

## RESEARCH PAPER

## AM-36 modulates the neutrophil inflammatory response and reduces breakdown of the blood brain barrier after endothelin-1 induced focal brain ischaemia

RM Weston<sup>1,2</sup>, B Jarrott<sup>2</sup>, Y Ishizuka<sup>1,3</sup> and JK Callaway<sup>2</sup><sup>1</sup>Department of Pharmacology, Monash University, Clayton, Australia; <sup>2</sup>Howard Florey Institute, The University of Melbourne, Parkville, Australia and <sup>3</sup>Department of Psychiatry, Miyazaki Medical College, Kihara, Kiyotake, Miyazaki, Japan

**Background and purpose:** Following transient focal stroke, rapid accumulation and activation of neutrophils in the ischaemic region is deleterious due to release of reactive oxygen species and myeloperoxidase (MPO). The purpose of this study was to examine whether AM-36, both a Na<sup>+</sup> channel blocker and an antioxidant, afforded neuroprotection by modulating neutrophil accumulation into brain, following endothelin-1 (ET-1) induced middle cerebral artery occlusion (MCAo) in conscious rats.

**Experimental approach:** AM-36 was administered at 3 and 24 h after ET-1-induced MCAo. Functional recovery was determined using grid-walking and cylinder tests. Image analysis of brain sections was used to determine infarct volume. The effect of AM-36 on neutrophil infiltration and their interaction with macrophages was examined in rats at 48 h following MCAo by both an MPO assay and double-label immunofluorescence. Blood brain barrier (BBB) breakdown was measured by the area stained by intravenous Evans Blue.

**Key results:** AM-36 reduced functional deficits in both tests such that no difference existed from pre-ischaemic values at 48 h. Neutrophil infiltration, assessed by MPO activity, and infarct volume were significantly reduced following AM-36 administration by 54 and 60% respectively. Similarly, immunofluorescence revealed that AM-36 reduced neutrophil infiltration by ~50% in selected brain regions, when compared to controls, and also modulated macrophage phagocytosis of neutrophils. Breakdown of the BBB was significantly reduced by 60% following AM-36 treatment.

**Conclusions and Implications:** These findings suggest that AM-36 can directly modulate the neutrophil inflammatory response and reduce BBB breakdown following MCAo.

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**Keywords:** AM-36; blood–brain barrier; cerebral ischaemia; endothelin; neutrophils; macrophages

**Abbreviations:** AM-36, [1-(2-(4-chloro-phenyl)-2-hydroxy)-ethyl-4-(3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl) methylpiperazine di-tartrate]; BW619C89, [4-amino-2-(4-methyl-1-piperazinyl)-5-(2,3,5-trichlorophenyl) pyrimidine]; BBB, blood brain barrier; BHT, 3,5-dibutyl-4-hydroxy-toluene; BN 80933, (S)-N-[4-[4-[(3,4-Dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-yl)carbonyl]-1-piperazinyl]phenyl]-2-thiophenecarboximidamide; ET-1, endothelin-1; ICAM-1, intracellular adhesion molecule-1; IR, immunoreactive; LY231617, 2,6-bis(1,1-Dimethylethyl)-4-[[[(1-ethyl)amino]methyl]phenol, Hydrochloride; MCA, middle cerebral artery; MCAo, middle cerebral artery occlusion; MPO, myeloperoxidase; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NGS, normal goat serum; PMN, polymorphonuclear neutrophil; ROS, reactive oxygen species; TTX, tetrodotoxin

## Introduction

The role of polymorphonuclear leukocytes (neutrophils) in the infarcted region following cerebral ischaemia remains

controversial (Hayward *et al.*, 1996; Emerich *et al.*, 2002), with some studies showing a neuroprotective effect after ablation of circulating neutrophils or blockade of their entry into the ischaemic region (for summary, see Beray-Berthart *et al.*, 2003). However, other studies have failed to show that reducing neutrophil infiltration limits the progression of the ischaemic injury (for review see Emerich *et al.*, 2002). Thus, Emerich *et al.* (2002) proposed that neutrophils enter

Correspondence: Dr JK Callaway, Brain Injury and Repair Team, Howard Florey Institute, The University of Melbourne, Parkville 3010, Australia.

E-mail: j.callaway@hfi.unimelb.edu.au

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the brain after the majority of ischaemic damage has occurred, mainly to remove dead cells and set the stage for tissue remodelling. Recently, Yu *et al.* (2004) have shown that adenosine (which accumulates in the ischaemic brain) can activate neutrophils, thereby enhancing the formation of inflammatory mediators via the respiratory burst, including superoxide anion. Additionally, neutrophil activation leads to degranulation and release of the enzyme myeloperoxidase (MPO), which leads to the production of hypochlorous acid, a potent oxidant (for review see Nathan, 2006). Findings from other studies have shown that inhibiting neutrophil infiltration into the ischaemic brain can reduce the formation of damaging ROS including ascorbyl radicals (Matsuo *et al.*, 1995) and superoxide anions (Fabian and Kent, 1999), along with limiting the depletion of cortical glutathione (Beray-Berthat *et al.*, 2003). Furthermore, van der Goes *et al.* (2001) demonstrated that superoxide anion can damage cerebral endothelial cells, disrupting the tight junctions of the blood-brain barrier (BBB), leading to increased BBB permeability. Coupled together, these findings directly implicate infiltrating neutrophils in the mechanisms of ischaemic injury (Iadecola, 2004). Furthermore, neutrophil adhesion to the endothelium can also lead to re-occlusion of the microvasculature, thus exacerbating ischaemia (see review by del Zoppo and Mabuchi, 2003).

Resolution of the neutrophil inflammatory response following cerebral ischaemia begins with the removal of neutrophils, either by necrosis or by apoptosis, followed by macrophage phagocytosis (Weston *et al.*, 2006). Neutrophils are constitutively programmed to undergo apoptosis, with the resulting apoptotic bodies being rapidly removed through phagocytic recognition by macrophages thus preventing the lytic release of cytotoxic and immunogenic intracellular contents into the surrounding microenvironment which can subsequently damage adjacent cells (Savill and Fadok, 2000). Macrophages can also induce apoptosis in neutrophils that infiltrate an injury site, in addition to engulfing them, and this action is considered to be beneficial because recognition and engulfment of neutrophils prevents an inflammatory response (Meszaros *et al.*, 2000).

Several drugs that target neutrophil infiltration have been developed as potential therapies for ischaemic stroke, being specifically designed to inhibit neutrophil adhesion and subsequent infiltration. Two such drugs were tested in clinical trials; a monoclonal antibody (enlimomab, R6.5) to intracellular adhesion molecule-1 (ICAM-1) and a humanized antibody (Hu23F2G or LeukArrest) to the CD18 integrin (Becker, 2002). Clinical trials with these drugs were unsuccessful due both to a lack of neuroprotective efficacy and side effects such as leukopenia, which occurs at the therapeutic doses required to achieve successful inhibition of the infiltration (Becker, 2002). There is thus a need to investigate compounds, which may inhibit the damaging effects of neutrophils through alternative mechanisms.

The novel arylalkylpiperazine compound AM-36 [1-(2-(4-chloro-phenyl)-2-hydroxy)-ethyl-4-(3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl) methylpiperazine], was designed to

incorporate multiple neuroprotective properties within one structure (Jarrott *et al.*, 1999), by integrating the anti-oxidant group, butylated hydroxytoluene (BHT) into a modified form of the polyamine antagonist, eliprodil. AM-36 has been shown to have antioxidant properties (Jarrott *et al.*, 1999; Callaway *et al.*, 2003), Na<sup>+</sup> channel blocking activity (Callaway *et al.*, 2004) and is capable of inhibiting neuronal apoptosis (Callaway *et al.*, 2001). Previous studies by Callaway *et al.* (1999) have demonstrated AM-36 to be neuroprotective, reducing both cortical and striatal damage when administered up to 3 h following transient forebrain ischaemia in conscious rats. An editorial comment on this paper (Clemens, 1999) emphasized the long window of therapeutic opportunity for AM-36 administration, and alluded to the possibility that mechanisms other than the sodium channel blockade and antioxidant properties may be involved in its action. Thus, we have investigated the possible effects of AM-36 administration on neutrophil infiltration into the ischaemic region following endothelin-1 (ET-1)-induced stroke in conscious rats. In addition, behavioural experiments were performed to investigate the effect of AM-36 on functional recovery and its correlation with neutrophil infiltration. The present data demonstrates that AM-36 modulates the neutrophil inflammatory response and reduces BBB breakdown following ET-1 induced stroke.

