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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.

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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

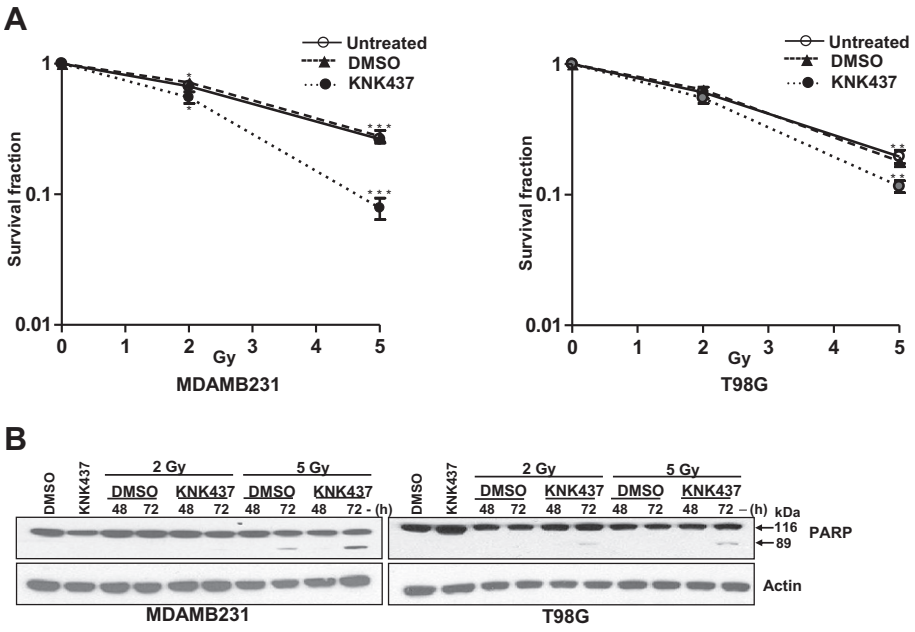

Fig. 1. KNK437 sensitizes human cancer cells to IR. (A) Clonogenic survival in irradiated cells. Survival fraction was determined by clonogenic cell survival assay as described in materials and methods. Survival fraction was normalized to corresponding un-irradiated control or KNK437 or DMSO-treated cells. Data point shows the mean of three independent experiments ± SEM. $^{*}p < 0.01$, $^{**}p < 0.001$ and $^{***}p < 0.0001$ B. Cleavage of PARP was determined by immunoblotting using PARP antibody.

HSP70 was analyzed by Western blotting. HSP70 is the major induced HSP during stress and its expression is a confirmation of the activation of stress response mediated by HSF1 [32]. Surprisingly, no significant change in the expression of HSP70 was observed in irradiated cells (Fig. 2A). On the other hand, heat shock upregulated the expression of HSP70 in both cell lines and the induced expression was abrogated in KNK437-treated cells (Supplementary Fig. 1C). In MDA-MB-231 cells, an increased concentration of KNK437 (100 μM) was required to inhibit heat stress-induced up regulation of HSP70 whereas induction of HSP27 was abrogated at 50 μM (Supplementary Fig. 1C). The basal level expression of HSP70 was not affected by KNK437 in these cell lines (Fig. 2A). Expression of HSP27 was also unchanged in both cell lines after radiation (Supplementary Fig. 1A). Increased expression of HSP27 observed at 24 h time point in MDA-MB-231 cells was instead not due to radiation since corresponding un-irradiated cells also exhibited the same (Supplementary Fig. 1B). Both cell lines exhibited high basal level expression of HSPs (Fig. 2A and Supplementary Fig. 1A). Interestingly, the expression of HSP27 remained unchanged in heat stressed T98G cells (Supplementary Fig. 1C).

