Prevention of in Vivo Excitotoxicity by a Family of Trialkylglycines, a Novel Class of Neuroprotectants

CARMINA MONTOLIU, MARC HUMET, JUAN-JOSÉ CANALES, JOZEF BURDA, ROSA PLANELL-S-CASES, FRANCISCO SÁNCHEZ-BAEZA, TERESA CARBONELL, ENRIQUE PÉREZ-PAYÁ, ANGEL MESSEGUER, ANTONIO FERRER-MONTIEL, and VICENTE FELIPO

Laboratory of Neurobiology, Instituto de Investigaciones Citológicas, Fundación Valenciana de investigaciones Biomedicas, Valencia, Spain (C.M., J.-J.C., J.B., V.F.); Institut d'Investigacions Químiques i Ambientals de Barcelona, Consejo Superior de Investigaciones Científicas, Barcelona, Spain (M.H., F.S.-B., A.M.); Centro de Biología Molecular y Celular, Universidad Miguel Hernández, Alicante, Spain (R.P.-C., A.F.-M.); and Departamento de Bioquímica y Biología Molecular, Universidad de Valencia, Valencia, Spain (T.C., E.P.-P.)

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ABSTRACT

Excitotoxicity has been implicated in the etiology of ischemic stroke and chronic neurodegenerative disorders. Hence, the development of novel neuroprotectant molecules that ameliorate excitotoxic brain damage is vigorously pursued. We used a neuroprotection-based cellular assay to screen a synthetic combinatorial library of *N*-alkylglycine trimers. Two compounds (6-1-2 and 6-1-10) that efficiently prevented excitotoxic neurodegeneration in vitro and in vivo were identified. Both molecules protected primary cultures of cerebellar neurons against glutamate-induced neuronal death with an efficiency equivalent to *N*-methyl-D-aspartate (NMDA) receptor antagonists. These trialkylglycines did not block appreciably the NMDA receptor channel, or attenuated glutamate-induced increase of Ca^{2+} , or affect the glutamate-nitric oxide-cGMP pathway. Intraperito-

neal injection of both peptoids in mice attenuated $\geq 80\%$ ammonia-induced, NMDA receptor-mediated animal death. Furthermore, these two molecules reduced by $\geq 50\%$ the neurodegeneration in striatum in a rat model of cerebral ischemia. Neuroprotection against ischemia was associated with decreased activation of caspase-3, reflecting prevention of apoptotic neuronal death. Collectively, the results reported indicate that these trialkylglycines are new neuroprotectant leads with important in vivo activity against excitotoxicity, and that they act on a novel, yet-unrecognized cellular target. These lead compounds may become tolerated drugs for the treatment of acute and chronic neurodegenerative diseases with fewer side effects than NMDA receptor antagonists.

Glutamate is considered the main excitatory neurotransmitter in mammals. However, excessive activation of glutamate receptors, particularly of the NMDA receptor subtype, leads to neuronal degeneration and death (Choi and Rothman, 1990). Glutamate neurotoxicity is involved in the neuronal damage found in cerebral ischemia, as well as in the pathogenesis of different neurodegenerative diseases, including amyotrophic lateral sclerosis, and Huntington, Alzheimer, and Parkinson diseases (Choi and Rothman, 1990; Kromer et al., 1998). Although the underlying mechanisms for the selective vulnerability of neurons are unknown, a widely held view considers that excessive activation of NMDA re-

ceptor mediates a massive influx of Ca^{2+} , which induces different effects, including alterations of the mitochondrial potential and formation of nitric oxide and cGMP. These alterations lead ultimately to cell death (Choi, 1987; Dawson et al., 1991; Dawson et al., 1993; Mattson et al., 1993; Montoliu et al., 1999).

Because of their central role in neurodegeneration, NMDA receptors have been considered prime therapeutic targets for the development of useful neuroprotective strategies (Bräuner-Osborne et al., 2000). Accordingly, a significant effort has been made to develop high-affinity and selective NMDA antagonists that target the different drug binding sites on this receptor. Although most of these molecules efficiently reduce glutamate neurotoxicity in vitro, their in vivo utility has been heavily questioned due to serious side effects at clinically effective doses (Choi and Rothman, 1990; Morris and Davis, 1995; Bräuner-Osborne et al., 2000). The high receptor affinity of known NMDA receptor antagonists appears to be a major shortcoming because these compounds

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; MK-801, (SR,10S)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine hydro-gen maleate.

bind to both pathologically activated and physiologically working NMDA receptor populations (Choi and Rothman, 1990; Kroemer et al., 1998). Because NMDA receptors are implicated in learning and memory, inhibition of glutamatergic neurotransmission may underlie the cognitive deficits provoked by high-affinity NMDA receptor antagonists (Morris and Davis, 1995). In addition, it has been reported that chronic administration of these antagonists enhances neuronal death (Ikonomidou et al., 2000; Olney et al., 2000). Therapeutic alternatives that may significantly overcome these deficiencies involve the use of uncompetitive NMDA antagonists (Lipton and Rosenburg, 1994; Ferrer-Montiel et al., 1998), NMDA receptor subtype-specific blockers (Chenard and Menniti, 1999; Bräuner-Osborne et al., 2000), or NMDA receptor-directed oral vaccines (During et al., 2000).

