Small molecules not direct activators of caspases

To the editor:

Apoptosis is executed by the effector caspases-3 and -7, which normally reside as latent precursors (zymogens) in most of the nucleated cells of the human body. The physiologic mechanism for their activation requires cleavage, and such cleavage is generated by a proteolytic attack by the apical caspase-8 or caspase-9, or by the caspase analog granzyme B. It therefore came as an exciting surprise when, in a previous issue of *Nature Chemical Biology*[1], Hergenrother and colleagues reported on a series of small molecules that could directly activate the zymogens of caspases-3 and -7, because such compounds could form the basis of new anti-neoplastic therapeutics. In the normal process, proteolysis of caspase zymogens results in a gain of activity of several orders of magnitude[2]. Hence, we were surprised that Putt and co-workers could only obtain a 3–4.5-fold relative increase in activity (as monitored by UV), especially since the authors observed >50% cleavage of procaspase-3 within 4 h by western blotting; this apparent discrepancy prompted us to examine the utility of PAC-1.

We obtained PAC-1, and several analogs that included both active (PAC-2; compound **2** in ref. 1) and inactive compounds (PAC-3; compound **3** in ref. 1), from ChemDiv and ChemBridge (the same vendor that supplied PAC-1 for the initial screen described in Putt *et al.*[1]). We fully characterized these compounds by LC-MS and NMR, confirming the structure and purity at >99%. In two independent laboratories, we conducted several types of assays, all of which represent well-established systems to measure and detect executioner caspase activation. (i) In assays similar to those described by Putt and co-workers, we tested each compound in both chromogenic (Ac-DEVD-pNA) and fluorogenic (Ac-DEVD-AMC) assays for their potency in activating purified recombinant procaspase-3 and procaspase-7 expressed in *Escherichia coli* (**Fig. 1a**). (ii) We treated procaspase-3 and -7 for 14 h with the panel of PAC molecules and analyzed the proteins for cleavage by gel electrophoresis (**Fig. 1b**). (iii) We used extracts from cell lines to determine whether the compounds could activate endogenous caspases in this frequently used model of cell-free apoptosis[3,4] (**Fig. 1c**). In all of these assays and others not described here, we did not detect any reproducible caspase activation or proteolytic processing over background levels. In contrast, the canonical activator granzyme B rapidly generated massive activation and complete processing of the caspase zymogens (**Fig. 1b**,**c**).

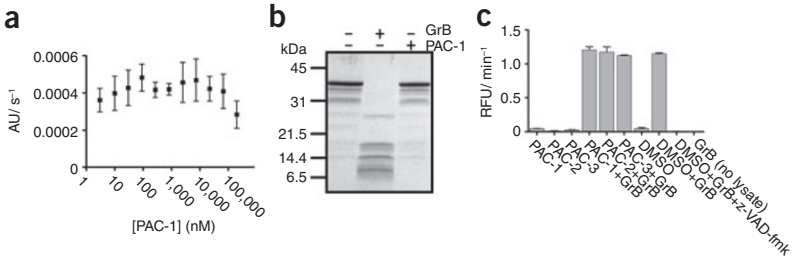

Figure 1 Lack of activation or processing of procaspase-3 by PACs. (**a**) Procaspase-3 was expressed in *E. coli* and purified as previously described[5]. The dose dependence of PAC-1 (ChemDiv) for procaspase-3 activation was determined by adding various concentrations of PAC-1 to procaspase-3 (100 ng ml^{-1}) in a 384-well plate in caspase activity buffer (consisting of 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1 mM EDTA, 0.1% CHAPS, 10% glycerol and 10 mM DTT) and incubating the mixtures at 37 °C for 12 h. Ac-DEVD-pNA in caspase activity buffer was added to the incubated samples and activity was monitored by reading absorbance at 405 nm (A_{405}) every 5 min for 6 h. The rates (absorbance units, AU s^{-1}) from triplicate runs were plotted against the concentration of PAC-1. (**b**) Procaspase-3 at 15 μg ml^{-1} was incubated with 50 μM PAC-1 or 12 nM granzyme B (GrB) as a positive control, in 20 mM HEPES, pH 7.2, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% sucrose for 14 h at 37 °C. Samples were separated by SDS-PAGE and stained with Coomassie blue. (**c**) Cell lysates from SK-MEL-5 cells were prepared and used as previously described[3,4]. The PAC compounds were incubated at 100 μM with 7.4 μg ml^{-1} cell lysate in the caspase buffer described in **a** for 5 h at 37 °C. Ac-DEVD-AMC substrate was added to a final concentration of 200 μM and activity was monitored by measuring fluorescence, in relative fluorescence units (RFU) per minute, every minute for 1 h at 380 nm excitation and 460 nm emission. GrB was used as a positive control for caspase zymogen activation. GrB was also co-incubated with compounds and lysate as a control to ensure that the compounds were not quenching the fluorescence readout. The caspase inhibitor Z-VAD-fmk was used as a control to demonstrate that the GrB activation observed was indeed due to caspase zymogen activation.

In addition, the authors suggested that MCF7 cells, which do not express procaspase-3, are resistant to PAC-1, supporting the hypothesis that PAC-1 exerts its activity by directly activating caspase-3 (ref. 1). We tested the effect of PAC-1 on MCF7 cells, but found that PAC-1 was able to kill these cells with an efficacy similar to that obtained with H226 and SK-MEL-5 cells (which contain substantial procaspase-3), arguing against a direct caspase-activating mechanism for the compound. In sum, we suggest that PAC-1 and related compounds do not activate executioner caspases by a direct mechanism, and we look forward to a further explanation of this compound's mode of action.

COMPETING INTERESTS STATEMENT
The authors declare no competing financial interests.

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