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Immediate-Early Gene Expression Responses to Sulfur Mustard Exposure

B.N. Ford, Y. Shei, P. Nelson, M. McWilliams, T. Weiss, T.W. Sawyer

Defence R&D Canada

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In conducting the research described in this report, the investigators adhered to the 'Guide to the Care and Use of Experimental Animals, Vol. I, 2nd Ed.' published by the Canadian Council on Animal Care.

Abstract

Sulfur mustard (bis-(2-chloroethyl) sulphide, HD) is a well-known chemical warfare agent which causes skin blisters, respiratory tract injury, and systemic toxic effects. The underlying molecular basis of HD toxicity is not well understood. Multiple hypotheses attempting to improve understanding of this problem have been proposed, including DNA damage and repair, inflammation, aberrant cytokine expression, direct protein modification to keratin fibres, and combinations of these effects. Despite such efforts, there is little consensus on the biological basis for the profound observed toxicity of HD. A particularly puzzling aspect is the terminal arrest of cultured cells at concentrations of HD substantially lower than those at which cytotoxic effects are seen, suggesting there may be multiple pathways through which HD can exert its biological effects. Previously, Sawyer and others have proposed receptor-based interactions as being at the root of HD induced cytotoxic effects. In order to explore this hypothesis, gene expression profiles of first passage cultures of human keratinocytes were developed using an oligonucleotide microarray platform. Analysis of data from six independent donors at multiple concentrations of HD and varying time points revealed a complex response. Combined data at adjacent time points enabled the identification of the mitogen-activated protein kinase (MAPK) network and genes associated with it, as primary functions responding to sulfur mustard. Published work on the role of apoptosis in HD cytotoxicity led to the hypothesis that mitochondrial permeability modulated as a downstream effect of the MAPK network might be involved in the HD response. Preliminary data from live-cell studies demonstrated an immediate-early mitochondrial membrane change which may be a critical early event in HD-induced cytotoxicity. These studies are a step towards refining our understanding of the toxic effects of HD in cells.

Résumé

Le gaz moutarde (sulfure de bis (2-chloroéthyle), HD, est un agent de guerre chimique bien connu causant des boursouflures de la peau, des dommages à l'appareil respiratoire et des effets toxiques systémiques. On ne comprend pas encore très bien l'origine moléculaire sous-jacente de la toxicité du HD. De multiples hypothèses ont été proposées dans la tentative d'approfondir la compréhension de ce problème dont le dommage et la réparation de l'ADN, l'inflammation, l'expression aberrante de cytokine, la modification directe de la protéine en fibres de kératine et les combinaisons de ces effets. En dépit de ces efforts, on ne s'accorde pas sur l'origine biologique de la profonde toxicité du HD qui a été observée. Un aspect particulièrement curieux est l'arrêt terminal de cellules cultivées à des concentrations de HD considérablement plus faibles que celles auxquelles on observe les effets cytotoxiques, suggérant qu'il existe peut-être des voies multiples à travers lesquelles le HD peut exercer ses effets biologiques. Sawyer et certains autres chercheurs proposent que les interactions à base de récepteurs puissent être à l'origine des effets cytotoxiques induits par le HD. Pour mieux explorer cette hypothèse, des profils d'expression de gènes des cultures du premier passage des kératinocytes humains ont été développés au moyen d'une plateforme de microréseaux d'oligonucléotides. Des analyses de données provenant de six donneurs indépendants ayant des concentrations multiples de HD à des moments donnés variés ont produit une réponse complexe. Les données combinées et les moments donnés adjacents ont permis d'identifier le réseau de la protéine kinase activée par des agents mitogènes (MAPK) et les gènes lui étant associés, comme étant les fonctions primaires de réponse au gaz moutarde. Des travaux publiés relatifs au rôle de l'apoptose dans la cytoxicité HD ont amené à l'hypothèse que la perméabilité mitochondriale modulée, comme une activité en aval sur le réseau MAPK, pourrait contribuer à la réponse du HD. Des données préliminaires provenant des études de cellules vivantes ont indiqué un changement rapide et immédiat de la membrane mitochondriale pouvant être un événement critique rapide de la cytotoxicité induite par l'HD. Ces études sont une étape vers le raffinement de la compréhension des effets toxiques du HD sur les cellules.

Executive summary

Immediate-Early Gene Expression Responses to Sulfur Mustard Exposure

Barry N. Ford; Yimin Shei; Peggy Nelson; Michael McWilliams; Thomas W. Sawyer; DRDC Suffield TM 2007-290; Defence R&D Canada – Suffield; December 2007.

Introduction: Sulfur mustard (HD) is a chemical warfare agent that causes skin blistering, lung damage, and systemic toxicity. Despite decades of research, the basic cellular mechanisms of sulfur mustard toxicity are not well understood. After exposure to toxic materials such as HD, cells and tissues show changes in the expression of many genes responsible for cell function, maintenance, and damage repair. Using large-scale analysis tools, it has been possible to analyze the expression of thousands of genes in cultured cells after sulfur mustard exposure. This information can be used to improve our understanding of the toxic effects of HD, with a view towards development of targeted medical countermeasures.

Results: Cultured normal human skin keratinocytes from six different donors were exposed to HD. RNA representing expressed genes was harvested at multiple time points after exposure. Reduced overall RNA yields at elevated HD concentrations, suggest that gene expression was profoundly and generally reduced after HD exposure, possibly due to damage within the genomic DNA. At lower concentrations, microarray analysis was used to measure the expression of thousands of individual genes. Expression from multiple signaling and biochemical pathways, as well as individual functions, were affected by sulfur mustard. In particular, genes involved in global regulation of gene expression, damage response signaling, and related functions were altered after HD exposure. Such responses are predicted to have an effect on cell death and cell division, as has been previously observed for HD-exposed cells.

Significance: HD has multiple and varied effects on cells and tissues, which correspond to the complexity of its effects within the body. Understanding the type and degree of response to sulfur mustard in keratinocytes is a step towards the development of new therapies.

