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Genetic Toxicity Test of 8-Hydroxyquinoline by Ames, Micronucleus, Comet Assays and Microarray Analysis

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Abstract

8-Hydroxyquinoline is used as antibacterial agent and antioxidant based on its function inducing the chelation of ferrous ion present in host resulting in production of chelated complex. This complex being transported to cell membrane of bacteria and fungi exerts antibacterial and antifungal action. In this study, we have carried out *in vitro* genetic toxicity tests and microarray analysis to understand the underlying mechanisms and the mode of action of toxicity of 8-hydroxyquinoline. TA1535 and TA98 cells were treated with 8-hydroxyquinoline to test its toxicity by basic genetic toxicity test, Ames and two new *in vitro* micronucleus and COMET assays were applied using CHO cells and L5178Y cells, respectively. In addition, microarray analysis of differentially expressed genes in L5178Y cells in response to 8-hydroxyquinoline were analyzed using Affymatrix genechip. The result of Ames test was that 8-hydroxyquinoline treatment increased the mutations in base substitution strain TA1535 and likewise, 8-hydroxyquinoline also increased mutations in frame shift TA98. 8-Hydroxyquinoline increased micronuclei in CHO cells and DNA damage in L5178Y. 8-Hdroxyquinoline resulted in positive response in all three tests showing its ability to induce not only mutation but also DNA damage. 783 Genes were initially selected as differentially expressed genes in response to 8-hydroxyquinoline by microarray analysis and 34 genes among them were over 4 times of log fold changed. These 34 genes could be candidate biomarkers of genetic toxic action of 8-hydroxyquinoline related to induction of mutation and/or induction of micronuclei and DNA damage. Further confirmation of these candidate markers related to their biological function will be useful to understand the detailed mode of action of 8-hydroxyquinoline.

Keywords: 8-hydroxyquinoline, Ames test, COMET assay, MN assay, Microarray, S9 fraction

8-Hydroxyquinoline is used as antibacterial agent and antioxidant based on its function inducing the chelation of ferrous ion present in host resulting in production of chelated complex. This complex being transported to cell membrane of bacteria and fungi exerts antibacterial and antifungal action there. Gene activity depends on cations for activities of the different DNA and RNA polymerases, which rely on supply of Mg^{2+} and Mn^{2+} to different extents[10,11,13]. Previous study indicated that a chelating agent, such as 8-hydroxyquinoline affects the process of DNA and RNA synthesis[5].

In Europe, 8-hydroxyquinoline is accepted for use as stabilizer for hydrogen peroxide in rinse-off and leave-on hair care preparations, with concentration limitations. It is metabolized and excreted in the urine as glucuronidated metabolites. And a paper reports the effectiveness of 0.1% (W/V) 8-hydroxyquinoline as stabilizers of stock solutions on peracetic acid[15].

8-Hydroxyquinoline was genotoxic in certain *Salmonella typhimurium* strains with metabolic activation and in a mouse lymphoma assay. There was some evidence of increased chromosome aberrations *in vitro* study and an increase in sister-chromatid exchanges (but not chromosome aberrations) in a *Drosophilia* sex-linked recessive lethal test, mouse bone marrow micronucleus test, a rat bone marrow and hepatocyte micronucleus test, and unscheduled DNA synthesis in rat hepatocytes. 8-Hydroxyquinoline did bind to DNA in the presence of liver enzymes. Although the International Agency for Research on Cancer concluded that the existing evidence is inadequate to determine carcinogenicity in animals, 8-hydroxyquinoline was noncarcinogenic in several rodent feeding studies, and newly available studies using genetically altered mice, in one case carrying the human c-Ha-ras gene, demonstrated that 8-hydroxyquinoline was not carcinogenic. In clinical tests, 8-hydroxyquinoline is neither an irritant nor a sensitizer

when tested at 1% in petrolatum[1].

8-Hydroxyquinoline was tested for their genotoxicity in CD1 male mice by using a bone marrow micronucleus assay. The cytotoxic effect of this compound was expressed as low polychromatic erythrocyte (PCE)/normochromatic erythrocyte (NCE) ratios with three does levels (25, 50, and 100 mg/kg) at 24 h after injection and as a high mortality rate in animals treated with the high does (100 mg/kg)[7].