## Methods

### Animals

Adult male Long-Evans rats weighing 280–300 g were purchased from Monash University Animal Services and a total of 58 rats were used in this study. Rats were group-housed (four rats to a cage) under diurnal lighting with temperature maintained between 18 and 24°C and given free access to food and water, until surgery, whereupon individual rats were housed in separate cages. All animal experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986, under the guidelines of the National Health and Medical Research Council for the Care and Use of Animals for Experimental Purposes in Australia.

### Surgical preparation

Rats were anaesthetized using pentobarbitone sodium (60 mg kg<sup>-1</sup>) by intraperitoneal injection (i.p.). Using the method of Sharkey *et al.* (1993) a 23-gauge stainless steel guide cannula was stereotaxically inserted into the piriform cortex 2 mm dorsal to the right middle cerebral artery (MCA) using the coordinates of Callaway *et al.* (1999), (0.2 mm anterior, -5.2 mm lateral, and -5.9 mm ventral, relative to Bregma, according to a stereotaxic atlas (Paxinos and Watson, 1986)). The cannula was secured in place with dental cement, and two small screws inserted into the skull, to assist the dental cement to adhere to the skull. The incision was closed with sutures. Rats had access to food and water *ad libitum* and were allowed to recover for at least 3 days before the induction of cerebral ischaemia.

### Induction of focal cerebral ischaemia

Cerebral ischaemia was induced in conscious rats by occlusion of the right MCA by an ET-1 injection (120 pmol in 6  $\mu$ l of saline over 6 min; American Peptide Company, Sunnyvale, CA, USA) via a 30-gauge injector needle that protruded 2 mm beyond the end of the previously implanted guide cannula. The injector was held in place by a polyethylene-tubing cuff, and the animal was placed into a clear Plexiglas box for video recording during the induction of ischaemia (Callaway *et al.*, 1999). Characteristic indications of middle cerebral artery occlusion (MCAo) included clenching or failure to extend the contralateral forelimb and counterclockwise circling. Rats that did not exhibit these behavioural signs were deemed not to have had any occlusion of the MCA and were excluded from any further study. Sham-occlusion rats underwent cannula implantation but received a saline injection instead of an ET-1 injection and did not exhibit any behavioural reactions to the injection that would indicate a stroke.

### AM-36 administration

To examine the effect of AM-36 treatment on neutrophil infiltration, rats were divided into three groups: (1) sham-MCAo ( $n=5$ ), (2) ET-1 + water (vehicle-treated) ( $n=6$ ) and (3) ET-1 + AM-36 ( $n=6$ ). The effects of AM-36 have been investigated in a number of studies at different therapeutic doses, including 1 mg kg<sup>-1</sup> i.p. (Callaway *et al.*, 2004), 1.8 mg kg<sup>-1</sup> i.p. (Callaway *et al.*, 2000) and 6 mg kg<sup>-1</sup> i.p. (Callaway *et al.*, 1999; Callaway *et al.*, 2003; Callaway *et al.*, 2004), with 6 mg kg<sup>-1</sup> i.p. found to have maximal neuroprotective effects in reducing ischaemic damage. Although higher doses of AM-36 have been examined, for instance 10 mg kg<sup>-1</sup> i.v., these very high doses appeared to have a central action which modulates cardiovascular autonomic function, indicated by bradycardia and hypotension. These effects, however, are not seen at a dose of 6 mg kg<sup>-1</sup> i.p. (unpublished observations). AM-36 is a lipophilic compound (Log D=4.5) and pharmacokinetic analysis of AM-36 revealed that it readily crosses the BBB with a brain to plasma ratio of 9:1 achieved within 30 min following administration, a ratio that is maintained as plasma concentrations decrease with  $t_{1/2}$  of 14 h (unpublished observations). Given these findings, AM-36 was administered at a dose of 6 mg kg<sup>-1</sup> i.p. at 3 h and 24 h after the ET-1 injection, while vehicle-treated rats were injected with an equal volume of water i.p. AM-36 ditartrate was dissolved in sterile water for injection and the dose was calculated as the base.

### Physiological parameters

Core body temperature was measured rectally before stroke, and at 30 min, 1, 2 and 3 h following stroke. For rats administered with AM-36, core body temperature was measured for a further 2 h after stroke, to ensure that AM-36 was not having any effect on body temperature. Each rat was weighed before surgery, before stroke induction and every day after stroke induction until the day rats were killed.

### Assessment of functional outcome

The grid-walking test (Soblosky *et al.*, 1996) and the cylinder test (Schallert *et al.*, 2000) were used as methods for evaluation of limb deficits following ET-1-induced MCAo. Behavioural tests were conducted, pre-surgery, pre-stroke (0 h), and each subsequent day until the time of death. Each rat acted as its own control, with behavioural results being compared to pre-stroke values within the same rat. The grid-walking test was used to measure motor coordination in the forelimbs and hindlimbs. For this test, rats were placed onto a plastic-coated wire grid (3  $\times$  3 cm) 20 cm above the ground, and video monitored over 3 min. Slow-motion video playback was used to examine rat movement across the grid. A slip of a rat's paw through the wire grid was counted as an error. The number of errors for each paw were counted and subsequently divided by the total number of steps made (for each paw) and thus expressed as the percentage of total steps. The cylinder test was used to assess limb use asymmetries between the contralateral and ipsilateral forepaws on rearing and landing following cerebral ischaemia. For this test, as previously described by Schallert *et al.* (2000), rats were placed into a clear plastic cylinder 20 cm in diameter and 30 cm high for a period of 2 min, and forelimb use was videotaped. The number of contacts made with the wall or floor of the cylinder (when rearing and landing, respectively) by ipsilateral, contralateral or both forepaws simultaneously were counted over a 2 min period and expressed as a percentage of total paw use.

### Brain tissue processing

At 48 h after ischaemia, rats were anaesthetized with pentobarbitone sodium (60 mg kg<sup>-1</sup> i.p.) and transcardially perfused with 250 ml of physiological saline (0.9%) at a perfusion pressure of 100 mm Hg. As previously described by Weston *et al.* (2006), brains were removed and using a brain matrix, cut into six 2 mm coronal slices from the olfactory bulbs, frozen and stored at -80°C until processing. Coronal cryostat sections (16  $\mu$ m thick) were then cut from each of the six slices, slide mounted, and used for both image analysis and immunohistochemistry. Approximately 10 sections were taken from each of the slices, with the majority of the brain remaining in the slices used in the biochemical assay for MPO activity.

### Determination of infarct volume

The infarct area was determined in unstained sections using the ballistic light method (Callaway *et al.*, 2000), which has been shown to permit rapid and reliable measurements of infarct damage. The infarct area was measured, using a computerized image analysis system (MCID M4 image analyzer, Imaging Research Inc., St Catherines, Ontario, Canada), by tracing around the area of damage in each brain section, which appeared dark in contrast to non-lesioned areas. Infarct volume was determined by integrating the cross-sectional area of damage at each stereotaxic level and the distance between each of the levels according to the method of Osborne *et al.* (1987). Correction for oedema of ischaemic area was calculated as described by Leach *et al.* (1993).

