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ORIGINAL CONTRIBUTION

Impact of JNK1, JNK2, and ligase Itch on reactive oxygen species formation and survival of prostate cancer cells treated with diallyl trisulfide

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Abstract
Purpose In our previous study, we demonstrated that diallyl trisulfide (DATS) induced iron-dependent G2-M arrest of prostate cancer cell cycle. Moreover, ferritin degradation and an increase of labile iron pool has been linked to the activation of the JNK signaling axis. In the present work, we extended this study to determine which of the c-jun kinases is responsible for ferritin degradation and the role of iron in DATS-induced cell death. We hypothesized that JNK1 activates Itch ligase which will lead to ferritin ubiquitination, an increase in iron-dependent ROS formation and cell death.

Methods PC-3 prostate cancer cells were used in this study. Cell viability, concentration of ROS, labile iron pool, and changes in ferritin and P-Itch and DNA damage were determined.

Results We observed that DATS induced ferritin degradation through JNK, Itch signaling axis. DATS did not induce neither ROS formation nor increase the LIP in JNK1-DN transfected cells. We also observed that DATS increased JNK-dependent activating phosphorylation of E3ligase Itch. The cells transfected with inactive form of Itch were more resistant against cytotoxicity of DATS and showed lower DATS-induced ferritin degradation. Desferrioxamine a specific iron chelator had no effect neither on cell viability nor DNA damage evaluated by comet assay.

Conclusions These results suggest that JNK1-dependent increase in LIP is mediated by Itch ubiquitin ligase.

Keywords Iron · Oxidative stress · Chemoprevention

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Introduction

Diallyl trisulfide (DATS) is a promising anticancer agent which is mainly present in Allium vegetables such as garlic [1]. DATS and many other naturally occurring anticancer agents are potentially antioxidant compounds. It has been shown that in vitro DATS and S-allylcysteine had strong antioxidant activity [2, 3]. Moreover, garlic and garlic extract has been shown to increase antioxidant potential of LDL and serum [4, 5]. There are also reports showing indirect antioxidant activity of organosulfur compounds (OSCs) including DATS [6]. This activity is related to ability of these compounds to induce antioxidant proteins like ferritin and GST [7, 8]. Surprisingly, it has been

reported that many anticancer chemicals present in food that are considered to be antioxidants, induced ROS formation in cancer cells [9]. The anticancer activity of DATS and other compounds are related to ROS formation in cancer cells. Antioxidants like NAC, EUK134, and others have been shown to reduce the pro-apoptotic activity of these compounds [10]. Therefore, understanding the mechanism of ROS formation has become crucial point as it can help to elucidate the anticancer activity of DATS and other chemicals. Recently, we have reported that DATS induced ROS formation by increasing ferritin degradation and labile iron pool in prostate cancer cells and that this process was controlled by the c-jun terminal kinase signaling pathway [11]. JNK belongs to kinases that are activated in stress conditions like osmotic stress, ischemia and reperfusion, radiation, mechanical stress, chemotherapy, and many others. In many experimental conditions, the JNK activation is crucial for induction of apoptosis e.g., previously it was shown that JNK inhibition reduced the DATS-induced apoptosis in prostate cancer cells. JNK controls the apoptotic pathways through different mechanisms e.g., by phosphorylating the Bcl2 leads to liberation of Bax protein and its translocation into mitochondria [12]. Moreover, there are several reports showing that JNK may affect the stability of proteins like c-jun, p53 or c-myc by regulating their ubiquitination and proteasomal degradation [13, 14]. In addition, activation of JNK by TNF-induced c-FLIP degradation an anti-apoptotic protein. JNK phosphorylates and activates E3 ligase Itch which then ubiquitninates the c-FLIP protein targeting it for degradation [15]. Recently, it has been reported that there is another possible mechanism by which JNK activation may increase cell death after TNF treatment. JNK has been shown to control the ROS formation. Cells with JNK1 and JNK2 knock-out have been completely resistant to TNF-induced ROS formation [16]. Previously, we have demonstrated that inactivation of JNKK2 or SEK1, which are kinases located upstream to JNK, prevent DATS-induced ROS formation [11]. These data indicated that JNK signaling axis controls ferritin degradation and the level of labile iron pool (LIP). LIP is defined as a pool of iron loosely chelated to low molecular weight compounds that can stimulate ROS formation [17]. Iron in reaction with hydrogen peroxide or lipid hydroperoxides gives rise to the hydroxyl, peroxyl, and alkoxyl radicals, respectively. LIP level inside a cell is strictly controlled. An increased free iron concentration leads to an elevated ferritin biosynthesis, which is an iron storage protein and decreases the amount of iron imported into the cell. Iron stored by ferritin is considered as a non-chelatable pool and its level is much higher than LIP [17]. Ferritin on one hand keeps iron out of free radical-generating reactions and on the other hand, it could be a source of iron in different experimental conditions. For example, the repression of ferritin expression has been shown to increase the LIP, leading to oxidative stress in human erythroleukemia cells [18]. There is an increasing number of evidence showing that iron is a signaling molecule leading among others to accelerated cell proliferation and activation of NF-κB [19]. In our previous report, we have shown that iron signaling plays an important role in DATS-induced G2/M arrest of prostate cancer cells. Both chelation of iron by DFO or blockage of ferritin degradation, were shown to protect from DATS-induced G2/M arrest [11].