Alternatively, molecules that prevent neuronal death by acting on other cellular targets involved in neurodegeneration onset may be therapeutically useful to ameliorate the harmful consequences of excitotoxicity. These compounds may be devoid of the adverse in vivo effects of well established NMDA antagonists. To address this issue, we have used a neuroprotection-based cellular assay to identify neuroprotectant molecules from a combinatorial library of oligo *N*-substituted glycines (Fig. 1). At variance with biological assays that focus on receptor activity, we have evaluated the efficiency of combinatorial mixtures preventing glutamate-induced neuronal death directly on primary cultures of cerebellar neurons. Library deconvolution identified a family of trialkylglycines that efficiently reduced excitotoxic cell death in cultured neurons (Fig. 2). Noteworthy, intraperitoneal administration of compounds *N*-[2-(*N*-pyrrolidinyl)ethyl]glycyl]-[*N*-(cyclopropyl)glycyl]-*N*-(*sec*-butyl)glycinamide (referred to as 6-1-2) and [*N*-[2-(*N*-pyrrolidinyl)ethyl]glycyl]-[*N*-(cyclopropyl)glycyl]-*N*-(2-phenethyl)glycinamide (denoted as 6-1-10) significantly protected mice against ammonia-induced, NMDA receptor-mediated animal death. In addition, these two peptoids remarkably prevented the neuronal apoptosis induced by cerebral ischemia in rats. Both compounds are novel neuroprotectants that act on an as yet-unrecognized cellular target. Because these neuroprotectant peptoids do not block the NMDA receptor, the psychotropic effects displayed by antagonists of this ionotropic receptor may be avoided.

Experimental Procedures

Materials

Basal Eagle's medium, fetal bovine serum, and gentamycin were from Invitrogen (Rockville, MD). DNase I (E.C. 3.1.21.1) and dispase II were from Roche Applied Science (Mannheim, Germany). Glutamate and NMDA were from Sigma Chemical (St. Louis, MO). Fluorescein diacetate, propidium iodide, and fluo-3/AM were from Molecular Probes (Leuven, Belgium). Culture plates were from Costar (Cambridge, MA). The kit for cGMP determination was from Amersham plc (Little Chalfont, Buckinghamshire, UK).

Fig. 1. Chemical structure of the library of *N*-alkylglycine trimers.

Synthesis of Library of *N*-Alkylglycines

A library of 10,648 compounds in 66 controlled mixtures was synthesized by using the positional scanning format in solid phase (Pinilla et al., 1992; Figliozzi et al., 1996). In our case, a collection of 22 commercially available primary amines was used for introducing the desired chemical diversity in the library. The details of this synthesis will be described elsewhere (M. Humet, T. Carbonell, F. Sánchez-Baeza, P. Mora, E. Cantón, M. Bobernado, E. Percz-Paya, and A. Messegueur, manuscript in preparation). Briefly, starting from Rink amide resin (Rapp Polymere, 0.7 meq) the eight-step synthetic pathway involved the initial release of the Fmoc protecting group. Then the successive steps of acylation with chloroacetyl chloride followed by the corresponding amination of the chloromethyl intermediate using the particular primary amine, or the equimolecular mixture of the 22 amines was conducted as appropriate. All these reactions were carried out in duplicate. Finally, the products were released from the resin by using a trifluoroacetic acid/dichloromethane/water mixture, solvents were evaporated, and the residues were lyophilized and dissolved in 10% dimethyl sulfoxide at the concentration of 10 mg/ml for screening of neuroprotection.