#### *Myeloperoxidase activity assay*

The remaining slices of brain, not used for histology and immunohistochemistry were used to determine MPO activity as previously described by Weston *et al.* (2006). Briefly, the ischaemic and non-ischaemic hemispheres (from the six slices), for each individual rat, were pooled separately for the assay, and wet weight recorded. Pooled tissues were homogenized (1:20, wt vol<sup>-1</sup>) in 5 mM phosphate buffer (pH 6; 4°C) and centrifuged at 30 000 *g* for 30 min (4°C). The supernatant was discarded and the pellet washed again as described above. After decanting the supernatant, the pellet was extracted by suspension in 0.5% hexadecyltrimethylammonium bromide (Sigma Chemical Co, St Louis, MO, USA), in 50 mM potassium phosphate buffer (pH 6, 25°C) for approximately 2 min at an original wet weight to volume ratio of 1:10. Three freeze-thaw cycles were then performed with sonications (10 s, 25°C) between cycles. After the final cycle, the tissues were incubated at 4°C for 20 min and centrifuged at 12 500 *g* for 15 min (4°C). A 0.1 ml sample of the supernatant was mixed with 2.9 ml of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg ml<sup>-1</sup> *o*-dianisidine dihydrochloride (Sigma Chemical Co, St Louis, MO, USA) and 0.0005% hydrogen peroxide. The change in absorbance was measured at 460 nm using a spectrophotometer, with absorbance recorded at 15 s intervals over a 3 min period. One unit of MPO activity is defined as the amount that degrades 1 μmol of peroxide min<sup>-1</sup> at 25°C. Tissue MPO activity was calculated using human MPO (DakoCytomation, Botany, Australia) as a standard, with units of MPO activity for each tissue sample being normalized on the basis of grams per wet weight of tissue.

#### *Double-labelled immunofluorescence for neutrophils and macrophages*

As previously described by Weston *et al.* (2006), slide-mounted sections were fixed in 4% paraformaldehyde for 30 min. All sections were subject to a pre-block in 10% normal goat serum (NGS), 0.5% bovine serum albumin and 0.3% Triton X-100 in phosphate buffered saline (PBS; 0.1 M, pH 7.4) for 1 h at room temperature, and then washed in PBS. All washes mentioned herein were 3 × 10 min. Sections were then incubated overnight at 4°C in rabbit polyclonal polymorphonuclear neutrophil (PMN) anti-sera at 1:100 000 (Accurate Chemicals, Westbury, NY, USA) for neutrophils and mouse ED1 monoclonal antibody at 1:300 (Accurate Chemicals, Westbury, NY, USA) for macrophages in PBS containing 2% NGS and 0.3% Triton X-100. Tissue sections were washed and subsequently incubated with Alexa Fluorophore 488 nm donkey anti-mouse IgG at 1:500 (Invitrogen, Mount Waverley, Australia) and Texas Red Fluorophore 595 nm goat anti-rabbit IgG at 1:500 (Vector Laboratories, Burlingame, CA, USA) in PBS containing 2% NGS and 0.3% Triton X-100 for 90 min at room temperature. Sections were again washed in PBS, prior to being coverslipped with anti-fade mountant (Vector Laboratories, Burlingame, CA, USA). In control experiments, primary antibodies were omitted to verify the absence of uncontrolled secondary antibody binding.

#### *Quantification of immunohistochemistry*

Immunoreactive (IR) cells, in dual-stained sections, were visualized in a 20 × field sections under an Olympus BX-51 fluorescence photomicroscope, and counted using UTHSCSA Image Tool (version 3) (The University of Texas, San Antonio, TX, USA). The effects of AM-36 were examined by counting the total number of IR neutrophils in the ischaemic and non-ischaemic hemispheres of six predetermined cerebral regions (striatum, motor cortex, forelimb, hindlimb, parietal and barrel cortical regions), across six stereotaxic levels (4.2 to -5.2 mm relative to Bregma; Paxinos and Watson, 1986). A mean was calculated for each cerebral region, of the total number PMN IR neutrophils and the total number of PMN IR neutrophils colocalized with ED1.

#### *Confocal imaging*

Slide-mounted sections stained with both PMN anti-sera and ED1 monoclonal antibody (see above) were imaged with a Leica TCS-NT (Argon laser, Leica Microsystems, Wetzlar, Germany) inverted confocal microscope using 100 ×/1.0 oil immersion lens. In dual-channel imaging, photomultiplier sensitivities were set to a level at which bleed-through effects from one channel to the other were negligible. For simultaneous imaging of PMN and ED1, immunostaining controls were used to adjust the photomultiplier levels separately for PMN and ED1, and the same settings were used during imaging of double immunostained sections.

#### *Evaluation of BBB integrity*

A separate group of rats was used to investigate the integrity of the BBB using Evans Blue extravasation, according to Belayev *et al.* (1996). Evans Blue binds to albumin, which normally is too large a molecule to cross the BBB, but which enters the brain when the BBB permeability increases following cerebral ischaemia (Uyama *et al.*, 1988). A time-course of Evans Blue extravasation into the brain was examined at 3 (*n* = 6), 24 (*n* = 6), 48 (*n* = 6) and 72 h (*n* = 6) after ET-1 induced MCAo in conscious rats. To investigate whether AM-36 affected Evans Blue extravasation, two additional groups (sham MCAo (*n* = 6) and AM-36 treated (*n* = 6)) were examined at 48 h. Evans Blue (2% in saline, 4 ml kg<sup>-1</sup>) was injected intravenously at 48 h after the onset of MCAo. The chest was subsequently opened under pentobarbitone sodium (120 mg kg<sup>-1</sup> i.p.) anaesthesia 2 h later. Rats were transcardially perfused with physiological saline (0.9%) at 100 mm Hg pressure. After decapitation, brains were removed and frozen over liquid nitrogen and stored at -80°C until processing. Coronal cryostat sections (16 μm thick) were cut and slide mounted, from 11 predetermined levels ranging from +4.2 mm to -4.8 mm relative to Bregma (Paxinos and Watson, 1986). Evans Blue extravasation was visualized using an Olympus BX-51 fluorescence photomicroscope. The area of Evans Blue extravasation was measured using imaging analysis system for each of the 11 levels (SCION software system, SCION Corporation, Frederick, MD, USA).

### Statistical analyses

Data are expressed as mean  $\pm$  s.e.m. Infarct area was examined by ANOVA followed by Student–Newman–Keuls method, and infarct volumes were compared by Student's *t*-test. ANOVA followed by Student–Newman–Keuls method was used for analysis of the MPO activity, the examination of BBB permeability and physiological parameters (temperature and body weight). Behavioural tests were analysed using ANOVA followed by Bonferroni post-test being used for analysis of individual differences. Cell counts of PMN and ED1 IR cells were assessed by Kruskal–Wallis nonparametric ANOVA followed by Dunn's post-test. Percentage differences between vehicle and AM-36-treated rats were compared by Kruskal–Wallis nonparametric ANOVA followed by Dunn's post-test. A value of  $P < 0.05$  was considered to be significant.

## Results

### Effect of AM-36 on functional outcomes

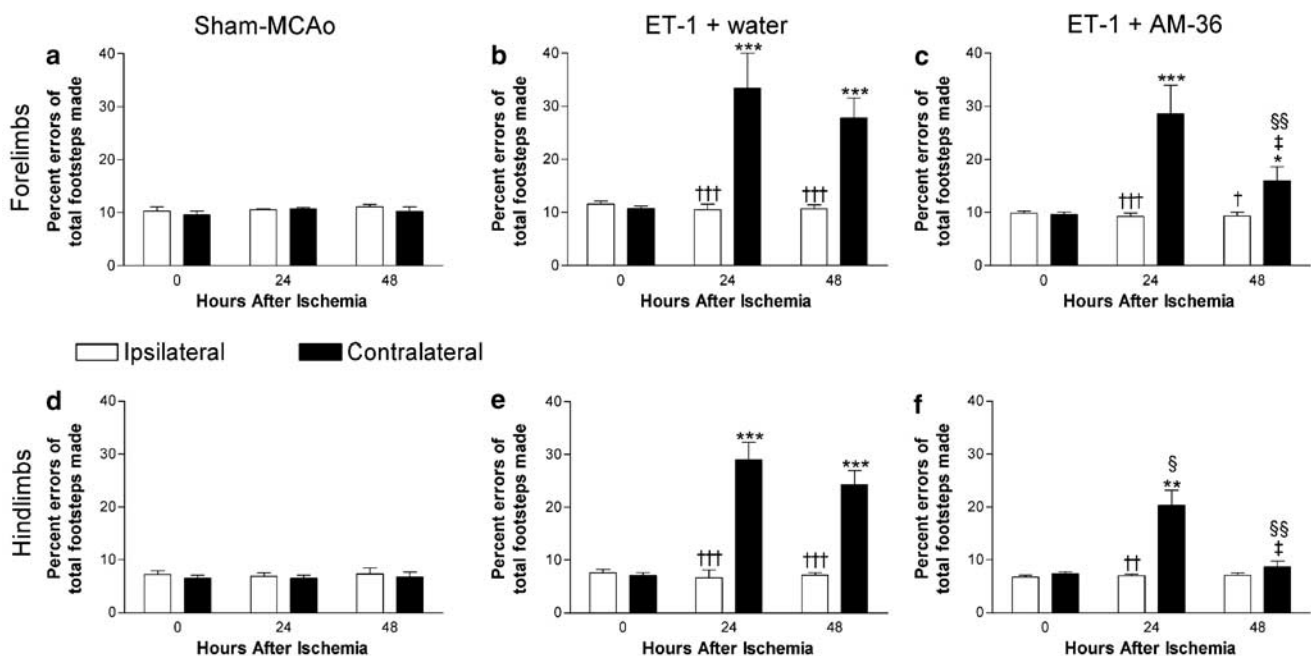
The grid-walking test and cylinder test were used to assess functional recovery following AM-36 administration. Grid-walking assessments revealed increased errors when stepping with the forelimb and hindlimb contralateral to the ET-1 MCAo (Figure 1b, c, e and f) following both vehicle and AM-36 treatment. AM-36 administration, however, significantly reduced the number of hindlimb errors at 24 h after ET-1 administration, while at 48 h, AM-36 reduced the number of errors in both the contralateral forelimb and hindlimb (Figure 1c and f) when compared to vehicle-treated rats (Figure 1b and e) (with the result at 48 h in the hindlimbs

