Clinical and Experimental Health Sciences

Antioxidant and Cytotoxic Activity Studies of Sulfur Containing Glycine Imine Derivatives MCF-7 and DLD-1 Cell Lines

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ABSTRACT

Objective: To investigate the antioxidant and cytotoxic activities of sulfur-containing glycine imine derivatives MCF-7 (human breast adenocarcinoma) and DLD-1 (colorectal adenocarcinoma) cell lines.

Methods: This study examined the antioxidant activities (25-200 µM) of sulfur-containing glycine imine derivatives via the DPPH, metal chelating and reduction methods. Furthermore the cytotoxic activity of MCF-7, MCF-12A (normal breast epithelial) and DLD-1, CCD-18CO (normal colon fibroblast) were examined with MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) and RTCA (Real-time Cell Analysis) assays.

Results: The antioxidant assay of the metal chelating activity showed significant results (71, 77 and 40% respectively) as compared to knowing synthetic antioxidant (trolox; 95.45, EDTA; 97.06 %). Reducing activity was found to be very low compared to the standard compounds. Compounds were shown to be moderated by DPPH (2,2-Diphenyl-1-picrylhydrazyl) activity, and the IC_{50} value ranged from 91 to 150. The IC_{50} values (100 µM) of the MTT and RTCA analyses were similar.

Conclusion: The study showed that the compounds had selective and significant antioxidant activities, and we also found that they had cytotoxic effects on MCF-7 and DLD-1 cells.

Keywords: Sulfur, Glycine Imine, MTT, RTCA, DPPH, Metal chelating

1. INTRODUCTION

According to the World Health Organization the most common types of carcinoma are breast, colon, prostate and lung cancer (1, 2). Breast cancer, which is a critical health problem worldwide, is the most common type of cancer among women and the deadliest cancer after lung cancer (3). The cancer with the third highest mortality rate and fourth highest incidence worldwide is cancer of the colon (4). Colorectal cancer accounts for approximately 10% of cancers in men and women (5).

Human life often involves disease, stressand various other difficulties. In order to protect ourselves against diseases and boost our immune systems, it is necessary for us to take supplemental foods in addition to the basic nourishment required to sustain life. Such protective or inhibitory compounds are called antioxidants. Antioxidants are substances that can suppress or reduce oxidative damage at low concentrations. For many years, antioxidants have been critical in pharmacological studies (6).

Free radicals and oxidants are produced physiologically and metabolically, and play a role in the human body's defence mechanism. These mechanisms depend on cell and tissue type and act antagonistically or synergistically. When produced in large quantities, they can seriously disrupt the structure of biological substrates such as proteins, lipids, lipoproteins and deoxyribonucleic acid (DNA). They have many different potential effects on cells and they easily emerge as anti-cancer. They have an effect on the cell cycle. As a result of this, they prevent cell deathin aging, apoptosis and necrosis and change the anti-cancer mechanism. They cause increased proliferation, angiogenesis and metastasis and suppression of apoptosis. When ROS (reactive oxygen species) increases in concentration, it has a toxic effect and causes chronic diseases. It has been stated that oxidative damage can be prevented by antioxidants in the cell, possibly at low concentrations (7).

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Glutathione (GSH), consisting of glutamic acid, cysteine and glycine, is an antioxidant tripeptide with a reducing form in the cell (8). High oxidative stress and low antioxidant capacity have relationship with glutathione antioxidant systems (9). An important function of glycine is also partially to prevent the antioxidant capacity, and to reduce the oxidative stress which occurs in heavy metals, peroxide or other medicinal toxins (10). Glycine imines with biologically significant activity are also relatively antidiabetic (11).

Glycine suppresses the formation of angiogenesis signals and the growth of tumor cells in endothelial cells. Glycine is seen as a promising addition to targeted cancer treatments used as a standard (12).

Sulfur is a major constituent of biological systems. This element is ordinarily integrated into proteins as a redox active cysteine residue. It is found in the structure of vital antioxidant molecules and glutathione, thioredoxin and glutaredoxin, which are necessary for life. Sulfur exists in all living cells and is a valuable component of many proteins necessary for a healthy life (13).

Many researchers have reported that glycine is protective against hepatotoxicity and nephrotoxicity caused by anoxia, ischemia and various xenobiotics (10, 14).

MSM (Methylsulfonethane) is a sulfur-containing organic compound known to have no toxic properties. MSM has been shown to significantly reduce cell viability in human breast cancer cells (15). Cases have been reported where proliferation in various cancer cells was suppressed in treatments with natural sulfur (16). It has also been reported for the first time that cell proliferation was inhibited by an inorganic sulfur in a study on breast cancer (17).

Here, we report the design and testing of the bis(alkylthio) imines, which are key compounds, used for the antioxidant (18) and anti-lipid peroxidation system (19).

