Glyceraldehyde-3-phosphate Dehydrogenase, the Putative Target of the Antiapoptotic Compounds CGP 3466 and R-(-)-Deprenyl*

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R-(-)-Deprenyl (Selegiline) represents one of the drugs currently used for the treatment of Parkinson's disease. This compound was shown to protect neurons or glia from programmed cell death in a variety of models. The mechanism of action of neuroprotection as well as inhibition of apoptosis remains elusive. CGP 3466 is a structurally related analog of R-(-)-deprenyl that exhibits virtually no monoamine oxidase type B inhibiting activity but is neuroprotective in the picomolar concentration range. We showed specific binding of CGP 3466 to glyceraldehyde-3-phosphate dehydrogenase by affinity binding, by affinity labeling, and by means of BIAcore® technology. Apoptosis assays based on the human neuroblastoma cell line PAJU established the importance of this interaction for mediating drug-induced inhibition of programmed cell death.

The cause of symptoms in Parkinson's disease is progressive loss of nigrostriatal dopaminergic neurons. The reason for this neuronal loss remains elusive. Among the currently favored possible explanations are oxidative stress as cause or consequence of mitochondrial dysfunction (i.e. deficiency of the respiratory chain due to a defect in complex I), environmental toxins, NMDA receptor-mediated excitotoxicity in combination with mild mitochondrial dysfunction, and radical formation due to excessive iron accumulation in the substantia nigra (1). All of these processes have been shown to be able to induce apoptotic cell death under appropriate circumstances (2–4). The presence of nigral dopaminergic cells undergoing apoptosis in the brains of parkinsonian patients has been demonstrated (5, 6). Nevertheless, a crucial role of apoptosis in the degeneration of dopaminergic neurons in Parkinson’s disease is not yet established, and the benefit of antiapoptotic treatment likewise awaits proof (7). The currently prevalent drug therapy for the treatment of Parkinson's disease consists of levodopa (L-3,4-dihydroxy-phenylalanine), a combination of levodopa and carbidopa (Sinemet/Sinemet CR), and monoamine oxidase type B inhibitors such as R-(-)-deprenyl (selegiline hydrochloride; Ref. 8) or a variety of dopamine agonists of different subtype specificity, alone or in combination with levodopa/carbidopa (9).

While it is still debated whether the beneficial effects of (-)-deprenyl in Parkinson's disease, e.g. as documented in the DATATOP study (10), are symptomatic in nature or indicate a neuroprotective component (e.g. see Ref. 11 as opposed to Ref. 1), neuroprotective effects of the compound have clearly been demonstrated in vivo in experimental animal models and in vitro in cellular systems. Thus, (-)-deprenyl was shown to rescue spinal motor neurons (12) and facial motor neurons (13) after axotomy, nigral dopaminergic neurons after systemic 1-methyl-4-phenyl-1,2,3,6 tetrahydroxypidine treatment (14), or hippocampal pyramidal neurons after systemic kainate acid treatment (15) or after unilateral carotid occlusion/traumatic hypoxia (16). In vitro, the compound has been reported to rescue PC12 cells from apoptotic cell death induced by trophic withdrawal (17), to rescue mesencephalic dopaminergic neurons from 1-methyl-4-phenylpyridinium toxicity (18) and glutamate excitotoxicity (19) or death by aging (20), and to rescue dopaminergic neurons in a co-culture of mesencephalic and striatal cells from 1-methyl-4-phenylpyridinium toxicity (21). While clearly monoamine oxidase type B inhibition by (-)-deprenyl is not responsible for these effects, de novo gene expression seems to be required (22). Target(s) and the mechanism of (-)-deprenyl’s neuroprotective effects are not known.

(-)-Deprenyl is metabolized to (-)-demethyleprenyl and further to (-)-amphetamine and (-)-methamphetamine, principally by P450 enzymes (for a review, see Ref. 23). The rescuing effect of (-)-deprenyl, but not that of (-)-demethyleprenyl, in trophically withdrawn PC12 cells in vitro and in facial motor neurons after axotomy in vivo, is blocked by P450 inhibitors, and (-)-amphetamine and (-)-methamphetamine antagonize the rescuing effect of (-)-deprenyl in these paradigms (24). Accordingly, its capacity to rescue neurons after oral administration is limited, and this may relate to the rather moderate benefit observed clinically, where (-)-deprenyl was administered orally, as opposed to the more clear cut effects in the above mentioned animal experiments, where it was given parenterally (24). Also, the complications of (-)-deprenyl's metabolism may hamper the search for target(s) and the mechanism of its neurorescuing effect.

