SHORT COMMUNICATION

Curcumin modifies $Apc^{min}$ apoptosis resistance and inhibits 2-amino 1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) induced tumour formation in $Apc^{min}$ mice

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Curcumin, the active ingredient of the rhizome of *Curcuma longa*, promotes apoptosis and may have chemopreventive properties. This study investigates the effects of curcumin on apoptosis and tumorigenesis in male $Apc^{min}$ mice treated with the human dietary carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). Intestinal epithelial apoptotic index in response to PhIP treatment was approximately twice as great in the wild-type C57BL/6 APC$^{+/+}$ strain than in $Apc^{min}$ mice (3.7% $Apc^{+/+}$ versus 1.9% $Apc^{min}$; $P < 0.001$). PhIP promoted tumour formation in $Apc^{min}$ proximal small intestine (4.6 tumours per mouse, PhIP treated versus 2.1 tumours per mouse, control untreated; $P < 0.05$). Curcumin enhanced PhIP-induced apoptosis (4.0% curcumin + PhIP versus 2.1% PhIP alone; $P < 0.01$) and inhibited PhIP-induced apoptosis in the proximal small intestine of $Apc^{min}$ mice (2.2 tumours per mouse, curcumin + PhIP versus 4.6 tumours per mouse PhIP alone; $P < 0.05$). This study shows that the $Apc^{min}$ genotype is associated with resistance to PhIP-induced apoptosis in intestinal epithelium. Curcumin attenuates $Apc^{min}$ resistance to PhIP-induced apoptosis and inhibits PhIP-induced tumorigenesis in proximal $Apc^{min}$ mouse small intestine.

Introduction

Heterocyclic amines (HCA) are formed by the cooking of meat and comprise an important class of human dietary carcinogens. PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) is the most abundant HCA in cooked meat (1) and is bioactivated to its genotoxic N-hydroxy derivative (2) by cytochrome P4501A2 (3). The intestinal or colonic epithelia are primary targets for dietary PhIP, which are metabolically competent for PhIP bioactivation (4) and may be particularly susceptible to genotoxic injury (2).

DNA damage resulting from dietary PhIP exposure may be converted to characteristic mutations in the adenomatous polyposis coli (APC) tumour suppressor gene (5). APC mutation is an early event in the evolution of familial adenomatous polyposis (FAP) (6) and sporadic human colorectal cancer (7), and promotes intracellular accumulation of β-catenin, increased expression of the AP-1 transcription complex (8) and a phenotype with altered apoptosis (9). The $Apc^{min}$ heterozygous mouse carries a germline nonsense mutation in codon 850 of $Apc$ (10) and mutational inactivation of the second $Apc$ allele promotes adenoma formation (11). These mice develop, on average, >50 adenomas over the entire length of the intestinal tract. Like FAP, $Apc^{min}$ is an autosomal dominant trait. Intrapерitoneal injection of PhIP has been shown to significantly increase the numbers of small tumours and cystic crypts in the proximal section of the small intestine of male $Apc^{min}$ mice, and the number of aberrant crypt foci in the colons of both males and females (12). In addition, neonatal exposure to PhIP directly or via breast milk resulted in an increase in tumour number in both small intestine and colon of $Apc^{min}$ mice (13).

Regulatory pathways of apoptosis may provide novel molecular targets for chemoprevention (9,14). Curcumin [1,7-bis (4-hydroxy-3-methoxyphenyl) 1,6-heptadiene-3,5-dione], the major pigment derived from turmeric, inhibits the growth of colon adenocarcinoma cell lines HT29 and HCT15 (15), suppresses activation of the transcription factor NFκB (16,17) and promotes apoptosis in neoplastic cells in vitro (18,19) and in rat colonic epithelium in vivo (20). Administration of curcumin in the diet suppresses azoxymethane-induced tumour incidence and multiplicity in male F344 rats (21) and inhibits benzol[a]pyrene-induced forestomach tumours and 12-O-tetradecanoylphorbol-13-acetate-induced skin tumours in mice (22). In addition, dietary curcumin has been shown to promote apoptosis and inhibit spontaneous tumour formation in $Apc^{min}$ mice (23). Development of a role for curcumin in clinical chemoprevention may be enhanced by studies in carcinogenesis models which mimic human disease. In spite of differences in the distribution of tumours between FAP patients and $Apc^{min}$ mice, the $Apc^{min}$ model is considered a useful one for the study of both FAP and sporadic intestinal neoplasms and has previously been used to assess the chemopreventive potential of agents such as aspirin (24) and sulindac (25).

