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**Pro-oxidant Induced DNA Damage in Human Lymphoblastoid Cells: Homeostatic Mechanisms of Genotoxic Tolerance**

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3 **Pro-oxidant Induced DNA Damage in Human Lymphoblastoid Cells: Homeostatic**  
4 **Mechanisms of Genotoxic Tolerance**  
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6

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27 **Running title: Pro-oxidant Genotoxicity and Thresholds levels**  
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**Abstract**

Oxidative stress contributes to many disease aetiologies including ageing, neurodegeneration, and cancer, partly through DNA damage induction (genotoxicity). Understanding the interactions of free radicals with DNA is fundamental to discern the mutation risks posed. In genetic toxicology, regulatory authorities view most genotoxins to exhibit a linear relationship between dose and mutagenic response. Yet, homeostatic mechanisms exist, including DNA repair, which allow cells to tolerate low levels of genotoxic exposure. Acceptance of thresholds for genotoxicity has widespread consequences in terms of understanding cancer risk and regulating human exposure to chemicals/ drugs. Three pro-oxidant chemicals, hydrogen peroxide ( $H_2O_2$ ), potassium bromate ( $KBrO_3$ ), and menadione, were examined for low dose-response curves in human lymphoblastoid cells. DNA repair and antioxidant capacity were assessed as possible threshold mechanisms.  $H_2O_2$  and  $KBrO_3$ , but not menadione, exhibited thresholded responses, containing a range of non-genotoxic low doses. Levels of the DNA glycosylase OGG1 were unchanged in response to pro-oxidant stress. DNA repair focussed gene expression arrays reported changes in *ATM* and *BRCA1*, involved in double strand break repair, in response to low dose pro-oxidant exposure, however, these alterations were not substantiated at the protein level. Determination of oxidatively induced DNA damage in  $H_2O_2$ -treated AHH-1 cells reported accumulation of thymine glycol above the genotoxic threshold. Further, the  $H_2O_2$  dose response curve was shifted by modulating the antioxidant glutathione. Hence, observed pro-oxidant thresholds were due to protective capacities of base excision repair enzymes and antioxidants against

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3 DNA damage, highlighting the importance of homeostatic mechanisms in “genotoxic  
4 tolerance”.  
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### 10 **Keywords**

11 Pro-oxidants; DNA damage; reactive oxygen species; DNA repair; OGG1; antioxidants;  
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13 glutathione; genotoxicology; thresholds  
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### 18 **Introduction**

19  
20 Assessing the genotoxic threat of chemicals is essential in gaining a better  
21 understanding of their carcinogenic potential to reduce any deleterious effects that may  
22 be produced through occupational and recreational exposures (Doak et al., 2007;  
23 Sedelnikova et al., 2010). Traditionally, regulatory authorities utilise a linear model to  
24 assess the safety of direct-acting genotoxins, whereby a mutagenic response at high doses  
25 is extrapolated to lower doses. The so-called “single hit, single target” hypothesis, infers  
26 there is no minimal safe exposure limit for such agents (Jenkins et al., 2005). This view,  
27 however, does not account for the plethora of homeostatic mechanisms which allow  
28 mammalian cells to tolerate low levels of genotoxins. The application of a threshold  
29 mechanism in toxicology is not new, it is widely accepted that indirect-acting genotoxins,  
30 which have non-DNA targets, may exhibit a threshold mode of action (MOA) (Elhajouji  
31 et al., 2011). To date, however, the effect has been established experimentally for a  
32 limited number of DNA reactive compounds (Doak et al., 2007; Jenkins et al., 2005;  
33 Platel et al., 2009). We recently demonstrated that genotoxic thresholds induced by the  
34 alkylating agents ethyl methane sulfonate (EMS) and methyl methane sulfonate (MMS)  
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3 are due to DNA repair by the base excision repair (BER) enzyme *N*-methylpurine DNA  
4 glycosylase (MPG; [GenBank ID:4350](#)) and DNA repair protein *O*<sup>6</sup>-methylguanine-DNA  
5 methyltransferase (MGMT; [GenBank ID:4255](#)), selectively removing DNA adducts at  
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10 low doses but becoming saturated (or repressed) at higher doses (Doak et al., 2008; Zair  
11 et al., 2011). The acceptance of dose-response thresholds for genotoxins may have  
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An important class of DNA reactive agents present in the human environment are pro-oxidants. Mammalian cells are constantly exposed to potentially damaging reactive oxygen species (ROS) arising from multiple sources (Evans et al., 2004; Loft et al., 2008). Cellular defences exist to combat attack from ROS, including the antioxidant glutathione (GSH) and the scavenging enzyme superoxide dismutase (Forman et al., 2009). Despite this, oxidative stress and damage to cellular macromolecules can arise when the number of ROS produced exceeds the antioxidant capacity of the cell.

Numerous types of DNA damage can potentially arise upon exposure to ROS, with thymine bases being the most susceptible to modification and thymine glycol representing an important thymine lesion formed after treatment by oxidising agents such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Basu et al., 1989).

A network of complex DNA repair pathways has thus evolved to avoid the perpetuation of such damage. Oxidised base lesions are repaired predominantly by the BER pathway. BER is mediated by damage-specific DNA glycosylases; with 8-oxoguanine DNA glycosylase (OGG1; [GenBank ID:4968](#)) repairing one of the most common forms of oxidatively generated DNA base damage, that is, 8-oxo-7,8-

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3 dihydroguanine (8-oxoG) (Boiteux and Radicella, 2000; Wallace, 2002). The role of  
4 repair in maintaining cellular homeostasis is highlighted by reports of BER gene  
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6 activation in response to genotoxic exposure (Powell et al., 2005). Further, mutation and  
7  
8 abnormal expression of DNA repair genes, such as *ATM* (ataxia telangiectasia mutated;  
9  
10 [GenBank ID:472](#)) and *BRCA1* (breast cancer 1, early onset; [GenBank ID:672](#)), have been  
11  
12 directly linked with genomic instability and cancer development (Hartman and Ford,  
13  
14 2003; Smirnov and Cheung, 2008).  
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20 The present study focused on DNA-pro-oxidant interaction and generated  
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22 genotoxic dose responses for three pro-oxidant chemicals, H<sub>2</sub>O<sub>2</sub>, menadione, and  
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24 potassium bromate (KBrO<sub>3</sub>). Two different mutagenic endpoints were assessed *in vitro* as  
25  
26 recommended by Committee on Mutagenicity (COM); the cytokinesis block  
27  
28 micronucleus (CBMN) assay and *HPRT* ([GenBank ID:3251](#)) forward mutation assay,  
29  
30 examining induction of chromosomal damage and frequency of point mutations,  
31  
32 respectively (Great Britain. Committee on Mutagenicity of Chemicals in Food, 2000).  
33  
34 The principle aims being to analyse low-range dose-response curves, to establish the  
35  
36 existence of possible genotoxic thresholds, and explore MOA of any observed thresholds  
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38 (tolerance). Compounds were chosen due to their known capacity to generate DNA  
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40 damage through differential generation of various ROS.  
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46 H<sub>2</sub>O<sub>2</sub> is a physiological constituent of living cells, continually produced by a  
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48 variety of cellular pathways, and has a wide range of external applications, for instance,  
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50 in bleaching and in the treatment of water and sewage (Jeong et al., 2010; Naik et al.,  
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52 2006). H<sub>2</sub>O<sub>2</sub> is a nonradical ROS but can react via radical-mediated routes, for example,  
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54 in the presence of ferrous ions undergoes Fenton's reaction to form the highly reactive  
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3 hydroxyl radical ( $\text{HO}^\bullet$ ) (Pryor, 1986). Menadione is a multivitamin component and a  
4 therapeutic agent for hypothermia and cancer. In the presence of flavoenzymes  
5 menadione may undergo reduction to a semiquinone; an extremely unstable compound  
6 that reacts rapidly with oxygen forming superoxide anion radical ( $\text{O}_2^{\bullet-}$ ) and other ROS  
7 (Chung et al., 1999; Nutter et al., 1992).  $\text{KBrO}_3$  has been used as a food additive  
8 primarily in bread making processes. EU countries now prohibit this application due to its  
9 proven carcinogenicity. The mechanism by which  $\text{KBrO}_3$  generates damaged DNA is not  
10 fully elucidated but is believed to involve reduction of bromate by thiols such as cellular  
11 glutathione to reactive intermediates including bromine radicals ( $\text{Br}^\bullet$ ) or oxides ( $\text{BrO}^\bullet$ ,  
12  $\text{BrO}_2^\bullet$ , etc.) (Ballmaier and Epe, 1995; Ballmaier and Epe, 2006).  
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## 29 **Materials and methods**

### 30 **Chemicals**

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32 Buthionine sulfoximine (BSO),  $\text{H}_2\text{O}_2$ ,  $\text{KBrO}_3$ , menadione, and N-acetylcysteine (NAC)  
33 were all purchased from Sigma (Dorset, UK). All chemical dilutions were freshly  
34 prepared from stock solutions with water.  
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### 43 **Cell culture**

