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KNK437, abrogates hypoxia-induced radioresistance by dual targeting of the AKT and HIF-1α survival pathways

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ABSTRACT

KNK437 is a benzylidene lactam compound known to inhibit stress-induced synthesis of heat shock proteins (HSPs). HSPs promote radioresistance and play a major role in stabilizing hypoxia inducible factor-1α (HIF-1α). HIF-1α is widely responsible for tumor resistance to radiation under hypoxic conditions. We hypothesized that KNK437 sensitizes cancer cells to radiation and overrides hypoxia-induced radioresistance via destabilizing HIF-1α. Treatment of human cancer cells MDA-MB-231 and T98G with KNK437 sensitized them to ionizing radiation (IR). Surprisingly, IR did not induce HSPs in these cell lines. As hypothesized, KNK437 abrogated the accumulation of HIF-1α in hypoxic cells. However, there was no induction of HSPs under hypoxic conditions. Moreover, the proteosome inhibitor MG132 did not restore HIF-1α levels in KNK437-treated cells. This suggested that the absence of HIF-1α in hypoxic cells was not due to the enhanced protein degradation. HIF-1α is mainly regulated at the level of post-transcription and AKT is known to modulate the translation of HIF-1α mRNA. Interestingly, pre-treatment of cells with KNK437 inhibited AKT signaling. Furthermore, down regulation of AKT by siRNA abrogated HIF-1α levels under hypoxia. Interestingly, KNK437 reduced cell survival in hypoxic conditions and inhibited hypoxia-induced resistance to radiation. Taken together, these data suggest that KNK437 is an effective radiosensitizer that targets multiple pro-survival stress response pathways.

1. Introduction

Stress ranging from heat stress to pathophysiological conditions induce HSPs [1]. These proteins function as molecular chaperones, highly conserved and cytoprotective [2]. Enhanced expression of HSPs in response to stress is mediated by the heat shock transcription factor 1 (HSF1) [2]. HSPs enhance DNA repair and inhibit cell death to activate an adaptive response to IR [3–6]. Although, a number of HSPs have been implicated in cellular resistance to IR, only HSP90 has been effectively targeted. The most widely used HSP90 inhibitors such as geldenamycin and its clinically relevant analog 17AAG, sensitize cancer cells to radiation [7,8]. However, these inhibitors activate HSF1 and subsequent induction of HSPs [9,10]. Induction of HSPs by HSP90 inhibitors in turn limits their efficacy as radiosensitizers.

Hypoxia induces genetic instability and tumor invasion, resulting in a more malignant phenotype [11] and is a major limiting factor in the efficacy of IR against solid tumors. HIF-1 is an oxygen sensitive heterodimeric transcription factor responsible for cellular adaptation to hypoxic conditions. HIF-1 comprises α and β components where the α component is regulated by oxygen dependent protein degradation [12]. HIF-1 contributes to radioresistance by modulating the expression of genes involving angiogenesis and cell proliferation [13,14]. Inhibition of HIF-1α by either genetic or pharmacological means sensitizes cancer cells to radiation [15,16]. HIF-1α is mostly regulated at post-transcriptional levels of translation and protein stability [17,18]. Various studies have reported that hypoxia induces HSPs which in turn stabilizes HIF-1α [19–21]. AKT signaling is an established upstream modulator of HIF-1α [18,21–23]. Selective inhibition of AKT abrogates the accumulation of HIF-1α under hypoxia [18,23].

KNK437 is a novel benzylidene lactam compound, isolated from an organic source library (Kaneka Corp., Osaka, Japan). KNK437 is characterized by its ability to inhibit stress induced synthesis of HSPs mediated by HSF1 [24]. The efficacy of KNK437 as a sensitizer to hyperthermia mediated cell killing has been validated *in vivo* [25]. When used along with the HSP90 inhibitor, 17-AAG, KNK437 abrogated the induction of HSP70 and exhibited synergy

Abbreviations: IR, ionizing radiation; DMSO, dimethyl sulfoxide; CAT, chloramphenicol acetyl transferase; PMMA, poly (methyl methacrylate); h, hours; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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to reduce the survival of HL-60 cells [10]. In the present study, we examined whether KNK437 sensitizes cancer cells to IR and overrides hypoxia-induced radioresistance.

2. Materials and methods

2.1. Chemicals and antibodies

All chemicals were purchased from Sigma unless indicated otherwise. KNK437 (heat shock protein inhibitor 1) was obtained from Calbiochem, Merck Chemicals Ltd., Nottingham, UK. Rabbit polyclonal antibodies against PARP, HIF-1α, HIF-1β, and Phospho-AKT (Ser473) and mouse monoclonal antibody against AKT were obtained from Cell Signaling Technology, Danvers, MA. Mouse monoclonal antibodies against HSP70, HSP27 were obtained Stressgen, Enzo Life Sciences, and Exeter, UK. Mouse monoclonal antibody against HSP90 and GLUT1 were purchased from Santacruz Biotechnology Inc. Santacruz, CA. and Abcam, Cambridge, UK, respectively.

