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Received: 25 October 2021 | Revised: 9 May 2022 | Accepted: 6 June 2022

DOI: 10.1002/jbt.23148

RESEARCH ARTICLE

WILEY

The effect of a bis-structured Schiff base on apoptosis, cytotoxicity, and DNA damage of breast cancer cells

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Funding information
Van Yuzuncu Yil University, Scientific Research Projects (YYU BAP), Grant/Award Number: [2015-SBE-YL358]

Abstract

Developing new anticancer agents are crucial for cancer treatment. Antiproliferative activity of L1H as a bis-structured Schiff base was subjected to preliminary research in eight different kinds of cell lines by the cell viability method using different concentrations to determine their inhibitory concentration. L1H demonstrated the highest cytotoxicity in human breast cancer cell line MCF-7. In this perspective, the MCF-7 cell line was cultured for the examination of different molecular techniques, including MTT, apoptosis analysis by enzyme-linked immunosorbent assay (ELISA), and comet assay. Moreover, the DNA ladder, acridine orange/ethidium bromide as another apoptotic cell analysis, markers of oxidative stress, and total antioxidant status, total thiol, and GSH as nonenzymatic antioxidants assay were conducted. The above techniques have proven that L1H is a growth inhibitor effect when compared to cisplatin as a positive control in human breast cancer cells, especially those affected by L1H. The findings clearly show that L1H evaluated in MCF-7 cell lines causes rising or induced apoptosis, DNA damage, diminished antioxidant status against the increase of oxidized protein, and prevents cell proliferation. Manifold evidence supported our hypothesis that L1H has a potential therapeutically improved effect against the MCF-7 cell line, and then without a doubt is a suitable candidate drug for investigating cancers next.

KEYWORDS
apoptosis, bis-structured Schiff base, breast cancer cell line, cisplatin, cytotoxicity, DNA damage, MCF-7

1 | INTRODUCTION

Cancer is a well-known dangerous disease over the world and is identified as abnormal cell growth and cell division while it can be also defined as uncontrolled proliferation irrespective of the normal rules of irregular functioning. Normal cells are constantly subject to signals that dictate whether the cell should divide, differentiate into another cell, or die.[1] Generally, tumors have the risk of potential information on cancers that have been shown in numerous categories of cancers. Since this disease, which is called an effective disease that threatens human life, is extremely risky, they should be warned about breast cancer. Breast cancer, which shows the abnormal progression and development of breast cells, is one of the most upsetting cancers in women. Cancer has enlarged its occurrence in current years.[2] However, the opinions of epidemiologists are taken about the risk of aggressive infection due to breast cancer, which increases and develops depending on age and environment. It is suggested that breast cancer is the first

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risk disease to cause death from cancer in women before lung cancer.

Decades ago, apoptosis, which is particularly of great relevance to disease, was defined as a very important and valid framework. In addition, in our research, it was observed that the MCF-7 cell line, one of the breast cancer cell lines, is very vital. Thereby, apoptosis is essential to highlight preservation and maintaining balance at the individual cellular damaged DNA. Apoptosis has a major target pointfor biological activity and progressive pharmacological by using novel drug for treatment.[3–5] Apoptosis is related to complex cases in numerous pathological processes, including cancer.[6] Consequently, signaling of apoptosis has been a target for entering pathways to pathological processes, particularly cancer. Apoptosis may result from cancer, virus infection, and autoimmune syndromes. However, for investigating the route of DNA damage is that cell inactivation usually induced and converted apoptosis. DNA injury ought to occur spontaneously through basic hydrolysis and natural stress for example inflammation. There are general genotoxic factors of the environment whose goals are to affect DNA double chains, for example polycyclic aromatic hydrocarbons, ionizing radiation and ultraviolet light.[7] It has been observed that the role played by DNA damage accumulated throughout their lives in breast cancer in women increases and develops.[8]

Our therapeutic assessment and examination selected optional medication utilized in opposition to the MCF-7 cell line. Checking the cellular responses of these drugs to health gives us a particularly good understanding of how we can work with these drugs and how appropriately and more efficiently they affect therapy patterns. Clearly, in our study, the novel ligand as anticancer cell line apoptosis was used for the treatment of many different kinds of cancerous cell lines, this is known as bis-structured Schiff base (L1H).

Schiff base was first reported by Hugo Schiff in 1964, a product that is formed by summarizing the reaction of primary amines with the carbonyl compound. The structure of Schiff base is sometimes called azomethine, which is imperative in restorative and pharmaceutics fields. It is joined with various transient metals and their broad range of natural initiates can make the intricate compound, then a proposable anticancer MCF-7 apoptosis.[9]

Cisplatin can be used for the treatment of cancers, such as lung cancer, bladder cancer, neck cancer, esophagus cancer, brain tumors, and breast cancer. Cisplatin was licensed for medical use in 1978. "L1H has been compared with cisplatin, which is known to be used in many different assays and in many new multi-ligand drug applications that work by binding to DNA and affecting its duplication. In our examination, we estimated the drug supervisory that was very important and beneficial in apoptosis. Cisplatin (CDDP) is extensive and is mostly used by the investigator as a result, in an inorganic composite, cured in chemoresistance trained in five, and in vitro cisplatin cured has a dose side effect in vivo and cancer cell can advance resistance to cisplatin.[10]

Therefore, in our study, two cell lines and two chemicals have been used, the breast cancer cell line (MCF-7) as the target cell and the normal prostate epithelial cell line (PNT1-A) as a control. The chemicals used as anticancer agents were bis-structured Schiff base L1H as a new agent and cisplatin as a control. This study aims to estimate the apoptotic, cytotoxic, and genotoxic effects of L1H by determining the MTT, enzyme-linked immunosorbent assay (ELISA), DNA laddering, comet assay, and acridine orange (AO) results on the breast cancer cell line MCF-7. Also, by measuring the total oxidant status (TOS), total antioxidant status (TAS), and ferric reducing antioxidant power (FRAP), we estimated the oxidative stress index (OSI). In this study, the anticancer potential of bis-structured Schiff base in breast cancer MCF-7 cell line was tried to be revealed as a result of the evidence of mutually confirming findings.

2 | MATERIALS AND METHODS

2.1 | Cell culture and reagent

During the preparation of the cell culture, especially MCF-7 cell lines were routinely cultured in Dulbecco's modified Eagle medium (DMEM) containing 100 µg/ml streptomycin, 100 IU/ml penicillin 2 µM (P/S) L-glutamine, and Fetal Bovine Serum (FBS) 10% in a humidified atmosphere with 37°C and 5% CO2 (Steri-Cycle-Thermo Scientific, USA). The medium was supplemented with L-glutamine because it is an unstable amino acid and was previously used at the concentration of 2 µM (0.292 g/L). In addition to the cells that were grown to gather, cells were detached and separated using trypsin/EDTA solution and sterilized. Cells were separated by trypsin in a 37°C and 5% CO_2 incubator and then isolated in suspension. To count cells, the suspension was covered with a coverslip when placed in a hemocytometer with multiple square fields. Cells were counted by inverted microscopes and calculated by the following formula:

$$\text{Cells /ml} = \text{average count per square} \times \text{dilution factor} \times 10.$$

L1H is a cytotoxic ligand in our study that was chosen for the inhibitory effect on breast cancer cells. The ligands containing the imine group prepared by the condensation of primary amines with the carbonyl group in the presence of organic acid as a catalyst and in the pH range (4–5) are called Schiff bases. There is a lot of literature information about the Schiff bases obtained by this method.[11–14] L1H is one of the Base Schiff classes critically used as part of our theory in MCF-7 cells, it turned out to be a good growth inhibitor compared with cisplatin as a positive control.

