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Biocompatibility study of P (N-isopropylacrylamide)-based nanocomposite and its cytotoxic effect on HeLa cells as a drug delivery system for Cisplatin

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ABSTRACT

Cisplatin (CDDP) is widely used as an effective drug in the treatment of various cancers. In spite of its therapeutic effects, there are disadvantages in using CDDP, including causing toxicity in healthy cells, also tumor cells become resistant in prolonged use. The use of magnetic nanogels (nanocomposite), as a drug delivery system, can be a potential strategy to overcome these disadvantages. In this study, the biocompatibility (hemocompatibility and cytocompatibility) of the magnetic, pH, and thermo-responsive poly (N-isopropylacrylamide-co-N, N-dimethylaminoethyl methacrylate-co-4-acrylamidofluorescein)-Fe_3O_4 nanocomposite was assessed in normal cells. Moreover, we evaluated the cytotoxicity, apoptosis, and drug-resistant genes expression of CDDP-loaded nanocomposite against free CDDP in the cervical cancer cell line (HeLa cells) to investigate its potentials as a vector for drug delivery. The results exhibited that the prepared nanocomposite had an appropriate hemocompatibility. Also, the nanocomposite displayed a good cytocompatibility using LDH test. In addition, the MTT assay of the blank nanocomposite, free CDDP, and CDDP-loaded nanocomposite in the HeLa cells indicated that the blank nanocomposite, had no cytotoxic effects on the cells. However, the Free CDDP and CDDP-loaded nanocomposites demonstrated considerable amount of cytotoxicity effect on the HeLa cells ($p < 0.05$). The results from the Real Time PCR illustrated that the CDDP-loaded nanocomposite could increase the expression of apoptosis genes and reduce the expression of drug resistance genes in the cancer cells. Our data suggested that the synthetized nanocomposite could be used as an effective and biocompatible drug delivery system for smart delivery and intravenous administration of CDDP. Literature mining-based network discovery proposed candidates for tumor sensitivity/resistance to Cisplatin, viz *NR4A1*, *NR1I2, TP73*, and *TP53* transcription factors as well as *EDN1*, *CD40*LG, *INS*, and *TNFSF11* secreted ligands.

1. Introduction

Cisplatin (Cis-diamine-dichloroplatinum (II): CDDP) is a drug used mostly in the treatment of solid tumors, especially cervical cancer [1–3]. Despite its widespread administration, cisplatin has major drawbacks, such as intrinsic or acquired resistance and severe toxicity in normal

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tissues which have limited its clinical applications [4,5]. Cisplatin's toxicity in normal tissues causes a number of side effects, such as renal toxicity, phlebitis, bone marrow suppression, and neurotoxicity [6,7]. Long-term exposure to cisplatin also leads to cellular resistance in tumor cells, reducing its therapeutic efficacy, which is considered as a major constraint in cancer treatment. The mechanisms involved in developing cellular resistance to cisplatin are complex and multifactorial. Cellular alterations, such as decreased drug influx, increased drug efflux, drug detoxification systems activation, drug targeting alteration, increased DNA damage repair, defective apoptosis, and the alteration of oncogene expression, are claimed to play a role in resistance to cisplatin [8–10]. Although the molecular basis behind the resistance to Cisplatin has not yet been determined, research have shown that various genes contribute to the development of drug resistance [11–13]. Nanotechnology-based drug delivery systems can overcome chemotherapy resistance through different mechanisms [11,14]. Firstly, nanomaterials (NMs) can pass through obstacles that cause drug resistance due to Enhanced Permeability and Retention (EPR) effect or active targeting mechanisms. They provide effective bio-distribution and increased intracellular concentration in the encapsulated drugs within the tumor sites. Secondly, under a controlled procedure, NMs release the encapsulated drugs when encountering the drug-resistant cancerous cells (e.g. acidic pH). Thirdly, due to size-exclusion effect, NMs can escape drug efflux via the Multidrug Resistance (MDR) transporters. Furthermore, the encapsulated drug clearance from cancer cells can be declined by dint of P-glycoprotein (P-gp) gene (an effective gene involved in generating protein pumps in drug efflux) downregulation, enabling the drug to retain an effective intracellular concentration within the resistant cells. Fourthly, NMs can reduce the expression of effective genes involved in drug resistance [15–17]. Hence, designing nano-based drug delivery systems can be considered as an effective approach for overcoming drug resistance [18,19]. In order to avoid Cisplatin's side effects and to prevent cellular resistance, Cisplatin can be encapsulated into nanoparticles prepared from amphiphilic copolymers (hydrogel) [20,21]. Nanogels (hydrogel nanoparticles) are hydrophilic cross-linked polymeric particles that form soft porous three-dimensional scaffolds at nanoscale size. They own the characteristics of both the hydrogels and nanoparticles. Also feature associated with nanoparticles concern high surface area, stability, specificity, drug encapsulation, and overall sizes in the range of cellular compartments. Developing of new stimulus-responsive nanogels, as drug delivery systems, for the treatment of cancer is crucial to enhance the therapeutic index, optimize the drug's effectiveness, and decrease the toxic side effects [22–24]. The stimulus-responsive systems carry a drug in a spatio-temporal or dosage-controlled manner. For example, the stimulus-responsive nanogels undergo a sol–gel transition because of different environmental stimuli, such as temperature, pH, solvents, biochemical agents, magnetic or electric field, electromagnetic radiation, and ultrasound [25–27]. As drug delivery systems, Nanogels must be biocompatible. The term "biocompatibility" concern different features of nanoparticles. Cytotocompatibility and hemocompatibility behaviors are two important aspects in evaluating the nanoparticles biocompatibility [28]. Certainly, any nanoparticles intended for *in vivo* applications must pass the biocompatibility tests prior to administration. In many studies, poly (N-isopropylacrylamide) (PNIPAM)-based copolymers are found to be the most commonly used stimulus-responsive nanogels for drug delivery [29–33]. Although the designed PNIPAM-based drug delivery systems might eventually be used to human, few studies have yet been conducted on their cell and blood compatibility [32]. For cytocompatibility, cytoplasmic membrane damage could be quantified by release of cytosolic enzyme lactate dehydrogenase (LDH), and the erythrocyte membrane damage could be assessed by the spectrophotometric measurement of erythrocytes released hemoglobin after nanoparticle treatment [28]. Due to variability in experimental approaches and lack of a standard procedure for test validation, drawing conclusions on such studies are complicated. Biocompatibility level of the prepared nanocomposite can be estimated using the available protocols [30,31]. In our previous report, we have successfully synthesized the magnetic, pH, and thermo-responsive nanocomposite of poly (N-isopropylacrylamide-co-N, N-dimethylaminoethyl methacrylate-co-4-acrylamidofluorescein)-Fe_3O_4 [P(NIPAM--co-DMAEMA-co-AFA)-Fe_3O_4] via free radical copolymerization, also their characterization and stimuli-responsive properties have been investigated in detail [34]. In the present work, studies on the cytotoxicity and *in vitro* biocompatibility of P(NIPAM-co-DMAEMA-co-AFA)-Fe_3O_4 nanocomposite were discussed, and an assessment of CDDP-loaded nanocomposite cytotoxicity was carried out on cervical cancer cells (HeLa cells) as compared with free CDDP. Moreover, the interaction network underlying Cisplatin resistance in the HeLa cells was investigated using literature mining and network analysis. In addition, the impact of the designed drug delivery system on gene expression involved in cell apoptosis and drug resistance in the HeLa cells was evaluated.

