Development of resistance to TRAIL, an apoptosis-inducing cytokine, is one of the major problems in its development for cancer treatment. Thus, pharmacological agents that are safe and can sensitize the tumor cells to TRAIL are urgently needed. We investigated whether gossypol, a BH3 mimic that is currently in the clinic, can potentiate TRAIL-induced apoptosis. Intracellular esterase activity, sub-G1 cell cycle arrest, and caspase-8, -9, and -3 activity assays revealed that gossypol potentiated TRAIL-induced apoptosis in human colon cancer cells. Gossypol-induced TRAIL death receptor (DR)3-4 (4) and TRAIL DR5 (5), and three antagonistic receptors, transmembrane decoy receptor (DcR)-1, transmembrane DcR2 (6, 7), and osteoprotegerin, a soluble receptor that lacks the transmembrane domain (8). The binding of TRAIL to DR4 and DR5 leads to caspase-8 and caspase-10 activation, which then activates downstream executioner caspase-3, -6, and -7 to mediate apoptosis (9). Because of the selectivity of TRAIL toward tumor cells, both TRAIL and agonistic antibodies against its receptor are currently being studied in clinical trials aimed at the treatment of various chemotherapies that express TRAIL to the cytotoxic mechanisms may be sensitized to TRAIL (11). Gossypol-dependent caspase activation was observed in a combination of DR5 expression and stimulated mitochondrial membranes of the Bcl-2 family members, Bcl-2, Bcl-xL, Bcl-w, and Bcl-1 to reduce anti-apoptotic protein expression. The use of anti-apoptotic agents has been shown to enhance the toxic effects of TRAIL (11, 18).

Among the candidate sensitizers is gossypol, a polyphenol derived from cottonseed oil that was once used as an anti-fertility agent and that has recently been shown to have anticancer properties (19). In part through their ability to bind to and inactivate BH3 domain-containing anti-apoptotic proteins (20), gossypol and its analogs inhibit the growth of a wide variety of human cancer cells in culture and xenograft models (21, 22).

We investigated whether gossypol potentiates TRAIL-induced cancer cell apoptosis, and if so, how gossypol potentiates this effect. We found that gossypol potentiates TRAIL-induced apoptosis through the ROS-ERK-CHOP-DR5 pathway.
apoptosis by up-regulating TRAIL receptor DR5 through the ROS (reactive oxygen species)-ERK-CHOP (CAAT/enhancer-binding protein homologous protein) pathway and by down-regulating the expression of cell survival proteins Bcl-xL, Bcl-2, XIAP, cFLIP, and survivin.

EXPERIMENTAL PROCEDURES

Materials—Gossypol (LKT Laboratories, Inc.) was dissolved in dimethyl sulfoxide (50 mM) and stored at −20 °C until needed. Soluble recombinant human TRAIL/Apo2L was purchased from PeproTech (Rocky Hill, NJ). Penicillin, streptomy-ycin, DMEM, RPMI 1640 medium, and FBS were obtained from Invitrogen. Tris, glycine, NaCl, SDS, bovine serum albumin, N-acetylcyesteine, glutathione, and antibody against β-actin were obtained from Sigma. Antibody against DR5 was purchased from ProSci Inc. Antibodies against DR4, poly(ADP-ribose) polymerase (PARP), Bcl-2, Bcl-xL, Bax, survivin, caspase-3, caspase-8, GRP78, CREB2 (ATF4), and eIF2α were obtained from Santa Cruz Biotechnology. Anti-XIAP antibody was purchased from BD Biosciences. Antibody against phospho-eIF2α was obtained from Cell Signaling.

Cell Lines—HCT116 (human colon adenocarcinoma), MDA-MB-231 (human breast adenocarcinoma), AsPC-1 (human pancreatic adenocarcinoma), SCC4 (human squamous cell carcinoma), H1299 (human lung adenocarcinoma), Hela (human cervix adenocarcinoma), Ku-7 (human bladder cancer), and KBM-5 (human chronic myeloid leukemia) cell lines were obtained from American Type Culture Collection. SEG-1 cells were kindly provided by Dr. Jaffer A. Ajani (The University of Texas Anderson Cancer Center). HCT116; however, we showed that the effect of gossypol was not restricted to these cells.

