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Anti-inflammatory activity *in vitro* and *in vivo* of the protein farnesyltransferase inhibitor, tipifarnib

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Running title: Anti-inflammatory activity of FTI tipifarnib

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Number of text pages: 30

Number of figures: 8

Number of tables: 0

Number of references: 24

Number of words in the Abstract: 248

Number of words in the Introduction: 428

Number of words in the Discussion: 1372

Abbreviations:

FTI, farnesyltransferase inhibitor;

LPS, lipopolysaccharide;

PBMC, peripheral blood mononuclear cell;

TNF, tumor necrosis factor;

MMP-9, matrix metalloproteinase 9;

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MCP-1, monocyte chemotactic protein 1;

MIP-1α, macrophage inflammatory protein 1 alpha;

IκB, inhibitory subunit of NF-κB;

NF-κB, nuclear factor κB;

ERK, extracellular signal-regulated kinase;

TLR4, toll-like receptor 4;

TLR2, toll-like receptor 2;

IKK, IκB kinase;

TRAF6, TNF receptor-associated factor 6;

TNFR, tumor necrosis factor receptor;

IRAK, IL-1 receptor-associated kinase;

TRIF, TIR domain-containing adaptor protein inducing IFN-β;

MAPK, mitogen-activated protein kinase;

TAK, transforming growth factor-β-activated kinase;

MD-2, myeloid differentiation protein-2

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Abstract

Protein farnesyltransferase inhibitors (FTIs) have shown clinical responses in hematologic malignancies, but the mechanisms are unclear. To better understand potential mechanisms of action, we have studied effects of the FTI, tipifarnib, on inflammatory responses *in vitro* and *in vivo*. In a human leukemia cell line, THP-1, tipifarnib inhibited LPS-induced transcription of chemokines (MCP-1, MCP–2), cytokines (IL-1β, IL-6, IFNβ), signaling pathway genes (MyD88, STAT-1), proteases (MMP-9) and receptors (urokinase receptor). Tipifarnib also inhibited LPS-induced secretion of MMP-9, IL-6, MCP-1 and IL-1β in THP-1 cells. In primary human PBMC, dose-dependent inhibition of LPS-induced TNF-α, IL-6, MCP-1 and IL-1β by tipifarnib was observed, with no evidence of cytotoxicity. Similar results were obtained *in vivo* in a murine model of LPS-induced inflammation, where pre-treatment with tipifarnib resulted in significant inhibition of TNF-α, IL-6, MCP-1, IL-1β, and MIP-1α production. Tipifarnib had no effect *in vitro* or *in vivo* on LPS-induced IL-8. Studies in THP-1 cells to address potential mechanism(s) showed that tipifarnib partially inhibited LPS-induced p38 phosphorylation. Tipifarnib significantly inhibited IκB-α degradation and p65 nuclear translocation induced by LPS, but not by TNF-α, IL-1α, or TLR2 ligand, suggesting that the target for inhibition of NF-κB activation was exclusive to the LPS/TLR4 signal pathway. The extent of IκB-α degradation inhibition did not correlate with inhibition of Ras farnesylation, indicating that Ras was not the target for the observed anti-inflammatory activity of tipifarnib. Our findings differ from those for other FTIs, which may have relevance for their dissimilar activity in specific tumor repertoires.

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Introduction

The links between cancer and inflammation are well established (reviewed by Balkwill and Mantovani, 2005). Many human and murine cancers are found in a microenvironment rich in cytokines, chemokines, and inflammatory enzymes. It is therefore of interest to investigate the effects of antitumor drugs on inflammation. Tipifarnib (R115777, Zarnestra™, (R)-6-amino[(4-chlorophenyl)(1-methyl-1H-imidazol-5-yl)methyl]-4-(3-chlorophenyl)-1-methyl-2(1H)-quinolinone)is an orally active inhibitor of protein farnesyltransferase. It has shown significant antitumor activity and is currently in clinical trials for acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) (Cortes et al., 2003). Farnesyltransferase is an enzyme that catalyzes the attachment of a farnesyl group, from farnesyl pyrophosphate, to the cysteine-thiol group of protein C-terminal CAAX consensus sequences (Moores et al., 1991; Reiss et al., 1991). A variety of cellular proteins are farnesylated (Schafer et al., 1992; Tamanoi et al., 2001), including Ras superfamily G proteins, nuclear lamins A and B, rhodopsin kinase, CENP-E and CENP-F, co-chaperone DnaJ/HDJ-2, prostacyclin receptor (O'Meara and Kinsella, 2004; O'Meara and Kinsella, 2005) and cytosolic phospholipase $A_2\gamma$ (Jenkins et al., 2003), Ras farnesylation is critical for oncogenic Ras signaling (Kato et. al., 1992) and Ras mutants are associated with ~30% of human cancers. Farnesyltransferase inhibitors (FTIs) were developed to prevent Ras farnesylation and cell membrane association and therefore block aberrant Ras function in cancer. However, further studies have shown that the response to FTIs does not correlate with Ras status, suggesting inhibition of farnesylation of other proteins might also contribute to the antitumor properties of FTIs (Cox and Der, 1997; Sebti and Hamilton, 2000).

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Recently a FTI has been shown to inhibit TNF-α induced NF–κB activation *in vitro* (Takada et al., 2004). Na *et al.* (Na et al., 2004) also showed inhibition by another FTI of LPS-induced NF–κB activation *in vitro*, and iNOS, COX-2, TNF-α and IL-1β expression *in vivo*. These findings suggested that FTIs may have anti-inflammatory activity through affecting IL-1R/TLR/TNFR signaling. We tested the effect of tipifarnib on inflammatory responses induced by LPS. The results of our study demonstrated inhibition by tipifarnib of LPS induction of a number of inflammatory mediators, *in vitro* and *in vivo*. We present the first evidence for downregulation by a FTI of LPS-induced MCP-1, IL-6, MMP-9, STAT1, MyD88 and MIP-1α. We also found that tipifarnib significantly inhibited IκB degradation and p65 translocation induced by LPS, but not by TNF-α, TLR2 ligand or IL-1α. In addition, inhibition of Ras farnesylation did not correlate with the extent of inhibition of IκB degradation. The results of our study suggest that tipifarnib affected pro-inflammatory responses through inhibiting farnesylation of one or more proteins other than Ras, that are involved in a pathway exclusive to LPS/TLR4 signaling.

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Materials and Methods

Materials

General chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Human THP-1 cells were obtained from the American Type Culture Collection (Manassas, VA). RPMI-1640 cell culture medium and Penicillin-Streptomycin were purchased from Sigma-Aldrich, and fetal bovine serum (FBS) from Gibco/Invitrogen (Carlsbad, CA). LPS, from Escherichia coli 026:B6, was obtained from Sigma-Aldrich. IL-1α and TNF-α were purchased from R&D systems (Minneapolis, MN). Pam3CSK4, the synthetic lipoprotein, was purchased from InvivoGen (San Diego, CA).

