Down-regulation of c-FLIP Enhances Death of Cancer Cells by Smac Mimetic Compound

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Abstract

Smac mimetic compounds (SMC) are novel small molecules being developed for cancer therapy. The mechanism of SMC-induced sensitivity in cancer cells depends on autocrine release of tumor necrosis factor (TNF)α; however, potential mechanisms of resistance remain unknown. Here, we investigated the molecular profile and cytotoxic responsiveness of a diverse panel of 51 cancer cell lines to combinations of a dimeric SMC (AEG40730), death ligand TNFα, and tumor necrosis factor-related apoptosis-inducing ligand. Synergy was seen in combination with death receptor agonists in some cells, although single-agent activity was limited to a few sensitive lines. Unexpectedly, the majority of cell lines resistant to combinations of SMC-AEG40730 and death ligands expressed caspase-8, FADD, RIP1, and ligand receptors necessary for apoptosis execution. Furthermore, TNFα-mediated ubiquitination of RIP1 was repressed by SMC-AEG40730 treatment, leading to the formation of the proapoptotic complex II. However, in resistant cancer cells, SMC-AEG40730 repressed TNFα-mediated c-jun-NH2-kinase activation and the levels of caspase-8 inhibitor c-FLIP were persistently elevated, in contrast to SMC-responsive cancer cells. Importantly, the silencing of c-FLIP restored SMC sensitivity in previously resistant cancer cells by allowing ligand-mediated activation of caspase-8 and caspase-3 to proceed. Together, these results provide mechanistic insight into the action of SMCs, demonstrating that the deciphering of the relevant molecular signature in cancer cells leads to the prediction of cancer cell responsiveness to SMC treatment. Furthermore, a majority of resistant cancer cells were sensitized to SMC-AEG40730 and TNFα by down-regulating c-FLIP, suggesting novel approaches in the use of SMCs and c-FLIP antagonists in treating cancer.

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Introduction

Smac mimetic compounds (SMC) are a class of small-molecule therapeutics currently in clinical trials for cancer (1). The targets of SMCs are the inhibitor of apoptosis (IAP) proteins that include cIAP1, cIAP2, and XIAP, all of which possess three baculoviral IAP repeat (BIR) domains and a ubiquitin E3 ligase RING domain. The divergent structures of SMCs originate from the conserved AVPI tetrapeptide NH2-terminal sequence of Smac that binds to the BIR domains of IAPs. When this feature was incorporated into a dimeric format, remarkable increases in binding potency were seen (2).

A common mode of action of SMCs involves the rapid activation of the ubiquitin ligase of cIAP1 and cIAP2, which leads to autoubiquitination and proteosomal degradation of these cIAPs (3–8). The loss of cIAPs allows the accumulation of their substrate NIK, an activator of the alternative NF-κB pathway (5, 9). In parallel, the absence of cIAPs facilitates the recruitment of RIP1 to the tumor necrosis factor receptor 1 (TNF-R1), promoting the activation of the classic NF-κB pathway (4). The induction of these NF-κB pathways leads to TNFα production. However, with cIAPs depleted, TNFα cannot promote RIP1 ubiquitination (3, 10, 11), and, as a consequence, RIP1 is released from the TNF-R1 complex. Subsequently, the death-inducing complex II containing RIP1, FADD, and caspase-8 is formed, activating the extrinsic apoptotic pathway (12).

Similar to complex II, a death-inducing signaling complex (DISC) that contains TRADD, FADD, and caspase-8 can also promote apoptosis. The activation of DISC requires the down-regulation of c-FLIP, an enzymatically dead caspase-8 homologue that competes for caspase-8 binding to FADD (13, 14). The down-regulation of c-FLIP can be achieved through c-jun-NH2-kinase (JNK)–mediated activation of the ubiquitin-proteasome pathway (15). Alternatively, c-FLIP can also be silenced by inhibiting the NF-κB signaling pathway. Paradoxically, the inhibitory role of c-FLIP toward caspase-8 is in conflict to the current model of SMC mode of action, which suggests that endogenous c-FLIP does not block complex II–mediated caspase-8 activation in SMC-sensitive cells (12).

