Pharmacological Reports 69 (2017) 1059–1066

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Original article

4-(Methylthio)butyl isothiocyanate inhibits the proliferation of breast cancer cells with different receptor status

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ARTICLE INFO

Article history:
Received 7 November 2016
Received in revised form 7 March 2017
Accepted 19 April 2017
Available online 23 April 2017

Keywords:
Erucin
Isothiocyanates
Breast cancer
Apoptosis
Necrosis

ABSTRACT

Background: Epidemiological studies indicate that the consumption of Brassicaceae plants, a rich source of biologically active isothiocyanates (ITCs), may effectively reduce cancer risk. In the current study, we evaluated the anticancer potential of 4-(methylthio)butyl ITC (erucin, ERN) against three phenotypically different breast cancer cell lines: MDA-MB-231, SKBR-3 and T47D.
Methods: The effect of ERN on the viability of breast cancer cells was evaluated using sulforhodamine B and clonogenic assays, and acridine orange/ethidium bromide staining. Cell cycle was investigated using flow cytometry. The status of signaling molecules was examined by western blot analysis.
Results: ERN decreased the viability of all tested cancer cell lines in a concentration-dependent manner; this effect was much weaker in normal breast cells (MCF-10A). ERN induced cell cycle arrest in the G2/M phase, down-regulated the phosphorylation of S6 ribosomal protein in all tested breast cancer cell lines, and reduced HER2 receptor levels in SKBR-3 cells. A 24-h treatment with lower concentrations of ERN (5–20 μM) induced apoptosis; higher ERN concentrations (40 μM) induced necrosis. The latter also irreversibly inhibited the proliferative potential of cancer cells.
Conclusion: ERN effectively inhibits proliferation of breast cancer cells irrespectively of their receptor status.

Introduction

Chemopreventive and anticancer properties of dietary agents have been widely investigated in recent years. One of the most extensively studied groups of plants rich in anticarcinogenic phytochemicals is the Brassicaceae family. It has been shown that the consumption of Brassicaceae family vegetables, such as broccoli, cauliflower, cabbage, or rocket salad, is associated with a lower risk of developing cancer [1,2]. Anticancer potential of these plants is associated with the activity of isothiocyanates (ITCs), produced during the hydrolysis of glucosinolates [2]. Both *in vitro* and *in vivo* studies revealed that the protective mechanism of ITCs involves, *inter alia*, inhibition of phase I carcinogen-activating enzymes, induction of phase II detoxification enzymes (such as quinone reductase and glutathione *S*-transferase), disruption of tubulin polymerization, induction of cell cycle arrest, and activation of apoptosis in cancer cells [3,4]. Moreover, it has been shown that dietary ITCs are well absorbed and have good bioavailability, rendering them promising candidates for anticancer therapies [5–7].

Erucin, 4-(methylthio)butyl ITC (ERN), occurs abundantly in the rocket salad (*Eruca sativa* Mill.) and is generated from glucoerucin by myrosinase, an enzyme released during chopping, mastication, or chewing of vegetables [8,9]. *In vivo*, ERN is interconverted with sulforaphane (SFN), one of the most widely studied ITCs [10]. Increasing evidence supports the anticancer effect of ERN on different cancer cell lines, including colon, lung, hepatoma, ovarian, breast, and leukemia cells [8,11–15], and in tumor xenograft mouse models [16,17]. Reports of ERN activity against different molecular subtypes of breast cancer are limited, however. Azarenko et al. [14] showed that ERN disturbs microtubule dynamics in MCF-7 breast cancer cells, leading to cell cycle arrest and the induction of apoptosis. A study by Li et al. [16] indicated that ERN induces mitochondrial fission and apoptosis in two human breast cancer cell lines (MCF-7 and MDA-MB-231) *via* mitochondrial translocation of cofilin.

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http://dx.doi.org/10.1016/j.pharep.2017.04.014
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In the current study, we investigated the effect of ERN on the viability and proliferation of three established breast cancer cell lines with different receptor status: SKBR-3 cells, overproducing the human epidermal growth factor receptor 2 (HER2); a T47D cell line, characterized by high level of estrogen (ER) and progesterone receptors (PR); and a triple-negative MDA-MB-231 cell line [18]. Our findings indicate that ERN effectively impairs the viability of cells representing different molecular subtypes of breast cancer, while exerting a much weaker effect on MCF-10A normal breast cells. Mechanisms underlying the antiproliferative action of ERN include cell cycle inhibition and induction of cell death.

