Synergistic effect of allyl isothiocyanate (AITC) on cisplatin efficacy in vitro and in vivo

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Received May 18, 2015; Accepted June 30, 2015; Epub July 15, 2015; Published August 1, 2015

Abstract: Although in vitro studies have shown that isothiocyanates (ITCs) can synergistically sensitize cancer cells to cisplatin treatment, the underlying mechanisms have not been well defined, and there are no in vivo demonstrations of this synergy. Here, we report the in vitro and in vivo data for the combination of allyl isothiocyanate (AITC), one of the most common naturally occurring ITCs, with cisplatin. Our study revealed that cisplatin and AITC combination synergistically inhibits cancer cell growth and colony formation, and enhances apoptosis in association with the downregulation of antiapoptotic proteins Bcl-2 and survivin. Importantly, the in vivo combination treatment suppresses human tumor growth in animal models without observable increases in toxicity (body weight loss) in comparison with single agent treatment. Furthermore, our data revealed that addition of AITC to cisplatin treatment changes the profile of G2/M arrest (e.g. increase in M phase cell number) and significantly extends the duration of G2/M arrest in comparison with cisplatin treatment alone. To explore the underlying mechanism, we found that AITC treatment rapidly depletes β-tubulin. Combination of AITC and cisplatin inhibits the expression of G2/M checkpoint-relevant proteins including CDC2, cyclin B1 and CDC25. Together, our findings reveal a novel mechanism for AITC enhancing cisplatin efficacy and provides the first in vivo evidence to support ITCs as potential candidates for developing new regimens to overcome platinum resistance.

Keywords: Cisplatin, AITC, combination index (CI), survivin, Bcl-2, microtubule, cancer cells, human tumor mouse model

Introduction

Cisplatin (cis-diaminedichloroplatinum II, CDDP) is one of the most widely used anticancer drugs [1]; however, its clinical efficacy is often limited by primary or secondary acquired resistance. Several mechanisms are involved in cisplatin resistance development, including reduced drug uptake, increased cellular thiol/folate levels and increased DNA repair [2-4]. Current studies show that activation of anti-apoptotic pathways may also contribute to the resistance phenotype [5-11]. A combination of cisplatin with other therapeutic agents to enhance tumor sensitivity and decrease unwanted systemic toxicity is an attractive area of study.

Previous studies showed that cisplatin and a new synthetic isothiocyanate (ITC) derivate, ethyl 4-isothiocyanatobutanoate (E-4IB), can synergistically inhibit cell growth in both ovarian cancer cell line A2780 and its cisplatin-resistant variant A2780/CP in vitro [12]. Further studies found that this synergistic effect is related to increased intracellular platinum accumulation, glutathione level depletion and mitochondrial membrane potential dissipation [13]. These events were also accompanied with changes in apoptosis and cell cycle related pathways [13]. Later studies found that not only E-4IB but also indol-3-ethyl isothiocyanate (homoITC), benzyl isothiocyanate (BITC) and phenethyl isothiocyanate (PEITC) could synergistically enhance cell sensitivity to cisplatin treatment in vitro, and confirmed that this syn-
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ergistic effect is independent of cisplatin sensi-
tivity [14-16]. Di Pasqua, et al. recently pub-
lished on the sensitization of lung cancer cells
to cisplatin by naturally occurring isothiocya-
nates, and showed that structural variations
among the isothiocyanates affected their ability
to sensitize cancer cells, and this correlated
with their ability to deplete β-tubulin [17].
Although accumulated evidence suggests that
several natural or synthetic ITCs could sensitize
cancer cells to cisplatin treatment, the detailed
underlying mechanisms remain largely unde-
fined. Furthermore, to the best of our knowl-
edge, there is no evaluation of the effect of ITCs
and cisplatin combination in tumor growth in vivo.

Allyl isothiocyanate (AITC) is one of the most
common naturally occurring ITCs and is found
in many cruciferous vegetables [18]. It has
been reported that AITC inhibits various types
of cancer cell growth through multiple mecha-
nisms such as apoptosis induction and cell
cycle arrest [19-22], and has very good oral bio-
availability [23, 24]. Further studies found that
short-term treatment with AITC inhibits cancer
cell growth with IC50 values in the low µM
range, even in drug resistant cancer cells [19,
25, 26]. Interestingly, AITC was shown to be
more toxic to cancer cells than to either normal
human epithelial cells or de-transformed
human cancer cells [21, 27, 28]. The IC50 value
of AITC in normal human bladder epithelial cells
is approximately 10 times higher than that in
human bladder cancer cells [28]. Studies have
also demonstrated that 10 micromoles AITC
given through intraperitoneal injection (three
times per week for three weeks) could signifi-
cantly inhibit PC-3 human prostate cancer
 xenografts in athymic mice with no apparent
toxicity [29], suggesting an effective concentra-
tion of AITC for tumor inhibition in vivo. An earlier study also showed that the peak
plasma concentrations of AITC in the blood of
both mice and rats, following a single oral dose
of [14C] AITC at 25 and 250 µmol/kg, were
approximately 40 µM and 500 µM, respectively
[23, 24], well above the range used to achieve
synergistic response with cisplatin in our study.
Additional studies revealed that ITCs could be
concentrated in cancer cells through GSH con-
jugation [30, 31], suggesting that intracellular
concentration of AITC can be much higher than
extracellular or plasma concentration. Taken
together, these prior studies suggest that the
minimum concentration of AITC required to fully
realize a synergistic effect is reachable in vivo
and that AITC is a good candidate for in vivo
combination study.

