Chemoprevention of aflatoxin B₁-induced carcinogenesis by indole-3-carbinol in rat liver—predicting the outcome using early biomarkers

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Indole-3-carbinol (I3C) was examined for its ability to inhibit aflatoxin B₁ (AFB₁)-induced hepatocarcinogenesis in male Fischer rats when administered either before or after the carcinogen. After 13 weeks, animals pretreated with I3C (0.5% in the diet) for 2 weeks prior to administration of AFB₁ and with continuing treatment during exposure to the carcinogen were protected from development of preneoplastic lesions, as determined by the classical markers γ-glutamyl-transpeptidase (GGT) and glutathione S-transferase (GST) P. In animals receiving AFB₁ for 6 weeks before treatment with I3C, there was no obvious protective effect at 13 weeks compared with animals receiving only AFB₁. Using cytokeratin 18 expression as a marker, animals fed AFB₁ alone had a small number of positive foci at 13 weeks. However, no cytokeratin-positive foci were visible in the majority of livers from either group receiving I3C in combination with AFB₁ and after 43 weeks all animals in these groups were protected from liver tumour formation. These results suggest that expression of cytokeratin 18, a later phenotypic change in foci than induction of GST-P and GGT, correlates more closely with tumour outcome in this model. I3C appeared to retard progression of AFB₁-induced carcinogenesis at both the initiation and promotion stages. Continuous treatment with I3C for 13 weeks caused significant induction of CYP1A1, 1A2, 3A and 2B1/2, GST γ c2, aflatoxin B₁ aldehyde reductase and quinone reductase. Such alteration of the drug metabolizing capacity of the liver by I3C contributes to blocking of initiation, while the observed inhibition of ornithine decarboxylase, a rate limiting enzyme in polyamine biosynthesis, and of tyrosine kinase activity may contribute to the suppressive effect of I3C.

Introduction

There are many studies describing the chemopreventive properties of dietary constituents with respect to a range of carcinogens in a number of tissues and several species. However, closer examination reveals that the chemopreventive effect may not be ubiquitous, in some cases manifest only in selected tissues or only at certain stages of the carcinogenic process.

Abbreviations: AFAR, aflatoxin B₁ aldehyde reductase; AFB₁, aflatoxin B₁; AFM₁, aflatoxin M₁; AFQ₁, aflatoxin Q₁; CK, cytokeratin; GGT, γ-glutamyl-transpeptidase; GST, glutathione S-transferase; H&E, haematoxylin and eosin; I3C, indole-3-carbinol; ODC, ornithine decarboxylase; QR, quinone reductase; TK, tyrosine kinase.

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Clearly, it is vitally important to address such details of chemopreventive efficacy for any compound which may be considered for clinical trial or in the more general context of giving dietary advice. Indole-3-carbinol (I3C), a constituent of cruciferous vegetables, is one such agent for which results in the literature are somewhat contradictory. For example, it was shown to inhibit aflatoxin B₁ (AFB₁)-induced hepatocarcinogenesis in trout when given prior to and together with the carcinogen, but to promote when given continuously after AFB₁ initiation (1). Promotional enhancement of tumour incidence was found to be significant when treatment was delayed for several weeks or months after initial AFB₁ challenge (2). These workers found that promotion was enhanced with length of exposure to I3C treatment and was less marked when I3C was given in alternating months or weeks or twice per week only. They concluded that the potential for dietary I3C to promote prior hepatic initiation events when fed continuously is approximately as great as its potential to inhibit concurrent AFB₁ initiation.

These data were supported by another study in which the number of preneoplastic, glutathione S-transferase (GST) P-positive foci induced in rats by diethylnitrosamine was found, in a medium term bioassay, to be significantly reduced following pre-initiation exposure to I3C, while post-initiation exposure caused an increase in both number and area of foci. These results were interpreted as demonstrating that I3C had a promoting effect on the post-initiation stages (3).

In contrast, Tanaka et al. (4) found that I3C inhibited rat tongue carcinogenesis (as measured by tumour incidence as well as preneoplastic lesions) in both the initiation and post-initiation phases when administered together with or following treatment with 4-nitroquinoline 1-oxide. In a study of mammary carcinogenesis induced by dimethylbenzanthracene, administration of I3C throughout both the initiation and promotion phases or during the initiation phase only was found to be highly protective. Administration both before and after methylidinitrosourea, a direct acting carcinogen, was also effective (5).