Future plans: Detected changes will be evaluated independently using other assay techniques, including protein expression changes, functional assays, and inhibitor responses.

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Immediate-Early Gene Expression Responses to Sulfur Mustard Exposure

Barry N. Ford; Yimin Shei; Peggy Nelson; Michael McWilliams; Thomas W. Sawyer; DRDC Suffield TM 2007-290; R & D pour la défense Canada – Suffield; Décembre 2007.

Introduction : Le gaz moutarde (HD) est un agent de guerre chimique qui cause des boursouflures de la peau, des dommages aux poumons et une toxicité systémique. Malgré des décennies de recherches, les mécanismes cellulaires de base de la toxicité du gaz moutarde ne sont pas bien compris. Les cellules et les tissus, après avoir été exposés à des matériaux toxiques tels que le HD, indiquent des changements dans l'expression de beaucoup de gènes responsables de la fonction des cellules, de leur entretien et de la réparation des dommages. Il a été possible d'analyser l'expression de milliers de gènes dans des cellules cultivées, après leur exposition au gaz moutarde, à l'aide d'outils d'analyse à grande échelle. Cette information peut être utilisée pour améliorer notre compréhension des effets toxiques du HD tout en développant des contremesures médicales ciblées.

Résultats : Des kératinocytes de peau humaine normale, cultivés à partir de six donneurs différents, ont été exposés au HD. L'ARN représentant des gènes exprimés a été récolté à de multiples moments donnés après l'exposition. La réduction générale des rendements de l'ARN, à des concentrations élevées de HD, suggèrent que l'expression de gènes était grandement et généralement réduite après l'exposition au HD, possiblement dû au dommage causé à l'intérieur de l'ADN génomique. Les analyses des microréseaux ont été utilisées pour mesurer l'expression de milliers de gènes individuels à de plus faibles concentrations. L'expression provenant de signalisations multiples et de voies de passage biochimiques ainsi que des fonctions individuelles ont été affectées par le gaz moutarde. Les gènes participant à la régulation globale d'expression de gènes, de la signalisation de réponse au dommage et des autres fonctions reliées, ont, en particulier, été altérés par l'exposition au HD. On prévoit que de telles réponses auront un effet sur la mort et la division des cellules, comme on l'a observé auparavant, chez les cellules exposées au HD.

Portée des résultats : Le HD a des effets multiples et variés sur les cellules et les tissus ce qui correspond à la complexité de ses effets sur le corps. Le fait de comprendre le type et le degré de réponse des kératinocytes au gaz moutarde est une étape vers le développement de nouvelles thérapies.

Perspectives d'avenir : Les changements détectés seront évalués indépendamment au moyen d'autres techniques de bio-essais, y compris les changements d'expression de protéines, les bio-essais fonctionnels et les réponses inhibitrices.

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Introduction

Sulfur mustard (HD, bis-2-chloroethylsulfide) is a vesicant chemical warfare agent with a poorly understood mechanism of toxic action and for which no effective specific therapeutic interventions exist. While the general chemistry of HD is well studied, the cellular mechanisms by which it exerts toxic effects are less clear. Published work on HD suggests that there may be dose-dependent effects wherein the toxic mechanisms might be different at lower vice higher doses [1–5]. If so, it is likely that multiple biological pathways represent potential therapeutic targets. The time course of effects after HD exposure may also be dose dependent [5–10]. If so, then the timing of the therapeutic intervention may also be critical. If there is a critical window of opportunity for treatment which is missed, the vesicant or systemic toxic effects manifest according to the dose.

Previous work examining HD toxicity has looked at the DNA damaging properties of this agent and its consequences in (for the most part) transformed or immortalized cell lines. HD represents probably the first mutagenic chemical ever identified and studied [6, 11]. At the level of DNA, sulfur mustard causes alkylation of bases and interstrand crosslinks, leading to mutations as the cell attempts to repair the lesion. Such lesions are thought to be highly toxic to cells, but efforts to connect the amount of damage to the degree of toxicity have not shown strong correlation between the observed toxicity and the detectable DNA damage [12]. This suggests that DNA damage, while undoubtedly important for some effects, is an incomplete model of HD toxicity. The usefulness of nitric oxide synthase (NOS) inhibitors, based on the hypothesis that the toxic effects of HD might be mediated by nitric oxide [13, 14], has also been published. Initially several NOS inhibitors were shown to have a range of protective properties in various experimental models [15–18]. Compounds with HD-protective properties fall into two general groups, those which are effective only when applied before HD exposure, and those which are still effective after HD exposure. It has been suggested that these groups of compounds could act synergistically in cell culture in protection against HD, implying that they act upon different molecular targets [19]. However, it has also been reported that contrary to predictions, d-isomers of the NOS inhibitors, expected to have no NOS inhibitory properties, were about equally protective as the NOS-inhibiting compounds [15, 19]. Thus NOS inhibition, although certainly occurring in the presence of inhibitors, did not adequately explain the protective effects against HD. Direct chemical interactions between HD and the protective NOS inhibitors have also been ruled out.

Cytotoxicity of HD has been reported to be due to apoptosis or necrosis of exposed cells, or some combination of effects [4, 20, 21]. It has also been shown that direct caspase inhibitors [22, 23] or inhibitors of upstream apoptosis events have some minor (but inconsistent) protective value against HD [24–26], but this has not led to development of useful therapeutics. Questions remain about the temporal limits of therapeutic efforts. Clearly at some point after exposure, the effect of HD exposure will be fully developed and no therapeutic effect can be expected, but the point at which this limit occurs is unknown.

