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Carcinogenicity of glycidamide in B6C3F$_1$ mice and F344/N rats from a two-year drinking water exposure

Frederick A. Beland[a,*], **Greg R. Olson**[b], **Maria C.B. Mendoza**[c], **M. Matilde Marques**[d], and **Daniel R. Doerge**[a]

[a]Division of Biochemical Toxicology, National Center for Toxicological Research, Jefferson, AR 72079, United States.

[b]Toxicologic Pathology Associates, National Center for Toxicological Research, Jefferson, AR 72079, United States.

[c]Division of Bioinformatics and Biostatistics, National Center for Toxicological Research, Jefferson, AR 72079, United States.

[d]Centro de Química Estrutural, Instituto Superior Técnico, Universidade de Lisboa, 1049-001 Lisboa, Portugal.

Keywords

Acrylamide; glycidamide; tumorigenicity; mice; rats; bioassay

1. Introduction

Acrylamide (Figure 1) is a high-production-volume chemical used in a variety of industrial applications (International Agency for Research on Cancer, 1994; Cosmetic Ingredient Review Expert Panel, 2005). Acrylamide is also found in baked and fried starchy foods (*e.g.*, French fries, potato chips, breads, and cereals; Rosén and Hellenäs, 2002; Tareke *et al.*, 2002), due to Maillard reactions involving reducing sugars, such as glucose, and asparagine, a major amino acid present in potatoes and cereals (Mottram *et al.*, 2002; Stadler *et al.*, 2002). Additional sources of acrylamide include coffee (Dybing *et al.*, 2005) and cigarettes (Bergmark, 1997; International Agency for Research on Cancer, 2004). As a consequence of its ubiquitous occurrence, there has been considerable interest in the carcinogenicity of acrylamide, which has led to a number of bioassays being conducted (Bull *et al.*, 1984a; Bull *et al.*, 1984b; Johnson *et al.*, 1986; Robinson *et al.*, 1986; Friedman *et al.*, 1995; Ølstørn *et al.*, 2007; Von Tungeln *et al.*, 2012; National Toxicology Program, 2012; Beland *et al.*, 2013; Maronpot *et al.*, 2015).

[*]**Correspondence to:** Division of Biochemical Toxicology (HFT-110), National Center for Toxicological Research, 3900 NCTR Road, Jefferson, AR 72079, U.S.A.; Telephone: (870)543-7205; FAX: (870)543-7136; frederick.beland@fda.hhs.gov.

Upon ingestion, acrylamide undergoes oxidation by cytochrome P450 2E1 to the epoxide glycidamide (Sumner *et al.*, 1999; Ghanayem *et al.*, 2005a; Figure 1). Although acrylamide will react with DNA, this occurs slowly (Solomon *et al.*, 1985); in contrast, the metabolite glycidamide is considerably more reactive. Several glycidamide-DNA adducts have been characterized (Segerbäck *et al.*, 1995; Solomon, 1999; Gamboa da Costa *et al.*, 2003; Backman *et al.*, 2004; Backman and Kronberg, 2007; Kotova *et al.*, 2011), and two of these, N7-(2-carbamoyl-2-hydroxyethyl)guanine (N7-GA-Gua) and N3-(2-carbamoyl-2-hydroxyethyl)adenine (N3-GA-Ade) (Figure 1), have been detected in mice and rats treated with acrylamide (Segerbäck *et al.*,1995; Gamboa da Costa *et al.*, 2003; Twaddle *et al.*, 2004; Doerge *et al.*, 2005a; Doerge *et al.*, 2005b; Doerge *et al.*, 2005c; Ghanayem *et al.*, 2005a; Manière *et al.*, 2005; Tareke *et al.*, 2006; Von Tungeln *et al.*, 2009; Zeiger *et al.*, 2009). These data suggest that glycidamide, due to its electrophilic properties, may be responsible for the multi-organ carcinogenic activity of acrylamide. This postulate is supported by a number of observations. Compared to wild-type mice, mice lacking cytochrome P450 2E1 and administered acrylamide have decreased levels of male germ cell mutagenicity, micronuclei, and glycidamide-derived DNA adducts (Ghanayem *et al.*, 2005a; Ghanayem *et al.*, 2005b; Ghanayem *et al.*, 2005c). Transgenic Big Blue mice and rats treated with equimolar doses of acrylamide and glycidamide have mutant frequencies and mutation spectra in the *cII* transgene consistent with the metabolic conversion of acrylamide to glycidamide (Manjanatha *et al.*, 2006; Wang *et al.*, 2010; Manjantha *et al.*, 2015). Neonatal B6C3F$_1$/*Tk*$^{+/-}$ mice treated on postnatal days 1, 8, and 15 with glycidamide have an increased mutant frequency at the hypoxanthine-guanine phosphoribosyltransferase gene in splenic T-lymphocytes. This does not occur in mice treated with equimolar doses of acrylamide, which was attributed to the limited capacity of neonatal mice to metabolize acrylamide to glycidamide (Von Tungeln *et al.*, 2009).

The carcinogenicity of glycidamide has been assessed in two studies (Ølstørn *et al.*, 2007; Von Tungeln *et al.*, 2012). In the Ølstørn *et al.* (2007) experiment, C57BL/6J *Min*/+ mice, a strain susceptible to intestinal neoplasia, and their wild-type littermates were administered subcutaneous injections of 10 or 50 mg glycidamide per kg body weight (bw) at one and two weeks after birth. In both strains, there was a dose-related induction of tumors of the small intestine, with the increase being significant at 50 mg glycidamide per kg bw. There was no increase in intestinal lesions when acrylamide was given in the same manner at 10 or 50 mg per kg bw. In the Von Tungeln *et al.* (2012) study, male B6C3F$_1$ mice injected intraperitoneally with 0.70 mmol glycidamide per kg bw on postnatal days 1, 8, and 15 had a significant increase in hepatocellular tumors that was associated with A → G and A → T mutations at codon 61 of the H-*ras* oncogene. As with the Ølstørn *et al.* (2007) experiment, mice administered an equimolar amount of acrylamide did not have an increased tumor incidence due to the limited capacity of newborn mice to convert acrylamide to glycidamide.

In a previous study, we assessed the carcinogenicity of acrylamide in B6C3F$_1$ mice and F344/N rats in order to provide data for use in quantitative risk assessments (National Toxicology Program, 2012; Beland *et al.*, 2013). In that experiment, we hypothesized that acrylamide was activated to an ultimate carcinogen through metabolism to glycidamide. To provide additional data for use in quantitative risk assessments and to test this hypothesis,

we have now examined the carcinogenicity of glycidamide in B6C3F$_1$ mice and F344/N rats treated chronically with glycidamide in the drinking water for two years.

2. Materials and methods

2.1 Chemicals

Glycidamide (CAS 5694-00–8) was purchased from Toronto Research Chemicals, New York, Ontario, Canada. The purity (> 98%) and identity of the glycidamide were confirmed by gas chromatography coupled with electron impact mass spectrometry, nuclear magnetic resonance spectroscopy, and gas chromatography using flame ionization detection.

2.2 Dose selection

The selection of doses for the two-year glycidamide drinking water study was based upon the effects observed in three-month drinking water studies, conducted simultaneously, with glycidamide (this study) and acrylamide (National Toxicology Program, 2012; Beland *et al.*, 2013). The administration of either 3.52 mM glycidamide or 3.52 mM acrylamide in the drinking water resulted in hind limb paresis and decreased body weight. Decreases in body weight were also observed with 1.41 mM acrylamide. In addition, rats administered 1.41 mM acrylamide displayed hind limb paresis. Since one of the goals of this study was to compare acrylamide with glycidamide, a high dose of 0.70 mM glycidamide was selected for the chronic two-year drinking water study, with the remaining doses being 0.0, 0.0875, 0.175, and 0.35 mM glycidamide. These doses were identical to those used in the two-year chronic bioassay with acrylamide (National Toxicology Program, 2012; Beland *et al.*, 2013).

2.3 Study design

The Institutional Animal Care and Use Committee at the National Center for Toxicological Research (NCTR) reviewed and approved the protocol for these bioassays.

Male and female F344/N Nctr rats and B6C3F$_1$/Nctr (C57BL/6N x C3H/HeN MTV$^-$) mice were obtained from the NCTR breeding colony. Treatment was initiated when the rats were four to five weeks of age and the mice were five to six weeks of age. The rats were housed two of the same sex per cage in polycarbonate cages with hardwood chip bedding. The mice were housed four of the same sex per cage in polycarbonate cages with hardwood chip bedding and micro-isolator tops. Irradiated Purina 5LG6 meal, which was selected for its low acrylamide content (<10 ppb), and Millipore-filtered tap water were provided *ad libitum*. The stability of glycidamide in the drinking water was assessed for 21 days and determined to be 104 ± 8% (mean ± s.d.; n = 6). Drinking water bottles were replaced weekly. The glycidamide concentrations of the drinking water solutions were determined bi-monthly during the course of the study and found to be acceptable (± 10%, except for the 0.0875 mM dose, where ± 20% was considered to be acceptable). The animal rooms were maintained on a 12-hour light-dark cycle. Environmental controls were set to maintain the temperature at 22 ± 4°C, with a relative humidity of 40 – 70%.

