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Research Paper

# Phytochemical-induced nucleolar stress results in the inhibition of breast cancer cell proliferation

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ABSTRACT

The nucleolus is a stress sensor and compromised nucleolar activity may be considered as an attractive anticancer strategy. In the present study, the effects of three plant-derived natural compounds, i.e., sulforaphane (SFN), ursolic acid (UA) and betulinic acid (BA) on nucleolar state were investigated in breast cancer cell lines of different receptor status, namely MCF-7, MDA-MB-231 and SK-BR-3 cells. Cytostatic action of phytochemicals against breast cancer cells was observed at low micromolar concentration window (5–20 μM) and mediated by elevated p21 levels, and cell proliferation of SFN-, UA- and BA-treated normal human mammary epithelial cells (HMEC) was unaffected. Phytochemical-mediated inhibition of cell proliferation was accompanied by increased levels of superoxide and protein carbonylation that lead to disorganization of A- and B-type lamin networks and alterations in the nuclear architecture. Phytochemicals promoted nucleolar stress as judged by the nucleoplasmic translocation of RNA polymerase I-specific transcription initiation factor RRN3/TIF-IA, inhibition of new rRNA synthesis and decrease in number of nucleoli. Phytochemicals also decreased the levels of NOP2, proliferation-associated nucleolar protein p120, and WDR12 required for maturation of 28S and 5.8S ribosomal RNAs and formation of the 60S ribosome, and phosphorylation of S6 ribosomal protein that may result in diminished translation and inhibition of cell proliferation. In summary, three novel ribotoxic stress stimuli were revealed with a potential to be used in nucleolus-focused anticancer therapy.

## 1. Introduction

More recently, non-ribosomal functions for the nucleolus have been established [1,2]. Beyond its primary role in ribosome biosynthesis, the nucleolus is also involved in the regulation of cell cycle progression and stress signaling [3–5]. Oxidative and ribotoxic stress stimuli have been reported to inhibit RNA polymerase I (Pol I) transcription by inactivation of the Pol I-specific transcription factor RRN3/TIF-IA [6]. The inactivation of TIF-IA is achieved by phosphorylation of TIF-IA by c-Jun N-terminal kinase (JNK) at a single threonine residue (Thr200) that result in both impaired interaction of TIF-IA with Pol I and SL/TIF-IB, thus preventing transcription initiation complex formation at the rDNA promoter, and relocation of TIF-IA from the nucleolus to the nucleoplasm where it is sequestered from Pol I [6]. Several stressors can also promote the nucleoplasmic translocation of nucleolar proteins such as ARF, L5, L11, L23 or B23/nucleophosmin that is considered as a hallmark of nucleolar stress [3,7–9]. Relocated proteins bind MDM2 (HDM2 in human, E3 ubiquitin ligase) that block the ubiquitinylation of p53 and induce p53-dependent cell cycle arrest and/or apoptosis [3,10]. Genetic inactivation of RRN3/TIF-IA may also result in nucleolar disruption, cell cycle arrest and p53-mediated apoptosis [11]. Moreover, p53-independent responses to nucleolar stress have been documented [12–16]. As more than a half of human cancers lack functional p53 [17], these p53-independent pathways could potentially reveal additional cancer therapies that are based on drugs targeting the rDNA transcription machinery and inducing nucleolar stress.

Dietary phytochemicals are considered as promising candidates for anticancer therapy [18] and the mechanisms of action of plant-derived anticancer drugs are numerous including apoptosis, autophagy, necrosis-like programmed cell death, mitotic catastrophe and cellular senescence [19]. However, the ability of dietary agents to provoke nucleolar stress response that would block the proliferation of cancer cells has not been addressed.

In the present study, we have investigated the mechanism of cytostatic activity of two pentacyclic triterpenoids, namely ursolic acid (UA) and betulinic acid (BA) and sulforaphane (SFN), an isothiocyanate, against phenotypically distinct breast cancer cells MCF-7 ($ER^+$, $PR^{+/-}$, $HER2^-$, wild type p53), MDA-MB-231 ($ER^-$, $PR^-$, $HER2^-$, mutant

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p53) and SK-BR-3 ($ER^-$, $PR^-$, $HER2^+$, mutant p53). We found that phytochemicals induced oxidant-based nucleolar stress that resulted in the nucleoplasmic translocation of RRN3/TIF-IA and inhibition of rRNA synthesis, and decreased phospho-S6 ribosomal protein signals leading to diminished translation efficiency and p21-mediated inhibition of cell proliferation.

## 2. Materials and methods

### 2.1. Reagents

The reagents used, if not otherwise mentioned, were purchased from Sigma-Aldrich (Poland) and were of analytical grade. Sulforaphane (1-isothiocyanato-4-(methylsulfinyl)-butane, SFN), ursolic acid (3β-hydroxy-12-ursen-28-ic acid, UA) and betulinic acid (3β-hydroxy-20(29)-lupaene-28-oic acid, BA) were dissolved in dimethyl sulfoxide (DMSO). DMSO concentrations did not exceed 0.1% and had no effect on parameters analyzed.

