Gene Expression Responses to Sulphur Mustard

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Sulphur mustard (bis-(2-chloroethyl) sulphide, HD) is a well-known agent of chemical warfare. HD causes skin blisters, respiratory tract damage, and other toxic effects. The underlying biochemical response leading to the observed vesicant damage by HD is not well understood. Multiple hypotheses attempting to gain traction on 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, nor indeed for its vesicant properties. A particularly puzzling aspect is the terminal arrest of cultured cells at doses of HD substantially lower than those at which cytotoxic effects are seen, suggesting that there may be multiple pathways through which HD can exert biological effects. Previously, Sawyer and others have proposed receptor-based interactions as being at the root of HD induced cytotoxic effects, and thus of the overt vesicant action. In order to explore this hypothesis, gene expression profiles of first passage cultures of human keratinocytes were developed using an oligonucleotide microarray platform. At concentrations exceeding 200 µM HD, total RNA yield and quality from harvested cells was consistently reduced. This suggested that global transcription was substantially affected, and that some published data may be measures of relative RNA degradation rather than modulated gene expression. Analysis of data from six independent donors at multiple concentrations and time points revealed a complex response. Multiple genes exhibited expression changes, but gene lists had few convergences at different time points. At lower concentrations of HD, combined data at adjacent time points enabled the identification of the mitogen-activated protein kinase (MAPK) pathway and genes associated with it, as primary functional groups responding to sulphur mustard. Data from the literature have tended to support this hypothesis. We have expanded this study with directed experiments on the mitochondrial role in HD-induced apoptosis, affected by the MAPK regulatory network, demonstrating early effects of HD on mitochondrial permeability. These studies are a step towards refining our understanding of the toxic effects of HD in cells.

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1. INTRODUCTION

Sulphur 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. Sulphur mustard is related in its vesicant properties to nitrogen mustards, widely used in chemotherapy against cancer. 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 be critical. If the window of opportunity for treatment is missed, the vesicant or systemic toxic effects manifest according to the dose.

Previous work in HD toxicity has looked at the DNA damaging properties of HD 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 sulphur mustard causes alkylation of bases and interstrand crosslinks. Such lesions are known 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. Previous work has also assessed the usefulness of nitric oxide synthase (NO) inhibitors, based on the hypothesis that the toxic effects of HD might be mediated by nitric oxide [13,14]. Initially this seemed like a promising approach, and multiple 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, Sawyer at al. also 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 the HD and the protective NOS inhibitors have also been ruled out.

The 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 the 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, we examined gene expression effects in cultured primary human keratinocytes after HD exposure. Using oligonucleotide microarray analysis of mRNA expression, we compared multiple donors at varying HD concentrations and time points after exposure. After statistical analysis and data review, the 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 family was consistently involved early in the HD response of NHEK, including several genes related to the function of p38MAPK (MAPK14). From the observed MAPK effects, we hypothesized that depolarization of mitochondria is 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 vesicant doses of HD.

2. MATERIALS AND METHODS

2.1 Cell Culture

Culture of primary human keratinocytes from neonatal foreskin explants has been previously documented by this laboratory (57,58). Primary cultures of human skin keratinocytes were prepared from neonatal foreskins following an established method and in accordance with approved Human Subjects guidelines. Tissue was usually obtained on the day of circumcision and incubated at 4 ºC 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 ºC. 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 x 10^5 cells/8 ml KSFM supplemented with gentamicin (50 µg/ml) and Fungizone (0.25 µg/ml) and incubated in a 37 ºC 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 x 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, ONT).

2.2 HD Exposure

Sulphur mustard was prepared and analyzed at the Canadian National Small Scale Synthesis 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 ºC 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 were allowed to incubate with the dye for the last 2 – 3 hr of the 48 hr treatment time period. The absorbance at two wavelengths (570 nm - 600 nm) were then read on a Thermomax titerplate reader (Molecular Devices, Sunnyvale, CA). Median lethal concentration (LC50) 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.

2.3 RNA Isolation

At varying time points after HD exposure ranging from 1 minute to 24 hours, 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 KGM with 20% serum. Cell suspensions were transferred to clean 15 ml polypropylene tubes, and cells collected by centrifugation at 600 RPM x 2 minutes. Cell pellets were transferred to clean microcentrifuge tubes and frozen at -80 ºC until use. For RNA isolation, cell pellets were thawed briefly on ice, then RNA was purified using the Versagene RNA Cell Kit (Intermedico, Markham, ONT). 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, ONT) capillary electrophoresis system.

