Trifluoromethyl Ketone-Based Inhibitors of Apoptosis in Cerebellar Granule Neurons

Masami KAWASE,*[,a] Katsuyoshi SUNAGA,[a] Satoru TANI,[a] Masayuki NIWA,[b] and Toshihiko UEMATSU[b]

Faculty of Pharmaceutical Sciences, Josai University,[a] Sakado, Saitama 350–0295, Japan and Department of Pharmacology, Gifu University School of Medicine,[b] Gifu 500–8705, Japan.
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A variety of aromatic trifluoromethyl ketone derivatives has been studied as inhibitors of apoptosis in cerebellar granule neurons (CGNs). Among them, α-trifluoromethyl diketone (2) and benzyl trifluoromethyl ketone (11) were found to be apoptosis inhibitors which can prevent a neurodegenerative disease. Compounds 2 and 11 showed neuroprotection effect on low K^+-induced apoptosis in CGNs. Furthermore, these compounds effectively suppressed DNA fragmentation accompanied with apoptosis. The neuroprotection mode of 2 and 11 was not related to inhibition of caspase-3.

Key words trifluoromethyl ketone; apoptosis; cerebellar granule neuron; neuroprotection; DNA fragmentation

Apoptosis is a genetically programmed form of cell death which is essential for normal development and health.[1)] Neurodegenerative diseases involve apoptotic cell death.[2)] Recent studies have suggested that the compounds which inhibit the apoptosis process can prevent a neurodegenerative disease.[3,4)] Consequently, apoptosis inhibitors can be considered as potential neurorescuing agents. In addition, specific apoptosis inhibitors might be useful tools to investigate the molecular events involved in cell death.

Trifluoromethyl ketones (TFMKs) are interesting compounds in the design of enzymatic inhibitors.[5—7)] The strong electron-withdrawing effect of the fluorine atom causes TFMKs to form stable hydrates or hemiketals in aqueous solution, whose tetrahedral geometry resembles the transition state of the water addition process to the carbonyl group of a peptide substrate. Consequently, TFMKs are potent inhibitors of a variety of serine esterases, juvenile hormone esterase, mammalian carbonyl esterases, or antennal esterases in insects.[8)]

Continuing our efforts in the development of new synthetic methods[9—12)] and new biological activity[13,14)] of TFMKs, we now want to report on a neuroprotective effect of TFMKs on apoptosis induced by low extracellular K^+ in cultured cerebellar granule neurons (CGNs).

MATERIALS AND METHODS

Cell Culture Culture enriched in granule neurons were obtained from 8-d-old Wistar rats as described previously.[15)] Cells were plated in basal medium Eagle (BME) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 25 mM KCl, and 50 μg/ml gentamicin on 48-well plate or 60-mmϕ dish coated with poly-L-lysine. Cells were plated at a density of 2.5×10^5/cm^2. Cytosine-β-D-arabinofuranoside (AraC, 10 μM) was added to the culture medium 18—22 h after plating to prevent proliferation of nonneuronal cells.

Treatment of Cultures and Assessment of Neuronal Survival After 12—13 d *in vitro* in 25 mM KCl medium, the culture medium was replaced with serum-free BME medium containing 5 mM KCl and supplemented with L-glutamine, gentamicin and AraC at the concentrations indicated above (LK). The test compounds were dissolved and diluted in ethanol, and 200-fold concentrated compounds were added directly to the low K^+/serum-free medium. Control cultures were maintained in serum-free BME medium supplemented with 25 mM KCl (HK). After 20—24 h maintained in LK or HK, neuronal survival was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. The assay relies on the ability of the mitochondria of live cells to reduce MTT to a water-insoluble blue formazan product. In brief, cultures were washed twice and BME medium containing 500 μg/ml MTT was added. After 90-min incubation at 37 °C, the reaction was stopped by adding a lysing buffer [20% sodium dodecyl sulfate (SDS) in 50% aqueous *N*,*N*-dimethylformamide solution, pH 4.7]. The absorbance was measured spectrophotometrically at 570 nm after a further overnight incubation at 37 °C. The percent survival was defined as [absorbance (experimental-blank)/absorbance (HK-blank)]$\times$100, and the blank was the value taken from wells without cells. Results obtained were analyzed by a one-way analysis of variance (ANOVA).