Dibenzo[b,f]oxepin-10-ylmethyl-prop-2-ynyl-amine (CGP 3466) is structurally related to (-)-deprenyl but exhibits virtually no monoamine oxidase type B or type A-inhibiting properties and is not metabolized to amphetamines. It showed neurorescuing properties qualitatively similar to, but about 100-fold more potent than, those of (-)-deprenyl in the same in....

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vivo and in vitro paradigms.\textsuperscript{2} It therefore seemed a useful candidate to be used for a search for potential targets for the neuroprotective, neurotrophic effects, and the target was identified as glyceraldehyde-3-phosphate dehydrogenase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Compounds (see Fig. 1) were synthesized in house. Rat hippocampi were isolated from Tif:RAIf(SPF) rats (Tierfarm Sisseln, Switzerland). Chemicals used for the BIAcore\textsuperscript{®} experiments were provided by Boehringer Mannheim. Rotenone was obtained from Sigma, and NGF was from Upstate Biotechnology, Inc. Phosphorothioate-modified oligonucleotides were synthesized by B. Craig & Grob (Germany).

**Preparation of Rat Hippocampus Extracts**—Hippocampi of 50 rats were washed with 100 ml of ice-cold phosphate-buffered saline for 1 min, and then 2 volumes of lysis buffer were added. Lysis buffer contained 50 mM Na-HEPES, 10% glycerol (v/v), 0.1 mM EDTA, 1 mM dithiothreitol, 2 mM benzamidine (Sigma, and NGF was from Upstate Biotechnology, Inc. Phosphorothioate-modified oligonucleotides were synthesized by B. Craig & Grob (Germany).)

**Affinity Precipitation of Rat Hippocampus Extracts or Rabbit Muscle Glyceraldehyde-3-phosphate Dehydrogenase**—To 1 ml of cytosolic or membrane fractions of rat hippocampus extract was added 50 μl of suspended resin containing 100 meq/ml demethyldeprenyl or CGP 3466 immobilized on Toyopearl AF amino 650 μm resin, respectively. Alternatively, to 36 μg of rabbit muscle GAPDH in 0.5 ml of lysis buffer 1 ML was added 10 μl of suspended resin. After incubation for 1 h at 4 °C in an end-over-end mixer, the suspension was centrifuged for 1 min at 12,000 × g, and the supernatant was discarded. The pellet was washed three times with 1 ml of 50 mM Na-HEPES, 10% glycerol, 500 mM NaCl, pH 7.5, for 20 min at 4 °C in an end-over-end mixer with intermittent centrifugation at 12,000 × g for 1 min and discarding of the supernatant. The washed resin was resuspended in 20–50 μL of SDS-PAGE sample buffer and heated for 5 min at 95 °C, and 20 μl was loaded on a 12.5% SDS-PAGE gel. SDS-PAGE was carried out as described (25). Gels were stained using BM Fast Stain according to the manufacturer’s instructions. In control experiments, affinity precipitation of the cytotoxic fraction in low salt lysis buffer with undervirulized and acetylated Toyopearl resin was investigated using the described procedure.

