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Selective chemical reagents to investigate the role of caspase 6 in apoptosis in acute leukemia T cells

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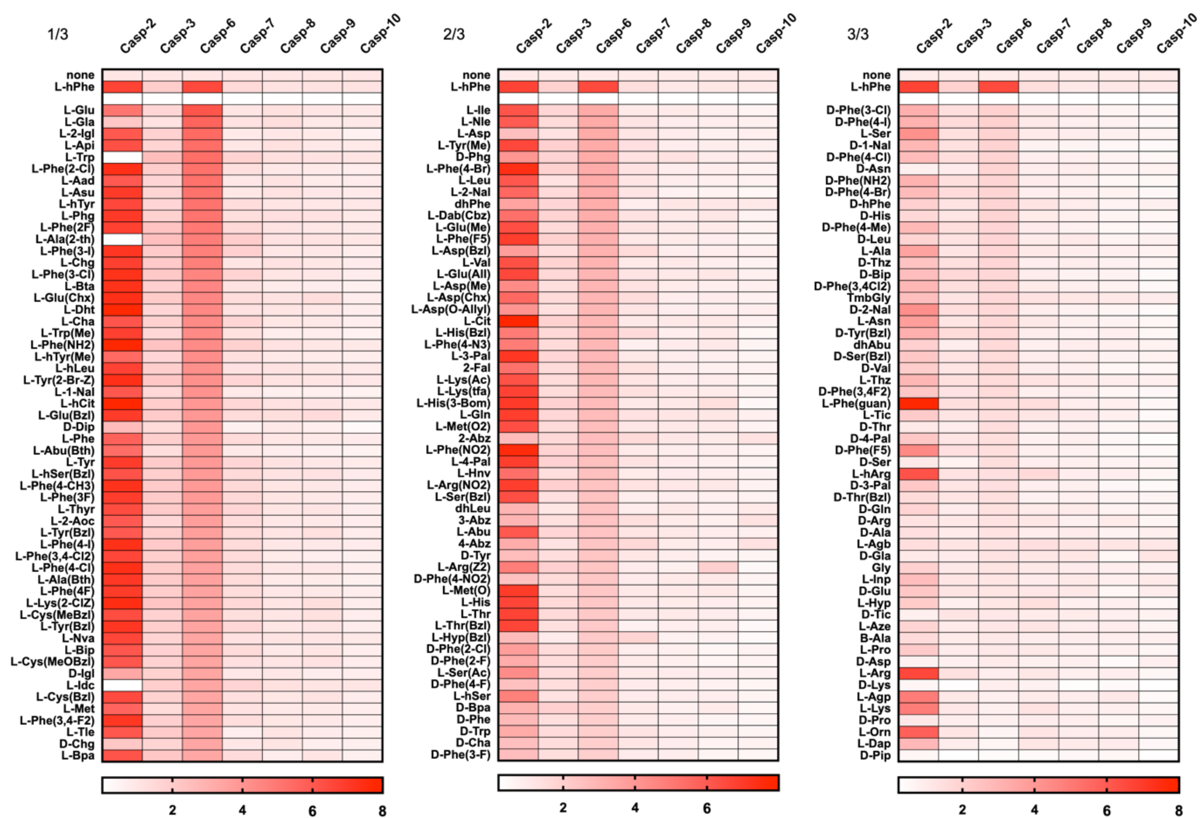


Figure S1. Caspases specificity at the P5 position. Human apoptotic caspases (-2, -3, -6, -7, -8, -9, -10) were screened at the P5 position with the combinatorial fluorogenic substrate library, Ac-P5-Mix-Glu-Mix-Asp-ACC. The relative rate of P5 substrate hydrolysis was divided by the hydrolysis rate of substrates mixture lacking P5 position (Ac-Mix-Glu-Mix-Asp-ACC, "*none = 1*") and the specificity profile was expressed as white-red heat map, where the substrates were ordered by caspase-6 preferences, from highest to lowest.

