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Chemoprevention of benzo(a)pyrene-induced colon polyps in *Apc^{Min}* mice by resveratrol

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Abstract

Human dietary exposure to benzo(a)pyrene [BaP] has generated interest with regard to the association of BaP with gastrointestinal carcinogenesis. Since colon cancer ranks third among cancer-related mortalities, it is necessary to evaluate the effect of phytochemicals on colon cancer initiation and progression. In this study we investigated the preventive effects of resveratrol (RVT) on BaP-induced colon carcinogenesis in *Apc^{Min}* mouse model. For the first group of mice, 100 µg BaP/kg bw was administered to mice in peanut oil via oral gavage over a 60 day period. For the second group, RVT was co-administered with BaP at a dose of 45 µg/kg. For the third group, RVT was administered for 1 week prior to BaP exposure for 60 days. Jejunum, colon and liver, were collected at 60 days post-BaP & RVT exposure; adenomas in jejunum and colon were counted and subjected to histopathology. Resveratrol reduced the number of colon adenomas in BaP + RVT-treated mice significantly compared to mice that received BaP alone. While dysplasia of varying degrees was noted in colon of BaP-treated mice, the dysplasias were of limited occurrence in RVT-treated mice. To ascertain whether the tumor inhibition is a result of altered BaP-induced toxicity of tumor cells, growth, apoptosis and proliferation of adenocarcinoma cells were assessed post treatment with RVT and BaP. Co-treatment with RVT increased apoptosis and decreased cell proliferation to a greater extent than with BaP alone. Overall, our observations reveal that RVT inhibits colon tumorigenesis when given together with BaP and holds promise as a therapeutic agent.

Keywords

Benzo(a)pyrene; Polycyclic aromatic hydrocarbons; resveratrol; colon carcinogenesis; *Apc^{Min}*; mouse

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1. Introduction

Colorectal cancer (CRC) is one of the most common cancers in the Western world. In the United States alone, nearly 150,000 new cases of CRC are reported every year and 56,000 deaths are attributed to this cancer. The overall incidence of colorectal cancer is higher in men (58.9/100,000 in 1987-91) than in women (40.4/100,000), and this holds for all age groups [1]. In 90% of the colon cancer cases, there is no familial history of colon cancer. Sporadic gene damage seems to play an important role in the development of tumors in the colon. It has been postulated that dietary and environmental factors might contribute to the sporadic gene mutations and therefore be involved in the induction of sporadic colon carcinomas [2].

One environmental compound which has been linked to dietary intake leading to the development of colon tumors is Benzo(a)pyrene. Benzo(a)pyrene (BaP) is a prototypical representative of the family of polycyclic aromatic hydrocarbons (PAH) chemicals. Cigarette smoke, automobile exhausts, charcoal-broiled meat, and industrial emissions contain considerable amount of BaP. When inhaled or ingested through water and diet, BaP becomes activated in biological systems to reactive metabolites and as a consequence can lead to the development of cancer [3]. A study conducted by Kazerouni et al. [4] revealed substantial amounts of BaP in bread, cereals, grains, vegetables and fruits. These authors also found elevated levels of BaP in meats. Contamination of a variety of foods, the average daily intake and the contribution of BaP dietary intake to toxicity and carcinogenesis from a risk assessment standpoint was reviewed in Ramesh et.al. [5]. All these studies highlight that sustained dietary exposure of humans to BaP most likely leads to the development of CRC. Evidence pertaining to the dietary intake of PAHs and their role in the development of digestive tract cancers in animal models and humans has recently been reviewed by our research group [6].

Epidemiological and animal model studies have shown that phytochemical ingredients of diet play a major role in disease prevention [7,8]. Nutritional prevention has been suggested to reduce the occurrence of colon cancer by ~60% [9]. Just as there are many carcinogenic chemicals of environmental origin or cooking-generated ones in human diet, the diet also contains chemicals, which are biologically active and proven to be effective against tumors in animal models and cell culture studies [10,11]. One such promising compound is resveratrol (RVT; 3,5,4'-trihydroxystilbene). Resveratrol is a phytoalexin and a polyphenolic compound present in grapes, peanuts and mulberries [12]. Because of its anti-carcinogenic and chemotherapeutic activities, studies have been undertaken to test its ability to block tumor initiation, promotion and progression [13]. A great majority of the available studies on RVT's chemopreventive and anti-carcinogenic effects on toxicity of environmental chemicals were conducted using liver microsomal preparations, mammary cell cultures of rodents, liver, hepatoma cells, and bronchial epithelial cells of humans. These studies showed decreases in tumor cell proliferation, increased apoptosis and decreases in cell proliferation [12,14].

