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Complete List of Authors:	Rakariyatham, Kanyasiri; Dalian Polytechnic University Yang, Xiao; Xihua University Gao, Zili; University of Massachusetts Amherst, Food Science Song, Mingyue; University of Massachusetts, Amherst, Food Science Han, Yanhui; University of Massachusetts, Amherst Chen, Xiang; Xihua University, College of Food and Bioengineering Xiao, Hang; University of Massachusetts Amherst, Food Science

Synergistic chemopreventive effect of allyl isothiocyanate and sulforaphane on non-small cell lung carcinoma cells

Kanyasiri Rakariyatham[2#], *Xiao Yang*[1,2#], *Zili Gao*[1], *Mingyue Song*[1], *Yanhui Han*[1], *Xianggui Chen*[1*], *Hang Xiao*[2*]

[1]School of Food and Bioengineering, Xihua University, Chengdu, P. R. China

[2]Department of Food Science, University of Massachusetts, Amherst, Massachusetts, 01003, United States

# these authors contributed equally to this work.

* Corresponding Authors:

Hang Xiao, (hangxiao@foodsci.umass.edu)

Xianggui Chen (chenxianggui@hotmail.com)

Abstract

Isothiocyanates from cruciferous vegetables are known for their potential anti-carcinogenic actives. These isothiocyanates are frequently consumed together as part of regular diet, but their combined effects on carcinogenesis have not been well studied. Herein, we tested the hypothesis that combination of two isothiocyanates, i.e. allyl isothiocyanate and sulforaphane produced a synergy in inhibiting the growth of A549 lung cancer cells. Our results showed that the combination treatment led to a stronger growth inhibition than the singular treatment. Isobologram analysis proved that enhanced inhibitory effect of the combination treatment was synergistic. Flow cytometry demonstrated that the combination treatment caused more extensive cell cycle arrest and apoptosis than the singular treatment with modified expression of key proteins regulating these cellular processes. The combined treatment resulted in the production of intracellular reactive oxygen species, which might contribute to the inhibitory effects on cancer cells. Moreover, a synergy between allyl isothiocyanate and sulforaphane was also observed in anti-cell migration. Collectively, our results have demonstrated the potential of different isothiocyanates used in combination to produce enhanced protective effects against carcinogenesis.

Keywords: Allyl isothiocyanate, Sulforaphane, Combination, Anticancer, A549

1. Introduction

Lung cancer is one of the most common cancers. Although the rates of incidence and death from lung cancer have decreased in the past few decades, this type of cancer has still been the number one cause of cancer death and a major health problem in many parts of the world[1]. Accumulating evidence suggested that chemoprevention with natural and/or synthetic compounds could be effective to prevent cancer from being initiated, promoted and/or progressed to the advanced malignant stages. Among different chemopreventive agents, dietary compounds from fruits and vegetables are of interest due to their multi-targeting activities, low toxicity, and low cost [2].

Isothiocyanates are well-known naturally occurring small molecules that are produced by enzymatic conversion of glucosinolates in cruciferous vegetables. Isothiocyanates have been suggested to be promising anti-cancer agents. Many of them including allyl isothiocyanate (AITC) and sulforaphane (SFN) display anticarcinogenic activity through various mechanisms including reducing activation of carcinogens, reducing cancer cell proliferation, inducing cell cycle arrest, leading to apoptosis, and decreasing invasion and metastasis [3, 4].

Combination of different cancer chemopreventive agents is a promising strategy where two or more compounds may effectively act against cancer growth by synergistic type of interaction and result in stronger inhibitory effects compared to those achieved by each compound individually [5]. The enhanced anti-cancer effects by combination could lead to lower dose requirement, reducing potential side effects and minimizing the development of drug resistance[6, 7]. Several isothiocyanates combinations have been tested on different cancers by combining among themselves or with other anti-cancer agents, and synergies have been observed

on the basis of the combination index (CI) or relevant statistical analyses. Gupta *et al*.[4] demonstrated that either benzyl- or phenyl isothiocyanates could sensitize platinum containing agents in lung cancer. Pappa *et al*.[8] reported that incorporation of sulforaphane and 3,3'-diindoylmethane in colon cancer dose-dependently provided synergistic anti-cell proliferation by arresting cell cycle at G2/M phase. However, the combined effects of particular isothiocyanates, AITC and SFN, on lung carcinogenesis have not been studied. Herein, we tested the hypothesis that the combination of AITC and SFN produced a synergy in inhibiting the growth of human non-small cell lung cancer cells. Therefore, we examined the effect of AITC and SFN individually and in combination on cancer cell survival and cell migration. To determine molecular pathways underlying the mechanisms of the combined treatment, we investigated expression of proteins associated with apoptosis, cell cycle arrest, cell invasion and metastasis.

2. Materials and methods

2.1. Cells culture conditions and treatments

Lung cancer A549 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and were cultured in RPMI-1640 medium supplemented with 5% heat-inactivated FBS and 100U/ml of penicillin and 0.1 mg/ml of streptomycin at 37°C with 5% CO_2. Dimethyl sulfoxide (DMSO) at finial concentration of 0.1 % v/v was used to prepare cell treatments which were AITC (98%, Sigma-Aldrich, St. Louis, MO, USA), and SFN (> 98 %, Quality phytochemicals, Edison, NJ, USA). Cells were treated with freshly prepared treatment in culture medium for 72 hours before subjecting to further analysis as described below.

84 **2.2. Measurement of cell viability**

85 Cytotoxicity of treatments on A549 cells were assessed by the enzymatic reduction of 3-(4,5-
86 dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma-Aldrich) as
87 previously described [9]. Briefly, 2000 cells/well grown in 96-well tissue culture plates were
88 exposed to indicated series concentrations of AITC, SFN, and their combination. After treatment,
89 cells were incubated for 1 hour with 0.5 mg/ml of MTT in cell culture medium and the
90 absorbance of resulting formazan product was measured at 570 nm using a microplate reader
91 (SpectraMax, Molecular Devices, Sunnyvale, CA, USA).
92
93 **2.3. Detection of apoptosis**
94 Treated cells (4 x 10^4 cells/well in a 6-well plate) were washed with iced-cold phosphate buffer
95 saline (PBS) and detached using trypsin (0.25% trypsin-EDTA; Mediatech, Manassas, VA,
96 USA). Analysis of apoptosis by flow-cytometry (BD LSRII, BD Biosciences, San Jose, CA,
97 USA) was accessed using dual staining, Annexin V fluorescein isothiocyanate (Annexin V-
98 FITC) and propidium iodide (PI) in Annexin V binding buffer (BioVision, Milpitas, CA, USA)
99 as previously described [10]. Early apoptotic cells were stained with Annexin V-FITC, while late
100 apoptotic cells were stained with both Annexin V-FITC and PI.
101
102 **2.4. Cell-cycle analysis**
103 Collected cells were fixed in 70% ethanol overnight at 4 °C. As previously described [10], cells
104 were suspended in PBS containing PI, and RNase (Sigma-Aldrich) in dark for 30. The
105 population of cells in each cell-cycle phase was determined using BD LSRII flow cytometer (BD
106 Biosciences), and data were processed using ModFit LT software.