Cell culture, RNA isolation and cytokine Analysis

THP-1 cells were cultured in RPMI-1640 medium, containing 10% FBS, 100 units/ml Penicillin, 10 μg/ml Streptomycin, at 37°C, under 5% CO_2. Medium components were certified to contain less than 0.3 endotoxin units/ml. For RNA isolation, THP-1 cells were cultured at $4.5x10^5$/ml in 0.5% FBS/RPMI-1640 medium in the absence or presence of 100ng/ml LPS plus or minus 5 μM tipifarnib. Cells were collected at 3, 6 and 12 hours for total RNA isolation using the RNeasy mini kit from QIAGEN (Valencia, CA). For cytokine secretion studies, THP-1 cells at $3.4 x10^5$/ml were cultured in 96-well plates in 0.5%FBS/RPMI 1640, in the absence or presence of 100ng/ml LPS plus or minus tipifarnib. Supernatants were collected at different time points for analysis of cytokine production.

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Microarray experiments and data analysis

5 µg of total RNA was linearly amplified to double-stranded cDNA using T7-based amplification. The cDNA was purified using the QIAquick 96 PCR purification kit (QIAGEN Valencia, CA), and used to generate amplified (a)RNA using AmpliScribe™ T7 High Yield Transcription Kit (Epicentre, Madison, WI). 10 µg of purified aRNA was reverse transcribed to cDNA using the SuperScript RTII kit (Invitrogen) and labeled by direct incorporation of Cy3-dCTP. The generated cDNA probe was used for hybridizing to cDNA microarrays. The human cDNA microarrays were custom made as described previously (Peterson et al., 2004), and contained approximately 8,000 human genes and ESTs. RNA preparation, cDNA probe synthesis and hybridization were performed as described by Shaw *et al.*(Shaw et al., 2003) Data normalization and preparation were performed as previously described (Peterson et al., 2004).

Reverse Transcriptase and Real-time quantitative PCR

DNase-treated total RNA (2-3 µg) was reverse transcribed to cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer's instructions. Real-time PCR was carried out with the LightCycler FastStart DNA Master^{plus} SYBR Green I kit (Roche Diagnostics) according to the manufacturer's instructions. Real-time PCR reaction conditions were as follows: The 20 µL final volume contained: 4 mM $MgCl_2$, 0.5 µM of each primer, 2 µL Master mix and 2 µL of cDNA. The PCR profile was as follows: (i) denaturation at 95 °C for 10 min. (ii) 45 cycles of 0 s at 95 °C, 5 s at 54-58°C (depending on Tm of the primers), 6-16 s (determined by the length of amplicon) at 72 °C. (iii)

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Melting curves for 0 s at 95°C, 15s at 65°C and 0 s at 95 °C. (iv) Cooling at 40°C for 30 s. Transition rates were: 20 °C/s for all steps except 0.1 °C/s for 95 °C segment 3 of the melting curves. For data analysis, the baseline adjustment was carried out in the "arithmetic" mode, and the fluorescence curve analysis was carried out in the "Second Derivative Maximum" mode of the LightCycler™ software (Version 3.5). The primers used for real-time PCR were as follows (5' to 3'):

IL-1β: sense GGATAACGAGGCTTATGTGCACG,

antisense GGACATGGAGAACACCACTTGTTG;

MCP-1: sense AGCCAGATGCAATCAATGCCC,

antisense, CCTTGGCCACAATGGTCTTGAA;

MMP-9: sense ACCGCTATGGTTACACTCGG,

antisense AGGGACCACAACTCGTCATC;

IL-8: sense CTGATTTCTGCAGCTCTGTGTGAA,

antisense TGGCATCTTCACTGATTCTTGGA;

STAT1: sense TTCAGAGCTCGTTTGTGGTG,

antisense AGAGGTCGTCTCGAGGTCAA;

MyD88: sense TGGGACCCAGCATTGAGGAGGATT,

antisense, AAACTGGATGTCGCTGGGGCAA;

IL6 sense, TGGATTCAATGAGGAGACTTGC,

antisense, CAGGAACTGGATCAGGACTT.

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Human PBMC isolation

Venous blood was drawn from healthy donors and treated with heparin. PBMCs were isolated by density-gradient sedimentation on Ficoll-Paque (Amersham Bioscience, Piscataway, NJ). PBMCs were washed twice with PBS, resuspended in RPMI cell culture medium, then plated at 3x10^5 per well in 96-well plates and incubated at 37°C, under 5% CO_2 for 2hr. Then, cells were pretreated with tipifarnib at 2 and 5 μM for 1hr, followed by incubation with 10 ng/ml LPS for 16hr. The supernatants were collected for cytokine assay. The groups of untreated and LPS-treated only were also included.

***In vivo* experiments**

The experiments were performed on female BALB/c mice (6-7 week old; Charles River Laboratories, Inc. MA) after review of the protocol and approval by the IACUC committee. Tipifarnib was dissolved in 20% cyclodextran, and 50 mg/kg were orally administered to mice at 24, 17 and 1 hour before intraperitoneal injection of 20 μg LPS per mouse, 1mg/kg. Control mice received vehicle (20% cyclodextran) only. Blood samples were collected by eye bleeding or cardiac puncture at 2 and 3 hours after LPS injection. Plasma samples were tested for cytokines using individual ELISA kits (R&D Systems) or Luminex analysis (Luminex Corporation, Austin, TX), according to the manufacturers' instructions.

SDS-PAGE and Western blot analysis

THP-1 cells in 0.5 % FBS/RPMI were pre-treated with or without tipifarnib for 18 hours, and subsequently stimulated with LPS (100 ng/ml), TNF-α (10 ng/ml), IL-1α (5 ng/ml)

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or Pam3CSK4 (1 μg/ml) for 30 minutes. Cells were harvested, washed three times in ice-cold PBS, and lysed in lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml Leupeptin, 1 mM PMSF). Nuclear extracts were prepared using the NE-PER kit (PIERCE). Cytoplasmic or nuclear lysates from each sample were mixed with an equal amount of 2x SDS-PAGE sample buffer (Invitrogen). Proteins were separated by electrophoresis in Tris-glycine 10-20% gradient gels (Novex), and transferred to nitrocellulose membranes (PROTRAN; Schleicher & Schuell). The membranes were blocked with 3% BSA in PBS-0.05%Tween-20, and sequentially incubated with primary antibodies and HRP-conjugated secondary antibodies (anti-rabbit IgG or anti-mouse IgG and IgM; PIERCE), followed by ECL detection (Amersham Biosciences). The primary antibodies used were rabbit polyclonal anti-IκBα, -p65 and -p38 (Santa Cruz Biotechnology), mouse monoclonal anti-H-Ras (Chemicon), mouse monoclonal anti-phospho-p38, rabbit polyclonal anti-phospho-Erk and anti-total Erk (Cell Signaling Technology).

