

RESEARCH ARTICLE

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Thiosemicarbazide Derivative of Captopril (8) imposes Cellular-Dependent Death Modalities on Breast Cancer Cell Lines

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Abstract

Objective: Breast cancer prevalence is continuing to rise worldwide. Despite the diversity of the current approaches and protocols to treat this heterogeneous disease, most of these face the challenges of side effects and resistance. Hence, novel and innovative approaches to the treatment of breast cancer are almost constantly needed. This study aimed to investigate the antiproliferative and death modalities induced by three thiosemicarbazide derivatives of captopril in two subtypes of breast cancer cell lines, the Estrogen- receptor positive MCF-7, and the Estrogen/progesterone-receptor-negative AMJ13. **Methods:** MTT assay was used to determine the cytotoxicity of the derivatives and their parent compound Captopril, Hematoxylin and Eosin (H&E) staining, Acridine Orange/ Ethidium Bromide (AO/ EtBr) staining, Caspase immunocytochemistry analysis and ROS generation by Human ROMO1 ELISA assays were conducted to explore the type of cellular death induced by these derivatives. **Results:** One of the derivatives denoted as (8) demonstrated the best antiproliferative profile recording the highest cytotoxic effect with IC₅₀ of (88.06 μ M) and (66.82 μ M) compared to that of captopril (849.8 μ M),(1075 μ M) in MCF-7 and AMJ13 breast cancer cells respectively. In MCF-7 cells, derivative (8) imposed an apoptotic cellular death with the involvement of *caspase-3*, and *caspase-9* and displayed a time-dependent ROS generation. In AMJ13 breast cancer cells, results revealed an extensive vacuole forming, non-apoptotic cellular death, without ROS generation, but with a significant implication of *caspase-9*. **Conclusion:** This study demonstrated the thiosemicarbazide derivative of captopril (8) as a promising antiproliferative agent against breast cancer cells displaying different cellular death modalities, signifying the versatility of the derivative and suggesting multitarget pathways. This study strongly recommends derivative (8) as a future leading molecule.

Keywords: Thiosemicarbazide Derivative of Captopril- Breast Cancer Cell Lines Antiproliferative- Death modalities

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Introduction

In 2020, female breast cancer took the lead over lung cancer in the number of newly diagnosed cases worldwide [1]. The disease is characterized as highly heterogeneous with diverse subtypes classified on different bases. Molecularly and depending on gene expression of estrogen ER, progesterone PR and epidermal growth factor 2 into: luminal A (ER and /or PR)+, HER2 -), luminal B ((ER and /or PR)+, HER2 \pm), HER2 overexpression (ER/PR)-HER2 +), and basal-like (triple negative) TNBC (ER/PR)- HER2-) [2, 3].

Over the years, many therapies have been developed for the treatment of all types of breast cancer, but almost all of them share the same challenges of side effects and resistance. In addition to that, the most aggressive with the worst prognosis TNBC still lacks standardized effective

treatment [4].

As such, the pursuit of novel approaches seems in constant demand, and one of these new approaches is the Renin-Angiotensin System (RAS), which is implicated in almost all cancer hallmarks [5], including breast [6, 7]. Moreover, recent attention has been driven towards Angiotensin Converting Enzyme inhibitors (ACEi) and Angiotensin II type 1 Receptor Blockers (ARBs) role in oncology as potential chemopreventative, adjuvant or even anticancer agents [5, 8, 9].

Captopril, the first member of the ACEi family with a characteristic sulfhydryl group [10] was among those that were investigated for its potential role in the treatment of breast cancer with positive results [11-13].

Recently, Al-Saad and colleagues (2019) synthesized novel derivatives of captopril that demonstrated higher antiplatelet [14] and ACE inhibition activity than their

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parent compound captopril [15]. The authors justified the improvement to the incorporation of the thiosemicarbazide moiety that replaced the carboxylic acid group of captopril with the perseverance of the characteristic thiol group [14].

Worth mentioning, thiosemicarbazide/thiosemicarbazone derivatives have biological activity against various types of cancer [16, 17]. These compounds are believed to induce their anticancer effects by inhibiting many enzymes and vital cell cycle targets, including ribonucleotide diphosphate reductase (RNR) [18, 19], tubulin-colchicine and epidermal growth factor receptor (EGFR) tyrosine kinase [17], and topoisomerase II [20].

Remarkable antitumor activity of these derivatives has been documented by many studies in both the positive and negative hormonal receptor breast cancer cell lines. In the positive hormonal receptor breast cancer cell line (MCF-7), Malki and his fellow researchers (2015) demonstrated the anticancer effect of one of their novel thiosemicarbazide derivatives (4c) through targeting JNK signaling [21]. Other studies [22-27] investigated the antitumor activity of these derivatives in the negative hormonal receptors of breast cancer cells through targeting various cell targets.

Having in mind the implication of RAS in breast cancer [28] and the anticancer activities of both captopril and thiosemicarbazide, this work sought to investigate three thiosemicarbazide derivatives of captopril designated as (5, 7, and 8) (Figure 1) in two types of breast cancer cell lines; the estrogen receptor-positive MCF-7 and the aggressive estrogen/progesterone receptor-negative AMJ13 [29]. The derivatives were also investigated on the transformed non-tumorigenic HBL-100 breast cell line.

