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Oxamusplatin: A cytotoxic Pt(II) complex of a nitrogen mustard with resistance to thiol based sequestration display enhanced selectivity towards cancer

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Abstract

Pt(II) drugs and nitrogen mustards display severe side effects, poor tumour selectivity and face growing resistance by cancer cells due to sequestration by the thiol-containing molecules (*viz.* glutathione (GSH) and the copper ATPases like ATP7A/7B). ATP7A and ATP7B sequester Pt(II) complexes which contribute to the dose inefficacy and resistance. Incorporation of bulky ligands and chelating leaving groups may prevent deactivation by thiols. In this work, we have synthesised four new Pt(II) complexes (**3-6**) of two carrier ligands, bis(2-hydroxyethyl)pyridylmethylamine (L1) and bis(2-chloroethyl)pyridylmethylamine (L2) with oxalato and cyclobutanedicarboxylato leaving groups. Among the four new complexes (**3-6**) the Pt(II) complex of L2 with oxalato leaving group (**5**, termed as, “oxamusplatin”) is cytotoxic. 'Oxamusplatin' is more resistant than cisplatin or oxaliplatin towards hydrolysis, thiol binding and sequestration by ATP7B. It targets cellular DNA and is capable of disrupting the microtubule network in the cytoskeleton. Oxamusplatin demonstrates better selectivity than oxaliplatin towards cancerous cells. It is ca. 4-10 times greater cytotoxic towards metastatic prostate carcinoma (DU-145, IC₅₀ = 21 ± 1 μM) and ca. 10-24 times greater cytotoxic towards breast adenocarcinoma (MCF-7, IC₅₀ = 8.1 ± 0.8 μM) compared to the three noncancerous cells investigated.

Introduction

Cancer affects billions of people¹ and the increasing occurrence of drug resistance makes treatment more difficult. Platinum drugs proved effective against testicular, ovarian, colon, non-small cell lung, prostate, breast, cervical and stomach cancer.² Cisplatin (CDDP) is used in the treatment of metastatic testicular cancer with more than 80% cure rate and is widely used against ovarian and bladder cancer.³ The nitrogen mustards are among the first chemotherapeutic agents against cancer, used in chronic lymphocytic leukaemia (CLL), non-

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Conflicts of interest

There are no conflicts to declare.

Hodgkin's lymphoma, Hodgkin's disease, breast, ovarian and testicular cancer.^{4, 5} However, CDDP or nitrogen mustards both exhibit high toxicity to normal cells leading to severe side effects.⁶ Attachment of various functionalities (*viz.* glucose, folate, targeting peptides and vitamins to Pt-drugs or nitrogen mustards) increases efficacy towards therapeutic targets and minimises systemic toxicity.^{4, 7–12} The structural properties of CDDP limits its use in the targeted delivery approach since after the dissociation of the other components, the active metal complex is still CDDP and hence would bear its drawbacks. Hence, the major disadvantages associated with CDDP are (i) the high systemic toxicity due to non-selectivity and ease of reactivity (ii) easy sequestration by copper transporter ATPases or other thiol donor molecules (*viz.* GSH, metallothioneine) leading to efflux out of the cancer cells. In spite of the disadvantages, the next CDDP equivalent is difficult to found.^{13, 14} Among the next generation FDA approved Pt drugs, carboplatin has a higher solution half-life and lower systemic toxicity, but it has the same active species as CDDP, so it is not active in CDDP resistant cells.^{15–19} In contrast, oxaliplatin with a *trans*-(R,R)-1,2-diaminocyclohexane (DACH) carrier ligand and oxalato leaving group show less systemic toxicity.^{13, 20–22} Oxaliplatin overcomes CDDP resistance due to the change in the carrier ligand that alters the type of DNA adducts formed.^{13, 20–22} This strategy has inspired the generation of many excellent Pt (II/IV) complexes.^{7, 11, 23} A wide variety of Pt(II) complexes *viz.* picoplatin, phenanthriplatin, BBR3464 (Figure 1) are known to kill cancer cells efficiently with a mechanism of action different from CDDP.^{24–27} However, the resistance to the copper ATPases (ATP7A or ATP7B) are not investigated for newly designed Pt complexes. On the other hand, mechanistic studies on CDDP sequestration by ATP7A or metallothionein's have been reported^{30, 31} (Scheme 1). The affinity of CDDP towards thiol motifs is linked to their sequestration by the copper ATPases, since these proteins use cysteine thiols (-SH) in their metal binding site (the MXCXXC motif, where M = methionine, C = cysteine and X = any other amino acid) to bind to the Pt-drugs.³¹ The nitrogen mustards are also susceptible to bind to thiol donors and are deactivated by thiol-containing molecules *viz.* metallothioneines, GSH due to uncontrolled reactivity.^{32–35} It has been shown that the reactivity of the nitrogen mustards can be controlled by coordinating the lone pair of the nitrogen with a metal ion.⁴ In some cases, there may be total loss of activity, nevertheless, it is an important methodology to control the reactivity of nitrogen mustard which might otherwise lead to high systemic toxicity. In this regard, our earlier works show that incorporation of the nitrogen mustard ligand bis(2-chloroethyl)pyridylmethylamine provides higher steric hindrance and improves the stability of a dichlorido-Pt(II) complex.^{36, 37} The ligand acts as a strong chelating N,N-donor to the Pt(II) displaying higher stability and high cytotoxicity. In addition to the above, the already known advantages of the kinetic inertness of carboplatin and oxaliplatin led us to improve our design to circumvent sequestration pathways as well as lower the normal cell toxicity.^{4, 11–14} Thus, incorporation of a chelating leaving group (*viz.* oxalate, cyclobutane-1,1-dicarboxylate) should make the complexes kinetically more inert towards deactivation by thiols. The kinetic inertness may also impart alternate targeting ability apart from DNA cross-linking. Thus, we coordinated the chelating nitrogen mustard bis(2-chloroethyl)pyridylmethyl-amine (L2) with Pt(II) and changed the leaving group to oxalato and cyclobutane-1,1-dicarboxylato (**6**, Scheme 2). The Pt(II) complex of the cyclobutane-1,1-dicarboxylato analogue is highly stable and loses cytotoxicity *in vitro* but the oxalato analogue (**5**), termed as 'oxamusplatin' shows excellent stability, selective

cytotoxicity, strong resistance to GSH and sequestration by ATP7B. This work presents the effect of changing the leaving group in a family of Pt(II) complexes with the same carrier ligand (L2) towards hydrolysis, DNA binding and thiol-based sequestration including that of the copper efflux protein ATP7B.