Therefore, one goal of this work was to establish if iron signaling plays a role in DATS-induced cell death. Neovascularization and hemorrhage are common features of malignant tumors. It was suggested that hemoglobin derived from extravasated RBC deposits heme-derived iron into the tumor, which could modulate the sensitivity of cancer cells to oxidant-mediated injury [20]. Thus, it can be expected that iron stores can modify sensitivity of prostate cancer cells to the DATS. Moreover, we hypothesized that JNK1 by activating ligase E3 Itch [15] will lead to ferritin ubiquitination and degradation. The present study provides compelling evidence to indicate that JNK1-dependent ROS generation in DATS-treated cells is mediated by enhanced ferritin degradation which leads to an increase in the labile iron. However, increased LIP is not essential to DATS-induced cell death.

Materials and methods

Reagents

Tissue culture media, antibiotic mixture and fetal bovine serum were obtained from Gibco Life Technologies (Warsaw, Poland). 6-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) was from Molecular Probes (Warsaw, Poland). The antibodies against ubiquitine, HA-tag and actin were from Santa Cruz Biotechnology (Heidelberg, Germany). The antibodies against total ferritin was purchased from Sigma-Aldrich Ltd. (Poznań, Poland). DATS was purchased from LKT.

Cell culture

The PC-3 cells were cultured in F12 K supplemented with 10% fetal bovine serum and antibiotics. The DU145 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, and antibiotics. The cells were maintained at 37 °C in an atmosphere of 95% air and 5% CO_2. Briefly, PC-3 or DU145 cells were seeded in T25 flasks, and allowed to attach by overnight incubation. The

medium was replaced with fresh complete medium containing desired concentrations of DATS. Stock solutions of DATS were prepared in DMSO, and an equal volume of DMSO (final concentration 0.05%) was added to the controls. After incubation at 37 °C for the desired time point, floating and adherent cells were collected.

Measurement of ROS

Intracellular ROS generation was measured by flow cytometric monitoring of oxidation of H_2DCFDA which is cleaved by nonspecific cellular esterases and oxidized in the presence of peroxides and e.g., iron [11].

Immunoblotting

The cells were treated with DATS as described above. Both floating and attached cells were collected; washed in PBS; resuspended in a lysis solution containing 50 mmol/L Tris–HCl (pH 8.0), 150 mmol/L NaCl, 0.1% Triton X-100, 10 μg/mL phenanthroline, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A, 1 mmol/L phenylmethylsulfonyl fluoride; and incubated for 40 min on ice with gentle shaking. The cell lysate was cleared by centrifugation at 18.000×*g* for 20 min. Lysate proteins were resolved in 12% SDS-PAGE and subjected to immunoblotting as described previously [11]. Changes in protein level were assessed by densitometric scanning of the bands and corrected for β-actin loading control.