Synthesis of Individual Neuroprotective Trialkylglycines

The results from the screening of the 66 mixtures of the peptoid library led to a deconvolution process from which the synthesis of 12 individual molecules was derived. These trialkylglycines were prepared on solid phase following the same synthetic scheme but using the *N*-hydroxysuccinimide ester of chloroacetic acid as acylating agent and the appropriate amines for the amination steps. The screening of these library members in the in vitro test led to the final selection of two trimers (612 and 6110; Fig. 2) as the most active candidates for performing the in vivo assays. Crude compounds 612 and 6110 obtained from the release of the resin were purified by preparative reversed phase high-performance liquid chromatography using a 250 × 20-mm column packed with 5-μm Kromasil C-8. Methanol and water, both containing 0.1% trifluoroacetic acid, were used as a mobile phases at 6 ml/min, under the following elution conditions: 20:80 methanol/water during 2 min and then gradient up to 100% methanol in 30 min. Eluates were collected, methanol was removed under vacuum, and the residues were lyophilized to render the desired compounds (>95% purity by high-performance liquid chromatography).

612. ¹H-NMR (500 MHz, 10°C in D₂O, mixture of conformers), δ from N-terminal to C-terminal: 2.04–1.89 (m, 4H, CH₂CH₂N pyrrolidine), 3.01–3.61 (m, 4H, CH₂N pyrrolidine), 3.52 (t, 2H, CH₂CH₂NH), 3.45 (t, 2H, CH₂NH), 4.31 (s, 2H, CH₂CO), 2.73 (m, 1H, CHN), 0.69–0.80 (m, 4H, (CH₂)₂CHN), 4.35 (s, 2H, CH₂CO), 3.73 (m, 1H, CH₃CHCH₂CH₃), 1.43 (m, 2H, CH₃CHCH₂CH₃), 0.76 (m, 3H, CH₃CHCH₂CH₃), 1.07 (m, 3H, CH₃CHCH₂CH₃), 3.72–3.89 (m, 2H, CH₂CO). ¹³C-NMR (500 MHz, 10°C in D₂O), δ from N-terminal to C-terminal: 24.8 (CH₂CH₂N pyrrolidine), 57.2 (CH₂N pyrrolidine), 52.2 (CH₂CH₂NH), 45.2 (CH₂NH), 48.0 (CH₂CO), 171.0 (CO), 10.1 ((CH₂)₂CHN), 32.5 (CHN), 51.9 (CH₂CO), 172.1 (CO), 56.8 (CH₃CHCH₂CH₃), 29.4 (CH₃CHCH₂CH₃), 12.5 (CH₃CHCH₂CH₃), 19.8 (CH₃CHCH₂CH₃), 46.0 (CH₂CO), 176.0 (CO). HRMS: MS Calculated for C₁₉H₃₆N₅O₃ 382.281815. Found: 382.282518.

6110. ¹H-NMR (500 MHz, 10°C in D₂O, mixture of conformers), δ from N-terminal to C-terminal: 2.01–2.17 (m, 4H, CH₂CH₂N pyrrolidine), 3.11–3.72 (m, 4H, CH₂N pyrrolidine), 3.59 (t, 2H, CH₂CH₂NH), 3.50 (t, 2H, CH₂NH), 4.31 and 4.38 (s, 2H, CH₂CO), 2.67 and 2.80 (m, 1H, CHN), 0.58–0.92 (m, 4H, (CH₂)₂CHN), 3.88 and 4.22 (s, 2H, CH₂CO), 7.30–7.42 (m, 5H, H Arom), 2.95–2.84 (m, 2H, CH₂CH₂NH), 3.59–3.67 (m, 2H, CH₂N), 4.05 (s, 2H, CH₂CO). ¹³C-NMR (500 MHz, 10°C in D₂O), δ from N-terminal to C-terminal: 21.7 (CH₂CH₂N), 54.0 (CH₂N), 48.8 (CH₂CH₂NH), 41.9 (CH₂NH), 48.0 (CH₂CO), 167.8 (CO), 7.0 ((CH₂)₂CHN), 29.4 (CHN), 48.3 and 48.4 (CH₂CO), 169.1 and 169.0 (CO), 49.4 (CH₂CH₂N), 32.6 (CH₂N), 137.3 (C arom), 128.3 (CH *orto* arom), 128.2 (CH *meta* arom), 126.3

Fig. 2. Structures of 6-1-2 and 6-1-10, two representative neuroprotective agents derived from the library of *N*-alkylglycine trimers.

(CH *para* arom), 47.9 (CH₂CO), 172.1 (CO). HRMS: MS calculated for C₂₃H₃₆N₅O₃ 430.281815. Found: 430.283409.

Primary Cultures of Neurons

Primary cultures of cerebellar neurons were prepared using cerebella from 7- to 8-day-old Wistar rats as described (Miñana et al., 1998). Neurons were grown at 37°C in 5% CO₂ atmosphere. To prevent proliferation of non-neuronal cells, 10 μM cytosine arabinoside was added 24 h after plating. Glucose, 5.6 mM final concentration, was added to the culture medium twice a week.