Gossypol Sensitizes Colon Cancer Cells to TRAIL-mediated Apoptosis—We used the LIVE/DEAD cell viability assay to investigate the effect of gossypol on TRAIL-induced apoptosis and found that a subtoxic dose of gossypol induced apoptosis in HCT116 cells in a dose-dependent manner. We also observed that TRAIL treatment alone induced apoptosis in 10% of the HCT116 cells. However, treating cells with TRAIL plus gossypol induced apoptosis in 51% of the HCT116 cells (Fig. 1A).

We also examined apoptosis by determining the size of the sub-G1 cell pool. We found that apoptosis was induced at 12.1% by gossypol, 17.4% by TRAIL, and 47% by the combination of the two (Fig. 1B).

Gossypol Potentiates TRAIL-induced Apoptosis

Propidium Iodide Staining for DNA Fragmentation—Cells were pretreated with gossypol for 8 h and then exposed to TRAIL for 24 h. Propidium iodide staining for DNA content analysis was performed as described previously (23). A total of 10,000 events were analyzed by flow cytometry using an excitation wavelength set at 488 nm and an emission wavelength set at 610 nm.

Measurement of Reactive Oxygen Species—To detect intracellular reactive oxygen species (ROS), we preincubated HCT116 cells with 20 μM dichlorofluorescein diacetate for 15 min at 37 °C before treating the cells with gossypol. After 30 min of incubation, the increase in fluorescence resulting from the oxidation of dichlorofluorescein diacetate to dichlorofluorescein was measured by flow cytometry as described previously (24). The mean fluorescence intensity at 530 nm was calculated for at least 10,000 cells at a flow rate of 250–300 cells/s.

Western Blot Analysis—After the specified treatments, cells were incubated on ice for 30 min in 0.5 ml of ice-cold whole-cell lysis buffer (10% Nonidet P-40, 5 mM NaCl, 1 mM HEPES, 0.1 mM EGTA, 0.5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, 1 mM sodium fluoride, 2 μg/ml aprotinin, and 2 μg/ml leupeptin). Proteins were then fractionated by SDS-PAGE and transferred to nitrocellulose membranes, blocked with 5% nonfat dry milk, and detected by enhanced chemiluminescence (GE Healthcare).

RESULTS

The objective of this study was to determine whether gossypol, a polyphenol derived from cottonseed oil, can modulate TRAIL-induced apoptosis and, if so, to determine the mechanism by which gossypol modulates the effect of TRAIL. Most studies were carried out in human colorectal cancer cell line HCT116; however, we showed that the effect of gossypol was not restricted to these cells.

Gossypol Sensitizes Colon Cancer Cells to TRAIL-mediated Apoptosis—We used the LIVE/DEAD cell viability assay to investigate the effect of gossypol on TRAIL-induced apoptosis and found that a subtoxic dose of gossypol induced apoptosis in HCT116 cells in a dose-dependent manner. We also observed that TRAIL treatment alone induced apoptosis in 10% of the HCT116 cells. However, treating cells with TRAIL plus gossypol induced apoptosis in 51% of the HCT116 cells (Fig. 1A).

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We examined the effect of gossypol on TRAIL-induced cytotoxicity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method, which detects mitochondrial activity. Briefly, cells were pretreated with various concentrations of gossypol for 8 h and then exposed to TRAIL for 24 h.
Gossypol Potentiates TRAIL-induced Apoptosis

