Polyethylenimine-based Magnetic Nanocomplexes Enhanced Cisplatin Toxicity on Ovarian Cancer Cells in Presence of Static Magnetic Field

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Research

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Abstract

Background

The drug resistance of cancer cells is a major problem in the chemotherapy. Cisplatin (CIS) is one of the most effective chemotherapeutics used for ovarian cancer. Resistance to different chemo-drugs has developed over times. Here, we investigated the experimental approach to increase the CIS cytotoxicity and overcoming cell resistance through nanoparticle-based combination treatments. Polyethylenimine (PEI)-based magnetic iron oxide nanocomplexes were used to transfer the drugs in both genetically matched CIS-resistant (A2780CP) and -sensitive (A2780) ovarian cancer cells in presence of 20 mT static magnetic field. Magnetic nanoparticles (MNPs) were synthesized and bonded to PEI cationic polymer that leads to formation of binary complexes (PM). In the following, binding of CIS to PM binary complexes two including ternary complexes PM/C (PEI-MNP/CIS) and PMC (PEI-MNP-CIS) formed.

Results

Our results showed that the CIS cytotoxicity increased at different concentrations of CIS and PEI in all of binary and ternary treatments overtimes. Furthermore, CIS induced S and G2/M phase’s cell cycle arrest as well as reactive oxygen species (ROS) production in the both cell lines. Ternary complexes enhanced apoptosis more than binary complexes in the treated-cells.

Conclusions

PEI-based magnetic noncomplex can be considered as a novel carrier for increasing the CIS cytotoxicity and probably overcoming drug resistance in ovarian cancer cells.

Background

Disruption in the cell cycle progression impacts on the cell homeostasis and physiological functions that can leads to cancer. Ovarian cancer represents fourth diagnosed cancer among women population in the world and more than any other cancers that threaten the female reproductive system. Many risk factors identified for ovarian cancer such as lifetime ovulatory cycles and etc., (1,2). Ovarian cancer is the leading cause of death in gynecologic cancers through the tumor spreading beyond the ovary at the time of diagnosis (3).

Cisplatin (CIS) as platinating agents most generally used to chemotherapy a wide range of cancers such as breast, lung, ovarian cancer and etc., (4–6). This compound inhibits DNA synthesis through the binding to guanine base and forming intra-strand DNA adducts (7). The drug resistance is the most important problem in the CIS treatment of ovarian tumor cells that easily occur in the early phase of chemotherapy(8,9). Various mechanisms have been proposed for drug resistance containing genes caused to drug resistance such as cooper transporter protein (10–12). Another mechanism that leading to CIS drug resistance is related to multidrug resistance (MDR) protein, which pumps the drug to
extracellular with consumption of energy (13–17). Generally, CIS resistance can be accomplished by number of cellular adaptations including uptake decrement, inactivation by glutathione, metallothionein and other anti-oxidants, and also increasing the gene expression of DNA repairing system (13,18–20). There is an ongoing attempt to overcome the drug resistance because it is necessary for efficient treatments. Indeed, there is no an effective approach for overcoming of drug resistance in the cancer therapy (21–25).

Zhang et al., using the Hsp90 inhibitors reverse the CIS resistance by modifying the expression of multiple drug resistance related genes (26). In the another study, Ai et al., showed that inhibition of HIF-1α induces reactive oxygen species (ROS) overproduction in CIS-resistant cells and resensitizes CIS-resistant ovarian cancer cells (27). Moreover, Hu et al., found that targeted-nanocarriers are able to deliver drug, oligonucleotide, peptide and DNA to tumor cells through enhancing of permeation and retention effects. Nanoparticle-based combination approaches used in clinical assessment to overcome drug resistance including control of over drug loading, temporary sequencing on drug release and co-encapsulation of drugs by various physicochemical properties (28,29).

