SYNAPSE 26:95–103 (1997)

Priming of Cultured Neurons With Sabeluzole Results in Long-Lasting Inhibition of Neurotoxin-Induced Tau Expression and Cell Death

DANIELA UBERTI,[1] CLAUDIA RIZZINI,[1] PAOLA GALLI,[1] MARINA PIZZI,[1] MARIAGRAZIA GRILLI,[1] ANNE LESAGE,[2] PIERFRANCO SPANO,[1] AND MAURIZIO MEMO[1]*

[1]*Division of Pharmacology, Department of Biomedical Sciences and Biotechnologies, School of Medicine, University of Brescia, Brescia, Italy*

[2]*Department of Biomemical Pharmacology, Janssen Research Foundation, Beerse, Belgium*

KEY WORDS cerebellar granule cells; human neuroblastoma SH-SY5Y; glutamate; doxorubicin; Tau proteins; PCR

ABSTRACT Sabeluzole was described to have antiischemic, antiepileptic, and cognitive-enhancing properties, and is currently under development for Alzheimer's disease. Recently, it was reported that repeated treatments with sabeluzole protect cultured rat hippocampal neurons against NMDA- and glutamate-induced neurotoxicity. We evaluated the possibility that sabeluzole elicits neuroprotection by acting, either directly or indirectly, on tau proteins. We found that repeated treatments during development of primary cultures of cerebellar granule cells with nanomolar concentrations of sabeluzole resulted in mature cells that were resistant to the excitotoxicity induced by glutamate. Also, sabeluzole treatment specifically prevented the glutamate-induced increase of tau expression without modifying the basal pattern of expression of tau proteins, as shown by measurement of mRNA and protein levels. In human neuroblastoma cell line SH-SY5Y, differentiated by treatment with retinoic acid, doxorubicin increased tau immunoreactivity, and later induced cell death. Both effects were prevented by sabeluzole. Our data indicate that increased tau expression is a common response to different types of cells to neurotoxic agents, and that sabeluzole-induced neuroprotection is functionally associated with the prevention of the injury-mediated increase of tau expression. **Synapse 26:95–103, 1997.**

INTRODUCTION

Sabeluzole is an experimental drug originally developed for its antiischemic, antiepileptic (Wauquier et al., 1986), and cognitive-enhancing properties (Clinke and Sahgal, 1986), and it is currently under development for Alzheimer's disease. Recently, it was reported that repeated treatments with sabeluzole protect cultured rat hippocampal neurons against NMDA- and glutamate-induced neurotoxicity (Pauwels et al., 1990). However, the site(s) as well as the mechanism(s) of action of sabeluzole responsible for its neuroprotective properties are up to now largely unknown.

We tested the possibility that sabeluzole may elicit neuroprotection by acting, either directly or indirectly, on tau expression. Tau proteins are cytoskeleton-associated, neural proteins promoting microtubule assembly and neurite polarization (Caceres and Kosik, 1990; Dubrin and Kirschner, 1986). The interest in tau proteins has recently been increased by the finding that tau proteins are the major component of neurofibrillary tangles, one of the hallmarks of the Alzheimer's disease brain (Braak et al., 1986). We previously found that inhibition of tau expression by tau oligonucleotide antisense treatment in primary cultures of cerebellar granule cells reduced neural vulnerability to glutamate-induced neurotoxicity (Pizzi et al., 1995a). Increased tau expression may thus be considered one of the crucial steps in the chain of intracellular events, induced by glutamate, leading to neural death.

In this report we show that increased tau expression is a converging effect induced by a variety of neurotoxic agents, and that the neuroprotective properties of sabeluzole, independent of type of experimental injury,

*Correspondence to: Prof. Maurizio Memo, Division of Pharmacology, Department of Biomedical Sciences and Biotechnologies, School of Medicine, University of Brescia, Via Valsabbina 19, 25123 Brescia, Italy.
E-mail: memo@master.cci.unibs.it

Received 23 May 1996; Accepted 3 October 1996.

are associated with its capability to prevent such an increase.

MATERIALS AND METHODS

Cell cultures

Primary cultures of cerebellar granule cells were prepared from 8-day-old Sprague-Dawley rat pups as previously described (Levi et al., 1984). Cells were plated onto poly-1-lysine-coated dishes and cultured in basal Eagle's medium containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, 50 μg/ml gentamicin, and 25 mM KCl, at a density of 1.5×10^5 cells/cm^2. Cytosine arabinoside (100 μM) was added to the cultures 18 h after seeding to prevent non-neuronal cell proliferation. Experiments were done after culturing the neurons for 12 days in vitro (DIV) unless otherwise indicated. Neuroblastoma cell line SH-SY5Y was routinely cultured in 1:1 Ham's F12 Dulbecco's modified Eagle's medium with 10% fetal calf serum, 2 mM glutamine, in 120-mm Falcon plastic petri dishes in humidified incubators at 37°C with 5% CO_2. For differentation, cultures were seeded at approximately 10^5 cells/dish, and retinoic acid was added to give a final concentration of 10^{-5} μM. Medium was changed on alternate days, and cultures were allowed to differentiate for at least 3 weeks.

Neurotoxicity assay

The culture-conditioned media of cerebellar granule cells were collected, and the cells were washed once with Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM glucose, and 5 mM HEPES, pH 7.4) and exposed to different concentrations of glutamate for 15 min. After this period, cells were washed three times with Locke's solution containing 1 mM $MgSO_4$ and returned to the original culture-conditioned media. Cells were cultured for an additional 24 h before intravital staining was performed. To evaluate the acute effect of sabeluzole, 0.001% dimethyl solfoxide (DMSO) or sabeluzole, prepared from a 100-fold concentrated drug solution in 0.1% DMSO, were added 20 min before the glutamate pulse. When the chronic effect of sabeluzole was investigated, vehicle or the drug, prepared as described above, was added to the cultures on DIV 5 and DIV 8. Cell viability was determined by fluorescein diacetate (15 μg/ml) and propidium iodite (80 μg/ml) staining as previously described (Jones and Senft, 1985).

Neurotoxicity experiments were also performed on differentiated neuroblastoma SH-SY5Y cells. The acute effects of sabeluzole were evaluated by pretreating the cells with vehicle or the drug 20 min before cell injury, while chronic effects were studied by adding vehicle or sabeluzole on DIV 14 and DIV 18. On DIV 21, 10 nM doxorubicin, an anthracycline antibiotic that induces DNA damage (Cole et al., 1985), was added to the medium; 24 h later, neurotoxicity was evaluated as percentage of total lactate dehydrogenase (LDH) activity. Extracellular and intracellular LDH activity was measured spectrophotometrically, following NADH-oxidation at 340 nm (Bergmeyer, 1974; Pauwels et al., 1989). Total LDH activity was defined as the sum of intracellular and extracellular LDH activity.

