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Effect of Lipoic Acid on the Biochemical Mechanisms of Resistance to Bortezomib in SH-SY5Y Neuroblastoma Cells

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Abstract Neuroblastoma (NB) is an extracranial solid cancer and the most common cancer in infancy. Despite the standard treatment for NB is based on the combination of chemotherapeutic drugs such as doxorubicin, vincristine, cyclophosphamide, and cisplatin, chemoresistance occurs over the time. The aim of the present research was to evaluate the effect of bortezomib (BTZ) (50 nM) on NB cell viability and how lipoic acid (ALA) (100 μM) modifies pharmacological response to this chemotherapeutic agent. Cell viability was assessed by ATP luminescence assay whereas expression of oxidative stress marker (i.e., heme oxygenase-1) and endoplasmic reticulum stress proteins was performed by real-time PCR, western blot, and immunofluorescence. Our data showed that BTZ treatment significantly reduced cell viability when compared to untreated cultures (about 40%). Interestingly, ALA significantly reduced the efficacy of BTZ (about 30%). Furthermore, BTZ significantly induced heme oxygenase-1 as a result of increased oxidative stress and such overexpression was prevented by concomitant treatment with ALA. Similarly, ALA significantly reduced BTZ-mediated endoplasmic reticulum stress as measured by reduction in BiP1 and IRE1α, ERO1α, and PDI expression. In conclusion, our data suggest that BTZ efficacy is dependent on cellular redox status and such mechanisms may be responsible of chemoresistance to this chemotherapeutic agent.

Keywords Neuroblastoma cell cultures · Lipoic acid · Bortezomib · HO-1 · ER-stress · Cancer

Introduction

Neuroblastoma (NB) is an extracranial solid cancer and the most common cancer in infancy [1]. It is a neuroendocrine tumor, and deriving from the neural crest component of the sympathetic nervous system (SNS), it is capable to switch from the highly aggressive chemoresistant disease to the spontaneous regression [2]. Despite the standard treatment for NB is based on the combination of chemotherapeutic drugs such as doxorubicin, vincristine, cyclophosphamide, and cisplatin, chemoresistance occurs over the time. Therefore, it is important to study and evaluate new therapeutic approaches for the treatment of this cancer.

Bortezomib (BTZ), a reversible inhibitor of the 26S proteasome, is used as counteracting drug against the multiple myeloma (MM) [3]. However, recent experimental evidences demonstrated that BTZ treatment is capable to overcome cancer cell resistance in different solid tumors, including NB [4]. In addition, it has been reported a synergistic effect when this drug was used in combination with doxorubicin in vitro [5]. However, BTZ treatment in NB cell lines induces overexpression of heme oxygenase 1 (HO-1), which in turn leads to BTZ resistance [6]. Similarly, our group showed that BTZ-induced

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HO-1 is involved in resistance to proteosome inhibitor and is involved in genomic instability of MM [7]. Heme oxygenases catalyze the degradation of heme into biliverdin, carbon monoxide (CO), and ferric iron. Such upregulation represents an intrinsic defense mechanism to maintain cellular homeostasis and enhance cell survival [8]. In some cancer cells, HO-1 is considered to play a major role as an essential survival factor, protecting against chemotherapy-induced reactive oxygen species (ROS) increase [9–14]. Interestingly, the role of HO-1 in cancer cells has been shown to be cell-specific since in some tumors its upregulation has been shown to be associated with cell cycle arrest and/or death whereas in other malignancies it was associated with tumor progression and survival [15, 16]. Therefore, it is important to evaluate the effects of pharmacological interventions causing a significant modification of redox status and resulting in a modification of cellular response to chemotherapy. Furthermore, it is well known that (+)lipoic acid (ALA) is a neuroprotective and antioxidant agent able to scavenge ROS, to regenerate endogenous antioxidants as well as to promote glutathione synthesis and it also serves as metals chelator [17, 18]. It is well known that oxidative stress is implicated in the development and evolution of a lot of diseases and there are many factors related to oxidative stress as well as to the development of several pathologies. In this regard, previous reports demonstrated that ALA prevented cell death induced by drugs depleting glutathione in NB cells [17]. In fact, it is well known that oxidative stress is implicated in the development and evolution of a lot of diseases and there are many factors related to oxidative stress as well as to the development of several pathologies. In addition, ROS are reactive molecules derived from the natural byproduct of the oxygen metabolism and having significant roles in the homeostasis and in the cell signaling. Moreover, among the antioxidants of the latest generation, lipoic acid is capable to fulfill many important functions of "scavenger" of hydroxyl radicals, hypochlorous acid, of oxygen singlet, and of peroxyl radicals.

