Published in final edited form as:

Anticancer Drugs. 2010 March ; 21(3): 297–305. doi:10.1097/CAD.0b013e3283350e43.

NF-kappaB is involved in SHetA2 circumvention of TNF-alpha resistance, but not induction of intrinsic apoptosis

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Abstract

Treatment of cancer with tumor necrosis factor- α (TNF α) is hindered by resistance and toxicity. The Flexible Heteroarotinoid (Flex-Het), SHetA2, sensitizes resistant ovarian cancer cells to TNF α -induced extrinsic apoptosis, and also induces intrinsic apoptosis as a single agent. This study tested the hypothesis that nuclear factor kappa B (NF- κ B) is involved in SHetA2-regulated intrinsic and extrinsic apoptosis. SHetA2 inhibited basal and TNF α - or hydrogen peroxide-induced NF- κ B activity through counter-regulation of upstream kinase (IKK) activity, inhibitor protein (I κ B α) phosphorylation, and p65 NF- κ B subunit nuclear translocation, but independently of reactive oxygen species (ROS) generation. Ectopic over-expression of p65, or treatment with TNF α receptor 1 (TNFR1) siRNA or a caspase 8 inhibitor, each attenuated synergistic apoptosis by SHetA2 and TNF α , but did not affect intrinsic apoptosis caused by SHetA2. In conclusion, NF- κ B repression is involved in SHetA2 circumvention of resistance to TNF α -induced extrinsic apoptosis, but not in SHetA2 induction of intrinsic apoptosis.

Keywords

Apoptosis; TNF α Resistance; NF- κ B; IKK repression; Ovarian Cancer

Introduction

Development of resistance to platinum-based chemotherapy is the major cause of ovarian cancer deaths. New treatment approaches include administration of death receptor ligands, such as Tumor Necrosis Factor Alpha (TNF α), to induce the extrinsic apoptosis pathway. TNF α was the first death receptor ligand to be considered as an antitumor drug, however the clinical trials failed due to TNF α resistance and unacceptable toxicity [1,2]. Currently multiple NCI-sponsored clinical trials are evaluating various ways of tumor-selective delivery of TNF α to reduce the toxicity (Protocols NCT00483509, NCT00181025 and NCT00496535, www.cancer.gov). SHetA2 is a Flexible Heteroarotinoid (Flex-Het) compound that has been shown to sensitize ovarian cancer cells to TNF α -induction of the extrinsic apoptosis pathway, but the mechanism remains to be elucidated [3].

Induction of apoptosis by TNF α binding to the TNF receptor 1 (TNF-R1) is hindered by simultaneous activation of the Nuclear Factor kappa B (NF- κ B) survival pathway [4,5]. NF-

κ B is an inducible dimeric transcription factor complex of subunits (p65 (RelA), RelB, Rel (c-Rel), p50/p105, and p52/p100) that are upregulated in many cancers [6–8]. The activity of NF- κ B is regulated by Inhibitor of NF- κ B (I κ B) proteins, which bind NF- κ B dimers and sequester them in the cytoplasm by masking the nuclear localization sequences [9]. The I κ B proteins in turn are regulated by the I κ B kinase (IKK) complex composed of two catalytic subunits: IKK α and IKK β , and a regulatory subunit IKK γ /NEMO [10]. TNF α and other cytokines induce I κ B α phosphorylation by IKK, thus targeting I κ B α for ubiquitin-dependent proteasomal degradation. The released NF- κ B subunits translocate into the nucleus where the dimers bind to consensus DNA sequences (κ B sites) in several hundred genes involved in immune response, growth, tumorigenesis, inflammation, carcinogenesis, and apoptosis [8,11,12].