With respect to the mechanism of action of I3C, there is substantial literature on its ability to block initiation, with rather less understood about its potential to inhibit or enhance promotion. Goeger et al. (6) showed that 0.2% I3C caused substantial changes in metabolism and distribution of AFB₁ in trout, including a reduction in in vivo hepatic DNA binding of injected AFB₁ by 70% and a 7-fold increase in aflatoxicol-M₁ glucuronide levels in bile. By carrying out a range of dose–response experiments, varying both I3C and AFB₁ concentrations, the same group also concluded that even at low levels, I3C may offer some protection against chemically induced neoplasia (7,8). Studies in rat showed that I3C is a powerful inducer of hepatic cytochrome P450s 1A1, 1A2 and 2B1/2, causing a dose-dependent increase in aflatoxin M₁ (AFM₁), aflatoxin Q₁ (AFQ₁) and also AFB₁ epoxide (9). Our results, which included a 30-fold induction of AFM₁ production...
by I3C-treated rat liver microsomes, are in agreement with these observations (10).

As a bifunctional blocking agent, I3C also induces GSTs, in particular GST Yc2 (otherwise known as GST A5) and AFB1 aldehyde reductase (AFAR), both of which are central to the detoxification of AFB1 epoxide in rodents (10–13). We therefore predicted that, despite an increased production of epoxide, animals pretreated for 2 weeks with I3C should be protected from AFB1-induced toxicity and carcinogenicity by increased phase II activity, in much the same way as we showed previously with ethoxyquin pretreatment (14,15). In the present intervention study in Fischer rats exposed to AFB1, we examined the effects on the liver of treatment with I3C at 0.5% in the diet, either with 2 weeks of pretreatment or when given following 6 weeks exposure to AFB1. In addition, we looked for pathological changes in other tissues which might be I3C-related or would indicate a change in organotropism for AFB1.

When assessing the ability of chemopreventive agents to inhibit tumour formation, a biomarker which predicts the outcome accurately at an early time point would be extremely useful. This is particularly relevant to intervention studies in humans. Classical markers employed in animal models to monitor the carcinogenic process by quantitation of altered cell foci have been γ-glutamyltranspeptidase (GGT) and GST-P. However, not all foci expressing these markers are destined to progress to tumours: in fact, many have the ability to regress (16). It is therefore important to identify the subset of foci which will progress. In a pilot study to address this question we used a combination of markers, which included loss of fibronectin receptor (17), and cytokeratins (CKs) 18 and 19 to follow the progress of altered hepatic foci induced by AFB1 in rat liver. We found that CK overexpression in hepatocytes was the last of the markers to appear, that it identified the smallest number of altered foci, all of which could be identified by the other markers, and that, on removal of AFB1 from the diet, these CK-positive foci were stable, whereas the number identified by other markers decreased (18). We suggest that overexpression of CK 18 or 19 might be a more reliable marker for predicting tumour outcome than either GGT or GST-P, at least in this system. In the present study, we therefore compared the ability of CK 18 with that of GGT, GST-P and AFAR to predict tumour formation.

### Materials and methods

#### Animals

Male Fischer F344 rats obtained from Harlan Olac UK (Bicester, UK), were housed in Morehead isolators (Morehead Animal Health, Edinburgh, UK) under negative pressure with a 12 h light–dark cycle, with a temperature range of 19–25°C and humidity of 40–60%. The animals were allowed 6 weeks to acclimatize.

#### Diets

Animals, at 19 weeks of age, were assigned to one of two experimental groups. Older animals were used to ensure that the levels of some phase II enzymes, in particular AFAR, were as low as possible, since these were found to decline gradually after weaning. In the first experiment (Figure 1), five groups of six rats were administered one of the following experimental diets for 13 weeks: group 1, RM1 control maintenance diet throughout; group 2, I3C, group 3, AFB1; group 4, I3C for 2 weeks, followed by AFB1; in addition to I3C; group 5, AFB1 for 6 weeks, followed by I3C in addition to AFB1. AFB1 (Sigma, Poole, UK) was mixed into powdered RM1 diet (Special Diet Services, Northwick, UK) with 2% arachis oil to give a final concentration of 2 p.p.m. I3C was treated similarly to give a final concentration of 0.5%. Diet was freshly prepared every week. Food and water were provided ad libitum. In the second experiment (Figure 1), five similarly treated groups were returned to the control diet after 24 weeks, until completion of the study at 43 weeks.