Animal models have increasingly been used in cancer prevention research as they are useful to developing biomarkers for early detection, surrogate endpoint biomarkers and also serve as screening tools to test the efficacy of anticarcinogenic compounds [15]. Towards this end, transgenic mouse models are developed through germ line manipulation by over expressing or deleting certain genes with the sole objective of generating mice that are more prone to developing cancer and mimic human cancer paradigms. In most patients with CRC, whether sporadic or inherited, there is a mutation in the Adenomatous Polyposis Coli (APC) tumor suppressor gene. The APC protein interacts with β -catenin in a multi-protein complex to regulate the level of expression of β -catenin [16]. Loss of normal APC protein function can

lead to an accumulation of β -catenin in the cytosol and the nucleus. This loss of function is associated with bi-allelic mutations of the *APC* gene [17]. These mutations are signatures of sporadic colorectal cancer and colorectal tumors that develop in Familial Adenomatous Polyposis (FAP) patients. FAP is a dominantly inherited disease that manifests itself by the development of polyps in the colon and the upper gastrointestinal tract, which ultimately, evolve into fatal aggressive tumors when left untreated. The *Apc^{Min}* mouse model has a mutated adenomatous polyposis coli (*Apc*) gene, similar to that in patients with familial adenomatous polyposis. The *Apc^{Min}* mice are born with a large number of small polyps of the upper GI tract, but fewer polyps in colon and have an average lifespan of 120 days [18]. This model is most promising as it mimics the rapid development of adenomatous polyps that affect humans and sporadic colorectal cancers and hence is widely used to elucidate the cellular and molecular mechanisms that underlie gastrointestinal tract (GI) cancers [19]. This model is ideal to evaluate the effects of diets and chemopreventive compounds on the rate and extent of colon cancer initiation and progression.

The purpose of the current study was to investigate the chemopreventive effects of resveratrol (RVT) on benzo(a)pyrene (BaP)-induced adenomas and pathology of the colon in *Apc^{Min}* mouse model. Since adenomas are biomarkers of tumor formation, examining the relationship between RVT exposure and adenoma development provides an understanding of the extent to which the target tissues are susceptible to damage from exposure to BaP alone and BaP in combination with RVT. In this study we show that RVT treatment caused a decrease in the incidence, size, and number of adenomas formed in the colon of mice exposed to BaP, compared to mice exposure to BaP alone.

2. Materials and Methods

2.1. Animal husbandry and BaP and RVT exposure

Five-week-old male *Apc^{Min}* mice (Jackson Labs, Bar Harbor, ME) weighing approximately 30 g were housed in groups of 2-3 per cage, maintained on a 12/12 hour light/dark cycle and allowed free access to rodent chow (NIH-31 open formula diet) and water. All animals were allowed a seven-day acclimation period prior to being randomly assigned to a control (n = 10 per each time point) or treatment group (n = 10 per each time point). Treatment consisted of a single dose (100 μ g/kg bw) of BaP (97% pure, Sigma Chemical Co., St. Louis, MO) dissolved in research grade peanut oil (Sigma). Resveratrol (45 μ g/kg bw; Sigma), dissolved in 10% ethanol and 90% deionized water, was given concurrently with BaP (for 60 days), or prior (daily for 1 week) to BaP exposure (for 60 days). The test chemicals (BaP & RVT) were administered through oral gavage (200 μ L volume). All animal studies carried out were in conformity with the policies of Institutional Animal Care and Use Committee of Meharry Medical College. As BaP is a potential carcinogen, it was handled in accordance with NIH guidelines [20].

All the mice from control and treatment groups were observed twice a day (including holidays and weekends) for moribundity and mortality. Mice body weight and food consumption were monitored periodically.

2.2. BaP and RVT dose relevance

Dietary exposures of humans to BaP vary. While some studies reported BaP intake of 2.8 μ g/person/day [21], others reported 8.4 μ g/person/day [22] and 17 μ g/person/day [23]. Because of the increasing environmental contamination by BaP, allowance was made for exceptionally high dietary exposures. For computing dietary intake of BaP by mice, which approximates the human dietary intake of BaP, the highest daily exposure of 17 μ g/person/day for an average male weighing 70 Kg was chosen. Using this value, the human intake of

BaP translates to 0.24 $\mu\text{g/kg}$ bw/day. Thus, the dose of 100 μg BaP /kg bw, when given to *Apc^{Min}* mice was equivalent to the human dietary intake of BaP for a period of 12 months.

The dose of RVT given to mice was also within the range of dietary levels of this compound in humans. Wang et al. [24] have shown that RVT levels range from 1.6 to 1040 nmol/g in grape products, 1.1 and 1.6 nmol/g in cranberry and grape juice, respectively. Thus, consumption of an 8 ounce portion of cranberry or grape juice by a healthy human will result in an intake of 0.24 to 0.37 $\mu\text{mol/g}$ RVT; consumption of a 230 grams portion of grape products will result in an intake of 0.24 mmol/g RVT. Thus, the dose of 45 μg BaP /kg bw, when given to *Apc^{Min}* mice was equivalent to human dietary intake of RVT for a period of 6 months.