In order to elucidate the molecular effects of HD exposure and to attempt rational therapeutic approaches, gene expression effects in cultured primary human keratinocytes after HD exposure were analyzed. Using first passage keratinocytes, oligonucleotide microarray analysis of mRNA expression, at varying HD concentrations and time points after exposure was performed. After

statistical analysis and data review, data from 50 and 100 µM HD exposures were collated, and a list of genes responding to the HD exposure was developed. Using a suite of analytical tools, we also determined that the MAPK network was consistently involved early in the HD response of normal human epidermal keratinocytes (NHEK), including several genes related to the function of p38MAPK (MAPK14). From the observed MAPK effects, it was hypothesized that depolarization of mitochondria might be an early irreversible event in HD-induced cell death which might represent a therapeutic target [26-28]. Although it is unlikely that early effects on keratinocytes in culture represent the entirety of HD effects or therapeutic opportunities, it is clear that the window of opportunity could be quite short at exposure concentrations of HD within the range expected to be vesicant on skin.

Materials and Methods

Cell Culture

Primary cultures of human skin keratinocytes were prepared from neonatal foreskins following established methods [29, 30], and in accordance with approved Human Subjects guidelines. Tissue was usually obtained on the day of circumcision and incubated at 4°C for 24 hr in 25 U/mL dispase (Collaborative Research, Bedford, MA). The epidermis was removed and the cells segregated by a further 5 min incubation in 0.25% trypsin at 37°C. Trypsin was deactivated by the addition of 20% serum in medium and the cell suspension was centrifuged. The cell pellet was resuspended in Keratinocyte Serum Free Medium (KSFM, Gibco BRL, Grand Island, NY) and filtered through sterilized 70 µM nylon mesh. 75 cm^2 flasks were seeded at 5×10^5 cells/8 mL KSFM supplemented with gentamicin (50 µg/ml) and Fungizone (0.25 µg/mL) and incubated in a 37°C humidified incubator in a 5% CO_2/95% air atmosphere. Culture media was replaced every 2–4 days, as required. First passage cultures were seeded from log growth primary cultures at a density of 1000 cells/well in 96-well Costar multiwell plates, or at 2.5×10^5 cells per 75 cm^2 culture flask. Cultures were treated with HD while in active log growth (3–4 days). For confocal analysis, cells were subcultured onto 35 mm glass coverslips in six-well dishes or into 35 mm clear-bottom dishes (Ibidi, Penetrating Innovations, Ingersoll, ON).

HD Exposure

HD was prepared and analyzed at the Canadian National Single Small Scale Facility at DRDC Suffield. HD was stored neat or in absolute ethanol until use. When cells reached approximately 60-70% confluence, HD exposures were carried out. On the day of experimental treatment, the cultures were fed with fresh medium prior to agent exposure. Cultures were treated with freshly prepared treatment medium so that the desired final HD concentration was reached at 0.25% ethanol (v/v). Cultures were placed back into incubators at 37°C until assayed for cell viability at varying time points after exposure. To assess cytotoxicity, alamarBlue™ (AccuMed International Inc., Westlake, OH) was added (10% v/v) and the cultures incubated with the dye for the last 2–3 hr of the treatment time period. The absorbance at two wavelengths (570 & 600 nm) was then read on a Thermomax titerplate reader (Molecular Devices, Sunnyvale, CA). Median lethal concentration (LC_{50}) values were determined graphically from experiments utilizing 6 wells per data point. All experiments were carried out 6 times with cells derived from different donors.

Cell Harvest and RNA Isolation

At intervals ranging from 30 minutes to 24 hours after HD exposure, cultures were terminated by removing media and rinsing cultures with PBS. Trypsin (0.25%) for 5 minutes was used to detach cells from the surface of the flask, followed by an equal volume of KSFM with 20% serum. Cell suspensions were transferred to clean 15 ml polypropylene tubes, and cells were collected by centrifugation at 600 RPM for 2 minutes. Cell pellets were transferred to microcentrifuge tubes and frozen at −80°C until use. For RNA isolation, cell pellets were thawed briefly on ice, then RNA was purified using the Versagene RNA Cell Kit (Intermedico, Markham, ON). Purified RNA was stored with RNA-Guard RNase inhibitor (0.5%, GE

Healthcare Lifesciences, Baie d'Urfé, QUE). RNA was quantitated using a RIBO-green fluorescence method (Molecular Probes, Eugene, OR). RNA quality was verified on denaturing agarose gels or by analysis on the Experion™ (Bio-Rad, Mississauga, ON) capillary electrophoresis system.

RNA Labeling and Hybridization

Purified RNA was thawed on ice, then labeled with Cy3-dCTP (GE Healthcare Lifesciences), in a cDNA reaction with 20 µM each dATP, dTTP, and dGTP, 16 µM dCTP, 16 µM Cy3-dCTP, 4 µM Anchor-T primer (Invitrogen, Carlsbad, CA), MMuLV buffer 1X as provided with the enzyme, and 2 U MMuLV reverse transcriptase (Invitrogen). This reaction mixture was incubated for 2 hours at 37°C.

After labeling, the cDNA product was briefly heated to 95°C, then added to a preheated (65°C) hybridization solution containing 5% dextran sulfate, and 0.1 % SDS and 50 µg/ml denatured sonicated salmon sperm DNA. This mixture was applied to the microarray slide (prepared by the Vancouver Prostate Cancer Research Centre Microarray Facility using the 21K human oligonucleotide library (Operon Genetics, Carlsbad, CA) in a Corning microarray hybridization chamber and incubated for 18 hours with gentle rotation in a hybridization oven at 45°C.

After hybridization, microarrays were immediately washed with 0.1% SDS at room temperature to rinse off excess hybridization solution, then rinsed with deionized water, and spun to dry. Slides were scanned in a GeneFocus DNAscope IV at 10 µm resolution. Images were digitized using GenePix (Agilent Technologies), and intensity reports collated in Excel (Microsoft Corp). Data review and analysis was performed using Genespring, ONTO-Express [31], Bioconductor, and other open source microarray packages. Pathway mapping was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg/) [32].