Bis-structured Schiff base L1H was synthesized by the condensation of 3,5-Dibromosalicylaldehyde (2.0 mmol) absolute methanol with 1,6-Hexanediamine (1.0 mmol) in 20 ml absolute methanol. Formic acid (two drops) was also added as a catalyst to adjust the pH to about 4–5. The mixture was refluxed for 4 h. The mixture was cooled to room temperature and the precipitated product was filtered off and dried. Then, the L1H was characterized using both analytical and spectroscopic methods. Briefly, the FT-IR spectrum of

the L1H compound shows the disappearance of the aldehyde carbonyl group C=O and the formation of an imine C=N absorption bonds at 1630 cm^{-1}. In the NMR spectrum of the L1H compound, the imine proton (C=N) appeared as a single signal corresponding to two protons at 8.18 ppm in the ^{1}H NMR spectrum. The OH proton was seen at 14.90 ppm. Also, in the ^{1}H NMR spectrum of L1H, aromatic protons were observed around 7.69 and 7.31 ppm, respectively, in the expected regions. Additional support to the L1H structure was provided by the ^{13}C NMR spectra, and the carbon signal was seen in the expected areas. The spectroscopic results for L1H indicate that the color is yellow with a purification percentage of 84%, and a melting point of 149–151°C. The elemental analysis showed the following formula ($C_{20}H_{20}Br_4N_2O_2$) with a molecular weight of (640.00 g/mol) (Figure 1).[15] The use of cell culture in the study was approved by the Ethics Committee of Van Yuzuncu Yil University.

2.2 | Cell viability assay (MTT assay) and IC_{50} measurement

The cytotoxicity of bis-structured Schiff base L1H was evaluated by the 3-(4,5-dimethyl thiazol-2-y1)-2, 5-diphenyl tetrazolium bromide (MTT) assay, which is essential for the split of tetrazolium salts by the enzymes is mitochondrial succinate-tetrazolium reductase in viable cells to form a formazan dye.[16]

MCF-7 cells were seeded in 96-well plates at a density of 5×10^3 cells/well and allowed to attach. Cells were exposed to different concentrations (2–200 µM) of L1H and after incubation at 37°C 5% CO_2 for 24 hours, the medium was removed. MTT (5 mg/ml) was added, and after 3 h of incubation at 37°C the resulting formazan crystal was dissolved in dimetil sülfoksit. Measurement of cell viabilities was explored by absorbance at 570 nm in a microplate reader (Varioskan Flash Multimode Reader; Thermo Fisher Scientific) and the decrease in the cell viability was expressed. The experiment was completed in triplicate and the average of the viable cells was calculated. Three individual wells were measured per treatment point and the percentage value obtained by comparison to the control group was expressed as 100%.

The dose–response curve of L1H was obtained by plotting the percentage of inhibition versus the concentrations using GraphPad Prism software (GraphPad Software). Nonlinear regression analysis was carried out and the IC_{50} values of all cell types used in this current study were calculated by performing similar procedures. The concentration of drugs that are needed for 50% prevention in vitro is represented by IC_{50}. Especially MCF-7 cells have a high sensitivity rate against the L1H ligand used on many cancer cell lines in our current study. Therefore, we decided that the next experiments, which are supported to be feasible, should be performed on the MCF-7 cells in question.

2.3 | Apoptotic analysis by ELISA

ELISA is a suitable enzyme-dependent immunosorbent assay to investigate and detect apoptosis of a sample in cell culture. An early DNA fragmentation is an event in apoptosis followed by the spread of nucleosomes into the cytoplasm. This test uses two immunoassays are monoclonal antibody particular for a pair nucleosome epitope to capture and detect cytoplasmic nucleosomes onto ELISA plate.[17]

Laboratory studies required to prepare the sample were easy, dilute cells and culture medium were adjusted to obtain proper cell concentration, and then the number of cells per test was determined and optimized. Camptothecin (CAM) was used as a known apoptosis-inducing agent to constitute a model of apoptosis. MCF-7 cells diluted first with the culture medium were then proliferated and a cell concentration of 1×10^5 cell/ml was obtained. Cell samples are transferred to tubes (500 µl/tube 5×10^4 cells/tube) and different concentrations of CAM (0–4 µg CAM/ml) are added to 500 µl culture medium. The sample contents of the microplate were carefully dispersed and mixed well before measuring at 405 nm against a substrate solution blank.

2.4 | SCGE (comet) assay

SCGE also called comet assay is a technique used to analyze and quantify DNA damage in single cells. Comet analysis, developed by Swedish researchers Johansson and Östling in 1984, is one of the sensitive and rapid genotoxicity techniques used in cancer research.

SCGE assay has two techniques that have successful applications when DNA is damaged in cancer cells; neutral comet assay and alkaline comet assay. The first technique, the neutral comet assay, detects double-stranded breaks, while the second application, the

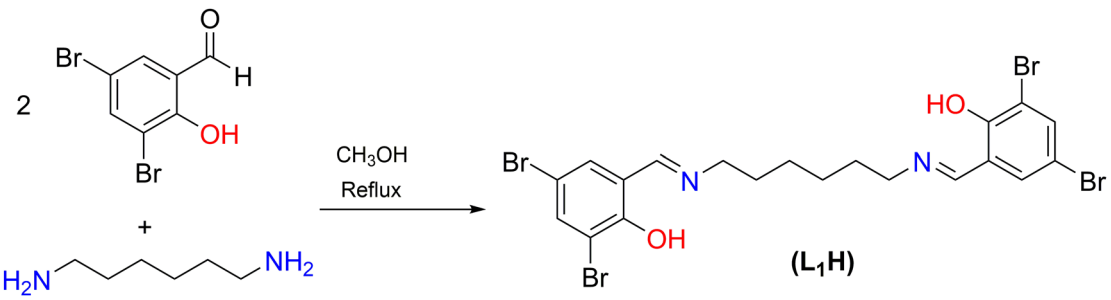


FIGURE 1 Synthetic routes and molecular structure of the (L1H)

alkaline comet assay, determines single-stranded and double-stranded DNA breaks. In Alkaline comet assay, after prepared lysis solution, low melting point agarose was added to a beaker of boiling water and placed in a 37°C water bath for 5 minutes with the lid loosened and then kept at 4°C for at least 20 minutes for cooling.

After the samples are prepared according to the protocol, comets were analyzed with a fluorescence microscope. Each cell nucleus takes a fluorescence value in the range 0–4 (arbitrary units: 0—undamaged, 4—maximally damaged).

2.5 | Apoptotic DNA fragmentation (DNA ladder) assay

Fragmentation of nuclear DNA into nucleosomal fragments is considered one of the hallmarks of apoptosis, called programmed cell death.[18] This process identified a particular characteristic DNase that split chromosomal DNA in a caspase-dependent manner. DNA can be revealed using agarose gel after preparing the purified DNA procedure. The Apoptotic DNA ladder kit (Roche Diagnostics GmbH) was used in DNA isolation to monitor apoptosis in MCF-7 cells. Briefly, MCF-7 cells (5×10^6 cells) were incubated alone or with L1H (10–100 μM) for 24 h, and then collected. DNA was isolated after cells were homogenized in lysis buffer. Finally, the extracted DNA was electrophoresed using 1% agarose gel and visualized by ultraviolet light following ethidium bromide (EB) staining and the photograph was taken.

