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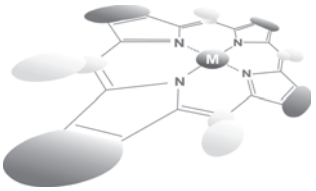

New water soluble magnesium phthalocyanine as a potential anticancer drug: Cytotoxic and apoptotic effect on different cancer cell lines

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ABSTRACT: Although phthalocyanines are usually used a photosensitizers for photodynamic therapy, these works focus on the directly cytotoxic effect of a new water-soluble magnesium phthalocyanine. The new water-soluble magnesium phthalocyanine **2** was synthesized, characterized and investigated for cytotoxic and apoptotic activities. The cytotoxic activities of the compound **2** were determined by using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay on human breast cancer cells (MDA-MB-231, MCF-7), human prostate cancer cells (PC-3), and human healthy lung fibroblast cells (WI-38). The cells were plated and treated 1 to 20 μM of different concentrations of the compound. MTT assay results indicated that the compound **2** has concentration and time-dependent cytotoxic activities against cancer cells. We also observed that the compound displayed lower toxicity against WI-38 healthy cells than cancer cells at 48 and 72 h. The compound showed a significant cytotoxic activity difference between breast cancer cells and WI-38 healthy cells at 48 and 72 h. Selectivity index of the compound **2** against MCF-7 for 72 h was calculated >15.62. We also studied the apoptotic and necrotic effect of compound **2** using TUNEL and PI staining, respectively. It was found that the synthesized compound **2** increased apoptotic and necrotic cells.

KEYWORDS: anticancer, phthalocyanine, magnesium, apoptosis.

INTRODUCTION

Many classes of drugs have been developed for cancer, a disease that poses a serious threat to human health. Today, the use of synthetic compounds in new therapeutic anticancer drugs has of growing interest. On the other hand, many developed drugs help the cancer cells to survive due to undesirable side effects such as lack of tumor specificity or multiple drug resistance for example [1]. Therefore, new drug development studies that are cancer type-specific and free from unwanted side effects are critical. Recently, studies on the relationship between cancer and apoptosis have been increasing. There is growing evidence in these studies that neoplastic transformation, progression, and metastasis processes alter normal apoptotic pathways, depending on the relationship between cancer and apoptosis [2, 3]. Providing many clues in anti-cancer treatment, apoptosis plays an important role in the development and maintenance of tissue homeostasis. It represents an effective mechanism by which harmful cells can be eliminated [4–6]. Phthalocyanine compounds are extensively researched in the pharmaceutical industry due to their potential to be used as photosensitizers in photodynamic therapy (PDT) [7–11], photodynamic antibacterial activities [12], and antioxidant activities [13]. A large part of photodynamic therapy applications is based on the opinion that it kills cancer cells. Photodynamic agents kill unwanted cells. Since the term "apoptosis" was introduced in 1972, cell killing mechanisms are generally classified as originating from apoptosis, necrosis or autophagy. [14, 15]. Apoptosis is a normal physiological process necessary for the control of tissue development and involution and tissue homeostasis.

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Apoptosis is a strictly regulated process of cell suicide, controlled by intracellular and extracellular signals, for the systematic disruption of the cell and the termination of residual cell components known as apoptotic bodies in a characteristic sequence. During apoptosis, leakage of intracellular material into the immediate environment is prevented, thereby preventing tissue inflammation [16, 17]. Loss of apoptosis regulation mechanisms in the treatment of diseases such as cancer may lead to the progression of the disease. In contrast, necrosis is caused by excessive cell damage [18, 19]. PDT participates in the treatment process with apoptosis, necrosis, or a combination of both mechanisms [20, 21]. However, most PDT agents are effective in inducing apoptosis. Thanks to this feature, it is useful in destroying unwanted cells or cells at a lower dose in the treatment of cancer and controlling the disease [22]. In studies conducted, it has been stated that various PDTs induce apoptosis in tumor cells, but its mechanism is not fully understood [23, 24].

On the other hand, it is also vital that the drugs being used in biological and biomedical applications are water-soluble. The water solubility of phthalocyanine compounds can be achieved by attaching groups to the appropriate substituent. Synthesizing the compound was made water-soluble by quaternization in this study. This feature will provide an advantage to the use of the synthesized compound in these areas. Magnesium, one of the essential metals for living organisms, also plays a very important role in biological, biomedical and anticancer applications [25]. Also, in the literature, magnesium phthalocyanine and its derivatives have proven to be promising photosensitizers in PDT due to their non-toxicity and optical properties [26–29]. In addition, it was thought that magnesium phthalocyanine might be biologically active due to its similarity with chlorophyll [30]. We therefore chose to study the magnesium phthalocyanine compound. Oxadiazole groups are a class of heterocyclic compounds that play an essential role in various pharmaceutical applications. Oxadiazole and its derivatives show quite good cytotoxic activity [31]. In particular, 1,3,4-oxadiazole derivatives are essential for their broad-spectrum biological activity [2, 32]. On the other hand, in our previous study, we determined that the oxadiazole-substituted double decker lutetium phthalocyanine compound can bind to DNA intercalatively. This result shows that this compound will accumulate more specifically in cancer cells where DNA replication is much higher than in healthy cells, and accordingly, it may have the potential to be used in cancer therapy [33]. There are also studies in the literature that oxadiazole derivatives have DNA binding properties [31, 34]. Similarly, in this study, it is thought that the water-soluble oxadiazole substituted magnesium phthalocyanine compound may have DNA binding properties and this situation is also suitable for the purpose of the study. It is expected that the new compound, which will be obtained by bringing together these two groups of compounds that are active in cancer studies, will show very high activity in this area. Also, the fact that the newly synthesized compound is water-soluble will be advantageous in use. In this study, we aimed to investigate the cytotoxic, apoptotic/necrotic effect on cancer cells of the newly synthesized magnesium phthalocyanine.