#### Tissue preparation

Animals were killed using CO2 and tissues removed immediately. Fresh liver samples were used to prepare microsomal and cytosolic fractions. Tissue slices were taken, in ice-cold acetone for immunohistochemistry and in buffered formalin for haematoxylin and eosin (H&E) histology, from the following organs: liver, kidney, spleen, heart, lung, pancreas, small and large intestine and testis. Remaining tissues were snap frozen in liquid N2.

#### Enzyme assays

Microsomal fractions were assayed for total P450 content (19) and metabolic capacity with respect to conversion of AFB1 to AFM1, AFQ1 and epoxide as described previously (15). Cytosols were assayed for many GST activities (20), for quinone reductase (QR) activity (21), for AFB1–GSH conjugation attributable to GST Yc2 activity (22) and for production of AFB1 dialcohol, a measure of AFAR activity (13). Assays were performed for all animals individually.

Ornithine decarboxylase (ODC) was measured by preparing crude extracts from frozen liver according to the method of Tanaka et al. (23). The reaction mixture consisted of 320 µl liver extract, 0.1 mM pyridoxal phosphate (Sigma), 0.22 mM L-ornithine (Sigma), containing 18.5 kBq [14C]ornithine hydrochloride (Amersham International, Little Chalfont, UK) in a final volume of 400 µl. CO2 was collected on filter papers soaked with 100 mM NaOH and suspended inside the reaction vessel. The reaction was allowed to proceed in a sealed vial for 1 h at 37°C, before addition of 200 µl 1 M perchloric acid and incubation for a further hour. Filter disks were counted in 10 ml Ultima Gold scintillation fluid (Canberra-Packard, Pangbourne, UK). Assays were performed on individual animals in duplicate and each sample was assayed on three separate occasions.
Prediction using early biomarkers

Table I. Capacity of liver microsome and cytosol samples to metabolize AFB₁ (13 week treatments)

<table>
<thead>
<tr>
<th>Group</th>
<th>Total P450</th>
<th>Tris diol</th>
<th>AFM₁</th>
<th>AFQ₁</th>
<th>CDNB/DCNB</th>
<th>Yc2</th>
<th>AFAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1/1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>I3C</td>
<td>2.6</td>
<td>2.1</td>
<td>7.2</td>
<td>1.9</td>
<td>1.9/1.9</td>
<td>9.4</td>
<td>3.1</td>
</tr>
<tr>
<td>AFB₁</td>
<td>1.3</td>
<td>0.9</td>
<td>1.0</td>
<td>1.1</td>
<td>1.3/1.7</td>
<td>6.8</td>
<td>5.7</td>
</tr>
<tr>
<td>AFB₁ + I3C</td>
<td>3.5</td>
<td>2.5</td>
<td>8.6</td>
<td>3.0</td>
<td>2.2/2.5</td>
<td>10.8</td>
<td>6.8</td>
</tr>
<tr>
<td>I3C + AFB₁</td>
<td>3.2</td>
<td>2.6</td>
<td>9.0</td>
<td>2.1</td>
<td>2.0/2.2</td>
<td>11.1</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Values are given as fold increase over controls (n = 6 for all groups). Mean control values (± SD) were as follows: total cytochrome P450, 0.41 ± 0.08 nmol/min/mg microsomal protein; AFB₁ epoxide (tris diol), 0.05 ± 0.01 nmol/min/mg microsomal protein; AFM₁, 0.025 ± 0.004 nmol/min/mg microsomal protein; AFQ₁, 0.04 ± 0.01 nmol/min/mg microsomal protein; GST (CDNB), 1.87 ± 0.25 μmol/min/mg cytosolic protein; GST (DCNB), 0.05 ± 0.01 μmol/min/mg cytosolic protein; GST Yc₂ (AFB₁-GSH), 0.02 ± 0.006 nmol/min/mg cytosolic protein; AFAR (AFB₁ dialcohol), 0.002 ± 0.001 nmol/min/mg cytosolic protein.

*Significantly different from controls (P < 0.05).

Fig. 2. Western blots of cytochromes P450 after 13 weeks treatment. Although each lane represents a single animal, blots for other animals in each group showed similar results. +ve control, microsomal sample supplied with the appropriate ECL kit.