Finally, the aim of the present study was to study and evaluate the effect of ALA on NB redox balance and how such effect may impact on NB response to BTZ.

Material and Methods

Cell Cultures and Treatments

NB cell lines were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C and 5% CO_2. ALA was added 24 h before the addition of BTZ (50 nM) for ALA/BTZ combined treatment. For estimation of the effect of BTZ on ER-stress markers and HO-1 expression, NB cells were seeded in six-well culture plate at density 5×10^5 cell per well and treated with BTZ alone and in combination with 5 mM 4-sodium phenylbutyrate (4PBA, Sigma-Aldrich, Milan, Italy) for 6 and 24 h, with 10 μM thapsigargin (Santa Cruz Biotechnology) alone and in combination with 5 mM 4-PBA for 24 h. For viability assay, NB cells were seeded on 96-well culture plate (Eppendorf, Milan, Italy) at density 1×10^4 cell per well, and subsequently treated with 100 μM of ALA. After 24 h, 50 nM BTZ alone and in combination with ALA was added to cell cultures for 24 h. All agents were diluted directly in cell culture medium.

Cell Viability Assay

Cell viability was assessed using ATPlite 1step assay (PerkinElmer, Milan, Italy) according to the manufacturers' protocol. Briefly, the 96-well black culture plate was taken from the incubator and equilibrated at room temperature for 30 min. Subsequently, to each well containing 100 μl of the cell suspension (5×10^5 cells/ml), 100 μl of reconstituted reagent was added and the plate was shaken for 20 min at 700 rpm using orbital shaker (Stuart Scientific, Staffordshire, UK). The luminescence was measured using Victor3 (PerkinElmer, Milan, Italy). Viability of the cells was expressed as percentage of vitality of untreated cells.

Gene Expression Analysis by Real-Time PCR (qRT-PCR)

RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA, USA). First strand cDNA was then synthesized with Applied Biosystem (Foster City, CA, USA) reverse transcription reagent [16, 19]. HO-1 mRNA expression was assessed by TaqMan Gene Expression, Applied Biosystem and quantified using a fluorescence-based real-time detection method by 7900HT Fast Real Time PCR System (Life technologies, Carlsbad, CA, USA). For each sample, the relative expression level of HO-1 (Hs01110250_m1), Ire1 alpha (Hs00980095_m1), and BIP/ GRP78 (Hs00607129_gH) mRNA was normalized using GAPDH (Hs02758991_g1) as an invariant control [20].

Western Blot Analysis

Briefly, for western blot analysis, 30 μg of protein was loaded onto a 12% polyacrylamide gel MiniPROTEAN® TGX™ (BIO-RAD, Milan, Italy) followed by electrotransfer to nitrocellulose membrane TransBlot® Turbo™ (BIO-RAD, Milan, Italy) using TransBlot® SE Semi-Dry Transfer Cell (BIO-RAD, Milan, Italy). Subsequently, membrane was blocked in Odyssey Blocking Buffer (Licor, Milan, Italy) for 1 h at room temperature. After blocking, membrane was three times washed in phosphate-buffered saline (PBS) for 5 min and incubated with primary antibodies against HO-1 (1:1000) (anti-rabbit, Cat. No. BML-HC3001-0025, Enzo Life Sciences, Milan, Italy), BiP (1:1000) (anti-rabbit, Cat. No. 3177S, Cell

