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Flupirtine increases the levels of glutathione and Bcl-2 in hNT (human Ntera/D1) neurons: mode of action of the drug-mediated anti-apoptotic effect

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Abstract

Flupirtine is a triaminopyridine analogue which has been successfully applied in clinics as a non-opiate analgesic drug. Previously we described that flupirtine acts like an *N*-methyl-D-aspartate (NMDA) receptor antagonist in neuronal cells both in vitro and in vivo. Here we show that flupirtine displays its anti-apoptotic effect also in hNT (human Ntera/D1) neurons. hNT neurons were induced to apoptosis applying glutamate (Glu; at concentrations $\geq$ 1 mM) or NMDA ($\geq$ 1 mM). During Glu/NMDA-mediated apoptosis the levels of the intracellular anti-apoptotic agents Bcl-2 and glutathione dropped by more than 50%. Flupirtine completely abolished this reduction of Bcl-2 and glutathione level at a concentration of 10 μM. In the presence of 3 μM flupirtine a > 6-fold increase of the Bcl-2 (B-cell leukemia/lymphoma-2) level was observed in hNT neurons. At the same concentration, the intracellular level of glutathione increased to 200%. We conclude that the Glu/NMDA-mediated neuronal cell death in vitro is controlled at least partially by Bcl-2 and glutathione. Neuronal cell death by Glu or NMDA in vitro can be overcome applying the drug flupirtine which is in clinical use.

Keywords: Flupirtine; Triamnopyridine; hNT neuron; Bcl-2; Neuroprotection; Apoptosis; Glutathione

1. Introduction

Apoptosis and programmed cell death are two forms of cellular death which are totally different from the process of necrosis (Glücksmann, 1965). In contrast to necrosis, both apoptosis and programmed cell death are seen among scattered cells, rather than in contiguous groups of cells. On the cellular level plasma membrane disruption is not the early feature (reviewed in Lo et al., 1995). In apoptosis and programmed cell death nuclear chromatin undergoes condensation during which a calcium-dependent endonuclease is activated. This process results in a specific cleavage of nuclear DNA at linker regions; DNA fragments of 180–200 base pairs in length and multiples of them are produced (Wyllie et al., 1980). Apoptosis and programmed cell death refer to distinct phenomena, which are not necessarily synonymous (Glücksmann, 1965). While programmed cell death implies cell death which has to occur during development of a metazoan organism, apoptosis refers to a series of morphological changes of cells eventually leading to cell death and it is not correlated with development. Often, apoptosis is associated with de novo gene expression (reviewed in Lo et al., 1995). Inducers of apoptosis can be some physiological activators (e.g., neurotransmitters), damage-related inducers (e.g., heat shock), therapy-associated agents (e.g., chemotherapeutic agents) or toxins (e.g., ß-amyloid peptide) (Thompson, 1995).

Kure et al. (1991) and we (Müller et al., 1992, 1993) described that the excitatory amino acid glutamate (Glu) and the amino acid derivative *N*-methyl-D-aspartate (NMDA) induce apoptosis in cortical neurons. Glu and

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NMDA activate the ligand-gated Ca^{2+} channels, the NMDA receptor complex (reviewed in Olney, 1993), in a variety of vertebrate and invertebrate neurons (Fass and Levitan, 1994). The excitatory amino acid Glu has besides normal physiological function also neurotoxic (excitotoxic) potential (reviewed in Olney, 1993) and for the latter reason an intensive screening for drugs able to abolish the Glu-mediated apoptosis has been initiated.

The drug, which acts like an NMDA receptor antagonist, flupirtine, was described by Schwarz et al. (1994), Perovic et al. (1994) and Osborne et al. (1994). Flupirtine is a centrally acting, non-opiate analgesic agent (Szelenyi et al., 1989), which is applied in the clinics under the trademark of Katadolon (Friedel and Fitton, 1993). Recently this drug was identified as an anti-apoptotic agent for neurons in vitro (Perovic et al., 1994). However, until now no binding site for flupirtine has been identified at the NMDA receptor complex (Osborne et al., 1996). In vitro flupirtine enhances basal levels of ATP (Osborne et al., 1996) and reduces the glutamate-induced rise of $(Ca^{2+})_i$ in neurons concentration dependently (Rupalla et al., 1995). In addition, flupirtine acts neuroprotectively at doses of 1–10 mg/kg in animal models of focal cerebral ischemia (mouse) (Rupalla et al., 1995) and global cerebral ischemia (rat) (Block et al., 1995), as well as of retinal ischemia (rat, rabbit) (Block et al., 1994; Osborne et al., 1996).

