Triptolide-mediated cell death in neuroblastoma occurs by both apoptosis and autophagy pathways and results in inhibition of nuclear factor–kappa B activity

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Abstract

BACKGROUND: Neuroblastoma is an aggressive pediatric malignancy with significant chemotherapeutic resistance. We assessed triptolide as a potential therapy.

METHODS: SH-SY5Y and IMR-32 neuroblastoma cell lines were treated with triptolide. Viability, intracellular calcium, caspase activation, protein, and mRNA levels were measured. Autophagy was evaluated with confocal microscopy. Nuclear factor–kappa B (NF-κB) activation was measured using a dual luciferase assay.

RESULTS: Triptolide treatment resulted in death in both cell lines within 72 hours, with sustained increases in intracellular calcium. IMR-32 cells underwent cell death by apoptosis. Conversely, light chain 3II (LC3II) protein levels were elevated in SH-SY5Y cells, which is consistent with autophagy. Confocal microscopy confirmed increased LC3 puncta in SH-SY5Y cells compared with control cells. Heat shock pathway protein and mRNA levels decreased with treatment. NF-κB assays demonstrated inhibition of tumor necrosis factor (TNF)-α–induced activity with triptolide.

CONCLUSIONS: Triptolide treatment induces cell death in neuroblastoma by different mechanisms with multiple pathways targeted. Triptolide may serve a potential chemotherapeutic role in advanced cases of neuroblastoma.

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Triptolide has been shown to inhibit the growth of cholangiocarcinoma cells in hamsters, as well as the growth of xenografts in human melanoma, bladder cancer, and breast cancer, and gastric carcinoma in nude mice. Our previous research has demonstrated that triptolide is markedly effective in inducing cell death in both pancreatic cancer and cholangiocarcinoma. Interestingly, both apoptosis and autophagy have been found to be induced in response to triptolide therapy in pancreatic cancer cell lines, perhaps implying a cell line–specific response to cellular insult. We have also previously investigated the effects of triptolide in vitro and in vivo in the neuroblastoma cell lines SKNSH (human) and N2a (murine). Triptolide resulted in apoptotic cell death in these cell lines in a time- and concentration-dependent fashion. In addition, treatment in orthotopic mouse models demonstrated inhibition of tumor growth.

Despite the developing research into the efficacy of triptolide in inducing cancer cell death, an exact mechanism of action remains to be identified. Two notable pathways have been the target of investigation: the heat shock response and nuclear factor–kappa B (NF-κB) pathways. The heat shock response serves as a protective response to cellular insult, leading to the synthesis of multiple cell-survival proteins, including heat shock proteins (HSPs). Given its prosurvival role, the pathway is thought likely to play a role in carcinogenesis by imparting a survival mechanism to cells that would otherwise undergo cell death. Investigation of this pathway in neuroblastoma has recently shown a strong relationship between cellular levels of HSP70 and HSP72 and the resistance of SH-SY5Y neuroblastoma cells to staurosporine-induced cell death: increased expression of HSP70 and HSP72 protects against cell death because of increased thermotolerance and acquisition of other neuroprotective features. We have previously demonstrated a link between the heat shock pathway and triptolide in that cellular apoptosis is induced with triptolide by inhibition of HSP70 expression. Inhibition of HSP70 increases intracellular calcium levels, which in turn leads to the loss of lysosomal integrity and ultimate cell death. The mechanism by which triptolide leads to inhibition of the HSP response in neuroblastoma needs to be further investigated.

NF-κB is active in most tumor cell lines, regulating cell survival, proliferation, angiogenesis, and invasion through its transcriptional activation. The genes transcribed affect multiple biologic processes, including immune and inflammatory responses. Triptolide has been used as a natural remedy in Eastern medicine for centuries, particularly in the treatment of autoimmune and inflammatory diseases, including rheumatoid arthritis. Given these anti-inflammatory medicinal purposes, it would be highly reasonable that the NF-κB pathway may be inhibited by triptolide, in effect turning off the innate machinery the cell requires for its survival. In addition, the pathway is generally inducible in normal cells but has been shown to be constitutively active in many cancer cell lines, including human neuroblastoma, providing a possible upregulated pathway that when inhibited may lead to cancer cell death. Although an abundance of research has been focused on the possible effects of triptolide in the NF-κB pathway, there is a lack of agreement on the main target for its effect.