2.3. Sample Collection

At the end of 60 days of exposure, mice were sacrificed and target tissues (liver, small intestine, and large intestine) were retrieved following the guidelines of Ruehl-Fehlert et al. [25] and preserved in 5% formalin for observation for gross pathological changes. The size, location and number of adenomas in the colon were documented. The large intestine was excised into proximal (cecum through transverse colon), distal (splenic flexure through sigmoid) and rectum (first 2 cm from anal opening) portions. Intestines were opened longitudinally and the mucosal layers were spread out over tissue paper. Adenomas were identified by naked eye as tumor-like excrescences which stood out from the surrounding mucosa. Surface staining with methylene blue was used to enhance the contrast between polyps and normal mucosa. The polyps were counted by one observer, blind to the treatment group, and dissected using a scalpel. The intestines were preserved for histopathological examination using the Swiss roll technique [26]. Intestinal samples were placed in a 50 mL tube containing 10% formalin. After 24 hrs, the samples were transferred to a 15 mL tube containing 70% ethanol. The H&E staining and sectioning was performed at Vanderbilt Ingram Cancer Center (VICC) Human Tissue Acquisition and Pathology Laboratory. In addition to studying the total distribution of polyps in jejunum and intestine, we also categorized the polyps on the basis of their size. The neoplastic lesions were evaluated by size (< 0.5 cm, > 1.0 cm) using a digital Vernier caliper, number (single and multiple), and type (adenoma with or without high grade dysplasia, or invasive adenocarcinoma). Degree of dysplasia and invasive carcinoma were evaluated using criteria as described by Boivin and colleagues [26]. The pathologist (MKW) who examined and assessed the slides had no prior knowledge of the treatment groups.

2.4. Mouse colon cell culture, BaP and RVT treatment

Mouse colon adenocarcinoma cell line (CT26.CL25) was purchased from the American Type Culture Collection (ATCC; Manassas, VA). This cell line was maintained as specified in the supplier's manual. It was cultivated in a T75 polystyrene cell culture flasks containing RPMI-1640 medium (ATCC) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin (Invitrogen) at 37°C in a 5% CO₂ incubator containing 50mL/LCO₂.

2.5. Growth assay

At specific time points (24, 48, 72 and 96hrs) mouse colon cells treated with BaP and/or RVT were detached from 6well plates using trypsin. After the cells had been trypsinized, 0.5 mL of suspended cells was added to 950mL of Isoton II (GMI, Inc., Ramsey, MN). Cells were then counted using a Beckman Coulter Z1 Cell Counter (GMI, Inc., Ramsey, MN). All cell counts were done in triplicate.

2.6. Apoptosis assay

To analyze apoptosis, cells were plated onto a 6 well plate at a density of 20,000 cells/well. The cells were synchronized using the serum starvation method by incubating the cells overnight at 37°C in starvation media (RPMI-1649 medium supplemented with 1%FBS, 1% penicillin-streptomycin). The cells were then either left untreated, vehicle-treated (DMSO) or treated with BaP (5µM) or RVT (10µM) individually or in combination (5µM BaP + 10µM RVT) for 24, 48, 72 and 96 hrs. At the end of the exposure periods, cells were harvested and apoptosis was determined by using the Caspase-3 colorimetric assay kit (MBL International Corporation, Woburn, MA). Cells treated with sodium butyrate (10 mM) served as a positive control (data not shown).

2.7. Cell cycle analysis

Mouse colon tumor cells were seeded (1 million cells per flask) in T-75 flasks. After 24 hours, to allow adherence to the flask, the cells were synchronized using RPMI as mentioned above. Once the cells were synchronized by serum starvation, they were treated with 5µM BaP, 10 µM RVT, and 5µM BaP + 10µM RVT. Untreated and DMSO-treated cells served as controls. After exposure to BaP and RVT for specific time points (24, 48, 72 and 96 hours), 2 million cells were taken, washed twice with Phosphate Buffered Saline (PBS, pH 7.4 containing 0.01% Bovine Serum Albumin), trypsinized and collected. They were then fixed in 100% cold ethanol, incubated at 4°C for 1 hour and stained with propidium iodide (Sigma-Aldrich). After staining, 20µL of 0.1% RNase in PBS was added to the cells and incubated at 4°C for 1 hr prior to measuring the fluorescence. The cell preparations were analyzed using a Fluorescence-Activated Cell Sorting (FACS) instrument (Becton Dickinson, Franklin Lakes, NJ) available in the Meharry BSL3/FACS core facility. The fluorescence was measured at an excitation wavelength of 488 nm with a blue laser and detected at channel FL2. All experiments were done in triplicate.

2.8. Statistical evaluation of data

All statistical analyses were done using the GraphPad Prism software (GraphPad software, La Jolla, CA) package. Adenoma data were analyzed by one-way ANOVA and Bonferroni *posthoc* test. Data for growth, apoptosis, and cell cycle changes for each time point were analyzed by one-way ANOVA, whereas differences among all treatment groups were

3. Results

3.1. Exposure to BaP or RVT does not cause changes to body weight or food consumption

No BaP or RVT treatment-related deaths occurred in mice during administration of these chemicals or their vehicle counter parts. Additionally, no treatment-related changes were seen (data not shown here) in either body weight or food consumption of mice administered with BaP/RVT compared to their control counterparts.