Intracellular calcium concentration measurement

Regulation of cytosolic free calcium concentration was investigated by microfluorimetry in single cerebellar granule neurons, according to Malgaroli et al. (1987) with minor modifications (Pizzi et al., 1995a). Briefly, cells were plated onto 100 μg/ml poly-l-lysine-coated glass coverslips and cultured as described above. On DIV 12, cells were loaded with the calcium-sensitive fluorescent dye fura-2, by a 60-min incubation at 37°C with its acetoxymethyl derivate (4 μM in Krebs-Ringer medium containing 1.3 mg/ml bovine serum albumin, pH 7.4), and then mounted in a 22-mm holder. Fura-2 emission was monitored using an inverted fluorescence microscope (Nike Diaphot, Ni Ron, Japan) associated with an intensified charge-coupled device camera (Mira-100 TE; Applied Imaging, Gateshead, UK) which recorded the 510-nm fluorescence emission in neurons excited through a narrow bandpass filter (340 and 380 nm). The background was subtracted, and the amount of free calcium within the cell bodies was calculated from the ratio of 340/380 nm obtained every 3–4 sec. Calibration was according to external standards of calcium and fura-2 (Connor, 1986). Fluorescence image acquisition and analysis were performed using the MIRAcal (Multiple Image Ratioing and Analysis with Calibration) system by Applied Imaging.

Polymerase chain reaction

A polymerase chain reaction (PCR)-derived method was used to determinate tau mRNA levels in cerebellar granule cells (Pizzi et al., 1995b) and in the neuroblastoma SH-SY5Y cell line. Total mRNA was isolated from the cultures by the method of RNAzol (Chirgwin et al., 1979; Chomczynski and Sacchi, 1987), extracted by chloroform-ethanol, and reverse-transcribed with Moloney murine leukemia virus reverse transcriptase in the presence of [^{32}P]dCTP, as recommended by the manufacturer (BRL). The resulting cDNA was quantitated by determining the amount of radioactivity incorporated into trichloroacetic acid-precipitable nucleic acids. PCR was done with Taq polymerase (Perkin-Elmer Cetus) in 100 μl of standard buffer containing 0.5 ng cDNA, [^{32}P]dCTP as radioactive tracer, and 1 μM specific primers. The following primers were used: 5′-AGCCGCCTACAGACTGCCCCTGTG-3′, corresponding to nucleotides 693–717 and referred to as A, and 3′-

ATTTCTGCTCCATGGTCTGTCTTG-5′, corresponding to nucleotides 1124–1147 and referred to as B, of the rat tau cDNA sequence (Kosik et al., 1989). Human tau cDNA sequencing was performed using the following specific primers: 5′-AGCCGCCTGCAGACAGCCCCC-GTG-3′, corresponding to nucleotides 546–570 and referred to as A′, and 3′-ATCTCCGCCCCGTGGTCT-GTCTTG-5′, corresponding to nucleotides and 885–909 referred to as B′. Thirty cycles of amplification were performed with a DNA Thermal Cycler (Perkin-Elmer Cetus) and a step program (94°C, 30 sec; 60°C, 30 sec; 72°C, 60 sec), followed by a 15-min final extension. PCR products were separated by electrophoresis, visualized by ethidium bromide staining, and cut out. The radioactivity incorporated into the bands was counted by scintillation spectrometry. The data are expressed as the ratio between the radioactivity incorporated by the two isoforms of PCR product.

Western blot analysis

Ten million granule cells were harvested in 100 µl of lysis buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 5 mM EDTA, 1 mM phenyl methyl sulphonyl fluoride, 0.5 µg/µl leupeptin, 5 µg/µl aprotinin, and 1 µg/ml pepstatin. The suspension was sonicated for 30 sec at full power and centrifuged at 15,000*g* for 30 min at 4°C. The resulting supernatant was isolated, and protein content was determined by a conventional method (BCA Protein Assay Kit, Pierce, Rockford, IL). Thirty µg of total proteins were electrophoresed on 10% sodium dodecylsulphate-polyacrylamide gel electrophoresis, and transferred to nitrocellulose paper (Schleicher and Schuell, Dassel, Germany). Filters were incubated at 4°C overnight with a phosphate-independent tau polyclonal antibody (anti-tau Sigma, 1:100) (Dubrin et al., 1984) in 3% nonfat dried milk (Sigma, St. Louis, USA). For immunodetection, a goat anti-rabbit alkaline phosphatase conjugate antibody (Promega, Madison, USA), 1:7,500 dilution, was used.

Immunocytochemistry

Tau immunoreactivity was measured 2 h after the glutamate pulse according to a previously described procedure (Mattson, 1990; Pizzi et al., 1995a) on the cerebellar granule cells, and 2 h after the addition of doxorubicin to differentiated SH-SY5Y cells. Briefly, cultures were fixed for 30 min in 50 mM phosphate-buffered saline (PBS) containing 4% paraformaldehyde. Following several rinses in PBS, cells were incubated for 20 min in Tris-buffered saline containing 0.5% hydrogen peroxide to block endogenous peroxidase staining. Cells were then permeabilized by a 5-min exposure to 0.2% Triton X-100 in PBS, and incubated for 24 h at 8°C with the primary antibody, an anti-tau monoclonal antibody Alz50 (1:100). The Alz50 epitope is at the amino terminal of tau. It comprises amino acids 2–10 of tau, a sequence that appears to be conformationally sensitive to hyperphosphorylation associated with Alzheimer's disease tau (Goedert et al., 1991; Goedert, 1993; Lang et al., 1992). After rinses, cells were processed using an avidin-biotin complex kit (ABC Elite Kit, Vector Laboratories, Burlingame, USA). Enzymatic reaction was developed as described by Mattson (1990). In order to verify the specificity of the cromogen reaction, some dishes were processed identically, except that cells were incubated with the primary antibody solvent. In these conditions, no immunostaining was generated. A blind analysis of immunoreactive cells was performed in all dishes. A minimum of 100 neurons was counted in at least three fields of three different culture dishes. A semiquantitative study of immunoreactivity was carried out using the Magiscan Image Analysis System (Joyce-Loebl Ltd., London, UK). The General Image Analysis Software (Genias) provided by Joyce-Loebl as standard with Magiscan gave us access to the image-processing and analysis functions of Magiscan. The microdensitometry analysis was performed as previously described (Mize et al., 1988) by measuring the following parameters in each cell body: 1) integrated optical density (ID), i.e., the sum of optical density (OD) for each pixel in the cell area considered; and 2) area, i.e., the sum of pixels over the image of the cell point set. The ratio of ID/area from each cell was processed for the statistical analysis. The data were evaluated as gray levels, expressed by a defined score: A = 0–8 $\times$ 10^{-2} OD/pixel; B = 9–16 $\times$ 10^{-2} OD/pixel; C = 17–30 $\times$ 10^{-2} OD/pixel. The statistical significance of differences between values was tested by analysis of variance. Data are presented as mean ± SEM of at least three experiments.