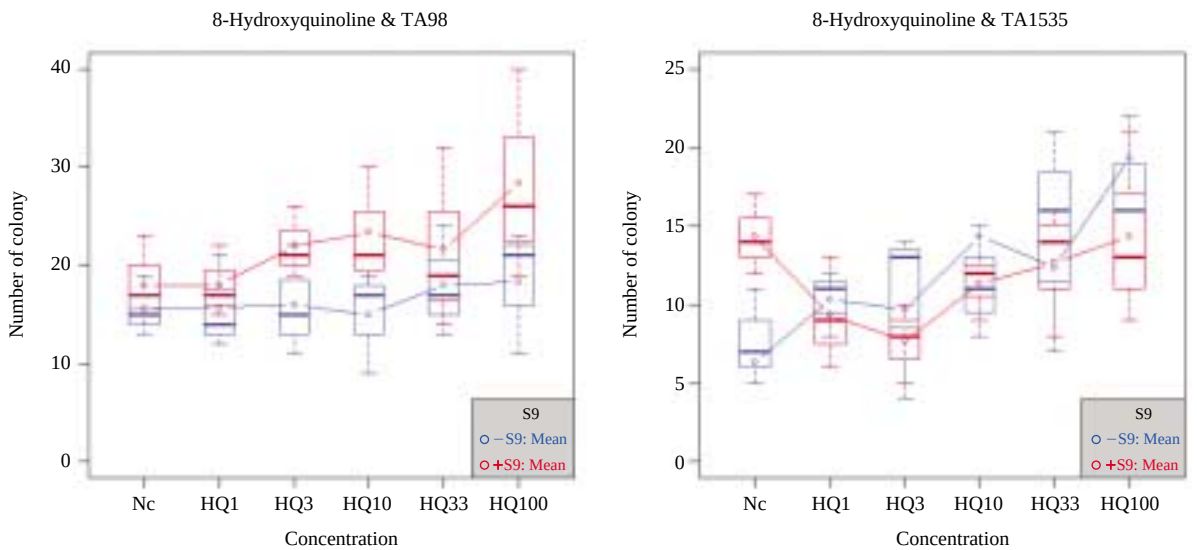
In vitro assays of the genotoxicity of 8-hydroxyquinoline had no measurable effect on either chromosome aberrations (CA) or sister chromatid exchanges (SCE) but did tent to prolong the cell cycle[12].

Recent result showed that Tris (8-quinolinolato-N1, O8) aluminum (AlQ) is metabolized to 8-hydroxyquinoline and then induced reverse mutations[3]. Tris (8-quinolinolato-N1, O8) aluminum (AlQ), an aluminum chelate of 8-hydroxyquinoline is an important charge transfer molecule in semiconducting imaging devices.

Although the genetic toxicity of 8-hydroxyquinoline has been reported, no further study has not been carried out to find out the underlying mechanism of genetic toxic action of 8-hydroxiquinoline. In this study, we have tested 8-hydroxyquinoline using Ames test, *in vitro* micronuclei assay in CHO cells, single cell gel/comet assay in L5178Y cells, microarray analysis of gene expression profiles in L5178Y cells in order to find out biomarker genes in response to genetic toxicity of 8-hydroxyquinoline.

