http://dergipark.org.tr/trkjnat
Trakya University Journal of Natural Sciences, 26(1): 1-8, 2025
ISSN 2528-9691
DOI: 10.23902/trkjnat.1549105

Research Article

Antiproliferative effect of silver compounds containing benzimidazole ring

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Cite this article as:

Bahar D., Akkoç S. & Özkan B.B. 2025. Antiproliferative effect of silver compounds containing benzimidazole ring. *Trakya Univ J Nat Sci*, 26(1): 1-8, DOI: 10.23902/trkjnat.1549105

Received: 13 September 2024, Accepted: 02 November 2024, Online First: 20 December 2024, Published: 15 April 2025

Edited by:
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Key words:
Apoptosis
Cytotoxic activity
Ag-NHC
Oxidative stress
Cancer

Abstract: The benzimidazole nucleus is a crucial pharmacophore in medicine, due to its significant biological activities. Recent studies have focused on the synthesis and biological activity of benzimidazole compounds. Therefore, in the present study, a series of *N*-heterocyclic silver compounds containing benzimidazole nuclei were prepared, and *in vitro* cell culture experiments were performed to evaluate their effects. The compounds were cultured with lung (A549), colon (CaCo2), breast (MCF7), prostate (PC3) cancer cell lines, and healthy lung (WI38) cell line, and their anticancer effects were investigated through the MTT cell proliferation test, apoptosis determination, and oxidative stress tests on these cell lines. The results revealed that the presence of drug candidates was more practical than that of commercially used cisplatin.

Özet: Benzimidazol çekirdeği önemli biyolojik aktiviteleri ile tıpta önemli bir farmakofordur. Son çalışmalar benzimidazol bileşiklerinin sentezi ve biyolojik aktivitesine odaklanmıştır. Bu nedenle, bu çalışmada benzimidazol çekirdekleri içeren bir dizi *N*-heterosiklik gümüş bileşiği hazırlamıştır. Bileşiklerin etkilerini incelemek için *in vitro* hücre kültür deneyleri yapılmıştır. Bileşikler akciğer (A549), kolon (CaCo2), meme (MCF7), prostat (PC3) kanser hücre hatları ve sağlıklı akciğer (WI38) hücre hattı ile kültüre edilmiş ve bileşiklerin antikanser etkileri bu hücreler üzerinde MTT hücre çoğalma testi, apoptoz tayini ve oksidatif stres testleri yapılarak araştırılmıştır. Sonuçlar ilaç adaylarının varlığının ticari olarak kullanılan sisplatinden daha pratik olduğunu ortaya koymuştur.

Introduction

Cancer is a growing global threat to human life, with increasing mortality rates due to numerous factors such as disease diversity and drug resistance (Block *et al.* 2015). Over 14 million cancer cases are reported worldwide annually, and this number is estimated to reach 19.3 million by 2025. Although cancer is a treatable disease, late diagnosis, risky surgical procedures, prolonged and severe side effects of chemotherapy, and resistance to chemotherapeutic drugs make treatment challenging (Chaicharoenaudomrung *et al.* 2019). The incidence and mortality rates of breast cancer are expected to show an increase pattern in recent future. Indeed, breast cancer accounted for 11.6% of all cancer cases worldwide in 2018. Colon cancer is the most common cancer affecting both sexes, with one in ten cancer patients diagnosed with metastatic colon cancer. Approximately 600,000 people die from metastatic colon cancer annually (Siegel *et al.* 2024).

While the effectiveness of cancer drugs is debatable, toxic risks have been observed in drugs developed against cancer, and resistance to cancer drugs is increasing. This underscores the urgent need for the development of new methods for disease treatment. Despite significant advancements in treatment methods and improvements, cancer remains unbeaten, fueling the ongoing demand for more effective drugs in this sector. The present research, focusing on the synthesis and determination of antiproliferative effects of silver compounds containing benzimidazole ring, has the potential to contribute significantly to this fight.

Benzimidazoles, with their 'Hem' structure, a key component of hemoglobin, and the presence of the 'heme ion' system, which is crucial for oxygen transport in the body, play a vital role in functions of some biological molecules, such as metalloproteins and B12 vitamins (Akkoç *et al.* 2019). Naturally occurring nucleotides containing adenine and guanine are isoesters of the benzimidazole ring, allowing interaction with biopolymers in organisms. Benzimidazole and its

derivatives are among the most synthesized and investigated heterocyclic compounds in reactivity (Gök *et al.* 2014, Akkoç *et al.* 2016a, Akkoç *et al.* 2017, Akkoç 2019). The anticancer effects of benzimidazole derivatives may arise from their cytotoxic effects on cancer cells (Bansal & Silakari 2012, Tonelli *et al.* 2014, Akkoç *et al.* 2016b, Gök *et al.* 2019).