Materials and Methods

Chemicals and cell lines

The derivatives were supplied by Dr. Hiba. Najeh. AL-Saad, Department of Pharmaceutical Chemistry, College of Pharmacy, Basrah University, Basrah, Iraq. Derivatives (5, 7 and 8) molecular weights are 357.79, 384.88 and 380.46 respectively, all derivatives are highly pure compounds with ¹H NMR analysis validation [14]. Captopril molecular weight (217.29) ($\geq 98\%$ HPLC) was provided from Sigma –Aldrich/ USA.

Cell Bank Unit of Experimental Therapy Department, ICCMGR, AL- Mustansiriyah University, Baghdad, Iraq supplied the cells (MCF-7, AMJ13 and HBL-100). AMJ13 and HBL-100 cells were maintained in RPMI-1640 medium (Gibco/USA), whereas MCF-7 was in MEM (U.S Biological, USA). All were supplied with (10%) fetal bovine serum (Bio west/ USA) and 1% of (Penicillin–Streptomycin) (Capricorn-Scientific, Germany) and Incubated at 37 °C and (5%) CO₂. Exponential growing cells were a prerequisite in every experiment.

Cytotoxicity

Cytotoxicity of the three-thiosemicarbazide derivatives and captopril was assessed by MTT assay. Simply, the procedure included seeding the 96 micro-plate with 1x 10⁴ cells and incubating at 37°C until adherence and confluence were achieved. Followed by 72 hours of

treatment with different concentration ranges (0– 2000 µM) for captopril and derivative (7), and (0-200 µM) for both derivatives (5) and (8) (the concentration ranges were selected based on a pilot study). Cells were then incubated for 4 hours with 50 µl of MTT solution (2 mg/ml), which was then removed and solubilized with 100 µl of DMSO for 20 minutes [30]. Optical densities were measured by an ELISA plate reader [31, 32]. Cytotoxicity was determined by the following equations [33].

$$\text{Cytotoxicity (\%)} = (\text{OD Control} - \text{OD sample}) / \text{OD Control} \times 100$$

Where OD Control = mean optical density of drug unexposed wells, OD sample mean optical density of drug-exposed wells, IC₅₀ was then calculated using GraphPad Prism®, version 9.

Examination of morphological changes by Hematoxylin and Eosin staining (H&E)

Morphological changes were documented by H&E staining. The procedure was performed according to [34] with slight modifications. IC₅₀ (88.08 µM) and (66.82 µM) of derivative (8) were used to treat MCF-7 and AMJ13 respectively. Fixation was done with 2 drops of formalin for two minutes, followed by the addition of Hematoxylin for another two minutes. The slides were then washed with tap water for two minutes. Afterwards, two drops of Eosin were added and left for two minutes. The slides were then washed with distilled water and dehydrated by a series of ethanol dilutions (70%, 90%, and 100%). Xylene and Canada balsam were used for clearing and mounting respectively. The examination was done with Scopetek/ USA Light microscope.

Assessment of apoptosis by the Acridine Orange/ Ethidium Bromide (AO/EtBr) staining

The assay was performed to assess the potential implication of apoptosis as a cellular death induced by derivative (8). Seeding of the cells were done in eight well chamber slides, followed by 72 hours of incubation with (88.06 µM) and (66.82) of derivative (8) in MCF-7 and AMJ13 respectively. Double washing with sterile phosphate buffer saline preceded staining with 100 µl of AO/EtBr [35]. A fluorescence microscope (Olympus/ Japan) was used for examination, and Fiji (Image J version 1.53) was used for analysis.

Immunocytochemistry

In charged slides, cells were seeded and incubated for 10 minutes with -20o methanol. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide, which was followed by thirty-minute incubation with normal goat serum. The slides were then incubated for one hour with primary antibodies dilution (anti-caspase-3, anti-caspase -9, and anti-PECAM-1 monoclonal IgG1) (Santa Cruz/ USA). Gentle washing with PBS was performed and repeated three times followed by incubation with a polymer helper that was once again followed by another PBS washing. 20- 30 minutes of incubation with secondary antibody dilution (polyperoxidase –anti-mouse/

rabbit IgG/ Elabscience /USA/ E-IR-R213) ensued afterwards, which was followed by a third cycle of PBS washing. Counter-staining with pre-prepared DAB solution was done, followed by mounting, sealing and examination [36]. A digital camera (Micros, Austria) was used to take the images, and Fiji (image J version 1.53c) was used for analysis.

Assessment of ROS generation

Generation of ROS was assessed by Human ROMO1 ELISA Kit (Elabscience®, USA), which is supplied with a micro ELISA plate pre-coated with human ROMO1 antibody. All procedures were performed according to the manufacturer's instructions. Binding with antibody occurs after incubation with the cell supernatant for 90 minutes at 2-8 Co. This was followed by one-hour incubation with Biotinylated Detection Ab working solution (Human ROMO1 specific) at 37 Co. Triplicate washing with a washing buffer was performed before and after incubation with (Avidin-Horseradish Peroxidase) HRP Conjugate working solution. In a dark room, the plate was supplied with substrate reagent and was incubated for 15 minutes. A stop solution was then added and the optical densities were measured by micro-plate reader at 450 nm.