Each dose group consisted of 48 animals per sex per species. Animal inspections were conducted twice daily; body weights, food consumption, and water consumption were measured weekly.

2.4 Necropsy and histopathology

Complete necropsies were performed on all animals, including those that died or became moribund prior to the scheduled terminal sacrifice. Tissues were examined grossly, removed, and preserved in 10% neutral buffered formalin, except the eyes and testes, which were placed in Davidson's fixative. The tissues were trimmed, processed, and embedded in Formula R® infiltrating medium (Leica Micro Biosystems Division, Richmond, IL), sectioned at approximately 5 μm, and stained with hematoxylin and eosin for microscopic evaluation. In a few cases, special staining procedures were applied to selected lesions to aid in characterizing the pathology changes.

A quality assessment pathologist evaluated slides of all proliferative lesions and target organs. Histopathology slides underpinning the diagnoses made by the study pathologist and the quality assessment pathologist were reviewed by a Pathology Working Group (PWG). Final diagnoses of reviewed lesions represented a consensus between the study pathologist, the quality assessment pathologist, and the PWG.

Acrylamide is neurotoxic in experimental animals (reviewed in National Toxicology Program, 2012), and the possibility exists that glycidamide is likewise. In view of this possibility, an additional pathology quality assessment review was conducted on sections of brain, spinal cord, and peripheral nerve by pathologists from an independent laboratory who have specialized expertise in neuropathology. During this review, all changes in the nervous system were documented, regardless of their severity. Based upon these very stringent criteria, additional lesions were detected. In light of these findings, an additional PWG, consisting of six experienced pathologists, was convened to evaluate the results. This subsequent PWG used criteria similar to those used in the neuropathology quality assessment and concurred that all of the lesions, regardless of their severity, be added to the pathology results.

2.5 Statistical analyses

The SAS Proc Life test procedure was used to obtain Kaplan-Meier (Kaplan and Meier, 1958) estimates of survival times. SAS Proc Phreg was used to conduct Cox proportional hazards regression analyses (Cox, 1972) for comparing dosed to control group hazard rates and testing for linear trends between the hazard and glycidamide dose.

SAS Proc Mixed was used to analyze the effect of glycidamide dose on animal body weight. A sex-stratified, repeated measures, mixed models analysis of variance (ANOVA), with dose and week main effects and a dose by week interaction effect, was fit to the data to obtain least squares estimates of mean body weight for each dose group from weeks 4 to 104 in four-week intervals. Dunnett's adjusted (Dunnett, 1955) pair-wise comparisons of dosed to control group body weight means were performed to determine if there was a difference between control and individual dosed group means.

Analyses of the effect of glycidamide dose on food and water consumption paralleled those of body weight. The results, however, were determined on a per cage basis, rather than on a per animal basis, and consumption periods were grouped into four-week study periods according to the observation date. The study period was used in lieu of week in the analysis model. Water consumption and body weight data were used to determine glycidamide exposure. Differences in mean food, water, and compound consumption between the previous two-year acrylamide bioassay (National Toxicology Program, 2012; Beland *et al.*, 2013) and the current bioassay were assessed using ANOVA techniques.

Continuity-corrected Poly-3 tests (Bailer and Portier, 1988), modified by Bieler and Williams (1993), were conducted to assess the age-adjusted prevalence of neoplastic and non-neoplastic lesions. P-Values for Poly-3 trend tests were one-sided. Differences in tumor incidences between the previous two-year acrylamide bioassay (National Toxicology Program, 2012; Beland *et al.*, 2013) and the current glycidamide bioassay were also assessed. Logistic regression, using Poly-3 weighted averages, was used to model the dose response of acrylamide and glycidamide and determine if the slopes of these regression lines were equal.

Benchmark doses (BMD) and the lower (BMDL) 95% confidence limits were calculated using Environmental Protection Agency Benchmark Dose software (version 2.1.1; http://www.epa.gov/ncea/bmds). The calculations were conducted using gamma, logistic, log-logistic, log-probit, multistage, probit, and Weibull models to fit the observed neoplastic incidences and the mean doses of glycidamide for the entire two-year study. The BMD_{10} was defined as the dose that caused a 10% excess risk of the specified adverse effect over that observed in the appropriate control group.

3. Results

Groups of 48 male and 48 female $B6C3F_1$ mice and 48 male and 48 female F344/N rats were administered 0, 0.0875, 0.175, 0.35, and 0.70 mM glycidamide in the drinking water for two years.

3.1 Body weights

3.1.1 $B6C3F_1$ mice—The administration of glycidamide in the drinking water to male and female $B6C3F_1$ mice caused only sporadic statistically significant changes in body weight, with the magnitude of the change never exceeding 5% of the mean control body weight at the same time point (Supplementary Figure S1).

3.1.2 F344/N rats—Glycidamide in the drinking water caused significant dose-related decreasing trends in body weight in male and female F344/N rats (Supplementary Figure S2). Pair-wise comparisons indicated that treatment with 0.70 mM glycidamide resulted in significant decreases in body weight gain beginning at week 8 in male rats. In female rats, treatment with 0.175, 0.35, or 0.70 mM glycidamide resulted in significant decreases in body weight gain beginning at week 4. At the end of the two-year period, male rats administered 0.70 mM glycidamide weighed 82% of the control group; female rats administered 0.70 mM glycidamide weighed 79% of the control group.

3.2 Food consumption

3.2.1 B6C3F$_1$ mice—Glycidamide in the drinking water caused dose-related increasing trends in food consumption beginning at week 84 in male B6C3F$_1$ mice and week 60 in female B6C3F$_1$ mice (Supplementary Figure S3).

3.2.2 F344/N rats—Glycidamide in the drinking water caused only sporadic dose-related trends in food consumption in male and female F344/N rats (Supplementary Figure S4).

3.3 Water consumption

3.3.1 B6C3F$_1$ mice—Glycidamide in the drinking water caused sporadic dose-related increasing trends in water consumption in male B6C3F$_1$ mice (Supplementary Figure S5A). In female B6C3F$_1$ mice, there was a dose-related increasing trend in water consumption beginning at week 76, with water consumption in the 0.70 mM glycidamide group being significantly greater than in the control group beginning at week 80 (Supplementary Figure S5B).

The mean amount of glycidamide consumed by male B6C3F$_1$ mice, calculated at four-week intervals, for the entire two-year experiment was 1.20, 2.65, 5.13, and 9.55 mg glycidamide per kg bw per day for the 0.0875, 0.175, 0.35, and 0.70 mM glycidamide dose groups, respectively. The corresponding values for female B6C3F$_1$ mice were 1.37, 2.89, 5.64, and 12.99 mg glycidamide per kg bw per day.

3.3.2 F344/N rats—Glycidamide in the drinking water did not affect water consumption in either male or female F344/N rats (Supplementary Figure S6). The mean amount of glycidamide consumed by male F344/N rats, calculated at four-week intervals, for the entire two-year experiment was 0.39, 0.79, 1.56, and 3.34 mg glycidamide per kg bw per day for the 0.0875, 0.175, 0.35, and 0.70 mM glycidamide dose groups, respectively. The corresponding values for female rats were 0.54, 1.08, 2.23, and 4.65 mg glycidamide per kg bw per day.

3.4 Survival

3.4.1 B6C3F$_1$ mice—Glycidamide in the drinking water caused a dose-related decreasing trend in survival in male and female B6C3F$_1$ mice (Figure 2). Compared to control mice, male B6C3F$_1$ mice administered 0.175, 0.35, and 0.70 mM glycidamide and female B6C3F$_1$ mice administered 0.35 and 0.70 mM glycidamide had decreased survival.

3.4.2 F344/N rats—Glycidamide in the drinking water caused a dose-related decreasing trend in survival in male and female F344/N rats (Figure 3). Compared to control rats, both sexes administered 0.35 and 0.70 mM glycidamide had decreased survival.

3.5 Neoplasms

3.5.1 B6C3F$_1$ mice—Male and female B6C3F$_1$ mice administered glycidamide in the drinking water had dose-related increases in Harderian gland adenoma, with the incidences being significant in both sexes at all doses of glycidamide (Table 1). Harderian gland

adenocarcinoma was also observed in one male mouse administered 0.70 mM glycidamide and one female mouse administered 0.175 mM glycidamide.

Dose-related increases in lung alveolar/bronchiolar adenoma occurred in both sexes of $B6C3F_1$ mice, with the incidence being significant at all doses of glycidamide in male mice and at 0.70 mM glycidamide in female mice (Table 1). Low incidences of alveolar/bronchiolar carcinoma (<5%) were also observed in both sexes.

Forestomach neoplasms occurred in both sexes of $B6C3F_1$ mice administered glycidamide, with the incidence being significant at 0.70 mM glycidamide (Table 1). Squamous cell carcinoma of the forestomach was also observed in two male mice administered 0.70 mM glycidamide.

Male $B6C3F_1$ mice exposed to glycidamide had a dose-related increase in squamous cell papilloma of the skin, with the incidence being significant at 0.70 mM glycidamide (Table 1). Squamous cell carcinoma of the skin also occurred in two male mice administered 0.70 mM glycidamide. Female $B6C3F_1$ mice had dose-related increases in malignant mesenchymal skin tumors (fibrosarcoma or combined fibrosarcoma or sarcoma; Table 1). The incidence of fibrosarcoma was significant at 0.70 mM glycidamide and the incidence of combined fibrosarcoma or sarcoma was significant at 0.35 and 0.70 mM glycidamide.