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Results

Effect of tipifarnib on LPS-induced gene transcription

Initial analysis of global effects for tipifarnib on transcription of inflammatory genes was performed by cDNA microarray analysis of RNA samples from THP-1 cells, treated with LPS, in the presence or absence of tipifarnib. Out of 94 genes showing at least 1.5-fold induction by LPS at 2 time points, 23 showed at least a 1.5-fold decrease in LPS-induced transcripts by tipifarnib at one or more time points (Fig. 1). Included in the genes down-regulated by the drug were chemokines (MCP-1 and –2), cytokines (IL-1β, IFNβ), signaling pathway genes (MyD88, STAT-1), receptors (urokinase receptor) and proteases (MMP-9).

The results of the microarray analysis were confirmed for selected genes by real-time PCR analysis (Fig. 2). Transcription of IL–1β was induced by LPS as early as 3 hours, and the induction was unaffected by tipifarnib up to this time point. However, after 6 hours, and to a greater extent, after 12 hours, the LPS-dependent induction was decreased by tipifarnib (Fig. 2a). MCP-1 was induced by LPS from 6 hours, and this induction was inhibited significantly by tipifarnib (Fig. 2b). MMP-9 induction by LPS increased up to 12 hours and was abolished by treatment of tipifarnib (Fig. 2c). In the case of IL-8, induction by LPS was evident as early as 3 hours, and the FTI showed no significant effect on this induction (Fig. 2d). STAT1 induction was inhibited by tipifarnib at 6 and 12 hours (Fig. 2e). Myeloid differentiation factor, MyD88, the adaptor for Toll-like receptor-mediated responses, was significantly upregulated by LPS at 6 and 12 hours, and

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this was almost completely inhibited by tipifarnib (Fig. 2f). Although microarray data for IL-6 transcription did not meet the criteria for inclusion in Figure 1, analysis by quantitative real-time PCR clearly showed inhibition by tipifarnib of LPS-induced IL-6 transcription at 6 and 12 hours (Fig. 2g). Thus, tipifarnib was able to inhibit LPS induction of numerous inflammatory genes.

Effect of tipifarnib on LPS-induced cytokine and MMP-9 production

To further investigate the effects of tipifarnib at the protein level, cytokine and MMP-9 secretion by THP-1 cells in response to LPS was examined. Cells were treated with a range of concentrations of tipifarnib in the presence and absence of LPS. Tipifarnib showed dose-dependent inhibition of MMP-9 and IL-6 secretion, reaching greater than 50 % inhibition for both mediators at 2 μM (Fig. 3). This concentration was chosen to examine the effects of tipifarnib treatment on LPS-induced inflammatory mediators at different time over a 48 hour period. Tipifarnib showed significant inhibition as early as 20 hours for MCP-1 and IL-6 , and 30 hours for IL-1β and MMP-9. (Fig. 4). Tipifarnib showed no significant inhibition of IL-8 production, consistent with the lack of effect for tipifarnib on IL-8 transcription (Fig. 2d). LPS-induced TNF-α also showed a trend towards reduction after treatment by tipifarnib for 20 hours (not shown), although the production of TNF-α by THP-1 cells was low and the inhibition did not reach statistical significance. At 2 μM, tipifarnib had no effect on cell viability or growth up to 48 hours (trypan blue exclusion, data not shown), as was also evident from the lack of inhibition of continuous IL-8 secretion.

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Effect of tipifarnib on LPS-induced cytokine production in human PBMC

We also tested the effect of tipifarnib on primary human PBMC. PBMCs were pretreated with tipifarnib at 2 and 5 μM for 1 hour, followed by incubation with LPS for 16 hours. Tipifarnib inhibited LPS-induced TNF-α, IL-1β and MCP-1 significantly at both 2 and 5 μM. IL-6 was significantly inhibited at 5 μM, while IL-8 inhibition did not reach statistical significance at either concentration (Fig. 5). Tipifarnib showed no inhibition of LPS-induced IL-10 secretion (data not shown). In the same experiment, the effect of tipifarnib at 2 and 5 μM on PBMC viability was also tested by a XTT assay and no significant cytotoxicity was observed (data not shown).

Effect of tipifarnib on LPS-induced cytokine production *in vivo*

To follow up on our *in vitro* observations, we tested the effect of tipifarnib pretreatment in a murine model of LPS-induced inflammation. The mice were pre-treated for 24 hours with the compound by oral administration at three time points, 24 hours, 17 hours, and 1 hour before LPS injection. Blood samples were taken 2 and 3 hours after LPS injection for analysis.

As shown in Figure 6, the most striking effect of the FTI was its highly significant inhibition of LPS-induced TNF-α production at 2 hours. Approximately 50% inhibition of IL-6 production, and almost complete inhibition of IL-1β production, was observed in tipifarnib-treated animals after 3 hours. Approximately 50% inhibition of MIP-1α and MCP-1 production by the FTI was observed at 2 hours. IL12-p40 and -p70 induction by

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LPS was also inhibited by tipifarnib at 3 hours, while IL-10 was not significantly changed at both time points (data not shown). No effects of tipifarnib on LPS-induced KC were observed, consistent with *in vitro* results for IL-8. Overall, the results show significant downregulation by tipifarnib of numerous LPS-induced cytokines and chemokines *in vivo*.

Effect of tipifarnib on TLR/IL-1R ligand and TNF-α induced signaling

It was recently reported that TNF-α induced NF-κB activity was inhibited by another FTI (SCH 66336) (Takada et al., 2004). To compare the effects of tipifarnib to this finding, we tested TNF-α induced NF-κB activation by luciferase reporter activity in HEK 293 cells. Cells were treated with tipifarnib overnight, followed by stimulation with TNF-α for 4 hours. In contrast to the results reported for SCH 66336, TNF-α induced NF-κB activity was not inhibited by tipifarnib at 2 or 5 μM (data not shown). We further examined the effect of tipifarnib on several signaling pathways in THP-1 cells. Cells were pre-treated with 2 μM tipifarnib for 18 hours, then stimulated with LPS or TNF-α for 30 minutes. Cell lysates were tested for p38 expression and p38 phosphorylation by Western blot analysis. Phosphorylation of p38 was significantly increased by LPS and TNF-α stimulation, but only the LPS-induced increase was partially reduced by tipifarnib treatment (Fig. 7a). No significant inhibition was observed for TNF-α induced p38 phosphorylation. P38 protein expression was not affected by LPS or TNF-α stimulation and/or tipifarnib treatment.

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As shown in the upper panels of Fig. 7 b and c, IκB-α degradation was significantly induced by TLR/IL-1R ligands (LPS, Pam3CSK4 and IL-1α) and TNF-α. Interestingly, tipifarnib significantly inhibited LPS-induced IκB degradation (Fig. 7b, lane 4, Fig. 7c, lane 6), but showed no inhibition of IκB degradation induced by TNF-α or IL-1α (Fig. 7b), or by Pam3CSK4 (Fig. 7c).We furthur determined the effect of tipifarnib on NF-κB translocation by immunoblotting of p65 in nuclear extracts. Tipifarnib almost completely suppressed nuclear translocation of p65 induced by LPS but not that induced by TNF-α and Pam3CSK4 (Fig. 7c, lower panel).

