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KATSUYOSHI SUNAGA, TORU TANAKA, SATORU TANI and MASAMI KAWASE

Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama 350-0295, Japan

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Trifluoromethyl Ketones Show Culture Age-dependent Inhibitory Effects on Low K^+-induced Apoptosis in Cerebellar Granule Neurons

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Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama 350-0295, Japan

Abstract. *We previously reported that two trifluoromethyl ketones, 3,3,3-trifluoro-1-phenyl-1,2-propanedione (**TF1**) and 1,1,1-trifluoro-3-phenyl-2-propanone (**TF2**), have neuroprotective effects against low K^+-induced apoptosis in cerebellar granule neurons (CGNs) exposed at 12-13 days in vitro (DIV). On the other hand, these compounds showed weak neuroprotective potency against 7 DIV CGNs. It is reported that actinomycin D (Act-D), cycloheximide (CHX), and caspase-3 inhibitors prevent the apoptosis of CGNs induced by K^+ deprivation. However, these experiments are generally performed using 7 DIV CGNs. We investigated and compared the anti-apoptotic efficacy of these drugs and newly-discovered **TF1** and **TF2** to protect DIV 7 and 12-13 CGNs from death induced by K^+ deprivation. Apoptosis of CGNs induced by K^+ withdrawal at 13 DIV was potently inhibited by Act-D and CHX similar to those at 7 DIV. Caspase-3 inhibitors moderately suppressed cell death during low K^+-induced apoptosis both exposed 7 and 13 DIV. Serine protease inhibitor N-tosyl-L-phenylalanyl chloromethylketone (TPCK) had no effect on K^+-deprivation-induced apoptosis of CGNs at both 7 and 12 DIV. This study showed that there are different pathways of apoptosis in CGNs depending on the culture age.*

Potassium (K^+) deprivation-induced apoptosis of cerebellar granule neurons (CGNs) represents one of the best *in vitro* models of neuronal apoptosis (1, 2). This culture system is also a good method for screening many compounds in order to find effective apoptosis inhibitors. The cultures of CGNs are formed by a homogenous population of granule neurons which can survive up to 15 days when they are maintained in fetal bovine serum supplemented with 25 mM K^+ (3). Apoptosis of CGNs can be induced by lowering the extracellular K^+ concentration from 25 to 5 mM, as evidenced by morphological and biochemical methods (1, 2, 4). Death of CGNs induced by switching to 5 mM K^+ can be prevented by several compounds, such as actinomycin D (Act-D) (4), cycloheximide (CHX) (4), fullaren derivatives (5), forskolin (6) and caspase-3 inhibitors (Z-DEVD and Z-VAD-FMK) (7), but not by the chymotrypsin inhibitor (TPCK), trypsin inhibitor (TLCK) and caspase-1 inhibitors (Ac-YVAD-CHO) (4). These experiments are generally performed using CGNs on days *in vitro* (DIV) 7-8. During our screening program of apoptosis inhibitors, we found that $PhCOCOCF_3$ (**TF1**) and $PhCH_2COCF_3$ (**TF2**) prevent the apoptosis of DIV 12-13 CGNs induced by K^+ deprivation, but not the apoptosis of DIV 7 CGNs (Figure 1). Compounds **TF1** and **TF2** showed similar neuroprotective potency to Act-D and CHX (8).

Therefore, we studied the effects of several drugs such as Act-D, CHX, caspase inhibitors, serine protease inhibitors and newly-discovered **TF1** and **TF2** on apoptosis of CGNs induced by K^+ deprivation at both DIV 7 and 12-13. The purpose of this study was to compare the anti-apoptotic efficacy of these drugs to protect DIV 7 and 12-13 CGNs from death induced by K^+ deprivation.

Materials and Methods

Cell culture. Cultures enriched in granule neurons were obtained from 8-day-old Wistar rats as described previously (9). The 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 coated with poly-L-lysine. The cells were plated at a density of 2.5 × 10^5/cm^2. Cytosine-ß-D-arabinofuranoside (AraC, 10 μM) was added to the culture medium 18-22 hours after plating to prevent proliferation of nonneuronal cells. Cultures generated by this method have been characterized and shown to contain >95% granule neurons.