Signaling Technology, Milan, Italy), Iron Responsive Element1α (IRE1α) (1:1000) (anti-rabbit, Cat. No. 3294S, Cell Signaling Technology, Milan, Italy), PDI (1:1000) (anti-rabbit, Cat. No. 5683S, Cell Signaling Technology, Milan, Italy), ERO (1:1000), and β-actin (1:1000) (anti-mouse, Cat. No. 4967S, Cell Signaling Technology, Milan, Italy), overnight at 4 °C [21]. Next day, membranes were three times washed in PBS for 5 min and incubated with infrared anti-mouse IRDye800CW (1:5000) and anti-rabbit IRDye700CW secondary antibodies (1:5000) in PBS/0.5% Tween-20 for 1 h at room temperature [22, 23]. All antibodies were diluted in Odyssey Blocking Buffer. The blots were visualized using Odyssey Infrared Imaging Scanner (Licor, Milan, Italy) and protein levels were quantified by densitometric analysis. Data were normalized to β-actin expression [24, 25].

Immunofluorescence

Cells were grown directly on coverslips before immunofluorescence. After washing with PBS, cells were fixed in 4% paraformaldehyde (Sigma-Aldrich, Milan, Italy) for 20 min at room temperature [26, 27]. Subsequently, cells were incubated with primary antibody against HO-1 (anti-rabbit, Cat. No. BMLHC3001-0025, Enzo Life Sciences, Milan, Italy) at dilution 1:200 and against β-actin (anti-mouse, Cat. No. 4967S, Cell Signaling Technology, Milan, Italy) at dilution 1:200, overnight at 4 °C. Next day, cells were washed three times in PBS for 5 min and incubated with secondary antibodies: TRITC (anti-mouse, Cat. No. sc-3796, Santa Cruz Biotechnology) at dilution 1:200, and FITC (anti-rabbit, Cat. No. sc-2012, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at dilution 1:200 for 1 h at room temperature. The slides were mounted with medium containing DAPI (4′, 6-diamidino-2phenylindole, Santa Cruz Biotechnology, Santa Cruz, CA, USA) to visualize nuclei [28]. The fluorescent images were obtained using a Zeiss Axio Imager Z1 Microscope with Apotome 2 system (Zeiss, Milan, Italy). As a control, the specificity of immunostaining was verified by omitting incubation with the primary or secondary antibody. Immunoreactivity was evaluated taking into account the signal-to-noise ratio of immunofluorescence.

Statistical Analysis

Statistical analyses were made by Prism Software (Graphpad Software Inc., La Jolla, CA, USA), (Graphpad Prism, data analysis software, RRID: rid_000081). The data are expressed as mean ± SEM. Statistical analysis was carried out by ANOVA test: it is used to compare the means of more than two samples. The significance of differences between means was analyzed by analysis of variance. A p value of less than 0.05 ($*p < 0.05$) was accepted as statistically significant between experimental and control groups.

Results

ALA Effects in BTZ-Induced Cytotoxicity in Neuroblastoma Cell Lines

We observed that BTZ 50 nM exhibited cytotoxicity in SH-SY5Y ($p < 0.0001$) (Fig. 1). Moreover, we tested the effects of ALA on SH-SY5Y cell proliferation alone or in combination with BTZ. Our data also showed that ALA alone had no significant effect on cell viability, whereas co-treatment with BTZ significantly decreased the cytotoxic effect of BTZ ($p < 0.0001$) (Fig. 1).

ALA Reduces HO-1 Expression Induced by BTZ and Increase Its Nuclear Translocation

In order to evaluate cellular oxidative stress response after BTZ treatment alone or in combination with ALA, we evaluated HO-1 expression. We observed a significant increase of HO-1 mRNA following treatment with BTZ 50 nM ($p < 0.0001$) (Fig. 2a). The evaluation of HO-1 protein expression by western blot assay further confirmed such effect (Fig. 2b, c). Interestingly, these set of experiments suggested a possible cleavage of HO-1 protein (Fig. 2b), which it is known to allow HO-1 translocation into the nuclear compartment where it may exerts its non-canonical functions (i.e., genetic instability, transcriptional activation of antioxidant genes). In order to confirm such evidence, we studied the nuclear localization of HO-1 in neuroblastoma cells by structured Illumination ZEISS Apotome 2 microscopy. We observed an increase of protein expression and nuclear localization signal of HO-1 in neuroblastoma cells after treatment with BTZ