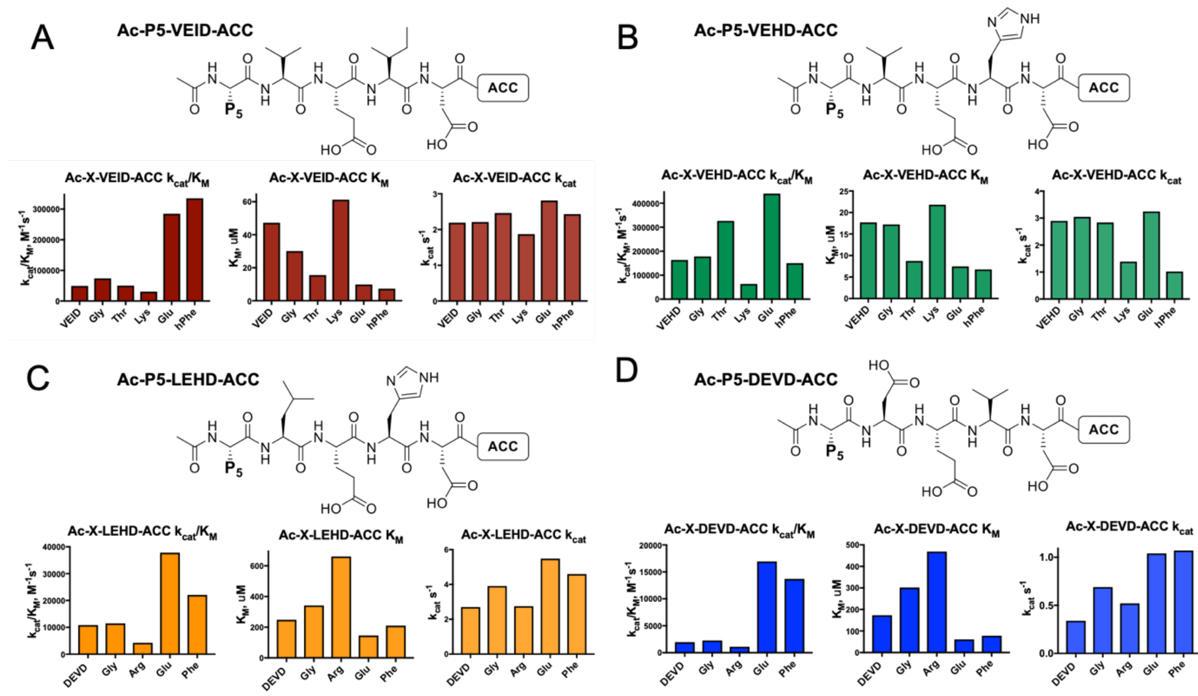
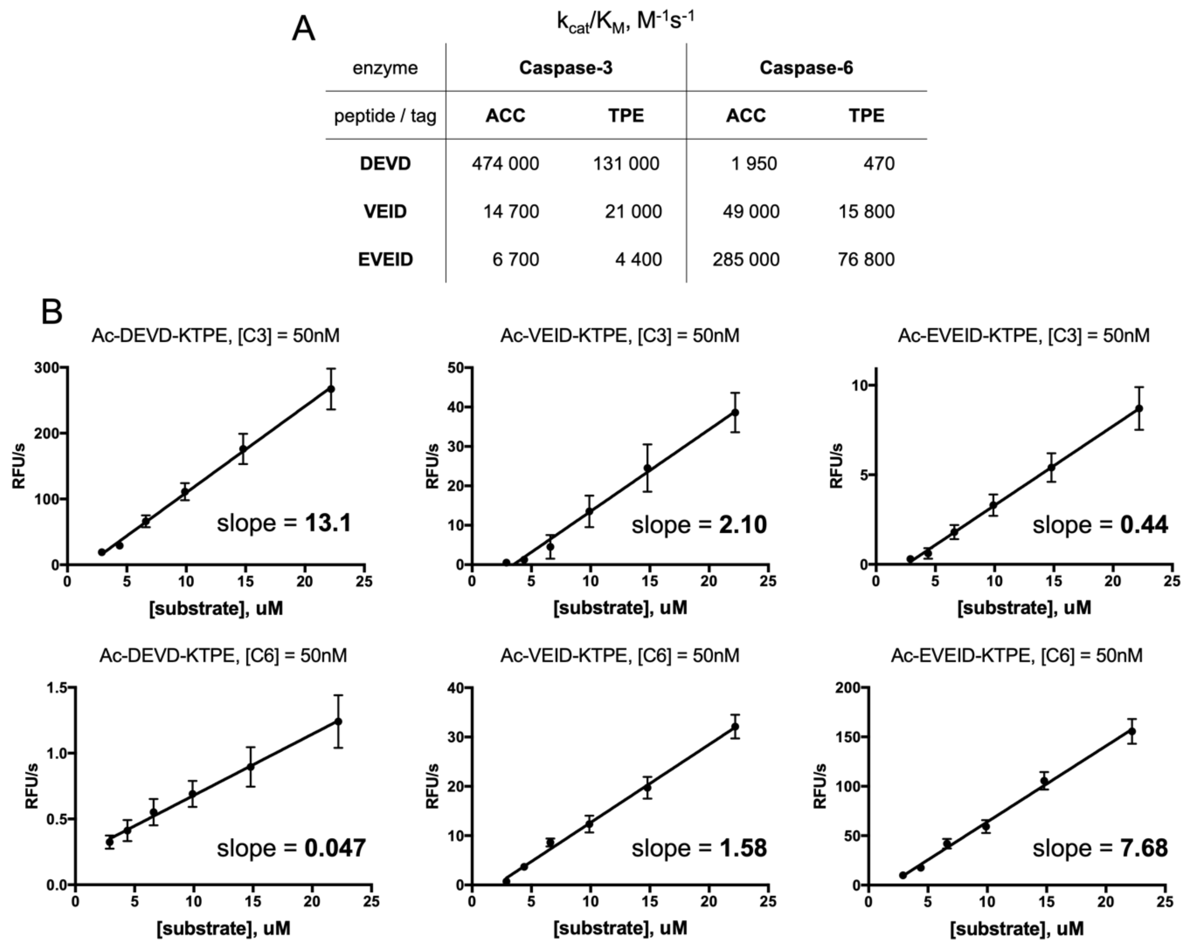