Assay of Protection against Glutamate Neurotoxicity

Glutamate toxicity in cerebellar neurons was assayed after 11 to 15 days of culture. Briefly, culture medium was removed and kept at 37°C (conditioned medium). Cells were washed and incubated at 37°C for 20 min with Mg²⁺-free Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO₃, 2.3 mM CaCl₂, 5.6 mM glucose, 5 mM HEPES, pH 7.4), containing 10 μM glycine. Thereafter, this solution was removed and cells were incubated with 1 mM glutamate in Mg²⁺-free Locke's solution without glycine at 37°C for 4 h. Preincubation with glycine was convenient to obtain more reproducible results when comparing different culture preparations. Individual peptoids were added 20 min before glutamate addition. Cells were washed with Mg²⁺-free Locke's solution and the conditioned medium previously removed was added again. Cell viability was measured 24 h later by staining with fluorescein diacetate and propidium iodide as previously described (Felipo et al., 1993). The percentage of surviving neurons was calculated by assessing the ratio of fluorescein diacetate/propidium iodide (green/red) staining directly under the microscope. At least 1200 cells were counted for each data point.

Determination of cGMP in Cultured Neurons

Neurons were used 11 to 15 days after seeding. Monolayers were washed three times with prewarmed Mg²⁺-free Locke's solution. The assay of NMDA-induced formation of cGMP was carried out in the same medium. Treatments with 1 mM NMDA were at 37°C for 5 min. cGMP was determined using the BIOTRAK cGMP enzyme immunoassay kit from Amersham plc as described (Montoliu et al., 1999). For each experiment, samples were measured in duplicate.

Determination of Free Intracellular Calcium

Changes in intracellular free Ca²⁺ were monitored in single neurons using an ACAS 570 confocal laser cytometer (Meridians Instruments, Okemos, MI). Primary cultures of cerebellar neurons were plated on 35-mm tissue culture dishes and loaded with 20 μM Fluo-3/AM in the culture medium at 37°C for 1 h. Thereafter, the cells were washed twice with prewarmed Mg²⁺-free Locke's solution, and 1 ml of the same solution was added. To study the effects of the peptoids on free intracellular Ca²⁺ levels, each peptoid was added to the medium, the fluorescence was recorded for the time desired, and 250 μM glutamate or NMDA was then added. Ca²⁺ levels were monitored continuously. Free intracellular calcium was quantified using the confocal laser cytometer as previously described (Marcaida et al., 1995a). Each experiment was repeated at least four times with three different neuronal cultures.

Prevention of Ammonia-Induced, NMDA Receptor-Mediated Death of Mice

Acute ammonia intoxication leads to excessive activation of NMDA receptors in brain (Hermenegildo et al., 2000), which is responsible for ammonia-induced death of animals (Marcaida et al., 1992; Hermenegildo et al., 1996). Male Swiss mice (25–35 g) were injected i.p. with 14 mmol/kg (3 μl/g) of ammonium acetate. To assess the protective effect of peptoids, these were injected i.p. 10 min before ammonium injection. The number of mice surviving 24 h after injection was counted.

Transient Bilateral Hemispheric Ischemia: Surgery and Treatments with Peptoids

For the transient forebrain ischemia experiments, we used the four-vessel occlusion model of Pulsinelli and Brierley (1979) with the modifications of Schmidt-Kastner et al. (1989). Briefly, rats were anesthetized with 120 μg/g ketamine and 15 μg/g xylazine and placed in a rat holder. Vertebral arteries were coagulated permanently at the level of the lateral vertebral foramen with the aid of an electrocauter device. After 24 h, we clamped temporarily (15 min) the two common carotids under halothane anesthesia. Rats received i.p. injections of the peptoids (50 μg/g; 2.5 μl/g) three times: 15 min before occlusion of the carotids, immediately after removal of the carotid clamp, and at 24 h of reperfusion. After a 7-day survival period, rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in phosphate buffer (0.1 M NaKPO₄). The brains were removed and stored in 30% sucrose in phosphate buffer until further processing.

Detection of Neurodegeneration by Silver Impregnation

For the detection of neuronal somata, axons, and terminals undergoing degeneration, free-floating 50-μm-thick sagittal sections were stained with a silver impregnation protocol (FD NeuroSilver kit I; FD Neurotechnologies, Baltimore, MD) (Gallyas et al., 1992; Beltramino et al., 1993). Degenerating neurons and axons appeared stained black against a background of relatively spared neural processes stained yellow/brown (Fig. 7).

