Simultaneous Assessment of the Efficacy and Toxicity of Marine Mollusc–Derived Brominated Indoles in an In Vivo Model for Early Stage Colon Cancer

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Abstract

The acute apoptotic response to genotoxic carcinogens animal model has been extensively used to assess the ability of drugs and natural products like dietary components to promote apoptosis in the colon and protect against colorectal cancer (CRC). This work aimed to use this model to identify the main chemopreventative agent in extracts from an Australian mollusc *Dicathais orbita*, while simultaneously providing information on their potential in vivo toxicity. After 2 weeks of daily oral gavage with bioactive extracts and purified brominated indoles, mice were injected with the chemical carcinogen azoxymethane (AOM; 10 mg/kg) and then killed 6 hours later. Efficacy was evaluated using immunohistochemical and hematoxylin staining, and toxicity was assessed via hematology, blood biochemistry, and liver histopathology. Comparison of saline- and AOM-injected controls revealed that potential toxic side effects can be interpreted from blood biochemistry and hematology using this short-term model, although AOM negatively affected the ability to detect histopathological effects in the liver. Purified 6-bromoisatin was identified as the main cancer preventive agent in the Muricidae extract, significantly enhancing apoptosis and reducing cell proliferation in the colonic crypts at 0.05 mg/g. There was no evidence of liver toxicity associated with 6-bromoisatin, whereas 0.1 mg/g of the brominated indole tyrindoleninone led to elevated aspartate aminotransferase levels and a reduction in red blood cells. As tyrindoleninone is converted to 6-bromoisatin by oxidation, this information will assist in the optimization and quality control of a chemopreventative nutraceutical from Muricidae. In conclusion, preliminary data on in vivo safety can be simultaneously collected when testing the efficacy of new natural products, such as 6-bromoisatin from Muricidae molluscs for early stage prevention of colon cancer.

Keywords

animal models, apoptosis, azoxymethane, brominated indoles, colon cancer, muricid extracts, toxicity

Introduction

In the general population worldwide, colorectal cancer (CRC) is the third most commonly diagnosed cancer, with about 1 million cases and >500 000 deaths annually.[1] In the United States, CRC is also the second leading cause of cancer death overall in men and women combined.[2] The lifetime risk of CRC is about 5% to 6%.[3] Most people with early colon cancer do not have any symptoms such that in many CRC patients their cancer is not detected until they present with metastasis and micrometastases.[4,5] This results in an increasing global mortality from CRC, which is approximately 50% of incidence.[1]

Almost 40% to 50% of patients relapse and ultimately die of metastatic disease after undergoing curative surgery

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alone.[6] The risk of treatment failure is reduced by 35% among patients with stage III colon cancer treated with chemotherapy, in comparison to surgery alone.[7] The combination of fluorouracil, irinotecan, and leucovorin (FOLFIRI) is currently the standard and first-line chemotherapy for metastatic CRC.[8] Nevertheless these therapies are far from curative and many serious side effects have been reported, such as peripheral sensory neuropathy,[9] myelosuppression, mucositis,[10-12] pulmonary embolus, myocardial infarction, and death.[13] As a result of the morbidity associated with chemotherapy and poor prognosis of the disease, along with the large economic burden CRC presents,[1] prevention of this disease is an important priority.

Lifestyle alteration is an important way to lower the risk of developing CRC, but implementing lifestyle changes is intrinsically difficult.[14,15] However, the utilization of natural products as a source of novel chemopreventatives holds some promise for reducing CRC risk.[16,17] It has been reported that the consumption of food with anticancer natural properties may result in a 10-fold decrease in the incidence of CRC.[18] However, in order to develop new natural products as medicinal foods or pharmaceutical agents, rigorous preclinical trials are required to meet both efficacy and safety standards. The acute apoptotic response to genotoxic carcinogens (AARGC) model has been developed for chemopreventive research; this model tests the ability of agents to reduce the incidence of CRC by inducing apoptosis of damaged colon cells.[19] To date this model has been mainly used to test acute apoptotic response of dietary components such as starches and oils[20-22] and nonsteroidal anti-inflammatory drugs.[23] AARGC models are not typically used to collect toxicity data, presumably due to the known effects of azoxymethane (AOM) on the liver.[24] However, in the short-term model for early stage prevention of CRC, rodents are killed just 6 hours after injection with AOM,[21,22] and therefore, it should be possible to simultaneously obtain some preliminary data on potential side effects of the extracts and compounds, if they can be detected over and above any effects of AOM. Maximizing the use of additional data from this model could provide an early indication of any products that may cause problematic side effects in longer-term animal models. Ultimately this could help reduce the total number of animals required for preclinical testing by allowing prioritization of compounds that are both effective and likely to be safe and thereby help satisfy the requirements of animal ethics committees.

The Muricidae are a family of predatory marine molluscs (whelks) that provide a source of high-protein meat for people throughout Asia, Europe, and Central and South America. They also form the basis of some rare traditional medicines that have been used for thousands of years.[25] Muricids are well known for the production of brominated indole derivatives, including the ancient dye Tyrian purple.[26] The brominated isatin and indole precursors of Tyrian purple from the Muricidae family and in particular the Australian species *Dicathais orbita*, are known for their anticancer activity (reviewed in Benkendorff et al[25]). In an in vitro study, Edwards et al[27] revealed that tyrindoleninone inhibited cell growth (IC$_{50}$ 39 μM) and induced apoptosis in female reproductive cancers cell lines, with 10- to 100-fold specificity over freshly isolated human granulosa cells. Semipurified 6-bromoisatin and tyrindoleninone in our previous in vitro study induced apoptosis and inhibited the proliferation of both HT29 and Caco2 cells.[28] Furthermore, Muricid extract containing a mixture of tyrindoleninone and 6-bromoisatin was found to be effective for enhancing colonic apoptotic index in a dose-dependent manner in mice.[29] However, further in vivo testing of the crude Muricidae extract has indicated the potential for idiosyncratic gastrointestinal and liver toxicity.[30] Thus, the aim of this new study was first to establish whether the short-term AARGC model can be used to assess any potential side effects caused by purified extracts using blood and liver analysis. Second, we aimed to determine the minimum dose of crude extract required to enhance the AARGC response (0.25 and 0.5 mg/g vs 0.125 and 1 mg/g doses previously tested[29]). Finally, we aimed to confirm which of the brominated indoles in crude muricid extracts are responsible for initiating apoptosis in AOM-damaged colon cells in vivo, along with any associated negative side effects, after separating into fractions enriched in tyrindoleninone and 6-bromoisatin.

