Carcinogenesis vol.10 no.8 pp.1419–1423, 1989

Covalent binding of [2-^{14}C]2-amino-3,8-dimethylimidazo[4,5-*f*]-quinoxaline (MeIQx) to mouse DNA *in vivo*

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Female BALB/c mice were administered intragastrically with equimolar amounts of either [2-^{14}C]2-amino-3,8-dimethyl-[4,5-*f*]quinoxaline (MeIQx) or 2-acetylamino[9-^{14}C]fluorene (2AAF). DNA was isolated from tissues of mice killed either 6 or 24 h after administration. Analysis of liver DNA nucleotide digests by HPLC analysis revealed that all of the radioactivity was attributable to adduct formation. The specific activities of DNA samples were converted to covalent binding indices (CBI, μmol adduct per mol DNA nucleotides/mmol chemical applied per kg animal body weight). CBI values of 25 and 9 were determined for 2AAF and MeIQx in the livers of mice killed 6 h after dosing. The values were in general agreement with the moderate carcinogenic potency of these compounds. The specific activities of DNA preparations obtained from the kidneys, spleens, stomachs, small intestines and large intestines of mice treated with MeIQx and killed 6 h after dosing were 5- to 35-times less than those obtained with the liver. DNA isolated from the lungs (a target organ for MeIQx tumorigenicity) of MeIQx-treated mice was not radiolabelled at the limit of detection (CBI <0.3). With the exception of the gastrointestinal tract, the specific activities of DNA samples isolated from mice killed 6 h after administration were higher than those from mice killed after 24 h.

Introduction

The cooking of proteinaceous foods leads to the generation of heterocyclic amines (1), a number of which have been shown to be genotoxic in short-term tests and carcinogenic in rodents (2,3). One of the more commonly occurring members of this group is 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx*), originally found in fried beef (4). Under experimental conditions MeIQx has been shown to occur (in fried beef) at amounts of 1.0 μg/kg (uncooked weight) (1).

In the mouse, carcinogenicity studies have shown that MeIQx induces tumours in the livers and lungs, and also leukaemias and lymphomas (5), while in the rat, it induced tumours in the liver, skin, zymbal and clitoral glands (6). Distribution experiments have revealed MeIQx to be rapidly absorbed from the gut and to be excreted in the bile and the urine (7,8). The mutagenic activity of MeIQx is dependent on metabolic activation to electrophilic intermediates able to bind covalently to DNA (4). *In vivo*, ^{32}P-post-labelling analysis has revealed a number of DNA adducts to be formed after administration of any of 12 heterocyclic amines including MeIQx (9). Parallel *in vitro* studies indicated that it was hydroxylation of the extracyclic amino group that gave rise to the proximate carcinogen.

We have determined the extent to which radiolabelled [2-^{14}C]-MeIQx binds to mouse-tissue DNA after a single intragastric dose. Tissues were chosen either on the basis of their apparent susceptibility to MeIQx-induced tumorigenicity, or on their degree of exposure to the compound. The use of radiolabelled MeIQx and a rigorous DNA isolation and purification protocol permitted the numbers of DNA adducts to be accurately determined. Consequently it was possible to determine covalent binding index (CBI) values (10) for the compound (a predictor of carcinogenic potency for carcinogens which act by binding to DNA) and so determine to what extent the carcinogenicity of MeIQx can be attributed to its DNA binding ability.

Materials and methods

Chemicals

Unlabelled and 2-^{14}C-labelled MeIQx (sp. act. 50 mCi/mmol) were purchased from Toronto Research Chemicals Inc. (Downsview, Canada). Unlabelled 2-acetylaminofluorene (2AAF) was purchased from Sigma Inc. (St Louis, MO, USA), [9-^{14}C]2AAF (sp. act. 47 mCi/mmol) was purchased from New England Nuclear. Dosing solutions were prepared using either 10 mM acetic acid (pH 4.0) for MeIQx or 50% (w/w) aqueous polyethylene glycol 300 (Merck, Darmstadt, FRG) for 2AAF. Both radiolabelled compounds had similar UV spectra and mobilities on silica-gel TLC (developing solvent: chloroform:methanol, 95:5) when compared to their unlabelled counterparts. Radioactive purity (as determined by TLC) was >98% for MeIQx and >97% for 2AAF.