**Identification of Proteins by Nanoelectrospray Mass Spectrometry**—Affinity-purified proteins were separated by SDS-PAGE as described. The gel was silver-stained, and excised bands were prepared for nanoelectrospray mass spectrometry using a recently developed procedure (26, 27). Briefly, the gel was silver-stained using a modified procedure to prevent covalent chemical modification of the proteins in the gel. Bands of interest were excised, reduced in gel, carboxymethylated, and digested in gel with trypsin followed by extraction of the generated peptides using 100 μl of 20 mM NH₄HCO₃ and 3 × 100 μl of 5% formic acid in 50% propanol/water (v/v). Extracted peptides were dried at reduced pressure in a SpeedVac and sequenced using a Sciex API III triple quadrupole mass spectrometer (Toronto, Canada). Electrospray needles were obtained from the Protein Analysis Company (Odense, Denmark). Dried protein digests were resuspended in 5% formic acid, concentrated, and desalted on a capillary similar to that used for spray- ing. It was packed with 100 nl of POROS R2 sorbent (Perseptive Biosystems, Framingham, MA) and calibrated with 5% formic acid. After loading the dissolved peptide digest, the capillary was washed with 20 μl of 5% formic acid, 5% methanol. The sample was eluted into the spraying capillary in 2 × 1 μl of 5% formic acid, 50% methanol. Q₁ scans were performed with unit mass resolution and mass steps of 0.1 Da. The orifice voltage was 65 V. For operation in the MS/MS mode, Q₁ was set to transmit approximately a mass window of 2 Da. The collision energy was tuned individually for peptides of interest to obtain maximum sequence information. Molecular weight information in combination with MS/MS-derived short sequence tags were used to search the newest SWISSPROT database and, if necessary, the translated GenBank™ data base with PeptideSearch version 2.9.2h running on a Macintosh computer.

**Binding of Rabbit Muscle GAPDH to Immobilized CGP 3466**—To 1 ml of cytosolic or membrane fractions of rat hippocampus extract was added 50 μl of 5% formic acid buffer, pH 9.0, 3:1 (v/v), was prepared, and 25 μl was immediately injected on the activated sensor chip. Unreacted active sites on the chip were subsequently blocked by injecting 70 μl of 1 M ethanolamine HCl, pH 8.5. The derivatized sensor chip was washed extensively with PBS (10 mM Na-HEPES, pH 7.5, 150 mM NaCl, 0.4 mM EDTA, 0.005% surfactant, P20). Binding experiments by injecting 35 μl of solutions containing rabbit muscle GAPDH plus various additives (see figure legends) at a flow of 5 μl/min. The sensor chip surface was regenerated by injection of 20 μl of 6 M guanidinium HCl in PBS at 5 μl/min. Prior to injection in the BIAcore® rabbit muscle, GAPDH was dialyzed overnight at 4 °C against HBS using Pierce Slide-A-Lyzer® 10 K dialysis cassettes. Unspecific binding was evaluated using flow cells on the chip with undervirulized surface or surface derivatized with ethanolamine.

**Photoaffinity Labeling of Rat Hippocampus Extracts and Rabbit Muscle GAPDH**—Cytosolic and membrane extract of rat hippocampi corresponding to 100 μg of total protein or 5 μg of rabbit muscle GAPDH were incubated with 5 nM 125I-PHCGP (2000 Ci/mmol) and 5 μM PHCGP (Fig. 1F) in 50 μl of lysis buffer L1 for 30 min at 37 °C. In competition experiments, proteins were preincubated for 30 min at 37 °C in the absence of photoaffinity ligand with the appropriate amounts of competitor. After incubation, samples were cooled on ice for 5–10 min and exposed to 254-nm UV light with an intensity of 3000 watts/cm² for 120 s. Immediately after exposure, samples were quenched with 25 μl of SDS-PAGE loading buffer, and heated at 95 °C for 5 min. Samples were analyzed on a 12.5% SDS-PAGE gel as described (25). The gel was subsequently vacuum-dried on paper and exposed for the indicated amount of time to Kodak Bio-Max MS or Kodak X-Omat AR film at 70 °C using an intensifying screen. Samples were separated together with prestained standard mixtures (Bio-Rad) as molecular weight standards.

**Immunostainning**—PAJU cell pellet was washed with phosphate-buffered saline and resuspended in 10 mM Na-Hepes, pH 7.4, containing 10 mM NaCl, 0.5 mM dithiothreitol and a mixture of protease inhibitors (Boehringer Mannheim). The cells were swollen on ice and lysed by repeated aspiration in a plastic syringe. After centrifugation at 4 °C for 15 min at 14,000 rpm, the supernatant was kept as cytosolic fraction, and the pellet was resuspended in 20 ml of 50 mM Na-Hepes, pH 7.9, 10% glycerol, 0.5 mM dithiothreitol containing the protease inhibitor mixture and 10 units/ml DNase I. The protein concentration was estimated with the Bio-Rad protein assay kit, calibrated with bovine serum albumin.

