

Aspirin Sensitizes Cancer Cells to TRAIL-Induced Apoptosis by Reducing Survivin Levels

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Abstract Purpose: Although tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and agonistic antibodies targeting its receptors are promising cancer therapies because of their tumor selectivity, many tumors are resistant to TRAIL-based therapies. We examined whether the nonsteroidal anti-inflammatory drug aspirin sensitized cancer cells to TRAIL agonists *in vitro* and *in vivo* and investigated the underlying mechanism.

Experimental Design: The effects of aspirin on sensitivity to TRAIL agonists and expression of apoptosis regulators was determined in human breast cancer cell lines and xenograft tumors. The specific role of survivin depletion in the TRAIL-sensitizing effects of aspirin was determined by silencing survivin.

Results: Aspirin sensitized human breast cancer cells, but not untransformed human mammary epithelial cells, to TRAIL-induced caspase activation and apoptosis by a cyclooxygenase-2-independent mechanism. Aspirin also sensitized breast cancer cells to apoptosis induced by a human agonistic TRAIL receptor-2 monoclonal antibody (lexatumumab). Aspirin treatment led to G₁ cell cycle arrest and a robust reduction in the levels of the antiapoptotic protein survivin by inducing its proteasomal degradation, but did not affect the levels of many other apoptosis regulators. Silencing survivin with small interfering RNAs sensitized breast cancer cells to TRAIL-induced apoptosis, underscoring the functional role of survivin depletion in the TRAIL-sensitizing actions of aspirin. Moreover, aspirin acted synergistically with TRAIL to promote apoptosis and reduce tumor burden in an orthotopic breast cancer xenograft model.

Conclusions: Aspirin sensitizes transformed breast epithelial cells to TRAIL-based therapies *in vitro* and *in vivo* by a novel mechanism involving survivin depletion. These findings provide the first *in vivo* evidence for the therapeutic utility of this combination.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) is a proapoptotic cytokine that preferentially induces apoptosis in transformed cells (1). TRAIL activates the extrinsic apoptotic pathway by binding to its cell surface death receptors TRAIL receptor-1 (TRAIL-R1)/DR4 or TRAIL receptor-2 (TRAIL-R2)/DR5, which each contain a cytoplasmic death domain that is required for apoptosis induction. Upon ligand binding, the death domain-containing protein FADD is

recruited to the death-inducing signaling complex (DISC), which in turn, leads to the recruitment and activation of apical procaspase-8 and procaspase-10 at the DISC via a homophilic interaction involving their respective death effector domains. In contrast, two decoy receptors DcR1/TRAIL-R3 and DcR2/TRAIL-R4, which lack functional death domains, act to neutralize the cytotoxicity of TRAIL by sequestering the ligand from its death receptors. In cells with robust activation of caspase-8 and caspase-10 (type I cells), these initiator caspases directly activate the executioner caspases (caspase-3, caspase-6, and caspase-7) and trigger apoptosis (2). In type II cells, the initiator caspases cleave the BH3-only protein Bid, which then translocates to the mitochondria to activate the intrinsic apoptotic pathway by inducing cytochrome *c* release by a Bax/Bak-dependent mechanism (3, 4). Cytosolic cytochrome *c* activates caspase-3 by inducing the Apaf-1-dependent activation of the apical procaspase-9 in the apoptosome (5). Importantly, the mitochondria play an essential role in TRAIL-induced apoptosis in type II cells: mutation or deletion of Bax results in resistance to TRAIL (6, 7).

Given the potential tumor selectivity of its proapoptotic actions, recombinant TRAIL has garnered a great deal of interest as a cancer therapy and is currently in early stage clinical trials. In preclinical models, TRAIL induces apoptosis in diverse cancer cells *in vitro* and suppresses primary tumor growth and metastases *in vivo* (8–10). In addition, the safety

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of recombinant TRAIL at doses up to 10 mg/kg/d for 7 days has been documented in non-human primates (8). Similarly, agonistic monoclonal antibodies (mAb) targeting TRAIL-R1 or TRAIL-R2 have been shown to induce apoptosis in cancer cells *in vitro* and in xenograft carcinomas *in vivo*, and these antibodies are well tolerated in rodents (11–15). Indeed, fully human agonistic mAbs specifically targeting TRAIL-R1 (mapatumumab) or TRAIL-R2 (lexatumumab) are currently in clinical trials. Collectively, these results suggest that recombinant TRAIL and/or agonistic mAbs targeting its death receptors may prove to be an efficacious and minimally toxic strategy to treat cancer.

One of the major obstacles confronting TRAIL-based cancer therapies is the intrinsic or acquired resistance of many human tumors to TRAIL-induced apoptosis (7, 8, 16). TRAIL-resistance is likely to be mediated by multiple defects in the TRAIL signaling pathway, including inactivating mutations in TRAIL-R1 and TRAIL-R2, loss of the initiator caspase-8 and the proapoptotic Bcl-2 family member Bax, and overexpression of antiapoptotic Bcl-2 family members such as Bcl-2, Bcl-x_L, and Mcl-1 (7, 17–22). Hence, optimal TRAIL-based therapies for many tumors will need to incorporate agents which sensitize cancer cells to TRAIL-induced apoptosis. For example, chemotherapy and radiation sensitize cancer cells and xenograft tumors to TRAIL- or TRAIL receptor antibody-induced apoptosis at least partly by increasing the expression of TRAIL-R2 and Bax (7, 8, 12, 15, 16). In addition, several potential chemopreventive agents and/or emerging cancer therapies, including PPAR γ ligands (thiazolidinediones and triterpenoids), resveratrol and inhibitors of histone deacetylase, the proteasome and cyclin-dependent kinases have been shown to promote TRAIL-induced apoptosis in cancer cells (13, 23–27). These latter agents are particularly attractive because they do not abrogate the tumor selectivity of TRAIL-based therapies, and they are likely to have less systemic toxicity than conventional cytotoxic agents or radiation.