2.2. Cell culture and treatments

MDA-MB-231 cells and T98G cells were maintained in DMEM (PAA, Pasching, Austria) and EMEM (Lonza, Cambridge, UK), respectively. The medium was supplemented with 10% Fetal Bovine Serum (Gibco, Paisley, UK) 50 U/ml penicillin (Gibco) and 50 mg/ml streptomycin (Gibco) and maintained at 37 °C in a humidified 5% CO_2 atmosphere. For treatment with KNK437, stock solutions were made in DMSO. The cells were treated with 50 μM of KNK437 or equivalent volume of carrier (DMSO) for 24 h. KNK437 or DMSO was removed and replaced with fresh complete medium before subjecting to IR or heat shock. Cells (1×10^6) were exposed to heat shock at 42 °C for 1 h in polystyrene culture flasks; after the flasks were sealed with parafilm, they were fully immersed in a thermo stated water bath and were allowed to recover at 37 °C for 6 h in a humidified chamber supplied with 5% CO_2. Irradiation was performed using 2 mm Cu filtered 225 kV X-ray source (XRAD225, Precision X-ray Inc. Branford, CT) at a dose rate of 1.71 Gy/min.

2.3. Clonogenic cell survival assay

Survival fraction was determined by clonogenic cell survival assay as published [26].

2.4. Luciferase reporter assay for HSF1 activation

HSP70 promoter region was released from HSP70 B-CAT reporter vector (Stressgen Biotechnologies Corp., Victoria, BC, Canada) by digesting with restriction enzymes, Bgl II and Hind III (New England Biolabs, Inc., Beverly). The resulting 1.4 kb fragment containing HSP70 promoter region was cloned upstream to luciferase gene in PGL3-B basic vector (Promega, Madison). Cells were plated at about 0.3×10^6 cells per 35 mm petri dish and cultured for 24 h before transfection. Cells were co-transfected with 1 μg of Renila and 2 μg of HSP70 Luciferase construct expression vectors using lipofectamine (InVitrogen, San Diego, CA) according to manufacture's protocol. After 24 h of transfection, cells were treated with KNK437 or DMSO as mentioned in the above section before subjected to heat shock or IR. The Luciferase activity was assayed according to manufacturer's instruction (Promega, Southampton, UK) and the luciferase activity was expressed as fold changes following normalization with Renila luciferase activity.

2.5. Western blotting

Following experimental treatments, cells (0.3×10^6) were removed from the culture plates or flasks or dishes by scraping. The whole cell lysate preparation and Western blotting were carried out as previously reported [27]. Nuclear extract for Western blotting were made using NE-PER Nuclear and Cytoplasmic extraction reagents (Thermo Scientific, IL) according manufacture's protocol. Stripping and reprobing of immunoblots were carried out with Restore Western blot stripping buffer (Thermo Scientific) according to manufacture's protocol.

2.6. RNA interference

MDA-MB-231 cells (0.2×10^6) were transfected with AKT or scrambled siRNAs as described previously [28]. The siRNA sequences used were following: Hs_AKT1_11, CCA UGA GCG ACG UGG CUA U; Hs_AKT1_5, UCA CAC CAC CUG ACC AAG A; AKT2 A, ACG GGC TAA AGT GAC CAT GAA; AKT2 B, CAA GCG TGG TGA ATA CAT CAA and scrambled siRNA, AGCAGCACGACTTCTTCAAG. siRNAs were obtained from Qiagen, West Sussex, UK.

2.7. Hypoxia studies

For hypoxia studies, cells (0.3×10^6) were cultured in 35 mm tissue culture petri dishes and treated with KNK437 or DMSO for 24 h. The medium was replaced with fresh medium and cells were then incubated at 37 °C in a hypoxic workstation (*In Vivo* 2 400, Ruskinn, and Bridgend, UK) with 0.1% O_2, 5% CO_2 and 94.9% N_2 for indicated time points. For radiation experiments cells were treated with KNK437 or DMSO and incubated in a hypoxic workstation for 24 h and transferred in airtight PMMA chambers (developed in-house) to an irradiator. The chamber was gassed under positive pressure with 94.9% N_2, 5% CO_2 and 0.1% O_2 for 1 h prior to and during radiation. Cells were irradiated at a dose rate of 0.69 Gy/min and the medium was replaced with fresh complete medium and clonogenic survival was measured as described in the above section.

3. Results

3.1. KNK437 sensitizes cancer cells to ionizing radiation

We hypothesized that KNK437 inhibits IR induced synthesis of HSPs, which in turn renders cells sensitive to radiotherapy. Two human tumor cell lines, MDA-MB-231 (breast) and T98G (glioma) were pre-treated with KNK437 (50 μM) as described in Section 2 before irradiation. Pre-treatment conditions were determined by dose response studies using the MTT assay (data not shown). As hypothesized, KNK437 reduced survival and clonogenicity of cells irradiated with single doses of 2 and 5 Gy (Fig. 1A). MDA-MB-231 cells were more sensitive to KNK437 mediated radiosensitization. KNK437 reduced cell survival to 33% and 99.7% at 2 and 5 Gy, respectively, compared to DMSO-treated cells. The decreased survival rate in KNK437-treated cells after IR was also correlated with increased cleavage of PARP, a substrate of caspase-3 (Fig. 1B).

3.2. IR does not activate HSF1-mediated stress response

IR is known to activate the upregulation of HSPs [29,30]. HSPs maintain genomic integrity during normal conditions as well as in the event of stress by chaperoning the molecules involved in DNA repair [3,31]. To evaluate the effect of KNK437 on the activation of HSF1 and subsequent induction of HSPs in irradiated cells, MDA-MB-231 and T98G cells were irradiated. The expression of