Our total synthesis of (Figure 1) began with the preparation of glycine ethyl ester, the key amino acid fragments required for the synthesis of bis-sulfur substituted glycine ethyl ester via the known procedure (18, 19).

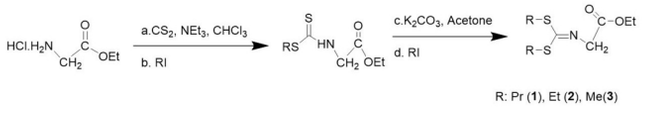

Figure 1. *Synthesis of ethyl 2-((bis(alkylthio)methylene)amino) acetate*

In this study, the antioxidant activities (25-200 µM) of the sulfur-alkyl side chains in glycine imine derivatives were determined via the free radical scavenging, reducing and metal chelating methods.

In addition, the cytotoxic activity of MCF-7, MCF-12A and DLD-1, CCD-18CO was examined by MTT and RTCA assays with varying concentrations (25-100 µM) of compounds for 24hr. The significance of differences between the data sets in the MTT assay was analyzed statistically by ANOVA for four cell lines using the SPSS 20.0 program.

2. METHODS

2.1. Chemistry

All commercial reagents and solvents were purified by standard procedures prior to use. The silica gel (100–200 Mesh) used for column chromatography were supplied and thin layer chromatography used for Merck silica gel 60 F254 precoated plates (0.25 mm). The ^{1}H and ^{13}C – NMR chemical shifts were measured relative to $CDCl_3$ on a Bruker-Spectrospin, Avance spectrometer. Mass spectrums were taken Agilent 7890-GCMS and the mass spectra showed the expected molecular ion peaks.Compounds **I**, **II** and **III** were prepared by known methods (20).

General Synthesis Procedure: Glycine ethyl ester hydrochloride (6 mmol) and carbon disulphide (6.2 mmol)in chloroform (50 mL) were mixed before triethylamine (12.4 mmol) was added dropwise at room temperature. After 1h alkyl iodine (7 mmol) was added, and then the solution was refluxed. After the reaction was completed, first it was extracted with water twice (30 mL), and secondly with ether and water. The residue was dissolved in acetone (20 mL) and alkyl iodine (6 mmol), then potassium carbonate (7 mmol) was added. The reaction solution was refluxed for 1h. The reaction mixture was extracted and residue was evaporated to obtain a light-yellow oil.

Compound I: yield 75%; oil; selected NMR values ^{1}H NMR (250Hz, $CDCl_3$)2.78, 2.82 (s, 6H; – SCH_3), 4.33 (t, *J* 7.4 Hz, 2H, – OCH_2) ppm.**Compound II**: yield 68%; oil; selected NMR values ^{1}H NMR (250Hz, $CDCl_3$) 3.35-3.48 (m, 4H, – SCH_2), 4.25 (t, *J* 7.4 Hz, 2H, – OCH_2) ppm.**Compound III**: yield 70%; oil; selected NMR values ^{1}H NMR (250Hz, $CDCl_3$) 3.37-3.51 (m, 4H, – SCH_2), 4.00-4.12 (m, 2H, – OCH_2)ppm.)

2.2. Antioxidant Activity

Doses of the compounds were studied at concentrations of 25, 50, 100, 150 and 200 µM. In addition, the antioxidant activity of the compounds was determined using free radical scavenging (21), metal chelating (22), and reducing (23).

2.2.1. Free Radical Scavenging (DPPH) Activity Assay

The free radical scavenging activity assay was performed according to (21). Three replicates of the compounds with different concentrations were studied. Butylated hydroxytoluene (BHT), Trolox and Butylated hydroxyanisole (BHA) (25-200 µg/mL) were used as standard. The activities of the compounds at 517 nm absorbance were measured. The free radical scavenging activity (%) = $[(A_0 – A_1) / A_0] \times 100$. ($A_0$ = control absorbance and A_1 = sample solution absorbance).

2.2.2. Metal Chelating Activity (Fe^{+2}Ions Chelating Activity) Assay

The chelate activity assay of the compounds was performed according to (22). Butylated hydroxytoluene (BHT), Trolox and Ethylenediaminetetraacetic acid (EDTA) (25-200 µg/mL) were used as standard. The activities of the compounds at 562 nm absorbance were measured. The metal chelating activity (%) = $[(A_0 – A_1) / A_0] \times 100$. ($A_0$ = control absorbance and A_1 = sample solution absorbance).

2.2.3. Reducing Activity Assay

The reducing activity of the compounds was performed according to (23). EDTA, Trolox and Gallic were used as standard (25-200 µg/mL). The activities of the compounds at 700 nm absorbance were measured and activity results were evaluated by absorbance measurements.