RESULTS AND DISCUSSION

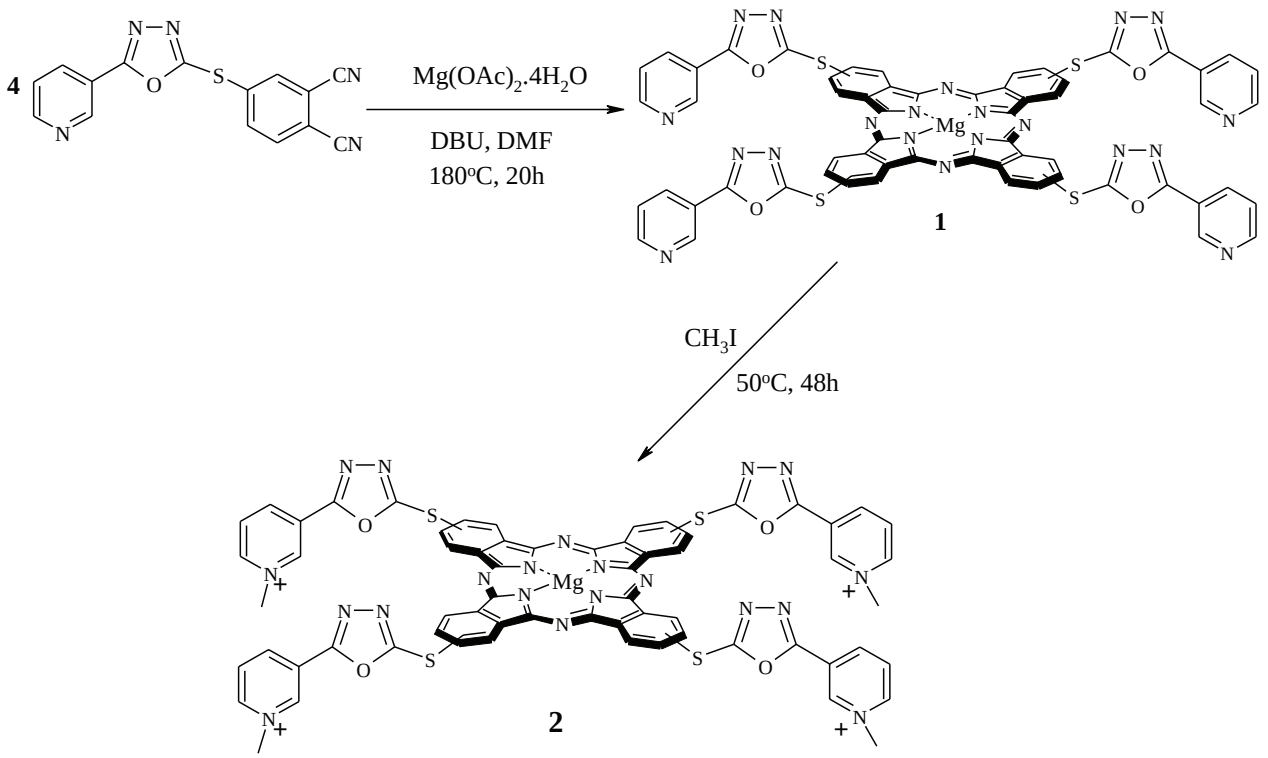
Synthesis

The new tetrasubstituted magnesium phthalocyanine compound **1** was synthesized by the tetramerization reaction of oxadiazole derivative substituted phthalonitrile [33] with magnesium acetate tetrahydrate in the basic medium. The new water-soluble tetrasubstituted magnesium phthalocyanine compound **2** was synthesized by the quaternization of compound **1** (Scheme 1). The water solubility of the water-insoluble compound **1** was increased by quaternization. As can be seen from the examples in the literature [35], quaternization has occurred over the pyridine nitrogen atom. The synthesized compounds were purified by solubility differences. At the end of the synthesis and purification processes carried out by determining the optimum conditions, compound **1** was obtained in 31% and compound **2** in 74% yield. The purity of compounds was characterized by ^{1}H-NMR, UV-vis, ATR-IR, MALDI-TOF MS and elemental analysis.

In the ATR-IR spectrum of compound **1**, it was observed that the –C≡N band observed at the characteristic 2241 cm^{-1} of the phthalonitrile compound disappeared as a result of the tetramerization reaction [36–38]. This confirms that the reaction is complete and the purity of the compound. ATR-IR spectra of synthesized new phthalocyanine compound **1** and **2** are shown the peaks at 1603–1589 cm^{-1} for the stretching vibration of C=C, at 1489–1443 cm^{-1} for the stretching vibration of C=N, at 1100–1081 cm^{-1} for the stretching vibration of N–N, at 764–683 cm^{-1} for the stretching vibration of C–S–C [37–40].

In the ^{1}H NMR spectrum of compounds **1** and **2**, the aromatic protons appeared between at 8.936–8.031 ppm and at 9.013–8.069 ppm, respectively. Also, in the ^{1}H NMR spectrum of compound **2** showed N-alkyl protons at 3.305 ppm. This data shows that the quaternization reaction has occurred [36–39]. The integral ratios of the ^{1}H-NMR peaks of the compounds **1** and **2** are compatible with the structures of the compounds. The elemental analysis results of compound **1** and **2** shows good agreement with calculation values.

In the UV-vis spectra of phthalocyanines, the characteristic Q-band for phthalocyanines is generally observed at 600–750 nm [7]. In the UV-vis spectra of compounds **1** and **2**, the characteristic Q-band was observed at 674 nm and 677 nm, respectively, and these bands confirm the formation of metal phthalocyanine.



Scheme 1. Synthesis of the water-soluble tetrasubstituted magnesium phthalocyanine compound.

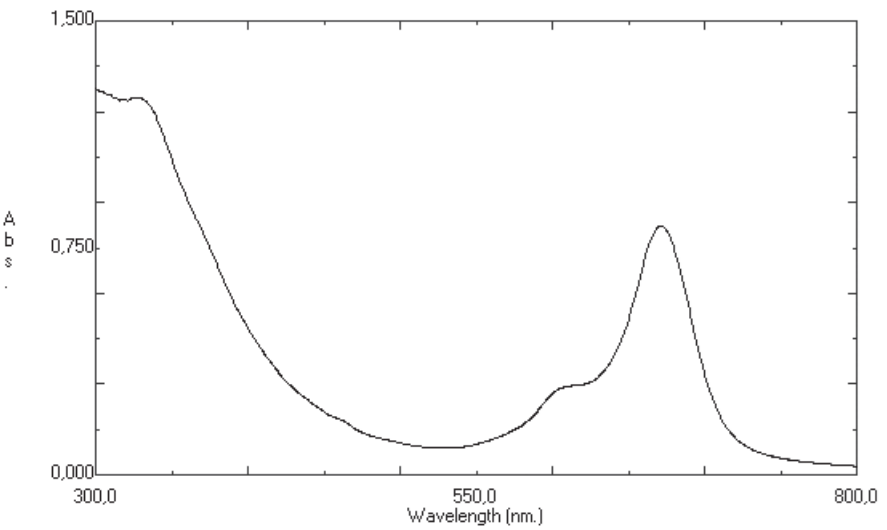


Fig. 1. UV-vis spectrum of compound **1** in DMSO (10^{-5} M).

The B-band for compounds **1** and **2** was appeared at around 328 nm and 329 nm, respectively (Fig. 1). All data confirm the structure of the compounds **1** and **2**.

Cytotoxic and apoptotic activities

The four cell lines used in this work were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). MDA-MB-231 (HTB-26, human breast adenocarcinoma cells), known as triple-negative cells that did not overexpress human epidermal growth factor receptor 2 (HER2), estrogen, and progesterone receptors. MCF-7 (HTB-22, human breast adenocarcinoma) are estrogen receptor (ER)-positive. PC-3 (CRL-1435, human prostate adenocarcinoma cells are androgen insensitive, and high metastatic potential also do not express p53 or p63 genes. WI-38 (CCL-75, human healthy lung fibroblast cells) were used as a model cell lines to the toxicity of compound against healthy cells. Cells were maintained at 37 °C in a humidified atmosphere and 5% CO_2. Cytotoxic

activity assay was done accordingly to the Skehan method [41]. The effects of the compound on the cell growth were evaluated by measuring the cell number variations after treatment for 24, 48, and 72 h. Figure 2 (a–d) shows the concentration- and time-dependent cytotoxic activity of the compound on MDA-MB-231, MCF-7, PC-3, and WI-38 cell lines, respectively. The IC_{50} (concentration of the test compound to achieve 50% of cell death) values for the compounds are listed in Table 1 for all cell lines. Compound exhibited time- and concentration-dependent inhibition on human breast and prostate cancer cells. The compound exerts differences in cytotoxic activity between all cell lines. IC_{50} values of compound >20 μM for 24 h, 10.36 to >20 μM for 48 h and 1.29 to >20 μM for 72 h. The compound displayed the following IC_{50} values against the MDA-MB-231 cell lines, >20, 10.36 ± 0.34, 5.90 ± 0.08; against the MCF-7 cells were >20, >20, and 1.28 ± 0.07 μM; and against PC-3 cells were >20, >20, and 20 μM for 24, 48 and 72 h respectively. Compound not have possessed IC_{50} values against WI-38 healthy lung cells in all-time points, at least at doses equal to 20 μM. Because the MDA-MB-231 and MCF-7 breast cancer cells have lower IC_{50} values according to the PC-3 cells, breast cancer cells more sensitive than the PC-3 prostate cancer cells.

