RESEARCH ARTICLE

Dietary intake of rosmarinic acid by *Apc*^{Min} mice, a model of colorectal carcinogenesis: levels of parent agent in the target tissue and effect on adenoma development

Ankur Karmokar, Timothy H. Marczylo, Hong Cai, William P. Steward, Andreas J. Gescher and Karen Brown

Department of Cancer Studies and Molecular Medicine, University of Leicester, Leicester, UK

Received: September 12, 2011
Revised: December 13, 2011
Accepted: January 9, 2012

Scope: Rosmarinic acid (RA), a constituent of culinary herbs is considered to possess cancer chemopreventive properties. It has been shown to inhibit the development of cancer in preclinical models but data are conflicting and whether it can protect against gastrointestinal malignancies in vivo has not been examined. This study aimed to investigate the effect of RA on the development of intestinal adenomas in the *Apc*^{Min} mouse model of colorectal carcinogenesis, and to correlate efficacy with levels of RA achieved in the plasma and gastrointestinal tract.

Methods and results: RA inhibited the growth of APC10.1 cells derived from *Apc*^{Min} mouse adenomas, with an IC_{50} of 43 μM. Consumption of dietary RA (0.3%) by *Apc*^{Min} mice for 8 weeks post weaning decreased adenoma burden by ∼35%, but the difference from controls was not significant. Although RA significantly decreased the frequency of large adenomas, the number of small ones increased. Using a novel validated HPLC assay, average levels of RA in the plasma and intestinal mucosa of these mice were found to be 1.1 μM and 38 nmol/g, respectively.

Conclusion: Chronic consumption of RA furnished quantifiable levels of parent compound in the plasma and intestinal tract of *Apc*^{Min} mice and may slow adenoma development.

Keywords:
Apc^{Min} mouse / Cancer chemoprevention / Colorectal cancer / HPLC / Rosmarinic acid

1 Introduction

Rosmarinic acid [RA, (2*R*)-2-(2″E″)-3-(3,4-dihydroxyphenyl)-1-oxo-2-propenyloxy-3-(3,4-dihydroxyphenyl) propanoic acid, for structure see Fig. 1A] is an ester of caffeic and 3,4-dihydrophenylacetic acids. It occurs abundantly in plants of the Lamiaceae family, including commonly used culinary herbs such as lemon balm, rosemary, oregano, sage, thyme and peppermint. In intact plants, RA is thought to accumulate constitutively, providing a defence against stress [1]. RA exerts several biological activities in vitro, which are potentially beneficial; it has been described as antiviral, antibacterial, antioxidant, anti-inflammatory and antiallergenic [2]. Moreover, RA has recently been reported to extend lifespan in *Caenorhabditis elegans*, in a concentration-dependent manner, suggestive of a hormetic dose-response [3]. RA and extracts of herbs containing RA have long been suspected to exert anticarcinogenic properties. RA displayed antimutagenic activity, as assessed by the micronucleus assay, in Swiss mice [4]. An extract of *Prunella vulgaris*, which contains RA together with other polyphenolic carboxylic acids, inhibited the growth of Lewis lung tumour cells implanted in mice [5]. In addition, the reduction in tumorigenesis in a murine two-stage skin carcinogenesis model with topical application of *Perilla frutescens* extract has also been attributed to the presence of RA [6]. As a single agent, RA compromised the migratory ability of MDA-MB-231BO bone-homing breast cancer cells in vitro [7]. In contrast to these protective effects, RA at up to 139 μM (50 μg/mL) increased the growth rate of K-562 leukaemia

Correspondence: Dr. Karen Brown, Department of Cancer Studies and Molecular Medicine, University of Leicester, Leicester LE2 7LX, UK
E-mail: kb20@le.ac.uk
Fax: +44-116-223-1855

Abbreviations: RA, rosmarinic acid

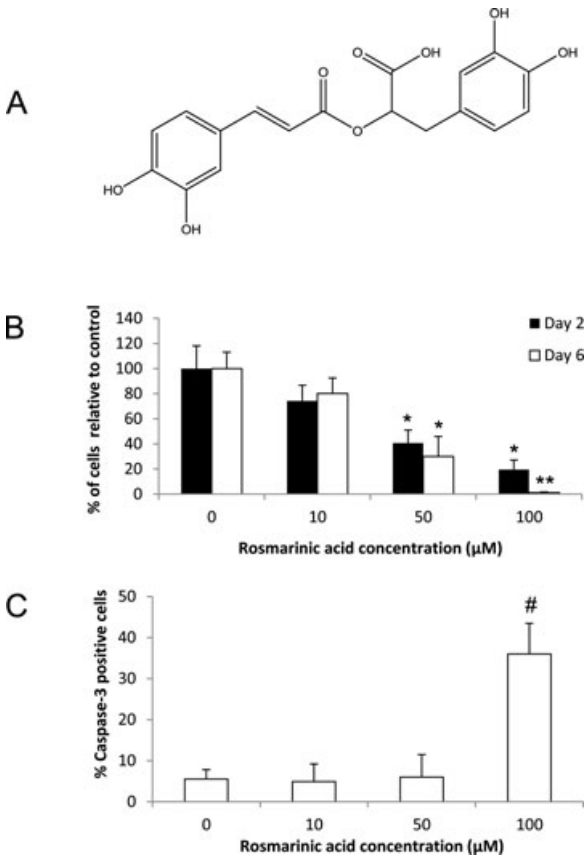

Figure 1. Structure of RA (A) and effect on growth (B) and apoptosis (C) of APC10.1 cells. Cells were exposed to RA at 10, 50 or 100 μM. Effects on cell number were assessed on days 2 and 6 of incubation, whilst the level of cleaved caspase-3, which is an indicator of apoptosis was measured by FACS analysis on day 6 only. Results, which are expressed as percentage of control incubations omitting RA, are the mean ± SD of three experiments, each conducted in triplicate. * and ** indicate the proportion of cells remaining is significantly lower than numbers in control incubations, where $p < 0.01$ and <0.001 respectively. # denotes that the degree of caspase-3 staining is significantly higher than the level in solvent control incubations ($p < 0.01$).

and MCF-7 breast carcinoma cells in vitro as much as two-fold [8]. Such a proliferative effect was not seen in cultures of the human-derived colorectal carcinoma cells HCT-15 and CO-115, in which RA concentrations of up to 100 μM failed to alter growth, although it induced apoptosis [9].

