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Proteasomal regulation of caspase-8 in cancer cell apoptosis

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Abstract

Previous studies demonstrated that proteasome inhibition sensitizes TRAIL resistant prostate cancer cells to TRAIL-mediated apoptosis via stabilization of the active p18 subunit of caspase-8. The present study investigated the impact of proteasome inhibition on caspase-8 stability, ubiquitination, trafficking, and activation in cancer cells. Using caspase-8 deficient neuroblastoma (NB7) cells for reconstituting non-cleavable mutant forms of caspase-8, we demonstrated that the non-cleavable forms of caspase-8 are capable of inducing apoptosis comparably to wild-type caspase-8, in response to proteasome inhibitor and GST-TRAIL. Moreover in the LNCaP human prostate cancer cells, caspase-8 polyubiquitination occurs after TRAIL stimulation and caspase-8 processing. Subcellular fractionation analysis revealed caspase-8 activity in both cytosol and plasma membrane fractions in both NB7 reconstituted caspase-8 cell lines, as well the LNCaP prostate cancer cells. The present results suggest that caspase-8 stabilization through proteasome inhibition leads to reactivation of the extrinsic pathway of apoptosis and identify E3 ligase mediating caspase-8 polyubiquitination, as a novel molecular target. Inhibition of this E3 ligase in combination with TRAIL towards restoring apoptosis signaling activation may have potential therapeutic significance in resistant tumors.

Keywords

Apoptosis; Caspase-8; Proteasome inhibitors

Introduction

Development of advanced castration-recurrent prostate cancer (CRPC) is a consequence of lack of an apoptotic response to androgen-ablation and chemotherapy [1, 2]. Overriding this resistance to apoptosis is the most critical therapeutic endpoint for prostate cancer patients. There are two pathways involved with apoptosis-termed intrinsic and extrinsic pathways [3, 4]. The extrinsic pathway of apoptosis involves the activation of death receptors via death ligands such as FAS, tumor necrosis factor (TNF) α , and TNF related apoptosis inducing ligand (TRAIL). The TRAIL induced apoptotic pathway initially involves TRAIL binding to

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death receptors 4 and/or 5. Upon binding, these receptors oligomerize, leading to the recruitment of adaptor protein FAS associated death domain (FADD) followed by initiator caspase-8 recruitment to form the death inducing signaling complex (DISC) [5]. After DISC formation, procaspase-8 is processed into the active p18 and p10 subunits, which form an active heterodimer that cleaves specific targets such as histone deacetylase 7 (HDAC7) [6], and executioner caspases (3 and 7), responsible for the apoptotic response [7, 8]. Other players are recruited to the DISC, such as cellular FLICE-inhibitory protein (c-FLIP), which consists of short and long forms [9, 10]. The short form of c-FLIP is capable of preventing caspase-8 processing by competitively binding and displacing caspase-8, due to its sequence homology with the caspase-8 pro-domain [11]. Moreover, c-FLIP can modulate caspase-8 activation and substrate specificity [12]. Mechanistically direct caspase-8 inhibition through c-FLIP interference at the DISC or downstream inhibition following caspase-8 processing mediated by the cellular inhibitors of apoptosis (cIAP) [13, 14]. Additional mechanisms resulting in caspase-8 down-regulation include gene deletion, methylation, [15, 16], phosphorylation or polyubiquitination [17–19].

Caspase-8 is polyubiquitinated either through K63 ubiquitin linkage [18], or through K48 linkage [19] and degraded by the proteasome, evidence enabling a potential platform in restoring cancer cell apoptosis using proteasome inhibitors to stabilize caspase-8. Proteasome inhibitors are pharmacologically promising via exploitation of their apoptosis inducing action [20, 21]. Velcade (PS-341/ Bortezomib) is an FDA-approved proteasome inhibitor used for the treatment of multiple myeloma [22, 23]. Velcade is a dipeptide boronic acid small molecule that blocks the chymotrypsin-like activity of the 20S particle [24]; the drug impacts several cellular growth regulatory processes such as, inhibiting cell cycle and NF- κ B activation [25]. Although Velcade is effective against multiple myeloma and other human cancers [26], lack of cell specificity and emerging tumor resistance, challenge its clinical use as an anti-cancer agent. In multiple myeloma patients the therapeutic response varied from initial resistance to acquired resistance to Velcade during treatment [27]. Efforts to overcome the therapeutic failure of Velcade led to interrogation of combination strategies of Velcade with different chemotherapeutic agents, like HDAC inhibitors, doxorubicin, [28, 29] and TRAIL [30], with promising therapeutic advantage.