8-Hydroxyquinoline Induced Gene Mutations in both TA98 and TA1535 Strains

Number of revertants/plate was assessed as a measure of gene mutation in both TA98 and TA1535 strains exposed to different concentrations of 8-hydroxyquinoline (Figure 1). In TA98 strain, the number of revertants/plate of 1.0 μg 2-nitrofluorene treated bacteria in the absence of S9 was 564±15.1 and the MF of cells exposed to 10 μg 2-aminofluorene in the presence of S9 was 399.7±10.50. The positive control chemicals, 2-nitrofluorene and 2-aminofluorene generated large increases in revertant. The number of revertants/plate of solvent control bacteria were 15.67±3.06 in the absence of S9 and 18.00±4.58 in the presence of S9. The number of revertants /plate (1, 3, 10, 33, 100 μg) treated bacteria was 15.67±4.73, 16.00±5.57, 15.00±5.29, 18.00±5.57, 18.33±6.43 in the absence of S9, 18.00±3.61, 22.00±3.61, 23.00±6.24, 21.67±9.29, 28.33±10.69 in the presence of S9, respectively. 8-Hydroxyquinoline treatments statistically significant increased in revertant numbers in TA98 with or without S9. In TA1535 strain, the number of revertants/plate of 1.5 μg sodium azide treated cells in the absence of S9 was 12.00±2.00 and the number of revertants/plate of bacteria exposed to 10 μg 2-aminofluorene in the presence of S9 was 20.67±5.03. The positive control chemicals, sodium azide and 2-aminofluorene resulted large increases in revertant numbers. The number of revertants/plate of solvent control bacteria were 7.67±



(A) TA98 (B) TA1535

Figure 1. The Mutagenicity of 8-Hydroxyquinoline tested in strain TA98 and TA1535. The Ames test was performed by the pre-incubation test method (Gatehouse *et al.* 1994) with or without metabolic activation using Salmonella typhimurium strains TA98 and TA1535 as described in methods. The data represent averages from three experiments with triplicate plates per dose. NC: negative control. Data are means, boxplots.

3.06 in the absence of S9 and 14.33±2.52 in the presence of S9. The number of revertants/plate of 8-hydroxyquinoline (1, 3, 10, 33, 100 μg) treated bacteria were 10.33±2.08, 10.33±5.51, 11.33±3.51, 14.67±7.09, 18.00±3.46 in the absence of S9, 9.33 ±3.51, 7.67±2.52, 11.33±2.08, 12.67±4.16, 14.33 ±6.11 in the presence of S9, respectively. 8-hydroxyquinoline treatments statistically significant increased in revertant numbers in TA1535 with or without S9. These 8-hydroxyquinoline dose-dependent increases were therefore considered to have provided clear evidence of mutagenic activity of 8-hydroxyquinoline in both TA98 and TA1535.

8-Hydroxyquinoline Induced DNA Damage in L5178Y Cells

The Olive Tail Moment was assessed as a measure of DNA damage in the comet assay in L5178Y mouse lymphoma cells exposed to different concentrations of 8-hydroxyquinoline (25-100 μg/mL) for 2 or 24 h (Figure 2). The Olive Tail Moment of MNNG -treated cells (100 μM, positive control in the absence of S9) was 4.13±3.08 and the OTM of cells exposed to B[a]P (50 μM, positive control in the presence of S9 metabolic activation system) was 2.79±2.17. The Olive Tail Moment of control cells was 4.76±3.50 in the absence of S9 and 52.59±45.21 in the presence of S9. Cells were exposed to 25, 50, 100 μg/mL 8-hydroxyquinoline for 2 h. The tail Moment induced by 8-hydroxyquinoline were 7.19±3.18, 13.88± 4.64, 28.27±8.05 in the absence of S9 and 21.97± 5.20, 38.73±6.24, 54.09±13.61 in the presence of S9, respectively. It thus caused a significant increase in DNA damage in comparison to the solvent control.

8-Hydroxyquinoline Induced Micronuclei in CHO-K1 Cells

CHO-K1 cell cultured RPMI medium and treated with cyclophosphamide (CPA) in the presence of S9. As expected, numbers of micronuclei were induced to be 2.5, 5, 10 μg/mL CPA 41.0±9.0, 39.0±11.0, 39.0±8.0, respectively. Cells were exposed 0.4, 0.8, 1.6 μg/mL 8-hydroxyquinoline for 4 h. Numbers of micronuclei of 8-hydroxyquinoline treated cells were 52.7±12.7, 82.7±18.6, 80.0±3.5 in the absence of S9 and 52.7±18.9, 69.7±13.5, 65.7±9.1 in the presence of S9, respectively. Increase in the numbers of micronuclei with 8-hydroxyquinoline treatment was