Animals and treatment regimes

Female Balb/cAnNCrlBR (BALB/c) mice were purchased at a weight of 18 g (36–56 days old) from Charles River Wiga GmbH (Sulzfeld, FRG). They were acclimatized for 1 week prior to use. Mice were housed separately in macrolone cages, with sawdust bedding under an alternate 12 h light/dark cycle. Food (Haltungsdiät nr 343, Klingental Mühle AG, Kaiseraugst, Switzerland) and water were available *ad libitum*.

Administration of the test compound or appropriate solvent was performed by intragastric gavage. Eight mice were used in each experiment, four mice received the test substance; two mice were administered with an equivalent amount of solvent (solvent control) and two mice remained untreated (controls against the possibility of radioactive contamination). In order to control for temporal variability, mice were dosed according to a strictly adhered-to schedule. Thus solvent controls were treated at 08.00 and 08.30, while test animals were dosed at 09.00 and at subsequent 30 min intervals and both food and water were available between treatment and killing. Mice were killed 6 or 24 h later. The average MeIQx treatments were: for the 6 h exposure, 50 μmol (3.5×10^9 d.p.m.) and for the 24 h experiment 64 μmol ($4.4. \times 10^9$ d.p.m.) per kg bodyweight, while for the 2AAF experiments they were 67 μmol (2.7×10^9 d.p.m.) and 69 μmol (2.3×10^9 d.p.m.) per kg bodyweight for the 6 and 24 h experiments, respectively.

Isolation of DNA and nuclear protein

Mice were anaesthetized with nembutal and killed by cardiac puncture. In the MeIQx study the liver, lungs, kidneys, spleen, stomach, small intestine (duodenum to ileocaecal junction) and large intestine (caecum to rectum) were removed, while in the 2AAF study only the liver and lungs were removed. All organs were rinsed in saline (gut contents were removed by flushing with saline), weighed, snap-frozen in liquid nitrogen and stored at −20°C prior to DNA isolation. Total blood content was calculated from the body weight using previously published data (11).

In the cases of the liver and the small intestine, DNA was extracted from organs taken from a pair of similarly treated mice. Chromatin was prepared from tissue homogenates by Nonidet P-40 (BDH, Poole, UK) precipitation and DNA purified to a constant specific radioactivity (where possible) using a previously published

*Abbreviations: 2AAF, 2-acetylaminofluorene; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; CBI, covalent binding index.

method (12). However, with other tissues, to facilitate better recovery, DNA was extracted from the combined tissues of two carcinogen-treated and two control mice (for this purpose control mice were either those treated with solvent or left untreated).

As a precaution against the possibility that radioactivity associated with DNA was an artifact due to work-up, liver chromatin from the pair of control mice (i.e. treated with neither the test compound nor solvent) was incubated for 15 min at 4°C in the supernatant derived from a hepatic homogenate of mice treated with the radiolabel (*in vitro* control). Chromatin was reisolated and DNA extracted in an identical manner to the other preparations. Purified DNA samples were dissolved in succinate buffer (20 nM Na-succinate, pH 6.0, 8 mM $CaCl_2$). The concentrations of DNA in the resultant solutions were determined by UV spectrophotometry at 260 nm and calculated assuming that 1 mg/ml DNA has an absorbance of 20. Radioactivity was measured by liquid scintillation counting, using 10 ml Instagel (Canberra-Packard, Zurich, Switzerland) and using the 12–156 keV channel. Nuclear protein was isolated and purified as described previously (12). The concentration of each protein solution was determined using the method of Lowry *et al.* (13). Radioactivity was determined as for DNA.

