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N-[3,4-dimethoxycinnamoyl]-anthranilic acid (tranilast) suppresses microglial inducible nitric oxide synthase (iNOS) expression and activity induced by interferon-γ (IFN-γ)

*,[1]Michael Platten, [1]Wolfgang Wick, [1]Jörg Wischhusen & [1]Michael Weller

[1]Laboratory of Molecular Neuro-Oncology, Department of Neurology, University of Tübingen, School of Medicine, Hoppe-Seyler Str. 3, 72076 Tübingen, Germany

1 Microglial cells up-regulate inducible nitric oxide synthase (iNOS) expression in response to various pro-inflammatory stimuli including interferon-γ (IFN-γ), allowing for the release of nitric oxide (NO). Tranilast (N-[3,4-dimethoxycinnamoyl]-anthranilic acid) is an antiallergic compound with suppressive effects on the activation of monocytes.

2 Here, we show that N9 murine microglial cells express iNOS mRNA and protein and release nitric oxide into the culture medium in response to IFN-γ (200 u ml^{-1}) as measured by Northern and Western blot analyses and Griess assay.

3 Exposure to non-toxic doses of tranilast (30–300 μM) leads to a concentration-dependent inhibition of IFN-γ-induced (200 u ml^{-1}) iNOS mRNA and protein expression. This is paralleled by a suppression of NO-release into the cell culture medium.

4 Inhibition of IFN-γ-induced iNOS mRNA expression by tranilast is paralleled by an inhibition of nuclear factor-κB (NF-κB) activation and phosphorylation of inhibitory κB (IκB) as determined by Western blot analyses and NF-κB reporter gene assay.

5 These results suggest that tranilast-mediated suppression of microglial iNOS activity induced by IFN-γ involves the inhibition of NF-κB-dependent iNOS mRNA expression.
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Keywords: Tranilast; microglia; nitric oxide; inducible nitric oxide synthase; interferon-γ; nuclear factor-κB; inhibitory-κB

Abbreviations: ALS, amyotrophic lateral sclerosis; CNS, central nervous system; COX-2, cyclo-oxygenase type 2; DEX, dexamethasone; IFN-γ, interferon-γ; IL-1β, interleukin-1β; IκB, inhibitory κB; iNOS, inducible nitric oxide synthase, LPS, lipopolysaccharide; NF-κB, nuclear factor κB; NO, nitric oxide; PGE_2, prostaglandin E_2; TGF-β, transforming growth factor-β; TNF-α, tumour necrosis factor-α; TNL, tranilast

Introduction

Microglial cells share many morphological and functional features with monocytes and macrophages and are thought to contribute to inflammatory processes in the brain. Microglia is activated during brain ischaemia, infection, demyelination, neurodegeneration and tumorigenesis (Gonzalez-Scarano & Baltuch, 1999). On a cellular level, activation of microglial cells, like monocytic cells, results in the expression of pro-inflammatory mediators such as cyclo-oxygenase type 2 (COX-2), tumour necrosis factor-α (TNF-α) and nitric oxide (NO). NO is a free radical generated from L-arginine by NO synthases (NOS) (Griffith & Stuehr, 1995). It is involved in a wide range of physiological and pathological processes including the regulation of vascular homeostasis, neurotransmission and inflammation (Nathan, 1992). Three isoforms of NOS have been characterized. Neuronal (type I or nNOS) and endothelial (type III or eNOS) NOS are constitutively expressed and are mainly regulated posttranscriptionally by calmodulin-dependent pathways. The inducible NOS isoform (type II or iNOS) is not expressed in healthy tissues but is rapidly expressed *de novo* in many cell types including astrocytes and microglia in response to lipopolysaccharide (LPS), a bacterial wall component, or inflammatory cytokines such as interleukin-1β (IL-1β), TNF-α or IFN-γ (Galea *et al*., 1992; Hu *et al*., 1995; Nathan & Xie, 1994). When produced in excess amounts NO may promote neuronal death by reacting with superoxide anion to generate peroxynitrite (Beckman *et al*., 1990). Consequently, overproduction of NO has been implicated in neurological disorders associated with neuronal damage including brain ischaemia, Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (ALS) (Chabrier *et al*., 1999). Accordingly, pharmacological inhibition of iNOS has been shown to attenuate brain damage in an animal model of brain ischaemia (Nagayama *et al*., 1998).

