Biochimica et Biophysica Acta 1862 (2016) 601–610

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Altered S-nitrosothiol homeostasis provides a survival advantage to breast cancer cells in HER2 tumors and reduces their sensitivity to trastuzumab

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ARTICLE INFO

Article history:
Received 14 October 2015
Received in revised form 21 January 2016
Accepted 4 February 2016
Available online 6 February 2016

Keywords:
Apoptosis
Breast cancer
S-nitrosoglutathione reductase
HER2/neu
Nitric oxide
S-nitrosylation

ABSTRACT

The monoclonal antibody trastuzumab against HER2/neu, which is overexpressed in 15–20% of breast cancers, has clinical efficacy but many patients do not respond to initial treatment or develop resistance during treatment. Nitric oxide (NO) regulates cell signaling by targeting specific cysteine residues in proteins, forming S-nitrosothiols (SNO) in a process known as S-nitrosylation. We previously reported that molecular characteristics in breast cancer may dictate the tumor response to impaired SNO homeostasis. In the present study, we explored the role of SNO homeostasis in HER2 breast tumors.

The antiproliferative action of trastuzumab in HER2-overexpressing BT-474 and SKBR-3 cells was suppressed when S-nitrosoglutathione reductase (GSNOR/ADH5) activity, which plays a key role in SNO homeostasis, was specifically inhibited with the pyrrole derivative compound N6022. Moreover, GSNOR inhibition restored the activation of survival signaling pathways involved in the resistance to anti-HER2 therapies (AKT, Src and c-Abl kinases and TrkA/NRTK1, TrkB/NRTK2, EphA1 and EphA3 receptors) and reduced the apoptotic effect of trastuzumab. Accordingly, GSNOR inhibition augmented the S-nitrosylation of apoptosis-related proteins, including Apaf-1, pSer73/63 c-Jun, calcineurin subunit α and HSF1. In agreement with in vitro data, immunohistochemical analyses of 51 breast tumors showed that HER2 expression was associated with lower expression of GSNOR protein. Moreover, gene expression analysis confirmed that high ADH5/GSNOR gene expression was associated with high patient survival rates in HER2 tumors.

In conclusion, our data provide evidence of molecular mechanisms contributing to the progression of HER2+ breast cancers and could facilitate the development of therapeutic options to counteract resistance to anti-HER2 therapies.

1. Introduction

Breast cancer is the most common malignancy in women and one of the leading causes of death among women. In 2014, the rate of incidence of breast cancer in the United States represented 29% of all cancer cases with 15% of total cancer deaths that year [43]. In Europe, the rate of incidence of breast cancer was 29%, with an estimated 17% of deaths from this tumor. About 15–20% of patients with breast cancer have HER2/neu (human epidermal growth factor receptor 2) positive tumors, and overexpression or amplification of HER2 has been shown to be an important predictor for both overall survival and for the time to relapse in these patients [31]. The use of the humanized monoclonal antibody trastuzumab as therapy directed against HER2 has shown considerable clinical efficacy and increased overall survival of patients

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http://dx.doi.org/10.1016/j.bbadis.2016.02.005
0925-4439/

with HER2 positive breast tumors [3]. Trastuzumab has been shown to induce tumor regression in about a third of patients with HER2-positive metastatic cancer, but this response is hardly sustainable only with trastuzumab as monotherapy [5]. Moreover, the overall response rate to trastuzumab remains modest since about 26% of patients respond when trastuzumab is administered as monotherapy, and 40–60% of patients will respond when used in combination with systemic chemotherapy [41,51]. Many patients do not respond to initial treatment with trastuzumab (de novo resistance), and many others develop resistance to trastuzumab after continued treatment (acquired resistance) [38,51]. Also, about 10% of patients develop distant recurrence after trastuzumab-based adjuvant chemotherapy, and all patients with metastatic breast cancer eventually develop disease progression. The causes of these failures to anti-HER2 treatment are not well known and more research is needed on those molecular mechanisms involved in HER2 signaling and their response to targeted therapies.

In recent years a number of studies have provided evidence that nitric oxide (NO) may regulate cell signaling by modifying target proteins through reaction with a thiol group in specific cysteine residues, forming a S-nitrosothiol (SNO) in a process commonly known as S-nitrosylation [29]. Several enzymatic mechanisms play important roles in SNO metabolism and therefore in the regulation of this post-translational modification [28,29]. One such factor is nitrosoglutathione reductase (GSNOR), a highly evolutionarily conserved enzyme that plays a key role in SNO homeostasis [26]. Altered SNO metabolism and S-nitrosylation of proteins play a key role in pathologies such as arthritis, diabetes, multiple sclerosis, asthma, cystic fibrosis, pre-eclampsia, and septic shock [17]. However, there are scarce studies addressing the participation of SNO metabolism in cancer.

We have previously shown that molecular characteristics in breast cancer may dictate the tumor response to impaired SNO homeostasis, and that the molecular mechanisms involved may also play a significant role in the development of resistance against hormonal therapies [11]. Significantly, the present study shows that anti-tumoral action exerted by trastuzumab in HER2-overexpressing breast cancer cells is suppressed when GSNOR activity is inhibited. Moreover, our study supports that altered SNO homeostasis provides a survival advantage to cancer cells in HER2 + tumors and may constitute a mechanism of resistance to anti-HER2 therapy in breast cancer.

2. Material and methods

2.1. Materials

N6022 was obtained from Axon Medchem (Groningen, Netherlands). A 50 mM stock solution of N6022 was prepared in DMSO and diluted in culture medium and added to cells as described in figure legends. The final concentration of DMSO was 0.001%, which was added alone at this concentration as vehicle control. The monoclonal antibody trastuzumab (Herceptin®, Roche), was prepared in 7.2 ml distilled water at 21 mg/ml stock concentration, aliquoted and stored at −20 °C. The necessary aliquots were thawed and diluted to the appropriate concentration in culture medium.

2.2. Cell culture

MCF-7, and BT-474 cells were from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and obtained through Sigma-Aldrich (Madrid, Spain). Cells were grown in MEM with Earle's salts (PAA Laboratories GmbH, Pasching, Austria), containing 15% FBS (PAA) and supplemented with 2 mM glutamine, 1% non-essential aminoacids, penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml). SKBR-3 cells were from the ATCC Cell Lines Services (CLS GmbH, Eppelheim, Germany), and were cultured in DMEM (Gibco™ Dulbecco's Modified Eagle Medium, Life Technologies, Carlsbad, CA, USA), containing 10% SFB and supplemented as above.

2.3. Cell proliferation and cell death assays

Cells were seeded in 96-wells plates, treated as described in figure legends and cell proliferation was assayed using the XTT Cell Proliferation Assay Kit (Roche, Basel. Switzerland) following the manufacturer's instructions. The dye produced by viable cells was spectrophotometrically detected (450–655 nm) using an ImarkTM Microplate Reader (Biorad, Hercules, Ca, USA). In each assay, cell proliferation was expressed as percentage of untreated cells. Apoptotic cell death was measured using FITC-conjugated Annexin V/propidium iodide assay (Bender MedSystems Inc., Vienna, Austria) following the manufacturer's recommendations. Flow cytometry was performed in a FACSCalibur (BD Biosciences, San Jose, California, USA) to quantify the percentage of apoptotic cells.