Results and Discussion

Syntheses and characterisation

L1, L2 and their corresponding Pt(II) complexes (**1-2**) were synthesized following the procedure reported by us earlier.³⁶ The Pt(II) complexes **3** and **4** were prepared from **1** by reacting with AgNO₃ followed by addition of sodium salt of the dicarboxylic acid. Complexes **5** and **6** were prepared from **2** using a similar method as **3** and **4**. The stretching frequency corresponding to the carbonyl group of oxalate or cyclobutanedicarboxylate appears in the range of 1600-1800 cm⁻¹ in all complexes. The yield of **5** and **6** is less compared to **3** and **4**. This may be attributed to the more washing needed to purify **5** and **6**. During washing, some complex might have been lost leading to lower yield. All the complexes were characterized by ¹H, ¹³C, ¹⁹⁵Pt NMR, ESI-HRMS, UV-vis and IR spectroscopy. The bulk purity of the complexes was also confirmed by elemental analysis.

Stability and binding study

The solution stability and binding studies were only performed for **5** and **6** since they were structurally close to each other. The complexes **3** and **4** did not have any mustard group and were not cytotoxic as well, so they were not studied for their stability and binding. The stability and binding studies of complexes **5** and **6** were investigated in 1:1 v/v DMSO-d₆ and phosphate buffer (20 mM, pD 7.4, 4 mM NaCl) by ¹H NMR (Figure 2 & Figure S1). The stability studies showed that both the complexes **5** and **6** do not hydrolyse up to 24 h (Figure 2 & Figure S1), which is further evident from the ESI-MS (Figure S2-S3 & Figure S4) done with less than 5 μM concentration of **5** and **6**. This is attributed to the chelate effect of the oxalato and cyclobutanedicarboxylato ligands respectively. ¹H NMR showed that on reacting 3:1 mole ratio of 9-EtG with **5**, it formed a small amount of 9-EtG adduct after 4 days, which is confirmed from the shift of H8 proton from 7.69 to 8.10 ppm (shown as H8', Figure 3a). At much lower concentrations (ca. 5 μM) the ESI-MS data shows that within 12 h there was formation of 9-EtG adduct (Figure 3b & Figure S5, Scheme 3). In contrast, the NMR studies of complex **6** showed that the complex did not react with 9-EtG even after 4 days or more since the chemical shift of the H8 proton remained the same (Figure S6). Complex **6** turned out to be so inert that it was non-toxic *in vitro*, as discussed later in the cytotoxicity section.

Since one of our objectives was to check whether the complexes are resistant to GSH binding hence we probed the reactivity of **5** and **6** with the cellular tripeptide GSH, which is found to be abundant in many cancer cells^{4, 38} and is responsible for the deactivation of platinum drugs.⁴ The GSH binding study with **5** shows that the Pt complex remained intact since there is no change in the chemical shifts in the ¹H NMR spectrum (Scheme 3 and Figure 4a). The high resolution ESI-MS data also suggests that there is no adduct formation of GSH with **5** (Scheme 3 and Figure 4b & Figure S7). In the ¹H NMR the shift in GSH

protons from their original position after a long period is due to the auto-oxidation of GSH over time (Figure 4a). Complex **6** which is more inert also displays no GSH bound complex as evident from the NMR but only auto-oxidation of GSH is observed (Figure S8).

Cytotoxicity

The ligands L1 and L2 did not exhibit any cytotoxicity against the cell lines investigated. The reason may be that L1 is not a nitrogen mustard ligand and L2 although a nitrogen mustard, it has the lone pair on the nitrogen free so is reacting with the media components and thus getting deactivated. The cytotoxicity of **3-6** were first investigated against breast adenocarcinoma (MCF-7). Complexes **3** and **4** were non-toxic up to 200 μM and they also did not have the mustard motif so they were not investigated any further. Complex **6** bearing a nitrogen mustard motif in the Pt complex demonstrated no *in vitro* toxicity against MCF-7, metastatic prostate carcinoma (DU-145), pancreatic carcinoma (MIA PaCa 2) and non-cancerous human foreskin fibroblast (HFF-1). So, we decided not to study this complex any further. Only oxamusplatin turned out to be the promising molecule in the series which showed best cytotoxicity against MCF-7 ($\text{IC}_{50} \approx 8 \mu\text{M}$) and was at least 10 times less toxic to non-cancerous cells *viz.* HFF-1 ($\text{IC}_{50} > 150 \mu\text{M}$, Figure S9) and Madin-Darby Canine kidney cell line (MDCK) ($\text{IC}_{50} > 200 \mu\text{M}$, Figure S10) and Chinese Hamster Ovary (CHO) cell line ($\text{IC}_{50} \approx 88 \mu\text{M}$, Table 1).

We investigated oxamusplatin against the metastatic prostate carcinoma (DU-145) and found that the complex displays an $\text{IC}_{50} \approx 21 \mu\text{M}$ which again shows the selectivity of oxamusplatin against pancreatic cancers (MIA PaCa 2 and PANC-1) and liver carcinoma (Hep G2) and found that it is toxic with IC_{50} range of ca. 25 - 36 μM (Table 1, Figure S11). Hence, oxamusplatin is much less toxic towards non-cancerous cell lines *viz.* human foreskin fibroblast (HFF-1) and canine kidney cell line (MDCK) and Chinese Hamster Ovary (CHO) cell line (Table 1). The above observations helped us conclude that the 'oxamusplatin' shows higher cytotoxicity towards cancerous cells than that of the non-cancerous cells. The selectivity in oxamusplatin may be due to its higher stability or some other reason, which is not clearly understood. The insight into the selectivity remains elusive in many small molecules which does not have any motif that can be held responsible for its selectivity.^{39, 40} However, this is a highly appreciable feature in a cancer chemotherapeutic agent and indicates a possibility of lower systemic toxicity compared to CDDP or oxaliplatin. The difference between complex **6** and oxamusplatin is only the leaving group yet **6** was not cytotoxic up to 150 μM . It was possible that the difference in cytotoxicity was due to difference in cellular accumulation apart from the kinetic inertness so we studied the cellular Pt content using ICP-MS upon treatment with complexes **3-6**.