Therefore, our study investigated the cytotoxic effects of a series of *N*-heterocyclic silver compounds containing benzimidazole nuclei on lung (A549), colon (CaCo2), breast (MCF7), and prostate (PC3) cancer cell lines and Wl38 healthy epithelial cell line.

Materials and Methods

Synthesis of silver metal compounds

The process of synthesizing the silver metal compounds involved stirring the synthesized benzimidazolium salt (2 mmol), silver oxide (1 mmol), and molecular sieve (5-10 beads) in dried dichloromethane (15 mL) at room temperature for 18 hours (Gök *et al.* 2014, Gök *et al.* 2019). The solution was then filtered through celite, and the resulting silver complexes were crystallized in a dichloromethane-diethyl ether mixture. The cis-platin that used as positive control drug were purchased from Sigma (SigmaAldrich, USA).

Evaluation of compounds' anticancer effect

For this purpose, breast (MCF-7) lung (A-549), prostate (PC-3), and colon (CaCo2) cancer cell lines, and WI38 healthy lung epithelial cell line were cultured. The culture media included 10% Fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin-streptomycin, and EMEM (Eagle's Minimum Essential Medium) for MCF-7 cell line, F-12K for A-549 and PC-3 cell lines, and RPMI 1640 for CaCo2 cell line.

Cells were thawed from liquid nitrogen, seeded into 75 cm^2 flasks, and placed in a 37°C, 5% CO_2 incubator. The culture medium was changed every 3-4 days. When the cell density in the culture dishes reached 90-95%, trypsinization was performed, and cells were subcultured to obtain a higher number. Excess cells were cryopreserved in a freezing medium containing 10% FBS, 80% culture medium, and 10% Dimethyl sulfoxide (DMSO), stored at -80°C, and then transferred to liquid nitrogen for further studies. Once an adequate number of cells were obtained, they were subjected to trypsinization again and seeded into 96-well culture plates at a density of $3x10^3$ cells per well for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After 24 hours of cell seeding, cells were cultured in triplicates with different concentrations of each silver metal compounds (200, 100, 50, 25, 12.5, and 6.25 μM) and following a 48-hour incubation, MTT solution prepared in Phosphate-buffered saline (PBS) was added to each well at a final concentration of 0.5 mg/mL, and the plates were further incubated for three hours. The medium containing formazan crystals formed within the cells was aspirated, and DMSO was added to dissolve the formazan crystals. The color developed after dissolving the formazan crystals in DMSO was measured at 560 nm using a Promega Glomax Elisa reader. Each test was performed at least in triplicate. The obtained absorbance values were analyzed using the GraphPad Prism statistical software, and the half-maximal inhibitory concentrations (IC_{50}) of the drugs on cells were determined (Aslan *et al.* 2020).

The impact of effective doses on apoptosis and reactive oxygen species (ROS) activity in cells was determined using the Muse™ Annexin V & Dead Cell Kit (Catalog No: MCH100105) and Muse® Oxidative Stress Kit (Catalog No: MCH100111), respectively. For this purpose, cells were seeded into 6-well culture plates, and silver metal compounds doses that induced IC_{50} values in the cells, as determined by MTT assay, were used. After a 48-hour incubation with drugs, apoptosis and oxidative stress tests were performed. The kits were used according to the manufacturer's instructions, and data were analyzed using TURCOSA statistical software. A dependent two-sample t-test was used to evaluate the effects of different drugs on the same cells.

Cells were stained with propidium iodide (PI) and Hoechst double staining to visualize apoptosis and cell membrane integrity. Like the MTT assay, cells were seeded into 96-well culture plates, and drug doses resulting in IC_{50} values were added. After 48 hours, cells were washed three times with DPBS, incubated with DPBS containing PI and Hoechst for 15 minutes in the dark and imaged using a fluorescence microscope at x20 objective (Nikon, Ti Eclipse).

Statistics

The half-maximal inhibitory concentrations (IC_{50}) values were analyzed using the GraphPad Prism statistical software and other statistical evaluations were made using a t-test.

Results

Preparation of Silver Metal Compounds

The open structures of benzimidazol ring conteined silver metal complexes were given in Fig. 1. The known complex, [1-phenyl-3-(2,4,6-trimethylbenzyl) benzimidazole-2-ylidene]chlorosilver(I), SE-231, was synthesized according to the literature (Gök *et al.* 2014). The known complexes, 1-(2-diethylaminoethyl)-3-(2,3,5,6-tetramethylbenzyl)benzimidazole-2-ylidene]chlorosilver(I), SE-208, and 1-(2-diethylaminoethyl)-3-(2,3,4,5,6-pentamethylbenzyl) benzimidazole-2-ylidene]chlorosilver(I), SE-209, were prepared according to the literature (Akkoç *et al.* 2014, Gök *et al.* 2014).

NMR data of the synthesized known compounds are given below. Both proton and carbon NMR data prove the accuracy of the structures.