Female $B6C3F_1$ mice administered glycidamide had dose-related increasing trends in adenoacanthoma, adenocarcinoma, and combined adenoacanthoma or adenocarcinoma of the mammary gland (Table 1). The incidence of adenoacanthoma was increased significantly in the 0.70 mM glycidamide dose group, and the incidence of adenocarcinoma and combined adenoacanthoma or adenocarcinoma was increased significantly in the 0.35 and 0.70 mM glycidamide dose groups. Female $B6C3F_1$ mice also had dose-related increasing trends in benign, malignant, and combined benign and malignant granulosa cell tumors of the ovary (Table 1).

3.5.2 F344/N rats—In both sexes of F344/N rats administered glycidamide in the drinking water, there was a dose-related increasing trend in the incidence of thyroid gland follicular cell adenoma, follicular cell carcinoma, and combined follicular cell adenoma or carcinoma (Table 2). In male F344/N rats, the incidence of follicular cell adenoma, follicular cell carcinoma, and combined follicular cell adenoma or carcinoma was significant at 0.70 mM glycidamide. In female F344/N rats, the incidence of follicular cell adenoma and follicular cell carcinoma was significant at 0.70 mM glycidamide, while the incidence of combined follicular cell adenoma or carcinoma was significant at 0.175, 0.35, and 0.70 mM glycidamide.

The administration of glycidamide was associated with a dose-related increase in squamous cell papilloma of the oral mucosa and tongue and combined squamous cell papilloma or carcinoma of the oral mucosa or tongue in male F344/N rats (Table 2). The incidence of squamous cell papilloma of the tongue and combined squamous cell papilloma or carcinoma of the oral mucosa or tongue was significant at 0.70 mM glycidamide. In female F344/N rats, there was a dose-related increase in squamous cell papilloma of the oral mucosa,

squamous cell carcinoma of the oral mucosa and combined squamous cell papilloma or carcinoma of the oral mucosa or tongue, with the incidence of combined squamous cell papilloma or carcinoma of the oral mucosa or tongue being significant at 0.70 mM glycidamide. Both sexes of F344/N rats also had dose related increases in the incidence of mononuclear cell leukemia, with the increase in incidence being significant at 0.70 mM glycidamide (Table 2).

Exposure to glycidamide in the drinking water was associated with development of malignant mesothelioma on membranes surrounding the epididymis and on testicular tunicae in male F344/N rats (Table 2). The incidence of malignant mesothelioma was significant in the epididymis, testes, and combined testes or epididymis at 0.35 and 0.70 mM glycidamide. Glycidamide in the drinking water also resulted in a dose-related increase in malignant Schwannoma in the heart of male F344/N rats, with the incidence being significant at 0.70 mM glycidamide (Table 2).

Female F344/N rats exposed to glycidamide in the drinking water had an increased occurrence of fibroadenomas in the mammary gland, with the incidence being significantly increased at all doses (Table 2). Female F344/N rats also had dose-related increases in clitoral gland carcinoma and forestomach squamous cell papilloma. The incidence of clitoral gland carcinoma was significant at 0.35 and 0.70 mM glycidamide, while the occurrence of forestomach squamous cell papilloma was significant at 0.70 mM glycidamide (Table 2).

3.6 Non-neoplastic lesions

3.6.1 B6C3F$_1$ mice—The drinking water administration of glycidamide to B6C3F$_1$ mice resulted in a dose-related increase in cataracts and corneal inflammation in both sexes (Table 3). The incidence of cataracts was increased in the 0.175, 0.35, and 0.70 mM glycidamide dose groups, while the incidence of corneal inflammation was increased at 0.70 mM glycidamide. Glycidamide administration resulted in a dose-related increasing trend in epithelium hyperplasia of the forestomach in both sexes of B6C3F$_1$ mice, with the incidence being significant at 0.70 mM glycidamide in male mice and 0.35 mM glycidamide in female mice. Both sexes of B6C3F$_1$ mice also had increasing dose-related trends in hematopoietic cell proliferation of the spleen, with the incidence being significant at 0.35 and 0.70 mM glycidamide.

Other treatment-related non-neoplastic lesions in male B6C3F$_1$ mice included alveolar epithelium hyperplasia of the lung (Table 3). Additional non-neoplastic lesions in female B6C3F$_1$ mice included angiectasis and necrosis of the liver and axonal degeneration of the spinal cord.

3.6.2 F344/N rats—Drinking water administration of glycidamide to F334/N rats resulted in a dose-related increase in brain gliosis in both sexes (Table 4). In male rats, the incidence of gliosis was increased in the 0.70 mM glycidamide dose group, while in female rats the incidence was increased at 0.175, 0.35, and 0.70 mM glycidamide. Male F344/N rats also had glycidamide-related increases in hepatocyte degeneration and necrosis in the liver. Additional non-neoplastic lesions associated with glycidamide exposure in female F344/N rats included axonal degeneration of the spinal cord and uterine endometrial hyperplasia.

4. Discussion

Acrylamide is a contaminant in baked and fried starchy foods, roasted coffee, and cigarette smoke as a result of Maillard reactions involving asparagine and reducing sugars. Previously we reported that acrylamide is a multi-organ carcinogen in male and female B6C3F$_1$ mice and F344/N rats (National Toxicology Program, 2012; Beland *et al.*, 2013), and we hypothesized that acrylamide was activated to an ultimate carcinogen through cytochrome P450-catalzyed oxidation to the epoxide glycidamide. We have now examined this hypothesis by comparing the carcinogenic effects of glycidamide administered at equimolar doses to the same strains of rodents.

4.1 B6C3F$_1$ mice

The administration of glycidamide in the drinking water to B6C3F$_1$ mice resulted in only sporadic changes in body weight (Supplementary Figure S1). The doses of glycidamide selected for the current bioassay (0, 0.0875, 0.175, 0.35, and 0.70 mM in the drinking water) were identical to those used in our previous bioassay with acrylamide, and there were only sporadic differences in body weights when comparisons were made between mice administered glycidamide and mice given acrylamide. The survival of the B6C3F$_1$ mice was affected by glycidamide, with significant decreases in survival being observed at the three highest dose levels in male mice and the two highest dose levels in female mice (Figure 2). Similar trends existed with B6C3F$_1$ mice given acrylamide. In addition to having comparable body weights and survival, B6C3F$_1$ mice exposed to glycidamide or acrylamide typically consumed similar (±10%) amounts of each of the compounds (on a μmol per kg body weight basis). These results indicate that any differences in the incidence of neoplasms between mice given glycidamide and those administered acrylamide are not a consequence of differences in body weights, survival, or amount of test compound consumed.

The most sensitive site for tumor induction in B6C3F$_1$ mice administered glycidamide in the drinking water was the Harderian gland (Table 1), and even at the lowest dose of glycidamide (0.0875 mM), the incidence of Harderian gland adenoma exceeded the range observed in control male and female B6C3F$_1$ mice in experiments conducted at the NCTR. As we reported previously, the Harderian gland was also the most sensitive site for tumor induction in the B6C3F$_1$ mice administered acrylamide in the drinking water (National Toxicology Program, 2012, Beland *et al.*, 2013), and a comparison of the bioassays (Supplementary Table S1) indicates that the tumor incidences were similar. These data, plus the fact that other low-molecular-weight carcinogens thought to be metabolized to electrophilic epoxides also target the Harderian gland in B6C3F$_1$ mice (Bucher *et al.*, 1990; Melnick and Sills, 2001; Melnick, 2002; Beland *et al.*, 2005a), strongly support the concept that the carcinogenic activity of acrylamide in the Harderian gland of B6C3F$_1$ mice is due to its metabolism to glycidamide.

In both sexes of B6C3F$_1$ mice, there were significant dose-dependent increases in alveolar/bronchiolar adenoma of the lung (Table 1), and the incidences in the 0.35 and 0.70 mM glycidamide dose groups exceeded the range observed in control B6C3F$_1$ mice in experiments conducted at the NCTR. As with Harderian gland adenoma, the incidence of alveolar/bronchiolar adenoma in B6C3F$_1$ mice administered glycidamide did not differ

significantly from the incidence in mice given acrylamide (Supplementary Table S1). Furthermore, Big Blue mice administered equimolar quantities of acrylamide or glycidamide had similar increases in the mutant frequencies at the *cII* transgene in their lungs (Manjanatha *et al.*, 2015). These results, coupled with the observation that glycidamide and acrylamide give very similar DNA adduct profiles in the lungs of B6C3F$_1$ mice and other strains of mice (Gamboa da Costa *et al.*, 2003; Doerge *et al.*, 2005c; Von Tungeln *et al.*, 2009) are consistent with the premise that the lung neoplasms induced in B6C3F$_1$ mice are due to acrylamide being metabolized to glycidamide.