RA is a tetraphenol, and many dietary-derived polyphenols have been shown to delay colorectal carcinogenesis in rodent models [10]; in fact, the cancer chemopreventive activity of some of these agents, such as curcumin and resveratrol, has been so promising they are currently undergoing clinical trials [11–13]. Whether RA can protect against gastrointestinal cancers in vivo has not yet been explored. Therefore, the aim of this study was to investigate the effect of dietary RA in the *Apc*^{Min} mouse, a model of colorectal carcinogenesis frequently employed in the preclinical selection of chemopreventive agents for further development [14]. Initially, the ability of RA to affect the survival of APC10.1 cells was assessed. The use of these cells, which were originally derived from *Apc*^{Min} mouse adenomas [15], has been proposed as an in vitro screen to predict effects of novel agents on *Apc*^{Min} adenoma development in vivo [16]. Additionally, the concentration of RA achievable in murine plasma and gastrointestinal mucosa after dietary ingestion was determined; this required development and validation of a novel HPLC assay. Finally, the consequence of RA consumption on adenoma development was evaluated in *Apc*^{Min} mice exposed to a dietary dose of 0.3%. Overall, the work was designed to help adjudge whether RA warrants further development as a putative colorectal cancer chemopreventive agent.

2 Materials and methods

2.1 Chemicals

Rosmarinic acid (purity 97%), Caffeic acid (purity 98%) and Coumaric acid (purity 98%) were obtained from Sigma-Aldrich (Poole, UK) in amorphous form. HPLC grade methanol, acetonitrile, acetone and 98% formic acid were purchased from Fisher Scientific Ltd. (Loughborough, UK). Water for analysis was generated from a Nano-pure water purification system (Barnstead, UK). Human plasma was obtained from the National Blood Transfusion Centre (Sheffield, UK).

2.2 Cell culture

APC10.1 cells were kindly provided by Dr. Carla De Giovanni (University of Bologna, Italy). Cells were cultured in low glucose media (Sigma, UK) supplemented with 20% fetal calf serum (Invitrogen, UK). RA (10, 50 or 100 μM) was added once at the start of the incubation, and cells were incubated (in triplicate) for up to 6 days. Cell numbers were determined on days 2 and 6 using a Z2 Coulter Particle Count and Size Analyser (Beckman Coulter, UK). IC_{50} values were calculated from the plot of cell number as percentage of control *versus* agent concentration at day 6, when cells were still in a linear growth phase. Values are the mean ± SD of three separate experiments.

2.3 Measurement of cleaved caspase-3 staining by flow cytometry

Cleaved caspase-3 was analysed in RA treated cells as a measure of apoptosis. Cells were first fixed in methanol and permeabilised with 0.5% Tween-20 in phosphate buffered saline (PBS), then incubated with a primary rabbit monoclonal cleaved caspase-3 antibody (Cell Signalling, UK) at a dilution of 1:10 for 40 min in the dark at room temperature.

The cells were washed twice (3% bovine serum albumin in PBS) and incubated with a secondary anti-rabbit conjugated Alexa Fluor@594 antibody (Invitrogen, UK) at a dilution of 1:50 for 30 min at 4°C. As a negative control, single staining with the secondary antibody was performed to eliminate any background staining. After the final incubation, cells were again washed twice before analysis on a BD FACS Aria II instrument (BD Biosciences).

2.4 Animal experiments

The animal experiment was carried out under animal project license PPL40/2496, granted to Leicester University by the UK Home Office. The experimental design was vetted by the Leicester University Local Ethical Committee for Animal Experimentation and met the standards required by the UKCCCR guidelines [17]. C57BL/6J Min/+ (*Apc*^{Min}) mice were bred in Leicester University Biomedical Services using animals originally obtained from Jackson Laboratory (Bar Harbor, ME, USA), and the *Apc*^{Min} genotype was confirmed by polymerase chain reaction [14]. Groups of 13–14 mice (male:female ~1:1) received standard AIN 93G diet or AIN diet supplemented with RA (0.3% in the diet, translating to a dose of ~9 mg per mouse per day, 360 mg/kg) from weeks 4 to 12 of the animals' lives. The choice of dose was based on the fact that dietary doses of naturally occurring polyphenols that attenuate colorectal carcinogenesis in preclinical models are generally of this order of magnitude; an example is curcumin in *Apc*^{Min} mice [10, 18]. Animals were killed by cardiac exsanguination under terminal anaesthesia, and the intestinal tract was removed and flushed with PBS. Plasma was obtained by centrifugation of blood. The multiplicity, location and size of intestinal adenomas were recorded as described previously [10] before mucosa samples were obtained by scraping with a spatula. All samples were flash frozen in liquid nitrogen and then were kept at −80°C until analysis.

2.5 Standard solutions

The RA stock solution (1 mg/mL) was prepared fresh in HPLC grade methanol on the day of analysis and diluted serially to provide working solutions in the range of 100–1000 ng/mL. Fixed volumes of working solutions were added to aliquots of plasma or homogenate of small intestinal mucosa from control animals. RA concentrations in the calibration standard and quality control samples were in the range of 50–1000 ng/mL and 100–5000 ng/mL for plasma and intestinal mucosa, respectively.

