

# Development of a systemically-active dual CXCR1/CXCR2 allosteric inhibitor and its efficacy in a model of transient cerebral ischemia in the rat

Angela Garau<sup>1</sup>, Riccardo Bertini<sup>2</sup>, Marco Mosca<sup>2</sup>, Cinzia Bizzarri<sup>2</sup>, Roberto Anacardio<sup>2</sup>, Sara Triulzi<sup>1</sup>, Marcello Allegretti<sup>2</sup>, Pietro Ghezzi<sup>1</sup>, Pia Villa<sup>1,3</sup>

<sup>1</sup> Laboratory of Neuroimmunology, "Mario Negri" Institute for Pharmacological Research, via Eritrea 62, 20157 Milan, Italy

<sup>2</sup> Dompé s.p.a, via Campo di Pile, 67100 L'Aquila, Italy

<sup>3</sup> CNR, Institute of Neuroscience, Cellular and Molecular Pharmacology, 20129 Milan, Italy

Correspondence : P. Ghezzi

<pietro.ghezzi@libero.it>

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**ABSTRACT.** The chemokine receptors CXCR1 and CXCR2 present on polymorphonuclear neutrophils (PMN), bind the chemokine CXC ligand 8 (CXCL8)/interleukin-8 (IL-8), and have a key role in PMN recruitment in inflammation. Based on the structure of reparixin, a small-molecular-weight allosteric inhibitor of CXCR1, we designed a dual inhibitor of CXCR1 and CXCR2 with a longer *in vivo* half-life, DF2156A. This molecule inhibited human and rat PMN migration in response to CXCR1 and CXCR2 ligands and showed an elimination half-life following i.v. administration, of 19 hours. In a rat model of cerebral ischemia/reperfusion induced by temporary (90 min) middle cerebral artery (MCA) occlusion, DF2156A (8 mg/kg, i.v., at the time of reperfusion) decreased the PMN infiltrate, infarct size and significantly improved neurological function. These results indicate that CXCR1/CXCR2 and their ligands have a role in the inflammatory component of cerebral ischemia, and that these pathways represent an important pharmacological target.

**Keywords:** chemokines, cerebral ischemia, neutrophils, inflammation, anti-inflammatory drugs

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Inflammatory processes have been implicated in the pathophysiology of cerebral ischemia, and particularly in ischemia/reperfusion (I/R) injury. Experimental studies have shown recruitment and influx into the lesioned brain of vascular leukocytes, mainly polymorphonuclear neutrophils (PMNs) in the early post-ischemic period [1], and monocytes/macrophages later, activation of resident brain cells and expression of proinflammatory cytokines, chemokines and adhesion molecules [1]. Activated PMN may contribute to brain injury by causing microvascular occlusion and production of a variety of toxic mediators, including cytokines, reactive oxygen and nitrogen metabolites, and lipid mediators [2]. The role of PMN infiltration in the development of ischemia-induced damage has been studied in transient cerebral ischemia using various strategies designed to reduce PMN accumulation. Many studies have shown neuroprotection [3, 4] but only a few – depending on the severity of the ischemia model – concluded that PMN did not contribute to cerebral infarct [5].

It has been postulated that PMN chemoattractant CXC chemokines, including CXC ligand 8 (CXCL8)/interleukin-8 (IL-8), are implicated in post-ischemic leukocyte accumulation and activation [6]. Systemic increases in CXCL8 have been reported in patients with

ischemic stroke [7], and a transient increase in cytokine-induced neutrophil chemoattractant (CINC), a CXCL8-like neutrophil chemokine related to CXCL8 in humans, was seen in ischemic brain areas starting 3h after focal ischemia-reperfusion in rats [8]. Some neuroprotection studies using an anti-chemokine approach have been successful in the rabbit [9] and rat [10, 11], suggesting the potential of therapy that targets CXCL8 action in cerebral ischemia.

Reparixin (formerly repertaxin) is a recently described, specific, allosteric inhibitor of the CXCL8 receptor CXCR1 [12, 13]. Of the two CXCL8 receptors, CXCR1 and CXCR2, reparixin had a 100-fold higher efficacy in inhibiting CXCR1 than CXCR2. The drug is active in animal models of hepatic I/R injury [12], renal post transplant I/R injury [14], and in intestinal I/R injury [15], and has been granted orphan drug status for the prevention of delayed graft function in organ transplantation by US and EU regulatory agencies. In a previous paper, we have shown that reparixin has a protective effect in a rat model of cerebral I/R induced by transient middle cerebral artery (MCA) occlusion, with a therapeutic window of 3.5 hours after ischemia [16]. In that study, reparixin (15 mg/kg) was administered systemically at the time of ischemia and then

every two hours on four occasions. Using this schedule, we were able to demonstrate a significant reduction in PMN accumulation in the ischemic hemisphere and a decreased infarct size. However, no neurological functions were evaluated in those experiments.

