Gossypol, a phytochemical with BH3-mimetic property, sensitizes cultured thoracic cancer cells to Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand

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Supplemental material is available online.

Objectives: Chemotherapeutic agents sensitize cancer cells to Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL) via recruitment of the mitochondria-dependent activation of caspase and induction of apoptosis. This study was designed to evaluate whether gossypol, a phytochemical compound with BH3-mimetic property that functions as an inhibitor of Bcl2/BclXL, would sensitize cultured thoracic cancer cells to this death-inducing ligand.

Methods: Cancer cell lines from the lung (H460, H322), the esophagus (TE2, TE12), and the pleura (H290, H211) or primary normal cells were treated with gossypol+Apo2L/TRAIL combinations. Cell viability and apoptosis were evaluated by (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assays, respectively. Caspase 9 and 3 specific proteolytic activity in combination-treated cells was determined by fluorometric enzymatic assay.

Results: Gossypol, selectively cytotoxic to cancer cells and not primary normal cells, significantly sensitized thoracic cancer cells to Apo2L/TRAIL as indicated by 1.5- to more than 10-fold reduction of Apo2L/TRAIL 50% inhibitory concentration values in cells treated with gossypol+Apo2L/TRAIL combinations. Whereas less than 20% of cancer cells exposed to either gossypol (5 μ mol/L) or Apo2L/TRAIL (20 ng/mL) were dead, more than 90% of cells treated with the drug combinations were apoptotic. Combination-induced cytotoxicity and apoptosis was completely abrogated either by overexpression of Bcl2 or by the selective caspase 9 inhibitor. This combination was not toxic to normal cells.

Conclusion: Gossypol profoundly sensitizes thoracic cancer cells to the cytotoxic effect of Apo2L/TRAIL via activation of the mitochondria-dependent death signaling pathway. This study provides evidence for the profound anticancer activity of this drug combination and should be further evaluated as a novel targeted molecular therapeutic for thoracic cancers.

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Better understanding of the molecular basis of carcinogenesis and elucidation of signal transduction pathways regulating cell growth and death in normal cells and their roles in the process of malignant transformation offers great opportunities for the development of novel molecularly targeted anticancer therapy.

Abbreviations and Acronyms

IC ₅₀	= inhibitory concentration of 50%
MTT	= (4,5-dimethylthiazo-2-yl)-2,5-diphenyl tetrazolium bromide
TNF	= tumor necrosis factor
TRAIL	= tumor necrosis factor–related apoptosis-inducing ligand
TUNEL	= terminal deoxynucleotidyltransferase–mediated dUTP nick-end labeling

Within this context, therapeutic strategies aiming at direct induction of cell death by activation of the TRAIL receptor-mediated signal transduction pathways using the recombinant protein Apo2L/TRAIL (the Zn⁺⁺-coordinated homotrimer of the extracellular domain of TRAIL)¹ or the human agonistic anti-DR4 monoclonal antibodies² have attracted a great deal of attention for clinical development, as these biologics are selectively cytotoxic to cancer cells.¹⁻⁴ In fact, they are currently being tested in phase 1 clinical trials. Binding of Apo2L/TRAIL to its cognate functional receptors DR4 and/or DR5 activates the apical caspases 8 and 10, which either directly (type I pathway) or indirectly, via the mitochondria-dependent death-signaling cascade (type II pathway), activate the downstream executioner caspases 3, 6, and 7 to mediate apoptotic cell death.⁵