Statistical analysis

Data was analysed using GraphPad Prism®, version 9. An unpaired t-test was used to compare between two groups with a significance of < 0.05, one-way ANOVA with Tukey's multiple comparison test was used to compare the means of more than two groups. All results in this work were presented as mean ± SD.

Results

Cytotoxicity

Thiosemicarbazide derivative (8) has the best antiproliferative profile compared to the other derivatives (5 and 7) and their parent compound captopril. Where

the derivative (8) has strongly inhibited the growth with an IC₅₀ of (88.06µM) and (66.82µM) in MCF-7 and AMJ13 breast cancer cells respectively with lesser effect (153.3µM) on the transformed non-tumorigenic HBL -100 breast cells (Figure 2A). Compared to that of captopril which displayed an IC₅₀ of (849.8 µM) and (1075µM) in MCF7 and AMJ13 respectively with no effect on non-tumorigenic breast cells (HBL-100) (Figure 2C). On the other hand, derivative (5) demonstrated improved cytotoxicity only on AMJ13 breast cancer cells with IC₅₀ of (96.28 µM) with less effect on both MCF-7 (256.6 µM) and HBL-100 (204 µM) (Figure 2B). While Derivative (7) showed no inhibitory effect on any of the three cell lines (data not shown).

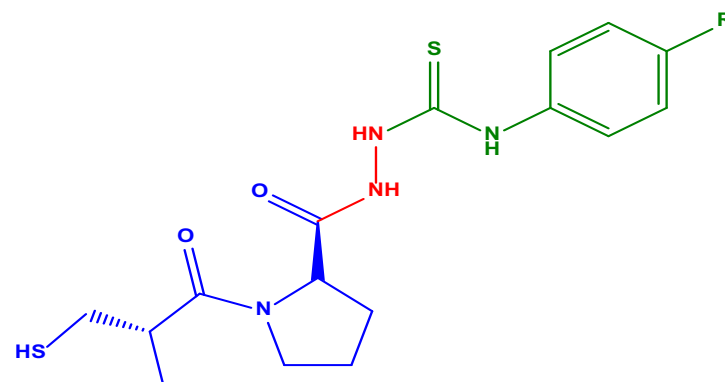
Identification of Cellular Death

Morphological changes by Hematoxylin and Eosin (H&E) staining

MCF-7 cells demonstrated mainly apoptotic features characterized by shrinkage of the cells and positional loss of organelles causing multiple opaque foci with slight vacuole degeneration (Figure 3A). AMJ13 displayed an atrophied pattern with extensive vacuole degeneration and some cytoplasm-free cells (Figure 3B).

Assessment of apoptosis by the Acridine Orange/ Ethidium Bromide (AO/EtBr) staining

The Acridine Orange/ Ethidium Bromide (AO/EtBr) staining was used for both quantitative and qualitative detection of apoptotic bodies induced by the derivative (8). In the MCF7 cell line, four stages of apoptosis were recorded, the viable cells having round nuclei and emitting green fluorescence. The early apoptotic cells with a greenish appearance demonstrate croissant-like chromatin condensation on the edges of irregularly shaped nuclei. The dead cells have the yellowish-orange appearance of rounded nuclei, and late apoptotic cells express condensed or fragmented chromatin of irregularly shaped nuclei with a yellow-orange stain (Figure 4a/ A2).



Thiosemicarbazide derivatives (5,7, &8)

Compound	R'
(5)	H
(7)	Cl
(8)	OCH ₃

Figure 1. Chemical Structure of Thiosemicarbazide Derivatives of Captopril (derivatives 5, 7 and 8)[14].