2.6 Sample preparation

Prior to analysis, samples of intestinal mucosa or plasma were thawed to room temperature. An aliquot (50 μL) of mouse intestinal mucosa homogenate (1:2 w/v ratio of mucosa and 1.15% potassium chloride,) was mixed with 950 μL of 1:1 (v/v) ice-cold acetone and methanol. The mixture was vortexed and left (−20°C, 20 min) to facilitate protein precipitation, then centrifuged (13 227 × g, 15 min). The clear supernatant was transferred to an Eppendorf tube and evaporated to dryness under N_2. The residue was reconstituted in methanol (70 μL) and injected onto the HPLC column (injection volume 50 μL). RA was extracted from spiked plasma using a solid-phase extraction (SPE) method. One volume of the plasma from mice that received RA or plasma from control mice spiked with RA was mixed with one volume of 5% *ortho*-phosphoric acid. The sample was loaded on an Oasis HLB cartridge (1 mL, Waters, Elstree, UK), which was pre-conditioned with 1 mL of methanol followed by 1 mL of de-ionized water. After washing with 1 mL of 20% methanol, elution was achieved with 1 mL of 1:1 (v/v) methanol:acetone. The eluate was dried under N_2, and the residue was reconstituted in 70 μL of 100% methanol. An aliquot (50 μL) of each sample was injected onto the HPLC column for analysis.

2.7 HPLC analysis

The HPLC system consisted of a Varian Prostar 230 pump and a Prostar 410 autosampler, along with a Prostar 325 UV–visible detector (Varian Analytical Instrument, CA, USA). HPLC separation was carried out on an Atlantis 3 μm dC_{18} column (4.6 × 150 mm, Waters, Elstree, UK) at a flow rate of 1.5 mL/min using a linear gradient from 95% mobile phase A (0.1% formic acid in water) and 5% mobile phase B (0.1% formic acid in acetonitrile) to 60% A and 40% B over 20 min. UV detection was at 330 nm, the maximal absorption wavelength of RA.

2.8 Method development

The methods for RA quantitation were developed and validated using murine gastrointestinal mucosa and human, rather than mouse plasma, due to limited availability of the latter. SPE was chosen over liquid extraction for plasma samples as it resulted in ~10-fold higher extraction efficiency. Recovery of RA from mucosa using liquid extraction exceeded >50%. Validation was performed in accordance with FDA guidelines (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf).

2.9 Statistical analysis

For determining the significance of data, a two sample paired Student's *t*-test was performed using Excel. A *p*-value of less than 0.05 (95% confidence interval) was considered as a significant difference between two sets of data.

3 Results

3.1 Effect of RA on APC10.1 cell growth

Exposure of APC10.1 cells for two days to concentrations of RA exceeding 10 μM had a dramatic effect on cell numbers; despite the fact RA was undetectable in cell culture media beyond 24 h (data not shown and 19) this effect persisted and was even more pronounced after 6 days of incubation (Fig. 1B). The IC_{50} for growth inhibition calculated on day 6, when the effect was maximal, was 42.8 ± 2.0 μM (mean ± SD, n = 3), and incubation with 100 μM RA reduced cell numbers by ~98% relative to the solvent-treated control cells. This growth inhibition was accompanied by a significant increase in apoptosis at the highest concentration, as reflected by cleaved caspase-3 staining measured using a FACS based assay (Fig. 1C). The extent of apoptosis detected after 6 days was ~5-fold greater in cells treated with 100 μM RA compared to background levels in control cells (27.8 ± 7.5% vs. 5.4 ± 2.3%, $p < 0.01$). These results suggest RA may have an effect on adenoma development in the *Apc*^{Min} mouse and warrants assessment of efficacy and pharmacokinetics in this model.

3.2 Validation of HPLC method for quantitation of RA

3.2.1 Selectivity and sensitivity

HPLC-UV chromatograms of extracts of murine plasma and gastrointestinal mucosa did not show any endogenous peak which might have interfered with the detection and quantitation of RA. The lower limits of RA quantitation were 140 nM (50 ng/mL) in plasma and 278 pmol/g (100 ng/g) for mucosa, with an acceptable signal-to-noise ratio of >8. Values for precision and accuracy at the lower limit of quantitation (LLOQ) were 13.3 and 101.5% and 12.6 and 91.5% for plasma and mucosa, respectively. These figures are in accordance with FDA guidelines that stipulate precision should be <20% and accuracy must be within the range 80–120% (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf) at the LLOQ. The limit of detection (LOD) of RA in plasma was 14 nM (5 ng/mL), which represents comparable sensitivity to published LC-MS/MS methods [20, 21].

3.2.2 Accuracy, precision and recovery

Accuracy and precision were calculated by performing five replicate analyses of plasma and mucosa samples spiked with RA at low, medium and high concentrations. The inter-day accuracy for the measured concentrations in spiked plasma and mucosa was 97–108% and 97–111% (Table 1), respectively, whilst the intra-day accuracy was 92–100% and 88–100%, respectively. The precision was <15% in either matrix. Recovery of RA was determined by comparing peak areas of spiked extracted samples with peak areas obtained by direct injection of standards. The average values for RA recovery from plasma and mucosa were 91 ± 6.9% and 52 ± 0.7%, respectively.