3.2. Resveratrol modulates the tumor burden and histopathology of BaP-exposed Apc^{Min} mice

Representative of mouse colon tumors in untreated, BaP-treated and BaP + RVT-treated mice are shown in Fig. 1. The number of adenomas were significantly decreased in jejunum and colon by 45% and 40% ($P < 0.001$) respectively (Fig. 2) in mice treated with RVT. The size distribution of polyps in jejunum and colon for BaP-, RVT-, BaP + RVT-treated mice are depicted in Fig. 3. Though no significant difference was observed in the size of adenomas in jejunum of BaP + RVT-treated mice, compared to that of BaP-treated mice (data not shown); the size of colon adenomas in BaP + RVT-treated mice was significantly less compared to mice that received BaP alone. Concurrent administration of BaP & RVT

yielded a greater reduction in total tumor numbers and tumor size compared to RVT treatment prior to BaP administration.

The histopathological features of colon from BaP-, RVT prior to BaP- and BaP + RVT treatment groups are shown in Fig. 4. There were no histopathologic differences in the colon of RVT-only treated mice compared to the untreated control groups. While a greater amount of dysplasia in the colon was observed in BaP-treated mice, the dysplasia was of limited occurrence in RVT-treated mice.

3.3. Resveratrol inhibits the growth of BaP-exposed mouse colon adenocarcinoma (CT26.CL25) cells

In order to see whether the growth inhibition of tumors in mice could be replicated in vitro conditions, the viability of mouse colon carcinoma cells exposed to BaP and RVT were monitored and the results are shown in Fig. 5. Compared to control (untreated cells), RVT-inhibited colon tumor cell growth at all treatment time points. However, the effect was prominent only at 48, 72 & 96 hrs ($p < 0.05$). Benzo(a)pyrene together with RVT caused a greater inhibition of growth of colon tumor cells than either compound alone at 48, 72 & 96 hrs and the results were statistically significant ($p < 0.05$). A progressive decrease in growth inhibition of these cells was noticed with an increase in duration of combined exposure to BaP and RVT.

3.4. Resveratrol induces apoptosis in BaP-exposed mouse colon adenocarcinoma cells

As apoptosis plays a pivotal role in cancer causation, the ability of RVT to induce apoptosis on its own and in the presence of BaP is investigated and the results are shown in Fig. 6. The activation of caspase-3 by 5 μ M BaP was very similar between treatment group and untreated controls at all-time points examined. Compared to untreated cells, RVT showed a marginal increase in apoptosis at 24 & 48 hrs, but at 72 & 96 hrs, the differences were statistically significant ($p < 0.01$ to $p < 0.001$). Compared to untreated cells, BaP showed a marginal increase in apoptosis at 24 & 72 hrs, but the differences were statistically significant ($p < 0.01$ to $p < 0.001$) only at 48 & 96 hrs. Even though cells treated with BaP in combination with RVT showed an increase in apoptosis compared to BaP alone at all-time points; the results were statistically significant at the 48 hrs exposure time point. Among the various time points used, BaP in combination with RVT showed a statistically significant increase over BaP alone in inducing apoptosis at all-time points studied except at the 72 hrs time point.

3.5. Resveratrol alters cell cycle profiles in BaP-exposed mouse colon adenocarcinoma cells

The distribution of colon cells in different phases of the cell cycle following exposure to RVT, BaP individually and in combination are shown in the form of a histogram in Fig. 7 and the cell cycle profile outputs from flow cytometry measurements are shown in Figs. 8-11. No significant change was observed in each cell cycle phase among the various treatment groups. Following exposure to RVT alone, the percentage of cells in the G₁- and S phases did not show much of a difference at 24 hour exposure; while at 48 and 72 hours, cells showed a slight increase in S phase relative to G₁ phase. At all the exposure periods, the percentage of cells showed a reduction in G₂ phase, which indicates that RVT blocks the cell cycle progression at G₂/M transitions.

When the cells were exposed to BaP, the percent cells that remained at the S phase showed an increase at 24, 48 and 72 hours and showed a decline at 96 hrs. On the other hand, the percentage of cells remaining in the G₂ phase registered a two-fold decline at all the time

points studied. Our results suggest that BaP reduced the transition or accumulation of cells in the G₂ phase.

Upon exposure of cells to a combination of BaP + RVT, the percentage of cells that remained at the S phase showed a decrease at 48-96 hrs time points tested compared to 24 hrs. Our results suggest that RVT when used in conjunction with BaP caused S-phase arrest and further reduced the transfer of cells in G₂/M phase.

However, overall assessment of cell cycle changes during 48, 72, and 96 hours for all treatment groups revealed an extremely significant decrease in the percent of cells progressing from G₁ to G₂. There is also a significant difference among the treatment groups in cell cycle progression observed during the 96 hour exposure period.