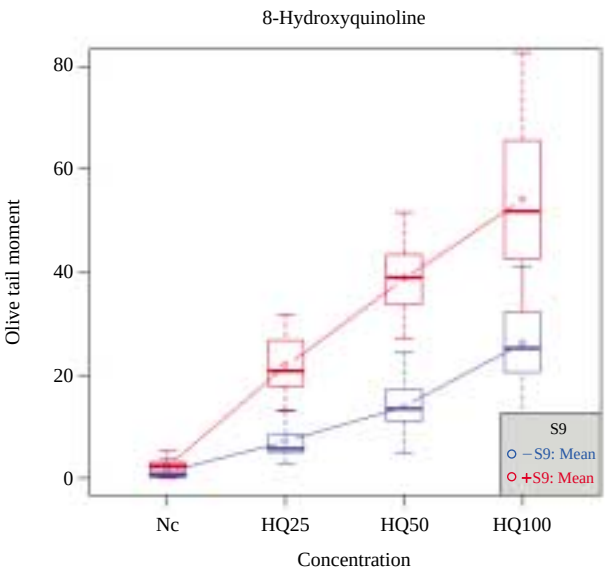


Figure 2. Olive tail moments by 8-Hydroxyquinoline in L5178Y mouse lymphoma cells. Tail moments were measured using comet assay according to Singh *et al.* (Singh *et al.* 1988) with slight modification as described in methods. Tail moments of L5178Y mouse lymphoma cells exposed to 25, 50, 100 μg/mL 8-hydroxyquinoline for 2 h. Negative control was medium. Positive controls were MNNG (100 μM) in the absence of S9 and B(a)P (50 μM) in the presence of S9 metabolic activation system, respectively. NC: negative control. PC: positive control, Data are means, boxplots.

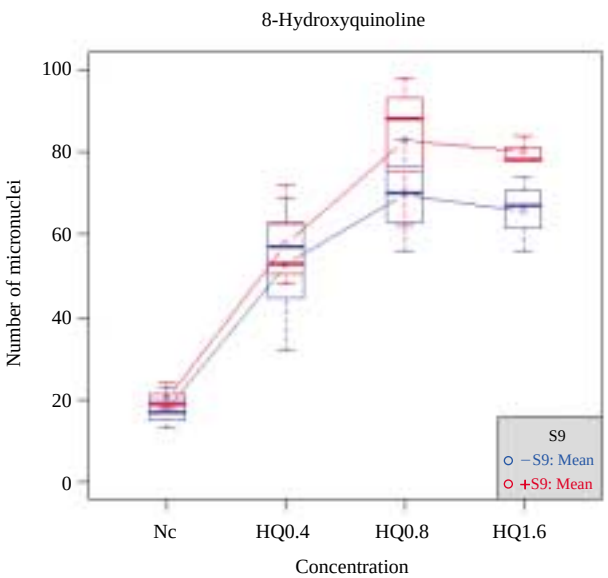


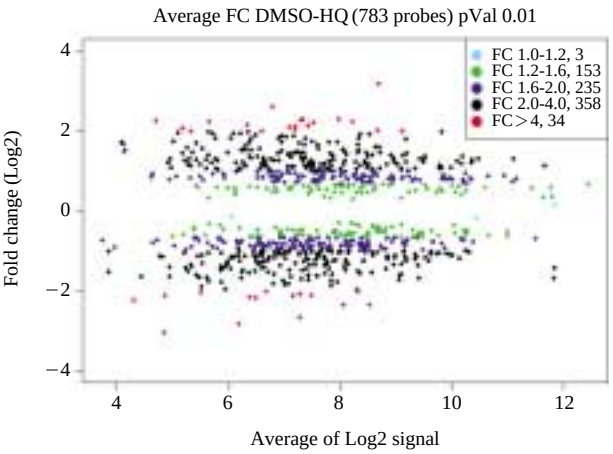
Figure 3. Micronucleus formation induction by 8-Hydroxyquinoline in CHO-K1 cells. The CBMN (cytokinesis-block micronucleus) assay was performed according to Fenech (Fenech, 2000) with modification and the recommendation of the 3rd International Workshop on Genotoxicity Testing (Kirsch-Volders *et al.* 2003) as described in methods. CHO-K1 cells were grown in 24-well plates and treated with 8-hydroxyquinoline (0.4, 0.8, 1.6 μg/mL) or cyclophosphamide (2.5, 5, 10 μg/mL) for 4 h with or without S9. Cells were stained with 0.24 mM acridine orange and micronuclei were scored under the fluorescence microscope at 1000 magnification. Data are means, boxplots.

statistically significantly and concentration-dependent (Figure 3).