Materials and Methods

Hypobranchial Gland Extraction, Flash Column Chromatography, and Chemical Analysis

A total of 1378 *Dicathais orbita* (1146 small ones from 2 to 4 cm and 222 large ones from 4 to 7 cm) were collected from a sea-based abalone farm at Elliston, South Australia (Permit No. 9902638) and frozen at −20°C. Hypobranchial glands were dissected from all frozen *Dicathais orbita* and soaked in chloroform and methanol (1:1, v/v, high-performance liquid chromatography grade; Sigma Aldrich) under agitation at room temperature. The extract was prepared according to the method previously described by Westley et al.[29] All extracts were combined to obtain a total weight of 7.83 g crude extract, which was then stored at −20°C until use. Flash silica chromatography pressurized with nitrogen gas was used to purify tyrindoleninone and 6-bromoisatin according to Esmaeelian et al.[28] The extract was passed through a silica column and the fractions were collected; briefly, tyrindoleninone was purified as a bright orange fraction using dichloromethane and hexane (1:4, v/v) and 6-bromoisatin was subsequently semipurified using 10% methanol in dichloromethane as the solvent system. Both fractions were dried and analyzed along with the crude

extract, by high-performance liquid chromatography (Waters Alliance) coupled to a mass spectrometer (MS; Micromass, Quatro micro) and stored at −20°C. Liquid chromatography (LC)/MS separation was performed on a Hydro-RP C18 column with parallel UV/Vis diode-array detection (300 and 600 nm), a flow rate of 1 mL/min using a gradient of acetonitrile in water with 0.1% formic acid, according to the methods established by Westley and Benkendorff.[31]

In Vivo Rodent Model

In this experiment, an established rodent model was used for early stage prevention of colon cancer.[19,21,29,32] Male mice (wild-type C57BL/6J), 10 weeks old, were obtained from the Animal Resource Centre, Perth, Western Australia. Animal ethics and protocol were approved by the Animal Welfare Committee at Flinders University (Approval Number: 751/10). The mice were maintained at the temperature of 22 ± 2°C and humidity of 80 ± 10% with a 12-hour light/dark cycle. The mice were given water and food (rodent chow) ad libitum and monitored daily for any sign of illness, stool consistency, rectal bleeding, and normal behavior such as grooming. All mice were weighed at days 1, 5, 10, and 14 of the experiment.

The mice were divided randomly into 7 treatment and 2 control groups (10 mice per group) and housed in 18 cages (5 mice per cage). Treatment groups included the following: (1) 0.5 mg/g crude extract, (2) 0.25 mg/g crude extract, (3) 0.1 mg/g tyrindoleninone, (4) 0.05 mg/g tyrindoleninone, (5) 0.25 mg/g tyrindoleninone, (6) 0.1 mg/g semipurified 6-bromoisatin, and (7) 0.05 mg/g semipurified 6-bromoisatin. The extracts and purified compounds were administered to mice by daily oral gavage in 100 µL sunflower oil (Golden Fields, New South Wales, Australia) supplemented with 0.02% vitamin E (Sigma Aldrich) for 2 weeks. There were also 2 control groups gavaged with sunflower oil (containing 0.02% vitamin E) only. At the end of the 2-week period each mouse in all of the treatment groups and one of the control groups was injected with a single intraperitoneal (ip) injection of AOM (Sigma Aldrich, Australia) at a dosage of 10 mg/kg bodyweight, to induce DNA damage. A second control group received a saline injection instead of the AOM. The mice were euthanized 6 hours later by cervical dislocation under ketamine/xylazine anesthesia. The colon was excised immediately postmortem. Two centimeters of distal colon were fixed in 10% buffered formalin (24 hours) and subsequently embedded in paraffin for histological and immunohistological examination.

Histopathological Evaluation of the Liver

Histopathological variables are indicative of toxicity induced by cytotoxic or antimitotic drugs.[33] The liver was therefore removed from each mouse and weighed. Liver weight was standardized by measuring the percentage of the liver weight divided by the body weight for each mouse. Livers were then fixed in 10% buffered formalin and embedded in paraffin. Four-micrometer sections of liver were stained with hematoxylin and eosin for histopathological examination of potential toxicity[34] under light microscopy (Olympus, BH-2). Hepatotoxicity indictors that were assessed included porphyrin, hepatocellular hyperplasia and hypertrophy, mallory bodies, hemosiderin, sinusoidal dilation, congestion, hemorrhage, lipofuscin, steatosis (fatty change), necrosis, and inflammation.[33,35]

Blood Analysis (Hematology and Biochemistry)

Before euthanizing the mice, blood samples (0.5-1 mL) were obtained by cardiac puncture into heparinized vacutainer tubes, under anesthesia and transferred to Gribbles Veterinary Pathology laboratory, Adelaide, South Australia, for hematology and biochemistry analysis. The samples were run through the Abbott Cell Dyn 3700 analyzer for hematology assessment and Siemens Advia 1800 chemistry analyzer for biochemistry analysis. The blood levels of liver enzymes including aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were assessed as indicators of hepatotoxicity.[36]

Measurement of Apoptosis Using Hematoxylin Staining

The apoptotic epithelial cells of distal colon sections were determined by hematoxylin staining as described by Hu et al.[32] Distal colon sections were embedded in paraffin, sectioned at 4 µm (3-4 sections per mouse), stained with hematoxylin, and examined under a light microscope (Olympus, BH-2) at ×400 magnification. Apoptotic cells were identified in 20 randomly chosen complete crypts by characteristic morphological changes of cell shrinkage, condensed chromatin, and sharply delineated cell borders surrounded by an unstained halo.[21,37] The percentage of apoptotic cells were calculated as the mean number of apoptotic cells/crypt, divided by total number of cells in the crypt and multiplied by 100. The height of each crypt was determined along with the position of apoptotic cells.[38]

Determination of Epithelial Proliferation (Immunohistochemical Staining)

Ki-67 is a cell cycle–associated antigen and regarded as a useful epithelial cell proliferation marker.[39] Distal colon segments were embedded in paraffin then sectioned at 4 µm. Sections were de-waxed in Histoclear and hydrogen peroxide (3%) was used for 15 minutes to quench the endogenous

peroxidase activity. Antigen retrieval was achieved by cooking the sections in a pressure cooker at 120°C for 1 hour in 0.1 mol/L citrate buffer (pH 6.5). Sections were incubated with a primary monoclonal Ki-67 antibody (1:1000; Dako) at 4°C overnight. For detection of the primary antibody, biotinylated secondary rabbit-anti-mouse antibody (1:200; Dako) for 30 minutes and avidin/biotinylated peroxidase complex (Signet Laboratories USA-HRP kit) for 20 minutes were used. Slides were visualized by incubating with 3′-diaminobenzamine (DAB) substrate (Signet Laboratories USA-HRP kit) for 3 minutes, followed by counterstaining for 1 minute in hematoxylin and examining under a light microscope (Olympus, BH-2) at ×400 magnification. The percentage of proliferating cells was calculated in the same way as the apoptosis index described above.

Statistical Analysis

Statistical analyses were performed using SPSS (Smart Viewer 15.0) and/or Prism GraphPad (version 5.2), and P values of $\leq.05$ were considered to be statistically significant. A one-way ANOVA test was performed to compare between treatments and controls across the different concentrations. Tukey HSD post hoc test was applied to detect which groups significantly differ. Levene test was used to check homogeneity of variances, and in all cases $P > .05$.

Results

Chemical Analysis

LC/MS analysis of *Dicathais orbita* crude extract revealed at least 5 peaks attributed to brominated indole compounds (Figure 1A). The dominant peak at t_R 6.37 minutes occurring with major ions in ESI-MS at m/z 224, 226 is indicative of the molecular mass of 6-bromoisatin. The mass spectrum of the peak at t_R 9.35 minutes with major ions in ESI-MS at m/z 302, 304 corresponds to tyrindolinone. The small peak at t_R 10.44 minutes is attributed to tyrindoxyl sulfate, with major ions in ES-MS at m/z 336, 338. The peak at t_R 11.00 minutes is indicative of the molecular weight of tyrindoleninone with major ions at m/z 255, 257, and a peak at t_R 11.71 minutes with ions in ESI-MS at m/z 511, 513, 515 corresponding to the molecular mass of tyriverdin with major fragment ions at m/z 417, 419, 421 formed by the elimination of dimethyl disulfide. LC/MS analysis of the purified compounds showed a single peak at t_R 11.00 minutes corresponding to tyrindoleninone (Figure 1B) and a dominant peak at t_R 6.37 minutes corresponding to 6-bromoisatin with several other minor peaks (Figure 1C).

