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Review Article

Identification and pharmacological characterization of GV150526, a novel glycine antagonist as a potent neuroprotective agent

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Introduction

Stroke is a severe disabling disease caused by a sudden reduction of cerebral blood flow (ischemic or hemorrhagic stroke) which causes a permanent impairment of neurological functions. Stroke is currently the third cause of death and the first cause of long-term disability in the Western world, mainly because the therapeutic approach to stroke is presently rather poor. In fact, once the ischemic condition has occurred, if the cause is not rapidly eliminated (within minutes), the neuronal tissue will begin to die; currently nothing can be done to halt the progression of damage.

In the last few years several institutions worldwide have concentrated their efforts on understanding the mechanism of cell death in stroke and identifying biological targets of potential therapeutic interest. It is currently accepted that when the cerebral blood flow is reduced below a certain threshold of perfusion (10 ml/min/g tissue), an irreversible excitotoxic cascade is quickly activated leading to neuronal cell death mainly by necrosis. This necrotic zone is called the "core" of the infarct and is surrounded by a injured zone called the “penumbra”. In the penumbra, the degree of perfusion is above the survival threshold but still lower than in normal tissue; thus, cells are still vital but not functioning. Penumbral cells can stay alive for a longer period of time compared to core cells, but they are always at risk of death until normal reperfusion is restored. When reperfusion takes time, even if aided by drugs, part of the penumbral zone will die, thus causing neurological impairment. Therefore, to maximize the neurological recovery of stroke patients it is essential to preserve for as long as possible the neuronal integrity of penumbral cells until the blood flow is restored.

Figure 1 illustrates a simplified version of the neurochemical events occurring in neuronal cells after the onset of stroke. It is now generally agreed that under ischemia (1-4), the reduction of oxygen-energy supply to cells causes a significant increase in glutamate (5, 6) concentration within the synaptic cleft. This leads to overstimulation of postsynaptic receptors, and in particular, of the NMDA receptor subtype. This causes a massive influx of Ca^{2+} into the postsynaptic neurons, leading to cell death (7) through the activation of different intracellular enzymes. Therefore, a pharmacological intervention which blocks the overactivation of the NMDA receptor has been suggested to have potential therapeutic benefit (8-10).

The NMDA receptor belongs to the family of ligand-gated ion channels, although it has unique features: a) conductance through the channel associated with the NMDA receptor is regulated in a voltage-dependent manner by extracellular Mg^{2+}; b) the simultaneous presence of glycine as a coagonist of glutamate (11-14) is required to activate the NMDA receptor complex.

In view of the greater therapeutic index seen for glycine antagonists compared to competitive NMDA antagonists and NMDA ion channel blockers (15-17), the glycinergic site associated with the NMDA receptor complex was perceived as an attractive target for blocking excitotoxicity following stroke. Considerable effort has been devoted during the last few years to identifying potent and selective glycine antagonists (18-23), and several classes of promising compounds (Fig. 2) have been widely investigated by different research groups. With few exceptions, poor *in vivo* activity was claimed (24, 25).

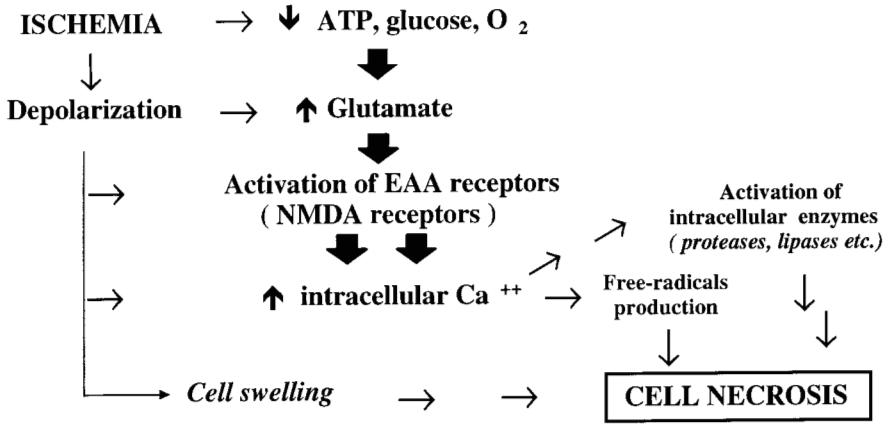


Fig. 1. Biochemical cascade following stroke.

