RESEARCH PAPER

Effects of zileuton and montelukast in mouse experimental spinal cord injury

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Background and purpose: 5-lipoxygenase (5-LO) is the key enzyme in leukotriene (LT) biosynthesis from arachidonic acid (AA). Here, we examined the role of the 5-LO-product, cysteinyl-LT (Cys-LT), with a 5-LO inhibitor (zileuton) and a Cys-LT, receptor antagonist (montelukast), in the inflammatory response and tissue injury associated with spinal cord injury (SCI).
Experimental approach: SCI was induced in mice by the application of vascular clips to the dura via a two-level T6 to T7 laminectomy for 1 min. Cord inflammation was assessed histologically and by measuring inflammatory mediators (ELISA) and apoptosis by annexin V, TUNEL, Fas ligand staining and Bax and Bcl-2 expression (immunohistochemistry and western blots). Motor function in hindlimbs was assessed by a locomotor rating scale, for 10 days after cord injury.
Key results: SCI in mice resulted in tissue damage, oedema, neutrophil infiltration, apoptosis, tumour necrosis-α (TNF-α) and cyclooxygenase-2 (COX-2) expression, prostaglandin E_2 (PGE$_2$) and leukotriene B_4 (LTB$_4$) production, and extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation in injured tissue. Treatment of the mice with zileuton or montelukast reduced the spinal cord inflammation and tissue injury, neutrophil infiltration, TNF-α, COX-2 and pERK1/2 expression, PGE$_2$ and LTB$_4$ production, and apoptosis. In separate experiments, zileuton or montelukast significantly improved the recovery of limb function over 10 days.
Conclusions and implications: Zileuton and montelukast produced a substantial reduction of inflammatory events associated with experimental SCI. Our data underline the important role of 5-LO and Cys-LT in neurotrauma.
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Introduction

Traumatic spinal cord injury (SCI) is an unexpected, catastrophic event, the consequences of which often persists for the life of the patient and influences not only the patient but also family members and society (Stover and Fine, 1987). It is known that SCI initiates a cascade of cellular and molecular events (Tator, 1995; Xu *et al.*, 2001; Hausmann, 2003; Beattie, 2004; Gris *et al.*, 2004) and that a progressive neuronal injury results from a combination of secondary injury factors, including ischaemia, biochemical alterations, apoptosis, excitotoxicity, neurotransmitter accumulation and lipid peroxidation/free radical injury (Carlson and Gorden, 2002). It is believed that inflammatory and immune responses are the major component of secondary injury and play a central role in regulating the pathogenesis of acute and chronic SCI (Anderson, 2002; Bethea and Dietrich, 2002; Yang *et al.*, 2004). Studies, mostly carried out *in vitro*, have disclosed a series of events related to the occurrence of neuronal damage following SCI: mitogen-activated protein kinase (MAPK) activation, initiation of inflammatory responses (liberation of pro-inflammatory mediators, such as nitric oxide, tumour necrosis factor-α (TNF-α), prostaglandins (PGs) and leukotrienes (LTs)) and degeneration of neurons. In particular, it has been reported that phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2)

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results in expression of genes mediating the inflammatory responses, such as those encoding for TNF-α and cyclooxygenase-2 (COX-2) (Jung *et al.*, 2005). Moreover, although the mechanisms are not fully understood, inflammatory responses leading to endothelial damage may be involved in the secondary injury process (Demopoulos *et al.*, 1978; Hamada *et al.*, 1996; Taoka *et al.*, 1997). In fact, neutrophils are the first leucocytes to arrive within the injured spinal cord (Carlson *et al.*, 1998; McTigue *et al.*, 1998) and can cause endothelial cell injury through the liberation of several inflammatory mediators (Xu *et al.*, 1990), such as cytokines (Profyris *et al.*, 2004), nitric oxide (Dietrich *et al.*, 2004), COX-2 (Adachi *et al.*, 2005) and 5-lipoxygenase (5-LO) metabolites (Suttorp *et al.*, 1987; Xu *et al.*, 1990; Mitsuhashi *et al.*, 1994). In particular, it has been demonstrated that neutrophil infiltration in SCI is proportional to both the magnitude of trauma and the amount of leukotriene B$_4$ (LTB$_4$), a product of 5-LO (Suttorp *et al.*, 1987; Xu *et al.*, 1990). An enhanced synthesis of leukotriene C$_4$ (LTC$_4$) in the injured spinal cord has also been reported (Mitsuhashi *et al.*, 1994; Nishisho *et al.*, 1996). For example, in the canine model, a significant increase in LTC$_4$ levels was observed and there was a good correlation between levels of LTC$_4$ in cerebrospinal fluid and the severity of neurological damage. Moreover, the time-dependent change in LTC$_4$ concentrations in seven patients with SCI was similar to that observed in the canine model (Nishisho *et al.*, 1996). Other 5-LO products are also involved in SCI, as 5-hydroxyeicosatetraenoic acid levels increase after experimental SCI in rabbits and it has been hypothesized that this increase may be involved in the pathophysiology of spinal cord trauma (Jacobs *et al.*, 1987; Shohami *et al.*, 1987).

One of the reasons why most of the treatments utilized in preclinical studies (Leker and Shohami, 2002) have had limited success in clinical trials is because of the complexity of the secondary degenerative response. In fact, many treatments affect only one aspect of this response, and a successful treatment will probably have to target several of these mechanisms (King *et al.*, 2006). A number of experimental animal models have been developed to simulate the pathophysiology of acute clinical spinal cord compression in humans (Beattie *et al.*, 2002). The most commonly used model is the compression model; in this model, injury is induced by applying either a weight or an aneurysm clip to the spinal cord (Tator and Fehlings, 1991). This experimental procedure is closer to the human situation in respect to the contusion model, since it replicates the persistence of cord compression that is commonly observed in human SCI (Tator, 1995). We have demonstrated in the compression model, by the use of 5-LO knockout mice, that the absence of this enzyme caused a substantial reduction of inflammatory events characteristic of SCI (Genovese *et al.*, 2005). As suppression of enzyme activity by gene deletion can give rise to compensatory mechanisms, we decided to investigate the role of 5-LO products using the same model of SCI. In particular, we examined cysteinyl-LTs (Cys-LTs) with a 5-LO inhibitor (zileuton) and a Cys-LT$_1$ receptor antagonist (montelukast).

Methods

Animals
Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (Ministerial Decree 116192) as well as with the European Economic Community regulations (Official Journal of E.C. L 358/1 12/18/1986). Male adult CD1 mice (25–30 g, Harlan Nossan, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water.

Spinal cord injury
Mice were anaesthetized using chloral hydrate (40 μg kg^{-1} body weight). A longitudinal incision was made on the midline of the back, exposing the paravertebral muscles. These muscles were dissected away, exposing T5–T8 vertebrae. The spinal cord was exposed via a two-level T6–T7 laminectomy, and SCI was produced by extradural compression of the spinal cord using an aneurysm clip with a closing force of 24 g. In all of the injured groups, the spinal cord was compressed for 1 min. Sham-injured animals were only subjected to laminectomy.

After surgery, 1 ml of saline was administered subcutaneously to replace the blood volume lost during the surgery. During recovery from anaesthesia, the mice were placed on a warm heating pad and covered with a warm towel. The mice were singly housed in a temperature-controlled room at 27 °C for a survival period of 10 days. Food and water were provided to the mice *ad libitum*. During this period, the bladders of the animals were manually voided twice a day until the mice were able to regain normal bladder function.

Experimental groups
Mice were randomly allocated into the following groups: (1) SCI + vehicle (saline solution), subjected to SCI and given vehicle i.p. 1 h and 6 h after injury ($n = 50$ mice, Table 1); (2) SCI + zileuton, subjected to SCI and given zileuton (50 mg kg^{-1} weight in saline solution p.o) 1 h and 6 h after injury ($n = 50$ mice, Table 1); (3) SCI + montelukast, subjected to SCI and given montelukast (5 mg kg^{-1} weight in saline i.p.) 1 and 6 h after injury ($n = 50$ mice, Table 1); (4)

Table 1 Number of animals used in the different experiments

Animal number (n)	Experiment type
10	Histological examination, immunohistochemical localization of MPO, TNF-α, Bax, Bcl-2 and FAS ligand, Annexin-V evaluation and TUNEL assay.
10	Myeloperoxidase activity
10	Measurement of TNF-α
10	Western blot analysis for Bax, Bcl-2, COX-2, pERK-1/2, ERK-2 and β-actin
10	Motor function
10	Measurement of PGE$_2$ and LTB$_4$

Abbreviation: ERK, extracellular signal-regulated kinase; LTB$_4$, leukotriene B$_4$; MPO, myeloperoxidase; PGE$_2$, prostaglandin E$_2$; TNF-α, tumour necrosis-α; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labelling.

sham + vehicle, subjected to a T6–T7 laminectomy and given vehicle i.p. 1 and 6 h after laminectomy ($n = 50$ mice, Table 1); (5) sham + zileuton, subjected to a T6–T7 laminectomy and given zileuton (50 mg kg^{-1}, p.o.) 1 and 6 h after laminectomy ($n = 50$ mice, Table 1) and (6) sham + montelukast, subjected to a T6–T7 laminectomy and given montelukast (5 mg kg^{-1}, i.p.) 1 h and 6 h after laminectomy ($n = 50$ mice, Table 1). In the experiments measuring motor function, the animals were treated with drugs 1 and 6 h after SCI daily until day 9.