RESULTS

Neuroprotective effects of sabeluzole in cerebellar granule cells

Neurotoxicity

The effects of sabeluzole on glutamate-mediated excitotoxicity in primary culture of cerebellar granule cells were evaluated using two different experimental paradigms: after acute treatment (i.e., 20 min before the glutamate pulse), and after repeated treatment (i.e., by exposing the cells to the drug twice during in vitro maturation). In the former case, 12-DIV cultures were pretreated for 20 min with vehicle, 5 µM MK 801, a noncompetitive antagonist on NMDA-type glutamate receptor, or sabeluzole (50 nM and 100 nM). Then, glutamate at a concentration of 50 µM or 100 µM was added to the dishes, and incubation was allowed for an additional 15 min. Cells were washed out of the glutamate, and viability was tested 24 h later. While MK 801 completely prevented glutamate-induced neuronal death, no protective effect was observed in the cultures pretreated with sabeluzole or vehicle (Table I).

TABLE I. Neuroprotection against glutamate-induced toxicity

Drug	Concentration (μM)	Viability (%)
----		100
Glutamate	50.0	60
Glutamate	100.0	25
Glutmate +	50.0	
MK 801	5.0	100
Glutamate +	100.0	
MK801	5.0	90
Glutamate +	50.0	
Sabeluzole	0.05	62
Glutamate +	100.0	
Sabeluzole	0.05	30
Glutamate +	50.0	
Sabeluzole	0.1	65
Glutamate +	100.0	
Sabeluzole	0.1	28

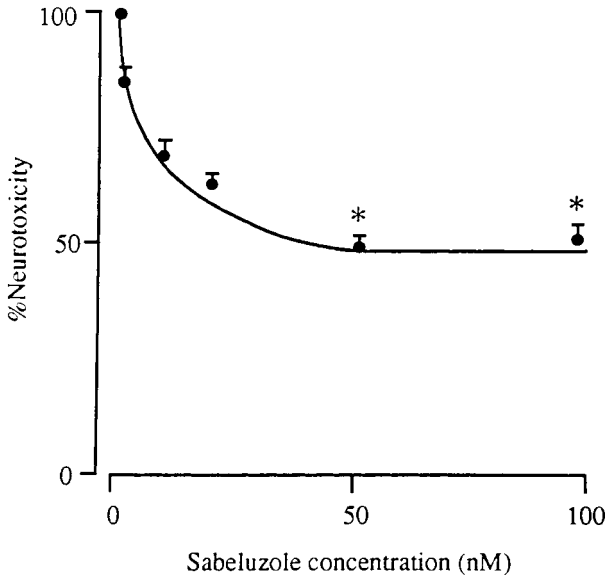

Fig. 1. Inhibition of glutamate-induced neurodegeneration by increasing concentrations of sabeluzole. Sabeluzole or vehicle were added to neurons on DIV 5 and DIV 8. Experiments were done on DIV 12. Data (mean ± SEM of five or six determinations) are from a representative experiment repeated with similar results in at least three separate preparations of neurons. Glutamate per se induced cell death in about 80% of untreated cells. *$P < 0.01$ vs. the values in vehicle-treated cells.

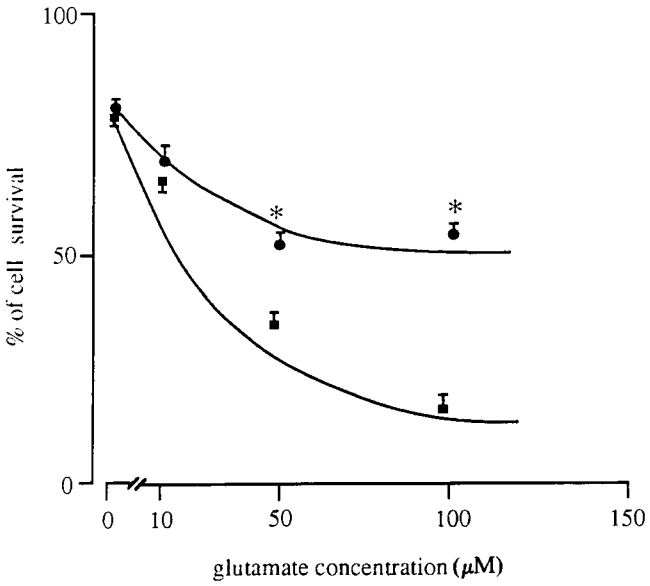

Fig. 2. Dose-dependent neurotoxic effects of glutamate in primary cultures of cerebellar granule cells pretreated with vehicle (squares) or 50 nM sabeluzole (circles). Sabeluzole or vehicle was added to neurons on DIV 5 and DIV 8. Experiments were done on DIV 12. Values represent percentage of cell survival 24 h after the glutamate pulse. Data (mean ± SEM of five or six determinations) are from a representative experiment repeated with similar results in at least three separate preparations of neurons. *$P < 0.01$ vs. the corresponding glutamate concentration values in vehicle-pretreated cells.

The effects of repeated treatment of sabeluzole on glutamate-induced neurotoxicity were investigated by exposing the cells to the drug during in vitro maturation, specifically at DIV 5 and DIV 8. Cells were cultured for an additional 4 days and on DIV 12, at which point they were fully differentiated, they were exposed to a 15-min glutamate pulse. This experimental paradigm will be referred to as "repeated treatment." Figure 1 shows the results obtained by measuring glutamate-induced neurotoxicity in cells repeatedly treated with vehicle or different concentrations of sabeluzole, ranging from 1–100 nM. The tested drug showed a dose-dependent neuroprotective effect. In particular, sabeluzole at the concentration of 50 nM reduced cell death by about 50%, in comparison to vehicle. The neurotoxic effects of increased concentrations of glutamate, ranging from 10–100 μM, were also evaluated in cerebellar granule cells repeatedly treated with vehicle or 50 nM sabeluzole. As shown in Figure 2, 50 μM and 100 μM glutamate reduced cell viability by 54% and 70%, respectively, in control cells. Repeated treatment with sabeluzole prevented the neurotoxicity induced by 50 μM and 100 μM glutamate by 32% and 48%, respectively.