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45 The human, male, near diploid lymphoblastoid cell line AHH-1 (ATCC, Middlesex, UK)  
46 was cultured in RPMI 1640 (Life Technologies, Paisley, UK) supplemented with 1% L-  
47 glutamine (Life Technologies) and 10% donor horse serum (BDGentest, Oxford, UK) in  
48 80-cm<sup>2</sup> flasks at 37°C, 5%  $\text{CO}_2$ . The cells were maintained at a concentration of 1 to 2 x  
49  $10^5$ /mL. AHH-1 cells were utilised in the study as they have been widely used in genetic  
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3 toxicology and represent a versatile and reproducible system for examining genotoxic  
4 agents and the induction of gene locus mutation, including the analysis of damage  
5 response pathways and cellular defences upon free radical exposure. AHH-1 contains  
6 native CYP1a1 activity, and despite harbouring a heterozygous p53 mutation at the codon  
7 281/282 interface within exon 8, retains the ability to undergo DNA damage induced  
8 apoptosis and has been reported to express phospho-p53 and p21 (Doak et al., 2008;  
9 Guest and Parry, 1999). Further, previous studies on thresholds have been completed  
10 utilising AHH-1, and have described stable background levels of chromosomal damage  
11 and point mutations (Doak et al., 2007; Zair et al., 2011).  
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### 27 **Forward mutation assays**

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29 We employed the *in vitro* *HPRT* assay to study induced point mutations. The assay was  
30 performed as previously described (Doak et al., 2007), with the following modifications,  
31  
32 AHH-1 cell suspensions (10mL), at  $5 \times 10^5$ /mL, were exposed to the test chemical in  
33  
34 80cm<sup>3</sup> flasks at the appropriate concentration, for 24h at 37°C, 5% CO<sub>2</sub>. For each dose,  
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36 fifteen 96-well plates for assessing mutation frequency and another five for plating  
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38 efficiency were set up. Each dose was performed in triplicate.  
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### 46 **Micronucleus assay**

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48 Micronuclei (MN) frequency was utilised to assess the level of chromosome aberration  
49 induction. AHH-1 (10mL) suspensions of cells at  $1 \times 10^5$ / mL were seeded for 24h at  
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51 37°C, 5% CO<sub>2</sub>. Replicate flasks (n=3, independently produced on different days) were  
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53 dosed with appropriately diluted test chemical (in duplicate) for 4h, after which cells  
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3 were centrifuged, washed once in PBS, and re-suspended in 10mL fresh media containing  
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5 6µg/mL cytochalasin B for one cell cycle (22h). Treated cells were harvested, re-  
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7  
8 re-suspended in 10mL of hypotonic solution (0.56% KCl), and centrifuged immediately.  
9  
10 Cell suspension was re-suspended in fixative 1 (methanol: acetic acid: 0.9% NaCl (5:1:6  
11  
12 parts)) and centrifuged after a 10min incubation period. Cells were transferred to fixative  
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14 2 (methanol: acetic acid (5:1 parts)) for a 10min incubation, centrifuged, washed 4 times,  
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16 and maintained in the final fixative 2-wash at 4°C for 16h. Fixed cells were centrifuged,  
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18 and 100µL dropped onto polished, fixed, and hydrated slides, stained with DAPI (4', 6-  
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20 diamido-2-phenylindole; 0.15µg/ mL final concentration), and viewed under a Carl Zeiss  
21  
22 Axiolmager fluorescent microscope. Slides were scored utilising the Metafer4 software  
23  
24 version 3.5 (MetaSystems, Altussheim, Germany). An optimal scoring criterion was  
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26 achieved from the development of specific classifiers adjusted to accommodate the  
27  
28 particular lymphoblast cell line (AHH-1), and to identify binucleate cells containing MN.  
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30 The criteria for identifying micronuclei were as previously described (Fenech, 2007). A  
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32 minimum of two thousand binucleated cells were scored per replicate, and each dose was  
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34 performed in triplicate (an average of 6000 binucleates per dose).  
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#### 44 **RNA isolation and gene expression analysis**

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46 Following exposure to the test pro-oxidants for 0h, 2h, 4h, and 24h time-points, total  
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48 cellular RNA was isolated from AHH-1 cells followed by DNase treatment using the  
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50 RNeasy mini kit (Qiagen, West Sussex, UK), and TURBO DNA-free kit (Ambion,  
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52 Huntingdon, UK), respectively, according to the manufacturer's instructions. Quantitative  
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54 PCR of mRNA was performed as a one-step reaction, where the entire reaction from  
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cDNA synthesis to real-time PCR amplification is performed in a single well, on a MyIQ™5 cyclor optics module (BioRad, Hertfordshire, UK). Commercially available Taqman® Gene Expression Assays and human endogenous controls were purchased from Applied Biosystems (UK); supplied as pre-mixed primers and FAM/ MGB probe. To perform one-step real time PCR, *OGGI* target gene (Hs00213454\_m1\*) and *HPRT* reference gene (4333768) probes were used in conjunction with QuantiFast Probe RT-PCR kit (Qiagen). Approximately 0.2µg RNA was used for cDNA synthesis and PCR in a reaction volume of 20µL, containing 10µl of 2x QuantiFast Probe RT-PCR Master Mix, 0.4µl QuantiFast RT Mix, and 1µl of Taqman probe mix (primers and probe at final concentrations of 900nM and 250nM, respectively). The following PCR reaction protocol was used: initially cDNA production occurred for 1 cycle at 50°C for 10min followed by 95°C for 5min. Subsequently, PCR was initiated for 40 cycles of 95°C for 10s and 61°C for 30s. Reactions were performed in triplicate and the level of gene expression is reported as the ratio between the mRNA level of the target gene and the HPRT reference gene using the relative standard curve method.

### Western Blot analysis

Following exposure to the test pro-oxidants for 0h, 2h, 4h, and 24h time-points, total cellular protein was isolated from AHH-1 cells. Cells were pelleted by centrifugation, washed in ice-cold PBS, then lysed in RIPA buffer (Sigma), supplemented with protease inhibitor cocktail (Sigma) at 4°C for 5min. Cells were further lysed by agitation and centrifugation, and the cell pellet discarded. Protein extracts (30ng) of AHH1 cells were resolved on 10% SDS-PAGE (Sodium dodecyl sulphate-polyacrylamide gel

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3 electrophoresis) at 120V, transferred onto polyvinylidene difluoride (PVDF) membranes  
4 (BioRad), and blocked for 1h at room temperature with 5% Bovine serum albumin (BSA)  
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6 in TBS-T (20mM Tris (pH 7.6), 125mM NaCl, 0.1% (v/v) Tween20). Membranes were  
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8 incubated with monoclonal mouse anti-OGG1 (Sigma), polyclonal rabbit anti-beta  
9  
10 tubulin (AbCam, Cambridge, UK), monoclonal rabbit anti-ATM (Cell Signaling  
11  
12 Technology, Massachusetts, USA), polyclonal rabbit anti-BRCA1 (Cell Signaling  
13  
14 Technology), or monoclonal rabbit anti-beta actin (Cell Signalling Technology)  
15  
16 antibodies overnight at 4°C. Following washing (4 × 5 min in TBS/T), membranes were  
17  
18 incubated with appropriate horseradish peroxidase (HRP) conjugated secondary  
19  
20 antibodies (AbCam). Protein bands were detected using the Immuno-Star Western C  
21  
22 chemiluminescence kit (BioRad). Membranes were visualised using the Bio-Rad  
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24 Chemidoc XRS and average band density analysis was performed using Quantity One  
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26 version 4.6.3 (BioRad).  
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### 36 **Human DNA repair PCR arrays**

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38 To assess the role of a wider range of DNA repair enzymes in the observed non-linear  
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40 damage responses to pro-oxidants, gene expression PCR arrays tailored for DNA repair  
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42 were utilised. AHH-1 cells were treated with specific doses above and below confirmed  
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44 threshold inflection points, which were 5µM and 25µM H<sub>2</sub>O<sub>2</sub>, 0.2mM and 0.8mM KBrO<sub>3</sub>,  
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46 and 0.5mM and 3.5mM menadione, for 4h and the RNA was extracted. RNA (1.6µg)  
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48 from each sample was reverse transcribed into cDNA using the RT<sup>2</sup> First Strand Kit  
49  
50 (Qiagen). Gene expression was performed utilising Human DNA Repair RT<sup>2</sup> Profiler™  
51  
52 PCR Arrays (PAHS-042A) and 2X SABiosciences RT<sup>2</sup> qPCR Master Mix (Qiagen) and  
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3 MyiQ real-time PCR Platform (BioRad) according to the manufacturer's instructions.  
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5 Gene expression was normalised using five housekeeping genes within the array and  
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8 quantified using the  $\Delta\Delta C_t$  method by accessing the PCR Array Data Analysis Web Portal  
9  
10 <http://www.SABiosciences.com/pcrarraydataanalysis.php>.  
11  
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### 13 14 15 **Gas Chromatography/Mass Spectrometry (GC/MS) Determination of Oxidatively** 16 17 18 **Induced DNA Damage**

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20 GC/MS with isotope dilution was used to determine the absolute levels of five different  
21  
22 oxidatively modified bases: 8-oxoG, thymine glycol (TG), 5-hydroxy-5-methylhydantoin  
23  
24 (5-OH-5-MeHyd), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FAPyG) and 4,6-  
25  
26 diamino-5-formamidopyrimidine (FAPyA). AHH-1 cells were seeded for 24h and treated  
27  
28 with 0, 5  $\mu\text{M}$ / mL and 25  $\mu\text{M}$ / mL  $\text{H}_2\text{O}_2$  for 4h. Following washing, DNA was extracted  
29  
30 from the treated cells using the DNeasy Blood & Tissue Kit (Qiagen). The DNA was  
31  
32 precipitated and GC/MS analyses were performed as previously described (Dizdaroglu,  
33  
34 1985; Jaruga et al., 2008).  
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### 41 **Alteration of cellular GSH levels**