Various phthalocyanines have been approved or under investigation for clinical practice and clinical trials [42]. Ghazal and co-workers synthesized a series of hexadeca-cationic zinc, magnesium, and metal-free phthalocyanines and tetrapyrazinoporphyrazines. They investigated their toxicity against HCT 116 colon cancer, MCF-7 breast cancer, and HeLa cervical cancer cells [43]. EC_{50} values of one of the magnesium-containing compounds were determined 1.18, 22.4, 3.16 μM for HeLa, MCF-7, HCT 116, respectively. Researchers were reported that some newly synthesized compounds have minimal EC_{50} values (12 nM), and new polycationic compounds showed approximately the same activity as the commercial PSs [43]. In another study, new manganese(III), cobalt(II), copper(II),

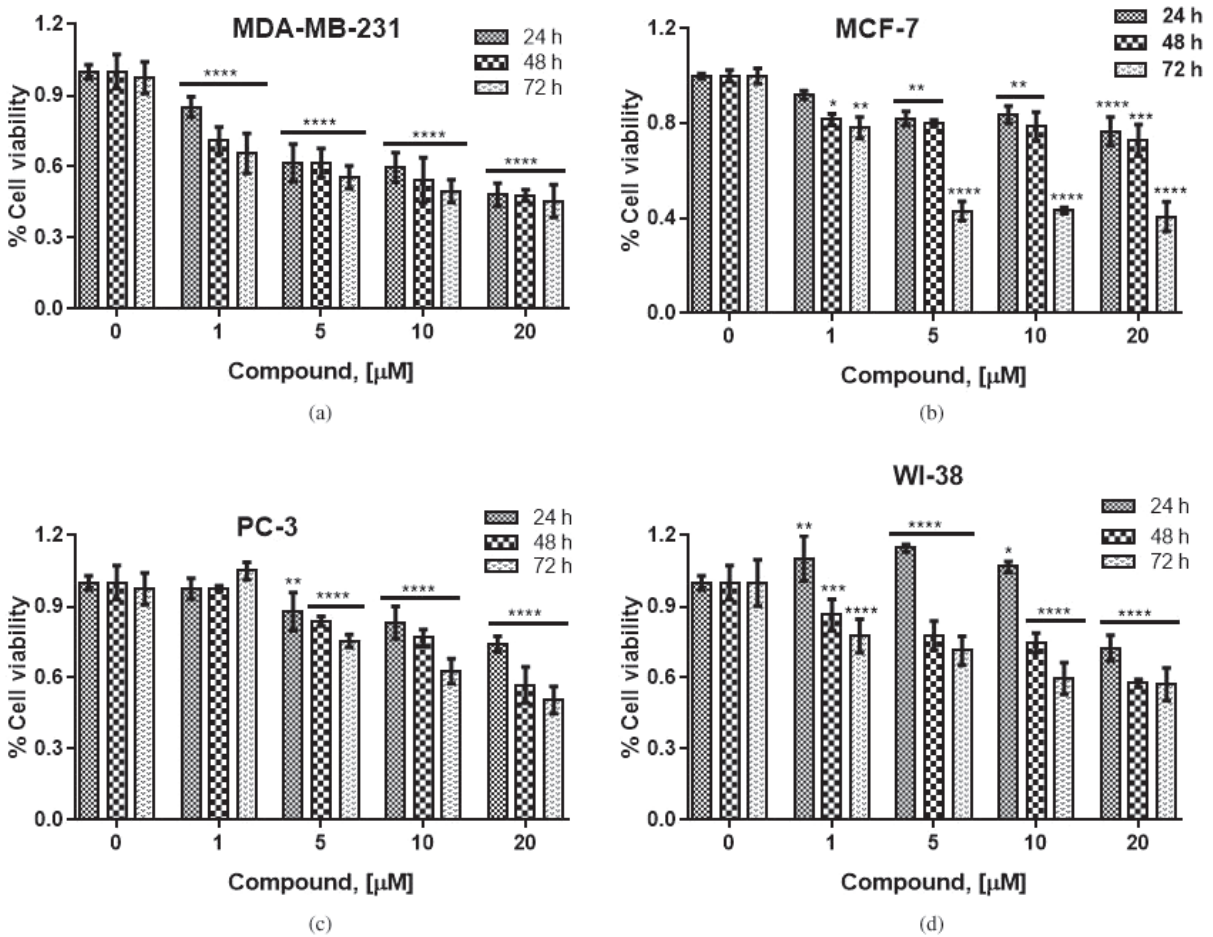


Fig. 2. The dose and time-dependence of cytotoxic activities of the compound against (a) MDA-MB-231, (b) MCF-7, (c) PC-3 cancer cells, and (d) WI-38 healthy cells. Control cells were treated with water. Data are representative of the mean of three separate experiments done in triplicate and are reported at the SEM. (*$p < 0.01$ *vs*. control, **$p < 0.005$ *vs*. control, ***$p < 0.0005$ *vs*. control, ****$p < 0.0001$ *vs*. control).

magnesium(II), zinc(II), and metal-free phthalocyanines, possessing 1,4,7-trioxanonyl substituents compounds were synthesized, and their biological activities were investigated [44]. Antiproliferative activities of the compounds have been investigated against human cervix carcinoma (HeLa), human melanoma SK-MEL-5, and human CD4+ T-lymphocyte (CEM) cells. IC_{50} values of the compound containing Mg^{2+} have been found that 7.7 μM and 14.6 μM for CEM, and HeLa, respectively. IC_{50} values of the compounds containing Co^{2+}, Cu^{2+}, and Zn^{2+} have been found that 7.1, 4.8, 5.4, and 13, 19, 6.8 for CEM and HeLa, respectively [44]. Dlugaszewska and co-workers synthesized a novel magnesium(II) phthalocyanine derivative with morpholiniumethoxy substituents and characterized. Antimicrobial and anti-cancer activities of the newly synthesized compound were also investigated. The anti-cancer activity of the compound was tested against human A549, HSC3, and H413 cancer cells, but no significant anti-cancer activity was observed [45]. It has been found that the compound displayed higher activity than cisplatin against MCF-7 (6.85 fold) and MDA-MB-231 (1.30 fold) for 72 h of administration time in breast cancer cells (Table 1).

