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Combined inhibition of Hsp90 and heme oxygenase-1 induces apoptosis and endoplasmic reticulum stress in melanoma

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ABSTRACT

Heat shock proteins are ubiquitous molecular chaperones involved in post-translational folding, stability, activation and maturation of many proteins that are essential mediators of signal transduction and cell cycle progression. Heat shock protein 90 (Hsp90) has recently emerged as an attractive therapeutic target in cancer treatment since it may act as a key regulator of various oncogene products and cell-signaling molecules. Heme oxygenase-1 (HO-1; also known as Hsp32) is an inducible enzyme participating in heme degradation and involved in oxidative stress resistance. Recent studies indicate that HO-1 activation may play a role in tumor development and progression. In the present study we investigated the chemotherapic effects of combining an Hsp90 inhibitor (NMS E973) and an HO-1 inhibitor (SnMP) on A375 melanoma cells. NMS E973 treatment was able to reduce cell viability and induce endoplasmic reticulum (ER) stress (i.e. Ire1α, ERO1, PDI, BIP and CHOP). Interestingly, no significant effect was observed in reactive oxygen species (ROS) formation. Finally, NMS E973 treatment resulted in a significant HO-1 overexpression, which in turn serves as a possible chemoresistance molecular mechanism. Interestingly, the combination of NMS E973 and SnMP produced an increase of ROS and reduced cell viability compared to NMS E973 treatment alone. The inhibitors combination exhibited higher ER stress, apoptosis as evidenced by bifunctional apoptosis regulator (BFAR) mRNA expression and lower phosphorylation of Akt when compared to NMS E973 alone. In conclusion, these data suggest that HO-1 inhibition potentiates NMS E973 toxicity and may be exploited as a strategy for melanoma treatment.

1. Introduction

Molecular chaperones are essential components of the cellular quality control. They can either aid in the folding and maintenance of newly translated proteins, or they can lead to the degradation of misfolded and destabilized proteins. The molecular chaperone Hsp90 plays a central role in a wide variety of cellular pathways and has been implicated in normal cell development, physiology, disease, and evolutionary processes (Zhao and Houry, 2005).

In particular, Hsp90 is essential for the conformational stability and activity of many key oncogenic proteins, including kinases such as ErbB2, B-Raf, Alk, Flt3, EGFR, RET, KIT, PDGFR, MET, AKT but also transcription factors, telomerase, and other proteins that participate in the activation of several biologic pathways whose dysfunctional activation have been collectively described as constituting the hallmark traits of cancer (Hanahan and Weinberg, 2011). Combined inhibition of proteasome and Hsp90 results in a synergistic cytotoxic effect leading to the accumulation of ubiquitinated, misfolded oncogenic proteins (Garcia-Carbonero et al., 2013). To this regard, it was shown that inhibition of Hsp90 induce apoptosis in multiple cancer cells through various pathways, such as mitochondria-mediated and death receptor-induced pathways (Wang et al., 2014). This latter effect has made Hsp90 a novel and attractive target for anticancer therapy and has led to the development of many Hsp90 inhibitors that inhibit the intrinsic ATPase activity of Hsp90 (Gallerne et al., 2013). These inhibitors have been shown to be pharmacologically active in various cancer types

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including melanoma (Acquaviva et al., 2014; Haarberg et al., 2013; Haggerty et al., 2014).

Melanoma is one such cancer type for which conflicting reports on the role of Hsps exist; the validation of Hsps in melanoma as therapeutic targets is of particular importance since Hsp inhibitors are currently being evaluated in the treatment of melanoma patients (Shipp et al., 2013). To this regard, previous reports showed that Hsp90 inhibitors enhance the radiosensitivity of tumor cells and the anticancer efficacy of chemotherapy was observed in a multicenter phase II clinical trial (Miyagawa et al., 2014). However, treatment of cancer cells with compounds such as Hsp90 inhibitors is usually accompanied by the upregulation of other heat shock proteins such as Hsp70 and Hsp27 (Chatterjee et al., 2013; Chauhan et al., 2003; Yasui et al., 2007), protecting cells from apoptosis and contributing to drug resistance. Moreover, heat shock proteins contribute to tumor survival and chemoresistance via their roles in multiple pathways known to be important in cancer (Zhang et al., 2014). Among the various Hsps, an important member heme oxygenase-1 (HO-1) has been detected as possible chemoresistant mechanisms in various cancer types (Furfaro et al., 2014; Tibullo et al., 2013; Wagener et al., 2013).