2.6 | Apoptotic cell analysis by using AO and EB

In this analysis, MCF-7 cells stained using AO and EB to detect apoptotic cells in breast cancer cells were then examined by fluorescence microscopy. While apoptotic cells containing dead bodies were detected in orange, necrotic cells emitted a red color. Living cells were determined due to the green fluorescence color. After ligand administration, MCF-7 cell lines were suspended with phosphate buffer saline (PBS). The cell suspension was accumulated by centrifugation. The cell suspension collected by centrifugation was washed three times with PBS. and then cells resuspended in 25 μg/ml of dye mixture; 100 μg/ml of AO and 100 μg/ml of EB prepared in PBS, and mixed gently. The added 10 μl mixed solution was stained on a microscope slide, covered with a coverslip and examined under 400x magnification using a fluorescence microscope.

We get to identify the cells by nuclear condensation of chromatin as a dense green patch or fragments easily. Late apoptotic cells containing fragmented chromatin were orange-colored and necrotic cells showed orange nuclei with intact structure.[19]

2.7 | Assay of intracellular TAS

Intracellular TAS level in MCF-7 cells was measured using commercially available kits (Rel Assay). The antioxidants in cell lysate reduce the dark blue-green 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical to the colorless reduced ABTS form. The absorbance change at 660 nm is related to the TAS level of MCF-7 cells. The units of the values obtained from the result of this test are expressed as mmol Trolox equiv/L.[20]

2.8 | Assay of intracellular TOS

Intracellular TOS level was measured using a novel automated colorimetric measurement method developed by Erel in 2005.[21] In this study, oxidants appear in the sample to oxidize the Fe^{2+} ion-O-dianisidine complex to Fe^{3+} in the oxidation reaction. Fe^{3+} makes a colored complex with xylenol orange in an acidic medium.[22] The color density associated with the total amount of oxidant molecules in the sample was measured using commercially available kits (Rel Assay). The assay is calibrated with hydrogen peroxide (HP), and the result is illustrated in terms of millimolar HP per L (mmol HP/L).

2.9 | Oxidative stress index (OSI)

The ratio of the TOS to the TAS level is described as the OSI.[23] OSI value was calculated according to the following formula:

$$\text{OSI} = ([\text{TOS,mmol HP equiv/L}] / [\text{TAS,mmol Trolox equiv/L}]) \times 10$$

2.10 | FRAP assay

FRAP analysis, known as antioxidant power, was applied as a simple and reliable colorimetric method.[24] o FRAP is an important parameter that based on reducing Fe^{3+} to Fe^{2+} and at low pH causes a colored ferrous–tripyridyl triazine complex. FRAP is monitored by measuring absorbance at 593 nm when the colorless Fe^{3+} turns into blue Fe^{2+} in MCF-7 cells treated ligand. To measure the FRAP value, 100 μl of our sample is added to 900 μl of deionized water, 2 ml FRAP reagent is then added into the cuvette for 30 min mixing in the dark room. The mixture is transferred to the wells plate and read in a spectrophotometer. FRAP levels were calculated using the equation below.

$$\text{FRAP value } \mu\text{M} = (\text{ABS. [sample]} \times \text{FRAP value of Std [}\mu\text{M]}) / \text{Abs. of Std}$$

2.11 | Total thiol assay

The measurement of the total thiol with the test kit provides an easy and accurate quantitation of the thiol. Simply load 100 μl of the reagent diluted 1:100 into a microplate well, then add 10 μl volume of sample, mix, then read the fluorescence. The assay has a linear

range of 0.05–5 μM thiol, making it more sensitive than colorimetric methods based on Ellman's reagent. Common contaminants are well tolerated in the assay. The assay was performed at room temperature, and the maximum fluorescence signal was attained within 5 min, which was stable for at least 1 h. Fluorescence measurement was done using a microplate reader.

2.12 | Glutathione (GSH) assay

This method was performed on cells prepared as $0.5–1 \times 10^6$ cells /well from ligand-treated and untreated MCF-7 cancer cells. Following the end of the application, the cells scraped in cold PBS were centrifuged. Cells were frozen at −80°C were thawed and homogenized in a cold buffer containing pH 6.0 and centrifuged at 8000*g* for 15 min at 4°C. Finally, the supernatant was transferred to a new tube and used for glutathione testing. Intracellular GSH levels were measured following the manufacturer's instructions (Glutathione assay kit). A standard curve was constructed using serial dilutions of the GSH standard solution. GSH concentration was normalized by milligrams of protein in each tube.

2.13 | Determination lipid peroxidation

This method is based on the colorimetric measurement of malondialdehyde (MDA), which is formed by the reaction of MDA in an acidic medium and high temperature (90–100°C). Since cell culture samples require washing before homogenization, cultured cells were washed several times. For lipid hydroperoxides (LOOH) measurement, it is recommended to prepare a homogenate of approximately 5×10^7 cells per ml for use in the test. Washed in ice-cold isotonic saline (0.9% NaCl), cells were prepared for homogenization. The measurements were carried out in accordance with the analysis protocol with the homogenates obtained after centrifugation (3000*g*, 4°C for 10 min).[25]

2.14 | Determination protein carbonyl assay

Protein carbonyl concentrations were determined in cell lysates defined according to the test protocol steps (Protein Carbonyl assay kit, 2012). Each sample obtained by analysis was transferred to a 96-well plate, and the absorbance was measured at 375 nm.

2.15 | Statistical analysis

All outcomes, including the measured concentrations of TAS, TOS, FRAP, Total Thiol, GSH, LOOH, and Protein carbonyl were evaluated statistically and the final results were expressed as mean ± SD. The results are presented as three replicates. Data in the experiments were analyzed using analyses of variance (one-way analysis of variance). The $p < 0.05$ value was considered statistically significant. All statistical analyses were performed using the MINITAB 17® package program for Windows.

3 | RESULTS

3.1 | Effect of L1H on cell proliferation

In our study, firstly, cytotoxicity assays were used to examine the antiproliferative activity of bis-structured Schiff base material (L1H) and cisplatin on various cell lines, including PNT1-A human normal prostate cells, MCF-7 human breast cancer cells, PC3 human prostate cancer cells, HT-29 human colon cancer cells, HELA human uterine cervical cells, DU-145 human prostate cancer cells, DLD-1 colorectal adenocarcinoma cells, and ECC-1 human endometrial epithelial cell. IC_{50} grade was used particularly to determine the efficiency of the ligand in inhibiting a particular metabolic function of the cancer cell.

IC_{50} values, known as the half-maximal inhibitory concentration value of all cell lines, were obtained by a specific measurement. Although the applied L1H ligand has different IC_{50} values for each of the cell lines aforementioned, it has proven to be more effective, especially in the breast cancer cell line, according to the following (Table 1).

3.2 | MTT assay in MCF-7 and PNT1-A cell line

The effect of L1H on inhibition of growth and proliferation in the MCF-7 cell line was determined via cell viability (MTT) assay at multiple concentrations. According to the results, L1H significantly halted the proliferation of MCF-7 cells and reduced cell viability at all concentrations in the range of 10–200 μM, and therefore, compared to zero doses as a negative control, *p*-values of in question doses were found to be <0.05. Significant reductions in viable MCF-7 cell count were demonstrated at all concentrations treated with cisplatin as a positive control (Figure 2). In general, the percentage of cell viability decreased synchronously in a dose-dependent manner.

TABLE 1 The IC_{50} values in MCF-7, PNT1-A, PC3, HT-29, HELA, DU-145, DLD-1, and ECC-1 cell lines treated with L1H and cisplatin

Cell lines	IC_{50} values (μmol/L)	
	L1H	Cisplatin
MCF-7 (Human breast cancer cells)	25.7	7.70
PNT1-A (Human normal prostate cells)	45.2	5.17
PC3 (Human prostate cancer cells)	69.8	-
HT-29 (Human colon cancer cells)	174.8	-
HeLa (Human cervical cancer cells)	117.7	-
DU-145 (Human prostate cancer cells)	63.8	-
DLD-1 (Colorectal adenocarcinoma cells)	43.5	-
ECC-1 (Human endometrial cancer cells)	58.5	-

Note: Data are averages for triple reading.

