IKKβ-mediated nuclear factor-κB activation attenuates smac mimetic–induced apoptosis in cancer cells

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Abstract

Smac mimetics (SM) have been recently reported to kill cancer cells through the extrinsic apoptosis pathway mediated by autocrine tumor necrosis factor (TNF). SM also activates nuclear factor-κB (NF-κB). However, how SM induces NF-κB and the role of NF-κB in SM-induced cancer cell death has not been well elucidated. We found that effective blockage of NF-κB had no detectable effect on SM compound 3 (SMC3)–induced TNF secretion, suggesting that the induction of TNF by SMC3 is independent of NF-κB. Conversely, SMC3-induced NF-κB activation was found to be mediated by autocrine TNF because this effect of SMC3 was effectively inhibited when TNF was blocked with either a TNF neutralizing antibody or TNF small interfering RNA. In addition, although SMC3 dramatically reduced c-IAP1 level, it had marginal effect on c-IAP2 expression, TNF-induced RIP modification, NF-κB activation, and downstream antiapoptosis NF-κB target expression. Furthermore, blocking NF-κB by targeting IKKβ or RelA substantially potentiated SMC3-induced cytotoxicity, suggesting that the NF-κB pathway inhibits SMC3-induced apoptosis in cancer cells. Our results show that through TNF autocrine, SM induces an IKKβ-mediated NF-κB activation pathway that protects cancer cells against SM-induced apoptosis, and thus, NF-κB blockage could be an effective approach for improving the anticancer value of SM. [Mol Cancer Ther 2009;8(6):1636–45]

Introduction

As a transcription factor that is activated by environmental stimuli and cellular stresses, nuclear factor-κB (NF-κB) plays an important role in numerous biological and pathologic processes (1). In cancer cells, NF-κB is frequently activated and contributes to proliferation, survival, invasion, and metastasis (2). NF-κB is generally considered as a cell survival factor that protects cancer cells against apoptosis because it up-regulates expression of antiapoptotic genes (3–5). However, emerging evidence from recent studies suggests that NF-κB may be proapoptotic because it is able to activate proapoptotic genes such as death receptor 5, Bax, Fas ligand, and p73 (6–8). How the contradictory cellular outcomes by NF-κB activation are regulated has not been well understood, but they could be cell type and stimulus specific (9). NF-κB can be activated by many anticancer therapeutics (9–11). Because chemotherapeutics kill cancer cells through apoptosis, understanding the role of chemotherapy-induced NF-κB in apoptosis regulation would help to improve their anticancer efficacy.

There are two, canonical and noncanonical, pathways that lead to NF-κB activation. In most cell types, the major one is the canonical pathway, which consists of IKK (IKKα as the catalytic subunit), inhibitor of κB (IκB), and NF-κB (typically a p65/p50 heterodimer) and is activated by proinflammatory cytokines such as tumor necrosis factor (TNF) and a variety of cellular stresses (3). The p65/p50 complex is retained in the cytoplasm by association with IκB. When this pathway is turned on by binding of TNF to the TNF receptor 1 (TNFR1), IKK is recruited to the TNFRI signaling complex through TRAF2 and activated through a RIP-mediated mechanism that involves MEKK3 (12, 13). IKKα is activated to phosphorylate IκB and trigger IκB rapid degradation. This process causes the nuclear translocation of p65/p50 to promote transcription of the target genes of NF-κB. Notably, TNF itself is also a NF-κB target in certain cell types (14). Numerous NF-κB targets activated through this pathway, such as cellular inhibitor of apoptosis 1 and 2 (c-IAP1 and c-IAP2), Bcl-xL, XIAP, and IEX-1L, have antiapoptotic properties (14). Specifically, c-IAP1 and c-IAP2 function as an apoptosis brake through direct binding and suppressing the effector caspases (15). Having an E3 ubiquitin ligase activity that modifies the key components of TNFR signaling complex, these IAP proteins also participate in TNF-induced NF-κB activation (16, 17). As a critical component for TNF-induced signaling, RIP is important for transmitting signals from TNFR1 to IKK for NF-κB activation (18–20). Ubiquitination of RIP by c-IAPs was thought to be important for recruitment and activation of IKK (16, 21–25). The noncanonical pathway is initiated by NF-κB-inducing kinase–mediated activation of IKKα,
which triggers processing of p100 to generate p52. Then, p52 forms a functional complex with RelB and translocates to the nucleus to enhance gene expression (3). Thus, IKKα, RelB, and p52 represent the unique gene targets for investigation and intervention of the noncanonical pathway.

