Al-Rafidain J Med Sci. 2024;7(1S):S64-69.
DOI: https://doi.org/10.54133/ajms.v7i(1S).926.iccpmu2024

Proceeding of the 5th International Conference, College of Pharmacy, Mustansiriyah University, Baghdad, April 2024 (ICCPMU2024)

Research Article

Online ISSN (2789-3219)

Investigating the Effect of EX-527 as SIRT1 Inhibitor in Breast Cancer Cell Line

Areege Ibrahim Salih*, Bahir Abdul-Razzaq Mshimesh, Basma Talib Al-Sudani

Department of Pharmacology and Toxicology, College of Pharmacy, Mustansiriyah University, Baghdad, Iraq

Received: 28 April 2024; Revised: 10 June 2024; Accepted: 12 June 2024

Abstract

Background: Breast cancer is one of the most common malignant and metastatic tumors worldwide that cause death in women. Therefore, preventing the growth and metastasis of cancerous cells is essential for enhancing the prognosis and efficacy of treatment for breast cancer. Sirtuin 1 (SIRT1) is a nicotinamide-adenine dinucleotide (NAD+)-dependent deacetylase that has been linked to a number of biological processes, including genomic stability, cell cycle, cell survival and cancer metastasis. EX-527 is a selective and potent SIRT1 inhibitor. Recent studies have revealed that SIRT1 has an oncogenic role in breast cancer. ***Objective***: To evaluate the effect of EX-527 on the MCF-7 breast cancer cell line. ***Methods***: MCF-7 was cultured in complete DMEM and treated with and without EX-527. Cell viability of the breast cancer cell line was evaluated by MTT assay and apoptosis by Annexin V/PI staining. Migration and invasion of breast cancer cells were determined by wound healing and transwell invasion assays, respectively. ***Results***: Results revealed that EX-527 at a concentration 25.30 µM was associated with a significant anti-proliferative effect and induction of apoptosis (98.3%) in breast cancer cells. Treatment with EX-527 was also associated with significant suppression of migration and invasion of MCF-7. ***Conclusions***: The current investigation showed that inhibition of SIRT1 by EX-527 inhibits proliferation, migration, invasion and apoptosis of human breast cancer cells.

Keywords: Breast cancer, EX-527, MCF-7, SIRT1 inhibitor.

التحقيق في تأثير EX-527 كمثبط SIRT1 في خط خلايا سرطان الثدي

الخلاصة

الخلفية: سرطان الثدي هو واحد من الأورام الخبيثة والنقيلي الأكثر شيوعا في جميع أنحاء العالم التي تسبب الوفاة لدى النساء. إن منع نمو الخلايا السرطانية وانتشارها أمر ضروري لتعزيز تشخيص وفعالية علاج سرطان الثدي. Sirtuin 1 (SIRT1) هو ديسيتيلاز يعتمد على نيكوتيناميد-أدينين ثنائي النوكليوتيد + NADتم ربطه بعدد من العمليات البيولوجية ، بما في ذلك الاستقرار الجيني ودورة الخلية وبقاء الخلية وورم خبيث للسرطان. EX-527 هو مثبط SIRT1 انتقائي وقوي. كشفت الدراسات الحديثة أن SIRT1 له دور في سرطان الثدي. **الهدف**: تقييم تأثير EX-527 على خط خلايا سرطان الثدي MCF-7. **الطرق**: تم استزراع MCF-7 في DMEM الكامل ومعالجته مع وبدون EX-527وتم تقييم صلاحية الخلية لخط خلايا سرطان الثدي عن طريق مقايسة MTT وموت الخلايا المبرمج عن طريق تلطيخ Annexin V/PIتم تحديد هجرة وغزو خلايا سرطان الثدي عن طريق التئام الجروح ومقايسات غزو transwell، على التوالي. **النتائج:** كشفت النتائج أن EX-527 بتركيز 25.30 ميكرومول كان مرتبطا بتأثير كبير مضاد للتكاثر وتحريض موت الخلايا المبرمج (98.3٪) في خلايا سرطان الثدي. ارتبط العلاج ب EX-527 أيضا بقمع كبير للهجرة وغزو MCF-7. **الاستنتاجات**: أظهرت الدراسة التحقيق الحالية أن تثبيط SIRT1 بواسطة EX-527 يمنع انتشار خلايا سرطان الثدي البشرية وهجرتها وغزوها وموت الخلايا المبرمج.