Intracellular calcium concentration

The levels of intracellular Ca^{++} concentrations ($[Ca^{++}]_i$) after a glutamate pulse were measured in cerebellar granule cells repeatedly treated with vehicle or sabeluzole. As shown in Figure 3a, 50 μM glutamate evoked a rapid $[Ca^{++}]_i$ rise followed by a sustained plateau ($[Ca^{++}]_i = 500 \pm 52$ nM) in all control neurons investigated (30 cells taken from two different cell preparations). Repeated treatment of cells with 50 nM sabeluzole, a concentration that was shown to induce 50% neuroprotection, did not alter neuron responsiveness to glutamate in terms of $[Ca^{++}]_i$ rise. As shown in Figure 3b, in 50 nM sabeluzole pretreated cells, glutamate induced a rapid $[Ca^{++}]_i$ elevation followed by a plateau with kinetics and intensity similar to those detected in untreated cells. Even higher concentrations of sabeluzole (100 nM) did not reduce the calcium response of cells to glutamate (Fig. 3c).

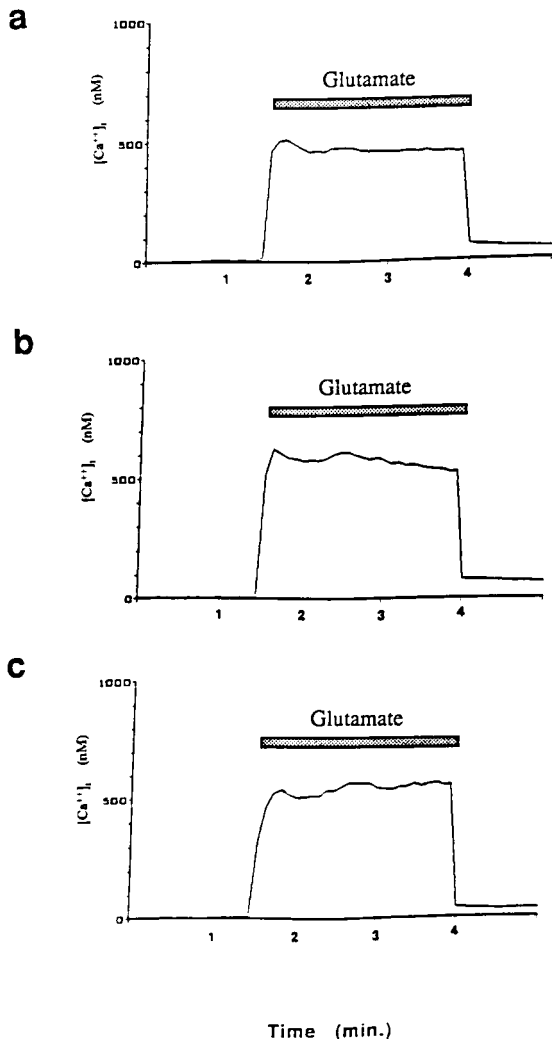

Fig. 3. Original recordings showing the glutamate-induced $[Ca^{++}]_i$ rise in cerebellar granule cells. **a:** Effect of 50 µM glutamate on $[Ca^{++}]_i$ in vehicle-pretreated neurons. **b:** Effect of 50 µM glutamate on $[Ca^{++}]_i$ in 50 nM sabeluzole-pretreated neurons. **c:** Effect of 50 µM glutamate on $[Ca^{++}]_i$ in 100 nM sabeluzole-pretreated neurons. Sabeluzole or vehicle was added to culture medium on DIV 5 and DIV 8. Experiments were done on DIV 12. Values are from representative neuron recordings. Bars represent time of glutamate exposure.

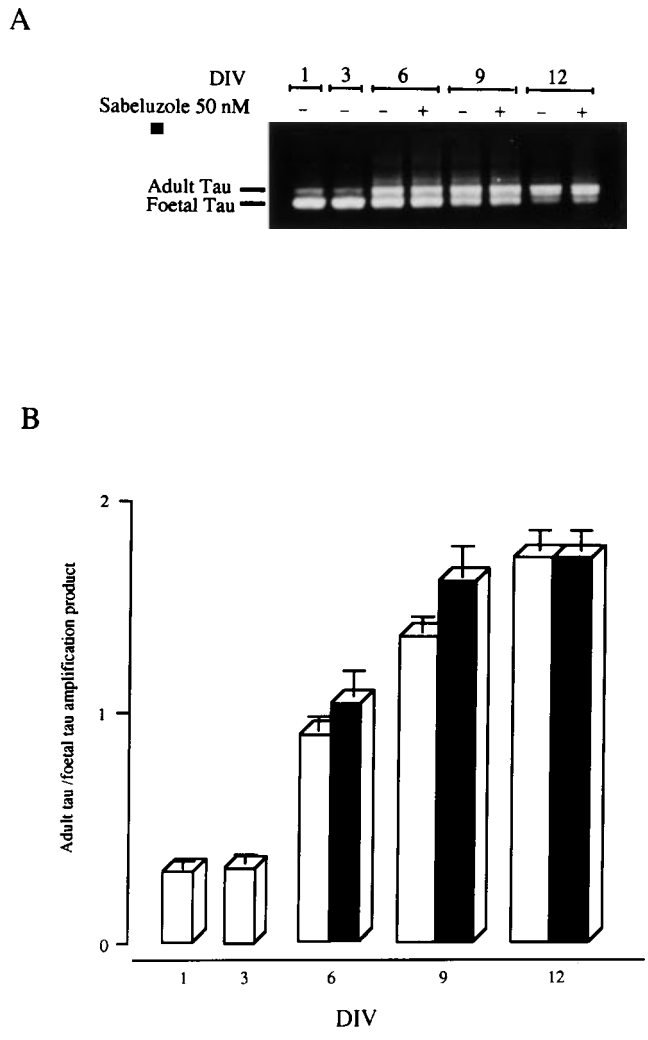

Fig. 4. Developmental changes of tau mRNA processing during in vitro maturation of cerebellar granule cells treated with vehicle (open bars) or 50 nM sabeluzole (shaded bars). **A:** PCR products using specific primers to amplify both the adult and fetal tau isoforms. Numbers at top correspond to DIV. Data are from a representative experiment. **B:** Semiquantitative analysis. Results are expressed as the ratio calculated by dividing the data from the mature isoform by the data from the fetal one. Data are cpm of $[^{32}P]$dCTP incorporated into the PCR-amplified bands, and represent the mean ± SEM of at least four experiments using two separate cDNA preparations from two different sets of cell cultures.

