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Hsp90 inhibition by PU-H71 induces apoptosis through endoplasmic reticulum stress and mitochondrial pathway in cancer cells and overcomes the resistance conferred by Bcl-2

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ABSTRACT

Heat shock protein 90 (Hsp90) has recently emerged as an attractive therapeutic target in cancer treatment because of its role in stabilizing the active form of a wide range of client oncoproteins. This study investigated the mechanism of apoptosis induced by the purine-scaffold Hsp90 inhibitor PU-H71 in different human cancer cell lines and examined the role of Bcl-2 and Bax in this process. We demonstrated that Hsp90 inhibition by PU-H71 generated endoplasmic reticulum (ER) stress and activated the Unfolded Protein Response (UPR) as evidenced by XBP1 mRNA splicing and up-regulation of Grp94, Grp78, ATF4 and CHOP. In response to PU-H71-induced ER stress, apoptosis was triggered in melanoma, cervix, colon, liver and lung cancer cells, but not in normal human fibroblasts. Apoptosis was executed through the mitochondrial pathway as shown by down-regulation of Bcl-2, up-regulation and activation of Bax, permeabilization of mitochondrial membranes, release of cytochrome c and activation of caspases. We also found that, in contrast to the ER stressor thapsigargin, PU-H71 induced apoptosis in cells overexpressing Bcl-2 and thus overcame the resistance conferred by this anti-apoptotic protein. In addition, although Bax deficiency rendered cells resistant to PU-H71, combined treatment with the anticancer drugs cisplatin or melphalan greatly sensitized these cells to PU-H71. Taken together, these data suggest that inhibition of Hsp90 by PU-H71 is a promising strategy for cancer treatment, particularly in the case of tumors resistant to conventional chemotherapy.

1. Introduction

The evolutionary conserved chaperone heat shock protein 90 (Hsp90) is essential for the maturation, activation and stability of cellular proteins, termed clients, mainly involved in the regulation of proliferation, cell cycle progression and survival [1,2]. Hsp90 is one of the most abundant molecular chaperones constituting 1–2% of total cellular proteins under non-stressed conditions and increasing to 4–6% under stress [3]. In cancer cells, the expression and activity of this ATP-dependent chaperone were demonstrated to increase [4], thus promoting the stabilization of the aberrant conformation of mutant proteins. 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. The most studied of these compounds are the natural antibiotic geldanamycin and its derivatives 17-N-Allylamino-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG). Clinical studies with these inhibitors have shown limited efficacy and unacceptable side effects often attributed to the benzoquinone moiety of these molecules [5]. To avoid off-target toxicity, Chiosis and collaborators have designed novel inhibitors using purine as a scaffold [6]. Among these, PU-H71 was identified as a potent inducer of apoptosis in breast tumor, B cell lymphoma, hepatocellular carcinoma and myeloma [7–10]. Although the mechanisms underlying the pro-apoptotic effects of PU-H71-induced apoptosis need further investigation, this Hsp90 inhibitor was reported to trigger caspase activation, PARP cleavage and Akt inactivation in multidrug resistant and triple-negative breast cancer cells [8,11].

Apoptosis is a highly regulated cell death program triggered through the extrinsic (or death receptors) pathway or the intrinsic (or mitochondrial) pathway. The mitochondrial pathway involves mitochondria as central integrators and coordinators of the apoptotic

Abbreviations: Cisp, cisplatin; ΔΨm, mitochondrial transmembrane potential; Hsp90, heat shock protein 90; IM, inner membrane; IP3R, inositol 1,4,5-triphosphate receptor; MLP, melphalan; MMP, mitochondrial membrane permeabilization; OM, outer membrane; PI, propidium iodide; PU-H71, 8-(6-iodobenzo[d][1,3]dioxol-5-ylthio)-9-(3-(isopropylamino)propyl)-9H-purin-6-amine; RT, room temperature; TG, thapsigargin; UPR, Unfolded Protein Response; vMIA, viral mitochondrial inhibitor of apoptosis