* ***Corresponding author***: Areege I. Salih, Department of Pharmacology and Toxicology, College of Pharmacy, Mustansiriyah University, Baghdad, Iraq; Email: areej_ibrahim@uomustansiriyah.edu.iq

Article citation: Salih AI, Mshimesh BA, Al-Sudani BT. Investigating the Effect of EX-527 as SIRT1 Inhibitor in Breast Cancer Cell Line. *Al-Rafidain J Med Sci*. 2024;7(1S):S64-69. doi: https://doi.org/10.54133/ajms.v7i(1S).926.iccpmu2024

INTRODUCTION

Right now, breast cancer comes in first in terms of female cancer rates, and it comes in second in terms of female cancer mortality. According to the most recent US cancer statistics report from 2018, breast cancer comprises 30% of the total number of cases of cancer among women, far greater than all other cancer types [1]. Certain developing nations are experiencing a rise in the occurrence of breast cancer, and their death rate is greater than that of advanced nations [2,3]. The majority of breast cancer metastases spread to the liver, lungs, and bones. Metastatic cancer is thought to be incurable, despite recent increases in survival rates [4,5]. For this reason, preventing the growth and metastasis of cancerous cells is essential for enhancing the prognosis

and efficacy of treatment for breast cancer. Endocrine expression of genes, such as the expression of the progesterone receptor (PR), human epidermal growth receptor 2 (HER2), and estrogen receptor (ER), is utilized for classifying human breast tumors. The two most frequently employed human breast tumor cell types are MCF-7 and MDA-MB-231. Both the progesterone receptor (PR) and the estrogen receptor (ER) are present in MCF-7 cells, but MDA-MB-231 cells are considered to have triple-negative breast cancer (TNBC) [6–8]. Sirtuin 1 (SIRT1), a class III histone deacetylase, is the mammalian analogue of the yeast protein silenced information regulator 2 (Sir2). It is dependent on nicotinamide adenine dinucleotide (NAD^+) [9]. In addition to deacetylating histones like H3 and H4, SIRT1 is capable of interacting with numerous transcription factors and their transcriptional cofactors, such as p53, NF-kB, the FOXO family, c-Myc, and others., to control apoptosis, DNA repair, cell growth, the cell cycle, and metabolic processes. As a result, SIRT1 is essential for the initiation, development, and drug resistance of tumors [9,10]. The majority of investigations conducted recently have shown that SIRT1 serves as an oncogene in cancer [11]. On the other hand, according to certain findings, SIRT1 has a bilateral effect on tumors; depending on the circumstances and the context of the study, it can either serve as an oncogene or a tumor inhibitor [12]. Researchers demonstrated that elevated NAD^+ synthesis in breast cancer cells served as a basis for SIRT1 to stimulate the growth of breast cancer cells by altering several transcription factors or oncogenes. SIRT1 also specifically targets the tumor suppressor gene p53 [13]. Deacetylation of p53, which is an apoptosis mediator, by SIRT1 links this enzyme to tumor progression [14]. It has been demonstrated that suppression of SIRT1 causes activation of p53 by acetylating it in breast and prostate malignancies, and this leads to cell cycle arrest and apoptosis [15]. Consequently, it is still unknown how precisely SIRT1 contributes to breast cancer and how it works. The most often utilized SIRT1 selective inhibitor in biological research is selistat, also known as EX-527. Through a 200-fold specificity above SIRT2 and SIRT3 in cells, EX-527 inhibits SIRT1 with an IC50 of 38 nM. Furthermore, EX-527 administration markedly elevated the acetylation of p53 [16]. Additionally, EX-527 has shown antitumor effects in a number of tumors, such as endometrial and lung cancers [17,18]. However, whether EX-527 exhibits antitumor effects in breast cancer and the underlying mechanism require further investigation. This study aims to provide more evidence on the antineoplastic effects of the SIRT1 inhibitor (EX-527) in the MCF-7 breast cancer cell line.

METHODS

Study design

In this study, MCF-7 (human breast cell carcinoma) was used as a model for breast cancer. MCF-7 was purchased from ATCC, USA. In this study, the effect of the SIRT1 inhibitor (EX-527) as a pharmacological substance in the breast cancer cell line (MCF-7) was studied as a potential approach for anticancer therapy for breast cancer. The methodology encompassed examining the anti-proliferative impact of EX-527 on the MCF-7 cell line using MTT, following treatment with varying concentrations of the EX-527 compound and Nicotinamide, which served as a reference compound; assessing cell apoptosis through flow cytometry; and assessing cell metastasis through wound healing and transwell invasion tests.