Mitochondrial Permeability Pore Analysis

Primary keratinocytes cultured in small dishes (Ibidi, Penetrating Innovations, Ingersoll, ON) were pretreated with JC-1 mitochondrial permeability indicator dye (Molecular Probes, Carlsbad, CA) predissolved in DMSO, for 30 minutes at 5 µg/ml. Following preincubation, cultures were rinsed twice with fresh media. Dishes were mounted on the confocal stage, and imaging initiated using Volocity (Improvision, Coventry, UK). Images were collected every 5 minutes for up to 90 minutes and collated as a stack of TIFF images. After an initial 5 minute control acquisition, HD was added to the final concentration required, or vehicle without HD in control samples. Images were then analyzed using ImageJ [33], to align frames and extract signal intensity data from the aligned frames in each color. Extracted intensities were plotted versus time in MS Excel. Transition of the mitochondrial pore, indicating mitochondrial membrane depolarization, is detected by a shift from a red punctate pattern towards green fluorescence with redistribution, and comparison of the pre or post-exposure images.

Results and Discussion

The initial design of this experiment involved HD exposures of up to 400 µM to keratinocytes in culture. Cytotoxicity data (Tables 1 and 2) revealed that survival (by alamarBlue assay) of individual donor samples exhibited quite different LC_{50} results at 24 hours, but by 48 hours, with one exception, the LC_{50} results were within a narrow range. Cytotoxicity assay at 48 hours after HD exposure of 200 µM or greater revealed less than 10% survival (Figure 1). This may suggest that genetic variation between individuals modulates initial response. Data from DNA repair-defective cell lines suggest that a wide variation in survival after HD exposure (as much as 5- to 50-fold) under experimental conditions could be due to differences in DNA repair [34–36].

Table 1: Relative cytotoxicity of HD in cultured keratinocytes at 24 hours.

DONOR	0 µM	50 µM	100 µM	200 µM	400 µM	LC_{50} µM
	Surviving fraction					
2HK41E	100.0	100.0	85.8	52.1	76.8	>400.0
2HK42D	100.0	100.0	100.0	57.0	70.7	>400.0
2HK43D	100.0	90.0	67.2	34.4	42.4	152.4
2HK53E	100.0	100.0	97.3	44.0	44.4	189.3
2HK74B	100.0	96.8	82.9	11.3	23.0	145.9
2HK80A	100.0	100.0	89.0	25.9	38.5	161.8
Average	100.0	97.8	87.0	37.5	49.3	>241.6
SD	0.00	3.68	10.72	15.63	18.68	112.84

Table 2: Relative cytotoxicity of HD in cultured keratinocytes at 48 hours.

DONOR	0 µM	50 µM	100 µM	200 µM	400 µM	LC_{50} µM
	Surviving fraction					
2HK41E	100.0	100.0	35.6	13.9	20.5	78.5
2HK42D	100.0	94.1	75.9	14.4	19.6	142.1
2HK43D	100.0	92.1	5.4	7.5	12.7	11.3
2HK53E	100.0	87.1	73.2	10.5	6.5	148.7
2HK74B	100.0	70.8	36.9	4.5	4.5	80.7
2HK80A	100.0	95.5	49.5	2.7	4.9	99.5
Average	100.0	89.9	46.1	8.9	11.5	93.5
SD	0.00	9.39	24.10	4.43	6.65	45.81

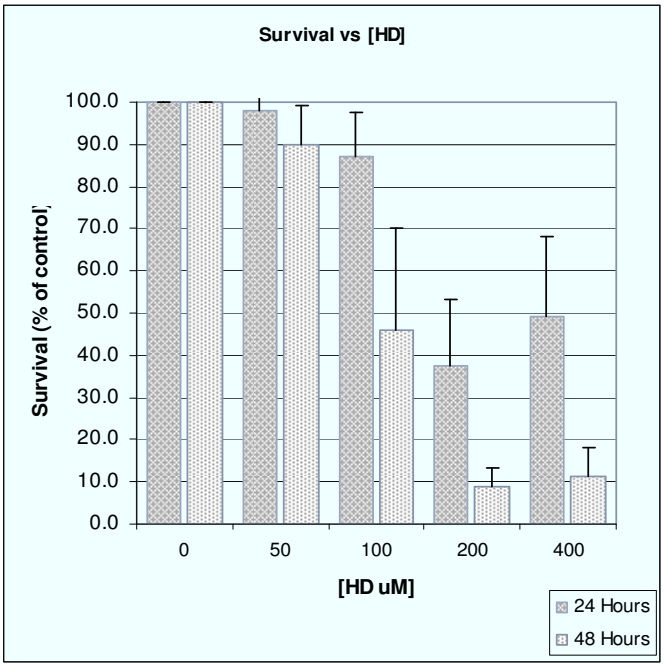

Figure 1: Relative survival of keratinocytes after HD exposure.

Upon review of the RNA extract quantitation data from exposed keratinocytes (all of which are mitotically arrested), it became clear that RNA yield from the cells after HD exposures of 200 to 400 µM was inversely related to the concentration of HD applied. It was considered likely that a large part of the loss was due to transcription arrest due to persistent DNA (or RNA) lesions at the higher doses, or due to direct interference with transcription. The response of transcription to HD has been previously reported [37–39], and the data at HD concentrations in excess of 100 µM are consistent with these observations. Published data on sulfur mustard and gene expression frequently utilize HD concentrations with reported strong effects on transcription [10, 37–41], beyond the range where RNA yield was diminished. This suggests that some of the gene expression differences reported may be due to differential degradation of mRNA species within the context of generally arrested transcription, and thus not representative of gene expression changes per se, which require a functional transcription system. Pre–labeling analysis of RNA extracts by agarose gels or capillary electrophoresis did not reveal obvious degradation of total RNA (data not shown) of ribosomal fragments within the RNA pool at any of the exposure concentrations.