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43 In order to modulate GSH levels prior to measuring pro-oxidant induced MN, GSH was  
44  
45 either supplemented or depleted. AHH-1 (10mL) suspensions of cells at  $1 \times 10^5$ / mL were  
46  
47 seeded for 24h at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ . Cells were dosed with 2mM NAC to promote  
48  
49 glutathione levels and increase cellular antioxidant status, or 0.5mM BSO an inhibitor of  
50  
51 glutathione synthesis, for 24h prior to pro-oxidant treatment and CBMN assay (as  
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3 previously described). The levels of GSH in treated cells were estimated utilising the  
4  
5 Glutathione Assay kit (Sigma) according to the manufacturer's instructions.  
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### 10 **Statistical analysis**

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12 For the genotoxicity data and DNA adduct data, a one-way Analysis of Variance  
13  
14 (ANOVA), followed by a Dunnett's posthoc test, was used to determine if any of the  
15  
16 treatment doses were significantly different from the zero dose, except when cells were  
17  
18 pre-treated with BSO or NAC, where a *T*-test was employed. The hockey stick modelling  
19  
20 of apparent thresholds was carried out using software, kindly provided by Lutz and Lutz  
21  
22 (Lutz and Lutz, 2009) and implemented in the R package. A *T*-test was performed to  
23  
24 establish any differences between relative protein and mRNA expression of *OGG1* after  
25  
26 pro-oxidant treatments at the different time points analysed. Independent sample *T* test  
27  
28 was utilised to analyse differences in normalised protein levels of ATM and BRCA1 after  
29  
30 treatment with H<sub>2</sub>O<sub>2</sub> (0-25µM) or KBrO<sub>3</sub> (0-0.8mM).  
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### 39 **Results**

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41 The cytokinesis blocked micronucleus (CBMN) and *HPRT* forward mutation  
42  
43 assays were used to assess the induction of chromosomal aberration (MN formation) and  
44  
45 frequency of point mutations, respectively, after exposure of AHH-1 lymphoblastoid cells  
46  
47 to low concentrations of the oxidising agents. The range of concentrations used was  
48  
49 determined following initial dose setting experiments analysing genotoxicity and  
50  
51 assessment of cytotoxicity utilising relative population doubling (RPD) calculations in  
52  
53 satellite cultures (data not shown). To ensure any observed genetic damage was not due  
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3 to cytotoxicity related mechanisms, only those concentrations leading to more than 50%  
4 cell viability were analysed (data not shown). Investigations into the MOA behind these  
5 threshold responses involved analysis of levels of DNA repair enzymes involved in the  
6 repair of oxidative damage, assessment of the levels of oxidatively modified bases in  
7 exposed DNA, and analysis of the effect of antioxidant status on the dose-response  
8 curves to chromosome damage. To ensure cellular exposure to ROS was occurring at low  
9 doses of pro-oxidants, oxidation of the non-fluorescent probe 2',7'-dichlorofluorescein-  
10 diacetate (DCFH-DA) to the fluorochrome 2,7-dichlorofluorescein (DCF) was used as an  
11 index to quantify the overall level of intracellular ROS produced by 0 to 100 $\mu$ M H<sub>2</sub>O<sub>2</sub>  
12 (Supplementary file S1).  
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### 29 **Gene mutation induction**

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31 The *HPRT* forward mutation assay was used here to investigate the mutagenic  
32 activity of H<sub>2</sub>O<sub>2</sub>, KBrO<sub>3</sub>, and menadione. All three pro-oxidants demonstrated a range of  
33 low doses with minimal levels of mutation induction in AHH-1 human lymphoblastoid  
34 cells as compared to solvent control data (Fig. 1). The first statistically significant  
35 increase in mutation frequency above background levels (lowest observed effect level;  
36 LOEL) was 18 $\mu$ M for H<sub>2</sub>O<sub>2</sub> ( $p= 0.004$ ), 0.5mM for KBrO<sub>3</sub> ( $p= 0.004$ ), and 2.9 $\mu$ M for  
37 menadione ( $p= 0.001$ ) utilising one-way ANOVA, followed by a Dunnett's posthoc test.  
38 Further analysis of these results applying a "hockey-stick model" developed by Lutz and  
39 Lutz (2009) rejected linearity of each dose response curve, whilst supporting a threshold  
40 model ( $p< 0.05$ ) and showed that H<sub>2</sub>O<sub>2</sub> had an inflection point (or threshold dose) at  
41 7.3 $\mu$ M with lower confidence interval (CI) of 2.04 $\mu$ M; KBrO<sub>3</sub> an inflection point of  
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3 0.18mM with lower CI of 0.08mM; and menadione an inflection point of 1.89 $\mu$ M with  
4 lower CI of 0.76mM (Fig 1.) (Lutz and Lutz, 2009).  
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### 10 **Chromosomal damage induction**

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12 The dose-response relationships, with respect to the induction of chromosome  
13 aberrations, obtained after pro-oxidant treatments with H<sub>2</sub>O<sub>2</sub>, KBrO<sub>3</sub>, and menadione are  
14 illustrated in Figure 2. LOEL concentrations of 25 $\mu$ M ( $p= 0.03$ ), 0.6mM ( $p= 0.007$ ), and  
15 2.9 $\mu$ M ( $p= 0.036$ ) were identified for exogenous treatment with H<sub>2</sub>O<sub>2</sub>, KBrO<sub>3</sub>, and  
16 menadione, respectively. Subsequent increases in concentration above each LOEL  
17 produced a progressive rise in cellular DNA damage, as detected by a higher incidence of  
18 MN. Hockey stick statistical modelling of the data using the Lutz approach rejected a  
19 linear fit to the dose response curves and confirmed an inflection point (threshold value)  
20 of 10.88 $\mu$ M with lower CI of 5.93 $\mu$ M and 0.35mM with lower CI of 0.22mM, for H<sub>2</sub>O<sub>2</sub>  
21 and KBrO<sub>3</sub>, respectively. The dose-response curve for menadione, despite indicating a  
22 non-linear response for the induction of genotoxic damage, favoured a linear model and  
23 did not achieve significance when hockey stick modelling was performed on the data.  
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### 43 **Base excision repair (BER) glycosylase cellular levels**

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45 In order to examine the protective mechanisms behind the genotoxic tolerance to  
46 low doses of pro-oxidants shown here, we focussed firstly on the role of DNA repair.  
47  
48 Previous work on thresholded responses for the alkylating agents has demonstrated  
49 upregulation of the DNA repair enzymes *MPG* and *MGMT* by EMS and MMS,  
50  
51 respectively, at doses below the threshold (Doak et al., 2008; Zair et al., 2011). Since, 8-  
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3 oxoG is a major DNA lesion formed as a consequence of ROS exposure and 8-oxoG in  
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5 DNA is primarily repaired by the DNA glycosylase OGG1, the mRNA and protein levels  
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7 of this enzyme were measured in response to specific pro-oxidant treatment (in this case  
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9 H<sub>2</sub>O<sub>2</sub>). *OGG1* expression levels in treated and untreated AHH-1 lymphoblastoid cells,  
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11 measured by real-time RT-PCR, are represented in Figure 3A. No significant modulation  
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13 in *OGG1* expression in response to H<sub>2</sub>O<sub>2</sub> treatment (0-50μM) was observed at any of the  
14  
15 time points (0-24h) studied. OGG1 protein levels in AHH-1 cells following oxidative  
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17 insult were also analysed by Western blotting and normalized to beta-tubulin expression  
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19 (Fig. 3B). Similarly to mRNA levels, treatment with H<sub>2</sub>O<sub>2</sub> (0-50μM) had no detectable  
20  
21 effect on the amount of OGG1 protein recovered from nuclear extracts at any of the  
22  
23 treatment periods (0-24h) monitored, comparable results were observed for treatment  
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25 with 1mM KBrO<sub>3</sub> (results not shown).  
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### 34 **Human DNA repair PCR arrays**

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36 To investigate the effects of pro-oxidant treatment on a wider range of DNA  
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38 repair genes within this interesting thresholded region, treated cells were analysed using  
39  
40 DNA repair-directed PCR gene expression arrays. Since only H<sub>2</sub>O<sub>2</sub> and KBrO<sub>3</sub> showed  
41  
42 thresholds for both point mutation and chromosome damage, these studies concentrated  
43  
44 on these two pro-oxidants. The PCR expression arrays focus on a selected panel of  
45  
46 eighty-four genes involved in the base-excision, nucleotide excision, mismatch, double-  
47  
48 strand break, and other DNA repair processes (Supplementary file S2). Numerous fold  
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50 changes in expression, compared to untreated control cells, were observed above and  
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52 below the threshold doses (Table 1). *ATM* for example (an important gene involved in the  
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3 signalling response to DNA damage), showed unchanged expression levels at  
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5 concentrations below the threshold doses, but was observed to be 2.13 fold (H<sub>2</sub>O<sub>2</sub>) and  
6  
7 4.0 fold (KBrO<sub>3</sub>) downregulated above the threshold dose for chromosome damage  
8  
9 induction, suggestive of a relatively higher degree of damage recognition below the  
10  
11 threshold. The cancer susceptibility gene *BRCA1*, involved in the repair of DNA double  
12  
13 strand breaks (DSBs), was upregulated by 2.39 and 2.04 fold (H<sub>2</sub>O<sub>2</sub> and KBrO<sub>3</sub>,  
14  
15 respectively) at concentrations below but not above the thresholds for chromosomal  
16  
17 damage in both chemicals. H<sub>2</sub>O<sub>2</sub> treated cells also exhibited a 2.69 fold increase in  
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19 expression of *APEX1* (APEX nuclease (multifunctional DNA repair enzyme) 1; [GenBank](#)  
20  
21 [ID:328](#)) at a concentration below, but not above, the threshold dose, indicative of a role  
22  
23 for BER in minimising chromosome damage at low H<sub>2</sub>O<sub>2</sub> doses. Further, the BER DNA  
24  
25 glycosylase *MUTYH* (mutY homolog (E.coli); [GenBank ID:4595](#)), which catalyses the  
26  
27 removal of adenine bases from the DNA backbone at sites where adenine is  
28  
29 inappropriately paired with guanine, cytosine, or 8-oxoG, was downregulated by 2.17  
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31 fold after exposure to 0.8mM KBrO<sub>3</sub>.  
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### 41 **Relative levels of DNA damage response proteins**

