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The neuroprotective activity of the glycine receptor antagonist GV150526: an in vivo study by magnetic resonance imaging

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Abstract

The neuroprotective activity of GV150526 (3-[2-(Phenylaminocarbonyl)ethenyl]-4,6-dichloroindole-2-carboxylic acid sodium salt), a selective glycine receptor antagonist of the NMDA receptor, has been evaluated by magnetic resonance imaging (MRI) in a rat model of middle cerebral artery occlusion. The aim of the work was to evaluate, using an in vivo method, whether GV150526 was able to reduce the extent of ischemic brain damage when administered both before and after (6 h) middle cerebral artery occlusion. GV150526 was administered at a dose of 3 mg/kg i.v. T2-weighted (T2W) and diffusion weighted (DW) images were acquired at 6, 24 and 144 h after the establishment of the cerebral ischemia. Substantial neuroprotection was demonstrated at all investigated time points when GV150526 was administered before the ischemic insult. The ischemic volume was reduced by 84% and 72%, compared to control values, when measured from T2W and DW images, acquired 24 h after middle cerebral artery occlusion. Administration of the same dose of GV150526, 6 h post-ischemia, also resulted in a significant ($p < 0.05$) neuroprotection. The ischemic volume was reduced by 48% from control values when measured from T2W images and by 45% when measured from DW images. No significant difference was found between volumes of brain ischemia obtained by either MRI or triphenyltetrazolium chloride staining. These data confirm the potential neuroprotective activity of the glycine receptor antagonist GV150526 when administered either before or up to 6 h after ischemia.

Keywords: Stroke; MRI (magnetic resonance imaging); (Rat); GV150526; Focal ischemia; Neurodegeneration

1. Introduction

NMDA receptor antagonists have been shown to reduce ischemic cerebral lesions in animal models of stroke (Simon et al., 1984; Park et al., 1988a,b; Graham et al., 1993). The neuroprotective action of these compounds is high when they are administered immediately after the ischemia, but decreases when administered a few hours after the onset of ischemia. A long time window of efficacy is important because the neuroprotective agent must be administered before tissue necrosis occurs, presumably when the injury is still reversible.

Recently, a novel selective glycine receptor antagonist of the NMDA receptor, GV150526 (3-[2-(Phenylaminocarbonyl)ethenyl]-4,6-dichloroindole-2-carboxylic acid sodium salt) has been reported (Ratti et al., 1995; Reggiani et al., 1995; Tomasini et al., 1998). It has been previously demonstrated that this compound is able to reduce the extent of the ischemic brain damage when administered within the first 6 h after ischemia induced by permanent middle cerebral artery occlusion in the rat (Bordi et al., 1997).

The long time window of GV150526 efficacy prompted us to further investigate its anti-neurodegenerative properties in vivo by magnetic resonance imaging (MRI), a sensitive, non-invasive technique, to measure the infarct volume and location after experimental focal cerebral ischemia induction (Fisher et al., 1992; Quast et al., 1993). Nowadays, MRI is the method of choice to follow-up the evolution of infarcts after pharmacological intervention. In view of this, MRI has been utilized for evaluating the neuroprotective efficacy of drugs, such as NMDA receptor antagonists or Ca^{2+} channel blockers, in animal models of

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focal ischemia (Sauter and Rudin, 1986; Minematzu et al., 1993a,b; Gill et al., 1995; Hasegawa et al., 1994; Lo et al., 1994).

In the present study, we have used MRI to assess the neuroprotective activity of GV150526 after pre- and post-ischemia administration. We utilized a protocol based on T2-weighted (T2W) and diffusion-weighted (DW) images. T2W imaging is currently utilized in the clinical management of stroke, but has the disadvantage of not detecting the lesion in the first few hours after the onset of ischemia (Yuh et al., 1991). DW imaging reflects cytotoxic edema and can identify areas of brain tissue that are ischemic and likely to progress to infarction within a short time after the onset of experimental stroke (Minematzu et al., 1993a,b; Lo et al., 1994; Le Bihan et al., 1986; Benveniste et al., 1992; Minematzu et al., 1992). We have compared the data obtained in vivo with those obtained post mortem with conventional triphenyltetrazolium chloride staining (Seidler, 1980).