Materials and methods

Reagents

ERN (purity ≥99%) was obtained from LKT Laboratories (St. Paul, MN, USA); 10 mM ERN stock solution was prepared in dimethyl sulfoxide (DMSO). RPMI 1640, MEM, and DMEM/F12 media, fetal bovine serum, and human insulin were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Horse serum, epidermal growth factor (EGF), hydrocortisone, penicillin/streptomycin mixture, DMSO, sulforhodamine B (SRB), thiazolyl blue tetrazolium bromide, acridine orange, ethidium bromide, propidium iodide, crystal violet were from Sigma (St. Louis, MO, USA). The antibodies against phospho-S6 (Ser 235) and PARP-1 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against HER2, phospho-HER2 (Tyr1221/1222), and S6 were from Cell Signaling (Danvers, MA, USA). Anti-β-actin, anti-mouse, and anti-rabbit antibodies conjugated with horse radish peroxidase were from Sigma.

Cell culture

SKBR-3 and T47D cells were cultured in RPMI 1640 medium with 10% fetal bovine serum. MDA-MB-231 cells were maintained in MEM medium supplemented with 10% fetal bovine serum, 0.1 mM essential amino acids and 1 mM sodium pyruvate. MCF-10A cells were cultured in DMEM/F12 medium with 5% horse serum, 20 ng/ml EGF, 10 μg/ml insulin, 0.5 mg/ml hydrocortisone. Each culture medium contained 1% penicillin/streptomycin mixture. The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Cell viability assay

For the cell growth assay, 4×10^3 cells per well were seeded into 96-well plate and incubated for 24 h. Next, the medium was removed and fresh medium, containing 5, 10, 20, 40, or 50 μM ERN, or corresponding quantities of DMSO (control), was added and the incubation continued for 24 h. The cells were fixed with 20% trichloroacetic acid (TCA), washed with dH_2O, and stained with 50 μl of 0.4% SRB. The wells were washed with TCA and dried. After the addition of 10 mM Tris base (pH 10.5, 150 μl/well), the absorbance was measured at 570 nm, with a reference filter of 660 nm, in Victor3 microplate reader (PerkinElmer Life and Analytical Sciences, Boston, MA). Each ERN concentration was tested in triplicate and the experiment was repeated three times.

Clonogenic assay

Cells (2×10^6) were plated in 10-cm plates. After 24 h, the medium was removed and fresh ERN-containing medium (5, 10, 20, 40, or 50 μM) was added. Following 24 h of ERN treatment, the

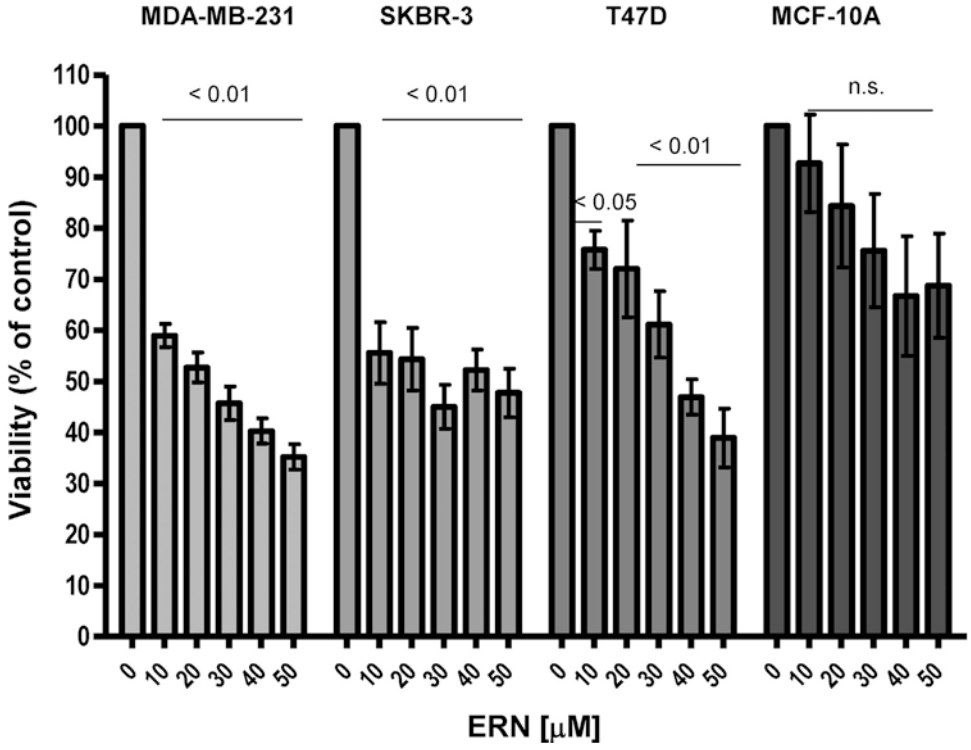

Fig. 1. ERN decreases the viability of breast cancer cells with different receptor status in a concentration-dependent manner, and is less potent against normal breast cells. MDA-MB-231, SKBR-3, T47D, and MCF-10A cells were treated with DMSO (0) or different concentrations of ERN for 24 h. Cell viability was evaluated by SRB assay as described in Materials and methods. Each point is a mean (±SE) of at least three experiments performed in triplicate. Data were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test (*p*-value *vs.* control cells); ns, not significant.

cells from each plate were trypsynized, counted, and plated (8×10^2) into two new plates in ERN-free medium. After 2 weeks, the cells were fixed with glutaraldehyde (6.0%, v/v) and stained with 0.5% crystal violet solution. Colonies consisting of at least 50 cells were counted.

