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Serine Protease Inhibitors *N*-α-Tosyl-L-Lysinyl-Chloromethylketone (TLCK) and *N*-Tosyl-L-Phenylalaninyl-Chloromethylketone (TPCK) Are Potent Inhibitors of Activated Caspase Proteases

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Abstract Serine protease inhibitors *N*-α-tosyl-L-lysinyl-chloromethylketone (TLCK) and *N*-tosyl-L-phenylalaninyl-chloromethylketone (TPCK) exhibit multiple effects on cell death pathways in mammalian cells. Thus, they are able to induce apoptosis by itself or promote cell death induced by other cytotoxic stimuli [King et al., 2004; Murn et al., 2004]. On the other hand, TLCK and TPCK were reported to prevent apoptosis by inhibiting the processing of caspases in response to some cell death inducing stimuli [Stefanis et al., 1997; Jones et al., 1998]. We observed that the pretreatment of HL-60 cells with TLCK or TPCK diminished caspases 3 and -7 (DEVDase) and caspase-6 (VEIDase) activity in response to various cell death inducing stimuli such as staurosporine (STS), etoposide (ETP), or N6-(2-isopentenyl)adenosine. In addition, TLCK but not TPCK inhibited collapse of mitochondrial transmembrane potential $\Delta\Psi m$ (delta psi) in dying HL-60 cells. Such effects used to be considered as protective, however, the protection was only presumable since neither TLCK nor TPCK actually prevented cells from death. Our results further indicated that serine protease inhibitors TLCK and particularly TPCK acted as efficient direct inhibitors of mature caspases. Indeed, experiments with human recombinant caspases provided unequivocal evidence that TLCK and TPCK are very potent but non-specific inhibitors of activated caspases, namely caspases 3, -6, and -7. Interestingly, TPCK exhibited similar efficiency towards human recombinant caspases to that found for panspecific caspase inhibitor Boc-D-CMK. Such properties of TLCK and TPCK, previously considered as specific inhibitors of serine proteases, might offer novel consistent explanation for several protective or protective-like effects on apoptotic cells. J. Cell. Biochem. 103: 1646–1656, 2008.

Key words: serine protease inhibitors; mature caspases; cell death; apoptosis; mitochondrial transmembrane potential

N-α-tosyl-L-lysinyl-chloromethylketone (TLCK) and *N*-tosyl-L-phenylalaninyl-chloromethylketone (TPCK) are irreversible serine protease inhibitors. TLCK inactivates trypsin and trypsin-like proteases but does not affect chymotrypsin. On the contrary, TPCK inhibits chymotrypsin and chymotrypsin-like proteases and it is not active against trypsin [Kostka et al., 1964; Harper and Powers, 1985]. The mechanism of inactivation has been already reported. It was demonstrated that histidine residue in the active center of trypsine and chymotrypsin are specifically alkylated by TLCK and TPCK, respectively [Malthouse and Scott, 1983]. TLCK and TPCK are also able to inactivate cysteine proteases such as bromelain, clostripain, and papain. X-ray structural analysis of papain–TPCK adducts revealed that instead of histidine, it is the cysteine residue of the active site that is irreversibly alkylated [Drenth et al., 1976; Jia et al., 1995].

Although TPCK and TLCK are mostly used in mixtures with other protease inhibitors throughout the protein isolation to prevent protein degradation, they also exhibit wide

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range of biochemical effects on cells. Thus, it was reported that TLCK and TPCK prevented activation of pp70(s6k), which is a mitogen-regulated serine/threonine kinase involved in the G1 to S phase transition of the cell cycle, by all stimuli tested [Grammer and Blenis, 1996]. In addition, they block the lipopolysacharide or cytokine-induced activation of nuclear factor κB [Breithaupt et al., 1996; D'Acquisto et al., 1997]. Besides that, inhibitors of serine proteases including TLCK and TPCK were found to suppress certain biochemical and morphological changes associated with apoptosis including nucleosomal DNA fragmentation, cell shrinkage, and complete nuclear collapse [Bruno et al., 1992; Weaver et al., 1993; Weis et al., 1995; Shimizu and Pommier, 1997]. In addition, TLCK and TPCK were able to block apoptosis by inhibiting the processing of caspases in some experimental models [Stefanis et al., 1997; Jones et al., 1998; Dong et al., 2000]. On the other hand, 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]. Antagonistic effects of TPCK on apoptotic pathways in human prostatic carcinoma cell lines was described recently by Rokhlin et al. [2004]. They demonstrated that TPCK inhibits TRAIL-induced caspase activity but potentiates wortmannin-dependent caspase activity [Rokhlin et al., 2004].

In this article we demonstrate that serine protease inhibitors TLCK and TPCK can abrogate some apoptotic hallmarks in cells exposed to cell death inducing stimuli. However, neither TLCK nor TPCK actually prevent cells from death. Owing to the fact that TLCK and TPCK can also act as potent direct inhibitors of mature caspases we suggest novel explanation for effects of these serine protease on cell death.

MATERIALS AND METHODS

Cell Culture

HL-60 cells were cultured in RPMI-1640, as described previously [Mlejnek and Kuglik, 2000]. Cells were maintained at density ranging from 8×10^4 to 5×10^5 cells per 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. Caspases 3 and -7 substrate Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) and Caspase-6 substrate Ac-Val-Glu-Ile-Asp-AMC (Ac-VEID-AMC) were obtained from Bachem (Bubendorf, Switzerland). Protease inhibitors were dissolved in DMSO. Active human recombinant caspases-6 and -7 were purchased from Alexis (Lausen, Switzerland) and active human recombinant caspase-3 was purchased from Sigma. Broad spectrum caspase inhibitor, Boc-D-CMK (Bachem) was dissolved in DMSO. To achieve maximum inhibitory effect, all protease inhibitors were applied at least 20 min prior to the cell treatment. Cell death inducing agents STS, 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%. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Duchefa (Haarlem, Netherlands).