We then tried to develop drugs acting as dual CXCR1/CXCR2 inhibitors and with a longer half-life. The reparixin binding site in CXCR1 and CXCR2 was extensively investigated; the lack of specific hydrophobic interaction in the allosteric site of CXCR2 accounts for marked selectivity of reparixin for CXCR1 [12]. On the basis of this observation, a molecular modeling-driven structure-activity relationship study led to the identification of a novel class of dual CXCR1/2 potent allosteric inhibitors (Moriconi *et al.*, manuscript in preparation).

The present study describes the characterization and pharmacological properties of a compound of this class of molecules, DF2156A. The selectivity of this molecule was investigated for its ability to inhibit the action of various chemoattractants in chemotaxis assays using human and rat PMN. Then, we performed pharmacokinetic studies in the rat. Since the molecule showed a longer half-life than reparixin, we evaluated its efficacy in a model of MCA occlusion in rats. For this purpose, we administered the drug as a single 8 mg/kg i.v. dose, 1.5 h after ischemia, and evaluated the infarct size at 24 h, and PMN infiltration. Additionally, we evaluated the neurological functions using two behavioral tests, De Rick's test and Bederson's test.

The results indicate the success of the strategy used to design a dual inhibitor of CXCR1 and CXCR2 with optimized pharmacokinetics to achieve neuroprotection through inhibition of chemokine-mediated inflammation.

## MATERIALS AND METHODS

### Materials

Human recombinant CXCL8, CXCL1 and rat recombinant CXCL2 were purchased from PeproTech (Rocky Hill, NJ, USA). Diff-Quik was from Harleco (Gibbstown, NJ, USA). Thioglycollate was from Difco (Detroit, MI, USA). DF 2156A (R(-)-2-[(4'-trifluoromethanesulphonyloxy)phenyl]-N-methanesulfonyl propionamide sodium salt) was routinely dissolved in saline at the indicated final concentrations (Bieffe Medical, Grosotto, SO, Italy). Micro Boyden chambers and polycarbonate filter were from Neuroprobe Inc (Pleasanton, CA, USA). All other chemicals and cell culture reagents were from Sigma (St Louis, MO, USA).

### Cells

Human PMNs were obtained from buffy coats of heparinized human peripheral blood from healthy volunteers, courtesy of Centro Trasfusionale, Ospedale S. Salvatore, L'Aquila, Italy. Human PMNs were prepared to 95% purity as previously described [17]. Rat PMNs were isolated from peritoneal cavities as previously described [12].

### Migration assay

Cell migration for human and rat PMNs was evaluated using a 48-well micro-chemotaxis chamber, as previously described [18].

Twenty-nine microliters of control medium (HBSS) or chemoattractant solution (hCXCL8, hCXCL1, hC5A, fMLP and rat CXCL2) were seeded in the lower compartment of the chemotaxis chamber. Fifty microliters of cell suspension ( $1.5 \times 10^6$ /mL and  $3 \times 10^6$ /mL for human and rat PMN, respectively), preincubated at 37 °C for 15 min in the presence or absence of different concentrations of DF 2156A or reparixin, were seeded in the upper compartment. Control samples received the appropriate vehicle. The two compartments of the chemotactic chamber were separated by a 5- $\mu$ m polycarbonate filter (PVP-free). The chamber was incubated at 37 °C in air with 5% CO<sub>2</sub>, for 60 min. At the end of incubation, filters were removed, fixed, stained with Diff-Quik and five oil immersion fields at high magnification (100X) were counted after sample coding.

### Animals

Male Crl:CD (SD)BR rats (Charles River, Calco, Italy) were fed *ad libitum* and housed under controlled conditions (21 °C  $\pm$  1 °C; 55% relative humidity; 12-hour light/dark cycle). Procedures involving animals and their care conformed to institutional guidelines that are in compliance with national (D.L. n. 116, G.U. suppl. 40; February 18, 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1; December 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996).

### Surgery and blood collection

The day before DF2156A administration, 4 rats were weighed and anaesthetized with 1.25 mL/kg i.m. of ketamine solution (50 mg/mL). The left jugular vein was cannulated and blood samples (about 400  $\mu$ L) were obtained 10 min., 1, 2, 6, 24, 48 and 72 h after i.v. injection, through the caudal vena, of DF 2156A (14 mg/kg). The withdrawn blood was replaced with 500  $\mu$ L of a heparin solution (0.28 mg/mL; 50.6 US units/mL).

### Determination of plasma levels of DF2156A

Plasma was obtained from heparinized blood by centrifugation at 2000 x g for 10 minutes. The bioanalytical method, based upon a high performance liquid chromatographic (HPLC) procedure, involved the extraction of DF 2156Y (the free acid of DF 2156A) and the internal standard, from aliquots of rat plasma [200 or 150  $\mu$ L mixed with hydrochloric acid (20  $\mu$ L, 5.8 M) and extracted with a 3 mL mixture of hexane/ethyl ether/ethyl acetate (5/4/1, v/v)]. The extracts were dried under vacuum and the residues were reconstituted in a mobile phase (200  $\mu$ L, 8:2, v/v). Twenty  $\mu$ L aliquots were injected into a Hypersil C18 BDS, 5  $\mu$ m analytical column. DF 2156Y and the internal standard were chromatographed using a mobile phase consisting of 0.05M phosphate buffer (pH3.8), acetonitrile and methanol (43/30/27), at a flow rate of 1.0 mL/min and detected by UV absorbance at 215 nm.

