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Journal Title: International Journal of Cancer
Volume: Volume 131, Number 6
Publisher: Wiley: 12 months | 2012-09-15, Pages 1435-1444
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1002/ijc.27344
Permanent URL: https://pid.emory.edu/ark:/25593/sq7vd

Final published version: http://dx.doi.org/10.1002/ijc.27344

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Accessed October 26, 2019 12:55 AM EDT
Chemoprevention of Familial Adenomatous Polyposis by Bromo-noscapine (EM011) in the Apc^{Min/+} Mouse Model

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Abstract

Germline mutation of the tumor suppressor gene, adenomatous polyposis coli (APC), is responsible for familial adenomatous polyposis (FAP) with nearly 100% risk for colon cancer at an early age. Although FAP is involved in only 1% of all colon cancer cases, over 80% of sporadic cancers harbor somatic mutations of APC. We show here that bromo-onoscapine (EM011), a rationally-designed synthetic derivative of a natural non-toxic tubulin-binding alkaloid-noscapine, that reduces the dynamics of microtubules, causes a reversible G_{2}/M arrest in wild type mouse embryonic fibroblasts (MEFs), but an aberrant exit from a brief mitotic block, followed by apoptosis in MEFs after APC deletion with siRNA. Furthermore, both β-catenin levels and activity fell to half the original levels with a concomitant reduction of cell proliferation-inducing cyclin D1, c-Myc, and induction of cytostatic protein p21 prior to caspase-3 activation. Additionally, we show a statistically significant reduction in the number of newly emerging intestinal polyps (to 35% compared with untreated mice) as well as the mean size of polyps (to 42% compared with untreated mice) in EM011-treated Apc^{Min/+} mice as compared to their sham-treated control littermates. The remaining polyps in the EM011 treated group of Apc^{Min/+} mice showed evidence of elevated apoptosis as revealed by immunohistochemistry. We failed to detect any evidence of histopathological and hematological toxicities following EM011 treatment. Taken together, our data are persuasive that a clinical trial of EM011 is possible for the prevention/amelioration of polyposis in FAP patients.

Keywords

Familial Adenomatous Polyposis (AFP); Colon Cancer; bromo noscapine; EM011; Noscapine; β-catenin
Introduction

Germline mutation of the tumor suppressor gene, adenomatous polyposis coli (APC), predisposes affected individuals to develop numerous polyps within the colon and intestine. This condition, familial adenomatous polyposis (FAP), puts these individuals at nearly 100% risk for acquiring colon cancer at an early age. Although FAP is involved in only 1% of all colon cancer cases, over 80% of sporadic cancers harbor somatic mutations of APC. This renders targeting APC mutations a sound strategy in designing rational chemopreventive approach for colon polyps or cancers.

APC is a large polypeptide with multiple domains binding to different cellular components and thought to play a role as a transient docking complex for β-catenin and its cognate kinase GSK3 via a microtubule plus-end interacting protein, EB1, onto microtubule (MT) plus-ends. In addition to this canonical pathway, APC has other independent means to target to MT plus ends. After being phosphorylated by GSK3 on this MT-tethered complex, β-catenin is degraded via ubiquitination coupled proteosome pathway. Additionally there are phosphorylation independent pathways of β-catenin degradation. In any event, the optimal cytoplasmic pool of β-catenin in normal cells is precariously maintained and balanced by its continual synthesis, post-translational modifications and subsequent degradation. Low levels of β-catenin are available to enter nucleus where it interacts with transcription factor (T cell factor-4, or Tcf-4) responsible for activating or repressing an array of downstream genes via specific TCF responsive elements. Among these are the cell proliferation genes cyclin D1 and c-Myc.

We reasoned that since a deletion of APC (as in APCsiRNA MEFs cells) lowered the probability of β-catenin phosphorylation and degradation, a concommittant increased time of MT-plus end existence in the cell cytoplasmic milieu could potentially rescue this defect. To this end, we have discovered that a natural alkaloid, noscapine, can bind tubulin with a 1:1 stoichiometry and reduce the transition of MT dynamics from growing to shortening phases and vice versa of otherwise relentlessly dynamic MTs thus increasing the residence time MT plus-ends in the cytoplasmic milieu. Furthermore, a rationally designed, orally available, synthetic bromo-derivative of noscapine, 9-bromonoscapine (EM011), binds tubulin with a higher affinity than the founding compound, noscapine, without changing total microtubule polymer mass.