Despite expressing adequate levels of functional receptors DR4/DR5 for Apo2L/TRAIL, significant percentages of cancer cells exhibit resistance to the cytotoxic effect of this ligand in vitro. The molecular basis of this phenomenon is complex and yet to be fully elucidated.⁶ It is well described that exposure of Apo2L/TRAIL-refractory cancer cells to standard cytotoxic chemotherapeutic agents such as cisplatin and paclitaxel or to experimental targeted anticancer drugs like the histone deacetylase inhibitors (such as depsipeptide or valproic acid) profoundly sensitizes these cells to this death-inducing ligand.⁷⁻⁹ Our laboratory,^{7,8} as well as others,^{9,10} has demonstrated that chemotherapeutic drugs sensitize cancer cells to Apo2L/TRAIL via recruitment of the mitochondria-dependent caspase activation cascade. As such, the next logical step in the development of efficient TRAIL-based combination therapy for cancer is to directly target the mitochondria to stimulate its apoptosis-inducing property. The apoptogenicity of the mitochondria is tightly regulated by members of the Bcl2 superfamily, which respond to various intrinsic and extrinsic death-promoting stimuli and ultimately decide the fate of the affected cells.¹¹ Suppression of antiapoptotic protein expression by antisense or small interfering RNA techniques has been shown to sensitize cancer cells to TRAIL.¹² However, this approach, while valuable in providing proof of concept, has limited clinical application, mainly because of the redundancy of the Bcl2 superfamily antiapoptotic

members, the long half-life of Bcl2/BclXL proteins, and the inefficient delivery of ribonucleic acid sequences to every cancer cells in vivo. The antiapoptotic proteins Bcl2 or BclXL sequester the proapoptotic proteins Bad, Bid, Bim, Bax, and Bak by selective interaction between the BH3 domains of these proteins with their own BH3-binding pockets to prevent their translocation to the mitochondria, where they perform their death-inducing function.¹³ Intense research and development of small molecule inhibitors of Bcl2/BclXL have identified multiple compounds that can interact with the BH3-binding pockets of these proteins to inhibit their antiapoptotic function.¹⁴ These drugs are commonly referred to as BH3 mimetics.

Gossypol, a phytochemical found in cottonseed oil, was initially developed as an antifertility agent and subsequently recognized to have anticancer property¹⁵ (and refs therein). This compound was tested in many small phase 1 clinical trials.¹⁶ Gossypol, furthermore, was identified to be a BH3 mimetic by computer-assisted molecular modeling, by nuclear magnetic resonance imaging study, and also by fluorescence-polarization assay.¹⁷ We hypothesize that gossypol, via its Bcl2/BclXL inhibitory activity as a BH3 mimetic, synergistically interacts with Apo2L/TRAIL to mediate profound cytotoxicity and apoptosis of cultured thoracic cancer cells (cancer cell lines derived from primary tumors of the lung, the esophagus, or the pleura) in vitro.

Materials and Methods**Cell Lines and Reagents**

Cultured non–small cell lung cancer cells H460 and H322, esophageal cancer cells TE2 and TE12, and malignant pleural mesothelioma H211 and H290 were maintained in RPMI 1640 cultured medium supplemented with fetal calf serum (10% v/v), L-glutamine (1 mmol/L), streptomycin (100 µg/mL), and penicillin (100 U/mL). Normal human primary fibroblast and normal human bronchial epithelia were purchased from Cambrex (Walkerville, Md) and grown in their special culture media as per instructions of the vendor. Apo2L/TRAIL was obtained from Genentech Inc (South San Francisco, Calif) via a Cooperative Research and Development Agreement. Bcl2–overexpressing stable transfectants of TE2 (TE2Bcl2), TE12 (TE12Bcl2) cells, as well as their respective vector controls, were created and characterized as previously described.⁸ Selective caspase 9 inhibitor z-LEHD-fmk was purchased from Calbiochem (San Diego, Calif). Gossypol was obtained from Sigma-Aldrich (St Louis, Mo), dissolved in dimethylsulfoxide (25 mmol/L stock), divided into aliquots, and stored in –20°C. Fresh drug was used for each experiment to avoid repeated freeze-thaws.

