LY303511 Enhances TRAIL Sensitivity of SHEP-1 Neuroblastoma Cells via Hydrogen Peroxide–Mediated Mitogen-Activated Protein Kinase Activation and Up-regulation of Death Receptors

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Abstract

We recently reported that LY294002 (LY29) and LY303511 (LY30) sensitized tumor cells to drug-induced apoptosis independent of the phosphoinositide 3-kinase/Akt pathway. Here, we investigated the mechanism of LY30-induced sensitization of human neuroblastoma cells to TRAIL-mediated apoptosis. We provide evidence that LY30-induced increase in intracellular H_2O_2 up-regulates the expression of TRAIL receptors (DR4 and DR5) in SHEP-1 cells by activating mitogen-activated protein kinases, resulting in a significant amplification of TRAIL-mediated caspase-8 processing and activity, cytosolic translocation of cytochrome *c*, and cell death. Involvement of the death receptors was further confirmed by the ability of blocking antibodies against DR4 and/or DR5 to inhibit LY30-induced TRAIL sensitization. Pharmacologic inhibition of c-Jun NH_2 terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) activation by SP600125 and PD98059, respectively, blocked LY30-induced increase in sensitization to TRAIL-mediated death. Finally, small interfering RNA–mediated gene silencing of JNK and ERK inhibited LY30-induced increase in surface expression of DR4 and DR5, respectively. These data show that JNK and ERK are two crucial players involved in H_2O_2-mediated increase in TRAIL sensitization of tumor cells upon exposure to LY30 and underscore a novel mode of action of this inactive analogue of LY29. Our findings could have implications for the use of LY30 and similar compounds for enhancing the apoptotic sensitivity of neuroblastoma cells that often become refractory to chemotherapy. [Cancer Res 2009;69(5):1941–50]

Introduction

Neuroblastomas are tumors of the sympathetic nervous system and account for a major proportion of neoplasia during infancy (1). Spontaneous regression and tumor maturation are frequent in infants and in low-grade tumors; however, patients over 1 year of age with metastatic disease continue to have poor prognosis and often develop drug resistance. Therefore, there is heightened interest in unraveling mechanisms underlying drug resistance in these tumors, as well as in developing alternative strategies for treatment (2).

TRAIL [tumor necrosis factor (TNF)–related apoptosis-inducing ligand], a member of the TNF family of proteins, is a promising candidate for therapy of many forms of cancer, as it selectively triggers apoptosis in transformed cells (3–7). Five receptors for TRAIL have been identified: DR4 (TRAIL-R1) and DR5 (TRAIL-R2) are capable of transducing apoptotic signal, whereas the other three (TRAIL-R3, TRAIL-R4, and OPG) are decoy receptors to block TRAIL-induced apoptosis (8–11). Interaction of TRAIL with DR4 and DR5 leads to recruitment of the adaptor protein FADD and initiator caspase-8 to the death initiating signaling complex (DISC), resulting in enzymatic activation of caspase-8, which in turn activates downstream caspase cascade in the presence or absence of mitochondrial amplification machinery (12). Deregulation of apoptotic-related signaling molecules, such as down-regulation of DR4/5, caspase-8, or Bax and/or overexpression of c-FLIP, Bcl-2, Bcl-x_L, or survivin, accounts for the resistance of various tumor cells to TRAIL-induced apoptosis (13, 14). Understandably, the relative tumor selectivity of TRAIL has generated enormous interest in unraveling effector mechanisms and the search for novel compounds that could resensitize tumor cells to TRAIL. In this regard, a number of studies have shown the amplifying effect of anticancer drugs or irradiation on TRAIL-mediated apoptosis via cell type–specific mechanisms (13, 15–17).

We previously reported that the phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (LY29) and its inactive analogue, LY303511 (LY30), strongly sensitized tumor cells to drug-induced apoptosis in a manner independent of the PI3K/Akt pathway (18). We linked the apoptosis sensitizing effect of these compounds to an increase in intracellular hydrogen peroxide (H_2O_2) production and amplification of the mitochondrial death pathway. More recently, we showed DR5 oligomerization and amplification of TRAIL signaling in ovarian cancer cells upon exposure to LY30 (19). Stimulated by these findings, we investigated the effect of preincubation of SHEP-1 cells with LY30 on TRAIL-induced apoptosis and questioned the specific role of intracellular H_2O_2 on apoptosis execution.

Reactive oxygen species (ROS), including superoxide, H_2O_2, and hydroxyl radicals, trigger a variety of cellular responses leading to cell growth, differentiation, or cell death (20–23). An important downstream mediator of ROS-induced signaling are the mitogen-activated protein kinases (MAPK; refs. 23–28), such as stress-activated protein kinase/c-Jun NH_2 terminal kinase (JNK), p38 MAPK, and extracellular signal-regulated kinase (ERK). MAPK activation, in turn, triggers diverse signaling cascades leading to cell proliferation, differentiation, or cell death (22, 26, 27, 29).

Here, we show that pretreatment of SHEP-1 cells with LY30 amplified TRAIL-induced apoptosis via intracellular H_2O_2-mediated

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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activation of p38, JNK, and ERK and up-regulation of the TRAIL receptors DR4 and DR5. These data show a novel mechanism of action of this inactive analogue of LY29, which could have implications for designing therapies to enhance apoptosis sensitivity of tumor cells.

Materials and Methods

Determination of cell viability. Human neuroblastoma SHEP-1 cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin. In a typical survival assay, SHEP-1 cells (8×10^4 per well) plated in 24-well plates for 24 h were exposed to LY30 (12.5, 25, and 50 μmol/L), TRAIL (25, 50, and 100 ng/mL), and a combination of the two (1 h preincubation with LY30 followed by TRAIL for 4 h). Cytotoxicity was determined by the crystal violet assay. After drug exposure, cells were washed with PBS and incubated for 20 min with crystal violet solution (200 μL). The excess crystal violet solution was washed away with distilled water, and the remaining crystals were dissolved with 20% acetic acid. Viability was determined by absorbance at 595-nm wavelength using an automated ELISA reader. Cell viability experiments were performed similarly with 2,000 units/mL of catalase, 4 μmol/L JNK inhibitor SP600125, 10 μmol/L p38 inhibitor SB202190, 20 μmol/L MAPK/ERK kinase (MEK) inhibitor PD98059 (Sigma-Aldrich), 50 μmol/L of caspase-8 inhibitor Z-IETD-FMK or pan-caspase inhibitor Z-VAD-FMK (R&D Systems), or death receptor blocking antibodies (4 μg/mL anti-DR4 or 1 μg/mL anti-DR5; Alexis Biochemicals), or in cells transfected with small interfering RNA (siRNA) for silencing JNK and ERK expression, respectively. Cells were preincubated for 1 h with LY30 and the respective inhibitor or catalase before the addition of TRAIL. Similar sensitizing effect of LY30 on TRAIL-induced apoptosis was carried out with SY5Y neuroblastoma, T98G glioblastoma, Jurkat leukemia, CEM myelogenous leukemia, HeLa ovarian carcinoma, and HT29 colorectal carcinoma cell lines.

Determination of the tumor cell colony-forming ability. SHEP-1 cells (0.8×10^4 per well) plated in 24-well plates for 24 h were treated with TRAIL (25 ng/mL) for 4 h with or without a 1-h pretreatment with LY30 (25 μmol/L). The cells were then washed and replated in 100-mm Petri dishes and incubated to allow colony formation for 10 d, after which they were washed once with PBS and stained with crystal violet solution (1 mL) and incubated for 20 min. The excess crystal violet solution was washed away with distilled water to visualize the clonogenic potential of the cells.

Propidium iodide staining for DNA fragmentation. Cells were pretreated with LY30 for 1 h and then exposed to TRAIL for 4 h. Propidium iodide (PI) staining for DNA content analysis was performed as described elsewhere (30). A total of 10,000 events were analyzed by flow cytometry using an excitation wavelength set at 488 nm and emission at 610 nm.