### Pharmacokinetics

Pharmacokinetic analysis was performed, after i.v. injection of 14 mg/kg of DF2156A, on the concentration-time profiles of DF 2156A in rat plasma with non-compartmental analysis using the Kinetica 2000™ Ver. 3.0 software.

The main pharmacokinetic parameters considered were: plasma concentration at time zero ( $C_0$ ), elimination rate constant ( $K_{el}$ ), half-life ( $t_{1/2}$ ), total body clearance (Cl), and steady-state distribution volume ( $V_{ss}$ ). The plasma area under the curve (AUC), from time 0 to infinite, was calculated using the trapezoidal rule.  $C_0$  and  $K_{el}$  were calculated from the y-intercept and slope of the regression line between  $\ln C$  and time up to 6 h, respectively.  $t_{1/2}$  was calculated as  $\ln 2/K_{el}$ . Cl was calculated as the dose divided by AUC.  $V_{ss}$  was calculated as dose x MRT/AUC. MRT is the mean resident time.

### Transient cerebral ischemia

Transient cerebral ischemia was induced in overnight-fasted rats (300-350 g) by 90 min occlusion of the middle cerebral artery (MCA) using the intraluminal suture method as previously described [16].

### Treatment

The compound DF 2156A was given as a single dose of 8 mg/kg intravenously (i.v.), 1.5h after ischemia, at the time of reperfusion. Saline was given to control ischemic rats.

### Neurological deficits

Twenty four hours after ischemia, and before sacrifice, the animals were controlled for neurological deficits according to Bederson's [19] and De Ryck's [20] tests. In the postural reflex test of Bederson, rats were scored as follows: grade 5, normal; grade 4-moderate, forelimb flexion and no other abnormality; grade 3 severe, reduced resistance to lateral push toward the paretic side (and forelimb flexion); grade 2 severe, same behavior as grade 3, with circling toward the paretic side when pulling the tail on the table; grade 1 severe, same behavior as grade 2, with spontaneous circling; grade 0, no activity.

The limb placing test developed by De Ryck, examines sensorimotor integration in limb-placing responses to visual, vibrissae, tactile, and proprioceptive stimuli. For each test, limb-placing scores were 0, no placing; 1 incomplete and/or delayed (> 2 seconds); or 2, immediate and complete placing. For each body side the maximum limb placing score was 16.

### Ischemic volume

To evaluate the extent of injury, the rats were killed 24h after ischemia. The brains were removed, transferred to cold saline and serial 1-mm thick sections through the entire brain were cut. Six alternate sections were incubated in a solution of 1% triphenyltetrazolium chloride (TTC) (w/v) in 154 mM NaCl for 30 min at 37 °C. The extent of injury was quantified using a computerized image analysis system (AIS version 3.0 software, Imaging Research, St. Catherine's, ON, Canada). The other six alternate sections

were frozen on dry ice and stored at -80 °C until MPO was measured as described [10].

### Statistical analysis

Statistical significance was assessed by Student's t-test and by a non-parametric test (Mann-Whitney's test) for neurological deficits, and a value of  $p < 0.05$  was considered statistically significant.

## RESULTS

### Characterization of DF2156A as a CXCR1/2 inhibitor

DF 2156Y (R(-)-2-[(4'-trifluoromethanesulphonyloxy)phenyl]-N-methanesulfonyl propionamide) is an optimized derivative of reparixin (R(-)-2-(4-isobutylphenyl)propionylmethanesulfonamide) and its chemical structure is shown in *figure 1*. Pretreatment of human PMNs with DF 2156A (the sodium salt of DF 2156Y) significantly reduced CXCL8-induced PMN migration stimulated by an optimal concentration (1 nM; [21]) of the agonist. Reduction of PMN migration was concentration-dependent being the  $IC_{50}$  0.2 nM (*figure 2A*). Similarly, DF 2156A inhibited PMN migration to CXCL1, a well-known specific agonist of CXCR2. As shown in *figure 2B*, DF 2156A inhibited CXCL1-induced PMN migration in the range of concentrations and with similar efficacy as for CXCL8-induced PMN migration ( $IC_{50}$  0.3 nM). Moreover, DF 2156A did not affect the migration to appropriate ligands of transfectants bearing the CC chemokine receptor and CCL2-induced leukocyte chemotaxis (Beccari A. *et al.* manuscript in preparation) or C5a, fMLP-mediated PMN chemotaxis (*figure 2C*). Finally, DF 2156A strongly reduced rat PMN migration stimulated by rat CXCL2 the inhibitory effect being observed within the range of concentrations, and with similar efficacy as for human PMNs stimulated by human CXCL8 or CXCL1 (*figure 2D*).

