Anticancer Properties of a New Platinum (IV) Agent on HT1080 Cancer Stem-Like Cells: An *in Vitro* Study

Seyede Masoome Sajjadi Mohri[1], Vahideh Montazeri[2], Shima Aliebrahimi[2], Anita Abedi[3], Marzieh Zare Dehnavi[3], Akram Jamshidzade[1], Seyed Nasser Ostad[4,5]

[1] *Department of Toxicology and Pharmacology, Faculty of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran*

[2] *Department of Artificial Intelligence, Smart University of Medical Sciences, Tehran, Iran*

[3] *Department of Chemistry, North Tehran Branch, Islamic Azad University, Tehran, Iran*

[4] *Department of Toxicology and Pharmacology, School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*

[5] *Toxicology and Poisoning Research Centre, School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*

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Abstract- According to the cancer stem cell (CSC) theory, a subpopulation of cells demonstrating stem-like cell properties is responsible for tumorigenicity, self-renewal capacity, therapeutic resistance, and recurrence. Due to the resistance of CSCs, it is necessary to develop drugs with appropriate efficacy. Although cisplatin is a potent antitumor agent widely used in the treatment of different cancers, its severe side effects and resistance remain a challenge in clinical practice. Research has shown that platinum (IV) has considerably fewer side effects and is associated with much less drug resistance. In this study, the toxicity and effectiveness of [Pt(dpyam)Cl_4], where dpyam is 2,2'-dipyridylamine as a platinum (IV) agent, was investigated to find a reliable alternative to cisplatin. For this purpose, cancer stem-like cells (CS-LCs) with $CD44^+/CD133^+$ phenotype were isolated from HT1080 cells. EJ138, HT1080, and CS-LCs derived from HT1080 cells were selected to compare the effectiveness of Pt (IV) complex versus cisplatin. MTT, apoptosis, and cell cycle analysis were carried out to evaluate drug toxicity. Sphere and colony formation assays confirmed the potentiality of Pt (IV) complex and cisplatin to target stemness characteristics of CS-LCs. Although toxicity results were in favor of cisplatin, the anticancer activity of the synthesized Pt (IV) complex was also considerable. Regarding other studies that proposed high selective toxicity of Pt (IV), it could be a candidate for additional improvements.

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Keywords: Cancer stem-like cells; Platinum (IV); Cisplatin; Cytotoxicity; Self-renewal

Introduction

Cancer is the second leading cause of mortality across the world. Despite all the efforts, the patient's long-term survival is not promising yet. One of the main challenges of cancer treatment is resistance, causing medical failure or tumor recurrence after initially successful treatment. Several studies have attributed tumor resistance to cancer stem cells (CSCs). The CSC theory is a hypothesis stating that there is a subpopulation in the tumor highlighted by self-renewal and differentiation capability. Indeed, they are responsible for generating all the mature cell types of a particular population via differentiation. It is suggested that being in a quiescent state, and overexpressing resistance genes, especially those involved in drug efflux transportation, are the main factors in drug resistance of CSCs (1-3). Since they have a pivotal role in therapeutic resistance, their elimination ameliorates response to chemotherapy and consequently improves patient survival. Therefore, it is necessary to find a new compound that has beneficial effects on CSCs to overcome chemotherapy resistance (4-6). Research has indicated that CS-like cells (CS-LCs) can be recognized and isolated from tumors using cell markers such as

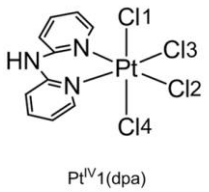
Corresponding Author: S.N. Ostad
Department of Toxicology and Pharmacology, School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran
Tel: +98 2166959105, Fax: +98 2166959105, E-mail address: ostadnas@tums.ac.ir

CD44, CD24, ESA, CD133, ALDH1, and c-Met. Specifically, in the fibrosarcoma cell line (HT1080), a high expression of CD133 can be utilized as a CS-LCs hallmark (7-9).