Table 1. Intra-day and inter-day accuracy and precision for determination of RA in mouse plasma and mucosa

Plasma	Concentration (ng/mL)	Accuracy (%)[a)]	Precision (% RSD)
Intra-day	50	98.9 ± 4.9	13.3
	100	96.6 ± 2.1	7.9
	1000	110.9 ± 0.01	9.8
Inter-day	50	97.4 ± 5.2	2.9
	100	108.8 ± 11.4	8.8
	1000	99.9 ± 0.1	14.7
Mucosa	Concentration (ng/g)	Accuracy (%)[a)]	Precision (% RSD)
Intra-day	100	88.3 ± 26.2	12.6
	1000	95.2 ± 6.5	7.8
	5000	100.1 ± 0.2	11.9
Inter-day	100	98.9 ± 15.3	12.1
	1000	96.6 ± 12.0	9.0
	5000	110.9 ± 10.0	12.0

a) Values are mean ± SD of $n = 5$ determinations. RSD, relative standard deviation.

3.2.3 Range and linearity

Calibration curves were constructed to cover the range of concentrations predicted to be achievable in mouse tissues and plasma following RA administration. The correlation between peak area and concentration was linear with $r^2 > 0.99$ for both plasma and mucosa at RA concentrations of 0.14–2.8 μM (0.05–1 μg/mL) and 0.28–55.6 nmol/g (0.1–20 μg/g), respectively. Regression equations from five independent experiments were $y = (6053 \pm 538)x - (36\,603 \pm 13\,608)$ for plasma and $y = (278 \pm 30)x - (18\,959 \pm 7243)$ for mucosa.

3.2.4 Stability

RA stability was assessed in the stock methanol solution as well as in stored plasma and mucosa. In stock methanol, the variation of RA content over a period of 24 h was <5%. Short-term stability was evaluated by analysing spiked, extracted samples (low, medium and high concentrations as listed below) kept in the autosampler at room temperature for up to 24 h, which represents the maximum time samples would be subject to these conditions during batch analysis. There was no evidence of degradation over time; the variation in RA content was <9% confirming samples are stable during the analysis procedure. Long-term stability was also

examined using spiked samples stored at −80°C for 7 days and extracted immediately prior to analysis. The variation in RA content when added to plasma at concentrations of 278 nM (100 ng/mL), 2.8 μM (1 μg/mL) and the LLOQ (140 nM, 50 ng/mL) was 5.8, 2.1 and 9.6%, respectively. The variation recorded for RA in the mucosa at concentrations of 278 pmol/g (100 ng/g), 2.8 nmol/g (1 μg/g) or 13.9 nmol/g (5 μg/g) was less than 6%. RA was therefore considered to be stable in stock solution and biological samples.

3.3 RA levels in murine plasma and gastrointestinal mucosa

In order to establish whether RA can inhibit intestinal adenoma development in vivo, *Apc*^{Min} mice were exposed to 0.3% RA in their diet, from 4 weeks of age. Control mice received unadulterated diet only. Extracts of plasma and intestinal mucosa obtained from the mice at termination of the experiment were analysed using the newly developed HPLC assay, to determine levels of RA achieved systemically and in the target tissue (Fig. 2). Plasma contained peaks corresponding to RA plus a number of potential metabolites not present in plasma from untreated animals. Two of the more polar species eluting before RA, were characterised as caffeic (peak a) and coumaric (peak b) acids, which are well established products of RA metabolism. Assignments were based on similarity of retention times to synthetic standards of the respective compounds (9.8 min for caffeic and 12.6 min for coumaric acid; results not shown) [22]. Peaks consistent with RA and caffeic acid were also seen in the mucosa extracts. Average steady-state concentrations of RA in the plasma and mucosa were 1.1 ± 1.4 μM (396 ± 504 ng/mL) and 37.7 ± 67.5 nmol/g (13.6 ± 24.3 μg/g), respectively. The high variability in tissue and plasma levels between mice may be attributed, at least in part, to the fact RA was freely available in the diet. Given that RA is rapidly absorbed from the gastrointestinal tract and has a relatively short plasma half-life in rodents [23,24], the levels detected may be heavily influenced by when the animal last ate and the amount consumed.

3.4 Effect of RA on *Apc*^{Min} adenoma development

The body weight of mice ingesting RA was not significantly different from those on control diet (Fig. 3), suggesting that RA did not affect food intake. Adenoma development was affected by RA consumption in a subtle fashion. RA decreased total adenoma burden measured throughout the small intestine and colon by 35% ($p = 0.06$), but had a greater influence in the colon, where adenoma volume was reduced by 54% ($p = 0.07$). However, both these effects just failed to reach statistical significance when compared to control mice (Fig 4A). Unexpectedly, the mean number of small adenomas (<1 mm diameter) present in the colon and small intestine was significantly elevated, from 7 adenomas per animal in those on the control diet to 16 in mice on RA (Fig. 4B). Conversely, the number of large adenomas (>3 mm) was halved, from an average of 1.6 per mouse in the control group to 0.8 in mice on RA ($p < 0.05$). There was no difference between mice consuming RA and those on control diet in the number of medium size adenomas (1–3 mm).

