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Anti-proliferative impact of three Schiff base platinum (II) complexes against human breast cancer cell line

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ABSTRACT: Treatment approaches for breast cancer (BC) are challenging in terms of tumor recurrence and drug resistance. We investigated the antitumor activity of three platinum (II) complexes bearing Schiff base ligands against breast cancer cells MCF-7. MCF-7 cells were treated with three platinum complexes and drug cytotoxicity was evaluated using an MTT assay. To quantify apoptosis and to confirm the mode of cell death, flowcytometry analysis using annexin V-FITC-PI kit and DNA fragmentation assay was used, respectively. The possible cytotoxic mechanism of action of these platinum compounds was also determined through mRNA expression analysis by the Real time PCR method. All complexes show a dose-dependent inhibitory effect on cell viability. flowcytometry analysis shows that the average percentage of apoptosis in 50 µM concentration of all complexes is 23±3.5% and DNA fragmentation assay confirmed apoptosis. Treatment of cells with complexes significantly decreases mRNA expression of topoisomerase 1 and 2 and Bcl-2 as compared to untreated control cells. Also, our results show that both caspase 6 and 9 genes expression levels were induced at high dose (50 µM) of complexes. Our findings indicate that three Schiff base platinum (II) complexes have potent antiproliferative effect against breast cancer cells and induce apoptosis via the mitochondrial pathway. Also, our data provide evidence for a possible role of Schiff base platinum complexes in inhibition of breast tumor progression through downregulation of HIF-1α, topoisomerase 1 and 2 genes. These data support the idea that these complexes would be promising anti-breast cancer drugs.

KEYWORDS: Platinum (II) complexes; schiff base ligands; apoptosis; MCF-7 cell line

1. INTRODUCTION

Breast cancer (BC), the most prevalent malignancy, is the leading cause of mortality among women throughout the world [1]. BC is a multifactorial disease and a variety of risk factors, including age, genetic factors, and early menarche contribute to its occurrence [1,2]. Early detection of BC by mammography screening method, can reduce the mortality rate [3]. Surgical resection methods combined with radiotherapy or chemotherapy are considered an efficient treatment for BC [4].

One of the widely used regimes in anticancer therapy is platinum-based compounds (PBCs) because of their ability to crosslink with DNA molecules, particularly N7 or O6 in purine bases. This can lead to cell cycle arrest and apoptosis or necrosis in tumor cells. Two well-known PBCs including cisplatin and carboplatin show a high antitumor effect against numerous cancer cells including breast, ovary, lung, and bladder [5-7]. Although PBCs were more effective in the treatment of breast cancer compared to other agents, their side effects and resistance are inevitable [8,9]. Drug resistance leads to cancer recurrence and metastasis and it is a major cause of treatment failure [10,11]. Therefore, it is essential to develop novel anticancer drugs with fewer side effects to target cancerous cells.

Recently, Schiff bases have shown therapeutic effects due to their extensive anti-inflammatory, antimicrobial, and anticancer properties [12]. It has been shown that Schiff base metal complexes also have a cytotoxic effect against cancerous cells [9,13,14]. The evaluation of anticancer property of Schiff base copper (II) and platinum (II) complexes against MCF-7 cell line showed that the complexes can decrease cell viability and arrest tumor growth [12,14,15].

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Platinum complexes bearing Schiff base ligands derived from 1,2-diamino-1,2-diarylethane could increase platinum cytotoxicity [16]. These compounds bind and break double-stranded DNA through hydrolytic and oxidative cleavage pathways, leading to DNA damage and cell apoptosis [12].

As the most common form of programmed cell death, the apoptosis process is crucial for maintaining the balance of cell death and cell growth. Dysregulation of the apoptosis pathway including disruption of caspase function and balance between pro and anti-apoptotic proteins plays a pivotal role in carcinogenesis. Restoration of apoptotic cell signaling, may have the potential to eliminate malignant cells [17,18].

Autophagy is an alternative form of programmed cell death [19]. The imbalance of the autophagy regulatory mechanisms leads to the accumulation of damaged organelles and proteins and development of complicated diseases such as cancer [19]. Liang et al. [20] have demonstrated that essential genes in the regulation of autophagy pathways such as beclin-1 can reduce mammary cell growth and induce autophagy in breast cancer cells. Koňariková et al. [21] indicated that Cu (II) complexes containing Schiff bases exert a strong cytotoxic effect against MCF-7 cell line and induce apoptosis, and autophagy by targeting the cell cycle.

