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Enhancement of arsenic trioxide cytotoxicity by dietary isothiocyanates in human leukemic cells via a reactive oxygen species dependent mechanism

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Abstract

Although clearly effective in acute promyelocytic leukemia (APL), arsenic trioxide (ATO) demonstrates little clinical benefit as a single agent in the treatment of non-APL hematological malignancies. We screened a library of 2000 marketed drugs and naturally occurring compounds to identify agents that potentiate the cytotoxic effects of ATO in leukemic cells. Here, we report the identification of 3 isothiocyanates (sulforaphane, erysolin and erucin) found in cruciferous vegetables as enhancers of ATO cytotoxicity. Both erysolin and sulforaphane significantly enhanced ATO-mediated cytotoxicity and apoptosis in a panel of leukemic cell lines; erucin activity was variable among cell types. Cellular exposure to sulforaphane in combination with ATO resulted in a dramatic increase in levels of reactive oxygen species (ROS) compared to treatment with either agent alone. Sulforaphane, alone or with ATO, decreased intracellular glutathione (GSH) content. Furthermore, addition of the free radical scavenger N-acetyl-L-cysteine (NAC) rescued cells from ATO/isothiocyanate-mediated cytotoxicity. Our data suggest that isothiocyanates enhance the cytotoxic effects of ATO through a ROS-dependent mechanism. Combinatorial treatment with isothiocyanates and ATO might provide a promising therapeutic approach for a variety of myeloid malignancies.

Keywords

Leukemia; Arsenic trioxide; Sulforaphane; Erysolin; Reactive oxygen species

Introduction

Arsenic trioxide (ATO) has a long history of use as an anti-cancer agent in traditional Chinese medicine [1]. More recently, the clinical efficacy of ATO has been well characterized in the treatment of acute promyelocytic leukemia (APL) [2]. Additionally, *in vitro* studies indicate that higher concentrations of ATO can inhibit cellular proliferation and induce apoptosis in a variety of non-APL cell types including myeloid leukemia, lymphocytic leukemia, multiple myeloma and lymphoma [3,4]. Despite the promising clinical results of ATO in patients with APL, clinical trials of ATO as a single agent in a variety of other hematological malignancies have not demonstrated significant benefit [5].

Dietary consumption of cruciferous vegetables such as broccoli and cabbage is thought to provide a chemopreventative benefit because they contain high concentrations of isothiocyanates [6]. Sulforaphane (4-methylsulfinylbutyl isothiocyanate), erysolin (4-methylsulfonylbutyl isothiocyanate) and erucin (4-methylthiobutyl isothiocyanate) are naturally

occurring members of the isothiocyanate family [7]. Sulforaphane is a well characterized inducer of several Phase II detoxification enzymes including glutathione-S-transferases and quinone reductase [8]. In addition to its chemopreventative effects, sulforaphane has also been reported to cause growth inhibition and induction of apoptosis in a variety of human cancer cell lines [9,10].

Here, we report that isothiocyanates are potent sensitizers of arsenic trioxide cytotoxicity in several cell lines representing various myeloid leukemias. Furthermore, we demonstrate that subtoxic concentrations of isothiocyanates strongly enhance ATO-mediated apoptosis by increasing cellular ROS levels. Combined treatment may provide a promising therapeutic approach to myeloid malignancies where ATO has not provided substantial benefit as a single agent.

Materials and Methods

Reagents

ATO, sulforaphane, erucin, erysolin and N-acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO).

Cell lines

The human leukemic cell lines HL-60 (acute myeloid leukemia), K-562 (erythroblastic chronic myelogenous leukemia), and U937 (promonocytic leukemia) cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, and 1% penicillin-streptomycin. All cells were cultured at 37°C in a 5% CO₂ humidified atmosphere.