The compound **2** was showed the critical difference in cytotoxicity between breast cancer cells and healthy cells in long exposure time. Because compound **2** has larger IC_{50} values for healthy cell lines, the compound was more cytotoxic toward breast cancer cells. In order to select the most sensitive cancer cell line, we further calculated the selectivity index (SI) of the compound. Selectivity index calculated [IC_{50} for healthy cell]/[IC_{50} for cancer cell]. Calculated selectivity index values for the compound were >2 and >3.38 against MDA-MB-231 cells for 48 and 72 h respectively. Selectivity index of the compound against MCF-7 for 72 h was found >15.62. Studies have shown that compounds with SI values of 5 and above represent more toxicity towards cancer cells compared to the healthy cells [52, 53]. On the other hand, similar to the oxadiazole-substituted double-decker lutetium phthalocyanine compound, which we determined to bind as a DNA intercalator in our previous study [33], led us to think that oxadiazole-substituted magnesium phthalocyanine compound in this study may also have DNA binding properties due to the presence of substituted oxadiazole groups. Also, apoptosis and necrosis are two crucial mechanisms in the elimination of cancer cells in newly produced compounds for possible cancer treatment. In apoptosis, the absence of inflammation and damage to healthy cells, and elimination of only cancer cells is one of the critical events in cancer treatment. In this study, the apoptotic and necrotic effect on different cancer cell lines of compound **2** was investigated. Different methods are used to determine and visualize apoptosis. The preferred method in this study is the TUNEL method. Terminal deoxynucleotidyl transferase (TdT) mediated dNTP nick-end labeling (TUNEL) is often used to detect in situ cell death. DNA strand breaks in apoptotic cells can be identified by labeling the free 3p-OH terminus with altered nucleotides (fluorescein-labeled dNTP) in a TdT enzymatic reaction [54].

Identification of necrosis in cell culture is defined by allowing the passage of dyes, such as propidium iodide, through the impaired plasma membrane of cells [55]. Various morphological definitions are made in the description of necrosis, such as cell plasma membrane changes, potential mitochondrial changes [56]. In this study, we marked apoptotic cells with the TUNEL method and necrotic cells with PI staining. Thus, we aimed to show apoptotic/necrotic cells at the same time. *Via* this double staining, stylishly microscopically, time-dependent apoptotic/necrotic cell changes of the newly synthesized drug active substance were demonstrated in different cancer cell lines. Few studies have highlighted the specific contribution of TUNEL/PI double staining to the definition of apoptosis/necrosis cells in cancer cells [57]. In our study, it was determined that the newly synthesized drug molecule gradually increased apoptosis in cancer cell lines compared to control at 24, 48, and 72 h (Figs 3–6). In parallel with our findings, it was stated in

Table 1. IC_{50} (μM) values of compounds against the selected human cancer cells and healthy cells[a] *in vitro* after 24, 48, and 72 h of incubation.

Cell lines	Compound 2 (IC_{50}, μM)[b]			Cisplatin (IC_{50}, μM)		
	24 h	48 h	72 h	24 h	48 h	72 h
MDA-MB-231	>20 ± 0.24	10.4 ± 0.34	5.90 ± 0.46	39.4 ± 1.30[46]	36.9 ± 0.90[46]	7.7 ± 3.21[47]
MCF-7	>20 ± 0.34	>20 ± 0.11	1.28 ± 0.07	28.2 ± 1.10[46]	24.9 ± 1.21[46]	8.78 ± 1.32[48]
PC-3	>20 ± 0.11	>20 ± 0.04	20 ± 0.04	104.2 ± 8.10[49]	34.4 ± 0.75[50]	12.8 ± 0.90[51]
WI-38[a]	>20 ± 0.20	>20 ± 0.23	>20 ± 0.14			

[a]Healthy cells. [b]Cell viability after treatment for 24, 48, and 72 h was determined by MTT assay as described in the Experimental section. Each IC_{50} value represents the mean ± SEM of three independent experiments ($n = 9$).

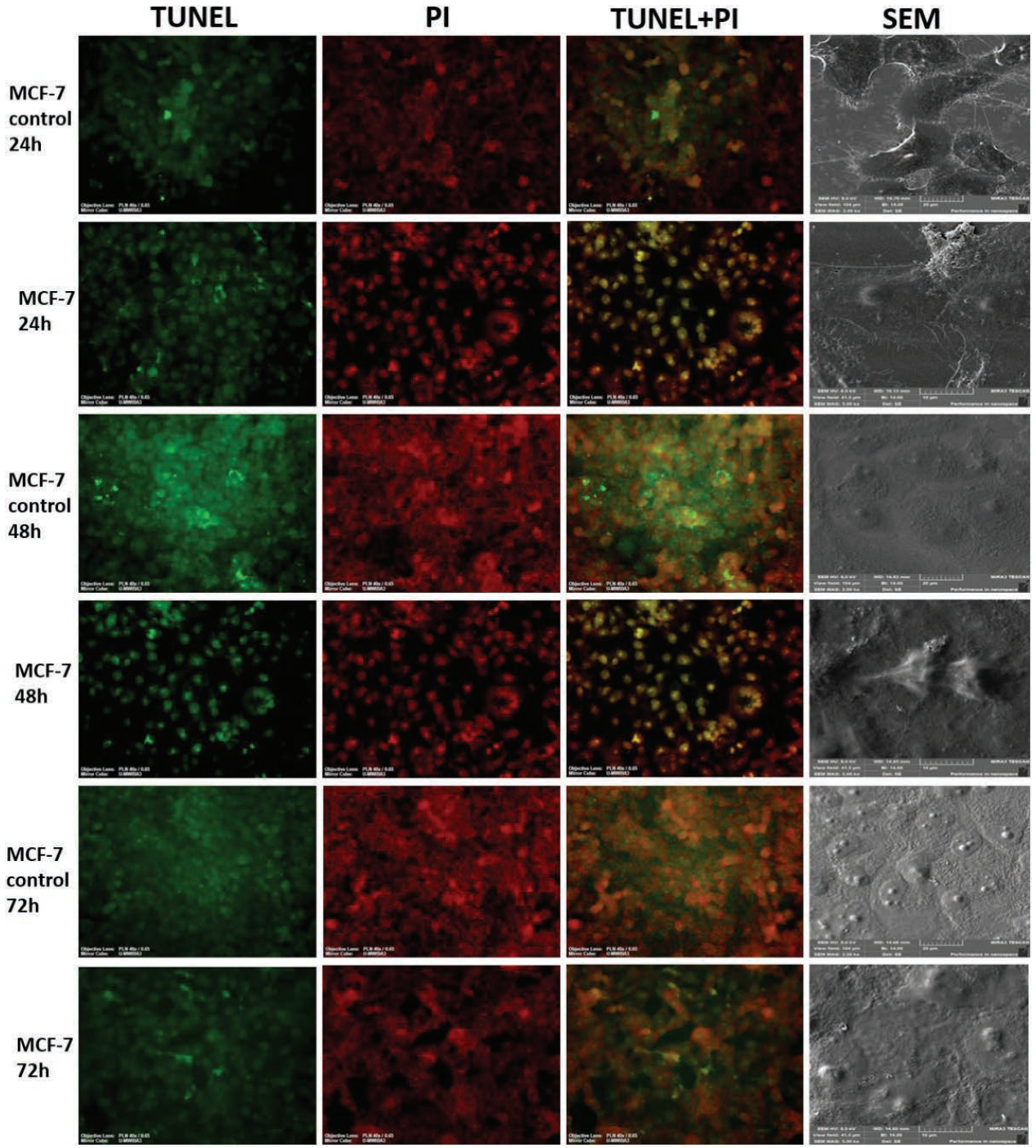


Fig. 3. Apoptotic and necrotic effects of compound **2** against MCF-7 breast cancer cells for 24, 48, and 72 h. Apoptotic cells were labeled with a TUNEL stain (green); necrotic cells were PI stained (red). They photographed under a light microscope (magnification 40X). SEM analysis was performed and photographed (Magnification 2000 KX) to observe the change in cell morphology in the experimental and control groups.