Cellular accumulation study

Cellular uptake studies for **3-6** were carried out in MCF-7 cells to measure the distribution of Pt content in the cells. The cells were treated with an equal concentration of **3-6** for 24 h and the platinum content of each set was determined by ICP-MS. The ICP-MS accumulation data showed that the accumulation of Pt decreased in the following order **5** > **3** > **4** $\approx$ **6** (Figure 5a). Data indicates that the oxalato derivatives (**3** & **5**) have the higher accumulation inside the cell than the cyclobutanedicarboxylate analogues (**4** & **6**) and hence the later

derivatives do not exhibit any cytotoxicity towards cancer cell lines. Among **3** and **5** the later one contains the mustard moiety where as in case of **3** the chloro group of mustard was replaced by hydroxyl group. Our earlier investigations have shown that whenever a pendant ethylhydroxy motif is present in the vicinity of a coordinated Pt(II) it may interfere with the reactivity by itself chelating to the Pt(II) through the release of the chloride ion.³⁶ Thus the pendant hydroxyl group in **3** may be binding to the Pt(II) centre leading to its deactivation. Hence, we found that **3** is not toxic but **5** is quite cytotoxic. Thus, the mustard ligand has a prominent role in cytotoxicity to prevent deactivation of the Pt(II).

Lipophilicity

The cellular accumulation of a chemotherapeutic agent is often correlated with its lipophilicity so we studied the lipophilicity (log D) of **3-6** by the shake-flask method in octanol/water mixture (Figure 5b). The lowest log D was observed for **3**, while **6** showed the highest log D value. Complex **3-5** is more hydrophilic in the order **3** > **4** > **5**, whereas **6** is moderately lipophilic. The lipophilicity pattern does not co-relate with the higher cellular accumulation or better cytotoxicity. The cytotoxicity is highest for **5** (oxamusplatin) and it also accumulates the most in cells but it is more hydrophilic than **6**. Complex **3** and **5** are quite different in lipophilicity yet they are more similar in terms of accumulation in cells. Complex **6** is a stable complex with lipophilicity within range for better accumulation but **6** shows lower accumulation in cells.

ATP7B sequestration study

Pt(II) drugs like CDDP is efficiently sequestered by cellular thiols and ATP7A/7B. It is well known that ATP7A/7B regulates the resistance of human cells to CDDP.^{33, 41} The oxamusplatin showed enhanced selectivity towards cancer cells and resistance to binding by the cellular thiol GSH. This led to the investigation whether efficacy of ATP7B to impart resistance towards oxamusplatin is better than that towards CDDP. The copper ATPase *viz.* ATP7A/7B contains six metal-binding motifs in its N-terminal (MXCXXC) containing thiol donors to sequester Cu(I), which also efficiently sequesters Pt(II) and both the proteins have similar metal-binding site.⁴² ATP7B localizes at the trans-Golgi network (TGN) under copper-deprived conditions and traffics to vesicles with a rise in copper concentration to export it out of the cells. The binding of Pt to ATP7B leads to vesicularization that traffics from the TGN towards cell periphery.³² We utilized trafficking response of ATP7B towards the complexes as a function of its sequestration and export.^{32, 33, 41}

Hence, the Golgi and the ATP7B were marked with two different coloured fluorescent tags [*viz.* Golgi (red) and ATP7B (green)] so that we are able to detect colocalization (yellow). Thus, allowing to study the trafficking response of ATP7B towards the complexes and compare with known Pt drugs *viz.* CDDP or oxaliplatin.^{32, 33, 41} We studied the effect of ATP7B on oxamusplatin (and compared with oxaliplatin) using the Hep G2 cell line which has significant endogenous ATP7B levels. Our immunofluorescence studies showed that cells treated with oxamusplatin showed minimal ATP7B trafficking (Figure 6). The resistance of oxamusplatin towards ATP7B seems to be even more than oxaliplatin (Figure 7). In case of oxamusplatin most of the ATP7B resided in the perinuclear compartment and co-localized with the TGN marker (Golgin 97) (Figure 6 & Figure S13). This effect is

similar to the effect of the copper chelator bathocuproinedisulfonate (BCS) (Figure 6-7 & Figure S13). Thus, oxamusplatin is not easily sequestered by ATP7B unlike CDDP, preventing its deactivation. The immunofluorescence data was quantified using a population of ca. 50 cells and measuring the Pearson correlation coefficient (PCC) of co-localization between ATP7B (green) and *trans*-Golgi network (red). Thus, suggesting that the strong chelation of the oxalate and the steric hindrance from the mustard motif together prevent the reactivity with the thiol motifs (MXCXXC) thus inhibiting sequestration by the N-terminal of ATP7B.

Effect of a P-glycoprotein(P-gp) inhibitor on cytotoxicity

Among the other pathways of deactivation, the P-gp seems to play a role in efflux of many drugs. Effect of a P-gp inhibitor on the cytotoxicity of a drug provides indication of the role of P-gp in the efflux of the drug.^{43, 44} If the cytotoxicity of the complex increases in presence of a P-gp inhibitor, then the P-gp may have a role in effluxing the drug, thus reducing its toxicity. Drugs like vinblastine and doxorubicin are known to be transported by P-gp and in presence of a P-gp inhibitor their toxicity is increased.^{44, 45} Verapamil is a first generation competitive inhibitor of P-gps which competes as a P-gp substrate and is used in studies to support the efflux through P-gp.^{45, 46} Verapamil itself is not toxic up to 100 μ M (Figure S14) against DU-145 cells. We used 10 μ M verapamil for studying the effect of P-gp on cytotoxicity, since this concentration shows substantial inhibition against P-gp.^{43, 45} The results show that, in presence of verapamil the oxaliplatin became 8 times more efficient in cell killing (Table S1 & Figure S15-S16) whereas oxamusplatin only became twice more efficient (Table S1 & Figure S15-S16). Verapamil also happens to be a calcium channel blocker.⁴⁷ So the result indicates that the cytotoxicity of oxamusplatin is less affected by a P-gp inhibitor or it may have pathways of action different from oxaliplatin and is not affected by a calcium channel blocker unlike the oxaliplatin.

Tubulin polymerization inhibition assay

We found that oxamusplatin shows moderate to good cytotoxicity against various cancer cell lines and is much less toxic to non-cancerous cells. The NMR and ESI-MS studies showed that oxamusplatin shows low binding with 9-EtG. Oxamusplatin is stable and may have other targets that contribute to the cytotoxicity hence we probed if oxamusplatin is disrupting the microtubule network which is the backbone of the cytoskeleton of the cell. We found that oxamusplatin disrupted the organization of the microtubule network in MCF-7 cells. Colchicine was taken as a positive control because it is a known tubulin polymerization inhibitor. The control MCF-7 cells showed normal filamentous microtubule array, where the cells treated with oxamusplatin (25 μ M) exhibited disruption of microtubule network which was also observed for colchicine treated cells (Figure 8). In 24 h, oxamusplatin does not kill a significant number of cells at a concentration of 25 μ M (Cell viability > 80%) still we see the microtubule disruption. Recently it was shown by Brabec et al. that certain Pt(IV) complexes inhibit the organization of microtubule.⁴⁸ Here in, we find that oxamusplatin not only targets DNA but disrupts the organization of microtubules affecting the cytoskeleton.

