Combined Inhibition of Hsp90 and Heme Oxygenase-1 induces Apoptosis and Endoplasmic Reticulum Stress in Melanoma

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Introduction

Molecular chaperones are essential components of a quality control machinery present in the cell. 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. In particular, Hsp90 is essential for the conformational stability and activity of many key oncoproteins, including kinases such as EGF-R, B-Raf, Akt, Flk, EGFR, NET, KIT, PDGFR, MET, AKT but also transcription factors like NFkappaB, and other proteins that participate in the activation of several biologic pathways whose dysfunctional activity have been collectively described as constituting the hallmark traits of cancer. (2)

The ability of this chaperone to stabilize the active form of a wide range of client oncoproteins 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. (3) These inhibitors have been shown to be pharmacologically active in various cancer types including melanoma (6-8).

Melanoma is one such cancer type for which conflicting reports on the role of hsp exist, but the validation of hsp as therapeutic targets is of particular importance because hsp inhibitors are currently being evaluated in the treatment of melanoma patients. (9) Furthermore, the ability of Hsp90 inhibitors to enhance the radiosensitivity of tumor cells and the anticancer efficacy of chemotherapies observed in a multicenter clinical trial, (10) and these drugs are now in phase II trials (10). 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 (11-13), protecting cells from apoptosis and contributing to drug resistance.

Aiming these hsp, other important members such as heme oxygenase-1 (H0-1) have been detected as possible chemoresistant mechanisms in various cancer types (15-17). Heme oxygenase (HO) is the enzyme which catalyses the regio-selective, oxidative degradation of heme, with the simultaneous release of carbon monoxide (CO), ferrous iron (Fe2+), 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. (18) 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 tumours and acute leukaemia (21)(17).

Methods

Cell culture and treatments.

Cell cultures were cultured in DMEM (Sigma-Aldrich) supplemented with 10 % FBS(EuroClone) and antibiotics 1X at 37°C under an atmosphere of 5% CO2, 95% air. Treatments were performed for 3, 6, 12, 24, 48 and 72 h with 1 μM NMS-E973 alone, or 10 μM SnMP alone, or a combination of drugs. Control groups received DMSO alone. Western blot analysis.

Primary polyclonal antibodies directed against HO-1, were purchased from Enzo Life Sciences; primary polyclonal antibodies directed against b-actin, Akt, p-Akt were purchased from Santa Cruz Biotechnology (Dallas, TX, USA); primary polyclonal antibodies directed against CHOP was purchased from Cell Signaling Technology (Beverly, MA, USA).

Protein detection was carried out using a secondary infrared fluorescent dye conjugated antibody absorbing at 800 nm or 700 nm. The bands were visualized using an Odyssey Infrared Imaging Scanner (LiCor Science Tec) and quantified by densitometric analysis performed after normalization with b-actin. Results were expressed as arbitrary units (AU).

Real-time PCR quantification.

Expression of HO-1, BFAF and GADPH were evaluated by real-time PCR. The quantitative real-time polymerase chain reaction (RT-PCR) was performed with the TaqMan gene expression assay (HMXD1_HO1_Hs00176665_m1; BFAF_H00275423_m1) on a Step One Real-Time PCR System according to the manufacturer’s recommended protocol (Applied Biosystems, Foster City, CA, USA).

Detection of ROS.

Determination of ROS was performed by using a fluorescence probe 20,70-dichlorodihydrofluorescein diacetate (DCFH-DA), month by month spectrophotometer (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.

Statistical analysis.

Differences among the groups were analyzed by the t test and ANOVA. Values were expressed as mean ± SEM, and differences between groups were considered to be significant at P < 0.05.

Results

In order to clarify a possible involved of HO-1 system in NMS973 treatment, we measured the protein and gene expression of HO-1 after HSP90 inhibitor treatment. As expected, NMS973 treatment resulted in a significantly increase of HO-1 mRNA and protein expression (Fig 1).

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 NMS973) resulted in a significant increase of ROS production after 6 and 12h. (Fig 3). Interestingly, this set of experiment showed that neither SnMP nor NMS973 treatment increase ROS production.

Effects of combination of Hsp90 and HO1 Inhibitors on the 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. This effect was enhanced by the combination of SnMP and NMS973. 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 downregulate the Ire1α expression (Fig 4).

Conclusions

We demonstrated that the combination of Hsp90 and HO1 inhibitors produced an increase in ROS formation and those increasing apoptosis when compared to NMS973 alone. The inhibitors together generated ER stress and induced apoptosis as evidenced by BIP/RNA expression, pronounced decrease in phosphorylation of Akt and up-regulation of ERO1, PDI, BIP and CHOP.

In conclusion, these data suggest that inhibition of Hsp90 and HO1 is a promising strategy for melanoma treatment.

References