

A Synthetic Derivative of the Natural Product Rocaglaol Is a Potent Inhibitor of Cytokine-Mediated Signaling and Shows Neuroprotective Activity in Vitro and in Animal Models of Parkinson's Disease and Traumatic Brain Injury

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ABSTRACT

Many acute and chronic neurodegenerative diseases are characterized by a localized inflammatory response and constitutive activation of the transcription factors nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) as well as their upstream activating signaling cascades. Ample evidence indicates the implication of these processes in the pathogenesis of several diseases of the central nervous system. In this study, we show that a synthetic derivative of the natural product rocaglaol (compound A) displays potent anti-inflammatory properties in human endothelial and murine glial cells in vitro. Compound A inhibited cytokine- and lipopolysaccharide-induced release of various cytokines/chemokines and of nitric oxide as well as expression of the adhesion molecule endothelial leukocyte adhesion molecule-1 and the inducible enzymes nitric-oxide synthase and cyclooxygenase-2. As shown by immunocytochem-

istry and immunoblotting, compound A inhibited NF- κ B and AP-1 activity in mixed glial cultures. Compound A exhibited neuroprotective activity in vitro and in vivo. 1-Methyl-4-phenylpyridinium-induced damage of mesencephalic dopaminergic neurons was significantly decreased, and long-term treatment of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-injected mice with compound A significantly and dose-dependently reduced dopaminergic neuronal cell death. In addition, short-term application of compound A to rats suffering from traumatic brain injury induced by subdural hematoma resulted in a significant reduction of the cerebral infarct volume. These results suggest that by inhibiting NF- κ B and AP-1 signaling, compound A is able to reduce tissue inflammation and neuronal cell death, resulting in significant neuroprotection in animal models of neurodegeneration.

Nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) are both ubiquitously expressed, dimeric transcription factors that regulate a wide range of cellular processes, including cell death, survival, proliferation, and differentiation. Currently five mammalian NF- κ B family members have been identified and cloned. These include NF- κ B1 (p50/p105), NF- κ B2 (p52/p100), RelA (p65), RelB, and c-Rel. In resting cells, NF- κ B is sequestered in the cytoplasm in an inactive form through its association with one of several inhibitory molecules, including I κ B- α , I κ B- β , I κ B- ϵ , p105, and p100. Activation of the NF- κ B signaling cascade results in a com-

plete degradation of I κ B or partial degradation of the carboxyl termini of p105 and p100 precursors, allowing the translocation of NF- κ B to the nucleus, where it induces transcription. Activated NF- κ B binds to specific DNA sequences in target genes, designated κ B elements, and regulates transcription of genes mediating inflammation, carcinogenesis, and pro- and antiapoptotic reactions. Aside from regulated nuclear availability, transcriptional activity is further controlled by modulatory phosphorylations (for review, Karin and Lin, 2002).

AP-1 proteins are sequence-specific transcription factors composed of homodimers or heterodimers of the Jun family (c-Jun, JunD, and JunB) or heterodimers of the Jun family member with any of the Fos family members (c-Fos, FosB,

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ABBREVIATIONS: NF- κ B, nuclear factor- κ B; AP-1, activator protein-1; AD, Alzheimer's disease; PD, Parkinson's disease; CNS, central nervous system; TNF, tumor necrosis factor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; BSA, bovine serum albumin; LPS, lipopolysaccharide; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; ICAM, intercellular adhesion molecule; ELAM, endothelial leukocyte adhesion molecule; HUVEC, human umbilical vein endothelial cell; PBS, phosphate-buffered saline; LDH, lactate dehydrogenase; MPP⁺, 1-methyl-4-phenylpyridinium; GBR 12909; COX, cyclo-oxygenase; INOS, inducible nitric-oxide synthase; MPTP; TH-ir, tyrosine hydroxylase immunoreactivity; SNc, substantia nigra pars compacta; SDH, subdural hematoma; LSD, least significant difference; MAP, mitogen-activated protein; TH, tyrosine hydroxylase; JNK, c-Jun N-terminal kinase; IKK, I κ B kinase.

Fra1, and Fra2) or other transcription factors, such as activating transcription factor 2 and nuclear factor of activated T cells, that mediate gene induction via binding to the 12-*O*-tetradecanoylphorbol-13-acetate response element or, for Jun-ATF dimers, via the cAMP-responsive element. Members of the AP-1 family are regulated at both the transcriptional and post-transcriptional levels by mitogen-activated protein kinases (for review, see Shaulian and Karin, 2002).

The acute and chronic phases of many neurodegenerative diseases are characterized by a localized inflammatory response and activation of NF- κ B and AP-1. For example, increased nuclear NF- κ B levels have been observed in dying neurons of brains exposed to trauma and ischemia as well as in brains of patients with Alzheimer's disease (AD) and Parkinson's disease (PD). Brain hypoxia and traumatic brain injury cause enhanced AP-1 activity and activation of upstream activating mitogen-activated protein kinase cascades in AD and PD (Hunot et al., 1997; Kaltschmidt et al., 1997; Schneider et al., 1999; Ferrer et al., 2001; Tong et al., 2003). In addition, a large number of cytokines and chemokines and their respective receptors are expressed in neurons and glia, either constitutively or induced by inflammatory mediators, and expression of many of these is regulated by NF- κ B and AP-1 activation. There is ample evidence that both, cytokines/chemokines and their receptors, are up-regulated under various neurodegenerative conditions and their implication in CNS pathogenesis has been demonstrated in vitro and in respective animal models of diseases (Knott et al., 2000; Allan and Rothwell, 2001; Hoozemans et al., 2002). Inhibition of NF- κ B and/or AP-1 activity may therefore be a promising approach to therapeutic treatment of acute and chronic neurodegenerative diseases.

Compound A is a synthetic monofluor derivative of the natural product rocaglaol, which, like other members of the structurally closely related family of rocaglamids, can be isolated from the leaves and blossoms of *Aglaiia* species (for review, see Proksch et al., 2001). Extracts of these plants are used in natural medicine in Southeast Asia and have been reported to exhibit anti-inflammatory and antitumor activity. Baumann and colleagues (2002) have provided evidence that rocaglaol and certain rocaglamide derivatives inhibit TNF α - and phorbol 12-myristate 13-acetate-induced NF- κ B signaling in Jurkat T cells in a cell type-specific manner. The aim of the current study therefore was to further characterize the putative anti-inflammatory properties of a closely related synthetic compound in different primary cell-based assays relevant to the nervous system and in animal models of CNS diseases. We report that compound A is a potent inhibitor of NF- κ B- and AP-1-mediated cellular activation in vitro and exhibits neuroprotective properties in neuronal tissue cultures as well as in animal models of brain damage.

Materials and Methods

Compound. Compound A [(1*R*,3*S*,3*aR*,8*bS*)-3*a*-(4-methoxyphenyl)-6,8-dimethoxy-3-(3-fluor-phenyl)-2,3,3*a*,8*b*-tetrahydrocyclopenta[*b*]benzofuran-1,8*b*-(1*H*)-diol] and its 1*S*-stereoisomer have been synthesized by the Bayer chemistry department. In all in vitro studies, compounds were dissolved in dimethyl sulfoxide and applied immediately before cell stimulation or challenge. Final dimethyl sulfoxide concentration was 0.1%.

Cell Cultures. If not otherwise stated, media and cell culture supplements were purchased from Sigma-Aldrich (Taufkirchen, Ger-

many) or Invitrogen (Karlsruhe, Germany). All media and additives were of low endotoxin content. Human umbilical vein endothelial cells (HUVEC) were obtained from CellSystems (St. Katharinen, Germany) and were expanded in EGM-2 medium (CellSystems). After reaching 60 to 80% confluence, cells were detached, dissociated, and plated in 96-well tissue culture plates (Corning, Wiesbaden, Germany) at a density of 5000 cells/well. Cells were cultured for an additional 6 days before use in cellular assays. Glial and neuronal cell cultures were established according to published standard protocols. Mixed glial cell cultures were prepared from P1 mouse (CFW-1) or rat (Wistar) brain, and cells were grown for 12 to 14 days in DMEM/Ham's F-12 medium containing 10% fetal calf serum (FCS) and 100 μ g/ml penicillin/streptomycin. Microglia growing on top of a confluent cell layer in mixed glial cultures were removed by rotary shaking and collected by centrifugation. Cells were resuspended in phenol red-free DMEM/astrocyte-conditioned DMEM [1:2 (v/v)] containing 10% heat-inactivated FCS and plated at a density of 50,000 cells/well in 96-well tissue culture plates. Microglia were used for experiments 2 days after plating. The remaining astrocytes were detached, dissociated, and seeded in 96-well tissue culture plates at a density of 80,000 cells/well, in six-well tissue culture plates at a density of 10^6 cells/well, or in eight-well chamber slides (NUNC A/S, Roskilde, Denmark) at a density of 10^5 cells/well. Astrocytes were maintained in phenol red-free DMEM/Ham's F-12 medium, 10% FCS, and penicillin/streptomycin, with reduction of FCS to 1% 1 day before use. Primary mesencephalic neurons were prepared from the brains of embryonic day 16 rats (Wistar). The ventral two thirds of the mesencephalic region were collected. The tissue pieces were dissociated by means of a papain dissociation kit (Cell Systems, St. Katharinen, Germany), and the resulting cell pellet was resuspended in culture medium consisting of DMEM/Ham's F-12 medium, 0.5% BSA, N2 additives, 6 mg/ml glucose, 2 mM glutamine, and 100 μ g/ml penicillin/streptomycin. Cells were plated in poly-D-lysine-coated 96-well tissue culture plates at a density of 80×10^3 cells/well or in poly-D-lysine-coated 8 well chamber slides at a density of 2×10^5 cells/well. Cultures were kept in a CO₂ incubator until use, and half of the culture medium was exchanged every 2 to 3 days.