We showed here that EM011 treatment of APC siRNA MEFs cells restored the regulated expression of β-catenin, in that it reduced β-catenin levels, its activity, as judged by decreased expression of reporter gene operating under the control of a Tcf-4 responsive promoter (owing to 3 cloned copies of the responsive element Tcf-4) as well as the canonical responsive cell proliferation-inducing cyclin D1 and c-Myc proteins. Perhaps most importantly, we showed that oral administration of EM011 in ApcMin/+ mice prevented the formation of new polyps and reduced the number and size of existing polyps significantly without any evidence of histopathological and hematological toxicities. These data were prerequisite prior to clinical trials of EM011 for the prevention/amelioration of polyposis in FAP patients.

Materials and Methods

Cells and reagents

Murine Embryonic Fibroblasts (MEFs) were derived from embryonic day 14.5 fetuses produced from intercrosses of wild-type C57BL/6J male and female (WT MEFs). To deplete APC using RNA interference (RNAi), MEFs cells were transfected with small interfering RNA (siRNA) directed against APC with Lipofectamine 2000 (Invitrogen, Carlsbad, CA,
USA) according to manufacturer’s instructions using 0.3 mM siRNA targeting canine APC (Ambion) or 0.3 mM non-targeting siRNA. Cells were harvested for immunoblot analysis or fixed for immunofluorescence at appropriate time after siRNA treatment. The sequences of siRNA oligonucleotides were: siAPC1: 59-GGAAUCAACCCUCAAAAGUtt-39, siAPC2:59-GCACACUGCAGAGAAUAtt-39, siAPC3:59-GCACACUGCAGAGAAUAtt-39 (Ambion). MEFs were maintained at 37°C with 5% CO₂ in DMEM /High Glucose supplemented with 10% FBS. HCT116 and DLD1 cells were obtained from ATCC and maintained at 37°C with 5% CO₂ in McCoy’s medium and RPMI 1640 medium respectively with 10% FBS. All media contained 100 μg/ml penicillin, 50 μg/ml streptomycin sulfate. EM011 was synthesized from noscapine (Sigma, St. Louis, MO) using a procedure described earlier.19

Plasmids
pRL-CMV (Promega, Madison, WI), pEGFP-NI (BD Biosciences Clontech), pTOPFLASH luciferase reporter (a gift from Dr. Bert Vogelstein)21.

Chemotherapeutic sensitivity assay, cell cycle and apoptosis analysis
Cell viability was determined using the exclusion of trypan blue by live cells and cell counting using a hemocytometer. Cell cycle and apoptosis studies were performed as previously described.17, 18, 20, 22 Briefly, EM011 treated cells (for 0, 12, 24, and 48 hours) were stained with propidium iodide (PI) followed by analysis on a FACSCalibur flow cytometer (Beckman Coulter, Inc., Fullerton, CA). For Annexin V staining, both Alexa Fluor 488–conjugated Annexin V and propidium iodide were used (Vybrant Apoptosis Assay Kit from Molecular Probes, Eugene, OR). Two-color flow cytometric analyses were performed on a FACSCalibur equipped with an argon ion laser.

Confocal analysis
Confocal immunofluorescent microscopy was performed as described.23 Briefly, cells grown on poly-L-lysine–coated glass coverslips were fixed with cold (−20°C) methanol, washed with PBS, and blocked with 2% bovine serum albumin (BSA)/PBS. Mouse monoclonal antibody against α-tubulin (DM1A; Sigma, St. Louis, MO) and a FITC-labeled goat anti-mouse secondary antibody and DNA binding fluorescent dye propidium iodide (0.5 μg/ml) was used prior to examination using a Zeiss 510 confocal microscope (using a 63x objective with a numerical aperture of 1.4).

Western blot analysis
For immunoblot analyses cells treated with EM011 (50μM), or DMSO were collected and extracted with 1× cell lysis buffer (Promega, Madison, WI, USA). Indicated amounts of total proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore), blocked, stained with primary and secondary antibodies, followed by enhanced chemiluminescence detection reagent (Pierce Biotechnology) and densitometry. The primary antibodies used were whole anti-β-catenin (Cell Signaling Inc.), anti-β-actin (Santa Cruz), anti-cyclin-D1 (Cell Signaling Inc.), anti-c-Myc (Santa Cruz), anti-p21 (Santa Cruz), anti-ki67 (Abcam) and anti-cleaved caspase-3 (Cell Signaling Inc.).

Luciferase assay
β-catenin activity was determined using a dual luciferase reporter assay system (Promega). ApcRNAiMEFs, HCT-116, DLD1 cells were cultured to 70–80% confluence in 24-well plates and transfected with pTOPFLASH (firefly luciferase reporter construct) and Renilla luciferase reporter to normalize for transfection efficiencies. An independent pCMV-EGFP construct was used to check the transfection efficiencies. All the tranfections were done using
Lipofectamine reagent (Invitrogen) following the manufacturer’s protocol. 24 hours post transfection, cells were treated with EM011 at a concentration of 50 μM or the vehicle. Luciferase activities were determined at 8h post drug treatment using the Dual Luciferase Assay System kit (Promega).