Cell cycle arrest by flow cytometry

The disruption of cytoskeleton would lead to arrest of cell cycle in the mitotic phase. On treating MCF-7 cells with oxamusplatin for a period 24 h we could find G2/M phase arrest to be the major one along with a small population of S-phase arrest (Table 2). The above results suggest that oxamusplatin can target the DNA as well as can inhibit tubulin polymerization in MCF-7 cells.

Pathway of cell killing

The pathways of cell killing via apoptosis were detected by Annexin-V/PE and 7AAD double staining assay in MCF-7 cells. The cells treated with IC₂₅ and IC₅₀ dose of oxamusplatin for 24 h showed the apoptotic cell killing. It induces ca. 48% and ca. 84% of apoptosis at IC₂₅ and IC₅₀ dosage respectively compared to ca. 6% in control (Figure 9a-9c). The apoptosis was further confirmed by DNA ladder assay, since it is a well-known hallmark for apoptosis detection.^{49, 50} The DNA extracted from MCF-7 cells, treated with oxamusplatin for 24 h, showed DNA ladder formation (Figure S17), which further confirms the cells were killed via apoptosis.

The apoptosis induction by oxamusplatin may be intrinsic or extrinsic in nature. The intrinsic pathway is controlled by mitochondria and this can be monitored by observing the change in mitochondrial membrane potential (MMP, Ψ_m). This change can be evaluated by using cationic dye JC-1, a highly specific probe for the detection of change in the membrane potential of mitochondria. JC-1 gives red fluorescence ($\lambda_{em} = 590$ nm) to its aggregation but upon depolarization of the mitochondria the aggregation does not happen and the monomer of JC-1 emits green fluorescence ($\lambda_{em} = 550$ nm) which suggests mitochondrial depolarization.

Oxamusplatin depolarizes the mitochondria in MCF-7 cells in a dose dependent fashion upon treatment with IC₂₅ and IC₅₀ concentration for 24 h (Figure 9d). The change in the mitochondrial membrane potential led us to study the caspase activation. During apoptosis the release of cytochrome C due to mitochondrial depolarization leads to activation of caspase 9 leading to a cascade which activates caspase 3/7.^{51, 52} The results of the activation of caspase 7 in MCF-7 cells treated with oxamusplatin confirms mitochondria mediated apoptotic pathway of cell killing in a dose dependent fashion (Figure 9e).

Experimental

Materials and methods

The solvents and chemicals used in this work were purchased from different commercial sources. 9-ethylguanine (9-EtG) and reduced glutathione (GSH) were purchased from Carbosynth and used as received. Verapamil was purchased from Sigma Aldrich. Solvents purchased were of analytical grade and were distilled prior to use. Spectroscopy grade solvents (Spectrochem India) were used in spectroscopy. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) and Penicillin-Streptomycin were purchased from USB and Hyclone respectively. Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Medium (MEM) and

Phosphate-Buffered Saline (PBS) were purchased from Invitrogen. 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) and propidium iodide (PI) were purchased from Sigma-Aldrich. ATP7B and tubulin antibodies were purchased from abcam. Golgin 97 antibodies and the secondary antibodies (anti-rabbit-Alexa Fluor 488 and anti-mouse- Alexa Fluor 568) were purchased from Thermo Fisher Scientific. 96-well microplates were purchased from Thermo Fisher Scientific. UV-visible data were recorded using Perkin Elmer lambda 35 spectrophotometer. FT-IR spectra were performed using Perkin-Elmer SPECTRUM RX I spectrometer in KBr pellets. ^1H , ^{13}C (proton decoupled) and ^{195}Pt (proton coupled) NMR spectra were measured using Bruker Avance III 500 MHz spectrometer at room temperature. All chemical shifts are reported in parts per million (ppm). ^{195}Pt NMR chemical shifts are described against standard $\text{K}_2[\text{PtCl}_4]$ chemical shift in D_2O at -1628.0 ppm. Elemental analysis was studied in Perkin-Elmer 2400 series II CHNS/O analyzer. High Resolution Electrospray Ionization Mass Spectrometry (ESI-HRMS) were performed using Bruker maXisITM instrument by +ve mode electrospray ionization. The synthetic yields reported are of isolated analytically pure compounds.

Kinetics and binding studies: NMR and ESI experiments

The sample for NMR experiments were prepared in 50% 20 mM phosphate buffer (prepared in D_2O , pD 7.4) in $\text{DMSO}-d_6$ and the data were recorded in JEOL ECS 400 MHz spectrophotometer in dark. The ESI MS samples were prepared 50% 5 mM phosphate buffer (pH 7.4, 4 mM NaCl) in MeOH diluted in HPLC grade methanol.

Cell lines and culture

Breast adenocarcinoma (MCF-7) and normal human foreskin fibroblast (HFF-1) were obtained from the Department of Biological Sciences, IISER Kolkata (purchased from ATCC). Human hepatocellular carcinoma (Hep G2), human pancreatic carcinoma (MIA PaCa 2 & PANC-1), brain metastasized prostate carcinoma (DU-145), Madin-Darby Canine kidney cell line (MDCK) and Chinese Hamster Ovary (CHO) cell lines were obtained from NCCS (Pune, India). MCF-7, MIA PaCa 2, PANC-1 and MDCK cells were maintained in the logarithmic phase in Dulbecco's Modified Eagles Medium (DMEM) while DU-145, Hep G2, HFF-1 and CHO cells were grown in Minimum Essential Medium (MEM). Both the media were supplemented with 10% fetal bovine serum (GIBCO) and antibiotics (100 units ml^{-1} penicillin and 100 mg ml^{-1} streptomycin). The cell culture was done in a humid atmosphere with 15% O_2 and 5% CO_2 at 37°C .

Cell viability assay

The cytotoxicity of the compounds against various cancer cell lines was evaluated based on MTT assay. Cells were seeded in 96-well microplate at a density of 4×10^3 viable cells per well in a volume of 200 μL of medium. It was subsequently incubated at 37°C in a 5% carbon dioxide atmosphere for 24 h. After incubation, the medium was renewed with a fresh one (200 μL) followed by addition of metal complexes at appropriate concentrations. Each concentration was loaded in triplicate. The stock solutions of compounds were prepared in DMF such that concentration of DMF in the well does not exceed 0.2%. After 72 h of subsequent incubation, the existing drug containing media was removed, followed by

addition of fresh media along with 20 μL of a 1 mg mL^{-1} MTT in 1X PBS (pH 7.2). On incubation of the plates for 3 h at 37°C in a humidified 5% CO_2 atmosphere, MTT was allowed to form formazan crystals in metabolically active cells. Finally, media was removed after incubation and replaced with 200 μL of DMSO in each well that would solubilize the formazan crystals. The absorbance for each well was recorded at 595 nm using a SpectraMax M2e plate reader. IC_{50} values represent the drug concentration at which 50% cells are inhibited compared to control, which was calculated by fitting nonlinear curves in GraphPad Prism 5. Each independent experiment was carried out in triplicate.

