Available online at www.sciencedirect.com
ScienceDirect
Immunobiology 212 (2007) 549–556
Immunobiology
www.elsevier.de/imbio

Triptolide binds covalently to a 90 kDa nuclear protein. Role of epoxides in binding and activity

Christine McCallum[a,*], Suzy Kwon[a], Penny Leavitt[a], Dong-Ming Shen[b], Wensheng Liu[c], Anne Gurnett[a]

[a]*Department of Infectious Diseases, Merck Research Laboratories, PO Box 2000, Rahway, NJ 07065, USA*
[b]*Department of Basic Chemistry, Merck Research Laboratories, PO Box 2000, Rahway, NJ 07065, USA*
[c]*Department of Drug Metabolism Labeling, Merck Research Laboratories, PO Box 2000, Rahway, NJ 07065, USA*

Received 27 October 2006; received in revised form 17 January 2007; accepted 21 February 2007

Abstract

Triptolide is a naturally occurring diterpene triepoxide whose anti-inflammatory effects correlate with transcriptional inhibition of various cytokines. Despite its use in herbal medicine for thousands of years, the cellular target and mode of action of this drug are unknown. [^{3}H]-triptolide was prepared and a filtration assay designed to measure binding to cells and cellular extracts. Triptolide bound specifically and irreversibly to a single, 90 kDa protein in nuclear extracts from stimulated and non-stimulated monocytic and epithelial cell lines. Thiol reactivity of one or more of the epoxides on triptolide was necessary for the covalent binding, since thiol oxidizing agents dithiodipyridine and diamide, and the thiol alkylating agent *N*-ethylmaleimide all reduced the binding of [^{3}H]-triptolide to nuclear extract. Neither glutathione nor the pro-oxidant *tert*-butylhydroperoxide affected the binding of [^{3}H]-triptolide to the nuclear protein, ruling out a general oxidant effect. The number of epoxide moieties correlated with the ability to compete with radiolabeled triptolide for binding to the nuclear extract and with the potency of inhibition of TNFα secretion from monocytes, IL-2 secretion from Jurkat cells, and with inhibition of RNA synthesis. The correlation between the structure–activity relationship and observed binding suggests that identification of the triptolide binding protein could provide insight into the cellular mode of action of this anti-inflammatory natural product.

Keywords: Epoxide; Filtration binding; TNFα; *Tripterygium wilfordii*; Triptolide

Introduction

Triptolide is a diterpene triepoxide isolated from the root of *Tripterygium wilfordii* Hook F (Kupchan et al., 1972). The potent anti-inflammatory and immunosuppressive effects of triptolide have been studied in vitro and in vivo, and have been attributed to inhibition of the transcription factor NF-κB (Qui et al., 1999; Lee et al., 1999, 2002). The in vivo immunosuppressive effect of triptolide correlates with inhibition of NFAT and

Abbreviations: DTDP, dithiodipyridine; FBS, fetal bovine serum; IL2, interleukin 2; LPS, lipopolysaccharide; NEM, *N*-ethylmaleimide; NFAT, Nuclear factor of activated T-cells; NF-κB, nuclear factor kappa B; PBS, phosphate buffered saline; PHA, phytohaemagglutinin; PMA, phorbol myristate acetate; TNFα, tumor necrosis factor alpha; *t*BPH, *tert*-butylhydroperoxide; XIAP, x-chromosome-linked inhibitor-of-apoptosis

*Corresponding author. Tel.: +1 732 594 2502; fax: +1 732 594 6708.
E-mail address: christine_mccallum@merck.com (C. McCallum).

0171-2985/$ - see front matter © 2007 Elsevier GmbH. All rights reserved.
doi:10.1016/j.imbio.2007.02.002

NF-κB in T-cells (Qui et al., 1999). Changes in the numbers of CD4+ and CD8+ cells in the Peyer's patch and periphery have been observed in triptolide treated rats in the collagen-induced arthritis model (Zhou et al., 2006). In vitro, triptolide suppresses production of various cytokines via inhibition of NF-κB (Qui et al., 1999; Dai et al., 2006; Liu et al., 2006). We have previously shown, however, that triptolide's effects in vitro are not limited to NF-κB inhibition, but rather are a result of general transcriptional inhibition (McCallum et al., 2005). Consistent with a general suppression of transcription, triptolide has recently been reported to suppress an HSP70 promoter-reporter construct by inhibiting the heat shock transcription factor HSF-1 transactivation function without inhibiting DNA binding (Westerheide et al., 2006), similar to that reported for triptolide inhibition of NF-κB transactivation (Lee et al., 1999). The compound has also been reported to inhibit proliferation of transformed cell lines (Wei and Adachi, 1991; Shamon et al., 1997) and to be pro-apoptotic, as a result of activation of caspase-3 and caspase-8 in a bcl-2 dependent manner (Wang et al., 2006).