1 μg of total protein/lane was separated electrophoretically on a 12.5% SDS-polyacrylamide gel and subsequently blotted on to a polyvinylidene difluoride membrane (Millipore Corp.). Immunoblots were probed with rabbit anti-GAPDH antibody from Chemicon as described (28).

**Cultivation of PAJU cells**—The PAJU tumor cell line was kindly provided by L. C. Andersson (University of Helsinki). The cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, in a surface-adherent manner. The cells were seeded at a density of 1.5 × 10⁵ cells/well in 24-well plates 24 h after seeding. Differentiation was enhanced by the addition of NGF to 100 ng/ml. 1 day after triggering differentiation, the cells were induced in apoptosis by adding 3 μM rotenone. CGP 3466 was added at 10⁻⁵ M 1 h before exposure to rotenone.

Cell death was quantified microscopically by nuclear DNA analysis; the cells were fixed with 3.7% formaldehyde for 30 min at room-
perature. After fixing, cells were washed twice with phosphate-buffered saline and stained with 1 μg/ml 4,6-diamidine-2-phenylindole-dihydrochloride (DAPI; Boehringer Mannheim). Cells were transfected with the Superfect kit (Qiagen) according to the manufacturer’s instructions. GFP-expressing cells were counted 2 days after transfection by means of fluorescence microscopy using a Leica DM IRB microscope.

RESULTS

Immobilized CGP 3466 binds glyceraldehyde-3-phosphate dehydrogenase in rat hippocampus extracts—Deprenyl-related compounds (Fig. 1) have been reported to rescue serum-deprived cultured PC12 cells from apoptosis. In an attempt to isolate potential target proteins of these compounds, extracts of neuronal rat cells were used in affinity precipitation experiments. Cytosolic and membrane fractions of rat hippocampal lysates were incubated with CGP 3466 or (−)-demethyldeprenyl immobilized to Toyopearl resin (Fig. 1, D and E) followed by extensive washing. Bound proteins were analyzed by SDS-PAGE. Binding to immobilized CGP 3466 appeared more selective than to immobilized (−)-demethyldeprenyl. Using immobilized CGP 3466, four protein bands at approximately 38, 43, 50, and 200 kDa that were prominent in the cytosolic extracts, appeared to be enriched compared with the total extracts (Fig. 2A). These were selected for identification by the nanoelectrospray mass spectrometry/sequence tag approach after silver staining and in gel tryptic digestion (26, 27). In general, a tandem mass spectrum of a selected peptide provided a short sequence stretch, which, together with molecular weight information, enabled us to identify the respective parent protein in publicly available sequence data bases. Identification was verified by applying the same procedure to the additional peptides present in the digest. On the basis of four individual peptides (Table I), the protein band at 38 kDa was recognized to represent GAPDH. The tryptic digests of the bands at 43, 50, and 200 kDa contained peptides corresponding to α- or β-actin or a mixture of both, α- and β-tubulin, and α- and β-spectrin respectively. The amino acid sequence for α- or β-spectrin in rat was not present in publicly available data bases; identification was based upon the presence of the identified amino acid sequences in α- and β-spectrin in related species. To evaluate unspecific binding to the resin, affinity precipitation of cytosolic extract with underivatized Toyopearl resin and acetylated Toyopearl resin was performed. An intense band at approximately 50 kDa on the SDS-PAGE gel at a position corresponding to that of tubulin and a weaker band at 43 kDa at the position of actin indicated unspecific binding to both underivatized Toyopearl resin and acetylated Toyopearl (Fig. 2B). Affinity precipitation experiments using commercially available purified rabbit muscle GAPDH were done to prove direct interaction of GAPDH with CGP 3466 and (−)-demethyldeprenyl and to exclude indirect binding via cytoskeletal proteins. Rabbit muscle GAPDH did bind to CGP 3466.

