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ORIGINAL ARTICLE

Neuroprotective arylpiperazine dopaminergic/serotonergic ligands suppress experimental autoimmune encephalomyelitis in rats

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Abstract

Arylpiperazine-based dopaminergic/serotonergic ligands exert neuroprotective activity. We examined the effect of arylpiperazine D_2/5-HT_{1A} ligands, N-{4-[2-(4-phenyl-piperazin-1-yl)-ethyl}-phenyl]-picolinamide (**6a**) and N-{3-[2-(4-phenyl-piperazin-1-yl)-ethyl]-phenyl}-picolinamide (**6b**), in experimental autoimmune encephalomyelitis (EAE), a model of neuroinflammation. Both compounds (10 mg/kg i.p.) reduced EAE clinical signs in spinal cord homogenate-immunized Dark Agouti rats. Compound **6b** was more efficient in delaying the disease onset and reducing the maximal clinical score, which correlated with its higher affinity for D_2 and 5-HT_{1A} receptors. The protection was retained if treatment was limited to the effector (from day 8 onwards), but not the induction phase (day 0–7) of EAE. Compound **6b** reduced CNS immune infiltration and expression of mRNA encoding the proinflammatory cytokines tumor necrosis factor, IL-6, IL-1, and GM-CSF, T_H1 cytokine IFN-γ, T_H17 cytokine IL-17, as well as the signature transcription factors of T_H1 (T-bet) and T_H17 (RORγt) cells. Arylpiperazine treatment reduced apoptosis and increased the activation of anti-apoptotic mediators Akt and p70S6 kinase in the CNS of EAE animals. The *in vitro* treatment with **6b** protected oligodendrocyte cell line OLN-93 and neuronal cell line PC12 from mitogen-activated normal T cells or myelin basic protein-activated encephalitogenic T cells. In conclusion, arylpiperazine dopaminergic/serotonergic ligands suppress EAE through a direct neuroprotective action and decrease in CNS inflammation.

Keywords: apoptosis, arylpiperazines, CNS inflammation, neuroprotection, oligodendrocytes.

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Abbreviations used: 6-OHDA, 6-hydroxydopamine; ADEM, acute disseminated encephalomyelitis; CFA, complete Freund's adjuvant; Con A, concanavalin A; EAE, experimental autoimmune encephalomyelitis; GM-CSF, granulocyte monocyte colony-stimulating factor; HE, hematoxylin–eosin; IFN, interferon; IL, interleukin; MBP, myelin basic protein; MNC, mononuclear cells; MS, multiple sclerosis; mTOR, mammalian target of rapamycin; NO, nitric oxide; S6K, p70S6 kinase; SCH, spinal cord homogenate; TGF, transforming growth factor; TNF, tumor necrosis factor; TUNEL, TdT-mediated dUTP nick-end labeling.

Biologically active compounds containing the arylpiperazine scaffold are a class of privileged structures which, due to their ability to act as dopamine and serotonine receptor ligands, hold promise as antipshychotic, antidepressant, and anxiolytic drugs (Lopez-Rodriguez *et al.* 1996; Taverne *et al.* 1998; Gonzalez-Gomez *et al.* 2003; Zajdel *et al.* 2013). Interestingly, arylpiperazine dopaminergic ligands are also endowed with neuroprotective ability. Aripiprazole, a clinically approved arylpiperazine-based atypical antipsychotic with partial dopaminergic/serotonergic agonist activity, has been shown to protect neurons from various toxic insults, both *in vitro* and *in vivo* (Cosi *et al.* 2005; Eren *et al.* 2007; Matsuo *et al.* 2010; Koprivica *et al.* 2011). Moreover, we have recently reported that in a series of 19 novel arylpiperazine-based dopaminergic/serotonergic ligands (Sukalovic *et al.* 2013), N-{4-[2-(4-phenyl-piperazin-1-yl)-ethyl]-phenyl}-picolinamide (**6a**) and N-{3-[2-(4-phenyl-piperazin-1-yl)-ethyl]-phenyl}-picolinamide (**6b**) were the most potent in exerting an *in vitro* neuroprotective activity against the free radical nitric oxide (NO) and neurotoxin 6-hydroxydopamine (6-OHDA) (Tovilovic *et al.* 2012, 2013). In addition, some arylpiperazine derivatives have displayed an anti-inflammatory activity, reducing the inflammation in carrageenan-induced hind paw edema model (Dundar *et al.* 2007), and inhibiting the production of proinflammatory cytokines tumor necrosis factor (TNF) and IL-1β in bacterial lipopolysaccharide-stimulated human leukocytes (Gouault *et al.* 2004). Based on the above data, it is plausible to assume that the combination of anti-inflammatory and neuroprotective action of arylpiperazines could potentially be useful in the treatment of inflammatory/autoimmune damage of the central nervous system (CNS).

The experimental autoimmune encephalomyelitis (EAE) is an animal model of brain inflammation with significant clinical and pathological similarities to the human CNS demyelinating diseases, including multiple sclerosis (MS) and acute disseminated encephalomyelitis (ADEM) (Javed and Khan 2014; Robinson *et al.* 2014). Following immunization with spinal cord homogenate or different myelin antigens, susceptible animals, usually rodents, develop lymphocyte infiltration of the CNS, an increase in blood-brain barrier permeability, and demyelination, all of which contribute to the flaccid paralysis as the most commonly observed clinical feature of the disease (Robinson *et al.* 2014). Depending on the antigen used and the genetic background, rodents can display a monophasic bout of EAE, a relapsing-remitting form, or chronic EAE. While chronic and relapsing-remitting forms of EAE may be more relevant for studying MS (Robinson *et al.* 2014), an acute monophasic EAE has been suggested to be far more similar to ADEM than MS (Sriram and Steiner 2005). Although EAE might not be a perfect mirror of human demyelinating diseases (Diem *et al.* 2007), it has been considered useful in understanding general immunological mechanisms and phenomena, as well as *in vivo* interactions between the immune and the nervous system (Farooqi *et al.* 2010). According to the generally accepted concept of EAE pathogenesis, encephalitogenic proteins prime and expand peripheral autoreactive $CD4^+$ T cells that migrate to the CNS and, upon reactivation by target antigen, secrete proinflammatory cytokines, thus producing inflammation and subsequent neurological signs (Kuerten and Lehmann 2011; Rangachari and Kuchroo 2013). While in some EAE models a demyelinating humoral immune response is more pronounced (Diem *et al.* 2007), the major culprits for causing neuroinflammation in EAE are interferon (IFN)-γ-producing T helper (T_H)1 and, most notably, interleukin (IL)-17-secreting T_H17 cells (El-behi *et al.* 2010; Petermann and Korn 2011). Recently, the atypical antipsychotic quetiapine, a dibenzodiazepine derivative containing piperazine ring and acting as dopamine/serotonine antagonist, has been found effective in reducing T-cell activation and subsequent CNS demyelination in a mouse model of EAE (Mei *et al.* 2012). However, the effect of arylpiperazines dopaminergic/serotonergic ligands on CNS inflammation/autoimmunity has not been explored so far.

In the present study, we investigated the potential therapeutic effect of arylpiperazines **6a** and **6b** in CNS inflammation in a rat model of acute EAE. Our results demonstrate that arylpiperazine treatment reduces CNS infiltration of immune cells and ameliorates clinical signs of EAE, through mechanisms possibly involving a direct protection of CNS from the immune-mediated damage.