In this study we assessed the effects of PhIP and curcumin on tumorigenesis and regulation of apoptosis in the $Apc^{min}$ mouse intestine. Our findings further support a role for curcumin in chemopreventive strategies against colorectal cancer.

Materials and methods

Chemicals and reagents

PhIP was obtained from NARD Pharmaceuticals (Amagasaki, Japan) and curcumin was obtained from Sigma-Aldrich (Dorset, UK). All other reagents were readily available commercially.

Animals

Four-week-old $Apc^{min}$ heterozygous C57BL/6J male mice and their male wild-type $Apc^{+/+}$ littermates were used in the study. Calculations of animal numbers were based upon previous studies of curcumin effects on azoxymethane colonic tumorigenesis where curcumin treatment reduced tumour number by 40% (20). On the assumption of comparable chemoprevention by curcumin in a PhIP tumorigenesis model, 10 animals in each group were calculated to provide 80% power for detection of a 40% difference of polyp number, with...
95% confidence. Animal numbers were kept to this minimum in accordance with the United Kingdom Coordinating Committee on Cancer Research guidelines (26).

Genotyping
Tail DNA genotyping was carried out by allele specific PCR (27). Tail tips were finely minced and suspended in 1 ml PCR digestion buffer (50 mM KCl, 10 mM Tris–HCl pH 7.5, 5 mM MgCl₂, 0.45% Nonidet P40, 0.45% Tween 20, 0.1 mg/ml gelatin). Aliquots of 10 μl of 20 mg/ml proteinase K were then added before digestion at 55°C for 2 h. Proteinase K was inactivated by incubation at 95°C for 10 min, then samples were centrifuged at 12 000 g for 1 min and the supernatants removed and stored at –20°C until PCR amplification. Genomic DNA (2 μl of the proteinase K digest) was amplified in a 25 μl reaction volume containing final concentrations of 12.5 μM APC-specific primers (fwd, 5′-TGACTACTCTTCAAGACCTTGCTTCTCTCATTGATTCTGATTCTCTGAGAAAGACAGAAGC), 50 mM Tris–HCl, 2.5 mM MgCl₂, 200 μM dNTPs, 0.01 U/μl Taq polymerase. Amplification was carried out for 4 min at 94°C followed by 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min before a final extension at 72°C for 10 min. An aliquot (10 μl) of the PCR reaction was then digested with HindIII (1 U) in a final volume of 20 μl at 37°C for 1 h. Wild-type (123 bp) and mutated (144 bp) bands were separated on an 8% acrylamide gel and visualized after staining with ethidium bromide.

Treatment regimes
The pelleted AIN-76 diet which lacks carcinogens was used as control and vehicle diet for delivery of curcumin (2000 p.p.m.) and PhIP (300 p.p.m.). Four groups of 10 Apcmin and wild-type Apc+/+ C57BL/6J mice received (i) control, (ii) curcumin only, (iii) PhIP only and (iv) curcumin + PhIP diets, respectively for 10 weeks. Animals were weighed weekly and checked daily for signs of ill health. Each animal was given 6 g diet daily. At 70 days, curcumin treatment were observed on PhIP-induced apoptosis animals were killed by CO2 inhalation and their intestinal tracts were removed in blocks for much of the variation of human cancer incidence may be associated with dietary differences (28). Meat intake has been implicated in the aetiology of colorectal cancer and heterocyclic amines, including PhIP, have come under close scrutiny (3). Cancer risk may also be ameliorated by dietary agents. Improved understanding of interactions between pro and anticarcinogens may enhance chemoprevention strategies.

Discussion
Much of the variation of human cancer incidence may be associated with dietary differences (28). Meat intake has been implicated in the aetiology of colorectal cancer and heterocyclic amines, including PhIP, have come under close scrutiny (3). Cancer risk may also be ameliorated by dietary agents. Improved understanding of interactions between pro and anticarcinogens may enhance chemoprevention strategies.