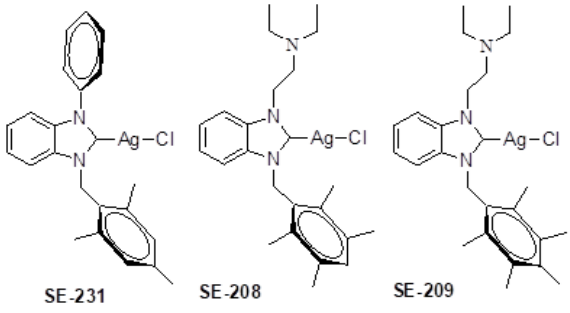

Fig. 1. Synthesized Ag-NHC complexes.

[1-Phenyl-3-(2,4,6-trimethylbenzyl)benzimidazol-2-ylidene]chlorosilver(I), SE-231

^{1}H NMR (300.13 MHz, DMSO-d_6); δ: 1.65 and 2.52 [s, 9 H, $NCH_2C_6H_2(CH_3)_3$-2,4,6]; 5.55 [s, 2 H, $NCH_2C_6H_2(CH_3)_3$-2,4,6]; 6.71–8.26 (m, 11 H, Ar-H). ^{13}C NMR (75.47 MHz, DMSO-d_6); δ: 20.4, 21.1, 55.3, 112.8, 125.0, 128.2, 130.4, 125.5, 129.8, 134.1, 135.2, 126.7, 134.3, 137.9, and 138.5 (Gök *et al.* 2014).

[1-(2-Diethylaminoethyl)-3-(2,3,5,6-tetramethylbenzyl)benzimidazol-2-ylidene]chlorosilver(I), SE-208

^{1}H NMR (300.13 MHz, DMSO-d_6), δ: 0.7–2.74 [m, 14 H, $NCH_2CH_2N(C_2H_5)_2$]; 2.07 and 3.35 [s, 12 H, $NCH_2C_6H(CH_3)_4$-2,3,5,6]; 5.54 [s, 2 H, $NCH_2C_6H(CH_3)_4$-2,3,5,6]; 6.97–7.93 (m, 5 H, Ar-H). ^{13}C NMR (75.47 MHz, DMSO-d_6), δ: 12.1, 20.8, 46.5, 47.3, 16.4, 48.6, 52.8, 112.2, 112.6, 124.4, 131.9, 132.5, 133.6, 133.8, 134.4, and 134.5 (Akkoç *et al.* 2014).

1-(2-Diethylaminoethyl)-3-(2,3,4,5,6-pentamethylbenzyl)benzimidazole-2-ylidene] chlorosilver(I), SE-209

^{1}H NMR (300.13 MHz, DMSO-d_6), δ: 0.92 [t, 6 H, *J*: 7.16 Hz, $NCH_2CH_2N(CH_2CH_3)_2$]; 2.21–2.35 [m, 15 H, $NCH_2C_6(CH_3)_5$]; 2.52 [q, 4 H, *J*: 7.14 Hz, $N(CH_2CH_3)_2$]; 2.84 and 4.39 (tt, 4 H, *J*: 6.61 and 6.59 Hz, $NCH_2CH_2NC_4H_{10}$); 5.48 [s, 2 H, $NCH_2C_6(CH_3)_5$]; 7.14–7.55 (m, 4 H, Ar-H). ^{13}C NMR (75.47 MHz, DMSO-d_6), δ: 12.1, 17.1, 17.2, 17.4, 47.6, 47.7, 49.0, 53.2, 111.3, 111.6, 123.9, 124.1, 126.6, 126.9, 132.9, 133.9, 134.2, and 137.3 (Akkoç *et al.* 2014).

Cell Culture and Evaluation of Compounds' Anticancer Effect

In the MTT assay, six doses of each drug candidate were added to the cells, with concentrations of 200, 100, 50, 25, 12.5, and 6.25 µM. The absorbance data obtained from the MTT assay were evaluated using the GraphPad Prism software to determine the IC_{50} value for each cell line. The IC_{50} values for the cells are presented in Table 1.

According to the MTT results, SE-208 containing 2-diethylaminoethyl and 2,3,5,6-tetramethylbenzyl substituents on the nitrogen atoms in the benzimidazole ring is the most cytotoxic compound at IC_{50}, with a value of 3.08 µM, against MCF7 cells. Overall, SE-208 was the most effective at the lowest dose for all cell lines. According to the literature, silver compounds exhibit high cytotoxicity due to their metal complex content.

The apoptosis test's result graph was given in Fig.2. According to the results of the apoptosis test using Annexin V, all compounds statistically significantly affected the cells. The initial evaluation was performed by comparing silver compounds with each other. According to this comparison, silver compounds reduced the number of viable cells in A549 and CaCo2 cell lines compared to organic compounds.