In the present study we describe that flupirtine augments the levels of Bcl-2 and glutathione in hNT (human Ntera/D1) neurons. For our experiments we have used these cells because they express neuronal Glu/NMDA receptor channels (Younkin et al., 1993). The human NT neurons have been differentiated from NT2 cells in the presence of retinoic acid (RA) (Pleasure and Lee, 1993; Younkin et al., 1993). Since earlier findings showed that both Bcl-2 and glutathione protect neuronal cells against apoptotic death (Bredesen, 1994), our data provide further support for the view that flupirtine is beneficial not only as an analgetic and myorelaxant drug. More specific, flupirtine might be also an active drug, useful for the treatment of such secondary brain damages which develop as a consequence of trauma to brain tissue as result of an increased release of the excitotoxin Glu.

2. Materials and methods

2.1. Materials

N-Methyl-D-aspartic acid (NMDA), poly-L-lysine (M_r > 300 000; P-5899), glycine, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) (MTT) (M-2128) and minimum essential medium-Eagle (without methionine, cysteine and L-glutamine) (MEM) (M-2289) were obtained from Sigma (St. Louis, MO, USA); terminal deoxynucleotide transferase-mediated dATP-biotin nick-end-labeling (TUNEL) kit from Boehringer-Mannheim (Mannheim, Germany); L-glutamine from Biochrom (Berlin, Germany); [^{35}S]methionine/cysteine from ICN-Radiochemicals (Irvine, CA, USA) and monoclonal antibodies against human Bcl-2 (mouse) from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Flupirtine maleate (2-amino-3-ethoxycarbonylamino-6-(4-fluoro-benzylamino)-pyridine maleate) (M_r 420.41) was obtained from ASTA-Medica (Frankfurt/M, Germany).

hNT (human Ntera/D1) neurons, hNT neuron conditioned medium and hNT neuron inhibition medium were obtained from Stratagene, Heidelberg, Germany.

2.2. hNT neurons

hNT neurons were grown as described (Younkin et al., 1993; Pleasure and Lee, 1993). They were cultivated in the first period (one week) in the hNT neuron inhibition medium and for the following 3 weeks in the hNT neuron conditioned medium as described in the instruction booklet from Stratagene.

2.3. Treatment

hNT neurons (4 weeks old) (Younkin et al., 1993) were treated with different concentrations of NMDA or Glu in salt solution, containing 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 15 mM glucose and 25 mM HEPES (pH 7.4). In the experiments with NMDA the saline buffer was additionally supplemented with 50 μM glycine.

The cells were incubated with NMDA or Glu for 30 min at 37°C. After incubation the salt solution was removed and replaced with DMEM/HG, containing 2 mM glutamine, 100 mU/l of insulin, 0.1 U/ml penicillin and 0.1 U/ml streptomycin; incubation was continued for 24 h.

Flupirtine was given to the cells 2 h prior to the addition of Glu or NMDA.

2.4. Evaluation of viable cells

The viability of total cells was determined with the MTT colorimetric assay system (Scudiero et al., 1988), followed by evaluation with an ELISA plate reader (Bio-Rad 3550, equipped with the program NCIMR IIIB).

2.5. Testing for DNA fragmentation

To analyze cells for DNA fragmentation, the terminal deoxynucleotide transferase-mediated dATP-biotin nick-end-labeling (TUNEL) assay was applied (Gavrieli et al., 1992).

