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Survivin Inhibitor YM-155 sensitizes TRAIL-resistant Glioma Cells to Apoptosis through Mcl-1 Down-regulation and by engaging the mitochondrial death pathway

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Running title: YM-155 sensitizes glioma cells to TRAIL

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ABBREVIATIONS:

AIF, apoptosis inducing factor; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; MTS, 3-[4,5-dimethylthiazol- 2yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H, tetrazolium; FITC, fluorescein isothiocyanate; IAP, inhibitor of apoptosis protein; NF-κB, nuclear factor κB; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TRAIL, tumor necrosis factor–related apoptosis inducing ligand; PI, propidium iodide.

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ABSTRACT

Induction of apoptosis by the death ligand tumor necrosis factor-related apoptosis inducing-ligand (TRAIL) is a promising antitumor therapy. However, not all tumor cells are sensitive to TRAIL, highlighting the need for strategies to overcome TRAIL resistance. Inhibitor of Apoptosis (IAP) family member survivin is constitutively activated in various cancers and blocks apoptotic signaling. Recently we demonstrated that YM-155, a small molecule inhibitor, not only downregulates survivin in gliomas, but also Mcl-1, and upregulates anti-apoptotic Noxa levels. Because Mcl-1 and survivin are critical mediators of resistance to various anti-cancer therapies, we questioned whether YM-155 could sensitize resistant glioma cells to TRAIL. To address this hypothesis, we combined YM-155 with TRAIL and examined effects on cell survival and apoptotic signaling. TRAIL or YM-155 individually induced minimal killing in highly resistant U373 and LNZ308 cell lines, but combining TRAIL with YM-155 triggered a synergistic pro-apoptotic response, mediated through mitochondrial dysfunction via activation of caspases 8, 9, 7, 3, PARP and Bid. Apoptosis induced by combination treatments was blocked by caspase-8 and pan-caspase inhibitors. In addition, knockdown of Mcl-1 by RNA-interference also overcame apoptotic resistance to TRAIL. Conversely, silencing Noxa by RNA-interference reduced the combined effects of YM-155 and TRAIL on apoptosis. Mechanistically, these findings indicate that YM-155 plays a role in counteracting glioma cell resistance to TRAIL-induced apoptosis, by downregulating Mcl-1 and survivin and amplifying mitochondrial signaling through intrinsic and extrinsic apoptotic pathways. The significantly enhanced antitumor activity of the combination of YM-155 and TRAIL may have applications for therapy of malignant glioma.

INTRODUCTION

Human malignant gliomas are aggressive tumors that generally respond poorly to current therapy with surgery, radiation, and conventional chemotherapy (Wen and Kesari 2008, DeAngelis 2001, Pollack 1994). Although molecularly targeted single agent therapy holds the promise of more effective treatment in many cancers, it has failed to offer long-term survival benefit in malignant gliomas (Pollack et al., 2007, Prados et al., 2009). Therefore, novel approaches are required, potentially taking into account multiagent combinations. Recently, we performed a siRNA screen to identify mechanisms underlying glioma cell survival. We identified that nuclear factor κB (NF-κB), proteasomal components, and Akt and Bcl2 family members (Kitchens et al., 2011, Thaker et al., 2010a, Thaker et al., 2010b, Thaker et al., 2009), when inhibited, enhanced cytotoxicity in glioma cells.

TRAIL (tumor necrosis factor–related apoptosis inducing ligand), is a promising agent due to its tumor-specific induction of apoptosis. The receptors for TRAIL, DR4 and DR5, contain a cytoplasmic “death-domain” capable of engaging the cell suicide apparatus through an adaptor molecule intermediate such as Fas-associated death domain protein, promoting death inducing signaling complex (DISC) assembly and activation of caspase 8 and further activation of caspase 3 (Gong and Almasan 2000b, Gong and Almasan 2000a). The TRAIL-mediated apoptotic process can be also activated by the mitochondrial pathway (Srivastava 2001). In such case, BID is cleaved by caspase 8, the apoptosome is assembled, caspase 9 is activated, and it further activates effector caspase 3 leading to accumulation of cytochrome c in the cytosol. Furthermore, the active truncated form of BID (tBID) triggers the mitochondrial activation of caspase-9 by inducing the homooligomerization and allosteric activation of Bak or Bax, two multidomain proapoptotic members of the Bcl-2 family (Wei et al., 2001).

Although most human cancer cell lines express death receptors for Apo2L/TRAIL, many remain resistant to TRAIL-induced death. Various mechanisms of resistance have been reported in different cancers (Wang et al., 2001, Yang et al., 2010, Russo et al., 2010). We, among others,

have indicated that dysregulation of the NF-κB, PKC, Bcl-2, and Akt pathways may be integrally involved in mediating TRAIL resistance in glioma (Jane et al., 2011, Premkumar et al., 2012, Opel et al., 2011, Okhrimenko et al., 2005). In a prior study, we demonstrated that TRAIL sensitivity was not correlated with the relative expression of functional TRAIL receptors or decoy receptors; however, bortezomib (NF-κB/proteasomal inhibitor) sensitized TRAIL-resistant glioma cells (Jane et al., 2011).

Survivin is a member of the IAP family (Salvesen and Duckett 2002) that may also contribute to TRAIL resistance. In recent studies, we and others have shown that survivin is strongly expressed in pediatric and adult gliomas (Chakravarti et al., 2004, Chakravarti et al., 2002, Okada et al., 2008). Survivin overexpression has been associated with cancer progression, multidrug resistance, poor prognosis and short survival in several tumor types (Karam et al., 2007, Reis et al., 2011, Lei et al., 2010, Tanaka et al., 2000, Troeger et al., 2007). Survivin can inhibit apoptosis by blocking a common step downstream of mitochondrial cytochrome c release by inhibiting terminal effectors caspase-3 and caspase-7, and by interfering with caspase-9 activity and processing (Bilancio et al., 2006, Shin et al., 2001, Asanuma et al., 2005, Xia et al., 2006).

YM-155 is a novel survivin suppressant that is currently in clinical trials (Kummar et al., 2009, Satoh et al., 2009). We have recently shown that YM-155 suppressed survivin expression, with little effect on expression levels of other IAP family members, and inhibited growth and viability of certain glioma cell lines, in addition to downregulating Mcl-1 levels (Jane et al., 2013). Recent studies showed that upregulation of survivin by gene transfer enhanced resistance to TRAIL-induced apoptosis (Raviv et al., 2011, Kim et al., 2011), whereas transfection with survivin antisense enhanced sensitivity to TRAIL-induced apoptosis (Li et al., 2005, Azuhata et al., 2006). Because Mcl-1 is also a critical mediator of cellular resistance to various anti-cancer therapies, including suppression of TRAIL-induced cell death (Kim et al., 2008, Ricci et al., 2007, Kobayashi et al., 2005, Oh et al., 2012), we questioned whether YM-155 could sensitize

resistant glioma cells to TRAIL, either by inhibition of survivin or Mcl-1 or both. In this report, we observed YM-155 sensitized glioma cells to TRAIL by promoting signaling through both the intrinsic and extrinsic apoptotic pathways. Our results demonstrate that therapeutic agents that downregulate Mcl-1 or survivin may promote the efficacy of TRAIL in the clinical setting.

MATERIALS AND METHODS

Cell Lines

The established malignant glioma cell lines U87, U373 LN229, A172 and T98G were obtained from the American Type Culture Collection (Manassas, VA). LN18, LNZ428 and LNZ308 were provided by Dr. Nicolas de Tribolet (Lausanne, Switzerland). Human astrocytes (HA) and growth media were obtained from ScienCell Research Laboratories (Carlsbad, CA). Cell culture conditions of these cell lines were as previously described (Jane et al., 2013, Jane et al., 2011, Premkumar et al., 2012).