Cisplatin (approved in 1997) is a potent bivalent platinum-based agent that has been widely used in solid tumors (10,11). After cisplatin administration, it is reduced into $[Pt(H_2O)Cl(NH_3)_2]^+$ and $[Pt(H_2O)_2(NH_3)_2]^{2+}$ cations in the cytosol. These mono and diaquo species of cisplatin attack the nucleophilic sites of a cell, which can be DNA or non-DNA targets. Subsequently, the cross-linked and damaged DNA inhibits replication and transcription, possibly triggering an apoptosis cascade in a cell (12,13). Despite the broad-spectrum efficacy and wide-spread use of cisplatin, severe side effects and resistance remain great challenges in its clinical use (13). It has been demonstrated that reduced drug accumulation, detoxification enhancement, and DNA repair pathways such as nucleotide excision repair (NER) and mismatch repair (MMR) have crucial roles in cisplatin resistance (14-19). Bladder cancer is one of the main situations where cisplatin resistance is frequently observed. It is the second most common genitourinary malignancy with a higher prevalence in men than in women, with a ratio of 3:1. The chemotherapy regimen for bladder cancer is based on cisplatin, either in the adjuvant or neoadjuvant protocol (20,21). The treatment outcome is still highly affected by cisplatin resistance (22). On the other hand, fibrosarcoma is a type of soft tissue sarcoma (STS) that involves the fibroblastic cells of deep soft tissues or adjunct to bones (23-25). It is classified into different types. An adult form of this sarcoma, which predominantly occurs between 25 and 79 years of age, is categorized as a highly malignant tumor by the WHO. Generally, chemotherapy is not a successful treatment for fibrosarcoma because of multi-drug resistance (MDR). In other words, a high percentage of patients that use doxorubicin with vincristine, actinomycin D, vinblastine, and etoposide as first-line drugs show drug resistance (25).

Platinum (IV) compounds have been corroborated to overcome cisplatin drawbacks (26-29). In these compounds, platinum is coordinated by four chloro ligands and a nitrogen donor aromatic chelate that can intercalate into nucleotides. Since it is known that stable platinum ligands remain intact during their action, it is expected that a bulky hydrophobic non-leaving group can increase drug uptake, which influences and normalizes the reduced drug accumulation as a resistance mechanism (30). Additionally, according to studies investigating the mechanism of resistance in satraplatin, as a platinum (V) agent, it is suggested that the symmetry of stable ligands (two amine groups of cisplatin in contrast with an amine and a cyclohexamine in satraplatin) can play a substantial role in platinum resistance. Contrary to cisplatin, satraplatin-afflicted adducts are not recognized by DNA mismatch repair proteins. This difference can provide a mechanism to overcome cisplatin resistance.

In the present research, we evaluated the effect of $[Pt(dpyam)Cl_4]$ where dpyam is (2,2'-dipyridylamine) as a platinum (IV) agent on EJ138, HT1080 parenteral cells, and CS-LCs derived from HT1080 cells to suggest an alternative platinum component with anticancer effects for bladder cancer and fibrosarcoma (Scheme 1). The complex had promising effects on a panel of cell lines. The ligand of this compound is dipyridylamine, which is bulky and lipophilic. It is believed that the presence of an NH group can cause pH sensitivity and enhance cell recognition. These characteristics are assumed to increase Pt (IV) complex uptake by cancer cells, leading to selective cytotoxicity towards cancer cells rather than the normal counterparts (28).