In the present study, we sought to determine
whether AITC could enhance cancer cell sensi-
tivity to cisplatin treatment in vitro and in vivo
and further explore its mechanisms.

**Material and methods**

**Cell culture and reagents**

Human ovarian cancer cell line 2008 (a gift
from Dr. Kunle Odunsi, Roswell Park Cancer
Institute), human lung cancer cell lines HOP62
(a gift from Dr. Daniel Chang, University of
Colorado) and A549 (ATCC) were maintained in
RPMI 1640 medium supplied with 10% fetal
bovine serum (FBS, Atlanta Biologicals,
Lawrenceville, GA), penicillin (100 units/ml)
and streptomycin (0.1 µg/ml) (Invitrogen, Grand
Island, NY). Cells were routinely subcultured
twice a week and maintained in a humidified
incubator with 5% CO2 at 37°C. Cisplatin was
purchased from VWR (West Chester, PA). AITC,
IGEPAL CA-630, phenylmethyl sulfonyl fluoride,
monoclonal anti-β-tubulin antibody, polyclonal
anti-actin antibody and goat peroxidase-conju-
gated anti-rabbit IgG antibody were purchased
from Sigma (St. Louis, MO). Survivin (FL-142),
cyclin B1 and polyclonal p-histone H3 (Ser10)
antibodies were purchased from Santa Cruz
(Santa Cruz, CA). Monoclonal Cdc2 and Cdc25C
antibodies were purchased from Epitomics
(Burlingame, CA). Alexa Fluor® 594 goat anti-
rabbit IgG and Alexa Fluor® 488 goat anti-
mouse IgG were purchased from Invitrogen
(Carlisle, CA). Antibodies against Bcl-2 and
activated caspase 3 (SA320) were purchased
from Cell Signaling (Beverly, MA). MTT
(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetra-
zolium bromide) and leupeptin were purchased
from USB (Cleveland, OH). Cell Death Detection
ELISA assay kit was purchased from Roche
(Indianapolis, IN).

**MTT cell viability assay**

The inhibitory effect of cisplatin (CDDP) and
AITC, alone and in combination, on cell growth
was determined by MTT cell viability assay.
Viable cells (2500 cells per well) were plated in
each well in 96-well plates. After an overnight
incubation, cells were treated with and without
cisplatin and/or AITC at various concentrations
and incubated for 96 hours. MTT, a colorimetric
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substrate, was added to a final concentration of 0.4 mg/ml to each well. Cells in 96-well plates were further incubated in a 5% CO₂ incubator at 37 °C for 4 hours, and then the medium was aspirated. The MTT metabolic product formazan was solubilized by adding 200 ul of DMSO to each well. Absorbance in the relevant wells was measured at 570 nm using an Ultra Microplate Reader (Bio-Tek Instruments). The experimental results were expressed as the mean ± standard deviation (SD). The statistical significance of differences was determined by Student’s t-test between two groups.

Cell colony formation assay

To determine the long-term effect of AITC and cisplatin (CDDP) combination, colony formation assays were carried out. 2008 cells and HOP62 cells were trypsinized and seeded into 6-well plates (200 cells per well). After being attached to the plate, the cells were treated with and without cisplatin and AITC, alone or in combination for 96 hours. Culture medium containing drugs in each well was replaced with fresh medium 96 hours after treatment. Cells were allowed to grow for 3 weeks. Colonies were then fixed with ice-cold methanol for 10 min and stained with 0.5% crystal violet solution in 25% methanol for 10 min. The stained 6-well plates were counted on a GS-800 Calibrated Densitometer using the Quantity One software (Bio-Rad, Hercules, CA) and reported as the average of triplicate wells.

Determination of combination index (CI) for AITC and cisplatin (CDDP) treatment combination

Effects of cisplatin and AITC combination on growth inhibition were analyzed by the Combination Index (CI) equation developed by Chou-Talalay [32, 33] using the CalcuSyn program (Biosoft, Cambridge, UK). The general equation for the classic isobologram is given by: CI = ([D₁/Dₜ₁]ₐ + [D₂/Dₜ₂]ₐ) where CI<1 indicates synergy; CI=1 indicates additive effect, and CI>1 indicates antagonism; [D₁/Dₜ₁]ₐ and [D₂/Dₜ₂]ₐ in the denominators are the doses (or concentrations) of D₁ (drug #1, for example, cisplatin) and D₂ (drug #2, for example, AITC) alone that gives x% inhibition, whereas [D₁/Dₜ₁]ₐ and [D₂/Dₜ₂]ₐ in the numerators are the doses of D₁ and D₂ in combination that also inhibits x% (i.e. isoeffective). The [D₁/Dₜ₁]ₐ and [D₂/Dₜ₂]ₐ can be readily calculated from the median-effect equation of Chou [34]:

\[ D_x = D_m \left( \frac{f_a}{1-f_a} \right)^{1/m} \]

where Dᵢ is the median-effect dose, f_a is the fraction affected, Dₘ is the median-effect dose signifying potency and m is the kinetic order signifying the shape of dose-effect curve. A 3-D plot of CI versus concentrations of both Drug #1 and Drug #2 was obtained for each treated cell line as descried before [35].