4. Discussion

Several studies have documented that BaP and other PAHs administered to various animal models contribute to colon tumor development (reviewed in [6]). Our results on RVT-induced decline in colon tumors are in agreement with those of other researchers who reported suppression of colon tumors by RVT in rodent models. Schneider et al. [27] noted a 70-100% reduction in the number of tumors in the small and large intestine of *Apc^{Min}* mice treated with RVT. These mice were not exposed to any carcinogen. Since most *Min* mice are born with minute polyps in their small intestines, in the absence of exposure to exogenous carcinogen, the reduction in tumor numbers is noteworthy. On the same lines, RVT administration has been reported to decrease the number of aberrant crypt foci (ACF) in the colon of rats [28]. Similarly, grape-derived polyphenols, of which RVT is a constituent, reduced the numbers of azoxymethane (AOM)-induced ACF by 49% in the colon of Wistar rats [29].

Resveratrol has also been reported to reduce dimethylhydrazine-induced ACF by 50% in the colon of rats [30]. Similarly, pterostilbene, a methylated analog of RVT has been shown to inhibit ACF per mouse colon by 4-3-54% and adenomas-carcinomas per mouse colon by 70% in AOM-treated mice [31]. Another study followed suit where in pterostilbene has been found to bring down colonic adenocarcinomas by 20% (compared to controls) in F-344 rats exposed to AOM [32]. Also, a reduction in invasive adenocarcinoma in test animals was noticed in this study. Experiments by Walter et al. [29] have also demonstrated the polyphenol inhibition of tumor growth by 31% in BACB/C mice subcutaneously implanted with C26 colon carcinoma cells. Further evidence in support of the preventive effect of RVT comes from the studies of Sengottuvelan et al. [33] who reported suppression of 1,2-dimethylhydrazine induced ACF in rats by RVT.

Dietary grape extract, which contains RVT has been shown to decrease the incidence and multiplicity of tumors in small intestines (30% reduction compared to controls) and colon (9% reduction compared to controls) in Fisher rats treated with AOM [34]. In addition to RVT-induced inhibition of BaP-induced colon tumors in *Apc^{Min}* mice as observed in our study, other investigators also reported inhibition of BaP-induced lung tumors in A/J mice by RVT [35]. Resveratrol has been reported to decrease adenoma formation in small intestines and colons of chemically untreated *Apc^{Min}* mice [36].

The afore-mentioned findings have clearly shown that RVT is effective against tumors of the small intestine and colon. In addition to decreasing tumorigenesis, RVT may also suppress inflammatory bowel disease. Given the fact that ulcerative colitis is a risk factor for colon cancer [37], studies were conducted to see whether RVT could suppress colitis and colon cancer associated with it [38]. A reduction in tumor incidence from 80% (AOM and dextran sodium sulfate treated mice) to 20% (AOM and DSS and RVT) was noted in a DSS

mouse model of colitis-driven colon cancer. In these studies, RVT was admixed in diet for administration. Another study, where a RVT derivative was intragastrically exposed to a similar mouse model of colitis also reported a reduction of 15-35% in tumor multiplicity compared to the AOM-treatment group [39]. On the other hand, studies conducted by Ziegler et al [40] have revealed that RVT does not modify tumor load in *Apc^{Min}* mice, despite administering high doses (0, 4, 20 & 90 mg/kg for 7 weeks). These mice were not exposed to any carcinogens prior to administering RVT. As a result of RVT administration, neither Cox-2 expression nor tumor number was affected in these mice. Whether the lack of consensus among various *in vivo* studies regarding RVT's effects on tumors is related to the mode of RVT administration and its bioavailability is not yet known. The conflicting reports call the need for conducting more *in vivo* studies with site-specific RVT delivery systems to tumor-prone or tumor bearing tissues to explore whether RVT renders any protective effect.

The debate regarding the bioavailability of RVT [41,42] notwithstanding, considerable accumulation of RVT in mouse intestinal tissues has been reported subsequent to oral administration [43,44] to elicit the presumed beneficial effects. At least 50% of the orally administered RVT was found to be absorbed from the gastrointestinal tract in rats [45,46]. Another study supported considerable bioavailability (more than 60%) of RVT in the GI tract of pigs (47). Also, biologically effective concentrations of RVT were shown to result from chronic dosing with this phytochemical as shown in humans [48,49]. Therefore, it is likely that biologically active concentrations of RVT could be achieved in *Apc^{Min}* mice in our subchronic dosing study.

Timing of RVT administration appears to be important in eliciting the anticarcinogenic effect. We have used RVT concurrently with BaP, and also prior to BaP administration. In order to inhibit tumor growth, RVT must be readily available in target tissues. Since carcinogenesis encompasses initiation, promotion and progression phases, chemopreventive agents like RVT can act at one or more phases to render their protective effect [50]. Given the rapid metabolism of RVT [51], prior treatment of mice with RVT in the present study may not have yielded enough 'biologically potent fraction of the administered RVT dose' to be readily available when BaP administration is commenced, and tumor formation is initiated, so that the tumor growth could be inhibited. On the other hand, during concurrent BaP & RVT administration, the biochemical or molecular pathways targeted by BaP could be modulated by RVT as indicated by the drop in tumor counts and tumor size in the present study. We also have investigated whether RVT administration post BaP subchronic exposure could bring down the tumor count and size. Resveratrol failed to reverse the BaP-induced carcinogenic effects (data not shown). These observations are consistent with a previous report where RVT administration post-tumor initiation phase had no effect on the lung tumors induced by BaP in A/J mice [35], which could be attributed to the insufficient bioavailable fraction of RVT at the target site [52] to undo the damage caused by BaP.