Materials and methods

EAE induction and monitoring

Female 8–10 week-old Dark Agouti rats were obtained from the Institute for Medical Research, Military Medical Academy (Belgrade, Serbia). The rats were housed in the local animal facility under conventional conditions with laboratory chow and water *ad libitum*. The animals' health status was monitored and any infections were excluded according to Federation of European Laboratory Animal Science Associations (FELASA) guidelines (Nicklas *et al.* 2002). For EAE induction, the animals were immunized by intradermal injection in the hind footpad of 100 μL of emulsion made by a mixture of equal volumes of rat spinal cord homogenate (SCH) in phosphate-buffered saline (PBS) (50% w/v) and complete Freund's adjuvant (CFA; Difco Laboratories, Detroit, MI, USA). The rats were monitored daily for clinical signs of EAE, and scored according to the following scale: 0, no clinical signs; 1, flaccid tail; 2, hind limb paresis; 3, hind limb paralysis; 4, moribund state or death (Badovinac *et al.* 1998). The following clinical parameters were used: onset of EAE, the first day clinical signs were observed; cumulative EAE score, the sum of all daily clinical scores; and maximum EAE score, the highest clinical score observed during disease. At the appropriate time-points (day 14, day 21, or day 28), the rats were sacrificed by cervical dislocation and the tissues (spinal cord, lymph nodes) were collected for further analysis. The study was carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All animal

experiments were approved by the Local Ethics Committee for Animal Experimentation (School of Medicine, University of Belgrade), and all efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques, if available.

Treatment with arylpiperazines

Arylpiperazine compounds **6a** and **6b** were synthesized as previously described (Tovilovic *et al.* 2012). All arylpiperazines were stored at +4°C as 10 mM stock solutions in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA), and were diluted in PBS immediately before use. The animals were randomized into control and treatment groups (≤ 3 rats per cage). The rats in the treatment group were injected daily with arylpiperazines (10 mg/kg i.p.) in three different settings: (i) starting on the day of immunization until the end of the experiment (day 21 or 28), (ii) during first seven days, and (iii) starting from the day 7 post-immunization until the end of the experiment. Control animals were injected intraperitoneally with PBS containing the appropriate amount of DMSO in PBS.

Isolation and flow cytometry analysis of mononuclear cells (MNC) from spinal cord

Mononuclear cells (MNC) were isolated from the spinal cords of immunized rats using a slight modification of the standard protocol (Beeton and Chandy 2007). Spinal cords of animals perfused with sterile PBS were dissociated through a wire mesh, and centrifuged at 700 *g* for 2 min. The pellets were then resuspended in 40% Percoll (Sigma-Aldrich) and centrifuged on a discontinuous 40%/70% Percoll gradient at 850 g for 25 min. The spinal cord MNC were collected from the 40%/70% interface and washed two times (900 g for 5 min) in Roswell Park Memorial Institute 1640 medium (Sigma-Aldrich) supplemented with 2% rat serum. The purity of MNC was > 95%, as determined by May-Grünwald-Giemsa staining (not shown). Cells were counted and stained for the presence of CD3, CD8, CD11b (BD Biosciences, San Jose, CA, USA), and CD4 (BioLegend, San Diego, CA, USA), and concomitantly for the intracellular content of IFN-γ (BioLegend), IL-17 (eBioscience, San Diego, CA, USA), and IL-10 (BD Biosciences) by using the fixation/permeabilization kit and anti-rat monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PercP), allophycocyanin (APc), V450, PECy7 and AlexaFluor 647, following the manufacturer's instructions. Intracellular staining was performed after a 4-h stimulation with phorbolmyristate acetate (100 ng/mL) and ionomycin (400 ng/mL) in the presence of brefeldin A (5 μM) (all from Sigma-Aldrich). The stained cells were counted using a FACSAria III flow cytometer (BD Biosciences), and the results were analyzed with the FlowJo software (LLC, Ashland, OR, USA).

Real-time RT-PCR

Total RNA was extracted using TRIZOL and 1 μg of RNA was reversely transcribed with M-MuLV reverse transcriptase and random hexamers (all from Life Technologies, Carlsbad, CA, USA). The real-time PCR analysis was performed in a Realplex Mastercycler (Eppendorf, Hamburg, Germany), using TaqMan Master Mix and the following TaqMan primers and probes (Life Technologies): IL-1 (Rn00580432_m1), IL-12p35 (Rn00584538_m1), IL-23p19 (Rn00590334_g1), IL-6 (Rn01410330_m1), IL-17A (Rn01757168_m1), transforming growth factor (TGF)-β1 (Rn00572010_m1), IFN-γ (Rn00594078_m1), TNF (Rn99999017_m1), IL-1 receptor antagonist (IL-1Ra) (Rn02586400_m1), granulocyte-macrophage colony-stimulating factor (GMCSF) (Rn01456850_m1), IL-10 (Rn 00563409_m1), T-bet (Rn01461633_m1), RORγt (Rn01533717_g1), and Foxp3 (Rn01525092_m1). The geometric mean of threshold cycle (Ct) values of housekeeping genes (18s ribosomal RNA) was subtracted from the Ct values of target genes to obtain ΔCt, and the relative gene expression was determined as $2^{-\Delta Ct}$.

Immunoblotting

Tissues were lysed in lysis buffer (30 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40) containing 1 mM phenylmethylsulfonyl fluoride, 1 mM Na-orthovanadate, 10 mM NaF, and protease inhibitor cocktail (all from Sigma-Aldrich) on ice for 30 min, centrifuged at 14 000 *g* for 15 min at +4°C, and the supernatants were collected. Equal amounts of protein from each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Following incubation with primary rabbit antibodies against poly (ADP ribose) polymerase (PARP), phospho-Akt (Ser473), phospho-p70S6 kinase 1 (p70S6K; Thr389), phospho-mammalian target of rapamycin (mTOR; Ser2448), or actin (Cell Signaling Technology, Cambridge, MA, USA), and peroxidase-conjugated goat anti-rabbit IgG (Southern Biotech, Birmingham, AL, USA) as the secondary antibody, specific protein bands were visualized using the enhanced chemiluminescence reagent (GE Healthcare, Pollards Wood, UK). The results were quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Histological examination of MNC infiltration and apoptosis

Surgically removed specimens of rat spinal cords were fixed in 4% paraformaldehyde and embedded in paraffin. Tissue sections (5 μm thick) were deparaffinized in xylol, dehydrated in increasing alcohol concentrations, and stained with hematoxylin–eosin (HE). Digital images of HE sections were made on a Leica DM4000B LED (Leica Microsystems, Wetzlar, Germany) microscope equipped with a Digital Camera (Leica DFC295) and LAS 4.4 Software. Morphometric analysis of areas with mononuclear cell infiltrate was done in Fiji (ImageJ 1.49b) using 'analyze measurements' tool.

DNA fragmentation assay for apoptosis detection

To identify fragmented DNA as a marker of apoptotic cell death, a TdT-mediated dUTP nick-end labeling (TUNEL) assay was performed on paraformaldehyde-fixed, paraffin-embedded lumbar spinal cord sections. After deparaffinization and permeabilization with 0.2% Triton X-100, cells were treated with 3% bovine serum albumin and with TUNEL reaction mixture (Roche, Mannheim, Germany). Then the converter for alkaline phosphatase was applied, and Fast red (both from Roche) was used to visualize the signal for light microscopy. Apoptotic cells, displaying granular red nuclear staining were counted in five random medium-power fields (× 200) at each cell slide.

Co-cultivation experiments

Rat oligodendrocyte cell line OLN-93 (Richter-Landsberg and Heinrich 1996) was a kind gift from Marcus Kipp (Institute of

Neuroanatomy, Faculty of Medicine, Aachen, Germany). Cells were maintained in Dulbecco's modified eagle medium containing 5% fetal calf serum and 1% penicillin/streptomycin (all from Sigma-Aldrich). Rat pheochromocytoma PC12 cell line was obtained from the European Collection of Cell Cultures (Salisbury, UK) and grown in high glucose Dulbecco's modified eagle medium containing 10% horse serum (Sigma-Aldrich), 5% FetalClone III serum (Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin/streptomycin. Single cell suspensions of lymphocytes were obtained from the cervical lymph nodes of non-immunized or draining lymph nodes of SCH-immunized rats. OLN-93 or PC12 cells were incubated with lymphocytes in Corning Transwell 3460 system (Corning Inc. Life Sciences, Tewksbury, MA, USA). OLN-93 and PC12 cells were incubated in 12-well plate (3×10^5 cells/well), while lymphocytes (1×10^6) were seeded in permeable inserts with 0.4 μM pores. The cells were incubated with 10 μM of arylpiperazines for 1 h, followed by stimulation with T-cell mitogen Concanavalin A (Con A; 5 μg/mL) or guinea pig myelin basic protein (MBP; 10 μg/mL) (both from Sigma-Aldrich) for 48 or 72 h, respectively. The concentration of arylpiperazines (10 μM) was chosen based on a previous study in which it did not exert any toxic effects on human SH-SY5Y neuronal cell line (Tovilovic *et al.* 2012). Control cells were incubated with the appropriate amount of solvent (DMSO).