Chemical library screening

The Spectrum library (Microsource Discovery Systems, Gaylordsville, CT), containing 2000 marketed drugs and naturally occurring compounds, was used to screen for enhancers of ATO growth inhibition in K-562 cells. Cells were seeded at a density of 50,000 cells/cm² in 96-well plates and allowed to adhere overnight. Library compounds were added to cell cultures at a final concentration of 1μM, either alone or in combination with 0.5μM ATO. After a 72 hour incubation period, cellular proliferation was measured using the CellTiter 96 AQueous non-radioactive cell proliferation assay (Promega, Madison, WI), according to manufacturer's instructions. This assay determines the number of living cells based upon the bioreduction of MTS tetrazolium compound into a colored formazan product. The quantity of formazan product, as measured by absorbance on a microplate reader (Biorad model 550) at 495 nm, is directly proportional to the number of living cells in culture. The absorbance of control wells exposed to vehicle alone defined 100% viability, and the effect of drugs on cellular proliferation was expressed as a percentage of cell viability relative to untreated cells. Drug interaction was analyzed by CalcuSyn software (Biosoft, Cambridge, United Kingdom). This software determines the interaction of two drugs (i.e., synergy, antagonism, or additivity) through calculations of the combination index (CI) based upon the multiple drug effect equation of Chou and Talalay [11,12]. A CI value >1 denotes antagonism, 1 is additivity, and <1 denotes synergy. Each CI value represents the mean of a least three independent experiments, with each datapoint performed in triplicate.

Measurement of apoptotic cells

Induction of apoptosis was determined using the SensoLyte Homogenous Rh110 Caspase 3/7 Assay Kit (AnaSpec, San Jose, CA). This kit is optimized to detect caspase-3/7 activity using a sensitive fluorogenic substrate, (Z-DEVD)-2-Rh110, whose signal can be monitored at

excitation/emission=496 nm/520 nm. K-562 cells were seeded at 1×10^6 cells per well in a 96-well microplate and allowed to adhere overnight. Indicated compounds were then added, and cells were incubated at 37°C for 18 hours. Quantification of apoptotic cells was performed per the manufacturer's instructions. Relative fluorescence units (RFU) are reported relative to untreated cells.

Immunoblotting

Cells were treated with 0.3 μ M ATO, 3 μ M of indicated isothiocyanate, or both for 18 hours. Cellular lysate was obtained and subject to western blotting as previously described [13]. Samples were probed with primary antibodies to cleaved poly (ADP-ribose) polymerase (PARP) and cleaved caspase 9 (Cell Signaling Technology, Boston, MA). Following wash cycles, membranes were probed with secondary donkey anti-rabbit IgG (Amersham, Piscataway, NJ). Immunoreactive bands were visualized using ECL detection reagent (PerkinElmer, Waltham, MA) and X-OMAT processing.

Measurement of intracellular ROS generation

Intracellular ROS generation was directly visualized by fluorescent microscopic examination of cells following staining with the oxidant-sensitive probe H₂DCFDA. Cells were incubated with indicated compound(s) for 3 hours. Intracellular ROS levels were determined using Image-iT™ LIVE green reactive oxygen species detection kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. DNA was counterstained with Hoechst 33342. Cells were examined using a Zeiss Axiophot upright phase contrast microscope.

Measurement of cellular glutathione levels

Cellular levels of glutathione (GSH) levels were determined using the HT Glutathione Assay kit from Trevigen (Gaithersburg, MD). Briefly, leukemic cells (1×10^6) were seeded in T25 flasks. After incubation for 24 hours, cells were exposed to indicated compounds for 3 hours at 37°C. The cells were collected, washed with PBS and counted. An equal number of cells from each treatment group were used for the determination of GSH according to the manufacturer's instructions.

Results

Dietary isothiocyanates identified as potentiators of ATO-mediated growth inhibition in leukemic cells through chemical library screening

In order to identify agents that sensitize leukemic cells to ATO, we screened K-562 cells with the commercially available Spectrum Collection in the presence and absence of 0.5 μ M ATO, a clinically achievable concentration that is minimally growth inhibitory to K-562 cells *in vitro*. We sought compounds that lacked growth inhibitory effects when used alone, but demonstrated at least a 30% increase in growth inhibition when combined with 0.5 μ M ATO.

Erysolin, a naturally occurring isothiocyanate, was found to sensitize K-562 leukemic cells to the growth inhibitory effects of ATO. As shown in Figure 1A, erysolin significantly inhibited cellular proliferation in a dose dependent manner when combined with ATO, compared to either agent alone. For example, the IC₅₀ of ATO is 0.98 μ M ($\pm$ 0.2) as a single agent, 0.19 μ M ($\pm$ 0.07) in combination with 3 μ M erysolin, and 0.07 μ M ($\pm$ 0.1) in combination with 5 μ M erysolin. Because 3 μ M erysolin displays synergy with ATO (Table 2) and is a clinically achievable concentration [14], this dose was chosen for use in subsequent studies.

