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Mechanism of the induction of endoplasmic reticulum stress by the anti-cancer agent, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT): Activation of PERK/eIF2α, IRE1α, ATF6 and calmodulin kinase

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ABSTRACT

The endoplasmic reticulum (ER) plays a major role in the synthesis, maturation and folding of proteins and is a critical calcium (Ca^{2+}) reservoir. Cellular stresses lead to an overwhelming accumulation of misfolded proteins in the ER, leading to ER stress and the activation of the unfolded protein response (UPR). In the stressful tumor microenvironment, the UPR maintains ER homeostasis and enables tumor survival. Thus, a novel strategy for cancer therapeutics is to overcome chronically activated ER stress by triggering pro-apoptotic pathways of the UPR. Considering this, the mechanisms by which the novel anti-cancer agent, Dp44mT, can target the ER stress response pathways were investigated in multiple cell-types. Our results demonstrate that the cytotoxic chelator, Dp44mT, which forms redox-active metal complexes, significantly: (1) increased ER stress-associated pro-apoptotic signaling molecules (*i.e.*, p-eIF2α, ATF4, CHOP); (2) increased IRE1α phosphorylation (p-IRE1α) and *XBP1* mRNA splicing; (3) reduced expression of ER stress-associated cell survival signaling molecules (*e.g.*, XBP1s and $p58^{IPK}$); (4) increased cleavage of the transcription factor, ATF6, which enhances expression of its downstream targets (*i.e.*, CHOP and BiP); and (5) increased phosphorylation of CaMKII that induces apoptosis. In contrast to Dp44mT, the iron chelator, DFO, which forms redox-inactive iron complexes, did not affect BiP, p-IRE1α, XBP1 or $p58^{IPK}$ levels. This study highlights the ability of a novel cancer therapeutic (*i.e.*, Dp44mT) to target the pro-apoptotic functions of the UPR *via* cellular metal sequestration and redox stress. Assessment of ER stress-mediated apoptosis is fundamental to the understanding of the pharmacology of chelation for cancer treatment.

1. Introduction

The endoplasmic reticulum (ER) is a crucial organelle that is responsible for the synthesis, maturation and folding of proteins [1]. Homeostasis of the ER is maintained by preventing toxic accumulation of unfolded or misfolded proteins and/or calcium (Ca^{2+}) depletion [1]. The unfolded protein response (UPR) is an ER-specific cellular stress response that has been found to be

Abbreviations: ASK1, apoptosis signal-regulating kinase 1; ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; BiP, binding immunoglobulin protein; BSO, buthionine sulfoximine; Ca^{2+}, calcium; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CHOP, C/EBP homologous protein; DFO, desferrioxamine; Dp2mT, di-2-pyridylketone 2-methyl-3-thiosemicarbazone; Dp44mT, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone; DpT, di-2-pyridylketone thiosemicarbazone; eIF2α, eukaryotic initiation factor-2α; ER, endoplasmic reticulum; FTH1, ferritin heavy chain 1; GSH, glutathione; HIF1α, hypoxia inducible factor-1α; IRE1, inositol-requiring enzyme 1; JNK, c-jun *N*-terminal kinase; MEM, Eagle's minimum essential medium; NAC, *N*-acetyl-L-cysteine; PERK, protein kinase RNA-like endoplasmic reticulum kinase; RT-qPCR, reverse transcription-quantitative real-time polymerase chain reaction; ROS, reactive oxygen species; SERCA, sarcoplasmic or endoplasmic reticulum Ca-ATPase family; TfR1, transferrin receptor 1; TRAF2, tumor necrosis factor receptor-associated factor 2; UPR, unfolded protein response; XBP1, X-box binding protein 1; XBP1u, unspliced XBP1; XBP1s, spliced XBP1.

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conserved in all mammalian species [1]. Disruption of ER homeostasis triggers the UPR, which arrests protein translation and activates signaling pathways for molecular chaperones to assist protein folding and to direct the degradation of misfolded proteins [1,2]. The prolongation of the UPR can also lead to apoptosis in a caspase-dependent manner [1,2].

Under resting conditions, the ER chaperone, binding immunoglobulin protein (BiP; also known as glucose-related protein 78, GRP78) binds to the luminal domains of ER membrane sensor proteins to render them inactive [3,4]. These membrane proteins trigger the main axes of the UPR and include: (1) protein kinase RNA-like endoplasmic reticulum kinase (PERK); (2) inositol-requiring enzyme 1 (IRE1); (3) activating transcription factor 6 (ATF6); and (4) the translocon [3,4] *via* phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). During the UPR, BiP dissociates from these complexes, resulting in autophosphorylation of PERK and IRE1α, translocation of ATF6 to the Golgi for cleavage and the release of Ca^{2+} from the ER *via* the translocon into the cytosol [4]. These alterations result in activation of the downstream signaling pathways of each of these axes, which is described in turn below [2].

In terms of the PERK axis, activated PERK phosphorylates eukaryotic initiation factor-2α (eIF2α), which then results in the attenuation of protein translation to reduce protein overload in the ER [3]. However, despite attenuation of protein translation, certain essential mRNAs are selectively translated, including the transcription factor, ATF4, which induces the expression of C/EBP homologous protein (CHOP) that can lead to apoptosis [1].

The second axis, which is mediated by the activation of IRE1α, results in the increased splicing of *X-box binding protein 1* (*XBP1*) and the activation of genes important for cell survival during ER stress *e.g.*, the ER chaperone, $p58^{IPK}$ [3]. In addition, IRE1α also has a pro-apoptotic role and can bind to the tumor necrosis factor receptor-associated factor 2 (TRAF2) and apoptosis signal-regulating kinase 1 (ASK1) to promote phosphorylation of c-jun *N*-terminal kinase (JNK) that can induce apoptosis [3].

