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Min Gi[1, #], Masaki Fujioka[1], Yukari Totsuka[2], Michiharu Matsumoto[3], Kenichi Masumura[4], Anna Kakehashi[1], Takashi Yamaguchi[1], Shoji Fukushima[3, 5], Hideki Wanibuchi[1, *]

[1] Department of Molecular Pathology, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585; Japan

[2] Division of Carcinogenesis and Cancer Prevention, National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan

[3] Japan Bioassay Research Center, Japan Organization of Occupational Health and Safety, Hadano, Kanagawa 257-0015, Japan

[4] Division of Genetics and Mutagenesis, National Institute of Health Sciences, 3-25-26 Tonomachi, Kawasaki-ku, Kawasaki, Kanagawa 210-9501, Japan

[5] Association for Promotion of Research on Risk Assessment, Nakagawa-ku, Nagoya 454-0869, Japan

[#] Current address: Department of Environmental Risk Assessment, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585; Japan

[*] To whom correspondence should be addressed: Hideki Wanibuchi, Department of Molecular Pathology, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585; Japan. Tel: +81 6-6645-3735; Fax: +81 6-6646-3096; E-mail: wani@med.osaka-cu.ac.jp

Abstract

Quantitative analysis of the mutagenicity and carcinogenicity of the low doses of genotoxic carcinogens present in food is of pressing concern. The purpose of the present study was to determine the mutagenicity and carcinogenicity of low doses of the dietary genotoxic carcinogen 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ). Male F344 *gpt* delta transgenic rats were fed diets supplemented with 0, 0.1, 1, 10, or 100 ppm IQ for 4 weeks. The frequencies of *gpt* transgene mutations in the liver were significantly increased in the 10 and 100 ppm groups. In addition, the mutation spectra was altered in the 1, 10, and 100 ppm groups: frequencies of G:C to T:A transversion were significantly increased in groups administered 1, 10, and 100 ppm IQ in a dose-dependent manner, and the frequencies of G:C to A:T transitions, A:T to T:A transversions, and A:T to C:G transversions were significantly increased in the 100 ppm group. Increased frequencies of single base pair deletions and Spi^- mutants in the liver, and an increase in glutathione *S*-transferase placental form (GST-P) positive foci, a preneoplastic lesion of the liver in rats, was also observed in the 100 ppm group. In contrast, neither mutations nor mutation spectra or GST-P positive foci were statistically altered by administration of IQ at 0.1 ppm. We estimated the point of departure (PoD) for the mutagenicity and carcinogenicity of IQ using the no-observed effect level approach and the Benchmark dose approach to

characterize the dose-response relationship of low doses of IQ. Our findings demonstrate the existence of no effect levels of IQ for both *in vivo* mutagenicity and hepatocarcinogenicity. The findings of the present study will facilitate an understanding of the carcinogenic effects of low doses of IQ and help to determine a margin of exposure that may be useful for practical human risk assessment.

Introduction

Exposure to environmental carcinogens is one of the most significant causes of human cancers. Determination of the dose-response relationship between carcinogen exposure and induction of cancer is one of the most important areas of chemical risk assessment. Especially of high priority is cancer risk assessment of dietary carcinogens.

2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ) is a well-known dietary genotoxic carcinogenic heterocyclic amine formed by high-temperature cooking of proteinaceous food such as meat and fish (1,2). IQ induces cancers of the liver, colon, and mammary and zymbal glands in rats; cancers of the liver, lung and forestomach in mice; and cancer of the liver in nonhuman primates (3-5). Based on sufficient evidence of carcinogenicity in experimental animals and limited evidence of carcinogenicity in humans, IQ is classified as a category 2A carcinogen (probably carcinogenic to humans) by the International Agency for Research on Cancer (6). Therefore, although the concentrations of IQ in food are low, they constitute a potential hazard, and there is concern regarding the carcinogenic effects of low doses of IQ.

Genotoxic carcinogens induce DNA damage, and even the lowest doses may cause mutations. Therefore, linear nonthreshold extrapolation is generally accepted for human health risk assessment and regulatory decision-making of DNA-reactive genotoxic

carcinogens, especially in cases where the mode of action has not been ascertained (7-9). The logical conclusion from linear non-threshold extrapolation is that some risk of carcinogenicity exists for any dose of a genotoxic carcinogen and that no safe dose exists. However, as experimental evidence continues to accumulate showing that the carcinogenic effects of genotoxic carcinogens can be negligible at low doses (10-20), the non-threshold approach has been challenged by quantitative approaches using point-of-departure (PoD) metrics (8,19-25). Using PoD metrics, with an acceptable daily intake and margin of exposure, is superior to non-threshold approaches for defining acceptable human exposure limits and assessing human risk (19-25).