Animals and treatment regimens

There were 14 mice in each group. EM011 or vehicle solution was administered by daily gavage. Body weight was monitored every day. After genotyping, Apc<sup>Min/+</sup> mice were randomly divided into control or test – Apc<sup>Min/+</sup> mice (Jackson Laboratory, Bar Harbor, ME) were mated to wild-type C57BL/6J females and the progeny were screened for the Apc<sup>Min/+</sup> genotype as described earlier<sup>21, 24</sup>. Mice with Apc<sup>Min/+</sup> genotype were assigned as drug treatment and control groups. Treated 1: EM011 oral treatment (150 mg/kg body weight) began after 8 weeks of age and continued daily for 12 weeks prior to the experimental end point (in 20 weeks); treated 2: EM011 oral administration began 21 days after birth at the same dose level for time matched period until the experimental end point, while groups and labeled with appropriate markers. Apc<sup>Min/+</sup> mice: Male C57BL/6J the control group received same amount of vehicle solution, DMSO.

Tissue collection and polyp analysis

At 20 weeks of age Apc<sup>Min/+</sup> mice (treated and control) were sacrificed and intestines were analyzed for number, location, and size of tumors under a dissecting microscope using a stereomicroscope and a measuring grid. Animals were euthanized and the entire GI tract was dissected out and flushed with PBS. The intestine was then removed from the colon and cut into three equal segments: proximal, medial, and distal. The colon and all three segments of the intestine were cut open longitudinally, stained with methylene blue prior to examination under dissecting microscope fitted with a micrometer to aid the measurements<sup>24, 25</sup>. The vertex and co-vertex of each elliptical lesion was measured. The area was then calculated by multiplying the vertex, co-vertex and π (3.14). Number and size of individual tumors were determined for each intestinal section<sup>24</sup> (see for example, Figure 3A). Adenomas identified in the small and large intestines were grouped by size (<0.1, 0.1 ~ 1.0, 1.0 ~ 3.0, and >3.0 mm). All tumor counting and measurements were performed by person who was blind to the treatments.

Histopathologic and immunohistochemical analysis

Histopathologic analysis of brain, lung, liver, kidney, duodenum, thymus, spleen, heart, nerve and tumors were performed as described previously<sup>18, 23</sup>. Immunohistochemistry for apoptosis was done as described previously<sup>24</sup>. Briefly, deparaffinized sections were blocked with PBS containing 10% nonfat dry milk, 0.01% Tween 20, and 10% normal horse serum for 1 h prior to staining with appropriate antibody and concentration in the blocking buffer for 1 h at room temperature prior to staining with an activated caspase-3 antibody and appropriate secondary antibodies as per supplier’s directions (Vector Laboratories, Inc.). Images were acquired using an Axioskop 2 plus microscope (Zeiss) equipped with an AxioCam MRC5 CCD camera (Zeiss).

Statistical analysis

Student’s <i>t</i> test (two tails) was used to analyze the difference between two groups. For comparison among three groups, ANOVA test was used.

Values were means±SE, and differences were significant when p < 0.01.
Results and Discussions

We have previously shown that EM011, with a bromo-group strategically replacing the acidic proton at position C-9 of noscapine, binds tubulin more efficiently and stalls the dynamic instability of cellular microtubules (MTs) effectively increasing the life span of microtubule plus ends\(^1\),\(^3\),\(^4\). As a result, the cellular process intimately dependent on fast MT-dynamics, i.e., progression of mitosis, is severely affected and the robust mitotic checkpoint mechanisms arrest mitoses. Since APC is involved in the robustness of these checkpoint mechanisms\(^5\), a defect in this pathway in colon cancers leads to a defective arrest and these cells aberrantly exit mitosis with copious aneuploidy, polyploidy and cell death\(^6\). In addition, APC-dependent cell signaling pathways also require microtubule integrity (particularly the presence of MT plus ends), primarily because a MT-plus end binding protein, EB-1, and a MT-associated kinase, GSK-3, are integral components of this pathway\(^7\). Also, there exist EB1 independent pathways of APC localization onto MT plus ends\(^8\), as well as β-catenin degradation pathways independent of its phosphorylation\(^9\),\(^\text{10}\),\(^\text{11}\).

