In vitro versus *in vivo* effects of triptolide: the role of transcriptional inhibition

Christine McCallum†, Suzy Kwon, Penny Leavitt, Wesley Shoop, Bruce Michael, Tom Felcetto, Dennis Zaller, Edward O'Neill, Betsy Frantz-Wattley, Chris Thompson, Gail Forrest, Ester Carballo-Jane & Anne Gurnett

†Author for correspondence
Merck Research Laboratories, Department of Human and Animal Infectious Disease Research, PO Box 2000, Rahway, NJ 07065, USA
Tel.: +1 732 594 2502
Fax: +1 732 594 6708
christine_mccallum@merck.com

Background: The mode of action of triptolide, the active ingredient of an anti-inflammatory Chinese herbal remedy, has been investigated *in vitro* and *in vivo*. Prior reports suggested that triptolide specifically inhibits the nuclear transcription factor (NF)-κB. . **Methods:** *In vitro* effects of triptoilde on cytokine release and cellular transcription were measured in A549 and THP-1 cells by enzyme-linked immunosorbent assay and incorporation of radiolabeled uridine, respectively. Levels and translocation of the transcription factor NF-kB *in vitro* were monitored by western blot. Arthritic mice were treated with triptolide, up to 0.5 mg/kg/day for 21 days, and inflammation was evaluated. Taqman analysis was performed on RNA isolated from the arthritic paws to determine relative levels of various cytokines in response to *in vivo* triptolide treatment. **Results:** *In vitro*, triptolide inhibited cellular transcription in A549 and THP-1 cells with IC_{50} values of 139 nM and 105 nM, respectively, similar to that for inhibition of cytokine release. Nuclear translocation of the transcription factor NF-κB was not inhibited, and IκB levels were reduced in response to triptolide exposure. Transcriptional inhibition was not limited to transcripts under the control of NF-κB, but rather appeared to be a general effect. Triptolide suppressed luciferase expression driven by NF-κB, AP-1, mouse mammary tumor virus and glucocorticoid response element with various stimuli. Nuclear run-on illustrated that *de novo* synthesis of RNA was inhibited by 50% in nuclei from cells treated with 50 ng/ml triptolide, while addition of triptolide to isolated nuclei had no effect on transcription. *In vivo* administration of triptolide reduced mouse plasma tumor necrosis factor-α levels with long-lasting results. However, monocytes isolated from these triptolide-treated mice showed no impaired RNA synthesis. Efficacious doses of triptolide in a collagen-induced arthritis model in mice reduced the transcript levels of interleukin (IL)-1b, IL-6, p38, and tumor necrosis factor-α in paws by only 5, 3.5, 1.8 and 1.6-fold, respectively, as determined by Taqman analysis. Mice treated with 0.5 mg/kg/day triptolide for 21 days had arthritis scores lower than those treated with methotrexate. These repeatedly treated mice exhibited no toxicity, and had blood cell counts within normal limits. **Conclusion**: Thus, despite the transcriptional inhibition in tissue culture, the *in vivo* mode of action of triptolide cannot be attributed to general inhibition of RNA synthesis, nor strictly to inhibition of NF-κB signaling, and remains to be elucidated.

Keywords: collagen-induced arthritis, cytokine, NF-κB, TNF-α, triptolide

future medicine part of fsg

The anti-inflammatory properties of extracts of the Chinese Thunder God Vine, *Tripterygium wilfordii Hook F* (TWHF) have been known to Chinese herbal medicine for several thousand years [1,reviewed in 2]. The active ingredients of TWHF have been identified, mostly from the roots [3], and include the diterpenoids triptolide and tripdiolide.

Chinese herbal extracts containing triptolide showed efficacy in collagen- and carageenan-induced arthritis models in mice and rats [4–6]. Purified triptolide was efficacious in the rat collagen-induced arthritis (CIA) model at 0.1 mg/kg/day, with no toxicity observed for up to 28 days [5]. Several human trials have also been conducted with TWHF extracts [7]. An ethanol/ethyl acetate extract of TWHF containing known amounts of triptolide was efficacious when administered orally for 20 weeks to treatment-refractory arthritis patients. At therapeutic doses, the extract was well tolerated by most patients, although there were some side effects, the most common of which was diarrhea [7].

While inhibition of cytokine production in response to triptolide can be demonstrated in isolated human cells, the mechanism of action of this compound remains to be elucidated. Several studies to elucidate the cellular effects of triptolide were published in the mid-1990s. Using partially purified triptolide, Yu and colleagues demonstrated inhibition of interleukin (IL)-1 production in isolated human monocytes [8]. In 1995, Gu and colleagues showed that immunosuppressive

activity of an extract from TWHF was coincident with triptolide and tripdiolide [9]. Tao and colleagues further illustrated that the triptolide component of the ethyl acetate extract accounted for its immunosuppressive activity *in vitro* [10]. The immunosuppressive properties were examined by a number of groups and a picture of these compounds as general inhibitors of cytokine production and activators of apoptosis has emerged [reviewed in 11]. Several studies have implicated interference of nuclear transcription factor (NF)-κB signaling in the mode of action of triptolide [12–14]. In particular, it was shown that triptolide interferes with the cycling of IκBa levels and subsequent nuclear translocation of NF-κB [15].

We report that triptolide inhibition of signaling through the NF-κBpathway *in vitro* resulted from general inhibition of transcription. The *in vitro* transcriptional inhibition and cytotoxicity were compared with the effects on RNA synthesis *in vivo*. Despite *in vivo* efficacy comparable with dexamethasone and superior to methotrexate, we could find no general transcriptional effects of triptolide in mice with CIA. Inhibition of cytokine release *in vivo* was not simply a result of nonspecific inhibition of transcription. We caution that the *in vivo* mode of action of triptolide cannot be directly inferred from *in vitro* studies of intracellular signaling.

Methods

Cytokine secretion assays

TNF-α secretion by THP-1 cells

THP-1 cells were grown in RPMI-1640 plus 10% fetal bovine serum (FBS) (Invitrogen), and plated at a density of 5 × 106/ml in 384-well plates which had been coated with 5 μg/ml of antihuman tumor necrosis factor (TNF)-α antibody (R&D Systems). Test samples were added, and cells were stimulated with 100 ng/ml lipopolysaccharide (LPS, Sigma) at 37°C for 4 h in the same medium. Cells were removed from plates and the plates washed three times with tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.5, 150 mM NaCl). 400 ng/ml antihuman TNF-biotin (R&D systems) in KPL wash buffer (Kirkgaard & Perry Laboratories) was added. After 1 h incubation at room temperature (RT), plates were washed three times with TBS. Streptavidin-europium (Perkin Elmer Life Sciences) at 1:1000 in europium assay buffer (Tris-buffered saline, 7 μg/ml diethylenetriamine-*N*,*N*,*N*(1),*N*(2),*N*(2)-pentaacetic acid, 0.01% sodium axide, 1% bovine serum albumin (BSA), and 0.05% Tween 20) was added to each well and incubated for 20 min at RT, followed by wash as above. Enhancer solution (Perkin Elmer Life Sciences) was added and after 1 h europium fluorescence was measured using a Wallac Victor plate reader.

Interleukin 2 secretion by Jurkat cells

Jurkat cells were grown in RPMI-1640 plus 10% FBS and plated in 96-well plates at a density of 5 × 106/ml. After pretreating with triptolide (Calbiochem) in the same medium for 1 h, one of three sets of stimuli was used for IL-2 secretion: anti-VB8 (250 ng/ml) plus anti-CD28 (250 ng/ml) (BD Pharmingen), phorbol myristate acetate (PMA) (3 ng/ml) plus ionomycin (5 μg/ml), or phytohaemagglutinin (PHA) (2 μg/ml) plus PMA (3 ng/ml) (all from EMD Biosciences). After stimulating for 4 h, plates were centrifuged for 20 min at 1100 × g and the supernatant collected. Levels of IL-2 in the supernatants were assayed using IL-2 enzyme-linked immunosorbent assay (ELISA) kit.