Cellular Assays. Cytokine/chemokine release was induced by addition of human recombinant IL-1 β (endotoxin content, <0.1 ng/ μ g; Biosource, Solingen, Germany), human recombinant TNF α (endotoxin content, <0.1 ng/ μ g; Biosource), or lipopolysaccharide (LPS; Sigma-Aldrich), and production of cytokines/chemokines was determined in cell culture supernatants by ELISA. ELISA kits for human IL-8, human IL-6, and rat TNF α were obtained from Biosource. ELISA kits for mouse IL-6, mouse MIP-2, rat MIP-2, and rat MCP-1 were purchased from R&D Systems (Wiesbaden, Germany). ELISAs were performed according to the manufacturer's instructions.

Quantitation of ICAM-1 and ELAM-1 expression was performed by a cell-based ELISA. In brief, HUVECs were stimulated by cytokine-treatment, the culture supernatant was removed after the indicated incubation period, and, after washing with phosphate-buffered saline (PBS), the cells were fixed by addition of ice-cold 4% paraformaldehyde in PBS. Thereafter, cells were washed again and unspecific binding sites were blocked by incubation with PBS, containing 1% bovine serum albumin (BSA) for 1 h at room temperature. Cells were then sequentially incubated with mouse monoclonal antibodies directed against either human ICAM-1 or human ELAM-1 (R&D Systems), respectively, and anti-mouse peroxidase-conjugated secondary antibodies (Chemicon, Hofheim, Germany). After washing, peroxidase activity was revealed by addition of substrate solution [2,2-azino-di(3-ethylbenzthiozinesulfonate)-(6)]; Roche, Mannheim, Germany] and monitored photometrically at 405 nm.

Quantitative measurement of nitric oxide formation was performed by a fluorimetric assay according to the suppliers instructions (Calbiochem, Bad Soden, Germany). In some experiments, nitrite, which is formed from released nitric oxide, was determined photometrically by the Griess reaction. One hundred microliters of the cell-free culture supernatant was mixed with Griess reagent [1%

sulfanilic acid in 5% H_3PO_4 and 0.1% *N*-(1-naphthyl)-ethylenediamine in H_2O , mixed at a ratio of 1:2] and incubated for 10 min. Optical density was read at 550 nm.

Cytotoxicity was determined by measurement of lactate dehydrogenase (LDH) release. The LDH assay was performed according to the manufacturer's instructions (Roche, Mannheim, Germany).

Dopamine uptake was determined as a measure of dopaminergic neuronal cell damage in primary mesencephalic neuronal cell cultures maintained for 7 days in vitro. After treatment with 1-methyl-4-phenylpyridinium (MPP^+ ; Sigma-Aldrich), cells were washed twice with uptake buffer (25 mM HEPES, pH 7.4, 125 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4 , 1.3 mM CaCl_2 , 1.2 mM MgSO_4 , and 5.6 mM glucose) and subsequently equilibrated in uptake buffer for 30 min at 37°C. Thereafter, the cell supernatant was removed, and 100 μl of uptake buffer containing 10 μM pargyline (a monoamine oxidase inhibitor; Sigma-Aldrich) and 100 μM ascorbic acid were added. After 5 min, the cell supernatant was removed, and uptake was started by addition of uptake buffer containing 100 μM ascorbic acid and 3 nM [^3H]dopamine (PerkinElmer Life and Analytical Sciences, Zaventem, Belgium). Unspecific binding of the radioligand was determined by incubation in the presence of the dopamine uptake inhibitor GBR 12909 (final concentration, 10 μM ; Sigma-Aldrich). Uptake was stopped by removal of the cell supernatant and addition of ice-cold uptake buffer. Cells were then washed once with uptake buffer (4°C) and solubilized in 140 μl of 0.1 M NaOH. Homogenates were transferred to scintillation vials, mixed with 3 ml of aquasol (Du Pont de Nemours Belgium BVBA, Mechelen, Belgium), and radioactivity was counted by scintillation counting. All incubations were carried out at 37°C, and dopamine uptake was measured 30 min after [^3H]dopamine addition.

Determination of MPP^+ toxicity by counting of tyrosine hydroxylase immunoreactivity (TH-ir) cells was performed in primary mesencephalic neuronal cultures plated in 96-well tissue culture plates. 3,3'-Diaminobenzidine (DAB) was used as a substrate for secondary antibody-conjugated peroxidase and immunoreactive cells/well were counted.

Preparation of Cell Extracts and Immunoblotting. After treatment for the indicated periods, cells were placed on ice and washed twice with ice-cold PBS. All subsequent steps were then carried out at 4°C. Cells were collected by scraping and centrifugation for 5 min at 500g. The supernatant was removed, cells were resuspended in 10 mM HEPES, pH 7.9, 1.5 mM MgCl_2 , 10 mM KCl, 0.1% Triton X-100, 5 mM NaF, 1 mM β -glycerophosphate, 0.5 mM phenylmethylsulfonyl fluoride, 1 $\mu\text{g/ml}$ aprotinin, 10 $\mu\text{g/ml}$ leupeptin, and incubated for 10 min on ice. After vortexing, homogenates were centrifuged for 5 min at 10,000g, and the supernatant was carefully removed (cytosolic protein fraction) and aliquoted. One aliquot was kept for determination of protein content by the BCA assay (Sigma-Aldrich) and the others stored at -80°C until use. For extraction of nuclear proteins, the pellet was washed twice, resuspended in 20 mM HEPES, pH 7.9, 1.5 mM MgCl_2 , 420 mM NaCl, 0.2 mM EDTA, 5 mM NaF, 1 mM β -glycerophosphate, 0.5 mM phenylmethylsulfonyl fluoride, 1 $\mu\text{g/ml}$ aprotinin, and 10 $\mu\text{g/ml}$ leupeptin, and incubated for 15 to 30 min on ice with intermittent vortexing. The homogenates were centrifuged for 5 min at 10,000g, and the supernatant was carefully removed (nuclear protein fraction) and aliquoted. One aliquot was kept for determination of protein content and the others stored at -80°C until use.

Cytosolic or nuclear proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Eschborn, Germany). Membranes were blocked for 1 h in Tris-buffered saline, pH 7.4, containing 0.05% Tween 20 and 5% BSA. After the blocking step, membranes were incubated with primary antibody overnight at 4°C, followed by the appropriate peroxidase-conjugated secondary antibody for 1.5 h at room temperature. Immunoblots were developed by enhanced chemiluminescence (Amersham Biosciences, Freiburg, Germany). Quantitation of protein band intensities was performed by filmless autora-

diographic analysis (STORM 860 PhosphorImager; Amersham Biosciences) using ImageQuant 5.0 (Amersham Biosciences).

Primary antibodies used were rabbit anti NF- κB p65 (Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti c-Jun/AP1 (New England Biolabs, Frankfurt, Germany), rabbit anti phospho-c-Jun (Ser 63) (Calbiochem, Bad Soden, Germany), rabbit anti iNOS (BD Pharmingen, Heidelberg, Germany), and mouse anti COX-2 (BD Pharmingen).

Immunocytochemistry. Immunostaining of cultured cells was essentially performed according to standard procedures. In brief, after washing with PBS, cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were then permeabilized with methanol (-20°C) for 5 min; washed in PBS containing 5% sucrose and 0.3% Triton X-100; and blocked with PBS, 1% goat serum, and 2% BSA (30 min, room temperature). Cells were incubated for 1 h with primary antibody diluted in blocking buffer at room temperature or over night at 4°C. After washing, cells were incubated with an appropriate Cy2- or Cy3-conjugated secondary antibody for 1 h at room temperature. Excess antibody was removed by washing, and cell nuclei were stained by Hoechst 33258 diluted in PBS for 10 min. After two further washes in PBS, cultures were mounted on slides with mounting medium (Sigma-Aldrich). In double staining experiments, incubation with primary antibodies was done in a single step over night at 4°C. Secondary antibody staining was performed sequentially. Antibodies used for immunocytochemistry were rabbit anti-tyrosine hydroxylase (Institute Jacques Boy SA, Reims, France), mouse anti-tyrosine hydroxylase (Chemicon), rabbit anti-dopamine (Chemicon), mouse anti-GFAP (Sigma-Aldrich), rabbit anti-NF- κB p65 (Santa Cruz Biotechnology), rabbit anti-c-Jun/AP1 (New England Biolabs), rabbit anti-phospho-c-Jun (Ser 63) (Calbiochem), rabbit anti-iNOS (BD Pharmingen), mouse anti-COX-2 (BD Pharmingen), secondary Cy2-conjugated antibodies (Amersham Biosciences), and secondary Cy3-conjugated antibodies (Sigma-Aldrich).