Studies published up to now on the neuroprotective effect of GV150526 in animal models were performed using classical, ex vivo methodologies (such as triphenyltetrazolium chloride staining). This work was carried out in order to confirm the results obtained ex vivo using an in vivo, non-invasive methodology whose findings could more easily be compared to those obtained in clinical trials. Aim of the work was also to extend the knowledge on the anti-neurodegenerative action of NMDA receptor antagonists and to explain the discrepancy that has been reported between data obtained using this class of compounds in animal models and in clinical trials (Lee et al., 1999; Stroke therapy academic industry roundtable (STAIRS), 1999; Gorelick, 2000; Lees et al., 2000). Compared to previous reports on the neuroprotective activity of GV150526, this work presents the advantage of allowing for study of time evolution of the ischemic lesion and also for assessment of the neuroprotection obtained at early time points after ischemia induction.

2. Material and methods

2.1. Surgery

Cerebral ischemia was induced by following the procedure published by Tamura et al. (1981) with minor modifications. Male Sprague Dawley rats ($n = 24$) weighing 300–400 g were anaesthetized with chloral hydrate (Fluka, 300 mg/kg i.p.). The animals were maintained normothermic by means of a homeothermic heating system (IMS "K-temp" control unit) coupled to a rectal thermistor probe. Rats were placed under an operating microscope in the lateral position and a curved 2-cm skin incision was made in the midpoint between the left orbit and the external auditory canal. An incision was made around the superior and posterior margins of the temporalis muscle that was scraped from the lateral aspect of the skull and reflected forward. A craniotomy was performed by drilling with a 018 round burr (Ash) at the junction between the medial wall and the roof of the infratemporal fossa. The position of the skull opening was about 3 mm anterior and 1 mm lateral to the foramen ovale. At this point, the dura was opened through a cruciate incision by means of a fine needle. The exposed right middle cerebral artery was coagulated by bipolar diathermy and then the hole was filled with absorbable bone sealant (Absele, Ethicon). The scalp incision was sutured and the animal was placed in a warm environment until recovery from anaesthesia.

2.2. Drug treatment

GV150526 sodium salt (batch VLPI/1831/35/9) was synthesized in the Chemistry Department, Glaxo-Wellcome, Verona. In preliminary studies, it demonstrated high affinity and selectivity for the glycine site associated with the NMDA receptor channel complex (Ratti et al., 1995). GV150526 showed no or slight inhibition of [^{3}H]CPP (4-(3-phosphonoproxyl)piperazine-2-carboxylic acid), [^{3}H]AMPA(α-amino-3-hydroxy-5-methyl-4-isoxazole), [^{3}H]kainic acid and [^{3}H]strychnine specific binding at doses of 10 and 100 μM. The compound is therefore more than 10,000-fold selective for the glycine site vs. these excitatory and inhibitory amino acid receptor binding sites. Up to 10 μM, GV150526 showed no significant displacement at the following receptors: adenosine A1 and A2 receptors, α_1-, α_2-, β_1-, β_2-adrenoceptors, dopamine D1 and D2, γ-aminobutyric acid $GABA_A$ and $GABA_B$, 5-HT_1 and 5-HT_{1C}, muscarinic M_1, M_2 and M_3, nicotinic, histamine H_1 and NMDA receptors; μ- and κ-opioid, bradykinin, neurokinin 1, 2 and 3, calcitonin gene-related peptide, neuropeptide Y, bombesin, somatostatine, vasopressin 1 and 2, vasoactive intestinal peptide and galanin peptide receptors; type T, L and N Ca^{2+} channel receptors and the benzodiazepine receptor regulatory site.