Immunoprecipitation-immunoblotting to determine ferritin ubiquitination

Cells were treated with DATS for specified time periods, washed twice with ice-cold PBS, and lysed as described above. Aliquots containing 400 μg of lysate protein were incubated with 3 μg of anti-ferritin antibody overnight at 4 °C. Protein A/G plus-agarose (30 μL; Santa Cruz Biotechnology) was then added to each sample, and the incubation was continued for an additional 3 h at 4 °C. The immunoprecipitated complexes were washed five times with lysis buffer and subjected to SDS-PAGE followed by immunoblotting using anti-ubiquitin antibody.

Determination of labile iron

The effect of DATS treatment on labile iron was determined by analysis of calcein fluorescence, a specific iron probe as described in [21]. Briefly, the cells were seeded at density of 1×10^5 per well, allowed to attach to cover glass overnight, and exposed to DATS in the absence or presence of 25 μmol/L desferrioxamine (2 h pretreatment). Cells were then stained with 5 μmol/L calcein in full medium and maintained at 37 °C in an atmosphere of 95% air and 5% CO_2 for 30 min, rinsed five times with PBS and calcein fluorescence was examined using a fluorescence microscope. In some experiments, 10 μmol/L SP600125 a JNK inhibitor was used (2 h pretreatment).

Transient transfection

Plasmids expressing catalytically inactive mutant of JNK1, JNK2 or Itch were kindly provided by Dr. Michael Karin [22, 23]. PC-3 cells were transfected with the plasmid encoding inactive JNK1, JNK2, Itch or empty pcDNA3.1 vector at 50–60% confluency using FuGENE 6 (Roche, Indianapolis, IN). After 24 h of transfection, cells were treated with DATS and processed for different assays.

Comet assay

The comet assay procedure used to measure the DNA strand breaks in individual cells was essentially the same as that described previously [24].

Evaluation of DNA damage

Cells were examined with an Axioskop 2 plus microscope (Carl Zeiss, Germany) equipped with an excitation filter of 515–560 nm and a magnification of ×20. Imaging was performed using a specialized analysis system ("Metasystem" Altlussheim, Germany) to determine tail moment (TM) which correlated with the degree of DNA damage in the single cell.

Results

DATS-mediated increase in labile iron pool is regulated by JNK1 but not JNK2

We have shown previously that the DATS-induced increase in LIP was attenuated in cells transfected with catalytically inactive JNKK2 or cells with down-regulated SEK1, that are an upstream kinases of c-jun terminal kinases (JNK) [11]. In order to establish if JNK kinases directly regulate the LIP level, first we used SP600125, a specific JNK inhibitor. The level of labile iron was measured by fluorescence microscopy after staining with iron-sensitive probe calcein, the fluorescence of which is quenched on binding to non ferritin-bound iron [25]. As expected, DATS treatment increased calcein fluorescence quenching what indicates that this is due to an increase in LIP (Fig. 1a). On the other hand, DATS-induced increase in LIP was not observed when cells were pretreated with SP600125.