At different time points, the animals ($n = 10$ mice from each group for each time point) were killed in order to evaluate the various parameters described below.

The dose of zileuton (50 mg kg^{-1}) was chosen according to our earlier studies (Failla *et al.*, 2006; Mazzon *et al.*, 2006), while the selected dose of montelukast (5 mg kg^{-1}) was chosen on the basis of published data (Saito *et al.*, 2004; Lee *et al.*, 2007).

Histological examination

Spinal cord biopsies were taken at 24 h following trauma. Tissue segments containing the lesion (1 cm on each side of the lesion) were paraffin embedded and cut into 5-µm-thick longitudinal sections. The tissue sections were deparaffinized with xylene, stained with haematoxylin/eosin and examined by light microscopy (Dialux 22 Leitz). The segments of each spinal cord were evaluated by an experienced histopathologist and all the histological studies were performed without knowledge of the treatments.

Grading of motor disturbance

The motor function of mice subjected to compression trauma was assessed once a day for 10 days after injury. Recovery from motor disturbance was graded using the modified murine Basso, Beattie and Bresnahan (BBB) (Basso *et al.*, 1995) hindlimb locomotor-rating scale (Joshi and Fehlings, 2002a, b). The following criteria were considered: 0 = no hindlimb movement; 1 = slight (<50% range of motion) movement of one to two joints; 2 = extensive (>50% range of motion) movement of one joint and slight movement of one other joint; 3 = extensive movement of two joints; 4 = slight movement in all three joints; 5 = slight movement of two joints and extensive movement of one joint; 6 = extensive movement of two joints and slight movement of one joint; 7 = extensive movement of all three joints; 8 = sweeping without weight support or plantar placement and no weight support; 9 = plantar placement with weight support in stance only or dorsal stepping with weight support; 10 = occasional (0–50% of the time) weight-supported plantar steps and no coordination (front/hindlimb coordination); 11 = frequent (50–94% of the time) to consistent (95–100% of the time) weight-supported plantar steps and no coordination; 12 = frequent to consistent weight-supported plantar steps and occasional coordination; 13 = frequent to consistent weight-supported plantar steps and frequent coordination; 14 = consistent weight-supported plantar steps, consistent coordination and predominant paw position is rotated during locomotion (lift off and

contact) or frequent plantar stepping, consistent coordination and occasional dorsal stepping; 15 = consistent plantar stepping and coordination, no/occasional toe clearance, paw position is parallel at initial contact; 16 = consistent plantar stepping and coordination (front/hindlimb coordination) and frequent toe clearance and predominant paw position is parallel at initial contact and rotated at lift off; 17 = consistent plantar stepping and coordination and frequent toe clearance and predominant paw position is parallel at initial contact and lift off; 18 = consistent plantar stepping and coordination and consistent toe clearance and predominant paw position is parallel at initial contact and rotated at lift off; 19 = consistent plantar stepping and coordination and consistent toe clearance and predominant paw position is parallel at initial contact and lift off; 20 = consistent plantar stepping, coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift off and trunk instability; 21 = consistent plantar stepping, coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift off and trunk stability.

Myeloperoxidase activity

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leucocyte (PMN) accumulation, was determined as previously described (Mullane *et al.*, 1985) 24 h after SCI. This time was chosen in agreement with other studies (Hamada *et al.*, 1996). At the specified time following SCI, spinal cord tissues were obtained and weighed and each piece homogenized in a solution containing 0.5% (w/v) hexadecyltrimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20 000 *g* at 4 °C. An aliquot of the supernatant was then allowed to react with a solution of 1.6 mM tetramethylbenzidine and 0.1 mM H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 µmol of peroxide min^{-1} at 37 °C and was expressed in U g^{-1} of wet tissue.

Immunohistochemical localization of MPO, TNF-α, Bax, Bcl-2 and FAS ligand

At 24 h after SCI, the tissues were fixed in 10% (w/v) phosphate-buffered saline (PBS)-buffered formaldehyde and 8 µm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeabilized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Nonspecific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin, respectively. Sections were incubated overnight with anti-MPO, anti-TNF-α, anti-Bax or anti-FAS ligand rabbit polyclonal antibody, and anti-Bcl-2 polyclonal antibody rat (1:500 in PBS, v/v). Sections were washed with PBS and incubated with secondary antibody. Specific labelling was detected with a

biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex. To verify the binding specificity for MPO, TNF-α, Bax, Bcl-2 and FAS ligand, some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations, no positive staining was found in the sections, indicating that the immunoreactions were positive in all of the experiments carried out.

Immunohistochemical photographs ($n = 5$ photos from each sample collected from each mice in each experimental group) were assessed by densitometry as described previously (Shea, 1994) by using Optilab Graftek software on a Macintosh personal computer.

Measurement of TNF-α

Portions of spinal cord tissues, collected at 24 h after SCI, were homogenized as previously described in PBS containing 2 mM of phenylmethyl sulphonyl fluoride to evaluate TNF-α tissue levels. The assay was carried out by using a colorimetric, commercial kit (Calbiochem-Novabiochem Corporation, CA, USA) according to the manufacturer's instructions. All TNFα determinations were performed in duplicate serial dilutions.

Annexin-V evaluation

The binding of annexin-V fluorescein isothiocyanate (FITC) (Ann-V) to externalized phosphatidylserine was used as a measurement of apoptosis in spinal cord tissue section with an Ann-V-propidium iodide (PI) apoptosis detection kit (Santa Cruz, DBA, Milan, Italy) according to the manufacturer's instructions. Briefly, normal viable cells in culture will stain negative for annexin-V FITC and negative for PI. Cells induced to undergo apoptosis will stain positive for annexin-V FITC and negative for PI as early as 1 h after stimulation (Schutte et al., 1998). Cells in later stages of apoptosis and necrotic cells will both stain positive for annexin-V FITC and PI. Sections were washed as before, mounted with 90% glycerol in PBS, and observed with an LSM 510 Zeiss laser confocal microscope equipped with a × 40 oil objective.

Terminal deoxynucleotidyltransferase-mediated UTP end labelling assay

These assays were conducted using a terminal deoxynucleotidyltransferase-mediated UTP end labelling assay (TUNEL) detection kit according to the manufacturer's instruction (Apotag, HRP kit; DBA srl). In brief, sections were incubated with 15 μg ml^{-1} proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was inactivated by 3% H_2O_2 for 5 min at room temperature and then washed with PBS. Sections were immersed in TdT buffer containing deoxynucleotidyl transferase and biotinylated deoxyuridine triphosphate in TdT buffer, incubated in a humid atmosphere at 37 °C for 90 min, and then washed with PBS. The sections were incubated at room temperature for 30 min with anti-horseradish peroxidase-conjugated antibody, and the signals were visualized with diaminobenzidine.

