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Research Article

Erastin Induces Ferroptosis and Apoptosis in MDA-MB-231 Breast Cancer Cell Line

Rua Abbas Naser*, Inam Sameh Arif, Basma Talib Al-Sudani
Department of Pharmacology and Toxicology, College of Pharmacy, Mustansiriyah University, Baghdad, Iraq
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Abstract

Background: Breast cancer (BC) is one of the most malignant types of cancer in women. Triple-negative breast cancer (TNBC) is a subtype of breast cancer with poor prognosis and high recurrence and invasive metastasis rates. Ferroptosis is a non-apoptotic form of cell death characterized by iron-dependent accumulation of reactive oxygen species (ROS). Although ferroptosis induced by erastin has been widely studied, the ability of erastin to induce apoptosis has not been extensively investigated. ***Objective***: To evaluate the effect of erastin on the viability of MDA-MB-231 breast cancer cells and also to investigate the potential of erastin to induce ferroptosis and apoptosis. ***Methods***: MDA-MB-231 breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and treated with erastin. Cell viability was assessed by 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay. The total cellular labile iron pool (LIP) was detected based on the calcein-acetoxymethyl ester (C-AM) method. Cellular ROS level was detected using an ROS fluorometric assay kit. Cell apoptosis was detected using an annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) apoptosis detection kit. ***Results***: Erastin inhibited the growth of MDA-MB-231 cells and significantly increased the levels of ROS, LIP, and MDA in MDA-MB-231 breast cancer cells. The results have also demonstrated that erastin had the ability to induce apoptosis. ***Conclusions***: Erastin induced two forms of cell death, ferroptosis and apoptosis, in MDA-MB-231 breast cancer cells. These findings suggest that erastin may be further investigated as a novel anti-TNBC agent.

Keywords: Apoptosis, Erastin, Ferroptosis, Triple negative breast cancer.

يحفز الإراستين حدوث الاستماتة الحديدية والموت الخلوي المبرمج في خلايا سرطان الثدي MDA-MB-231

الخلاصة

الخلفية: يُعد سرطان الثدي (BC) أكثر أنواع السرطان خبثًا لدى النساء. ويُشكل سرطان الثدي الثلاثي السلبي (TNBC) أحد أنواع سرطان الثدي، ويتميز بتشخيص سيء ومعدلات عالية من التكرار والانتشار الغزوي. الاستماتة الحديدية هي شكل من أشكال موت الخلايا غير المبرمج، يتميز بتراكم مركبات الأوكسجين الفعالة (ROS) المعتمدة على الحديد. ورغم أن الاستماتة الحديدية الناجمة عن الإراستين قد خضعت للعديد من الدراسات، إلا أن قدرته على تحفيز الموت الخلوي المبرمج لم تُدرس بشكل موسع. **الهدف**: تقييم تأثير الإراستين على حيوية خلايا سرطان الثدي من نوعMDA-MB-23 ، والتحقق من قدرة الإراستين على تحفيز كل من **الاستماتة الحديدية** والموت الخلوي المبرمج. **الطرائق**: تمت تنمية خلايا سرطان الثدي MDA-MB-231 في وسط دُلكو المعدل (DMEM) وعولجت بالإراستين. تم تقييم حيوية الخلايا باستخدام اختبار (4،5-ثنائي ميثيل ثيازول-2-يل)-2،5-ثنائي فينيل تترازوليوم بروميد (MTT).وتم قياس مخزون الحديد الخلوي (LIP) باستخدام طريقة كالسيين-أسيتوكسي ميثيل إستر(C-AM) ، بينما تم تحديد مستوى ROS باستخدام مجموعة الكشف الفلوريومتري الخاصة بـ ROS. وتم الكشف عن موت الخلايا المبرمج باستخدام مجموعة كشف الاستماتة المحتوية على أنيكسين-V فلوروسئين أيزوثيوسيانات (FITC) ويوديد البروبيديوم(PI). **النتائج**: أعاق الإراستين نمو خلايا سرطان الثديMDA-MB-231 ، كما أدى إلى زيادة معنوية في مستويات كل من: ROS، LIP وMDA في هذه الخلايا. وأوضحت النتائج قدرته على تحفيز الموت الخلوي المبرمج. **الاستنتاجات**: حفز الإراستين نوعين من موت الخلايا في خلايا سرطان الثديMDA-MB-231 ، وهما الاستماتة الحديدية والموت الخلوي المبرمج. أن الإراستين قد يكون مرشحًا واعدًا للدراسة كعامل علاجي جديد لسرطان الثدي الثلاثي السلبي(TNBC) ، مما يستدعي بحثا معمقا لتقييم فعاليته العلاجية وآليته الجزيئية بالتفصيل.

