CH_3)_2(NH_3)_2$] yields a ^{13}C labeled acetate that can be detected by [^{1}H,^{13}C]HSQC.

Toward this end, *ctc*-[$PtCl_2(OCO^{13}CH_3)_2(NH_3)_2$] (**1**) was prepared as described, using ^{13}C labeled acetic anhydride for the carboxylation reaction, and was characterized by ^{195}Pt NMR spectroscopy (1136 ppm) and ESIMS (418.9 monoisotopic peak).[4] The reduction of *ctc*-[$PtCl_2(OCO^{13}CH_3)_2(NH_3)_2$] yields *cis*-[$PtCl_2(NH_3)_2$] and free $OCO^{13}CH_3$ (Scheme 1), which can be detected by [^{1}H,^{13}C]HSQC.

The [^{1}H,^{13}C]HSQC spectrum showed that there is a clear difference between the ^{1}H chemical shifts of the Pt(IV) bound axial acetate (2.13 ppm) and the free acetate (1.94 ppm at pH 7), in agreement with the results of Ranford.[5] Cell extracts were prepared by lysing 160 million cells in 1.6 mL of deionized water followed by centrifugation to get rid of the insoluble material and by reducing the volume to 225 μL. D_2O (25 μL) was added. The sample (100 μM *ctc*-[$PtCl_2(OCO^{13}CH_3)_2(NH_3)_2$] in 250 μL of extracts, pH ∼6.8) was placed in a Shigemi NMR tube, and a spectrum was measured every 10 min on a Varian Inova 500 at 37 °C. Initially a strong peak of **1** was observed at 2.13, 21.22 ppm, which was converted into the free acetate at 1.94, 22.54 ppm (Figure 2).

The peaks in the [^{1}H,^{13}C]HSQC spectra were integrated, and the percentage of the Pt(IV) complex *ctc*-[$PtCl_2(OCO^{13}CH_3)_2(NH_3)_2$] was plotted as a function of time. The plots of the reduction of **1** by the extracts of A2780, A2780cisR, and HT-29 are depicted in Figure 3. Extracts from the different cell lines reduce *ctc*-[$PtCl_2(OCO^{13}CH_3)_2(NH_3)_2$] at different rates. The $t_{1/2}$ values for the reduction of **1** are 36, 90, and 130 min for

* To whom correspondence should be addressed. Phone: ++972 2 675 8702. Fax: ++972 2 675 7076. E-mail: dang@ekmd.huji.ac.il.

† Affiliated with the David R. Bloom Center for Pharmacy at The Hebrew University of Jerusalem, Israel.

[a] Abbreviations: HSQC, heteronuclear single quantum correlation; ESIMS, electrospray ionization mass spectrometry; GSH, glutathione; BSO, buthionine sulfoximine; MWCO, molecular weight cutoff.

10.1021/jm070740j CCC: $37.00
Published on Web 10/20/2007

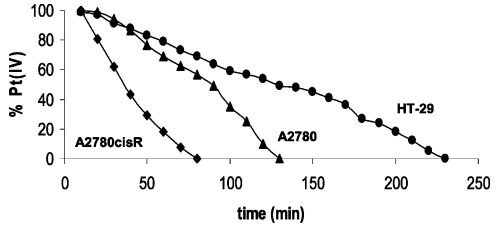


Figure 3. Plot showing the decrease of the peaks of *ctc*-[$PtCl_2(OCO^{13}CH_3)_2(NH_3)_2$] as a function of time by the whole extracts of native A2780cisR, A2780, and HT-29 (diamonds, triangles, and circles, respectively). Each point represents an average of three measurements.

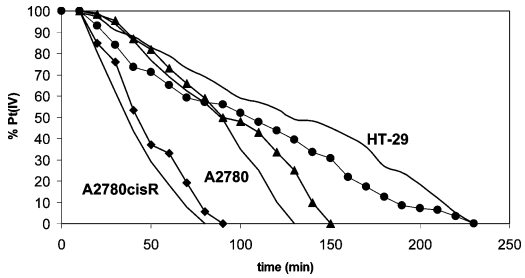


Figure 4. Plot showing the comparison of the reduction of *ctc*-[$PtCl_2(OCO^{13}CH_3)_2(NH_3)_2$] by native cells (lines) and cells pretreated with BSO: A2780cisR (solid diamond), A2780 (solid triangle), and HT-29 (solid circle).