Total protein extraction and western blot analysis for Bax, Bcl-2, COX-2, pERK-1/2, ERK-2 and β-actin

Tissue samples from SCI-injured animals ($n = 10$) after 24 h were homogenized in a buffer containing 20 mM HEPES pH 7.6, 1.5 mM MgCl$_2$, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 15 μg ml^{-1} trypsin inhibitor, 3 μg ml^{-1} pepstatin, 2 μg ml^{-1} leupeptin, 40 μM benzamidine, 1% Nonidet P-40, 20% glycerol and 50 mM NaF. The homogenates were centrifuged at 10 000 g for 15 min and at 4 °C, and the supernatant was collected and protein concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories GmbH, Munchen). Equal amounts of protein (50 μg) were mixed with gel loading buffer (50 mM Tris, 10% sodium dodecyl sulfate, 10% glycerol, 10% 2-mercaptoethanol and 2 mg ml^{-1} bromophenol) in a ratio of 1:1, boiled for 3 min and centrifuged at 10 000 g for 10 min. Each sample was loaded and electrophoresed on a 10% sodium dodecyl sulfate–polyacrylamide gel. The proteins were transferred on to nitrocellulose membranes. The membranes were blocked with 0.1% PBS-Tween containing 5% non-fat dry milk for ERK-2, Bax, Bcl-2, COX-2 and β-actin, whereas with 0.1% PBS-Tween containing 5% non-fat dry milk and 50 mM NaF for pERK-1/2. After the blocking, the membranes were incubated with the relative primary antibody overnight at 4 °C. Rabbit monoclonal antibodies, anti-Bax, anti-bcl2 (Santa Cruz Biotechnology) and anti-COX-2 (Upstate cell signalling solutions, New York), and mouse monoclonal antibodies, anti-ERK-2 (Santa Cruz Biotechnology), were diluted 1:1000 in 0.1% PBS-Tween, 5% non-fat dry milk; mouse monoclonal antibody anti-pERK1/2 (Santa Cruz Biotechnology) was diluted 1:1000 in 0.1% PBS-Tween, 5% non-fat dry milk, 50 mM NaF; mouse monoclonal antibody anti-β-actin (Sigma-Aldrich, Milan, Italy) was diluted 1:10 000 in 0.1% PBS-Tween, 5% BSA. After the incubation, the membranes were washed six times with 0.1% PBS-Tween and were incubated for 1.5 h at room temperature with horseradish peroxidase-conjugated anti-mouse secondary antibodies (Dako Cytomation, Denmark) diluted 1:1000 in 0.1% PBS-Tween containing 5% non-fat dry milk. The membranes were washed and protein bands were detected by an enhanced chemiluminescence system (Amersham Pharmacia, Amersham Biosciences, UK). Densitometric analysis was performed with a Fluor S quantitative imaging system (Bio-Rad Laboratories).

Determination of PGE$_2$ and LTB$_4$ levels in mouse spinal cords

The spinal cords were collected at 24 h after SCI from the animals (two to six spinal cords were pooled together) and immediately homogenized in methanol. The mediators in the tissues were extracted in 5 ml methanol for 3–5 days at −80 °C. To remove the tissue residues, the extracts were centrifuged at 1800 g for 20 min at 4 °C. Then the sample solutions were dried to remove methanol. The dried samples were suspended in an assay buffer that was supplied with each ELISA kit (Assay Designs). To determine the levels of PGE$_2$ and LTB$_4$ within the sensitive (that is, linear) ranges of the standard curves, the samples were diluted a further 1- to 20-fold and assayed at multiple dilutions (Marusic et al., 2005).

Statistical procedures

All values in the figures are expressed as mean ± s.e.mean of *n* observations. For the *in vivo* studies, *n* represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative at least three experiments (histological or immunohistochemical staining) performed on different experimental days on the tissues section collected from all the animals in each group ($n = 10$). The results were analysed by one-way ANOVA followed by a Bonferroni *post hoc* test for multiple comparisons; $P < 0.05$ was considered significant. Basso, Beattie and Bresnahan scale data were analysed by the Mann–Whitney test and considered significant when $P < 0.05$.

Drugs and chemical reagents

Zileuton and montelukast were obtained from Sequoia Research Products (Oxford, UK). Biotin and avidin were obtained from DBA (Milan, Italy). The antibodies against Bax, bcl2, pERK1/2 and ERK-2 were obtained from Santa Cruz Biotechnology Inc. (Milan, Italy). The antibody against COX-2 was from Upstate (NY). All other reagents and compounds used were obtained from Sigma-Aldrich.

Results

Effects of zileuton and montelukast on the severity of spinal cord trauma
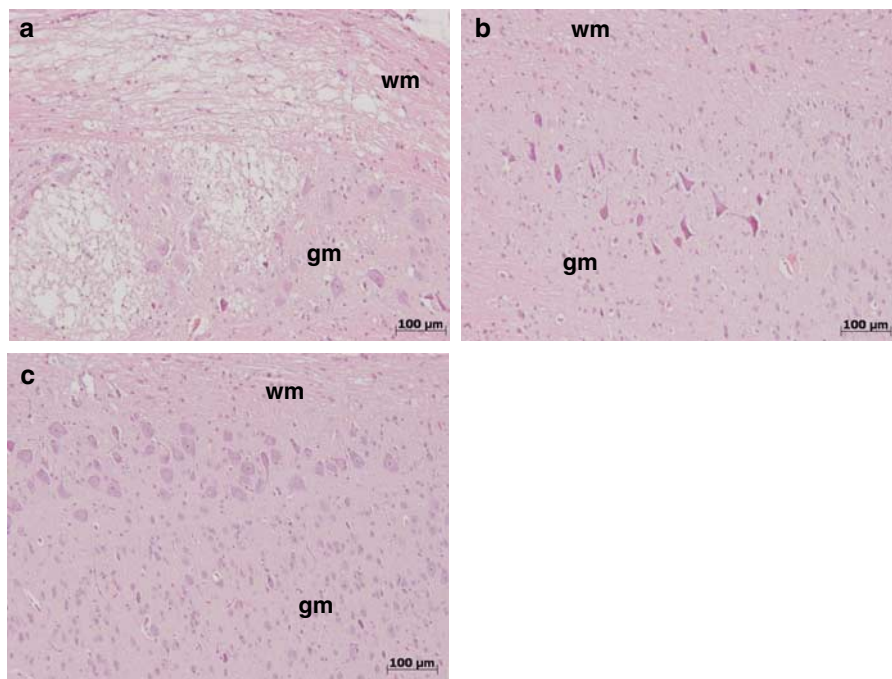
Twenty-four hours after the trauma, there was significant damage to the spinal cord at the perilesional zone, as assessed by the presence of oedema, as well as an alteration of the white matter observed in SCI + vehicle-treated mice (Figure 1a) when compared with spinal cord tissue collected from sham + vehicle-treated mice. A significant protection of the SCI was observed in the tissue collected from SCI + zileuton-treated mice ($50\,\mathrm{mg\,kg^{-1}}$ 1 and 6 h after injury) (Figure 1b) or SCI + montelukast-treated-mice ($5\,\mathrm{mg\,kg^{-1}}$ weight 1 and 6 h after injury) (Figure 1c).

To evaluate whether histological damage to the spinal cord was associated with a loss of motor function, the modified Basso, Beattie and Bresnahan hindlimb locomotor-rating scale score was evaluated. Although motor function was only slightly impaired in sham mice groups (Figure 2a), SCI + vehicle-treated mice had significant deficits in hindlimb movement (Figure 2b). In contrast, a significant ($P < 0.001$) amelioration of hindlimb motor disturbances was observed in SCI + zileuton-treated or montelukast-treated-mice (Figure 2b).

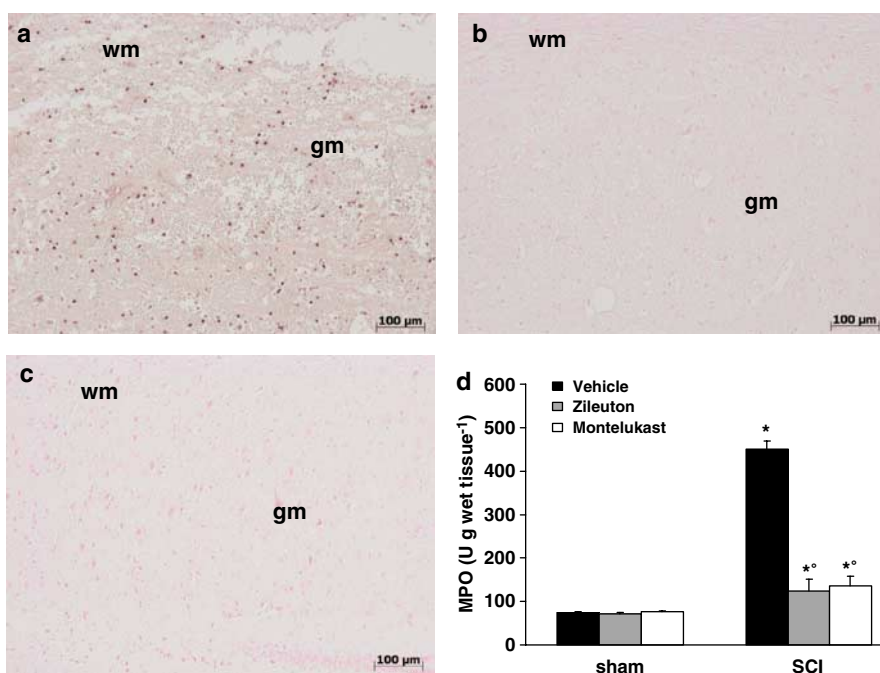## Effects of zileuton and montelukast on neutrophil infiltration