2.4. Cell cycle analysis

Cells ($0.5–1 \times 10^6$ cells) were trypsinized and resuspended in PBS. Ice-cold 100% ethanol was added in a drop-wise manner while gently vortexing and incubated for 20 min at room temperature. Samples were centrifuged at $300 \times g$ for 5 min, resuspended in PBS containing 50 μg/ml propidium iodide plus 100 μg/ml RNase A and incubated for 20 min at room temperature protected from light. Analysis and measurement of propidium iodide fluorescence were performed on a FACSCalibur (BD Biosciences) flow cytometer (FACS; BD, Franklin Lakes, NJ, USA).

2.5. Immunoblotting

Cells grown in 60 mm dishes were harvested with cold PBS and after centrifugation ($1500 \times g$, 4 °C, 5 min), the cell pellet was incubated 15 min on ice with 1 ml lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1.5 mM MgCl2, 10% glycerol, 1% NP40, 0.1 M dithiothreitol (DTT), 0.1 M phenylmethylsulfonyl fluoride (PMSF), 1% *v*/*v* protease inhibitor cocktail (SERVA, Heidelberg, Germany), and 1% *v*/*v* phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich)], and centrifuged at $15,000 \times g$ for 15 min at 4 °C. Cell lysates were stored at 80 °C until analysis. Total protein content of the lysates was determined by a standard Bradford assay using the reagent from Bio-Rad Laboratories (Hercules, CA). Proteins were separated on SDS polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was probed with primary antibodies dissolved in TTBS followed by incubation with secondary antibody conjugated with HRP, chemiluminescent reaction with ECL Plus Western Blotting Detection System or ECL Advance Western Blotting Detection Kit (GE Healthcare Life Sciences, Little Chalfont, UK). Images were captured on a ChemiDoc XRS Imaging System (BioRad Hercules, CA, USA). Sources of antibodies were as follows: polyclonal anti-ADH5 (GSNOR) was from Origene (Rockville, MD, USA), monoclonal anti-phospho-Akt (Ser473), polyclonal anti-Akt, monoclonal anti-phospho-ERK1/2 (Thr202/Tyr204), polyclonal anti-ERK1/2, and monoclonal anti-Cyclin D1 were from Cell Signaling (Beverly, MA, USA). Monoclonal anti-HER2, polyclonal anti-actin, and secondary antibodies conjugated with horseradish peroxidase (HRP) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody array (PathScan® RTK Signaling Antibody Array Kit, Cell Signaling Technology, USA) was incubated with 75 μg of cell lysate protein following the manufacturer's instructions. After samples incubation, phosphorylated proteins were detected on the nitrocellulose membrane by chemiluminescence using ImageQuant LAS4000 equipment (GE Healthcare Life Sciences, Piscataway, NJ, USA). The relative chemiluminescence of each spot was quantified by densitometry using the Quantity One software (Biorad). Data from three independent experiments were normalized to the negative controls included in the array.

2.6. Detection of protein S-nitrosylation by the biotin switch method

The procedure was performed as previously described [11]. Briefly, cell lysates obtained in lysis solution (50 mM Tris–HCl, pH 7.4, 300 mM NaCl, 5 mM EDTA, 0.1 mM neocuproine, 1% Triton X-100 and 1 mM PMSF plus 1% *v*/*v* protease and phosphatase inhibitor cocktails) were incubated with 20 mM methyl methanethiosulfonate (Sigma) followed by acetone precipitation. Precipitates were centrifuged and resuspended in HENS buffer (250 mM HEPES pH 7.7, 1 mM EDTA, 0.1 mM neocuproine, and 1% SDS) and then incubated with 1.2 mM ascorbic acid and 4 mM N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (Biotin-HPDP, Pierce, Rockford, IL) for 1 h. Because biotin-HPDP is cleavable under the reduced conditions, prepared samples were loaded onto SDS-PAGE gels without DTT. All steps preceding SDS-PAGE were carried out in the dark. Biotinylated samples were then detected by immunoblotting using a primary monoclonal anti-biotin antibody (Sigma-Aldrich). To couple biotin-switch assay to the antibody microarray, the former S-nitrosylated proteins labeled with biotin were captured with Cy3-labeled streptavidin (150 μg protein/5 ml incubation buffer and 100 μl of Streptavidin-Cy3 conjugate (Sigma Aldrich), and then incubated for 1 h on the nitrocellulose coated antibodies microarray slides (Panorama® Antibody Array — XPRESS Profiler725 Kit Sigma-Aldrich), according to the protocol described by Numajiri et al. [33]. After 5 washes in washing buffer, slides were dried for 20 min and scanned with a microarray scanner (Axon 4000B, Molecular Devices, Sunnyvale, CA, USA) using the recommendations and settings described by the manufacturer of the array. This microarray of antibodies allow the simultaneous detection of 725 different proteins.

2.7. Immunohistochemical analysis

Immunohistochemical analyses were performed in a paraffin tissue array of breast tumors (Biochain, Newark, CA, USA). Paraffin sections (4 μm) on poly-L-lysine-coated slides were used after drying for 30 min at 60 °C. The sections were dewaxed in xylene, rehydrated in ethanol and incubated at 100 °C in ChemMate™ Target Retrieval Solution (Dako, Barcelona, Spain) pH 6.0 for 20 min. After washing in PBS, the sections were incubated for 10 min in 3% hydrogen peroxide to block endogenous peroxidase, and then incubated overnight with 1:10 dilution of polyclonal anti-ADH5 (Human protein Atlas, Sigma-Aldrich) at 4 °C. After washing 5 min in PBS, the slides were incubated 30 min with a HRP-labeled polymer (DAKO Envision™ System) and developed for 3 min using diaminobenzidine. Finally, the slides were counterstained with hematoxylin and mounted in Eukitt mounting medium. Microscopy images were obtained using a Coolscope digital microscope (Nikon, Tokyo, Japan). Sections were independently scored by two experienced pathologists.

2.8. Statistical analysis

Reported values are the means ± SEM (n = 3) and statistical comparisons were determined with two-tailed Student's t tests. Associations between HER2 status and GSNOR expression in tumors were assessed by chi-square test. All *p* values resulted from two-sided tests and were considered significant when $p < 0.05$. Kaplan–Meier curves from gene expression and survival data from breast cancer patients were made using a freely available on-line database and Kaplan Meier plotter tool (http://kmplot.com/analysis/) which integrates gene expression and clinical data [20].