Our primary objective was to assess the response to triptolide therapy in more aggressive neuroblastoma cell lines, specifically SH-SY5Y (human, not MYCN amplified) and IMR-32 (human, MYCN amplified), given the far worse prognosis for patients with more advanced tumors. We hypothesized that the differences in responsiveness of various neuroblastoma cell lines to an attempted treatment, in this case with triptolide, may be secondary to alternative forms of cell death used by the various cell lines. Additionally, we sought to delineate the mechanism of triptolide’s effects. In this study, we demonstrated that triptolide treatment indeed induces cell death in more aggressive neuroblastoma cell lines in vitro. We further showed that different cell death mechanisms are induced in response to triptolide therapy, with both apoptosis and autophagy observed. Our study also showed that triptolide targets a number of cell survival–associated pathways, including the HSP and NF-κB pathways, ultimately resulting in cell death.

Methods

Cell culture

Neuroblastoma cells were generously provided by Dr Loh at the University of Minnesota (SH-SY5Y cells) and Dr J. Yang at Baylor College of Medicine (IMR-32 cells). The SH-SY5Y cells were cultured in Dulbecco Modified Eagle’s Medium/Nutrient F-12 Ham (Life Technologies, Grand Island, NY) containing 10% fetal bovine serum and 1% penicillin-streptomycin. The IMR-32 cells were cultured in Minimum Essential Medium (Life Technologies, Grand Island, NY) with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂. Experiments were completed on cells with less than 8 passages.

Treatment of cells with triptolide

Calbiochem triptolide (EMD Millipore Chemicals, Billerica, MA) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) and added to the cells at the indicated concentrations in serum-free medium (SFM). Cells treated with SFM alone served as controls.

Determination of cell viability

Cells (1 × 10⁴/well) were seeded into 96-well plates and allowed to adhere for 48 hours at 37°C. After treatment with triptolide at varying concentrations for 24 to 72 hours, cell viability was determined by the Dojindo

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Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc, Rockville, MD). Briefly, 10 μL of the tetrazolium substrate was added to each well of the plate and was allowed to incubate at 37°C for 2 hours, after which the absorbance at 450 nm was measured.

**Caspase 3 and caspase 7 activity assays**

Cells (1 × 10^4/well) were seeded into 96-well white opaque plates, as well as into a corresponding optically clear 96-well plate, and allowed to adhere for 48 hours at 37°C. The cells were then treated with varying concentrations of triptolide for 24 to 48 hours. At the end of the incubation time, 100 μL of the appropriate Caspase-Glo reagent (Caspase Glo 3/7, Promega, Madison, WI) was added to each well containing 100 μL of blank negative control or treated cells in SFM. Plates were incubated in the dark for 1 hour at room temperature (RT). The luminescence was then read. The corresponding 96-well clear plate was used to measure the number of viable cells with the Dojindo Cell Counting Kit-8 reagent, and caspase activity was normalized to these values.

**Measurement of intracellular calcium ion levels**

Cells (1 × 10^5/well) were plated into 96-well black plates, allowed to adhere for 48 hours at 37°C, and then treated with varying doses of triptolide. At the set collection time, the cells were prepared with a Calcium Orange AM kit (Invitrogen, Life Technologies Corp, Grand Island, NY) for determination of results. Briefly, the cells were washed in phosphate-buffered saline (PBS), and 1.5 μL of 5 mmol/L Calcium Orange (in 20% pluronic acid in DMSO) in SFM was added to each well. The plate was incubated for 1 hour at RT, after which the media was removed and the cells washed further with PBS. Fresh PBS was added and the plate was read on a luminometer. A corresponding 96-well clear plate was used for viability determination, and the results were standardized to these values.