Another key regulatory factor in tumor growth is hypoxia. Angiogenesis, glycolysis, immortalization, genetic instability, tissue invasion, and metastasis are some pathways regulated by hypoxia [22]. Hypoxia-inducible factor-1α (HIF-1α) as a transcription factor, is induced by hypoxia and plays a pivotal role in primary mammary tumor growth and metastasis. It can be targeted by anticancer drugs to block the downstream function of genes that have potentially important roles in tumor therapy [22,23]. Furthermore, topoisomerases are proved to be potential targets for therapeutic approaches in cancers. Following events such as replication and transcription that modify the DNA topology, these enzymes untangle and restore native DNA [24,25]. Anticancer agents targeting topoisomerases contribute to replication fork arrest and double-strand break formation [25]. As a result, cellular DNA damage and even apoptosis may accrue [24,25].

There have been several studies on the function of human topoisomerase-inhibiting metal-based complexes, including some platinum and copper complexes [24].

In the present work, we investigate the cytotoxicity of platinum (II) complexes bearing Schiff base ligands with three different molecular weights (536.6, 550.6, 564.6 g/mol) against MCF-7 breast cancer cells. In addition, the potential molecular mechanism of the cytotoxic property of these synthetic agents was studied.

2. RESULTS

2.1. Anticancer effect of three platinum complexes against MCF-7 cells

The cytotoxic effect of platinum complexes against BC cells (MCF-7) after treatment of cells with different concentrations (1.56-100 µM) of complexes was performed using an MTT assay. All complexes show a dose-dependent inhibitory effect on cell viability (Figure 1). The IC_{50} values of A, B, and C complexes, and carboplatin for 72 h treatment in the MCF-7 cell line are shown in Table 1. Our result demonstrates that complex A, B, and C have lower IC_{50} values in comparison to carboplatin as the standard chemotherapy drug. A lower dose of each complex is needed for killing breast cancer cells effectively than carboplatin. As shown in Table 1, complex B has the lowest IC_{50} value, indicating it has the most cytotoxic effect against MCF-7 cells in comparison to other complexes. In Figure 1, the pattern of cells response to antitumor property of complex A, B, C, and carboplatin was shown.

2.2. Effect of three platinum complexes on mRNA expression levels of topoisomerase1, topoisomerase2, HIF-1a, apoptotic and autophagy related genes

mRNA expression levels of selected genes were detected after treatment of BC cells with different concentrations (12.5, 25, 50 µM) of A, B, and C complexes for 24h. As illustrated in Figure 2, topoisomerase 1 and 2 expression levels were significantly downregulated after treatment of cells with all concentrations of complex A and high concentrations (25 and 50 µM) of complex B and C. HIF-1a gene downregulation was mostly significant in 50 µM concentration of three complexes. Although, treatment with complexes could not affect Bax gene expression level, significantly decreased expression of Bcl-2 was noted in all concentrations of complex A and 50 µM of complex C. Also, our results show that both caspase 6 and 9 genes expression levels were induced at a high dose (50 µM) of A and C complexes. However, mRNA expression of other caspases including caspase 3 and 8 was not affected by complexes. Treatment of MCF-7 cells with different

concentrations of complexes could not significantly affect gene expression level of the autophagy marker, beclin-1.

