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Mutagenicity of 2-amino-3-methylimidazo[4,5-*f*]quinoline in colon and liver of Big Blue rats: role of DNA adducts, strand breaks, DNA repair and oxidative stress

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The contribution of oxidative stress, different types of DNA damage and expression of DNA repair enzymes in colon and liver mutagenesis induced by 2-amino-3-methylimidazo [4,5-*f*]quinoline (IQ) was investigated in four groups of six Big Blue rats fed diets with 0, 20, 70, and 200 mg IQ/kg for 3 weeks. There were dose-response relationships of DNA adducts (^{32}P-postlabeling) and DNA strand breaks (comet assay) in colon and liver tissues, with the highest levels of DNA adducts and strand breaks in the colon. There was dose-dependent induction of mutations in both the colon and the liver, and the same IQ dose produced two-fold more *cII* mutations in the liver compared with the colon. The IQ-induced mutation spectrum in the colon was not significantly different to that of control rats. The expression of *ERCC1* and *OGG1* was higher in the colon than liver, and was unaffected by the IQ diet. Investigations of oxidative stress biomarkers produced inconclusive results. Oxidative DNA damage detected by the endonuclease III enzyme and 7-hydro-8-oxo-2′-deoxyguanosine in colon, liver and/or urine was unaltered by IQ. However, there was increased level of γ-glutamyl semialdehyde in liver proteins, indicating a higher rate of protein oxidation in the liver following IQ administration. In plasma and erythrocytes there were unaltered levels of oxidized protein, malondialdehyde, and antioxidant enzyme activities (superoxide dismutase, glutathione peroxidase, catalase, glutathione reductase) indicating no systemic oxidative stress. However, the level of total vitamin C was increased in plasma, with the largest fraction being in the reduced form. In conclusion, our results indicate that DNA adducts rather than oxidative stress are responsible for the initiation of IQ-induced carcinogenesis of the liver and colon. A lower frequency of mutations in the colon than in the liver could be related to higher expression of DNA repair enzymes in the former.

Abbreviations: AAS, 2-amino adipic semialdehyde; CAT, catalase; GGS, γ-glutamyl semialdehyde; GR, glutathione reductase; GPx, glutathione peroxidase; HAA, heterocyclic aromatic amine; HPLC, high pressure liquid chromatography; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; 8-oxodG, 7-hydro-8-oxo-2′-deoxyguanosine; ROS, reactive oxygen species, RBC, red blood cells, SOD, superoxide dismutase; WBC, white blood cells.

Introduction

2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) is a carcinogenic heterocyclic aromatic amine (HAA) which may be formed during cooking of meat and fish as a condensation product of creatine (or the cyclic form, creatinine) with amino acids and proteins (1). IQ and other HAAs are among the most potent mutagens in the Ames test, and are carcinogenic in several target organs in long-term rodent feeding bioassays (2). IQ is the most potent carcinogen of the tested HAAs with evidence for carcinogenicity in the liver, small and large intestine, Zymbal gland, and the skin of male rats (3,4).

It is widely accepted that the HAAs are carcinogenic by forming adducts between the metabolically activated metabolites and DNA (5). Recent results indicate that the carcinogenic action of HAAs and other mutagens such as aflatoxin, benzo[*a*]pyrene, and nitrosamines also may involve reactive oxygen species (ROS) or a general oxidative stress situation (6). ROS are thought to be involved in the carcinogenic process, although it is not clear whether it is a primary cause of cancer or a secondary effect (7). For instance, it has been shown that metabolism of HAAs by NADPH-cytochrome P-450 reductase or cytochrome b5 reductase *in vitro* results in generation of superoxide radicals, with 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and IQ showing the highest level (8,9).

The direct consequence of ROS interaction with DNA is the generation of oxidative DNA damage, such as 7-hydro-8-oxo-2′-deoxyguanosine (8-oxodG). It has been shown that 8-oxodG is a mutagenic DNA lesion in mammalian cells (10,11), indicating that it may be a relevant premutagenic DNA lesion in relation to initiation of cancer. Increased 8-oxodG level has been observed in the liver of rats fed with MeIQx for 1 week, whereas similar levels of 8-oxodG in MeIQx-treated and control rats have been reported in 6-weeks feeding experiments (12,13). In rat feeding studies naturally occurring and synthetic antioxidants inhibit the formation of IQ-induced preneoplastic (placental GST-P positive) hepatic foci (14,15), and hepatocarcinogenesis in rats (16). Hence the available evidence from previous publications suggests that oxidative stress is not a mechanism in the initiation of HAA-induced liver carcinogenesis, even though antioxidants appear to have a protective effect. It has been suggested that the effect of antioxidants is likely to be chemopreventive in the liver because of their ability to block metabolism of HAAs (17).