Heme oxygenase (HO) is the enzyme, which catalyzes the regio-selective, oxidative degradation of heme, with the simultaneous release of carbon monoxide (CO), ferrous iron (Fe^{2+}), and biliverdin, this last further reduced to bilirubin by biliverdin reductase. To date two enzyme isoforms have been identified: HO-1 and HO-2. HO-1, the most studied isoform also know as heat shock protein 32 (Hsp32), is an inducible 32-kDa enzyme that can be stress-induced by a variety of stimuli such as heavy metals, reactive oxygen species and, particularly, heat shock (Barbagallo et al., 2012; Novo et al., 2011). Since HO-1 is induced as a protective mechanism in response to various stimuli, targeted induction of this stress-response enzyme may be considered an important therapeutic strategy for protection against inflammatory processes and oxidative tissue damage (Barbagallo et al., 2013, 2014; Kushida et al., 2002; Li Volti et al., 2007; Sacerdoti et al., 2005). HO-1 expression has been reported as an important protective endogenous mechanism against physical, chemical and biological stress under various experimental and clinical conditions such as solid tumors and acute leukemia (Gleixner et al., 2009; Tibullo et al., 2013).

The aim of the present study was to assess the possible significance of the HO system as a possible chemoresistant pathway to Hsp90 inhibitors in Melanoma cells.

2. Materials and methods

2.1. Cell culture and treatments

Human A375 cells, commercially available melanoma cell line (ATCC, USA), were cultured in Dulbecco's modified Eagle's medium – high glucose (Sigma–Aldrich, Milan, Italy) supplemented with 10% fetal bovine serum (EuroClone, Milan, Italy) and antibiotics (10,000 U/ml penicillin G, 10 mg/ml streptomycin sulfate) at 37 °C under an atmosphere of 5% CO_2, 95% air.

Treatments were performed for 6, 12 and 24 h either with 1 μM NMS E973 alone (Selleckchem, USA), a specific Hsp90 inhibitor, or 10 μM SnMP alone, or a combination of drugs. NMS-E973 was dissolved in DMSO (Sigma–Aldrich) and stored in the dark at −20 °C. For the experiments, NMS-E973 stock solution was diluted to the desired concentrations in medium immediately before use. The same concentration of DMSO was used for vehicle as a control.

The HO activity inhibitor tin mesoporphyrin (SnMP), was prepared just before use and dissolved in medium.

2.2. Western blot analysis

Western Blot analysis was performed as previously descripted (Parenti et al., 2014). Briefly, cells were cultured in T75 flasks, washed with DPBS (EuroClone) and then trypsinized (0.05% trypsin w/v with 0.02% EDTA, EuroClone). Pellets were resuspended in DPBS and then lysed by sonication. After protein quantification, samples (80 or 100 μg/well) were mixed with sample loading buffer (Bio-Rad), boiled for 5 min and then loaded into 8 or 12% SDS-polyacrylamide (SDS-PAGE) gels and subjected to electrophoresis (120 V, 90 min).

The separated proteins were transferred to Immobilon-FL Transfer Membrane (Millipore) using a Trans-Blot Turbo Transfer System (Bio-Rad) (1.2A, 50 min).

After transfer, blots were incubated with Li-Cor Blocking Buffer for 1 h, followed by overnight incubation with 1:1000 dilution of the primary antibody. Primary polyclonal antibodies directed against HO-1, were purchased from Enzo Life Sciences (Farmingdale, NY, catalog number SPA896); primary polyclonal antibodies directed against β-actin (catalog number sc-47778), Akt (catalog number sc-377457), p-Akt (catalog number sc-33437) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA); primary polyclonal antibodies directed against ERO1 (catalog number 3264), PDI (catalog number 3501), Ire1α (catalog number 3294), BIP (catalog number 3177) and CHOP (catalog number 2895) were purchased from Cell Signaling Technology (Beverly, MA, USA).

After washing with DPBS, blots were incubated for 1 h with secondary antibody (1:1000). Protein detection was carried out using a secondary infrared fluorescent dye conjugated antibody absorbing at 800 nm (catalog number 92632210) or 700 nm (catalog number 92668021). The blots were visualized using an Odyssey Infrared Imaging Scanner (Li-Cor Science Tec) and quantified by densitometric analysis performed after normalization with β-actin. Results were expressed as arbitrary units (AU).