Reagents and Antibodies

Soluble human recombinant SuperKillerTRAIL (referred as TRAIL in this manuscript) was purchased from Enzo Biochemicals (Enzo Life Sciences, Farmingdale, NY). YM-155 was purchased from Chemie Tek (Indianapolis, IN). Caspase inhibitors (z-VAD-FMK, z-IETD-FMK, z-DEVD-FMK, and z-LEHD-FMK) were purchased from R & D Systems (Minneapolis, MN). The following antibodies were used: Mcl-1 (#4572), Bak (3814), Bax (#2774), Bid (#2002), Cytochrome c (#4280), cleaved PARP (#9546), cleaved Caspase-3 (#9664), cleaved caspase-8 (#9496), cleaved caspase-9 (#9501), and ß-Actin (#4970) were from Cell Signaling Technology (Beverly, MA). Noxa (sc-26917) and AIF (sc-5586) were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-Bax (#556467) and monoclonal anti-Bak (AM03) was from BD Pharmingen (San Diego, CA) and Calbiochem respectively.

Cell Proliferation and Cytotoxicity Assay

Cells were seeded in 96-well plates (5,000 cells per well) in 100 μl of growth medium and incubated at 37°C for 24 hours before addition of inhibitors or vehicle for 3 days.Cells were washed, and viable cells quantitated using a colorimetric assay (CellTiter96 Aqueous NonRadioactive Cell Proliferation Assay; Promega, Madison, WI) as described previously (Jane et al., 2013). The IC50 value was calculated as the mean drug concentration required to inhibit cell proliferation by 50% compared with vehicle controls.

Annexin V Apoptosis Assay

Apoptosis was evaluated using a fluorescent annexin V/propidium iodide assay kit (Molecular Probes, Invitrogen) as described previously (Jane et al., 2013, Jane et al., 2011, Premkumar et al., 2012). Cells were treated with or without inhibitors for various intervals, harvested and pelleted by centrifugation (1,000 rpm for 5 min), washed in ice-cold PBS, and resuspended in the annexin V-FITC/ propidium iodide reagent in the dark for 15 min before flow cytometric analysis, using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). .

DiOC6 labeling and detection of mitochondrial membrane depolarization

Mitochondrial membrane depolarization was measured as described previously (Jane et al., 2013, Jane et al., 2011, Premkumar et al., 2012). Nonadherent cells were collected, and attached cells were trypsinized and resuspended in PBS. Cells were loaded with 50 nmol/L 3',3'-dihexyloxacarbo-cyanine iodide (DiOC6, Invitrogen), which accumulates in intact mitochondria, at 37°C for 15 min. Cells were then spun at 3,000 x *g*, rinsed with PBS and resuspended. Fluorescence intensity was detected by flow cytometry and analyzed with CellQuest (Becton Dickinson) and FlowJo (Tree Star, Inc., Ashland, OR) analysis software.

Immunoprecipitation and Western blotting analysis

Cells were washed in cold PBS and lysed in buffer containing 30 mM HEPES, 10% glycerol, 1% Triton X-100, 100 mmol/L NaCl, 10 mmol/L $MgCl_2$, 5 mM EDTA, 2mM Na_3VO_4, 2 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride, 0.8 μmol/L aprotinin, 50 μmol/L bestatin, 15 μmol/L E-64, 20 μmol/L

leupeptin, and 10 μmol/L pepstatin A for 15 min on ice. Samples were centrifuged at 12,000*g* for 15 min, supernatants were isolated, and protein was quantified using Protein Assay Reagent (Pierce Chemical, Rockford, IL). Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto a nylon membrane (Invitrogen). Nonspecific antibody binding was blocked by incubation of the membranes with 4% bovine serum albumin in Tris-buffered saline (TBS)/Tween 20 (0.1%). The membranes were incubated with primary antibody overnight at 4°C, then washed in TBS/Tween 20 and incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody in TBS/Tween 20 at room temperature for 1 h. Proteins were visualized by Western Blot Chemiluminescence Reagent (Cell Signaling). Where indicated, the membranes were reprobed with antibodies against β-actin to ensure equal loading and transfer of proteins.

For immunoprecipitation, cell extracts were prepared by lysing 5 x 10^6 cells on ice for 30 min in CHAPS lysis buffer (10 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1% CHAPS, protease, phosphatase inhibitors). Lysates were clarified by centrifugation and equal amounts of protein extracts were incubated overnight with primary antibody. Dynabeads Protein G (Invitrogen) was added for 2 hours, followed by magnetic separation of the immunoprecipitated fraction; Western blot analysis was conducted as outlined above. Scanning densitometry was performed using acquisition into Adobe Photoshop (Adobe Systems, Inc) followed by image analysis (UN-SCAN-IT gel, version 6.1; Silk Scientific).

Subcellular Fractionation

Cells were treated with or without inhibitors and cytosolic proteins were fractionated as described previously (Premkumar et al., 2012). Cells were resuspended in lysis buffer containing 0.025% digitonin, sucrose (250 mM), HEPES (20 mM; pH 7.4), MgCl2 (5 mM), KCl (10 mM), EDTA (1 mM), phenylmethylsulfonyl fluoride (1 mM), 10 μg/mL aprotinin, 10 μg/mL leupeptin. After 10 min incubation at 4°C, cells were centrifuged (2 min at 13,000 x g) and the supernatant (cytosolic fraction) was removed and frozen at -80°C for subsequent use.

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Transient transfection

Optimal 29mer-pRS-shRNA constructs were obtained from Origene (Rockville, MD). Sequences specific for human Mcl-1 (ACC TAG AAG GTG GCA TCA GGA ATG TGC TG), Noxa (GGA GGT GCT ACA CAA TGT GGC GTC GGC AC) and control sequences (GCA CTA CCA GAG CTA ACT CAG ATA GTA CT) (non-target shRNA) were used for this study. Glioma cells were seeded in six-well plates and allowed to reach 70% confluence. Transfection of targeting or control shRNA was performed using FuGene 6 as per the manufacturer's recommendations (Roche Applied Science, Indianapolis, IN). One μg of Mcl-1 or Noxa or non-targeting shRNA in 100 μL Opti-MEM medium was mixed with 2 μL of FuGene 6, and incubated at room temperature for 20 min, followed by addition of complete medium to make the total volume up to 2 mL. After 48 h, media was changed and cells were incubated with inhibitors or vehicle for 24 h. Assessment of cell viability (annexin V binding), loss of mitochondrial membrane potential or Western blot analysis was carried out as described above.

Statistical analysis

Unless otherwise indicated, data are expressed as mean ± S.D. The significance of differences between experimental conditions was determined using a two-tailed Student's *t* test. Differences were considered significant at p values <0.05 or less.

