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Dibenzoxepines as treatments for neurodegenerative diseases*

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Abstract: In recent years, apoptotic cell death has been implicated with different progressive neurodegenerative diseases such as Parkinson's disease, Huntington's disease, Amyotrophic Lateral Sclerosis or Alzheimer's disease. The hypothesis emerged, that a drug preventing apoptosis may slow or even halt the disease progression. (–)-Deprenyl was reported to rescue neurons from cell death in different *in vitro* and *in vivo* systems. However, deprenyl suffers the antagonizing actions of its major metabolites. We set up a screening for compounds with neurorescuing properties, lacking deprenyl's metabolic problems. 10-Aminomethyl-dibenzo[b,f]oxepin derivatives were identified to show marked effects in a survival assay of trophically-withdrawn PC12 cells. Dibenzo[b,f]oxepines bearing different aminomethyl side-chains and aromatic substituents were prepared in a multistep synthesis, and a structure-activity relationship was established. In particular the N-methyl-N-propargylaminomethyl derivative, CGP 3466, shows neurorescuing properties at concentrations as low as 10^{-13} M in different *in vitro* test systems. *In vivo*, CGP 3466 prevents the death of dopaminergic cells in the mouse substantia nigra after MPTP-lesion. It also rescues mouse facial motor neurons after axotomy and increases the life-span of mice with progressive motor neuronopathy. Glyceraldehyde-3-phosphate dehydrogenase was identified as the putative molecular target of CGP 3466-derivatives by means of affinity binding and photoaffinity labeling.

INTRODUCTION

The pathology underlying the symptoms of neurodegenerative diseases is mostly known, e.g. progressive loss of nigrostriatal dopaminergic neurons in Parkinson's disease or degeneration of motoneurons in Amyotrophic Lateral Sclerosis (ALS). However, the cause of these neuronal deaths remains elusive. The spectrum of hypotheses reaches from environmental toxins, oxidative stress, excitotoxicity, mitochondrial dysfunction to genetic predisposition. Ultimately, all these processes may lead to cell death.

In recent years, the hypothesis has emerged that programmed cell death, also referred to as apoptosis, may play a critical role in the pathogenesis of neurodegenerative diseases [1]. In contrast to necrosis caused by the noxious effect of harsh environmental impacts, apoptotic cell death is a highly organized and 'clean' degradation of a cell. In relation to major neurodegenerative disorders there is accumulating evidence from assessment of human postmortem tissue, suggesting that apoptosis is involved [2–8]. Therefore, a treatment preventing neuronal apoptosis in the adult brain is believed to be beneficial in progressive neurodegenerative conditions.

The anti-parkinsonian drug (–)-deprenyl (Selegiline) **1** was reported to rescue different types of neurons from apoptotic cell death *in vitro* in a manner unrelated to its monoamine oxidase-B (MAO-B)

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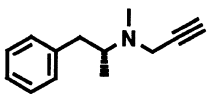
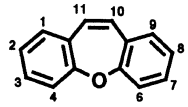
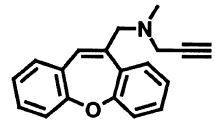


R-(-)-deprenyl (1)



dibenzo[b,f]oxepine



CGP 3466

inhibiting properties [9–12]. Moreover, deprenyl rescues nigral dopaminergic neurons after MPTP treatment *in vivo* [13–15], facial motor neurons after axotomy [16] and hippocampal pyramidal neurons after systemic kainate treatment [17] or after unilateral carotid occlusion with transient hypoxia [18].

Deprenyl is readily metabolized to methamphetamine and amphetamine, particularly after oral administration [19]. These two compounds have been shown to antagonize deprenyl's rescuing effects *in vitro* and *in vivo* [20]. This may limit the therapeutic exploitability of (–)-deprenyl in neurodegenerative diseases.

It was therefore our goal to find compounds with equal or better neurorescuing properties than (–)-deprenyl, but unable to give rise to antagonistic metabolites.