Tranilast (N-[3,4-dimethoxycinnamoyl] anthranilic acid) is an orally active anti-allergic compound which is clinically effective in the control of autoimmune diseases such as bronchial asthma and atopic dermatitis, presumably by inhibiting the release of various chemical mediators from mast cells (Azuma *et al*., 1976). In addition, tranilast has been shown to prevent restenosis after percutaneous transluminal angioplasty in patients with coronary heart disease (Tamai *et al*., 1999). Recently, our laboratory has defined a role for tranilast as a novel anti-glioma agent (Platten *et al*., 2001). The

*Author for correspondence;
E-mail: michael.platten@uni-tuebingen.de

molecular effects of tranilast are not completely understood. The inhibitory effects of tranilast on collagen accumulation, proliferation and migration of different cell types may be attributed to antagonizing TGF-β (Fukuyama *et al*., 1996; Miyazawa *et al*., 1995). The suppressive effects on fibrosis may involve the inhibition of activated macrophages known to release NO (Kawano & Noma, 1993; Mori *et al*., 1995; Suzawa *et al*., 1992). On the other hand, there are lines of evidence that tranilast may be a positive regulator of iNOS expression and activity in vascular smooth muscle cells and rat mesangial cells (Hishikawa *et al*., 1996).

Here we investigate the effects of tranilast on iNOS expression and activity in microglial cells.

Methods

Reagents and cell culture

Tranilast was a generous gift of Kissei Pharmaceuticals (Nagano, Japan). Murine recombinant IFN-γ was purchased from Boehringer (Mannheim, Germany). Dexamethasone (DEX) was obtained from Sigma (Deisenhofen, Germany). N9 murine microglial cells were a kind gift from P. Ricciardi-Castagnoli (Milano, Italy) (Righi *et al*., 1989). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2 mM glutamine and penicillin (100 u ml^{-1})/streptomycin (100 μg ml^{-1}), and maintained at 37°C in an atmosphere of 5% CO_2. Viability was assessed by crystal violet staining.

iNOS activity

iNOS activity was assessed by the Griess assay as described elsewhere (Rieger *et al*., 1998). Briefly, conditioned supernatant (100 μl) was incubated with an equal volume of 1% sulphanilamide/0.1% naphthyl ethylene diamine dihydrochloride/2.5% H_3PO_4 (Griess reagent) (Green *et al*., 1982), incubated for 5 min at room temperature, and the absorbance at 546 nm was determined. Solutions of $NaNO_2$ diluted in DMEM served as standards. The absorbance readings resulting from DMEM alone were subtracted from the sample values. Cell density was assessed using crystal violet staining to control for differences in cell proliferation. iNOS activity is thus expressed here as NO_2 accumulated per 48 h per 10^5 cells.

Immunoblot analysis

For the preparation of whole cell lysates, the cells were rinsed in phosphate-buffered saline (PBS), harvested, centrifuged at $1200 \times g$, lysed in 0.1 M TRIS-HCl (pH 7.2) containing 0.1% NP40, 0.1 mM EDTA and 5 μg ml^{-1} PMSF for 40 min on ice, and centrifuged at $10{,}000 \times g$ for 10 min. Protein concentration was determined using Biorad reagents with photometric analysis. Proteins (20 μg lane^{-1}) were separated by 10–12% SDS–PAGE and electroblotted on nitrocellulose. Equal protein loading was controlled by Ponceau S staining. After blocking for 1 h in PBS supplemented with 5% skimmed milk and 0.1% Tween 20, immunodetection was performed using anti-iNOS rabbit polyclonal antibody (1 : 1000, Cayman, Ann Arbor, MI, U.S.A.) and anti-phospho-IκBα mouse monoclonal antibody (1 : 1000, New England Biolabs, Frankfurt am Main, Germany). Bands were visualized using horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1 : 4000, Santa Cruz Biotechnology) or anti-mouse IgG (1 : 4000, Amersham) and enhanced chemoluminescence (ECL) (Amersham).