showing no significant difference from sham-occluded rats). Furthermore, AM-36 administration significantly reduced the number of errors made when stepping with contralateral forelimbs and hindlimbs at 48 h when compared with 24 h (Figure 1c and f). Sham-MCAo rats showed negligible differences with time or between paws (Figure 1a and d).

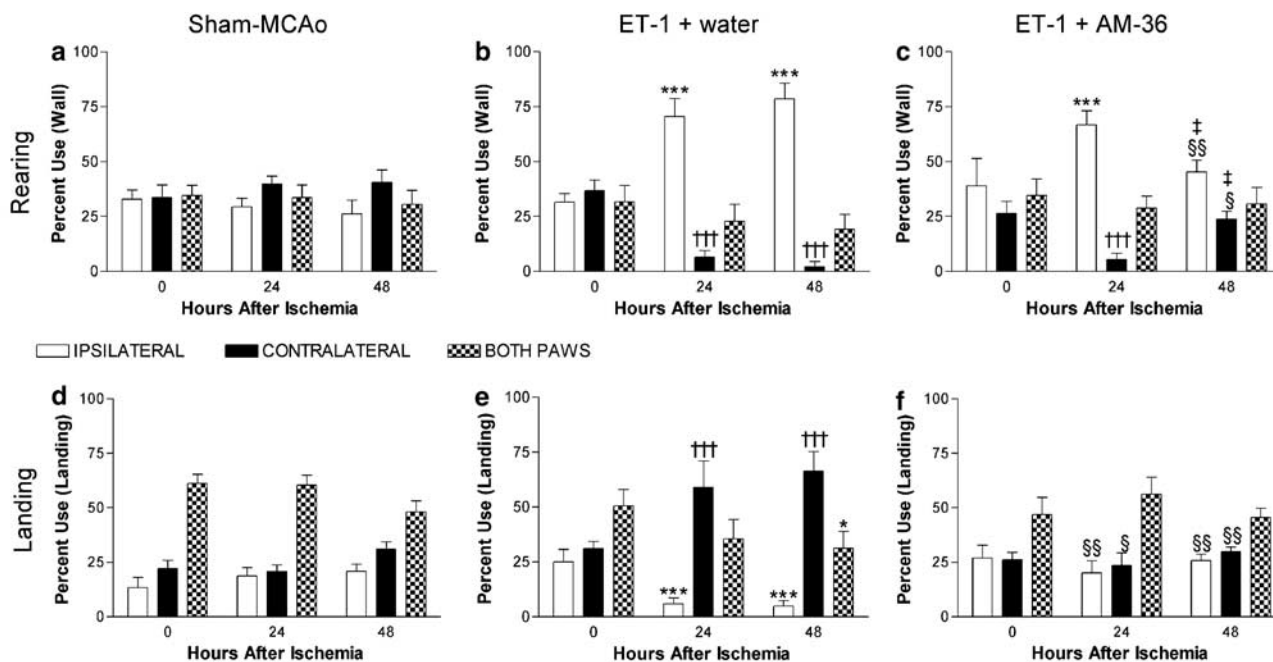
Sham-MCAo rats showed no limb use asymmetries when either rearing or landing in the cylinder test (Figure 2a and d). Following MCAo, however, rats showed significant limb use asymmetries when rearing and landing, with increased use of the ipsilateral forelimb on rearing and an increased use of the contralateral forelimb on landing. Vehicle-treated rats showed significant limb use asymmetries when rearing at both 24 and 48 h when compared to pre-ischaemia forelimb use (Figure 2b), while AM-36-treated rats only showed significant limb use asymmetries at 24 h, with forelimb use at 48 h showing no difference from pre-ischaemia values (Figure 2c). The landing preference of AM-36-treated rats (Figure 2f) showed no significant change from pre-ischaemia preferences or sham-occlusion rats. Conversely, vehicle-treated rats showed significant limb use asymmetries on landing at both 24 and 48 h, as indicated by the increased contralateral forelimb preference (Figure 2e).

### Effect of AM-36 on histopathological outcome

The infarct area (Figure 3a and b) and the infarct volume (Figure 3c) of both the cortex and striatum were significantly reduced by approximately 60% following AM-36 administration, when compared with vehicle-treated rats. In particular, AM-36 administration significantly reduced the size of



**Figure 1** Effects of AM-36 on grid-walking. Data are expressed as errors made as a percentage of total steps taken with contralateral and ipsilateral forelimbs (a–c) and hindlimbs (d–f) after cerebral ischaemia induced by ET-1. Each rat acted as its own control, and results after ischaemia were compared with pre-ischaemia scores (0 h after ischaemia). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  relative to the 0 h score in the same limb; † $P < 0.05$ , †† $P < 0.01$ , ††† $P < 0.001$  compared with opposite limb at the same time; ‡ $P < 0.01$  relative to the result at 24 h in the same limb; § $P < 0.01$ , §§ $P < 0.001$  compared with ET-1 + water group in the same limb (ANOVA followed by Bonferroni post-test). Data are mean  $\pm$  s.e.m.  $n = 5–6$  in each group.



**Figure 2** Effects of AM-36 on limb use asymmetries when rearing (a–c) and landing (d–f) in the cylinder test after ET-1 induced MCAo. Data are mean  $\pm$  s.e.m. expressed as a percentage of total forelimb use. Each rat acted as its own control, and results after stroke were compared with pre-ischæmia scores (0 h after ischaemia). \*\*\* $P < 0.001$  ipsilateral paw relative to 0 h score in same paw; ††† $P < 0.001$  contralateral paw relative to 0 h in the same paw; \* $P < 0.05$  both paws compared to 0 h; § $P < 0.01$ , §§ $P < 0.001$  AM-36-treated rats compared to vehicle rats at the same time; ‡ $P < 0.01$  relative to the result at 24 h in the same limb (ANOVA followed by Bonferroni post-test).  $n = 5–6$  in each group.

the infarct within the forelimb, hindlimb and parietal regions of the cortex, ipsilateral to occluded artery, while the barrel region of the somatosensory cortex demonstrated consistent damage in both vehicle and AM-36-treated rats (data not shown). Sham-MCAo rats only showed damage associated with the injection tract. Representative pictures taken from image analysis, shown in Figure 3d (0.20 mm relative to Bregma; Paxinos and Watson, 1986), indicate the reduction in infarct area following AM-36 administration, especially in the sensorimotor cortex.