We hypothesized that SHetA2 sensitizes cancer cells to TNF α by interfering with the NF- κ B survival pathway. In support of this hypothesis, SHetA2 has been shown to counter-regulate expression of NF- κ B-controlled genes [13–15] and induce reactive oxygen species (ROS) generation that has the potential to regulate redox-sensitive transcription factors, such as NF- κ B [16,17]. Furthermore, a systems biology analysis of microarray data implicated the TNF α pathway as a major player in the SHetA2 mechanism of action [18]. Previous studies demonstrated that SHetA2 induces apoptosis in cancer cells, with very little effects on normal cells, by directly targeting mitochondria and the Bcl-2 family of proteins leading to induction of the caspase 9-dependent intrinsic apoptosis pathway [16,17,19]. TNF α on the other hand initiates the caspase 8-dependent extrinsic apoptosis pathway [20]. Although the intrinsic and extrinsic pathways are initiated through separate mechanisms, they converge through activation of the Bid protein [21]. The objective of this study was to determine if SHetA2 treatment alters NF- κ B signaling and potential down-stream effects on intrinsic apoptosis and sensitization to TNF α -driven extrinsic apoptosis in ovarian cancer cells.

Materials and methods

Reagents and Cell Lines

SHetA2 was synthesized by Dr K. Darrel Berlin [22], dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C . Human recombinant TNF α (R&D systems, Minneapolis, MN), TRAIL (Peprotech, Rocky Hill, NJ), Caspase 8/FLICE Inhibitor (Z-IETD-FMK) (Medical & Biological Laboratories Ltd, Japan) and MG132 (Calbiochem, San Diego, CA) were stored according to manufacturer's instructions. A2780 ovarian cancer cells (Michael Birrer, National Cancer Institute, Bethesda, MD) and SK-OV-3 ovarian cancer cells (ATCC, Manassas, VA) were cultured in RPMI 1640 supplemented with 10% FBS, 1mmol/L HEPES buffer, 1mmol/L sodium Pyruvate, and 1X antimycotic/antibiotic.

Plasmids, transfections and luciferase reporter assays

Cultures were co-transfected with a 10:1 ratio of NF- κ B specific reporter plasmid and pNF- κ B-luc (Dr Wei Qun Ding, University of Oklahoma Health Sciences Center) to evaluate NF- κ B transcriptional activity and pRenilla Luciferase (pRL)-thymidine kinase (TK) (Dr A.L. Olson University of Oklahoma Health Sciences Center) as a transfection control using Metafectene Pro (Biontex, Germany). For p65 over-expression, 3 μ g pCytomegalovirus (pCMV)4 or pCMV4-p65 (Dr WC Greene, University of San Francisco, CA) were co-transfected with pNF- κ B-luc and pRL-TK to assess NF- κ B activity or transfected alone to determine effects on apoptosis or cell viability. Twenty-four hours after transfection, cells were trypsinized and re-plated onto 12-well plates. After further 24-hour incubation, cultures were treated with 10 μ M SHetA2 for various times. In some instances, 20 ng/ml TNF α was added for the last 30 minutes of treatment or varying concentrations of H₂O₂ was added for the last 240 minutes. Cells were prepared and evaluated for luciferase activity as described [13]. Firefly luciferase activity was normalized to Renilla luciferase activity and expressed as fold of

luciferase activity in untreated cells. For additional controls the pTATA-box binding protein (pTBP)-luc or cFos-luc (both from Dr. D.L. Johnson, University of Southern California) were co-transfected with pRL-TK plasmid. For antioxidants studies, A2780 cultures were pretreated with butylated hydroxyanisole (BHA); 50 μ M), MnTBAP (100 μ M), Trolox (0.5 mM), or NAC (5 mM) for 24 hours before SHetA2 or DMSO treatments. All experimental results are presented as the average and standard error of three independent experiments performed in triplicate.

Western blotting

Whole cell extracts prepared from cultures using M-PER (Pierce, Rockford, IL) were evaluated by Western blots using the following primary antibodies: IKK α , GAPDH, PARP-1, β -actin, (Santa Cruz Biotechnology, Santa Cruz, CA); I κ B α ser 32/36, total I κ B α , IKK ser 176/180, Bid, Caspase 8, Caspase 9, and Caspase 3 (Cell Signaling Technology, Danvers, MA) as previously described [15]. Results presented are representative blots of three independent experiments producing similar results.

