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Genotoxicity studies of glycidol fatty acid ester (glycidol linoleate) and glycidol

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ABSTRACT

Glycidol fatty acid esters (GEs) are found in refined edible oils. Safety concerns have been alleged due to the possible release of glycidol (G), an animal carcinogen.

We evaluated the genotoxic potential of glycidol linoleate (GL), a primary GE found in an edible oil (diacylglycerol oil), and G, using three established genotoxicity tests (a bacterial reverse mutation test, an *in vitro* chromosomal aberration test, and an *in vivo* bone marrow micronucleus test) under GLP conditions complying with all OECD guidelines.

In the bacterial reverse mutation test, GL and G showed positive responses. The positive responses of GL were less than those of G and observed only in strains detecting point mutations where G showed remarkably positive responses. G was involved in the positive response of GL. In the chromosomal aberration test, GL did not induce chromosome aberrations whereas G induced structural chromosome aberrations in the presence and absence of metabolic activation. In the bone marrow micronucleus test, neither GL nor G induced significant increases of micronucleated immature (polychromatic) erythrocytes in bone marrow of test animals.

Based on the above results as well as pertinent information on toxicokinetics, GL itself does not play a key role in genotoxic action.

1. Introduction

In Europe, questions have arisen concerning the safety of glycidol fatty acid esters (GEs) contained in refined edible oils (Matthäus, 2011; Schilter et al., 2011; Pudel et al., 2011). In March 2009, the German Federal Institute for Risk Assessment (BfR) issued an opinion that the known animal carcinogen, glycidol (G), may be released from GEs during digestion in humans and infants who are fed exclusively industrially prepared infant milk formula and would absorb harmful level of G based on a worst-case scenario (BfR, 2009a,b; Bakhiya et al., 2011). Since no reliable analytical method to detect GEs in oils and little information on the conversion of GEs into G in the human body were available, BfR expressed the need for further research.

Diacylglycerol (DAG) oil is a unique edible oil containing >80% (w/w) DAG that has preventive effects on body fat accumulation (Nagao et al., 2000; Flickinger and Matsuo, 2003; Nishide et al., 2004; Yasunaga et al., 2004). In June 2009, a small amount of GEs was found in DAG oil, and the levels were significantly higher than in other commercial edible oils. According to an analytical report by Masukawa et al. (2010), GE levels in DAG oil were measured using five synthetic GE standards (palmitic, stearic, oleic, linoleic, and linolenic G-esters). The most common GE was glycidol linoleate (GL) (132 μg/g), followed by glycidol oleate (96 μg/g) with minor amounts (6.0 μg/g) of glycidol palmitate. GL (9.0 μg/g) and glycidol oleate (10.2 μg/g) were identified as major GEs in one commercial edible oil that primarily consisted of triacylglycerol. (Masukawa et al., 2010).

Toxicological and carcinogenesis studies of G have been extensively evaluated in the National Toxicology Program (NTP) and the International Agency Research on Cancer (IARC) monograph (NTP,

Abbreviations: CP, cyclophosphamide monohydrate; G, glycidol; GEs, glycidol fatty acid esters; GLP, Good Laboratory Practice; OECD, Organization for Economic Co-operation and Development; MN, micronucleus; ME, mature erythrocyte; IME, immature erythrocyte; MNIME, micronucleated IME; MMS, methyl methanesulphonate; ENNG, N-Ethyl-N-nitro-N-nitrosoguanidine; 2NF, 2-nitrofluorene; AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; 2AA, 2-aminoanthracene; 9AA, 9-aminoacridine hydrochloride hydrate; NaN_3, sodium azide; CMC-Na, carboxymethyl cellulose sodium aqueous solution; MMC, mitomycin C; B(a)P, benzo(a)pyrene.

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1990; IARC, 2000). In the NTP study, the carcinogenicity of G was tested by oral administration in both B6C3F1 mice and F344/N rats. It was concluded that there was clear evidence of carcinogenic activity in both mice and rats. Although no epidemiological data relevant to the carcinogenicity of G in humans are available, based on sufficient evidence in experimental animals as well as the direct-acting mode of action of G that was demonstrated in a wide range of *in vitro* and *in vivo* systems, the IARC concluded that G is probably carcinogenic to humans (Group 2A) (IARC, 2000).