HSF1 exists as monomers in the cytoplasm and undergo a multi step activation process upon stress. During stress, HSF1 homotrimerizes, translocates to the nucleus, becomes hyperphosphorylated and induces HSP expression by binding to *GAA* repeats (heat shock elements) present in the promoter regions of HSP genes [32]. Activation of HSF1 *in vivo* was monitored by using a Luciferase reporter gene under HSP70 promoter. Interestingly, HSF1 was not activated in irradiated cells whereas heat stressed cells exhibited a 2000-fold increase in Luciferase activity (Fig. 2B). The slight Luciferase activity observed in KNK437-treated and irradiated cells were rather not significant considering the high affinity of HSF1 for HSP70 promoter used in the assay. Heat stress-induced activation of HSF1 was significantly inhibited in KNK437-treated cells (Fig. 2B). Taken together, IR does not activate HSF1-mediated stress response and expression of HSP70 remained unchanged in KNK437-treated cells.

3.3. KNK437 inhibits AKT, HIF-1α pro-survival pathways and overrides hypoxia induced radioresistance

Activation of HIF-1α and expression of its target genes are required for tumor survival in a hypoxic environment [11]. HSPs stabilize HIF-1α in hypoxic cells [19,21]. We hypothesized that KNK437 inhibits induction of HSPs under hypoxia and thereby modulates HIF-1α stability. Surprisingly, pre-treatment of cells with KNK437 inhibited the accumulation of HIF-1α in hypoxic cells (Fig. 3A, Supplementary Fig. 2B). However, in contrast to previous reports [19–21] neither HSP70 nor HSP90 were induced in hypoxic cells (Fig. 3B). This indicated that KNK437 negatively modulates HIF-1α levels, a novel function independent of its established role as an inhibitor of stress response. Glucose transporter 1 (GLUT 1), a transcriptional target of HIF-1α was not induced in KNK437-treated cells under hypoxia (Fig. 3B). Pre-treatment of cells with proteosome inhibitor MG132 did not restore HIF-1α to a significant level (Fig. 3A). This suggested that the absence of HIF-1α in hypoxic cells was not due the enhanced protein degradation. As expected, MG132 blocked the degradation of HIF-1α in DMSO-treated cells as inferred by its presence in normoxic cells, which was further increased under hypoxic conditions (Fig. 3A).

AKT modulates the translation of HIF-1α mRNA [22,23]. Interestingly, Western blot analysis revealed a strong inhibition of AKT in KNK437-treated cells as inferred by the reduced phosphorylation at serine 473 (Fig. 3B). However, the total level of AKT was not altered by KNK437 (Fig. 3B). Similarly, AKT signaling was attenuated in irradiated cells that were treated with KNK437 (Fig. 3C, Supplementary Fig. 2A). Furthermore, down regulation of AKT by siRNA inhibited the expression of HIF-1α in hypoxic cells (Fig. 3D). Taken together, these data suggested that KNK437 targets the pro-survival AKT signaling pathway which is essential for radioresistance and indispensable for hypoxia induced accumulation of HIF-1α.

Hypoxia promotes tumor transition towards a more malignant phenotype often characterized as highly metastatic and resistant