Northern blot analysis

Total RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany). Total RNA (10 μg) was separated on 1.2% agarose gels and blotted onto nylon membranes (Amersham). The filters were hybridized according to standard procedures (Platten *et al*., 2001) with a ^{32}P-labelled murine cDNA probe for iNOS. For the generation of the iNOS probe, N9 cells were stimulated with IFN-γ (200 u ml^{-1}) for 24 h. Total RNA (5 μg) was subjected to reverse transcription using SuperScript II (Gibco-BRL, Gaithersburg, MD, U.S.A.) and oligo-dT priming (Amersham Pharmacia Biotech, Uppsala, Sweden). iNOS fragments were PCR-amplified using primers 5′-AAGCTGCATGTGACATCGAC-3′ and 5′-ATGTGTCTG-CAGATGTGCTG-3′ corresponding to nucleotides 386–405 and 839–858 of murine iNOS cDNA. Equal loading was assured by ethidium bromide staining.

NF-κB reporter assay

N9 murine microglial cells were seeded in a 96-well plate (8×10^3 well^{-1}) and allowed to adhere for 24 h. The cells were transfected with an NF-κB cis-reporter gene plasmid (PathDetect® no. 219077, Stratagene) which encodes firefly luciferase in a NF-κB-dependent-manner. For each transfection, 0.2 μg DNA and 0.6 μl FuGene (Roche, Mannheim, Germany) were used. The cells were either co-transfected with 0.02 μg of the pFC-Mekk positive control plasmid, included in the NF-κB cis-reporting system from Stratagene, or with the pcDNA-3 plasmid, to maintain a constant amount of total DNA. In order to assess the unspecific background, a control transfection with pcDNA-3 only was included in all experiments. After 24 h the cells were washed twice with PBS (0.12 M NaCl, 0.01 M $NaH_2PO_4 \times H_2O$, 0.031 M K_2HPO_4) and lysed using 40 μl well^{-1} of Cell Lysis Buffer (mM): tricine pH 7.8 40, NaCl 50, EDTA 2, $MgSO_4$ 1, DTT 5, 1% Triton® X-100, all from Sigma. To optimize lysis, a freeze-thaw cycle was performed. Then the lysate was transferred to a LumiNunc TM plate (Nunc, Roskilde, Denmark). Luciferase assay reagent (100 μl) containing (mM): tricine pH 7.8 40, $MgSO_4$ 10, EDTA 0.5, DTT 10, coenzyme A 0.5 and beetle luciferin 0.5, (0.5 M ATP), (all from Sigma) were added and luminescence was measured in a LumimatPlus (EG&G Berthold, Pforzheim, Germany). Background was subtracted from all values and the remaining luciferase activity was expressed in per cent of the positive control (pFC-Mekk transfection). Thus, the results become independent from transfection efficiency. The value of the positive control remained largely unchanged through all treatment groups.

Statistical analyses

Experiments were usually performed in triplicate and repeated three times. The significance was evaluated by *t*-test or ANOVA at $P<0.05$, $P<0.01$ or $P<0.001$.

Results

Tranilast suppresses microglial iNOS protein expression and activity induced by IFN-γ

While N9 microglial cells displayed no constitutive iNOS protein expression, incubation with IFN-γ (200 u ml^{-1}) induced strong iNOS protein expression in N9 cells in a time-dependent manner as measured by immunoblot analysis. iNOS protein was weakly detectable 4 h after stimulation, strongly induced after 8 h and lasted for at least 24 h (Figure 1A). Addition of tranilast (300 μM) greatly suppressed IFN-γ-induced iNOS protein expression as did dexamethasone (500 nM) (Figure 1B). No detectable amounts of NO_2 were measured in the conditioned medium of untreated N9 cells indicating that there was no constitutive iNOS activity (data not shown). When stimulated with IFN-γ (200 u ml^{-1}) the cells released 13 μM ($\pm$3 μM) NO_2 per 48 h per 10^5 cells. Addition of tranilast led to a concentration-dependent inhibition of NO release with a reduction by 65% at 300 μM. In comparison, addition of 500 nM dexamethasone reduced the amount of NO_2 by 75% (Figure 1C).

Tranilast suppresses microglial iNOS mRNA expression induced by IFN-γ

IFN-γ (200 u ml^{-1}) induced strong iNOS mRNA expression in N9 cells in a time-dependent manner as assessed by Northern Blot (Figure 2A). Coincubation with tranilast (300 μM) greatly suppressed iNOS mRNA expression, indicating that tranilast inhibits iNOS induction on a transcriptional level (Figure 2B).