Smac (second mitochondria-derived activator of caspase, also called direct IAP binding protein with low isoelectric point) is a key proapoptotic protein released from mitochondria during the activation of the intrinsic apoptosis pathway (26–28). The proapoptotic activity of Smac is executed through suppression of the IAP family members c-IAP1, c-IAP2, and XIAP to release the brake for apoptosis (27, 28). Many cancer cells are insensitive to apoptosis. Thus, it is assumed that reducing the apoptosis threshold by modulating apoptosis-regulating molecules such as Smac will sensitize cells to anticancer chemotherapy (29). Although other cancer cell–killing mechanisms were reported (30, 31), Smac mimetic (SM)–caused apoptosis in cancer cells through induction of autocrine TNF seems to be the major SM-triggered cytotoxicity pathway (32–35). SM also activates NF-κB, which may involve both the canonical and noncanonical pathways (33, 34). Because TNF is a potential NF-κB target and NF-κB blocks TNF-induced apoptosis, there is a paradox about the role of NF-κB in SM-induced cytotoxicity in cancer cells.

We systematically investigated the mechanism by which SM induces NF-κB activation and the role of NF-κB in SM-induced cytotoxicity using a well-characterized SM [SM compound 3 (SMC3); refs. 23, 32, 36]. In this report, we provide evidence showing that SMC3 activates NF-κB mainly through the canonical pathway by inducing autocrine TNF and that NF-κB blocks TNF-induced apoptosis, there is a paradox about the role of NF-κB in SM-induced cytotoxicity in cancer cells.

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**Materials and Methods**

**Reagents and Antibody**

SMC3 was kindly provided by Dr. Xiaodong Wang (University of Texas Southwest Medical Center, Dallas, TX). Human TNF, interleukin-1β, and anti-TNFFR1 were purchased from R&D Systems. Inhibitors for IKK (IKK inhibitor II) were from Calbiochem. Actinomycin D was purchased from Sigma. The following antibodies were used for Western blot: anti-c-IAP2, anti-caspase-8, and anti-caspase-3 (Pharmingen); anti–poly(ADP-ribose) polymerase (BioSource); anti–Bcl-xL (Cell Signaling); anti–RIP and anti–MnSOD (BD Biosciences); anti-IKKβ (Upstate); anti-ubiquitin, anti–IκBα, anti–RelA, anti–RelB, and anti–NF-κB p52 (Santa Cruz Biotechnology); anti–c-IAP1 (R&D Systems); and anti–β-tubulin (Sigma). Small interfering RNA (siRNA) for IKKβ, RelA, and RelB and TNF, and negative control siRNA were purchased from Dharmacon. The human TNF detection ELISA kit was purchased from eBioscience, Inc.

**Cell Culture**

The human lung cancer cell line H23, human hepatoma cell lines HepG2 and Huh-7, and human breast cancer cell line MCF-7 were obtained from the American Type Culture Collection. H23 cells were grown in RPMI 1640 with 10% fetal bovine serum, 1 mmol/L glutamate, 100 units/mL penicillin, and 100 μg/mL streptomycin. HepG2, Huh-7, and MCF-7 cells were cultured in DMEM with 4.5 g/L glucose, 10% fetal bovine serum, 1 mmol/L glutamate, 100 units/mL penicillin, and 100 μg/mL streptomycin.

**Cytotoxicity Assay**

Cytotoxicity was determined using a lactate dehydrogenase (LDH) release-base cytotoxicity detection kit (Promega). Cells were seeded in 48-well plates at 70% to 80% confluence. After culture overnight, cells were treated as indicated in each figure legend. LDH release was determined and cell death was calculated as described previously (37, 38).