FIGURE 1. Gossypol-enhanced TRAIL induces HCT116 cell death. A, HCT116 cells were treated with the indicated concentrations of gossypol for 8 h and rinsed with PBS. Cells were then treated with TRAIL (25 ng/ml) for 24 h. Cell death was determined by the LIVE/DEAD cell viability assay. B, cells were treated with the indicated concentrations of gossypol for 8 h and then treated with TRAIL (25 ng/ml) for 24 h. Cells were stained with propidium iodide, and the sub-G1 fraction was analyzed by flow cytometry. C, cells were pretreated with various concentrations of gossypol (GP) for 8 h, the medium was removed, and the cells were exposed to TRAIL (25 ng/ml) for an additional 24 h. Cell viability was then analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. D, cells were pretreated with the indicated concentrations of gossypol for 8 h and then rinsed. The cells were then treated with TRAIL (25 ng/ml) for 24 h. Whole-cell extracts were prepared and analyzed by Western blotting using antibodies against caspase-9, caspase-8, caspase-3, and PARP.
HCT116 cells were moderately sensitive to either gossypol or TRAIL alone. However, pretreatment with gossypol significantly enhanced TRAIL-induced cytotoxicity in a dose-dependent manner (Fig. 1C).

We also investigated whether gossypol enhances TRAIL-induced activation of caspase-8, -9, and -3 and consequent PARP cleavage and found that gossypol enhanced TRAIL-induced activation of all three caspases, thus leading to increased PARP cleavage (Fig. 1D). These results suggest that gossypol enhances TRAIL-induced apoptosis.

**Gossypol Down-regulates the Expression of Various Cell Survival Proteins**—Because numerous anti-apoptotic proteins such as survivin, cFLIP, XIAP, Bcl-2, and Bcl-xL have been shown to regulate TRAIL-induced apoptosis (25, 26), we investigated whether gossypol potentiates TRAIL-induced apoptosis by regulating these cell survival proteins. HCT116 cells were exposed to different concentrations of gossypol for 24 h and then examined for Bcl-xL, Bcl-2, survivin, XIAP, cFLIP, cIAP-1 (cellular inhibitor of apoptosis protein 1), and TRAF1 (TNF receptor-associated factor 1) expression. Gossypol inhibited Bcl-xL, Bcl-2, survivin, and XIAP expression (Fig. 2A). The effect of gossypol on the expression of Bcl-xL and Bcl-2 was quite dramatic, whereas the down-regulation of cIAP-1, cFLIP, and TRAF1 was not very pronounced. These results suggest that the gossypol-induced down-regulation of certain cell survival proteins could be one mechanism by which TRAIL-induced apoptosis is potentiated.

**Gossypol Up-regulates the Expression of Bax**—We next examined whether gossypol could modulate the expression of pro-apoptotic proteins. We found that gossypol dramatically up-regulated the expression of Bax in a dose-dependent manner (Fig. 2B).

**Gossypol Induces the Expression of TRAIL Receptor DR5**—To determine whether gossypol can modulate DR4 and/or DR5 expression in HCT116 cells, we treated HCT116 cells with gossypol for 24 h and then used Western blot analysis to examine the cells for TRAIL receptors and CHOP expression. Gossypol increased DR5 expression in a dose-dependent manner but had no effect on DR4 expression or DcR1 or DcR2 induction (Fig. 3A). Gossypol also induced DR5 expression in a time-dependent manner (Fig. 3B). These findings suggest that DR5 up-regulation could be a mechanism by which gossypol enhances the apoptotic effects of TRAIL in HCT116 cells.
To determine whether gossypol induces expression of the death receptor on the cell surface, we analyzed the cell-surface expression of DR5 and DR4 in cells using the flow cytometry method. After cell exposure to gossypol, the level of DR5 on the cell surface increased (Fig. 3C, left panel). However, the level of DR4 on the cell surface was not significant (Fig. 3C, right panel). Collectively, these results indicate that gossypol up-regulates the expression of DR5 on the cell surface.

**Up-regulation of DR5 by Gossypol Is Not Cell Type-specific**—We also investigated whether up-regulation of DR5 by gossypol is specific to HCT116 cells or also occurs in other cell types. Gossypol induced DR5 expression in pancreatic cancer cells (AsPC-1), esophageal cancer cells (SEG-1), head and neck cells (SCC4), breast cancer cells (MDA-MB-231), lung cancer cells (H1299), cervical cancer cells (HeLa), bladder cancer cells (Ku-7), and leukemia cells (KBM-5) (Fig. 3D). Together, these findings suggest that the up-regulation of DR5 by gossypol is not specific to cell type.