Studies demonstrated that curcumin-loaded nanoparticles induce apoptotic cell death through regulation of the MDR functions and ROS levels in CIS-resistant human ovarian cancer cells (30). Polyethyleneimine (PEI) polymer has been used for transfer of gene, protein, and anticancer drugs (31–35). Yang et al., showed that chemo-radioresistance of glioblastoma was decreased using microRNA145 with cationic polyurethane-short branch PEI (PU-PEI), which is effectively suppressed the expression of drug-resistance and anti-apoptotic genes that consequently leads to a novel therapeutic approach for malignant brain tumors (36).

Magnetic fields (MFs) have effects on the biological systems and may increase the production of ROS, which may leads to oxidative stresses in the DNA, protein and lipids as well as cause genetic mutations, apoptosis cell death and etc., (37,38). Cell proliferation is impacted through treatments with both anticancer agents and MFs that enhance the anticancer effect of chemotherapeutics, which may serve as a novel combination approach in the cancer therapy (38). In this study, we examined the magnetic iron oxide nanoparticles (MNPs) with PEI cationic polymer to increase the CIS cytotoxicity and overcome drug-resistance. These drug-loaded PEI-based MNPs were transferred by homogenous static magnetic field (SMF) into sensitive and resistant ovarian cancer cell lines. PEI-based magnetic nanocomplexes can boosted the drug delivery as well as cytotoxicity, and may decreased the cell drug resistance.

**Results**

**Formation of magnetic nanoparticles**

As results as shown in Fig. 1, the FTIR spectrum of MNPs indicated on two peaks. The first absorption is at 3420 cm\(^{-1}\), which is related to the hydration bond formed with hydroxyl groups and the second absorption is at 572 cm\(^{-1}\), which is due to vibrational band of Fe-O in MNPs. These peaks confirm the
accuracy of MNPs synthesis. In the PM binary complex IR spectrum, in addition to MNPs peaks, 2924, 1633, and 1029 cm\(^{-1}\) wavelengths indicate C-H, C-C, and C-N bonds of PEI polymer, respectively that confirmed the accuracy of PM binary complex formation. The FTIR spectrum of PMC ternary complexes, peaks at 867 cm\(^{-1}\) wavelength in addition to the peaks of MNPs and PM show the N-H bond of CIS in conjugation with other molecules were less than 500 cm\(^{-1}\), which was not visible in our spectrum (41). Furthermore, the results of DLS as presented in Table 1 showed the size corresponding of MNPs ~ 69 ± 5 nm, PM binary complex ~ 88 ± 15 nm and PMC ternary complex ~ 151 ± 21 nm, respectively, which seems that is suitable as delivery agents. Furthermore, zeta potential results indicated that the MNPs surface charge was about -25 eV, which negative surface charges create an interconnection through electrostatic interaction with positive charges of PEI.

**Determination the cytotoxicity of cisplatin-conjugated magnetic nanoparticles**

We assessed the cell viability of A2780/CP and A2780 cells in the different treatments at 24 and 48 h following the end point of treatments using MTT staining and microplate reader. As shown in Fig. 2a, b, CIS decreased the cell viability of A2780 compared to A2780/CP cells at the 48 h higher than 24 h, respectively. In addition, PEI can significantly decreased the viability of both cells compared to untreated cells in the two exposure times (Fig. 2c, d). The combination effects of CIS and PEI were determined as seen in Fig. 3, so that PC binary complex significantly increased the cytotoxicity in the different concentrations in both cell lines. In following treatments, the effect of 20 mT SMF exposure was evaluated on the cell viability in treated to PM binary complex (Fig. 4). The results showed that MNPs just has been enable to decrease the cell viability in the PM complexes in presence of SMF.