For early apoptosis, the most effective cells for silver compounds were MCF7, PC3, and Wl38, respectively. The lowest early apoptosis values were observed in the CaCo2 cell line. An increase in late apoptosis values was observed in MCF7, CaCo2, and A549 cells. In the Wl38 cell line, the SE-208 compound primarily increased late apoptosis. CaCo2 and A549 cells had the highest number of dead cells.

When the results were evaluated cell-by-cell, the Wl38 epithelial lung line was the most affected by the compounds. The study included this line to test whether the compounds were specific to cancer cells. According to the apoptosis test, the SE209 compound containing 2-diethylaminoethyl and 2,3,4,5,6-pentamethylbenzyl substituents on the nitrogen atoms in the benzimidazole ring showed more cancer cell specificity than others.

Regarding individual apoptosis test results for the produced compound, the SE209 compound was effective. SE209 significantly reduced viability in CaCo2 and A549 cells and increased apoptosis significantly in the same cell lines. Overall, CaCo2 and A549 cells were the most affected by the compounds and showed similar reactions.

The oxidative stress test results were given in Fig3. According to the oxidative stress test results, silver metal compounds increased the ROS activity more than Cis-platin on A549 (%ROS+ activity: $_{\text{Cis-platin}}56$; $_{\text{SE231}}77$; $_{\text{SE208}}64$; $_{\text{SE209}}59$), PC3 (%ROS+ activity: $_{\text{Cis-platin}}60$; $_{\text{SE209}}61$), and Wl38 (%ROS+ activity: $_{\text{Cis-platin}}17$; $_{\text{SE231}}77$; $_{\text{SE208}}19$; $_{\text{SE209}}69$) cell lines statistically significantly (p>0.05). On CaCo2 and MCF7 cell lines, compounds affected the cells positively and reduced the ROS activity when compared to the control.

Based on the results obtained from PI-Hoechst double staining, PI staining was observed in the cells as a result of the compounds' apoptotic effects. The integrity of the cell membranes was maintained, and no disruption in cell integrity was observed (Figs 4-8).

Table 1. IC_{50} values of complexes.

Complexes	**IC_{50} (µM)**				
	A549	CaCo2	MCF-7	PC3	Wl38
SE-231	38.95	22.22	49.62	163.90	6.83
SE-208	4.79	5.01	3.08	10.18	3.34
SE-209	14.49	9.60	17.74	45.48	40.34
Cisplatin	21.05	16.53	28.22	72.17	51.65

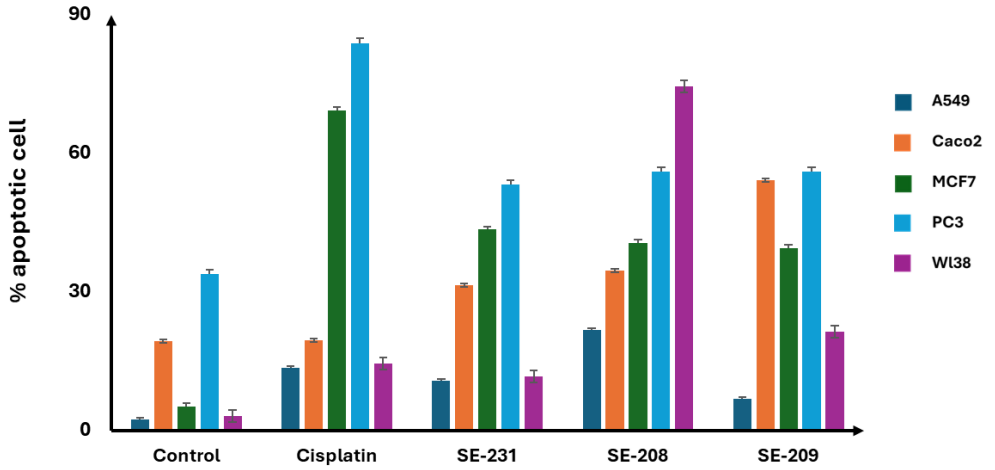

Fig. 2. Percentages of the cancer cell lines' apoptosis in the absence/presence of compounds SE-231, SE-208, SE-209.