Tyrosine kinase (TK) activity was also measured using extracts prepared from frozen tissue samples, essentially as described by Sharma et al. (25), except that the assay buffer contained 10 μM ZnCl₂ and the reaction was initiated by adding 0.5 μCi [γ-32P]ATP (Amersham International) per assay. Assays were performed in duplicate on each of four animals per treatment group on two separate occasions.

Western blots

These were carried out on microsomal tissue fractions from individual animals using antibodies against CYP1A1, 1A2, 3A, 4A, 2B1/2 and 2E1 (Amersham International) and against CYP2C11 (a gift from Prof. Roland Wolf) and on cytosols for GST-P (26), GST Yc₂ (L.Weir, R.James and G.Neal, unpublished data) and AFAR (27), using 10 μg/lane for microsomal protein and 20 μg for cytosols, blotting onto nitrocellulose membranes and with detection using an ECL kit (Amersham International). An alkaline phosphatase-conjugated secondary antibody (Sigma) was used to detect GST-P.

Histochemistry and immunocytochemistry

Liver sections (2–3 mm) were fixed in ice-cold acetone and used, as described previously (28), to examine the localization of the enzymes GST-P and AFAR, using appropriate antisera diluted 1:100, or CK 18, using monoclonal antibody (29) in meat culture supernatant. GGT activity was localized histochemically (30).

Statistical analyses

Unless otherwise stated, statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett’s test, with a 5% critical value.

Results

Protective effect of I3C on preneoplasia after 13 weeks treatment

After 13 weeks there was no effect on body weight gain with any of the diets. However, all the groups exposed to I3C showed a significant increase in liver weight when this was expressed as a percentage of body weight.

Induction of phase I and II drug metabolizing enzymes. The effect of the various diets on hepatic enzymes was examined after 13 weeks and compared with previous results obtained after feeding I3C for 2 weeks (10). Western blot analysis (Figure 2) showed that in all groups receiving I3C there was induction of CYP1A1, 1A2, 3A, 2B1/2 and, to a lesser extent, 4A, but not of 2C11 or 2E1. The group fed AFB₁ alone showed no induction of these isozymes, in fact there appeared to be a slight loss of 2C11 and 2E1. All treatment groups showed some induction of AFAR, while only those animals receiving AFB₁ or AFB₁ followed by I3C showed any induction of GST-P over control values (Figure 3 and Table I). In the group fed only AFB₁, GST-P was localized in altered foci, while in the group receiving I3C after initiation with AFB₁, much of the liver stained positive for this enzyme, making it impossible to count discrete foci. Using a monoclonal antibody against GST Yc₂, induction of this subunit was visible in all groups receiving I3C and, to lesser extent, in animals receiving AFB₁ alone, where it was localized in the foci. The results obtained from western blots were corroborated by HPLC analyses of the capacity of microsomes to metabolize AFB₁ to AFM₁, AFQ₁ and epoxide and of liver cytosols to conjugate AFB₁ epoxide to GSH (predominantly a measure of GST Yc₂ activity) and convert AFB₁ dihydrodiol to dialcohol (AFAR activity) (Table I). The effect of continuous treatment with I3C for 13 weeks on both phase I and II enzyme activities was similar to that observed after only 2 weeks treatment (10). QR activity was also induced by I3C. Compared with control animals (0.107 ± 0.050 μmol/min/mg protein), the levels were as follows: I3C alone, 3.3-fold; AFB₁ alone, 1.6-fold; I3C followed by AFB₁, 3.2-fold; AFB₁ followed by I3C, 5.5-fold. These increases were all highly significant (P < 0.001), except for the group receiving AFB₁ alone.
Immunocytochemistry and quantitation of phenotypically altered foci. From acetone-fixed, semi-serial liver sections which had been stained immunocytochemically for GST-P (Figure 4), AFAR and CK 18 (Figure 5) or histochemically for GGT, the number of foci positive for each marker was calculated and expressed per cm² of liver (Table II). In the AFB1-treated group at 13 weeks the number of CK-positive foci was ~8-fold lower than for GGT and 30- to 40-fold lower than for GST-P and AFAR. In the group where I3C was administered 6 weeks after the start of AFB1 treatment, the numbers of AFAR-positive and CK-positive foci were significantly reduced. Half the animals in the group contained no CK-positive foci. In contrast, GGT-positive foci were more numerous than with AFB1 alone. As mentioned above, so much of the liver was positive for GST-P in this group (both zonal and focal staining) that it was no longer possible to count individual foci. Predictions using GGT, GST-P or AFAR as early markers showed that animals in the groups treated with AFB1 and AFB1 + I3C contained significant numbers of phenotypically altered foci and if these represented true preneoplastic lesions, then these animals should all develop tumours. However, no CK 18-positive foci were observed when animals were pretreated with I3C, and the group receiving I3C after AFB1 also had very few foci (Figure 5 and Table II), so that based on this marker, a low incidence of tumours would be predicted in both these groups, with significant tumour incidence only in the group treated with carcinogen alone.