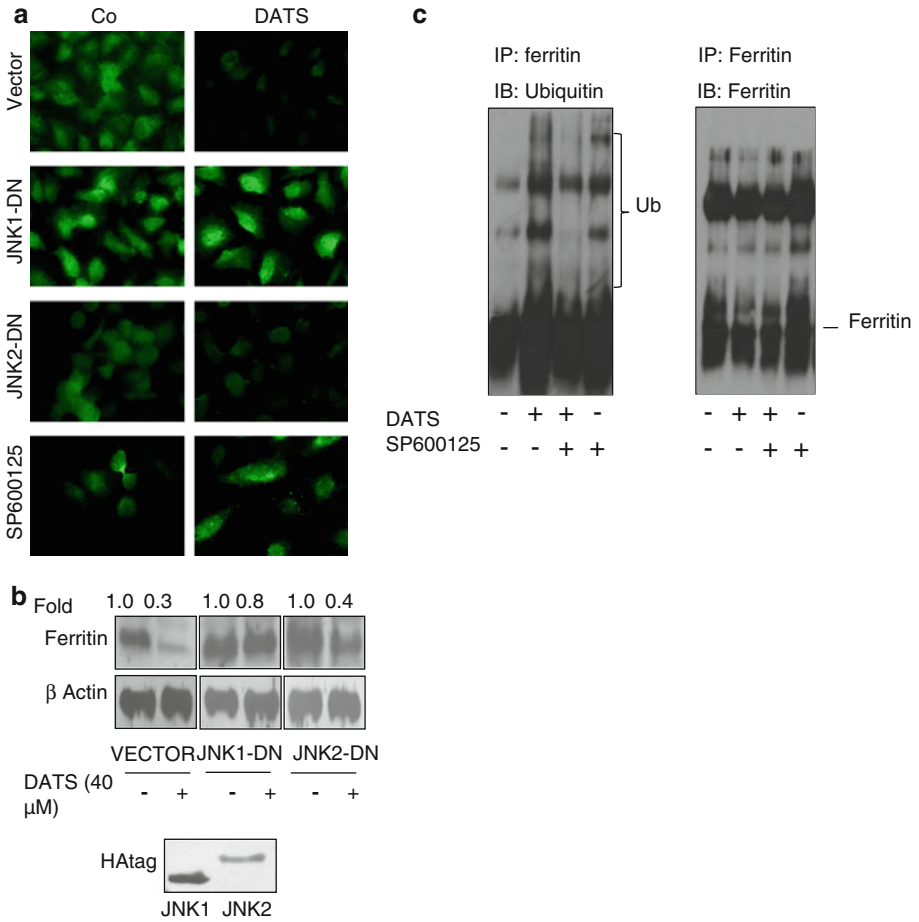

Fig. 1 DATS-induced JNK-dependent increase in the labile iron pool. **a** Fluorescence microscopy for calcein fluorescence in PC-3 cells treated with DMSO for 4 h (control), 40 μmol/L DATS for 4 h, 40 μmol/L DATS for 4 h in the presence of 10 μM of the JNK inhibitor SP600125 (2 h pretreatment). PC-3 cells transfected with an empty vector or plasmid encoding catalytically inactive mutant of JNK1 or JNK2 following a 4 h treatment with PBS (control) or 40 μmol/L DATS. Similar results were observed in replicate experiments. **b** Immunoblotting for ferritin using lysates from PC-3 cells transfected with an empty vector, catalytically inactive mutant of JNK1 or JNK2 following a 4 h treatment with DMSO (control) or 40 μmol/L DATS. The blots were stripped and re-probed with anti-actin antibody to ensure equal protein loading. Immunoblotting for HA-tag using lysates from PC-3 cells transfected with catalytically inactive mutant of JNK1 or JNK2 HA-taged. The *numbers* on *top* of the immunoreactive bands represent change in levels relative to DMSO-treated control. **c** Ubiquitination of ferritin is elevated in DATS-treated cells. Aliquots of lysate proteins (500 μg) from PC-3 cells treated with DMSO (control) or 40 μmol/L DATS for 2 h in the presence or absence of JNK inhibitor SP600125 were used for immunoprecipitation using anti-ferritin antibody followed by immunoblotting using anti-ubiquitin antibody or anti-ferritin antibody, respectively. Similar results were obtained in replicate experiments

The data shown above indicate that JNKs are directly involved in the control of the LIP level in diallyl trisulfate-treated cells. In order to find out which of JNK kinases is responsible for the control of LIP in prostate cancer cells treated with DATS, we used PC-3 cells transiently transfected with a vector expressing dominant negative catalytically inactive mutant of JNK1 (JNK1-DN) or JNK2 (JNK2-DN) [22, 23]. DATS treatment caused quenching of calcein fluorescence in cells transfected with an empty vector what confirms our earlier observation. However, PC-3 cells transfected with a vector expressing JNK1- DN were resistant to DATS-induced quenching of calcein fluorescence. On the other hand, cells expressing JNK2-DN showed the same sensitivity to DATS as that transfected with an empty vector (Fig. 1a).