Theoretically DNA lesions are turned into mutations *in vivo* if they are not removed from the genome by DNA repair. Usually, most of the DNA damage is rapidly repaired and only a very small fraction results in mutations. ERCC1 plays an essential role in the nucleotide excision repair process as a part of the 5′-incision complex (18). The base excision repair enzyme, OGG1, removes mainly oxidized guanine from the DNA (19). Recently, expression of several base excision repair genes, including *OGG1*, was shown to be upregulated in the liver of rats and mice by administration of a carcinogenic

peroxisome proliferator (20). Accordingly, changes in expression of DNA repair enzymes may modify the mutagenic effect of HAAs.

The aim of this study was to investigate the role of the different types of DNA damage and repair in the genotoxicity and mutagenicity *in vivo* of orally administered IQ. To this end IQ-dosed Big Blue rats were studied by means of a panel of biomarkers detecting oxidative stress, DNA damage, expression of DNA repair enzymes and mutations in colon and liver. Markers of DNA damage included strand breakage, bulky adducts and 8-oxodG. Oxidative stress markers from target organs and in the blood included oxidative protein modifications at proline and arginine (GGS), and at lysine (AAS) sites, vitamin C oxidation, and the activity of antioxidant enzymes.

Materials and methods

Animals and exposure protocol

Twenty-four male Big Blue (Fischer) rats, ~8 weeks of age (50 g) (Stratagene, AH Diagnostics, Århus, Denmark), were acclimatized for 7 days while maintained on Altromin pellets (Lage, Germany). The animals were assigned to four groups of six animals, receiving 0, 20, 70 or 200 mg/kg IQ in an Altromin diet. IQ was purchased from Toronto Research Chemicals, North York, Ont., Canada, and mixed with Altromin 2110 standard rat feed (Lage, Germany). Animals were kept in a 12 h light and 12 h dark cycle at an average temperature of 22°C and at 55% humidity. The diets were administered for a total of 21 days. Throughout the study the animals had free access to the diet and water and their food consumption was assessed daily. A 24 h urine sample was collected on day 20 and stored at –80°C. At day 21, all rats were healthy, and each animal was anesthetized with carbon dioxide/oxygen, decapitated and blood was collected. Liver and colon were removed, weighed and submerged in liquid nitrogen until storage at –80°C. The heparinized blood samples were separated into plasma, white blood cells (WBC) and red blood cells (RBC) by centrifugation on Ficoll Paque. The washed RBC, WBC and plasma were also stored at –80°C.

Determination of oxidized amino acids, malondialdehyde, and antioxidant enzyme activity

The preparation of cytosolic proteins from the liver by tissue homogenization and centrifugation was carried out as described by Kim and Lee (21). Oxidized protein (AAS and GGS) levels in proteins were analyzed as described by Daneshvar *et al.* (22). Antioxidant enzyme activities were determined by automated assays for superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and glutathione reductase (GR) in blood hemolysate, performed on a Cobas Mira analyzer. SOD and hemoglobin were determined using commercially available kits (Randox). GR was determined by the method of Goldberg and Spooner (23). GPx and CAT activity were determined according to the methods described by Wheeler *et al.* (24). The enzymatic activities in red blood cells were calculated relative to the amount of hemoglobin. Total malondialdehyde in plasma was determined by high pressure liquid chromatography (HPLC) as described by Lauridsen and Mortensen (25).

Determination of vitamin C status

The level of ascorbic acid and dihydroascorbic acid was determined in the liver (mM units) and plasma (μM units) as described previously (26).

Detection of DNA strand breaks, endonuclease III sensitive sites by the alkaline comet assay, and 8-oxodG by HPLC

The isolation of liver cells was essentially carried out as described previously (27). Cells from the colon were scraped off the mucosa with a glass microscope slide and placed in ice-cold Merchant-EDTA solution (0.14 M NaCl, 1.47 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 10 mM Na_2EDTA, pH 7.4) (28). The detection of DNA strand breaks was carried out as described previously (27). The level of endonuclease III sensitive sites was obtained as the difference in scores of parallel slides incubated with and without endonuclease III enzymes at 37°C for 45 min (endonuclease III enzyme was a kind gift from Serge Boiteaux, UMR217 Centre National de la Recherche Scientifique et Commissariat a l'Energie Atomique, France). A total of 50 nuclei was scored for each sample, using the Kinetics Imaging Limited version 4, Liverpool, UK software system to determine the amount of DNA migrated from the comet head to the tail.

The level of 8-oxodG in nuclear DNA from colon and tissue was analyzed as described elsewhere (29). In urine, 8-oxodG was detected by HPLC-tamdem mass spectroscopy (30).