DNA Fragmentation Assay

DNA fragmentation to nucleosomal level was assessed using standard agarose gel electrophoresis [Mlejnek and Kuglik, 2000].

Assessment of Mitochondrial Transmembrane Potentials

Cells were incubated for 15 min in 50 nmol/L $DiOC_6(3)$ at 37°C, as described previously [Zamzami et al., 1995]. Stained cells were immediately analyzed on a FACScan cytofluorometer (Becton Dickinson, San Jose, CA) with argon laser excitation at 488 nm and emission at 525 nm. Atleast 10,000 cells in each sample were analyzed. The control experiment was performed in untreated cells and in cells treated with 50 μmol/L carbamoyl cyanide *m*-chlorophenylhydrazone (*m*ClCCP, an uncoupling agent that abolishes $\Delta\Psi m$) for 20 min at 37°C.

Determination of Cell Survival and Proliferation

The MTT assay was used for estimation of cell viability and growth as originally described by Mosmann [1983]. Cells were incubated in the presence of 0.5 mg MTT/ml (final concentration) for 1 h at 37°C, then pelleted by centrifugation and extracted by isopropanol acidified with 0.01 M HCl. Absorbance was read at 570 nm in

extracts clarified by centrifugation. The amount of formazane produced is proportional to the number of live and metabolically active cells.

ATP Determination by Bioluminometry

Approximately 5×10^5 cells were extracted with ice-cold trichloracetic acid (TCA, 4%, w/v) for 15 min at 0°C. Precipitate and debris were removed by centrifugation (18,000*g* for 10 min at 2°C). TCA extracts were neutralized with 0.1 M Tris/HCl, pH = 7.2 (extract to buffer 1:9, v/v) and then mixed with the ATP bioluminiscent assay kit (Sigma) according to manufacturer's instructions. The quantitation was based on a calibration curve obtained using externally added ATP standard solution.

Protein Quantification

Protein content in extracts was determined by the method of Bradford [1976] using bovine serum albumin as a standard.

Measurement of Caspase Enzymatic Activity in Cell Extracts

Caspase enzymatic activities were determined using fluorescent substrates, as described previously [Mlejnek, 2001] with slight modifications. Briefly, cells were washed in PBS and lysed in lysis buffer [50 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.2% Chaps, and proteinase inhibitor cocktail (Roche)] for 20 min at 4°C. Lysates were centrifuged for 15 min at 30,000*g*. The assay was carried out in 96-well plates. Aliquots of lysates (25–50 μg of total protein) were mixed with assay buffer (25 mM PIPES/KOH, 2 mM EGTA, 2 mM $MgCl_2$, and 5 mM DTT, pH 7.3—for caspases 3 and -7 (DEVDase activity), and 25 mM HEPES, 10% glycerol, 2 mM EGTA, 2 mM $MgCl_2$, 0.1% (w/v) Chaps, and 5 mM DTT, pH 7.2—for caspase-6 (VEIDase activity)) in a final volume of 200 μl. Reactions were initiated by adding substrates, Ac-DEVD-AMC (50 μM) and Ac-VEID-AMC (50 μM), for assessment of DEVDase (caspases 3 and/or -7) and VEIDase (caspase-6) activity, respectively. Samples were incubated for 30 min at 30°C before the fluorescence was determined at 360/465 nm. A non-specific substrate cleavage, which was determined as residual substrate cleavage in the presence of 1 μM Ac-DEVD-CHO (for caspases 3 and -7), or 1 μM Ac-VEID-CHO (for caspase-6), was subtracted from every measured value of activity. Caspase enzymatic activities are expressed in arbitrary units (a.u.) = as a fluorescence of released 7-amino-4-methylcoumarin that corresponds to the cleaved substrate (either Ac-DEVD-AMC or Ac-VEID-AMC) normalized to amount of proteins.

Measurement of Enzymatic Activity of Recombinant Caspases

Assays were performed in 96-well plates. Reaction mixtures consisted of 10 ng of recombinant caspase-3, 20 ng of caspase-6, or 20 ng of caspase-7 (corresponding to approximately 1.2, 0.25, or 0.5 U, respectively), appropriate assay buffer [20 mM HEPES, pH 7.4, 0.1% (w/v) Chaps, 5 mM DTT, 2 mM EDTA for caspase-3 and 20 mM PIPES, 100 mM sodium chloride, 10 mM DTT, 1 mM EDTA, 0.1% (w/v) Chaps, 10% sucrose, pH 7.2 for caspases 6 and -7] were prepared on ice. Protease inhibitors were applied to assay mixtures on ice and incubation proceeded for 15 min. Assay mixtures were transferred to a room temperature and reactions were initiated by adding of 50 μM Ac-DEVD-AMC and Ac-VEID-AMC, which served as specific substrates for caspases 3 and -7, and -6, respectively. Samples were incubated for 30 min at room temperature before the fluorescence was determined at 360/465 nm.