**Measurement of Autocrine TNF Secretion by ELISA**

Cells were plated onto 12-well plates at 70% to 80% confluence. After culture overnight, cells were treated as described in the figure legends. The culture media were collected and concentration of TNF was detected by ELISA analysis with the human TNF-α ELISA kit following the instruction of the manufacturer (eBioscience).

**Western Blot and Immunoprecipitation**

Cells were harvested and lysed in M2 buffer [20 mmol/L Tris-HCl (pH 7.6), 0.5% NP40, 250 mmol/L NaCl, 3 mmol/L EGTA, 3 mmol/L EDTA, 2 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L β-glycerophosphate, 1 mmol/L sodium vanadate, 1 μg/mL leupeptin]. Equal amounts of protein extracts were resolved in 12% SDS-PAGE and the proteins of interest were probed by Western blot and visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham; refs. 39, 40).

For immunoprecipitation, cells were cultured in 100-mm dishes, treated as indicated in figure legends, and lysed in M2 buffer. Immunoprecipitation was done as described previously (12, 41). Briefly, 20 μL of protein A agarose beads (50%) were coupled to 1 μg of TNFR1 antibody in PBS for 2 h at room temperature. Then, 1 mg cell lysates were added and incubated with the beads by rotating overnight at 4°C. The beads were washed seven times with M2 buffer. The immunoprecipitants were eluted off the beads using 2× electrophoresis sample buffer. The samples were boiled for 5 min and loaded up with 12% SDS-PAGE gel. Ubiquitinated RIP was detected by Western blot with an anti-RIP antibody.

**Transfection, Luciferase Report Assay, and RNA Interference**

Cells grown in 24-well plates were transfected with p5x-κB-Luc and pRSV-LacZ with FuGENE 6 according to the manufacturer’s instruction (Roche). Twenty-four hours after transfection, cells were treated as indicated in each figure legend. Luciferase activity was measured using a luciferase assay kit (Promega) and normalized to β-galactosidase activity (11, 20). siRNA was transfected with INTERFERin.
Forty-eight hours after transfection, cells were treated with SMC3 as described in figure legends and then followed by Western blot or cell death assay (11).

**Reverse Transcription-PCR**

Total RNA was extracted with the RNeasy kit (Qiagen). An equal volume of cDNA product was used in the PCR. The primers used were as follows: MnSOD, AGTTGCTGGAAGCCATCAAACGTG and TAAGGCCTGTGTTCTTGAGTG; Bcl-xL, TGGGCTCACTCTTCAGTCGAAAT and ATGTAGTGTTCTCCTGTTG; and β-actin, CCTGGCTTCCCTGGCAT and AGGAGCAATGATCTTGTAG.

Statistical Analysis

Data are summarized with mean ± SD. The association between design factors and cell response was examined by either one- or two-way ANOVA. To assess the potential for interactions between factors, we included interaction terms in the models. When tests indicated an interaction, 95% confidence intervals for the differences in means were obtained to assess the magnitude of the interaction. In all analyses, \( P < 0.05 \) was considered statistically significant.

**Results**

**Transcription-Independent TNF Autocrine in SMC3-Induced Cytotoxicity in Cancer Cells**

We found that the lung cancer cell line H23 is sensitive to SMC3-induced cytotoxicity. SMC3 kills this cell line in a dose-dependent manner, starting at the concentration as low as 10 nmol/L (Fig. 1A). The cytotoxicity induced by SMC3 was mainly apoptotic because SMC3 effectively activated caspase-8 and caspase-3 and the cleavage of the caspase-3 substrate poly(ADP-ribose) polymerase (Supplementary Fig. S1A). The fact that the SMC3-induced apoptosis was efficiently blocked by a caspase-8 inhibitor implies that the extrinsic apoptosis pathway was the main cell death pathway triggered by SMC3 (Supplementary Fig. S1B). We further detected SMC3-induced TNF secretion in the culture medium as early as 2 hours after treatment (Fig. 1B, top).