Tau expression

A semiquantitative PCR-derived method was applied to evaluate tau mRNA content in primary cultures of cerebellar granule cells during in vitro maturation. PCR analysis yielded two distinct cDNA bands corresponding to about 360 bp and 455 bp. The longer PCR product corresponded to that theoretically expected on the basis of the tau sequence, while the shorter one reflected tau mRNA species lacking one 93-bp repeat. These two bands have been respectively referred to as "mature" and "fetal" tau mRNA isoforms. As shown in Figure 4A, granule cells virtually entirely express the fetal tau isoforms on DIV 1 and DIV 3, both the adult and the fetal tau isoforms on DIV 6 and DIV 9, and predominantly the adult tau isoforms on DIV 12. The ratio between adult and fetal isoforms increases with time in culture, the fetal isoform tending to decrease and virtually disappearing by DIV 12. The PCR products derived from cultures repeatedly treated with 50 nM sabeluzole did not show any difference compared to age-matched, vehicle-treated cells. Furthermore, data obtained by calculating the amount of radioactivity incorporated into the bands on different DIV did not point out any difference between treated and untreated cells (Fig. 4B).

Immunoblot analysis was used to verify whether repeated treatment with sabeluzole could induce modi-

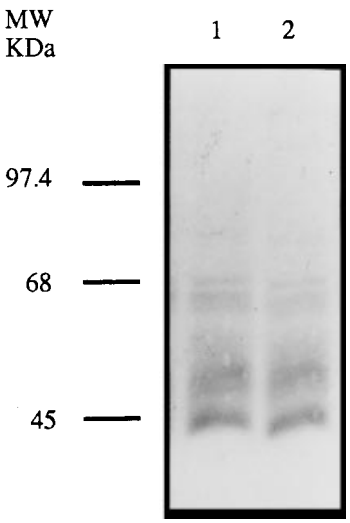

Fig. 5. Western blot analysis with anti-tau antibody of protein extracts from cerebellar granule cells pretreated with vehicle (**lane 1**) or 50 nM sabeluzole (**lane 2**). The size of molecular weight markers is indicated at left. Sabeluzole or vehicle was added to neurons on DIV 5 and DIV 8. Experiments were done on DIV 12. Data are from a representative experiment. Similar results were obtained using protein extracts from three separate cell preparations.

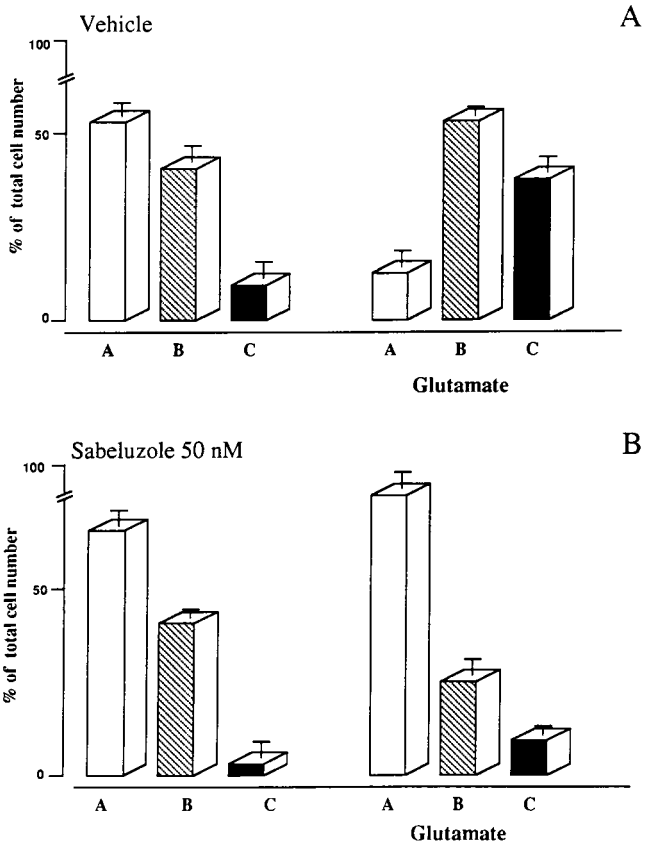

Fig. 6. Glutamate-induced increase of tau immunoreactivity in rat cerebellar granule cells pretreated with vehicle (**A**) or 50 nM sabeluzole (**B**). Cells were immunostained with anti-tau monoclonal antibody Alz-50. Sabeluzole or vehicle was added to neurons on DIV 5 and DIV 8. Experiments were done on DIV 12. Columns represent the mean ± SEM of the percentage of cells for each level of gray with A < B < C, expressed as integrated density/area. Details in Materials and Methods.

fication in protein levels. A representative immunoblot is shown in Figure 5. Heat-stable extracts were obtained from granule cells repeatedly treated with vehicle (Fig. 5, lane 1) or 50 nM sabeluzole (Fig. 5, lane 2). The immunostaining performed with the phosphate-independent tau polyclonal antibody (Binder et al., 1985) detected five sharp bands with apparent MW ranging from 68–45 kDa. The anti-tau antibody has been largely described for bovine and rat tissue by Binder et al. (1985), and recognizes all of the known electrophoretic tau isoforms on immunoblots of mammalian brain. Densitometric analysis of the immunoblot shown in Figure 5 revealed no difference in the immunostained bands in lane 1 in comparison with those of lane 2, (data not shown).

Immunocytochemistry

As previously described, application of 50 μM glutamate to cerebellar granule cells induced a significant increase in the level of tau immunoreactivity, which has been functionally associated with neuronal death (Pizzi et al., 1995a). In the present study we examined the effect of repeated treatment of sabeluzole on the glutamate-induced increase of tau expression in cerebellar granule cells. In particular, immunocytochemistry for tau proteins was carried out with Alz50, a mouse monoclonal antibody raised against Alzheimer's disease brain homogenates. Cells were repeatedly treated with vehicle or 50 nM sabeluzole and challenged with 50 μM glutamate. The glutamate pulse increased tau immunoreactivity in most of the vehicle-treated neurons. Repeated treatment of cells with sabeluzole significantly reduced the capability of glutamate to increase Alz50 immunostaining. As shown in Figure 6, semiquantitative analysis of Alz50 immunoreactivity revealed a 60% decrease of gray intensity in cells repeatedly treated with sabeluzole, compared with vehicle-treated cells.