**Measurement of heat shock factor protein 1, heat shock protein 70, and heat shock protein 27 levels by Western blotting**

Cells (2 × 10^6) were seeded in 10-cm plates and allowed to adhere for 48 hours at 37°C before treatment. Triptolide was then added at varying concentrations, and plates were collected at varying time points. HSF1, HSP70, and HSP27 was examined with real-time quantitative PCR, using the QuantiTect SYBR green PCR kit (Qiagen Inc) according to the manufacturer’s instructions and were treated with DNase-1 on the column. Total RNA (1 μg) was reverse transcribed. The expression of HSF1, HSP70, and HSP27 was examined with real-time quantitative PCR, using the QuantiTect SYBR green PCR kit (Qiagen Inc) according to the manufacturer’s instructions using an Applied Biosystems 7300 real-time PCR system (Applied Biosystems, Life Technologies). All data were normalized to the housekeeping gene 18S (18S Quant iTect Primer Assay; Qiagen).

**Quantitative real-time polymerase chain reaction for heat shock factor protein 1, heat shock protein 70, and heat shock protein 27**

Cells (1 × 10^5) were plated into 6-well plates and allowed to adhere for 48 hours at 37°C before treatment. Triptolide was then added at varying concentrations and plates were collected at varying time points. RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Inc, Valencia, CA) according to the manufacturer’s instructions and were treated with DNase-1 on the column. Total RNA (1 μg) was reverse transcribed. The expression of HSF1, HSP70, and HSP27 was examined with real-time quantitative PCR, using the QuantiTect SYBR green PCR kit (Qiagen Inc) according to the manufacturer’s instructions using an Applied Biosystems 7300 real-time PCR system (Applied Biosystems, Life Technologies). All data were normalized to the housekeeping gene 18S (18S QuantiTect Primer Assay; Qiagen).

**Immunofluorescence for light chain 3**

Cells (6 × 10^5) were plated in Lab-Tek Chamber Slides, allowed to incubate at 37°C for 48 hours for cell adherence, and then treated with 100 nmol/L triptolide (or SFM for control) for 20 hours. The cells were then fixed with 2% paraformaldehyde at RT for 15 minutes and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 5 minutes (also at RT). The cells were blocked with 1% bovine serum albumin for 1 hour at RT before staining with anti-LC3B antibody (rabbit polyclonal antibody; Cell Signaling Technology) at a 1:200 dilution in blocking buffer at 4°C overnight. Alexa Fluor 488 (donkey antirabbit antibody; Invitrogen, Life Sciences) was then used as a secondary antibody at a 1:500 dilution in blocking buffer for 1 hour at RT. Slides were mounted with Prolong Gold Antifade Reagent (Invitrogen, Life Sciences).
Sciences) and allowed to dry for 24 hours before microscopic examination. Immunofluorescence images were obtained on a Nikon Eclipse Ti confocal microscope (Nikon Instrument, Melville, NY) using a 40× oil immersion objective. The LC3 dots were quantified using the Image J software command “analyze particles,” which counts and measures objects in binary or thresholded images.

**Dual luciferase assay for nuclear factor–kappa B activity**

Cells (8 × 10⁴) were seeded into 24-well plates and allowed to adhere for 48 hours at 37°C. The cells were then treated with 100 nmol/L triptolide or various controls: SFM, tumor necrosis factor (TNF)-α (10 ng/mL), 100 nmol/L triptolide with TNF-α. The plates were collected after 6 hours, and further collection was completed using Promega Dual Luciferase Reporter Assay kit (Promega) according to the manufacturer’s protocol. A TD-20/20 luminometer was used for determination of results (Turner Designs, Sunnyvale, CA).

**Statistical analysis**

All experiments were repeated 3 independent times. Values are expressed as the mean ± standard error of the mean (SEM). The significance between the control and each experimental test condition was analyzed by the unpaired Student t test, and a P value of less than .05 was considered statistically significant. One-way analysis of variance (ANOVA) was performed to compare mean values between treatment groups at different time points. Post hoc analysis was performed using the Bonferroni multiple comparison test. Results from dual luciferase assays and immunofluorescence microscopy were qualitative and statistical analyses were not appropriate.