Immunocytochemistry

Cells were seeded at 20,000 cells per chamber in an 8-chamber slide, grown overnight and treated with 10 μ M SHetA2 for 16 hours. TNF α (20ng/ml) was added for the last 20 minutes of treatment. After treatment, immunocytochemistry was performed using p65 primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and Alexa-488 conjugated secondary antibody (Invitrogen, Carlsbad, CA) with propidium iodide (PI) nuclear staining. Results presented are representative photomicrographs of three independent experiments producing similar results.

Preparation of IKK complex and Immunocomplex Kinase assay

IKK activity was evaluated using the protocol described by Lou and Kaplowitz [23]. Briefly, whole cell extracts (250 μ g) were immunoprecipitation (IP) with the IKK α antibody and protein G-agarose (Roche, Indianapolis, IN). The IKK activity of precipitates was measured using 1 μ g of GST-fused I κ B α (Millipore, Billerica, MA) as substrate in the presence of 20 μ l kinase buffer supplemented with 0.3mM ATP in a 30-minute incubation at 30°C. I κ B α phosphorylation by IKK was visualized by Western blotting of kinase reaction components using ser 32/36 phospho-specific antibody (Cell Signaling Technology, Danvers, MA). Results presented are representative blots of three independent experiments producing similar results.

siRNA experiments

Cultures were transfected with siRNA from the NF- κ B signaling pathway SiRNA Array (SABiosciences, Frederick, MD) using the manufacturer's reverse transfection protocol. Sixteen hours after transfection, media was changed and cells were treated with 10 μ M SHetA2 alone or in combination with 20 ng/ml TNF α for 24 hours. Control cultures were treated with DMSO solvent only. Apoptosis was measured using DNA fragmentation cell death ELISA kit (Roche, Indianapolis, IN). Results are presented are representative of two independent repeats of the experiment producing similar results.

Cell viability and apoptosis assays

Twenty-four hours after transfection with pCMV4-p65 plasmids or mock-transfection, cultures were treated with a range of SHetA2 concentrations alone or in combination with 20 ng/ml TNF α for 24 hours. Cell Viability was measured with MTS reagent (Promega, Madison, WI) and expressed as fold of the untreated control. Apoptosis was detected using Annexin V FITC/PI from the Vybrant Apoptosis assay kit #3 (Invitrogen, Carlsbad, CA) and evaluated by flow cytometry using a Becton Dickinson FACS Caliber automated bench- top flow cytometer at

an excitation wavelength of 488 and observation wavelengths of 530 and 575nm. All results are presented as the average and standard error of two to three independent experiments performed in duplicate.

Data analysis and statistics

Graphpad Prism software and Microsoft Excel were used to plot the graphs and test for statistical significance using two-tailed paired t-test and significance was established at 95% confidence ($P < 0.05$). For comparing more than three groups at once, one way ANOVA with Bonferroni post test was used to compare all pairs of groups with significance established at $p < 0.05$.

Results

SHetA2 inhibits basal, TNF α - and H₂O₂-induced NF- κ B activity

Regulation of NF- κ B activity was evaluated in A2780 ovarian cancer cells transfected with NF- κ B reporter and transfection control plasmids. SHetA2 suppressed basal NF- κ B activity in a dose- and time-dependent manner within the earliest time point of 30 minutes and lowest dose of 2.5 μ M evaluated (Fig. 1a and b, respectively). Induction of NF- κ B activity through two different mechanisms, TNF α (Fig. 1c) and hydrogen peroxide (H₂O₂) (Fig. 1d), was also repressed by SHetA2. Lack of SHetA2 repression of different promoter sequences in the c-Fos and TBP driven reporter plasmids demonstrated that this repression was specific for NF- κ B sites and not due to a global inhibition of transcription (Fig. 1c). Similar results were observed for the SK-OV-3 ovarian cancer cell line (data not shown).