To obtain systemically active glycine antagonists as potential agents for the treatment of stroke, we explored a novel series of 2-carboxyindole derivatives. GV150526 (Fig. 3) was identified (26-30) as a powerful neuroprotective agent in an animal model of cerebral ischemia, with a wide therapeutic window. This paper deals with the discovery process as well as the pharmacological profile of this potent and selective glycine antagonist acting at the strychnine-insensitive glycine binding site associated with the NMDA receptor.

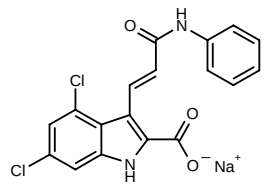


Fig. 3. Chemical structure of GV150526.

Screening sequence

The biological evaluation of the new chemical entities (NCE) included: a) binding assay to evaluate the affinity for the glycine site and selectivity for the glutamate receptors in rat cortex (K_i), b) *in vitro* functional antagonism studies to evaluate both potency and activity in rat cortex (K_b), c) *in vivo* anticonvulsant activity in mouse (ED_{50}), and d) *in vivo* model (MCAo) of focal cerebral ischemia in rat (ED_{50}).

The *in vitro* affinity of the NCEs for the glycinergic site was assessed by inhibition of the binding of [^{3}H]-glycine to crude synaptic membranes prepared from adult rat cerebral cortex, as reported by Kishimoto *et al.* (31).

Functional antagonism at the glycine binding site was measured by the ability of the NCEs to antagonize the glycine-induced enhancement of the binding of the channel blocking agent [^{3}H]-TCP (32) in a glycine-sensitive extensively washed rat cortical preparation (33).

The selectivity of the NCEs to the glutamate binding site of the NMDA receptor channel complex, AMPA and kainate inotropic glutamate receptors was assessed by inhibition of the binding to rat cortical membranes of [^{3}H]-CPP, [^{3}H]-AMPA and [^{3}H]-kainic acid. Experiments were performed as reported by Van Amsterdam *et al.* (34), Giberti *et al.* (35) and Honoré *et al.* (36).

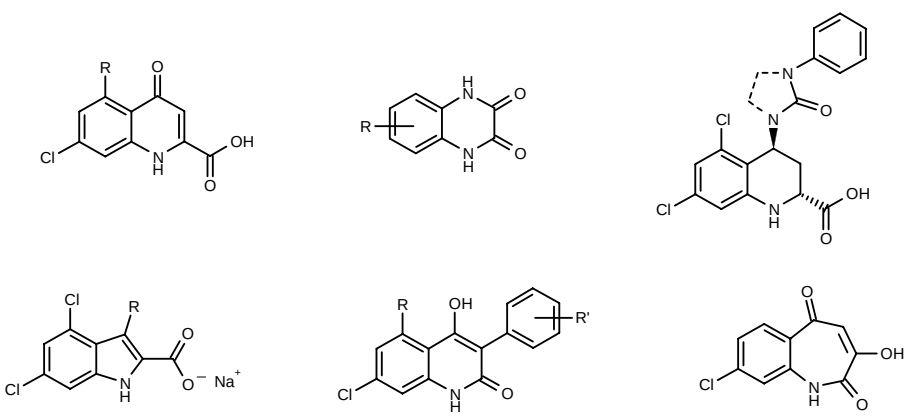


Fig. 2. Chemical structures of different classes of glycine antagonists identified.

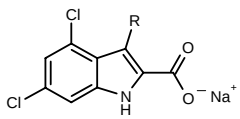
The second step of the screening process deals with the evaluation of the *in vivo* profile. Since the characterization of NCEs in the animal stroke model of middle cerebral artery occlusion (MCAo) (37) was time-consuming and therefore unsuitable for screening purposes, the *in vitro* most potent and selective indole-2-carboxylates were tested first in the NMDA-induced convulsion model (15-17) in mice, and in some cases in rats. This rapid *in vivo* evaluation was introduced as a surrogate for stroke models, starting from the basic assumption that NMDA receptor overactivation is the key event in neurodegeneration following cerebral ischemia. The ability of the novel glycine antagonists to counteract NMDA-induced convulsions was used as an endpoint of the model. Full dose-response studies were performed and ED_{50} values were generated.