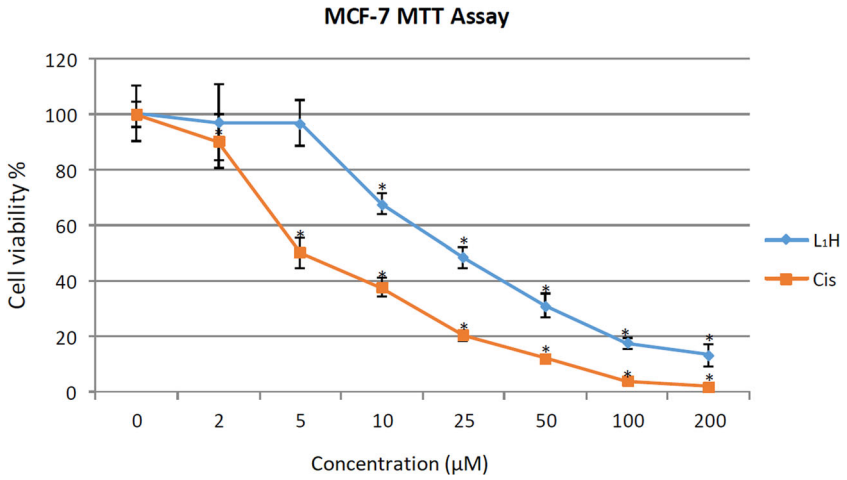


FIGURE 2 Effects of L1H and Cisplatin on MCF-7 cell viability for 24 h (*effective dose)

In the present study, the MTT test was accomplished as a cell-based metabolic activity analysis to screen the cytotoxicity of the ligand. Not all doses of L1H, but their concentrations in the range of 25–200 μM, have significantly impaired the cell viability of human PNT1A cells in Figure 3. Meanwhile, cisplatin produced a significant antiproliferative effect even at the lowest dose (2 μM) administered in healthy PNT1A cells.

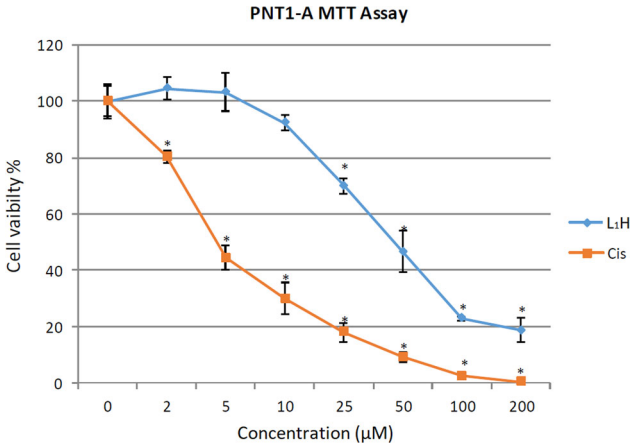


FIGURE 3 Effects of L1H and Cisplatin on PNT1-A viability for 24 h (*effective dose)

3.3 | Apoptotic analysis of MCF-7 by ELISA

The elucidation of the apoptosis was checked by ELISA when a new ligand was treated in MCF-7 cells. Elucidation of apoptotic examination on the growth of MCF-7 cells was controlled by ELISA when we utilized the novel ligand L1H to measure apoptosis, DNA fracture, and histone discharge from the core amid the apoptosis procedure.

The ELISA test of all doses (5, 10, 25, 50, and 100 μM) of the ligand on MCF-7 cells, as shown in Figure 4, indicates that apoptosis is triggered and increases significantly compared to the control group ($p < 0.05$). Apoptosis, which started to be induced with the first dose (5 μM) of the ligand, reached the level of apoptosis caused by cisplatin-induced DNA fragmentation in MCF-7 cells due to the dose increase.

3.4 | SCGE (comet) in MCF-7

The SCGE known as the comet assay is an important approach to using a small-scale gel electrophoresis to assess DNA strand breakage in cells.[26] SCGE analysis was used to distinguish the genotoxicity of the ligand in the cell lines, especially since it can examine DNA chain breaks in a single cell. The genotoxic effect of the L1H on the MCF-7 cells was revealed DNA damage in all concentrations through comet assay (Figure 5). L1H doses (10, 25, 50, and 100 μM) in this experiment had the potential for fragmented DNA damage, significant at $p < 0.05$. The ligand had reached the genotoxic capacity of cisplatin only after 25–100 μM administration.

3.5 | Determination of intracellular TAS in MCF-7 cell line

Our results showed that the new ligand at various concentrations treated for the MCF-7 cell line had a TAS reduction close to cisplatin. Overall, this analysis only provides strong evidence that the 50–100 μM doses of the ligand and the cisplatin have the same efficacy. This showed that the antioxidant ratio in the cell decreased depending on the gradually increasing concentrations. All concentrations of L1H caused a statistically significant decrease in total antioxidant capacity, ie TAS level ($p < 0.05$) (Figure 6).

3.6 | Determination of intracellular TOS in MCF-7 cell line

In this study, the intracellular TOS level was determined in breast cancer cells after different concentrations of ligand administration. A significant increase was observed at all doses of the ligand applied in MCF-7 cell lines compared to the control. Consequently, due to this

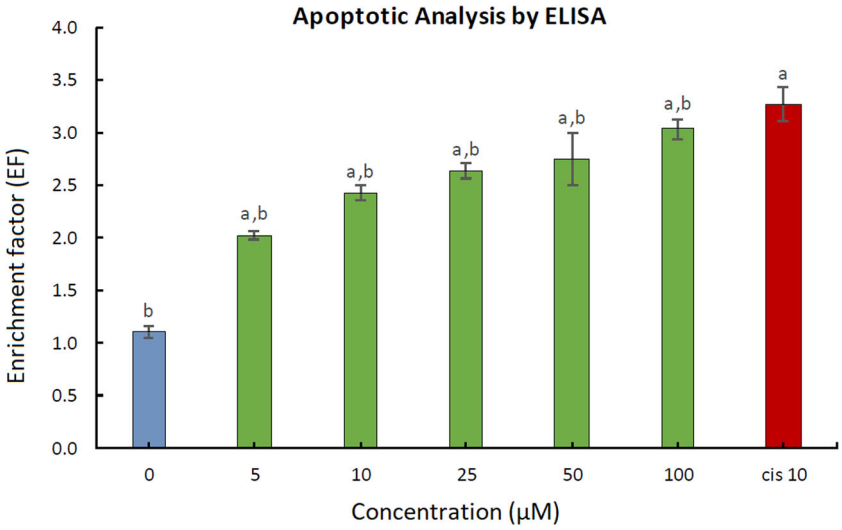


FIGURE 4 Apoptotic effect of various concentrations of L1H on MCF-7 cell line DNA damage measured by ELISA (*p*-value [a]: compared with zero group and *p*-value [b]: compared with the cis group).