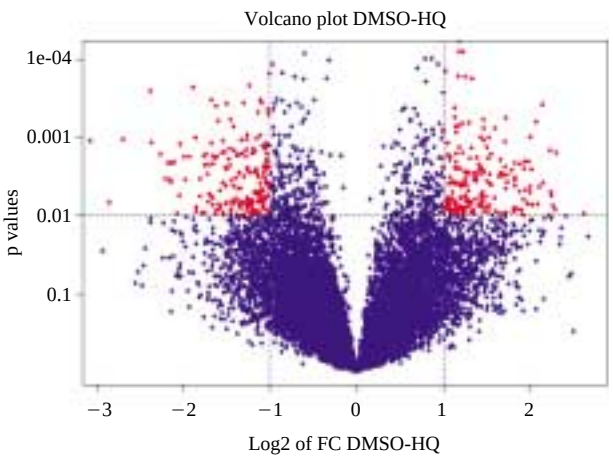
Microarray Analysis of Differentially Expressed Genes with 8-hydroxyquinoline Treatment in L5178Y Cells

Gene expression profiling offers a powerful approach for identifying differentially expressed gene and identifying mechanism.

Differentially expressed genes from L5178Y cells treated with 8-hydroxyquinoline (100 μg/mL) was analyzed by microarray using Affymetrix Mouse Genome 430 2.0 GeneChip arrays[2]. 783 genes were specifically regulated and their folds of change were greater than 2 of log formation. Among them 34 genes were selected after the *t*-tests and performed Volcano plot analysis (Figure 4). Figure 5 showed the results of clustering analysis of 8-hydroxyquinoline regulated genes. Table 2 showed related pathway information with 8-hydroxyquinoline treatment and the genes list in Figure 6 showed genes which expression were up and do regulated with 8-hydroxyquinoline treatment. If these genes expression would be related to genetic toxicity of 8-hydroxyquinoline, it would need further study.



(A) Stratified fold change



(B) 8-Hydroxiquinoline/control (VolcanoPlot)

Figure 4. Number of genes regulated by 8-Hydroxyquinoline in L5178Y cells. The microarray analysis was performed according to Affymetrix Inc. (Affymetrix Inc., 2002) with modification as described in methods. L5178Y mouse lymphoma cells were treated with 8-hydroxyquinoline (100 μg/mL), and total RNA was isolated by TRIzol. After the hybridization and staining arrays were scanned with an Affymetrix scanner 3000, and data were obtained using the GeneChip operating software (GCOS, version 1.2.0.037). Profiles were analyzed by Volcano Plot (B).

