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Antiproliferative and Apoptotic Role of Novel Synthesized Cu(II) Complex with 3-(3-(4-fluorophenyl)Triaz-1-en-1-yl) Benzenesulfonamide in Common Cancer Models

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Abstract. *Background/Aim: Chemotherapeutic treatment options are often ineffective due to the development of resistance in cancer cells. Therefore, developing new anti-cancer agents is crucial for cancer treatment. Some triazine derivatives, their complexes and Copper(II) have anti-cancer effects on cancer cells. In this study, we aimed to determine the anti-proliferative effect of the novel synthesized Cu(II) complex with 3-(3-(4-fluorophenyl)triaz-1-en-1-yl) benzene-sulfonamide compound on the common cancer cell lines HeLa, MDA-MB-231, A2780 and MCF7. Materials and Methods: Common cancers cell lines were treated with copper complexes. Cell viability and apoptotic gene expression were examined. Results: Novel synthesized copper complex led to decreased viability of all cell lines. It also induced apoptosis via increasing the expression of caspase-3, caspase-9, Bax and p53 proteins and decreasing ERK expression. Conclusion: The novel synthesized copper complex has a significant inhibitory effect on the viability of cancer cell lines and can be considered as an antitumor agent for further studies.*

Breast, cervical and ovarian cancers are the most common female cancer types worldwide. According to the World Cancer Research Fund International, breast cancer contributed to more than 25% and cervical cancer contributed to almost 8% of all newly-diagnosed cancers in 2012 (1). Cancer of the ovaries is the leading cause of gynecological cancer deaths in women worldwide. It is difficult to diagnose ovarian cancer in its early stages. Thus, most patients (90%) are diagnosed at advanced stages, the survival rate is less than 30% (2, 3). Platinum- and taxane-based adjuvant chemotherapies are used for the treatment of breast, cervical and ovarian cancers especially in advanced stages (4). However, most patients develop resistance to chemotherapy (5). Therefore, developing novel therapeutic strategies for the treatment of cancers is an urgent issue.

To date, many chemical molecules have been reported to inhibit proliferation of cancer cells, which may potentially be further developed to antitumor agents. Sulfonamides form an important class of drugs with various pharmacological agents that have antibacterial, anticarboxylic anhydrase, diuretic, hypoglycemic and antithyroid activity (6, 7). 1,3-Triazenes ($RN=N-NR_1R_2$) are very useful and diverse class of compounds because they contain adaptable reactive groups against many synthetic reaction transformations (8). The use of metal complexes such as platinum-based drugs has also recently been increased in cancer treatment. They can bind to DNA and cause distortion of its structure leading to inhibition of DNA replication and transcription. Therefore, metal complexes can become suitable candidates for anticancer drug development (9, 10). Copper has critical roles in all living organisms. It is a cofactor in redox reactions and participates in many metabolic processes (11, 12). Copper compounds have an important advantage because they display their cancer-specific cytotoxic activity through inhibiting the ubiquitin proteasome activity and/or increasing reactive oxygen species depending on the metabolic differences between normal and cancer cells (11, 13, 14). Therefore, their selective chemotoxicity makes copper complexes excellent candidates for the development of anticancer drugs.

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In this study, we evaluated the cytotoxic effects of novel synthesized Cu (II) Complex with 3-(3-(4-fluorophenyl) Triaz-1-en-1-yl) Benzenesulfonamide on common cancer cell lines. Its apoptotic action was also analyzed in HeLa and A2780 cell lines.

Materials and Methods

Synthesis of Triazene Ligand (L_1) [3-(3-(4-fluorophenyl)triaz-1-en-1-yl) benzenesulfonamide]. A solution of 3-aminobenzenesulfonamide (5 mmol) in 1.5 ml hydrochloric acid and 5 ml water was cooled to 0-5˚C. Sodium nitrite (7 mmol) in 3 ml of water was added dropwise to this solution for 15-20 min under continuous stirring. After stirring the mixture for about 30 min at 0-5˚C, diazonium solution was added to 4-fluoroaniline solution (prepared by 5 mmol 4-fluoroaniline in 5 ml of MeOH) by adjusting the pH to 6-7 with simultaneous addition of saturated sodium acetate. Then, the reaction mixture was stirred for 3 h at 0-5˚C and left overnight at room temperature in dark. The yellow colored solution was filtered off, washed several times with cold water, crystallized from ethanol and the final desired compound was dried under vacuum overnight. Yellow solid; yield 92%; UV-visible (DMSO, nm): 216, 233, 309, 360, 397. IR (KBr, cm^{-1}): 3339, 3247 (N-H), 3066 (Ar-H), 1655 (C=N), 1595 (C=C), 1497 (N=N), 1158 (N-N). 1H NMR (300 MHz, DMSO-d6) δ 8.07 (s, 1H, NH), 7.95 (s, 1H), 7.88 (s, 1H), 7.71 (s, 1H), 7.69 (s, 1H), 7.42 (s, 2H, NH2), 6.65 (d, J=8.4 Hz, 2H), 6.53 (d, J=8.4 Hz, 2H).