Treatment of cultures and assessment of neuronal survival. After 7 or 12-13 days *in vitro* (DIV) 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 hours maintained in LK or HK, neuronal survival was

Correspondence to: Dr. Katsuyoshi Sunaga, Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama 350-0295, Japan. Tel: +81-492-86-2233, Fax: +81-492-71-7984, e-mail: ksunaga@josai.ac.jp

Key Words: Trifluoromethyl ketone, neuroprotection, apoptosis, cerebellar granule neuron, culture age.

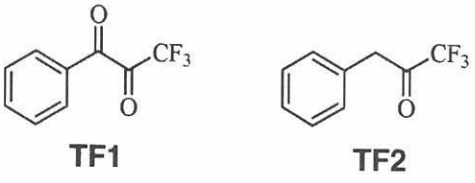

Figure 1. *Chemical structures of TF1 and TF2.*

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, the cultures were washed twice and added to BME medium containing 500 μg/mL MTT. After a 90-minute incubation at 37°C, the reaction was stopped by adding lysing buffer (20% 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 (experimetal-blank)/absorbance (control-blank)] × 100, and the blank was the value taken from wells without cells.

DNA fragmentation analysis. Total genomic DNA was extracted from cultured CGNs and the extent of DNA fragmentation was analyzed by agarose gel electrophoresis as described (10). After treatment with RNase A (50 μg/mL) and proteinase K (0.1 mg/mL) at 37°C for 30 minutes and 0.2% SDS at 56°C for 60 minutes, soluble DNA was subjected to electrophoresis in a 1.2% agarose gel and visualized by ethidium bromide staining.

Chemicals. 3,3,3-Trifluoro-1-phenyl-1,2-propanedione (**TF1**) and 1,1,1-trifluoro-3-phenyl-2-propanone (**TF2**) were obtained from Aldrich, Japan. Carboxybenzoyl-L-valyl-L-ß-methyl-aspart-1-ylfluoromethane (Z-VAD-FMK), acetyl-Try-Val-Ala-Asp-aldehyde (Ac-YVAD-CHO) and carboxybenzoyl-L-aspartyl-L-glutamyl-L-valyl-L-aspart-1-ylfluoromethane (Z-DEVD-FMK) were purchased from Calbiochem-Novabiochem (San Diego, CA, USA). Actinomycin-D, cycloheximide and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were obtained from Sigma (St. Louis, MO, USA).

Statistical analysis. The data are expressed as means ± SEM. Statistical significance was determined by using an analysis of variance (ANOVA), followed by Dunnett's or Bonferroni's post-tests, as indicated in the figure legends.

Results

CGNs were cultured on DIV 7 and 12-13 in depolarizing levels of K^+ (25 mM) in the presence of serum. We used an MTT assay to examine the lifetime in culturing of CGNs (Figure 2). CGNs augmented MTT reduction activity during culture, which culminated at 8 DIV. The cells maintained healthy aspects at least 14 DIV. Thereafter, the cells abruptly died in the subsequent 2 days. These observations were also confirmed by morphological examination. Simultaneous lowering of the K^+ concentration on DIV 7 and 12-13, to 5 mM and removal of serum (K^+/serum withdrawal) led to a loss of neuronal viability. Morphologically, both DIV 7 and 12-13 CGNs remained almost normal in appearance for several hours after K^+/serum withdrawal, after which the