We previously reported that combination of proteasome inhibition with TNF- α related apoptosis inducing ligand (TRAIL), induces apoptosis in TRAIL resistant human prostate cancer cells in vitro and in vivo [31], potentially via caspase-8 p18 subunit stabilization [30, 32], evidence implicating caspase-8 degradation under the control of the 26S proteasome. This study determined the functional consequences of proteasome inhibition on caspase-8 trafficking, ubiquitination and activity in cancer cells. The results indicate that caspase-8 processing into subunits preceded caspase-8 polyubiquitination, implicating polyubiquitination as a new significant aspect of caspase-8 regulation during apoptosis induction.

Materials and methods

Cell culture and reagents

Human prostate cancer lines, LNCaP and PC-3 were cultured in Dulbecco's modified eagles media (DMEM) (Media Tech Inc, Manassas, VA), or F12K media (Media Tech Inc, Manassas, VA). Lung cancer cell line H460 (a generous gift from Dr. John Yannelli at University of Kentucky, Lexington KY), Jurkat T cell line was purchased from the American Type Culture Collection (ATCC) and Neuroblastoma cell line (NB7) caspase-8 deficient cell line (caspase-8 constructs and NB7 cell line were a very generous gift from Drs. Andrew Oberst and Douglas Green (St. Jude's Children's Research Hospital, Memphis, TN) were cultured in RPMI (Mediatech). Media was supplemented with 10 % FBS

(Cellagro), 1 % penicillin (10,000 IU/ml)/streptomycin (10,000 lg/ml). Epoxomicin was generously provided by Dr. Kyung Bo Kim (College of Pharmacy, University of Kentucky). Velcade (Millennium Pharmaceuticals, Cambridge, MA) was kindly donated by the Markey Cancer Center Pharmacy. The GST-TRAIL fusion protein is purified using affinity chromatography as previously described [31, 32]. Caspase-8 reconstitution was carried out as previously described [33]. NB7 cells were transduced with a retro-virus encoding either wild-type or non-cleavable mutant caspase-8 constructs. Caspase-8 expression was standardized via flow cytometry (Beckman Coulter MoFlo, Ft. Collins, CO) at Flow Cytometry Core Facility at the University of Kentucky.

Cell viability and apoptosis evaluation

Cell viability was assessed using MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)), after treatment with GST-TRAIL or Epoxomicin. Results were read using a spectrophotometer at 570 – 690 nm. For evaluation of apoptosis we employed Annexin V, APC/PI (Invitrogen, Eugene, OR) staining. Cells were seeded in 24-well plates and treated with GST-TRAIL or Epoxomicin separately or in combination. Following treatment, cells were washed with Annexin V binding buffer, resuspended in Annexin binding buffer with PI, and Annexin V APC was added to samples, as recommended by the manufacturer. Staining was analyzed by flow cytometry (FACSCalibur, BD Immunocytometry Systems, San Jose, CA) at the Flow Cytometry Core Facility, University of Kentucky. Annexin V positive and viable cells were quantified using CellQuest Pro software (BD Immunocytometry Systems, San Jose, CA) in 20,000 gated events. All experiments were performed in triplicate.

Half-life evaluation

Cells (2×10^6 /well) were plated into 6-well plates and were pre-treated with proteasome inhibitor or not, followed by GST-TRAIL stimulation (100 ng/ml, 2 h). Cells were subsequently treated with cycloheximide or caspase inhibitor Z-VAD-FMK and harvested at indicated time points. Caspase-8 expression was analyzed via Western blot using the Caspase-8 IC12 or cleaved caspase-8 (ASP 384) antibodies (Cell Signaling Technology, Danvers, MA). Western blots were quantified using densitometry performed with NIH Image J software.