At HD exposure concentrations of 100 µM or less, gene expression data were first normalized using q-spline normalization in the Bioconductor package of R [43–45]. Using the normalized output, data were first pruned with a high-pass filter (double background) to remove in-common low-intensity signals. Although this process carries the risk of removing genes which are fully repressed in controls and fully expressed in the treatments (or vice versa), it is likely that expression values calculated from such genes would exhibit falsely large ratios of change. After high-pass filtering approximately 14,300 genes remained. Differentially expressed genes at each

concentration were then identified using t-statistics for difference of means in the replicate data. Within the gene lists, at each HD concentration, few simple convergences were detected. However ontology analysis using Onto-Express [31] revealed differential expression of multiple classes (Table 3 and 4). It is apparent that at either 50 or 100 µM HD, transcriptional functions are widely affected. At 50 µM there appear to be a greater number of groups differentially affected, but this diversity is also driven by the prefiltering and statistical tests applied (high-pass and t–test), which may lead to different gene lists being generated for different concentrations.

Mapping gene expression results to the KEGG pathways [32] typically resulted in a small fraction of genes within any given pathway. However, when mapping onto the mitogen-activated protein kinase (MAPK) pathway using the KEGG toolset [32], it was determined that members of the MAPK regulatory network were disproportionately represented (Figure 2). This had not been seen in the ontology analysis, likely because of the different functional aspects of the pathway which were involved or annotated. The MAPK pathway has been previously implicated in HD toxicity [47, 48], and studies exploring the response of HD toxicity to p38MAPK inhibitors have subsequently been published.

Table 3: Ontology clusters with corrected p value <0.05, at 100 µM HD, 1 hour.

Category	Gene Ontology : Genes
Regulation of transcription	0006355: KIAA0194, ZNF84, PLAGL2, TBL1XR1, TRIM66, SF1, MAF1, RFX3, ETV6, DPF1, SOX2, MYSM1, PCGF6, RERE, PAX1, NR3C2, ATXN3, MITF, CASZ1, EGR1, NPAS3, THRAP2, CCNL2, MCM7, E2F2, ZNF606, BRPF3, ZNF350, ONECUT2, ZNF557, ZNF559, RAX, JMJD1C, KHSRP, POU2F1, MYCBP2, PRRX2, C20orf20, TCF2
Wnt receptor signalling cascade	0016055: CSNK2A2, CSNK2A1, BCL9
Intracellular signaling cascade	007242: ROCK1, PIK3R2, BLNK, PLCD4, MAGI3, PIP5K3, PLCB1, DCAMKL2, NCF1
Lipid catabolism	0061402: PLCD4, APOC2, PLCB1
Transcription	0006350: ZNF84, PLAGL2, TBL1XR1, SF1, SIN3A, MAF1, ETV6, POLR1B, KLF12, DPF1, SOX2, PCGF6, PAX1, NR3C2, ATXN3, CASZ1, EGR1, THRAP2, CCNL2, NCOA4, MCM7, E2F2, ZNF606, ZNF350, TP53BP1, CRSP3, ZNF557, ZNF559, KHSRP, POLR2G, MYCBP2
Development	0007275: RERE, PAX1, ARIH2, ARIH2, MITF, ANGPTL2, NGFB, RAX, LIF, PRRX2, DOPEY2
Nervous system development	0007399: KCNN3, FABP7, ST8SIA2, ATXN3, EFNB1, APBA1, PCDHB17, NGFR, PCDHA6, DBN1, CACNA1A
Positive regulation of transcription	0045893: NCOA3, NCOA4, TP53BP1, TXNDC
mRNA export	0006406: MAGOH, HRB, KHSRP
Phosphate transport	0006817: COL13A1, COL9A2, COL18A1, ADIPOQ

Table 4: Ontology clusters with corrected p value <0.05, at 50 μM HD, 1 hour.

Category	Ontology Cluster ID: Genes
Transcription	0006355: PITX3, ZNF43, KCNH2, HDAC8, ZFHX1B, EZH2, RELA, PER1, PHF16, MITF, HDAC3, LHX6, HESX1, MCM7, AIRE, GATA2, ZBTB37, DLX2, ZNF250, NHLH2, MSL3L1, SIRT6, LHX2, PHF8, TCEB2, FOXC2
Immune response	0006955: IL26, LST1, SECTM1, SCAP1, AFP, DEFB4, HLA-DQA2
Nucleosome assembly	0006334: H2AFX, H2DFV
Mismatch repair	0006298: MSH4, MSH5
Organ Morphogenesis	0009887: SYK, PITX3, DGCR2, GNPAT
Muscle contraction	0006939: KCNMB1, CNN3
I-kappaB kinase/NF-kappaB cascade	0043123: RELA, SECTM1, ZDHHC13
Innate immune response	0045893: NCOA3, NCOA4, TP53BP1, TXNDC
Chromatin assembly	0006333: HDAC8, MSL3L1
Cell cycle	0000074: PCTK2, VEGFB, PLK3, HDAC3, MATK, NOL1, CCNF, MAGEC2
Notch signalling	0007219: APH1B, RBPSUH
Humoral response	0019735: IL26, MGST2, IGSF2
Mitochondrial e transport	0006120: NDUFV3, NDUFS4, NDUFB8
Development	0007275: CUTL1, PITX3, ARIH2, MITF, HESX1, GCNT2, SHH, NNAT, DLX2, MSL3L1
Cell-cell signalling	0007267: IL26, MGST2, AREG, SHH, IAPP, STC2, ADORA2
Cholesterol biosynthesis	0006695: MVD, FDFT1, HMGCR
G-protein signaling	0007186: C1S, PTGER1, APLP2, GPR157, DEFB4, ADORA2A
Brain development	0007420: LHX6, HESX1, NNAT, DLX2
Induction of apoptosis	0008629: LGALS12, CUL4A
Epidermis development	0008544: KRT10, KRT2, KRT1
Ubiquitin Cycle	0006512: USP10, ARIH2, UBE1, SMURF2, DCST1, FBXO22, UEVLD, FBXO31, TCEB2, CUL4A
Muscle contraction	0006936: KCNH2, SLC8A1, CHRND, RYR2
Cell adhesion	0007156: AMIGO1, PCDHGC3, PCDHA6
Response to drug	0042493: MVP, ABCB4, CSAG2
Neurotransmitter secretion	0007269: SYN3, NOS1AP
Cell motility	0006928: KAL1, ELMO1, VASP, CAPZB