In spite of the potential of this novel class of therapeutics, SMCs as single agents are effective only in a small percentage of cancer cell lines tested to date, with enhanced activity being seen in the presence of an apoptotic trigger engaging the extrinsic death receptor pathway (16). As part of the immune surveillance system, death receptor ligands such as TNFα and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) are expressed by immune cells that can eliminate cancerous cells by triggering extrinsic apoptosis through the death receptors expressed on cancer cells (17, 18). In the presence of either TNFα or TRAIL, apoptosis induction by SMCs is promoted synergistically (2). The extent of synergy, however, has not been extensively tested. Moreover, as most cancer cell lines are refractory to SMC-mediated apoptosis, delineating the molecular basis for resistance to TNFα and TRAIL may also facilitate the identification of complementary therapeutic strategies as apoptotic triggers for SMCs.

In this study, we investigated the mechanism of SMC resistance using a prototypic SMC of the dimeric class, AEG40730, which displays potent low nanomolar binding to the BIR3 domains of XIAP, cIAP1, and cIAP2 (3). In common with other SMCs, AEG40730 causes ubiquitin-mediated loss of cIAP1 and cIAP2; however, AEG40730, as well as SM-164, also induces the loss of XIAP when higher
concentrations are used at longer exposure periods (3–5, 7). We analyzed a diverse panel of cancer cell lines for their cytotoxic responsiveness to SMC-AEG40730, TNFα, and TRAIL. We show that in this panel, a majority of cancer cell lines are resistant to SMC-AEG40730 alone, but about half of the panel is responsive when cotreated with TNFα or TRAIL. Interestingly, components of the machinery necessary for TNFα- or TRAIL-mediated apoptosis seem to be present in most of the SMC-resistant cancer cells. Intriguingly, in resistant cancer cells, SMC-AEG40730 represses the TNFα-mediated activation of JNK and the level of c-FLIP persists. The knockdown of c-FLIP restores the ability of SMC-AEG40730 to promote apoptosis in these formerly resistant cancer cells, demonstrating...
that endogenous c-FLIP blocks SMC-induced caspase-8 activation. Therefore, to the extent that SMC-AEG40730 is representative of the mechanism of SMC-induced killing, the molecular profiling of the extrinsic apoptosis pathway predicts SMC responsiveness in cancer cells. Moreover, suppression of c-FLIP expression, in combination with SMC treatment, warrants further investigation as it has the potential of increasing the scope and efficacy of this experimental cancer therapy.

Materials and Methods

Reagents. SMC AEG40730 as previously described (3) was synthesized by Vibrant Pharma, Inc. Recombinant human TNFα and soluble TRAIL were obtained from BIOMOL International.

Transfection of small interfering RNA. Annealed double-stranded ON-TARGETplus SMARTpool small interfering RNA (siRNA) for c-FLIP (GUGCCGGGAUGUUGCUAUA, CAAGCAGUCUGUUCAAGGA, CAUG-GUAAUCCCAAGAUCCUAGUAUCCGUAUA, CCAAGAACUGCCUGUAUA) and nontargeting Accell SMARTpool were purchased from Dharmacon. Cells were forward or reversed transfected at the indicated concentration for each siRNA using DharmaFECT I reagent (Dharmacon) according to the manufacturer's protocol.

Protein preparation and immunoprecipitation. Protein samples were prepared as described before (19). Protein content was determined by Bio-Rad Protein Assay using bovine serum albumin as a standard. For endogenous caspase-8 immunoprecipitation, –1 × 10⁷ cells were washed twice with PBS and lysed in a buffer supplemented with 10% glycerol on ice for 15 min. Endogenous caspase-8 complex was immunoprecipitated overnight at 4°C with 4 μg of antibody, and complexes were recovered with 40 μL Protein G-agarose. For endogenous TNF-R1 complex immunoprecipitation, 1 × 10⁷ cells were washed twice with PBS and lysed in a buffer [20 mmol/L Tris-HCl (pH 7.5), containing 0.2% NP10, 10% glycerol and 150 mmol/L NaCl] on ice for 15 min. The TNF-R1 complex was immunoprecipitated (anti–TNF-R1, R&D Systems) overnight at 4°C with 1 μg of antibody, and complexes were recovered with...
Results

The majority of cancer cell lines are resistant to the SMC AEG40730 as a single agent. We initially tested SMC-AEG40730 on a known responsive breast carcinoma cell line, MDA-MB-231, and four previously untested neuroblastoma cell lines, Lan-1, Lan-5, SH-SY5Y, and SK-N-BE(2). Although MDA-MB-231 cells responded robustly to SMC-AEG40730 with an IC50 of 1.32 nmol/L, the four neuroblastoma cell lines failed to respond to SMC-AEG40730 even at 2 μmol/L (Supplementary Fig. S1).