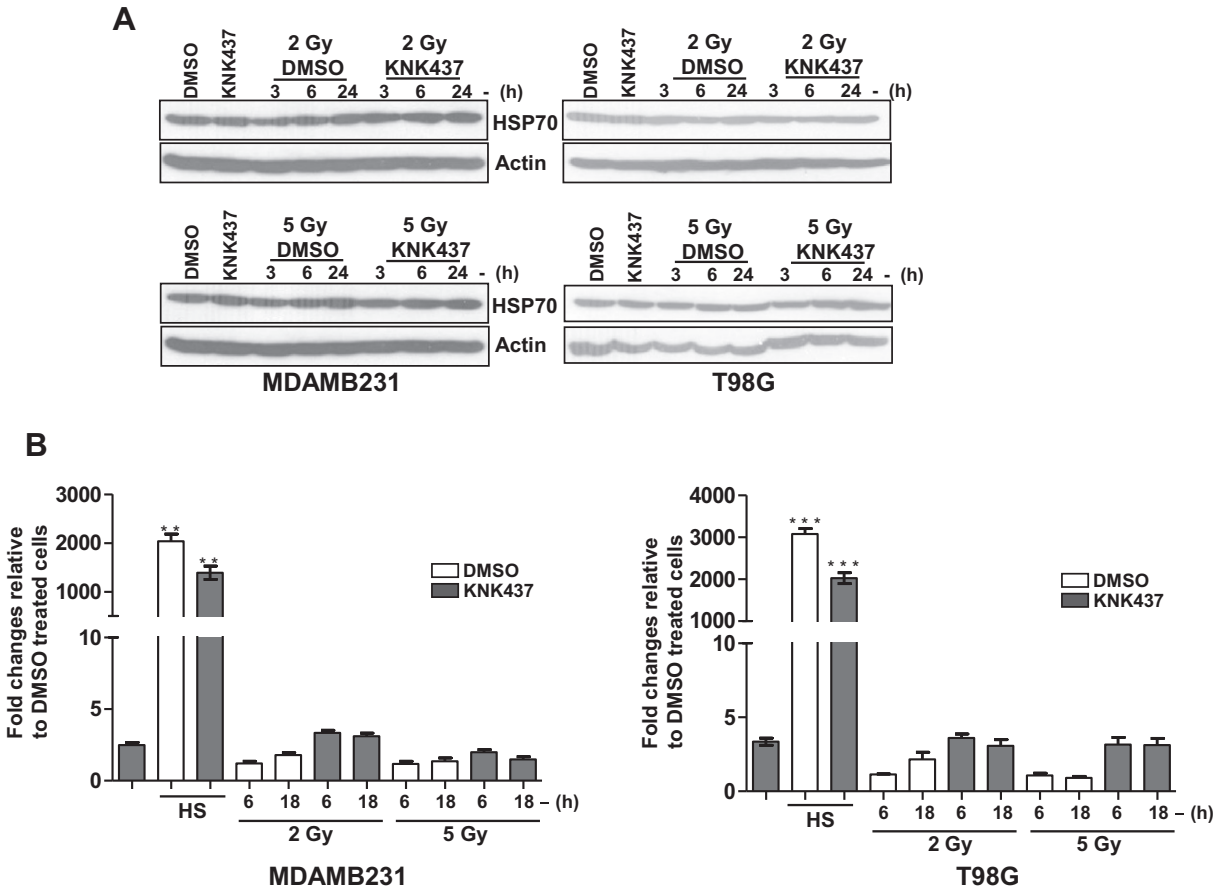

Fig. 2. IR does not activate HSF1 mediated stress response in cancer cells. (A) The expression of HSP70 was determined by immunoblotting using HSP70 antibody (B) HSF1 activity was determined by HSP70-Luciferase reporter assay. MDA-MB-231 and T98G cells were transfected with HSP70-firefly reporter DNA and internal control Renila Luciferase DNA. KNK437 or DMSO-treated cells were heat stressed or irradiated as described in materials and methods before measuring the Luciferase activity. HSP70-Luciferase activity was normalized to Renila Luciferase activity and the fold changes relative to DMSO-treated samples were plotted. HS – Heat stressed. Data point shows the mean of three independent experiments ± SEM. $^{**}p < 0.001$ and $^{***}p < 0.0001$.

to therapy [11]. Selective inhibition of HIF-1α renders cancer cells more sensitive to therapy [15,16]. Since KNK437 inhibited the accumulation of HIF-1α, we investigated whether KNK437 overrides hypoxia induced resistant to radiation. MDA-MB-231 cells were cultured in a hypoxic chamber for 24 h with KNK437 as mentioned in materials and methods before subjected to IR. Interestingly, DMSO-treated hypoxic cells exhibited increased clonogenicity and survival after IR (Fig. 3E). Exposure to hypoxia resulted in a 46% increase in cell survival relative to the normoxic population after irradiation. However, hypoxia induced resistance to radiation was completely abrogated in KNK437-treated cells (Fig. 3E). There was reduced survival in KNK437-treated cells under hypoxic conditions (Fig. 3E). The data suggested that KNK437 targeted the AKT-HIF-1α survival pathway, which is vital in promoting cell survival and radioresistance under hypoxic conditions.