To identify additional TRAIL-sensitizing agents, we examined the hypothesis that aspirin (acetylsalicylic acid, ASA) might act synergistically with TRAIL and/or TRAIL receptor agonist antibodies to induce apoptosis. Aspirin is a nonsteroidal anti-inflammatory drug which has been reported to reduce the risk of colorectal adenomas and carcinomas, breast cancer, and other malignancies, particularly long-term and higher dose use of aspirin (28–31). Aspirin inhibits both cyclooxygenase (COX)-1 and COX-2 enzymes that catalyze the rate-limiting step in prostaglandin synthesis (32). In addition, 5 mmol/L of aspirin induces G₁ cell cycle arrest and triggers COX-independent apoptosis in cancer cells at least in part by activating Bax and releasing cytochrome *c* from mitochondria (33, 34). We postulated that these latter mitochondrial apoptotic effects of aspirin would sensitize cancer cells to death receptor-mediated apoptosis initiated by TRAIL by amplifying caspase activation. Although aspirin has recently been shown to promote TRAIL-induced apoptosis *in vitro* (35), we report here that aspirin sensitizes breast cancer cells to TRAIL-induced caspase activation and apoptosis at least in part by a novel mechanism involving the proteasomal degradation of the antiapoptotic protein survivin. Survivin is a member of the IAP (inhibitor of apoptosis) family that is preferentially expressed in transformed cells and confers resistance to diverse apoptotic stimuli, including TRAIL

(23, 25, 27, 36). We also show for the first time that aspirin acts synergistically with TRAIL to promote apoptosis and suppress human breast xenograft tumor growth *in vivo*, thereby underscoring the potential therapeutic utility of this combination.

Materials and Methods

Cell lines and reagents. Human MDA-MB-435 and T47D breast carcinoma cells (American Type Culture Collection) were grown in DMEM (Mediatech) supplemented with 4.5 g/L of glucose and 4 mmol/L of L-glutamine, 100 units/mL of penicillin/streptomycin and 10% FCS (Invitrogen), whereas HCT116 colon cancer cells were grown in McCoy's 5A medium (Invitrogen) supplemented with 100 units/mL of penicillin/streptomycin and 10% FCS. Although the origin of MDA-MB-435 cells has been debated, recent studies suggest that they are breast cancer cells that have undergone lineage infidelity (37). Human mammary epithelial cells (HMEC; Cambrex) were grown as described in the manufacturer's instructions. All cells were grown in 5% CO₂ atmosphere at 37°C. Aspirin (ASA) was purchased from Sigma-Aldrich and dissolved in 100% ethanol for *in vitro* studies. Recombinant TRAIL (amino acids 95-281) was expressed in *Escherichia coli* and purified as described (23, 38). Human TRAIL-R1 (mapatumumab) and TRAIL-R2 (lexatumumab) agonistic antibodies of IgG₁ isotype were kindly provided by Dr. Robin Humphreys (Human Genome Sciences, Rockville, MD; refs. 13–15).

Induction and scoring of apoptosis. Cells were preincubated with ASA (0–5 mmol/L) for 48 h and then treated with TRAIL (0–2.5 μ g/mL), mapatumumab, or lexatumumab (0 or 2.5 μ g/mL) for 16 h. Apoptosis was measured by two independent methods. First, the percentage of apoptotic (condensed/fragmented) nuclei were scored by Hoechst (Sigma-Aldrich) staining and fluorescence microscopy as described (39). Three independent experiments were done, counting at least 200 nuclei in each experiment. Apoptotic cells were also identified by flow cytometry-based Annexin V-labeling using the Annexin-PE Apoptosis Detection Kit 1 (BD Bioscience) according to the manufacturer's instructions.

Cell cycle analyses. Cells were treated with vehicle or ASA (1 mmol/L or 5 mmol/L) for 48 h. Cells were washed twice in PBS and then incubated overnight in 70% ethanol/30% PBS at -20°C. The fixed cells were then washed twice and incubated with a propidium iodide solution (50 μ g/mL propidium iodide, 0.2 mg/mL RNase A, and 0.1% Triton X-100 in PBS) for 20 min at 37°C. The cell cycle distribution of cells was determined by flow cytometry-based analysis of DNA content using ModFit software.

Crystal violet cell survival assays. Cells were plated on six-well plates (3 \times 10⁵ cells/well), allowed to adhere overnight, preincubated with vehicle or 5 mmol/L of ASA for 48 h, and then treated with vehicle or 2.5 μ g/mL of TRAIL for 16 h. Cells were then washed with PBS, and fresh growth medium was added. The medium was changed every other day for 5 additional days. Surviving cells were fixed and stained with crystal violet solution (40% ethanol, 60% PBS, and 0.5% crystal violet).

Immunoblotting. Cells were lysed in a modified radioimmunoprecipitation assay buffer (50 mmol/L Tris, 0.1% SDS, 150 mmol/L NaCl, 0.5% sodium deoxycholate, and 1% NP40). Proteins were detected by immunoblotting as described (40) with the following antibodies: TRAIL-R1, TRAIL-R2, DcR2 (Stressgen), tubulin (Sigma-Aldrich), caspase-3, RIP, Bcl-x_L, FADD, TRADD, DcR1, FLIP, Mcl-1, caspase-8, and Bak (BD Biosciences), XIAP and survivin (R & D Systems), Bcl-2 and Bax (Santa Cruz Biotechnology). For proteasome inhibitor experiments, cells were preincubated with vehicle or 5 mmol/L of ASA for 48 h and then treated with the proteasomal inhibitor epoxomicin (0–200 nmol/L; EMD Biosciences; ref. 41) for 16 h (longer treatments with epoxomicin resulted in cell death).

Real-time reverse transcription-PCR. Total RNA was prepared from MDA-MB-435 cells treated with vehicle or 5 mmol/L of ASA for 64 h using the RNeasy mini kit (Qiagen). RNA was used to synthesize cDNA using the Superscript First-Strand Synthesis System for reverse transcription-PCR (Invitrogen). Survivin mRNA and glyceraldehyde-3-phosphate dehydrogenase mRNA (for normalization) were amplified by real-time PCR using TaqMan universal PCR master mix and an ABI PRISM 7000 sequence detection system instrument (Applied Biosystems) according to the manufacturer's instructions. A predesigned 20× mix of primers and TaqMan MGB probes for survivin and glyceraldehyde-3-phosphate dehydrogenase were purchased from ABI. cDNA (100 ng), primers (900 nmol/L), and probes (250 nmol/L) were used in PCR reactions (50°C for 2 min, 95°C for 1 min, and 40 cycles of 95°C for 15 s, and 60°C for 1 min).

Survivin silencing using small interfering RNAs. MDA-MB-435 cells (2.5×10^5 cells/well) were plated on six-well plates and transiently transfected with 50 nmol/L of Smartpool small interfering RNAs (siRNA) targeting human survivin (Dharmacon) or a nonsilencing siRNA targeting luciferase (Dharmacon NS2) using OligofectAMINE reagent (Invitrogen) according to the manufacturer's instructions. Survivin levels were determined by immunoblotting at various time points after transfection. For apoptosis experiments, the medium was changed 72 h after siRNA transfection, and cells were treated with vehicle, 2.5 µg/mL of TRAIL or 2.5 µg/mL of lexatumumab for 16 h. Apoptotic nuclei were scored as described earlier (Induction and scoring of apoptosis).