2.3. Cytotoxic Activity

2.3.1. Cell Lines

In *in vitro* cell culture studies MCF-7 (ATCC® HTB22™) (human breast adenocarcinoma), MCF-12A (ATCC® CRL-10782™) (normal breast epithelium), DLD-1 (colon cancer) (ATCC® CCL221™) and CCD-18CO (normal colon epithelium) (ATCC® CRL-1459™) cell lines were used. Cancer cell lines indicated with ATCC numbers were obtained from the cell culture collection at Gebze Technical University. The study did not include human subjects, and ethics committee approval was therefore not required.

2.3.2. Cell Culture

In the study, MCF-7, MCF-12A, DLD-1 and CCD-18CO cell lines DMEM (Dulbecco's Modified Eagle Environment), EMEM (Eagle's Minimum Essential Medium) and RPMI-1640 (Roswell Park Memorial Institute) mediums containing 10 % fetal bovine serum (FBS) and/or horse serum were cultured with a 5 % CO_2 incubator. These were produced by incubation for 24 hours. Cells were grown to 80% saturation, followed by washing with phosphate buffered saline (PBS). 1X Trypsin-EDTA was used to pass the cells (24).

2.3.3. MTT Assay

MTT analysis is a method in which cell proliferation is determined based on the colorimetric measurement of color change occurring in cells incubated with enzymatic activity due to formazan dyes or MTT reduction. The cytotoxic or proliferative effects of any therapeutic agent to be used on this cell can be determined by this method.

The analysis of the possible cytotoxic effect of the sulfur-containing glycine imine derivatives compounds on MCF-7, MCF-12A, DLD-1 and CCD-18CO cell lines was performed according to the manufacturer's instructions for use with the MTT (Sigma). The resulting color change is caused by the reduction of the tetrazolium salt in activated cell mitochondria of yellow-colored formazan salts. The absorbance value of these compounds is proportional to the metabolic activity.

100 µl of RPMI medium was prepared in a 96-well plate ($1x10^4$ / well) 1 day before the MTT assay was applied and the wells were cultured. The microplate was kept in an incubator at 37 °C and 5% CO_2 for 24 h to allow the cells to adhere to the surface. Sulfur-containing glycine imine derivatives compounds prepared in serial dilutions (1.56-3.12-6.25-12.5-25-100 µM) were added to the wells after 24h of incubation. After incubation, 100 µl of MTT (5 mg/mL) solution was added to the cells for 2h and then 100 µL of DMSO (dimethyl sulfoxide) was added to the wells to terminate the reaction. Incubated cells, microplate reader spectrophotometer and 570 nm absorbance value measurements were taken in three replicates. The dose and % cell viability curve were determined with the help of the Microsoft Excel program and 50% suppressive concentration (IC_{50}) was calculated by logarithmic slope graph (Cytotoxicity = test absorbance value / control absorbance average value x 100). Experiments were repeated three times for each compound concentration and solution. According to these results, the cytotoxic effects of sulfur-containing glycine imine derivatives compounds in cell lines were evaluated and the dose-response relationship was defined (25).

Statistics

The significance of the differences between the data for the MCF-7 and MCF-12A, DLD-1 and CCD-18CO cell lines was analyzed statistically with ANOVA using SPSS 20.0 program. The conclusions were indicated as ID50 ± SE (standard error of the mean) for cell lines.

2.3.4. Real-Time Cell Analyzer (iCELLigence) Assay

The study used MCF-7, MCF-12A, DLD-1 and CCD-18CO cells. The mediums of DMEM, EMEM and RPMI-1640 were incubated at 37 °C and 5% CO_2. 10% FBS and/or horse serum was added to the media. $1x10^4$ cells / 400 µL medium were loaded into the E-plates of the iCELLigence RTCA device. Cells were left to adhere to the E-plates for 24 hours, 25-50-100 µM concentrations of the compounds were added to the E-plates at the end of 24 hours and monitored in real time for 24 hours. The viability of the cells was calculated by comparing the 25-50-100 µM concentrations of the compounds according to the negative control (26).

3. RESULTS

3.1. Chemistry

The three bis(alkylthio)imines, ethyl 2-((bis(methylthio) methylene)amino)acetate, ethyl 2-((bis(ethylthio)methylene) amino)acetate, and ethyl 2 ((bis(propylthio)methylene) amino)acetate, which were given the names **I**, **II**, and **III** respectively. were synthesized according to the methods found in the literature (18, 19).

3.2. Antioxidant Activity

The DPPH activity **III** › **II** › **I** and the metal chelating activity **II** › **I** › **III** were determined. The reductive activity results were found to be very low compared to standard compounds. The IC_{50} value is defined as the half inhibition concentration in inhibiting antioxidant activity. Antioxidant activities have an IC_{50} value of 91 to 150 (Table 1-3) (Figure 2-4).