3. Results

3.1. The inhibition of GSNOR activity reduces the anti-tumoral effect of trastuzumab in HER2 overexpressing breast cancer cells

Nitrosoglutathione reductase activity (GSNOR/ADH5) plays a key role in maintaining SNO homeostasis [26]. Therefore, we decided to

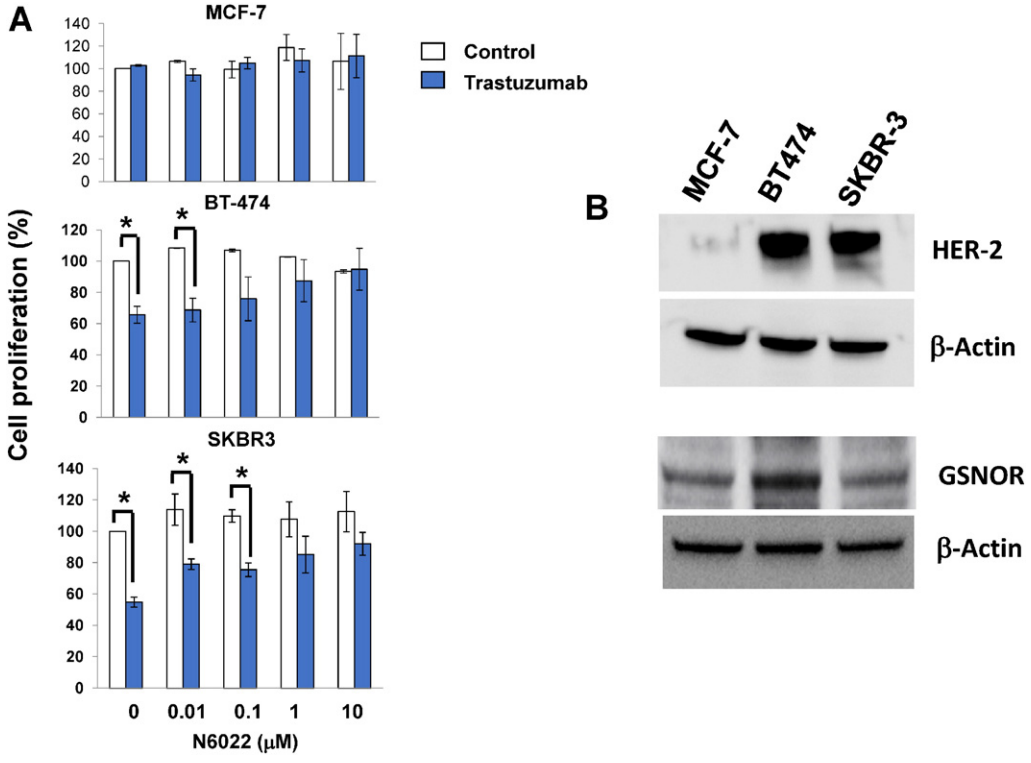

Fig. 1. The antiproliferative effect of trastuzumab in HER2 + breast cancer cells is abolished when GSNOR enzyme system is specifically inhibited. (A) MCF-7, BT-474 and SKBR-3 cells were exposed to the indicated treatments and cell proliferation was determined after 72 h. Cell proliferation is expressed as percentage of control. Data are means ± standard error of the mean of three independent experiments (*$p < 0.05$). (B) The expression of HER2 and GSNOR in MCF-7, BT-474 and SKBR-3 cells was analyzed by Western blot using the corresponding antibodies.

study the effect of inhibiting this enzyme on the response of breast cancer to anti-HER2 therapy. To this end, cells were treated with N6022, which is a pyrrole derivative compound with potent inhibitory activity on GSNOR [12,19]. To do this, BT-474 and SKBR-3 breast cancer cells, which overexpress HER2, were treated with the monoclonal antibody trastuzumab, in the absence or presence of different doses of N6022. As shown in Fig. 1A, the antiproliferative action exerted by trastuzumab in HER-2 overexpressing cells was significantly suppressed when the GSNOR activity was inhibited. On the other hand, the antiproliferative action of anti-HER2 drug was not observed in MCF-7 cells that express GSNOR but do not overexpress HER-2 receptor (Fig. 1A and B).

To further explore the mechanisms participating in this resistance to anti-HER2 therapy when GSNOR is inhibited, cell signaling pathways involved in cell proliferation and survival were analyzed. As shown in Fig. 2, analysis by Western blot revealed that trastuzumab did not inhibit ERK1/2 activation or cyclin D1 expression. In fact, trastuzumab exposure increased ERK1/2 phosphorylation, an effect previously reported to be associated with a feedback loop switched on by activation of EGFR/HER2 and HER2/HER4 dimerisation in response to trastuzumab treatment [18]. On the contrary, anti-HER2 therapy did exert a potent inhibitory effect on AKT survival pathway, as indicated by the lower levels of the phosphorylated form of this protein in cells treated with trastuzumab. However, GSNOR inhibition partially restored AKT phosphorylation in trastuzumab-treated HER2 + breast cancer cells. Receptor tyrosine kinases (RTKs) and their signaling pathways, play an important role in regulating most key cellular processes, including cell cycle, cell migration, metabolism, cell survival, and cell proliferation and differentiation. Therefore we next analyzed in our experimental conditions the activation of RTKs and signaling kinases using an array of antibodies against various signaling kinases. As shown in Fig. 3, N6022 decreased the inhibition by trastuzumab of TrkA/NRTK1, TrkB/NRTK2, EphA1 and EphA3 receptors and Src, c-Abl, AKT (Thr 308) and AKT (Ser 473) signaling kinases. Therefore, impairing of GSNOR system abolishes the inhibition by trastuzumab of cell proliferation and survival signaling pathways in HER2 overexpressing breast cancer cells.

To confirm these results, the rate of apoptotic cell death was analyzed in BT-474 cells undergoing anti-HER2 therapy in the presence or absence of N6022. As shown in Fig. 4, the treatment with N6022 inhibitor abolished the apoptotic effect of trastuzumab in BT-474 cells. Significantly, similar results were obtained in SK-BR-3 breast cancer cells, that also overexpress HER2 receptor.

G1 cell cycle arrest is one of the known effects of trastuzumab [3,45]. Indeed, when BT474 cells were exposed to this monoclonal antibody, a significant increase in the percentage of G1 phase cells, with a concomitant reduction in the percentage of cells in S and G2/M phases (Fig. 5). However, the GSNOR inhibition in these cells did not reverse these cell cycle alterations, although partially recovered the number of cells in G2/M phase, possibly due to the anti-apoptotic effect in this experimental model and confirming that this compound does not induce proliferation of cells treated with trastuzumab. Similar, although less pronounced alterations were obtained in the case of SKBR-3 cells (Fig. 5). Compared to BT-474, SKBR-3 cells showed somewhat lower GSNOR expression (Fig. 1), which could explain their lower response to GSNOR inhibition in terms of apoptosis and cell cycle changes.