Since somatic mutation is considered responsible for carcinogenesis with stepwise accumulation of alterations in cancer-related genes leading to malignant neoplasia (26), correlation of the *in vivo* genotoxic potency with the carcinogenic potency of genotoxic carcinogens has important implications for risk assessment. We previously demonstrated the existence of no effect levels of IQ for hepatocarcinogenicity in F344 rats (13). However, a dose-response relationship for *in vivo* mutagenicity of IQ has not been evaluated in rats. In the present study, we determined the low dose mutagenicity of IQ in the liver of F344 *gpt* delta transgenic rats. This rat model is well established and widely used for determination of organ-specific mutagenesis induced by various carcinogens

(27-29). To evaluate the low dose hepatocarcinogenicity of IQ in F344 *gpt* delta transgenic rats, we examined induction of glutathione *S*-transferase placental form (GST-P) positive foci, which is a well-established preneoplastic lesion of the liver in rats and has been accepted as a useful end-point marker in assessment of carcinogenic effects of environmentally relevant concentrations of carcinogens (10). To estimate exposure-related risk, we determined the PoDs for the mutagenicity and hepatocarcinogenicity endpoints of IQ by two quantitative approaches: the no-observed-effect level (NOEL) and the benchmark dose (BMD) approaches.

Materials and Methods

Chemicals and diet

IQ was purchased from Nard Institute Ltd. (Osaka, Japan) with a purity of 99.9%. Basal diet (powdered MF, Oriental Yeast Co. Tokyo, Japan) and the diets supplemented with IQ were prepared by Oriental Yeast Co., Japan.

Animals

Five-week old male F344/NSlc *gpt* delta transgenic rats were supplied by Japan SLC Inc. (Shizuoka, Japan). Animal studies were approved by the Institutional Animal Care and Use Committee of Osaka City University Graduate School of Medicine and conducted in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, 2006). Animals were housed in polycarbonate cages in experimental animal rooms with a targeted temperature of 22 ± 3°C, relative humidity of 55 ± 5%, and a 12-h light/dark cycle. Diet and drinking water were available *ad libitum* throughout the study. Body weight, food consumption, and water intake were measured weekly.

Experimental protocol

A total of 25 male F344 *gpt* delta transgenic rats, 6 weeks of age at the commencement of the experiment, were divided into 5 groups of 5 rats each and fed diets supplemented with 0, 0.1, 1, 10, or 100 ppm IQ for 4 weeks and then maintained on basal diet for three days as recommended by the OECD test guideline for Transgenic Rodent Somatic and Germ cell Gene Mutation Assays (TG488) (30).Three days after the end of the 4-week treatment, rats were euthanized by inhalation of an overdose of isoflurane (Abbott Japan Co., Ltd., Tokyo, Japan) using a Small Animal Anesthetizer (MK-A110D, Muromachi Kikai Co., LTD., Tokyo, Japan) coupled with an Anesthetic Gas Scavenging System (MK-T 100E, Muromachi Kikai Co., LTD., Tokyo, Japan). At necropsy, livers were excised and weighed. A total of 3 sections of liver tissue (one section each from the left lateral lobe, right middle lobe, and caudate lobe) were fixed in 10% phosphate buffered formalin, embedded in paraffin, and processed for hematoxylin/eosin and immunohistochemical staining. The remaining liver tissues were snap frozen with liquid nitrogen and stored at -80 °C for mutation assays.

in vivo mutation assays

The *gpt* and Spi$^-$ assays were conducted according to the published protocols by Nohmi *et al* (31). Genomic DNA was extracted from frozen liver tissue using the RecoverEase DNA Isolation kit according to the manufacturer's protocol (Agilent Technologies, Santa Clara, CA). Lambda EG10 DNA in the genomic DNA was rescued as the lambda phage using the Transpack packaging extract (Agilent Technologies).

In the *gpt* assay, packaged phages were transfected into *Escherichia coli* YG6020 expressing Cre recombinase and cultured on selection plates containing chloramphenicol and 6-thioguanine (6-TG) for mutant selection. The *gpt* mutants (6-TG–resistant (6-TG^R) phenotype) were isolated and re-streaked on selection plates to confirm the 6-TG^R phenotype. All confirmed *gpt* mutants were recovered and sequenced; identical mutations from the same rat were counted as one mutant. The mutation frequency (MF) of the *gpt* gene in the liver was calculated by dividing the number of confirmed 6-TG^R colonies by the number of rescued plasmids (chloramphenicol–resistant (Cm^R) colonies). DNA sequencing of the *gpt* gene was performed with the BigDye Terminater Cycle Sequencing Ready Reaction (Applied Biosystems, Inc., Carlsbad, CA, USA) on an Applied Biosystems PRISM 310 Genetic Analyzer. A:T to T:A transversions in the 299th bp of the *gpt* gene is a germline mutation in *gpt* delta F344 rat regardless of the experimental

treatment (32,33); therefore, A:T to T:A transversions in the 299th bp of the *gpt* gene were excluded from the MF and mutation spectra.