From these initial analyses, we chose 100 nmol/L as the standard SMC-AEG40730 concentration for subsequent investigations. Our preliminary findings suggested that there might be a wide range of SMC-AEG40730 responsiveness in a spectrum of cancer cells. In addition to standalone efficacy, SMCs also potently synergize with TNFα and TRAIL (2, 3). We therefore screened a diverse panel of cancer cells, including glioblastomas, lung, neuroblastoma, ovarian, and pancreatic cancer cell lines, for a measure of cytotoxic responsiveness at 24 hours to fixed dose combinations of SMC-AEG40730 and TNFα or TRAIL.
TRAIL (Supplementary Fig. S2A–E). Of the 51 cancer cell lines examined, 4% were sensitive (viability <50%), 6% were intermediate responders (viability 50–75%), and 90% were resistant (viability >75%) to SMC-AEG40730 alone (Supplementary Fig. S2A). None of the cell lines examined was sensitive to TNFα alone, and 20% were sensitive to TRAIL (Supplementary Fig. S2B and C). When cell lines were treated with the combination of SMC-AEG40730 and TNFα, or TRAIL, the sensitive populations increased to 48% and 55%, respectively (Supplementary Fig. S2D and E). TNFα and TRAIL are triggers for the extrinsic apoptotic pathway, and,

Figure 4. Processing and down-regulation of c-FLIP in Panc1 cells in response to SMC-AEG40730 and TNFα. Panc1 (A), A2780-CP (B), A549 (C), and U343 (D) cells were treated with a vehicle or 100 nmol/L SMC-AEG40730 for 24 h, and then these cells were exposed to 100 ng/mL TNFα. At the indicated times, cells were harvested and subjected to Western immunoblotting with antibodies recognizing c-FLIP, cIAP1, cIAP2, and XIAP. β-Actin was used as a loading control. In SMC-treated Panc1, c-FLIP was first processed to its p43 form and then degraded. In A2780-CP, A549, and U343 cells, c-FLIP level remains constant.
as expected, cell lines that were responsive to cotreatment of SMC-AEG40730 and TNFα also tended to respond to SMC-AEG40730 in the presence of TRAIL. As such, 22 of the 24 cell lines that were sensitized by SMC-AEG40730 to exogenous TNFα were likewise susceptible to TRAIL addition. The overall results were tabulated and are shown in Supplementary Table S1. Our findings indicate that the majority of cancer cell lines across the spectrum are refractory to SMC-AEG40730 treatment, and about half are sensitive when an extrinsic apoptotic pathway trigger is added.

Core components of the TNFα- and TRAIL-mediated apoptotic pathway are present in the majority of SMC-AEG40730-resistant cancer cell lines examined. The unresponsiveness of certain cancer cell lines to SMC-AEG40730, even in the presence of an extrinsic apoptotic trigger, prompted us to investigate the basis of this resistance. The execution of TNFα-mediated apoptosis requires RIP1 or TRADD, whereas both TNFα- and TRAIL-mediated apoptosis require their respective receptors (i.e., TNF-RI and DR5) as well as caspase-8 and FADD. We therefore profiled the entire panel of the 51 cancer cell lines for the presence of these components of the extrinsic apoptotic pathway. Remarkably, the apoptotic machinery was present in the vast majority of the cell lines (86%), with the notable exception of six neuroblastoma cell lines and the H520 non–small cell lung carcinoma that lacked critical components such as caspase-8, TNF-RI, or DR5 (Fig. 1A–C). The targets of SMC, specifically cIAP1, cIAP2, and XIAP, were present in the entire panel investigated. No correlation was found between the levels of cIAP1, cIAP2, and XIAP, suggesting that the level of these inhibitors does not correlate with the sensitivity to SMC-AEG40730.

