Biochimica et Biophysica Acta 1842 (2014) 613–622

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

HO-1 up-regulation: A key point in high-risk neuroblastoma resistance to bortezomib ☆

Anna Lisa Furfaro [a], Sabrina Piras [a], Mario Passalacqua [a], Cinzia Domenicotti [a], Alessia Parodi [b], Daniela Fenoglio [b], Maria Adelaide Pronzato [a], Umberto Maria Marinari [a], Lorenzo Moretta [c], Nicola Traverso [a], Mariapaola Nitti [a,*]

[a] Department of Experimental Medicine, University of Genoa, 2, L.B. Alberti Street, I-16132 Genoa, Italy
[b] Center of Excellence for Biomedical Research, Department of Internal Medicine, University of Genoa, 16132 Genoa, Italy
[c] Giannina Gaslini Institute, 16147 Genoa, Italy

ARTICLE INFO

Article history:
Received 31 July 2013
Received in revised form 17 December 2013
Accepted 19 December 2013
Available online 28 December 2013

Keywords:
Redox signaling
Chemoresistance
Heme oxygenase-1
Neuroblastoma

ABSTRACT

High-risk neuroblastoma (NB) is characterized by the development of chemoresistance, and bortezomib (BTZ), a selective inhibitor of proteasome, has been proposed in order to overcome drug resistance. Considering the involvement of the nuclear factor-erythroid-derived 2-like 2 (Nrf2) and heme oxygenase-1 (HO-1) in the antioxidant and detoxifying ability of cancer cells, in this study we have investigated their role in differently aggressive NB cell lines treated with BTZ, focusing on the modulation of HO-1 to improve sensitivity to therapy. We have shown that MYCN amplified HTLA-230 cells were slightly sensitive to BTZ treatment, due to the activation of Nrf2 that led to an impressive up-regulation of HO-1. BTZ-treated HTLA-230 cells down-regulated p53 and up-regulated p21, favoring cell survival. The inhibition of HO-1 activity obtained by Zinc (II) protoprophyrin IX (ZnPPIX) was able to significantly increase the pro-apoptotic effect of BTZ in a p53- and p21-independent way. However, MYCN non-amplified SH-SY5Y cells showed a greater sensitivity to BTZ in relation to their inability to up-regulate HO-1. Therefore, we have shown that HO-1 inhibition improves the sensitivity of aggressive NB to proteasome inhibition-based therapy, suggesting that HO-1 up-regulation can be used as a marker of chemoresistance in NB. These results open up a new scenario in developing a combined therapy to overcome chemoresistance in high-risk neuroblastoma.

1. Introduction

Neuroblastoma (NB) is the most common extra-cranial solid tumor in childhood. It accounts for 8–10% of all pediatric cancers and 15% of childhood cancer mortality [1]. One of the clinical hallmarks of neuroblastoma is heterogeneity, varying from the highly aggressive chemoresistant disease to the spontaneous regression in infants [2]. Despite advances in the treatment of other childhood tumors, high-risk neuroblastoma remains one of the most difficult cancers to cure with long-term survival still less than 40%. A typical feature of high-risk disease is MYCN amplification, which occurs in around 20–25% of neuroblastoma, associated with rapid tumor progression, chemoresistance and poor prognosis [3].

The availability of antioxidants is recognized as one of the critical mechanisms able to provide cancer cells with resistance to anticancer therapies [4]. In drug resistance, nuclear factor-erythroid-derived 2-like 2 (Nrf2) is reported to play a key role [5]. Nrf2 is activated in response to electrophylic stimuli and oxidative stress and regulates the expression of many antioxidant and detoxifying genes involved in the development of chemoresistance [6]. Among Nrf2 target genes, heme oxygenase-1 (HO-1) is considered a master regulator of antioxidant response. Heme oxygenase is the first and rate-limiting enzyme in the degradation of heme into biliverdin, carbon monoxide (CO), and free iron [7]. HO-1, the inducible form of the HO system, is a 32-kDa stress protein and is present at low levels in most mammalian tissues [8]. Its expression is induced by a wide variety of stress stimuli, including its substrate, heavy metals, UV irradiation, reactive oxygen species (ROS), nitric oxide, and inflammatory cytokines [9–11]. HO-1 and its metabolic products are involved in the maintenance of cellular homeostasis and play a key role in the adaptive response to cellular stress [12]. Recent experimental evidence has shown the involvement of HO-1 in cancer cell biology, with a dual role. On one hand, HO-1 protects healthy cells from transformation into neoplastic cells by counteracting ROS mediated