We set out to test if wild type murine embryonic fibroblasts (MEFs) after depletion of cellular APC (Apc siRNA MEFs, Apc siRNA) by siRNA respond differentially to EM011 than wild type MEFs (WT MEFs). To do this we first verified that transfection of MEFs cells with siRNAs targeting APC do reduce the level of APC as shown in Figure 1B, then we tested whether there was any difference in apoptosis between the APC\(^\text{wt}\) and APC knockdowns. Cell cycle and apoptosis analysis showed no apparent changes in three independent tests. Representative data were shown in supplementary figure 1. After this, we treated both cell lines with either vehicle solution-DMSO, or varying concentrations of EM011 (in DMSO) for 48 hours. Viable and dead cells were counted in triplicate using exclusion of the vital dye, trypan blue. As shown in Figure 1A, the IC\(_50\) curve of WT MEFs was clearly different than that of Apc siRNA MEFs and they revealed a marked difference in their susceptibility to EM011, in that these cells were 4.7 times more sensitive with a steeper death-curve than WT MEFs (IC\(_50\) =135.2 \(\mu\)M for WT MEFs v/s IC\(_50\) = 28.6 \(\mu\)M for Apc siRNA MEFs). This provided a clear therapeutic window (5–50 \(\mu\)M) for selective killing of Apc siRNA MEFs while causing minimal damage to the WT MEFs (Figure 1A).

Fluorecent-activated sell sorting (FACS) analyses of DNA content showed a decline in G1 and a concomitant rise in G2/M of both WT MEFs (Figure 1Ci,iii) and Apc siRNA MEFs (Figure 1Ci,iii). However, in contrast to the WT MEFs, Apc siRNA MEFs showed a distinct shift of cells to a sub-G1 DNA content of <2N at 48h EM011-treatment which suggested that DNA-degradation was associated with apoptosis. Careful confocal microscopic examination of the MT arrays and the chromosomes in drug-treated cells revealed a typical mitotic arrest. We found that bipolar prometaphase spindles with tightly condensed chromosomes in WT MEFs were completely reversed as the cells resumed normal mitoses as early as 24 hours after drug-withdrawal (Figure 1D, upper panels). In contrast, highly disorganized, often multipolar mitoses were visible with less condensed chromosomes at 48 hours of drug treatment in Apc siRNA MEFs, which did not recover after the drug removal but rather appeared to die 48-hours post treatment (Figure 1D, lower panels).

To determine the extent of the apoptotic onset caused by EM011 in these cells types, we analyzed the early apoptotic cells that externalize the normally internal membrane lipid, phosphatidylserine (PS), by binding to a fluorescently conjugated PS-binding protein, Annexin V-Alexa Fluor488. These cells can be distinguished from the late apoptotic cells that also allow the DNA dye, propidium iodide (PI) to penetrate the cell membranes allowing intracellular DNA to bind it. As shown in Figure 1Ei-ii, 73% of Apc siRNA MEFs were in early and late stages of apoptosis after a 48 hour EM011 treatment (50 \(\mu\)M) as
compare to a modest 21.6% WT MEFs under identical treatment regimen (Figure 1Ei-ii). We concluded that EM011 caused a selective cell death (apoptosis) in \( Apc \) siRNA MEFs when compared to the WT MEFs.

In addition to its direct involvement in the proper maintenance of the robust mitotic checkpoint by APC via the MAD2 pathway\(^{27} \), APC is also involved in \( \beta \)-catenin/Tcf4 mediated transcriptional regulation within the nucleus\(^{28} \). Increased cytoplasmic levels of \( \beta \)-catenin also increase its intra-nuclear pool, which interacts with a transcriptional activator, Tcf4. Tcf4/\( \beta \)-catenin is responsible for activating an array of downstream genes such as cyclin D1 and c-Myc via specific Tcf-responsive elements\(^{32} \). The cytoplasmic levels of \( \beta \)-catenin are maintained by exquisite regulation of its degradation mediated by APC signaling complexes assembled on cellular MTs via a MT plus end tracking proteins EB1 and APC itself\(^{28} \). The complex brings together \( \beta \)-catenin in the vicinity of a kinase GSK3 resulting in extensive phosphorylation of \( \beta \)-catenin\(^{8, 9} \). The phosphorylated \( \beta \)-catenin loses its affinity to this complex and targeted to protein degradation pathway via its ubiquitination by BRCA1\(^{33} \). We first wanted to determine if EM011 treatment has an effect on \( \beta \)-catenin activity by using HCT116 cells, that harbor an in-frame deletion of one phosphorylation site (Ser\(^{45} \)) in \( \beta \)-catenin that renders it partially constitutively active\(^{34–36} \), but has wild type APC. As shown in Figure 2Aiii, these cells do show a 31% decline in the \( \beta \)-catenin activity as early as 8 hours of EM011 treatment. However, in cell lines that are deficient in APC function either due to a mutational inactivation such as in DLD1 cells\(^{37} \) or due to a deletion by siRNA as in \( Apc \) siRNA MEFs, the \( \beta \)-catenin activity was significantly lowered (p<0.01) to 48% and 59% respectively (Figure 2Aiii). These results were consistent with the hypothesis that increasing the cytoplasmic abundance of MT plus ends by treatment with EM011 restores the appropriate down-regulation \( \beta \)-catenin levels and activity. As predicted, we found that more than two-fold decrease of the cytoplastic \( \beta \)-catenin levels followed the treatment of \( Apc \) siRNA MEFs by EM011 (Figure 2B). Our interpretation was also consistent with the observation that \( Apc \) siRNA MEFs cells grew fewer MT plus ends after cold induced MT-depolymerization compared to \( Apc \) WT MEFs cells\(^{38} \). In addition, since \( \beta \)-catenin is a negative regulator of CDKs inhibitor p21\(^{39} \), p21 levels might rise upon restored downregulation of \( \beta \)-catenin inhibiting cycle progression and allowing cellular apoptosis\(^{40} \). The rise in p21 in colorectal carcinoma cells (HCT116) has previously been shown to be associated with the induction of apoptosis\(^{41} \).

