# Uptake-release by MSCs of a cationic platinum(II) complex active *in vitro* on human malignant cancer cell lines

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## Abstract

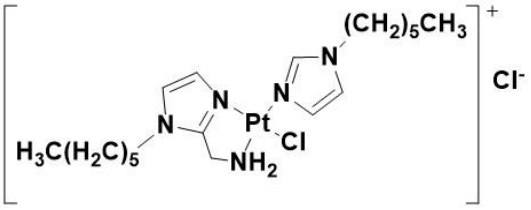
In this study, the *in vitro* stability of cisplatin (CisPt) and cationic platinum(II)-complex (caPt(II)-complex) and their *in vitro* activity (antiproliferative and anti-angiogenic properties) were investigated against three aggressive human tumor cell lines. caPt(II)-complex shown a high stability until 9 days of treatment and displayed a significant and higher activity than CisPt against both NCI-H28 mesothelioma (19.37 ± 9.57 μM *versus* 34.66 ± 7.65 μM for CisPt) and U87 MG glioblastoma (19.85 ± 0.97 μM *versus* 54.14 ± 3.19 for CisPt). Mesenchymal Stromal Cells (AT-MSCs) showed a significant different sensitivity ($IC_{50}$= 71.9 ± 15.1 μM for caPt(II)-complex and 8.7 ± 4.5 μM for CisPt) to the antiproliferative activity of caPt(II)-complex and CisPt. The ability of MSCs to uptake both the drugs in a similar amount of 2.49 pM /cell, suggested a possible development of new therapies based on cell mediated drug delivery.

*Keywords*: Cisplatin, Cationic platinum (II)-complex, mesothelioma, glioblastoma, mesenchymal stromal cells, drug delivery.

## 1. Introduction

In the last decades the field of cancer therapeutic treatment has been marked by important advancements thanks to the introduction of new surgical procedures and new chemotherapeutic drugs. Unfortunately, the 4th most common cause of cancer-related death is due to three different tumors still lacking for a proper response: malignant pleural mesothelioma (MPM),[1] glioblastoma multiforme (GBM)[2] and pancreatic adenocarcinoma (PAC)[3]. These types of solid tumor are in fact difficult to defeat because they spread aggressively and resulted highly resistant to conventional chemotherapeutic agents other than the fact that they exhibited a high propensity to recur.
MPM is a malignant neoplasm of the pleura related to asbestos exposure. It tends to grow over the serosal surface and finally encases the lung, causing death by asphyxiation. Currently the standard first-line treatment is a platinum-based doublet containing a third-generation antifolate like pemetrexed (PMX) but any second-line treatments for MPM has been approved yet.[4-6]
GBM is a highly malignant and aggressive primary brain tumor. Despite of an arsenal of therapeutic interventions, the prognosis of glioblastoma remains very poor. Cisplatin based therapy is one of the most important chemotherapy treatments for GBM and it is repurposed as the second line against GBM albeit its efficacy is limited by drug resistance and undesirable side effects including neurotoxicity that limits its efficacy.[7, 8]

PAC has recently emerged as one of the most aggressive tumors with a death rate that has remained relatively stable in the last ten years, thus providing a limited progress in this field. In the treatment of pancreatic cancer, platinum derivatives are frequently used in combination schedules with gemcitabine (GCB) in several phase II trials.[9, 10]
These tumors remain some of the most lethal thus evoking the need for new therapeutic treatments in order to improve patient survival and quality of life. To date, the use of platinum drugs for the treatment of these pathologies has shown minimal success mainly due to a failure in the delivery to the tumor site so that high doses of the platinum chemotherapeutic agent is required for a positive response to the therapy. Over the last thirty years, the research field of platinum anticancer drugs has experienced an extensive development with the recognition of the potential anticancer activity of platinum compounds whose novel structural properties developed for active platinum drugs allowed to enlarge the spectrum of activity, to overcome cellular resistance and to lower toxicity. In this regard, violating the apparently demanded neutrality, many cationic monofunctional platinum-based anticancer agents were synthetized and characterized.[11] This focusing is because, in comparison with the bifunctional cisplatin, monofunctional compounds display a distinct mechanism of action and a different antitumor profile taking into consideration their ability to effectively bind to DNA and to inhibit transcription both *in vitro* and *in vivo*. They are often characterized by the general formula cis-$[Pt(NH_3)_2(L)Cl]^+$, where L is an *N*-heterocycle and the choice for L is usually for a small *N*-heterocyclic amine ligand with a molecular weight less than 200, such as pyridine (for pyriplatin) or phenanthridine (for phenanthriplatin). Indeed, the choice of the *N*-heterocycle ligand has been suggested as the main responsible for determining the anticancer activity spectrum and the relative mechanism of monofunctional platinum complexes.[12] Upon the above premises, many cationic platinum complexes have been identified with different biological behavior as compared to cisplatin but endowed with equivalent or higher potential to kill cancerous cells.[13]
Recently, our research group has synthesized a series of cationic bulky triamine platinum compounds of general formula [Pt(N-N')N'Cl]$X^-$ where *N-N'* is an aminomethylimidazole ligand[14] and the *N'* an imidazole ring, both bearing the same alkyl group at the *N1* position. Notably, when the alkyl group is a long linear carbon chain (C6) as in complex **2c**, the cationic platinum (II) compound showed a very effective and potent cytotoxic effect in triple-negative breast cancer cells and in cell lines partially resistant to cisplatin.[15] Its cytotoxic capability closed to its completely different pharmacodynamic and cellular uptake behavior than cisplatin, makes it as a valid candidate for evaluating cytotoxicity in these three tumor cell lines.