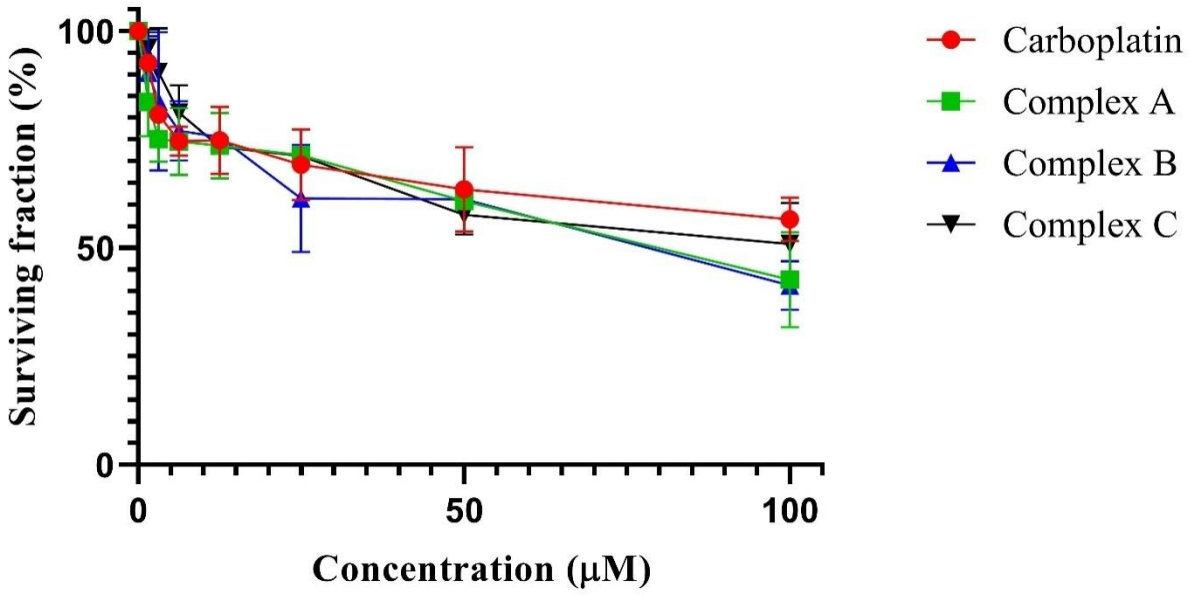

Figure 1. Surviving fraction of MCF-7 cell lines after treatment with three platinum complexes. An MTT assay was used to evaluate the dose-response curve for the effect of different concentrations of A, B, and C complexes, and carboplatin on MCF-7 cells for 24 hours. Values are mean ± S.D, each performed in triplicate.

Table 1. IC_{50} values of carboplatin and three platinum complexes in MCF-7 cell line

	IC_{50} value, μM						
cell line	Carboplatin	Complex-A	Complex-B	Complex-C	P-value[a]	P-value[b]	P-value[c]
MCF-7	186.81±4.99	117.10±2.54	66.72±6.98	95.34±3.67	0.0087	0.0003	0.0009

[a]P-value for Carboplatin vs. Complex-A. [b]P-value for Carboplatin vs. Complex-B. [c]P-value for Carboplatin vs. Complex-C

2.3. Apoptosis detection by flowcytometry following treatment of cell with platinum complexes

Flowcytometry was carried out to determine the mode of cell death after exposure of cells to different concentrations (12.5, 25, 50 μM) of A, B, and C complexes for 24 h. As shown in Figure 3 the average percentage of apoptosis in 50 μM concentration of all three complexes is 23±3.5%. Cells represent 27.1±1%, 21.8±0.36%, and 20.7±0.46% apoptosis when treated with A, B, and C complexes (50 μM), respectively in comparison to cells without complexes treatment.

2.4. Apoptosis detection by DNA laddering

DNA laddering is a hallmark of apoptosis. Flowcytometric analysis shows (Figure 3), complex A could potentiate apoptosis more effectively than other complexes. To confirm apoptosis and gain more insight into the mode of cell death, MCF-7 cells were treated with different concentrations (12.5, 25, 50, 100 μM) of complex A and then a DNA laddering assay was done. The result showed an increase in the DNA fragmentation in a dose-dependent manner in the MCF-7 cell line (Figure 4). As it is shown in Figure 4, with increasing complex concentration, the amount of DNA fragmentation has also increased. The highest amount of DNA fragmentation was observed at the highest concentration of complex A (100 μM).

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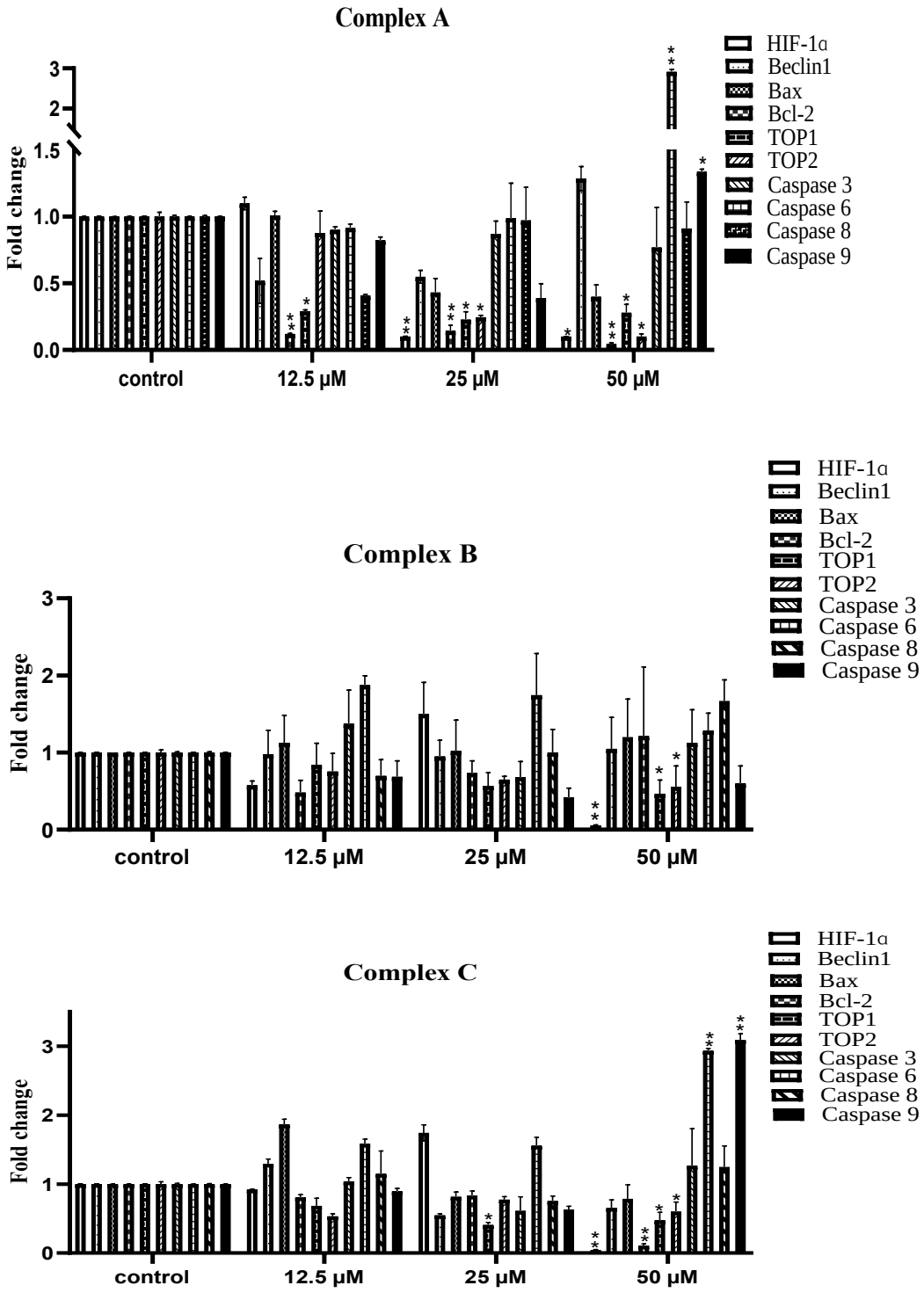