2.6. Metabolic labelling and quantitative immunoprecipitation of Bcl-2

hNT neurons were metabolically labelled for 6 h in MEM (without methionine, cysteine and L-glutamine) supplemented with 1% bovine serum albumin in the presence of 100 μCi/ml of [^{35}S]methionine/cysteine. The cells were harvested, lysed in the presence of 0.4% of Na-lauryl sulfate (SDS) and trichloroacetic acid. Precipitable counts were immunoprecipitated with monoclonal anti-Bcl-2 antibody as described (Oltvai et al., 1993; Dole et al., 1994). The resulting immunoprecipitate was size separated on a 12.5% SDS-polyacrylamide gel and processed as described (Castle et al., 1993).

For a semiquantitative analysis of the bands present on the autoradiograph, the bands were scanned with an integrating densitometer (Shimadzu CS-910/C-R1A).

2.7. Immunostaining procedure

Cells were fixed with methanol, containing 0.02% ethylene glycol bis(ß-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid at −20°C as described (Bachmann et al., 1986). Subsequently, the cells were incubated with monoclonal Bcl-2 (human) antibodies; the immunocomplexes were visualized with anti-mouse fluorescein isothiocyanate-conjugated immunoglobulin (Ig-FITC labelled).

2.8. Determination of glutathione content

Glutathione was determined spectrophotometrically using 1-chloro-2,4-dinitrobenzene as described (Romero and Romá, 1995).

2.9. Statistics

The results were analyzed by paired Student's *t*-test (Sachs, 1984).

3. Results

3.1. Effect of Glu and NMDA on viability of hNT neurons

hNT neurons were treated with different concentrations of Glu or NMDA. As shown in Table 1 Glu and NMDA at concentrations of 1 mM or higher displayed significant reduction of viable cells ($P < 0.001$).

In an earlier study (Perovic et al., 1994) we reported that primary neurons from rat embryos undergo DNA fragmentation in a manner typical for apoptosis. DNA was isolated and analyzed by agarose gel electrophoresis; fragments of ≈ 180 base pairs and multiples of them have been resolved.

In the present study the TUNEL assay was applied to determine if DNA fragmentation in hNT neurons can also be identified by that technique. As seen in Fig. 1a, in control culture a considerable number of cells stained positive with respect to TUNEL labeling. A quantitative analysis revealed that the controls show a percentage of 22% of positive cells (Table 2). Addition of 1 mM of Glu (Fig. 1b) or of NMDA increased the percentage of TUNEL-positive cells to 82% and 87%, respectively (Table 2).

3.2. Neuroprotective effect of flupirtine against Glu- and NMDA-caused neurotoxicity

hNT neurons were incubated with flupirtine in order to determine the potential neuroprotective capacity of the drug. In the absence of Glu or NMDA and in the presence of 10 μM of flupirtine the viability – as analyzed by the MTT colorimetric assay – remained approximately 100% (Table 1).

When these cells were incubated with Glu or NMDA the significant decrease of viability was almost totally prevented by 10 μM flupirtine. The percentage of viable cells increased significantly from 41.3% in the assays with

Table 1
Effect of flupirtine on Glu and NMDA-mediated cytotoxicity on hNT neurons

hNT neurons			hNT neurons		
Glu (mM)	Flupirtine (μM)	Percentage of viable cells	NMDA (mM)	Flupirtine (μM)	Percentage of viable cells
0.1	0	95.8 ± 5.7	0.1	0	98.2 ± 7.4
1	0	52.1 ± 8.2	1	0	63.0 ± 8.9
10	0	41.3 ± 10.6	10	0	56.8 ± 8.5
0	10	106.4 ± 6.2	0	10	104.3 ± 6.6
0.1	10	101.3 ± 6.2	0.1	10	106.4 ± 6.8
1	10	96.3 ± 5.6 [a]	1	10	94.1 ± 7.3 [a]
10	10	88.9 ± 8.1 [a]	10	10	82.4 ± 7.7 [a]

These cells were incubated in the absence or presence of the compounds as described under Materials and methods. Cell viability was assayed following the MTT procedure. The absorbance in the controls (without Glu or NMDA and flupirtine) was set to 100%. Ten parallel experiments were performed.
[a] $P < 0.001$ (Student's *t*-test versus assay with Glu or NMDA alone).