* ***Corresponding author***: Rua A. Naser, Department of Pharmacology and Toxicology, College of Pharmacy, Mustansiriyah University, Baghdad, Iraq; Email: rua.alhamdy@uomustansiriyah.edu.iq
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INTRODUCTION

Breast cancer (BC) is one of the most aggressive types of cancer in females and is the primary cause of cancer-related mortality in women within developed nations and represents the leading cause of fatal malignancy worldwide [1,2]. In 2023, BC was the most frequent type of cancer globally, accounting for more than 12.5% of all new cases [3]. Triple-negative breast cancer (TNBC), a subtype of breast cancer associated with poor prognosis and high recurrence and invasive metastasis rates, accounts for approximately 15–20% of all cases of breast cancer [4]. TNBC faces clinical challenges and lacks effective targeted therapy because it does not express progesterone receptor, estrogen receptor, and human epidermal growth factor receptor-2 (HER2) [5,6]. Therefore, there is a crucial demand to identify a promising new strategy for the treatment of TNBC. Ferroptosis is a programmed form of cell death that differs from apoptosis, necrosis, and autophagy. It is characterized by an iron-dependent intracellular buildup of lipid reactive oxygen species (ROS), leading to damage of cellular membranes through

lipid peroxidation [7,8]. According to studies, highly invasive TNBC cells are susceptible to ferroptosis and especially to ferroptosis inducers (FINs). This vulnerability is due to several metabolic factors, including an expanded labile iron pool (LIP), high levels of polyunsaturated fatty acids (PUFA), and a compromised glutathione peroxidase 4 (GPX4)-reduced glutathione (GSH) defense system. A classic inducer of ferroptosis, erastin, acts through inhibition of system Xc^-, resulting in GPX4 and GSH depletion [9]. Erastin is found to induce ferroptosis in various types of human cancers [10]. Recent studies have revealed that lipid peroxidation caused by ROS-induced lipid peroxidation promotes apoptosis [11]. The non-apoptotic cell death induced by erastin has been widely studied [12]; however, the ability of erastin to induce apoptosis has not been extensively investigated. MDA-MB-231 cells are considered to have TNBC [13], and in this study these cells have been used as an in vitro model for TNBC. In this study we aimed to assess the effect of erastin on the viability of MDA-MB-231 breast cancer cells and also to determine the potential of erastin to induce ferroptosis and apoptosis.

METHODS

Cell line and culture conditions

Human MDA-MB-231 breast cancer cells were obtained from (ATCC, USA). The Cells were maintained at 37°C/ 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with low glucose (Capricorn Scientific, GmbH), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. When the cells reached 80–90% confluence, erastin was added.

Cell viability assay

4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the cytotoxicity of erastin on MDA-MB-231 cells. The cells were seeded in 96-well plates at a density of $5x10^3$/well and incubated at 37˚C with 5% CO_2. When confluence reached 80-90%, cells were treated with increasing concentrations of erastin (MyBiosource, Inc., USA) prepared by serial dilution (1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.9, and 1.95) µM and incubated for 24 hours. Afterwards, the medium was removed, and MTT (Bidepharm, Shanghai, China) was added at a concentration of 5 mg/mL for 4 h. The supernatants were then removed from the wells, followed by dissolving formazan crystals with dimethyl sulfoxide (DMSO) [14]. The solution absorbance was measured at 530 nm with a 630 nm reference wavelength using a Promega, USA microplate reader. The percentage of erastin-treated cells compared to erastin-free control cells, which were used as negative controls, was used to figure out the cellular relative viability. The percentage of cell viability was calculated as the following equation:

Cell viability % = [OD530, 630 (sample) / OD530, 630 (control)] × 100 [15]

Cytotoxicity % = 100 - Cell viability % [15]

Apoptosis assay

Cell apoptosis was detected using an annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) apoptosis detection kit (Elabscience). MDA-231 cells were seeded at a density of 0.2 x 10^6 cells/well in 6-well plates and incubated for 24 hr after treatment with 40.63 µM of erastin. After trypsinization, the cells were washed twice with cold PBS. 1X annexin V binding buffer (100 µl) is then added to resuspend the cells. According to the manufacturer's protocol, 2.5 µl of Annexin V-FITC and 2.5 µl of PI were added to the cell suspension and left for 15 minutes in the dark at room temperature in the dark. Cells were assessed by flow cytometry (BD FACSVerse™), and the percentages of apoptotic cells were calculated using FlowJo 10.2 software (TreeStar, Ashland, USA) [15].