Cell culture

MCF-7 was cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose, supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic (Capricorn Scientific, GmbH). Then, it was maintained in a fully humidified cell culture incubator at 37 °C with 5% CO_2. After seeding the cells in 75 cm3 culture flasks, the monolayers were washed with phosphate-buffered saline (PBS) (Capricorn Scientific, GmbH) and briefly incubated with 0.05% trypsin/EDTA (Capricorn Scientific, GmbH) to facilitate cell passage. Before being resuspended in culture medium for plating or counting, the trypsinized cells were separated by centrifugation in a centrifuge (Hettich, Germany).

Cell proliferation assay

The colorimetric MTT assay was used to detect cell proliferation [19]. Following trypsinization, MCF-7 suspension was prepared and seeded into a 96-well plate at a density of 5×10^3 cells/well for 72 hours of incubation. Drug therapy was introduced when the confluency reached 80–90%. On the day of treatment, serial dilutions of EX-527 and Nicotinamide were added to each of the 96-well plates at concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.125, and 1.56 µM. After treatment, the plates were incubated for 72 hours in an incubator (Hermle, Germany) at 37 °C with 5% CO_2. Following the incubation period, MTT (Bidepharm, Shanghai, China) was added and incubated for 4 hours. Then, the formazan crystals were dissolved in 100 µl of DMSO (Thomas Baker, India), and a multiplate reader was used to detect the absorbance at 560 nm (Promega, USA) [20,21].

Annexin V/PI apoptosis assay

Cell apoptosis was achieved using an annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) apoptosis detection kit (Elabscience). Briefly, on a six-well plate, MCF-7 cells were seeded at a density of 1×10^4 cells/2.0 mL/well. Cells were reconstituted with a new medium when confluency reached 70%, and they were subsequently subjected to EX-527 and Nicotinamide, followed by incubation for 48 hours.

Then, treated cells were detached with trypsin and washed twice with pre-cold 1X PBS. After that, the cell pellets were resuspended with 1X binding buffer (100 µl). Next, following the manufacturer's instructions, 2.5 µl of Annexin V-PE was added to the cell suspension and left for 15 minutes at room temperature in the dark. Staining cells were assessed by flow cytometry (BD FACSVerseTM), and the proportion of cells that were apoptotic was calculated utilizing FlowJo 10.2 software (TreeStar, Ashland, OR, USA) [22].

Wound healing assay

A test of wound healing was performed as described by Kozlova *et al*. [23]. MCF-7 cells were seeded in 2 ml of complete DMEM media for every well in 6-well plates at a density of 1×10^5 cells per well/2.0 ml and cultured in normal conditions. Every group possessed three triplicate wells, comprising MCF-7 (the blank control group), and the other two groups were treated with EX-527 and Nicotinamide. When the confluency reached 90%–100%, a 200-µl pipette tip was used to create an artificial wound on the outer layer of the cells and washed gently three times with 1 ml PBS. Subsequently, media supplemented with 2.5% fetal bovine serum (FBS) containing EX-527 and nicotine was added to the wells. Then, photographs from the inverted microscope were captured, and the width of the performed wounds was measured after 0, 24 and 72 hours.

Transwell invasion assay

In order to evaluate the correlation between SIRT1 gene expression and invasion ability, a cell invasion assay was performed using an extracellular matrix (ECM) 24-well cell invasion kit (8 µm pore-size, BD Biosciences, USA). In summary, according to the manufacturer's instructions, 300 µL of warm serum-free DMEM medium was used to suspend 1×10^5 MCF-7 cells. EX-527 and Nicotinamide were added directly to the cell suspension, and then the cells were plated to the inside of each insert. Media containing 10% fetal bovine serum was added to the lower well of the invasion plate. Followed by incubation for 48 h at 37 °C with 5% CO2. Subsequently, the cells on the interior of the insert were removed using a cotton swab, and the cells on the lower side were stained with crystal violet. The number of MCF-7 cells invaded in the lower chamber was photographed and counted using a microscope (Optika, Italy), and then the OD was measured at 560 nm by using a plate reader (Promega, USA) [24].

Statistical analysis

All statistical analysis of the data was performed using the Prism 8 software. A comparison between all groups within the same plate of MTT, apoptosis and migration assays were evaluated by one-way ANOVA with Tukey (Prism 8 software). The invasion assay was evaluated using Students' *t*-test. Statistically significant values are considered at $p< 0.05$.

