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Mutagenesis of Mouse Intestine *in Vivo* Using the *Dlb-1* Specific Locus Test: Studies with 1,2-Dimethylhydrazine, Dimethylnitrosamine, and the Dietary Mutagen 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline[1]

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ABSTRACT

The ability of three model carcinogens, 1,2-dimethylhydrazine, dimethylnitrosamine, and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, to induce mutation in a novel *in vivo* assay in mouse intestine has been examined. The assay is based on mutations at the *Dlb-1* locus which determines the tissue specific pattern of expression of the binding site for the lectin *Dolichos biflorus* agglutinin. In C57BL/6J × SWR F_1 mice *Dlb-1* mutants are recognized as clones of epithelial cells not staining with a peroxidase conjugate of *D. biflorus* agglutinin. Chronic administration of 1,2-dimethylhydrazine (20 mg/kg/week s.c. for 10 weeks) induced *Dlb-1* mutants, whereas administration of a single dose did not. Similarly, chronic dimethylnitrosamine treatment p.o. (0.001% in drinking water for 8 weeks) induced *Dlb-1* mutants, but acute administration did not. In contrast, neither chronic nor acute treatment of the mice with 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline induced *Dlb-1* mutations. The activities of 1,2-dimethylhydrazine, dimethylnitrosamine, and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline in the *Dlb-1* assay more accurately reflect their carcinogenic potential than do many *in vitro* bioassays.

INTRODUCTION

Evidence from epidemiological studies that a substantial proportion of human cancer is attributable to diet and the environment (1) has stimulated considerable effort to determine the primary agents of the disease. Short-term mutagenicity tests, such as the Ames *Salmonella typhimurium* mutagenicity assay (2), have helped identify chemicals which may be carcinogenic in humans. However, there are numerous examples of compounds which, although potent carcinogens in animals, are not mutagenic or at best only weakly mutagenic in the Ames test, *e.g.*, 1,2-dimethylhydrazine and dimethylnitrosamine (3). Conversely, some chemicals that exhibit considerable mutagenicity in the Ames test are only weak carcinogens in animals.

Hydrazines are found as natural constituents of vegetables and are used in industry, agriculture, and medicine (4). 1,2-Dimethylhydrazine has been used extensively as a model carcinogen, giving rise to tumors mainly in the colon, but also in the lungs and blood vessels of mice (4). Nitrosamines have been detected in foods, in gastrointestinal contents, in blood, and in urine and are present in cigarette smoke (5). They can also be formed *in vivo* by the nitrosation of amines by nitrite, which can be derived from nitrate. Dimethylnitrosamine has been widely used as a representative model carcinogen, producing tumors of the lungs, liver, and kidneys of rodents (5). Both 1,2-dimethylhydrazine and dimethylnitrosamine are potent procarcinogens yet are negative or only weakly mutagenic in the Ames test (3). Another class of genotoxic chemical shown to be present in the environment is the heterocyclic aromatic amines (aminoazaarenes), which are formed when food is cooked. The aminoazaarenes have attracted considerable interest over the last decade since they are some of the most potent mutagens ever tested in the Ames *Salmonella* assay (6). These compounds, which include IQ,[3] MeIQ, MeIQx, and DiMeIQx have, however, given negative or at best only weakly positive results in mammalian cell based mutagenicity assays (7–9) and in specific locus mutations in mouse embryos (10). They are relatively weak carcinogens in rodents (6).

There could be many reasons for the disparity between the mutagenicity and carcinogenicity of these different classes of compounds in the various tests, including the assays applied to date having been inappropriate. The heterocyclic amines, hydrazines, and nitrosamines are largely active in inducing epithelial tumors in rodents and few *in vitro* or *in vivo* assays measure mutation using cells of epithelial origin. The carcinogenic heterocyclic amines and hydrazines have, however, been shown to induce nonspecific genotoxic damage in mouse intestine as determined by the nuclear abnormality test (11, 12). Thus an assay based on the recognition of mutagens in epithelia *in vivo* may be more relevant to these classes of mutagen.