Finally, the most active compounds, both in terms of affinity for the glycine binding site and *in vivo* activity in the NMDA-induced convulsion model in mice, were tested both pre- and postischemia in the MCAo model in rats.

Identification of GV150526

To identify glycine site antagonists endowed with high affinity and good receptor selectivity, an indole-2-carboxylate was chosen as the key template. The choice of this nucleus was based mainly on the preliminary evidence (38-44) that the affinity for the glycine binding site of this class of compounds could be modulated by introducing a suitable C-3 position, thus maintaining a high selectivity towards the different glutamate receptor binding sites. In this way, the indole derivatives bearing different H-bond accepting groups and terminal lipophilic substituents within the C-3 side chain were designed (Table I).

Table I: Substitution at the C-3 position.



Entry	R	pK_i*
1	H	5.7
2	CH_2CH_2COOH	7.4
3	$CH_2CH_2CONHPh$	7.6
4	CH=CHCOOH	7.7
5	CH=CHCOO-*t*-Bu	6.3
6	CH=CHCONHPh	8.5
7	CH=CHCONH-c-C_6H_5	8.0
8	CH=CHCONH$C_{10}H_7$	7.4
9	CH=CHCONHCH_2Ph	6.9
10	CH=CHSO_2NHPh	6.1

*Inhibition of binding of [^{3}H]-glycine

In addition to the known derivatives 1 and 2 (19), these compounds were prepared and evaluated in terms of affinity for the glycine binding site. The results of this preliminary characterization (Table I) can be summarized as follows.

a) The affinity at the glycine binding site of the different compounds tested was found to be greater than the unsubstituted analog **1** (R=H), confirming the crucial role of the H-bond acceptor group present within the C-3 side chain.

b) The affinity of compounds substituted at the C-3 position with an α,β-unsaturated side chain was found to be greater than the corresponding saturated analogs. This effect could be explained both in terms of higher electron density on the oxygen atom of the carbonyl group and improved H-bond donor character of the indole NH group.

c) The need for a correct spatial orientation and H-bond accepting character of the H-bond accepting group (C-3 side chain) was confirmed by the lower affinity of the sulfonamide derivative **10** with respect to the amide analog **6**.

d) The homologation of the terminal phenyl ring, belonging to the C-3 side chain, resulted in a reduction of affinity as a consequence of both the increased steric bulk and electronic reasons (presence of a phenyl ring not directly conjugated with the nitrogen of the amide group.

e) The *tert*-butyl ester derivative **5** showed a significant reduction in affinity giving additional information on both the electronic requirement of the carbonyl group and the steric bulk of the terminal lipophilic substituents.

In view of these results, the presence of the α,β-unsaturated side chain at the C-3 position of the indole nucleus seems to be crucial to maximize the affinity for the glycine binding site of this indole template, further confirming the predictability of the proposed pharmacophoric model of the glycine binding site (45, 46).

Substitution of the terminal phenyl ring

After the identification of GV150526, the most potent compound belonging to this first series of indole-2-carboxylates, the effect of the substitution of the aromatic ring belonging to the C-3 side chain was carefully investigated.

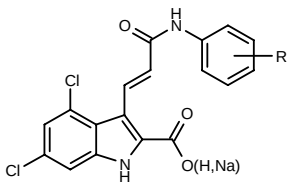
Following the Hansch approach, a set of traditional descriptors (47-49) was chosen and the aromatic substituents were described in terms of their electronic and steric effects and lipophilicity. The series of substituted analogs of compound **6** (Table II) was chosen ensuring maximum variance and minimum colinearity between parameters characterizing the physicochemical properties of the substituents. Multiple regression analysis (MRA)) (50, 51) was used to analyze the data (52, 53). The following statistically significant equation was obtained:

$$pK_i = - 0.53\ MR_{omp} - 0.39\ \pi_{omp} - 0.82\ \sigma_{para} + 8.23$$

$$(n = 25,\ R^2 = 0.84,\ s = 0.28,\ F = 37,\ p < 0.0001,\ R^2_{cv} = 0.1)$$

where MR_{omp} is the total bulk of the *ortho*, *meta* and *para* substituents, π_{omp} is the global lipophilicity of the *ortho*, *meta* and *para* substituents, and para is Hammett's σ_{para}. According to Equation 1, the affinity at the glycine binding site increases as total bulk (MR_{omp}) and lipophilicity (π_{omp}) of substituents decrease and as the electron donor resonance effect of the para (σ_{para}) substituent increases. Therefore, the terminal aromatic ring of the α,β-unsaturated C-3 side chain should lie in a nonhydrophobic pocket of limited size, further refining the proposed pharmacophoric model (45, 46) of the glycine binding site associated with the NMDA receptor. The electronic effect (σ_{para}) is more difficult to interpret in terms of drug receptor interaction. It may be related to the improvement of the hydrogen binding acceptor ability of the carbonyl group present within the α,β-unsaturated side chain.

Table II: Substitution at the terminal aromatic ring.



Entry	Ro	Rm	Rp	Pk_i*
6	H	H	H	8.5
11	H	H	NH_2	8.9
12	H	NH2	H	8.3
13	NH_2	H	H	8.5
14	H	H	OH	8.7
15	H	OH	H	8.4
16	OH	H	H	8.4
17	NO_2	H	H	7.6
18	H	OCH_3	OCH_3	8.1
19	CH_3	H	OCH_3	7.7
20	NO_2	H	F	7.5
21	F	NO_2	H	8.3
22	F	H	F	8.3
23	H	H	COOH	7.2
24	H	NO_2	H	8.0
25	H	H	$N(CH_3)_2$	7.9
26	H	H	NO_2	7.0
27	H	H	F	8.2
28	H	H	OCH_2CH_3	8.3
29	i-C_3H_7	H	H	7.3
30	H	NO_2	Cl	6.9
31	H	H	$N(C_2H_5)_2$	7.0
32	H	H	CF_3	6.8
33	H	H	NHC_6H_5	6.7

*Inhibition of binding of [^{3}H]-glycine

Indole-2-carboxylates: synthetic route

GV150526 was easily prepared starting from the known 3-unsubstituted indole derivative (39) following the general synthetic route described in Scheme 1.

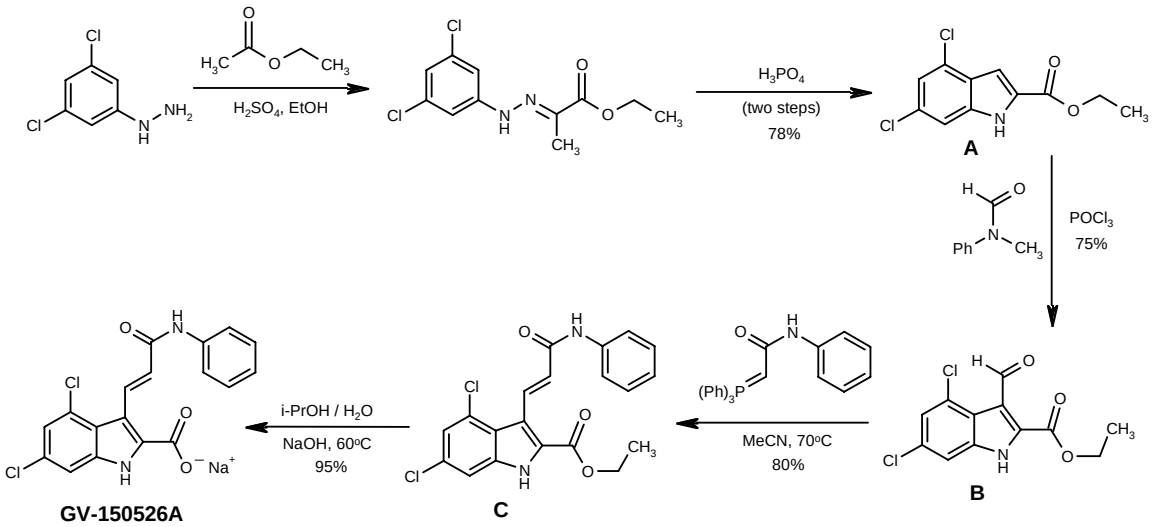
The known indole derivative **A** was formylated at the C-3 position according to the well known Vilsmaier-Haack procedure obtaining the intermediate **B** in high yield. The subsequent Wittig-type olefination reaction afforded the compound **C** with high regio control in the formation of the olefinic moiety. The hydrolysis of the ethyl ester group afforded GV150526 as sodium salt derivative. Alternatively, for the synthesis of analogs of GV150526 substituted at the terminal phenyl ring belonging to the C-3 side chain as shown in Scheme 2, compound **A** was transformed into the intermediate **D** in high yield. Following chemoselective deprotection of the *tert*-butyl ester group, the free carboxylic acid derivative **E** was submitted to amidation reaction using different synthetic methods (54). In particular, the activation of the carboxyl group through the formation of the corresponding 2-pyridyl thioester, generated *in situ* by the mild oxidation-reduction condensation reaction (55) in the presence of 2,2'-dipyridyl disulphide and PPh, was found to be highly efficient, also in the case of poor nucleophilic aromatic amines, affording the desired amide derivatives **G** in high yield (either from the isolated 2-pyridyl thioester intermediate **F** or from the acid **E** using a one-pot procedure). Finally, target compounds **H** were easily prepared by basic hydrolysis of the 2-carboxyethyl ester protecting group.