^{32}P-postlabeling

DNA was extracted by standard phenol/chloroform extraction procedure, and the ^{32}P-postlabeling assay was carried out as described previously, using butanol extraction as enrichment procedure (31). A standard consisting of *in vitro* benzo[*a*]pyrene-diol-epoxide modified calf thymus DNA was used to correct for day to day variation in the assay. The results are expressed as adducts/10^8 nucleotides, based on the mean of two independent assays.

Quantification of ERCC1 and OGG1 mRNA levels

Total RNA was purified from 10 mg liver or from 5×10^6 colon cells using a Qiagen total RNA purification kit as recommended by the manufacturer. The RNA was DNase treated as recommended by Qiagen. Quality control showed that all genomic contaminations were removed by the DNAse treatment. The integrity of the RNA was checked by gel electrophoresis. Two hundred ng RNA was used for cDNA synthesis in a reaction volume of 10 μl using the Taqman Gold RT-PCR kit as recommended by Applied Biosystems. For quantification of the mRNA levels Taqman probes were used. For *ERCC1* the following oligonucleotides were used: forward primer: 53F: 5′-CCT GGG AAG GAC GAG GAA A-3′, reverse primer: 121R: 5′-TGG GAT AAC AAA CTT CTT CCT GGT-3′, taqman probe 74T: 5′-FAM-CGG CCA CAG CCC TCA GGA CC-Tamra-3′ (TAGCopenhagen, Denmark). For *OGG1* the following oligonucleotides were used: taqman probe: 5′-FAM-TCA TGC CCT GGC TGG TCC AGA AG-TAMRA-'3, forward primer: 5′-ACT TAT CAT GGC TTC CCA AAC C-'3, reverse primer: 5′-CAA CTT CCT GAG GTG GGT CTC T-'3.

The PCR reactions were performed in duplicate or triplicate in the ABI 7700 Sequence Detection System in 15 μl reactions containing 200 nM primers, 300 nM or 150 nM Taqman probe for *ERCC1* and *OGG1*, respectively, 0.1–0.5 μl cDNA in 1 × Mastermix (Applied Biosystems). For normalization 18↑S was quantified in a separate PCR reaction (Applied Biosystems) in duplicate or triplicate. For each animal the average value of *ERCC1* quantifications was divided by the average value of the 18↑S quantifications.

The signal from *ERCC1, OGG1* and 18↑S were linear over 100-fold dilution. Likewise, normalization of the mRNA signals to 18↑S yielded the same signal over 100-fold dilution. The variation coefficient was 18% on repeated measurements of the same sample (mean 13.7×10^{-6}, and standard deviation 2.5×10^{-6}). The variation coefficient of triplicates was on the average 15%.

Mutation frequency

About 20 million cells, isolated as described for the comet assay, were filtered through a cell strainer (Falcon, Becton Dickinson, Franklin Lakes, New Jersey, USA) and DNA were purified by the RecoverEase™ DNA isolation kit (Stratagene. La Jolla, USA). DNA from about 60 mg frozen liver was prepared by the RecoverEase™ as described (Stratagene, La Jolla, USA).

Eight microliters DNA preparation was packaged with Transpack packaging extract (Stratagene, La Jolla, USA). If the packaging mixture was viscous after the recommended standard packaging time of 180 min, the mixture was incubated for another 60 min. If the mixture was still viscous after this time additional Transpack reagents were added and the mixture was incubated for another 60 min. This phage preparation was used to infect *E. coli* G1250 (*hfl*$^-$). Phages with mutations at the *cII* locus were identified by plaque formation under selective growth conditions at 24°C and the total number of infective phages was determined by plaque formation under unselective growth conditions at 37°C as described (λ Select-cII™ Mutation Detection System for the Big Blue Rodents, Stratagene, La Jolla, USA).

The c*II* mutations from the rats that had received 0 and 200 mg IQ/kg, were sequenced principally as described by Jakubczak *et al.* (32). For generating sequencing templates the primers used were 5′-CCG CTC TTA CAC ATT CCA GC-3′ and 5′-CCT CTG CCG AAG TTG AGT AT-3′. All templates were sequenced with the primer 5′-CCA CAC CTA TGG TGT ATG-3′. Some of the templates were also sequenced with the primer 5′-CTC CTG TTG ATA GAT CCA G-3′ for resolving a few ambiguities.

Statistics

The data were tested for homogeneity of the variance within dose groups using Levene's test and for normality using Shapiro–Wilk's W test. Log-transformations, using the base of 10, were performed for the biomarkers, which did not have homogeneity of variance. The biomarkers that fulfilled the criteria of variance homogeneity were analyzed by post hoc ANOVA analysis as the least square difference, using 5% level as significance level. For three of the biomarkers (DNA adducts in colon and liver AAS and GGS level) log transformations did not result in homogeneity of variation, and these data sets were analyzed by the nonparametric Kruskal–Wallis test. The statistical analysis was performed in Statistica 5.0 for Windows, StatSoft (1997), Tulsa, OK, USA).