**Figure 1.** SMC3-induced transcription-independent TNF autocrine is required for SMC3-induced cytotoxicity in cancer cells. A, H23 cells were treated with indicated concentrations of SMC3 for 36 h. Cell death was measured by LDH leakage assay. Column, mean; bars, SD. B, top, H23 cells were treated with SMC3 (50 nmol/L) or DMSO for the indicated times. The concentrations of TNF in cell culture medium were measured by ELISA. Bottom, H23 cells were mock transfected or transfected with 5 nmol/L of TNF siRNA or negative control siRNA. Forty-eight hours after transfection, the cells were treated as indicated. Cell death was measured as described in A. **,** \( P < 0.01 \). C, top, TNF mRNA was detected by reverse transcription-PCR. β-Actin was detected as an input control. Bottom, H23 cells were pretreated with actinomycin D (Act-D; 10 μmol/L) for 30 min followed by exposure to SMC3 (50 nmol/L) or interleukin-1β (IL-1β; 5 ng/mL) for 8 h. TNF was detected as described in B. D, HepG2, Huh-7, and MCF-7 cells were treated with indicated concentration of SMC3 for 36 h. Cell death was measured as described in A.
neutralizing antibody that blocks TNF binding to its receptor dramatically inhibited SMC3-induced cell death (Fig. 1B, bottom; Supplementary Fig. S2). Interestingly, SMC3 had no detectable effect on TNF message RNA expression, implying that the induction of TNF by SMC3 may not require activation of transcription on the tnf gene (Fig. 1C, top). Pretreatment of the cells with the transcription inhibitor actinomycin D had no effect on SMC3-induced TNF secretion (Fig. 1C, bottom). As a control, actinomycin D effectively blocked interleukin-1β-induced TNF increase in the culture medium (Fig. 1C, bottom). These results suggest that SMC3-induced TNF autocrine is transcription independent. The effect of TNF siRNA is likely through shutting off the constitutive TNF expression. Similar observations were made in the hepatoma cell lines HepG2 and Huh-7 and breast cancer cell line MCF-7, although the effective doses of SMC3 were much higher in these cells (Fig. 1D; data not shown). In agreement with and supplementary to previous reports (32–34), these results suggest that SMC3 induces apoptosis through TNF autocrine, which is independent of transcription, in cells derived from lung, breast, and liver tumors.

The Noncanonical Pathway Contributes Marginally to SMC3-Induced NF-κB Activation and Is Dispensable for SMC3-Induced TNF Secretion

Previous reports suggested that some SMs stimulate both the canonical and noncanonical NF-κB activation pathways (33, 34). However, the contribution of each pathway to SM-induced NF-κB activation was not determined. Thus, we sought to examine the mechanism by which SMC3 induces NF-κB activation and the role of NF-κB in SM-induced cytotoxicity. SMC3 induced NF-κB activation (Fig. 2A) and stimulated the expression of antiapoptotic NF-κB targets at both the protein and mRNA levels in all the tested SMC3-sensitive cell lines (Fig. 2B). Consistent with previous reports (33, 34), SMC3 was able to stimulate the noncanonical pathway, which was shown as generating the NF-κB p52 subunit by cleavage of the p100 precursor. The activation of the noncanonical pathway was quite moderate because no reduction of p100 was detected throughout the course of treatment and the p52 fragment could be detected only after long-time exposure (Fig. 2C, top and middle). Then, we examined the contribution of the noncanonical pathway to the overall NF-κB activation by specifically blocking this pathway with siRNA, targeting the key component RelB, and with a NF-κB luciferase reporter assay that is sensitive to block the noncanonical pathway (Supplementary Fig. S3). Additionally, there was no detectable effect of RelB siRNA on the SMC3-induced expression of the NF-κB target gene MnSOD (data not shown). The involvement of the noncanonical pathway in SMC3-induced TNF secretion was also tested with RelB siRNA. The results show that RelB is dispensable for SMC3-induced TNF secretion (Fig. 2D, bottom). These results suggest that although SMC3 stimulates the processing of p100, the noncanonical pathway contributes marginally to the SMC3-induced overall NF-κB activation and TNF secretion.
The Canonical Pathway Mediates SMC3-Induced NF-κB Activation but Is Not Required for SMC3-Induced TNF Secretion