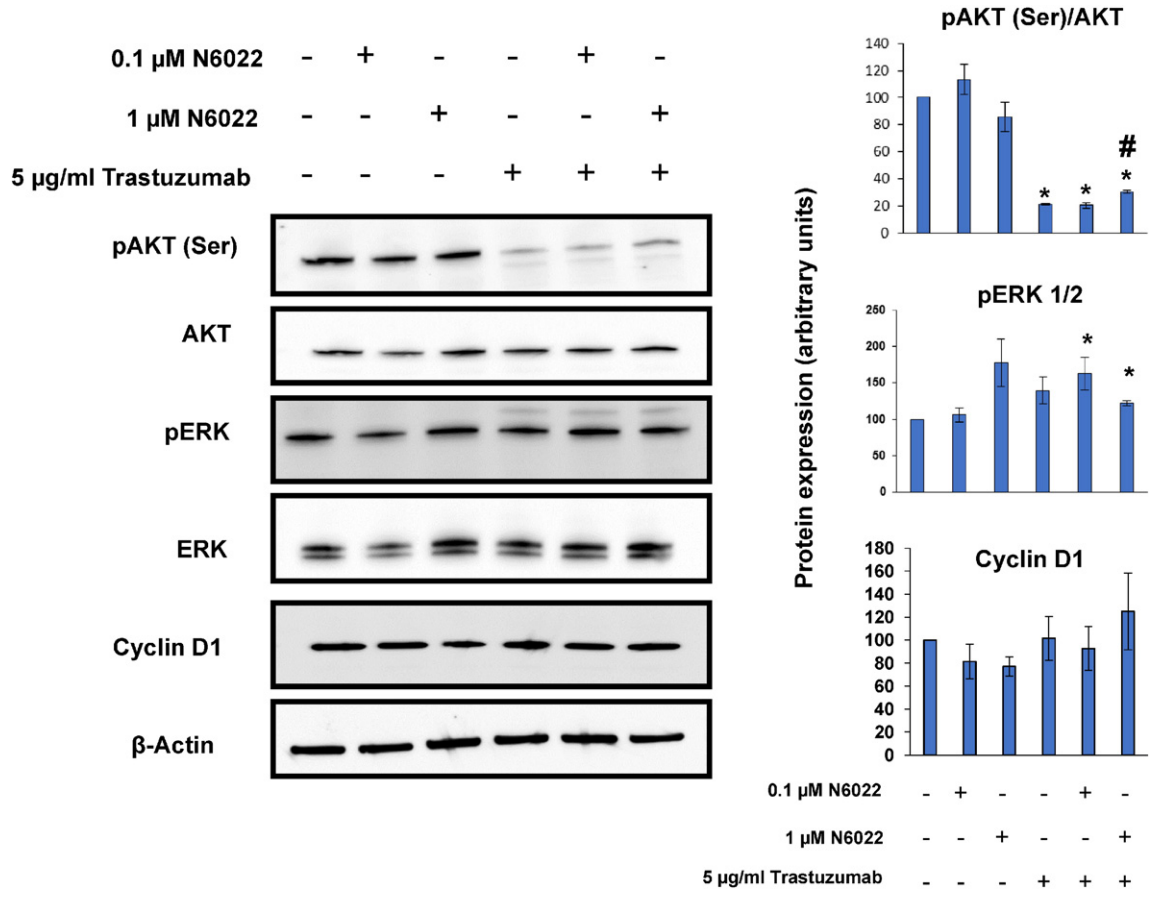

Fig. 2. The inhibition of GSNOR activity partially restores AKT activation in trastuzumab-treated BT-474 cells. Cells were exposed for 6 h to the indicated treatments and AKT and ERK 1/2 phosphorylation, and cyclin D1 expression were determined by Western blot using specific antibodies. The corresponding densitometric analyses of the protein bands detected in Western blots and normalized against β-actin are shown. Data are means ± standard error of the mean of three independent experiments (*$p < 0.05$: compared to control cells; #$p < 0.05$: compared to cells treated with trastuzumab alone).

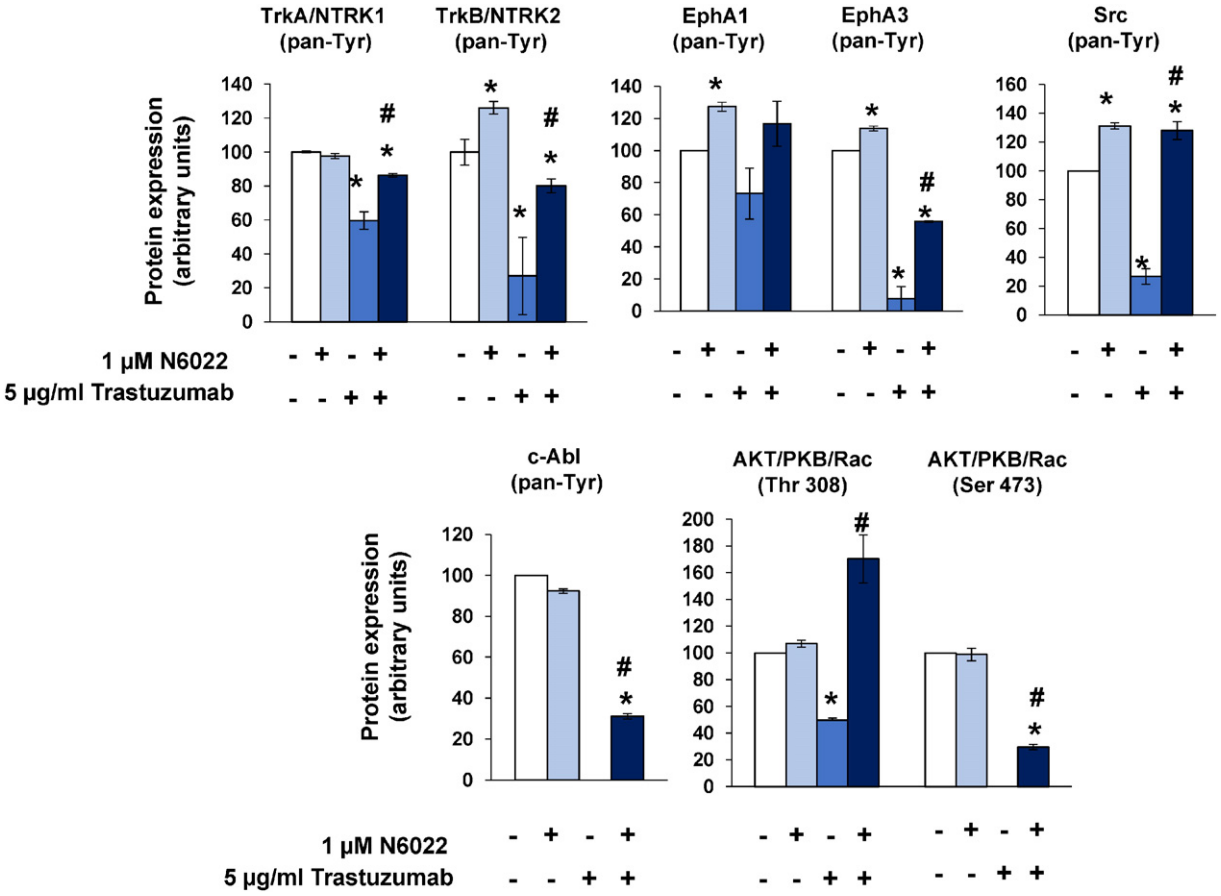

Fig. 3. GSNOR inhibition restores the activation of survival signaling pathways in cells treated with trastuzumab. BT-474 cells were treated for 6 h with the indicated treatments and the expression of signaling kinases was analyzed using an antibody array of RTK signaling kinases, as described in the Materials and methods section. Data were normalized to untreated cells and are expressed as mean ± standard error of the mean of three independent experiments (*$p < 0.05$: compared to control cells; #$p < 0.05$: compared to cells treated with trastuzumab alone).