ROS are not involved in SHetA2 suppression of NF- κ B activity

Since NF- κ B activity can be regulated by the redox state of the cell, and SHetA2 can induce reactive oxygen species (ROS) generation ([16,22], we hypothesized that SHetA2 repression of NF- κ B is a result of increased intercellular ROS. To test this, NF- κ B reporter assays were performed in the presence and absence of various antioxidants. Several ROS scavengers did not prevent SHetA2 inhibition of basal (Fig. 2a) or TNF α -induced (Fig. 2b) NF- κ B activity. Although NAC, a precursor of the natural cellular tripeptide antioxidant, glutathione, prevented SHetA2 repression of basal NF- κ B activity (Fig. 2a), it had no effect on repression of TNF α -induced NF- κ B activity (Fig. 2b) or induction of apoptosis (Fig 2c and d).

Upstream events leading to SHetA2 inhibition of NF- κ B

Phosphorylation of I κ B α on serine 32 and serine 36 triggers degradation of I κ B α and release of NF- κ B repression. Western blot analysis of cytoplasmic extracts demonstrated that TNF α treatment of A2780 ovarian cancer cells resulted in phosphorylation of I κ B α and reduction in total I κ B α levels, while SHetA2 inhibited these effects at all time-points evaluated (Fig. 3a). This inhibition was followed by preservation of I κ B α total levels only at the 30-minute time-point suggesting an I κ B α -independent mechanism in inhibition of NF- κ B activity upon prolonged exposure with SHetA2.

Inhibition of IKK activity was hypothesized to be responsible for SHetA2 inhibition of basal and induced I κ B α phosphorylation because activation of this kinase is required for both TNF α - and H₂O₂-induced phosphorylation of I κ B α and p65. An immune complex assay demonstrated that SHetA2 treatment of intact cells suppressed TNF α -induced IKK activity at each time point evaluated (Fig. 3b). Basal IKK activity in the absence of TNF α was also repressed by SHetA2 (data not shown). The inhibition appears to be indirect however since SHetA2 did not alter IKK activity when added directly to the kinase assay *in vitro* (Fig. 3c).

Because I κ B α phosphorylation and subsequent degradation is known to release the cytoplasmic retention of NF- κ B subunits, it was anticipated that SHetA2 repression of this phosphorylation would prevent nuclear accumulation of NF- κ B subunits. Immunocytochemistry revealed that the p65 protein was predominantly localized in the cytoplasm in both DMSO and SHetA2 treated cells, and that TNF α induced p65 nuclear translocation, while SHetA2 inhibited this translocation (Fig. 3d).

Evaluation of intrinsic and extrinsic apoptosis pathway components and crosstalk in SHetA2 regulated apoptosis

The activation and crosstalk of the intrinsic and extrinsic apoptosis pathways in the apoptosis mechanisms induced by SHetA2, as a single agent, and when used as a TNF α sensitizer, were evaluated. TNF α , as a single agent, did not induce apoptosis in either the A2780 or SK-OV-3 ovarian cancer cell lines, as depicted by absence of Annexin V staining to detect apoptosis (Fig. 4a). Lack of cleavage (indicating activation) of PARP-1, caspase 8, caspase 9 and caspase 3 in cultures treated with TNF α only confirmed resistance of the A2780 cell line (Fig. 4b) and SK-OV-3 cell line (data not shown). Simultaneous treatment of cells with TNF α and SHetA2 resulted in potentiation of SHetA2-induced apoptosis in both cell lines (Fig. 4a) and induction of PARP-1, caspase 3 and caspase 8 cleavage in the A2780 cell line (Fig. 4b) and SK-OV-3 cell line (data not shown). The weak cleavage of caspase 9 observed in A2780 cultures treated with SHetA2 only was enhanced by addition of TNF α , most likely due to cleavage of Bid (Fig. 4b), which is known to connect the extrinsic to the intrinsic apoptosis pathway [21].