$Pt^{IV}1(dpa)$

Scheme 1. Chemical structure of synthesized platinum (IV) complex

Materials and Methods

Chemical and reagents

$[Pt(dpyam)Cl_4]$ was kindly synthesized by Dr. Abedi *et al*., at Islamic Azad University, North Tehran Branch. Cisplatin was obtained from Accord (Accord Healthcare Limited, United Kingdom). MTT dye was purchased from Carl Roth (Karlsruhe, Germany). CD133-APC and CD44-FITC antibodies were obtained from eBioscience (San Diego, CA). Epithelial growth factor (EGF) and B27 supplement were acquired from Gibco (Invitrogen, CA, USA). B-FGF was purchased from Royan Institute (Tehran, Iran).

Cell culture

EJ138 (Human bladder carcinoma) and HT1080 (fibrosarcoma) cell lines were obtained from the Pasteur Institute (Tehran, Iran). Cancerous cells were cultured in

the RPMI 1640 medium (Biowest, Nuaillé, France) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA) and 1% penicillin/streptomycin (Biosera, East Sussex, UK) and incubated in 5% CO_2 at 37° C. The medium was replaced with a fresh one every 2-3 days, and the cells were trypsinized and recultured when reaching 70-80% confluency.

Flow cytometry analysis

To assess the CS-LCs population of HT1080 cells, the surface marker of the cells was evaluated with flow cytometry. For this purpose, HT1080 cells were cultured, trypsinized, and washed with PBS. Cell pellets were resuspended and incubated in a solution containing antibodies against CD44 and CD133 and 1% BSA in PBS for 30 min at 4° C in dark conditions. Then, the cells were washed with PBS to remove unbounded antibodies, evaluated with a FACS caliber system (BD Biosciences, San Jose, CA, USA), and analyzed using the FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Cell sorting

8×10^6 HT1080 cells were trypsinized after centrifugation for 5 minutes. The pellet was resuspended in the MACS running buffer. Blocking reagent and CD133 microbeads were added to cell suspensions according to the MACS protocol for 30 min at 4° C. Irrigation was done with 1 ml of running buffer, and $CD133^+$ cells were separated by positive selection on LS columns according to the manufacturer's instructions. HT1080 cells were analyzed by flow cytometry prior to and after sorting. All reagents were purchased from Miltenyi Biotec (Auburn, CA, USA).

MTT assay

MTT assay was performed to evaluate the cytotoxicity of cisplatin and Pt (IV) complex on cancerous cells. Metabolically active cells can reduce MTT salt to insoluble formazan that is purple. The color is detected by a Multi-scan MS spectrophotometer. EJ138, HT1080, and its CS-LCs were seeded at a density of 1×10^4 cells/well in a 96-well plate. After 24 h, the cells were treated with cisplatin and Pt (IV) complex for 48 h. Twenty microliters of MTT solution (5 mg/ml) were added to each well and incubated for an additional 4 h at 37° C. Then, 100 µl of DMSO (Merck, Germany) was added to the wells, and absorbance was measured at 570/690 nm on a plate reader (Anthos 2020, UK). Graphs were plotted, and the IC_{50} value was calculated for each drug.

Apoptosis detection

To determine the proportion of early and late apoptosis, staining with Annexin-V/ PI was done. For this purpose, 5×10^5 cells/well of EJ138, HT1080, and its CS-LCs were cultured in a 6-well plate overnight. Then, the cells were treated with the IC_{50} concentration of cisplatin and Pt (IV) complex for 48 h. Trypsinized cells were resuspended in 100 µl binding buffer, and the suspension was incubated with 4 µl of PI (2 µg/ml; Sigma) and 3.5 µl of Annexin V-FITC (Invitrogen, USA) for 15 min. A binding buffer was added to obtain a 500 µl cell solution. The solution was assessed by a flow cytometer.

Cell cycle analysis

5×10^5 cells/well of EJ138, HT1080, and its CS-LCs were cultured and treated similarly to the apoptosis detection phase. Then, the cells were fixed with cold 70% ethanol for 15 min at 4° C. After washing, the cell pellet was stained with a solution containing PI (100 mg/ml), ribonuclease (50 mg/ml), and 0.05% (v/v) Triton X-100 in PBS for 40 min at 37° C. The stained cells were evaluated and analyzed with a flow cytometer.