In Vivo Model: General Observations

There were no signs of illness (ie, no diarrhea, constipation, dysphagia, hematemesis, or loss of appetite) in any of the
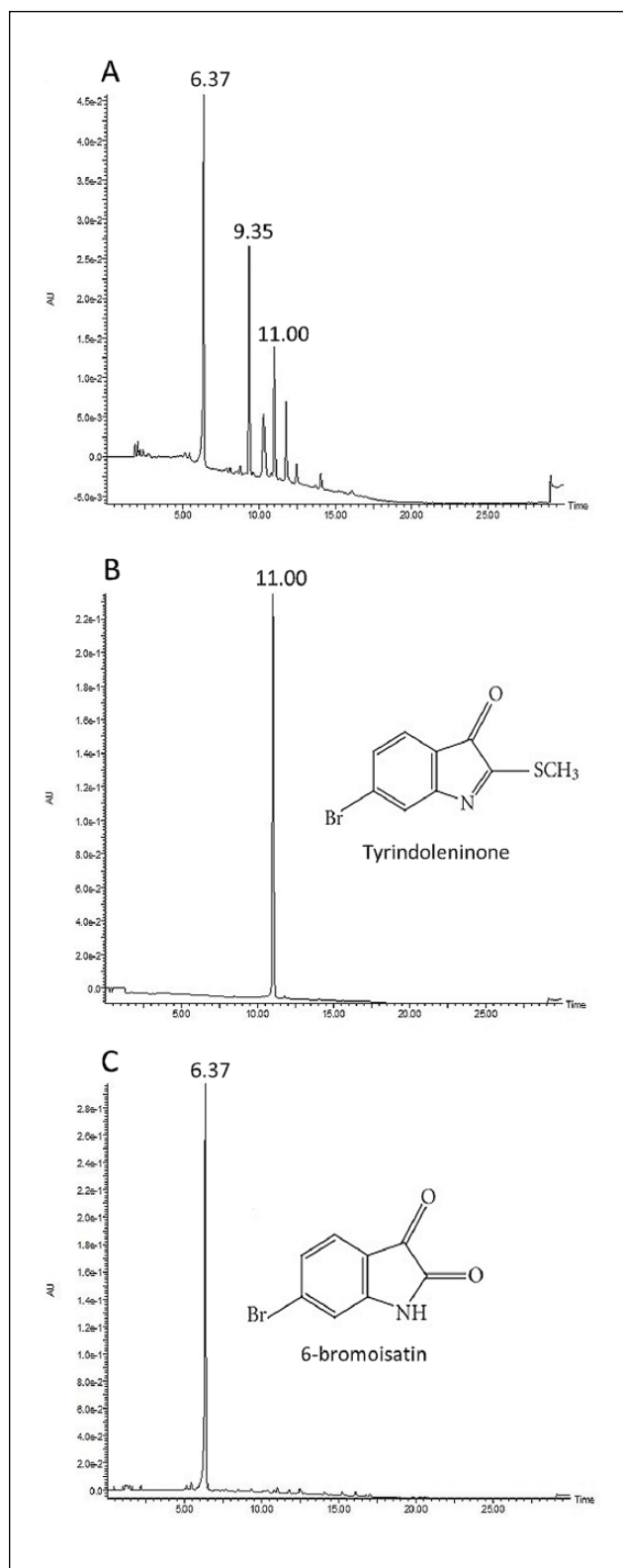

Figure 1. Liquid chromatography/mass spectrometry analysis of (A) crude extract from hypobranchial gland of *Dicathais orbita*, (B) purified tyrindoleninone, and (C) semipurified 6-bromoisatin.

Table 1. Body and Liver Weights of Animals During the Experiment[a,b].

	Weight (g)					
	Body (Day 1)	Body (Day 5)	Body (Day 10)	Body (Day 14)	Total Weight Gain	Liver	Liver/Body (%)
Saline control	24.6 ± 1.1	25.0 ± 0.9	25.2 ± 1.0	25.1 ± 1.1	0.5 ± 0.4	1.1 ± 0.2	4.2 ± 0.6**
AOM control	24.2 ± 1.4	24.5 ± 1.4	25.1 ± 1.6	25.0 ± 1.6	0.7 ± 0.5	1.3 ± 0.1	5.3 ± 0.5
TYR 0.025 mg/g	22.9 ± 1.0	23.0 ± 1.1	23.9 ± 1.4	24.5 ± 1.7	1.6 ± 1.2	1.1 ± 0.1	4.7 ± 0.7
TYR 0.05 mg/g	23.9 ± 1.0	24.1 ± 1.2	24.8 ± 1.3	24.6 ± 1.2	0.7 ± 0.5	1.3 ± 0.1	5.2 ± 0.4
TYR 0.1 mg/g	23.0 ± 1.6	23.3 ± 1.5	23.6 ± 1.5	23.7 ± 1.6	0.6 ± 2.0	1.2 ± 0.2	5.3 ± 0.9
6-BRO 0.05 mg/g	24.9 ± 0.9	25.1 ± 1.2	25.5 ± 1.2	25.5 ± 1.3	0.6 ± 0.9	1.2 ± 0.1	4.8 ± 0.4
6-BRO 0.1 mg/g	25.9 ± 1.1	25.8 ± 1.3	26.2 ± 1.4	26.4 ± 1.4	0.5 ± 0.5	1.4 ± 0.1	5.2 ± 0.2
CE 0.25 mg/g	24.7 ± 1.2	25.3 ± 1.3	25.7 ± 1.6	26.1 ± 1.6	1.4 ± 0.6	1.4 ± 0.1	5.3 ± 0.5
CE 0.5 mg/g	24.0 ± 2.0	24.5 ± 2.1	24.5 ± 2.4	25.1 ± 2.3	1.1 ± 0.4	1.3 ± 0.2	5.2 ± 0.5

Abbreviations: TYR, tyrindoleninone; 6-BRO, semipurified 6-bromoisatin; CE, crude extract; AOM, azoxymethane.
[a]N = 10 mice in all groups.
[b]Comparison of mean (±SE) progressive body weight (g) in control and treatment mice on different experimental days. Liver weight (g) and percent of liver weight/body weight were calculated on the day of kill.
**$P \le .01$ between the AOM- and saline-injected oil controls.

treatment groups or control animals throughout the study. All animals groups were oral gavaged daily with sunflower oil only or sunflower oil containing treatments. The body weights of all mice increased steadily over the trial duration (Table 1); no significant differences in mean total weight gain was revealed either between the saline control and AOM control, or the AOM control group and treatment groups by analysis of variance (Table 1). The livers of all mice were excised and weighed at the end of the experiment. The results showed a significant increase in the percent liver to body weight (~25%) for the controls injected with AOM compared to saline ($P \le .01$). No significant change in the liver weight was revealed after AOM injection between oil control and any treatment group (Table 1).