Statistical analysis

All the IC_{50} data given are mean of three independent experiments carried out in each cell lines, where, in each experiment each concentration was assayed in triplicate. The statistical analyses were done using Graph pad prism® software 5.0 with student's t-test.

Metal accumulation study in MCF-7 cells by ICP-MS

In 90 mm sterile tissue culture petri-dish 1.2×10^5 numbers of MCF-7 cells were seeded and grown for 48 h followed by treatment with equimolar (10 μM) concentration of complex solutions (**3**, **4**, **5** and **6**) for an additional 24 h. Subsequently the media was discarded and cells were washed using 1X-PBS (pH 7.2). The cells were counted accurately after trypsinization for each drug treated sample. 1×10^6 number of cells from each sample were centrifuged to form cell pellet. The cell pellets were washed twice by re-dispensing in 1X PBS (pH 7.2) followed by centrifugation. Cell pellets were then digested with 200 μL of extra pure (70% v/v) nitric acid (Sigma-Aldrich) at 100°C for 12 h. Finally, the digested cell suspension was diluted using Milli-Q water and the platinum content in the samples were analyzed on a Thermo Scientific XSERIES 2 ICP-MS instrument. Platinum standard solutions were freshly prepared before the experiment while analysis for all the samples were carried out in triplicates and the standard deviations were calculated.

Lipophilicity

The distribution coefficients of the complexes in an octanol-water system were determined using the standard shake-flask method. Each set was performed in triplicate and the absorbance of the organic and aqueous layers were recorded in a UV-vis spectrophotometer after proper dilution. The concentration of the substances in each layer was calculated using the respective molar extinction coefficients of **3-6** and oxaliplatin. After that the distribution coefficient values ($\log D_{o/w}$) were obtained from the ratio.

Immunofluorescence assay for mobilization of ATP7B in HepG2 cells

1×10^5 numbers of Hep G2 cells were grown over glass cover slips (Corning Life Sciences) for 48 h. Cells were then treated with bathocuproinedisulfonate (BCS) (50 μM), copper (50 μM), oxaliplatin (20 μM) and oxamusplatin (20 μM) for 2 h. After 2 h media containing drugs were removed and washed with 1X PBS. Cells were then fixed with 4% (w/v) paraformaldehyde in PBS for 10 min and subsequently quenched with 50 mM NH_4Cl . After washing with 1X PBS for three times, cells were blocked with 3% bovine serum albumin (BSA) in PBS containing 0.1% Tween 20 (PBST) for 20 min at room temperature. Then

cells were incubated with primary antibody ATP7B and Golgin 97 in 1:400 dilution for 2 h, followed by washing with 1X PBS for three times. After that the cells were incubated with secondary antibody in 1:1000 dilutions for 2h in dark. Cells were mounted on slides for imaging using the Fluoroshield mounting medium containing DAPI after 3 times washing with 1X PBS. All images were acquired in Leica SP8 confocal microscope at 63X oil immersion objective.

Effect on tubulin polymerization

A total of 5×10^4 MCF-7 cells were seeded over glass coverslips (Corning Life Sciences) for 48 h. Cells were then treated with 25 μ M oxamuplatin and 100 nM colchicine (positive control) for 24 h. After 24 h, media containing drugs were removed and washed with 1X PBS. Cells were then fixed with 4% (w/v) paraformaldehyde for 15 min and subsequently quenched with 50 mM NH_4Cl . After being washed three times with 1X PBS, cells were blocked with 2% (w/v) bovine serum albumin (BSA) in PBS containing 0.1% Tween 20 (PBST) for 20 min at room temperature. After removing the BSA, cells were incubated with the primary antibody against α -tubulin (anti- α tubulin antibody, EP1332Y, rabbit monoclonal microtubulin marker) in a 1:400 dilution for 2 h, followed by washing three times with 1X PBST and 1X PBS. Secondary antibody incubation was done with goat anti-rabbit IgG H&L (Alexa Fluor 488) in 1:1000 dilutions for 2 h in the dark at room temperature. After being washed with PBST and PBS, cells were mounted on slides for imaging using the Fluoroshield mounting medium containing DAPI. The emission of Alexa Fluor 488 ($\lambda_{\text{max}} = 520$ nm) was used to visualize the microtubular network within the cells. The DAPI enabled the visualization of the nucleus. All images were taken with a Leica SP8 confocal microscope with a 63X objective.

Cell cycle analysis by flow cytometry

MCF-7 cells were seeded at 1.2×10^5 cells per plate in a 90 mm petri dish containing 12 mL DMEM and incubated at 37°C in a 5% carbon dioxide atmosphere for 48 h. After incubation, media was replaced with fresh media along with appropriate concentration of complex solutions and incubated for 24 h at previously mentioned culturing condition. Subsequently, drug containing media was removed and cells were harvested by trypsinization, washed twice with cold 1X PBS (pH 7.2) and were fixed using chilled 70% ethanol and stored at 4°C for 12 h. The cell pellets were re-suspended in 1X PBS solution comprising of PI (55 $\mu\text{g mL}^{-1}$) and RNase A (100 $\mu\text{g mL}^{-1}$) and incubated at 37°C for half an hour in dark for DNA staining. Finally, the homogenized cell samples were analyzed in a BD Biosciences FACSCalibur flow cytometer and the resulting DNA histograms were quantified using the CellQuestPro software (BD).