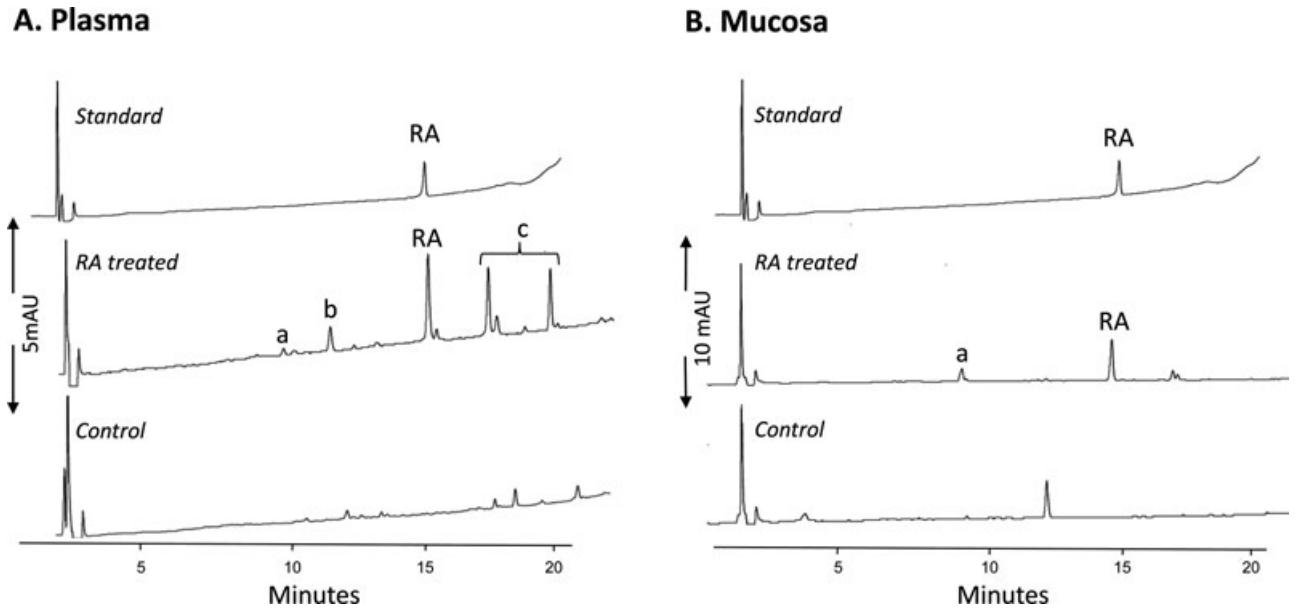

Figure 2. Representative HPLC-UV chromatograms of RA (500 ng/mL) in methanol (top traces), and extracts of plasma (A) or gastrointestinal mucosa (B) of mice that received control diet (bottom traces) or diet containing 0.3% RA (middle traces). Metabolite peaks with shorter retention times than RA may be caffeic (a) and coumaric acid (b), those with longer retention times may be methylated RA (c). AU = absorbance units.

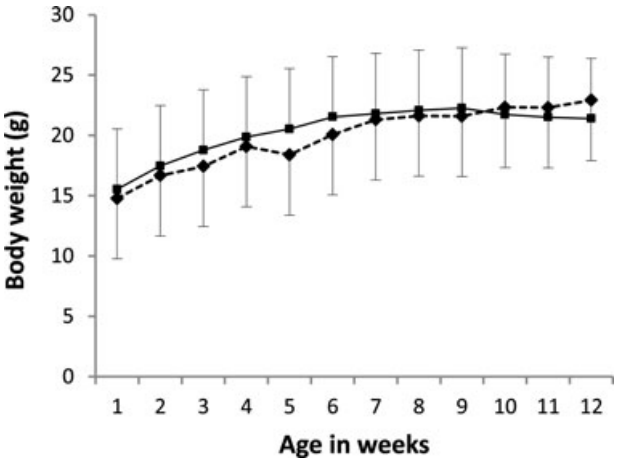

Figure 3. Body weight of *Apc*^{Min} mice which received AIN95G diet (rhombi, broken line) or diet containing RA (0.3%) (squares, solid line) for 8 weeks post weaning. Values are the mean ± SD of 13–14 animals per group.

4 Discussion

The results of this study suggest RA can potentially decelerate tumour development in the *Apc*^{Min} mouse, but the effect is relatively small. In mice which received RA at a dietary dose of 0.3%, tumour burden was reduced by 35%, but the difference compared to control mice did not reach statistical significance. Furthermore, RA consumption decreased the number of large adenomas, but increased the number of small adenomas. This observation suggests RA may inhibit the growth of established adenomas rather than preventing their formation; the increase in small tumours may therefore be due to the treatment restricting adenoma size to less than 1 mm in diameter. Consistent with an overall growth-compromising effect in vivo, RA reduced the proliferation of *Apc*^{Min} adenoma cells in vitro at concentrations which reportedly failed to affect the growth of human-derived colorectal cancer cells [9]. This activity was evident even though RA was unstable in cell culture media and was no longer detectable after 24 h, which suggests the initial exposure was sufficient