Table 1. *DPPH activity (free radical scavenging) and IC_{50} of compounds* ***I, II and III***

Compounds	Concentration (µM)					
	25	50	100	150	200	IC_{50}
I	36.727	41.467	44.495	51.376	53.975	150.01
II	29.969	51.834	53.975	61.071	63.761	91.733
III	7.4159	46.116	63.180	68.455	78.746	96.986
BHT	61.840	74.570	78.360	85.620	94.690	
Trolox	59.590	70.520	72.540	81.360	90.570	
BHA	50.450	53.970	60.990	76.430	82.470	

Table 2. *Chelating activity of Metal (Fe^{2+} ions chelating activity) of compounds* ***I, II and III***

Compounds	Concentration (µM)				
	25	50	100	150	200
I	42.933	53.069	62.961	67.567	71.662
II	12.043	31.603	42.311	56.298	77.356
III	17.198	26.615	29.500	33.956	40.388
BHT	77.000	81.250	83.090	94.200	97.500
Trolox	54.270	67.840	74.250	81.530	95.450
EDTA	70.850	72.320	79.470	81.090	97.060

Table 3. *Reducing activity of compounds* ***I, II and III***

Compounds	Concentration (µM)				
	25	50	100	150	200
I	0.050	0.080	0.104	0.113	0.123
II	0.137	0.155	0.213	0.212	0.238
III	0.168	0.216	0.274	0.267	0.317
EDTA	2.178	1.503	3.865	3.925	4.355
Trolox	1.506	2.765	2.537	3.689	2.701
GALLİC	1.273	1.511	1.876	3.597	3.756

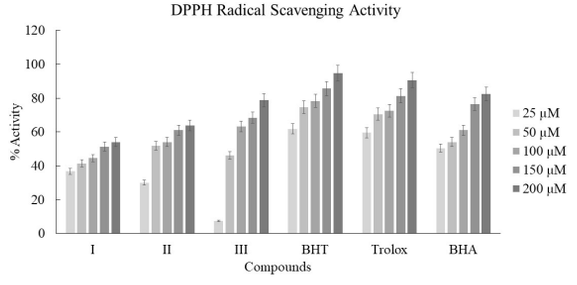

Figure 2. *DPPH activities of compounds and BHT, Trolox and BHA as standards*

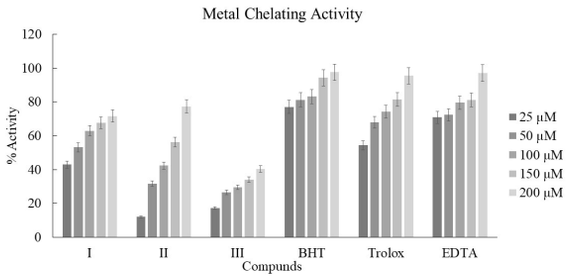

Figure 3. *Metal activity of compounds and BHT, Trolox and EDTA as standards*

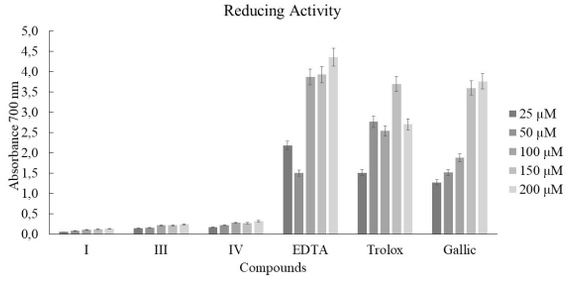

Figure 4. *Reducing activities of compounds and EDTA Trolox and Gallic as standards*

3.3. Cytotoxic Activity

Three compounds were investigated by comparison of two normal and two cancer cell lines. Compounds were evaluated for their cytotoxic effects against MCF-7, DLD-1 cancer cell lines and MCF-12A, CCD-18CO normal cell lines. For this purpose, cells were cultured with compounds (**I-II-III**) at 37 °C for 24 h at concentrations of 25, 50, 100 µM (the best doses obtained in MTT assays). Cell viability was measured at 570 nm, the optical density (OD) value. Cytotoxic activity was calculated using the formula (%) = (average experimental OD value / average control OD value) x 100 %. Values were stated as 50 % inhibitory concentration (IC_{50}). The compounds synthesized in the study are particularly noteworthy as anti-cancer agents.

Based on the MTT assay the cytotoxic activities of the compounds for MCF-7 cell line were determined as **II** › **III** › **I**. The cytotoxic activities of the compounds for DLD-1 cell line were determined as **II** › **I** › **III**. In fact, the cytotoxic activity of the compounds **I** and **III** were approximately the same (Figure 5 and 6).

Based on the RTCA assay the cytotoxic activities of the compounds for MCF-7 cell line were determined as **II** › **III** › **I**. The cytotoxic activities of the compounds for DLD-1 cell line were determined as **II** › **III** › **I**. It can even be said that compound **I** did not affect cell proliferation (Figure 7 and 8).