Determination of cell viability

At the end of the co-cultivation, the cell culture inserts were removed and the viability of OLN-93 and PC12 cells was determined by colorimetric assessment (570 nm) of mitochondrial dehydrogenase-mediated conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to purple formazan, as previously described (Mijatovic *et al.* 2004). The results are presented as % viability of the untreated control, which was set to 100%.

Statistical analysis

The differences in EAE clinical score were analyzed by mixed ANOVA with repeated measures, followed by Dunnett's test for multiple comparisons. In other experiments, Student's *t*-test or one-way ANOVA followed by Tukey's test was used to assess the differences between two or more independent groups, respectively. The data were presented as mean ± SEM values. A p value of < 0.05 was considered significant.

Results

Arylpiperazine treatment reduces clinical signs of EAE

The chemical structures of arylpiperazines **6a** and **6b** are presented in Fig. 1. Both compounds were first tested in non-immunized animals for the signs of acute toxicity (respiratory distress, impaired body weight gain, anorexia, weakness, apathy, and death), as well as for the effects that could interfere with the monitoring of EAE clinical signs (rigidity, sedation, and change of the tail tonus). The doses over 20 mg/kg induced abdominal writhing in some of the rats, so the dose of 10 mg/kg, which did not cause any obvious side effects, was chosen for further experiments. SCH/CFA-

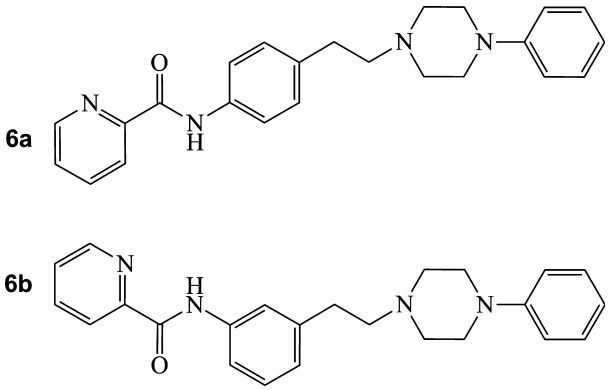

Fig. 1 Chemical structures of arylpiperazines **6a** and **6b**.

immunized animals developed classical monophasic acute disease with the symptom-free inductive phase (day 0–7 post-immunization) and the effector phase (from day 8 onwards, with the disease peak approx. at day 14), followed by recovery phase. The rats were treated with arylpiperazines daily from the day of EAE induction until the end of the experiment (day 28). As presented in Fig. 2a, both treatments significantly reduced the EAE clinical score at several time-points. The analysis of the body mass change demonstrated that arylpiperazine treatment prevented the disease-associated weight loss, thus further confirming its protective effect (Fig. 2b). An additional analysis revealed that the mean cumulative clinical score was significantly lower in arylpiperazine-treated rats compared to EAE controls (Table 1). The mean maximum score was also reduced by arylpiperazines, but the difference was statistically significant only for **6b** (Table 1.) Similarly, the disease onset was significantly delayed in rats treated with **6b**, while the difference was not significant for the treatment with **6a** (Table 1). Because of the better efficiency, **6b** was selected for the further study. Since the clinical score did not significantly change from day 22–28, the experiment duration was reduced to 21 day to minimize animal suffering.

Arypiperazine treatment exerts protection during the effector, but not inductive phase of EAE

To get some insight into the mechanisms of the observed protection in EAE, we administered **6b** during the inductive (day 0–7) or effector phase (from day 8 onwards) of the disease. In contrast to the continuous treatment (Fig. 2a), no protection was observed in rats treated with **6b** during the inductive phase of EAE (Fig. 2c). On the other hand, animals that received **6b** during the effector phase of EAE developed a significantly milder disease (Fig. 2d), as reflected by a significantly later mean onset (12.3 ± 0.3 vs. 11.3 ± 0.3), and lower mean cumulative score (8.4 ± 1.1 vs. 13.4 ± 1.2) and mean maximum score (1.3 ± 0.2 vs. 2.0 ± 0.2), compared to control EAE rats ($p < 0.05$). The observed