In the Spi$^-$ assay, packaged phages were incubated with *E. coli* XL-1 Blue MRA for survival titration and *E. coli* XL-1 Blue MRA P2 for mutant selection. Infected cells were mixed with molten lambda-trypticase agar and poured onto lambda-trypticase agar plates. The next day, plaques (Spi$^-$ candidates) were punched out with sterilized glass pipettes and the agar plugs were suspended in SM buffer. The Spi$^-$ phenotype was confirmed by spotting the suspensions on three types of plates in which XL-1 Blue MRA, XL-1 Blue MRA P2, or WL95 P2 strains were spread with lambda-trypticase soft agar. True Spi$^-$ mutants, which made clear plaques on all of the plates, were counted.

Immunohistochemical analysis

Paraffin sections of the livers of all animals were examined for GST-P positive foci by immunohistochemical staining using the avidin–biotin–peroxidase complex (ABC) method. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in distilled water for 5 min. After blocking non-specific binding with goat serum at 37°C for 30 min, sections were incubated with rabbit polyclonal anti-GST-P antibody (#311, Medical and Biological Laboratories Co., Ltd., Nagoya, Japan) diluted 1:1000 overnight at 4°C.

Immunoreactivity was detected using a Rabbit IgG VECSTAIN ABC Kit (PK-4001, Vector Laboratories, Burlingame, CA, USA) and 3,3′-diaminobenzidine hydrochloride (Sigma-Aldrich Co., St Louis, MO, USA).

Evaluation of GST-P positive foci in the liver

GST-P positive hepatocellular foci composed of 2 or more cells were counted under a light microscope (10,13,17,18). Total areas of livers were measured using a color image processor IPAP (Sumica Technos, Osaka, Japan) and the number of GST-P positive foci per square centimeter of liver tissue was calculated.

Derivation of PoDs

The PoD marks the beginning of low-dose extrapolation. In the NOEL approach, the PoD is the highest dose at which no statistically significant differences in response are observed compared with the background response. In the benchmark dose (BMD) approach, continuous models were used to fit dose-response data for IQ-DNA adducts, MF, and GST-P positive foci. BMD analyses were conducted using the PROAST software developed by the Dutch National Institute for Public Health and the Environment (version 65.5). Dose-response data were analyzed using the exponential

model or the Hill model, consistent with EFSA guidelines (34,35). The benchmark response (BMR), also known as the critical effect size (CES), is a percentage change in the response relative to the control. It has been argued that BMR values need to be scaled according to endpoint-specific theoretical maxima for comparison of BMDs across endpoints (36,37). The BMR for IQ-DNA adducts and mutations for the current analyses were defined as a 50% increase in the response (BMD_{50}), as recent analyses indicate that BMR values in the range of 40–50% are more appropriate for the interpretation of transgenic rodent mutagenicity dose–response data (38). A BMR of 10% (BMD_{10}) was used for GST-P positive foci, as we have used in previous studies (19,39). The BMDL and BMDU values represent the lower and upper bounds of the two-sided 95% confidence limit of the BMD. The $BMDL_{50}$ was used as the PoD for IQ-DNA adducts and mutations, and $BMDL_{10}$ was used as the PoD for GST-P positive foci.

Statistical analysis

All mean values are reported as mean ± SD. Statistical analyses were performed using the Statlight program (Yukms Co., Ltd., Tokyo, Japan). The values of MF and DNA adducts were log-transformed (Log10) prior to statistical analysis. Homogeneity of variance was tested by the Bartlett test. Differences in mean values between control (0 ppm) and treatment groups were analyzed by the one-tailed Dunnett's Multiple

Comparison Test when variance was homogeneous and the one-tailed Steel's test when variance was heterogeneous. *p* values less than 0.05 were considered significant.

Results

General findings and liver histopathology

Data on final body weights, liver weights, water intake, food consumption, and IQ intake are summarized in Table 1. Final body weights showed a tendency to decrease in the 100 ppm group compared to the control group (0 ppm group), along with a slight suppression of water intake and food consumption. The intake of IQ was approximately proportional to the doses supplemented in the diet. A non-significant slight increase in relative but not absolute liver weight was observed in the 100 ppm group compared to control group, possibly related to the lower body weights in this group.

There were no treatment-related histopathological changes in the livers of any of the IQ-treated groups.

in vivo mutation assay

Data on mutation frequencies (MFs) and mutation spectrum of the *gpt* transgene in the liver are summarized in Tables 2 and 3, respectively. The *gpt* MFs were significantly increased in the groups administered 10 and 100 ppm IQ compared to the control group. The predominant type of base substitution was the G:C to T:A transversion, and the

incidence of this transversion was significantly increased in the groups administered 1, 10, and 100 ppm IQ compared to the control group. Frequencies of G:C to A:T transitions and A:T to T:A and A:T to C:G transversions, and single base pair deletions were also significantly increased in the 100 ppm group. There were no significant differences between the 0.1 ppm group and the control group in MF or the mutation spectrum in the *gpt* transgene.