Livers of animals treated with I3C alone also exhibited periportal induction of GGT and, with antibody against GST-P, the bile ducts appeared very prominent (Figure 4a).

Measurement of hepatic ornithine decarboxylase activity showed that this was depressed in all groups treated with I3C relative to the untreated group. Treatment with AFB1 alone had no significant effect on control levels of activity (Table III). Significant decreases in TK activities were also observed in all the groups receiving I3C, compared with the control group (Table III).

Protective effect of I3C on tumour development after 43 weeks

After 43 weeks, during which time all surviving animals had been returned to control diet for the final 22 weeks, there were no significant differences in body weight, but the group which had received AFB1 alone had an increase in liver weight when
Fig. 5. Immunocytochemistry for CK 18 after 13 weeks treatment. Monoclonal antibody against CK 18 showed staining only in the biliary epithelial cells in control livers and in animals treated with I3C (arrows) (a). In AFB1-treated livers some foci overexpressing CK 18 in the cytoplasm of hepatocytes were present (b), while after pretreatment with I3C (c) or addition of I3C 6 weeks after AFB1 (d) no or very few such foci developed. Positively stained biliary hyperplasia, a sign of AFB1 toxicity, was still prominent in livers treated with I3C in the post-initiation phase (arrows in d). Magnification ×120.

Table III. ODC and TK activities after 13 weeks treatment

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>ODC activity (pmol CO₂ produced/h/mg protein) (n)</th>
<th>TK activity (pmol [γ-32P]ATP incorporated/mg protein/10 min) (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.00 (6)</td>
<td>0.150 (8)</td>
</tr>
<tr>
<td>I3C</td>
<td>4.30a (5)</td>
<td>0.125b (8)</td>
</tr>
<tr>
<td>AFB1</td>
<td>13.18 (6)</td>
<td>0.127 (8)</td>
</tr>
<tr>
<td>I3C + AFB1</td>
<td>4.88a (6)</td>
<td>0.119b (8)</td>
</tr>
<tr>
<td>AFB1 + I3C</td>
<td>5.20a (6)</td>
<td>0.107b (8)</td>
</tr>
</tbody>
</table>

aSignificantly different from control or AFB1-treated groups (P < 0.05). SD for the group = 4.42 (ANOVA general linear model, followed by Fisher’s test).

expressed as a percentage of body weight, reflecting the development of tumours in this tissue. One animal from this group was killed at 35 weeks because of progressive body weight loss and was found to have a particularly large liver tumour. The liver weight was 28.5 g, compared with an average control value at 43 weeks of 16.8 ± 1.4 g. This animal also had extensive metastases in the lung.

Histological findings. Examination of buffered formalin-fixed liver sections from control animals showed several signs of ageing, including slight biliary fibrosis and proliferation, small granulomas and vacuolated hepatocytes in zone 3 (centrilobular areas). Animals which had received only I3C had livers similar to controls, but with less apparent biliary fibrosis and proliferation and an increase in fat accumulation. In the group treated with AFB1 (n = 5), as expected, there was a variety of advancing altered cell foci of various morphologies, portal tract inflammation and biliary hyperplasia. In addition, all animals had developed several hepatic tumours (Table IV). When AFB1 was followed by treatment with I3C, there was a reduction in the numbers of large foci seen with AFB1 alone, but there were still considerable numbers of small mixed clear/ vacuolated cell foci, some of which were positive for GGT and GST-P. No macroscopic tumours were visible. Animals fed the I3C diet before exposure to AFB1 appeared to be almost completely protected from induction not only of tumours, but also of altered hepatic foci, as judged by CK 18, GST-P and GGT staining (Table IV).