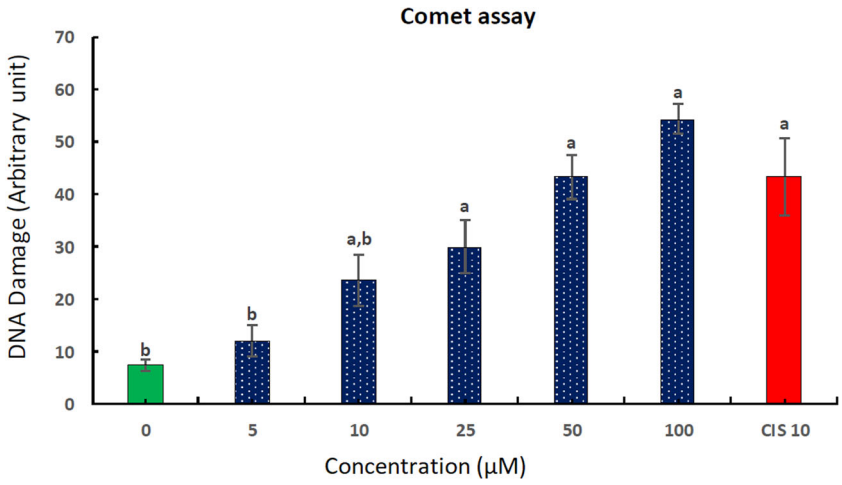


FIGURE 5 Comet assay result of MCF-7 treated by L1H and cisplatin at different doses (*p*-value [a]: compared with zero group and *p*-value [b]: compared with cisplatin group).

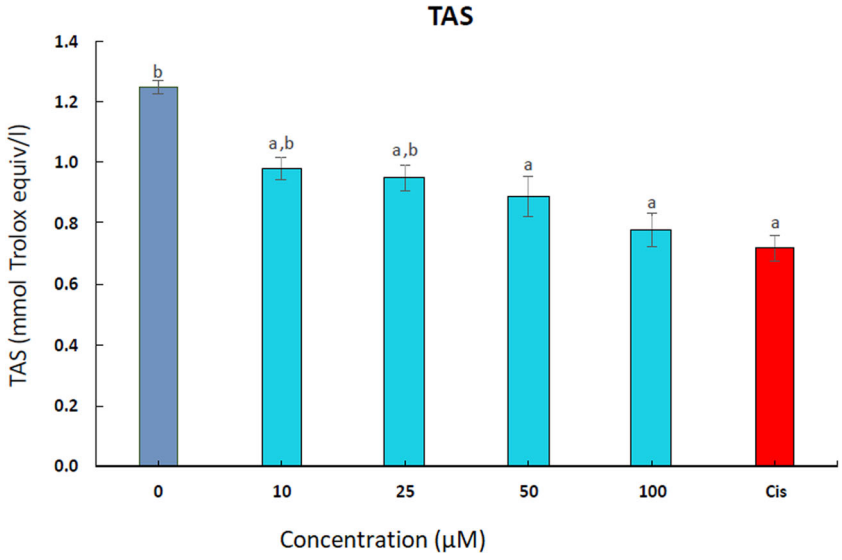


FIGURE 6 TAS value of MCF-7 treated with different doses of L1H and cisplatin (*p*-value [a]: is compared with zero group and *p*-value [b]: is compared with cisplatin group). TAS, total antioxidant status.

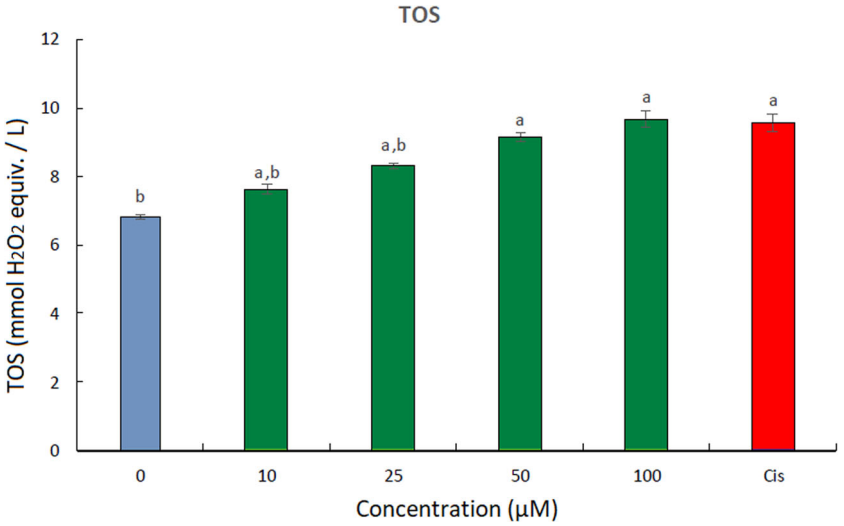


FIGURE 7 TOS values of MCF-7 treated by L1H and cisplatin in various doses (*p*-value [a]: compared with zero group and *p*-value [b]: compared with cisplatin group). TOS, total oxidant status.

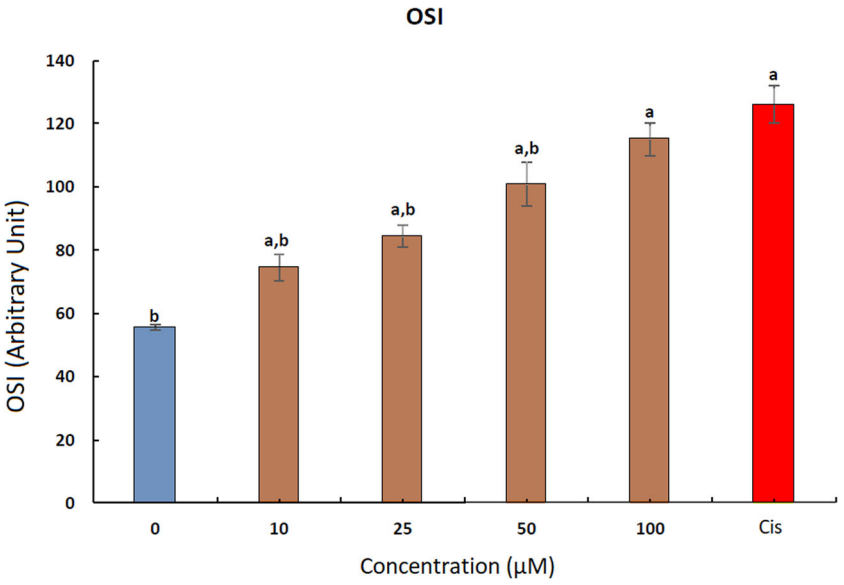


FIGURE 8 OSI values of MCF-7 treated with L1H and cisplatin at different doses (*p*-value [a]: compared with zero group and *p*-value [b]: compared with the cisplatin group). OSI, oxidative stress index.

significant increase, *p*-values were also lower than 0.05. The TOS level in the cells started to increase continuously from the first dose of the ligand and after reaching the cisplatin level with 50 µM doses, it was seen that it reached the peak with a dose of 100 µM ($p < 0.001$) (Figure 7).

3.7 | OSI in MCF-7 cell line

Based on TOS and TAS levels, the evaluation of the OSI in MCF-7 cell lines was performed. After this point, the ratios of the rising values of TOS and the decreasing trend values of TAS were determinants in the OSI value. As shown in Figure 8, our data show that all of the L1H doses have a significant effect on generating oxidative stress on MCF-7 cell lines ($p < 0.05$). OSI values of the ligand had a similar effect to cisplatin only because it achieved a high level in 100 µM dosing applications (Figure 8).

3.8 | FRAP assay of MCF-7 cell line

As a basic assessment and a repeatable test, FRAP has been used as one of the different vital techniques to evaluate the substance targeting the cancer cell. In the FRAP tests, we evaluated the antioxidant power-reducing effect of various concentrations of the ligand in the MCF-7 cell lines, as shown in Figure 9. All L1H concentrations (10, 25, 50, and 100 µM) produced statistically significant results compared to control after being measured by FRAP ($p < 0.05$). However, the last two doses of the ligand, as well as cisplatin, were able to produce the same effect (Figure 9).