TNF-α stimulation of IL-6 secretion by A549 cells

A549 cells were grown in F-12 Ham's media (Invitrogen) plus 10% FBS and plated in 96-well plates at a density of 5 × 105 cells/well 1 day before assay. Cells were pretreated with 50 ng/ml triptolide for 1 h followed by 4 h stimulation with 20 ng/ml TNF-α at 37°C. Media was removed and IL-6 in the supernatant was assayed using the IL-6 ELISA kit.

TNF-α secretion by human peripheral blood mononuclear cells

The peripheral blood mononuclear cell (PBMC) layer was isolated from heparinized human blood by addition of an equal volume of Hanks Balanced Salt Solution (HBSS, Invitrogen). The mixture was underlayed with a half volume of lymphocyte separation medium (ICN Biomedicals) and centrifuged at 500 × g for 20 min. The PBMC layer was removed and washed with HBSS and subsequent centrifugation at 500 × g for 10 min. Pelletted PBMCs were washed three more times. Isolated PBMCs were resuspended in RPMI-1640 with 25 mM HEPES (Invitrogen) and 5% serum from the same blood donor. Cells were plated in 96-well flat bottom microtiter plates at a density of 2 × 105cells/ml, and immediately stimulated with 100ng/ml LPS with or without drug treatment (final dimethylsulfoxide [DMSO] concentration of 1%) and incubated overnight at 37°C/5% CO_2. Monocyte supernatants were collected by filtration. Secreted LPS-stimulated TNF-α was assayed by ELISA as described above with modifications for 96-well format.

Cytotoxicity assays

Cell viability was determined using the MTT microplate assay. Cells were plated at 2.5 × 106 cells/ml in RPMI-1640 supplemented with 10% FBS, and exposed to compounds for 4 or 16 h. A solution of 20 ûl of 5 mg/ml MTT solution in PBS was added and cells were incubated another 4 h. The insoluble formazaan crystals were pelleted by centrifugation at 1150 × g for 5 min, and the supernatants were aspirated. The crystals were solubilized in 200 ûL DMSO with shaking, and quantified by absorbance at 540 nm.

LPS challenge in mice

LPS challenge was measured as described previously [16]. Mice were dosed intravenously with 0.1, 1 or 10 mg/kg triptolide 2, 4 or 6 h prior to intraperitoneal injection of 10 ûg of LPS per mouse. Mouse plasma was harvested 90 mins after LPS administration, and plasma TNF-α levels were assayed by ELISA, as above.

Collagen-induced arthritis in mice

Male B10RIII mice, approximately 8 weeks of age, were injected subcutaneously at the base of the tail with an emulsion of bovine Type II collagen (Sigma) and Freunds Complete Adjuvant (BD Biosciences). Each mouse received 100 ûg Type II collagen in 100 ûL emulsion once on day 0. No booster injection was given. Following administration of the bovine collagen emulsion, groups of ten mice were assigned randomly and the groups were allocated at random to one of the following experimental treatments: vehicle, 0.01, 0.1, 0.5 and 1.0 mg/kg/day triptolide. Mice were dosed daily for 21 days starting at day 7. All drugs were dissolved sequentially in DMSO and then 0.05% methylcellulose (0.1:1.99). Each mouse received a 0.2 ml solution via oral gavage daily. Mice were housed individually and given food and water *ad lib*. In addition, each mouse was given gelatin 3 days a week directly into the corner of their cage to aid hydration. All studies were carried out in accordance with the Merck Research Laboratories Institutional Animal Care and Use Committee. Evaluation of inflammation was started at day 0 and carried out three times a week until day 21. At each evaluation, all four paws of each mouse were graded based on the following scale:

- 0: No inflammation
- 1: Inflammation in at least one digit progressing to the metacarpus or metatarsus
- 2: Inflammation progressing to the carpus or tarsus
- 3: Inflammation progressing above the carpus or tarsus
- 4: Mild-to-moderate ankylosis of the carpus or tarsus
- 5: Severe ankylosis of the carpus or tarsus (functional fusion of the joint)

Inflammation in each paw of each mouse was recorded and a summary for each mouse was made by adding the scores of the four paws for each observation period (maximum score for an individual mouse at a given observation point was 20). The data are presented as the mean score for each mouse in a treatment group for each observation period.

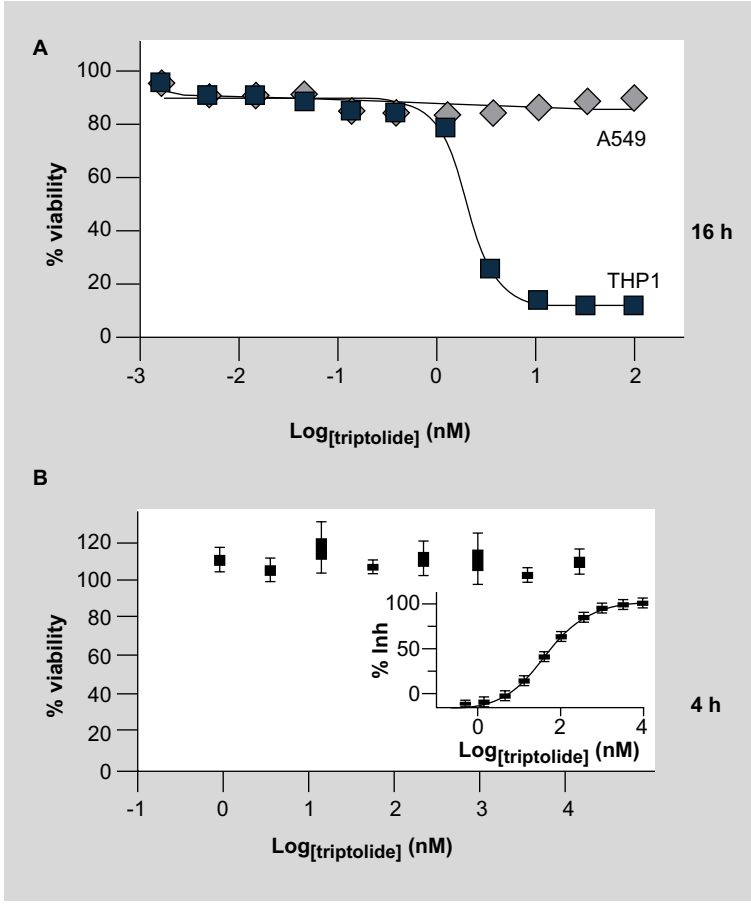
Preparation of nuclear extract

For binding studies and inflammatory protein level immunoblotting experiments, A549 cells were densely seeded into T-175 flasks and allowed to grow for 24 h at 37°C to 80–85% confluence prior to drug treatment and cytokine stimulation. Cells were treated with varying concentrations of triptolide one hour prior to addition of 20 ng/ml TNF-α (Upstate), all in RPMI-1640 plus 10% FBS. DMSO was added to appropriate flasks for vehicle control. Cells were harvested by trypsinization and washed with cold PBS. Cytosolic and nuclear extracts were prepared using NePER nuclear extraction kit (Pierce Biotechnology) following manufacturer's instruction with an additional wash of the nuclear pellet prior to nuclear lysis. Protein levels were determined using the Micro BCA Protein Assay (Pierce). The fractionation quality was monitored by western blot with antibodies to cytoplasmic and nuclear markers, heat shock protein (Hsp)90 (Sigma) and Oct 1 (Santa Cruz Biotechnology), respectively. Determination of signaling protein levels in nuclear and cytoplasmic extracts were performed by western blot analysis using anti-IκBa and anti-NF-κB antibodies (Santa Cruz). Phosphorylated IκBa levels were detected using antibodies purchased from Calbiochem.

Metabolic labeling

The putative effects of triptolide on *in vitro* transcription and translation were measured by the incorporation of ^{3}H-uridine or ^{35}S-Met/Cys, respectively, in 96-well format. A549 cells were seeded at 5 × 104/well one day prior to assay initiation. Cellular nucleotide pools were depleted by incubating cells in media with 5% dialyzed serum 2 h before the addition of radiolabel and experimental treatment. Cells were then incubated in the depleted media with 1 ûCi /well ^{3}H-uridine

Figure 1. Triptolide effects on cell viability.



