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# Identification of a Novel and Potent Nrf2 inhibitor as a Radiosensitizer with High Throughput Screening

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## 1. Introduction

Lung cancer is the leading cause of cancer death in both men and women in the United States. Non-small cell lung cancer (NSCLC) accounts for more than 75% of all lung cancers and radiotherapy (RT) is the general treatment modality for these lung cancer patients. Nuclear factor erythroid-2–related factor 2 (Nrf2) is a redox-sensitive transcription factor that regulates the expression of genes encoding electrophiles and xenobiotic detoxification enzymes and efflux proteins, which confer cytoprotection against oxidative stress and xenobiotics in normal cells (1, 2). Kelch-like ECH-associated protein (Keap1) sequesters Nrf2 and leads to proteasomal degradation of Nrf2 in non-stressed condition. Keap1 is often found with biallelic mutation in NSCLC cell lines and NSCLC patients, results in constitutive activation of Nrf2 function, and contributes to resistance of chemotherapy (CT) or RT (3, 4). We thus postulated that inhibition of Nrf2 in cancer cells could increase sensitivity to RT. Our primary results show that IM3829, a putative Nrf2 inhibitor, enhances the efficacy of RT and CT in H1299 lung cancer cell.

## 2. Materials and Methods

### *2.1. Cell culture*

HEK293 (Human embryonic kidney cell line) was maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KA, USA) and 100 units/mL penicillin and 100 μg/mL streptomycin solution (Mediatech, Inc., Herndon, VA) and was incubated at 37°C in a humidified atmosphere with 5% $CO_2$.

### *2.2. Chemical library*

A library consists of 3 sub-libraries, which are 1,040 natural single products, 1,040 US drug collection compounds and 5,040 single synthetic compounds, and was purchased from ChemBridge Co. (San Diego, USA).

### *2.3. Luciferase assay*

Transient transfections were carried out using Lipofecamine 2000™ (Invitrogen) according to the manufacturer's specifications. HEK293 cells were cotransfected with 4XARE-luciferase reporter gene and CMV-Renilla as a transfection control in 24-well tissue culture plates. HEK293 cells stably expressing 4XARE-luc were established and were plated in 96-well plates. Cells in each well were then treated with a different compound from the chemical library for 16 h at 10 μmol concentration. Luciferase activity was measured with the Dual Luciferase Assay System (Promega, Madison, WI, USA). Experiments were performed in duplicate wells and the luciferase activity of active compounds was determined repeatedly at least three times.

### *2.4. Clonogenic assay*

The radiation response curve was obtained using a clonogenic assay. In brief, cells were plated in culture dishes at 200 to 800 cells and irradiated with indicated doses using $^{137}Cs$ γ-rays at a dose rate of 3.0 Gy/min. After 7-9 days, cells were fixed and stained with 1% methylene blue in absolute methanol, and colonies consisting of more than 50 cells were counted.

### *2.5. Immunoblotting*

Cell lysates were prepared by extracting proteins with RIPA buffer [50 mM Tris-Cl (pH7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA] supplemented with protease inhibitors [1 mM PMSF, 1 μM/mL aprotinin, 1μg/mL leupeptin, and 1 mM $Na_3VO_4$]. Equal amounts of the proteins were separated on 8 or 10 % SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, CA). The membranes were blocked with 5% skim milk in Tris-buffered saline and then incubated with primary antibodies for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary antibody, and proteins were visualized by enhanced chemiluminescence (ECL) reagents according to the manufacturer's recommendation (Amersham Biosciences, England). The experiments were repeated at least three times.

## 3. Results

### *3.1. High throughput screening (HTS) based on luciferase assay*

The single compound chemical library was replicated in a 96-well format, and compounds were dissolved in a final concentration of 1% DMSO. HEK293 cells stably expressing ARE-luciferase reporter gene were increased

luciferase activity 35 fold higher than pGL3 control vector transfected cells. Through the primary screening, 20 kinds of compounds were chosen for down-regulation of luciferase activity. Among them, we first investigated IM3829 with $IC_{50}$ (4.6 μM) as an Nrf2 inhibitor.

*3.2. Effect of IM3829 on Nrf2 and its target gene expression in H1299 lung cancer cell*

To determine whether IM3829 inhibit Nrf2 and its target protein HO-1 expression, H1299 cells which have defective keap1 and highly express Nrf2 protein level were treated with IM3829 for 24 hours. IM3829 was significantly decreased Nrf2 and HO-1 expression.

*3.3. Nrf2 Hits radiosensitize and chemosensitize H1299 lung cancer cell and U87-MG glioma cell.*

We next prove our hypothesis that inhibition of Nrf2 in tumor cells might enhance sensitivity of RT, the clonogenic survival of H1299 lung cancer cell and U87 glioma cells with IM3829 was determined. Pretreatment of IM3829 demonstrated radiosensitive enhancing effect in both cell lines. To investigate chemosensitizing effect of IM3829, H1299 and U87-MG were treated with IM3829 for 1 h before 2.5 μM doxorubicin (adriamycin) treatment and were assayed the cytotoxicity with CCK-8 2 days after the treatment. Chemosensitization was evident at 5 μM and 10 μM of IM3829 in H1299 and U87-MG cells.

## 4. Conclusions

Many lung cancer patients and lung cancer cell lines have dysfunctional keap1 and more express Nrf2, which is a key transcription factor of detoxifying enzymes against oxidative stress or electrophiles. The aim of this study was to identify novel small chemicals, which can inhibit Nrf2 activity for sensitization to radiotherapy or chemotherapy, with high throughput screening.

We established a 96-well microtiter plate-based luciferase reporter gene assay system with antioxidant response element (ARE) in HEK293 cells, which Nrf2 can bind, and screened over 7,000 single compounds. The active compound IM3829 significantly inhibits Nrf2 and HO-1 protein expressions. IM3928 exhibits not only radiosensitization but chemosensitization in H1299 lung cancer cells in a dose dependent manner.

These results suggest that IM3829 is a novel and potent Nrf2 target inhibitor and might be useful in radiotherapy or chemotherapy against cancer cells with high expressing Nrf2.

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