Cell cycle analysis

The effect of ERN on cell cycle distribution was determined by flow cytometry. Briefly, cells (5×10^5) were seeded into 6-cm plates; 24 h later, ERN (20 μM) or DMSO (the control) was added for 8, 16, or 24 h. The cells were treated in triplicate. After the treatment, floating and adherent cells were collected, washed with PBS and fixed with 70% ethanol. The cells were treated with RNase A and propidium iodide at final concentrations of 80 and 50 μg/ml, respectively, for 30 min at room temperature, and analyzed using a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Acridine orange/ethidium bromide staining

Cells (1×10^5) were seeded on microscope slides inserted into a 12-well plate. After a 24-h treatment with ERN, the cells were

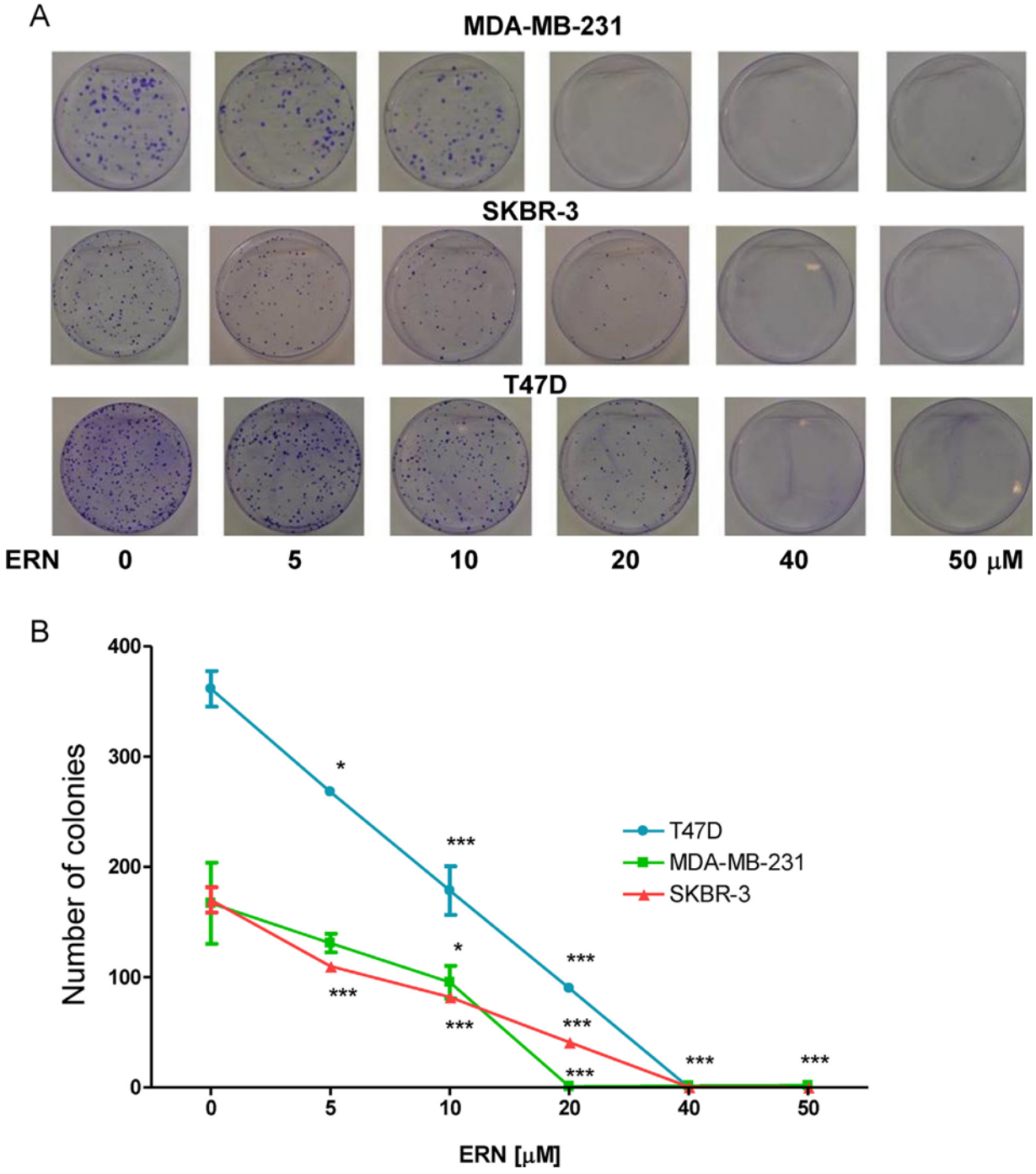

Fig. 2. ERN inhibits clonogenicity of breast cancer cells. Cells were exposed to 5, 10, 20, 40, or 50 μM ERN for 24 h. Following the treatment, the cells were re-plated at lower confluency and allowed to grow for 2 weeks in a drug-free medium. **A.** Representative images from two replicates. **B.** Colonies arising from cells that retained the proliferative potential were counted and the results are shown as a mean ± SE of duplicates. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparison test, and $p < 0.05$ (*) or $p < 0.01$ (***) *vs.* control.