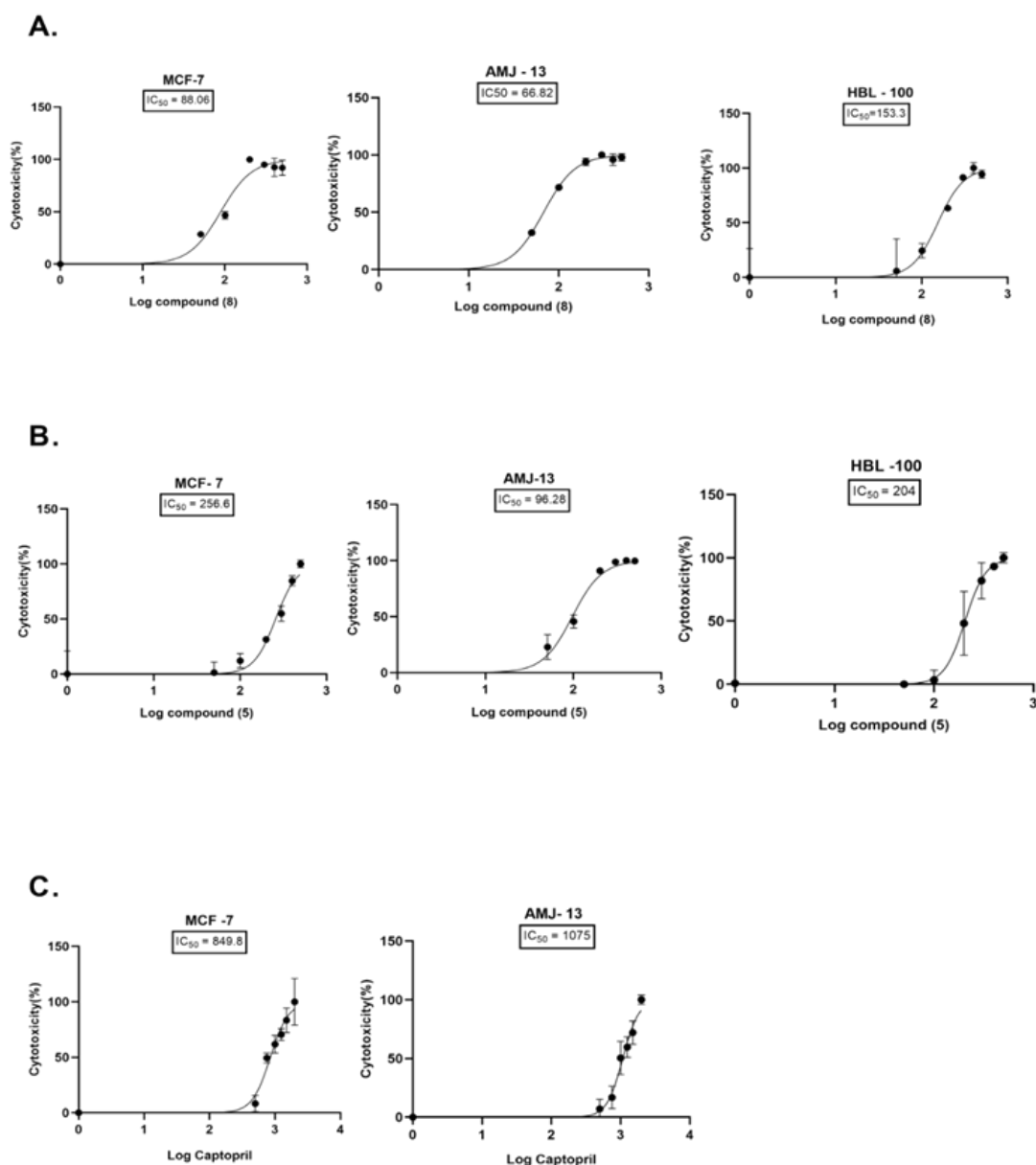


Figure 2. Illustration of IC_{50} of A) thiosemicarbazide derivative (8). B) thiosemicarbazide derivative (5) and C) captopril in the estrogen receptor-positive MCF-7, ER/PR receptor negative AMJ13 breast cancer cell lines and in the non-tumorigenic breast cell line HBL-100. All results shown here were done as (n= 4, 3 and 2) with quadruplicate per experiment.

The analysis demonstrated a significant increase in the mean apoptotic cells (62.99%) compared to live cells (37.1%) in the MCF-7 cell line, and when compared to control cells there was a significant difference between the mean apoptotic cells with a p-value of (0.0001) (Figure 4b). These results indicate apoptosis as a type of cellular death produced in MCF-7 cells treated with a derivative (8).

On the other hand, in the AMJ13 cell line, the analysis revealed an insignificant number of apoptotic cells with a percentage of (23.6), and no significant difference was recorded between the mean apoptotic cells treated with derivative (8) to that of the control (untreated cells) (Figure 4b). Majority of the (23.6%) cells were either dead or late apoptotic (Figure 4a/B2).

Immunocytochemistry analysis

Monoclonal IgG1 were directed against *caspase -3*, *caspase-9*, and cell surface marker *PECAM-1* to document their expression in the cells.

The results revealed a significant increase in *caspase (3)* (P-value 0.0082) and *caspase -9* (P-value 0.0284) in MCF-7 cells treated with derivative (8) compared to the normal ones (Figure 5a/A2, A1/b) and (Figure 5a/B2, B1/b) respectively. In addition to that, derivative (8) imposed non-significant increase in *PECAM-1* (CD-31) compared to non-treated cells (Figure 5a/C2, C1/b).

While in the AMJ13 cell line, the Derivative of captopril (8) imposed a statistically significant increase in *caspase -9* (p-value 0.0003) (Figure 6b) illustrated as dark brown pigmentation around the cells (Figure 6a/B2) compared to very light scattered brown pigmentation