2.4 RNA Labelling and Hybridization

Purified RNA was thawed on ice, then labelled 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 ºC.

After labelling, the cDNA product was briefly heated to 95 ºC, then added to a preheated (65 ºC) hybridization solution containing 5% dextran sulphate, 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 ºC.

After hybridization, microarrays were immediately transferred to 0.1% SDS at room temperature to rinse off excess hybridization solution, then rinsed with deionised 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 [29], Bioconductor, and other open source microarray packages.

2.5 Mitochondrial Permeability Pore Analysis

Primary keratinocytes cultured in small dishes (Ibidi) were pre-loaded with JC-1 mitochondrial permeability indicator dye (Molecular Probes, Carlsbad, CA) dissolved in DMSO, for 30 minutes at 5 µg/ml. Following incubation, 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 in ethanol was added to the final concentration required, or ethanol without HD in control samples. Images were then analysed using ImageJ [30], to align frames and extract signal intensity data from the aligned frames in each colour. Extracted intensities were plotted versus time in MS Excel. Transition of the mitochondrial pore is detected by a shift from red towards green fluorescence with redistribution, and comparison of the pre- or post-HD exposure images.

3. RESULTS AND DISCUSSION

The initial design of this experiment involved HD exposures of up to 400 µM to keratinocytes in culture. Cytotoxicity data (Table 1) revealed that survival (by alamarBlue assay) at 24 hours after HD exposure of 200 µM or greater resulted in less than 10% survival (Figure 1). Individual donor samples exhibited quite different LC50 results at 24 hours, but by 48 hours, with one exception, LC50 results were within one standard deviation of the mean. This may indicate 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 5-50 fold) under experimental conditions could be due to differences in DNA repair [31-33].

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. Pre-labelling analysis of RNA extracts did not reveal obvious

degradation of total RNA or ribosomal fragments within the RNA pool at any of the exposure concentrations. 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 concentrations, or due to direct interference with transcription. The response of transcription to HD has been previously reported [34-37], and the data at HD concentrations in excess of 100 µM are consistent with these observations. Published data on sulphur mustard and gene expression frequently utilize HD concentrations which have reported strong effects on transcription [10,35,36,38,39], suggesting that some of the differences reported therein 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.

At HD exposure concentrations of 100 µM or less, gene expression data were first normalized using q-spline normalization in the Bioconductor package of the R statistics package [40]. 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 hi-pass filtering approximately 14,300 genes remained. Differentially expressed genes at each dose were 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. (Due to their length, the gene list data are not included here. The data lists may be obtained from the authors.) However ontology analysis using Onto-Express [29] revealed differential expression of multiple ontological classes (Table 2 and 3, end of section), which did reveal overlaps. From the summary tables, 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 ontology groups differentially affected, but this diversity is also driven by the statistical prefiltering applied (t-test and hi-pass), which may lead to different gene lists being tested at different doses.

A

DONOR	0 µM	50 µM	100 µM	200 µM	400 µM	LC50 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

B

DONOR	0 µM	50 µM	100 µM	200 µM	400 µM	LC50 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

Table 1: Relative cytotoxicity of HD in cultured keratinocytes. A: 24 hours. B: 48 hours