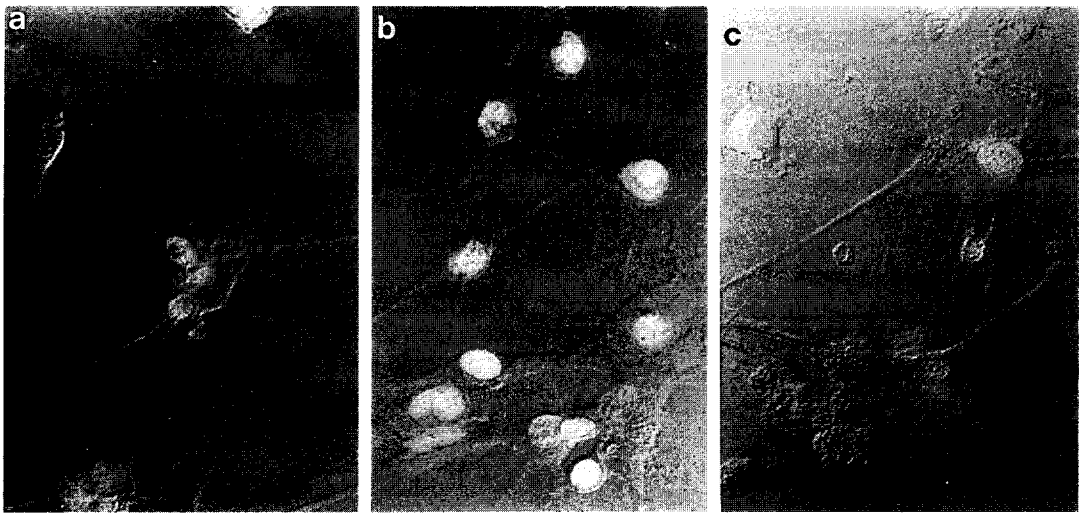

Fig. 1. Analysis of DNA fragmentation of hNT neurons by the TUNEL technique. The cells were incubated either in the absence (a) or in the presence of 1 mM of Glu for 30 min (b). In one series of experiments (c) the hNT neurons were incubated first with 10 μM of flupirtine and subsequently with 1 mM of Glu. Magnification: ×400.

Glu (56.8% with NMDA) to 88.9% (82.4%) in the presence of the drug ($P < 0.001$) (Table 1).

The cytoprotective effect caused by flupirtine on hNT neurons which had been incubated with 1 mM of Glu or NMDA could also be documented applying the TUNEL assay (Table 2; Fig. 1c). Increasing concentrations of flupirtine had no influence on the number of TUNEL-positive cells. In the experiments with Glu and flupirtine, 1 μM to 10 μM of the drug caused a reduction of the percentage of fragmented cells from 82% (absence of flupirtine) to 69% (1 μM) and to 34% (10 μM). A similar neuroprotective effect was documented also in the studies with NMDA (Table 2); at 10 μM flupirtine a reduction from 87% to 44% was seen.

3.3. Effect of flupirtine on the expression of Bcl-2

The expression of Bcl-2 in hNT neurons was analyzed by two techniques. Firstly, Bcl-2 was localized by the