The level of ERK phosphorylation was similar in cells treated with different stimuli, and tipifarnib pre-treatment reduced the ERK phosphorylation to a similar extent under all conditions, without affecting the level of total ERK protein (Fig. 7b, center panels). We also examined the effect of tipifarnib on H-Ras farnesylation (Fig. 7b, lower panel). The level of farnesylated (or processed (P)) H-Ras was not significantly changed in the absence or presence of inflammatory stimuli. Tipifarnib partially inhibited H-Ras farnesylation, as shown by the presence of unprocessed (U) Ras, to the same degree in the different treatment groups, and this inhibition correlated with the extent of inhibition of ERK phosphorylation (Fig. 7b, second panel).

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Discussion

In the present study we investigated the anti-inflammatory activity of the FTI, tipifarnib, both *in vitro* and *in vivo*. Tipifarnib demonstrated inhibition of both transcription and secretion of IL-6, IL−1β, MCP-1 and MMP-9 induced by LPS treatment of THP-1 cells, as well as dose-dependent inhibition of LPS-induced TNF-α, MCP-1, IL−1β and IL-6 production in primary human PBMC, without any evidence for cytotoxicity. We also observed inhibition by tipifarnib in THP-1 cells of LPS-induced MyD88 and STAT1 transcription, providing the first evidence for a role of farnesyltransferase in regulation of these genes. Global anti-inflammatory effects of tipifarnib were also observed *in vivo*, including downregulation of LPS-induced TNF-α, IL-6, MCP-1, IL-1β and MIP-1α.

Tipifarnib had contrasting effects between LPS-induced IL-8 and other mediators both in THP-1 and human PBMC. No inhibition of LPS-induced IL-8 transcription or secretion was observed in our studies for tipifarnib, at concentrations that were highly effective at inhibiting IL-1β, IL-6 and MCP-1 production. Consistent with the *in vitro* data, no inhibition of production of the murine homolog, KC, was observed *in vivo*. The results suggest that the target(s) of tipifarnib may be less crucial for optimal expression of IL-8, than for that of other cytokines or chemokines.

A recent study has shown inhibition *in vitro* and *in vivo* of LPS-induced nitric oxide, PGE_2, TNF-α and IL-1β by the FTI, LB42708 (Na et al., 2004). Our present study represents the first observation of FTI-mediated inhibition of LPS-induced IL-6, MCP-1

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and MIP-1α *in vivo*. We also observed inhibition *in vivo* by tipifarnib of LPS-induced IL12-p40 and -p70 . While Na *et al.* showed inhibition by LB42708 of TNF-α and IL-1β production *in vivo*, the LPS-induced cytokine levels were 10- to 100-fold lower than we obtained. The cytokines were measured 12 hours after LPS administration in their study (Na et al., 2004), which is long after the peak between 1 and 3 hours for LPS-induced TNFα and IL-1β in this model. We also investigated the effect of tipifarnib on IL-10, which is known as an anti-inflammatory mediator, and have shown that tipifarnib had no effect on LPS-induced IL-10 production, both in human PBMC and *in vivo*.

Inhibition of inflammatory mediators by tipifarnib appeared to require pre-treatment time. For example, cytokines such as IL-1β showed significant transcriptional induction by LPS as early as 3 hours *in vitro*. When drug treatment was started at the same time as the LPS stimulation, the earliest signs of transcriptional inhibition were only observed after 6 hours, and of cytokine release only after 22 hours. In contrast, when animals were pre-treated with the drug for 24 hours before LPS challenge *in vivo*, inhibition of cytokine release could be observed as early as one hour after the challenge (data not shown). The lag time in the inhibition of inflammatory responses may indicate a requirement to deplete intracellular pools of particular prenylated proteins.

Previous animal studies with tipifarnib have shown inhibition of cachexia in a tumor xenograft model, and reduction of disease scores in dextran sodium sulphate-induced colitis and collagen-induced arthritis, but the mechanism for these effects was unknown (David End, unpublished observations). The major roles of TNF-α, IL-6, and IL-1β in the

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pathogenesis of these diseases suggest that their inhibition by tipifarnib may have been responsible for the observed amelioration. While we did not measure the plasma concentrations of tipifarnib at which we observed inhibition of inflammatory mediators *in vivo*, the doses where we observed anti-inflammatory effects in the LPS-induced inflammation model were similar to those used previously for tumoricidal effects in mouse xenograft studies (End et al., 2001).

It is of interest to note that in Phase I studies with tipifarnib (300-900 mg/day) in myelodysplastic syndrome, the only correlation with positive response to treatment was a 42% median decrease in serum levels of TNF-α (Kurzrock et al., 2003). Tipifarnib, 300 mg twice a day was the maximum tolerated dose in a number of studies (Head and Johnston, 2003). At this dose, plasma levels reached approximately 2 μM (Karp et al., 2001), at which concentration significant inhibition of inflammation was obtained *in vitro* in our present study. Thus, sufficient plasma concentrations of tipifarnib for anti-inflammatory effects were reached in clinical trials where positive responses were observed, but existing data are insufficient to allow any direct correlation.

To explore the underlying mechanism for the anti-inflammatory activity of tipifarnib, we examined the effect of tipifarnib on different pro-inflammatory signaling pathways. The FTI, SCH 66336, was previously shown to inhibit TNF-α-induced IκB-α phosphorylation, degradation and NF-κB activity (Takada et al., 2004). However, in our study, tipifarnib did not affect TNF-α induced NF-κB activity as measured by a luciferase reporter assay. Tipifarnib also showed no inhibition of TNF-α induced IκB-α

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degradation or NF-κB nuclear translocation in THP-1 cells. Similarly, tipifarnib did not inhibit TNF-α-induced p38 phosphorylation. The reason for the lack of inhibition of TNF-mediated signaling by tipifarnib, in contrast to SCH66336 is not clear, but it is interesting to note that these two FTIs have also shown different efficacy in clinical trials for different tumor types.

The Ras/Raf/MAPK pathway has been well studied. GTP-bound Ras binds to Raf, causing Raf activation and relocation to plasma membrane. Activated Raf phosphorylates and activates MEK1 and MEK2, and MEK1/2 phosphorylates and activates ERK1/2. We found that tipifarnib partially inhibited ERK1/2 phosphorylation, and that the extent of inhibition correlated with inhibition of Ras farnesylation, both in the absence and in the presence of inflammatory stimuli. These data confirm that tipifarnib suppresses ERK1/2 phosphorylation through inhibition of Ras farnesylation.