Nucleotide analysis

When conditions permitted, DNA was further analysed to determine how much radioactivity could be attributed to adduct formation as opposed to bioincorporation. Essentially, DNA was digested to its component nucleotides using micrococcal nuclease (Sigma) and calf spleen phosphodiesterase (Boeringer-Mannheim GmbH) as described previously (14). The resultant digest was analysed by HPLC. The digest was loaded onto a Lichrosorb 18 column and developed for 10 min with 10 mM ammonium formate containing 4% methanol (pH 3.8). After 10 min a linear methanol gradient was applied, such that, 50 min after commencement of the run, the column was being developed with 100% methanol. The flow rate was 3.5 ml/min. After passing through a UV detector (254 nm), fractions were collected (usually at 2 min intervals). Fractions were combined with 10 ml Instagel and radioactivity determined as described below. Radioactivity co-eluting with fractions having UV absorbance was attributed to bioincorporation, only radioactivity associated with fractions having no UV-absorbance was considered to be due to adducts.

Radioactivity determinations and calculations

The radioactive contents of all preparations were determined by liquid scintillation counting. In the cases of DNA, nucleotide analysis fractions and protein preparations, each sample was counted for 30 min three times. A DNA sample was considered to have a significant amount of radioactivity if the difference between the sample mean-count and that of the corresponding hepatic *in vitro* control DNA was >2 SD c.p.m. Calibration experiments (examination of the statistics of radioactive decay, vial to vial variation, etc.) at this institute revealed this value to be 1.8 c.p.m. for ^{14}C. With the exceptions of the liver and the small intestine, the specific activity of isolated DNA was corrected for the presence of unlabelled DNA originating from the tissues of untreated mice. The proportion of DNA from control mice in a preparation was considered to be equal to that of the weight contributed by that tissue. Specific radioactivities were thus corrected for by multiplying with the appropriate factor.

The covalent binding index (CBI, reference 10) was calculated from the CBI' (10). The latter is defined as:

$$CBI' = \frac{\text{d.p.m./mg DNA}}{\text{d.p.m./kg bodyweight}}$$

(converting to molar units)

$$CBI = \frac{\mu\text{mol chemical bound/mol DNA nucleotide}}{\text{mmol chemical applied/kg bodyweight}}$$

$CBI = CBI' \times 3.09 \times 10^8$
(assuming 1 mol DNA nucleotides weighs 309 daltons).

Results

Uptake of test substances

Radioactivity was detected in all of the tissue homogenates and blood samples prepared from mice treated with either of the test compounds at both time points. In the 6 h experiment, amounts of radioactivity in the liver homogenates from MeIQx-treated mice were lower than their 2AAF counterparts (1.4 versus 2.9%) although blood values were similar (1.3%). After 24 h, the amounts of radioactivity were lower throughout. With this longer interval between dosing and killing, amounts of radioactivity in liver homogenates were similar (0.34 versus 0.41%) for the two test compounds. However, amounts of radioactivity in blood taken from 2AAF treated mice (0.14%) were lower than in the corresponding MeIQx (0.59%) experiment.

Binding of test substances to DNA

Liver. Radioactivity was associated with the DNA from livers of mice treated with either compound (Table I). Since the specific radioactivity of the DNA preparations did not appreciably change on reisolation, it was assumed that the radioactivity represented covalently bound or bioincorporated radiolabel. Analysis by HPLC of DNA digests (Figure 1a and b) revealed that in every case all recoverable radioactivity was only detected in fractions having zero UV absorbance. This indicates that all of the radioactivity associated with the DNA was due to covalent binding. A conversion of the specific radioactivities of hepatic DNA to covalent binding index values is shown in Figure 2. 2AAF (CBI = 25 at 6 h) was about three times more potent than MeIQx (CBI = 8.6). The values at 24 h were lower (Table I; Figure 2),

Table I. DNA specific activities and CBI obtained from tissues of mice killed 6 or 24 h after treatment with MeIQx (data for 2AAF shown in parentheses)

