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Synthesis and Biological Evaluation of Novel IM3829 (4-(2-Cyclohexylethoxy)aniline) Derivatives as Potent Radiosensitizers

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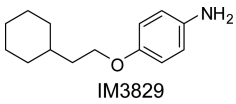
Nuclear factor-erythroid 2-related factor 2 (Nrf2) regulates the expression of over 200 genes of antioxidant and phase II drug-metabolizing enzymes, and is highly expressed in non-small cell lung cancer (NSCLC). Nine derivatives of 4-(2-cyclohexylethoxy)aniline were designed. Our previous study demonstrated that IM3829 increases radiosensitivity of several lung cancer cells *in vitro* and *in vivo*. Here, biological effects of IM3829 derivatives (**2a-2i**) were evaluated. Compound **2g** derivative effectively inhibits mRNA and protein expression of Nrf2 and HO-1. In addition, we observed over two fold enhancement in IR-induced cell death, from 2.90 ± 0.22 to 6.02 ± 0.87, in H1299 cancer cell-line. Among the nine derivatives, compound **2g** derivative exhibited the highest enhancement of radiosensitizing effect via inhibition of Nrf2 activity.

Key Words : Radiosensitizer, Nrf2 inhibitor, Reactive oxygen species, Radiotherapy, Free radicals

Introduction

Radiotherapy is an effective treatment modality for solid tumors that can accompany surgery. The dosage of radiotherapy on the tumor is limited due to the side effects on the surrounding normal tissues. On the other hand, fractionated ionizing radiation (IR) can lead to acquisition of radioresistance cancer cells, which results in decreasing treatment efficiency. Therefore, efforts have been made to develop radiosensitizer or combination therapeutic agents with enhanced efficacy of radiotherapy.

Cancer cells harbor highly developed antioxidant systems to maintain redox homeostasis that ensures cell survival with aberrant metabolism.[1,2] A transcription factor within the nucleus known as nuclear factor-erythroid 2-related factor 2 (Nrf2), regulates the expression of over 200 genes of antioxidant and phase II drug-metabolizing enzymes, and is highly expressed in non-small cell lung cancer (NSCLC) and its derived cell lines.[3] Cancers with high Nrf2 expression levels are chemo- and radio-resistance because of increased expression of cytoprotective genes.[4,5] Recently, Wang *et al.* reported that all-trans retinoic acid (ATRA) reduces the ability of Nrf2 to mediate induction of ARE-driven genes by cancer chemopreventive agents.[6] An enhanced Nrf2 activity promotes formation and chemoresistance of colon cancer where the PI3K inhibitor LY294002 abrogated the Nrf2 activity.[7] However, antioxidants can prevent cancer through activation of Nrf2. The antioxidant activity of natural products such as sulforaphane and gallic acid shows the ability to induce Nrf2 activation.[8] Nrf2 function could play an important role in a large variety of biological processes and should be taken into account in cancer prevention or treatment.



IM3829

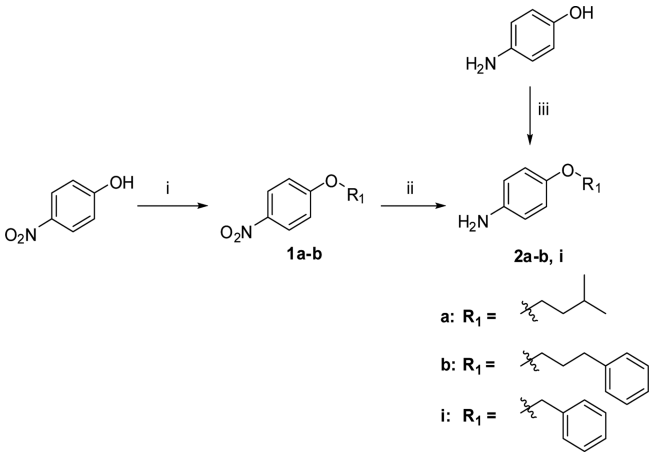
Figure 1. IM3829 Structure.

We previously identified that inhibition of Nrf2 might be an effective strategy to increase the radio-sensitivity of lung cancer. 4-(2-cyclohexylethoxy)aniline, designated IM3829 (Figure 1), could be a putative radio-sensitizer by inhibiting Nrf2 mNRA and protein expression.[9] In H1299 xenografts mice, combined IM3829 and radiation inhibits tumor growth by 47% and 27% compared with vehicle or radiation treated animals, respectively. Herein we describe an extended structure-activity relationship study of IM3829 to identify a more active and stable compounds in this series.[10,11] With the objective of investigating the effect of phenol-based substituents on activity, we designed and constructed a series of **2a-i**.