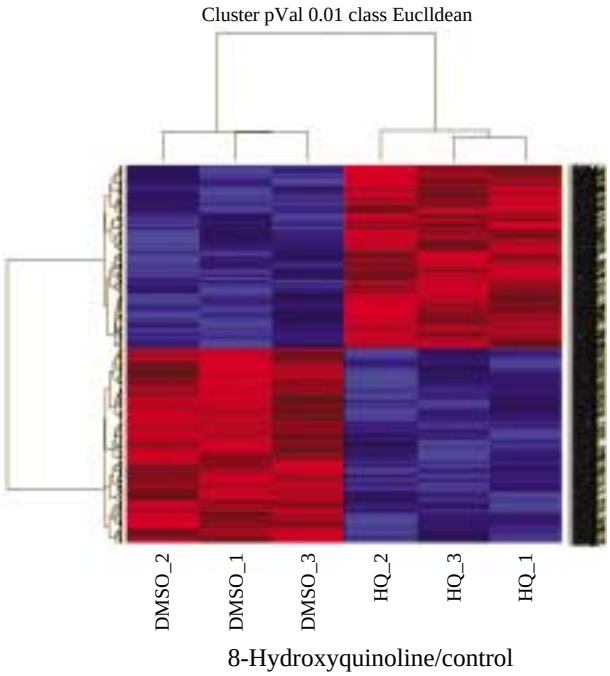


Figure 5. Results of hierarchical clustering by 8-Hydroxyquinoline. The microarray analysis was performed according to Affymetrix Inc. (Affymetrix Inc., 2002) with modification as described in methods. L5178Y mouse lymphoma cells were treated with 8-hydroxyquinoline (100 μg/mL), and total RNA was isolated by TRIzol. After the hybridization and staining arrays were scanned with an Affymetrix scanner 3000, and data were obtained using the GeneChip operating software (GCOS, version 1.2.0.037). The data were analyzed by hierarchical clustering, and green represents down regulation of the transcripts; black, no change; red, up regulation of the transcript.

Discussion

Genetic toxicity of 8-hydroxyquinoline was verified through Ames test, Comet assay, *In vitro* micronu-

Table 1. Significant test result of One-way (test Dose effect), Two-way (interaction between Dose and S9) ANOVA.

Test type	S9	One-way ANOVA	Two-way ANOVA		
Ames TA98	HQ without S9	model: $P<0.40$, coefficient: 0.55, $P<0.3$	Dose	S9	Dose × S9
	HQ with S9	model : $P<0.05$, coefficient: 1.81, $P<0.05$*	0.49405	0.01413*	0.83772
Ames TA1535	HQ without S9	model: $P<0.00$**, coefficient: 0.39, $P<0.51$	Dose	S9	Dose × S9
	HQ with S9	model: $P<0.00$*, coefficient: 1.88, $P<0.00$**	0.009152*	0.713899	0.070040
Comet assay	HQ without S9	model: $P<0.00$***, coefficient: 20.40, $P<0.00$**	Dose	S9	Dose × S9
	HQ with S9	model: $P<0.00$**, coefficient: 16.1, $P<0.00$*	2.2e-16**	2.2e-16***	2.2e-16**
Micronuclei test	HQ without S9	model: $P<0.00$***, coefficient: 1.67, $P<0.00$**	Dose	S9	Dose × S9
	HQ with S9	model: $P<0.00$***, coefficient: 4.44, $P<0.00$**	1.309e-06***	0.09595	0.7955

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1''1

cleus assay. Differentially expressed genes by 8-hydroxyquinoline was analyzed by microarray analysis.

8-Hydroxyquinoline is positive in Ames test that was performed using *Salmonella typhimurium* strains TA98 and TA1535. Comet assay was performed in L5178Y mouse lymphoma cell with and without metabolic activation. 8-hydroxyquinoline caused a significant increase does-dependent in DNA damage and the result was confirmed with 3 replicate experiments.

In vitro micronucleus assay was carried out using CHO-K1 cell and 3 different concentrations with and without metabolic activation. The result of assay was positive response that is 8-hydroxyquinoline increased micronuclei in CHO cell.

The cell was treated 8-hydroxyquinoline and the gene expression profile from microarray was analyzed data manipulation and preprocessing. Then Hierarchical clustering was conducted with meaningful genes.

8-Hydroxyquinoline-inducement by the level of concentration was evaluated with one-way ANOVA for each test with S9 and without S9 respectively and inference of direct or indirect effect of 8-hydroxyquinoline to DNA was evaluated with two-way ANOVA for each test with S9 and without S9 respectively in Table 1. As the result, expected 8-hydroxyquinoline toxicity was indirect DNA frame shift (TA98), indirect DNA base substitution (TA1535), direct and indirect DNA damage (comet, MN assay).

The gene expression profile provides us a better understanding of underlying mechanisms for 8-hydroxyquinoline-induced genetic toxicity. Integration of gene expression changes with known pathological changes can be used to formulate a mechanistic scheme for 8-hydroxyquinoline-induced genetic toxicity as Table 2 and Figure 6.

Table 2. Pathway information of expressed gene by 8-Hydroxyquinoline treatment from 392 (FC > 2.0) of 783 ($P<0.01$).