Orthotopic breast cancer xenograft experiments. Pieces of human MDA-MB-435 breast carcinoma xenografts (1 mm³) were implanted subcutaneously into both mammary fat pads of 4- to 5-week old female athymic *nu/nu* mice (Harlan Sprague-Dawley) as described (23). Three weeks later, mice (10 per group) were randomly assigned to one of six treatment groups: vehicle (0.75% methylcellulose; Sigma-Aldrich), ASA 100 mg/kg/d by oral gavage, ASA 400 mg/kg/d by oral gavage, TRAIL 10 mg/kg/d i.p., ASA 100 mg/kg/d orally + TRAIL 10 mg/kg/d i.p., or ASA 400 mg/kg/d orally + TRAIL 10 mg/kg/d i.p. For these experiments, ASA was ground into a fine powder in a mortar and pestle, suspended in 0.75% methylcellulose, and administered to mice by oral gavage. Mice were treated for 3 weeks, and tumor volume was measured weekly as described (23). The induction of apoptosis in formalin-fixed, paraffin-embedded breast cancer xenografts was measured by terminal nucleotidyl transferase-mediated nick end labeling (TUNEL) assay (In Situ Cell Death Detection kit, TMR Red; Roche). These experiments were approved by the Animal Care and Use Committee of Northwestern University.

Statistical analysis. Statistical significance was determined by ANOVAs with Bonferroni posttests or two-tailed unpaired *t* tests with Welch's correction (xenograft experiments) using Prism 4 (GraphPad Software). *P* < 0.05 was considered statistically significant.

Results

Aspirin promotes TRAIL-induced apoptosis of human breast carcinoma cells in vitro. To determine whether ASA sensitizes breast cancer cells to TRAIL, estrogen receptor (ER)-negative human MDA-MB-435 breast cancer cells were preincubated with ASA (0-5 mmol/L) for 48 hours and then treated with TRAIL (0-2.5 µg/mL) for 16 hours. Consistent with our previous findings (23), MDA-MB-435 cells were resistant to TRAIL-induced apoptosis when this cytokine was used as a single agent at concentrations as high as 2.5 µg/mL (Fig. 1A, left). MDA-MB-435 cells were also highly resistant to ASA alone at concentrations up to 5 mmol/L. Strikingly, ASA (at 5 mmol/L but not at 1 mmol/L) robustly sensitized MDA-MB-435 cells to TRAIL-induced apoptosis. Synergistic effects of 5 mmol/L ASA were observed at concentrations of TRAIL as low as 0.1 µg/mL, and the

combination of 5 mmol/L of ASA and 2.5 µg/mL of TRAIL resulted in >70% of cells undergoing apoptosis. Preincubation with ASA was essential and shorter preincubation times were associated with less robust sensitization to TRAIL-induced apoptosis (data not shown). Flow cytometry-based Annexin V labeling of apoptotic cells revealed comparable findings: MDA-MB-435 cells were resistant to TRAIL or ASA alone, but were sensitive to the combined treatment (Fig. 1A, right). ASA also dramatically promoted TRAIL-induced apoptosis of ER-positive T47D breast cancer cells, which similar to MDA-MB-435 cells, were resistant to either agent alone (Fig. 1B, left). In contrast, the combination of 5 mmol/L of ASA and 2.5 µg/mL of TRAIL induced minimal apoptosis in untransformed HMECs (Fig. 1B, right), suggesting that ASA may not abrogate the potential tumor selectivity of TRAIL. Collectively, these results indicate that high-dose ASA sensitizes ER-negative and ER-positive breast cancer cells, but not untransformed HMECs, to TRAIL-induced apoptosis *in vitro*.

We next examined whether ASA sensitized breast cancer cells to apoptosis induced by human agonistic mAbs targeting TRAIL-R1 (mapatumumab) or TRAIL-R2 (lexatumumab; refs. 13-15). To this end, human T47D breast cancer cells were preincubated with vehicle or 5 mmol/L of ASA for 48 hours and then treated with mapatumumab (0 or 2.5 µg/mL), lexatumumab (0 or 2.5 µg/mL), or both mAbs (0 or 2.5 µg/mL each). T47D cells were resistant to mapatumumab (abbreviated "Mapa") or lexatumumab (abbreviated "Lexa") alone or in combination and to ASA alone (Fig. 1C). However, ASA preincubation resulted in a striking sensitization of these cells to lexatumumab-induced apoptosis, inducing levels of cell death comparable to the combination of ASA and TRAIL (Fig. 1B, left). In contrast, ASA did not sensitize T47D cells to mapatumumab-induced apoptosis (Fig. 1C), even though these cells express TRAIL-R1 (data not shown). Similar results were obtained in MDA-MB-435 cells (data not shown). Moreover, the addition of mapatumumab did not significantly increase apoptosis induction by ASA and lexatumumab. Taken together, these results indicate that ASA sensitizes breast cancer cells to apoptosis induced by an agonistic mAb targeting TRAIL-R2.

To determine whether the TRAIL-sensitizing effects of ASA were mediated by COX-2 inhibition, we preincubated COX-2-deficient HCT116 colon carcinoma cells with vehicle or 5 mmol/L of ASA for 48 hours and then treated cells with TRAIL (0-0.5 µg/mL) for 16 hours. Although HCT116 cells were partially sensitive to TRAIL alone, ASA dramatically enhanced TRAIL-induced apoptosis in these COX-2-deficient cells (Fig. 1D). These findings show that the TRAIL-sensitizing actions of ASA are COX-2-independent.

Aspirin induces G₁ cell cycle arrest and cooperates with TRAIL to reduce long-term cell survival. Because several drugs which induce G₁ cell cycle arrest have been shown to promote TRAIL-induced apoptosis, and aspirin has been reported to trigger G₁ arrest (23, 25, 33), we postulated that ASA might sensitize breast cancer cells to TRAIL by this mechanism. Treatment of MDA-MB-435 cells with 5 mmol/L of ASA for 48 hours resulted in a robust G₁ arrest, whereas 1 mmol/L of ASA did not affect cell cycle distribution (Fig. 2A) or promote TRAIL-induced apoptosis (Fig. 1A). Similar results were observed in T47D cells (data not shown). To determine whether the enhanced apoptosis observed by the combination of ASA and TRAIL resulted in greater long-term reductions in cell survival than treatment with either agent alone, we