Next we evaluated the pharmacokinetic profile of DF 2156A in the rat. Plasma samples were collected 10 min., 1, 2, 6, 24, 48 and 72 h after intravenous injection of DF 2156A (14 mg/kg). In order to obtain the pharmacokinetic profile of the compound, plasma samples were analyzed by a validated HPLC method. As shown in *figure 3* and *table 1*, the elimination of DF 2156Y in rats following i.v. administration was very slow, with a mean elimination half-life of approximately 19 hours. The mean total body clearance (5.8 mL/h/kg) was low relative to the hepatic (3300 mL/h/kg) and renal (2200 mL/h/kg) blood flow

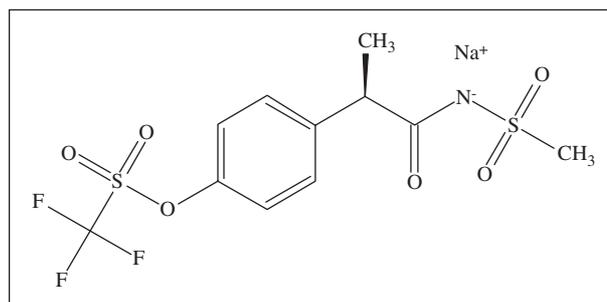
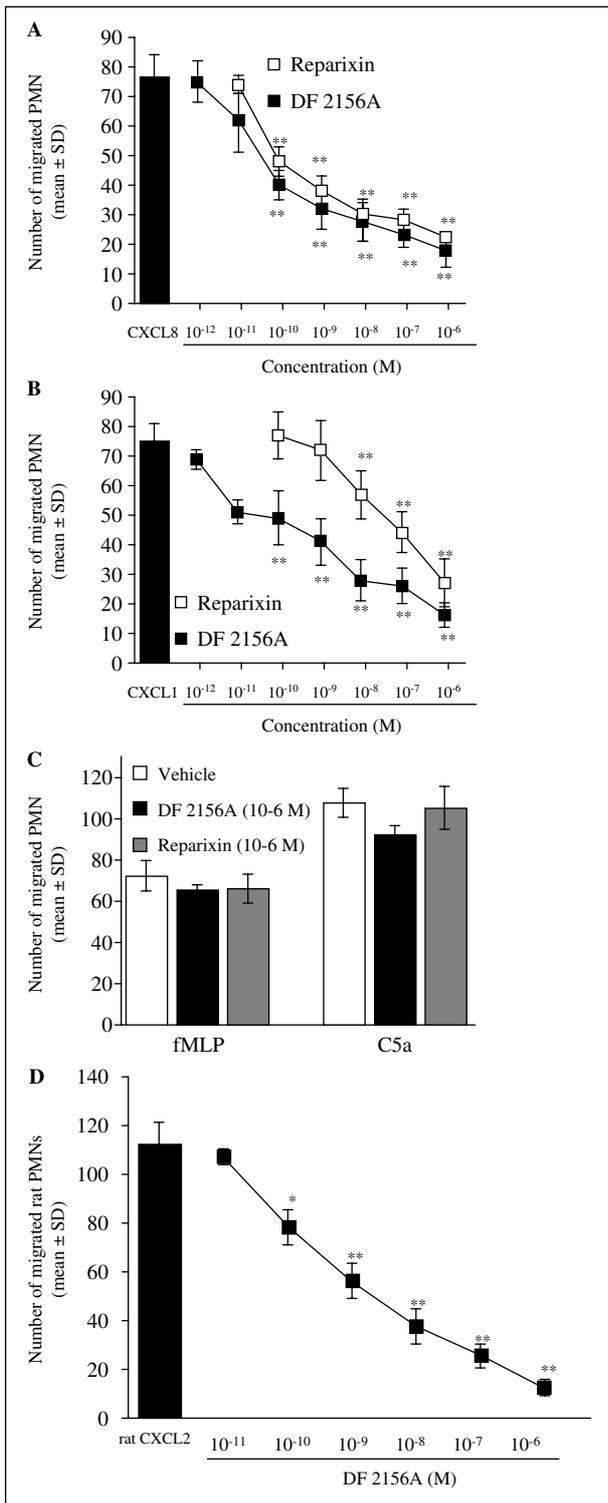


Figure 1

Structure of DF 2156A.



**Figure 2**

Effect of DF 2156A on CXCL8-, CXCL1-, C5a-, fMLP- and rat CXCL2-induced PMN migration. Human or rat PMNs were preincubated at 37 °C for 15 min with indicated concentrations of DF 2156A or reparixin. PMNs were then tested for their ability to migrate in response to 1 nM CXCL8 (panel A), 10 nM CXCL1 (panel B), 1 nM C5a or 10 nM fMLP (panel C) or 2.5 nM rat CXCL2 (panel D). PMN migration was determined as described in Material and Methods. Data are expressed as the mean ± SD of six independent experiments. Spontaneous human and rat PMN migration was 13 ± 8 and 16 ± 4, respectively. \* p < 0.05 and \*\* p < 0.01 versus appropriate agonist (vehicle pretreated) groups by Student's test and Mann-Whitney U test.

rates of the rat [22]. The distribution of the compound was low ( $V_{ss} = 157.3$  mL/kg) considering the total body water (670 mL/kg) in the rat, suggesting that a single treatment every 24 h is sufficient to evaluate its efficacy in experimental rat models.