2.5. Examination of intracellular ROS accumulation

ROS in cells were monitored by a modified method from Wang *et al*. [11]. Cells were stained with 10 μM 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich) in the dark for 30 minutes followed by fluorescent detection using flow-cytometry.

2.6. Cell migration assay

A wound healing assay adapted from Zhou *et al*.[12] was performed to observe cancer cell migration. A549 (1.0×10^5 cells) were seeded in 24-well plate and allowed to grow to a confluent monolayer. Prior to scratch using a 200 μL- pipette tip, cells were washed with cold PBS. Medium containing treatment was added to each well followed by gap-width measurement using digital pictures taken from a transparent microscope (Eclipse TS100, Nikon, Melville, NY, USA) and SPOT Basic software at the beginning and the end of treatment (72 hours). For the purpose of visual enhancement, cells were dyed with crystal violet. Percent change in wound width reflected percent cells migration.

2.7. Western blotting

Whole cell lysates were prepared as previously described [10]. Attached cells on the culture plates were washed with cold PBS prior to the addition of RIPA buffer containing protease and phosphatase inhibitors (Boston BioProducts, Ashland, MA, USA). Cells were collected using cell scrapers into Eppendorf tubes and placed on ice for 20 minutes. Cell suspensions were then sonicated and lysed on ice for a further 20 minutes. Supernatants were collected after centrifugation at 20,817 x g for 10 minutes and used to determine protein concentrations by

bicinchoninic acid (BCA) protein assay. Equal amounts of proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (GVS Filter Technology, Indianapolis, IN). Blocking buffer in PBS was used to block non-specific binding of antibodies prior to immunodetection using specific antibodies at the manufacturer's recommended concentrations. Protein bands were visualized on blots probing with secondary antibodies using Odyssey system (LI-COR, Lincoln, NE, USA). Antibodies for cleaved caspaes-3, caspase-3, cleaved PARP, PARP, Survivin, Bcl-xL, Cyclin B1, p21, STAT3 and MMP9 were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies for p53, COX-2, and p-STAT3 were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). β-Actin antibody obtained from Sigma-Aldrich (St. Louis, MO, USA) was used as a loading control.

2.8. Analyses of Synergy

Synergistic effects of AITC/SFN combinations were analyzed based on Chou and Talalay's method[6] with modifications using R software. This model is used for constant ratio drug combinations. When the combination dose of d1 and d2 provides the same effect x as Drug1 alone at dose $D_{x,1}$ and Drug2 alone at dose $D_{x,2}$, the combination index (equation 1) indicates synergism, additivity, or antagonism of the combinatorial effect when the index <1, =1, or >1, respectively.

$$\text{Combination index} = d_1/D_{x,1} + d_2/D_{x,2} \quad (1)$$

2.9 Correlation Analysis

Pearson correlation analysis was performed to investigate the association between different factors on the inhibition of cell proliferation related to oxidative stress after being exposed to treatments. Correlations were considered significant when P value was less than 0.05.

2.10. Statistical analysis

All cell culture experiments were repeated for at least three times with similar results. Statistical comparisons were made using one-way analysis of variance (ANOVA) and P value of less than 0.05 was considered significant.

3. Results

3.1. Synergistic growth inhibition of non-small cell lung cancer cells by AITC/SFN combination

Using MTT assay, the effect of single compound of AITC and SFN on A549 lung cancer cell viability was determined in comparison to their combined treatment with a constant ratio of AITC: SFN at 1.25: 1 based on their IC_{50} values which were 12.6 ± 1.2, and 10.3 ± 0.6 μM, respectively. Figure 1A shows a concentration-dependent efficacy of both single and combined treatments that they decreased cell viability after 72 hours. AITC (2.5 – 12.5 μM) or SFN (2 – 10 μM) alone decreased cell proliferation from 3.2% to 50.9% and from 4.2% to 49.9%, respectively. Utilization of AITC and SFN co-treatment provided stronger anti-proliferation than that of a single treatment, which was reflected by less viable cells and less concentration requirement. Half-dose combination between AITC and SFN (6.25 μM AITC with 5 μM SFN)

provided as high as 58.6 % inhibition of cell viability. Based on Chou and Talalay's method[6], we further determined mode of interaction between the two compounds by median-effect plot and isobologram analyses. The median effect plot (Figure 1B) demonstrated reduced IC_{50} values of the combined treatments (5.53 ± 0.31 μM AITC and 4.43 ± 0.24 μM SFN) in comparison to the IC_{50} values of each compound. Isobologram (Figure 1C) confirmed the synergistic effect from the combined treatment with the combination index ranging from 0.82 – 0.94 (Figure 1A).

3.2. Induction of extensive apoptosis in lung cancer cells by AITC/SFN combination

Early and late apoptotic cells were relatively quantified by flow cytometry with Annexin V/PI co-staining after 72-hour treatment. Figure 2A which are representative images of Annexin-V (x-axis)/PI (y-axis) intensity dot plots of A549 cells, showed significantly increased dot intensity in Q2 (late apoptosis) and Q4 (early apoptosis) region and decreased dot intensity in Q3 region (non-apoptotic cells) in the AITC/SFN combined treatment group. Percent apoptotic cells were obtained from the Annexin-V/PI dot plots. As shown in figure 2B, numbers of both early and late apoptotic cells increased from control in dose-dependent manner under single and combined treatments. Single treatment of AITC (12.5 μM) significantly increased numbers of early apoptotic cells (8%) while the single treatment of SFN (10 μM) significantly increased numbers of both early and late apoptotic cells by 8, and 13%, respectively. Combination treatment, especially at higher concentrations demonstrated a synergy by clearly increasing numbers of cells in late-stage apoptosis over those in early-stage apoptosis, which was 15% maximum from total population. A synergy in total apoptosis (CI = 0.61-0.79) was observed at as low doses as 6.25 μM AITC with 5 μM SFN that they could increase 34% total apoptotic

cells. The higher combined doses (12.5 μM AITC with 10 μM SFN) increased more apoptotic cells to 52% in total in comparison to control without treatment.