MPTP Animal Model. Animal care was according to protocols and guidelines approved by the European Council Directive of November 24, 1986 (86/609/EEC). All efforts were made to minimize animal suffering. Fifty 8-week-old male C57/BL6 mice (Janvier, Le Genest-St-Isle, France) were housed under standardized conditions (temperature, $23 \pm 2^\circ\text{C}$; relative humidity, $55 \pm 5\%$; 12-h light/dark cycle, lights on at 7 AM) with free access to food and water. Mice were acclimated for at least 1 week in the animal room before the experiment started. Animals were randomly assigned to one of the following five groups: intact ($n = 10$), MPTP intoxication plus vehicle ($n = 10$), MPTP intoxication plus compound A at the following doses: 0.3, 1.0, 3.0 mg/kg ($n = 10$ each). For MPTP intoxication, mice received daily injections of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (Sigma-Aldrich) dissolved in saline solution (0.9% NaCl) for 3 consecutive days. In addition, animals were injected with compound A dissolved in 10% solutol, 15% ethanol, 75% distilled water, or vehicle twice daily with an interval of 6 h for a total of 10 days. These injections always preceded the MPTP administration. All drugs were applied intraperitoneally in a volume of 5 ml/kg. On day 11, mice were deeply anesthetized with 40 mg/kg i.p. tribromethanol (Aldrich, Steinheim, Germany) and perfused transcardially with saline solution (0.9% NaCl) followed by 4% paraformaldehyde. Brains were removed, postfixed in 4% paraformaldehyde for 1 h, cryoprotected in 20% sucrose/PBS for 24 h, and frozen in 2-methylbutane. Cryostat sections (25 μm) encompassing the entire midbrain were collected free floating and processed for visualization of TH-ir. Immunostaining was performed as described by Nelson et al. (1996).

Sections stained for tyrosine hydroxylase were analyzed according to the procedure described by Bézard et al. (1997). For each animal, a section through the substantia nigra pars compacta (SNc) corresponding to a level of bregma -2.92 mm – -3.08 mm in the mouse brain atlas of Franklin and Paxinos (1977) was chosen. Counting of TH-ir cells was done in a blinded fashion with regard to the experimental condition and was aided by an imaging system. The number

of TH-ir SNc neurons of one hemisphere was determined by two independent examiners, and the average of both measurements was used for further data processing.

The mean and S.E.M. of the number of TH-ir SNc neurons were calculated for each treatment group. Results were analyzed using a one-way analysis of variance followed by Fisher's least-significant-difference test. Significance level was defined to be $p < 0.05$.

Animal Model of Traumatic Brain Injury. Unilateral subdural hematoma (SDH) in rats was used as animal model of traumatic brain injury. Animal care was according to protocols and guidelines approved by the European Council Directive of November 24, 1986 (86/609/EEC), and all efforts were made to minimize animal suffering. Forty male Wistar rats were subjected to surgery, which was performed as described by Eijkenboom et al. (2000). Immediately after surgery (2 and 4 h), 30 animals received i.v. injections of compound A dissolved in 0.9% NaCl (2 ml/kg). Ten animals were vehicle-injected. Animals were allowed to survive for another 7 days. Thereafter, animals were killed by decapitation, and brains were removed and immediately frozen in *n*-methylbutane at -40°C . After sectioning (20 μm), tissue sections were stained with cresyl violet, and the infarct volume was assessed by computer-assisted image analysis. Results were statistically analyzed by one-way analysis of variance with post hoc analysis (Fisher's LSD test).

Results

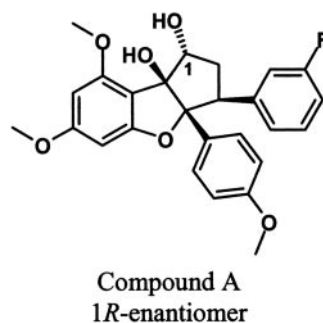
Inhibition of Cytokine- and LPS-Induced Cell Activation.

Compound A, a monofluor derivative of the naturally

occurring 1*H*-cyclopenta[*b*]benzofuran rocaglaol, was tested for its ability to modulate cytokine- or LPS-induced cell activation in different, non-neuronal cell types (Table 1). Whereas glial cells are directly involved in immune reactions within the brain parenchyma, umbilical vein endothelial cells were chosen as a simplified model system exhibiting basic activation properties similar to those of cerebral vascular endothelial cells. As part of the blood-brain-barrier, activated vascular endothelium has been implicated in mediating migration of peripheral immune cells into the brain in acute and chronic neurodegenerative diseases.

Activation of HUVECs was determined by ELISA-based measurement of IL-1 β and TNF α -induced cell surface adhesion molecule expression (ICAM-1, ELAM-1) and cytokine/chemokine release (IL-6, IL-8) (Fig. 1). Both IL-1 β and TNF α stimulated adhesion molecule expression and IL-6/IL-8-release in a dose- and time-dependent manner. Cells were treated with different concentrations of compound A immediately before IL-1 β (10 ng/ml) or TNF α (10 ng/ml) addition, and the effect of treatment was determined 6 h later, when adhesion molecule expression and IL-6 release were nearly maximal and IL-8 release reached half-maximal levels. Compound A potently inhibited IL-6 and IL-8 release and ELAM-1 expression independent of the stimulus used (Fig. 2). Inhibitory efficacy with respect to ELAM-1 expression,

TABLE 1
Enantio-selective inhibition of cytokine- and LPS-induced cell activation
Mean EC₅₀ values were calculated from at least two independent experiments.



	Stimulus	Mean EC ₅₀		Species
		1 <i>R</i> -Enantiomer	1 <i>S</i> -Enantiomer	
		<i>nM</i>	<i>nM</i>	
HUVEC				
ELAM-1 expression	IL-1 β	17	N.D.	Human
	TNF α	39	N.D.	
ICAM-1 expression	IL-1 β	>10,000	N.D.	
	TNF α	>10,000	N.D.	
IL-6 release	IL-1 β	208	>10,000	Human
	TNF α	311	N.D.	
IL-8 release	IL-1 β	28	2450	
	TNF α	13	N.D.	
Astrocytes				
IL-6 release	IL-1 β	286	>10,000	Mouse
MCP-1 release	IL-1 β	4	206	Rat
MIP-2 release	IL-1 β	20 ^a	2335 ^a	Mouse
	IL-1 β	>10,000	N.D.	Rat
NO release	LPS	3	274	Mouse
	LPS	3	257	Rat
Microglia				
NO release	IFN γ /LPS	3	1024	Rat
TNF α release	LPS	39	N.D.	Rat

^a Stimulation.
N.D., not determined.

however, was less than for IL-6 and IL-8 release, respectively (maximal inhibition of ELAM-1 expression, 60 to 70%; maximal inhibition of IL-6/IL-8 release, 80–90%). Little or no inhibition of ICAM-1 expression was observed for compound A at concentrations below 10 μ M (Fig. 2). Significant inhibi-

tion was found only for higher compound concentrations, and the inhibitory effect paralleled increased LDH release, indicating that at these concentrations, compound A may exert unspecific or toxic effects (Fig. 2).