3.9 | AO and EB apoptotic analysis in MCF-7 cell line

The use of both colors (AO/EB) is extremely important to recolor molecular changes and hence the apoptotic, necrotic status.[27] In our

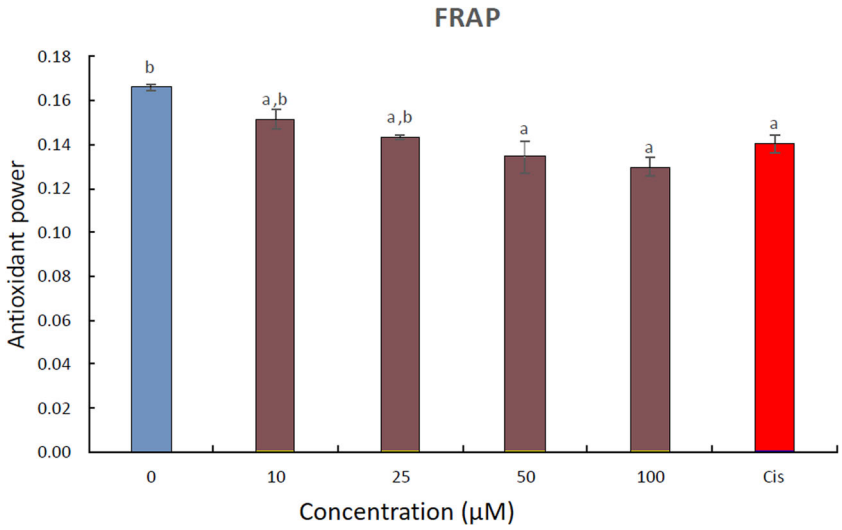


FIGURE 9 FRAP values of MCF-7 treated by L1H and cisplatin at different doses (*p*-value [a]: compared with zero group and *p*-value [b]: compared with cisplatin group). FRAP, ferric reducing antioxidant power.

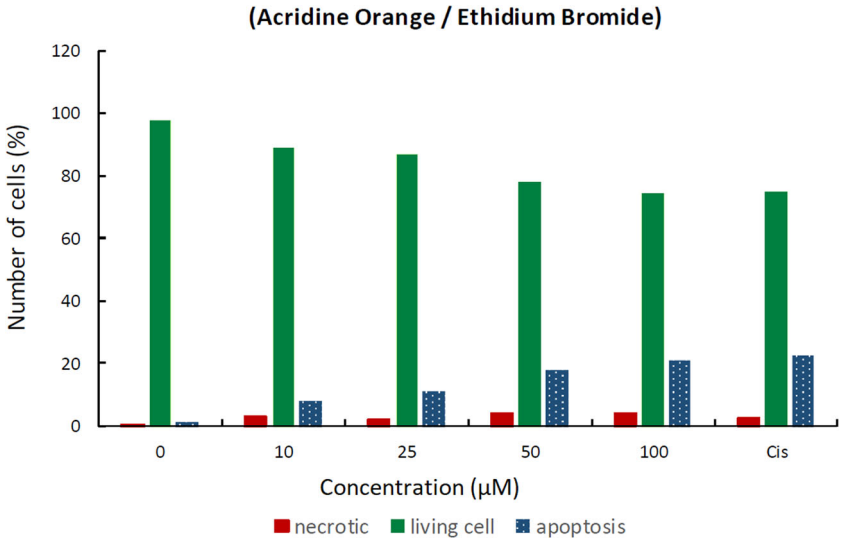


FIGURE 10 Views AO/EB values in MCF-7 treated with different doses of L1H and cisplatin. AO, acridine orange; EB, ethidium bromide.

experiment, the ability of L1H as a ligand to induce apoptosis in MCF-7 cell lines was shown as a percentage evaluation. In a dose-dependent manner, apoptotic cell numbers increased respectively, then viable cell numbers decreased and necrosis cell numbers did not change. Remarkably, cisplatin levels had a similar effect on the 100 μM ligand level (Figure 10). This can be attributed to the fact that L1H is undoubtedly an effective material to disrupt MCF-7 cells when the ligand concentration is increased. Eventually, the level of individual cell apoptosis percentages was distinctly increased. This proves that the decrease in the number of living cells is caused not only by necrotic cells but mainly by apoptotic cells.

3.10 | DNA fragmentation (DNA ladder) of MCF-7 cells

Biochemical apoptotic assays provide easy detection of apoptotic DNA fragmentation in cells. Numerous base pairs that are fragmented by leading to internucleosomal DNA cleavage are called DNA ladders.[28] DNA damage in MCF-7 cells treated with various doses of L1H was measured by DNA ladder segmentation. After 10 μM concentration, there was no significant elongation, but less than zero dose elongation occurred. However, the longitudinal extensions of DNA fragmentation were noticed at doses of 25, 50, and 100 μM. As a result, DNA fragments realized by the ligand have approached the elongation step of the DNA fragments formed by the cisplatin (Figure 11).

3.11 | Total thiol concentrations of MCF-7 cells

Total thiol, as an antioxidant found in our entire body, struggles in the oxidizing environment formed by free radicals.[29] Figure 12 shows the effectiveness of the L1H ligand in different concentrations that are administrated in MCF-7 cell lines. In light of our results, ligand doses (50 and 100 μM) applied to human MCF-7 cells were shown to

significantly reduce total thiol levels compared to control. Even though there was a gradual decrease in the total thiol level in the last doses, it was only able to approach cisplatin efficiency.

3.12 | GSH of MCF-7 cell line

GSH can be found as a protective agent in all tissues, which detoxifies the body.[30] When all the doses of the ligand administered in MCF-7 cells were compared with the control, it had significantly decreased GSH level, and $p < 0.05$ was seen in all results (Figure 13). In this sense, cisplatin dramatically lowered the GSH level in MCF-7 cells compared to control. The GSH level tended to decrease steadily to approach cisplatin at doses of 10–100 μM of the ligand, but could not reach it. In this sense, cisplatin dramatically lowered the GSH level in MCF-7 cells compared to control.

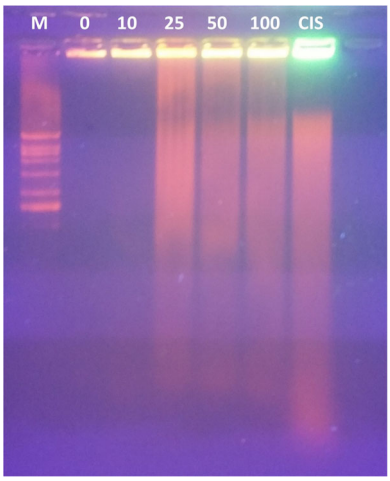


FIGURE 11 DNA ladder in MCF-7 cells treated with different doses of L1H and cisplatin

3.13 | Lipid peroxidation in MCF-7 cell line

LOOH is imperative for a biomarker to discover and evaluate early-stage lipid peroxidation and free radical in the cell level damage.[31] Lipid oxidation is increased in a dose-dependent manner through the administration of the ligand in MCF-7 cells. At the last two doses of L1H (50 and 100 μM), LOOH values increased significantly compared to the zero dose ($p < 0.05$). Thus, it reached the level of lipid hydroperoxide induced by cisplatin administered as a positive control in MCF-7 cell lines (Figure 14).

3.14 | Protein carbonyl assay of MCF-7 cell line

Protein carbonyl levels are the most commonly used biomarker of protein oxidation, and their accumulation is being studied as a risk factor for a variety of human diseases, including cancer.[32] This test seemed to be very useful for apoptosis due to rapid protein peroxidation using a new ligand, which can be considered a cure for MCF-7 cells. One of the characteristic roles of reactive oxygen species is that it can damage DNA, lipids, and protein. When the initial concentration (10 and 25 μM) was investigated, it was determined that the protein carbonyl values started to rise and eventually cisplatin reached its effective value (Figure 15).

