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Genetic Toxicity Test of Glycidol by Ames, Micronucleus, Comet Assays and Microarray Analysis

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Abstract – The primary use for glycidol is as a stabilizer in the manufacture of vinylpolymers, however, it is also used as an intermediate in the production of pharmaceuticals, as an additives for oil and synthetic hydraulic fluids, and as a diluting agent is same epoxy resins. In this study, we have carried out in vitro genetic toxicity test of glycidol and microarray analysis of differentially expressed genes in response to glycidol. The result of Ames test showed mutations with glycidol treatment in base substitution strain TA1535 both with and without exogenous metabolic activation. Likewise, glycidol showed mutations in frame shift TA98 both with and without exogenous metabolic activation. The result of COMET assay in L5178Y cells with glycidol treatment showed DNA damage both with and without exogenous metabolic activation. Glycidol increased micronuclei in CHO cells both with and without exogenous metabolic activation. 150 Genes were selected as differentially expressed genes in response to glycidol by microarray analysis and these genes would be candidate biomarkers of genetic toxic action of glycidol.

Keywords □ Glycidol, Ames test, COMET assay, MN assay, Microarray, S9 fraction

INTRODUCTION

Glycidol is a colorless, viscous liquid soluble in both water and organic solvents. The primary use for glycidol is as a stabilizer in the manufacture of vinylpolymers, however, it is also used as an intermediate in the production of pharmaceuticals, as an additives for oil and synthetic hydraulic fluids, and as a diluting agent is same epoxy resins. Over 5 million kilograms of glycidol compounds as produced and used (Irwin *et al.*, 1996). Glycidol has been evaluated for teratogenicity in both rats and mice. Intra-amniotic injection of glycidol into pregnant Sprague Dawley rats on day 13 of gestation caused embryolethality and induced malformation in a significant number of fetuses (Slott and Htales, 1985). No evidence of teratogenicity was observed in a study in which pregnant CD-1 mice received 100, 150, or 200 mg/kg glycidol by gavage during days 6-15 of gestation (Marks *et al.*, 1982). However, exposure to glycidol showed antifertility activity, such as reduction in sperm motility and infertility in male rats (Jackson H. *et al.*, 1970).

There is evidence to demonstrate that glycidol is carcinogenic in experimental animal model. When glycidol was administered to syrinan golden hamsters (20 males and 20 females) by gavage (12 mg twice a week for 60 weeks), more tumors were observed in the glycidol exposed hamsters than in control group (Lijinsky and Kovatch, 1985). In another study in which glycidol was administered by gavage to F344/N rats (37.5 and 75 mg/kg) and B6C3F1 mice (25 or 50 mg/kg) daily for 2 years, dose-related increases in the incidences of neoplasms in numerous tissues in both species were observed (Irwin *et al.*, 1996). The dermal carcinogenicity of glycidol has been evaluated in a study in which 5% solution in acetone was applied to the backs of female ICR/Ha Swiss mice, three times a week for 2 years (Van Duuren et al., 1967). Glycidol application produced no visible reaction on the skin, however, similar application of a 10% solution of glycidaldehyde to 41 mice induced the formation of papillomas in 6 mice and carcinoma in 3 mice.

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(NTP) in rats and mice, diglycidyl resorcinol ether, a glycidol derivative, administered by gavage in corn oil for 2 years induced hyperkeratosis, hyperplasia, and neoplasms of the fore-stomach in both species (NTP, 1986). In 2 year inhalation studies, 1,2-expoxybutane induced papillary adenomas of the nasal cavity in male and female rats and alveolar/bronchiolar neoplasms in male rats (NTP, 1988). In inhalation studies, ethylene oxide induced neoplasms of the lung and harderian gland in both male and female mice (NTP, 1987). Glycidol has produced positive results in several tests of genotoxicity. A number of reports have documented the induction of gene muatations in Salmonella base substitution strains TA100 and TA1535 with or without S9 activation (McCann *et al.*, 1975; Wade *et al.*, 1979; Simmon *et al.*, 1979; Thompson *et al.*, 1981; Kaplan *et al.*, 1982; Mamber *et al.*, 1984).

Although the genetic toxicity of glycidol has been reported, no further study has not been carried out to find out the underlying mechanism of genetic toxic action of glycidol. In this study, we have tested glycidol 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 reponse to genetic toxicity of glycidol.