Figure S2. Detailed analysis of caspase 6 subsite cooperativity. To accurately examine the substrate-caspase 6 interactions and potential subsite cooperativity, we performed a comprehensive analysis of caspase 6 cleavage preferences towards four sets of substrates bearing various tetrapeptide motifs: VEID (**Panel A**), VEHD (**Panel B**), LEHD (**Panel C**), and DEVD (**Panel D**). Each panel represents different substrates with fixed P1-P4 amino acid residues and one of five P5 amino acid residues (G, T, K, E, hF in Panels A, B, and G, R, E, F in Panels C, D). The kinetic parameters (k_{cat}/K_M, K_M, and k_{cat}) are presented on the y-axis, whereas the P5 amino acids are presented on the x-axis. In each set of substrates, a tetrapeptide substrate lacking a P5 amino acid was used as a control.



A

k_{cat}/K_M, $M^{-1}s^{-1}$

enzyme	Caspase-3		Caspase-6	
peptide / tag	ACC	TPE	ACC	TPE
DEVD	474 000	131 000	1 950	470
VEID	14 700	21 000	49 000	15 800
EVEID	6 700	4 400	285 000	76 800

Figure S3. The kinetic analysis of AIE substrates. A The table with k_{cat}/K_M parameters of DEVD-X, VEID-X, and EVEID-X substrates toward recombinant caspase 3 and caspase 6 (X stands for ACC or TPE tag). Data indicate that TPE-labeled peptides are potent substrates for caspases. **B** The velocity of substrate hydrolysis by recombinant caspase 3 and caspase 6 (at 50nM). Slope value (RFU/s*μM) was used to calculate k_{cat}/K_M value ($M^{-1}s^{-1}$).

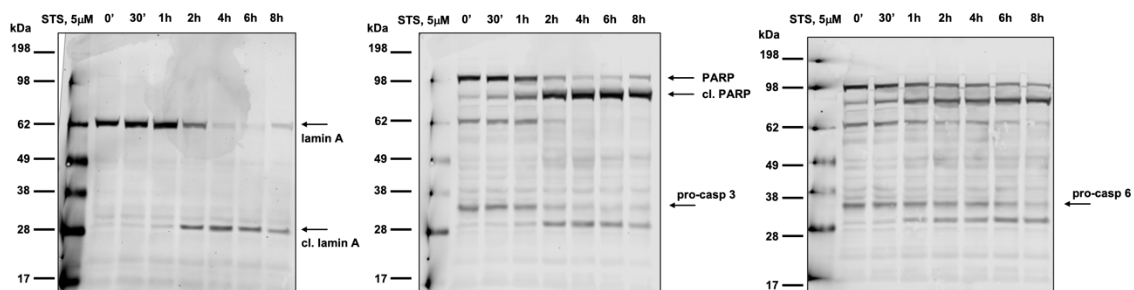


Figure S4 Caspases activation in STS-treated Jurkat T cells. The activation of pro-caspase 3 (p32) and pro-caspase 6 (p35) into mature forms, and the processing of PARP (p116 → p89) and lamin A (p74 → p28) substrates demonstrate hierarchical activation of executioner caspases.