Detection of Neurons Showing Activated Caspase-3

Free-floating 25-μm-thick sagittal sections of the same brains were treated with standard single-antigen immunohistochemical methods. Sections were exposed to hydrogen peroxide, blocked, and incubated overnight with antiserum raised against the large fragment of activated caspase-3, resulting from cleavage after aspartic acid at position 175 (1:100; Cell Signaling Technology Inc., Beverly, MA). Sections were then incubated with biotinylated secondary antiserum and with the avidin-biotin complex (ABC kit; Vector Laboratories, Burlingame, CA). The horseradish peroxidase reaction products were visualized with diaminobenzidine and intensified with nickel sulfate, appearing as discrete black precipitates (Fig. 8).

Quantification

For the sections stained with the silver impregnation method, we estimated the area in the dorsal striatum showing dense silver precipitates. Between three and five sections were averaged per animal. For the sections processed for immunohistochemistry, caspase-3-positive cells were counted within a rectangular area of ca. 1.5 mm² at the level of the dorsal striatum, caudally (Fig. 7). At least four adjacent sections were averaged per animal. Estimations of ischemic injury as measured by the silver method and counts for active caspase-3 immunoreactivity were carried out blind to the experimental treatments.

Statistics

Results of Figs. 3, 4, and 6 were analyzed by one-way analysis of variance followed by post hoc Dunnett's test or Student's *t* test using the PRISM program (version 2.0; GraphPad Software, San Diego, CA) and the results of Fig. 8 were analyzed by Tukey-Kramer test.

All animal experiments were carried out in accordance to the European Union Guidelines for Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering.

Results

Identification of Novel Neuroprotectant Molecules from a Library of Trialkylglycines. A positional scanning, mixture-based combinatorial library composed of trimers of *N*-alkylglycines was screened to identify molecules that prevent glutamate-induced neuronal death in primary cultures of cerebellar neurons. The design of this library contained two distinctive features. First, the use of the positional scanning format strategy for its construction. Second, the selection of the diversity included the use primary amines bearing an additional tertiary amino moiety. The introduction of these amines conducted to trimers containing additional protonable fragments, which could complement the activity pattern and bioavailability of the library members. The general structure of the trimers is shown in Fig. 1. The library was organized in 66 mixtures, each containing 484 molecules, giving rise to a chemical diversity of 10,648 individual trialkylglycines. Screening of the 66 mixtures

Fig. 3. Protective effect of peptides against glutamate neurotoxicity in primary cultures of cerebellar neurons. Primary cultures of cerebellar neurons were used 10 to 14 days after seeding. Neurons were treated with 1 mM glutamate after preincubation for 15 min with 50 μ g/ml of the indicated peptides. Neuronal viability was determined as indicated under *Experimental Procedures*. At least 1200 cells/point were counted in each assay. Values are the mean $\pm$ standard deviations of six assays using five different cultures. Values that are significantly different from controls ($p < 0.001$) are indicated by asterisks.

identified the preferred diversity at the three separate positions (R_1 , R_2 , and R_3) of the oligomer (Fig. 1). At the R_1 position the selected amines were cyclopropyl (denoted as amine 1) and 2-*N*-pyrrolidinyl (denoted as amine 6); at the R_2 position were cyclopropyl and 2-(*N*-morpholino)ethyl (denoted as amine 18); and at the R_3 position were *sec*-butyl (denoted as amine 2), 2-phenylethyl (amine 10), and 2,4-(aminosulphonylphenyl)ethyl (amine 17).

When used in concert, these results suggest the chemical identity of potential neuroprotectant molecules. Hence, we synthesized a family of the individual peptoids containing the different combinations of the above-mentioned amines and assayed their activity preventing glutamate-induced neuronal death. The structures of two of these compounds are shown in Fig. 2. As illustrated in Fig. 3, several of these peptoids exhibited remarkable neuroprotective activity at 50 μ g/ml (≈ 100 μ M).

Because most of the active peptoid exhibit a similar neuroprotective efficacy, further characterization was focused on two of these peptoids, 6-1-2 and 6-1-10. These compounds were purified and rigorously characterized by analytical and spectroscopic means.

Dose-response studies showed that concentrations as low as 1 μ g/ml (≈ 2 μ M) prevented 20% neuronal culture neurodegeneration. The effect was maximal at 50 μ g/ml (≈ 100 μ M) with a 70% neuroprotection (Fig. 4A). For compound 6-1-2 the IC_{50} (concentration of peptoid that prevented half of maximal neurodegeneration) was ≈ 8 μ M and for 6-1-10 was ≈ 15 μ M. Therefore, these newly identified trialkylglycine-based molecules are potent neuroprotectants.