LPS, Pam3CSK4, IL-1 and TNF-α each interact with their own receptors to trigger the corresponding inflammatory signaling cascades (see Fig. 8). TNF-α signaling links to IKK through TNFR/RIP (Aggarwal, 2003). LPS/TLR4, Pam3CSK4/TLR2, and IL-1/IL-1R signaling all link to IKK through the shared MyD88/IRAK/TRAF6/TAK pathway (Akira and Takeda, 2004). IKK phosphorylates IκB, which leads to the ubiquitination and degradation of IκB, and thus enables the translocation of NF-κB to the nucleus and induction of target gene expression such as TNF-α, IL-1β, IL-6 and others. Besides the common MyD88 dependent pathway shared with IL-1R and TLR2, the interaction of LPS and TLR4 also induces IκB degradation through MyD88-independent pathways, *e.g.*

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through TRIF and TRAM (Akira and Takeda, 2004). We found that tipifarnib inhibited IκB-α degradation induced by LPS, but not by TNF-α, Pam3CSK4 or IL-1, suggesting that the target protein for tipifarnib is not involved in TLR2/IL-1R and TNFR signal pathways, but is involved in a pathway exclusive to LPS/TLR4 signaling.

A recent study has shown that Ras is involved in CpG oligonucleotide signaling as an early event, by associating with TLR9 and promoting IRAK/TRAF6 complex formation (Xu et al., 2003). Another study has shown that Ras controls TRAF-6-dependent induction of NF-κB (Caunt et al., 2001). We found that inhibition of Ras farnesylation was consistently observed in control, LPS-, IL-1α- and TNF-α treated cells, but inhibition of IκB-α degradation and NF-κB translocation was only observed in the LPS-treated cells. This lack of correlation between inhibition of Ras farnesylation and inhibition of NF-κB signaling indicates that Ras is unlikely to be the target protein mediating the anti-inflammatory effects of tipifarnib. Candidate targets for tipifarnib could be the function or expression of TLR4 itself, or associated proteins such as CD14 or MD-2, or other molecules unique to the LPS signaling pathway, either upstream or independent of MyD88.

In conclusion, we present the first evidence for anti-inflammatory effects of the FTI, tipifarnib, and expand and highlight the potential for farnesyltransferase inhibition as a therapeutic approach to inflammation. We show inhibition by tipifarnib of a number of LPS-induced genes not previously known to be dependent on protein farnesylation. In particular, tipifarnib inhibited LPS induction of MCP-1, IL-6, MMP-9, MIP-1α, Stat1

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and MyD88, in addition to TNF-α and IL-1β, all of which have been implicated in inflammatory diseases. Our data suggest that the target protein for these effects of tipifarnib is likely to be a component of the TLR4 pathway, upstream or independent of MyD88, and is unlikely to be Ras, in contrast to the conclusions of Na *et al.* for the FTI, LB42708. Elucidation of the identity of the target protein or proteins may provide new targets for anti-inflammatory therapy. Furthermore, some of the therapeutic efficacy of FTIs in cancer may be related to inhibition of inflammatory mediators, which have been shown to contribute to cancer progression.

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Figure Legends

Figure 1. Microarray analysis of the effect of tipifarnib on LPS-induced genes

THP-1 cells were cultured in the absence (no treatment) or presence of LPS (100 ng/ml), plus or minus tipifarnib (5 μM). RNA was isolated at the indicated times for cDNA preparation as described in "Materials and Methods". Genes were selected as LPS-induced if the ratio between LPS-treated and non-treated samples was greater than 1.5-fold, at more than two time points. Gene expression during treatment with LPS and the FTI (tipifarnib/LPS) was compared with LPS alone at the corresponding time points. Genes that showed at least 1.5-fold decrease in induction by tipifarnib, at one or more time points were selected for inclusion in the figure.

Fold-change values between LPS-treated and non-treated samples are listed and colored in orange for greater than 1.5-fold higher expression, and in red for greater than 2-fold higher expression. Fold-change values between tipifarnib/LPS and LPS are listed and colored in light blue for more than 1.5-fold lower expression, and in dark blue for more than 2-fold lower expression. The results are representative of two separate experiments.

Figure 2. The effect of tipifarnib on LPS-induced transcription of IL-1β (a), MCP-1 (b), MMP-9 (c), IL-8 (d), STAT1 (e), MyD88 (f) and IL-6 (g). THP-1 cells were cultured in the absence (no treatment) or presence of LPS (100 ng/ml), plus or minus tipifarnib (5 μM). RNA was isolated at the indicated times for cDNA preparation as described in "Materials and Methods". Relative mRNA levels compared to GAPDH were determined by real-time PCR. The results are representative of two separate experiments.

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Figure 3. Dose dependent effect of tipifarnib on LPS-induced MMP-9 (a) and IL-6 (b) secretion. THP-1 cells were cultured in the absence (no treatment) or presence of 100 ng/ml LPS, minus or plus tipifarnib at concentrations starting from 5 μM, and 3-fold serial dilutions down to 0.06 μM. After 48hr, supernatants were collected for ELISA assays. % control of protein secretion by tipifarnib is determined by comparing LPS/tipifarnib-treated with LPS-treated only. Values are presented as the mean ± S.D. for triplicate wells.

Figure 4. The effect of tipifarnib on LPS-induced secretion of MCP-1 (a), IL-6 (b), IL-1β (c), IL-8 (d), MMP-9 (e). THP-1 cells were cultured in the absence (no treatment) or presence of 100 ng/ml LPS, minus or plus 2 μM tipifarnib. At the indicated time points, supernatants were analyzed for cytokines, chemokines or MMP-9 by ELISA. Data points are means ± S.D. for triplicate wells. (*, $P < 0.05$; **, $P < 0.01$, by Student's *t* test.) The results are representative of three separate experiments.

Figure 5. The effect of tipifarnib on LPS-induced cytokine secretion by human PBMC. PBMC were isolated and pre-incubated in the absence (no treatment) or presence of 2 and 5 μM tipifarnib for 1 hour. Cells were then treated with 10 ng/ml LPS for 16 hours. Supernatants were analyzed for cytokines by ELISA. Values are presented as the means ± S.D, for triplicate wells. (*, $P < 0.05$; **, $P < 0.01$, by Student's *t* test). The results are representative of three separate experiments.

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Figure 6. The effect of tipifarnib on LPS-induced cytokine production *in vivo*. BALB/c mice (n = 4 or 5 per group) were treated orally with tipifarnib in 20% cyclodextran, at a dose of 50 mg/kg, 24 hours, 17 hours and 1 hour before LPS injection. LPS 20 μg, or 1mg/kg was administered intraperitoneally. Plasma samples were collected at 2 and 3 hours after LPS injection, and tested for cytokines using ELISA or Luminex as described in "Materials and Methods". Data are presented as the mean ± S.D. (*, $P < 0.05$; **, $P < 0.01$, by Student's *t* test.) The results are representative of two separate experiments.

Figure 7. **Effect of tipifarnib on TLR/IL-1R ligands and TNF-α -induced signaling.** THP-1 cells were cultured in the absence or presence of 2 μM tipifarnib for 18 hours, then incubated with LPS and TNF-α (a), LPS, TNF-α and IL–1α, (b), or Pam3CSK4, LPS and TNF-α (c) for 30 minutes. Cytoplasmic lysates, or nuclear extracts (only for p65) were analyzed by Western blotting, using antibodies against p38, phosphorylated p38, total ERK1/2, phospho-ERK1/2, IκB, p65 and H-Ras, as described in "Materials and Methods".(U, unprocessed; P, processed).