KEGG pathway	Involved genes count	Involved genes/Total count (%)	Modified fisher exact P-value, Ease score
MMU00240: PYRIMIDINE ETABOLISM	9	2.50%	0.0008***
MMU04110:CELL CYCLE	7	1.94%	0.0312*
MMU00670:ONE CARBON POOL BY FOLATE	4	1.11%	0.0060**

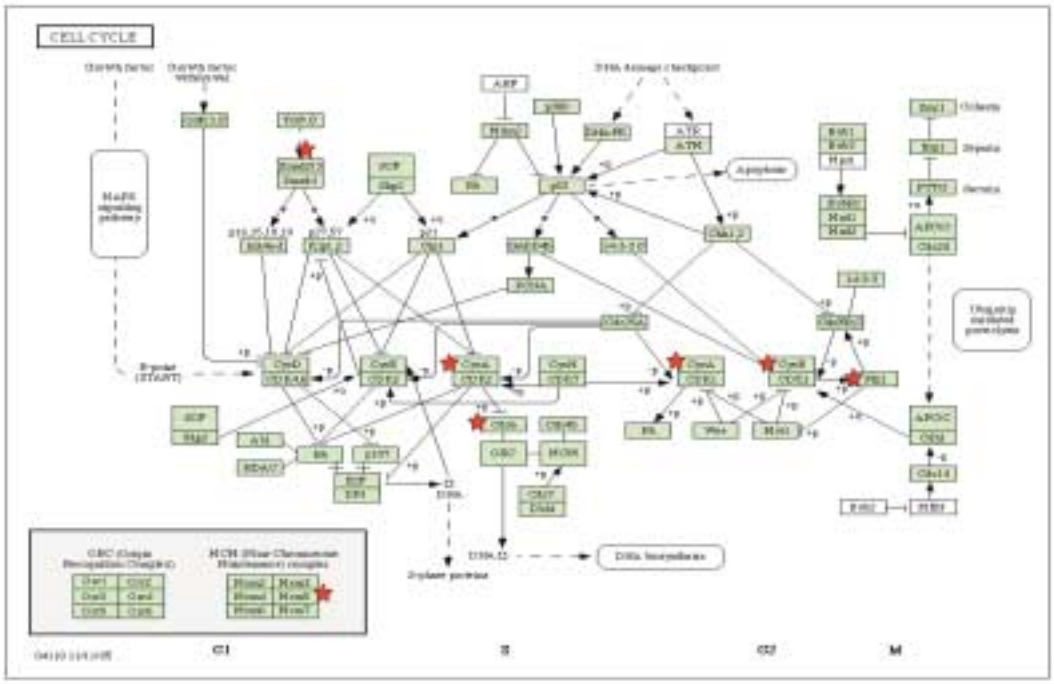
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1''1

Further confirmation of these candidate markers related to their biological function will be useful to understand the detailed mode of action of 8-hydroxyquinoline.

Methods

Materials

8-Hydroxiquinoline, 2-aminofluorene, 2-nitrofluorene, sodium azide, 1-methyl-3-nitro-1-nitrosogu-



Related Gene Description
ORIGIN RECOGNITION COMPLEX, SUBUNIT 3-LIKE (S. CEREVISIAE)
MINICHROMOSOME MAINTENANCE DEFICIENT 5, CELL DIVISION CYCLE 46 (S.CEREVISIAE)
V-ABL ABELSON MURINE LEUKEMIA ONCOGENE 1
POLO-LIKE KINASE 1 (DROSOPHILA)
PITUITARY TUMOR-TRANSFORMING 1
EXTRA SPINDLE POLES-LIKE 1 (S. CEREVISIAE)
GROWTH ARREST AND DNA-DAMAGE-INDUCIBLE 45 GAMMA
MAD HOMOLOG 3 (DROSOPHILA)
RIKEN CDNA 2310042N09 GENE
CELL DIVISION CYCLE 6 HOMOLOG (S. CEREVISIAE)
CYCLIN B2
CYCLIN A2

Figure 6. Cell Cycle of expressed gene by 8-Hydroxyquinoline treatment.

anidin (MNNG), benzo(a)pyrene and cyclophosphamide were obtained from Sigma chemical Co. (St. Louis, MO, USA). The S9 fraction was purchased from Moltox® S9 (Canbiotech, USA).