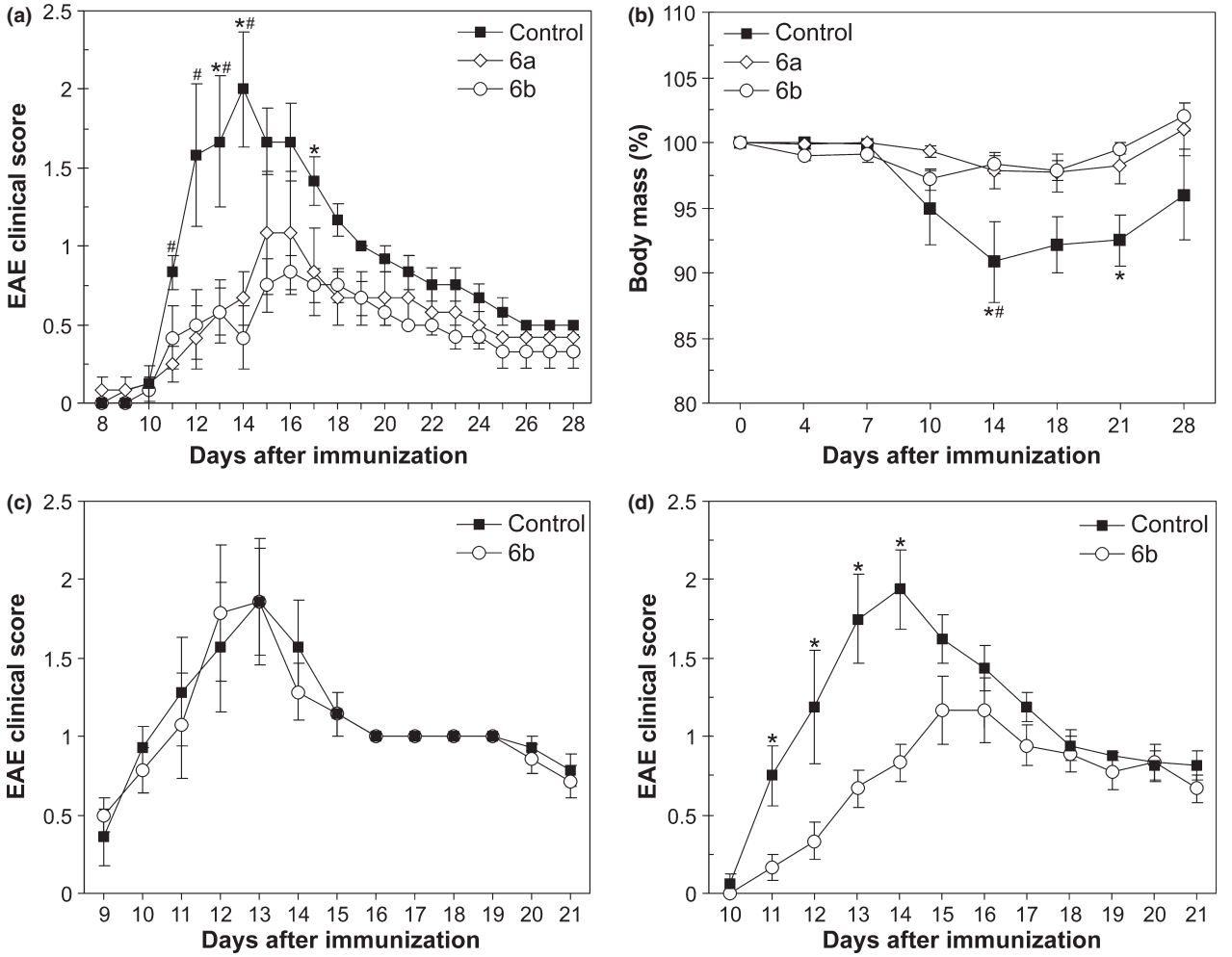

Fig. 2 Arylpiperazines reduce clinical signs of experimental autoimmune encephalomyelitis (EAE) by acting at the effector phase of the disease. (a and b) The rats were treated daily with phosphate-buffered saline (PBS)/DMSO (control; $n = 8$) or arylpiperazines (10 mg/kg; $n = 8$ per group) from the day of spinal cord homogenate (SCH)-complete Freund's adjuvant (CFA) immunization (day 0) until the end of experiment (day 28). The clinical signs (a) and body mass change (b) were monitored at the indicated time-points ($^{\#}p < 0.05$ and $^{*}p < 0.05$ vs. **6a** and **6b** treatment, respectively). (c and d) The rats immunized with SCH-CFA were treated daily with arylpiperazines (10 mg/kg) from the day 0–7 (c) or day 8–21 (d), and the clinical signs of the disease were recorded ($n = 8$ rats per group; $^{*}p < 0.05$ vs. **6b** treatment).

Table 1 The effect of arylpiperazines on EAE clinical features

	Cumulative clinical score	Maximum clinical score	Day of onset
Control	19.3 ± 2.0	2.1 ± 0.4	10.5 ± 0.2
6a	11.2 ± 2.0*	1.2 ± 0.4	11.8 ± 1.0
6b	9.5 ± 2.0*	0.8 ± 0.1*	12.7 ± 1.0*

The SCH-CFA-immunized rats were treated daily with PBS/DMSO (control; $n = 8$) or arylpiperazines **6a** or **6b** (10 mg/kg; $n = 8$ per group) for 28 days. The disease was monitored and the clinical parameters were calculated ($^{*}p < 0.05$).

protection during the effector, but not inductive phase of the disease indicates that arylpiperazines might interfere directly with the immune-mediated CNS damage, rather than activation/expansion of autoimmune cells at the periphery. Consistent with the delayed onset, the disease in arylpiperazine group reached its peak later than in control animals (day 15–16 vs. day 13–14, respectively; Fig. 2a, c and d). Nevertheless, we decided to perform further mechanistic analyses at the same time-point (day 14 post-immunization) in order for them to reflect not only the overall reduction, but also the delayed onset/progression of clinical signs.

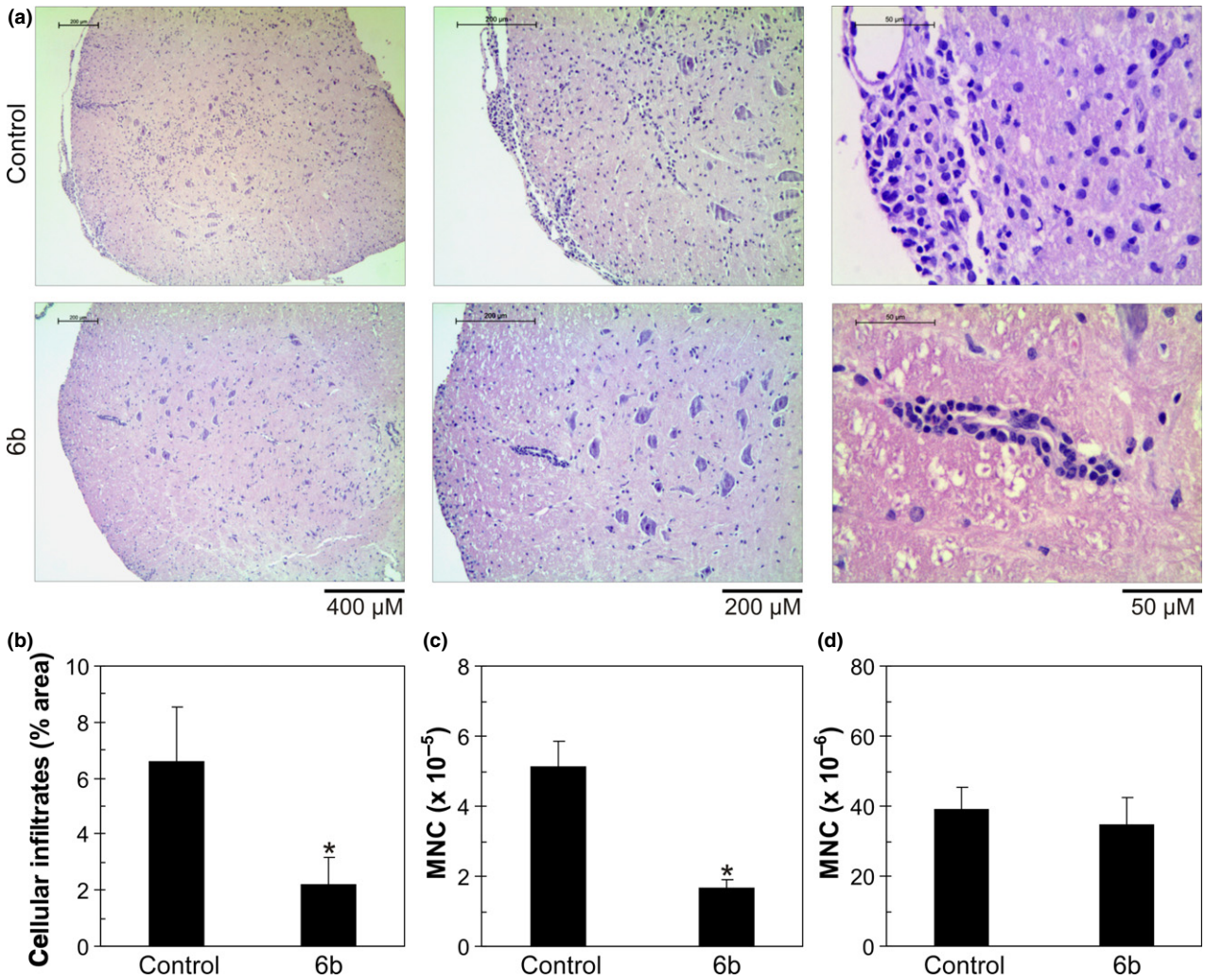

Fig. 3 Arylpiperazine treatment reduces CNS immune infiltration in experimental autoimmune encephalomyelitis (EAE). (a and b) At day 14 post-immunization, the spinal cord lumbar sections were analyzed by immunocytochemistry for the presence of immune cell infiltrates in spinal cord homogenate (SCH)-complete Freund's adjuvant (CFA)-immunized control ($n = 5$) and **6b**-treated rats (10 mg/kg/day; $n = 5$). The representative micrographs are shown in (a), while the results of the morphometric analysis are presented in (b) (*$p < 0.05$). (c and d) mononuclear cells (MNC) counts in the spinal cord perfusates (c) or draining (popliteal) lymph nodes (d) obtained from SCH-CFA-immunized control ($n = 5$) and **6b**-treated rats (10 mg/kg/day; $n = 5$) are presented (*$p < 0.05$).

