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Sulforaphene, an isothiocyanate present in radish plants, inhibits proliferation of human breast cancer cells

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ABSTRACT

Background: Isothiocyanates derived from the *Brassicaceae* plants possess chemopreventive and anticancer activities. One of them is sulforaphene (SF), which is abundant in *Rhapanus sativus* seeds. The underlying mechanism of its anticancer activity is still underexplored.
Purpose: SF properties make it an interesting candidate for cancer prevention and therapy. Thus, it is crucial to characterize the mechanism of its activity.
Study design: We investigated the mechanism of antiproliferative activity of SF in breast cancer cells differing in growth factor receptors status and lacking functional p53.
Methods: Viability of SKBR-3 and MDA-MB-231 breast cancer cells treated with SF was determined by SRB and clonogenic assays. Cell cycle, cell death and oxidative stress were analyzed by flow cytometry or microscopy. The levels of apoptosis and autophagy markers were assessed by immunoblotting.
Results: SF efficiently decreased the viability of breast cancer cells, while normal cells (MCF10A) were less sensitive to the analyzed isothiocyanate. SF induced G2/M cell cycle arrest, as well as disturbed cytoskeletal organization and reduced clonogenic potential of the cancer cells. SF induced apoptosis in a concentration-dependent manner which was associated with the oxidative stress, mitochondria dysfunction, increased Bax:Bcl2 ratio and ADRP levels. SF also potentiated autophagy which played a cytoprotective role.
Conclusions: SF exhibits cytotoxic activity against breast cancer cells even at relatively low concentrations (5–10 μM). This is associated with induction of the cell cycle arrest and apoptosis. SF might be considered as a potent anticancer agent.

Introduction

According to the GLOBOCAN estimates, nearly 1.7 million women were diagnosed with breast cancer in 2012. This type of cancer is also the most frequent cause of cancer-related deaths in women in the less developed regions, and the second cause of cancer deaths in the more developed regions (Ferlay et al., 2015). Therapies that are currently available are only partially effective and often cause serious side effects. Therefore, a search for new anticancer drugs and more effective treatment strategies is now considered a priority.

Vegetables from the Brassicaceae family are a rich source of glucosinolates. They are enzymatically hydrolyzed by a plant myrosinase to isothiocyanates and other products (Wu et al., 2009). Isothiocyanates (ITC) have been studied intensively in recent years, mainly because of their chemopreventive and anticancer activities. Both, *in vitro* and *in vivo* studies indicate that these properties are associated with modulation of phase I and II biotransformation enzyme activities, induction of the cell cycle arrest and/or apoptosis of the cancer cells of different origin, as well as inhibition of angiogenesis and metastasis (for review, see (Gupta et al., 2014)). Sulforaphane (1-isothiocyanato-4-methylsulfinylbutane, SFN), produced by the hydrolytic conversion of glucoraphanin after ingestion of cruciferous vegetables, such as broc-

Abbreviations: ADRP, Adipose Differentiation-Related Protein; AO, acridine orange; DAPI, 4′,6-diamidino-2-phenylindole; EB, ethidium bromide; IC_{50}, concentration required to reduce cell viability by 50%; NAC, N-acetyl-L-cysteine; PARP, Poly (ADP-ribose) polymerase; PBS, phosphate buffered saline; SF, sulforaphene; SFN, sulforaphane; SRB, sulforhodamine B

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coli and broccoli sprouts, has been extensively studied and its chemopreventive activities are well established in a wide variety of cancer cells (Sestili and Fimognari, 2015; Tortorella et al., 2015). A number of phase I and II clinical trials on SFN are in progress or have been completed to assess its safety, tolerance, pharmacokinetics and therapeutic benefit in healthy human subjects and in the context of cancer (Tortorella et al., 2015). Nevertheless, the level of its consumption in an average diet, stability as a supplement, and difficulty in administration of a therapeutic dose, limit the potential for its clinical use.

Glucoraphenin (4-methylsulfinyl-3-butenyl glucosinolate) is an important component of radishes (*Raphanus sativus* L.), especially their seeds. It is hydrolyzed by myrosinase to sulforaphene (4-isothiocyanato-1-methylsulfinylbut-1-ene, SF). SF is a structural analog of SFN which differs only in having one double bond in the alkyl chain. However, in contrast to SFN, the activity of SF against cancer cells is poorly understood. Only a few reports exist that describe the anticancer activity of extracts from different parts of the radish plant. For instance, it has been documented that a hexane extract of R. sativus roots induced apoptosis of HeLa, A549, MCF-7 and PC-3 cells, which was associated with the upregulated expression of genes coding for pro-apoptotic Bax and caspase 3, and downregulated expression of genes of anti-apoptotic Bcl-2 and Bcl-xL (Beevi et al., 2010). On the other hand, an ethanol extract of aerial parts of *R. sativus* inhibited growth of MDA-MB-231 breast cancer cells, which was accompanied by reduced expression of ErbB2, ErbB3 and Akt (Kim et al., 2011). These findings are important, however, they do not explore which of the extract's ingredients is responsible for these activities. Anticancer potential of pure sulforaphene has been investigated in a few studies using cancer cell lines of different origin (Byun et al., 2016; Kim et al., 2014; Mondal et al., 2016; Papi et al., 2008; Wang et al., 2014). It has been also shown that it sensitized breast and ovarian cancer cells to lapatinib and cisplatin, respectively (Biswas et al., 2015; Kaczynska et al., 2015). Nevertheless, these limited studies indicate that sulforaphene has a greater anticancer potential than sulforaphane or some other clinical drugs. Besides, a main source of SF, radishes - contrary to other cruciferous vegetables - are generally eaten raw, thus generation of this ITC might be more effective in context of chemoprevention.