Determination of caspase activities. SHEP-1 cells were preincubated with LY30 (25 μmol/L) for 1 h and then incubated with TRAIL (50 ng/mL) for 4 h. Cells were then washed with 1× PBS, resuspended in chilled cell lysis buffer (BD Pharmingen), and incubated on ice for 10 min. Reaction buffer (50 μL; 2×; 10 mmol/L HEPES, 2 mmol/L EDTA, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 10 mmol/L DTT) and fluorogenic caspase-specific substrates (1 μL; Ac-VDVAD-AFC for caspase-2, DEVD-AFC for caspase-3, VEID-AFC for caspase-6, LETD-AFC for caspase-8, and LEHD-AFC for caspase-9; Alexis) were added to each sample and incubated at 37°C for 1 h. Enzymatic activity was determined by the relative fluorescence intensity at 505 nm after excitation at 400 nm using a spectrofluorometer.

Flow cytometric analysis of H_2O_2. Intracellular concentration of H_2O_2 was determined by staining with the redox-sensitive dye 5-(and -6)-chloromethyl-2′,7′-dichlorofluorescin diacetate (CM-H_2DCFDA; Molecular Probes), which becomes fluorescent when oxidized by H_2O_2 and its free radical products (30). After treatment, cells were washed with PBS, loaded with 5 μmol/L of CM-H_2DCFDA at 37°C for 15 min, and analyzed by flow cytometry using an excitation wavelength of 488 nm. At least 10,000 events were analyzed.

siRNA-mediated silencing of JNK, ERK, and p38. For total ERK, JNK, or p38 knockdown using specific siRNAs, SHEP-1 cells were plated in six-well plates for 24 h before transfection. On the day of transfection, the medium was replaced with antibiotic-free DMEM. Transient transfections were performed using Dharmafect and 100 nmol/L of ERK1, ERK2, JNK, or p38 SMARTpool siRNA (Dharmacon RNA Technologies). The control cells were transfected with scrambled siRNA. At 24 h after transfection, the medium was replaced with complete DMEM and cells were incubated further for 48 h. After which, the cells were treated with LY30 before assaying for protein expression by Western blotting, as well as the effect on DR4 and DR5 cell surface expression and cell viability.

Western blot analyses. After treatment of SHEP-1 cells, they were washed with cold 1× PBS and lysed by adding lysis buffer [50 mmol/L Tris-CI, 150 mmol/L NaCI, 0.25% Na-dexoycholate, 1% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L activated sodium orthovandate (Na_3VO_4), 1 mmol/L NaF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin]. Total protein (60–150 μg) was separated by 10% to 12% PAGE transferred to polyvinylidene difluoride membrane and then blocked with 5% nonfat milk in PBST (PBS with 0.5% Tween 20). For detection of cytochrome *c* release, cytosolic and mitochondrial fractions were prepared as described elsewhere (31). Antibodies were used to probe for phosphorylated Akt^{Ser473}, phosphorylated and total JNK1/2 (p-JNK), phosphorylated and total ERK1/2 (p-ERK), phosphorylated and total p38 (p-p38), caspase-8, cytochrome *c* (Cell Signaling), DR4, DR5, and MAPK phosphotase-1 (MKP-1; Santa Cruz). Anti–β-actin (Sigma-Aldrich) was used as a loading control.

Analysis of DR4 and DR5 surface expression. SHEP-1 cells (2.5×10^5) were treated with LY30 and TRAIL as described and washed with 1× PBS supplemented with 0.5% fetal bovine serum (FBS) after detachment with EDTA. Cells were then stained with phycoerythrin (PE)-conjugated mouse monoclonal anti-human DR5 or DR4 (clone 71908 and 69036, respectively, R&D Systems) for 45 min at 4°C according to manufacturer's instructions before washing and resuspension in a fluorescence-activated cell sorting buffer (1× PBS + 0.5% FBS) for flow cytomteric analysis using an excitation wavelength of 488. PE-conjugated mouse IgG2B was used as an isotype control.

Statistical analysis. Data are expressed as mean ± SE. Statistical differences were determined by two-way ANOVA and Student's *t* test. A P value of <0.05 was considered significant. The effects of drug combinations at the IC_{25} (inhibitory concentration, 25%) level were analyzed by the isobologram method. The x and y axes represent doses of LY30 and TRAIL, respectively. The hypotenuse is the line of additivity, which represents the dose of each individual drug required to produce an effect like IC_{25}. Of note, LY30 (50 μmol/L) acts as an enhancer of TRAIL sensitivity without inducing cell death on its own; hence, the line of additivity is drawn beyond the 50 μmol/L point on the *Y* axis. The dotted line and dashed line represent the drug combination point, which is the dose of the drugs in combination showing 25% inhibition of cell viability. Synergism between the two compounds is strongly indicated when the drug combination points fall to the left of the line of additivity (32, 33).

Results

LY30 increases sensitivity of SHEP-1 cells to TRAIL. SHEP-1 cells were exposed to varying concentrations of LY30, TRAIL, and a combination of the two (1-h preincubation with LY30 followed by TRAIL for 4 hours). SHEP-1 cells were responsive to TRAIL (~10%, ~15%, and ~30% reduction in the surviving fraction at 25, 50, and 100 ng/mL, respectively); however, treatment with LY30 (12.5, 25, or 50 μmol/L) had no effect on cell viability (Fig. 1*A*). Interestingly, incubation of cells with LY30 (25 μmol/L) for 1 hour followed by 4 hours exposure to 50 ng/mL of TRAIL had a strong synergistic effect (~40% reduction in viable cells with LY30 + TRAIL versus ~15% with TRAIL alone). To confirm synergism, the dose-response interactions at the IC_{25} level were analyzed by the isobologram method. Synergism between the two compounds is shown as the drug combination points fall to the left of the line of additivity