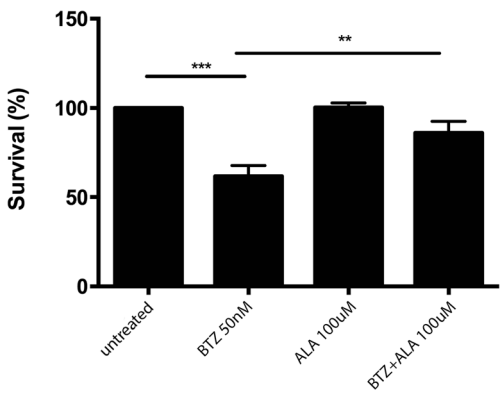

Fig. 1 Determination of cell viability of NB cell lines. ATPLite analysis for cell viability evaluation of NB cell lines untreated, treated with BTZ (50 nM) alone, treated with ALA (100 μM) alone and treated with BTZ + ALA in combination. The data are expressed as mean ± SEM (standard error of the mean). The significance of differences between means was analyzed by analysis of variance. ($*p < 0.05$) ($**p < 0.01$) ($***p < 0.001$)

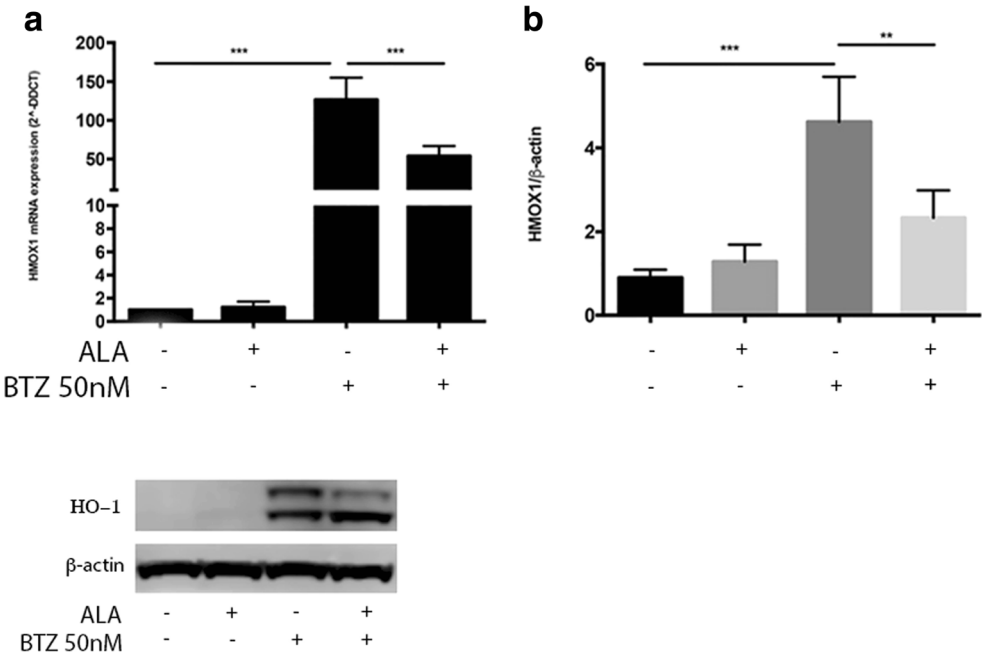

Fig. 2 Evaluation of HO-1 gene and protein expression on NB cell lines. **a** Evaluation of HO-1 gene expression after treatment with ALA (100 μM) alone, with BTZ (50 nM) alone, and with BTZ + ALA in combination, in NB cell lines. **b** Evaluation of HO-1 protein expression after treatment with ALA (100 μM) alone, with BTZ (50 nM) alone, and with BTZ + ALA in combination, in NB cell lines. The data are expressed as mean ± SEM. The significance of differences between means was analyzed by analysis of variance. (*$p < 0.05$) (**$p < 0.01$) (***$p < 0.001$)