Following stroke induction there was no significant change in core rectal temperature between vehicle ( $38.6 \pm 0.1^\circ\text{C}$ ), AM-36 ( $38.3 \pm 0.2^\circ\text{C}$ ) or sham-MCAo rats ( $38.4 \pm 0.2^\circ\text{C}$ ). AM-36 had no effect on body temperature  $37.5 \pm 0.2^\circ\text{C}$  (pre-AM-36 administration) versus  $37.5 \pm 0.1^\circ\text{C}$  (post-AM-36). Vehicle and AM-36-treated rats showed a significantly decreased body weight at both 24 ( $-18.7 \pm 7.5$  versus  $-11.5 \pm 4.9$  g) and 48 h ( $-23.2 \pm 8.5$  versus  $-14.2 \pm 5.1$  g) respectively, after stroke induction. Vehicle-treated rats showed a significantly greater decrease in body weight than AM-36-treated rats, compared with pre-stroke body weights. No change in body weight was seen in sham-occlusion rats after saline injection.

#### BBB opening

Measurement of the area stained by Evans Blue, given *i.v.*, was used to determine the time course of BBB opening, following ET-1-induced transient focal ischaemia. The area stained by Evans Blue significantly increased from 3 to 48 h, where tissue Evans Blue extravasation was maximal in

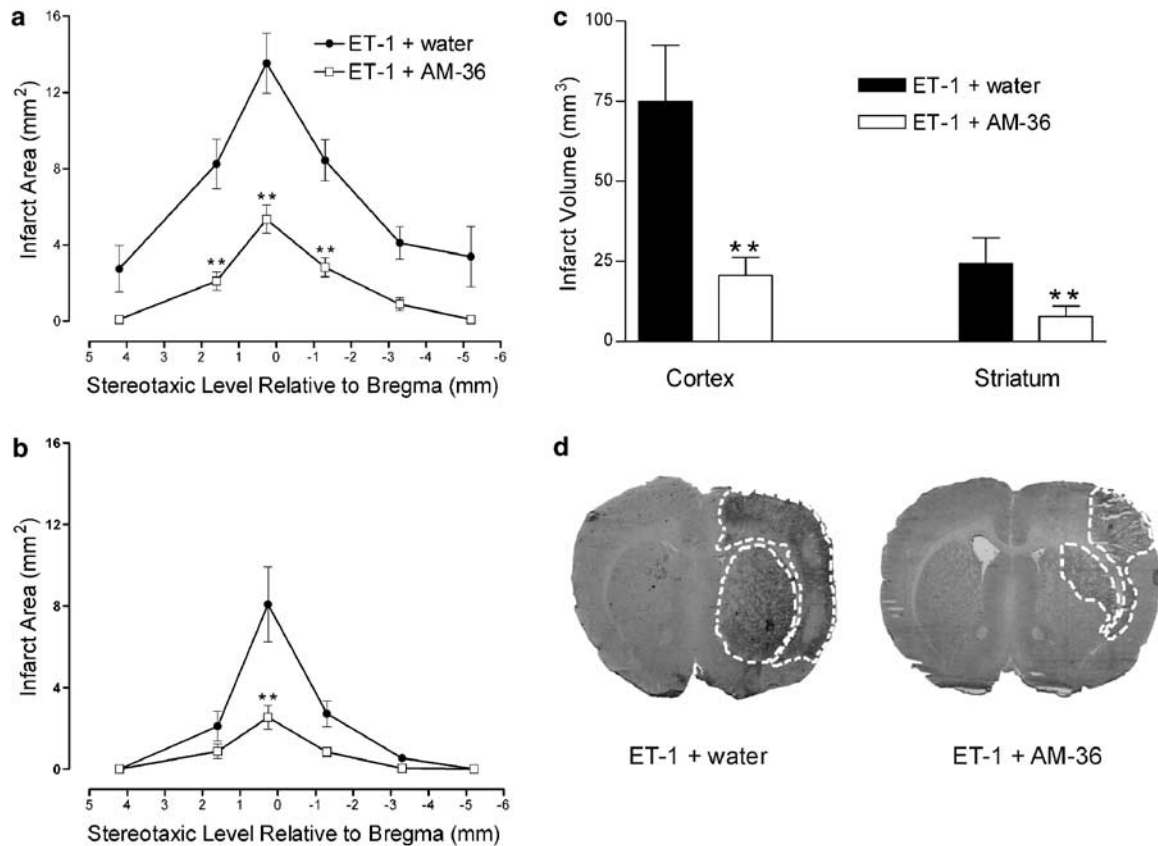
the ischaemic hemisphere, after the onset of MCAo (Figure 4a). At 72 h, Evans Blue extravasation was less than that at 48 h (Figure 4a). AM-36 administration significantly reduced the extravasation of Evans blue by  $\sim 60\%$  when compared to vehicle-treated rats (Figure 4a). Representative images (Figure 4b–d) show the increase in Evans Blue extravasation at 48 h in vehicle rats (Figure 4c) when compared to sham-occluded rats (Figure 4b). AM-36 administration reduced the extravasation of Evans Blue (Figure 4d), seen as a reduced area of fluorescence, when compared to vehicle-treated rats.

#### Effect of AM-36 on myeloperoxidase activity

AM-36 administration significantly reduced MPO activity in the ischaemic hemisphere by 54% when compared to vehicle-treated rats (Figure 5). Both ischaemic hemispheres of AM-36-treated and vehicle-treated rats displayed significantly greater MPO activity when compared to the non-ischaemic hemisphere. Vehicle-treated rats displayed a significantly greater MPO activity in the non-ischaemic hemisphere when compared with sham-MCAo rats. MPO activity was negligible in both hemispheres of sham-MCAo rats, and in the non-ischaemic hemisphere of AM-36-treated rats.

#### Effect of AM-36 on neutrophil inflammatory response

AM-36-treated rats showed a significantly reduced number of PMN IR cells in all forebrain regions examined, including the barrel cortical region that showed consistent damage



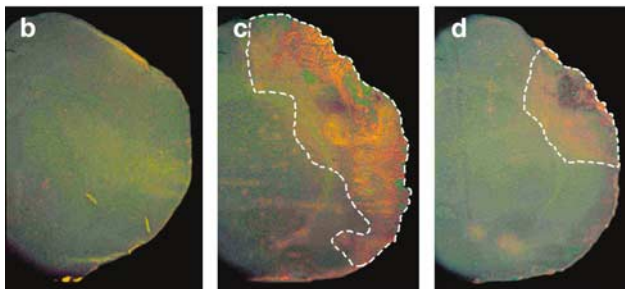
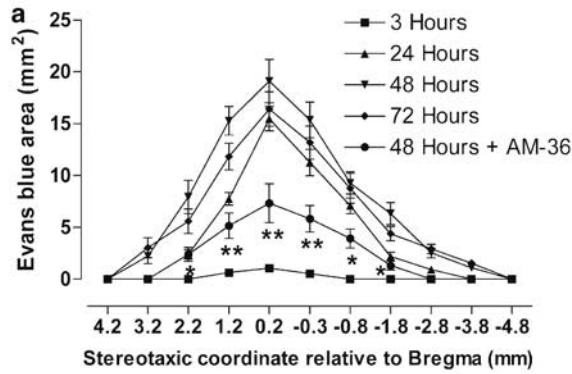
**Figure 3** Effect of AM-36 on infarct area (**a**, **b**) and total infarct volume (**c**) in both the cortex (**a**) and striatum (**b**) induced by ET-1, with data presented as mean  $\pm$  s.e.m. of infarct area measured at six predetermined coronal planes through the brain.  $**P < 0.01$  (**a**, **b**) compared with vehicle-treated control rats (Two way repeated measures ANOVA followed by Student–Newman–Keuls method) for infarct area.  $**P < 0.01$  (**c**) compared with vehicle-treated control rats (Student's *t*-test). Infarct area and volume is significantly reduced following AM-36 treatment. Representative images generated from unstained sections, using the MCID, showing reduced infarct size from vehicle-treated (ET-1 + water) rats following treatment with AM-36 (**d**). Infarct area in right hemisphere induced by ET-1 can be visualized as the dark area and is marked by the white dotted line on each of the sections.  $n = 5$ – $6$  rats in each treatment group.

between AM-36- and vehicle-treated rats, when compared to vehicle-treated rats (Figure 6a). AM-36 treatment significantly affected the interaction of neutrophils and macrophages, seen as differences in the colocalization of PMN and ED1 IR between vehicle- and AM-36-treated rats (Figure 6b and c). AM-36 treatment significantly reduced the total number of PMN IR cells that were colocalized with ED1 (Figure 6b), which corresponded to a 10% decrease in the percentage of PMN cells colocalized with ED1 when compared with vehicle-treated control rats (Table 1). AM-36 treatment, when compared to vehicle-treated rats, also significantly reduced the total number of PMN IR cells that were not colocalized with ED1 in all cerebral regions except the barrel region, where no significant difference existed (Figure 6c). However, the percentage of the total PMN IR cells not colocalized with ED1 was significantly increased by approximately 10% following AM-36 treatment, when compared to vehicle-treated rats (Table 1), except in the motor cortex, where no difference existed. Representative images of the dual labelling between PMN and ED1 demonstrate the engulfment of neutrophils by phagocytic macrophages in the cortex of ET-1 controls at 48 h, as seen in the merged images in Figure 7.