Sphere-formation assay

The HT1080 cells expressing CD133 were enriched using CD133 microbeads with the MACS method, and cancer stem-like cells were purified and cultured. CS-LCs (2×10^5) were treated with IC_{50} concentration in a complete medium and then reseeded (1×10^4) into a 6-well plate pre-coated with 1% low melt agarose to make a low attachment surface. The cells were inoculated into serum-free DMEM/F12 (Invitrogen Life Technologies, Carlsbad, CA, USA) medium enriched with 2% B27, EGF (20 ng/ml), and bFGF (20 ng/ml). Every other day, 20 ng/ml of EGF and bFGF was added to each well. After 7 days of treatment, spherical colonies were quantified using light microscopy.

Colony-formation assay

300 cells/well of HT1080 CS-LCs that were previously treated with IC_{50} concentrations of cisplatin and Pt (IV) complex for 48 h were cultured in the wells of a 6-well plate. After a week, the cells were fixed with acetone: methanol solution (1:9) for 20 min and stained with 0.25% crystal violet for 15 min. Finally, the colonies were counted under light microscopy.

Statistical analysis

The data are reported as the mean±standard deviation (SD). The GraphPad Prism version 6.07 (San Diego, CA) was administered to evaluate the differences between

treatment groups using the Tukey post hoc test. $P<0.05$ were considered significant.

Results

Characterization of isolated $CD44^+/CD133^+$ subpopulation

The flow cytometry result for putative CSC markers in HT1080 cells was 4.3% for $CD44^+/CD133^+$. Since approximately 90% of the cells were $CD44^+$, cell sorting was performed only for CD133. The cells were purified and characterized as HT1080 CS-LCs with 18.6% $CD44^+/CD133^+$ phenotype (Figure 1).

Cytotoxicity of Pt (IV) and cisplatin on cancer cells

The cells were treated with various concentrations of cisplatin (0.875-56 µg/ml) and Pt (IV) complex (31.25-1000 µg/ml) for 48 h. As illustrated in Figure 2, the IC50 concentration of cisplatin was 8.5±1.5, 6.3±0.5, and 25.4±0.3 µM in EJ138, HT1080, and HT1080 CS-LCs, respectively. Less cytotoxic effects were achieved for Pt (IV) complex in all tested cancerous cell lines. The IC50 values of 280±29, 81.9±5.2, and 157±4 µM were obtained for Pt (IV) complex in EJ138, HT1080, and HT1080 CS-LCs, respectively. In addition, CS-LCs demonstrated significant drug resistance; in fact, cisplatin and Pt (IV) complex both exhibited higher IC50 values in CS-LCs compared to parenteral HT1080, suggesting that the CD133+ subpopulation can be considered as HT1080 CS-LCs. The 50% inhibitory concentration of cell proliferation was subsequently used for sphere and colony formation assays as well as flow cytometry analysis.