3.2. The inhibition of GSNOR activity augments protein S-nitrosylation levels in breast cancer cells

To confirm the disruption of S-nitrosothiol homeostasis after GSNOR inhibition, protein S-nitrosylation levels were analyzed in BT-474 cells treated with N6022. As shown in Fig. 6, treatment of BT-474 cells with the GSNOR inhibitor caused a significant increase in the levels of S-nitrosoproteins detectable by the biotin switch assay. Moreover, only in the presence of ascorbate the inhibition of GSNOR in BT-474 cells augmented the intensity of protein bands

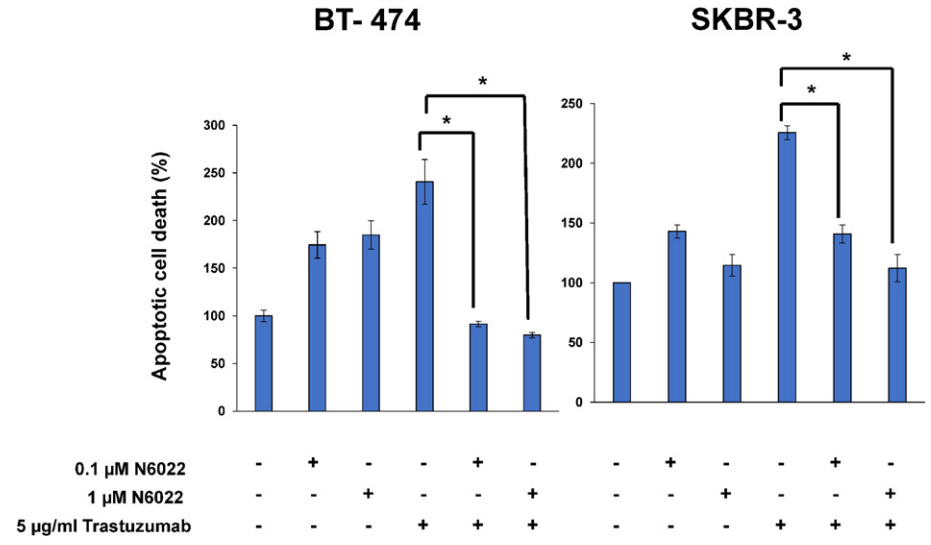

Fig. 4. GSNOR inhibition reduces the apoptotic effect of trastuzumab in HER+ breast cancer cells. Cells were exposed to the indicated treatments and the rate of apoptotic cell death was determined after 48 and 72 h for BT-474 and SKBR-3 cells respectively. Apoptotic cell death is expressed as percentage of untreated cells. Data are means ± standard error of the mean of three independent experiments (*$p < 0.05$).

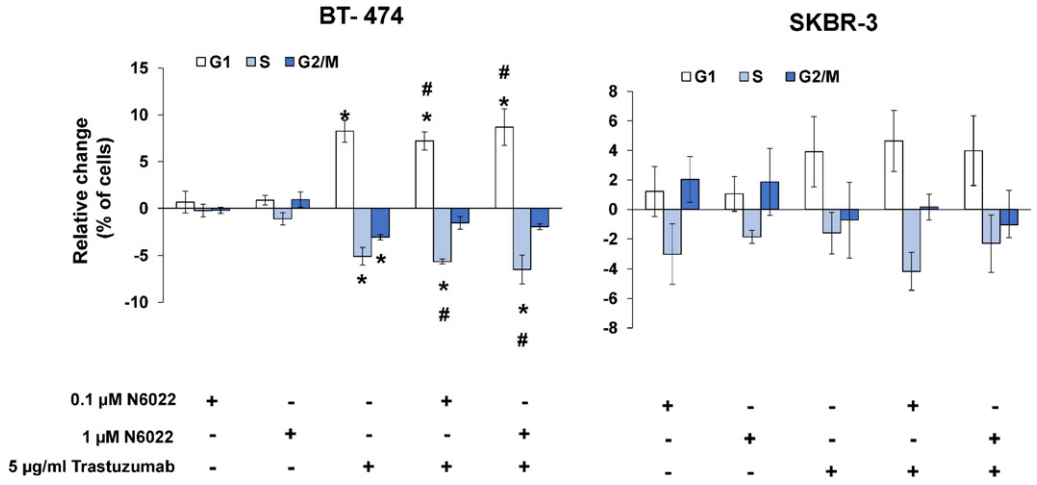

Fig. 5. Cell cycle alterations in cells treated with trastuzumab in the presence or absence of N6022. BT-474 and SKBR-3 cells were subjected to the indicated treatments for 48 h and cell cycle analysis was performed using flow cytometry □ *p 0.05.; compared to untreated cells with trastuzumab. Data are mean ± standard error of the mean of three independent experiments (*$p < 0.05$: compared to control cells; #$p < 0.05$: compared to trastuzumab-treated cells).

detected in the assay. Next, we decided to identify those proteins with increased S-nitrosylation levels when the GSNOR activity is inhibited in BT-474 cells. However, one of the main limitations of the biotin-switch method as a proteomic tool is that the assay is biased toward the identification of abundant proteins [29]. For that reason, and as an alternative, the biotin-switch assay was coupled to an antibody array. In this approach (see Materials and methods), after application of the biotin switch method, the biotinylated proteins were captured using streptavidin labeled with Cy3 fluorophore and subsequently incubated with an antibody array. As shown in Fig. 7 and Table 1, APAF1 (apoptosis protease-activating factor-1), c-Jun (phosphorylated at Ser 63 and Ser 73), the α subunit of calcineurin, CUGBP1 (CUG triplet repeat, RNA binding protein 1), HSF1 (heat shock factor 1) and neurofilament 160, showed higher S-nitrosylation levels when GSNOR activity was inhibited in BT-474 cells. Notably, most of these proteins play important roles in the regulation and modulation of various aspects of cellular apoptosis machinery [4,13,16,22,24,35,44,46]. Therefore, these results confirm that the alteration through S-nitrosylation of processes involved in cell death may explain the antiapoptotic effects of GSNOR inhibition in breast cancer cells treated with trastuzumab.

3.3. GSNOR expression was associated with high survival rates in HER2 tumors

The in vitro results described above suggest that altered SNOs homeostasis, could provide an adaptive advantage for some types of breast cancer overexpressing HER2. Therefore, we conducted a study of the association between HER2 and GSNOR expression in breast cancer. First, GSNOR expression was analyzed by immunohistochemistry in a panel of 51 breast cancer tumors, whose clinical characteristics are summarized in Table 2. As shown in Fig. 8A, GSNOR expression was mainly detected in the tumor stroma. According to the expression of HER2 and GSNOR immunodetection, the analyzed tumors were classified as $HER2^{-}/GSNOR^{-}$ (n = 11), $HER2^{-}/GSNOR^{+}$ (n = 29), $HER2^{+}/GSNOR^{-}$ (n = 7) and $HER2^{+}/GSNOR^{+}$ (n = 4). Significantly, most tumors which did not express HER2 showed GSNOR expression, while. Conversely. the majority of tumors overexpressing HER2 were negative for GSNOR expression (Fig. 8B).