To further elucidate the molecular basis of this event, expressions of proteins associated with apoptosis pathways were compared through immunoblotting. As shown in Figure 2C, both single and combined treatment dose-dependently decreased expression of survivin, an anti-apoptotic protein, and increased expression level of pro-apoptotic proteins, p53, cleaved caspase-3, and cleaved PARP. The highest concentrations of the combined treatment at 12.5 μM AITC with 10 μM SFN, obviously increased expression of pro-apoptotic proteins, especially cleaved PARP that were 70.5-fold increased while the expression of PARP did not significantly change, suggesting constant abundance of PARP in cells. The expression of cleaved caspase-3 and caspase-3 were significantly affected by both single and combined treatments. The ratios between these two protein expressions (cleaved caspase-3/caspase-3) does-dependently increased, especially by the highest combination treatment. In addition, Bcl-xL which is a member of Bcl-2 family knowing as an anti-apoptotic regulator also had relatively constant expression under treatments compared to the control. The results of pro- and anti-apoptotic protein expressions were consistent with Annexin V/PI co-staining analysis that demonstrated enhanced apoptotic effects from the combined treatment. The results suggested that the combination of AITC and SFN improved the anti-proliferation of A549 lung cancer cells through increasing number of apoptotic cells, especially at the late-stage apoptosis.

3.3. Combination of AITC and SFN led to G2/M phase cell cycle arrest in non-small cell lung cancer cells

To gain further insight into the mechanism of their anti-proliferative activities, A549 cells were treated with either AITC (3.125, 6.25,12.5 μM) or SFN (2.5, 5,10 μM) alone or in combination, and their effect on cell cycle progression and distributions were assessed after 72 hours. In figure 3A, representative images of A549 cell cycle histogram showed significantly increased G2/M phase arrest in the AITC/SFN combined treatment group. Percent cells population in each phase were calculated from the cell cycle histogram. As shown in Figure 3B, in comparison to control, single treatment of AITC or SFN at 6.25, or 5 μM, respectively did not significantly change cell-cycle progression. Unlike their lower concentrations, 12.5 μM AITC decreased G0/G1 cell population and 10 μM SFN increased G2/M phase arrest with a decrease in S-phase population. AITC and SFN combined treatment at concentrations of 12.5 and 10 μM, respectively significantly increased G2/M phase arrest up to 47% and lowered G0/G1 population to 37%.

Although there was no synergy from the combined treatment on the cell cycle arrest, the expression of cyclin B1, which is necessary during G2/M phase of cell cycle, was significantly decreased (9-fold lower than the control; Figure 3C). These data were supported by a dose-dependent increase of p21 protein expression. This G2/M phase negative regulator was maximally increased up to 5-fold under high-dose combined treatment (12.5 μM AITC with 10 μM SFN). This protein expression information is consistent with the results from flow cytometry analysis of PI-stained cells, and suggested that the combination treatment of AITC and SFN increased G2/M phase arrest in A549 cells.

3.4. Combined treatment of AITC and SFN increased intracellular ROS

ROS-induced oxidative stress was assayed in DCFH-DA-stained A549 cells after 72-hour treatment using flow cytometry (Figure 4). ROS was monitored only in the population of lived-cells due to non-stainable property of death cells. There was a significant increase of ROS levels in A549 treated with combined treatment though the increments were not synergistic. The concentrations of 6.25 μM AITC with 5 μM SFN, and 12.5 μM AITC with 10 μM SFN increased ROS 1.9-, and 2.9-fold, respectively. Combined treatment at lower concentrations than those indicated doses as well as single treatment (as high dose as 12.5 μM AITC or 10 μM SFN) did not significantly change ROS in lived A549 cells in comparison to control.

Considering correlation analysis (Table 1) between ROS level in A549 and either apoptosis or cell cycle arrest that was constructed based on Pearson correlation, there was a significantly strong positive correlation between intracellular ROS and apoptosis, especially the late apoptosis as well as a correlation between ROS and G2/M phase cell arrest. Slightly less correlation was observed between ROS and early apoptosis. A Negative correlation was found between ROS and G0/G1 phase arrest while no significant correlation was observed between ROS and S phase of cells. The correlation analysis confirmed the consistency of results and suggested that apoptosis and G2/M phase arrest under combined treatment were mediated through ROS signaling.

3.5. Synergistic inhibition of cancer cell migration by AIN/SFN combination

The inhibitory effect of AITC and SFN on migration of A549 cells through wound healing assay was examined by comparing the wound width right after treatment application in comparison to the wound width after 72-hour treatment. Figure 5A shows representative images taken at hour-0 in comparison to hour-72 using 4× magnification. When A549 cells were

incubated with AITC and SFN in either single or combined treatment, cell migration was inhibited in a dose-dependent manner (Figure 5B). Treatment of AITC at the concentrations of 6.25, and 12.5 μM significantly decreased wound healing by 13, and 22%, respectively. SFN at 10 μM also significantly decreased wound healing by 26%. Low-does combination at 3.125 μM AITC/2.5 μM SFN started to provide a synergy. The maximal anti-cell migration effect from the highest combinatorial concentrations (12.5 μM AITC with 10 μM SFN) used in this study was 48% with the interaction index of 0.59.

After studying cell migration, which is an integral part of metastasis, we further examined expression of proteins that play important roles in lung cancer metastasis including COX-2, p-STAT3 and MMP9 by Western blotting. Treatment of AITC or SFN alone in A549 cells reduced the expression levels of COX-2 and p-STAT3 in a dose-dependent manner as compared to the expression of untreated control, while the expression of STAT3 was constant under different conditions. Medium and high concentrations of AITC and SFN in combination significantly decreased MMP9 expression by 0.29-, and 0.4-fold, respectively. The results from Western blotting were consistent with that observed from cell migration assay that combination treatment between AITC and SFN, especially at higher concentrations improved anti-metastatic property in A549 lung cancer cells.

4. Discussion

This study demonstrated for the first time the synergistic effect of two isothiocyanate type of compounds, i.e. AITC and SFN in inhibiting non-small cell lung cancer cells. First, we determined the anti-proliferative potential of AITC and SFN alone on non-small cell lung cancer A549 cells. IC_{50} values after 72-hour treatment of AITC and SFN were 12.64 ± 1.19, and 10.29 ±

285 0.66 μM, respectively, suggesting that SFN slightly had higher efficacy than AITC to inhibit
286 A549 cell growth. In correspondence with our results, SFN also had lower IC_{50} doses than AITC
287 in inhibiting growth of 8226/S myeloma and HepG2 cells after being treated for 3 days [13]. In
288 addition, the cytotoxic effects of isothiocynates were selective. AITC and SFN did not
289 demonstrate toxicity in non-malignant cells at the concentrations that they could inhibit growth
290 of cancers [14, 15]. Furthermore, they have been shown to possess antioxidant property in healthy
291 cells by lowering ROS through phase II detoxification proteins [16-18].