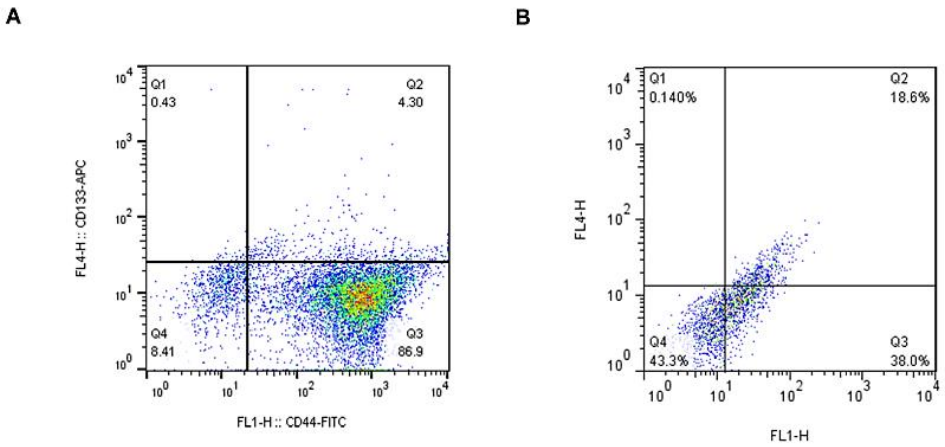


Figure 1. Flow cytometric analysis of the CD44+/CD133+ HT1080 CS-LCs before (A; Q2: 4.30%) and after (B; Q2:18.6%) sorting using MACS method

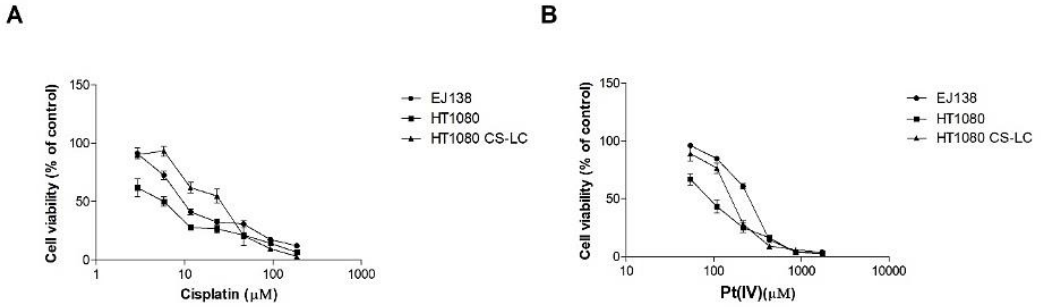


Figure 2. Exposure to various concentrations of drugs prevented EJ138, HT1080, and HT1080 CS-LCs proliferation in a dose-dependent manner after 48 h incubation. (A) Cisplatin; (B) Pt (IV) complex

Apoptosis analysis after exposure to Pt (IV) and cisplatin

Cell death induced by the IC_{50} dose of cisplatin and Pt (IV) complex was investigated using the Annexin-V/PI assay, and the number of apoptotic cells was evaluated by flow cytometry. As illustrated in Figure 3, cisplatin toxicity was mainly through apoptosis in EJ138 cells; however, necrosis had a bolder role in cell death of the group treated with complex Pt (IV). Cisplatin and Pt (IV) complex enhanced early and late apoptosis significantly compared to the control.

As for the HT1080 cells, a marked necrosis was detected for both drugs. While cisplatin caused 32% cell necrosis, Pt (IV) complex increased it to 40%, which was statistically significant. As for CS-LCs, Pt (IV) complex led to noticeable late apoptosis (47.7% vs. 4.07% in the control group), whereas cisplatin-induced early apoptosis (33.65% vs. 2.23). The rate of necrosis was 12% for cisplatin and 16% for Pt (IV) complex in CS-LCs. Overall, apoptosis was more effective in CS-LCs cell death induced by cisplatin and Pt (IV).

