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Serine Protease Inhibitors *N*-α-tosyl-L-Lysinyl-Chloromethylketone (TLCK) and *N*-Tosyl-L-Phenylalaninyl-Chloromethylketone (TPCK) Do Not Inhibit Caspase-3 and Caspase-7 Processing in Cells Exposed to Pro-Apoptotic Inducing Stimuli

Ivo Frydrych and Petr Mlejnek*

Faculty of Medicine and Dentistry, Department of Biology, Palacky University Olomouc, Hnevotinska 3, Olomouc 77515, Czech Republic

ABSTRACT

We recently demonstrated that TLCK and TPCK could act as potent but nonspecific inhibitors of mature caspases [Frydrych and Mlejnek [2008] J Cell Biochem 103:1646–1656]. The question whether TLCK and TPCK inhibit simultaneously caspase activation and/or processing remained, however, open. In this article, we demonstrated that TPCK even enhanced caspase-3 and caspase-7 processing although it substantially inhibited caspase-3 and caspase-7 enzymatic (DEVDase) activity in HL-60 cells exposed to various cell death inducing stimuli. Under the same conditions, TLCK had no effect or affected caspase-3 and caspase-7 processing marginally depending on cell treatment used. Importantly, TLCK substantially inhibited caspase-3 and caspase-7 enzymatic (DEVDase) activity irrespectively to the treatment used. Interestingly, treatment of cells with toxic concentrations of TPCK alone was accompanied by full caspase-3 and -7 processing even if it induced necrosis. In contrast, treatment of cells with concentrations of TLCK that caused necrosis was accompanied by only partial caspase-3 and caspase-7 processing. Our results clearly indicated that TPCK and TLCK did not inhibit caspase-3 and -7 enzymatic activity by prevention of their activation and/or processing. J. Cell. Biochem. 105: 1501–1506, 2008.

KEY WORDS: SERINE PROTEASE INHIBITORS; MATURE CASPASES; CASPASE-3 PROCESSING; CASPASE-7 PROCESSING; APOPTOSIS; NECROSIS

A crucial biochemical feature of apoptosis is proteolytic cleavage of a limited number of cellular proteins by caspases [Cohen, 1997]. However, the involvement of additional proteases, such as serine proteases, was also described in different experimental models of apoptotic cell death. Evidence that serine proteases participate in apoptotic process were based on the observation that specific inhibitors of serine proteases could affect apoptotic process [Johnson, 2000]. Only recently, it was demonstrated that serine proteases are activated during apoptosis [Grabarek et al., 2002a,b]. It seems that activation of caspases is an upstream event required for activation of serine proteases [Grabarek et al., 2002a,b].

However, the data that apply to the effects of serine protease inhibitors, TLCK, and TPCK, on cell death induction are still controversial. Thus, TLCK [Murn et al., 2004; Mlejnek, 2005] and in particular TPCK were reported to induce cell death themselves [Wu et al., 1996; Drexler, 1997; Murn et al., 2004; Mlejnek, 2005] or at least enhanced apoptosis induced by other cytotoxic agents [King et al., 2004; Okada et al., 2004]. On the other hand, it was reported that TLCK and TPCK could either abrogate some apoptotic hallmarks such as cell shrinkage, nucleosomal DNA fragmentation [Bruno et al., 1992; Weaver et al., 1993; Weis et al., 1995; Shimizu and Pommier, 1997], or even prevent cells from death in some experimental models [Stefanis et al., 1997; Jones et al., 1998; Dong et al., 2000]. It was suggested that TLCK and TPCK blocked apoptosis by inhibiting caspase processing [Stefanis et al., 1997; Jones et al., 1998; Dong et al., 2000]. Interestingly, antagonistic effects of TLCK and TPCK on apoptotic pathways were observed across the same concentration range [Wu et al., 1996; Drexler, 1997; Stefanis et al., 1997; Jones et al., 1998; Dong et al., 2000; Murn et al., 2004; King et al., 2004; Okada et al., 2004; Mlejnek, 2005].