Apoptosis detection: Annexin-V/PE assay

Apoptosis was detected using the PE-Annexin V and 7-AAD dual staining apoptosis detection kit (BD Pharmingen) by flow cytometry according to the manufacturer's protocol. A total of 5×10^5 MCF-7 cells were seeded into a 35 mm sterile tissue culture Petri dish using 2 mL of DMEM. Then, cells were incubated at 37 °C in a 5% carbon dioxide atmosphere for another 48 h. Subsequently, the medium was changed and cells were treated with different concentrations (IC_{25} and IC_{50}) of oxamuplatin for 24 h. Cells were then

harvested with cold 1× PBS containing 0.1 mM EDTA and subsequently washed with cold 1× PBS twice. The cells were finally re-suspended in Annexin V binding buffer. Cells were then incubated with both Annexin V-PE and 7-AAD for 15 min in the dark at 25 °C. Data were analysed in a BD Biosciences FACS Verse flow cytometer within 1 h sample preparation

DNA ladder assay for apoptosis detection

DNA ladder assay was performed for apoptosis detection using a literature procedure.⁵³ Briefly, MCF-7 cells (1.2×10^5 per plate) were seeded in a 90 mm tissue culture petri dish. After 48 h, the media was changed and different concentrations of complex solutions were added. After 24 h of incubation, media was removed and the cells were collected in a tube. Then the cells were washed with 1X PBS (pH 7.2) and the cells along with washings were collected in the same tube and mixed with the harvested cells after trypsinization. The cells were then washed twice with 1X PBS (pH 7.2) and centrifuged at 2500 rpm for 10 minutes at 25°C. 500 μ L of lysis buffer (20 mM Tris-HCl, 0.4 mM EDTA, 0.25% Triton-X 100, pH 8.0) was added and incubated at room temperature for 15 minutes. The lysed cells were centrifuged at 14000 rpm for 10 minutes at 4°C and the supernatant was mixed well with 500 μ L of 1:1 (v/v) mixture of phenol and chloroform. After centrifugation at 14000 rpm for 10 minutes at 4°C, the aqueous layer was pipetted out carefully and mixed with 55 μ L of 5 M NaCl solution and 550 μ L of isopropanol. The mixture was incubated at -20°C for overnight. The resultant solution was then centrifuged at 14000 rpm for 10 minutes at 4°C and the obtained pellet was washed with 70% ice-cold ethanol and finally air dried. The dried pellet was re-suspended in 40 μ L of 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and then 8 μ L of RNase solution (150 μ g mL⁻¹) was added. After centrifugation at 5000 rpm for 10 minutes, the supernatant was loaded in 1.6% agarose gel containing ethidium bromide (1 μ g mL⁻¹) and run for 3 h at 60 V in 1X TBE buffer. Untreated cells were used as controls whereas 50bp DNA ladder was used to track the migration of bands and fragmentation sizes on the agarose gel. Visible bands on gel were observed and picture taken on exposure to UV light through gel documentation system of Bio-Rad.

JC-1 staining assay for detection of mitochondrial membrane potential change

Seeding of cells, drug treatment and final harvesting of cells after 24 h of drug exposure were carried out using same procedure followed during the DNA ladder assay. The harvested cells were then collected by centrifugation at 2500 rpm at 25°C for 5 min, washed with 1X PBS (pH 7.2) by subsequent centrifugation and finally the cells were mixed with 500 μ L of JC-1 (10 μ g mL⁻¹ in 10% FBS supplemented 1X PBS). The cell suspension was subjected to 30 min incubation at 37°C. The stained cells were collected by centrifugation and resuspended in 1X PBS (pH 7.2) for analysis in BD Biosciences FACSVerse flow cytometer measuring green and red fluorescence intensities.

Caspase 7 activation assay

The effect of oxamuplatin in activation of caspase 7 was investigated against MCF-7 cells using Caspase 3/7 colorimetric detection kit (Sigma). The manufacturer's protocol was followed throughout the assay. In brief, 1.2×10^5 number of MCF-7 cells were seeded at 100 mm sterile tissue culture petri dish for 48 h. Cells were then treated with both IC₂₅ and

IC₅₀ concentrations of oxamuplatin for 24 h. Release of p-nitroaniline was monitored with time after caspase 7 substrate treatment with the cell lysate. The assay was performed following 96 well plate method and data recorded using ELISA plate reader at 405nm. Standard curve was drawn using known concentration of pNA (p-nitroaniline) to estimate amount of pNA released by Caspase 7.

Synthetic procedure

General procedure for synthesis of oxalato and cyclobutane-1,1-dicarboxylato derivatives (3, 4, 5 & 6)

Complex **1** or **2** were synthesized using our previously reported procedure.³⁶ **1** or **2** were added with AgNO₃ (1.9 equivalents) in deionized water. After stirring for 48 h at room temperature, the reaction mixture was filtered with a 0.22-micron syringe filter to remove AgCl. The filtered yellow solution was now treated with an aqueous solution of 1 equivalent of disodium oxalate (ox) or cyclobutane-1,1-dicarboxylate (cbdca) and stirred for another 12 h at room temperature. The white precipitate formed was filtered, washed twice with cold water, acetonitrile & diethyl ether respectively and air dried.

Caution: The nitrogen mustard ligands and the complexes are to be handled with care since they are potent DNA modifying agents

[Pt(L1)(ox)] (3). Yield 72%. ¹H NMR (500 MHz, DMSO-*d*₆) δ: 8.38 (d, 1H, *J* = 5.0 Hz, PyH6), 8.20 (td, 1H, *J*₁ = 7.7 Hz, *J*₂ = 1.2 Hz, PyH4), 7.72 (d, 1H, *J* = 8.0 Hz, PyH3), 7.53 (td, 1H, *J* = 6.7 Hz, *J*₂ = 1.0 Hz, PyH5), 5.07 (t, 2H, *J* = 4.5 Hz, OH), 4.62 (s, 2H, PyCH₂N), 4.18-4.13 (m, 2H, CH₂OH), 3.99-3.94 (m, 2H, CH₂OH), 3.23-3.19 (m, 2H, CH₂N), 2.99-2.94 (m, 2H, CH₂N) (Figure S18); ¹³C NMR (125 MHz, DMSO-*d*₆) δ: 165.3, 165.2 (2 × CO), 162.6 (PyC2), 146.8 (PyC6), 139.6 (PyC4), 124.7 (PyC5), 122.6 (PyC3), 68.2 (PyCH₂N), 64.2 (CH₂N), 58.2 (CH₂OH) (Figure S19); ¹⁹⁵Pt NMR (107.5 MHz) δ: -1778.0; FT-IR (KBr, cm⁻¹): 1707 (s), 1048 (s), 768 (s), 561 (w), 461 (w); UV-vis in DMF [λ_{max} , nm (ϵ , M⁻¹cm⁻¹): 290 (5790); ESI-HRMS (methanol) *m/z* (calc.): 502.055 (502.054) [C₁₂H₁₆N₂O₆PtNa⁺]; Elemental analysis calc (%) for C₁₂H₁₆N₂O₆Pt: C 30.07, H 3.36, N 5.84, found: C 29.98, H 3.35, N 5.81.