![Fig. 1. Deprenyl-related compounds used in target identification. A, (−)-deprenyl; B, (−)-demethyldeprenyl; C, CGP 3466; D and E, CGP 3466; (−)-demethyldeprenyl, respectively, as attached to Toyopearl resin; F, photoaffinity-labeled CGP 3466 derivative; G, CGP 3466 with spacer for amine coupling to a CM-Sepharose chip in the BIAcore®.](image)

![Fig. 2. SDS-PAGE analysis of affinity-precipitated proteins. Protein solutions were incubated with Toyopearl resin and analyzed on 12.5% SDS-PAGE gels as described under "Experimental Procedures." A, rat hippocampus extracts on CGP 3466-derivatized Toyopearl resin (see Fig. 1D). Lane 1, cytosolic extract in lysis buffer L1; lane 2, cytosolic extract in lysis buffer L2; lane 3, membrane extract in lysis buffer L1; lane 4, membrane extract in lysis buffer L2; lane 5, molecular weight standard. B, cytosolic rat hippocampus extract in buffer L1. Lane 1, molecular weight standard; lane 2, CGP 3466-derivatized Toyopearl resin (see Fig. 1D); lane 3, underivatized Toyopearl resin; lane 4, acetylated Toyopearl resin. C, rabbit muscle GAPDH in buffer L1. Lane 1, molecular weight standard; lane 2, underivatized Toyopearl resin; lane 3, acetylated Toyopearl resin; lane 4, CGP 3466-derivatized Toyopearl resin (Fig. 1D).](image)

| Peptide | Sequence       |
|---------|---------------|
| 1       | GAAQNIIPASTGAAK |
| 2       | VGVDGFGR       |
| 3       | VIFELDGK       |
| 4       | LTGMAFR        |

![Table I](image)

Identification of glyceraldehyde-3-phosphate dehydrogenase-derived peptides by electrospray mass spectrometry

Data bank searches using mass spectrometric data as described under "Experimental Procedures" identified the 38-kDa band that affinity-precipitated with immobilized CGP 3466 as glyceraldehyde-3-phosphate dehydrogenase. The aspartic acid residues in peptides 2 and 3 are in conflict with the reported amino acid sequence, which has asparagine residues in these positions. Most likely, this results from deamidation, which has been reported before for Asn-Gly sequences.
immobilized on Toyopearl, whereas no binding to underivatized or acetylated Toyopearl was observed (Fig. 2C).

**Photoaffinity Labeling Demonstrates Specific Association of GAPDH and Labeled CGP 3466 in Solution**—To further substantiate the affinity precipitation results and to exclude artifacts arising from immobilization of ligands, photoaffinity labeling, a method relying on solution interactions, was performed. Photoaffinity labeling was carried out using a derivative of CGP 3466 containing a photo reactive aryl azide (PHCGP) substituted with 125I linked through a spacer (Fig. 1F). Rat hippocampus extract or purified rabbit muscle GAPDH was incubated with PHCGP at 37 °C, cross-linked by photoactivation at 254 nm, and subsequently analyzed by SDS-PAGE. Labeled proteins were detected by exposing the dried SDS-PAGE gels to x-ray film. Bands with variable intensities at approximately 200, 70, 50, 38, 30, and 27 kDa in the cytosolic extract and at approximately 50, 40, and 36 kDa in the membrane extract were observed (Fig. 3A). In the same experiment, purified rabbit muscle GAPDH generated a labeled band at the same position as the 38-kDa band in the cytosolic extract, suggesting that labeled GAPDH is present in the rat hippocampus extract. Identically treated samples that were not UV-exposed generated only very faint bands, indicating that labeling was essentially UV-dependent (Fig. 3A). The identities of the other bands were not investigated. Since GAPDH was the only protein consistently present in both affinity precipitation and photoaffinity labeling experiments and did not show unspecific binding in the affinity precipitation experiments, this interaction was further investigated. Nonradioactive PHCGP and NAD+ were found to effectively compete with PHCGP for labeling of purified rabbit muscle GAPDH, demonstrating the specificity of the interaction. (Fig. 3, B and C).