RESULTS

The viability of breast cancer cell lines was evaluated by an MTT assay to determine the concentrations of EX-527 and nicotinamide that cause 50% inhibition in the viability of MCF7. As shown in Figure 1, the proliferation of MCF7 was significantly inhibited when treated with EX-527.

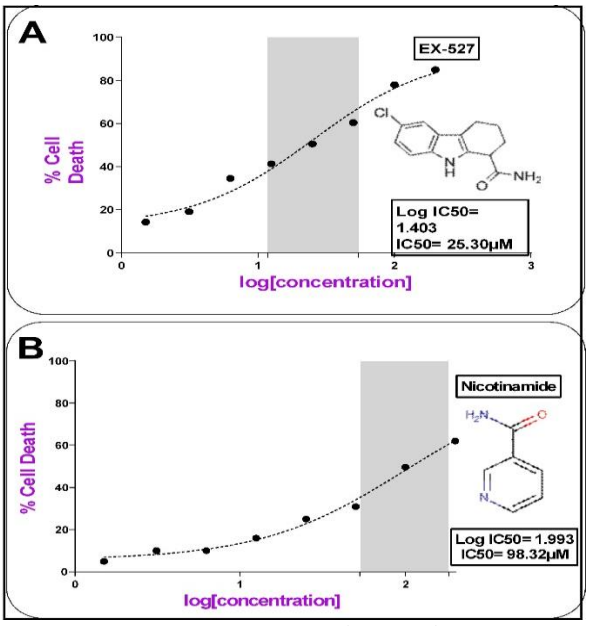

Figure 1: Dose-response curves of IC50 for EX-527 and Nicotinamide in MCF-7cells. (A) MCF-7 cells were treated for 72 h with 1.56, 3.12, 6.25, 12.5, 25, 50, 100 and 200 µM dose ranges of EX-527. (B) MCF-7 cells were treated for 72 h with 1.56, 3.12, 6.25, 12.5, 25, 50, 100 and 200 µM dose ranges of Nicotinamide. The dose response was plotted over log transformed concentrations. Results for triplicate data.

Nicotinamide showed lower potency than EX-527 in inhibiting proliferation of MCF7 with an IC50 <50 µM. In order to investigate whether the inhibition effect of EX-527 on the growth of MCF-7 could be associated with the induction of apoptosis, conjugated annexin V FITC/PI was used, which is able to distinguish between apoptotic cell death. Then, the result was measured by flow cytometry (Figure 2). The annexin V FITC/PI staining shows the cell undergoes early and late apoptosis. As shown in Figure 2A, the average of cells that undergo early apoptosis treated with EX-527 was 23.5%, compared to Nicotinamide (53.6%). The same pattern has also been seen in the population with late apoptosis. The average of the cells that undergo late apoptosis treated with EX-527 was 74%, compared with nicotine (4.2%). As shown in Figure 3, the average wound size of MCF-7 treated with EX-527 was 38.7, 29.2, and 23.26 mm, compared with control (38, 14.7, and 4.3 mm) and with Nicotinamide-treated cells (38.36, 16.7, and 7.2 mm) at 0, 24, and 48 h, respectively.

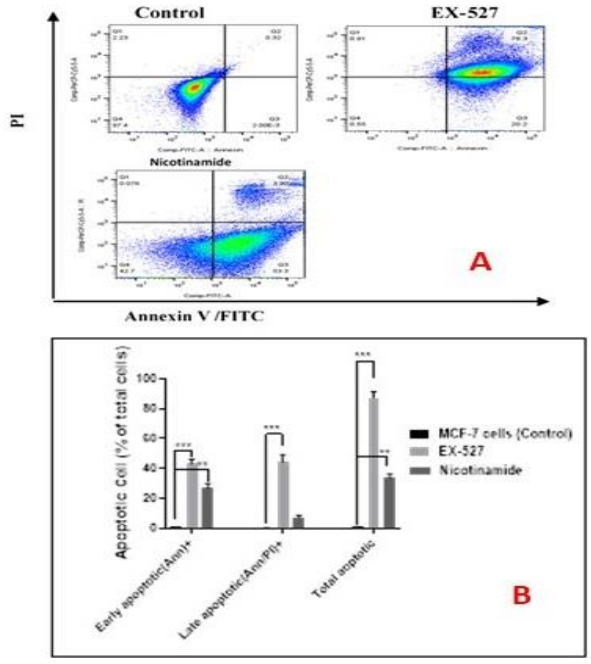

Figure 2. Flow cytometry analysis of MCF-7 with annexin V /PI after 48 h of treatment with EX-527 and Nicotinamide. (A) Represent flow cytometry analysis of apoptotic cells of MCF-7 stained with Annexin V and propidium iodide (PI) after treatment with EX-527 (25.30 µM) and Nicotinamide (98.32 µM) for 48 h. (B) Graphical representation of apoptotic cell undergo early and late apoptosis. *** Significant variation in comparison with blank control group (without treatment). FITC, fluorescein isothiocyanate; PI, propidium iodide.