### Efficacy of DF2156A in cerebral ischemia

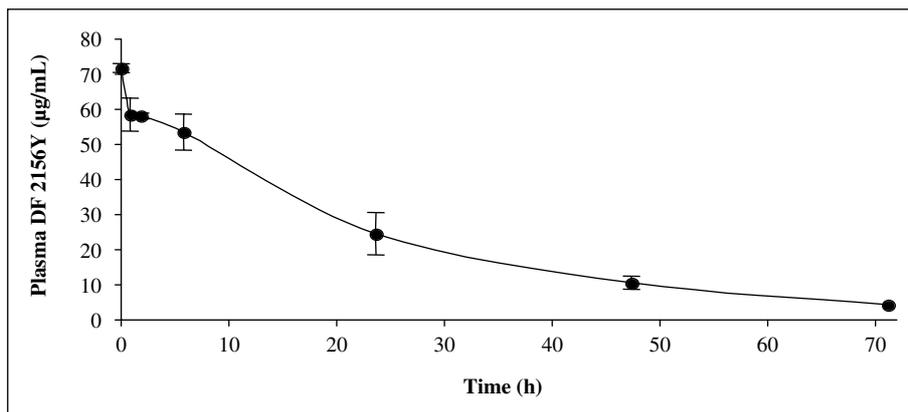
DF 2156A (8 mg/kg i.v. at the time of reperfusion) significantly reduced the MPO activity and ischemic volume by 38% and 35%, respectively, in a rat model of transient cerebral ischemia (table 2). Representative images of TTC-stained slices show a smaller infarct area in DF 2156A-treated rats than in saline-treated rats (figure 4). The reduced ischemic volume 24h after ischemia was associated with a significantly improved neurological function as indicated by the limb-placement test (De Ryck's test), which evaluate integration of sensory input with motor responses (table 3). The same rats were also tested using the Bederson postural reflex test, but this parameter showed only a trend towards a slight improvement, which was not significant (table 3).

## DISCUSSION

The present study indicates that DF 2156A is a potent CXCR1/CXCR2 dual inhibitor. Its activity *in vitro* on human and rat PMN is similar to that of the more CXCR1-specific inhibitor, reparixin, a small molecule, which, by non-competitive allosteric block of the receptor, inhibits a wide range of CXCL-8-mediated activities related to leukocyte recruitment and functional activation in inflammatory sites. Two high-affinity CXCL8/IL-8 receptors have been described in human PMN, CXCR1 and CXCR2. Of them, CXCR1 appears to have a dominant role as suggested by experiments showing that antibodies to CXCR1 inhibit most of the IL-8 chemotactic response, while anti-CXCR2 have a smaller effect [23]. On the other hand, inhibiting CXCR2 in mice and rats, with antibodies or by gene knock-out, diminishes several inflammatory responses, suggesting that it contributes to the action of IL-8-like chemokines (KC, GRO). Furthermore, CXCR2-specific ligands, such as GRO- $\alpha$ /CXCL1, GRO- $\beta$ /CXCL2, NAP-2/CXCL7 and ENA-78/CXCL5 [24, 25] that have been described in men, have a role. Given the redundancy of this chemokine network, it could be important to achieve inhibition of both CXC receptors.

Its long half-life allowed us to test its efficacy *in vivo* in a model of cerebral ischemia/reperfusion using a single dose. The results obtained with a single dose of DF2156A are very similar, in terms of extent of reduction of infiltrate and decreased infarct size, to those previously reported with reparixin administered at the time of ischemia and then every 2 h on four occasions, indicating the importance of achieving a longer half-life. Thus, the markedly different pharmacokinetics profile is accompanied by an *in vivo* efficacy. While this may not be a key factor in the treatment of stroke, where drugs can be given as infusions, this may become important in more chronic diseases requiring prolonged treatment.

More importantly, in this study we were able to show a significant improvement in the neurological functions, which was not investigated in our previous report with

**Figure 3**

Pharmacokinetic profile of DF 2156A in the rat. Plasma concentrations of DF 2156Y were determined after a single i.v. administration of DF 2156A (14 mg/kg). Plasma levels of DF 2156Y were determined at different times after treatment, as described in *Materials and Methods* section. Each experimental determination is the mean  $\pm$  SD of four rats.

**Table 1**  
Pharmacokinetic parameters for DF 2156A

Parameter	
C <sub>0</sub> (µg/mL)	72.07 $\pm$ 4.78
AUC <sub>tot</sub> (h $\times$ µg/mL)	1767 $\pm$ 319
Kel (1/h)	0.037 $\pm$ 0.0042
t <sub>1/2</sub> (h)	19.0 $\pm$ 2.6
V <sub>ss</sub> (mL/kg)	157.3 $\pm$ 19.8
Cl (mL/h/kg)	5.82 $\pm$ 1.08

Data are the mean  $\pm$  SD of 4 rats.