Synthesis of $CuL_1(CH_3COO)_2$. The $CuL_1(CH_3COO)_2$ were prepared using chemical precipitation of ion Cu^{2+} with a molar ratio of 1:1. Typically, Copper (II) acetate monohydrate (1 mmol) was dissolved in ethanol (10 ml) at room temperature. L1 (1 mmol) dissolved in ethanol (10 ml), was added into the resulting solution drop by drop. The mixture was refluxed for 3 h and after cooling, the resulting solution partially evaporated. The precipitate was separated by filtration, washed with 1:1 ethanol/water mixture and the pure complex was dried in a desiccator over anhydrous calcium chloride at room temperature. Brown solid; yield 41%; UV-visible (DMSO, nm): 232, 261, 305, 359, 460. IR (KBr, cm^{-1}): 3,374 (N-H), 3,077(Ar-H), 1,666 (C=N), 1,589 (C=C), 1437 (N=N), 1,162 (N - N). 1H NMR (300 MHz, DMSO-d_6) δ 7.91 (s, 1H), 7.85 (s, 1H), 7.72 (s, 1H), 7.70 (s, 1H), 7.40 (s, 2H, NH2), 6.61 (d, J=8.4 Hz, 2H), 6.58 (d, J=8.4 Hz, 2H).

Cell culture and reagents. HeLa, MCF 7, MDA-MB231 and A2780 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 mg/ml penicillin, 50 mg/ml streptomycin, and 1 mM L-glutamine. Reagents were obtained from the following sources: monoclonal anti-ERK1/2, anti-p53, anti-Bax, anti-beta actin antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Monoclonal anti-caspase 9 and anti-caspase 3 antibodies were from Cell Signaling (Beverly, MA, USA); anti-rabbit HRP and anti-mouse HRP were purchased from BioRad (Hercules, CA, USA).

Treatments and MTT analysis. Cell viability was tested by MTT analysis. The cells were plated at a density of 5 thousand cells per well in a 96-well microtiter plates with 6 replicates. Cells were treated with different concentrations of compound and DMSO as a vehicle for 72 h. At the end of 72 h, 20 μl of MTT (5 mg/ml) solution was added for 4 h at 37˚C in an incubator, then medium was removed and DMSO (100 μl) was added to dissolve the formazan crystals. The plates were shielded from light using a foil and left in an orbital shaker maintained at 600 revolutions/min for 5 min. The amount of MTT formazan product formed was determined by measuring absorbance (1) at 540 nm, with 690 nm as the reference wavelength.

Western blotting. Sixty to seventy percent confluent cells in six-well plates were treated with 100 μM Copper complex for 48 h. After 48 h, cells were homogenized in Triton X-100 buffer containing 50 mM HEPES, pH 7.0, 150 mM NaCl, 10% glycerol, 1.2% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 0.15 units/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A. For western blot analysis 75 microgram of proteins were fractionated by SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked in 5% non-fat milk powder in PBST. The blots were labeled with ERK, caspase 3, caspase 9, Bax and p53 antibodies. Beta actin was used as loading control for these blots. Signal intensities on blots were determined using the enhanced chemiluminescent detection system. Signal intensities of the blots were calculated with Image J software program.