We have recently developed an *in vivo* specific locus test in mouse intestine (13) which may be an appropriate assay for the detection of mutagens. The assay is based on recognition of mutations affecting one allele at the polymorphic *Dlb-1* locus which determines the tissue specific expression of the binding site for the lectin *Dolichos biflorus* agglutinin in the intestinal epithelium of some mouse strains. The assay has been validated in experiments with direct acting agents such as ethylnitrosourea and γ-radiation (13, 14) and is sensitive due to a large target cell population. However, the majority of dietary and environmental carcinogens require metabolic activation to exert their carcinogenic effects and we have now tested the procarcinogens 1,2-dimethylhydrazine, dimethylnitrosamine, and MeIQx in the *Dlb-1* assay as representative compounds of three of the most important classes of genotoxins.

MATERIALS AND METHODS

Chemicals. 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) was synthesized (15) and administered in aqueous solution (distilled water for oral gavage and isotonic saline for i.p. injection). NO_2-MeIQx was synthesized as described previously (16) and administered (i.p.) in a solution of dimethyl sulfoxide (30%, v/v) in water. 1,2-Dimethylhydrazine hydrochloride (Aldrich Chemical Co., Ltd., Gil-

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[3] The abbreviations used are: IQ, 2-amino-3-methyl-imidazo[4,5-*f*]quinoline; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; DiMeIQx, 2-amino-3,4,8-trimethylimidazo-[4,5-*f*]quinoxaline; NO_2-MeIQx, 2-nitro-3,8-dimethylimidazo[4,5-*f*]quinoxaline; DBA, *Dolichos biflorus* agglutinin; DBA-Px, peroxidase conjugate of DBA; DMH, 1,2-dimethylhydrazine; DMH, dimethylnitrosamine; SI, small intestine; i.g., intragastrically.

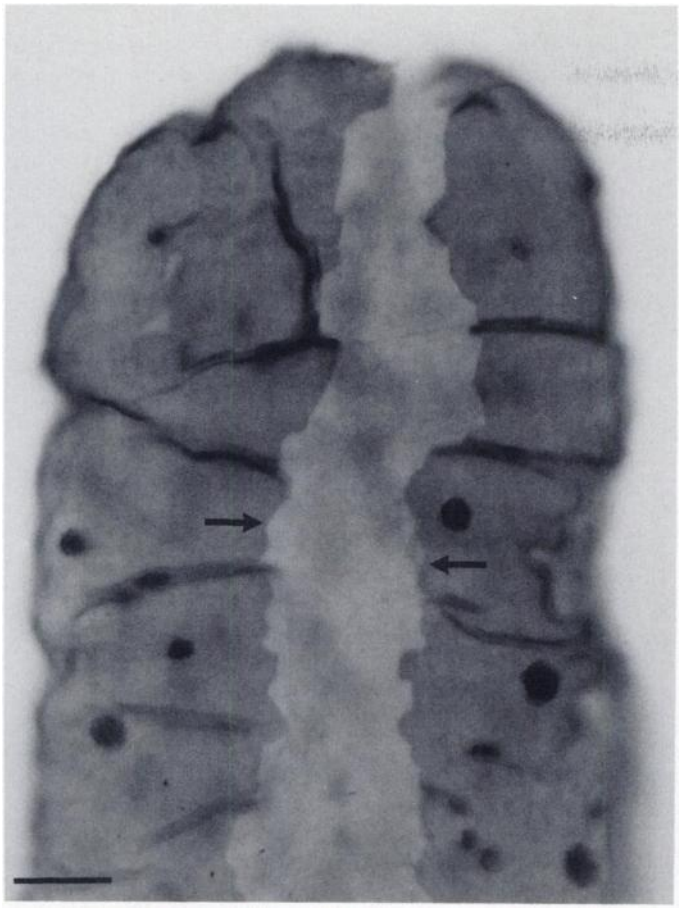

Fig. 1. An unstained ribbon of epithelial cells (*arrows*) on a villus from a whole mount of small intestine prepared from a C57BL/6J × SWR F_1 mouse stained with DBA-Px. Ribbons arise as a result of mutation affecting the *Dlb-1* locus in a crypt stem cell. See text for details. *Bar*, 0.1 mm.

lingham, Dorset, United Kingdom) was dissolved in isotonic saline containing EDTA (40 μM) adjusted to pH 6.5 and administered by s.c. injection. Dimethylnitrosamine (Sigma Chemical Co., Ltd., Poole, Dorset, United Kingdom) was dissolved in distilled or drinking (tap) water for single dose and chronic studies, respectively.

