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Research Article

Chemoprevention of Colon and Small Intestinal Tumorigenesis in *APC*$^{min/+}$ Mice By SHetA2 (NSC721689) without Toxicity

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Abstract

The occurrence of intestinal polyps in people at high risk for developing colorectal cancer provides an opportunity to test the efficacy of chemoprevention agents. In this situation of treating otherwise healthy people, the potential for toxicity must be minimal. The small-molecule flexible heteroarotinoid (Flex-Het), called SHetA2, has chemoprevention activity in organotypic cultures *in vitro* and lack of toxicity at doses capable of inhibiting xenograft tumor growth *in vivo*. The objective of this study was to evaluate SHetA2 chemoprevention activity and toxicity in the *APC*$^{min/+}$ murine model. Oral administration of SHetA2 at 30 and 60 mg/kg five days per week for 12 weeks significantly reduced development of intestinal polyps by 40% to 60% depending on the dose and sex of the treatment group. Immunohistochemical and Western blot analysis of polyps showed reduced levels of cyclin D1 and proliferating cell nuclear antigen in both SHetA2 treatment groups. Western blot analysis also showed SHetA2 induction of E-cadherin, Bax, and caspase-3 cleavage along with reduction in Bcl-2, COX-2, and VEGF, consistent with SHetA2 regulation of apoptosis, inflammation, and angiogenesis. Neither dose caused weight loss nor gross toxicity in *APC*$^{min/+}$ or wild-type littermates. Magnetic resonance imaging (MRI) of cardiac function showed no evidence of SHetA2 toxicity. SHetA2 did not alter left ventricular wall thickness. In summary, SHetA2 exerts chemoprevention activity without overt or cardiac toxicity in the *APC*$^{min/+}$ model. SHetA2 modulation of biomarkers in colon polyps identifies potential pharmacodynamic endpoints for SHetA2 clinical trials. *Cancer Prev Res; 6(9); 908–16. ©2013 AACR.*

Introduction

An ideal chemoprevention agent is orally bioavailable and will reduce the incidence of cancer while exerting no side effects. On the basis of preclinical data, the small-molecule compounds called flexible heteroarotinoids (Flex-Hets, Fig. 1A) have the potential to meet these criteria (1–18). *In vitro*, Flex-Hets regulate growth, differentiation, and apoptosis in cancer cells, whereas the effects on normal cells are limited to growth inhibition (1, 3, 6, 7, 12–15). In organotypic culture, they reverse the cancerous phenotype of ovarian and kidney cancer cell lines and primary cultures (1, 13). Sulfur Heteroarotinoid A2 (SHetA2, Fig. 1A) was chosen as the lead Flex-Het because it exerted maximal cancer cell line cytotoxicity in comparison with other Flex-Hets, while not harming nontransformed cells (1, 4, 7). SHetA2 prevented carcinogen-induced transformation of human endometrial organotypic cultures (8). *In vivo*, SHetA2 inhibited xenograft tumor angiogenesis and growth without evidence of gross toxicity (4, 12, 13).

The National Cancer Institute (NCI) Rapid Access to Intervention Development (RAID) and Rapid Access to Preventive Intervention Development programs completed the preclinical testing needed for an Investigational New Drug application to the U.S. Food and Drug Administration for the initiation of SHetA2 clinical trials (5, 11, 17, 18). The ID given to SHetA2 in these studies is NSC721689. Metabolism studies identified hydroxylated metabolites that are less active than the parent SHetA2 structure (11). Twenty-eight-day toxicity studies reported lowest observed adverse effect levels (LOAELs) of oral SHetA2 in rats to be 2,000 mg/kg/day (administered in 1% aqueous methylcellulose/0.2% Tween 80) and the no-observed adverse effect level (NOAEL) in dogs to be greater than the 1,500 mg/kg/day highest dose studied (administered in 30% aqueous Solutol® HS 15; ref. 18). The pharmacokinetic studies showed that SHetA2 is bioavailable and appears to have a maximum absorption at

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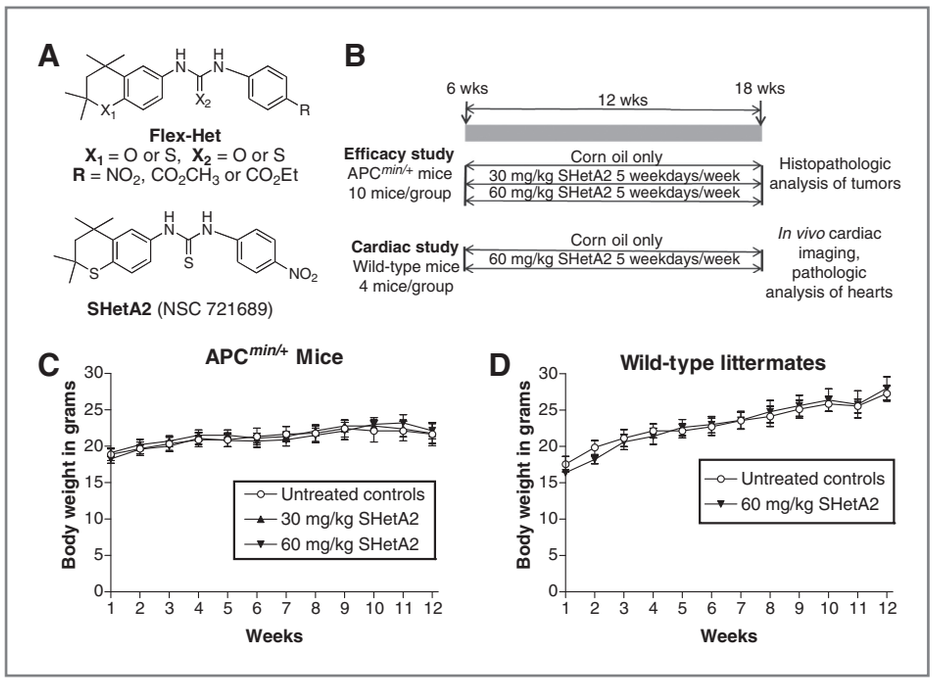