The mass of PEI that used in the nanocomplexes is an important parameter. The mass ratio of PEI/CIS as shown in Fig. 5, at different concentrations of PEI and constant concentration of 2.5 µg/ml CIS that decreased the cell viability of A2780 more than A2780/CP cells. Indeed, PEI boosted the CIS toxicity in the both cells especially in A2780 cells at 48 h. Moreover, in the Fig. 6, results indicated that cytotoxicity of PM/C and PMC at mass ratio of 0.4 including 2.5 µg/ml of CIS, 1 µg/ml of PEI and 1 µg/ml MNPs enhanced in presence of 20 mT SMF in both cell lines. Furthermore, the effects of PMC on the cell viability were more than PM/C complexes in presence and absence of SMF. The IC\(_{50}\) values of CIS, PEI, PC, PM, PM/C and PMC were calculated in A2780/CP and A2780 cells at 24 and 48 h (Table 2). These results suggested that PMC complexes were more effective from PM/C complexes for the cytotoxicity effects of CIS.

**Cisplatin increased intracellular ROS production**

We measured the intracellular ROS accumulations of A2780/CP and A2780 cells in presence and absence of 20 mT SMF treated to CIS, PEI and ternary complexes (PM/C and PMC) at the same
concentration for 48 h through fluorescent probe DCFH-DA and flow cytometry (Supplementary Fig. S1). As shown in Fig. 7, ROS levels significantly increased in all of these treatments compared to untreated cells. Moreover, PM/C and PMC complexes induced increment of ROS production more than alone CIS-treated cells.

Cisplatin induced S and G2/M phase cell cycle arrest

We evaluated the cell cycle distribution of A2780/CP and A2780 cells in presence and absence of 20 mT SMF treated to CIS, PEI and ternary complexes (PM/C and PMC) at the same concentration of 2.5 μM CIS, 1 μM PEI and 1 μM MNP for 48 h through PI staining and flow cytometry (Supplementary Fig. S2). As shown in Fig. 8, SMF caused S phase arrest in A2780/CP and G2/M phase arrest in A2780 cells. CIS triggered S and G2/M phase arrest in the both cell lines. Furthermore, PEI led to G2/M arrest in presence of SMF-treated A2780 cells more than A2780/CP cells. Besides, PMC and PM/C treatments induced S and G2/M phase in presence of SMF in the both cell lines.

Cisplatin promoted apoptosis in cancer cells

We investigated the apoptosis rate of A2780/CP and A2780 cells in presence and absence of SMF (20 mT) treated to CIS, PEI and PM/C and PMC ternary complexes at the same concentration for 48 h through annexin/PI and flow cytometry (Supplementary Fig. S3). As seen in Fig. 9, SMF can modulated the induction of apoptotic cell death in the A2780 cells compared the A2780/CP cells. SMF do not show any changes in the levels of apoptosis in CIS and PEI treatments in both cell lines. The apoptosis rate significantly increased in the PMC and PM/C treatments compared to alone CIS and PEI treatments. The PMC ternary complex showed a high level of apoptosis more than other treatments in A2780 cell line. Indeed, PMC was more effective than PMC for inducing apoptosis cell death.

Discussion

This study aimed to investigate the effects of PEI-based magnetic nanocomplexes on ovarian cancer cells in presence of SMF, and the probably mechanisms of this effects. Cancers are caused by a series of mutations in the genes and changes in the cell physiology and functions. Here, the ternary complexes of CIS increased cytotoxicity and cell accumulation in S and G2/M phase’s cell cycle as well as ROS production and apoptosis cell death (34). Many efforts made to use the polymeric nanocomplexes to increase the cytotoxicity and efficiency of delivery systems enhancing drug toxicity and overcome chemodrug resistance. Tseng et al., reported that CIS-incorporated gelatin nanocomplexes can be used for cancer chemotherapy (46). Lipid and polymer-based nanoparticles siRNA delivery systems are also developed by Mainini et al., to cancer therapy (47). Polymeric nanoparticles synthesized based on host–guest interaction between β-cyclodextrin and benzimidazole used for liver cancer-targeted therapy and its ability to induce cell apoptosis was found to be remarkable (48). Lim et al., used redox-responsive polymeric nanocomplexes for delivery of cytotoxic protein and chemotherapeutics. They synthetized a
nanocomplexes including PEI cross-linked by oxaliplatin (IV) pro-drug to generate ROS in the cell. The ROS can stimulates the controlled-drug release and effects on of protein reactivity (49). We similarly synthetized a noncomplex including PEI cross-linked by CIS and MNPs as ternary complexes (PMC) and characterized by FTIR (Fig. 1) and DLS (Table 1), which enhanced the CIS cytotoxicity and successfully overcome drug resistance. As shown in Fig. 2, CIS and PEI decreased the cell viability of A2780 more than A2780/CP cells. The cytotoxicity increased in the PC (Fig. 3) and PM (Fig. 4) binary complexes, and also finally PMC ternary complex (Fig. 6). CIS causes cell death by conjugation and disrupt the nuclear (nDNA) and mitochondrial DNA (mtDNA), which inhibits transcription and replication of nDNA and mtDNA (50).