Results and Discussion

Chemistry. Synthesis of new series of IM3829 derivatives (**2a-b**, **i**) were carried out by starting from commercially available 4-nitrophenol and 4-amino phenol (Scheme 1). The corresponding nitrobenzenes (**1a-b**) were prepared by alkylation with 1-bromo-3-methylbutane or (3-bromopropyl)benzene resulting in the alkylated derivatives (**1a-b**). Another IM3829 derivative (**2i**) was synthesized by alkylation with benzyl chloride. Reduction of 4-nitro groups of **1a-b** in the presence of palladium resulted in IM3829 deriva-

[a]These authors contributed equally to this work.



Scheme 1. *Reagents and Conditions*; (i) 1-bromo-3-methylbutane or (3-bromopropyl)benzene, Cs_2CO_3, DMF, rt. (ii) Pd/C, MeOH (iii) benzyl chloride, Potassium *tert*-butoxide, DMF.

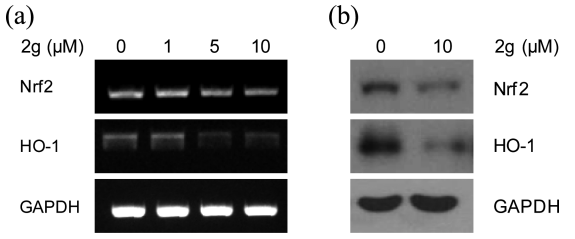


Figure 2. IM3829 derivatives inhibit mRNA and protein expression of Nrf2 and HO-1 in H1299 lung cancer cells. H1299 cells were treated with the indicated concentrations of **2g** and incubated for 24 h to detect mRNA expression of Nrf2 and its target gene HO-1 by RT-PCR (a) and their protein expression by Western blotting (b). DMSO treatment and GAPDH were used as a vehicle control and a loading control, respectively.

tives (**2a-b**, **i**). The other IM3829 derivatives (**2c-h**) were purchased as reagents.

Biological Assays. To determine whether IM3829 derivatives inhibit Nrf2 activity, chemical compounds were evaluated with a luciferase assay using pARE-Luc reporter gene in response to binding of antioxidant-response transcription factors including Nrf2. H1299 cells were cotransfected with pARE-Luc and pRL CMV-Renilla as an internal control and treated with each compounds at a concentration of 10 μM in the presence or absence of 80 μM tertiary butylhydroquinone (*t*BHQ), which is an antioxidant and an effective Nrf2 inducer. pARE-Luc luciferase activity in cell lysates was normalized to that of pRL CMV-Renilla luciferase activity and expressed as fold-change from DMSO control.

As shown in Table 1, IM3829 and compound **2g** reduced 77% and 87% of *t*BHQ-induced luciferase activity, respectively, indicating that IM3829 derivative **2g** was more potent in Nrf2 inhibition compared with IM3829.

To further confirm the inhibitory effect of compound **2g**, mRNA and protein of Nrf2 and its down target gene HO-1 were identified by RT-PCR and western blotting, respectively. Consistent with the result of ARE luciferase activity, compound **2g** significantly decreased both Nrf2 and HO-1 mRNA and protein expressions (Figure 2).

We next examined whether IM3829 derivatives enhance IR-induced cell death in H1299 cells. The apoptotic and necrotic cell death were detected with Annexin V/PI staining. Combined treatment of the cells with IR and compound **2g** resulted in a 2-fold increase in cell death, compared with IR–only-treated cells. Compound **2g** alone also increased cell toxicity by 2.9 folds compared with DMSO treated cells (Table 1). Similar radiosensitizing activity of compound **2g** and IM3829 may be stemming from the experiment using only a single dose for screening. Based on the results of luciferase assay, the IC_{50} of compound **2g** could be lower than IM3829 and further studies are required. Although compound **2b** exhibited a 2-fold increase in IR-induced cell death, it was excluded from the study because it did not regulate Nrf2-responsive luciferase activity.

Compound **2a**, with an isopentyl group, showed the complete loss of the anti-cancer activity in comparison to IM3829. It suggests that cyclohexyl, as a functional group, plays a key role in the inhibition of Nrf2 activity. To determine whether the amine group is important for the activity, the amine groups of compounds **2f** and **2h** were modified. They were compared with unchanged R_2 groups to examine the effect of the modification on the activity. The activity was dramatically reduced when the amine group was substituted for another group. This shows that amine is an indispensable functional group for the activity. In addition, the activity of aromatic ring compounds **2b**, **2h** and **2i** decreases as the chain length increases. This means that the length of the liker affects the activity. No effect was observed for compound **2d**, when its activity was investigated in interaction with molecules in intermediate linker through hydroxyl. Likewise, no effect was observed for various heterocyclic compounds **2c-d** and **2e**.