**Scheme 1.** Cationic platinum(II)-complex (caPt(II)-complex)

As previously reported mesenchymal stromal cells (MSCs) from different tissues are able to uptake drugs (eg.: Paclitaxel, Gemcitabine, Doxorubicin et al.)[16-20] and release them both as free molecule and exosome associated drugs.[21] The drug is released *in vitro* in amounts effective against cancer cells and also *in vivo* the drug loaded MSCs may be used as a physiological tool for drug delivery by injecting them both *in situ* and by systemic way.[22] These characteristics of MSCs suggest new strategies to apply in advanced cell therapy, in particular for treating cancer. Their potential application is in part depending on the solubility of the drug in medium and their stability during the drug loading procedure. The same platinum-based drugs have very limited solubility in culture medium and also can suffer degradation process by pH modification and temperature.[23] The present study evaluated these biological characteristics of platinum based drugs by comparing a new cationic platinum(II) complex (caPt (II)-complex) to cisplatin (CisPt). Our results evidenced that caPt (II)-complex has a very high stability when incubated at 37 °C in cell culture medium and that its *in vitro* activity against glioblastoma and mesothelioma was significantly higher than that exerted by CisPt. We also demonstrated that caPt (II)-complex can be easy incorporate and release by MSCs without loss of its anticancer activity.

## 2. Materials & Methods

### 2.1. Drugs and tumor cell lines

Rates of Cisplatin (CisPt) and caPt (II)-complex were prepared at the concentration of 4 mg/ml in dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA). Working solutions were freshly prepared according to the experimental design by serial dilutions in complete culture medium. The chemotherapeutic drug Paclitaxel (PTX, kindly provided by Freseneius-Kabi, Italy; stock solution for infusion 6 mg/ml), was used as standard drug for antiproliferation assays. The *in vitro* activity of CisPt, caPt (II)-complex and PTX was tested against three tumor cell lines: malignant pleural mesothelioma (MPM) cell line NCI-H28[24], glioblastoma cell line U87 MG[25] and pancreatic adenocarcinoma cell line CFPAC-1,[26] used as laboratory standard cancer cell line. Cells lines were maintained by 1:5 weekly dilution in Roswell Park Memorial Institute medium (RPMI) medium (NCIH28), Dulbecco's Modified Eagle (DMEM LG) medium (U87 MG) and Iscove's Modified Dulbecco's Medium (IMDM) medium (CFPAC-1), supplemented by Foetal bovine serum (FBS) 10%. All reagents for culture were provided by Euroclone, UK.

### 2.2. Adipose Tissue Mesenchymal stromal cells (AT-MSCs)

As starting biological material were used lipoaspirate samples of adult donors after signed informed consent of no objection for the use for research of surgical tissues (otherwise destined for destruction) in accordance with the Declaration of Helsinki. The approval for their use was obtained from the Institutional Ethical Committee of Milan University (n.59/15, C.E. UNIMI, 09.1115). The Adipose Tissue Mesenchymal stromal cells (AT-MSCs) were isolated from 20 ml of lipoaspirate processed as previously described by using Lipogems device (Lipogems® International Spa)[27] and 2 ml for a sample of lipogems (LG) were processed for MSC isolation as previously described.[28] Briefly, the sample was disaggregated by enzymatic digestion with 200 U/ml of collagenase tipe I (Life technologies, USA), then was centrifuged (1000xg, 15 min) and the floating fraction was separated from the cellular pellet. Cellular pellet was plated on 25cm² flask (Euroclone, UK) and expanded in StemMACS medium (Miltenyi Biotec, Germany) until passage 3. Primary cultures were analyzed for their proliferation rate (Population doubling time), clonogenicity (CFU-F assay), expression of the typical mesenchymal stem cell markers and multi-differentiative ability towards mesodermal lineages (osteogenic, adipogenic and chondrogenic differentiation).

### 2.3. Drug sensitivity of tumor cells and AT-MSCs to Cisplatin (CisPt), caPt (II)-complex and PTX

The effect of CisPt and caPt (II)-complex against cell proliferation has been studied in 96 multiwell plates (Sarstedt, Germany). Briefly, 1:2 serial dilutions of pure drug (from 0.15 to 20 µg/ml) were prepared in 100 µl of culture medium/well and then to each well were added 2,000 tumor cells. As standard internal positive control, the sensitivity of tumor cells to paclitaxel (PTX) were determined by adding the drugs at increasing two folds concentrations from 0.78 to 50 ng/ml. After 7 days of culture (anti-proliferative assay) at 37°C and 5% $CO_2$, cell growth was evaluated by MTT assay (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium) as previously described.[29, 30] Against AT-MSCs, also cytotoxicity assay (24 hours at 37°C, 5% $CO_2$) was performed at increasing concentrations of 0.8, 4, 20, 25, 50, 75, 100 µg/ml. The inhibitory concentration ($IC_{50}$) was determined according to the Reed & Muench formula.[31] AT-MSCs were treated for 24 hours with 75 µM CisPt or CaPt(II) complex respectively and the cell cycle was analyzed according to the procedure previously reported.[19]

### 2.4. Drug loading of AT-MSCs with CisPt and caPt (II)-complex

The drug loading of AT-MSCs was performed by priming the cells according to a standardized procedure that use high drug dosage as previously described.[29] Briefly, subconfluent cultures ($2 \times 10^4$ cells/cm²) were exposed to 75 µM CisPt or CaPt (II)-complex for 24 hours. Then, the cells were washed twice with PBS, detached with trypsin and washed twice in Hank's solution (HBSS, Euroclone UK). $5x10^5$ drug-primed cells (AT-MSCs/CisPt, AT-MSCs/ caPt (II)-complex) were then seeded in a 25 cm² flask in 3 ml of IMDM with 10% FBS and 2 mM L-glutamine (Euroclone, UK) to release the drug. After 24 hours, conditioned media (CM), (CM 24h/CisPt, CM 24h/ caPt (II)-complex) were collected and tested *in vitro* for their anti-proliferative activity on CFPAC-1 cells (used as standard assay). Conditioned Media from unprimed MSCs were used as control. To determine the amount of drug internalized, $5x10^5$ cells collected after the uptaking phase were lysed by three sonication cycles of 0.4 second pulse cycle and 30% amplitude each (Labsonic U Braun). Lysates from CisPt and caPt(II) primed cells (LYS/CisPt, LYS/caPt (II)-complex) and unprimed AT-MSCs were tested for their anti-proliferative activity.

