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Research Article

SHIP2 Silencing Enhances Erastin Cytotoxicity in MDA-MB-231 Breast Cancer Cell Line

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Abstract

Background: Breast cancer (BC) is a serious health risk to women worldwide. Triple-negative breast cancer (TNBC) is a highly heterogeneous type of breast cancer that is highly malignant, recurrent, and invasive. Despite cancer treatment having developed rapidly in the past decades, TNBC is still having challenges. Erastin is an inducer of ferroptosis and inhibits tumor cell growth, thus making it a promising strategy for cancer therapy. The SH2-containing 5′ inositol phosphatase 2 (SHIP2) is overexpressed in BC, where it is associated with poor prognosis. ***Objective***: To address the effect of erastin, SHIP2 silencing, and the combination of both on the viability of MDA-MB-231 BC cells and the impact on the protein kinase B (Akt)/mechanistic target of rapamycin (mTOR) pathway. ***Methods***: MDA-MB-231 BC cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and treated with erastin, SHIP2-siRNA, and their combination. Cell viability was assessed by 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay. Western blot was used to estimate the levels of phospho-Akt and p70 ribosomal protein S6 kinase (p70S6k). ***Results***: The results showed that each of erastin, SHIP2-siRNA, and their combination resulted in growth inhibition of MDA-MB-231 cells and suppressed phospho-Akt but not p70S6K. ***Conclusions***: SHIP2 silencing enhanced the cytotoxicity of erastin in MDA-MB-231 BC cells. Akt, but not mTOR, was involved in the signaling pathway of erastin and SHIP2-siRNA. The combination of both treatments has shown enhanced suppression of phospho-Akt. The results indicate that the combination of erastin and SHIP2-siRNA gives a promising strategy for the treatment of TNBC.

Keywords: Erastin, MDA-MB-231, SHIP2-siRNA, Triple-negative breast cancer.

كتم تعبير SHIP2 يعزز السمية الخلوية للإيراستين في خلايا سرطان الثدي MDA-MB-231

الخلاصة

الخلفية: يمثل سرطان الثدي الثلاثي السلبي (TNBC) نوع شديد التباين من سرطانات الثدي، يتميز بدرجة عالية من الخباثة، ومعدلات انتكاس مرتفعة، وقدرة غزوية كبيرة. بالرغم من التطور السريع لعلاج السرطان ، لا يزال TNBC يشكل تحديا علاجيا. يُعدّ الإيراستين مُحفّزًا معروفًا للموت الحديدي وتشير الدلائل المتزايدة إلى أن الموت الحديدي يُثبّط نمو الخلايا السرطانية، مما يجعله استراتيجية واعدة لعلاج السرطان. يُظهر إنزيم فوسفاتاز الإينوزيتول 5′ المحتوي على SH2 (SHIP2) فرط التعبيرفي سرطان الثدي حيث يرتبط بالتشخيص السيء. **الهدف:** دراسة تأثير الإيراستين و/أو كتم التعبير الجيني ل SHIP2 وتأثير كلاهما على قابلية خلايا سرطان الثدي MDA-MB-231 للبقاء، وكذلك دراسة تأثير ذلك على مسار بروتين كاينيز B (Akt)/ الهدف الميكانيكي للراباميسين (mTOR). **الطرائق:** زُرعت خلايا سرطان الثدي MDA-MB-231 في وسط إيجل المُعدّل من دولبيكو (DMEM) وعولجت بالإيراستين أو SHIP2-siRNA أو كليهما. تم تقييم حيوية الخلايا باستخدام اختبار بروميد (4,5- ثنائي ميثيل ثيازول-2-يل)-2,5 ثنائي فينيل تترازوليوم (MTT). حُلّل تعبير فوسفو Akt و p70 البروتين الريبوسومي 6 كاينيز (p70S6k) باستخدام لطخة ويسترن. **النتائج:** أظهرت النتائج أن الإيراستين أو SHIP2-siRNA وكذلك كليهما أدّى إلى تثبيط نمو خلايا MDA-MB-231. بالإضافة إلى ذلك، وجد أن الإيراستين و/أو SHIP2-siRNA أدى بشكل ملحوظ الى تثبيط فوسفو-Akt، ولكن دون تأثير على p70S6K. **الاستنتاجات:** عزّز إسكات SHIP2 السمية الخلوية للإيراستين في خلايا سرطان الثدي MDA-MB-231. تضمن المسار الإشاري للسمية الخلوية لكل من للإيراستين و كتم التعبير الجيني ل SHIP2 وكذلك كليهما وجود Akt وليس mTOR.

