

# Sulfur Mustard Induces Markers of Terminal Differentiation and Apoptosis in Keratinocytes Via a $\text{Ca}^{2+}$ -Calmodulin and Caspase-Dependent Pathway

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**Sulfur mustard (SM) induces vesication via poorly understood pathways. The blisters that are formed result primarily from the detachment of the epidermis from the dermis at the level of the basement membrane. In addition, there is toxicity to the basal cells, although no careful study has been performed to determine the precise mode of cell death biochemically. We describe here two potential mechanisms by which SM causes basal cell death and detachment: namely, induction of terminal differentiation and apoptosis. In the presence of 100  $\mu\text{M}$  SM, terminal differentiation was rapidly induced in primary human keratinocytes that included the expression of the differentiation-specific markers K1 and K10 and the cross-linking of the cornified envelope precursor protein involucrin. The expression of the attachment protein, fibronectin, was also reduced in a time- and dose-dependent fashion. Features common to both differentiation and apoptosis were also induced in 100  $\mu\text{M}$  SM, including the**

**rapid induction of p53 and the reduction of Bcl-2. At higher concentrations of SM (i.e., 300  $\mu\text{M}$ ), formation of the characteristic nucleosome-sized DNA ladders, TUNEL-positive staining of cells, activation of the cysteine protease caspase-3/apopain, and cleavage of the death substrate poly(ADP-ribose) polymerase, were observed both *in vivo* and *in vitro*. Both the differentiation and the apoptotic processes appeared to be calmodulin dependent, because the calmodulin inhibitor W-7 blocked the expression of the differentiation-specific markers, as well as the apoptotic response, in a concentration-dependent fashion. In addition, the intracellular  $\text{Ca}^{2+}$  chelator, BAPTA-AM, blocked the differentiation response and attenuated the apoptotic response. These results suggest a strategy for designing inhibitors of SM vesication via the  $\text{Ca}^{2+}$ -calmodulin or caspase-3/PARP pathway. *Key words:* BAPTA/caspase-3/poly(ADP-ribose) polymerase/W-7. *J Invest Dermatol* 111:64–71, 1998**

**S**ulfur mustard [bis-(2-chloroethyl) sulfide; SM] causes blisters in the skin via poorly understood mechanisms. Because SM is a strong alkylating agent, its ability to induce DNA damage via apurinic sites and endonucleolytic activation has been advanced as one possible pathway leading to vesication (Papirmeister *et al*, 1985). Similar to other agents that induce DNA strand breakage, SM activates the nuclear protein poly(ADP-ribose) polymerase (PARP), which drastically depletes levels of cellular nicotinamide adenine dinucleotide and adenosinetriphosphate (Wielckens *et al*, 1982; Alvarez *et al*, 1986), a mechanism proposed to induce cell death (Berger *et al*, 1983). We have recently examined this mechanism using a human skin graft derived from human keratinocytes stably transfected with a PARP anti-sense inducible vector (Rosenthal *et al*, 1995). Recent studies have further implicated PARP as an important player in apoptosis. Proteolytic cleavage of PARP was first demonstrated in

chemotherapy-induced apoptosis (Kaufmann *et al*, 1993) and the specific proteolysis of PARP is now closely associated with apoptosis in different systems (Neamati *et al*, 1995; Nicholson *et al*, 1995; Tewari *et al*, 1995). We recently showed that the reversible stage of apoptosis is characterized by the transient activation of PARP, and poly(ADP-ribosylation) of nuclear proteins followed by the breakdown of poly(ADP-ribose) and PARP (Rosenthal *et al*, 1997b).

$\text{Ca}^{2+}$  also plays an important role in apoptosis, as well as in the maintenance and homeostasis of the skin, and SM has been shown to elevate intracellular levels of  $\text{Ca}^{2+}$  in keratinocytes (Ray *et al*, 1995; Mol and Smith, 1996). Several laboratories, including our own, have shown that terminal differentiation can be induced in both murine and human keratinocytes by the elevation of extracellular  $\text{Ca}^{2+}$  (Hennings *et al*, 1980; Stanley and Yuspa, 1983; Rosenthal *et al*, 1991b). This in turn results in an increase in intracellular free  $\text{Ca}^{2+}$  ( $\text{Ca}_i$ ).  $\text{Ca}_i$  appears to be an important signal for terminal differentiation, because agents that chelate and buffer  $\text{Ca}_i$  can block markers of terminal differentiation (Li *et al*, 1995).  $\text{Ca}^{2+}$  has also been shown to play a role in apoptosis in a number of systems. Studies by Kaiser and Edelman (1977) showed the first evidence that  $\text{Ca}_i$  may trigger apoptosis in glucocorticoid-stimulated thymocytes. Since then, several other studies have confirmed this role for  $\text{Ca}^{2+}$ . The observed  $\text{Ca}_i$  increase during apoptosis appears to occur by two different mechanisms. The first mechanism involves the activation of protein tyrosine kinases, leading to the activation of phospholipase C, the formation of IP3, and  $\text{Ca}^{2+}$

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Abbreviations:  $\text{Ca}_i$ , intracellular free calcium; NHEK, normal human epidermal keratinocytes; PARP, poly(ADP-ribose) polymerase, SM, sulfur mustard.

mobilization (Takata *et al.*, 1995; Silvennoinen *et al.*, 1996). The second pathway involves oxidative stress, which can occur in response to cytotoxic agents, such as SM, that generate reactive oxygen species. Oxygen radicals can damage  $\text{Ca}^{2+}$  transport systems localized in the endoplasmic reticulum (ER), mitochondria, and plasma membrane, leading to a disruption in  $\text{Ca}^{2+}$  homeostasis and a sustained increase in  $\text{Ca}_i$  (Orrenius *et al.*, 1989).

$\text{Ca}^{2+}$ -buffering experiments have supported the role of  $\text{Ca}^{2+}$  in the etiology of SM-induced cytotoxicity (Ray *et al.*, 1996). Experiments utilizing specific inhibitors of calmodulin have also demonstrated the importance of  $\text{Ca}^{2+}$ -calmodulin complexes in programmed cell death. Cyclosporine A-sensitivity of apoptosis in certain systems also suggests a role for  $\text{Ca}^{2+}$ -calmodulin complexes in programmed cell death. Cyclosporine binds to a family of cytosolic receptors (cyclophilins); the complex then binds to and suppresses the serine/threonine phosphatase calcineurin, which in turn is regulated by  $\text{Ca}^{2+}$ -calmodulin complexes in programmed cell death (Shi *et al.*, 1989).  $\text{Ca}^{2+}$  also plays a role in the induction of the endonuclease responsible for the internucleosomal DNA cleavage, yielding the characteristic apoptotic DNA ladders (Shiokawa *et al.*, 1994).