Results of the Spi$^-$ mutation assay are summarized in Table 4. The frequency of Spi$^-$ mutants in the liver was significantly increased in the 100 ppm group compared to the control group. There were no significant differences between groups administered 0.1 ppm, 1 ppm, or 10 ppm IQ and the control group.

GST-P-positive foci induction in the livers

As summarized in Table 2, the number of GST-P positive foci per unit area in the livers in the 100 ppm group was significantly increased compared to the control group. There were no significant differences between the groups administered 0.1 ppm, 1 ppm, or 10 ppm IQ and the control group.

Derivation of PoDs

To determine the PoDs we analyzed the data on mutagenicity and GST-P positive foci induction in the liver from the present 4-week study, and the data on IQ-DNA adduct formation (4-week administration) and GST-P positive foci induction (16-week administration) from our previous studies, which used wild-type F344 rats (Supplementary Table 1) (13), by the NOEL approach and the BMD approach using the PROAST software package. The derived PoDs are summarized in Table 5. The incidence of G:C to T:A transversion was significantly increased in groups administered 1, 10, and 100 ppm in a dose-dependent manner (Table 3). Other types of mutations (Table 3), total mutations (Table 2), and Spi^- mutations (Table 4) were not increased in the 1 ppm group. Therefore, the PoD value for mutations was determined using the data of the G:C to T:A transversion, the most sensitive indicator of IQ-induced mutations. The PoD values determined by the NOEL approach were 0.1 ppm for mutation ($Mutation_{4\ weeks}$) and 10 ppm for GST-P positive foci ($GST\text{-}P\ positive\ foci_{4\ weeks}$) in the present 4-week study; and 0.01 ppm for DNA-adduct formation ($DNA\ adduct_{4\ weeks}$) and 1 ppm for GST-P positive foci ($GST\text{-}P\ positive\ foci_{16\ weeks}$) in the previous study (13) (Table 5). The PoD ranking by the NOEL approach is IQ-DNA $adduct_{4\ weeks}$ < $Mutation_{4\ weeks}$ < GST-P positive $foci_{16\ weeks}$ < GST-P positive $foci_{4\ weeks}$. The PoD values derived by the BMD approach using

PROAST (Figure 1) were 0.03 ppm for mutation (Mutation $_{4\ weeks}$), and 0.29 ppm for GST-P positive foci (GST-P positive foci $_{4\ weeks}$) in the present 4-week study; and 9.1E-03 ppm for IQ-DNA adduct formation (IQ-DNA adduct $_{16\ weeks}$) and 1.4 ppm for GST-P positive foci (GST-P positive foci $_{16\ weeks}$) in the previous study (13). The PoD ranking by the BMD approach under the criteria used in the present study is IQ-DNA adduct $_{4\ weeks}$ < Mutation $_{4\ weeks}$ < GST-P positive foci $_{4\ weeks}$ < GST-P positive foci $_{16\ weeks}$.

Discussion

Dose-response relationships for genotoxic carcinogens have been a topic of intense scientific and public debate. It is gradually shifting away from a linear non-threshold approach towards quantitative approaches using PoD metrics for defining acceptable exposure limits and assessing the human risk of genotoxic carcinogens (8,19-23). The findings of the present study argue for the presence of no effect levels of IQ for mutagenicity and hepatocarcinogenicity: 1) only in the group administered 100 ppm were statistically significant increases in mutations and also altered mutation spectrum and also GST-P positive foci observed; 2) none of the measured parameters of mutagenicity and carcinogenicity was statistically altered by administration of IQ at a dose of 0.1 ppm. Therefore, these results indicate that IQ only has mutagenic and carcinogenic effects above an experimentally identifiable dose: in the studies presented here the BMD-identified PoD for mutation was 0.03 ppm and the lower BMD-identified PoD for GST-P positive foci, a preneoplastic lesion of the liver in rats, was 0.29 ppm (see Table 5). These results are also in line with our previous findings of the existence of no effect levels of another genotoxic heterocyclic amine, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), for both hepatocarcinogenicity and *in vivo* mutagenicity in various rat carcinogenesis models (12,17,18,40,41).

Currently, there are several statistical approaches available for deriving a PoD, including the NOEL, BMD, no-observed-genotoxic-effect-level (NOGEL), and breaking-point dose (BPD) approaches (42). Recent comparative studies of the genotoxicity of alkylating agents indicated that the BMD approach yields more conservative PoDs than the NOGEL or BPD approaches (42). In the present study, the PoD values for IQ derived from the NOEL and BMD approaches are markedly different. It is well known that PoD values derived from the NOEL approach are dependent on dose selection since the PoD is limited to one of the doses included in the study. PoDs derived from the NOEL approach are also highly dependent on sample size and the statistical sensitivity of the study. Limitations of the NOEL approach include: it does not allow for estimation of the probability of a response for dose levels not included in the study, making it difficult to compare separate studies, and the PoD will tend to be higher in studies with smaller numbers of animals per group (22,34,43-46). For example, the PoD value derived by the NOEL approach for GST-P positive foci was 10 ppm in the present study where the sample size was 5 rats per group, but was 1 ppm in the previous study where the sample size was 120 or 240 rats per group (13). Advantages of the BMD approach have been well documented (22,34,43-45) (47) and include the points that (1) when using the BMD approach, the PoD is not constrained to a dose used in the study;