In addition to Harderian gland and lung adenoma, the drinking water exposure to glycidamide resulted in significant dose-related increases in forestomach and skin neoplasms in both sexes of B6C3F$_1$ mice and mammary gland neoplasms in female B6C3F$_1$ mice. Forestomach neoplasms were also observed in male B6C3F$_1$ mice administered acrylamide in the drinking water (National Toxicology Program, 2012; Beland *et al.*, 2013), and the incidence of these tumors did not differ significantly between the two compounds (Supplementary Table S1). Likewise, skin and mammary gland neoplasms occurred in female B6C3F$_1$ mice treated with acrylamide, and as was the case with forestomach neoplasms in male mice, the incidence of these neoplasms did not differ significantly between female mice given glycidamide and those treated with acrylamide (Supplementary Table S1). Other low-molecular-weight epoxides (*e.g.*, glycidol; Irwin *et al.*, 1996) or alkenes that are thought to be metabolized to electrophilic epoxides (*e.g.*, 1,3-butadiene, chloroprene, and urethane; Melnick and Sills, 2001; Melnick, 2002; Beland *et al.*, 2005a) also induce mammary gland neoplasms in female B6C3F$_1$ mice.

Male B6C3F$_1$ mice treated neonatally with glycidamide developed a high incidence of combined hepatocellular adenoma or carcinoma (Von Tungeln *et al.*, 2012). In other studies, high levels of N7-GA-Gua and N3-GA-Ade have been detected in liver DNA from mice treated as adults with glycidamide (Gamboa da Costa *et al.*, 2003; Doerge *et al.*, 2005a; Doerge *et al.*, 2005c; Tareke *et al.*, 2006) and an increase in mutant frequency has been observed in the *cII* transgene in the livers of adult Big Blue mice dosed orally with glycidamide for four weeks (Manjanatha *et al.*, 2006). These data suggested that glycidamide had the potential to be hepatocarcinogenic in the current bioassays. Liver necrosis was observed in female B6C3F$_1$ mice (Table 3) administered glycidamide; nonetheless, an increased incidence of hepatocellular tumors was not observed. B6C3F$_1$ mice treated with acrylamide also did not have increased incidences of liver neoplasms (National Toxicology Program, 2012; Beland *et al.*, 2013). While unexpected, there is precedent for the induction of liver tumors in mice treated as newborns but not as adults. The administration of benzo[*a*]pyrene to neonatal B6C3F$_1$ mice results in an increased incidence of liver tumors (Wiseman *et al.*, 1987; Flammang *et al*, 1997); however, although substantial levels of benzo[*a*]pyrene-derived DNA adducts were detected in the livers of adult B6C3F$_1$ mice fed diets containing benzo[*a*]pyrene (Beland *et al.*, 2005b), this did not lead to an increased hepatic tumor incidence in a two-year chronic bioassay (Culp *et al.*, 1998). Likewise, treating newborn B6C3F$_1$ mice with aflatoxin B$_1$ resulted in the induction of liver tumors (Vessolinovitch *et al.*, 1972), whereas liver tumors were not induced in various strains of adult mice fed diets containing aflatoxin B$_1$ (Wogan, 1969).

4.2 F344/N rats

The administration of glycidamide in the drinking water to F344/N rats resulted in significant dose-related decreases in body weight, with the effect being most pronounced in the 0.70 mM glycidamide dose group (Supplementary Figure S2). F344/N rats administered acrylamide in the drinking water for two years also had significant dose-related decreases in body weight (National Toxicology Program, 2012; Beland *et al.*, 2013), and a comparison of body weights between these two bioassays indicated that the mean body weights for all dose groups through the entire two year treatment period were typically within 5% of one another.

The survival of the F344/N rats also was affected by glycidamide, with significant decreases in survival being observed at the 0.35 and 0.70 mM doses (Figure 3). Similar trends existed with F344/N rats given acrylamide (National Toxicology Program, 2012; Beland *et al.*, 2013). Furthermore, F344/N rats exposed to glycidamide or acrylamide typically consumed similar (±10%) amounts of each of the compounds (on a μmol per kg body weight basis), thus permitting a direct comparison between the neoplasms arising as a result of the treatments.

Glycidamide induced follicular cell adenoma or carcinoma of the thyroid gland in both male and female F344/N rats. In male rats, the incidence of combined follicular cell adenoma or carcinoma was significantly increased at 0.70 mM glycidamide and in female rats the incidence was increased significantly at 0.175, 0.35, and 0.70 mM glycidamide (Table 2). In both sexes, the incidence in each of the glycidamide dose groups exceeded the historical range observed in control F344/N rats at the NCTR. Follicular cell carcinoma also was detected in all the glycidamide dose groups of F344/N rats, with the exception of female rats administered 0.0875 mM glycidamide. Follicular cell carcinoma of the thyroid gland has not been observed in either male or female control F344/N rats in bioassays conducted at the NCTR. Follicular cell adenoma or carcinoma has been reported in F344 and Wistar Han rats given acrylamide (Johnson *et al.*, 1986; Friedman *et al.*, 1995; National Toxicology Program, 2012; Beland *et al.*, 2013; Maronpot *et al.*, 2015). The incidence of combined follicular cell adenoma or carcinoma of the thyroid gland induced by acrylamide in the bioassay conducted at NCTR did not differ statistically from that induced by glycidamide (Supplementary Table S1). In addition to having similar incidences of follicular cell neoplasms, F344 rats treated with equimolar levels of acrylamide or glycidamide have similar levels of N7-GA-Gua in their thyroid gland DNA (Doerge *et al.*, 2005c). Furthermore, Big Blue rats administered equimolar quantities of acrylamide or glycidamide have increased mutant frequencies at the *cII* transgene of their thyroid glands (Mei *et al.*, 2010). The totality of these data is consistent with the conversion of acrylamide to glycidamide being an important step in the induction of follicular cell tumors in the thyroid gland of F344/N rats.

The most sensitive site for tumor induction in female F344/N rats administered glycidamide in the drinking water was the mammary gland, where there was a significant increase in fibroadenoma at all dose levels of glycidamide (Table 2). Furthermore, the incidence of mammary gland fibroadenoma in each of the glycidamide dose groups exceeded the range observed in control female F344/N rats at the NCTR. The mammary gland was also the most sensitive site for tumor induction in female F344/N Nctr rats administered acrylamide in the drinking water (National Toxicology Program, 2012; Beland *et al.*, 2013) and the

fibroadenoma incidences observed with glycidamide were very similar to those occurring with acrylamide (Supplementary Table S1).

In addition to having similar incidences of mammary gland fibroadenomas, female F344 rats administered a single intraperitoneal injection of 0.7 mmol acrylamide or glycidamide per kg body weight form high levels of N7-GA-Gua in their mammary gland DNA (Doerge *et al.*, 2005c), which supports the concept that the fibroadenomas result from a genotoxic mechanism as a consequence of the metabolic conversion of acrylamide to glycidamide. This interpretation conflicts with the fact that Big Blue rats treated with 0.12 mmol glycidamide or acrylamide per kg body weight per day for two months did not have an increased mutant frequency in the *cII* transgene in the mammary gland (Mei *et al.*, 2010); however, this may be a consequence of the high spontaneous mutant frequencies that occur with transgenic mutation assays in general. Other low-molecular-weight epoxides, such as glycidol, and alkenes that are thought to be metabolized to epoxides, such as 1,3-butadiene and chloroprene, also induce mammary gland tumors in female F344 rats (Irwin *et al.*, 1996; Melnick and Sills, 2001; Melnick, 2002). In addition, an increased incidence in mammary gland fibroadenoma has recently been reported in female Wistar Han rats administered acrylamide for two years (Maronpot *et al.*, 2015).

Female F344/N rats treated with glycidamide in the drinking water also had increased incidences of clitoral gland carcinoma and oral cavity neoplasms (primarily squamous cell papilloma of the oral mucosa or tongue; Table 2) that, in each of the glycidamide dose groups, exceeded the NCTR historical range for control female F344/N rats. These neoplasms were also observed in female F344/N rats administered acrylamide in the drinking water, with incidences very similar to those recorded in the glycidamide bioassay (Supplementary Table S1). Oral cavity neoplasms (primarily squamous cell papilloma) also occurred in male F344/N rats given glycidamide (Table 2). The incidence in the 0.70 mM glycidamide group was significant and the incidence in all dose groups, including the control group, exceeded the historical control range for male F344/N rats at the NCTR. These neoplasms were also observed in male F344/N rats administered acrylamide, but the incidences were not significant (National Toxicology Program, 2012).

Female F344/N rats treated with 0.70 mM glycidamide had a low, but statistically significant, occurrence of forestomach papilloma (Table 2), the incidence of which exceeded the NCTR historical control range. Mononuclear cell leukemia was also observed in all dose groups of male and female F344/N rats, with the incidence in the 0.70 mM glycidamide group (Table 2) exceeding the NCTR historical control range for female F344/N rats.

In addition to thyroid gland neoplasms, oral cavity neoplasms, and leukemia, malignant mesothelioma of the epididymis or testes was observed in male F344/N rats administered glycidamide (Table 2). The incidence of these neoplasms was significant at 0.35 and 0.70 mM glycidamide and exceeded the NCTR historical control range. Malignant mesothelioma of the epididymis or testes has been reported in F344 rats given acrylamide (Johnson *et al.*, 1986; Friedman *et al.*, 1995; National Toxicology Program, 2012; Beland *et al*, 2013) and the incidence of these neoplasms was similar to that induced by acrylamide at NCTR except at the 0.70 mM dose, where the incidence was greater with glycidamide (Supplementary

Table S1). The higher tumor incidence in rats treated with 0.70 mM glycidamide compared to 0.70 mM acrylamide may reflect the saturation of enzymatic oxidation that occurs at high doses of acrylamide in rats (Bergmark et al., 1991). Higher levels of N7-GA-Gua have also been detected in testicular DNA from rats administered glycidamide as compared to acrylamide (Doerge et al., 2005c), which again may be a consequence of metabolic saturation occurring with acrylamide.