Glial cells respond to cytokine- or LPS-induced activation

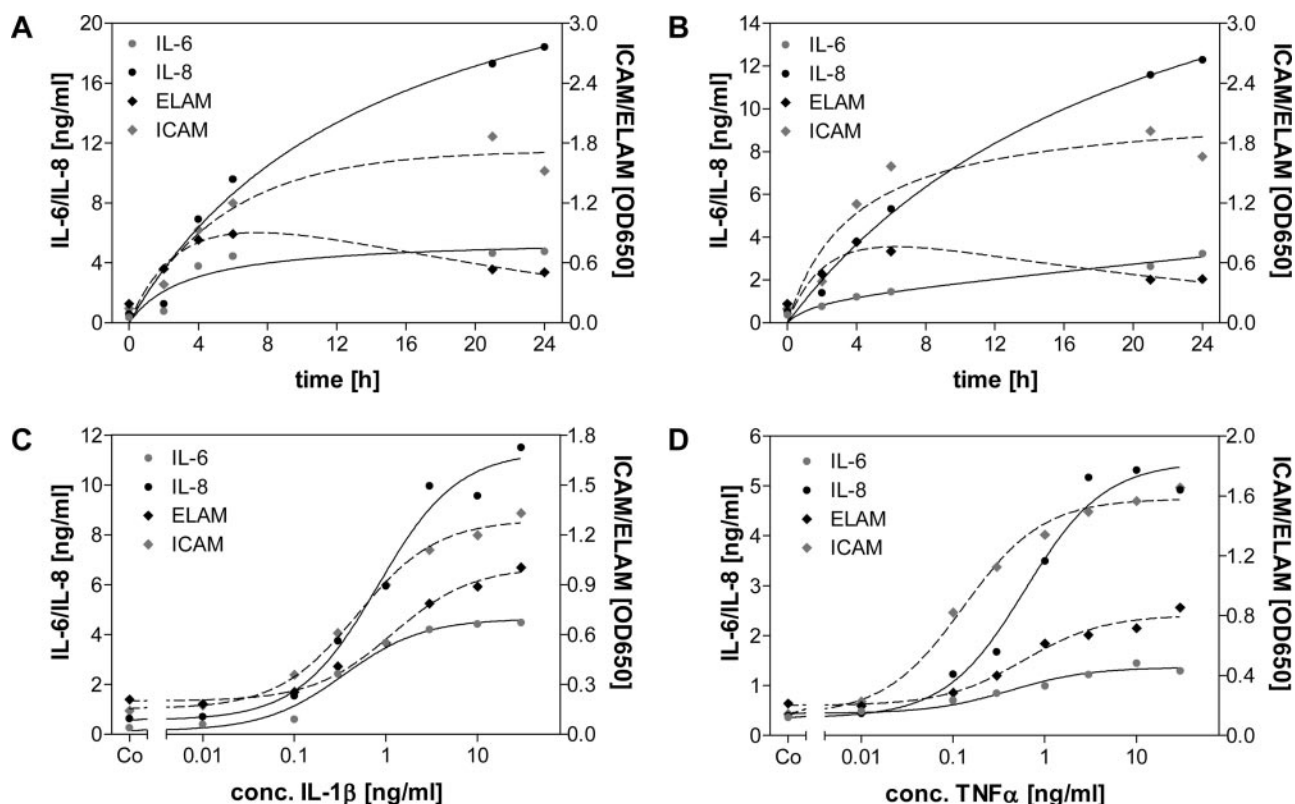


Fig. 1. Time- and dose-response curves of IL-1 β - (A and C) and TNF α -induced (B and D) adhesion molecule expression and cytokine/chemokine release from HUVEC. Adhesion molecule expression (ELAM-1, ICAM-1) was determined by cell-based ELISA, and the respective optical densities are indicated at the right axis. IL-6 and IL-8 release was measured in culture supernatants by ELISA, and concentrations were calculated by means of a respective standard curve. Data are means from representative experiments carried out in quadruplicates. Standard deviations were less than 10%.

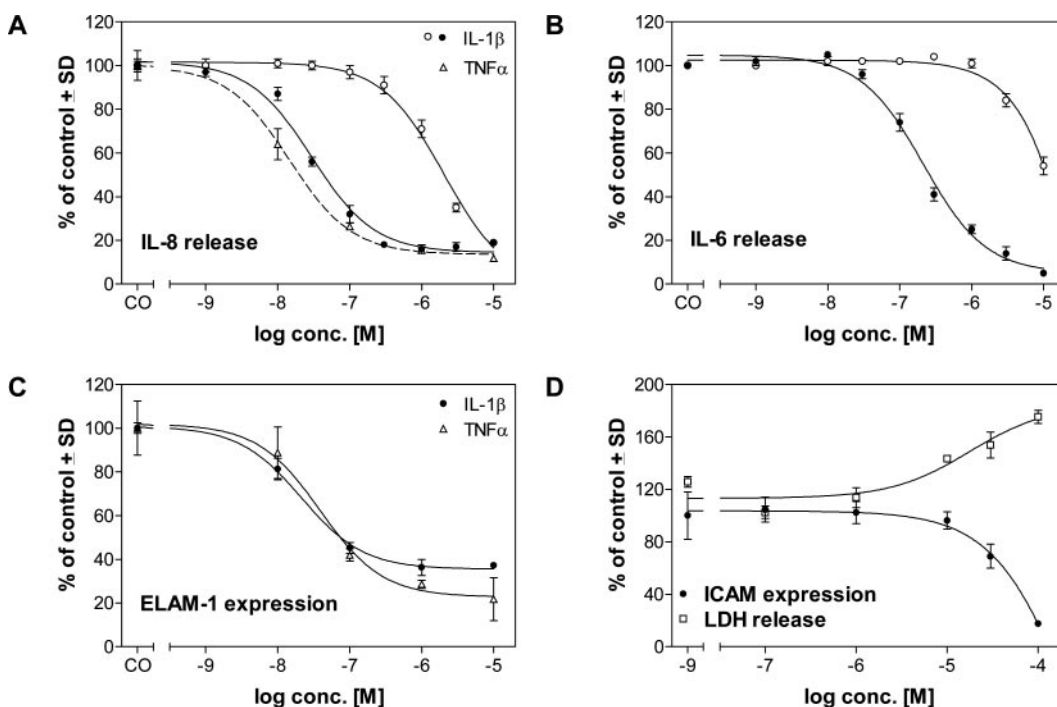


Fig. 2. Inhibition of ELAM-1/ICAM-1 expression and IL-6/IL-8 release from HUVEC by compound A and its 1S-enantiomer. Cytokine/chemokine release and adhesion molecule expression was induced by addition of 10 ng/ml IL-1 β and TNF α (Δ in A and C), respectively. Compound A (●) and its 1S-enantiomer (○) were applied at the indicated concentration immediately before cell stimulation. IL-8 release (A), IL-6 release (B), and ELAM-1 expression (C) were determined by ELISA 6 h after IL-1 β and TNF α addition. ICAM-1 expression (D, ●) and LDH release (D, □) were measured 24 h after stimulation. Controls determined in the absence of compound A or its 1S-enantiomer were set at 100% and all other values calculated in relation. Data are means \pm S.D. from one representative experiment carried out in quadruplicates.

by release of various cytokines, chemokines, and nitric oxide (NO). Enriched astrocyte and microglia cultures were prepared, and the effect of compound A on glia activation was investigated. Treatment of mouse or rat brain astrocytes with IL-1 β resulted in a time- and dose-dependent release of IL-6, MIP-2, and MCP-1 (Fig. 3). IL-1 β in combination with IFN γ as well as LPS also induced astroglial NO release (Fig. 3). Enriched astrocyte cultures were treated with compound A immediately before stimulation by IL-1 β or LPS and cytokine release was determined 6 h later. Formation of nitrite/nitrate as a result of NO release was measured 24 h after LPS challenge. Compound A had no effect (rat astrocytes; Fig. 3) or even a stimulatory effect (mouse astrocytes; Table 1) on MIP-2 release. However, the presence of compound A resulted in dose-dependent inhibition of IL-6, MCP-1, and NO release; MCP-1 and NO release (Fig. 3, Table 1) were more potently inhibited than IL-6 release (Table 1). Moreover, compound A blocked the induction of iNOS in astrocyte enriched cultures as shown by Western analysis of cell homogenates and immunocytochemistry (Fig. 4). Under basal conditions, iNOS protein was detectable neither by immunoblotting nor by immunostaining of cells. Treatment of cultures with 100 ng/ml LPS resulted in iNOS induction in approximately 50% of the cells 24 h after LPS addition. Double-staining experiments showed that a portion of the iNOS-ir cells did not express GFAP, indicating that these

cells probably were microglia or immature astroblasts. Induction of iNOS was completely blocked by the presence of compound A (Fig. 4). Likewise, compound A treatment resulted in a dose-dependent inhibition of iNOS protein expression as detected by immunoblot, 24 and 48 h after LPS addition. Compound A also prevented LPS-mediated induction of COX-2 (Fig. 4). Although some cells, probably microglia, expressed COX-2 under basal conditions, it was not detected in resting astrocytes by immunocytochemistry but was induced in approximately 70% of the cells 24 h after LPS addition. Immunostaining of cells was reduced nearly to background levels by the continuous presence of 1 μ M compound A (Fig. 4). These results were confirmed by immunoblot of astrocyte homogenates prepared from mixed glial cultures. Dose-dependent inhibition of COX-2 protein expression by compound A was observed at 24 h and was even more pronounced at 48 h after LPS addition (Fig. 4).