(2) the BMD approach takes the full shape of the dose–response curve into account, thereby incorporating more dose-response information into the determination of the PoD; (3) because it incorporates all of the study data, it is better at taking into account statistical uncertainties, for example those associated with inter-animal differences and study size; (4) it allows for cross-study comparison; (5) one consequence of these points is that the BMD approach also makes more efficient use of animals; and (6) a BMD can be calculated even when a NOEL is missing from the study. While determination of the best approach is beyond the scope of the present study, the PoDs for IQ-DNA adduct formation and mutagenicity derived by the BMD approach using PROAST are conservative.

It has been argued that BMR values need to be scaled according to endpoint-specific theoretical maxima for comparison of BMDs across endpoints (36,37). In the present study, the $BMDL_{50}$ was used as the PoD for mutation, as recent analyses indicate that BMR values in the range of 40–50% are more appropriate for the interpretation of transgenic rodent mutagenicity dose–response data (38). However, the appropriate BMRs for DNA adduct and GST-P positive foci remain to be established in the animal studies. When the $BMDL_{50}$ is used as the PoD for IQ-DNA adducts and the $BMDL_{10}$ is used as the PoD for GST-P positive foci (19,39), the PoDs for earlier key events tend to be lower

than the PoDs for events closer to the apical endpoint of cancer induction: IQ-DNA adduct < Mutation < GST-P positive foci. While further studies are necessary to establish consensus BMRs for each endpoint and to verify the ranking across the endpoints, the above-mentioned ranking is in agreement with our earlier findings on the genotoxic hepatocarcinogen 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline in animal models that indicated the existence of a practical threshold with respect to its carcinogenicity, and that it induced formation of DNA adducts at low doses, gave rise to gene mutations at higher doses, and induced the development of preneoplastic lesions at the highest doses (17,25).

In conclusion, we demonstrated the existence of no effect levels of IQ for both hepatocarcinogenicity and *in vivo* mutagenicity, and show that it induced DNA adduct formation at very low doses, gave rise to gene mutations at higher doses, and induced development of preneoplastic lesions at the highest doses. The findings of the present study will facilitate an understanding of the carcinogenic effects of low doses of IQ and also help to determine a margin of exposure that may be useful for practical human risk assessment.

Conflict of Interest statement

The authors declare that they have no conflicts of interest.

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Figure legend

Fig. 1.

Dose-response plots and derived BMD values for total *gpt* mutations (A), G:C to T:A transversion mutations in the *gpt* gene (B), Spi− mutations (C), and GST-P positive foci (D) in the present 4 weeks experiment, and IQ-DNA adduct (E) and GST-P positive foci (F) in the previous 4 and 16 week experiment. The terms “critical effect size” (CES), “critical effect dose” (CED), “critical effect lower confidence level” (CEDL), and “critical effect upper confidence level” (CEDU) in the PROAST software are used synonymously with the terms BMR, BMD, BMDL, and BMDU, respectively.

Fig. 1.

