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Platinum(II) Complexes with Bioactive Carrier Ligands Having High Affinity for the Translocator Protein

Nicola Margiotta,[†] Nunzio Denora,[‡] Rosa Ostuni,[†] Valentino Laquintana,[‡] Amy Anderson,[§] Steven W. Johnson,[§] Giuseppe Trapani,*[,‡] and Giovanni Natile*[,†]

[†]*Pharmaco-Chemistry Department, Bioinorganic Division, University "A. Moro" of Bari, Via E. Orabona 4, 70125 Bari, Italy,* [‡]*Pharmaco-Chemistry Department, Pharmaceutical Technology Division, University "A. Moro" of Bari, Via E. Orabona 4, 70125 Bari, Italy, and* [§]*Department of Hematology/Oncology, University of Pennsylvania, 421 Curie Boulevard, Philadelphia, Pennsylvania 19104*

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Peripheral benzodiazepine receptors (PBRs, also named TSPO) are overexpressed in many tumor types, with the grade of TSPO overexpression correlating with the malignancy of the tumor. For this reason, TSPO-binding ligands have been widely explored as carriers for receptor-mediated drug delivery. In this paper we have selected a ligand with nanomolar affinity for TSPO, [2-(4-chlorophenyl)-8-aminoimidazo[1,2-*a*]pyridin-3-yl]-*N*,*N*-di-*n*-propylacetamide (**3**), for preparing platinum adducts that are structural analogues to picoplatin, *cis*-[$PtCl_2(NH_3)$(2-picoline)] (AMD0473, **6**), a platinum analogue currently in advanced clinical investigation. In vitro studies assessing receptor binding and cytotoxicity against human and rat glioma cells have shown that the new compounds *cis*-[$PtX_2(NH_3)$\{[2-(4-chlorophenyl)-8-aminoimidazo[1,2-*a*]pyridin-3-yl]-*N*,*N*-di-*n*-propylacetamide\}] (X = I, **4**; X = Cl, **5**) keep high affinity and selectivity for TSPO (nanomolar concentration) and are as cytotoxic as cisplatin. Moreover, they appear to be equally active against sensitive and cisplatin-resistant A2780 cells. Similar to cisplatin, these compounds induce apoptosis but show a favorable 10- to 100-fold enhanced accumulation in the glioma cells.

Introduction

Cisplatin (*cis*-diamminedichloridoplatinum(II)) is one of the five antitumoral agents most used in clinical therapy and is the only antineoplastic drug with highly curative effects in a solid malignancy such as the testicular cancer.[1,2] Toxicity (ototoxicity, neuropathy, myelosuppression, renal and hepatic toxicity) and intrinsic or acquired resistance limit the use of cisplatin to the treatment of ovarian, head and neck, bladder, and testicular tumors.[3,4]

Several cisplatin analogues have been prepared and tested in the past 3 decades with the aim of discovering complexes with better pharmacological properties. Most of these studies were inspired by the early structure−activity relationships described by Cleare and Hoeschele.[5,6] However, platinum drugs that have not fulfilled at least one of the classic structure−activity relationships have been investigated, with a few of them displaying good antineoplastic activity.[7−12] This tremendous research effort has led to the approval of few other platinum drugs that are currently in clinical use.[10]

The approach to target platinum drugs via an organ- or receptor-specific carrier has been also extensively exploited. Biologically active ligands such as DNA intercalators, doxorubicin, estrogen analogues, amino acids, ferrocene, phosphonates, and sugars have been selected as molecular carriers to obtain a specific delivery/accumulation of the antitumor drug to the target cells (or organs) or to obtain synergistic pharmacological activities.[13−15]

We have pursued a drug targeting approach exploiting receptors overexpressed in some kinds of tumors, and in particular, we have prepared platinum compounds carrying ligands specific for the peripheral benzodiazepine receptor (PBR[a]).[16] PBRs (recently renamed "translocator protein 18 kDa (TSPO)")[17] are abundant in peripheral tissues and also in glial cells of the central nervous system (CNS). The functions of TSPOs are wholly distinct from those of the well-known central benzodiazepine receptors (CBRs) which are associated with GABA-A receptors and mediate the classical sedative anxiolytic and anticonvulsant properties of benzodiazepines. TSPOs are located primarily on the outer mitochondrial membrane and are involved in the control of apoptosis. They in fact belong to a multiproteic complex known as "mitochondrial permeability transition (MPT) pore",[18] which plays a key role in controlling the apoptotic process. TSPO-binding ligands may stimulate the opening of MPT pores and trigger the cascade of events leading to apoptosis. The observation

*To whom correspondence should be addressed. For G.T.: phone, +39-080-5442764; fax, +39-080-5442754; e-mail, trapani@farmchim.uniba.it.. For G.N.: phone, +39-080-5442774; fax, +39-080-5442230; e-mail, natile@farmchim.uniba.it.

[a] Abbreviations: CBR, central benzodiazepine receptor; CNS, central nervous system; COSY, correlation spectroscopy; DMF, *N*,*N*-dimethylformamide; ESI-MS, electrospray mass spectrometry; EtOH, ethanol; Et_2O, diethyl ether; GABA, γ-aminobutyric acid; GSH, γ-glutamylcysteinylglycine (glutathione); HSQC, heteronuclear single quantum coherence; IC_{50}, median inhibition concentration; IR, infrared spectroscopy; MPT, mitochondrial permeability pore; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; PBR, peripheral benzodiazepine receptor; PBS, phosphate buffered saline; SD, standard deviation; TMS, tetramethylsilane; TOCSY, total correlation spectroscopy; TSPO, translocator protein 18 kDa.

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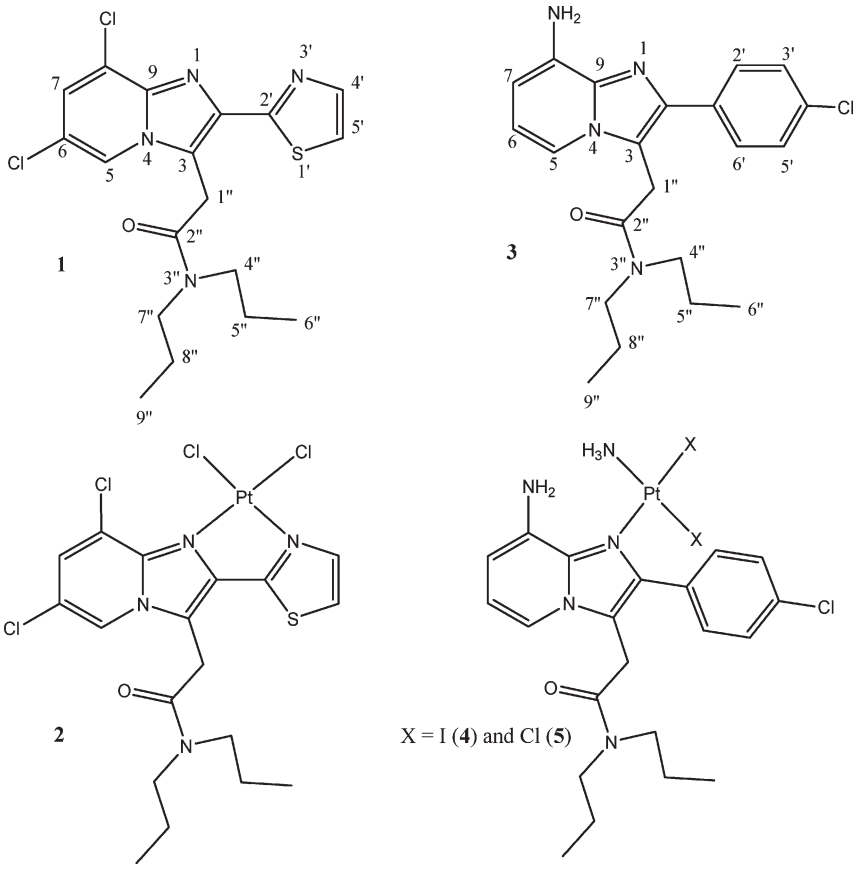