### 2.5. *In vitro* anti-proliferative assay of CM and LYSATE of AT-MSCs loaded with CisPt or caPt (II)-complex

The inhibitory effect of CM and cell lysates from drug loaded AT-MSCs were evaluated on CFPAC-1 proliferation by the MTT assay[29, 32] and the inhibitory concentration $IC_{50}$ was determined according to the Reed and Muench formula.[31] The anti-tumoral activity of CM 24h/CisPt, CM 24h/ caPt (II)-complex, LYS/CisPt, LYS/caPt (II)-complex were compared to that of the pure drugs (CisPt, caPt (II)-complex) alone and expressed as drug equivalent concentration (DEC), according to the following algorithm: DEC (µg/ml) = $IC_{50}$ Drug(µg/ml) × 100/ $IC_{50}$ CM or LYS (µl/well). The

$IC_{50}$ Drug is the concentration (μg/ml) of pure drug able to inhibit CFPAC-1 proliferation by 50%; $IC_{50}$CM or LYS is the volume (μl/well) of CM or LYS inhibiting CFPAC-1 proliferation by 50%. To evaluate the drug released by a single primed cell (CDR = cell drug release), an arbitrary internal parameter based on the ratio between the DEC and the number of seeded cells [CDR (pg/cell) = DEC (ng/ml) × $10^3$ × CM or LYS volume (ml)/ number of seeded cells] was used.

### 2.6. Determination of Pt concentration in cell lysate (LYS) and in Conditioned Medium (CM) from drug loaded AT-MSCs

Both the lysates (LYS/cisPt, LYS/caPt (II)-complex) and the conditioned media (CM 24 h/Cis-Pt, CM 24h/ caPt (II)-complex) were dissolved in 1% $HNO_3$. Platinum was quantified by inductively coupled plasma mass spectrometry (ICP-MS) (BRUKER aurora M90 ICP-MS, MA, USA) according the previously described method.[33, 34] Cellular metal levels were expressed as the mean of three independent determinations as μg Pt per L of lysate or medium. The amount of Pt was transformed into caPt(II)-complex or CisPt, through these formula: caPt(II)-complex or CisPt = (Pt g/L) / MW Pt (g/moli) * MW caPt(II)-complex or CisPt. The concentration found (ng/ml) of caPt(II)-complex or CisPt was then evaluated as picoMolar per single AT-MSCs.

### 2.7. Anti-angiogenic potential of CaPt(II)-Complex and CisPt

To assess the anti-angiogenic potential of CaPt(II)-complex and CisPt, we investigated their inhibitory activity on Human Umbilical Vein Endothelial Cells (HUVECs) (LONZA Walkersville, MD. USA). HUVECs were routinely maintained in EGM bullet kit (LONZA Walkersville, MD. USA) plus 10% FBS. HUVECs proliferation assay was performed as previously described.[35] Briefly, HUVECs were harvested from culture flasks by trypsin. After enzyme inactivation and centrifugation, cells were re-suspended in EGM medium (LONZA Walkersville, MD. USA) +0.2% BSA and counted. To evaluate the growth response to CaPt(II)-complex and CisPt, 0.5 ml of HUVECs ($10^4$ cells) were seeded into a 24-multiwell plate (Corning, NY, USA) coated with collagen type I. After adhesion, medium was aspirated and replaced with EGM control medium +10% FBS (CTRL) supplemented or not with different concentrations of CaPt(II)-complex and CisPt (from 1 to 40μM). Experiments were repeated using positive control growth medium consisted in EGM Bullet kit complete medium which produce a potent increment of HUVECs proliferation. After 72h, the cells were washed, fixed and stained. Cells were counted with a calibrated ocular eyepiece in 10 different fields (400x magnification). Every test was run in triplicate. To test the effect of CaPt(II)-complex and CisPt on HUVECs tube formation, we used growth factor reduced-matrigel assay (Sigma) as described by Kleinman et al.[36] Briefly, around 50μl of matrigel was placed into cold (4 °C) 96-multiwell plate (Corning, NY, USA) at 37 °C for 30 min until jellification. HUVECs were then seeded on matrigel at a concentration of $10^4$ cells/well in 50μl of EGM growth medium (1:1) in presence or absence of different μM concentration of CaPt(II)-complex and CisPt. After 24 h, the number of HUVEC tube formations were counted with an inverted microscope (10x magnification) and reported as n° of tube structures/field. HUVECs proliferation and tube formation experiments were repeated to evaluate the stability of CaPt(II)-complex and CisPt. The drugs once diluted in the CTRL medium or in EGM Bullet kit complete medium were kept for 24-72 h at 37 °C before applying to the HUVECs.