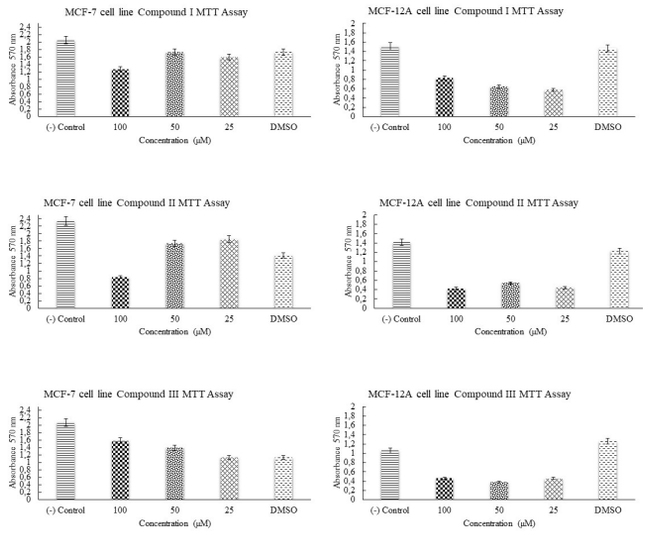

Figure 5. *MTT assay of increasing concentrations of compounds on MCF-7 and MCF-12A cells.*

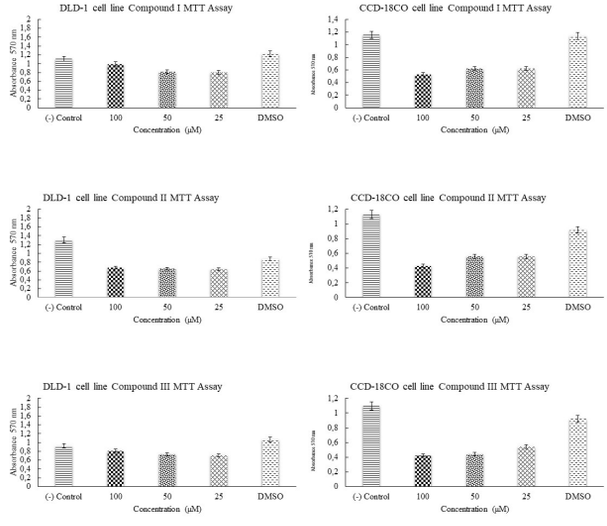

Figure 6. *MTT assay of increasing concentrations of compounds on DLD-1 and CCD-18CO cells.*

Compound **II** exhibited IC_{50} values approximately better than compound **I** and **III** under the same conditions, indicating improved selectivity for cancer cells.

Also, MCF-12A and CCD-18CO were found to affect cell proliferation and reduce cell proliferation in normal cell lines. Compounds **I**, **II** and **III** exhibited moderate cytotoxicity toward the normal cell lines.

In addition, cytotoxic activities (MTT and RTCA assays) of sulfur-containing glycine imine derivatives were compared with antioxidant activities, and the cytotoxic activities were found to be similar to the antioxidant activity results.

The MTT assay concentrations of compounds **I**, **II** and **III** in four cell lines were compared. As a result of ANOVA analyses (SPSS 20.0), there was a significant difference between the compounds and concentrations. Post hoc analyzes were done to understand the relationships between the groups. First, the homogeneity of the variance test result was examined and tests were applied depending on these results. Games-Howell and Tukey tests were used for multiple comparisons of compounds **I, II** and **III** in MCF-7 and MCF-12A, DLD-1 and CCD-18CO cell lines *(p <0.005)* (Table 4 and 5) (Figure 9 and 10).