**DR5 Induction by Gossypol Is Needed for TRAIL-induced Apoptosis**—To determine the role of DR5 in TRAIL-induced apoptosis, we used siRNA specific to DR5 to down-regulate the expression of this receptor. Transfection of cells with siRNA for DR5 but not with the control siRNA reduced gossypol-induced DR5 expression (Fig. 4A). However, DR5 siRNA had a minimal effect on the gossypol-induced up-regulation of DR4.

We next used an esterase staining assay (the LIVE/DEAD cell viability assay) to examine whether suppression of DR5 expression by siRNA could abrogate the effect of gossypol on TRAIL-induced apoptosis. Our results showed that the effect of gossypol on TRAIL-induced apoptosis was effectively abolished in cells transfected with DR5 siRNA (Fig. 4B, lower panel). Taken together, these results indicate that induction of TRAIL receptor DR5 is critical for sensitization of tumor cells to the effect of gossypol on TRAIL-induced apoptosis.

**Gossypol-induced DR5 Up-regulation Is Mediated through Induction of CHOP**—Several studies have shown that the up-regulation of DR5 is mediated through p53, peroxisome prolif-
ATOR-activated receptor-γ, and CHOP (27–29). We next investigated how these transcription factors are involved in gossypol-induced DR5 up-regulation. Our results showed that gossypol induced CHOP expression (Fig. 5A). However, gossypol had no significant effect on the expression of peroxisome proliferator-activated receptor-γ and p53 (Fig. 5A).

Gene Silencing of CHOP Abrogates Gossypol-induced Up-regulation of DR5 Expression and Its Effect on TRAIL-induced Apoptosis—To elucidate the role of CHOP in gossypol-induced up-regulation of the death receptor, we examined gene silencing of CHOP by siRNA. Whereas gossypol up-regulated the expression of DR5 in non-transfected and control-transfected (scrambled RNA) cells, transfection with CHOP siRNA significantly suppressed gossypol-induced DR5 up-regulation (Fig. 5B, upper panel). CHOP siRNA had no effect on gossypol-induced DR4 expression (Fig. 5B, middle panel).

We next used an esterase staining assay to examine whether the suppression of CHOP by siRNA could abrogate the sensitizing effects of gossypol on TRAIL-induced apoptosis. Gossypol significantly reduced the effects (from 58 to 24%) on TRAIL-induced apoptosis (Fig. 5C, middle panel), whereas treatment with control siRNA had no effect (Fig. 5C, lower panel). These results indicate that CHOP plays a major role in DR5 up-regulation and contributes to the sensitizing effect of gossypol on TRAIL-induced apoptosis.

Gossypol-induced TRAIL Receptor Up-regulation Requires ERK but Not p38 MAPK and JNK—Because various studies have suggested that MAPK activation plays a role in TRAIL receptor induction (30, 31), we investigated whether the activation of ERK1/2, p38 MAPK, or JNK plays a role in gossypol-induced DR5 induction. We first investigated the gossypol-induced activation of these kinases. Gossypol activated JNK, ERK1/2, and p38 MAPK in a dose-dependent manner (Fig. 6A).

Next, to investigate whether JNK, ERK1/2, or p38 MAPK activation is involved in gossypol-induced DR5 induction, we used the pharmacological inhibitors of JNK (SP600125), p38 MAPK (SB202190), and ERK1/2 (PD98059). Of note, the inhibitor of ERK1/2 (PD98059) suppressed gossypol-induced DR5 as well as CHOP induction (Fig. 6B, right panel); however, the p38 MAPK and JNK inhibitors had no effect on gossypol-induced DR5 induction (Fig. 6B), which suggests that ERK (but not p38 MAPK and JNK) is needed for death receptor induction.