The MTT cytotoxicity test was run as described in the Methods section. **A.** THP-1 or A549 cells were incubated with triptolide for 16h. **B**. THP-1 cells were incubated with triptolide for 4h. **Inset:** ELISA results for TNF-α secretion in the same cells after 4 h. ELISA: Enzyme-linked immunosorbent assay; TNF: Tumor necrosis factor.

(Amersham), or 1 ûCi ^{35}S-Met/Cys promix/well (Amersham) in the presence or absence of 20 ng/ml TNF-α and 50 ng/ml triptolide (added 1 h prior to TNF-α). Positive controls consisting of 50 ng/ml α-amanitin (Sigma) or 20 µg/ml puromycin (Sigma) were included. After 4 h TNF-α stimulation at 37°C, cells were washed twice with cold PBS, followed by addition of 50 ûL lysis buffer (50 mM Hepes, pH 7.4, 500 mM NaCl, 0.5% NP40, 0.3 mg/ml sheared salmon sperm DNA). 50 ûL cold 20% TCA was added. Samples were transferred to a Millipore 96-well filter plate then washed twice with 5% TCA followed by one wash with 70% ethanol. Filters were dried, scintillation fluid was added, and incorporation of ^{3}H or ^{35}S was quantified by scintillation counting.

To measure the effect of triptolide on replication, A549 cells were seeded in six-well plates at 2 × 105cells/well 1 day before assay. Nucleotides were depleted by incubation in media with 5% dialyzed serum for 2 h. The addition of 1 ûCi ^{3}H-thymidine (Amersham) / well in the presence or absence of triptolide and TNF-α to the cells was performed as described above for transcription and translation studies. Cells were washed twice with cold PBS to stop the reaction. cold 5% TCA at a measure of 2 ml was added to the wells. After 30 min on ice, the TCA was aspirated and the wells washed with 2 ml cold PBS. The precipitated DNA was solubilized by addition of 0.5 ml 0.5 N NaOH/0.5% sodium dodecyl sulfate (SDS) at room temperature, and transferred to scintillation vials for quantitation of ^{3}H incorporated into DNA.

For studies with rat PBMCs, rats were dosed four times a day with 0.1 mg/kg/day triptolide for 5 days and bled 2 h after the last dose. The PBMC monolayer was isolated as described above. Cells from triptolide-treated and -untreated rats were plated in 96-well plates at 1 × 105 cells/well. Metabolic labeling of RNA was performed as described above for A549 cells.

Nuclear run-on

Nuclear run-on experiments were performed as published [17] with modifications described briefly. A549 cells at 80% confluency in RPMI-1640 plus 10% FBS were pretreated with 50 ng/ml triptolide or α-amanitin for 1 h, then stimulated with 20 ng/ml TNF-α for 4 h. Cells were harvested using the RNeasy Mini/Midi kit according to manufacturers instructions. Monolayers were harvested by trypsinization. Pelleted cells were resuspended gently in cold RLN buffer (50 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% IGEPAL (Sigma), 1000 U/ml RNAsin (Promega), and 1 mM dithiothreitol (DTT) and incubated on ice for 5 min. Lysates were centrifuged at 400 × g at 4°C for 5 min and pelletted nuclei were washed with RLN buffer. Purified nuclei were incubated with rNTP mixture and 100 ûCi ^{33}P UTP (Amersham) for an hour at 30°C. Three volumes of TRIZol (Invitrogen) were added to one volume of the nuclear run-on reaction mixture and nuclei. Samples were homogenized using polytron and disposable omnitips. Following chloroform extraction, (0.2 ml chloroform per 1 ml TRIZol), ethanol was added to the aqueous phase (2.8 ml of 100% ethanol per 0.5 ml of aqueus phase) and QIAGEN mini or midi protocol was followed with the addition of the sample to the RNeasy column. A total of 1 ûl of the resulting purified RNA was mixed with scintillation fluid and counted.

Table 1. Triptolide inhibition of cytokine release from monocytes and cell lines.

Cell	Stimulation	Cytokine Release	IC_{50} (nM)
THP-1	LPS	TNF-α	83
hPBMC	LPS	TNF-α	3.7
mPBMC	LPS	TNF-α	1.1
Mouse Macrophage	LPS	IL-6	10
Mouse Macrophage	LPS	TNF-α	0.74
Jurkat	αCD4/αCD28	IL-2	9.8
Jurkat	PMA/PHA	IL-2	162
A549	TNF-α	IL-6	97 (50% inh)

IL: Interleukin; PBMC: Peripheral blood mononuclear cell; PMA: Phorbol myristate acetate; THP: Human monocytic cell line; TNF: Tumor necrosis factor.

RTPCR TaqMan analysis

At the completion of the CIA mouse study, paws were harvested and flash-frozen in liquid nitrogen, pulverized using a Bessman pulverizer, homogenized for 2 min in TRIZol (approx. 1 ml/100 mg tissue) and RNA was extracted as described for nuclear run-on. Integrity of the RNA was assessed using Agilent 2100 Bioanalyzer. Amplicons for TNF-α, p38a, IL-6, IL-1, and RankL were purchased from Applied Biosystems and reverse transcriptase polymerase chain reaction (RT-PCR) was measured on an ABI Prism 7700 sequence detection system. Relative quantification was achieved using the comparative CT method [18]. Efficiency of amplification was tested for each TaqMan probe relative to the controls (GAPDH, and ^{18}S RNA).

Results

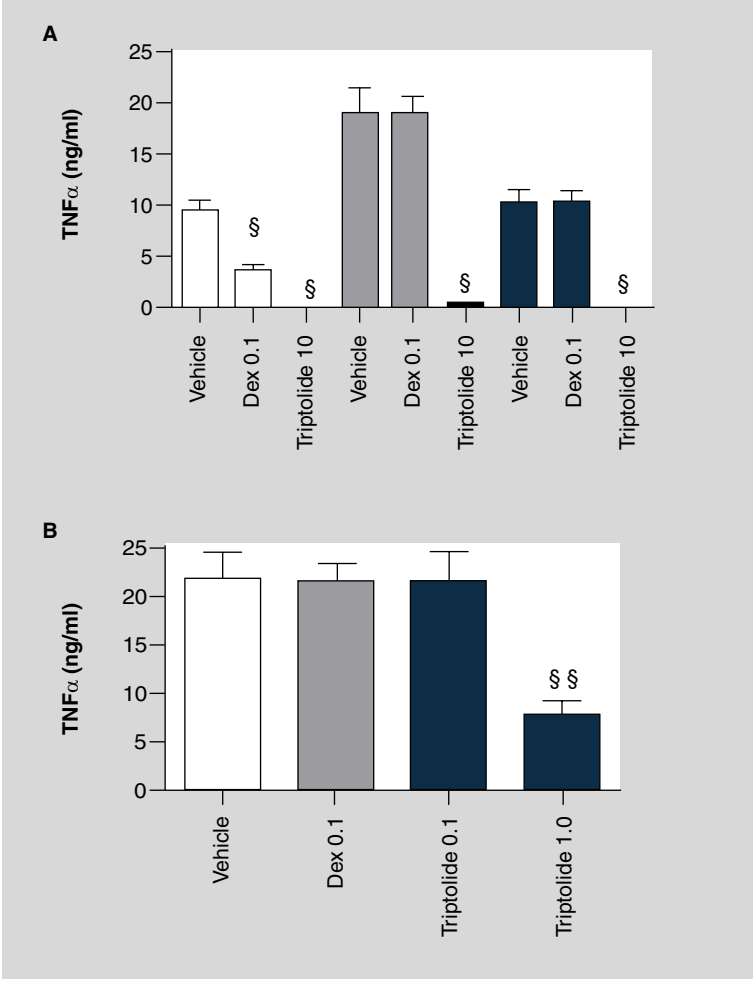
Triptolide inhibits cytokine release in various cells and cell lines. In a high-throughput screen (HTS) for inhibitors of TNF-α secretion, the natural product triptolide was found to be a potent inhibitor. Triptolide inhibited LPS-induced release of TNF-α from the monocytic cell line THP-1, with an IC_{50} value of 83 nM (**Table 1**). This result was consistent with previous reports in mouse macrophages [19] and with inhibition of LPS stimulation of IL-1 release in monocytes [8]. The inhibition of cytokine release was not limited to cells stimulated with LPS, since in human peripheral blood monocytic cells, triptolide inhibited TNF-α-stimulated secretion of IL-6 with an IC_{50} value of 3.7 nM (**Table 1**), in general agreement with previous studies [14]. Similar effects were observed in murine PBMCs and macrophages, with inhibition of both TNF-α and IL-6 release (**Table 1**). Stimulation of IL-2 expression in Jurkat cells via anti-CD4/anti-CD28 or via PMA/PHA was also inhibited by triptolide (**Table 1**), consistent with previous studies in peripheral blood lymphocytes [15].