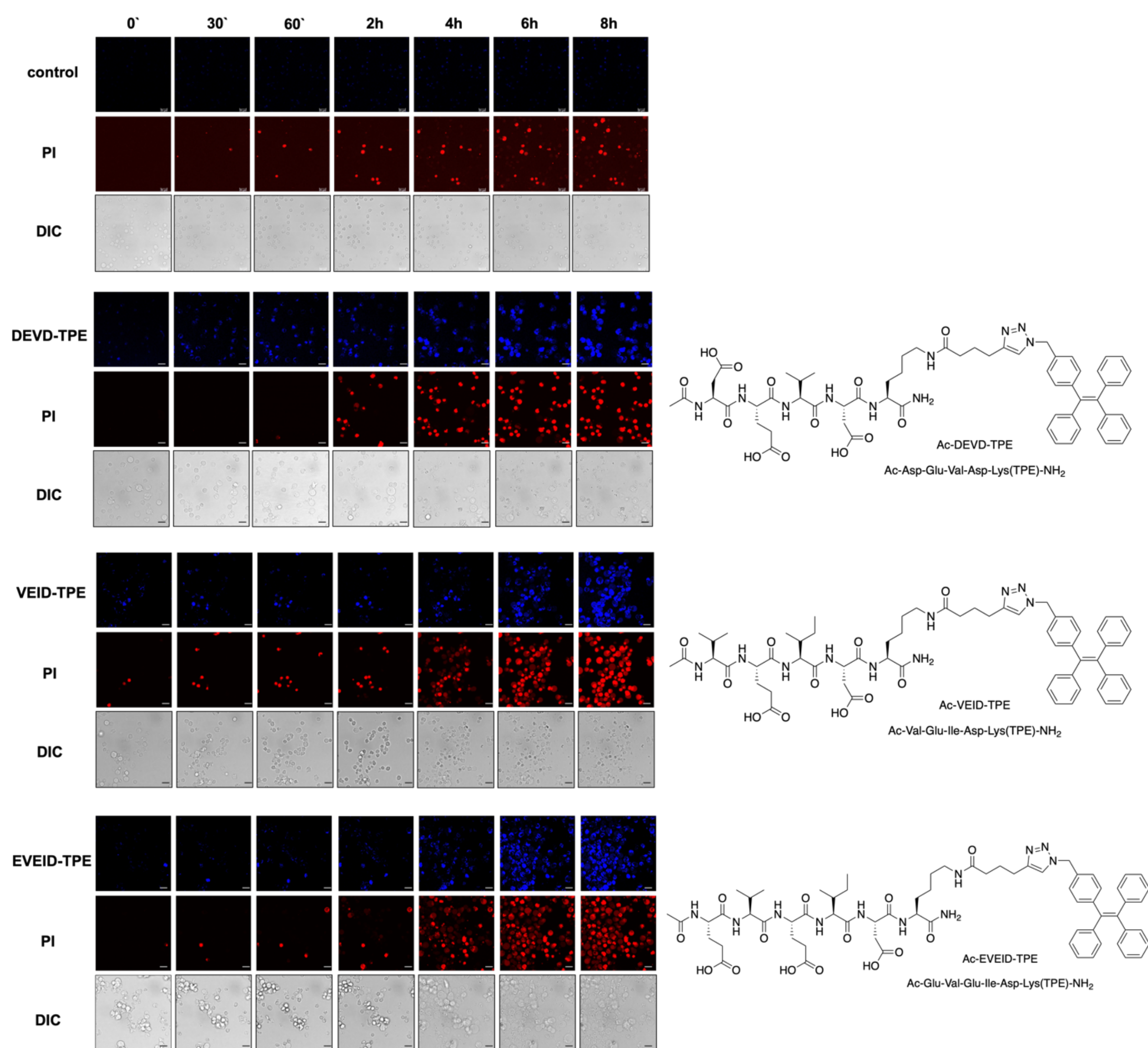


Figure S5. Real-time imaging of caspase activation in Jurkat T cells using various fluorogens. Ac-DEVD-TPE was designed to monitor the activation of caspases 3/7, whereas Ac-EVEID-TPE was designed to monitor the activation of caspase 6. The Ac-VEID-TPE substrates detects the activity of caspases 3, 6, and 7. TPE substrates were preincubated with Jurkat T cells for 2 hours prior to staurosporine stimulation.