Cationic polymers bind to negatively charged proteins in the cells such as heat shock proteins, glutathione s-transferases, which are involved in apoptosis. This is the most important mechanism of toxicity by PEI polymers that may enhance the CIS cytotoxicity in the PC, PM and PMC complexes (51,52). Recently, Karimi et al., reported a usage of CoFe$_2$O$_4$/MNPs as an effective carrier for epirubicin anticancer drug delivery (53). As shown in Fig. 7, ROS production significantly increased in all of the treatments. In addition, PMC and PM/C complexes induced enhancement of ROS production more than CIS. Free radicals have essential roles in the cell physiological and proliferation processes in low levels. In contrast, high accumulation of ROS leads to oxidative damages in the cell components and cell death (13). Indeed, the platinum in CIS induces the ROS production and damages in DNA, proteins and lipids, and also ultimately promotes the apoptosis (54)(4 P). PEI polymer in the PMC and PM/C complexes inhibits a number of antioxidant enzymes such as glutathione s-transferases which is a ROS scavenger. That may cause the increase of ROS content and therefore induction of apoptosis.

CIS induced S and G2/M phase cell cycle cell accumulation in A2780/CP and A2780 cells. PEI led to G2/M arrest in presence of SMF-treated A2780 cells more than A2780/CP cells. PMC and PM/C treatments induced S and G2/M phase cell cycle arrest by decrease of cell population in G0 / G1 phase in presence of SMF in the both cell lines (Fig. 8). Enhancement of ROS levels increase the oxidation of nucleotides in the nucleotide pool and DNA molecules. Free radicals cause a variety of damages, including single- or double-stranded breaks in DNA, and eventually activate proteins such phosphorylated form of checkpoint kinases ATM in the cells. The ATM phosphorylates the Cdc25, p53 and E2F1 and initiates the repair process or apoptosis in the damaged-cells that their inactivation induce G2/M phase's cell cycles arrest (55–58).

As seen in Fig. 9, the apoptosis rate significantly increased in the PMC and PM/C treatments compared to CIS and PEI, only. In addition, the PMC ternary complex showed a high level of apoptosis compared to other treatments in A2780 cell line. The PMC was more effective than PM/C for inducing apoptosis cell death. The PMC complexes induce apoptosis through two mechanisms to including inhibitory potential of PEI on negatively charged proteins and also CIS conjugated to PM that promoted the efficiency of drug's transfer by PMC complexes while the PM/C complexes used only the first mechanism.

Although SMF exposure did not show any effects on the CIS and PEI treatments but SMF influences on the cell functions in treatment of MNPs through a mechanical force that caused the penetration of the
cell membrane and induced more apoptosis (59).

In the PM/C complexes, first the PEI polymer and MNPs entered to the cell without CIS. The PEI polymer had enough opportunity to inhibit the negatively charged proteins. Drug resistance decreased by neutralizing some of them in A2780/CP cells. While, in the PMC complexes, all components entered to the cells at the same time, so the efficiency of PEI was less than PM/C complexes. In A2780 cells, negatively charged proteins did not exceed. Therefore, the changes of IC50 rate at CIS in A2780/CP cells was greater than A2780 cells as shown in Table 2.