Figure 8. **Proposed target(s) for tipifarnib inhibition of LPS-induced inflammatory signaling pathways.** Signals from TLR/IL-R through the MyD88-dependent pathway, or from TNFR1 through RIP/MEKK, induce the activation of the IKK complex. IKK phosphorylates IκB, inducing its degradation, which enables translocation of NF-κB to the nucleus and induction of target gene expression. LPS binding to TLR4 also induces activation of NF-κB through MyD88-independent pathways. The data in the present

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study suggest that the target for anti-inflammatory effects of tipifarnib is a component of pathways exclusive to LPS/TLR4 signaling, as indicated in **BOLD**.

(TLR2-L = TLR2 ligands).

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Figure 1

GeneName	Fold-change (LPS vs control)			Fold-change (tipifarnib/LPS vs LPS)			GeneDescription
	3hr	6hr	12hr	3hr	6hr	12hr	
CCL2	1.41	9.85	9.85	-1.32	-2.83	-3.25	monocyte chemotactic protein 1 (MCP-1)
CCL8	1.00	3.48	2.30	1.00	-2.30	-2.30	monocyte chemotactic protein 2 (MCP-2)
CCL4	5.28	8.00	5.66	1.87	1.07	-1.52	small inducible cytokine A4 (homologous to mouse Mip-1b) (SCYA4)
CXCL11	1.15	7.46	3.03	-1.07	-2.83	-1.52	small inducible cytokine subfamily B, member 11 (SCYB11)
IL1B	5.66	8.00	6.96	1.32	-1.87	-2.46	interleukin 1, beta
IL1RN	1.00	3.25	1.87	-1.15	-1.62	-1.15	interleukin-1 receptor antagonist
STAT1	1.15	4.29	3.73	-1.32	-2.14	1.00	signal transducer and activator of transcription 1
IFNB1	1.23	3.25	6.06	-1.15	-1.62	-3.03	interferon, beta 1, fibroblast
MX1	1.07	4.92	3.03	-1.07	-1.74	1.32	myxovirus (influenza) resistance 1
IGFBP3	1.15	1.62	4.00	-1.07	1.00	-1.52	insulin-like growth factor-binding protein-3 gene
MYD88	1.07	3.25	1.74	-1.15	-2.14	-1.23	myeloid differentiation primary response protein
TAP1	1.32	4.92	2.14	1.15	-1.74	-1.15	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)
TAP2	1.07	2.00	1.87	-1.15	-1.62	-1.32	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)
ABCB8	1.00	2.30	1.52	1.00	-1.62	-1.23	ATP-binding cassette, sub-family B (MDR/TAP), member 8
PLAUR	1.15	1.62	1.87	-1.23	-1.62	-1.87	plasminogen activator, urokinase receptor
C3AR1	1.15	2.00	2.30	1.07	-1.23	-1.62	C3a anaphylatoxin chemotactic receptor (C3a-R)
B2M	1.07	1.62	1.52	1.00	-1.23	-1.62	beta-2-microglobulin
LAP3	1.00	2.46	2.30	-1.07	-1.62	-1.23	leucine aminopeptidase
MMP9	-1.07	1.52	3.48	-1.07	-1.41	-1.74	type IV collagenase, matrix metalloproteinase 9
TRIM22	1.00	2.83	3.03	1.00	-1.62	-1.07	tripartite motif-containing 22
LGR7	-1.07	1.62	1.52	-1.15	-1.41	-1.62	leucine-rich repeat-containing G protein-coupled receptor 7
GNPNAT1	1.00	3.25	2.14	1.00	-2.00	-1.41	glucosamine-phosphate N-acetyltransferase 1
LY6E	1.00	3.03	3.73	-1.15	-1.52	1.15	lymphocyte antigen 6 complex, locus E