The drug was dissolved in a minimum amount of dimethyl-sulfoxide (DMSO, Merck) and then diluted to a final volume with distilled water. The drug was injected in the tail vein at the dose of 3 mg/kg. The drug or vehicle was administered either before (5 min) or after (6 h) middle cerebral artery occlusion in a single dose. The pharmacokinetics of GV150526 was previously described (Hoke et al., 2000). The dose of GV150526 was selected on the basis of previously reported results, obtained in the same experimental model, that showed the volume of cerebral infarct, after post-ischemia administration of 3 mg/kg i.v., was about 78% smaller compared to the untreated group (Bordi et al., 1997). Doses up to 10 mg/kg i.v. did not further improve significantly the neuroprotective activity. Therefore, 3 mg/kg i.v. should be considered as the most effective dose. In addition, these studies demonstrated that at a dose of 3 mg/kg i.v., no significant change was found, compared to vehicle, in

classical physiological variables that could affect brain damage, such as temperature, blood pressure, pCO^2, $[HCO^{3-}]$ or pH (Bordi et al., 1997). Moreover, this dose is 10-fold higher than the ED_{50} value calculated in the evaluation of the anticonvulsant activity in the model of NMDA-induced convulsion in rat (Di Fabio et al., 1997). On these bases, we can assume that the neuroprotective activity observed at this dose, i.e. 3 mg/kg i.v., might be the consequence of an effective blockade of the glycine site at NMDA receptors in the brain. At this dose, a functional recovery was electrophysiologically demonstrated, 7 days after ischemia (Bordi et al., 1997).

2.3. MRI experiments

Animals were anaesthetized with fentanyldroperidol at 2.5 ± 0.5 mg/kg i.p. (Leptofen, Farmitalia Carlo Erba), placed in the prone position and fixed to a cage radio frequency coil (Oxford 4.7-T) for MRI acquisitions. MRI experiments were performed on a NMR SIS 200/330 spectrometer (Varian). The exact slice plane was standardized by first obtaining preview spin-echo images. Each T2W and DW scan imaged 11 transversal slices, 0.5 mm thick and with 1.0 mm center-to-center separation over a 10-mm field of view. Spin-echo DW images were acquired with a repetition time (TR) of 1000 ms and an echo time (TE) of 70 ms. Diffusion-sensitive gradient pulses of 40 ms duration and 2 G/cm strength were applied along the z direction (the slice selection direction). With these parameters, a diffusion weighting factor of 500 s/mm^2 was obtained. T2W images were acquired with TR = 1000 ms and TE = 70 ms. Total acquisition time was 15 min for both T2W and DW scans. Images were acquired 6, 24 and 144 h after middle cerebral artery occlusion, in order to monitor the early and late phases of the cerebral ischemia.

2.4. Histology

Immediately after the last MRI scan, rats belonging to the control group were killed under chloral hydrate anaesthesia. Brains were removed, cooled in ice-cold isotonic phosphate buffered saline (pH = 7.4), and dissected into 0.5 mm axial sections using a motorized vibratome at the following coordinates from the interaural line (expressed as mm): 2.2, 3.8, 5.7, 7.2, 8.1, 9.7, 11.2, 12.2, 13.2 (Paxinos and Watson, 1986). These brain slices were immersed in a solution containing 2% triphenyltetrazolium chloride in isotonic phosphate-buffered saline at 37°C for 30 min and then stored in 10% neutral-buffered formalin (Seidler, 1980).

2.5. Evaluation of brain ischemia and statistical analysis

The area of cerebral damage in each axial section obtained from MRI studies and triphenyltetrazolium chloride stain was measured by an image analyzer (Imaging Research, Canada). The image of the whole section was digitized and the infarct size for each section was calculated and expressed as mm^2. Accordingly, infarct volumes were calculated by the trapezoidal rule method. Statistical parameters were calculated as mean standard error (S.E.). The statistical significance of differences was performed