4. Discussion

Cancer cells express high basal level of HSPs [33]. Extensive studies have been reported on the protective role of HSPs against radiation [3–6]. A probable reason for the lack of induced synthesis of HSPs in irradiated cells under this study is the high basal level expression of both HSP70 and HSP27. Monomeric forms of HSF1 exist in complex with HSPs in the cytoplasm under normal conditions [32]. During stress, HSPs, which have a high affinity for denatured proteins dissociate from the complex and this in turn activates HSF1 for further synthesis of HSPs [32]. In this study, in a high background level of HSPs, the stress resulted from IR would not have dissociated HSF1–HSP complex.

KNK437-mediated cytotoxicity to IR is indeed an off target from its known function. This was corroborated with the findings that KNK437 targeted the AKT pathway, which plays a major role in radioresistance [34]. Inhibiting AKT has a multifaceted positive outcome in cancer therapy. In addition to being a major component of cell survival and proliferation signaling complex, AKT modulates tumor survival under hypoxic conditions [18,23]. AKT is phosphorylated at Thr308 and Ser473 by PDK1 [35] and mTORC2 [36], respectively for its activation. KNK437 is known to inhibit mTORC1 *via* disruption of mTORC-raptor complex [37]. However, the inhibition of mTORC1 was attributed to the reduced expression of HSP70 by KNK437 [37]. As described in the previous sections, there was no induction of HSPs either in irradiated or in hypoxic cells. Neither was there any change in the basal level expression of HSP70 in KNK437-treated cells. Hence, KNK437 might possibly target the upstream activators of AKT or AKT might be a target by itself. Further studies are required to establish the mechanism behind the inhibition of AKT by KNK437.

Hypoxia is known to induce HSPs [19,21]. Contrary to the previous reports, in our study, HSPs were not induced in cancer cells under hypoxia. This suggested that the existing large pool of HSPs might be sufficient to stabilize HIF-1α. Our study also demonstrated that AKT is indispensable for hypoxia induced