a study that zinc(II) phthalocyanine substance increased apoptosis in cancer cells, and the reason was caused by damage to organelles such as endoplasmic reticulum, Golgi, lysosome, and mitochondria [58]. PDT agents are harmless molecules. However, it is emphasized that with molecular oxygen, singlet oxygen, and other types of reactive oxygen damage cell organelles with oxidative cell damage and cell death [15]. In this study, TUNEL/PI staining results applied to determine apoptosis and necrosis was scored semi-quantitatively in Table 2, and the results are given in Figs 7–8. Accordingly, apoptotic and necrotic cells were found to be significantly higher in MDA-MB-231, MCF-7, and PC-3 cancer cell lines compared to their controls at 24, 48, and 72 h. As

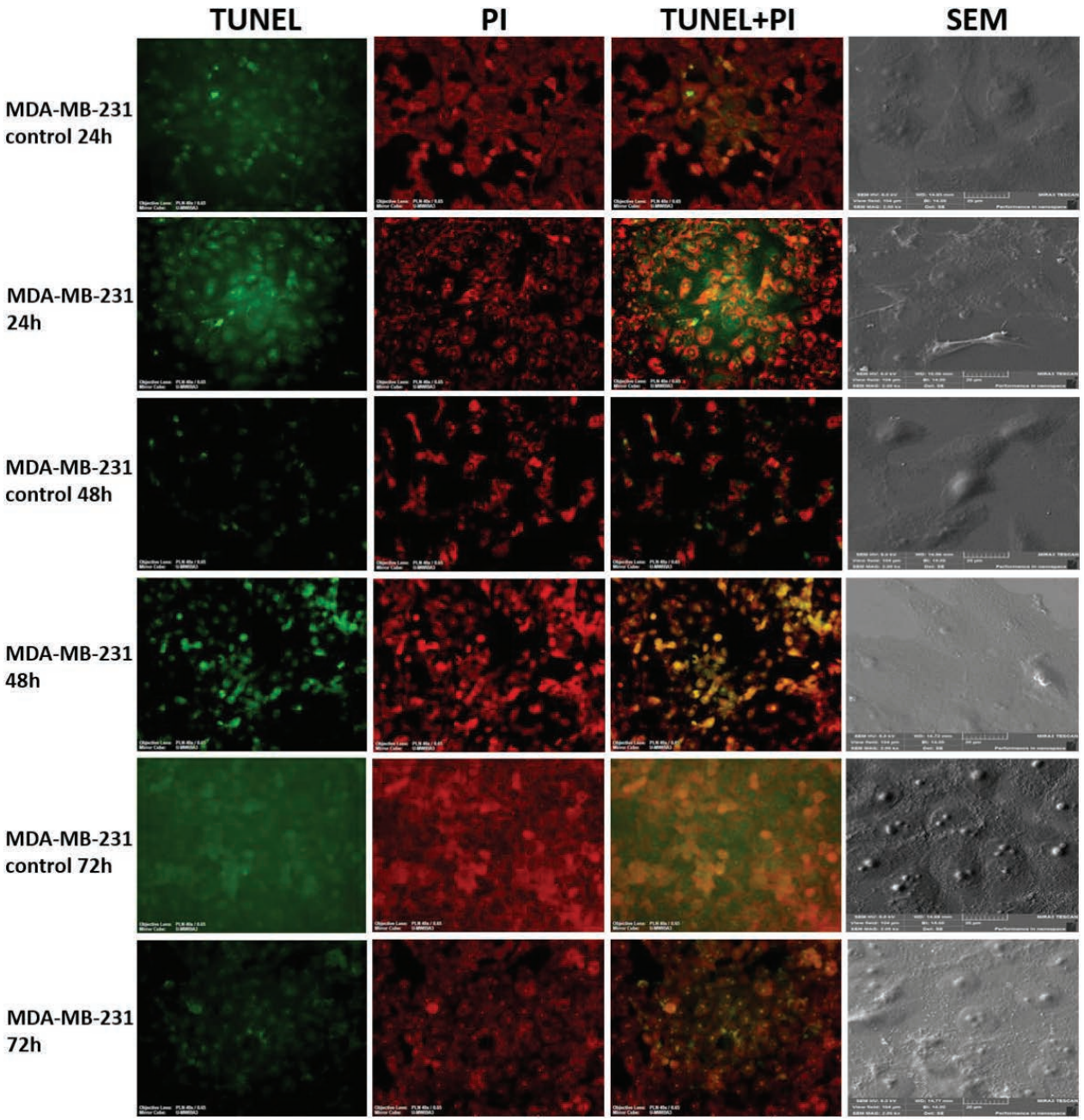


Fig. 4. Apoptotic and necrotic effects of compound **2** against MDA-MB-231 breast cancer cells for 24, 48, and 72 h. Apoptotic cells were marked by TUNEL staining (green), necrotic cells were stained by PI staining (red) and photographed under a light microscope (magnification 40X). SEM analysis was performed and photographed (Magnification 2000 KX) to observe the change in cell morphology in the experimental and control groups.

emphasized in the literature [59], the agent used in this study can be thought to cause mitochondrial disruption in MCF-7 cells, increase chromatin condensation in the nucleus, and accelerate proapoptotic mechanisms. In our study, cell morphologies changed with SEM analysis compared to the control groups depending on the time. This change was observed in the form of shrinkage in cells and disruption in the plasma membrane. The PDT agent used may be significantly altering cell morphology as well as inducing apoptosis due to reactive oxygen derivatives accumulating in the cell. Control of cell death mechanisms is essential for tumor development and limitation. Some PDTs use necrotic pathways to shrink the tumor. However, since necrosis triggers inflammation, it is not the primary mechanism of choice for controlling the tumor. For this reason, PDT agents that induce apoptotic mechanisms are preferred in clinical applications [60, 61]. In a study on aggressive tumors such as melanoma, it was emphasized that PC13, a PDT agent, contributes to the treatment process by killing