MPO activity was significantly ($P < 0.001$) elevated at 24 h after SCI in vehicle-treated mice compared with spinal cord tissue collected from sham + vehicle-treated mice (Figure 3d). In zileuton- or montelukast-treated mice, spinal cord MPO activity, at 24 h, was significantly ($P < 0.001$) attenuated compared with those of SCI + vehicle-treated animals. In addition, tissue sections obtained at 24 h from SCI + vehicle-treated mice demonstrated positive staining for MPO, mainly localized in the infiltrated inflammatory cells in the injured area (Figures 3a and 4). Moreover, in mice

Figure 1 Effect of zileuton and montelukast on histological alterations of the spinal cord tissue 24 h after injury. (**a**) Twenty-four hours after trauma, a significant damage to the spinal cord from SCI + vehicle-treated mice at the perilesional area was assessed by the presence of oedema and alteration of the white matter. A significant protection from the SCI was observed in the tissue collected from SCI + zileuton (**b**) or montelukast-treated mice (**c**). This figure is representative of at least three experiments performed on different experimental days. wm, white matter; gm, grey matter. No histological alteration was observed in the spinal cord tissues from sham-operated mice (data not shown). SCI, spinal cord injury.
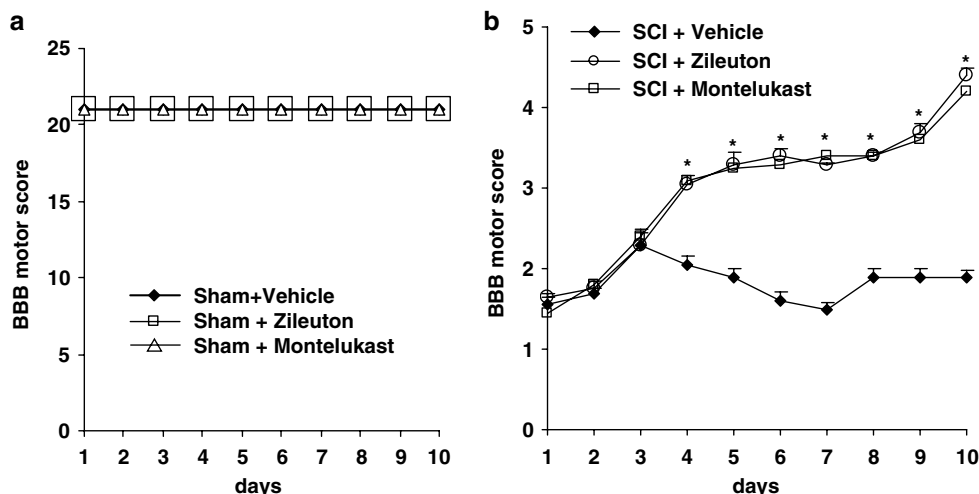
Figure 2 Effect of zileuton and montelukast on hindlimb motor dysfunction after SCI. The degree of motor dysfunction of (**a**) Sham groups and (**b**) SCI groups was assessed every day for 10 days after SCI induction by Basso–Beattie–Bresnahan criteria (Basso *et al.*, 1995). Treatment with zileuton or montelukast reduces the loss of motor function after SCI. Values shown are mean ± s.e.mean of 10 mice for each group. *$P < 0.01$ versus SCI + vehicle. SCI, spinal cord injury.


Figure 3 Effect of zileuton and montelukast on immunohistochemical localization and activity of MPO. A significant positive staining and a significant increase of MPO activity was observed in the spinal cord tissues collected from SCI + vehicle-treated mice (**a**, **d**). In mice treated with zileuton (**b**, **d**) or montelukast (**c**, **d**), the MPO staining and activity was visibly and significantly reduced in comparison with the SCI + vehicle-treated mice. No positive staining for MPO was observed in spinal cord tissues collected from sham-operated mice (data not shown). Image is representative of at least three experiments performed on different experimental days. Data are means ± s.e.mean of 10 mice for each group. *$P < 0.01$ versus sham-operated mice; °$P < 0.01$ versus SCI + vehicle-treated mice. wm, white matter; gm, grey matter; MPO, myeloperoxidase; SCI, spinal cord injury.

treated with zileuton (Figures 3b and 4) or montelukast (Figures 3c and 4), the staining for MPO was visibly reduced in comparison with that observed in SCI + vehicle-treated mice. There was no staining for MPO in spinal cord tissues obtained from the sham group of mice (data not shown).

Effect of zileuton and montelukast on TNF-α levels
Immunohistological analysis of the spinal cord after SCI was performed to determine whether zileuton and montelukast may modulate the secondary inflammatory reaction also through the regulation of the secretion of cytokines, such as TNF-α. A substantial increase of TNF-α (Figures 4, 5a and d)
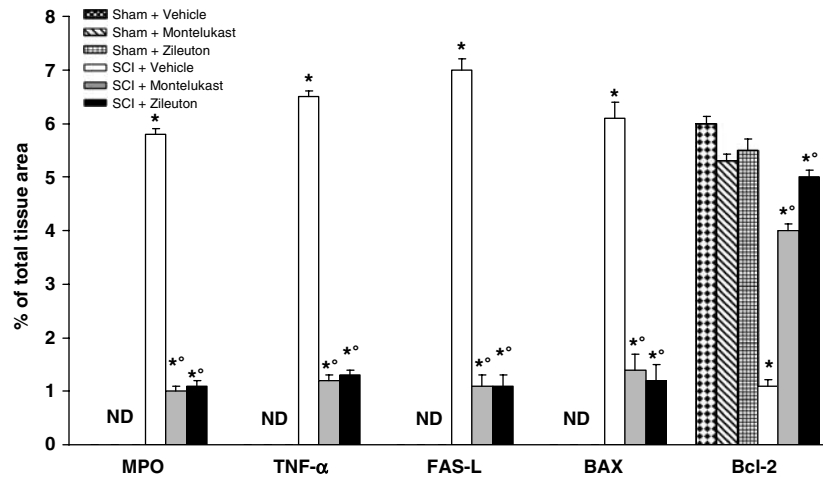
Figure 4 Typical densitometry evaluation. Densitometry analysis of immunocytochemistry photographs ($n = 5$ photos from each sample collected from all mice in each experimental group) for MPO, TNF-α, FAS-L, Bax and Bcl-2 from spinal cord tissues was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as % of total tissue area. *$P < 0.01$ versus Sham; °$P < 0.01$ versus SCI. ND, not detectable; MPO, myeloperoxidase; SCI, spinal cord injury; TNF-α, tumour necrosis-α.
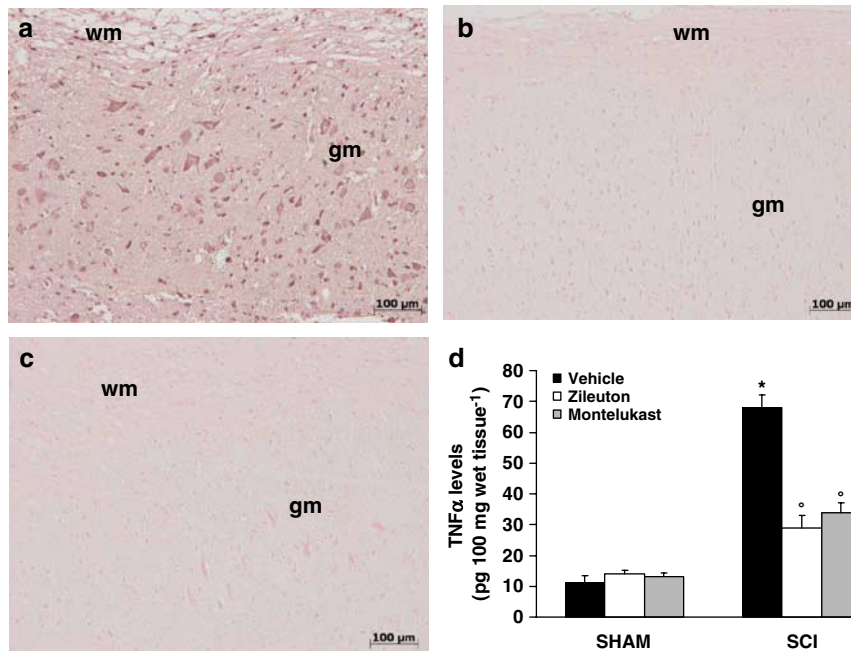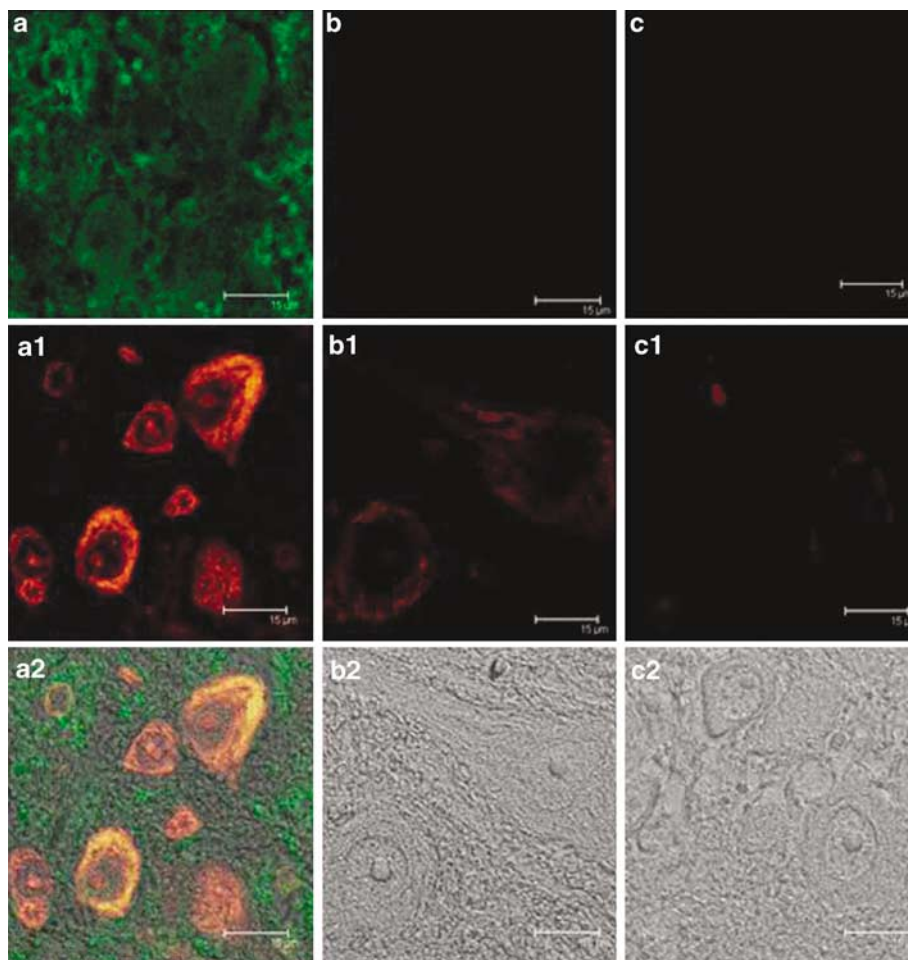