Figure 2. Effects of three platinum complexes (A, B, and C) on mRNA expression levels of genes in breast cancer MCF-7 cell line. Cells were treated with 12.5, 25, and 50 µMof Schiff base platinum complexes for 24 h, and then total RNA was extracted and used for RT-qPCR. Results are expressed as the mean ± SD. ($p< 0.05$ *, $p< 0.001$ ** as compared to control).

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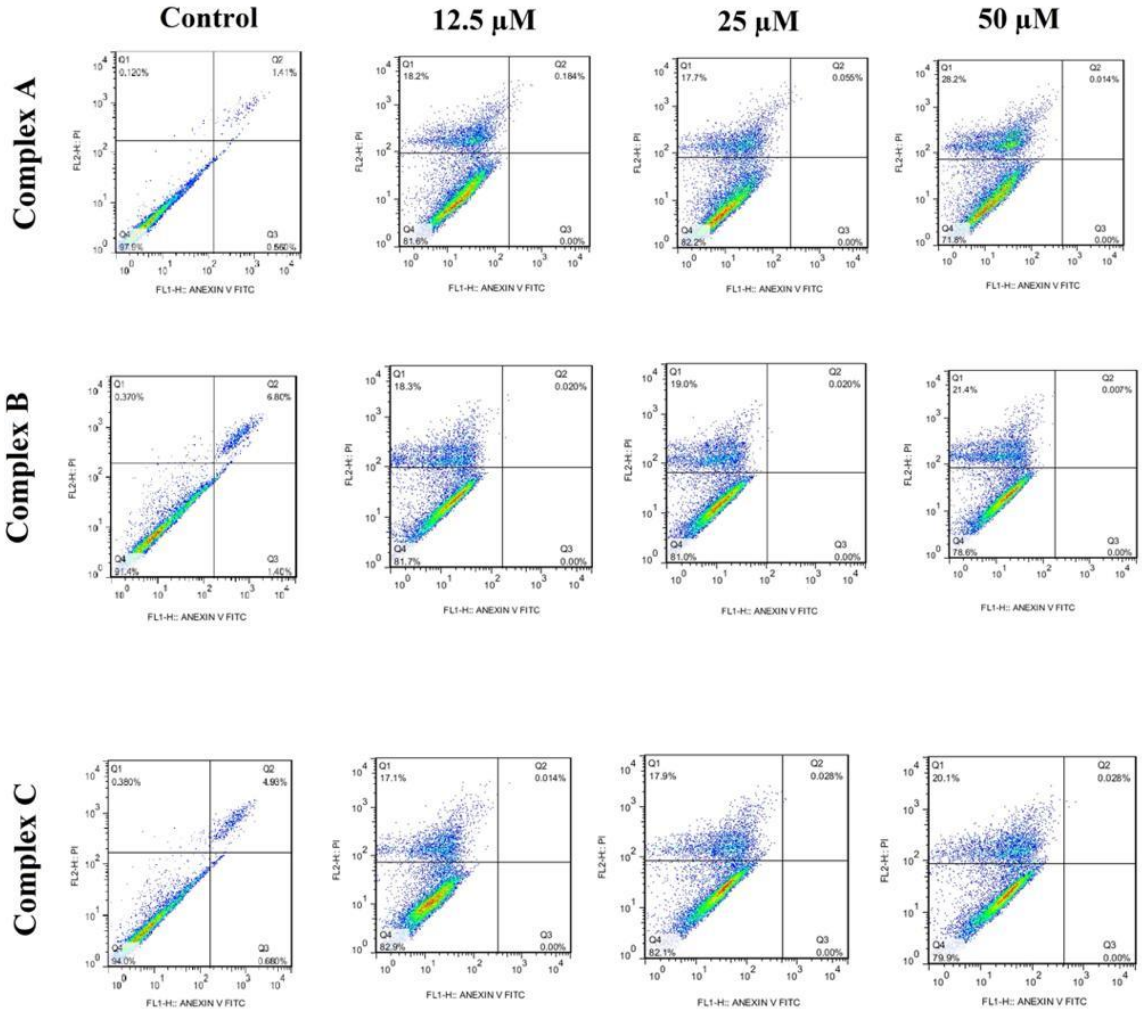