Figure 5. SMC-AEG40730 blunts JNK activation in resistant cells in response to TNFα. Panc1 (A), A2780-CP (B), A549 (C), and U343 (D) cells were treated with a vehicle or 100 nmol/L SMC-AEG40730 for 24 h, and then these cells were exposed to 100 ng/ml TNFα. At the indicated times, cells were harvested and subjected to Western immunoblotting with antibodies recognizing active phospho-JNK and total JNK.
the IAPs in these cell lines and their responsiveness to SMC-AEG40730. As perhaps expected, these cell lines that were missing components of the extrinsic apoptotic pathway were also among those that are the most resistant to SMC-AEG40730 and relevant ligand triggers. Notwithstanding this finding, about a third of all the cell lines examined were resistant to SMC-AEG40730 and death ligand triggers but still possessed core components of the extrinsic apoptotic pathway. Therefore, lesions in the apoptotic pathway explained SMC unresponsiveness in some cell lines, but the basis of resistance for most others remained unresolved.

SMC-AEG40730 blocks TNFα-mediated ubiquitination of RIP1 and delays TNFα-mediated NF-κB signaling in resistant cell lines. A consequence of cIAP1/2 removal is the reduction of RIP1 ubiquitination in response to TNFα (3, 10, 11). In cancer cells that are sensitive to SMC, the lack of RIP1 ubiquitination allows for the release of RIP1 from TNF-R1 to activate caspase-8 in the death complex II (3, 12). The status of TNFα-mediated ubiquitination of RIP1 in resistant and intermediate sensitive cancer cells after SMC treatment, however, is not known. Therefore, we next analyzed the ubiquitination of RIP1 in three SMC-resistant cell lines (ovarian carcinoma A2780-CP, lung carcinoma A549, and glioblastoma U343), after exposure to SMC-AEG40730 and TNFα. We immunoprecipitated the TNF-R1 complex and then probed for its constituents. Exposure to TNFα alone led to the accumulation of ubiquitinated RIP1 in the TNF-R1 complex (Fig. 2 and data not shown). The ablation of cIAPs by SMC-AEG40730 diminished RIP1 ubiquitination in response to TNFα (3, 10, 11). In cancer cells that are sensitive to SMC, the lack of RIP1 ubiquitination allows for the release of RIP1 from TNF-R1 to activate caspase-8 in the death complex II (3, 12). The status of TNFα-mediated ubiquitination of RIP1 in resistant and intermediate sensitive cancer cells after SMC treatment, however, is not known. Therefore, we next analyzed the ubiquitination of RIP1 in three SMC-resistant cell lines (ovarian carcinoma A2780-CP, lung carcinoma A549, and glioblastoma U343), after exposure to SMC-AEG40730 and TNFα. We immunoprecipitated the TNF-R1 complex and then probed for its constituents. Exposure to TNFα alone led to the accumulation of ubiquitinated RIP1 in the TNF-R1 complex (Fig. 2 and data not shown). The ablation of cIAPs by SMC-AEG40730 diminished

![Image]

Figure 6. Endogenous c-FLIP protects resistant cancer cells from SMC AEG40730-mediated cell death. A2780-CP (A) and U343 (B) cells were transfected with siRNA against a c-FLIP or a nontargeting (NT) siRNA as a control. At 24 h after siRNA-mediated silencing, these cells were exposed to combinations of 100 nmol/L SMC-AEG40730, 100 ng/mL TNFα, or TRAIL for the indicated times. Cells were harvested and immunoblotted for caspase-8, cleaved caspase-3, c-FLIP, cIAP1, cIAP2, and XIAP. For the probing of cleaved caspase-8, higher laser intensity was used to detect the IR fluorescent signals. ß-Actin was used as a loading control. The absence of c-FLIP facilitated the activation of caspase-8 and caspase-3 in response to SMC-AEG40730 and death receptor ligands. A2780-CP (C) and U343 (D) cells were silenced with c-FLIP and then exposed to the combinations of SMC-AEG40730, TNFα, and TRAIL. After 24 h (A2780-CP) or 48 h (U343) of drug exposure, cell viability was determined by trypan blue exclusion for A2780-CP cells and Alamar blue for U343 cells. Percentage viability relative to nontargeting RNAi and vehicle ± SD (n = 4) was plotted. The down-regulation of c-FLIP reversed the resistance of these cells to the cotreatment of SMC-AEG40730 and death receptor ligands.
ubiquititation of RIP1 at the TNF-R1 complex (Fig. 2A and data not shown), consistent with the finding that cIAPs are the ubiquitin E3 ligases for RIP1. To confirm whether the SMC-induced loss of ubiquitination of RIP1 indeed enhanced the formation of complex II containing RIP1 and caspase-8, we immunoprecipitated caspase-8 following SMC-AEG40730 treatment. We found that following exposure to SMC-AEG40730, caspase-8 increased its association with RIP1 (Fig. 2B and C). These results show that in cancer cells that are resistant to SMC and TNFα, SMC treatment still represses the ubiquitination of RIP1.