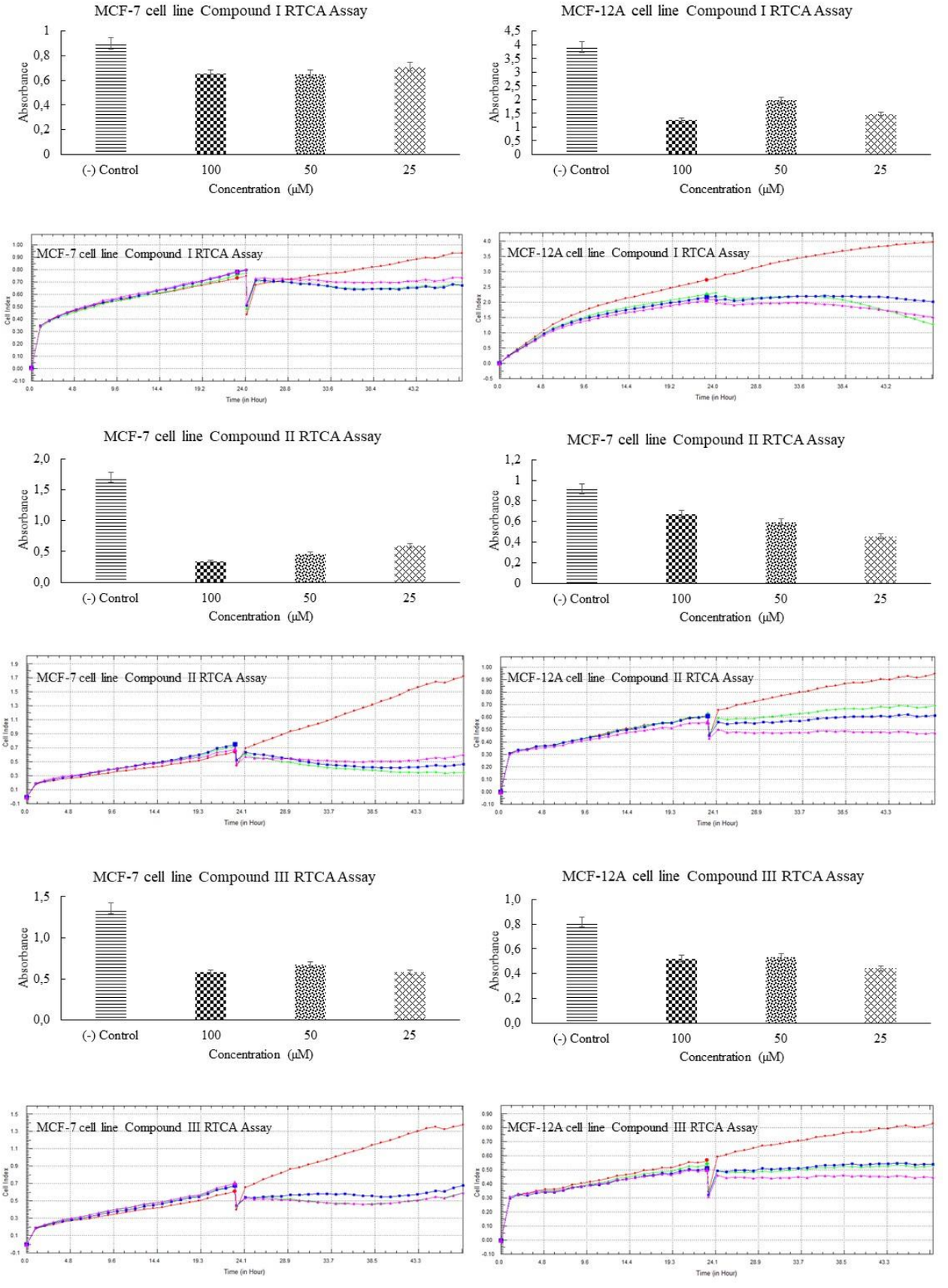

Figure 7. *Real-time cell analysis of increasing concentrations of compounds on MCF-7 and MCF-12A cells. (Red; Negative control, Green; 100 µM, Blue; 50 µM, Pink; 25 µM).*

