Arsenic Trioxide Increases Apoptosis of SK-N-BE (2) Cells Partially by Inducing GPX4-mediated Ferroptosis

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Research Article

Keywords: Neuroblastoma, Arsenic trioxide, Ferroptosis, GPX4, Quantitative proteomic analysis

Posted Date: February 9th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1271272/v1>

Abstract

Neuroblastoma (NB) is the most common extracranial tumor in central nervous system threatening children's health with limited therapeutic options. Arsenic trioxide (ATO) has been identified the cytotoxicity in NB cells but the potential mechanism remains unclear. In this study, we attempted to obtain some insight into the mechanisms of cell death induced by ATO in NB cells using proteomic technology. Proteomic analyses found that ATO can affect the signaling pathway associated with ferroptosis, including the upregulation of iron absorption (FTL, FTH1, HO-1), ferritinophagy (LC3, P62, ATG7, NCOA4) and modifier of glutathione synthesis (GCLM); downregulation of glutamine synthetase (GS) and GPX4, which was the critical inhibitor of ferroptosis. Western blot analysis revealing GPX4 expression in SK-N-BE (2) cells decreased after treatment with ATO (7.3 μ M), resulting in a loss of GPX4 activity. Our study revealed that ATO may induce ferroptosis in neuroblastoma cell SK-N-BE (2) by facilitating the downregulation of GPX4, ultimately resulting in iron-dependent oxidative death.

Introduction

Quantitative proteomics is predominantly conducted using mass spectrometry (MS)-based technologies[1], where proteins are extracted and digested into peptides by a sequence-specific enzyme, and quantified by different quantification strategies such as label-free quantification (LFQ)[2]. Quantitative proteomics can identify novel protein as well as differentially expressed, which is a powerful technique for revealing the underlying mechanisms of cancer and therapeutic targets. Based on the advantages that serum/plasma has a vast array of proteins complexes and ease of serial sample collection, proteomics has been applied in detection, diagnosis, prognostic judgment, or management of cancers[3–5].

Neuroblastoma (NB) is the most common extracranial tumor in childhood with the 7–8% incidence rate and approximately 15% mortality rate accounts for of all pediatric cancer deaths. Several recent reports highlight the potential use of a proteomics in investigating the pathobiology of NB. Murillo et al.[6] used iTRAQ-based proteomics to analyze potential differentiation markers which were similar to neuronal differentiation aspects, and select MARCKS, STMN1, AIFM1, SMN1, AGRN and CTNND1 to be used as candidates applied in mature NB phenotype. LFQ proteomic profiling revealed the proteins involved in cell communication and signal transduction in exosomes which released by the MYCN-amplified NB cells[7], suggesting that exosomes play an role in chemoresistance of MYCN-driven aggressive NB. In a study by Lorena et al.[8], protein expression patterns of distant metastases in NB xenograft mouse model were identified by Quantitative proteomics. Hwang et al.[9] found a total of 12 proteins differentially regulated after LGR5 knockdown, including Wnt/ β -catenin signaling and hnRNP family, indicating that LGR5 and its downstream signaling may play a critical role in NB by hyperactivation of alternative pre-mRNA processing.

According to clinical manifestation, biological characteristics and age, NB patients can be divided into very low risk, low risk, intermediate risk, or high risk, respectively. High-risk NB has a poor prognosis with 5

years overall survival (OS) rate of less than 15% after the multimodality therapy[10] and exceed 80% recurrence rate within 2 years[11]. Therefore, there is an urgent need to identify novel drugs or therapeutic approaches for patients with High-risk NB. Proteome analysis has been used to identify the molecular mechanisms of potential drugs for NB. For example, Halakos et al.[12] found that cysteine protease inhibitor K777 combined with 13-cis RA could stimulate NB cell lines differentiation by multiple signaling pathways, whereas RET, GDF15, APP, ADAM10, and PLAT may be a novel biomarker of differentiation. Oliva et al.[13] found that upregulation of GLS2 in NB cell line SH-SY5Y induced an antiproliferative response with upregulation of tumor suppressor p53 and p21 protein and cell cycle arresting at the G2/M phase. AURKA and AURKB were significantly associated with MYCN-amplified and high-risk NB. Aurora kinase inhibitor tozasertib was found to suppress tumor cells migration and invasion ability in NB Th-MYCN mouse model via regulated the actin cytoskeleton as well as altered fatty acid and carbohydrate metabolic processes[14]. Therefore, proteomic analysis can help in drug discovery, unraveling drug mechanisms of action and drug clinical application.