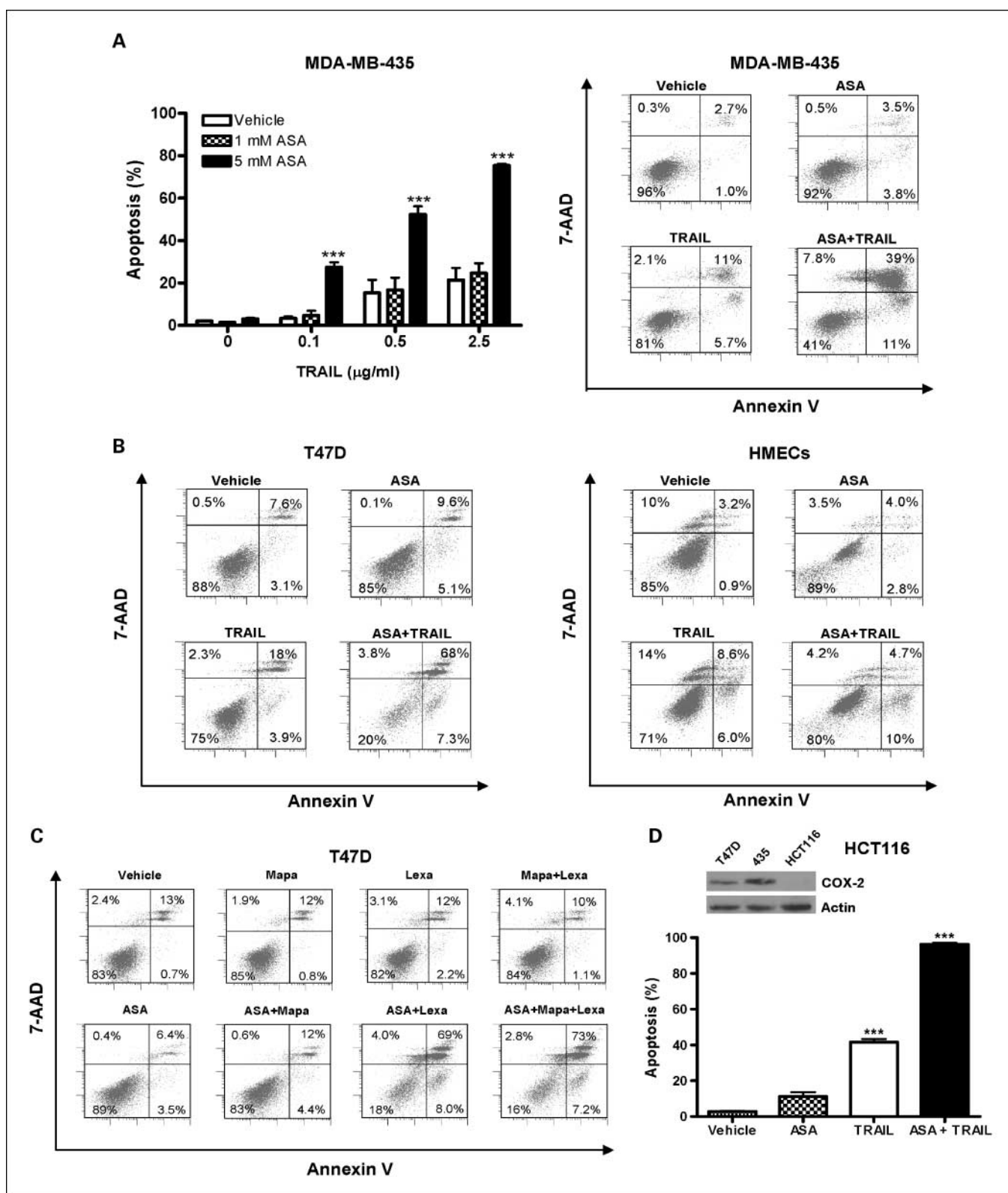


Fig. 1. Aspirin promotes TRAIL-induced apoptosis of human breast cancer cells, but not HMECs, *in vitro*. **A**, MDA-MB-435 breast carcinoma cells were preincubated with vehicle or ASA (1 or 5 mmol/L) for 48 h and then treated with TRAIL (0–2.5 µg/mL) for 16 h (left). Apoptotic nuclei were scored (columns, mean; bars, SE; $n = 3$). ***, $P < 0.001$ vs. vehicle-treated cells. **A**, MDA-MB-435 cells (right); **B**, T47D breast cancer cells (left) or HMECs (right) were preincubated with vehicle or 5 mmol/L of ASA for 48 h and then treated with TRAIL (0 or 2.5 µg/mL) for 16 h. Apoptosis was measured by Annexin V labeling. **C**, T47D cells were pretreated with vehicle or 5 mmol/L of ASA for 48 h and then treated with the TRAIL-R1 agonistic mAb mapatumumab (Mapa, 0 or 2.5 µg/mL), the TRAIL-R2 agonistic mAb lexatumumab (Lexa, 0 or 2.5 µg/mL), or both mAbs (0 or 2.5 µg/mL each) for 16 h. Apoptosis was measured by Annexin V labeling. **D**, ASA sensitizes COX-2-deficient HCT116 colon cancer cells to TRAIL-induced apoptosis. HCT116 cells were pretreated with ASA (0–5 mmol/L) for 48 h and then treated with TRAIL (0–0.5 µg/mL) for 16 h. Apoptotic nuclei were scored (columns, mean; bars, SE; $n = 3$). ***, $P < 0.001$ vs. vehicle-treated cells.

preincubated T47D cells with vehicle or 5 mmol/L of ASA for 48 hours, treated them with vehicle or 2.5 $\mu\text{g}/\text{mL}$ of TRAIL for 16 hours, washed the cells, and grew them for 5 additional days in the absence of drugs. Under these conditions, cells treated with vehicle, ASA alone, or TRAIL alone remained viable and continued to grow following the removal of these agents (Fig. 2B). In contrast, the combination of ASA and TRAIL led to the virtual elimination of all viable cells, indicating that the TRAIL-sensitizing actions of ASA are accompanied by robust long-term reductions in cell survival.

Aspirin promotes TRAIL-induced caspase activation and reduces survivin protein levels. To examine whether ASA promotes TRAIL-induced caspase activation, we incubated MDA-MB-435 breast cancer cells with vehicle control (C) or 5 mmol/L of ASA for 64 hours, 2.5 $\mu\text{g}/\text{mL}$ of TRAIL for 16 hours, or 5 mmol/L of ASA for 48 hours followed by 2.5 $\mu\text{g}/\text{mL}$ of TRAIL for 16 hours. Combined treatment with ASA and TRAIL induced proteolytic cleavage of procaspase-8 and procaspase-3 and the caspase substrates BID and PARP (detected by diminished intensity of each full-length protein; Fig. 3A). In contrast, little caspase activation or caspase substrate cleavage was observed in cells treated with either ASA or TRAIL alone. To elucidate the mechanisms by which ASA promotes TRAIL-induced caspase activation, we examined the effects of treating MDA-MB-435 cells with 5 mmol/L of ASA for 64 hours on the expression of DISC proteins (Fig. 3B) or apoptosis regulators (Fig. 3C) by immunoblotting. Although ASA had minimal to no effect on the expression levels of the vast majority of these proteins, ASA treatment profoundly reduced the levels of survivin, an antiapoptotic protein previously implicated as a mediator of TRAIL-resistance in diverse cancers (23, 25, 27, 36). Importantly, 1 mmol/L of ASA did not reduce survivin levels (Fig. 3D), induce G_1 arrest (Fig. 2A), or promote TRAIL-induced apoptosis (Fig. 1A). Moreover, the time course of survivin depletion by 5 mmol/L of ASA (Fig. 3D) coincided closely with its TRAIL-sensitizing effects: maximal survivin depletion and TRAIL-sensitization were observed after prolonged exposure (64 hours) to ASA. These results suggest that the TRAIL-sensitizing effects of ASA might be due, at least in part, to the observed reduction in survivin protein levels.