Activation of NF-κB can affect the survival of cancer cells (21). In mouse embryonic fibroblasts (MEF), the loss of cIAPs reduced the ubiquitination of RIP1, which blunted the activation of TNFα-mediated classic NF-κB pathway and sensitizes cells to apoptosis (10, 11). Given that we found in resistant cancer cells that SMC-AEG40730 has the ability to knock down cIAPs, but that reduced ubiquitination of RIP1 still occurs (Fig. 2A and data not shown), we further examined the effect of SMC on TNFα-mediated classical NF-κB pathway. We measured the activation of NF-κB by the degradation of IκBα, in both SMC-resistant cell lines as well as in the highly responsive pancreatic Panc1 cells to the cotreatment of SMC and TNFα (5% viability; Supplementary Fig. S2D). SMC treatment blunted the activation of the TNFα-mediated classical NF-κB pathway in U343, A2780-CP, and GI-ME-N cells (Fig. 2D; Supplementary Fig. S3A and B). In A549 and Panc1 cells, SMC-AEG40730 pretreatment modestly delayed TNFα-mediated activation of the classical NF-κB pathway (Supplementary Fig. S3C and D). Although SMC-AEG40730 pretreatment delayed or blunted the activation of the classical NF-κB pathway, the extent of the signal reduction does not seem to correlate with loss of cell viability. This finding in cancer cells is in marked contrast to the robust suppression of TNFα-mediated classical NF-κB activation and sensitization to TNFα-mediated cell death in MEFs deficient of cIAP1 and cIAP2 (10, 11). Overall, these results indicate that the degree of blunting of the classical NF-κB pathway following TNFα addition is not a unique predictor for the efficacy of SMC in killing cancer cells.

c-FLIP is not decreased in response to SMC-AEG40730 and death receptor ligands in resistant cell lines. To further investigate the underlying basis for SMC-resistance, we analyzed the effects of SMC-AEG40730, TNFα, and TRAIL on the expression profile of pathway-associated proteins. We first confirmed the ability of SMC-AEG40730 to reduce the levels of cIAP1, cIAP2, and XIAP (Fig. 3A). The level of full-length caspase-8 remained constant in the resistant cell lines examined following the cotreatment of SMC-AEG40730 and TRAIL (Fig. 3B). A decrease in full-length caspase-8 seen in GI-ME-N is indicative of processing and activation of caspase-8, consistent with GI-ME-N cells showing a response to the cotreatment of SMC-AEG40730 and TRAIL (Fig. 3B). Following TNFα treatment, the levels of TRADD decreased in all four cell lines examined (Fig. 3B). This decrease was mirrored by a corresponding drop in RIP1 protein level in A2780-CP and A549 cells. These results show that TNFα treatment tends to induce a modest down-regulation of the components of complex II and the DISC, which may partially account for resistance to SMC in combination with this death receptor ligand.

c-FLIP is an important apoptosis regulator that functions as an inhibitor to caspase-8–mediated events. Given that caspase-8 expression in resistant cells seems to be suppressed, we analyzed the protein level of c-FLIP. In SMC-resistant U343 cells, the level of c-FLIP remained constant irrespective of its exposure to SMC-AEG40730 and TRAIL (Fig. 3C). In SMC-resistant U343 cells, c-FLIP level was steady in response to SMC-AEG40730 alone and remained relatively high when cotreated with TRAIL (Fig. 3C). In the presence of SMC-AEG40730 and TRAIL, we detected a decrease in c-FLIP level in GI-ME-N cells (Fig. 3C), which coincided with caspase-8 activation (Fig. 3D) and the decrease in cell viability (Supplementary Fig. S2D). These results suggest that persistent c-FLIP expression impedes SMC-mediated apoptosis.

Next, we extended our analysis to earlier time points following treatments of SMC-AEG40730 and TNFα. In sensitive Panc1 cells, exposure to TNFα alone resulted in an accumulation of c-FLIP starting at 2 hours, which became marked by 4 hours (Fig. 4A). When Panc1 cells were pretreated with SMC, the subsequent exposure to TNFα resulted in the processing of c-FLIP to its p43 form by 60 minutes. The c-FLIP(p43) form, which retained anti-caspase-8 function, transiently accumulated but became weakly detectable by 8 hours. In contrast, the levels of c-FLIP in resistant cells (A2780-CP, A549, and U343) remained constant throughout the 8-hour time course (Fig. 4B–D). These results indicate that, in response to SMC-AEG40730 and TNFα treatment, levels of c-FLIP positively correlated with the sustained viability of resistant cells, and the down-regulation of c-FLIP preceded death in sensitive cells.