Neuroprotective effects of sabeluzole in human neuroblastoma cell line SH-SY5Y

Neurotoxicity

The neuroprotective effects of sabeluzole were also tested in the human neuroblastoma cell line SH-SY5Y, differentiated in neuron-like cells by treatment with retinoic acid for 3 weeks. Since these cells do not express functional glutamate receptors, doxorubicin was chosen as cytotoxic agent. As in cerebellar granule cells, the neuroprotective effects of sabeluzole were evaluated using two different experimental paradigms: after acute treatment (i.e., 20 min before doxorubicin treatment) or after repeated treatment (i.e., by expos-

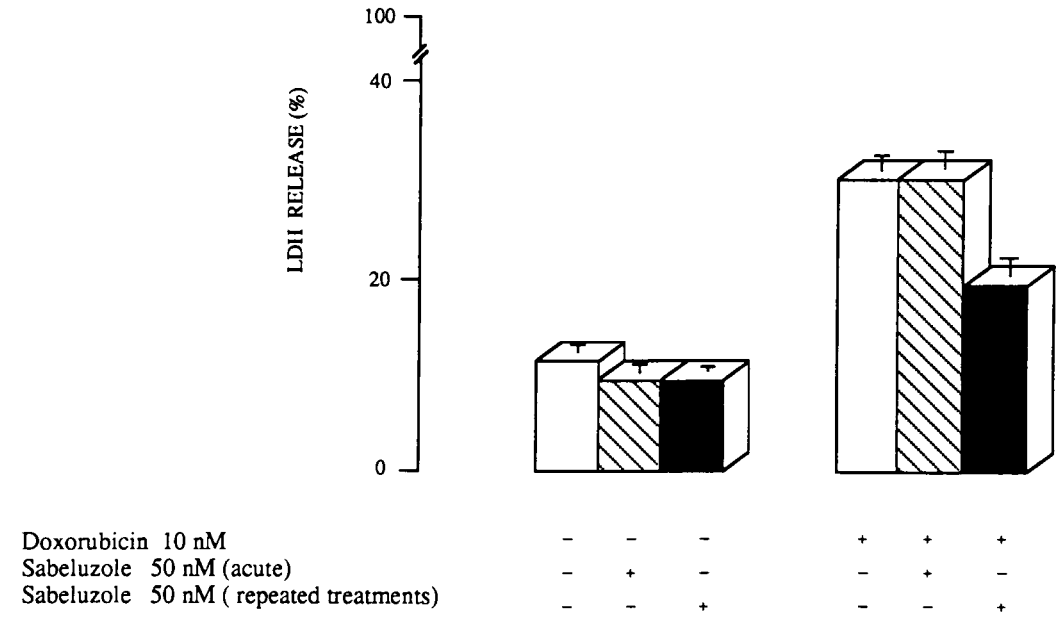

Fig. 7. Doxorubicin-induced LDH release in human neuroblastoma cell line SH-SY5Y pretreated with vehicle (open bars), 50 nM sabeluzole in acute (shaded bars), or 50 nM sabeluzole on DIV 14 and 18 (solid bars). LDH release was measured 24 h after 10 nM doxorubicin treatment. Data (mean ± SEM of five or six determinations) are from a representative experiment repeated with similar results in at least three preparations of cells. Details in Materials and Methods.

ing cells to the drug twice during in vitro differentiation, before doxorubicin treatment). In the former case, differentiated SH-SY5Y cells were pretreated with vehicle or 50 nM sabeluzole for 20 min, and then 10 nM doxorubicin were added to the dishes and left for an additional 24 h. As shown in Figure 7, sabeluzole treatment was unable to prevent the neurotoxic effects induced by doxorubicin.

To test the effect of repeated sabeluzole treatment, cells were exposed to vehicle or 50 nM sabeluzole during their differentiation, specifically on DIV 14 and DIV 18. On DIV 21, the cultures were incubated with 10 μM doxorubicin for 24 h and then tested for viability. As shown in Figure 7, sabeluzole was able to significantly prevent the neurotoxicity induced by doxorubicin. In particular, repeated sabeluzole treatment inhibited doxorubicin-induced LDH release by 30%.

Tau expression

PCR analysis was performed to evaluate tau mRNA content in undifferentiated and differentiated SH-SY5Y cells. Agarose gel electrophoresis of PCR products revealed two distinct cDNA bands. This pattern was similar to that described for cerebellar granule cells: a long and a short isoform, respectively defined as adult and fetal tau mRNA isoforms. Undifferentiated SY5Y cells expressed predominantly the fetal tau isoform (Fig. 8, lane 3). After 3 weeks of retinoic acid treatment, two mRNA species, respectively encoding the fetal and the mature tau isoforms, were detected. In differentiated cells, the fetal tau mRNA isoform appeared to be more abundant than the adult one (Fig. 8, lane 2). Two additional weeks of retinoic acid treatment did not modify this pattern (data not shown). Measurement of tau mRNA levels in differentiated SH-SY5Y cells that were pretreated with 50 nM sabeluzole on DIV 14 and DIV 18 gave PCR products similar in size and quantity to those found in control cells (Fig. 8, lane 1).

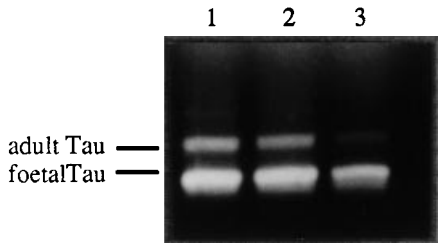

Fig. 8. PCR analysis of tau mRNA isoforms from human neuroblastoma SH-SY5Y cells. **Lane 1,** retinoic acid-differentiated cells pretreated with vehicle on DIV 14 and DIV 18. **Lane 2,** retinoic acid-differentiated cells pretreated with 50 nM sabeluzole on DIV 14 and DIV 18. **Lane 3,** undifferentiated cells. Arrows indicate PCR products originating from the fetal and adult tau mRNA isoforms. Data are from a representative experiment. Details in Materials and Methods.

Immunocytochemistry

SH-SY5Y cells were incubated with 10 nM doxorubicin for 2 h, and then immunocytochemical analysis was performed with Alz50 antibody. Doxorubicin induced a significant increase of tau immunoreactivity, similar to that induced by glutamate in cerebellar granule cells.