Polyubiquitin immunoprecipitation (IP) analysis

LNCaP cells (6×10^6) were treated either separately or in combination with GST-TRAIL (400 ng/ml) or Epoxomicin (1 μ M) for 8 h. Cells were subsequently harvested in (1 % NP-40 in PBS and protein content was determined using the BCA assay (Pierce). Prior to immunoprecipitation, cell lysates were used as input. Reciprocal IP was performed by using the Ubiquitin Enrichment Kit, which is designed to pull-down polyubiquitinated proteins followed by probing for the protein of interest (Thermo Fisher Scientific, Rockford, IL) and caspase-8 expression was determined via Western blot analysis using the IC12 caspase-8 monoclonal antibody (Cell Signaling Technology, Danvers, MA).

Immunocytochemical staining

Slides (EZslide, Millipore, Temecula, CA) were coated with Fibronectin (Sigma, St. Louis, MO). LNCaP cells (8×10^4 /well) were treated with GST-TRAIL (100ng/ml), Epoxomicin (1 μ M), or in combination for various time periods. Upon termination of treatment, cells were fixed with 4 % (v/v) paraformaldehyde, permeabilized with SAP buffer, blocked with BSA and probed with respective primary antibodies, against caspase-8 (R&D, Minneapolis, MN); Na^+K^+ ATPase (Millipore Temecula CA), and 26S Proteasome 20S particle (Calbiochem, San Diego, CA). Cells were subsequently exposed to either 488 Alexa Fluor

goat-anti-mouse or 594 Alexa Fluor goat-anti rabbit, (Invitrogen, Eugene, OR) conjugated secondary antibodies. Following exposure to the secondary antibody, sections were mounted using Vectashield (Vector laboratories Inc, Burlingame, CA). Images were photographed at 63× magnification using Leica TSP SP5 Confocal microscope (University of Kentucky Imaging Facility).

Subcellular fractionation

Cells, LNCaP (1.2×10^7) or NB7 (1.6×10^7) were treated with GST-TRAIL (400 ng/ml) and/or (1 μ M) Epoxomicin. Following treatment, cells were harvested in sucrose buffer A, subjected to homogenization and centrifugation (1,000×*g*; 10 min) and cell lysates were subjected to 30 % Percoll gradient ultracentrifugation in a Beckman Coulter Optima L-90 K Ultracentrifuge [1 h at 26,000 RPM (61,000×*g*; 4 °C], for the collection of cytosol and plasma membrane fractions. Inner membrane fractions were collected subsequently by adding 1× buffer to 30 % (v/v) Percoll (GE Healthcare)/2× sucrose buffer A followed by ultracentrifugation (1 h, 200,000×*g* at 4 °C). Fraction purity was assessed using actin, (cytosolic), Na⁺/K⁺ ATPase (plasma membrane), and Calnexin (internal membrane) as markers. Cellular fractions were analyzed by Western blotting and for caspase activity.

Caspase-8 activity assay

LNCaP (1.6×10^7) were plated and treated either separately or in combination with GST-TRAIL (100 ng/ml) or Epoxomicin (1 μ M) for the times indicated. Whole lysates or cellular fractions (cytosol and plasma membrane) were analyzed for caspase-8 activity using the Caspase-8-glo luminescence kit (Promega, Madison, WI) according to the manufacturer's protocol and measured using Veritas™ Microplate luminometer (Promega Corp., Sunnyvale, CA). NB7 parental or caspase-8 reconstituted cells were treated with GST-TRAIL (100 ng/ml), Epoxomicin (1 μ M) singly or in combination for 4 h. Caspase-8 immunoprecipitation was performed in whole cell lysates or cytosol/plasma membrane fractions. The caspase-8 glo reagent (Promega, Madison, WI) was added to beads containing caspase-8 and activity was measured using Veritas™ Microplate luminometer (Promega Corp., Sunnyvale, CA). All experiments were carried out in triplicate.

Statistical analysis

Data were analyzed using unpaired Student t test analysis. Statistical significance of the numerical values among different treatment groups was set at $p < 0.01$.