DNA Fragmentation Analysis Total genomic DNA was extracted and the extent of DNA fragmentation was analyzed by agarose gel electrophoresis as described.[16)] After treatment with RNase A (50 μg/ml) and Proteinase K (0.1 mg/ml) at 37 °C for 30 min and 0.2% SDS at 56 °C for 60 min, soluble DNA was subjected to electrophoresis in a 1.2% agarose gel and visualized by ethidium bromide staining.

Preparation of Neutrophils Human neutrophils in blood were isolated by Dextran-Histopaque method as previously described[17)] with minor modifications.[18)] Purification of neutrophils was performed to minimize exposure of the cells to bacterial endotoxin. Purity of neutrophils was greater than 95%. Cell number was counted by a Coulter counter model ZM (Coulter, U.S.A.), and diluted in RPMI 1640 medium to the final required concentrations and kept on ice until examined.

Measurement of Caspase-3 Activity Neutrophils were harvested after being exposed to TNF-α (100 ng/ml)/cycloheximide (1 μg/ml) for 3 h, and resuspended in hypotonic lysis buffer (25 mM HEPES, pH 7.5, containing 5 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 5 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A and 10 μg/ml leupeptin). Then cells were lysed by subjecting them to four

* To whom correspondence should be addressed. e-mail: kawasema@josai.ac.jp

cycles of freezing and thawing. After centrifugation (15000×***g***, for 20 min at 4 °C) of the cell lysates, supernatant was used as caspase-3 activated fraction. The fraction was incubated with test compound at 30 °C for 10 min, then caspase-3 substrate, as described previously.[19,20] Caspase-3 activities were expressed as the amount of liberated AMC (7-amino-4-methylcoumarin) cleaved from Ac-L-aspartyl-L-glutamyl-L-valyl-L-aspartyl (DEVD)-AMC, measured by using spectrofluorometer (Fluoroskan, Dainippon Pharmaceutical Co., Ltd., Japan).

Materials Trifluoromethyl ketones (**4**, **12**) were synthesized by reactions of mandelic acid or 3-phenyllacetic acid with trifluoroacetic anhydride (TFAA).[10] Compounds (**13**, **14**) were prepared by treatment of the corresponding *N*-alkyl-*N*-methoxycarbonylphenylalanines with TFAA.[11] Compound **15** (mp 81—84 °C) was obtained in 91% yield by the catalytic reduction of *N*-(2,6-dichlorobenzoyl)-2-trifluoroacetylpyrrolidine.[9] Other compounds (**1**—**3**, **5**—**11**) are commercially available.

RESULTS AND DISCUSSION

We screened our in-house library of TFMKs, most of them previously synthesized in our laboratory.[9—12] To our knowledge, this is the first report on the screening of TFMKs for their neuroprotective activity (Table 1 and Fig. 1). Potassium (K^+) deprivation-induced apoptosis of CGNs represents one of the best *in vitro* models of neuronal apoptosis.[21,22] The cultures of CGNs are formed by a homogenous population of granule neurons which can survive up to 15 d when they are maintained in fetal bovine serum-containing BME supplemented with 25 mM K^+.[23] Apoptosis of CGNs can be induced by lowering the extracellular K^+ concentration from 25 to 5 mM, as evidenced by morphological and biochemical methods.[21,22,24] A switch to low K^+ concentration decreases viability of CGNs by >50% when measured after 24 h. Neuronal survival was determined by the MTT assay. The apoptosis after K^+ deprivation was reported previously to be blocked by treatment with actinomycin D (Act-D),[24] cycloheximide (CHX),[24] forskolin,[25] C_3-fullero-*tris*-methanodicarboxylic acid,[26] and caspase-3 inhibitors.[27]

As a result of screening to obtain possible lead structures bearing a trifluoroacetyl group, α-trifluoromethyl diketone (**2**) and trifluoromethyl ketone (**11**) were identified as the most potent inhibitor of apoptosis in CGNs. In an effort to further define this novel inhibitory activity, a series of structurally similar compounds was screened in order to determine the importance of the α-trifluoromethyl diketone group. The trifluoromethyl ketones tested were classified as follows: ketones (**1**, **11**), α-diketone (**2**), β-diketone (**3**), α-hydroxyketones (**4**, **12**), α-amido ketones (**13**—**15**), and alcohols (**10**) (Table 1). Among them, α-diketone (**2**) and $PhCH_2COCF_3$ (**11**) rescued most CGNs from death caused by low K^+ and its protection potency was similar to those of Act-D and CHX.[24]