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*Correspondence to: Dr. Petr Mlejnek, Faculty of Medicine, Department of Biology, Palacky University, Hnevotinska 3, Olomouc 77515, Czech Republic. E-mail: mlejnek_petr @volny.cz
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In the previous article we documented that TLCK and TPCK could act as potent direct but nonspecific inhibitors of mature caspase-3, caspase-6, and caspase-7 [Frydrych and Mlejnek, 2008]. However, in that article we did not exclude the possibility that diminished DEVDase and VEIDase activity was at least partly on account of inhibition of caspase processing and/or activation [Frydrych and Mlejnek, 2008]. In this article we demonstrate that serine protease inhibitors TLCK and TPCK exhibited no or only marginal effect on caspase-3 and -7 processing in cells exposed to cell death-inducing stimuli. Therefore, the observed decrease in DEVDase activity cannot be explained by inhibitory effects of TLCK and TPCK on caspase -3 and -7 processing.

MATERIALS AND METHODS

CELL CULTURE

HL-60 cells were cultured in RPMI-1640, as described previously [Frydrych and Mlejnek, 2008]. Cells were maintained at density ranging from 8×10^4 to 5×10^5 cells/ml. The cell density was determined using hemocytometer. Cells were obtained from ECACC.

CHEMICALS AND CELL TREATMENT

TLCK and TPCK (Sigma, St. Louis, MO) were dissolved in DMSO. Caspase-3 substrate Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) and Caspase-3 inhibitor Ac-Asp-Glu-Val-Asp-CHO (Ac-DEVD-CHO) were obtained from Bachem (Bubendorf, Switzerland). To achieve maximum inhibitory effect, all protease inhibitors were applied at least 60 min prior to the cell treatment. Cell death inducing agents staurosporine (STS), etoposide (ETP), and N6-(2-isopentenyl)adenosine (IPA) all purchased from Sigma, were dissolved in DMSO. The final concentration of DMSO in culture medium was approximately 0.1%.

MEASUREMENT OF CASPASE-3/7 ENZYMATIC ACTIVITY IN CELL EXTRACTS

Caspase-3/7 enzymatic activity (DEVDase activity), was determined using fluorescent substrate, as described previously [Frydrych and Mlejnek, 2008].

IMMUNOBLOT ANALYSIS (WESTERN BLOT ANALYSIS)

Pelleted cells were extracted by lysis buffer (50 mM Tris/HCl buffer pH 8.1 containing 1% NP-40, 150 mM NaCl, 50 mM NaF, 5 mM EDTA, and 5 mM sodium pyrophosphate, supplemented with protease (Roche, Mannheim, Germany) and phosphatase (Sigma) inhibitor cocktails). Cell extracts were mixed with 4× Laemmli buffer, and the samples were heated to 96°C for 5 min. Samples equivalent to 30 μg protein were analyzed by Western blot using a polyclonal anti-caspase-3 antibody (1:1,000) and polyclonal anti-caspase-7 (1:1,000) antibody, both recognizing pro- and active forms of protein (Cell Signaling Technology, Denvers, MA), monoclonal anti-β-actin (1:2,000; Sigma). The signal was detected using a horseradish peroxidase-conjugated secondary antibody (1:1,5000; Dako, Glostrup, Denmark). Products were visualized using an enhanced chemiluminesence (ECL; Amersham, Little Chalfont, UK).

RESULTS

We reported recently that serine protease inhibitors TLCK and TPCK suppressed DEVDase (caspase-3 and caspase-7) and VEIDase (caspase-6) activity in cells exposed to cell death inducing stimuli such as STS, ETP, or IPA. Our results suggested that TLCK and particularly TPCK could act as potent inhibitors of mature caspases, specifically, caspase-3, caspase-6, and caspase-7 [Frydrych and Mlejnek, 2008].

To extend our previously published data, we studied the process of activation and/or processing of main executioner caspases, namely caspase-3 and caspase-7, in HL-60 cells exposed to either STS, ETP, or IPA in detail. We used immunoblotting technique, which provides information about the presence of both precursor and active form of caspase-3 and caspase-7, in combination with biochemical assay for DEVDase enzymatic activity to estimate possible contribution of impaired caspase activation and/or processing to the observed decreased caspase enzymatic activity. The cells were analyzed at 6 and 18 h after the treatment since the results obtained from the longer period might be altered by caspase leakage due to damage of plasma membrane. Immunohistochemical analysis of precursor and active forms of caspase-3 and caspase-7 clearly indicated that TPCK even enhanced caspase-3 and caspase-7 processing regardless of cell treatment used (Figs. 1–3). However, TPCK inhibited substantially the DEVDase activity (Figs. 1–3). Similarly, TLCK exhibited only a marginal effect on caspase-3 and caspase-7 processing. Thus, TLCK did not inhibit caspase-3 and caspase-7 processing in cells treated with STS (Fig. 3). Processing of caspase-3 and caspase-7 was affected only negligibly in cells treated with ETP or IPA (Figs. 1 and 2). Importantly, TLCK substantially inhibited DEVDase activity regardless of cell treatment used (Figs. 1–3).