Gene Silencing of ERK Abolishes DR5 Up-regulation and TRAIL Sensitization—To determine whether silencing of ERK blocks DR5 induction and abrogates TRAIL-induced cell death, we transfected HCT116 cells with control siRNA and specific siRNA against ERK1. After 48 h, cells were exposed to gossypol
for 24 h and harvested, and lysates were subjected to Western blotting. The results indicated that the expression of ERK1 was significantly reduced in cells treated with specific siRNA compared with that in untreated cells or cells treated with control siRNA (Fig. 6C, left panel). The reduction in ERK1 expression by the siRNA correlated with suppression of gossypol-induced up-regulation of CHOP and DR5; however, the deletion of ERK1 had no effect on DR4 expression (Fig. 6C, left panel). Furthermore, siRNA suppression of ERK1 expression significantly reduced gossypol enhancement of TRAIL-induced apoptosis. However, control siRNA had no effect (Fig. 6C, right panel). These results suggest that CHOP-dependent DR5 up-regulation contributes to the sensitizing effect of gossypol on TRAIL-induced apoptosis.

Gossypol-induced TRAIL Receptor Up-regulation and Apoptosis Potentiation Are ROS-dependent—Because numerous studies have implicated ROS in TRAIL receptor induction, we investigated whether gossypol mediates its effects through ROS. To investigate the ability of gossypol to induce ROS, we treated cells with gossypol and used dichlorofluorescein diacetate as a probe to measure the increase in ROS levels in cells. Gossypol-induced ROS generation, and ROS mediates gossypol-induced DR5 up-regulation. A and B, HCT116 cells (1 × 10^6 cells/well) were labeled with dichlorofluorescein diacetate, treated with the indicated doses of gossypol for 1 h, and examined for ROS production. The ROS mean fluorescence intensity (MFI) increased with gossypol dose. B, HCT116 cells were pretreated with various concentrations of N-acetylcysteine (NAC) for 1 h and then treated with 5 μM gossypol for 24 h. Whole-cell extracts were prepared, and Western blotting was used to analyze relevant antibodies. p, phosphorylated. C, N-acetylcysteine reversed gossypol and TRAIL-induced cell death. HCT116 cells were pretreated with N-acetylcysteine for 1 h and then treated with gossypol for 8 h. The cells were washed with PBS and treated with TRAIL for 24 h. The LIVE/DEAD cell viability assay was used to determine cell death. D, N-acetylcysteine suppressed caspase activation and PARP cleavage induced by TRAIL and gossypol. HCT116 cells were pretreated with N-acetylcysteine for 1 h and then treated with gossypol for 8 h. Next, the cells were washed with PBS and treated with TRAIL for 24 h. Whole-cell extracts were prepared, and Western blotting was used to analyze the extracts for caspase and PARP expression. β-Actin was used as a loading control.

Next, we investigated whether ROS plays any role in gossypol-induced TRAIL potentiation. As shown in Fig. 7C, gossypol significantly enhanced TRAIL-induced apoptosis in HCT116 cells, and pretreatment of cells with N-acetylcysteine markedly reduced gossypol-induced enhancement from 51 to 19%. Then, we determined whether N-acetylcysteine could reverse gossypol-enhanced caspase activation. We found that N-acetylcysteine abolished the effect of gossypol on TRAIL-induced caspase activation, and parap cleavage (Fig. 7D), suggesting that ROS plays a critical role in mediating the effects of gossypol on TRAIL-induced apoptosis.
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Although both TRAIL and agonistic antibodies to TRAIL receptors are currently in clinical trials for treatment of cancer patients, resistance of tumor cells to apoptosis is one of the major hurdles in the usefulness of this cytokine. Thus, in this study, we investigated the ability of gossypol, a polyphenol derived from cotton seed oil and once tested in men as an anti-fertility drug, to modulate TRAIL signaling in cancer cells, and our findings indicate that gossypol potentiates TRAIL-induced apoptosis in cancer cells by inducing DR5 expression and by down-regulating cell survival proteins linked to tumor cell resistance to TRAIL. Gossypol up-regulation of DR5 appears to be mediated through activation of the ROS-ERK-CHOP pathway (Fig. 8C).