Isoelectric pH of most proteins involved in CIS resistance is less than pH ~ 7.2. These proteins have negative charge in neutral pH, which makes them a good target for binding to PEI. As shown in Table 3, the net charge of proteins involved in pre-target resistance that is negative. One of these proteins is the glutathione S-transferase enzyme, which its expression increases in CIS resistance (60, 61). The net charge and isoelectric pH of this enzyme is 3 and 5.3, respectively. The glutathione S-transferase enzymes are important scavengers of ROS that inhibition of them induced ROS accumulation, G2/M phase cell cycle arrest and apoptosis (52,62). PEI can binds to these negative-charged enzymes (51). Other negative biological molecules such as mRNA can also be targets of polymer binding. So that, proteins that are highly expressed in CIS resistance have more mRNA content in the cytoplasm. It seems that PEI may inhibits the scavengers of ROS in pre-target mechanism and translation of drug resistance proteins. Furthermore, the high buffering and endosomal escape of PEI trigger to endocytosis transferring. Endosomal escape that is the entry of carrier into the nucleus before it is prematurely destroyed by endosomes and intracellular lysosomes (63,64). Therefore, PMC complexes had less presence in the cytoplasm of cells. These complexes less recognized by lysosome or multi-drug resistance mechanisms and also less exposed to antioxidants such as glutathione, metallothionein and etc. In conclusion, PEI-based magnetic nanocomplexes may be a novel strategy to enhance the CIS cytotoxicity and possibly overcome the drug resistance in cancer cells.

Methods

Chemical reagents

Roswell park memorial institute 1640 (RPMI-1640) medium and fetal bovine serum (FBS) were purchased from Gibco. Penicillin-streptomycin and trypsin-EDTA were obtained from Bioidea. Cisplatin was purchased from Oncotic Pharma production GmbH, Germany. 25 kDa branched polyethylenimine was obtained from Sigma-Aldrich. 2′,7′-dichlorofluorescein diacetate (DCFDA) (ab113851) was purchased from Abcam. Propidium iodide (PI), (4H₂O.FeCl₂), (6H₂O.FeCl₃) and (NaOH) were obtained from Merck. Annexin V apoptosis detection kit was purchased from eBioscience, USA.

Synthesis and characterization of magnetic nanoparticles
The Fe₃O₄ MNPs synthesized by co-precipitation approach. Physical properties of nanoparticles and size-dependent parameters such as reaction temperature, pH of suspension, initial molar concentration were investigated (39). In this approach, nanoparticles synthesized from (4H₂O.FeCl₂), (6H₂O.FeCl₃) and (NaOH) with high purity and distilled water. The (4H₂O.FeCl₂) and (6H₂O.FeCl₃) solutions were mixed in the respective stoichiometry (i.e., ratio Fe (II):Fe (III) = 1:2) in presence of OH⁻ deposited through the following reaction in equation (1).

\[
\text{Fe}^{2+} + \text{Fe}^{3+} + 8\text{OH}^- \rightarrow \text{Fe(OH)}_2 + 2\text{Fe(OH)}_3 \rightarrow \text{Fe}_3 \text{O}_4 + 4\text{H}_2\text{O} \quad (1)
\]

Distilled water was initially deoxygenated by ultrasonic transducer and nitrogen gas. Then, iron salts II and III were added. The NaOH solution was also deoxygenated by the ultrasonic apparatus. The 12 ml of saline solution was added to 120 ml of NaOH solution under nitrogen atmosphere, and the solution was homogenized for 10 h at 10,000 rpm for 30 min. After that, the particles were washed three times with distilled water and once with acetone, and dried under vacuum conditions (40,41).

The binary complexes (PEI-CIS (PC)) and PEI-MNPs (PM)) were synthesized at 37°C for 1 h. for formation of ternary complexes (PEI-MNP/CIS (PM/C)) CIS bounded to PM binary complexes. Moreover, PEI, MNP and CIS bounded together at the same time and (PEI-MNP-CIS (PMC)) ternary complexes were synthesized at 37°C for 1 h.