[Pt(L1)(cbdca)] (4). Yield 68%. ¹H NMR (500 MHz, DMSO-*d*₆) δ: 8.42 (dd, 1H, *J*₁ = 5.0 Hz, *J*₂ = 0.5 Hz, PyH6), 8.18 (td, 1H, *J*₁ = 7.7 Hz, *J*₂ = 1.5 Hz, PyH4), 7.67 (d, 1H, *J* = 7.5 Hz, PyH3), 7.55 (td, 1H, *J*₁ = 6.7 Hz, *J*₂ = 1.0 Hz, PyH5), 5.04 (t, 2H, *J* = 5.0 Hz, OH) 4.60 (s, 2H, PyCH₂N), 4.17-4.11 (m, 2H, CH₂OH), 3.99-3.94 (m, 2H, CH₂OH), 3.17-3.12 (m, 2H, CH₂N), 2.94-2.89 (m, 2H, CH₂N), 2.68-2.60 (m, 4H, CyclobutaneCH₂), 1.76-1.63 (m, 2H, CyclobutaneCH₂) (Figure S22); ¹³C NMR (125 MHz, DMSO-*d*₆) δ: 176.7 (2 × CO), 163.0 (PyC2), 145.6 (PyC6), 139.3 (PyC4), 124.2 (PyC5), 122.2 (PyC3), 67.8 (PyCH₂N), 64.2 (CH₂N), 58.0 (CH₂OH), 55.2 (Cyclobutane), 30.1 (CyclobutaneCH₂), 14.8 (CyclobutaneCH₂) (Figure S23); ¹⁹⁵Pt NMR (107.5 MHz) δ: -1715.4; FT-IR (KBr, cm⁻¹): 1653 (s), 1039 (s), 777 (s), 552 (w), 470 (w); UV-vis in DMF [λ_{max} , nm (ϵ , M⁻¹cm⁻¹): 290 (5740); ESI-HRMS (methanol) *m/z* (calc.): 556.0985 (556.1018) [C₁₆H₂₂N₂O₆PtNa⁺]; Elemental analysis calc (%) for C₁₆H₂₂N₂O₆Pt: C 36.03, H 4.16, N 5.25, found: C 35.99, H 4.17, N 5.23.

[Pt(L2)(ox)] (Oxamusplatin, 5). Yield 52%. ^1H NMR (500 MHz, DMSO- d_6) δ : 8.38 (d, 1H, $J = 5.5$ Hz, PyH6), 8.23 (td, 1H, $J_1 = 7.7$ Hz, $J_2 = 1.0$ Hz, PyH4), 7.74 (d, 1H, $J = 7.5$ Hz, PyH3), 7.57 (t, 1H, $J = 6.7$ Hz, PyH5), 4.59 (s, 2H, PyCH₂N), 4.31-4.26 (m, 2H, CH₂Cl), 4.24-4.19 (m, 2H, CH₂Cl), 3.54-3.48 (m, 2H, CH₂N), 3.30-3.23 (m, 2H, CH₂N) (Figure S26); ^{13}C NMR (125 MHz, DMSO- d_6) δ : 165.1, 164.9 (2 $\times$ CO), 161.2 (PyC2), 147.1 (PyC6), 140.0 (PyC4), 125.0 (PyC5), 122.9 (PyC3), 66.9 (PyCH₂N), 62.3 (CH₂N), 38.6 (CH₂Cl) (Figure S27); ^{195}Pt NMR (107.5 MHz) δ : - 1789.5; FT-IR (KBr, cm^{-1}): 1716 (s), 1364 (s), 768 (s), 561 (w), 470 (w); UV-vis in DMF [λ_{max} , nm (ϵ , $\text{M}^{-1}\text{cm}^{-1}$): 287 (6170); ESI-HRMS (methanol) m/z (calc.): 537.9871 (537.9876) [$\text{C}_{12}\text{H}_{14}\text{Cl}_2\text{N}_2\text{O}_4\text{PtNa}^+$]; Elemental analysis calc (%) for $\text{C}_{12}\text{H}_{14}\text{Cl}_2\text{N}_2\text{O}_4\text{Pt}$: C 27.92, H 2.73, N 5.43, found: C 27.98, H 2.72, N 5.45.

[Pt(L2)(cbdca)] (6). Yield 50%. ^1H NMR (500 MHz, DMSO- d_6) δ : 8.43 (d, 1H, $J = 5.5$ Hz, PyH6), 8.22 (t, 1H, $J = 7.7$ Hz, PyH4), 7.68 (d, 1H, $J = 8.0$ Hz, PyH3), 7.60 (t, 1H, $J = 6.7$ Hz, PyH5), 4.58 (s, 2H, PyCH₂N), 4.27 (m, 4H, CH₂Cl), 3.47-3.42 (m, 2H, CH₂N), 3.23-3.17 (m, 2H, CH₂N), 2.71-2.66 (m, 2H, CyclobutaneCH₂), 2.63-2.57 (m, 2H, CyclobutaneCH₂), 1.77-1.67 (m, 2H, CyclobutaneCH₂) (Figure S30); ^{13}C NMR (125 MHz, DMSO- d_6) δ : 176.6, 176.4 (2 $\times$ CO), 161.4 (PyC2), 146.0 (PyC6), 139.7 (PyC4), 124.6 (PyC5), 122.6 (PyC3), 66.5 (PyCH₂N), 62.2 (CH₂N), 55.3 (Cyclobutane), 38.3 (CH₂Cl), 30.1 (CyclobutaneCH₂), 14.9 (CyclobutaneCH₂) (Figure S31); ^{195}Pt NMR (107.5 MHz) δ : - 1729.9; FT-IR (KBr, cm^{-1}): 1680 (s), 1346 (s), 768 (s), 561 (w), 470 (w); UV-vis in DMF [λ_{max} , nm (ϵ , $\text{M}^{-1}\text{cm}^{-1}$): 291 (5670); ESI-HRMS (methanol) m/z (calc.): 592.0340 (592.0346) [$\text{C}_{16}\text{H}_{20}\text{Cl}_2\text{N}_2\text{O}_4\text{PtNa}^+$]; Elemental analysis calc (%) for $\text{C}_{16}\text{H}_{20}\text{Cl}_2\text{N}_2\text{O}_4\text{Pt}$: C 33.70, H 3.53, N 4.91, found: C 33.76, H 3.55, N 4.94.