SCREENING USING THE PC12 CELL SURVIVAL ASSAY

Compounds structurally related to deprenyl were searched in the corporate archives. The screening was performed using the survival assay of trophically-withdrawn PC12 cells [9]. These cells were partially differentiated by maintaining them for six days in the presence of serum (10% horse serum and 5% fetal bovine serum) and nerve growth factor (NGF). Both, serum and NGF were replaced by minimal essential medium, thus inducing apoptotic cell death. Test compounds at 10^{-7} M and 10^{-9} M were added immediately after NGF/serum deprivation. Surviving cells containing intact nuclei were counted 24 h later. Each experiment was carried out in quadruplicates. The cell counts of the serum/NGF-withdrawn controls without compound were set to 100%. All results were normalized to the mean value of the (–)-deprenyl-effect at 10^{-9} M (203%). Compounds with scores <170% were considered of no further interest. 10-Aminomethyl-dibenzo[b,f]oxepin derivatives were found to potently inhibit apoptosis in the PC12 assay (see Table 1).

SYNTHESIS OF DIBENZO[B,F]OXEPINES

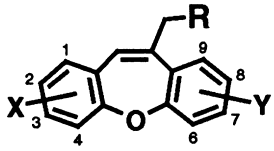
Subsequently, this class of compounds was further investigated. Dibenzo[b,f]oxepines were synthesized using a modified literature protocol [21–24] (see Scheme 1). The phenoxyphenyl-benzaldehyde derivatives **2** were obtained from ortho-fluorobenzaldehydes and phenols under mild alkaline conditions. Azlactonization with hippuric acid, followed by ring-closure under strongly acidic conditions led to the dibenzo[b,f]oxepin-10-carboxylic acids **5**. Depending on the ring-substituents X and Y the azlactone route gave highly variable yields. As an alternative pathway the aldehyde **2** was reduced to an alcohol, brominated and converted into the phenoxyphenyl-acetonitril derivatives **3** in high yields. Condensation with diethyloxalate giving **4**, and intramolecular Friedel–Craft type acylation under harsh acidic condition completed this route to the dibenzo[b,f]oxepin-10-carboxylic acid derivatives **5**. The reduction of the carboxylic acid group of **5** using $LiA1H_4$ resulted in the unwanted saturation of the 10,11-double bond. Clean reduction to the alcohols **6** was achieved after formation of mixed aldehydes using isobutyl-chloroformiate, followed by borohydride-reduction in water with nearly quantitative yields. The allylic bromides **7** were obtained smoothly using N-bromosuccinimide and triphenylphosphine in THF. The synthesis was completed by amination with different primary or secondary amines giving 10-aminomethyl-dibenzo[b,f]oxepines **8** in overall yields of up to 40%.

STRUCTURE–ACTIVITY RELATIONSHIPS IN THE PC12 ASSAY

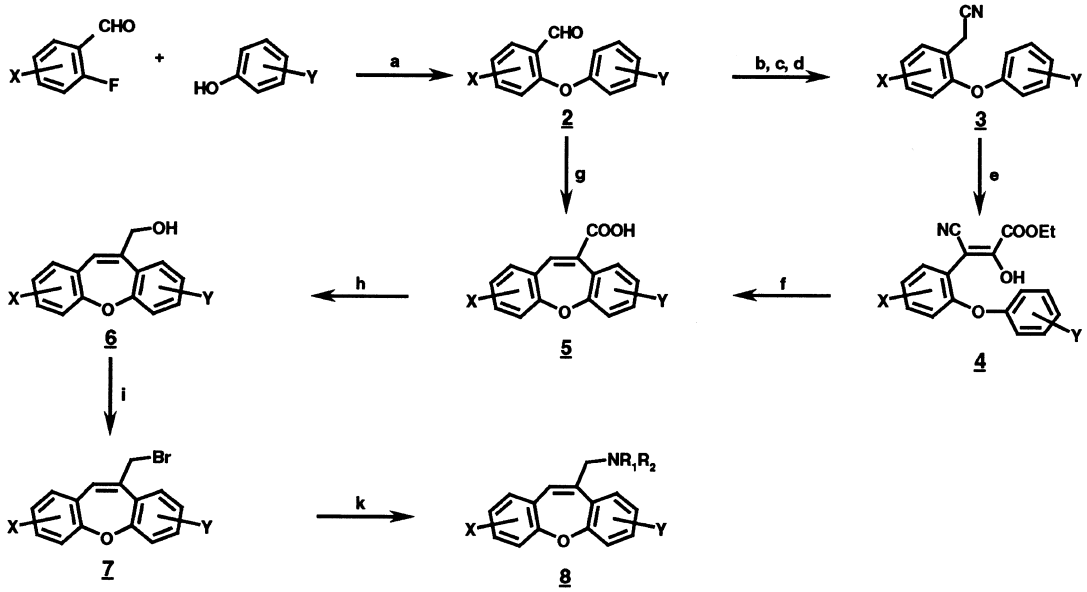
Neurorescuing activities were assessed in the CP12 cell survival assay after trophic withdrawal (see description above and data in Table 1). In the series of unsubstituted 10-aminomethyl-dibenzo[b,f] oxepines (X, Y = H) the primary amine **8a** and the N-methyl derivative **8b** showed strong activities, whereas the dimethylamino compound **8c** was considerably less active. Derivatives with unsaturated