2.3. Real-time PCR quantification

Expression of HO-1, BFAR, and 18S were evaluated by realtime PCR (Gazzolo et al., 2009; Salamone et al., 2012). Cultured cell layers in different conditions were rinsed with cold DPBS and immediately lysed using Trizol Reagent (Qiagen). Total RNA was isolated, treated with RNase-free DNase I, and quantified by UV spectrophotometry. For RT-PCR analysis of mRNA expression, 1.0 μg of total RNA (in 20 μl reaction volume) was reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The resultant cDNA was then used as the template for PCR amplification.

The quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the TaqMan gene expression assay (HMOX1 Hs00157965_m1; BFAR Hs00275423_m1; 18S Hs99999901_s1) on a Step One Real-Time PCR System according to the manufacturer's recommended protocol (Applied Biosystems, Foster City, CA, USA). Each reaction was run in triplicate. The comparative threshold cycle (CT) method was used to calculate the amplification fold as specified by the manufacturer. A value of 10 ng of reverse transcribed RNA samples was amplified by using the TaqMan Universal PCR Master Mix.

2.4. Detection of ROS

Determination of ROS was performed by using a fluorescent probe 20,70-dichlorofluorescein diacetate (DCFH-DA) (Masella et al., 2012). The fluorescence [corresponding to the oxidized radical species 20,70-dichlorofluorescein (DCF)] was monitored spectrofluorometrically (excitation, λ = 488 nm; emission, λ = 525 nm). The total protein content was evaluated for each

sample and the results are reported as fluorescence intensity/mg protein.

2.5. Apoptosis assessment by annexin V

To evaluate the possible apoptotic effect of treatments, cells were collected, washed twice with cold PBS, and resuspended in 1X binding buffer at a concentration of 1×10^6 cells/ml. Then 5 μl of FITC annexin V (FITC annexin V Apoptosis Detection Kit, BD Pharmingen) were added, cells were gently vortexed and incubated for 15 min at room temperature in the dark. Four hundred microliters of 1X binding buffer were added. Flow-cytometric analysis was performed within 1 h using a FC500 Beckman Coulter. At least 15,000 cells were acquired for each sample.

2.6. Immunocytochemistry

Immunocytochemistry for ER stress was performed on A375 following anti-calnexin specific antibody (Cell Signaling, catalog number 2679), and then incubated with a species-specific FITC-conjugated secondary antibody (Chemicon, Milan, Italy catalog

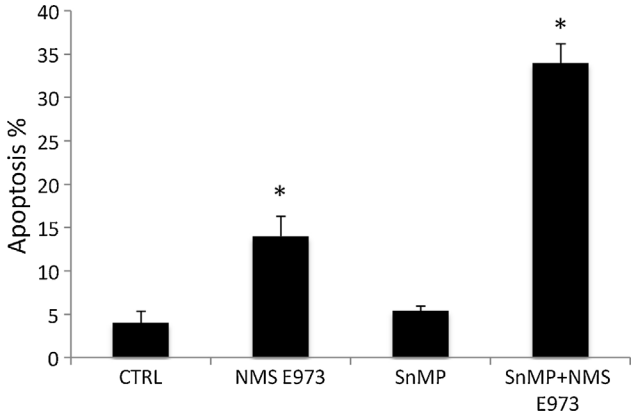

Fig. 1. Percentage of Apoptotic cell death measured by annexin V at 24 h after treatment. All values are mean ± SEM of four experiments ($n = 4$) in duplicate (*$P < 0.05$ versus control).