## Discussion

The present study demonstrated that AM-36, a Na<sup>+</sup> channel blocker and antioxidant, reduces infarction and functional deficits following transient focal cerebral ischaemia. Furthermore, our results show for the first time that AM-36 reduces breakdown of the BBB and modulates the neutrophil inflammatory response by decreasing the ratio of ED-1 positive macrophages and PMN positive neutrophils colocalized cells, as compared to the ET-1 control group, and by reducing neutrophil accumulation in the brain parenchyma.

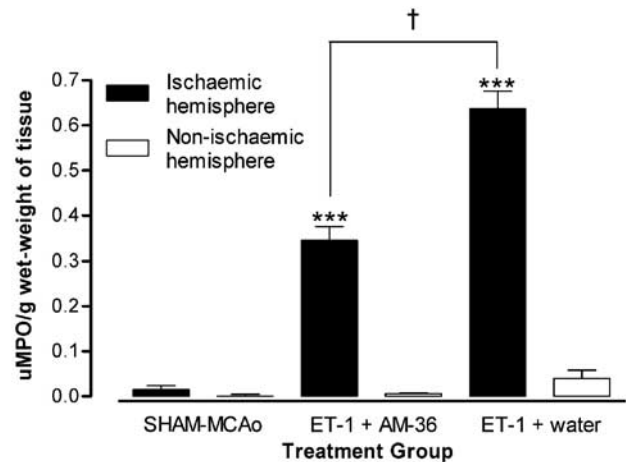
We have previously shown that the temporal profile of neutrophil infiltration into the brain parenchyma increases acutely (0–72 h) with the progression of the ischaemic injury in a conscious model of stroke (Weston *et al.*, 2006). Therefore, the effects of AM-36 were examined at 48 h post-stroke, when neutrophil infiltration into the ischaemic region was still increasing. Neutrophil infiltration, as assessed by MPO activity and infarct volume measurements, were significantly reduced following AM-36 administration at 48 h after stroke, by 54 and 60%, respectively. The reduction in MPO activity and infarct volume following AM-36 administration compares favourably with other



**Figure 4** Time course of Evans Blue extravasation in rat brain from 3 to 72 h after ET-1 induced MCAo (a) including the effect of AM-36 when administered up to 48 h, with data presented as mean  $\pm$  s.e.m. of area stained by Evans Blue. The area stained by Evans Blue significantly increased from 3 to 48 h, and then decreased at 72 h ( $P < 0.001$ , 3 versus 24 h;  $P < 0.05$ , 24 versus 48 h;  $P < 0.05$ , 48 versus 72 h). \* $P < 0.05$ , \*\* $P < 0.01$  AM-36 compared to ET-1 group at 48 h (Two way repeated measures ANOVA followed by Student–Newman–Keuls method).  $n = 6$  rats in each group. Representative sections through the ipsilateral cortex ( $\times 2$ ) viewed following Evans Blue injection at 48 h (b–d), showing reduced Evans Blue extravasation with AM-36 administration (d) when compared with vehicle-treated rats (c). There was no extravasation of Evans Blue in the sham-MCAo rats (b). Area of Evans Blue extravasation is indicated by white dotted line.

compounds designed to reduce neutrophil infiltration, such as an anti-CD18 monoclonal antibody (WT3) which showed a reduction in MPO activity and infarct volume by 75 and 61%, respectively, using the filament model of MCAo in rats (Matsuo *et al.*, 1994). AM-36 was also shown to afford profound neuroprotection to both the cortex and striatum, which is important as the striatum is considered to be the core of the ischaemic injury. These results, however, did not indicate whether AM-36 directly targets the neutrophil inflammatory response, or whether the observed reductions in neutrophil infiltration were secondary to reductions in infarct volume. Consequently, we utilized the more powerful investigatory tool of double-label immunofluorescence (Weston *et al.*, 2006) to specifically and selectively examine the accumulation of neutrophils (PMN anti-sera) and macrophages (ED-1) within selected cerebral regions of the ischaemic core and penumbra.

In penumbral regions, which included the striatum, forelimb and hindlimb regions of the cortex, motor and parietal cortex, AM-36 protected against neuronal damage and also reduced neutrophil accumulation, with neutrophils confined to the margins of the ischaemic damage.

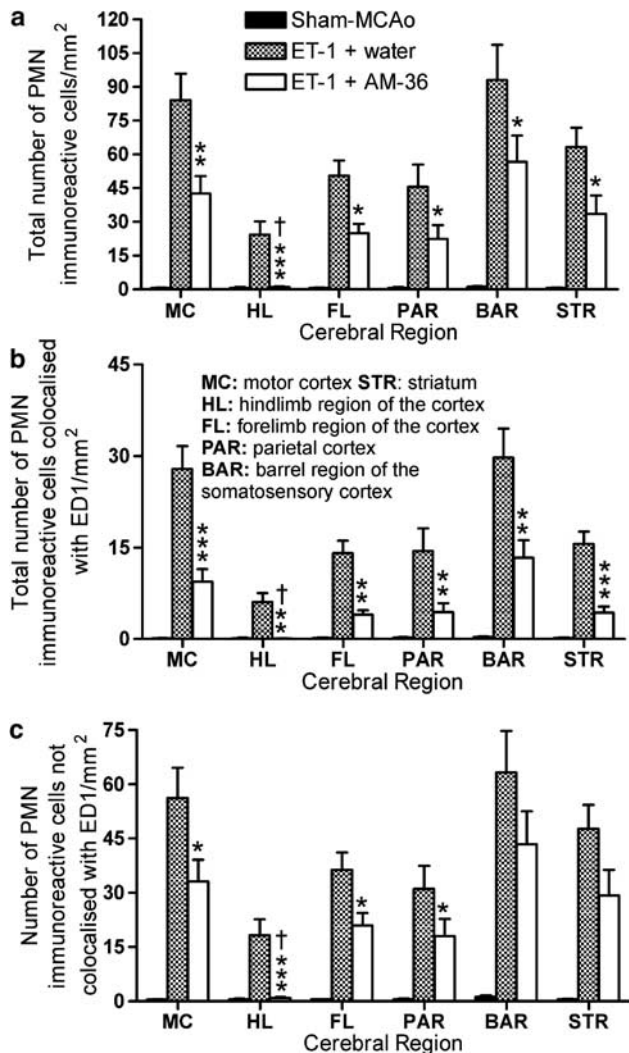


**Figure 5** MPO activity was measured in the ischaemic and non-ischaemic hemispheres of ET-1 + water, ET-1 + AM-36 and sham-MCAo groups. Values are mean  $\pm$  s.e.m., expressed as units of MPO activity per gram wet-weight of brain tissue, for  $n = 5$ –6 rats in each group. AM-36 treatment significantly reduced the MPO activity in the ischaemic hemisphere when compared to the ET-1 + water group. \*\*\* $P < 0.0001$  ischaemic hemisphere versus non-ischaemic hemisphere for ET-1 + AM-36 and ET-1 + water groups; † $P < 0.05$  AM-36 treatment group compared with the vehicle-treated group (ANOVA followed by Student–Newman–Keuls method).