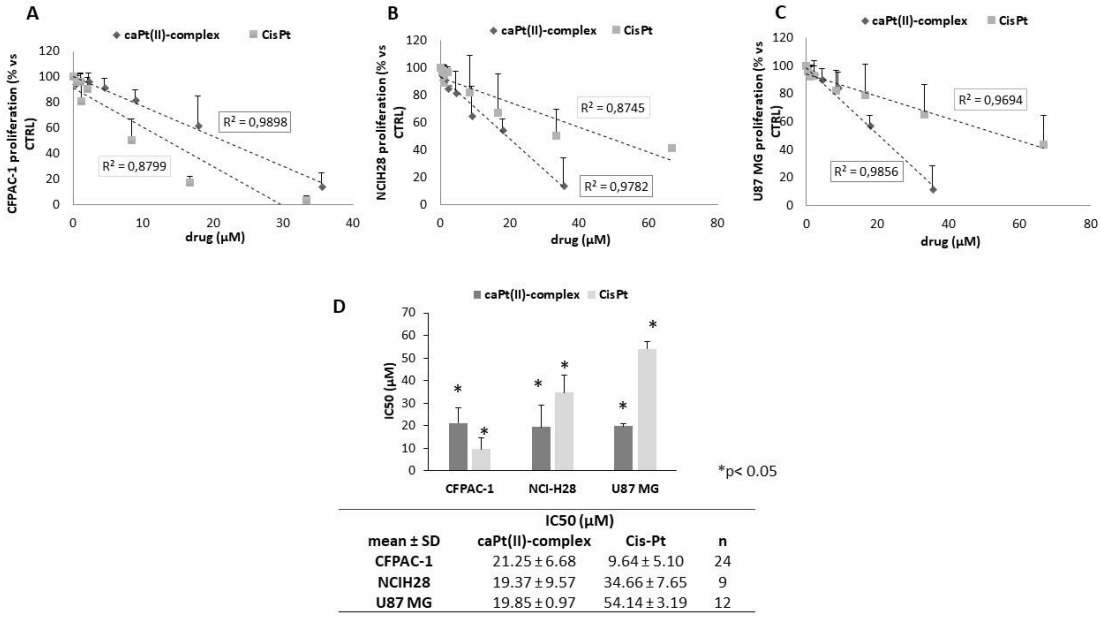
### 2.8. Statistical analysis

Data are expressed as average ± standard deviation (SD). Differences between mean values were evaluated according to Student's t-test performed by GRAPHPADINSTAT program (GraphPad Software Inc., San Diego, CA, USA). p values ≤ 0.05 were considered statistically significant. The linearity of response and the correlation were studied using regression analysis, by Excel 2013 software (Microsoft, Inc.)

## 3. Results and Discussion

### 3.1. *In vitro* anticancer activity of CisPt and caPt (II)-complex

The activity of CisPt and caPt(II)-complex, evaluated *in vitro* against three human cancer cell lines (pancreatic carcinoma CFPAC-1, glioblastoma U87 and mesothelioma NCI-H28), showed a dose-response kinetics of inhibition with a significant coefficient of correlation ($R^2$) (**Figure 1**). However, against CFPAC-1 proliferation, CisPt was significantly ($p<0.01$) more active ($IC_{50}$ = 9.64 ± 5.10 μM) than caPt(II)-complex ($IC_{50}$= 21.25 ± 6.68 μM). A significant higher activity ($p>0.05$) of caPt(II)-complex was seen both against NCI-H28 cells ($IC_{50}$ = 19.37 ± 9.57 μM *versus* 34.66 ± 7.65 μM for CisPt) and against U87 MG ($IC_{50}$ = 19.85 ± 0.97 μM *versus* 54.14 ± 3.19 for CisPt).




| IC50 (μM) | | | |
|---|---|---|---|
| mean ± SD | caPt(II)-complex | Cis-Pt | n |
| CFPAC-1 | 21.25 ± 6.68 | 9.64 ± 5.10 | 24 |
| NCIH28 | 19.37 ± 9.57 | 34.66 ± 7.65 | 9 |
| U87 MG | 19.85 ± 0.97 | 54.14 ± 3.19 | 12 |

**Figure 1: *In vitro* anticancer activity of CisPt and caPt (II)-complex:** The effect of increasing concentrations of caPt(II)-complex or CisPt was evaluated by a 7-day antiproliferation MTT assay on different tumor cell lines. A: pancreatic adenocarcinoma CFPAC-1; B: mesothelioma NCIH28; C: glioblastoma U87MG. The effect was expressed as a percentage of the optical density measured in cultures that did not receive drugs (100% proliferation). Linear regression with $R^2$ value was reported. Histogram and box D report the $IC_{50}$ values are expressed as mean ± standard deviation (SD) of "n" independent experiments. The means were compared by Student's t-test (*p < 0.05).

### 3.2. Anti-angiogenic potential of caPt(II)-complex and CisPt

It has been shown that CisPt has some anti-angiogenic potential.[37] In order to verify whether also caPt (II)-complex could have anti-angiogenic activities *in vitro*, we investigated their effect on HUVECs proliferation and tube-structures formation on matrigel. To this end, CisPt and caPt (II)-complex were diluted in CTRL medium or in EGM-bullet kit medium and then tested on HUVECs to evaluate anti-proliferative activity at 72 h incubation. Under our experimental conditions, as shown in **Figure 2**, the addition of 10 to 30 μM of CisPt diluted in CTRL medium reduced significantly HUVEC proliferation (**Figure 2A**), caPt(II)-complex behaved similarly but was less effective at 10 μM concentration. Inhibition of HUVECs proliferation by CisPt was even more evident when experiments were repeated by culturing HUVECs in EGM-bullet kit medium which strongly enhance endothelial growth. In this case the addition of caPt(II)-complex was effective as well as CisPt. The activity of CisPt and caPt(II)-complex to inhibit capillary-like tube formations on matrigel was practically similar at each tested concentration (**Figure 2B**). When proliferation and tube-structures formation assay were repeated upon pre-incubating at 37 °C for 72 h both CisPt and caPt(II)-complex diluted in EGM bullet kit medium, we found that caPt(II)-complex maintained completely its inhibitory activity, in contrast to CisPt that lost its efficacy to inhibit both HUVECs proliferation (**Figure 2C**) and cords formations (**Figure 2D**). These data suggested that caPt(II)-complex and CisPt have a similar anti-angiogenic activity, however caPt(II)-complex is a very stable molecule in contrast to CisPt.