Cytotoxicity and Apoptosis Assays

Cells were seeded in 96-well microtiter plates at predetermined plating densities appropriate for each cell line (1.0 to 2.0 × 10⁴ cells/well) to achieve 80% confluence at the time cells were to be treated with Apo2L/TRAIL. After an overnight incubation, cells were treated with either gossypol (2.5, 5.0, or 10.0 µmol/L) or Apo2L/TRAIL (5 to 100 ng/mL) or 1-hour pretreatment with

gossypol followed by addition of Apo2L/TRAIL (as 20 μ L of 10X stock) for a total of 200 μ L per well. Cell viability after treatment with Apo2L/TRAIL alone or with gossypol+Apo2L/TRAIL combinations was calculated as percentage of untreated controls or of gossypol-treated controls (to normalize for growth-inhibitory effect of gossypol, which ranged from 20% to 50% depending on the duration of exposure to this drug), respectively. The Apo2L/TRAIL 50% inhibitory concentration (IC_{50}) values were estimated from respective dose-response curves. Apoptosis after treatments with Apo2L/TRAIL (20 ng/mL) alone or in combination with gossypol (5.0 μ mol/L or 10.0 μ mol/L) was determined by the TUNEL*-based ApoBrdU assay (both from BD-Pharmingen, Torrance, Calif).

Caspase Activity Assay

Specific enzymatic activity of caspases 9 and 3 at intervals after the onset of treatment with Apo2L/TRAIL (20 ng/mL) with or without gossypol (5 μ mol/L) in TE2, TE12, and H460 cells was measured by enzymatic assays using fluorochrome-labeled substrates (R&D Systems, Inc, Minneapolis, Minn). The specific caspase activity, normalized for total proteins of cell lysates (BCA technique; Pierce Biotechnology, Rockford, Ill), was then expressed as fold of the baseline caspase activity of untreated control cells.

Statistical Analysis

Experiments were performed in quadruplicates unless otherwise indicated. Supra-additive cytotoxicity or apoptosis is defined as the cytotoxicity or apoptosis induced by the drug combinations that is, by statistical analysis, significantly greater than the sum of cytotoxicity or apoptosis induced by individual drug treatment. Results were presented as means $\pm$ standard errors of the means. The Student *t* test was performed when indicated.

Results

Antiproliferative Effect of Gossypol on Thoracic Cancer Cells

Continuous exposure of 6 cultured cancer cells to gossypol for 24 hours resulted in a significant dose-dependent reduction of cell proliferation as determined by the MTT[†] at 36 hours after the onset of drug treatment (Figure 1). The viability of primary normal cells, on the other hand, was not significantly affected by gossypol. Gossypol induced mild cell cycle arrest at G1/S checkpoint in TE2, TE12, and H322 cells but not in H460, H211, or H290 cells (data not shown). Moreover, gossypol mediated apoptosis of thoracic cancer cells, the magnitude of which was clearly dependent on the drug concentrations and the duration of drug exposure (Figure E1).

Figure 1. The effect of gossypol on the viability of thoracic cancer cells and primary normal cells. Cells were treated with gossypol for 24 hours and cell viability was determined by MTT assay at 36 hours after the onset of drug treatment. Data are presented as means $\pm$ SEM of 4 independent experiments. NHBE, Normal human bronchial epithelia; NHEK, normal human epidermal keratinocyte; micM, micromoles per liter.

Supra-additive Enhancement of Cytotoxicity by the Gossypol+Apo2L/TRAIL Combination