With the COCOR series, the non-fluorinated ketones were as follows: $PhCOCOCH_3$ (**5**), PhCOCHO (**6**), $PhCOCO_2H$ (**7**), $PhCOCO_2CH_3$ (**8**), $PhCOCH_2COCH_3$ (**9**). The protection potency decreased in the order: **8**>**7**≫**5**, **6**, **9**. It is noted that non-fluorinated α-diketone (**5**) did not exhibit protection activity. These data suggest the importance of the α-trifluoromethyl diketone moiety for the apoptosis-inhibition. The EC_{50} values of **2**, **4**, **7**, **8**, and **11** were 1.20, 2.26, 2.03, 0.44, and 1.73 μM, respectively.

A electrophoresis pattern of genomic DNA extracted from LK-exposed CGNs for 24 h were drastically fragmented and effectively suppressed by treatment of compounds **2** (30 μM) and **11** (30 μM) (Fig. 2). The results suggest that these compounds suppress the apoptosis induced by lowering extracel-

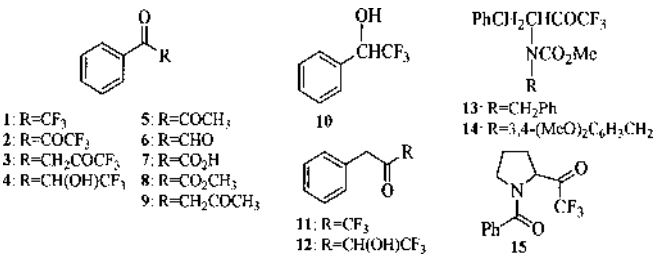

Fig. 1. Structures of TFMKs (**1**—**15**)

Table 1. Effects of Trifluoromethyl Ketones on K^+ Deprivation-Induced Apoptosis of CGNs

Compound	Viability (%) normalized to high K^+ (25 mM)					
	0	1 μM	3 μM	10 μM	30 μM	100 μM
1	53.8±4.8	56.1±4.2	55.5±5.1	55.5±4.3	56.7±4.3	58.9±4.7
2	50.0±2.1	54.1±5.5	65.7±7.5	71.4±6.2	72.0±5.5	66.0±4.0
3	43.4±2.8	42.1±10.6	48.2±6.2	51.5±4.7	52.8±11.4	38.9±7.6
4	48.9±4.1	52.8±4.8	58.7±4.3	62.6±3.8	58.4±1.9	64.1±6.5
5	51.8±3.4	52.3±4.7	51.4±4.8	53.0±5.1	49.8±5.1	50.1±5.2
6	50.8±3.9	53.4±6.4	52.7±6.0	49.6±10.0	36.9±7.1	19.6±2.0*
7	50.2±3.6	54.5±6.0	52.9±7.7	60.9±5.0	61.2±5.7	57.6±3.9
8	50.7±3.0	60.4±2.8	60.3±4.3	65.4±5.9	61.9±4.7	61.7±4.9
9	53.2±4.5	54.0±4.0	48.5±5.3	40.3±3.4	24.9±1.0*	23.0±0.8*
10	50.0±4.6	57.1±6.5	52.6±6.0	57.3±5.5	48.9±4.4	51.8±5.0
11	50.7±2.7	58.3±3.2	70.5±5.6	74.8±5.7	78.1±3.8	77.3±3.1
12	47.0±6.5	51.9±0.2	54.4±5.9	53.8±9.1	57.7±2.2	43.8±5.9
13	45.5±8.7	51.5±8.1	47.4±9.2	58.3±11.0	62.3±1.3	33.1±15.2
14	45.5±8.7	45.4±0.0	48.7±10.5	46.0±9.7	42.2±6.9	41.1±13.0
15	47.0±6.5	46.9±5.1	47.1±4.2	45.4±3.1	39.8±17.6	34.8±26.7
TPCK	43.4±2.8	45.8±2.9	45.7±4.1	9.4±22.4	3.9±17.9	9.3±37.5

* $p<0.05$ compared with the vehicle control, using ANOVA analysis.