MATERIALS AND METHODS

Materials

Glycidol, 2-aminofluorene, 2-nitrofluorene, sodium azide, methanesulfonic acid methyl ester, benzo(a)pyrene (BaP) and cyclophosphamide were obtained from Sigma chemical Co. (St. Louis, MO, USA). The S9 fraction was purchased from Moltox® S9 (Canbiotech, U.S.A.).

Ames test

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. The tester strains were cultured overnight in nutrient broth medium 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 glycidol (10, 33.3, 100, 333.3, 1000 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 (Kasamatsu *et al,* 2005).

Comet assay

Comet assay was carried out according to Singh et al. (Singh *et al.*, 1988) with slight modification. L5178Y mouse lymphoma cells were seeded in 12 well plates and were exposed to 1000, 2000, 4000 ìg/ml glycidol for 2 h. For the positive controls, cells were exposed to 150 ìM methyl methanesulfonate (MMS) in the absence of S9, 50 ìM 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 1.5h at 4°C and then for 20min 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 ìg/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 (Fenech, 2000) with modification, and the recommendation of the 3rd International Workshop on Genotoxicity Testing (Kirsch-Volders *et al.*, 2003). CHO-K1 cells were grown in 24-well plates and treated with glycidol (7.5, 15, 30 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 6.7 mM 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. (Affymetrix Inc., 2002) with modification. L5178Y mouse lymphoma cells were plated in RPMI-1640 medium into 12-well plate. After 2 h of treatment with glycidol (400 μg/ml), cells were resuspended in media without glycidol and cultured for 20 h. 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).

RESULTS

Glycidol induced gene mutations in both TA98 and TA1535 strains

The mutant frequency(MF) was assessed as a measure of gene mutation in both TA98 and TA1535 strains exposed to different concentrations of glycidol (Fig. 1). In TA98 strain, the MF of 1.0 μg 2-nitrofluorene treated bacteria in the absence of S9 was 58.00±6.00 and the MF of bacteria exposed to 10 μg 2-aminofluorene in the presence of S9 was 114.00±16.52. The positive control chemicals, 2-nitrofluorene and 2-aminofluorene generated large increases in revertant. The MF of solvent control bacteria were 27.33±4.51 in the absence of S9 and 26.00±9.54 in the presence of S9. The MF of glycidol (10, 33.3, 100, 333.3, 1000 μg) treated bacteria were 16.00±6.24, 15.00±7.00, 36.00±8.54, 52.33±7.37, 62.67±10.07 in the absence of S9, 223.00±65.05, 380.50±21.92, 509.67±49.80, 658.67±92.65, 851.33±132.38 in the presence of S9, respectively. Glycidol treatments significantly increased revertant numbers in TA98 with or without S9. In TA1535 strain, the MF of 1.5 μg sodium azide treated bacteria in the absence of S9 was 339.33±18.56 and the MF of bacteria exposed to 10 μg 2-aminofluorene in the presence of S9 was 273.00±18.33. The positive control chemicals, sodium azide and 2-aminofluorene showed large increases in revertant numbers. The MF of solvent control bacteria were 23.67±4.51 in the absence of S9 and 31.33±7.37 in the presence of S9. The MF of glycidol (10, 33.3, 100, 333.3, 1000 μg) treated bacteria were 77.33±5.69, 201.33±7.23, 355.33±31.07, 618.67±40.05, 704.00±15.62 in the absence of S9, 220.33±31.66, 312.33±44.12, 406.00±78.92, 865.67±87.13, 1368.67±206.11 in the presence of S9, respectively. Glycidol treatments significantly increased revertant numbers in TA1535 with or without S9. These glylcidol dose-dependent increases were therefore considered to have provided clear evidence of mutagenic activity of glycidol in both TA98 and TA1535.