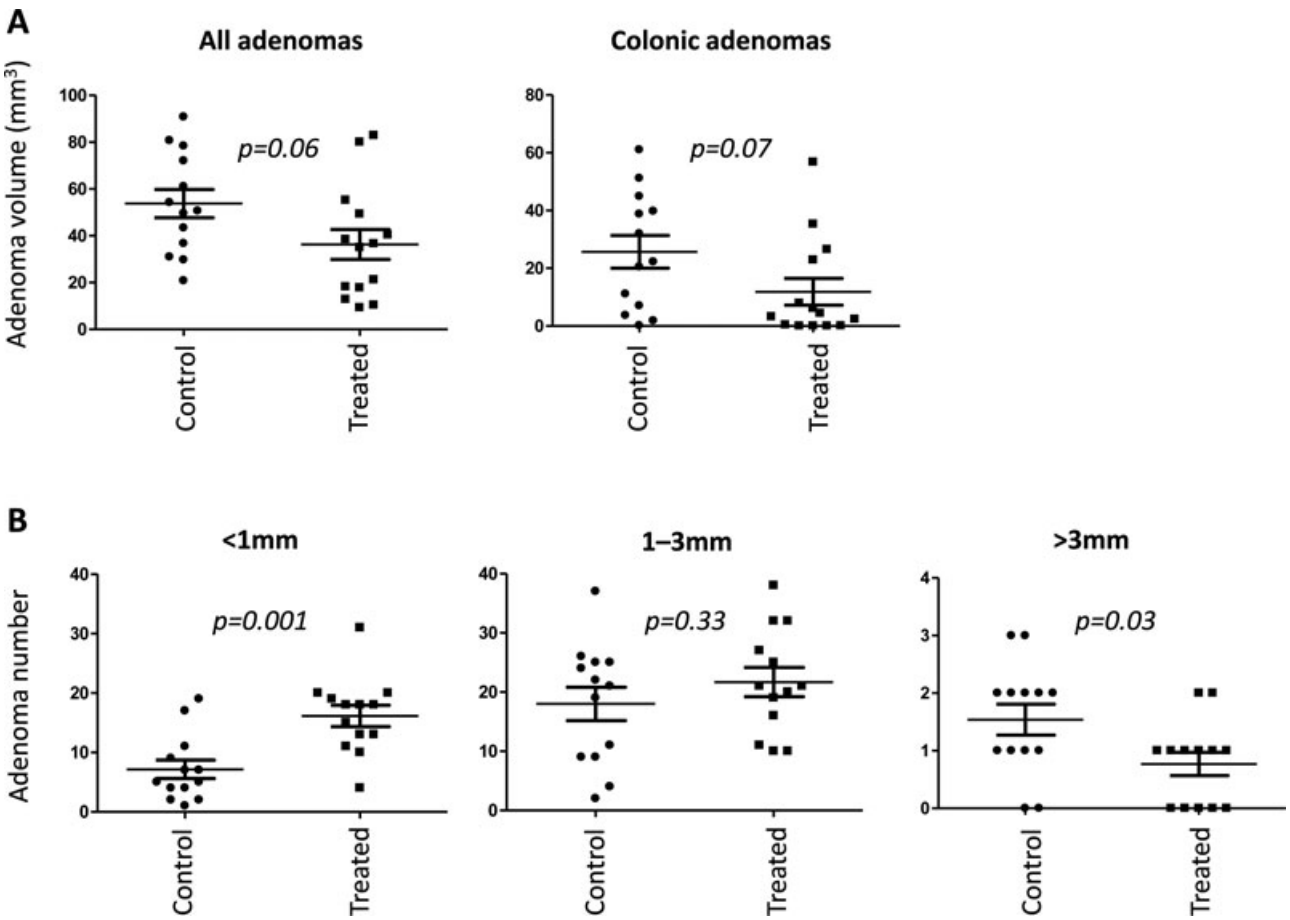

Figure 4. Total adenoma burden in the small intestine plus colon and in the colon alone (A) and number of small, medium and large sized adenomas (B) in *Apc*^{Min} mice that received AIN93G diet (control) or AIN fortified with RA (0.3%) (treated) from weaning to 12 weeks of age. Burden corresponds to the volume of all tumours per mouse. Bars reflect the mean ± SD of 13 mice per group.

to cause prolonged molecular changes resulting in greatly reduced cell numbers at day 6. Alternatively, RA degradation products may contribute to the activity, indeed, it has recently been demonstrated that it undergoes rapid oxidation in vitro to generate H_2O_2, which may itself elicit biological changes [19]. The inhibitory effect of RA on adenoma development shown here was subtle and inferior to that exerted by curcumin at a similar dietary dose, which significantly reduced Apc^{Min} adenoma number by 40% [10]. Other phytochemicals, including the anthocyanins oenocyanin and cyanidin-3-glucoside, plus the rice bran constituent tricin and its fully *O*-methylated analogue 3′,4′,5′,5,7-pentamethoxyflavone have also proven more potent with respect to reducing adenoma burden and/or total numbers in the Apc^{Min} model, when administered at a comparable dose [25–28]. However, examples also exist of putative cancer chemopreventive agents with less activity than RA in this model, such as resveratrol and apigenin [28,29]. Although only one dose level (0.3%) was chosen for evaluation in the present investigation, this is towards the upper limit of what is typically studied in the Apc^{Min} mouse for diet derived compounds and equates to an intake of over 2 g per day for a 70 kg person [30]. Even if this quantity was found to be safe in humans it is likely that ingestion of amounts exceeding this may be associated with reduced compliance, particularly in the chemoprevention setting, due to the number of capsules/tablets required to achieve this dose. For these reasons higher doses were not pursued in the Apc^{Min} mice, as it is unlikely they would be translatable to the clinic. Furthermore, increasing evidence suggests that diet-derived polyphenols such as resveratrol, may exhibit U-shaped dose-response curves for a variety of biological activities [31, 32]. Given the recent account of a hormetic response for the life-extension properties of RA in *C. elegans*, where concentrations of 200–300 μM were effective but lower concentrations were not and 600 μM actually decreased lifespan [3], it is conceivable that RA at doses below 0.3% might elicit a more robust inhibition of tumour development than that observed here. The concentrations generated in mouse tissues (∼1–185 μM, assuming 1 g is equivalent to 1 mL) approached the range that prolonged life in *C. elegans*; however, it is likely that different concentrations may prove optimal for each type of activity, whilst species differences will also play a role, it is therefore difficult to directly extrapolate regarding the ideal dose for cancer prevention. Wide-ranging efficacy studies with RA in the Apc^{Min} mouse model will shed definitive light on its potential ability to curtail adenoma development. Pharmacological properties of RA which may mediate anticarcinogenic activity include inhibition of cyclooxygenase (COX) enzyme activity [33], modulation of AP-1 activity and COX-2 gene expression [34], and inhibition of tumour necrosis factor (TNF)-alpha-induced NF-kappa B activation [35] and of extracellular signal-regulated kinase (ERK) phosphorylation [9]. RA has also been shown to protect cells against aflatoxin- and ochratoxin A-induced cytotoxicity by reducing the production of reactive oxygen species and apoptosis caused by the toxins [36]. Reassuringly, RA did not adversely affect Apc^{Min} mouse physiology in the present study, as borne out by the finding that RA consumption did not decrease mouse body weight.