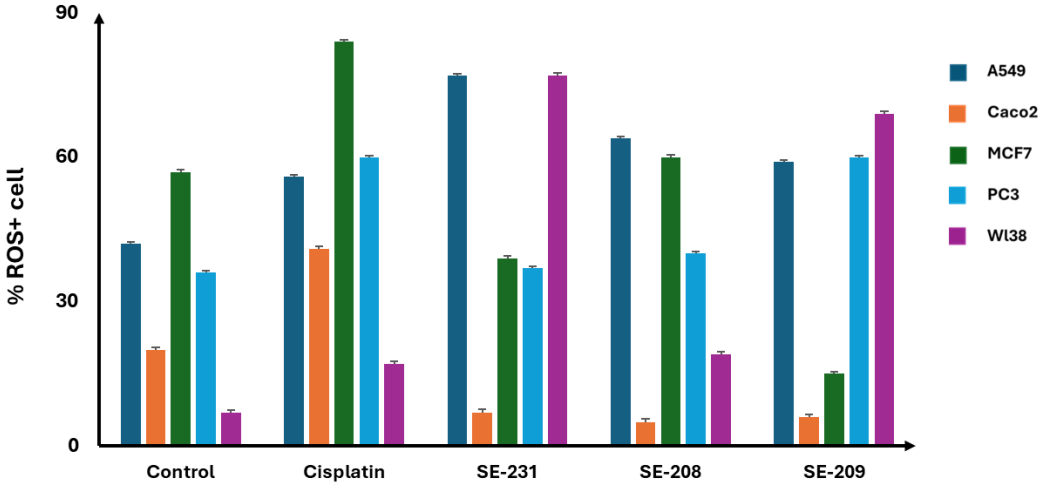

Fig. 3. Percentages of the ROS+ cells in the absence/presence of compounds SE-231, SE-208, SE-209.

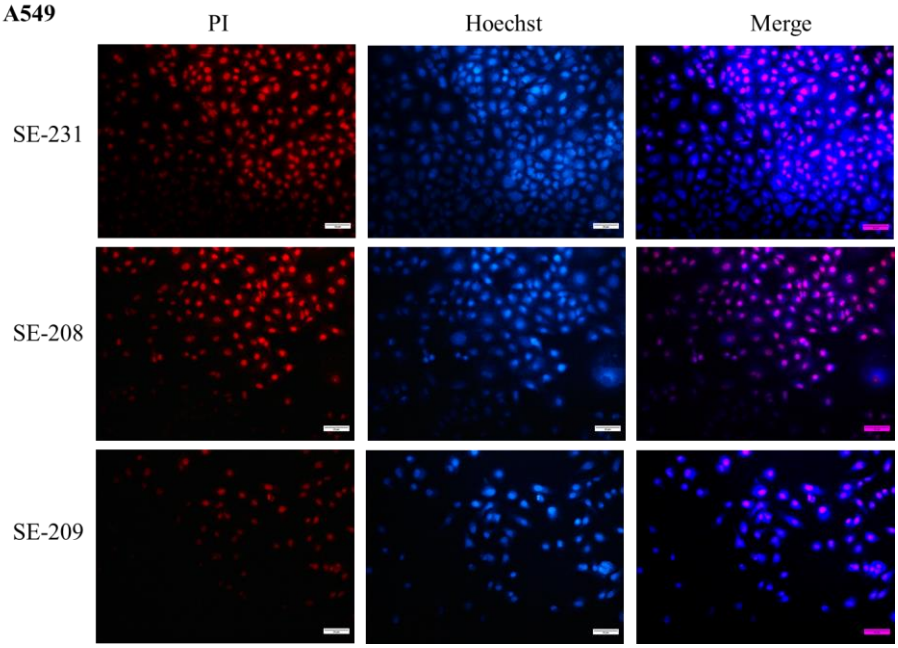

Fig. 4. The fluorescence microscopy images of A549 cell line in the presence of compounds SE-231, SE-208, and SE-209, scale bars=100µM.