The concordance in the incidence in malignant mesothelioma of the epididymis or testes that occurred at the lower doses of glycidamide and acrylamide supports the concept that the testicular tumors are a result of acrylamide being converted to glycidamide. Although these data suggest a genotoxic mechanism for the induction of testicular tumors, Big Blue rats treated with either acrylamide or glycidamide did not show an increased mutant frequency in the testes (Mei *et al.*, 2010); however this may be a consequence of the fact that the entire testicular tissue rather than the target tunica vaginalis was assessed.

As an alternative to a genotoxic mechanism, malignant mesothelioma of the tunica vaginalis has been proposed to be a consequence of Leydig cell tumor proliferation resulting from carcinogen-induced decreases in testosterone and increases in luteinizing hormone (Shipp *et al.*, 2006; Maronpot *et al.*, 2009). Maronpot *et al.* (2015) recently reported that acrylamide did not induce malignant mesothelioma in male Wistar Han rats, a strain with a very low incidence of spontaneous Leydig cell tumors compared to that observed in F344/N rats. F344 rats administered relatively high doses of acrylamide (≥ 10 mg acrylamide per kg bw) do have decreased serum levels of testosterone and increased serum levels of luteinizing hormone; nonetheless, Leydig cell proliferation was not evident (Camacho *et al.*, 2012).

Male F344/N rats administered glycidamide developed malignant Schwannoma of the heart (Table 2), with the incidence in all dose groups, including the control group, exceeding the historical control range at the NCTR. This neoplasm also occurred in male F344/N rats treated with acrylamide, at an incidence similar to that found in the current bioassay with glycidamide (Supplementary Table S1). An increased incidence of adenoma or carcinoma of the pancreatic islets was observed in male F344/N rats treated with acrylamide (National Toxicology Program, 2012; Beland *et al.*, 2013). This did not occur with glycidamide.

During the histopathological examinations, special attention was given to the brain and spinal cord tumors of glial cell origin that have been reported in F344 rats administered acrylamide (Johnson *et al.*, 1986). These tumors were not observed in two subsequent bioassays with acrylamide in F344 rats (Friedman *et al.*, 1995; National Toxicology Program, 2012; Beland *et al.*, 2013) nor were they observed in the current bioassay with glycidamide. A treatment-related non-neoplastic brain lesion was gliosis, which was detected in both male and female F344/N rats (Table 4). A non-neoplastic lesion reported in F344 and Wistar Han rats during previous two-year bioassays with acrylamide was peripheral nerve degeneration (Johnson *et al.*, 1986; Friedman *et al.*, 1995; National Toxicology Program, 2012; Beland *et al.*, 2013; Maronpot *et al.*, 2015). A dose-related prevalence of this lesion was not observed in F344/N rats in the current study with glycidamide, although it should be noted that a high incidence of the lesion was detected in all dose groups, including the controls.

4.3 Benchmark dose comparisons

At the initiation of this study we hypothesized that acrylamide is carcinogenic due to its metabolic conversion to glycidamide. To evaluate this hypothesis, benchmark dose modeling was conducted to estimate the doses of glycidamide that would give a 10% excess risk for specific neoplasms (BMD_{10}); these doses were then compared to BMD_{10} values previously determined for acrylamide (Beland *et al.*, 2013). In $B6C3F_1$ mice, the most sensitive site for tumor induction by glycidamide was the Harderian gland, with a BMD_{10} for Harderian gland adenoma of 5.24 to 5.91 μmol glycidamide per kg body weight per day in male mice and 4.55 μmol glycidamide per kg body weight per day in female mice (Supplementary Table S2). In F344/N rats, the most sensitive site for tumor induction was the mammary gland in female rats (BMD_{10} of 2.39 μmol glycidamide per kg body weight per day for fibroadenoma) and the epididymis or testes in male rats (BMD_{10} of 11.23 to 17.94 μmol glycidamide per kg body weight per day for malignant mesothelioma) (Supplementary Table S2).

A comparison of these data with those previously reported for acrylamide (Beland *et al.*, 2013) indicates that both chemicals have similar potencies in the target tissues (Table 5). For example, the BMD_{10} for Harderian gland adenoma in male $B6C3F_1$ mice was 5.51 to 5.91 μmol per kg body weight per day for glycidamide as compared to 5.14 to 5.39 μmol per kg body weight per day for acrylamide. Likewise, in female $B6C3F_1$ mice the BMD_{10} for Harderian gland adenoma was 4.55 μmol per kg body weight per day for glycidamide as compared to 6.65 μmol per kg body weight per day for acrylamide. In female F344/N rats, the BMD_{10} for mammary gland fibroadenoma was 2.39 μmol per kg body weight per day for glycidamide and 7.74 μmol per kg body weight per day for acrylamide, and in male F344/N rats, the BMD_{10} for malignant mesothelioma of the epididymis or testes was 11.23 to 17.94 μmol per kg body weight per day for glycidamide and 29.90 to 30.66 μmol per kg body weight per day for acrylamide.

5. Conclusions

Male and female $B6C3F_1$ mice and F344/N rats were exposed to glycidamide in the drinking water for two years. In male and female $B6C3F_1$ mice, there were significant dose-related increases in tumors of the Harderian gland, lung, forestomach, and skin. Female $B6C3F_1$ mice also had a significantly increased incidence of tumors of the mammary gland and ovary. In male and female F344/N rats, there were significant increases in thyroid gland and oral cavity neoplasms, and mononuclear cell leukemia. Male F344/N rats also had significant dose-related increases in tumors of the epididymis/testes and heart, while female F344/N rats demonstrated significant increases in tumors of the mammary gland, clitoral gland, and forestomach.

In $B6C3F_1$ mice, the most sensitive site for tumor induction by glycidamide was the Harderian gland, with a BMD_{10} of 5.51 to 5.91 μmol glycidamide per kg body weight per day in male mice and 4.55 μmol glycidamide per kg body weight per day in female mice. In F344/N rats, the most sensitive site for tumor induction was the mammary gland in female rats (BMD_{10} of 2.39 μmol glycidamide per kg body weight per day) and the epididymis/testes in male rats (BMD_{10} of 11.23 to 17.94 μmol glycidamide per kg body weight per

day). Similar values were obtained in B6C3F$_1$ mice and F344/N rats administered acrylamide in the drinking water for two years. These data indicate that, under the conditions of these bioassays, acrylamide is efficiently metabolized to glycidamide in both sexes of both species and that the carcinogenic activity of acrylamide is due to its metabolic conversion into glycidamide.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ANOVA	analysis of variance
BMD	benchmark dose
BMDL	lower limit of benchmark dose
N3-GA-Ade	N3-(2-carbamoyl-2-hydroxyethyl)adenine
N7-GA-Gua	N7-(2-carbamoyl-2-hydroxyethyl)guanine
NCTR	National Center for Toxicological Research
PWG	Pathology Working Group

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Highlights

- Glycidamide is an electrophilic metabolite of the food contaminant acrylamide.
- The carcinogenicity of glycidamide was assessed in a two-year bioassay.
- The tumors observed from glycidamide correspond to those found with acrylamide.
- The carcinogenicity of acrylamide is due to metabolic conversion to glycidamide.