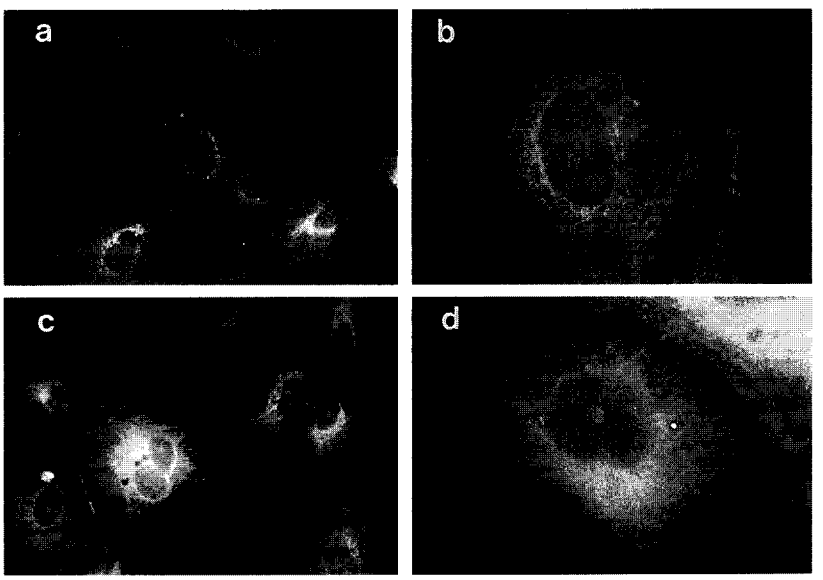

Fig. 2. Fluorescence localization of Bcl-2 in hNT neurons. The cells were treated with 0 (a and b) or 10 μM of flupirtine (c and d) for 2 h and then incubated with monoclonal antibodies against Bcl-2. The immunocomplex was visualized using FITC-conjugated anti-mouse Ig. Magnifications: a and c: ×600; b and d: ×1500.

Table 2
Effect of flupirtine on DNA fragmentation in nuclei from hNT neurons after incubation for 30 min with 1 mM of Glu or NMDA

Flupirtine (μM)	Fragmented cells (%)		
	None	Glu (1 mM)	NMDA (1 mM)
0	22	82	87
1	25	69	74
3	29	28	32
10	18	34	44

Different concentrations of flupirtine were added to the cells. DNA fragmentation was determined by the TUNEL technique. 250 cells each were examined; the percentage of TUNEL-positive cells is given.

microfluorescence method. Cells, either non-treated or pre-treated with 10 μM of flupirtine for 2 h were fixed and incubated with antibodies against Bcl-2. Subsequently the immunocomplexes were visualized by fluorescence microscopy. As shown in Fig. 2a and b, a punctate to fibrillar cytoplasmic distribution of Bcl-2-staining was seen. When cells were treated with flupirtine a very intense staining pattern for Bcl-2 was seen (Fig. 2c and d). This finding suggests that the drug induces Bcl-2 expression.

In order to support this conclusion a further approach, immunoprecipitation of metabolically labelled Bcl-2, was undertaken. hNT neurons were incubated with [^{35}S]methionine/cysteine. The immunocomplexes obtained with monoclonal anti-Bcl-2 antibodies were precipitated and size separated (Fig. 3). As seen on the autoradiograph, the 26-kDa Bcl-2 protein was visualized in extracts from the control samples together with a 31 kDa band, observed already earlier by Oltvai et al. (1993) (lane a). Setting the intensity of the 26-kDa Bcl-2 band to 100%, cells incubated with 1 mM of Glu showed a substantially suppressed expression of Bcl-2 by ≈ 80% (lane c). Incubation of hNT neurons with Glu together with increasing concentrations of flupirtine (3 and 10 μM) resulted in a > 6-fold increase of the Bcl-2 level (lanes d and e).

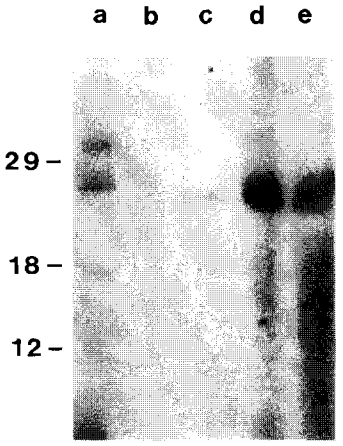

Fig. 3. Cell lysates of [^{35}S]methionine/cysteine-labelled hNT neurons were immunoprecipitated with anti-Bcl-2 antibodies; the immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis. The extracts of the following incubations were analyzed: untreated cells (lane a); control sample, incubated (20 min) in the presence of 2 μg/ml of pronase (lane b); sample from neurons incubated in the presence of 1 mM of Glu (lane c); samples from neurons preincubated with 3 μM or 10 μM of flupirtine and subsequently with 1 mM of Glu as described under Materials and methods (lane d and e). Bcl-2 migrates at a M_r of 26000; the size of the band is given in kDa.