Measurement of labile iron pool

The total cellular LIP was detected based on the calcein-acetoxymethyI ester (C-AM) method. After treatment with 40.63 µM of erastin, cells were collected using trypsin and then washed twice with PBS and incubated with 2 µM of (C-AM) (MyBiosource, Inc., USA) for 30 min at 37 °C. After that, the cells were washed with PBS, followed by incubation with or without deferoxamine (5 µM) for 1 hour at 37 °C. The cells were analyzed by a multiplate reader (Promega, USA). Calcein was excited at 488 nm, and fluorescence was measured at 525 nm. The level of LIP was calculated according to the difference in cellular mean fluorescence between the cells that have been incubated with deferoxamine and those incubated without deferoxamine [16].

Measurement of reactive oxygen species

Cellular ROS level was detected using the ROS fluorometric assay kit (Elabscience) containing the fluorescent probe DCFH-DA (2,7-dichlorofluorescein). Briefly, cells were seeded in 6-well plates and cultured with 40.63 µM of erastin. After treatment, cells were washed with PBS, and then DCFH-DA was added at 37 °C for 30 minutes in the dark. After that, cells were collected, and the fluorescence intensity of DCF (dichlorofluorescein) was tested by microplate reader (Promega) at Ex/Em = 502 nm/525 nm.

Measurement of malondialdehyde

The MDA colorimetric assay kit (Elabscience) was used to measure cellular MDA level based on thiobarbituric acid (TBA) reactivity. Briefly, MDA-MB-231 cells were seeded into 6-well plates. After

treatment with 40.63 µM of erastin for 24 hours, cells were lysed. After protein quantification of the lysate, MDA working solution was added and heated in a 100 °C water bath for 40 minutes. Next, the supernatant was collected after centrifuging at 1000 rpm for 10 minutes and then measured at 532 nm with a microplate reader (Promega, UAS). The data of each sample were normalized, and the MDA content was calculated according to the manufacturer's instructions.

Statistical analysis

Data analysis was performed using GraphPad Prism software version 8.0 (GraphPad Software, Inc., San Diego, CA, USA). All data were represented as the mean ± standard deviation (SD). Nonlinear regression analysis of the sigmoidal dose-response curve is performed to determine the value of half-maximal inhibitory concentration (IC50). The t-test has been used to compare two groups. A two-way analysis of variance (two-way ANOVA) was used to compare more than two groups. $p< 0.05$ was considered to be statistically significant.

RESULTS

The MTT assay was used to assess the effect of erastin on the viability of MDA-MB-231 cells. The results revealed that erastin inhibited the growth of MDA-MB-231 cells in a concentration-dependent manner, as shown in Figure 1.

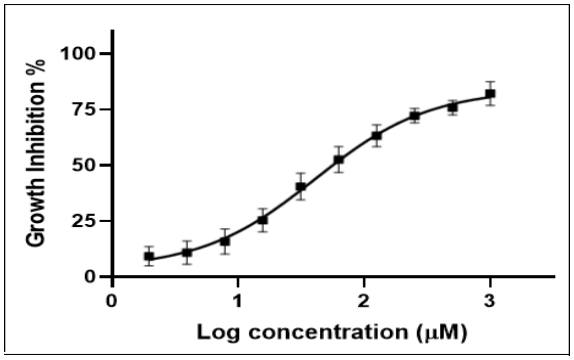

Figure 1: Effect of erastin on viability of MDA-321 breast cancer cells. The cells were treated with increasing concentration of erastin (1000, 500, 250, 125, 26.5, 31.25, 15.63, 7.81, 3.9 and 1.95) µM of erastin for 24 hr. The cell viability was determined using MTT assay. The data are presented as mean ± SD of three independent experiments, n=3.