The canonical pathway was then examined by detection of the hallmark of this pathway, IκB phosphorylation, and degradation. The results showed that SMC3 triggered phosphorylation of IκBα, which was detected as early as 2 hours and peaked at 4 to 8 hours after treatment (Fig. 3A). IκBα degradation was also seen following IκBα phosphorylation (Fig. 3A). siRNA targeting IKKβ or RelA, two key components of the canonical pathway, almost completely blocked SMC3-induced NF-κB activation (Fig. 3B, left). Together with the finding that blocking the noncanonical pathway had no effect on SMC3-induced NF-κB activity (Fig. 2D, top), these results suggest that the canonical, but not the noncanonical, pathway is the main pathway for SMC3-induced NF-κB activation. The chemical IKK inhibitor, which blocks both the canonical and noncanonical pathways through suppressing IKKβ and IKKα, inhibited the SMC3-induced NF-κB activation in a similar efficiency as that of IKKβ or RelA siRNA (Fig. 3B, right), further supporting the conclusion that the canonical pathway is the main pathway for SMC3-induced overall NF-κB activation. To examine if NF-κB plays a role in SMC3-induced autocrine TNF, the H23, HepG2, Huh-7, and MCF-7 cells were transfected with siRNA targeting IKKβ or RelA to block SMC3-induced NF-κB activation and TNF in the culture medium was detected by ELISA. IKKβ or RelA siRNA had no effect on SMC3-induced TNF secretion in all cell lines (Fig. 3C). The knockdown of IKKβ or RelA was confirmed by Western blot (Supplementary Fig. S4) and blocking of SMC3-induced NF-κB activation was confirmed by luciferase reporter assay (Fig. 3B; data not shown). Consistent with the results with siRNA, the IKK inhibitor that blocks both the canonical and noncanonical NF-κB activation pathways did not affect TNF autocrine triggered by SMC3 (Fig. 3D). Taken together with the results that SMC3-induced TNF autocrine was independent of transcription, these experiments establish that NF-κB is dispensable for SMC3-induced TNF autocrine in cancer cells.

SMC3-Induced NF-κB Activation in Cancer Cells Requires TNF Secretion

Because SMC3 induces NF-κB activation through the canonical pathway and triggers TNF autocrine, we hypothesize that SMC3 induces NF-κB activation through TNF secretion. Supporting this notion, SMC3-induced IκBα phosphorylation and degradation (4–8 hours; Fig. 3A) and expression of NF-κB targets (8–16 hours; Fig. 2B) started at late time points, which was well correlated to the time course of TNF secretion (Fig. 1B). To further test this

Figure 3. The canonical pathway mediates SMC3-induced NF-κB activation but is not required for SMC3-induced TNF secretion. A, H23 cells were treated with SMC3 (50 nmol/L) for various times as indicated. Phosphorylated and total IκBα were detected by Western blot. β-Tubulin was detected as an input control. B, left, H23 cells were mock transfected or transfected with 5 nmol/L of RelA, IKKβ, or negative control siRNA. Forty-eight hours after transfection, the cells were cotransfected with p5×B-Luc and pRSV-LacZ. Twenty-four hours after transfection, the cells were pretreated with IKK inhibitor II (10 μmol/L) for 1 h followed by SMC3 (50 nmol/L) treatment for 24 h or left untreated. Luciferase activity was detected and normalized to β-galactosidase activity. *, P < 0.05. Right, H23 cells were cotransfected with p5×B-Luc and pRSV-LacZ. Twenty-four hours after transfection, the cells were pretreated with IKK inhibitor II (10 μmol/L) for 1 h followed by SMC3 (50 nmol/L) treatment for 24 h or left untreated. The concentrations of TNF in conditioned cell culture medium were measured by ELISA. D, H23, HepG2, Huh-7, and MCF-7 cells were pretreated with IKK inhibitor II (10 μmol/L) for 1 h followed by SMC3 (50 or 100 nmol/L as indicated) treatment for 24 h or left untreated. The concentrations of TNF in conditioned cell culture medium were measured by ELISA.
hypothesis, we first examined if the TNF-induced NF-κB activation pathway is normal in SMC3-treated cells. Consistent with the previous report (23), although SMC3 significantly suppressed the expression of c-IAP1, it only partially reduced the c-IAP2 level (Fig. 4A). Because both c-IAP1 and c-IAP2 are involved in TNF-induced NF-κB activation through regulating RIP modification (16, 17), the retaining of c-IAP2 expression may be sufficient to allow the transmission of the NF-κB activation signal from TNFR1 to RIP. Indeed, SMC3 did not reduce recruitment of TNF-induced RIP to TNFR1 and the subsequent modification of RIP (Fig. 4B). Consistently, SMC3 had no detectable effect on TNF-induced IκBα degradation and NF-κB activation (Fig. 4C and D). These results suggest that the TNF-induced NF-κB activation pathway remains intact in SMC3-treated cells, allowing the secreted TNF to stimulate this pathway.