Arylpiperazine treatment reduces CNS infiltration of immune cells in EAE

We next assessed the influence of arylpiperazine treatment on the size of immune cell infiltrates in the CNS of diseased animals. The microscopic analysis of HE-stained lumbar spinal cord sections, performed at day 14 post-immunization, revealed an extensive subpial/perivascular cellular infiltration in control animals with EAE (Fig. 3a). Inflammatory cells were also present in the spinal cord anterior horns and in the spinal cord white matter, while the analysis at higher magnification demonstrated that the infiltrates mainly consisted of MNC with sporadic neutrophils (Fig. 3a). On the other hand, perivascular cellular infiltrates, as well as those observed in the superficial parts of the white matter at day 14 of EAE induction, were clearly more discrete in rats treated with **6b** (Fig. 3a). Accordingly, the morphometric analysis demonstrated that the spinal cord area containing infiltrated cells was reduced in arylpiperazine-treated animals (Fig. 3b). To further confirm arylpiperazine-mediated reduction in the size of CNS immune infiltrates, animals were sacrificed at day 14 post-immunization, and MNC obtained by spinal cord perfusion were counted. In accordance with the histochemistry data, there was a significant reduction in the number of CNS-infiltrating MNC observed in **6b**-treated rats compared to the control group (Fig. 3c). On the other hand, there was no significant

change in the MNC number in the draining (popliteal) lymph nodes of **6b**-injected animals (Fig. 3d). The observed decrease in CNS immune infiltration is consistent with the delayed and reduced neurological symptomatology in arylpiperazine-treated EAE rats.

Arylpiperazine treatment reduces the expression of inflammatory mediators in the CNS tissue, but not CNS-infiltrated MNC in EAE

We next investigated how the arylpiperazine-mediated reduction in CNS immune infiltration in EAE correlates with the expression of inflammatory mediators/regulators in the CNS tissue. To that aim we analyzed the spinal cord levels of mRNA for the proinflammatory cytokines TNF, IL-6, IL-1, and GMCSF, T_H1 cytokines IL-12 and IFN-γ, T_H17 cytokines IL-23 and IL-17, anti-inflammatory/immunosuppressive mediators IL-10, TGF-β, and IL-1Ra, as well as signature transcription factors of T_H1 (T-bet), T_H17 (RORγt), and regulatory T cells (Foxp3). The real-time RT-PCR analysis revealed that the mRNA expression of all mediators, except IL-12 and IL-23, was significantly increased at day 14 in the spinal cords of rats with EAE, compared to non-immunized animals (data not shown). In accordance with the reduction of the disease severity in the same experiment (Fig. 4a), the treatment with **6b** significantly decreased the CNS levels of all investigated mRNAs, except those for IL-12 and IL-23 (Fig. 4b–d). In contrast to the results obtained with the whole spinal cord tissue containing the infiltrated MNC, no significant differences were observed between control and **6b**-treated animals when mRNA levels of proinflammatory/anti-inflammatory mediators were compared in MNC isolated from the spinal cord (Fig. 4e). Accordingly, the treatment with **6b** did not significantly alter the composition of MNC infiltrates obtained from the CNS at day 14 post-immunization. Namely, the flow cytometry analysis revealed that the frequencies of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD4^+$ cells producing IFN-γ, IL-10, or IL-17, or $CD8^+$ cells producing IFN-γ or IL-10, did not significantly differ in spinal cord MNC infiltrates of control, compared to **6b**-treated rats (Fig. 5). Therefore, the observed decrease in the expression of proinflammatory/anti-inflammatory mediators in the CNS of arylpiperazine-treated animals is due to a delay and decrease in MNC infiltration, rather than specific changes in gene expression or cellular composition of the infiltrates.

Arylpiperazine treatment reduces apoptosis and increases the activity of Akt/S6K signaling pathway in the CNS of EAE rats

To evaluate the effect of arylpiperazine treatment on the apoptotic death of CNS cells during EAE, we performed TUNEL assay for DNA fragmentation. While no apoptotic cells were observed in the spinal cord of healthy animals at day 14 post-immunization, the TUNEL-positive nuclei were found in cells located in the white mater of spinal cord samples from EAE animals (Fig. 6a). According to their position and size, the apoptotic cells were mostly glial cells, presumably oligodendrocytes. Consistent with the protective effect of arylpiperazines, TUNEL-positive cellular nuclei were significantly less frequent in the spinal cord sections from **6b**-treated EAE animals (Fig. 6a). The arylpiperazine-mediated reduction of apoptotic DNA damage was further confirmed by a decrease in PARP cleavage (Fig. 6b), which facilitates apoptotic demise by blocking the DNA-repairing activity of PARP, and serves as a marker of cells undergoing apoptosis (Oliver *et al.* 1998). We also examined the ability of arylpiperazine treatment to modulate phosphorylation of the anti-apoptotic kinase Akt and its downstream effectors mTOR and p70S6K in the CNS of rats with EAE. The immunoblot analysis of spinal cord homogenates demonstrated that **6b**-treated rats, compared to control EAE animals, displayed an increased phosphorylation of Akt and p70S6K at day 14 post-immunization, while the phosphorylation of mTOR was not significantly affected (Fig. 6). These data indicate that the activation of anti-apoptotic Akt/p70S6K signaling might contribute to the delayed onset/progression and overall suppression of EAE in arylpiperazine-treated animals.

Arylpiperazine treatment protects oligodendrocyte- and neuron-like cell lines from T-cell-dependent damage *in vitro*

As we have previously shown that arylpiperazine treatment exerts an *in vitro* neuroprotective activity against neurotoxins 6-OHDA and NO (Tovilovic *et al.* 2012, 2013), we examined if it can antagonize immune-mediated damage of oligodendrocytes and neurons. To address this issue, we employed a co-cultivation system in which rat oligodendrocyte cell line OLN-93 and rat PC12 neuron-like cell line were incubated with rat lymph node MNC stimulated with T-cell mitogen Con A or auto-antigen MBP. Both Con A-activated normal MNC and MBP-stimulated MNC from SCH/CFA-immunized rats markedly reduced the viability of OLN-93 and PC12 cells, as demonstrated by MTT assay (Fig. 7a–d). The treatment with **6b** significantly protected both cell lines from the damage induced by activated MNC (Fig. 7a–d), without affecting MNC expression of proinflammatory or anti-inflammatory mediators (Fig. 7e). Therefore, arylpiperazine treatment can directly protect oligodendrocytes and neurons from immune-mediated damage without exerting immunomodulatory activity.

Discussion

The present study for the first time demonstrates the ability of novel arylpiperazine derivatives to ameliorate immune-mediated CNS damage, delaying the onset/progression and reducing the maximal disease score in a rat model of EAE.