To test if the positively responsive target genes cyclin-D1 and c-Myc, and the negatively responsive gene, p21, respond accordingly with the restoration of partial loss of function by depleted APC activity by EM011 treatment, we performed quantitative western blotting. Figure 2C showed indeed the cyclin D1 and c-Myc proteins were down while p21 levels were elevated in response to EM011 treatment. Furthermore, the caspase-3 was activated (as measured by the rising levels of cleaved active caspase-3).

Taken together, all of these data were in line with the cellular observations of EM011 treatment of cells in Figure 1, i.e., the \( Apc \) siRNA MEFs cells were driven to apoptosis by EM011 treatment more efficiently than the wild type MEFs cells. Our results, however, cannot rule out a possibility that the observed effects of EM011-treatment on \( \beta \)-catenin could reflect a consequence of prolonged mitotic arrest or activation of known apoptotic pathways by EM011 due to mechanisms independent of APC, or its downstream effectors other than \( \beta \)-catenin. Whatever the precise mechanistic pathways responsible, we cannot escape the conclusion that EM011-treatment is effective in inducing mitotic arrest followed by apoptosis in cells with compromised APC function.

We next wanted to test if EM011-treatment prevents the growth of polyps and the formation of new polyps in \( Apc^{Min/+} \) mice. The majority of intestinal adenomas were in the small
The histopathology and morphology of adenomatous changes included dysplasia, nuclear enlargement, increased mitotic rate, and the expansion of crypts showing loss of the normal columnar architecture (Fig. 3A, B). There was no difference in the dysplastic grade of adenoma between the control and treated mice—all were low-grade, non-invasive, tubular or tubulovillous adenomas. Figure 3A showed the en face panoramic image of methylene blue stained inner surface of dissected intestine. The pronounced decrease due to EM011 treatment both in the number and overall areas of intestinal lesions were readily visible. Representative bright filed micrographs of hematoxylin and eosine stained 5μm cross sections from the intestine of vehicle treated control group (Figure 3B, left panel) and EM011 treated group (Figure 3B, right panel) showed mucosa muscularis, the connective tissue and the epidermis of the microvilli were readily discernible, and hyperplastic tissue lesions often affected the entirety of multiple adjacent (Figure 3B). In EM011 treated animals however, the lesions were not clustered and, when found, seem much more restricted to the individual microvilli and were subdued in appearance (Figure 3B, right panel).

