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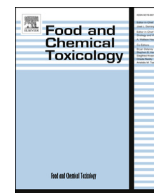

The genotoxic potency of glycidol established from micronucleus frequency and hemoglobin adduct levels in mice

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ABSTRACT

Glycidol is a genotoxic animal carcinogen that has raised concern due to its presence in food, as glycidyl fatty acid esters. Here we investigated the genotoxicity of glycidol in BalbC mice (0–120 mg/kg) by monitoring the induction of micronuclei in peripheral blood as a marker of chromosomal damage. The scoring of the micronuclei was assessed by flow cytometry. In the treated mice, the internal dose of glycidol, expressed as area under the concentration-time curve, AUC (mol $\times$ L^{-1} $\times$ h; Mh), was measured by dihydroxypropyl adducts to hemoglobin (Hb). The study showed that glycidol induced linear dose-dependent increases of Hb adducts (20 pmol/g Hb per mg/kg) and of micronuclei frequencies (12‰ per mMh).

Compared to calculations based on administered dose, an improved dose-response relationship was observed when considering internal dose, achieved through the applied combination of sensitive techniques used for the scoring of micronuclei and AUC estimation of glycidol in the same mice. By comparing with earlier studies on micronuclei induction in mice exposed to ionizing radiation we estimated the radiation dose equivalent (rad-eq.) of glycidol to be *ca* 15 rad-eq./mMh.

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1. Introduction

Glycidol (2,3-epoxy-1-propanol) (Fig. 1) is well characterized with regard to its genotoxicity and carcinogenicity, both from *in vitro* and *in vivo* studies. Positive results from mutagenicity tests in bacteria (Ames) (Thompson et al., 1980; Ikeda et al., 2012) and in mammalian cells have been observed (El Ramy et al., 2007; Aasa et al., 2016). Glycidol is carcinogenic in mice and rats (NTP, 1990) and is classified by IARC as probably carcinogenic to humans (group 2A) (IARC, 2000). Human exposure to glycidol is of concern as there is intake from diet where glycidol is present as a process-induced contaminant as glycidyl fatty acid esters. The esters have been shown to occur in various refined, edible vegetable oils, e.g., coconut oil and palm oil (Kuhlmann, 2011; MacMahon et al., 2013). Palm oil is added to a variety of food products, such as pastries (NFA, 2008). In 2009, the German Federal Institute of Risk Assessment (BfR) raised concern that glycidol may be released from glycidyl fatty acid esters in infant formula (BfR, 2009). The cancer risk in terms of margin of exposure (MoE) for glycidol released from different food products has recently been evaluated by the European Food Safety Authority (EFSA, 2016). The recommendation from the EFSA was that more studies are needed with the aim of reducing uncertainties in the risk assessment.

The aim of the present study was to investigate the genotoxic potency of glycidol to induce micronucleus (MN) formation in male BalbC mice, and how this effect relates to the internal dose. As a quantitative marker of chromosomal instability, the frequency of micronucleated polychromatic erythrocytes (fMPCE) were measured by a sensitive flow cytometry technique using two lasers (method described by Grawé et al., 1992 and Abramsson-Zetterberg et al., 1996). To most accurately understand the dose-response

Abbreviations: ACN, acetonitrile; EO, ethylene oxide; FITC, fluorescein isothiocyanate; fMPCE, frequency of micronucleated polychromatic erythrocytes; Gly, glycidol; Hb, hemoglobin; i.p., intraperitoneal; MN, micronucleus; MRM, multiple reaction monitoring; p.o., per oral; PO, propylene oxide; rad-eq., radiation dose equivalent.

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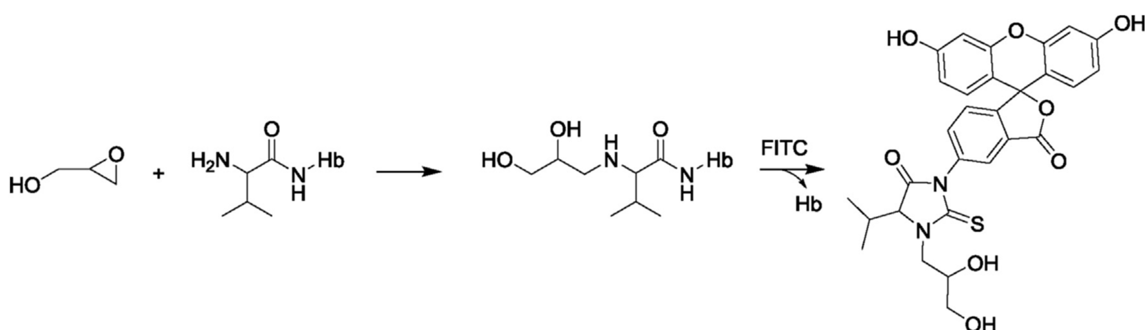

Fig. 1. Glycidol reacts with the N-terminal valine in hemoglobin (Hb) and yields a *N*-(2,3-dihydroxypropyl)-valine adduct (Gly-Val-FTH). After derivatization and detachment from Hb with FITC (fluorescein isothiocyanate) the formed adduct was analyzed by LC-MS/MS.

relationship *in vivo*, the internal dose should be measured in the treated mice to account for individual differences in pharmacokinetics. Specifically, and as a measure for short-lived genotoxic intermediates, we estimated the area under the concentration-time curve (AUC) from measurements of stable glycidol adducts to the N-terminal valine in hemoglobin (Hb) (Fig. 1) using the adduct FI*R*E procedure (Rydberg, 2009; von Stedingk et al., 2010).