Tranilast suppresses NF-kB activation induced by IFN-γ in N9 microglia

Since NF-κB is an essential transcription factor for the induction of iNOS gene expression, we next asked whether tranilast modulates the activation of NF-κB in response to IFN-γ. At 24 h after transfection with an NF-κB reporter gene plasmid, incubation with IFN-γ (200 u ml^{-1}) led to a time-dependent activation of luciferase activity. Of note, there was a small but significant increase of luciferase activity 4 h after stimulation with IFN-γ which peaked after 30 h. There was an induction of protein expression of phosphorylated IκB by IFN-γ preceeding the activation of NF-κB reporter gene activity as measured by immunoblot analysis (Figure 3A). When coincubated with tranilast, there was a concentration-dependent suppression of IκB phosphorylation and NF-κB reporter gene activity (Figure 3B).

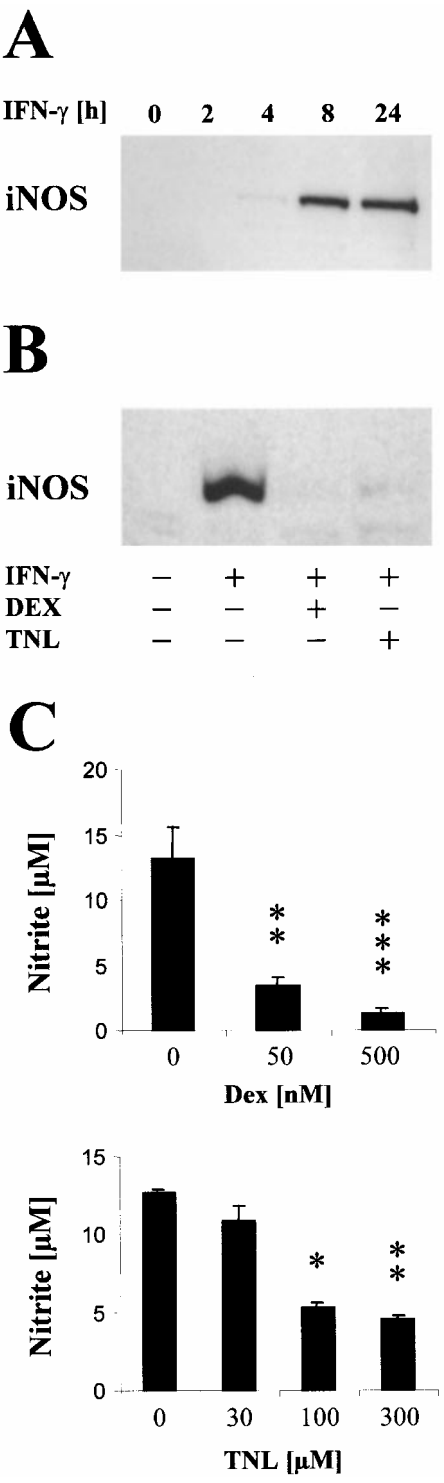

Figure 1 Tranilast suppresses iNOS protein expression and activity in N9 microglia in response to IFN-γ. (A) N9 cells were incubated with IFN-γ (200 u ml^{-1}) and protein lysates were prepared at the time points indicated. Immunoblot analysis was performed using an anti-iNOS antibody revealing a band at $\sim$120 kDa. (B) N9 cells were incubated with medium alone, IFN-γ alone (200 u ml^{-1}) or coincubated with IFN-γ and tranilast (TNL, 300 μM) or dexamethasone (DEX, 500 nM) for 24 h. (C) N9 cells were stimulated with IFN-γ alone or in combination with dexamethasone (DEX, upper panel) or tranilast (TNL, lower panel) at the concentrations indicated. Supernatant was collected after 48 h and nitrite was measured using the Griess assay. Values are expressed as NO_2 accumulated per 10^5 cells (mean and s.e.mean, $n=3$, $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

Discussion

De novo expression of iNOS and the release of large amounts of NO by microglia is considered to play a significant role in the pathogenesis of various neurological disorders. For instance, induction of microglial NO synthesis is thought to contribute to the neuronal damage in AIDS dementia (Adamson *et al*., 1996), Alzheimer's disease (Goodwin *et al*., 1995) and Parkinson's disease (Dehmer *et al*., 2000). Further, microglial-derived NO is thought to play a crucial

role in oligodendrocyte cytotoxicity and demyelination in multiple sclerosis (Merrill *et al*., 1993). Inhibition of iNOS activity in microglial cells may thus represent an interesting target to ameliorate cytotoxic effects in various diseases associated with increased NO production.