2. Materials and methods

The human cervical cancer cell line (HeLa cells) and the normal cell line (3T3 mouse fibroblasts) were purchased from Pasteur Institute, Iran. Cis-diamine-dichloroplatinum (II) was procured from Mylan Laboratories (France) based on a clinical formulation of 1 mg/ml in 0.9% saline solution. Fetal bovine serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) were obtained from Gibco. Dimethyl thiazol diphenyl tetrazolium bromide (MTT), penicillin–streptomycin, LDH Cytotoxicity Assay kit (DQ 1340-K), and Collagen were prepared from Sigma. The Dimethyl sulfoxide (DMSO) and Triton X-100 were purchased from Merck. TRIZOL reagent was purchased from Ambion (USA). cDNA synthase kit was procured from Thermo (USA). All the primers and master mix were purchased from BioNEER, Korea.

2.1. Hemolysis assay

The effect of P(NIPAM-co-DMAEMA-co-AFA)-Fe_3O_4 nanocomposite on erythrocyte membrane stability was investigated using *in vitro* hemolysis assay in compliance with the available standard [35], with minor modifications. Briefly, human RBC (Red Blood Cell) was separated from 5 ml of fresh citrated human blood through centrifugation at 700 rpm for 10 min. It was then washed with normal saline several times until the supernatant became colorless. The purified RBC was resuspended in normal saline to obtain the primary volume. Then, 1 ml of the RBC suspension was incubated with 100 μl of different concentrations of the drug-free P (NIPAM-co-DMAEMA-co-AFA)-Fe_3O_4 nanocomposite solution (ranging from 1 to 50 mg/ml) for 2 h in an incubator shaker at 37 °C. The samples were then centrifuged at 700 rpm for 10 min. Each concentration comprised three test tubes. The hemolysis amount was measured by UV–Visible (Ultraviolet–Visible) spectrophotometry of the supernatant at 540 nm absorbance. The normal saline and the distilled water were used as the negative and the positive controls, respectively. The percentage of hemolysis (HP%) was calculated based on the following equation:

$$\%\ \mathrm{HP} = (\mathrm{ABS\ sample} - \mathrm{ABS\ saline}) / (\mathrm{ABS\ distilled\ water} - \mathrm{ABS\ saline}) \times 100$$

2.2. Platelet aggregation assay

Blood was collected from a healthy volunteer who had not taken any drugs 2 weeks prior to the study, just to avoid possible effects on the platelet functions. Platelet-rich plasma (PRP) was prepared by centrifuging the citrated whole blood at 280 rpm for 15 min, at room temperature. Stirred constantly, 500 μl of various concentrations of the nanocomposite solution (ranging from 1 to 50 mg/ml) were incubated with 1 ml PRP for 15 min at 37 °C. Collagen (2 μg/ml) and PBS were used

as the positive and negative controls, respectively. The platelet aggregation in each test tube was measured by UV–Visible spectrophotometry at 500 nm absorbance. Also, the platelet aggregation in the positive control, which was added to the PRP, was measured in the absence and presence of the nanocomposite (50 mg/ml).

2.3. Lactate dehydrogenase (LDH) release assay

One method to evaluate cytocompatibility is to measure the degree of membrane damage in normal cells. The LDH release assay measures the membrane integrity or damage as a function of the cytoplasmic LDH enzyme quantity that permeates the culture medium [36]. This assay was used to measure the *in vitro* nanocomposite cytocompatibility using normal cell line (3T3 mouse fibroblast cells). 1×10^4 cells per well of a 12-well plate were seeded into 2 ml Dulbecco's Modified Eagle Medium (DMEM) supplemented with FBS (10%), penicillin (100 Unit/ml), and streptomycin (100 μg/ml). Next, they were cultivated for 24 h in a humidified atmosphere with 5% of CO_2 at 37 °C. Then, the cells were washed with 2 ml phosphate buffered saline (PBS, pH 7.4) and treated with 2 ml of different concentrations of the nanocomposite solutions in PBS (ranging from 1 to 50 mg/ml). After 4 h incubation, 50 μl samples were withdrawn, and the LDH-content was measured using an available commercial LDH Cytotoxicity Assay kit. In this assay kit, Nicotinamide Adenine Dinucleotide (NAD) reduction was determined by UV–Visible spectrophotometry at 340 nm in the presence of lactate and LDH. PBS was used as the negative control (leading to 0% LDH release), and 0.1% (w/w) Triton X-100 solution in PBS was used as the positive control (leading to 100% LDH release). The LDH-release value was equal to the amount of released LDH to the total LDH contained in the intact cells. Experiments were performed in triplicate.

2.4. Cell culture

HeLa cells, obtained from the human cervical carcinoma cells, were grown in a 25 cm^2 flask in DMEM medium, supplemented with 10% FBS and antibiotic mixture (penicillin:100 Unit/ml; streptomycin:100 μg/ml). The cells were placed inside an incubator in a 5% CO_2 humidified atmosphere at 37 °C. The medium was renewed twice a week.

2.5. The preparation of various concentrations of the drug from CDDP-loaded nanocomposite

To prepare CDDP-loaded nanocomposite, CDDP was loaded into P (NIPAM-co-DMAEMA-co-AFA)-Fe_3O_4 nanocomposite based on the swelling method, as explained later [34]. Briefly, 10 ml of CDDP in 0.9% saline solution (1 mg/ml) was added dropwise into 5 ml of the nanocomposite aqueous solution. The solution was kept in a shaker incubator for 48 h at 5 °C, allowing for swelling of the nanocomposite to encapsulate. Considering that Cisplatin's encapsulation efficiency in the nanocomposite was 65%, before using the CDDP-loaded nanocomposite, the amount of lyophilized nanocomposite was calculated to obtain the required concentration of CDDP. Then, it was reconstituted in phosphate-buffered saline (PBS) and diluted in the culture medium.