Animals. C57BL/6J × SWR F_1 mice were bred from C57BL/6J females and SWR males obtained from Olac UK, Ltd. (Bicester, Oxon, United Kingdom). All mice were maintained under standard animal house conditions of a 12-h day/night cycle. Water and diet (SDS No. 1 modified expanded diet; Special Diet Services, Essex, United Kingdom) were supplied *ad libitum*. Mice were allocated to treatment groups as shown, were between 8 and 12 weeks of age at the commencement of treatment, and were killed 12–15 days after the completion of treatment. This posttreatment period allows sufficient time for mutations in the stem cell compartment to be quantitatively recognized on the villus surface (13).

***Dlb-1* Assay.** The *Dlb-1* assay is a specific locus test for somatic mutation. The *Dlb-1* locus, on mouse chromosome 11 (17), determines the tissue specific pattern of expression of the binding site for the lectin DBA. Homozygous *Dlb-1*^{b} mice (*e.g.*, C57BL/6J) express the binding site on intestinal epithelium but not on vascular endothelium. Homozygous *Dlb-1*^{a} mice (*e.g.*, SWR) display the converse pattern of expression (18). DBA binding is codominant and in whole mounts of intestine from heterozygous *Dlb-1*^{b}/*Dlb-1*^{a} (C57BL/6J × SWR F_1) mice, mutations affecting the single *Dlb-1*^{b} allele can be recognized by white ribbons (Fig. 1) of unstained epithelia on villi in preparations stained brown with DBA-Px. Ribbons arise due to the migration of DBA-Px negative cells from small intestinal crypts in which a stem cell has been mutated at the single *Dlb-1*^{b} allele and its subsequent progeny has undergone clonal expansion in the amplifying compartments of the crypt. DBA-Px negative villus ribbons induced by treatment regimens are indistinguishable from the low background of ribbons arising by occasional spontaneous mutations in adult mice (13).

Treatment of Tissues. Animals were killed by cervical dislocation. Where killed concurrently with others in control or different dosage/treatment groups each animal was allocated a random code number and the intestinal whole mounts were scored blind. Thus only animals killed 12 weeks after the end of chronic DMH treatment and after 16 and 20 weeks of continuous treatment of DMN were not scored blind. Whole mounts of SI for the visualization of villus ribbons were prepared as described previously (13). Briefly, the SI was removed into cold phosphate buffered saline and pinned out on histological wax with the luminal (villus) side uppermost. Following fixation for 30 min in 10% formol-saline, whole mounts were washed in phosphate buffered saline and mucin was removed using dithiothreitol (19). Following overnight incubation with the DBA-Px conjugate (18) whole mount preparations were developed using 3′,3′-diaminobenzidine to visualize peroxidase as a brown reaction product (18). Whole mounts commonly underwent a second round of staining with DBA-Px before examination.

Quantification of DBA-Px Negative Events. Whole mounts were examined for DBA-Px negative villus ribbons starting at a point 25% along the length of the SI from the pylorus using a Kyowa dissecting microscope at ×45. Ribbons were recognized as continuous negative staining columns of cells running from the bottom to the top of the villi (13). Fifty fields delineated by the square field of an eyepiece graticule (Agar Aids; L5054) were scored for the presence of DBA-Px negative villus ribbons. As far as was possible the fields were contiguous. Multiple negative ribbons on adjacent villi and apparently arising from a common center were scored as a single event. Duplicate counts were made of the number of villi (usually 200–250) in the first and last graticule defined microscope field and the total number of villi scored (usually between 10,000 and 14,000) were estimated from the mean of these four observations. For each mouse the number of events scored was corrected to 10^4 villi using the formula

$$\text{Total no. of events} \times \frac{10^4}{\text{Mean no. of villi/field} \times 50}$$

This value was subsequently used to determine the means for all mice in a treatment group.

Ames *S. typhimurium* Mutagenicity Test. Bacterial mutagenicity was determined using the *S. typhimurium* test as described by Maron and Ames (2), using strain TA98. Hepatic microsomal fractions were prepared from C57BL/6J × SWR F_1 mice and were used to activate the mutagens. A 20-min preincubation of mutagen, bacteria, and hepatic microsomal fraction was used prior to plating out (2).