RESULTS

Differential apoptotic responses of glioma cell lines to TRAIL. Our recent study demonstrated a wide range of TRAIL sensitivity to apoptosis induction in glioma cell lines (Jane et al., 2011). As shown in Fig. 1A, annexin V/PI flow cytometry analysis clearly demonstrated that the percentage of apoptotic cells was increased to ~80% when LN18 and T98G (TRAIL-sensitive) cells were treated with TRAIL (with dramatic apoptotic responses to concentrations as low as 5 ng/ml for 24 h), whereas U373 and LNZ308 cells were resistant to TRAIL (~12% cell death at 50 ng/ml of TRAIL). Western blot analysis [time course (Fig. 1B) and dose response (Fig. 1C)

studies] clearly demonstrate activation of the caspase cascade with cleavage of the initiator caspase-8 and the main effector caspase-3 in LN18 and T98G (TRAIL sensitive) cells. For example, after 6 h of treatment (TRAIL, 10 ng/ml), the exogenous caspase-8 was activated by proteolytic cleavage as shown by the appearance of the cleaved intermediate (43 kDa) and the active caspase-8 (18 kDa) fragments. As a result of caspase-3 activity, cleavage of its substrate PARP (poly-ADP-ribose polymerase) was seen in TRAIL-sensitive cell lines (LN18 and T98G) but not in TRAIL-resistant LNZ308 cell line. The active form of caspase-9 (37 kDa) and the reduction of procaspase-10 were observed after TRAIL treatment in LN18 and T98G cell lines. The downstream caspases-7 and -3 as well as the proapoptotic Bcl-2 family member protein Bid were also cleaved (Fig. 1D). However, no Bid cleavage was observed in LNZ308 and U373 (TRAIL-resistant) cell lines (Fig. 1D). As caspases have a key role in TRAIL-mediated apoptosis, we preincubated LN18 cells with caspase-specific inhibitors (z-DEVD-fmk, caspase 3 inhibitor; z-IETD-fmk, caspase 8 inhibitor and z-VAD-fmk, pan caspase inhibitor) and examined the effect by Western blot analysis. Our results indicate that caspase-8 inhibition suppressed TRAIL-mediated caspase 3 and 7 cleavage suggesting that caspase-8 is necessary to engage the caspase effector cascade activation in response to TRAIL (Fig. 1E). The cleavage of Bid and caspase-9 induced by TRAIL and cell death protection by z-LEHD-fmk (caspase 9 inhibitor) suggests that the mitochondrial apoptotic pathway is also activated (data not shown). To confirm this, the release of cytochrome c, and apoptosis-inducing factor (AIF) was measured after TRAIL treatment. The results showed that the amount of cytochrome c and AIF in the cytosolic fraction was increased in LN18 and T98G (TRAIL-sensitive, Fig. 1F) but not in TRAIL-resistant LNZ308 (Fig. 1F) and U373 (data not shown) cell lines. Together these results confirm that TRAIL induces the activation of the mitochondrial pathway in TRAIL-sensitive glioma cell lines.

YM-155 synergizes with TRAIL to induce apoptosis in TRAIL-resistant glioma cell lines. In our recent studies, we demonstrated that the survivin inhibitor YM-155 exhibited significant activity against glioma cells (Jane et al., 2013). Because Inhibitor of Apoptosis Protein (IAP)

family proteins block TRAIL-mediated apoptotic signaling through inhibition of caspase activity (Azuhata et al., 2006, Azuhata et al., 2001, Kim et al., 2004, Gaiser et al., 2008, Nakao et al., 2006), we hypothesized that combining YM-155 and TRAIL would potentiate apoptosis induction in TRAIL-resistant gliomas. To explore the possibility of sensitizing TRAIL-induced cytotoxicity in human glioma cells by YM-155, we treated TRAIL resistant cells (LNZ308 and U373) with TRAIL in the presence or absence of YM-155 for 24 h. Then, cell death was assessed by annexin V/PI flow cytometry and Western blot analysis. Although TRAIL or YM-155 alone was minimally effective, cotreatment with YM-155 significantly enhanced the effect of TRAIL-induced apoptosis. As shown in Fig. 2A, the number of viable cells only decreased to 92% with YM-155 (25 nmol/L) and 91% with TRAIL (25 ng/ml) in LNZ308. Similarly limited effects were seen in U373. In contrast, when cells were cotreated with YM-155 and TRAIL, the number of viable cells reduced to 30% (Fig. 2A). Western blot analysis demonstrated that combination of YM-155 and TRAIL potently induced activation of caspase-3, caspase-7 and PARP cleavage in both LNZ308 and U373 cell lines (Fig. 2B). The combination of YM-155 and TRAIL strongly induced caspase-3 processing with 17 and 15 kDa cleavage products and 89 kDa cleavage product of PARP in TRAIL-resistant glioma cells. Bid and caspase-8 were also activated (Fig. 2C). Incubation with caspase 9 inhibitor (z-LEHD-fmk) was able to partially salvage LNZ308 cells from YM-155 + TRAIL-induced apoptosis (~50% protection from YM-155 + TRAIL-induced apoptosis, Fig. 2D). Chemical inhibitors of caspase-8 (z-IETD), caspase-3 (z-DEVD-fmk) and pan-caspase inhibitor (z-VAD-fmk) also significantly blocked the apoptotic activity of combination therapy with YM-155 and TRAIL (Fig. 2D), suggesting that both the intrinsic and extrinsic pathways play a role in cell death induced by these agents. Overall, these results indicate that YM-155 substantially increases the apoptotic potential of TRAIL-resistant glioma cells. We further addressed whether treatment with YM-155, TRAIL or both would be toxic to normal human astrocytes (HA). Annexin V/PI (Fig. 2E, upper panel) and cell proliferation (assessed by MTS assay; Fig. 2E, lower panel) assay clearly demonstrate that both

YM-155 and TRAIL were not toxic to non-neoplastic astrocytes, suggesting that the combination of YM-155 and TRAIL may selectively kill malignant glioma cells.

YM-155 enhances TRAIL-mediated signaling by engaging the mitochondrial death pathway. Having shown that YM-155-induced sensitization to TRAIL involved caspase activation; we next investigated if the amplification of death signaling was a function of mitochondrial outer membrane permeabilization (MOMP). To analyze this hypothesis, we measured the disruption of the transmembrane mitochondrial potential ($\Delta\Psi m$) using the fluorescent dye DiOC6 in glioma cells after TRAIL plus YM-155 treatment. No reduction of the $\Delta\Psi m$ was observed with TRAIL resistant LNZ308 cells treated with TRAIL (Fig. 3A) or YM-155 alone (Fig. 3B); whereas co-treatments with YM-155 substantially increased the number of cells with a reduced $\Delta\Psi m$ (Fig. 3C). A similar result was observed in U373 cell line (data not shown). The disruption of $\Delta\psi m$ was largely prevented by the caspase-8 inhibitor (z-IETD-fmk), caspase-3 inhibitor (z-DEVD-fmk) and the pan-caspase inhibitor z-VAD-fmk in these cells (Fig. 3D). Western blot analysis of the kinetics of the release of mitochondrial amplification factors revealed that YM-155 + TRAIL treatment resulted in a significant time-dependent increase of cytosolic cyt c and AIF levels, whereas TRAIL or YM-155 alone had minimal effect on the cytosolic translocation of these proteins (Fig. 3E). Taken together, these results show that the disruption of the $\Delta\psi m$ induced by TRAIL + YM-155 is highly dependent on caspase activation and mitochondrial membrane potential is a crucial event driving initiator caspase activation and apoptosis.

Cotreatment of YM-155 and TRAIL induces Bax and Bak conformational changes. Because activation of Bax or Bak is essential for the loss of mitochondrial membrane potential, given that cells lacking both proteins fail to undergo mitochondrial membrane outer membrane potential loss and apoptosis in response to diverse intrinsic stimuli (Wei et al., 2001), we examined the effect of TRAIL on Bax activation in glioma cells by analyzing activity-related conformational changes by immunoprecipitation followed by Western blot analysis with antibodies recognizing

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active Bax or Bak (monoclonal anti-bax, 6A7, Sigma or monoclonal anti-Bak, Ab-1, Calbiochem). LN18 and T98G (TRAIL-sensitive) cells exposed to TRAIL displayed a significant increase in Bax conformational change (Fig. 4A). When TRAIL-resistant cells were exposed to TRAIL or YM-155, a minimal Bax and Bak conformational change was observed. In contrast, cells co-exposed to YM-155 and TRAIL displayed a significant increase in Bax (Fig. 4B) and a modest increase in Bak (Fig. 4C) conformational change compared with cells treated with YM-155 or TRAIL alone, suggesting that activation of Bak and, particularly, Bax may be responsible for the marked induction of apoptosis in cells co-exposed to YM-155 and TRAIL.