As early as the 1970s, Chinese scholars discovered that Arsenic Trioxide (ATO) can induce acute promyelocytic leukemia (APL) tumor cell differentiation and apoptosis [15]. Since then, dozens of studies showed the broad-spectrum anti-tumor activities of ATO[16–20]. One of the interesting findings was that ATO increasing the levels of malonaldehyde (MDA) and reactive oxygen species (ROS), while decreasing glutathione (GSH); The accumulation of ROS could trigger oxidative stress and damage mitochondrial membrane potential in spermatogonia [21]and pulmonary fibroblast cell [22], therefore activate downstream caspase-dependent apoptosis pathways[23];this allowed ATO to be inferred can treat cancer effectively through this mechanism. Our previous studies confirmed the anti-tumor growth effect of ATO in NB cells. ATO could inhibit the NB cells growth in vitro by means of arresting NB cells in G2/M phase[24], increasing TrkA/C receptors which related to the good prognosis of NB[25] and reducing multidrug-resistance protein[26]. Our previous work [27]found that ATO can restore the sensitivity of chemotherapy in some relapsed/refractory NB patients. Preliminary data[28] of our ongoing clinical trial (ChiCTR1800014748, NCT03503864) also showed that ATO combined with chemotherapy can significantly improve induction response in 4/M neuroblastoma without serious adverse effects. To further improve the clinical therapeutic effect and explore the mechanisms underlying ATO treatment, in this study, we compared SK-N-BE (2) cells proteome before and after ATO treatment by using LFQ proteomic analysis. In view of the results issued from the proteomic analyses, complementary experiments were conducted to gain a deeper insight into the mechanisms of ATO killing NB cells.

Results

ATO inhibit the proliferation of SK-N-BE (2) cells

The neuroblastoma cell line SK-N-BE (2) were cultured with gradient concentration of ATO for 24hours. Consistent with our previous research[24, 25], the results revealed that ATO inhibited SK-N-BE(2) cells

(Fig. 1). ATO inhibited the growth of SK-N-BE(2) cells in a concentration-dependent manner from 4 μ M to 16 μ M and the IC₅₀ of ATO were 7.3 μ M.

Protein intensity information indicated before and after ATO incubation

To compare the protein expression profiles from SK-N-BE (2) cells before and after ATO treatment, the mass spectrometry-based on liquid chromatography (LC-MS) was adopted for conducting the label-free proteome quantitation (LFQ) to identify protein intensities information. The ratio of LFQ between ATO-treated and negative control (NC) samples upon two times was considered to indicate a significant difference. After 24 hours of ATO (IC₅₀ 7.3 μ M) incubation, we identified 6095 proteins where 291 and 386 proteins were significantly upregulated and downregulated in ATO-treated group, respectively. The proteins were classified by Gene Ontology (GO) annotation based on three categories: cellular component, molecular function and biological process. For each category, the enrichment of the differentially expressed protein against all identified proteins have detected (Fig. 2). Differentially expressed proteins were mainly composed of nucleus (38%), cytoplasm (27%), extracellular (10%), mitochondria (8%) and plasma membrane (8%). Molecular functions include binding (55%), catalytic activity (18%) and molecular function regulators (11%). Differential proteins were involved in the cellular processes (17%), biological regulation (13%), single-organism process (13%), response to stimulus (10%) and metabolic process (9%).