Numerous recent studies have suggested that the ultimate targets for many of these signaling pathways that lead to apoptosis are a family of cysteine proteases, known as "caspases," named for their preference for aspartate at their substrate cleavage site (Alnemri *et al.*, 1996). In collaboration with others, our laboratory has been particularly active in studying the characterization of caspase-3 (also known as "apopain"), which appears to be a converging point for different apoptotic pathways (Nicholson *et al.*, 1995). In several apoptotic systems, caspase-3 cleaves key proteins involved in the structure and integrity of the cell, including PARP (Nicholson *et al.*, 1995; Tewari *et al.*, 1995; Casciola-Rosen *et al.*, 1996; Song *et al.*, 1996).

In this study, we show that SM induces both terminal differentiation and apoptosis in human keratinocytes. Further, we demonstrate that these processes are  $\text{Ca}^{2+}$  and/or calmodulin dependent, and involve the activation of caspase-3 as well as the activation and specific cleavage of PARP. These responses may, in part, explain the death and detachment of basal cells of the epidermis that occurs following exposure to SM.

## MATERIALS AND METHODS

**Cells** Normal human epidermal keratinocytes (NHEK) were obtained as primary cultures from Clonetics (San Diego, CA) and maintained in serum-free keratinocyte growth medium. NHEK were grown in 75  $\text{cm}^2$  tissue culture flasks to 60–80% confluency, then exposed to SM diluted in keratinocyte growth medium to final concentrations of 100  $\mu\text{M}$  or 300  $\mu\text{M}$ . Media was not changed for the duration of the experiments. Cell viability was measured by the ability of cells to exclude trypan blue. For all studies, similar results were obtained from three independent experiments utilizing human primary adult keratinocytes and from three independent experiments utilizing human primary neonatal keratinocytes.

**Chemicals** SM (>98% purity) was obtained from the US Army Edgewood Research, Development and Engineering Center. Glycine, N,N'-[1,2-ethane-diylbis(oxy-2,1-phenylene)]bis[N-[2-(acetyloxy)methoxy]-2-oxoethyl]-bis(acetyloxy)methyl ester (BAPTA-AM) was purchased from Molecular Probes (Eugene, OR), as was Pluronic F-127, used to facilitate loading of BAPTA-AM into cells. N-(6-Aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W-7) was obtained from Sigma (St. Louis, MO). The tetrapeptide aldehyde inhibitor of caspase-3 (AcDEVD-CHO) was obtained from Biomol (Plymouth Meeting, PA).

## Antibodies

**Immunoblotting** The following antibodies were obtained from Sigma: (i) mouse monoclonal antibody (clone 8.60) that recognizes both K1 and K10 keratins; (ii) monoclonal antibody (clone SY5) against the 68 kDa cornified envelope precursor, involucrin; (iii) a mixture of three monoclonal antibodies (clones 2D1, 1F11, and 6D4), recognizing a 17 kDa band corresponding to calmodulin; and (iv) affinity purified rabbit anti-serum against the attachment protein, fibronectin, recognizing both a 220 kDa and a 94 kDa form of the protein. Rabbit affinity-purified anti-peptide anti-sera specific for human K1 (AF87) was from Babco (Richmond, CA). Mouse monoclonal antibody (clone DO-1) recognizing human p53 is from Calbiochem (Cambridge, MA). Monoclonal

anti-human Bcl-2 antibody (clone 4D7) is from Biomol. Rabbit anti-peptide anti-sera specific for the N-terminal propeptide sequence of pro-caspase-3/apopain (CPP32) was obtained from Transduction Labs (Lexington, KY). Rabbit anti-sera against the p17 subunit of CPP32 was obtained from Donald Nicholson (Merck Frosst Center for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada). Rabbit anti-sera recognizing both full-length PARP, as well as the 89 kDa apoptotic cleavage product of PARP, was a kind gift from Eric Ackerman (Pacific NW National Laboratories, Richland, WA). Guinea pig anti-sera specific for poly(ADP-ribose) has been described (Rosenthal *et al.*, 1995).

**Fluorescence-activated cell sorter analysis** FITC-conjugated anti-human cytokeratin ("CK") antibody (MNF116), recognizing keratins 10, 17, and 18, was from DAKO (Carpinteria, CA).

**Immunoblot analysis** For immunoblot analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis-separated proteins were transferred to nitrocellulose filters. Proteins were measured (DCA protein assay; BioRad), and normalized prior to gel-loading, and all filters were stained with Ponceau-S, in order to reduce the possibility of loading artifacts. The details for using rabbit anti-serum to human PARP (Ding *et al.*, 1992) and guinea pig anti-serum to poly(ADP-ribose) (Rosenthal *et al.*, 1995) for immunoblot analysis have been described previously in detail. Immune complexes were visualized by electrochemiluminescence (Amersham Life Sciences, Arlington Heights, IL).

**Fluorescence-activated cell sorter analysis** At the designated time points, monolayer culture medium was decanted, trypsin-ethylenediamine tetraacetic acid was added for 5 min, and the cells were removed from the flasks by scraping. The cell suspension was mixed with trypsin neutralizing solution, washed in keratinocyte growth medium, and fixed with 1% formaldehyde for 15 min followed by 70% ethanol. Fixed cells were stored at  $-200^\circ\text{C}$  until stained for cytometry. Flow cytometric analyses were conducted on a Becton-Dickinson (Franklin Lakes, NJ) FACStar Plus cytometer using a 100 mW air-cooled argon laser at 488 nm.

**PARP cleavage assay** The full-length cDNA clone for PARP (pcD-12) (Alkhatib *et al.*, 1987) was excised and ligated into the XhoI site of pBluescript-II SK+ (Stratagene, La Jolla, CA), and then used to drive the synthesis of PARP labeled with [ $^{35}\text{S}$ ]methionine (Dupont-NEN, Wilmington, DE) by coupled T7 transcription/translation in a reticulocyte lysate system (Promega, Madison, WI). [ $^{35}\text{S}$ ]PARP was separated from the other constituents by gel filtration chromatography on a Superdex-75 FPLC column (Pharmacia, Piscataway, NJ;  $1 \times 30$  cm) in 10 mM HEPES-KOH (pH 7.4), 2 mM ethylenediamine tetraacetic acid, 0.1% (wt/vol) CHAPS, and 5 mM dithiothreitol.