RESULTS

Treatment of HL-60 cells with STS, ETP, or IPA resulted in cell death that exhibited biochemical apoptotic hallmarks, including endonuclease (Fig. 1) and caspase activation (Fig. 2). Using TLCK and TPCK, we addressed the question whether these serine specific protease inhibitors could actually prevent cells from death. Pretreatment of HL-60 cells with TLCK and TPCK completely inhibited oligonucleosomal DNA fragmentation (Fig. 1) and diminished DEVDase and VEIDase activity (Fig. 2). Our results indicated that the DEVDase activity was inhibited to almost similar extent with respect to cell treatment (Fig. 2). Thus, TLCK and TPCK reduced DEVDase activity to 35–45% in cells treated with IPA or ETP, and to 40–50% in cells treated with STS (Fig. 2). VEIDase activity was inhibited almost uniformly irrespectively to cell treatment and decreased to 45–55% (Fig. 2). We further observed, that TLCK reduced collapse of the mitochondrial transmembrane potential $\Delta\Psi m$

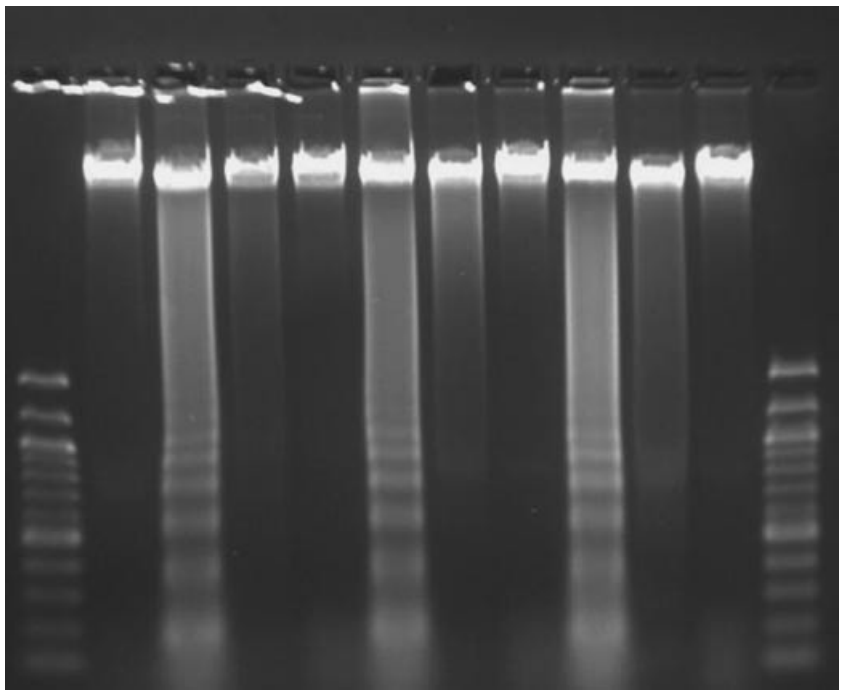

Fig. 1. Effect of TLCK and TPCK on nucleosomal DNA fragmentation in dying HL-60 cells. DNA was extracted 18 h after the cell treatment with 0.5 μM STS (**lane 3**), 0.5 μM STS + 100 μM TLCK (**lane 4**), 0.5 μM STS + 25 μM TPCK (**lane 5**), 5 μM ETP (**lane 6**), 5 μM ETP + 100 μM TLCK (**lane 7**), 5 μM ETP + 25 μM TPCK (**lane 8**); 10 μM IPA (**lane 9**), 10 μM IPA + 100 μM TLCK (**lane 10**), 10 μM IPA + 25 μM TPCK (**lane 11**). Untreated cells were taken as a control (**lane 2**), DNA molecular weight markers (**lanes 1**,**12**).

in cells treated with ETP, or IPA but not with STS (Fig. 3). On the contrary, TPCK even promoted collapse of the mitochondrial transmembrane potential ΔΨm for all cell death inducing agents used (Fig. 3). Abrogation of DNA cleavage, prevention of caspase activation, and especially inhibition of collapse of mitochondrial transmembrane potential ΔΨm in cells exposed to the cell death inducing stimuli could be considered as manifestation of protective effects, however, we found that serine protease inhibitors, TLCK and also TPCK, actually failed to prevent cells from death. Indeed, the cell viability and proliferation (Fig. 4) as well as the ATP production (Fig. 5) were not revived upon transfer of cells, that were subjected to combined treatment with cytotoxic agents plus TLCK or TPCK, to a standard growth medium.

To test whether TLCK and TPCK inhibited caspase activation or could act as direct inhibitors of activated caspases, we added these inhibitors after the treatment to the cell extracts prepared from apoptotic cells, which were treated with either STS, ETP, or IPA. We observed that TLCK and TPCK could efficiently reduce caspase activity even when added after the cell treatment (Fig. 6). The inhibitory effect of TPCK on caspase activity was almost equal, regardless to cell treatment (Fig. 6). DEVDase activity was reduced to 40–50% and VEIDase activity was reduced to 50–60% (Fig. 6). In contrast, TLCK exhibited under this experimental setting substantially higher inhibitory effect on caspases. Thus, DEVDase activity was reduced to 4–6% and VEIDase activity was reduced to 20–25% (Fig. 6). These results strongly suggested that TLCK and TPCK might exhibit direct inhibitory effect on mature caspases. Finally, we have used human recombinant caspases 3, -6, and -7 to further prove that TLCK and TPCK acted as direct inhibitors of already activated caspases. Our results unambiguously revealed that TLCK and particularly TPCK could act as potent inhibitors of mature caspases 3, -6, and -7 (Fig. 7). Interestingly, inhibitory effect of TPCK was comparable to that found in pan specific caspase inhibitor Boc-D-CMK (Fig. 7). The quantitative comparison of inhibitor efficiency of TLCK and TPCK with Boc-D-CMK on mature caspases is given in