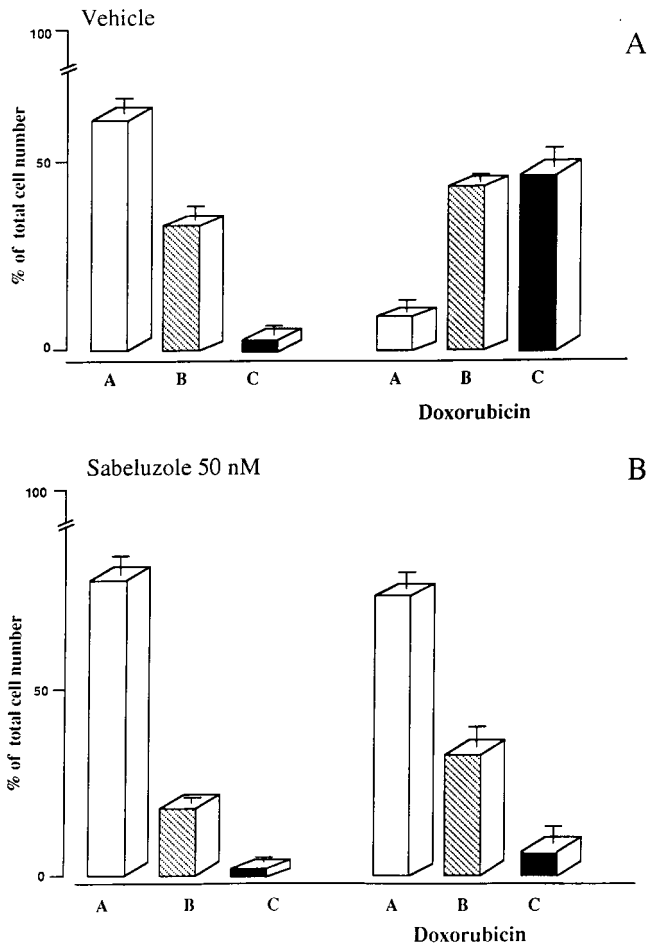

Fig. 9. Doxorubicin-induced increase of tau immunoreactivity in differentiated human neuroblastoma SH-SY5Y cells pretreated with vehicle (**A**) or 50 nM sabeluzole (**B**). Cells were immunostained with anti-tau monoclonal antibody Alz50. Sabeluzole or vehicle was added to cells on DIV 14 and DIV 18. Experiments were done on DIV 21. Columns represent the mean ± SEM of the percentage of cells for each level of gray with A < B < C, expressed as integrated density/area. Details in Materials and Methods.

Fifty nM sabeluzole, added to the culture on DIV 14 and DIV 18 during differentation, were able to prevent the glutamate-induced increase of tau expression. As shown in Figure 9, semiquantitative analysis of Alz50 immunoreactivity revealed a 65% decrease in gray intensity in cells repeatedly treated with sabeluzole, compared with vehicle-treated cells.

DISCUSSION

The present data show that when either primary neurons from rat brain or differentiated human neuroblastoma cell lines are exposed to low concentrations of sabeluzole, they become more resistant to neurotoxicity. Apparently, these cells are primed by two pulses of sabeluzole to develop long-lasting modification in those intracellular pathways, that are intrinsically designed to organize a positive functional response to an environmental injury.

In particular, treatment of primary cultures of cerebellar granule cells during in vitro development with nanomolar concentrations of sabeluzole results in mature cells that are resistant to the excitotoxicity induced by glutamate. There are several observations supporting the hypothesis that sabeluzole may not act at glutamate receptor level: 1) sabeluzole was inactive in preventing glutamate-induced neural death after acute treatment, 2) at neuroprotective concentrations, it did not alter the capability of glutamate to increase intracellular calcium levels, and 3) it elicited its neuroprotective activity in an experimental paradigm in which neurotoxicity was induced by a drug, doxorubicin, which does not interact with plasma membrane receptors (see below).

The neuroprotective properties elicited by sabeluzole were more evident at a higher concentration of glutamate. Moreover, at maximally effective concentrations, the degree of neuroprotection induced by sabeluzole never reached 100%. Taken together, these observations suggest that glutamate-induced neurodegeneration may either involve distinct pathways leading to cell death or unravel different sets of neurons with different thresholds to neurotoxic stimuli. Whatever the case, our data suggest that sabeluzole is either active in preventing a specific intracellular pathway leading to cell death, or else neuroprotective only on a subpopulation of neurons.

Functionally, sabeluzole treatment prevented the glutamate-elicited increase of tau immunoreactivity detected with Alz50 antibody 2 h after the neurotoxic pulse. The involvement of tau in the mechanism of action of sabeluzole has been indirectly suggested by previous studies showing that tau protein epitopes recognized by the Alz50 antibody induced by hyperstimulation of various protein kinases in neuroblastoma cells and in hippocampal neurons were inhibited by sabeluzole (Geerts et al., 1993). On this basis, we were interested in investigating if the addition of sabeluzole at specific stages of granule cell in vitro development might change the pattern of expression of tau protein. First, we tested the possibility that sabeluzole treatment may modify tau mRNA levels during granule cell maturation. We found that, in the first day of culturing, granule cells expressed predominantly the tau mRNA short isoform, on DIV 6 they expressed both the short and the long isoforms, and on DIV 12 the cells almost exclusively expressed the long tau mRNA isoform. The ratio of the two tau mRNA isoforms could thus be used as a marker of neural maturation. Sabeluzole treatment apparently did not modify the basal pattern of expression of tau proteins during development, as shown by measurement of the mRNA levels of the two different tau isoforms. Moreover, Western blot analysis with a phosphate-independent polyclonal tau

antibody showed that treatment with sabeluzole during cell maturation did not alter the pattern of tau expression in terms of protein levels and posttranslational modifications.

We then performed a second set of experiments using a different experimental model, the human neuroblastoma cell line SH-SY5Y, differentiated in neuron-like cells after treatment with retinoic acid. Since these cells do not express functional glutamate receptors, doxorubicin was used as an experimental tool to induce neurotoxicity. Doxorubicin is an anthracycline antibiotic, a cytotoxic drug that causes DNA damage (Argasinski et al., 1989; Tewey et al., 1994). We found that doxorubicin was toxic to differentiated SH-SY5Y cells. Interestingly, this drug, like glutamate, increased tau immunoreactivity 2 h after treatment. Both doxorubicin-induced cell death and neurotoxicity were prevented by exposing the cells to sabeluzole during differentiation.

The mechanism(s) responsible for the sabeluzole neuroprotective priming effect is still unclear. The drug was indeed able to prevent neurotoxicity induced by agents acting on different mechanisms and/or at different cellular levels. Sabeluzole did not modify the program of development dictating the differential expression of the various tau isoforms, in terms of both mRNA and protein levels. However, sabeluzole was able to prevent the increase in tau immunoreactivity induced by the two neurotoxic agents glutamate and doxorubicin.

