

# Nitrogen Mustard Induces Mitochondria-Mediated Apoptosis Associated with Endoplasmic Reticulum Stress-Regulated MAPK Signaling in Human HaCaT Keratinocytes

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Vesicating agents, including sulfur mustard (SM, bis(2-chloroethyl) sulfide) and nitrogen mustard mechlorethamine (HN2, bis(2-chloroethyl)methylamine), are bifunctional alkylating agents that are highly reactive in the skin causing extensive tissue damage and blistering. Previously, we reported that treatment of HaCaT human keratinocytes with HN2 caused a block in the S phase of the cell cycle and cell cycle dependent DNA damage signaling, processes contributing to cytotoxicity. In the present studies, we evaluated molecular mechanisms underlying HN2 toxicity. Multi-color flow cytometric analysis revealed that HN2 treatment caused a time- and concentration-dependent induction of apoptosis. This was associated with selective activation of caspase 3, caspase 6, caspase 7 and caspase 9, but not caspase 8 or caspase 10, suggesting that HN2 triggers mitochondrial-mediated apoptosis. This is supported by findings that HN2 also caused increases in levels of Bax and p62, and decreases in Bcl-xL. In contrast, HN2 had little or no effect on expression of LC3B and Beclin-1, markers of autophagy. Expression of cleaved caspase 3 and cleaved PARP was found to be homogenous throughout the cell cycle, suggesting that HN2-induced apoptosis is cell cycle independent. HN2 also triggered endoplasmic reticulum (ER) stress and activation of mitogen-activated protein kinase (MAPK) signaling in HaCaT cells, which was identified by enhanced phosphorylation of several key molecules including eukaryotic initiation factor 2 $\alpha$  (eIF2 $\alpha$ ), p38, ERK1/2 and c-Jun N-terminal kinases (JNKs) and up-regulation of ATF4 expression. GSK2606414, an ER stress inhibitor, reduced phosphorylation of these proteins. Conversely, MAPK inhibitors, including SB203580, PD98059 and SP600125, blocked phosphorylation of p38, ERK1/2 and JNKs, but not eIF2 $\alpha$ . These data indicate that ER stress regulates MAPK signaling in HN2-treated HaCaT cells. A pan-caspase inhibitor, z-VAD-FMK, was also found to suppress HN2-induced ER stress and MAPK activation and to attenuate cytotoxicity and block apoptosis. Taken together, these data demonstrated that ER stress and MAPK signaling play an important role in HN2-induced apoptosis, contributing to vesicant-induced cytotoxicity and tissue injury. *Support: NIH grants AR055073, NS108956, and ES005022.*