**Binding of GAPDH to CGP 3466 in the BIAcore®**—The single protein consistently identified by both affinity precipitation and photoaffinity labeling as selectively interacting with CGP 3466 was GAPDH. To further characterize this interaction kinetically, purified rabbit muscle GAPDH was investigated using BIAcore® technology. A CGP 3466 derivative with an appropriate spacer (Fig. 1G) was covalently linked by amine coupling to a CM-Sepharose chip. Experiments were started by injecting a solution of rabbit muscle GAPDH in binding buffer, allowing association to take place, followed by a pulse of eluent during which bound GAPDH could dissociate. The derivatized surface was regenerated by injecting 6 mM guanidinium HCl. A typical sensogram for different concentrations of GAPDH shows concentration-dependent binding (Fig. 4A). Using BIAcore® evaluation software (version 2.1), the dissociation phase displayed a good fit ($\chi^2 = 0.06$) with a model assuming simultaneous dissociation of two binding complexes with dissociation constants on the order of $10^{-2}$ and $10^{-5}$. The association phase of the binding curve did not fit adequately to any of the available theoretical models; therefore, an association constant could not be determined, suggesting complex association kinetics. Binding experiments in flow cells with unmodified surface or derivatized with ethanol amine indicated that unspecific binding was marginal (data not shown). Binding of GAPDH to immobilized CGP 3466 in the BIAcore® was dramatically reduced after dialysis against HBS with or without 2 mM NAD+ followed by equilibration at 37 °C for 15 min. Indicated are amounts of GAPDH (expressed as relative response relative to 0 mM NAD+ set at 100) bound at the start of the dissociation phase (8 min).

**PAJU Cells Are Protected from Apoptosis by CGP 3466**—The
human neuroblastoma cell line PAJU was established as an in vitro model system to investigate CGP 3466 functionally. In a typical culture of PAJU cells, a small percentage of the cells spontaneously stop to proliferate and exhibit neural sprouting (29). The proportion of cells undergoing differentiation can be increased to almost 100% by the addition of phorbol esters (e.g. phorbol 12-myristate 13-acetate). We examined PAJU cells presence or absence of phorbol 12-myristate 13-acetate morphologically. More than 90% of cells in a given culture show neural sprouting in the presence of phorbol 12-myristate 13-acetate. However, most of the remaining cells undergo apoptosis as visualized by staining of condensed chromosomal DNA with DAPI. Such spontaneous induction of apoptosis by phorbol 12-myristate 13-acetate rendered the semiquantitative analysis of apoptosis effectors difficult. Alternative procedures to induce differentiation were tested to reduce the background of cell death. Experimental conditions involving a 24-h exposure to NGF have proven to effectively differentiate cells with no apparent dead cells.

NGF-differentiated PAJU cells were treated in a number of ways known to induce apoptosis in cellular model systems: rotenone, high concentrations of ara-C, carbamoyl cyanide m-chlorophenylhydrazone, or peroxide. For most compounds, the concentration range yielding reproducible and quantifiable numbers of apoptotic cells was very narrow. The mitochondrial complex I inhibitor rotenone was selected as the apoptosis inducer most suitable for subsequent experiments as illustrated in Fig. 5. The conditions for rotenone-induced apoptosis were optimized (3 μM) so that approximately 50% of the cells die within 20 h after toxin addition as observed by DAPI staining (Fig. 5, B and D) and by following the loss of neural processes morphologically (Fig. 5, A and C).

CGP 3466 exhibits antiapoptotic effects in vitro in trophically withdrawn PC12 cells and in cerebellar granule cells treated with ara-C. We therefore analyzed PAJU cells partially forced into apoptosis by rotenone in the presence versus absence of CGP 3466 by DAPI staining. Up to 40% more cells survived when treated with compound in a concentration-dependent manner. Concentrations in the range of 1 nM were most effective (Fig. 5E). The degree of confluency of the cell culture was critically influencing CGP 3466-mediated protection. Similar protective properties of CGP 3466 were seen upon induction of apoptosis with 3 mM ara-C (not shown). The number of neural processes and the length of them seen in CGP 3466-treated cells was lower than in NGF-treated control cells, in agreement with the observation that the drug was delaying

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3 W. G. Tatton, R. M. Chalmers-Redman, and N. A. Tatton, manuscript in preparation.
4 I. A. Paterson and C. Voll, manuscript in preparation.
rather than preventing apoptosis. In conclusion, PAJU cells were established as a model system to investigate the mechanism of drug-mediated protection from apoptosis.