### 2.2. Cell culture

Human breast cancer cells MCF-7, MDA-MB-231 and SK-BR-3 were obtained from ATCC (Manassas, VA, USA). Cells (10,000 cells/$cm^2$) were cultured at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotic and antimycotic mix solution (100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B) in a humidified atmosphere in the presence of 5% $CO_2$ until they reached confluence. Typically, cells were passaged by trypsinization and maintained in DMEM. Normal human mammary epithelial cells (HMEC) were obtained from Lonza (Basel, Switzerland). Cells (10,000 cells/$cm^2$) were cultured in Mammary Epithelial Growth Medium (MEGM) supplemented with BPE, hydrocortisone, hEGF, insulin and gentamicin/ amphotericin B according to manufacturer's instructions.

### 2.3. Cell proliferation

DNA content was assessed using CyQUANT® Cell Proliferation Assay Kit (Thermo Fisher Scientific). Briefly, the cells were cultured in a 96-well plate (5000 cells per well) and treated with SFN, UA and BA (5, 10, 20 μM) for 24 h and cells were then frozen, thawed and CyQUANT® GR dye/cell lysis buffer was added. After 5-min incubation in the dark, fluorescence (CyQUANT® GR dye bound to nucleic acids) was measured using a fluorescence microplate reader ($\lambda_{ex}$=480 nm, $\lambda_{em}$=520 nm). DNA content was normalized to control.

### 2.4. Cell viability

Cell viability was assessed using Muse™ Cell Analyzer and Muse™ Count and Viability Kit according to manufacturer's instructions (Merck Millipore, Poland). Briefly, the cells were cultured in a 6-well plate (10,000 cells/$cm^2$) and treated with SFN, UA and BA (5, 10, 20 μM) for 24 h and viable and non-viable cells were then differentially stained based on their permeability to the two DNA-binding dyes present in the reagent. The calculations were performed automatically and viability profiles (dot plots) were displayed using the Muse™ Count and Viability Software Module.

### 2.5. Immunostaining

An immunostaining protocol was used as previously described [20]. Briefly, the cells were cultured in a 96-well plate (5000 cells per well) and treated with SFN, UA and BA (5, 10, 20 μM) for 24 h and cells were then fixed and incubated with the primary antibodies anti-Ki67 (1:500), anti-p21 (1:400), anti-lamin A/C (1:100), anti-lamin B1 (1:500), anti-cofilin (1:200), anti-RRN3 (1:200), anti-NOP2 (1:1000), anti-WDR12 (1:200), anti-S6 ribosomal protein (1:50), anti-phospho-S6 ribosomal protein (Ser235/236) (1:100), anti-nucleolar antigen (Thermo Fisher Scientific, Novus Biologicals) and secondary antibodies conjugated to Texas Red or FITC (1:1000) (Thermo Fisher Scientific). Nuclei were visualized using Hoechst 33342 staining, F-actin was assayed using phalloidin staining or β-actin was detected using anti-β-actin antibody and nucleolus was immuno-stained using anti-nucleolar antigen antibody (Thermo Fisher Scientific). Digital cell images were captured using an imaging cytometer In Cell Analyzer 2000 (GE Healthcare, UK) equipped with a high performance CCD camera. Quantitative analysis was conducted using In Cell Analyzer software (GE Healthcare). In general, immuno-fluorescent signals are presented as relative fluorescence units (RFUs). Ki67 signals were scored and normalized to control. Cells with aberrant lamin signals were scored [%]. Compartment-specific immuno-fluorescent signals were also considered and are presented as relative fluorescence units (RFUs) or were scored [%] or were normalized to control.

### 2.6. Superoxide levels

After SFN, UA and BA treatments, intracellular total superoxide levels were measured using a fluorogenic probe dihydroethidium and imaging cytometry (In Cell Analyzer 2000 equipped with a high performance CCD camera, GE Healthcare, UK). Briefly, the cells were incubated in DPBS containing 5 μM dihydroethidium for 15 min in the dark, cells were then washed and intracellular fluorescent signals were acquired and quantified using In Cell Analyzer 2000 Software (GE Healthcare). The level of superoxide is presented as relative fluorescence units (RFUs).

### 2.7. Protein carbonylation

Actin, lamin A/C and total nuclear protein carbonylation was considered. Protein derivatization was conducted according to Lazarus et al. [21]. SFN-, UA- and BA-treated, fixed and derivatized cells were immuno-stained using anti-β-actin antibody (1:500) or anti-lamin A/C antibody (1:100) (Thermo Fisher Scientific) or stained using Hoechst 33342 staining and incubated with anti-DNP antibody (1:200) (Abcam) and the secondary antibodies conjugated to FITC or TR (1:1000) (Thermo Fisher Scientific). Digital cell images were captured using imaging cytometry (In Cell Analyzer 2000 equipped with a high performance CCD camera, GE Healthcare, UK). Co-localization analysis was performed using In Cell Analyzer 2000 Software (GE Healthcare). Protein carbonylation is presented as relative fluorescence units (RFUs).

### 2.8. Immunodetection of nascent RNA (5-fluorouridine labeling)

After SFN, UA and BA treatments, cells were incubated with a halogenated RNA precursor, 2 mM 5-fluorouridine (5-FU) for 15 min and fixed in 3.7% formaldehyde in PBST (PBS with 0.01% Triton X-100). Indirect immunofluorescence with an anti-BrdU antibody (1:500, BD Biosciences) and the appropriate secondary antibody coupled to FITC (1:1000, BD Biosciences) were used to detect halogenated RNA [22]. 5-FU in the nucleolus is presented as relative fluorescence units (RFUs).

### 2.9. Western blotting

Whole cell protein extracts were prepared according to Lewinska