On the other hand, little information has been available on the carcinogenicity and genotoxicity of GEs. One of the GEs, glycidyl (glycidol) oleate, has been tested in a limited study in mice in which subcutaneous injection produced a low incidence of local sarcomas. Glycidol stearate was also tested in mice by subcutaneous injection; it produced no significant increase in the incidence of local tumors (Walpole, 1958). IARC previously examined these results for evaluation of carcinogenicity and judged that these were insufficient to reach firm conclusions. Accordingly, glycidyl oleate and glycidyl stearate have been classified as Group 3 (The agent is not classifiable with regard to its carcinogenicity to humans) (IARC, 1976a,b, 1987). The carcinogenicity and/or genotoxicity of GL remains uncertain.

As a first step addressing the alleged safety concerns of GEs, in the present study, we evaluated the genotoxic potential of GL and compared its genotoxicity profile to G. In a manner consistent with internationally accepted guidelines (e.g. ICH guidelines, 1997), three established genotoxicity tests were conducted, i.e., a bacterial reverse mutation test, a chromosomal aberration test in cultured Chinese hamster lung cells (CHL/IU), and a bone marrow micronucleus (MN) test in ICR CD mice.

2. Materials and methods

2.1. Study design

The genotoxicity tests conducted in the present study were designed to meet the Good Laboratory Practice (GLP) standards outlined in the Organization for Economic Co-operation and Development (OECD) guidelines.

2.2. Preparation of G and GL

G (purity 100%) was purchased from Tokyo Chemical Industry, Japan. GL (purity 96.7%) was synthesized by the Wakayama Laboratories of Kao Corporation as described previously (Masukawa et al., 2010).

2.3. Bacterial reverse mutation test (Ames test)

Bacterial reverse mutation tests were performed according to OECD Test Guideline 471. In this test, histidine dependent auxotrophic mutants of *Salmonella typhimurium*, strains TA98, TA100, TA1535, and TA1537, and a tryptophan-dependent auxotrophic mutant of *Escherichia coli*, strain WP2*uvrA*, were exposed to the test substance dissolved in water for injection (for G) or in DMSO (for GL). Treatments with only water for injection or DMSO were used as negative controls.

The test employed a pre-incubation method (Gatehouse et al., 1994) and was performed in the presence or absence of metabolic activation (S9 mix, see below). Specifically, 0.5 mL of 0.1 M sodium phosphate buffer (pH 7.4) (absence of metabolic activation) or 0.5 mL of S9 mix (presence of metabolic activation) and 0.1 mL of test substance solution were added to a test tube. Then, 0.1 mL of bacterial suspension was added and incubated for 20 min at 37 °C. After incubation, 2.0 mL of top agar was added to the mix and the mixture was poured onto a minimal glucose agar plate. The plates were incubated for 48 h at 37 °C. Following the incubation, the number of revertant colonies was counted for evaluation.

The study consisted of two independent tests: evaluation of the dose range and a main test. A response was classified as positive if the test substance caused a dose-dependent increase of at least twofold in the mean number of revertants per plate compared to the negative control value, in conjunction with confirmation that the two independent tests were reproducible.

S. typhimurium, TA100, was obtained from the National Institute of Health Sciences, Tokyo, Japan. Other strains of *S. typhimurium* were obtained from Professor Bruce N. Ames, University of California, Berkeley. The strain of *E. coli*, WP2*uvrA* was obtained from the Institute of Medical Science, the University of Tokyo, Tokyo, Japan. The S9 fraction (Oriental Yeast Co. Ltd., Japan) was prepared from phenobarbital and 5,6-benzoflavone-induced Sprague–Dawley rats. The S9 mix was prepared by mixing with 9 volume of Cofactor-I (Oriental Yeast Co. Ltd., Japan) dissolved in water as a NADPH regenerating system to one volume of the S9 fraction. Chemicals for positive controls, 2-(2-furyl)-3-(5-nitro-2furyl)acrylamide (AF-2), 2-aminoanthracene (2AA), and sodium azide (NaN_3) were purchased from Wako Pure Chemical Industries, Ltd., Japan, and 2-methoxy-6-chloro-9-[3-(2-chloroethyl)aminopropylamino]acridine·2HCl(ICR-191) was purchased from Polysciences, Inc. Benzo[a]pyrene (B[a]P) was purchased from Tokyo Chemical Industry Co. Ltd., Japan.

