

A Convenient Fluorometric Method to Study Sulfur Mustard-Induced Apoptosis In Human Epidermal Keratinocytes Monolayer Microplate Culture

Radharaman Ray*, Stephanie Hauck, Rachel Kramer and Betty Benton

Biochemical Pharmacology Branch, Pharmacology Division
US Army Medical Research Institute of Chemical Defense,
Aberdeen Proving Ground, MD 21010-5400

Key Words: Fluorometric apoptosis assay; Sulfur mustard; Apoptosis; Human epidermal keratinocytes; Monolayer culture

Disclaimer: The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the US Army or the Department of Defense.

Report Documentation Page			Form Approved OMB No. 0704-0188		
Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.					
1. REPORT DATE 16 NOV 2004		2. REPORT TYPE N/A		3. DATES COVERED -	
4. TITLE AND SUBTITLE A Convenient Fluorometric Method to Study Sulfur Mustard-Induced Apoptosis In Human Epidermal Keratinocytes Monolayer Microplate Culture			5a. CONTRACT NUMBER		
			5b. GRANT NUMBER		
			5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S)			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Biochemical Pharmacology Branch, Pharmacology Division US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010-5400			8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)			10. SPONSOR/MONITOR'S ACRONYM(S)		
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release, distribution unlimited					
13. SUPPLEMENTARY NOTES See also ADM001849, 2004 Scientific Conference on Chemical and Biological Defense Research. Held in Hunt Valley, Maryland on 15-17 November 2004.					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 12	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			

ABSTRACT

Sulfur mustard (SM (bis-(2-chloroethyl) sulfide)), a vesicant, is a chemical warfare as well as a potential terrorism agent. SM-induced skin blistering is believed to be due to epidermal-dermal detachment as a result of epidermal basal cell death via apoptosis and/or necrosis. Regarding the role of apoptosis in SM pathology in animal skin, the results obtained in several laboratories, including ours, suggest the following: (a) cell death due to SM begins via apoptosis that proceeds to necrosis via an apoptotic-necrotic continuum and (b) inhibiting apoptosis decreases SM-induced microvesication *in vivo*. To study the mechanisms of SM-induced apoptosis and its prevention *in vitro*, we have established a convenient fluorometric apoptosis assay using monolayer human epidermal keratinocytes (HEK) adaptable for multi-well plates (24-, 96-, or 384-well) and high throughput applications. This assay allows replication and multiple types of experimental manipulation in sister cultures so that the apoptotic mechanisms and the effects of test compounds can be compared statistically. SM affects diverse cellular mechanisms, including endoplasmic reticulum (ER) Ca^{2+} homeostasis, mitochondrial functions, energy metabolism, and death receptors, each of which can independently trigger apoptosis. However, the biochemical pathway in any of these apoptotic mechanisms is characterized by a pathway specific sequence of caspases, among which caspase-3 is a key member. Therefore, we exposed 80-90% confluent HEK cultures to SM and monitored apoptosis by measuring the fluorescence generated due to hydrolysis of a fluorogenic caspase-3 substrate (acetyl- or benzyl oxycarbonyl-Asp-Glu-Val-Asp-fluorochrome, also designated as AC-or Z-DEVD- fluorochrome) added to the assay medium. Fluorescence was measured using a plate reader. We used two types of substrates, one (Sigma-Aldrich, CASP-3-F) required cell disruption and the other (Beckman-Coulter CellProbeTM HT Caspase-3/7 Whole Cell Assay Kit) was cell permeable. The latter substrate was useful in experiments such as determining the time-course of apoptosis immediately following SM exposure without disruption, e.g., due to cell processing. In SM-exposed HEK, fluorescence generated from the fluorogenic caspase-3 substrate hydrolysis increased in a time (0 – 24 hr) and concentration (0.05, 0.1, 0.15, 0.2, 0.3, 0.5 mM) dependent manner. SM caused maximum fluorescence at about 0.5 mM. However, at 2 mM SM, fluorescence decreased compared with 0.5 mM, which remains to be explained. Following 0.3 mM SM exposure, which is considered to be the *in vitro* equivalent of a vesicating dose *in vivo*, a small fluorescence increase was observed at 6 to 8 hr, which was markedly higher at 12 hr. At 24 hr, all SM concentrations increased fluorescence. Fluorescence increase due to SM was prevented 100% by a caspase-3 specific peptide inhibitor (AC-DEVD-CHO), but less effectively by a general caspase inhibitor (Z-VAD-FMK), indicating that the fluorescence increase was due to caspase-3-mediated apoptosis. These results suggest potential applications of this method to study apoptosis mechanisms and possibly those involving other caspase substrates.