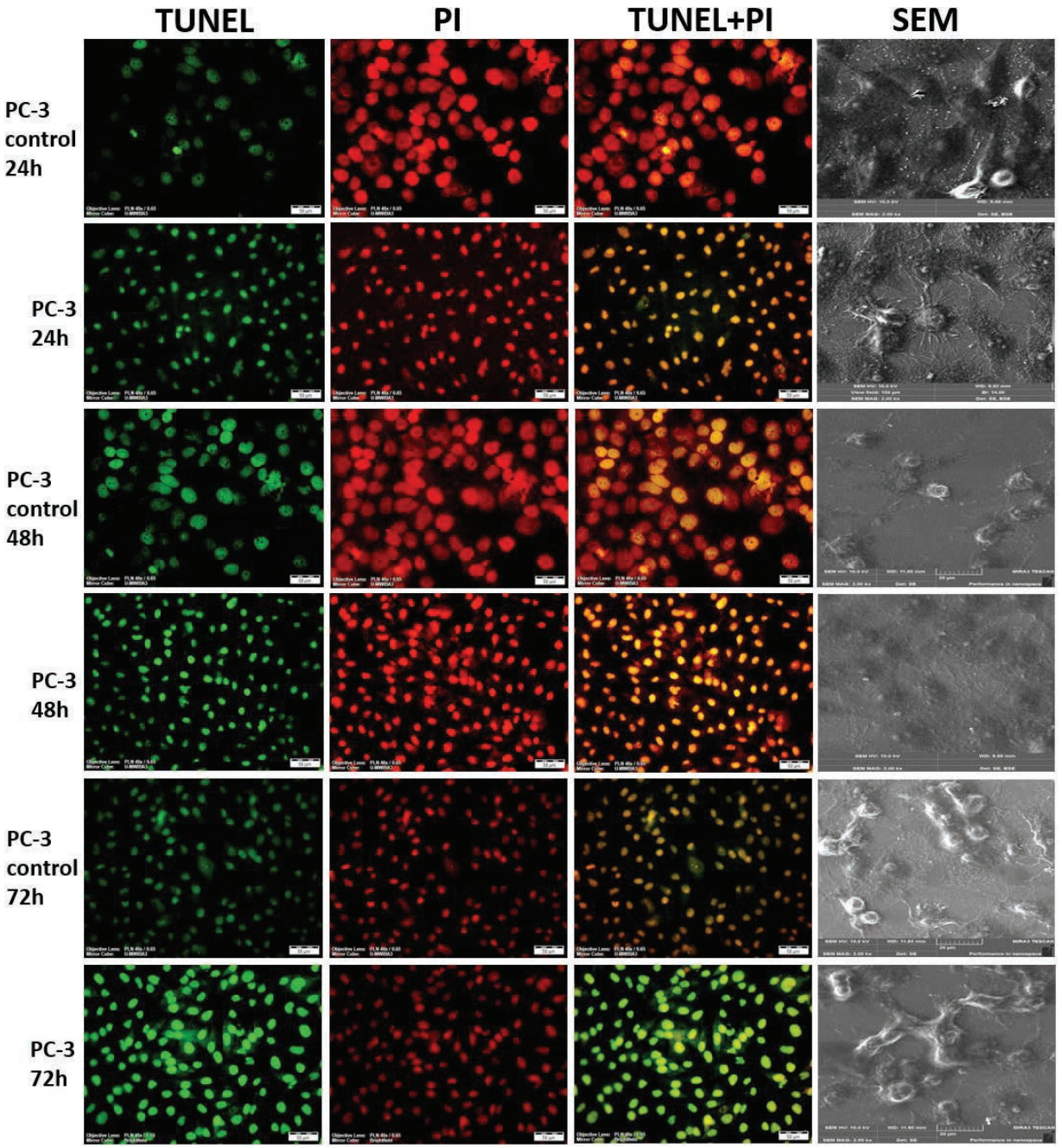


Fig. 5. Apoptotic and necrotic effects of compound **2** against PC-3 prostate cancer cells for 24, 48, and 72 h. Apoptotic cells were marked with TUNEL staining (green), necrotic cells with PI staining (red), and photographed under a light microscope (magnification 40X). SEM analysis was performed and photographed (Magnification 2000 KX) to observe the change in cell morphology in the experimental and control groups.

cancer cells by primarily apoptosis and then necrosis, in the treatment of melanoma cells [15].

EXPERIMENTAL

Chemistry

All solvents used in synthesis reactions carried out under nitrogen atmosphere were dried by molecular sieves or suitable methods [62]. The oxadiazole derivative substituted phthalonitrile derivative as starting material and 4-nitrophthalonitrile were synthesized according to the literature [33, 63]. UV-vis spectra were recorded in the Shimadzu UV-1800 UV-vis spectrophotometer, respectively. ^{1}H-NMR spectra were taken in DMSO-d_6 using JEOL Resonance ECZ400S 400 MHz spectrometer. SEM images were examined with a TESCAN® MIRA3 XMU (Brno, Czechia) brand scanning electron microscope.

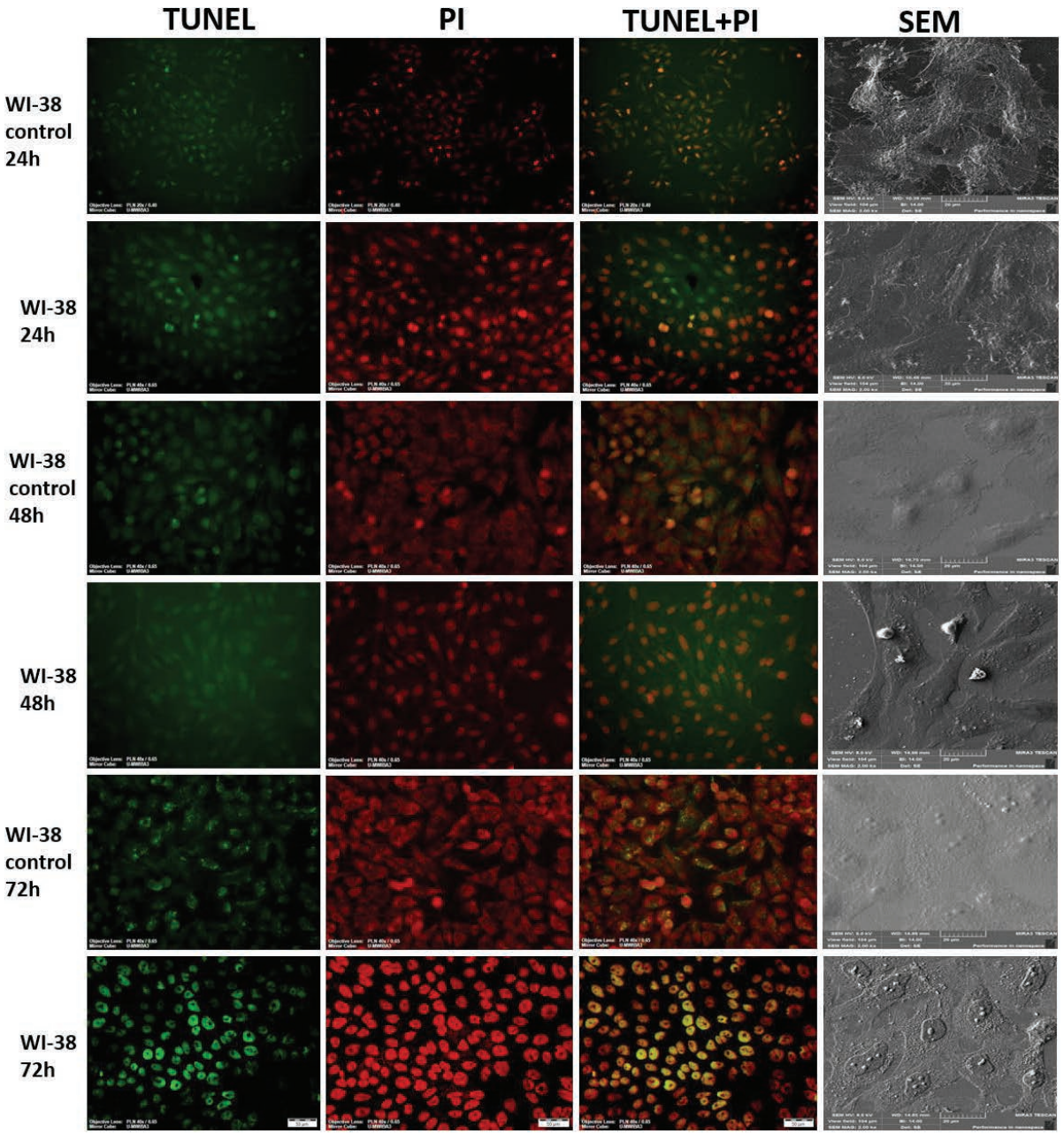


Fig. 6. Apoptotic and necrotic effects of compound **2** against WI-38 healthy cells for 24, 48, and 72 h. Apoptotic cells were marked by TUNEL staining (green), necrotic cells were stained by PI staining (red) and photographed under a light microscope (magnification 40X). SEM analysis was performed and photographed (Magnification 2000 KX) to observe the change in cell morphology in the experimental and control groups.