Figure 1. Experimental details and lack of toxicity. A, chemical structures of Flex-Hets and SHetA2. B, experimental design for the evaluation of SHetA2 chemoprevention activity in $APC^{min/+}$ mice and cardiac toxicity in their wild-type littermates. C, changes in body weight over time in $APC^{min/+}$ mice gavaged with the indicated doses of SHetA2 or the same volume of corn oil (Control). D, body weights of wild-type mice gavaged with high dose SHetA2 at the time of cardiac imaging.

100 mg/kg/d (5, 11, 18). Thus, SHetA2 appears to have a good safety profile as an oral chemoprevention agent; however, before entering clinical trials, *in vivo* chemoprevention activity needs to be demonstrated.

Colorectal cancer is a rationale cancer to target for chemoprevention studies because it has a high incidence of preneoplastic lesions and cancerous tumors. There are more than a million new cases of colorectal cancer each year making it the second most common cancer in the Western world (19). Colon cancers are thought to arise due to a series of histopathologic and molecular changes that transform normal colonic epithelial cells into colorectal carcinoma, with an adenomatous polyp as an intermediate step in this process (20). Risk factors include age, diet, and genetic predisposition, including hereditary polyposis and nonpolyposis syndromes (21). Individuals who inherit a defective allele of the *adenomatous polyposis coli* (*APC*) gene suffer from familial adenomatous polyposis (FAP), in which the intestinal epithelium is studded with hundreds of benign polyps, some of which progress to colon adenocarcinoma (22). Furthermore, most of sporadic colorectal cancers exhibit *APC* mutations (23).

Animal models of intestinal tumorigenesis are needed to study the pathogenesis and to develop strategies to control the malignancy including chemoprevention. In this regard, the $APC^{min/+}$ mouse, one of the most studied models of intestinal tumorigenesis, harbors a dominant germline mutation at codon 850 of the mouse homolog of human *APC* gene, which is similar to the mutation in patients with FAP. $APC^{min/+}$ mice develop multiple adenomas throughout the whole intestinal tract that are primarily small intestinal polyps (tubular adenomas) and fewer colon tumors (adenomas and adenocarcinomas; ref. 24). As the $APC^{min/+}$ mouse model is similar to the human FAP, it is extensively used in chemoprevention studies (25).

In addition to studies of chemoprevention activity, animal models offer the opportunity to evaluate potential toxicities that could be induced by the chemoprevention agent. The mechanism of SHetA2 action suggests some potential harm that might come from long-term use of SHetA2 in the general population. Mitochondrial swelling and loss of membrane potential is apparent within 15 minutes of treating human cancer cell lines with SHetA2 and other Flex-Hets (7). This suggests that SHetA2 might cause harm to organs, such as the heart, that are highly dependent upon mitochondrial respiration. Mitochondria in nontransformed human epithelial cells, however, do not swell upon SHetA2 treatment, even when treatment is extended up to 24 hours (7). While this lack of effect on mitochondria in normal cells suggests that SHetA2 would not be harmful to the heart, a more definitive study in animals is needed.

The objective of this study was to determine whether oral SHetA2 has sufficient chemopreventive activity and lack of toxicity to justify initiation of clinical trials. The hypothesis is that oral SHetA2 will reduce incidence and sizes of colon polyps and intestinal tumors in the $APC^{min/+}$ mouse model without causing gross or cardiac toxicity.

Materials and Methods

Breeding and genotyping of $APC^{min/+}$ mice

All animal experiments were conducted in accordance with the institutional guidelines of the American Council on Animal Care and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Oklahoma Health Sciences Center (OUHSC, Oklahoma City, OK). Male $APC^{min/+}$ (C57BL/6J) and female wild-type

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littermate mice were purchased initially from The Jackson Laboratory as founders, and our own breeding colony was established in the OUHSC rodent barrier facility and genotyped by the PCR method using primers (IMR0033 50-GCC ATC CCT TCA CGT TAG-30, IMR0034 50-TTC CAC TTT GGC ATA AGG C-30, IMR0758 50-TTC TGA GAA AGA CAG AAG TTA-30) according to vendor's instructions. All mice were housed, 4 per cage, in ventilated cages under standardized conditions (21°C, 60% relative humidity, 12-hour light/12-hour dark cycle, 20 air changes per hour). All mice were allowed *ad libitum* access to AIN76A diet and automated tap water purified by reverse osmosis.

