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ORIGINAL ARTICLE

Novel read through agent: ZKN-0013 demonstrates efficacy in APC^{min} model of familial adenomatous polyposis

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Abstract

Familial adenomatous polyposis (FAP) is a precancerous, colorectal disease characterized by hundreds to thousands of adenomatous polyps caused by mutations in the tumor suppressor gene adenomatous polyposis coli (APC). Approximately 30% of these mutations are premature termination codons (PTC), resulting in the production of a truncated, dysfunctional APC protein. Consequently, the β-catenin degradation complex fails to form in the cytoplasm, leading to elevated nuclear levels of β-catenin and unregulated β-catenin/wnt-pathway signaling. We present in vitro and in vivo data demonstrating that the novel macrolide, ZKN-0013, promotes read through of premature stop codons, leading to functional restoration of full-length APC protein. Human colorectal carcinoma SW403 and SW1417 cells harboring PTC mutations in the APC gene showed reduced levels of nuclear β-catenin and c-myc upon treatment with ZKN-0013, indicating that the macrolide-mediated read through of premature stop codons produced bioactive APC protein and inhibited the β-catenin/wnt-pathway. In a mouse model of adenomatous polyposis coli, treatment of APC^{min} mice with ZKN-0013 caused a significant decrease in intestinal polyps, adenomas, and associated anemia, resulting in increased survival. Immunohistochemistry revealed decreased nuclear β-catenin staining in the epithelial cells of the polyps in ZKN-0013-treated APC^{min} mice, confirming the impact on the β-catenin/wnt-pathway. These results indicate that ZKN-0013 may have therapeutic potential for the treatment of FAP caused by nonsense mutations in the APC gene.

Key messages

- ZKN-0013 inhibited the growth of human colon carcinoma cells with APC nonsense mutations.
- ZKN-0013 promoted read through of premature stop codons in the APC gene.
- In APC^{min} mice, ZKN-0013 treatment reduced intestinal polyps and their progression to adenomas.
- ZKN-0013 treatment in APC^{min} mice resulted in reduced anemia and increased survival.

Keywords Familial adenomatous polyposis · Macrolide · Nonsense mutations · Adenomatous polyposis coli · β-catenin

Introduction

FAP is characterized by the development of numerous (100–1,000) nonmalignant adenomas in the colon and rectum starting in adolescence that eventually progress to colorectal cancer in mid-life if left untreated. This is commonly associated with clinical symptoms such as rectal bleeding and resultant anemia in ~ 40% of patients [1]. The primary driver of the disease is deficient APC protein activity resulting from germline mutations in the gene. The germline mutations are detected in 80% of FAP patients and are inherited in an autosomal dominant pattern [2–4]. Mutations can be insertions, deletions, or nonsense that result in the loss of function of the APC protein and are primarily located in the 5′ region of the 8.5-kb coding region [5, 6]. Premature stop codons in the APC gene are generally caused by nonsense (30%) or frameshift (68%) mutations, resulting in the synthesis of a truncated, dysfunctional protein, and are causative in the majority of FAP patients [2–5]. In addition to colorectal carcinoma, the absence of a functional APC protein is linked to a predisposition for other diseases such as

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desmoid tumors and Turcot syndrome, which is associated with brain tumors [7].

The APC gene encodes for a large 312 kDa protein composed of multiple functional domains that are involved with cell division, adhesion, and cellular polarization during embryonic development [8, 9]. The general structure of the protein consists of 4 domains, which permit the protein to interact and form active complexes with other proteins such as β-catenin, axis inhibition protein (AXIN), and microtubule plus-end binding protein (EB1) [8–10]. APC acts as a tumor suppressor gene, and the protein negatively regulates the β-catenin/wnt-signaling pathway by mediating the degradation of β-catenin in the cytoplasm [11]. The central region of APC between codon 1284 and codon 1580 is referred to as the mutation cluster region (MCR), and approximately 60% of all APC mutations occur in this area, with a majority being nonsense or frameshift mutations generating a truncated protein [4, 12, 13]. The MCR is involved with β-catenin ubiquitination, and in the absence of this function, cytoplasmic levels of β-catenin increase [8, 9]. Subsequently, β-catenin is translocated to the nucleus, resulting in elevated nuclear levels and increased activation of β-catenin/wnt-pathway genes such as c-myc and other proto-oncogenes [14–16]. Somatic mutations in the APC gene occur in 80% of colon cancers, of which 30% are nonsense [2, 12]. These somatic mutations are known to play a critical role in cancer initiation [12, 17].