Figure 5 Effect of zileuton and montelukast on spinal cord levels and immunohistochemical localization of TNF-α. SCI caused, at 24 h, an increase in the release of TNF-α in spinal cord tissues (**a, d**). Treatment with zileuton (**b, d**) or montelukast (**c, d**) significantly inhibited the SCI-induced increase of TNF-α. No positive staining for TNF-α was observed in spinal cord tissues obtained from the sham group of mice (data not shown). Figure is representative of at least three experiments performed on different experimental days. wm, white matter; gm, grey matter. Data are means ± s.e.mean of 10 mice for each group. *$P < 0.01$ versus sham-operated mice; °$P < 0.01$ versus SCI + vehicle-treated mice. SCI, spinal cord injury; TNF-α, tumour necrosis-α.

formation was found in spinal cord samples collected from SCI + vehicle-treated mice at 24 h after SCI. Spinal cord levels of TNF-α were significantly attenuated in mice treated with zileuton or montelukast (Figures 4 and 5b–d). There was no staining for TNF-α in spinal cord obtained from sham groups of mice (data not shown).

Effect of zileuton and montelukast on apoptosis in spinal cord tissue after injury
To test whether the tissue damage was associated with apoptotic cell death, we measured annexin V staining in the

spinal cord tissues. Almost no apoptotic cells were detectable in the spinal cord tissue from sham groups of mice (data not shown). However, at 24 h after SCI, tissues obtained from SCI + vehicle-treated mice demonstrated a marked appearance of positive staining for annexin-V (Figure 6a), indicative of cells undergoing apoptosis. In addition, some cells showed a positive intracellular staining to PI, an index of cells in the late stage of apoptosis (Figure 6a1). On the contrary, spinal cord tissues section from mice treated with zileuton or montelukast demonstrated no cells in the earlier stage of apoptosis (Figures 6b and c, respectively) and a decreased

Figure 6 Effect of zileuton and montelukast on apoptosis. At 24 h after SCI, spinal cord tissues obtained from SCI + vehicle-treated mice demonstrated a marked appearance of positive stain for annexin V FITC (**a**), an index of cells that are induced to undergo apoptosis. Some cells showed a positive intracellular staining to PI (**a1**), an index of cells in the late stage of apoptosis. On the contrary, spinal cord tissues section from mice treated with zileuton or montelukast demonstrate no cells in the earlier stage (**b** and **c**, respectively) of apoptosis and fewer cells (positive to PI) in the later stages of apoptosis (**b1** and **c1**, respectively). (**a2**, **b2**, and **c2**) Staining combination of a and a1, b and b1, and c and c1, respectively. The figure is representative of at least three experiments performed on different experimental days. FITC, fluorescein isothiocyanate; PI, propidium iodide SCI, spinal cord injury.

presence of cells (positive for PI) in the later stages of apoptosis (Figures 6b1 and c1, respectively). The Figures 6a2, b2 and c2 staining are the combination of a and a1, b and b1, and c and c1, respectively.

Moreover, to confirm whether tissue damage was associated with apoptotic cell death, we also measured TUNEL-like staining in the perilesional spinal cord tissue. Almost no apoptotic cells were detectable in the spinal cord tissue from sham-operated mice (data not shown). At 24 h after the trauma, tissues obtained from SCI + vehicle-treated mice demonstrated a marked appearance of dark-brown (TUNEL-positive) apoptotic cells and intercellular apoptotic fragments (Figure 7a). In contrast, tissues obtained from mice treated with zileuton (Figure 7b) or montelukast (Figure 7c) contained a smaller number of TUNEL-positive cells. Section D demonstrates the positive staining in the Kit-positive control tissue.

Immunohistological staining for FAS Ligand in the spinal cord was also determined 24 h after injury. Sections of spinal cord from sham + vehicle-treated mice did not stain for FAS Ligand (data not shown), whereas spinal cord sections

obtained from SCI + vehicle-treated mice exhibited positive staining for FAS Ligand (Figures 4 and 8a) mainly localized in inflammatory cells as well as in nuclei of Schwann cells in the white (wm) and grey (gm) matter of the spinal cord tissues. Treatment of mice subjected to SCI with zileuton (Figures 4 and 8b) or montelukast (Figures 4 and 8c) reduced the degree of positive staining for FAS Ligand in the spinal cord. There was no staining for FAS Ligand in spinal cord tissues obtained from the sham group of mice (data not shown).
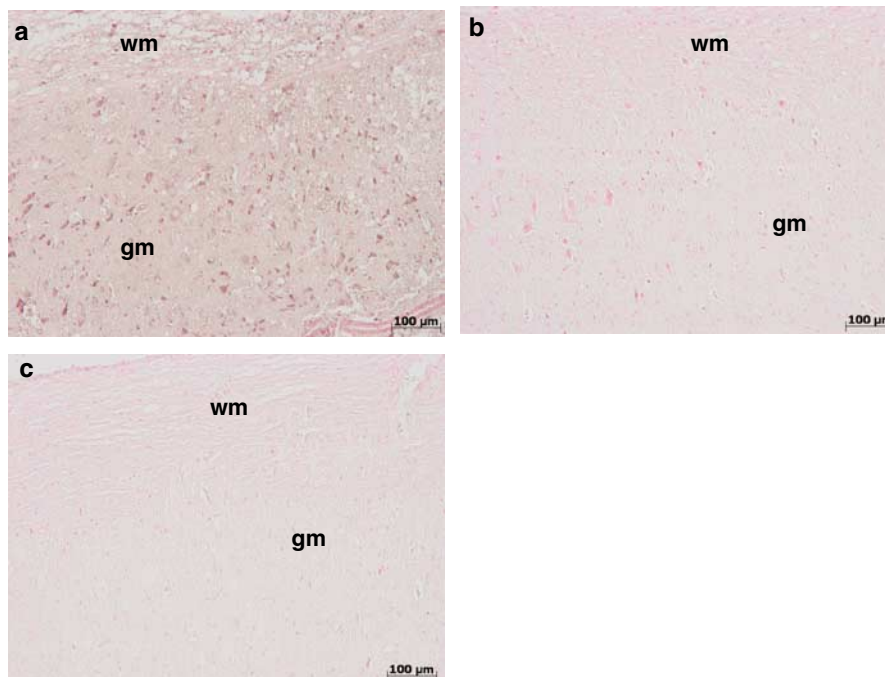
Effect of zileuton and montelukast on Bax and Bcl-2 immunohistochemistry and expression