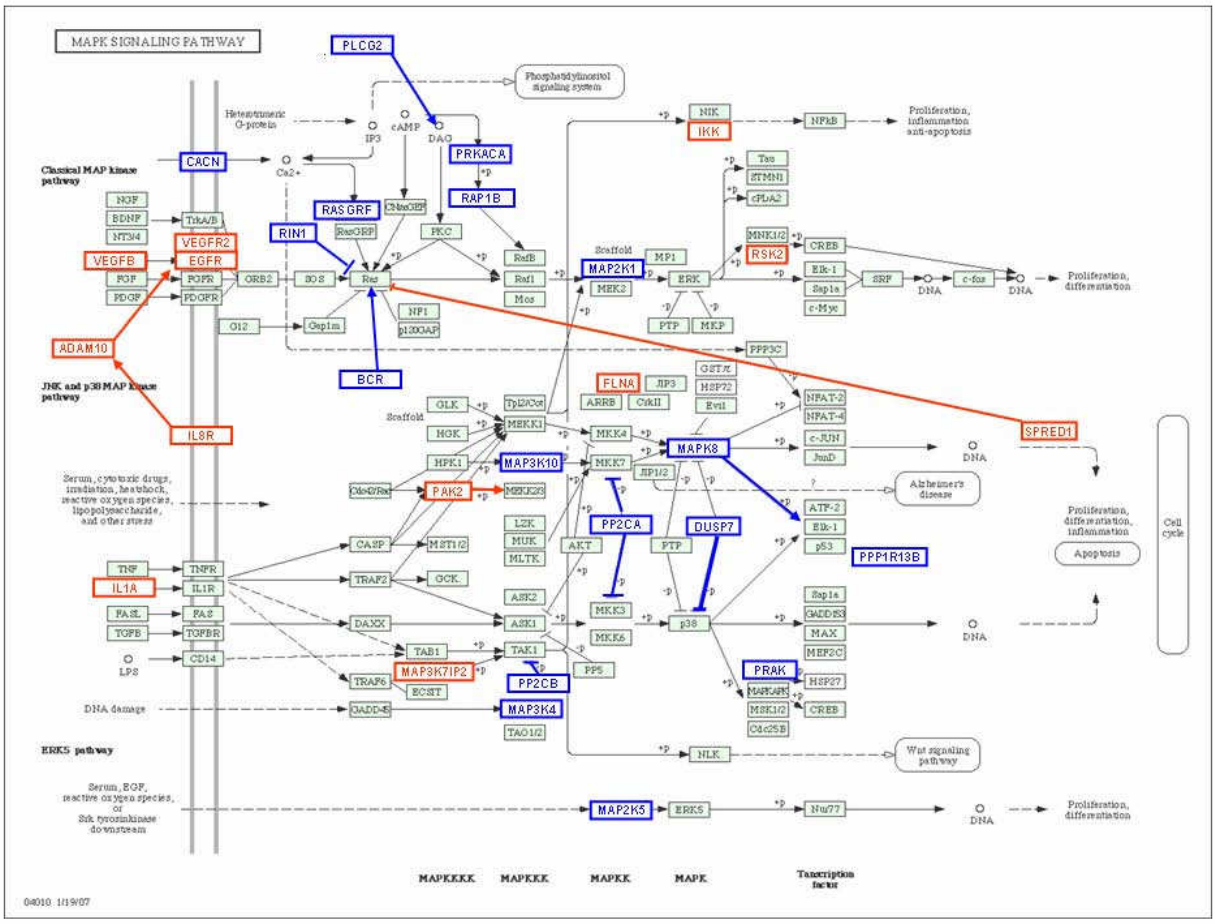

Figure 2: Differentially expressed genes in the MAPK signaling network. Blue boxes indicate down regulation, red boxes indicate up-regulation.

Interestingly, PPM1A (aka PP2A), a multifunctional signaling protein phosphatase with several effects in and around the MAPK network, also differentially regulated after HD exposure, has been shown to have a role in HD toxicity [49]. It was also reported that okadaic acid and calyculin A, profound inhibitors of PP2A, could rescue exposed cells from cell cycle arrest, a toxic effect of HD at sub-vesicant doses [50, 51]. While this result has not yet been replicated, the connection is an intriguing one. Paradoxically, okadaic acid also causes apoptosis via the mitochondrial/caspase/ PARP pathway, in a p38(MAPK14)-dependent response [52, 53].

One of the downstream effects of p38MAPK (MAPK14) is a change in the permeability pore of mitochondria towards depolarization of the mitochondrial membrane. One proposed mechanism is activation of p38MAPK by some insult, which mediates the translocation of Bax from cytoplasm to the mitochondrion as an immediate-early proapoptosis response [54, 55]. Bax in turn interacts with Bid, another proapototic protein, modifying the Bid interaction with the mitochondrial permeability transition pore (MPTP), increasing its permeability to ions, and leading to a loss of polarization across the mitochondrial membrane. Loss of polarization means that mitochondrial energy (ATP) productions fails, and the cell experiences a net loss of ATP. Energy depletion is itself a positive inducer of apoptosis, so the MPTP response tends to increase the apoptotic response [56]. An alternative model suggests that p38MAPK activation may cause

early cytochrome C release from the mitochondria, which in turn leads to caspase activation, and through a cascade of events leads to loss of mitochondrial polarization via changes in the MPTP [58]. Associated effects of the depolarization include activation of other proapoptotic functions, PARP and caspase activation, which have also been reported individually as consequences of HD exposure [58,59]. p38MAPK inhibitors have been reported to have some effect in rescuing cells from downstream effects of HD-induced cytoxicity [47]. These observations suggest the hypothesis that mitochondrial effects (e.g depolarization), driven by the early response seen in the MAPK network, might be detected as early events after HD exposure.