Consistent with previous studies demonstrating that SHetA2 acts through the intrinsic apoptosis pathway [16], a caspase 8 inhibitor did not prevent SHetA2 induced apoptosis (Fig. 4c). The ability of the caspase 8 inhibitor to partially attenuate apoptosis induced by simultaneous SHetA2 and TNF α treatment confirmed the participation of the extrinsic pathway in the synergistic induction of apoptosis (Fig. 4c). The residual potentiation of apoptosis is most likely due to contribution from caspase 10, another initiator caspase for the extrinsic apoptotic pathway (29). To complement this data knockdown of TNFR1, the cognate receptor for TNF α by a validated siRNA reduced potentiation of apoptosis (Fig. 4d). The partial effect may be due to a low 10% knockdown of TNFR1.

Role of NF- κ B repression in TNF α sensitization, but not intrinsic apoptosis

If SHetA2 induction of apoptosis is mediated by inhibition of the NF- κ B cell survival pathway as our studies suggests, then over-expression of p65 to elevate NF- κ B activity should modulate apoptosis induction by SHetA2 alone and when combined with TNF α . To test this hypothesis, ovarian cancer cells were transfected with a constitutive p65 cDNA expression vector. Over-expression of p65 in ovarian cancer cells resulted in increased p65 protein expression and enhanced NF- κ B transcriptional activity (Fig. 5a). SHetA2 treatment inhibited NF- κ B transcriptional activity in both mock and p65 overexpressing cells (Fig. 5a). The level of NF- κ B activity in p65 transfected cells was significantly higher than mock transfected cells even when treated with SHetA2, thus providing a model to test our hypothesis (Fig. 5a). Under these conditions ectopic over-expression of p65 did not repress loss of cell viability and apoptosis induced by SHetA2 as a single agent (Fig. 5c), which is consistent with finding that NAC reversal of SHetA2 NF- κ B repression did not attenuate SHetA2 induced apoptosis (Fig. 2d). When SHetA2 was combined with TNF α treatment however, the p65-induced elevated NF- κ B activity prevented the enhanced decrease in cell viability caused by combination of the two agents (Fig. 5b), indicating a vital role of NF- κ B repression in the mechanism of SHetA2 sensitization of ovarian cancer cells to TNF α . These results were confirmed in at the level of apoptosis (Fig. 5c) and in the SK-OV-3 ovarian cancer cell line (data not shown). To further support the role of NF- κ B inhibition in sensitization of ovarian cancer cells to TNF α , validated

siRNA from the NF- κ B signaling pathway array against p65 and p50 NF- κ B subunits significantly sensitized A2780 cells to TNF α -induced cell death (Fig 5d).

Discussion

The results of this study demonstrate that SHetA2 suppresses NF- κ B transactivation activity in ovarian cancer cell lines. In support of our hypothesis that this NF- κ B repression is involved in the mechanism of SHetA2 regulated extrinsic apoptosis, prevention of the reduced levels of NF- κ B activity by over-expression of the p65 NF- κ B protein subunit also suppressed the synergistic induction of apoptosis when SHetA2 was used in combination with TNF α . In opposition to the hypothesis that NF- κ B repression is involved in SHetA2 regulation of intrinsic apoptosis, the elevated NF- κ B activity did not attenuate apoptosis caused by SHetA2 as a single agent. These different effects are consistent with the different pathways induced by SHetA2 and TNF α that lead ultimately to the apoptotic death of the cell.