	Tissue						
	Liver	Spleen	Lung	Kidney	Stomach	Small intestine	Large intestine
Specific activity (d.p.m./mg DNA)							
6 h	97 ± 22 (221 ± 3)	16 ± 1	<6.3 (30 ± 1)	7.8 ± 1.8	23 ± 3	12 ± 4	60 ± 20
24 h	64 ± 14 (135 ± 7)	7.1 ± 1	<3.2 (ND)	9.5 ± 0.9	38 ± 5	10 ± 1	12 ± 1
CBI							
6 h	8.6 ± 2.0 (25.2 ± 2.2)	1.44 ± 0.02	<0.7 (3.5 ± 0.3)	0.70 ± 0.15	2.0 ± 0.4	1.3 ± 0.7	5.3 ± 3.7
24 h	4.6 ± 1.0 (18.6 ± 1.6)	0.50 ± 0.06	<0.3 (ND)	0.44 ± 0.03	2.8 ± 0.3	0.74 ± 0.64	0.86 ± 0.11

Data are expressed as mean ± SD for two tissue pools, CBI values were determined as described in Materials and methods. ND, not determined.

nominal half lives of the adducts were determined to be between 19 and 39 h.

Extrahepatic tissues. Treatment of mice with MeIQx and killing 6 h later led to radioactivity being associated with DNA from the spleen, kidney and sections of the gastrointestinal tract, enabling the determination of CBI values (Table I). No radioactivity was found to be associated with lung DNA (in contrast, radioactivity was found with DNA from the lungs of mice treated with 2AAF and killed 6 h later). Although it was generally