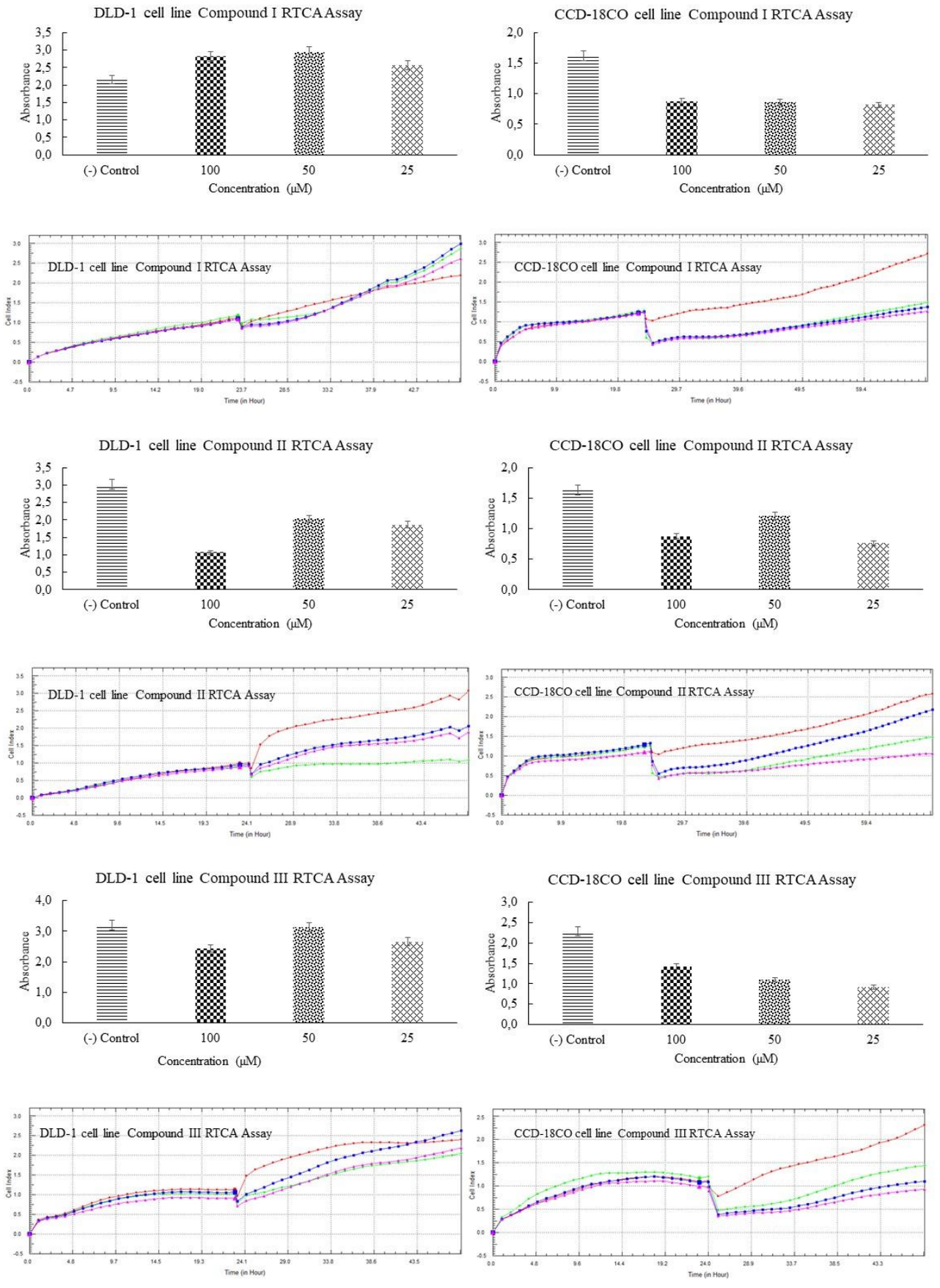

Figure 8. *Real-time cell analysis of increasing concentrations of compounds on DLD-1 and CCD-18CO cells. (Red; Negative control, Green; 100 µM, Blue; 50 µM, Pink; 25 µM).*

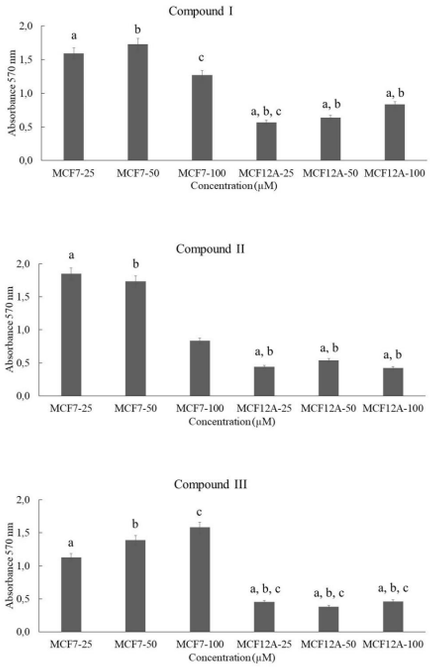

Figure 9. *Comparison between MCF-7 and MCF-12A cell lines in terms of cell viability. a, b, c indicate significant difference for compounds*

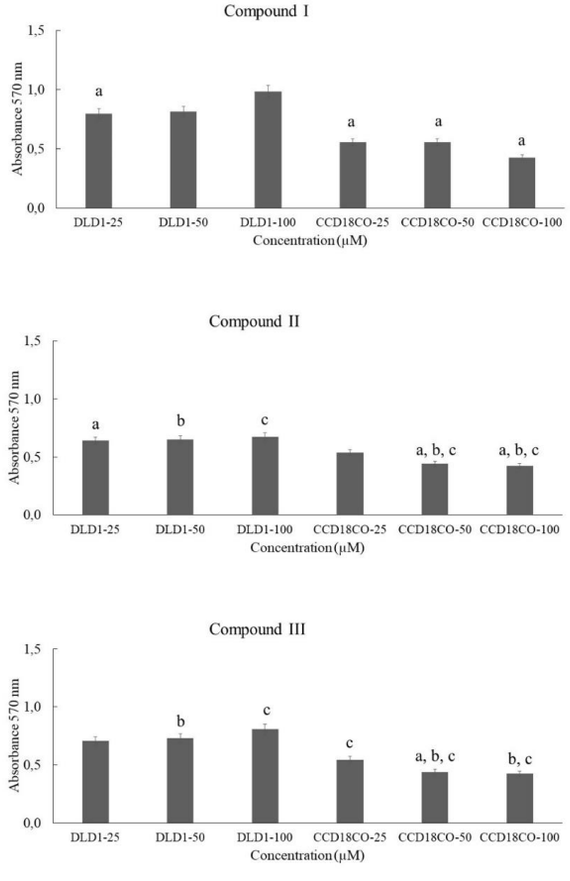

Figure 10. *Comparison between DLD-1 and CCD-18CO cell lines in terms of cell viability. a, b, c indicate significant difference for compounds*