Results

Caspase-8 activation is independent of caspase-8 processing

It was previously demonstrated that combination of TRAIL with a proteasome inhibitor restored TRAIL sensitivity in TRAIL resistant human prostate cancer cells [31], and led to stabilization of caspase-8 p18 subunit [32]. To determine the impact of proteasome inhibition on caspase-8 activity we used non-cleavable caspase-8 constructs (Fig. 1a). The caspase-8 non-cleavable mutant constructs, have aspartic (D) to alanine (A) point mutations to prevent caspase-8 cleavage at either the caspase-8 p18 (D210, D216) named D3A mutant or caspase-8 p10 (D384) cleavage sites (termed the D3A, D2A respectively) [33]. Preventing cleavage at D384 results in a complete non-cleavable form of caspase-8 as this first cleavage event is required for D210, D216 processing [5, 34]. Blocking cleavage at residues D210 and 216 would prevent p18 cleavage from the pro-domain however, this construct would still have the ability for the p10 to be cleaved. After successful reconstitution of wild-type and non-cleavable caspase-8 mutant constructs in NB7 caspase-8 deficient cells (Fig. 1b), caspase-8 activity was comparatively analyzed between the wild-

type and non-cleavable constructs in response to treatment. Analysis of caspase-8 activity in whole cell lysates, and subcellular fractions (Fig. 1c, d respectively), revealed low caspase-8 activity in response to GST-TRAIL alone, while caspase-8 activity increased in response to the combination of Epoxomicin with TRAIL. Interestingly enough, the caspase-8 non-cleavable mutants possess higher activity levels than the wild-type caspase-8 (Fig. 1c) ($p < 0.01$). Caspase-8 activity was highest in the cytosol and lowest at the plasma membrane for the wild-type caspase-8, while the non-cleavable constructs had cytosolic activity and higher at the membrane (Fig. 1d).

Caspase-8 processing and stabilization by proteasome in apoptosis regulation

The functional consequences of caspase-8 processing on cell death were determined on the basis of cell viability and apoptosis induction outcomes in the wild-type and non-cleavable caspase-8 constructs. As shown on Fig. 2a and b NB7 cells without caspase-8 do not die in response to treatment with either GST-TRAIL or Epoxomicin (given as single agents) or in combination; there was however a significant decrease in cell viability in NB7 caspase-8 expressing cells (Fig. 2a). Treatment with a lower dose of GST-TRAIL resulted in the non-cleavable mutants inducing apoptosis comparable to wild-type caspase-8 (Fig. 2b). Combination of GST-TRAIL and Epoxomicin led to a significant induction of apoptosis (Fig. 2c, $p = 0.0001$).

The results summarized on Fig. 3 indicate that proteasome inhibition confers caspase-8 stabilization in different cancer cells; PC-3 human prostate cancer cells (Fig. 3a), Jurkat T cells (Fig. 3b), and H460 lung cancer cell lines (Fig. 3c). Simultaneous addition of either GST-TRAIL and proteasome inhibitor, or proteasome inhibitor pretreatment followed by GST-TRAIL stimulation leads to caspase-8 p18 subunit accumulation in all three cell lines (Fig. 3). To determine whether accumulation of the caspase-8 p18 subunit is a result of proteasome inhibition (and not due to increased processing), the half-life of p18 and p10 subunits was determined in the presence/absence of proteasome inhibitor. We found that proteasome inhibition stabilizes caspase-8 p18 subunit, and extends its half-life (Fig. 3d, e), while p10 expression temporally persisted regardless of proteasome inhibition (Fig. 3f).

The ubiquitination status of pro-caspase-8 or cleaved caspase-8, was determined next in the LNCaP human prostate cancer cells in response to proteasome inhibitors (Velcade or Epoxomicin) separately, or in combination with GST-TRAIL. Cell lysates were subjected to caspase-8 immunoprecipitation and Western blotting probing for polyubiquitin (Fig. 4a, b). As shown on Fig. 4, polyubiquitin smears were detected in the GST-TRAIL and GST-TRAIL/Velcade treated cells, but not in the untreated control or Velcade only-treated cells. An additional immunoprecipitation approach involved the Ubiquitin Enrichment kit to pull down polyubiquitinated proteins and probing for caspase-8; the observations summarized on Fig. 4d are identical to data shown b (Fig. 4).