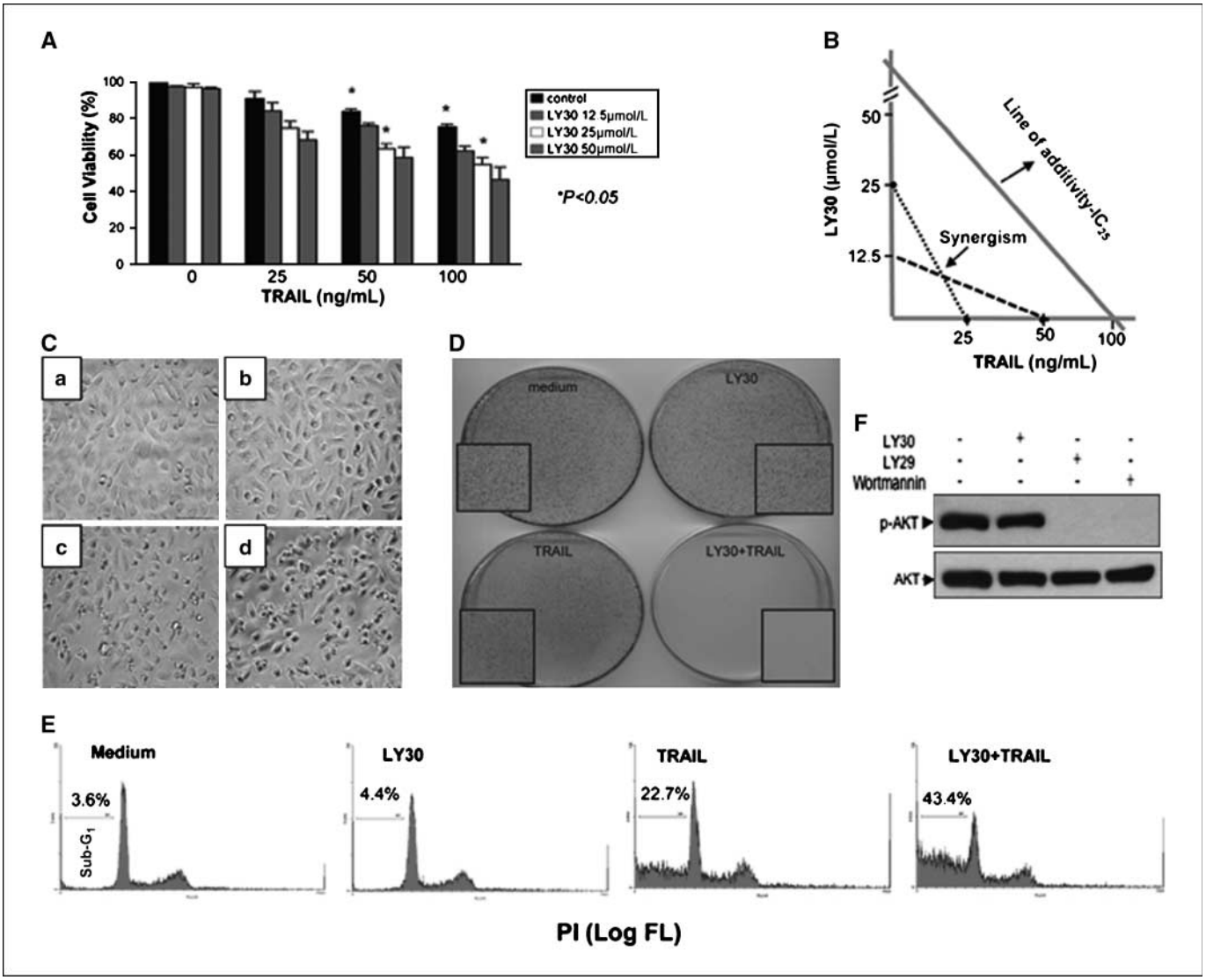

Figure 1. LY30 sensitizes SHEP-1 cells to TRAIL. *A,* SHEP-1 cells (8×10^4 per well) were plated in 24-well plates for 24 h, pretreated with LY30 (12.5, 25, or 50 μmol/L) for 1 h before the addition of TRAIL (25, 50, or 100 ng/mL). Four hours after TRAIL treatment, cell viability was determined by crystal violet staining, as described in Materials and Methods. *B,* an isobologram analysis representing the synergy between the two drugs at various doses. *C,* photomicrograph of SHEP-1 cells: *a,* control; *b,* LY30 25 μmol/L; *c,* TRAIL 50 ng/mL; *d,* LY30 25 μmol/L + TRAIL 50 ng/mL. *D,* SHEP-1 cells were treated with TRAIL (25 ng/mL) for 4 h with or without pretreatment with LY30 (25 μmol/L). The cells were then reseeded in 100-mm Petri dishes and allowed to form colonies for 10 d after which they were stained with crystal violet, as described in Materials and Methods. Interestingly, the combined treatment with LY30 and TRAIL completely inhibits the colony-forming ability of SHEP-1 cells. *E,* cell cycle profiles by flow cytometry using PI staining after treatment of cells with TRAIL (50 ng/mL) for 4 h with or without pretreatment with LY30 (25 μmol/L) for 1 h. *F,* Western blot analysis of the effect of 1 h incubation with LY29 or LY30 (25 μmol/L) or wortmannin (200 nmol/L) on Akt phosphorylation, as described in Materials and Methods. Data are representative of at least three independent experiments.

(Fig. 1*B*). These data were further corroborated by morphologic examination of cells, which showed significant increase in cell death upon LY30 + TRAIL treatment (Fig. 1*C, d*) compared with TRAIL alone (Fig. 1*C, c*). In addition to the enhanced sensitization of SHEP-1 cells to TRAIL by LY30, the two drugs in combination completely inhibited the colony-forming ability of tumor cells even when TRAIL concentration was lowered to 25 ng/mL (Fig. 1*D*). Consistent with the results, PI staining also showed strong sensitizing effect of LY30 on TRAIL-induced apoptosis, as evidenced by the increase in subdiploid fraction (sub-G_1, 43% versus 22% with TRAIL alone; Fig. 1*E*). Furthermore, to confirm that this effect of LY30 was not a function of inhibition of PI3K/Akt pathway, the phosphorylation status of Akt was probed in cell lysates. Whereas, LY29 and wortmannin completely blocked Akt phosphorylation, LY30 had no effect on the PI3K/Akt pathway (Fig. 1*F*).

LY30-induced sensitization to TRAIL is mediated by caspase-8 activation. To obtain insight into the signaling pathway involved in the death amplification activity of LY30, the involvement of caspase proteases was investigated. As expected, exposure of cells to TRAIL (50 ng/mL) resulted in activation of caspase-8, caspase-2, caspase-6, caspase-9, and caspase-3; however single-agent treatment with LY30 (50 μmol/L) did not significantly induce caspase activation (Fig. 2*A*). Interestingly, pretreatment of cells with LY30 significantly amplified TRAIL-induced activation of caspase-8, caspase-2, caspase-9, and caspase-3 (Fig. 2*A*).

Because caspase-8 is a crucial downstream mediator of death receptor signaling, we asked if the sensitizing effect of LY30 was a