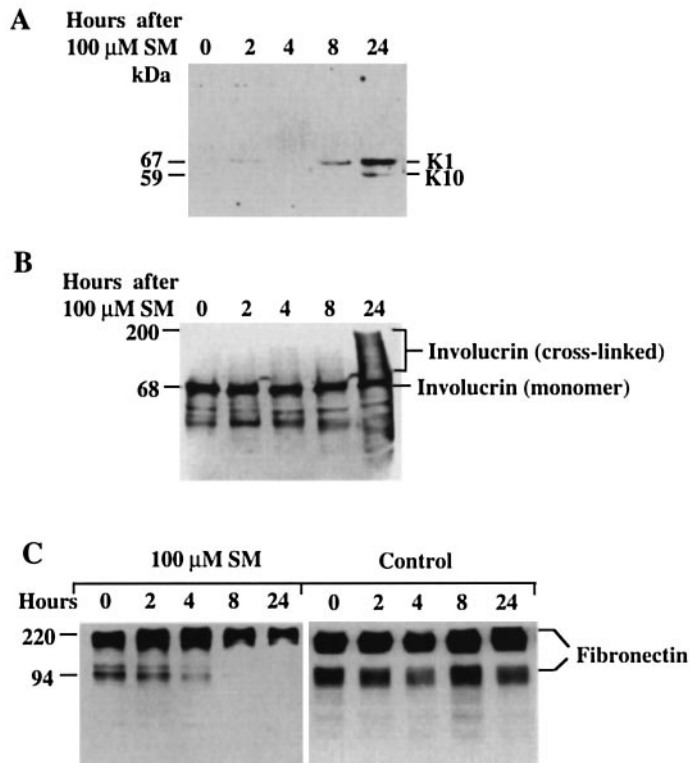
Cytosolic extracts were prepared from NHEK by scraping phosphate-buffered saline-washed monolayers in 10 mM HEPES/KOH (pH 7.4), 2 mM ethylenediamine tetraacetic acid, 0.1% CHAPS, 5 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride, 10  $\mu\text{g}$  pepstatin A per ml, 20  $\mu\text{g}$  leupeptin per ml, and 10  $\mu\text{g}$  aprotinin per ml (at  $1 \times 10^8$  cells per ml). The post-100,000  $\times g$  supernatant was recovered after centrifugation.

PARP cleavage activity was measured in mixtures containing 5  $\mu\text{g}$  protein from the cytosol fractions of keratinocytes. Assay mixtures also contained purified [ $^{35}\text{S}$ ]PARP ( $\approx 5 \times 10^4$  cpm), 50 mM PIPES-KOH, 2 mM ethylenediamine tetraacetic acid, 0.1% (wt/vol) CHAPS, and 5 mM dithiothreitol in a total volume of 25  $\mu\text{l}$ . Incubations were performed at  $37^\circ\text{C}$  for 1 h, and terminated by the addition of 25  $\mu\text{l}$  of 2 $\times$ sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer containing 4% sodium dodecyl sulfate, 4%  $\beta$ -mercaptoethanol, 10% glycerol, 0.125 M Tris-HCl (pH 6.8), and 0.02% bromophenol blue. Samples were resolved by 10% sodium dodecyl sulfate-polyacrylamide gels.

PARP cleavage products were visualized either by fluorography, or else the 89 kDa cleavage product of [ $^{35}\text{S}$ ]PARP was quantitated relative to the full-length PARP using a Storm 840 PhosphorImage analyzer (Molecular Dynamics, Sunnyvale, CA). Quantitation included a correction for background, as well as for the difference in methionine residues present in the 89 kDa fragment (18 met residues) versus full-length PARP (25 met residues).

**DNA isolation** Cells were lysed for 2 h in 10 mM Tris-Cl pH 7.5, 10 mM ethylenediamine tetraacetic acid, 0.5% sodium dodecyl sulfate, containing 144  $\mu\text{g}$  proteinase K per ml, and 500  $\mu\text{g}$  RNase A per ml (Boehringer Mannheim, Indianapolis, IN). Lysates were extracted twice with phenol:chloroform (1:1), and precipitated by the addition of ethanol to 70%. Precipitates were resuspended in distilled water. Glycerol was added to 10% and DNA was resolved on 1% agarose gels and visualized by ethidium bromide staining.

**[ $^{35}\text{S}$ ]Methionine labeling** To measure total protein synthesis, cells were pulse-labeled with [ $^{35}\text{S}$ ]methionine (10  $\mu\text{Ci}$  per ml; Dupont-NEN) for 1 h.



**Figure 1. Modulation of differentiation markers and attachment proteins by SM.** NHEK were treated with 100  $\mu$ M SM for the indicated times, harvested, and total cell extracts were immunoblotted using antibodies specific for K1 + K10 (A), involucrin (B), or fibronectin (C). For all studies, similar results were obtained from three independent experiments utilizing human primary adult keratinocytes and three independent experiments utilizing human primary neonatal keratinocytes.

Cells were then washed twice with phosphate-buffered saline, and harvested. Cells were lysed in 10% trichloroacetic acid, and the precipitated protein was collected on glass filters. Filters were washed successively with 10% trichloroacetic acid, 70% ethanol, and absolute ethanol, and dried. Protein-incorporated [ $^{35}$ S]methionine on filters was measured by scintillation spectroscopy.