The third axis mediated by the activation of the transcription factor, ATF6, occurs after its proteolytic cleavage in the Golgi to generate cleaved ATF6, which then undergoes nuclear translocation to transcribe genes involved in apoptosis (*e.g.*, *DNA-damage-inducible transcript 3* [gene encoding CHOP]) and/or those responsible for cell survival (*e.g.*, ER chaperones such as *heat shock 70 kDa protein 5* [gene encoding BiP], *calreticulin*, *calnexin*, *etc.*; [1,3]).

The fourth axis is activated by Ca^{2+} release from the ER into the cytosol and is mediated by the translocon [4]. The increased cytosolic Ca^{2+} then binds to calmodulin to activate CaMKII signaling, leading to ER stress-induced cell apoptosis through activation of caspase-12/caspase-3 and the mitochondrial apoptosis pathway [5]. To some extent, the cell's fate is decided by the balance between survival and apoptotic signaling, and the specific ER stressor plays a crucial role in tuning these signals [1,3].

Iron is a critical nutrient for cells, which is required for many processes, such as DNA synthesis and energy transduction [6]. Excessive iron on the other hand, can cause cellular damage due to the generation of cytotoxic reactive oxygen species (ROS; [6,7]). Hence, cellular iron levels are intricately regulated by a variety of molecular mechanisms and its alteration may cause activation of cellular stress pathways [8]. Recently, some fragmentary evidence of a potential intersection between the alteration of intracellular iron levels and ER stress has emerged. For example: (i) iron-loaded cells have increased transcript levels of ER chaperones, namely, BiP, calreticulin 3 and calnexin [9]; (ii) iron-overloaded rats show increased BiP expression in the heart and liver [10]; and (iii) it has been demonstrated that the expression of several molecules integrally involved in ER stress are regulated by cellular iron levels, including PERK/eIF2α [11] and CHOP [12]. However, there has been no attempt to comprehensively investigate the ER stress pathway in relation to understanding the effects of cellular iron depletion. This is significant considering that iron chelation therapy for iron-loading diseases (*e.g.*, β-thalassemia) is widely utilized [7,13] and iron-deficiency affects more than ~2 billion people worldwide [14]. Despite this, little is known regarding the response of cells to iron deficiency-induced stress.

Desferrioxamine (DFO; Fig. 1A) is an iron chelator used for the treatment of iron overload diseases [6,7]. DFO does not show potent anti-tumor activity, as it is poorly membrane-permeant and its mechanism of action involves binding cellular iron, resulting in a redox-inert iron complex [6,7]. On the other hand, novel chelators based on the di-2-pyridylketone thiosemicarbazone (DpT) scaffold, such as di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT; Fig. 1B), induce iron sequestration and also form redox-active metal complexes that demonstrate potent and selective anti-tumor activity [6,15–20]. Notably, Dp44mT and its analogs possess broad anti-cancer and anti-metastatic activity *in vitro* and *in vivo* against a variety of aggressive solid tumors [6,16,17,21–23]. In fact, clinical trials of one of these agents will soon commence [22], highlighting the importance of understanding the mechanism of action of these ligands and their effect on the ER stress pathway. Indeed, it has been reported that iron depletion induced by Dp44mT and its metal complexes causes apoptosis by generating cytotoxic ROS [17,24,25] and by inducing DNA strand breaks [26]. Importantly, the redox active complexes formed result in lysosomal damage that plays a significant role in tumor cell cytotoxicity [24,25].

The alterations in gene expression after iron depletion are complex [27–30]. Currently, it is unclear if ER stress-mediated apoptotic pathways play a crucial role in iron depletion-induced cell death. Investigating the molecular regulation of iron depletion on Ca^{2+} homeostasis and ER stress-mediated cell apoptosis is fundamental to the mechanistic understanding of the pharmacology of iron chelation, particularly for cancer treatment.

In the current investigation, we demonstrate for the first time that iron sequestration mediated by the chelator, Dp44mT, which forms redox-active metal complexes, robustly induces ER stress and thereby activates the UPR signaling pathways in multiple cell-types. In contrast, the effect of DFO, which only induces cellular iron depletion, is far less active in stimulating ER stress. This suggests using iron chelators that induce ER stress and apoptosis could serve as a novel approach for developing new chemotherapeutics. Collectively, these results are important for understanding the role of iron chelation and redox cycling in cell death mediated by the ER stress signaling response.

2. Materials and methods

2.1. Reagents

Di-2-pyridylketone 2-methyl-3-thiosemicarbazone (Dp2mT; Fig. 1C) and Dp44mT were synthesized and characterized using standard procedures [15]. Briefly, equimolar equivalents of di-2-pyridylketone and either 2-methyl-3-thiosemicarbazide or 4,4-dimethyl-3-thiosemicarbazide (all purchased from Sigma–Aldrich, St. Louis, MO) were refluxed in EtOH in the presence of glacial acetic acid (5 drops) for 2 h [15]. After cooling, the precipitate was collected by vacuum filtration and washed in EtOH to afford the final thiosemicarbazone [15]. DFO, buthionine sulfoximine (BSO), *N*-acetyl-L-cysteine (NAC), Tunicamycin (Fig. 1D) and Thapsigargin (Fig. 1E) were purchased from Sigma–Aldrich (St. Louis, MO).