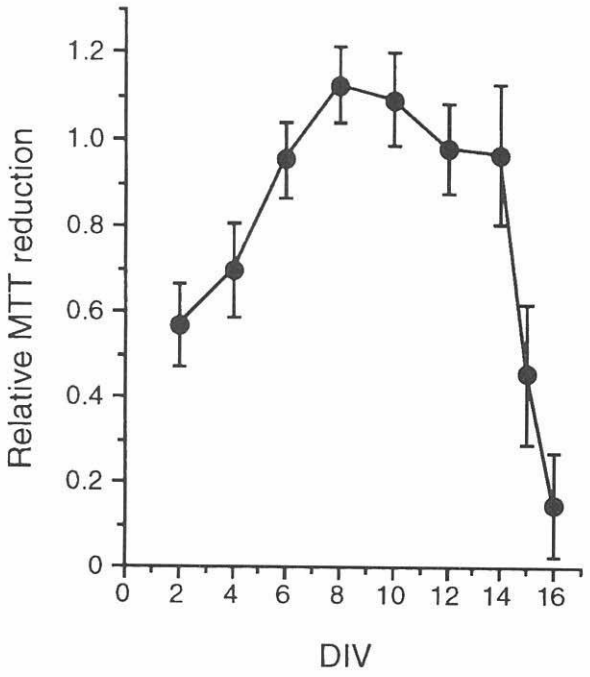

Figure 2. *Growth curve of CGN cultured under normal conditions. CGNs were assessed for mitochondrial activity by using the MTT assay at the indicated culture period as described in Materials and Methods. The data presented are means ± SEM of three independent experiments.*

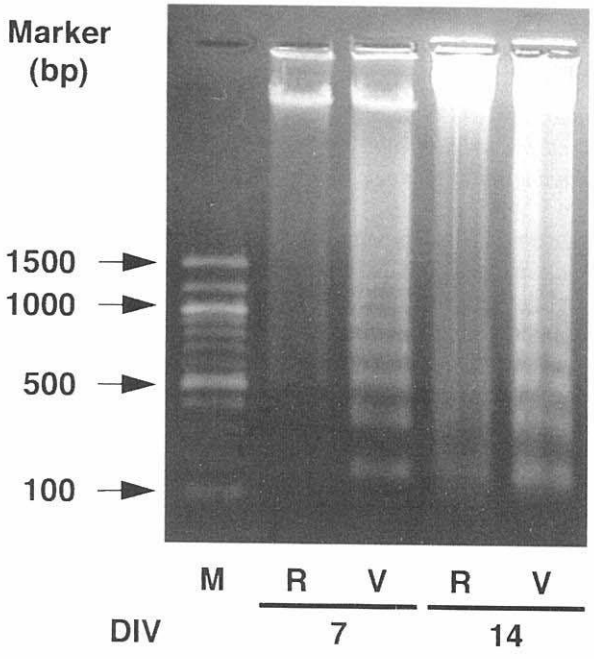

Figure 3. *Comparison of extent of genomic DNA fragmentation on apoptosis induced by low K^+ at 7 DIV and 13 DIV. CGNs received replacement to low K^+ and serum-free medium at 7 or 13 DIV. Genomic DNA was extracted and subjected to electrophoresis as described in Materials and Methods. Lane R: unexposed control, V: low K^+-exposed.*