Electrothermal 9100 digital melting point apparatus was used to determine the melting point. Olympus BX51 Japan fluorescence microscopy was used for cell photos.

Synthesis

Synthesis of oxadiazole derivative tetrasubstituted magnesium phthalocyanine (1)

5-(3-pyridyl)-1,3,4-oxadiazole derivative substituted phthalonitrile compound (100.0 mg, 0.34 mmol) and magnesium acetate tetrahydrate (36.5 mg, 0.17 mmol) was mixed at room temperature under nitrogen gas in DMF (3 mL). DBU was added to this mixture and heated at 180 °C for 20 h. After the reaction was completed, the mixture was cooled, the organic phase was precipitated with MeOH and filtered. The crude product was washed with water (2 × 5 mL), MeOH (4 × 5 mL), and dried. Then the green solid was washed with acetone (10 mL) using the soxhlet apparatus and dried in vacuum. The purified green compound was soluble in THF, DMF and

Table 2. Apoptotic and necrotic effects of compound **2** against MCF-7, MDA-MB-231, and PC-3 cancer cells and WI-38 cells, for 24, 48, and 72 h. Apoptotic cells were stained with TUNEL, necrotic cells were PI, and 5 different regions were selected from the stained area and evaluated semi-quantitatively (1: slightly, 2: minimal, 3:mild, 4: moderate, 5: marked).

Cell line	TUNEL/PI Assay	Time		
		24 h	48 h	72 h
MCF-7 Control	Apoptotic cells	2,1,1,1,2	2,2,1,1,2	2,2,2,2,1
	Necrotic cells	2,1,1,1,1	2,2,1,1,1	2,2,2,1,1
MCF-7 **Experiment**	Apoptotic cells	3,4,4,4,4	5,5,5,4,4	5,4,4,3,5,
	Necrotic cells	3,3,2,4,3	3,3,4,5,5	4,5,3,4,3
MDA-MB-231 Control	Apoptotic cells	1,2,1,2,2	1,2,2,2,2	2,1,3,3,2
	Necrotic cells	1,1,2,2,1	2,2,3,2,1	2,2,2,3,2
MDA-MB-231 **Experiment**	Apoptotic cells	3,5,4,4,4	4,5,5,5,5	3,4,5,5,5
	Necrotic cells	4,4,3,3,3	4,4,4,4,4	4,5,5,3,4
PC-3 Control	Apoptotic cells	3,2,1,1,1	1,2,1,2,3	3,3,2,1,1
	Necrotic cells	2,1,2,1,2	3,1,2,2,2	1,3,3,2,2
PC-3 **Experiment**	Apoptotic cells	4,3,4,4,4	5,4,4,4,4	5,5,5,4,5
	Necrotic cells	3,3,3,4,4	3,4,4,4,4	4,4,4,5,3
WI-38 Control	Apoptotic cells	2,1,1,1,1	2,2,1,1,1	2,1,2,1,3
	Necrotic cells	1,1,1,1,1	2,1,1,1,1	2,1,2,1,1
WI-38 **Experiment**	Apoptotic cells	2,2,2,3,2	3,3,3,2,2	4,4,3,4,5
	Necrotic cells	2,2,1,2,2	2,2,2,3,3	3,3,4,3,5

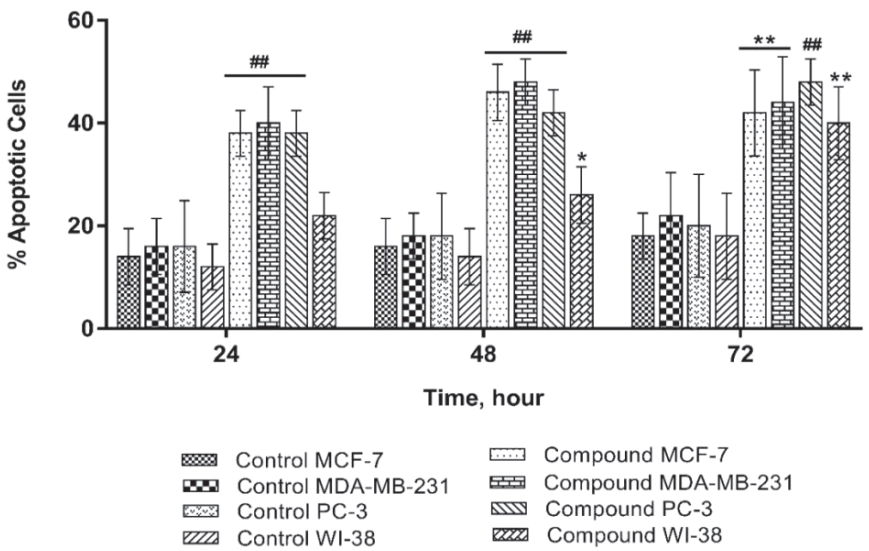


Fig. 7. Apoptotic effects of the compound **2** against MCF-7, MDA-MB-231, and PC-3 cancer cells and WI-38 cells, for 24, 48, and 72 h. Data are representative of the mean of three separate experiments ($n = 3$) and are reported at the SEM. (*$p < 0.05$ *vs*. control, **$p < 0.005$ *vs*. control, $^{\#}p < 0{,}0005$ *vs*. control, $^{\#\#}p < 0{,}0001$ *vs*. control.

DMSO. Yield 31% (33.0 mg). Mp: >300 °C. ^{1}H-NMR (400 MHz, DMSO-d_6) δ = 8.936–8.711 (br, 12H, Pc Ar-H); 8.627–8.031 (br, 16H, pyridyl Ar-H). UV-vis (DMSO) λ_{max}/nm 674, 608, 328. IR (ATR) υ (cm^{-1}) 3049, 1603, 1589, 1489, 1443, 1100, 1081, 764, 751. Anal. Calc. for $C_{60}H_{28}N_{20}O_4S_4Mg$: C 57.87; H 2.27; N 22.50; S 10.28%, found: C 57.18; H 2.14; N 22.82; S 10.54%. MALDI-TOF MS m/z: 1247 $[M + H]^+$.