Induction of the intrinsic apoptosis pathway by SHetA2 has been well characterized and validated in multiple cancer cell types [13,16,17,24,25], while binding to TNF α to its cell surface death receptors is known to induce the extrinsic apoptosis pathway [20]. The ability of a caspase 8 inhibitor to prevent the synergistic level of apoptosis, but not the level of apoptosis induced by SHetA2 as a single agent, confirms that the enhanced apoptosis induced by the SHetA2 and TNF α combination is due to induction of the extrinsic apoptosis pathway, which adds to the level of apoptosis caused by SHetA2-induction of the intrinsic apoptosis pathway. These results are consistent with the findings of others that various NF- κ B inhibitors can sensitize other types of cancer cell lines to death receptor ligands [26–28], and that induction of intrinsic apoptosis by curcumin does not involve NF- κ B repression [29]. While constitutive NF- κ B activation has been reported to prevent induction of intrinsic apoptosis in lymphoma cell lines [30], our results indicate that the level of basal NF- κ B activity in ovarian cancer cell lines does not cause resistance to intrinsic apoptosis.

The mechanism of SHetA2 NF- κ B repression appears to involve upstream signaling components, as this drug caused repression of IKK phosphorylation of I κ B α leading to inhibition of translocation of the p65 NF- κ B subunit to the nucleus where it can regulate gene expression. Expression of several NF- κ B controlled genes, including E-Cadherin, VEGF and Cyclin D1 have been shown to be counter-regulated by SHetA2 [13–15].

The mechanism of SHetA2 IKK inhibition appears to be indirect in that SHetA2 only caused repression when administered to intact cells and not when tested *in vitro*, suggesting that a metabolic product of SHetA2 may be responsible for the repression, or that SHetA2 may inhibit a kinase upstream of IKK. The NF- κ B-inducing kinase (NIK) is a likely candidate for the next up-stream level of control, since SHetA2 inhibits induction of NF- κ B by both TNF α and H₂O₂, which activate NF- κ B through different mechanisms that converge at activation NIK, which then activates the IKK complex [31,32]. Alterations in IKK phosphorylation may not be entirely responsible for mediating SHetA2 regulation of IKK activity however, because another mechanism of IKK activation has been observed. IKK phosphorylation has been shown to be insufficient for induction of IKK activity by G protein-coupled receptors, which can increase IKK activity by stimulating physical association of the IKK complex with CARMA3 (caspase recruitment domain (CARD)-associated and membrane associated guanylate kinase domain (MAGUK)-containing protein 3) [33,34].

Although NF- κ B activity can be regulated by the redox state of the cell, it is unlikely that the known generation of intracellular ROS by SHetA2 is involved in the NF κ B repression or regulation of apoptosis. Carefully controlled anti-oxidant studies demonstrated that induction of intrinsic apoptosis by SHetA2 can proceed despite quenching of the mitochondrial and

cytoplasmic ROS generated [16]. The ROS generation therefore appears to be a consequence, and not a cause, of the mitochondrial swelling and apoptosis induced by SHetA2 in cancer cells. Although metabolism studies have demonstrated that SHetA2 binds to the natural cellular tripeptide antioxidant, glutathione [35], this activity is unlikely to be responsible for the ROS generated because glutathione is present in mM concentrations, while only μM SHetA2 concentrations are required for induction of mitochondrial swelling, ROS generation and intrinsic apoptosis [13,16,19,36]. The ability of the glutathione precursor, NAC, to prevent SHetA2-repression of basal, but not induced, NF- κB activity levels could be explained by the ability of NAC to prevent oxidation of cys 62 on the p50 protein and thereby prevent the decreased DNA binding caused by this oxidation [37]. While this suggests that SHetA2 causes p50 oxidation, the inability of general ROS scavengers to prevent SHetA2 repression of basal NF- κB activity discounts this possibility.