(Fig. 3) compared with untreated cells. Cytoplasmic localization of HO-1 was disrupted when BTZ and ALA were used in combination (Fig. 3). Moreover, ALA did not induce HO-1 expression, but in combination with BTZ, ALA was able to reverse BTZ-induced HO-1 expression and promoted its nuclear localization (Fig. 3). In order to confirm ER-stress-induced HO-1 expression, we performed the real-time PCR of the UPR related genes after treatment with thapsigargin alone or in combination with a chemical chaperon 4-PBA. We observed that the thapsigargin treatment of neuroblastoma cells induced UPR related genes expressions such as HO-1 and this effects was reverted by 4-PBA (Fig. 4).

ALA Modulates ER-Stress Signaling

Our data showed that BTZ was able to induce protein expression of ER-stress markers BiP1, IRE1α, ERO1α, and PDI ($p < 0.0001$) compared to control. By contrast, when NB cell lines were exposed to BTZ in combination with ALA, we observed a concomitant reduction of ER-stress protein levels ($p < 0.001$) in respect to BTZ alone (Fig. 5).

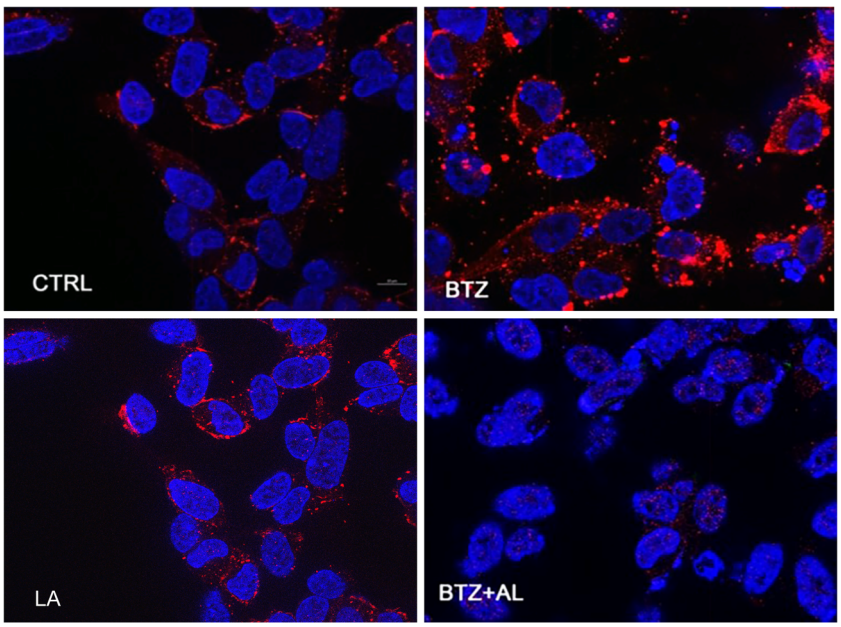

Fig. 3 Microscopy analysis of HO-1 localization in NB cell lines. The detection of HO-1 was performed by incubation with anti-rabbit secondary antibody followed by monoclonal antibody conjugated with TRITC (*red*). The counter-staining of the cells was performed using the nuclear dye DAPI (*blue*)