incubated with a mixture of acridine orange and ethidium bromide (final concentration of each, 4 μg/ml) in PBS (pH 7.4) for 5 min, and visualized using a Nikon Eclipse E800 fluorescence microscope.

Western blotting

Cells (2.5×10^6) were seeded into 10-cm plates and allowed to attach for 24 h. Next, ERN in was added to final concentrations: 5, 10, 20, or 40 μM. After 24 h, the cells were collected and lysed in a buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris (pH 7.5), 0.5 mM EDTA, and a phosphatase and protease inhibitor cocktail (Roche Diagnostics, Warsaw, Poland). Cell lysates were obtained by centrifugation (4 °C, 15,493 × *g*, 25 min). Protein concentration was determined in each sample using the Bradford method [19]. The proteins were separated by SDS-PAGE and electrotransferred onto PVDF membranes. The membranes were blocked with 5% nonfat dry milk in TBS-Tween (1%) and incubated with the primary antibodies overnight at 4 °C. Next, the membranes were probed with the appropriate peroxidase-conjugated secondary antibodies and visualized using the Pierce ECL western blotting substrate (Thermo Scientific). Each protein was detected two or three times in independently prepared lysates. Densitometry analysis was carried out using Quantity One software.

Statistical analysis

Data were analyzed using GraphPad Prism or STATISTICA software. Student's *t*-test or one-way ANOVA followed by Dunnett's multiple comparison test was used to determine statistical significance of difference in the measured variables between tested groups.

Results

ERN decreases the viability of different breast cancer cell lines but not of normal breast cells

To investigate whether the anticancer potential of ERN depends on the phenotype of cells, we used a panel of breast cancer cell lines differing in their growth factor (HER2) and hormone (ER and PR) receptor status, i.e., MDA-MB-231 ($HER2^-$, ER^-, PR^-), T47D ($HER2^-$, ER^+, PR^+), and SKBR-3 ($HER2^+$, ER^-, PR^-), as well as MCF-10A normal breast cells. The cells were treated with increasing concentrations of ERN for 24 h and their viability was assessed by SRB assay. As shown in Fig. 1, cellular survival was reduced in all investigated breast cancer cell lines in an ERN dose-dependent manner; ER-positive T47D cells were least sensitive to ERN. Importantly, the compound was less potent against normal breast cells (Fig. 1).

ERN irreversibly inhibits the proliferative potential of breast cancer cells

Disease recurrence remains one of the major challenges in cancer therapy and the ability of an anticancer agent to induce cell reproductive death might prevent cancer relapse. To determine whether ERN treatment led to reproductive death of breast cancer cells, we performed a clonogenic assay. The cells were treated with different concentrations of ERN (5, 10, 20, 40 or 50 μM) for 24 h and then recovered in a drug-free medium for 2 weeks. We found that ERN treatment substantially reduced the clonogenic potential of all treated breast cancer cell lines (Fig. 2). MDA-MB-231 was the most sensitive cell line and all cells lost the ability to proliferate after a 24-h treatment with 20 μM ERN. At least 40 μM ERN was required to irreversibly suppress the proliferation of SKBR-3 and T47D cells (Fig. 2).

ERN affects cell cycle progression and induces cell death

To elucidate which processes are modulated by ERN in breast cancer cells and account for their decreased survival, we analyzed cycle progression, induction of apoptosis and necrosis in these cells. First, SKBR-3, T47D and MDA-MB-231 cells were treated with 20 μM ERN for different time periods. After staining with propidium iodide, cell cycle was analyzed using flow cytometry. The results indicated that an 8-h ERN treatment resulted in an increase in the G0/G1 cell fraction (MDA-MB-231 and T47D) or S phase cells (SKBR-3). After a 16-h treatment, we observed significant ($p < 0.05$) increase in fraction of cells arrested in G2/M stage, which dropped after a longer, 24-h, exposure to ERN (Table 1). This observation may have indicated that longer ERN exposure leads to cell death. To verify this hypothesis, we performed acridine orange/ethidium bromide staining of cells treated with ERN for 24 h. Representative images are shown in Fig. 3A. In this assay, the viable cells have a uniformly green nucleus, early apoptotic cells have a bright green nucleus with condensed chromatin, late apoptotic cells have a condensed and orange-stained chromatin; and the nucleus of necrotic cells is structurally normal but stains orange [20]. Our results strongly indicated that up to 20 μM ERN increases the apoptotic fraction of cells in all the tested cell lines in a concentration-dependent manner. Interestingly, in cells treated with a higher concentration of ERN (40 μM), the apoptotic subpopulation of cells was smaller than in case of samples treated with 20 μM ERN, and at the same time the necrotic fraction of cells was highest (Fig. 3B).