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INTRODUCTION

Breast cancer (BC) is a serious health risk to women worldwide. According to recent studies, BC accounted for over 12.5% of all new cancer cases worldwide as of 2023, making it the most common type of cancer worldwide [1]. Breast cancer is the primary cause of cancer-related mortality in women within developed nations and represents the leading cause of fatal malignancy worldwide [2]. Triple-negative BC (TNBC) is a highly heterogeneous type of BC that is clinically characterized by aggressive, highly proliferative cells and poor prognosis [3,4]. The clinical landscape of TNBC is currently significantly affected by immunotherapy, targeted therapy, and radiotherapy [5]. Despite treatment having developed rapidly in the past decades to prevent tumor growth and metastasis, TNBC is still having challenges because of the disease's heterogeneity, poor prognosis, and lack of well-defined molecular targets [6,7]. Taking into account the limitations associated with existing pharmacological therapies, often resulting in resistance against apoptosis, the investigation of non-apoptotic cell death has emerged to find a new strategy in both fundamental research and clinical studies [8].

Ferroptosis is a specialized form of non-apoptotic cell death characterized by the iron-dependent buildup of lipid peroxides, which ultimately leads to the disruption of cell membranes and cell death [9,10]. Growing evidence suggests that ferroptosis inhibits tumor cell growth, thus making it a promising strategy for cancer therapy [11]. Erastin is a well-known inducer for ferroptosis that inhibits the glutamate/cystine antiporter (system XC–), which in turn affects the formation of reduced glutathione (GSH) by inhibiting cellular uptake of cystine. This eventually leads to oxidative damage and ferroptosis [12]. The phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mechanistic target of rapamycin (mTOR) signaling pathway is frequently over-activated in human cancers and promotes BC growth, survival, proliferation, and angiogenesis, thus making it an interesting target of therapy [13]. p70 ribosomal protein S6 kinase (p70S6K) is a critical downstream effector of the oncogenic PI3K/Akt/mTOR pathway [14]. mTOR complex 1 (mTORC1) phosphorylates P70S6K at threonine 389 (Thr389); thus, the phosphorylation status of p70S6K is commonly used as an indicator of mTOR activity [15,16]. The SH2-containing 5′ inositol phosphatases, SHIP1 and SHIP2, are regulators of the PI3K/Akt signaling pathway that has a crucial role in the progression of cancer. It has been found SHIP2 is overexpressed in a variety of cancers, including colon, breast, and glioma, where it is associated with poor prognosis [17]. SHIP2 has been reported to promote tumor formation and metastasis in MDA-MB-231 cells [18]. It has been suggested that SHIP2 could contribute to the formation of breast cancers through activation of the Akt signaling as well as through regulation of the turnover of epidermal growth factor receptors (EGFRs) [19]. MDA-MB-231 cells are considered to have TNBC [20], and in this study these cells have been used as an in vitro model for TNBC. In this study we aimed to address the effect of erastin, SHIP2 silencing, and the combination of both treatments on the viability of MDA-MB-231 BC cells and also to investigate the impact on the Akt/mTOR pathway.

METHODS

Cell line and culture conditions

The MDA-MB-231 cell line was purchased from (ATCC, USA). The Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with low glucose (Capricorn Scientific, GmbH), supplemented with 10% fatal bovine serum (FBS) and 1% penicillin/streptomycin (Capricorn Scientific, GmbH). The cells were maintained at 37°C in a humidified incubator with 5% CO_2.