The intrinsic sensitivity to Apo2L/TRAIL varies significantly between cultured thoracic cancer cells lines, with H460, H322, and H290 being more susceptible to this death-inducing ligand (Apo2L/TRAIL IC_{50} values of 50 ± 3 , 40 ± 2 , and 53 ± 3 ng/mL respectively) while TE2, TE12, and H211 are Apo2L/TRAIL-resistant cells (Apo2L/TRAIL $IC_{50} > 100$ ng/mL). All of the cancer cell lines used in this study are type II cells.^{7,8} Concurrent treatment of cultured thoracic cancer cells to gossypol (2.5-10.0 μ mol/L) and Apo2L/TRAIL (5-100 ng/mL) resulted in significant enhancement of Apo2L/TRAIL-mediated cytotoxicity. Gossypol alone, at the treatment conditions used, mediated a cell line-dependent mild-to-moderate reduction of cell viability (20%-50%). The gossypol-mediated sensitization of cancer cells to Apo2L/TRAIL was most easily appreciated when the growth-inhibitory effect of the gossypol+Apo2L/TRAIL combinations was normalized for the gossypol-mediated cytotoxicity, as presented in the respective dose-response curves shown in Figure 2A for TE2, H460, and H211 cells. With Apo2L/TRAIL IC_{50} values used as indicators of cellular sensitivity to this ligand, it was apparent that there was 1.5- to greater than 10-fold reduction of Apo2L/TRAIL IC_{50} values in gossypol-treated cells in a dose-dependent manner (Figure 2B). This effect was observed in all cultured thoracic cancer cells regardless of their intrinsic sensitivity to Apo2L/TRAIL and particularly most pronounced in Apo2L/TRAIL-resistant TE2, TE12, and H211 cell lines. Comparable gossypol-mediated enhancement of Apo2L/TRAIL sensitivity was observed in cells treated with either 12 or 24 hours of gossypol+Apo2L/TRAIL, with no further reduc-

*TUNEL = terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling.

[†]MTT = (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

Figure 2A. Gossypol sensitizes cancer cells but not primary normal cells to Apo2L/TRAIL-mediated cytotoxicity. Cells were treated with Apo2L/TRAIL, gossypol, or gossypol+Apo2L/TRAIL for 24 hours, and cell viability was assessed by MTT at 36 hours. Data are presented as means $\pm$ SEM of 4 independent experiments. *NHBE*, Normal human bronchial epithelia; *micM*, micromoles per liter.

Figure 2B. Apo2L/TRAIL IC₅₀ values of cultured cancer cells treated with either Apo2L/TRAIL alone or gossypol+Apo2L/TRAIL combinations. These values were estimated from the respective dose-response curves and used as indicators of cellular sensitivity to Apo2L/TRAIL. Data are presented as means $\pm$ SEM of 4 independent experiments; #*P* < .0001 and +*P* < .01 versus Apo2L/TRAIL alone by analysis of variance and Bonferroni pairwise analysis. *micM*, Micromoles per liter.

tion of Apo2L/TRAIL IC₅₀ after 36 hours of exposure to the drug combination except in H211 cells (Figure E2). Whereas gossypol (5 μ mol/L) alone or Apo2L/TRAIL (20 ng/mL) alone induced less than 20% apoptosis in representative H460, TE12, and H211 cancer cells, massive supra-additive induction of apoptosis of more than 90% was observed in combination-treated cells (Figure 3). Most im-

portant, neither Apo2L/TRAIL alone nor gossypol+Apo2L/TRAIL combination induced apoptosis of primary human fibroblasts and normal human bronchial epithelia (Figures 2A and 3).

Mitochondria-dependent Death Signaling Cascade Plays Important Role in Enhanced Cytotoxicity by Gossypol+Apo2L/TRAIL

As gossypol is a BH3-mimetic drug, it most likely sensitizes cultured thoracic cancer cells to Apo2L/TRAIL via activation of mitochondria and recruitment of the type II death signaling pathway. Time-course evaluation of caspase activation in TE12 cells treated with gossypol, Apo2L/TRAIL, or gossypol+Apo2L/TRAIL combination indicated significant supra-additive enhancement of the specific proteolytic activity of caspase 9 (apical caspase of the mitochondria-mediated caspase activation cascade) and the downstream executioner caspase 3 (Figure E3). Such caspase activation directly contributed to Apo2L/TRAIL- or gossypol+Apo2L/TRAIL-mediated cytotoxicity and apoptosis, as these were significantly abrogated by either the general caspase inhibitor z-VAD-fmk or the caspase 9 inhibitor z-LEHD-fmk (Figure 4 and Figure E4). To further confirm the involvement of the mitochondria-mediated (type II) pathway in this process, we similarly treated Bcl2-overexpressing stable transfectants TE2Bcl2 and TE12Bcl2 cells with Apo2L/TRAIL or gossypol+Apo2L/TRAIL combinations. Bcl2 overexpression completely protected TE2 or TE12 cells from Apo2L/TRAIL-mediated cytotoxicity (as in TE12 cells) and gossypol+Apo2L/TRAIL-mediated cytotoxicity (in both TE2 and TE12 cells) (Figure 5).