In particular, the hindlimb region of the cortex showed consistent ischaemic damage in ET-1 control rats, whereas AM-36-treated rats that showed no damage in this region. Conversely, the barrel region of the somatosensory cortex, which represents the ischaemic core, demonstrated consistent ischaemic damage in both vehicle- and AM-36-treated rats. Furthermore, no significant difference in infarct volume within the barrel region of the cortex was seen when compared with ET-1 control rats, although AM-36 administration did reduce neutrophil accumulation. This result indicates that AM-36 is able to modulate the accumulation of neutrophils into the brain and that its effects are not merely the result of reductions in infarct size, as seen in the barrel region of the somatosensory cortex. We previously showed that the resolution of neutrophil accumulation in rat brain parenchyma following transient cerebral ischaemia is achieved through phagocytosis of neutrophils by macrophages and that a significant correlation between neutrophil infiltration and infarct formation exists (Weston *et al.*, 2006). AM-36 also significantly reduced the extent of colocalization between ED-1 and PMN positive stained cells, indicating the engulfment of neutrophils by macrophages was also modulated following AM-36 administration.

The cellular basis underlying the neuroprotective effects of AM-36 in modulating the inflammatory response of neutrophils following cerebral ischaemia is unknown. Meszaros *et al.* (1999) demonstrated that macrophages utilize distinct receptor patterns to recognize and ingest only neutrophils undergoing apoptotic cell death. The reduced colocalization of PMN and ED1 IR cells following AM-36 treatment may indicate that neutrophil apoptosis was impaired, and thus macrophage receptor recognition and engulfment of neutrophils was subsequently limited (Fadok *et al.*, 2001), although additional research would be required to elucidate the





**Figure 6** Cell counts of polymorphonuclear neutrophil (PMN) anti-sera and ED1 immunoreactive (IR) cells following double staining at 48 h after ET-1 induced middle cerebral artery occlusion (MCAo) in selected cerebral regions. PMN IR cells were increased at 48 h after MCAo. (a) Total number of PMN IR cells at 48 h following MCAo in different cerebral cortex regions. AM-36 treatment significantly reduced the total number of PMN IR cells in all of the regions investigated when compared to vehicle-treated rats. AM-36-treated rats were significantly different from sham-occluded rats in all regions except the hindlimb region of the cortex. (b) The total number of PMN IR cells that were colocalized with ED1. AM-36 administration reduced the total number of PMN IR cells that were colocalized with ED1 in all cerebral regions examined, except the hindlimb region of the cortex, which again showed no difference from sham-occluded rats. (c) The total number of PMN IR cells not colocalized with ED1 was also reduced by AM-36, however, there was no significant difference between ET-1 and ET-1+AM-36 treatment in the barrel region of the somatosensory cortex and the striatum. Bars are presented as mean  $\pm$  s.e.m. of the number of IR cells in each cerebral region. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  AM-36 compared with vehicle-treated rats; † $P > 0.05$  AM-36 compared with sham-occluded control rats (Kruskal–Wallis ANOVA with Dunn's post-test).  $n = 6$  for both ET-1 + water and ET-1 + AM-36-treated rats and  $n = 5$  for sham-occluded control rats.

importance of this pathway. More specifically, the Na<sup>+</sup> channel blocking and antioxidant properties of AM-36, which have previously been shown to inhibit neuronal

apoptosis (Callaway *et al.*, 2001), could be involved in modulating the neutrophil inflammatory response.

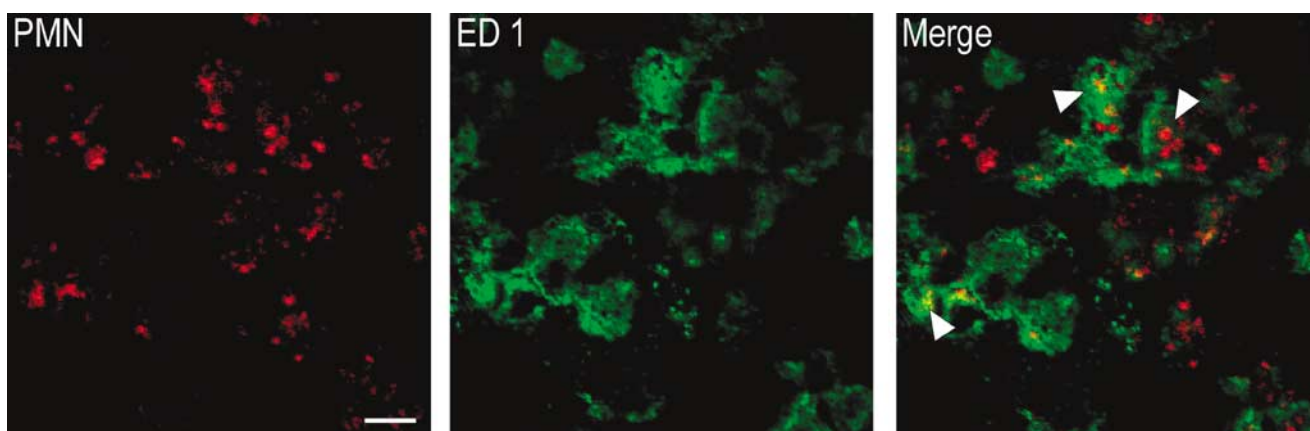
The Na<sup>+</sup> channel blockers, including TTX, have been shown to inhibit ATP depletion following cerebral ischaemia (Gleitz *et al.*, 1996). Inflammatory mediators such as adenosine, which has been shown to increase and prolong neutrophil activation through adenosine A<sub>2A</sub> receptor activation (Yu *et al.*, 2004), are produced as ATP is hydrolysed and depleted following MCAo (Rudolphi *et al.*, 1992). By maintaining ionic homeostasis and thus preventing ATP depletion (Gleitz *et al.*, 1996), through Na<sup>+</sup> channel blocking action, AM-36 could inhibit the formation of pro-inflammatory mediators thereby limiting neutrophil activation. Indeed, AM-36 exhibits potent Na<sup>+</sup> channel blocking activity, both *in vitro* and *in vivo*, which is comparable to that of tetrodotoxin (TTX) and sipatrigine, and has been shown to inhibit excess activation of slow-inactivating Na<sup>+</sup> channels (Callaway *et al.*, 2004). *In vitro* studies using cultured cerebellar granule cells demonstrated that AM-36 completely inhibited cell death caused by exposure to the Na<sup>+</sup> channel opener veratridine, with this effect being more potent and effective than other Na<sup>+</sup> channel blockers such as sipatrigine and dibucaine (Callaway *et al.*, 2001; Callaway *et al.*, 2004). Additionally, these Na<sup>+</sup> blocking properties could directly act on neutrophils to inhibit adherence to post-capillary venules as well as inhibit neutrophil activation, as similar effects have been demonstrated in studies using the Na<sup>+</sup> channel blocker lidocaine. Lidocaine was shown to inhibit neutrophil adherence (Scott *et al.*, 1993), the production of superoxide anions (Peck *et al.*, 1985; Vitola *et al.*, 1997) and MPO secretion from azurophilic granules (Fittschen and Henson, 1991) resulting in decreased infarct formation in the myocardium. Reductions in neutrophil accumulation and MPO activity demonstrated in the present study following AM-36 treatment were more potent than those described for lidocaine (Vitola *et al.*, 1997; Lan *et al.*, 2004), and may indicate the dual properties of AM-36 are more efficacious.