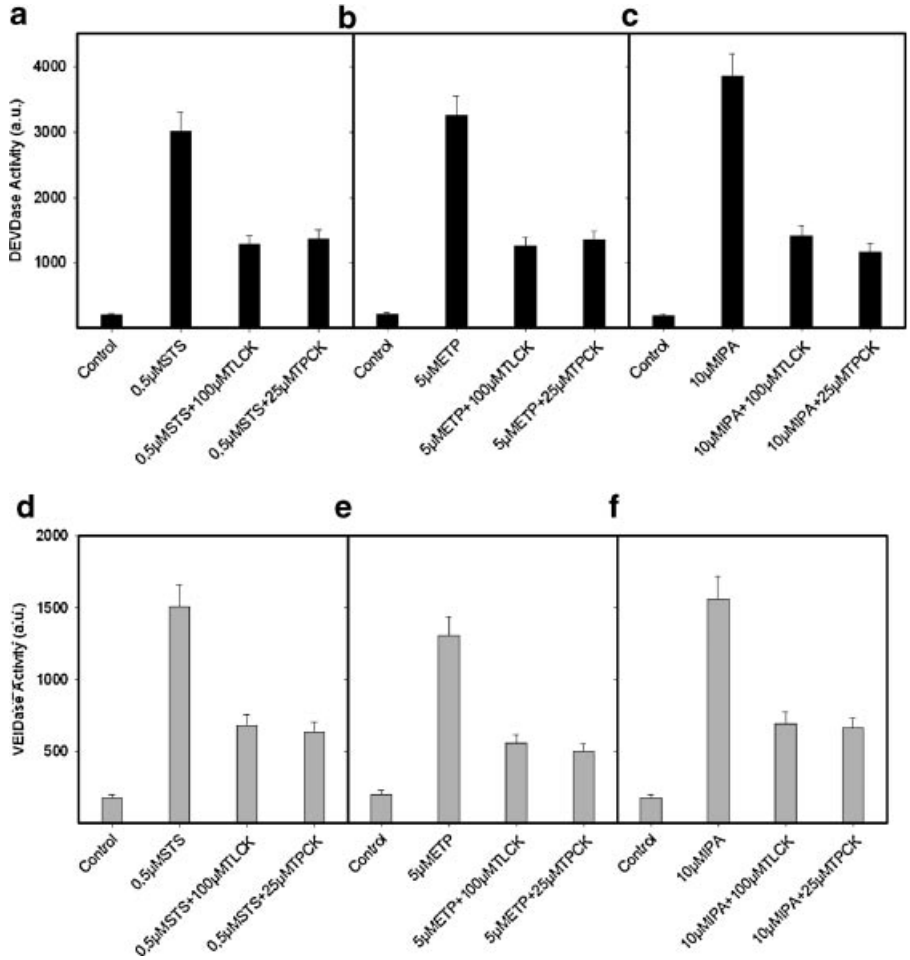

Fig. 2. Effect of TLCK and TPCK on caspase activation in dying HL-60 cells. Cells were treated with STS (**panels a**,**d**), ETP (**panels b**,**e**), and IPA (**panels c**,**f**) in the presence or absence of TLCK or TPCK, as indicated. Caspase activity was determined in cell lysates 18 h after the treatment; caspase-3 and/or caspase-7 = DEVDase activity (panels a–c), caspase-6 = VEIDase activity (panels d–f). Untreated cells were taken as a control. The experimental points represent mean values from three replicate experiments with standard deviations.

the Table I. The IC_{50} values indicated that both serine protease inhibitors, TLCK or TPCK, acted as non-selective caspase inhibitors (Table I).

DISCUSSION

Serine protease inhibitors, TLCK and TPCK were reported to interfere with many physiological processes in mammalian cells [Breithaupt et al., 1996; Grammer and Blenis, 1996; D'Acquisto et al., 1997]. Probably the most conflicting data apply to the effects of TLCK and TPCK on apoptosis induction. While some authors demonstrated that serine protease inhibitors, including TLCK and TPCK, blocked apoptosis by inhibition of caspase processing [Stefanis et al., 1997; Jones et al., 1998; Dong et al., 2000], others argued that TLCK and TPCK alone were capable to induce apoptosis [Wu et al., 1996; Drexler, 1997; Murn et al., 2004; Mlejnek, 2005] or at least enhance cell death induced by other cytotoxic agents [King et al., 2004]. Interestingly, both effects could be observed almost across the same range of TLCK and TPCK concentrations [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; Mlejnek, 2005]. In this work we readdressed the question whether serine protease inhibitors TLCK and TPCK could provide cells a strong cytoprotective effect against cell death inducing stimuli by means of inhibition of caspase processing and/or activation. STS, ETP, and IPA, which induce cell death with distinct morphological and biochemical apoptotic