Downregulation of Mcl-1 and Noxa activation are crucial events in TRAIL-induced apoptosis. Recently, several groups have shown that Mcl-1 down-regulation (Ricci et al., 2007, Rosato et al., 2007, Meng et al., 2007) or Noxa upregulation (Naumann et al., 2011, Opel et al., 2011, Shankar et al., 2008) by various means dramatically enhances TRAIL lethality in diverse human tumor cells. As we (Jane et al., 2013) observed YM-155 synergistically enhanced ABT-737–induced apoptosis at least in part by down-regulating Mcl-1 expression, we examined the functional relevance of Mcl-1 and Noxa on TRAIL-induced apoptosis. As shown in Fig. 5A, Western blot analysis demonstrated that YM-155 induced Noxa protein expression and caused downregulation of Mcl-1 in a dose-dependent manner .resulting Mcl-1, the antiapoptotic protein that Noxa binds, was down-regulated in this cell line, resulting in a net increase in Noxa relative to its target. YM-155 had little effect on other IAP family member proteins (Fig. 5B). In addition, Bcl-2, Bim and Bcl-xL expression were relatively unchanged (data not shown). Because Noxa exerts its proapoptotic effects through binding to the pro-survival protein Mcl-1, resulting in dissociation and activation of Bax and Bak, followed by mitochondrial outer membrane permeabilization and induction of apoptosis (Okumura et al., 2008), we examined the effect of YM-155 treatment on the interaction between Noxa and its high-affinity partner Mcl-1 (Chen et al., 2005). Immunoprecipitation followed by Western blot analysis revealed that an increased Mcl-1-Noxa complex was detected after YM-155 treatment (Fig. 5C). We also examined whether

up-regulation of Noxa can activate Bak by displacing it from Mcl-1. As shown in Fig. 5C, treatment with YM-155 released Bak from its interaction with Mcl-1. These observations suggest that induction of Noxa by YM-155 can sequester Mcl-1 and also release Bak from Mcl-1, which may contribute to the enhanced apoptotic effect of TRAIL in glioma cells.

To validate the hypothesis that Mcl-1 downregulation and Noxa induction are required for TRAIL-induced apoptosis, we performed RNA interference experiments to knock down the expression of these proteins. Cells were transfected with Noxa- or Mcl-1-specific or non-target shRNA as described in Materials and Methods. Whereas Mcl-1 shRNA mimicked the inhibitory effect on Mcl-1 expression obtained with YM-155, Noxa shRNA counteracted the Noxa induction obtained with this agent (Fig. 5D)]. To examine the role of Mcl-1 and Noxa on cell viability, transiently transfected cells were treated as indicated and apoptosis was detected by Annexin V/PI staining by flow cytometry. As shown in Fig. 5D, decrease in Mcl-1 significantly increased TRAIL-induced apoptosis; whereas decrease in Noxa levels resulted in reduced TRAIL cytotoxicity. These data show that modulation of Mcl-1 and Noxa by YM-155 could play a pivotal role in apoptotic susceptibility to TRAIL in glioma cell lines.

DISCUSSION

TRAIL induces apoptosis of many cancer cell lines in vitro, and its tumoricidal activity has been confirmed in different animal models of human cancer. Although most human cancer cell lines express death receptors for TRAIL (e.g., DR4 and DR5), many remain resistant to TRAIL. Therefore, identifying the signals responsible for protecting tumor cells against TRAIL-induced apoptosis may have a major effect on using TRAIL therapeutically. Considering its roles in cell proliferation and apoptosis, survivin is a promising therapeutic target in this regard. Inhibitors targeting this pathway may reduce cellular anti-apoptotic activity by down-regulating survivin expression, thereby increasing the efficacy of co-therapies. Recent studies showed that upregulation of survivin by gene transfer enhanced resistance to TRAIL-induced apoptosis

(Raviv et al., 2011, Kim et al., 2011), whereas transfection with survivin antisense rendered resistant cells susceptible to TRAIL-induced apoptosis (Li et al., 2005, Azuhata et al., 2006). In addition, because it was shown that Mcl-1 down-regulation could cooperate with TRAIL (Kim et al., 2008, Meng et al., 2007) and that its inhibition could be required for apoptosis induction and promoted by YM-155 treatment (Premkumar et al., 2013), we hypothesized that combining YM-155 and TRAIL could achieve synergistic cytotoxicity in TRAIL-resistant glioma cells.

In this report, we showed that YM-155 has such an action, suppressing survivin and Mcl-1 expression, and at clinically achievable concentrations plays a significant role in enhancing apoptosis in TRAIL-resistant glioma cells mediated by the loss of mitochondrial membrane polarization in a caspase-dependent manner. We observed both caspase-8 and caspase-9 activation with YM-155 and TRAIL, suggesting that both intrinsic and extrinsic caspase pathways are involved. Our data suggesting that caspase inhibition could block YM-155 and TRAIL-induced apoptosis are consistent with a process that may involve caspase pathway activation as a fundamental component of the response.

Interestingly, among prosurvival Bcl-2 family proteins Mcl-1 is the only member downregulated by YM-155 (Jane et al., 2013). Mcl-1 plays a prominent role in the inhibition of apoptosis, mediating its effects primarily through interaction with pro-apoptotic members of the Bcl-2 family at the level of the mitochondria. Mcl-1 is a critical molecule for glioma cell survival (Rieger et al., 1998), making YM-155 a potentially useful therapeutic agent. Previously, we have shown that Mcl-1 is a major determinant of ABT-737 toxicity and that YM-155-mediated downregulation of survivin and Mcl-1 significantly enhanced ABT-737 efficacy (Jane et al., 2013). Regarding the mechanism, Tang et al (Tang et al., 2011) identified that treatment with YM-155 results in the downregulation of Mcl-1 at the transcription level. In agreement with prior reports, Bcl-2 and Mcl-1 exert inhibitory functions on TRAIL-induced apoptosis (Kim et al., 2008, Ricci et al., 2007, Kobayashi et al., 2005, Oh et al., 2012, Chanvorachote and Pongrakhananon 2013, Premkumar et al., 2012), which is consistent with our observation that

Mcl-1 downregulation by YM-155 or by genetic manipulation (shRNA studies) plays a pivotal role in TRAIL-induced apoptosis. In parallel with the decrease in Mcl-1, we observed an increase of the BH-3–only protein NOXA, an antagonist of Mcl-1, suggesting that Noxa-upregulation in response to YM-155 may serve to post-transcriptionally inactivate Mcl-1, causing displacement of proapoptotic proteins bound to the Mcl-1 protein. Because downregulation of Noxa (by genetic interference) inhibited YM-155 and TRAIL-induced apoptosis to some extent, a role of Noxa in YM-155 + TRAIL-mediated apoptosis can be assumed. This is consistent with our previous reports that the antiglioma activity of bortezomib is the result of Noxa up-regulation and Mcl-1 cleavage/degradation (Premkumar et al., 2013, Premkumar et al., 2012).