**Results**

**Triptolide induces cell death in imr-32 neuroblastoma cells by apoptosis**

A time- and concentration-dependent response to triptolide treatment in both cell lines was demonstrated in vitro at minimal concentrations (Fig. 1A). Cell death occurred in less than 72 hours at a 100-nmol/L concentration in both cell lines, with 45% cell death in IMR-32 cells and 50% cell death in SH-SY5Y cells. One-way ANOVA using a Bonferroni multiple comparison test showed that there was a significant difference between the SH-SY5Y untreated group and the groups treated with 50 to 200 nmol/L triptolide for 24 hours, but that there was no significant difference in the groups treated with 100 nmol/L and those treated with 200 nmol/L. At 48 and 72 hours after treatment, the
untreated group was significantly different from the groups treated with 50 to 200 nmol/L, but there was no significant difference between the 50 nmol/L, 100 nmol/L, and 200 nmol/L groups. These data suggest that lower concentrations of triptolide were more effective in causing cell death when treatment was for a longer duration. Similar results were obtained with the IMR-32 cell line, in which no significant differences were obtained between the 50 nmol/L and the 200 nmol/L treatment groups at all time points studied (24, 48, and 72 hours). We therefore conclude that the effect of triptolide on cell viability in either cell line plateaued at a concentration of 50 nmol/L (Supplemental Fig. 1).

The mechanism of cell death in each cell line was then investigated, beginning with assays for caspase-3 and caspase-7 activation. Notably, the IMR-32 cell line showed a time- and concentration-dependent increase in caspase-3 activity, mirroring the viability response. A caspase activation level at 600% of control was observed within 48 hours (at 50 nmol/L). One-way ANOVA showed no statistical difference in the cells treated for 24 hours, but at 48 hours after treatment there was a significant difference in caspase activity at all concentrations tested (25 to 100 nmol/L triptolide). These results suggested that triptolide-mediated cell death occurred by apoptosis in this cell line. However, the SH-SY5Y cell line did not demonstrate a significant change in caspase activity ($P = 0.8039$ at 24 hours and $P = 0.9481$ at 48 hours), suggesting that another method of cell death may be operational in these cells (Fig. 1B).

**Triptolide induces cell death by autophagy in sh-sy5y neuroblastoma cells**

Because our data suggested that the cell death in the SH-SY5Y cell line is not by caspase-dependent apoptosis, we first investigated a caspase-independent mechanism through the protein expression of apoptosis-inducing factor (AIF). This was found not to change with increasing concentrations of triptolide within a 48-hour timed collection (Supplemental Fig. 2). We next looked into another form of cell death: autophagy. We first measured LC3II protein expression, a hallmark of autophagy, by Western blotting. LC3 is present in the cell in 2 forms: LC3I is a cytosolic form and LC3II is a membrane-bound form. When autophagy is induced, LC3I is conjugated to phosphatidylethanolamine to form LC3II, which is seen as a faster migrating band by Western blotting. Because the amount of LC3II correlates with the number of autophagosomes formed, it is a good indicator of the status of autophagy.17 Remarkably, triptolide treatment (50 nmol/L) of SH-SY5Y cells for 6 and 24 hours resulted in increased LC3II levels in the later period by Western blotting, suggesting activation of autophagy (Fig. 2A).

As another method of autophagy investigation, LC3 can also be detected by immunofluorescence: the presence of LC3II by confocal microscopy is confirmed with the observation of aggregates or punctate collections, which indicates autophagosome formation. In contrast, the unconjugated cytosolic LC3I will show a more diffuse staining pattern.17 As a confirmatory test for autophagy occurrence in SH-SY5Y cells in response to triptolide therapy, confocal microscopy was completed on treated cells and compared with an untreated control. Our results showed a homogeneous cytosolic distribution of LC3 in the untreated SH-SY5Y cells, whereas a notable punctate pattern was noted in the SH-SY5Y cells after 20 hours of triptolide treatment, again confirming the induction of autophagy in this cell line by triptolide (Fig. 2C). The number of LC3 puncta were quantified, with 9.2 ± 3.9 puncta in the treated SH-SY5Y cells (n = 50 cells) in comparison to 0.06 ± 0.02 puncta in the control SH-SY5Y cells (n = 50 cells). This finding was not observed in the IMR-32 neuroblastoma cell line, with little to no puncta quantified and a minimal diffuse pattern observed in both the control (n = 35 cells) and treated (n = 35 cells) groups (Fig. 2B,C).