Aspirin does not affect survivin mRNA levels but induces its proteasomal degradation. To determine the mechanisms by which ASA reduces survivin protein levels, we first examined the effect of ASA on survivin gene expression by real-time reverse transcription-PCR. Treatment of MDA-MB-435 cells with 5 mmol/L of ASA for 64 hours did not significantly affect survivin mRNA levels compared with vehicle-treated cells (Fig. 4A). However, the reduction in survivin protein levels induced by ASA was suppressed by the proteasome inhibitor epoxomicin (41) in a dose-dependent manner, with maximal suppression by 200 nmol/L of epoxomicin (Fig. 4B). These results indicate that ASA regulates survivin protein levels by a posttranscriptional mechanism that requires the proteasome.

Silencing survivin promotes TRAIL-induced apoptosis in vitro. To examine the specific contribution of survivin to the TRAIL-sensitizing actions of ASA, we selectively inhibited the expression of survivin by RNA interference using siRNAs. MDA-MB-435 cells were transiently transfected with siRNAs targeting human survivin or a nonsilencing siRNA. Survivin

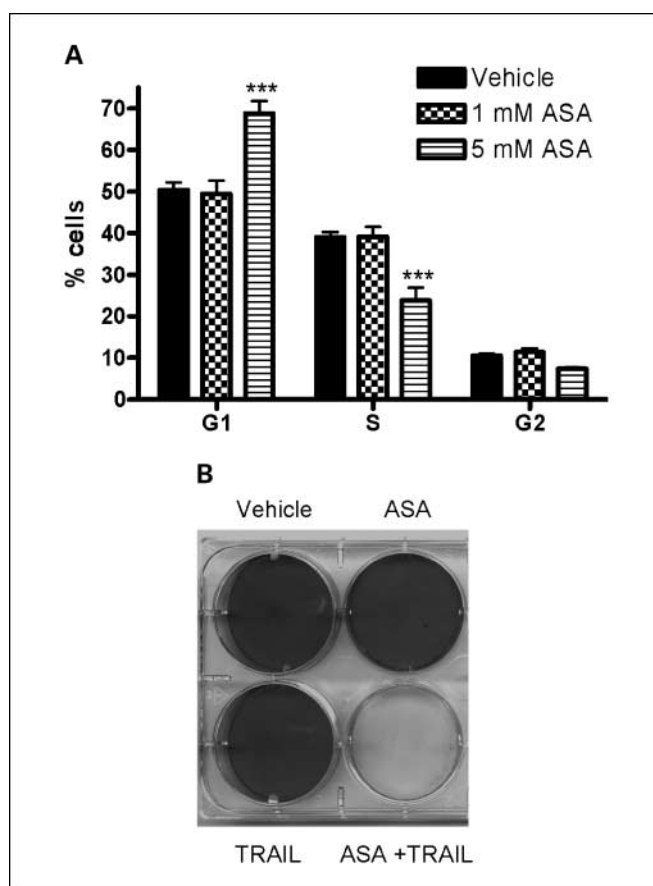


Fig. 2. Aspirin induces G_1 cell cycle arrest and cooperates with TRAIL to reduce long-term cell survival. **A**, MDA-MB-435 cells were treated with vehicle or ASA (1 or 5 mmol/L) for 48 h. The cell cycle distribution of treated cells was determined by flow cytometry – based analysis of DNA content (columns, mean; bars, SE; $n = 3$). ***, $P < 0.001$ vs. vehicle-treated cells. **B**, T47D cells were preincubated with vehicle or 5 mmol/L of ASA for 48 h, and then treated with vehicle or 2.5 $\mu\text{g}/\text{mL}$ of TRAIL for 16 h. Cells were washed and cultured for 5 additional days in the absence of drugs. Viable cells were stained with crystal violet. Representative of three independent experiments.

siRNAs markedly reduced the expression of survivin, whereas the nonsilencing siRNA had no effect on survivin levels (Fig. 5A). Moreover, silencing survivin sensitized MDA-MB-435 cells to TRAIL- or lexatumumab-induced apoptosis, but did not induce apoptosis in the absence of TRAIL agonists (Fig. 5B). Stable silencing of survivin using short hairpin RNAs also sensitized breast cancer cells to TRAIL-induced apoptosis (data not shown). These results indicate that survivin negatively regulates TRAIL agonist-induced apoptosis and that the TRAIL-sensitizing effects of ASA are partly mediated by the observed reduction of survivin levels.

Aspirin sensitizes human breast xenograft tumors to TRAIL in vivo. To determine the antitumor efficacy of ASA and TRAIL *in vivo*, we treated female athymic nude mice with orthotopic MDA-MB-435 xenograft tumors with vehicle, ASA alone (100 or 400 mg/kg/d by oral gavage), TRAIL alone (10 mg/kg/d i.p.), or ASA (100 or 400 mg/kg/d) and TRAIL (10 mg/kg/d) for 3 weeks. Neither ASA nor TRAIL alone suppressed tumor growth compared with vehicle-treated mice (Fig. 6A). In contrast, the combination of high-dose ASA (400 mg/kg/d) and TRAIL robustly inhibited tumor growth,

whereas low-dose ASA (100 mg/kg/d) and TRAIL did not significantly affect tumor growth. High-dose ASA also sensitized xenograft tumors to TRAIL-induced apoptosis as determined by TUNEL staining (Fig. 6B). Moreover, high-dose ASA and TRAIL treatment reduced survivin levels in the breast xenograft tumors compared with the levels observed in vehicle-treated mice (Fig. 6C), consistent with the observed *in vitro* effects of ASA. Taken together, these findings indicate that ASA promotes TRAIL-induced apoptosis *in vivo*, thereby suggesting that this combination may be an effective therapy for breast cancer.