Results

Synthesis of compound. The synthesis of novel triazene ligand (L1), 3-(3-(4-fluorophenyl)triaz-1-en-1-yl) benzenesulfonamide, was carried out with some modifications of the literature procedures and outlined in Figure 1 (15-17). Briefly, the diazonium salt derived from 3-aminobenzenesulfonamide was coupled with 4-fluoroaniline, leading to the formation of triazene. The Cu (II) complex was prepared by reaction of the respective triazene ligand in a particular solvent with Cu (II) solution at reflux temperature with stirring (18). The structures of triazene ligand (L1) and Cu complex were confirmed by using several analytical and spectral data (see experimental part for details).

Cytotoxic effect of compound in HeLa, MDA-MB231, MCF7 and A2780 cell lines. To determine the *in vitro* cytotoxic activity of Cu (II) Complex with 3-(3-(4-fluorophenyl) Triaz-1-en-1-yl) Benzenesulfonamide in cancer cell lines, the cells were first treated with different concentrations of inhibitor and DMSO as a vehicle (0, 2, 5, 10, 25, 50, 100, 200 μM) for 48 h. The MTT assay was used to test for the viability of the treated cells. Optimum dosages of the compound causing 50% of cancer cell growth (IC_{50} values) were calculated using GraphPad Prism and are shown in Table I. Inhibitor showed its cytotoxic activity (IC_{50}) at 42.35 μM concentration in MCF7, at 50.42 μM in MDA-MB231, at 52.44 μM in HeLa and at 71.79 μM in A2780 cancer cell lines.

The inhibitory effect of Cu (II) Complex with 3-(3-(4-fluorophenyl) Triaz-1-en-1-yl) Benzenesulfonamide was also evaluated on the viabilities of these cell lines. Treatment with inhibitor led to a decrease in the survival of

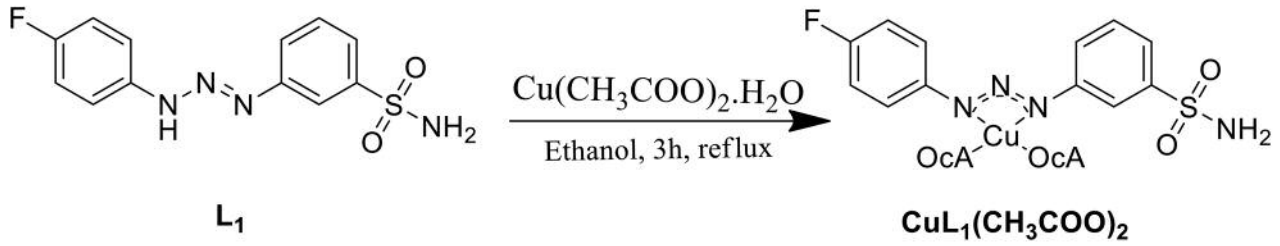

Figure 1. *The synthesis of the Cu complex of novel triazene ligand (L1).*

all cell lines in a concentration-dependent manner. The viability of the all cell lines was significantly reduced after exposure to 50 μM inhibitor compared to vehicle (Figure 2). Treatment with 100 μM inhibitor led to a two-fold decrease in the survival rates of MCF7, MDA-MB231 and HeLa cells. Growth inhibition with 100 μM Cu (II) Complex with 3-(3-(4-fluorophenyl) Triaz-1-en-1-yl) Benzenesulfonamide was 69% in MCF7 cell line, 50% in MDA-MB231 and HeLa cell line and 27% in A2780 cell line. MCF7 cells were more sensitive to the inhibitor compared to A2780, MDA-MB231 and HeLa cells (Figure 2).

Table I. *Inhibitory effect of Cu (II) Complex with 3-(3-(4-fluorophenyl) Triaz-1-en-1-yl) Benzenesulfonamide on cell viability showen as IC_{50} values.*

Cell lines	Calculated IC_{50} values (48 h)
MCF7	42.35 μM
MDA-MB210	50.42 μM
HeLa	52.44 μM
A2780	71.79 μM