Cytotoxicity of triptolide

Since triptolide has been shown to be pro-apoptotic in tumor cells [13] the role of toxicity in the observed decrease in cytokine production was investigated. In MTT cell viability assays it was apparent that triptolide indeed induced some toxicity. In our standard cytotoxicity assay, exposure of the lung epithelial cell line, A549, to 100 nM triptolide for 16 h had no effect on cell viability while the number of viable THP-1 cells was reduced by 90% at concentrations above 10 nM triptolide (**Figure 1A**). Triptolide had no effect on THP-1 viability up to 14 µM when exposed for only 4 h (**Figure 1**), yet the same cells were inhibited in their production of TNF-α with an IC_{50} value of 83 nM, during this time (**Figure 1B inset**).

Triptolide inhibits cytokine release *in vivo*. In our LPS challenge model, mice were dosed with triptolide, dexamethasone, or vehicle, up to 6 h prior to LPS challenge. The level of TNF-α in the plasma was measured 90 min after LPS administration. A dose of 10 mg/kg triptolide resulted in complete obliteration of the LPS response in this acute model of mouse inflammation, and the triptolide effect lasted for at least 6 h (**Figure 2**). A total of 1 mg/kg triptolide reduced plasma TNF-α values by 60% compared with vehicle, even when administered 6 h prior to initiation of the immune response. There was no effect of triptolide at 0.1 mg/kg in these acute studies (**Figure 2B**). While 0.1 mg/kg dexamethasone inhibited TNF-α levels by 60%, this anti-inflammatory effect was short-lived, with a return to normal levels of TNF-α observed by 4 h (**Figure 2A**).

Figure 2. Triptolide has a long-lasting effect *in vivo*.



A. 2 h (open bars), 4 h (gray bars), or 6 h (black bars) after dosing mice intravenously with dexamethasone or triptolide, mice were challenged intraperitoneally with 10 µg LPS. Plasma TNF-α levels were measured 90 mins post LPS-challenge by ELISA. **B.** Mice were exposed to dexamethasone or triptolide for 4 h prior to LPS challenge. Doses listed are mg/kg. Bars are mean ± standard deviation for triplicates.
ELISA: Enzyme-linked immunosorbent assay; LPS: Lipopolysaccharide; TNF: Tumor necrosis factor. $^{\S}p < 0.001$, $^{\S\S}p < 0.01$.

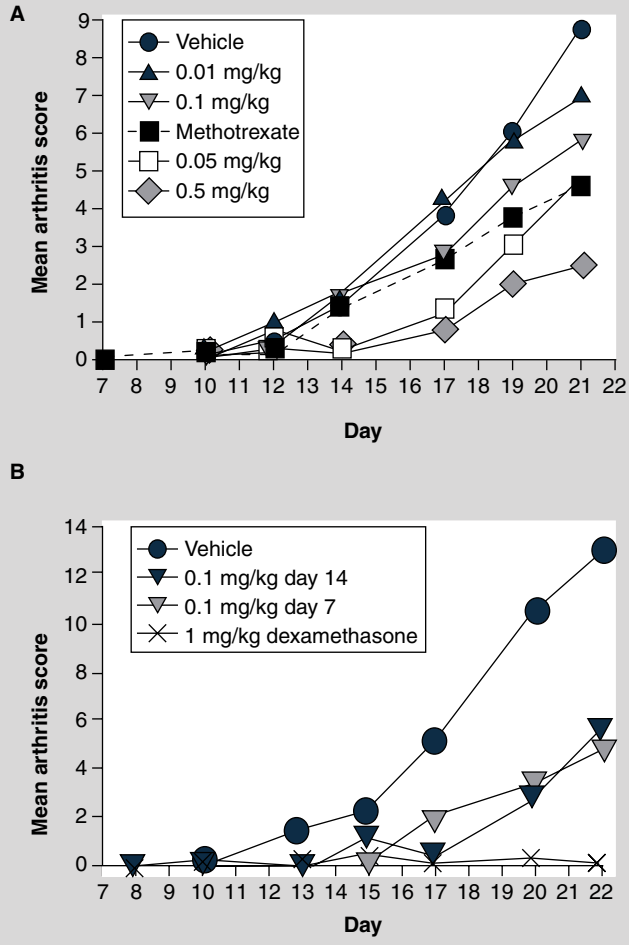
Triptolide inhibits collagen-induced arthritis in mice

Triptolide was efficacious in our CIA murine model when dosed orally once a day, starting 7 days after collagen injection (**Figure 3**). Arthritis was first observed in the vehicle-treated group at day 12 and escalated continuously to the end of the trial at day 21. Methotrexate, the positive control administered at 1 mg/kg once each day, produced a mean palliative reduction relative to vehicle control throughout the trial and ended with a 46% overall reduction in arthritis scores. The high dose of triptolide (0.5 mg/kg/day) showed superior reduction of arthritis scores even to the methotrexate positive control group throughout the trial and produced a terminal mean reduction in arthritic scores of 71.3% relative to vehicle-treated control. The lowest dose group of triptolide (0.1 mg/kg/day) behaved similar to the vehicle control throughout most of the trial, but even that group showed a 20% reduction in arthritis at the terminus. Throughout the course of the study one animal died in the vehicle control group and one died in the 0.1 mg / kg triptolide group, but did not appear to be treatment-related. The ability of triptolide to reverse the symptoms of CIA was also investigated in a separate study, by starting the triptolide dosing on day 14, two days after clinical symptoms were apparent. At 0.1 mg/kg/day, mice treated earlier (starting at day 7) or later (starting at day 14) reacted identically, with a 61% reduction in arthritis scores at the end of the test (day 22) (**Figure 3B**). Initiation of triptolide treatment on day 7 did however, delay the onset of arthritic symptoms by 2 days, from day 15 to day 17 (**Figure 3B**). Blood was drawn from the mice at the end of the study and blood cell analysis was performed. Despite the cytotoxicity observed in the 16 h *in vitro* assay mentioned above, the complete blood cell counts appeared within normal ranges. There were no overall differences in the numbers of total white blood cells, red blood cells, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, or platelet count. While blood from triptolide-treated mice (0.5 mg/kg/day) had a 35% increase in lymphocytes, there was a 31% reduction in the percentage of neutrophils and a 58% reduction in eosinophils (data not shown).

Role of NF-κB signaling in triptolide mode of action

It has previously been reported that triptolide inhibits cytokine release by inhibiting the action of the transcription factor NF-κB [12–15]. Using A549 cells, we observed that triptolide does not inhibit NF-κB p65 translocation to the nucleus (**Figure 4A, lower-right panel**). On the contrary, nuclear translocation of NF-κB is enhanced in the presence of 500 ng/ml triptolide in A549 cells, both in the presence and absence of TNF-α stimulation, in agreement with signaling effects observed in T-cells [11]. This validated the use of A549 cells in investigating triptolide effects on intracellular signaling to cytokine release. A549 cells were used for these studies since NF-κB signaling in these cells has been extensively studied [13], and triptolide cytotoxicity

Figure 3 Triptolide efficacy in mouse collagen-induced arthritis model.