## RESULTS

**SM induces markers of terminal differentiation in both primary and immortalized keratinocytes** To determine if SM altered keratin expression, NHEK were exposed to 100  $\mu$ M SM, fixed after 24 h, and then subjected to fluorescence-activated cell sorter analysis, using the broad-range-reactive cytokeratin (CK) antibody as a tag. Following SM exposure, the number of CK+ cells increased significantly (data not shown). Because the CK antibody recognizes the suprabasal keratin K10, we were curious to determine if SM altered the expression of any differentiation-specific proteins. Immunoblot analysis with specific anti-sera revealed that both K1 and K10 were induced in the presence of 100  $\mu$ M SM (Fig 1A). The viability of NHEK throughout the time course was greater than 90% as measured by dye exclusion. We also examined the expression of involucrin, a precursor protein that becomes cross-linked in the fully differentiated cornified envelope (Yaffe *et al*, 1993; Robinson *et al*, 1996; Steinert and Marekov, 1997). In extracts derived from untreated cells, involucrin migrated as a 68 kDa monomer form. Following 24 h exposure to SM, the staining pattern shifted to higher molecular weight forms (Fig 1B), suggesting that the protein is cross-linked in response to SM.

We next examined the levels of fibronectin expressed in NHEK following SM treatment, for two reasons. First, fibronectin is expressed in basal cells, but is suppressed in suprabasal cells *in vivo*, and in response to differentiating agents *in vitro* (Adams and Watt, 1990; Nicholson and Watt, 1991). In turn, contact with fibronectin also inhibits keratinocyte differentiation (Staiano-Coico and Higgins, 1992; Drozdoff and Pledger, 1993; Watt *et al*, 1993). Second, fibronectin is

a major component of the basal lamina, and forms an attachment site for the  $\alpha 5 \beta 1$  integrin of the basal cells (Adams and Watt, 1990). Thus, suppression of this protein by SM could in part explain the detachment of basal cells from the basal lamina during vesication *in vivo*. Fibronectin is produced in keratinocytes (as well as fibroblasts), in two isoforms. Both the 220 kDa and the 94 kDa forms of fibronectin were reduced with time after SM exposure. In contrast, untreated NHEK showed no decrease in the levels of fibronectin (Fig 1C).

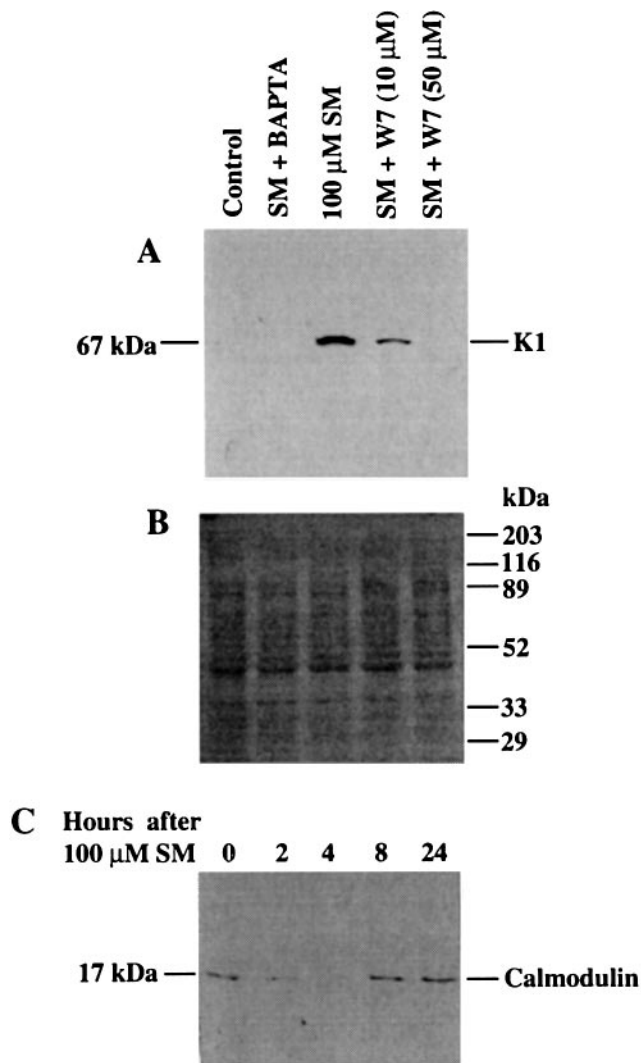
Because previous studies have shown that SM can induce an increase in  $\text{Ca}_i$  (Ray *et al*, 1995), and because we have observed that a rise in  $\text{Ca}_i$  is associated with the normal terminal differentiation response of keratinocytes (Yuspa *et al*, 1989; Rosenthal *et al*, 1991), the expression of these markers suggested a role for  $\text{Ca}^{2+}$  in the SM response that was observed. Furthermore, we have shown that the K1 gene contains specific  $\text{Ca}^{2+}$ -inducible enhancer sequences located 3' to the gene (Huff *et al*, 1993) and expression of K1 (and other differentiation-specific genes) can be blocked by the  $\text{Ca}_i$  chelator BAPTA (Li *et al*, 1995). BAPTA also enhances the survival of keratinocytes in the presence of SM (Ray *et al*, 1996). We therefore preincubated keratinocytes with 20  $\mu$ M BAPTA-AM (+ Pluronic F-127; see *Materials and Methods*) for 30 min prior to SM treatment. When NHEK were subsequently treated for 24 h with 100  $\mu$ M SM, keratin K1 was suppressed (Fig 2A). Although BAPTA treatment suppressed total protein synthesis by 50% after 24 h (not shown), this effect was not enough to account for the complete suppression of K1.  $\text{Ca}^{2+}$  may induce differentiation via its role in the activation of protein kinase C (Dlugosz and Yuspa, 1993). In addition,  $\text{Ca}^{2+}$ -calmodulin complexes are also generated that modulate this protein kinase C response (Chakravarthy *et al*, 1995). We therefore determined whether the calmodulin inhibitor W-7 could alter the differentiation response to SM. Figure 2(A) shows that a 30 min pretreatment with W-7 prior to exposure to 100  $\mu$ M SM inhibited the expression of K1. Protein calibration prior to gel-loading, followed by Ponceau-S-staining of the immunoblot (Fig 2B), eliminated the possibility of loading artifact, indicating that SM induces the expression of this differentiation-specific marker via a  $\text{Ca}^{2+}$ -calmodulin-dependent pathway. Interestingly, calmodulin itself was downregulated by SM (Fig 2C).

**SM suppresses Bcl-2 and induces p53** To examine possible mechanisms by which SM altered the differentiation response, we initially examined the expression of p53, which has been postulated to play important roles in both the differentiation and the apoptotic responses. Immunoblot analysis showed a significant increase in the protein levels of p53 after exposure to 100  $\mu$ M SM, and that this increase in p53 levels occurs within 2 h (Fig 3A).