In an attempt to identify the molecular target of triptolide responsible for the transcriptional inhibition, we have probed the binding of [^{3}H]-triptolide in monocytic and epithelial cells. We report here that triptolide binds specifically and irreversibly to an as-yet unidentified nuclear protein. The roles of the three triptolide epoxide moieties in this irreversible binding, and the relationship of such interactions with the inhibition of transcription and cytokine secretion, are discussed.

Materials and methods

Cytokine secretion assays

TNFα secretion by THP-1 cells: THP-1 cells were grown in RPMI-1640 plus 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), and plated at a density of 5×10^6/mL in 384-well plates which had been coated with 5 μg/mL anti-human TNFα antibody (R&D systems, Minneapolis, MN). Test samples were added, and cells were stimulated with 100 ng/mL LPS (Sigma, St Louis, MO) at 37 °C for 4 h. Cells were removed and the plates were washed three times with Tris-buffered saline (TBS). A total of 400 ng/mL anti-human TNFα-biotin (R&D systems) in KPL wash buffer (Kirkgaard & Perry Laboratories, Inc.) was added. After 1 h incubation at room temperature plates were washed three times with TBS. Streptavidin-europium (Perkin Elmer, Shelton, CT) at 1:1000 in europium assay buffer (TBS, 7 μg/mL DTPA, 0.01% sodium azide, 1% BSA, and 0.05% Tween 20) was added to each well and incubated for 20 min, followed by wash as above. Enhancer solution (Perkin–Elmer) was added and after 1 h, europium fluorescence was measured using a Wallac Victor plate reader.

IL2 secretion by Jurkat cells: Jurkat cells were grown in RPMI-1640 plus 10% FBS and plated in 96-well plates at a density of 5×10^6/mL. After pre-treating with triptolide (Calbiochem/EMD Biosciences, San Diego, CA) for 1 h, cells were stimulated with phytohaemagglutinin (PHA) (2 μg/mL) plus PMA (3 ng/mL) (EMD Biosciences). After stimulating for 4 h, plates were centrifuged for 20 min at $1100 \times g$ and the supernatant was collected. Levels of IL-2 in the supernatants were assayed using IL-2 ELISA kit (GE healthcare Biosciences, Piscataway, NJ).

Preparation of cell lysates and nuclear extract

A549 cells were densely seeded into T-175 flasks and allowed to grow for 24 h to 80–85% confluence prior to cytokine stimulation in RPMI-1640 plus 10% FBS. Cells were treated with 20 ng/mL TNFα (Upstate) for 4 h, then harvested by trypsinization and washed with cold PBS. Cytosolic and nuclear extracts were prepared using NePER nuclear extraction kit (Pierce Biotechnology, Inc., Rockford, IL) following manufacturer's instructions with an additional wash of the nuclear pellet prior to nuclear lysis. Protein levels were determined using Pierce Micro BCA Protein Assay. The fractionation quality was monitored by Western Blot with antibodies to cytoplasmic and nuclear markers, Hsp90 (Sigma) and Oct 1 (Santa Cruz Biotechnology, Santa Cruz, CA), respectively.

THP1 cells were cultured in RPMI-1640 without phenol red plus 10% FBS. Cells were treated with 250 ng/mL LPS for 4 h, harvested, washed three times with cold PBS, then mixed vigorously with an equal volume lysis buffer (20 mM HEPES, 2% SDS, 10% glycerol, and protease inhibitor) and incubated on ice 20 min. The THP1 lysates were clarified by centrifugation at $14{,}000 \times g$ for 10 min at 4 °C.

^{3}H-labeling of triptolide

Triptolide was oxidized to triptonide using the Dess-Martin periodinane, then tritiated according to the reduction procedure of Yang et al. (2000) employing $NaBT_4$ and $Eu(FOD)_3$ in methanol. $Eu(FOD)_3$ (0.5 mg) was added to a stirred solution of triptonide (1 mg, 2.79×10^{-3} mmol) in anhydrous methanol (200 μL). The reaction mixture was stirred for 20 min and sodium borotritide (1 Ci, 80 Ci/mmol) was added at room temperature. After 4 h, the reaction mixture was filtered using a Whatman auto-vial syringe-less filter and rinsed with methanol. The labile tritium in the combined filtrates