Propidium iodide (PI) staining and flow cytometric analysis

Cancer cells were treated with or without AITC and cisplatin (CDDP) alone or in combination for 12, 16, 24, 48 and 72 hours, then harvested by trypsinization and washed with PBS. Cells (~1 x 10⁶) were resuspended in 5 ml 70% ethanol. After the initial fixation, cells were suspended in 0.5 ml PBS containing 25 μg/ml PI, 0.2% Triton X-100 and 40 μg/ml RNase A, incubated for at least 30 minutes at 4°C with PI and then analyzed by flow cytometry as previously described [36]. Data from flow cytometry were analyzed using WinList software (Verity Software House Inc., Topsham, ME) and presented as a percentage of total cells in G2/M phase. Triplicate assays were performed. The experimental results were expressed as the mean ± standard deviation (SD). The statistical significance of differences was determined by Student’s t-test between two groups.

Cell death detection ELISA assay

Apoptosis was measured by quantification of the histone-complex DNA fragments (mono- and oligonucleosomes) using the Cell Death Detection ELISA assay kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions. 2008 cells were seeded in triplicates in 96-well plates and treated with and without AITC and cisplatin (alone or in combination) for 48 hours. The levels of mono- and oligonucleosomes released into the cytoplasm were measured at 405 nm, using an ABTS solution as a blank control. The Enrichment factor was calculated using the following formula:

\[ \text{Enrichment factor} = \frac{A_{405 \text{ drug treated samples}}} {A_{405 \text{ no treatment}}} \]

The experimental results were expressed as the mean ± standard deviation (SD). The statistical significance of differences was determined by Student’s t-test between two groups.
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Figure 1. Combination of cisplatin (CDDP) with AITC inhibits cancer cell growth. A and B. 2008 ovarian cancer cells and Hop62 lung cancer cells were treated with different concentrations of AITC and cisplatin alone or in combination. After 96-hour treatment, MTT assay was used to determine cell growth status. Each bar is the mean ± SD from at least 3 independent parallel experiments. C and D. Colony formation assays. Two hundred 2008 cells or Hop62 cells were seeded per well in six-well plates and treated with indicated doses of cisplatin and/or AITC for 96 hours and then cultured in medium without drugs. Photographs were taken three weeks after treatment. E and F. Colonies were stained and counted three weeks after treatment. Each bar is the mean ± SD from 3 independent triplicates. *Significant difference compared to single drug treatment alone (P<0.01); other P values are marked on the relevant images. Cisplatin for 2.5 µg/ml, 5 µg/ml and 10 µg/ml is equivalent to cisplatin for 8.3 µM, 16.7 µM and 33.3 µM.

Triple staining of DNA, p-histone H3 and β-tubulin, and fluorescence microscopy observation

2008 cells and A549 cancer cells were seeded on round glass cover slips which were coated with 2% gelatin in 12-well plates. Sixteen hours after AITC and/or cisplatin treatment, cells were fixed with 4% paraformaldehyde in PBS for triple staining of DNA, β-tubulin and p-histone H3 (Ser10), a mitotic marker. After one hour fixation, cells were blocked/permeabilized with PBS containing 2% BSA and 0.2% Triton X-100 for 30 minutes, and then incubated in PBS containing 1% BSA, an anti-β-tubulin antibody (1:2000) and an anti-p-histone H3 antibody (1:2000) for 60 min at 37°C. After washing with PBS, cells were incubated in PBS containing Alexa Fluor® 594 goat anti-rabbit IgG (1:2000) and Alexa Fluor® 488 goat anti-mouse IgG antibody (1:2000) for 45 min at room temperature, followed by staining with 4,6-diamidino-2-phenylindole (DAPI) at a final concentration of 0.5 µg/ml in PBS for 15 min. Cover slips were mounted onto glass slides with Gel/MountTM solution (Biomedia, Foster City, CA). Cell morphology was visualized and digitally captured using a Zeiss Axiovert 100 M Fluorescence Microscopy System.