In order to probe this hypothesis around the MAPK pathway, and to test previous reports about the involvement of mitochondrial mechanisms in HD cytoxicity, we analyzed the temporal change of mitochondrial pore permeability via confocal microscopy with the fluorescent dye JC-1. JC-1 has been shown be a good indicator of mitochondrial pore permeability changes caused by various cell stressors [59,60]. Loss of mitochondrial polarization is generally considered an irreversible effect, leading to cell death via apoptosis or necrosis. Hydrogen peroxide also causes rapid membrane depolarization in the mitochondria detectable by JC-1 [60-62], and this is considered diagnostic of significant cytoxicity. In the presence of HD, rapid transition of the dye from red to green was observed, indicating changes in pore permeability leading to loss of mitochondrial polarization (Figure 3) within 50 minutes. Quantitation of green to red fluorescence from stored images, indicates loss of polarity of the mitochondrial membrane (Figure 4). The rapid depolarization observed after HD exposure suggests that efforts to protect cells from HD-induced cytotoxicity, should be prophylactic or initiated very soon after exposure in order to have maximal effectiveness. At higher HD concentrations the observed depolarization effect was less, suggesting the involvment of an alternate mode of apoptosis or necrosis which was independent of mitochondria. It is also notable that despite the immediate-early mitochondrial effects observed at 50 μM HD, the initial cytotoxicity data at 24 hours revealed greater than 90% survival. This suggests that immediate-early effects such as loss of mitochondrial membrane polarity may be followed by a latent phase in which cells committed to apoptosis are trancriptionally active, expressing functions required for orderly cell death. It remains to be tested whether HD exposure causes early release of cytochome C from the mitochondria, or whether other markers of apoptosis (e.g. caspase activation) are detected later.

Cell death via the MAPK mechanism is not a direct track from insult to apoptosis. Indeed, it appears that among the identified mechanisms of apoptosis, certain points in each pathway act as cross-connections between mechanisms [62–64]. The interactions within the MAPK signaling network (Figure 2) illustrates the degree to which feed-back, feed-forward and branching events create a network of response. Multiple interactions with other signaling mechanisms also exist but are not indicated in Figure 2.

If the vesicant or systemic effects of HD arise predominantly from or in response to the initial cytotoxic events, this supports the contention that rapid intervention after exposure will be essential to minimize overt damage. It may also be possible to intervene against downstream effects of HD-induced mitochondrial changes. Previous reports have indicated that post-exposure hypothermia of exposed regions of skin substantially reduces the severity of HD-induced damage if initiated within minutes of exposure [5], but it remains unresolved at a molecular level whether this is due to reduction of direct cytoxicity, or to prevention of subsequent tissue inflammation.

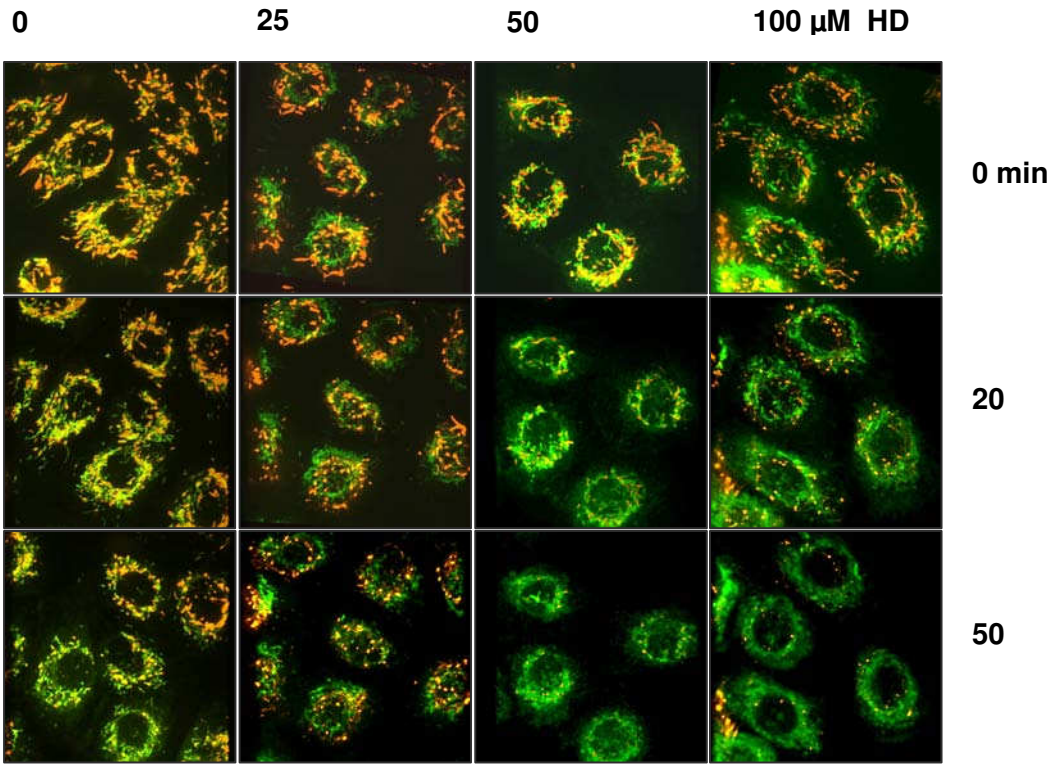

Figure 3: JC-1 fluorescence images: HD concentration versus time.