To confirm these observations, we examined cellular levels of an apoptotic marker, i.e., the caspase-specific cleavage of PARP-1 (Fig. 4). This protein was detected in cells treated with low concentrations of ERN (5 μM, in SKBR-3 and T47D cells; and 10 μM, in MDA-MB-231); however, the most apparent cleavage of PARP-1 was observed in cells treated with 20 μM ERN. Higher concentration of ERN (40 μM) did not potentiate the cleavage of PARP-1 (Fig. 4), which was consistent with the results of acridine

Table 1
The effect of 20 μM ERN on cell cycle distribution of breast cancer cells. Data are a mean ± SE; *indicates a significant difference compared with the respective control ($p < 0.05$) by Student's *t*-test.

Time [h]		G0/G1	S	G2/M
MD-MB-231				
8	Control	48 ± 0.4	16 ± 0.4	33 ± 0.7
	ERN	55 ± 0.6*	14 ± 0.2*	27 ± 0.8*
16	Control	59 ± 1	14 ± 0.3	25 ± 0.6
	ERN	41 ± 0.3*	16 ± 0.4*	40 ± 0.7*
24	Control	50 ± 2.5	16 ± 0.4	31.3 ± 2.4
	ERN	53 ± 1	14 ± 0.7	29 ± 0.2
SKBR-3				
8	Control	69 ± 0.5	8 ± 0.3	23 ± 0.6
	ERN	67 ± 1*	16 ± 0.2*	16 ± 0.8*
16	Control	71 ± 0.2	11 ± 0.6	18 ± 0.6
	ERN	61 ± 1.6*	10 ± 0.2	28 ± 1.6*
24	Control	74 ± 0.5	7 ± 0.1	18 ± 0.4
	ERN	64 ± 0.8*	12 ± 0.2*	21 ± 1
T47D				
8	Control	47 ± 0.2	16 ± 0.5	35 ± 0.6
	ERN	51 ± 3.3	17 ± 0.2	31 ± 2.5
16	Control	52 ± 1.6	16 ± 0.2	32 ± 1.3
	ERN	41 ± 1*	12 ± 0.2*	46 ± 1*
24	Control	53 ± 1.2	16 ± 0.4	32 ± 1
	ERN	53 ± 1.6	19 ± 1.6	28 ± 3.1