Efficacy studies in $APC^{min/+}$ mice

The experimental protocol is summarized in Fig. 1B. At 6 weeks of age, groups of mice were gavaged with SHetA2 (obtained from the NCI RAID Program, compound NSC721689, and dissolved in corn oil) 5 weekdays per week at 2 different dose levels (30 and 60 mg/kg) for 12 weeks. A control untreated group was gavaged with the same volume of corn oil. There were 10 mice in each treatment group. To monitor for toxicity, male wild-type C57BL/6J mice were gavaged with 60 mg/kg SHetA2 or the same volume of corn oil solvent in parallel. There were 4 wild-type mice per treatment group. The animals were weighed once weekly and monitored daily for signs of weight loss or lethargy that might indicate intestinal obstruction or anemia. At the end of 12 weeks, the animals were sacrificed by CO_2 euthanasia and all organs were examined for gross pathologic observations. Intestinal tumors were examined and counted under a dissection microscope by two independent investigators who were blinded with respect to the treatment group. Tumor size was determined by measuring along the longest diameter using digital calipers to the nearest 100 μm. Colonic and other small intestinal tumors that require further histopathologic evaluation were fixed in 10% neutral-buffered formalin, embedded in paraffin blocks, and processed by routine hematoxylin and eosin (H&E) staining. In addition, multiple samples of tumors from the small intestines, colons, and normal colonic mucosa were harvested and stored in liquid nitrogen for analysis of cell proliferation, apoptosis, and inflammatory markers.

In vivo cardiac imaging

MRI analysis of cardiac cycles was conducted in the Oklahoma Medical Research Foundation MRI facility. The *in vivo* cardiac imaging was accessed by MRI on a 4.7-T. Oxford Magnet using a Bruker Avance console and a ParaVision (Bruker BioSpin MRI Inc.) MRI system equipped with Master gradients and a five-element cardiac phased array receiver coil. A small-animal instrument monitoring and gating system for respiration rate and electrocardiogram (ECG)-synchronized triggering was adapted to the system. Dorsal and sagittal images were acquired using a cardiac gated gradient echo (GEFI_TOMO) sequence. On the basis of these views, transverse images were prescribed and were collected with the GEFI_TOMO sequence. The following parameters were used: FOV = 4.0 × 3.0 cm, matrix = 256 × 128; repetition time = 0.158 ms; recovering time = 13.22 ms; echo time = 2.0 ms; pulse angle = 301. Slice thickness was 2.0 mm. The image analysis was conducted offline using the NIH Image J software version 1.33n (NIH, Bethesda, MD). The left ventricle wall thickness was measured at end-diastole and end-systole on a series of three slices showing the maximum size of the two papillary muscles while they were still connected with the myocardium. The cardiac output was determined by the average stroke volume obtained from all slices and the average heart rate obtained from the immediate postimaging period (cardiac output = stroke volume × heart rate). In all datasets, one experienced observer manually traced the endocardial and the epicardial contours of the left ventricle. The global myocardium thickness was determined by the difference between the epicardial and endocardial areas.

Immunohistochemistry

Paraffin embedded, formalin-fixed sections were dewaxed and rehydrated through a series of graded alcohols. Sections were treated for 30 minutes with 0.6% hydrogen peroxide in methanol to destroy endogenous peroxidase before antigen retrieval. Antigen was retrieved by microwaving sections for 10 minutes in 10 mmol/L sodium citrate buffer. Nonspecific binding was inhibited by incubation in a blocking solution (10 mmol/L Tris-HCl pH 7.4, 0.1 mol/L $MgCl_2$, 0.5% Tween 20, 1% bovine serum albumin, 5% serum) for 1 hour at room temperature. Rabbit polyclonal antibodies (cyclin D1 and PCNA, Santa Cruz Biotechnology) were diluted 1:100 in blocking solution and applied at 4°C overnight. Sections were washed with PBS buffer and incubated with appropriate biotinylated secondary antibody for 1 hour at room temperature followed by washing and streptavidin–peroxidase complex for 1 hour. Sections were subsequently washed and stained with 3,3′-diaminobenzidine tetrahydrochloride substrate (Sigma). Nuclei were counterstained with Mayer hematoxylin (Sigma), washed in PBS, dehydrated through a gradient of alcohols, cleared in xylene, and mounted.

Western blot analysis

Small intestinal polyps isolated from individual mice were combined to obtain sufficient tissue (5–6 samples per group). Polyps were homogenized in 1:3 volume of 100 mmol/L Tris-HCl buffer (pH 7.2) with 2 mmol/L $CaCl_2$. After centrifugation at 10,000 × *g* for 15 min at 4°C, the protein concentrations of the supernatants were determined and equal amounts were electrophoresed into SDS-PAGE gels and then electroplated onto polyvinylidene difluoride nitrocellulose membranes. These membranes were blocked for 1 hour at room temperature with 5% skim milk and probed with primary antibodies for 1 hour. The primary antibodies COX-2 (Cayman Chemicals), β-catenin, PCNA, cyclin D1, VEGF, Bcl-2, BAX, E-cadherin, and caspase-3 (Santa Cruz Biotechnology) were used at a dilution of 1:500. Blots were washed three times and incubated with secondary antibodies conjugated with

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horseradish peroxidase (Santa Cruz Biotechnology) at a dilution of 1:2,500 for 1 hour. The membranes were washed thrice and incubated with Super-Signal West Pico Chemiluminescent Substrate (Pierce Chemical Co.) for 5 minutes and exposed to Kodak XAR5 photographic film. Intensities of each band were scanned by a computing densitometer. The actin antibody (Santa Cruz Biotechnology) was used as a loading control at a dilution of 1:1,000 for all Western blots.