Cell transfection

All transfections were carried out using the i-Fect transfection kit (MyBiosource, Inc., USA). To determine the optimum conditions for transfection, the cells were transfected with 40, 60, and 80 pmol of SHIP2-siRNA (Santa Cruz Biotechnology, Inc., USA) in a 6-well plate. Furthermore, SHIP2-siRNA was transfected at 24 h, 48 h, and 72 hr of incubation time to identify the optimum time required for transfection of SHIP2-siRNA in MDA-MB-231 cells. Cells were cultured in a 6-well plate at a seeding density of 0.2 X 106 cells/well with antibiotic-free medium and incubated at 37 °C in a CO2 incubator. When confluence reached 60–80%, cells were transfected with SHIP2-siRNA and incubated for 5 h at 37 °C. After this time, the medium was replaced with fresh medium alone or with fresh medium containing erastin for combined therapy groups and then incubated for 24, 48 and 72 hr. Cells transfected with control siRNA (Santa Cruz Biotechnology, Inc., USA) were considered as a control group [21], while the cells treated with SHIP2 siRNA were the gene silencing group (si-SHIP2).

Assessment of SHIP2 mRNA expression

The quantitative real time (qRT)-polymerase chain reaction (PCR) technique was used to quantify the mRNA expression of SHIP2 in transfected cells. Total RNA was extracted using the Promega SV total RNA isolation system (catalogue number: Z3105). The extracted RNA was quantified using a NanoDrop Microvolume Spectrophotometer [22]. The RT-PCR was carried out using the QIAGEN Rotor-Gene Q RT-PCR System (Germany). The expression levels and fold changes of the GAPDH and SHIP2 genes were assessed using the TransStart® Top Green qPCR Super Mix kit, and the cycle threshold (Ct) has been measured. Each reaction is carried out twice. Primers used were synthesized by Alpha AND, Canada. Sequences of the primers used were as follows: SHIP2 (forward primer, 5′ ACGTGACATCCTGGTTCACA-3′; reverse primer, 5′- GCGGTAATCCAGATCCGTAA -3′) and GAPDH (forward primer, 5′-GGCCTCCAAGGAGTAAGACC-3′; reverse primer: 5′-AGGGGTCTACATGGCAACTG-3′) and it was used as a reference. Reaction conditions of qRT-PCR were as follows: SHIP2 (initial denaturation at 94°C for 30 sec, then 40 cycles of denaturation at 94°C for 20 sec, annealing at 55°C for 30 sec, and extension at 72°C for 20 sec) and GAPDH (initial denaturation at 94°C for 30 sec, then 40 cycles of denaturation at 94°C for 10 sec, annealing at 54°C for 15 sec, and extension at 72°C for 20 sec). The data were expressed as relative mRNA levels and were normalized against the internal control gene, GAPDH. Relative expressions of candidate genes were calculated by the $2^{-\Delta\Delta CT}$ method [23].

Cell viability assay

Cell viability was assessed using the (4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using the Promega Cell-Titer 96 Nonradioactive Cell Proliferation Assay [24]. The MTT assay was used to determine the cytotoxic effect of erastin, SHIP2 suppression, and the combination of both treatments on MDA-MB-231 cells. The cells were seeded into 96-well plates at a seeding density of

5x103/well and incubated at 37°C with 5% CO2. When confluence reached 80-90%, cells were treated with different 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 three different times (24, 48, and 72 hr.). Afterwards, the medium was removed, and MTT (Bidepharm, Shanghai, China) was added at a concentration of 5 mg/mL for 4 hr. The supernatants were then removed from the wells, followed by dissolving formazan crystals with dimethyl sulfoxide (DMSO) [25] (Thomas Baker, India). The solution absorbance was measured at 530 nm with a 630 nm reference wavelength using a Promega, USA microplate to determine the cytotoxicity of SHIP2 silencing. MDA-MB-231 were seeded in 96-well plates at a seeding density of 5x103/well and incubated at 37˚C with 5% CO_2. Cells were transfected with SHIP2-siRNA when confluence reached 60-70%, and then incubated for 24 hr. The cell viability was assessed following the same steps mentioned above. Additionally, the effect of the combination of erastin and SHIP2-siRNA on cell viability was also evaluated using the MTT assay. Cellular relative viability was calculated as the percentage of treated cells to treatment-free control cells, which were considered as negative controls. The percentage of cell viability was calculated as the following equation:

Cell viability % = [OD530, 630 (sample) / OD530, 630 (control)] × 100 [24]

Cytotoxicity % = 100 - Cell viability % [24]