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43 To further investigate the gene expression changes observed during the PCR gene  
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45 array experiments, and to ascertain whether such expression changes were consistently  
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47 observed at the protein level, the amount of ATM and BRCA1 protein recovered from  
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49 nuclear extracts after pro-oxidant treatment was determined in AHH-1 cells. Cells were  
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51 dosed with H<sub>2</sub>O<sub>2</sub> and KBrO<sub>3</sub> at concentrations above and below the thresholds for  
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53 chromosome damage induction, and relative protein levels were deduced by Western  
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3 blotting via the examination of band intensities of ATM or BRCA1 normalised to beta-  
4 actin loading controls (Fig. 4). Unlike the alterations in *ATM* and *BRCA1* mRNA  
5 expression observed following treatment with concentrations of H<sub>2</sub>O<sub>2</sub> above (25µM) or  
6 below (5µM) the threshold, no significant threshold-dependent differences in the levels of  
7 these DNA repair proteins were detected (Fig. 4A). Treatment with 0.2mM KBrO<sub>3</sub>  
8 (below the threshold) appeared to induce an increase in protein levels of both ATM and  
9 BRCA1 (Fig. 4B), which then decreased at 0.8mM KBrO<sub>3</sub> (above the threshold) to levels  
10 comparable to untreated samples, however these differences were not deemed significant  
11 by independent sample *T* test.  
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### 27 **GC/MS Determination of Oxidatively Induced DNA Damage**

28 Four out of the five modified bases were detected in the AHH-1 DNA extracts (Fig. 5).  
29 The FAPyA lesion was not detectable in either the control or the H<sub>2</sub>O<sub>2</sub>-treated samples,  
30 most likely due to the low amount (~ 30µg) of extracted DNA/sample available for MS  
31 analysis. Of the four detected lesions, the levels of the 8-oxoG, FAPyG and 5-OH-5-  
32 MeHyd lesions in the H<sub>2</sub>O<sub>2</sub>-treated samples were not significantly different from the  
33 background levels in the control samples. The TG lesion, however, was found to be  
34 significantly (*p*= 0.0259) increased in the 25µM H<sub>2</sub>O<sub>2</sub>-treated sample (Fig. 5D), in  
35 comparison to the TG background level in the control samples. The level of TG in the  
36 5µM H<sub>2</sub>O<sub>2</sub>-treated sample was not significantly different from the TG levels in the  
37 controls. The accumulation of TG in the 25µM H<sub>2</sub>O<sub>2</sub>-treated sample could potentially  
38 have biological relevance as TG is a cytotoxic lesion that is a known block to DNA  
39 polymerase.  
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### Impact of GSH on pro-oxidant induced DNA damage

GSH is the principal non-protein thiol involved in the antioxidant cellular defence, playing a critical role in protecting cells from oxidative damage and the toxicity of xenobiotic electrophiles, as well as maintaining redox homeostasis (Forman et al., 2009). To elucidate the function of antioxidants in the observed genotoxic threshold effects described above, intra-cellular levels of GSH were modified and the shapes of the resultant dose-response curves for H<sub>2</sub>O<sub>2</sub>, with respect to chromosomal damage, were assessed. AHH-1 cells were pre-treated with BSO (0.5mM), a specific GSH synthesis inhibitor, or NAC (2.0mM), a GSH precursor, for 24h and the effects on H<sub>2</sub>O<sub>2</sub>-induced DNA damage were determined by the CBMN assay (Fig. 6). BSO pre-treatment altered the shape of the H<sub>2</sub>O<sub>2</sub> dose-response curve significantly from a thresholded curve to a linear dose-response which saturates at higher doses. BSO pre-treatment reduced the first dose to induce a statistically significant increase in genetic damage from 18μM to 8μM ( $p= 0.0008$ ), which indicates that GSH depletion by BSO (confirmed utilising a GSH assay) reduces protection against oxidatively induced DNA damage, at low dose treatments. Furthermore, statistically significant increases in micronuclei frequency were observed in BSO pre-treated cells at 10μM ( $p< 0.001$ ) and 12μM ( $p< 0.01$ ) H<sub>2</sub>O<sub>2</sub> as compared to cells without pre-treatment. In contrast, NAC pre-treated cells had significantly lower levels of micronuclei induction at 18μM ( $p< 0.001$ ) and 25μM ( $p< 0.01$ ) H<sub>2</sub>O<sub>2</sub>, as compared to H<sub>2</sub>O<sub>2</sub> cells without pre-treatment suggestive of a protective effect of enhanced GSH status.

## Discussion

Elevated cellular levels of ROS can imbalance homeostasis and create oxidative stress, and chronic exposure to this stress can cause permanent genomic changes.

Accumulation of oxidative lesions has been associated with ageing and a host of human diseases including cancer, chronic inflammation, atherosclerosis, and neurodegenerative diseases such as Alzheimer's disease (Sedelnikova et al., 2010). The use of oxidative DNA damage measurements in populations may have important implications for human health risk assessment, distinguish relevant environmental exposures, and predict frequency of disease (Hatt et al., 2008; Loft et al., 2008).

Traditionally, characterisation of DNA-reactive agents has involved the assumption of a linear relationship between genotoxin exposure and induction of mutagenic modifications (Henderson et al., 2000). This view, however, does not account for homeostatic mechanisms that potentially counteract DNA damage induced after genotoxic exposure. Knowledge of the dose-response relationship of a given chemical is paramount; if a threshold exists, safe levels can be calculated, if there is a linear dose-response, there are no safe exposure levels. This can have economic implications; impacting the availability and use of certain compounds.

To investigate the biological significance of low-dose exposures, a robust analysis of mutation and chromosomal damage induction was performed with the DNA-reactive compounds  $H_2O_2$ ,  $KBrO_3$ , and menadione. All three pro-oxidants displayed a range of low doses with no statistically significant increase above background levels of DNA damage induction. Objective analysis of the data employing "hockey stick" statistical modelling demonstrated inflection (threshold) points for each of the dose relationships,