Statistical analysis

Differences in the body weights and numbers of tumors among different groups were compared on the basis of one-way ANOVA and a Tukey posttest to determine P values between each of the groups. Differences were considered significant at the $P < 0.05$ level. Data were analyzed using GraphPad Prism. For the cardiac imaging data, The Newman–Keuls procedure was used to access the significance of differences between means. Significance was set at the 95% confidence interval. Data were analyzed using SAS (SAS System for Windows, ver. 9.3, SAS Institute Inc.).

Results

SHetA2 did not cause overt toxicity

APC$^{min/+}$ mice were divided into three treatment groups of 10 mice each. A low-dose group was gavaged with 30 mg/kg of SHetA2, the high-dose group was gavaged with 60 mg/kg, and the untreated control group was gavaged with the same volume of corn oil used in the treatment groups. Treatments were administered 5 weekdays per week. Total body weight was measured in the *APC*$^{min/+}$ mice and compared between treatment groups as an indication of general toxicity. There were no significant differences in the body weights or growth between the three treatment groups of *APC*$^{min/+}$ mice (Fig. 1C). Wild-type C57BL/6J littermates gavaged with the higher 60 mg/kg dose also did not exhibit significant differences in body weight compared with the untreated control group of wild-type mice (Fig 1D). None of the animals that were treated with SHetA2 exhibited any observable toxicities or gross histopathologic changes. Neither of the SHetA2 doses assayed caused intestinal ulceration or acute toxicity.

SHetA2 did not exert cardiac toxicity

The group of wild-type mice gavaged with the higher 60 mg/kg dose of SHetA2 was evaluated for cardiac toxicity in comparison with the control untreated group of wild-type mice using MRI. The investigators were blinded to knowing the treatment groups to which the animals belonged. The stroke volume (volume of blood pumped from the heart with each beat) and cardiac output (volume of blood pumped by the heart per minute) were not significantly different between any two groups, although they were lower in the female than in the male groups (Fig. 2A and B, respectively). The ejection fraction (the fraction of blood pumped out of a ventricle with each heart beat) was not significantly different among groups, indicating that the cardiac work efficiency was not affected by SHetA2 (Fig. 2C). In addition, SHetA2 did not affect the left ventricle wall thickness (LV, Fig. 2D).

SHetA2 suppressed small intestinal polyposis and colon tumorigenesis in *APC*$^{min/+}$ mice

To test the chemoprevention activity of SHetA2, intestinal polyp formation was evaluated at the end of the 12-week treatment period in the groups of *APC*$^{min/+}$mice gavaged with 0, 30, or 60 mg/kg of SHetA2 using the same volume of corn oil for each group. The untreated control groups of male and female mice spontaneously developed on average 55.5 ± 3.9 and 47 ± 7.7 intestinal polyps, respectively. Each dose of SHetA2 significantly reduced the average number of polyps per mouse in both sexes (mean $\pm$ SD tumors for 30 mg/kg and 60 mg/kg; 19.0 ± 6.5 and 21.7 ± 7.1 in male mice respectively; and 22 ± 3 and 28 ± 6.6, respectively in female mice) in a statistically significant manner (Fig. 3A and B). There were no significant differences between the two treatment groups. As is characteristic of this model, mice developed fewer colon tumors in comparison with small intestinal polyps. Both treatment doses significantly reduced the colon tumor multiplicity in comparison with untreated controls in males (Fig. 3C). SHetA2 reduction of colon tumors in mice was not statistically significant in females (Fig. 3D).

To determine whether there was a regional effect of SHetA2 on polyp multiplicity in these treatment groups, polyp distribution was determined by comparing the number of polyps in the three regions of the small intestine, namely duodenum, jejunum, and ileum (Fig. 4A and B). Most of the polyps were observed in the jejunum, which is consistent with our previous findings, and both doses of SHetA2 significantly reduced polyp incidence in this region in both males and females. Duodenum polyps were significantly reduced by both treatment doses in females; however, only the higher dose was statistically significant in males. The fewest numbers of polyps developed in the ileum, and the only significant reduction of polyps in this region was for the 30 mg/kg dose in males.

The effects of SHetA2 were also evaluated specifically with regard to polyp size by categorizing the polyps into large (>2.0 mm), medium (>1–2.0 mm), and small (<1.0 mm; Fig. 4C and D). In male mice, polyp incidences in all size categories were significantly reduced by both SHetA2 doses. In female mice, the polyp incidences in large and medium size categories were significantly reduced by both SHetA2 doses; however, there were no significant differences in polyp incidence in the smallest size category between groups.

SHetA2 modulates polyp biomarkers of proliferation, apoptosis, differentiation, inflammation, and angiogenesis

Tissue sections of the polyps were immunohistochemically stained for expression of cyclin D1 and proliferating cell nuclear antigen (PCNA) as biomarkers of proliferation. Compared with controls, tumors from SHetA2-treated mice had decreased cyclin D1- and PCNA-positive cells (Fig. 5A).