Results
Effects of dietary PhIP on intestinal epithelial apoptosis in wild-type Apc+/+ and Apcmin mice
Rates of ISEL detected apoptosis were compared in proximal, middle and distal thirds of Apc+/+ and Apcmin mouse small intestine. No differences of apoptosis were observed between untreated Apc+/+ and Apcmin mucosa (Table I). Kruskal–Wallis analysis of variance demonstrated significant differences of apoptosis between treatment groups (P < 0.001). PhIP induced epithelial apoptosis in all three anatomical levels of the small intestine in both mouse strains (Figure 1), although higher levels were observed in APC+/+ than in Apcmin mice (P < 0.001). Lower levels of PhIP-induced apoptosis were observed in Apcmin adenomas than in Apcmin unaffected mucosa (Table I) (P < 0.001).

Dietary PhIP increased tumour formation in the proximal third of Apcmin mouse small intestine (P < 0.05; Table II). Tumour size was greater in PhIP-treated than control Apcmin mice (P < 0.001; Table III). No tumours were seen in the intestines of any wild-type Apc+/+ mice.

Effects of curcumin on PhIP-induced apoptosis and tumour formation
Curcumin treatment enhanced PhIP-induced apoptosis (P < 0.01; Table I) and inhibited PhIP-induced tumorigenesis in the proximal small intestine of Apcmin mice. Tumour number (P < 0.05) was lower in curcumin + PhIP versus PhIP-treated Apcmin mice (Table II). No effects of curcumin treatment were observed on PhIP-induced apoptosis or tumour formation in Apcmin mid or distal small intestine. No effects of curcumin treatment were observed on PhIP-induced apoptosis in Apc+/+ mouse small intestine.

Discussion
Much of the variation of human cancer incidence may be associated with dietary differences (28). Meat intake has been implicated in the aetiology of colorectal cancer and heterocyclic amines, including PhIP, have come under close scrutiny (3). Cancer risk may also be ameliorated by dietary agents. Improved understanding of interactions between pro and anticarcinogens may enhance chemoprevention strategies.

In this study we show that dietary administration of curcumin inhibits PhIP-induced tumorigenesis and promotes apoptosis in the proximal section of the small intestine. In addition, induction of apoptosis following administration of PhIP is shown to be more pronounced in the normal intestinal mucosa of wild-type mice than in that of Apcmin mice, suggesting that the APC status of a cell may have an effect on its ability to respond to PhIP-induced DNA damage by inducing apoptosis. Furthermore, we show that in proximal small intestine this resistance to PhIP-induced apoptosis in the Apcmin mouse can be reversed by administration of curcumin in the diet.

The increase in tumour number in only the proximal part of the small intestine of Apcmin mice after exposure to PhIP is in accordance with a previous study which showed that administration of PhIP by intraperitoneal injection resulted in a similar region-specific effect, although only an increase in the number of small tumours was observed (12). We also show that PhIP is associated with increased tumour size, an effect again only seen in proximal small intestine. Mechanisms responsible for this regional specificity of PhIP are unclear. Our results suggest that it is not due to regional differences in the mutagenic effect of PhIP since the rate of PhIP-induced apoptosis is not significantly altered along the length of the small intestine of both Apcmin and Apc+/+ mice. Indeed, it has previously been shown that there is no difference in mutability of PhIP between the proximal and distal section of the small intestine of mice exposed to the carcinogen (29). PhIP-induced
Apc show apoptotic cells. number of tumours in the proximal small intestine of in situ Apoptosis in (4) Curcumin treatment enhanced PhIP apoptosis in (b) PhIP treatment induced higher levels of apoptosis in uninvolved (a) PhIP treatment induced higher levels of apoptosis throughout the small intestine in (c) Apc Apcmin apoptotic indices of normal mucosa and adenomas of Table I.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Diet</th>
<th>Proximal SI</th>
<th>Middle SI</th>
<th>Distal SI</th>
<th>Total SI</th>
<th>Adenomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apc+/+</td>
<td>Control</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td>Apc+/+</td>
<td>Curcumin</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.2</td>
<td>0.2 ± 0.2</td>
<td>0.1 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td>Apc+/+</td>
<td>PhIP</td>
<td>3.7 ± 2.0a</td>
<td>3.9 ± 1.4a</td>
<td>3.4 ± 2.0a</td>
<td>3.7 ± 1.7b</td>
<td>-</td>
</tr>
<tr>
<td>Apcmin</td>
<td>Control</td>
<td>0.2 ± 0.3</td>
<td>0.2 ± 0.2</td>
<td>0.4 ± 0.4</td>
<td>0.2 ± 0.2</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>Apcmin</td>
<td>Curcumin</td>
<td>0.4 ± 0.6</td>
<td>0.3 ± 0.1</td>
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<td>0.2 ± 0.3</td>
<td>0.1 ± 0.1</td>
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<tr>
<td>Apcmin</td>
<td>PhIP</td>
<td>2.1 ± 1.7a</td>
<td>1.6 ± 0.6a</td>
<td>2.2 ± 1.0b</td>
<td>2.0 ± 0.9b</td>
<td>0.4 ± 0.3b</td>
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<tr>
<td>Apcmin</td>
<td>Curcumin + PhIP</td>
<td>4.0 ± 1.3c</td>
<td>2.3 ± 1.4</td>
<td>2.0 ± 1.4</td>
<td>2.9 ± 1.6</td>
<td>0.6 ± 0.4</td>
</tr>
</tbody>
</table>