Western blot analysis

Western Blot Detection Kit (Elabscience, USA) was used. RIPA lysis buffer containing a protease inhibitor (PMSF) and a phosphatase inhibitor (Na_3VO_4) was used to isolate protein content of cells. Protein content was determined using the bicinchoninic acid (BCA) protein colorimetric assay kit. Equal quantities of total proteins were loaded onto gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The semi-dry method was used to transfer protein content from the gel to a polyvinylidene difluoride (PVDF) membrane [26]. After that, the membranes were blocked with 5% skimmed milk for 3 h and then incubated with primary antibodies specific to phospho-Akt (Ser473), p70S6k (phospho-threonine 389) and β-actin (MyBiosource, Inc., USA) at 4ºC overnight. Following the incubation, anti-rabbit secondary antibody (Elabscience, USA) was added. The ChemiDoc® XRS+ imaging system (Bio-Rad, USA) is utilized to visualize protein bands. The protein expression of phospho-Akt (Ser473), p70S6k, and β-Actin was quantified using ImageJ 1.53e software (National Institutes of Health, USA).

Statistical analysis

Data analysis was performed using GraphPad Prism software version 8.0 (GraphPad Software, Inc., San Diego, CA, USA). All results 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). One-way analysis of variance (one-way ANOVA) was used to compare more than two groups. A two-way analysis of variance (two-way ANOVA) was processed for comparison of more than two groups at different times using Tukey's multiple comparisons test. $p<0.05$ was considered statistically significant.

RESULTS

Compared to the control group, the expression of the SHIP2 gene was significantly downregulated in a dose-dependent manner at 40, 60, and 80 pmol of siRNA ($p< 0.01$, $p< 0.01$ and $p< 0.001$, respectively), with the highest reduction obtained at 80 pmol, as shown in Figure 1A. It was observed that the relative SHIP2 mRNA expression was 56.5±2.121%, 47.50±3.536%, and 32.50±7.778%, respectively. Furthermore, in order to identify the optimum time for transfection of SHIP2-siRNA, the cells were transfected with 80 pmol at 24, 48, and 72 hr of incubation time. Compared to the control, SHIP2-siRNA significantly reduced SHIP2 gene expression at both 24 and 48 hr post-transfection ($p< 0.01$), as shown in Figure 1B.

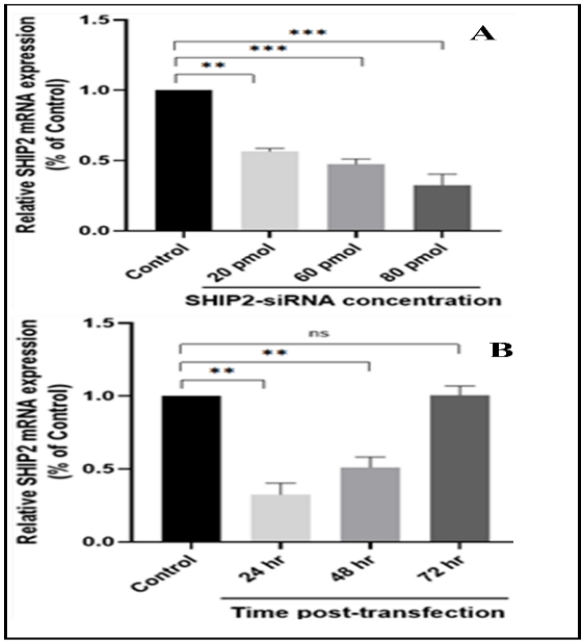
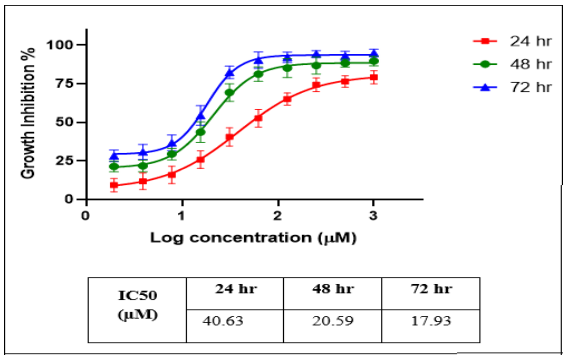