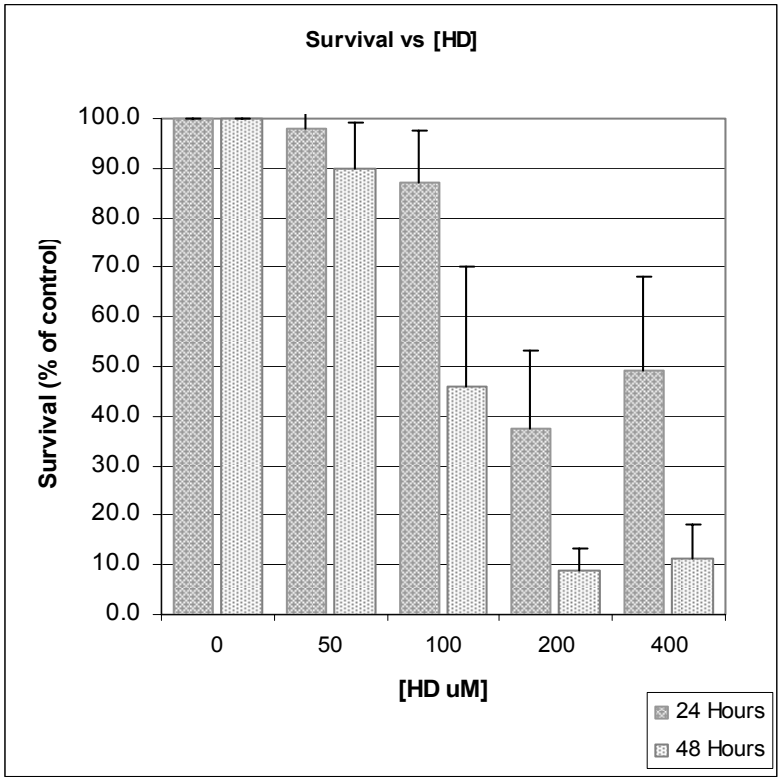

Figure 1: Relative survival of keratinocytes after HD exposure

Mapping gene expression results to the KEGG (Kyoto Encyclopedia of Genes and Genomes [41]) pathways typically resulted in a small fraction of genes within any given pathway. However, when mapping onto the MAPK pathway using the KEGG toolset [41], 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. The MAPK pathway has been previously implicated in HD toxicity [42,43], and studies exploring the response of HD toxicity to p38MAPK inhibitors have subsequently been published. Interestingly, PPM1A (aka PP2A), a multifunctional signalling protein phosphatase with several effects in and around the MAPK network, also differentially regulated after HD exposure, has been shown to have an interesting role in HD toxicity [44]. It was previously 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 [45]. 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 [46,47]. MAPK-dependent apoptosis also leads to PARP cleavage and activation, frequently reported as a indicator of HD toxicity [23,31,48,49].

One of the downstream effects of p38MAPK (MAPK14) is a change in the permeability pore of mitochondria towards depolarization of the mitochondrial membrane. Associated effects of the depolarization include activation of proapoptotic functions (bax, bcl etc.), PARP and caspase activation, all of which have been

reported individually as consequences of HD exposure [23,26,49]. p38MAPK inhibitors have been reported to have some effect in rescuing cells from HD-induced cytotoxicity [42].

In order to probe our hypothesis around the MAPK pathway, and to test previous reports about mitochondrial mechanisms in HD cytotoxicity, we analyzed the temporal change of mitochondrial pore permeability via confocal microscopy with the fluorescent dye JC-1. This vital dye has been shown be a good indicator of mitochondrial pore activity [50,51]. 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). 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 [52-54], and this is considered diagnostic of significant cytotoxicity. 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. Notably higher HD concentrations lessen the depolarization effect on mitochondria, possibly due to a transition to another mode of (mitochondria-independent) apoptosis, or necrosis. Cell death via the MAPK network 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 [55,56]. The network of interactions within the MAPK signalling mechanisms (Figure 2) illustrates the degree to which feed-back, feed-forward and branching events create a network of response. Multiple interactions with other signalling mechanisms also exist but are not indicated here.

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 cytotoxicity, prevention of subsequent tissue inflammation, or to immobilization of a dermal HD reservoir.

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, 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, thus the toxic effects of an agent such as sulphur mustard 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 sulphur mustard-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.