2.6. Cytotoxicity assay

The cytotoxicity of the blank nanocomposite, free CDDP, and CDDP-loaded nanocomposite with the HeLa cells were studied using the MTT assay in 24, 48, and 72 h treatment. The HeLa cells were seeded into a 96-well plate (3500 viable cells per well) and were kept for 24 h inside the incubator in a 5% CO_2 humidified atmosphere at 37 °C. The cells were then treated by different concentrations of the blank nanocomposite, the free CDDP, and the CDDP-loaded nanocomposite (3/12–100 μM CDDP for 24 and 48 h incubation, and 0/78–100 μM CDDP for 72 h incubation). Noteworthy, the amount of blank nanocomposite was equal to the amount of CDDP-loaded nanocomposite. After 24, 48, and 72 h incubation, 2 mg/ml of the MTT solution was added to the wells, which were incubated for 4 h at 37 °C. With contents being removed from all the wells, 200 μl of DMSO and 25 μl of Sorensen were added to each well. The absorbance was read in a microplate reader at 570 nm. Each experiment was repeated in triplicate.

2.7. The preparation of the resistant HeLa cells and determination of resistance

The resistant cells were prepared through the periodic exposure of the HeLa cells to different drug concentrations. The cells were cultured stepwise based on the increasing concentrations of the CDDP-loaded nanocomposite and free CDDP. The CDDP's concentration (free or encapsulated) was increased stepwise. Initially, the CDDP was exposed to 0.5 μM concentration, and the cell line was under exposure 4 times every 3 days. Between the cycles, the cells were allowed for growth recovery for 8 weeks. After completing the four treatment cycles, the dose was doubled and the process was repeated until the concentration reached 8 μM. For the Cisplatin-resistant cells by CDDP-loaded nanocomposite (HeLa/R-CDDP.NC), cells cultured with the blank nanocomposite in the same amount as CDDP-loaded nanocomposite in experimental cultures, were used as controls. Cells cultured in the Cisplatin-free media were used as the controls for the Cisplatin-resistant cells by free CDDP (HeLa/R-CDDP). The resistant cells were preserved inside a monolayer culture in DMEM media, and 1 μM Cisplatin was added to the media to retain resistant phenotype. The resistant cells lost resistance within 10–12 months. Prior to the experiment, the resistant cells were cultured continuously in the media, with no Cisplatin, for 2 weeks. The acquired cell resistance level for both the free CDDP and the CDDP-loaded nanocomposite was determined as the IC_{50} ratio of the resistant cells to that of the parental cells. MTT was used to assay Cytotoxicity.

2.8. RNA extraction and cDNA synthesis

RNA extractions were carried out using Trizol reagent according to Chomczynski and Sacchi [37] After the extraction, the RNA concentration was determined by nanodrop (ND-1000, Thermo Scientific, USA) with light absorbance (at the ratio of A260/A280). cDNA synthase kit was used to perform the cDNA synthesis (reverse transcription) on 200 ng of the total RNA in a reaction mixture that contained 1 μl of Oligo dT Primer, 2 μl of dNTP Mix, 1 μl of ribolock RNase Inhibitor, and 1 μl of revert aid MulV Reverse Transcriptase in 4 μl of 5X Reaction Buffer in a final volume of 20 μl. Samples were incubated using Thermo cycler (PEQLAB, Germany) at 25 °C for 5 min, 60 °C for 60 min for reverse transcription, and 70 °C for 5 min for enzyme inactivation, respectively.

2.9. Real-time PCR

Real-time PCR, with *β-actin* expression as the internal control, was used to investigate the gene expression of *P53*, *BCl2, Bax* (involved in cellular apoptosis), *NAPA,* and *CITED2* (upregulated genes in the HeLa cells, resistant to cisplatin). Primer 5 Express software (Applied Biosystems) was used to select the primers (see Table 1).

The reaction was performed in a final volume of 20 μl with Syber Green PCR master mix based on the manufacturer's instructions. Corbett Roter (GENE 6000, USA) was used to incubate 5 pmol/μl of each primer at 95 °C for 5 min for enzyme activation, at 95 °C for 20 s (40 cycles) for denaturation, at 60 °C for 20 s for annealing, and at 72 °C for 20 s for extension. Each experiment was performed in triplicate, and determination of relative expression levels was as follow. For each sample, the Ct of the gene was normalized with the Ct of the *β-actin* gene. Then, the sample underwent a secondary normalization with untreated cells (control). Relative quantitative expression of the interest gene was calculated as follows:

Table 1
Primers used in Real Time- PCR.

Target transcript	Sequence (5′- 3′)
P_{53}:	
Forward Primer	5′ GTTCCGAGAGCTGAATGAGG 3′
Reverse Primer	5′ ACTTCAGGTGGCTGGAGTGA 3′
Bax	
Forward Primer	5′ CTGAGCAGATCATGAAGACAGG 3′
Reverse Primer	5′ CTCCATGTTACTGTCCAGTTCG 3′
BCl2:	5′ GTGGATGACTGAGTACCTGAAC 3′
Forward Primer	5′ GAGACAGCCAGGAGAAATCAA 3′
Reverse Primer	
NAPA	
Forward Primer	5′ CTGTTTGATGCGAGCAATCG 3′
Reverse Primer	5′ GTCCACCAACTCTGTCTCATAG 3′
CITED2	
Forward Primer	5′ CAACCAGTATTTCAACCATCACC 3′
Reverse Primer	5′ CTGGTTTGTCCCGTTCATCTG 3′
β-actin	
Forward Primer	5′ GGCACCCAGCACAATGAAGA 3′
Reverse Primer	5′ CGACTGCTGTCACCTTCACC 3′

$$\Delta Ct = Ct_{\text{gene}} - Ct_{\beta\text{-}actin}$$

$$\Delta\Delta Ct = \Delta Ct_{\text{test sample}} - \Delta Ct_{\text{control sample}}$$

$$\text{Relative expression} = 2^{-\Delta\Delta Ct}$$

To evaluate the relative expression of the genes involved in cellular apoptosis, HeLa cells were divided into two groups, namely the control and the test. The test group was treated with free CDDP and CDDP-loaded nanocomposite at concentrations corresponding to IC_{50} level at 37 °C for 48 h.

2.10. Literature mining-based network discovery of Cisplatin drug resistance in HeLa cells

Pathway Studio v12.4 (2020) [34,38] was used to implement the literature mining-based network analysis and network visualization, as previously described [35,36]. In order to construct the Pathway Studio interactions database, MedScan, a natural language processing engine [39], was used to mine the literature and extract the relationships among PubMed abstracts. The relationships were extracted from 692 full-text Elsevier journal articles and 976 full text articles published by other journals. Thirteen million relations were extracted via the Pathway Studio and 2300 pathways were extracted from the literature. The Pathway Studio is a rich database with a wide array of entities that contain 1,057,236 small molecules and drugs, 141779 proteins/genes, 14960 cell processes, and 157,344 genetic variants. The database is updated weekly.

For network analysis, proteins were selected based on three criteria, already addressed in the literature: a) direct interaction with Cisplatin, b) involvement in drug resistance, and c) expression in HeLa cells.