Figure 1: Silencing of SHIP2 by siRNA in MDA-MB-231 breast cancer cells. (A) The SHIP2 mRNA expression in different concentrations of 20, 60 and 80 pmol/well. mRNA levels were detected using qRT-PCR at 24 hr post-transfection. The data are represented by mean ± SD (n = 2); $^{**}p < 0.01$, $^{***}p < 0.001$ versus control. B) Percentage of SHIP2 mRNA expression at 24, 48 and 72 hr post-transfection. Cells were transfected with 80 pmol of siRNA/well. Levels were detected by RT-PCR. The data are represented by mean ± SD (n = 2); $^{**}p < 0.01$, $^{***}p < 0.001$ versus control.

The relative expression of SHIP2 was 32.5±7.778%, 51±7.071%, and 100.5±6.364%, respectively. The MTT assay was performed to assess the effect of erastin, SHIP2-siRNA, and their combination on the viability of MDA-MB-231 cells. The results revealed that erastin inhibited the growth of MDA-MB-231 cells in a concentration- and time-dependent manner, as shown in Figure 2. The IC50 values of erastin were 40.63, 20.59, and 17.93 µM after incubation for 24, 48, and 72 hr, respectively. To determine the effect of transfection time of SHIP2-siRNA on the viability of MDA-MB-321, cells were transfected with 80 pmol at 24, 48, and 72 hr post-transfection. At each time, the cytotoxicity effect of SHIP2-siRNA has been compared to two controls: the untreated cells in DMEM media only and the negative control group (NC-SHIP2), where cells were transfected with a nontargeting siRNA using i-Fect transfecting reagent.



IC50 (µM)	24 hr	48 hr	72 hr
	40.63	20.59	17.93

Figure 2: Effect of erastin on viability of MDA-321 breast cancer cells. The cells were treated with increasing concentration of erastin (1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.9 and 1.95) µM for 24, 48 and 72 hr. For each assay n=3. The cell viability was determined using MTT assay. The data are presented as mean ± SD of three independent experiments.

No significant difference was observed among all the control groups ($p > 0.05$). Compared to the control group (untreated cells), a significant difference was observed in the viability of the cells at 24 hr post-transfection ($p < 0.01$). Besides, there was no significant difference between 48 hours and 72 hr post-transfection ($p > 0.05$) (Figure 3).

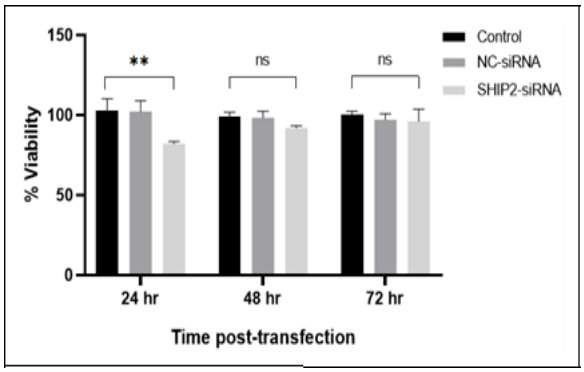


Figure 3: Effect of SHIP2-siRNA on viability of MDA-321 cells at 24, 48, and 72 hr post-transfection. The control group: untreated cells in DMEM media; NC-siRNA: negative control group where cells were transfected with a nontargeting siRNA; and SHIP2-siRNA: cells were transfected with SHIP2-siRNA using transfection reagent. Cell viability was determined by MTT assay. For each assay, n= 2. The data are represented by mean ± SD of two independent experiments; ns, not significant, $^{**}p < 0.01$ versus control.

Moreover, the effect of the combination of erastin and SHIP2-siRNA on the viability of MDA-MB-231 cells has also been investigated at 24, 48, and 72 hr post-transfection. It has been observed that the combination significantly inhibited viability compared to erastin at 24 hours post-transfection ($p < 0.0001$) (Figure 4). While there was no significant effect at 48 and 72 hours post-transfection ($p > 0.05$). Therefore, 80 pmol of SHIP2-siRNA at 24 hr post-transfection was considered the optimum condition for transfection, and a Western blot was carried out under these conditions. In order to figure out whether the Akt/mTOR pathway is involved in the cytotoxicity of erastin/and SHIP2-siRNA in MDA-MB-231, the expression of phospho-Akt and p70S6k was analyzed by Western blot.