A2780cisR, A2780, and HT-29, respectively. This demonstrates that the reduction rates depend not only on the reduction potential but also on the contents of the cancer cell.

Interestingly the reduction is fastest in the resistant ovarian cancer cell A2780cisR. A2780cisR is a cisplatin resistant cell line derived from A2780 by exposure to cisplatin, and it encompasses all known mechanisms of acquired resistance, including increased production of GSH.[6] GSH has been proposed as one of the cellular reducing agents that can activate Pt(IV) prodrugs. But are the higher GSH levels in A2780cisR really responsible for the faster reduction of *ctc*-[$PtCl_2(OCO^{13}CH_3)_2(NH_3)_2$] in A2780cisR compared with the other cell lines?

To address the question, we performed two experiments. In the first set of experiments, the cells were incubated for 24 h with 250 μM BSO (an inhibitor of GSH synthesis) to reduce the cellular GSH levels. The cells were extracted as described, and the GSH levels were determined by an HPLC equipped with an electrochemical detector.[7] The cells that were pretreated with BSO had GSH levels that were 14-fold lower than those found in the native cells. The reduction profiles of **1** by the BSO pretreated cells were compared with those of the native cells. Despite the large differences in GSH levels between the extracts of the native and BSO treated cells, the reduction profiles are very similar (Figure 4). The $t_{1/2}$ values are 42, 90, and 105 min for the BSO treated A2780cisR, A2780, and HT-29 respectively.

In the second set of experiments, the cell extracts were separated into a low MW fraction and a high MW fraction by a Centricon with a MWCO of 3000 Da, and the ability of each fraction to reduce *ctc*-[$PtCl_2(OCO^{13}CH_3)_2(NH_3)_2$] was measured. All the low molecular weight antioxidants, including GSH, should be in the low MW fraction. We quantified the GSH levels in the low and high MW fractions and found that 96% of the GSH was in the low MW fraction (~2.2 mM in the NMR sample). For each of the three cell lines the reduction of *ctc*-[$PtCl_2(OCO^{13}CH_3)_2(NH_3)_2$] by the high and low MW fractions was studied. The results are depicted in Figure 5. The reduction

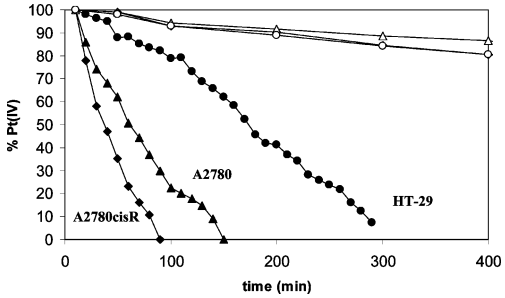


Figure 5. Plot showing the reduction of *ctc*-[$PtCl_2(OCO^{13}CH_3)_2(NH_3)_2$] as a function of time by the high MW fractions of A2780cisR (solid diamond), A2780 (solid triangle), and HT-29 (solid circle) and for the low MW fractions (open diamonds, triangles, and circles, respectively).

by the high MW fraction has a similar pattern to that exhibited by the whole extracts with $t_{1/2}$ of 35, 60, and 170 min for A2780cisR, A2780, and HT-29, respectively. Surprisingly, the low MW fraction seems to be quite inefficient at reducing **1** (20% reduction in the peak of **1** after 500 min). The results from the pretreatment with BSO and the fractionation experiments suggest that GSH does not play a major role in the reduction of *ctc*-[$PtCl_2(OCO^{13}CH_3)_2(NH_3)_2$].