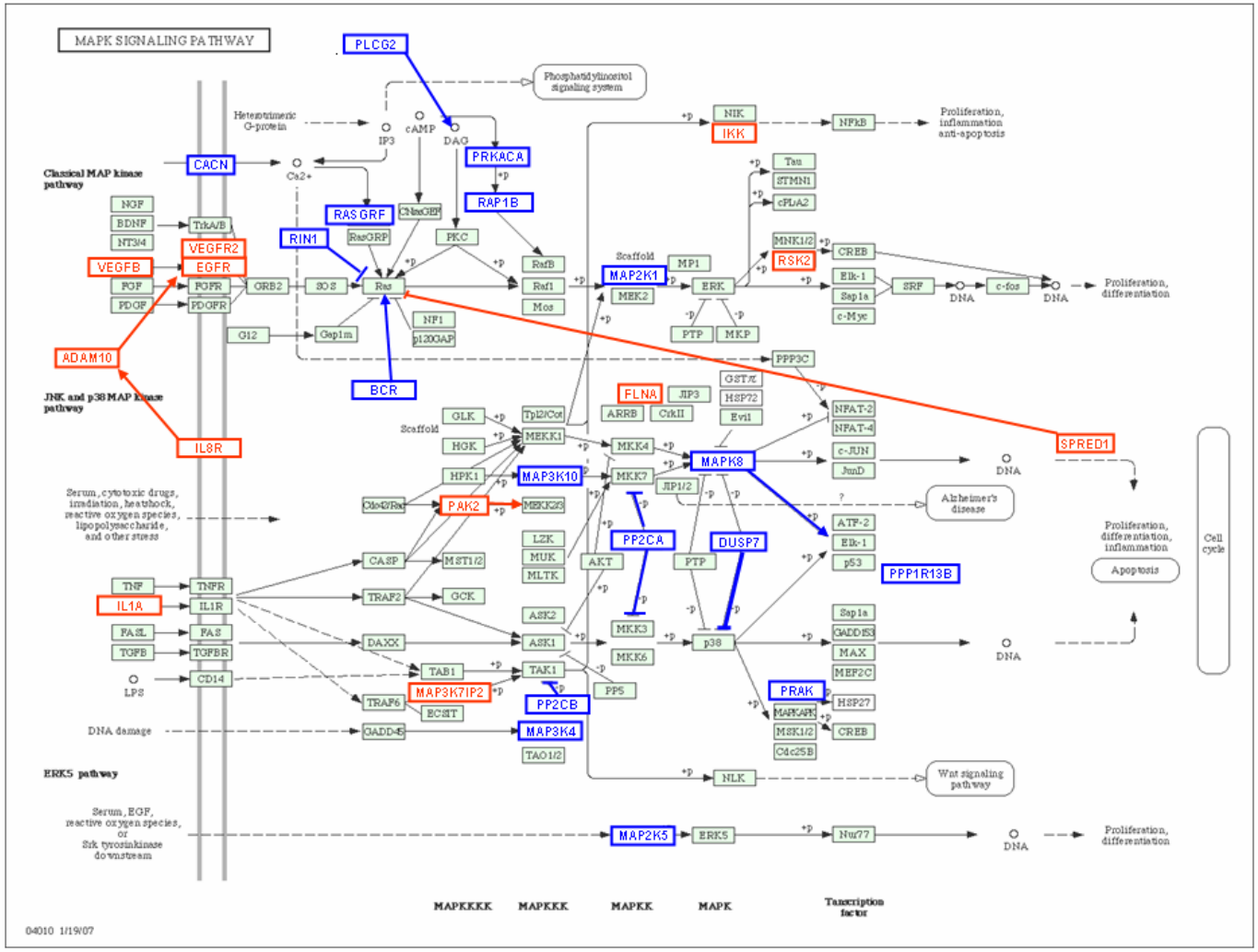

Figure 2: Differentially expressed genes in the MAPK signalling network. Blue boxes indicate down regulation, red boxes indicate up-regulation.