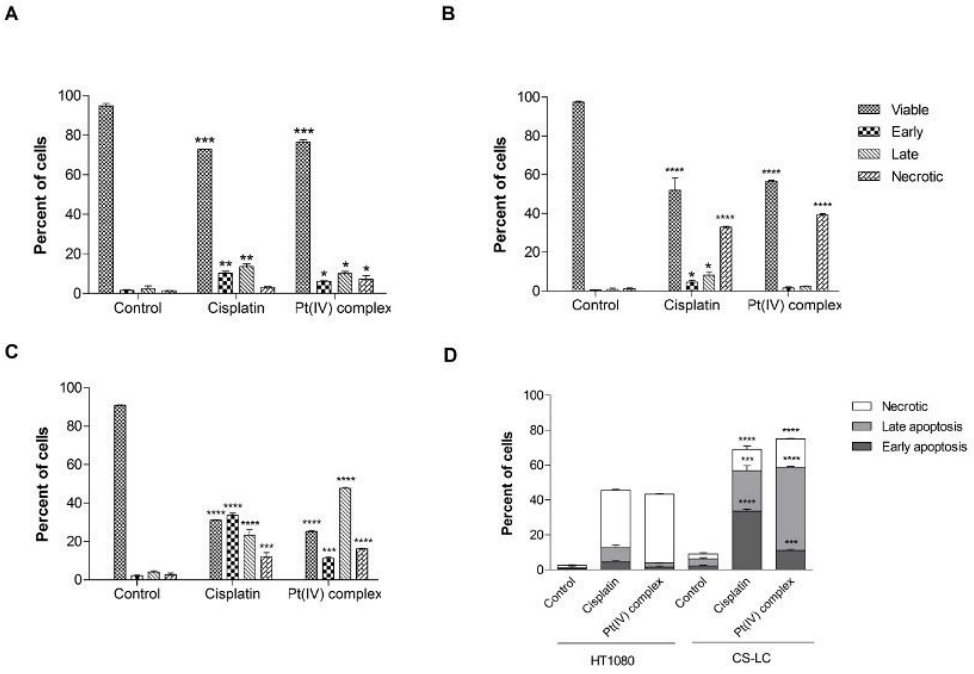


Figure 3. EJ138, HT1080, and related CS-LCs apoptotic analysis by flow cytometry. Cells were treated with cisplatin and Pt (IV) complex at the IC_{50} concentration. (A) EJ138. (B) HT1080. (C) CS-LCs. *implies significant difference relative to control group with $P<0.05$, **$P<0.01$, ***$P<0.001$, and ****$P<0.0001$ and. (D) Comparison between HT1080 and CS-LCs. ***$P<0.001$ and ****$P<0.0001$ as compared to corresponding section

Cell cycle distribution after drug exposure

The effect of cisplatin and Pt (IV) complex on cell cycle progression was assessed quantitatively using flow cytometry after exposure to the IC_{50} concentration. According to Figure 4, both cisplatin and Pt (IV) complex induced a slight increase in the G2/M phase in the EJ138 cells. Fluctuations in other phases were not significant. As for the HT1080 cells, cisplatin and Pt (IV) significantly arrested the cells in the S phase. Although a slight accumulation was found in the S phase following cisplatin and Pt (IV) complex treatment in the CS-LCs, the difference was not significant.