Discussion

Most human breast carcinoma cell lines are highly resistant to TRAIL-induced apoptosis even though they express its death receptors, TRAIL-R1 and TRAIL-R2 (16, 38), indicating that TRAIL-resistance in breast cancer is likely mediated by defects in the TRAIL signaling pathway downstream of death receptor activation. From a therapeutic standpoint, the high prevalence of TRAIL-resistance points to the need to identify agents which

sensitize breast tumors to TRAIL-induced apoptosis without compromising the tumor selectivity and limited systemic toxicity of TRAIL. We have shown that ASA is a TRAIL-sensitizing agent *in vitro* and *in vivo* that fulfills many of these criteria. Specifically, we have shown that ASA (5 mmol/L) sensitizes ER-positive T47D and ER-negative MDA-MB-435 breast cancer cells, but not normal HMECs, to TRAIL-induced apoptosis. Both T47D and MDA-MB-435 breast cancer cells harbor a mutation in *TP53* (42, 43), indicating that the combination of ASA and TRAIL is effective against cancer cells with defects in p53-dependent apoptosis. Moreover, we show that ASA sensitizes breast cancer cells to lexatumumab, a fully human agonistic mAb targeting TRAIL-R2 (13, 15). Intriguingly, we observed that ASA does not promote apoptosis induced by an agonistic antibody targeting TRAIL-R1 (mapatumumab) even though the breast cancer cell lines used in this study express TRAIL-R1. These findings suggest that TRAIL-R2 is the principal death receptor used by TRAIL agonists in breast cancer cells sensitized to these agents by ASA. The molecular mechanisms underlying the observed differential effect of ASA on TRAIL-R1- and TRAIL-R2-dependent apoptosis are

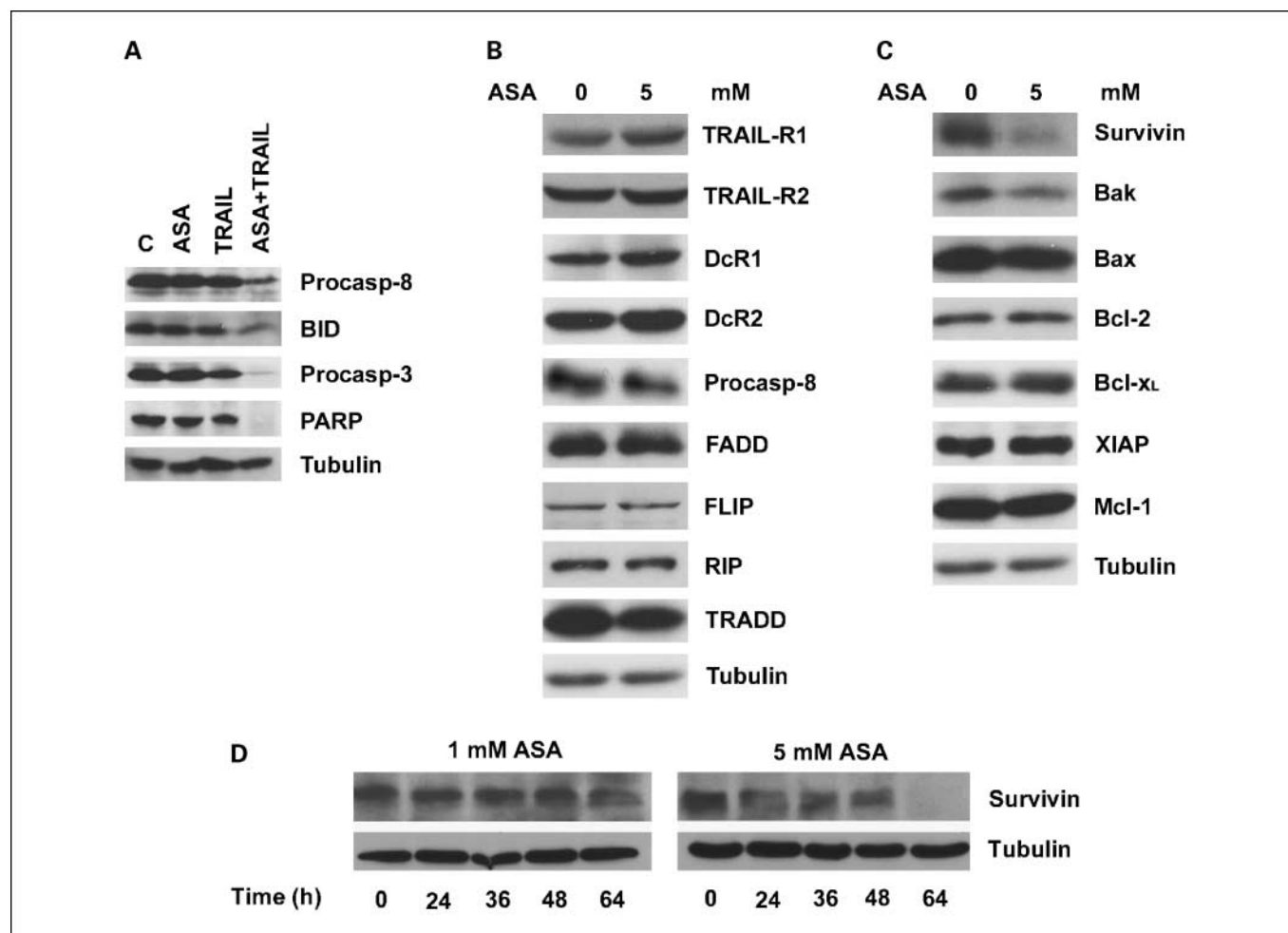


Fig. 3. Aspirin promotes TRAIL-induced caspase activation and selectively reduces survivin protein levels. *A*, MDA-MB-435 cells were treated with vehicle control (*C*), 5 mmol/L of ASA alone for 64 h, 2.5 μ g/mL of TRAIL alone for 16 h, or 5 mmol/L of ASA for 48 h followed by 2.5 μ g/mL of TRAIL for 16 h. Procaspase-8, BID, procaspase-3, and PARP were detected by immunoblotting. Tubulin was used as a control for protein loading. *B* and *C*, MDA-MB-435 cells were treated with ASA (0 or 5 mmol/L) for 64 h and the expression levels of DISC proteins (*B*) or other apoptosis regulators (*C*) were measured by immunoblotting. *D*, MDA-MB-435 cells were treated with ASA (1 or 5 mmol/L) for the indicated number of hours, and survivin levels were determined by immunoblotting.