Enriched rat microglial cultures were prepared, and the effect of compound A on LPS-stimulated NO and TNF α release was measured. Before induction of NO release by addition of LPS, cells were primed by IFN γ (500 U/ml) for 16 h. LPS potently stimulated microglial NO and TNF α release in a dose- and time-dependent manner (Fig. 3). Compound A was added immediately before addition of LPS. NO formation was determined 6 h thereafter, whereas TNF α release was measured 5 h after LPS addition, a time point at which TNF α

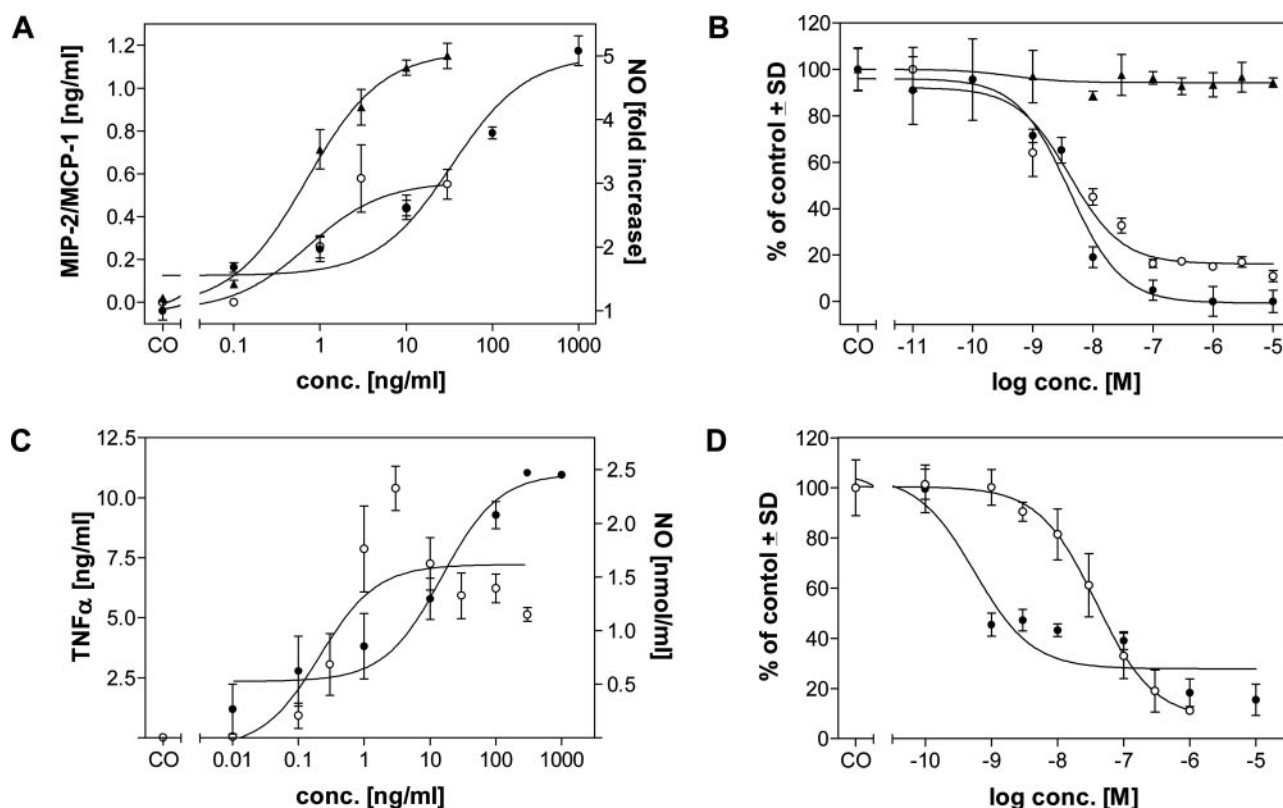


Fig. 3. Rat glial chemokine, TNF α and NO release, and effect of compound A treatment. Astroglial chemokine release was induced by addition of IL-1 β (indicated concentrations in A; 10 ng/ml in B) and NO release by addition of LPS (indicated concentrations in A; 100 ng/ml in B). MCP-1 (A and B, \circ) and MIP-2 (A and B, \blacktriangle) content was determined in culture supernatants 6 h after stimulation by ELISA. NO release (A and B, \bullet) was determined 24 h after LPS addition. Microglial TNF α release was triggered by LPS addition (indicated concentrations in C; 100 ng/ml in D) and determined in culture supernatants 5 h thereafter by ELISA. Microglial NO release was induced by LPS (indicated concentrations in C; 1 μ M in D) after cells have been treated with IFN γ (500 U/ml) for 16 h. NO formation was fluorometrically measured in culture supernatants 6 h after LPS addition. A and C, dose-dependent release of chemokines, TNF α , and NO; B and D, effect of compound A added to cultures immediately before stimulation. Data are means \pm S.D. from one representative experiment each carried out in quadruplicates.

release was maximal. Compound A potently and efficiently inhibited microglial NO formation as well as TNF α release (Fig. 3, Table 1). Inhibition was dose-dependent and in both cases amounted to more than 80% at a compound A concentration of 1 μ M.

The effects of compound A were stereoselective. Thus, although still active and equally effective, the 1*S*-stereoisomer of compound A was less potent by at least a factor of 5, indicating that compound A may hit a specific molecular target (Table 1, Fig. 2).

Compound A Inhibits NF- κ B and c-Jun Signaling in Astrocytes. To investigate which signaling cascades are modulated by compound A, immunoblot and immunocytochemical studies were performed. As shown, compound A blocked IL-1 β , TNF α , and LPS-induced gene expression. Intracellular signaling pathways common to all three types of stimuli are the activation of mitogen-activated protein (MAP) kinase cascades and of the transcription factor NF- κ B. Therefore, nuclear extracts were prepared from LPS-stimulated and vehicle- or compound A-treated astrocytes. Nuclear protein fractions were analyzed by immunoblot for the presence of the p65 subunit of NF- κ B, c-Jun, and Ser63-phosphorylated c-Jun (Fig. 5). Addition of LPS (100 ng/ml) to astrocyte cultures resulted in a time-dependent accumulation of NF- κ B p65 and phosphorylation of c-Jun. In addition, nuclear c-Jun protein increased approximately 3-fold within a 90-min period. Treatment of astrocytes with 1 μ M compound A prevented LPS-induced nuclear accumulation of NF- κ B p65 as well as increases in c-Jun protein and c-Jun phosphorylation (Fig. 5). Inhibition of nuclear accumulation of NF- κ B p65, however, may only be partial with increasing

incubation time. These findings were corroborated by immunocytochemistry (Fig. 5). Under basal conditions, only a few cells showed nuclear staining for NF- κ B p65. Nuclear translocation of NF- κ B was induced by addition of 100 ng/ml LPS and significantly inhibited by preincubation of cells with 1 μ M compound A. Likewise, no phosphorylated c-Jun was found in astrocyte nuclei in untreated cultures but was frequently present 60 min after LPS addition. Immunostaining was nearly completely abolished in cultures treated with 1 μ M compound A. Taken together, these results clearly demonstrate that compound A inhibits both NF- κ B and c-Jun nuclear signaling.

Compound A Protects Dopaminergic Neurons from MPP⁺ Toxicity in Vitro and in Vivo. Primary rat mesencephalic neurons were prepared from embryonic day 16 brain and kept for 7 days in vitro until use. At this point, approximately 0.5 to 1% of the neurons were dopaminergic as judged by tyrosine hydroxylase (TH) and dopamine immunostaining (Fig. 6). TH-immunoreactive (TH-ir) neurons exhibited the typical bi-/tripolar shape of catecholaminergic neurons with partially extensive branching of the primary neurites. Dopamine uptake is a selective functional characteristic of dopaminergic neurons and was therefore employed as a sensitive measure reflecting dopaminergic neuronal cell numbers and/or physiological status. During the first 45 min, dopamine uptake in mesencephalic cultures as measured by addition of radiolabeled dopamine was linear (data not shown). Approximately 10% of the added radioactivity was recovered from the cell pellet under the conditions of this study. From this, the cells took up more than 90%. The remaining less than 10% were nonspecifically bound as de-