For interpreting the activity of putative cancer chemopreventive agents in preclinical in vivo models and extrapolating to doses potentially useful in humans, it is pivotal to correlate pharmacologically active species in the target organ and general circulation with efficacy. To that end, suitable validated methods for measuring concentrations of agent in blood and tissues are required. Various analytical methods for the determination of RA in biological matrices of mammals have been published [20, 23, 37]. However they lack the sensitivity required for measurement of RA in mouse plasma, which is often only available in small quantities. The HPLC-UV method described here allows the rapid, selective and specific determination of RA levels in both murine plasma and intestinal mucosa. The average target organ concentration of RA detected in Apc^{Min} mice, 38 nmol/g (38 μM), is close to the IC_{50} for growth inhibition of APC10.1 adenoma cells derived from these animals (43 μM), which suggests an antiproliferative effect may have contributed to the reduction in tumour burden and number of large adenomas observed in vivo. The concentration of RA achieved in the intestinal mucosa following chronic exposure is similar to levels of resveratrol and curcumin observed in Apc^{Min} mice as a result of analogous experimental protocols. These agents, when administered with the diet at concentrations similar to those used for RA, generated mean steady state levels of 111 and 36 nmol/g [10,29]. In both cases these intestinal concentrations were accompanied by a reduction in adenoma development.

In summary, consumption of RA in mice at 0.3% was not associated with any visible signs of toxicity. It furnished quantifiable levels of parent compound in the plasma and intestinal tract. There was an indication that RA consumption slowed adenoma development in a mouse model of colorectal carcinogenesis, although further work is required to confirm this notion. Such studies will help adjudge whether RA warrants further development as a colorectal cancer chemopreventive intervention.

This work was supported by a Programme Grant from Cancer Research UK (GO100874).

The authors have declared no conflict of interest.

5 References

[1] Clifford, M. N., Chlorogenic acids and other cinnamates. Nature, occurrence and dietary burden. *J. Sci. Food Agric.* 1999, *79*, 362–372.

[2] Parnham, M. J., Kesselring, K., Rosmarinic acid. *Drugs Fut.* 1985, *10*, 756–757.

[3] Pietsch, K., Saul, N., Chakrabarti, S., Stürzenbaum, S. R. et al., Hormetins, antioxidants and prooxidants: defining quercetin-, caffeic acid- and rosmarinic acid-mediated life extension in *C. elegans*. *Biogerontology* 2011, *12*, 329–347.

[4] Furtado, M. A., Almeida, L. C. F., Furtado, R. A., Cunha, W. R. et al., Antimutagenicity of rosmarinic acid in Swiss mice evaluated by the micronucleus assay. *Mutat. Res.* 2008, *657*, 150–154.

[5] Feng, L. A., Jia, X. B., Zhu, M. M., Chen, Y. et al., Antioxidant activities of total phenols of Prunella vulgaris L. in vitro and in tumor-bearing mice. *Molecules* 2010, *15*, 9145–9156.

[6] Osakabe, N., Yasuda, A., Natsume, M., Yoshikawa, T., Rosmarinic acid inhibits epidermal inflammatory responses: anticarcinogenic effect of Perilla frutescens extract in the murine two-stage skin model. *Carcinogenesis* 2004, *25*(4), 549–557.

[7] Xu, Y. C., Jiang, Z. J., Ji, G. A., Liu, J. W., Inhibition of bone metastasis from breast carcinoma by rosmarinic acid. *Planta Med.* 2010, *76*, 956–962.

[8] Yesil-Celiktas, O., Sevimli, C., Bedir, E., Vardar-Sukan, F., Inhibitory effects of rosemary extracts, carnosic acid and rosmarinic acid on the growth of various human cancer cell lines. *Plant Foods Hum. Nutr.* 2010, *65*, 158–163.

[9] Xavier, C. P. R., Lima, C. F., Fernandes-Ferreira, M., Pereira-Wilson, C., Salvia Fruticosa, Salvia Officinalis, and rosmarinic acid induce apoptosis and inhibit proliferation of human colorectal cell lines: The role in MAPK/ERK pathway. *Nutr. Cancer* 2009, *61*, 564–571.

[10] Perkins, S., Verschoyle, R. D., Hill, K., Parveen, I. et al., Chemopreventive efficacy and pharmacokinetics of curcumin in the min/ +mouse, a model of familial adenomatous polyposis. *Cancer Epidemiol. Biomarkers Prev.* 2002, *11*, 535–540.

[11] Patel K. R., Brown, V. A., Jones, D. J. L., Britton, R. G. et al., Clinical pharmacology of resveratrol and its metabolites in colorectal cancer patients. *Cancer Res.* 2010, *70*, 7392–7399.

[12] Patel, K. R., Scott, E., Brown, V. A., Gescher, A. J. et al., Clinical trials of resveratrol. *Ann. N. Y. Acad. Sci.* 2011, *1215*, 161–169.

[13] Garcea, G., Berry, D. P., Jones, D. J. L., Singh, R. et al., Consumption of the putative chemopreventive agent curcumin by cancer patients: assessment of curcumin levels in the colorectum and their pharmacodynamic consequences. *Cancer Epidemiol. Biomarkers Prev.* 2005, *14*, 120–125.

[14] Luongo, C., Moser, A. R., Gledhill, S., Dove, W. F., Loss of Apc+ in intestinal adenomas from Min mice. *Cancer Res.* 1994, *54*, 5947–5952.

[15] De Giovanni, C., Landuzzi, L., Nicoletti, G., Astolfi, A. et al., Apc10.1: an ApcMin/+ intestinal cell line with retention of heterozygosity. *Int. J. Cancer* 2004, *109*, 200–206.