Table 1 PC12 cell survival assay: structure–activity relationships of substituted dibenzo[b,f]oxepines

	R (no ring substituents (X, Y = H)	% eff. 10^{-7} M	% eff. 10^{-9} M
8a	NH_2	227	234
8b	$NHCH_3$	240	193
8c	$N(CH_3)_2$	140	121
8d	N-Me-N-allylamine	198	182
8e	N-propargylamine	n.d.	207
CGP 3466	N-Me-N-propargylamine	212	243
8f	N-Me-N-phenylamine	111	91
8g	N-Me-N-benzylamine	135	146
8h	pyrrolidine	n.d.	208
8i	morpholine	132	160
8j	piperidine	169	169
8k	3-pyrroline	171	175
8l	1,2,3,6-tetrahydropyridine	158	159
8m	1,2,3,4-tetrahydroisoquinoline	111	89
8n	1,2,3,4-tetrahydroquinoline	122	105



	R	ring substituents X, Y	% eff. 10^{-7} M	% eff. 10^{-9} M
8o	N-Me-N-propargylamine	1-F	167	147
8p	pyrrolidine	1-F	147	184
8p	$N(CH_3)_2$	2-Cl	n.d.	162
8r	pyrrolidine	3-CF_3	200	191
8s	N-Me-propargylamine	6-Cl	199	170
8t	pyrrolidine	6-Cl	175	210
8u	N-Me-N-propargylamine	7-C≡C$(CH_2)_3CO_2Me$	237	239
8v	N-Me-N-propargylamine	8-Cl	180	169
1	**R-(–)-deprenyl** (as reference compound)		181	203



Scheme 1 General synthetic procedure for ring-substituted dibenzo[b,f]oxepines. (a) K_2CO_3; (b) LAH; (c) HBr; (d) NaCN; (e) Na, diethyloxalate; (f) H_2SO_4; (g) hippuric acid, NaOAc; Ac_2O, H_2SO_4; (h) 1. isobutyl-chloroformiate, 2. $NaBH_4$; (i) NBS; (k) HNR_1R_2.

amino-side chains **8d–f** proved to be very potent. In particular compound **8f** (CGP 3466) was more potent than (–)-deprenyl **1** and showed significant neurorescuing effects even at concentrations of 10^{-13} M. The biological profile of CGP 3466 is reported below. N-Side-chains bearing aromatic moieties were generally found to be inactive (**8g–h**, **k–l**). Small cyclic amines (**8j–m**) showed moderate effects with the exception of the pyrrolidine derivative **8i**, which was equally active to (–)-deprenyl at 10^{-9} M.

Dibenzo[b,f]oxepines bearing aromatic ring-substituents were generally investigated having either a N-methyl-N-propargylaminomethyl or a pyrrolidinomethyl side-chain in position 10. Small ring substituents, such as a fluorine or chlorine atom, in the aromatic positions 1, 2 and 8 led to moderate activities of these compounds (**8p–r**, **8w**). Introduction of a trifluoromethyl group in position 3 (**8s**), or a chlorine atom in position 6 (**8t–q**) resulted in highly active derivatives. Interestingly, the compound **8u**, having a long hexynoic acid side chain in position 7 resulted in an even increased rescuing effect. This derivative has been used as a key-intermediate in the synthesis of tool compounds for the target identification program of 10-aminomethyl-dibenzo[b,f]oxepines [22].

Subsequently, the key-compound CGP 3466 was investigated on other *in vitro* assays of neuronal cell death and in different animal models, indicative for neurodegenerative diseases.