Proteasome inhibition impacts caspase-8 intracellular trafficking

The consequences of proteasome inhibition on caspase-8 intracellular localization and trafficking in LNCaP cells after treatment with Epoxomicin and GST-TRAIL as separate agents or in combination, were determined in nuclear, cytosolic and plasma membrane fractions. Figure 5a reveals the identity and purity of each fraction. Comparative analysis of caspase-8 localization and expression in response to TRAIL alone vs TRAIL and epoxomicin is shown on Fig. 5b. Controls for this experiment were the non-treated predominantly elevated in the plasma membrane. This LNCaP and epoxomicin only treated LNCaP. A relatively accumulation of the p43/41 intermediate and p18 subunit in high caspase-8 expression (full-length and cleaved forms) the plasma membrane and cytosol fractions was significantly was detected in cytosol and plasma membrane fractions enhanced

in the presence of the proteasome inhibitor (Fig. 5b); the p43/41 intermediate and p18 subunit were (Fig. 5b). We next examined the effect of proteasome inhibition on caspase-8 activity in prostate cancer cells LNCaP. As shown on Fig. 5c, caspase-8 p43/41 expression in response to the combination treatment of epoxomicin and GST-TRAIL was higher than exposure to GST-TRAIL alone treatment. There was an increase in plasma membrane associated caspase-8 activity with GST-TRAIL treatment alone, and a significant induction in caspase-8 activity in response to the combination treatment of GST-TRAIL and epoxomicin (Fig. 5c) ($p = 0.001$ and $p = 0.0006$). Figure 5d is a representative time course showing caspase-8 expression after 2, 4, 8, and 12 h of treatment in the cytosolic and plasma membrane fractions, with time-dependent caspase-8 accumulation in both fractions. The temporal increase in caspase-8 activity in both fractions was followed by a significant reduction in activity for the plasma membrane fraction (Fig. 5e). Statistically significant difference was set at a p value of 0.0001.

Caspase-8 localization to the plasma membrane

The results on the proteasomal-mediated subcellular distribution of caspase-8 were validated using confocal microscopy, by assessing its co-localization with a plasma membrane associated protein, Na^+/K^+ ATPase (Fig. 6). Prostate cancer cells LNCaP were treated and the cellular localization of caspase-8 was determined by fluorescence staining with the Na^+/K^+ ATPase (membrane marker). Representative images shown on Fig. 6, revealed a significant co-localization between caspase-8 and Na^+/K^+ ATPase, in untreated and treated prostate cancer cells, indicating the presence of caspase-8 on plasma membrane, as well the cytosol. The 20S particle of the 26S proteasome did not co-localize with the Na^+/K^+ ATPase (control).

Discussion

Growing evidence supports a dynamic functional interaction between proteasome inhibition and caspase-8 mediated apoptosis in cancer cells of diverse origin [30, 35, 36]. Consequently the combination of TRAIL and Velcade emerges as a promising chemotherapeutic strategy due to the enhanced efficacy of selectively inducing specific apoptosis in tumor cells [31, 37, 38], but not targeting normal cells [39, 40]. The present study demonstrates that proteasome inhibition increases cleaved caspase-8 stability and activity, as well as caspase-8 protein accumulation at the plasma membrane and in cytosol. Further cellular localization profiling identified that the presence of caspase-8 p43/41 and p18 cleavage products in both the cytosolic and membrane fractions, with the p43/41 and p18 subunit was predominantly detected at the plasma membrane. Mechanistic dissection of caspase-8 regulation revealed that caspase-8 processing is not required for caspase-8 activation and apoptosis induction. Unexpectedly, the decrease in cell viability observed with the D2A non-cleavable construct, was comparable to the D3A and wild-type caspase-8 constructs. Using a lower dose of TRAIL doses of GST-TRAIL in combination with Epoxomicin revealed that D2A and D3A were more effective in inducing apoptosis. Apoptosis was however significantly higher in response to GST-TRAIL and Epoxomicin, compared to the TRAIL treatment, while D2A and D3A NB7 cells undergo apoptosis to a level comparable to the wild-type NB7 cells. The non-cleavable mutant forms of caspase-8 may be more effective in inducing apoptosis due to their possible resistance to proteasome regulation. Overall apoptosis induction was significantly higher in the GST-TRAIL and proteasome inhibitor treated cells (compared to TRAIL only) and D2A and D3A NB7 cell apoptosis induction was comparable to the wild-type NB7 cells. The present findings indicate a significant induction in caspase-8 activity in response to the combination treatment of TRAIL with the proteasome inhibitor (compared to TRAIL alone exposure). The marked increase after 12 h in caspase-8 activity in both cytosolic and plasma membrane

fractions, could be due to the release of caspase-8 into the cytosol as evidenced by the increase in p43–41 expression.