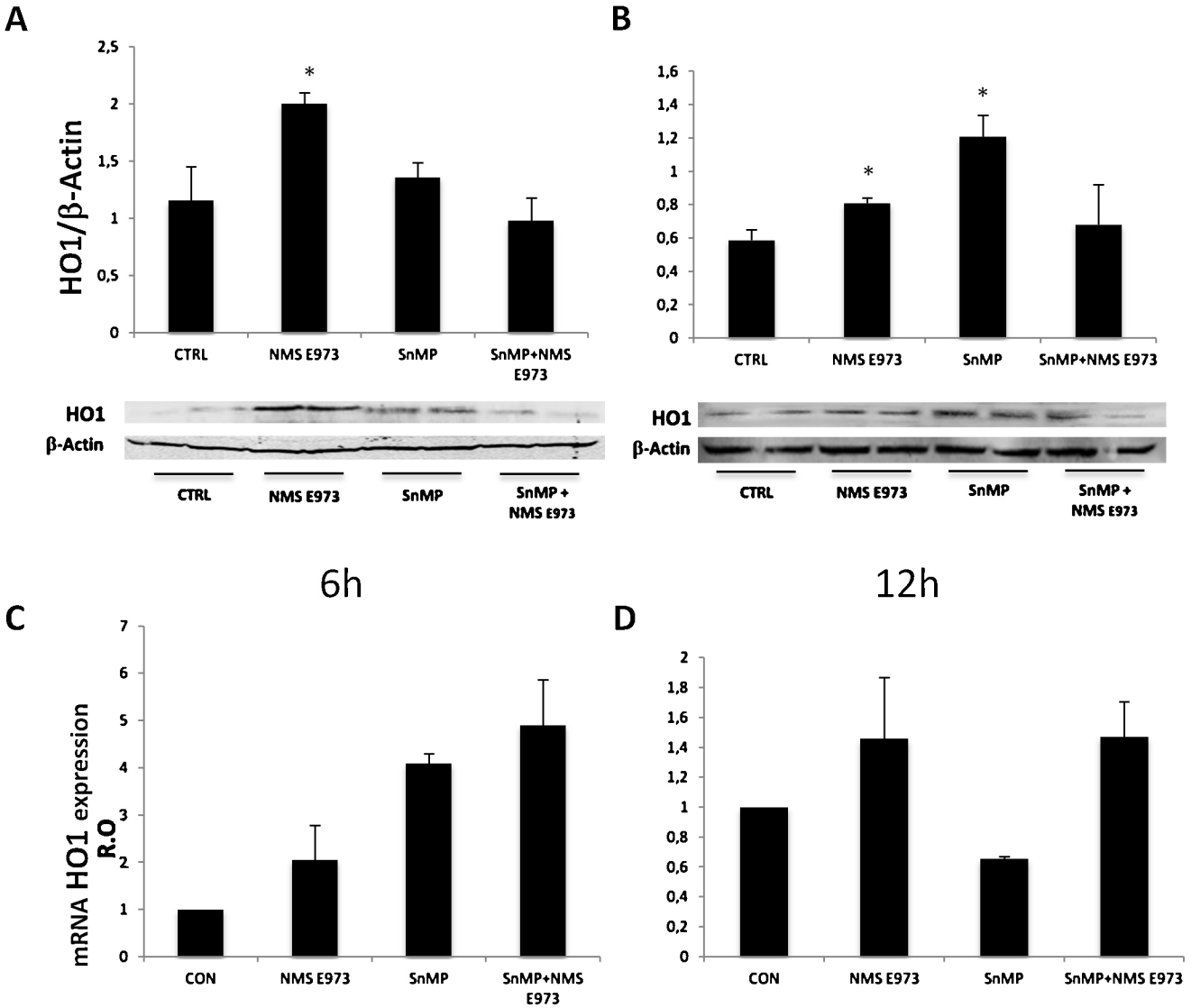

Fig. 2. (A and B) Heme oxygenase 1 protein expression (6 and 12 h) was evaluated by immunoblotting with specific antibody. Densitometric analysis was performed after normalization with actin. (C and D) Heme oxygenase 1 gene expression (6 and 12 h) was evaluated by Real Time PCR. All values are expressed as mean ± SEM of four experiments ($n = 4$) in duplicate (*$P < 0.05$ versus control).

