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Letter to the Editor

Cathepsin B-independent abrogation of cell death by CA-074-OMe upstream of lysosomal breakdown

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Dear Editor,

Recent progress provided compelling evidence for the major role of lysosomal cathepsin proteases in cell death pathways especially when caspase activity is suppressed.[1,2] Cathepsin B, a ubiquitous endopeptidase and ectodipeptidase, was shown to be a component of TNF-α cell death signaling,[3] as well as an executor protease in caspase-compromised cells induced to undergo apoptosis[4] or necrosis.[5]

L-*trans*-epoxysuccinyl-Ile-Pro-OH propylamide (CA-074) is a highly specific inhibitor of cathepsin B.[6] The methylated variant, CA-074-OMe, was shown to penetrate into cells more easily than the parental molecule,[7] whereas it loosened its cathepsin B specificity reacting with other, unidentified cathepsins as well.[8]

In this study, we presented evidences showing that CA-074-OMe abrogated both apoptotic and necrotic cell death (at $ID_{50} \sim 10\,\mu M$) in caspase-inhibited leukemia cells induced by staurosporine. However, abrogation of cell death occurred at a much higher (around three orders of magnitude higher) concentration of CA-074-OMe than required for inhibition of RR-ase dipeptidase activity (at $ID_{50} \sim 10\,nM$), a feature of cathepsin B. Furthermore, Z-FA-FMK, another cathepsin (including cathepsin B) inhibitor, did not abrogate cell death but switched apoptosis to necrosis in the caspase-compromised cells. Based on these results, we came to the conclusion that CA-074-OMe has a cathepsin B-independent target that (1) plays a role in some forms of cell death upstream of lysosomal breakdown and (2) not targeted by Z-FA-FMK.

HL-60 cells exposed to staurosporine (Sigma) showed the sign of typical apoptotic morphology (not shown) and biochemical changes including nucleosomal DNA fragmentation that was detected as sub-G1 population by flow cytometry[9] (Figure 1a). Most of the cells had fragmented DNA after treatment with staurosporine alone (Figure 1a and f). The nonselective, cell-permeable caspase inhibitor Z-VAD (OMe)-FMK (Z-Val-Ala-DL-Asp-(OMe)-fluoromethylketone, Bachem) at 50 μM concentration – where no residual DEVD-ase activity was detected[10] (not shown) – did inhibit apoptosis compared to staurosporine-treated cells ($P<10^{-5}$), but switched the cell death type from apoptosis to necrosis ($P<10^{-3}$), detected by propidium iodide (PI) uptake in nonfixed cells (Figure 1a and f). Apoptotic and necrotic cells provided separate, nonoverlapping cell populations that were confirmed by acridine orange–PI viable staining and fluorescence microscope observation (not shown). Overall cell death (apoptosis + necrosis) was also significantly diminished by the caspase inhibitor ($P<0{,}05$), but without major effect (mean percentage of cell death for staurosporine: 92.1; for staurosporine + Z-VAD(OMe)-FMK: 62.3; $n=7$) (Figure 1f).

To explore if various cellular compartments are involved in DEVD-ase-independent cell death, acidity of endolysosomes and the inner membrane potential of mitochondria were measured by appropriate fluorescent dyes and flow cytometry (Figure 1b–e). A remarkable decline of acidic compartments was detected by acridine orange in the major population of staurosporine + Z-VAD(OMe)-FMK-treated cells (Figure 1b). In parallel, mitochondrial membrane potential decreased notably in most cells detected by $DiOC_6(3)$ staining (Figure 1d). Recent data indicate that release of lysosomal enzymes to the cytosole is incidental to lysosomal breakdown and loss of acidity.[11] The released cathepsins in the cytoplasm can activate Bid, resulting in mitochondrial damage.[12] This prompted us to study the possible involvement of cathepsins in cell death induced by staurosporine in the presence of caspase inhibition.

We measured the dipeptidase activity of cathepsins by using Z-RR-AMC (Z-Arg-Arg-7-amino-4-methylcoumarin, Bachem) and Z-FR-AMC (z-Phe-Arg-7-amino-4-methylcoumarin, Bachem) as fluorescent substrates according to Rozman-Pungercar *et al.*[13] Z-RR-AMC is considered as a specific substrate for cathepsin B, while Z-FR-AMC is cleaved by cathepsin B, L and S.[14] Linear RR-ase activity was detected in the control and Z-VAD(OMe)-FMK-treated cells (not shown). Not surprisingly, Z-VAD(OMe)-FMK (50 μM) markedly decreased detectable RR-ase activity after 4 h of treatment.[13,15] However, cathepsin B activity could partially recover at the time when apoptosis and necrosis rise at 8 h ($\Delta F/\Delta t_{cont} = 0.540 \pm 0.142$; $\Delta F/\Delta t_{ZVAD,4\,h} = 0.042 \pm 0.012$, $P<0{,}001$ to $\Delta F/\Delta t_{cont}$; $\Delta F/\Delta t_{ZVAD,8\,h} = 0.145 \pm 0.036$, $P<0{,}005$ to $\Delta F/\Delta t_{ZVAD,4\,h}$ (nM AMC/min/10^5 cells, mean $\pm$ S.D., $n=2$, with three parallels)). Therefore, the role of cathepsin B cannot be entirely excluded from the caspase-independent death process.