Crosstalk between the extrinsic and intrinsic pathways has been previously reported to be mediated by activated BID (truncated Bid, tBid), a BH3-only proapoptotic BCL-2 protein, observed to initiate cytochrome c release from mitochondria, thereby initiating intrinsic apoptosis. Similarly, proapoptotic tBid activates the death receptor pathway initiator caspase 8, displacing Bax and Bak and allowing them to oligomerize and induce mitochondrial cytochrome c release, an important early step in mitochondrial-mediated apoptosis (Tait and Green 2010). Our study demonstrated that cotreatment with YM-155 and TRAIL induces BID activation, cytochrome c release and mitochondrial membrane dysfunction. Inhibiting caspase activity with caspase inhibitors blocks mitochondrial depolarization seen with YM-155 and TRAIL and resultant cell death. Furthermore, Mcl-1 downregulation by YM-155 may elicit apoptosis via Bak released from Mcl-1/Bak complexes. We also demonstrated that Bak and Bax, major proapoptotic effectors, undergo conformational changes to mediate mitochondrial outer membrane permeabilization. Bak and Bax conformational change is known to follow caspase-8 activation and is accompanied by pore formation in the outer mitochondrial membrane and the release of cytochrome c from mitochondria (Tait and Green 2010). Together, Bak and Bax conformational change, activation of Bid, and loss of mitochondrial membrane potential may provide a mechanistic explanation for the enhanced cell death induced by YM-155 in TRAIL-

resistant glioma cell lines. In summary, combined treatment with YM-155 and TRAIL might be a promising experimental therapy because YM-155 sensitizes glioma cells to TRAIL-induced apoptosis via various mechanisms, thereby minimizing the risk of acquired tumor cell resistance.

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Conflict of Interest: None

Authorship contributions:

Participated in research design: Daniel R. Premkumar and Ian F. Pollack

Conducted experiments: Esther P. Jane, Daniel R. Premkumar, and Kim A. Foster

Performed data analysis: Esther P. Jane, Daniel R. Premkumar, Kim A. Foster and Ian F. Pollack

Wrote or contributed to the writing of the manuscript: Daniel R. Premkumar and Ian F. Pollack

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FIGURE LEGENDS

Figure 1 Differential apoptotic responses of glioma cell lines to TRAIL. A. TRAIL sensitive (LN18 and T98G) and TRAIL resistant (LNZ308 and U373) malignant human glioma cell lines were seeded at 60% confluence, allowed to attach overnight, and treated with the indicated concentrations of TRAIL for 24 h. Control cells received an equivalent amount of DMSO (O). Apoptosis (upper panel, representative annexin V binding histogram; lower left panel, bar chart representing three independent experiments) was analyzed by flow cytometry. The percentages of cells in each quadrant are indicated. Annexin V is plotted on the x-axis, and PI is plotted on the y-axis. Cells in the lower left quadrant reflect live cells; cells in the lower right quadrant (annexin V positive) represent early apoptotic cells; cells in the upper right quadrant (annexin V/PI positive) represent late apoptotic cells; cells in the upper left quadrant (PI positive) represent dead cells. Data are representative of triplicate studies from three independent experiments (** $p < 0.005$). **B** and **C**. Glioma cells were seeded as described in A. After treatment with TRAIL for the indicated concentration or duration, cell extracts were prepared, and equal amounts of protein were separated by SDS-PAGE and subjected to Western blotting analysis with the indicated antibodies. β-actin served as the loading control. **D**. Logarithmically growing TRAIL sensitive (LN18 and T98G) and TRAIL resistant (LNZ308 and U373) malignant human glioma cell lines were treated with indicated concentrations of TRAIL for 6 h and Western blot analysis was performed with anti-Bid antibody. β-actin served as the loading control. **E.** LN18 cells were pretreated with 25μmol/L z-VAD-fmk (pan caspase inhibitor), z-IETD-fmk (caspase-8 inhibitor) or z-DEVD-fmk (caspase-3 inhibitor) for 2 h followed by TRAIL (10 ng/ml) for 6 h. Western blot analysis was performed with the indicated antibodies. **F**. TRAIL sensitive (LN18 and T98G) and TRAIL resistant (LNZ308) cells were incubated with TRAIL (10 ng/ml) for the indicated duration. Control cells received equal amount of DMSO (0). Cytosolic extract was prepared and

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equal amounts of protein were separated by SDS–PAGE and subjected to Western blotting analysis with the indicated antibodies.

Figure 2 YM-155 synergizes with TRAIL to induce apoptosis in TRAIL-resistant glioma cell lines. A. TRAIL resistant (LNZ308 and U373) cells were seeded at 60% confluence, allowed to attach overnight, and treated with indicated concentrations of YM-155 with or without TRAIL (25 ng/ml) for 24 h. Control cells received an equivalent amount of DMSO (0). Apoptosis was analyzed by flow cytometry. The percentages of cells in each quadrant are indicated. Bar chart represents data from three independent experiments (** $p < 0.005$). **B**. LNZ308 and U373 cells were treated with YM-155 (25 nmol/L) or TRAIL (25 ng/ml) or the combination of both for 24 h. Equal amounts of protein were separated by SDS–PAGE and subjected to Western blotting analysis with the indicated antibodies. **C**. LNZ308 cells were pretreated with 25μmol/L z-IETD (caspase-8 inhibitor), z-DEVD (caspase-3 inhibitor), or z-VAD-fmk (pan caspase inhibitor) for 2 h followed by YM-155 (25 nmol/L) + TRAIL (25 ng/ml) for 24 h. Whole cell extracts were prepared as described in the Materials and Methods Section. Equal amounts of protein were separated by SDS–PAGE and subjected to Western blotting analysis with the indicated antibodies. **D**. LNZ308 cells were pretreated with caspase inhibitors, as in C, followed by YM-155 (25 nmol/L) + TRAIL (25 ng/ml) [Y + T] for 24 h. Apoptosis was analyzed by flow cytometry. Data are representative of triplicate studies from three independent experiments (** $p < 0.005$). **E**. Non-neoplastic human astrocytes (HA) were seeded at 60% confluence, allowed to attach overnight, and treated with TRAIL (25 ng/ml) or YM-155 (25 nmol/L) or the combination of both (T + Y) for 24 h. Control cells received an equivalent amount of DMSO. Apoptosis was analyzed by flow cytometry. The percentages of cells in each quadrant are indicated (upper panel). In parallel, human astrocytes were incubated with varying concentrations of TRAIL with or without YM-155 (25nmol/L). Control cells were treated with equivalent concentrations of vehicle (DMSO). After a 72 h treatment, cell proliferation was assessed semiquantitatively by

spectrophotometric measurement of MTS bioreduction. Points represent the mean of three measurements ± S.D (lower panel).