Spinal cord tissues were taken at 24 h after SCI to determine the immunohistological staining for Bax and Bcl-2. Sections of spinal cord from sham + vehicle-treated mice showed a negative stain for Bax (data not shown), whereas those obtained from SCI + vehicle-treated mice exhibited positive staining for Bax (Figures 4 and 9a). The degree of positive staining for Bax was reduced in spinal cord tissues collected
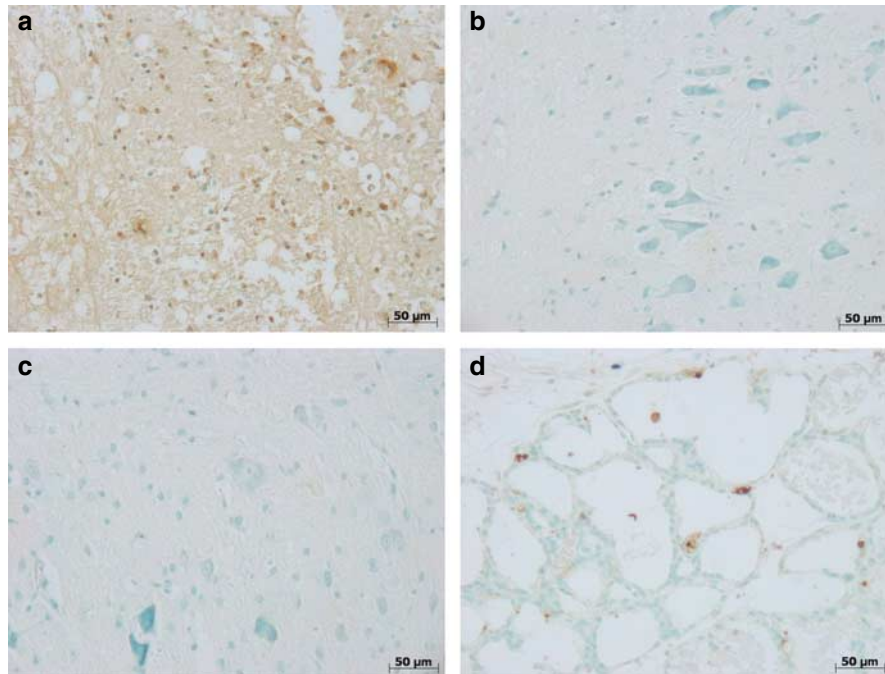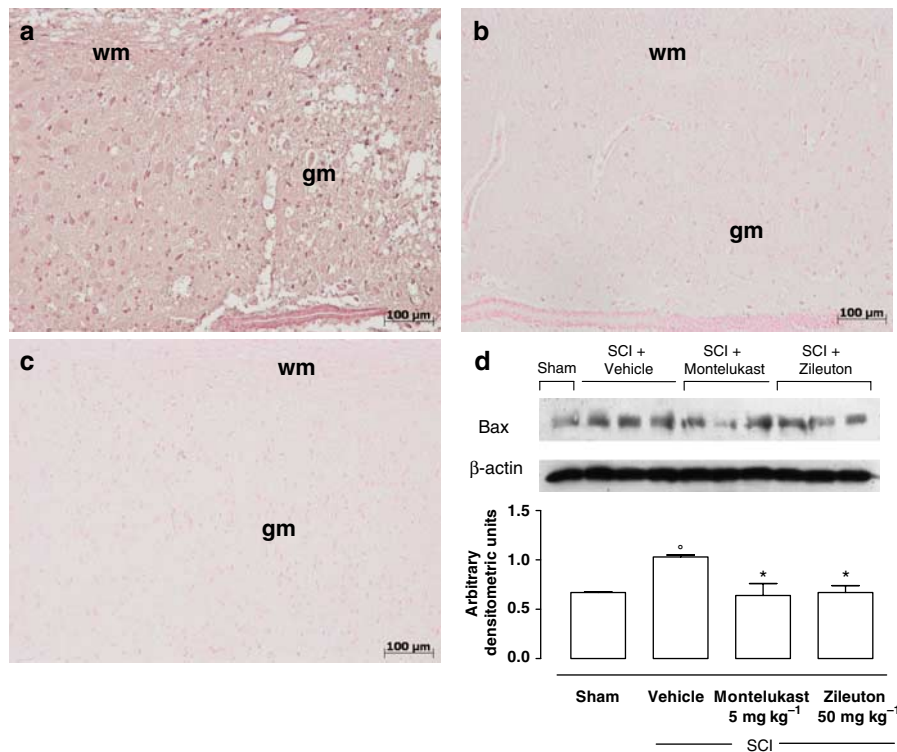
Figure 7 Effect of zileuton and montelukast on TUNEL staining in perilesional spinal cord tissue. At 24 h after the trauma, tissues obtained from SCI + vehicle-treated mice demonstrated a marked appearance of dark-brown apoptotic cells and intercellular apoptotic fragments (**a**). In contrast, tissues obtained from mice treated with zileuton (**b**) or montelukast (**c**) demonstrated a small number of apoptotic cells or fragments. Almost no apoptotic cells were detectable in the spinal cord tissue of sham-operated mice (data not shown). Panel **d** demonstrates the positive staining in the Kit-positive control tissue. The figure is representative of at least three experiments performed on different experimental days. SCI, spinal cord injury; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labelling assay.

Figure 8 Effect of zileuton and montelukast on immunohistological staining for FAS Ligand. At 24 h after the trauma spinal cord sections obtained from SCI + vehicle-treated mice exhibited positive staining for FAS Ligand (**a**) mainly localized in inflammatory cells as well as in nuclei of Schwann cells in the white (wm) and grey (gm) matter of the spinal cord tissues. Treatment of mice subjected to SCI with zileuton (**b**) or montelukast (**c**) reduced the degree of positive staining for FAS Ligand in the spinal cord. There was no staining for FAS Ligand in spinal cord tissues obtained from the sham group of mice (data not shown). SCI, spinal cord injury.

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Figure 9 Effect of zileuton and montelukast on Bax levels. At 24 h, SCI caused an increase of Bax expression in vehicle-treated mice (**a**). On the contrary, the degree of positive staining for Bax was reduced in spinal cord tissues collected from mice treated with zileuton (**b**) or montelukast (**c**). No positive staining for Bax was observed in the tissue section from sham-operated mice (data not shown). Figure is representative of at least three experiments performed on different experimental days. (**d**) Western blot analysis of extracts of spinal cord tissue collected at 24 h after injury. Basal levels of Bax was present in the tissue from sham-operated mice. Bax band is more evident in the tissue from SCI + vehicle-treated mice. The treatment with zileuton or montelukast reduced Bax levels. Data were normalized on the basis of β-actin levels. Densitometric analysis of protein expression represents the mean ± s.e.mean of 10 spinal cord tissues. $°P < 0.01$ versus sham-operated mice; $*P < 0.05$ versus SCI + vehicle. wm, white matter; gm, grey matter; SCI, spinal cord injury.

from mice treated with zileuton (Figures 4 and 9b) or montelukast (Figures 4 and 9c). Moreover, the appearance of Bax in homogenates of spinal cord tissues was investigated by western blot at 24 h after SCI. A basal level of Bax was detectable in the homogenized spinal cord tissues from sham + vehicle-treated animals (Figure 9d). Bax levels were significantly increased in the spinal cord tissues of SCI + vehicle-treated mice (Figure 9d). The treatment of mice with zileuton or montelukast prevented the SCI-mediated Bax expression (Figure 9d).