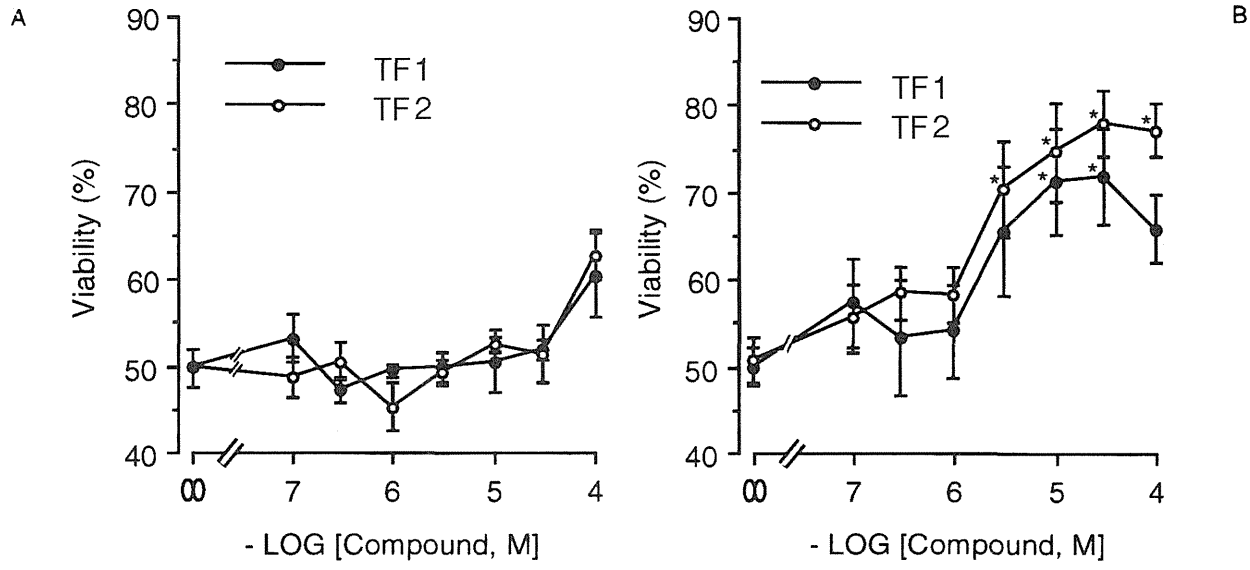

Figure 4. *Dose-response relationship for effects of **TF1** and **TF2** on neuroprotection against low K^+-induced apoptosis exposed at 7 or 13 DIV. CGNs were exposed to low K^+ at 7 DIV (A) or 13 DIV (B) and treated with the indicated concentrations of **TF1** or **TF2**. After a 24-hour exposure to low K^+, the cell viability was assessed by MTT assay as described in Materials and Methods. The data presented are means ± SEM of 4-6 independent experiments. *p<0.05 compared with vehicle control, using one-way ANOVA analysis.*

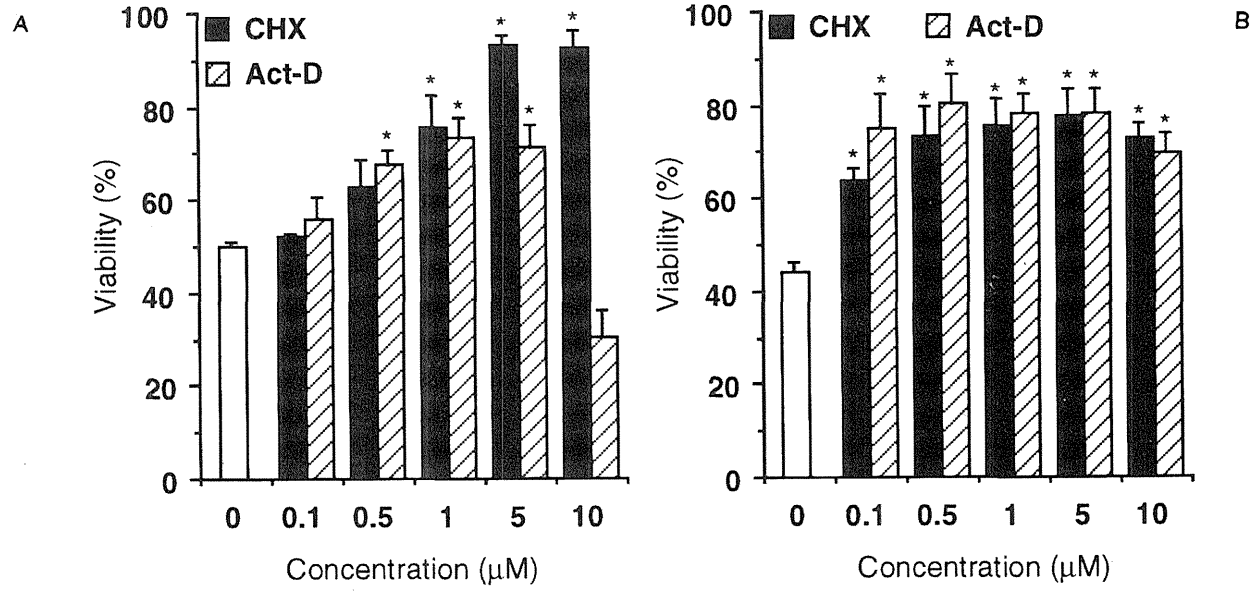

Figure 5. *Comparison of neuroprotective effects of CHX and Act-D on apoptosis induced at 7 DIV and 13 DIV. CGNs cultured 7 (A) or 13 (B) days received replacement to low K^+ and serum-free medium and were treated with the indicated concentrations of CHX or Act-D. After 24 hours, the cell viabilities were estimated by MTT assay as described in Materials and Methods. The data presented are means ± SEM of 4-6 independent experiments. *p<0.05 compared with vehicle control, using Student's t-test.*