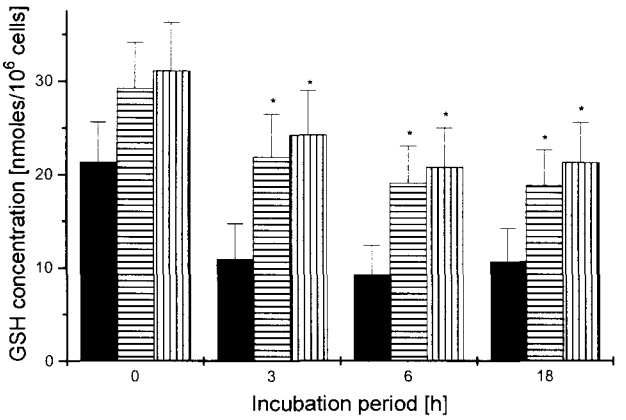

Fig. 4. Concentration of glutathione (GSH) in hNT neurons incubated with 1 mM of Glu in the absence (solid bars) or presence of 1 μM (horizontally lined bars) or 10 μM of flupirtine (vertically lined bars). The determinations were performed 0, 3, 6 and 18 h after addition of Glu. Results are expressed as means from 10 determinations ± S.D. * $P <$ 0.001 (versus assays with Glu alone).

3.4. Effect of flupirtine on the intracellular level of glutathione

The intracellular level of glutathione in hNT neurons (controls) was found to be 21.9 ± 4.8 nmol/10^6 cells (Fig. 4; time 0 in the absence of flupirtine). Incubation of cells in the presence of 1 mM of Glu resulted in a significant reduction of the glutathione level to 11.2 ± 4.2 nmol/10^6 cells ($P < 0.001$) already after a 3-h incubation period. During a further incubation time for up to 18 h this level remained constant (Fig. 4). When the cells were preincubated for 2 h with 1 μM (10 μM) flupirtine the glutathione level increased to 29.0 ± 4.9 (30.8 ± 5.1) nmol/10^6 cells (Fig. 4; time 0).

4. Discussion

The sensitivity of the hNT neurons was tested by exposure to different concentrations of Glu and NMDA. A significant reduction of cell number to 41% (57%) was obtained with 10 mM of Glu (NMDA). At 1 mM of Glu

the reduction was 52% (for NMDA: 63%). The data of Younkin et al. (1993) show that 1 mM of Glu caused a reduction of cell viability to 65%, a value which is in the range of our data. With respect to NMDA no toxicity experiments have been performed by Younkin et al. (1993). In the present study we could show that flupirtine significantly abolishes the neurotoxic activity displayed by Glu or NMDA.

In the central part of this report we demonstrate that flupirtine causes an augmentation of the level of Bcl-2 and glutathione in hNT neurons. The proto-oncogene *bcl-2* was identified by Tsujimoto et al. (1984) through its location at the chromosomal breakpoint of B-cell lymphomas carrying t(14;18) translocations. These translocations result in a movement of the *bcl-2* gene from 18q21 into juxtaposition with the immunoglobulin heavy chain locus at 14q32 (Reed et al., 1989). The consequence is a dysregulation of *bcl-2* gene expression. The physiological role of *bcl-2* was discovered by Vaux et al. (1988); they showed that overexpression of *bcl-2* in interleukin 3-dependent pro-B cell lines allowed cells to survive also in the absence of the cytokine. In the absence of *bcl-2* expression the cells are induced to undergo apoptosis (Hockenbery et al., 1990). Hence, the normal function of Bcl-2 in cells, which have the *bcl-2* gene at locus 18q21, is to promote cell survival in tissues that are controlled by activation of an apoptotic program.

The mechanism by which the Bcl-2 protein inhibits cell death is not yet fully elucidated. It is intracellularly located within the inner cytoplasmic membrane (Tsujimoto and Croce, 1988) and the inner mitochondrial membrane (Hockenbery et al., 1990) but also at the nuclear membrane (Chen-Levy et al., 1989). It has been suggested that Bcl-2 inhibits neuronal cell death at a point distal to the rise of intracellular free Ca^{2+} (Zhong et al., 1993). This finding is interesting in view of previous data which revealed that the coat protein of the human immunodeficiency virus type 1 (HIV-1), the 120-kDa glycoprotein (gp120), increased the intracellular Ca^{2+} level in neurons due to an activation of the NMDA-receptor-ionophore complex; this effect could be blocked by NMDA receptor antagonists (Ushijima et al., 1993). This result indicates that an alteration of Ca^{2+} level is required for the induction of apoptosis in neurons; however, cell death can be prevented by inducers of *bcl-2* expression, like flupirtine.