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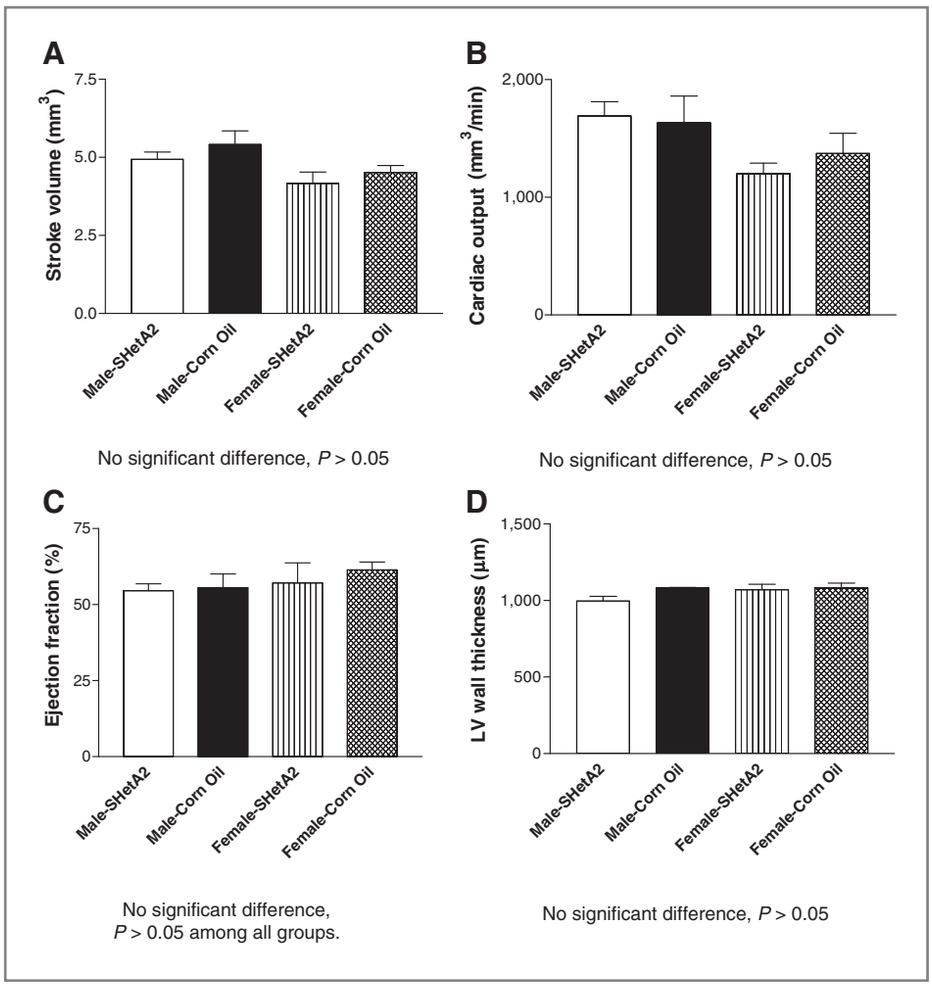

Figure 2. *In vivo* MRI analysis of cardiac function. SHetA2 did not affect: A, stroke volume; B, cardiac output; C, ejection fraction; or D, LV wall thickness. No significant difference was found between any two groups ($P > 0.05$).

These results were confirmed by Western blot analysis of pooled small intestinal tumors, which showed dose-responsive decreases in cyclin D1 and PCNA expression (Fig. 5B). Western blot analysis was also used to verify SHetA2 regulation of apoptosis, differentiation, inflammation, and angiogenesis biomarkers in polyp tissue. SHetA2 induction of apoptosis was shown by increased levels of the proapoptotic BAX protein and decreased levels of the anti-apoptotic Bcl-2 protein at both treatment doses (Fig 5C). In addition to the increased Bax/Bcl-2 ratio, cleaved caspase-3, another biomarker of apoptosis, was observed in both treatment groups (Fig 5C). Western blot analysis of pooled tumors also showed a dose-responsive upregulation of E-cadherin as a biomarker of epithelial differentiation (Fig. 5B) and a dose-responsive inhibition of COX-2 as a biomarker of inflammation (Fig. 5C). Expression of VEGF, as a biomarker of angiogenesis regulation, was dose dependently decreased in the SHetA2 treatment groups (Fig. 5C).

Discussion

The results of this study confirm that the small molecule called SHetA2 (NSC 721689) possesses *in vivo* chemoprevention activity without evidence of toxicity. Both doses of SHetA2 significantly reduced the incidence and sizes of small intestinal polyps in both males and females. Colon tumors were significantly reduced by both treatment doses in male mice; however, the reductions in female mice were not statistically significant, which could be due to the low numbers not having sufficient power to detect significance. In a similar study, we found that dietary Bexarotene, a retinoid x receptor (RXR)-selective drug, exerted greater reduction of small intestinal tumors in $APC^{min/+}$ males in comparison with females (26). Multiple studies have reported gender differences in the development of intestinal tumors in the $APC^{min/+}$ model, with females developing greater numbers of small intestinal tumors and males developing greater numbers of colon tumors in comparison with the respective opposite sex (reviewed in ref. 27). While a number of factors could be responsible for this difference, a primary culprit is the observed suppressive effect of estrogen on intestinal tumorigenesis. Hormone replacement has been shown to reduce colorectal cancer incidence in women, while reducing hormones by removing the ovaries of $Apc^{min/+}$ mice increases tumor development (28, 29). The observation of differences in the responses of males and females to chemoprevention drugs indicates