292 Based on the IC_{50} values of AITC and SFN on A549 cells, a combination between AITC
293 and SFN at ratio of 1.25:1 was used in comparison to the single treatment. Our analysis using
294 Chou and Talalay's model[6] displayed the similar degree of synergism with the combination
295 index ranging from 0.82 – 0.94 over concentrations varied in this study. Through combination
296 index analyses, AITC and SFN combined treatment exhibited synergism by lowering
297 concentrations of AITC and SFN 2 – 2.9 -fold compared to the results of each single compound.
298 This moderate to slight interaction was possibly due to characteristics of natural bioactives that
299 are multi-targeting but milder in comparison to pharmaceutical drugs. Supporting evidence
300 showed that majority of natural compounds in combination provided 2 – 10 fold anticancer
301 improvements [19]. As being shown in figure 1, 72-hour treatment of the mixture between AITC
302 and SFN synergistically inhibited growth of A549 cells. In contrast, the combined treatment with
303 one compound presented at a time (either 36-hour AITC followed by 36-hour SFN or SFN
304 followed by AITC) did not demonstrate any synergy (data not shown). These data suggested that
305 both compounds needed to be applied at the same time to allow enhancement of
306 chemopreventive effect of these two isothiocyanates.

We further demonstrated that the combination of AITC and SFN synergistically increased apoptotic cells, particularly in late apoptosis. When comparing the values of CI obtained from cell survival MTT assay and the values obtained from flow cytometric apoptosis assay, we found a stronger synergy from anti-apoptotic activity (CI = 0.61 ± 0.03) in comparison to the anti-proliferative activity of A549 cells (CI = 0.82 ± 0.02) using MTT assay. This information suggested that the isothiocyanate AITC and SFN in combination played important roles to control cell growth at least through apoptosis pathway. However, there are other factors such as cell cycle arrest, necrosis, autophagy, as well as phase II detoxification system that might also affect the overall efficacy of the treatments on A549 cell survival. The validity of this result was demonstrated by Western Blotting, which treatments clearly increased pro-apoptotic and decreased anti-apoptotic proteins expression. A transcription factor, p53, is known to regulate apoptosis upon the increase of its expression through the activation of downstream proteins such as caspase-3, PARP, Bcl-xL, and survivin [20]. Isothiocyanates both single and combined treatments dose-dependently increased the expression of p53 and other pro-apoptotic proteins. The increase of p53 led to an obvious decrease of the inactive form caspase-3 and to increase expression of the active cleaved caspase-3 under combination treatment at high concentrations. PARP which has dual role in both DNA repair and apoptosis relatively expressed at constant levels under all treatments. Corresponding with the expression of cleaved caspase-3, cleaved PARP was also induced by the combination treatment. Protein expression of an anti-apoptotic protein, survivin which has a function to inhibit caspases [21], was decreased, especially by the high-dose combination. However, the expression of Bcl-xL which is also an anti-apoptotic protein was not under-regulated by isothiocyanate treatments. Up to this point, our information

329 suggested that isothiocaynate treatments induced apoptosis through p53 transcription factor and
330 some of its downstream proteins including cleaved-caspase3, cleaved-PARP, and survivin.
331 Regarding p53 expression that did not only regulate apoptotic event but also led to an
332 effect on cell cycle arrest, p53 could signal growth arrest of cell at a checkpoint to allow DNA
333 damage to be repaired before DNA replication or to lead cell arrest before entering mitosis and
334 undergo apoptosis when the damage was irreparable [20, 22]. Our results demonstrated a trend of
335 cells in G2/M phase increase under both single and combined treatments upon dose increment,
336 particularly the high-dose combination that significantly increased cells in G2/M phase and
337 decreased cells in G0/G1 phase. Although there was no synergy obtained on G2/M phase arrest,
338 the data was corresponding to the previous experiment that the combination of 12.5 μM AITC
339 with 10 μM SFN could synergistically induce apoptosis. Taking these data together, the
340 combination treatment once reaching certain concentration at the ratio used in this study possibly
341 induced DNA damage as being indicated in several studies[4, 23, 24], and led to cell cycle arrest at
342 G2/M phase and apoptosis. At the molecular level, this was supported by the efficient inhibition
343 of the expression of cyclin B1, a regulatory protein in mitosis while a protein marker of G1 phase
344 (cyclin D1), and a marker of S phase (cyclin E) were increased (data not shown). In addition, p21
345 which is one of the inhibitors of cyclin-dependent kinase that regulates cells mitosis phase was
346 also increased in expression.
347 The increase of intracellular ROS under AITC and SFN combination treatment was
348 correlated with cell cycle arrest and apoptosis. Single treatment did not significantly affect the
349 ROS level possibly according to their low doses. This information was consistent with other
350 studies using SFN and other isothiocyanates on many cancer cell lines including lung cancer [25-
351 28]. These data indicated that high doses of isothiocyanates could increase ROS and depleted

reduced glutathione leading to cell cycle arrest and apoptosis induction. Therefore, through ROS generation causing DNA damage, the combination of AITC and SFN mediated G2/M phase cell cycle arrest and late apoptosis.

Apart from cell viability, cell migration was also observed under treatments as an indicator of anti-metastatic/invasive property. Our results showed that A549 migration was significantly and synergistically delayed under AITC/SFN combination treatment. Higher combined concentrations demonstrated stronger synergy by lowering CI values. Expression of COX-2 was decreased by isothiocyanates AITC and SFN, especially when they were combined. Reducing COX-2 expression could lower the level of prostaglandin E2 production, leading to a less promotion of tumor growth due to prostaglandin E2 activating pathways that control cell proliferation, migration, apoptosis, and/or angiogenesis [29]. Besides COX-2, STAT3 also regulates the expression of various genes involving proliferation, apoptosis, angiogenesis, invasion, and metastasis [30, 31]. Here, we showed that the combination treatment clearly decreased phosphorylated STAT3, an active form, as well as MMP9 which has a function in metastatsis to facilitate cells penetration through extracellular matrix [32].

Our findings showed that the combined treatment of isothiocyanates particularly AITC and SFN synergistically acted as chemopreventive agents in the inhibition of cancer proliferation and progression. These synergistic effects could be due to the fact of low doses of compounds utilization which could minimize the development of drug resistance [7]. In cancer cells, there are transporter proteins which the increase of their expressions involved in the mechanism of drug resistance. BCRP is one of them which was found to be unaffected by AITC, SFN, and their combination (data not shown). These data suggested that our isothiocyanate treatments did not increase drug resistance in A549 which corresponded with a study in breast and lung cancer

cells [33]. The use of more than one compound as a treatment may also act through different mechanisms and provide an efficient outcome. However, more information is still necessary for a better understanding in the mechanistic actions behind the synergy of compounds in combination. Additionally, the concentration rages of AITC (1.25-12.5 μM) and SFN (1-10 μM) used throughout this study were reasonable in comparison to the concentration of AITC and SFN found in blood of rats and mice after oral application of the compounds [14, 34]. This suggests a high possibility to obtain similar synergy in an *in vivo* model as well.