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3 except for chromosomal damage induction in menadione, which conformed to a linear  
4 model. Menadione, may thus, be considered a non-thresholded compound overall. The  
5 linear relationship of damage induction observed after menadione exposure may be  
6 explained by the reported production of a high frequency of DNA strand scission by the  
7 compound mediated by HO<sup>•</sup>, that may be too extensive for the repair machinery to  
8 correct, even at low doses (Nutter et al., 1992). Although DNA strand breaks occur after  
9 exposure to H<sub>2</sub>O<sub>2</sub> and KBrO<sub>3</sub>, other oxidative lesions such as 8-oxoG and TG occur  
10 preferentially (Cadet et al., 2010; Kawanishi and Murata, 2006).  
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22 Thresholds were reported at lower concentrations (1.5 to 2 fold) for point  
23 mutations than for MN induction, which may be explained by the higher probability of a  
24 single oxidative lesion formation yielding a base substitution, compared to a DSB arising  
25 from multiple clustered lesions. Indeed, abortive BER processing of radical damage can  
26 form DSBs when the position of lesions are closely opposed on the two strands (Wallace,  
27 2002).  
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36 Several mechanisms may be responsible for contributing to genotoxic thresholds  
37 in response to ROS, however, DNA repair is likely to be the primary mode of defence.  
38 Repair pathways may well successfully remove newly formed adducts at low doses, and  
39 if the rate of lesion repair is faster than its rate of formation, a no observable effect limit  
40 (NOEL) will result. We have previously noted that thresholds for alkylating agents (EMS  
41 and MMS) are accompanied by increases in the expression of DNA repair genes *MGMT*  
42 and *MPG* (Doak et al., 2008; Zair et al., 2011).  
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53 A well studied biomarker of oxidative damage, and a key repairable DNA lesion  
54 induced by the pro-oxidants studied, is 8-oxoG. 8-oxoG is a premutagenic DNA lesion  
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3 due to its propensity to mispair with adenine, generate errors in replication, and G:C to  
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5 T:A transversions. It is a substrate for the BER pathway, initiated by the OGG1 enzyme  
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7 (Boiteux and Radicella, 2000; Nishimura, 2002). To investigate the potential of OGG1 as  
8  
9 a thresholded mode of action (MOA), gene and protein expression of OGG1 were  
10  
11 examined. No modulation in OGG1 levels was observed in response to oxidative stress;  
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13 which is in agreement with other studies (Mistry and Herbert, 2003; Saitoh et al., 2001).  
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15 Furthermore, *OGG1* has been described as a “house keeping” gene with a constant level  
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17 of expression throughout the cell cycle (Dhenaut et al., 2000). Basal, not inducible,  
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19 expression of *OGG1* therefore, may play a role in the maintenance of homeostasis in the  
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21 presence of low levels of pro-oxidants.  
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27 A lack of OGG1 induction may reflect the redundancy that exists between BER  
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29 glycosylases, and indeed between other pathways to repair oxidative lesions. Further  
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31 investigation into the responses of other DNA repair genes to pro-oxidant stress was  
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33 needed, and to fulfil this requirement the gene expression profile of eighty-four key DNA  
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35 repair enzymes was compared at doses above and below the thresholds of chromosome  
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37 damage induction observed for H<sub>2</sub>O<sub>2</sub> and KBrO<sub>3</sub> (S2). For most of the nine genes with  
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39 altered expression there was no clear pattern for the two chemicals and therefore it is  
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41 difficult to propose a direct mechanistic link in which a threshold level of exposure  
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43 determines a switch in the expression of the DNA damage response program. Despite  
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45 this, two repair genes showed interesting results; *ATM*, a central component of the DSB  
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47 repair pathway in mammalian cells, was downregulated above the threshold doses for  
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49 damage induction and *BRCA1*, a nuclear phosphoprotein that plays a role in maintaining  
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3 genomic stability, was upregulated below the threshold doses for damage induction in  
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5 both H<sub>2</sub>O<sub>2</sub> and KBrO<sub>3</sub>.  
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8 Analysis of protein levels of these DNA damage response genes following  
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10 exposure to pro-oxidants, however, did not substantiate the PCR array findings, and no  
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12 significant alterations in the protein levels of ATM or BRCA1 were observed above or  
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14 below the genotoxic thresholds. Thus, although DSB may occur under oxidative stress  
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16 conditions, such as when ROS induced DNA damage interferes with either DNA  
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18 replication or transcription such as during the processing of bulky DNA adducts such as  
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20 FAPyG produced from ring opening of guanine upon exposure to HO<sup>•</sup>, it is not involved  
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22 in the MOA of genotoxic thresholds observed for pro-oxidants. In agreement with this  
23  
24 conclusion, in the present study, FAPyG lesions were observed at similar levels at doses  
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26 above and below the H<sub>2</sub>O<sub>2</sub> threshold and thus appear to be repaired effectively or are not  
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28 formed upon exposure to low doses. In contrast, TG levels were significantly higher in  
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30 25µM H<sub>2</sub>O<sub>2</sub>-, but not in 5µM H<sub>2</sub>O<sub>2</sub>-treated samples, versus untreated controls.  
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36 TG is the most common thymine lesion found after treatment by oxidising agents and  
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38 exerts significant distortion on the duplex DNA molecule, blocking replication. There are  
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40 instances, however, whereby DNA polymerases can by-pass TG and a low level of  
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42 misincorporation of guanine opposite thymine glycol occurs, giving rise to mutation  
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44 (Wallace, 2002). The presence of higher levels of TG above the threshold suggests a role  
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46 for this lesion, and BER in the observed genotoxic thresholds. The BER glycosylases  
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48 NEIL1 (nei endonuclease VIII-like 1; [GenBank ID:79661](#)) and NTHL1 (nth  
49  
50 endonuclease III-like 1; [GenBank ID: 4913](#)) may successfully remove TG at low doses  
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53 but above the threshold the glycosylases are ‘saturated’, lesions start to escape repair,  
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3 becoming fixed permanent defects, and subsequent increases in dose result in a more  
4 linear increase in damage. Alternatively, formation of TG may be reduced below the  
5 threshold due to lower exposure to ROS.  
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10 DNA repair represents but one tier of protection against oxidatively generated  
11 DNA damage present in multicellular organisms. Antioxidants may also contribute to the  
12 above described thresholds. Alteration of the antioxidant status of cells via the  
13 manipulation of GSH levels, transformed the shape of the dose-response curve of H<sub>2</sub>O<sub>2</sub>  
14 induced chromosomal damage. For example, inhibition of GSH by BSO altered the shape  
15 of the curve from a non-linear threshold to a more linear response, and reduced the lowest  
16 dose to induce a statistically significant increase in genetic damage from 25 μM to 8 μM  
17 H<sub>2</sub>O<sub>2</sub>. Furthermore, boosting GSH levels with NAC shifted the dose response to the right,  
18 with treated cells showing significantly lower levels of MN induction at 18 μM and 25 μM  
19 H<sub>2</sub>O<sub>2</sub>, as compared to H<sub>2</sub>O<sub>2</sub> cells without pre-treatment. Such effects suggest a causal role  
20 for GSH in the genotoxic thresholds of pro-oxidants, competing with DNA to accept  
21 electrons from ROS, removing their oxidative capacity and potential to create mutagenic  
22 lesions. Altering the redox status *in vitro* by increasing the levels of antioxidants has  
23 beneficial, protective effects against pro-oxidant agents.  
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44 Although it is difficult to extrapolate from the *in vitro* data described here to an *in*  
45 *vivo* setting, the existence of a NOEL implies at least a pragmatic threshold for  
46 carcinogenicity of H<sub>2</sub>O<sub>2</sub> and KBrO<sub>3</sub>. Genotoxic tolerance to low levels of pro-oxidant  
47 chemicals appears to be due, in part, to basal BER DNA repair plus the protective  
48 capacity of antioxidants against DNA damage. The abundance of repair pathways and  
49 significant redundancy achieved by overlapping substrates in maintaining REDOX  
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homeostasis suggest that the persistence of oxidative DNA damage is extremely detrimental to cells. Theoretically, as genetic alterations do not arise at very low doses, the risk of carcinogenesis (and also several degenerative chronic diseases) is unlikely to occur after exposure to concentrations below the LOEL, as no biologically significant effects are observed experimentally. This outcome has implications on the numerous uses of pro-oxidant chemicals including as cosmetic bleaches, as cancer treatment agents and in food production. Furthermore, the present study strengthens the evidence of the existence of thresholds for direct-acting genotoxins.

### Supplementary data

Intracellular ROS production by H<sub>2</sub>O<sub>2</sub> was measured using the oxidation-sensitive fluoroprobe 2',7'-dichlorofluorescein diacetate (DCFH-DA, Invitrogen molecular probes). DCFH-DA assay is based on the principle that upon cellular internalisation, the DA portion of the hydrophobic dye is cleaved by esterases. The resulting DCFH is oxidised to its highly fluorescent product DCF by cellular oxidants, thereby indicating the level of intracellular ROS. DCFH-DA was dissolved in DMSO to a concentration of 1M and was further diluted to the appropriate concentration with Hepes-buffered (20 mM) Hanks balanced salt solution with glucose (5mM) (pH 7.4). A 2µM aliquot of DCFH-DA was added to the wells of a 96-well plate previously loaded with AHH-1 cells and 0 to 100µM H<sub>2</sub>O<sub>2</sub>, and fluorescence was measured over 30min with fluorescence excitation and emission at 480 and 520 nm, respectively. The supplementary graph (S1) shows the relative fluorescence after exposure of AHH-1 cells to 0 to 100µM H<sub>2</sub>O<sub>2</sub> (mean of triplicate experiments). There were non-significant increases in the quantity of ROS

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3 production measured over background (untreated) levels after exposure to doses within  
4  
5 the range of the threshold of H<sub>2</sub>O<sub>2</sub>. ROS production was observed at significantly higher  
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7 levels than background samples after exposure to 15µM of H<sub>2</sub>O<sub>2</sub>, a dose higher than the  
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9 threshold observed within the study, by One-way ANOVA, followed by Dunnett's post  
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11 hoc analysis ( $p= 0.032$ ).  
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14 Complete analysis of the DNA repair PCR expression arrays detailing the expression  
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16 levels of eighty-four human repair genes after exposure to H<sub>2</sub>O<sub>2</sub> and KBrO<sub>3</sub> doses above  
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18 and below the threshold inflection point (output from hockey stick statistical modelling)  
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20 is supplied as a supplementary table (S2).  
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28  
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### 34 **NIST Disclaimer**

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36 Certain commercial equipment, instruments and materials are identified in this paper to  
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38 specify an experimental procedure as completely as possible. In no case does the  
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40 identification of particular equipment or materials imply a recommendation or  
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42 endorsement by the National Institute of Standards and Technology nor does it imply that  
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44 the materials, instruments, or equipment are necessarily the best available for the purpose.  
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**Table 1. Alterations in DNA repair gene expression above and below the threshold for chromosome damage induction.**

Gene symbol	Fold change in gene expression			
	5 $\mu$ M H <sub>2</sub> O <sub>2</sub>	25 $\mu$ M H <sub>2</sub> O <sub>2</sub>	0.2mM KBrO <sub>3</sub>	0.8mM KBrO <sub>3</sub>
APEX1	2.69	1.14	n/a	2.57
ATM	0.52	-0.47	1.08	-0.25
BRCA1	2.39	1.71	2.04	1.89
ERCC4	0.55	0.74	0.66	-0.5
FEN1	-0.46	0.83	0.61	-0.44
LIG3	0.53	0.7	0.62	-0.47
MUTYH	0.54	0.74	0.62	-0.46
PNKP	-0.41	0.71	0.53	-0.34
TOP3B	0.51	0.79	0.55	-0.43

## Figure legends

### Figure 1. Gene mutation frequency in response to pro- oxidants.

Dose-response relationships of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), potassium bromate ( $\text{KBrO}_3$ ), and menadione, in the AHH-1 cell line with respect to *HPRT* gene mutation frequency. Hockey stick statistical modelling analysis has been applied to each data set to calculate the inflection point (IP), probability for non- linearity ( $p$ ) and  $Y$ - intercept. (A)  $\text{H}_2\text{O}_2$ , threshold, IP=  $7.28\mu\text{M}$ , lowerIP/CI=  $2.04\mu\text{M}$ ,  $Y= 1.95$ , and  $p= 0.038$ . (B)  $\text{KBrO}_3$ , threshold, IP=  $0.18\text{mM}$ , lowerIP/CI=  $0.08\text{mM}$ ,  $Y= 6.11$ , and  $p= 0.004$ . (C) Menadione, threshold, IP=  $1.89\mu\text{M}$ , lowerIP/CI=  $0.76\mu\text{M}$ ,  $Y= 5.05$ , and  $p= 0.004$ .