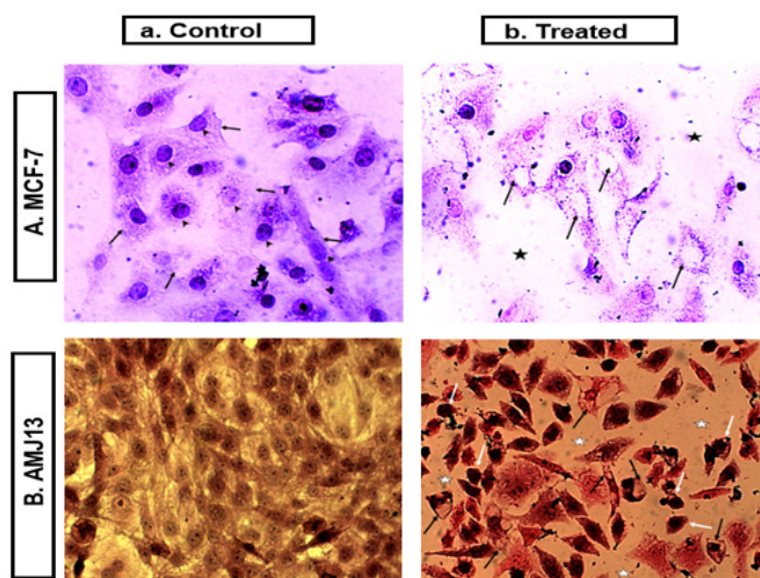


Figure 3. The Cell line Morphological Changes Following 72 hrs Exposure to Compound (8) A. MCF-7 (IC_{50} 88.06). a) Control (untreated MCF-7 cell line) arrows indicate normal cells, whereas the arrowhead refers to a normal nucleus. b) The arrows refer to the vacuoles, whereas the asterisk refers to cell-free spaces. B) AMJ13 (IC_{50} 66.82). a) Control (untreated AMJ-13 cell line). b) The black arrows refer to the vacuoles, the white ones refer to atrophied cells and the asterisk refers to cell-free space. All cells were stained with Hematoxylin and eosin staining and were imaged by light microscope 40 X.

control cells (Figure 6a/B1).

Contrary to *caspase -9*, the derivative had an insignificant increase in *caspase -3* (Figure 6a/ A2, b) and *PECAM-1*. (Figure 6a/C2.b) compared to controls (Figure 6a/A1) and (Figure 6a/C1) respectively.

Assessment of ROS-inducing activity of the derivative

Two experiments were conducted, one included

72 hours of incubation of the cells with different concentrations of derivative (8) (IC_{50} , up and down the IC_{50}), and the second was performed by treatment of the cells with fixed concentration (IC_{50} of the derivative) at different time intervals (2, 6, and 12 hours).

In the MCF7 cell line, the first experiment's highest level of ROS was achieved at the lowest concentration (25 μ M), whereas MCF-7 basal ROS level was documented

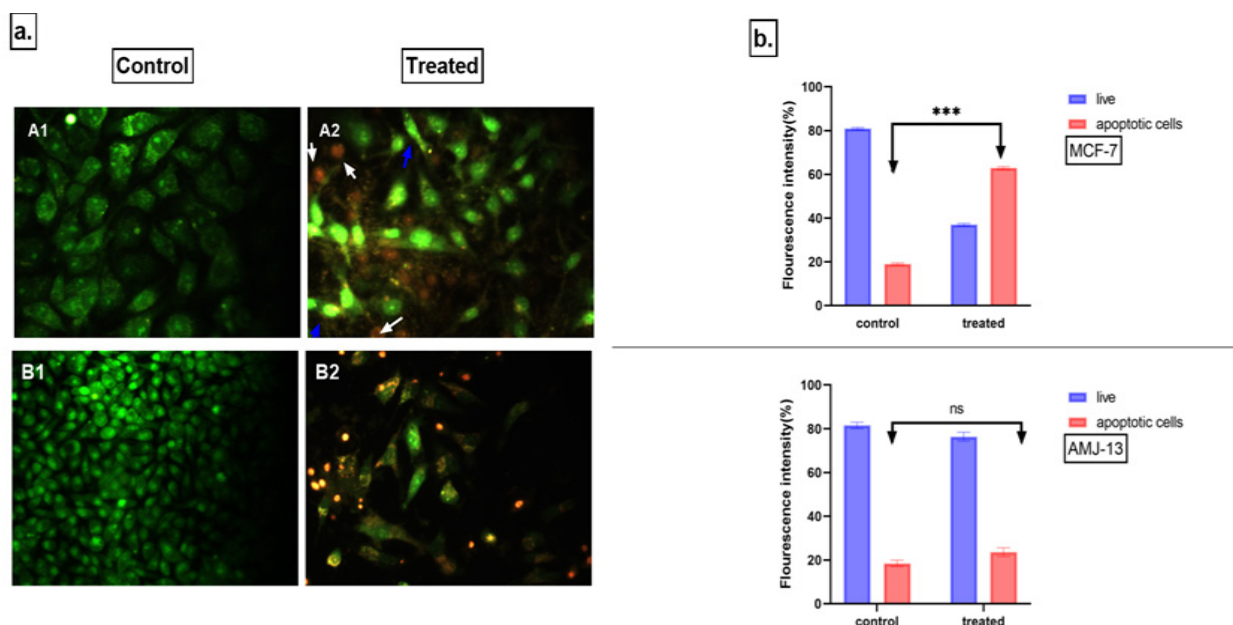


Figure 4. AO/EtBr Double Staining Assay for Assessment of Apoptosis. a) A1 control MCF-7 cells, A2 MCF-7 treated cells with derivative (8) (IC_{50} = 88.06 μ M). The blue arrows refer to early apoptotic cells, while the white ones refer to the dead cells. B1) control AMJ-13 cells, B2) AMJ-13 treated cells with derivative (8) (IC_{50} =66.82 μ M). All images were visualized under a fluorescent microscope 40x. b) Statistical analysis comparing mean (percentage) apoptotic cells between control and derivative (8) treated cells calculated from AO/EtBr staining assay. An unpaired t-test was performed and the results represented are mean \pm SD.