Hambley et al. have shown that *ctc*-[$PtCl_2(OCOCH_3)_2(NH_3)_2$] is reduced in A2780 cells and that after 2 h only 33% remains as Pt(IV).[8] Studies of the biotransformation products of satraplatin revealed that after a 4 h incubation with SKOV3 cells only 11% of the drug remained intact (73% when the experiment was done with CH1 cells).[9] Although Galanski and Keppler showed that Pt(IV) complexes can bind directly to GMP,[10] low MW antioxidants are assumed to play an important role in the metabolism of Pt(IV) anticancer agents. Elding calculated that the half-life for the reduction of satraplatin with 5 mM ascorbic acid (15-fold excess) is approximately 12 min at 37 °C and pH 7.4.[3c]

Kratochwil and Bednarski report that a 50 μM solution of *ct*-[$PtCl_2(OCOCH_3)_2(en)$] in 3 mM GSH is stable for 24 h at 37 °C. They suggest that other as yet unidentified processes may be involved in the reduction of Pt(IV) complexes in cell culture.[11] Ranford compared the reduction of *ctc*-[$PtCl_2(OCOCH_3)_2(NH_3)_2$] by L-Cys and L-Met, finding that reduction by the thiol is significantly faster, and on the basis of typical concentrations in blood and cells, he predicted that the predominant reductant of Pt(IV) complexes will be GSH.[5] He concludes that the reduction rates are dramatically slower than for previous models of Pt(IV) systems. Kasparkova reports that GSH and MT do not play a major role in cellular deactivation of a Pt(IV) complex, implying that they are not readily reduced by them.[12] In contrast, Raynaud et al. studied the intracellular metabolism of satraplatin, finding that the nature and percentage of the biotransformation products depended on the cellular GSH levels. They also report that one of the biotransformation products contains a GSH adduct, though this adduct was not fully characterized and it is not known if it is a Pt(IV) or a Pt(II) complex. Their data support the view that GSH conjugation is an important inactivation pathway for satraplatin.[13] They do not claim or provide any evidence that the reduction was carried out by GSH.

The prevalent assumption is that the reduction is carried out by low molecular weight reductants, and little attention was devoted to the possibility that the reduction might be carried out by proteins. In a very interesting recent paper McKeage showed that satraplatin can be reduced by hemoglobin and cytochrome *c* in the presence of NADH (satraplatin disappeared with a half-life of 36 min in a solution containing hemoglobin

and NADH), suggesting that the reduction is done by transferring electrons from the metal center to the Pt(IV).[14] In the same work it is reported that satraplatin is stable in GSH in the presence or absence of NADH and after 2 h of incubation the recovery of satraplatin from GSH was 99% and from GSH with NADH, 95%. These results are consistent with those reported by Bednarski.

In agreement with the findings of Raynaud that the same Pt(IV) compound is transformed differently by different cell lines,[13] we found that aqueous extracts from different cell lines reduce the same Pt(IV) complex at different rates. While we have not identified the components of the extracts that reduce *ctc*-$[PtCl_2(OCO^{13}CH_3)_2(NH_3)_2]$, it seems reasonable to conclude that it is not the low MW reducing agents, including GSH, that are primarily responsible for the reduction of **1**. Because there are many cellular components that are capable of reducing Pt(IV) complexes and because the components responsible for the reduction of Pt(IV) complexes have not been identified and may vary from one cell line to another, care should be taken when using simple model systems for predicting cellular reduction rates. Even the low MW fractions of the aqueous extracts, which were presumed to be responsible for the reduction of Pt(IV) complexes, display reduction properties that are very different from those of the whole cell extracts. It seems reasonable to conclude that aqueous solutions (37 °C, pH 7.4) containing a single reducing agent (such as GSH, Cys, or ascorbic acid) may not be good models for predicting the reduction of Pt(IV) complexes inside cancer cells.

In summary, we have shown that the rate of reduction of *ctc*-$[PtCl_2(OCO^{13}CH_3)_2(NH_3)_2]$ depends on the contents of the cancer cells and that the reduction is not carried out by the low MW antioxidants and specifically not by GSH. The reduction is done by molecules with MW > 3000 Da. We are currently studying the effects of the equatorial ligands on the reduction by cancer cell extracts by looking at the reduction of *ctc*-$[PtCl_2(OCO^{13}CH_3)(NH_3)(Am)]$ complexes.

Acknowledgment. D.G. acknowledges partial support by the Israel Science Foundation (Grant 565/03).