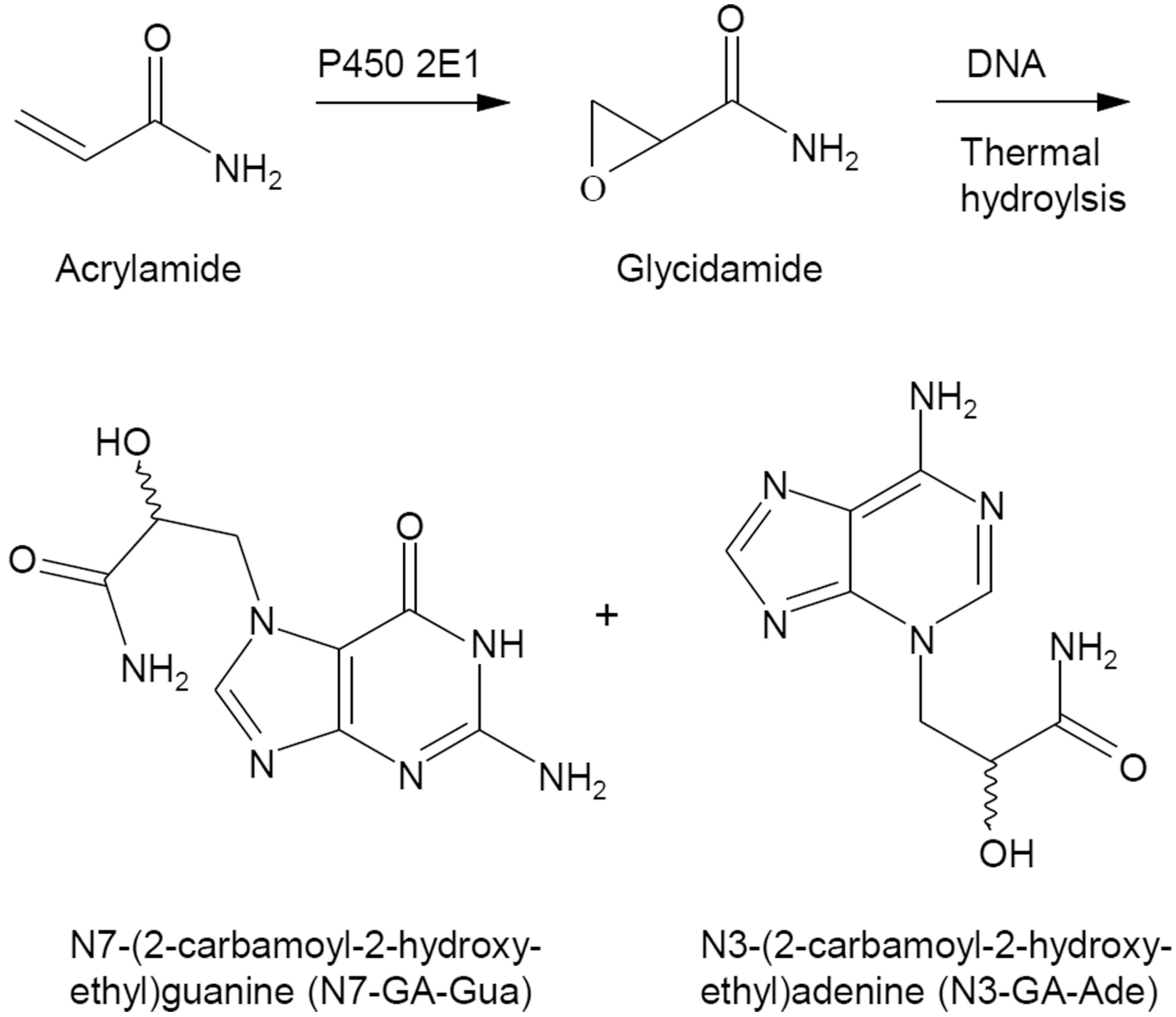

Figure 1.
Structures of acrylamide and glycidamide, and the DNA adducts resulting from the reaction of glycidamide with DNA. N7-GA-Gua and N3-GA-Ade are released from the DNA upon thermal hydrolysis.

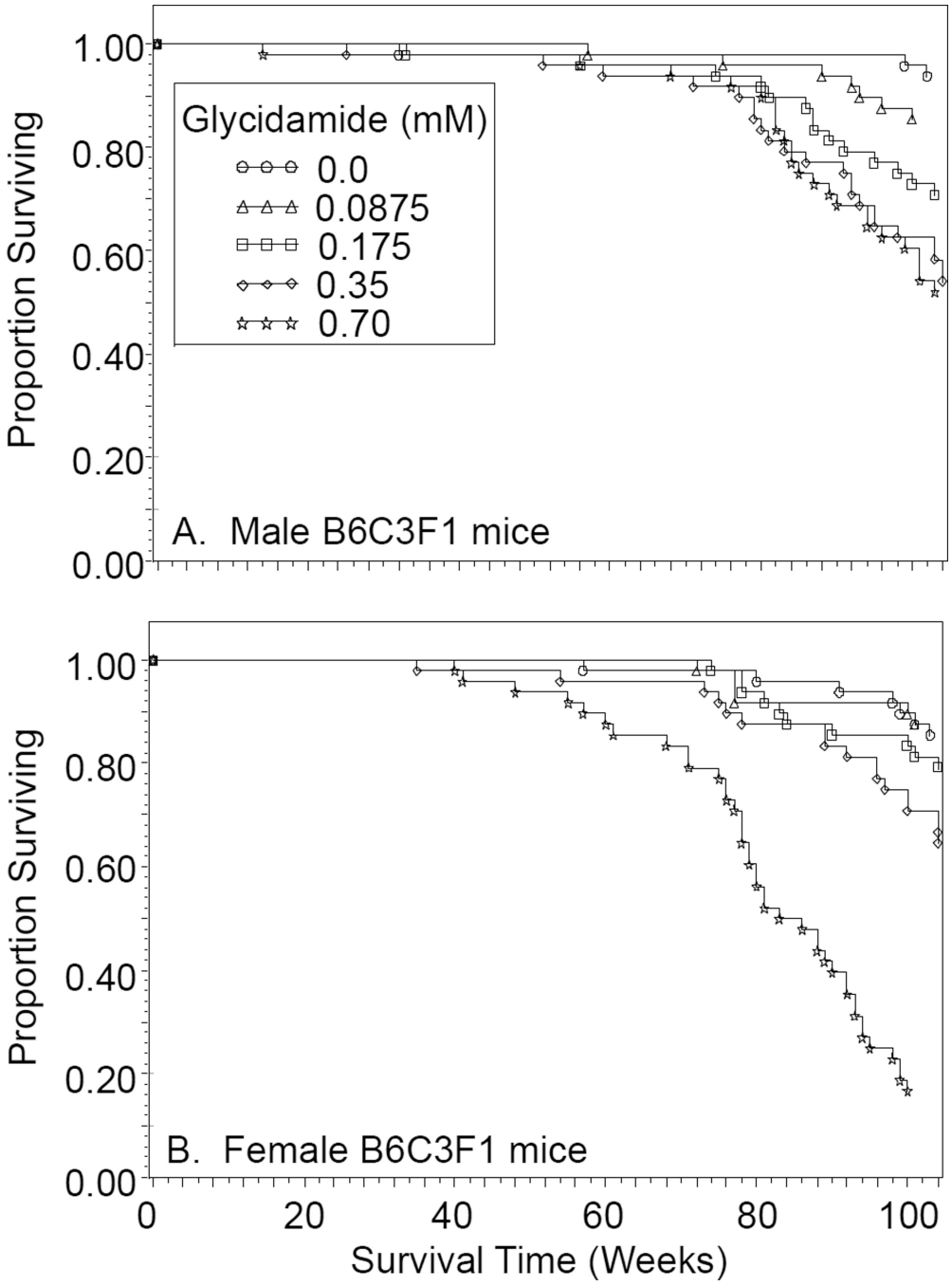

Figure 2.

Survival of male (A) and female (B) B6C3F$_1$ mice administered 0, 0.0875, 0.175, 0.35, or 0.70 mM glycidamide in the drinking water for two years as a function of the number of weeks on treatment.

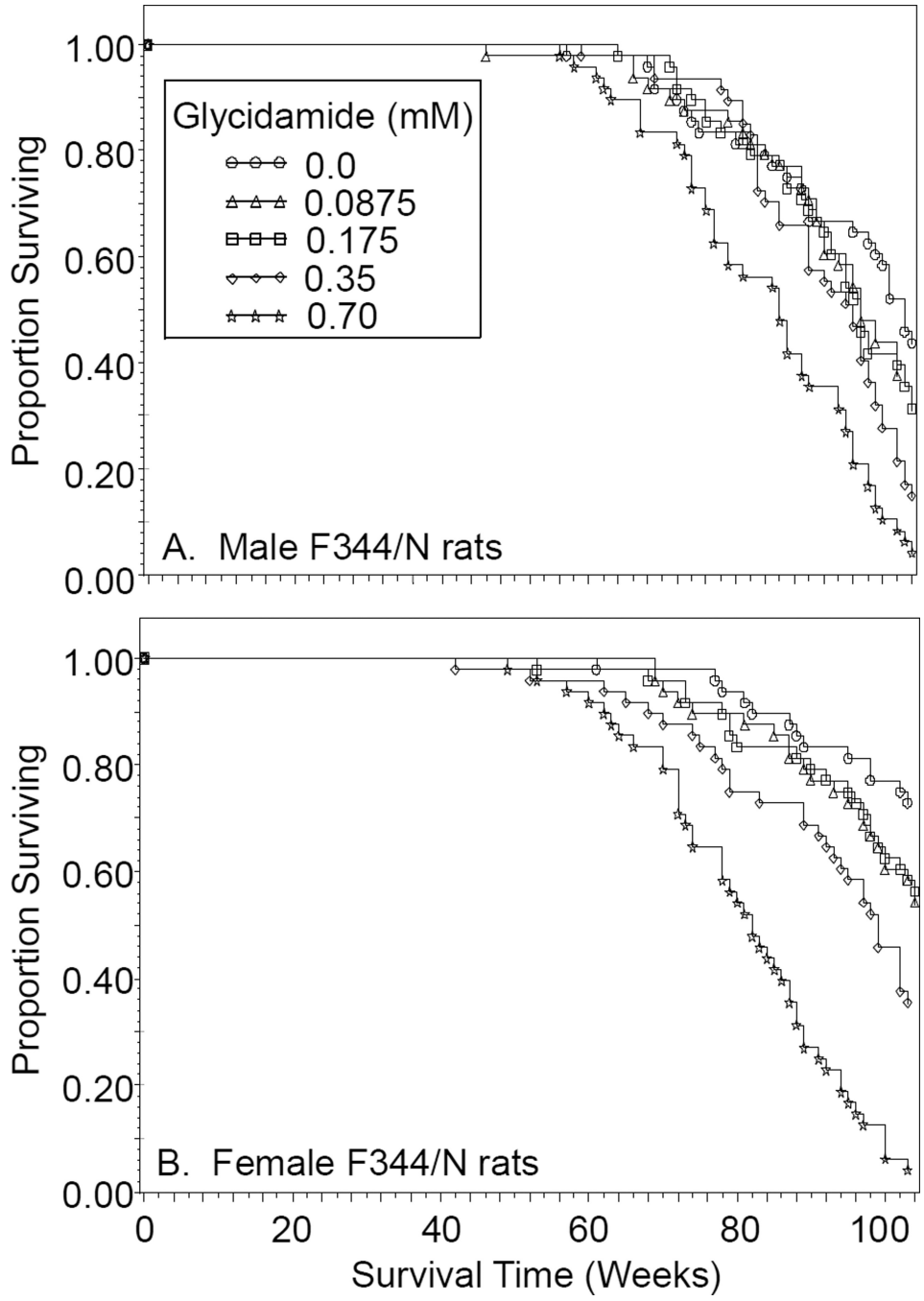

Figure 3.