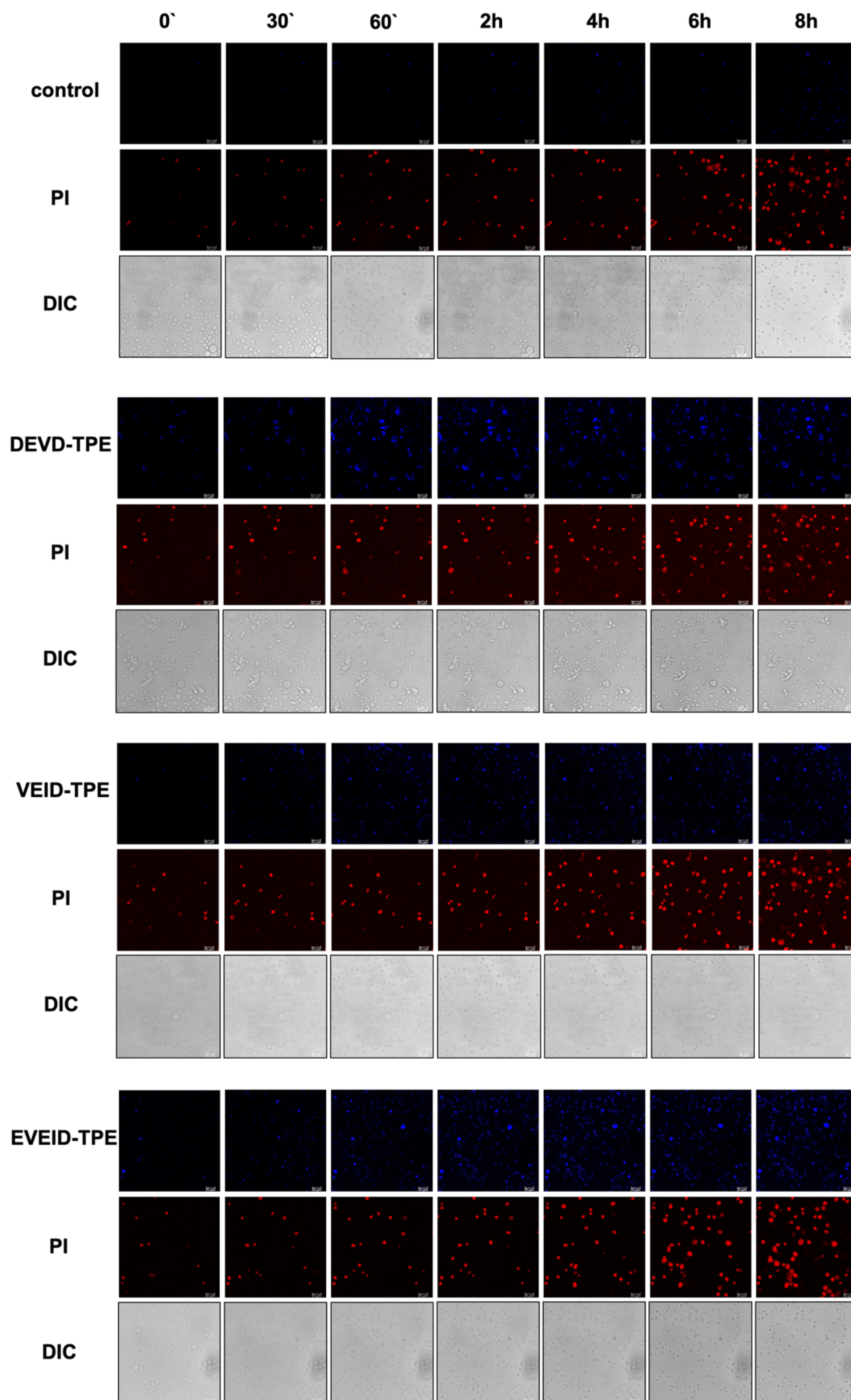


Figure S6. Real-time imaging of caspase activation in Jurkat T cells using various fluorogens. Ac-DEVD-TPE was designed to monitor the activation of caspases 3/7, whereas Ac-EVEID-TPE was designed to monitor the activation of caspase 6. The Ac-VEID-TPE substrates detects the activity of caspases 3, 6, and 7. TPE substrates were preincubated with Jurkat T cells for 10 hours prior to staurosporine stimulation.