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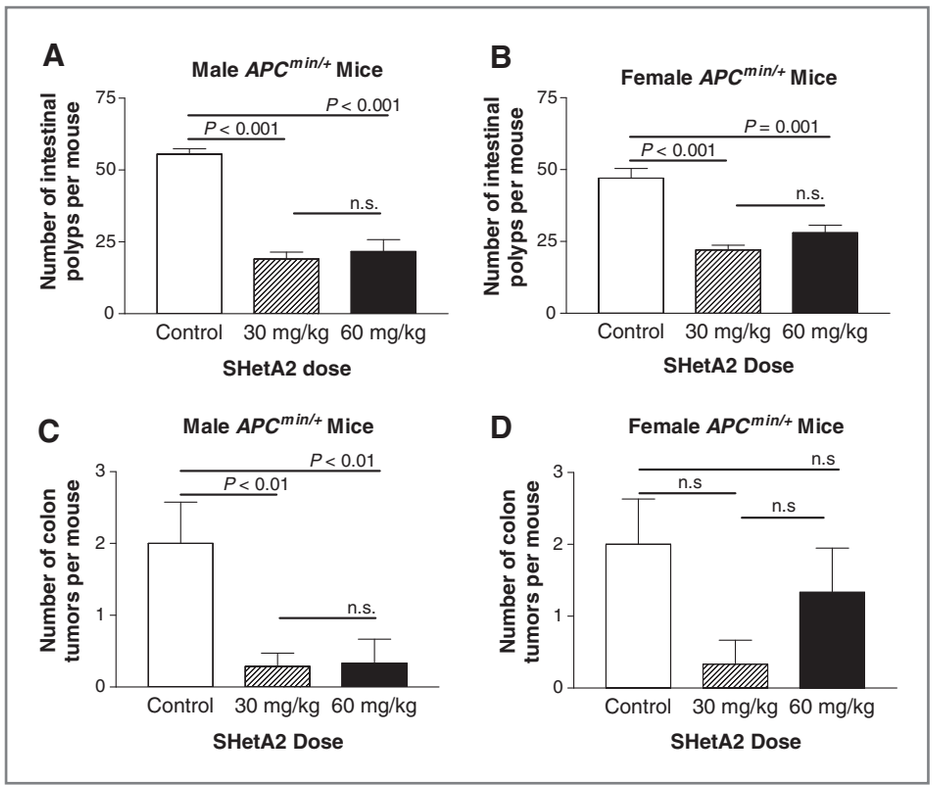

Figure 3. Effects of SHetA2 on intestinal polyp and colon tumor incidence. Average and SE of the number of intestinal polyps in: A, male mice and B, female mice. Average and standard error of the number of colon tumors in: C, male mice and D, female mice. *P* values were derived using ANOVA Tukey post test. n.s., not significant, $P > 0.05$.

that males and females may need to be dosed differently to obtain optimal chemoprevention activity in each sex. The chemopreventive effect of SHetA2 on intestinal polyp development occurred throughout the various regions of the intestine and range of sizes.

No overt toxicities were noted in either wild-type or $APC^{min/+}$ mice, and no adverse effects on cardiac function were observed in a detailed evaluation of SHetA2-treated wild-type mice compared with untreated controls. It was important to evaluate cardiac toxicity, because this organ is especially dependent upon mitochondrial function, and SHetA2 causes mitochondrial swelling and loss of membrane potential in cancer cells (7). Although cardiac tissue is not cancerous, the high rate of mitochondrial respiration may cause similar mitochondrial sensitivity to that of cancer cells. The lack of cardiac toxicity is consistent with the resistance of mitochondria in normal cells to SHetA2 and suggests that mitochondrial defects in cancer cells involve more than just hyperactivity. Extensive preclinical evaluation of toxicity in 28-day toxicity models reported the LOAEL of SHetA2 to be 2,000 mg/kg/d in rats and the NOAEL to be 1,500 mg/kg/d in dogs (18). In the dog study, no toxicity of SHetA2 was seen in any tested dose groups. Thus, the doses used in this study, 30 mg/kg and 60 mg/kg given 5 days per week, were well below the LOAEL and NOAEL.

The mechanism of SHetA2 chemoprevention activity includes inhibition of proliferation and angiogenesis and induction of apoptosis and differentiation as previously observed in studies of human cancer cell lines *in vitro* and *in vivo* (1, 2, 4, 7–10, 12–16). In this study, small intestinal polyps from both SHetA2-treatment groups exhibited reduced levels of the proliferation biomarkers, cyclin D1, and PCNA. Previous studies documented SHetA2 induction of G_1 cell-cycle arrest in both normal epithelial and endothelial cells and cancer cell lines (12, 13, 14). A mechanistic study in ovarian cancer cell lines showed that this G_1 arrest is caused by phosphorylation and ubiquitination of cyclin D1 leading to its degradation and downstream effects on Rb phosphorylation and signal transduction, while overexpression of wild-type or a degradation-resistant mutant of cyclin D1 attenuated SHetA2-induced G_1 arrest (14). The reduction of cyclin D1 validates that this mechanism is associated with colorectal cancer chemoprevention and supports using cyclin D1 as a pharmacodynamic endpoint in planned SHetA2 clinical trials.