β-Catenin expression immunohistochemistry was performed in both control and EM011 treated ApcMin/+ mice. Immunohistochemistry of tumors revealed nuclear and cytoplasmic accumulation of β-catenin within dysplastic cells all over (Fig. 3C, left panel). We found β-catenin decreased in proliferative regions of normal crypts and adenomatous polyps in EM011 treated adenomas compared with the vehicle treated control (Fig. 3C, right panel). The vehicle treated control and EM011 treated normal tissues also showed a similar decline in the β-catenin staining of treated group, see Supplementary Figure 2. Ki67 staining in vehicle-treated ApcMin/+ mice of the normal tumor surrounding tissues showed dark staining within the crypts (Figure 3D, left panel), while Ki67 staining was significantly reduced in EM011-treated ApcMin/+ mice (Figure 3D, right panel). Using an antibody specific to activated (cleaved) anti-caspase-3, the immunohistochemical analyses of these lesioned-tissues revealed the normal apoptotic zones along the apices of intestinal villi in both the vehicle treated control and EM011 treated animals (arrows). In sharp contrast to the untreated control animals however, the EM011 treated animals showed additional copious apoptotic foci abnormally in the basal areas of the villus (Figure 3E). For the quantitative analyses of β-Catenin expression from immunohistochemistry, we chose three polyps each from different sections of the small intestine (proximal, medial, and distal). A total of fourteen mice from EM011 treatment groups (7 from treatment group-1 and 7 from group treatment group-2) and twelve mice from vehicle only control group were analyzed. Altogether there were 32 tumors in treated group and 26 tumors in control group that were analyzed. The number of crypts with clearly identifiable accumulation of β-Catenin within polyps were counted for each polyp at 400x magnification by a technician blind to the treatments group types. Polyps with less than five β-catenin-positive crypts (< 5 foci) were considered small polyps, while polyps with five or more than five β-catenin-positive crypts (≥5 foci) were considered large. The number of these small and large polyps was averaged for each animal. As shown in Figure 1F, EM011 treatment had a significant reduction of β-catenin positive foci on both large (4.6±0.7 vs. 8.3±1.0; p=0.0282) and small (5.2±0.9 vs. 10.4±1.3; p=0.0225) category polyps when compared with data from vehicle treated controls. These data revealed that β-catenin expression was prominent in control (vehicle-treated) ApcMin/+ mouse intestinal polyps, and precancerous lesions also have abundant β-catenin expression. EM011 treatment that decreased intestinal polyp burden also decreased β-catenin content and nuclear localization.

To distinguish between neopolyposis post-treatment versus the growth of apparently existing polyps, we have followed two treatment regimens as mentioned in the materials and methods. The total number of adenomas showed a significant decline in both treatment
groups as compared with the sham-treated controls (Figure 4A). The size distribution bins of lesions were displayed along the abscissa (X-axis) and the numbers of adenomas along the ordinate (Y-axis). Most notable features were significant decrease of the big lesions (>3 mm² and >3 ~ 1.0 mm²), which remained small, and showed up as an apparent concomitant increase in small size bin (<0.1 mm²) (Figure 4B). The statistical analysis of variance revealed highly significant prevention of the adenoma load across the proximal intestine from 32.2 ± 2.3 mm² to 13.7 ± 1.4 mm² (treated 1) and 11.8 ± 0.8 mm² (Treated 2) (p<0.001), medial intestine from 21.9 ± 5.0 mm² to 9.1 ± 3.3 mm² (treated 1) and 7.3 ± 1.3 mm² (Treated 2) (p<0.01) and distal intestine from 12.5 ± 2.9 mm² to 5.2 ± 0.6 mm² (Treated 1) and 5.2 ± 0.8 mm² (Treated 2) (p<0.01) (Figure 4C). There was no visible difference between groups of treated 1 and treated 2. The colonic adenomas are well known to be rare in Apc<sup>Min/+</sup> mouse models. Therefore, we did not have statistically meaningful numbers of adenomas in colon. The occasional adenoma within the colon (0–5) did not reveal any readily apparent differences (Figure 4C).

Finally, we wished to test if EM011 causes any obvious histopathologic abnormalities in normal tissues. To do this, we analyzed hematoxylin and eosin stained 5 μm sections of paraffin-embedded brain, lung, liver, kidney, thymus, spleen, heart, and sciatic nerve tissues. Blinded observations of these two groups did not reveal any significant differences in the tissue architecture as seen in Figure 5.

In general, histopathologically visible changes in the tissue architecture were only apparent after an extended period of exposure to even well known toxic agents. Although comforting, it was not entirely satisfactory level of analyses for organ toxicities. Perhaps the most sensitive and immediately influenced parameters of toxicity lie in the physiological and systemic outcome of vital organ functions by assessing the organ function as revealed by blood chemistry. Therefore, we next assessed organ-associated toxicity by measuring organ functions in vehicle-treated and EM011-treated groups. Liver function tests (total bilirubin, alanine transaminase, aspartate aminotransferase and alkaline phosphatase levels) and renal function tests (blood urea nitrogen and creatinine levels) were similar between drug-treated and vehicle-treated groups (Figure 6A). In addition, systemic homeostasis (albumin, total protein, glucose) (Figure 6B) and electrolyte balances (Na⁺, K⁺, TCO₂, Cl⁻) (Figure 6C) also showed no distinguishable profiles among the three groups.

The hematopoietic system takes the lead among tissues that harbor cells with frequent divisions hence it is most susceptible to toxicity by anti-mitotic drugs. This problem can easily be monitored with minimally invasive total blood cell counts from small peripheral blood samples. As shown in Figure 6D, we failed to detect any significant difference in WBC, RBC counts, hemoglobin concentration, hematocrit, and platelet counts (Figure 6D).