Double staining of p-histone H3 and DNA

Sixteen hours after drug treatment (see above), cells were double-stained for DNA and p-his-
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A

Percentage of G2/M phase cells

control
CDDP 5 μg/ml
CDDP 10 μg/ml
AITC 10 μM
CDDP 5 μg/ml + AITC 10 μM
CDDP 10 μg/ml + AITC 10 μM

8h 12h 18h 24h 48h 72h

2008

B

A549

Control CDDP Control CDDP

AITC CDDP + AITC AITC AITC+CDDP

2008

C

p-H3
Actin

2008
Control CDDP5 CDDP10 AITC10 AITC+CDDP5 AITC+CDDP10

D

E

A549 Control CDDP

G1: 78%
S: 1.6%
G2/M: 18.5%

G1: 68%
S: 3.2%
G2/M: 28%

G1: 58%
S: 8.8
G2/M: 29.8%

G1: 28%
S: 3.43%
G2/M: 66.85%
Synergistic interaction of AITC with cisplatin

Figure 2. Combinational regimen of AITC and cisplatin (CDDP) changes the time-dependent G2/M arrest profile compared to single drug treatment. A. 2008 cells were treated with or without AITC and/or cisplatin for the duration as shown. Cells (~1 x 10^6) were fixed and prepared for analysis with flow cytometry (20,000 events per sample) as noted in Materials and Methods. Raw data were then analyzed using WinList software. B and D. 2008 cells or A549 cells were seeded on round glass cover slips coated with 2% gelatin in 12-well plates and treated with or without AITC and cisplatin for 24 hours. Cells were then incubated first with a p-histone H3 antibody and then with a fluorescent secondary antibody (pink), and finally stained with DAPI (blue) and visualized under fluorescent microscope. C. Expression of p-H3 in 2008 cells was determined by Western blotting. Actin is presented to verify protein loading. E. Cell cycle distribution in A549 cells was determined by flow cytometry at 16 hours. Cisplatin for 5 μg/ml and 10 μg/ml is equivalent to cisplatin for 16.7 μM and 33.3 μM.

tone H3. For double staining of DNA and p-histone H3, cells were fixed/blotted as described above, and then incubated in PBS containing 1% BSA and an anti-p-histone H3 antibody (1:2000) for 60 min at room temperature. After washing with PBS, cells were incubated in PBS containing Alexa Fluor® 594 goat anti-rabbit IgG (1:2000) for 45 min at room temperature, followed by staining with DAPI at a final concentration of 0.5 μg/ml in PBS for 10 min. Coverslips were mounted on glass slides with Gel/Mount™ solution (Biomedia, Foster City, CA). Cell morphology was visualized and digitally captured as described above.

Western blot analysis

Cells treated with and without cisplatin (CDDP) and/or AITC were lysed in RIPA buffer containing 150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0. Fifty μg total protein from each sample were heated at 95°C for 5 minutes after mixing with equal volume of 2X SDS loading buffer. Samples were separated on 12-15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels and electrotransferred to Pure Nitrocellulose Membranes (Bio-Rad, Hercules, CA). The membrane was then blocked in 5% skim milk in TBS-T containing 1% Tween 20) at room temperature for 2-3 hours. Next, the membrane was incubated with different primary antibodies in TBS-T containing 5% BSA overnight at 4°C in the range of dilutions from 1:500 to 1:2000. After washing with TBS-T, the membrane was incubated in TBS-T buffer containing 5% skim milk and corresponding secondary antibody (1:5000) for 45-60 minutes at room temperature with shaking. Protein of interest was detected using Western Lightning Plus-ECL (Perkin Elmer, Waltham, MA) and visualized by various times (30-120 seconds) of exposure. Actin was detected as the internal control to normalize total protein loading for each sample.

Human tumor xenograft mouse model and treatment

All experiments were performed following the IACUC-approved mouse protocol.

Animal work followed our previously published protocols [37]. Briefly, A549 lung cancer cells were first used to establish tumor mass. Specifically, tumor cells grown in culture medium were harvested by trypsinization, washed twice in ice-cold PBS, and adjusted to 2 × 10^7 viable cells/ml. A 0.1-0.2 ml cell suspension was injected subcutaneously into the right flank of female athymic nude mice to establish sufficient tumor size in about 3 weeks. After tumor tissues were isolated from established tumors, a piece of non-necrotic tumor tissues (30-40 mg) were subcutaneously transplanted into the flank area of 20 female athymic nude mice. Mice were then randomly divided into 4 groups (5 mice per group): a control group, an AITC group, a cisplatin group and a combination group. Based on the previous studies of AITC [29] and in consideration of the clinical relevance, we chose per oral (p.o.) instead of using the intraperitoneal (i.p.) route for AITC administration at a dose of 50 mg/kg formulated in vegetable oil, once every other day for two weeks starting from the day on which 30-40 mg tumor masses were subcutaneously implanted at the flank area on individual mice. Seven days after tumor implantation (defined as day 0) on which xenograft tumors reached 200-250 mm³, a one-time sub-maximal tolerated dose (MTD, 6 mg/kg) of cisplatin in a clinically used formulation was injected via the mouse tail vein. Tumor length (L) and width (W) were measured using a vernier caliper every other day on Days 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 28. The tumor volume (v) was calculated using the formula: v=0.5 (L × W²). The mean tumor volume at each time point was derived from 5 mice in each group.