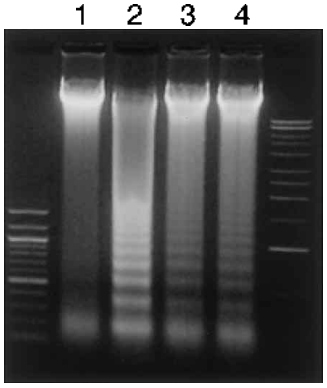

Fig. 2. Effects of Compounds **2** and **11** on Low K^+ (LK)-Induced DNA Fragmentation in Cultured Cerebellar Granule Cells

Lane 1, unexposed to LK medium; Lane 2, exposed to LK for 24 h; Lane 3 and 4, exposed to LK and treated with compounds **2** (30 μM) and **11** (30 μM), respectively.

lular K^+ concentration in CGNs.

In agreement with previous reports,[24] both Act-D (0.5 μg/ml) and CHX (5 μg/ml) rescued most CGNs from death caused by low K^+. As Act-D and CHX are thought to act at the upstream of several apoptotic cascades, they may exhibit powerful neuroprotective effects. Since **2** and **11** showed potent neuroprotection, it is interesting to know where TFMKs effect this potency.

Caspases, a family of cystein proteases, play a critical role in execution of apoptosis and are responsible for many of the biological and morphological changes associated with apoptosis.[28,29] Recent report suggested that caspase-3 activity is up-regulated and specific caspase-3 inhibitors moderately suppressed cell death during low K^+-induced apoptosis in CGNs.[27] Thus, the caspase inhibitors, carboxybenzoxy-L-aspartyl-L-glutamyl-L-valyl-L-aspart-1-ylfluoromethane (Z-DEVD-FMK, caspase-3 inhibitor) and carboxybenzoxy-L-valyl-L-alanyl-L-β-methyl-aspart-1-ylfluoromethane (Z-VAD-FMK, non-selective caspase inhibitor) used at 200 μM, diminished death by 50—60%, whereas 200 μM acetyl-Tyr-Val-Ala-Asp-aldehyde (Ac-YVAD-CHO, caspase-1 inhibitor) was not protective in our assay systems, as previously shown.[27] These inhibitors are the peptides including the amino acid sequence with an enzymatic cleavage site. Therefore, it is useful if the simple compounds (**2** and **11**) have inhibitory activity on caspases. On the other hand, the chymotrypsin inhibitor *N*-tosyl-L-phenylalanyl chloromethylketone (TPCK) had no effect on K^+-deprivation-induced apoptosis at concentrations tolerated by CGNs (Table 1).[24] Therefore, the apoptosis-inhibitory activity of TFMKs was suspected to be related to the inhibition of caspase-3. We examined the inhibitory activity of TFMKs against caspase-3 activated fraction from neutrophils.[20] However, the neuroprotective activity of TFMKs was unrelated to the caspase-3 activity.

Previous studies from our laboratories have shown that α-trifluoromethyl acyloins (**4**, **12**) can induce apoptosis of human cancer cells *in vitro*, whose induction was mediated by activation of the caspase pathway.[14] Two structurally related $PhCOCOCF_3$ (**2**) and $PhCOCH(OH)CF_3$ (**4**) differ in their apoptosis-modulating activity. These distinct inhibitory modes of action by **2** and **4** are interesting. It could be suggested the existence of several pathways and/or regulation mechanisms of apoptosis by cell type and physiological state. A correlation between the structural and biochemical differences of **2** and **4** may serve as a model system for understanding apoptosis.

In conclusion, the present study demonstrated that α-trifluoromethyl diketone (**2**) and 1,1,1-trifluoro-3-phenyl-2-propanone (**11**) are neuroprotective in an *in vitro* model of K^+ deprivation-induced apoptosis of CGNs and may be attractive lead compounds for further development as a neurorescuing agent. The extensive structure-relationship in this type of compound, including the inhibition mechanism of apoptosis, will be reported in due course.

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