One could argue that proteasome inhibition may prevent the degradation of the fully processed caspase-8 dimer or stabilize the p43/41 intermediate form at the DISC. In-depth elucidation of caspase-8 control mechanisms may lead to the development of agents that stabilize the DISC with either full-length caspase-8 or p43/41 intermediate, towards impairing rapid caspase-8 degradation and increasing caspase-8-mediated apoptosis. Such an argument points to the therapeutic value of caspase-8 stabilizing agents as specific triggers for apoptosis that could circumvent issues associated with toxicity, tumor specificity and cancer cell resistance.

We recognize that the present study does not define whether caspase-8 polyubiquitination occurs at the DISC causing its release into the cytosol, or in the cytosol subsequent to its release from the DISC. However, the functional significance of caspase-8 polyubiquitination, whether at the DISC or in the cytosol, provides an opportunity for interrogating a new regulatory mechanism of caspase-8 by polyubiquitination and defines the specific E3 ligase responsible for caspase-8 K48 linked polyubiquitination. Existing evidence suggests that caspase-8 polyubiquitination can be either K63 linked on the p10 subunit leading to caspase-8 activation, or K48 linkage resulting in caspase-8 degradation [18, 19]. Our results indicate that processed caspase-8 is polyubiquitinated on the p18 subunit rather than the p10 subunit, in contrast with previous reports [18]. Since the IC12 caspase-8 antibody targets an epitope in the p18 region, caspase-8 polyubiquitination would suggest that p18 is a site targeted for polyubiquitination, in addition to the p10 subunit reported by Jin et al. [18]. One should also consider that we are pursuing endogenous caspase-8, rather than non-cleavable constructs, or caspase-8 fragments. The focus was on caspase-8 polyubiquitination in activation of the extrinsic apoptosis pathway, rather than non-apoptotic pathways, as EGF signaling [19]. Moreover, the cellular platform hosting the kinetics for caspase-8 processing, polyubiquitination, and 26S proteasome degradation maybe different in diverse cell types; another compounding factor is that the entire pool of pro- caspase-8 is not completely processed into cleavage products. Our findings indicate that p18 fragment stabilization is a result of proteasome inhibition and not of manipulation of c-FLIP in response to Velcade or MG132 [41, 42]. The cellular site for caspase-8 polyubiquitination is currently being pursued.

The present results establish that non-cleavable mutants D3A and D2A are capable of activation in the presence of GST-TRAIL alone and this activity was markedly increased by the combination of GST-TRAIL and Epoxomicin. Two activity patterns are thus revealed, (i) in the cytosol caspase-8 activity decreased depending on the cleavage mutation, and (ii) in the plasma membrane the wild-type caspase-8 had the lowest activity, while paradoxically the non-cleavable D2A and D3A exhibited higher activity. Potential caspase-8 processing at the DISC, is followed by cycling back into the cytosol. The current understanding of the functional regulation of caspase-8 is that processing is required for caspase-8 activation in death receptor-mediated apoptosis [9, 33, 43]. These findings further support the concept that non-cleavable caspase-8 either at the D210, D216 or D384 position is capable of becoming active and inducing apoptosis. Jin et al. [18], recently identified CUL3 as an E3 ligase that modulates caspase-8 activation in accordance with work from our group that showed E3 ligases, *Seven in absentia homolog* (Siah2) and SH3RF1 (POSH) [44] modulate caspase-8 activity, without affecting caspase-8 processing or stability. Our data identify E3 ligases as new molecular targets responsible for caspase-8 degradation via their ability to mediate its polyubiquitination. E3 ligase confers a powerful trigger for caspase-8 release from the DISC, following the initial cleavage at aspartic acid residue 384. A preferred cleavage intermediate of caspase-8 (likely the p43/41) serving as a threshold in inducing

apoptosis, rather than complete processing into the p18/10 heterodimer may control its activity. Identification of novel inhibitors stabilizing caspase-8 (at the DISC) towards impairing cancer growth via apoptosis induction is of major therapeutic significance as a potential anti-cancer strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

IP	Immunoprecipitation
PI	Propidium iodide
TRAIL	TNF- α related apoptosis inducing ligand
HDAC7	Histone deacetylase 7
DISC	Death inducing signaling complex
FADD	Fas associated death domain
NB7	Neuroblastoma 7
GST	Glutathione S-transferase
TNF-α	Tumor necrosis factor- α
ATCC	American type culture collection
MTT	3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
CRPC	Castration-recurrent prostate cancer
APC	Allophycocyanin
SH3RF1	Plenty of SH3, POSH
PBS	Phosphate buffered saline
c-FLIP	Cellular FLICE-inhibitory protein
NMS	Normal mouse serum
Siah2	Seven in absentia homolog

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Fig. 1.