The APC^{min} (multiple intestinal neoplasia) mouse model of FAP was developed in C57BL/6 mice by exposure to the mutagen N-ethyl-N-nitrosourea and is a widely used model of the disease [18, 19]. Pathology is due to a nonsense mutation at codon 850 of the murine homolog of the APC gene, resulting in a nonfunctional, truncated protein [18, 20]. This mutation is similar to the mutations in the APC gene carried in human FAP patients [21]. The APC^{min} model utilizes mice that are heterozygous for the mutation (homozygous APC^{min} mice are nonviable), having an average lifespan of 119 days with chronic anemia caused by intestinal polyps as the major causative factor of lethality [18]. Moribund APC^{min} mice present with numerous (~30/GI tract) adenomas, mainly located in the small intestines [18–20]. Adenomas are detectable in APC^{min} mice at 5 weeks of age, and these early lesions appear as crypts crowded with cells of similar morphology observed in low-grade colonic lesions found in FAP patients [19, 21]. Additionally, accumulation of nuclear β-catenin can be observed in adenomas in APC^{min} mice by immunohistochemistry [19]. Disruption of nuclear translocation of β-catenin leads to reduced expression of genes that are transcriptionally co-regulated by β-catenin, such as c-myc, resulting in decreased polyp formation in APC^{min} mice [22].

Macrolides and other ribosome-targeting antibiotics have been shown to induce read through of premature stop codons in eukaryotic cells, leading to translation of the full-length mRNA. Investigations by Zilberberg et al. [23] demonstrated that tylosin could suppress translation termination generated by nonsense mutations in the APC gene using colorectal cancer cell lines and in vivo colon carcinoma models. Other studies have demonstrated that treatment with gentamycin, an aminoglycoside, and erythromycin, a macrolide, suppressed nonsense mutations in the APC gene in human colon carcinoma cells, inhibited the in vivo growth of human colorectal xenografts possessing APC nonsense mutations, and reduced the number of intestinal polyps in a mouse model of FAP [23, 24]. In a clinical study of FAP patients harboring APC nonsense mutations, Kariv et al. [25] showed that erythromycin resulted in a decrease in adenoma burden. Recent investigations utilizing aminoglycosides demonstrated that these antibiotics could promote read through of premature stop codons in the APC and cystic fibrosis transmembrane conductance regulator genes resulting in partial alleviation of the disease state [23]. In general, the degree of full-length, functional protein induced by macrolide and aminoglycoside-mediated read through is in the range of 2–10% of normal levels, which has been shown to be sufficient to restore normal cell function [26, 27]. However, the aminoglycoside and macrolide antibiotics that have demonstrated read through have weak potency, inadequate target tissue exposure, and adverse effects that limit chronic administration, thereby precluding their clinical use in FAP therapy [28–30].

We synthesized a series of macrolide compounds with the aim of targeting the mammalian ribosome to modulate the read through of premature stop codons. In the present study, we explored the potential of ZKN-0013 to induce read through of premature stop codons in the APC gene. Studies in human colon carcinoma cells with known APC nonsense mutations exhibited decreased levels of nuclear β-catenin and a reduction in c-myc protein after ZKN-0013 treatment, signifying restoration of functional APC protein. Treatment of APC^{min} mice for 8 weeks resulted in a reduction of intestinal polyps, anemia, and spleen size, which translated into improved survival. Histopathology of the intestines confirmed that treatment with ZKN-0013 resulted in decreases in overall lesion area, progression to adenocarcinoma, and nuclear localization of β-catenin.

Materials and methods

Cell lines

Human colon cancer cell lines SW1417, SW403, and HCT116 were purchased from American Type Culture Collection (Manassas, VA). SW1417 and SW403 cells were cultured in Dulbecco Modified Eagle Medium (DMEM

high glucose with GlutaMAX, Gibco), and HCT116 cells were cultured in McCoy's 5A Medium (Sigma-Aldrich). All media was supplemented with 10% fetal bovine serum (FBS, heat inactivated, Gibco) and 1% penicillin–streptomycin (Gibco). Cells were maintained in a humidified incubator at 37 °C with 5% of CO_2 and passaged once a week.

Cell growth inhibition

SW1417 and SW403 cells were plated at a density of 2.4×10^3 cells/well in a 96-well plate in triplicate. HCT116 cells were plated at a density of 5.0×10^2 cells/well in a 96-well plate. After 24 h, cells were treated with either DMSO or ZKN-0013 (concentrations ranged from 100 to 0.02 μM). SW1417 and SW403 cell viability was assessed 6 days post-treatment, and HCT116 cell viability was assessed 2 days post treatment using CellTiter-Glo 2.0 (Promega, Madison, WI). The concentrations of ZKN-0013, which inhibited cell growth by 50% as compared to vehicle-treated cells (GI50s), were calculated using GraphPad Prism software version 9 (GraphPad Software, La Jolla, CA).