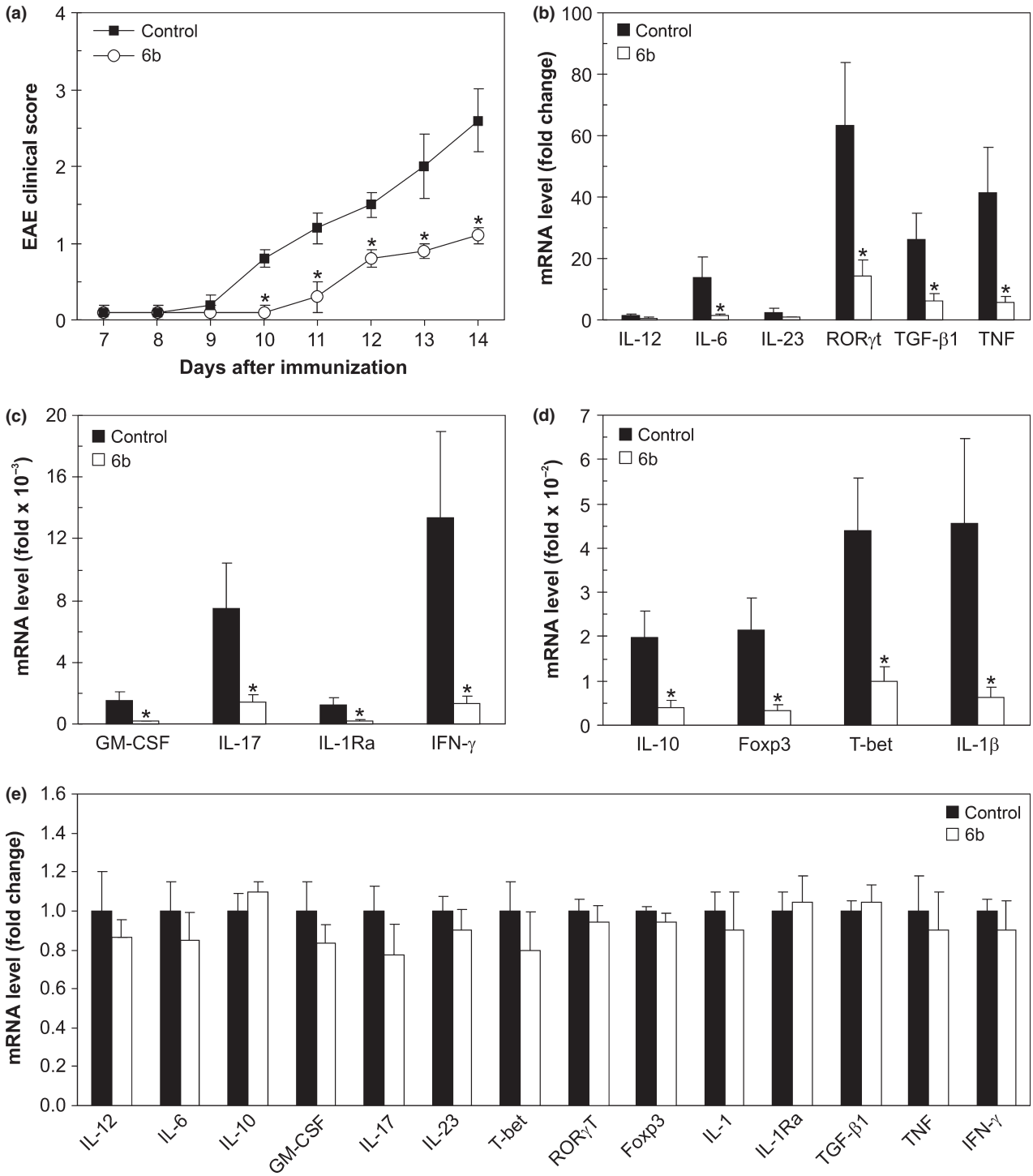

Fig. 4 Arylpiperazine treatment reduces the expression of mRNA for the inflammatory mediators in the CNS tissue, but not CNS-infiltrated mononuclear cells (MNC). (a–e) The rats were immunized with spinal cord homogenate (SCH)-complete Freund's adjuvant (CFA), receiving phosphate-buffered saline (PBS)/DMSO (control, $n = 8$) or **6b** (10 mg/kg/day, $n = 8$). The clinical signs were monitored until day 14 (a), when RNA was isolated for the RT-PCR determination of proinflammatory/anti-inflammatory mediators and transcription factors in the spinal cord lumbar sections (b–d) or CNS-infiltrated MNC (e) (*$p < 0.05$). The results are presented relative to the values obtained in untreated (b–d) or control experimental autoimmune encephalomyelitis (EAE) animals (e).

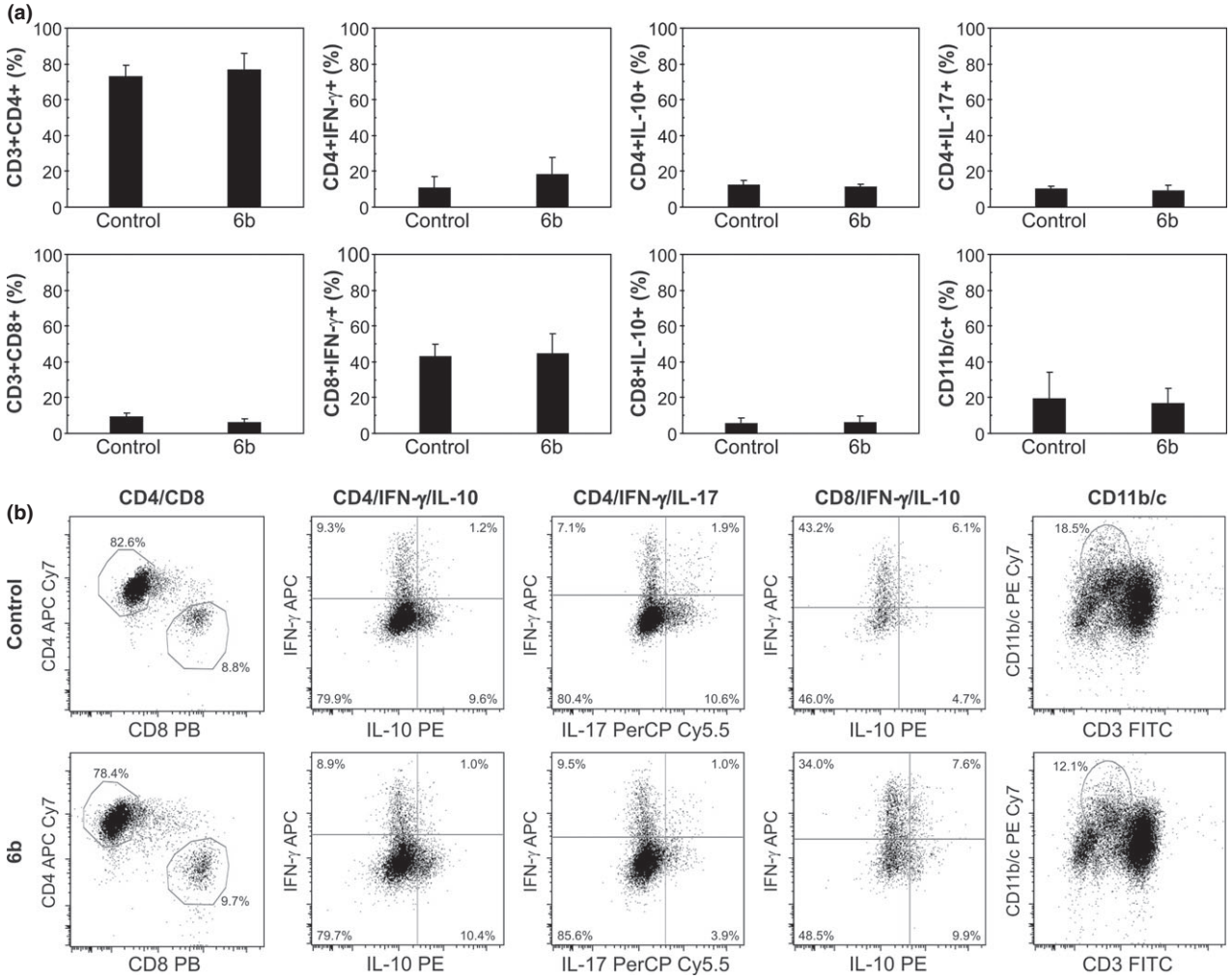

Fig. 5 Arylpiperazine treatment does not affect cellular composition of mononuclear cells (MNC) infiltrates and their cytokine production. (a and b) At day 14 post-immunization, the presence of various MNC subpopulations was quantified by flow cytometry in spinal cord infiltrates from spinal cord homogenate (SCH)-complete Freund's adjuvant (CFA)-immunized control and **6b**-treated rats (10 mg/kg/day; $n = 8$ per group; $^*p < 0.05$). The representative dot plots are shown in (b).

The protective effect was mediated by the reduction in CNS immune infiltration in the absence of any overt immunosuppressive/immunomodulatory action.

The protective effect in EAE of quetiapine, a dibenzodiazepine derivative with the piperazine ring, was apparently mediated through inhibiting the proliferation of autoimmune T cells (Mei *et al.* 2012). However, there is a line of evidence arguing against direct immunomodulatory action of arylpiperazines in our experiments. Firstly, the absence of the protective effect in the inductive phase indicates the inability of the drugs to interfere with the activation and/or expansion of the encephalitogenic cells at the periphery. Secondly, the results of the RT-PCR analysis of CNS-infiltrated or *in vitro* stimulated MNC confirm that arylpiperazines failed to affect the expression of crucial transcription factors and cytokines (TNF, IL-1, GMCSF, IFN-γ, IL-17) involved in CNS inflammation. Thirdly, the flow cytometry analysis of major encephalitogenic/regulatory immune cell populations clearly suggests that arylpiperazines did not act by altering the cellular composition of the inflammatory CNS infiltrates. Taken together, these data indicate that the protective effect of arylpiperazine derivatives was not due to a direct interference with peripheral activation/expansion, or target tissue reactivation of encephalitogenic T_H1 and T_H17 cells.