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Figure 2

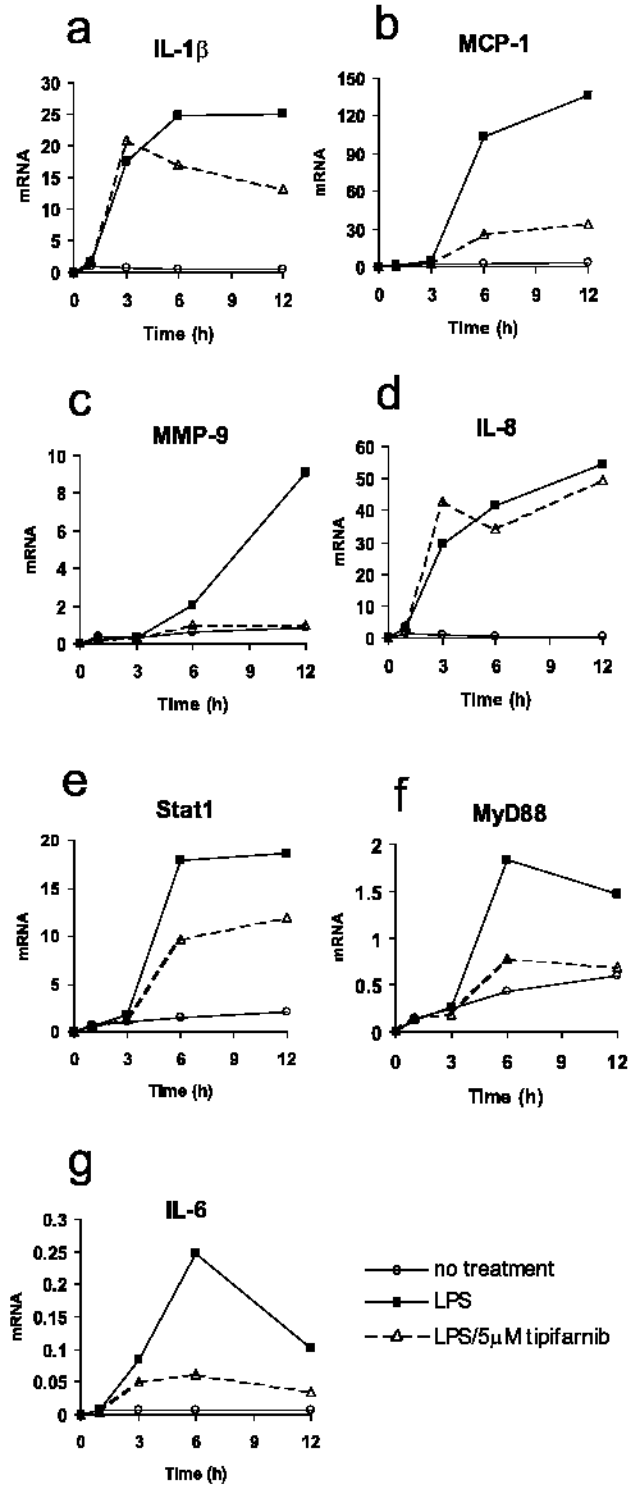

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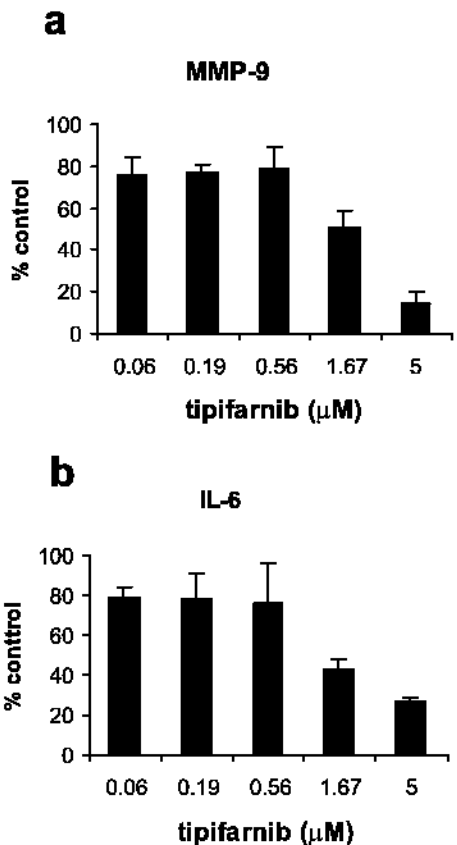
Figure 3



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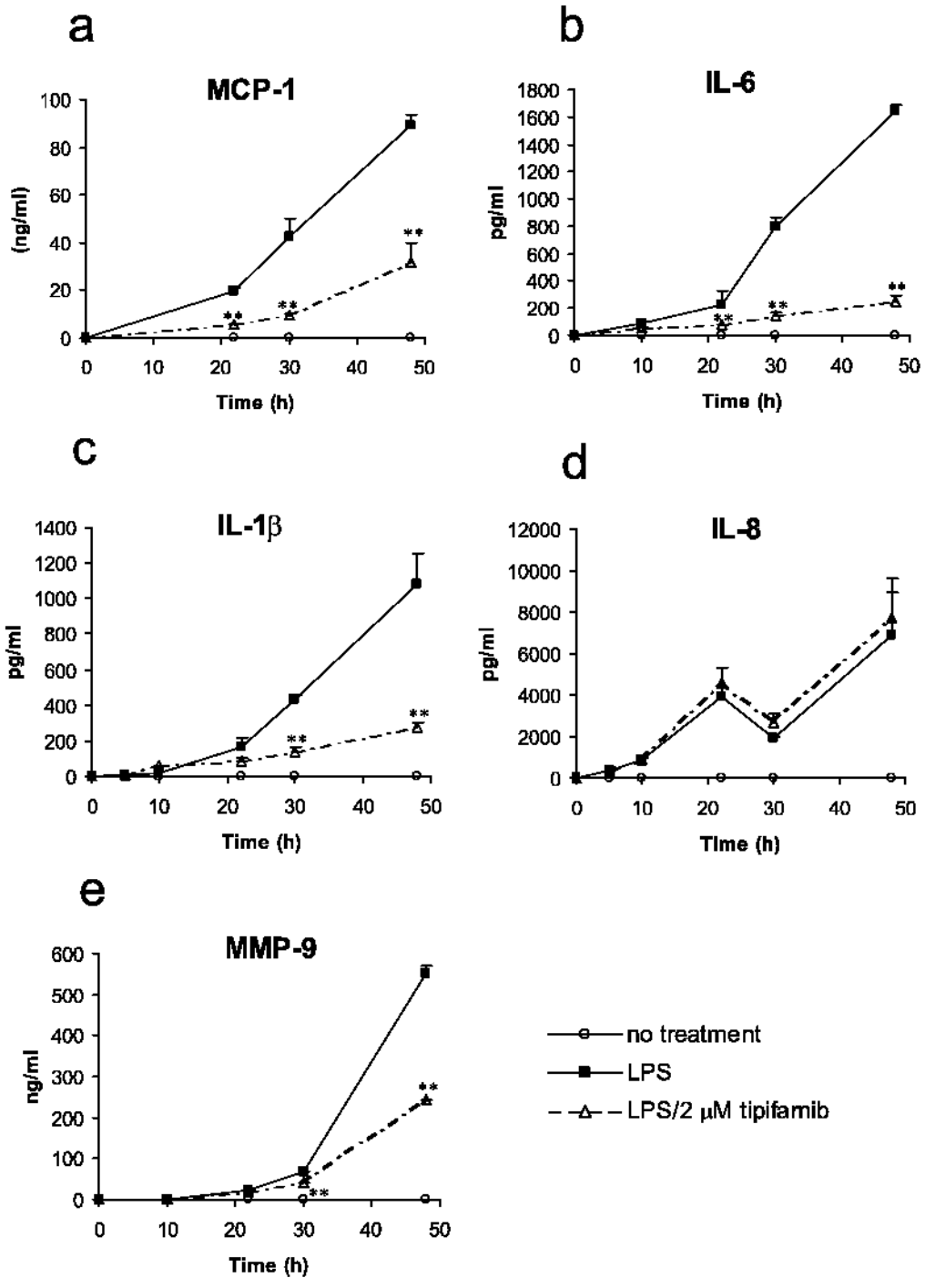

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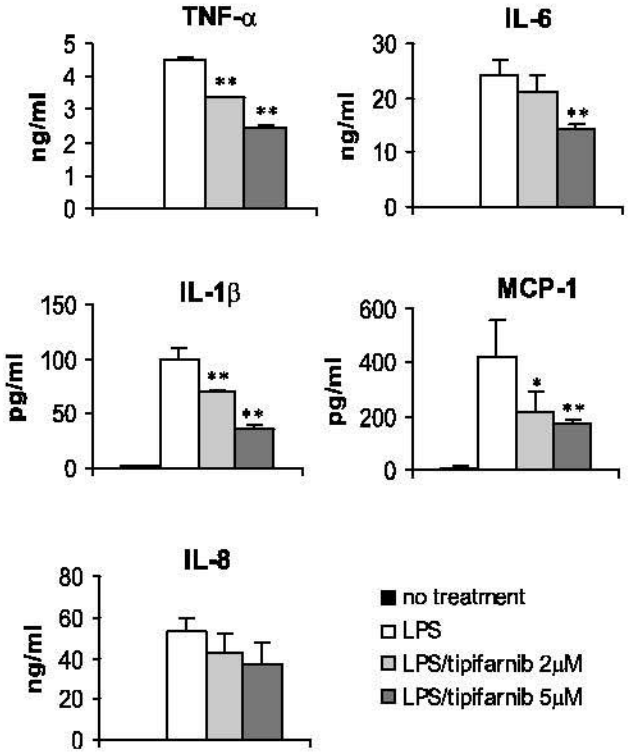
Figure 5