Figure 1. Sketches of the TSPO ligands **1** and **3** and of the Pt complexes **2**, **4**, and **5**.

that an overexpression of TSPOs occurs in many tumor types,[19−21] such as brain, liver, hepatic, glial, and mammary tumors, breast carcinoma, and ovarian and colorectal cancers, led us to choose TSPO-binding ligands for targeting purposes. Furthermore, the grade of TSPO overexpression appears to correlate with the malignancy of the tumor. Given that TSPO-binding ligands as receptor-mediated carriers for antitumor drugs have been evaluated, TSPO ligands can actively be used for chemosensitization of solid tumors.[22−26]

In our recent paper[16] we reported on a Pt complex with the ligand 2-[6,8-dichloro-2-(1,3-thiazol-2-yl)-*H*-imidazo[1,2-*a*]pyridin-3-yl]-*N*,*N*-di-*n*-propylacetamide (**1**, Figure 1), which is endowed with high affinity and selectivity for TSPO and belongs to a series of potent and selective TSPO ligands containing the imidazopyridine nucleus.[27−29] This molecule, **1**, characterized by a thiazole ring in position 2 of the imidazopyridine nucleus, demonstrated to act as a chelating agent toward platinum, so forming a large planar aromatic polycyclic system with potential intercalative properties toward DNA (a feature that could help to circumvent cisplatin resistance).[30] The obtained complex, *cis*-[$PtCl_2$\{2-[6,8-dichloro-2-(1,3-thiazol-2-yl)-*H*-imidazo[1,2-*a*]pyridin-3-yl]-*N*,*N*-di-*n*-propylacetamide\}] (**2**, Figure 1), combines the alkylating properties of the metal residue with the high affinity (in nanomolar concentration) and selectivity of **1** for TSPO-overexpressing tissues. Additionally, in solvents of low dielectric constants, the complex underwent a dimerization process via formation of noncovalent intermolecular interaction, which further supports the potential intercalating ability of this platinum substrate toward DNA.

As is characteristic of platinum complexes with sterically hindered heterocyclic ligands,[31] **2** exhibits poor aqueous solubility, compelling us to design more water-soluble Pt compounds with TSPO ligands. To this extent, in this paper we report the synthesis and characterization of two new Pt compounds with another ligand displaying high affinity and selectivity for the translocator protein, [2-(4-chlorophenyl)-8-aminoimidazo-[1,2-*a*]pyridin-3-yl]-*N*,*N*-di-*n*-propylacetamide (**3**, Figure 1). The two new Pt complexes, with general formula *cis*-[PtX_2-(NH_3)\{[2-(4-chlorophenyl)-8-aminoimidazo[1,2-*a*]pyridin-3-yl]-*N*,*N*-di-*n*-propylacetamide\}] (X = I (**4**), Cl (**5**); Figure 1) have been fully characterized by 1D and 2D NMR techniques, and the results indicate that **3** acts as monodentate ligand toward Pt. As a result of the NH_3 ligand on platinum, **4** and **5** have improved water solubility in comparison to **2**. Moreover, the presence of an ammine group and a N-donor heterocycle in cis-positions renders the new complexes similar to *cis*-[$PtCl_2$(NH_3)(2-picoline)], also known as picoplatin (AMD0473, **6**), a platinum analogue currently in advanced clinical investigation. AMD0473 has an improved safety profile in comparison with other clinically used platinum drugs and can overcome cisplatin resistance.[32]

This paper summarizes the complete chemical characterization of compounds **4** and **5** and in vitro assessments, including their stability in the presence of bionucleophiles such as glutathione (GSH), receptor binding, cytotoxicity against human and rat glioma cells, and against sensitive and resistant human ovarian carcinoma cell lines, and their ability to induce apoptosis and cellular accumulation.

Results and Discussion

The peripheral benzodiazepine receptor, also referred to as the translocator protein 18 kDa, is overexpressed in many tumor cells such as brain, liver, and hepatic tumors. In a

Table 1. ^{1}H Chemical Shifts (ppm, Acetone-d_6) of **3**, **4**, and **5**

H	3	4	5
2′/6′	7.76	8.06	8.08
5	7.55	7.61	7.62
3′/5′	7.47	7.51	7.53
NH_2	5.27	7.09	7.25
6	6.66	6.84	6.85
7	6.37	6.55	6.61
1″	4.21	3.97	4.03
NH_3		3.68	3.65
4″/7″	3.43−3.27	3.29−3.23	3.34−3.20
5″/8″	1.72−1.50	1.56−1.49	1.63−1.44
6″/9″	0.91- 0.79	0.85−0.78	0.87−0.74

Table 2. Chemical Shifts (^{1}H/^{15}N, ppm) of the Amminic and Ammonia Groups for Free **3** and Complexed Species **4** and **5**

compd	solvent	NH_2	NH_3
3	acetone-d_6	5.27/27.70	
4	acetone-d_6	7.09/39.80	3.67/ −81.56
5	DMF-d_7	7.64/39.19	4.10/ −67.28

previous study we linked the TSPO ligand **1** to platinum for evaluating its potential to target antitumor platinum drugs to tissues overexpressing TSPO. The Pt adduct, **2**, maintained the affinity and selectivity toward TSPO. However, preliminary cytotoxicity data carried out on cell lines sensitive to cisplatin (such as the human ovarian cancer cells A2780) showed that **2** was 10-fold less active than cisplatin. Similarly, **2** was less active than cisplatin against the human breast adenocarcinoma MCF7 cell line, expressing a low level of TPSO.[33] The conclusion of this initial study was that while the bifunctional coordination to platinum did not alter significantly the affinity of **1** for the TSPO receptor, in contrast, the structure of the platinum residue was profoundly modified and its antitumor activity significantly inhibited.

We have now extended the investigation to another TSPO ligand, **3**, whose affinity and selectivity for TSPO is similar to (or better than) that of **1** but is expected to act as monodentate ligand toward Pt and to form a complex strictly analogous to picoplatin, a drug in advanced clinical investigation.

The TSPO ligand **3** was first thoroughly characterized by means of 2D COSY (not shown), NOESY, and [^{1}H−^{15}N]-HSQC NMR experiments (Tables 1 and 2). The 1D ^{1}H NMR spectrum of a solution of **3** in acetone-d_6 is reported in Figure 2. The doublets at 7.76 and 7.47 ppm were assigned to the protons of the 4-Cl-phenyl substituent in position 2 of the imidazopyridine ring system. These signals exhibit a coupling constant typical for two protons in vicinal positions ($^3J_{H-H}$ = 8.59 Hz) and have the typical pattern for a 1,4-disubstituted phenyl ring. The doublet at 7.47 ppm was assigned to protons 3′/5′, which are expected to be shielded by the chloro substituent, while the doublet at 7.76 ppm is assigned to protons 2′/6′. The latter assignment is also supported by the 2D-NOESY showing a spatial correlation between the signal at 7.76 ppm and the signal at 4.21 ppm assigned to the methylene proton 1″ (cross peak A in Figure 3). The signal at 4.21 ppm has a NOESY cross peak with the doublet at 7.55 ppm which can be assigned to the proton in position 5 of the imidazopyridine ring system. The low field of the latter signal is a consequence of the inductive effect of the nitrogen atom in position 4.

The triplet at 6.66 ppm, showing coupling with proton 5 ($^3J_{H-H}$ = 6.94 Hz), was assigned to proton 6. Proton 6 is also coupled with the proton yielding the remaining shielded doublet in the aromatic region (6.37 ppm); thus, the latter doublet is assigned to proton 7.

The broad signal at 5.27 ppm is easily assigned to the primary aminic group in position 8. The multiplet in the range 3.43−3.27 ppm is assigned to the methylene protons 4″/7″ which are expected to be less shielded than protons 5″/8″ being adjacent to the amidic nitrogen. As a consequence, the multiplet in the range 1.72−1.50 ppm is assigned to protons 5″/8″. The most shielded multiplet in the range 0.91−0.79 ppm is assigned to methyl protons 6″/9″. Protons 4″, 5″, and 6″ are not coincident with protons 7″, 8″, and 9″, respectively, because of the hindered rotation about the C(2″)−N(3″) amidic bond.