[16] Sale, S., Fong, I., De Giovanni, C., Landuzzi, L. et al., APC10.1 cells as a model for assessing the efficacy of potential chemopreventive agents in the *Apc^{Min}* mouse model in vivo. *Eur. J. Cancer* 2009, *45*, 2731–2735.

[17] Workman, P., Twentyman, P., Balkwill, F., Balmain, A. et al., United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia (second edition). *Br. J. Cancer* 1998, *77*, 1–10.

[18] Mahmoud, N. N., Carothers, A. M., Grunberger, D., Bilinski, R. T. et al., Plant phenolics decrease intestinal tumors in an animal model of familial adenomatous polyposis. *Carcinogenesis* 2000, *21*, 921–937.

[19] Long, L. H., Hoi, H., Halliwell, B., Instability of, and generation of hydrogen peroxide by, phenolic compounds in cell culture media. *Arch. Biochem. Biophys.* 2010, *501*, 162–169.

[20] Li, X., Yu, C., Cai, Y., Liu, G. et al., Simultaneous determination of six phenolic constituents of danshen in human serum using liquid chromatography/tandem mass spectrometry. *J. Chromatogr.* 2005, *820*, 41–47.

[21] Li, X., Yu, C., Sun, W., Liu, G. et al., Simultaneous determination of magnesium lithospermate B, rosmarinic acid, and lithospermic acid in beagle dog serum by liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 2004, *18*, 2878–2882.

[22] Baba, S., Osakabe, N., Natsume, M., Yasuda, A. et al., Absorption, metabolism, degradation and urinary excretion of rosmarinic acid after intake of Perilla frutescens extract in humans. *Eur. J. Nutr.* 2005, *44*, 1–9.

[23] Baba, S., Osakabe, N., Natsume, M. & Terao, J., Orally administered rosmarinic acid is present as the conjugated and/or methylated forms in plasma, and is degraded and metabolized to conjugated forms of caffeic acid, ferulic acid and m-coumaric acid. *Life Sci.* 2004, *75*, 165–178.

[24] Konishi, Y., Hitomi, Y., Yoshida, M., Yoshioka, E., Pharmacokinetic study of caffeic and rosmarinic acids in rats after oral administration. *J. Agric. Food Chem.* 2005, *53*, 4740–4746.

[25] Cai, H., Marczylo, T. H., Teller, N., Brown, K. et al., Anthocyanin-rich red grape extract impedes adenoma development in the *Apc^{Min}* mouse: pharmacodynamic changes and anthocyanin levels in the murine biophase. *Eur. J. Cancer* 2010, *46*, 811–817.

[26] Cooke, D., Schwarz, M., Boocock, D., Winterhalter, P. et al., Effect of cyanidin-3-glucoside and an anthocyanin mixture from bilberry on adenoma development in the *Apc^{Min}* mouse model on intestinal carcinogenesis – relationship with tissue anthocyanin levels. *Int. J. Cancer* 2006, *119*, 2213–2220.

[27] Cai, H., Al-Fayez, M., Tunstall, R. G., Platton, S. et al., The rice bran constituent tricin potently inhibits cyclooxygenase enzymes and interferes with intestinal carcinogenesis in *Apc^{Min}* mice. *Mol. Cancer Ther.* 2005, *4*, 1287–1292.

[28] Cai, H., Sale, S., Schmid, R., Britton, R. G. et al., Flavones as colorectal cancer chemopreventive agents-phenol-*O*-methylation enhances efficacy. *Cancer Prev. Res.* 2009, *2*, 743–750.

[29] Sale, S., Tunstall, R. G., Ruparelia, K. C., Potter, G. A. et al., Comparison of the effects of the chemopreventive agent resveratrol and its synthetic analog trans 3,4,5,4′-tetramethoxystilbene (DMU 212) on adenoma development in the *ApcMin*+ mouse and cyclooxygenase-2 in human-derived colon cancer cells. *Int. J. Cancer.* 2005, *115*, 194–201.

[30] Reagan-Shaw, S., Nihal, M., Ahmad, N., Dose translation from animal to human studies revisited. *FASEB J.* 2008, *22*, 659–661.

[31] Calabrese, E. J., Mattson, M. P., Calabrese, V., Dose response biology: the case of resveratrol. *Hum. Exp. Toxicol.* 2010, *29*, 1034–1037.

[32] Scott, E. N., Gescher, A. J., Steward, W. P., Brown, K., Development of dietary phytochemical chemopreventive agents: biomarkers and choice of dose for early clinical trials. *Cancer Prev. Res. (Phila)* 2009, *2*(6), 525–530.

[33] Osakabe, N., Takano, H., Sanbongi, C., Yasuda, A. et al., Anti-inflammatory and anti-allergic effect of rosmarinic acid; inhibition of seasonal allergic rhinoconjunctivitis and its mechanism. *Biofactors* 2004, *21*, 127–131.

[34] Scheckel, K., Degner, S. C., Romagnolo, D. F., Rosmarinic acid antagonizes activator protein-1–dependent activation of cyclooxygenase-2 expression in human cancer and non-malignant cell lines. *J. Nutr.* 2008, *138*, 2098–2105.

[35] Moon, D. O., Kim, M. O., Lee, J. D., Choi, Y. H. et al., Rosmarinic acid sensitizes cell death through suppression of TNF-alpha-induced NF-kappa B activation and ROS generation in human leukemia U937 cells. *Cancer Lett.* 2010, *288*, 183–191.

[36] Renzulli, C., Galvano, F., Pierdomenico, L., Speroni, E. et al., Effects of rosmarinic acid against aflatoxin B1 and ochratoxin-A-induced cell damage in a human hepatoma cell line (Hep G2). *J. Appl. Toxicol.* 2004, *24*, 289–296.

[37] Petersen, M., Simmonds, M. S., Rosmarinic acid. *Phytochemistry* 2003, *62*, 121–125.