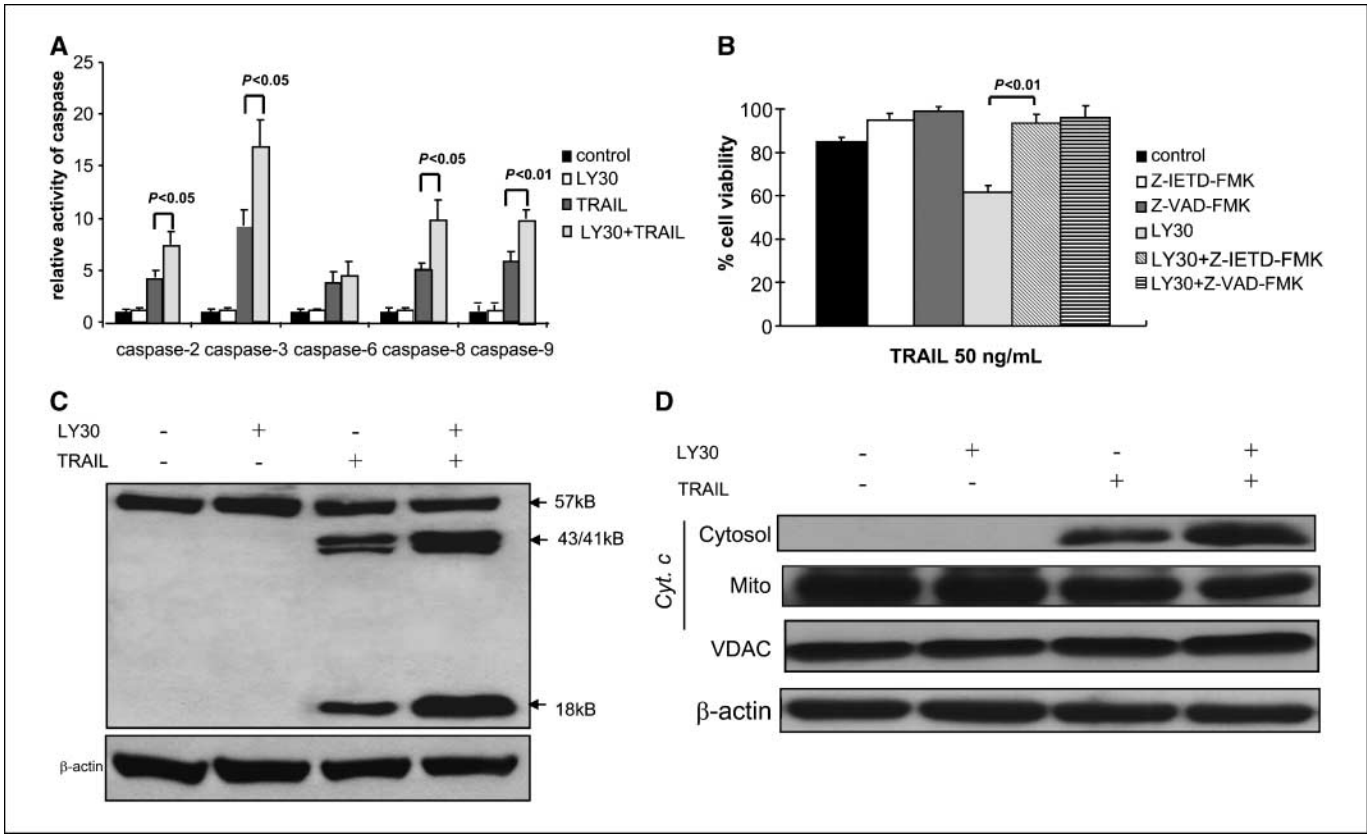

Figure 2. Amplification of TRAIL signaling by LY30 is mediated by caspase-8 activation. *A,* SHEP-1 cells in six-well plates were incubated with TRAIL (50 ng/mL) for 4 h in the presence or absence of 1-h pretreatment with LY30 (25 μmol/L). A total of 50 μg protein in 2× reaction buffer was incubated with caspase-specific substrates (1 μL; Ac-VDVAD-AFC for caspase-2, DEVD-AFC for caspase-3, VEID-AFC for caspase-6, LETD-AFC for caspase-8, and LEHD-AFC for caspase-9). Caspase activity was determined as described in Materials and Methods. *B,* cells (2.0×10^4 per well) were plated in 96-well plate for 24 h and treated with TRAIL (50 ng/mL) for 4 h with or without 1 h pretreatment with LY30 (25 μmol/L) ± Z-IETD-FMK or Z-VAD-FMK (50 μmol/L). *C,* SHEP-1 cells ~90% confluence were treated for 4 h with LY30 (25 μmol/L), TRAIL (50 ng/mL), or TRAIL (50 ng/mL) after 1 h pretreatment with LY30 (25 μmol/L). Total protein (100 μg) was subjected to SDS-PAGE and Western blotting for caspase-8 processing. *D,* SHEP-1 cells at ~90% confluence were incubated with TRAIL (50 ng/mL) for 4 h with or without 1 h pretreatment with LY30 (25 μmol/L) and release of cytochrome *c* from the mitochondria to the cytosol was detected by Western blotting, as described in Materials and Methods. Data are representative of at least three independent experiments.

function of amplification of TRAIL-induced caspase 8 activity. Indeed, the presence of the caspase-8 tetrapeptide inhibitor Z-IETD-FMK (50 μmol/L) not only blocked TRAIL signaling but also inhibited ($P < 0.01$) the sensitizing effect of LY30 on TRAIL-induced cell death (Fig. 2*B*). Similar results were obtained with the pan-caspase tetrapeptide inhibitor Z-VAD-FMK (50 μmol/L; Fig. 2*B*). Furthermore, whereas the overall expression of caspase-8 did not change significantly upon exposure to either LY30, TRAIL, or their combination, TRAIL-induced processing of procaspase-8 was significantly amplified in cells treated with LY30 and TRAIL (Fig. 2*C*). The increase in TRAIL-induced caspase-8 processing and activity induced by pretreatment with LY30 was accompanied by a significant increase in the release of cytochrome *c* to the cytosol, indicating induction of mitochondrial outer membrane permeabilization (Fig. 2*D*). These data and the significant increase in caspase-9 activity ($P < 0.01$) indicate engagement of the mitochondrial (intrinsic) pathway, in addition to the strong amplification of death receptor–mediated (extrinsic) death signaling.

LY30-induced sensitization to TRAIL is mediated by intracellular H_2O_2. We previously showed that LY30 treatment sensitized tumor cells to drug-induced apoptosis via intracellular ROS production (18). Similar to our earlier report with prostate carcinoma cells, incubation of SHEP-1 cells with LY30 (25 μmol/L) resulted in a significant increase in DCF fluorescence by flow cytometry, which was blocked by the H_2O_2 scavenger catalase (Fig. 3*A* and *B*). More importantly, LY30-induced increase in sensitivity of SHEP-1 cells to TRAIL was significantly ($P < 0.01$) inhibited in the presence of catalase, thus underscoring the involvement of intracellular H_2O_2 in the TRAIL sensitizing effect of LY30 (Fig. 3*C*).

JNK and ERK are involved in LY30-induced sensitization to TRAIL. Having linked the sensitizing effect of LY30 to intracellular H_2O_2 production, we next set out to investigate the downstream effector pathway(s), in particular the involvement of MAPKs. SHEP-1 cells were exposed to LY30 (25 μmol/L) for 15 to 120 minutes, followed by Western blot analysis of p-p38, p-JNK, or p-ERK. Interestingly, exposure to LY30 for as short as 15 minutes resulted in significant activation of all three MAPKs, with very similar activation kinetics (Fig. 4*A*); peaked at 15 to 30 minutes, and returned to basal levels within 2 hours of treatment. Intrigued by these findings, we tested the role of MAPK in the sensitizing effect of LY30 on TRAIL-induced cell death. To do so, cells were treated with JNK inhibitor SP600125 (4 μmol/L), p38 inhibitor SB202190 (10 μmol/L), or MEK inhibitor PD98059 (20 μmol/L) for 1 hour before the addition of TRAIL. Whereas, the presence of MAPK inhibitors did not affect cellular response to TRAIL alone, pharmacologic inhibition of JNK and ERK significantly blocked

LY30-induced increase in sensitivity of SHEP-1 cells to TRAIL (Fig. 4*B*). The p38MAPK inhibitor SB202190 was unable to rescue cells from the sensitizing effect of LY30. The specific effect of each inhibitor on respective MAPK was confirmed by Western blotting (Fig. 4*C*). Collectively, these data are the first to report the effect of LY30 on MAPK activation and link intracellular ERK and JNK activation to the sensitizing effect of LY30 on TRAIL-induced cell death in SHEP-1 cells.

LY30-induced MAPK activation is downstream of H_2O_2 production. Thus far, our data showed involvement of MAPKs in LY30-induced sensitization of SHEP-1 cells to TRAIL. We next investigated if LY30-induced H_2O_2 production is the upstream signal responsible for activation of p38, JNK, and ERK. To do so, SHEP-1 cells were treated with LY30 (25 μmol/L) in the presence or absence of the H_2O_2 scavenger catalase (2,000 units/mL) for 30 minutes, and activation of p38, JNK, and ERK was detected by Western blotting. The presence of catalase significantly blocked LY30-induced phosphorylation of p38, ERK, and JNK (Fig. 4*C, i–iii*). These results implicate intracellular H_2O_2 production in the activation of MAPKs upon exposure of SHEP-1 cells to LY30.

LY30 induces up-regulation of DR4 and DR5 mediated by JNK and ERK activation. Having shown that LY30-mediated amplification of TRAIL signaling was mediated by H_2O_2 and MAPK activation, we were interested in deciphering the effect of LY30 treatment on TRAIL receptors DR4 and DR5. Interestingly, exposure of SHEP-1 cells to LY30 resulted in a time-dependent increase in the expression of both DR4 and DR5 (Fig. 5*A*). Given that LY30 activated MAPKs, we next investigated the involvement of MAPK in the up-regulation of DR4 and DR5 expression by LY30. To do so, SHEP-1 cells were exposed to LY30 in the presence or absence of p38, JNK, or ERK inhibitor. Of note, pharmacologic inhibition of JNK (SP600125) blocked LY30-induced up-regulation of DR4 (no effect on DR5), whereas presence of the ERK inhibitor PD98059 prevented DR5 (no effect on DR4) up-regulation upon LY30 treatment (Fig. 5*B*). Unlike JNK and ERK inhibitors, the inability of the p38 inhibitor SB202190 to block the effect of LY30 on death receptor expression suggested that p38 activation was not a critical signaling event in LY30-induced TRAIL sensitization (Fig. 5*B*), which is in line with our data in Fig. 4*B*. Taken together, these data indicate that TRAIL sensitization of SHEP-1 cells induced by LY30 was a function of JNK-mediated and ERK-mediated up-regulation of death receptor expression, resulting in increased caspase-8 processing/activity and amplification of TRAIL-evoked death signaling.