Survival of male (A) and female (B) F344/N rats administered 0, 0.0875, 0.175, 0.35, or 0.70 mM glycidamide in the drinking water for two years as a function of the number of weeks on treatment.

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Table 1

Incidence of neoplasms in male and female B6C3F$_1$ mice administered 0, 0.0875, 0.175, 0.35, or 0.70 mM glycidamide in the drinking water for two years[a].

Neoplasm	Sex	Glycidamide (mM)				
		0	**0.0875**	**0.175**	**0.35**	**0.70**
Harderian gland adenoma	Male	3/47 (6%)***	17/47 (36%)***	23/47 (49%)***	32/46 (70%)***	42/47 (89%)***
	Female	2/45 (4%)***	19/47 (40%)***	20/47 (43%)***	24/46 (52%)***	40/46 (87%)***
Lung alveolar/bronchiolar adenoma	Male	0/47 (0%)***	7/46 (15%)**	7/47 (15%)**	13/47 (28%)***	17/47 (36%)***
	Female	3/46 (7%)**	5/48 (10%)	3/47 (6%)	7/47 (15%)	9/44 (20%)**
Stomach (forestomach) squamous cell papilloma	Male	0/47 (0%)***	2/45 (4%)	3/48 (6%)	2/45 (4%)	10/41 (24%)***
	Female	1/45 (2%)***	1/45 (2%)	1/47 (2%)	5/45 (11%)	9/44 (20%)***
Skin squamous cell papilloma	Male	0/47 (0%)***	1/48 (2%)	2/47 (4%)	1/47 (2%)	8/46 (17%)***
Skin fibrosarcoma	Female	0/45 (0%)***	1/48 (2%)	2/47 (4%)	2/47 (4%)	9/45 (20%)***
Skin fibrosarcoma or sarcoma	Female	0/45 (0%)***	1/48 (2%)	3/47 (6%)	5/47 (11%)**	12/45 (27%)***
Mammary gland adenoacanthoma	Female	0/45 (0%)***	0/48 (0%)	0/47 (0%)	1/47 (2%)	8/45 (18%)***
Mammary gland adenocarcinoma	Female	1/45 (2%)***	1/48 (2%)	2/47 (4%)	9/47 (19%)**	11/45 (24%)***
Mammary gland adenoacanthoma or adenocarcinoma	Female	1/45 (2%)***	1/48 (2%)	2/47 (4%)	9/47 (19%)**	18/45 (40%)***
Ovary benign granulosa cell tumor	Female	0/45 (0%)*	0/47 (0%)	0/47 (0%)	1/46 (2%)	2/44 (5%)
Ovary malignant granulosa cell tumor	Female	0/45 (0%)*	0/47 (0%)	0/47 (0%)	2/46 (4%)	1/44 (2%)
Ovary benign or malignant granulosa cell tumor	Female	0/45 (0%)***	0/47 (0%)	0/47 (0%)	3/46 (7%)	3/44 (7%)

[a]The data are reported as the number of animals with a neoplasm per number of animals examined microscopically and (in parentheses) the % incidence. Statistical analyses for dose-related trends and differences in incidence were conducted by survival-adjusted Poly-3 tests.

An asterisk (*) associated with the 0 mM glycidamide incidence indicates a significant (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$) dose-related trend with respect to glycidamide. An asterisk (*) associated with a specific treatment indicates a significant (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$) difference compared to the 0 mM glycidamide incidence.

Food Chem Toxicol. Author manuscript; available in PMC 2016 December 01.

Table 2

Incidence of neoplasms in male and female F344/N rats administered 0, 0.0875, 0.175, 0.35, or 0.70 mM glycidamide in the drinking water for two years[a].

Neoplasm	Sex	Glycidamide (mM)				
		0	**0.0875**	**0.175**	**0.35**	**0.70**
Thyroid gland follicular cell adenoma	Male	2/47 (4%) ***	1/42 (2%)	3/48 (6%)	3/47 (6%)	8/46 (17%) **
	Female	0/48 (0%) *	3/48 (6%)	3/46 (7%)	1/46 (2%)	5/47 (11%) **
Thyroid gland follicular cell carcinoma	Male	0/47 (0%) **	2/42 (5%)	3/48 (6%)	1/47 (2%)	5/46 (11%) **
	Female	0/48 (0%) **	0/48 (0%)	2/46 (4%)	3/46 (7%)	3/47 (6%) *
Thyroid gland follicular cell adenoma or carcinoma	Male	2/47 (4%) ***	3/42 (7%)	6/48 (13%)	4/47 (9%)	13/46 (28%) ***
	Female	0/48 (0%) ***	3/48 (6%)	5/46 (11%) *	4/46 (9%) *	8/47 (17%) ***
Oral mucosa squamous cell papilloma	Male	1/48 (2%) *	1/48 (2%)	0/48 (0%)	2/47 (4%)	3/48 (6%)
	Female	1/48 (2%) *	1/48 (2%)	2/48 (4%)	0/48 (0%)	4/48 (8%)
Oral mucosa squamous cell carcinoma	Male	1/48 (2%)	0/48 (0%)	1/48 (2%)	1/47 (2%)	0/48 (0%)
	Female	0/48 (0%) *	0/48 (0%)	0/48 (0%)	1/48 (2%)	2/48 (4%)
Tongue squamous cell papilloma	Male	0/48 (0%) **	1/48 (2%)	0/48 (0%)	1/47 (2%)	4/48 (8%) *
	Female	0/48 (0%)	1/48 (2%)	0/48 (0%)	1/48 (2%)	0/48 (0%)
Tongue squamous cell carcinoma	Male	0/48 (0%)	0/48 (0%)	1/48 (2%)	0/47 (0%)	0/48 (0%)
	Female	0/48 (0%)	0/48 (0%)	0/48 (0%)	0/48 (0%)	1/48 (2%)
Oral mucosa or tongue squamous cell papilloma or carcinoma	Male	2/48 (4%) **	2/48 (4%)	2/48 (4%)	3/47 (6%)	7/48 (15%) *
	Female	1/48 (2%) ***	2/48 (4%)	2/48 (4%)	2/48 (4%)	7/48 (15%) **
Mononuclear cell leukemia	Male	21/48 (44%) **	26/48 (54%)	27/48 (56%)	27/47 (57%)	31/48 (65%) **
	Female	14/48 (29%) ***	11/48 (23%)	21/48 (44%)	19/48 (40%)	27/48 (56%) ***
Epididymis malignant mesothelioma	Male	0/48 (0%) ***	1/45 (2%)	3/48 (6%)	10/47 (21%) ***	17/47 (36%) ***
Testes malignant mesothelioma	Male	0/48 (0%) ***	1/47 (2%)	3/48 (6%)	6/47 (13%) **	13/48 (27%) ***

Neoplasm	Sex	Glycidamide (mM)				
		0	0.0875	0.175	0.35	0.70
Epididymis or testes malignant mesothelioma	Male	0/48 (0%) ***	1/48 (2%)	3/48 (6%)	10/47 (21%) ***	17/48 (35%) ***
Heart malignant Schwannoma	Male	2/48 (4%) **	3/48 (6%)	3/48 (6%)	7/47 (15%)	8/48 (17%) *
Mammary gland fibroadenoma	Female	16/48 (33%) ***	26/48 (54%) *	35/48 (73%) ***	33/48 (69%) ***	36/48 (75%) ***
Clitoral gland adenoma	Female	6/48 (13%)	3/48 (6%)	6/48 (13%)	3/48 (6%)	5/47 (11%)
Clitoral gland carcinoma	Female	4/48 (8%) ***	6/48 (13%)	7/48 (15%)	11/48 (23%) *	14/47 (30%) ***
Clitoral gland squamous cell papilloma	Female	0/48 (0%) **	0/48 (0%)	0/48 (0%)	1/48 (2%)	2/47 (4%)
Clitoral gland squamous cell carcinoma	Female	2/48 (4%)	0/48 (0%)	0/48 (0%)	0/48 (0%)	0/47 (0%)
Clitoral gland adenoma, carcinoma, or squamous cell papilloma or carcinoma	Female	11/48 (23%) ***	9/48 (19%)	13/48 (27%)	14/48 (29%)	20/47 (43%) ***
Stomach (forestomach) squamous cell papilloma	Female	0/48 (0%) *	1/48 (2%)	0/48 (0%)	0/47 (0%)	3/46 (7%) *

[a]The data are reported as the number of animals with a neoplasm per number of animals examined microscopically and (in parentheses) the % incidence. Statistical analyses for dose-related trends and differences in incidence were conducted by survival-adjusted Poly-3 tests.