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process and is characterized by mitochondrial membrane permeabilization (MMP), which is due to the opening of pores formed by Bcl-2 family members, cardiolipin and/or the mitochondrial permeability transition pore (PTP). MMP leads to dissipation of the mitochondrial transmembrane potential ($\Delta\Psi m$), release of pro-apoptotic proteins (e.g. cytochrome c, AIF) from the intermembrane space to the cytosol, subsequent caspase cascade activation, DNA fragmentation and cell dismantling [12]. Bcl-2 family proteins, which have either pro- (Bax, Bak, Bad…) or anti-apoptotic (Bcl-2, Bcl-x_L…) activities, have been involved as crucial regulators of MMP. The mitochondrial pathway of apoptosis is usually triggered by intracellular stimuli (viral proteins, chemotherapeutic drugs…) or signals emanating from other organelles such as the endoplasmic reticulum (ER) [13].

Disruption of ER homeostasis induces ER stress which in turn leads to the Unfolded Protein Response (UPR), a pro-survival process induced to restore normal ER function. The UPR is initiated by activation of three proximal sensors: the PERK kinase, the transcription factor ATF6 and the kinase/endoribonuclease IRE1, which are normally kept inactive through the interaction with the ER-resident chaperone Grp78/BiP [14]. Upon activation, these sensors lead to the increase of ER chaperones (e.g. Grp78 and Grp94) and to the transcription of UPR target genes (e.g. ATF4, XBP1 and CHOP). However, in the case of prolonged or severe ER stress, the mitochondrial apoptotic process is triggered to eliminate damaged cells [15]. ER stress-dependent apoptosis was reported to be mediated mainly by the pro-apoptotic transcription factor CHOP, pro-apoptotic members of the Bcl-2 family and direct calcium transfer from ER to mitochondria at contact sites between the two organelles [16,17]. Therefore, targeting ER stress and components of the UPR is a promising strategy for cancer treatment. Interestingly, Hsp90 has been demonstrated to be an important component of the transcriptional arm of the UPR, as it associates with IRE1 and PERK to maintain their stability [18]. In addition, inhibition of the molecular chaperone function of Hsp90 by geldanamycin or 17-AAG was reported to induce ER stress-mediated apoptosis in different cell lines [19–21].

Little is known about the molecular mechanisms implicated in the apoptotic pathway triggered by Hsp90 inhibition in response to PU-H71. In this study, we investigated the involvement of ER stress and mitochondrial pathways in PU-H71-induced apoptosis in different human cancer cell lines. We also determined the role of Bcl-2 and Bax and the ability of anticancer drugs cisplatin and melphalan to sensitize resistant cancer cells to PU-H71 treatment.

2. Material and methods

2.1. Cells, cell culture and reagents

Human HeLa cervical cancer cells stably transfected with the human *bcl-2* gene or with the cytomegalovirus UL37 exon 1 gene coding for vMIA or with the pcDNA3.1 vector containing the neomycin resistance gene were generously given by Dr V. Goldmacher (ImmunoGen, Cambridge, MA, USA). Human colon cancer cell lines HCT116 Bax wild type and Bax K.O. (HCT116 Bax−) were generously provided by Dr B. Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD, USA). Human melanoma cell line Mel501 was kindly given by Dr L. Larue (Institut Curie, Orsay, France). Normal human fibroblasts, generously provided by Dr A. Lombes (Institut Cochin, Paris, France), were obtained from the tissue repository of the AFM (Association Française contre les Myopathies). Human lung (A549) and human hepatocellular (HepG2) carcinoma cell lines were purchased from ECACC (Salisbury, UK). Cells were cultured in DMEM:F12 medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 1% glutamax (Invitrogen, Villebon sur Yvette, France) and 10% FBS (Lonza, Levallois-Perret, France), at 37 °C under 5% CO_2/95% air. PU-H71 (#P0030) was purchased from Sigma-Aldrich (Lyon, France). The caspase inhibitor z-VAD-fmk was obtained from Bachem (GmbH, Germany). Calpain inhibitors ALLN and ALLM were from Merck Millipore (Fontenay-sous-Bois, France). All other chemicals were purchased from Sigma-Aldrich. DEVD-NucView™ 488 caspase-3 substrate was from Interchim (Montluçon, France). All other fluorescent probes were from Invitrogen.