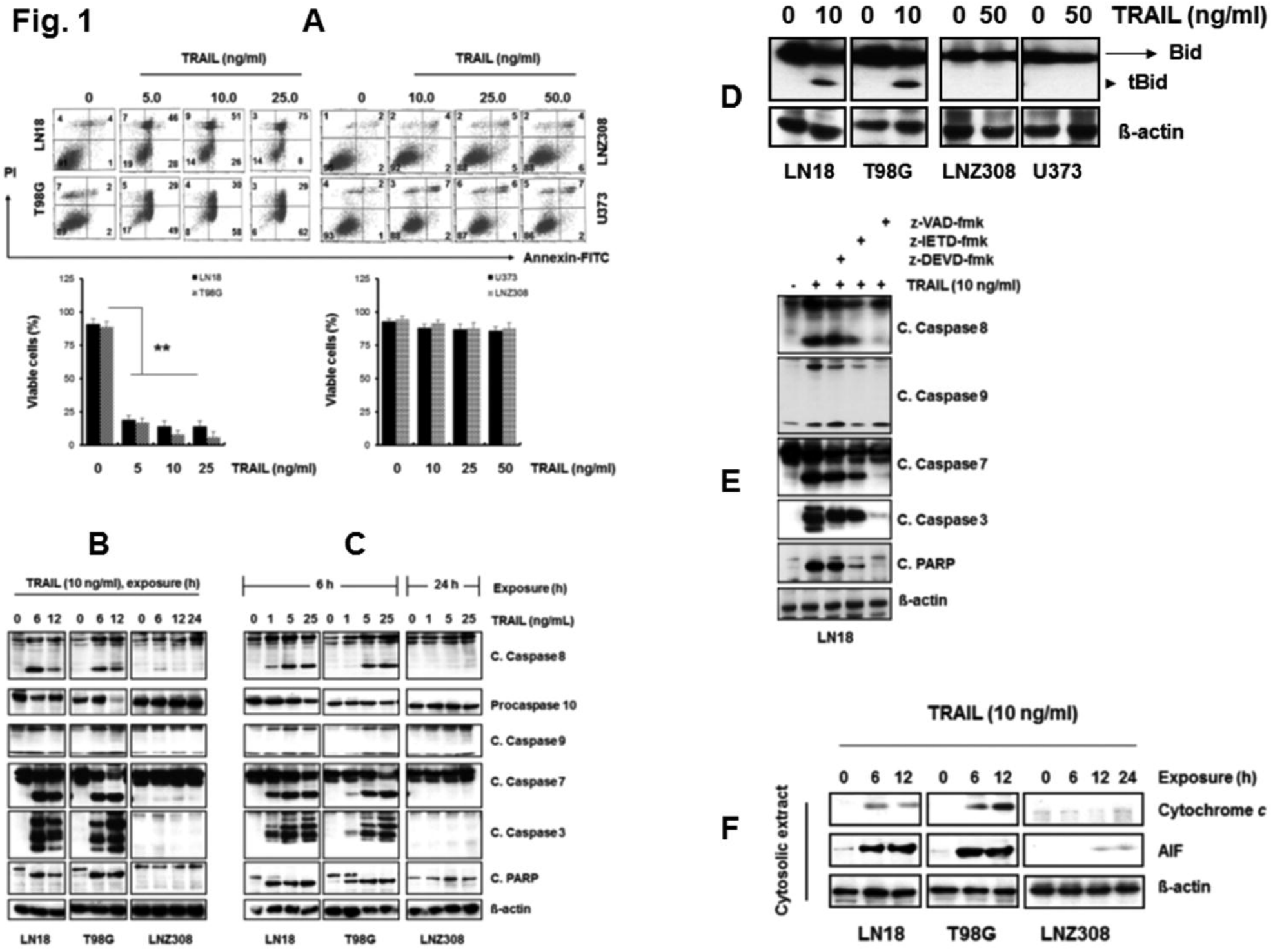
Figure 3 YM-155 enhances TRAIL-mediated signaling by engaging the mitochondrial death pathway. LNZ308 cells were treated with indicated concentrations of TRAIL (**A**), YM-155 (**B**), or both (**C**) for 24 h. Control cells received equivalent amounts of DMSO. The integrity of the mitochondrial membranes was examined by DiOC6 staining as described in the Materials and Methods. The decrease in fluorescence intensity reflected the loss of $\Delta\psi m$. Histogram (upper panel) and bar chart (lower panel) represent the mean number of $\Delta\psi m$ cells acquired from three independent experiments. (** $p < 0.05$, compared to control). **D**. LNZ308 cells were pretreated with 25μmol/L z-IETD-fmk (caspase-8 inhibitor), z-DEVD-fmk (caspase-3 inhibitor), or z-VAD-fmk (pan caspase inhibitor) for 2 h followed by YM-155 (25 nmol/L) + TRAIL (25 ng/ml) for 24 h. Loss of mitochondrial membrane potential was determined by DiOC6 staining and flow cytometry. Histogram (left panel) and bar chart (right panel) represent the mean number of $\Delta\psi m$ cells acquired from three independent experiments (** $p < 0.005$, compared to control). **E**. TRAIL resistant LNZ308 cells were incubated with YM-155 (25 nmol/L) or TRAIL (25 ng/ml) or the combination of both for the indicated duration. Control cells received equal amounts of DMSO. Cytosolic extract was prepared as described in the Materials and Methods Section. Equal amounts of protein were separated by SDS–PAGE and subjected to Western blotting analysis with the indicated antibodies.

Figure 4 Cotreatment of YM-155 and TRAIL induces Bax and Bak conformational changes. A. TRAIL sensitive LN18 and T98G cells were treated with TRAIL (10 ng/ml) for indicated duration and lysed with 1% CHAPS buffer. An equal amount of protein (400 μg) was immunoprecipitated (IP) with monoclonal anti-Bax (6A7, Sigma) antibody and then immunoblotted with polyclonal anti-Bax antibody (Cell Signaling Technology). **B**. TRAIL

resistant LNZ308 and U373 cells were incubated with TRAIL (25 ng/ml) or YM-155 (25 nmol/L) and subjected to IP and immunoblotting as in A. **C**. LNZ308 cells were treated with the indicated concentrations of TRAIL with or without YM-155 (25 nmol/L) for 24 h. Bak immunoprecipitates using monoclonal anti-Bak (Ab-1, Calbiochem) were subjected to Western blot (WB) analysis using polyclonal anti-Bak antibody (Cell Signaling Technology). Samples of whole cell lysates (10% input) were subjected to Western blot analysis to monitor the total level of Bak protein.

Figure 5 Downregulation of Mcl-1 and Noxa activation are crucial events in TRAIL-induced apoptosis. **A** and **B.** Logarithmically growing glioma cells were treated with the indicated concentrations of YM-155 for 24 h and Western blot analysis was performed with the indicated antibodies. **C**. LNZ308 and U373 cells were treated with indicated concentrations of YM-155 for 24 h. An equal amount of protein (400 μg) was immunoprecipitated (IP) with Mcl-1 antibody and subjected to Western blot analysis using the indicated antibodies. **D.** LNZ308 cells were transfected with non-target (NT) or Noxa or Mcl-1 shRNA. Forty-eight hours post-transfection, cells were treated with the indicated concentrations of TRAIL or YM-155 or the combination of both for 24 h, and viability was assessed by Annexin V/PI apoptosis assay (** $p < 0.005$ versus control). In parallel, cell lysates were collected and protein was subjected to Western blot analysis using anti- Noxa or anti-Mcl-1 antibody. Immunoblots were stripped and reprobed with β-actin. Data are representative of triplicate studies from three independent experiments.