ROS have also been shown to affect neutrophil activation and infiltration following cerebral ischaemia by inducing the activation of transcription factors such as nuclear factor kappa B, which increases the expression of adhesion molecules leading to increased neutrophil infiltration (Howard *et al.*, 1998; Christman *et al.*, 2000). Previous studies using antioxidants, such as BN 80933, have shown reduced neutrophil activation and infiltration (Ding-Zhou *et al.*, 2003). AM-36 has also been demonstrated to possess antioxidant properties (Callaway *et al.*, 2003). In rat brain homogenates, AM-36 was found to inhibit lipid peroxidation, with its activity comparing favourably to that of known antioxidants such as BHT, Trolox and LY231617 (Jarrott *et al.*, 1999). Further, Callaway *et al.* (2003) showed a reduction in ROS metabolites in the striatum of rats with AM-36 treatment following cerebral ischaemia, demonstrating this drug has direct redox effects within the brain parenchyma. Hence, AM-36 could act directly on neutrophils to prevent the respiratory burst (Nathan, 2006) and reduce the release of ROS following ischaemia-reperfusion in a similar way to propofol (Mikawa *et al.*, 1998), or indirectly by reducing ROS-induced neuronal damage and

**Table 1** Neutrophil – macrophage interactions

Brain region	% of total PMN IR cells colocalized with ED1		% of total PMN IR cells not colocalized with ED1	
	ET-1 + water	ET-1 + AM-36	ET-1 + water	ET-1 + AM-36
Hindlimb cortex	24 ± 1.7	3 ± 2.5***	76 ± 1.7	97 ± 2.5***
Forelimb cortex	27 ± 0.8	16 ± 0.6***	73 ± 0.8	85 ± 0.9**
Striatum	25 ± 0.7	11 ± 1.2***	75 ± 3.2	89 ± 1.3*
Motor cortex	31 ± 2.0	19 ± 2.2***	69 ± 2.0	75 ± 4.3
Parietal cortex	29 ± 1.9	16 ± 3.3**	71 ± 2.0	84 ± 3.3**
Barrel cortex	32 ± 2.0	25 ± 2.8*	68 ± 2.0	75 ± 2.8

Changes in percentage following AM-36 administration in the number of PMN IR cells colocalized or not colocalized with ED1 in different cerebral regions. AM-36 treatment significantly reduced the total number PMN IR cells that were colocalized with ED1, by approximately 10–15% when compared with vehicle-treated rats. Conversely, the percentage of the total number of PMN IR cells not colocalized with ED1 was increased by approximately 10–15% following AM-36 treatment in all cerebral regions except the motor cortex, where no significant difference existed. Data is presented as mean ± s.e.m. \* $P < 0.05$ , \*\* $P < 0.001$ , \*\*\* $P < 0.0001$  AM-36 compared with vehicle-treated rats (Kruskal–Wallis ANOVA with Dunn's post-test).  $n = 5–6$  in each group.



**Figure 7** Confocal imaging of double immunostained sections shows colocalization of PMN (red) IR bodies within ED1 (green) IR cells in the cortex of an ET-1 + water control rat at 48 h following cerebral ischaemia. This image represents macrophage (green) engulfment of apoptotic neutrophils (red), indicated by the arrows. Scale bar represents 50  $\mu\text{m}$ . PMN, polymorphonuclear neutrophil; IR, immunoreactive.

oxidative stress (Chan, 2001) and subsequent neutrophil infiltration.

Neutrophil appearance in the microvasculature following cerebral ischaemia has also been shown to increase the permeability of the BBB via the release of superoxide anion (Fagan *et al.*, 2004). It is well established that ROS can disrupt vascular integrity and increase microvascular permeability by injuring endothelial cells (Chan *et al.*, 1984; Nelson *et al.*, 1992; Gasche *et al.*, 2001). Previous studies utilizing antioxidant therapies have shown that decreases in ROS with following cerebral ischaemia can attenuate BBB disruption (Kim *et al.*, 2001; Ding-Zhou *et al.*, 2003). Examination of BBB integrity following ET-1-induced MCAo in this study, revealed a similar time course of Evans Blue extravasation to that found by Belayev *et al.* (1996) following transient MCAo in rats, with BBB permeability increasing from 3 to 48 h within the ipsilateral hemisphere, where it peaks, before decreasing at 72 h. The degree of Evans Blue extravasation into brain tissue, in the present study, was also significantly higher in the ipsilateral hemisphere compared to the contralateral hemisphere in all rats following ET-1-induced MCAo.

Treatment with AM-36 reduced BBB breakdown, seen by the reduced extravasation of Evans Blue into the brain

parenchyma. This effect could in part be mediated through the antioxidant effect of AM-36 on superoxide anions and hydroxyl radicals, which are released from neutrophils and contribute to BBB opening (Nelson *et al.*, 1992). Similar reductions in neutrophil accumulation and BBB breakdown following transient cerebral ischaemia have been seen using the compound BN 80933, which combines neuronal nitric oxide synthase inhibition and antioxidant properties (Ding-Zhou *et al.*, 2003). By preserving the integrity of the BBB, AM-36 may also be inhibiting neutrophil adherence and subsequently decreasing trans-endothelial migration of neutrophils (Ainsworth *et al.*, 1996). Interestingly, accumulation of neutrophils into the brain parenchyma closely correlates with the area stained by Evans Blue, in both AM-36 and control rats, adding further support to the hypothesis that AM-36 alters neutrophil adherence at the BBB and subsequently limits infiltration. Alternatively, AM-36 may indirectly affect neutrophils by decreasing inflammatory mediators and oxidative stress through antioxidant actions.

As increased permeability of the BBB and neutrophil infiltration have been correlated with ischaemic damage and neurological deficits (Albayrak *et al.*, 1997; Weston *et al.*, 2006), it was therefore considered important to assess if

AM-36 had a neuroprotective effect on behavioural outcomes. Thus, we utilized the cylinder test to investigate limb use asymmetries in the forelimbs. The cylinder test showed that rats had no preference for either limb when rearing or landing pre-ischaemia. Following MCAo, rats showed an increased reliance on the ipsilateral (unimpaired) forelimb on rearing and for exploratory movements along the cylinder wall indicating limb use asymmetry, which is consistent with the results observed by Schallert *et al.* (2000) at acute times after stroke induction. Conversely, rats showed an increased preference for the contralateral (impaired) forelimb when landing. Rats treated with AM-36 only showed significant limb use asymmetries when rearing at 24 h, with no significant forelimb preference observed at 48 h when rearing, or while landing at either 24 or 48 h as compared with sham-occluded rats. Thus, AM-36 reduced forelimb deficits in rats following stroke, as seen by the absence of limb use asymmetries in the cylinder test when compared to vehicle-treated rats.

In contrast to the cylinder test, the grid-walking test is an effective method for assessment of motor coordination and functional recovery in all four limbs following cerebral ischaemia (Rogers *et al.*, 1997). Both vehicle and AM-36-treated rats showed increased numbers of errors associated with the contralateral forelimb and hindlimb, which is consistent with other studies (Soblosky *et al.*, 1996; Rogers *et al.*, 1997). AM-36 administration significantly reduced the number of forelimb and hindlimb errors compared with vehicle-treated rats at 48 h. Thus AM-36 was able to reduce functional deficits and improve motor coordination in both the forelimbs and hindlimbs, a finding not previously reported. Furthermore, the pronounced protection afforded by AM-36 was associated with a decrease in the infarct volume in both cortical and subcortical regions involved in motor coordination; including the striatum, forelimb and hindlimb regions of the cortex, motor and parietal cortex. Previous studies have shown that reductions or prevention of infarct formation in these regions of the cortex can be correlated with improved performance in behavioural paradigms (Rogers *et al.*, 1997; Virley *et al.*, 2000).

Importantly, AM-36 was shown to be neuroprotective following delayed administration in this model, and it should be emphasized that while recovery in this study was only investigated up to 48 h post-ischaemia, sustained protective effects have been demonstrated in more chronic studies conducted in our laboratory (unpublished observations). Thus, the profound neuroprotection and functional recovery with this drug is a permanent effect and not merely the result of spontaneous recovery that has been observed in previous studies (Schallert *et al.*, 2003).

In conclusion, the present data confirms the effectiveness of AM-36 in reducing infarct size and functional deficits following transient cerebral ischaemia. More importantly, this data demonstrates that AM-36 can reduce the neutrophil inflammatory response and BBB breakdown after focal stroke. The mechanism by which AM-36 exerts this action is unknown, but it is likely that AM-36 reduces disruption to the BBB, thus inhibiting trans-endothelial migration of neutrophils through a combination of its properties. The present study adds support to a pathogenic role of neutro-

phils following cerebral ischaemia and provides the basis for additional investigation into development of drugs to limit neutrophil entry into the brain parenchyma.

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## Conflicts of interest

The authors state no conflict of interest.

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