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Sulforaphene Enhances The Efficacy of Photodynamic Therapy In Anaplastic Thyroid Cancer Through Ras/RAF/MEK/ERK Pathway Suppression

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ABSTRACT

Sulforaphene (SFE), a natural isothiocyanate from cruciferous vegetables has shown a potential anticancer effect against cervical and lung cancer. Palliative treatments like photodynamic therapy (PDT) are being implemented for a long time however, the results are still not promising in case of aggressive cancers like anaplastic thyroid cancer. The objective of this work is to establish an alternative method with the combination of photofrin-PDT and sulforaphene, a natural isothiocyanate from cruciferous vegetables, against human anaplastic thyroid cancer to enhance the efficacy of PDT. In this study, cell viability of FRO cells due to combination treatment was analyzed by MTT assay, Cell cycle arrest, MMP depolarization and ROS generation, analyzed by flow cytometry. Western blot analysis of various proliferative proteins was performed to assess the activity of combination treatment against FRO cells. From the results, sulforaphene alone showed no cytotoxicity against normal cells, however, combination of sulforaphene and photofrin mediated PDT showed a noticeable decrease in cell proliferation against FRO cells. Combination treatment synergistically caused cell cycle arrest via ROS generation and MMP depolarization. The expressions of Ras, MEK, ERK, B-Raf proteins significantly modulated due to combination treatment. PDT and SFE can induce apoptosis in anaplastic thyroid cancer cells individually but while treated in combination, it enhanced the apoptotic and anti-proliferative effect, much higher than the individual doses. In summary, our work designates sulforaphene as a unique natural enhancer of efficacy with PDT against anaplastic thyroid cancer.

1. Introduction

Sulforaphene, an isothiocyanate mainly available in cruciferous vegetables, reportedly inhibits tumorigenesis through MAP kinase path [1]. Isothiocyanates are a group of glucosinolates, which are commonly present in cruciferous vegetables in high amount. Sulforaphene is an analog of another isothiocyanate sulforpahane (4-methylsulfinylbutyl isothiocyanate SFA) which acts as the principal inducer of phase II enzymes, has exhibited promising anticancer properties [2–5]. Though significant effect on malignant cells with no toxicities leads the sulforaphane into several clinical trials however, high isolation cost of natural sulforaphane restricts its application. Whereas, sulforaphene (4-methylsufinyl-3-butenyl isothiocyanate, SFE) is found in cruciferous vegetables in rich quantity [5]. High purity and low cost of isolation with significant anti-cancer property SFE can be a promising choice of researchers for further investigation [3,5]. In previous reports, it has been shown that SFE exhibits synergistic inhibitory effect on cell proliferation with ovarian cancer cells and cervical cancer cell line [4] however; its effect on anaplastic thyroid cancer has not been reported until now. On the other hand, photodynamic therapy or PDT is being used in tumor therapy for certain malignancies as palliative therapy. Photodynamic therapy (PDT) is used to treat several types of cancer by the administration of a light absorbing chemical known as photosensitizer and light of a specific wavelength. The photosensitizer is activated by absorbing light and produces singlet oxygen, causing damage to the tumor cells [6]. The mechanism of the anticancer effect of photodynamic therapy has been studied where generation of singlet oxygen and free radicals were reported to promote the secondary necrosis of the cancer cells [7]. Hematoporphyrin or photofrin photosensitizers are one of the commonly used photosensitizers in PDT [8]. Therefore, photofrin sensitizer is an ideal choice for the study with SFE. Anaplastic thyroid cancer (ATC) is a rare form of endocrine malignancy,

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with rapid increasing tendency throughout the world. With a median survival of 6 months, ATC contributes poor prognosis mainly due to undifferentiated character and drug resistance [9–10]. Majority of the cases accounts for well-differentiated papillary and follicular thyroid cancer where very fewer cases have been registered for poorly differentiated thyroid cancer and anaplastic thyroid cancer [11]. Despite of having primary chemotherapeutic drugs like Carboplatin and Cisplatin, high side effect and aggressive ATC behavior are demoralizing the mortality rate gradually. Therefore implementing a non-toxic treatment procedure, which will improve patient life while killing the malignancy, is in great need.

In this present study, combination of SFE and PDT was compared with the individual dose to assess, whether SFE can enhance the efficacy of PDT against anaplastic thyroid cancer FRO cells. By this combination therapy, not only the efficacy of PDT will be enhanced but also the adverse side effects of the chemotherapy drugs can be averted. SFE being a naturally extracted drug, has no toxicity against normal cells, whereas, photofrin photosensitizer or the laser itself has very minimal toxicity against normal cells. Therefore, combination of SFE and photofrin-PDT can implement a promising mode of treatment of anaplastic thyroid cancer with an enhanced efficacy and minimal side effects also.