**Table 2**  
Effect of DF 2156A on cerebral MPO and ischemic volume

Experimental group	MPO ( $\Delta$ A/min/mg protein) (%)	Ischemic volume (mm <sup>3</sup> ) (%)
Naive	0.015 $\pm$ 0.007 (n = 6)	-
Ischemic control	0.465 $\pm$ 0.190 (100%) (n = 7)	272 $\pm$ 94 (100%)(n = 9)
DF 2156A-treated §	0.289 $\pm$ 0.138* (62%) (n = 9)	176 $\pm$ 69* (65%)(n = 10)

The results are the mean  $\pm$  SD of 7-10 rats.

§ The rats were treated with DF 2156A (8 mg/kg i.v. at the time of reperfusion). \*p < 0.05 versus ischemic control (t-test).

**Table 3**  
Effect of DF 2156A on neurological deficits

Experimental group	De Ryck's score	Bederson's score
Naïve	16 (n = 3)	5
Ischemic control	0.57 $\pm$ 0.79 (n = 7)	1.71 $\pm$ 0.95 (n = 7)
DF 2156A-treated§	5.70 $\pm$ 2.21 *(n = 10)	2.50 $\pm$ 1.35 (n = 10)

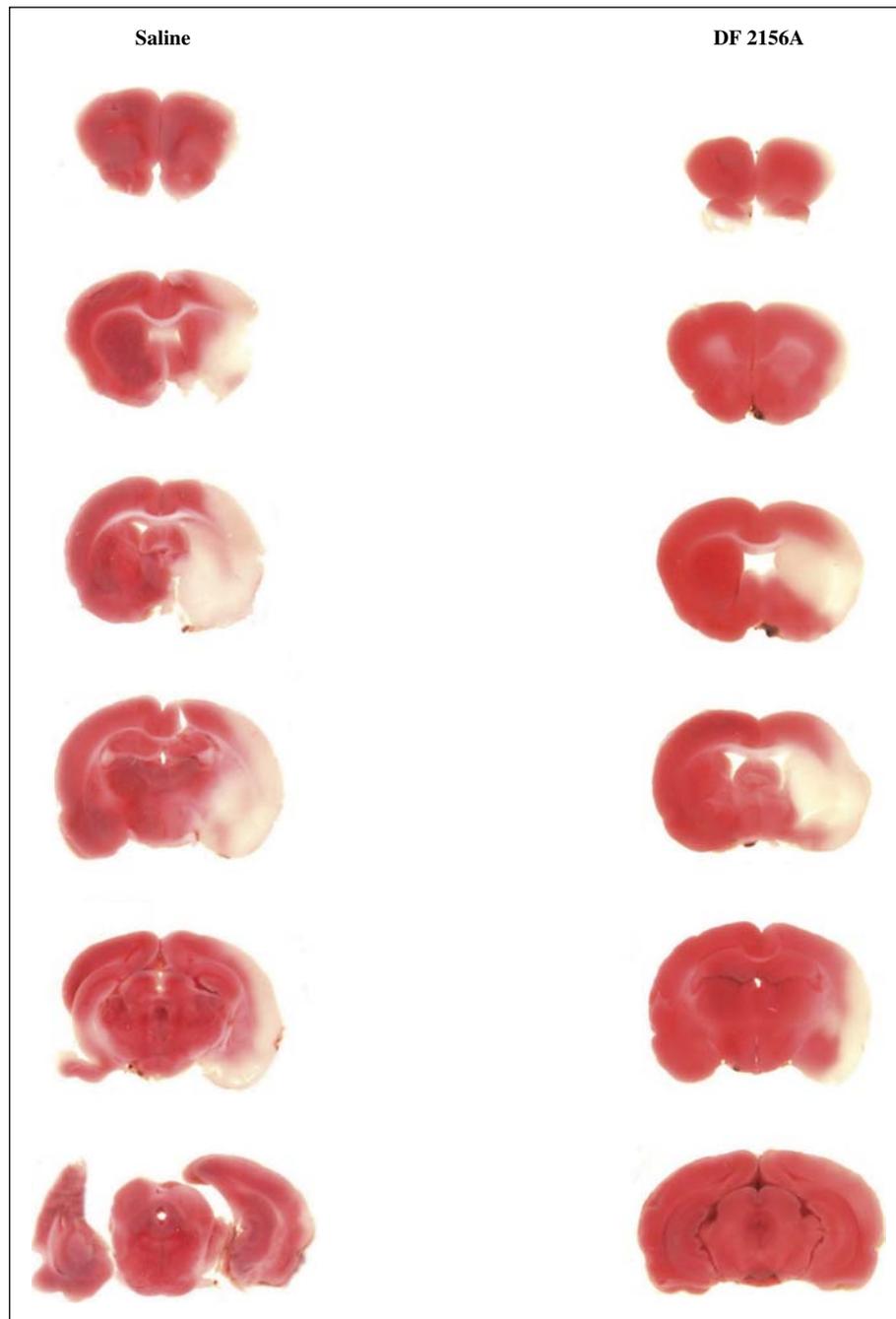
The results are the mean  $\pm$  SD of 7-10 rats.