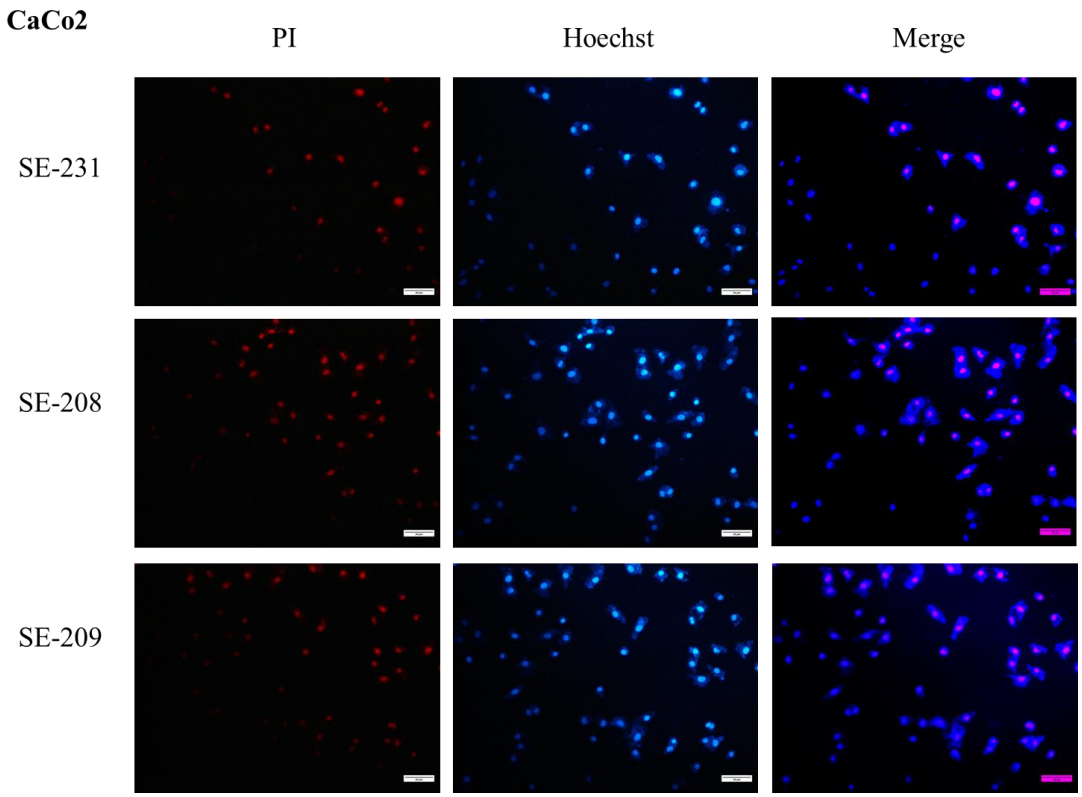

Fig. 5. The fluorescence microscopy images of the CaCo2 cell line in the presence of compounds SE-231, SE-208, and SE-209, scale bars=100µM.

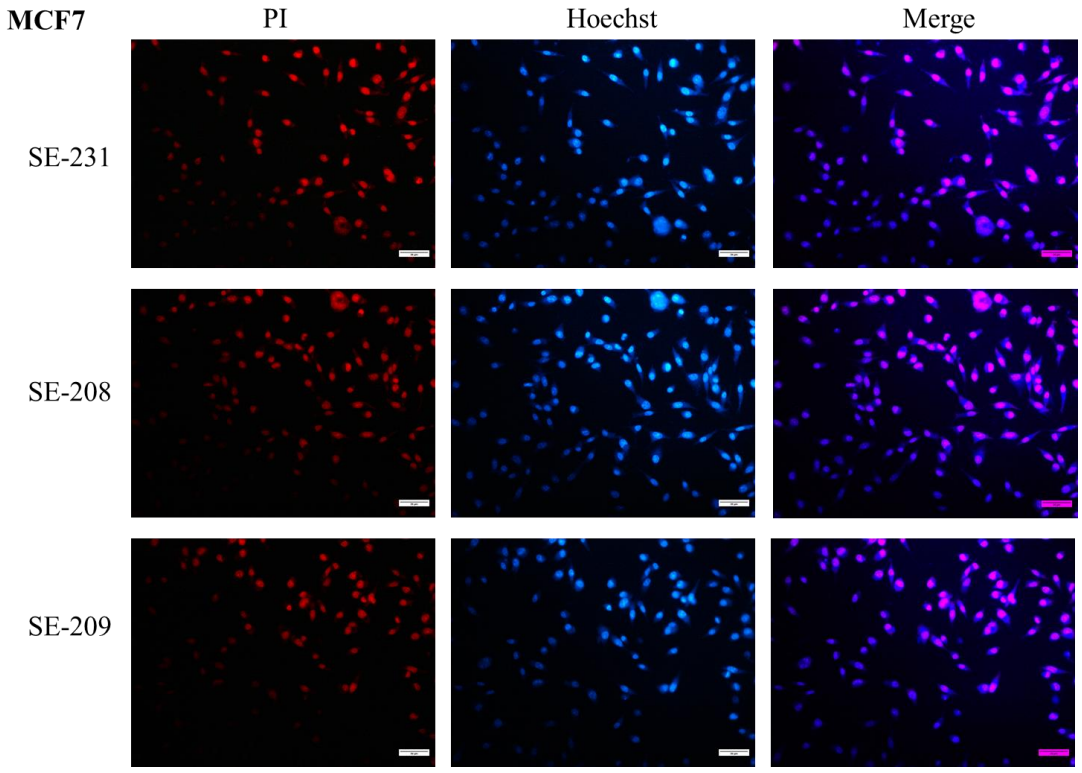

Fig. 6. The fluorescence microscopy images of the MCF-7 cell line in the presence of compounds SE-231, SE-208, and SE-209, scale bars=100µM.