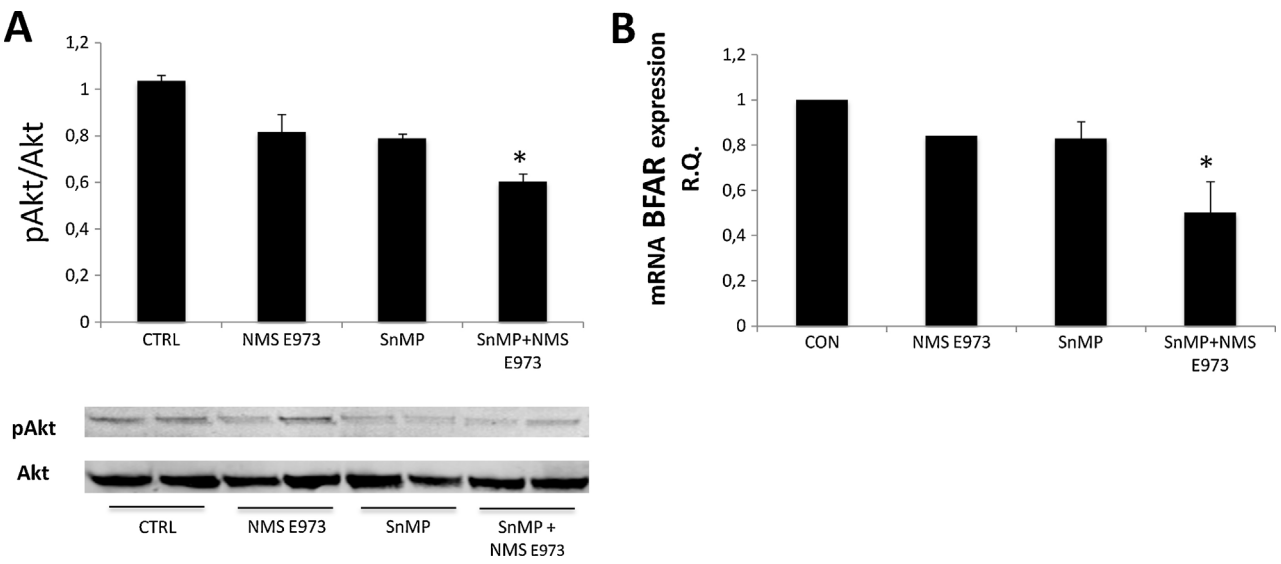

Fig. 3. (A) Phosphorylated Akt was measured by Western Blot at 24 h after treatment. Densitometric analysis was performed after normalization with total Akt. BFAR gene expression was analyzed by Real Time PCR at 24 h after treatment (B). All values are expressed as mean ± SEM of four experiments ($n = 4$) in duplicate (*$P < 0.05$ versus control).

number AP132F). Specimens were washed thoroughly in between incubations and counterstained with DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride) (Sigma–Aldrich). The sections were mounted with polyvinyl alcohol mounting medium with DABCO (Sigma–Aldrich) and visualized under fluorescence microscope.

2.7. Statistical analysis

Differences among the groups were analyzed by the t test and ANOVA (Prism 5, GraphPad, La Jolla, CA). Values were expressed as mean ± SEM, and differences between groups were considered to be significant at $P < 0.05$. Variances in the different groups entered the parametric ANOVA analyses were tested and found not significantly different.

3. Results

3.1. Effect of NMS E973 on apoptosis and HO-1 expression

Hsp90 inhibitor treatment resulted in a significant increase of apoptotic cell death as measured by annexin V in A375 cell at 24 h after treatment (Fig. 1). In order to clarify the impact of the HO system in NMS E973 toxicity, we measured HO-1 protein and gene expression following NMS E973 treatment. Our data showed that NMS E973 treatment resulted in a significantly increase of HO-1 mRNA and protein expression (Fig. 2). Given the protective effects of HO-1 in various cancer types we therefore hypothesize that HO-1 upregulation may serve as a possible chemoresistance mechanism to NMS E973 treatment.

3.2. Effects of combination of Hsp90 and HO1 inhibitors on apoptosis

In order to further clarify the role of HO-1 on A375 cell death, we inhibited HO activity by the use of a competitive inhibitor, namely SnMP. This set of experiment showed that no significant effect was observed on apoptotic cell death following treatment with SnMP alone (Fig. 1A). Interestingly, the combination of the inhibitors (NMS E973 and SnMP) resulted in a significant increase of apoptotic cell death respect to treatment with NMS E973 alone (Fig. 1A). Similarly, only the inhibitors combination was able to decrease phosphorylation of AKT protein (Fig. 3A) and BFAR gene expression (Fig. 3B), known as markers of cell survival.

3.3. ROS production and inhibitors treatment

In order to assess the molecular mechanisms underlying apoptotic cell death, we measured ROS production. This set of experiments showed that only the combination of inhibitors treatment (SnMP and NMS E973) resulted in a significant increase of ROS production after 6 and 12 h (Fig. 4). Interestingly, our data showed that neither SnMP nor NMS E973 treatment alone increased ROS production.