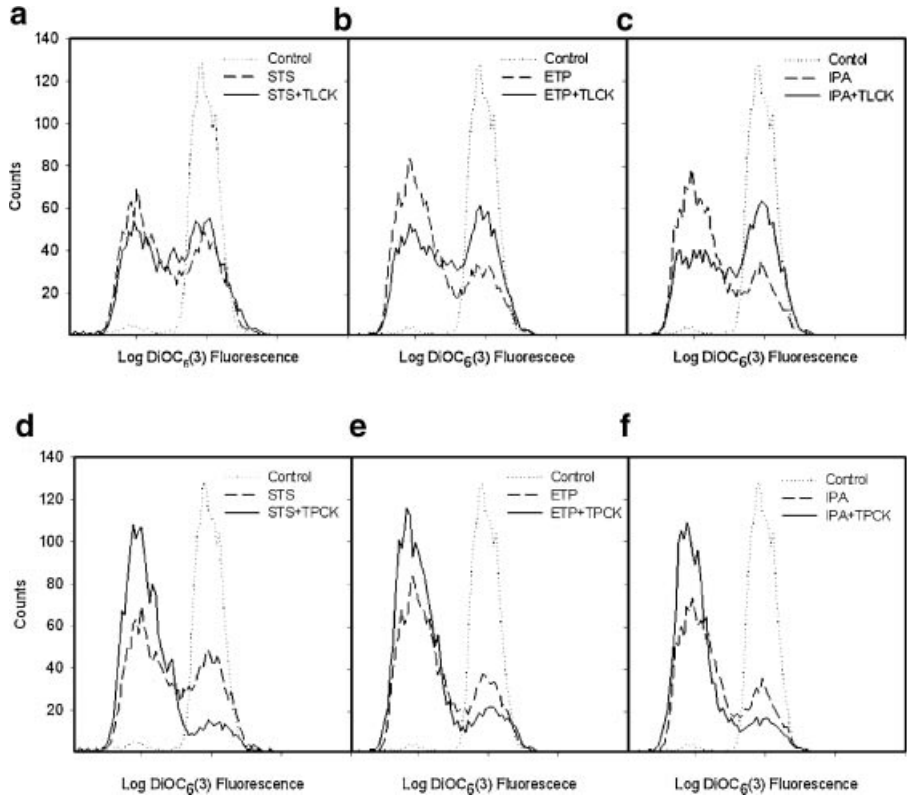

Fig. 3. Effect of TLCK and TPCK on mitochondrial transmembrane potential ΔΨ in dying HL-60 cells. Cells were treated with STS (**panels a**,**d**), ETP (**panels b**,**e**), and IPA (**panels c**,**f**) in the presence or absence of TLCK or TPCK, as indicated. ΔΨ was determined 18 h after the cell treatment as described in Materials and Methods Section. Untreated cells were taken as a control. Histograms represent typical results.

hallmarks [Martins et al., 1997; Zhang et al., 2004; Mlejnek and Dolezel, 2005], were chosen as cytotoxic agents.

Similarly to other authors [Bruno et al., 1992; Weaver et al., 1993], we observed that TLCK and TPCK very efficiently inhibited nucleosomal fragmentation of nuclear DNA in cells exposed to pro-apoptotic stimuli (Fig. 1). This may indicate that TLCK and TPCK abrogated caspase activation. Indeed, caspases were reported to be involved also in DNase activation during apoptosis [Porter and Janicke, 1999]. Namely, caspase-3 initiates apoptotic DNA fragmentation by proteolytically inactivating DFF45 (DNA fragmentation factor-45)/ICAD (inhibitor of caspase-activated DNase), which releases active nuclease DFF40/CAD (caspase-activated DNase) as demonstrated by Inohara et al. [1999]. However, abrogation of nucleosomal DNA fragmentation by TLCK or TPCK actually did not prevent cells from death. Indeed, the nucleosomal DNA fragmentation is a rather late apoptotic event, which is downstream of cell death commitment [Collins et al., 1997].

As we demonstrated in this study, even assessment of mitochondrial transmembrane potential ΔΨm within 18 h after the treatment might fail to reflect cell survival adequately (compare Fig. 3 with Figs. 4 and 5). This is a particularly important observation because the collapse of mitochondrial transmembrane potential ΔΨm can occur as a consequence of the mitochondrial permeability transition (MPT), which is obviously considered as an upstream event of caspase activation. In addition, the MPT appears to be a common mechanism shared in cell death pathways including necrosis, apoptosis, and autophagy [Lemasters et al., 2002]. Therefore, the collapse of mitochondrial transmembrane potential ΔΨm is obviously considered as a reliable indicator of cell death/cell survival. However, caspase inhibition by panspecific caspase inhibitor Z-VAD-FMK could delay this event without increasing cell survival [Hirsch et al., 1997]. Thus, the finding

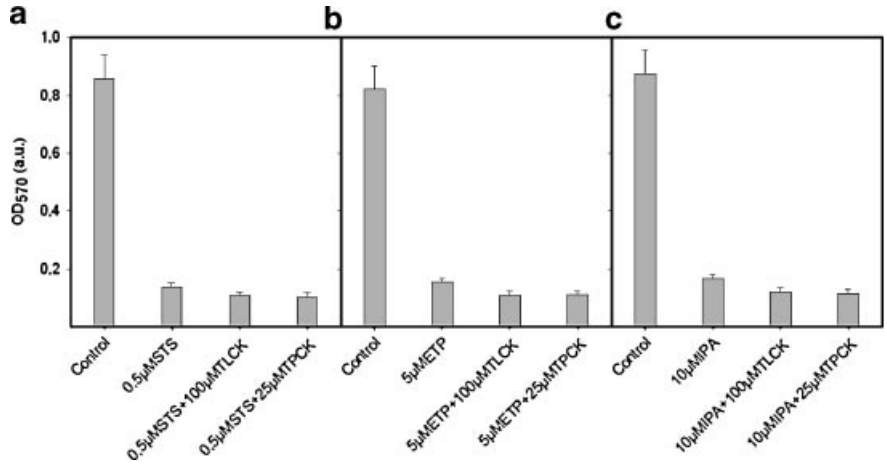

Fig. 4. Effect of TLCK and TPCK on proliferation potential of dying HL-60 cells. Cells were treated for 18 h with STS (**panel a**), ETP (**panel b**), and IPA (**panel c**) in the presence or absence of TLCK or TPCK, as indicated. Then cells were washed and transferred into standard medium, which contained neither drug nor serine protease inhibitors, and incubation proceeded another 24 h prior to MTT assay. Untreated cells were taken as a control. The experimental points represent mean values from three replicate experiments with standard deviations.