**Triptolide downregulates heat shock gene expression**

Treatment of SH-SY5Y and IMR-32 neuroblastoma cell lines for short intervals (less than 24 hours) with triptolide concentrations of 100 nmol/L resulted in a significant decrease in mRNA levels of HSF1, HSP70, and HSP27 compared with untreated controls. Although levels of the transcription factor HSF1 were depleted to approximately half-fold of the control (in IMR-32 cells), levels of HSP70 and HSP27 were seen to deplete further at the 24-hour period, which is consistent with a downstream effect (Fig. 3A).

Analysis of HSP expression by Western blotting in triptolide-treated cells (50 to 100 nmol/L) showed depletion in both neuroblastoma cell lines within 72 hours in comparison with untreated controls (Fig. 3B). This depletion was similar in both cell lines despite the difference in the mechanism of cell death. Western blot findings were confirmed with densitometry analysis (Supplemental Fig. 3). Levels were seen to drop at a time frame consistent with mRNA analysis and with the time frame of reduced cellular viability.

**Triptolide treatment results in large intracellular calcium increases**

On confirmation of reduced HSP70 levels in both cell lines, and given that we previously demonstrated resultant increased intracellular calcium levels with HSP70 inhibition in pancreatic cancer, we next investigated intracellular calcium levels in response to triptolide treatment in our neuroblastoma cell lines. Despite the difference in the ultimate cell death pathways, both cell lines again
demonstrated a time- and concentration-dependent increase in intracellular calcium levels with triptolide treatment to levels greater than 200% of control at 72 hours (Fig. 3C), which was consistent with our previous results.

Triptolide results in inhibition of nuclear factor–kappa B activity

To further investigate the downstream effects of triptolide, the NF-κB pathway was investigated in both neuroblastoma cell lines using a dual luciferase-based promoter activity assay. Triptolide decreased NF-κB activation with 100 nmol/L of triptolide treatment at a 6-hour time point in both cell lines. In a representative result from 3 separate experiments, triptolide resulted in a 0.1% NF-κB activation (SH-SY5Y cells) or complete lack of activation (IMR-32 cells) compared with the untreated control.

Cellular stresses have been shown to increase NF-κB activity. Therefore, we next examined the effects of triptolide combined with TNF-α, a well-known activator of NF-κB. This was again performed in both cell lines, with a 100-nmol/L triptolide treatment at a 6-hour time point. Relative to a 100% TNF-α response in each cell line, the combination treatment resulted in substantial blunting of the TNF-α elevation, with only 9% activation in SH-SY5Y cells and 20% activation in IMR-32 cells, which is consistent with an inhibitory effect on the pathway.

Comments

Neuroblastoma remains a clinical enigma with highly variable severity that ultimately leads to an equally variable prognosis. In the most benign form, it may regress completely with minimal to no treatment. Often, surgical resection may be the most important modality in localized cases. The more aggressive tumors, particularly when diagnosed in patients with metastatic disease who are older than 18 months of age, may convey a lethal diagnosis.
despite multimodal therapy. Tumors with MYCN amplification, observed in up to 20% of cases, are associated with poor outcomes. This status is routinely used in clinical practice to determine the therapeutic intensity as well as to gauge its potential response. However, successful therapeutic options remain limited, and additional chemotherapeutic agents are needed to target these advanced cases.

Our laboratory has previously investigated the efficacy of triptolide in pancreatic cancer and cholangiocarcinoma. In addition, we have studied the effects of triptolide both in vitro and in vivo in the neuroblastoma cell lines SKNSH (human) and N2a (murine). In these cell lines, triptolide resulted in significant cell death in vitro in a time- and concentration-dependent fashion.