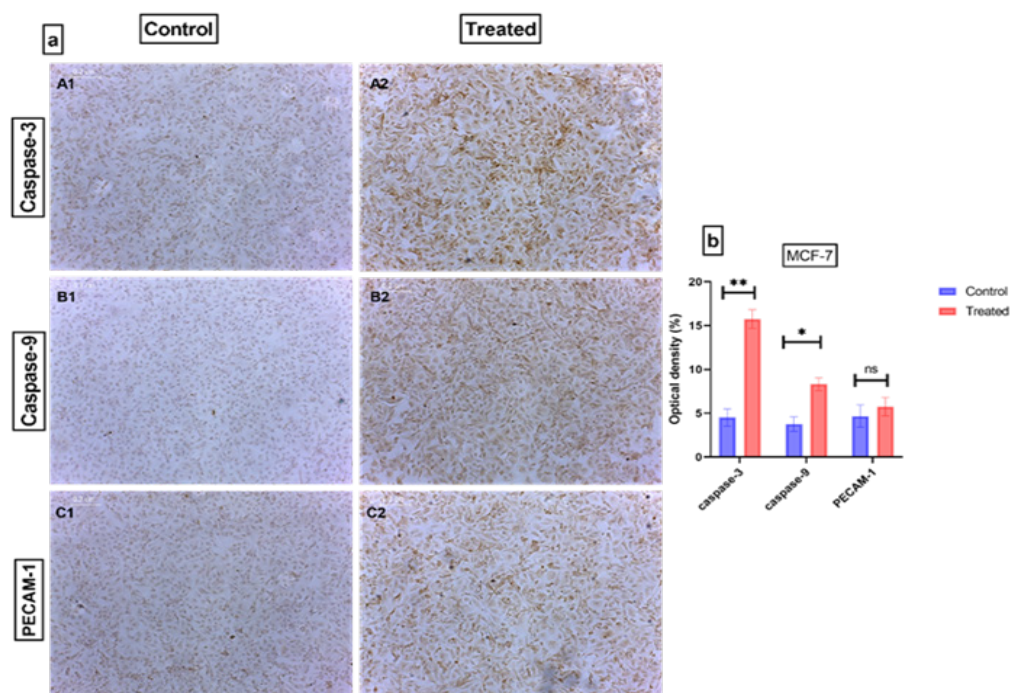


Figure 5. a/ Immunocytochemistry Images of MCF-7 Cells Treated with (88.06 μM) of Compound (8). Expression of *caspase-3* in A1 control cells, A2 treated cells. Expression of *caspase-9* in B1 control cells, B2 in treated cells expressing significant increase represented by the dark brown staining around the cells. Expression of *PECAM-1* in C1 control and C2 treated cells. b/ Statistical analysis of quantitative expression of *caspase-3*, *caspase-9* and *PECAM-1* demonstrating a significant increase in *caspase-3* (p-value 0.0082) and *caspase-9* (p-value 0.0284). Analysis was performed using 3-5 immunocytochemistry images analyzed by Fiji (Image J version 1.53). An unpaired t test was done and all results shown here are mean ± SD.