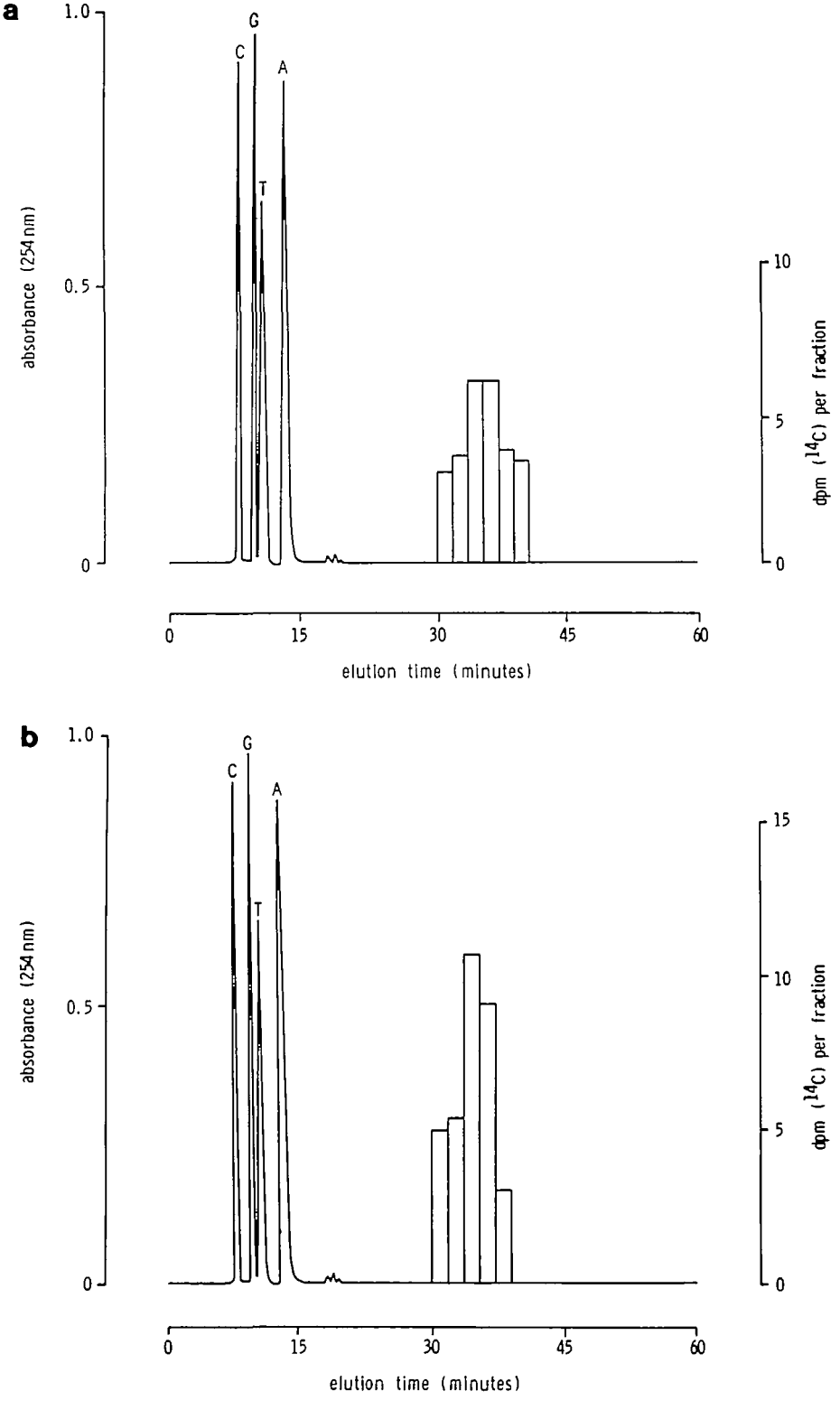

Fig. 1. HPLC analysis of nucleotides obtained from the enzymatic hydrolysis of hepatic DNA from mice treated with either MeIQx (a) or 2AAF (b) and killed 6 h after treatment. The plot shows the UV absorbance of the eluate, while the bars show the radioactivity of the collected fractions (if significantly above control DNA). Letters indicate the order of nucleotide (deoxynucleoside-3′-phosphate) elution: A, adenine; C, cytosine; G, guanine; T, thymidine.

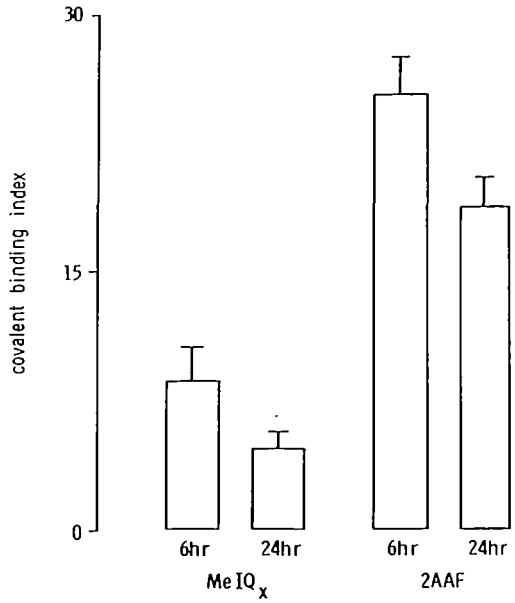

Fig. 2. Covalent binding indices determined for hepatic DNA isolated from mice treated (*per os*) with either MeIQx or 2AAF and killed 6 h or 24 h after administration. Data are presented as the means and standard deviations (bars) from two mouse liver pools (two livers per pool).

possible to purify DNA to a constant specific activity, it was not possible to perform HPLC analysis of the nucleotides. This was due to the small sample size and low specific activity. Although it was not possible to show conclusively that all radioactivity was due to covalent binding, it was assumed that the extrahepatic DNA radioactivity was also a consequence of nucleotide–adduct formation. In the case of the gastrointestinal tract there was, additional, circumstantial, evidence to support this idea. Analysis of DNA biosynthetic activity in the mouse (15) reveals the small intestine to have the greatest activity followed by (in descending order) large intestine, stomach and liver. The (descending) order of CBI values at 6 h was: liver, large intestine, stomach and small intestine (Table I). If radioactivity did represent biosynthesis it would be expected that the order of CBI values would be similar to that of biosynthesis. For all of the extrahepatic tissues the CBI values were lower than those obtained for the corresponding liver preparations. As in the case of the liver the CBI values obtained for the spleen and kidneys were higher in mice killed 6 h after dosing compared with the 24 h values. This result contrasted with sections of the gastrointestinal tract which appeared to be unchanged.