The present study seeks to investigate the effects of tranilast, an orally active anti-allergic drug, on the expression and activity of iNOS in murine N9 microglial cells. In macrophages, high doses of IFN-γ initiate the synthesis of NO through the engagement of response elements in the promotor region of the iNOS gene. Induction of iNOS in murine macrophages by IFN-γ alone appears to be strictly dependent on the activation of the transcription factor NF-κB (Lowenstein *et al*., 1993). Activation of NF-κB requires

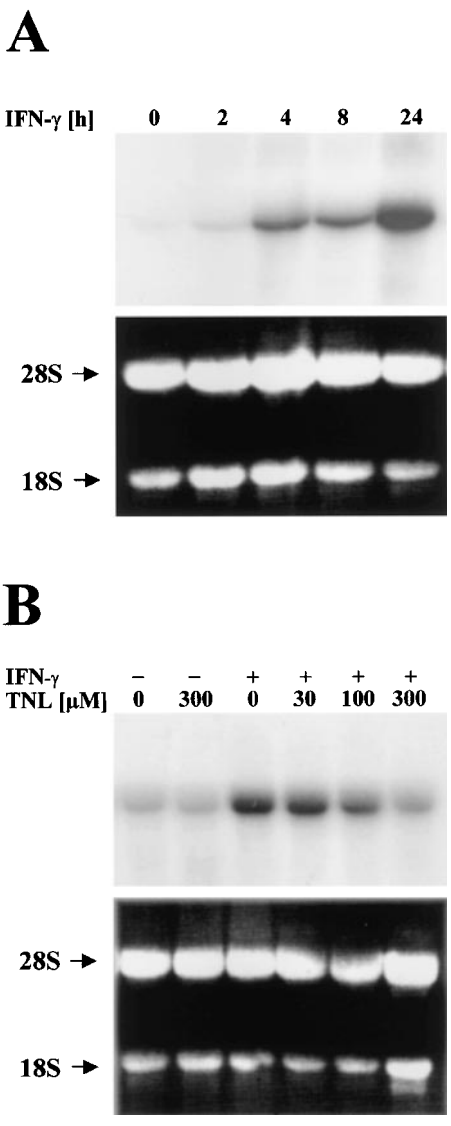

Figure 2 Tranilast suppresses iNOS mRNA expression in N9 microglia in response to IFN-γ. (A) N9 cells were incubated with IFN-γ (200 u ml^{-1}) and mRNA was prepared at the time points indicated. Northern blot analysis was performed using an iNOS cDNA probe revealing a band running at $\sim$1.2 kB. Equal loading was assured by ethidium bromide staining of the 18 S and 28 S ribosomal fractions. (B) N9 cells were incubated with tranilast (30–300 μM) and stimulated with IFN-γ (200 u ml^{-1}) for 24 h. Northern blot analysis was performed as in (A).