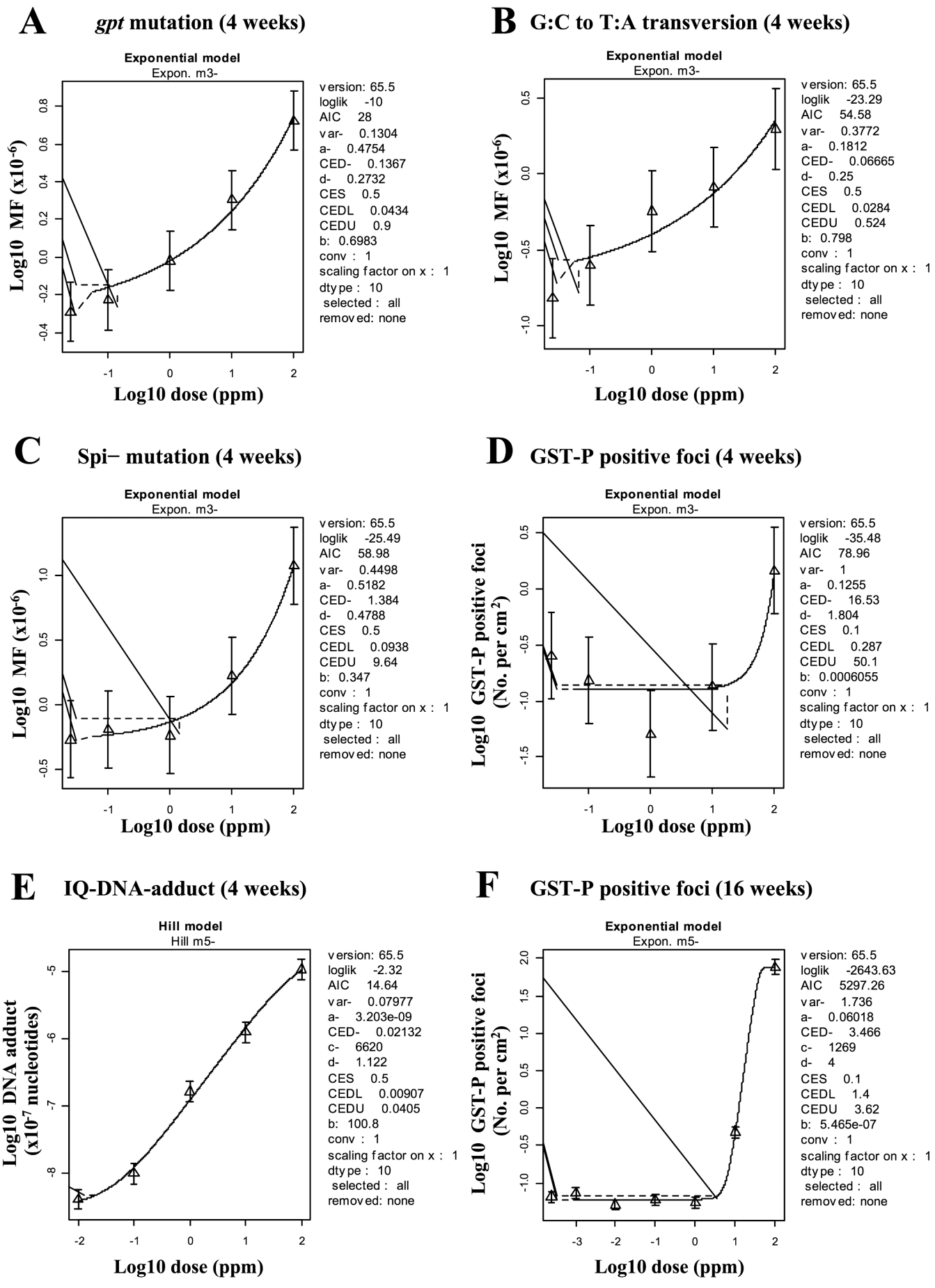
A
gpt mutation (4 weeks)
Exponential model
Expon. m3-
version: 65.5
loglik -10
AIC 28
var- 0.1304
a- 0.4754
CED- 0.1367
d- 0.2732
CES 0.5
CEDL 0.0434
CEDU 0.9
b: 0.6983
conv : 1
scaling factor on x : 1
dtype : 10
selected : all
removed: none
Log10 MF (x10-6)
Log10 dose (ppm)
B
G:C to T:A transversion (4 weeks)
Exponential model
Expon. m3-
version: 65.5
loglik -23.29
AIC 54.58
var- 0.3772
a- 0.1812
CED- 0.06665
d- 0.25
CES 0.5
CEDL 0.0284
CEDU 0.524
b: 0.798
conv : 1
scaling factor on x : 1
dtype : 10
selected : all
removed: none
Log10 MF (x10-6)
Log10 dose (ppm)
C
Spi− mutation (4 weeks)
Exponential model
Expon. m3-
version: 65.5
loglik -25.49
AIC 58.98
var- 0.4498
a- 0.5182
CED- 1.384
d- 0.4788
CES 0.5
CEDL 0.0938
CEDU 9.64
b: 0.347
conv : 1
scaling factor on x : 1
dtype : 10
selected : all
removed: none
Log10 MF (x10-6)
Log10 dose (ppm)
D
GST-P positive foci (4 weeks)
Exponential model
Expon. m3-
version: 65.5
loglik -35.48
AIC 78.96
var- 1
a- 0.1255
CED- 16.53
d- 1.804
CES 0.1
CEDL 0.287
CEDU 50.1
b: 0.0006055
conv : 1
scaling factor on x : 1
dtype : 10
selected : all
removed: none
Log10 GST-P positive foci (No. per cm2)
Log10 dose (ppm)
E
IQ-DNA-adduct (4 weeks)
Hill model
Hill m5-
version: 65.5
loglik -2.32
AIC 14.64
var- 0.07977
a- 3.203e-09
CED- 0.02132
c- 6620
d- 1.122
CES 0.5
CEDL 0.00907
CEDU 0.0405
b: 100.8
conv : 1
scaling factor on x : 1
dtype : 10
selected : all
removed: none
Log10 DNA adduct (x10-7 nucleotides)
Log10 dose (ppm)
F
GST-P positive foci (16 weeks)
Exponential model
Expon. m5-
version: 65.5
loglik -2643.63
AIC 5297.26
var- 1.736
a- 0.06018
CED- 3.466
c- 1269
d- 4
CES 0.1
CEDL 1.4
CEDU 3.62
b: 5.465e-07
conv : 1
scaling factor on x : 1
dtype : 10
selected : all
removed: none
Log10 GST-P positive foci (No. per cm2)
Log10 dose (ppm)