We also examined the levels of the *bcl-2* gene product, which inhibits both keratinocyte differentiation and apoptosis. Bcl-2 levels are high in basal keratinocytes and are reduced in the differentiating layers of the epidermis (Hockenberry *et al*, 1991). Furthermore, expression of *bcl-2* anti-sense RNA can lower endogenous levels of Bcl-2 and induce markers of terminal differentiation in mouse keratinocytes (Marthinuss *et al*, 1995). Following SM treatment, there is a time-dependent decrease in Bcl-2 protein levels in NHEK as determined by immunoblot analysis (Fig 3B).

**SM induces apoptosis via caspase-3** The striking decrease in Bcl-2 levels and the increase in p53 levels suggested that in addition to modulating differentiation, SM may induce apoptosis as well. Thus, we assayed for markers of apoptosis following SM treatment. A hallmark of apoptosis in several cell types is the appearance of nucleosome-sized ladders due to the presence of a  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonuclease that is induced in apoptotic cells. DNA isolated from NHEK treated with 0 or 100  $\mu$ M SM was intact; however, at 300  $\mu$ M SM, NHEK showed nucleosome-sized ladders analyzed by agarose gel electrophoresis, although some nonspecific fragmentation was also apparent (Fig 4). Accordingly, trypan blue exclusion at 24 h was 98% in control cells, 90% following 100  $\mu$ M SM treatment, and 60% after 300  $\mu$ M SM (not shown). W-7 suppressed DNA fragmentation, whereas BAPTA partially inhibited DNA cleavage (see below).

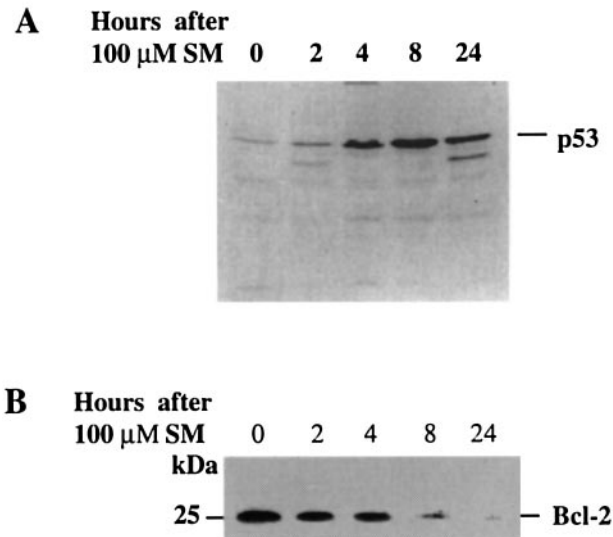
We have recently determined that the activation of PARP plays a role in the etiology of apoptosis in osteosarcoma cells induced to



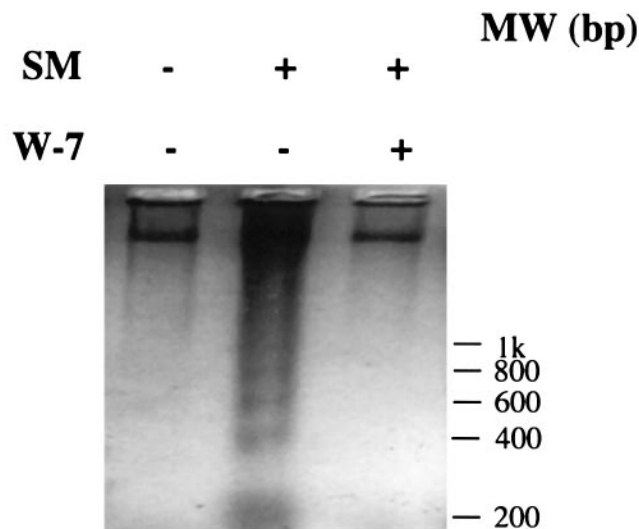
**Figure 2.  $\text{Ca}^{2+}$  and calmodulin are required for the induction of markers of terminal differentiation by SM.** NHEK were treated with SM as above, either with or without pretreatment with 20  $\mu$ M BAPTA-AM or W-7 (A). Cell extracts were immunoblotted using antibodies specific for K1 (A), or calmodulin (C). (B) Ponceau-S stain of total cell protein on nitrocellulose prior to immunostaining shown in (A).

undergo programmed cell death, in which poly(ADP-ribosylation) corresponds with the early reversible stages of apoptosis (Rosenthal *et al*, 1997b). We therefore determined whether we could detect increased levels of poly(ADP-ribosylation) following exposure of NHEK to SM. Because PARP itself is the main acceptor protein for poly(ADP-ribosylation), via intermolecular "automodification" (Mendoza-Alvarez and Alvarez-Gonzalez, 1993), the presence of a 116 kDa antipoly(ADP-ribose)-crossreactive band is a sensitive indicator of poly(ADP-ribosylation) within the nucleus. Anti-sera specific for poly(ADP-ribose) did in fact detect a strong band at 116 kDa in extracts of primary keratinocytes treated with all concentrations of SM tested, whereas no such band was present in extracts of control keratinocytes, indicating that SM induces DNA strand breaks and PARP is activated. **Figure 5(A)** shows that, at 100  $\mu$ M SM, activation of PARP occurs and automodification is apparent by 2 h. The molecular weight of automodified PARP was similar to that of unmodified PARP (116 kDa), indicating that the average polymer length is relatively short at this time point. After this time, the level of poly(ADP-ribose) decreases precipitously, similar to our previous observations using osteosarcoma cells induced to undergo apoptosis (Rosenthal *et al*, 1997b).

We previously determined that this characteristic rise and rapid



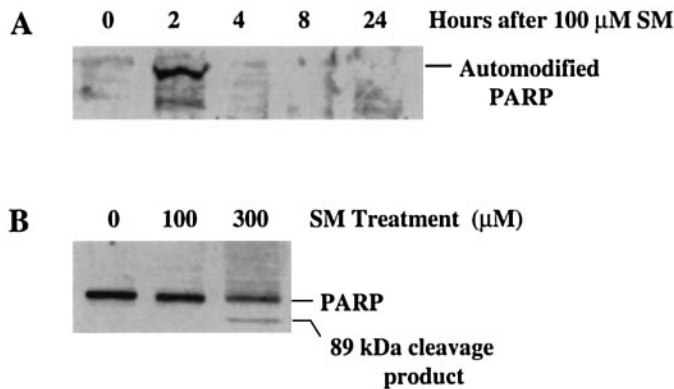
**Figure 3. SM induces an increase in p53 levels, and a decrease in Bcl-2.** NHEK were treated with 100  $\mu$ M SM for the indicated times. Total cell extracts were derived, resolved by polyacrylamide gel electrophoresis, and subjected to immunoblot analysis using anti-sera specific for p53 (A) or Bcl-2 (B).