Trialkylglycine-Based Neuroprotectants Do Not Inhibit Activation of NMDA Receptor. To gain insights on the mechanism underlying the neuroprotective activity of these trialkylglycines, we investigated whether they act as antagonists of NMDA receptors. We determined whether these peptoids reduced the raise in intracellular Ca^{2+} induced by activation of NMDA receptor by L-glutamate or

Fig. 4. Concentration dependence of the protective effect of peptides on glutamate neurotoxicity in primary cultures of cerebellar neurons. Primary cultures of cerebellar neurons were used 10 to 14 days after seeding. Neurons were treated with 1 mM glutamate after preincubation for 15 min with the indicated concentrations of the different peptides and cell viability was determined as indicated under *Experimental Procedures*. At least 1200 cells/point were counted in each assay. Values are the mean $\pm$ standard deviations of five to six assays using four to six different cultures. Values that are significantly different from controls ($p < 0.001$) are indicated by asterisks.

NMDA. As depicted in Fig. 5, the NMDA receptor-mediated increase in intracellular Ca^{2+} was not significantly altered by addition of compounds 6-1-2 and 6-1-10 to the extracellular medium before and during treatment with the agonist. The lack of inhibitory effect was independent of the agonist used because similar results were obtained with glutamate or NMDA. In contrast, addition of MK-801 drastically prevented Ca^{2+} influx (Fig. 5). Consistent with this notion, both peptoids did not significantly block the glutamate-evoked ionic currents from recombinant rat brain NMDA receptors heterologously expressed in *Xenopus* oocytes ($25 \pm 5\%$ at 50 μM ; data not shown). These findings imply that these peptoids do not inhibit NMDA receptor activity.

Trialkylglycines Do Not Alter Glutamate-Induced cGMP Production in Neurons. Activation of the NMDA receptor leads to production of cGMP through the glutamate-nitric oxide-cGMP pathway. It has been shown that inhibition of this signaling pathway prevents glutamate neurotoxicity (Dawson et al., 1991; Lafon-Cazal et al., 1993; Marcaida et al., 1995b) and that inhibition of glutamate-induced for-

mation of cGMP by using inhibitors of soluble guanylate cyclase prevents glutamate-induced neuronal death in cerebellar neurons in culture (Hermenegildo et al., 1998; Montoliu et al., 1999). To assess whether the protective peptoids interfere with any step of this pathway, we next investigated whether identified trialkylglycines decreased the production of cGMP in cultured cerebellar neurons treated with NMDA. As shown in Fig. 6, none of the peptoids affected the NMDA-induced production of cGMP. Therefore, the neuroprotective activity exerted by these molecules is not due to inhibition of any of the steps of this signaling pathway.

Trialkylglycines Prevent Ammonia-Induced, NMDA Receptor-Mediated Death of Mice. To assess the protective activity of identified peptoids in vivo, we next investigated whether compounds 6-1-2 and 6-1-10 were able to prevent death in an animal model of glutamate-mediated neurotoxicity. Intraperitoneal administration of high doses of ammonium acetate leads to animal death. Ammonia lethality is mediated by excessive activation of NMDA receptors (Hermenegildo et al., 1996, 2000). We used this model to evaluate

Fig. 5. Peptoids do not prevent glutamate- or NMDA-induced increase in free intracellular Ca^{2+} in neurons. Primary cultures of cerebellar neurons were prepared using 35-mm-diameter tissue culture dishes and were used 10 to 14 days after seeding. Free intracellular calcium content was determined with fluo-3/AM using a confocal microscope. For controls the basal calcium level was recorded for 200 s, then the agonist (250 μM NMDA or glutamate) was added (indicated by vertical line), and the fluorescence was recorded for the indicated time. For assaying the effect of peptoids or MK-801 the basal calcium level was recorded for 200 s. Peptoid (50 $\mu\text{g/ml}$) or MK-801 (1 μM) was then added (first vertical line), and the fluorescence was recorded for 600 s, followed by addition of the agonist (second vertical line).