2.2. Flow cytometry analysis

For flow cytometry analysis of mitochondrial transmembrane potential ($\Delta\Psi m$), cells were stained with 100 nM $DiOC_6(3)$ for 20 min at 37 °C. Necrosis was estimated by adding 10 μg/ml of propidium iodide (PI) just before analysis. PTP opening was assessed as described previously [22]. Briefly, cells were preincubated for 15 min at 37 °C with 1 μM calcein-AM/100 μM $CoCl_2$ in HBSS (Invitrogen) supplemented with 1 mM HEPES, pH 7.3. HBSS was then replaced by complete culture medium during apoptosis induction. The fluorescent probe fluorescein diacetate (FDA) was used to assess cell viability. After treatment, cells were incubated for 5 min at 37 °C with FDA at 0.2 μg/ml. Caspase-3 activation was assessed using DEVD-NucView 488 caspase-3 substrate. After treatment, cells were incubated with 5 μM DEVD-NucView 488 for 30 min at RT before cytometric analysis. Fluorescence of cells was analyzed on a Cell Lab Quanta MPL cytometer (Beckman Coulter, Villepinte, France).

2.3. Sample preparation for transmission electron microscopy

Cells were fixed with 2% glutaraldehyde/0.1 M sodium cacodylate for 1 h at RT, pelleted and rinsed with 0.1 M sodium cacodylate/0.2 M sucrose for 1 h. Samples were epon-embedded, sliced, mounted on slides and the images were obtained at the electron microscopy facility of INRA (Jouy-en-Josas, France).

2.4. Immunofluorescence analysis

To examine the intracellular localization of cytochrome c and the activation of Bax, cells were seeded on slides in six-well multidishes, treated with PU-H71, washed in PBS 1×, fixed in 3.7% paraformaldehyde for 10 min at RT and permeabilized for 3 min in acetone at −20 °C. Cells were saturated in PBS/3% bovine serum albumin (BSA) for 30 min and incubated for 1 h with anti-cytochrome c (mAb 6H2.B4, BD Biosciences, Le Pont de Claix, France) or anti-activated Bax (6A7, Sigma-Aldrich) in PBS/1% BSA at RT. After two washes, the secondary antibody (Jackson ImmunoResearch, Suffolk, England) was added in PBS/1% BSA. To detect apoptotic cells, nuclei were stained with 2.5 μg/ml Hoechst 33348 for 5 min. Micrographs were taken on a Leica fluorescence microscope (Leica Microsystems, Nanterre, France).

2.5. RNA isolation and real-time reverse transcription polymerase chain reaction

RNA was isolated from cultured cells using Zymo Research QuickRNA MiniPrep according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed using BioRad iScript reverse transcription kit. For real-time PCR, cDNA was amplified by the "two-step" SsoFast EvaGreen supermix (BioRad), heated at 95 °C for 30 s, then 50 cycles of denaturation at 95 °C during 2 s and hybridization/elongation at 60 °C during 5 s. The PCR primers were obtained from Eurofins: 5′GTCCCTCCAACAACAGCAAG3′(F) and 5′AGGTCATCTGGCATGGTTTC3′(R) for *atf4*; 5′TGCTGAGTCCGCAGCAGGTG3′(F) and 5′GCTGGCAGGCTCTGGGGAAG3′(R) for spliced *xbp1* as described by van Schadewijk et al. [23]; 5′AGCGACAGAGCCAAAATCAG3′(F) and 5′ACAAGTTGGCAAGCTGGTCT 3′(R) for *chop*. The results were quantified according to the Cq value method, where Cq is defined as the quantification cycle of PCR at which the amplified product is detected. The ratio $(1+E_{target\ gene})^{\wedge}-(\text{Cq sample}-\text{Cq control})_{target\ gene}/(1+E_{reference\ gene})^{\wedge}-(\text{Cq sample}-\text{Cq control})_{reference\ gene}$ was