4 | DISCUSSION

In this study, we checked and investigated the anticancer drug potential of the cytotoxic new ligand L1H. Our experimental tests used to examine the efficacy of L1H on cancer cell lines showed that the human MCF-7 cell line was affected by sensitivity. As a priority study, the L1H we used on various cell lines inhibited proliferation, especially in the MCF-7 cell line. It was measured by accurate and

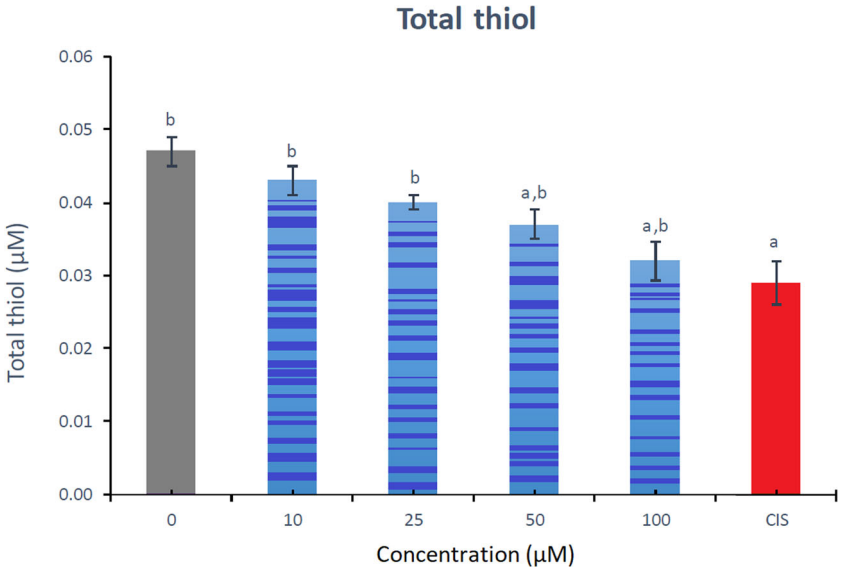


FIGURE 12 Total thiol value of MCF-7 treated by L1H and cisplatin at different doses (*p*-value [a]: compared with zero group and *p*-value [b]: compared with the cisplatin group).

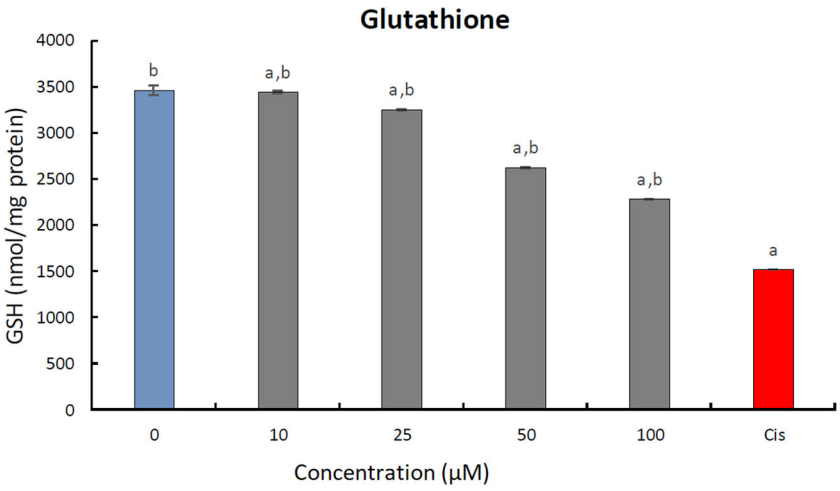


FIGURE 13 Glutathione levels of MCF-7 treated by L1H and cisplatin in various doses (*p*-value [a]: compared with zero group and *p*-value [b]: compared with cisplatin group).

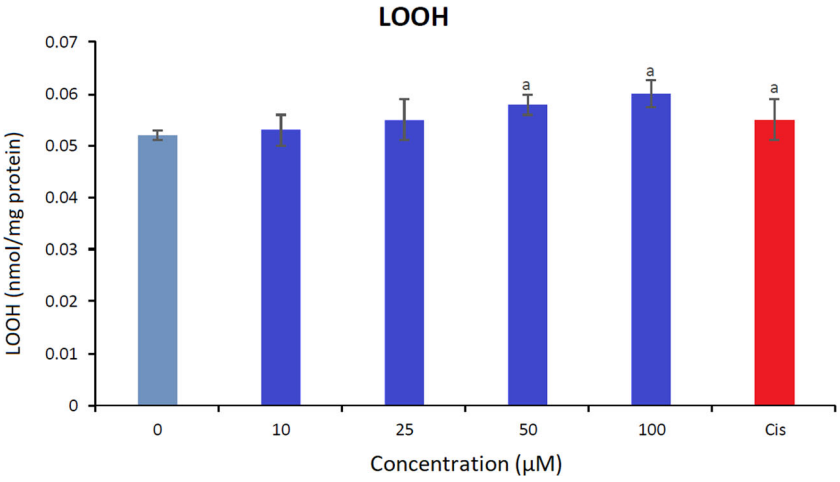


FIGURE 14 LOOH values of MCF-7 treated by L1H and cisplatin at various doses (*p*-value [a]: compared with zero doses and *p*-value [b]: compared with cisplatin doses).

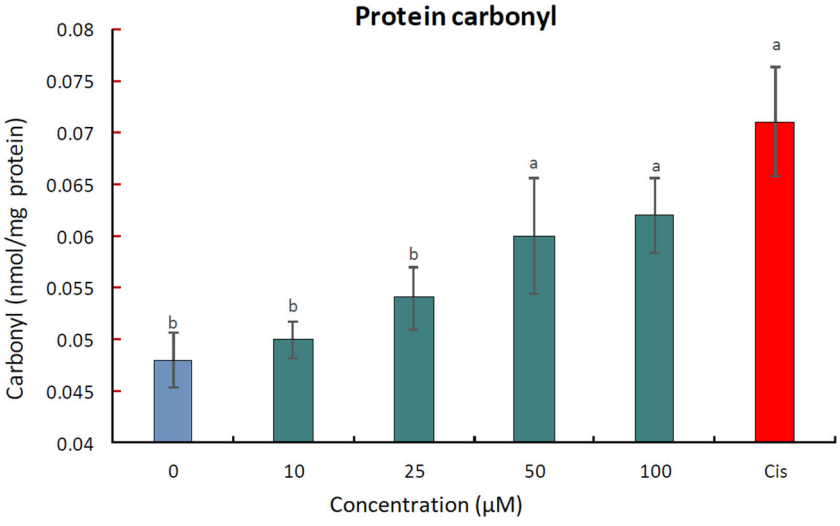


FIGURE 15 Protein carbonyl of MCF-7 treated by L1H and cisplatin in different doses (*p*-value [a]: compared with zero group and *p*-value [b]: compared with cisplatin group).

comprehensive IC_{50} tests that cisplatin, which affected both MCF-7 and PNTI-A cell lines, did not allow cell proliferation. Another research report using arsenic trioxide (As_2O_3) and the MCF-7 cell line was recorded. Thus, As_2O_3 inhibits proliferation in the MCF-7 cell line, in its 99.5% lyophilized form, which was demonstrated by IC_{50} measurement.[33]

v Our study led us to conclude that different L1H doses showed to induce apoptosis and that the underlying reason for the suppression of proliferation revealed in the MTT test occurred accordingly. Thereby, significant p-values indicating reduced cell viability of the MTT assay in the MCF-7 cell line were seen at all doses ranging from 10 to 200 μM compared to the negative control.[34,35]