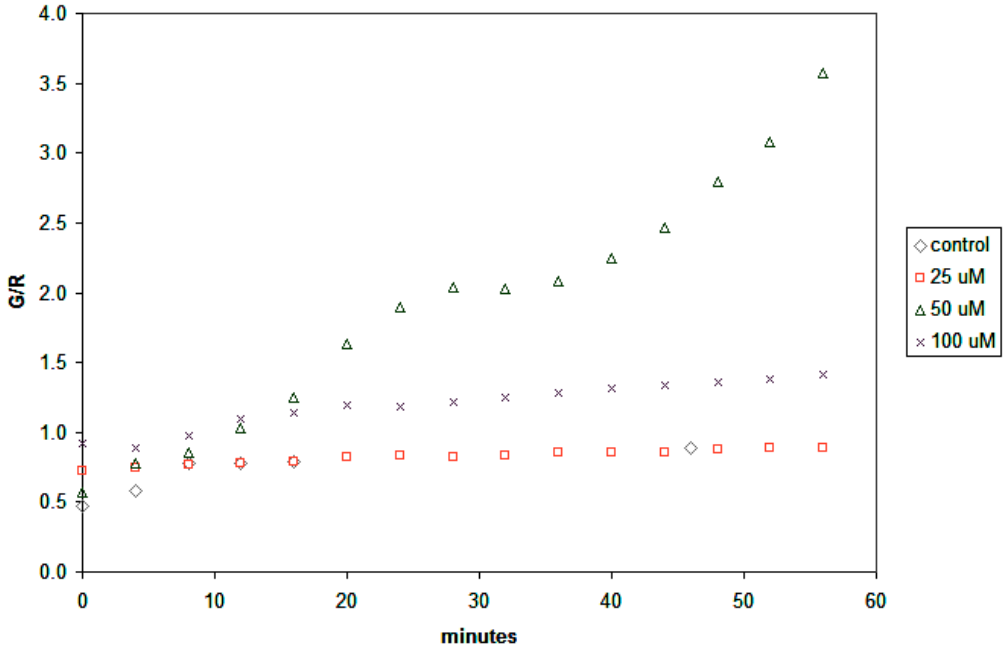

Figure 4: Green to red fluorescence ratio vs time at varying concentrations of HD.

Assuming that the precipitating response after HD exposure is a single receptor or molecular interaction, the network of molecular interactions in response to toxic insult can lead to different outcomes depending on the kinetics of the downstream interactions. Thus single-event changes as functional probes (e.g. knock-out cell lines) are unlikely to elucidate the entirety of the response mechanism.

In some cases inhibition of individual functions along the pathway may not have the effect of preventing apoptosis, but rather cause a temporary or terminal change in the course of events which might be interpreted as a novel pathway. Since the mechanisms and degree of switching between connected parts of the network are not well understood, and may well be dependent on cell cycle, energy status, or differentiation of the cell, a wide variety of apparently different outcomes might be observed which are subtle variations on the central theme. If more than one or two switch-points exist within the network, and the direction of switching is life-history dependent, it may well prove to be impossible to predict the outcome of specific pathways in a general experimental system.

On the other hand, it is also possible that a single inhibitory molecule can affect more than one function in the cell, thus the pleiotropic effects of an agent such as HD might be prevented by a broad spectrum inhibitor (*e.g.* a “dirty” inhibitor) rather than a single molecular interaction. Future efforts in the area of HD-induced cytotoxicity will attempt to probe the involvement of the MAPK pathway and its regulatory partners, to test whether modulation of different functions of the network can be exploited to prevent the downstream cytotoxic or apoptotic effect of HD exposure.

Conclusions

Sulfur mustard is a highly toxic chemical, with immediate-early effects ranging from mitotic arrest to overt cytotoxicity at micromolar exposure concentrations. The molecular basis of the toxic effects of HD remain poorly understood. In this work, effects observed very soon after HD exposure include changes in expression of genes involved in the MAPK signaling network and changes in the permeability of the mitochondrial membrane. Both of these have reported involvement in cell death pathways including apoptosis and necrosis. In the case of HD, it is possible that the exposure concentration of HD may modulate which mode of cell death occurs, depending on the nature of the signaling networks which are activated. It should be possible to examine these effects using specific signaling and apoptotic mechanism inhibitor molecules, that interfere with specific reactions along the various pathways.

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List of Symbols/Abbreviations/Acronyms/Initialisms

RNA	ribonucleic acid
DNA	deoxyribonucleic acid
MAPK	mitogen activated protein kinase
mRNA	messenger RNA
d{n}TP	deoxy{A; adenosine: C; cytosine: G; guanosine: T; thymidine} triphosphate
cDNA	complementary DNA; product of reverse transcription reaction
Cy3	fluorescent dye of the cyanine dye family, Cy3 has 555 nm excitation and 570 nm emission

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Sulfur mustard (bis-(2-chloroethyl) sulphide, HD) is a well-known chemical warfare agent which causes skin blisters, respiratory tract injury, and systemic toxic effects. The underlying molecular basis of HD toxicity is not well understood. Multiple hypotheses attempting to improve understanding of this problem have been proposed, including DNA damage and repair, inflammation, aberrant cytokine expression, direct protein modification to keratin fibres, and combinations of these effects. Despite such efforts, there is little consensus on the biological basis for the profound observed toxicity of HD. A particularly puzzling aspect is the terminal arrest of cultured cells at concentrations of HD substantially lower than those at which cytotoxic effects are seen, suggesting there may be multiple pathways through which HD can exert its biological effects. Previously, Sawyer and others have proposed receptor-based interactions as being at the root of HD induced cytotoxic effects. In order to explore this hypothesis, gene expression profiles of first passage cultures of human keratinocytes were developed using an oligonucleotide microarray platform. Analysis of data from six independent donors at multiple concentrations of HD and varying time points revealed a complex response. Combined data at adjacent time points enabled the identification of the mitogen-activated protein kinase (MAPK) network and genes associated with it, as primary functions responding to sulfur mustard. Published work on the role of apoptosis in HD cytotoxicity led to the hypothesis that mitochondrial permeability modulated as a downstream effect of the MAPK network might be involved in the HD response. Preliminary data from live-cell studies demonstrated an immediate-early mitochondrial membrane change which may be a critical early event in HD-induced cytotoxicity. These studies are a step towards refining our understanding of the toxic effects of HD in cells.

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microarray, sulfur mustard, MAPK pathway, therapeutics

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