INTRODUCTION

The term apoptosis is used to describe cell death via modulation of a defined set of biochemical parameters in a cascade of events leading to apoptotic morphological expressions. The major morphological features are cellular shrinkage, nuclear and cytoplasmic condensation, and cellular disintegration followed by prolific budding to produce apoptotic bodies that are characterized by membrane-bound chromatin fragments.^[1,2] Apoptosis contributes to the mechanisms of removal of unwanted cells in normal physiological processes, e.g., during organ development, sloughing off of the endometrium in early menstruation period, immune response, etc. Apoptosis also is the mechanism of cell death responsible for pathological conditions such as cancer, Alzheimer's disease, ischemic cardiac damage, oxidative stress, autoimmune syndromes,^[3] or chemical toxicity, e.g., due to the highly reactive alkylating compound sulfur mustard (SM).^[4] SM reacts with multiple targets in the cell and, therefore, causes toxicity via different mechanisms such as DNA damage, disturbance in intracellular calcium homeostasis, abnormal cellular energy metabolism to include oxidative stress, and up-regulation of death receptors (Fas) and Fas-ligand.^[5] Any of these mechanisms may independently contribute to apoptosis by initially activating a pathway that is characteristic of the mechanism involved, but later converging to a common pathway culminating into the final apoptotic manifestations. There are reports showing that in cultured human epidermal keratinocytes (HEK), SM induces apoptosis via at least two separate mechanisms: (a) an intrinsic mechanism involving mitochondria, Ca^{2+} -calmodulin (Ca^{2+} -CaM), and caspase-3/poly (ADP-ribose) polymerase (PARP), and (b) an extrinsic mechanism involving Fas (death) receptors and Fas ligand (FasL).^[4,6] These reports also show that inhibiting the Ca^{2+} -CaM or the Fas response attenuates the biochemical markers of apoptosis and pathology due to SM. However, it is possible that other mechanisms, e.g., involving (a) endoplasmic reticulum (ER) Ca^{2+} and (b) abnormal energy metabolism, may also contribute to SM-induced apoptosis. Our goal is to understand thoroughly the mechanisms of apoptosis due to SM via each of the pathways discussed above, and to utilize the knowledge in developing medical countermeasures against SM toxicity and pathology.

In apoptosis, a consistent feature is the induction of a series of cytosolic proteases, which specifically cleave their substrates and lead to apoptotic morphology.^[7-10] These proteases are designated as caspases because they are cysteine proteases and cleave their substrates after aspartic acid residues (cysteine aspartase). Caspases remain dormant in the cell, but when activated by an apoptotic stimulus, they act by proteolytic processing, thus modulating the function of their substrates. The substrate can be the activated caspase itself (autocatalysis) or another caspase. This way, caspases function not only as proteases, but also as specific signaling molecules in apoptosis. Different types of caspases initiate different apoptotic pathways. However, caspase-3, also called apopain, or CPP32, has been shown to be a key mediator of the final biochemical and morphological steps in apoptosis.^[8] In apoptosis, the roles of different caspases have been shown to be pathway-specific as well as sequential in the steps involved in the pathway.^[5] Therefore, it seems possible to understand the mechanisms of apoptosis, e.g., due to SM, by studying the caspases involved. At present, this approach is facilitated by the availability of (a) some fluorogenic peptide substrates, which are specific for different caspases, and (b) some peptide caspase inhibitors, specific for different caspases. It should be noted that besides being useful to study apoptotic mechanisms, these inhibitors might also serve as prospective anti-apoptotic medical countermeasures. In this report, we demonstrate the

usefulness of a convenient fluorometric caspase-3 assay to study SM-induced apoptosis in monolayer human epidermal keratinocytes (HEK) grown in multi-well plates.