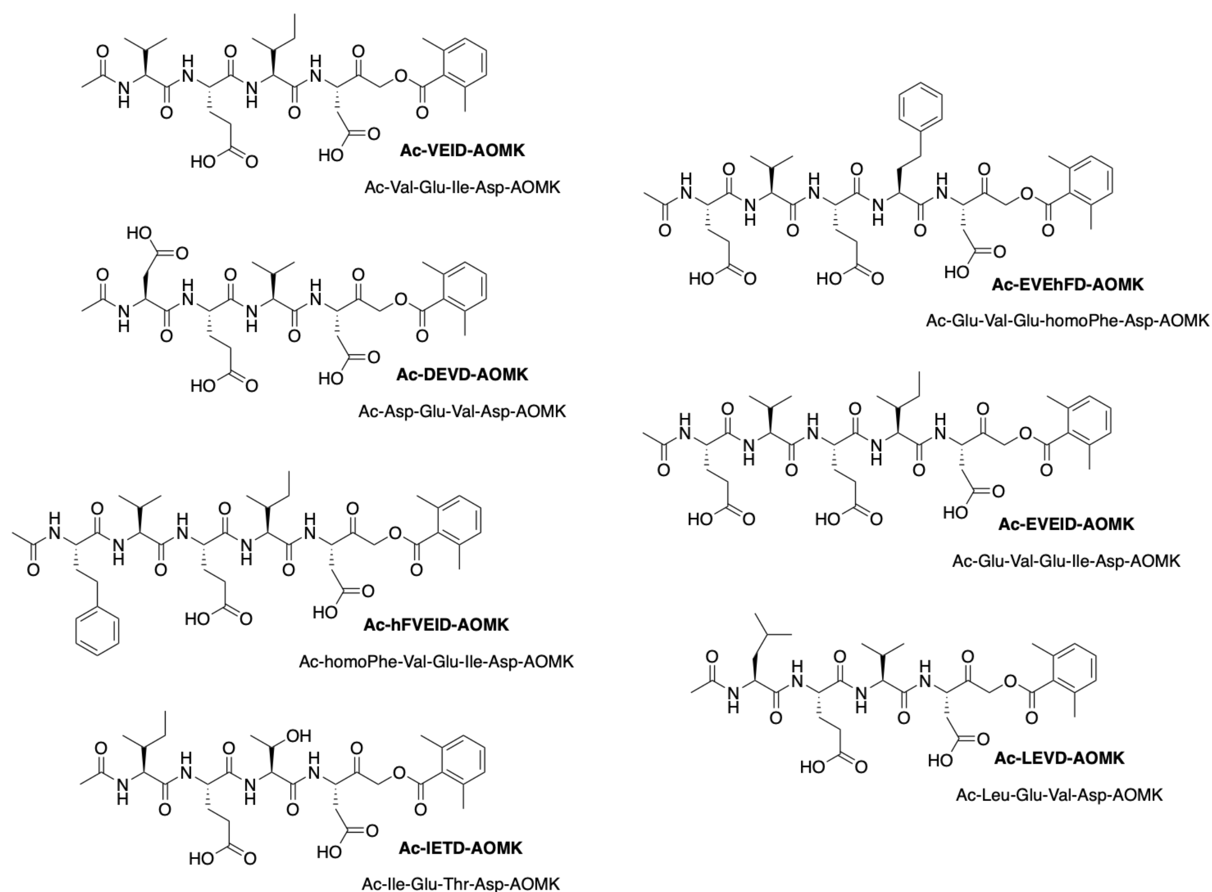


Figure S7. Structure of caspase inhibitors bearing AOMK electrophilic warhead. AOMK is acyloxymethyl ketone (2,6-dimethylbenzyloxymethyl ketone). hF (homoPhe) is homo-phenylalanine.

inhibitor	K_{obs}/I, $M^{-1}s^{-1}$					
	Casp-3	Casp-6	Casp-7	Casp-8	Casp-9	Casp-10
VEID	573 000	1 440 000	63 000	524 000	37 800	113 000
DEVD	6 900 000	345 000	1 230 000	576 000	5 700	68 500
hFVEID	1 020 000	1 990 000	49 600	51 100	24 000	78 400
IETD	252 000	542 000	32 000	573 000	15 200	136 000
LEVD	430 000	285 000	112 000	635 000	15 000	78 000
EVEhFD	1 120 000	1 650 000	72 500	461 000	26 200	86 500
EVEID	48 000	472 000	10 100	71 000	7 300	12 200

Figure S8. Kinetic parameters of tetra- and pentapeptide inhibitors toward recombinant human caspases. The general structure of inhibitors is Ac-(P5)-P4-P3-P2-Asp-AOMK.

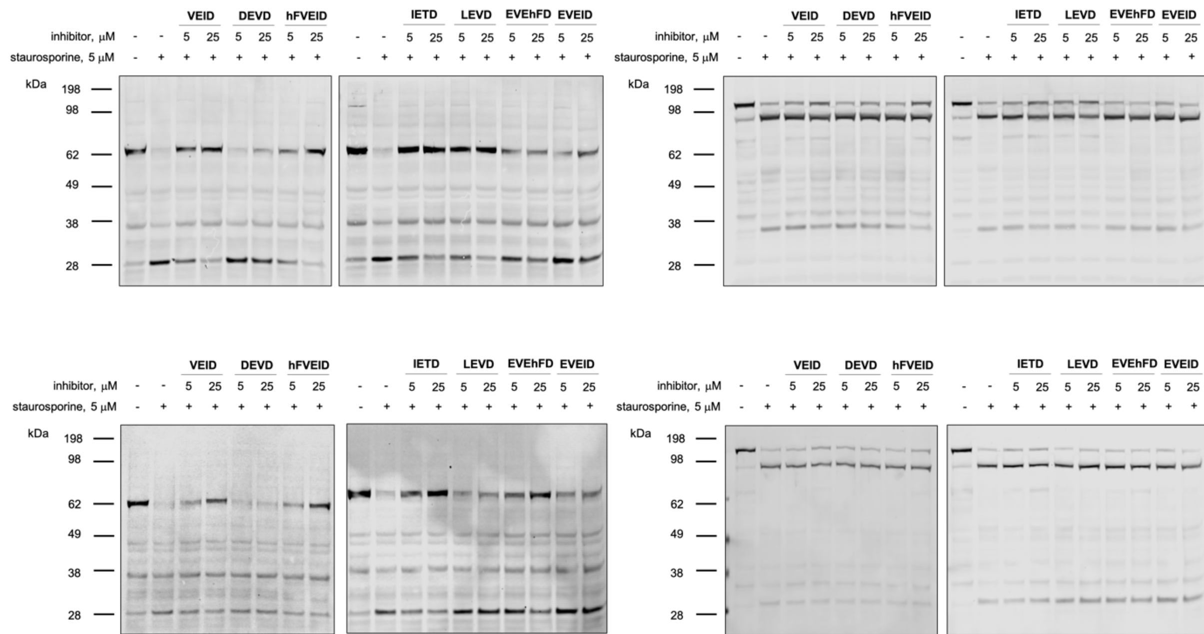


Figure S9. Analysis of caspase 6 activity by covalent inhibitors. The influence of small molecule inhibitors on PARP and lamin A processing by caspases 3 and 6 in staurosporine-treated Jurkat T cells. The most potent caspase 6 inhibitors almost completely blocked lamin A cleavage, whereas most of caspase 3 inhibitors failed to prevent from PARP processing.