EFFECTS OF CGP 3466 AGAINST MPTP-TOXICITY

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is known to induce parkinsonian symptoms in humans and other primates [25,26]. MPTP is oxidized by MAO-B to its active metabolite 1-Methyl-4-phenylpyridinium (MPP^+) [27]. MPP^+ is selectively taken up into dopaminergic cells via the dopamine transporter and subsequently accumulated in mitochondria, resulting in energy break-down and cell death. MPTP-toxicity was also demonstrated in mice [13,28] leading to reduced numbers of tyrosine-hydroxylase-positive (TH+) cells in the substantia nigra. Finally, MPP^+-induced apoptotic cell death was reported for different *in vitro* neuron culture systems [29–31]. Currently, MPTP/MPP^+-toxicity is the best-established model-system for Parkinson's disease.

Rescue of rat embryonic mesencephalic dopaminergic cells in culture from MPP^+-induced cell death

Rat mesencephalic dopaminergic cells taken at embryonic day 15 were treated on the 4th day in culture with 0.5 μM MPP^+, the active metabolite of MPTP, in the presence or absence of CGP 3466 (free base). The compound was re-added after 24 h. After another 24 h in culture, tyrosine-hydroxylase-positive (TH+) cells were counted after immunostaining. CGP 3466 at 10^{-7} and 10^{-9} M rescued about two-thirds of the cells, to a similar extent after both concentrations (Fig. 1).

Effects of CGP 3466 on survival of dopaminergic cells in mouse substantia nigra after treatment with MPTP

MPTP dopaminergic neurotoxicity is an accepted model of Parkinson's disease. C57/B1/6J mice received MPTP (twice 30 mg/kg s.c., 72 h apart) and were subsequently treated twice daily for 18 days with 0.00142, 0.0142, 0.142 and 1.42 mg/kg p.o. (corresponding to 0.001, 0.01, 0.1 and 1 mg/kg free base) CGP 3466B (maleate salt), beginning 48 h after the second MPTP injection. The animals were sacrificed on the last day of treatment and tyrosine hydroxylase-positive (TH+) cells were counted in serial sections of the substantia nigra. Data are means of the sums of cells ± SEM counted in 22 (p.o. experiment) or 17 (s.c. experiment) sections spaced by 50 μm. The substantia nigras of 5–9 animals were evaluated.

Nigral TH+ cells were significantly spared, to approximately the same extent, at the doses of 0.0142 and 0.142 mg/kg p.o. of the compound (Fig. 2, left panel). A marginal effect was seen at 0.00142 and none at all at 1.42 mg/kg p.o., indicating a biphasic dose-response curve *in vivo*. In a parallel experiment, a similar and significant effect of CGP 3466 (free base) administered at 0.1 mg/kg s.c. according to the same treatment schedule was observed. Although the comparative compound, (–)-deprenyl, seemed to have a similar effect, it did not reach statistical significance (Fig. 2, right panel). Because nigral TH+ cells are post-mitotic and therefore do not divide, CGP 3466B was not tested in the absence of MPTP.

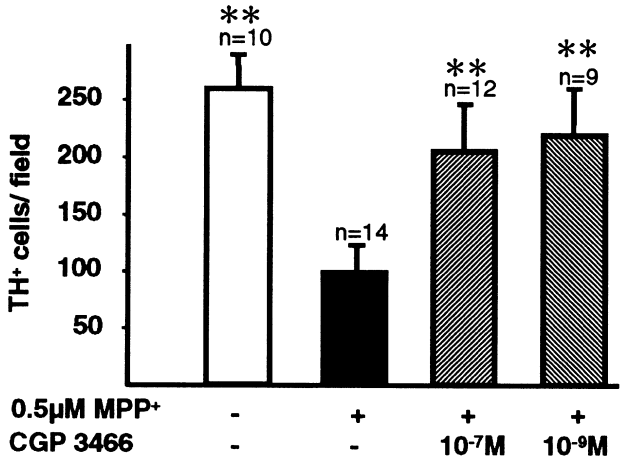


Fig. 1 Rescue by CGP 3466 of rat embryonic mesencephalic dopaminergic cells from death induced by MPP^+. Data are means ± SD of tyrosine hydroxylase-positive cells per counting field. $^{**}P < 0.01$ vs. MPP^+.