Intrigued by the effect of LY30 on death receptor up-regulation, we next set out to confirm that TRAIL sensitization by LY30 was a function of amplification of death receptor signaling. To do so, death receptor stimulation was blocked by preincubation of cells with blocking anti-DR4 and/or anti-DR5 before LY30 or TRAIL or LY30 + TRAIL treatment. Indeed, blocking either of the receptors significantly rescued cell death induced by TRAIL alone and LY30 + TRAIL, whereas simultaneous blocking of both DR4 and DR5

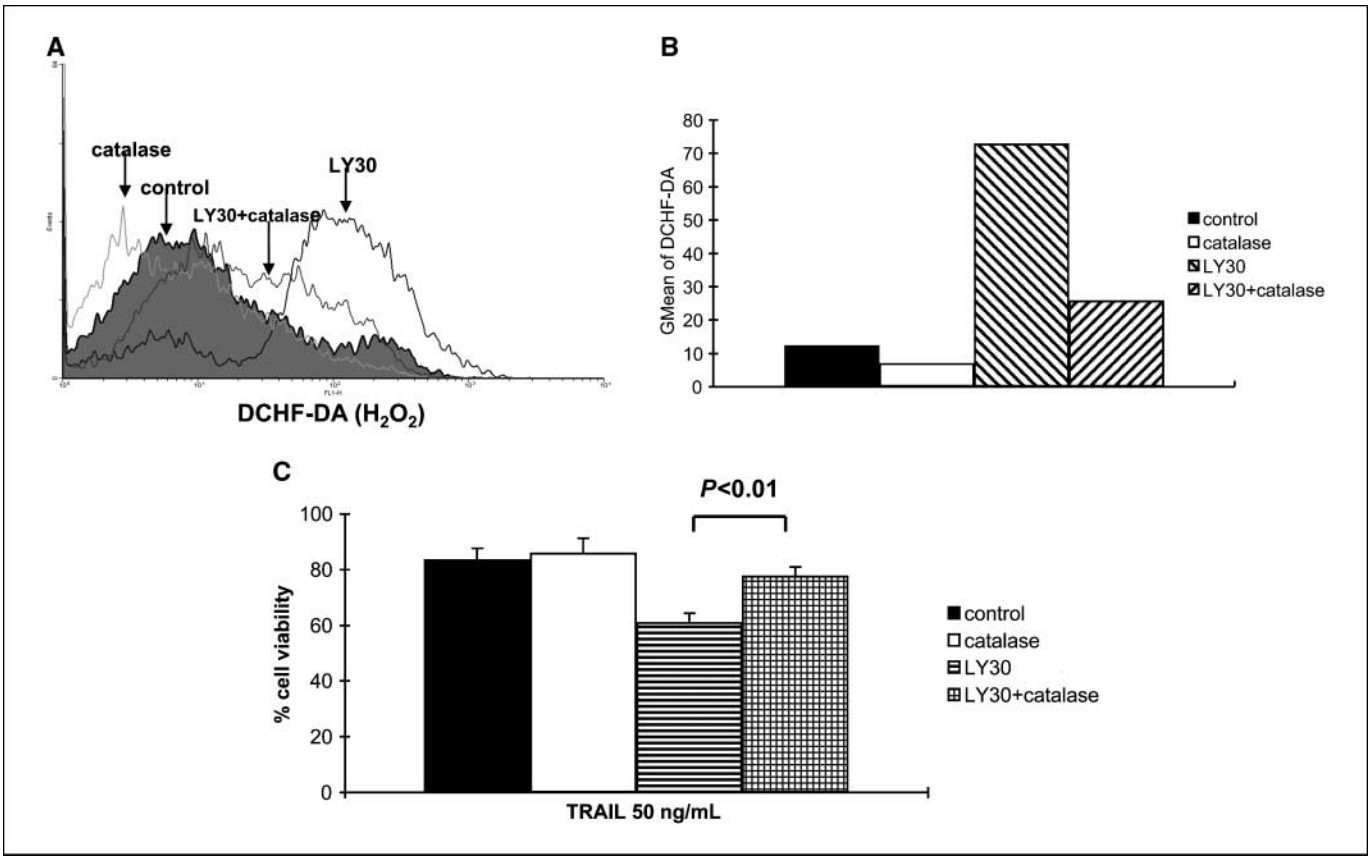

Figure 3. LY30-induced TRAIL sensitization is mediated by H_2O_2. *A,* SHEP-1 cells were treated with LY30 (25 μmol/L) for 30 min in the presence or absence of catalase (2,000 units/mL) and loaded with DCHF-DA (5 μmol/L) for 15 min. Intracellular H_2O_2 generated was indicated by the shift in fluorescence as detected by flow cytometry. *B,* quantitative representation of intracellular H_2O_2 (mean fluorescence). *C,* SHEP-1 cells (2.0×10^4 per well) were plated in 96-well plate for 24 h and treated with TRAIL (50 ng/mL) ± LY30 (25 μmol/L) for 4 h in the presence or absence of catalase (2,000 units/mL), and cell viability was determined by crystal violet staining. Data are representative of at least three independent experiments.