We then examined whether SMC3-induced NF-κB activation requires TNF. TNF siRNA almost completely blocked SMC3-induced NF-κB activation, which is associated with effective suppression of SMC3-triggered TNF secretion (Fig. 5A and B). In addition, the TNF neutralizing antibody also suppressed SMC3-induced NF-κB activation and expression of NF-κB targets (Fig. 5C). The dependence of SMC3-induced NF-κB on TNF was also observed in HepG2, Huh-7, and MCF-7 cells (Fig. 5D). Furthermore, the incapability of activating NF-κB of SMC3 was associated with its failure in TNF induction in the lung cancer cell line A549 (data not shown). Taken together, these results strongly suggest that the SMC3-induced NF-κB activation is dependent on autocrine TNF.

**Discussion**

This report delineates our systematic investigation into the mechanism of NF-κB activation by SM and its role in SM-induced cancer cell death. The results show that although SMC3 can stimulate a slight processing of p100,
the noncanonical pathway marginally contributes to the overall NF-κB activity induced by SMC3. The NF-κB activation by SMC3 is mainly through the canonical pathway that is dependent on autocrine TNF. Although SMC3 suppresses c-IAP1 expression, it does not interfere with TNF-induced NF-κB activation or expression of antiapoptotic NF-κB targets. SMC3-induced autocrine TNF is NF-κB independent and blockade of NF-κB efficiently sensitized SMC3-induced cytotoxicity in different cancer cell types. Thus, blockade of NF-κB activation suppresses cell survival while does not compromise the apoptosis pathway, shifting the outcome of the SM-responding cells to death (Fig. 6D).

Consistent with recent reports on other SMs (33, 34), SMC3 stimulated both the canonical and noncanonical NF-κB activation pathways. However, we found that the noncanonical pathway was only moderately activated and contributed marginally to the overall NF-κB activity, which was shown by specific blockage of this pathway with RelB or IKKα siRNA and an IKK inhibitor. The result agrees with the observations that the canonical NF-κB pathway is the main pathway in cancer cells (43, 44). Thus, the pathophysiological role of the noncanonical pathway in the response of a cell to SM remains to be determined. Although the activation of the noncanonical NF-κB pathway was determined to be through suppression of c-IAP1-mediated degradation of NF-κB-inducing kinase (33), how the canonical NF-κB activation pathway is activated by SM has not been determined. Our results show that activation of the canonical NF-κB pathway by SMC3 is achieved by induction of autocrine TNF because inhibition of TNF by either a neutralizing antibody or siRNA against TNF effectively blocked SMC3-induced NF-κB activity.

We provide evidence generated with different approaches showing that autocrine TNF does not require NF-κB. First, the IKK inhibitor II, which blocks both the canonical and noncanonical pathways, had no effect on SMC3-induced NF-κB activity. Second, although it effectively suppressed the SMC3-induced NF-κB activation, siRNA targeting either IKKβ or RelA did not show a detectable effect on SMC3-triggered NF-κB activity. Third, the RelB and IKKα siRNA, which block the noncanonical pathway, had no effect on autocrine TNF. Additionally, there was no detectable change of NF-κB mRNA following SMC3 treatment (Fig. 1C, top) and suppression of transcription did not inhibit SMC3-induced NF-κB secretion (Fig. 1C, bottom), further supporting that transcription of NF-κB gene is not involved in SMC3-induced TNF.