Figure 3. Profound and supra-additive induction of apoptosis by the gossypol+Apo2L/TRAIL combinations in representative cancer cell lines H460, H211, and TE12. This combination does not induce apoptosis in primary normal cells. Data are presented as means $\pm$ SEM of 4 independent experiments. G, Gossypol; NHBE, Normal human bronchial epithelia; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling; micM, micromoles per liter.

Discussion

The intrinsic sensitivity of cancer cells to TRAIL can be enhanced by treating them with standard cytotoxic chemotherapeutics or with investigational drugs or gamma radiation.¹⁸ The molecular basis of such enhancement effect is complex, cell dependent, and not completely elucidated. Several possibilities have been postulated as the basis of chemotherapy-induced potentiation of TRAIL cytotoxicity:

upregulation of receptor expression, elevation of TRAIL-initiated signal transduction through the receptor complex and/or the intracellular pathways, and downregulation of natural inhibitor proteins of the caspase cascade after treatment of cancer cells with sensitizers. Whereas the molecular basis of enhanced TRAIL cytotoxicity by these combinations is diverse, functional and/or phenotypic modulation of the mitochondria-regulated caspase-activation cascade leading to amplification of the Apo2L/TRAIL-induced apoptosis appears to be the common theme. Our current working model of chemotherapy-induced sensitization of cancer cells to Apo2L/TRAIL is as follows: Apo2L/TRAIL engagement of its functional receptors DR4/DR5, via the formation of the death-induced signaling complex, results in an initial minute activation of caspase 8, which activates the mitochondria via cleavage of BID to form tBID. In sensitive type II cells, this would be adequate to engage the mitochondrial arm of the caspase activation cascade and the amplification feedback loop to potentiate caspase 8 activation. In resistant cells, the chemosensitizer “primes” the mitochondria via phenotypic or functional alteration of proapoptotic and antiapoptotic proteins of the Bcl2 superfamily to make the mitochondria more susceptible to tBID and thus effectively engage the mitochondria-dependent caspase activation pathway.^{7,8} Proofs of principle of such model have been demonstrated by sensitization of cancer cells to TRAIL by targeting the mitochondria using BclXL small interfering RNA¹² or the mitochondriotropic cytotoxic drug betulinic acid.¹⁹ The gossypol+Apo2L/TRAIL drug combination was thus designed on the basis of the observation that

Figure 4. Significant reduction of gossypol+Apo2L/TRAIL-mediated apoptosis by the general caspase inhibitor Z-VAD-fmk or the caspase 9 inhibitor Z-LEHD-fmk in H211 and TE12 cells. Representative data of 3 independent experiments with similar results are shown.

Figure 5. Overexpression of Bcl2 protects cancer cells against the profound gossypol+Apo2L/TRAIL-mediated cytotoxicity in TE2 and TE12 Bcl2-overexpressing stable transfectants. Vector control stable transfectants were equally susceptible to the cytotoxic effect of this drug combination (data not shown). Data are presented as means $\pm$ SEM of 4 independent experiments; [#] $P < .001$ between gossypol+Apo2L/TRAIL in parental cells versus in Bcl2-overexpressing stable transfectants. G, Gossypol; GFP, green fluorescent protein.

mitochondria are essential for the chemotherapy-induced potentiation of Apo2L/TRAIL cytotoxicity and the hypothesis that functional inhibition of Bcl2/BclXL using BH3-mimetic drugs like gossypol would sensitize cancer cells to this death-inducing ligand. Indeed, gossypol synergistically interacts with Apo2L/TRAIL to induce profound induction of apoptosis in cultured thoracic cancer cells and, most important, not in primary normal cells. Experiments are also in progress to elucidate the exact molecular mechanism(s) by which gossypol interacts with Apo2L/TRAIL to mediate profound cytotoxicity in cancer cells.