Ferritin degradation in PC-3 cells treated with DATS is controlled by JNK1

Ferritin is an iron storage protein which is responsible for the control of LIP. When ferritin-containing iron undergoes proteolytic degradation, the LIP expands [18]. Therefore, our next goal was to check if JNK1, the kinase that controls LIP, also regulates ferritin degradation. As

may be seen in Fig. 1b, DATS induced a decrease in ferritin level in PC-3 cells transfected with an empty vector or a vector expressing JNK2-DN. However, in cells expressing JNK1-DN the ferritin level was not affected by DATS treatment. Immunoblotting for HA-tag was used to assess the expression level of ectopic JNK1-DN and JNK2-DN. Moreover, we observed that DATS treatment increased ferritin ubiquitination and that this process was inhibited by the JNK inhibitor SP600125 (Fig. 1c).

DATS-induced increase in ROS formation is JNK1-dependent

As cells expressing JNK1-DN, are resistant to DATS-induced ferritin degradation and increase in LIP we expected that it will also abolish DATS-induced ROS formation. In fact, in these cells there was no increase in ROS formation after DATS treatment. On the other hand, cells transfected with an empty vector or a vector coding for JNK2-DN showed a pronounced increase in ROS formation (Fig. 2a) which is in agreement with data presented in Fig. 1a. Overall, these data show that an increase in ROS formation in DATS-treated cells parallels with changes in LIP.

DATS-induced ferritin degradation is controlled by E3 ubiquitin ligase Itch

Recently, it has been shown that JNK1 activates E3 ubiquitin ligase Itch by its phosphorylation. Therefore, we speculated that Itch could be responsible for ferritin ubiquitination and degradation. We observed that DATS induced Itch phosphorylation and it was blocked by JNK inhibitor SP600125 (Fig. 2b). These data suggest that in PC-3 cells JNK mediates DATS-induced Itch activation. In order to confirm our assumption that Itch ligase plays a role in ferritin degradation, PC-3 cells were transfected with vector expressing dominant negative Itch (Itch-DN). In vector transfected cells, DATS induced ferritin degradation. In cells transfected with Itch-DN, DATS-induced ferritin degradation is significantly reduced. Similar observation has been done on DU145 cells (Fig. 2d). Overall, these data suggest that DATS-induced ferritin degradation is mediated by Itch ligase.

Iron is not involved in DATS-induced cell death

Next, we raised a question whether DATS-mediated inhibition of PC-3 cell viability was iron dependent. We addressed this question by determining the effect of DATS