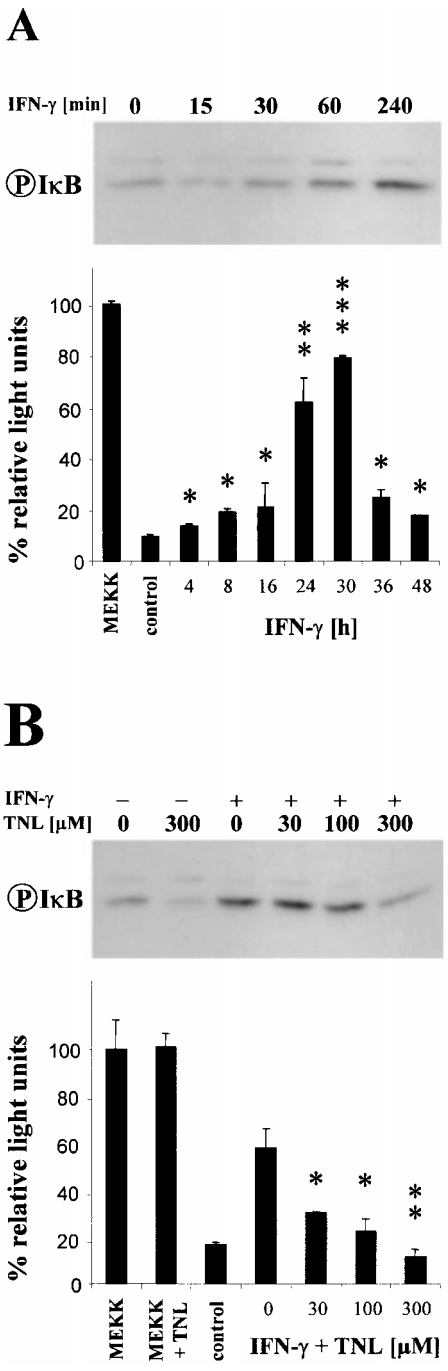

Figure 3 Tranilast suppresses NF-κB activation and IκBα phosphorylation in N9 microglia in response to IFN-γ. N9 cells were incubated with IFN-γ (200 u ml^{-1}) with or without a 4 h preincubation with tranilast (30–300 μM) and protein lysates were prepared at the time points indicated. Immunoblot analysis was performed using an anti-phospho-IκBα antibody (A, B, upper panels). In parallel, N9 cells were transiently transfected with an NF-κB reporter plasmid or MEKK plasmid serving as a positive control. After 24 h cells were incubated with IFN-γ (200 u ml^{-1}) with or without a 4 h preincubation with tranilast (30–300 μM). Luciferase activity was determined as described. Values are expressed as per cent relative light units of the positive control. Data are expressed as mean and s.e.mean ($n=3$; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, effect of IFN-γ compared to control (A); $^{*}P<0.05$, $^{**}P<0.01$, effect of IFN-γ +TNL compared with IFN-γ alone (B)) (A, B, lower panel).

phosphorylation on specific serine residues and degradation of IκBα which then dissociates from the NF-κB/IκB complex and allows NF-κB to translocate to the nucleus and bind to κB motifs (Mercurio *et al*., 1997; Woronicz *et al*., 1997).

We find, that incubation of N9 microglial cells with IFN-γ (200 u ml^{-1}) leads to an induction of iNOS mRNA and protein expression accompanied by a release of NO into the cell culture medium (Figures 1A and 2A). After transient transfection with an NF-κB reporter gene plasmid, there was a time-dependent induction of luciferase activity upon stimulation with IFN-γ indicating an activation of transcriptional activity of NF-κB. Activation of NF-κB by IFN-γ appears to be achieved by degradation of IκB since IFN-γ induces phosphorylation of IκBα in N9 microglia (Figure 3A).

We further show that the anti-allergic drug tranilast inhibits the expression of iNOS (Figures 1B and 2B) and the release of NO from N9 microglia (Figure 1C). Since NF-κB activation is involved in iNOS induction we tested the hypothesis that tranilast blocks NF-κB activation by transiently transfecting N9 cells with an NF-κB reporter plasmid. Our data suggest that the inhibition of iNOS mRNA expression involves suppression of NF-κB activation (Figure 3B). This is supported by several studies demonstrating inhibitory effects of tranilast on the transcription of other genes transcriptionally activated by NF-κB binding, such as IL-5 and COX-2 (Hiratochi *et al*., 2000; Inoue *et al*., 1997).

How tranilast blocks IFN-γ-induced NF-κB activation, however, is not clear. Like most inhibitors of NF-κB activation, tranilast prevents IFN-γ-induced phosphorylation and degradation of IκBα (Figure 3B). The signalling events involved in phosphorylation of IkB include multiple protein kinases belonging to the family of MAPK (Karin & Delhase, 1998), PKC and PKB (Bauer *et al*., 2001), all of which may be suppressed by tranilast (Koyama *et al*., 1999; Watanabe *et al*., 2000). The exact mechanisms leading to the suppression of NF-κB activation by tranilast require further investigation.

Taken together, we demonstrate that tranilast suppresses the induction of iNOS expression and activity by IFN-γ in N9 microglia, possibly as a result of preventing NF-κB activation, and thus identify tranilast as a novel therapeutic agent for various neurological conditions associated with increased NO production, including multiple sclerosis, cerebral ischaemia, Alzheimer's disease and AIDS dementia.

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