Signaling pathway regulated by ATO in SK-N-BE (2) cell

To further know information on molecular interaction networks, Encyclopedia of Genes and Genomes (KEGG) database was used to identify enriched pathways which were classified into hierarchical categories according to the KEGG website (<http://www.kegg.jp/kegg/mapper.html>). In accordance with the enrichment of pathway analysis, we identified pathways in mineral absorption, ferroptosis and autophagy as significant biological processes in SK-N-BE(2) cells after ATO treatment (Fig. 3). Proteins differentially upregulated were ferritin light (FTL), heavy chain (FTH1), Heme oxygenase (HO)-1 and glutamate cysteine ligase (GCL) in mineral absorption and ferroptosis, as well as autophagy related proteins microtubule-associated proteins 1A/1B light chain 3 beta (LC3/MAP1LC3B), autophagy protein (ATG7), nuclear receptor coactivator 4 (NCOA4) and P62. On the contrary, the expression of GS and GPX4 were significantly reduced (Fig. 4).

Effects of ATO on the GPX4 in SK-N-BE (2) cell

As shown in Fig. 5, western blotting revealed that GPX4 expression was profoundly decreased under 7.3 μ M (IC₅₀) ATO treatment for 24 hours. The enzyme activity of GPX4 were downregulated in 9 μ M ATO treatment more notably than in control group without ATO (Fig. 6A). It was also observed that GSH appeared decreased in 9 and 12 μ M ATO treatment in comparison to 3 μ M group (Fig. 6B).

Discussion

As one of the oldest drugs, ATO has shown efficacy against hematologic malignancies and various solid tumors. Previous studies have shown that ATO inhibited NB cells growth via activation of apoptosis-related proteins[29]. Lowest dose of ATO could induce the oxidative stress and cause the alterations of BCL2, BID and BCL-x/L, and activation of caspase-3 and-9, resulting in activation of apoptotic signaling pathways[30]. However, the detailed molecular mechanisms of ATO action are still largely unknown. To identify novel mechanism of ATO action in NB cells, we investigated the changes in the proteome after ATO treatment in SK-N-BE (2) cells. It had been reported that ATO induced ROS production[21], and in the present study we identified a group of proteins related to ferroptosis and ferritinophagy, such as GPX4, ferritin (FTL, FTH1), LC3, P62, ATG7 and NCOA4.

Ferroptosis is a form of regulated cell death which is distinct from other identified types of cell death such as apoptosis and necrosis. It is characterized by the accumulation of lipid ROS derived from iron overload, first demonstrated by Stockwell et al. in 2012[31, 32]. In general, the ferroptosis is mainly induced by lipid peroxidation, which is triggered by inactivation of the GPX4 because of the glutathione (GSH) exhaust, or by iron overload leading to ROS production[33]. GPX4 activity is essential to maintain lipid homeostasis in the cell, prevent the accumulation of toxic lipid peroxides and thereby block the onset of Fenton chemistry catalyzed by intracellular iron, preventing ferroptosis from happening[34]. Our proteomic results showed that GPX4 protein expression in ATO group decreased significantly, and western blotting findings were consistent with proteomic analysis. Moreover, at higher concentrations of ATO, the amount of intracellular GSH dropped, meanwhile, a decrease in the enzyme activity of GPX4 was noticed. Glutamine (Gln) is a nonessential amino acid with the roles in tricarboxylic acid (TCA) cycle, promoting cell proliferation as a carbon and nitrogen sources of the tumor. Glutamine synthetase (GS) metabolizes glutamate (Glu) to Gln while Glu is synthesized from Gln by glutaminase (GLS)[35, 36]. GSH consists of Glu, cysteine and glycine, and it is synthesized by the rate limiting enzymes glutamyl cysteine ligase (GCL). GCL is composed of two subunits, catalytic (GCLC) and modifier (GCLM)[37]. Our proteomic results showed that the expression of GS protein was significantly decreased while the GCLM upregulated after ATO exposure; in the meanwhile, reduced GSH and GPX4 activity were found to be significantly low, speculating that ATO may reduce the intracellular Gln synthesis and dampen down intracellular GSH, finally resulting in the inhibition of GPX4 activity.