Histopathology Evaluation of the Liver

Using light microscopy, the predominant histopathological alteration in the liver was the presence of numerous cytoplasmic vacuoles consistent with microvesicular steatosis (fatty liver), with varying degrees of sinusoidal dilation and congestion (Figure 2). This effect was visible 6 hours after injection with AOM, but not in the saline control (Figure 2C). There were no further changes with regard to microvesicular steatosis (fatty liver), sinusoidal dilatation, or red blood cell extravasation into the space of Disse that could be discerned in the mice oral gavaged with either compound or extract, as compared to AOM injected controls, which were oral gavaged with oil only.

Liver Enzyme Analysis

Toxicity was monitored by quantitative analysis of the liver enzymes AST, ALT, and ALP, enzymes that are known biochemical markers of liver toxicity. ALP level was similar between the 2 control groups and all treatment groups. ALT and AST levels were higher in AOM-injected controls compared to saline-injected controls (Figure 3), but these were not significantly different. No significant differences were found in the ALT and AST blood levels between the AOM-injected controls and all treatment groups, except for one tyrindoleninone group (0.1 mg/g). In this group a combined effect of AOM and tyrindoleninone was observed on the AST levels, and mice treated with tyrindoleninone (0.1 mg/g) showed a significant AST blood level increase in comparison with the AOM-injected oil control ($P \le .05$).

Hematological and Biochemical Blood Analysis

Hematology results from the mouse blood samples including red blood count, hemoglobin, hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white cell count, neutrophil number, lymphocytes, and monocytes are summarized in Table 2. Following 2 weeks of oil control gavage only, AOM injection led to a significant reduction of lymphocytes compared to saline injection ($P \le .01$). By contrast, the number of neutrophils increased significantly 6 hours after injection with AOM compared to saline ($P \le .05$). In the treatment groups, red cell count and hemoglobin concentration showed a significant reduction in the mice treated daily with 0.1 mg/g tyrindoleninone in comparison with AOM oil only control ($P \le .05$), whereas monocytes were significantly elevated in mice treated daily with the lower dose of 0.05 mg/g tyrindoleninone. White cell count and the number of neutrophils were reduced significantly in the group treated with the lower concentration 0.05 mg/g 6-bromoisatin compared to the oil only control after injection with AOM ($P \le .05$). However, the number of neutrophils in the 6-bromoisatin
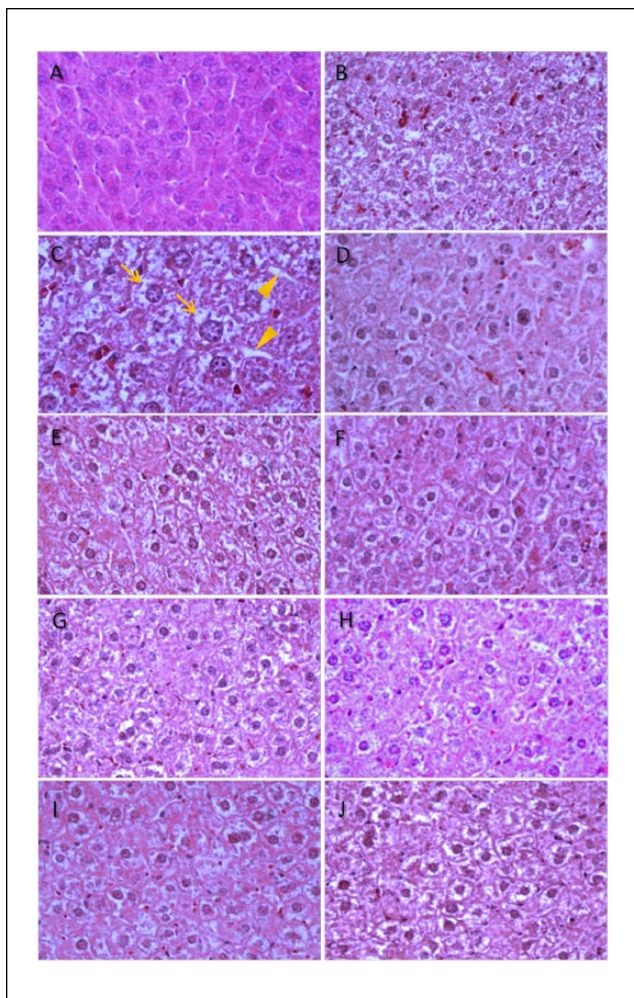
Figure 2. Microscopic images of sectioned liver tissue (original magnification ×200 except "C," which is ×400) from mice in control and treatment groups stained by hematoxylin and eosin. (A) Saline control; (B) AOM control; (C) AOM control (×400); (D) tyrindoleninone 0.025 mg/g; (E) tyrindoleninone 0.05 mg/g; (F) tyrindoleninone 0.1 mg/g; (G) semipurified 6-bromoisatin 0.05 mg/g; (H) semipurified 6-bromoisatin 0.1 mg/g; (I) crude extract 0.25 mg/g; (J) crude extract 0.5 mg/g. Microvesicular steatosis (shown by arrow) was the predominant histopathological finding with varying degrees of sinusoidal dilatation (shown by arrow head) and congestion in all AOM-injected mice after 6 hours.

treatment group (0.05 mg/g) was not significantly different to that observed in the saline injected control.

The concentration of blood biochemical parameters such as sodium, potassium, sodium/potassium ratio (Na/K), urea, creatinine, calcium, protein, albumin, and globulin were also measured (Table 3). There was a significant elevation ($P \leq .001$) in the urea level of the oil control after AOM injection (11.81 ± 1.39 mmol/L) in comparison to saline injection (9.01 ± 1.62 mmol/L). Globulin protein level was decreased significantly, but not with a clear dose-dependent

response, in the mice administered tyrindoleninone (Table 3). Conversely, 6-bromoisatin (0.05 mg/g) increased the level of globulin in comparison to the oil control after AOM injection.

Apoptotic Index and Crypt Height

Apoptotic cells were rarely detected in the crypts of the distal colon following saline injection, whereas in response to AOM injection, a low background level of apoptosis was initiated (see Figure 4A and Supplementary Figure 1A and B, available online at http://ict.sagepub.com/supplemental). Apoptosis significantly increased in the distal colon of mice administered semipurified 6-bromoisatin, at both concentrations of 0.05 mg/g and 0.1 mg/g ($P \leq .001$) compared to the oil control (by 2.5-fold) after AOM injection. A significant increase in the apoptotic index after AOM injection was also revealed between oil gavaged control mice and those administered 0.25 mg/g ($P \leq .05$) and 0.5 mg/g ($P \leq .001$) of crude extract, with a clear dose effect (Figure 4A). In contrast, there was no significant difference in apoptotic index after AOM injection between mice treated with tyrindoleninone and those gavaged oil only. Mean crypt height in distal colon was similar between the AOM injected and saline injected control (Figure 4B). However, after AOM injection, 2 treatment groups showed a significant decrease in mean crypt height, 0.025 mg/g tyrindoleninone ($P \leq .05$) and 0.5 mg/g crude extract ($P \leq .05$), in comparison to the oil control (Figure 4B).

Proliferation Index

Mean proliferation index in the distal colon was not significantly different between the AOM- and saline-injected oil controls (Figure 4C). However, after AOM injection, cell proliferation was reduced significantly in mice treated with semipurified 6-bromoisatin at concentrations of 0.05 mg/g and 0.1 mg/g compared to the oil only control ($P \leq .001$; see Figure 4C and Supplementary Figure 1C and D, available online at http://ict.sagepub.com/supplemental). Similarly after AOM injection, tyrindoleninone administration at 0.05 ($P \leq .01$) and 0.1 mg/g ($P \leq .001$) resulted in a significant reduction of proliferation compared to the oil only control (Figure 4C). Tyrindoleninone at the 0.025 mg/g lowest concentration had no significant effect on the proliferation index (Figure 4C). A significant reduction in proliferative cells was also observed in mice treated with the crude extract at both concentrations of 0.25 and 0.5 mg/g ($P \leq .001$; Figure 4C) after AOM injection.