Both NF-κB–mediated transcription and JNK-mediated ubiquitin-proteasome activation regulate the steady-state protein levels of c-FLIP (15). The ability of SMC-AEG40730 to blunt the activation of the TNFα-mediated classic NF-κB pathway differs to varying degrees in the resistant and sensitive cell lines (Fig. 2D and Supplementary Fig. S3), indicating that NF-κB suppression alone is unlikely to account for changes in the level of c-FLIP. The effect of SMC-AEG40730 on JNK activation in cancer cells, however, has not yet been studied in depth. We therefore examined the activation of JNK in response to SMC-AEG40730 and TNFα treatment. In Panc1 cells, exposure to TNFα induced both an early, transient (10–30 minutes), as well as a late, sustained (2–8 hours) activation of JNK (Fig. 5A). When Panc1 cells were pretreated with SMC-AEG40730, TNFα-mediated early transient activation of JNK2 and JNK3 was abolished, whereas early transient activation of JNK1 was markedly decreased (Fig. 5A). In contrast to the repression of early transient activation of JNK, TNFα-mediated late activation of JNK persisted in spite of SMC-AEG40730 pretreatment (Fig. 5A). This sustained activation of JNK coincided with the loss of c-FLIP (p43) (Fig. 4A). In cell lines resistant to SMC-AEG40730 and TNFα (A2780-CP, A549 and U343), TNFα activated the JNK pathway to varying degrees (Fig. 5B–D). In all three cell lines, SMC-AEG40730 pretreatment repressed JNK activation (Fig. 5B–D), suggesting that the JNK pathway may regulate the steady-state protein levels of c-FLIP.

Endogenous c-FLIP protects resistant cancer cells from SMC-AEG40730–mediated cell death. The correlation between c-FLIP levels and cell viability implies that endogenous c-FLIP may protect cancer cells from SMC and death receptor ligand activation of the extrinsic apoptotic pathway. We therefore tested this hypothesis by silencing c-FLIP with siRNA in cancer cells, before exposure to SMC-AEG40730 and death receptor ligands. In A2780-CP cells, the depletion of c-FLIP led to the processing and activation of caspase-8 by 2 hours following cotreatment of SMC-AEG40730 with TRAIL and by 4 hours with SMC-AEG40730 and TNFα (Fig. 6A). We also detected the downstream processing of caspase-3, consistent with the activation of caspase cascade. Similarly, in c-FLIP–depleted U343, A549, and GI-ME-N cells, activation of caspase-8 and caspase-3 was seen as early as...
2 hours after cotreatment with SMC-AEG40730 and TNFα, or TRAIL (Fig. 6B; Supplementary Fig. S4A and B). The activation of the caspase cascade in the absence of c-FLIP in these cell lines suggests that cancer cells will be susceptible to SMC-mediated death receptor ligand–induced cell death if c-FLIP is repressed. Next, we determined the role of c-FLIP in cell viability in response to SMC and death receptor ligands. In all four cell lines, the loss of c-FLIP led to a significant increase in cell death in response to SMC-AEG40730 or death receptor ligand treatment alone or in combination (Fig. 6C and D; Supplementary Fig. S4C and D). Furthermore, c-FLIP down-regulation also accelerates the cell death of the sensitive Panc1 cells in response to SMC and TNFα (data not shown).

To further establish that c-FLIP is indeed the final impediment to SMC-mediated cell death, we investigated the effects of c-FLIP silencing in other resistant cancer cell lines, the selection of which was based on the criteria that their extrinsic apoptotic pathway is intact. Without exception, all cancer cell lines examined were sensitized to killing by the combination of SMC-AEG40730 and TNFα following siRNA targeting c-FLIP (Supplementary Fig. S5). These results show that endogenous c-FLIP prevents the induction of cell death by SMC and death receptor ligand treatment.