MATERIALS AND METHODS

Chemicals and Cells

SM (> 98% pure) was obtained from the US Army Research, Development and Engineering Command, Aberdeen Proving Ground, MD, USA. Frozen stock normal human epidermal keratinocyte (HEK) culture and keratinocyte growth medium (KGM) were from CAMBREX, Walkersville, MD. The caspase-3 assay kits containing fluorogenic caspase-3 substrates were obtained from two separate sources. One substrate required cell lysis and was purchased from Sigma-Aldrich, Saint Louis, MO (Product code: CASP-3-F). The other cell permeable substrate did not require cell lysis and was procured from Beckman-Coulter, Fullerton, CA (Product code: Beckman-Coulter CellProbe™ HT Caspase-3/7 Whole Cell Assay Kit). All other chemicals were of the purest grade available.

Cell Culture

Frozen stock HEK (passage 2) were cultured in 150 cm² tissue culture flasks in KGM according to company instructions to initiate the culture. When these monolayer cells became 70-80% confluent they were sub-cultured to 80-90% confluency in multi-well plates (96- or 24-well) and used in the experiments. To maintain consistency, HEK from a single donor and sub-cultured only to passage 3 were used.

Exposure of HEK to SM

Frozen stock^[11] of SM in KGM was thawed and vortexed to obtain a 4 mM solution. Exposure of HEK to SM was done as described previously.^[12] The stock 4 mM SM solution was diluted in multi-well plates containing monolayer HEK in KGM for specified concentrations and kept for times as indicated.

Caspase-3 Assay

The caspase-3 fluorometric assays were done according to the instructions provided with the assay kits, but with necessary modifications required for developing the method described here. This enzyme assay was based on the hydrolysis of the caspase-3 peptide substrate (acetyl-Asp-Glu-Val-Asp or AC-DEVD) conjugated to a fluorochrome at the C-terminal Asp, resulting in the release of the fluorescent moiety. The fluorescence (absolute units) was measured using the CytoFluor^R Multi-Well Plate Reader Series 4000 spectrofluorometer from PerSeptive Biosystems, Framingham, MA.

Caspases are present in the cytosol. In the assay kit from Sigma-Aldrich, the substrate AC-DEVD-AMC (7-amido-4-methylcoumarin) is not cell permeable and, therefore, the use of this substrate requires cell lysis. Cell lysis was accomplished by sonication of monolayer cultures in 1x lysis buffer added to the wells of the culture plate as described by Gross *et al.*^[13] We used the Misonix Sonicator^R 3000 purchased from Misononix, Farmingdale, NY. The sonicator was equipped with a microplate horn housed inside an acoustic enclosure. During sonication, the microplate horn was chilled by circulating water at 0-2⁰ C using a temperature controlled water circulator from Thermo Neslab, Portsmouth, NH. To avoid any contamination of cultures by cooling water, the microplate containing monolayer cultures in lysis buffer was tightly sealed using an adhesive plastic sheet. Sonication of cells was done for 30-second intervals at the highest power setting on the sonicator dial followed by a 60-second cooling period; the sonication/cooling cycles were repeated four times. At the end, cell disruption was confirmed by light microscopic observation.

The substrate in the Beckman-Coulter proprietary caspase-3/7 assay kit is cell permeable and, therefore, no cell lysis was required. This assay is based on the cleavage of the non-fluorescent bisamide substrate Z-DEVD-R110 (bis-(N-CBZ-L-aspartyl-L-glutamyl-L-Valyl-L-aspartic acid amide derivative of rhodamine 110).

It should be noted that the use of the Sigma-Aldrich substrate would require a cell processing (lysis) time at the end of each time interval following cell exposure to an apoptosis-inducing agent such as SM. On the contrary, the Beckman-Coulter substrate could be added along with the apoptosis inducer, and the time-course of caspase-3/7 activation could be monitored beginning immediately after SM addition and continuing without interruption.