2.11. Statistical analysis

Three independent experiments were conducted, and the data were reported as means values ± Standard Deviation (SD). An unpaired *t*-test was carried out to analyze the between-group means comparisons. Differences were considered significant at p-values less than 0.05. SPSS software was used to analyze the data.

3. Results and discussion

3.1. Physicochemical characteristics of synthesized nanocomposite as a drug delivery system

As previously reported, the magnetic, pH, and thermo-responsive nanocomposite of P(NIPAM-co-DMAEMA-co-AFA)-Fe_3O_4 was synthesized using free radical emulsion copolymerization. Briefly, Cisplatin was loaded into the nanocomposite via equilibrium swelling method. The lower critical solution temperature (LCST) of the synthesized nanocomposite was approximately 40 °C. The CDDP encapsulation efficiency (EE) was approximately 65%. The CDDP-loaded nanocomposite demonstrated thermal and pH-responsive drug release. The most drug release was observed at the low pH and high temperature (above its LCST) which are conditions relevant to cancer medium. Field-emission scanning electron microscopy (FE-SEM) indicated that the size of CDDP-loaded nanocomposite was approximately 160 nm. The magnetic saturation of the CDDP-loaded nanocomposite represented an appropriate level, showing its capability to produce sufficient heat for application in hyperthermia. The dual pH and thermo-responsive nanocomposite showed efficiency for combined application within the local hyperthermia and controlled drug delivery [34].

The zeta-potential analysis revealed CDDP-loaded nanocomposite bearing a positive charge (in the range of 2–5 mV) due to DMAEMA moieties in the nanoparticles. The positive charge of the surface has the capacity to facilitate the production of a repulsion force, which stabilizes the synthesized particles against aggregation [34].

Regarding the cellular uptake of the designed drug delivery system, the nanocomposite entry into the cells is fulfilled with two stages. In the first stage, the nanocomposite is attached to the cells' surface; and in the second stage, they enter the cells through a specific endocytosis pathway. Since the cell membrane has a negative charge, the nanocomposite particles are expected to have an effective endocytosis due to their positive charge. It is difficult to identify the exact mechanism of endocytosis and cellular uptake, because they are cell-dependent processes [39,40].

3.2. Hemolytic toxicity of nanocomposite

Erythrocytes exist abundantly in human blood. Damage to Erythrocytes results in the release of hemoglobin (an iron-containing-protein), causing anemia, jaundice, and other pathological conditions (e.g., renal failure) [30,41]. Nanocomposite characteristics such as small size, large surface area, and unique physiochemical properties can facilitate their interaction with red blood cells [42,43]. The amphiphilic nanocomposite copolymers, prepared in this study, were found to be capable of entering the lipids or phospholipid membranes and destabilize them [44]. Hence, the determination of hemolytic properties is among the most common and valuable tests for assessing the adverse effects of the prepared nanocomposite on blood components. Consequently, it is argued that hemolysis assay can provide further insights into hemocompatibility in future *in vivo* applications.

Fig. 1 shows the hemolytic activities of the blank nanocomposite solutions in terms of different concentrations. Although the nanocomposite's concentrations were high, they did not cause significant erythrocyte lysis (<5%) the same also applies to lower concentrations.

The hemolysis percentage of the nanocomposite was found to be independent of its concentration; it was lower than 5%. According to the Guiding Principles of the Hemolysis Test [H]GPT4-1, the samples were considered as non-hemolytic if the hemolysis percentage was less than 5%. As a result, the blank nanocomposite did not induce hemolysis. Our data underpinned that the nanocomposite was appropriate for drug delivery and intravenous applications.

3.3. Platelet activation by nanocomposite

Platelet activation and aggregation have important roles in the body's defense mechanisms against exogenous materials or pathogens [45]; they are essential to vascular hemostasis and thrombosis [46]. However, in terms of toxicity, they can lead to the development of thrombotic disorders and atherosclerosis [47,48]. Also, the determination of nanoparticles' interactions with blood platelets is highly

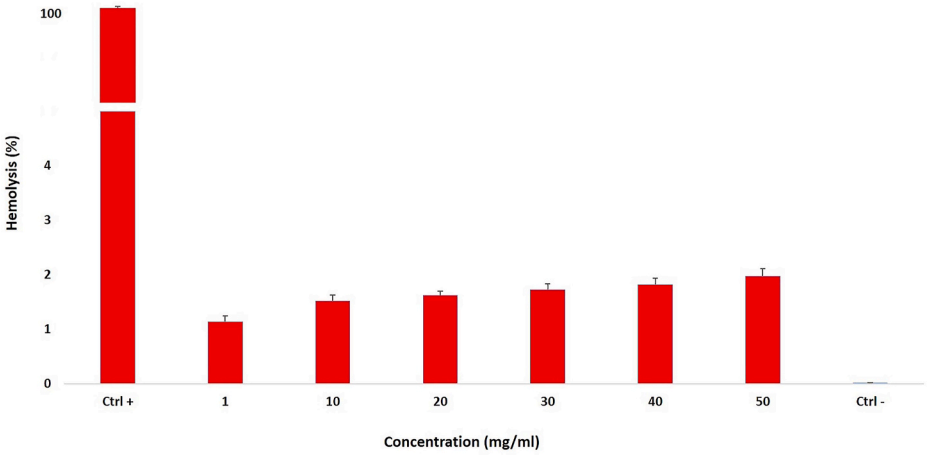

Fig. 1. Hemolysis of blank nanocomposite solutions in different concentrations (ranging from 1 to 50 mg/ml). Data represent mean ± SD (N = 3).

important. Fig. 2 shows the results of platelet aggregation survey and the effect of various concentrations of the nanocomposite solution on the platelet aggregation. In concentrations 1–50 mg/ml, the nanocomposite failed to induce any detectable platelet aggregation. In each sample, the aggregation was measured in terms of the absorbance at 500 nm. Collagen was incubated with platelets as the positive control of platelet aggregation in the presence and absence of the nanocomposite (50 mg/ml). As a result, the nanocomposite prevented the collagen-induced platelet aggregation, and in the presence of the nanocomposite, platelet aggregation was significantly less than that of pure collagen. These results are consistent with the hypothesis that interaction of nanocomposite with blood components is dictated by their composition, size, surface hydrophobicity, and charge. The nanoparticles' size has an important effect on platelet aggregation, as larger nanoparticles induced platelet aggregation more easily. Furthermore, nanoparticles with more hydrophobic surfaces led to a greater platelet activation and aggregation. In the same vein, negatively charged nanoparticles induced a platelet aggregation greater than cationic or neutral nanoparticles [49,50].