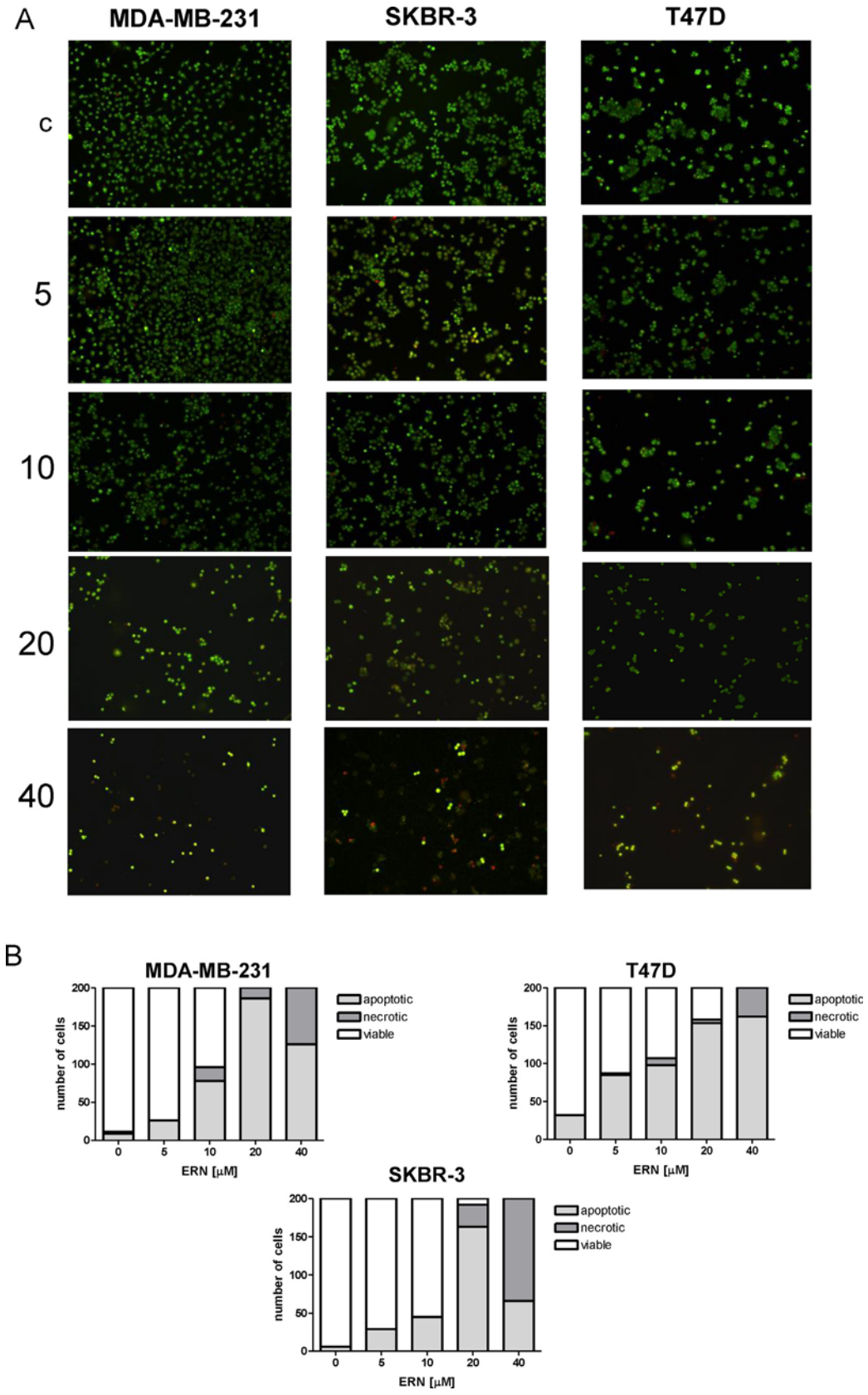

Fig. 3. ERN treatment induces apoptosis or necrosis depending on ERN concentration. MDA-MB-231, SKBR-3, and T47D cells were treated with DMSO (0) or different concentrations of ERN for 24 h, and stained with acridine orange/ethidium bromide. **A.** Representative images of cells observed under fluorescence microscope. Magnification, 200×. **B.** Numbers of viable, apoptotic, and necrotic cells in a population of 200 randomly selected cells.

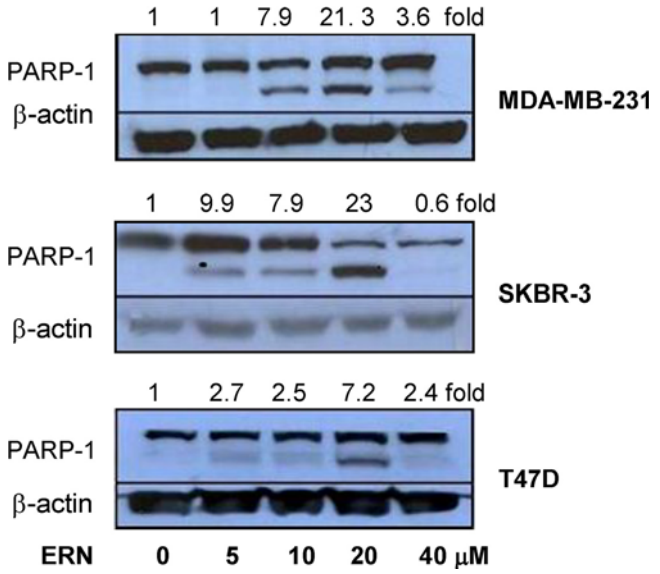

Fig. 4. The effect of increasing ERN concentrations on the induction of apoptosis. MDA-MB-231, SKBR-3, and T47D cells were treated with DMSO (0) or different concentrations of ERN for 24 h. Cleaved PARP-1 was evaluated by immunoblotting with specific antibodies. The blots were stripped and re-probed with anti-β-actin antibody to ensure equal protein loading. Densitometric data of cleaved PARP-1 band intensity (the faster migrating band) after correction for loading control are given above the respective immunoreactive bands. Similar results were observed in at least two independent experiments.

orange/ethidium bromide staining and suggested that this concentration of ERN induces cell death other than *via* apoptosis.

ERN decreases the levels of prosurvival proteins

The proliferation of cells depends on the activity of hormone and growth factor receptors that activate downstream signal transduction pathways, such as mitogen-activated protein kinase (MAPK) and the phosphoinositide-3-kinase (PI3 K)/Akt/mTOR pathways. The common substrate of both pathways is the ribosomal S6 protein, which plays a crucial role in the regulation of translation. We analyzed the degree of S6 phosphorylation (p-S6) at Ser-235, a position recognized by RSK and S6K, in ERN-treated breast cancer cells. As shown in Fig. 5A, 5–20 μM ERN concentrations led to decreased p-S6 levels in a dose-dependent manner. Interestingly, 40 μM ERN increased p-S6 levels, albeit in SKBR-3 and T47D cells it did not reach the levels observed in control cells (Fig. 5A).