Also significant was our finding that RVT could not only reduce the tumor burden, but also the tumor progression as evident by histopathology. Benzo(a)pyrene and other PAHs have been reported to cause adenomatous polyps with low- to high grade dysplasia and invasive adenocarcinomas in the gastrointestinal tract (reviewed in Diggs et al. [6]). The pathological changes in target tissues induced by these toxicants are associated with production of highly reactive free radicals, and initiation of oxidative damage [53,54]. In contrast, in the present study, mice treated with resveratrol which possesses antioxidant properties [55] showed noticeable improvement in histopathological characteristics with reduced number of lesions and low-grade dysplasia. A similar histopathological recovery effect of RVT has been reported in C57BL/6 mice model of colon cancer associated with colitis [38]. These findings indicate that resveratrol may protect mouse colon cells against cell damage induced by reactive oxygen species (ROS) or lipid peroxidation similar to that reported for other target

tissues. Therefore, our study provides evidence that RVT is able to rescue the altered architecture of BaP-exposed colon tissue.

The decrease in tumor size, number and pathological changes are indicative of the anticarcinogenic effect of RVT. Several lines of evidence suggest that the net increase in number of initiated cells and growth of tumors are dependent on how chemical carcinogens and/or their metabolic products modulate the rates of cell proliferation and apoptosis [56-58]. To find out whether RVT treatment enables the tumor cells to recover from toxic insult, we have used mouse adenocarcinoma cells exposed to BaP and RVT individually and in combination to investigate the effect of RVT on growth, apoptosis and cell cycle changes in mouse colon cells. Published reports indicate that after RVT oral administration (at varying doses and exposure durations) 3-30 μM of this compound was registered in plasma and/or target tissues of rodents [43,59]. Hence we have used physiologically relevant - concentrations of 10 μM to expose mouse colon cells in order to study apoptosis and cell cycle changes. Instead of using conventional dosing procedures, if site-specific delivery systems (pectin-based formulations that circumvent RVT being metabolized by enzymes of the upper GI tract; [60]) are used to deposit RVT in target tissues such as colon, the exposure concentration used by us (10 μM) and other investigators (50-150 μM ; [61]) could very well be achieved in the colon.

Our studies have revealed that BaP is toxic to mouse colon cells, which is in agreement with cytotoxicity of this chemical and other PAHs reported for human colon tumor cell lines [62-64]. Even though studies have shown that RVT caused inhibition of cell growth and proliferation in different human colon tumor cell lines such as Caco-2 [27,65], Hct-116 [65,66], HT-29 [61] and SW480 [42,61], the cells were not exposed to BaP or PAHs in any of these studies. Our findings in a co-exposure system have shown that RVT exerts an inhibitory effect on colon cancer cell growth, which explains the decrease in polyp size observed in mice treated with RVT. This result is consistent with a previous report which indicated a concordance between the ability of RVT to inhibit the growth of Apc 10.1 colon cells and adenoma development in vivo in the Apc^{Min} mouse model [67].

Our findings also show that RVT increases the rate of apoptosis in tumor cells and slow the tumor progression. Imbalances between cell death and proliferation culminate in tumor formation [68]. Since disruption of this balance can lead to malignant transformation of cells, apoptosis serves as a checkpoint thereby regulating tissue homeostasis [69]. Therefore, our studies and those of others advocate that modulation of apoptosis using RVT is a promising strategy to control chemical-induced cancers [70,71]. Our findings are in accordance with those of others that reported RVT and/or its derivatives stimulated apoptosis in colon cancer cell lines [72] and animal models [73,74]. Cell culture and animal model studies where RVT was used along with BaP also generated similar findings. For e.g. co-exposure of human bronchial epithelial cells to both RVT (10-50 μM) and BaP (1 μM) showed inhibition of BaP-DNA adduct formation [75]. Similarly, co-treatment of RVT (50 mg/kg bw/wk) and BaP (5mg/kg bw/wk) were shown to increase apoptosis and inhibit BaP-DNA adduct formation in lung tissues in a Balb-c mouse model [76]. Enhancement of apoptosis by RVT may have implications in ameliorating the tumor burden in Apc^{Min} mice, especially in long term exposure scenarios (similar to that used in the present study) through modulation of proinflammatory cytokine production. In this regard it needs to be mentioned that BaP has been reported to cause colonic inflammation [77] and tumor development [78]. Martin et al. [73] reported that RVT stimulates apoptosis during early colonic inflammation in a rat model. Larossa et al. [79] also reported that low doses of RVT prodrugs have a dampening effect on colon inflammation in mice.