RESULTS

Dlb-1 Assay

1,2-Dimethylhydrazine. At single doses of 1–15 mg/kg s.c., the model colon carcinogen DMH was unable to induce a significant increase in the number of *Dlb-1* mutations (Table

Table 1 *Effects of single doses of DMH, DMN, and MeIQx on the induction of intestinal Dlb-1 mutations in female mice*

Treatment	Dose (mg/kg)	No. of mice	Total ribbons	Villi scored	Mean ± SD[a]
DMH (s.c.)	0	4	15	52,800	2.9 ± 3.4
	1	6	15	80,000	1.9 ± 1.1
	7.5	6	21	77,900	2.7 ± 0.9
	15	6	23	78,400	2.9 ± 1.7
DMN (p.o.)	10	6	30	78,000	3.9 ± 1.1
MeIQx (oral gavage)	0	10	32	127,400	2.5 ± 1.4
	20	6	15	80,800	1.8 ± 1.6
	50	5	15	67,100	2.2 ± 1.7
	100	6	25	80,300	3.2 ± 1.5

[a] Mean number of DBA-Px negative villus ribbons/mouse/10^4 villi.

Table 2 *Effect of chronic DMH treatment on the induction of intestinal Dlb-1 mutations*

All female mice treated with DMH or vehicle control (EDTA/saline). See "Materials and Methods."

Treatment	Mice	No. of mice	Time killed[a]	Total ribbons	Villi scored	Mean ± SD[b]
Control	F_1[c]	6	2	33	75,600	4.3 ± 1.3
DMH[d]	F_1	6	2	123	76,300	17.6 ± 5.2
DMH[d]	F_1	6	12	189	60,800	31.4 ± 9.4
DMH[d]	B6[e]	3	2	0	32,500	0

[a] Time (weeks) after last injection of DMH or vehicle.
[b] Mean number of DBA-Px negative villus ribbons/mouse/10^4 villi.
[c] F_1, C57BL/6J × SWR F_1.
[d] 20 mg/kg/week s.c. for 10 weeks.
[e] C57BL/6J (*Dlb-1*[b] homozygote). Due to the presence of two copies of the *Dlb-1*[b] allele, no DBA-Px negative ribbons are expected.

Table 3 *Effect of chronic DMN treatment on the induction of intestinal Dlb-1 mutations*

All mice were males.

Treatment	Time[a]	No. of mice	Total ribbons	Villi scored	Mean ± SD[b]
	8	4	25	41,900	6.0 ± 1.4
DMN[c]	8	4	71	41,500	17.6 ± 4.9
	12	4	43	48,200	8.9 ± 1.2
DMN	12	4	92	47,100	19.7 ± 1.8
DMN	16	4	134	48,900	27.4 ± 10.4
DMN	20	12	377	129,300	29.4 ± 11.3

[a] Duration of treatment in weeks.
[b] Mean number of DBA-Px negative ribbons/mouse/10^4 villi.
[c] DMN (0.001%) in drinking water.

1). However, when administered chronically, using a regimen known to induce colonic tumors in the longer term (weekly single injections of 20 mg/kg s.c.), DMH induced a significant increase in *Dlb-1* mutations. *Dlb-1* mutations induced by 10 weeks of DMH treatment persisted until at least 12 weeks after termination of treatment (Table 2). C57BL/6J (*Dlb-1*[b] homozygote) mice, treated with DMH using the same protocol, showed no DBA-Px negative ribbons (Table 2). This result is predicted due to the low probability of obtaining two independent mutations of *Dlb-1* in a single stem cell.

Dimethylnitrosamine. A single dose of DMN (10 mg/kg) administered i.g. was unable to induce a detectable increase in *Dlb-1* mutations (Table 1). Chronic treatment with DMN in the drinking water [0.001%], however, induced a significant increase in *Dlb-1* mutations by 8 weeks of treatment [17 ± 4.9 (SD) *Dlb-1* mutations/10^4 villi/mouse] as compared to age matched controls [6.0 ± 1.4 *Dlb-1* mutations/10^4 villi/mouse] [Table 3]. The increased frequency of *Dlb-1* mutations due to DMN treatment was evident at all sampling times throughout the experiment (Table 3).