Discussion

SMCs are a promising novel cancer therapeutic class, but their standalone in vitro efficacy is restricted to a small subset of cancer cell lines. Although the presence of TNFα or TRAIL enhances the ability of SMC to induce apoptosis, many cancer cell lines remain resistant or only modestly responsive to SMC treatment for unknown reasons. Here, through the use of a prototypic SMC, AEG40730, we delineate the basis for SMC resistance (Supplementary Fig. S6) and establish an indicator of sensitivity toward SMC based on the molecular profile of cancer cells.

The responsiveness of cancer cells to the combinations of SMC-AEG40730 and death receptor ligands can be categorized into four groups. The first group represents those that are outright sensitive to SMC because of the induction of a TNFα autocrine loop (16). The second group represents those that are not affected by SMC alone but would succumb to apoptosis when concurrently exposed to exogenous death receptor ligands. The third group is resistant to SMC and death receptor ligands because they lack core components necessary for the extrinsic apoptotic pathway. The fourth group, those we discovered and elucidated in this study, includes cancer cells that are nominally resistant to the combination of SMC and death receptor ligands, yet their extrinsic apoptotic machinery is present. However, when we silence c-FLIP in these cells, they too become susceptible to SMC-induced apoptotic challenge.

We have discovered that the silencing of c-FLIP in resistant cancer cells reverses their resistance toward the concomitant exposure to SMC and death receptor ligands, providing that their extrinsic apoptotic pathway is intact. In MEFs, exposure to TNFα activates the classic NF-κB pathway, which promotes transcriptional up-regulation of c-FLIP (22). Furthermore, TNFα-mediated NF-κB activation can up-regulate mitogen-activated protein kinase phosphatases, which in turn inhibits JNK (23). Inhibited JNK can no longer phosphorylate ITCH, an ubiquitin E3 ligase that can mediate c-FLIP turnover (15, 24). We noted that in resistant cancer cells, SMC repressed JNK activation in response to TNFα. In contrast, JNK activation was not blunted in SMC-responsive cells, in accord with the involvement of JNK activity in c-FLIP metabolism. Our study thus provides significant mechanistic insight into the mode of action of SMC and the basis of SMC resistance (Supplementary Fig. S6).

This present study has profound implications on the utilization of SMC. Cancer is a disease of defects in multiple pathways. Some of these pathways contribute to the ability of cancer cells to evade apoptosis induced by the immune surveillance and cancer therapeutics. As agents designed to enhance or reactivate apoptosis programs, SMCs target the IAPs, which are fundamental factors of cell death resistance in cancer cells. Death receptor ligands are constituents of the tumor microenvironment (25, 26), and based on synergies, will be expected to synergize with SMCs in a clinical setting. Our results and previous studies (2, 3) show that SMC synergizes with TRAIL, suggesting that the combination of TRAIL agonistic antibodies with SMCs is an appealing future therapeutic avenue.

Here, we provide compelling evidence to support a new paradigm in which the loss of c-FLIP is pivotal to SMC-induced death receptor ligand–mediated apoptosis. Hence, the ability of cancer cells to resist SMC-mediated cell death by up-regulating c-FLIP or avoiding its degradation may illustrate a potential challenge facing the broad implementation of this class of drug. Combination chemotherapy is widely used clinically and is also a likely development path for the SMCs. Hence, a viable therapeutic approach could exploit the endogenous presence of death receptor ligands by combining SMCs with a chemotherapeutic that affects c-FLIP levels. For instance, bortezomib, a proteasomal inhibitor that is currently in use for treating multiple myeloma, can down-regulate c-FLIP independently of its effect on NF-κB activity (27). Paclitaxel, a mitotic inhibitor widely used in cancer chemotherapy, can also down-regulate c-FLIP (28). Therefore, our results suggest that a strategy that combines the use of SMC with a c-FLIP antagonist might have a greater therapeutic effect than a standalone treatment.

In conclusion, our study shows that silencing one single gene, CFLAR, which encodes for c-FLIP, can reengage the apoptotic pathway in SMC-resistant cancer cells. In the advent of personalized cancer therapeutics, our study endorses the emerging concept of biomarker-guided therapeutic choices, in which the molecular signature dictates optimal outcome. Such paradigms would be leveraged considerably by the collection of data on c-FLIP responsiveness to SMCs in ongoing clinical trials.

Disclosure of Potential Conflicts of Interest

R.G. Korneluk: founder and shareholder, Ægera Therapeutics, Inc. The other authors disclosed no potential conflicts of interest.

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References

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