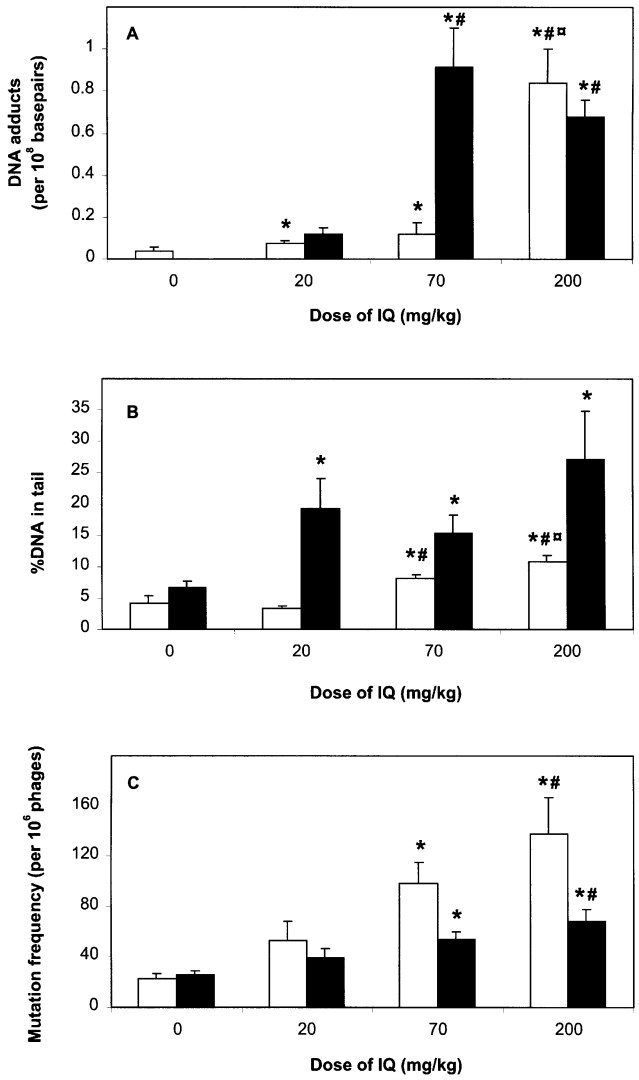

Fig. 1. DNA damage and mutations in the liver (open bars) and colon (solid bars) of Big Blue rats given IQ in the feed for 3 weeks. (**A**) DNA adducts by the ^{32}P postlabeling assay; (**B**) DNA strand breaks by the comet assay; and (**C**) mutation frequency at the *cII* transgene. *statistically significant from control. #Statistically significant from 20 mg/kg *statistically significant from 70 mg/kg. Bars represent the means and SEM of six experiments.

Results

All of the animals were healthy at the end of the 21-day feeding period with no signs of toxicity. The rats fed the highest dose of IQ gained weight more slowly during the three week period than the other groups (i.e. 35 g for the 200 mg IQ/kg feed group compared with 46–53 g for the other groups) and consumed less feed (i.e. 119 g for the 200 mg IQ/kg feed group compared with 134–135 g for the other groups). The final body weights of the rats and their liver weights did not differ between the groups (means and standard deviations for liver weights were 10.9 ± 1.2, 11.2 ± 0.6, 11.6 ± 1.0, 10.7 ± 1.5 for 0, 20, 70, and 200 mg IQ, respectively). The average dose of IQ was 9, 32 and 79 mg IQ, ↑kg feed for the rats receiving 20, 70 and 200 mg IQ/kg feed, respectively, based on the weight of the rats at day 21.

Figure 1 depicts the dose-response relationship of DNA adducts, DNA strand breaks, and mutations in the colon mucosa and liver. When the rats were killed after 21 days on a diet containing IQ, significantly more DNA adducts were detected by the ^{32}P-postlabeling assay at 70 mg/kg and 200 mg/kg in the colon and liver, respectively. DNA strand breaks were induced significantly at 20 and 70 mg IQ/kg in the colon and liver, respectively. The level of strand breaks and DNA adducts were increased more and at lower doses in the colon compared with liver, whereas the level of mutations was increased more in the liver than in the colon ($P < 0.05$ in multifactorial ANOVA). There were dose-dependent increases in the mutation frequencies in both the colon and liver, which attained statistical significance at 70 mg/kg in both organs. Table I outlines the mutations induced by 200 mg IQ/kg in the colon. There was a tendency of more G:C→T:A transversions in the rats fed with 200 mg IQ/kg compared with the controls (21% to 30%) and fewer G:C→A:T transitions (31% to 20%), but the difference was not statistically significant (Table II).