In summary, the present study provided evidence supporting potential of the combined treatment of AITC and SFN that they synergistically multi-targeted the system of proliferation and metastasis of A549 non-small cell lung cancer cells. We also demonstrated cell cycle arrest and apoptosis mediated by the treatments through intracellular ROS signaling. These results demonstrated the synergy from AITC and SFN combined treatment that could be useful for further *in vivo* and clinical studies as well as being a guidance to prevent lung cancer.

Conflict of interest

The authors declare that there is *no conflict of interest.*

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Abbreviations

AITC, allyl isothiocyanate; Bcl-xL, B-cell lymphoma-extra-large; CI, combination index; COX-2, cyclooxygenase2; MMP9, matrix metalloproteinase9; PARP, poly ADP ribose polymerase; p-STAT3, phosphorylated STAT3; ROS, reactive oxygen species; STAT3, signal transducer and activator of transcription3; SFN, sulforaphane

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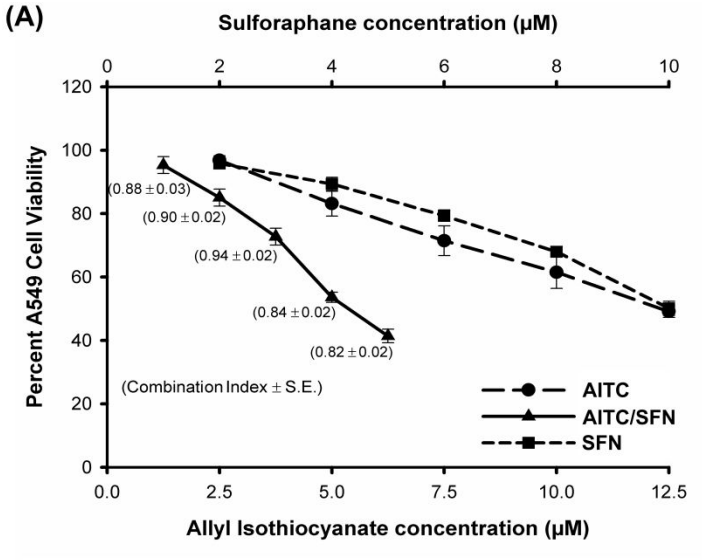
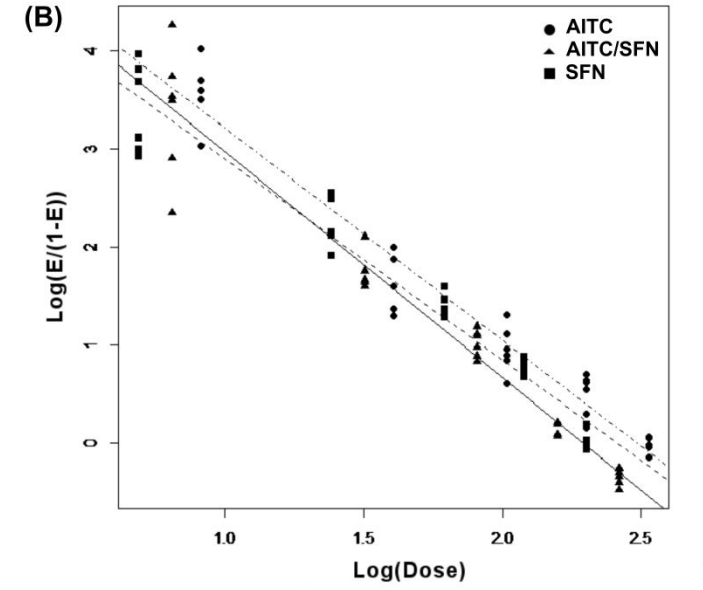
Table 1. Correlation analysis of oxidative stress and apoptosis or cell cycle arrest

factor	Pearson correlation coefficient	p value
early apoptosis	0.795	0.006
late apoptosis	0.957	< 0.001
total apoptosis	0.932	< 0.001
G0/G1 phase	-0.757	0.011
S phase	-0.558	0.93
G2/M phase	0.810	0.004


(A)
Sulforaphane concentration (μM)
0
2
4
6
8
10
120
100
80
60
40
20
0
Percent A549 Cell Viability
(0.88 ± 0.03)
(0.90 ± 0.02)
(0.94 ± 0.02)
(0.84 ± 0.02)
(0.82 ± 0.02)
(Combination Index ± S.E.)
AITC
AITC/SFN
SFN
0.0
2.5
5.0
7.5
10.0
12.5
Allyl Isothiocyanate concentration (μM)


(B)
AITC
AITC/SFN
SFN
Log(E/(1-E))
4
3
2
1
0
1.0
1.5
2.0
2.5
Log(Dose)

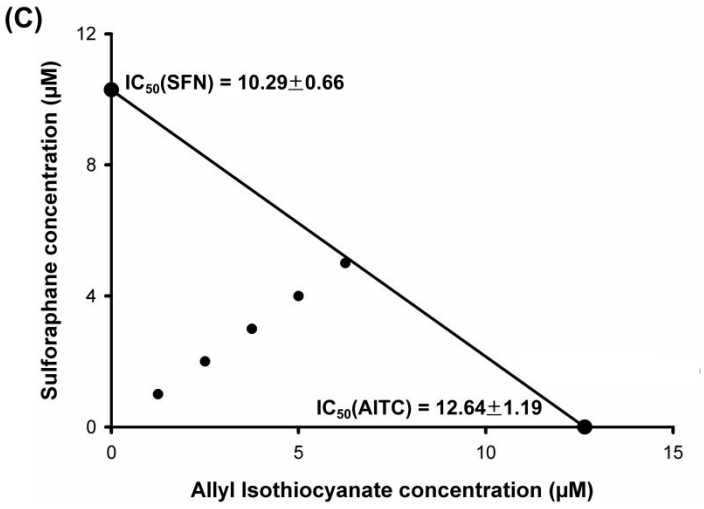

(C)
12
8
4
0
Sulforaphane concentration (μM)
IC50(SFN) = 10.29±0.66
IC50(AITC) = 12.64±1.19
0
5
10
15
Allyl Isothiocyanate concentration (μM)

Figure 1. Growth inhibitory effects of AITC, SFN, and their combined treatment on non-small cell lung cancer A549 cells. Cells were treated for 72 hours before viability measurement by MTT assay. Data are shown as mean ± SD (n = 6). Combination indexes are shown in parentheses (A). Median-effect plot (B) and isobologram analyses (C) of synergy between the combination of AITC and SFN at different concentrations (1.25 μM AITC + 1 μM SFN, 2.5 μM AITC + 2 μM SFN, 3.75 μM AITC + 3 μM SFN, 5 μM AITC + 4 μM SFN, and 6.25 μM AITC + 5 μM SFN) with in the ratio of 1.25:1 were constructed using Chou and Talalay's method.