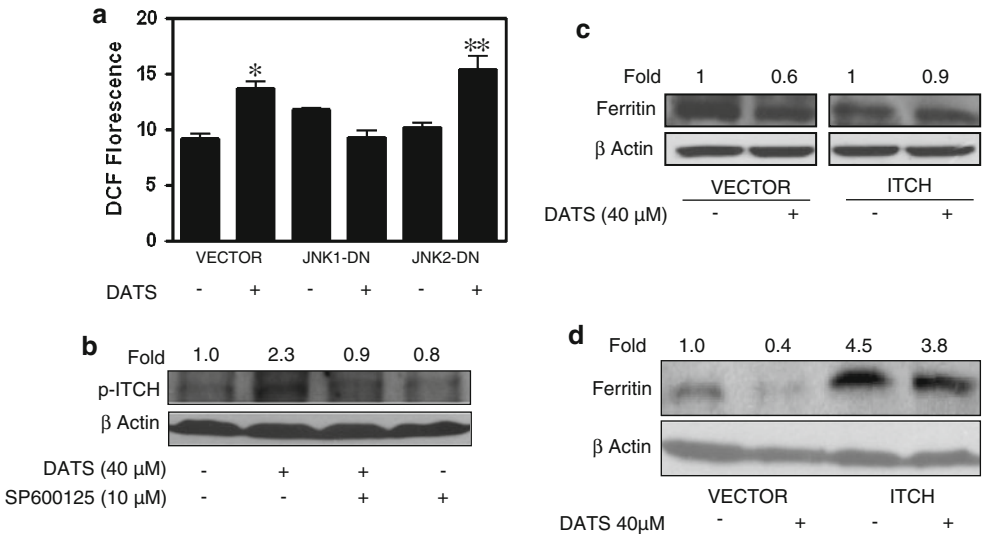

Fig. 2 DATS-induced ROS generation is JNK1 dependent. **a** ROS generation in PC-3 cells transfected with an empty vector, catalytically inactive mutant of JNK1 or JNK2 following a 4 h treatment with DMSO (control) or 40 μmol/L DATS. Data are mean ± SE ($n = 3$). $^{a}p < 0.05$, significantly different as compared to DMSO-treated control by one-way ANOVA followed by Bonferroni's multiple comparison test. **b** Immunoblotting for P-Itch using lysates from PC-3 cells treated with DMSO for 4 h (control), 40 μmol/L DATS for 4 h, 40 μmol/L DATS for 4 h in the presence of 10 μM JNK inhibitor SP600125 (2 h pretreatment). **c** Immunoblotting for ferritin using lysates from PC-3 cells transfected with empty vector or catalytically inactive mutant of Itch following a 4 h treatment with DMSO (control) or 40 μmol/L DATS. The blots were stripped and re-probed with anti-actin antibody to ensure equal protein loading. Similar results were observed in four replicate experiments. **d** Immunoblotting for ferritin using lysates from DU145 cells transfected with empty vector or catalytically inactive mutant of Itch following a 4 h treatment with DMSO (control) or 40 μmol/L DATS. The blots were stripped and re-probed with anti-actin antibody to ensure equal protein loading. The *numbers* on *top* of the immunoreactive bands represent change in levels relative to DMSO-treated control

treatment on viability of cells pretreated with iron chelators. We also studied the role of iron on changes in cell viability induced by DATS. As we reported before DATS-induced G2/M arrest was iron dependent [11], therefore we hypothesized that iron will also stimulate cell death. The sulforhodamine B assay, surprisingly, showed that specific iron chelator desferrioxamine (DFO) did not protect form DATS cytotoxicity (Fig. 3a). Moreover, other iron chelator thujaplicins had no protective action too (data not shown).To confirm these data, PC-3 cells were preloaded with iron by treatment with hemin as described before [20]. Again, DATS cytotoxicity was not affected by hemin pretreatment (Fig. 3b). Experiments performed on another prostate cancer cell line DU145 gave similar results (not shown). To confirm this data, viability of PC-3 cells was also assessed by trypan blue dye exclusion assay.

As JNK1 mediates ferritin degradation and increase in LIP, we hypothesized that expression of JNK1-DN should protect against DATS cytotoxicity more efficiently than expression of JNK2-DN. Nevertheless, cells expressing JNK1-DN were protected from DATS toxicity to the same degree as cells expressing JNK2-DN (Fig. 3c). These results support the notion that JNK activation indeed is essential for DATS cytotoxicity [12] but it is not related to JNK1-mediated ferritin degradation and iron-dependent ROS formation. As we have shown above, the Itch ligase is activated in the DATS-treated cells, therefore we also evaluated the role of the ligase in cytotoxic effects of DATS. As shown in Fig. 3c, PC-3 cells transfected with vector expressing dominant negative Itch (Itch-DN) were significantly protected. These data indicate that the ligase plays an important role in the DATS-induced cell death.

DATS-induced DNA damage in PC-3 cells is not ameliorated by iron chelation

Assessment of the DNA damage was carried out using the comet assay. TM is the product of tail length and percentage DNA in tail; thus TM represents both the amount of DNA migrated into the tail and the distance migrated. The TM is reported as a valid marker of single-strand DNA breakage [26]. As reported in Fig. 3d, DATS induced significant increase in TM in PC-3 cells but DFO had no effect. These data indicate that labile iron do not play a role in DATS-induced DNA damage.