The results from the immunohistochemical analysis suggested a lower GSNOR expression in HER2 positive tumors, compared with other subtypes of breast tumors. Therefore we decided to explore whether GSNOR expression in HER2 positive breast tumors also had

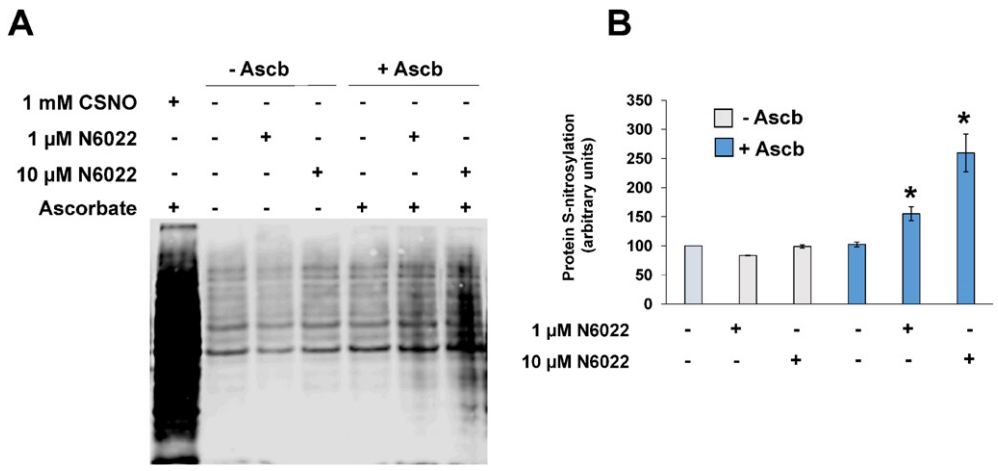

Fig. 6. GSNOR inhibition in BT-474 cells augments protein S-nitrosylation. (A) BT-474 cells were subjected for 6 h to the indicated treatments and cell lysates were analyzed using the biotin-switch assay, in the absence or in the presence of ascorbate (Ascb), for the detection of S-nitrosylated proteins. (B) The corresponding densitometric analysis of all the protein bands detected by biotin switch method, normalized to Ponceau staining of total protein. Data are mean ± standard error of the mean of three independent experiments (*$p < 0.05$: compared to untreated cells).

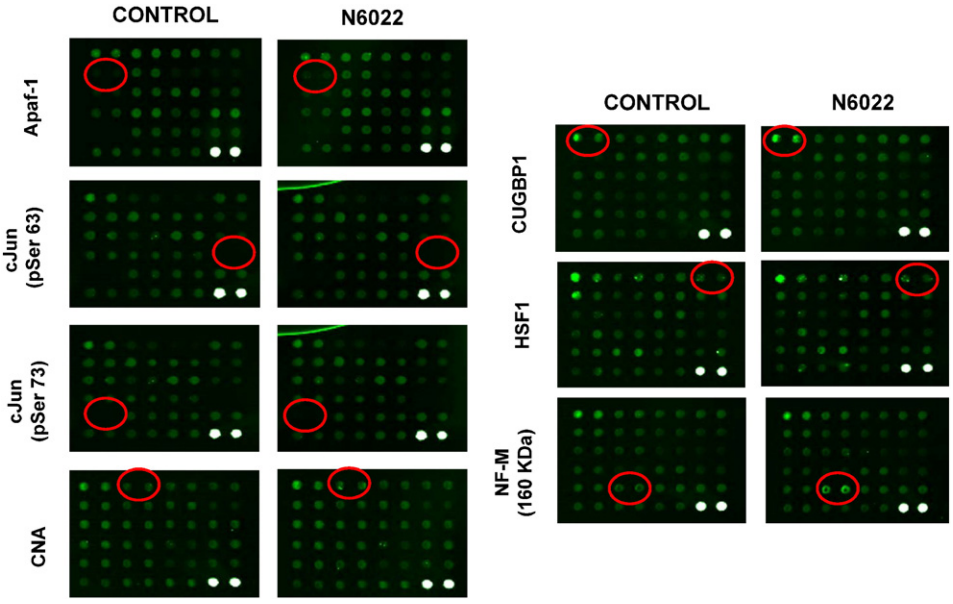

Fig. 7. GSNOR inhibition induces S-nitrosylation of proteins involved in the regulation and modulation of cellular apoptosis. BT-474 cells were cultured for 6 h in the presence or absence of 10 μM N6022, and cell lysates were then subjected to a biotin-switch assay coupled to an antibody array, as described in Materials and methods. Array regions corresponding to proteins that showed a significant change in fluorescence intensity after N6022 treatment are represented. Apaf-1: (apoptosis protease-activating factor-1); cJun (pSer73/63): activated c-Jun protein (phosphorylated on Ser63 and Ser73 residues); CNA: α subunit of calcineurin; CUGBP1: CUG triplet repeat, RNA binding protein 1; HSF1: heat shock factor 1; NF-M (160 KDa): neurofilament 160.

impact on patient survival. To this end, breast cancer patients survival rate was analyzed according to the expression in tumor of ADH5 gene which encodes for GSNOR. An on-line tool was used to analyze the relevance of the gene of interest and to obtain Kaplan–Meier curves from gene expression and survival data of nearly 2000 patients [20]. As shown in Fig. 9, ADH5/GSNOR gene expression only had impact on survival in HER2 breast cancer. Specifically, those HER2 breast cancer patients with increased expression of ADH5/GSNOR in their tumors showed significantly higher survival rates than those with lower expression of this gene. Therefore, these survival analysis data are in agreement with GSNOR expression data in tumors, and suggest that a minor GSNOR expression provides an adaptive advantage for HER2-positive tumors, resulting in lower patient survival rates.

4. Discussion

GSNOR is a key enzyme system maintaining cell SNO homeostasis [7,26,27]. Recent studies suggest that N6022, a pyrrole derivative class with potent activity GSNOR inhibiting activity, has beneficial effects in animal models of diseases such as asthma, obstructive pulmonary disease and chronic inflammatory bowel disease, as low levels of GSNO and other SNO have been implicated in the pathogenesis of such diseases [12,19]. We have previously reported that the alteration of SNO homeostasis by means of thioredoxin reductase (TrxR) inhibition exerted a proliferative effect in estrogen receptor positive breast cancer cells with an intact p53 protein [11]. However, the specific inhibition of GSNOR activity with N6022 had no effect on the proliferation of breast cancer cells. The different mechanisms through which these two enzyme systems maintain SNO homeostasis may explain their differential effects on cell proliferation. In this regard, previous studies in our group showed that the specific alteration of these enzyme systems in hepatoma cells induces different profiles of S-nitrosylated proteins [27]. Significantly, the results of the present study indicate that the antiproliferative action exerted by trastuzumab in breast cancer cells that overexpress HER2 is suppressed when GSNOR activity is inhibited. Therefore, altered SNO homeostasis provides a survival advantage to cancer cells in HER2+ tumors and may constitute a mechanism of resistance to this targeted therapy in HER2+ breast cancer.