In addition, sections of spinal cord from sham-operated mice demonstrated positive staining for Bcl-2 (data not shown). Spinal cord sections obtained from SCI + vehicle-treated mice exhibited less staining for Bcl-2 (Figures 4 and 10a). The loss of positive staining for Bcl-2 was significantly reduced in spinal cord tissues collected from mice treated with zileuton (Figures 4 and 10b) or montelukast (Figures 4 and 10c). The same results were observed by western blot analysis. In fact, a high basal level of Bcl-2 expression was detected in spinal cord homogenates from the tissue of sham + vehicle-treated mice (Figure 10d). The Bcl-2 expression was significantly diminished in whole extracts obtained from spinal cord tissues of vehicle-treated mice 24 h after SCI. Treatment of mice with zileuton or montelukast significantly increased the expression of Bcl-2 (Figure 10d).
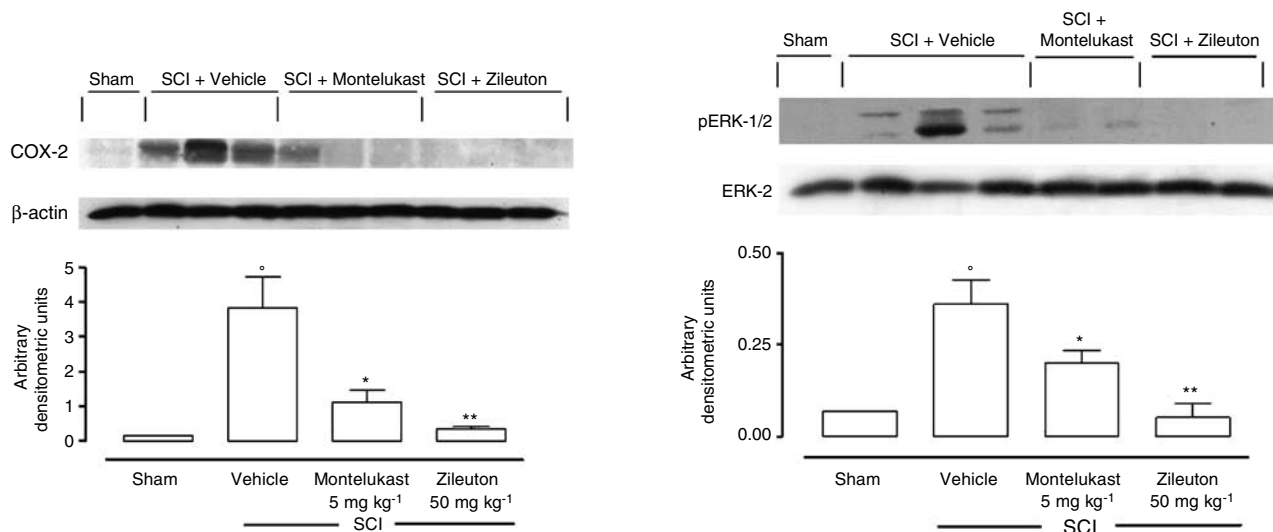
Effect of zileuton and montelukast on COX-2 expression and on activation of MAPK signal transduction pathway

As phosphorylation of ERK1/2 results in expression of genes, such as that encoding for COX-2, mediating the inflammatory responses characteristic of SCI, the expression of this enzyme and ERK1/2 phosphorylation in homogenates of spinal cord tissues was investigated by western blot at 24 h after SCI. A basal level of COX-2 was detectable in the homogenized spinal cord tissues from sham-operated animals (Figure 11). COX-2 levels were significantly increased in the spinal cord tissues of SCI + vehicle-treated mice. The treatment of mice with zileuton or montelukast reduced the SCI-mediated COX-2 expression (Figure 11).

To investigate whether the increase in COX-2 expression, observed in our experimental conditions, corresponded to an activation of signal transduction pathways involved in the regulation of COX-2 expression, we analysed the activation of MAPK pathways in particular the phosphorylation of ERK1/2 24 h after SCI. As shown in Figure 11, pERK1/2 levels were significantly increased in the spinal cord tissues of SCI + vehicle-treated mice. The treatment of mice with zileuton or montelukast significantly reduced the level of pERK1/2 (Figure 12).
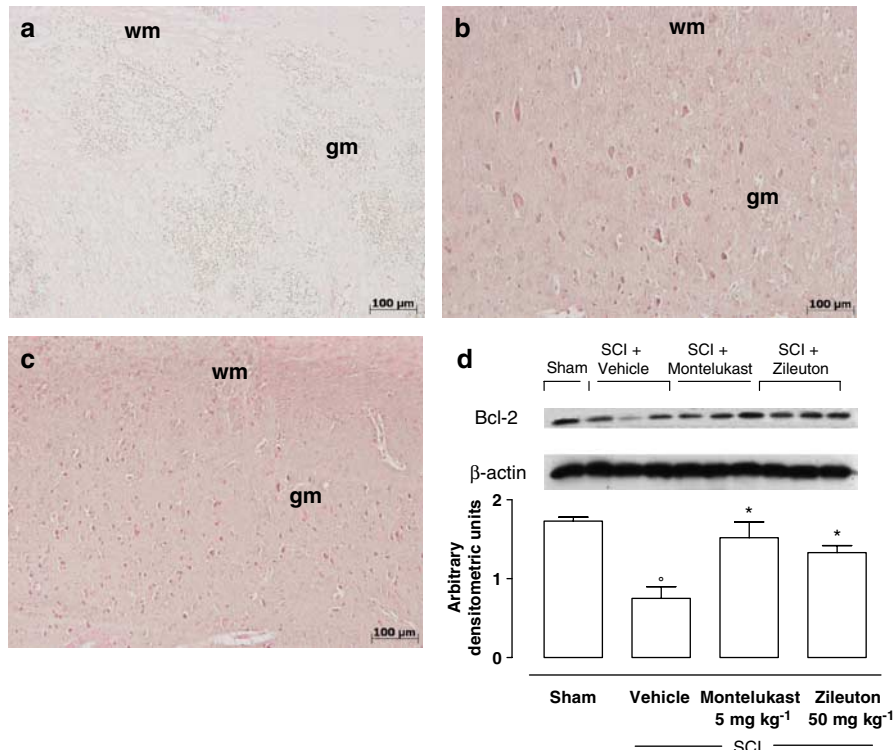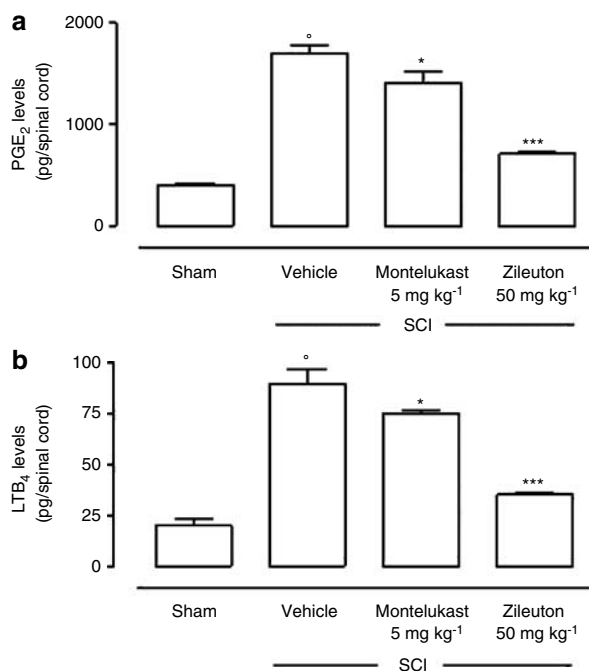
Figure 10 Effect of zileuton and montelukast on the Bcl-2 levels. At 24 h, SCI caused a decrease of the release of Bcl-2 expression in vehicle-treated mice (a). The degree of positive staining for Bcl-2 was increased in spinal cord tissues collected from mice treated with zileuton (b) or montelukast (c). Figure is representative of at least three experiments performed on different experimental days. (d) Western blot analysis of extracts of spinal cord tissue collected at 24 h after injury. Basal levels of Bcl-2 were present in the tissue from sham-operated mice. Bcl-2 band is less evident in the tissue from SCI + vehicle-treated mice. The treatment with zileuton or montelukast increased Bcl-2 levels. Data were normalized on the basis of β-actin levels. Densitometric analysis of protein expression represents the mean ± s.e.mean of 10 spinal cord tissues. $^{\circ}P<0.01$ versus sham; *$P<0.05$ versus SCI + vehicle. wm, white matter; gm, grey matter; SCI, spinal cord injury.