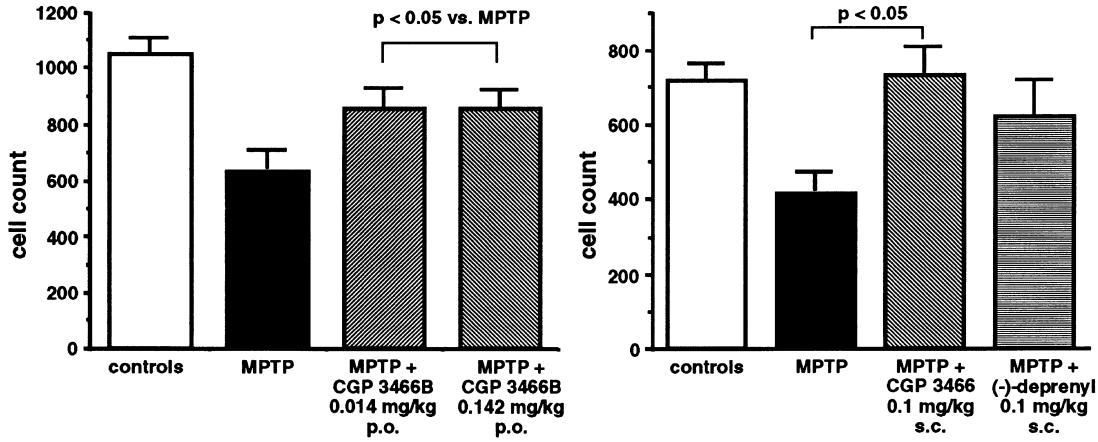


Fig. 2 Effects of CGP 3466 on nigrostriatal dopaminergic cells in MPTP-lesioned mice.

EFFECTS OF CGP 3466 ON MOTOR NEURONS *IN VIVO*

Loss of motor neurons is the underlying pathology of some neurodegenerative diseases such as Amyotrophic Lateral Sclerosis. In very young rats the axotomy of the facial nerve leads to apoptotic degeneration of the corresponding nerve bodies in the facial nucleus due to lack of trophic support [32,33]. This model has been used to investigate the effects of CGP 3466 on motor neurons histologically. In the progressive motor neuronopathy mouse model [34–37] the mice develop ALS-like symptoms during development and die very early. Life-span was assessed as a marker for the effect of the test compounds.

Facial motor neuron axotomy

Rat pups were axotomized by transection of the right facial nerve, immediately distal to its exit from the stylomastoid foramen, at postnatal day 14 (P14) and treated once daily with CGP 3466A (oxalate salt) either s.c. or p.o. for 21 days. Animals were sacrificed on the last day of treatment. Motor neuron somata containing definite nucleoli in Nissl stained sections were counted in the facial nucleus.

CGP 3466A increased motor neuron survival significantly at and above 0.0004 mg/kg s.c. or 0.004 mg/kg p.o., nearly doubling it at 0.04 mg/kg with both routes of administration. In comparison, (–)-deprenyl administered p.o. twice daily significantly increased motor neuron survival only at doses of and above 0.1 mg/kg. (–)-Deprenyl did not reach the maximal effect of CGP 3466A even at a dose of 10 mg/kg (Fig. 3).

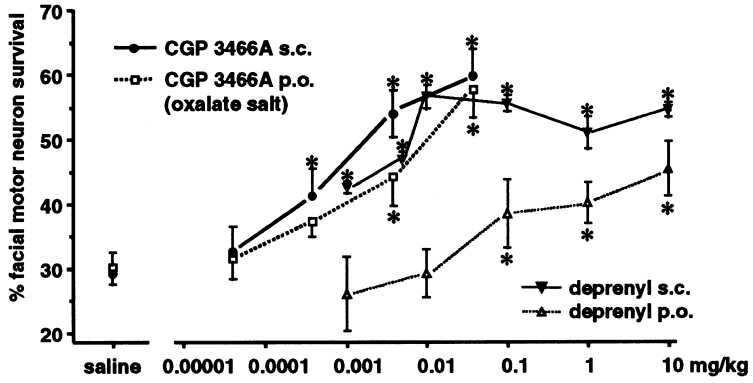


Fig. 3 Increased survival of facial motor neurons in rats axotomized at P14 and treated s.c. or p.o. with CGP 3466A (oxalate salt) once daily or with (-)-deprenyl s.c. or p.o. twice daily. Error bars are standard errors of the mean (SEM $n = 7$–8). Values marked with asterisks differ significantly from saline controls at $P < 0.05$. Control facial nuclei contained 1010 ± 143 (mean ± SD) neurons in the s.c. experiment and 1019 ± 184 neurons in the p.o. experiment.