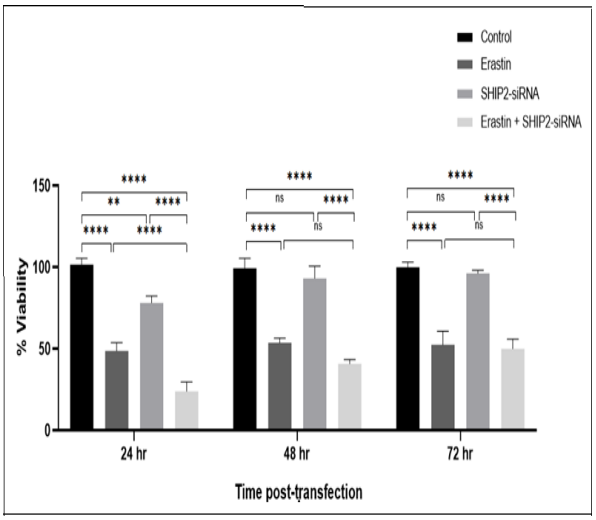


Figure 4: Effect of erastin, SHIP2-siRNA and their combination on viability of MDA-321 cells after 24, 48 and 72 hr from treatment. Cell viability was determined by MTT assay. For each assay, n= 2. The data are represented by mean ± SD of two independent experiments: ns, not significant, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.000$ versus control.

It has been found that individual treatments and their combination significantly reduced phospho-Akt level compared to the control ($p < 0.05$ and $p < 0.01$, respectively, compared to the control), as shown in Figure 5.

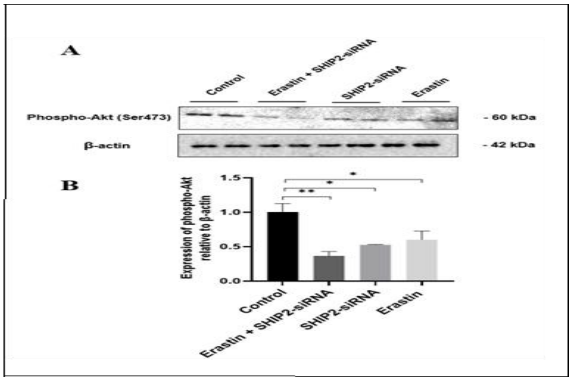


Figure 5: The effect of erastin, SHIP2-siRNA and their combination on phospho-Akt protein expression in MDA-MB-231 cells analyzed by Western blot. **A**) Cells were treated with erastin/and or SHIP2-siRNA for 24 hr. β-actin was used as a control. **B**) Protein bands were quantified by ImageJ software. The data are represented by mean ± SD (n= 2); $^{*}p < 0.05$, $^{**}p < 0.01$ versus control.

However, there was no significant change in the levels of p70S6K in all treated groups compared to the control ($p > 0.05$) as shown in Figure 6.

DISCUSSION

Breast cancer is an increasingly common type of female cancer that contributes greatly to the disease burden globally [4]. Despite advancements in basic research and treatment of TNBC, poor clinical outcomes are caused by a combination of the aggressive nature of this type of cancer, delayed diagnosis, and a lack of suitable endocrine and targeted therapy [4]. Therefore, there is an essential need to find new promising targets for treatment. Numerous studies suggest that ferroptosis plays an essential role in tumor suppression and thus provides a new target for cancer therapy [27].