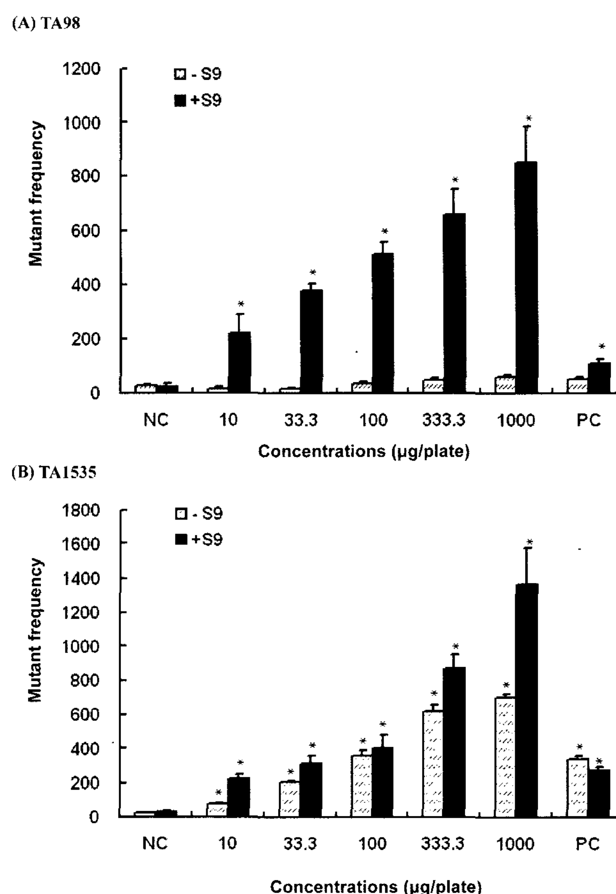

Fig. 1. The mutagenicity of glycidol tested in strain TA98 and TA1535. The Ames test was performed by the pre-incubation test method (Gatehouse et al., 1994) with or without metabolic activation using Salmonella *typhimurium strains* TA98 and TA1535 as described in methods. The data represent averages from three experiments with triplicate plates per dose. NC: negative control. PC: positive control (-S9: 1.5 μg/plate sodium azide, +S9: 10 μg/plate 2-aminofluorene)

Glycidol induced DNA damage in L5178Y cells

The Olive Tail Moment (OTM) was assessed as a measure of DNA damage in the comet assay in L5178Y mouse lymphoma cells exposed to different concentrations of glycidol (1000-4000 μg/ml) for 2 h (Fig. 2). The OTM of MMS-treated cells (150 μM, positive control in the absence of S9) was 29.79±3.84 and the OTM of cells exposed to B[a]P (50 μM, positive control in the presence of S9 metabolic activation system) was 46.75±9.76. The OTM of control cells was 0.21±0.30 in the absence of S9 and 1.56±1.66 in the presence of S9. Cells were exposed to 1000, 2000, 4000 μg/ml glycidol for 2 h. OTMs induced by glycidol were 8.44±2.93, 19.91±5.57, 49.62±11.7 in the absence of S9 and 21.13±5.23, 31.69±7.71, 38.00±3.40 in the presence of S9, respectively. It thus caused a significant increase

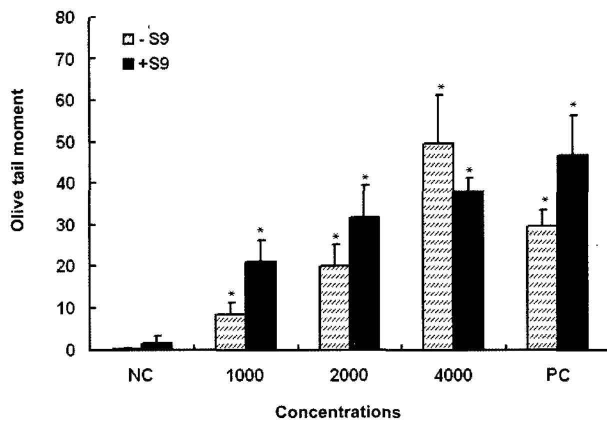

Fig. 2. Olive tail moments by glycidol in L5178Y mouse lymphoma cells. Olive tail moments were measured using comet assay according to Singh *et al.* (Singh *et al.*, 1988) with slight modification as described in methods. Olive tail moments of L5178Y mouse lymphoma cells exposed to 1000, 2000, 4000 μg/ml glycidol for 2h. Negative control was medium. Positive controls were MMS (150 μM) in the absence of S9 and BaP (50 μM) in the presence of S9 metabolic activation system, respectively. NC: negative control. PC: positive control, Data are means ± S.D. (n=15)