Apoptotic effects of Cu (II) complex with 3-(3-(4-fluorophenyl) Triaz-1-en-1-yl) Benzenesulfonamide. According to our MTT results, we wanted to determine whether our compound induced antiproliferative activity *via* apoptosis. Therefore, the expression levels of caspase 3, caspase 9, Bax and p53 proteins was examined. In order to evaluate growth inhibition at the molecular level, we also analyzed the expression of ERK. HeLa and A2780 cell lines were used for the analysis. Western blot analysis showed that our compounds caused an increase in the expression of apoptotic proteins compared to their controls (Figure 3). Caspase 9 expression was elevated 8-fold in HeLa cells and 13-fold in A2780 cells (Figure 3a). Expression of caspase 3 increased 6-fold in HeLa cells and -12-fold in A2780 cells compared to untreated controls (Figure 3b). Although expression levels of Bax significantly increased in HeLa cells, it did not show any changes in A2780 cells (Figure 3c). The expression level of p53 was also significantly higher in both cell lines compared to controls (Figure 3d). Unlike expression of apoptotic proteins, expression levels of ERK decreased in both cells (Figure 3e). ERK expression dramatically decreased in HeLa but not as much in A2780 cells.

Discussion

The purpose of this *in vitro* study was to investigate the effects of novel synthesized Cu (II) Complex with 3-(3-(4-fluorophenyl) Triaz-1-en-1-yl) Benzenesulfonamide on the proliferation of cancer cells. Our results suggested that our novel synthesized copper compound significantly induced apoptosis and also inhibited the proliferation of the cancer cells. The survival rates of the cells were different between the cell lines. MCF7 cells were more sensitive compared to the other cell lines.

Copper compounds and their anticancer features have been shown by many studies in different cancer cell lines. Geromichalos *et al*. showed the cytotoxic effects of copper (II) chelate complex on different cancer cell lines (19). Their molecules successfully lead to a reduction in viability of cancer cells. They determined the IC_{50} values of Cu (II) complex as 76.5 μM in HeLa cells in 48 h treatment and 27.2 μM in MCF7 cells in 24 h (19). According to our results, HeLa and MCF7 cells were more sensitive to Cu (II) Complex with 3-(3-(4-fluorophenyl) Triaz-1-en-1-yl) Benzenesulfonamide.

Copper-cyclam and copper–cyclam/Polymer complexes have also shown antitumoral activity on HeLa cells (20). They inhibited the proliferation of the cells at 100 mg/ml concentration. We observed that Cu (II) complex with 3-(3-(4-fluorophenyl) Triaz-1-en-1-yl) benzenesulfonamide was more toxic in HeLa cells than Copper-cyclam and copper–cyclam/Polymer complexes. Based on our results, we conclude that Cu (II) complex with 3-(3-(4-fluorophenyl) Triaz-1-en-1-yl) Benzenesulfonamide behaves as a good antitumor agent on different cancer cell lines.

Based on our findings, we also wanted to clarify the molecular effects of Cu (II) complex with 3-(3-(4-fluorophenyl) Triaz-1-en-1-yl) benzenesulfonamide on