3.4. Cytocompatibility of nanocomposite

As the cell membrane is a potential site for cationic nanocomposite interaction, the LDH test unraveled the nanocomposite's destructive effect on cell's membrane [28,51]. The nanocomposite *in vitro* effects on the cell membrane were studied on 3T3 mouse fibroblast cells based on different concentrations. The negative control, without nanocomposite (only PBS), showed that the cells were stable during the LDH experiment. As shown in Fig. 3, the nanocomposite in concentrations 1–50 mg/ml failed to affect the cell membrane integrity, exhibiting scarce plasma membrane toxicity. The amount of LDH release remained below 5% within the entire experimental concentrations.

3.5. In vitro cytotoxicity to HeLa cells

MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) was used to investigate the cytotoxicity of the blank nanocomposite, free CDDP, and CDDP-loaded P(NIPAM-co-DMAEMA-co-AFA)-Fe_3O_4 nanocomposite to against the HeLa cells. The MTT assay was employed to evaluate the intracellular effects on mitochondria and the metabolic activities. Based on the selective ability of viable cells to

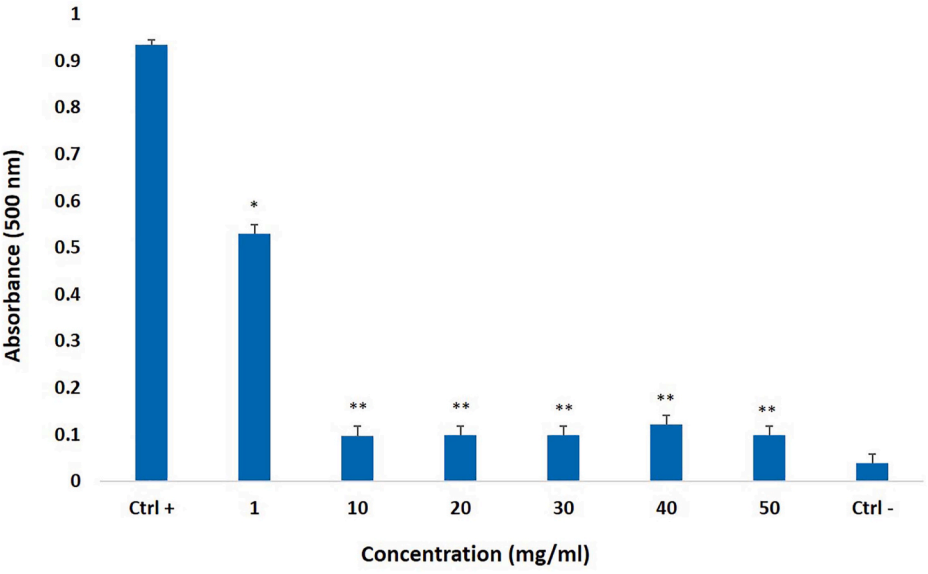

Fig. 2. Effect of various concentrations of nanocomposite solution (ranging from 1 to 50 mg/ml) on platelet aggregation. PBS (the negative control) and collagen (Col) (the positive control) were incubated with platelets in the presence and absence of nanocomposite (50 mg/ml). Data represent mean ± SD (N = 3). Absorbance change measured at 500 nm *: $p < 0/05$, **: $p < 0/001$ Compared with the positive control.

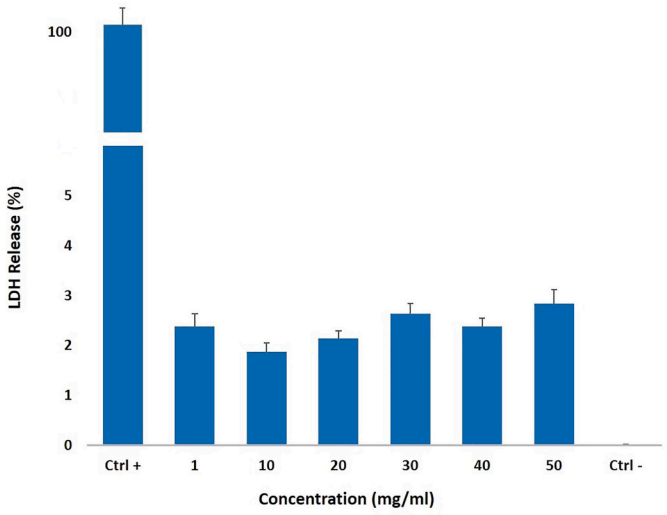

Fig. 3. LDH-release value caused by nanocomposite in 1–50 mg/ml concentrations studied on 3T3 mouse fibroblast cells. Data represent mean ± SD (N = 3). Abbreviations; LDH: Lactate Dehydrogenase.

reduce MTT into purple formazan, the colorimetric MTT test was used to assess the cytotoxicity. Figs. 4 and 5 compare the *in vitro* cytotoxicity of the blank nanocomposite, free CDDP, and CDDP-loaded nanocomposite against the HeLa cells, as determined by the MTT assay. After 24 h of incubation with the blank nanocomposite, the HeLa cells viability was retained to approximately 100%, implying that the blank nanocomposite had no effect on the cytotoxicity (see Fig. 4). As shown in Fig. 5, free CDDP and CDDP-loaded nanocomposite significantly decreased the viability of the cells in a dose- and time-dependent manner. Also, the CDDP-loaded nanocomposite exhibited higher *in vitro* cytotoxicity than the free CDDP during 48 and 72 h incubation times ($p < 0.05$), underpinning the effectiveness of the designed drug delivery system in promoting the cytotoxicity and efficacy of CDDP on cancer cells.

Electrostatic interactions between the magnetic nanoparticles (negative charge) and CDDP (positive charge) in the CDDP-loaded nanocomposite led to a controlled and sustained release of CDDP from the prepared nanocomposite. Consequently, within 24 h of incubation, the increase in the CDDP-loaded nanocomposite cytotoxicity was found to be insignificant ($p > 0.05$) as compared with the free CDDP (Fig. 5A). Because of the CDDP deactivation in the biological environment by nitrogen and sulfur biomolecules; the half-life of CDDP *in vitro* is about 2 h after administration [52]. In this study, the free CDDP showed relatively stable cytotoxic effect on the HeLa cells at the incubation outset. As the incubation time increased, the test results were affected by a number of confounding factors, including the continuous growth of the remaining cells, nutrients reduction in the cell culture media, and hypoxia. Consequently, the CDDP released from the CDDP-loaded nanocomposite was biologically active over time, showing more cytotoxic effects than the free CDDP during the 48 and 72 h incubation times.