The ^{15}N chemical shift (27.70 ppm, Table 2) of the aminic group in position 8 was derived from a [^{1}H−^{15}N]-HSQC 2D experiment (Figure 4) performed in natural abundance of ^{15}N and is consistent with the chemical shift for an aromatic primary amine.

Compound **4** was synthesized by direct reaction of **3** with K[PtI$_3$(NH$_3$)] (Scheme 1). The use of the iodo species has two advantages: (i) It is more reactive than the chloro species, K[PtCl$_3$(NH$_3$)], and (ii) allows a better control of the reaction stereochemistry, since the more trans-labilizing effect of the iodido ligand (compared to ammine) favors the formation of the cis-product. The reaction was performed in absolute ethanol by refluxing for 24 h, and the resulting product (**4**) was purified by column chromatography on silica gel.

Compound **4** was characterized by elemental analysis, ESI-MS, 1D ^{1}H and ^{195}Pt NMR (acetone-d_6), and 2D [^{1}H−^{15}N]-HSQC, COSY, and NOESY NMR experiments (DMF-d_7). The ^{1}H NMR spectrum of compound **4** in acetone-d_6 (Figure 5) shows a pattern of signals similar to that of the free ligand **3** with small differences in the chemical shifts of the imidazopyridine ring system (Table 1). For instance, H(7) (6.55 ppm) and H(6) (6.84 ppm) are both deshielded by 0.18 ppm with respect to the free ligand while protons 2′/6′ are deshielded by 0.31 ppm. A large deshielding (1.82 ppm) is observed for the NH_2 group in position 8. Such a huge deshielding of NH_2 strongly supports coordination of platinum to N1. In fact, platinum coordination to N1 causes the NH_2 group to approach the metal center from an axial position and suffer a deshielding effect.[34]

Two cross peaks falling at 3.67/−81.56 and 7.09/39.80 ppm (^{1}H/^{15}N) were observed in the [^{1}H−^{15}N]-HSQC 2D-NMR spectrum (Figure 6). The first cross peak is assigned to the coordinated NH_3 (chemical shift typical for a NH_3 ligand coordinated to Pt(II) in a square planar geometry and having a halido ligand in trans position).[34] The second cross peak (7.09/39.80 ppm, ^{1}H/^{15}N) is assigned to the NH_2 group of coordinated **3**. The positive ^{15}N chemical shift clearly indicates that this amino group is not coordinated to Pt but instead suffers a deshielding stemming from its approaching to platinum from an axial position.

Further evidence in favor of coordination of **3** through N1 came from the [^{1}H−^{195}Pt]-HMQC 2D-NMR experiment (Figure 6). Only one cross peak, falling at 3.68/−3291 ppm (^{1}H/^{195}Pt), was observed (correlation between the protons of the coordinated NH_3 and the Pt atom). The ^{195}Pt NMR chemical shift is in a range typical for a Pt atom in a PtI$_2$N$_2$ coordination environment (−3291 ppm).[35,36] All these spectroscopic results confirm that **3** acts as monodentate ligand toward Pt.

The dichlorido derivative **5** was prepared by substitution of chloride for iodide. The usual dehalogenation reaction,

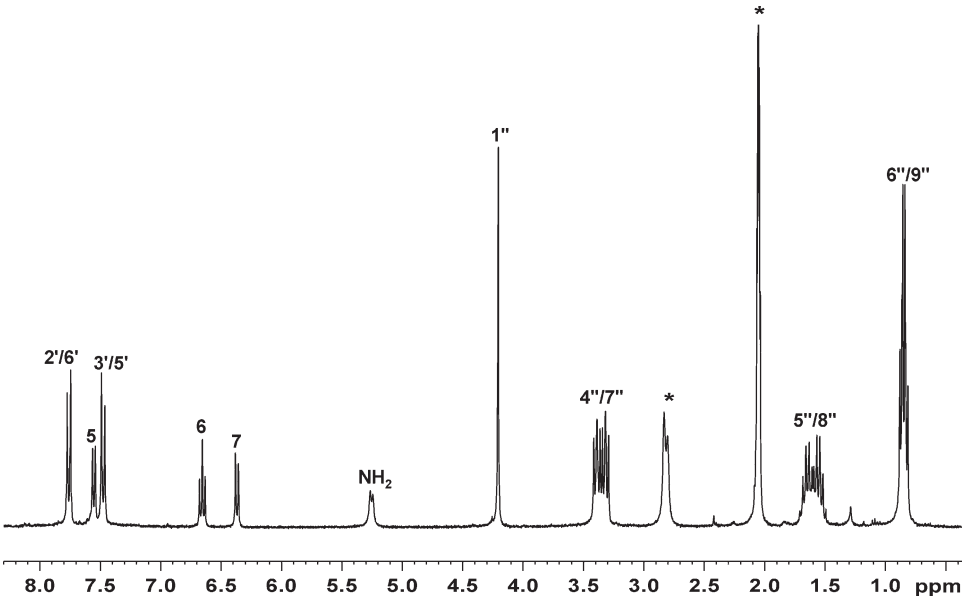


Figure 2. ^{1}H NMR of **3** in acetone-d_6. Numbering of protons is reported in Figure 1. Asterisks indicate residual solvent peaks (protic acetone and water).

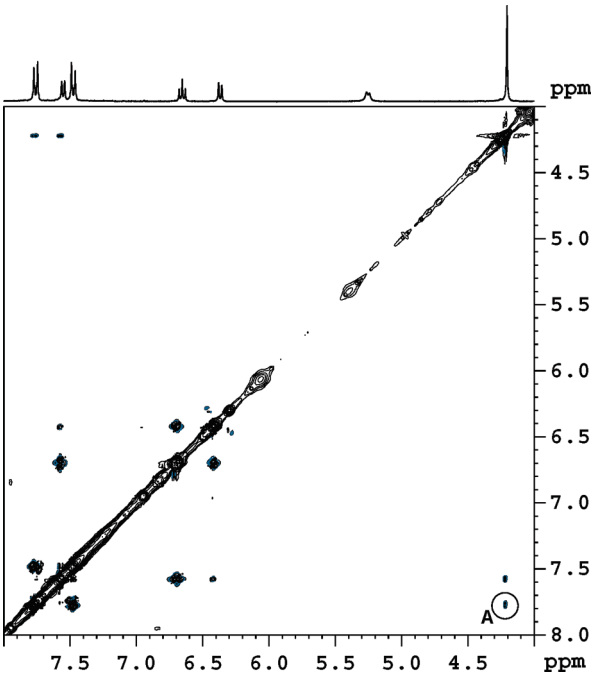


Figure 3. Aromatic region of the 2D-NOESY of **3** in acetone-d_6.

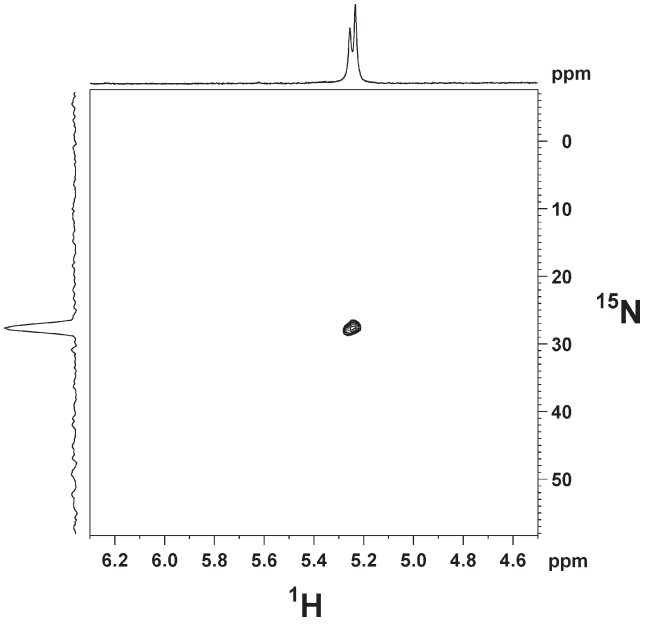


Figure 4. [^{1}H−^{15}N]-HSQC of **3** in acetone-d_6.

carried out in platinum chemistry by treatment with $AgNO_3$ or Ag_2SO_4, did not lead to complete displacement of the iodido ligands. Therefore, we performed a reaction with Ag(CF_3SO_3), in acetone, which results in complete removal of the iodido ligands and their substitution with triflato. The triflato derivative [Pt(O_3SCF_3)$_2$(NH_3){[2-(4-chlorophenyl)-8-aminoimidazo[1,2-*a*]pyridin-3-yl]-*N*,*N*-di-*n*-propylacetamide}] was then treated with LiCl first in acetone (in which the Pt complex is soluble but LiCl is not soluble) and then in EtOH (in which the Pt complex is not soluble but LiCl is soluble).