A. Mice were treated with vehicle (filled circles), triptolide at 0.5 mg/kg (diamonds), 0.1 mg/kg (downward triangles), 0.05 mg/kg (open squares), 0.01 mg/kg (triangles), or with methotrexate (filled squares) orally, once a day, starting 7 days after collagen injection, as described in Methods. **B** Triptolide dosing (0.1 mg/kg) was started 7 days (light triangles) or 14 days (dark triangles) after collagen injection.

was not an issue (Figure 1A). A novel finding was that following the degradation of IκBa after stimulation of cells, 500 ng/ml triptolide prevented the synthesis of new IκBa, even after 4 h (Figure 4A, top panel). This is in contrast to the effects seen on NF-κB and IκBa in response to dexamethasone (Figure 4A). The decrease in cytoplasmic IκBa, and increased translocation of NF-κB to the nucleus were observed in stimulated cells at concentrations of triptolide at or above 20 ng/ml (Figure 4B). Much higher concentrations of triptolide (250 ng/ml) were required to observe any effects on IκBa and NF-κB in unstimulated cells (Figure 4B).

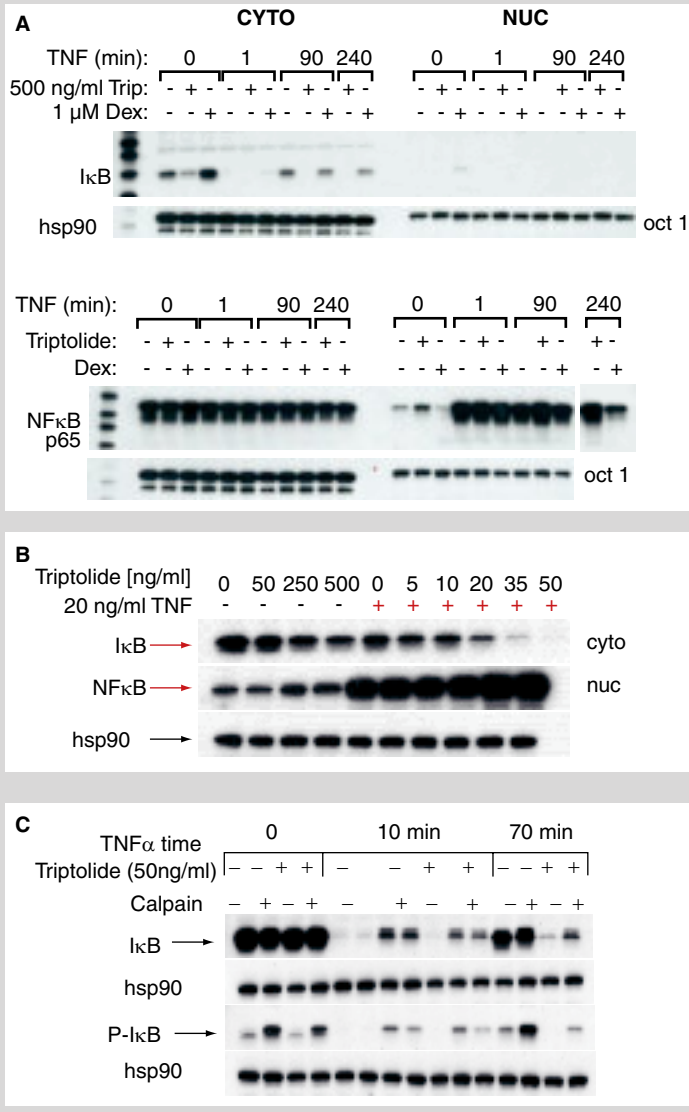
It was questioned whether the lack of IκBa in the cytoplasm and nucleus was due to inhibition of new synthesis of IκBa, or due to the rapid phosphorylation, ubiquitination and degradation of newly synthesized IκBa. In the absence or presence of triptolide, the degradation of phospho-IκBa caused by 10 min TNF-α stimulation was inhibited by the addition of the proteosome inhibitor calpain (Figure 4C). In the absence of calpain the phosphorylated IκBa was not detected at 10 min poststimulation, presumably due to rapid ubiquitination and degradation. The increased level of phospho-IκBa observed in the presence of calpain confirmed the inhibition of the proteosome in this experiment. A 70 min stimulation of triptolide-treated cells led to reduction of IκBa levels even when the proteosome was inhibited (compare "- Triptolide + Calpain" and "+ Triptolide + Calpain" lanes in Figure 4C), suggesting that triptolide was mainly inhibiting *de novo* protein synthesis of IκBa

Triptolide is a general transcription inhibitor in vitro

Since triptolide inhibited *de novo* IκBa synthesis, transcriptional inhibition in response to triptolide was investigated. A549 cells were transfected with reporter constructs containing the following promoters: mouse mammary tumor virus (MMTV, the natural mouse mammary tumor virus promoter), 3 × glucocorticoid response element (GRE, consisting of three copies of the glucocorticoid receptor binding sites, a TATA element and a transcription start site), NF-κB (six copies of binding site, a TATA element and a transcription start site), and AP1 (three copies of binding site, a TATA element and a transcription start site). All of the reporter constructs tested showed a reduction in gene expression at similar concentrations of triptolide for 4 h, regardless of stimulus used (Table 2).

We therefore asked whether this transcriptional inhibition was restricted to a subset of genes, or whether triptolide was a nonspecific inhibitor of RNA synthesis. In A549 cells, 4h treatment with triptolide inhibited the TNF-α-stimulated incorporation of ^{3}H-uridine into RNA, with an IC_{50} value of 50 ng/ml, similar to that at which the reduction in cytokine synthesis was observed (Figure 5). Under the same conditions, triptolide had no direct effect on protein synthesis. Triptolide had a similar effect in nonstimulated cells (Figure 5), suggesting that triptolide is indeed a general transcription inhibitor in the cell lines tested. Similar results on RNA synthesis were

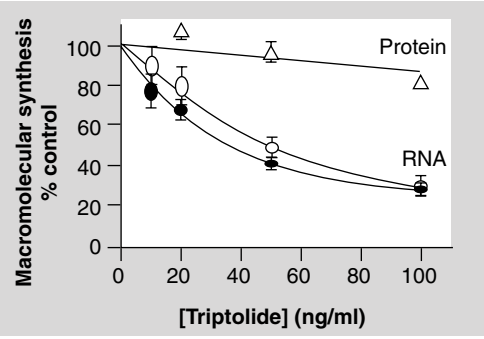
Figure 4. Triptolide inhibits synthesis of IκBa.



A549 cells were **A:** Pretreated with triptolide or dexamethasone for 1 h prior to 20 ng/ml TNF-α stimulation for various times as indicated; **B:** Treated with various concentrations of triptolide as indicated, for 1 h prior to 4 h stimulation with 20 ng/ml TNF-α. **C:** Incubated with or without triptolide in the presence or absence of calpain and stimulated with 20 ng/ml TNF-α for 0, 10, or 70 min, as indicated. Cells were fractionated and cytosol and nucleosol were subjected to SDS-PAGE and western blotted with anti-IκBa, antiphospho-IκBa, or anti-TNF-κB p65 as indicated. Fractionation was monitored by western blotting with anti-hsp90 and anti-oct1.
hsp: Heat shock protein; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TNF:Tumor necrosis factor.

observed in THP-1 cells. This inhibitory effect on transcription was not limited to immortalized cells, since the same effect was observed in primary rat monocytes (**Figure 7**).

Figure 5. Triptolide inhibits transcription.



A549 cells were incubated with triptolide for 4 h in the presence or absence of TNF-α. Incorporation of ^{3}H-uridine or ^{35}S-Met/Cys was measured as described in the Methods section. Circles: ^{3}H-uridine incorporation in nonstimulated (open circles) and stimulated cells (filled circles). Triangles: ^{35}S-Met/Cys incorporation into stimulated cells. Values are mean ± standard deviation of triplicate measurements.