Discussion

This study demonstrates for the first time that the short-term AARGC rodent model for colon cancer prevention can be
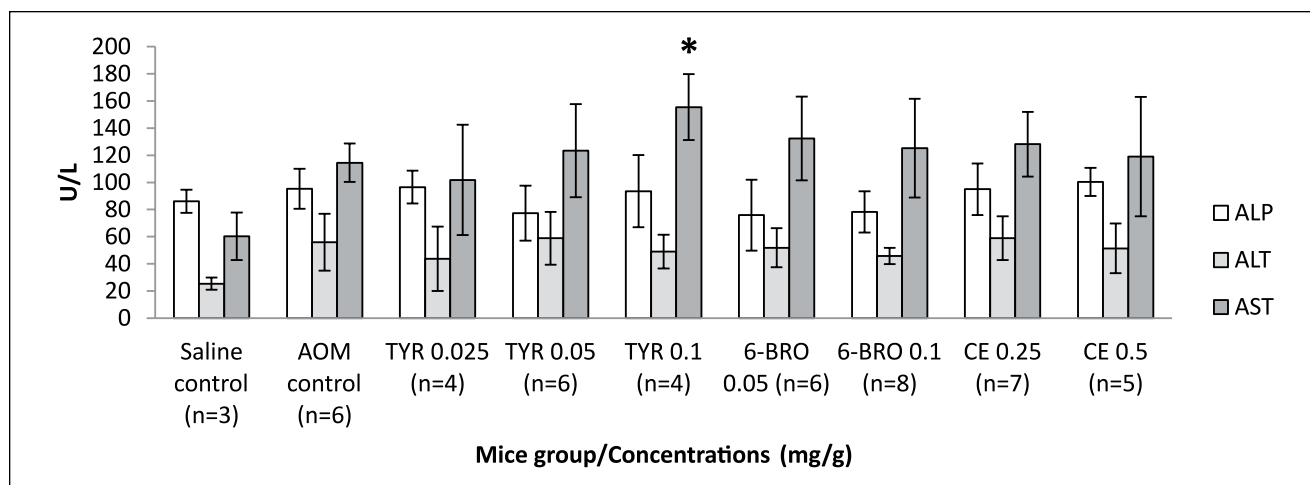
Figure 3. Levels of the liver enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) in blood (U/L) from control and treatment groups including purified tyrindoleninone (TYR), semipurified 6-bromoisatin (6-BRO), and crude extract (CE). $P \leq .05$ (*) for AST levels in 0.1 mg/mL TYR treatment group compared to the AOM-injected oil control.

used to simultaneously collect preclinical in vivo data on efficacy and safety by including AOM and saline injection controls. Although some histopathological effects specific to AOM were detected in the liver, the effects of the extracts and compounds on liver function could be discerned from the levels of liver enzymes in blood. Furthermore, despite the fact that the carcinogen AOM appears to induce inflammatory neutrophils, synergistic and immune-modulating effects of the bioactive compounds could be detected by hematological assessments. This combined model revealed that both the crude muricid extract and semipurified 6-bromoisatin significantly increased the apoptotic index after administration of a DNA-damaging agent, and also inhibited cell proliferation in the distal colon of mice, without any sign of toxicity. LC/MS analysis confirmed 6-bromoisatin as the major compound in both the crude extract and semipurified 6-bromoisatin fraction and the enhanced bioactivity within the semipurified fraction indicates that 6-bromoisatin is the main chemopreventative agent in the muricid extract. In contrast, purified tyrindoleninone did not increase the apoptotic index after AOM injection, but significantly reduced the cell proliferation index in the distal colon, in a dose-dependent manner. In addition, there was some evidence for toxicity in the liver and mild anemia in the mice corresponding to the administration of tyrindoleninone. This information on the potential side effects is very useful for development and quality control of an optimized nutraceutical from Muricidae hypobranchial glands. Tyrindoleninone is easily oxidized into 6-bromoisatin,[26] so controlled oxidation of the extract could help reduce the previously reported idiosyncratic hepatotoxicity[30] and improve the overall safety and efficacy of these marine mollusc extracts.

Liver histopathology showed evidence of hepatocyte damage within 6 hours due to the AOM injection, which was consistent with microvesicular steatosis and varying degree of sinusoidal dilation and congestion. These qualitative changes were observed in the oil control following a single AOM injection and no further effect was discernible in the mice treated for 14 days with either compound or extract in addition to the single AOM injection. Microvesicular steatosis with varying degrees of hemorrhagic congestion and necrosis was previously shown in 15-week-old male C57BL/6 mice following the injection of 100 mg/kg AOM and sacrificing the animals after reaching the coma stage of encephalopathy.[24] In our study, no sign of necrosis or hemorrhage in the liver were observed 6 hours after injection using one tenth of this dose (10 mg/kg). However, some other hepatocyte alterations observed in mice livers in our study were consistent with those observed by Bémeur et al.[24] In another histological study by Matkowskyj et al,[40] the mice showed microvesicular steatosis after 2 hours and sinusoidal dilatation after 4 hours of high-dose AOM administration (100 mg/kg), while centrilobular necrosis was only apparent 20 hours after AOM injection. Macroscopically in our study, the most striking alteration in the liver was the significant increase in the percent liver to body weight in the oil controls compared to saline, just 6 hours after AOM injection. Hepatic enlargement in the liver has been reported in mice receiving 100 mg/kg AOM,[41] and our study confirms that this effect can occur even at the lower dose of 10 mg/kg. Consequently, it is not possible to extract information on the potential histopathological damage to the liver or changes in liver weights in treatments tested using the AARGC rodent model for colon cancer, due to the rapid overriding short-term effects of AOM.

Table 2. Hematological Analysis of Mouse Blood[a].