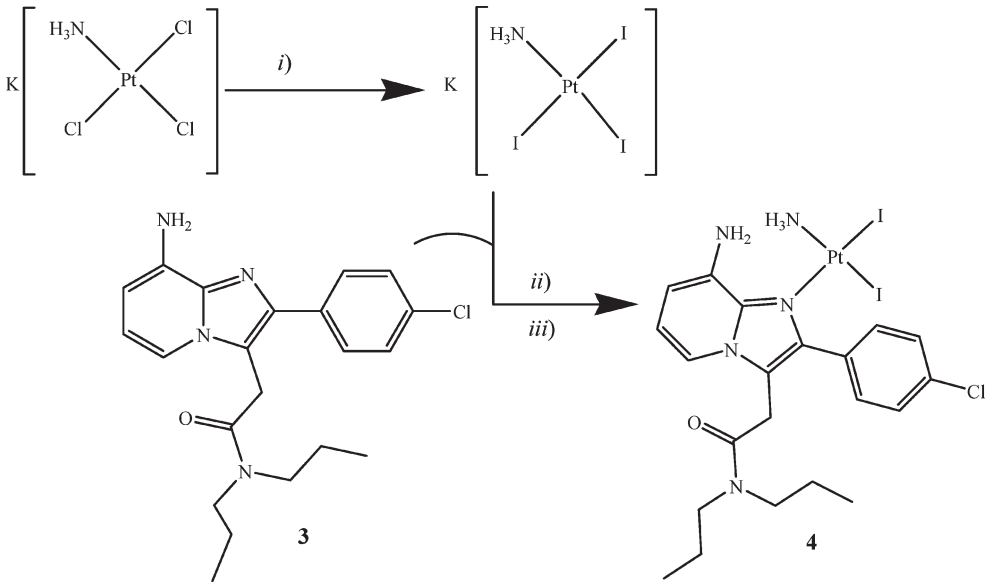
Compound **5** was characterized by elemental analysis, ESI-MS, and 1D ^{1}H (acetone-d_6) and ^{195}Pt (DMF-d_7) and 2D [^{1}H−^{15}N]-HSQC, TOCSY, and NOESY experiments (DMF-d_7).

The ^{1}H NMR spectrum of complex **5** showed a pattern of signals similar to that of **4**. The ^{1}H chemical shifts (Table 1) do not appear to be influenced by the nature of the halido ligands. The change in chemical shifts are in the range of 0.01−0.06 ppm for the protons of the imidazopyridine moiety, while it is slightly bigger for the NH_2 protons (0.16 ppm).

The ^{195}Pt-NMR spectrum (Figure 7) exhibits a signal at −1975 ppm, 1316 ppm at lower field with respect to that of compound **4**, a clear evidence of complete substitution of the iodido by chlorido ligands. In fact the ^{195}Pt chemical shift of compound **5** is typical for a platinum atom in a $PtCl_2N_2$ coordination environment.[35,36]

The 2D [^{1}H−^{15}N]-HSQC spectrum of compound **5** in DMF-d_7 (Figure 8) shows two signals falling at 4.10/−67.28 and 7.64/39.19 ppm (^{1}H/^{15}N). The first signal is assigned to coordinated NH_3 and reveals that substitution of chloride for iodide causes a deshielding of the ^{15}N nucleus by 14.28 ppm

Scheme 1. Synthesis of **4**[a]



[a] (i) EtOH/H_2O (50:50, v/v), KI, 50 °C; (ii) abs EtOH, reflux 24 h; (iii) column chromatography.

(Table 2). Different from the NH_3 signal, the second signal, assigned to the NH_2 group of coordinated **3**, does not exhibit significant changes with respect to the iodido species (7.64/39.19 compared to 7.09/39.80 ppm, $^1H/^{15}N$).

Stability studies were carried out on compound **5** which, as already pointed out, is strictly analogous to picoplatin. The stability was assessed by monitoring the signals falling in the aromatic region of the spectrum. After 6 days in isotonic buffered solution at pH 7.4 and 37 °C, there was no formation of new sets of signals. Furthermore, there was no detection of free ammonia, which would have appeared at ~6.5 ppm. Moreover, **3** remained coordinated, since the addition of free **3** into the NMR tube led to a new set of signals belonging to the free ligand. We conclude that the chloride concentration used in our experiment is sufficiently high to prevent aquation, as it is the case for cisplatin in the bloodstream.

Pt^{II} compounds have a strong thermodynamic preference for binding to sulfur donor ligands. Hence, before antitumor platinum drugs reach DNA in the nucleus of tumor cells, they may interact with various sulfur-containing substrates. An endogenous thiol, which could sequester platinum complexes after their entering the cell, is glutathione (GSH).[37] In the present study, we tested the stability of compound **5** in the presence of GSH using HPLC. Following literature suggestions,[37] compound **5** at 33 μM was mixed with GSH at a physiologically relevant concentration of 5 mM at 37 °C. The observed half-life was 23.9 ± 0.6 h for compound **5** in comparison with a half-life of 1.5 ± 0.1 h found for cisplatin.[37] This 15-fold improved stability of **5** over cisplatin implies stability in solutions containing sulfur donors, a feature that confirms the similarity of this compound to picoplatin.

Radioligand Binding Assays. The affinity of compounds **4** and **5** for TSPO and CBR was assessed by measuring their ability to prevent binding to the rat cerebral cortex of [^{3}H]-1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide ([^{3}H]-PK11195, [^{3}H]-**7**) and [^{3}H]-5-(2-fluorophenyl)-1-methyl-7-nitro-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one ([^{3}H]-flunitrazepam, [^{3}H]-**8**), respectively. Their effects were compared to those of unlabeled **7**. The results are shown in Table 3. The binding data clearly demonstrate that both compounds **4** and **5** are endowed with high affinity and selectivity for TSPO at the nanomolar level. Although free **3** has ~1 order of magnitude greater affinity for TSPO compared to its Pt derivatives **4** and **5**, both Pt compounds maintain their selectivity for TSPO as measured by the high selectivity indices (defined as the ratio IC_{50}(CBR)/IC_{50}(TSPO)) which is greater than 5000. This result is particularly encouraging in view of an in vivo application of the two compounds as drugs specifically targeted to TSPO-overexpressing cancers.

Cytotoxicity Assays. Table 3 summarizes the cytotoxicity of compounds **4** and **5** against human SF188 and SF126 and rat C6 and RG2 glioma cells selected for their high expression level of TSPO. Cisplatin was used as a control for **4** and **5**. Compounds **4** and **5** are extremely effective, as shown by their IC_{50} values which are essentially equal to those of cisplatin and, in some cases, even better (IC_{50} values of 1.53 ± 0.6 and 3.07 ± 0.8 μM for **4** and cisplatin, respectively, toward RG2 cells). It is interesting to note that the iodido derivative **4** is somewhat better than the chlorido derivative **5** and as effective as cisplatin.

The cytotoxic activities of cisplatin and compounds **4** and **5** were also determined against the cisplatin sensitive A2780 and the cisplatin resistant A2780cisR human ovarian carcinoma cell lines, commonly used to test cytotoxic activity of cisplatin analogues.[38] A2780cisR cells are resistant to cisplatin due to a combination of decreased uptake, enhanced DNA repair/tolerance, and enhanced GSH levels.[38] As shown in Table 3, compounds **4** and **5** are effective toward both A2780 and A2780cisR cell lines as shown by their IC_{50} values. Interestingly, compounds **4** and **5**, unlike cisplatin, are equally active toward the sensitive and the resistant cell lines.