In summary, we found that increased tau expression is a common response of different types of cells to neurotoxic agents, and that neuroprotection is functionally associated with the prevention of the injury-mediated increase of tau expression.

ACKNOWLEDGMENTS

We thank J.E. Leysen for critical review of the manuscript, and P. Davies for kindly supplying Alz50 antibody. This work was supported by a grant from the Consiglio Nazionale della Ricerca, Italy.

REFERENCES

Argasinski, A., Sternberg, H., Fingado, B., and Huynh, P. (1989) Doxorubicin affects tau protein metabolism in human neuroblastoma cells. Neurochem. Res., 14:927–931.

Bergmeyer, H.U. (1974) Biochemical reagents. In: Methods of Enzymatic Analysis, 2nd ed. H.U. Bergmeyer, ed. Academic Press, New York, p. 480.

Binder, L.I., Frankfurter, A., and Rubhun, L.I. (1985) The distribution of tau in the mammalian central nervous system. J. Cell Biol., 101:1371–1378.

Braak, H., Braak, E., and Grundke-Iqbal, K. (1986) Occurrence of neuropil threads in the senile human brain and in Alzheimer's disease: A third location of paired helical filaments, outside of neurofibrillary tangles and neuritic plaques. Neurosci. Lett., 65:351–355.

Caceres, A., and Kosik, K.S. (1990) Inhibition of neurite polarity by tau antisense oligonucleotides in primary cerebellar neurons. Nature, 343:461–464.

Chirgwin, J.M., Przybylz, A.E., MacDonald, R.J., and Rutter, W.J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry, 18:5294–5299.

Chomczynski, P., and Sacchi, N. (1987) Single step method of RNA isolation by acid guanidium thiocyanate-phenol-cloroform extraction. Anal. Biochem., 162:156–159.

Clincke, G., and Saghal, A. (1986) R58735, a novel antihypoxic drug improves memory in rats. Drug Dev. Res., 8:381–385.

Cole, G.M., Wu, K., and Timiras, P. (1985) A culture model for age-related human neurofibrillary pathology. Int. J. Dev. Neurosci., 3:23–32.

Connor, J.A. (1986) Digital imaging of free calcium change and of spatial gradient in growing processes in single, mammalian central nervous system cells. Proc. Natl. Acad. Sci. USA, 83:6179–6183.

Dubrin, D.G., and Kirschner, M.W. (1986) Tau protein function in living cells. J. Cell Biol., 103:2739–2746.

Dubrin, D.G., Caput, D., and Kirschner, M.W. (1984) Studies of the expression of the microtubule-associated protein, tau, during mouse development, with newly isolated complementary DNA probes. J. Cell Biol., 98:1090–1097.

Geerts, H., Nuydenns, R., Nuyens, R., and Cornelissen, F. (1993). Sabeluzole, a prototype drug targeted at the aberrant cytoskeleton. Proceedings of the Fourth International Conference on Alzheimer's Disease, Chicago, Abstract No. 100.

Goedert, M. (1993) Tau protein and the neurofibrillary pathology of Alzheimer's disease. TINS, 6:149–154.

Goedert, M., Spillantini, M., and Jakes, R. (1991) Localization of the Alz50 epitope in recombinant human microtubule-associated protein tau. Neurosci. Lett., 126:149–154.

Jones, K.H., and Senft, J.A. (1985). An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodite. J. Histochem. Cytochem., 33:77–84.

Kosik, K.S., Orecchio, L.D., Bakalis, S., and Neve, R. (1989) Developmentally regulated expression of specific tau sequences. Neuron, 2:1389–1397.

Lang, D.L., Szendrei, G.I., Lee, V.M.Y., and Otuos, L. (1992) Immunological and conformational characterization of a phosphorylated immuno-dominant epitope on the paired helical filament found in Alzheimer's disease. Biochem. Biophys. Res. Commun., 187:783–790.

Levi, G., Aloisi, F., Ciotti, M.T., and Gallo, V. (1984) Autoradiographic localization and depolarization-induced release of acidic amino acid in differentiating cerebellar granule cell cultures. Brain Res., 290:77–86.

Malgaroli, A., Milani, D., Meldolesi, J., and Pozzan, T. (1987) Fura-2 measurement of cytosolic free Ca^{2+} in monolayers and suspensions of various types of animal cells. J. Cell Biol., 105:2145–2155.

Mattson, M.P. (1990) Antigenic changes similar to those seen in neurofibrillary tangles are elicited by glutamate and Ca^{2+} influx in cultured hippocampal neurons. Neuron, 4:105–117.

Mize, R.R., Holdefer, R.N., and Nabors, L.B. (1988) Quantitative immunocytochemistry using an image analyzer. I. Hardware evaluation, image processing and data analysis. J. Neurosci. Methods, 26:1–24.

Pauwels, P.J., Van Assouw, H.P., Leysen, J.E., and Janssen, P.A.J. (1989) Ca^{2+}-mediated neuronal death in rat brain neuronal cultures by veratridine: Protection by flunarizine. Mol. Pharmacol., 36:525–531.

Pauwels, P.J., Leysen, J.E., and Janssen, P.A.J. (1990) Resistance of sabeluzole-treated rat brain neuronal cultures to neuronal damage. Neurosci. Res. Commun., 6:19–25.

Pizzi, M., Valerio, A., Arrighi, V., Galli, P., Belloni, M., Ribola, M., Alberici, A., Spano, P.F., and Memo, M. (1995a) Inhibition of glutamate-induced neurotoxicity by a tau antisense oligonucleotide in primary culture of rat cerebellar granule cells. Eur. J. Neurosci., 7:1603–1613.

Pizzi, M., Valerio, A., Belloni, M., Arrighi, V., Alberici, A., Liberini, P., Spano, P.F., and Memo, M. (1995b) Differential expression of fetal and mature tau isoforms in primary cultures of rat cerebellar granule cells during differentiation *in vitro.* Mol. Brain Res., 34:38–44.

Tewey, K.M., Chen, G.L., Nelson, E.M., and Liu, L.F. (1994) Interactive anticancer drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. J. Biol. Chem., 259:9182–9187.

Wauquier, A., Clincke, G., Ashton, D., De Ryck, M., Fransen, J., and Van Clemen, G. (1986) R58735: A new antihypoxic drug with anti-convulsant properties and possible effects on cognitive functions. Drug Dev. Res., 8:373–382.