To further examine the clinical applicability of this sensitization to other leukemic malignancies, we examined the effects of combinatorial erysolin and ATO treatment in HL-60

and U-937 cells. In both cell lines, 3 μ M erysolin significantly enhanced the growth inhibitory effects of ATO (Figures 1C, D).

Erysolin is a member of the isothiocyanate family of chemopreventative compounds found in cruciferous vegetables such as broccoli and cauliflower. Other naturally occurring isothiocyanates include sulforaphane and erucin. In addition to its chemoprotective activity, recent studies have suggested that sulforaphane may also suppress tumor growth through cell cycle arrest and induction of apoptosis [15]. Because erysolin was able to sensitize leukemic cells to ATO, we sought to characterize the ability of two other structurally related isothiocyanates - sulforaphane and erucin - to enhance ATO-mediated growth inhibition in leukemic cells (Table 1). Similar to erysolin, 3 μ M sulforaphane significantly enhanced the growth inhibitory effects of ATO in a synergistic fashion in all leukemic cell lines examined (Figure 1, Table 2). By contrast, 3 μ M erucin demonstrated variable effects on ATO-mediated growth inhibition (Figure 1), and hence was not subjected to further study.

Erysolin and sulforaphane enhance induction of apoptosis by ATO

Because ATO/sulforaphane and ATO/erysolin combination treatment resulted in reduced cellular proliferation, we evaluated the induction of apoptosis in K-562 cells using a fluorometric based assay that monitors the cleavage of caspase 3 and caspase 7 as general marker of apoptosis. Figure 2a shows that K-562 cells treated with the combination of ATO and erysolin or sulforaphane displayed significantly higher levels of cleaved caspase 3 and caspase 7 as compared to those treated with each agent individually. Induction of apoptosis through the intrinsic, mitochondrial pathway was further investigated in all cell lines via assessment of cleavage of caspase 9 and poly-ADP ribose polymerase (PARP). As shown in Figure 2b, significantly higher levels of cleaved caspase 9 and PARP protein were present in all cell lines exposed to the combination of ATO/erysolin or ATO/sulforaphane than with either agent alone. These data indicate that combinatorial ATO/isothiocyanate treatment enhances apoptotic induction in leukemic cells via the intrinsic pathway.

Sulforaphane enhances the cytotoxic effects of ATO through a ROS dependent mechanism

Both ATO and sulforaphane are known to induce the production of ROS as single agents [16,17]. Therefore, we sought to assess the role of ROS generation in ATO/sulforaphane cytotoxicity using the oxidant sensitive probe carboxy- H_2 DCFDA. Carboxy- H_2 DCFDA enters live cells where esterases deacetylate the molecule. In the presence of ROS, the reduced fluorescein compound is oxidized and emits a bright green fluorescent signal. When K-562 leukemic cells were treated with ATO and sulforaphane as single agents at subtoxic concentrations, only minimal levels of cellular ROS were observed (Figure 3). By contrast, the ATO/sulforaphane combination resulted in significantly higher levels of intracellular ROS (Figure 3), suggesting that the combined effect of ATO/sulforaphane is mediated by ROS. Similar results were obtained with U-937 and HL-60 cells (data not shown).

To further demonstrate that enhancement of ATO-mediated cytotoxicity by sulforaphane in leukemic cells is mediated by ROS, we evaluated the effects of ATO/sulforaphane treatment on cellular proliferation and apoptosis in the presence of the free radical scavenger, N-acetyl-l-cysteine (NAC). At a concentration of 100 μ M, NAC reversed ATO/sulforaphane-induced growth inhibition (Figure 4). Furthermore, NAC also attenuated the apoptotic response of combination treatment as evidenced by decreased levels of cleaved PARP (Figure 4). The addition of NAC restored cellular proliferation and prevented apoptosis only to levels comparable to those observed in cells treated with ATO alone (Figure 4). Taken together, these data suggest that the cytotoxic effects of the combination of ATO and sulforaphane on leukemic cells are mediated by oxidative stress.