Fig. 6. Effects of peptoids on NMDA-induced formation of cGMP in neurons. Neuronal cultures were used 10 to 14 days after seeding. Cells were washed in Mg^{2+} -free Locke's solution and preincubated with the same solution for 20 min at 37°C. Then 50 $\mu g/ml$ of the indicated peptoids were added and incubated for 15 min before addition of NMDA (1 mM final concentration). Basal and NMDA-induced formation of cGMP were measured as indicated under *Experimental Procedures*. For each experiment, the basal level of formation cGMP in control neurons was considered as 100%. Values are the mean $\pm$ standard deviations of duplicate samples from five experiments using four different cultures. All values with NMDA alone NMDA + peptoid are significantly different ($p < 0.001$) from basal.

the in vivo protective activity of trialkylglycines 6-1-2 and 6-1-10 (Table 1). Notably, intraperitoneal injection of 50 $\mu g/g$ peptoid 6-1-2, 10 min before injection of ammonia, afforded a

TABLE 1
Peptoids 6-1-2 and 6-1-10 prevent ammonia-induced, NMDA receptor-mediated death of mice

Male Swiss mice weighing 25 to 35 g were used. Peptoids were injected intraperitoneally 10 min before ammonium injection. Ammonium acetate (14 mmol/kg) was also injected intraperitoneally. The concentrations of the solutions were adjusted to reach the desired dose by injecting 3 μl per gram of body weight. The number of surviving mice was counted 24 h after ammonium injection. All animals surviving at this time remained alive for several weeks until they were sacrificed.

Compound Injected	Number of Mice Surviving/Injected	Survival
		% total
None	5/30	16
6-1-10	24/29	82
6-1-2	24/24	100

complete protection against ammonia-induced death of mice (24 of 24 mice survived to ammonia intoxication), whereas only 16% of mice injected with saline survived. Similarly, treatment with 50 $\mu g/g$ peptoid 6-1-10 protected 82% of mice. These results are comparable to those reported for NMDA receptor antagonists (Hermenegildo et al., 1996, 2000). Thus, identified peptoids exhibit in vivo neuroprotectant activity.

Trialkylglycines Prevent Ischemic Neurodegeneration in Rats. To further characterize the in vivo activity of these peptoids, we evaluated their beneficial effect in preventing the neuronal damage provoked by cerebral ischemia. For these experiments, peptoids (50 $\mu g/g$) were administered to rats 15 min before provoking the ischemic insult, as well as 1 and 24 h after the ischemia onset. Animals were sacrificed 7 days later and the extent of brain damage was evaluated. As shown in Fig. 7, the ischemic insult provoked by transient occlusion of the carotids produced extensive degeneration in the dorsal and caudal aspects of the caudoputamen, mainly

Fig. 7. Peptoids 6-1-2 and 6-1-10 prevent neurodegeneration induced in striatum by ischemia. Rats were subjected to transient ischemia, with or without treatment with peptoids 6-1-2 or 6-1-10 as described under *Experimental Procedures*. Degeneration neurons were visualized by the silver impregnation method and given the dark staining shown in c. a, diagram representing the rectangular area of the caudoputamen considered for quantification purposes. b, control rat. c, rat subjected to ischemia without treatments. d, rat treated with peptoid 6-1-10 and subjected to ischemia. e, rat treated with peptoid 6-1-2 and subjected to ischemia. Similar results were obtained in two different rats per group. CX, cerebral cortex; STR, striatum; HIP, hippocampus.

in the immediate environs of the lateral ventricle. In animals exposed to transient ischemia, areas of dark silver stain extended rostrally in the lateral, sensorimotor area of the caudoputamen. The administration of the peptoids notably prevented the neuronal damage provoked by ischemia, as revealed by the significant decrease in silver accumulation in the dorsal caudoputamen. Peptoid 6-1-10 reduced the estimated area of insult by 65% and peptoid 6-1-2 by 82% (Fig. 7).

It has been reported that many neurons undergo apoptosis after ischemia (Choi and Rothman, 1990). We questioned whether selected peptoids may also attenuate ischemia-induced neuronal apoptosis. For this task, we monitored the content of activated caspase-3 in the striatum of control and peptoid-treated rats that suffered an ischemic attack. As shown in Fig. 8, ischemia induced a significant activation of caspase-3 in control animals, reflecting induction of apoptosis in a large number of neurons. In marked contrast, animals treated with 50 $\mu\text{g/g}$ i.p. peptoids exhibited a significant reduction in caspase-3 activation in the same brain region. Noticeably, peptoid 6-1-2 decreased by 74% the number of apoptotic neurons, and peptoid 6-1-10 by 50% (Fig. 8E). Therefore, these trialkylglycines reduce the infarct volume after transient ischemia in rats.

Discussion

Neurodegeneration is a multifaceted process involving, among others, the unbalance of the glutamatergic system.