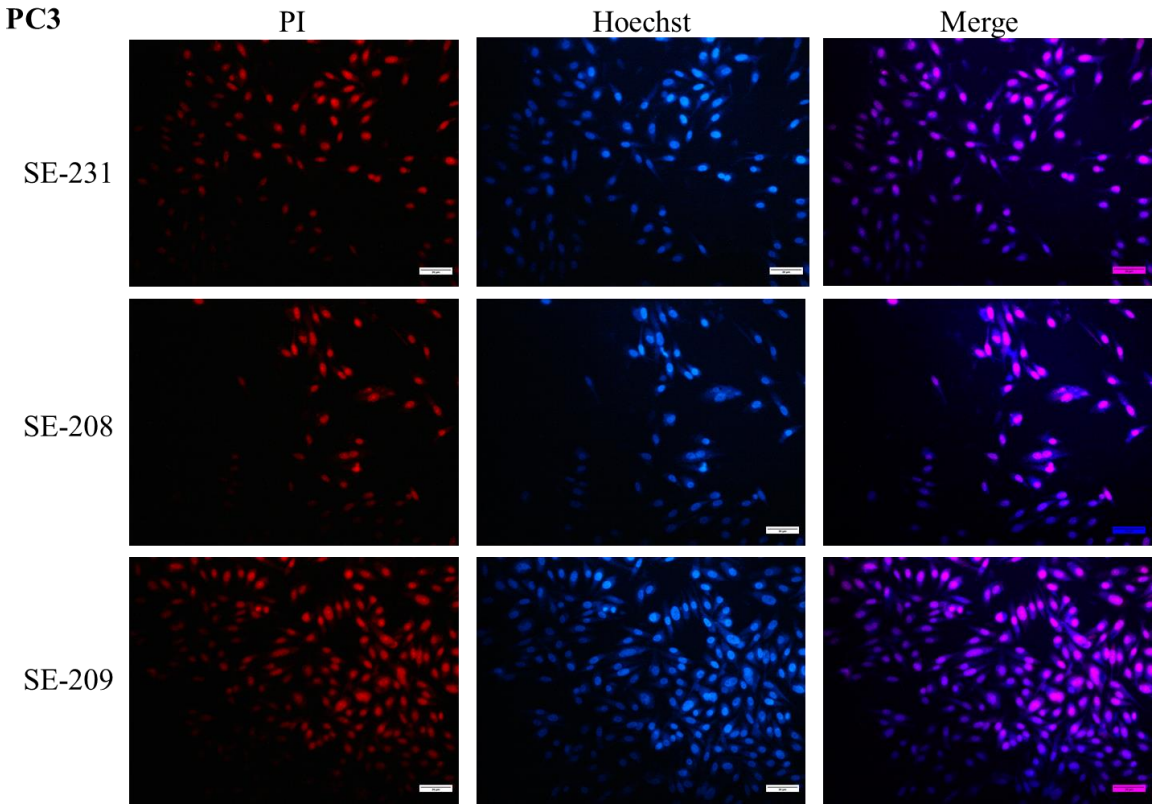

Fig. 7. The fluorescence microscopy images of the PC3 cell line in the presence of compounds SE-231, SE-208, and SE-209, scale bars=100µM.