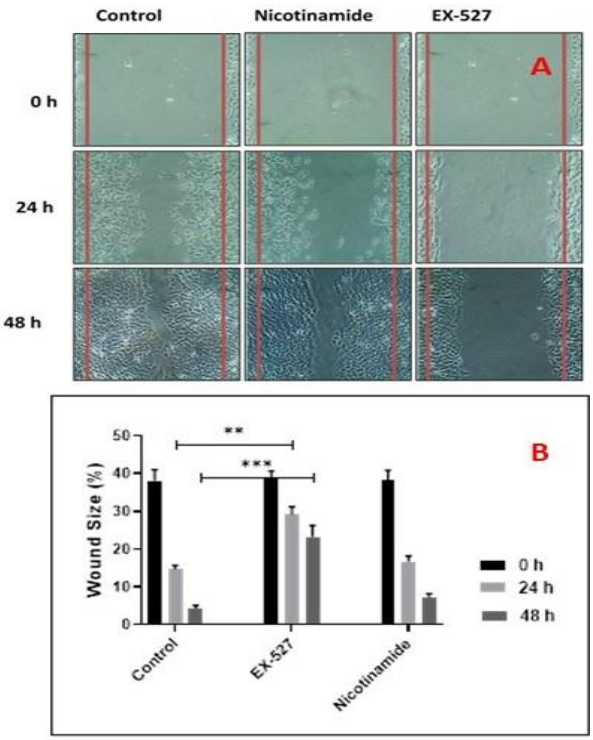

Figure 3: The inhibitory effect of EX-527 on migration of MCF-7. (A) Images of migrated MCF-7 that were treated with EX-527 (25.30 µM) and Nicotinamide (98.32 µM). (B) Ratio of wound size among different groups. There was a significant difference between the EX-527 treated group and the Nicotinamide treated group in terms of the speed of wound closure when compared with the control group. The graph displays the average ± standard deviation for a minimum of three separate tests. Scale bar equals to 100 µm.

These data indicate that EX-527 significantly inhibits the migration of MCF-7 ($p < 0.05$) in contrast to nicotine. Results of the transwell invasion assay revealed that the number of cells of MCF-7 (Figure 4) that had invaded was effectively decreased when treated with EX-527 compared to the control. These data indicated that EX-527 dramatically decreased cell invasion in MCF-7 cells.

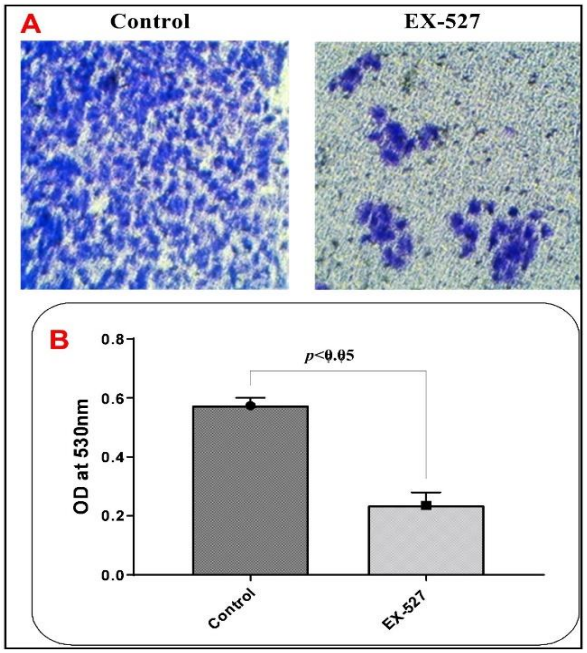

Figure 4: Transwell invasion assay assessed cell invasion of MCF-7 cells. (**A**) Images of invaded MCF-7 cells that were treated with EX-527 (25.30 µM) in the presence of control group; (**B**) Statistical analysis of invaded MCF-7 was performed by measuring the OD at 560 nm. Values displayed as the means ± S.D.