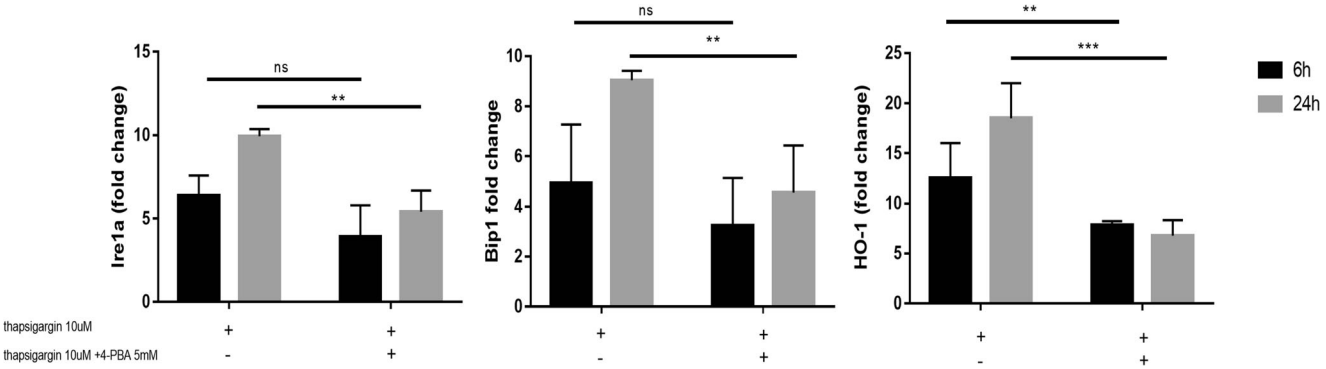

Fig. 4 Gene expression chaperon levels after thapsigargin treatment. Comparison of gene expression chaperon levels of BIP1, Ire1a, and HO-1 in neuoblastoma cell lines treated with thapsigargin (10 μM) alone, and in combination with a chemical chaperon 4-PBA (5 mM). The data are expressed as mean ± SEM. The significance of differences between means was analyzed by analysis of variance. (*$p < 0.05$) (**$p < 0.01$) (***$p < 0.001$)

Discussion

In the present study, we showed the role of ALA on the development of chemoresistance in NB cells treated with BTZ. Our data indicate that ALA, in combination with BTZ, acts as chemical chaperone reducing the stress response induced by proteasome inhibition. Firstly, we showed that BTZ significantly reduces cell viability. These data are consistent with previous observations showing that BTZ, a boronic acid dipeptide inhibitor of the 26S proteasome [12], downregulates the expression of several antiapoptotic factors as well as it induces caspase-dependent apoptosis [29, 30]. It is well known that proteasome inhibitors induce ER-stress, resulting in a UPR and escalation of ROS that leads to cell death. In cancer cells, HO-1 plays a primary role as essential survival factor, protecting against chemotherapy-induced increase in ROS [9, 11, 14]. These observations were confirmed in the present study. Under our experimental conditions, HO-1 upregulation was observed following BTZ treatment on all tested NB cell lines, suggesting a protective role against BTZ-induced ROS. Concomitantly to HO-1 upregulation, we showed a significant induction of ER-stress. Other papers demonstrated that BTZ enhances