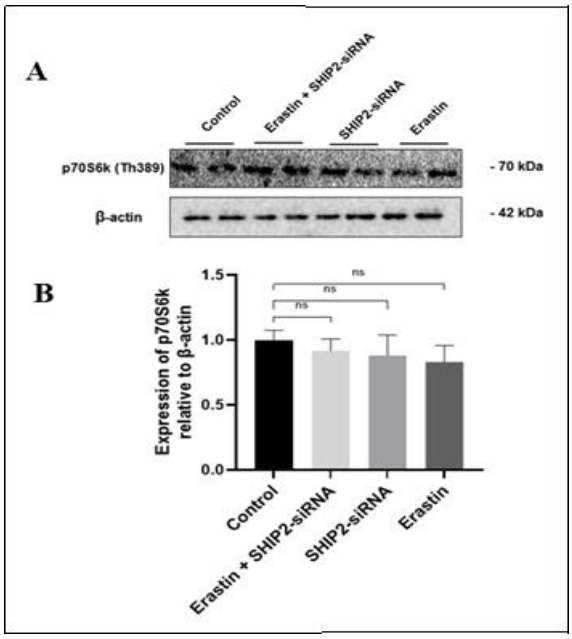


Figure 6: Protein expression of p70S6k in MDA-MB-231 cells after treatment with erastin, SHIP2-siRNA and their combination. **A**) Cells were treated with erastin/and or SHIP2-siRNA for 24 hr. β-actin was used as a control; **B**) Protein bands were quantified by ImageJ software. The data are represented by mean ± SD (n= 2); ns, not significant.

Moreover, it has been found that highly TNBC cells are particularly vulnerable to ferroptosis-inducing compounds (FINs) [28]. Erastin was found to induce ferroptosis in various cancer cells, such as hepatocellular carcinoma (HCC), NSCLC non-small-cell lung cancer, melanoma, ovarian cancer, and thyroid cancer [29]. The present study revealed that the viability of MDA-MB-231 cells has decreased in a concentration- and time-dependent manner following erastin treatment. IC50 of erastin was 40.63 µM after 24 hr of treatment. This finding was close to the result reported in a previous study conducted also on MDA-MB-231 cells in which IC50 of erastin was found to be 40 µM after 24 hr [30]. Phosphatases are generally considered as tumor suppressors; however, there is growing evidence that phosphatases can also act as inducers for signaling, thereby presenting them as possible oncogenes and targets for treatment [31]. SHIP2 is a lipid phosphatase acting on the PI3K-PKB-mTOR pathway [32]. It converts the PI3K product PI(3,4,5)P3 to PI(3,4)P2 to create a mixture of both these inositol phospholipids at the plasma membrane. It has been demonstrated that PI(3,4)P2 directly increases Akt activation and survival in many types of cancers [17]. The aim of this study was also to investigate the effect of SHIP2 silencing on MDA-MB-231 cells. Today, siRNA-mediated silencing, one of the targeted gene silencing techniques, is presented as a powerful tool against cancer [33]. This present study established SHIP2 silencing by siRNA resulted in significant suppression of SHIP2 expression after 24 hr from transfection. In a previous study, the oncogenic role of SHIPs on MDA-MB-231 cells has been investigated. The results of that study have revealed that SHIP2 depletion decreased cell migration [18]. Moreover, Tang *et al*. reported that knockdown of SHIP2 decreased proliferation and increased apoptosis, which implies that SHIP2 played a key role in the progression of cancer [34]. Consistently, the present study demonstrated that SHIP2 silencing by siRNA significantly reduced the viability of MDA-MB-231 cells compared to untreated cells at 24 hr post-transfection. Additionally, the results demonstrated that SHIP silencing significantly enhanced the cytotoxicity of erastin on MDA-MB-231 cells. PI3K/Akt/mTOR signaling is one of the most commonly dysregulated pathways in cancer patients that plays crucial roles in cancer development [28]. In this study, western blotting revealed that erastin and/or SHIP2 silencing significantly reduced protein expression levels of phosphor-Akt but not p70S6k. Moreover, the results revealed that SHIP2 silencing synergized with erastin to reduce phospho-Akt. This suggests that the ability of erastin and/or SHIP2 siRNA to kill tumors may be due to the inhibition of phosphor-Akt. However, no significant change has occurred in the level of p7S6K in all treated cells compared to the control. Thus, it implies that erastin and/or SHIP2 siRNA did not affect mTOR.

Conclusion

The outcomes of this study implied that the SHIP2 silencing enhanced the cytotoxicity of erastin in the MDA-MB-231 cell line. Additionally, that combination also significantly reduced the level of phospho-Akt but not p70S6k. This means that mTOR is not part of the signaling pathway that causes cytotoxicity for erastin, SHIP2 silencing, or both of them together. Finally, the results indicate that the combination of erastin and SHIP2 siRNA gives a promising cytotoxic strategy for TNBC cells. However, further work is required to investigate the underlying pathway for this strategy.

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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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