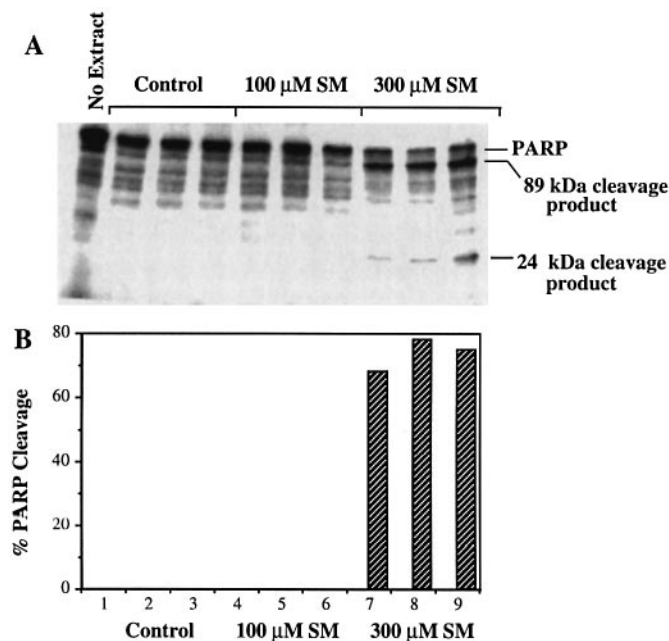
**Figure 4. SM treatment induces DNA fragmentation in keratinocytes.** NHEK were treated with 100  $\mu$ M or 300  $\mu$ M SM, with or without W-7 or BAPTA. Total genomic DNA was isolated, resolved by agarose gel electrophoresis, and stained with ethidium bromide.

decline in poly(ADP-ribose) levels could be attributed not only to poly(ADP-ribose) glycohydrolase activity (Wielckens *et al*, 1983), but also to the proteolytic cleavage of PARP into the characteristic 89 kDa and 24 kDa fragments, the latter of which contains the  $\text{Zn}^{2+}$  finger region and DNA-binding domain. We therefore performed western analysis to monitor the cleavage of PARP using an antibody that recognizes both the full-length 116 kDa protein as well as the 89 kDa fragment of PARP. **Figure 5(B)** shows a significant conversion of full-length PARP to the 89 kDa fragment following 300  $\mu$ M SM treatment.

We have previously described an ICE-like protease similar to the Ced-3 protein of *C. elegans* and closely associated with apoptosis (Nicholson *et al*, 1995), now known as "caspase-3" (Alnemri *et al*, 1996). Accordingly, a sensitive technique to verify that SM induces apoptosis is to determine the activation of caspase-3 from its precursor (pro-caspase-3; CPP32) via the use of *in vitro* translated PARP. We therefore used a combination transcription/translation system to radiolabel full-length PARP (see *Materials and Methods*) that was subsequently incubated with extracts derived from keratinocytes treated



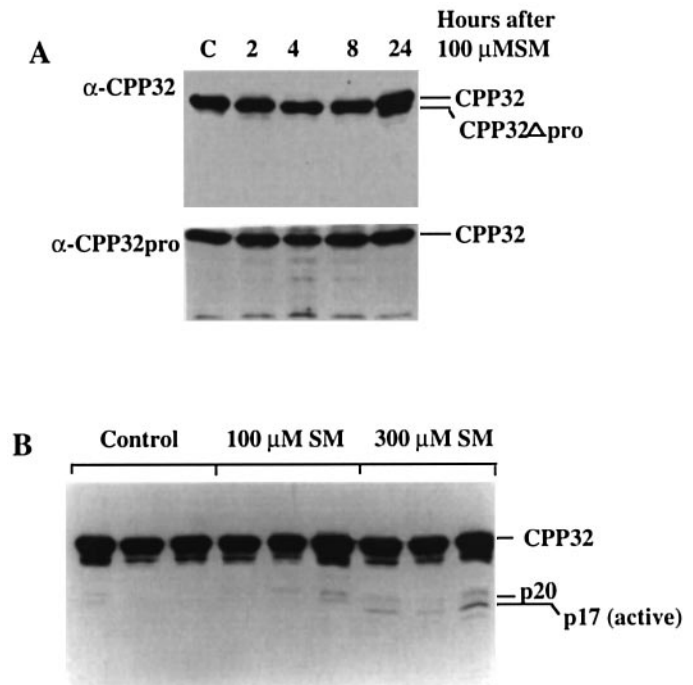
**Figure 5. SM treatment induces *in vivo* PARP activation and cleavage in keratinocytes.** NHEK were treated with 100  $\mu$ M (A, B) or 300  $\mu$ M (B) SM. Total cell extracts were derived, resolved by polyacrylamide gel electrophoresis, and subjected to immunoblot analysis using anti-sera specific for poly(ADP-ribose) (A), or for both the full-length and the 89 kDa cleavage product of PARP (B).



**Figure 6. Extracts of keratinocytes treated with SM show *in vitro* PARP-cleavage activity.** Triplicate cultures of NHEK were treated with 100  $\mu$ M or 300  $\mu$ M SM. Cytoplasmic extracts were then derived after 24 h and assayed for caspase-3 activity, using [ $^{35}$ S]PARP as a substrate (Materials and Methods). (B) Quantitation of PARP-cleavage activity by phosphorimage analysis.

with SM. **Figure 6(A)** shows that PARP cleavage activity is clearly seen in NHEK in 300  $\mu$ M SM (but not 100  $\mu$ M SM) after 24 h, as evidenced by the strong appearance of the 24 kDa and 89 kDa cleavage products. **Figure 6(B)** shows, by quantitative phosphorimage analysis, the relative PARP cleavage activities that result from the treatment of NHEK with SM for 24 h. The high level of PARP-cleavage activity observed in 300  $\mu$ M SM is indicative that this vesicant is also a strong inducer of apoptosis in primary keratinocytes, and that apoptosis is occurring via a caspase-3-like pathway.