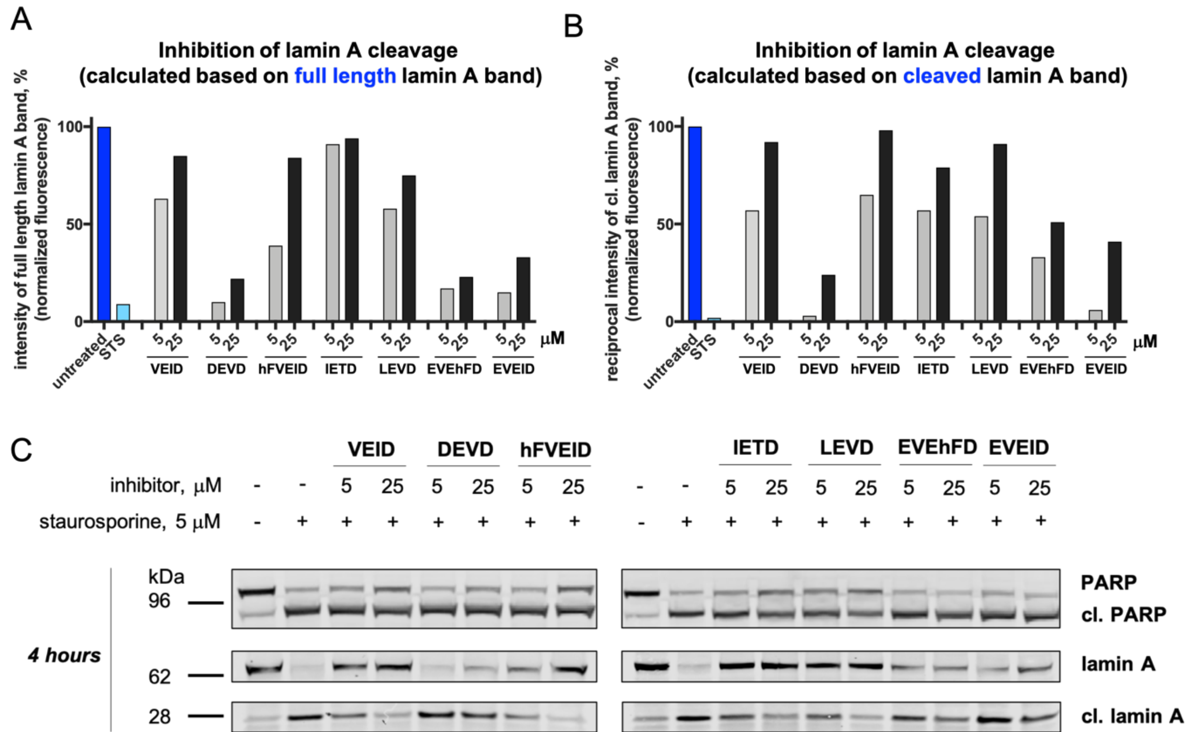


Figure S10. Analysis of caspase 6 activation and lamin A cleavage with a set of covalent inhibitors – quantitative Western blot analysis. A The fluorescence of WB bands indicating full-length lamin A was counted, normalized (untreated vs. STS-treated), and utilized to calculate the % of inhibition of lamin A cleavage. **B** The fluorescence of WB bands indicating cleaved lamin A was counted, normalized (untreated vs. STS-treated), and utilized to calculate the % of inhibition of lamin A cleavage. **C** indicates the bands of full-length and cleaved lamin A that were taken for the analysis.