Conclusion

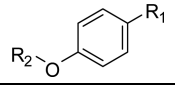
In summary, nine novel IM3829 derivatives were designed with diverse functional groups and were evaluated for the ability to inhibit Nrf2 and increase the cytotoxicity of lung cancer cells. We identified an effective IM3829 derivative by a structure-activity relationship study. Our results demonstrate that the treatment with compound **2g** inhibits Nrf2 binding activity to ARE consensus sequences. It also inhibits mRNA and protein expression of Nrf2 and HO-1 more effectively than the mother compound.

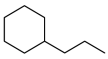
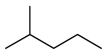
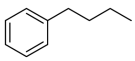
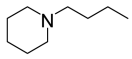
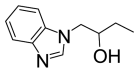
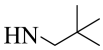
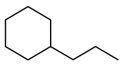
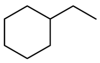
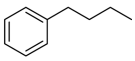
In addition, we observed the enhancement in IR-induced cell death in H1299 cells using compound **2g**. These data suggested that combination of compound **2g** and IR might be a useful radiosensitizing treatment, guiding the development of newly improved IM3289 derivatives through SAR models.

Experimental

Cell Culture. The human NSCLC cell line NCI-H1299 (H1299) were maintained in RPMI supplemented with 10%

Table 1. Structure-activity relationship of aniline analogues



No.	Structure		Fold-change of relative ARE-luciferase activity (10 μM)		Fold-change of cell death (10 μM)	
	R1	R2	Compound	Combined treatment (Compound + *t*BHQ)	Compound	Combined treatment (Compound + IR)
Vehicle (DMSO)	–	–	1.00 ± 0.10	6.22 ± 0.58	1.00 ± 0.20	2.99 ± 0.10
IM3829	NH_2		0.31 ± 0.04	1.68 ± 0.14	3.46 ± 0.32	6.32 ± 0.39
2a	NH_2		1.31 ± 0.08	6.75 ± 0.66	1.91 ± 0.16	4.76 ± 0.16
2b	NH_2		1.64 ± 0.13	6.02 ± 0.69	1.56 ± 0.23	6.03 ± 0.74
2c	NH_2		1.23 ± 0.15	6.57 ± 0.43	1.42 ± 0.15	4.30 ± 0.01
2d	NH_2		1.27 ± 0.09	5.76 ± 0.88	1.40 ± 0.54	3.47 ± 0.15
2e	NH_2		1.05 ± 0.09	5.10 ± 0.11	1.60 ± 0.05	3.76 ± 0.24
2f			1.53 ± 0.19	6.19 ± 0.60	1.22 ± 0.09	3.13 ± 0.30
2g	NH_2		0.16 ± 0.05	0.81 ± 0.15	2.90 ± 0.22	6.02 ± 0.87
2h	NO_2		1.31 ± 0.24	4.84 ± 0.64	1.26 ± 0.19	2.87 ± 0.19
2i	NH_2		0.91 ± 0.01	4.36 ± 0.57	1.11 ± 0.06	2.72 ± 0.12

fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA), 100 units/mL penicillin and 100 μg/mL streptomycin solution (Mediatech, Inc., Herndon, VA, USA) at 37 °C in a humidified 5% CO_2 atmosphere.

Luciferase Assay. pARE-Luc was prepared as described previously.[6] H1299 cells were cotransfected with the pARE-Luc reporter construct and pRL CMV-Renilla (Promega, Madison, WI, USA), as an internal control, using Lipofectamine 2000 (Invitrogen, Chicago, IL USA) according to the manufacturer's specifications. Briefly, the cells were plated into 96-well plates at a density of 1000 cells/well, incubated for 18 h, and then treated for an additional 18 h with each of IM3829 derivatives in the presence or absence of 80 μM tertiary butylhydroquinone (*t*BHQ; Sigma-Aldrich, St Louis, MO, USA). The cells were lysed with 100 μL of cell lysis buffer (Promega), after which ARE–binding-dependent transcriptional activity was determined using a Dual-Luciferase assay system (Promega) and a luminometer (Victor ×2; Perkin Elmer, Waltham, MA, USA). The firefly luciferase activity of the reporter was expressed relative to that of cotransfected CMV-Renilla luciferase to normalize for transfection efficiency.