3.6. Expression of genes involved in cell apoptosis

The tumor suppressor gene *P53* was activated in response to various cellular stress types in order to influence the sensitivity of the cancerous cells to diverse chemotherapeutic drugs. For example, the protein p53 was involved in modulating the activity of the Nucleotide Excision Repair (NER) pathway in response to Cisplatin-induced DNA damage. Increased reparation of DNA double-strand breaks, was considered as an important mechanism in Cisplatin resistance [53]. Cisplatin caused inhibition of DNA replication fork progression in the dividing cells by forming inter-strand crosslinks leading to activation of NER pathway and Cisplatin resistance. *P53* activates several genes that affect the apoptosis process (e.g., *P21*) with arrested cell cycle and induced apoptotic [45,46]. The apoptotic gene expression levels including *P53, Bax*, and *Bcl*-2 mRNA were analyzed after treating the CDDP and CDDP-loaded nanocomposites with IC_{50} concentration for 48 h. The Ct values of these genes were normalized against mRNA level of *β-actin,* as the housekeeping gene. As illustrated in Fig. 6, we observed a significant increase ($p < 0.001$) in the expression levels of *P53* and pro-apoptotic *Bax* gene, and a significant decrease ($p < 0.05$) in the expression level of anti-apoptotic *Bcl*-2 mRNA in the CDDP-loaded nanocomposite--treated cells as compared with the free CDDP-treated cells ($p < 0.05$).

3.7. Expression of genes associated with drug resistance in CDDP-resistant HeLa cells

A genome-wide analysis of the Cisplatin-resistant HeLa cells showed upregulation in the expression of 9 genes, including *NAPA, CITED2, CABIN1, ADM, HIST1H1A, EHD1, MARK2, PTPN21*, and *MVD* [9,54]. Among these genes, N-ethylmaleimide-sensitive factor attachment protein alpha (*NAPA*) and CBP/P300-interacting transactivator protein with Glu/Asp-rich carboxy-terminal domain (*CITED2*) are of particular interest in the acquisition of Cisplatin resistance for constructing a connection with the tumor suppressor *P53*. Irrespective of their location in the cellular compartments, these two gene products communicate with the cancerous cells. While *CITED2* encodes a nuclear protein and regulates its transcription, *NAPA* encodes a cytoplasmic protein and functions as an intracellular transportation means [9,44].

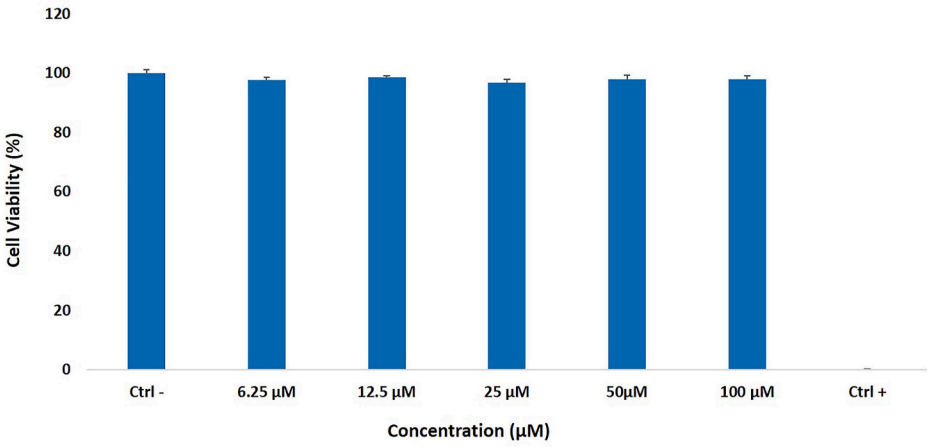

Fig. 4. Cytotoxic effect of different concentrations of blank nanocomposite to HeLa cell line for 24 h' incubation. Data represent mean ± SD (N = 3).

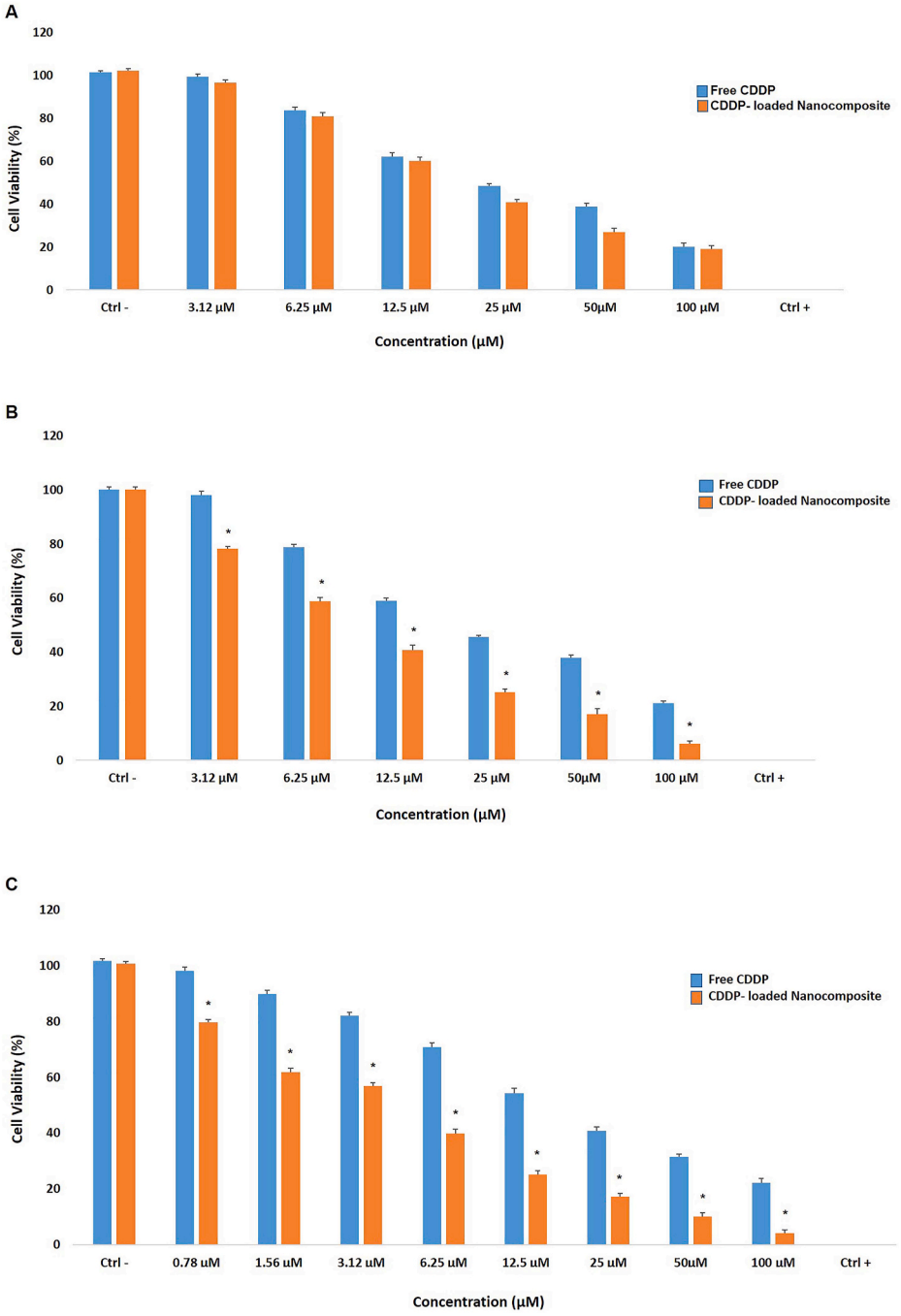

Fig. 5. Cytotoxic effect of different concentrations of free CDDP and CDDP-loaded nanocomposite to HeLa cell line at 24 h (A), 48 h (B), and 72 h (C) incubation times. Data represent mean ± SD (N = 3). *: p < 0/05, as compared with free CDDP. Abbreviations; CDDP: Cisplatin.