All assays were done using monolayer cells grown in 96-well plates. Exposure of cells to different dilute SM concentrations was done according to approved procedures, which included exposing cells to SM at room temperature inside a total exhaust chemical/biological hood and then keeping the exposed cells inside this hood for at least 1 hr for venting of volatile gases.

In assays using the Beckman-Coulter substrate, cells were exposed to SM in a total volume of 100 μ L, which also contained 20 μ L substrate solution added just prior to SM. Cells were incubated for specified times and fluorescence was read at excitation and emission wavelengths of 485 nm and 530 nm, respectively.

In assays using the Sigma-Aldrich substrate, cells were exposed to desired SM concentrations, with appropriate controls, in fresh KGM in a total volume of 80 μ L. SM-exposed cells were then incubated inside a cell culture incubator overnight for 18-24 hr. Following this incubation, 20 μ L of 5x lysis buffer was added to each well, and the cells were disrupted by sonication as described above. The substrate was then added to a total volume of 150 μ L, and fluorescence was read at increasing times at excitation and emission wavelengths of 360 nm and 460 nm, respectively.

Inhibitor Study

The effects of caspase inhibitors on SM-induced caspase-3 activation were studied by using the Beckman-Coulter substrate to avoid any possible effect of cell sonication on caspase-3 activity. Cells were first incubated with the inhibitors in KGM at 37°C inside a cell culture incubator for 30 min prior to SM addition. These SM-exposed cells were further incubated inside a cell culture incubator overnight, the caspase-3 substrate was added and fluorescence was monitored at specified times after substrate addition.

Data analysis.

The results of assays performed in replicates of eight were averaged and expressed as the mean $\pm$ standard error of the mean (SEM). For each assay type, the differences between treatment groups at each time point were determined using a one way Analysis of Variance (ANOVA) followed by post hoc comparison of treatment group means using the Tukey's test. In addition, for each assay type, the differences between observation time points within each treatment group were compared using a mixed model ANOVA with time as a fixed factor and wells as a random factor followed by post hoc comparison of observation time means using the Tukey's test. Statistical significance was defined as $p < 0.05$.

RESULTS AND DISCUSSION

Experiments were designed to study the caspase-3 activation (apoptosis) due to SM in monolayer HEK cultures grown in multi-well plates, using the fluorogenic caspase-3 substrates AC-DEVD-AMC or Z-DEVD-R110 described above. The time-course of enzyme activation, the effects of caspase inhibitors, and the SM concentration dependence are shown in *figures 1, 2 and 3*, respectively.

The data shown in *figure 1* were generated in an experiment in which replicate HEK cultures were exposed to increasing concentrations of SM (0.05 to 0.3 mM) in KGM and the caspase-3 substrate Z-DEVD-R110 (Beckman-Coulter) had been added to the cells just prior to SM. Fluorescence increase following SM exposure was monitored at specified times throughout the day until 12 hours and at 24 hours next morning. The results are shown as percent fluorescence relative to SM-unexposed controls. In SM-exposed HEK, fluorescence generated from the fluorogenic caspase-3 substrate hydrolysis increased in a time (0 - 24 hr) and concentration (0.05, 0.1, 0.15, 0.2, 0.3, mM) dependent manner. Following 0.3 mM SM exposure, which is considered to be the *in vitro* equivalent of a vesicating dose *in vivo*, a very small but statistically significant increase in fluorescence was observed at 6 to 8 hr, which was markedly higher at 12 hr. At 24 hr, all SM concentrations increased fluorescence. These results are in agreement with those reported by Rosenthal *et al.* on the concentration and time dependence of SM-induced caspase-3 processing (conversion of inactive 32 KDa procaspase-3 to active 17 KDa caspase-3) and activation as well as other apoptosis indicators in HEK studied by the Western blotting method.^[4] The time-course and concentration dependence of SM-induced caspase-3 activation

in HEK observed by our fluorometric method, therefore, appear to be valid indicators of apoptosis. These results also show that in 0.3 mM SM-exposed HEK, an overnight incubation is needed using this method to see a high level of caspase-3 activity.