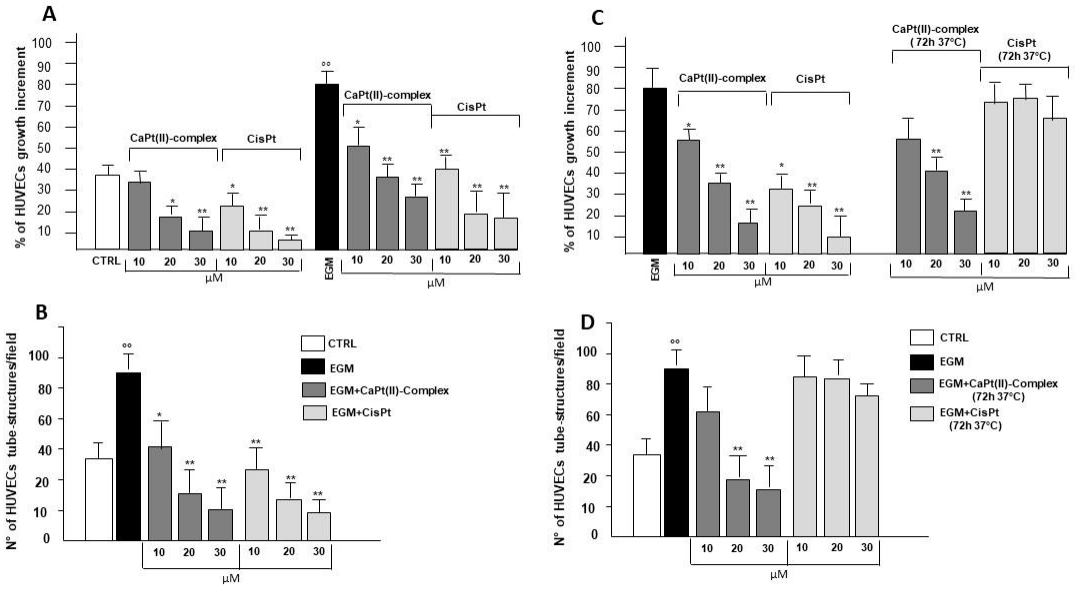



**Figure 2. Anti-Angiogenic properties of CisPt and caPt(II)-complex.** The anti-angiogenic properties of CisPt and caPt(II)-complex were investigated by adding μM concentrations of both compounds diluted either in CTRL medium or in EGM- bullet kit medium. In (A) the inhibitory effect of CisPt and caPt(II)-complex on HUVECs proliferation. In (B) the quantification of cord formations on matrigel by HUVECs under the influence of CisPt and caPt(II)-complex at 24 h. To note that caPt(II)-complex and CisPt diluted in EGM-bullet kit medium a medium that stimulate both HUVECs proliferation and cords formations, demonstrated a similar anti-angiogenic efficacy. In (C) and (D) the effect on HUVECs proliferation and cords formation respectively, after pre-incubating at 37 °C for 72 h of CisPt and caPt(II)-complex diluted in EGM bullet kit medium. To note, that CisPt lost completely its anti-angiogenic activity, whereas it was maintained by caPt(II)-complex compound (* $p<0.05$; ** $p<0.01$ *vs* CTRL medium or EGM-bullet kit medium; °° $p<0.01$ *vs* CTRL medium).

### 3.3. Mesenchymal stromal cells (MSCs) sensitivity to CisPt and caPt(II)-complex

The two compounds were tested both for their cytotoxic and antiproliferative activity on AT MSCs (**Figure 3A-B**). As evidenced by the linear regression analysis, by considering the cytotoxicity at 24 h, MSCs showed to have a similar sensitivity both to caPt(II)-complex and CisPt (91.1 ± 29.4 μM *versus* 82.2 ± 54.2 μM). On the contrary, in the antiproliferation test the $IC_{50}$ for the two drugs were significantly ($p< 0.05$) different being 69.9 ± 5.5 μM for caPt(II)-complex and 11.4 ± 5.2 μM for CisPt. **Figure 3C** reported the cell cycle of AT-MSCs treated for 24 h with the two molecules at 75 μM showing any significant modification of the cell cycle.