The level of 8-oxodG in the urine, and endonuclease III sensitive sites and 8-oxodG in the colon mucosa and liver cells were not significantly changed by the IQ administration (Table III). The expression of DNA repair genes, determined by quantification of the mRNA level of *ERCC1* and *OGG1*, was not significantly changed by IQ in colon cells and liver tissue (Table IV). Exclusion of one outlier causing the high mean level of OGG1 after IQ 70 mg/kg feed did not change the statistical test (mean and SD after excluding the outlier was 55 ± 28, $P = 0.40$).

At increasing IQ doses the concentration of total vitamin C increased in the liver ($r = 0.42$, $P < 0.05$, linear regression) and plasma ($r = 0.47$, $P < 0.05$, linear regression). The major fraction of total vitamin C was ascorbic acid, and there was no effect seen on the oxidized form, dihydroascorbic acid (Table V). The malondialdehyde concentration in plasma was unchanged (Table V). In the liver, IQ administration was associated with increased levels of GGS, whereas AAS was unaffected in both liver and plasma (Table VI). No effect of IQ on the activity of antioxidant enzymes in RBC was detected (Table VII).

Discussion

The present study demonstrated that IQ induced more DNA adducts and strand breaks in colon than in the liver whereas the opposite was found with for the mutation frequencies. However, there was no increase in oxidative DNA damage, and the mutation spectra in colon cells were compatible with bulky adducts to guanine in DNA.

Previous studies have indicated that the relationship between tumorigenicity and IQ-induced DNA adducts in rat colon and liver is complex (33). Also, there were no direct correlations between DNA adducts, mutation frequency and cancer incidence in different organs of MeIQ-treated Big Blue mice (34,35). The lack of such simple relationships suggests a more complex manner in which HAA is carcinogenic. The present study could not support involvement of oxidative DNA damage in the effects of HAA as suggested by earlier findings (12).

The role of DNA repair processes was investigated by mRNA expression of *ERCC1* and *OGG1* in colon and liver tissue. The ERCC1 protein is involved in the nucleotide excision repair process and OGG1 is a base excision repair enzyme that excises oxidative DNA lesions from the DNA strand. Some studies have indicated that exposure to ROS

Table I. Mutations in the *cII* gene of phages rescued from colon cells of Big Blue rats fed Altromin diets with or without IQ

	Position	Mutation	Sequence	Amino acid change
Altromin				
Control rat 1	3	G→T	AT**G**→AT**T**	Met→Ile
	25	G→T	**G**AG→**T**AG	Glu→Stop
	34	C→T	**C**GA→**T**GA	Arg→Stop
	89	C→T	G**C**G→G**T**G	Ala→Val
	179	G→C	T**G**G→T**C**G	Trp→Ser
	179	Inds. G	1 of 6	–
	179	Inds. G	1 of 6	–
	179	Inds. G	1 of 6	–
	212	C→T	G**C**G→G**T**G	Ala→Val
	223	G→A	**G**CT→**A**CT	Ala→Thr
	241	Del A	1 of 6	–
	241	Del A	1 of 6	–
Control rat 2	-13	G→A	AG**G**→AG**A**	Arg→Arg
	24	C→G	AA**C**→AA**G**	Asn→Lys
	25	G→A	**G**AG→**A**AG	Glu→Arg
	35	G→A	C**G**A→C**A**A	Arg→Gln
	64	G→A	**G**CA→**A**CA	Ala→Thr
	160	C→G	**C**TG→**G**TG	Leu→Val
	179	Inds. G	1 of 6	–
Control rat 3		Not found		
	29	C→A	G**C**T→G**A**T	Ala→Asp
	34	C→T	**C**GA→**T**GA	Arg→Stop
	47	C→G	G**C**G→G**G**G	Ala→Gly
	65	C→G	G**C**A→G**G**A	Ala→Gly
	179	Inds. G	1 of 6	–
	241	Del A	1 of 6	–
Control rat 4	4	G→C	**G**TT→**C**TT	Val→Leu
	40	G→A	**G**AG→**A**AG	Glu→Lys
	62	T→C	A**T**C→A**C**C	Arg→Thr
	103	G→A	**G**TT→**A**TT	Vla→Ile
	128	G→T	T**G**G→T**T**G	Trp→Leu
	184	G→C	**G**TC→**C**TC	Val→Leu
	214	C→T	**C**GA→**T**GA	Arg→Stop
Control rat 5	-3	C→G	**C**AT→**G**AT	His→Asp
	179	Inds. G	1 of 6	–
	179	Inds. G	1 of 6	–
	179	Del G	1 of 6	–
	214	C→T	**C**GA→**T**GA	Arg→Stop
	241	Del A	1 of 6	–
	241	Del A	1 of 6	–
	248	G→C	C**G**C→C**C**C	Arg→Pro
Control rat 6	28	G→T	**G**CT→**T**CT	Ala→Ser
	29	C→G	G**C**T→G**G**T	Ala→Gly
	57	C→A	AA**C**→AA**A**	Asn→Lys
	74	G→T	G**G**A→G**T**A	Gly→Val
	74	G→T	G**G**A→G**T**A	Gly→Val
	74	G→T	G**G**A→G**T**A	Gly→Val
	103	G→A	**G**TT→**A**TT	Val→Ile
	150	G→T	AA**G**→AA**T**	Lys→Asn
	155	C→G	T**C**A→T**G**A	Ser→Stop
	193	G→A	**G**AC→**A**AC	Asp→Asn
200 mg IQ/kg Altromin				
IQ rat 1	2	T→C	A**T**G→A**C**G	Met→Thr
	28	G→C	**G**CT→**C**CT	Ala→Pro
	64	G→A	**G**CA→**A**CA	Ala→Thr
	67	A→G	**A**TG→**G**TG	Met→Val
	193	G→T	**G**AC→**T**AC	Asp→Tyr
	215	G→C	C**G**A→C**C**A	Arg→Pro
	280	Del C	**C**AG	–
IQ rat 2	–14	G→T	A**G**G→A**T**G	Arg→Met
	29	C→T	G**C**T→G**T**T	Ala→Val
	47	C→G	G**C**G→G**G**G	Ala→Gly
	101	G→C	G**G**C→G**C**C	Gly→Ala
	113	C→T	T**C**G→T**T**G	Ser→Leu
	179	G→C	T**G**G→T**C**G	Trp→Ser
	212	C→T	G**C**G→G**T**G	Ala→Val