Table 1. Body and liver weights, water intake and food consumption, and IQ intake

IQ (ppm)	No. of rats	Final body weights (g)	Liver		Water intake (g/rat/day)	Food consumption (g/rat/day)	IQ intake	
			Absolute weight (g)	Relative weight (%)			Daily intake (mg⁄kg b.w.)	Total (mg⁄kg b.w.)
0	5	212 ± 19	6.7 ± 0.7	3.2 ± 0.1	22.8	16.1	0	0
0.1	5	209 ± 18	6.5 ± 0.6	3.2 ± 0.1	22.5	14.1	0.01	0.25
1	5	207 ± 13	6.3 ± 0.6	3.1 ± 0.1	22.2	14.9	0.10	2.55
10	5	207 ± 10	6.3 ± 0.6	3.2 ± 0.4	24.2	14.8	0.91	25.58
100	5	201 ± 10	6.6 ± 0.4	3.4 ± 0.1	21.4	14.9	9.23	258.47

Table 2. *gpt* transgene MFs and induction of GST-P positive foci in the livers of F344 *gpt* delta transgenic rats administered IQ for 4 weeks

IQ (ppm)	Rat No.	Cm^R colonies	6-TG^R and Cm^R colonies[a]	MF ($\times 10^{-5}$)	Average MF ($\times 10^{-5}$)	GST-P positive foci (No./cm^2)
0	111	912500	6	0.66		
	112	496250	3	0.60		
	113	753750	5	0.66		
	114	933125	2	0.21		
	115	1422500	8	0.56	0.54 ± 0.19	0.34 ± 0.31
0.1	211	623125	3	0.48		
	212	111250	1	0.90		
	213	238750	2	0.84		
	214	540625	2	0.37		
	215	541875	3	0.55	0.63 ± 0.23	0.26 ± 0.36
1	311	308750	4	1.30		
	312	337500	4	1.19		
	313	246875	2	0.81		
	314	1743125	6	0.34		
	315	59375	1	1.68	1.06 ± 0.51	0.12 ± 0.26
10	411	591250	8	1.35		
	412	855000	14	1.64		
	413	275000	8	2.91		
	414	992500	27	2.72		
	415	1388750	27	1.94	2.11 ± 0.68*	0.23 ± 0.32
100	511	1982500	85	4.29		
	512	359375	10	2.78		
	513	1488125	68	4.57		
	514	911875	80	8.77		
	515	301875	27	8.94	5.87 ± 2.81*	1.50 ± 0.38*

[a] Number of colonies with independent mutations (%).

Significantly different from the control group (0 ppm) at *$p < 0.001$.

Table 3. Mutation spectra of the *gpt* transgene in the livers of F344 *gpt* delta transgenic rats administered IQ for 4 weeks

	IQ (ppm)				
Type of mutation	0	0.1	1	10	100
Transition					
A:T to G:C	2 (8.3) [a]	0	0	5 (6.0)	12 (4.4)
	0.07 ± 0.09 [b]	0	0	0.09 ± 0.13	0.25 ± 0.28
G:C to A:T	6 (25.0)	3 (27.3)	2 (11.8)	8 (9.5)	43 (15.9)
	0.12 ± 0.08	0.11 ± 0.16	0.13 ± 0.29	0.16 ± 0.31	1.00 ± 0.51*
Transversion					
G:C to T:A	8 (33.3)	7 (63.6)	11 (64.7)	38 (45.2)	92 (34.1)
	0.20 ± 0.17	0.34 ± 0.31	0.72 ± 0.56*	0.91 ± 0.45*	2.27 ± 1.29*
G:C to C:G	1 (4.2)	0	1 (5.9)	7 (8.3)	22 (8.1)
	0.01 ± 0.03	0	0.08 ± 0.18	0.21 ± 0.15	0.41 ± 0.42
A:T to T:A	3 (12.5)	0	0	4 (4.8)	10 (3.7)
	0.05 ± 0.07	0	0	0.07 ± 0.10	0.23 ± 0.08*
A:T to C:G	1 (4.2)	1 (9.1)	2 (11.8)	9 (10.7)	27 (10.0)
	0.04 ± 0.09	0.18 ± 0.40	0.12 ± 0.27	0.22 ± 0.15	0.57 ± 0.47*
Deletion					
Single bp	2 (8.3)	0	1 (5.9)	7 (8.3)	41 (15.2)
	0.04 ± 0.05	0	0.01 ± 0.03	0.25 ± 0.35	0.80 ± 0.32*
≥ 2bps	1 (4.2)	0	0	0	13 (4.8)
	0.01 ± 0.03	0	0	0	0.18 ± 0.17
Insertion					
Single bp	0	0	0	6 (7.1)	10 (3.7)
	0	0	0	0.21 ± 0.30	0.16 ± 0.23
Total	24 (100)	11 (100)	17 (100)	84 (100)	270 (100)
	0.54 ± 0.19	0.63 ± 0.23	1.06 ± 0.51	2.11 ± 0.68**	5.87 ± 2.81**

[a] Number of colonies with independent mutations (%).