Fig. 1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U373
Viable cells (%)
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T98G
U373
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C. Caspase 3
C. PARP
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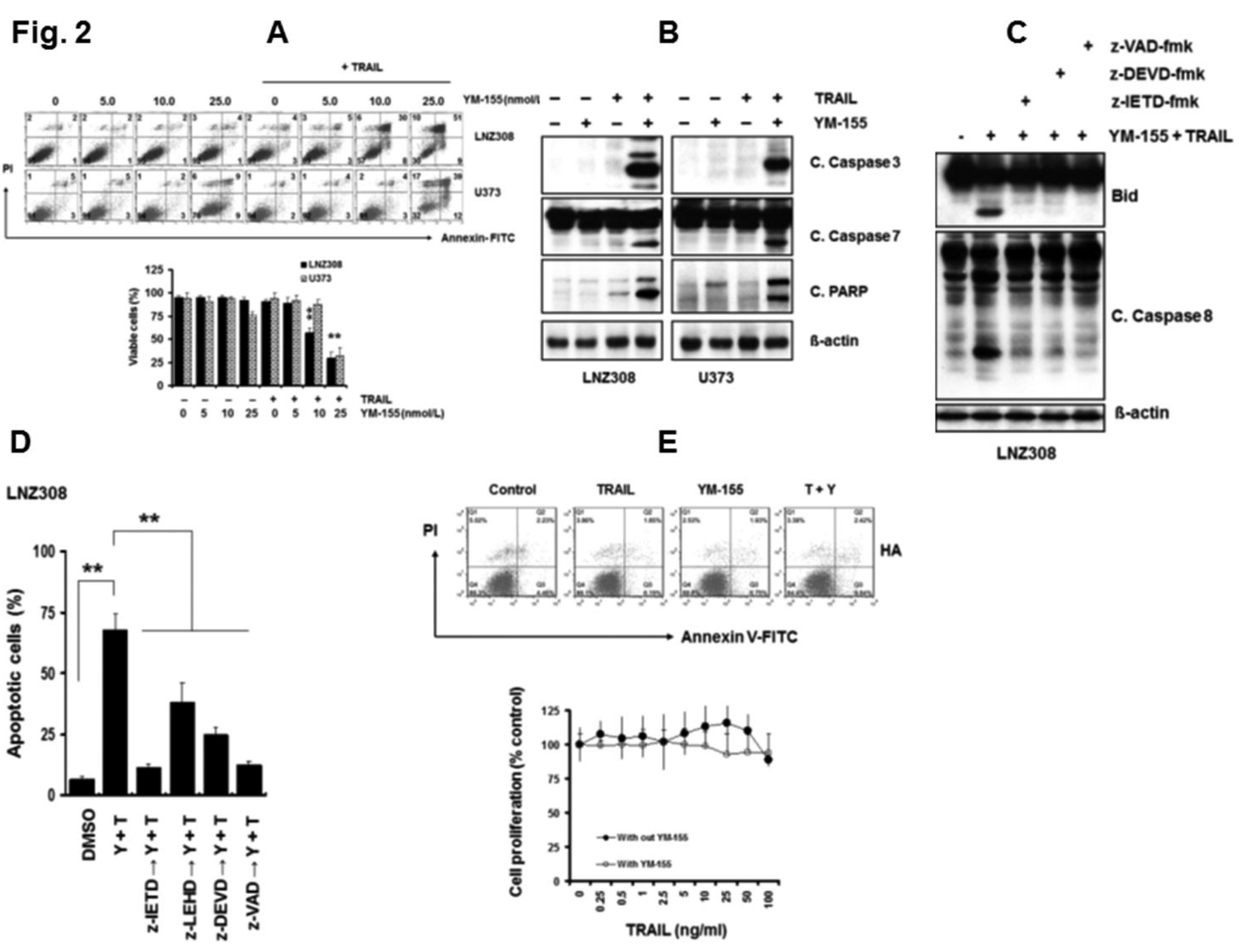
Fig. 2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Viable cells (%)
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z-IETD → Y + T
z-LEHD → Y + T
z-DEVD → Y + T
z-VAD → Y + T
E
Control
TRAIL
YM-155
T + Y
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HA
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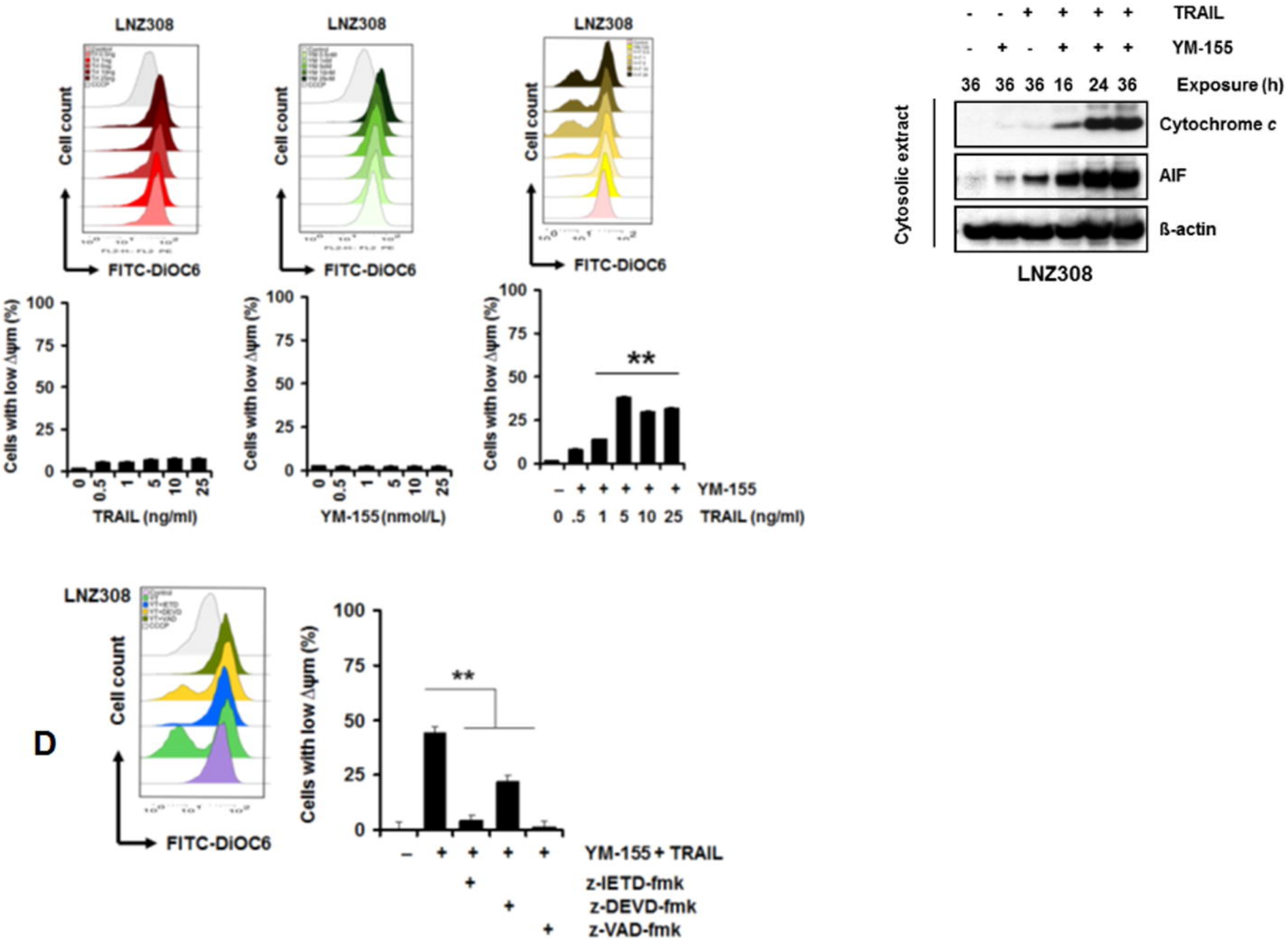
With out YM-155
With YM-155
TRAIL (ng/ml)