The upregulation of *NAPA* expression leads to an increased cellular Cisplatin resistance because of the reduction in endoplasmic reticulum (ER) stress, apoptosis suppression, and the decrease in Cisplatin effect on the cell [9].

Depending on the content and the environmental stimulus, the cellular transactivation and modification of p53 protein can positively/negatively be regulated by CBP/p300. For instance, the phosphorylation of p53N-terminal residues lead to the interaction of p53with CBP/p300 and its acetylation at the C-terminus, increasing the stability and sequencing of p53as a special DNA-binding function. As a CBP/P300-interacting transactivation protein, *CITED2* is essential for the modification of genes involved in cellular growth and oncogenesis. Therefore, it is proposed that *CITED2* can alter a cell's sensitivity to Cisplatin by affecting the *P53* stability [44,45,54].

As shown in Fig. 7, the increased expression of the mRNA in *NAPA* and *CITED2* genes in HeLa/R-CDDP and HeLa/R-CDDP.NC were 12.7-fold and 9.4-fold, respectively ($p < 0.05$).

3.8. Cisplatin resistance cellular evaluation in the prepared CDDP-resistant HeLa cells

MTT assay within 48 h' treatment was used to evaluate the cytotoxicity of Cisplatin on prepared resistant cell lines and determine the degree of drug resistance. Table 2 illustrated, the degree of drug resistance compared drug-sensitive cells of the HeLa/R-CDDP and HeLa/R-CDDP.NC is 18.6- and 1.1-fold, respectively. The results, in parallel

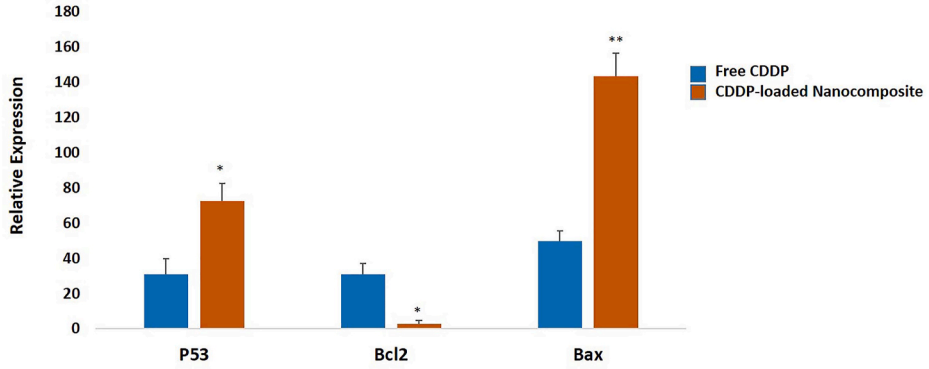

Fig. 6. Relative expression of *Bax, Bcl2*, and *P53* genes in HeLa within 48 h' incubation time. Treatment with Free CDDP and CDDP-loaded nanocomposite in IC50 concentration. The data are presented as mean ± SD (N = 3). (*P < 0.05, **P < 0.001). Abbreviations; CDDP: Cisplatin.

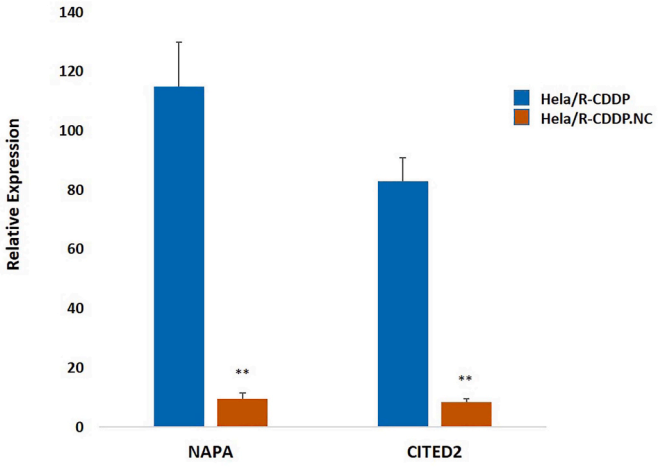

Fig. 7. Relative expression of NAPA and CITED2 genes in HeLa within 48 h' incubation time. Treatment with HeLa/R-CCDP and HeLa/R-CDDP-NC. The data are presented as mean ± SD (N = 3) (**P < 0.001). Abbreviations; R: Resistant, NC: Nanocomposite.

Table 2
Cell lines, IC_{50} value (μM), and degree of drug resistance in HeLa cells after the 48 hourstreatment

Cell lines	IC_{50} (μM)	Degree of drug resistance compared to drug-sensitive cells
HeLa (drug-sensitive)	14.1 ± 1.1	–
HeLa/R-CDDP	262.5 ± 0.99	18.6 fold
HeLa/R-CDDP.NC	16.3 ± 1.06	1.1 fold

with the study of drug-resistant genes expression, conformed that the prepared nanocomposite could be considered as possible drug delivery system for chronic cancer therapy without enticing resistance in cancerous cells. In line with our finding, it has been suggested that rapid increase in intracellular drug concentration and maintenance of high drug concentration in cancer cells can decline their drug resistance [15, 55].

3.9. Gene interaction network underlying the cisplatin drug resistance in the HeLa cells

Fig. 8 represents the HeLa cells' interaction network underlying the expression of genes that play a role in drug resistance and interaction with Cisplatin. Supplementary 1 to 3 illustrate the networks entities, their underlying relationships, and the references extracted from the literature. Table 3 presents the key components of the Cisplatin drug resistance network, including the transcription factors, microRNAs, transporters, ligands, and receptors. Cisplatin can directly upregulate *TFAM, NR1I2, TP73,* and *TP53*. These transcription factors are expressed in the HeLa cells and are involved in the regulation of drug resistance. As a transcription factor expressed in the HeLa cells, *NR4A1* plays a key role in Cisplatin-induced apoptosis by repressing tumorigenesis. Thus, *NR4A1* can be considered a biomarker of Cisplatin success in repressing cancer.

Cisplatin interacts with a number of HeLa cells' transporters, videlicet *VDAC1*, *SLC31A1*, and *SLC9A1* s. While Cisplatin positively regulates *VDAC1*, it represses the expression of *SLC31A1* and *SLC9A1* (see Table 3).