*PhIP treatment induced higher levels of apoptosis throughout the small intestine in Apc+/+ versus Apcmin intestinal mucosa (P < 0.001 by Mann–Whitney test).

Apcmin of wild-type mice than in that of heterozygous mutant Apcmin mice which in turn was greater than in homozygous mutant Apcmin adenomas, suggesting that resistance to PhIP-induced apoptosis may be related to the mutational status of Apc alleles in the cell. Similarly, expression of APC in human colorectal cancer cells containing endogenous inactive APC alleles inhibits cell growth by the induction of apoptosis (9).

Curcumin has previously been shown to inhibit tumorigenesis in animal models (21,22) and to promote apoptosis both in vivo and in vitro (18–20). Curcumin given to female Apcmin mice resulted in a decrease in intestinal tumour number and an increase in apoptosis (23). In the present study we administered a 2-fold higher dose of curcumin to male Apcmin mice, but found no effect on spontaneous apoptosis or tumour number. It is possible that the sex of test animals could influence the response to curcumin. However, when given in combination with PhIP, curcumin significantly reduced the number of tumours in the proximal small intestine of Apcmin mice compared with PhIP alone, so that tumour numbers were similar to those in mice receiving control diet. This decrease in tumour number was accompanied by a corresponding increase in apoptotic index. In contrast, in the middle and distal sections of the small intestine, apoptotic indices in PhIP/curcumin-treated animals remained unchanged from those in the cell.

### Table I. Apoptotic indices of normal mucosa and adenomas of Apc+/+ and Apcmin mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Diet</th>
<th>Proximal SI</th>
<th>Middle SI</th>
<th>Distal SI</th>
<th>Total SI</th>
<th>Adenomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apc+/+</td>
<td>Control</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td>Apc+/+</td>
<td>Curcumin</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.2</td>
<td>0.2 ± 0.2</td>
<td>0.1 ± 0.2</td>
<td>-</td>
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<tr>
<td>Apc+/+</td>
<td>PhIP</td>
<td>3.7 ± 2.0a</td>
<td>3.9 ± 1.4a</td>
<td>3.4 ± 2.0a</td>
<td>3.7 ± 1.7b</td>
<td>-</td>
</tr>
<tr>
<td>Apcmin</td>
<td>Control</td>
<td>0.2 ± 0.3</td>
<td>0.2 ± 0.2</td>
<td>0.4 ± 0.4</td>
<td>0.2 ± 0.2</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>Apcmin</td>
<td>Curcumin</td>
<td>0.4 ± 0.6</td>
<td>0.3 ± 0.1</td>
<td>0.1 ± 0.2</td>
<td>0.2 ± 0.3</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Apcmin</td>
<td>PhIP</td>
<td>2.1 ± 1.7a</td>
<td>1.6 ± 0.6a</td>
<td>2.2 ± 1.0b</td>
<td>2.0 ± 0.9b</td>
<td>0.4 ± 0.3b</td>
</tr>
<tr>
<td>Apcmin</td>
<td>Curcumin + PhIP</td>
<td>4.0 ± 1.3c</td>
<td>2.3 ± 1.4</td>
<td>2.0 ± 1.4</td>
<td>2.9 ± 1.6</td>
<td>0.6 ± 0.4</td>
</tr>
</tbody>
</table>

*PhIP treatment induced higher levels of apoptosis in uninvolved Apcmin intestinal mucosa than in Apcmin adenomas (P < 0.001 by Mann–Whitney test).

*Curcumin treatment enhanced PhIP apoptosis in Apcmin proximal small intestinal mucosa (P < 0.01 by Mann–Whitney test). Apoptosis values expressed as percentages.