The apoptotic activity of SHetA2 was also validated in biomarker analysis of polyps in this study. Similar to studies of ovarian, kidney, and lung cancer cell lines, SHetA2 reduced levels of the antiapoptotic Bcl-2 protein in colon polyps in this study (7, 9, 13). Tipping the balance further towards apoptosis, the proapoptotic Bax protein was elevated in the SHetA2 treatment groups in comparison with the untreated controls. This is in contrast with the studies of human cancer cell lines treated with SHetA2 which found no upregulation of Bax in ovarian and kidney cancer cell lines and, depending on the specific cell line, no effect or downregulation, of Bax in lung cancer cell lines (7, 9, 13). The inconsistency between upregulation of Bax levels in $APC^{min/+}$ polyps and no effect to downregulation in human

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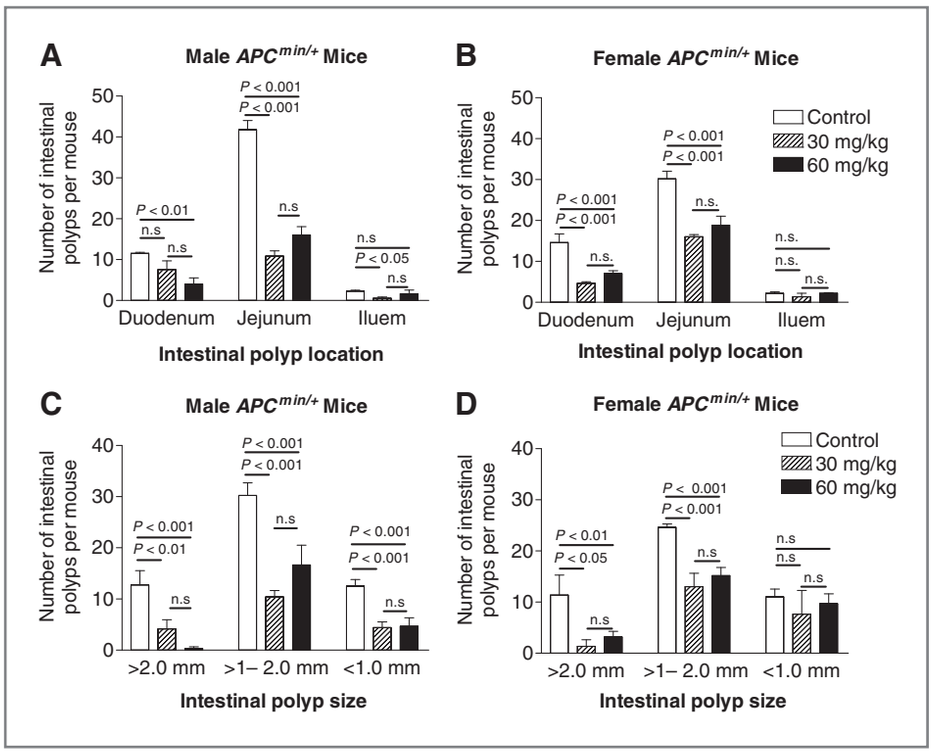

Figure 4. Chemoprevention effects of SHetA2 categorized by intestinal location and polyp size. Average and SE of the number of intestinal polyps categorized by: A, intestinal polyp location in male mice and B, intestinal polyp location in female mice. C, polyp size in male mice and D, polyp size in female mice. *P* values were derived using ANOVA Tukey post test. n.s., not significant, $P > 0.05$.

cancer cell lines could be due to differences inherent to human in comparison with murine cells or due to differences inherent to *in vitro* human cancer cell lines in comparison with *in vivo* preneoplastic murine polyps. Regardless of the difference in Bax levels between the studies, the increase in the Bax/Bcl-2 ratio is consistent and is indicative of SHetA2 induction of apoptosis in the polyps. Further support for SHetA2 induction of apoptosis in the polyps is indicated by the induction of caspase-3 cleavage in polyps from the treated mice.

Induction of differentiation by SHetA2 was previously observed as formation of glands in organotypic cultures of human ovarian cancer cell lines and primary cultures of human ovarian cancer cells and as formation of tubules in