	Red Cell Count (×10^12/L)	Hemoglobin (g/L)	Hct (L/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	White Cell Count (×10^9/L)	Neutrophils (×10^9/L)	Lymphocytes (×10^9/L)	Monocytes (×10^9/L)
Saline control (n = 3)	8.9 ± 0.2	135.6 ± 3.5	0.43 ± 0.0	48.3 ± 1.5	15.3 ± 0.5	312.6 ± 3.7	6.4 ± 1.7	0.5 ± 0.3*	5.5 ± 2.0**	0.2 ± 0.1
AOM control (n = 6)	9.1 ± 0.2	135.8 ± 1.9	0.42 ± 0.01	46.3 ± 1.3	15.0 ± 0.0	322.1 ± 6.6	5.1 ± 1.1	2.2 ± 1.0	2.6 ± 0.9	0.2 ± 0.1
TYR 0.025 mg/g (n = 3)	8.6 ± 0.4	131.7 ± 5.3	0.41 ± 0.01	48.7 ± 0.9	15.2 ± 0.5	316.7 ± 2.5	4.3 ± 0.5	0.9 ± 0.9	3.3 ± 1.0	0.1 ± 0.1
TYR 0.05 mg/g (n = 5)	8.8 ± 0.2	133.1 ± 4.5	0.41 ± 0.01	47.3 ± 0.5	15.0 ± 0.0	319.6 ± 3.7	5.3 ± 0.5	1.3 ± 0.5	3.2 ± 0.7	0.6 ± 0.3*
TYR 0.1 mg/g (n = 5)	8.2 ± 0.7*	124.8 ± 8.9*	0.40 ± 0.02	48.2 ± 1.9	15.2 ± 1.3	321.5 ± 4.7	5.3 ± 2.1	2.3 ± 1.0	2.7 ± 1.1	0.2 ± 0.3
6-BRO 0.05 mg/g (n = 9)	9.4 ± 0.4	140.0 ± 7.6	0.44 ± 0.01	46.6 ± 0.7	14.7 ± 0.4	317.7 ± 6.2	2.8 ± 1.3*	0.8 ± 0.6*	1.8 ± 0.5	0.1 ± 0.1
6-BRO 0.1 mg/g (n = 9)	9.0 ± 0.2	136.1 ± 3.2	0.42 ± 0.01	47.0 ± 0.7	15.0 ± 0.0	321.7 ± 2.5	5.1 ± 1.1	2.1 ± 0.4	2.5 ± 0.7	0.4 ± 0.1
CE 0.25 mg/g (n = 7)	8.6 ± 0.2	132.0 ± 3.5	0.41 ± 0.01	47.1 ± 1.2	15.1 ± 0.3	321.5 ± 7.4	4.2 ± 1.0	1.7 ± 0.6	2.3 ± 0.3	0.2 ± 0.1
CE 0.5 mg/g (n = 5)	8.9 ± 0.1	130.0 ± 4.7	0.42 ± 0.007	47.2 ± 0.8	14.6 ± 0.5	314.0 ± 8.1	5.6 ± 0.4	2.0 ± 0.5	3.3 ± 0.8	0.2 ± 0.1

Abbreviations: Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; AOM, azoxymethane; TYR, tyrindoleninone; 6-BRO, semipurified 6-bromoisatin; CE, crude extract.

[a]At the end of a 2-week period of oral gavage with oil or crude muricid mollusc extract or purified compounds in oil and 6 hours after injection with 10 mg/kg azoxymethane (AOM) or saline, blood was collected from mice in each experimental group via cardiac bleed and analyzed.

*$P \leq .05$ and **$P \leq .01$ between the AOM-injected oil control and either the saline-injected oil control or a treatment group.

Table 3. Biochemical Analysis of Mouse Blood[a].

	Sodium (mmol/L)	Potassium (mmol/L)	NA/K	Urea (mmol/L)	Creat. (µmol/L)	Calcium (mmol/L)	Protein (g/L)	Albumin (g/L)	Globulin (g/L)
Saline control (n = 10)	143.0 ± 1.7	6.1 ± 1.4	24.6 ± 6.5	9.0 ± 1.6***	12.3 ± 2.0	2.2 ± 0.0	43.4 ± 1.6	27.3 ± 1.2	16.1 ± 0.8
AOM control (n = 7)	144.0 ± 3.9	6.3 ± 2.0	25.3 ± 10.1	11.8 ± 1.3	15.8 ± 3.5	2.1 ± 0.1	46.7 ± 2.3	29.4 ± 1.9	17.2 ± 0.9
TYR 0.025 mg/g (n = 10)	139.9 ± 7.0	6.4 ± 3.7	22.3 ± 14.5	10.0 ± 0.6	18.8 ± 10.9	1.9 ± 0.2	47.0 ± 4.2	31.2 ± 4.4	15.8 ± 0.6*
TYR 0.05 mg/g (n = 10)	141.9 ± 6.2	6.7 ± 3.7	27.3 ± 9.5	10.8 ± 1.5	14.6 ± 4.8	2.0 ± 0.2	48.2 ± 5.1	31.3 ± 4.6	16.9 ± 0.9
TYR 0.1 mg/g (n = 4)	143.0 ± 2.4	6.1 ± 2.7	27.0 ± 11.2	10.2 ± 2.1	15.2 ± 3.5	2.1 ± 0.0	43.7 ± 3.7	28.2 ± 3.3	15.5 ± 0.5*
6-BRO 0.05 mg/g (n = 10)	141.1 ± 2.6	5.8 ± 0.7	24.4 ± 2.7	10.0 ± 1.3	12.4 ± 1.0	2.1 ± 0.0	49.3 ± 1.5	30.5 ± 1.0	18.8 ± 0.7*
6-BRO 0.1 mg/g (n = 9)	143.5 ± 1.5	5.9 ± 0.4	22.2 ± 7.4	11.0 ± 0.8	13.2 ± 1.0	2.1 ± 0.0	47.4 ± 2.0	29.2 ± 1.3	18.2 ± 0.8
CE 0.25 mg/g (n = 10)	142.5 ± 4.4	6.6 ± 3.1	25.2 ± 9.1	10.4 ± 1.0	17.8 ± 8.2	2.0 ± 0.1	46.7 ± 3.3	30.0 ± 3.3	16.6 ± 1.2
CE 0.5 mg/g (n = 7)	142.4 ± 4.3	6.5 ± 2.9	24.8 ± 7.9	10.4 ± 0.9	15.2 ± 8.0	2.1 ± 0.1	47.5 ± 1.8	30.2 ± 2.1	17.2 ± 0.9

Abbreviations: AOM, azoxymethane; TYR, tyrindoleninone; 6-BRO, semipurified 6-bromoisatin; CE, crude extract.

[a]At the end of a 2-week period of oral gavage with oil or crude muricid mollusc extract or purified compounds in oil and 6 hours after injection with 10 mg/kg AOM or saline, blood was collected from mice in each experimental group via cardiac bleed and biochemical parameters were measured.