To further explore the role of cathepsin B, we applied CA-074 that is usually assumed to be a specific inhibitor of cathepsin B. Methylation of the proline carboxyl group makes CA-074 more cell membrane permeable and in the cytoplasm the methyl group is removed by aspecific esterases, releasing the active, specific enantiomer. When CA-074-OMe (Bachem

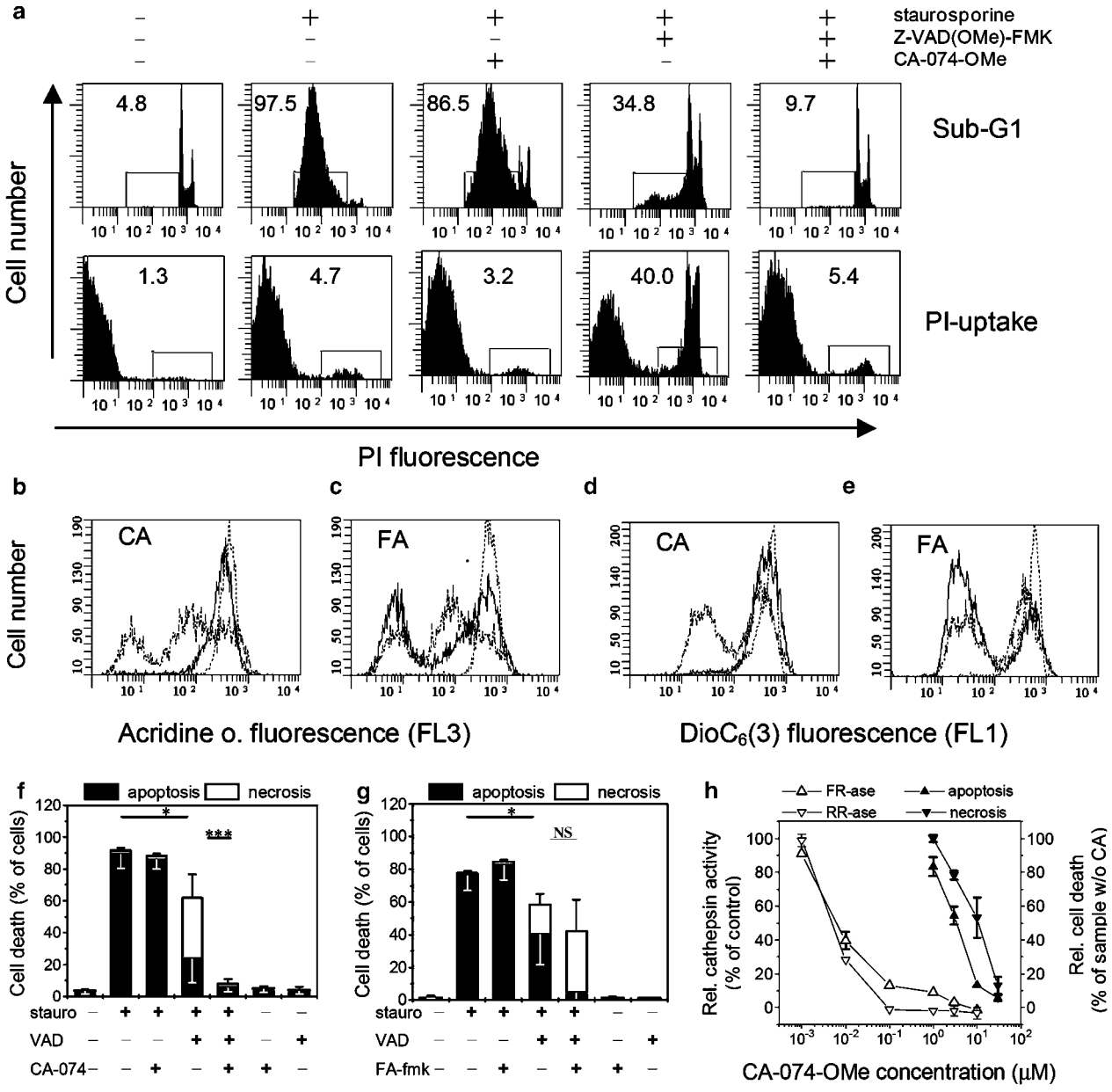

Figure 1 CA-074-OMe has a non-RR-ase (cathepsin B) target mediating caspase-independent cell death. HL-60 cells – cultured in RPMI1640 + 10% FBS – were exposed to protease inhibitors Z-VAD(OMe)-FMK (50 μM), CA-074-OMe (40 μM or as indicated) or Z-FA-FMK (1 μM) 1 h before treatment with staurosporine (1 μM) for further 8 h in 24- or 48-well plates. Data were presented as mean ± S.D. of at least three experiments. Significance was calculated by Student's *t*-test with two tails and homoscedastic distributions. The indicated significances are: not significant (NS): $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (**a**) *Detection of cell death*: Cell death was determined by flow cytometry (FACScan, Becton-Dickinson) using nonfixed cells labeled with PI for necrosis detection, according to Petak *et al.*,[19] and ethanol-fixed cells stained with ethidium bromide for apoptosis detection, according to Gong *et al.*[9] (**b–e)** Modulation of lysosomal acidification (**b**, **c**) or mitochondrial membrane potential (**d**, **e**) by cathepsin inhibitors. Treatments were: vehicle (DMSO) (… dotted line); staurosporine + Z-VAD(OMe)-FMK (·–·–· dotted-dashed line); staurosporine + Z-VAD(OMe)-FMK + either cathepsin inhibitor: CA-074-OMe (CA) or Z-FA-FMK (FA) (— solid lines). Cells were stained with either acridine orange, according to Zhao *et al.*,[20] (**b**, **c**) or $DiOC_6(3)$ (3,3′-dihexyloxacarbocyanine iodide), according to Nagy *et al.