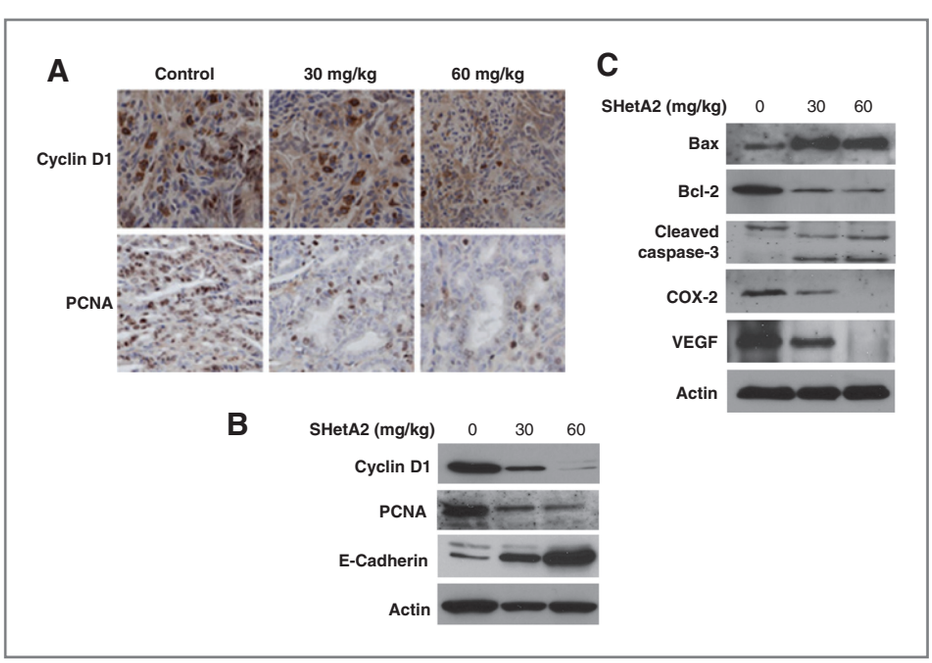

Figure 5. Effects of SHetA2 on biomarkers in intestinal tumors. A, representative polyps from each treatment group evaluated for biomarkers of proliferation by immunohistochemical analysis. B and C, Western blot analysis of biomarker expression in pooled protein extracts from 3 individual polyps from each treatment group.

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organotypic cultures and xenografts of a human kidney cancer cell line in SHetA2-treated groups, but not in untreated control groups (1, 13). These differentiated structures were associated with induction of mucin-1 (Muc-1) in ovarian cancer cells and E-cadherin in kidney cancer cells (1, 13). In this study, elevated levels of E-cadherin protein were observed in polyps from SHetA2-treated mice in comparison with untreated controls. E-cadherin is a transmembrane protein expressed in epithelial cells that forms adherence junctions between cells by binding to other E-cadherin molecules on adjacent cells (30). Loss of E-cadherin expression is common in human colorectal tumors and is known to facilitate cell migration and invasion (31). Thus, upregulation of E-cadherin by SHetA2 could contribute to reversal of the cancerous phenotype. The binding of E-cadherin proteins to each other is dependent upon calcium and on binding of the β-catenin protein to the intracellular domain of E-cadherin (30). When β-catenin is bound to E-cadherin at the inner plasma membrane, it mediates connection to the actin cytoskeleton, but upon reduction of E-cadherin, it is degraded (32). In situations common in colorectal cancer, namely altered APC or Wnt signaling, β-catenin accumulates in the cytoplasm and nucleus (32). Elevated levels of cytoplasmic and nuclear β-catenin are associated with tumor invasion and worse patient prognosis (33–35). In the nucleus, β-catenin dimerizes with the lymphoid enhancer-binding factor-1 (LEF-1) transcription factor to regulate multiple genes including cyclin D1, which is upregulated by β-catenin/LEF-1 (36). Thus, the elevated levels of E-cadherin in SHetA2-treated polyps could contribute to the reduced cyclin D1 levels by sequestering β-catenin on the cell membrane away from the nucleus where it would elevate cyclin D1 gene transcription.

Other beneficial effects of SHetA2 treatment observed were the reduction of COX-2 and VEGF proteins in small intestinal polyps. COX-2 has been targeted in the development of chemoprevention agents because it is overexpressed in most colorectal tumors in association with chronic inflammation and worse patient survival (37). VEGF is a proangiogenic cytokine commonly found elevated in patients with cancer, which has prompted extensive development of cancer therapeutics targeted at the VEGF pathway (38). Elevated levels of COX-2 and VEGF are often coexpressed within the same colon tumor and associated with elevated lymphangiogenesis and worse patient prognosis (39, 40).

The two doses evaluated in this study, 30 and 60 mg/kg, exerted similar levels of reductions on tumor incidence; however, dose-dependent effects were observed for most of the biomarkers evaluated in polyp tissues. This difference may be due to dose-dependent effects being masked by the larger number of factors required to regulate tumor incidence in comparison with the lesser number of factors required to regulate levels of individual molecules. SHetA2 is a highly hydrophobic molecule and appears to have maximum limits of absorption in the colon (18). Suspension of SHetA2 in Solutol HS15 (now called Kolliphor HS15) has been shown to increase bioavailability of SHetA2 in comparison with suspension of SHetA2 in carboxymethylcellulose by about 10-fold (18). Thus, the doses and frequency of administration, in addition to lower than optimal formulation, may have caused poor bioavailability of SHetA2 in this study leading to blood levels that were too low to observe dose-dependent effects on the intestinal tissue. It is not known whether the SHetA2 exposure in the intestine of mice could directly affect intestinal tumors without first being taken up into the blood stream.

In conclusion, oral administration of the small-molecule Flex-Het, SHetA2, exerts significant chemoprevention activity without evidence of toxicity in an $Apc^{min/+}$ murine model of colon cancer. Detailed analysis of cardiac function of wild-type littermates showed no evidence of cardiotoxicity at the highest dose used. The mechanism of chemoprevention is associated with reduction in levels of biomarkers of proliferation, differentiation, and inflammation and induction of biomarkers of apoptosis. These results justify further evaluation of SHetA2 in clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: D.M. Benbrook, A. Mohammed, C.V. Rao, S. Guruswamy
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.M. Benbrook, S. Guruswamy, Y. Wang, Z. Sun, A. Mohammed, Y. Zhang, Q. Li, C.V. Rao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.M. Benbrook, Y. Wang, Z. Sun, C.V. Rao
Writing, review, and/or revision of the manuscript: D.M. Benbrook, Z. Sun, C.V. Rao
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.M. Benbrook, Q. Li, C.V. Rao
Study supervision: A. Mohammed, Y. Zhang, C.V. Rao

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