Figure 3. Flowcytometric assay of apoptosis induction using Schiff base platinum complexes in MCF-7 cells. The cells were treated with different concentrations of compounds for 24 h and apoptotic cells were evaluated by flowcytometry using annexin V-FITC/PI staining. Each experiment was done in triplicate.

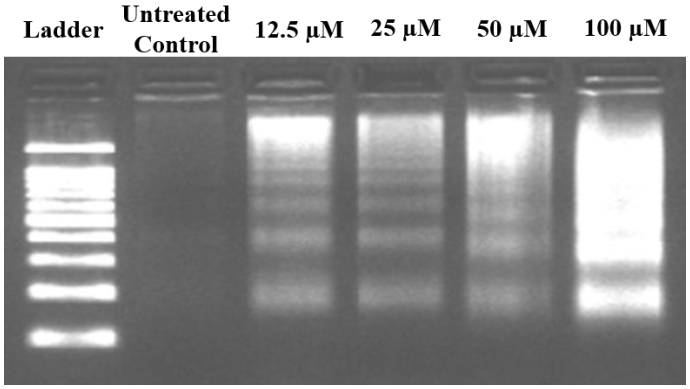

Figure 4. DNA laddering assay for apoptosis detection. Agarose gel electrophoresis of fragmented nucleosomal DNA induced by complex A in MCF-7 cell line. The MCF-F cell line was treated with different concentration of complex A for 24 h and then DNA fragmentation was assessed.

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3. DISCUSSION

Breast cancer is one of the major causes of death worldwide [1]. To date, approaches for tumor eradication and prolonging the survival of patients with BC are surgery, chemotherapy, and radiotherapy. However, in terms of tumor recurrence and drug resistance, these methods are challengeable [27].

Recently Schiff base complexes have paid attention in medical and pharmaceutical fields due to their biological activities such as anticancer property [28]. The anticancer activity of schiff base complexes has been reported in numerous types of cancers [14,29].

In the present work, the anticancer properties of three platinum (II) complexes bearing schiff base ligands against MCF-7 breast cancer cells were investigated. These compounds were previously synthesized and characterized. Our results demonstrate that treatment of MCF-7 cells with various concentrations (1.56-100 µM) of these complexes decreases cell viability in dose-dependent manner (Figure 1), confirming its cytotoxicity against BC cells.

As shown in Table 1, the IC50 values of platinum complexes were significantly lower in comparison to the standard chemotherapy drug carboplatin. A very low concentration of complexes was required to arrest tumor growth and induce apoptosis. In agreement with our study, the antitumor property of the platinum complexes was assessed by MTT assay against the HL-60, BGC-823, Hela, and HepG2 cell lines [29]. Their findings revealed that the complexes could have selectivity kill cancerous cells [29]. The anticancer activity of the Schiff base platinum complex was also evaluated by Gupta et al. [12] using an MTT assay against the MCF-7 cell line and the result showed that the complex is capable to decrease cell viability and arrest tumor growth.

To find the number of cells that underwent apoptosis upon treatment with different concentrations (12.5, 25, 50 µM) of A, B, and C complexes, annexin V/PI staining using a flow cytometer was used. As shown in Figure 3, treatment of cells with complexes revealed a significant increase in the proportion of apoptotic cells. Complex A was more potent than other complexes to induce apoptosis. As reported before, [12] MCF-7 cells stained with annexin V and propidium iodide demonstrated the early and late apoptosis and necrosis after exposure of cells to platinum (II) complexes of Schiff base ligands. Also, in Gupta et al work, after incubation of MCF-7 cells with 5, 25, and 50 µM concentrations of complexes, the distribution of different cell cycle phases was substantially altered compared to untreated control [12].