Preliminary pharmacological characterization of GV150526

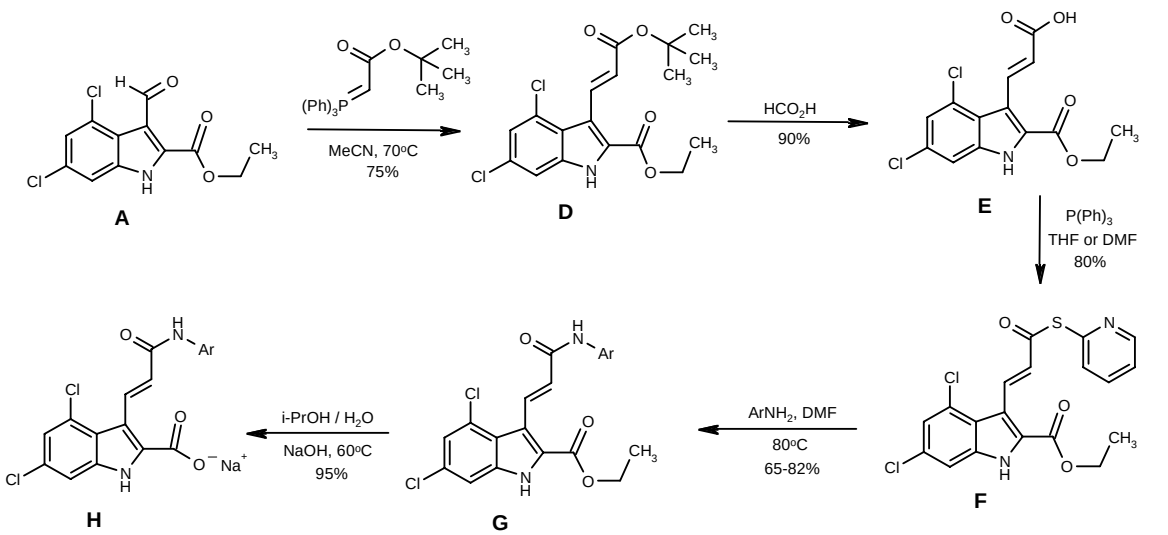
Compounds endowed with high affinity at the glycine binding site (pK_i >8) were further characterized following the initial screening sequence (activity in the *in vitro* functional assay, selectivity in binding assays and preliminary *in vivo* pharmacological profile in the NMDA-induced convulsions model in mice, by i.v. route). As shown in Table III, GV150526 was found to be highly potent in NMDA-induced convulsions both in mice (ED_{50} = 0.06 mg/kg) and rats (ED_{50} = 0.38 mg/kg). This anticonvulsant effect was completely reversed when the glycine agonist D-serine was given intracerebroventricularly, confirming the glycinergic mechanism of action for this novel class of compounds.