Cytotoxic Assay. Cytotoxic assay was carried out using an Annexin V-FITC assay kit according to the manufacturer's protocol (BD PharMingen, San Diego, CA, USA). Briefly, 10,000 cells were plated into 60-mm plates and pretreated with 10 μM IM3829 for 2 h, then exposed to 5 Gy of ^{137}Cs γ-radiation. The cells were harvested and incubated with 5 μL of fluorescein isothiocyanate (FITC)-conjugated Annexin V and 10 μM propidium iodide (PI) for 15 min. Fluorescence analyses were performed using a FACSort flow cytometer (Becton Dickinson, San Jose, CA, USA).

Western Blot Analysis. Cell lysates were prepared by extracting proteins with RIPA buffer (50 mM Tris-Cl pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride [PMSF], 1 μM/mL aprotinin, 1 μg/mL leupeptin, and 1 mM Na3VO4). Equal amounts of proteins were separated by sodium dodecyl sulfate-polymerase gel electrophoresis (SDS-PAGE) on 8% or 12% gels, and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% skim milk in Tris-buffered saline, followed by incubation with primary antibodies for 3 h at room temperature (RT). Blots were developed with peroxidase-conjugated secondary antibody and immunoreactive proteins were visualized with enhanced chemiluminescence (ECL) reagents, according to

the manufacturer's recommendations (Amersham, GE Healthcare, Buckingamshire, UK). Experiments were repeated at least three times.

Real-time RT-PCR. Total RNA was extracted from H1299 cells and reverse transcribed as described for semi-quantitative RT-PCR. Real-time PCR was done using SYBR Green (Fermentas, Burlington, ON, Canada) and a Chromo4 Four-Color Real-Time PCR Detector (BioRad, Hercules, CA, USA) according to the manufacturer's guidelines. Thermocycling conditions for real-time PCR were as follow: one cycle of denaturation at 95 °C for 3 min, followed by 40 cycles of amplification at 95 °C for 15 s and 60 °C for 20 s. The following primer pairs were used: HO-1, 5'-AAG ATT GCC CAG AAA GCC CTG GAC-3' (forward) and 5'-AAC TGT CGC CAC CAG AAA GCT GAG-3' (reverse); and GAPDH, 5'-ACC ACA GTC CAT GCC ATC AC-3' (forward) and 5'-TCC ACC ACC CTG TTG CTG TA-3' (reverse). Experiments were repeated at least three times.

General. All of the commercial chemicals and solvents were of reagent grade and were used without further purification. All reactions were carried out under an atmosphere of dried argon in flame-dried glassware. Proton nuclear magnetic resonance (^{1}H NMR) spectra were determined on a Varian (400 MHz) spectrometer. Compounds **2c-h** were purchased.

General Procedure for Alkylation (1a-b). Cesium carbonate (1.5 equiv) and 1-bromo-3-methylbutane or (3-bromopropyl)benzene (1.2 equiv) was added to the solution of 4-nitrophenol (1 equiv) in DMF (5 volume). The reaction mixture was stirred overnight at room temperature and quenched with water. The solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel.

General Procedure for Reduction (2a-b). A solution of nitrobenzenes (1 equiv) in methanol was treated with 5% Pd/C (5% w/w). The reaction was subjected to hydrogenation under the hydrogen pressure of 1 atm at room temperature and the reaction mixture was stirred overnight. After completion of the reaction, the mixture was filtered through a Celite pad and concentrated. The resulting residue was purified by silica gel.

4-(Isopentyloxy)aniline (2a): ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.76-6.72 (m, 2H), 6.65-6.61 (m, 2H), 3.90 (t, J = 6.8 Hz, 2H), 3.40 (brs, 2H), 1.85-1.76 (m, 1H), 1.66-1.61 (m, 2H).

4-(3-Phenylpropoxy)aniline (2b): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30-7.26 (m, 2H), 7.22-7.17 (m, 3H), 6.76-6.72 (m, 2H), 6.66-6.62 (m, 2H), 3.89 (t, J = 6.4 Hz, 2H), 3.43 (brs, 2H), 2.79 (t, J = 7.2 Hz, 2H), 2.09-2.03 (m, 2H).

4-(Benzyloxy)aniline (2i). Potassium tert-butoxide (1.0 equiv) was added to the solution of 4-amino phenol (1 equiv) in DMF (5 volume) in ice bath for 30 min at 0 °C. After stirring for 30 min, Benzyl chloride (1.0 equiv) was added to the mixture solution in ice bath and stirred for 1 h at room temperature. After aqueous work-up, the residue was purified by column chromatography on silica gel. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.25-7.43 (m, 3H), 6.81 (d, J = 7.74 Hz, 1H), 6.64 (d, J = 7.60 Hz, 1H), 4.99 (s, 1H), 3.42 (br s, 1H).

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