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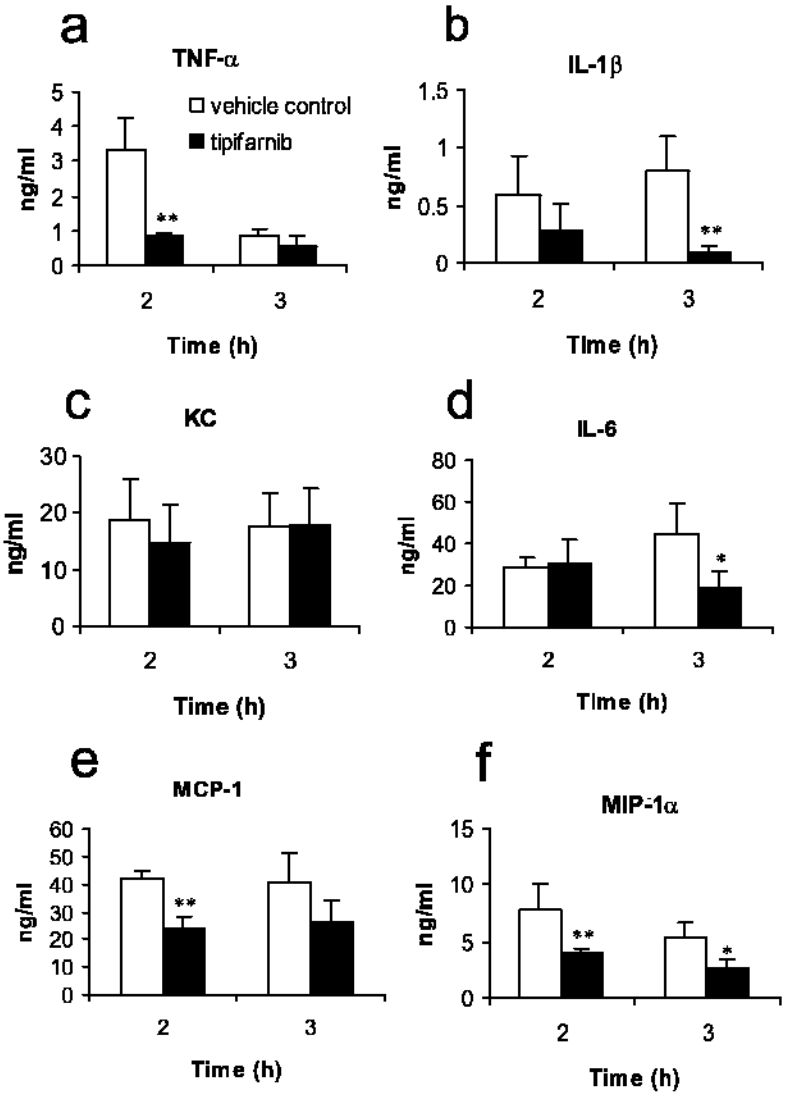

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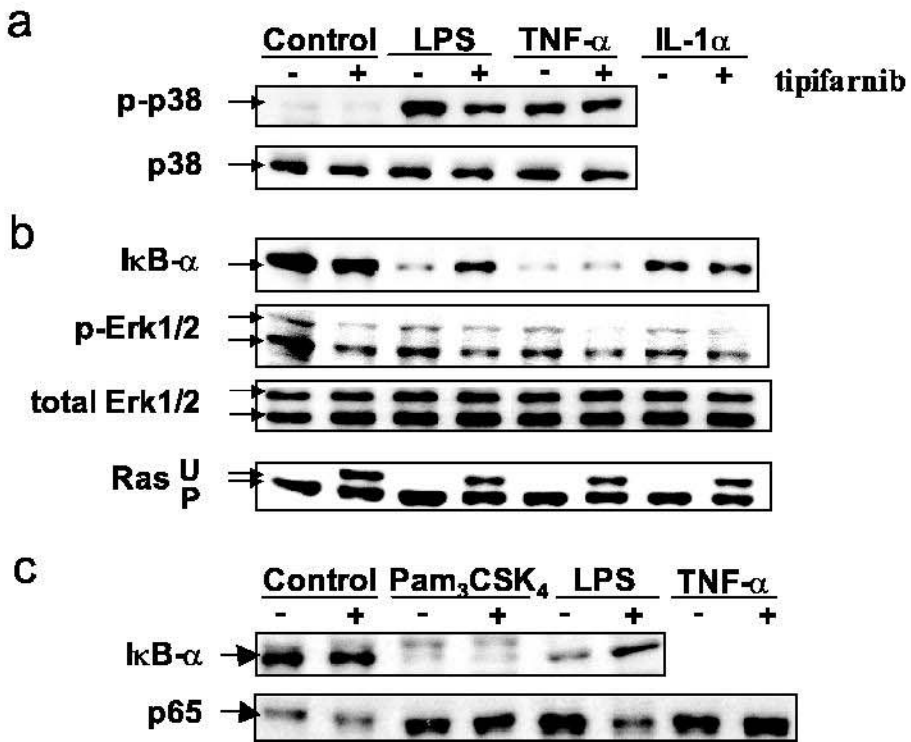

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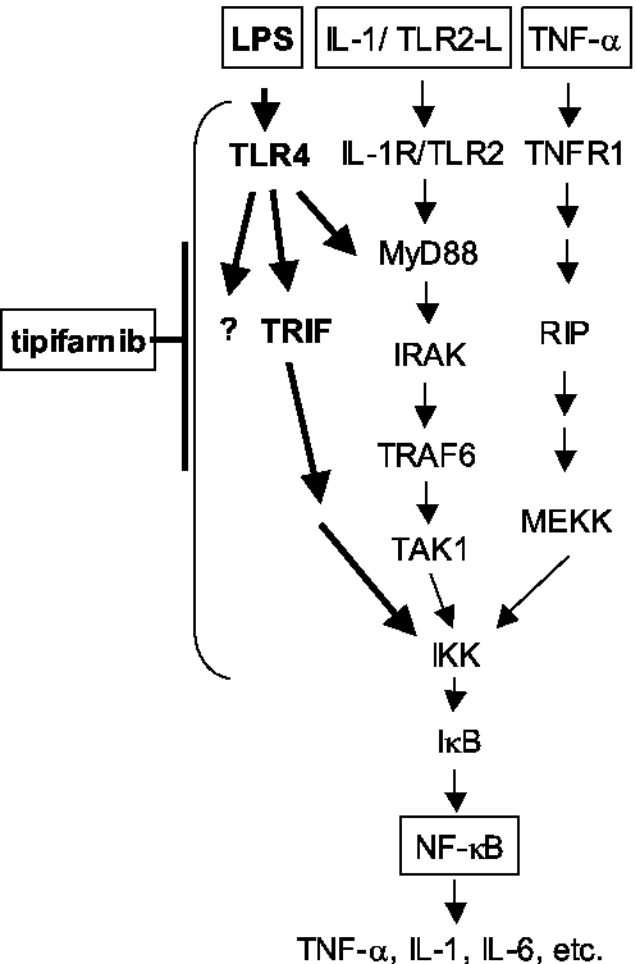
Figure 8