In our results, the cell growth inhibitory role in the dose-dependent administration of L1H in nonneoplastic human PNT1-A cell lines is investigated and demonstrated in the MTT assay, which is used as a marker that reflects the reduction of live cell metabolism. The biological activities of anticancer drugs occur only when they are tested in vitro and especially in cytotoxicity studies on live cell lines. Only more intensive doses of the ligand administered at various concentrations affected the PNT1A cell line in the MTT assay. Whereas all doses of cisplatin, used as a positive control in this study, have reduced cell proliferation in PNTA-1 cells, which are healthy control cells by suppressing cell viability in Figure 3. Moreover, a study has reported that high and low calcium (Ca^{2+}) reduced the viability of PNT1A cell lines compared to the physiological dose of calcium.[36]

Our experiment has indicated that reliable and leisurely depicted apoptosis on MCF-7 cell lines at gradually increasing concentrations of the ligand after being analyzed by ELISA. Apoptotic activity was not only dominant in parallel with increasing doses, but all ligand concentrations administered without exception also had a significant effect. Considering all of this ELISA evidence, it seems that the programmed cell death trend was able to reach the highest levels in the cisplatin group as a positive control.

In another study investigating Taxol, a different chemotherapeutic agent used in the MCF-7 cell line, it was shown to modulate the progression of apoptosis induced in the MCF-7 cell line. Increasing doses of Taxol mentioned to sensitize cancerous cells have been reported to cause apoptosis as measured by ELISA analysis, supporting our current research.[37]

We studied an innovative detection of DNA damage caused by various number concentrations of L1H on the MCF-7 cell line evaluated by comet assay. Nevertheless, L1H concentrations have an accurate therapy potential for cancerous cells by causing fragmented DNA damage in the process leading to apoptosis. A prominent DNA breakage has also emerged in the MCF-7 cell lines, as shown in Figure 4 utilizing cisplatin. This finding is also consistent with a study by other authors, in which they studied the induction of single- and double-strand breaks of DNA damage in MCF-7. Current findings may partially explain the genotoxicity of Ligand, which was assessed using comet assays.[38]

For the current research, the ligand was limited to TAS and TOS values only in terms of total oxidant and antioxidant status, then OSI values were calculated by proportioning TOS values to TAS values to demonstrate the importance of oxidative stress in the MCF-7 cell line. All doses of the ligand L1H (10, 25, 50, and 100 μM) were measured separately for TAS and TOS, while TAS values in human breast cancer cell lines were undoubtedly lessened at all L1H doses, the TOS values ascended at the entire concentration of L1H. Subsequently, OSI values were working based on these results were demonstrated to increase in all L1H doses in MCF-7 cells. This result is an index showing that denoted to move up oxidation, similar to the results of cisplatin, used as a positive control group in MCF-7 cell lines in Figure 8. A prior study investigated the sensitivity of breast cancer cells to rapamycin and found that oxidative stress was enhanced with the administration. Conversely, raising antioxidant levels after treatment might be a reason for the development of rapamycin resistance in MCF-7.[39] Our results are consistent with Ali and his colleagues[40] who reported that the preparations thienopyrimidine derivatives possess significant anticancer activity in MCF-7 cells through regulation of free radicals generation.

In our results, FRAP values measured after administration of all doses of the L1H in the MCF-7 cell lines were shown to gradually decrease compared to control. When the values of FRAP known as a powerful antioxidant that can be reduced ferric to ferrous in the last dose are compared with cisplatin, the results show that it is as effective as cisplatin (Figure 9). It is possible to state that the interaction, which was effective in the slowing of cellular growth resulting in reduced MCF-7 cancer cell proliferation, could be decreased antioxidants and increased oxidants.

Dual AO/EB staining was used to examine basic morphological changes of apoptotic cells in MCF-7 cells treated with the L1H. Besides that, as shown in Figure 10, it provided quantification of the distinction between living cells, apoptotic cells, and necrotic cells. In a dose-dependent manner, the number of apoptotic cells increased compared to the number of viable cells that were detected to decrease, while necrotic cell numbers did not alter notably. In an article by the researchers, the results of the measurement of apoptosis with AO/EB in MCF-7 cells applied ethanolic extract of *Withania somnifera* and *Tinospora cordifolia* were similar to our results.[41]

The appearance of a longitudinal extension image in the gel indicates that DNA damage occurs due to DNA degradation. Thus, we verified the internucleosomal cleavage of genomic DNA, which is considered one of the distinguishing features of apoptosis, by DNA fragmentation analysis in L1H-administered MCF-7 cells as presented in Figure 11. In the article showing the DNA ladder by the researcher in the MCF-7 cell line using cladribine, it was stated that the addition of 500 μM cladribine caused apoptosis due to DNA degradation, which is more extensive than other doses.[34]

Overall, the total thiol values of the MCF-7 cell line were measured after treatment and were seen to be reduced at all L1H doses. The oxidative effect of the ligand may have reduced the amount of total thiol by consuming the thiol during neutralizing the free radicals formed by it. Thus, a reduced antioxidant condition compared to the control may have led the cell to the apoptosis pathway. Reports from other researchers, indicating that total thiol

(sulfhydryl) decreases due to oxidative stress when topotecan is used in the same MCF-7 cells, support our study results.[42]

In the current study, it should not be surprising to say that the amount of GSH consumed as a result of dose-dependent ligand treatment followed the total thiol decrease in a similar way, as seen in the Figure 13.

Data analysis of the LOOH test identified the most effective concentrations in lipid peroxidation levels that occurred after applying L1H to MCF-7 cells (Figure 14). Both L1H levels (50 and 100 μM) are as effective as cisplatin in these cancer cells. It has been reported by the researchers who treated the flaxseed oil in the MCF-7 cell line that the application in these cells caused apoptosis due to the increased LOOH values.[43]

In our study, protein carbonyl values increased significantly in all dose-dependent administration of the ligand and especially in the last dose. It is compatible with the report showing the correlation between increased carbonyl values after topotecan application and the formation of apoptosis in MCF-7 breast cancer cells.[42]

5 | CONCLUSIONS

As a result of the novel ligand L1H mediating DNA damage, oxidative stress and protein carbonyl increase, we revealed that the ligand has the ability to increase or induce apoptosis in MCF-7 cells, which is an attractive target for anticancer drugs. Besides this, the chain of events resulting in increased lipid peroxidation due to weakening in FRAP and decreases in total thiol and glutathione levels carries molecular traces of the interaction of the ligand in the cellular mechanism. Our results will depict that a good medical chemopreventive with potential pharmaceutical principles should be investigated as an option for the treatment of the MCF-7 cell line, so in the future, we can deal with a different application model and processing system laboratory in vitro and in vivo using the novel ligand compound L1H, as well as investigation for progressive L1H, may investigate another assay that uses various cancer cell lines.

AUTHOR CONTRIBUTIONS

Yasin Tülüce and Azhee Ibrahim Hussein designed the experiments. Ahmet Kiliç and Mustafa Durgun synthesized the applied substance. Yasin Tülüce, Azhee Ibrahim Hussein, and İsmail Koyuncu performed the experiments. Yasin Tülüce and Azhee Ibrahim Hussein wrote the article.

ACKNOWLEDGMENT

This study was supported by Van Yuzuncu Yil University, Scientific Research Projects (YYU BAP) by the Project number [2015-SBE-YL358].

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Y. Tülüce, A. I. Hussein, İ. Koyuncu, A. Kiliç, M. Durgun, *J. Biochem. Mol. Toxicol.* **2022**, e23148. https://doi.org/10.1002/jbt.23148