Prolonged exposure of neurons to moderate-to-high concentrations of L-glutamate irreversibly culminates in neuronal damage. It is generally accepted that the influx of Ca^{2+} as a result of excessive activation of the NMDA receptor underlies the toxic actions of glutamate in many systems. (S)- α -Amino-2,3-dihydro-5-methyl-3-oxo-4-isoxazolepropanoic acid receptors also mediate glutamate neurotoxicity under certain conditions (Choi and Rothman, 1990; Lee and Choi, 1999). The pivotal role assigned to NMDA receptors in neurodegeneration has signaled this channel protein as prime target for neuroprotectant drug development. However, much of the initial enthusiasm has declined because of poor performance of NMDA receptor antagonists in clinical trials (Choi and Rothman, 1990; Garthwaite, 1995). Apparently, the clinical unsuitability of these compounds may arise, at least partially, from their psychotomimetic effects, especially for slow-developing neurodegeneration (Choi and Rothman, 1990; Chase and Oh, 2000). Moreover, it has been reported that NMDA antagonists may enhance neuronal death in vivo (Ikonomidou et al., 1999; Olney et al., 2000). Accordingly, new therapeutic approaches that attenuate excitotoxicity should be evaluated and developed. To pursue this goal, we used a neuroprotection-based cellular assay suitable to screen combinatorial libraries composed either of mixtures or of individual compounds. At variance with target-directed screenings, our assay directly evaluates the efficiency of molecules attenuating glutamate-induced neuronal death. A sa-

Fig. 8. Peptoids 6-1-2 and 6-1-10 prevent activation of caspase-3 induced in striatum by ischemia. Rats were subjected to transient ischemia, with or without treatment with peptoids 6-1-2 or 6-1-10 as described under *Experimental Procedures*. Distribution of activated caspase-3 in parasagittal sections of the caudoputamen after ischemia treatments was detected using a specific antibody as described under *Experimental Procedures*. a, control rat. b, rat subjected to ischemia without treatments. c, rat treated with peptoid 6-1-10 and subjected to ischemia. d, rat treated with peptoid 6-1-2 and subjected to ischemia. Similar results were obtained in two different rats per group. e, inhibition of ischemia-induced expression of caspase-3 by peptoid treatments. Analysis of variance showed a significant effect of treatment [$F(3,9) = 12.976, p < 0.001$]. *, $p < 0.05$; **, $p < 0.01$ (Tukey-Kramer test).

lient application of this cellular assay is the identification of a novel class of trialkylglycine-based neuroprotectant molecules that significantly prevents excitotoxic neuronal death in vitro and in vivo models of neurodegeneration. Oligomers of *N*-substituted glycines provide a class of small, non-natural molecules that are proteolytically stable and have potent biological activities (Ostergaard and Holm, 1997). A major advantage of using short oligomers is that they usually display acceptable tissue penetration properties and better pharmacological conformities (Lipinski et al., 1997; Newton, 1999).

Two peptoids, 6-1-2 and 6-1-10, protected primary neuronal cultures against the deleterious glutamate neurotoxicity with efficiencies that rival those characteristics of NMDA receptor antagonists. Of significance, both peptoids at 50 μ g/g displayed remarkable neuroprotective activity in two in vivo models of acute excitotoxicity, namely, acute ammonia intoxication and transient ischemia. Thus, these novel peptoids may be considered leads for drug development.

The neuroprotective activity exerted by these peptoids does not appear to be mediated by blockade of the NMDA receptor or by attenuation of glutamate/NMDA-induced Ca^{2+} influx. These observations suggest that newly identified molecules may interfere with signaling steps downstream in the glutamate-nitric oxide-cGMP cascade. However, our data demonstrate that trialkylglycines did not alter glutamate-induced neuronal cGMP synthesis, thus ruling out inhibition of nitric oxide synthase or guanylate cyclase. Furthermore, we have also observed that neither of these compounds inhibit the enzymatic activity of caspase-3 (data not shown). The question that arises is, What is the molecular mechanism underlying the neuroprotective activity of selected peptoids? Our studies show that these peptoids prevent neuronal death without attenuating glutamate-mediated neuronal Ca^{2+} overload. Additional studies are required to unequivocally uncover the molecular entity targeted by these compounds.

The development of this new class of neuroprotectant molecules may provide novel therapies for the treatment of disorders involving excessive activation of glutamate receptors such as neuronal damage in ischemia and several neurodegenerative and psychiatric disorders. Because these molecules do not block the NMDA receptor they may not exhibit the unwanted side effects that appear after chronic administration of NMDA receptor antagonists. Furthermore, because these peptoids appear to block ischemia-induced apoptosis they might be also useful in a therapeutic approach directed to dual inhibition of excitotoxic necrosis and apoptosis (Lee and Choi, 1999).

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Address correspondence to: Vicente Felipo, Laboratory of Neurobiology, Instituto de Investigaciones Citológicas, FVIB, Amadeo de Saboya 4, 46010 Valencia, Spain. E-mail: vfelipo@ochoa.fib.es