### Figure 2. Chromosomal damage in response to pro- oxidants.

Dose-response relationships of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), potassium bromate ( $\text{KBrO}_3$ ), and menadione, in the AHH-1 cell line with respect to micronucleus frequency. Hockey stick statistical modelling analysis has been applied to each data set to calculate the inflection point (IP), probability for non- linearity ( $p$ ) and  $Y$ - intercept. (A)  $\text{H}_2\text{O}_2$ , threshold, IP=  $10.88\mu\text{M}$ , lower IP/CI=  $5.93\mu\text{M}$ ,  $Y= 1.32$ , and  $p= 0.023$ . (B)  $\text{KBrO}_3$ , threshold, IP=  $0.35\text{mM}$ , lower IP/CI=  $0.22\text{mM}$ ,  $Y= 1.05$ , and  $p= 0.002$ . (C) Menadione, linear, and  $p= 0.35$ . *Mn/Bn%*: percentage of binucleated cells containing one or more micronuclei.

### Figure 3. Effect of hydrogen peroxide on OGG1 levels.

(A) Effect of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) on *OGG1* expression in AHH-1 cells. Cells were treated with 0-  $50\mu\text{M}$  of  $\text{H}_2\text{O}_2$  and total RNA extracted between 0 and 24h. Levels of

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*OGG1* mRNA were assessed by real time RT PCR. Values were normalized to levels of the constitutively expressed housekeeping gene, *HPRT*, and represent the mean ( $\pm$  SD) fold change from control levels at each time point. Each data point represents three independent measurements. (B) Western blot of OGG1 protein and loading control  $\beta$ -tubulin in AHH-1 cells following treatment with 25 $\mu$ M H<sub>2</sub>O<sub>2</sub> for 0, 2, 4, and 24h; L: ladder.

**Figure 4. Effects of pro-oxidants on protein levels of ATM and BRCA1.**

(A) Effect of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) on ATM and BRCA1 protein levels recovered from AHH-1 cells treated for 4 hours with concentrations above (25 $\mu$ M) and below (5 $\mu$ M) the genotoxic threshold for DNA damage induction. (B) Effect of potassium bromate (KBrO<sub>3</sub>) on ATM and BRCA1 protein levels recovered from AHH-1 cells treated for 4 hours with concentrations above (0.2mM) and below (0.8mM) the genotoxic threshold for DNA damage induction. *Normalised protein level*: Relative protein concentration calculated using membrane band intensities normalised to beta-actin loading control band intensities. *Bars*: standard deviation.

**Figure 5. Determination of oxidatively induced DNA damage in hydrogen peroxide-treated AHH-1 cells.**

(A) Accumulation of 8-oxo-7,8-dihydroguanine (8-oxoG) in relation to exposure dose, (B) Accumulation of 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FAPyG) in relation to exposure dose, (C) Accumulation of 5-hydroxy-5-methylhydantoin (5-OH-5-MeHyd) in relation to exposure dose, (D) Accumulation of thymine glycol (TG) in

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3 relation to exposure dose. \*: ( $p < 0.05$ ) indicates statistically significant result compared  
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5 to the control samples using one-way Analysis of Variance (ANOVA) followed by  
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7 Dunnett's multiple comparison test. All data points represent the mean of  $\geq 4$   
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9 independent measurements. Uncertainties are standard deviations.  
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16 **Figure 6. Pro-oxidant dose response following alteration of cellular glutathione**  
17  
18 **antioxidant levels.**

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20 Effect of BSO (0.5mM) or NAC (2.0mM) pre-treatment on the frequency of hydrogen  
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22 peroxide ( $H_2O_2$ ) induced micronuclei in AHH-1 cells; \*: At 8  $\mu M H_2O_2$ , first significant  
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24 increase in micronuclei (MN) frequency above the control in BSO pre- treated cells ( $p=$   
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26 0.0008) as determined by *T*-test. &: At 10 $\mu M H_2O_2$ , BSO pre- treated cells demonstrated  
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28 a statistically significant increase in percentage MN as compared to non pre-treated and  
29  
30 NAC pre- treated cells ( $p < 0.001$  and  $p < 0.05$ , respectively); \*\*: At 12 $\mu M H_2O_2$ , BSO  
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32 pre- treated cells showed a statistically significant elevation in MN as compared to non  
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34 pre- treated cells ( $p < 0.01$ ); \$: At 18 $\mu M H_2O_2$ , first statistically significant increase in MN  
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36 frequency above the control in non pre- treated cells ( $p < 0.0001$ ); also, non pre-treated  
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38 cells exhibited statistically significant higher levels of MN to NAC pre-treated cells  
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40 ( $p < 0.001$ ); \*\*\*: At 25 $\mu M H_2O_2$ , non pre-treated cells had statistically significant higher  
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42 levels of micronuclei than NAC pre-treated cells ( $p < 0.01$ ). *Mn/Bn%*: percentage of  
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44 binucleated cells containing one or more micronuclei.  
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53 **Supplementary Figure S1. Effect of hydrogen peroxide ( $H_2O_2$ ) on DCFH-DA**  
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55 **fluorescence.**  
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3 Intracellular ROS production by H<sub>2</sub>O<sub>2</sub> was measured using the oxidation-sensitive  
4 fluoroprobe 2',7'-dichlorofluorescein diacetate (DCFH-DA). \* ( $p < 0.05$ ) and \*\* ( $p < 0.005$ )  
5 indicates statistically significant result compared to the control zero samples using one-  
6 way Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison test.  
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8 Each bar represents the mean of three independent measurements. Uncertainties are  
9 standard deviations.  
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20 **Supplementary Figure S2. Effect of pro-oxidants on the expression levels of human**  
21 **repair genes.**  
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24 Table depicting the complete analysis of the DNA repair PCR expression arrays detailing  
25 expression levels of eighty-four human repair genes after exposure to 5 $\mu$ M- and 25 $\mu$ M-  
26 H<sub>2</sub>O<sub>2</sub> and 0.2mM- and 0.8mM-KBrO<sub>3</sub> doses (above and below the threshold inflection  
27 point, respectively) relative to control (untreated) samples.  
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## Abbreviations

5-OH-5-MeHyd= 5-hydroxy-5-methylhydantoin

8-oxoG= 8-oxo-7,8-dihydroguanine

ATM= ataxia telangiectasia mutated

Bn= binucleated cells

BRCA1= breast cancer 1

BSA= bovine serum albumin

BSO= buthionine sulfoximine

CBMN= cytokinesis block micronucleus assay

CI= confidence interval

CO<sub>2</sub>= carbon dioxide

DAPI= 4',6-diamido-2-phenylindole

DCFH-DA= 2',7'-dichlorofluorescein diacetate

EMS= ethyl methane sulphonate

FAPyA= 4,6-diamino-5-formamidopyrimidine

FAPyG= 2,6-diamino-4-hydroxy-5-formamidopyrimidine

GSH= glutathione

h= hours

H<sub>2</sub>O<sub>2</sub>= hydrogen peroxide

HAT= hypoxanthine-aminopterin-thymidine

*HPRT*= hypoxanthine phosphoribosyltransferase

HO•= hydroxyl radical

HRP= horse radish peroxidase

1  
2  
3 IP= inflection point

4  
5 KBrO<sub>3</sub>= potassium bromate

6  
7  
8 KCl= potassium chloride

9  
10 LOEL= low observed effect level

11  
12 MGMT= O<sup>6</sup>-methylguanine-DNA methyltransferase

13  
14 MPG= N-methylpurine-DNA glycosylase

15  
16 MMS= methyl methane sulphonate

17  
18 MN= micronuclei

19  
20 MOA= modes of action

21  
22 NAC= N-acetylcysteine

23  
24 NaCl= sodium chloride

25  
26 \*NO= nitric oxide

27  
28 \*NO<sub>2</sub>= nitrogen dioxide radical

29  
30 NOEL= no observed effect level

31  
32 O<sub>2</sub><sup>•-</sup>= superoxide anion radical

33  
34 OGG1= 8-oxoguanine DNA glycosylase 1

35  
36 *P*= probability

37  
38 PAGE= polyacrylamide gel electrophoresis

39  
40 PBS= phosphate buffered saline

41  
42 PVDF= polyvinylidene difluoride

43  
44 ROS= reactive oxygen species

45  
46 RPD= relative population doubling

47  
48 SDS= sodium dodecyl sulphate

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2  
3 TBS= Tris-buffered saline  
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5 TG= thymine glycol  
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8 TRIS= Tris(hydroxymethyl)aminomethane  
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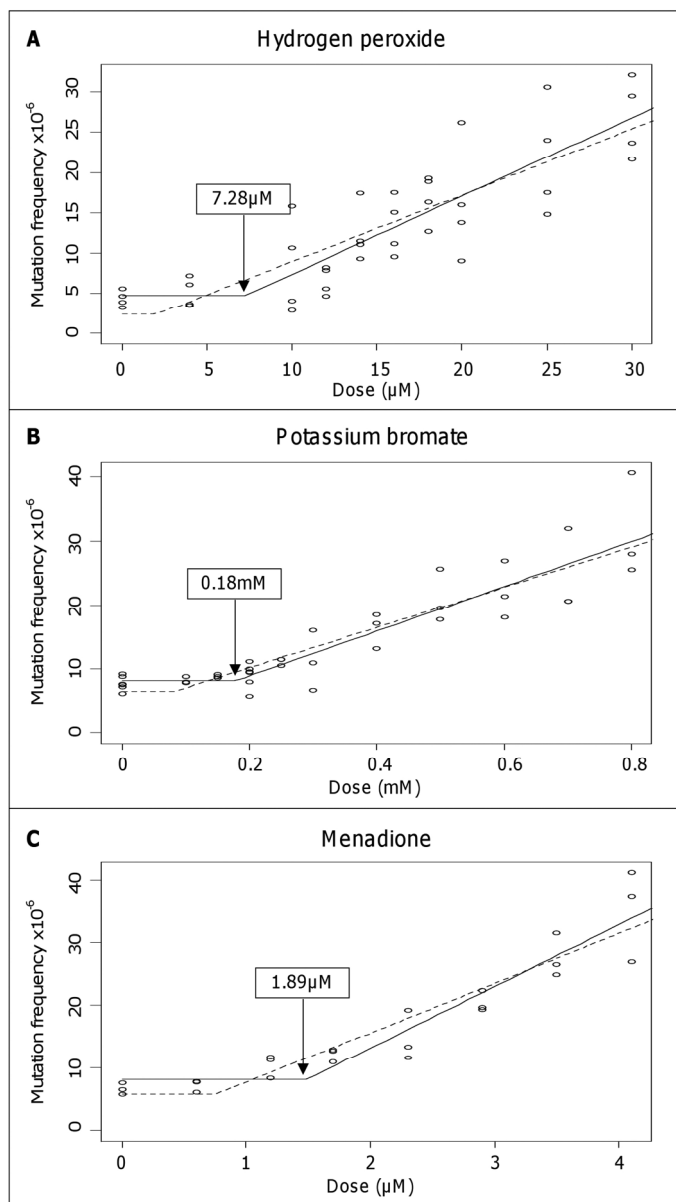