*[21] (**d**, **e**) (vehicle-treated cells did not show significantly altered staining compared to samples treated with CA-074-OMe only or Z-FA-FMK only). (**f–g)** Modulation of cell death by cathepsin inhibitors. Samples were processed as described at Figure 1a; CA-074-OMe (**f**, $n = 7$) or Z-FA-FMK (**g**, $n = 3$), stauro: staurosporine; VAD: Z-VAD(OMe)-FMK; CA-074: CA-074-OMe; FA-fmk: Z-FA-FMK. Significances were calculated for the overall percentage of cell death for column 2 *versus* column 4 and column 4 *versus* column 5). (**h)** Correlation of inactivation of cathepsin B and inhibition of cell death by CA-074-OMe dilutions. Left ordinate: protease activity is expressed as percentage of control (DMSO-treated) cells after 6 h treatment with CA-074-OMe dilutions only; right ordinate: relative percentage of cell death induced by staurosporine + Z-VAD(OMe)-FMK treatment in the presence of CA-074-OMe dilutions (in the absence of CA-074-OMe, the two cell death types (apoptosis or necrosis) were considered as 100%, each; cell death was detected as described in (**a**)). Cathepsin B activity was determined using fluorescent substrates Z-RR-AMC and Z-FR-AMC according to Rozman-Pungercar *et al.*[13] Briefly, 2–5×10^5 cells – exposed to various treatments – were washed two times in PBS plus 5 mM glucose, resuspended in 100 μl cathepsin detection buffer (CDB: 100 mM phosphate buffer, pH 6.0; 0.1% PEG; 5 mM DTT; 0.1% Triton X-100), substrates were added at 20 μM final concentration in 100 μl CDB. Fluorescence was detected for 15 min by a Fluoroskan plate reader (Labsystems) using a 390 nm excitation and a 460 nm emission optical filter set. The increase of fluorescence was linear in this time scale and the indicated values are calculated as the slopes of these lines (ΔFluorescence/Δtime, nM AMC/min/10^5 cells)

or Sigma) was added at 40 μM concentration, the proportion of dead cells did not change in staurosporine-treated cells, but was substantially suppressed in the staurosporine + Z-VAD(OMe)-FMK-treated ones (Figure 1a and f). Furthermore, CA-074-OMe reversed the decline of both lysosomal acidification (Figure 1b) and mitochondrial membrane potential (Figure 1d), indicating that the target of CA-074-OMe is functionally located upstream of the lysosomal breakdown and the mitochondrial depolarization.