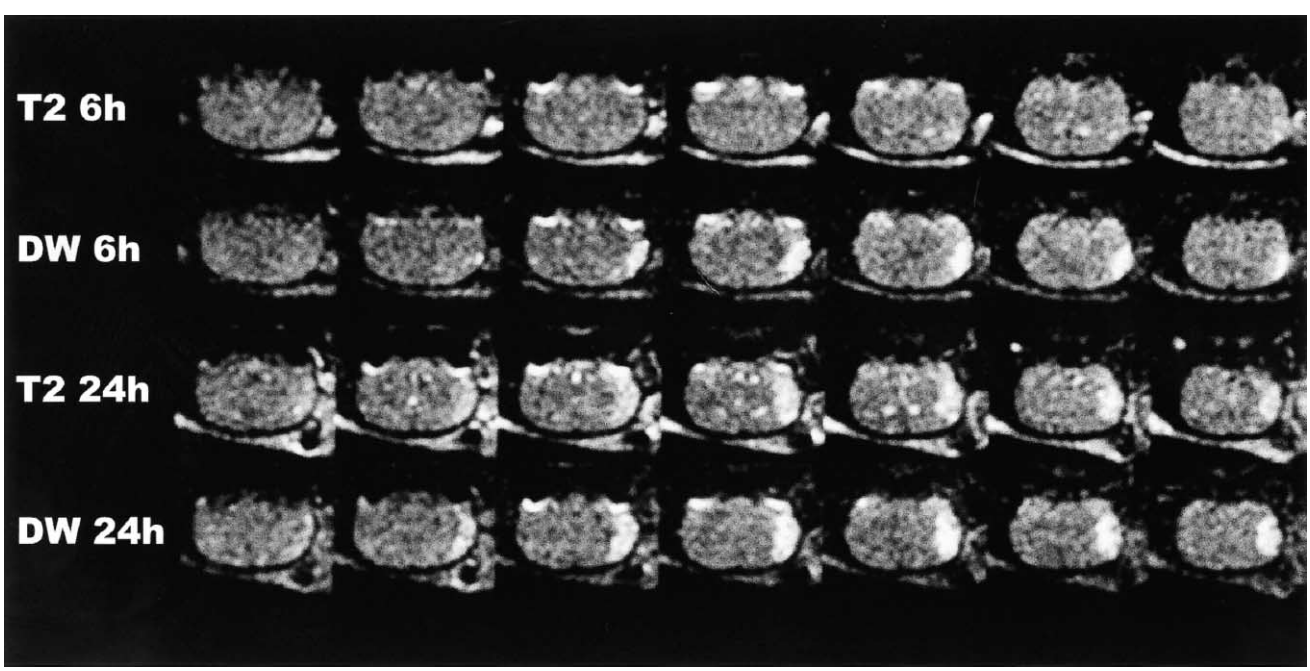

Fig. 1. Comparison between DW and T2W acquisitions, 6 and 24 h after ischemia induction in one animal belonging to the control group. DW imaging appears to be more sensitive then T2W imaging at the earlier time point.