Because there is evidence in the literature that agents which are apparently chemopreventive in one tissue cause damage or promote tumours in another (see Discussion), we examined a number of non-target tissues, namely kidney, lung, heart, spleen, pancreas, small and large intestine and testis. There were no obvious differences from control animals with the following exceptions. In the group fed AFB1, one animal was found dead, the cause of which was not established, and one which was killed early, as mentioned above, was found to have extensive metastasis of hepatocellular carcinoma to the lung. In the group fed AFB1 followed by I3C, one animal had a cyst on its hind limb. Another had a gut-associated tumour, which was diagnosed as a lipoma and thought unlikely to be treatment-related.
Data from this study unequivocally show that I3C has significant chemopreventive potential and is capable of retarding tumour formation, whether the animals were pretreated for 2 weeks before receiving carcinogen or whether they received AFB1 for 6 weeks before treatment with I3C. Results at 13 weeks using GGT or GST-P as markers of foci were in agreement with those of Kim et al. (3), who postulated that I3C given after the carcinogen actually caused promotion of these lesions. However, in the present study, when expression of these early markers was compared with the numbers of tumours which developed by 43 weeks there was clearly a lack of correlation. It appeared that CK 18 expression was more useful in predicting the outcome at an early time point, and as such merits further validation.

Several studies provide clues as to the role of CKs in carcinogenesis. Transfection of CK 8 and 18 cDNAs into several cell lines conferred a multiple drug resistance phenotype on cells exposed to a variety of drugs (31,32), similar to the resistance phenotype which develops in vivo in altered foci. Phosphorylation of CKs can be increased by the tumour promoter 12-O-tetradecanoylphorbol-13-acetate, an activator of protein kinase C, leading to some rearrangement of the intermediate filament network (33). Recently, CK 8 overexpression was reported in some altered foci in the Solt-Farber-resistant hepatocyte model, using diethylaminoethylamine in combination with partial hepatectomy and acetylaminofluorene to induce hepatocarcinogenesis (34), in agreement with our previous findings (29). Another tumour promoter, microcystin-LR, an inhibitor of protein phosphatases 1 and 2A, also caused hyperphosphorylation of CK 8 and 18 in rat hepatocytes, associated with morphological changes and intermediate filament rearrangement (35). Such hyperphosphorylation could be suppressed by carotenoids (36). Transglutaminase-induced cross-linking of CK polypeptides in liver and hepatoma cells has been suggested to be closely associated with cell degeneration and death (37). Using primary rat hepatocyte cultures, an inverse relationship has been shown between cytoskeletal (CK) and liver-specific protein expression (38). Because altered CK expression in epidermoid carcinomas induced in hamster buccal pouch by 7,12-dimethylbenz[a]anthracene was inhibited by antioxidant administration (GSH or vitamin E), this group has also suggested that it could be a useful biomarker in analysis of chemoprevention (39). The effect of the antitumour drug liarozole has been partly attributed to the decrease in CK content in rat prostate carcinoma (40). Thus, changes in CK expression, perhaps involving phosphorylation, might be more closely associated with malignant transformation than altered expression of drug metabolizing enzymes (which are also readily induced by non-carcinogens) and as such be predictive of carcinogenic or chemopreventive activity.

I3C is a bifunctional blocking agent, inducing both phase I and II drug metabolizing enzymes. Concern is often expressed that compounds which induce cytochromes P450 are undesirable as chemopreventive agents because they may inadvertently cause unwanted activation of carcinogens. Activation of P450s could be beneficial in diverting AFB1 from the epoxide pathway towards the formation of less toxic, less carcinogenic metabolites, such as AFM1 and AFQ1, but since I3C also increases production of the carcinogenic AFB1 epoxide, the protective outcome in this case must be primarily determined by the capacity for detoxification via phase II enzymes, in particular GST Yc2 and AFAR.

One further problem with chemoprevention regimens is that sometimes there is a protective effect in one tissue, but an enhanced toxicity or carcinogenicity elsewhere in the body (see for example ref. 14,41,42). However, in this study careful examination of a number of other tissues suggested that there were no adverse effects in non-target organs over the time course employed.