Ames Test

The Ames test was performed by the pre-incubation test method[6] with or without metabolic activation using *Salmonella typhimurium* strains TA98 and TA1535. The tester strains were cultured overnight in 0.8% oxide nutrient broth at 37˚C. To the 0.1 mL of bacterial suspension, 0.5 mL of 0.1 M sodium phosphate buffer (pH 7.4) or 0.5 mL of S9 was added and then 0.1 mL of 8-hydroxyquinoline (1, 3, 10, 33, 100 μg/plate) or positive control chemicals such as 2-aminofluorene, 2-nitrofluorene and sodium azide were added and incubated for 20 min at 37˚C. After incubation, 2.0 mL of top agar was added to mix and the mixture was poured onto a minimal glucose agar plate. 48 hours after the incubation at 37˚C, the numbers of revertant colonies were counted[8].

Comet Assay

Comet Assay was carried out according to Singh *et*

al.[14] with slight modification. L5178Y mouse lymphoma cells were grown at 37˚C in a 5% CO_2 incubation. L5178Y mouse lymphoma cells were seeded in 12 well plates (1×10^6 cells/mL) and were exposed to 25, 50, 100 μg/mL 8-hydroxyquinoline for 2 h. Positive controls were 100 μM 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) in the absence of S9 metabolic activation, 50 μM benzo(a)pyrene (BaP) in the presence of S9 metabolic activation. 20 μL of cell suspension were mounted in 1% agarose on slide glass. Slides were immersed in a cold lysing solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, pH 10, 1% (v/v) Triton X-100 and 10% (v/v) DMSO) for 1.5 h at 4˚C and then for 20 min in the electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH > 13). Slides were electrophoresed and neutralized using Tris buffer (0.4 M Tris, pH 7.5) and stained with ethidium bromide (20 μg/mL). Cells were analyzed using a Comet Image Analysis System, Version 5.5 (Kinetic Imaging Ltd., Andor Bioimaging Division, Nottingham, UK).

In vitro Cytokinesis Block Micronucleus Assay

The CBMN (cytokinesis-block micronucleus) assay was performed according to Fenech 4 with modification, and the recommendation of the 3rd International Workshop on Genotoxicity Testing[9]. CHO-K1 cells were grown in 24-well plates and treated with 8-hydroxyquinoline (0.4, 0.8, 1.6 μg/mL) or cyclophosphamide (2.5, 5, 10 μg/mL) for 4 h with or without S9. After the treatment, cells were washed with PBS and further incubated for 20 h in the medium containing 3 μg/mL cytochalasin B. Cells were harvested and spread on glass slide, and fixed with 100% methanol for 5 min and stained with 0.24 mM acridine orange in 1/150 M phosphate buffer (pH 6.8) for 3 min. Micronuclei were scored under the fluorescence microscope at 1000 magnification.

Microarray

The microarray analysis was performed according to Affymetrix Inc.[2] with modification. L5178Y mouse lymphoma cells were plated in RPMI-1640 medium into 12-well plate (1×10^6 cells/mL). After 24 h of treatment with 8-hydroxyquinoline (100 μg/mL), total RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA) and purified by a RNeasy mini kit (QIAGEN, Hilden, Germany). Total RNA (1 μg) was amplified using the Affymetrix one-cycle cDNA synthesis protocol. For each array, 15 μg of amplified biotin-cRNAs was fragmented and hybridized to the Affymetrix Mouse Genome 430 2.0 GeneChip arrays (Affymetrix, Santa Clara, CA) for 16 h at 45˚C in a rotating hybridization oven. Slides were stained with streptavidin/phycoerythrin and washed for antibody amplification. Arrays were scanned with an Affymetrix scanner 3000, and data were obtained using the GeneChip operating software (GCOS, version 1.2.0.037).

Statistical Analysis

All numerical data were expressed as the average of the values obtained ±S.D. and their significance determined by conducting a paired Student's *t*-test.

Acknowledgements

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