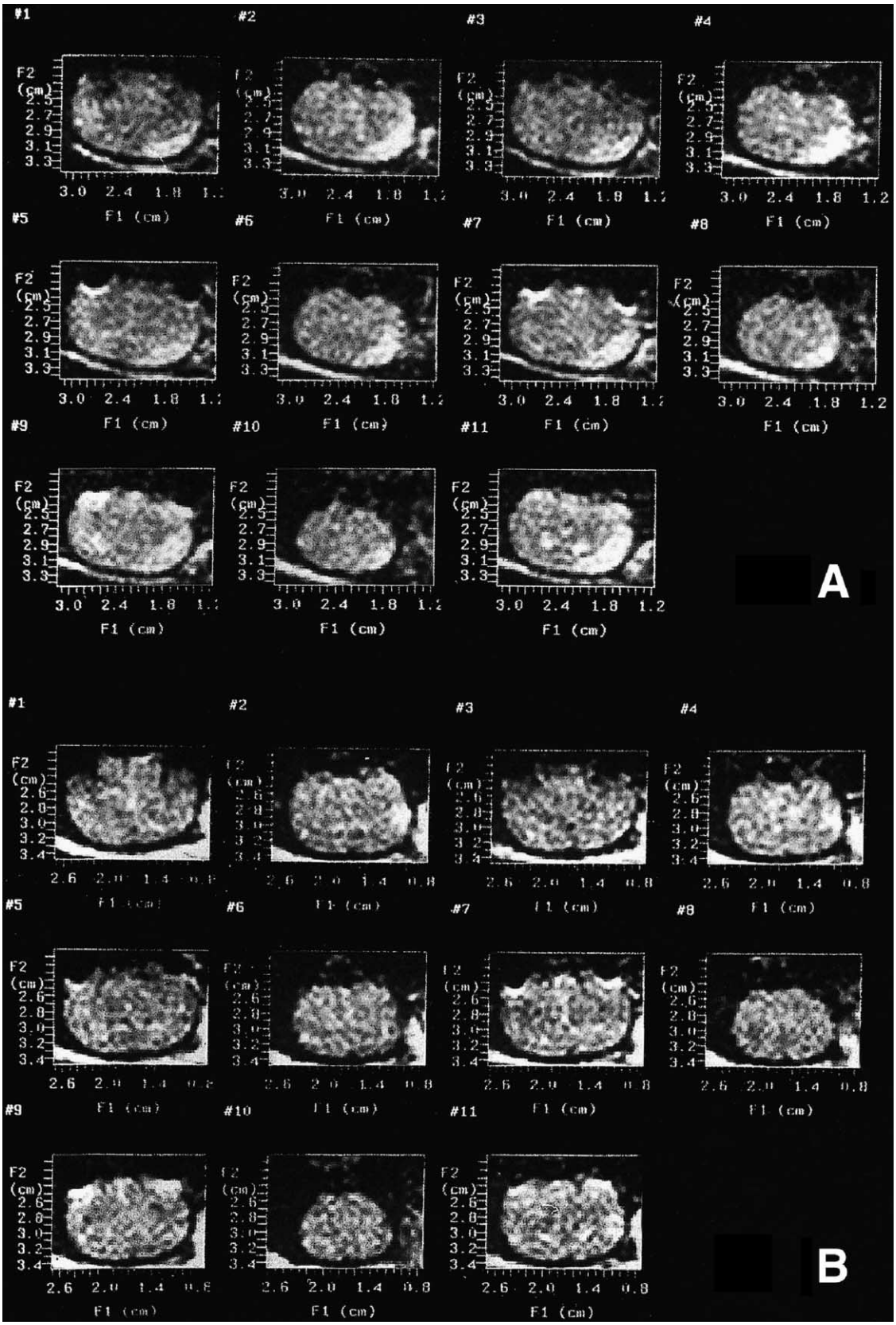

Fig. 2. Axial T2W images acquired 24 h after ischemia induction for a rat belonging to the control group (A) and a rat belonging to the treated group (B). The slices are shown in the interleaved order in which they are acquired (i.e. before the even-order and after the odd-order slices).

by using one-way analysis of variance (ANOVA) followed by Dunnett test implemented on the RS1 computer program (BBN Software Products).

3. Results

Fig. 1 shows MRI slices acquired with T2W and DW sequences at different time points after ischemia in the same animal. It is clearly apparent that, at the earlier time point (6 h), the region of the brain that appears hyperintense in DW images is much wider than the region that appears hyperintense in T2W images. At the later time point (24 h), T2W and DW images detect approximately the same hyperintense regions. One-hundred and forty-four hours after ischemia induction (images not shown), DW images fail to detect the infarcted area that, on the contrary, is still clearly visible in T2W images. It is apparent, from Fig. 1, that the damaged area is mainly localized in the brain cortex.

In Fig. 2 we show representative T2W images acquired 24 h after ischemia induction in rats belonging to the vehicle- (Fig. 2A) and GV150526-treated (Fig. 2B) groups. In general, the volume of infarct in animals treated with GV150526 appeared smaller than in the vehicle-treated group, at all investigated time points.