The effects of some peptide caspase inhibitors on 0.3 mM SM-induced hydrolysis of the caspase-3 substrate AC-DEVD-R110 (Beckman-Coulter) in HEK are shown in *Fig. 2*. In these experiments, as described in Methods, the cells in KGM were first incubated inside a cell culture incubator without or with the caspase-3 specific inhibitor AC-DEVD-CHO (acetyl-Asp-Glu-Val-Asp-aldehyde, 0.1 mM) or the general caspase inhibitor Z-VAD-FMK (benzyl oxycarbonyl-Val-Ala-Asp-fluoromethylketone, 0.01 mM) for 30 minutes prior to 0.3 mM SM exposure. The cells were then incubated overnight and caspase-3 assays were conducted. The results (*Fig. 2*) are shown as absolute fluorescence units $\pm$ SEM. SM-unexposed control cells incubated in KGM overnight had some caspase-3 activity indicating spontaneous apoptosis in HEK cultures without stimulation. Fluorescence increase due to 0.3 mM SM was prevented 100% by the caspase-3 specific peptide inhibitor (AC-DEVD-CHO), but less effectively by the general caspase inhibitor (Z-VAD-FMK) indicating that the fluorescence increase was due to caspase-3-mediated apoptosis.

We also examined the SM concentration dependence of caspase-3 activation at concentrations higher than 0.3 mM using 0.5, 1 and 2 mM. In these experiments, cells were exposed to different SM concentrations in KGM according to approved procedures and then incubated overnight inside a cell culture incubator for 18 hours. The cells were lysed in lysis buffer by sonication as described under Methods, and then the caspase-3 substrate AC-DEVD-AMC (Sigma-Aldrich) was added to cultures to monitor the kinetics of substrate hydrolysis between 0 to 8 hours. The results (*Fig. 3*) presented as percent fluorescence relative to SM-unexposed controls showed that when caspase-3 was almost fully activated during overnight incubation of HEK cultures following exposure to specified SM concentrations, there was a time-dependent increase in substrate hydrolysis that reached a plateau at around 4 hours. Fluorogenic substrate hydrolysis due to caspase-3 activity increased progressively, being maximum at 0.5 mM. The extent and the kinetics of substrate hydrolysis at 1 mM SM were almost identical with those at 0.5 mM SM, and in some experiments, 1 mM SM produced values slightly lower than those at 0.5 mM SM as shown in *Fig. 3*. However, at 2 mM SM, fluorescence decreased compared with 0.5 mM, which remains to be explained. One possible reason could be that at a SM concentration as high as 2 mM, the cells were metabolically compromised.

The results presented in this report support the following conclusions. The fluorometric method described here to study chemically induced apoptosis using fluorogenic caspase-3 substrates and monolayer cell cultures in multi-well plates is convenient, adaptable for high throughput applications, if needed, and suitable for evaluations of test compounds as inhibitors of apoptosis. This assay clearly demonstrates apoptosis, as indicated by caspase-3 activation, in cultured HEK due to SM in a SM concentration- and time-dependent manner. Since apoptosis is believed to be an important process in many cases of chemical toxicity, e.g., due to SM, and a number of diseases including cancer, this method provides a valuable means to conduct research on the role of apoptosis in respective situations and drug development. These results also suggest potential applications of this method to study the roles in apoptosis of caspases other

than caspase-3 provided (a) suitable fluorogenic substrates are available and (b) the subject caspases are stimulated enough to generate measurable fluorescent signals.