Taken together, our data showed that EM011 was an orally available, non-toxic, easily synthesized agent that effectively reduced the formation and progression of polyposis in genetically predisposed Apc<sup>Min/+/</sup> mice. Identical human genotype represents FAP with nearly 100% risk for colon cancer necessitating radical surgery in early age compromising the quality of life for the patient. Prevention or even a significant delay in the progression of the disease will necessarily improve the quality of life prior to surgery, and will prevent further progression of disease post-surgery. Thus clinical trials of EM011 are possible for the prevention/amelioration of polyposis in FAP patients.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
Acknowledgments

Grant support: NIH grants CA-095317-01A2 (H.C. Joshi), DK52230, DK64399, and CA84197 (V.W. Yang).

We thank Rekha Pai, Bing Yu, Mandayam O. Nandan, the members of the Joshi and the other members of the Yang and Joshi laboratories for experimental assistance. We wish to thank Dr. Meenakshi Gupta, pathologist for evaluating animal tissue sections in a blinded manner.

References


Figure 1.
EM011, with a bromo-group strategically replacing the acidic proton on the C-9 position of noscapine in the therapeutic window (5–50 μM range) caused a selective cell death (apoptosis) in Apc siRNA MEFs as compared to WT MEFs. A. Six-well plate was seeded with 3×10⁵ cells/well of both WT MEFs and Apc siRNA MEFs. After 16 hours of incubation, cells were treated with vehicle solution DMSO or varying concentrations of EM011 (in DMSO, 25 μM) for 48 hours. Viable and dead cell-count was obtained using the Trypan blue (0.02%) exclusion assay. B. Depletion of cellular APC by siRNA. APC levels following siRNA transfection for 24 hours (APC siRNA#1) and 48 hours (APC siRNA#2) were assessed by immunoblot analysis in cell lysates. Actin was used as a loading control. C. FACS analyses of cells for DNA content (PI staining) showed a decline in G1 and a concomittent rise in G2-M and sub-G1 population of wild type MEFs (i), and Apc siRNA MEFs (ii, quantified for both in iii). D. Confocal immunofluorescence analyses of cells treated as in A, except the drug was removed and cells were allowed to recover in fresh medium for 24 and 48 hours post-treatment. Microtubules and DNA were visualized using fluorescent anti-alpha-tubulin antibody (green) and fluorescent DNA dye propidium iodide (red). Both cell lines began to show mitotic arrest as early as 24 hours after drug treatment. While the wild type cells sustained the apparently normal mitotic arrest up to 48 hours, the Apc siRNA MEFs began to show disorganized arrest and the beginning of chromatin decondensation at 48 hours of treatment. Wild type cells recovered fully by 24 hours in fresh medium and resumed mitosis (upper panels), while Apc siRNA MEFs displayed cell death. E. FACS analyses of stained Apc siRNA MEFs showed 51% Alexa Fluor-positivity (early apoptosis) and 20% cells stain positive for both PI and Alexa Fluor (late apoptosis or dead) after 48 hours of EM011-treatment, as compared to those of the wild type MEFs (8.8% early apoptotic and 12.8% late apoptotic respectively)(Ei,ii).
Figure 2.
EM011-treatment lowered β-catenin levels and its activity in Apc siRNA MEFs. Ai. Schematic representation of the plasmids used for cotransfection assays: a reporter gene luciferase under the control of three cloned copies of a conserved Tcf4/β-catenin responsive element (TF), another reporter Renilla driven by a constitutive (β-catenin independent) promoter (CMV), and a GFP expression construct. Aii, transfection efficiencies were measured by counting the GFP positive cells (~70%) in all three cell types tested (HCT116, DLD1, Apc siRNA MEFs). The dual Renilla and the luciferase bioluminescence were measured simultaneously in cells (using dual-Luciferase® Reporter Assay) and the ratio between the two signals was plotted for each of the three cell lines treated with DMSO alone or 50μM of EM011 for eight hours. The luciferase activity was lowered by drug treatment in all three drug-treated cell types examined. The most striking and highly significant reduction (P>0.001) was observed in DLD1 (harboring APC mutation) and Apc siRNA MEFs (APC deletion) (Aiii). B, quantitative western blot measurements showed the lowered total levels of β-catenin. To ensure the measurements in the linear range of detection, 15, 10, 6.67 and 4.45 μg of cellular protein from cells treated either with the vehicle, DMSO (lanes 1–4) or EM 011 (25μM in DMSO, lanes 5–8) for 48hrs, was loaded in the respective wells. Protein extracts were probed with antibody against β-catenin. Inset showed the graphical presentation of the densitometry. Results were normalized to β-actin levels. C. Quantitative western blot analysis of cyclin D1, c-Myc, p21, cleaved (activated) caspase-3, and β-actin as a loading control. Densitometric analysis revealed a decrease in cyclinD1 (2.30-fold), c-Myc (3.45-fold), and an increase in p21 (7.50 fold) and cleaved caspase 3 (4.38 fold) levels in EM011 treated cells, as compared to cells treated with the vehicle solution DMSO alone.