that TLCK diminished the collapse of mitochondrial transmembrane potential $\Delta\Psi m$ in cells treated with ETP or IPA was not surprising considering the fact that it acted also as a non-specific caspase inhibitor (Fig. 7, Table I). The finding that TLCK did not inhibit collapse of $\Delta\Psi m$ in cells treated with STS can be explained by the fact that STS induces both caspase-dependent and caspase-independent apoptotic pathways [Zhang et al., 2004]. The reason, why this effect exhibited only TLCK but not TPCK (Fig. 3), remains to be elucidated. One of the possible explanations is that TPCK itself is very toxic [unpublished results, Mlejnek, 2005].

One important problem concerning the application of "inhibitors of apoptosis" is the experimental verification of cell death inhibition. For example, caspase inhibitors suppress many morphological and biochemical apoptotic hallmarks, what has been many times wrongly interpreted as inhibition of cell death without clear demonstration of cellular survival. Indeed, caspase inhibitors obviously do not prevent cells from death, cells still die but they undergo either delayed cell death with some

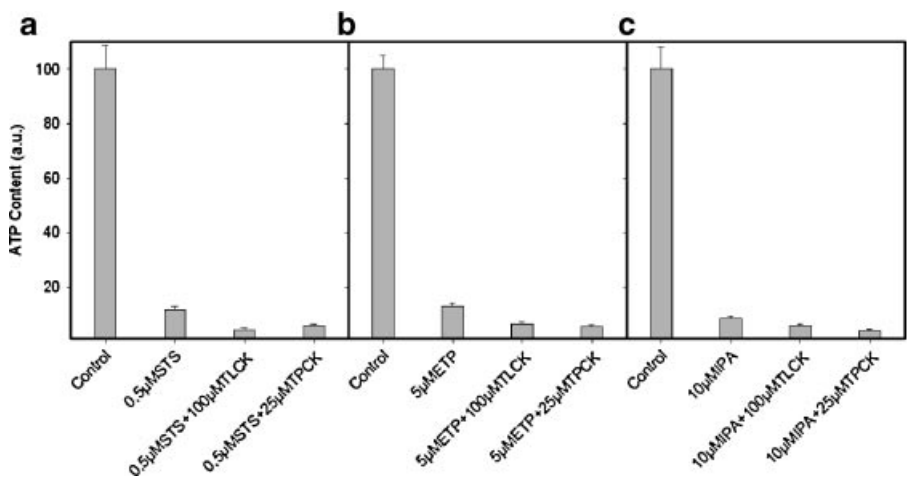

Fig. 5. Effect of TLCK and TPCK on ATP production in dying HL-60 cells. Cells were treated for 18 h with STS (**panel a**), ETP (**panel b**), and IPA (**panel c**) in the presence or absence of TLCK or TPCK, as indicated. Then cells were washed and transferred into standard medium, which contained neither drug nor serine protease inhibitors, and incubation proceeded another 24 h prior to ATP determination. Untreated cells were taken as a control. The experimental points represent mean values from three replicate experiments with standard deviations.