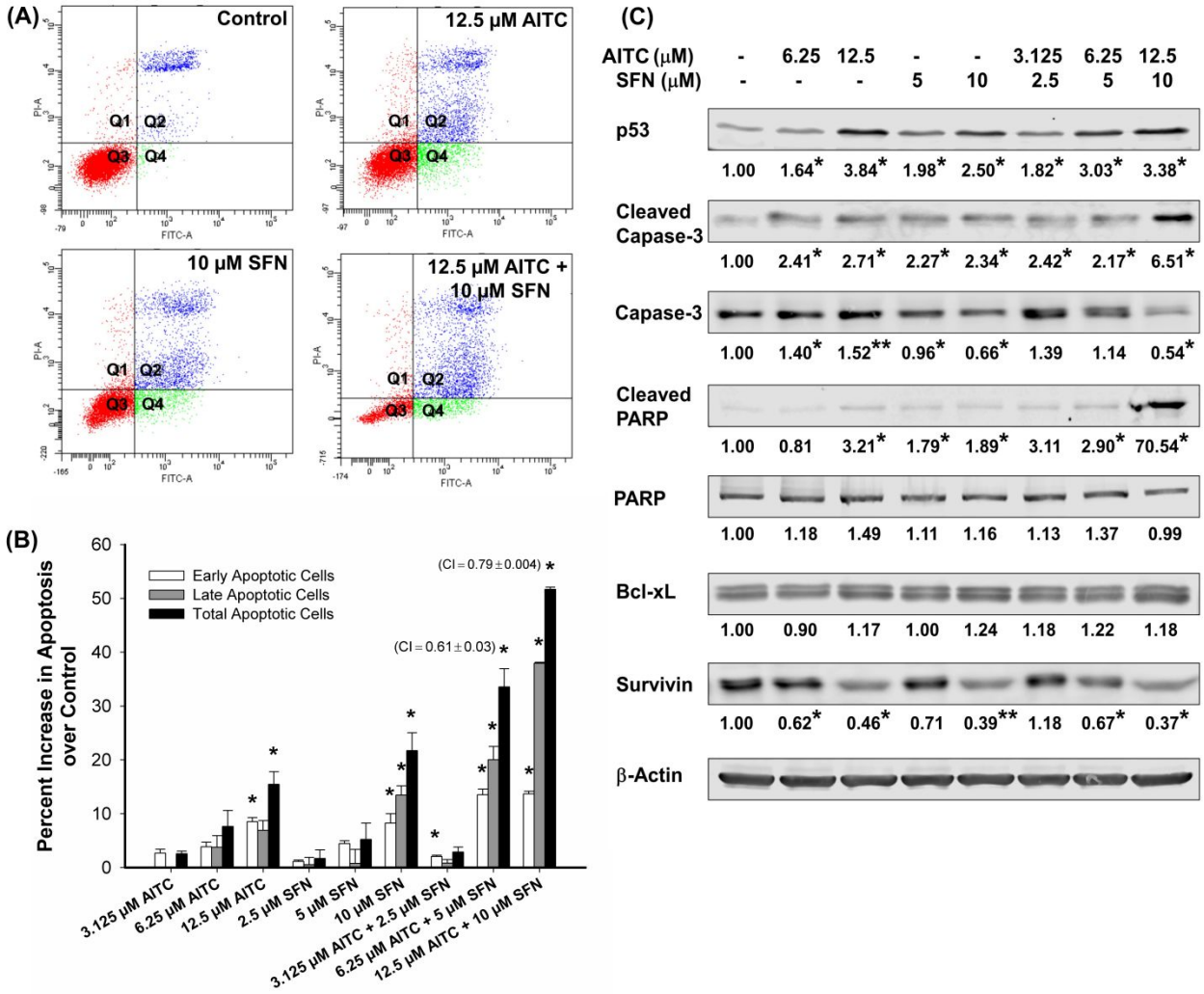
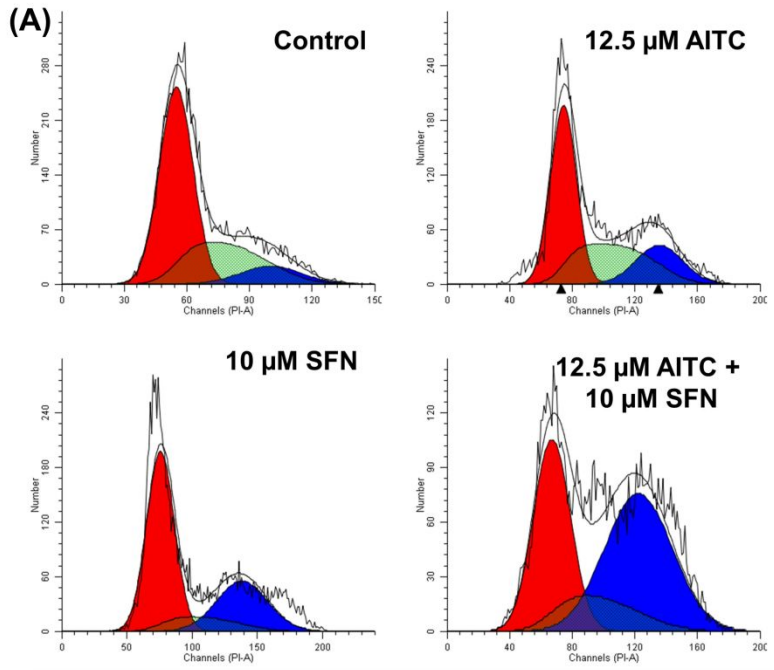
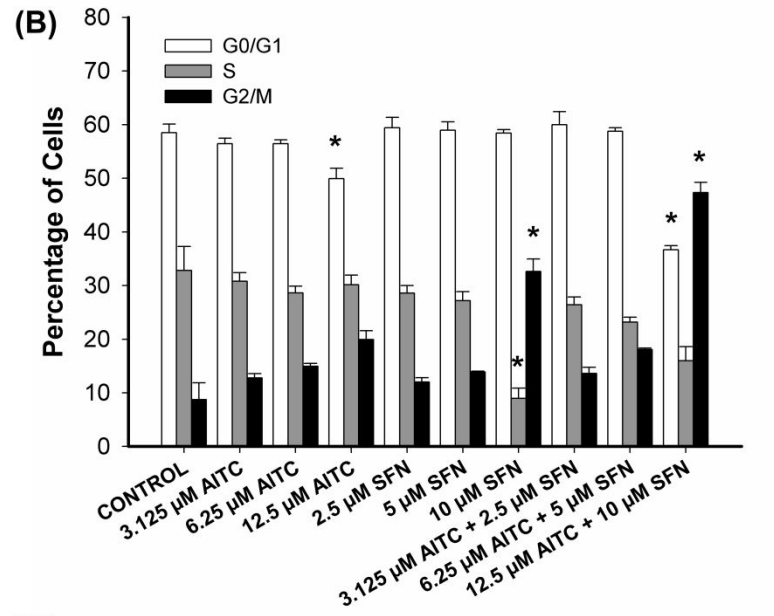