The IC50 value of erastin was 40.63 µM after 24 hours from treatment. The highest concentration used (1000 µM) of erastin has resulted in 80.7±4.53% growth inhibition. Compared to the control untreated cells, treatment with erastin resulted in a significant increase in ROS ($p< 0.001$), LIP ($p< 0.001$), and MDA level ($p< 0.01$) after 24 hr as shown in Figure 2. Using flow cytometry, assessment of apoptosis using Annexin-V FITC/PI in MDA-MB-231 breast cancer cells following treatment with 40.63 µM of erastin for 24 hours was illustrated in Figure 3.

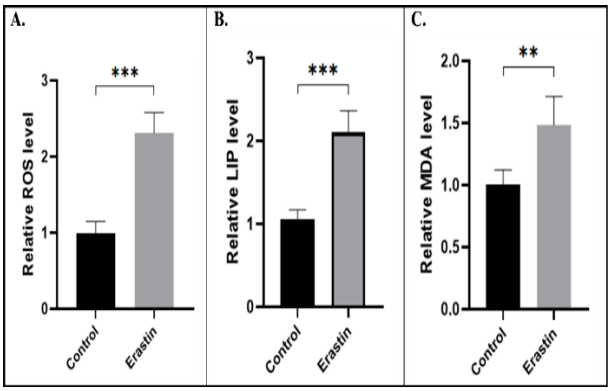

Figure 2: Erastin induces ferroptosis in MDA-MB-231 breast cancer cells. Cells were treated with 40.63 µM of erastin for 24 hr. Data are presented as mean ± SD, n=3. **A)** Quantification of cellular ROS level. **B)** Quantification of cellular LIP level using the calcein-AM (C-AM) method. **C)** Quantification of cellular MDA level. $**p< 0.01$, $***p< 0.001$.

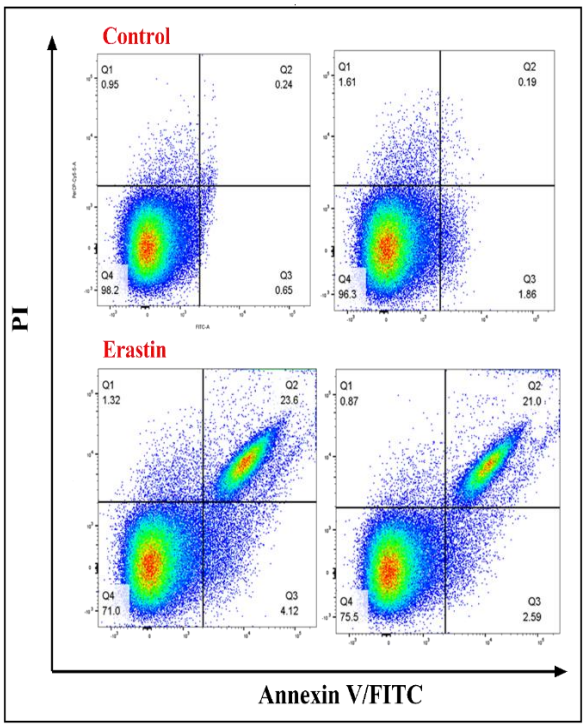

Figure 3: Assessment of apoptosis using Annexin-V FITC/PI in MDA-MB-231 breast cancer cells following treatment with 40.63 µM of erastin for 24 hours**.** Flowcytometry analysis after treatment with erastin.

The results of flow cytometry revealed that erastin induced apoptosis in MDA-MB-231 breast cancer cells, as shown in Figure 4. Compared to the control untreated cells, erastin significantly increased the proportion of late and total apoptosis ($p< 0.0001$). However, for early apoptosis, no significant difference was observed between the two groups.

DISCUSSION

TNBC is the most aggressive type of breast cancer and still clinically a challenging disease [17]. Because TNBC lacks well-defined molecular targets found in other subtypes of breast cancer, the effectiveness of conventional chemotherapy and targeted therapies is noticeably limited [18].