We found that gossypol could enhance TRAIL-induced apoptosis in human colorectal cancer cells. These results are in agreement with those of Yeow et al. (33), who reported that gossypol sensitizes colorectal cancer cells to TRAIL but did not address the mechanisms of sensitization. In this study, we identified several sensitization mechanisms. One of the mechanisms identified was the regulation of anti-apoptotic proteins. We down-regulated the expression of Bcl-2, Bcl-xL, and survivin (38). Embelin, an XIAP inhibitor, has also been shown to sensitize human glioma cells to TRAIL-mediated apoptosis by inducing the proteasomal degradation of survivin (38). Embelin, an XIAP inhibitor, has also been shown to enhance TRAIL-mediated apoptosis in malignant glioma cells (39).

We found that, in addition to down-regulating cell survival proteins, gossypol selectively induced DR5 expression. We have shown that DR5 up-regulation is critical for sensitization of cells to TRAIL, as gene silencing of the receptor (DR5) abolished TRAIL-induced apoptosis. Thus, DR5 up-regulation can sensitize cells to TRAIL-induced apoptosis (40). Numerous mechanisms have been described for induction of the death receptor, including ROS generation, p53 induction, and NF-κB. DDIT3 (DNA damage-inducible transcript 3), p21, and p53 have been shown to sensitize cells to TRAIL-induced apoptosis (40). Moreover, p53 has been shown to sensitize cells to TRAIL-induced apoptosis (40). Numerous mechanisms have been described for induction of the death receptor, including ROS generation, p53 induction, and NF-κB. DDIT3 (DNA damage-inducible transcript 3), p21, and p53 have been shown to sensitize cells to TRAIL-induced apoptosis (40). Moreover, p53 has been shown to sensitize cells to TRAIL-induced apoptosis (40). Numerous mechanisms have been described for induction of the death receptor, including ROS generation, p53 induction, and NF-κB. DDIT3 (DNA damage-inducible transcript 3), p21, and p53 have been shown to sensitize cells to TRAIL-induced apoptosis (40). Moreover, p53 has been shown to sensitize cells to TRAIL-induced apoptosis (40). Numerous mechanisms have been described for induction of the death receptor, including ROS generation, p53 induction, and NF-κB. DDIT3 (DNA damage-inducible transcript 3), p21, and p53 have been shown to sensitize cells to TRAIL-induced apoptosis (40). Moreover, p53 has been shown to sensitize cells to TRAIL-induced apoptosis (40). Numerous mechanisms have been described for induction of the death receptor, including ROS generation, p53 induction, and NF-κB. DDIT3 (DNA damage-inducible transcript 3), p21, and p53 have been shown to sensitize cells to TRAIL-induced apoptosis (40). Moreover, p53 has been shown to sensitize cells to TRAIL-induced apoptosis (40). Numerous mechanisms have been described for induction of the death receptor, including ROS generation, p53 induction, and NF-κB. DDIT3 (DNA damage-inducible transcript 3), p21, and p53 have been shown to sensitize cells to TRAIL-induced apoptosis (40). Moreover, p53 has been shown to sensitize cells to TRAIL-induced apoptosis (40).

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to those of a previous study showing that the induction of death receptors by LY303511 (a PI3K inhibitor) and zerbunome requires ERK1/2 activation (41). However, both of these studies showed that JNK is also required for death receptor induction.

In this study, DR5 up-regulation was also mediated through CHOP induction. We found that gossypol induced CHOP and that the gene silencing of CHOP by siRNA blocked the effect of gossypol on the induction of death receptors and on TRAIL-induced apoptosis. Our findings are similar to those of other studies that indicated that CHOP binds to the DR5 promoter and up-regulates this receptor expression (29, 42, 43). Although studies that indicated that CHOP binds to the DR5 promoter induced apoptosis. Our findings are similar to those of other studies that indicated that CHOP binds to the DR5 promoter and up-regulates this receptor expression (29, 42, 43). Although studies that indicated that CHOP binds to the DR5 promoter induced apoptosis. Our findings are similar to those of other studies that indicated that CHOP binds to the DR5 promoter and up-regulates this receptor expression (29, 42, 43).

Various BH3 mimetics, including gossypol, are now in clinical trials for treatment of cancer (51). The results of this study indicate that gossypol contributes to the sensitivity of tumor cells to TRAIL through numerous mechanisms (Fig. 8C). Additional studies of gossypol in animals are needed to fully realize the potential of gossypol therapy for cancer patients.

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