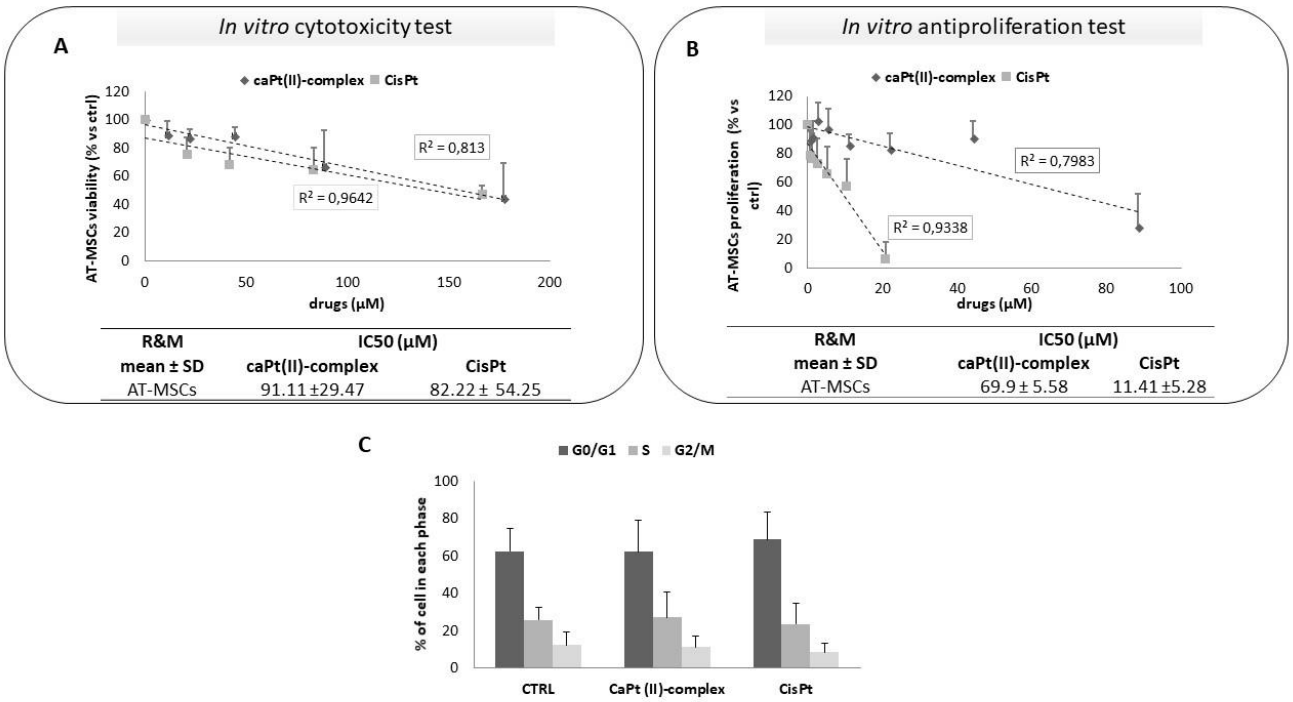
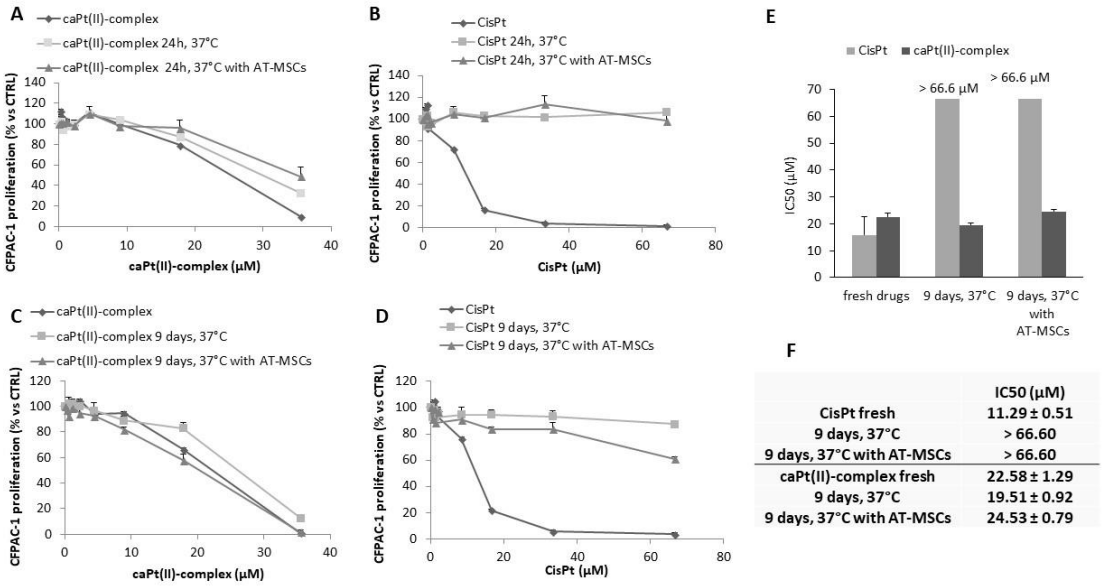



**Figure 3. Mesenchymal stromal cells (AT-MSCs) sensitivity to CisPt and caPt(II)-complex and cell cycle analysis: A:** The cytotoxic activity was evaluated after 24 h of treatment with different concentrations of caPt(II)-complex or CisPt (from 0 to 200 μM) and expressed as cell viability (% of control cells). Linear regression with $R^2$ value was reported. The box reports the $IC_{50}$ values **B**: The effect of increasing concentrations (from 0 to 85 μM) of caPt(II)-complex or CisPt was also evaluated by a 7-day anti-proliferation assay. The effect is expressed as percentage of the optical density measured in cultures that did not receive drugs (100% proliferation). Linear regression with $R^2$ value was reported. $IC_{50}$ values are expressed as mean ± standard deviation (SD) of four independent experiments. **C**: The histogram shows the effects of caPt(II)-complex or CisPt treatments (75 μM) on AT-MSCs after 24 h of treatment. The percentages of cells counted in each different cell phase (G0/G1, S and G2/M) are reported and compared to those found in untreated cells (CTRL). The values are expressed as mean ± standard deviation (SD) of eight independent experiments.