Discussion

There are already many scientific data that DATS and other OSCs are potent anticancer agents [27, 28]. Recent data published by others and our own demonstrated that they are able to induce cancer cell death by ROS-dependent mechanism as different kind of antioxidants have been shown to exert protection [10]. Therefore, one of the goals of this study was to establish whether iron-dependent ROS generation participates in DATS-induced prostate cancer cell death. Data demonstrating that DATS-induced ROS formation seems to be in contradiction to several earlier reports demonstrating that OSCs are potent antioxidants both in vitro and in vivo conditions [29]. In agreement with these data, we previously reported that DATS reduced ROS formation in cells devoid of SEK1**,** a kinase that activates c-jun terminal kinase [11]. These data suggest that SEK1 or downstream kinases are involved in ROS formation. Here, we show that DATS-induced ROS formation was completely abolished in cells transfected with JNK1-DN. Moreover, in such cells, DATS did not induce changes in LIP. Therefore, these data strongly indicate that JNK1 regulates iron-dependent ROS formation. As ferritin is the main iron storage protein, on one hand it protects the cell from iron toxicity by sequestering iron, on the other hand it could be an important source of reactive iron [17]. There are several data showing that reducing agents can liberate iron from ferritin in vitro condition; however, there is no convincing evidence that such mechanism operates in living cells. Ferritin proteolytic degradation both by lisosomes or proteasomes certainly leads to the liberation of iron [30]. Here, we observed that DATS-induced ferritin degradation in cells transfected with an empty vector or transfected with JNK2-DN. However, in cells transfected with JNK1-DN the ferritin was not affected by DATS treatment. These data indicate that JNK1 activation is crucial for DATS-induced ferritin degradation. Recently, it has been shown that activated JNK1 increases cFLIP ubiquitination and stimulates its proteasomal degradation by activation of the E3 ubiquitin ligase Itch [15]. Therefore, we hypothesized that JNK1 stimulates ferritin degradation by activating the Itch ligase. Indeed, DATS treatment increased Itch phosphorylation, which was blocked by JNK inhibitor SP600125, what confirms earlier reports. Moreover, cells transiently transfected with plasmid coding for an inactive mutant of Itch, DATS-induced ferritin degradation was significantly attenuated. These data suggest that Itch is involved in ferritin degradation. In addition, we observed an increase in ferritin ubiquitination in DATS-treated cells and this process was blocked by a JNK inhibitor, which suggests that indeed JNK1 and Itch ligase may regulate this process.

The inhibitory effect of iron chelator on ROS formation indicates that iron plays an important role in this process [11]. Previously, it was shown that antioxidants reduced diallyl disulfide (DADS) and DATS-induced cell death [31]. Therefore, we hypothesized that iron chelation will protect the cells form DATS toxicity. First of all, we confirmed our earlier observation that DFO reversed the