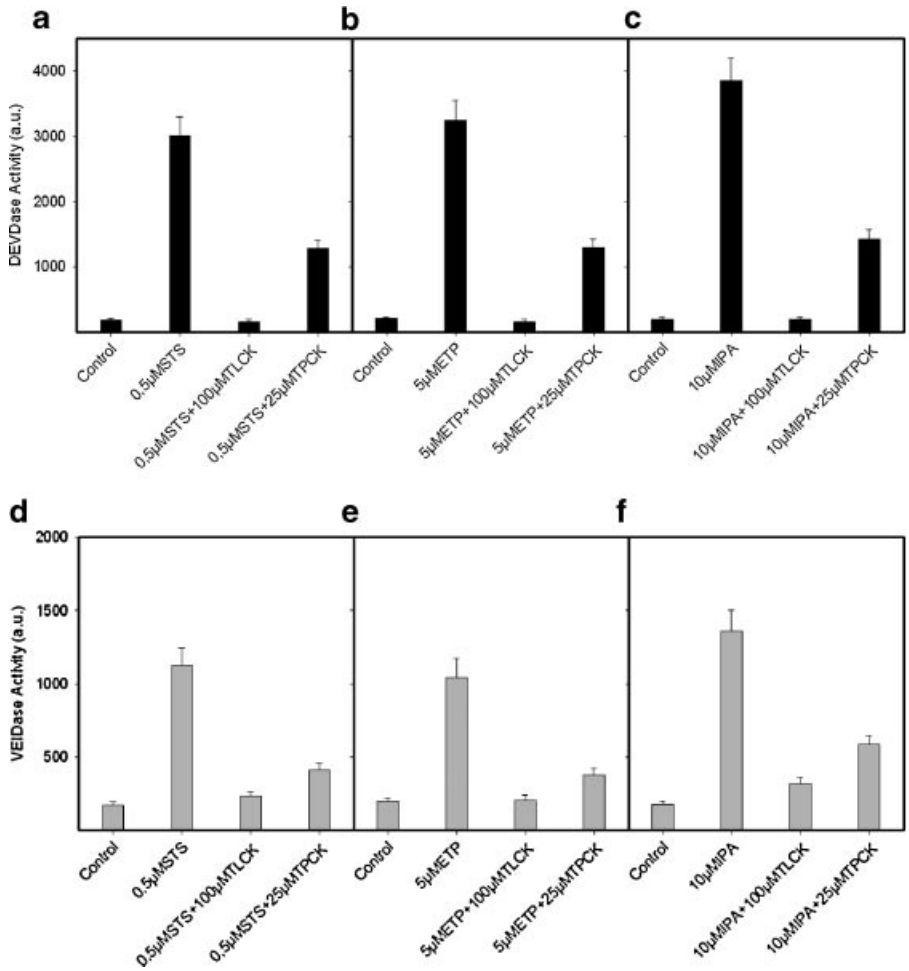

Fig. 6. Effect of TLCK and TPCK on caspase enzymatic activity in cell lysates of apoptotic HL-60 cells. TLCK or TPCK were added to the cell lysates prepared from cells that were treated for 18 h with STS (**panels a**,**d**), ETP (**panels b**,**e**), and IPA (**panels c**,**f**), as indicated. Untreated cells were taken as a control. Caspase-3 and/or caspase-7 = DEVDase activity (panels a–c), caspase-6 = VEIDase activity (panels d–f). The experimental points represent mean values from three replicate experiments with standard deviations.

apoptotic features or die via necrosis [Hirsch et al., 1997; Kitanaka and Kuchino, 1999]. Therefore, we evaluated cell death and/or cell survival using two independent methods 24 h after the 18-h treatment (Figs. 4 and 5). Our data unambiguously showed that neither TLCK nor TPCK provided cells any protection against cell death inducing stimuli (Figs. 4 and 5). Based on these results we offer an alternative explanation for the above-mentioned effects of TLCK and TPCK. Since TLCK and TPCK acted as direct and non-selective caspase inhibitors they may prevent some morphological and biochemical apoptotic hallmarks. TLCK and TPCK may even retard cell death but they actually can not provide cells long-term survival.

Our results indicated that TLCK and TPCK suppressed DEVDase (caspases 3 and -7) and VEIDase (caspase-6) activity in cells exposed to cell death inducing stimuli such as STS, ETP, or IPA (Fig. 2). Such finding is obviously interpreted as inhibition of caspase activation. Indeed, it was demonstrated that serine protease inhibitors, including TLCK and TPCK, inhibited caspase processing [Stefanis et al., 1997; Jones et al., 1998; Dong et al., 2000] However, diminished caspase activity could be a result of either impaired caspase processing and activation, or of the direct inhibition of mature caspases. The later possibility was largely overlooked despite the fact that TLCK and TPCK are able to inactivate in addition to serine proteases also cysteine proteases such