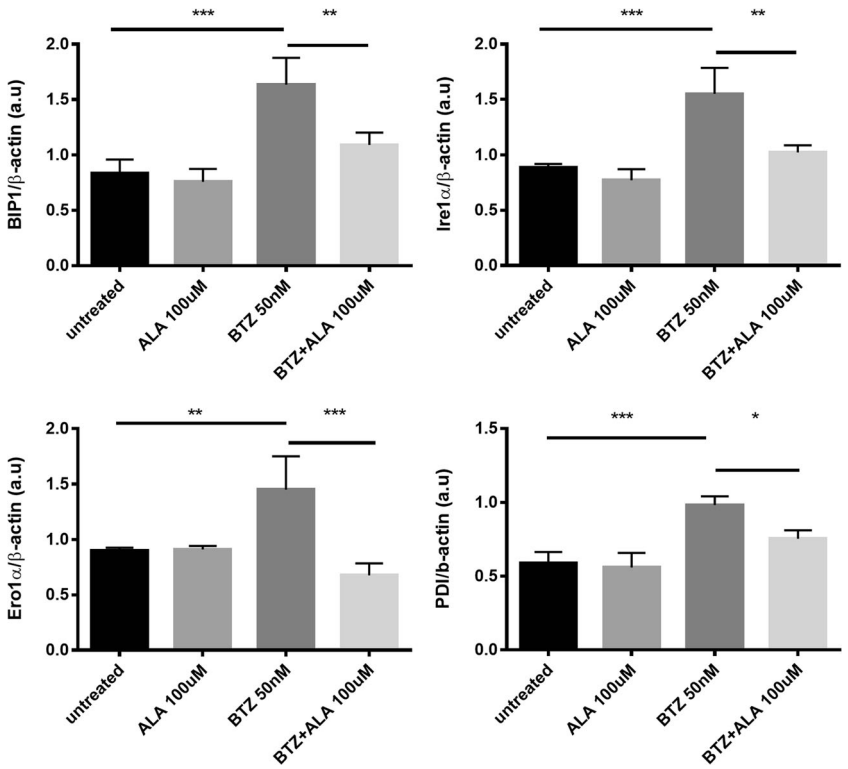

Fig. 5 Protein expression chaperon levels of BIP1, Ire1, Ero1, and PDI in neuoblastoma cell lines. Comparison of protein expression of BIP1, Ire1, Ero1, and PDI in neuoblastoma cell lines treated with BTZ (50 nM) alone, with ALA (100 μM) alone, and in combination with ALA: BTZ + ALA (100 μM). The data are expressed as mean ± SEM. The significance of differences between means was analyzed by analysis of variance. (*$p < 0.05$) (**$p < 0.01$) (***$p < 0.001$)

ER-stress [31, 32] and that HO-1 upregulation is dependent on ER-stress [7]. In order to demonstrate the implications of ALA in the mechanism of cell resistance to the cytotoxic effect induced by BTZ, we co-treated NB cells with ALA 100 μM. Our data shown an increase of cell viability in cells treated with both BTZ and ALA. The co-treatment with BTZ + ALA induced a downregulation of HO-1 and ER-stress respect to NB cells treated with BTZ alone. Previous researchers demonstrated that HO-1 is overexpressed in NB cells BTZ-resistant [33], although we shown both a significantly downregulation of HO-1 and cell survival induced by ALA. At the same time and in the same condition, the immunofluorescence assay showed a nuclear localization of HO-1 compared to NB cells treated with BTZ alone. The nuclear localization of HO-1 has been demonstrated in different situations [34, 35] and may serve to upregulate cytoprotective genes against oxidative stress [36]. In this regard, it has been previously demonstrated that nuclear HO-1 is capable to protect leukemic cells from drug-induced toxicity and it could be also implicated as a regulator of DNA repair activities [37, 38]. Thus we supposed that ALA induces cytoprotection in NB cell lines promoting intracellular HO-1 compartmentalization rather than its enzymatic activity. Some researchers [39] showed that nuclear HO-1 modulates the activation of Nrf2 and induces an adaptive reprogramming that stimulates antioxidant defenses. In order to confirm the pivotal role of HO-1 as chemical chaperone and its antioxidant properties, we treated NB cells with thapsigargin that was able to induce all ER-stress proteins and HO-1 and this effect was reversed by addition of 4-PBA. In conclusion, the mechanisms of cytoprotection of ALA against NB cells treated with BTZ seem to be complex. Our hypothesis is that antioxidant properties of ALA under our experimental conditions are not due to upregulation of HO-1 in response to stress induction by BTZ rather its nuclear localization. Recently, some interesting researches [7] demonstrated that protective effect of HO-1 on drug-induced cytotoxicity in leukemic and myeloma cells does not involve its enzymatic by-products, but rather its nuclear translocation following proteolytic cleavage. Furthermore, they showed that HO-1 upregulation is dependent on ER-stress suggesting a link between oxidative stress and UPR. Whether nuclear HO-1 can regulate the transcription of genes implicated in drug resistance wait for further investigations. All these data demonstrated that ALA protects NB cells by stress and damage induced by BTZ since it reduces ER-stress and activates autophagy as mechanism of cell survival. This work confirms the neuroprotective effects of ALA in neurological field and suggests that it should not use in treatment of neuroblastoma disease, since reduces both redox escalation and cellular damage induced by BTZ.

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