Table I. Continued.

IQ rat 3	25	G→T	**G**AG→**T**AG	Glu→Stop
	106	Del G	**G**AT	–
	179	G→C	T**G**G→T**C**G	Trp→Ser
	193	G→A	**G**AC→**A**AC	Asp→Thr
	212	C→A	G**C**G→G**A**G	Ala→Glu
	214	C→T	**C**GA→**T**GA	Arg→Stop
IQ rat 4	34	C→ T	**C**GA→**T**GA	Arg→Stop
	88	G→T	**G**CG→**T**CG	Ala→Ser
	89	C→A	G**C**G→G**A**G	Ala→Glu
	179	G→T	T**G**G→T**T**G	Trp→Leu
	179	Inds. G	1 of 6	–
	186	Del C	GT**C**	–
	212	C→A	G**C**G→G**A**G	Ala→Glu
IQ rat 5	28	G→T	G**C**T→**T**CT	Ala→Ser
	29	C→A	G**C**T→G**A**T	Leu→Val
	40	G→A	**G**AG→**A**AG	Glu→Lys
	179	Del G	1 of 6	–
	179	Inds. G	1 of 6	–
	185	T→G	G**T**C→G**G**C	Val→Gly
	202	G→C	**G**CT→**C**CT	Ala→Pro
IQ rat 6	-18	C→G	**C**TA→**G**TA	Leu→Val
	34	C→G	**C**GA→**G**GA	Arg→Gly
	64	G→T	**G**CA→**T**CA	Ala→Ser
	117	G→C	CA**G**→CA**C**	Gln→His
	119	T→C	A**T**C→A**C**C	Ile→Thr
	214	C→T	**C**GA→**T**GA	Arg→Stop

Table II. Number and type of mutations in phages isolated from colons of rats fed 200 mg IQ/kg feed and control feed

Type of mutation		Control	IQ (200 mg/kg feed)
Transition	GC→AT	15 (31%)	8 (20%)
	A:T→G:C	1 (2%)	3 (7.5%)
Transversion	G:C→T:A	10 (21%)	12 (30%)
	G:C→C:G	10 (21%)	10 (25%)
	A:T→T:A	0	0
	T:A→C:G	0	1 (2.5%)
Insertion	7 (15%)	2 (5%)	
Deletion	5 (10%)	4 (10%)	
	Total	40	50

generating agents, e.g. peroxisome proliferators and diesel exhaust particles can upregulate the base excision repair enzyme expression in rodent tissues (20,36). To the best of our knowledge expression of *ERCC1* mRNA has not been investigated in animals exposed to DNA damaging agents. In the present study IQ did not increase the expression of *ERCC1* or *OGG1*, although modest increases in colon cells at mid dose levels could not be excluded due to variation in the data. However a lack of *OGG1* mRNA expression seems reasonable considering that there was no change in 8-oxodG in nuclear DNA or in the urine, the latter representing the sum of 8-oxodG repair, sanitation of the nucleotide pool, cell turnover and mitochondrial turnover in the whole body (37). The unaffected expression of *ERCC1* suggests that the NER system is operating at sufficient capacity in the colon and liver of rats, or that IQ does not induce DNA repair. The higher level of DNA strand breaks in the colon may reflect transient breaks that are generated by the excision repair enzymes, or direct DNA damage. Previously, it has been reported that feeding of MeIQ for 13 days produced more DNA strand breaks in the colon than in the liver of rats (38). Interestingly, the mRNA expression of the DNA repair enzymes was several times higher in the colon cells than in the liver. This difference