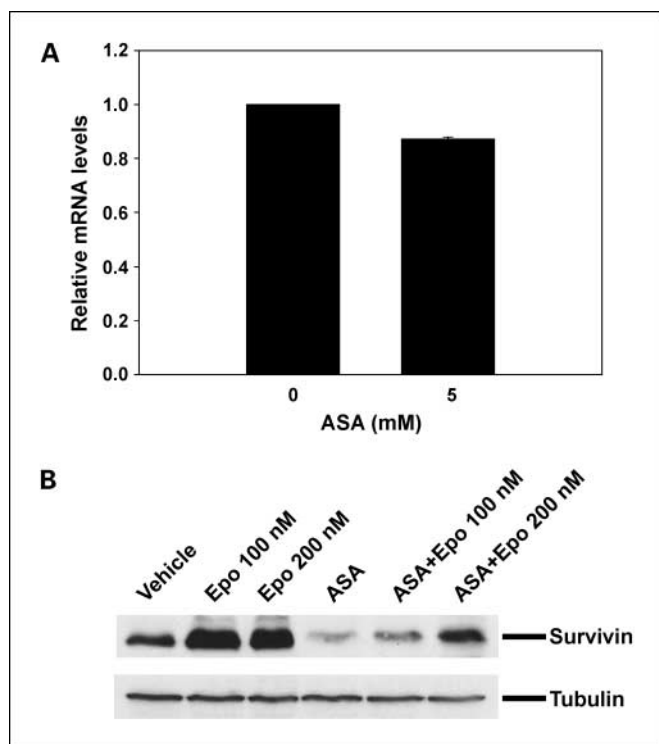


Fig. 4. Aspirin reduces survivin protein levels by inducing its proteasomal degradation. **A**, MDA-MB-435 cells were treated with vehicle or 5 mmol/L of ASA for 64 h. Survivin mRNA levels were measured by quantitative real-time reverse transcription-PCR. Columns, mean of survivin mRNA levels relative to vehicle-treated cells; bars, SE ($n = 3$). **B**, MDA-MB-435 cells were preincubated with vehicle or 5 mmol/L of ASA for 48 h, and then treated with the proteasome inhibitor epoxomicin (0–200 nmol/L) for 16 h. Survivin expression was examined by immunoblotting.

unclear. ASA does not affect the expression level of TRAIL-R2, but may affect its localization, posttranslational modification, or other specific components of the TRAIL-R2 signaling complex. Although ASA was recently reported to sensitize prostate and colon cancer cells to TRAIL *in vitro* (35), we have shown for the first time that ASA promotes TRAIL-induced apoptosis and inhibits xenograft tumor growth in mice, thereby providing critical *in vivo* proof of concept data supporting this novel combination cancer therapy. Importantly, the doses of ASA used in our xenograft studies (up to 400 mg/kg/d) suppress intestinal and mammary tumors when given to mice for their entire life span (44), confirming the feasibility of this dosing even for prolonged periods. Collectively, our results indicate that ASA selectively sensitizes transformed cells to TRAIL-based therapies *in vitro* and *in vivo* by a TRAIL-R2-dependent mechanism, and they suggest that this combination may be an effective cancer therapy that warrants additional study.

We have also shown that ASA sensitizes breast cancer cells to TRAIL-induced caspase activation and apoptosis at least in part by a mechanism involving G_1 cell cycle arrest and survivin depletion. Specifically, we have shown that ASA concentrations (5 mmol/L but not 1 mmol/L) which sensitize cancer cells to TRAIL also induce G_1 cell cycle arrest and robustly reduce the protein levels of survivin, but do not significantly affect the expression levels of many other apoptosis regulators or DISC components. Importantly, the TRAIL-sensitizing effects of ASA against breast xenograft

tumors *in vivo* were also accompanied by a reduction in survivin levels. Survivin expression is cell cycle-dependent, with the highest levels at G_2 -M, followed by a rapid reduction in survivin levels at G_1 mediated in part by proteasomal degradation of survivin (45). We observed that ASA does not affect survivin mRNA levels but acts by a posttranscriptional proteasome-dependent mechanism to reduce survivin protein levels. Hence, our results suggest that ASA reduces survivin levels by inducing G_1 arrest, which in turn, triggers the cell cycle-dependent proteasomal degradation of survivin (33, 45). The functional relevance of ASA-induced survivin degradation for its TRAIL-sensitizing actions is underscored by our observation that silencing survivin promotes TRAIL- or lexatumumab-induced apoptosis, albeit less dramatically than ASA treatment. This latter discrepancy may reflect the more modest reduction of survivin levels by RNA interference than by ASA treatment and/or the potential effects of ASA on other yet to be identified apoptosis regulators. Nevertheless, our findings provide unequivocal evidence that survivin is a negative regulator of TRAIL agonist-induced apoptosis and a functionally important target of ASA's TRAIL-sensitizing actions. These results are consistent with prior published reports from our group and others demonstrating that survivin confers protection against TRAIL and that suppressing survivin expression by a variety of strategies promotes TRAIL-induced apoptosis (23, 25, 27). Although the precise mechanism(s) by which survivin inhibits apoptosis remains controversial, recent work suggests that survivin sequesters Smac and/or procaspase-9, thereby suppressing caspase-3 activation (46, 47). In addition, survivin binds and stabilizes XIAP, which inhibits caspase-9 activation (48). Because