Proteomic data also showed that there was significant variation in ferritin (FTL, FTH1) proteins levels upon ATO exposure, following the increase in HO-1 protein[38]. The heme oxygenase (HO) is a major intracellular source of iron through detoxifying heme into biliverdin, releasing carbon monoxide and Fe^{2+} [39]. It has been reported that erastin induces HO-1-dependent ferroptotic cell death in fibrosarcoma cells by take part in iron supplement and lipid peroxidation[40, 41]. The process of autophagosomes degrade ferritin and release free iron called as “ferritinophagy”[42–44]; Lysosomal iron leading to lysosomal bursting and cell death via Fenton-like reactions [45]. Indeed, the significant up-expression of LC3, P62, ATG7 and NCOA4 indicated the autophagy initiation after ATO exposure. NCOA4 was highly enriched in autophagosomes, revealing ferritin heavy and light chains and mediating the transport of ferritin to the

lysosome via the autophagosome. Our results suggested that ferritinophagy may be involved in ATO-induced ferroptosis, but further experimental validation is needed to validate this.

In recent researches, ferroptosis-inducing agents could be developed as cancer therapy strategies: Sorafenib was found as an inducer of ferroptosis in hepatocellular carcinoma (HCC) cell lines and its cytotoxic effect could be prevented by iron chelation[46]. Sulfasalazine, used to anti-inflammatory, could induces ferroptotic cell death in glioma cells[47]. Antimalarial drug artesunate been reported can selectively killed HNC cells by generate ROS and lead to oxidative stress in cancer cells[48]. N Genget al. identified that the oncogenic RAS-selective lethal small molecule erastin induced ferroptosis in NB cells and decreased the expression of Fpn gene and protein. Fpn siRNA transfection in NB cells could enhance anticancer activity of erastin by increasing iron-dependent lipid ROS accumulation[49]. It also reported that arsenic induced ferroptotic cell death in neuron of mouse model by accumulation of reactive oxygen species and lipid peroxidation products, disruption of Fe^{2+} homeostasis, this is consistent with our in vitro experimental results [50]. Our study further confirmed a decrease in the levels of GPX4 protein in parallel with the loss of its enzyme activity after ATO exposure. The significant finding of our study is that ferroptosis activated by ATO may contribute to the major form of SK-N-BE (2) cell death. Expression levels of key proteins in the ferroptotic molecular pathway were up-regulated in response to ATO exposure. Moreover, ATO-induced ferroptosis was probably in a manner by ferritinophagy involving increasing iron accumulation.

Conclusion

Here, we found ATO may as a ferroptosis-inducing agent in neuroblastoma, which may act through the suppression of GPX4 protein and activation of ferritinophagy. Further research is needed to confirm our conjecture.

Materials And Methods

Cell lines and culture

Human SK-N-BE (2) cells were obtained from the Sun Yat-sen University (Guangzhou, China). SK-N-BE (2) cells were cultured in DMEM medium supplemented with 10% FBS, 100 $\mu\text{g}/\text{ml}$ penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin at 37°C and 5% CO_2 . Subsequent to growing in a monolayer and multilayers with 80% confluency, the cells were transferred to 96-well plates at a density of 1×10^4 cells or 6-well plates at a density of 1×10^5 cells. The ATO was produced by Harbin Medical University Pharmaceutical Company (Harbin, China).

Cytotoxicity assays

Growth inhibition of SK-N-BE (2) cells was assessed using Cell Counting Kit-8 (CCK-8) (APExBio, K1018). For the CCK-8 assay, a total of 1×10^4 cells were seeded in 96-well microculture plates and treated with

ATO ($1, 2^{1-27} \mu\text{M}$) for 24 hours. Then, 10 μl /100 μl of the CCK-8 solution were pipetted into each well. After incubating for 1 hour, the absorbance of plate was measured at 450 nm using plate reader Molecular Devices, LLC, Sunnyvale, CA, USA) to calculate percentages of survival cells. The cell proliferation rate was calculated as the percentage of CCK-8 absorption as follows: $[(\text{As}-\text{Ab})/(\text{Ac}-\text{Ab})] \times 100\%$. As: Absorbance of drug treated sample. Ab: absorbance of blank control sample (without cells). Ac: absorbance of control sample.