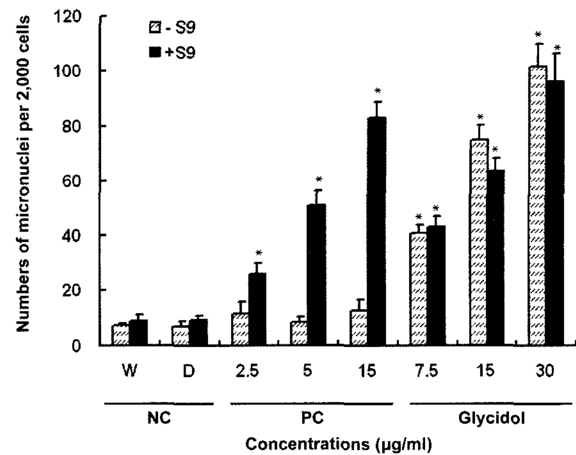

Fig. 3. Micronucleus formation by glycidol in CHO-K1 cells. The CBMN (cytokinesis-block micronucleus) assay was performed according to Fenech (Fenech, 2000) with modification, and the recommendation of the 3rd International Workshop on Genotoxicity Testing (Kirsch-Volders *et al.*, 2003) as described in methods. CHO-K1 cells were grown in 24-well plates and treated with glycidol (7.5, 15, 30 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 ± S.D. n=3 *: Statistically different from concurrent control at $P<0.05$. NC: negative control. PC: positive control (cyclophosphmide 2.5, 5 and 10 μg/ml).

in DNA damage in comparison to the solvent control and the lowest effective concentration was 1000 μg/ml.

Glylcidol induced micronuclei in CHO-K1 cells

CHO-K1 cells cultured in 24-well plate using RPMI medium and treated with cyclophosphamide(CPA) in the presence of S9. As expected, numbers of micronuclei of cells treated with 2.5, 5, 10 μg/ml CPA were 25.67±4.04, 51.00±5.29, 83.00±5.57, respectively. Numbers of micronuclei of 7.5, 15, 30 μg/ml glycidol treated cells were 41.00±4.00, 75.00±5.57, 101.33±13.50 in the absence of S9 and 44.33±4.51, 63.33±2.08, 96.00±7.55 in the presence of S9, respectively. Increase in the numbers of micronuclei with glycidol treatment was statistically significant and concentration-dependent (Fig. 3).

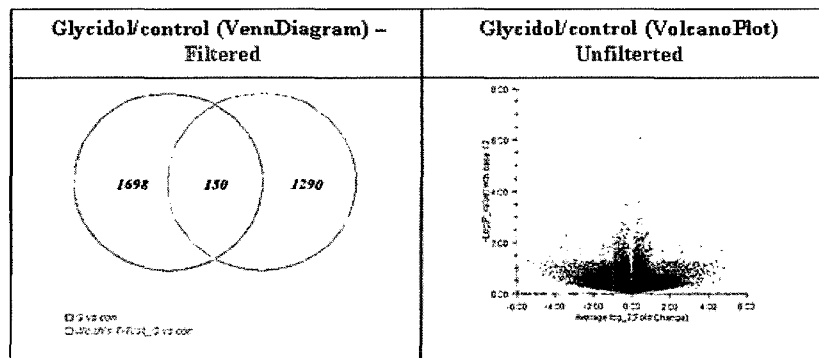

Fig. 4. Numbers of glycidol regulated genes in L5178Y cells. The microarray analysis was performed according to Affymetrix Inc. (Affymetrix Inc., 2002) with modification as described in methods. L5178Y mouse lymphoma cells were treated with glycidol (400 μg/ml), and total RNA was isolated by TRIzol. 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 VennDiagram (A) and Volcano Plot (B).

Microarray analysis of differentially expressed genes with glycidol treatment in L5178Y cells

Differentially expressed genes from L5178Y cells treated with glycidol (400 μg/ml) were analyzed by microarray using Affymetrix Mouse Genome 430 2.0 GeneChip arrays. 1848 genes were glycidol specifically regulated and their fold of change were greater than Log 2. Among them 150 genes were selected after the Welch's T-test and Volcano plot analysis (Fig. 4). Figure 5 showed the results of clustering analysis of glycidol regulated genes. Table I showed genes which expressied were increased with glycidol treatment. If these genes expression would be related to genetic toxicity of glycidol, it would need further study.