An effect on stable transcripts, such as the housekeeping genes generally used as controls in transcriptional assays, may not be observed in these short assays. Therefore, rather than using the lack of effect on GAPDH expression to support a hypothesis that triptolide is a specific inhibitor of inflammation-related gene transcription, we looked at triptolide's effect on *de novo* RNA synthesis by performing nuclear run-on experiments and asking whether the total amount of mRNA synthesized is affected by triptolide. In fact, it appears that the incorporation of ^{33}P-UTP into RNA in nuclei isolated from triptolide-treated cells was reduced compared to untreated cells (**Figure 6A**). Interestingly, nuclei isolated from mock-treated cells were insensitive to triptolide treatment in the run-on experiment itself, suggesting that the inhibition of transcription requires signaling outside the nucleus (**Figure 6B**). This is consistent with the lack of effect of triptolide in a cell-free transcription-translation system using rabbit reticulocyte lysate.

Triptolide does not appear to inhibit transcription in vivo

It is difficult to resolve how a general transcription inhibitor could be efficacious and nontoxic in animal models of arthritis. Transcription levels were therefore measured in blood from triptolide-treated animals, to see whether cells involved in the immune response were adversely affected. Blood

Table 2. Triptolide suppresses activity of various promoters.

Promoter	Stimulation	Luciferase expression IC_{50} (nM)
MMTV	Dexamethasone	39
3X GRE	Dexamethasone	26
NF-κB	TNF-α	44
NF-κB	Phorbol myristate acetate	45
AP-1	TNF-α	68
AP-1	Phorbol myristate acetate	27

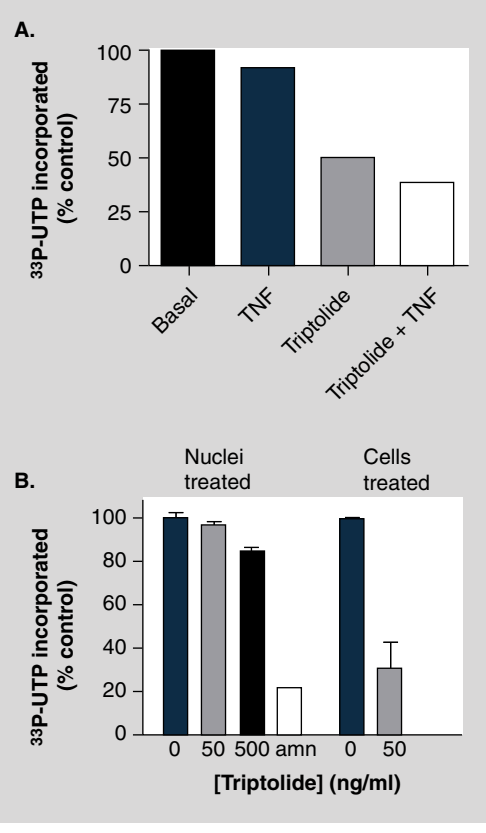
GRE: Glucocorticoid response element; MMTV: Mouse mammary tumor virus; NF-κB: Nuclear transcription factor; TNF: Tumor necrosis factor.

from rats treated for 5 days with 0.1 mg/kg/day triptolide was drawn 1 h after the last dose, and compared to blood from untreated rats. PBMCs were prepared and tested for ^{3}H-uridine incorporation. The PBMCs from untreated rats were capable of being both stimulated by TNF-α and inhibited by triptolide (**Figure 7A**). Interestingly, the washed PBMCs from triptolide-treated rats showed no significant difference in RNA synthesis compared to PBMCs from untreated rats, and could still be inhibited by further addition of triptolide to the isolated cells, suggesting that exposure to triptolide *in vivo* had no long lasting effects on the monocytes (**Figure 7A**). No toxicity or effects on blood cells was observed as might be expected if general transcriptional inhibition had occurred *in vivo* (**Figure 7B**). It is possible that a subset of transcripts was down-regulated by triptolide and was masked in the overall measurement.

Levels of specific transcripts in the CIA model

RT-PCR TaqMan analysis on paws from the treated and untreated mice in our CIA study was performed to specifically ask which transcripts were down-regulated in arthritic joints. As shown in **Figure 8A**, transcript levels in paws from triptolide-treated mice were only slightly reduced, compared with vehicle-treated controls. IL-1b transcripts showed the greatest reduction at 5.3-fold. TNF-α p38 and IL-6 mRNA levels were only reduced by 1.6, 1.8 and 3.5-fold, respectively. RankL transcript levels were not significantly different from untreated paws (**Figure 8A**). Dexamethasone treatment had a more drastic effect on RNA levels, with 13-fold reduction in IL-6, 16-fold reduction in TNF-α 17-fold reduction in RankL, and 29-fold reduction in IL-1b transcript levels

Figure 6. Triptolide inhibits *de novo* RNA synthesis at a step prior to initiation.



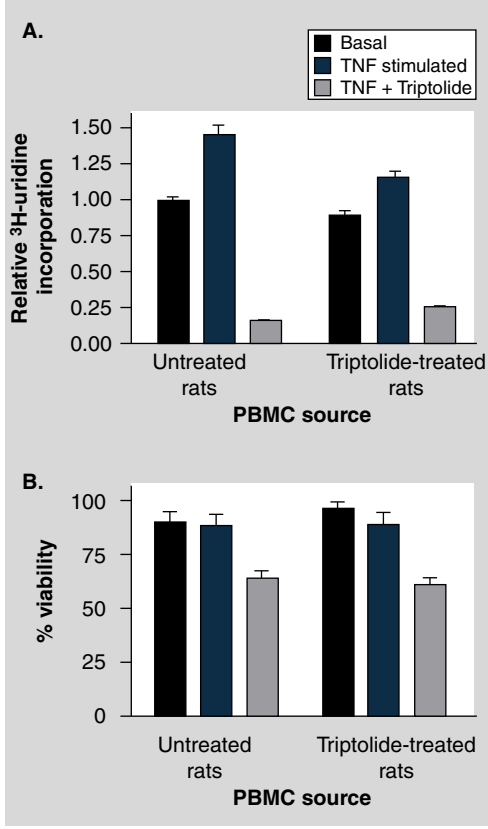
A: A549 cells were pretreated with or without 50 ng/ml triptolide for 1 h, then stimulated or not with 20 ng/ml TNF-α for 4 h. Nuclei were purified and run-on was performed by incubating with rNTP mixture and 100 µCi ^{33}P-UTP for one hour at 30°C. RNA was isolated and incorporation of ^{33}P-UTP into RNA determined as described in the Methods section. **B:** Nuclei were isolated from untreated cells, and run-on was measured in the absence or presence of 50 or 500 ng/ml triptolide, or 50 ng/ml α-amanitin (amn). Values are mean ± standard deviation of triplicate measurements.

(**Figure 8B**). The small effect of triptolide on RNA levels *in vivo* contrasts with the *in vitro* evidence of general transcriptional inhibition.

Discussion

Despite the efficacy of triptolide, and the use of *Tripterygium wilfordii Hook F* extract in Chinese medicine, the exact mode of action of this drug remains unknown. Triptolide has been implicated in signaling through NF-κB. Normally in response to stresses, signaling through the LPS or TNF-α receptors leads to phosphorylation,

Figure 7. Five-day treatment *in vivo* with triptolide does not impair transcription in isolated monocytes.



A: Monocytes from rats treated with 0.1 mg/kg/day for 5 days or from control untreated rats, were incubated with ^{3}H-uridine in the presence or absence of 20 ng/ml TNF-α and 50 ng/ml triptolide for 4 h at 37°C. RNA was precipitated and ^{3}H incorporation measured as described in the Methods section. **B:** Aliquots of the samples in A were tested for cell viability using the MTT assay as described in the Methods section. Values are mean ± standard deviation of triplicate measurements.