The accuracy of MNPs and formation of binary and ternary complexes were confirmed by the Fourier transform infrared spectroscopy (FTIR) and dynamic light scattering analysis. FTIR spectra was done for dried sample of MNP, PM binary and PMC ternary complexes by FTIR spectrometer (Thermo scientific NICOLET IR100) in wave range of 4000 - 400 cm⁻¹ with a resolution of 4 cm⁻¹. In brief, the dried sample was placed on a silicon substrate transparent to infrared, and spectra were measured by transmittance method. Then, spectrums of synthesized products were plotted through Essential FTIR software. The size and surface charge distribution of PEI, MNP, PM and PMC ternary complexes in solutions and suspensions was determined using Dynamic light scattering (DLS Zeta Sizer, a nano-ZS model, UK). Briefly, 1 mg/ml solution of nanoparticles made in deionized-water and placed in the ultrasonic bath for 30 min. The sample was filtered with a 0.25 μm to remove larger and accumulated particles. Then, the particle size distribution and surface charge were analyzed by Zetasizer software (version 7.11).

**Cell culture**

The CIS-resistant human ovarian carcinoma A2780/CP and sensitive A2780 cells were obtained from National Cell Bank of Iran (NCBI). A2780/CP is a sub-line of A2780 that gained CIS resistance in the in vitro (27). The cells were allowed to grow in RPMI-1640 in the neutral PH (7.2–7.4) supplemented with 10% (v/v) heat-inactivated (50 °C, 30 min) fetal bovine serum (FBS) and 2 mM glutamine, 100 units/mL of penicillin and 100 mg/mL of streptomycin at 37 °C and 5% CO₂ in a humidified incubator. The cells
were trypsinized (0.025% trypsin, 0.02% EDTA) after they grown until 70–80% confluent. Prior to treatments, cells were allowed to reattach overnight.

Magnetic field exposure

We used permanent cobalt magnets to apply 20 millitesla (mT) homogenous SMF. The magnitude of this magnetic field was calculated by a Teslameter (13610.93, PHYWE, Gottingen, Germany) with a probe type of Hall Effect in respect to its cross-sectional area and thickness. We exposed 20 mT SMF to cells through placing magnets under bottom of cell culture plates during this study.

Cell treatments

The A2780/CP and A2780 cells were treated by CIS and PEI at concentrations of 5, 10, 25, 50 and 100 μg/ml, PC binary complex included concentrations of PEI (1, 2.5, 5 and 10 μg/ml) and CIS (2.5, 5, 7.5 μg/ml) and PM binary complex included concentrations of PEI (2.5, 5 and 10 μg/ml) and MNPs (1 μg/ml) in presence and absence of 20 mT SMF for 24 and 48 h. Moreover, cells were treated at mass ratio of PEI/CIS (0.4, 0.5, 1, 2 and 4 w/w) for 24 and 48 h. In addition, cells treated with PM/C and PMC ternary complexes at concentrations of 1 μg/ml PEI, 1 μg/ml MNPs and 2.5 μg/ml CIS in presence and absence of SMF for 48 h.

Cell viability assay

The cell viability of A2780/CP and A2780 cells were measured by tetrazolium-based colorimetric assay (MTT assay). Briefly, cells (10⁴ cells/ well) were seeded into 96-well culture plate (SPL Life Sciences Co., Ltd. Korea) and incubated in a total volume of 100 μL supplemented RPMI at 37 °C and 5% CO2 in a humidified incubator. Cells were initially allowed to attach overnight. Following the treatments, 100 μL FBS-free RPMI containing of 0.5 mg/mL MTT was added to each well and kept at 37 °C for 4 h in the dark. Then, formazan was dissolved by 100 μL/well DMSO. The relative number of living cells in each group was measured by microplate reader (uQuant MQX200, BioTek, USA) at 570 nm. Cell viability results were shown as percentage compared to control cells. Sensitivity of selected cell types evaluated by the half-maximal inhibitory concentration (IC50).