MeIQx. As shown in Table 1, single doses of MeIQx (by oral gavage) up to 100 mg/kg were unable to induce a detectable increase in *Dlb-1* mutations in the small intestines of female C57BL/6J × SWR F_1 mice.

We have previously shown that MeIQx is rapidly absorbed and distributed in mice and is quickly eliminated (20) with a substantial proportion of the dose being metabolized and excreted in urine and feces within the first 24 h. We therefore treated mice with consecutive daily doses of MeIQx (20 mg/kg) to prolong the exposure period. Female mice receiving this dosage regimen for 10 days showed no increase in the frequency of *Dlb-1* mutations as compared to control animals (Table 4).

NO_2-MeIQx. MeIQx is progenotoxic and requires metabolic activation by the cytochrome P-450-dependent mixed function oxidase system before it will cause mutation of prokaryotic target populations. In contrast, the MeIQx analogue, 2-nitro-3,8-dimethylimidazo[4,5-*f*]quinoxaline (NO_2-MeIQx), is directly mutagenic in prokaryotes (21). Treatment of C57BL/6J × SWR F_1 mice with NO_2-MeIQx (20 mg/kg/day i.p.) for 7 consecutive days was also unable to induce *Dlb-1* mutations (Table 4).

Ames *S. typhimurium* Test. In view of the fact that multiple doses of MeIQx failed to induce *Dlb-1* mutations, the possibility that the C57BL/6J × SWR F_1 mice were unable to activate MeIQx to a genotoxic derivative(s) was considered. To test this, hepatic microsomes prepared from F_1 mice were used to assess the activation of MeIQx to genotoxic derivatives in the Ames test. As shown in Fig. 2, these microsomes were able to activate MeIQx to produce a dose dependent increase in the frequency of *S. typhimurium* (strain TA98) reversion. These data show that hepatic microsomes from F_1 mice are very efficient in activating MeIQx to a bacterial mutagen.

DISCUSSION

The utility of the *Dlb-1* assay for the detection of epithelial mutagens has been extended and further validated with respect to two model carcinogens which require metabolic activation, namely DMH and DMN. Although neither DMH nor DMN produced mutation after acute exposure, both caused mutations at the *Dlb-1* locus when given chronically. Interestingly, DMH and DMN are primarily tissue specific carcinogens, affecting mouse colon and lung, respectively, which indicates that mutation can occur at tissues other than the recognized tumour

Table 4 *Effect of multiple treatments of MeIQx and NO_2-MeIQx on the induction of Dlb-1 mutations*

Treatment	Time[a] (days)	No. of mice	Total ribbons	Total villi	Mean ± SD[b]
Saline	10	6	10	61,400	1.7 ± 0.9
MeIQx (20 mg/kg/day)	10	6	19	63,400	3.0 ± 1.7
DMSO/saline	7	6	18	68,500	2.7 ± 1.7
NO_2-MeIQx (20 mg/kg/day)	7	6	14	63,200	2.2 ± 1.5

[a] Days of treatment.
[b] Mean number of DBA-Px negative villus ribbons/mouse/10^4 villi.

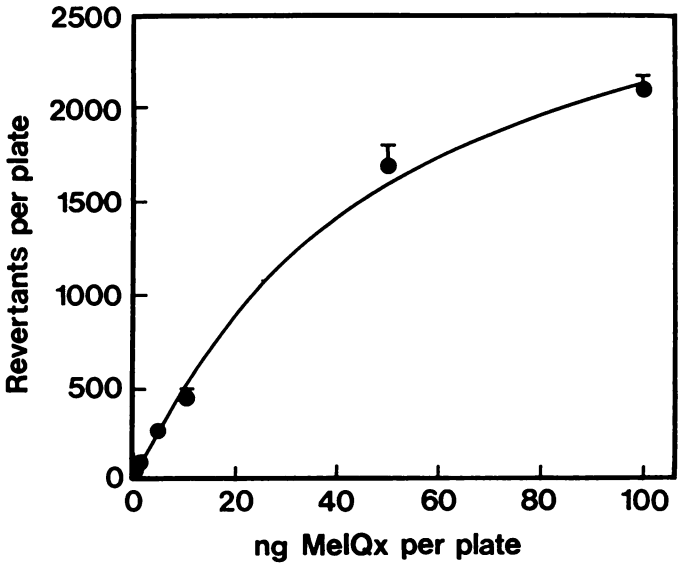

Fig. 2. Mutagenicity of MeIQx in the Ames test (*S. typhimurium* TA98). MeIQx was activated using hepatic microsomal fraction (0.5 mg/plate) from C57BL/6J × SWR F_1 mice. Values are mean ± SE (*bars*), $n = 6$. In the absence of microsomes no mutations above the spontaneous revertant rate (32 ± 1 revertant colonies) were observed.

site. In contrast MeIQx and NO_2-MeIQx were unable to induce mutations.