Antitumor efficacy was assessed by 1) maximum tumor growth inhibition (MTGI), which is
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the mean tumor weight difference for treated group (MTWTG) versus mean tumor weight for untreated control group (MTWCG) at the same time [the calculated formula is \( MTGI = \frac{(MTWTG - MTWCG)}{MTWCG} \times 100\% \)]; 2) the tumor doubling time (TDT), which was defined as the mean time for the tumor reaching twice its initial weight from the time beginning the treatment (Day 0); 3) partial tumor response (PR), which was expressed as when tumor weight was reduced at least 50% initial tumor size on Day 0; and 4) complete tumor response (CR), which was defined as inability to detect tumor via palpitation at the initial tumor-transplanted site. All images were made using Sigma Plot software. The differences among different

Figure 3. G2/M arrest resulted from AITC and cisplatin (CDDP) combination treatment is associated with G2/M profile changes and microtubule depletion. (A and E) 2008 ovarian cancer cells and A549 lung cancer cells were treated with AITC for 0, 8, 16, 24, 48, and 72 hours and then lysed. Immunoblotting was carried out with antibodies for β-tubulin and p-Histone H3 in 2008 cells (A) and A549 (E). (B) 2008 cells were also treated with AITC at 1, 10, and 100 µM for 24 hours and then lysed. Immunoblotting was carried out with antibodies for β-tubulin and p-Histone H3 to determine the response to varying doses of AITC. (C and F) Investigation of morphological changes related to AITC and cisplatin treatment. 2008 cells and A549 cells were treated with or without AITC and/or cisplatin for 18 hours. Cells were then incubated first with antibodies to p-histone H3 and β-tubulin and then with secondary fluorescent antibodies (pink=p-histone H3, green=β-tubulin) and finally stained with DAPI (blue) and visualized under a fluorescent microscope. (D) 2008 ovarian cancer cells were treated with different concentrations of AITC and cisplatin alone or in combination for 48 hours and then lysed. Immunoblotting was performed for G2/M transition pathway-relevant proteins β-tubulin, CDC2, CDC25, and cyclin B1. Actin protein expression shown in (A-E) is the internal controls for verifying protein loading.
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**Figure 4.** AITC enhances pro-apoptotic effects induced by cisplatin (CDDP) treatment. 2008 cells were treated with or without AITC and/or cisplatin for 48 hours and then subjected to the following analysis. A. Cells were subjected to flow cytometry to detect sub-G1 cells. B. Cells were lysed and analyzed by an ELISA as per manufacturer’s instructions (Cell Death Detection ELISA). Individual bars shown in A and B are the mean ± SD from 3-5 independent parallel assays. C. Cells were lysed and analyzed by immunoblotting for Bcl-2, survivin, and active caspase 3 levels. Cleaved/activated caspase 3 is present at the 17 kD band. Actin is the internal control to verify protein-loading profiles. Cisplatin for 5 µg/ml and 10 µg/ml is equivalent to cisplatin for 16.7 µM and 33.3 µM.

Results

AITC significantly enhances cancer cell growth inhibition induced by cisplatin (CDDP) in MTT and colony formation assays.

To evaluate the effects of AITC and cisplatin combination in cell growth in vitro, an MTT viability assay was carried out 72 hours after drug treatment. We found that combination of cisplatin with AITC significantly inhibits cell growth in comparison to cisplatin or AITC treatment alone in both 2008 ovarian cancer cells and HOP62 lung cancer cells (P<0.05) (**Figure 1A** and **1B**). We next studied the ability of cancer cells to...
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Figure 5. The combination index (CI) profile of cisplatin (CDDP) and AITC treatment on growth inhibition. A549 (A) and Hop62 (B) lung cancer cells were treated with cisplatin and AITC at the doses as shown for 72 hours and subjected to MTT assays, and the results were analyzed by for CI using the CalcuSyn software (Biosoft). Each bar represents the corresponding CI from a specific AITC and cisplatin combination treatment. Of note, CI>1 means antagonist effects from the combination treatment; CI=1 means additive effects from the combination treatment; and CI<1 means synergistic effects from the combination treatment; and CI<0.5 means highly synergistic effects from the combination treatment.

Form colonies on 6-well plates in presence or absence of cisplatin and AITC, alone and in combination. Consistent with the MTT assay results, combination of cisplatin with AITC resulted in much less colony growth than in AITC and cisplatin treatment alone (Figure 1C and 1D). Quantitative analysis of colony formation data showed that inhibition of cancer cell colony formation by the combination of AITC and cisplatin could be a synergistic effect (Figure 1E and 1F).