We also determined the effect of ERN on HER2 receptor status in the SKBR-3 cell line, where this receptor is overproduced. Our results indicated that the total amount of the receptor gradually decreased with increasing ERN concentrations (5–40 μM), while a drop in phosphorylation of HER-2 (modification crucial for its activity) was seen in cells treated with 10–40 μM ERN (Fig. 4B).

Discussion

Recently, biologically active plant-derived agents, such as ITCs, have generated a great deal of interests due to their chemopreventive and anticancer activity [3,4,21]. Numerous reports show that aliphatic ITCs, particularly SFN, may inhibit cancer development at all stages of cancerogenesis [22–27]. ERN is an aliphatic ITC closely related to SFN. Due to the molecular similarity to SFN and interconversion of the two ITCs *in vivo*, ERN has also become an interesting study subject in the context of anticancer therapies. *In vitro* studies using different cancer cell lines and *in vivo* tumor xenograft mice models all confirm antiproliferative activity of ERN

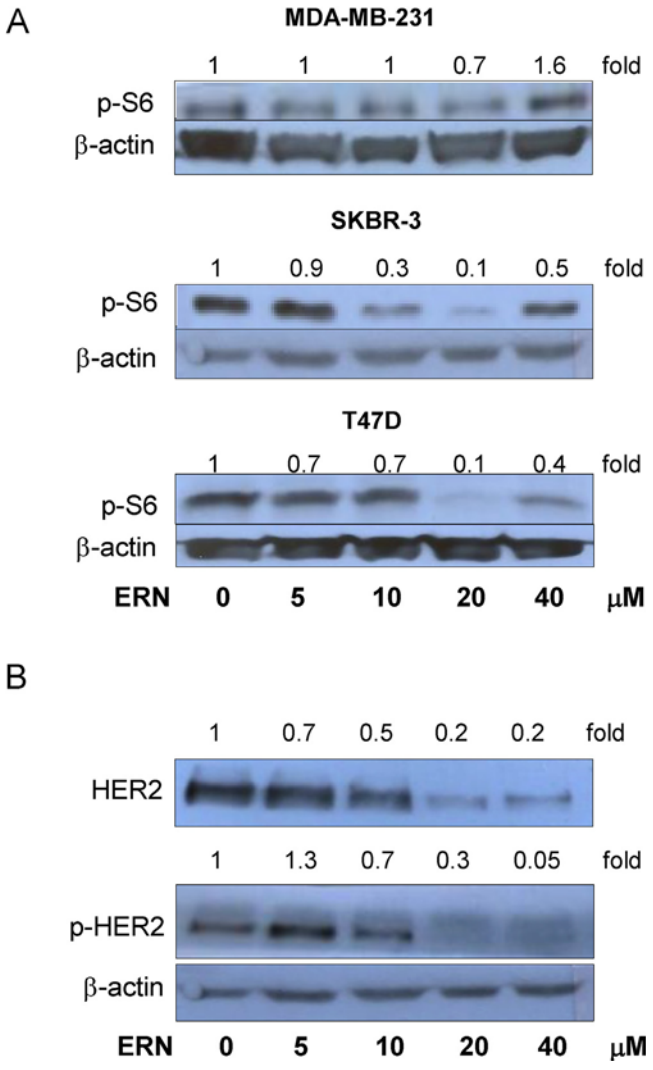

Fig. 5. ERN decreases the phosphorylation of S6 ribosomal protein in MDA-MB-231, SKBR-3, and T46D cells (**A**) and reduces the levels of phosphorylated and total HER2 receptor in SKBR-2 cell line (**B**). Representative immunoblots for p-S6 (Ser-235), p-HER2 (Tyr1221/1222), and total HER2 of lysates from cells treated for 24 h with 5, 10, 20, or 40 μM ERN. The blots were stripped and re-probed with anti-β-actin antibody to ensure equal protein loading. Densitometric data after correction for loading control are given above the respective immunoreactive bands. Similar results were observed in at least two independent experiments.

[9,11,14–17,28–30]. Nevertheless, only three reports showing anticancer activity of ERN against breast cancer cells have been published [14,16,31]. The effect of ERN on cell viability, cell cycle, apoptotic and necrotic cell death, or intracellular signaling in phenotypically divergent breast cancer cells has not yet been investigated.

In the current study, we show that ERN effectively decreased the viability of breast cancer cells with different HER2, ER, and PR receptor status. Interestingly, triple-negative MDA-MB-231 cells, considered to be highly aggressive [18,32], were most sensitive to ERN. On the other hand, MCF-10A, normal breast cells, were more resistant to ERN than cancer cell lines analyzed in this study. This selective activity is consistent with previous reports. E.g., Abbaoui et al. [17] showed that human bladder cancer cell lines RT4, J82, and UMUC3 are more sensitive to ERN than normal human bladder urothelial cells. Moreover, *in vivo* experiments indicated no side effects in mice that had been administered therapeutic doses of ERN. Fimognari et al. [33] showed that ERN selectively affects cell cycle progression and induces apoptosis in human leukemia cells

but not in non-transformed lymphocytes T. These observations are relevant to future studies in human.