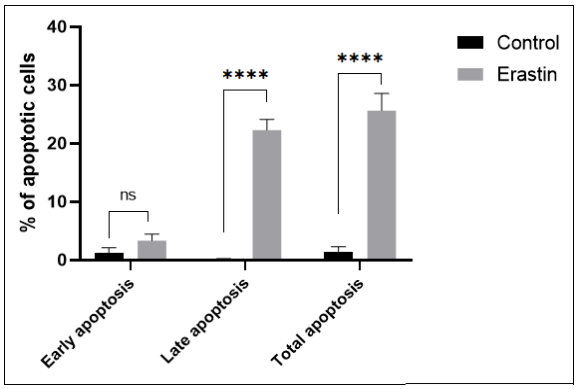

Figure 4: Assessment of apoptosis using Annexin-V FITC/PI in MDA-MB-231 breast cancer cells following treatment with 40.63 µM of erastin for 24 hours. Graphical presentation for the percentages of early and late apoptosis in MDA-231 cells treated with erastin compared to the control group. The data are presented as mean ± SD (n=2); ns: not significant, ****$p< 0.0001$.

Therefore, there is a crucial demand to find new effective therapeutic strategies for treatment. Inducers that trigger ferroptosis, an iron-mediated form of cell death, such as erastin and its related compounds, have emerged as a promising cancer treatment in recent years [8]. Ferroptosis has been shown to inhibit the growth of cells in various cancers, including breast, prostate, pancreatic, and liver cancers [19]. It has been reported that erastin efficiently induces cell death in a variety of cancers, such as liver cancer and rhabdomyosarcoma cells [20]. Moreover, TNBC might be more susceptible to ferroptosis than other subtypes of breast cancer [21]. Yu *et al*. reported that erastin induces ferroptosis in MDA-MB-231 breast cancer cells [22]. Consistently, the present study demonstrated that erastin reduced the viability of MDA-MB-231 cells in a concentration-dependent manner. The IC50 of erastin was 40.63 µM after 24 hr of treatment. This finding was very close to the result reported by Li et al. in a study conducted also on MDA-MB-231 cells in which the IC50 of erastin was found to be 40 µM after 24 hours [20]. In ferroptosis, LIP was found to be associated with the generation of ROS ferroptosis by the Fenton reaction [7,23]. ROS plays a crucial role in cell death. PUFAs are present in high levels in cell membranes, and they are particularly susceptible to ROS damage, resulting in lipid peroxidation [11]. MDA is the byproduct of lipid peroxidation and is considered a significant indicator for lipid peroxidation in ferroptosis [24]. This present study has revealed that erastin significantly increased the levels of ROS, LIP, and MDA after 24 hr. These results confirm that erastin induced ferroptosis, which is characterized by increased buildup of ROS, iron, and lipid peroxidation [21]. Nuclear receptor coactivator 4 (NCOA4) controls ferritinophagy, which breaks down ferritin through autophagy. This leads to more LIP [25,26]. Numerous studies have demonstrated that NCOA4-mediated ferritinophagy contributes to the ferroptosis that erastin induces [27]. The Fe^{2+} in the LIP is used in the Fenton reaction to generate ROS, mainly hydroxyl radicals. This results in membrane lipid peroxidation leading to ferroptosis [28]. Several studies have suggested that apoptosis is converted to ferroptosis under certain conditions and that ferroptosis promotes cellular susceptibility to apoptosis [29]. The excess amount of ROS in ferroptosis, which propagates the lipid peroxidation chain, can cause damage to biomembranes through lipid peroxidation, resulting in various types of cell death, including apoptosis [30]. In two previous studies, erastin was found to induce apoptosis in gastric and colorectal cancer cells [12,31]. Consistently, the flow cytometry analysis in the present study highlighted a significant increase in late apoptosis in MDA-MB-231 cells within 24 hr after treatment with erastin compared to the control. However, more work is necessary to understand the underlying molecular mechanism of erastin-induced apoptosis. Cancer cells are believed to be more susceptible to oxidative stress due to their comparatively high ROS levels compared to normal cells, and this sensitivity has emerged as a key therapeutic target for cancer treatment [32]. However, further studies are needed to assess the cytotoxicity of erastin on normal cells in order to determine its safety.

Conclusion

Erastin was able to provoke two forms of cell death, ferroptosis and apoptosis, in MDA-MB-231 breast cancer cells. The increased levels of ROS, LIP, and lipid peroxidation have confirmed the ferroptotic cell death. Additionally, the results have demonstrated that erastin also has the capability to induce apoptosis. This study suggests that erastin warrants further investigation as a potential novel therapeutic agent for treating TNBC.

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Conflict of interests

The authors declared no conflict of interest.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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