* Corresponding author. Tel.: +39 010 3538831; fax: +39 010 3538836.
E-mail addresses: annalisa.furfaro@unige.it (A.L. Furfaro), piras.sabri@tiscali.it (S. Piras), Mario.Passalacqua@unige.it (M. Passalacqua), Cinzia.Domenicotti@unige.it (C. Domenicotti), alessiaparodi@yahoo.it (A. Parodi), Daniela.Fenoglio@unige.it (D. Fenoglio), maidep@unige.it (M.A. Pronzato), umm@unige.it (U.M. Marinari), Lorenzo.Moretta@ospedale-gaslini.ge.it (L. Moretta), Nicola.Traverso@unige.it (N. Traverso), Mariapaola.Nitti@unige.it (M. Nitti).

0925-4439/$ – see front matter © 2013 The Authors. Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbadis.2013.12.008

carcinogenesis. On the other hand, HO-1 protects cancer cells, improving their survival and their resistance to anticancer treatment [13]. High levels of HO-1 have been observed in various human solid tumors (e.g. renal, prostatic and pancreatic) [14–16]. Moreover, HO-1 expression in tumor cells can be further increased by anticancer treatments (chemo-, radio-, and photodynamic therapy) [16] and it has been hypothesized that HO-1 and its products may have an important role in the development of a resistant phenotype [17,18].

Among the new therapeutic strategies designed to overcome cancer cell resistance, the use of proteasome inhibitors has been proposed [19,20].

Bortezomib (BTZ), a selective and reversible inhibitor of the 26S proteasome, the major intracellular pathway of protein degradation, has shown impressive clinical results in anticancer therapeutic approaches. BTZ was approved in 2004 by the US FDA for the treatment of multiple myeloma [21]. Recent experimental evidence has shown that BTZ treatment is able to overcome cancer cell resistance in different solid tumors [22].

In this study we have investigated the effects of bortezomib treatment on neuroblastoma cell lines. We used two NB cell lines: SH-SY5Y, MYCN non-amplified, and HTLA-230, MYCN amplified, in order to mimic two differently aggressive conditions and to compare their sensitivity to bortezomib. In particular, Nrf2/HO-1 pathway has been studied in both cell lines, focusing on the effect of HO-1 modulation to improve BTZ efficacy.

2. Materials and methods

2.1. Cell culture and treatments

MYCN non-amplified SH-SY5Y cells and MYCN amplified, stage IV, HTLA-230 human neuroblastoma cells were obtained from Prof. V. Pistoia (G. Gaslini Institute, Genoa, Italy). Both cell lines were maintained in RPMI 1640 medium (Euroclone, Milan, Italy) supplemented with 10% FBS (Euroclone), 2 mM glutamine (Sigma-Aldrich, Milan, Italy), 1% penicillin/streptomycin (Sigma-Aldrich) and 1% amphotericin B (Sigma-Aldrich), at 37 °C in a 5% CO_2 humid atmosphere and subcultured every 4 days at 1:5.

Both cell lines were treated for 24 h with 5, 10, 20 and 40 nM bortezomib (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Some samples were co-treated with 2.5 μM Zinc (II) protoporphyrin IX (Sigma-Aldrich).

2.2. MTT assay

Cell viability was evaluated by using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) assay. Cells were plated in 96-well plates and after 24 h of bortezomib treatment, incubated with 5 μg/ml MTT for 3 h at 37 °C. Insoluble formazan salts were dissolved in DMSO. The absorbance at 570 nm was measured with a spectrophotometric plate reader (EL-808 BIO-TEK Instruments Inc.). Mean values from each treatment were calculated as a percentage relative to the untreated control cells.

2.3. Detection of cells death by FACS analysis

After treatment, cells were stained with Annexin V-FITC and Propidium Iodide according to the manufacturer's instructions (BioVision, Mountain View, CA, USA). Stained samples were analyzed by flow cytometry using a FACSCanto II flow cytometer (Becton Dickinson Italia, BD, Milan, Italy) equipped by FacsDiva software (BD). At least 10,000 events were analyzed. Each experiment was performed three times.