The results from these studies support a model, in which repression of NF- κB signaling sensitizes cells to TNF α -induced extrinsic apoptosis, while SHetA2 induction of intrinsic apoptosis can occur regardless of NF- κB status (Fig. 6). Upon binding to its cognate receptor, TNFR1, TNF α induces a survival pathway governed by NF- κB transcription factors, which blocks extrinsic apoptosis. SHetA2 inhibits TNF α induction of the NF- κB pathway by inhibiting IKK-induced-phosphorylation and subsequent proteasomal degradation of I $\kappa\text{B}\alpha$ thereby retaining the p65 NF- κB subunit in the cytoplasm. The reduced NF- κB transcription factor activity removes repression of extrinsic caspase 8-dependent apoptosis by TNF α .

In summary, these results demonstrate that SHetA2 repression of NF- κB activity circumvents resistance to TNF α -induced extrinsic apoptosis, thereby enhancing the level of apoptosis caused by a NF- κB -independent intrinsic pathway induced by SHetA2 as a single agent. SHetA2, which has not shown any general toxicity, liver toxicity, skin irritancy, mutagenicity or teratogenicity in several animal models [13,36,38], and therefore could be used to reduce the effective dose of TNF α thereby reducing inflammation and organ injury observed at high concentrations of TNF α . These results also suggest that SHetA2 may sensitize ovarian cancer cells to other death receptor ligands currently in clinical trials, such as TNF related apoptosis inducing ligand (TRAIL) and agonistic antibodies to TRAIL.

Acknowledgments

This study was supported by a grant from the United States National Institutes of Health (NIH) National Cancer Institute (NCI) grant CA106713. We would like to thank the following: Dr WC Green from University of California, San Francisco, Gladstone Institute of Virology and Immunology for providing the p65 expression construct used in these studies; Dr Wei-Qun Ding from University of Oklahoma Health Sciences Center Department of Pathology for the advice and training in performing Luciferase reporter assays; Jim Henthorn, the Director of University of Oklahoma Health Sciences Center Flow Cytometry Core lab for training and advice in flow cytometry.

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Fig. 1. Effects of SHetA2 on basal and inducible NF-κB activity. (a–b)

Cultures co-transfected with the NF-κB inducible Firefly luciferase reporter plasmid (pNF-κB-luc) and the transfection control Renilla luciferase reporter plasmid (pRL-TK) were treated with 10 μM SHetA2 for the indicated times (a) or treated with the indicated doses of SHetA2 for 12 hours (b) and tested for luciferase activity. (c) Cultures co-transfected with NF-κB, TBP- or c-Fos-driven luciferase reporter plasmid and the pRL-TK transfection control plasmid were treated for 4 hours with 10 μM SHetA2. TNFα (10 ng/ml) was added for the last 30 minutes of incubation prior to evaluation of luciferase activity. (d) Cells co-transfected with pNF-κB-luc reporter and pRL-TK were treated with different doses of H₂O₂, with or without 10 μM SHetA2 for 4 hours prior to evaluation of luciferase activity. Firefly luciferase activity was normalized to Renilla Luciferase activity and expressed as fold of luciferase activity in untreated cells.

*p<0.05, **p<0.01 ***p<0.001

Fig 2. Evaluation of ROS in NF-κB induction and apoptosis in A2780 cells. (a) Cells co-transfected with pNF-κB-luc reporter and pRL-TK were treated different antioxidants a in the presence and absence of 10 μM SHetA2 for 24 hours prior to evaluation of luciferase activity. **(b)** Same as a, except that TNFα was added for the last 30 minutes of incubation prior to evaluation of luciferase activity. **(c)** Cells were treated with 10 μM SHetA2 or DMSO solvent only (Control) for 24 hours with or without 5 mM NAC and evaluated for apoptosis using annexin FITC/Propidium Iodide staining and flow cytometry. **(d)** Quantification of two independent experiments performed in duplicate as described in d are presented as average and standard error and expressed as fold activity of the untreated control. *p<0.05, **p<0.01 ***p<0.001

Fig. 3. SHetA2 repression of IκBα Phosphorylation and degradation, IKK activity and p65 nuclear translocation. (a–c)