Table 4. *In vitro cell viability of the cell lines were investigated by MTT assay after treating MCF-7 and MCF-12A cell lines with varying concentrations of compounds for 24 hr. The acquired data were evaluated using SPSS 20.0 analysis and defined as IC_{50} values.*

Compounds	Concentration (µM)	Cell lines ID50 [µm] ± SE	
		MCF-7	MCF12A
I	25	1.592±0.143	0.568±0.095
	50	1.731±0.123	0.64±0.058
	100	1.272±0.255	0.832±0.129
II	25	1.848±0.243	0.439±0.014
	50	1.733±0.040	0.539±0.025
	100	0.835±0.046	0.424±0.008
III	25	1.130±0.179	0.454±0.015
	50	1.391±0.202	0.381±0.010
	100	1.583±0.214	0.462±0.018

Table 5. *In vitro cell viability of the cell lines were investigated by MTT assay after treating DLD-1 and CCD-18CO cell lines with varying concentrations of compounds for 24 hr. The acquired data were evaluated using SPSS 20.0 analysis and defined as IC_{50} values.*

Compounds	Concentration (µM)	Cell lines ID50 [µm] ± SE	
		DLD-1	CCD-18CO
I	25	0.799±0.022	0.556±0.037
	50	0.816±0.096	0.556±0.029
	100	0.985±0.075	0.427±0.004
II	25	0.641±0.057	0.537±0.008
	50	0.649±0.065	0.441±0.006
	100	0.673±0.039	0.423±0.003
III	25	0.706±0.076	0.544±0.003
	50	0.731±0.032	0.440±0.003
	100	0.809±0.031	0.423±0.011

4. DISCUSSION

A study was conducted on the design, characterization and biological activity of the glycine derivatives, and the results showed cytotoxicity to 293T (27). In the current study, three different compounds derived from glycine imine containing sulfur were synthesized. Antioxidant and cytotoxic activity studies of the compounds were designed and carried out using five different methods. The compounds were shown to exhibit high DPPH and Metal chelating activity and high cytotoxic activity in MCF-7 and DLD-1 cells. It has been reported that glycine has protective properties against the state of shock caused by blood loss or endotoxin, reduces alcohol levels in the stomach and provides healing against alcohol-induced hepatitis. It has also been determined that it reverses the liver damage caused by hepatotoxic drugs, suppresses programmed cell death (apoptosis) and reduces the nephrotoxicity caused by the drugs, and prevents hypoxia and the formation of free radicals in the kidney (28). In the current study, three different antioxidant activity studies and two different cytotoxic activity studies were performed using two different cell lines. It was observed that glycine derived compounds exhibit anti-cancer activity in MCF-7 and DLD-1 cells. Apoptotic activity studies of glycine derived compounds are planned as the next step. MSM, a natural compound containing sulfur, has been reported to significantly reduce

human breast cancer cells (15). In the current study, the anti-tumor effects of the compounds were investigated using breast and colon cancer cells. It was determined that the compounds show high cytotoxic activity in breast and colon cancer cells. It has been reported that many patients with cancer have been given antioxidant supplements during cancer treatment to alleviate toxic effects and that these supplements contribute to an improvement in their condition, even if only to a small extent (29). Given that antioxidant and cytotoxic activity studies form the basis of cancer studies, the original compounds and methods used in this study confirm this purpose.

The information in the literature on synthesized compounds and biological activity studies is limited. Therefore, studies of compounds and activity are rare. The current study shows that sulfur-containing compounds in MCF-7 and DLD-1 cell lines have both antioxidant and cytotoxicity activities. In terms of comparing the compounds' antioxidant and cytotoxic activities, it was found that the compounds gave parallel results. Compound **II** had prominent selective properties in both the cytotoxic activity studies and the antioxidant studies.

5. CONCLUSION

We evaluated the antioxidant activities as DPPH, metal chelating, and cytotoxic activity using the MTT and RTCA methods of methyl, ethyl, propyl analogs of sulfur-containing glycine imine derivatives for describing the effect of a sulfur-linked alkyl group.

As a result of the study, it is thought that compound **II** has anti-oxidative and cytotoxic properties, preventing cell proliferation and reducing oxidative damage.

It is difficult to find information or studies about sulfur-containing glycine imine derivatives in the literature. This study is specific both for derivatives of compounds and cancer cells.

We concluded that this new drug candidate could reduce oxidative damage and had an effect on MCF-7 and DLD-1 cancer cells.

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