It can be concluded that, in contrast to the platinum compound **2**, containing chelated **1** which was found to be scarcely cytotoxic in a panel of nine cell lines (some of them overexpressing TSPO), compounds **4** and **5**, both having monodentate **3**, are very effective and promising candidates for in vivo evaluation. Additionally, it is noted that coordination of **3** not only targeted the platinum drug to tumor tissues overexpressing TSPO but also secured a synergistic

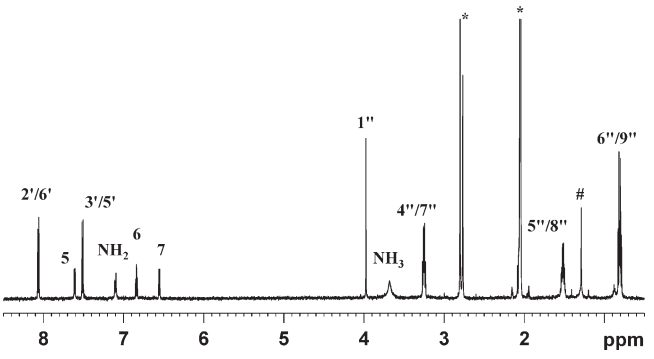


Figure 5. ^{1}H NMR of **4** (acetone-d_6). Numbering of protons is as in Figure 1. Asterisks indicate residual solvent peaks (protic acetone and water), and the hash indicates an impurity of the solvent.

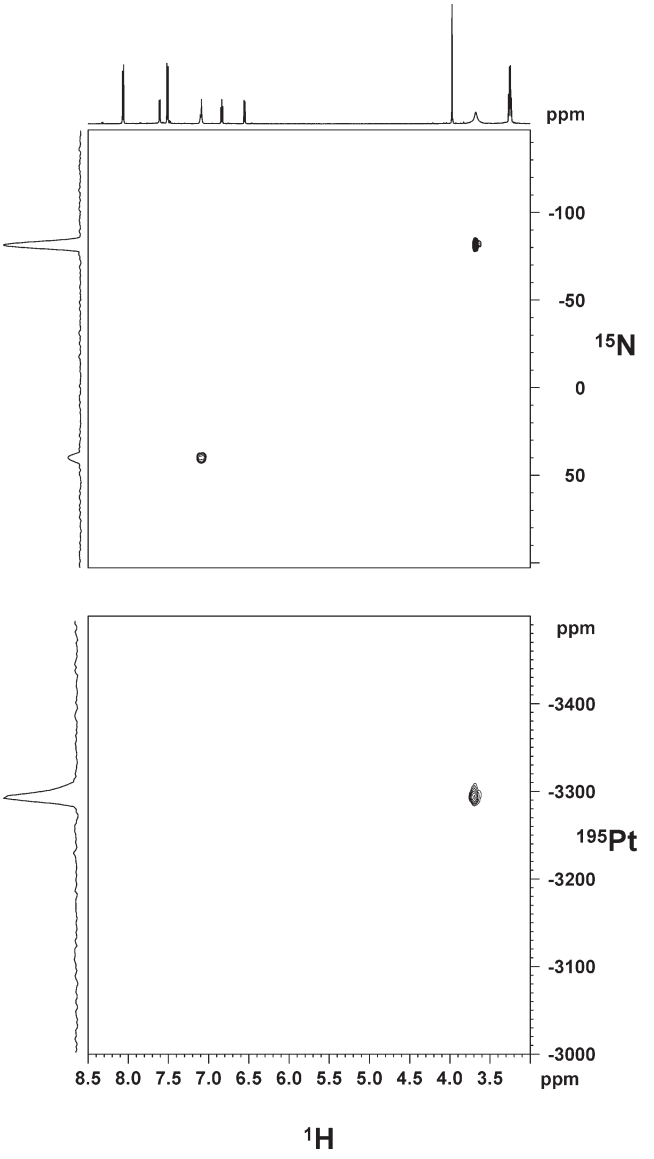


Figure 6. Natural abundance [^{1}H−^{15}N]-HSQC (top) and [^{1}H, ^{195}Pt]-HMQC (bottom) 2D-NMR spectra of **4** (acetone-d_6).

effect between the alkylating properties of the platinum residue and the apoptosis inducing activity of **3**.[25] Compound **3** itself was previously found to be cytotoxic against rat C6 glioma cells;[39] however, its activity was more than 1 order of magnitude lower than that of compounds **4** and **5**.

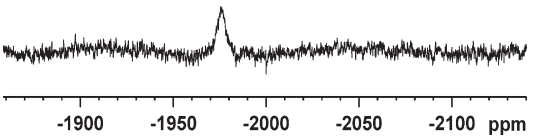


Figure 7. ^{195}Pt NMR of **5** (DMF-d_7).

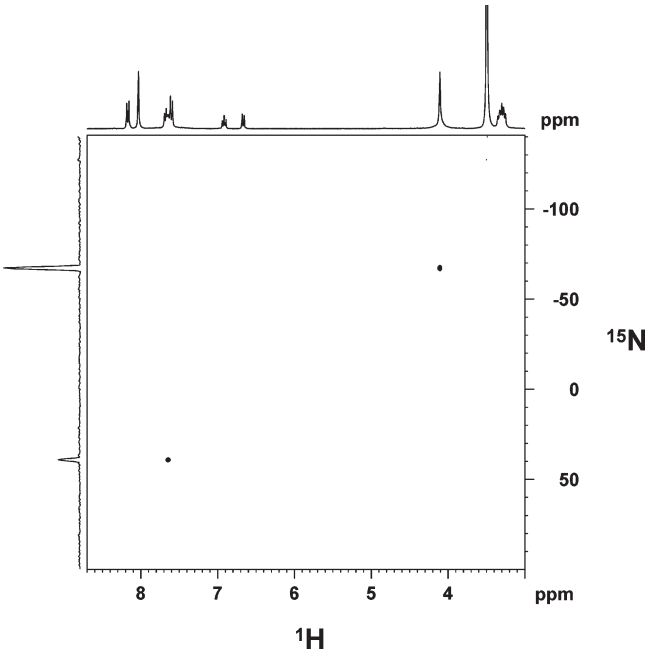


Figure 8. [^{1}H−^{15}N]-HSQC of **5** (DMF-d_7).

Cellular Uptake. To examine the cellular uptake, C6 glioma cells were exposed to a concentration (1 μM) close to the IC_{50} of compounds **4** and **5** and cisplatin for a short (4 h) and a long time (24 h), and the intracellular platinum was measured by inductively coupled plasma mass spectrometry. Interestingly, the results presented in Table 4 suggest that the uptake of compounds **4** and **5** was approximately 155- and 224-fold greater than that of cisplatin after just 4 h of incubation and became about 33 and 58 times greater than that of cisplatin after 24 h. The observed increase in cancer cell accumulation of compounds **4** and **5** is likely due to both the increased lipophilicity of the platinum complex leading to enhanced transport across the cellular membrane and to the selective target of tumor cells TSPO-mediated. This increase in lipophilicity of the platinum complexes compared to the parent cisplatin was confirmed by their greater capacity factors ($\log k'$) observed from HPLC analysis (data not shown).