We next further verified that SM induces apoptosis by determining whether the observed caspase-3 activity *in vitro* could be associated with the processing of pro-caspase-3/CPP32 into its active protease form. During apoptosis, procaspase-3/CPP32 is processed into 17 kDa and 12 kDa peptides, with the removal of a pro-peptide sequence from the N-terminus. The 17 kDa and 12 kDa fragments then form the active proteolytic heterodimer. Using an antibody that recognizes both the active (p17) and the inactive (CPP32) forms of caspase-3, a



**Figure 7. SM induces processing of procaspase-3/CPP32 to its active form.** Triplicate cultures of NHEK were treated with 100  $\mu$ M or 300  $\mu$ M SM for the indicated times (A), or for 24 h (B). Total cell extracts were derived, resolved by polyacrylamide gel electrophoresis, and subjected to immunoblot analysis using anti-sera specific for the p17 subunit (A, top; B), or the N-terminal pro-peptide sequence (A, bottom) of caspase-3.

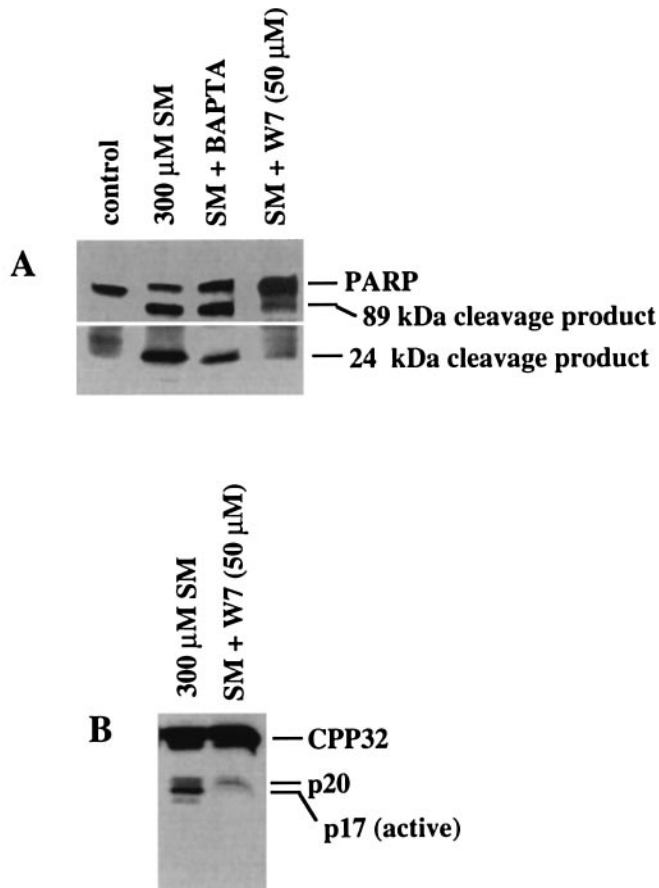
slightly smaller form of CPP32 was observed 24 h after the cells were exposed to 100  $\mu$ M SM, equivalent in size to CPP32 minus the pro-peptide sequence, suggesting that processing of the N-terminus of the precursor protein was occurring (**Fig 7A, top**). To confirm this, the same extracts were analyzed utilizing an antibody that is specific for the pro-sequence that is removed when caspase-3 is processed into its active form. The disappearance of the slightly smaller MW band previously observed at 24 h in 100  $\mu$ M SM (**Fig 7A, bottom**), indicates that this band is missing the propeptide sequence, and is thus the result of CPP32 N-terminal processing. Following treatment with 300  $\mu$ M SM, NHEK showed complete processing of a portion of CPP32 into the active p17 form (**Fig 7B**). A small amount of the partially processed p20, which represents p17 and the N-terminal pro-sequence, is also observed. Thus, in both 100  $\mu$ M and 300  $\mu$ M SM, markers of apoptosis are induced, although complete activation of caspase-3, PARP cleavage, and DNA fragmentation are only observed at the higher concentration of SM.

**A  $\text{Ca}^{2+}$  calmodulin-dependent pathway for SM-induced apoptosis** To determine if SM-induced apoptosis was proceeding via  $\text{Ca}^{2+}$ -calmodulin dependent pathways, BAPTA and W-7 were utilized as pretreatment agents. BAPTA had a small effect on *in vitro* PARP-cleavage activity, whereas greater suppression was observed following W-7 pretreatment (**Fig 8A**). W-7 also suppressed the level of DNA fragmentation (**Fig 4**).

We next determined whether this W-7-sensitive repression was related to the processing of CPP32 into its active form. In control NHEK, one of the two subunits of the active form of apoptosis, p17, can clearly be detected 24 h following treatment with 300  $\mu$ M SM; however, p17 is completely suppressed by 50  $\mu$ M W-7 (**Fig 8B**). Thus, SM induces apoptosis via a calmodulin-dependent pathway that involves the activation of caspase-3.

## DISCUSSION

SM vesication clearly involves both cytotoxicity and detachment of the epidermal basal layer *in vivo*. Using a cell culture model in this



**Figure 8. Caspase-3 activity and processing induced by SM is suppressed by inhibitors of  $\text{Ca}_i$  and calmodulin.** Cells were treated with 300  $\mu\text{M}$  SM for 24 h with or without a 30 min pretreatment with BAPTA-AM or W-7. Cytoplasmic extracts were then derived and assayed for caspase-3 activity, using [ $^{35}\text{S}$ ]PARP as a substrate. (B) Extracts of cells treated with 300  $\mu\text{M}$  SM, plus a 30 min W-7 pretreatment, were resolved by polyacrylamide gel electrophoresis, and subjected to immunoblot analysis using anti-sera specific for p17.

study, we have described two potential mechanisms for SM-induced keratinocyte basal cell death and detachment: induction of terminal differentiation and apoptosis. SM induced the differentiation-specific markers K1 and K10, cross-linking of the cornified envelope precursor protein involucrin, and suppressed fibronectin. SM also induced markers of apoptosis in NHEK, including the transient elevation of poly(ADP-ribose), the processing of CPP32 into caspase-3, and the specific cleavage of PARP both *in vivo* and in cell-free PARP-cleavage assays. Differentiation markers were suppressed by the intracellular  $\text{Ca}_i^{2+}$ -chelator BAPTA-AM, and the calmodulin inhibitor W-7, whereas markers of apoptosis were suppressed by W-7. Thus, both the differentiation and the apoptotic responses appear to be  $\text{Ca}_i^{2+}$ -calmodulin dependent.