*$P \le .05$ and **$P \le .001$ between the AOM-injected oil control and either the saline-injected oil control or a treatment group.
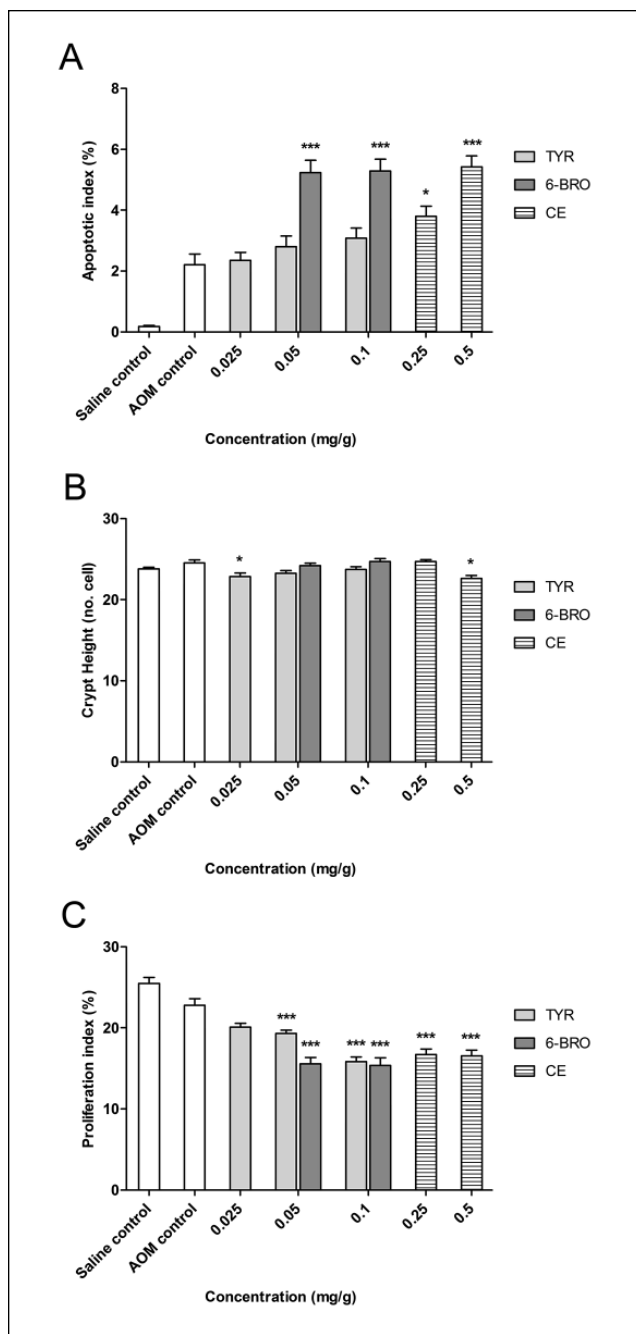
Figure 4. Apoptosis, crypt height and proliferation in the epithelial cells of crypts in the distal colon after 14-day oral gavage of different concentrations of tyrindoleninone (TYR), semipurified 6-bromoisatin (6-BRO), and crude extract (CE). The purified compounds were tested at lower concentrations than the crude extract. All treatments and the AOM control were injected with 10 mg/kg AOM, a genotoxic carcinogen, 6 hours prior to kill. (A) Apoptotic index, (B) crypt height, (C) proliferating epithelial cells were detected via immunostaining with the Ki-67 primary monoclonal antibody. Data are means ± SE for 10 mice per group (10 full crypts/animal). $P \leq 0.05$ (*), $P \leq .01$ (**), and $P \leq .001$ (***) between each treatment group and the AOM-injected oil control.

Nevertheless, it is possible to obtain a preliminary assessment of hepatotoxicity in the AARGC model using biochemical indicators in the blood. No significant differences in the level of the liver enzymes ALP, ALT, and AST were found between AOM- and saline-injected oil control mice in our study. Conversely, in a study by Bélanger et al,[42] a significant increase in the blood level of ALT and AST was detected after the injection of a very high dose of AOM (100 mg/kg) in C57BL6 male mice, followed by the development of acute liver injury, encephalopathy, coma, and death in the animals. In our study, only a slight but nonsignificant increase in ALT and AST levels was detected 6 hours following the injection of the lower dose of AOM (10 mg/kg) in the oil control group. Similarly, another study by Matkowskyj et al[40] showed no significant differences in the levels of ALT in the blood of C57BL/6J mice following the injection of 0.1 mg/g AOM (56 ± 5 U/L) after 4 hours, compared with a saline control (64 ± 14 U/L). However the same authors showed 20 hours after AOM administration the levels of ALT increased significantly with the presence of centrilobular necrosis in hepatocytes (5196 ± 126 U/L).[40] This indicates a longer period of time is required to induce necrosis in hepatocytes after administration of AOM and also highlights the correlation between hepatic necrosis and increased level of liver enzymes, especially ALT, in the mice blood. This finding, along with the treatment results from our study, demonstrate that blood liver enzymes can provide an early indicator of potential hepatotoxicity and that it is possible to obtain preliminary information on the effects of chemopreventative agents on the liver using the short-term AOM colon cancer model.

The muricid extracts and 6-bromoisatin appear to have little to no liver toxicity at the chemopreventative doses that were tested. Following AOM injection, no significant differences in the level of the liver enzymes ALP, ALT, or AST were found between oil-only controls and mice treated with oil containing the muricid extract or purified 6-bromoisatin. However, the combination of a single AOM injection and 2 weeks daily tyrindoleninone oral gavage (0.1 mg/mL) caused a significant increase in AST, indicating the administration of tyrindoleninone along with a DNA-damaging agent can lead to liver toxicity. AOM is metabolized by endogenous enzymes in the liver, primarily cytochrome P450 2E1 (CYP2E1) via hydroxylation of the methyl group to the genotoxic carcinogen methylazoxymethanol.[43,44] The tyrindoleninone metabolism pathway has not yet been elucidated. However, of the enzymes needed for endogenous indole metabolism in the intestinal mucosa, CYP2C19[45] plays a minor role in indole oxidation[46] and glutathione-S-transferase (GST)[47] can mediate debromination in phase 2, after oxidation by CYP2E1.[48] Therefore, tyrindoleninone could indirectly affect CYP2E1-associated AOM metabolism and increase the immediate toxic side effects of AOM in the liver. However, this study also supports the suggestion

that in vivo metabolism of indoles such as tyrindoleninone produce by-products, such as indoxyl sulfate,[30] that could directly affect the liver. Methanthiol generation during hepatic metabolism of tyrindoleninone may also contribute to steatohepatitis.[30] These metabolic by-products are not expected to form from 6-bromoisatin, which is consistent with the lack of significant effects on liver enzymes in mice treated with this compound. Although the crude extract containing tyrindoleninone showed no significant effect on liver enzymes, our previous study showed significant dose-dependent histopathological effects on the liver.[30] This study identifies tyrindoleninone as the most likely compound responsible for the idiosyncratic liver damage in muricid extract, thus suggesting that semipurification is required for nutraceutical development.

The other notable change in biochemical factors was the decreased level of globulin protein in the mice administered tyrindoleninone, although this alteration was not dose-dependent. Globulin and albumin proteins are 2 main serum proteins in the blood that are produced by the liver.[49] Therefore, decreased production of globulin could provide further evidence of the liver damage associated with the administration of tyrindoleninone. The mice treated with the highest concentration of tyrindoleninone (0.1 mg/g) also had a significantly lower red cell counts and hemoglobin levels (12.4 ± 0.8 g/dL) compared to the oil-only controls following AOM injection, thus indicating mild anemia in these mice. According to the chart for anemia severity in male mice established by the World Health Organization, the concentration ranges for mild anemia is 10.7 to 12.4 g/dL.[50,51] Interestingly when AOM was injected after 2 weeks of daily oral gavage with the medium dose of tyrindoleninone (0.05 mg/mL), there was also an increase in monocyte numbers compared to the AOM-only control, suggesting some potentially complex effects on the immune system. All this information from the blood parameters provides further evidence for toxic side effects associated with oral administration of tyrindoleninone that was apparent over and above any effects of the AOM.

By comparison, following injection with AOM, the mice administered the oil control showed signs of lymphopenia (low lymphocytes) and neutrophilia (high neutrophils) relative to the saline-injected oil control. This result suggests that AOM has direct effects on the immune system within 6 hours of injection. Neutrophils have a protective role in response to infection in the host[52] and can increase as part of the general stress response. For example, stress generated by poisons (eg, the heavy metal arsenic) can increase the blood level of neutrophils in mice.[53] In a study by Haratym-Maj,[54] poisoning of the mice with the low dose of deltamethrin significantly elevated neutrophil levels in blood compared to the control group. Therefore, occurrence of neutrophilia in the AOM-injected oil control is likely to be related to stress from injecting the DNA damaging AOM in mice.