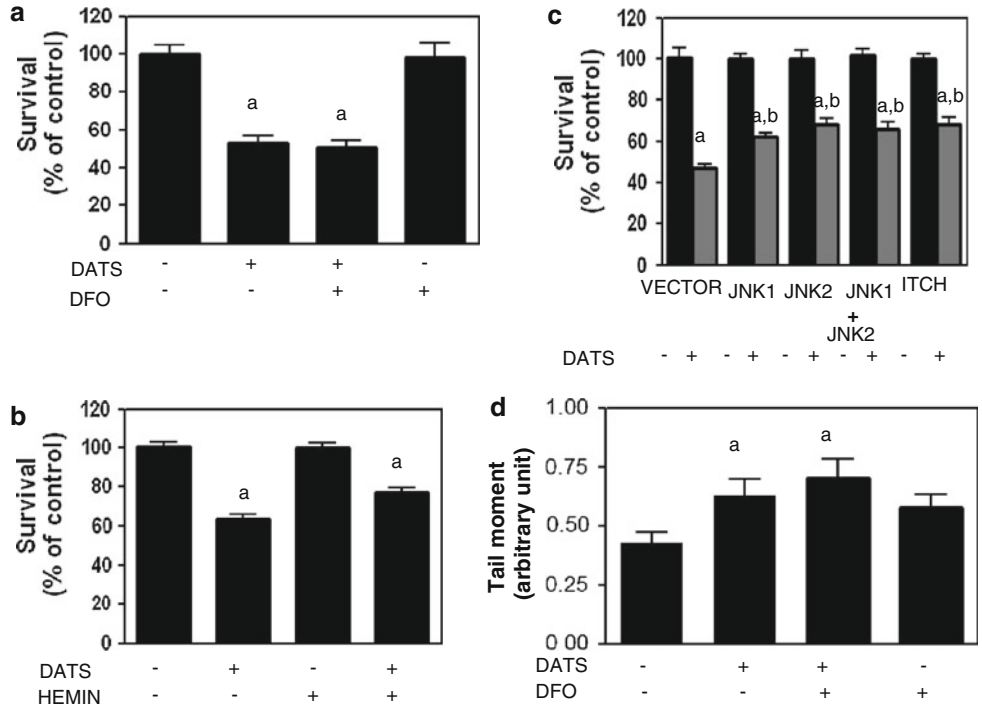

Fig. 3 Iron is not involved in DATS-induced cell death. **a** Survival of PC-3 cells treated for 24 h with 40 μM DATS with or without a 2 h pretreatment with 25 μM DFO as determined by sulforhodamine B assay. Data are mean ± SE ($n = 5$). $^{a}p < 0.05$, significantly different compared with DMSO-treated control by a one-way ANOVA followed by Bonferroni's multiple comparison test. **b** Survival of PC-3 cells treated for 24 h with 40 μM DATS with or without a 24 h pretreatment with 2.5 μM hemin as determined by sulforhodamine B assay. Data are mean ± SE ($n = 5$). $^{a}p < 0.05$, significantly different compared with DMSO-treated control by a one-way ANOVA followed by Bonferroni's multiple comparison test. **c** Survival of PC-3 cells expressing an empty vector or a catalytically inactive mutant of JNK1, JNK2 or Itch following a 24 h treatment with DMSO (control) or 40 μmol/l DATS as determined by sulforhodamine B assay. Similar results were observed in three independent experiments. Data are mean ± SE ($n = 5$). $^{a}p < 0.05$, significantly different compared with DMSO-treated control; $^{b}p < 0.05$ significantly different compared with vector transfected DATS treated by a one-way ANOVA followed by Bonferroni's multiple comparison test. **d** Iron chelation does not protects from DATS-induced DNA damage. The 'TM' parameter is reported using the comet assay in PC-3 cells treated for 12 h with 40 μM DATS treatment with or without a 2 h pretreatment with 25 μM DFO. Data are presented as mean ± SEM and refer to at least three individual experiments, 3 slides/experiment, 450 scores/experiment. $^{a}p < 0.05$, significantly different with DMSO-treated control by a Kruskal–Wallis statistic followed by Dunn's Multiple Comparison Test

DATS-induced cell cycle arrest (not shown), a process that is also ROS dependent. In addition to DFO, the synthetic antioxidant EUK 134 has been shown to ameliorate cell cycle arrest and cell death [10]. Unexpectedly, despite of the effects of DFO on the cell cycle and ROS formation [11] it had no influence on DATS-induced cell death. Previously, it was shown that DATS induced Chk 1 activation, which indicates a genotoxic stress in prostate cancer cells [32]. In order to prove that DATS induced DNA damage, we performed the comet assay. Obtained data clearly show that DATS induced DNA damage; however, DFO had no protective effect. These data are consistent with viability data and all together indicate that iron plays a signaling role in inducing cell cycle arrest but do not participate in DATS-induced cell death. The lack of effect of DFO or pretreatment with hemin on DATS-induced cell death indicate that in addition to iron-dependent ROS formation, other free radicals are formed thus triggering cell death signals. It has been shown previously that breast cancer cells (BT-20) became more sensitive to hydrogen peroxide but colon cancer cells (Caco-2) did not after hemin pretreatment [20].

It is worth to note that most of the data presented here were also replicated on another prostate cancer cells DU145 and the results were the same (data not show).

Contrary to our data, in many cancer cells an increase in LIP leads to augmented cell proliferation [33, 34]. Possibly, it is associated with high JNK activity in many rapidly proliferating cancer cells.

It is interesting to note that JNK1-dependent ROS formation could promote carcinogenesis. For example mice lacking JNK1 exhibited a marked decrease in gastric carcinogenesis induced by *N*-methyl-*N*-nitrosourea, relative to their wild-type counterparts. The tumor development has been associated with increased production of ROS [35]. On another experimental model, it was shown that JNK1

activation promotes ROS accumulation, liver damage, and carcinogenesis [36].

In conclusion, our results suggest that JNK1-dependent ROS formation in DATS-treated cells is related to Itch-dependent ferritin degradation, increase in LIP, and iron-dependent ROS formation. On one hand, iron-dependent ROS formation triggers signals leading to G2-M arrest on the other hand our data suggests that cytotoxic effects of DATS are not iron dependent.

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