It is necessary to note that 50 μM TPCK, that itself caused almost 100% necrosis (not shown), induced significant caspase-3 and caspase-7 processing (Fig. 4). In contrast, 200 μM TLCK, that itself also caused almost 100% necrosis (not shown), induced only partial caspase-3 and caspase-7 processing (Fig. 4).

In conclusion, our results clearly indicated that diminished DVDase activity (i.e., caspase-3 and/or caspase-7 enzymatic activity) by TPCK and TLCK could hardly be explained by inhibitory effects of these serine protease inhibitors on caspase-3 and caspase-7 processing in HL-60 cells exposed to various cell death inducing stimuli.

DISCUSSION

In our recently published article we demonstrated that TLCK and TPCK could compromise some morphological and biochemical apoptotic hallmarks in cells exposed to various cell death inducing stimuli such as STS, ETP, or IPA. Still, neither TLCK nor TPCK actually prevented cells from death [Frydrych and Mlejnek, 2008]. Among the observed effects, inhibition of executioner caspase enzymatic activity by TLCK and TPCK in dying HL-60 cells was probably the most important one. In general, the decrease in caspase enzymatic activity could be either due to inhibition of their

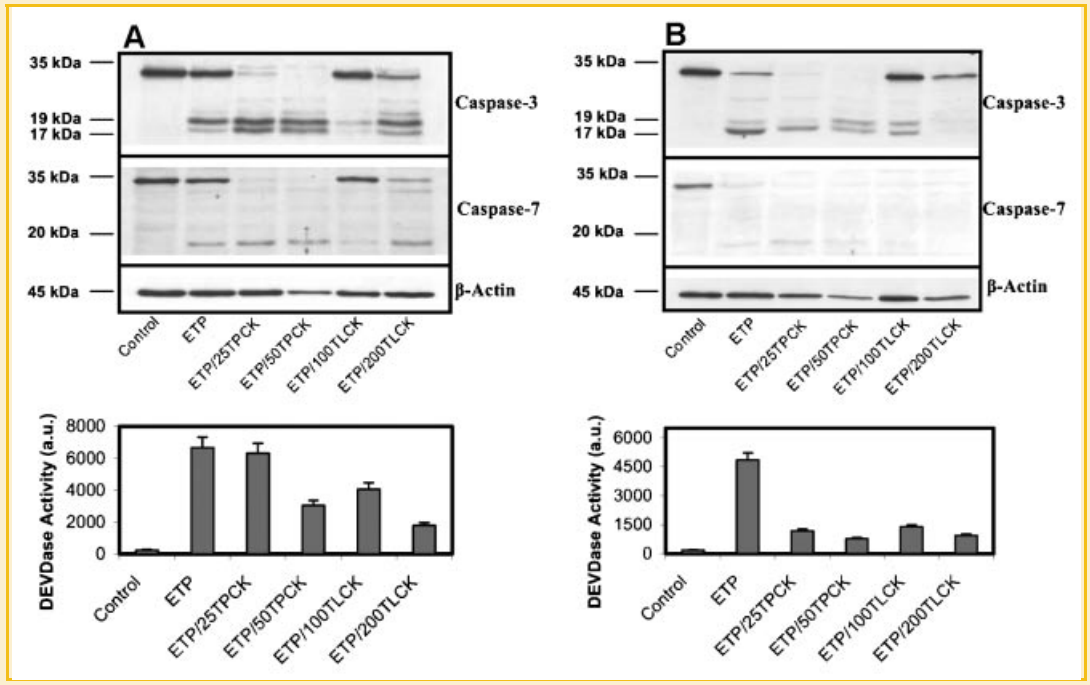