Table III. Level of oxidative DNA damage

Dose of IQ (mg/kg feed)	0	20	70	200
8-oxodG (urine)[a]	133.8 ± 30.7 (6)	138.5 ± 25.2 (6)	126.9 ± 20.1 (6)	122.9 ± 31.5 (6)
8-oxodG (colon)[a]	0.55 ± 0.20 (6)	0.48 ± 0.09 (6)	0.50 ± 0.26 (5)	0.41 ± 0.20 (6)
8-oxodG (liver)[a]	0.30 ± 0.14 (6)	0.38 ± 0.06 (6)	0.31 ± 0.14 (6)	0.42 ± 0.17 (6)
EndoIII sites (colon)[b]	2.8 ± 2.0 (6)	1.5 ± 4.8 (6)	–2.7 ± 4.3 (6)	1.8 ± 7.0 (6)
EndoIII sites (liver)[b]	2.4 ± 2.7 (6)	1.3 ± 1.2 (6)	1.5 ± 2.0 (6)	1.1 ± 1.9 (6)

[a]8-oxodG/10^5 dG ratio.
[b]% DNA in tail (endoIII sites, the difference in score between slides treated with and without endonuclease III enzyme). Data are the mean and standard deviation. The number of determinations is shown in parentheses.

Table IV. Level of mRNA of the DNA repair genes *ERCC1* and *OGG1* in liver and colon cells normalized to 18↑S ribosomal RNA ($\times 10^{-6}$)[a]

Dose of IQ (mg/kg feed)	0	20	70	200
Relative ERCC1 level (liver)	7.62 ± 3.19 (6)	5.67 ± 3.94 (6)	6.5 ± 2.63 (6)	6.68 ± 2.88 (6)
Relative OGG1 level (liver)	6.1 ± 1.4 (6)	5.7 ± 1.2 (6)	5.6 ± 0.8 (6)	7.6 ± 8.9 (6)
Relative ERCC1 level (colon)	11.81 ± 3.69 (6)	18.65 ± 20.9 (6)	19.9 ± 11.25 (6)	6.88 ± 4.3 (5)
Relative OGG1 level (colon)	39.6 ± 19.3 (6)	48.4 ± 14.2 (6)	94.2 ± 99.3 (6)	33.7 ± 24.8 (6)

[a]Data are the mean and standard deviation. The number of determinations is shown in parentheses.

Table V. Levels of vitamin C in liver and plasma

Dose of IQ (mg/kg feed)	0	20	70	200
Total vitamin C (liver) (mM)	2.10 ± 0.6 (6)	2.39 ± 0.2 (5)	2.54 ± 0.8 (6)	2.80 ± 0.4 (5)
DHAA (liver) (mM)	0.05 ± 0.1 (6)	0.03 ± 0.11 (6)	0.02 ± 0.10 (6)	0.05 ± 0.09 (6)
Total vitamin C (plasma) (μM)	14.2 ± 3.9 (6)	26.2 ± 12.4 (5)	31.5 ± 17.7* (6)	40.0 ± 27.1** (5)
DHAA (plasma) (μM)	3.9 ± 1.0 (6)	1.8 ± 2.9 (6)	0.4 ± 3.5 (6)	0.7 ± 2.1 (6)

Data are the mean and standard deviation. The number of determinations is indicated in parentheses. *$P < 0.05$, **$P < 0.01$. DHAA: Dihydroascorbic acid.

Table VI. Oxidative damage to lipids and proteins

Dose of IQ (mg/kg feed)	0	20	70	200
MDA (plasma)[a]	322 ± 89	271 ± 45	301 ± 84	311 ± 136
AAS (plasma)[a]	139 ± 7	147 ± 7	143 ± 99	145 ± 8
GGS (liver)[a]	81.7 ± 6.2	112 ± 28	131 ± 40	117 ± 24*
AAS (liver)[a]	82.7 ± 4.8	96.0 ± 18.3	96.3 ± 24.1	88.8 ± 24.3

[a]Units are pmol/mg protein. MDA: Malondialdehyde; AAS: amino adipic semialdehyde; GGS: γ-glutamyl semialdehyde. Each group consists of six animals. *$P < 0.05$.