To check whether this relatively high concentration of CA-074-OMe is specific for cathepsin B or not, the required concentration for inhibition of RR-ase and FR-ase activity was correlated with that required for suppression of cell death using CA-074-OMe dilutions. Surprisingly, both dipeptidase activities were inhibited at almost three orders less concentration (IC_{50}(RR-ase) = 9 ± 3 nM; IC_{50}(FR-ase) = 37 ± 33 nM) than cell death (IC_{50} apoptosis = 5.2 ± 1.5 μM, IC_{50} necrosis = 15.6 ± 3.2 μM); (mean ± S.D., $n = 3$; $P < 0.004$ compared the inhibition of either protease activity to inhibition of either form of cell death) (Figure 1h). The concentration required for prevention of lysosomal deacidification and mitochondrial depolarization by CA-074-OMe matched with the concentration required for abrogation of other signs of cell death, such as DNA fragmentation or permeabilization of the cytoplasmic membrane, indicating close connection among these phenomena. These results strongly imply that CA-074-OMe (or CA-074 itself) inhibited cell death by binding to a target other than cathepsin B.

To compare with CA-074-OMe, we applied Z-FA-FMK (Z-Phe-Ala-fluoromethylketone, Bachem), an inhibitor reacting with several cathepsins including cathepsin B. When Z-FA-FMK was added at 1 μM concentration, the proportion of dead cells did not change significantly compared either to the staurosporine-treated cells (similarly to CA-074-OMe) or to the staurosporine + Z-VAD(OMe)-FMK-treated ones (oppositely to CA-074-OMe) (Figure 1g). However, detailed analysis of the form of cell death revealed that Z-FA-FMK significantly decreased the apoptotic form ($P < 0.003$) but enhanced the necrotic form ($P < 0.02$) in staurosporine + Z-VAD(OMe)-FMK-treated samples.

Furthermore, Z-FA-FMK did not reverse decline of either lysosomal acidification or mitochondrial membrane potential, rather enlarged the population of cells with prominent declines (Figure 1c and e). The Z-FA-FMK-induced phenomena did not relate to its possible toxic effect because (1) there was no concentration dependence between 0.3 and 30 μM of Z-FA-FMK and (2) co-treatment with CA-074-OMe did not abolish the protective effect of CA-074-OMe on staurosporine + Z-VAD(OMe)-FMK-treated cells.

To explore the role of cathepsin D in this cell death process, we applied pepstatin A. Pretreatment with pepstatin A (Sigma) for up to 4 h and up to 100 μM did not protect cells treated with either staurosporine or staurosporine + Z-VAD(OMe)-FMK (not shown).

In summary, the following four reasons strongly imply that *CA*-074-OMe acted as a pro-survival compound because of blocking a noncathepsin B target in HL-60 cells (and U937 cells – not shown) treated with staurosporine + Z-VAD(OMe)-FMK: (1) The concentration of CA-074-OMe required to inactivate cytoplasmic cathepsin B activity is three orders less than required for the survival of leukemia cells; (2) Z-VAD(OMe)-FMK alone is a strong cathepsin B inhibitor at the applied concentration; (3) Z-FA-FMK did not protect cells but only switched apoptosis to necrosis, which is not related to its toxicity; (4) Bogyo *et al.*[8] demonstrated that demethylation of a CA-074-OMe-like compound may be slower than its binding to aspecific cathepsin targets.

Our results do not exclude the possibility that inactivation of cathepsin B is a necessary effect of CA-074-OMe to inhibit cell death, but it is surely not a sufficient one. To address this question, experiments should be performed on cells expressing downregulated cathepsin B activity.

Both dipeptidase activity (RR-ase and FR-ase) were suppressed similarly by CA-074-OMe (Figure 1h). This suggests that cathepsin B was responsible for the great majority of FR-ase activity as well. CA-074 is specific to cathepsin B with around 5 orders of magnitude compared to cathepsin L or H, *ex vivo*,[6] while the methylated form possibly crossreacts with several cathepsins and maybe noncathepsins,[8] indicating that the methylated form can be responsible for abrogating cell death. This phenomenon closely resembles the case of Z-VAD(OMe)-FMK, when modification of a specific inhibitor by esterification resulted in aspecific binding – in this case – to cathepsins.[13,15]

Our results call for careful interpretation of the experimental data obtained with CA-074-OMe. Recently, the influence of treatment with 10–100 μM CA-074-OMe on cell death was interpreted as specific cathepsin B effect, without checking the relevant concentration required for inactivation of the protease.[5,16–18] In HL-60 cells, 1 μM CA-074-OMe fully inhibited the extractable cathepsin B activity – even after 1 h treatment – indicating that higher concentration may be irrelevant to cathepsin B. This case is similar to the instance of Z-VAD(OMe)-FMK when it was shown that DEVD-ase activity was suppressed at a much lower concentration than was usually used in earlier studies.[4]

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