The activity of GV150526 in the functional assay was consistent with the glycine antagonist mechanism, as shown by the competitive antagonism on the glycine-mediated enhancement of the NMDA response in a range of potency comparable to its affinity in binding assays

Scheme 1



Scheme 2



(pA_2 = 8.13, A_2 = 7 nM). Finally, this glycine antagonist was found to be highly selective since no activity was found when tested at 10 μM on binding assays for 40 different brain neurotransmitter receptors (including additional sites on the NMDA receptor).

Neuroprotective action of GV150526

Based on the promising results described above, GV150526 progressed further along the screening sequence to assess its neuroprotective profile.

The neuroprotective action of GV150526 was tested in the permanent MCA occlusion model in rats. This method is the most widely used to generate a reproducible and consistent focal ischemia. The unilateral occlusion of the MCA induces a well-localized brain infarct, mainly distributed in the cerebral cortex, in particular in the area of dysgranular, insular and parietal cortex. To examine the neuroprotective effects of GV150526, groups of animals were intravenously administered the compound both pre- and postischemia. The results in Table IV show a clear neuroprotective effect of GV150526 after i.v. administration.

In the preischemia protocol, a dose-dependent effect of GV 150526 was found in the dose range of 0.3-3 mg/kg i.v. A maximal protection of 78% was observed at 3 mg/kg and an ED_{50} value of 0.76 mg/kg was found, which was in good agreement with the convulsion data in rats (ED_{50} = 0.38 mg/kg). An additional experiment at 10 mg/kg i.v.

Table III: Summary of the results of the preliminary pharmacological characterization of GV150526.

Type of study	Assay	Results	Remarks
Affinity	[^{3}H]-Glyciine binding	pK_i = 8.49 ± 0.02	Rat cortical membranes
Selectivity	Receptogram (40 brain receptors)	No displacement at 10 μM	
Functional activity	Glycine modulation of NMDA-mediated [3H]-TCP binding	pA_2 = 8.13	Comoetitive antagonism
Systemic activity	NMDA-induced convulsions in mice	ED_{50} = 0.06 mg/kg i.v. (0.005-0.42)	Reversed by glycine agonist (D-serine)
	NMDA-induced convulsions in rats	ED_{50} = 0.38 mg/kg i.v. (0.03-1.34)	

Table IV: Summary of the pre- and postischemia effect of GV150526 in the MCAo model in rats.

Type of Study	Protocol	Infarct volume (mm^3 ± SEM)[a]	Damage reduction (%)	Remarks
	Dose (mg/kg i.v.)			
Preischemia (5 min)	Vehicle	72.6 ± 17.4	0%	
			19%	
	0.3	59.1 ± 15.7	73%	ED_{50} = 0.76
	1	19.4 ± 5.3*		(0.25-1.51)
	3	16.8 ± 2.7*		
Post ischemia (3 mg/kg i.v.)	Vehicle	78.3 ± 14.5	0	
	1**	13.4 ± 1.8*	83%	
	3**	27.7 ± 5.9*	65%	
	6**	36.8 ± 9.5*	53%	

Measured 24 h after occlusion; *p < 0.05 vs. Vehicle; **time after occlusion.

was performed, but this dose did not further improve the neuroprotective effect. These results indicated that 3 mg/kg i.v. should be considered as the most effective dose.

In the postischemia studies, different time points after occlusion (1, 3, 6 h) and three different doses were considered. As seen in Table IV, there was a significant effect for all postocclusion injection times, even when administration of GV150526 was delayed up to 6 h. A single bolus dose of 3 mg/kg reduced the infarct volume by 83, 65 and 53% at 1, 3 and 6 h postadministration, respectively. No significant effects were seen with doses of 0.3 and 1 mg/kg. It should be pointed out that with the 3 mg/kg dose, compared with vehicle, no significant change was observed in the physiological variables which could affect brain damage (*i.e.*, temperature, blood flow).