Fig. 1. Effect of TLCK and TPCK on caspase-3 and caspase-7 activation, and on DEVDase activity in HL-60 cells exposed to ETP. Cells were treated with 5 μM ETP in combination with TPCK (25 and 50 μM) or TLCK (100 and 200 μM), as indicated. Caspase-3 and caspase-7 processing was studied at 6 h (panel A) and 18 h (panel B) after the treatment. Untreated cells were taken as a control. Anti-β-actin antibody was used to normalize protein loading among samples. Pictures represent typical results. DEVDase (caspase-3/-7) activity was determined in cell lysates 6 h (panel A) and 18 h (panel B) after the treatment. Untreated cells were taken as a control. The experimental points represent mean values from three replicate experiments with standard deviations.

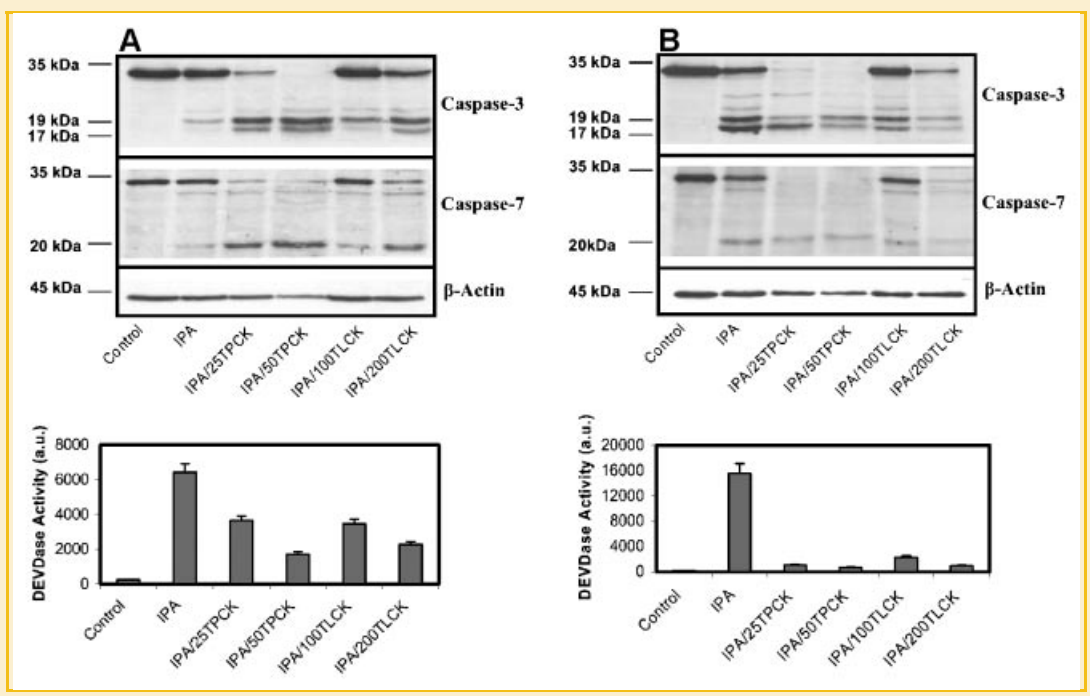

Fig. 2. Effect of TLCK and TPCK on caspase-3 and caspase-7 activation, and on DEVDase activity in HL-60 cells exposed to IPA. Cells were treated with 10 μM IPA in combination with TPCK (25 and 50 μM) or TLCK (100 and 200 μM), as indicated. Caspase-3 and caspase-7 processing was studied at 6 h (panel A) and 18 h (panel B) after the treatment. Untreated cells were taken as a control. Anti-β-actin antibody was used to normalize protein loading among samples. Pictures represent typical results. DEVDase (caspase-3/-7) activity was determined in cell lysates 6 h (panel A) and 18 h (panel B) after the treatment. Untreated cells were taken as a control. The experimental points represent mean values from three replicate experiments with standard deviations.