Protection of the group receiving AFB1 followed by I3C suggested that this compound may have the ability to suppress tumour progression as well as block tumour initiation. In the present study, we did not include a group of animals treated with AFB1 for only 6 weeks, with no further treatment during the 43 weeks. However, such a group included in another study indicated that 50% of the animals had developed tumours of 3 mm or greater in size and all animals had numerous foci or nodules after 50 weeks. Previously it was shown that Fischer rats which received AFB1 for <6 weeks were at negligible risk of developing tumours, while in those treated for 6 weeks or longer there was between 75 and 100% tumour incidence (43,44).

Possible mechanisms whereby I3C may protect during promotional stages have been suggested by in vitro studies of Sharma et al. (25), which showed inhibition of TK and ODC activities. Increases in TK activity, resulting in increased protein phosphorylation, are associated with cell proliferation.

### Table IV. Formation of altered hepatic foci and tumours after 43 weeks

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumour no.</th>
<th>Tumour size</th>
<th>GGT-positive foci</th>
<th>CK 18-positive foci</th>
<th>GST-P area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 6)</td>
<td>0.0</td>
<td>0.2 ± 0.4^e</td>
<td>0.0^b</td>
<td>1.1 ± 1.0^d</td>
<td></td>
</tr>
<tr>
<td>I3C (n = 6)</td>
<td>0.0</td>
<td>0.2 ± 0.4^b</td>
<td>0.0^b</td>
<td>0.4 ± 0.6^b</td>
<td></td>
</tr>
<tr>
<td>AFB1 (n = 4)</td>
<td>6.5 (4–9)^c</td>
<td>2–23 mm</td>
<td>92 ± 19</td>
<td>27 ± 10</td>
<td>94.1 ± 3.2</td>
</tr>
<tr>
<td>AFB1 + I3C (n = 5)</td>
<td>0.0</td>
<td>36 ± 18^b</td>
<td>2.7 ± 3.2^b</td>
<td>17.9 ± 7.4^b</td>
<td></td>
</tr>
<tr>
<td>I3C + AFB1 (n = 5)</td>
<td>0.0</td>
<td>22 ± 19^b</td>
<td>0.0^b</td>
<td>1.9 ± 0.4^b</td>
<td></td>
</tr>
</tbody>
</table>

^aThere were six rats in groups 1, 2, 4 and 5, none of which developed tumours. In contrast, group 3 (AFB1) had five rats, all of which developed tumours, although one rat died early with an exceptionally large tumour, which precluded the possibility of counting individual lesions. Differences in the incidence of tumours among groups were statistically significant (P < 0.0001) using Fisher’s exact test calculated with the StatXact statistical package (Cytel Software, Cambridge, MA). A comparison of group 3 with any other group also showed a statistically significant difference (P = 0.002) using the same statistical method.

^bSignificantly different from group receiving AFB1 alone (P < 0.001).

^cFoci/cm² liver ≥ SD.

^dIn AFB1-treated animals GST-P expression was too widespread to count individual foci. Therefore, results are expressed as percentage of liver area expressing this enzyme.

^eAverage number of macroscopic tumours per animal (range).
while a decrease in levels occurs during terminal differentiation. Overexpression is associated with a number of growth factor receptors and oncogenes and with the transforming ability of retroviruses and may be required to maintain the transformed state. Inhibition could therefore be involved in suppression or reversal of carcinogenic processes. After 13 weeks treatment there was no increase in TK activity in animals receiving AFB1 alone, but there was decreased activity in the presence of I3C, compared with control levels. ODC is the rate limiting enzyme in the synthesis of polyamines, required for cell proliferation and transformation, and is induced by various tumour promoters. Results from the present study show that I3C is also capable of reducing this activity in liver in vivo, suggesting that loss of both TK and ODC activity may be involved in the protective effect observed when I3C was given after AFB1.

Consideration of I3C for preclinical trials as a chemopreventive agent against breast cancer was prompted in part by a 3 month trial in women which showed that administration increases the 2-hydroxyestrone:estriol ratio in a sustained manner (45). Such an alteration in the metabolite balance, which is less favourable for cell proliferation, has been postulated to play a significant role in decreasing the risk of breast cancer development at the post-initiation stage. A similar mechanism was proposed to explain the inhibition of spontaneous endometrial adenocarcinoma in rats (46). However, more recent evidence suggests that I3C can cause arrest of cells in the G1 phase of the cell cycle, independently of oestrogen receptor status. (47). Further experiments are required to ascertain whether I3C is acting as a suppressing agent in the liver model described in this study or whether blocking of further initiating events following the first 6 weeks of treatment is sufficient to prevent tumour formation.

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