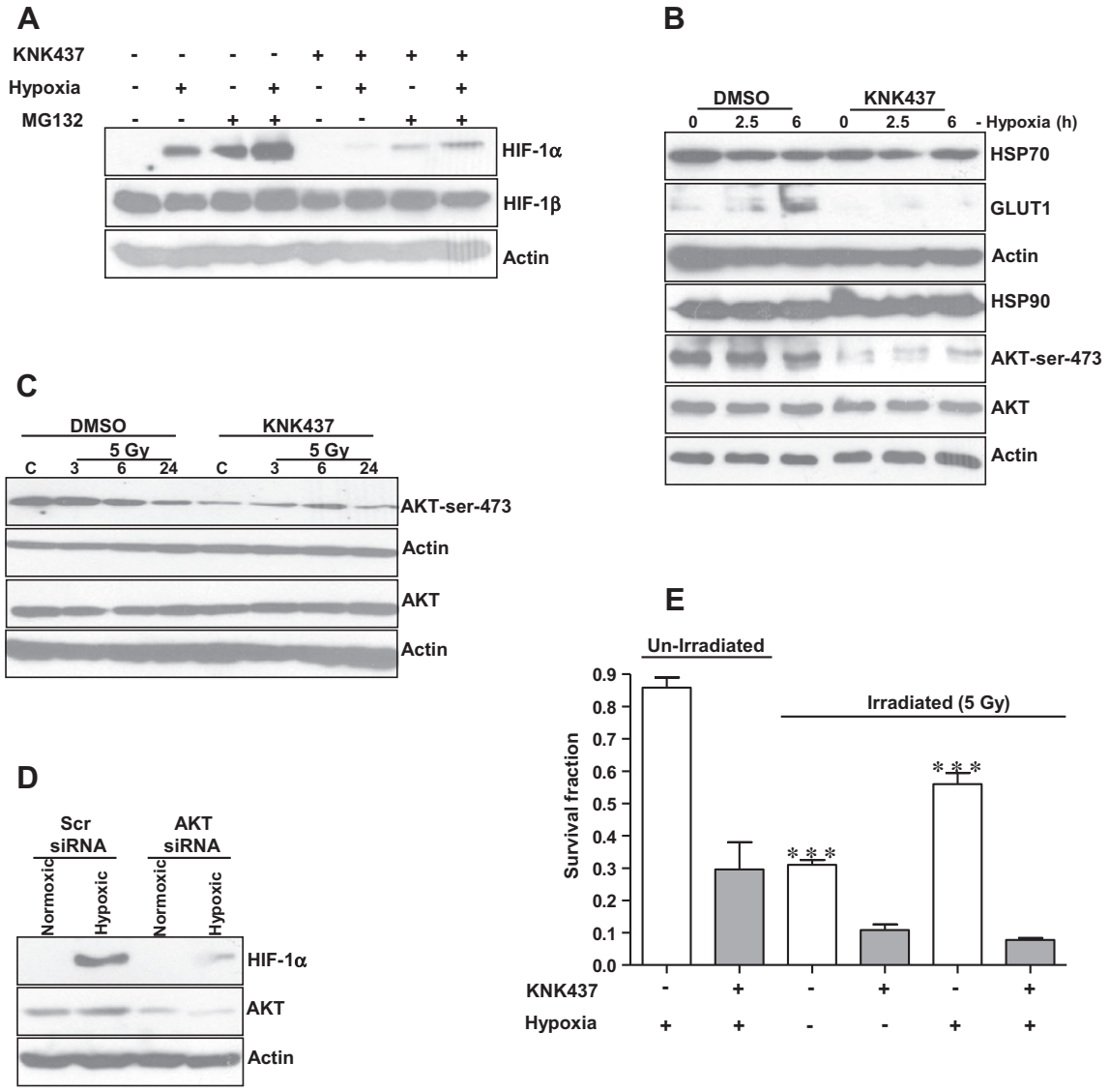

Fig. 3. KNK437 inhibits AKT, HIF-1α survival pathway and overrides hypoxia induced resistance to radiation in MDA-MB-231 cells. (A) KNK437 or DMSO- treated cells were exposed to hypoxia (0.1% O_2, 5% CO_2 and 94.9% N_2) for 2.5 h as described in materials and methods. Cells were lysed and the whole cell lysate (60 μg/well) were subjected to SDS–PAGE and immunoblots were performed with anti-HIF-1α, anti- HIF-1β and Actin. Cells were pre-treated with MG132 at 5 μM for 2 h after treating with DMSO or KNK437 before subjected to hypoxia. (B) Cells were exposed to hypoxia similar to A and immunoblotting was performed with HSP70, GLUT1, and HSP90, serine 473 AKT, AKT and Actin at indicated time points. (C) Immunoblot for phospho serine 473 AKT, AKT and Actin. (D) Cells were transfected with AKT siRNA or scrambled (Scr) siRNA as described in materials and methods before exposing to hypoxia similar to A and immunoblots were performed for HIF-1α, AKT and Actin. (E) KNK437 or DMSO-treated cells were subjected to hypoxia for 24 h and irradiated in hypoxic conditions as described in materials and methods. Survival fraction was determined by clonogenic cell survival assay and normalized to corresponding KNK437 or DMSO-treated cells. Data point shows the mean of two independent experiments (in triplicate) ± SEM. $^{***}p < 0.0001$.

accumulation of HIF-1α. Other than hypoxic conditions, HIF1α is induced in response to cytokines and radiation [22,38]. AKT/mTORC pathways are essential for the translation of HIF-1α mRNA in these conditions [22,38]. Inhibition of mTORC pathway or knockdown of AKT abrogates the accumulation of HIF-1α [22]. However, in our investigations, IR neither activates AKT (Fig. 3C) nor there was any accumulation of HIF-1α in irradiated cells (data not shown). Nevertheless, our study suggests that KNK437 could be an effective radiosensitizer in clinical contexts where stress and survival pathways are aberrantly activated. The suitability of KNK437 as a radiosensitizer is further supported by its established role as an inhibitor of HSF1. HSF1 is implicated in carcinogenesis and tumor progression and hence a target for cancer therapy [39]. In conclusion, KNK437 inhibits three key survival pathways (HSF1, AKT, HIF-1α) essential for tumor survival and promotion, therefore is an excellent candidate to be considered for future therapeutic applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.04.040.

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