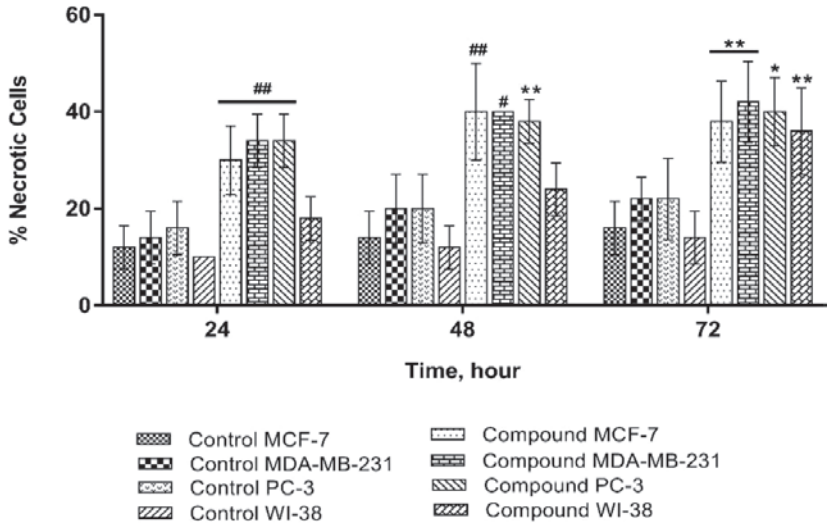


Fig. 8. Necrotic effects of the compound **2** against MCF-7, MDA-MB-231, and PC-3 cancer cells and WI-38 cells, for 24, 48, and 72 h. Data are representative of the mean of three separate experiments ($n = 3$) and are reported at the SEM. (*$p < 0.05$ *vs*. control, **$p < 0.005$ *vs*. control, $^{\#}p < 0{,}0005$ *vs*. control, $^{\#\#}p < 0{,}0001$ *vs*. control.

Synthesis of quaternized oxadiazole derivative tetrasubstituted magnesium phthalocyanine (2)

Compound **1** (100 mg, 0.08 mmol) was dissolved in THF (1 mL) in a nitrogen atmosphere and methyl iodide (1 mL) was added to the dark green solution. Then this mixture was stirred at 50 °C for 48 h. After the reaction was completed, this mixture was cooled, precipitated with diethylether, filtered, washed with diethylether (3 × 3 mL) and dried. The purified compound was soluble in water. Yield 74% (78.0 mg). Mp: >300 °C. ^{1}H-NMR (400 MHz, DMSO-d_6) δ = 9.013–8.792 (br, 12H, Pc Ar-H); 8.707–8.069 (br, 16H, pyridyl Ar-H); 3.305 (s, 12H, pyridyl N-CH_3). UV-vis (DMSO) λ_{max}/nm 677, 611, 329. IR (ATR) υ (cm^{-1}) 3054, 1605, 1594, 1488, 1456, 1142, 749, 683. Anal. Calc. for $C_{64}H_{40}N_{20}O_4S_4MgI_4$: C 42.40; H 2.22; N 15.45; S 7.06%, found: C 42.15; H 2.24; N 15.27; S 7.24%.

Cell culture

The cells lines MDA-MB-231 (HTB-26, human breast adenocarcinoma), MCF-7 (HTB-22, human breast adenocarcinoma), PC-3 (CRL-1435, human prostate adenocarcinoma), WI-38 (CCL-75, human healthy lung fibroblast cells), F-12K Medium (30-2004), Eagles Minimum Essential Medium (EMEM, 30-2003), fetal bovine serum (FBS, 30-2020) and penicillin and streptomycin (30-2300) were purchased from American Type Culture Collection (ATTC, Manassas, VA). Dulbecco's Modified Eagle's Medium (DMEM, D6429) and Trypsin-EDTA solution (T-3924) were purchased from Sigma Aldrich. (Sigma-Aldrich Chemie GmbH , Steinheim, Deutschland).

MTT assay

MDA-MB-231 and MCF-7 breast cancer cell lines were grown in DMEM medium, PC-3 prostate cancer cells and WI-38 human healthy lung fibroblast cells grown in F-12 and EMEM medium, respectively. All medium supplemented with 10% FBS and 1% penicillin/streptomycin solution. MDA-MB-231, MCF-7, PC-3, and WI-38 cells (1×10^5 cells/mL) were plated on 96-well plates and allowed to adhere under 5% CO_2 at 37 °C humidified air condition for 24 h. Compounds dissolved in water. Cells were treated with 1 μM, 5 μM, 10 μM and 20 μM of the compound for 24, 48, and 72 h. Control wells were supplemented with water (0.5% v/v). Cell viability was assessed by using the MTT. Briefly, at the end of the time period, 10 μL of 5 mg/mL MTT solution (w/v, prepared in PBS) was added to each well and kept at incubator at 37 °C for further 2 h. After removal of the medium, 100 μL of DMSO was added to each well to dissolve the formazan crystals formed by the reaction of dehydrogenase in a living cell with MTT dye. Absorbances were measured at 570 nm (Biotek, Epoch, USA). All concentration was worked three independent experiments and triplicate ($n = 9$). IC_{50} values were calculated for GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA).

Terminal deoxynucleotide transferase mediated dUTP nick end labeling (TUNEL) assay

A terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) to visualize apoptotic cells was performed according to the manufacturer's instructions (Roche Diagnostics, Germany). The cells were plated in sterile coverslips placed in 6-well culture dishes, each with 10 000 cells. After 24 h, the cells were administered the appropriate amount of IC50. Cells were washed with PBS at 24, 48, and 72 h after drug administration and fixed using 4% paraformaldehyde (Merc, Germany). The fixed cells were washed with PBS and kept on ice with 0.1% Triton X-100 (Sigma, USA)

for 5 min. Afterward, 50 µl of terminal deoxynucleotide transferase was incubated with tip labeling solution at 60 °C, and the DNA fragmented due to apoptosis was marked with a fluorescent dye. To separate necrotic cells from apoptotic cells, they were observed using a fluorescence microscope (Olympus BX51), staining with 1 mg/mL propidium iodide (PI, Sigma, USA) for 5 min at room temperature in the dark [64]. Apoptotic cells were examined under fluorescence microscopy with a green fluorescent dye, and necrotic cells were photographed at 40x magnification. Unlike necrosis, apoptotic cells were calculated by counting manually from 3 different areas.

SEM analysis

The cells were plated in sterile coverslips placed in 6-well culture dishes, each with 10 000 cells. After 24 h, the cells were administered the appropriate amount of IC50. Cells were washed with PBS at 24, 48, and 72 h after drug administration and fixed using 4% paraformaldehyde (Merc, Germany). The fixed cells were washed with PBS and passed through increasing alcohol series, gold plated with a coating device, and examined in SEM.

CONCLUSIONS

In this study, the new water-soluble magnesium phthalocyanine was synthesized, characterized, and investigated to cytotoxic and apoptotic activities. The cytotoxic activity of the water-soluble magnesium phthalocyanine was determined by MTT assay against MDA-MB-231, and MCF-7 human breast cancer cells, PC-3 human prostate cancer cells. Human healthy lung fibroblast cells (WI-38) were used as healthy cells. Cytotoxic activity of the water-soluble compound **2** has varied depending on the exposure time and cell line type. The most important result was the compound **2** displayed lower toxicity against WI-38 healthy cells than cancer cells at 48 and 72 h. Selectivity index of the new compound against MCF-7 for 72 h was calculated >15.62. The newly synthesized drug molecule used in this study induced apoptosis and necrosis in cancer cell lines depending on time. In the statistical evaluation, it was found that the newly synthesized molecule leads cancer cells to the death process in an apoptotic way. Particularly the apoptosis rate of MCF-7 and MDA-MB-231 cell lines is higher compared to the control groups of the same cells at all time points. These results clearly showed that newly synthesized molecule has high potential to be used as anti-cancer agents, especially breast cancer. Our future studies will focus on the determination of the apoptotic mechanism of the compound against breast cancer cells.

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Supporting information

Figures S1–S3 are given in the supplementary material. This material is available free of charge *via* the Internet at Internet at https://www.worldscientific.com/doi/suppl/10.1142/S1088424621500863.

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