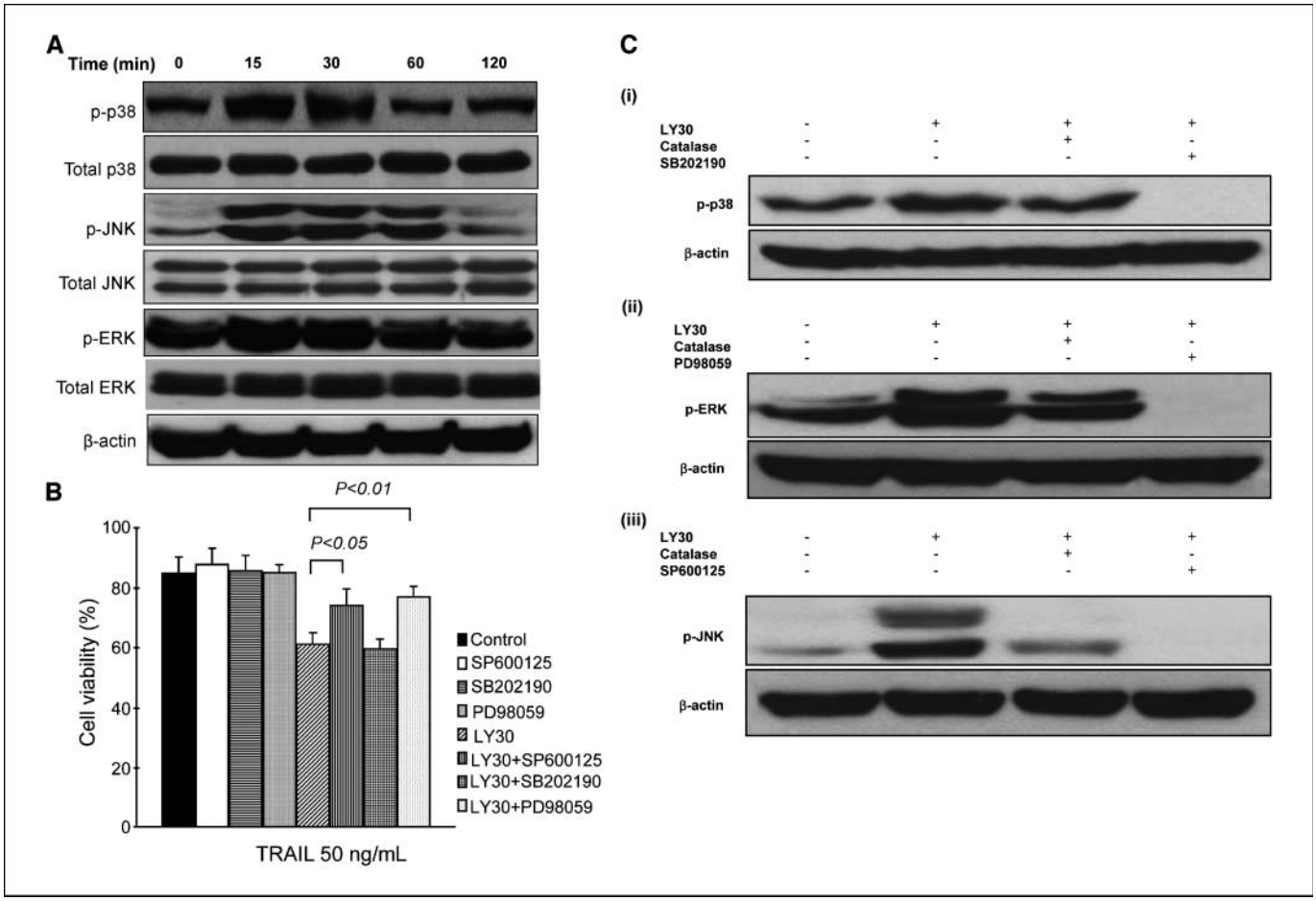

Figure 4. LY30-induced amplification of TRAIL signaling is mediated by JNK and ERK activation, which is blocked by catalase and respective inhibitors. *A,* time course of MAPK activation. SHEP-1 cells at ~90% confluence were treated with LY30 (25 μmol/L) for up to 120 min. Activation of p38, JNK, and ERK was detected by Western blotting and antibodies specific for total and phosphorylated forms of p38, JNK, and ERK. *B,* SHEP-1 cells (2.0×10^4 per well) were plated in 96-well plates for 24 h and treated with TRAIL (50 ng/mL) for 4 h with or without 1 h prior exposure to JNK inhibitor SP600125 (4 μmol/L), p38 inhibitor SB202190 (10 μmol/L), or MEK inhibitor PD98059 (20 μmol/L), and cell viability was determined by crystal violet staining. The effect of these inhibitors was also assessed on LY30-induced sensitization to TRAIL. Data shown are representative of at least three independent observations. *C,* SHEP-1 cells at ~90% confluence were treated with LY30 (25 μmol/L) ± catalase (2,000 units/mL) or LY30 (25 μmol/L) ± MAPK inhibitor (SB202190 10 μmol/L, PD98059 20 μmol/L, or SP600125 4 μmol/L) for 30 min and then activation of p38 (*i*), ERK (*ii*) and JNK (*iii*) was detected by Western blotting using 120 μg of total protein. Data are representative of at least three independent experiments.

completely rescued SHEP-1 cells from the sensitizing effect of LY30 on TRAIL-induced apoptosis (Fig. 5*C*).

H_2O_2 is the upstream signal in LY30-induced up-regulation of DR4 and DR5. LY30-induced increase in intracellular H_2O_2 triggered MAPK activation, which was required for the up-regulation of DR4 and DR5. To provide further confirmation of this signaling hierarchy, we investigated the effect of scavenging H_2O_2 (2,000 units/mL of catalase) on the cell surface expression of DR4 and DR5 induced by LY30 (25 μmol/L) using PE-conjugated anti-DR4 or anti-DR5. Results show that LY30 substantially increased the surface expression of DR4 and DR5, which was completely inhibited by catalase (Fig. 5*D*), thus strongly implicating H_2O_2 as a critical effector mechanism in LY30-induced death receptor activation and TRAIL sensitization.

Knockdown of JNK or ERK blocks LY30-induced up-regulation of surface DR4 or DR5, respectively. Using pharmacologic inhibitors, we provide evidence to link JNK to DR4 and ERK to DR5 up-regulation in response to LY30 in this system. To confirm these findings, SHEP-1 cells were transfected with specific siRNAs generated against each of the specific target genes (siERK1, siERK2, siJNK, si-p38) after the vendor's protocol. Cells were then harvested, and lysates were subjected to Western blotting for verification of the knockdown of individual proteins. Indeed, results show very efficient knockdown after 72 hours of transfection with the respective siRNAs (Fig. 6*A*). Next, the effect of MAPK gene silencing on LY30-mediated up-regulation of cell surface DR4 and DR5 was evaluated. Results clearly show that siJNK significantly blocked the effect of LY30 on DR4 expression (2.1× increase in surface expression in control cells versus 1.5× in siJNK cells), whereas neither siERK1/2 nor si-p38 had any noticeable effect (Fig. 6*B*). In contrast, the up-regulation of DR5 induced by LY30 was completely blocked by siERK1/2 (1.4× increase in surface DR5 in control cells versus 0.8–1× in siERK cells), whereas siJNK or si-p38 had absolutely no effect (Fig. 6*C*). Furthermore, whereas siERK1/2 virtually completely blocked the TRAIL sensitizing effect of LY30 (Supplementary Fig. S1), siJNK had a significant inhibitory effect, but si-p38 did not have any effect on this signaling pathway (data not shown).

It should be pointed out that the TRAIL sensitizing effect of LY30 was not restricted to SHEP-1 cells, as similar combination (albeit at

different concentrations) resulted in a significant increase in TRAIL responsiveness of a variety of cancer cell lines, such as T98G glioblastoma, Jurkat leukemia, SH-SY-5Y neuroblastoma, HeLa ovarian carcinoma, CEM leukemia, and HT29 colorectal carcinoma (Supplementary Figs. S2–S4). Furthermore, similar to the results obtained with SHEP-1 cells, LY30-induced sensitization in these cell lines was associated with intracellular H_2O_2 production and up-regulation of death receptors.

Discussion

LY30-induced activation of MAPKs is mediated by H_2O_2 production. We report here the death-amplifying activity of LY30, an inactive analogue of LY29, on the sensitivity of SHEP-1 cells to TRAIL-mediated apoptosis. In an earlier communication, we provided evidence that pretreatment with these compounds significantly increased the sensitivity of tumor cells to drug-induced apoptosis via intracellular ROS production but independent of PI3K/Akt pathway (18). Similar to our reported findings, exposure of SHEP-1 neuroblastoma cells to LY30 resulted in a significant and early increase in intracellular H_2O_2 production and amplified TRAIL-mediated death signaling. The intermediacy of H_2O_2 in the increased sensitization to TRAIL was further confirmed by the ability of catalase to block LY30-induced amplification of TRAIL signaling. Moreover, data presented here provide a mechanistic link between LY30-induced H_2O_2 production and sensitization to TRAIL via phosphorylation of p38, JNK, and ERK.

The ability of intracellular ROS to trigger activation of MAPKs has been reported in many systems (22, 23, 34). For example, a critical role for activated MAPKs in H_2O_2-induced apoptosis is supported by data showing that siRNA mediated silencing of MKP-1, a member of the MAPK phosphatase family that functions as a negative regulator of MAPK signaling, amplifies H_2O_2-mediated cell death. Alternatively, cells overexpressing MKP-1 were refractory to the death-inducing effect of H_2O_2 (23). Corroborating the critical role of intracellular H_2O_2 in the death sensitizing activity of LY30