Figure 3.
EM011 treatment decreased adenomas, diminished nuclear and cytoplasmic β-catenin, showed loss of proliferation and increased apoptosis in ApcMin/+ mice. A, en face panoramic low-magnification image of methylene blue stained inner surface of dissected intestine (Bar=5 mm). Arrows in the left panel indicate abundant individual lesions in the vehicle-treated control animals and a lesion in the EM011-treated animals on the right. B, Representative bright filed micrographs of hematoxylin and eosin stained 5µm cross sections from the intestine of vehicle treated control group (left panel) and EM011 treated group (right panel) (Bar = 20µm). C, β-Catenin staining of polyps following EM011 treatment (Bar=10µm). D, Ki67 staining in vehicle-treated mice (left panel) and EM011-treated mice (right panel) (Bar=10µm). E, Caspase-3 staining in polyps from the intestine of vehicle treated control group (left panel) and EM011 treated group (right panel) (Bar = 20µm). F: Quantitative immunohistochemical analysis revealed EM011 lowered both the total levels as well as nuclear β-Catenin in polyps. Following EM011 treatment, β-Catenin foci were counted in polyps located in the ileum of ApcMin/+ mice after EM011 or vehicle control treatment. Polyps were categorized as having a small number of foci (< 5 foci) or a
large number of foci (≥5 foci). Data are means±SE. Data were analyzed with independent t-tests within each polyp size category. Significance was set at P< 0.05.
Figure 4.
EM011 treatment prevented the formation and growth of polyps in \( \text{Apc}^{\text{Min/+}} \) mice. A, the total number of adenomas showed a significant decline in both treatment groups as compared with the vehicle-treated controls. The two treatment groups were--Treated 1: EM011 oral treatment (150 mg/kg body weight) began after 8 weeks of age and continued daily for 12 weeks prior to the experimental end point (till 20 weeks); treated 2: EM011 oral treatment began 21 days after birth at the same dose level for the entire 20 weeks period until the experimental end point. B. The size distribution bins of lesions were displayed along the abscissa (X-axis) and the numbers of adenomas along the ordinate (Y-axis). Most notable feature was significant decrease in the big lesions (>3 mm\(^2\) and >3 ~ 1.0 mm\(^2\)), which remained small, and showed up as an apparent concomitant increase in small size bin (<0.1 mm\(^2\)). C. The total adenoma load (the cumulative affected area in all the lesions) across different segments of the GI tract i.e., the proximal intestine (from 32.2 ± 2.3 mm\(^2\) to 13.7 ± 1.4 mm\(^2\) (treated 1) and 11.8 ± 0.8 mm\(^2\) (Treated 2) (p<0.001), medial intestine from 21.9 ± 5.0 mm\(^2\) to 9.1 ± 3.3 mm\(^2\) (treated 1) and 7.3 ± 1.3 mm\(^2\) (Treated 2) (p<0.01) and distal intestine from 12.5 ± 2.9 mm\(^2\) to 5.2 ± 0.6 mm\(^2\) (Treated 1) and 5.2 ± 0.8 mm\(^2\) (Treated 2) (p<0.01).
Figure 5.
EM011 treatment did not cause any pathologic abnormalities in normal tissue architecture. A. Hematoxylin and eosin staining of paraffin-embedded 5 μm sections of the brain, lung, liver, kidney, thymus, spleen, heart, and sciatic nerve (objective, 100 x/0.35 NA). Bar=20μm
Serological biochemical markers of organ function and hematological cell counts failed to reveal any toxicity of the oral EM011 treatment in mice. A. Liver function tests (TBil, total bilirubin; ALT, alanine transaminase; AST, aspartate aminotransferase; ALP, alkaline phosphatase) and renal function tests (BUN, blood urea nitrogen; creatinine) were statistically indistinguishable between EM011-treated and vehicle-treated groups (A). In addition, electrolyte balances (Na+, K+, TCO2, Cl−) (B), and systemic homeostasis (albumin, total protein, glucose) (C) also showed no distinguishable profiles among the control and two treatment groups. We also did a complete blood count analysis and found that EM011 treatment did not alter WBC, RBC counts, hemoglobin concentrations, hematocrit or platelet counts in different groups (D). All showed indistinguishable profiles among all variables examined for vehicle-treated control, treated 1 and treated 2 groups.