3.4. Effects of combination of Hsp90 and HO inhibitors on endoplasmic reticulum stress

Hsp90 inhibitor treatment was able to induce endoplasmic reticulum (ER) stress. In particular expression of protein markers of reticulum stress such as BIP and CHOP resulted significantly overexpressed (Fig. 5). This effect was enhanced by of the combination of SnMP and NMS E973. In fact, the combination of inhibitors resulted in a marked overexpression of BIP and CHOP, and were marked expressed other markers such as ERO1, PDI and down regulate the Ire1α expression (Fig. 5). These results were further confirmed by

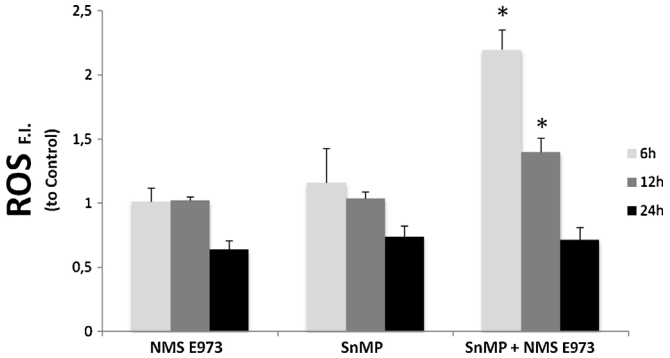

Fig. 4. ROS formation. The data are expressed as fold of increase respect to the control (untreated cells) of each time point. All values are expressed as mean ± SEM of four experiments ($n = 4$) in duplicate (*$P < 0.05$ versus control).