**GAPDH Antisense Oligonucleotides Protect from Apoptosis—** Photoaffinity labeling and BIAcore® experiments established a specific interaction between CGP 3466 and GAPDH. To approach the functional relevance of this interaction with respect to regulating apoptosis, PAJU cells were treated with GAPDH antisense oligonucleotides. The antisense oligonucleotide was designed to span the ATG initiation codon. The antisense oligonucleotide was able to significantly protect PAJU cells from rotenone-induced (Fig. 6C) and ara-C-induced apoptosis (not shown) in a concentration-dependent manner. In comparison, adding identical concentrations of the corresponding sense oligonucleotides did not significantly change the number of apoptotic cells upon rotenone challenge. The addition of CGP 3466 together with antisense oligonucleotide did not lead to further increase of surviving cells, in agreement with the hypothesis that both affect the same pathway.

The fate of GAPDH protein upon rotenone treatment was followed immunologically. The amount of cytosolic GAPDH did not change significantly under any conditions within relevant time frames (0–24 h, Fig. 6B). In contrast, the amount of GAPDH associated with the nucleus increased 2–3-fold in the first 8 h after rotenone addition (Fig. 6A). The addition of antisense oligonucleotide prevented this rotenone-initiated accumulation of nuclear GAPDH.

**CGP 3466 Protects from Cell Death Induced by GAPDH Overexpression—** Antisense oligonucleotides against GAPDH were designed to span the ATG initiation codon. The antisense oligonucleotide was able to significantly protect PAJU cells from rotenone-induced (Fig. 6C) and ara-C-induced apoptosis (not shown) in a concentration-dependent manner. In comparison, adding identical concentrations of the corresponding sense oligonucleotides did not significantly change the number of apoptotic cells upon rotenone challenge. The addition of CGP 3466 together with antisense oligonucleotide did not lead to further increase of surviving cells, in agreement with the hypothesis that both affect the same pathway.

**DISCUSSION**

The identification of target(s) of deprenyl-related compounds such as CGP 3466 is a prerequisite for the elucidation of its mechanism of action in protecting neuronal cells from apoptosis. The specific association of CGP 3466 with GAPDH in rat hippocampus extracts and with purified GAPDH from rabbit muscle is reported here. The only protein consistently identified by means of both affinity precipitation and photoaffinity labeling using immobilized and photoaffinity-labeled CGP 3466, respectively, is GAPDH. In addition, purified rabbit muscle GAPDH demonstrated affinity for immobilized CGP 3466 in BIAcore® assays. The specificity of the interaction is indicated by the inhibition of binding by NAD⁺, the natural coenzyme of GAPDH. This effect was observed in photoaffinity labeling and BIAcore® experiments alike. No significant influence of CGP 3466 at physiologically reasonable concentrations was seen on GAPDH-catalyzed dehydrogenase activity.

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5 E. Kragten, I. Lalande, K. Zimmermann, S. Roggo, P. Schindler, D. Müller, J. van Oostrum, P. Waldmeier, and P. Fürst, unpublished observations.
The binding of NAD\(^+\) to the four binding sites in tetrameric GAPDH has been shown to involve negative cooperativity (30, 31). In this respect, GAPDH is believed to behave as a dimer of dimers where binding of one NAD\(^+\) molecule results in conformational changes and diminished affinity for NAD\(^+\) in the second monomer (32). The observed effect of NAD\(^+\) on the affinity of GAPDH for CGP 3466 can be direct, by competition for the same binding site, or indirect, through conformational changes. The NAD\(^+\) binding site in GAPDH consists of a fold known as the Rossmann fold, which is shared by many NAD- and FAD-binding enzymes (33). Interestingly, amino acid sequence analysis and mutational analysis indicated the presence of a similar structure in monoamine oxidase type B (34), an FAD-binding enzyme to which also deprenyl binds. Deprenyl presumably binds in the proximity of FAD in monoamine oxidase type B, since it irreversibly inhibits the enzyme by reacting with the covalently bound FAD molecule. This is suggestive of binding of CGP 3466 to a similar fold in GAPDH.