A comparative study with reference compounds such as MK-801 (NMDA channel blocker) and 5,7-dichloro kynurenic acid (glycine antagonist) was performed (45, 46). The results of this study are shown in Figure 4. GV150526 had a better neuroprotective profile compared to both reference compounds. Preischemia, GV150526 was found to be roughly equieffective to MK-801 (78% *vs.* 72% maximal effect), but when tested postischemia, a much longer therapeutic window (6 h *vs.* 1 h) was observed. GV150526 was more efficacious than 5,7-dichloro kynurenic acid (78% *vs.* 51%). As shown in Figure 5, it seems that administration of GV150526

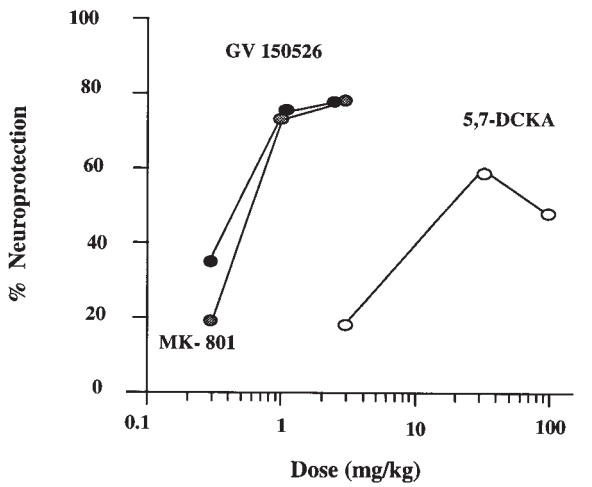


Fig. 4. Preischemia neuroprotective profile of GV150526 in the middle cerebral artery occlusion model in rats.

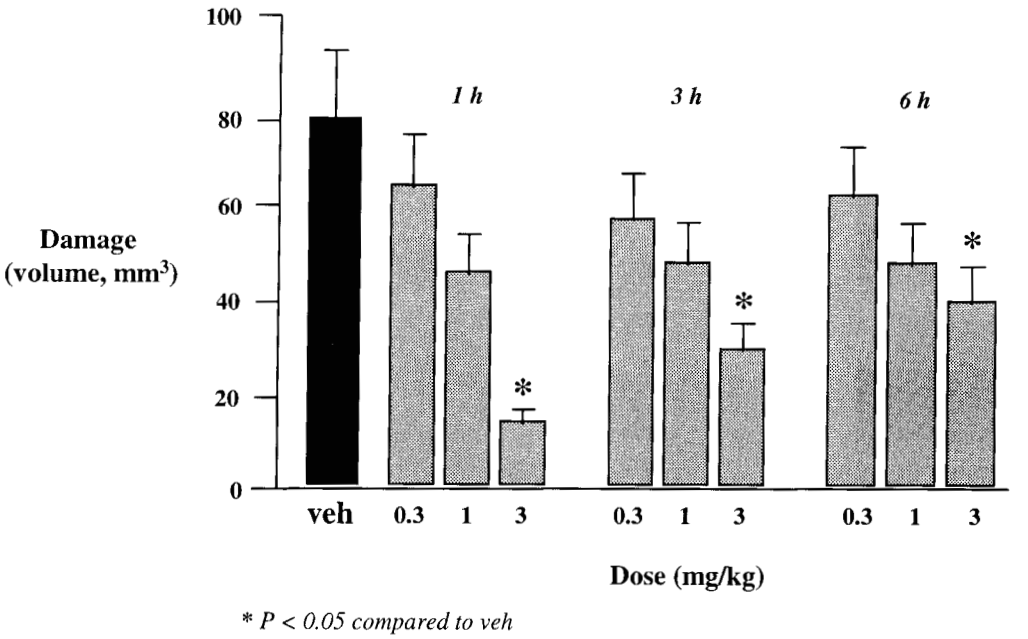


Fig. 5. Postischemia neuroprotective profile of GV150526 in the middle cerebral artery occlusion model in rats.

postischemia can cause a progressive decline of the drug's neuroprotective efficacy, despite maintaining a significant effect at all time points tested. Since stroke is a dynamic event, by delaying GV150526 administration, more brain tissue was lost reducing the number of salvageable neurons. In this scenario, the observed decline could simply reflect the rate of damage progression. To test this hypothesis, an experiment was performed in which all animals were treated 6 h after occlusion: half received vehicle (control group) and half 3 mg/kg i.v of GV150526. Animals from both groups were analyzed for damage size 24 h after occlusion (Fig. 6).