This is the first study where the internal dose of glycidol is measured in animals where a genotoxic endpoint has been monitored simultaneously, thereby giving more accurate quantitative information on genotoxic potency which should be useful for evaluation of the cancer risk coefficient.

2. Materials and methods

2.1. Chemicals

Glycidol was purchased from Acros Organics (Geel, Belgium). Propylene oxide, used as internal standard, cyanoacetic acid and ammonium hydroxide were purchased from Fluka (Buchs, Switzerland). *N*-(2,3-dihydroxypropyl)-valine, used for synthesis of analytical standard for the adduct measurement, and acrylamide-d_7-Val-FTH, used as internal standard were synthesized earlier within the research group (Hindsø Landin et al., 2000; von Stedingk et al., 2010). Fluorescein isothiocyanate (FITC) was purchased from Karl Industries (Aurora, OH, USA). Potassium hydrogen carbonate was purchased from Merck (Darmstadt, Germany). Fluothane was obtained from AstraZeneca (Göteborg, Sweden) and Percoll from Pharmacia Biosystems (Uppsala, Sweden). The fluorescent dye thiazole orange was obtained from Molecular Probes (Eugene, USA). Hydroxycobalamin hydrochloride, cobolt (II)nitrate ($Co(NO_3)_2$), sodium borohydride, the fluorescent dye Hoechst 33342 and all other chemicals (analytical grade) were purchased from Sigma Aldrich (St Louis, MO, USA).

2.2. Animal treatment and sample collection

Male BalbC mice aged 8 weeks and weighing about 25 g (Scanbur BK, Sollentuna, Sweden) were used. The animals were kept under controlled conditions in an animal facility with free access to standard diet and tap water. The ethical application for the experiment has been reviewed and approved by Uppsala Ethical Committee on animal experiment, application C322/12. The mice were administered glycidol, dissolved in PBS, via intraperitoneal (i.p.) injection, 10 μl/g b.w. The administered doses of glycidol were 30, 60, 90, 120 mg/kg in addition to vehicle only (dose levels chosen to be at comparable levels as a previous study (NTP, 1990). Three animals per dose group were used, except at the highest dose where five animals were used.

Blood samples were collected, under anesthesia with Fluothane, from the orbital plexus at 45 h after i.p. injections. This is within the time-course where the peak in the fMPCE in peripheral blood has been observed previously (Abramsson-Zetterberg et al., 1996, 2003). Immediately after blood sampling, mice were sacrificed by cervical dislocation. Three parallel aliquots (5 μl) of blood were layered for purification on a 65% Percoll gradient and centrifuged for 20 min at 600 g before fixation and analysis as described in section 2.3. Remaining blood samples were stored at −20 °C until sample preparation for measurement of Hb adducts.

2.3. Micronucleus assay

The procedures used for fixation, fluorochrome staining and flow cytometry have been described elsewhere (Abramsson-Zetterberg et al., 1995; Grawé et al., 1992, 1993). Pelleted cells from centrifugation, as described above, were fixed in a solution of glutaraldehyde and stored for one day at 4 °C. On the day of analysis the fixative was discarded and the remaining cell pellet was stained in a buffer prepared by adding fluorescent dyes, Hoechst 33342 (HO 342; DNA dye) and Thiazole orange (TO; RNA dye), to PBS.

The stained samples were analyzed on a dual laser FACStar Plus flow cytometer (Beckton Dickinson, Sunnyvale, CA, USA) equipped with an argon ion laser operating at both multiline UV (350 nm) and 488 nm. The setting and equipment of the flow cytometer have been described several times before, e.g., by Grawé (2005). From each mouse a mean of 180 000 polychromatic erythrocytes (PCE), i.e. young erythrocytes, were analyzed for frequency of MN. CellQuest software (BD Biosciences) was used for data acquisition and analysis.

2.4. Synthesis of Gly-Val-FTH standard for the Hb adduct measurement

The analytical standard, the fluorescein thiohydantoin derivative of the glycidol adduct to valine (Gly-Val-FTH) (Fig. 1), was synthesized according to the protocol described by von Stedingk et al. (2010). The earlier synthesized *N*-(2,3-dihydroxypropyl)-valine (purity $\geq$ 95% on ^{1}H NMR) (55.5 mg, 290 μmol) (Hindsø Landin et al., 2000) was dissolved in 0.125 M $KHCO_3$ (600 μL, 75 μmol). Acetonitrile (ACN) (400 μL) was added followed by the addition of FITC (110 mg, 282 μmol) in DMF (660 μL). The reaction solution was mixed and heated overnight (37 °C at 500 rpm). The derivatization was terminated by the addition of 1 M HCl (160 μL, 160 μmol).

Gly-Val-FTH was separated from byproducts using semi-preparative HPLC-UV. A C_{18} column (10 mm × 250 mm, 5 μm from Hichrom) was used for the chromatographic separation and the mobile phase ACN:H_2O (50:50) was run in isocratic mode at a

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