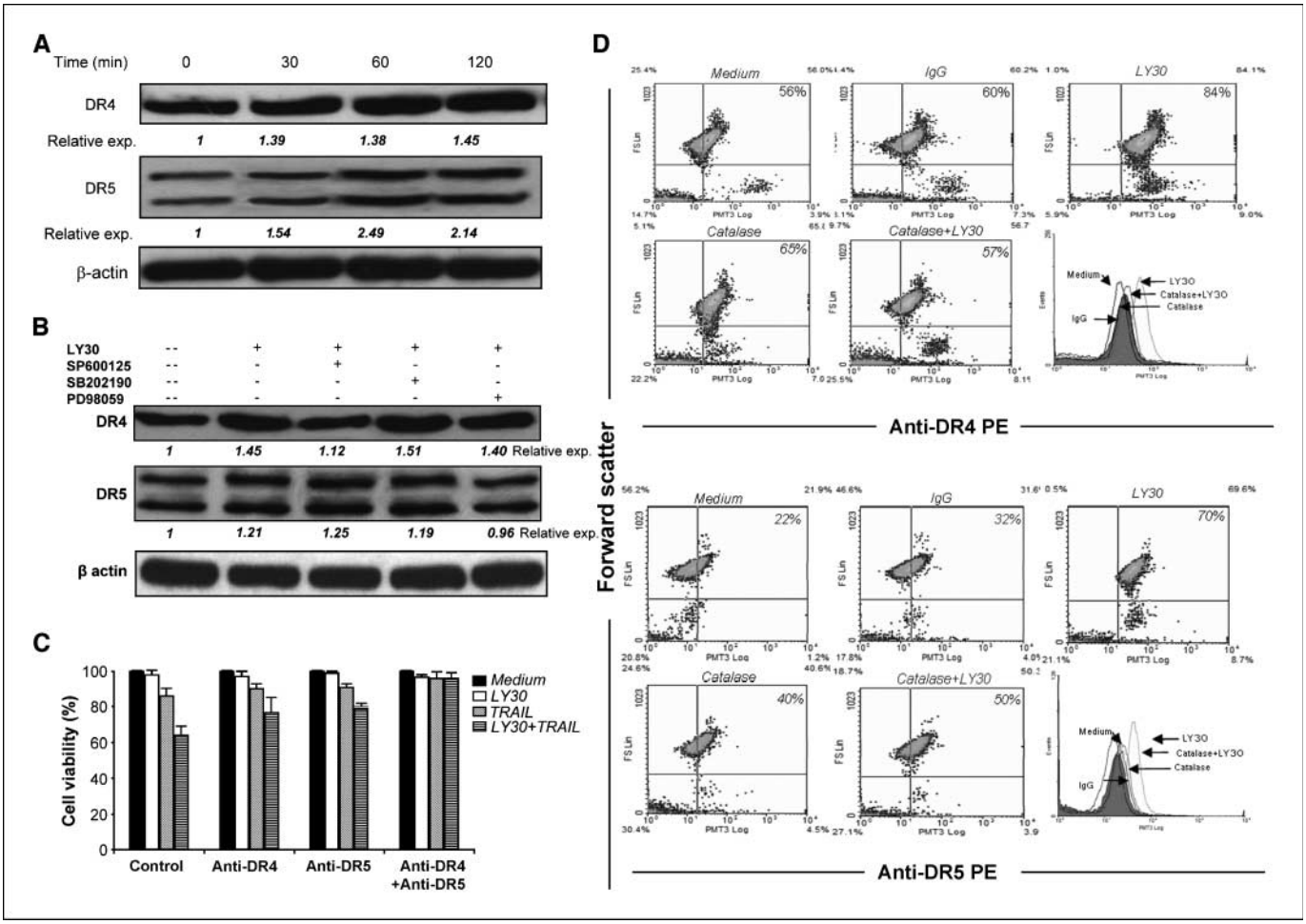

Figure 5. Up-regulation of DR4 and DR5 by LY30 is mediated by ROS-dependent JNK and ERK activation, respectively. *A,* time course of DR4 and DR5 expression in response to LY30. SHEP-1 cells at ~90% confluence were treated for up to 2 h with LY30 (25 μmol/L) and 100 μg of total protein was subjected to SDS-PAGE and Western blot analysis using anti-DR4 and anti-DR5. *B,* cells were treated for 1 h with LY30 (25 μmol/L) in the presence of absence of SP600125 (4 μmol/L), SB202190 (10 μmol/L), or PD98059 (20 μmol/L), and 100 μg of total protein were analyzed by Western blotting for DR4 and DR5. Data presented are representative of at least three independent experiments, and the relative differences in expression were obtained by densitometry and appear as fold increase with 1 being the control expression in untreated cells. *C,* to confirm the contribution of death receptors in TRAIL-mediated cell death, SHEP-1 cells were preincubated with or without monoclonal antibodies blocking DR4 and DR5 receptors, followed by treatment with respective drugs. The apoptotic cells are calculated as a percentage of the respective untreated cells. *D,* SHEP-1 cells at ~90% confluence were treated with LY30 alone, catalase (2,000 units/mL) alone, or a combination of both and analyzed for the surface expression of DR4 and DR5 using PE-conjugated mouse monoclonal anti-human DR4 and DR5 (10 μg/mL). Nonspecific mouse IgG2B was used as an isotype control. A representative histogram underscores the involvement of H_2O_2 in LY30-induced up-regulation of death receptors.