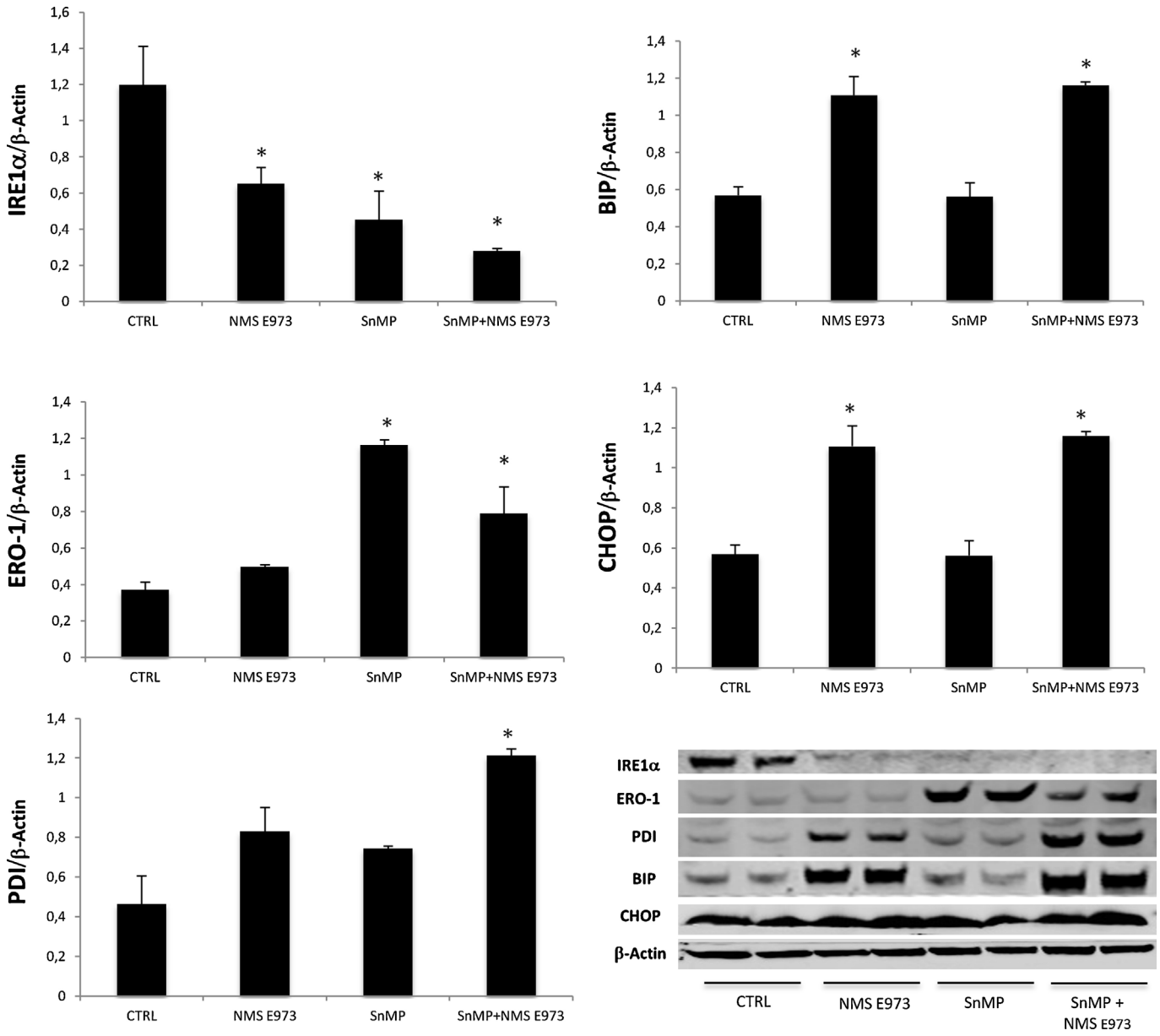

Fig. 5. Expression of Endothelium Reticulum Stress Markers (IRE1α, ERO-1, PDI, BIP and CHOP) by Western Blot Analysis. Densitometric analysis was performed after normalization with actin. All values are expressed as mean ± SEM of four experiments ($n = 4$) in duplicate (*$P < 0.05$ versus control).

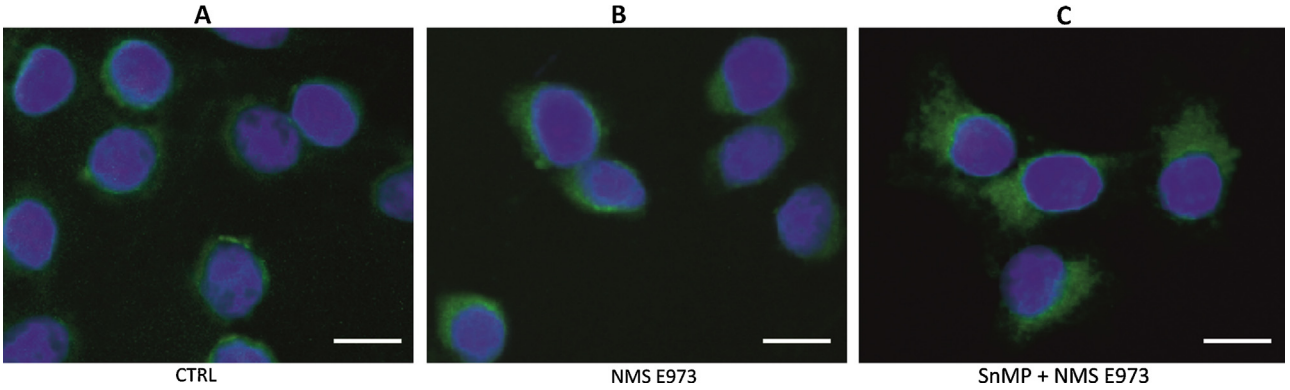

Fig. 6. Immunocytochemical analysis of calnexin, an ER-stress marker, in untreated A375 cell cultures (A) and following NMS E973 (1 μM for 24 h) treatment alone (B) and in combination with SnMP (10 μM for 24 h) (C). Calnexin detection detection was performed by incubation with anti-rabbit monoclonal antibody followed by secondary antibody conjugated to FITC (green). Counterstaining of cells was performed by using the nuclear dye, DAPI (blue) (Scale bars 10 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

immunocytochemical analysis showing that calnexin, a marker of ER stress, is overexpressed following NMS E973 treatment (Fig. 6). Similarly, SnMP in combination with NMS E973 further exacerbated ER stress.