REFERENCE

1. Kerr, J. F. R.; Wyllie, A. H.; Currie, A. R. Apoptosis: A Basic Biological Phenomenon with Wide-Ranging Implications in Tissue Kinetics. *Br. J. Cancer* **1972**, 26: 239-257.
2. Trump, B. F.; Berezsky, I. K. The Reactions of Cells to Lethal Injury: Oncosis and Necrosis – The Role of Calcium. In *When Cells Die*; Lockshin, R. A.; Zakeri, Z.; Tilly, J. L. Eds.; Wiley-Liss: New York, **1998**; 57-96.
3. Hollinger, M. A. Series Foreword. In *Apoptosis Methods in Pharmacology and Toxicology – Approaches to Measurement and Quantification*; Davis, M. A. Ed.; Humana Press: Totowa, New Jersey, **2002**; 5.
4. Rosenthal, D. S.; Rosenthal, C. M. G.; Iyer, S.; Smith, W.; Ray, R.; and Smulson, M. E. Sulfur Mustard Induces Terminal Differentiation and Apoptosis in Keratinocytes Via a Ca²⁺-Calmodulin and Apopain-Dependent Pathway. *J. Invest. Dermatol.* **1998**, 111(1): 64-71.
5. Ray, R.; Bhat, K. R.; Benton, B. J.; Burke, M. E.; Dierking, E.; Roman, J. M.; Anderson, D. R.; Petrali, J. P.; Smith, W. J.; Rosenthal, D. S.; Ray, P. In *Mechanism-Based Intervention of Vesicant Toxicity, Proceedings of the 3rd Singapore International Symposium on Protection Against Toxic Substances (SISPAT)*, Singapore, December 2-6, 2002; DSO National Laboratories, 2002.
6. Rosenthal, D. S.; Valena, A.; Choi, F-P.; Schlege, I. R.; Ray, R.; Benton, B.; Anderson, D.; Smith, W. J.; Simbulan-Rosenthal, C. M. Expression of Dominant-Negative Fas-Associated Death Domain Blocks Human Keratinocyte Apoptosis and Vesication Induced by Sulfur Mustard. *J. Biol. Chem.* **2003**, 278(10): 8531-8540.
7. Keppler-Hafkemeyer, A.; Brinkman, U.; Pastan, I. Role of Caspases in Immunotoxin-Induced Apoptosis of Cancer Cells. *Biochemistry* **1998**, 37(48): 16934-16942.
8. Kumar, S. Regulation of Caspase Activation in Apoptosis: Implications in Pathogenesis and Treatment of Disease. *Clin. Exp. Pharmacol. Physiol.* **1999**, 26(4): 295-303.
9. Porter, A. G.; Janicke, R. U. Emerging Role of Caspase-3 in Apoptosis. *Cell Death Differ.* **1999**, 6(2): 99-104.
10. Stennicke, H. R.; Salvesen, G. S. Biochemical Characteristics of Caspases-3, -6, -7, and -8. *J. Biol. Chem.* **1997**, 272(41): 25719-25723.
11. Broomfield, C. A.; Gross, C. L. Stability of Sulfur Mustard in a Two-Phase Storage Configuration. *Proc. 1989 Medical Defense Bioscience Review*, AD B139550, United

States Army Medical Research Institute of Chemical Defense: Aberdeen Proving Ground, MD **1989**, 433-436.

12. Ray, R.; Legere, R. H.; Majerus, B. J.; Petrali, J. P. Sulfur Mustard-Induced Increase in Intracellular Free Calcium Level and Arachidonic Acid Release From Cell Membrane. *Toxicol. Appl. Pharmacol.* **1995**, *131*: 44-52.
13. Gross, C. L.; Clark, O. E.; Nealley, E. W.; Nipwoda, M. T.; Smith, W. J. Lysis of Adherent Human Epidermal Keratinocytes In Situ by a Misonix Tissue Culture Plate Sonicator. *The Toxicologist* **2004**, *78(1-S)*: 265.