The recent discovery of small-molecule chemical inhibitors of Bcl2/BclXL by virtue of their ability to interact with the BH3-binding pocket of these proteins has suggested a new approach for cancer therapy. These compounds exhibit strong anticancer activity in many tumor cells, especially in those expressing high levels of Bcl2/BclXL.¹⁵ They also potentiate the tumoricidal effects of standard cytotoxic chemotherapeutics or radiotherapy^{20,21} as well as of Apo2L/TRAIL, as reported herein by our group or by other investigators.^{22,23} Gossypol interacts with the BH3-binding pockets of 4 antiapoptotic proteins, Bcl2, BclXL, BclW, and Bfl1, displacing BH3 peptide with an IC₅₀ of about 0.5 μ mol/L.¹⁴ Naturally occurring gossypol exists as a racemic mixture of (+) and (–) enantiomers. Interestingly, the (–) enantiomer of gossypol possesses higher affinity for Bcl2 and BclXL, reduced serum binding, and greater anticancer activity in in vitro assays than the (+) enantiomer or racemic gossypol.¹⁵ Gossypol, a polyphenolic containing two aldehyde groups, is a highly reactive compound, which may explain some of toxicities originally seen in phase 1 clinical trials of this drug in addition to producing unfavorable pharmacologic properties.¹⁴ Structural modification of gos-

sypol by removal of these aldehyde groups creates apogossypol, a semisynthetic BH3-mimetic drug with a better pharmacologic profile. The (–) gossypol and apogossypol are being jointly developed as Bcl2-targeted anticancer drugs by the National Cancer Institute with Ascenta Therapeutics, Inc (San Diego, Calif), and the Burnham Institute (La Jolla, Calif), respectively.¹⁴ Evaluation of the therapeutic efficacy and toxicity profile of the (–)gossypol + Apo2L/TRAIL combination in vitro and in vivo animal model of nude mice bearing human cancer xenografts is the current focus of our laboratory effort to facilitate translation of the gossypol+Apo2L/TRAIL drug combination into clinical application. Moreover, our findings form the basis for further evaluation of this combination strategy using synthetic compounds specifically designed as BH3 mimetics such as BH3I-2' or HA14-1 in cultured thoracic cancer cells.

In summary, we report for the first time profound cytotoxicity and apoptosis mediated by the gossypol+Apo2L/TRAIL combination in cultured thoracic cancer cells via a process that is caspase-mediated and dependent on the mitochondria-regulated death signaling pathway. More important, this drug combination is not toxic to primary normal cells. Our study, in conjunction with other reports cited in this manuscript, provides a strong rationale for further development of Apo2L/TRAIL-based therapy in combination with BH3 mimetics as novel targeted molecular therapeutic for cancers.

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Discussion

Dr David R. Jones (Charlottesville, Va). I congratulate you on a nice presentation.

You hypothesized that gossypol would enhance Apo2L and TRAIL-mediated apoptosis through its ability to inhibit Bcl2 and perhaps BclXL. Do you have any evidence that this is indeed the pathway? For instance, have you performed any siRNA experiments with either Bcl2 or BclXL that would be more confirmatory of your hypothesis?

Second, gossypol has been shown to increase both Fas and Fas ligand expression, which has been suggested to account for at least 75% of apoptosis in certain non-small cell lung cancer cell lines. Do you think this may be the mechanism through which gossypol is truly working, and, if not, why not?