Treatment of C6 Glioma Cells with Cisplatin and Compounds 4 and 5 Induces Mitochondrial and Nucleus Morphology Modification. Nuclear morphological changes during apoptosis are very distinct and are characterized by convoluted nuclei with cavitations and clumps of chromatin abutting the inner regions of the nuclear envelope between the nuclear pores. Cisplatin−DNA adducts cause various cellular responses, such as replication arrest, transcription inhibition, cell-cycle arrest, DNA repair, and apoptosis.[14] Furthermore, TSPO ligands act on mitochondria, exerting a pro-apoptotic activity. Therefore, we analyzed the structure of these organelles and of the nuclei in cells treated with cisplatin and compounds **4** and **5**. MitoTracker Red and DAPI dyes were used as mitochondrial and nucleus specific markers, respectively. The C6 glioma cells were treated with

Table 3. Affinities of **1**, **2**, **3**, **4**, and **5** for CBR and TSPO (PBR) from Rat Cerebral Cortex and Cytotoxicity of **4**, **5**, and Cisplatin toward SF188, SF126, C6, RG2, A2780, and A2780cisR Cancer Cell Lines, with **3** Tested Only against the C6 Cell Line

	IC_{50} (nM)		IC_{50} (μM)					
compd	TSPO (PBR)	CBR	SF188[a]	SF126[a]	C6[a]	RG2[a]	A2780[b]	A2780cisR[b,c]
1	2.07	$>10^5$						
2[d]	4.6	$>10^5$						
3	1.6	$>10^5$			19.71 ± 1.16			
4	18	$>10^5$	1.50 ± 0.46	1.47 ± 0.65	1.14 ± 0.75	1.53 ± 0.6	6.38 ± 1.09	5.39 ± 1.11 (0.8)
5	12.65	$>10^5$	3.67 ± 1.46	2.57 ± 0.58	1.73 ± 0.06	2.93 ± 0.7	3.56 ± 0.90	3.95 ± 0.75 (1.1)
cisplatin			1.80 ± 0.92	1.87 ± 0.15	0.73 ± 0.51	3.07 ± 0.8	2.94 ± 0.43	9.17 ± 0.43 (3.1)

[a] Cells were seeded at a density of ~1000−2000 cells/well into 96-well microtiter plates. Following overnight incubation, cells were treated with a range of drug concentrations (0.03−10 μM). Data are the mean ± SD of three independent experiments performed in duplicate. [b] Cells were seeded in 96-well plates at a density of ~10 000 cells/well and incubated at 37 °C in a humidified atmosphere with 5% CO_2. Cells were incubated in the presence of different concentrations of the tested compounds (0.1−50 μM) for a period of 72 h. [c] Resistance factor, defined as IC_{50}(resistant)/IC_{50}(sensitive), is given in parentheses. [d] In a preliminary investigation (data not reported) this compound showed cytotoxicity values (IC_{50}) greater than 10 μM against a panel of nine tumor cell lines (some of them overexpressing TSPO).

Table 4. Uptake by C6 Glioma Cells of Cisplatin and Compounds **4** and **5**

	uptake by C6 glioma cells (μmol of Pt/L)[a]		
	cisplatin	**4**	**5**
after 4 h treatment	0.0015 ± 0.0003	0.2254 ± 0.0173	0.3257 ± 0.0123
after 24 h treatment	0.0073 ± 0.0011	0.2409 ± 0.0171	0.4235 ± 0.011

[a] Cells were seeded in 60 mm tissue culture dishes at a density of ~30 000 cells/cm^2.

cisplatin or with compounds **4** and **5**, and the organelle structure modifications were evaluated using a digital imaging system. Figure 9 shows representative images of control C6 glioma cells where the typical nucleus and the tubular interconnected mitochondrial network are evident. In contrast, cells treated with 10 μM cisplatin, **4**, and **5** exhibit morphological alteration of the organelles and of the nucleus after 24 h (data not shown) and 48 h of incubation. In particular, in cells treated with cisplatin, **4**, and **5**, the nuclei and the mitochondria appear fragmented with MitoTracker Red diffused in the cytosol. Interestingly, inside the treated cells, the nearly circular fluorescent structures start to appear and their number and dimension to increase at 48 h of incubation, suggesting that the origin of these structures is related to a nuclear fragmentation. Therefore, it can be concluded that compounds **4** and **5**, like cisplatin, are able to induce apoptosis in cancer cells.

Conclusions

In a previous paper it was shown that coordination of a platinum moiety to the imidazopyridine residue of a TSPO ligand (**1**) does not alter its affinity and selectivity for TSPO, indicating that the specific interaction with the receptor is not precluded. However, the bidentate coordination of **1**, with consequent complete loss of the ammine ligands (compared to cisplatin), renders the platinum adduct, **2**, scarcely cytotoxic. Inspired from the high activity of the fourth-generation platinum drug picoplatin,[32,40] we have selected a new TSPO ligand, **3**, which can coordinate to platinum in a monodentate fashion to form complexes **4** and **5** which are strictly analogous to picoplatin. We point out the strict analogy existing between the imidazopyridine nucleus of **3** and the adenine nucleobase. On the basis of this analogy, **3**, like adenine, was expected to react monofunctionally with platinum substrates and, of the two nitrogen atoms in peri positions, only the endocyclic one was expected to be able to act as donor atom toward platinum under normal conditions.

Picoplatin was reported to be quite stable toward deactivation from GSH and other sulfur containing biomolecules because of steric hindrance exerted by the 2-picolinic moiety. Also, complex **5**, differing from picoplatin in having monodentate **3** in place of 2-picoline, was quite stable in the presence of GSH. Additionally, the new complexes are reasonably soluble in water and are stable in PBS for over 1 week.

Complexes **4** and **5** exhibit two excellent properties: (i) high affinity and selectivity (typical of the **3** ligand) for TSPO and (ii) high cytotoxicity (typical of platinum drugs) for glioma and other human tumor cell lines. Therefore, complexes **4** and **5** are highly cytotoxic against several human and rat glioma cell lines and able to induce apoptosis. Moreover, the two compounds were equally cytotoxic against the sensitive A2780 and the cisplatin resistant A2780cisR human ovarian carcinoma cell lines. Interestingly, the uptake of the new complexes by C6 glioma cells was much greater than that of cisplatin. Therefore, the new compounds appear to be very promising for a further evaluation toward brain tumors (and any type of TSPO-overexpressing tumor), hoping that a "receptor-mediated" drug targeting can lead to an improved clinical effect.

Experimental Section

Chemicals. Commercial reagent grade chemicals and solvents were used as received and without further purification. Silver trifluoromethanesulfonate (CF_3SO_3Ag) and lithium chloride (LiCl) were purchased from Sigma-Aldrich (Milan, Italy). The purity of synthesized compounds was higher than 95% as established by combustion analysis.

Instrumentation and Measurements. Elemental analyses were carried out with a Carlo Erba model 1106 elemental analyzer. IR spectra were obtained on a Perkin-Elemer Fourier transform IR spectrophotometer, using KBr pellets. ^{1}H and ^{195}Pt NMR 1D spectra and [^{1}H−^{15}N]-HSQC (natural abundance ^{15}N), [^{1}H,^{195}Pt]-HMQC, COSY, and NOESY 2D experiments were recorded on Bruker Avance DPX 300 MHz and Avance II 600 MHz instruments. Standard Bruker pulse sequences were used for the NMR experiments using gradient selected versions when necessary. In particular, 1D ^{1}H NMR spectra with water suppression were obtained by using the standard Bruker sequence with excitation sculpting.[41] ^{1}H chemical shifts were referenced to TMS by using the residual protic peak of the solvent as internal reference (2.05 ppm for acetone-d_6 and 8.03 ppm for *N*,*N*-dimethylformamide-d_7). ^{195}Pt chemical shifts were referenced to external $K_2[PtCl_4]$ in D_2O placed at −1615 ppm. ^{15}N chemical shifts were referenced to external $^{15}NH_4Cl$ (1M in HCl 1 M) placed at 0 ppm.

Electrospray mass spectrometry (ESI-MS) was performed with an electrospray interface and an ion trap mass spectrometer (1100 series LC/MSD trap system, Agilent, Palo Alto, CA).

Inductively coupled plasma mass spectrometry (ICP-MS) was performed with a Varian 820-MS ICP mass spectrometer.