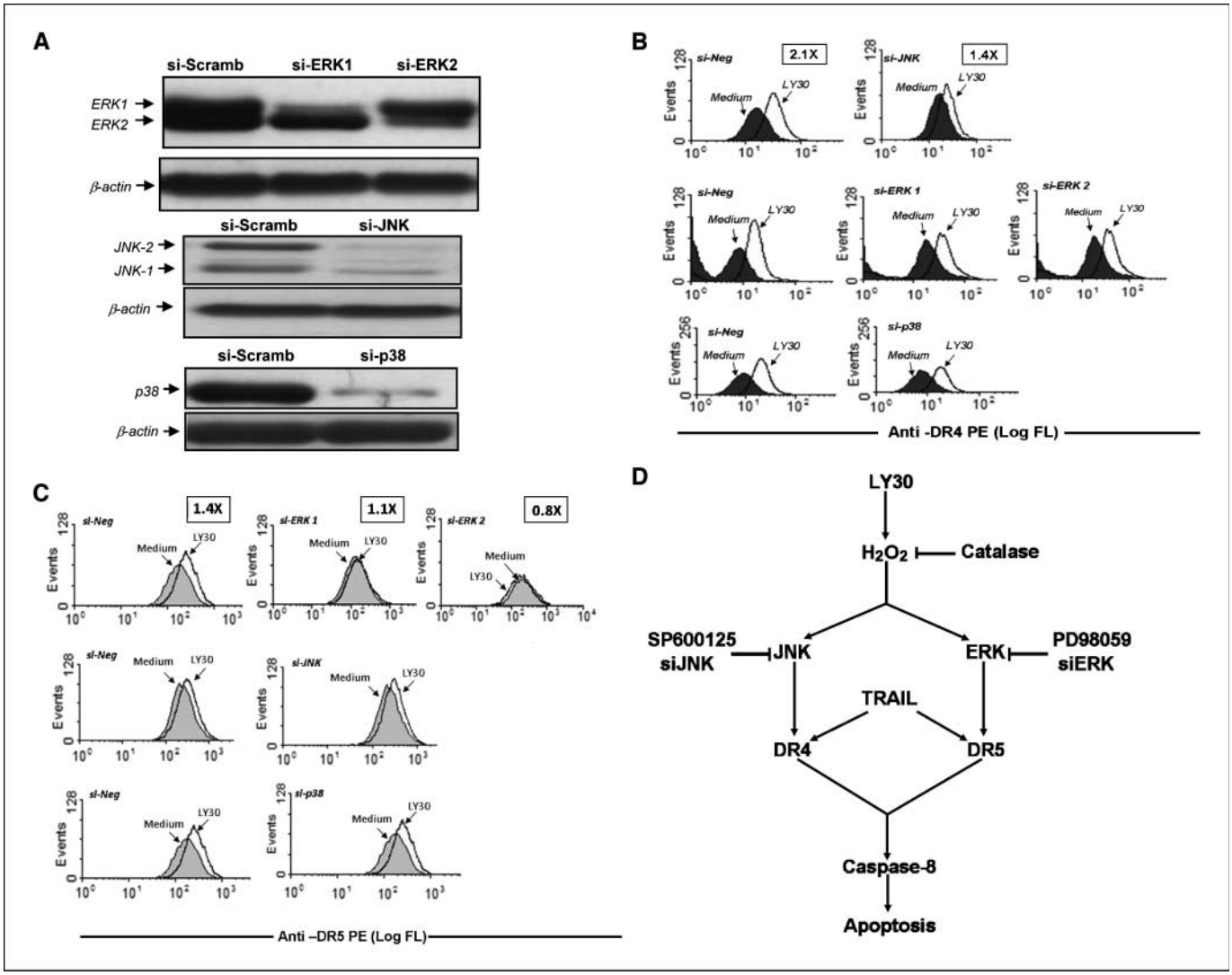

Figure 6. Silencing of JNK or ERK block LY30-induced surface expression of DR4 or DR5, respectively. SHEP-1 cells were transfected with 100 nmol/L of ERK1, ERK2, JNK, or p38 SMARTpool siRNA as described in Materials and Methods. *A,* total cell lysates were subjected to Western blot analysis using anti-JNK, anti-ERK, and anti-p38. β-Actin was probed as a loading control. *B* and *C,* siRNA-transfected cells (siERK, siJNK, or sip38) were treated with LY30 (25 μmol/L) for 2 h, and surface expression of DR4 and DR5 was assessed by flow cytometry using specific antibodies, as described in Materials and Methods. *D,* schematic representation of signaling pathways involved in LY30-induced TRAIL sensitization of SHEP-1 cells. LY30-induced H_2O_2 production stimulates JNK and ERK activation, and the activated JNK and ERK are responsible for DR4 and DR5 up-regulation, respectively. Subsequent exposure of cells to TRAIL facilitates recruitment of caspase-8, its processing, and downstream caspase cascade to trigger apoptosis.

are data showing that the presence of catalase not only inhibited TRAIL sensitization but also blocked LY30-induced MAPK activation and death receptor up-regulation, thus placing H_2O_2 upstream of MAPKs in our system.

LY30 amplifies TRAIL signaling via up-regulation of DR4 and DR5. TRAIL-mediated apoptosis follows a pathway similar to FasL and TNFα, whereby ligation of the death receptors (DR4 and DR5 in case of TRAIL) is necessary to trigger recruitment processing/activation of caspase-8, which can then drive the caspase cascade with (type II) or without (type I) the need for amplification from the mitochondria (3). In this regard, selective up-regulation of genes for DR4 and/or DR5 (35, 36), caspase-8 (37), FADD, BH3 protein Bid (38), or the mitochondrial protein Smac/Diablo (39, 40) have been documented as mechanism(s) underlying increased sensitization to TRAIL. Of note, LY30-induced sensitization to TRAIL signaling was accompanied by robust amplification of caspase proteases (caspase-8, caspase-2, caspase-9, and caspase-3) and release of cytochrome *c* from the mitochondria. The critical role of caspase-8 in the death-amplifying effect of LY30 was corroborated by the significantly stronger processing of procaspase-8 in cells treated with LY30 + TRAIL compared with TRAIL or LY30 alone. Furthermore, inclusion of the tetrapeptide inhibitor of caspase-8, IETD-fmk (as well as the pan caspase inhibitor zVAD-fmk), completely blocked the sensitizing effect of LY30 on TRAIL signaling. Of note, exposure of cells to similar concentrations of LY30 alone did not induce procaspase-8 processing or downstream caspase activation, thus indicating that the sensitizing effect on TRAIL-induced apoptosis was not an additive effect of LY30 and TRAIL but indeed an amplification of TRAIL signaling induced by recruitment and/or amplification of essential components of death receptor signaling upon pretreatment with LY30. These data are in agreement with earlier reports

demonstrating the TRAIL sensitizing effect of nonrelated naturally occurring compounds, curcumin and sulforaphane, via up-regulation of DR5 both at the mRNA and protein levels (41, 42).

Involvement of JNK and ERK in LY30-induced up-regulation of TRAIL receptors. Because intracellular ROS have been linked to the activation of the MAPK pathway, as well as the up-regulation of death receptors in a variety of experimental systems, and the fact that ROS production, MAPK activation, and death receptor up-regulation were observed in cells upon exposure to LY30, the hierarchical order of these events was investigated in SHEP1 cells. Having shown that LY30-induced MAPK activation was ROS-dependent, we questioned whether the activation of JNK and ERK was the cause or a downstream effect of up-regulation of the TRAIL receptors. Interestingly, the presence of the ERK inhibitor PD98059 blocked LY30-induced increase in the protein levels of DR5 (but had no effect on DR4), whereas the JNK inhibitor SP600125 blocked DR4 up-regulation without affecting DR5. The presence of the p38 inhibitor SB202190 neither had an effect on LY30-induced TRAIL sensitization nor death receptor expression. These data show the role of death receptors in LY30-mediated sensitization of SHEP-1 cells in this model. The fact that a short exposure to LY30 triggered significant increase in the cell surface expression of DR4 and DR5 is particularly interesting and provides a logical explanation for the amplification of TRAIL signaling. The involvement of the JNK and ERK pathway in differentially controlling the effect of LY30 on DR4 and DR5, respectively, was further corroborated by siRNA-mediated gene silencing, which clearly showed a rescue effect of siJNK on DR4 and that of siERK1/2 on DR5 up-regulation induced by LY30 (Fig. 6*B* and *C*). These data indicate that the activation of the MAPK pathway, in particular JNK and ERK, was an upstream event responsible for the increase in the expression of TRAIL receptors. Indeed, analysis of the kinetics of MAPK activation and death receptor up-regulation upon LY30 treatment revealed that JNK and ERK activation peaked much earlier (15–30 minutes; Fig. 4*A*) than DR4 and DR5 up-regulation (60–120 minutes; Fig. 5*A*).

The upstream role of MAPK, such as JNK, in extrinsic death receptor–mediated signaling is corroborated by studies using methyl-2-cyano-3,12-dioxooleana-1,9-dien-28-oate (CDDO-Me) in human lung cancer cells (23, 43). CDDO-Me not only activated caspase-8 but also induced expression of DR5 and significantly augmented TRAIL-induced apoptosis, regardless of p53. Interestingly, similar to LY30, CDDO-Me rapidly triggered JNK activation before DR5 up-regulation and the JNK-specific inhibitor SP600125 blocked CDDO-Me–induced increases in JNK activation, DR5 up-regulation, and caspase-8 activation. Furthermore, involvement of MEK-dependent ERK activation and ERK-mediated up-regulation of DR4 and DR5 have also been implicated in Ras-induced sensitization of colon carcinoma cells to TRAIL (44). These reports and our data presented here underscore the critical role of MAPK signaling pathway in the sensitization of tumor cells to TRAIL-induced apoptosis via up-regulation of DR4 and DR5 and could have tremendous implications for TRAIL-based therapeutic strategies.

LY30 and related compounds as novel sensitizers or amplifiers of TRAIL signaling. The myriad ways by which TRAIL-mediated death could be enhanced suggest the presence of multiple factors influencing the effector mechanisms underlying this receptor-dependent death signaling in tumor cells. Our findings suggest an interesting possibility in designing chemotherapeutic strategies aimed at enhancing the response of tumor cells to TRAIL. At the same time, the potency of LY30 as shown here and our earlier study serves to highlight the potential PI3K-independent effects of the widely used sister compound, the PI3K inhibitor LY29, given the structural similarity between these two. To that end, recent studies have also shown the PI3K-independent activity of LY30 in various systems, such as the ability to block K(V) currents in rat β cells (45), inhibition of the expression of monocyte chemoattractant protein-1 in human umbilical vein endothelial cells (46), inhibition of lipopolysaccharide-induced iNOS expression via inhibition of NF-κB activation in macrophage cells (47), and inhibition of cell proliferation via mammalian target of rapamycin (mTOR)–dependent and non–mTOR-dependent mechanisms in human lung epithelial adenocarcinoma cells (48). Our studies provide yet another (novel) mechanism of action of LY30 (and related compounds) involving the intracellular generation of H_2O_2 and downstream activation of MAPK signaling for enhancing cellular response to TRAIL signaling, as summarized in Fig. 6*D*. The differential control of DR4 and DR5 by JNK and ERK and the cellular source of ROS are currently the focus of our ongoing studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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