It has been proposed by Bredesen (1994) that *bcl-2* expression results in a decrease in reactive oxygen species. These cytotoxic species (Thompson, 1995) are formed during arachidonic acid metabolism (Lancaster et al., 1989). Arachidonic acid has been described to play a pathophysiological role in epilepsy and stroke as a diffusible second messenger in the central nervous system. This action is assumed to be due to an enhanced extraneuronal Glu concentration (Dickie et al., 1994). Recently we described that HIV-1 gp120 causes a stimulation of phospholipase A_2 which in turn increases the production of arachidonic acid; subsequently arachidonic acid sensitizes the NMDA-receptor-ionophore complex (Ushijima et al., 1995).

A second, intracellular component lowering reactive oxygen species is glutathione. The hypothesis outlined by Bredesen (1994) suggests that the anti-apoptotic agents Bcl-2 and glutathione inhibit apoptosis by decreasing the production or increasing the scavenging of reactive oxygen species. In light of these data we propose that flupirtine displays its anti-apoptotic property by increasing the levels of Bcl-2 and glutathione in neurons, whereby the oxidative stress caused by Glu and NMDA is prevented.

The neuroprotective effect of flupirtine has already been proven in animal models of cerebral and retinal ischemia (Block et al., 1995; Rupalla et al., 1995; Osborne et al., 1996). Since flupirtine does not bind to the NMDA receptor complex (Osborne et al., 1996) the incerased level of Bcl-2 may be one explanation for the NMDA antagonistic effect seen in several experiments (Schwarz et al., 1994; Osborne et al., 1994), such as the reduction of glutamate-induced intracellular Ca^{2+} increase in neurons (Rupalla et al., 1995) or the prevention of the NMDA-induced neurotoxicity (Perovic et al., 1994). Further investigations of the (Bcl-2- and glutathione-mediated) effect of flupirtine on energy metabolism (increased ATP levels by flupirtine; Osborne et al., 1996) and on intracellular Ca^{2+} homeostasis, especially the regulation of the Ca^{2+} release in endoplasmatic reticulum and mitochondria, are necessary and ongoing.

In the present study we describe that the levels of these two molecules, Bcl-2 and glutathione, are enhanced by flupirtine. The concentration of the drug required to augment the levels of these compounds in hNT neurons is 10 μM; this concentration is also sufficient to protect the hNT neurons against Glu- and NMDA-caused apoptosis. The TUNEL technique was applied to measure DNA fragmentation. In an earlier report we documented NMDA-induced apoptosis in neurons both by the DNA sedimentation technique and by agarose gel electrophoretical analysis (Müller et al., 1992; Perovic et al., 1994).

Flupirtine is already in clinical use. Adverse reactions are minimal in incidence, with drowsiness as the most frequently reported reaction (aproximately 10% of the patients; McMahon et al., 1987). Pharmakokinetic experiments revealed that at the therapeutic daily dose of 600 mg of flupirtine administered orally to patients (Friedel and Fitton, 1993) a plasma level of 2.5 μg/ml (6 μM) is obtained (Maier-Lenz and Thieme, 1983); levels of flupirtine in the cerebrospinal fluid are almost as high as in the plasma (Obermeier et al., 1985). Hence, the concentrations required in the present in vitro studies to normalize the glutathione level in Glu/NMDA-treated hNT neurons and to increase the amount of Bcl-2 in those cells are close to those found in vivo.

Extrapolating from the in vitro data summarized here to the in vivo situation just mentioned, we have strong reasons to assume that flupirtine is a drug which increases the

intracellular anti-apoptotic agents Bcl-2 and glutathione also in humans. Hence, flupirtine has the potential to be used rationally for the treatment of those diseases which are caused by apoptotic death of cells due to a lack of induction of Bcl-2 and glutathione.

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