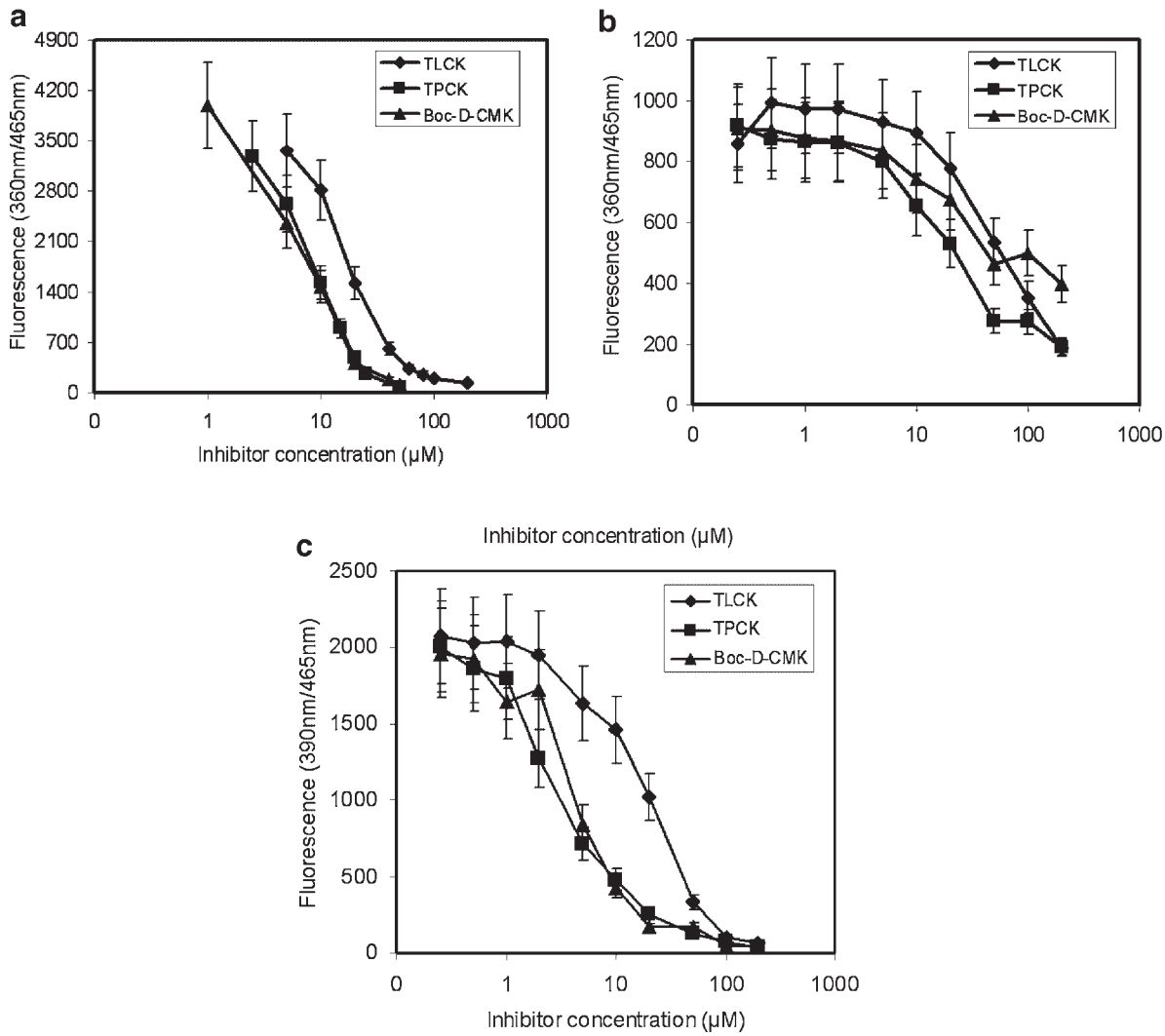

Fig. 7. Effect of TLCK and TPCK on enzymatic activity of recombinant caspases. Assay mixtures containing appropriate activity of human recombinant caspase-3 (**a**), caspase-6 (**b**), and caspase-7 (**c**) were preincubated with particular protease inhibitor for 15 min on ice before the assay started, (for details, see Materials and Methods Section). Caspase enzymatic activity in the presence of TLCK (filled circles), caspase enzymatic activity in the presence of TPCK (filled squares), caspase enzymatic activity in the presence of Boc-D-CMK (filled triangles). Caspase activity without inhibitor was taken as 100%. The experimental points represent mean values from three replicate experiments with standard deviations.

as bromelain, clostripain, or papain [Drenth et al., 1976; Jia et al., 1995]. To our knowledge, there is only one published paper where authors addressed this question. Thus, Dong et al. [2000] demonstrated that TPCK and TLCK were unable to inhibit DEVDase activity in crude extracts of apoptotic cells with active caspases 3, -8, and -9. However, they did not used recombinant caspases to provide direct evidence for their conclusion [Dong et al., 2000]. In contrast, we found that caspase activity was efficiently inhibited even in cell lysates made from apoptotic cells (Fig. 6). This observation suggested that serine protease inhibitors TLCK and TPCK could serve also as potent direct inhibitors of activated caspases. It is necessary to note that we have no adequate explanation for relatively weak inhibitory effect of TPCK and very strong inhibitory effect of TLCK on caspase enzymatic activity in cell lysates (Fig. 6). Owing to the fact that the strong inhibitory effect of TLCK on caspases corresponded very well to the data obtained with recombinant caspases (Fig. 7, Table I), we can speculate that the relatively low inhibitory effect of TPCK on caspases was paradoxically due to its higher chemical reactivity. Indeed, TPCK similarly to TLCK can react also with

TABLE I. Effect of TLCK, TPCK, and Boc-D-CMK on Enzymatic Activity of Human Recombinant Caspases

Inhibitor	IC_{50} (caspase-3) (μM)	IC_{50} (caspase-6) (μM)	IC_{50} (caspase-7) (μM)
TLCK	12.0	54.5	19.3
TPCK	6.9	17.5	2.7
Boc-D-CMK	6.0	20.2	4.1

other proteins [Breithaupt et al., 1996; Grammer and Blenis, 1996; D'Acquisto et al., 1997] and the phenylalanyl residue makes TPCK more hydrophobic compared to lysinyl in TLCK. Therefore TPCK molecule can interact with other proteins more easily.

Experiments with human recombinant caspases provided a direct evidence that both serine protease inhibitors, TLCK and TPCK could also act as potent but non-selective inhibitors of mature caspases 3, -6, and -7 (Fig. 7, Table I). Inhibitory effect of TPCK was approximately two times, three times, and six times stronger than that of TLCK to caspases 3, -6, and -7, respectively (Table I). Interestingly, the inhibitory effect of TPCK on mature caspases was comparable to that found for panspecific caspase inhibitor Boc-D-CMK (Fig. 7, Table I). Results obtained with recombinant caspases are in a good agreement with the data obtained for whole cells (Fig. 2), despite the facts that: (i) inhibitory efficiency of TLCK and TPCK, when applied before the cell treatment, were lower than those found for recombinant caspases (compare Fig. 2 to Fig. 7 and Table I); (ii) it was not possible to distinguish the enzymatic activity of caspase-3 from that of caspase-7 in cell lysates using the artificial fluorescence substrate Ac-DEVD-AMC (compare Fig. 2 to Fig. 7). Relatively low inhibitory efficiency of TLCK and TPCK, when applied before the cell treatment, could be explain by the facts that: (i) neither TLCK nor TPCK is stable compound [e.g., Shaw and Glower, 1970]; (ii) TLCK and TPCK are highly reactive compounds that could react with other proteins [Breithaupt et al., 1996; Grammer and Blenis, 1996; D'Acquisto et al., 1997].

In conclusion, we have unambiguously demonstrated for the first time that TLCK and TPCK may act also as direct and non-specific inhibitors of activated caspases, at least caspases 3, -6, and -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.

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