Quantitation of intracellular ROS accumulation

Intracellular ROS levels under normal and stress conditions were detected using 2′,7′-dichlorofluorescein diacetate (DCFDA) assay kit. The A2780/CP and A2780 cells were treated in 1 μg/ml of PEI and 2.5 μg/ml of CIS as well as PM/C and PMC complexes (at concentrations of 1 μg/ml of PEI, 1 μg/ml of MNP and 2.5 μg/ml of CIS) compared to untreated cells in the presence and absence of SMF for 48 h, which were prepared in supplemented RPMI media with 10% FBS in the 6-well cell culture plate (SPL Life...
Sciences Co., Ltd. Korea). After treatments, cells were prepared immediately as recommended by the manufacturer. Briefly, the cells were washed with PBS. Then the samples were suspended in the conical test tube with 20 µM DCFDA in the 1X buffer and incubated at 37 °C in dark for 30–45 min. Measurement of ROS production was monitored immediately by FACScalibur Becton-Dickinson flow cytometry (Franklin Lakes, NJ). The DCFDA flow cytometric data were analyzed by flowjo software (version 7.6.1) (27,42,43).

**Cell cycle analysis**

The A2780/CP and A2780 cells were treated to 1 µg/ml of PEI and 2.5 µg/ml of CIS as well as PM/C and PMC complexes (at concentrations of 1 µg/ml of PEI, 1 µg/ml of MNP and 2.5 µg/ml of CIS) in presence and absence of SMF for 48 h, which were prepared in supplemented RPMI media with 10% FBS in the 6-well cell culture plate (SPL Life Sciences Co., Ltd. Korea) and incubated at 37 °C and 5% CO₂ in a humidified incubator. After that, cells were trypsinized and collected through centrifugation at 400g for 5 min. Then, cells were resuspend in 0.5 ml of PBS and fixd by adding 4.5 ml of 70% (v/v) cold ethanol, and centrifuged at 400g for 5 min. After that, cells were washed in 5 ml PBS, centrifuged at 400g for 5 min and incubate at room temperature for 5 min, and recentrifuged at 400g for 5 min, respectively. Then, supernatant was removed and resuspended in 1 ml of DNA staining solution. The prepared cells were incubated for at least 30 min at room temperature in the dark. The obtained cell suspension was analyzed by FACScalibur Becton-Dickinson flow cytometry (Franklin Lakes, NJ). Data were collected from at least 104 cells. The flow cytometric data were analyzed by flowjo software (version 7.6.1) (44).

**Detection of cell apoptosis**

Apoptosis was detected using annexin V-FITC/PI staining. The A2780/CP and A2780 cells were treated in 1 µg/ml of PEI and 2.5 µg/ml of CIS as well as PM/C and PMC complexes (at concentrations of 1 µg/ml of PEI, 1 µg/ml of MNPs and 2.5 µg/ml of CIS) in presence and absence of SMF for 48 h, which were prepared in supplemented RPMI media with 10% FBS in the 6-well cell culture plate (SPL Life Sciences Co., Ltd. Korea) and incubated at 37°C and 5% CO₂ in a humidified incubator. After that, cells were collected and labeled by annexin V/PI in 1X binding buffer for 15 min. Then, the apoptotic and necrotic cells were evaluated by FACScalibur Becton-Dickinson flow cytometry (Franklin Lakes, NJ). The flow cytometric data were analyzed by flowjo software (version 7.6.1). Total number of apoptotic and necrotic cells were defined as sum of Annexin V+/PI- and Annexin V+/PI+ populations including Q1 = Necrosis; Q2 = Late Apoptosis; Q3 = Early Apoptosis; Q4 = Live Cells (45).