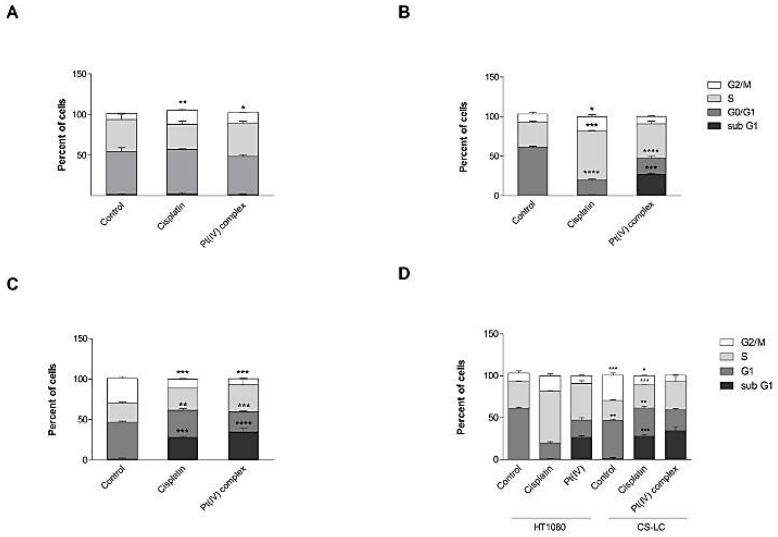


Figure 4. EJ138, HT1080, and the related CS-LCs cell cycle analysis via flow cytometric analysis. Cells were treated with cisplatin and Pt (IV) complex at the IC_{50} concentration. (A) EJ138; (B) HT1080; (C) CS-LCs; * implies significant difference relative to control group with $P<0.05$, ** $P<0.01$, *** $P<0.001$, and **** $P<0.0001$; (D) Comparison of cell cycle distribution between HT1080, and CS-LCs.* $P<0.05$, ** $P<0.01$, and *** $P<0.001$ as compared to corresponding phase

Effect of Pt (IV) and cisplatin on self-renewal potential of HT1080 CS-LCs

Sphere and colony formation assays were performed to investigate the effect of cisplatin and complex Pt (IV) on the stemness features of CS-LCs. In fact, sphere- and colony-forming assays exhibit self-renewal potentiality and can be used to distinguish between cancer and cancer stem cells functionally. According to Figure 5, both drugs significantly impaired the formation of colonies and spheres in number and size. The number of spheres decreased from 37±2.6 in the control group to 1 and 16.3±1.5 in the presence of cisplatin and Pt (IV), respectively. In addition, the number of colonies number decreased from 90.6±2.5 in the control group to 1 and 51±5 after the administration of cisplatin and Pt, respectively (IV).

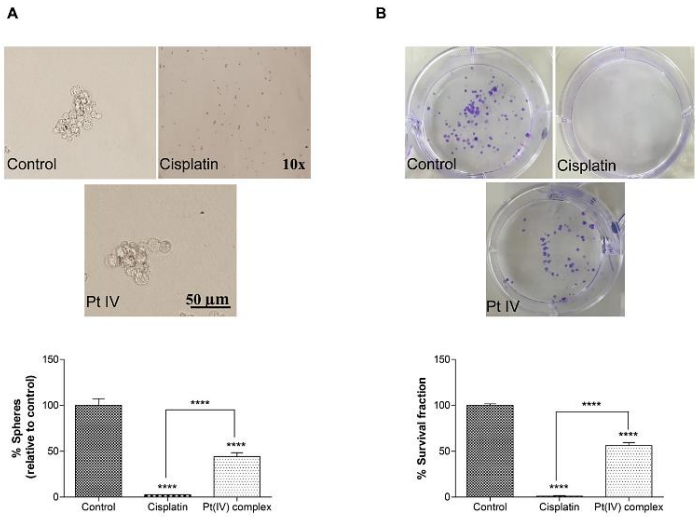


Figure 5. Impairment in the sphere- and colony-formation capacity by cisplatin and Pt (IV) complex in HT1080 CS-LCs. (A) Cells were treated with cisplatin and Pt (IV) complex at the IC_{50} dose for 48 h. Cells were harvested and seeded under spheroidal culture conditions for 7 days, as mentioned previously, for sphere assay, 20x magnification. (B) Likewise, cells treated at this concentration were recultured for 7 days. **** $P<0.0001$ relative to the control group

Discussion

Some researchers attribute the failure in cancer treatment to the CSC hypothesis. Stem cells are functionally described as self-renewing, multipotent cells that exhibit multilineage differentiation (18,19). It is believed that there are stem cells with unique features of proliferation and differentiation in a tumor (2). In other words, tumors develop from heterogenic subpopulations in which the CSC population is less differentiated and can differentiate from other cells. Furthermore, CSCs have a marked capability to recover in critical situations, which is known as self-renewal capability (2,31). Similar to stem cells, the CSCs growth rate is slower compared to other cells, and these cells lack the distinguishing factors for cytotoxic drugs (5,31). In fact, it is believed that targeting CSCs can prevent cancer progression (31,32).