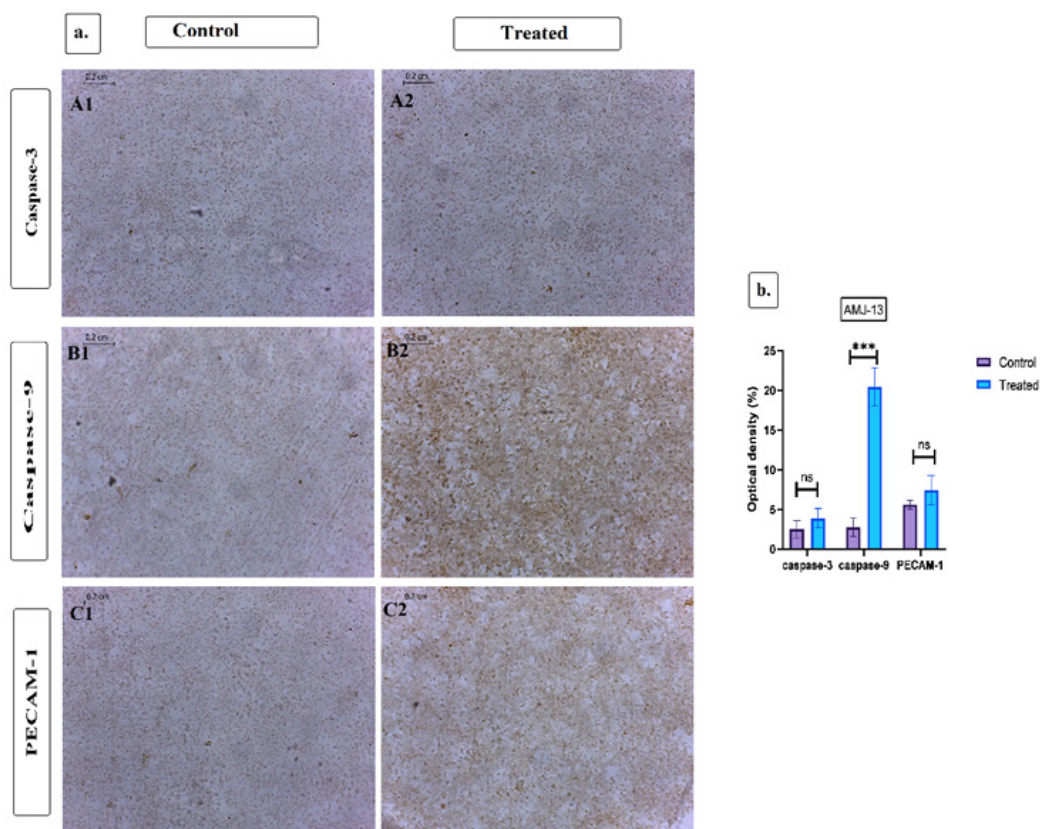


Figure 6. a/ Immunocytochemistry Images of AMJ13 Cells Treated with (66.92 μM) Derivative (8). Expression of *caspase-3* in A1 control cells, A2 treated cells. Expression of *caspase-9* in B1 control cells, B2 in treated cells expressing significant increase represented by the dark brown staining around the cells. Expression of *PECAM-1* in C1 control and C2 treated cells. b/ Statistical analysis of quantitative expression of *caspase-3*, *caspase-9* and *PECAM-1* from immunocytochemistry test. An unpaired t-test was done and the results represented here are mean ± SD.

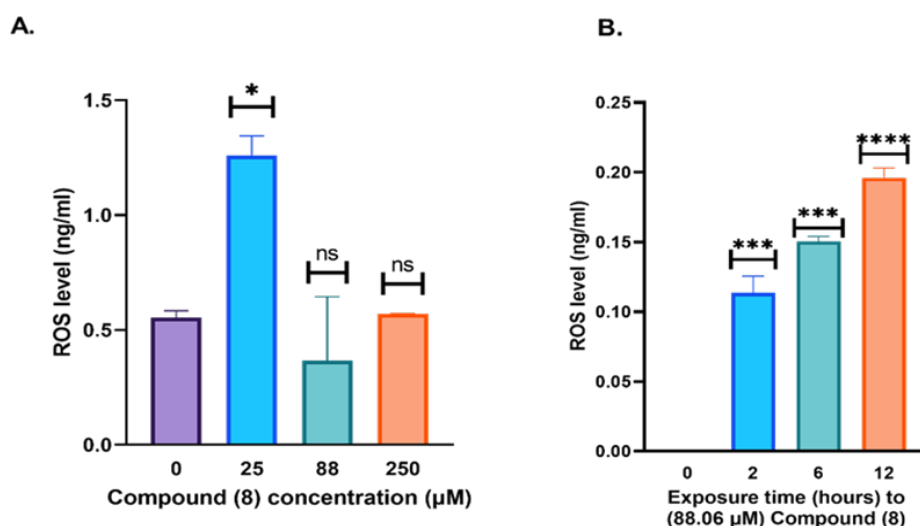


Figure 7. Generation of ROS in MCF-7 Cell Line after Treatment with Derivative (8). A) Cells were treated with three different concentrations (IC_{50} , below, and above the IC_{50}) of compound (8) for 72 hours. B) Cells were exposed to only 88.06 (μM) of derivative (8) for 2, 6, and 12 hours. Results are from two experiments with two replicates. Data presented here are mean + SD.

at both the IC_{50} (88.06 μM) and the highest concentration (250 μM) (Figure 7A).

On the other hand, the second experiment, documented a time-dependent increase in ROS level to reach its maximum following 12 hours of incubation (Figure 7B), indicating that the ROS level decreased gradually to reach the basal level after 72 hours. In the AMJ13 cell line, results demonstrated no change in basal ROS level (data not shown).

Discussion

Globally, Breast cancer prevalence is increasing and its heterogeneity renders many therapies almost useless in combating certain sub-types of this disease.

Over the years, many therapies and personalized treatments have been developed, however many challenges still arise. As such, novel and innovative approaches for the treatment of breast cancer seem like an endless pursuit (3). Among the newly pursued approaches are the Renin-Angiotensin System inhibitors [5] and thiosemicarbazide/ thiosemicarbazone derivatives [16, 17]. In this research, the two approaches have been joined by employing novel derivatives of the ACEi (Captopril) that include thiosemicarbazide moiety and were previously found to have an improved ACE inhibition activity over their parent compound captopril [15]

This work investigated three derivatives namely (5, 7, and 8) in the estrogen receptor-positive (MCF-7) and the highly aggressive estrogen/ progesterone receptor-negative (AMJ13) breast cancer cell lines. Results demonstrated derivative (8) having the best antiproliferative profile affecting both cell lines with much less effect on the non-tumorigenic breast cell line HBL-100. The distinct profile of the cytotoxicity imposed by the three derivatives can be explained based on their chemical structure. The substitution at the phenyl moiety is necessary for the

anti-breast cancer function, where the 4 methoxy moiety of derivative (8) documented the highest inhibition of proliferation on all cell lines, whereas, the chlorine of derivative (7) abrogated this function. It is worth mentioning that this structure-activity relationship (SAR) was not uncommon for thiosemicarbazide derivatives and was documented by others [37].