One of the signaling pathways activated by the HER2 receptor is the PI3K/AKT signaling cascade, that is involved in cell proliferation and survival [14,40]. AKT activation stimulates cell cycle progression, survival, migration and metabolism through phosphorylation of many physiological substrates [36]. Also, it has been shown that AKT plays a very important role in the suppression of apoptosis through HER2 receptor [1]. It is known that AKT is constitutively activated in breast cancer cells overexpressing HER2 and one of the main mechanisms of action of trastuzumab is the inhibition of PI3K and AKT [14,45]. Indeed, in our study the treatment of HER2+ breast cancer cells with trastuzumab exercised a potent inhibitory effect on AKT survival pathway, as indicated by the lower levels of the phosphorylated form of this protein. However, the inhibition of GSNOR activity significantly impaired this capability of trastuzumab to reduce AKT phosphorylation. High concentrations of NO in neurodegenerative diseases have been reported to increase the S-nitrosylation of PTEN and/or AKT modulating this signaling cascade [23]. Since in the present study neither PTEN nor AKT were detected as S-nitrosylated proteins after GSNOR inhibition, recovery of AKT activation in cells treated by trastuzumab may be due to other SNO-mediated modifications, such as S-glutathionylation of specific cysteine residues of PTEN catalytic domain [39,49]. This inhibition of the phosphatase activity of PTEN, which is the

Table 1
S-nitrosylated proteins detected using the biotin-switch assay coupled to an antibody array.

	Fluorescent intensity (arbitrary units)		
Proteins	C (Vehicle)	T (N6022)	T/C
APAF-1	172.5	348.0	2.02
c-Jun (pSer63)	20.5	49.5	2.41
c-Jun (pSer73)	19.5	43.5	2.23
CNA	792.5	1542.0	1.95
CUGBP1	3356.5	7249.5	2.16
HSF1	888.0	1680.5	1.89
NF-M (160 KDa)	1037.5	3353.0	3.23

Mean fluorescence values (arbitrary units) obtained from the analysis of lysates from BT474 cells treated (T) or not (C) with N6022 are shown. Those proteins with a fluorescent intensity ratio (T/C) ≥1.7 are listed.

Table 2
Pathological characteristics of breast tumors.

Characteristics	HER2− (n = 40)	HER2+ (n = 11)	p value
Histology	40	11	
Invasive ductal	40 (100.0)	11 (100.0)	
Invasive lobular	0	0	
Tumor size	38	11	0.1510
T1	0	1 (9.09)	
T2	37 (92.5)	10 (90.9)	
T3	1 (2.5)	0	
T4	0	0	
Nodal status	38	11	0.4475
N0	20 (50.0)	8 (75.7)	
N1	9 (22.5)	1 (9.09)	
N ≥ 2	9 (22.5)	2 (18.2)	
Metastasis	38	0	0.9856
M0	31 (77.5)	9 (81.8)	
M1	7 (17.5)	2 (18.2)	
Stage	40	11	0.1304
I	0	1 (9.09)	
II	26 (65.0)	8 (75.7)	
III	7 (17.5)	0	
IV	7 (17.5)	2 (18.2)	

Pathological details of the breast tumors analyzed were compared according to their HER status. All parameters were well matched between the two study groups. n = number of cases.

natural inhibitor of PI3K/AKT pathway, therefore would contribute to a partial recovery of this cell survival pathway in conditions of altered SNO homeostasis. Furthermore, it is known that PI3K/AKT activation is required for suppression of cell apoptosis mediated by HER2 [1]. In our study the inhibition of GSNOR activity in HER2+ breast cancer cells reduced the apoptotic effect of trastuzumab, which supports the hypothesis that altered SNO homeostasis in these cells reactivates mechanisms of cell survival.

The decrease in activation of tyrosine kinase receptors (TrkA/NRTK1 and TrkB/NRTK2) following treatment with trastuzumab is in agreement with other studies showing that the TrkA expression in breast cancer cells is associated with HER2 activation [48]. Also, the role of other tyrosine kinase receptors, such as Eph receptors and their ephrin ligands, is also important in HER2 breast cancer. Thus, EphA2 has been shown to be overexpressed in 60–80% of all cases of breast cancer and is also involved in the development of resistance to trastuzumab in HER2+ breast tumors [9,21,34,52]. Moreover, EphA2 has been described to form a complex with HER2 in human carcinoma cells, resulting in promotion of tumorigenesis and metastatic progression [10]. Interestingly, it is known that prolonged exposure to treatment with trastuzumab activates Src kinase that phosphorylates EphA2, and therefore PI3K/AKT survival pathway [21,52]. In breast cancer cells tyrosine kinase c-Src is activated by binding to HER2 and trastuzumab can inhibit their activity by preventing this union [6]. In the present study, treatment with trastuzumab decreased the activation of Src and c-abl, which is other tyrosine kinase signaling through HER2 that is involved in the progression and migration of breast cancer cells, and is also activated through Src [47]. Studies in animal models of breast cancer [8,30] and in human tumors [25,51] have shown that Src is a key modulator in the development of resistance to trastuzumab. Importantly, we have shown that inhibition of GSNOR abolished the inactivation of these tyrosine kinases by trastuzumab, confirming that altered SNO homeostasis promotes mechanisms of resistance to anti-HER2 therapy in breast cancer cells.

We have shown that the specific inhibition of GSNOR activity in HER2+ breast cancer led to increased levels of S-nitrosylated proteins. Moreover, the majority of these S-nitrosylated proteins are key participants in different aspects of regulation and modulation of cell apoptosis. It is known that S-nitrosylation is involved in the apoptotic signaling cascade, and that NO is able to regulate apoptosis by S-nitrosylation of pro-apoptotic and anti-apoptotic proteins [2]. In the intrinsic pathway of apoptosis, cytochrome c is released from mitochondria to the cytosol, where it binds to Apaf-1 and forms an activation complex with procaspase-9 (apoptosome), leading to its activation and cleavage in the form of active caspase-9, which in turn activates effector caspases such as caspase-3, triggering cell apoptosis. S-nitrosylation of Apaf-1 after GSNOR inhibition may affect apoptosome functionality and therefore impairs the activation of the apoptotic pathway. Notably, it has been reported that NO donors inhibit correct assembly of Apaf-1 into an active apoptosome complex, markedly attenuating caspase-recruitment domain interactions between Apaf-1 and procaspase-9 [50]. Active c-Jun protein (phosphorylated at Ser63 and Ser73 residues) was another protein with high levels of S-nitrosylation after N6022 treatment. The c-Jun protein is a component of the transcription factor AP-1, which can promote or inhibit apoptosis, depending on the tissue, the developmental stage and apoptotic stimulus [32]. In fact, AP-1 was one of the first mammalian transcription factors to demonstrate redox-sensitive DNA binding with this redox sensitivity conferred by a