Finally, if you believe you can move this preclinical data toward a phase 1 trial, can you tell us whether there are any combined or additive toxicities of these two biologic agents?

Dr Yeow. Thank you, Dr. Jones.

To answer your first question, gossypol inhibiting Bcl2/BclXL, it has not been shown by us but it has been shown by previous authors using colon carcinoma that gossypol does interact as a BH3 mimetic, does bind to the pocket of Bcl2/BclXL. They showed it through one assay that demonstrates that if two proteins come together, there is fluorescence. They were also able to show it in another one using x-ray crystallography. Thus it has been shown that gossypol will bind to the pocket.

Second, in terms of gossypol affecting 70% of non-small cell lung carcinomas and that being the sole agent, we are using a concentration of gossypol that does not do that. We are aiming for suboptimal doses, and we are using suboptimal doses of both gossypol and TRAIL. We hope that they will combine and selectively target the cancer cells.

Third, the ability to take it to a phase 1 trial, the gossypol that we are using is a mix of positive and negative enantiomers, and it is not as potent as the negative enantiomer, which is the agent that has been taken to clinical trials. There is a better secondary, tertiary, third generation of gossypol that could potentially be taken to clinical trials. We do not anticipate gossypol as a mix to be taken there at that level.

Figure E1. Gossypol-mediated induction of apoptosis. Cells were exposed to gossypol (5.0 or 10.0 $\mu\text{mol/L}$) for either 24 hours or 48 hours and harvested at 48 hours after the onset of gossypol treatment for quantitation of apoptosis using the TUNEL-based ApoBrdU assay. Data are presented as means $\pm$ SEM of 4 independent experiments, # $P < .001$ versus gossypol 5.0 $\mu\text{mol/L} \times 48$ hours, + $P < .01$ versus gossypol 10.0 $\mu\text{mol/L} \times 24$ hours, * $P < .001$ versus gossypol 5.0 $\mu\text{mol/L} \times 24$ hours. *G*, Gossypol; *TUNEL*, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling; *micM*, micromoles per liter.

Figure E2. The effect of the duration of gossypol+Apo2L/TRAIL treatment on gossypol-mediated reduction of Apo2L/TRAIL IC₅₀ values in representative H211, H460, and TE12 cells. Cells were treated with gossypol+Apo2L/TRAIL combination for 12 hours, 24 hours, or 36 hours, and cell viability was quantified by MTT at 36 hours after the onset of Apo2L/TRAIL exposure. Data are presented as means $\pm$ SEM of 4 independent experiments; # $P = .0061$ by ANOVA with statistically significant difference ($P < .01$) only between gossypol+Apo2L/TRAIL 12 hours and 36 hours; ** $P = .071$ and + $P = .077$ by ANOVA and $P > .05$ by Bonferroni pairwise analysis. *G*, Gossypol.

Figure E3. Supra-additive activation of caspases 9 and 3 specific proteolytic activity in H460, TE2, and TE12 cells treated with the gossypol+Apo2L/TRAIL combination. Cells were treated with gossypol (5.0 μ mol/L), Apo2L/TRAIL (10 ng/mL for H460 cells or 20 ng/mL for TE2 and TE12 cells), or gossypol+Apo2L/TRAIL combination. Representative data of 3 independent experiments are shown here. G, Gossypol.

Figure E4. Abrogation of gossypol+Apo2L/TRAIL-mediated cytotoxicity by Z-VAD-fmk. Cells were treated with 24 hours of gossypol, Apo2L/TRAIL, or gossypol+Apo2L/TRAIL with or without prior exposure to Z-VAD-fmk (60 μ m). Cell viability was determined by MTT 36 hours after the onset of drug exposure. Data are presented as means $\pm$ SEM of 4 independent experiments; #*P* < .0001, +*P* = .0006 between gossypol+Apo2L/TRAIL with and without Z-VAD-fmk.