Whole cell extracts were prepared from A2780 cells treated with 10 μM SHetA2 or DMSO solvent control (Untreated) for indicated time points. **(a)** 100 μg of extracts were analyzed for IκBα total and ser 32/36 IκBα by western blotting. **(b)** Whole cell extracts were immunoprecipitated with IKKα antibody or IgG isotype control antibody and subjected to the immune complex kinase assay. Phosphorylation of IκBα was detected by Western blot analysis of immune complex assay products using ser 32/36 IκBα antibody were compared to IKKα levels in the cell extract, which were evaluated by Western blot analysis using the same volume of whole cell extracts used in the assay. **(c)** To test direct effects of SHetA2 on IKK activity, untreated whole cell extracts were used to immunoprecipitate IKK enzyme. Kinase buffer containing the indicated concentrations of SHetA2 was used in immune complex kinase assay as described in b. **(d)** A2780 cells treated with 10 μM SHetA2 for 24 hours with or without exposure to 10ng/ml TNFα for the last 30 min were fixed, and subjected to immunocytochemistry of p65.

Fig. 4. Evaluation of apoptosis pathway components and cross-talk in SHetA2- and TNF α -induced apoptosis. (a)

A2780 (top graph) and SK-OV-3 (bottom graph) cells were treated with 10 μ M SHetA2 with or without the indicated concentrations of TNF α for 24 hours and evaluated for apoptosis as described in 2c and d.. **(b)** A2780 cells were treated with indicated concentrations of SHetA2 with or without 20 ng/ml TNF α for 24 hours. Protein extracts (100 μ g) were prepared and subjected to Western blot analysis with antibodies against indicated proteins. **(c)** A2780 cells were treated with 10 μ M SHetA2 with or without 20 ng/ml TNF α or Caspase 8 inhibitor (Casp 8i) for 24 hours and evaluated for apoptosis as described in 2c and d. **(d)** A2780 cells transfected with validated siRNA against TNFR1 or scrambled siRNA were treated with DMSO solvent only or 10 μ M SHetA2 with or without 20 ng/ml TNF α 24 hours after transfection for 24 hours and evaluated for apoptosis.

Fig. 5. Effects of p65 on SHetA2- and TNF α -induced apoptosis. (a)

Cells co-transfected with pNF- κ B-Luc and pRL-TK either alone (Control) or with empty vector (pCMV4) or p65 expression vector (pCMV4-p65) were treated with DMSO solvent only (-SHetA2) or 10 μ M SHetA2 (+SHetA2) for 16 hours and evaluated for luciferase activity. Firefly luciferase activity was normalized to Renilla luciferase activity. Inset shows Western blot confirming p65 expression in pCMV4-p65 transfected cells. **(b)** Cells were transfected with pCMV4-p65 or pCMV4 and treated with indicated concentrations of SHetA2 with or without 20 ng/ml TNF α for 24 hours and evaluated with an MTS assay in triplicate. Fold survival was derived by dividing the average OD of each treatment by the OD of the control treated with solvent only. **(c)** Parallel cultures treated as in panel b were analyzed for apoptosis using Annexin FITC/PI staining as described in 2c and d. **(d)** A2780 cells transfected with validated siRNA against p50, p65 and control scrambled siRNA were treated with 20 ng/ml TNF α for 24 hours, and then evaluated for apoptosis with a DNA fragmentation assay.

Fig. 6. Working model

Solid lines: TNF α activities. Dashed lines: SHetA2 activities. In ovarian cancer cells, binding of TNF α to its cell surface receptor; TNFR1 induces NF- κ B activity. SHetA2 inhibits NF- κ B activation through inhibition of IKK phosphorylation of I κ B α allowing TNF α -induced apoptosis mediated by caspase 8 to occur in cell death receptor ligands-resistant cells. Activation of TNF α -induced extrinsic apoptosis and SHetA2-induced intrinsic apoptosis results in synergistic induction of apoptosis.