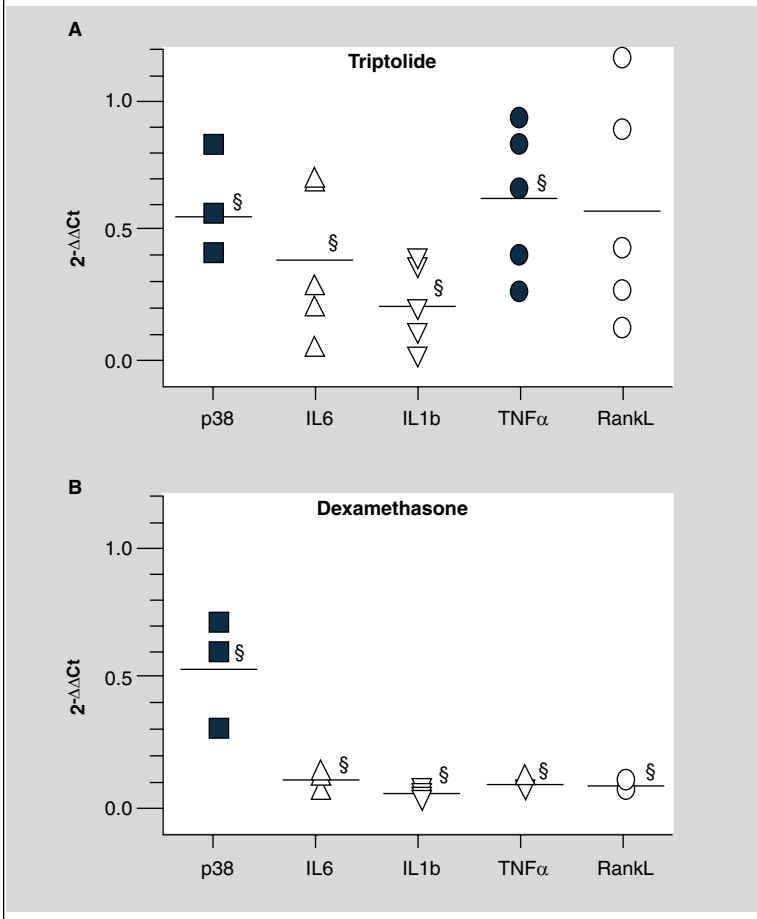
ubiquitination and degradation of the inhibitory factor IκBa. This frees NF-κB from the NF-κB-IκBa cytoplasmic complex, and allows translocation of NF-κB to the nucleus where association with other factors in the promoter drives synthesis of many of the genes involved in the inflammatory response [20–23]. The delayed re-synthesis of IκBa observed here is not sufficient to explain the anti-inflammatory effects of triptolide, since it is not consistent with decreased expression of cytokines, but rather would increase nuclear translocation of NF-κB. Triptolide treatment of cells has been reported to reduce cytokine transcription despite nuclear localization of NF-κB [15]. Furthermore, it has been found that triptolide does not block DNA binding of NF-κB, but inhibits transcriptional activation of NF-κB in A549 cells [13]. General transcriptional inhibition could explain both the lack of re-synthesis of IκBa, and decreased cytokine release observed.

While previous reports state that triptolide does not affect constitutive transcription, our evidence suggests that triptolide suppressed several different strong promoters in isolated cells. Inhibition of dexamethasone stimulation of the MMTV and 3 × GRE promoters suggested that triptolide could act through the glucocorticoid receptor in addition to NF-κB and AP-1. Triptolide had no effect, however, on binding of ^{3}H-dexamethasone to the glucocorticoid receptor in A549 cells, and it blocked IL-2 release in a glucocorticoid-insensitive cell line (results not shown), ruling out a specific interaction between triptolide and the glucocorticoid receptor. Thus, interpretation of results using expression systems with multiple copies of strong promoters such as these must be made with caution. Ultimately, the genomic actions of transcription factors, including NF-κB, are influenced by the stimuli applied and the promoter contexts in which they bind. [25]. The complexity in the genetic response to NF-κB complicates the interpretation of heterologous promoter assays.

Lack of effect on housekeeping genes such as GAPDH is generally used as evidence for signaling specificity. However, since one would not necessarily expect to detect effects on long half-life proteins that pre-existed the triptolide treatment in the cells, the nuclear run-on experiments were required to clearly demonstrate that triptolide treatment inhibits *de novo* RNA synthesis. The observed inhibition must occur prior to transcription initiation, since triptolide had no effect when added to isolated nuclei. Our results elaborate the findings of prior reports which suggested that triptolide inhibits the early stages of transcriptional activation [15] and are in contrast to the suggestion that triptolide exerts its immunosuppressive effects on NF-κB activation after the development of high-affinity specific DNA binding [11].

The transcriptional inhibition observed *in vitro* does not appear to be responsible for the anti-inflammatory effects of triptolide *in vivo*. If triptolide was acting as a general transcription inhibitor *in vivo*, one would expect toxicity to be observed. However, mice showed no signs of toxicity, even after 28 days at

Figure 8. TaqMan analysis of cytokine levels in paws from arthritic mice.



Transcript levels were measured in paws from mice at the end of the CIA study, as described in methods. Paws from triptolide-treated mice compared to control, vehicle-treated mice. A value of 1.0 represents no difference relative to control. Each point represents one paw and the horizontal bars denote the median value. Symbol (§) indicates statistically significant differences between treated and nontreated samples ($p < 0.01$).
CIA: Collagen-induced arthritis.

0.5 mg/kg triptolide per day. Furthermore, a general transcription inhibitor would result in overall reduction in mRNA levels *in vivo*, which were not observed. While we only measured levels of a small number of transcripts, our TaqMan results agree with those obtained using complementary DNA microarray [26]. Less than one percent of the genes investigated by Du and colleagues were suppressed by triptolide, and genes involved in signaling to cytokines were notably down-regulated. In contrast to the idea that triptolide is a general transcription inhibitor, 43 genes were upregulated in their study. This further supports the hypothesis that the general transcription inhibition by triptolide observed *in vitro* does not occur *in vivo*, and therefore is not necessary for the anti-inflammatory effects observed *in vivo*. *In vivo* administration of triptolide does correlate with decreased levels of pro-inflammatory cytokines [this paper,4,6], and decreased transcription of the nitric oxide synthase and cyclooxygenase II genes [27–29]. However, neither specific inhibition of NF-κB signaling nor general transcriptional inhibition appear to entirely explain the *in vivo* mode of action of this natural product which has been used for centuries. While triptolide has been shown to be efficacious in human trials, its use as an alternative or adjunct to steroid treatment should be treated with caution since the mode of action has not yet been determined. It is unknown whether triptolide treatment would lead to the increased risk of opportunistic infection as observed with methotrexate and steroids, or to other adverse effects such as those observed with long-term steroid use.

Conclusions

Triptolide inhibition of cytokine release *in vitro* is due to general transcriptional inhibition at a step prior to initiation. The *in vivo* immunosuppressive activity of triptolide does not correlate with general transcriptional inhibition, as measured by transcript levels in tissues, or transcriptional activity of isolated monocytes. The *in vivo* mode of action of triptolide cannot by directly inferred from *in vitro* studies of intracellular signaling, and remains to be elucidated.

Bibliography

Papers of special note have been highlighted as:
• of interest
•• of considerable interest

1. Lipsky PE, Tao X. A potential newtreatment
• for rheumatoid arthritis: thundergod vine. *Semin. Arthritis Rheum.* 26, 713–723 (1997).
2. Chen BJ. Triptolide, a novel
• immunosuppressive and anti-inflammatory agent purified from a Chinese herb Tripterygium wilfordii Hook F. *Leuk. Lymphoma* 42, 253–265 (2001).
3. Kupchan SM, Court WA, Dailey RG Jr., Gilmore C, Bryan RF. Triptolide and tripdiolide, novel antileukemic diterpenoid triepoxides from Tripterygium wilfordii. *J. Am. Chem. Soc.* 94, 7194–7195 (1972).
4. Asano K, Matsuishi J, Yu Y, Kasahara T,
• Hisamitsu T. Suppressive effects of Tripterygium wilfordii Hook F, a traditional Chinese medicine, on collagen arthritis in mice. *Immunopharmacology* 39, 117–126 (1998).
5. Gu WZ, Brandwein SR. Inhibition of type II
• collagen-induced arthritis in rats by triptolide. *Int. J. Immunopharm.* 20, 389–400 (1998).