Figure 2. Effect of AITC, SFN, and their combination on apoptosis. Cancer cells were treated for 72 hours, followed by apoptosis measurement with flow cytometry after Annexin-V/PI co-staining. (A) Representative images of Annexin-V/PI intensity dot plots of A549 cells showed significantly increased dot intensity in Q2 (late apoptosis) and Q4 (early apoptosis) region and decreased dot intensity in Q3 region (non-apoptotic cells) in the AITC-SFN combined treatment group. (B) Percent apoptotic cells were calculated from the Annexin-V/PI dot plots. Results are presented as mean ± SD (n = 3; *p < 0.05). Combination index (CI) ± SE are in parentheses. (C) Expression of relating proteins were monitored by Western Blotting. The protein band intensities underneath the blots were quantified using Image Studio software. Standard deviations (within ± 20% of the mean) were not shown. Asterisks indicate statistical significance (*p < 0.05, **p < 0.001; n = 3) in comparison to non-treated control. β-Actin served as an internal loading control.


(A)
Control
12.5 μM AITC
10 μM SFN
12.5 μM AITC +
10 μM SFN
Number
Channels (PI-A)


(B)
G0/G1
S
G2/M
Percentage of Cells
CONTROL
3.125 μM AITC
6.25 μM AITC
12.5 μM AITC
2.5 μM SFN
5 μM SFN
10 μM SFN
3.125 μM AITC + 2.5 μM SFN
6.25 μM AITC + 5 μM SFN
12.5 μM AITC + 10 μM SFN

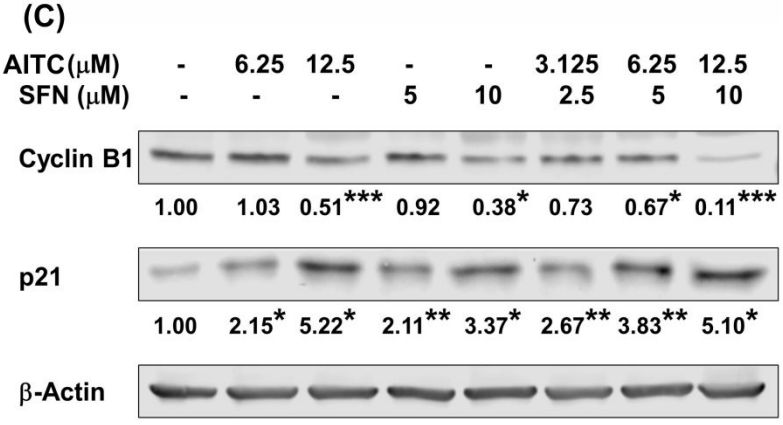

(C)
AITC (μM) - 6.25 12.5 - - 3.125 6.25 12.5
SFN (μM) - - - 5 10 2.5 5 10
Cyclin B1
1.00 1.03 0.51*** 0.92 0.38* 0.73 0.67* 0.11***
p21
1.00 2.15* 5.22* 2.11** 3.37* 2.67** 3.83** 5.10*
β-Actin

Figure 3. Effect of AITC, SFN, and their combination on cell cycle after 72-hour treatments. Cancer cells were fixed with ethanol, treated with RNAse and PI before determining cell cycle progression by flow cytometry. (A) Representative images of A549 cell cycle histogram showed significantly increased G2/M phase arrest in the AITC-SFN combined treatment group. (B) Percent cells population in each phase were calculated from the cell cycle histogram. Results are presented as mean ± SD ($n = 3$; $^*P < 0.05$). (C) Expression of cyclin B1 and p21 which relates to G2/M phase arrest were determined using Western Blotting. The protein band intensities underneath the blots were quantified using Image Studio software. Standard deviations (within ± 20% of the mean) were not shown. Asterisks indicate statistical significance ($^*p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$; $n = 3$) in comparison to non-treated control. β-Actin served as an internal loading control.