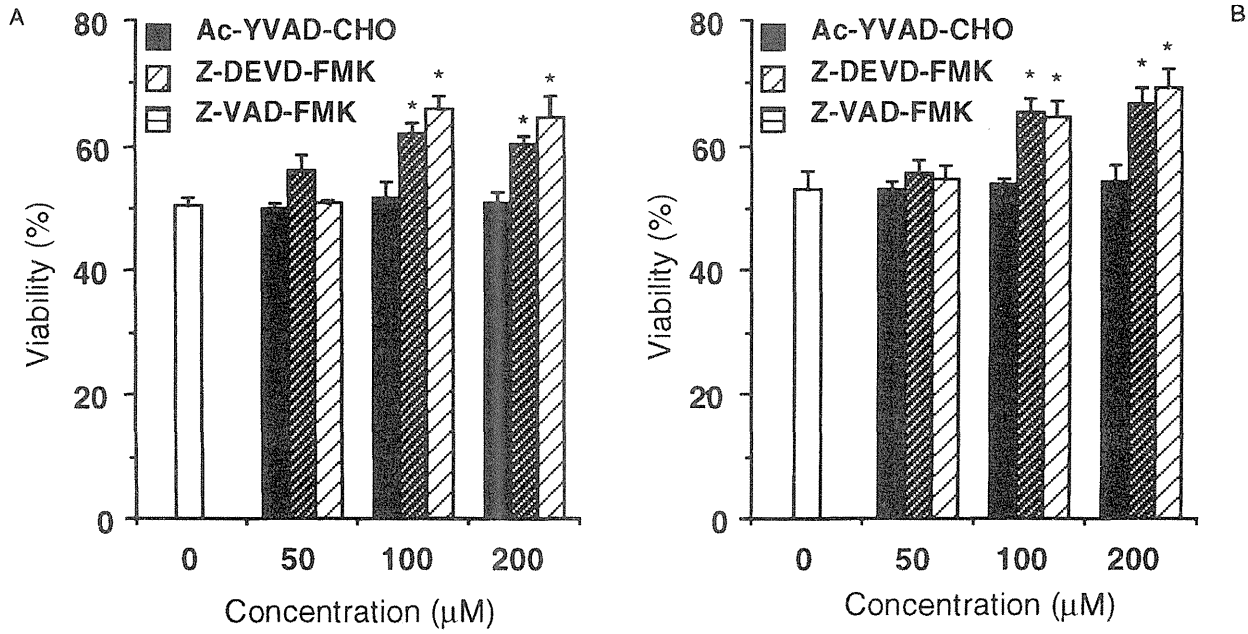

Figure 6. *Comparison of neuroprotective effects of caspase inhibitors on apoptosis induced at 7 DIV and 13 DIV. CGNs cultured 7 (A) or 13 (B) days received replacement to low K^+ and serum-free medium and were treated with the indicated concentrations of Ac-YVAD-CHO, Z-DEVD-FMK or Z-VAD-FMK. After 24 hours, the cell viabilities were estimated by MTT assay as described in Materials and Methods. The data presented are means ± SEM of 4-6 independent experiments. *$p<0.05$ compared with vehicle control, using Student's t-test.*

viability of CGNs decreased by >50% when measured after 24 hours, as similarly described by others (1, 4).

Fragmentation of genomic DNA is a hallmark of apoptosis and such fragmentation is induced in DIV 7 CGNs by switching to a low K^+ medium (1). This is also illustrated in Figure 3, showing the same DNA fragmentation in K^+-deprived DIV 12-13 CGNs.