Table VII. Activity of antioxidant enzymes in red blood cells

Dose of IQ (mg/kg feed)	0	20	70	200
Catalase[a]	119 ± 35 (5)	98.7 ± 43.4 (6)	91.8 ± 57.0 (5)	112 ± 72 (6)
Glutathione peroxidase[a]	2.3 ± 0.6 (6)	2.4 ± 0.9 (6)	2.5 ± 1.3 (5)	3.03 ± 1.9 (6)
Glutathione reductase[a]	11.4 ± 3.1 (5)	12.5 ± 4.0 (6)	12.9 ± 6.4 (5)	18.2 ± 13.2 (6)
Superoxide dismutase[a]	15.4 ± 5.3 (6)	18.9 ± 7.6 (6)	18.8 ± 10.0 (5)	21.9 ± 13.6 (6)

[a]Units/g hemoglobin (per 10^{-3} for glutathione peroxidase and superoxide dismutase). Data are the mean and standard deviation. The number of determinations is shown in parentheses.

could contribute to explain why mutations were induced at lower levels of IQ in the colon than the liver despite the fact that strand breaks and DNA adducts were induced at lower levels of IQ in the liver than in the colon.

The mutation frequencies of IQ in our study were remarkably similar to the levels reported with the *LacI* gene and a different experimental protocol by Bol *et al.* (39) and to those reported by Davis *et al.* using the MutaMice (40). The mutations at the highest IQ dose in the colon indicated a higher frequency of G:C to T:A mutations, similar to the mutation spectrum for IQ in the liver (39). This suggests that the mechanism is similar in the colon and liver. Most of the *cII* mutations were

at nucleotides also mutated in the rats that were fed the Altromin diet or have been reported before in untreated control Big Blue mice or Mutamice in the *cII* mutant database (http:dgm1pc13.nihs.go.jp/www/cII/cIIhome.html). However, there were fewer mutations at CpG sites in the IQ treated rats (7/40) compared with the rats fed Altromin (17/51). There were no obvious hotspots in gene, except for two 2 G→A mutations at nucleotide 64; a mutation that was reported as 5 of 70 mutations induced by MeIQx in the liver of Big Blue mice in *cII* mutation database. This mutation is rare in untreated Big Blue and Mutamice, but strangely, is a common spontaneous and MeIQx mutation in tissues and tumors Big Blue/p53 knock out mice (*cII* mutation database). It has been reported that IQ induced single base deletions may be targeted to stretches of Gs (39); three of five of our deletions were at stretches of Gs. Compared with the rats fed Altromin there was no apparent increase in the base substitution mutations at GC sites (14 mutations were detected in both treatment groups). The GC sites in the *LacI* gene have been reported to be targeted for mutations in Big Blue rats given IQ (39) and Mutamice given MeIQ (41). The mutation spectrum of IQ is similar to formation of many bulky adducts at guanine in DNA (42). However, some ROS induced DNA lesions, in particular 8-oxodG generate a similar spectrum (43). It has been speculated that cancer that is caused by inflammatory conditions, which also may involve ROS related etiology, typically is associated with G to A transitions (44). Accordingly, the IQ-induced mutation spectra do not support involvement of oxidative DNA damage. Indeed, recent studies by Hirose *et al.* indicate that the chemopreventive effect of antioxidants toward hepatocarcinogenesis probably are due changes in metabolic enzymes rather than an antioxidant property (17). As for oxidative stress, we found more oxidative protein modifications in the liver following IQ administration, detected by GGS, but not ASS. Previous *in vitro* studies have shown that ROS formed by Fenton reactions generated GGS more than AAS, whereas AAS was formed by peroxidase-catalyzed protein oxidations (22). The IQ treated rats had a small increase in vitamin C in the liver. Since the content of oxidized vitamin C was unchanged by the IQ administration, this could be due to toxicity as observed in severe hepatotoxicity (45,46). Similarly, the plasma vitamin C concentration was increased up to 3-fold, whereas all the biomarkers of oxidative stress were negative, including oxidized vitamin C content, antioxidant enzyme activity, and malondialdehyde. Thus, there appears to be little evidence of oxidative stress associated with IQ in the liver or plasma of rats.

In conclusion, IQ induced more DNA adducts and strand breaks in the colon than in the liver whereas the opposite was found with respect to mutations. The mutation spectra in colon cells were similar to many agents forming bulky adducts to guanine in DNA. Although signs of increased protein oxidation were found in the liver of IQ-treated rats, there was no formation of oxidative DNA damage and unaltered repair gene expression in that target organ. Accordingly, the results do not support that ROS or oxidative stress are major factors for the mutagenic effects of IQ after a 3-week exposure.

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