Cisplatin also interacts with secreted ligands, such as *EDN1*, *CD40*LG, *INS*, *TNFSF11,* and *PRL*. These extra cellular ligands can be considered as biomarkers of resistance or tumor sensitivity to Cisplatin (see Fig. 8).

Data from the literature mining suggest that Cisplatin repress the expressions of *EDN1*, *CD40*LG, and *TNFSF11*. As these ligands are positively correlated with drug resistance (see Table 3), any decline in their expression, be it at the level of gene or protein, can be considered as the biomarker of Cisplatin effectiveness on tumor control. Table 3, indicates that expressions of *INS* and *PRL* can be considered as the biomarker of tumor resistance to Cisplatin.

4. Conclusion

The present study argues that the prepared nanocomposite, as a drug delivery system, was non-toxic and biocompatible. As compared with the free CDDP, CDDP-loaded nanocomposite showed greater cytotoxicity effect on the HeLa cells, which were found to be dose- and time-dependent. Our data suggested that nanocomposite could be deemed as an effective smart drug delivery carrier for intravenous application and long-term use of Cisplatin without promoting drug resistance in cancerous cells. The performed literature mining-based network discovery elucidated the molecular mechanism underlying the Cisplatin resistance in the HeLa cells. We also found that decreased expression of *EDN1*, *CD40*LG, and *TNFSF11* ligands and increased expression of *NR4A1* transcription factor could be considered as biomarkers of tumor sensitivity to Cisplatin.

Author contributions

Zahra Shakoori: Resources, Software, Data curation, Writing- Original draft preparation, Investigation, Formal analysis.

Roghiyeh Pashaei-Asl: Writing- Reviewing and Editing, Software, Validation, Formal analysis, Investigation.

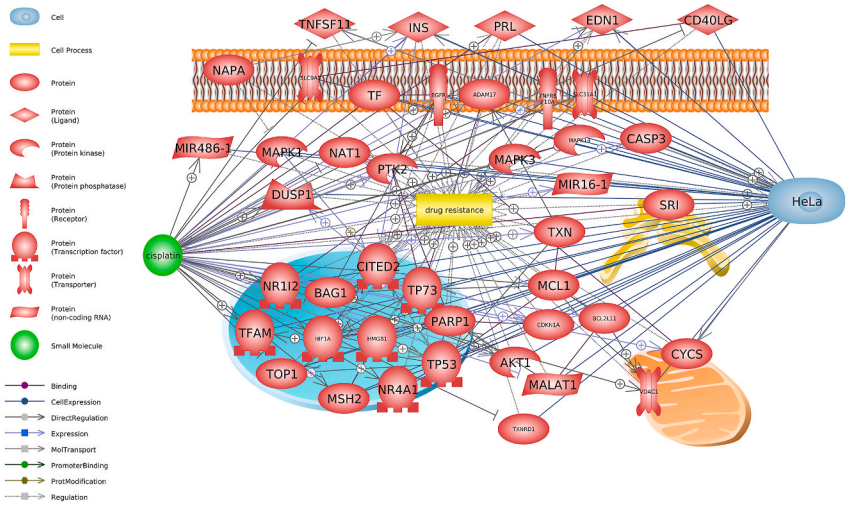

Fig. 8. Interaction network of HeLa cells expressed genes involved in drug resistance and interaction with Cisplatin.

Table 3
Key components of Cisplatin drug resistance network, including transcription factors, microRNAs, transporters, ligands, and receptors.

Name	Description	Class of gene/ protein	Interaction with Hela cells	Interaction with drug resistance	Interaction with Cisplatin
TFAM	transcription factor A, mitochondrial	Transcription factor	Cell Expression: HeLa —— TFAM	Regulation: TFAM —>drug resistance	Positive Direct Regulation: cisplatin –+> TFAM
NR4A1	nuclear receptor subfamily 4 group A member 1	Transcription factor	Cell Expression: HeLa —— NR4A1	negative Regulation: NR4A1 —| drug resistance	Direct Regulation: cisplatin — > NR4A1
TP53	tumor protein p53	Transcription factor	Cell Expression: TP53 —— HeLa	Regulation: TP53 —>drug resistance21224	Positive Direct Regulation: cisplatin –+> TP53
TP73	tumor protein p73	Transcription factor	Cell Expression: TP73 —— HeLa	Regulation: TP73 —>drug resistance	Positive Direct Regulation: cisplatin –+> TP73
HIF1A	hypoxia inducible factor 1 subunit alpha	Transcription factor	Cell Expression: HeLa —— HIF1A	Positive Regulation: HIF1A –+> drug resistance	Negative Expression: cisplatin —| HIF1A
HMGB1	high mobility group box 1	Transcription factor	MolTransport: HeLa —>HMGB1	Cell Expression: HMGB1 —— HeLa	Positive Direct Regulation: cisplatin –+> HMGB1
NR1I2	nuclear receptor subfamily 1 group I member 2	Transcription factor	Cell Expression: NR1I2 —— HeLa	Positive Regulation: NR1I2 –+> drug resistance	Positive Direct Regulation: cisplatin –+> NR1I2
VDAC1	voltage dependent anion channel 1	Transporter	Cell Expression: HeLa —— VDAC1	Regulation: VDAC1 —>drug resistance	Positive Direct Regulation: cisplatin –+> VDAC1
SLC31A1	solute carrier family 31 member 1	Transporter	Cell Expression: HeLa —— SLC31A1	Regulation: SLC31A1 —>drug resistance	Negative Expression: cisplatin —| SLC31A1
SLC9A1	solute carrier family 9 member A1	Transporter	Cell Expression: HeLa —— SLC9A1	Regulation: SLC9A1 —>drug resistance	Negative Direct Regulation: cisplatin —| SLC9A1
EDN1	endothelin 1	Ligand	Cell Expression: EDN1 —— HeLa	Positive Regulation: EDN1 –+> drug resistance	Negative Expression: cisplatin —| EDN1
CD40LG	CD40 ligand	Ligand	Cell Expression: HeLa —— CD40LG	Positive Regulation: CD40LG –+> drug resistance	Negative Direct Regulation: cisplatin —| CD40LG
INS	Insulin	Ligand	Cell Expression: HeLa —— INS	Positive Regulation: INS –+> drug resistance	Positive Expression: cisplatin –+> INS
TNFSF11	TNF superfamily member 11	Ligand	Cell Expression: TNFSF11 —— HeLa	Positive Regulation: TNFSF11 –+> drug resistance	Negative Direct Regulation: cisplatin —| TNFSF11
PRL	Prolactin	Ligand	Cell Expression: HeLa —— PRL	Regulation: PRL —>drug resistance	Positive Regulation: cisplatin –+> PRL

Maryam Pashaiasl: Project administration, Conceptualization, Methodology, Validation, Supervision.

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Declaration of competing interest

The author declared that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jddst.2022.103254.

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