Because SM induces an increase in  $\text{Ca}_i$  (Ray *et al.*, 1995; Mol and Smith, 1996), it seems likely that this elevation in  $\text{Ca}_i$  accounts for the induction of the markers of terminal differentiation observed in this study, because terminal differentiation of keratinocytes is closely associated with an elevation in  $\text{Ca}_i^{2+}$  (Hennings *et al.*, 1980; Stanley and Yuspa, 1983; Kruszewski *et al.*, 1991a, b; Rosenthal *et al.*, 1991; Li *et al.*, 1995). The suppression of these markers by BAPTA also lends support to the idea that the rise in  $\text{Ca}_i$  is an important event in SM-induced expression of differentiation markers (Fig 2). How SM induces a rise in  $\text{Ca}_i$  is unknown, but may stem from its ability to alkylate several molecules within the cell, including glutathione (Gross *et al.*, 1993) or other molecules involved in cellular homeostasis, ultimately resulting in disruptions of plasma, ER, and mitochondrial membranes. Disruption of these membranes could easily lead to perturbations in  $\text{Ca}_i$  within the cell.

The role of SM in the induction of apoptosis may also involve  $\text{Ca}_i$  and/or  $\text{Ca}_i^{2+}$ -calmodulin complexes, because these molecules have been shown to be involved in apoptosis in other systems (Kaiser and Edelman, 1977; Shi *et al.*, 1989). The suppression of apoptotic markers by the  $\text{Ca}_i$  buffer BAPTA and the calmodulin inhibitor W-7 in this study supports this idea. It is also likely that DNA strand breaks and the activation of PARP play a role in the SM-induced expression of apoptotic markers in keratinocytes. SM is a strong alkylating agent with a high affinity for DNA, and has been shown to induce DNA strand breaks and consequently activate PARP. PARP inhibitors can extend the lifespan of lymphocytes treated with SM (Meier and Johnson, 1992; Meier, 1996), and inhibitors of PARP have previously been reported to significantly affect the extent of apoptosis in response to other agents (Rice *et al.*, 1992; Ghibelli *et al.*, 1994; Monti *et al.*, 1994; Kuo *et al.*, 1996). PARP may thus be an important signaling molecule for cell death either via the lowering of NAD and/or ATP levels (Wielckens *et al.*, 1982; Berger *et al.*, 1983; Alvarez *et al.*, 1986), or by poly(ADP-ribosylation) of other key cellular proteins involved in apoptosis, such as p53 (Whitacre *et al.*, 1995) and the  $\text{Ca}_i^{2+}/\text{Mg}_i^{2+}$  dependent nuclease involved in the apoptotic cleavage of DNA (Rice *et al.*, 1992).

The early and short-lived poly(ADP-ribosylation), followed by caspase-3 activation and PARP cleavage detected in this study (Figs 5–7), is consistent with our previous findings during spontaneous apoptosis in the osteosarcoma system (Rosenthal *et al.*, 1997a,b). The synthesis of poly(ADP-ribose) from nicotinamide adenine dinucleotide increased early after initiation of apoptosis. The abundance of both poly(ADP-ribose) and PARP then decreased markedly, corresponding to the appearance of the proteolytic cleavage product containing the DNA-binding domain of PARP; no poly(ADP-ribose) was observed during this time, in spite of the fact that there was massive DNA internucleosomal degradation. This coincided with earlier data indicating that caspase-3 activity maximized in osteosarcoma cells at this time. These data suggested that there appears to be not only a requirement for destruction of PARP during apoptosis, but also a stage in early apoptosis that appeared to require the presence of PARP protein and presumably poly(ADP-ribosylation) of certain proteins. Recently, we utilized antisense RNA expression to deplete endogenous PARP in order to examine the requirement of this protein early in apoptosis, and found that the apoptotic response was modulated (Simbulan-Rosenthal *et al.*, 1998). Likewise, in recent PARP-knockout animals, apoptosis was found to be altered (de Murcia *et al.*, 1997).

The involvement of such varied molecules as  $\text{Ca}_i^{2+}$ , calmodulin, p53, Bcl-2, and caspase-3 suggests a complex network involved in SM-induced apoptosis; however, it seems that the activation of caspase-3 (or the closely related caspase-7) may be a final converging point for apoptosis, because (i) procaspase-3/CPP32 knockout mice show altered programmed cell death in the nervous system (Kuida *et al.*, 1996), (ii) caspase-3 (and -7) is a target for granzyme B-mediated apoptosis, as well as apoptosis mediated by other caspases (Darmon *et al.*, 1995, 1996; Chinnaiyan *et al.*, 1996; Gu *et al.*, 1996; Quan *et al.*, 1996), and (iii) a tetrapeptide aldehyde (Ac-DEVD-CHO) that inhibits caspase-3 activity blocks apoptotic events in isolated nuclei (Nicholson *et al.*, 1995). In this study, the convergence of these signals at the level of caspase-3 activation is supported by the facts that: (i) p53 and Bcl-2, which act upstream of caspase-3, are strongly and rapidly modulated by SM; (ii) inhibitors of  $\text{Ca}_i^{2+}$  and calmodulin, which have been found in previous studies to prolong the lifespan of keratinocytes exposed to SM (Ray *et al.*, 1996), and to block apoptosis in this study, prevent the processing and activation of caspase-3; and (iii) in preliminary experiments, NHEK pretreated with AcDEVD-CHO showed both inhibition of *in vitro* PARP-cleavage activity and a reduced apoptotic index in response to SM, as determined by TUNEL labeling (not shown).

An understanding of the mechanisms for SM vesication will hopefully lead to strategies for prevention or treatment of SM toxicity. This study suggests that inhibition of calmodulin (upstream) or caspase-3 (downstream) may protect the epidermis from SM-induced apoptosis. Although the mechanism for their protection has not been described, calmodulin inhibitors have already been successfully employed in the

treatment of both thermal burns and frostbite (Beitner *et al*, 1989a, b), and may prove effective for SM as well, either alone or in combination with caspase-3 inhibitors.

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