Label-Free Comparative Proteomics Analysis

1. Protein Digestion and TMT Labeling

Sample was sonicated three times in lysis buffer and centrifuged at 12000g for 10 min at 4°C. The protein concentration of supernatant was determined with BCA kit. Then, the protein solution was reduced and alkylated. The trypsin was added at 1:50 trypsin-to-protein mass ratio for the first digestion overnight and 1:100 trypsin-to-protein mass ratio for a second 4h digestion. Digested peptides were then dried and reconstituted with 0.5 M TEAB. Finally, the sample were labeled according to the manufacturer's protocol for TMT kit/iTRAQ kit.

2. LC-MS/MS Analysis

As for separation of peptide fragment by EASY-nLC 1000 UPLC, the tryptic peptides were dissolved in 0.1% formic acid (solvent A), then separated on the analytical column with a gradient elution at a constant flow rate of 400 nL/min. The gradient was comprised of an increase from 6–23% solvent B (0.1% formic acid in 98% acetonitrile) over 26 min, 23–35% in 8 min and climbing to 80% in 3 min then holding at 80% for the last 3 min. The eluted peptides were subjected to a nanospray ionization (NSI) source followed by tandem mass spectrometry (MS/MS) in Q Exactive™ Plus coupled online to the ultra-performance liquid chromatography (UPLC) with 2.0 kV electrospray voltage. Resolution was 17,500 at 350-1800 m/z for tandem mass spectrometry scans. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans with 15.0s dynamic exclusion. Automatic gain control (AGC) was set at 5E4. Fixed first mass was set as 100 m/z. The resulting MS/MS data were processed using Maxquant search engine (v.1.5.2.8). Tandem mass spectra were searched against human uniprot database concatenated with reverse decoy database.

GPX4 activity assay and Gluthione determination

The activity of GPX4 was determined using a Glutathione Peroxidase Assay Kit (KeyGEN BioTECH). Intracellular reduced form glutathione (GSH) were assessed using a GSH Detection Assay Kit II (KeyGEN BioTECH) according to the manufacturer's instructions.

Western blot analysis to investigate the expression of GPX4

After treatment with appropriate drug concentration, protein lysate was prepared in lysis buffer and the protein concentration was determined by BCA protein assay. SDS-PAGE gel, and electro-transferred to

PVDF membrane. The membrane was washed in TBST and blocked with 5% nonfat dry milk in TBST. Membranes were incubated overnight at 4°C with GPX4 primary antibody (1:1000). Peroxidase-conjugated secondary antibody (1:1000) was used as the secondary antibody. After electrophoresis, proteins were transferred to a nitrocellulose membrane and probed by corresponding antibodies.

Declarations

Acknowledgements

This work was supported by Grant 2017A030313806 and 2020A1515010127 from the Natural Science Foundation of Guangdong Province; Grant SYS-C-202007 from Sun Yat-Sen Clinical Research Cultivating Program.

Conflict of interest

The authors declare that there is no potential competing interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

Figure 1

Inhibitory effects of ATO on SK-N-BE(2) cells proliferation.SK-N-BE(2) cells were treated with the indicated concentrations of ATO(1, 2¹~2⁷μM) for 24 hours.

Figure 2

Enrichment of Gene Ontology analysis. Proteins were classified by GO annotation into three categories: biological process, cellular compartment and molecular function.

Figure 3

The pathway network regulated by ATO in SK-N-BE (2) cell. KEGG database was used to identify enriched pathways by a two-tailed Fisher’s exact test to test the enrichment of the differentially expressed protein against all identified proteins. The pathway with a corrected p-value < 0.05 was considered significant.

Figure 4

Differential protein expression of SK-N-BE (2) in mineral absorption, ferroptosis and autophagy pathways before and after ATO treatment.

Figure 5

GPX4 expression is decreased by incubating the SK-N-BE (2) cells in the presence of ATO (7.3 μ M).
*P<0.05, compared with 24hours control.

Figure 6

SK-N-BE (2) cells were treated with various concentrations of ATO for 24 hours. (A) The enzyme activity of GPX4 were downregulated in 9 μ M ATO. (B) The level of GSH decreased in high concentrations of ATO.