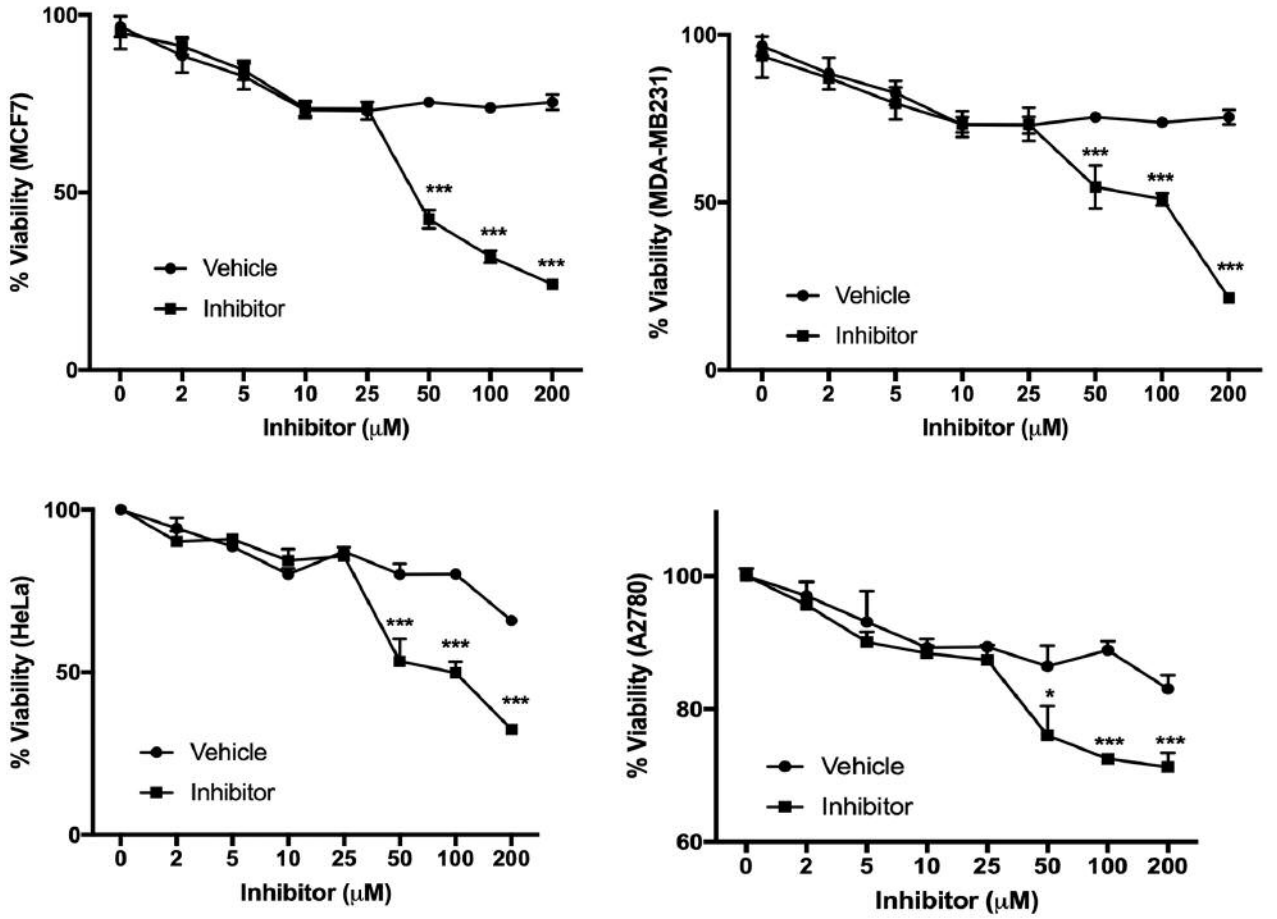

Figure 2. *Dose effects of Cu(II) Complex with 3-(3-(4-fluorophenyl) Triaz-1-en-1-yl) Benzenesulfonamide against common cancer cell lines after 48 h treatment. (*p=0.0008, ***p<0.0001).*

apoptosis. Our MTT analysis showed that HeLa cells were sensitive to our compounds however A2780 was more resistance compared to the other cell types. Therefore, we selected HeLa and A2780 as representative cell lines. Treatment with Cu (II) complex with 3-(3-(4-fluorophenyl) Triaz-1-en-1-yl) benzenesulfonamide caused an increase in the expression of apoptotic proteins and supressed ERK expression. Interestingly, even though A2780 cells were less sensitive to our compound compared to the other cells, expression of apoptotic markers also increased in this cell line too. Expression of ERK protein did not significantly change in A2780 cell line, so A2780 cells were less sensitive to the compound.

Copper can generate reactive oxygen species (ROS) in the cell and elevation of ROS causes DNA damage (21-23). This process results in activation of p53 (24). Our western blot result confirmed that Cu (II) complex with 3-(3-(4-fluorophenyl) Triaz-1-en-1-yl) benzenesulfonamide induced apoptosis *via* increasing the expression of p53. During apoptosis, elevation in p53 expression leads to increase in Bax expression and Bax induces the release of Cytochrome c from the mitochondrial outer membrane. This process results in caspase 9 and caspase 3 activation (25). In HeLa cells, the expression of Bax significantly increased compared to untreated control, however Bax expression level did not change in A2780 cells. These results suggested that our compound activates different apoptotic signalling pathways depending on the cell type.

In summary, our study showed that novel synthesized Cu (II) complex with 3-(3-(4-fluorophenyl) Triaz-1-en-1-yl) benzenesulfonamide decreased the viability of the common cancer cell lines and induced apoptosis in A2780 and HeLa cells. We suggest that cytotoxicity of our compound may generate excessive ROS and induce apoptosis depended on cell type.

In conclusion, Cu (II) complex with 3-(3-(4-fluorophenyl) Triaz-1-en-1-yl) benzenesulfonamide can be considered as a novel antitumor agent.