References

(1) (a) Hall, M. D.; Hambley, T. W. Platinum(IV) antitumour compounds: their bioinorganic chemistry. *Coord. Chem. Rev.* **2002**, *232* (1−2), 49−67. (b) Wong, E.; Giandomenico, C. M. Current status of platinum-based antitumor drugs. *Chem. Rev.* **1999**, *99* (9), 2451−2466. (c) Galanski, M.; Keppler, B. K. Searching for the magic bullet: anticancer platinum drugs which can be accumulated or activated in the tumor tissue. *Anticancer Agents Med. Chem.* **2007**, *7* (1), 55−73. (d) Latif, T.; Wood, L.; Connell, C.; Smith, D. C.; Vaughn, D.; Lebwohl, D.; Peereboom, D. Phase II study of oral bis-(aceto)amminedichloro(cyclohexamine)platinum(IV) (JM-216, BMS-182751) given daily ×5 in hormone refractory prostate cancer (HRPC). *Invest. New Drugs* **2005**, *23* (1), 79−84.

(2) (a) Talman, E. G.; Kidani, Y.; Mohrmann, L.; Reedijk, J., Can Pt(IV)-amine complexes act as "prodrugs"? *Inorg. Chim. Acta* **1998**, 283 (1), 251−255. (b) Blatter, E. E.; Vollano, J. F.; Krishnan, B. S.; Dabrowiak, J. C. Interaction of the antitumor agents *cis*,*cis*,*trans*-$Pt^{IV}(NH_3)_2Cl_2(OH)_2$ and *cis*,*cis*,*trans*-$Pt^{IV}[(CH_3)_2CHNH_2]_2Cl_2(OH)_2$ and their reduction products with PM2 DNA. *Biochemistry* **1984**, *23* (21), 4817−4820. (c) Novakova, O.; Vrana, O.; Kiseleva, V. I.; Brabec, V. DNA Interactions of antitumor platinum(IV) complexes. *Eur. J. Biochem.* **1995**, *228* (3), 616−624.

(3) (a) Ellis, L. T.; Er, H. M.; Hambley, T. W. The influence of the axial ligands of a series of platinum(IV) anticancer complexes on their reduction to platinum(II) and reaction with DNA. *Aust. J. Chem.* **1995**, *48* (4), 793−806. (b) Choi, S.; Filotto, C.; Bisanzo, M.; Delaney, S.; Lagasee, D.; Whitworth, J. L.; Jusko, A.; Li, C. R.; Wood, N. A.; Willingham, J.; Schwenker, A.; Spaulding, K. Reduction and anticancer activity of platinum(IV) complexes. *Inorg. Chem.* **1998**, *37* (10), 2500−2504. (c) Lemma, K.; Sargeson, A. M.; Elding, L. I. Kinetics and mechanism for reduction of oral anticancer platinum(IV) dicarboxylate compounds by L-ascorbate ions. *J. Chem. Soc., Dalton Trans.* **2000**, *7*, 1167−1172. (d) Weaver, E. L.; Bose, R. N., Platinum(II) catalysis and radical intervention in reductions of platinum(IV) antitumor drugs by ascorbic acid. *J. Inorg. Biochem.* **2003**, *95* (4), 231−239. (e) Arendse, M. J.; Anderson, G. K.; Majola, R. N.; Rath, N. P. Synthesis and reactions of platinum(IV) complexes with sodium ascorbate. *Inorg. Chim. Acta* **2002**, *340*, 65−69. (f) Kratochwil, N. A.; Bednarski, P. J. Effect of thiols exported by cancer cells on the stability and growth-inhibitory activity of Pt(IV) complexes. *J. Cancer Res. Clin. Oncol.* **1999**, *125* (12), 690−696.

(4) Giandomenico, C. M.; Abrams, M. J.; Murrer, B. A.; Vollano, J. F.; Rheinheimer, M. I.; Wyer, S. B.; Bossard, G. E.; Higgins, J. D. Carboxylation of kinetically inert platinum(IV) hydroxy complexes, an entree into orally-active platinum(IV) antitumor agents. *Inorg. Chem.* **1995**, *34* (5), 1015−1021.

(5) Chen, L.; Lee, P. F.; Ranford, J. D.; Vittal, J. J.; Wong, S. Y. Reduction of the anti-cancer drug analogue cis,trans,cis[$PtCl_2$-$(OCOCH_3)(2)(NH_3)(2)$] by L-cysteine and L-methionine and its crystal structure. *J. Chem. Soc., Dalton Trans.* **1999**, *8*, 1209−1212.