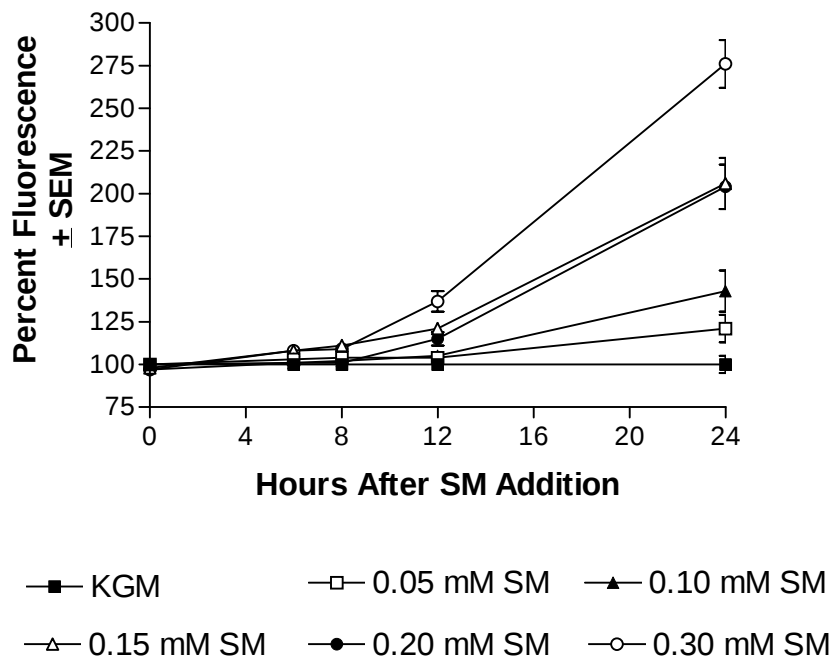


Figure1. Time-course of caspase-3 activation in HEK following exposure to increasing SM concentrations. Monolayer HEK cultures in a 96-well plate were exposed to different SM concentrations with the cell permeable caspase-3 substrate Z-DEVD-R110 added just prior to SM. SM exposures were done inside a total exhaust chemical/biological hood at room temperature according to approved procedures. After one hour inside the hood, the cells were transferred to a 37°C cell culture incubator. Fluorescence generated due to substrate hydrolysis was measured at specified times shown in the figure using a plate reader/spectrofluorometer as described under Methods. Percent fluorescence values in experimental samples compared with SM-unexposed controls as 100% are mean $\pm$ SEM of eight replicate determinations at each SM concentration. The absence of SEM bars means that the values are too small to be visible. Statistical significance was defined as $p < 0.05$ vs. SM-unexposed control group as described under Methods.