Platinum is one of the most common agents used in chemotherapy regimens. Pt can form a complex with its (II) or (IV) capacity. Cisplatin is the most important platinum-based chemotherapy that contains Pt (II). Although it is a potent agent, its side effects, including nephrotoxicity and gastrointestinal toxicity, restrict its use. Another limitation is innate, and acquired drug resistance observed in other approved platinum-containing drugs (33,34). Bladder cancer, a potentially lethal malignancy, exhibits cisplatin resistance progressively (11,35). Besides, chemoresistance is considered a great challenge in fibrosarcoma, a tumor with a high mortality rate despite its rare incidence (25), and finding an effective substance seems to be crucial. In recent years, studies have shown that Pt (IV) is effective, even in resistance to other platinum-containing agents. In addition, researchers have found that some drugs containing Pt (IV) act much more selectively, causing fewer adverse side effects (27,28,33). $[Pt(dpyam)Cl_4]$ complex showed considerable selectivity in a previous study (28). Hence, in the present research, we assessed its efficacy in EJ138, HT1080, and HT1080 CS-LCs. Due to the high prevalence of metastases in fibrosarcoma, we isolated CS-LCs from HT1080 cells. Feng BH *et al.*, showed that $CD133^+$ subpopulation was associated with stem cell properties (7); therefore, we used the MACS method to enrich this fraction of cells. Cell surface marker flow cytometry showed that the $CD133^+$ subpopulation increased from 4.3% up to 18.6%. These stem-like cells had higher IC_{50} values than parental cells in the MTT test, confirming resistance in this population functionally. Cell proliferation assay indicated that cisplatin was significantly more potent than $[Pt(dpyam)Cl_4]$ complex on cancer and CS-LCs. Intriguingly, we previously demonstrated that contrary to cisplatin, $[Pt(dpyam)Cl_4]$ complex did not induce any toxicity in NIH-3T3 (normal mouse fibroblast cells) and had considerable anticancer activity in HT-29 (human colorectal carcinoma) cells (36). Based on the above results, we evaluated cell death by flow cytometry. While Shahsavar *et al*., reported the superiority of the Pt (IV) complex in apoptosis induction in a panel of cell lines (28), our results showed that necrosis was the major mechanism of cell death for both drugs in HT1080 cells; however, programmed cell death was more noticeable for cisplatin and Pt (IV) complex in CS-LCs. Moreover, in EJ138, cisplatin toxicity was mainly through apoptosis; nonetheless, necrosis had a bolder role in cell death of the group treated with complex Pt (IV).

Considering the suggested mechanism of action for platinum, including DNA and protein adducts that hinder cell proliferation, oxidative stress, and P53 activation, cell death can occur in all cell phases (13,14). Consequently, cell cycle arrest was observed variously in differently treated cells. Briefly, accumulation occurred in the G2/M phase in the EJ138 cell line, while it occurred in the S phase of the cell cycle in HT1080 cells and CS-LCs. These findings were consistent with the results of a study by Di Zhang *et al.*, in which cisplatin resulted in significant arrests in S and G2/M phases in a resistant ovarian cancer cell line (37).

According to Hiromasa Fujii *et al.*, sphere formation is an ability that can be contributed to stem cell characteristics. They observed that the ability to form a sarcosphere was associated with cell resistance (38). Accordingly, to investigate the efficacy of the drugs on stemness features of CSCs functionally, colony and sphere formation assays were performed. The assays revealed that the self-renewal capability was markedly diminished by drugs; however, this reduction was greater for cisplatin. In fact, Pt (IV) complex reduced the quantity and size of spheres and colonies, while cells exposed to cisplatin could not form colonies or spheres.

Despite selective cytotoxicity, the synthesized Pt (IV) complex was not as promising as expected. Because of the undeniable advantages of platinum (IV), it is necessary to improve the structure of the synthesized compound to develop a satisfactory drug. In fact, it is necessary to consider the toxicity of cisplatin versus the selectivity and lack of resistance of platinum (IV).

Acknowledgments

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