Sulforaphane depletes cellular GSH

Intracellular GSH has been shown to play an important role in mediating ATO cytotoxicity by protecting cells from ROS generated during ATO exposure [18]. Because sensitivity to ATO has been reported to correlate with intracellular GSH levels [19] and because sulforaphane has been shown to modulate intracellular GSH levels [17], we investigated whether GSH depletion may play a role in sulforaphane's ability to sensitize leukemic cells to ATO. Treatment with ATO alone produced minimal effects on the level of intracellular GSH (Figure 5). In contrast, leukemic cells treated with sulforaphane alone or in combination with ATO showed a dramatic decrease in the level of intracellular GSH (Figure 5); thereby suggesting that sulforaphane acts as an ATO sensitizing agent through depletion of intracellular GSH.

Discussion

The remarkable success of ATO in the treatment of APL has stimulated an increasing number of preclinical studies evaluating the potential for broadening the clinical utility of ATO. Unfortunately, ATO has demonstrated only limited activity as a single agent in other hematological malignancies. In order to identify agents that increase the cytotoxic activity of ATO, we screened a library of over 2000 marketed drugs and naturally occurring products. Here, we report the identification of erysolin and sulforaphane, two dietary isothiocyanates, as ATO sensitizing agents in a panel of leukemic cell lines representing a variety of different myeloid malignancies. Our studies reveal that erysolin and sulforaphane significantly potentiate ATO-mediated cytotoxicity through enhanced growth inhibition and increased apoptotic induction through the intrinsic, mitochondrial-mediated pathway (Figure 2). When leukemic cells are treated in combination, a significant increase in cellular ROS levels is observed (Figure 3). The enhanced cytotoxic effects of combined ATO/sulforaphane combinatorial treatment are reversed upon addition of the free radical scavenger, NAC (Figure 4). Therefore, it is suggested that sulforaphane acts as an ATO-potentiator through an ROS dependent mechanism. To our knowledge this is the first report of potentiation of ATO cytotoxicity by isothiocyanates.

Interestingly, although enhanced ATO cytotoxicity was observed with erysolin and sulforaphane in all leukemic cell lines examined, variable effects were observed with the structurally similar erucin (Table 1). As shown in Figure 1, erucin was highly effective in K-562 cells, moderately effective in HL-60 cells, and not effective in U-937 cells. A variety of possible explanations could be postulated for this observation. The presence of a sulfinyl or sulfonyl group such as those found in sulforaphane and erysolin, respectively, may be an important structural factor for ATO potentiation by isothiocyanates in certain cell types. Moreover, cell-type specific molecular or genetic factors may play a role in determining the ability of specific isothiocyanates to act as enhancers of ATO cytotoxicity. For example, glutathione transferases (GSTs) are dimeric enzymes that facilitate isothiocyanate metabolism by catalyzing their conjugation with glutathione (GSH). The cytosolic GSTs are derived from at least 17 subunits within seven classes, and studies have revealed enhanced isothiocyanate activity with specific allelic combinations [20]. Also of note, isothiocyanates did not significantly enhance ATO-mediated growth inhibition in NB-4 (acute promyelocytic leukemia) cells (data not shown) which are significantly more sensitive to ATO due to the presence of the t(15;17) translocation [21]. The leukemic cell lines used in this study do not contain the resulting PML-RAR α fusion protein which is rapidly degraded by ATO and has been shown to be a key mediator in APL sensitivity to ATO [18,22].

ATO is a well known inducer of oxidative stress [23]. More recently, reports have indicated that sulforaphane also induces the production of ROS [17,24]. In the current study, we demonstrate markedly enhanced cellular ROS production in leukemic cells treated with both agents as compared to either agent alone (Figure 3). Combined treatment in the presence of

the free radical scavenger NAC reversed the inhibition of cell growth and the induction of apoptosis, indicating that ATO/sulforaphane combination treatment enhances ATO-mediated cytotoxicity through a ROS dependent mechanism (Figure 4). Interestingly, in the presence of NAC, cellular proliferation and apoptosis were only restored to levels similar to those observed with ATO treatment alone (Figure 4). These data suggest that a proportion of ATO cytotoxicity is ROS independent, consistent with previously reported ROS independent mechanisms of action for ATO including modulation of cell cycle regulatory proteins and/or inhibition of tubulin polymerization [4,25,26].