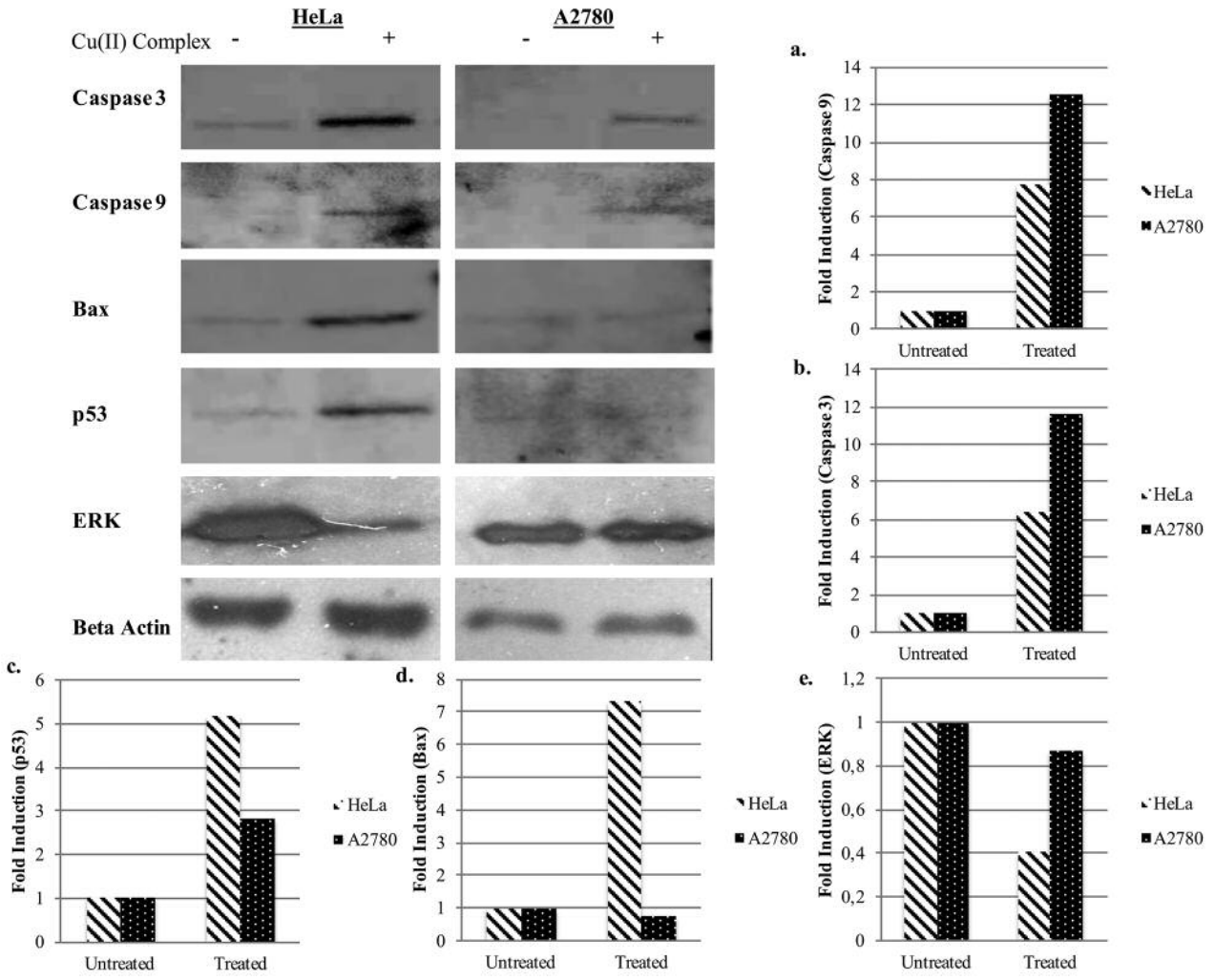

Figure 3. *Apoptotic effects of Cu(II) Complex with 3-(3-(4-fluorophenyl) Triaz-1-en-1-yl) Benzenesulfonamide on A2780 and HeLa cell line. a. Expression level of caspase 9 after normalization with beta actin. b. Expression level of caspase 3 after normalization with beta actin. c. Expression level of p53 after normalization with beta actin. d. Expression level of Bax after normalization with beta actin. c. Expression level of ERK after normalization with beta actin.*

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