Figure 1. Gene mutation frequency in response to pro- oxidants.

Dose-response relationships of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), potassium bromate ( $\text{KBrO}_3$ ), and menadione, in the AHH-1 cell line with respect to HPRT gene mutation frequency. Hockey stick statistical modelling analysis has been applied to each data set to calculate the inflection point (IP), probability for non- linearity ( $p$ ) and Y- intercept. (A)  $\text{H}_2\text{O}_2$ , threshold, IP= 7.28  $\mu\text{M}$ , lowerIP/CI= 2.04  $\mu\text{M}$ , Y= 1.95, and  $p= 0.038$ . (B)  $\text{KBrO}_3$ , threshold, IP= 0.18 mM, lowerIP/CI= 0.08 mM, Y= 6.11, and  $p= 0.004$ . (C) Menadione, threshold, IP= 1.89  $\mu\text{M}$ , lowerIP/CI= 0.76  $\mu\text{M}$ , Y= 5.05, and  $p= 0.004$ .

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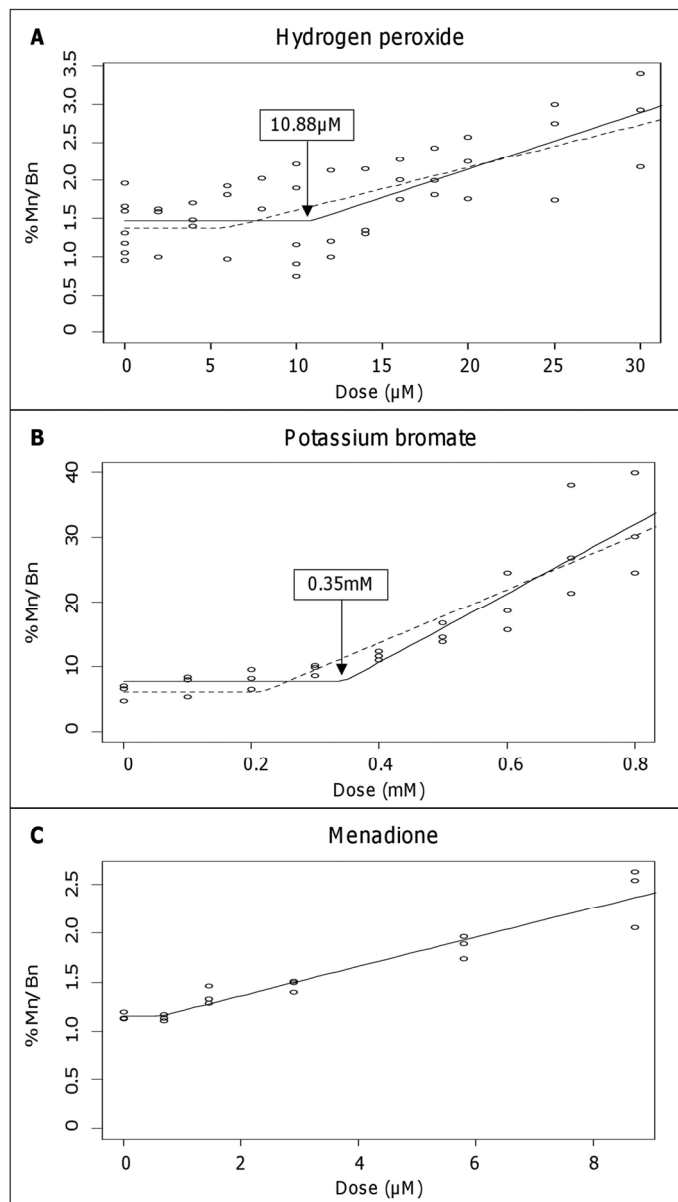


Figure 2. Chromosomal damage in response to pro- oxidants.

Dose-response relationships of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), potassium bromate ( $\text{KBrO}_3$ ), and menadione, in the AHH-1 cell line with respect to micronucleus frequency. Hockey stick statistical modelling analysis has been applied to each data set to calculate the inflection point (IP), probability for non- linearity ( $p$ ) and Y-intercept. (A)  $\text{H}_2\text{O}_2$ , threshold, IP= 10.88 $\mu\text{M}$ , lower IP/CI= 5.93 $\mu\text{M}$ ,  $Y = 1.32$ , and  $p = 0.023$ . (B)  $\text{KBrO}_3$ , threshold, IP= 0.35mM, lower IP/CI= 0.22mM,  $Y = 1.05$ , and  $p = 0.002$ . (C) Menadione, linear, and  $p = 0.35$ . Mn/Bn%: percentage of binucleated cells containing one or more micronuclei.

155x272mm (300 x 300 DPI)

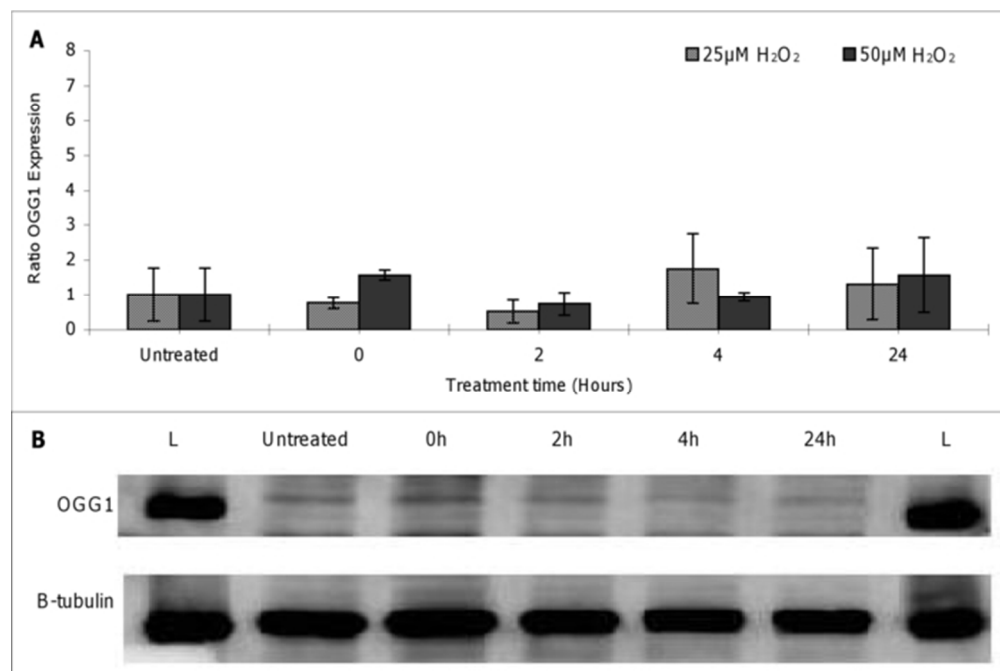


Figure 3. Effect of hydrogen peroxide on OGG1 levels.

(A) Effect of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) on OGG1 expression in AHH-1 cells. Cells were treated with 0- 50µM of H<sub>2</sub>O<sub>2</sub> and total RNA extracted between 0 and 24h. Levels of OGG1 mRNA were assessed by real time RT PCR. Values were normalized to levels of the constitutively expressed housekeeping gene, HPRT, and represent the mean ( $\pm$  SD) fold change from control levels at each time point. Each data point represents three independent measurements. (B) Western blot of OGG1 protein and loading control B-tubulin in AHH-1 cells following treatment with 25µM H<sub>2</sub>O<sub>2</sub> for 0, 2, 4, and 24h; L: ladder.  
59x39mm (300 x 300 DPI)