§ The rats were treated with DF 2156A at a dose of 8 mg/kg i.v. at the time of reperfusion. \*p < 0.05 versus ischemic control (Mann-Whitney's test).

reparixin [16], indicating that the observed decrease in the infarct size may be pharmacologically relevant.

The present data, along with those showing a reduced ischemic damage by reducing PMN infiltration using anti-PMN antibodies, [4, 26, 27], anti-intercellular adhesion molecule-1 (ICAM-1) antibodies [28], and CD18-deficient mice [29], confirm the role of PMN in cerebral ischemia/reperfusion injury [1, 2].

Finally, it is important to note that induction of leukocyte migration is not the only role of chemokines. CXCL8 in particular, stimulates the release of PMN granules and the respiratory burst of these cells [30, 31], and may also have inflammatory and noxious actions independent of chemotaxis. In addition, reparixin also inhibits CXCL8-mediated signaling, including PMN degranulation, adhesion and CD11b upregulation [13]. These data support the concept



**Figure 4**

Representative images of TTC-stained brain coronal sections 24 h after MCA occlusion. Ischemia was induced for 90 min followed by reperfusion; red is viable tissue and white is non-viable, infarcted tissue. Rats treated with DF 2156A (8 mg/kg i.v. at the time of reperfusion) (right) showed a smaller infarct area than saline-treated control rats (left).

that pharmacological inhibition of chemokine receptors may be a valid therapeutic strategy in cerebral ischemia.

#### REFERENCES

1. Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci* 1999; 22: 391.
2. Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L. Neutrophils: molecules, functions and pathophysiological aspects. *Lab Invest* 2000; 80: 617.
3. Jiang N, Chopp M, Chahwala S. Neutrophil inhibitory factor treatment of focal cerebral ischemia in the rat. *Brain Res* 1998; 788: 25.
4. Matsuo Y, Onodera H, Shiga Y, Nakamura M, Ninomiya M, Kihara T, Kogure K. Correlation between myeloperoxidase-quantified neutrophil accumulation and ischemic brain injury in the rat. Effects of neutrophil depletion. *Stroke* 1994; 25: 1469.
5. Beray-Berthet V, Palmier B, Plotkine M, Margail I. Neutrophils do not contribute to infarction, oxidative stress, and NO synthase activity in severe brain ischemia. *Exp Neurol* 2003; 182: 446.
6. Ransohoff RM, Tani M. Do chemokines mediate leukocyte recruitment in post-traumatic CNS inflammation? *Trends Neurosci* 1998; 21: 154.
7. Kostulas N, Kivisakk P, Huang Y, Matusevicius D, Kostulas V, Link H. Ischemic stroke is associated with a systemic increase of blood mononuclear cells expressing interleukin-8 mRNA. *Stroke* 1998; 29: 462.