In the vehicle-treated group of animals, the infarct volumes calculated from the T2W images were 10.3 ± 5.1 ($n = 6$), 93.1 ± 18.5 ($n = 7$) and 87.6 ± 19.6 ($n = 7$) mm^3 (mean $\pm$ S.E.) at 6, 24 and 144 h after ischemia, respectively. The same values, calculated from DW images, were 43.5 ± 14.5 and 100.8 ± 20.3 mm^3 at 6 and 24 h, respectively. After the last MRI examination, rats were sacrificed in order to estimate the volume of ischemic damage with triphenyltetrazolium chloride staining. The calculated value of 63.8 ± 12.6 mm^3 was not significantly different ($p > 0.05$) from that previously measured by T2W images. A significant correlation ($r = 0.86$; $p < 0.01$) was found between the infarct volumes measured with triphenyltetrazolium chloride staining and the ones measured by T2W sequences.

Pre-ischemia administration of GV150526 (3 mg/kg i.v.) resulted in a significant neuroprotective activity ($p < 0.01$ vs. vehicle-treated rats). Ischemic brain volumes calculated from T2W scans were 1.1 ± 0.5 ($n = 8$), 14.7 ± 5.9 ($n = 7$) and 16.4 ± 6.5 ($n = 5$) mm^3, respectively, at 6, 24 and 144 h after middle cerebral artery occlusion. The same values as calculated from DW images were 8.4 ± 3.7 and 28.2 ± 12.2 mm^3 at 6 and 24 h post-ischemia, respectively.

Post-ischemia administration of a single dose of GV150526 (3 mg/kg i.v.) 6 h after middle cerebral artery occlusion resulted in a significant neuroprotection ($p < 0.05$ vs. vehicle-treated rats). The infarct volume, measured from T2W and DW images 24 h after ischemia induction, were 45.7 ± 13.9 mm^3 ($n = 7$) (48% reduction from the control value) and 55.1 ± 16.9 mm^3 ($n = 7$) (45% of reduction from the control values), respectively.

4. Discussion

4.1. MRI techniques

In this study, in vivo MRI techniques were used to assess the neuroprotective activity of the glycine receptor antagonist, GV150526. In agreement with previously reported results (Gill et al., 1995; Allegrini and Sauer, 1992), the volumes of ischemic brain damage, as measured by MRI, were in agreement with histological findings, although the absolute values obtained with the triphenyltetrazolium chloride stain were slightly smaller than those obtained with MRI. This difference could be attributed to the shrinkage of the tissue after removing the brain or to an overestimation of infarct volume by MRI during the development of brain edema (Lin et al., 1993). One-hundred and forty-four hours after middle cerebral artery occlusion, the absence in DW images of a uniform hyperintensity in the infarcted area might be due to the complete breakdown of cells leaving fluid-filled spaces with unrestricted diffusion, as previously reported (Quast et al., 1993).

4.2. Protective action of pre-treatment

GV150526 has been reported to reduce ischemic volume, as measured by triphenyltetrazolium chloride staining, when administered either pre- or post-ischemia (Bordi et al., 1997). The present study shows that GV150526, administered before middle cerebral artery occlusion at a dose of 3 mg/kg i.v., reduces the volume of the ischemic infarct by 72% and 84% when measured 24 h after the ischemic insult, using DW and T2W MRI, respectively. This percentage of neuroprotection is close to the 75% reduction observed with triphenyltetrazolium chloride staining 24 h after middle cerebral artery occlusion (Bordi et al., 1997). In the present work, DW imaging allowed us to detect lesions also at time points (6 h after ischemia) when triphenyltetrazolium chloride staining does not provide reliable information. The present study demonstrates that pre-treatment with GV150526 significantly reduces the infarct from 43.5 to 8.4 mm^3 (about 80% of reduction) in the first 6 h. This degree of neuroprotection appears to be high when compared to that provided by other compounds in rat or other species (Park et al., 1988a; Minematzu et al., 1993a,b; Hasegawa et al., 1994; Ozyurt et al., 1988).