To confirm apoptosis induction by complexes, a DNA fragmentation assay was also used. The results show that treatment of MCF-7 cells with different concentrations of one of complexes (complex A) could markedly induce DNA fragmentation patterns compared to untreated control cells (Figure 4). Therefore, both annexin V/PI analysis and DNA fragmentation methods confirm that these complexes can induce cell death by inducing apoptosis.

In the present work, the underlying molecular mechanism of action of three complexes were evaluated. The transcript levels of different genes were determined using quantitative real time PCR assay following treatment of MCF-7 cells with various concentrations of each complex.

Aberrant caspase gene expression levels have a major role in different types of cancers [30]. Death receptor (extrinsic) and mitochondrial (intrinsic) pathways are two pathways that mediate the apoptosis regulated by caspase initiators (caspase-2,-8,-9 and-10) and caspase effectors (caspase-3, -6, and-7) [30, 31]. The extrinsic pathway is activated in response to ligand binding of superfamily members of the death receptor, resulting in caspase-8 activation. On the other hand, by the mitochondrial release of cytochrome C, the intrinsic pathway is activated, leading to the activation of caspase-9. In the following, caspase-9 and caspase-8 cause cleavage and activation of caspase-3, -6, and -7, resulting in apoptosis [30].

It is also mentioned previously that dysregulation of caspase activity in breast cancer is involved in chemotherapeutic resistance [32]. Zhong-YingMa et al. showed that MCF-7 cells exposure to Schiff base copper (II) complex depolarized the mitochondrial membrane which results mitochondrial cytochrome C release and subsequently combining with the apoptotic protease activating factor-1 (Apaf-1), ATP/dATP, and procaspase-9, which ultimately leads to apoptosis. Also, Bax protein is critical for the regulation of apoptosis mediated by mitochondria. Bax protein leads to opening of the mitochondria permeability transition pores and release of cytochrome C into the cytosol. Bax and caspase-3 protein expression were greatly increased over a 48-h treatment with the Schiff base copper (II) complex [14]. Our results showed that complex-A and -C exert their effect via the mitochondrial pathway by up-regulating caspase 9 and subsequently caspase 6 gene expression especially in high dosages of complexes. However, the extrinsic pathway did not play a major role after platinum Schiff bases complexes therapy. Also, the apoptotic genes expression level was not influenced by complex-B. Noteworthy, DNA is the main intracellular target of

anticancer agents, and its damage in cancerous cells results in cell death. It is considered that the ability of Schiff bases complexes is based on their tendency to bind and cleave double-stranded DNA in physiological conditions [12].

In cancerous cells, dysregulation of certain components of the mitochondria-mediated apoptosis pathway may occur. Increased pro-survival Bcl-2 gene expression has been seen in cancers such as breast cancer and causes therapeutic resistance. Enhanced Bcl-2 expression blocks the DNA cleavage, and nuclear condensation, and subsequently blocks apoptosis. Many efforts have been done to develop drugs targeting the Bcl-2 pro-survival family members [33]. Our results show that complex-A and -C could decrease Bcl-2 expression in a significant manner. It is important to note that decreased Bcl-2 expression may increase the susceptibility of breast cancer cells to chemotherapeutic agents.

It has been demonstrated that autophagy inhibition could significantly improve drug response and decrease tumor chemoresistance. Therefore, many efforts are being made to find anticancer drugs that can inhibit autophagy. In breast cancer, autophagy plays a key role in disease progression. A decrease in beclin-1 gene expression as a marker of autophagy has been shown in MCF-7 breast cancer cells, previously [34]. In the present work the cytotoxic effect of platinum complexes does not happen through induction of autophagic cell death, because beclin-1 gene expression level was not different between complexes-treated and control untreated cells.

Topoisomerases, the important enzymes involved in the regulation of DNA topological state, are another promising target for anti-cancer drugs [35]. Targeting topoisomerases bring about permanent DNA damage and cell death by interfering with DNA complex [35]. To investigate whether our complexes can exert their antiproliferative effects against the MCF-7 cell line by interfering with DNA complex formation, mRNA expression levels of topoisomerases I and II genes were detected. Our results show that these complexes significantly inhibit topoisomerases I and II gene expression. Interestingly all three platinum complexes could downregulate mRNA expression of topoisomerases. The effect of copper (II) and titanocene complexes against human breast cancer cell lines have been shown to block the activity of topoisomerases I and II [24,35].