In this work we compared the antiproliferative potential of SF against two different breast cancer cell lines (MDA-MB-231 and SKBR-3) and investigated the molecular mechanism of its anticancer activity. Our results clearly show that SF efficiently reduces the survival and clonogenicity of breast cancer cells, which is connected with the inhibition of cell cycle and induction of cell death.

Materials and methods

Reagents

Sulforaphene (purity ≥ 98%) was obtained from LKT Laboratories (St. Paul, MN). Culture media, FBS and human insulin were purchased from Thermo Fisher Scientific (Waltham, MA). Muse kits were purchased from EMD Millipore Bioscience (Billerica, MA). Media supplements, SRB, NAC, chloroquine, DAPI, anti-tubulin, anti-rabbit, anti-mouse, anti-β-actin antibodies conjugated with HPR and anti-mouse-TRITC antibody were purchased from Sigma-Aldrich (St. Louis, MO). PARP antibodies were obtained from Cell Signaling Technology (Danvers, MA), anti-LC3 antibodies were from Medical and Biological Laboratories CO., LTD. (Woburn, MA) and antibodies against Bcl-2, Bax and ADRP were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

SKBR-3 and MDA-MB-231 cells were maintained as described (Pawlik et al., 2013). MCF10A cells (non-tumorigenic human breast epithelial cells) were maintained in DMEM/F-12 medium supplemented with 5% equine serum, 20 ng/ml of epidermal growth factor, 10 μg/ml of human insulin, 0.5 μg/ml of hydrocortisone, 2 mM L-glutamine and 1% penicillin/streptomycin.

Cell viability assay

4×10^3 cells were seeded per well in a 96-well plate. After 24 h, the cells were exposed to SF (1–50 μM) for 24 h. Viability of cells was assessed using the SRB assay. Briefly, 100 μl per well of 10% (w/v) aqueous solution of ice-cold trichloroacetic acid was added for 1 h. Plates were washed with water, allowed to air dry and stained with 100 μl of 0.4% sulforhodamine B solution in 1% acetic acid for 15 min. The cells were washed 5 times with 1% acetic acid and dried. After addition of 10 mM Tris base (pH 10.5) the absorbance was measured at 570 nm with a reference filter of 660 nm in a Victor3 microplate reader.

Western blot analysis

1.5×10^6 cells were seeded on 100 mm dishes, grown overnight and treated with SF. For investigation of autophagy flux cells were pre-treated with 25 or 50 μM chloroquine for 1 h. After 24 h the cells were harvested, lysed for 30 min on ice with the lysis solution (50 mM Tris (pH 7.5), 1% Triton X-100, 150 mM NaCl, 0.5 mM EDTA, protease and phosphatase inhibitor cocktails (Roche Diagnostics, Germany) and centrifuged (12,000 rpm, 4 °C, 30 min). Immunoblots were performed as previously described (Pawlik et al., 2013).

Cell cycle and cell death analysis

5×10^5 cells were seeded on 60 mm dishes and incubated overnight. The cells were treated with SF (2.5–20 μM) for 24 h. Both, floating and attached cells were collected and washed with ice cold PBS. Next, cells were stained with the Muse® Cell Cycle Kit according to the manufacturer's instructions, and analyzed by flow cytometry. To determine the percentage of dead cells after treatment with SF and chloroquine, the Muse® Annexin-V & Dead Cell Assay Kit was applied according to the manufacturer's protocol. Briefly, 2.5×10^5 cells were seeded per well in 6 well-plates. After 24 h, the cells were pre-treated with 25 or 50 μM chloroquine for 1 h and exposed to 20 μM sulforaphene for 24 h Both, medium and trypsynized cells were collected and centrifuged for 10 min, 300 x g. The amounts of cells in the early (Annexin-V^+/7-AAD^-) and late stages of apoptosis (Annexin-V^+/7-AAD^+) or necrotic cells (Annexin-V^-/7-AAD^+) were determined using the Muse™ Cell Analyzer.

Detection of ROS in cells

5×10^5 cells were seeded on 60 mm dish and incubated overnight. The cells were treated with SF (5 μM or 10 μM) for 12 h. Both, floating and attached cells were collected and washed with PBS, proceeded with the Muse® Oxidative Stress Kit according to manufacturer's instruction, and analyzed by Muse™ Cell Analyzer. 3000 counts were measured per sample.

EB/AO staining

Cells were seeded on coverslips placed in a 12-well plate, at a density of 5×10^5 cells per well. After 24 h, the cells were treated with either a vehicle or 10 or 20 μM SF for 24 h The cells were stained with a mixture of acridine orange (4 μg/ml) and ethidium bromide (4 μg/ml) in PBS, for 5 min. The slides were washed with warm PBS, attached to a microscope slide and examined under a fluorescent microscope.

Immunofluorescence staining of tubulin

2.5×10^5 cells were seeded on coverslips in 6-wells plates and incubated overnight. The cells were treated with sulforaphene