Caspase-8 processing is not required for activation. **a** Schematic diagram showing the caspase-8 non-cleavable mutant constructs used. **b** Western blots demonstrating reconstitution and respective cleavage events of wild-type and mutant caspase-8 in NB7 cells. **c** Caspase-8 activity in NB7 cells was assessed after treatment with Epoxomicin and/or GST-TRAIL as indicated. Whole lysates were subjected to immunoprecipitation and activity was measured using caspase-8 containing beads. **d** Reveals caspase-8 activity as measured in cytosol and plasma membrane fractions

Fig. 2.

Induction of apoptosis is independent of caspase-8 processing in neuroblastoma cells. **a** and **b** indicate the cell viability in NB7 cells (assessed by MTT), in response to combination of Epoxomicin (1uM) and GST-TRAIL (50 ng and 100 ng/ml) for 6 h. **c** Apoptosis was measured in NB7 cells after treatment and staining with Annexin V/PI followed by FACS analysis. Values of apoptosis and caspase-8 activity represent the mean $\pm$ standard error of mean (SEM) from three independent experiments performed in triplicate

Fig. 3.

Stabilization of caspase-8 p18 subunit, by proteasome inhibition. **a**, **b**, and **c** human prostate cancer cells PC-3 (**a**), Jurkat (**b**), and lung cancer H460 cells (**c**), were treated with the proteasome inhibitor MG132 (10μM) and apoptosis inducer, GST-TRAIL (100 ng/ml). Cells were harvested and cell lysates were subjected to western blotting for caspase-8 expression. Proteasome inhibition leads to caspase-8 stabilization in all three cell lines. **d** and **e** Indicate the results of the half-life evaluation for caspase-8 p18 subunit and p10 subunit, respectively

Fig. 4.

Polyubiquitination of processed caspase-8. Caspase-8 and polyubiquitin immunoprecipitation was conducted in LNCaP cells after treatment with GST-TRAIL (100 ng/ml), or Epoxomicin (100 nM), separately or in combination (8 h). **a** and **c** Indicate caspase-8 expression in response to Epoxomicin and GST-TRAIL (by western blotting), prior to caspase-8 or ubiquitin pull down, respectively Normal mouse serum (NMS) was used as a control to show pull-down specificity with the caspase-8 specific antibody. **b** and **d** Western blots of caspase-8 polyubiquitination subsequent to caspase-8 or ubiquitin immunoprecipitation respectively. Results are representative of three independent experiments

Fig. 5.

Proteasome inhibition leads to cleaved caspase-8 accumulation at plasma membrane. **a** Markers indicating integrity of fractions, cytosol (C), plasma membrane (M) and internal membrane (I) **b** Caspase-8 expression in cytosol and plasma membrane fractions; expression in controls are fraction markers indicating purity and equal loading. **c** The effect of proteasome inhibition on caspase-8 expression in cytosol and membrane fractions **d** Time course analysis of caspase-8 trafficking, revealing increased expression of the p43/41 intermediate in the membrane fraction. **e** Represents a characteristic temporal profile of caspase-8 activity at 2, 4, 8, and 12 h in response to TRAIL alone, Epoxomycin or the combination (same temporal expression profile as **d**). Values represent mean (from triplicate measurements) $\pm$ SEM, obtained from three independent experiments. Statistical significance is set at $p < 0.0001$ (indicated by asterisk)

Fig. 6. Localization of caspase-8 with Na^+/K^+ ATPase to plasma membrane. **a** and **b** LNCaP cells were stained for 26S proteasome (*red*) and for the plasma membrane marker, Na^+/K^+ ATPase (*green*) as described in “Materials and methods”. **c** and **d** indicate untreated control or treated LNCaP prostate cancer cells, respectively, stained with caspase-8 antibody (*red*), and Na^+/K^+ ATPase (*green*); composite localization (*yellow*). Representative images shown at $\times 63$ magnification. Representative images from three independent experiments, performed in duplicate