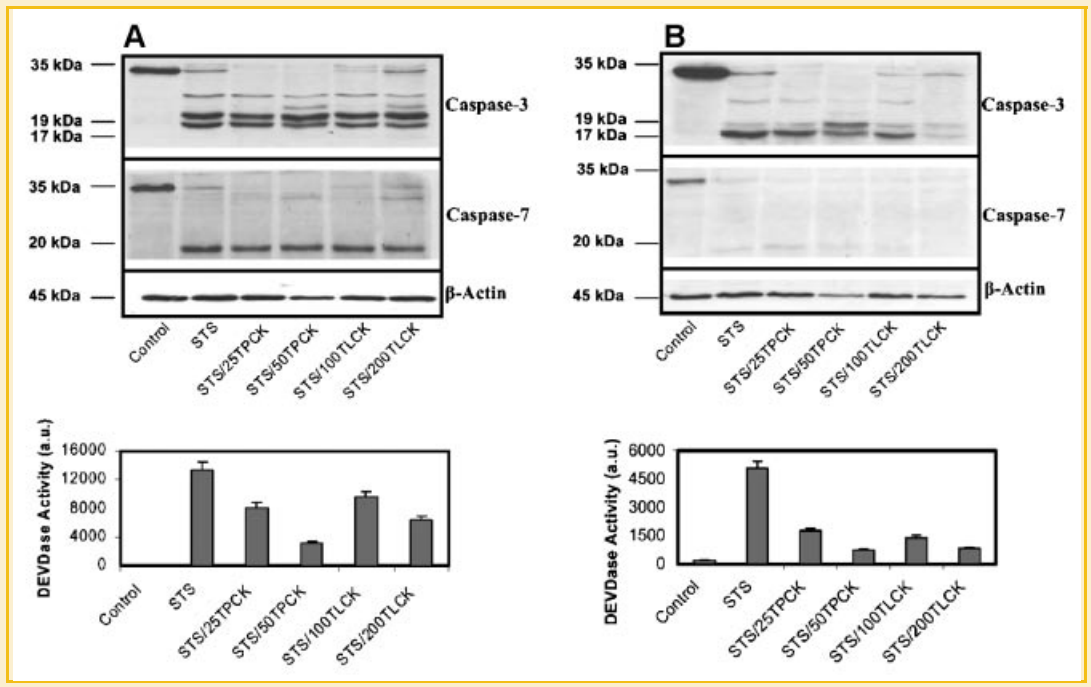

Fig. 3. Effect of TLCK and TPCK on caspase-3 and caspase-7 activation, and on DEVDase activity in HL-60 cells exposed to STS. Cells were treated with 0.5 μM STS in combination with TPCK (25 and 50 μM) or TLCK (100 and 200 μM), as indicated. Caspase-3 and caspase-7 processing was studied at 6 h (panel A) and 18 h (panel B) after the treatment. Untreated cells were taken as a control. Anti-β-actin antibody was used to normalize protein loading among samples. Pictures represent typical results. DEVDase (caspase-3/-7) activity was determined in cell lysates 6 h (panel A) and 18 h (panel B) after the treatment. Untreated cells were taken as a control. The experimental points represent mean values from three replicate experiments with standard deviations.