Figure 5. SMC3-induced NF-κB activation is TNF dependent. A, H23 cells were mock transfected or transfected with 5 nmol/L of TNF siRNA or negative control siRNA. Forty-eight hours after transfection, the cells were cotransfected with p5×κB-Luc and pRSV-LacZ. After incubation for another 24 h, the cells were treated with SMC3 (50 nmol/L) for 24 h or left untreated. Luciferase activity was detected and normalized to β-galactosidase activity. *, P < 0.05. B, H23 cells were treated as in A. The concentration of TNF in culture medium was determined by ELISA. **, P < 0.01. C, left, H23 cells were cotransfected with p5×κB-Luc and pRSV-LacZ. Twenty-four hours after transfection, the cells were pretreated with TNF neutralizing antibodies (1 μg/mL) or control antibody (1 μg/mL) for 1 h followed by 50 nmol/L SMC3 treatment for 24 h. Luciferase activity was detected as in A. *, P < 0.05. Right, H23 cells were pretreated with TNF neutralizing antibodies (1 μg/mL) or control antibody (1 μg/mL) for 1 h followed by 50 nmol/L SMC3 treatment for 24 h or left untreated. Bcl-xL and MnSOD were detected by Western blot. β-Tubulin was detected as an input control. D, HepG2, Huh-7, and MCF-7 cells were transfected, treated, and analyzed as described in C. **, P < 0.01.
autocrine. This is inconsistent with a recent report showing that the TNF protein expression in rhabdomyosarcoma (Kym1) and ovarian cancer (SKOV-3) cells induced by a SM was dependent on NF-κB (34). The discrepancy is likely due to different SMs used (34, 36) because, similar as in other tested cell lines, NF-κB blockage had no effect on SMC3-induced TNF autocrine, whereas significantly enhanced SMC3-induced cytotoxicity and actinomycin D had no effect on SMC3-induced TNF induction in SKOV-3 cells (Supplementary Fig. S5). As it does not involve transcriptional regulation, SMC3-induced TNF autocrine is likely through stimulating TNF from the cell membrane because protein synthesis inhibitor cycloheximide had no effect on SMC3-induced TNF secretion, whereas TACE inhibitor, which specifically suppresses TNF release, significantly suppressed this effect of SMC3 (Supplementary Fig. S6).

We found that although SMC3 induced effective cytotoxicity, it had no inhibitory effect on TNF-induced NF-κB activation, which is consistent with a recent report using the same compound by Wang and colleagues (23). Indeed, although SMC3 caused dramatic down-regulation of c-IAP1, it had minimal effect on c-IAP2 expression. The modification of RIP and activation of the NF-κB targets were retained intact in SMC3-treated cells, allowing the activation of NF-κB by autocrine TNF (Fig. 4). Additionally, modification of RIP was found in cells after long-time (8 hours) SMC3 exposure (data not shown), presumably through autocrine TNF. This is not contradictory to the reports that high doses of SM cause extensive RIP deubiquitination and switch the TNF-induced signaling from RIP-mediated NF-κB activation to caspase-8 activation that results in apoptosis (23, 35). It is reasonable to speculate that under our conditions, apoptosis caused by SMC3 occurs mainly through the c-FLIP–regulated caspase-8 activation pathway because RIP modification was retained and NF-κB activation was unaffected (23).

We further determined the effect of NF-κB blockage on SMC3-induced cancer cell death. This is because SMC3 kills...
cancer cells mainly through autocrine TNF (32–34), NF-κB is activated by SMC3 through TNF, and NF-κB blocks TNF-induced apoptosis (5, 37, 45, 46). The results showed a substantial potentiation of SMC3-induced cell death by blocking the canonical NF-κB activation pathway with either chemical IKK inhibitor or siRNA targeting IKKβ or RelA. This potentiation is well correlated to the suppression of SMC3-induced expression of the anti-apoptotic NF-κB targets. Thus, combination of NF-κB blocking means that target IKKβ or RelA and SM could be a useful strategy to improve the anticancer efficacy of SM. This approach may also reduce the adverse effects of SM by reducing the SM dosages without lowering the cancer cell–killing activity. Further in vivo studies are needed to verify the usefulness of NF-κB blockage in improving the anticancer efficacy of SM.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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45. Liu ZG, Hsu H, Goeddel DV, Karin M. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. Cell 1996;87:565–76.

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