Because GAPDH is a homotetramer, it can contain multiple binding sites for CGP 3466. This, combined with possible interference by NAD\(^+\) binding with negative cooperativity to the individual sites, can result in complex binding characteristics. This may well explain why it was not possible to fit the association phase in the BIAcore\(^\circledR\) to a simple kinetic model. Although the dissociation phase fitted to a model describing simultaneous dissociation of two complexes, this does not necessarily reflect the actual molecular binding mechanism. The slow dissociation, however, suggests a very tight binding between immobilized CGP 3466 and GAPDH.

If binding to GAPDH mediates the antiapoptotic action of deprenyl-related compounds, its biological function in programmed cell death has to be demonstrated. GAPDH has long been thought of as a housekeeping enzyme whose sole function lies in the glycolytic pathway. However, concomitant with the work described here, several publications have appeared that suggest a role for GAPDH in apoptosis of neuronal cells. Ishitani and co-workers have recently described up-regulation of GAPDH mRNA and an increase of GAPDH protein in the particulate fraction of cell extracts during age-induced apoptosis of mature cerebellar (35) and cerebrocortical neurons (36) and ara-C-induced apoptosis of cultured cerebellar neurons (37). In each of these cellular assays, apoptosis was significantly delayed by antisense GAPDH oligonucleotides. This delay was accompanied by a reversal of GAPDH mRNA overexpression to basal levels. Here, we describe a cellular neuronal apoptosis assay using PAJU cells and apoptosis induced by rotenone that confirms the above observations, strongly suggesting that the up-regulation of GAPDH mRNA and the increase in GAPDH protein content in the particulate fraction of apoptotic cell extracts is a general phenomenon in neuronal cells undergoing apoptosis. In addition, CGP 3466 actively rescues PAJU cells from rotenone-induced apoptosis demonstrating the occurrence of both phenomena in the same apoptosis assay. Moreover, CGP 3466 protects PAJU cells from cell death spurred by GAPDH overexpression. Altogether, this is strongly suggestive of an involvement of GAPDH in both neuronal apoptosis and the mechanism by which deprenyl-related compounds rescue cells from apoptosis.

The large number of functions unrelated to its glycolytic activity that have been ascribed to GAPDH recently (38) makes it an attractive candidate to mediate rescue of neuronal cells from apoptosis by deprenyl-related compounds but complications defining which potential function of GAPDH is influenced. Among the recently described features of GAPDH are specific tRNA binding (39), AU-rich mRNA binding (40), uracil-DNA-glycolase activity of the human monomeric form (41), and isoform-specific catalytic action in plasmenylethanolamine-selective membrane fusion in rabbit brain cytosol (42). In addition, GAPDH is a possible activator of transcription in neurons (43), GAPDH/uracil-DNA-glycolase expression is reported to be cell cycle-regulated in human cells (44, 45), and binding of GAPDH to proteins implicated in neurodegeneration has been reported (46). In accordance with some of these activities, GAPDH has been localized in the nucleus in oligodendrocytes (44), and an antibody against the monomer of human GAPDH displaying uracil-DNA-glycolase activity showed a preferential nuclear or perinuclear localization during cell proliferation of fibroblasts (47). GAPDH occurs in several isoforms; e.g., 16 individual isoforms have been observed in rabbit brain (42). These can result from posttranslational modifications, but, given the large number of genes that encode sequences strongly similar to GAPDH (48, 49), they can also represent mutations in the amino acid sequence. Preferential binding to or interference with the activity of a particular cell-specific isoform could explain the observation that the activity of deprenyl-related compounds appears to be restricted to neuronal and glial cells.

The diverse functions of GAPDH make it difficult to define the possible consequence of binding of deprenyl-related compounds to GAPDH in neuronal cells, and further studies are necessary to define the function of GAPDH in apoptosis. One hypothesis would assign GAPDH a central function as a link between neuronal apoptosis and the energy status of neurons. Alternatively, GAPDH interacts, directly or indirectly, with neuron-specific transcription factors. A further role could involve specific mRNA translocation between cellular compartments.

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