Interestingly, when semipurified 6-bromoisatin (0.05 mg/g) was administered for 2 weeks prior to the AOM injection, this resulted in neutrophil numbers in the normal range (similar to saline-injected oil controls). This indicates that purified 6-bromoisatin may also have an anti-inflammatory effect that counteracts the neutrophilia associated with AOM injection. This is consistent with the known anti-inflammatory activity of isatin, which has been shown to inhibit inducible nitric oxide synthase, cyclooxygenase-2, and tumor necrosis factor, resulting in a reduction of prostaglandin E2 in mouse macrophages.[55] In contrast, 0.05 mg/g purified 6-bromoisatin did not counteract the decrease of lymphocytes that occurs after AOM injection and actually lead to significantly less white blood cells compared to the AOM control. However, this was a dose-dependent effect, and in our previous studies using synthetic 6-bromoistain, there was a significant increase on white blood cells at the same dose.[56] These variable effects on white blood cells between purified and synthetic 6-bromoisatin suggest that further studies should be conducted on the influence of the muricid bioactive brominated indoles on the vertebrate immune system.

The most striking alteration among the biochemical factors was the significant increase of the blood urea levels in oil-only controls, when AOM was injected compared to saline. Urea, which is the end product of protein metabolism, is produced from ammonia in urea cycle[57] and excreted through the kidney in urine. Consequently, the rate of renal urea excretion directly affects the maintenance of nitrogen balance in blood.[58] In a study by Bémeur et al,[24] AOM-injected mice showed a significant 5.1-fold increase in ammonia concentration of the serum compared to the saline-injected mice. The increased concentration of ammonia caused by AOM injection in Bémeur et al[24] is consistent with the increased concentration of blood urea induced by AOM injection in our study. Urea serum concentration is used as a marker of renal function and is increased by renal injury.[59] Thus, our study indicates AOM results in rapid injury (within 6 hours) to the kidney, as well as to the liver. However, mice treated with the crude muricid extract and compounds did not show any increase in urea compared to the oil controls, indicating no further toxicity of these compounds in the kidney after 2 weeks of administration. In our previous study on synthetic 6-bromoisatin, a diuretic effect was found with increased sodium/potassium ratios in all 3 doses that were tested (0.1-0.025 mg/g).[56] However, no significant effects on the ion balance was observed using the naturally derived semipurified 6-bromoistain in this study. This suggests that differences in the minor impurities of the naturally derived and synthetic compound could alter some in vivo effects.

The inhibition of CRC has been previously linked with AARGC enhancement.[19,60,61] The work presented here demonstrates that the crude extract (0.25 and 0.5 mg/g) has a dose-dependent effect, inducing apoptosis, in the distal colon of mice and at the same time reducing cell proliferation in the colon. Previous work on the muricid extract in our laboratory reported a significant increase in the apoptotic index in the distal colon at the high 1.0 mg/g dose, but no effect on cell proliferation, whereas at the lower dose of 0.125 mg/g, there was no significant effect on apoptosis, but an unexpected increase in cell proliferation.[29] Thus, this latest in vivo study has allowed us to identify the minimum crude dose required for significant protective effects (0.25 mg/g). It is important to optimize the lowest effective dose for potential nutraceutical development of natural extracts. At the same time this study has confirmed that the chemopreventative effects are primarily associated with a purified fraction containing mostly 6-bromoisatin, which is active at even lower doses than the crude extract.

The in vivo results observed with the semipurified 6-bromoisatin are consistent with our previous in vitro work, which showed that naturally derived 6-bromoisatin could induce apoptosis via activating caspase-3 and -7 enzymes and reduce cell proliferation in both human colon adenocarcinoma (HT29) and human epithelial colorectal adenocarcinoma (Caco-2).[28] By comparison, synthetic 6-bromoisatin did not effectively induce apoptosis in CRC cells in vitro, but when tested in vivo, it did show the ability to significantly increase the apoptosis index and reduce cell proliferation in the distal colon of mice at the concentration of 0.05 mg/g,[56] in a similar fashion to the muricid-derived 6-bromoisatin reported here. Some structurally similar isatin and indole compounds have been also shown to induce apoptosis in a range of cancer cell lines.[62-64] For example, 5,6,7-tribromoisatin at a concentration of 8 µM induced apoptosis through activation of caspase-3/-7 in the Jurkat cell line.[62] Together these data highlight the potential for developing simple naturally derived brominated isatin derivatives for the prevention or treatment of cancer.

Tyrindoleninone, on the other hand, did not significantly increase the apoptosis index in the distal colon of mice and in vivo it also elicited some toxic effects. In vitro studies in our laboratory revealed that 195 µM (0.05 mg/mL) tyrindoleninone significantly induced apoptosis by activating caspase-3 and -7 activity in HT29 cell line, while this compound caused necrosis but not apoptosis in Caco-2 cells.[28] In contrast, Edwards et al demonstrated that 20 µM tyrindoleninone significantly induced apoptosis in the KGN cell line.[27] Combined these results indicate that the apoptotic effects of tyrindoleninone in vitro are cancer cell line or tissue type specific, and in combination with the lack of in vivo activity and potential for toxic effects on the liver and red blood cells observed in this study, this compound is of lower priority for future development as a colon cancer preventative agent. Tyrindoleninone is a relatively unstable compound that is easily oxidized to form 6-bromoisatin,[26] and it appears to be degraded on exposure to simulated gastric fluid.[29] By comparison, 6-bromoisatin is a relatively stable compound that increases in the extract after exposure to digestive fluid,[29] and it can be easily quantified using a validated method for quality control.[65] Consequently, by confirming the beneficial effects of 6-bromoisatin, as opposed to tyrindoleninone, the present study provides an important advance for the strategic development of Muricidae molluscs as functional foods and nutraceuticals. Recent studies have confirmed that 6-bromoisatin and tyrindoleninone can be selectively concentrated by supercritical fluid extraction,[66] thus providing an efficient alternative to traditional solvent extraction that is safe for nutraceutical production. Following on from this study, future work can focus on optimizing the purification and formulation of 6-bromoistain from *Dicathais orbita* for nutraceutical development and testing in longer term animal trials.

Conclusion

In conclusion, our study has confirmed the ability to simultaneously collect preliminary toxicity information in a short-term AOM model for colon cancer prevention using appropriate saline and AOM controls. More specifically, we have shown that it is possible to monitor for general signs of ill health in the 2 weeks prior to AOM injection, in addition to collecting useful end-point data on blood cell counts, blood biochemistry, and plasma liver enzymes, despite the fact that AOM causes histopathological damage to the liver. Using this approach, we have determined that both semipurified 6-bromoisatin and a crude extract from Muricidae molluscs enhanced the apoptotic response to a genotoxic carcinogen in the crypt of the distal colon, without any apparent toxic side-effects. 6-Bromoisatin was found to be the main chemopreventative agent in the extract, significantly enhancing apoptosis and reducing cell proliferation in the colonic crypts at the lowest dose of 0.05 mg/g. Our results also showed some potentially toxic effects of purified tyrinoleninone on red blood cells and on liver enzymes (when combined with AOM), but without any efficacy in increasing AARGC. By comparison, 6-bromisatin, in addition to its efficacy, appears to have some immune-modulatory effects and is relatively more stable, and thus is a more useful target for optimizing as a nutraceutical from muricid extracts for further development as a CRC preventative. Longer term animal models are required to determine the effect of crude extract and 6-bromoisatin on pre-neoplastic lesions or ACF formation. Further studies to assess any possible side effects corresponding to the long-term administration of these compounds would also be of interest.

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