Combination of AITC and cisplatin (CDDP) modulates cancer cell G2/M transition and G2/M arrest profiles

The G2/M checkpoint plays a key role in the response to cisplatin treatment [38-42]. To further explore if G2/M arrest is involved in combination-enhanced cell growth inhibition, we evaluated the changes in DNA distribution after drug treatment using flow cytometry. Our data showed that in AITC single drug treatment group, significant G2/M arrest starts at 8 hours and reaches the peak at 12-16 hours after treatment (P<0.05) (Figure 2A). The number of cells in G2/M phase begins to significantly decrease at 24 hours post-treatment. In the 10 µg/ml (33.3 µM) cisplatin group, the number of cells in G2/M begins increasing 16 hours after treatment, reaches its peak at 48 hours and begins to decrease 72 hours after cisplatin treatment. In the 5 µg/ml (16.7 µM) cisplatin group, increasing in G2/M phase cells begins at 16 hours and decreases 48 hours after treatment. However, G2/M arrest in the combination group appears at 8 hours and reaches its peak 12 hours after combination treatment, similar to the pattern seen in AITC treatment alone. In the late stage combination treatment, G2/M arrest levels stay high, much like the pattern observed in cisplatin treatment, indicating that the duration of G2/M arrest in AITC and cisplatin combination group has been extended in comparison with single drug treatment alone, which is likely important for the synergistic effect of combination treatment.

To better understand the extended G2/M arrest induced by combination of AITC and cisplatin (CDDP), we stained cells with DAPI and phospho-histone H3 (p-H3, a marker showing cells in the M phase), and explored the possibility that AITC and cisplatin combinations increase cell numbers in the M phase using fluorescent microscope. We observed round, p-H3-positive cells in the AITC treatment groups (Figure 2B). However, in the cisplatin treatment...
group, p-H3 positive cells which reflect the mitotic (M) phase cells clearly decreased (Figure 2B). In the combination group, there is no observable decrease in p-H3 positive cells compared to control, and there is an increase in M phase cells in comparison with cisplatin-treated cells alone (Figure 2B). These findings were further confirmed by western blots of p-H3. The results suggest that in the combination group, p-H3 is unchanged at 5 µg/ml (16.7 µM) cisplatin and slightly decreased in 10 µg/ml (33.3 µM) cisplatin as compared with no treatment. However, there is an observable increase in comparison to cisplatin treatment alone (Figure 2C). Intriguingly, flow cytometry-fractionated cells for cell cycle distribution, both cisplatin (CDDP) and AITC showed a cell arrest in the G2/M phase in comparison with control (Figure 2E). This suggests that there is a largely G2 arrest after cisplatin treatment alone. We know that there are only few M phase cells after cisplatin (CDDP) treatment (Figure 2B and 2D). Generally speaking, when the cells were arrested only in G2 phase, there will be an M phase decrease just like that shown in cisplatin treatment. However, in combination group, M phase cells have not been significantly decreased (Figure 2B), suggesting combination of AITC and cisplatin could arrest the cells in both G2 and M phase. Similar results were observed in A549 cells (Figure 2D and 2E).

G2/M arrest induced by AITC and cisplatin (CDDP) combination treatment is associated with signaling pathway change, microtubule depletion and dysfunction

Next, we searched for an appropriate time point after treatment to investigate the mechanism that is responsible for G2/M arrest in AITC and cisplatin combination. As shown above, AITC, cisplatin, and their combination, induce G2/M arrest in different time-dependent patterns. However, all regimens have significant effect on G2/M arrest 16 hours after treatment (P<0.05). We therefore concluded that the 16-hour time point offers a good window to study the mechanism of drug combination. It has been reported that some ITCs analogues could induce microtubule depletion [43]. We sought to determine whether cellular skeletal system dysfunction is involved in M phase arrest after AITC-containing regimen treatment. We first tested if AITC alone could deplete the microtubule in cancer cells. As shown in Figure 3A and 3B, in 2008 ovarian cancer cells, AITC alone could reduce β-tubulin expression in a dose- and time-dependent pattern. The level of β-tubulin decreased after 8 hours of AITC treatment, and recovered at 48 hours (Figure 3A and 3E). The β-tubulin decrease is associated with p-H3 increase (Figure 3A, 3E) and G2/M arrest (Figure 2E). This suggests that AITC might induce microtubule depletion and/or dysfunction. To further demonstrate that microtubule dysfunction and/or depletion is involved in G2/M phase arrest, we triple stained 2008 ovarian cancer cells with DAPI, p-H3 and β-tubulin after 16 hour treatment (Figure 3C). Regardless of the number of mitotic cells in different groups, during mitosis in both control and cisplatin groups we see a certain amount of polar microtubules in the spindles of cells. However, in AITC-containing groups, the amount of β-tubulin is decreased and these cells failed to establish microtubule polarity necessary for mitotic division (Figure 3C). This finding has been further confirmed by western blot assay (Figure 3D, top panel). In AITC alone and the AITC-cisplatin combination group, β-tubulin expression was decreased, while expression of p-H3 increased in comparison with cisplatin treatment alone. We further investigated whether AITC and cisplatin combination could induce changes in molecular signaling associated with the G2/M checkpoint. Combination treatment with AITC and cisplatin decreased CDC2, Cyclin B1 and CDC25C expression, more than single drug treatment alone (Figure 3D), suggesting that AITC-induced microtubule depletion and dysfunction is associated G2/M phase arrest. β-tubulin depletion and microtubule dysfunction were also observed in A549 cells (Figure 3E and 3F).