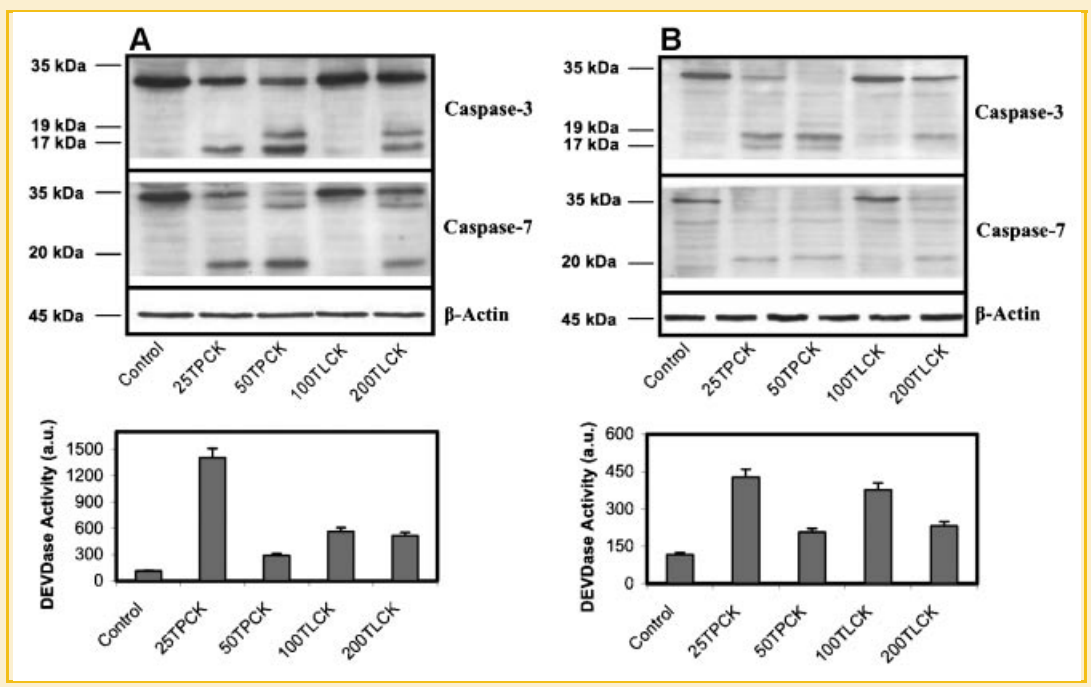

Fig. 4. Effect of TLCK and TPCK on caspase-3 and capsase-7 activation in HL-60 cells. Cells were treated with TPCK (25 and 50 μM) or TLCK (100 and 200 μM), as indicated. Caspase-3 and caspase-7 processing was studied at 6 h (panel A) and 18 h (panel B) after the treatment. Untreated cells were taken as a control. Anti-β-actin antibody was used to normalize protein loading among samples. Pictures represent typical results. DEVDase (caspase-3/-7) activity was determined in cell lysates 6 h (panel A) and 18 h (panel B) after the treatment. Untreated cells were taken as a control. The experimental points represent mean values from three replicate experiments with standard deviations.

activation and/or processing, or due to direct inhibition of already activated caspases. Eventually, it could be due to combination of both events. Our results suggested that serine protease inhibitors TLCK and particularly TPCK could act as efficient but nonspecific inhibitors of mature caspase-3, caspase-6, and capsase-7 [Frydrych and Mlejnek, 2008]. However, we did not address the question whether the observed decreased enzymatic activity of executioner caspases was also due to the inhibitory effect of TLCK and TPCK on their activation and/or processing [Frydrych and Mlejnek, 2008].

To extend our previously published data, we studied the process of activation and/or processing of main executioner caspases, namely caspase-3 and caspase-7, in HL-60 cells exposed to either STS, ETP, or IPA in detail. Our data clearly indicated that TPCK affected neither caspase-3 nor caspase-7 processing (Figs. 1–3) while it significantly inhibited DEVDase enzymatic activity (Figs. 1–3). In fact, TPCK even enhanced caspase-3 and caspase-7 activation. This was particularly distinct at 6 h after the drug addition (Figs. 1–3), when the results were not affected by possible protein leakage from dead cells (not shown). Similarly, TLCK did not prevent caspase-3 or caspase-7 processing even if 100 μM but not 200 μM TLCK marginally inhibited caspase-3 and caspase-7 processing in cells exposed to ETP or IPA (Figs. 1 and 2). However, TLCK significantly inhibited DEVDase activity proportionally to the concentration applied regardless of the treatment used (Figs. 1–3). Our results clearly indicated that TLCK and particularly TPCK did not interfere with processing of executioner caspases, namely caspase-3 and caspase-7 (Figs. 1–3). Therefore, we concluded that the observed diminished DEVDase activity was due to the direct inhibitory effect of TLCK and TPCK on mature caspases at least in our experimental system. This conclusion is in a good agreement with our recently published data [Frydrych and Mlejnek, 2008]. Our results are also consistent with earlier observations proving that serine protease inhibitors TLCK and TPCK could irreversibly alkylate cysteine residues of the active site of cysteine proteases such as bromelain, clostripain, and papain [Drenth et al., 1976; Jia et al., 1995]. It is necessary to note that we currently do not know which protease is responsible for caspase-3 and caspase-7 processing upon treatment with lethal and supralethal concentrations of TLCK or TPCK.