Conclusions

The nitrogen mustard ligand bis(2-chloroethyl)pyridylmethylamine (L2) based Pt(II) complexes with the oxalato and cyclobutanedicarboxylato leaving groups, show that both the carrier ligand and the leaving group is important in controlling the cytotoxicity of Pt(II) complexes. The oxalato leaving group bearing, oxamusplatin (**5**) is more cytotoxic to cancer cells compared to the non-cancerous cells. The change of the leaving group to the cyclobutanedicarboxylato (**6**) makes the complex very stable but reduces its cellular accumulation as well as cytotoxicity. Thus, the oxalato leaving group is optimal in rendering stability, better cellular accumulation and cytotoxicity. The oxamusplatin is an example of a Pt(II) complex capable of targeting both DNA and the cytoskeleton with multiple pathways of action. While we have pointed out a few targets of oxamusplatin, the list is not exhaustive. Encouragingly, oxamusplatin is more selective towards cancer cells, is highly resistant to sequestration by GSH or ATP7B. To the best of our knowledge, the above combinations in one molecule make oxamusplatin unique. The Pt(II) complexes presented, definitely warrants further work in this area to generate stable and selective metal-based small molecule chemotherapeutic agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Selected Pt(II) complexes with enhanced toxicity in cancer or different mechanism of action compared to CDDP.

Figure 2. Stability study of oxamusplatin (**5**) in 1:1 (v/v) DMSO- d_6 and phosphate buffer (20 mM, pH 7.4, 4 mM NaCl), monitored by ^1H NMR.

Figure 3.

(a) ^1H NMR of interaction of oxamusplatin (5) with 3 mol equivalent of 9-EtG in 1:1 (v/v) DMSO- d_6 and phosphate buffer (20 mM, pD 7.4, 4 mM NaCl) showing adduct formation with 9-EtG after 4 days. Where 'S' = intact complex; '@' = 9-EtG bound complex; HS' = shift of the H8 proton of free 9-EtG due to binding with oxamusplatin. (b) ESI-MS spectra of 9-ethylguanine (9-EtG) interaction with oxamusplatin after incubation for 12 h with 5 mol equivalents of 9-EtG in 50% 5 mM phosphate buffer (pH 7.4, 4 mM NaCl) in MeOH

mixture displaying intact complex and formation of 9-EtG adduct. Please refer to Figure S5 for more details and isotopic resolution.

Figure 4.

(a) ^1H NMR data of the interaction of oxam platinum (5) with 3 mol equivalents of GSH in 1:1 (v/v) phosphate buffer (20 mM, pH 7.4 containing 4 mM NaCl): DMSO- d_6 showing oxam platinum does not react with GSH ('*' intact complex 5; 'S' free GSH; '@' GSH dimer due to auto-oxidation). (b) ESI-MS spectra of Glutathione (GSH) interaction with oxam platinum with 3 mol equivalents of GSH in 50% 5 mM phosphate buffer (pH 7.4, 4 mM NaCl) in MeOH mixture after 24 h of incubation showing there is no GSH bound complex observed.

Figure 5.

(a) Cellular accumulation study of equimolar concentration of complexes **3-6** in MCF-7 cells, (b) lipophilicity study of complexes **3-6** in octanol-water mixture.

Figure 6.

Immunofluorescence studies in Hep G2 cells showing the mobilization of ATP7B from the *trans*-Golgi network (TGN) upon administration of (a) bathocuproinedisulfonate (BCS) (50 μ M), (b) CuCl₂ (50 μ M), (c) oxaliplatin (20 μ M) and (d) oxamusplatin (20 μ M) after treatment for 2 h. Scale bar represents 20 μ m. A larger area representation was shown in Figure S13 for readers.

Figure 7.

Quantification of ATP7B sequestration in Hep G2 cells. PCC stands for Pearson correlation coefficient.

Figure 8.

Immunofluorescence studies showing organization of microtubule network in MCF-7 cells after an incubation period of 24 h in presence of (a) 0.1% DMSO, (b) Colchicine (100 nM) and (c) oxamuplatin (25 μ M). Microtubules were visualized with a monoclonal anti- α -tubulin antibody (green) interacting with a secondary antibody tagged with AlexFluor 488 ($\lambda_{em} = 520$ nm). The nucleus was stained with DAPI (blue). Images were acquired with a Leica SP8 confocal microscope with a 63X objective.

Figure 9.

Investigation of apoptosis by flow cytometry analysis of MCF-7 cells after exposure to (a) 0.1% DMSO (Control), (b) IC₂₅ and (c) IC₅₀ of oxamusplatin for 24 h after Annexin V-PE/7-AAD dual staining. Lower left (LL) quadrant: intact cells, lower right (LR) quadrant: early apoptotic cells, upper right (UR) quadrant: late apoptotic cells, upper left (UL) quadrant: dead cells, (d) Dose dependent increase in depolarization of mitochondria in MCF-7 cells after treatment with oxamusplatin for 24 h, (e) colorimetric determination showing caspase-7 activation in MCF-7 cell line with oxamusplatin in two different concentrations for 24 h.

Scheme 1.

A general Scheme of cellular entry and efflux of Pt-based drugs, using cisplatin as an example, showing the role of copper transporter (CTR1) and ATP7A/ATP7B along with other cellular thiol donor based small molecules and other proteins.

Scheme 2.

Synthetic procedure for the preparation of ligand and complexes **3-6**. (a) cis-[Pt(DMSO)₂Cl₂], DCM, 12 h, RT; (b) SOCl₂, 48 h, RT; (c) cis-[Pt(DMSO)₂Cl₂], DCM, Et₃N, 48 h, RT; (d) AgNO₃, disodium oxalate, H₂O, RT; (e) AgNO₃, NaOH, disodium cyclobutane-1,1-dicarboxylate, H₂O, RT.

Scheme 3.

Proposed reaction pathways of oxamusplatin (**5**) for hydrolysis and binding with 9-EtG and GSH based on ^1H NMR and ESI-MS studies.

Table 1

IC₅₀ values (μM)^a of oxamusplatin and **6** in a selected panel of cell lines in comparison with oxaliplatin

Complexes	MCF-7	DU-145	MIA PaCa 2	Hep G2	PANC-1	HFF-1	MDCK	CHO
Oxamusplatin (5)	8.1 ± 0.8	21.0 ± 1.0	24.7 ± 0.1	32.0 ± 2.0	36.0 ± 2.0	> 150	>200	88.0 ± 3.0
6	>150	>100	>150	ND ^b	ND ^b	>150	ND ^b	ND ^b
Oxaliplatin	0.6 ± 0.1	4.5 ± 0.3	5.7 ± 0.2	9.8 ± 0.3	3.1 ± 0.2	7.0 ± 0.6	ND ^b	ND ^b

^a data are shown as mean of at least three independent experiments ± standard deviation (S.D).

^b ND stands for not determined.

Table 2

In vitro cell cycle inhibition of oxamusplatin in MCF-7, suggesting G2/M and S phase arrest. Data represent the percentage of cell populations present in each phase.

	G0/G1	G2/M	S	SubG1
Control	58	16	21	5.0
Oxamusplatin, IC ₂₅	33	36	22	9.0
Oxamusplatin, IC ₅₀	29	43	26	2.0