Control experiments. In order to ensure that the radioactivity associated with DNA was not due to work-up techniques or contaminating protein, other determinations were made. The radioactivity determined for hepatic DNA isolated from either solvent- or *in vitro*-control experiments were 13.2 and 13.5 c.p.m., respectively (MeIQx, 24-h experiment). Succinate buffer alone resulted in 13.6 c.p.m. total count. This indicates that neither the work-up procedure nor the solvent contributed to DNA radioactivity in treated mice.

Chromatin protein isolated from the liver showed specific activities of between 400 and 900 d.p.m./mg, i.e. they were only about 10-fold higher than for DNA. Similar results were obtained for other tissues. It has previously been shown (12) that using this method of DNA isolation, protein contamination accounts for no more than 0.2% by weight. Thus contamination due to protein cannot account for the radioactivity measured in the DNA samples.

Discussion

We have observed that intragastric administration of radiolabelled MeIQx or 2AAF to mice leads to radioactivity being co-isolated with DNA from a number of tissues 6 or 24 h after treatment. In all tissues it was possible to purify DNA to a constant specific activity. When hepatic DNA was digested to its component nucleotides and subjected to HPLC analysis it was not possible to detect radioactivity in the UV-absorbing regions of the eluates (Figure 1). Thus it can be concluded that the radiolabel was covalently bound in the form of adducts. The hepatic CBI values obtained were compatible with both compounds being carcinogens of medium potency (10).

Binding of MeIQx to the DNA of extrahepatic tissues (with the exception of the lung) was also observed. The results obtained with the lung were surprising, since the lung was a target site in the mouse carcinogenesis experiment (5). Four reasons can be put forward to explain this observation. (i) Strain differences, this study used BALB/c mice as opposed to CDF_1 used in the carcinogenicity study. (ii) Only a small population of lung cells (e.g. clara cells) are capable of performing the metabolic processes necessary for the conversion of MeIQx to an active carcinogen. Since DNA is extracted from the complete tissue, it is possible that adducted DNA is 'diluted' to such a level that it cannot be detected by our methods. (iii) The limit of detection of DNA binding (CBI ≤ 0.3 at 24 h, averaged over all cells) might not be low enough to exclude tumour formation in target cells within the lung. (iv) MeIQx induces lung tumours by an adduct independent process. It has been demonstrated that 2AAF can act as a promoter (16) and MeIQx may have a similar property.

Binding by MeIQx to hepatic or lung DNA was not as great as by 2AAF. This may in part be due to the different solvents used and/or inherent differences in pharmacokinetics. Six hours after administration, the radioactivity in the liver was approximately twice as high with 2AAF (see results). Nevertheless, the adducts produced by these two compounds appeared to have different properties. Up to 24 h after treatment adducts formed by 2AAF persisted longer than by MeIQx. Assuming first order kinetics, the nominal half-lives for MeIQx and 2AAF induced adducts had a ratio of 1:2. The apparently faster rate of disappearance of MeIQx adducts may be due to one or a combination of factors. Adducts produced by MeIQx may be repaired at a faster rate than those produced by 2AAF. Alternatively MeIQx-induced adducts may decay to form a second, more toxic lesion. It is known that both compounds produce guanine C-8 adducts (17,18). Furthermore it has been demonstrated that some guanine C-8 adducts are labile, leading to the generation of 8-hydroxyguanine (18). Since the half-life of MeIQx appears to be lower than that of 2AAF, it is possible that the MeIQx adduct unlike that of 2AAF has this property.

Acknowledgements

This work was partly supported by funds provided by the UK Ministry of Agriculture, Fisheries and Food and a Fellowship in Toxicology (PGT) provided by the European Science Foundation and European Medical Research Councils to A.J.A.

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Received on January 12, 1989; revised on May 16, 1989; accepted on May 24, 1989