Uncontrolled cell proliferation is one of the hallmarks of carcinogenesis. Upon BaP exposure, the percentages of cells remaining at the G₁ phase were less than those of other treatment groups, which is in line with the notion that G₁ phase serves as a restriction or checkpoint to govern the cell-cycle transition [80]. On the other hand, the percentage of cells remaining at the S phase showed a slight increase at 24, 48 hrs and maintained at the same level until 72 hrs and declined thereafter. Since progression beyond the G₁ gate will cause the cell to undergo mitosis, after carrying out DNA synthesis and replication [81], the considerable percent of cells remaining at the S phase increase the fixation of mutations and the likelihood of tumor development [82]. Normally one would expect to see a defense mechanism by cells to undergo arrest in G₁ phase. In the present study cells were accumulated both in G₁ and S phases which appears to be a consequence of cells skipping the DNA damage G₁ checkpoint and proceeding into the S phase. This 'stealth effect' of evading cellular defense has earlier been shown with diol epoxides of BaP and benzo[g]chrysene both in transformed and malignant cell lines, which may lead to frequency of mutations in daughter cells [83], a situation that favors progression of tumors. In contrast to G₁ and S phases, less than 20% of the cells remained at G₂ phase at all-time points. In this phase BaP did not show any difference with other treatment groups in terms of the percentage of cells positioned. The smaller G₂ phase is an indication of cell growth reaching a stationary phase at this stage. The cell cycle behavior of BaP, especially the prolonged arrest at S phase observed in our study is similar to that observed for human hepatoma [84], mouse hepatoma Hepa 1c1c7 cells [85], MCF-7 breast cancer cells [86] and non-neoplastic rat liver epithelial WB-F344 cells [87].

The cell cycle distribution for RVT-treated colon cells showed a trend similar to that of BaP. Resveratrol has been shown to inhibit cell proliferation by blocking cell cycle progression [88,89]. In the present study RVT blocked the cell cycle both at G₁ and S phases. Literature reports also indicate that RVT blocks different stages of the cell cycle which depends on the cancer cell type. For e.g. RVT-induced cell accumulation in G₁ phase has been reported in human epidermal carcinoma A 431 cells [88] and bladder cancer T24 cells [90]. Resveratrol-induced cell accumulation in S phase has been reported in Caco2 colon cancer cells [91], MCF-7 breast cancer cells [92] and human leukemia U937 cells [89]. Even in the same type of cells, RVT may have a role in all the 3 phases of the cell cycle as shown by Whyte et al [93] for A549 lung cancer cells. While accumulation of RVT-treated cells at G₁ phase could be attributed to apoptosis [88], considerable positioning of RVT-treated cells in S-phase could be attributed to activation of S-phase checkpoint either as a protective response against reactive metabolite binding with DNA to cause DNA damage [94] or removal of DNA lesions by eliminating injured cells through apoptosis [95]. The latter explanation draws support from our findings in the present study where RVT alone and RVT in combination with BaP induced apoptosis by 1-2 fold at 48, 72 & 96 post-exposure time points.

One explanation which we would like to propose for the RVT-induced cell cycle block at the G₁ and S phases is as follows. Even though the cells were initially blocked at G₁, the blockage may be leaky, allowing cells to reenter the cell cycle and resume progression, which was blocked again at S phase. Also, the possibility of apoptosis induction by RVT occurring independent of G₁ phase arrest cannot be ruled out. Of relevance in this context is the reported down regulation of genes in cell cycle progression in *Apc^{Min}* mouse intestines upon oral exposure to RVT [27]. Therefore, the differential effects of RVT on colon cell cycle could be attributed to changes in the expression of cell cycle regulatory proteins [96] which we have not explored in this study. Nonetheless, our data on apoptosis and cell growth together with cell cycle support our contention that RVT is a cell cycle blocker. We therefore suspect that the modulation of balance between proliferation and apoptosis of BaP-

treated colon cells by RVT could be useful in delaying the tumor onset and slowing down tumor progression as well.

In summary, results from our *in vitro* studies further corroborated our *in vivo* findings that RVT when used in conjunction with BaP slows down tumor progression. Taken together, our findings lend support to the hypothesis that RVT is a promising anticancer agent. Though several pathways are involved through which RVT exerts its chemopreventive effect, of special interest to us are the mechanisms by which RVT ameliorates the cancers induced by chemical carcinogens such as BaP. As metabolism of environmental or dietary toxicants drive the carcinogenic process, attempts are underway in our laboratory to investigate which BaP biotransformation pathways are targeted by RVT to delineate the mechanistic underpinnings of RVT-induced chemoprevention of chemical carcinogenesis.

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Figure 1.
Representative pictures of colon polyps of Apc^{Min} mouse exposed to A) 100 μg/kg BaP/kg bw; B) 100 μg/kg BaP + 45 μg/kg RVT.

Figure 2.

Distribution of polyps in jejunum and colon of *Apc^{Min}* mice treated with 100 μ g BaP and/or 45 μ g RVT /kg bw. Values are expressed as mean + SE. Asterisks indicate statistical significance between mice that received BaP alone and mice that received BaP + RVT or just the vehicles. * p < 0.05 compared to control; # p < 0.05 compared to BaP treatment.

Figure 3.