**Statistical analysis**

GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, USA) was used for data graphing and estimating the values of inhibitory concentration 50% (IC50) for any types of cytotoxicity. All experiments were performed with three independent repetitions. Data were showed as mean ± standard deviation (SD)
and were analyzed by factorial analysis of variance (ANOVA) followed by Tukey’s post hoc tests. Differences were assessed to be significant p-value was less than 0.05.

**Abbreviations**

CIS: Cisplatin  
PEI: Polyethylenimine  
MNPs: Magnetic nanoparticles  
MDR: multidrug resistance

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article and its supplementary information files.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ Contributions**

F.A. performed the experiments and analyzed the data and also wrote the original draft. B.H.V. and M.S. conceived and designed the study and methodology. P.A. as corresponding author conducted research,
sponsored the study and made all the arrangements. All authors reviewed the final version of the manuscript.

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**Tables**

Due to technical limitations, tables are only available as a download in the Supplemental Files section.

**Figures**
Figure 1

The FTIR spectrum of (a) MNPs, (b) PEI-MNPs (PM) and (c) PEI-MNP-CIS (PMC) in the range of 400-4000 cm$^{-1}$ wavelengths. The FTIR spectrum of MNPs is visible on two peaks. The first absorption peak is in the range of 3420 cm$^{-1}$ wavelength that is related to the hydration bond formed with hydroxyl groups and the second absorption peak is within the range of 572 cm$^{-1}$, which is due to the vibrational band of Fe-O in the nanoparticles. These peaks confirm the accuracy of nanoparticle synthesis. In the PM binary complex, in
addition to MNPs-dependent peaks (3433 and 569 cm\(^{-1}\)), the wavelengths of 2924, 1633, and 1029 cm\(^{-1}\) indicate C-H, C-C, and C-N bonds of PEI polymer, respectively. These results confirmed the accuracy of PM binary complex formation. In the PMC ternary complex spectrum, in addition the peaks of MNP and PM, peaks are in the range of 867 cm\(^{-1}\), which indicated the N-H bond of the cisplatin drugs.

Figure 6

The cell viability results of (a) A2780/CP and (b) A2780 cells treated with (PEI-MNPs/CIS (PM/C)) consist of cisplatin (CIS)-treatment (2.5 μg/ml) after 1 h of PEI-MNPs(PM) binary complex treatment at concentrations of 1 μg/ml polyethylenimine (PEI) and 1 μg/ml Fe3O4 magnetite nanoparticles (MNPs),
and also three-component magnetic nanocomplex (PEI-MNP-CIS (PMC)) at the same concentrations of PM/C in presence and absence of 20 mT static magnetic field (SMF) for 48 h. Cell viability values were determined by MTT assay and microplate reader, and results are expressed as the percentage of viable cells. Data are shown as mean ± SD (n = 3).****P<0.0001 show significant differences relative to unexposed cells (CTRL), and letters (β, P<0.01; χ, P<0.001; γ, P <0.0001 show significant differences between treated cells (2 × 2 × 2 × 2 factorial ANOVA flowed by post-hoc Newman–Keuls multiple comparison tests).
Figure 7

The intracellular ROS generation of (a) A2780/CP and (b) A2780 cells treated to 1 μg/ml of polyethylenimine (PEI), 2.5 μg/ml of cisplatin (CIS), and PM/C and PMC three-component magnetic nanocomplexes at same concentrations (1 μg/ml PEI, 1 μg/ml MNPs and 2.5 μg/ml CIS) in presence and absence of 20 mT static magnetic field (SMF) for 48 h. Cells were collected and the ROS generation was evaluated by using oxidized DCFDA and Flow-Cytometry analysis. Data are shown as mean ± SD (n = 3). ***P<0.001; ****P<0.0001 show significant differences relative to unexposed cells (CTRL), and letters (α,P<0.05; χ,P<0.001; γ,P <0.0001 show significant differences between treated cells (2 × 2 × 2 × 2 factorial ANOVA flowed by post-hoc Newman–Keuls multiple comparison tests).

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