The transcription factor HIF-1α plays a crucial role in adapting cells to the hypoxic environment by regulating angiogenesis, cell survival, and drug resistance [36]. Zhang et al. [37] reported that MCF-7 cells in hypoxic conditions showed higher proliferation, invasion, and migration activities as compared to the non-cancerous cells [36]. Knockdown of HIF-1 alpha has been shown to reduce migration and invasion in various types of breast cancer and inhibiting the expression of HIF1α may be a therapeutical approach against tumor progression [38]. Treatment of MCF-7 cells with 50μM of three platinum complexes markedly reduced gene expression of HIF-1 α compared to control untreated cells. Our data provide evidence for the potential role of Schiff base platinum complexes on human tumor progression by inhibiting the HIF-1α gene expression.

4. CONCLUSION

Our findings indicate that Schiff base platinum compounds have a cytotoxic effect against MCF-7 cell line and induce apoptosis via the mitochondrial pathway. Also, our data provide evidence for a possible role of Schiff base platinum complexes in inhibiting human breast tumor progression by down-regulating the expression of HIF-1α, topoisomerase 1, and topoisomerase 2 genes. It is worth mentioning that decreasing Bcl-2 gene expression after treatment of cells with Schiff base platinum complexes may help cells escape from drug resistance. These data support the idea that these complexes would be promising anti-breast cancer drugs.

5. MATERIALS AND METHODS

5.1. Materials

Three schiff base platinum (II) complexes including complex A (MW 536.6 g/mol), complex B (MW 550.6 g/mol), and complex C (MW 564.6 g/mol) were synthesized and characterized in Chemistry and Chemical Engineering Research center of Iran by a similar method which reported previously [9]. The structure of these compounds was shown in Figure 5. MTT reagent was obtained from Sigma (Darmstadt, Germany). Reagents for cell culture were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

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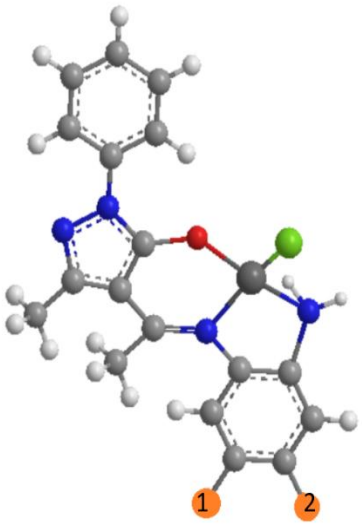

Figure 5. Structure of Schiff base compounds: complex A [Pt(SHB247)Cl] (1 and 2: H), complex b [Pt(SHB245)Cl] (1: CH3 and 2:H) and complex c [Pt(SHB246)Cl] (1 and 2 :CH3).

5.2. Cell culture

Human breast cancer MCF-7 cell line was obtained from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100U/ml penicillin, and 100µg/ml streptomycin. Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO2.

5.3. MTT assay

The growth inhibitory effect of complex A, complex B, complex C, and carboplatin as a standard chemotherapy drug26 was tested by performing a cell cytotoxicity assay using the MTT reagent. Briefly, MCF-7 cells were plated in 100 µl RPMI medium at a density of 2500 cells/well in an exponential growth phase. Cells were incubated overnight and treated with increasing concentration (1.56-100 µM) of each compound and then incubated at 37˚C for 72h. Then 20µl MTT reagent (0.5 mg/ml) was added to each well and, the plate was incubated for 4h at 37˚C. The medium was removed and replaced with DMSO (150 µl/well) to dissolve the formazan crystals. In the following, the absorbance was read by a microplate reader at 570 nm to determine the growth inhibitions of cells. IC50 of each compound was calculated using GraphPad Prism software (version 8.00; GraphPad Software, San Diego, CA, USA).

5.4. RNA extraction and quantitative Real-time PCR

MCF-7 cells were seeded at the density of 400000 cells per well in 6 well plates. After treatment with different concentrations (12.5, 25, and 50 µM) of each complex for 24h, total RNA was extracted using a RiboEx LSTM (GeneAll Biotechnology, Seoul, Korea). Nanodrop 1000 instrument (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA) and agarose gel electrophoresis was used to determine RNA quantity and quality, respectively. 1µg of total RNA was used for cDNA synthesis by cDNA synthesis Kit (TaKaRa, Tokyo, Japan). The expression level of apoptosis-related genes (Bax, Bcl-2, caspase3, caspase6, caspase8, and caspase9), topoisomerase 1 (TOP1), topoisomerase2 (TOP2), Hif-1 α, beclin1, and GAPDH (as an internal control) were investigated by quantitative real-time PCR (Corbett RotorGene RG 6000 instrument, Qiagen, Inc.) in duplicate. The pairs of primers for each gene are shown in Table 2. Quantitative Real-time PCR was performed using 2X SYBR Green Mix (RealQ Plus 2x Master Mix Green – Ampliqon, Denmark). The PCR program was: 95 ºC for 30 sec followed by 40 cycles of 95 ºC for 5 sec, annealing at 56 ºC (TOP1, Bax, caspase 3, and caspase 9), 61 ºC (Bcl-2), 52˚C (HIF 1α), 59 ºC (TOP2), 60 ºC (caspase 8 and GAPDH), and 62 ºC (caspase 6) for 30 sec and extension at 60 ºC for 30 sec. The relative expression levels of genes were determined using 2-ΔΔCt method and normalized to the level of GAPDH.