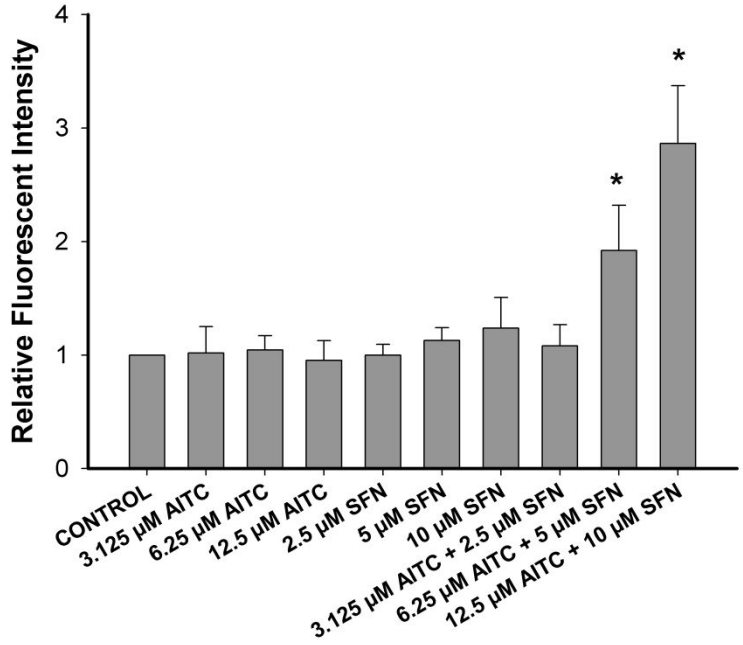
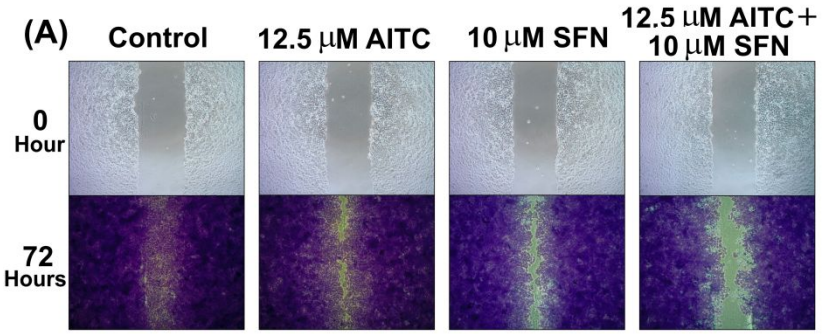
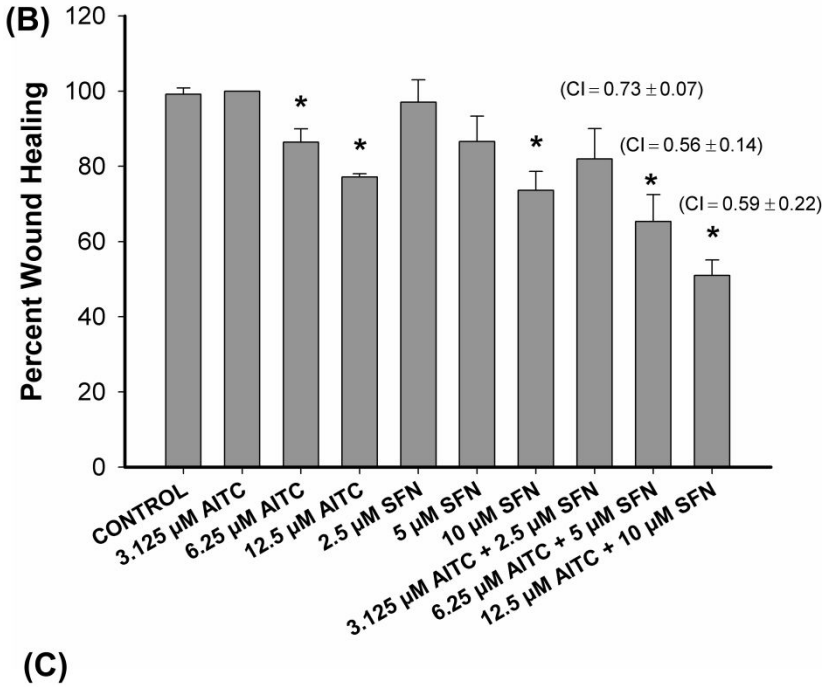


Figure 4. Effect of AITC, SFN, and their combination on cellular ROS. Cancer cells were incubated with the indicated treatments for 72 hours and stained with DCFH-DA before detection by flow cytometry. Results are presented as mean ± SD (n = 3; *p < 0.05).


(A)
Control
12.5 μM AITC
10 μM SFN
12.5 μM AITC+ 10 μM SFN
0 Hour
72 Hours


(B)
Percent Wound Healing
120
100
80
60
40
20
0
(CI = 0.73 ± 0.07)
(CI = 0.56 ± 0.14)
(CI = 0.59 ± 0.22)
CONTROL
3.125 μM AITC
6.25 μM AITC
12.5 μM AITC
2.5 μM SFN
5 μM SFN
10 μM SFN
3.125 μM AITC + 2.5 μM SFN
6.25 μM AITC + 5 μM SFN
12.5 μM AITC + 10 μM SFN

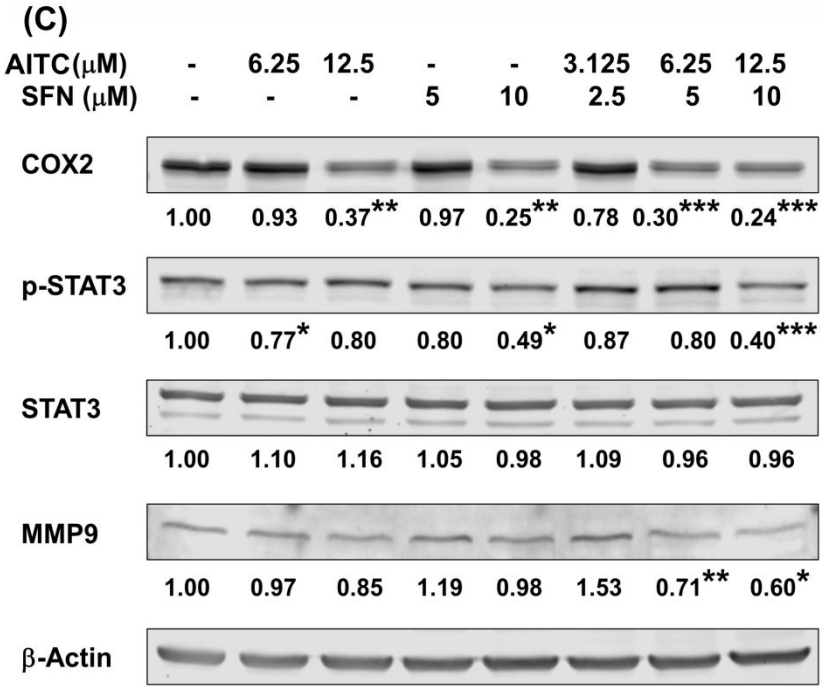

(C)
AITC(μM) - 6.25 12.5 - - 3.125 6.25 12.5
SFN (μM) - - - 5 10 2.5 5 10
COX2
1.00 0.93 0.37** 0.97 0.25** 0.78 0.30*** 0.24***
p-STAT3
1.00 0.77* 0.80 0.80 0.49* 0.87 0.80 0.40***
STAT3
1.00 1.10 1.16 1.05 0.98 1.09 0.96 0.96
MMP9
1.00 0.97 0.85 1.19 0.98 1.53 0.71** 0.60*
β-Actin

Figure 5. Effect of AITC, SFN, and their combination on cell migration after 72-hour treatments. (A) Representative images taken at hour-0 in comparison to hour-72 using 4× magnification showed significant inhibitory wound healing by the combination treatment. (B) Percent wound healing was calculated from width of the wound. Results are presented as mean ± SD ($n = 4$; $^{*}p < 0.05$). (C) Expression of proteins related to cell migration were determined using Western Blotting. The protein band intensities underneath the blots were quantified using Image Studio software. Standard deviations (within ± 20% of the mean) were not shown. Asterisks indicate statistical significance ($^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$; $n = 3$) in comparison to non-treated control. β-Actin served as an internal loading control.

Graphical Abstract