Figure 3  mRNA and protein levels in the heat shock protein pathway are decreased after triptolide treatment, with a sustained increase in intracellular calcium levels. (A) Triptolide treatment (100 nmol/L) of SH-SYSY and IMR-32 neuroblastoma cell lines for 24 hours resulted in a significant decrease in expression of HSF1, HSP70, and HSP27 mRNA levels compared with untreated controls. Data is representative of results obtained from 3 separate quantitative PCR analyses. (B) Representative Western blots show triptolide treatment (50 and 100 nmol/L) results in a depletion of the heat shock protein transcription factor, HSF1, as well as downstream proteins HSP70 and HSP27. Actin served as a loading control. (C) Both cell lines demonstrated a time- and dose-dependent increase in intracellular calcium levels with triptolide treatment to levels greater than 200% of control at 72 hours (with 100 nmol/L triptolide). Data are expressed as the mean ± SEM of 3 independent experiments. *P < .05 (t test) as compared with controls.
Further evaluation in an orthotopic mouse model showed inhibition of tumor growth. With these promising findings, we sought to investigate the effect of triptolide on more aggressive neuroblastoma cell lines and potentially elucidate the mechanism by which triptolide induces cell death in neuroblastoma cancer cells.

In this study, we demonstrated the potent effects of triptolide on 2 human neuroblastoma cell lines: SH-SY5Y (not MYCN amplified) and IMR-32 (MYCN amplified). We showed that triptolide does indeed induce substantial cell death in a time- and concentration-dependent fashion at early time points in vitro. Intriguingly, the more resistant MYCN-amplified IMR-32 cell line showed a nearly equivalent death response in direct comparison to the SH-SY5Y cell line, which is not MYCN amplified. This suggests that triptolide could emerge as a novel therapy against more aggressive MYCN-amplified forms of neuroblastoma.

Interestingly, further investigation into the mechanism of cell death showed that the cell lines underwent death by alternative pathways. Apoptosis was observed in the IMR-32 cell line, as evidenced by robust caspase-3 activation. These findings were not duplicated in the SH-SY5Y cell line, prompting further investigation. Because autophagy has been seen in response to various anticancer therapies, we examined whether triptolide induces autophagy in cells that show a nonapoptotic cell death. First, we evaluated LC3 protein expression, a hallmark protein of autophagy. We observed elevated protein expression of LC3II, a membrane-bound form of the protein that is found with autophagosome formation, in response to triptolide treatment in SH-SY5Y neuroblastoma cells. LC3 can also be detected by immunofluorescence; in this study, the induction of LC3II in SH-SY5Y cells was seen within 20 hours of treatment with triptolide, indicating the formation of autophagosomes and therefore autophagy. Further, evaluation of the previously determined apoptotic IMR-32 cell line showed only a homogeneously diffuse pattern by confocal microscopy, indicative of a more expected cytosolic LC3I presence.

In this study we also evaluated the potential downstream effects of triptolide therapy, beginning with the HSP pathway. Overexpression of HSP70 has been observed in multiple cancers and is believed to contribute to carcinogenesis by imparting a resistance to cell death. Recent literature in neuroblastoma has demonstrated a strong relationship between cellular levels of HSP70 and HSP72 and the resistance of SH-SY5Y neuroblastoma cells to induced death, which is thought to occur primarily because of increased thermotolerance. In our previous neuroblastoma research, we demonstrated downregulation of HSP70, a key protein in the pathway that is also seen to decrease in pancreatic cancer. Furthermore, a study from our group has shown that HSPs may protect the cells from cell death by attenuation of cytosolic calcium levels. Interestingly, we observed that triptolide treatment in both IMR-32 and SH-SY5Y cells led to downregulation of the heat shock response pathway: levels of mRNA for HSF1, the transcription factor for HSP70 and HSP27, are depleted to levels 50% of control within 24 hours. Further downstream, the effects are seen in depletion of mRNA and protein levels of HSP70 and HSP27. Although the effects on the pathway are clear, the mechanism by which the effects are initiated remains unclear.