### Table II. Small intestinal adenoma formation in Apcmin mice

<table>
<thead>
<tr>
<th>Diet</th>
<th>Proximal SI</th>
<th>Middle SI</th>
<th>Distal SI</th>
<th>Total SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.1 ± 2.5</td>
<td>6.0 ± 5.9</td>
<td>6.1 ± 5.2</td>
<td>14.1 ± 11.6</td>
</tr>
<tr>
<td>Curcumin</td>
<td>1.5 ± 2.2</td>
<td>6.5 ± 6.2</td>
<td>5.1 ± 6.7</td>
<td>13.2 ± 15.1</td>
</tr>
<tr>
<td>PhIP</td>
<td>4.6 ± 2.7a</td>
<td>7.8 ± 10.2</td>
<td>4.6 ± 10.3</td>
<td>17.1 ± 22.5</td>
</tr>
<tr>
<td>Curcumin + PhIP</td>
<td>2.2 ± 1.5b</td>
<td>6.7 ± 6.6</td>
<td>7.4 ± 5.6</td>
<td>16.3 ± 12.3</td>
</tr>
</tbody>
</table>

*PhIP promotes adenoma formation in Apcmin proximal small intestine (P < 0.05 by Mann–Whitney test).

*Curcumin inhibits PhIP tumorigenesis in Apcmin proximal small intestine (P < 0.05 by Mann–Whitney U test).

### Table III. Apcmin adenoma size

<table>
<thead>
<tr>
<th>Diet</th>
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<th>Middle SI</th>
<th>Distal SI</th>
<th>Total SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.5 ± 0.6</td>
<td>2.0 ± 0.8</td>
<td>1.7 ± 0.6</td>
<td>1.9 ± 0.7</td>
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<tr>
<td>Curcumin</td>
<td>2.9 ± 1.0</td>
<td>2.0 ± 0.7</td>
<td>1.8 ± 0.9</td>
<td>2.1 ± 0.9</td>
</tr>
<tr>
<td>PhIP</td>
<td>3.8 ± 1.7a</td>
<td>2.1 ± 0.8</td>
<td>2.1 ± 1.0</td>
<td>2.8 ± 1.5</td>
</tr>
<tr>
<td>PhIP + curcumin</td>
<td>3.2 ± 1.1</td>
<td>1.9 ± 1.0</td>
<td>1.7 ± 0.6</td>
<td>2.0 ± 1.1</td>
</tr>
</tbody>
</table>

*PhIP treatment is associated with increased tumor size in Apcmin proximal small intestine (P < 0.001 by Mann–Whitney test). Curcumin and PhIP treatment is associated with lower adenoma size than PhIP treatment alone in Apcmin small intestine (P < 0.001 by Mann–Whitney test).

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Fig. 1. Apoptosis in Apc+/+ and Apcmin mucosa. Apoptosis in (A) unaffected APC+/+ mucosa, (B) Apcmin mucosa, (C) Apcmin adenoma after PhIP treatment. Apoptosis was detected by in situ end labelling. Arrows show apoptotic cells.
receiving PhIP alone. This suggests that the protective effect of curcumin against PhIP-induced tumorigenesis is achieved, at least in part, by the reversal of resistance of Apc<sup>min</sup> intestinal epithelium to PhIP-induced apoptosis. Curcumin treatment may inhibit apoptosis resistance associated with Apc mutation through alteration of the equilibrium of NFκB and p53. These transcription factors have been shown to regulate each other’s ability to stimulate gene expression in a process that is controlled by their relative levels (30). Curcumin inhibits TNF-induced NFκB activation in human intestinal cell lines (31), and has been shown to increase p53 expression in basal cell carcinoma cells (32). Activation of NFκB by DNA damage appears to be mediated through the same signalling intermediates as activation by cytokines such as TNF. Both result in the induction of IkB kinase dependent degradation of IkB in the cytoplasm, resulting in the release and activation of NFκB (33). This pathway may be implicated in curcumin effects on PhIP-induced apoptosis and tumorigenesis in Apc<sup>min</sup> mice.

In conclusion, our findings support a role for curcumin in the inhibition of adenoma formation and progression, from a background of heterozygous APC mutant epithelium. These findings may facilitate chemopreventive strategies, in the human corollary of FAP.

Acknowledgements

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References

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