(6) (a) Behrens, B. C.; Hamilton, T. C.; Masuda, H.; Grotzinger, K. R.; Whang-Peng, J.; Louie, K. G.; Knutsen, T.; McKoy, W. M.; Young, R. C.; Ozols, R. F. Characterization of a cis-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. *Cancer Res.* **1987**, *47* (2), 414−418. (b) Kelland, L. R.; Abel, G.; McKeage, M. J.; Jones, M.; Goddard, P. M.; Valenti, M.; Murrer, B. A.; Harrap, K. R. Preclinical antitumor evaluation of bis-acetato-ammine-dichloro-cyclohexylamine platinum(IV): an orally active platinum drug. *Cancer Res.* **1993**, *53* (11), 2581−2586.

(7) (a) Spadaro, A.; Bousquet, E.; Santagati, N. A.; Vittorio, F.; Ronsisvalle, G. Simple analysis of glutathione in human colon carcinoma cells and epidermoid human larynx carcinoma cells by HPLC with electrochemical detection. *Chromatographia* **2005**, *62* (1−2), 11−15. (b) Mitchell, J. B.; Russo, A.; Biaglow, J. E.; Mcpherson, S. J. Cellular glutathione depletion by diethyl maleate or buthionine sulfoximine and its effects on the oxygen enhancement ratio. *Radiat. Res.* **1983**, *94* (3), 612−612.

(8) (a) Hall, M. D.; Foran, G. J.; Zhang, M.; Beale, P. J.; Hambley, T. W. XANES determination of the platinum oxidation state distribution in cancer cells treated with platinum(IV) anticancer agents. *J. Am. Chem. Soc.* **2003**, *125* (25), 7524−7525. (b) Hall, M. D.; Dillon, C. T.; Zhang, M.; Beale, P.; Cai, Z. H.; Lai, B.; Stampfl, A. P. J.; Hambley, T. W. The cellular distribution and oxidation state of platinum(II) and platinum(IV) antitumour complexes in cancer cells. *J. Biol. Inorg. Chem.* **2003**, *8* (7), 726−732. (c) Hall, M. D.; Alderden, R. A.; Zhang, M.; Beale, P. J.; Cai, Z. H.; Lai, B.; Stampfl, A. P. J.; Hambley, T. W. The fate of platinum(II) and platinum(IV) anti-cancer agents in cancer cells and tumours. *J. Struct. Biol.* **2006**, *155* (1), 38−44.

(9) Mistry, P.; Loh, S. Y.; Kelland, L. R.; Harrap, K. R. Effect of buthionine sulfoximine on Pt^{II} and Pt^{IV} drug accumulation and the formation of glutathione conjugates in human ovarian-carcinoma cell lines. *Int. J. Cancer* **1993**, *55* (5), 848−856.

(10) Galanski, M.; Keppler, B. K. Is reduction required for antitumour activity of platinum(IV) compounds? Characterisation of a platinum-(IV)-nucleotide adduct [enPt(OCOCH3)(3)(5′-GMP)] by NMR spectroscopy and ESI-MS. *Inorg. Chim. Acta* **2000**, *300*, 783−789.

(11) Kratochwil, N. A.; Bednarski, P. J. Relationships between reduction properties and cancer cell growth inhibitory activities of cis-dichloro- and cis-diiodo-Pt(IV)-ethylenediamines. *Arch. Pharm. (Weinheim, Ger.)* **1999**, *332* (8), 279−285.

(12) Kasparkova, J.; Novakova, O.; Vrana, O.; Intini, F.; Natile, G.; Brabec, V. Molecular aspects of antitumor effects of a new platinum-(IV) drug. *Mol. Pharmacol.* **2006**, *70* (5), 1708−1719.

(13) Raynaud, F. I.; Odell, D. E.; Kelland, L. R. Intracellular metabolism of the orally active platinum drug JM216: influence of glutathione levels. *Br. J. Cancer* **1996**, *74* (3), 380−386.

(14) Carr, J. L.; Tingle, M. D.; McKeage, M. J. Satraplatin activation by haemoglobin, cytochrome C and liver microsomes in vitro. *Cancer Chemother. Pharmacol.* **2006**, *57* (4), 483−490.

JM070740J