DISCUSSION

Breast cancer is one of the most prevalent tumors among women around the world. Besides, in the early stages, a hematogenous spread develops easily, which is a severe threat to the health of women. Presently, there are a number of treatments for breast cancer, such as surgery, chemotherapy, radiotherapy, and targeted biotherapy, that have the potential to eliminate cancer cells and cause injuries [25]. Yet certain breast cancers continue to resist standard treatment; this could be because they activate certain signaling pathways such as pro-survival, pro-proliferative, and anti-apoptotic factors. Together, these variables lead to medication resistance, which modifies the clinical susceptibility to monotherapy. In addition to the serious issue of medication resistance, current medications have adverse effects, which are also another challenging issue in cancer treatment [26,27]. As a result, it is urgent to develop new therapeutic targets to effectively treat breast cancer. SIRT1 overexpression, a mammalian NAD^+ -dependent deacetylase, is frequently observed in a variety of malignancies and is typically linked to poor prognosis and insufficient medical results [28]. A previous study demonstrated that there was a correlation between

SIRT1 upregulation and lymph node metastasis, as well as a decline in the rate of 5-year survival in breast tumors, revealing that SIRT1 has an oncogenic role in breast cancer [29]. There has also been evidence that elevated SIRT1 expression promotes the development of breast cancer through direct interaction with Akt activity [30]. Therefore, SIRT1 has been recommended as a specific target for eliminating tumor cells and decreasing their survival. The present study showed that treatment with the selective SIRT1 inhibitor EX-527 compound at a concentration 25.30 µM demonstrated a significant anti-proliferative effect against MCF-7, in contrast with Nicotinamide (Figure 1). The latter showed no significant effect on the viability of MCF-7 cells at concentrations less than 50 µM, while when concentrations > 50 µM were used, the viability began to decline with time. Consistent with these findings, it was shown that treatment of MCF-7 cells with Cambinol, a compound with SIRT1 inhibitory effects, either alone or in combination with Paclitaxel, inhibits the proliferation of breast cancer cells [31]. Moreover, the inhibitory effects of micro-22 and micro-590-3p on SIRT1 have been proven to effectively inhibit the proliferation and development of breast cancer cells [32,33]. It has been observed that inhibition of SIRT1 gene expression causes growth arrest and triggers apoptosis in human epithelial malignancies [34]. Thus, as a consequence of SIRT1 suppression and to further understand the mechanism of inhibition of cell proliferation of MCF-7 cells by EX-527, cell death was quantified in terms of the percentage of apoptotic cells by flow cytometry. This study revealed that the percent of apoptotic cells in MCF-7 treated with EX-527 was 98.3%, which is higher than that of the control group and the nicotine-treated group (57.8%). Consistent with current research, a recent study showed that targeting SIRT1 can effectively decrease cell survival and induce apoptosis in breast cancer cells [35,36]. On the other hand, another study showed that the EX-527 compound, at a concentration 50 µM, only induced cell cycle arrest with no detectable MCF-7 cell death [37]. Cancer metastasis and migratory ability are the most common reasons for mortality in patients suffering from breast cancer [38]. Several investigations have shown that elevated SIRT1 expression is strongly linked to metastasis, migration, and malignant cell invasion [39]. Therefore, to evaluate whether treatment with EX-527 could suppress the migration and invasion of MCF-7, a wound healing and transwell invasion assay was performed. As previously demonstrated, the wound healing assay was performed by making a mechanical wound in the monolayer of cultured cells. The results demonstrated that the cell migration of MCF-7 after treatment with EX-527 was significantly slower than that of the control group and the nicotine-treated group. Consistent with these data, a previous study revealed that the EX-527 compound inhibited cell growth and migration of HeLa cells by suppressing the activity of the HSF1 protein [40]. A transwell invasion assay showed that inhibition of SIRT1 by EX-527 significantly inhibited the invasion of MCF-7. This is in tune with the previous study that reported that miR-34a inhibits proliferation, migration, and invasion of breast cancer cells by targeting several genes, including E2F3, CD44, and SIRT1 [41].

Conclusion

The compound EX-527 inhibited SIRT1 significantly, reducing proliferation, migration, invasion, and induced apoptosis in MCF-7 cells. Therefore, it may represent a promising therapeutic option for breast cancer.

ACKNOWLEDGMENT

The authors thank the College of Pharmacy, Mustansiriyah University for supporting this project.

Conflict of interests

No conflict of interests was declared by the authors.

Funding source

The authors did not receive any source of fund.

Data sharing statement

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

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