DISCUSSION

In this study, we have tested glycidol for *in vitro* genetic toxicity and also looked for differentially expressed genes by glycidol by micronarray analysis. Glycidol gave positive result on Ames test (Fig. 1), caused DNA damage based on COMET assay (Fig. 2) and induced micronuclei in CHOK1 cells (Fig. 3). Glycidol induced gene mutations in *Saccharomyces cerevisiae* (Izard, 1973), *S. Pombe* (Heslot, 1962; Migliore *et al.*, 1982) and *Neurospora crassa* (Kolmark and Giles, 1955) in the abscence of exogenous activation. In test with mammalian cells, glycidol induced unscheduled DNA synthesis in human W138 cells and mouse L5178Y/TK cells in the absence of S9 (Thompson *et al.*, 1981) and induced chromosomal aberration and sister chromatid exchanges in human lymphocytes in the absence of S9 (Norppa *et al.*, 1981). Glycidol administered to Wistar rats by intraperitoneal injection induced chromosomal aberrations in bone marrow cells of both males and females (Thompson and Gibson, 1984). Results from this study are consistent with those were reported by others in the literatures.

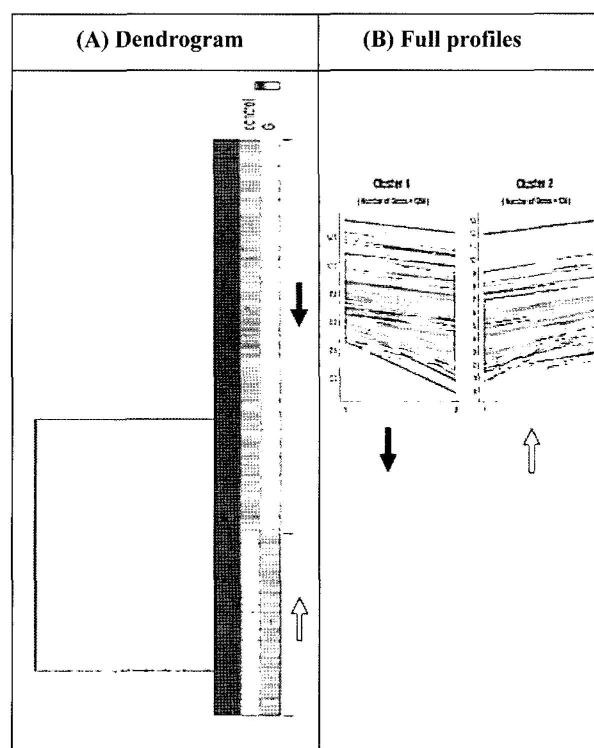

Fig. 5. Results of hierarchical clustering of genes regulated by glycidol. The microarray analysis was performed according to Affymetrix Inc. (Affymetrix Inc., 2002) with modification as described in methods. L5178Y mouse lymphoma cells were treated with glycidol (400 μg/ml), and total RNA was isolated 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.

Pharmaceutical drug discovery teams have made decisions on compound development based largely on toxicological data derived from short-term, range-finding compound exposures, which provide very limited evidence for long-term organ-spe-

Table I. Results of gene ontology analysis.

Accession No	Gene symol	Gene description	Fold change
NM_028175	Lrrc8e	leucine rich repeat containing 8 family, member E	35.34
NM_009253	Serpina3m	serine (or cysteine) peptidase inhibitor, clade A, member 3M	20.5
NM_009700	Aqp4	aquaporin 4	6.41
NM_019455	Ptgds2	prostaglandin D2 synthase 2, hematopoietic	4.32
NM_013761	Srr	serine racemase	3.28
NM_009964	Cryab	crystallin, alpha B	2.63
NM_023133	Rps19	ribosomal protein S19	2.21
NM_010489	Hyal2	hyaluronoglucosaminidase 2	2.18
NM_023120	Gnb1l	guanine nucleotide binding protein (G protein), beta polypeptide 1-like	2.15
NM_175305	Lrrc19	leucine rich repeat containing 19	2.03

cific toxicity, such toxicities may also become apparent during safety pharmacology studies at lower doses.

Systems for testing genetic toxicity are components of carcinogenic and genetic risk assessment. Present routine genetic toxicity testing is based on at least 20 years of development during which many different tests systems have been introduced and used. Today, it is clear that no single test is capable of detecting all genetic toxic agents. Therefore, the usual approach is to perform a standard battery of in vitro and in vivo tests for genetic toxicity.

ACKNOWLEDGMENTS

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