The most likely mechanism underlying the beneficial action of arylpiperazines in EAE is the non-selective reduction of immune cell infiltration into the CNS, affecting both encephalitogenic T_H1 and T_H17 cells, as well as those producing proinflammatory cytokines TNF, IL-6, GMCSF, and IL-1, or immunoregulatory/immunosuppressive IL-10, TGF-β, and IL-1Ra. Since the analysis of CNS infiltration

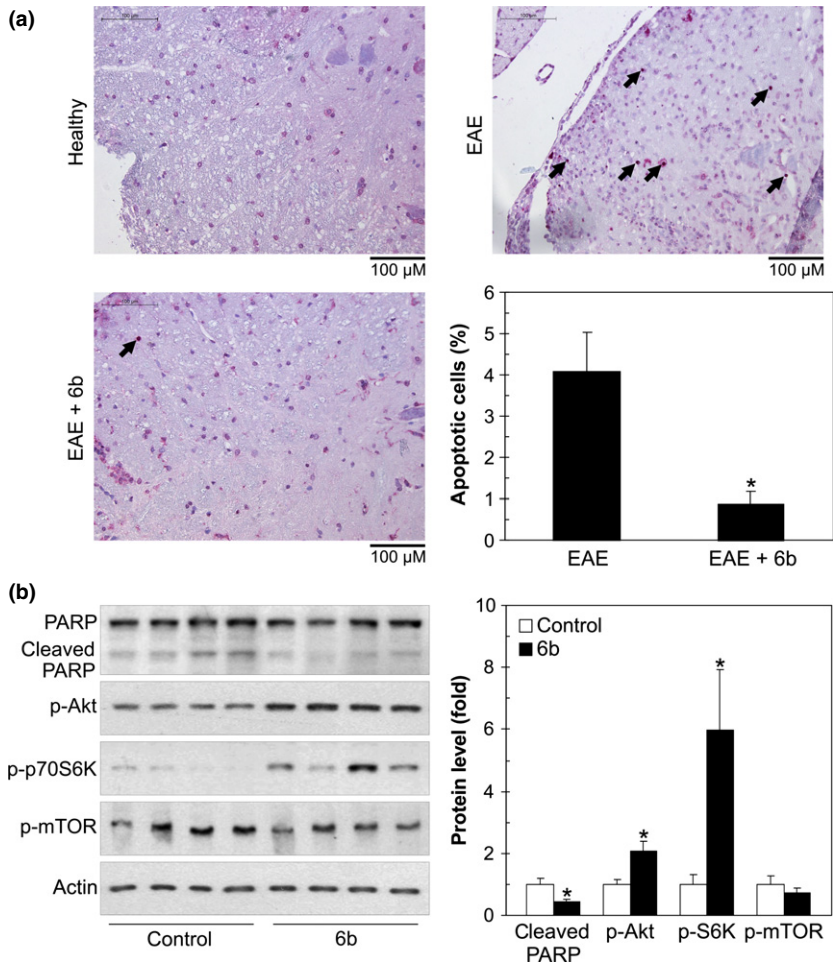

Fig. 6 Arylpiperazine treatment reduces apoptosis and increases Akt/p70S6K activity in the CNS of experimental autoimmune encephalomyelitis (EAE) rats. (a) Apoptotic nuclei (arrows) were detected by TdT-mediated dUTP nick-end labeling (TUNEL) staining in the spinal cord sections of healthy ($n = 3$), spinal cord homogenate (SCH)-complete Freund's adjuvant (CFA)-immunized control ($n = 5$), and **6b**-treated rats (10 mg/kg/day; $n = 5$) at day 14 post-immunization. The representative micrographs and the percentages of apoptotic cells are shown (*$p < 0.05$). (b) The cleavage of PARP and activation status (phosphorylation) of Akt, p70S6K, and mTOR at day 14 post-immunization were examined by immunoblotting in the lumbar spinal cord tissue from SCH-CFA-immunized control ($n = 4$) and **6b**-treated rats (10 mg/kg/day; $n = 4$). The densitometry data are presented relative to actin signals (*$p < 0.05$).

and cytokine expression in both control and arylpiperazine-treated group was performed at the same time-point, the observed differences presumably reflected not only the overall reduction, but also delayed onset/progression of neurological symptoms. As the arylpiperazine treatment was unable to affect the disease in the inductive phase or alter the expression of various inflammation-related molecules in the infiltrated cells, it seems plausible that the protective action was actually exerted in the target tissue rather than infiltrating cells themselves. For example, the possibility that arylpiperazines could affect the adhesion properties of CNS vascular endothelial cells and/or chemokine/proinflammatory cytokine production by CNS resident cells seems worthy of investigation. In addition, the observed anti-apoptotic action of arylpiperazines in the CNS of EAE animals, as well as our *in vitro* data, suggest that the direct protection of oligodendrocytes and neurons from immune-mediated damage might contribute to the reduced neuroinflammation. Namely, both oligodendrocyte and neuronal death have been observed in CNS during MS/EAE (Das *et al.* 2008a,b; Guyton *et al.* 2010; Herz *et al.* 2010; Fang *et al.* 2013), and the damage-associated molecular patterns released from injured CNS cells (heat shock proteins, adenine nucleotides, high-mobility group box 1, uric acid, and RNA), or formed in the extracellular matrix (hyaluronan fragments, tenascins, fibronectin, and sulfated proteoglycans), can perpetuate CNS inflammation (Miranda-Hernandez and Baxter 2013; Gaudet and Popovich 2014; Kigerl *et al.* 2014). It is therefore conceivable that the direct protection of oligodendrocytes and neurons by arylpiperazines, in addition to its immediate beneficial effect, contributed to a decrease in CNS inflammation by reducing the release of proinflammatory damage-associated molecular patterns.

There is a question of the molecular mechanism(s) underlying the arylpiperazine-mediated direct protection of CNS cells from the damage incurred by activated immune cells. We have recently reported that the activation of the prosurvival kinase Akt was responsible for arylpiperazine-mediated protection of SH-SY5Y cells exposed to a neurotoxic free radical NO (Tovilovic *et al.* 2012). Akt can block FOXO/p53-dependent transcription of 'death' genes, increase NF-κB-mediated transcription of survival genes, and directly inactivate pro-apoptotic Bcl-2 family members such as Bcl-2-associated death promoter (Brunet *et al.* 2001;