4. Discussion

Previous studies demonstrated that Hsps play important roles in cancer including melanoma, but few clear relationships have been defined and conflicting data have been reported (Shipp et al., 2013). Interestingly, expression of Hsp90 and HO-1 are up regulated in tumors, compared with normal tissue, and tumor cells are particularly sensitive to Hsp90 inhibition (Blagosklonny et al., 2001); similarly, we showed that HO-1 inhibition counteracted pharmacology resistance in leukemia cells (Tibullo et al., 2013). Hsp90 is an attractive cancer therapeutic target due to its association with approximately 200 client proteins, most of which are directly involved in cancer progression (Kamal et al., 2004; Workman, 2004). Inhibition of HSP90 not only suppresses tumorigenesis, but also sensitizes tumor cells toward anticancer drugs (Sobhan et al., 2012). In particular, Hsp90s are ATPases that exert their chaperone role through a complex cycle regulated by the binding and hydrolysis of ATP, and by several co-chaperones such as Hsp70, Hsp40, HOP, and p23 (McConnell and McAlpine, 2013; Yamamoto et al., 2014). However, upon Hsp90 inhibition, cells trigger a series of biochemical pathways (i.e. Hsp70 up regulation) to maintain redox balance and thus conferring chemoresistance to cancer cells (Powers and Workman, 2006). Among various biochemical mechanisms triggered, the HO system is emerging as a key mechanism in various cancer cells (Tibullo et al., 2013). HO-1 is an oxidative stress-response enzyme, which has the ability to reduce intracellular damage (Novo et al., 2011). In particular, HO-1 beneficial effects include anti-inflammation, neuron and cardiovascular protection and anti-senescence (Barbagallo et al., 2008; Cao et al., 2012; Lanteri et al., 2006; Li Volti et al., 2003; Marrazzo et al., 2014). Aberrant oxidative stress-mediated DNA mutation, protein degradation, and organelle damage are the main contributors to neoplastic transformation. Therefore, the induction of HO-1 may decrease tumorigenesis via reducing the extent of intracellular oxidants (Lin et al., 2008, 2010).

In the present study we evaluated the chemotherapic effect of NMS E973, a novel isoxazole-derived class of Hsp90 inhibitors, which has been shown to bind Hsp90α with subnanomolar affinity and high selectivity toward kinases, as well as other ATPases (Fogliatto et al., 2013). NMS-E973 is also able to induce tumor shrinkage in different human tumor xenografts, and it is highly active in models of resistance to kinase inhibitors (Brasca et al., 2013).

Our data show that inhibition of Hsp90, by NMS E973, was able to induce overexpression of HO-1 to partially counteract Hsp90 inhibition. Furthermore, we showed that inhibition of Hsp90 and HO-1 was able to induce apoptosis via ER stress pathway in A375 melanoma cells. In particular, our data showed that inhibition of Hsp90 alone induced apoptosis and ER stress in cell cultures, as showed by significantly overexpression of protein markers of ER stress such as BIP and CHOP. These data are consistent with previous observations showing that Hsp90 inhibition leads to ER stress (Gallerne et al., 2013; Wang et al., 2014).

Furthermore, it was demonstrated that the elevated HO-1 limits the oxidative dysregulation that causes misfolding of ER proteins, decreases the unfolded protein response and reduces several markers of ER stress by reducing ROS (Kim et al., 2012; Lee et al., 2007). In fact, NMS E973 alone did not affect ERO1 protein expression whereas SnMP alone significantly up regulated this protein expression, thus suggesting a link between HO system and ERO1. ERO1 is an important source of ER oxidative stress, its oxidative activity is related to the production of H_2O_2 and consequently burdens cells with potentially toxic reactive oxygen species (ROS) (Zito, 2015). However, the combination of Hsp90 and HO inhibitors exhibited higher ER stress, apoptosis as evidenced by BFAR mRNA expression and lower phosphorylation of Akt, known as markers of cell survival, when compared to NMS E973 treatment alone. Moreover the combination of NMS E973 and SnMP treatment resulted in an overexpression of others markers of ER stress such as ERO1 and PDI.

Our results are consistent with previous reports (Marcu et al., 2002) showing that Hsp90 activity is required to maintain Ire1α stability. Similarly, HO inhibition alone resulted in a significant reduction of Ire1α. It is possible to hypothesize that HO activity also serves as an important determinant for possible chaperone activity of HO-1. To this regard, several reports from our research group suggested that HO-1 may serve as a chaperone under different physiological conditions (Li Volti et al., 2010; Vanella et al., 2013). In addiction, only the inhibitors combination was able to product ROS. The production of ROS has been reported to be a cause or a consequence of ER stress and of the initiation of the mitochondrial pathway of apoptosis (Le Bras et al., 2005; Santos et al., 2009).

In conclusion, our data demonstrated that inhibitors combination of Hsp90 and HO-1 induces cell death through a ROS-dependent mechanism involving ER stress and activation of apoptotic pathway in human melanoma cells.

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