Following the time course of the brain damage depicted in Figure 6, when treatments were given at 6 h because of damage progression, the amount of salvageable neurons was reduced by about 50% (*i.e.*, 50% neurons were already lost at the time of administration). In vehicle-treated rats, damage progressed up to its full extension (achieved within 9-12 h). In contrast, in GV150526-treated rats, damage was completely halted. The amount of preserved neurons measured at 24 h was slightly above the amount of salvageable neurons at the time of administration.

These results suggest that when GV150526 was injected as a single i.v. bolus, a complete blockade of

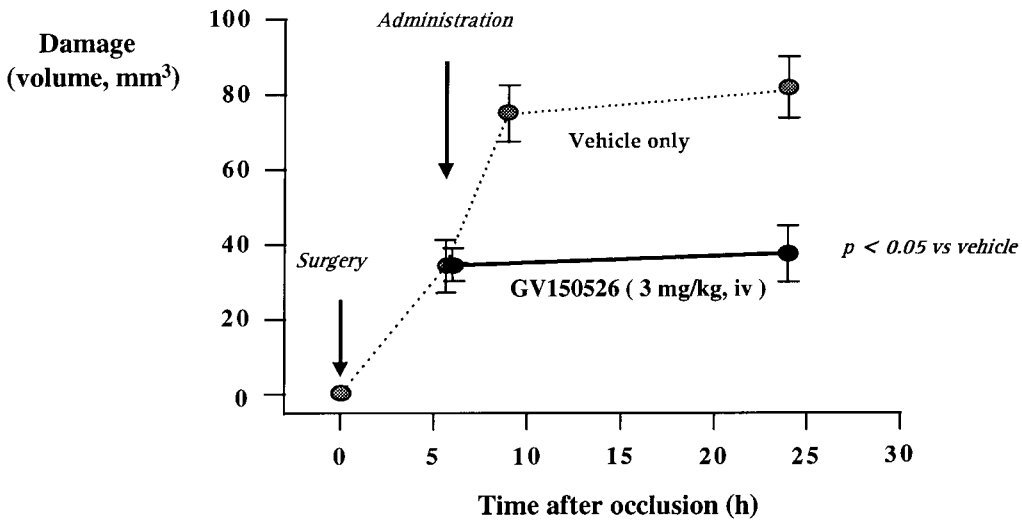


Fig. 6. Neuroprotective effect of GV150526 at different times after middle cerebral artery occlusion.

damage progression was achieved, and as hypothesized above, the apparent decline in efficacy of GV150526 with time was due to normal damage progression. Therefore, the compound fully retains its activity even at delayed postocclusion times.

Conclusions

Stroke is associated with a high unmet need for medical treatment due to the high incidence, particularly in Western countries, and the absence of effective neuroprotective drugs able to block the progression of brain damage after stroke onset. In this regard, great hope has been placed in the excitatory amino acid approach, and several NMDA antagonists are among the most advanced compounds in development as potential drugs.

The recognition of the key role of glycine in the modulation of the NMDA receptor and the clear side effects profile (7) observed for glycine antagonists with respect to competitive and noncompetitive NMDA antagonists, has prompted us to design and evaluate new ligands of the glycine binding site associated with the NMDA receptor. The indole-2-carboxylate derivative GV150526 has been identified as a potent, selective and systemically active glycine antagonist, showing an excellent neuroprotective profile in animal models of stroke (MCAo model in rats) when administered both pre- and postischemia. This compound was found to be devoid of relevant side effects, confirming the hypothesis that the modulation of the glycine binding site associated with the NMDA receptor might be a safer approach for blocking the ischemia-induced neurodegeneration after onset of stroke. In view of its outstanding profile, GV150526 represents a good tool to confirm the glycinergic hypothesis in man and, if successful, could become an important drug for the treatment of acute stroke.

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