Inhibition of cell growth via combination of cisplatin (CDDP) with AITC is accompanied with cell death, caspase 3 activation and decreases in survivin and Bcl-2 expression.

To study the biological mechanisms by which AITC and cisplatin combination decreases cell viability, we first investigated whether combination treatment was inducing cell death. After 72 hours of combination treatment, cell death was identified in 2008 ovarian cancer cells using flow cytometry (sub-G1 DNA content) (Figure 4A) and a cell death ELISA assay that quantified cytoplasmic DNA-histone complexes (Figure 4B). We found that combination of cis-
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Figure 6. Inhibition of human tumor xenograft growth in animal models by AITC, cisplatin (CDDP) alone and in combination. Experimental procedures are described in the Methods section. A549 lung cancer cell line-derived tumor xenograft in nude mouse models was used. (A) effects of AITC, cisplatin alone or in combination on the inhibition of human tumor xenograft growth. (B) mouse body weight profiles during treatment in the experiments. The value in each time point is the mean tumor size (A) or mean body weight (B) with SD derived from 5 mice.

Synergistic inhibitory effects of cisplatin (CDDP) and AITC combinational treatment in lung cancer cell growth

The results shown from Figure 1C and 1D clearly suggest a possible synergistic effect upon the combination treatment of cisplatin with AITC. We therefore used the Combination Index (CI) equation method developed by Chou-Talalay [32, 33] to study the combinational synergistic effects. Our studies revealed that in all the dose ranges used in the combination of the two agents (AITC and cisplatin) for treatment of either A549 and Hop62 lung cancer cells, we obtained the Cls≤1 (Figure 5). While only in a few combinational cases the Cl is approaching 1, most combinational cases showed the Cl is significantly <1. Furthermore, we obtained the Cl much smaller than 0.5 for the combinational treatment of AITC at 10-20 µM and cisplatin at 8-16 µM (Figure 5), suggesting a high synergistic effect can be reached.

Combination of cisplatin (CDDP) with AITC enhances the sensitivity of tumor cells to cisplatin treatment in lung cancer xenograft tumor models.

In order to demonstrate that AITC is a good candidate for in vivo combination with cisplatin (CDDP), we determined whether AITC could sensitize tumor cells to cisplatin treatment using human tumor xenograft mouse models. As we expected and also consistent with the data shown in Figure 5 for the CI study, combination of cisplatin with AITC significantly increases the tumor growth inhibition in comparison with each compound treatment alone (P<0.05) (Figure 6A), while combination treatment did not increase toxicity/body weight loss (Figure 6B). Data obtained in our xenograft animal model are summarized in Table 1. The maximum tumor growth inhibitions (MTRI) are 46.6 ± 18.3, 30.2 ± 10.1 and 94.3 ± 4.8 for AITC alone, cisplatin alone and their combination in turn, respectively. The tumor doubling times (TDT) are 9.3 ± 1.1, 9.7 ± 1.2 and more than 24 days (Table 1) in AITC, cisplatin and their combination in turn, respectively. In the combination group, 60% of mice had at least 50% reduction in tumor volume, and 20% of mice had no palpable tumors at the end of the study. Importantly, combination of cisplatin with AITC could significantly enhance the capacity of tumor growth inhibition without a significant increase in systemic toxicity/body weight loss (P<0.05) (Figure 6; Table 1).

Discussion

Consistent with the in vitro studies from other ITCs [12-16], we found that combination of cis-
Synergistic interaction of AITC with cisplatin

platin with AITC significantly sensitizes cancer cells to cisplatin-induced cell growth inhibition in both MTT and colony formation assays (Figure 1). We further revealed the synergistic effect between AITC and cisplatin using combination-index (CI) analysis (Figure 5). We defined the dose ranges for realizing synergistic combination effects for both cisplatin and AITC which is reachable in vivo [23, 24], suggesting that this combination could fully realize synergistic function in vivo. To make our protocol easier to translate into a clinical model, one time tail vein i.v. injection of cisplatin and daily oral administration of AITC was used for combination treatment in our studies. We found that treatment in human tumor animal models with AITC and cisplatin combination could significantly increase the capacity for tumor growth inhibition in comparison with AITC or cisplatin treatment alone (Figure 6A). Surprisingly, unlike other combination regimens, there is no significant increase in toxicity (both weight loss) in the combination group (Figure 6B).

In the present study, we also explored whether the addition of AITC could enhance the pro-apoptotic effect of cisplatin treatment. Our data showed that combination of AITC and cisplatin could significantly increase sub-G1 dying cells, DNA damage and caspase activation with the inhibition of Bcl-2 and survivin expression (Figure 4), suggesting that combination of AITC and cisplatin could significantly increase pro-apoptotic response in comparison with single drug treatment alone. This finding is consistent with our previous studies that revealed that drug resistance or sensitivity is associated with anti-apoptotic protein expression [44-48]. Substantial evidence shows that G2/M arrest plays a key role in cell growth inhibition induced by cisplatin treatment [38-42]. In our study we found that AITC and cisplatin combination not only changes the time course of G2/M arrest but also induces both G2 and M phase arrest with extending time (Figure 2). Furthermore, upon G2/M decrease at 72 hours after AITC and cisplatin combination treatment, there is a significant increase in sub-G1 cells and DNA damage; in contrast, for the cisplatin treatment alone, upon the decrease in G2/M cells, there is much less sub-G1 and DNA damage compared with AITC and cisplatin combination treatment (Figure 2). These observations further indicate that both cell cycle arrest and apoptosis induction are involved in cell growth inhibition induced by combination of AITC and cisplatin.