DMH, DMN, and MeIQx were selected for screening in the *Dlb-1* test because they give results in short term bioassays that do not accurately reflect their carcinogenicity *in vivo*. The positive results for both DMH and DMN in the *Dlb-1* assay more accurately predict the carcinogenic potential of these compounds than does their negative or weakly positive (DMH and DMN, respectively) (3) performance in prokaryote tests.

The negative result with MeIQx is more difficult to interpret inasmuch as it may not be activated to a genotoxin in C57BL/6J × SWR F_1 mice or may not be bioavailable in intestinal epithelium. However, we have previously shown that MeIQx is rapidly absorbed from the gastrointestinal tract with the small intestine exposed to high concentrations of MeIQx and/or its metabolites (20). Further, the Ames test result shows that hepatic microsomes from F_1 mice can efficiently activate MeIQx to a mutagen, indicating that F_1 mice can probably activate this chemical.

The IQ compounds are extremely potent bacterial mutagens. In mammalian cells they are much less genotoxic although they appear to be more active in assays measuring nonspecific genomic damage (7, 22, 23) than specific locus mutations (9, 10). MeIQx has been shown to be a relatively weak carcinogen in rodents (24–26) and in mice long term feeding (84 weeks) of around 11 mg/kg/day is needed to induce a 10- and 2-fold increase, respectively (25). Clearly, MeIQx does not have the carcinogenic potential indicated by its performance in prokaryote tests. Its lack of activity in the *Dlb-1* assay may more accurately reflect the true carcinogenicity of MeIQx although more prolonged treatment at the dosage level used in the chronic study might indicate some mutagenic activity of MeIQx in this assay. (The limitation in the present study was in the amount of material available.) However, it is of interest that the chemical carcinogens identified as positive in the *Dlb-1* assay to date, both direct and indirect acting, are alkylating agents whereas MeIQx is an arylating agent and confirmation of this interpretation will depend on subsequent validation of the assay with arylating agents such as methylcholanthrene and benzo(*a*)pyrene which are potent carcinogens.

In vitro assays for evaluating the genotoxicity of putative environmental carcinogens almost invariably use cells of non-epithelial origin or of well established cell lines in which many epithelial characteristics have been lost. The *Dlb-1* model may be more appropriate as a mutagenicity test because it detects mutation *in vivo* in mouse small intestine, an epithelium at direct risk from dietary mutagens. The present study illustrates the ability of this test to detect carcinogens (DMN) given p.o. The specific DNA lesions detected by the *Dlb-1* assay are not known. However, the assay gives positive results with ethylnitrosourea (13) which induces primarily point mutations and γ-radiation (14) which induces large scale genomic damage, indicating that the assay can detect a wide spectrum of DNA lesions.

The IQ compounds, including MeIQx, are found in a variety of cooked foods (27) at levels of a few ppb (28, 29), and given their extreme potency in bacterial mutagenicity assays, they represent an obvious hazard for inducing neoplasia in humans. However, the relative risk posed by these and many other compounds has yet to be determined and a short term *in vivo* assay of somatic mutation such as the *Dlb-1* assay could be of use in evaluating this risk. Possibly, the minimal activity of MeIQx and other IQ compounds in mammalian cell assays correctly reflects the relatively weak carcinogenicity of these compounds. Certainly, in validating the *Dlb-1* assay with the carcinogens DMH and DMN, the present study suggests that the lack of activity shown by MeIQx in many *in vitro* assays is not merely due to the inappropriate nature of the cellular target. Despite this weak activity, IQ compounds and other dietary and environmental constituents may induce cancer in humans after very long and continuous exposure due to their ubiquitous presence. In this case it will prove difficult to devise adequate procedures for accurate assessment of their risk.

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