2.4. Chromosomal aberration test in cultured cells

Chromosome aberration tests were performed according to OECD Test Guideline 473. G and GL were dissolved in saline or DMSO respectively and tested up to the concentration that showed more than a 50% reduction in viable cell numbers or 0.01 M of a test substance, whichever was lower.

Chinese hamster lung cells (CHL/IU) were plated at 4×10^3 cells/mL and incubated for 72 h. The culture medium was then replaced with fresh medium including a test substance. For short-term treatment, cells were treated with each test substance for six h with either additional culture medium (without metabolic activation) or S9 mix at a final concentration of 16.7% (with metabolic activation). After a six h exposure, the cells were washed and then incubated in fresh medium for a further 18 h. For continuous treatment, the cells were treated with each dose for 24 or 48 h. Colcemid was added (0.1 μg/mL final concentration) two h prior to harvesting. The cells were then harvested, swollen in 0.075 M KCl solution, fixed in methanol: acetic acid, placed on clean glass slides and Giemsa (3%) stained. One hundred metaphases per slide (200 metaphases per dose) were analyzed. Results were evaluated according to the following criteria (Matsuoka et al., 1991): negative, <5%; uncertain, 5–10%; positive, >10% for structural or numerical chromosomal aberration frequencies.

The S9 mix was prepared in a manner similar to that employed in the bacterial reverse mutation test. Chemicals for positive controls, mitomycin C (MMC) and benzo[a]pyrene (B[a]P), were purchased from, Kyowa Hakko Kirin Co., Ltd. and Tokyo Chemical Industry Co. Ltd., Japan, respectively.

2.5. Bone marrow micronucleus test in mice

Bone marrow micronucleus tests were performed according to OECD Test Guideline 474. G dissolved in water for injection or GL suspended in olive oil was administered to eight week old male ICR (Crlj:CD-1) mice in two oral gavages separated by 24 h. Preliminary testing was conducted according to procedures of the MN test to find appropriate dosage levels (non-GLP condition). The three animals administered G at a dosage level of 400 mg/kg or above died and one of three animals showed signs of toxicity at 200 mg/kg. All five animals that were administered GL at a dosage level of 2000 mg/kg died and one of five animals died at 1000 mg/kg. Accordingly, the dosage levels for G were set at 50, 100, and 200 mg/kg, while for GL, 250, 500, and 1000 mg/kg. The highest dose in each case was one-half of the lethal dosage level. Each negative control group received only vehicle used for each test substance. Mice in the positive control group were given two oral gavages of cyclophosphamide monohydrate (CP) (Sigma Aldrich Corporation, USA) dissolved in water for injection at a dosage of 20 mg/kg body weight separated for 24 h.

Bone marrow smears were prepared from each treatment group 24 h after the last administration. Each smear was stained with acridine orange and examined using fluorescent microscopy. Mature (normochromatic) erythrocytes (MEs) and immature (polychromatic) erythrocytes (IMEs) were identified according to the method of Hayashi et al. (1983). The number of micronucleated IMEs (MNIMEs) in 2000 IMEs was then counted. The percentage of IMEs was also determined by examination of 1000 erythrocytes from each animal. A response was classified as positive if the test substance caused a significant dose-dependent increase in the total number of MNIMEs.

The statistical method of Kastenbaum and Bowman was applied to compare the number of MNIMEs in each test substance-treated group with that in the control group at the upper-tailed significance levels of 5% and 1% (Kastenbaum and Bowman, 1970). If there was a significant increase in the number of MNIMEs in any test substance-treated group(s), the Cochran-Armitage trend test was conducted using the SAS System for preclinical study.

The IMEs ratio was analyzed statistically using the Toxicological Data Processing System (MiTOX, Mitsui Zosen Systems Research Inc.). First, all the data excluding the positive control group were tested by Bartlett's test for homogeneity of variance among the groups. Williams' test or Shirley-Williams test was applied to determine the statistical differences between the test substance-treated groups and the control group, when the variance was homogeneous or heterogeneous, respectively. If there were no significant differences with the Williams' test, Dunnett's test was performed to compare the mean value in each test substance-treated group with that in the control group. If there were no significant differences with the Shirley-Williams test, the Steel test was applied to compare the mean rank in each test substance-treated group with that in the control group. With respect to analysis of the positive control group, the *F*-test was applied to determine the homogeneity of variance between the positive control group and the negative con-