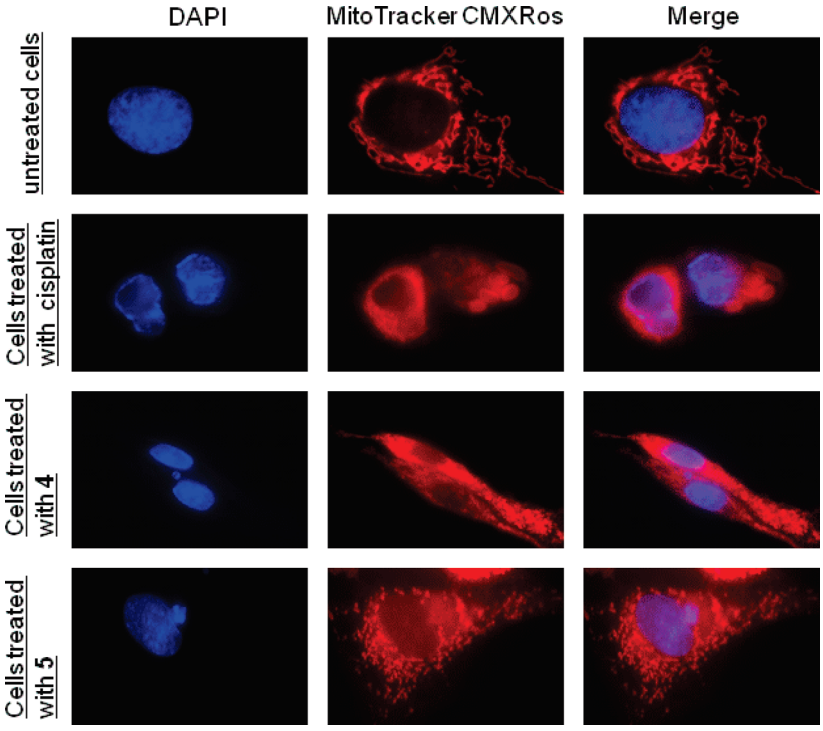


Figure 9. Morphological analysis of the mitochondrial network structure and of the nucleus in C6 glioma cells. Cells seeded (~100 000 cells/well) onto 24 mm coverslips were treated with 10 μM **4**, **5**, or cisplatin at 37 °C in a 5% CO_2 atmosphere. Mitochondrial structure was evaluated after 24 h (data not shown) or 48 h, after incubation of cells with 25 nM MitoTracker Red CMXRos and with 1 μg/mL DAPI. As a control, mitochondria of untreated cells are shown. Images are representative of three independent experiments in which more than 10 cells were examined.

Synthesis of TSPO Ligands. Compound [2-(4-chlorophenyl)-8-aminoimidazo[1,2-*a*]pyridin-3-yl]-*N*,*N*-di-*n*-propylacetamide (**3**) was synthesized following the slightly modified procedure previously reported.[28] Briefly, commercial 2,3-diaminopyridine (1.5 mol) was condensed with 3-bromo-4-(4-chlorophenyl)-4-oxo-*N*,*N*-dipropylbutanamide (1 mol) at 120 °C under vacuum for 6 h.

Synthesis of the Platinum Complexes. Cisplatin (*cis*-$[PtCl_2(NH_3)_2]$) was prepared according to the method of Dhara.[42] $K[PtCl_3(NH_3)]$ was prepared from $NH_4[PtCl_3(NH_3)]$ obtained by the method of Oksanen and Leskelä with slight modifications.[43] Briefly, *cis*-$[PtCl_2(NH_3)_2]$ (1.30 g, 4.37 mmol) was dissolved in a concentrated HCl/H_2O solution (24 mL, 1:2 v/v) and refluxed for 7 h. The resulting red solution was cooled to room temperature and left standing overnight. Unreacted *cis*-$[PtCl_2(NH_3)_2]$ (which precipitates at room temperature) was isolated by filtration of the solution, and the filtrate was evaporated under reduced pressure. The red residue was treated with methanol, and the resulting suspension was filtered. The filtrate was evaporated under reduced pressure. The dissolution−filtration−evaporation cycle was repeated three times in order to obtain a dark-red solid of pure $NH_4[PtCl_3(NH_3)]$. This compound was dissolved in methanol (20 mL) and treated with an excess of KOH (0.56 g, 10.0 mmol) at 0 °C, which induced the immediate formation of a yellow precipitate. After the mixture was stirred for 10 min, the pale-yellow solid ($K[PtCl_3(NH_3)]$) was isolated by filtration, washed with cold absolute ethanol containing a few drops of concentrated HCl, then with diethyl ether, and finally dried under vacuum (obtained 1.4 g, 79% yield). Anal. Calcd for $K[PtCl_3(NH_3)]\cdot H_2O$ (Cl_3H_5KNOPt): H, 1.34; N, 3.73. Found: H, 1.23; N, 3.56.

Synthesis of *cis*-[$PtI_2(NH_3)$]{[2-(4-chlorophenyl)-8-aminoimidazo[1,2-*a*]pyridin-3-yl]-*N*,*N*-di-*n*-propylacetamide}] (4). $K[PtCl_3(NH_3)]$ (84 mg, 0.24 mmol) was dissolved in ethanol/H_2O (30 mL; 1:1, v/v) and the resulting solution was treated with KI (82 mg, 0.71 mmol). After being stirred at room temperature for 5 min the mixture was evaporated under reduced pressure keeping the temperature at 50 °C. The resulting dark-red residue was treated with absolute ethanol where KCl is insoluble and can be removed by filtration. To the dark-orange filtrate of $K[PtI_3(NH_3)]$ was added **3** (91 mg, 0.236 mmol, also dissolved in 5 mL of absolute ethanol), and the resulting solution was refluxed for 24 h. After cooling to room temperature, the solution was taken to dryness under reduced pressure. The obtained brown solid was treated with CH_2Cl_2, and the resulting suspension was filtered. The filtrate was evaporated under reduced pressure and the resulting dark residue was purified by column chromatography on silica gel (CH_2Cl_2/Et_2O, 4:1 v/v, as eluent) to give the desired compound (R_f = 0.59, measured on TLC). An amount of 73.8 mg of **1** (40% yield) was obtained. Anal. Calcd for $\mathbf{1}\cdot{}^1/_2Et_2O$ ($C_{21}H_{29}N_5OCl_2Pt\cdot{}^1/_2C_4H_{10}O$): C, 31.68; H, 3.81; N, 8.03. Found: C, 31.58; H, 3.59; N, 7.87. ESI-MS: m/z $[M + Na]^+$ 873.7 Da.

Synthesis of *cis*-[$PtCl_2(NH_3)$]{[2-(4-chlorophenyl)-8-aminoimidazo[1,2-*a*]pyridin-3-yl]-*N*,*N*-di-*n*-propylacetamide}] (5). A solution of silver trifluoromethanesulfonate (CF_3SO_3Ag) (80 mg, 0.313 mmol) in acetone (2 mL) was added to a solution of **4** (132 mg, 0.155 mmol) in the same solvent (50 mL). After being stirred in the dark for 15 min, the resulting suspension was filtered through Celite to remove AgI. The filtrate was treated with LiCl (44.5 mg, 1.04 mmol), and the resulting suspension (LiCl is not soluble in acetone) was evaporated to dryness under reduced pressure. The resulting solid was treated with absolute EtOH, and after the mixture was stirred for 10 min, the solvent was evaporated under reduced pressure at 40 °C. The procedure (treatment with EtOH−stirring−evaporation) was repeated twice in order to favor the substitution of Cl^- for $CF_3SO_3^-$. Finally, the solid residue was suspended in absolute ethanol where both LiCl and $Li(CF_3SO_3)$ are soluble while the reaction product **5** is insoluble. Compound **5** was isolated by filtration of the mother solution, washed with cold H_2O and ethanol, and dried under vacuum. An amount of 73 mg of **5** was obtained (70% yield). Anal. Calcd for $\mathbf{5}\cdot H_2O$ ($C_{21}H_{28}Cl_3N_5OPt\cdot H_2O$): C,

28.63; H, 3.32; N, 7.95. Found: C, 28.64; H, 3.23; N, 7.55. ESI-MS: m/z $[M + Na]^+$ 689.8 Da.

Stability of Compound 5 in Buffered Solution. The stability of compound **5** in buffered solution was assessed by water-suppressed 1D ^{1}H NMR experiments. An amount of ~4 mg of **5** was dissolved in 200 μL of DMF-d_7 and diluted to 1 mL with isotonic (~25 mM NaCl) phosphate buffer (50 mM, pH 7.4) containing 10% of D_2O.