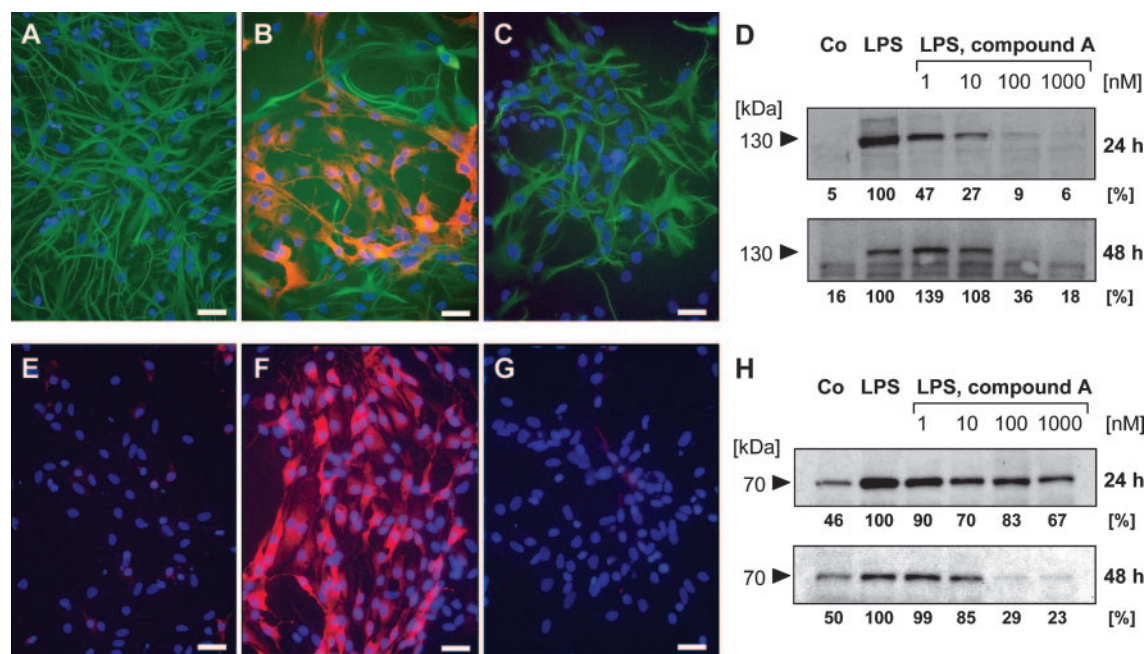


Fig. 4. Inhibition of iNOS and COX-2 expression in rat astroglial cultures by compound A. A–C, overlay images of enriched mixed glial cultures immunostained for iNOS (red) and GFAP (green). A, vehicle-treated cultures; B and C, cultures treated for 24 h with 1 μ g/ml LPS in the absence (B) and presence of 1 μ M compound A (C). Nuclei were stained by Hoechst 33258. D, immunoblot of protein extracts prepared from astroglial cultures 24 and 48 h after LPS (100 ng/ml) treatment in the presence and absence of the indicated concentrations of compound A and stained for iNOS. Relative band intensities are indicated beneath the images. E and F, images of enriched mixed glial cultures immunostained for COX-2 (red). E, vehicle-treated cultures; F and G, cultures treated for 24 h with 1 μ g/ml LPS in the absence (F) and presence of 1 μ M compound A (G). Nuclei were stained by Hoechst 33258. H, immunoblot of protein extracts prepared from astroglial cultures 24 and 48 h after LPS (100 ng/ml) treatment in the presence and absence of the indicated concentrations of compound A and stained for COX-2. Relative band intensities are indicated beneath the images. Scale bars in A–G, 20 μ m.

terminated by measuring uptake at 4°C or in the presence of GBR 12909, a selective dopamine transport inhibitor (data not shown). For all other experiments, dopamine uptake was measured at 37°C during a 30-min interval, and nonspecific binding was determined by addition of GBR 12909. The magnitude of specific dopamine uptake was dependent on age of the cultures and initial plating density (data not shown). From day 6 in culture, dopamine uptake starts to reach a

constant level, indicating that neuronal maturation ceased. Addition of MPP⁺ to mesencephalic neurons grown for 7 days in culture caused a dose- and time-dependent decrease in dopaminergic cell counts (TH-ir neurons) as well as in dopamine uptake (Fig. 6). The highest concentration of MPP⁺ used resulted in a reduction in dopamine uptake of more than 80%. However, the reduction of absolute cell counts of TH-ir cells was less pronounced at all MPP⁺ concentrations

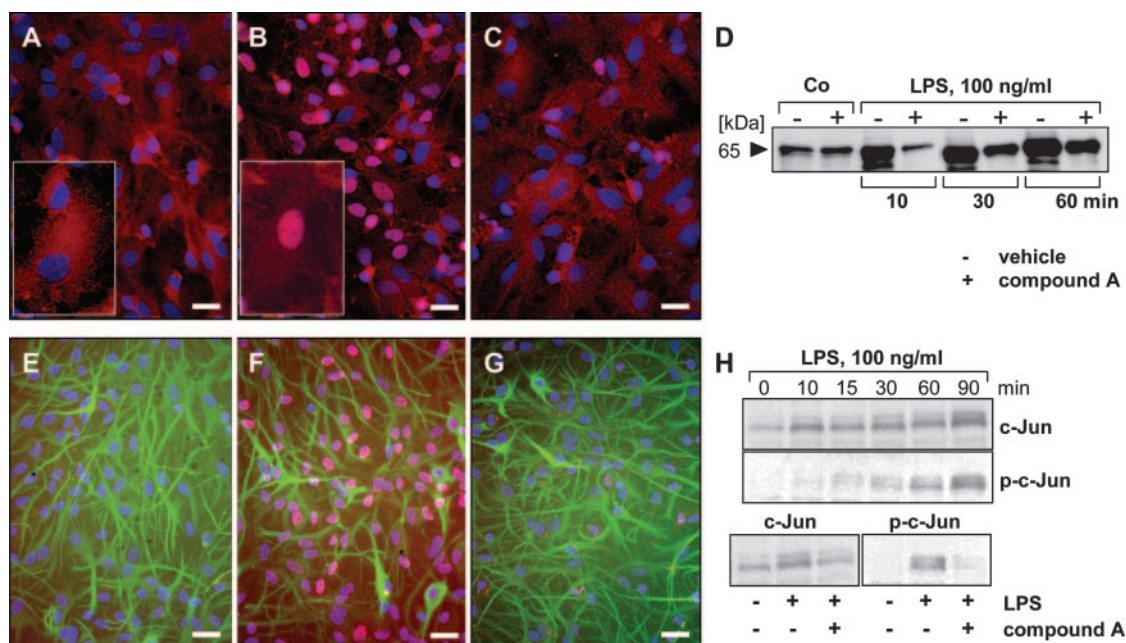


Fig. 5. Inhibition of NF-κB nuclear translocation and c-Jun phosphorylation by compound A. A–C, enriched astroglial cultures were immunostained for NF-κB p65 (red) and nuclei counterstained with Hoechst 33258. A, vehicle-treated cultures; B and C, cultures treated with 100 ng/ml LPS for 30 min in the absence (B) and presence of 1 μM compound A (C). D, NF-κB p65 immunoblot of nuclear extracts prepared at the indicated periods from unstimulated (Co) and LPS-stimulated enriched astroglial cultures in the presence and absence of 1 μM compound A. E–G, overlay images of enriched astroglial cultures immunostained for GFAP (green) and Ser63-phospho-c-Jun (red). Nuclei were counterstained with Hoechst 33258. E, vehicle-treated cultures; F and G, cultures treated with 100 ng/ml LPS for 60 min in the absence (F) and presence of 1 μM compound A (G). H, immunoblot of nuclear extracts prepared from enriched astroglial cultures stained for c-Jun and Ser63-phospho-c-Jun. Top, time-dependent up-regulation of c-Jun protein expression and phosphorylation after LPS stimulation. Bottom, inhibition of c-Jun up-regulation and phosphorylation by 1 μM compound A as determined 60 min after LPS addition. Scale bars in A–G, 20 μm.

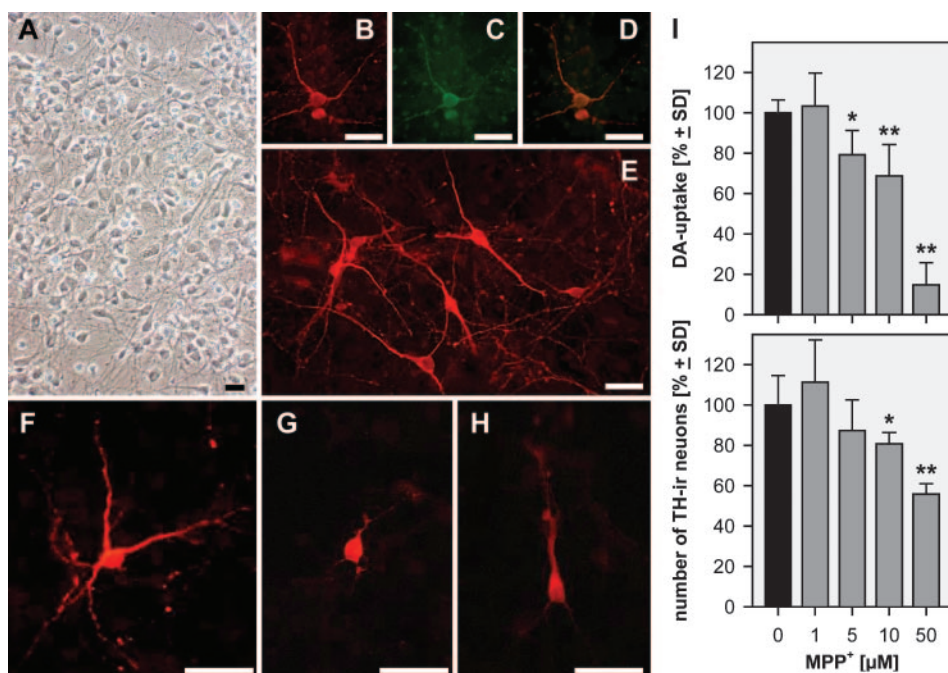


Fig. 6. Characterization of primary mesencephalic neuronal cultures and effect of MPP⁺ treatment. Rat mesencephalic neuronal cultures grown for 7 days in vitro (A, phase contrast image) were immunostained for the presence of tyrosine hydroxylase (TH) (C and E–H) and dopamine (B). TH-immunoreactive neurons were also positive for dopamine (B, C, and overlay D). Treatment of cultures with 50 μM MPP⁺ for 24 h induced neurite retraction (G, H) and eventually cell death. I, mesencephalic cultures were treated with the indicated concentrations of MPP⁺ and dopamine-uptake (top) and number of TH-immunoreactive neurons (bottom), respectively, were determined. Cell counts and dopamine-uptake of vehicle-treated controls were set 100% and the other values calculated in relation. Data are means ± S.D. of one representative experiment carried out in 6-fold replicates. *, $p < 0.05$; **, $p < 0.01$ (Student's *t* test). Scale bars in A–H, 50 μm.

tested. Therefore, reduction of dopamine uptake reflected both the loss of dopaminergic neurons and cellular damage. The latter was also indicated by pronounced morphological changes of TH-ir neurons after MPP⁺ challenge (loss of neurites, reduction of branches; Fig. 6).