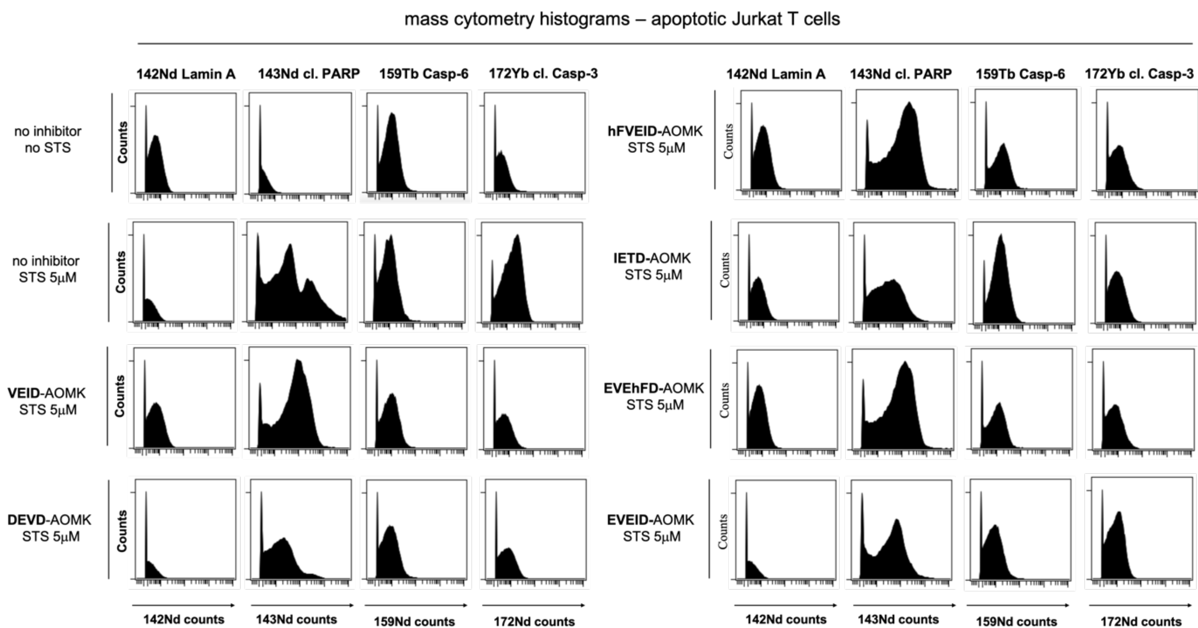


Figure S11. Analysis of apoptotic Jurkat T cells by mass cytometry. Histograms demonstrate the labeling of four apoptotic proteins: lamin A, cleaved PARP, caspase 6 and cleaved caspase 3 in staurosporine-treated

Jurkat T cells in the presence of various inhibitors. Data demonstrate that VEID, hFVEID and IETD inhibitors bind caspase 6 thus block lamin A processing, whereas DEVD and VEID inhibitors block pro-caspase 3 activation and PARP processing.

Compound	$[m/z + H]^+$ calculated	$[m/z + H]^+$ measured
ACC substrates Ac-peptide-ACC		
Ac-VEID-ACC	717.31	717.36
Ac-GVEID-ACC	774.33	774.39
Ac-TVEID-ACC	818.36	818.43
Ac-KVEID-ACC	845.40	845.48
Ac-EVEID-ACC	846.35	846.38
Ac-hFVEID-ACC	878.39	878.45
Ac-VEHD-ACC	741.28	741.34
Ac-GVEHD-ACC	798.30	798.35
Ac-TVEHD-ACC	842.33	842.39
Ac-KVEHD-ACC	869.37	869.48
Ac-EVEHD-ACC	870.32	870.38
Ac-hFVEHD-ACC	902.36	902.39
Ac-LEHD-ACC	755.30	755.34
Ac-GLEHD-ACC	812.32	812.33
Ac-RLEHD-ACC	911.40	911.45
Ac-ELEHD-ACC	884.34	884.35
Ac-FLEHD-ACC	902.37	902.42
Ac-DEVD-ACC	719.25	719.31
Ac-GDEVD-ACC	776.27	776.33
Ac-RDEVD-ACC	875.35	875.39
Ac-EDEVD-ACC	848.30	848.34
Ac-FDEVD-ACC	866.32	866.37
TPE substrates Ac-peptide-Lys(TPE)-NH_2		
Ac-DEVD-K(TPE)-NH_2	1127.52	1127.59
Ac-VEID-K(TPE)-NH_2	1125.58	1125.66
Ac-EVEID-K(TPE)-NH_2	1254.62 627.81 $[m/2 + H]^+$	627.89 $[m/2 + H]^+$
Ac-hFVEID-K(TPE)-NH_2	1286.66 643.83 $[m/2 + H]^+$	643.88 $[m/2 + H]^+$
AOMK inhibitors, Ac-peptide-AOMK		
Ac-VEID-AOMK	663.32	663.37
Ac-DEVD-AOMK	665.27	665.32
Ac-hFVEID-AOMK	824.41	824.45
Ac-IETD-AOMK	665.30	665.33
Ac-LEVD-AOMK	663.32	663.36
Ac-EVEhFD-AOMK	840.37	840.41
Ac-EVEID-AOMK	729.37	729.40

Table S1. Molecular mass of caspase substrates and inhibitors. All compounds were purified on RP-HPLC and displayed at least 95% of purity (measured with LC-MS, 220 nm).