The ability of several apoptosis inhibitors to limit CGN death induced by K^+ deprivation as a function of culture age was studied. First, newly-discovered apoptosis inhibitors **TF1** and **TF2** blocked the death of CGNs induced by K^+ withdrawal at DIV 12-13, but not at DIV 7. The dose-response relationships of neuroprotective effects by **TF1** and **TF2** are shown in Figure 4. When apoptosis was induced at 13 DIV, both **TF1** and **TF2** potently protected neuronal apoptosis in dose-dependent manner, in which maximum protection was approximately 66% at 10 μM and 56% at 100 μM, respectively. In the case of **TF1**, excess of 30 μM reduced neuroprotective effects, showing a bell-shape curve. On the other hand, when apoptosis was induced at 7 DIV, neuroprotection by **TF1** and **TF2** was very weak, approximately only 30% protection at 100 μM concentration. These protective effects of **TF** compounds were not affected by supplementation of glucose at 10 DIV, which prolonged cell survival more than a further seven days (data not shown). Second, apoptosis of CGN induced by K^+ withdrawal at both DIV 7 and 12-13 was inhibited by Act-D (a RNA transcription inhibitor) and CHX (a protein synthesis inhibitor) (Figure 5). Third, Z-VAD-FMK, a non-selective caspase inhibitor and Z-DEVD-FMK, a selective inhibitor of caspase-3 (CPP32/Apopain) moderately suppressed cell death from low K^+-induced apoptosis in DIV 7 and 12 CGNs (Figure 6). In contrast, Ac-YVAD-CHO, a selective inhibitor of caspase-1, did not block apoptosis induced by low K^+ in DIV 7 and 12-13 CGNs. Fourth, the chymotrypsin inhibitor, *N*-tosyl-L-phenylalanyl chloromethylketone (TPCK), had no effect on K^+ deprivation-induced apoptosis of DIV 7 (8) and 12-13 CGNs at concentrations tolerated by the CGNs (data not shown).

Discussion

Apoptosis is prominent in normal developing CNS, in certain genetic diseases and after some forms of CNS injury (11, 12). Neurodegenerative diseases also involve apoptotic cell death (13). Recent studies have suggested that apoptosis inhibitors can prevent a neurodegenerative disease (14, 15). Consequently, apoptosis inhibitors can be considered as potential neurorescuing agents. As the cultures of CGNs are formed by a homogeneous population of granule neurons and large amounts of CGNs are easily obtained, this culture system can be suitable for screening effective apoptosis inhibitors.

During our program for screening apoptosis inhibitors, we found that some **TF** compounds blocked the apoptosis of

DIV 12-13 CGNs, but not the apoptosis of DIV 7 CGNs. This indicated that K^+-deprived CGNs showed increasing sensitivity to **TF** compounds between DIV 7 and 12-13.

We undertook some experiments to elucidate the molecular mechanisms underlying the differences between apoptosis induced at 7 and 12-13 DIV. We showed that Act-D, CHX and caspase-3 inhibitors rescued both 7 and 12-13 DIV from death by K^+ deprivation; however, caspase-1 inhibitor and serine protease inhibitors did not prevent apoptosis of either 7 or 12-13 DIV. These results indicated that apoptosis induced at both 7 and 12-13 DIV similarly require new protein synthesis and activation of caspase-3. However, the apoptosis-inhibitory activity of **TF1** and **TF2** was unrelated to the inhibition of caspase-3 (8).

It is speculated that some pathways to apoptosis of matured CGNs (7 DIV) would disappear and/or a new pathway appears between 7 and 12-13 DIV. In this regard, considerable results have been reported that responsiveness of CGNs to neurotrophins change (16-18) and $p75^{NTR}$ falls drastically during cerebellar development (16). Furthermore, it is reported that up-regulation of cyclin D1 mRNA is accompanied with apoptosis of immature but not mature CGNs (19). These results suggest that there are different pathways of neuronal apoptosis depending on differentiation or aging state of the cultured neurons.

However, the possibility of changes in the permeability of the cell membrane for **TF** compounds by the culture ages (7 and 12-13 DIV) can not be ruled out. In this regard, a high dose (100 μM) of **TF** compounds slightly rescued CGNs from low K^+-induced apoptosis on 7 DIV culture (Figure 2). Moreover, the neuroprotective property of other neuroprotective agents, such as Act-D, CHX, Z-DEVD-FMK and Z-VAD-FMK, which are thought to act at intracellular compartment, remained unchanged during aging. It is reported that TPCK blocked caspase-3, –8 and –9 activation and apoptosis during hypoxia-reoxygenation (20); however in our experiments, TPCK had no protective effect on low K^+-induced apoptosis at both 7 and 12-13 DIV. These results suggested that membrane permeability for various agents could be related to expression of these biochemical effects. Although various factors in low K^+-induced apoptosis of CGNs are considered, this culture system would be useful for research of various mechanisms of apoptosis and screening of new apoptosis-preventing agents by evaluation at some developmental stage (7 and 12-13 DIV) of the culture.

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