Increased NF- κ B nuclear translocation and c-Jun N-terminal kinase (JNK) activity have been reported as a consequence of MPP⁺ challenge, both in vitro and in vivo (MPP⁺ is the active metabolite of MPTP). Therefore, the neuroprotective properties of compound A were investigated in primary dopaminergic neurons and in the MPTP mouse animal model. In primary cell cultures, dopamine uptake was taken as a measure of dopaminergic neuronal cell injury. Treatment of mesencephalic neurons with compound A immediately before MPP⁺ application reduced dopaminergic cell damage in a dose-dependent manner as determined 24 h after MPP⁺ addition (Fig. 7). The neuroprotective efficacy of compound A was dependent on the MPP⁺ concentration. At the lowest concentration used (1 μ M), which resulted in reduction of dopamine uptake by approximately 30%, compound A completely prevented dopaminergic neuron damage. At a MPP⁺ concentration of 50 μ M, which reduced dopamine uptake by approximately 70%, compound A was still able to protect 50% of the dopaminergic neurons from degeneration (Fig. 7). The EC₅₀ values ranged between 15 and 40 nM depending on the MPP⁺ concentration used.

In vivo efficacy of compound A was determined in the low dose (4 mg/kg, i.p., daily for 3 days) MPTP mouse animal model of PD. Application of smaller doses of the neurotoxin over longer periods introduces a chronic component, leading to progressive neurodegeneration that mimics the typical disease evolution in PD. Under these conditions, MPTP treatment of mice resulted in a 30 to 50% bilateral loss of TH-ir cells in the SNc 11 days after toxin application (Fig. 8A). Compound A was administered twice daily by i.p. injection. Pharmacokinetic analysis revealed that after i.p. injection in mice, the compound passed the blood-brain barrier and was eliminated with a terminal half-life of approxi-

mately 50 min (data not shown). Treatment started at the first day of MPTP injection and lasted for 10 days. On day 11, degeneration of dopaminergic neurons was determined. Compound A dose dependently prevented dopaminergic neuronal cell loss. Maximal efficacy was observed at a dose of 3 mg/kg, with 76% of neurons being protected from cell death (Fig. 8A). This result was confirmed in a second experiment in which MPTP-induced cell loss was more pronounced (55% cell loss; 51% neuroprotection by compound A; data not shown). Compound A neither blocked dopamine transport nor inhibited monoamine oxidases A or B in vitro (data not shown).

Compound A Is Neuroprotective in a Rat Model of Traumatic Brain Injury. To investigate whether compound A also exhibits therapeutic efficacy under acute neurodegenerative conditions, the compound was administered to rats subjected to traumatic brain injury by introduction of an SDH. After injection of autologous blood into the subdural space, regional cerebral blood flow decreases while, at the same time, the local glucose utilization and extracellular levels of excitatory amino acids increase in the cortical area beneath the blood clot and in the ipsilateral hippocampus. The tissue underlying the hematoma becomes ischemic, leading to cortical and hippocampal cell damage and death (Eijkenboom et al., 2000). Compound A (0.1, 1, and 10 μ g/kg) was applied by multiple i.v. bolus injections immediately, 2 h, and 4 h after surgery. After a 7-day survival period, animals were killed by decapitation, the brains were removed, and the volume of the resulting infarct was determined. At that point, brain swelling as a consequence of brain edema formation was no longer evident. As shown in Fig. 8B, at a dose of 10 μ g/kg, compound A significantly reduced the infarct volume by 48%.

Discussion

Compound A is a synthetic mono-fluor derivative of the natural product rocaglaol. Like other members of the struc-

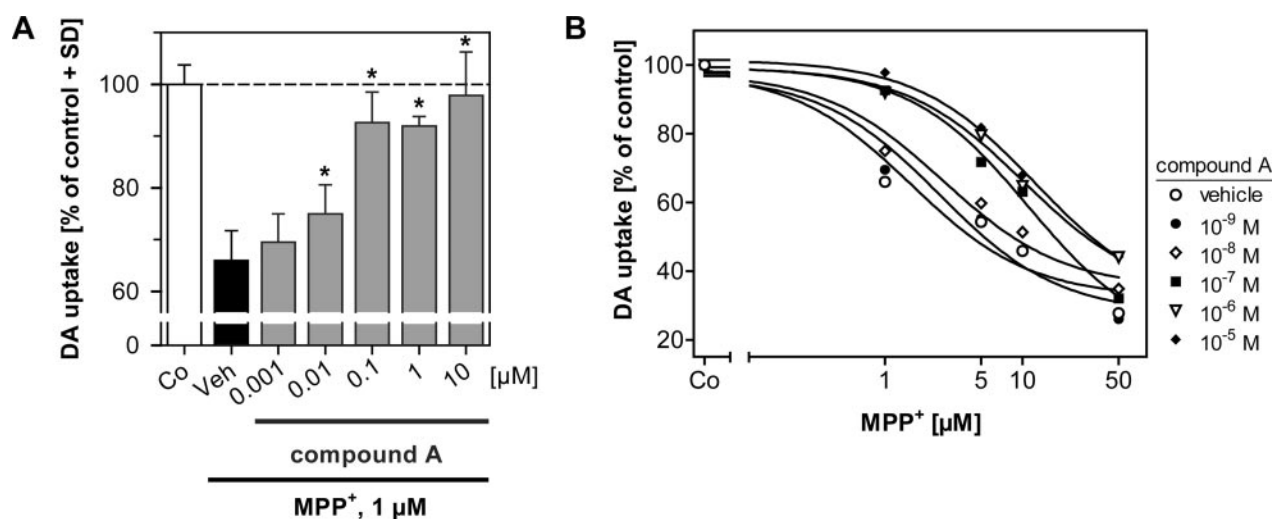


Fig. 7. Protective effect of compound A on MPP⁺-treated primary mesencephalic neurons. Primary rat mesencephalic neuronal cultures grown for 7 days in vitro were treated with the indicated concentrations of MPP⁺ in the presence and absence of various concentrations of compound A. Dopamine-uptake was measured 24 h after MPP⁺ addition. Vehicle-treated controls were set at 100% and the other values calculated in relation. A, dose-dependent amelioration of MPP⁺-induced (1 μ M) reduction of dopamine-uptake by compound A. B, shift of the MPP⁺ dose-response curve to the right by the presence of increased concentrations of compound A. Data are means from one representative experiment carried out in 6-fold replicates. *, $p < 0.05$ compared with MPP⁺-treated control (Student's t test).

turally closely related family of rocaglamids, rocaglaol can be isolated from the leaves and blossoms of *Aglaia* species. Extracts of these plants are used in natural medicine in South-east Asia and have been reported to exhibit anti-inflammatory and antitumor activity. In this study, we showed that compound A potently inhibited IL-1 β -, TNF α -, and LPS-induced expression of different cytokines and chemokines in human endothelial and/or murine glial cells. Moreover, compound A inhibited endothelial ELAM-1 as well as glial iNOS and COX-2 expression. For iNOS, this was shown both at the protein level and the activity level as determined by measurement of cellular nitric oxide production. The effects of compound A are specific, as indicated by the diminished or absent activity of the 1*S*-enantiomer and the lack of inhibition of ICAM-1 expression in endothelial cells or MIP-2 release from mouse astrocytes. It is interesting that MIP-2 release from rat astrocytes was stimulated, indicating species differences in the regulation of astrocyte MIP-2 gene expression.

Compound A inhibits gene expression induced by three different stimuli, namely IL-1 β , TNF α , and LPS, with comparable potency. Triggering of the respective receptors induces similar signaling cascades culminating in the activation of I κ B kinase (IKK) complex and MAP kinases, in particular of stress-activated protein kinases, which lead to a NF- κ B- and AP1-dependent transcriptional response (for review, see Medzhitov, 2001; Wajant et al., 2001; Janssens and Beyaert, 2002). In accordance with a recent report (Baumann et al., 2002), we found that compound A inhibited nuclear translocation of the NF- κ B p65 subunit in primary rat brain astrocytes after LPS treatment. NF- κ B activation was demonstrated as early as 10 min after LPS addition, both by immunoblot of astrocyte nuclear extracts and by immunocytochemistry. Inhibition of the NF- κ B-induced transcriptional activity after TNF α stimulation was also confirmed in a human adenocarcinoma cell line (SW1353) bearing an integrated NF- κ B-dependent reporter construct (data not shown). In addition, compound A prevented LPS-induced up-regulation and Ser-63 phosphorylation of c-Jun in astro-

glial cells. AP-1 activity is regulated by transcriptional regulation of its components and phosphorylation at specific sites. Phosphorylation of c-Jun by the JNK family of MAPKs at Ser73 and Ser63 located within its transactivation domain stimulates its ability to activate transcription either as homo- or heterodimer. The activated c-Jun can then autoregulate its own expression through a c-Jun/AP-1 enhancer element in its promoter (for review, see Shaulian and Karin, 2002). Thus, compound A may prevent cytokine- and LPS-induced expression of adhesion molecules, cytokines/chemokines, iNOS, and COX-2 by blocking both NF- κ B- and AP-1-mediated signaling. Expression of all gene products inhibited by compound A is primarily regulated at the level of gene transcription and the respective promoter regions contain functional binding sites for both inducible transcription factors (Yamamoto et al., 1995; Read et al., 1997; Marks-Konczalik et al., 1998; Ping et al., 1999; Roebuck, 1999).