Table 2. Primer sequences for quantitative Real-time PCR

Genes	Forward primer	Reverse primer
Bax	5'-GCCTCCTCTCCTACTTTG-3'	5'-CTCAGCCCATCTTCTTCC-3'
Bcl-2	5'-CTGCACCTGACGCCCTTCACC-3'	5'-CACATGACCCCACCGAACTCAAAGA-3'
Topoisomerase 1	5'-CAACTGTAGCAAAGATGCC-3'	5'-GTAACCTTGTTATCATGCCG-3'
Topoisomerase 2	5'-ATGTATCACCTTTCAGCCT-3'	5'-TTCATCCAACTTGTCCTTC-3'
HIF-1α	5'-AGGAAATGAGAGAAATGCTTA-3'	5'-GGTTGGTTACTGTTGGTAT-3'
Beclin1	5'-TGGACACGAGTTTCAAGAT-3'	5'-CTGGCGAGGAGTTTCAATA-3'
Caspase3	5'- CAGTGGAGGCCGACTTCTTG-3'	5'- TGGCACAAAGCGACTGGAT-3'
Caspase6	5'- CGATGTGCCAGTCATTCCTT-3'	5'- CTCTAAGGAGGAGCCATAT-3'
Caspase8	5'- GGATGGCCACTGTGAATAACTG-3'	5'- TCGAGGACATCGCTCTCTCA-3'
Caspase9	5'- TGTCCTACTCTACTTTCCCAGGTTTT-3'	5'- GTGAGCCCACTGCTCAAAGAT-3'
GAPDH	5'-GGTGTGAACCATGAGAAGTAT-3'	5'-AGTCCTTCCACGATACCAA-3'

5.5. Apoptosis quantification by flow cytometry

Flowcytometry analysis using annexin V-FITC-PI kit (Biolegend, USA) was used to quantify apoptosis of MCF-7 cells after treating with the Schiff base platinum complexes. Briefly, 106 cells/well were treated with different concentrations (12.5, 25, and 50 µM) of A, B, and C complexes for 24h. Subsequently, cells were washed twice with cell staining solution containing PBS plus 2% v/v fetal bovine serum (FBS). Cells were then added to the flow cytometry tubes and incubated with Annexin V-FITC and PI for 15 minutes in dark. The cells were then suspended in 400 µl of cell staining solution and cell apoptosis was measured by BD FACSCalibur flowcytometry. Finally, the results were analyzed using Flowjo 7.6 software.

5.6. Apoptosis detection by DNA laddering

DNA laddering was done to confirm the occurrence of apoptosis. Cells were seeded at a density of 5x106 in a 10 mm dish and treated with different concentrations (12.5, 25, 50 µM) of complex A and incubated for 48 h. Subsequently, adherent cells were collected and washed with 1×PBS, centrifuged at 1000 g for 5 min at 4 °C, and lysed in 0.5 ml of 10 mM Tris-HCl + 1 mM EDTA + 0.2% Triton X-100. Cells were centrifuged at 12,000 g for 20 min at 4 °C. The supernatant was treated with RNase A at a final concentration of 100µg/ml and incubated at 37°C for 1h. 500µl of phenol/chloroform/Isoamyl alcohol (25:24:1), 25µl cold 5M NaCl, and 2 volumes of 100% ethanol were added. Cells were incubated overnight at -20 °C and centrifuged at 12,000 g for 30 min at 4 °C. Pellets were washed with 70% cold ethanol and dissolved in 50 ml ddH2o. The DNA sample was run on 2% agarose gel and stained with ethidium bromide. DNA laddering pattern was observed under a UV illuminator.

5.7. Statistical analyses

Data were analyzed using the GraphPad Prism software (version 8.00; GraphPad Software, San Diego, CA, USA), and expressed as the mean ± standard deviation (SD). For all the measurements, one-way ANOVA was used to assess the statistical significance between groups. p-value <0.05 was statistically significant.

Author contributions: Concept – E.E.; Design –E.E.; Supervision – S. M., AR. N., E. E.; Resources – P.B., E.E.; Materials – E.E.; Data Collection and/or Processing – P. B., S. M., AR. N., E. E., M. ZJ.; Analysis and/or Interpretation – P. B., S. M., AR. N., E. E., M. ZJ.; Literature Search – S. M., AR. N., E. E., M. ZJ.; Writing – E.E.; Critical Reviews – E.E.

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