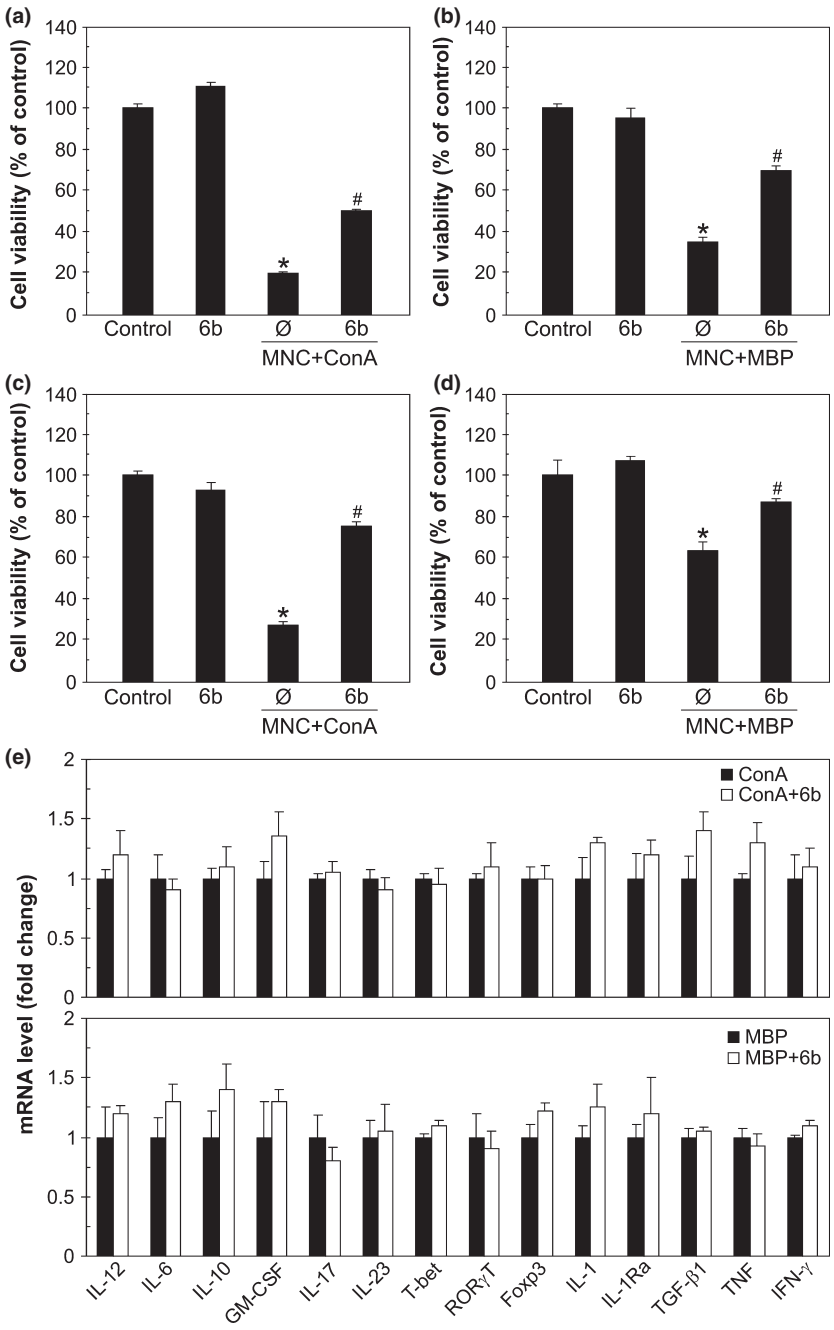

Fig. 7 Arylpiperazines protect oligodendrocyte and neuronal cell lines from T cell-dependent damage *in vitro*, without exerting immunomodulatory activity. (a–d) Rat oligodendrocyte cell line OLN-93 (a and b) and rat neuron-like cell line PC12 (c and d) were incubated with normal lymph node mononuclear cells (MNC) stimulated with T-cell mitogen Con A (5 μg/mL) (a and c), or draining lymph node from spinal cord homogenate (SCH)-complete Freund's adjuvant (CFA)-immunized rats, stimulated with CNS antigen myelin basic protein (MBP) (10 μg/mL) (b and d), in the presence or absence of **6b** (20 μM). The viability of OLN-93 and PC12 cells was determined by MTT assay, and the results of triplicate measurements from a representative of three experiments are presented (*$p < 0.05$ vs. control cells; $^{\#}p < 0.05$ vs. corresponding treatment without **6b**). (e) Lymph node MNC (5×10^6) from healthy or SCH-CFA-immunized rats ($n = 5$ per group) were stimulated with Con A (5 μg/mL) or MBP (10 μg/mL), respectively, and RNA was isolated for the RT-PCR determination of proinflammatory/anti-inflammatory mediators and transcription factors. The results are presented relative to the values obtained in cells treated with Con A or MBP alone.

Diem *et al.* 2007), while its downstream effector p70S6K maintains transcription and translation at the levels required for neuronal survival under apoptotic stress (Wu *et al.* 2004; Chen *et al.* 2010). In the present study, arylpiperazine treatment increased the phosphorylation of both Akt and p70S6K in the CNS of EAE rats. This is consistent with the recent finding that the engagement of estrogen receptor β on oligodendrocytes promoted remyelination in EAE through activation of Akt/p70S6K signaling (Khalaj *et al.* 2013; Kumar *et al.* 2013), and suggests that the activation of Akt and p70S6K in the CNS at least partly contributed to the beneficial effect of arylpiperazines in EAE. It should be noted that mTOR, the kinase that phosphorylates p70S6K upon activation by Akt (Magnuson *et al.* 2012), is required for the development of encephalitogenic T_H1 and T_H17 cells (Delgoffe *et al.* 2011). Accordingly, pharmacological inhibition of mTOR/S6K signaling with rapamycin suppress EAE by modulating both effector and regulatory T-cell function (Donia *et al.* 2009; Esposito *et al.* 2010). Therefore, it appears that mTOR/S6K axis might play a double role in neuroinflammation, promoting oligodendrocyte and neuronal survival on the one hand, while driving the pathogenic T-cell

responses on the other hand (Dello *et al.* 2013). Since arylpiperazine treatment in our study markedly reduced MNC infiltration, it is likely that CNS cells, rather than infiltrated T cells, were the source of the increased Akt/p70S6K activity in the CNS tissue. Interestingly, the activation of mTOR was not increased by arylpiperazine treatment, which could be explained by the ability of Akt to phosphorylate p70S6K independently of mTOR (Jaeschke *et al.* 2002), and/or by the involvement of other intracellular signaling pathways able to activate p70S6K independently of Akt/mTOR axis (Fang *et al.* 2007; Deguil *et al.* 2008; Liu *et al.* 2013). Nevertheless, arylpiperazine-mediated modulation of Akt/mTOR/p70S6K signaling in various types of CNS and immune cells, as well as its role in arylpiperazine protection from neuroinflammation, remain to be investigated in more detail.

Finally, it should be noted that the arylpiperazines investigated in the present study act as D_2 and 5-HT_{1A} receptor ligands (Sukalovic *et al.* 2013), displaying partial D_2 agonist activity (Tovilovic *et al.*, unpublished results). In the present study, a somewhat better efficiency of **6b**, reflected in the later onset and lower maximal disease score (Table 1), correlated with its higher binding affinities for D_2 (K_i 1400 and 71.6 nM for **6a** and **6b**, respectively) and 5-HT_{1A} receptors (K_i 304.9 and 2.4 nM) (Sukalovic *et al.* 2013). Therefore, it is possible that dopamine/serotonine receptor binding in arylpiperazine-mediated beneficial effects in neuroinflammation. This is consistent with the findings that D_2 partial agonist bromocriptine and the interference with serotonergic activity reduce CNS inflammation and clinical severity of EAE (Dijkstra *et al.* 1994; Freire-Garabal *et al.* 2003; Yuan *et al.* 2012), while D_2 antagonism exacerbates the disease (Nakano *et al.* 2008). On the other hand, the *in vitro* neuroprotective action of some dopaminergic ligands, including the arylipiperazines investigated here, was apparently independent of dopamine receptor binding (Ramirez *et al.* 2003; Gu *et al.* 2004; Matsuo *et al.* 2010; Tovilovic *et al.* 2012, 2013). Nevertheless, since dopaminergic dysfunction could contribute to anxiety and depression found both in EAE and MS (Feinstein 2007; Gentile *et al.* 2015), arylpiperazine-mediated interaction with dopaminergic/serotonergic signaling, even if not directly involved in the prevention of neuroinflammatory damage, might help alleviate the accompanying mood disturbances. It remains on future studies to clarify the exact role of different types of dopamine/serotonine receptors in the neuroprotective and behavioral effects of arylpiperazines in neuroinflammation.

In conclusion, our results indicate that arylpiperazine dopaminergic/serotonergic ligands suppress EAE through mechanisms involving Akt/p70S6K-mediated neuroprotection and subsequent decrease in CNS immune infiltration, rather than direct immunomodulatory action. These data support further exploration of arylpiperazine compounds as possible adjuncts to immune-targeted therapy of ADEM, MS, and other neuroinflammatory disorders.

Acknowledgments and conflict of interest disclosure

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