### 3.4. Stability of CisPt and caPt(II)-complex to treatment at 37 °C

Both CisPt and caPt(II)-complex were studied for their stability following the incubation at 37 °C in the complete cell culture medium both in the absence and the presence of a monolayer of MSCs. The stability has been evaluated by determining the dose response inhibiting kinetics against CFPAC-1 proliferation (**Figure 4A-D**). After 24 h of incubation CisPt completely lost its anticancer activity under both two incubation conditions. caPt(II)-complex maintained its ability to inhibit cancer cell proliferation with a dose response kinetics like that of the fresh drug both if incubated in the absence and presence of MSCs monolayer. By comparing $IC_{50}$ of the caPt(II)-complex no significant difference ($p<0.05$) was observed (**Figure 4E-F**).




| | IC50 (µM) |
|---|---|
| CisPt fresh | 11.29 ± 0.51 |
| 9 days, 37°C | > 66.60 |
| 9 days, 37°C with AT-MSCs | > 66.60 |
| caPt(II)-complex fresh | 22.58 ± 1.29 |
| 9 days, 37°C | 19.51 ± 0.92 |
| 9 days, 37°C with AT-MSCs | 24.53 ± 0.79 |

**Figure 4: Stability of Cis-Pt and caPt (II)-complex at 37°C of incubation.** The effect of increasing concentrations of caPt(II)-complex (**A** and **C**) or CisPt (**B** and **D**) was evaluated by a 7-day antiproliferation MTT assay on CFPAC-1. Fresh prepared drugs or drugs incubated for 24 h or 9 days at 37 °C were tested. Incubation was performed in medium and in the presence of AT-MSCs. The histogram **E** and box **F** report the $IC_{50}$ values expressed as mean ± standard deviation (SD) of four independent experiments.

## 3.5. Uptake/release of CisPt and caPt (II)-complex by AT-MSCs

**Biological dosage**: the anti-cancer activity of cell lysate (LYS/CisPt, LYS/caPt (II)-complex) and the corresponding conditioned medium (CM 24h/CisPt, CM 24h/caPt (II)-complex) obtained from drug-primed MSCs were tested against our standard laboratory cancer cell line CFPAC-1 (**Figure 5A-B**). Both CM 24h/CisPt and CM 24h/caPt (II)-complex did not express activity on the cancer proliferation until the dosage of 100 µl/well tested.

**Analytical dosage**: to evaluate the amount of the two drugs uptaken and released by MSCs an ICP spectrometry analysis was performed both on cell lysates and CM. MSCs were able to incorporate both the drugs in amounts that do not differ statistically (2.3 ± 0.11 pM/cell for caPt(II)-complex *versus* 1.63 ± 0.7 pM/cell for CisPt). The analysis of conditioned medium showed a release of 0.72 ± 0.14 pM/cell for caPt(II)-complex and 1.55 ± 0.97 for CisPt. By considering the drug released at 24 h as percentage of the amount incorporated, it is evident that the drug loaded MSCs released the 35.7% of the caPt(II)-complex and the 95 % of the CisPt. By mimicking a possible *in vivo* treatment "*in situ*" of a tumor mass (of about 1 $cm^3$) with drug loaded MSCs (**Figure 5D**) we can estimate that for delivering caPt(II)-complex to a concentration near to 3 x $IC_{50}$ value, we need about 24 x $10^6$ drug loaded MSCs.

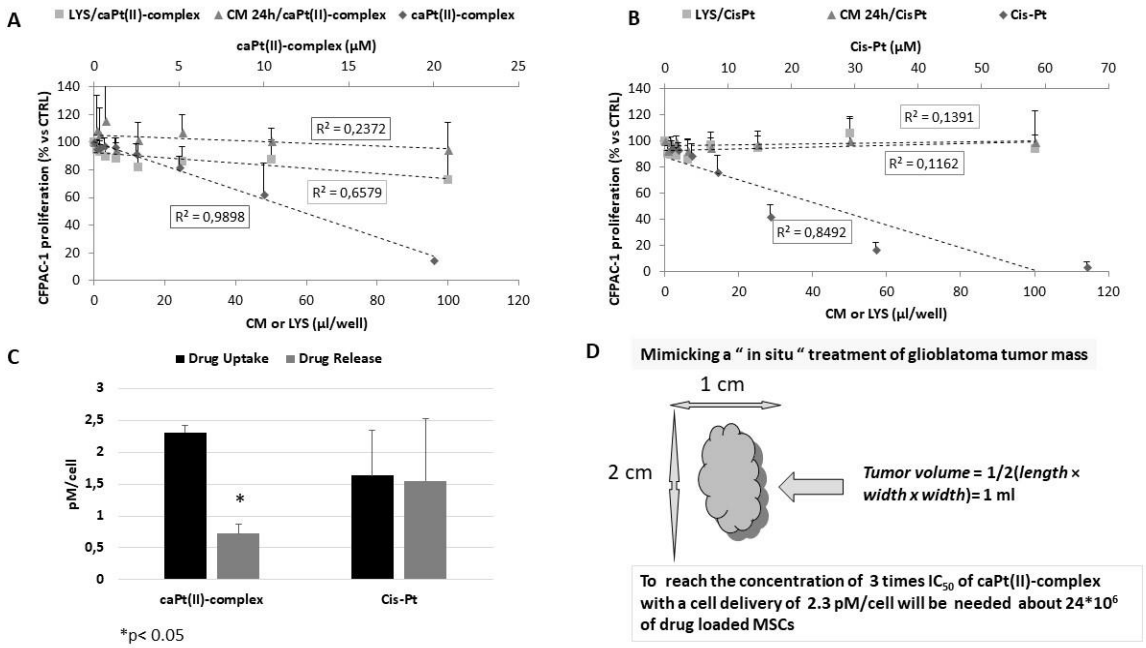



**Figure 5: Uptake/release of CisPt and caPt(II)-complex by AT-MSCs:** The activity of cell lysates (LYS) and 24 h conditioned media (CM) from AT-MSCs loaded with caPt(II)-complex (**A**) (LYS/caPt(II)-complex; CM 24 h/caPt(II)-complex) or with CisPt (**B**) (LYS/CisPt; CM 24 h/ Cispt) was evaluated against CFPAC-1 proliferation in comparison to the pure drugs. The concentration-dependent inhibition was normalized on the inhibition produced by lysate from unloaded AT-MSCs. Linear regression with $R^2$ value was reported. The histogram **C** reports drug uptake and release values expressed at pM/cell through inductively coupled plasma mass spectrometry (ICP-MS). The values are expressed as mean ± standard deviation (SD) of four independent experiments. The means were compared by Student's t-test (*p < 0.05). Figure **D** mimics an "in situ" treatment of glioblastoma tumor mass with drug loaded MSCs.