Executive Summary

- *In vitro*, triptolide inhibited cellular transcription in A549 and THP-1 cells with IC_{50} values of 139 nM and 105 nM, respectively, similar to that for inhibition of cytokine release.
- Following degradation of IκBa after stimulation of cells, 500 ng/ml triptolide prevented *de novo* synthesis of IκBa, even after 4 h.
- Triptolide suppressed luciferase expression driven not only by nuclear transcription factor (NF)-κB, but also by AP-1, mouse mammary tumor virus (MMTV) and glucocorticoid response element (GRE) promoters with various stimuli.
- *In vivo* administration of triptolide reduced mouse plasma tumor necrosis factor (TNF)-α levels with long lasting results, however monocytes isolated from these mice showed no impaired RNA synthesis.
- Efficacious doses of triptolide in a mouse collagen-induced arthritis model (0.5 mg/kg/day) had only modest effects on transcript levels measured by Taqman, and exhibited blood cell counts within normal limits, with no toxicity observed.
- The general transcriptional inhibition observed *in vitro* in response to triptolide treatment does not account for the *in vivo* efficacy of this anti-inflammatory compound.
- The *in vivo* mode of action of triptolide is not simply due to inhibition of NFκB signaling, and remains to be fully elucidated.

6. Tao X, Ma L, Mo Y, Lipsky PE. Suppression of carrageenan-induced inflammation *in vivo* by an extract of the Chinese herbal remedy Tripterygium wilfordii Hook F. *Inflamm. Res.* 48, 139–148 (1999).
7. Tao X, Younger J, Fan FZ, Wang B, Lipsky PE. Benefit of an extract of Tripterygium wilfordii Hook F in patients with rheumatoid arthritis: a double-blind, placebo-controlled study. *Arthritis Rheum.* 46, 1735–1743 (2002).
8. Yu KT, Nuss G, Boyce R, Jariwala N, Owens G, Pennetti A, Chan W, Zhang DC, Chang MN, Zilberstein A. Inhibition of IL-1 release from human monocytes and suppression of streptococcal cell wall and adjuvant-induced arthritis in rats by an extract of Tripterygium wilfordii Hook. *Gen. Pharmacol.* 25, 1115–1122 (1994).
9. Gu WZ, Chen R, Brandwein S, McAlpine J, Burres N. Isolation, purification, and characterization of immunosuppressive compounds from tripterygium: triptolide and tripdiolide. *Int. J. Immunopharmacol.* 17, 351–356 (1995).
10. Tao X, Cai J, Lipsky PE. The identity of immunosuppressive components of the ethyl acetate extract and chloroform methanol extract (T2) of Tripterygium Wilfordii hook.F. *J. Pharm. Exp. Therapeutics* 272, 1305–1312 (1995).
11. Qiu D, Ko PN. Immunosuppressive and anti-inflammatory mechanisms of triptolide, the principal active diterpenoid from the Chinese medicinal herb Tripterygium wilfordii Hook f. *Drugs R&D* 4, 1–18 (2003).
12. Liu H, Liu ZH, Chen ZH, Yang JW, Li LS. Triptolide: a potent inhibitor of NF-kB in T-lymphocytes. *Acta Pharmacol. Sin.* 21, 782–786 (2000).
13. Lee KY, Chang WT, Qiu D, Kao PN, Rosen D. PG490 (Triptolide) cooperates with tumor necrosis factor-a to induce apoptosis in tumor cells. *J. Biol. Chem.* 274, 13451–13455 (1999).
14. Zhao G, Vaszar LT, Qiu D, Shi L, Ko PN. Anti-inflammatory effects of triptolide in human bronchial epithelial cells. *Am. J. Physiol. Lung Cell Mol. Physiol.* 239, L958-L966 (2000).
15. Qiu D, Zhao G, Aoki Y, Shi L, Uyei A, Nazarian S, Ng JCH, Ko PN. Immunosuppressant PG490 (Triptolide) Inhibits T-cell Interleukin-2 Expression at the Level of Purine-box/Nuclear Factor of Activated T-cells and NF-kB Transcriptional Activation. *J Biol. Chem.* 274, 13443–13450 (1999).
16. Gonzales JC, Johnson DC, Morrison D, Freudenberg MA, Galanos C, Silverstein R. Endogenous and exogenous glucocorticoids have different roles in modulating endotoxin lethality in D-galactosamine-sensitized mice. *Infect. Immun.* 61, 970–974 (1993).
17. Greenberg ME, Bender TP. Identification of newly transcribed RNA. In: *Current Protocols in Molecular Biology* Ausubel FM, Brent R, Kingston RE, Moore D, Seidman JG, Smith JA, Struhl K (Eds), John Wiley & Sons, Inc., NY, USA (1997).
18. Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C. An overview of real-time quantitative PCR: Applications to quantify cytokine gene expression. *Methods* 25, 386–401 (2001).
19. Lin N, Sato T, Ito A. Triptolide, a novel diterpenoid triepoxide from Tripterygium wilfordii Hook. f, suppresses the production and gene expression of pro-matrix metalloproteinases 1 and 3 and augments those of tissue inhibitors of metalloproteinases 1 and 2 in human synovial fibroblasts. *Arthritis Rheum.* 44, 2193–2200 (2001).
20. Tao X, Lipsky PI. The Chinese anti-inflammatory and immunosuppressive herbal remedy Tripterygium wilfordii Hook F. *Rheum. Dis. Clin. North Am.* 26(1), 29–50 (2000).
21. Li Q, Verma IM. NF-kB regulation in the immune system. *Nature Rev. Immunol.* 2, 725–734 (2002).
22. Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: The control of NF-kB activity. *Annu. Rev. Immunol.* 18, 621–663 (2000).
23. Hoffmann A, Levchenko A, Scott ML, Baltimore D. The IkBa- NF-kB signaling module: Temporal control and selective gene activation. *Science* 298, 1241–1245 (2002).
24. Turpin P, Hay RT, Dargemont C. Characterization of IkBa nuclear import pathway. *J. Biol. Chem.* 274, 6804–6812 (1999).
25. Tian B, Brasier AR. Identification of a nuclear factor kappa B-dependent gene network. *Recent Prog. Horm. Res.* 58, 95–130 (2003).
26. Du ZY, Li ZY, Li YC, Wang SY. Analysis of triptolide-regulated gene expression in Jurkat cells by complementary DNA microarray. *Acta Pharmacol. Sin.* 24, 864–872 (2003).
27. Wang B, Ma L, Tao X, Lipsky PE. Triptolide, an active component of the Chinese herbal remedy Tripterygium wilfordii Hook F, inhibits production of nitric oxide by decreasing inducible nitric oxide synthase gene transcription. *Arthritis Rheum.* 50, 2995–3003 (2004).

28. Shao XT, Feng L, Yao HP, Sun WJ, Zhang LH. Effect of Triptolide on TNF-alpha-induced activation of NF-kappaB and expression of COX-2 and iNOS in human rheumatoid arthritis synovial fibroblasts. *Zhejiang Da Xue Xue Bao Yi Xue Ban.* 33, 160–165 (2004).
29. Tao X, Schulze-Koops H, Ma L, Cai J, Mao Y, Lipsky PE. Effects of Tripterygium wilfordii hook F extracts on induction of cyclooxygease 2 activity and prostaglandin E2 production. *Arthritis Rheum.* 41, 130–138 (1998).

Affiliations

Christine McCallum
Merck Research Laboratories,
Department of Human and Animal Infectious Disease Research,
PO Box 2000, Rahway,
New Jersey 07065, USA
Tel.: +1 732 594 2502
Fax: +1 732 594 6708
christine_mccallum@merck.com

Suzy Kwon, Penny Leavitt, Wesley Shoop, Bruce Michael, Tom Felcetto and Anne Gurnett
Merck Research Laboratories,
Department of Human and Animal Infectious Disease Research, PO Box 2000, Rahway,
New Jersey 07065, USA

Dennis Zaller, Edward O'Neill, Betsy Frantz-Wattley, Chris Thompson and Gail Forrest
Merck Research Laboratories,
Department of Immunology,
PO Box 2000, Rahway,
New Jersey 07065, USA

Ester Carballo-Jane
Merck Research Laboratories,
Department of Immunology,
PO Box 2000, Rahway,
New Jersey 07065, USA