Although a full understanding of the mechanism underlying the synergistic interaction between cisplatin and AITC requires more studies, both drugs have been reported to interact with tubulin. Cisplatin could change microtubule assembly dynamics [49, 50], and AITC and its analogues could inhibit the expression of alpha- and beta-tubulin proteins [51, 52]. We speculate that the microtubule depletion and dysfunction may be involved in synergistic function. We found that AITC decreases β-tubulin levels, while cisplatin alone in our experiment has not induced a significant β-tubulin decrease (Figure 3). This is possibly because we are limiting the dosing range to clinically relevant concentrations [49]. In the combination of AITC and cisplatin group, β-tubulin was observably decreased, and time course studies for tubulin

### Table 1. Antitumor activity and toxicity of CDDP ± AITC in nude mice bearing human lung A549 xenografts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Antitumor activity</th>
<th>Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTGI (%)</td>
<td>TDT (day)</td>
</tr>
<tr>
<td>A549 lung cancer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (vehicle)</td>
<td>-</td>
<td>5.7 ± 0.2</td>
</tr>
<tr>
<td>CDDP 6 mg/kg i.v. x 1</td>
<td></td>
<td>30.2 ± 10.1</td>
</tr>
<tr>
<td>AITC 50 mg/kg p.o.every 2 days x 8</td>
<td>46.4 ± 18.3</td>
<td>9.3 ± 1.1</td>
</tr>
<tr>
<td>CDDP 6 mg/kg + AITC 50 mg/kg</td>
<td>94.3 ± 4.8</td>
<td>&gt;24</td>
</tr>
</tbody>
</table>

Note: Antitumor activity and toxicity of AITC ± CDDP (cisplatin) in nude mice bearing human lung A549 xenografts. MTGI: maximum tumor growth inhibition; TDT: tumor doubling time; PR: partial tumor response; CR: complete tumor response; MWL: maximum weight loss of pretreatment body weight. Treatment was initiated 6 days after the tumor transplantation when the tumor weight reaching ~ 200-220 mg (mm³ in volume).
Synergistic interaction of AITC with cisplatin

depletion induced by AITC showed that β-tubulin decrease is negatively associated with p-H3 increase (Figure 3), suggesting that decreased β-tubulin levels might be involved in G2/M arrest after AITC and cisplatin combination treatment. In the triple staining β-tubulin, p-H3 and DAPI, we found that AITC alone or the AITC and cisplatin combination group, showed that not only has amount of microtubule protein been decreased but also the spindle architecture has been changed (Figure 3C and 3F), suggesting that microtubule depletion and dysfunction was involved in M phase arrest induced by combination of AITC and cisplatin. Our further studies revealed that combination of AITC and cisplatin could decrease CDC2, CDC25 and cyclin B1 expression levels (Figure 3D). Taken together, we propose that the combination of AITC and cisplatin induces both G2 and M arrest through depletion of microtubules, microtubule dysfunction and G2/M checkpoint change.

Our data is in agreement with reports by other groups. ITCs enhance the sensitivity of a variety of cancer cells to cisplatin treatment [19, 25, 26]. It is possible to take advantage of the synergistic effects for combinational treatment of both newly diagnosed patients as well as patients failing primary chemotherapy. Most importantly, our in vivo data showed that AITC could significantly enhance the anticancer function of cisplatin without increasing the toxicity of the treatment. Therefore, this combination might provide a novel therapeutic strategy in solid tumor treatment in general.

Acknowledgements

This work was sponsored in part by NIH Grants, R01CA109481 and R01CA133241 to FL, R01CA164574 to YZ, P50CA058187 to DC, and by shared resources supported by NCI Cancer Center Core Support Grant to Roswell Park Cancer Institute (P30CA160565), and by a Mesothelioma Foundation (Alexandria, VA) grant to FL. We thank other members in the Li Lab for helpful discussion of data during lab meeting. We also thank other faculty and staff scientists including PhD students for their supporting and friendship during carrying out the studies of our projects. We also thank Ms. Katherine Plante for editorial revision of this manuscript.

Disclosure of conflict of Interest

None.

Abbreviations

AITC, allyl isothiocyanate; CI, combination index; cisplatin (CDDP), cis-diaminedichloroplatinum II; DAPI, 4,6-diamidino-2-phenylindole; ITCs, isothiocyanates; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; PI, propidium iodide; SD, standard deviation; SDS, sodium dodecyl sulfate.

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