Figure 11 Effect of zileuton and montelukast on the COX-2 expression. Western blot analysis of extracts of spinal cord tissue collected at 24 h after injury. SCI caused an increase of COX-2 expression in vehicle-treated mice. The treatment with zileuton or montelukast reduced COX-2 levels. Densitometric analysis of protein expression represents the mean ± s.e.mean of 10 spinal cord tissues. Data were normalized on the basis of β-actin levels. $^{\circ}P<0.01$ versus sham; *$P<0.05$, **$P<0.01$ versus SCI + vehicle. COX-2, cyclooxygenase-2; SCI, spinal cord injury.

Figure 12 Effect of zileuton and montelukast on ERK1/2 activation. Western blot analysis of extracts of spinal cord tissue collected at 24 h after injury. SCI caused an increase of the ERK1/2 phosphorylation in vehicle-treated mice. The treatment with zileuton or montelukast reduced pERK1/2 levels. Densitometric analysis of protein expression represents the mean ± s.e.mean of 10 spinal cord tissues. Data were normalized on the basis of ERK-2 levels. $^{\circ}P<0.01$ versus sham; *$P<0.05$, **$P<0.01$ versus SCI + vehicle. ERK1/2, extracellular signal-regulated kinase 1/2; SCI, spinal cord injury.

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Figure 13 Effect of zileuton and montelukast on levels of PGE_2 and LTB_4 in spinal cord. SCI caused, at 24 h, an increase in the release of PGE_2 (**a**) and LTB_4 (**b**) in spinal cord tissues. Treatment with zileuton or montelukast significantly inhibited the SCI-induced increase of PGE_2 (**a**) and LTB_4 (**b**). Data are means $\pm$ s.e.mean of 10 mice for each group. $°P < 0.01$ versus sham-operated mice; $*P < 0.05$; $***P < 0.01$ versus SCI + vehicle-treated mice. LTB_4, leukotriene B_4; PGE_2, prostaglandin E_2; SCI, spinal cord injury.

Effect of zileuton and montelukast on the production of PGE_2 and LTB_4

An increase of PGE_2 and LTB_4 formation was found in spinal cord samples collected from SCI + vehicle-treated mice at 24 h after SCI. Spinal cord levels of PGE_2 and LTB_4 were significantly attenuated in mice treated with zileuton or montelukast (Figures 13a and b).

Discussion

The greater part of SCI in civilian life arises from fracture or dislocation of the vertebral column. Modern neuroscience techniques have led investigators to a better understanding of the importance of both primary (passive) and secondary (active) events in causing progressive loss of neural tissue. There are three stages of SCI where pharmacotherapy may have a therapeutic role (Greene et al., 1994, 1996). In the acute stage, treatments aimed towards diminishing the immune or inflammatory response, excitotoxicity and lipid peroxidation may limit secondary mechanisms of injury (Bracken et al., 1990). In the subacute stage, initiation of neurotrophic therapies may help to reconstitute the damaged tissue (Geisler et al., 1991, 1992). Interventions in the chronic stage of SCI will more than likely involve neurotrophic substances in combination with tissue or mesenchymal stem cell transplantation (Cheng et al., 1996; Bregman et al., 1997a, b, 1998; Diener and Bregman, 1998a, b). Nevertheless, only limited therapeutic measures

are currently available for the treatment of SCI and only an extensive knowledge of the molecular and cellular mechanisms involved in the etiopathogenesis of SCI will lead to a crucial therapeutic intervention.

In this report, we demonstrate that a 5-LO inhibitor, zileuton, and a $Cys-LT_1$ antagonist, montelukast, exert beneficial effects in a mouse model of SCI that mimics the human pathology. The main findings of the current study are that treatment with zileuton or montelukast improved motor recovery and reduced tissue injury, cell infiltration at the injured site, apoptotic cell death, ERK1/2 activation, and TNF-α and COX-2 levels in the injured tissue.

These results not only confirm our previous report showing that mice with a targeted deletion of the 5-LO gene (5-LO-deficient mice) are protected against experimental SCI (Genovese et al., 2005), but underline the role of Cys-LTs, although the role of other 5-LO metabolites cannot be excluded. (Sugaya et al., 2000; Bertolini et al., 2002; Hensley et al., 2006). In fact, it has been suggested that an over-expressed/overactive 5-LO pathway may lead to neurodegeneration by promoting neutrophil migration. It is well known that LTB_4 and Cys-LTs are potent chemotactic factors involved in recruitment of cells at the inflammation site (Ford-Hutchinson et al., 1980; Levine et al., 1984; Bisgaard and Kristensen, 1985; Virchow et al., 2001). In particular, Cys-LTs exert their effects through binding $Cys-LT_1$ receptors (Blain and Sirois, 2000). Moreover, in mice treated with zileuton or montelukast, a good correlation between the significant reduction of leucocyte infiltration, LT production and reduced tissue damage, evaluated by histological examination, was observed. In addition, mice treated with zileuton or montelukast showed an improvement in motor function.

Experimental evidence suggests that LTs may promote inflammation also through the regulation of other pro-inflammatory factors, such as TNF-α (Ichiyama et al., 2007; Tugtepe et al., 2007) and COX-2 (Rossi et al., 2005), which seem to be involved in neurodegeneration. Moreover, it has been demonstrated that TNF-α and COX-2 are clearly involved in the pathogenesis of experimental SCI in rats and mice (Yan et al., 2001; Bao et al., 2004; Adachi et al., 2005). In the present study, we have demonstrated, by immunohistochemistry, a significant increase of positive staining for TNF-α in SCI + vehicle mice compared with sham-operated animals. With regard to COX-2, we have recently demonstrated that in a model of inflammation in vitro, such as elicited peritoneal macrophages from mice activated with lipopolysaccharide/interferon-γ, LTC_4, but not the other LTs, increased COX-2 expression and PG production (Rossi et al., 2005). The signal transduction mechanism involved in this upregulation was related to activation of the MAPK family of protein serine/threonine kinases, which includes ERK-1 and ERK-2 (Garrington and Johnson, 1999). In particular, the MAPK activation observed in the study was correlated with the phosphorylation of ERK1/2 through activation of the $Cys-LT_1$ receptor. Similar results have been obtained in the present study as demonstrated by the decrease of COX-2, PGE_2 and pERK1/2 levels in SCI + zileuton- or SCI + montelukast-treated mice, compared to SCI + vehicle-treated mice.

The LTs are regulatory signals for neural cell proliferation and differentiation (Wada *et al.*, 2006) and apoptosis is an important mediator of secondary damage after SCI (Beattie *et al.*, 2002). In this study, as observed previously in 5-LO knockout mice subjected to SCI (Genovese *et al.*, 2005), the inhibition of the 5-LO pathway, by the use of zileuton or blockade of Cys-LT action by montelukast, reduced cell apoptosis (as assessed by annexin V, TUNEL and Fas ligand staining) after SCI induction in spinal cord tissue. This effect could be due to the inhibition of neutrophil infiltration. In other studies, decreased neutrophil infiltration due to the absence of 5-LO (Cuzzocrea *et al.*, 2005) may have contributed to the attenuation of pro-apoptotic mediators, such as reactive oxygen species (Xu *et al.*, 2005). Moreover, it is well known that Bax, a proapoptotic protein, plays an important role in the development of cell death (Chittenden *et al.*, 1995) and in central nervous system injury (Bar-Peled *et al.*, 1999). Likewise, it has been shown that the administration of Bcl–xL fusion protein (Bcl-2 is the most expressed antiapoptotic molecule in the adult central nervous system) into injured spinal cords significantly increased neuronal survival, suggesting that SCI-induced changes in Bcl–xL contribute considerably to neuronal death (Nesic-Taylor *et al.*, 2005). On the basis of this evidence, we have identified apoptotic transcriptional changes in mice subjected to SCI. These include upregulation of proapoptotic Bax and down-regulation of antiapoptotic Bcl-2, as assessed by Western blot assay and immunohistochemical staining. In particular, we demonstrated that zileuton and montelukast reduced the SCI-induced transcriptional changes related to apoptosis.

In conclusion, this study demonstrated that zileuton and montelukast improved motor recovery and reduced tissue injury, cell infiltration at the injured site, apoptotic cell death, pERK1/2 expression, and TNF-α and COX-2 level in injured tissue. Taken together, these results provide evidence that zileuton and montelukast were able to produce a substantial reduction of inflammatory events associated with experimental SCI and underline the important role of 5-LO and Cys-LTs in this model.

Acknowledgements

Conflict of interest

The authors state no conflict of interest.

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