Progressive motor neuronopathy mice

The pathological changes seen in the motor neurons of mice with progressive motor neuronopathy (pmn) closely resemble those of human motor neuron disease. Pmn mice show a severe loss of myelinated motor fibres and a significant loss of facial motor neuron cell bodies. The mice develop weakness of the hindlimbs at 14–16 days of age and die during the 6th week of life. Implanted polymer-encapsulated CNTF producing cells have been reported to rescue motor neurons and increase the life-span of the animals by 40% [36]. These results were the basis for the initiation of clinical trials in amyotrophic lateral sclerosis (ALS) patients with this preparation.

The possible beneficial effects of CGP 3466B (maleate salt) in pmn mice were compared with those of CNTF. CGP 3466B was administered to pmn mice p.o. every second day at 3.9 or 39 μg/kg, beginning at the occurrence of the first symptoms (14–16 days of age) and maintained for life. Evaluation of the survival data showed that the compound prolonged life to a similar extent as polymer-encapsulated CNTF producing cells at both doses (Fig. 4). Mean survival of untreated pmn control mice was 42 ± 1 days.

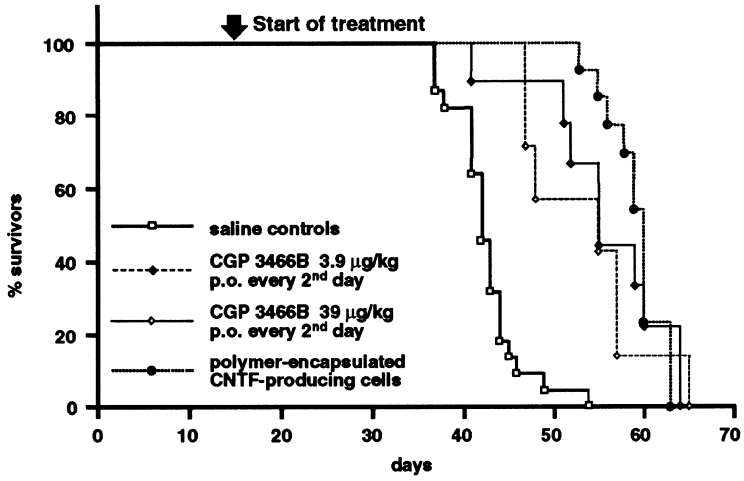


Fig. 4 Increased survival of pmn mice by CGP 3466B. Treatment was started when the first symptoms were noted (days 14–16, indicated by bold arrow). Saline controls: $n = 25$. Significance by Tukey–Kramers test: 3.9 μg/kg CGP 3466B p.o.: $P < 0.05$ vs. vehicle controls, $n = 7$, 39 μg/kg CGP 3466B p.o.: $P < 0.01$ vs. vehicle controls, $n = 9$ implanted polymer-encapsulated CNTF producing cells: $P < 0.001$ vs. untreated controls, $n = 13$.

Mice treated with 3.9 μg/kg lived for 54 ± 2 days, those treated with 39 μg/kg for 56 ± 2 days, and those with implanted polymer-encapsulated CNTF producing cells for 59 ± 1 days.

TARGET IDENTIFICATION PROGRAM

A special target identification program was initiated to elucidate the molecular target of dibenzo[b,f] oxepines such as CGP 3466. The synthesis of utilized tool compounds and the results of the investigations have been reported previously [22,38]. In summary, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was identified as the putative target molecule for derivatives of CGP 3466. Specific binding of CGP 3466 to GAPDH was consistently shown by affinity binding, photoaffinity labeling and BIAcore technology®. GAPDH has been demonstrated to induce apoptosis in different neuronal systems [39–43]. It was suggested recently, that tetrameric, but not dimeric GAPDH can facilitate apoptosis. It was proposed, that the antiapoptotic action of CGP 3466 is due to the conversion of GAPDH from the tetramer to its dimeric form and stabilization thereof [44].

CONCLUSIONS

Dibenzo[b,f]oxepin derivatives were found in a screening for antiapoptotic, neurorescuing compounds. In particular, CGP 3466 (N-methyl-N-propargyl-10-aminomethyl-dibenzo[b,f]oxepin) was identified as a highly potent substance preventing neuronal cell death in different *in vitro* and *in vivo* systems.

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