We next turned our focus on a potential interaction of triptolide with NF-κB. The NF-κB pathway is active in most tumor types, regulating cell survival, proliferation, angiogenesis, and invasion through its transcriptional activation. The genes transcribed ultimately affect multiple biologic processes, including immune and inflammatory responses. Triptolide has been used for centuries in Eastern medicine, with its application primarily in the treatment of autoimmune and inflammatory diseases. With its anti-inflammatory effects historically established, it would be
highly reasonable that the NF-κB pathway may be inhibited by triptolide, in effect turning off the innate machinery the cell requires for its survival. Therefore, targeting this pathway may prove to be of therapeutic potential. Triptolide has been investigated in previous reports: Xu et al. reported that triptolide increased the binding activity of NF-κB to DNA sites in colorectal cancer cells; however, transcriptional activity was inhibited. These findings were also substantiated in studies conducted by Qiu et al. (in T cells) and Zhao et al. (with bronchial epithelial cells). However, many other mechanisms of NF-κB pathway inhibition by triptolide have been reported, including either reduction of the active p65 subunit levels, or inhibition of p65 binding activity and inhibition of both p65 and IκBα expression in hepatocellular cells. Although many would not argue a likely effect of triptolide in this pathway, the exact mechanism remains questionable and certainly untested in neuroblastoma.

To investigate a possible effect on the NF-κB pathway, a dual luciferase-based promoter reporter assay was completed with each cell line. We showed that triptolide treatment resulted in very little to no NF-κB activation in comparison to a robust TNF-α control response. Further, when triptolide treatment was combined with TNF-α in vitro, a significant reduction in NF-κB activity was seen, which is consistent with an inhibitory effect. These findings corroborate previous literature and establish a possible further mechanism in neuroblastoma.

With the mechanism of death in each cell line, as well as some of the downstream effects, established, we completed our study by investigating the intracellular calcium levels in the treated cells. Several pharmacologic agents (cisplatin, staurosporine) function by disrupting intracellular calcium homeostasis, leading to their effect on apoptotic cell death within the cell. There is abundant evidence to suggest that endoplasmic reticulum stress leads to calcium movement to the mitochondria, resulting in membrane permeabilization, cytochrome c release, and the ultimate caspase-3 activation that is indicative of the apoptotic pathway. A link between autophagy and calcium has been discussed in the literature more recently, with endoplasmic reticulum stress also inducing autophagy through calcium mobilization. Reports have shown that formation of autophagosome formation may indeed be calcium dependent. In this study, we demonstrated that regardless of the ultimate cell death pathway, ie, apoptosis vs autophagy, both cell lines again demonstrated a time- and concentration-dependent increase in intracellular calcium levels with triptolide treatment to levels greater than 200% of control at 72 hours. This suggests that elevated cytosolic calcium could be one of the terminal events and the common thread in triptolide-induced cell death. Why cytosolic calcium elevation will induce apoptosis in one cell type and autophagy in the other is unclear and worth investigating in the future.

This study ultimately begins to tie a mechanism to the triptolide-induced cell death observed in neuroblastoma (Fig. 4). We have demonstrated reduced activity of NF-κB in both SH-SY5Y and IMR-32 neuroblastoma cell lines in response to triptolide treatment. In addition, we have shown depletion of players in the heat shock pathway: triptolide results in reduced mRNA levels and protein expression of HSF1, which is considered a major transcription factor for additional proteins in the pathway. This downstream effect was demonstrated with reduced levels of mRNA and protein expression in additional pathway proteins, ie, HSP27 and HSP70. Triptolide may directly target this pathway; however, HSP70 levels would be expected to decrease as a result of the NF-κB pathway inhibition, because NF-κB serves additionally as a transcription factor for HSP70 (UCSC Genome Bioinformatics Site). Regardless, HSP70 is shown to decrease in SH-SY5Y and IMR-32 neuroblastoma cell lines in response to escalating triptolide concentrations as well as to duration of treatment. We have previously demonstrated that triptolide therapy results in increased intracellular calcium as a result of HSP70 inhibition in pancreatic cancer cells and that ultimately this leads to cellular death. We have again demonstrated this response, now in neuroblastoma, with escalation of intracellular calcium at sustained levels after triptolide treatment. Ultimately, we believe this event triggers cellular death, with a mechanism innate to the cell being induced.

Conclusions

Neuroblastoma is a pediatric tumor with a wide range of severity, requiring a wider range of therapeutic options. Although surgical therapy may provide a cure in some cases, the more advanced cases will require the development of additional therapeutic modalities to provide an equally promising response. Triptolide induces cell death even in the advanced neuroblastoma cell lines, which may provide future therapeutic potential.

Supplementary material

Supplementary material related to this article can be found in the online version at http://dx.doi.org/10.1016/j.amj surg.2013.01.008.

References