During the preparation of this paper, Choi et al reported that sulforaphane generates reactive oxygen species leading to mitochondrial perturbation and subsequent apoptosis in U-937 cells [27]. Although we observed minimal ROS production when leukemic cells including U-937 are treated with concentrations of sulforaphane similar to those used by Choi et al, we failed to detect any substantial growth inhibition or induction of apoptosis. In numerous reports characterizing the antiproliferative activity of sulforaphane in a variety of cell types including leukemic cells, the growth inhibitory and apoptotic effects are only observed at concentrations of sulforaphane in the range of 10 – 25 μ M [10,28–30]. Our studies are consistent with these reports in that we observe minimal antiproliferative effects of sulforaphane at concentrations below 5 μ M (Figures 1 and 2), but do observe growth inhibition and induction of apoptosis with higher concentrations of sulforaphane (data not shown). The discrepancy between findings could be attributed to differences in culture conditions which have been reported to have an effect on isothiocyanate toxicity [31].

Our data suggest that sulforaphane acts as an ATO sensitizing agent through depletion of intracellular GSH. GSH has been shown to play an important role in mediating ATO cytotoxicity. Many reports suggest that sensitivity of leukemic cells to ATO correlates to intracellular GSH levels, with cells expressing higher levels of GSH appearing to be more resistant to ATO [19,32]. GSH function with respect to ATO toxicity is two-fold. First, GSH is a key component of cellular detoxification systems responsible for removing ATO from the cell. Second, it protects cells from ROS produced upon cellular exposure to ATO [18]. Therefore, it is surmised that depletion of intracellular GSH levels by sulforaphane reduces the oxidative threshold of the cell thereby sensitizing it to ATO. Indeed, other agents known to deplete intracellular GSH including buthionine sulfoximine and ascorbic acid have been shown to act as ATO sensitizers [33,34]. Interestingly, we did not observe isothiocyanate-mediated enhancement of the cytotoxicity of cisplatin or taxol, agents which are not as exquisitely sensitive as ATO to intracellular GSH levels (data not shown).

Isothiocyanates are found at high levels in cruciferous vegetables such as broccoli, cabbage, cauliflower, and Brussels sprouts. Given the interest in these compounds as chemopreventative agents, recent studies have examined the pharmacokinetics of sulforaphane. Collectively, published data indicate that sulforaphane can be absorbed, achieve micromolar concentrations in the blood, and accumulate in tissues [15]. Some reports in rats have estimated peak plasma concentrations of sulforaphane to be as high as 20 μ M [35]. However, other studies looking at sulforaphane levels after dietary consumption in humans have reported levels closer to 3 μ M [14]. For our studies, we chose to use the more conservative estimate in order to ensure that our observations were clinically relevant and achievable through diet alone. It should also be noted that the dose of ATO that we employed is also clinically achievable [18]. Given the remarkable enhancement of ATO cytotoxicity by isothiocyanates in a panel of leukemic cells along with the safety and ease of isothiocyanate administration through diet, combined therapy may provide a beneficial therapeutic option that warrants further clinical investigation.

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Figure 1.

Figure 2.

Figure 3.

A. **B.** **Figure 4.**

Figure 5.

Table 1

Erucin	$\text{CH}_3\text{-S-(CH}_2\text{)}_4\text{-N=C=S}$
Sulforaphane	$\text{CH}_3\text{-S(=O)-S-(CH}_2\text{)}_4\text{-N=C=S}$
Erysolin	$\text{CH}_3\text{-S(=O)-S(=O)-S-(CH}_2\text{)}_4\text{-N=C=S}$

Table 2

Cell Line	Drug Combination	CI	Interpretation
K-562	3 μ M Erysolin, 0.3 μ M ATO	0.338	Synergistic
K-562	3 μ M Sulforaphane, 0.3 μ M ATO	0.425	Synergistic
K-562	3 μ M Erucin, 0.3 μ M ATO	0.669	Synergistic
HL-60	3 μ M Erysolin, 0.3 μ M ATO	0.659	Synergistic
HL-60	3 μ M Sulforaphane, 0.3 μ M ATO	0.495	Synergistic
HL-60	3 μ M Erucin, 0.3 μ M ATO	0.92	Weakly Synergistic
U-937	3 μ M Erysolin, 0.3 μ M ATO	0.548	Synergistic
U-937	3 μ M Sulforaphane, 0.3 μ M ATO	0.35	Synergistic
U-937	3 μ M Erucin, 0.3 μ M ATO	2.793	Antagonistic