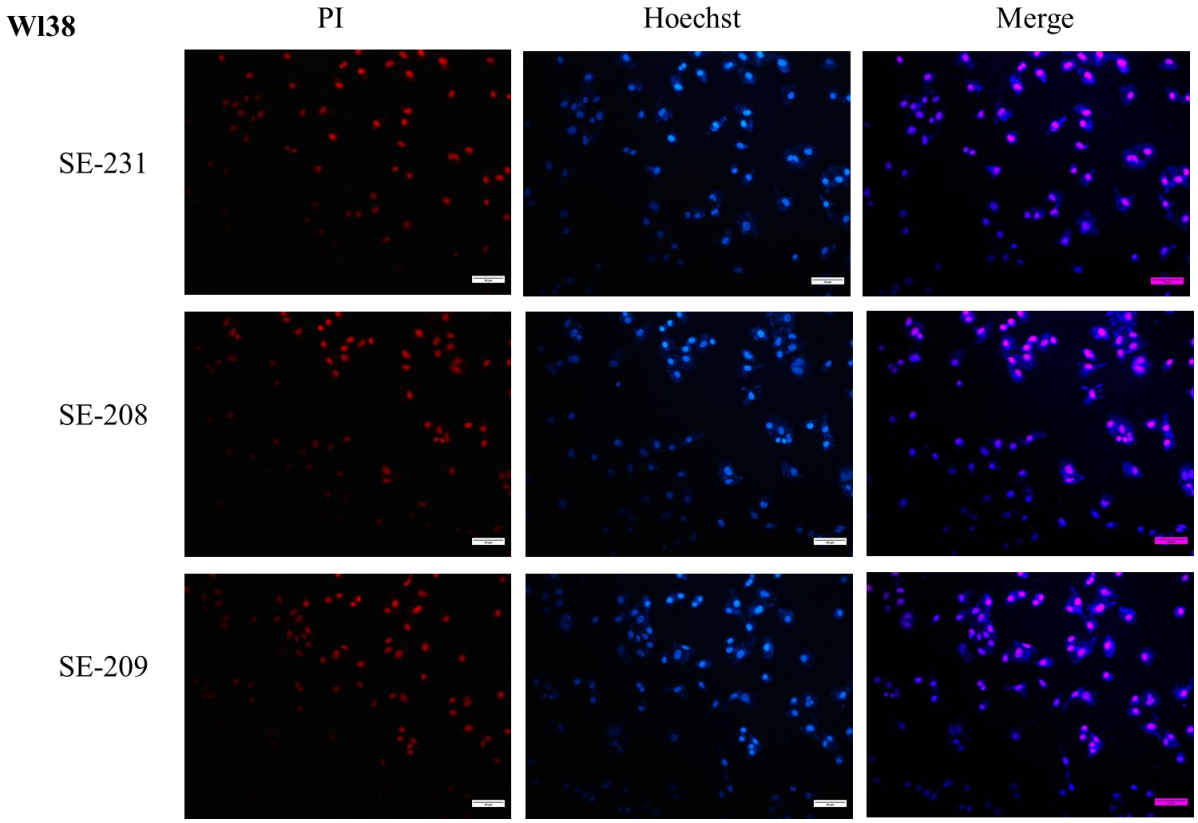

Fig. 8. The fluorescence microscopy images of the Wl38 cell line in the presence of compounds SE-231, SE-208, and SE-209, scale bars=100µM.

Discussion

Cancer is a threat to human life, with increasing mortality rates globally. The cancer drugs have toxic risks and resistance to cancer drugs is increasing. Because of this, the development of new drugs for disease treatment is urgent need. Benzimidazole containing drugs has cytotoxic effects on cancer cells (Bansal & Silakari 2012, Tonelli *et al*. 2014, Akkoç *et al.* 2016b, Gök *et al.* 2019). The benzimidazole ring's privileged skeletal structure has led to the development of many essential drugs used in various therapeutic areas, such as antihistamines, anthelmintics, proton pump inhibitors, and angiotensin receptor antagonists (Abdelgawad *et al.* 2019, Shingalapur *et al*. 2010). Due to their various biological activity properties, benzimidazole-based heterocyclic compounds hold a significant place in chemistry (Taha *et al*. 2018, Özil *et al.* 2018).

With these underscores we investigated the silver metal complexes that contained benzimidazole nuclei on lung (A549), colon (CaCo2), breast (MCF7), and prostate (PC3) cancer cell lines and Wl38 healthy epithelial cell line. Our results show the three silver metal complexes SE-231, SE-208 and SE-209 have antiproliferative effects on cell lines. In some studies in the literature, are in line with our study.

Liu *et al*. (2012) investigated the anticancer effect of benzimidazole derivatives synthesized on A549, HCT116, A375, HepG2, and PC-9 cancer cell lines using the MTT method and reported that the synthesized compounds had high anticancer activity. Thimmegowda *et al.* (2008), who examined the effects of benzimidazole-like compounds on the MDA-MB-231 breast cancer cell line, stated that these compounds had a vigorous antiproliferative activity. Mavrova *et al*. (2013) used benzimidazole-containing compounds and HT-29, MDA-MB231, HeLa, HepG2, and Lep-3 cancer lines in their study, and found that the compounds they synthesized were more effective in a different cell line, and all compounds had particularly effective antiproliferative effects against Lep-3.

In this study, three silver-containing compounds were produced. These compounds were compared regarding MTT, apoptosis, reactive oxygen species (ROS) activity, and cell membrane integrity in four cancers and one regular cell line. According to the apoptosis analysis, the most effective drug was SE209. CaCo2 and A549 cells were identified as the most affected cells by the drugs. According to the apoptosis data, again, the SE209 compound was more selective against cancer cells than cisplatin.

Regarding the oxidative stress analysis, cancer cells were more resistant to oxidative stress than normal cells. According to the fluorescent staining results, apoptosis markers were observed in the cells, and no disruption in cell membrane structures was observed. In conclusion, three benzimidazole containing silver metal complexes that may have anticancer properties were promising as anticancer drugs. *In vivo* animal studies may bring these compounds closer to clinical use.

Acknowledgement

We would like to thank GENKOK, Erciyes University Research Fund (Eru BAP) and the Proofreading & Editing Office of the Dean for Research of Erciyes University for copyediting and proofreading service for an earlier version of the manuscript.

Ethics Committee Approval: Since the article does not contain any studies with human or animal subject, its approval to the ethics committee was not required.

Data Sharing Statement: All data are available within the study.

Author Contributions: Concept: D.H., S.A., B.B.O., Design: D.H., S.A., Execution: D.H., S.A., B.B.O., Material supplying: D.H., S.A., Data acquisition: D.H., S.A., B.B.O., Data analysis/interpretation: D.H., S.A., B.B.O., Writing: D.B., Critical review: D.B

Conflict of Interest: The authors have no conflicts of interest to declare.

Funding: The study was supported by the Erciyes University Research Fund with project number TKB-2020-10346.

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