2.4. Cell proliferation assay

Cell proliferation was evaluated by staining cells with carboxyl fluorescein succinimidyl ester (CFDA-SE, Invitrogen, Milan, Italy), a lipophilic dye that reacts with amino groups on peptides and proteins forming a stable amide bond, and detection by flow cytometry analysis [18,23]. Cells were seeded in six-well plates, washed and incubated with 5 μM CFDA-SE in 10 mM PBS in the dark at 37 °C in 5% CO_2 for 5 min. At the end of incubation, the cells were washed three times with 10 mM PBS supplemented with 1% FBS. Then, the samples were exposed to the treatments described in Section 2.1. After 24 h cells were washed and scraped-off in PBS and the intensity of CFDA-SE fluorescence was evaluated by flow cytometry. The proliferation of CFDA-SE-labeled cells was estimated by the progressive halving of cellular fluorescence as every cell division was completed. Samples were analyzed using a FACSCanto II flow cytometer and FacsDiva software (BD). The flow cytometry data files were analyzed using the Proliferation Wizard module of the ModFit LT 3.2 software (Verity Software House Inc., Topsham, ME, USA). Each experiment was performed three times.

2.5. RNA extraction and RT-PCR

Total RNA was extracted using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions and was then reverse transcribed into cDNA by random hexamer primers and SuperScript™ II Reverse Transcriptase (Invitrogen). Amplification of cDNA by polymerase chain reaction was performed using Platinum Taq DNA Polymerase (Invitrogen) and specific primers for human GCLC, GCLM, HO-1, NQO1, x-CT, p21 and MYCN. Ribosomal 18S expression was used as the housekeeping gene. Primer sequences used (Tib Mol Biol, Genoa, Italy) were: **GCLC** Fw 5′ ATG GAG GTG CAA TTA ACA GAC 3′; GCLC Rv 5′ ACT GCA TTG CCA CCT TTG CA 3′ (206 bp); **GCLM** Fw 5′ CCA GAT GTC TTG GAA TGC 3′; GCLM Rv 5′ TGC AGT CAA ATC TGG TGG 3′ (408 bp); **HO-1** Fw 5′ GCT CAA CAT CCA GCT CTT TGA GG 3′; HO-1 Rv 5′ GAC AAA GTT CAT GGC CCT GGG A 3′ (284 bp); **NQO1** Fw 5′ CAC TGA TCG TAC TGG CTC A 3′; NQO1 Rv 5′ GCA GAA TGC CAC TCT GAA T 3′ (516 bp); **x-CT** Fw 5′ CGT CCT TTC AAG GTG CCA CTG 3′; x-CT Rv 5′ TGT CTC CCC TTG GGC AGA TTG 3′ (295 bp); **p21** Fw 5′ GTC CAG CGA CCT TCC TCA TCC A 3′; p21 Rv 5′ CCA TAG CCT CTA CTG CCA CCA TC 3′ (108 bp); **MYCN** Fw 5′ CCT GAG CGA TTC AGA TGA TG 3′; MYCN Rv 5′ GGC TCA AGC TCT TAG CCT TT 3′ (337 bp); **18s** Fw 5′ GGG GCC CGA AGC GTT TAC T 3′; 18s Rv 5′ GGT CGG AAC TAC GAC GGT ATC 3′ (296 bp). PCR products were separated by electrophoresis on 2% agarose gel pre-stained with ethidium bromide, visualized under UV light and quantified by densitometric analysis by using a specific software (GelDoc, BioRad, Milan, Italy).

2.6. Total protein extraction

Total protein extraction was performed using RIPA buffer as previously described [18].

2.7. Subcellular fractioning

Cytosolic and nuclear protein extraction was performed by using Nuclear Extract Kit (Active Motif, Belgium) following the manufacturer's instruction. Protein concentration of each subcellular fraction was measured using the BCA test (Pierce, Thermo Fisher Scientific, Rockford, USA).

2.8. Western Blot

Proteins were denatured in Laemmli buffer and then subjected to 10% or Any-kD SDS-polyacrylamide gel electrophoresis 200 V for 50 min (Mini Protean TGX Gel, Bio-Rad, Milan, Italy), followed by electroblotting (100 V for 50 min) on PVDF membrane (Pierce).