8. Yamasaki Y, Matsuo Y, Matsuura N, Onodera H, Itoyama Y, Kogure K. Transient increase of cytokine-induced neutrophil chemoattractant, a member of the interleukin-8 family, in ischemic brain areas after focal ischemia in rats. *Stroke* 1995; 26: 318-22; (discussion 322).
9. Matsumoto T, Ikeda K, Mukaida N, Harada A, Matsumoto Y, Yamashita J, Matsushima K. Prevention of cerebral edema and infarct in cerebral reperfusion injury by an antibody to interleukin-8. *Lab Invest* 1997; 77: 119.
10. Yamasaki Y, Matsuo Y, Zagorski J, Matsuura N, Onodera H, Itoyama Y, Kogure K. New therapeutic possibility of blocking cytokine-induced neutrophil chemoattractant on transient ischemic brain damage in rats. *Brain Res* 1997; 759: 103.
11. Yamagami S, Tamura M, Hayashi M, Endo N, Tanabe H, Katsura Y, Komoriya K. Differential production of MCP-1 and cytokine-induced neutrophil chemoattractant in the ischemic brain after transient focal ischemia in rats. *J Leukoc Biol* 1999; 65: 744.
12. Bertini R, Allegretti M, Bizzarri C, Moriconi A, Locati M, Zampella G, Cervellera MN, Di Cioccio V, Cesta MC, Galliera E, Martinez FO, Di Bitondo R, Troiani G, Sabbatini V, D'Anniballe G, Anacardio R, Cutrin JC, Cavalieri B, Mainiero F, Strippoli R, Villa P, Di Girolamo M, Martin F, Gentile M, Santoni A, Corda D, Poli G, Mantovani A, Ghezzi P, Colotta F. Noncompetitive allosteric inhibitors of the inflammatory chemokine receptors CXCR1 and CXCR2: prevention of reperfusion injury. *Proc Natl Acad Sci USA* 2004; 101: 11791.
13. Casilli F, Bianchini A, Gloaguen I, Biordi L, Alesse E, Festuccia C, Cavalieri B, Strippoli R, Cervellera MN, Di Bitondo R, Ferretti E, Mainiero F, Bizzarri C, Colotta F, Bertini R. Inhibition of interleukin-8 (CXCL8/IL-8) responses by repertaxin, a new inhibitor of the chemokine receptors CXCR1 and CXCR2. *Biochem Pharmacol* 2005; 69: 385.
14. Cugini D, Azzollini N, Gagliardini E, Cassis P, Bertini R, Colotta F, Noris M, Remuzzi G, Benigni A. Inhibition of the chemokine receptor CXCR2 prevents kidney graft function deterioration due to ischemia/reperfusion. *Kidney Int* 2005; 67: 1753.
15. Souza DG, Bertini R, Vieira AT, Cunha FQ, Poole S, Allegretti M, Colotta F, Teixeira MM. Repertaxin, a novel inhibitor of rat CXCR2 function, inhibits inflammatory responses that follow intestinal ischaemia and reperfusion injury. *Br J Pharmacol* 2004; 143: 132.
16. Garau A, Bertini R, Colotta F, Casilli F, Bigini P, Cagnotto A, Mennini T, Ghezzi P, Villa P. Neuroprotection with the CXCL8 inhibitor repertaxin in transient brain ischemia. *Cytokine* 2005; 30: 125.
17. McPhail LC, Snyderman R. Activation of the respiratory burst enzyme in human polymorphonuclear leukocytes by chemoattractants and other soluble stimuli. Evidence that the same oxidase is activated by different transductional mechanisms. *J Clin Invest* 1983; 72: 192.
18. Falk W, Goodwin Jr. RH, Leonard EJ. A 48-well microchemotaxis assembly for rapid and accurate measurement of leukocyte migration. *J Immunol Methods* 1980; 33: 239.
19. Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. *Stroke* 1986; 17: 472.
20. De Ryck M, Van Reempts J, Borgers M, Wauquier A, Janssen PA. Photochemical stroke model: flunarizine prevents sensorimotor deficits after neocortical infarcts in rats. *Stroke* 1989; 20: 1383.
21. Bizzarri C, Pagliei S, Brandolini L, Mascagni P, Caselli G, Transidico P, Sozzani S, Bertini R. Selective inhibition of interleukin-8-induced neutrophil chemotaxis by ketoprofen isomers. *Biochem Pharmacol* 2001; 61: 1429.
22. Davies B, Morris T. Physiological parameters in laboratory animals and humans. *Pharm Res* 1993; 10: 1093.
23. Hammond ME, Lapointe GR, Feucht PH, Hilt S, Gallegos CA, Gordon CA, Giedlin MA, Mullenbach G, Tekamp-Olson P. IL-8 induces neutrophil chemotaxis predominantly via type I IL-8 receptors. *J Immunol* 1995; 155: 1428.
24. Baggiolini M, Dewald B, Moser B. Interleukin-8 and related chemotactic cytokines--CXC and CC chemokines. *Adv Immunol* 1994; 55: 97.
25. Ben-Baruch A, Michiel DF, Oppenheim JJ. Signals and receptors involved in recruitment of inflammatory cells. *J Biol Chem* 1995; 270: 11703.
26. Connolly Jr. ES, Winfree CJ, Springer TA, Naka Y, Liao H, Yan SD, Stern DM, Solomon RA, Gutierrez-Ramos JC, Pinsky DJ. Cerebral protection in homozygous null ICAM-1 mice after middle cerebral artery occlusion. Role of neutrophil adhesion in the pathogenesis of stroke. *J Clin Invest* 1996; 97: 209.
27. Shimakura A, Kamanaka Y, Ikeda Y, Kondo K, Suzuki Y, Umemura K. Neutrophil elastase inhibition reduces cerebral ischemic damage in the middle cerebral artery occlusion. *Brain Res* 2000; 858: 55.
28. Zhang RL, Chopp M, Jiang N, Tang WX, Probst J, Manning AM, Anderson DC. Anti-intercellular adhesion molecule-1 antibody reduces ischemic cell damage after transient but not permanent middle cerebral artery occlusion in the Wistar rat. *Stroke* 1995; 26: 1438.
29. Prestigiacomo CJ, Kim SC, Connolly Jr. ES, Liao H, Yan SF, Pinsky DJ. CD18-mediated neutrophil recruitment contributes to the pathogenesis of reperfused but not nonreperfused stroke. *Stroke* 1999; 30: 1110.
30. Peveri P, Walz A, Dewald B, Baggiolini M. A novel neutrophil-activating factor produced by human mononuclear phagocytes. *J Exp Med* 1988; 167: 1547.
31. Baggiolini M, Imboden P, Detmers P. Neutrophil activation and the effects of interleukin-8/neutrophil-activating peptide 1 (IL-8/NAP-1). *Cytokines* 1992; 4: 1.