2. Materials and Methods

2.1. Chemicals and Reagents

The photosensitizer used in the experiment was purchased from Axcan Pharma, Inc. (Mont-Saint-Hilaire, QC, Canada). Stock solution was prepared with DPBS and stored in dark at − 20 °C. Sulforaphene [(*S*)-4-Isothiocyanato-1-methylsulfinylbut-1-ene] was purchased from LKT laboratories (St. Paul, MN, USA). 3-[4,5-dimethylthiazol-2-yl] – 2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Hoechst 33342, propidium iodide (PI), RIPA buffer, protease and phosphatase inhibitors were purchased from Sigma (Saint Louis, MO, USA). Rhodamine 123 were obtained from Invitrogen (Eugene, OR, USA). Bradford Reagent was supplied by Bio-Rad (Hercules, CA, USA). Required media DMEM and Dulbecco's phosphate-buffered saline or DPBS were supplied by Welgene (Daegu, South Korea), fetal bovine serum (FBS) from Biological Industries and streptomycin/penicillin from Thermo Scientific (South Logan, UT, USA). Apoptosis regulating proteins like Epidermal growth factor receptor (EGFR), Poly (ADPRibose) polymerase (PARP), Caspase 3, 9, Apoptosis Inducing factor or AIF, Bax, Bcl-2, PI3K, β-Actin and cytochrome *c* were purchased from Santa Cruz Biotechnology (SantaCruz, CA, USA).

2.2. Cell Culture and Treatments

Anaplastic thyroid cancer cell line, FRO were collected from Korean Cell Line Bank, Seoul. Cells were grown in RPMI 1640 culture medium supplemented with 10% heat-inactivated FBS and 1% streptomycin/penicillin and maintained at 37 °C in 5% CO_2 humid environment. Cells were harvested with 0.025% trypsin-EDTA in RPMI media, were plated in required cell numbers and allowed to grow for 24 h before treatment. The cells were further incubated with different concentrations of photofrin (0–50 μg/mL) in presence or absence of sulforaphene (0–50 μg/mL) in dark condition.

2.3. PDT Treatment

FRO, anaplastic thyroid cancer cells were pretreated with the different concentrations of photofrin (0–50 μg/mL) in RPMI 1640 media. After 3 h, the plate was washed off with warm DPBS and fresh media added into each well before laser irradiation. The cells were irradiated by keeping the culture flask under a set of 630 nm diode laser with an energy density of 10.8 J/cm^2 for 15 min. The laser system constitutes of an array of LED with a dimension of 13.5 cm × 8.5 cm. The laser power of the system was calibrated by a laser-power energy meter (Gentec EO, OR, USA). After irradiation, cells were further incubated for 3, 6, 12 and 24 h at 37 °C.

2.4. MTT Assay

Cell viability was determined by MTT assay. FRO cells were seeded in 96-well flat bottom microtiter culture plates at a density of 5×10^4 cells/well and incubated for 24 h before treatment. Cells were further treated with sulforaphene (0–50 μg/mL) and/or photofrin for 3, 6, 12 & 24 h with 5% CO_2 at 37 °C. Cells were irradiated with laser as described before. After 24 h incubation, fresh MTT solution (0.2 mg/mL) was added to each well of the culture plate. After 4 h of further incubation at 37 °C, formazan crystals were dissolved by detergent solution like DMSO and the absorbance was measured at 570 nm using Asys UVM 340 Microplate reader, Biochrom. Percentage of cell viability was calculated as the comparison between the absorbance of the treated cells with that of control cells. The mean values with standard deviation were calculated from three independent experiment results.

2.5. Determination of Synergism Between Sulforaphene and PDT

When two different drugs are used in combination for treatment, the combination mixture acts as a third drug for the dose-effect relationship. Synergism between two drugs can be calculated by the combination index (CI) theorem and plot derived according to Ahn et al. [12].

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2}$$

In the equation, (D_x) indicates D_1 or drug one 'alone' showing x% inhibition and similarly $(D_x)_2$ signifies for D_2 or drug two 'alone' which causes x% inhibition. If CI or the sum of these two fractions is < 1 then the experiment shows synergism. To evaluate the synergism between the drugs used for combination study, the CI value is calculated from the drug inhibition data using CompuSyn Version 1.0 software (ComboSyn Inc., NJ).

Further DRI or dose reduction index has been introduced keeping in mind the major objective of combination therapy i.e. to reduce the toxicity by lowering drug dose without affecting the overall efficacy. DRI is actually a measure of how many times the dose of each drug in a synergistic combination can be lowered at a given research condition level compared with the individual drug dose. To get a strong synergism DRI must be > 1.

Summarizing, with no overlapping toxicity towards the host, $CI < 1$ and $DRI < 1$, the combination group of two or more drugs can show strong synergism [12].

2.6. Cell Cycle Analysis

24 h after the drug treatment, drug treated and non-treated cells were harvested and fixed by adding ice-cold 70% ethanol and RNase treatment. Cells were later incubated for 30 min at room temperature and kept at 4 °C overnight. Next day cells were centrifuged at 5000 rpm for 5 min. Cell pellets obtained after centrifugation, were further washed with DPBS and stained with PI (5 μL) for 20 min. The percentage of fluorescence intensity was determined by flow cytometry using an FL2-A filter (BD FACS Accuri C6).

2.7. Intracellular ROS Accumulation

ROS accumulation can be estimated quantitatively by deploying cells to flow-cytometric methods. Cells were treated with SFE and PDT as described before. After drug treatment, cells were fixed with 70% ethanol and further incubated with 10 μM Rhodamine 123 for 20 min in dark. Cells were further analyzed for ROS generation using a flow