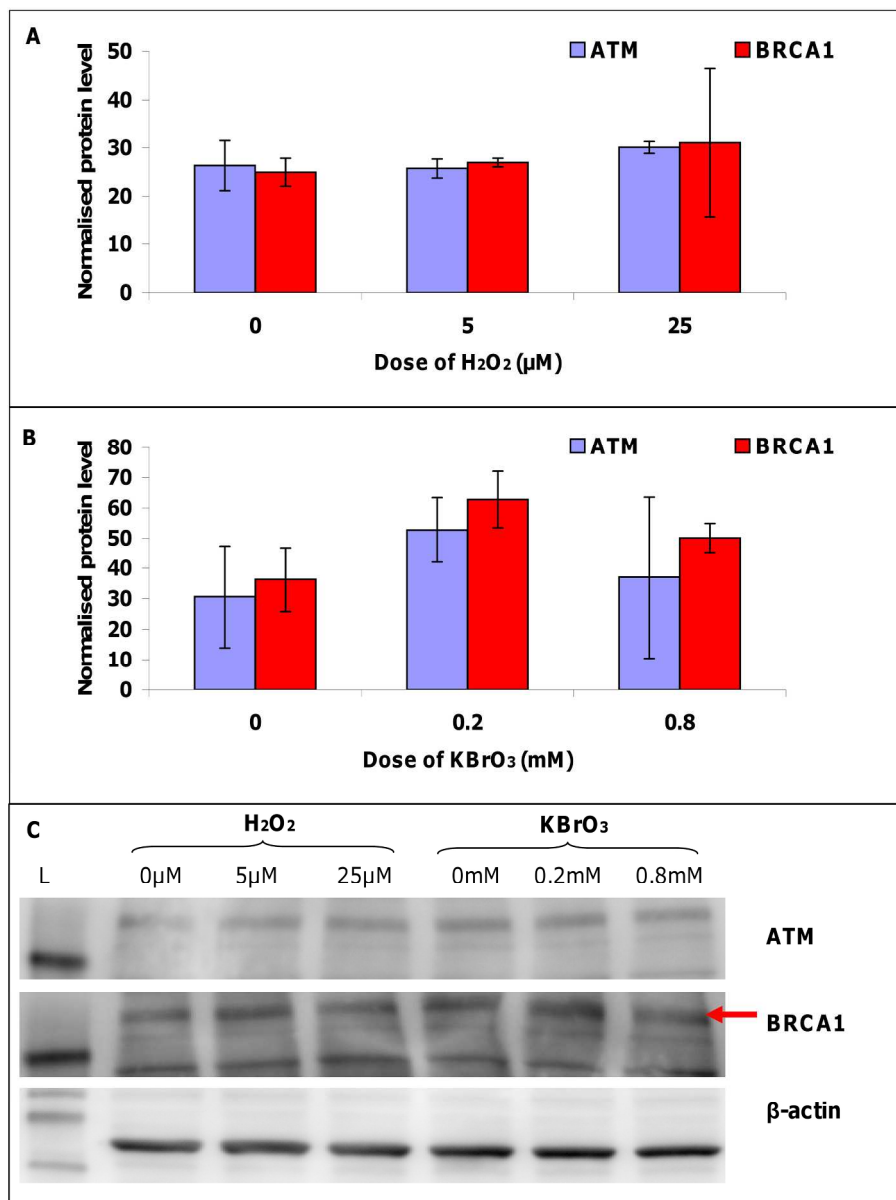


Figure 4. Effects of pro-oxidants on protein levels of ATM and BRCA1.

(A) Effect of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) on ATM and BRCA1 protein levels recovered from AHH-1 cells treated for 4 hours with concentrations above (25μM) and below (5μM) the genotoxic threshold for DNA damage induction. (B) Effect of potassium bromate (KBrO<sub>3</sub>) on ATM and BRCA1 protein levels recovered from AHH-1 cells treated for 4 hours with concentrations above (0.2mM) and below (0.8mM) the genotoxic threshold for DNA damage induction. Normalised protein level: Relative protein concentration calculated using membrane band intensities normalised to beta-actin loading control band intensities. Bars: standard deviation.

209x279mm (300 x 300 DPI)

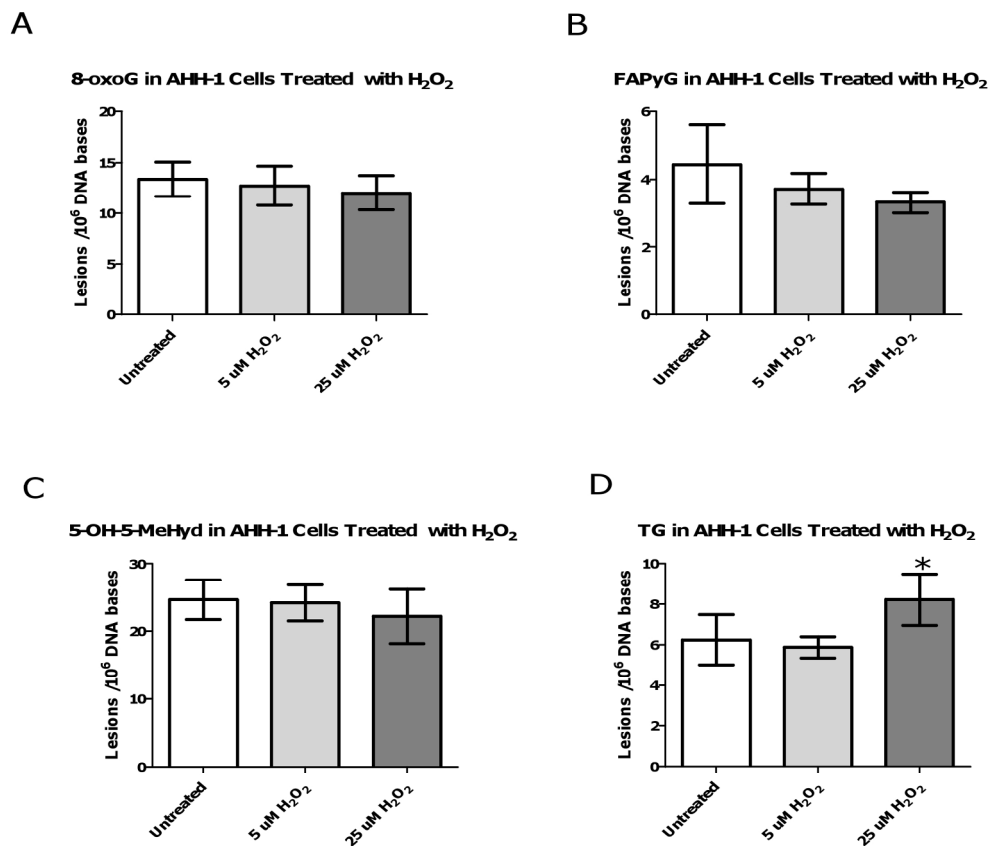


Figure 5. Determination of oxidatively induced DNA damage in hydrogen peroxide-treated AHH-1 cells. (A) Accumulation of 8-oxo-7,8-dihydroguanine (8-oxoG) in relation to exposure dose, (B) Accumulation of 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FAPyG) in relation to exposure dose, (C) Accumulation of 5-hydroxy-5-methylhydantoin (5-OH-5-MeHyd) in relation to exposure dose, (D) Accumulation of thymine glycol (TG) in relation to exposure dose. \*: ( $p < 0.05$ ) indicates statistically significant result compared to the control samples using one-way Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison test. All data points represent the mean of  $\geq 4$  independent measurements. Uncertainties are standard deviations.

187x161mm (300 x 300 DPI)

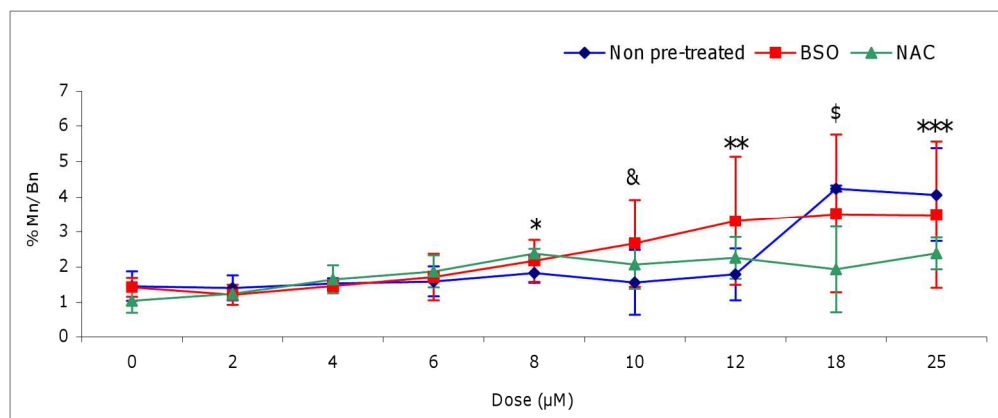


Figure 6. Pro-oxidant dose response following alteration of cellular glutathione antioxidant levels. Effect of BSO (0.5mM) or NAC (2.0mM) pre-treatment on the frequency of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) induced micronuclei in AHH-1 cells; \*: At 8 µM H<sub>2</sub>O<sub>2</sub>, first significant increase in micronuclei (MN) frequency above the control in BSO pre-treated cells ( $p=0.0008$ ) as determined by T-test. &: At 10µM H<sub>2</sub>O<sub>2</sub>, BSO pre-treated cells demonstrated a statistically significant increase in percentage MN as compared to non pre-treated and NAC pre-treated cells ( $p<0.001$  and  $p<0.05$ , respectively); \*\*: At 12µM H<sub>2</sub>O<sub>2</sub>, BSO pre-treated cells showed a statistically significant elevation in MN as compared to non pre-treated cells ( $p<0.01$ ); \$: At 18µM H<sub>2</sub>O<sub>2</sub>, first statistically significant increase in MN frequency above the control in non pre-treated cells ( $p<0.0001$ ); also, non pre-treated cells exhibited statistically significant higher levels of MN to NAC pre-treated cells ( $p<0.001$ ); \*\*\*: At 25µM H<sub>2</sub>O<sub>2</sub>, non pre-treated cells had statistically significant higher levels of micronuclei than NAC pre-treated cells ( $p<0.01$ ). Mn/Bn%: percentage of binucleated cells containing one or more micronuclei.

169x70mm (300 x 300 DPI)