Moreover, the no substitution of derivative (5) might be cell-specific where it exerted a strong effect on the ER/ PR receptor negative AMJ13 with little effect and even can be referred to as not affecting MCF-7 when compared to transformed cells. To further elaborate, the presence of an electron-withdrawing group at the para-phenyl position will lead to the loss of activity as displayed with the MTT assay. Thus, for hydrophobic head unsubstituted, or substituted with electron donated group was essential for activity against breast cancer cell lines.

Worth mentioning, that these derivatives demonstrated different antiproliferative profiles when tested in other cancer cell lines, including HCT-116, A549, HeLa and HepG2 (unpublished data). These investigations confirmed that the derivatives are indeed versatile and behave depending on the type of cell involved.

To elucidate the cytotoxicity of derivative (8), further investigations focused on the type of cellular death and the potential mode of action exerted by this derivative. Results revealed the cellular-dependent behaviour of the derivative, where in MCF-7 the derivative induced apoptotic type of death with the involvement of caspase-3, caspase-9 and ROS generation. On the other hand, apoptosis's distinct morphological profile was absent in AMJ13 cell lines, which demonstrated an extensive vacuole-forming type of death, without ROS generation, but with the significant implication of caspase-9.

This can be explained by the fact that caspase-9 activity is not limited to apoptosis; caspase-9 is implicated in the induction of paraptosis (a type of programmed non-

apoptotic vacuole-inducing cell death) [38].

Numerous studies have described thiosemicarbazide/thiosemicarbazone derivatives exhibiting several types of cellular death, including apoptosis [22, 39, 40], mitosis-like death [41], autophagy [42, 43], and paraptosis [44, 45]. As well as, exhibiting multiple cell death types at the same time as apoptosis and autophagy [43, 46, 47].

Notably, molecular docking studies revealed the derivative as a potential paraptosis-inducing agent through targeting the most important signalling pathways involved (JNK1, p38 and MEK-2) (supplementary material).

Hence, derivative (8) might have induced ROS production in the MCF-7 cell line because the cell is vulnerable to ROS-induced apoptosis while exhibiting another form of cell death independent from ROS in AMJ13; what type of death remains to be elucidated with further investigations. It is valuable to say that this is not the first time that a thiosemicarbazide derivative induces cell death that is neither apoptotic nor necrotic without ROS production. Cavazzoni and his colleagues witnessed the same behaviour from their thiosemicarbazone derivative (referred to as copper(II) complex 5) [48]. Hager et al. [45] also documented a paraptotic cell death with their compound Me2NNMe2 that elicited its cytotoxicity independently from ROS generation, rather it was associated with an increased level of the oxidised form of glutathione.

It is noteworthy that this cellular-dependent activity of derivative (8) was also witnessed in previous investigations of ours as an antiangiogenic agent. The derivative proved to be a promising antiangiogenic agent targeting multiple factors in the angiogenesis of breast cancer cells in particular the MCF-7 cell line with a different effect on AMJ13 cell lines [49].

In conclusion, this study has demonstrated the thiosemicarbazide derivative of captopril (8) as a very promising molecule against breast cancer cell lines. The derivative displayed a highly improved antiproliferative activity than captopril against either cell, the ER receptor positive MCF-7 and the highly aggressive ER/PR receptor negative AMJ13 breast cancer cells. The derivative caused an apoptotic death with the potential implication of caspase-3 and *caspase-9* along with time-dependent ROS generation in MCF-7, and an extensive vacuole forming, non-apoptotic cellular death, without ROS generation, but with significant implication of *caspase-9* in AMJ13. Whether this cellular-dependent behaviour of the derivative is related to the distinct characteristics of each cell line or is based on its versatility of targets that may or may not involve RAS components, the derivative proved to be a promising antiproliferative agent against breast cancer cells and is certainly recommended as a leading molecule in future investigations.

Limitations

The study could have included more detailed techniques regarding the two types of death, such as Western blot, TEM, flow cytometry. Lack of the availability of these techniques at the time of commencing the investigations, greatly limited that possibility. Currently, these techniques are available and

the proceeding studies of these derivatives will include those techniques.

Author Contribution Statement

Conceptualization, Baan AL-Jasani and Hiba AL-Saad; Data curation, Baan AL-Jasani; Formal analysis, Baan AL-Jasani; Investigation, Baan AL-Jasani; Methodology, Baan AL-Jasani; Project administration, Baan AL-Jasani and Hayder Sahib; Resources, Baan AL-Jasani and Hiba AL-Saad; Supervision, Hayder Sahib; Validation, Hayder Sahib; Visualization, Baan AL-Jasani, Hayder Sahib and Hiba AL-Saad; Writing – original draft, Baan AL-Jasani; Writing – review & editing, Baan AL-Jasani

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Ethical declaration

Ethical clearance was granted from all the required institutions and laboratories

Data Availability Statement

Data is within the article and can be provided by the corresponding author upon reasonable request

Conflicts of Interest

The authors declare no conflict of interest

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