One of the most serious limitations of anticancer therapies is disease recurrence. Cancer cells, that are resistant to therapy and survive the treatment, may resume proliferation and this can lead to the progression of disease. Hence, we asked whether the antiproliferative effect of ERN was irreversible. Based on the presented results, we conclude that ERN substantially reduces clonogenic potential of all tested cell lines. The MDA-MB-231 cell line was most sensitive to ERN: cells completely lost their ability to proliferate after a 24-h exposure to 20 μM ERN. Higher concentration of ERN (40 μM) was required to irreversibly suppress the clonogenic potential of SKBR-3 and T47D cells.

The antiproliferative effect of ERN against breast cancer cells stems from cell cycle arrest and cell death induction. Short-term treatment (16 h) mainly resulted in cell cycle perturbations, with G2/M arrest in each of the investigated cell lines. Longer, 24-h, ERN treatment induced apoptosis in a dose-dependent manner, with 20 μM concentration being the most effective. Furthermore, higher concentrations of ERN (40 μM) induced necrotic, not apoptotic, death in the treated cells. Cell cycle arrest at the G2/M phase and the induction of apoptosis seem to constitute an universal ERN activity against different cancer cell types. E.g., Jakubikova et al. [12] showed that SFN and ERN increase a G2/M-arrested cell subpopulation, and induce apoptosis in human myeloid leukemia cells (HL60) and in multidrug resistant HL60/ADR cells. Moreover, ERN appears to be more efficient than SFN. Other data suggest the occurrence of apoptotic events in HepG2 human hepatoma cells after a 6-h exposure to 10 μM ERN. The induction of apoptosis was accompanied by a time-dependent cell cycle arrest at the G2/M phase [34]. The molecular mechanism underpinning this process has not yet been completely delineated, however, it may be connected to a suppression of microtubule dynamics by ERN and disturbances in microtubule-dependent cell functions, such as mitosis [14]. Similarly, other *in vitro* experiments indicated that cell cycle arrest may be associated with a down-regulation of Cdc25C and disruption of mitotic spindles in cells treated with a broccoli sprout extract [35]. Melchini et al. [8] suggested that the activity of ERN against A549 human lung adenocarcinoma cells might be associated with a markedly enhanced p53 and p21 protein expression. Although cell lines used in the current study harbor missense mutations in the gene coding for p53, it cannot be excluded that ERN elevates the level of p21 cell cycle inhibitor independently of p53 status.

Overproduction of ER or HER2 may lead to an over-activation of prosurvival signal transduction pathways, such as PI3K/Akt/mTOR/S6K and MAPK. Alterations in intracellular signaling of these pathways result in enhanced cell proliferation, angiogenesis, migration, and adhesion, as well as inhibition of apoptosis. Cooperation of both pathways has been shown to contribute to glioblastoma formation by stimulating the recruitment of specific mRNAs to ribosomes [36]. Ribosomal protein S6, a component of the 40S ribosomal subunit, is phosphorylated upon mitogenic stimulation of cells and this correlates with enhanced protein synthesis in dividing cells. For instance, phosphorylation of S6 at Ser-235/236 was found to regulate the affinity of S6 for the 7-methylguanosine cap complex during translation [37]. Both PI3 K/Akt/mTOR/S6K and Ras/Raf/MEK/ERK/RSK pathways control the phosphorylation of S6. In the current study, we showed that phosphorylation of ribosomal S6 protein is reduced due to the activity of ERN. Interestingly, in cells treated with 40 μM ERN, the levels of p-S6 were higher than in cells treated with lower concentrations of ERN, suggesting weaker inhibition of translation. Anabolic processes, especially translation, require energy, and therefore, ATP depletion under stress conditions might contribute to necrosis [38]. This may explain the mechanism of the induction of necrosis by 40 μM ERN.

In the current study, we also showed that ERN down-regulates HER2 protein levels and phosphorylation of this receptor in the SKBR-3 cell line. This is consistent with previous studies of bladder cancer cells [17]. The comparable activity of ERN in all tested breast cancer cell lines differing in receptor status indicates that this compound, similarly to SFN, acts at different levels of prosurvival pathways, not only at the receptor level [39].

Our data emphasize the anticancer activity of ERN. Further investigations focusing on ERN as a potential agent against different molecular subtypes of breast cancer are warranted.

Conflict of interest

There are no conflicts of interest.

Acknowledgements

We are grateful to Dr. Anna Piotrowska (Medical University of Gdańsk) for technical assistance with flow cytometer. This study was financially supported by the National Science Centre (Poland), project no. N N301 601740.

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