Size distribution of polyps in jejunum and colon of *Apc^{Min}* mice treated with 100 μ g BaP and/or 45 μ g/kg bw. Values are expressed as mean + SE. Asterisks indicate statistical significance between mice that received BaP alone and mice that received BaP + RVT or just vehicles. * p < 0.05 compared to control; # p < 0.05 compared to BaP treatment.

Figure 4.

Representative images of hematoxylin and eosin stained colon histopathology in *Apc^{Min}* mice treated with A) control (vehicles for BaP and RVT treated simultaneously); B) control (vehicle for RVT treated for 7 days prior to treating with vehicle for BaP for 60 days; C & D) 100 μ g BaP/kg bw for sixty days; E) 100 μ g B(a)P/kg bw and 45 μ g RVT/kg bw simultaneously for sixty days; F) 45 μ g RVT/kg bw for 7 days prior to treating with 100 μ g BaP/kg bw for sixty days via oral gavage. Control (A & B) mice (original magnification 200 X) show normal colonic histology (A) or small adenomas in colon with no high grade dysplasia (B). Colon of BaP-treated mice show invasive carcinoma (C, 200X, arrow) and high grade dysplasia (D, 200X, arrow), whereas the colons of BaP and RVT-treated mice (E & F, 200X) show small adenomas without high grade dysplasia. Benzo(a)pyrene was administered through research grade peanut oil, while RVT was administered in 10% ethanol and 90% deionized water. BaP-benzo(a)pyrene; RVT-resveratrol.

Figure 5.

Growth of mouse colon cancer (CL-25) in presence of resveratrol and benzo(a)pyrene. Cells were treated for 24-96 hours with 5 μ M of BaP and 10 μ M of RVT either simultaneously or individually. Each value represents mean + SEM of at least 5 observations. Asterisk represents $p < 0.05$ when compared to control; # $p < 0.05$ when compared to 24 hrs exposure period. Samples from untreated group were assayed as representative samples for DMSO-treated and no treatment groups inasmuch as their growth responses were not different. Data presented here are from an experiment that was repeated three times with similar results.

Figure 6.

Apoptosis of mouse colon (CL-25) cells treated for 24 - 96 hours with 5 μ M of BaP and 10 μ M of RVT either simultaneously or individually. * $p < 0.05$ when compared to untreated group concentrations. Fold change in apoptosis refers to fold increase in caspase activity of apoptotic cells over that of non-induced cells. The data were normalized by subtracting values of background controls (containing no substrate or cell lysate) from the experimental data prior to calculating the fold increase, as recommended by the manufacturer. Samples from untreated group were assayed as representative samples for vehicle (DMSO)-treated and no treatment groups inasmuch as their apoptotic responses were not different. Data shown here are from an experiment that was repeated three times with similar results.

Figure 7.

Time-dependent distribution of fraction of cells in various phases subsequent to exposure of the cells to BaP and RVT. Cells were treated for 24-96 hours with 5 μM of BaP and 10 μM of RVT either simultaneously or individually. The DNA content of the cells was analyzed by flow cytometry. The fraction of cells in G₁-, S-, and G₂-phases were counted and the percentages of cells in respective phases were determined. Samples from untreated group were assayed as representative samples for vehicle (DMSO)-treated and no treatment groups inasmuch as their cell cycle responses were not different. Data from the histograms was obtained from three independent experiments that generated similar results.

Figure 8.

Cell cycle phase distribution in mouse colon cells exposed to 5 μ M of BaP and 10 μ M of RVT either simultaneously or individually for 24 hrs. The DNA content of the cells was analyzed by flow cytometry. Samples from untreated group were assayed as representative samples for vehicle (DMSO)-treated and no treatment groups inasmuch as their cell cycle responses were not different. Data shown here was obtained from a representative profile of one of three independent experiments performed that generated similar results.

Figure 9.

Cell cycle phase distribution in mouse colon cells exposed to 5 μ M of BaP and 10 μ M of RVT either simultaneously or individually for 48 hrs. The DNA content of the cells was analyzed by flow cytometry. Samples from untreated group were assayed as representative samples for vehicle (DMSO)-treated and no treatment groups inasmuch as their cell cycle responses were not different. Data shown here was obtained from a representative profile of one of three independent experiments performed that generated similar results.

Figure 10.

Cell cycle phase distribution in mouse colon cells exposed to 5 μ M of BaP and 10 μ M of RVT either simultaneously or individually for 72 hrs. The DNA content of the cells was analyzed by flow cytometry. Samples from untreated group were assayed as representative samples for vehicle (DMSO)-treated and no treatment groups inasmuch as their cell cycle responses were not different. Data shown here was obtained from a representative profile of one of three independent experiments performed that generated similar results.

Figure 11.

Cell cycle phase distribution in mouse colon cells exposed to 5 μ M of BaP and 10 μ M of RVT either simultaneously or individually for 96 hrs. The DNA content of the cells was analyzed by flow cytometry. Samples from untreated group were assayed as representative samples for vehicle (DMSO)-treated and no treatment groups inasmuch as their cell cycle responses were not different. Data shown here was obtained from a representative profile of one of three independent experiments performed that generated similar results.