An asterisk (*) associated with the 0 mM glycidamide incidence indicates a significant (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$) dose-related trend with respect to glycidamide. An asterisk (*) associated with a specific treatment indicates a significant (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$) difference compared to the 0 mM glycidamide incidence.

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Table 3

Incidence of non-neoplastic lesions in male and female B6C3F$_1$ mice administered 0, 0.0875, 0.175, 0.35, or 0.70 mM glycidamide in the drinking water for two years[a].

Non-neoplastic lesion	Sex	Glycidamide (mM)				
		0	0.0875	0.175	0.35	0.70
Eye cataracts	Male	1/47 (2%) ***	3/45 (7%)	7/46 (15%) *	8/44 (18%) **	17/42 (40%) ***
	Female	1/45 (2%) ***	2/44 (5%)	8/47 (17%) *	8/44 (18%) **	9/43 (21%) ***
Eye corneal inflammation	Male	0/47 (0%) ***	0/45 (0%)	2/46 (4%)	0/44 (0%)	8/42 (19%) ***
	Female	0/45 (0%) **	2/44 (5%)	1/47 (2%)	3/44 (7%)	5/43 (12%) **
Stomach (forestomach) epithelium hyperplasia	Male	5/47 (11%) ***	2/45 (4%)	5/48 (10%)	5/45 (11%)	12/41 (29%) **
	Female	4/45 (9%) *	4/45 (9%)	10/47 (21%)	11/45 (24%) *	5/44 (11%)
Spleen hematopoietic cell proliferation	Male	6/47 (13%) ***	6/47 (13%)	12/47 (26%)	14/46 (30%) *	17/44 (39%) ***
	Female	6/46 (13%) ***	10/47 (21%)	11/47 (23%)	14/47 (30%) *	29/45 (64%) ***
Lung alveolar epithelium hyperplasia	Male	0/47 (0%) **	1/46 (2%)	4/47 (9%) *	3/47 (6%)	6/47 (13%) **
Liver angiectasis	Female	0/47 (0%) ***	0/48 (0%)	1/47 (2%)	0/46 (0%)	5/43 (12%) **
Liver necrosis	Female	0/47 (0%) ***	0/48 (0%)	0/47 (0%)	0/46 (0%)	5/43 (12%) **
Spinal cord (cervical) axonal degeneration	Female	4/45 (9%) **	9/44 (20%)	10/47 (21%)	9/45 (20%)	10/43 (23%) **

[a]The data are reported as the number of animals with a non-neoplastic lesion per number of animals examined microscopically and (in parentheses) the % incidence. Statistical analyses for dose-related trends and differences in incidence were conducted by survival-adjusted Poly-3 tests.

An asterisk (*) associated with the 0 mM glycidamide incidence indicates a significant (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$) dose-related trend with respect to glycidamide. An asterisk (*) associated with a specific treatment indicates a significant (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$) difference compared to the 0 mM glycidamide incidence.

Table 4

Incidence of non-neoplastic lesions in male and female F344/N rats administered 0, 0.0875, 0.175, 0.35, or 0.70 mM glycidamide in the drinking water for two years[a].

Non-neoplastic lesion	Sex	Glycidamide (mM)				
		0	**0.0875**	**0.175**	**0.35**	**0.70**
Brain gliosis	Male	0/48 (0%) **	1/48 (2%)	0/48 (0%)	0/47 (0%)	4/48 (8%) *
	Female	0/48 (0%) **	0/48 (0%)	4/48 (8%) *	4/48 (8%) *	4/48 (8%) *
Liver hepatocyte degeneration	Male	2/47 (4%) **	6/47 (13%)	6/48 (13%)	10/47 (21%) **	8/47 (17%) **
Liver necrosis	Male	1/47 (2%) *	5/47 (11%)	2/48 (4%)	7/47 (15%) *	5/47 (11%) *
Spinal cord (lumbar) axonal degeneration	Female	5/48 (10%) *	6/48 (13%)	5/47 (11%)	6/48 (13%)	9/48 (19%) *
Uterus endometrium cystic hyperplasia	Female	11/48 (23%) ***	17/48 (35%)	14/48 (29%)	14/48 (29%)	23/48 (48%) ***

[a]The data are reported as the number of animals with a non-neoplastic lesion per number of animals examined microscopically and (in parentheses) the % incidence. Statistical analyses for dose-related trends and differences in incidence were conducted by survival-adjusted Poly-3 tests.

An asterisk (*) associated with the 0 mM glycidamide incidence indicates a significant (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$) dose-related trend with respect to glycidamide. An asterisk (*) associated with a specific treatment indicates a significant (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$) difference compared to the 0 mM glycidamide incidence.

Table 5

Comparison of BMD_{10} for selected neoplasms in male and female $B6C3F_1$ mice and F344/N rats administered 0, 0.0875, 0.175, 0.35, or 0.70 mM glycidamide or acrylamide in the drinking water for two years.

Species	Neoplasm	Sex	Model	Glycidamide (BMD_{10}, μmol/kg body weight/day)[a]	Acrylamide (BMD_{10}, μmol/kg body weight/day)[b]
$B6C3F_1$ mice	Harderian gland, adenoma	Male	Log-Logistic	5.51	5.14
			Log-Probit	5.91	5.39
		Female	Log-Logistic	4.55	6.65
$B6C3F_1$ mice	Lung, alveolar/ bronchiolar adenoma	Male	Log-Logistic	17.16	29.59
		Female	Gamma	99.36	27.69
			Logistic	112.72	56.06
			Log-Logistic	99.08	27.15
			Log-Probit	100.00	26.89
			Multistage	98.98	27.69
			Probit	110.13	51.97
			Weibull	99.33	27.69
$B6C3F_1$ mice	Stomach (forestomach), squamous cell papilloma	Male	Gamma	52.55	63.98
			Logistic	76.50	105.74
			Log-Logistic	51.96	62.30
			Log-Probit	49.72	60.51
			Multistage	55.10	63.98
			Probit	73.15	101.39
			Weibull	52.55	63.98
$B6C3F_1$ mice	Mammary gland, adenoacanthoma	Female	Gamma	117.96	140.56
			Logistic	129.97	146.10
			Log-Logistic	119.46	142.80
			Log-Probit	115.08	172.55
			Multistage	116.96	140.56
			Probit	125.67	145.74

Species	Neoplasm	Sex	Model	Glycidamide (BMD_{10}, μmol/kg body weight/day)[a]	Acrylamide (BMD_{10}, μmol/kg body weight/day)[b]
			Weibull	120.56	140.56
$B6C3F_1$ mice	Mammary gland, adenoacanthoma or adenocarcinoma	Female	Gamma	53.46	31.22
			Log-Logistic	52.72	28.31
			Log-Probit	51.98	24.23
			Multistage	55.63	31.22
			Weibull	53.41	31.22
F344/N rats	Thyroid gland, follicular cell carcinoma	Male	Gamma	40.32	28.57
			Logistic	42.24	35.95
			Log-Logistic	39.76	27.87
			Log-Probit	70.34	26.72
			Multistage	40.32	28.57
			Probit	42.65	35.00
			Weibull	40.32	28.57
F344/N rats	Thyroid gland, follicular cell adenoma or carcinoma	Male	Gamma	19.33	20.37
			Logistic	22.62	28.19
			Log-Logistic	20.77	19.47
			Multistage	20.85	20.37
			Probit	21.65	27.14
			Weibull	20.50	20.37
		Female	Gamma	27.00	54.15
			Logistic	42.74	60.24
			Log-Logistic	24.05	54.80
			Multistage	27.00	54.15
			Probit	41.31	59.36
			Weibull	27.00	54.15
F344/N rats	Heart, malignant Schwannoma	Male	Gamma	25.06	34.13
			Logistic	31.09	37.68

Species	Neoplasm	Sex	Model	Glycidamide (BMD_{10}, µmol/kg body weight/day)[a]	Acrylamide (BMD_{10}, µmol/kg body weight/day)[b]
			Log-Logistic	24.40	33.91
			Log-Probit	23.23	34.76
			Multistage	25.06	34.13
			Probit	30.17	37.23
			Weibull	25.06	34.13
F344/N rats	Epididymis or testes, malignant mesothelioma	Male	Gamma	11.71	29.90
			Multistage	11.59	30.66
			Probit	17.94	30.36
			Weibull	11.69	30.06
F344/N rats	Mammary gland, fibroadenoma	Female	Log-Logistic	2.39	7.74
F344/N rats	Oral mucosa or tongue, squamous cell papilloma or carcinoma	Female	Gamma	50.94	49.49
			Logistic	48.94	58.01
			Log-Logistic	51.03	48.42
			Log-Probit	50.82	61.93
			Multistage	49.97	49.49
			Probit	48.73	57.54
			Weibull	51.09	49.49

[a]The BMD_{10} for glycidamide in µmol/kg body weight/day are from the data presented in Supplementary Table S2.

[b]The BMD_{10} for acrylamide, in µmol/kg body weight/day, were calculated from the data presented in Beland *et al.* (2013).