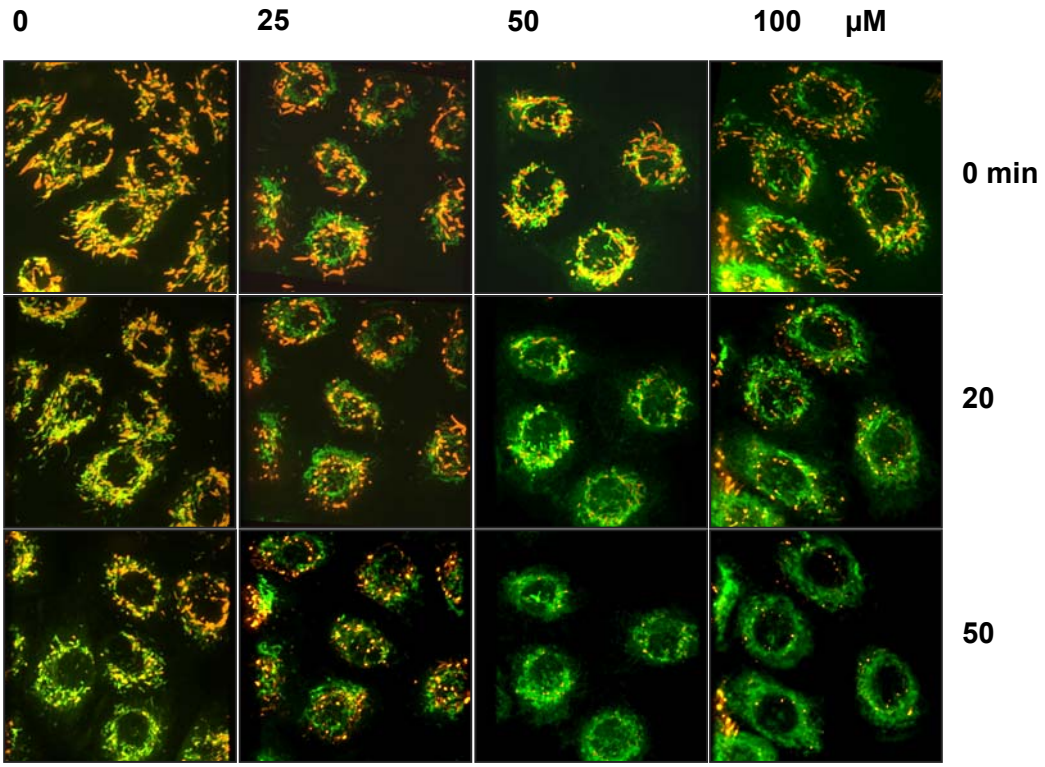

Figure 3: JC-1 fluorescence images versus HD and time.

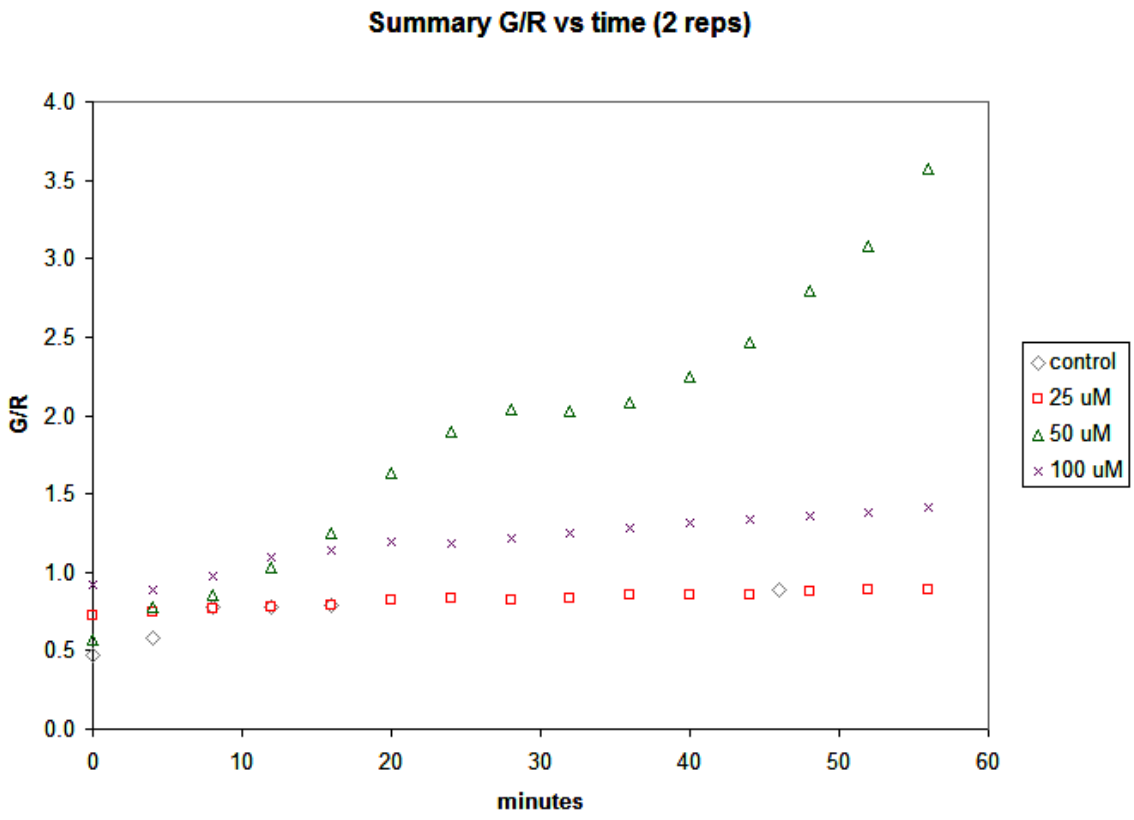

Figure 4: Green to red fluorescence ratio vs. time at varying [HD].

Table 2: 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 signalling cascade	007242: ROCK1, PIK3R2, BLNK, PLCD4, MAGI3, PIP5K3, PLCB1, DCAMKL2, NCF1
Lipd 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 3: Ontology clusters with corrected p value <0.05, at 50 µM HD, 1 hour.

Category	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 immue 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

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