Fig. 3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YM-155
TRAIL (ng/ml)
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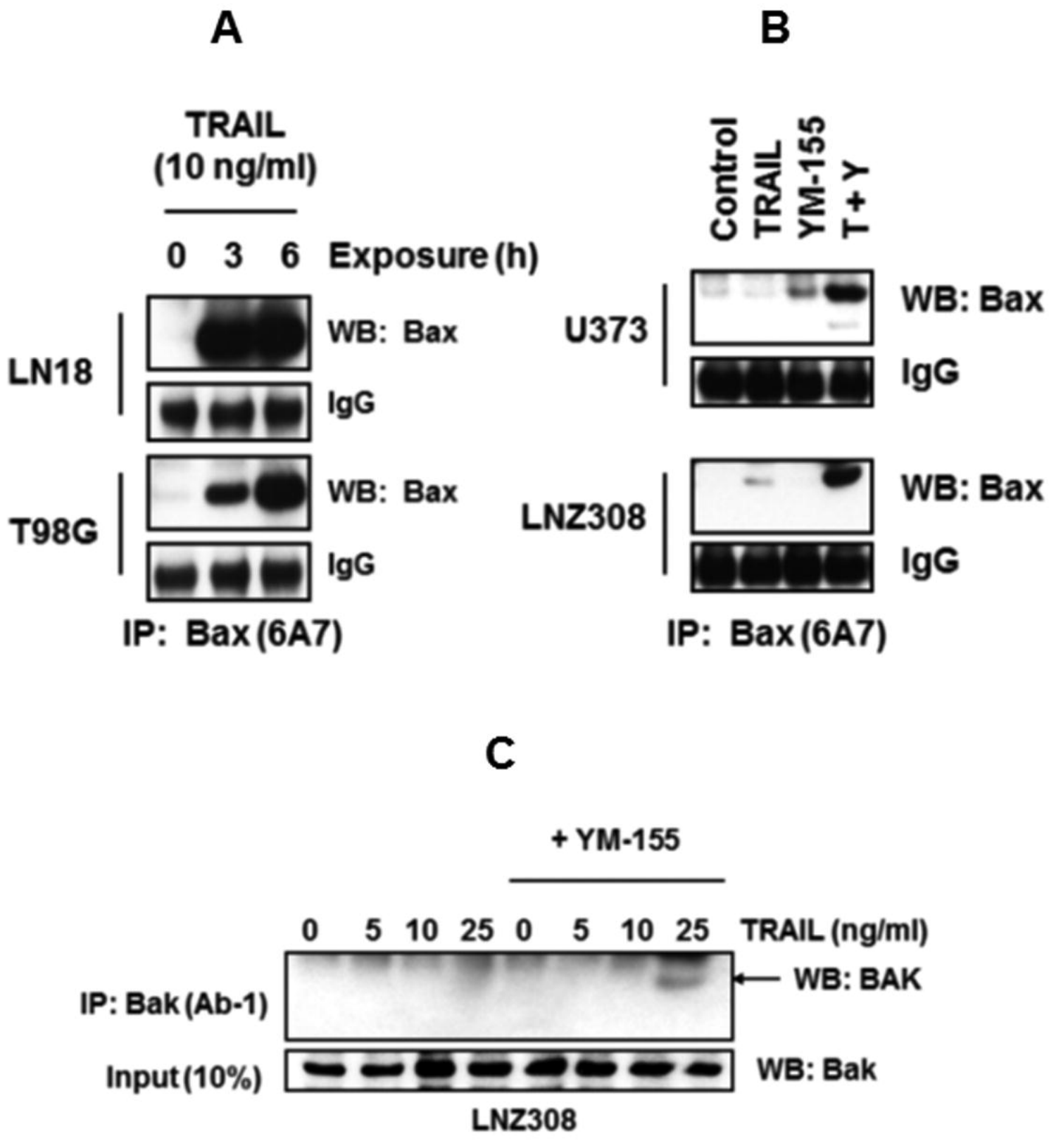
AIF
ß-actin

Fig. 4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Control
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YM-155
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U373
WB: Bax
IgG
LNZ308
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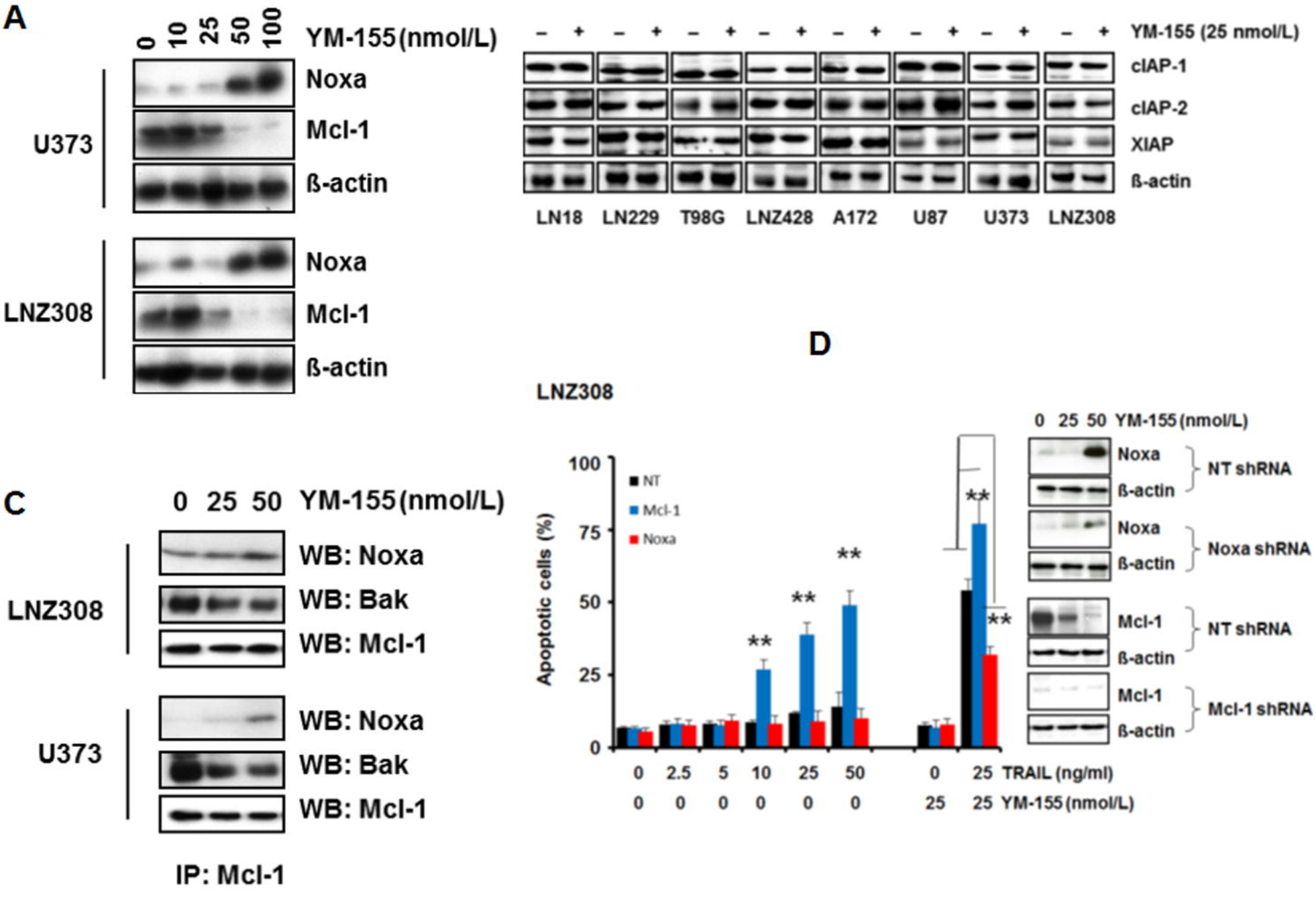
WB: Bak
LNZ308

Fig. 5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YM-155(nmol/L)
U373
Noxa
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LNZ308
Noxa
Mcl-1
ß-actin
B
– + – + – + – + – + – + – + – + YM-155 (25 nmol/L)
cIAP-1
cIAP-2
XIAP
ß-actin
LN18 LN229 T98G LNZ428 A172 U87 U373 LNZ308
C
0 25 50 YM-155(nmol/L)
LNZ308
WB: Noxa
WB: Bak
WB: Mcl-1
U373
WB: Noxa
WB: Bak
WB: Mcl-1
IP: Mcl-1
D
LNZ308
0 25 50 YM-155(nmol/L)
Apoptotic cells (%)
100
75
50
25
0
NT
Mcl-1
Noxa
**
0 2.5 5 10 25 50 0 25 TRAIL (ng/ml)
0 0 0 0 0 0 25 25 YM-155(nmol/L)
Noxa
ß-actin
NT shRNA
Noxa
ß-actin
Noxa shRNA
Mcl-1
ß-actin
NT shRNA
Mcl-1
ß-actin
Mcl-1 shRNA