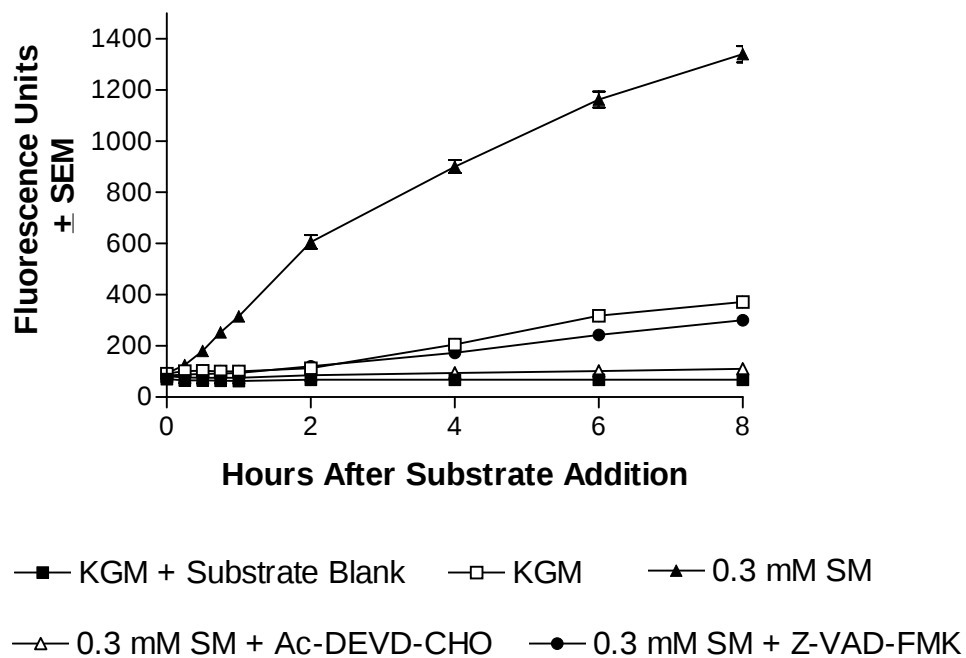


Figure 2. Effects of peptide caspase-3 inhibitors on 0.3 mM SM-induced caspase-3 activation. HEK cultures in KGM were first incubated inside a cell culture incubator without or with the caspase-3 specific inhibitor AC-DEVD-CHO (0.1 mM) or the general caspase inhibitor Z-VAD-FMK (0.01 mM) for 30 min prior to 0.3 mM SM exposure inside a total exhaust chemical/biological hood at room temperature according to approved procedures. SM-exposed cells were then incubated overnight, and caspase-3 assays were conducted by adding the cell permeable caspase-3 substrate Z-DEVD-R110 to cultures as shown. Fluorescence was measured at specified times after substrate addition. The results shown as absolute fluorescence units are mean $\pm$ SEM of eight replicate determinations. The absence of SEM bars means that the values are too small to be visible. Statistical significance was defined as $p < 0.05$ vs. corresponding control values obtained in experiments without substrate.

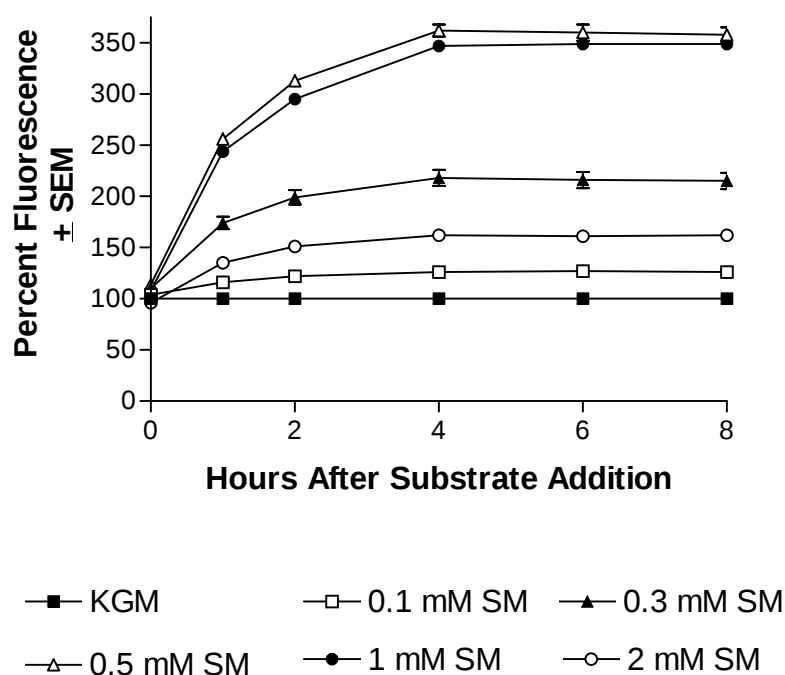


Figure 3. SM concentration dependence of caspase-3 activation in HEK. Monolayer HEK cultures in a 96-well plate were exposed to different SM concentrations inside a total exhaust chemical/biological hood at room temperature according to approved procedures as described in Methods. SM-exposed cells were incubated inside a 37°C cell culture incubator overnight for 18 hours. Following this incubation overnight, the cells were lysed in lysis buffer by sonication and the caspase-3 substrate AC-DEVD-AMC was added to monitor the kinetics of substrate hydrolysis as measured by increase in fluorescence with time. The cells were placed inside the 37°C cell culture incubator in between readings. Percent fluorescence increase with time compared with SM-unexposed controls as 100% are shown as mean $\pm$ SEM of eight replicate determinations at each SM concentration. The absence of SEM bars means that the values are too small to be visible. Statistical significance was defined as $p < 0.05$ vs. SM-unexposed control group as described under Methods.