Our results evidenced that caPt(II)-complex exerts a remarkable anticancer activity *in vitro* against the three cancer cells lines studied (**Figure 1**). While the activity against PAC cells exerted by caPt(II)-complex is lower than that exerted by CisPt a significant ($p<0.05$) higher activity of this new molecule was evidenced both against MPM and GBM. It is known that, beside the anticancer activity, for an optimal chemotherapeutic action is very important the solubility and the stability influencing the management of the molecules and increasing or reducing their action. Unfortunately, platinum-based drugs have very limited solubility in culture medium and also can suffer degradation process by pH modification and temperature.[23] Concerning their stability, our findings evidenced that caPt(II)-complex is a very stable compound if compared to CisPt. The treatment of the two drugs at 37 °C demonstrated that whereas CisPt lost its anticancer activity after only 24 h, caPt(II)-complex maintained its unaltered activity after a long time of incubation (**Figure 4**). In addition, although GBM angiogenic activity is not the primary target CisPt chemotherapy, we here confirm that CisPt can inhibit angiogenesis.[37] However, while CisPt lost rapidly its efficacy after pre-incubation at 37 °C, caPt(II)-complex maintained its capability to inhibit both HUVECs proliferation and cords formation even after 72 h incubation **(Figure 2)**. The cancer activity is maintained also if caPt(II)-complex is incubated in the presence of a monolayer of MSCs confirming that the molecule did not suffer modification due to cell metabolism and significant variation of pH of the culture medium produced by the cell growth (**Figure 4E**). Our results on CisPt agreed with literature data showing that this molecule is rapidly and dramatically degraded by pH changes and temperature.[22] The finding concerning the high stability of caPt(II)-complex is very important in order to apply the procedure of drug loading previously set up in our laboratory[18] that is based on the MSCs treatment for 24 h with high concentration of drug. To better set up the drug loading procedure, we also tested the sensitivity of MSCs to both caPt(II)-complex and CisPt because the cell viability and function during the procedure is important for an optimal drug incorporation. The results (**Figure 3**) demonstrated the same sensitivity of AT-MSCs to the cytocidal effect of the two molecules that confirmed our previous data on smooth muscle cells.[14] However, AT-MSCs were significantly more resistant (or less sensitive) to the antiproliferative action of caPt(II)-complex than to that exerted by CisPt. The reason of this difference is not clear. In fact, the cell cycle remained unaffected after the cell treatment with 75 μM concentration of the drugs that, on the contrary, produced a significant different inhibition of cell proliferation (**Figure 3C**).

Of course, this aspect needs to be better investigated because it seems to be related to a different biological response of MSCs to the two drugs concerning the connection between the mechanisms of proliferation and cell death pathways in mesenchymal stromal cells. By applying the standard procedure of MSCs drug loading, the efficiency of the MSCs incorporation of the two drugs was evaluated by testing cell lysates. The amount of drug released was measured in the conditioned medium of drug loaded MSCs sub-cultured for 24 h. The analytical data on lysates confirmed a similar efficient incorporation of both the molecules by AT-MSCs (**Figure 3C**) but a different capability of the cells to release them. The different behavior of the cells in releasing the drugs is not clear but it probably due to fact that caPt(II)-complex was not able to interact with nuclear DNA as previously reported.[14] This is also supported by the observation (not reported here) that CisPt loaded MSCs, differently from caPt(II)-complex loaded MSCs, had more difficulty to adhere to the substrate when sub-cultured to evaluate the drug release. This could be related to a pre-apoptotic program that allowed the cells to release higher amounts of incorporated drug. As reported in the results, both lysates and CM tested did not express *in vitro* any anticancer activity that is explained by the amount of drug that in our system the cells can carry and delivery. In fact, based on the analytical data, we calculated that the maximal amount (100 µl/well) tested of lysate or CM contains 2 µM of the drugs that is a too low concentration inadequate to exert on the cancer cells a detectable inhibition (being the $IC_{50}$ of 9.64 ± 5.1 µM).

## 4. Conclusion

In conclusion, our data report for the first time that caPt(II)-complex is a molecule that in vitro is more active than CisPt against two very aggressive tumors: MPM and GBM. The high stability of caPt(II)-complex other than favoring it possible clinical management suggests also a possible application for loading MSCs to use for advanced cell therapy based on cells mediated drug delivery. Loco-regional treatment of tumor with drug loaded MSCs could contribute to increase the selectivity and efficacy of chemotherapy and at the same time to reduce significantly the toxicity of CisPt following systemic treatment. Large scale production of drug loaded MSCs are in progress in our laboratory to manage *in vivo* preclinical studies that will help to understand if it will be possible to develop an advanced cell therapy to apply as adjuvant in traditional chemo-surgical and radiotherapy.

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**List of abbreviations**

1. MPM, malignant pleural mesothelioma;
2. GBM, glioblastoma multiforme;
3. PAC, pancreatic adenocarcinoma;
4. GCB, gemcitabine;
5. CisPt, cisplatin;
6. MSCs, mesenchymal stromal cells;
7. caPt(II)-complex, cationic platinum(II) complex;
8. CFPAC-1, pancreatic carcinoma cell lines;
9. U87, glioblastoma;
10. NCI-H28, mesothelioma.

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