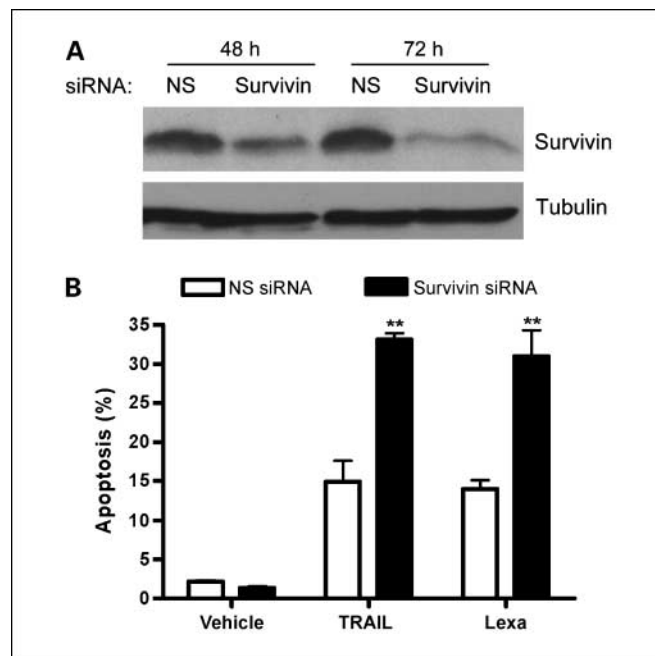
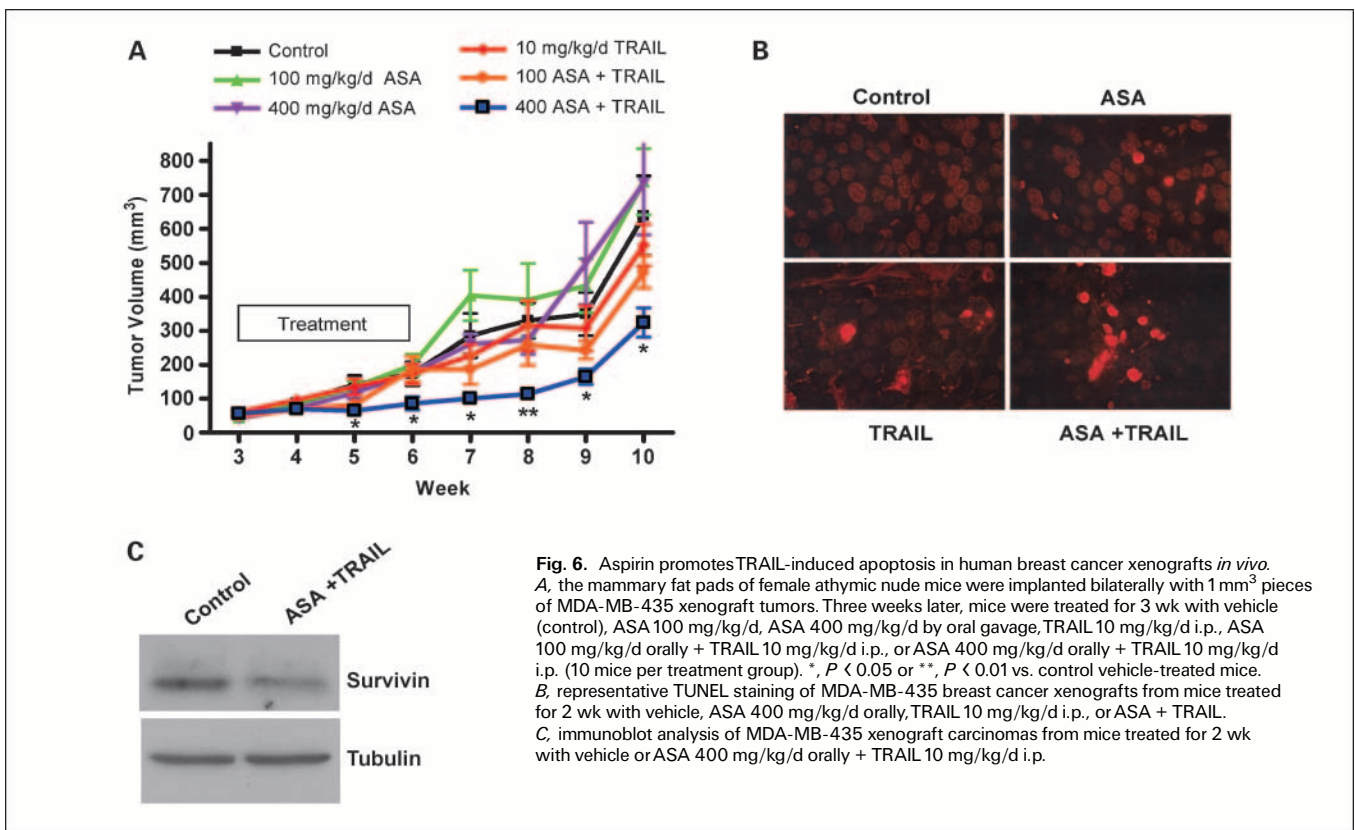


Fig. 5. Silencing survivin promotes TRAIL-induced apoptosis *in vitro*. **A**, MDA-MB-435 cells were transiently transfected with siRNAs targeting human survivin or a nonsilencing (NS) siRNA. Survivin levels were determined by immunoblotting at 48 and 72 h after transfection. **B**, MDA-MB-435 cells were transfected as in (A). Seventy-two hours later, cells were treated with vehicle, 2.5 μ g/mL of TRAIL, or 2.5 μ g/mL of lexatumumab (Lexa) for 16 h, and apoptotic nuclei were scored (columns, mean; bars, SE; $n = 3$). **, $P < 0.01$ vs. vehicle-treated cells.



the intrinsic apoptotic pathway plays an essential role in TRAIL-induced apoptosis in some cell types (6, 7), the inhibition of this pathway by survivin likely renders these cells resistant to TRAIL. Taken together, our findings indicate that the TRAIL-sensitizing effects of ASA are mediated at least in part by the proteasomal degradation of survivin.

As noted, Kim et al. have shown that ASA (0.1-10 mmol/L) promotes TRAIL-induced apoptosis in prostate and colon cancer cells *in vitro* by repressing *Bcl-2* gene expression by a COX-2-independent mechanism (35). However, we did not observe significant changes in the expression of any *Bcl-2* family member, including *Bcl-2*, after treating breast cancer cells with 5 mmol/L of ASA for 64 hours, whereas Kim et al. did not examine survivin levels. This disparity may reflect differences in the duration of ASA treatment (64 hours versus 20-24 hours in the Kim et al. study) or in the cell types examined. Intriguingly, a very recent publication from the same group also implicated survivin depletion in TRAIL-sensitization by ASA in prostate cancer cells, although ASA repressed survivin expression by a transcriptional mechanism (49). It is entirely plausible that the TRAIL-sensitizing actions of ASA may reflect the effects of ASA on the expression of multiple apoptosis regulators, including survivin, *Bcl-2*, and potentially others. In agreement with Kim et al., our findings suggest that the TRAIL-sensitizing actions of ASA are independent of COX-2 inhibition because ASA promotes TRAIL-induced apoptosis in COX-2-deficient HCT116 colon cancer cells. Indeed, Kim et al. showed that silencing COX-2 had no effect on TRAIL-sensitization by ASA (35). Consistent with these findings, the TRAIL-sensitizing actions of COX-2

inhibitors have been reported to be an off-target effect of these drugs that is independent of COX-2 inhibition (50).

In summary, this report provides the first *in vivo* proof of concept data demonstrating the efficacy of the combination of ASA and TRAIL to reduce tumor burden in mice that reflects ASA's ability to promote TRAIL-induced apoptosis *in vivo*. We also show that the TRAIL-sensitizing effects of ASA are tumor-specific, p53-independent, and mediated by the proteasome-dependent degradation of survivin, a key negative regulator of TRAIL-induced apoptosis. Indeed, several other TRAIL-sensitizing agents with different molecular targets, including cyclin-dependent kinase inhibitors, PPAR γ ligands, and resveratrol promote TRAIL-induced apoptosis at least in part by reducing survivin levels (23, 25, 27), thereby underscoring the importance of overcoming the antiapoptotic blockade imposed by survivin. Finally, we have shown that ASA treatment or survivin silencing also sensitizes breast cancer cells to apoptosis induced by an agonistic antibody targeting TRAIL-R2 but not TRAIL-R1, suggesting that this fully human TRAIL-R2 mAb (lexatumumab) may be a viable therapeutic alternative to TRAIL in treating cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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