The bifurcation of LPS-, IL-1- and TNF α -induced NF- κ B and AP-1 signaling cascades probably occurs immediately downstream of TRAF adapter proteins (TRAF2 for TNF α , TRAF6 for LPS and IL-1) and their associated effectors (for review, see Wajant et al., 2001). In transfection experiments, certain MAPK kinase kinases (e.g., MEKK1, MEKK3) have been shown to be able to phosphorylate and thereby activate both IKK α or IKK β and MKK4 or MKK7, finally leading to NF- κ B and AP-1 activation, respectively. However, it has been difficult to establish definitively a role in NF- κ B and AP-1 activation for either of these kinases under physiological conditions (for review, see Kyriakis and Avruch, 2001). An alternative mechanism of TRAF-dependent activation of NF- κ B was provided by Deng et al. (2000). They demonstrated in an in vitro assay that Ubc13 and Uev1A catalyze the synthesis of a lysine 63-linked polyubiquitin chain on TRAF6 (Deng et al., 2000), which enables TRAF6-mediated activation of TAK1 (Wang et al., 2001). Once activated, TAK1 can directly phosphorylate IKK β and MKK3/4/6, leading to the activation of both the JNK/p38 and NF- κ B signaling pathways (Wang et al., 2001; Takaesu et al., 2003). It is remarkable that IL-1-, TNF α - and TRAF2-mediated NF- κ B

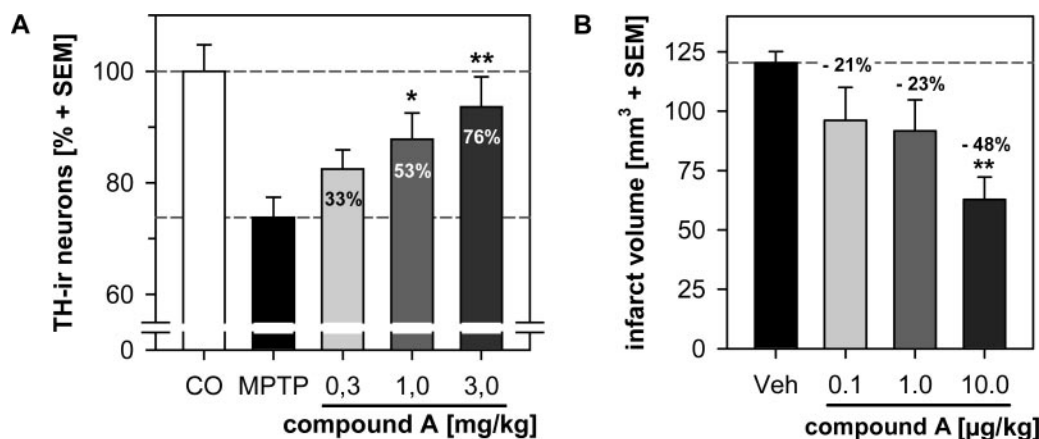


Fig. 8. Neuroprotection by compound A in the MPTP mouse model of Parkinson's disease (A) and in the rat SDH model of traumatic brain injury (B). A, male C57/BL6 mice ($n = 10$ /group) received daily injections of MPTP (4 mg/kg, i.p.) for 3 days. Animals were i.p. injected twice daily with the indicated doses of compound A or vehicle starting immediately before the first MPTP injection and lasting for 10 days. On day 11, mice were anesthetized, brains were removed, and TH-immunoreactive cell counts determined in the substantia nigra pars compacta. The relative percentage neuroprotection is indicated. *, $p < 0.05$; **, $p < 0.01$ compared with MPTP-treated controls (Fisher's LSD test). B, male Wistar rats were subjected to SDH and vehicle or compound A were administered by multiple i.v. bolus injections at the indicated doses immediately, 2 h, and 4 h after surgery ($n = 10$ animals/group). Seven days thereafter animals were killed by decapitation and the infarct volume was determined. The infarct volume and the relative percentage of neuroprotection are indicated. **, $p < 0.01$ compared with vehicle-treated animals (Fisher's LSD test).

activation were all blocked by expression of a dominant-negative, catalytically inactive Ubc13 (Deng et al., 2000). The important role of lysine 63-polyubiquitination in both TRAF2 and TRAF6 signaling has since then also been demonstrated by others (Shi and Kehrl, 2003; Tang et al., 2003; Sun et al., 2004). Whether compound A targets one of the above-mentioned activation mechanisms, however, remains to be determined. Support for an inhibitory activity upstream of IKK and downstream of TRAFs comes from the study by Baumann et al. (2002). Using transient transfections, they showed that the structurally related class of naturally occurring rocgamid derivatives almost completely blocked TRAF2-mediated NF- κ B activation in Jurkat T cells but only partially affect downstream elements of the signaling cascades when overexpressed. However, in their study, no effect of the respective rocgamids on AP-1 activity was observed.

Compound A did not inhibit expression of all NF- κ B regulated genes under investigation, such as ICAM-1 or MIP-2. For these, activation of IKK by alternative signaling cascades apart from the canonical TRAF-MAP3K pathway may be essential. One such pathway may comprise members of the protein kinase C family, in particular PKC- ζ , which has been shown to be critically involved in TNF α -induced ICAM-1 expression in endothelial cells (Rahman et al., 2000). Therefore, it is conceivable that the lack of inhibition of ICAM-1 or MIP-2 expression by compound A may be caused by the lack of inhibition of alternative signaling cascades culminating in IKK activation. Further experimental work is needed, however, to further clarify this issue.

A common feature of many acute and chronic neurodegenerative diseases, including traumatic and ischemic brain injury, AD, and PD, is the presence of a localized inflammatory tissue response and an accompanying activation of NF- κ B and stress-activated MAP kinases, in particular of JNK, both in neurons and glial cells (Hunot et al., 1997; Kaltschmidt et al., 1997; Schneider et al., 1999; Hirsch, 2000; Allan and Rothwell, 2001; Ferrer et al., 2001; Hoozemans et al., 2002; Zhu et al., 2003). JNK activation in turn causes an increase in c-Jun/AP-1 protein as well as in AP-1 activity (Tong et al., 2003). Because we found that compound A inhibited NF- κ B and AP-1 activity in glial cells in vitro, resulting in a dose-dependent reduction of NF- κ B and AP-1-triggered gene expression, the neuroprotective activity of compound A in a rat animal model of traumatic brain injury (SDH) and in the mouse MPTP animal model of PD was studied. In both cases significant neuroprotection was observed. Both conditions are characterized by early microglia and astroglia activation and increased production of pro-inflammatory cytokines, chemokines, and inflammatory mediators such as nitric oxide or arachidonic acid derivatives, all of which have been implicated in pathogenesis of acute and chronic neurodegeneration (Knott et al., 2000; Nagatsu et al., 2000; Teismann and Ferger, 2001; Basu et al., 2002; Hoozemans et al., 2002). Therefore, neuroprotective activity of compound A may be caused by its ability to inhibit the formation of these mediators of inflammation. In addition, compound A may directly protect the affected neuronal cell population from damage by inhibiting NF- κ B and AP-1 activation. In favor of such an additional direct effect of compound A on neuronal survival are our findings that the compound protected dopaminergic neurons from MPP⁺ toxicity in primary mesencephalic cultures. Others have shown that under these conditions, both

the JNK and NF- κ B signaling pathways are activated (Cassarino et al., 2000; Gearan et al., 2001). Whereas several reports suggest a pro-apoptotic and cell death-inducing activity of increased JNK and concomitant c-Jun/AP-1 activation, the precise role of NF- κ B within the nervous system is less clear (for review, see Karin and Lin, 2002). Several studies have found that NF- κ B activity facilitates neuronal survival, yet others report that NF- κ B activation is required for neuronal death. However, as shown by Pizzi et al. (2002), the balance between different NF- κ B/Rel proteins within a single neuron may account for either cell survival or cell death. Which NF- κ B dimer composition is activated may at least in part depend on the kind, duration, and strength (concentration) of the inducing stimulus.

In summary, herein we have characterized compound A as a potent inhibitor of IL-1 β , TNF α , and LPS signaling. Both, NF- κ B- and AP-1-induced transcriptional responses are inhibited, suggesting a common molecular target within the early receptor-mediated signal transduction cascades, upstream of IKK and MKK4/7. The neuroprotection elicited by compound A in animal models of brain damage underlines the importance of the respective signaling cascades in the pathogenesis of neurodegenerative diseases and establishes compound A as new therapeutic compound for treatment of brain injury.

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