TPCK and TLCK are both toxic compounds that induce cell death themselves at high concentrations [Wu et al., 1996; Drexler, 1997; Murn et al., 2004; Mlejnek, 2005]. Therefore, we tested how these compounds could contribute to caspase-3 and caspase-7 processing per se. Our results indicated that TPCK induced extensive caspase-3 and caspase-7 processing (Fig. 4) even at concentrations, which induced necrotic cell death (not shown). In contrast, concentrations of TLCK that caused necrotic cell death induced only partial caspase-3 and caspase-7 processing (Fig. 4). In accordance with previously obtained results [Frydrych and Mlejnek, 2008], DEVDase enzymatic activity increased extensively only with lower concentrations of TPCK and TLCK respectively (Fig. 4). Results obtained with TPCK correspond to those we recently obtained with nonspecific caspase inhibitor Boc-Asp-CMK [Frydrych et al., 2008]. Thus, Boc-Asp-CMK is a cytotoxic compound that induces cell death accompanied by full caspase-3 processing. Interestingly, concentrations up to 10 μM induced typical apoptosis with distinct caspase-3 activity; higher concentrations that significantly inhibited caspase-3 activity led to gradual increase in necrotic cell death, albeit the caspase-3 processing remained unaffected [Frydrych et al., 2008].

Our results are in agreement with other authors who demonstrated that TPCK and TLCK are cytotoxic themselves or at least potentiate cytotoxic effects of other drugs [Wu et al., 1996; Drexler, 1997; Murn et al., 2004; King et al., 2004; Okada et al., 2004; Mlejnek, 2005]. It is necessary to note that the anti-proliferative and anti-tumorigenic effects of TPCK and TLCK have been known for more than three decades [Troll et al., 1970; Hirschhorn et al., 1971; Chou et al., 1974; Su et al., 1991; Heussler et al., 1999]. However, mechanisms how these serine protease inhibitors acted have been unknown until the early nineties when several cellular targets were identified [Wu et al., 1996]. Inhibition of Akt signaling pathway is a mechanism that explains both antiproliferative and pro-apoptotic effect of TPCK [Ballif et al., 2001]. This later explanation might be of general validity and it is compatible with our results (not shown).

On the other hand, our results are markedly different from those published by other authors who argue that serine protease inhibitors TLCK and/or TPCK prevent cells from death by inhibiting caspase processing [Stefanis et al., 1997; Jones et al., 1998; Dong et al., 2000; Rokhlin et al., 2004]. However, closer analysis of these results does not always provide a consistent picture about how these inhibitors act in fact. Thus, Dong et al., 2000 suggested that TPCK prevents apoptosis by inhibition of caspase-9 processing and by suppression of downstream caspase activation. However, their findings are in a sharp contrast with observation of other authors who demonstrated that TPCK potentiates mitochondria mediated apoptosis [Rokhlin et al., 2004]. Their results also contradict observation of Ballif et al. [2001]. Work published by Jones et al. [1998] suggested that TPCK but not TLCK inhibits Fas induced apoptosis by inhibiting caspase-3 and caspase-7 processing. These results are in a sharp contrast to that described by Rokhlin et al. [2004] who described antagonistic effects of TPCK on apoptotic pathways in human prostatic carcinoma cell lines. They reported that TPCK inhibits TRAIL-induced caspase activation while it does not affect Fas-induced caspase activation but potentiates mitochondria mediated caspase activation [Rokhlin et al., 2004]. In addition, it is not clear whether TPCK inhibited caspase-8 activation or caspase-8 enzymatic activity [Jones et al., 1998]. Interestingly, only two articles consistently demonstrated relationship between inhibition of caspase processing and cell death prevention [Stefanis et al., 1997; Rokhlin et al., 2004]. Nevertheless, the described experimental systems did not comprise mitochondria mediated pathway directly [Stefanis et al., 1997; Rokhlin et al., 2004].

In conclusion, we have unambiguously demonstrated that TLCK and TPCK inhibited executioner caspase activity, namely caspase-3 and caspase-7, while did not affect their processing and/or activation in Hl-60 cells. These results further support our idea that serine protease inhibitors TLCK and particularly TPCK acted as efficient direct inhibitors of mature caspases, at least caspase-3 and caspase-7. Consistently with this finding TLCK and TPCK diminish some apoptotic hallmarks but they actually did not prevent cells from death similarly to other panspecific caspase inhibitors. We obtained similar results also for U937 cells (unpublished results),

indicating that the described effect is probably not restricted just to the cells used in the presented work.

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