4.3. Post-ischemia treatment

Several studies suggest that the sequence of events that leads to cell death may be blocked for several hours after

the primary insult with different classes of drugs, such as AMPA antagonists, Ca^{2+}-channel blockers or κ-opioid receptor agonists (Smith and Meldrum, 1992; Buchan et al., 1993; Xue et al., 1994; Widmayer et al., 1994; Moller et al., 1995; Tatlisumak et al., 1998). NMDA receptor antagonists generally have a short therapeutic time window. However, it has been reported that some NMDA receptor antagonists reduce the volume of ischemic brain damage when the treatment is initiated 30 min (Takano et al., 1997) or 1 h after occlusion (Steinberg et al., 1989; Chen et al., 1991). The neuronal degeneration induced by intracerebral injections of NMDA can be attenuated by treatment with MK-801 (5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate) up to 5 h after administration of the agonist (Foster et al., 1987a,b). In a cat model of focal cerebral ischemia, Park et al. (1988b) provided evidence that the volume of ischemic damage in cerebral hemisphere and cortex can be significantly reduced with MK-801, even when the treatment is initiated 2 h after the induction of ischemia. The authors stated that the excitotoxic events leading to cell death might be reversible for several hours after the primary insult. In the present work, post-ischemia administration of GV150526 was associated with 45% and 48% neuroprotection, as measured with DW and T2W images, respectively. This amount of neuroprotection is similar to that observed previously (53% neuroprotection) (Bordi et al., 1997). Thus, it can be concluded that GV150526, under these experimental conditions, has a therapeutic window of 6 h post-ischemia treatment. It appears that a single treatment with this compound is able to block progression of neuronal damage to irreversible necrosis in a significant portion of the salvageable tissue.

In previous studies using a cat model of middle cerebral artery occlusion, the magnitude of the reduction in the volume of ischemic tissue was similar both when MK-801 was administered prior to or 2 h after the occlusion (Park et al., 1988b; Ozyurt et al., 1988). In the present study, we found that delaying treatment by 6 h results in a lower level of protection (48% vs. 84% when measured on T2W images, 24 h after middle cerebral artery occlusion). Despite this decrease in neuroprotection, these studies demonstrate that NMDA receptor antagonists can have a long therapeutic time window similar to drugs of other classes (Xue et al., 1994; Widmayer et al., 1994).

5. Conclusions

A main problem in the discovery of new drugs against stroke is the discrepancy between studies on animal models and clinical trials. Several neuroprotectants (and in particular NMDA receptor antagonists) obtained negative results in clinical trials, despite promising results in experimental animals (Gorelick, 2000). A similar discrepancy was also reported for GV150526 (Gavestinel), which in a recent clinical trial did not improve the outcome of acute ischemic stroke (Lees et al., 2000). To date, the cause of this discrepancy is still matter of debate (Lee et al., 1999; Stroke therapy academic industry roundtable (STAIRS), 1999). A suggested hypothesis is that in most of the animal studies, an inappropriate time window after onset of the stroke was chosen since in most of the studies the drug was administered in a 1–2-h time interval after ischemia. An alternative hypothesis is that the size of the lesion calculated at post-mortem evaluation does not always correlate well with functional impairment, while this correlation is more robust with diffusion-perfusion MRI.

In the present study, we demonstrate that this discrepancy is not simply due to an inappropriate time-window, as the neuroprotective effect in the rat is also evident after delayed treatment, similarly to clinical trials. In addition, we demonstrated that the effect observed in post-mortem evaluation is well correlated to in vivo MRI data. Therefore, an anti-neurodegenerative activity of NMDA receptor antagonists on cortical lesion of the rat is confirmed and the discrepancy with the results obtained in clinical trials is probably due to other causes such as the presence of sub-cortical lesions in the latter. In our opinion, a more precise definition of the lesion pattern in human patients by MRI methods could be useful to select study groups with homogeneous type of stroke.

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