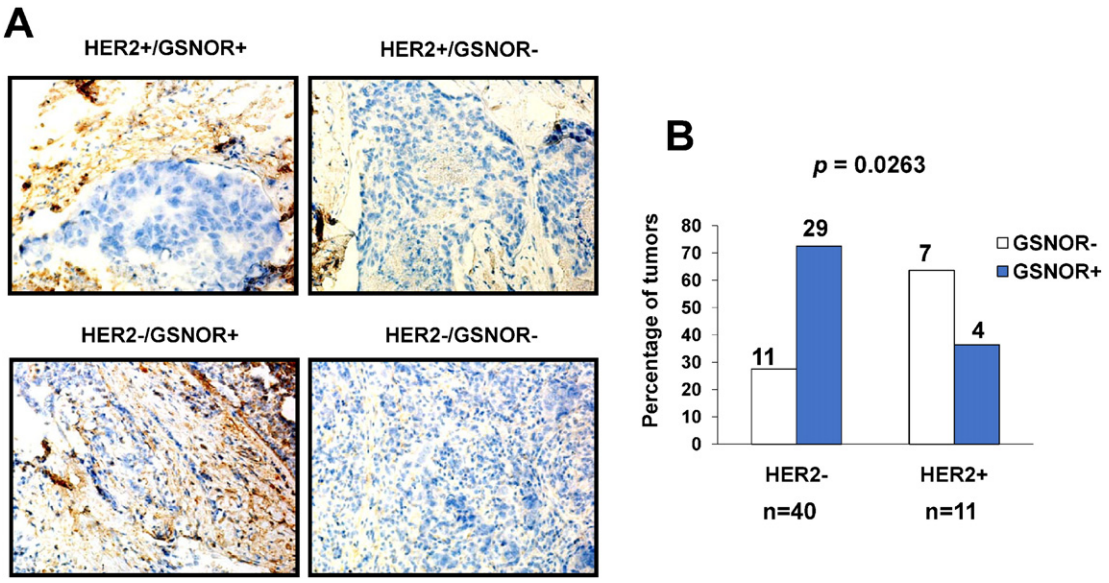

Fig. 8. HER2 expression in breast tumors is significantly associated with lower GSNOR expression. (A) GSNOR expression was analyzed by immunohistochemistry (IHC) in a panel of 51 breast tumors (40 HER2−, 11 HER2+). Representative IHC images of HER2+/GSNOR+, HER2+/GSNOR−, HER2−/GSNOR+ and HER2−/GSNOR− breast tumors are shown. Original magnification: 20×. (B) The frequency distribution according to GSNOR expression was significantly different (p = 0.0263) in HER2− and HER2+ tumors.

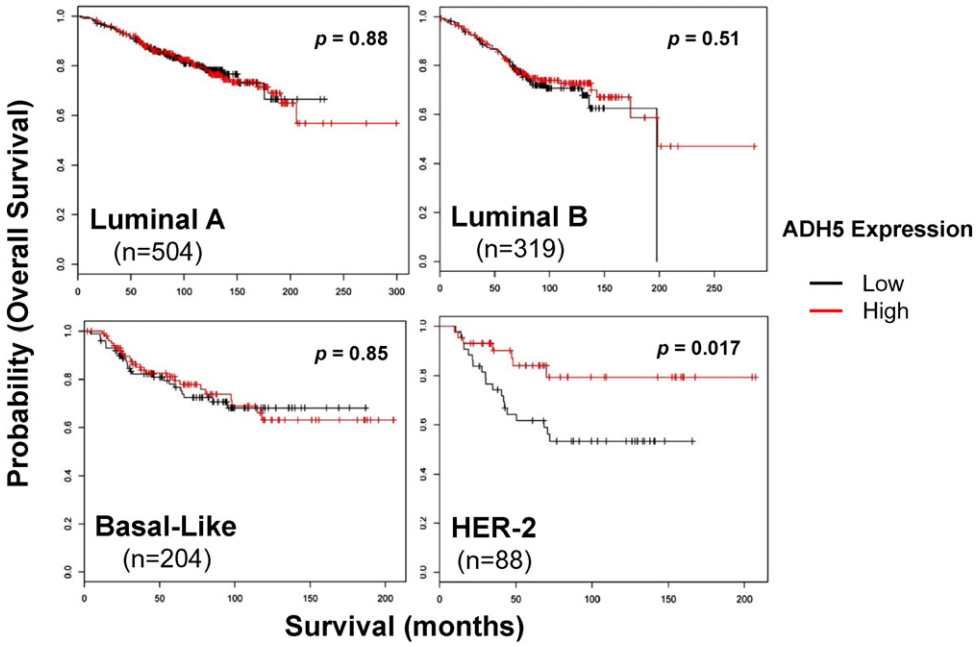

Fig. 9. An increased expression of GSNOR is associated with better survival rates in patients with HER2+ breast tumors. A higher expression of GSNOR is significantly associated with overall survival of patients with HER2+ tumors, but not with other breast cancer subtypes. Red or black curves correspond to survival rates in patients with tumors with high or low ADH5 (GSNOR) gene expression levels, respectively. Kaplan–Meier curves were made using the on-line Kaplan Meier plotter tool (http://kmplot.com/analysis/).

conserved Cys in the domain binding domain of c-Jun, and S-nitrosylation of this Cys residue inhibits DNA binding of this transcription factor [42]. Moreover, the phosphorylation of c-jun at Ser-63 and Ser-73 by c-Jun N-terminal kinases (JNK) can trigger both proapoptotic and antiapoptotic effects [24] and NO is known to inhibit the interaction between JNK and c-jun by S-nitrosylation [37].

Our immunohistochemical analysis suggested a lower GSNOR expression in HER2 positive tumors, compared with other subtypes of breast tumors. GSNOR expression was mainly detected in the tumor stroma, which is becoming increasingly recognized as a source of paracrine growth factors central to the establishment of a microenvironment favorable to tumor growth and progression. Indeed, a NO gradient within the tumor microenvironment has been shown to influence tumor progression through orchestrated molecular interactions between tumor cells and stroma [15]. Similarly, a lower GSNOR activity in tumor stroma may increase the uptake of S-nitrosothiols by epithelial tumor cells and therefore alter their nitrosative status.

Overall, our results indicate that alteration by S-nitrosylation of processes involved in cell death could explain the antiapoptotic effect of GSNOR inhibition in HER2+ breast cancer cells treated with trastuzumab. Furthermore, these data also suggest that, at least in the context of anti-HER2 therapy in HER2+ breast cancer, decreased activity GSNOR also provide an adaptive advantage to this type of tumors. Indeed, and in agreement with the results obtained in vitro, our immunohistochemical analysis showed that, in contrast to HER2 tumors, most HER2 overexpressing tumors were negative for GSNOR expression. Also, the analysis of gene expression data confirmed that expression of ADH5/GSNOR gene only had an impact on patient survival in HER2+ subtype tumors, where the increased expression of this gene was significantly associated with higher survival rates.

5. Conclusions

Our study emphasizes the importance of SNO metabolism in cancer and shows that an altered SNO homeostasis provides a survival advantage to breast cancer cells in HER2 tumors. These findings have clinical relevance, as they support that the expression of ADH5 and/or GSNOR activity, or other factors participating in the control o SNO homeostasis, may be important prognostic factors in HER2 positive breast tumors and also predictive of response to anti-HER2 therapy. Also, they provide evidence of molecular mechanisms contributing to the progression of HER2+ breast cancers and could facilitate the development of therapeutic options to counteract their eventual resistance to targeted therapies.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

Supported by grants PI10-00428 and PI13-00553 from the Plan Estatal de I+D+I, ISCIII-Subdirección General de Evaluación y Fomento de la Investigación, Spanish Government and FEDER (European fund for regional development) funds.

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