Stability of Compound 5 in the Presence of Glutathione. The stability of compound **5** in the presence of glutathione (GSH) was investigated at 37 °C in 50 mM isotonic (~25 mM NaCl) phosphate buffer at pH 7.4. Approximately 1 mg of compound **5** was dissolved in 1 mL of DMF, and an amount of 22.6 μL of the resulting solution was added to 1 mL of a 50 mM isotonic phosphate buffer at pH 7.4 containing 5 mM GSH, giving a final concentration of **5** of 33 μM. Aliquots of 30 μL were withdrawn from time to time and immediately analyzed by HPLC. Analyses were performed with a Waters Associates model 600 pump equipped with a Waters 990 variable wavelength UV detector and Empower software. A reversed phase Symmetry C18 (150 cm × 4.6 mm, 5 μm particles) column in conjunction with a SecurityGuard Phenomenex precolumn was eluted with mixtures of methanol and deionized water 75/25 (v/v). The volume injected was 20 μL. The flow rate was 1 mL/min, and the column effluent was monitored continuously at 254 nm. Quantification of the compound was performed by measuring peak areas relative to the standard eluted under similar conditions. The pseudo-first-order rate constant for the degradation of compound **5** was determined from the slopes of linear plots of the logarithms of residual starting material against time. All experiments were in triplicate.

In Vitro Receptor Binding Assays. Binding assays were performed according to the methods described previously.[27−29] Briefly, male Sprague−Dawley CD rats at 30 days of age were killed, the brain was rapidly removed, the cerebral cortex was dissected, and all tissues were stored at −80 °C.

[^{3}H]-7 Binding. The tissues were thawed and homogenized in 50 volumes of Dulbecco's phosphate buffered saline (PBS), pH 7.4, at 4 °C with a Polytron PT 10 (setting 5, for 20s). The homogenate was centrifuged at 40000g for 30 min, and the pellet was resuspended in 50 volumes of PBS and recentrifuged. The new pellet was resuspended in 20 volumes of PBS and used for the assay. [^{3}H]-**7** (New England Nuclear) binding was performed using 1000 μL containing tissue homogenate (0.15−0.20 mg of protein), 100 μL of [^{3}H]-**7** (specific activity of 85.5 Ci/mmol) leading to a final concentration of 1 nM, 5 μL of drug solution or solvent, and 795 μL of PBS buffer (pH 7.4 at 25 °C). Incubations (25 °C) were initiated by addition of tissue homogenate and were terminated 90 min later by rapid filtration through a glass-fiber filter strip (Wathman GF/B), which was rinsed with five 4 mL of ice-cold PBS buffer using a cell harvester filtration manifold (Brandel). Filter bound radioactivity was quantified by liquid scintillation spectrometry. Nonspecific binding was defined as binding in the presence of 10 μM unlabeled **7** (Sigma).

[^{3}H]-8 Binding. The tissues were thawed and homogenized with a Polytron PT 10 in 50 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) and centrifuged twice at 20000g for 10 min. The pellet was reconstituted in 50 volumes of Tris-HCl buffer and was used for the binding assay. Aliquots of 400 μL of tissue homogenate (0.4−0.5 mg of protein) were incubated in the presence of [^{3}H]-**8** (New England Nuclear) at a final concentration of 0.5 nM, in a total incubation volume of 1000 μL. The drug was added in 100 μL aliquots. After 60 min of incubation at 0 °C, the test sample was rapidly filtered through a glass-fiber filter strip (Whatman GF/B). The filter was then rinsed with 2−4 mL portions of ice-cold 50 mM Tris-HCl buffer (pH 7.4). The radioactivity bound to the filter was quantitated by liquid scintillation spectrometry. Nonspecific binding was determined as binding in the presence of 5 μM diazepam and represented about 10% of total binding.

Cytotoxicity Assays. These assays were carried out against human SF188 and SF126, rat C6 and RG2 glioma cells expressing high levels of TSPO. Furthermore, the cytotoxicity of cisplatin and compounds **4** and **5** was evaluated on the cisplatin sensitive A2780 and the cisplatin resistant A2780cisR human ovarian carcinoma cell lines (kindly provided by Prof. Mauro Coluccia, University of Bari, Italy). Compounds **4** and **5** were dissolved in DMF prior to their dilution to predetermined experimental concentration with cell culture medium. The percentage of the organic solvent used to dissolve the test compound did not exceed 1% (v/v) of the total solution volume. We verified that this amount (1% v/v) did not affect cell viability.

Human SF188 and SF126, rat C6 and RG2 glioma cell lines were maintained at 37 °C in a humidified incubator containing 5% CO_2 in RPMI 1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% (v/v) fetal calf serum (Invitrogen), 100 μg/mL streptomycin, 100 units/mL penicillin, and 0.3 mg/mL glutamine. Cisplatin sensitive A2780 and cisplatin resistant A2780cisR human ovarian carcinoma cell lines were cultured in DMEM nutrient supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cytotoxicity (IC_{50}) values for the compounds were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[44] Cells were dispensed into 96-well microtiter plates. Following overnight incubation, cells were treated with a range of drug concentrations. The plates were further incubated at 37 °C for 72 h. Then an amount of 40 μL of 5 mg/mL MTT was added to each well and the plates were incubated for an additional 2 h at 37 °C. The cells were then lysed by addition of 100 μL of aqueous 20% (w/v) SDS and 50% (v/v) *N*,*N*-dimethylformamide (pH 4.7) and incubated overnight at room temperature. The absorbance at 570 nm was determined using a Bio-Tek EL_X800 microplate reader (Bio-Tek Instruments, Winooski, VT). The reported values are the average of triplicate determinations made on at least two separate experiments.

Cellular Platinum Uptake. Cellular uptake of cisplatin, **4**, and **5** by C6 glioma cells was measured. The cells were seeded in 60 mm tissue culture dishes at a density of ~30 000 cells/cm^2. After 1 day of incubation at 37 °C in a humidified atmosphere with 5% CO_2, the culture medium was replaced with 2 mL of medium containing the tested compounds at 1 μM and incubated for 4 or 24 h. Compounds **4** and **5** were dissolved in DMF prior to their dilution to the predetermined experimental concentration with cell culture medium. The percentage of the organic solvent used to dissolve the test compound never exceeded 1% (v/v) of the total. At the end of the incubation period, the cell monolayer was washed twice with ice-cold PBS and then digested with 2 mL of HNO_3(67%)/H_2O_2(30%), 1:1 (v/v), solution for 4 h at 60 °C. The platinum content was determined by ICP-MS. All the experiments were performed in triplicate.

Fluorescence Microscopy. Wide field fluorescence of cells was analyzed through an inverted Zeiss Axiovert 200 microscope (Zeiss, Milano, Italy) equipped with a 63×/1.4 oil objective. In particular, C6 glioma cells were incubated in the presence and absence of cisplatin, **4**, and **5**. Mitochondrial morphology and nuclear morphology of control cells and cells treated with tested compounds were imaged 24−48 h after the treatment, subsequent to incubation for 15−30 min at 37 °C in a 5% CO_2 atmosphere with 25 nM MitoTracker Red CMXRos and 1 μg/mL DAPI (Molecular Probes) used as mitochondrial and nuclear markers, respectively. Excitation of MitoTracker Red and of DAPI as well as the selection of their respective emission fluorescences was accomplished with appropriate filters mounted on Lambda 10−2 filter wheel controllers (Sutter Instruments, Novato, CA). Fluorescence images were captured by a CoolSNAP HQ CCD camera (Roper Scientific, Trenton, NJ) using the Metamorph software (Universal Imaging Corporation). In each experiment, times of acquisition and light exposure were kept the same in order to compare emitted signal intensities.

Statistical Analysis. All data are presented as the mean ± SEM. The statistical analysis was accomplished using one-way analysis of variance (ANOVA) followed by the Tukey post hoc tests (GraphPad Prism, version 4, for Windows, GraphPad Software, San Diego, CA). Differences were considered statistically significant at $p < 0.05$.

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