[b] Mutation frequency, $\times 10^{-5}$.

Significantly different from the control group (0 ppm) at *p< 0.05, **p< 0.001, respectively.

Table 4. Spi− mutant frequencies in the livers of F344 *gpt* delta transgenic rats administered IQ for 4 weeks

IQ (ppm)	Rat No.	Plaques within XL-1 Blue MRA	Plaques within XL-1 Blue MRA (P2)	Mutant frequency ($\times 10^{-5}$)	Average mutant frequency ($\times 10^{-5}$)
0	111	520000	4	0.77	
	112	545000	3	0.55	
	113	445000	2	0.45	
	114	737500	1	0.14	
	115	662000	9	1.36	0.65 ± 0.46
0.1	211	500000	1	0.20	
	212	102500	1	0.98	
	213	152000	2	1.32	
	214	361000	1	0.28	
	215	180500	2	1.11	0.78 ± 0.51
1.0	311	204500	2	0.98	
	312	688000	3	0.44	
	313	175000	2	1.14	
	314	220000	1	0.45	
	315	350000	1	0.29	0.66 ± 0.37
10	411	265500	4	1.51	
	412	595500	7	1.18	
	413	500000	3	0.60	
	414	265500	26	9.79	
	415	613000	7	1.14	2.84 ± 3.9
100	511	696500	10	1.44	
	512	135500	48	35.42	
	513	468500	35	7.47	
	514	234500	63	26.87	
	515	418500	36	8.60	15.96 ± 14.45*

Significantly different from the control group (0 ppm) at *$p < 0.001$.

Table 5. NOEL values and BMD modeling results for various endpoints

Endpoint	NOEL (ppm) (PoD)	BMD (ppm)		
Present 4-week study in F344 *gpt* delta transgenic rats				
		$BMDL_{50}$ (PoD)	BMD_{50}	$BMDU_{50}$
gpt mutation (total)	1	0.04	0.14	0.90
Spi− mutation	10	0.09	1.38	9.64
G:C to T:A transversion	0.1*	0.03*	0.07	0.52
		$BMDL_{10}$ (PoD)	BMD_{10}	$BMDU_{10}$
GST-P positive foci	10	0.29	16.53	50.10
Previous 4-week study in wild-type F344 rats (Ref. #13) [a]				
		$BMDL_{50}$ (PoD)	BMD_{50}	$BMDU_{50}$
IQ-DNA adduct	0.01[b]	9.1E-03	2.1E-02	4.1E-02
Previous 16-week study in wild-type F344 rats (Ref. #13) [a]				
		$BMDL_{10}$ (PoD)	BMD_{10}	$BMDU_{10}$
GST-P positive foci	1	1.40	3.47	3.62

* Only data from the G:C to T:A transversions was used as the PoDs for mutations.
[a] Data from the previous 16-week study in wild-type F344 rats are summarized in the supplementary Table 1.
[b] The levels of IQ-DNA adduct in the livers of the 0 and 0.001 ppm groups were under the detectable limit. For NOEL and BMD analyses, we used values of the limit of detection (1 adduct/10^9 nucleotides) for the 0 and 0.001 ppm groups. IQ-DNA adducts were significantly increased in the livers of rats administered IQ at 0.01 ppm and above compared to the control (0 ppm) group.

Supplementary Table 1 IQ-DNA adduct levels in liver DNA and GST-P positive foci in the livers in the previous 16-week study in wild-type F344 rats (Wei, M. et al., 2011, Ref. 13) [a]

Group	IQ (ppm)	No. of rats	Adduct level (x 10^{-7} nucleotides)	No. of rats	No, of GST-P positive foci (No. /cm^2)
1	0	3	0.01[b]	240	0.15 ± 0.31
2	0.001	3	0.01[b]	240	0.16 ± 0.31
3	0.01	3	0.045 ± 0.020*	240	0.26 ± 1.30
4	0.1	3	0.104 ± 0.004*	240	0.15 ± 0.35
5	1	3	1.74 ± 0.46*	240	0.14 ± 0.33
6	10	3	12.72 ± 3.37*	240	0.74 ± 0.88*
7	100	3	107.09 ± 22.77*	120	88.03 ± 50.41*

[a] Typing mistakes in the number of rats analyzed and the SD values of IQ-DNA adduct levels in the 1, 10, and 100 ppm groups in Wei. M. et al., 2011, have been corrected in the present table.
[b] Detection limit.
* Significantly different from group 1.