Immunoblotting

Cells were plated at a density of 4×10^6 cells/100 mm dish. After 48 h, cells were treated with either DMSO or ZKN-0013 (50 μM). A total of 48 h post-treatment, cell pellets were harvested and split into two aliquots. One aliquot was used to prepare whole cell lysates (RIPA lysis and extraction buffer, Thermo Scientific), while the other aliquot was used to generate nuclear and cytoplasmic protein extracts (NE-PER Nuclear and Cytoplasmic Extraction Reagents, Thermo Scientific). Protein concentrations were determined using Pierce BCA Protein Assay Kit (Thermo Scientific). Proteins were separated on 4–12% gradient gels (NuPAGE Bis–Tris protein gels, Invitrogen, Thermo Scientific) using SDS running buffer (NuPAGE MES SDS Running Buffer, Invitrogen). Proteins were transferred to polyvinylidene difluoride membranes using an iBlot 2 system (Thermo Scientific). Primary antibodies used were non-phospho β-catenin (Ser33/37/Thr41) (1:1000, catalog 8814, Cell Signaling Technology, Danvers, MA), c-Myc (1:1000, catalog ab32072, Abcam, Waltham, MA), MEK1/2 (1:1000, catalog 8727, Cell Signaling Technology), Histone H3 (1:1000, catalog 4499, Cell Signaling Technology), and α-tubulin (1:10000, catalog T6199, Millipore-Sigma, Burlington, MA). Secondary antibodies used were anti-rabbit, IRDye800CW (1:20000, catalog 926–32213, LI-COR, Lincoln, NA) and anti-mouse, IRDye680CW (1:20000, catalog 926–680070, LI-COR). Blots were read on LI-COR Odyssey® CLx Imaging System and analyzed using LI-COR Image Studio software. Briefly, a rectangle was drawn and copied for all bands migrating at the predicted molecular weight on the blot. This ensured that the same size of rectangle was used to encompass the exact area containing the band of interest for protein quantitation. Final intensities were calculated by subtracting the background (auto-detected by Image Studio software) from the intensities of bands of interest. Normalization was achieved using MEK1/2 for the cytoplasmic fraction and histone H3 for the nuclear fraction using the following formula: $\text{intensity}_{\text{protein target}}/\text{intensity}_{\text{loading control}}$.

Animal studies

Pharmacokinetic studies were performed at WuXi AppTec Laboratory Testing Division (Cranbury, NJ) in male CD-1 mice, 6–8 weeks of age, sourced from Hilltop Lab Animals, Inc. (Scottdale, PA). Mice were allowed free access to water and rodent chow pellets (LabDiet, Certified Rodent Diet #5002). Animals received a single oral dose of ZKN-0013 (100 mg/kg body weight) from a sterile stock solution of 10 mg/ml ZKN-0013 in saline. Blood samples were collected in EDTA tubes from peripheral veins at 0.083, 0.250, 0.500, 1.00, 2.00, 4.00, 8.00, and 24.0 h time points post-dosing and processed for plasma. After the last blood collection, mice were euthanized, backs were shaved, and a 1 cm^2 skin sample was excised and weighed. A 2 cm section of jejunum was harvested, rinsed with saline, and weighed. Tissue samples and plasma were stored at − 70 °C. Thawed skin samples were finely minced, extracted with acetonitrile, centrifuged at 3000 × g, and supernatants were collected for LC–MS/MS analysis. Water was added to thawed intestinal tissue at a 6:1 ratio (ml water/mg tissue), homogenized, and processed for LC–MS/MS analysis. A SCIEX Triple Quad 6500 + LC–MS/MS system (SCIEX, Framingham, MA) was used to analyze plasma, jejunum homogenate, and skin/acetonitrile samples. Briefly, proteins were precipitated using IS solution (100 ng/mL labetalol and 100 ng/mL tolbutamide in acetonitrile) and centrifuged at 3900 × g. Supernatant was mixed with an equal volume of water, and 2.00–4.00 mL was injected for LC–MS/MS analysis. Calibration curves in the range of 1.00–3000 ng/ml were generated in corresponding tissue preparations from naïve CD-1 mice. Data analysis was performed using Phoenix WinNonlin software (Certara, Princeton, NJ).

APC^{min} female mice of C57BL/6 strain from Jackson Laboratory (Bar Harbor, ME), 10 weeks of age with body weight greater than 15 g, were used. Mice were housed using an individually ventilated caging system and allowed free access to sterile rodent chow (Teklad 2919) and water. Clinical observations and body weights were recorded daily, and mice with body weight loss greater than 20% were removed from the study. The study was conducted in compliance with the UK Animals Scientific Procedures Act 1986 and was

performed at Crown Biosciences UK Ltd (Loughborough, UK). ZKN-0013 was formulated in sterile saline to a working concentration of 5 mg/ml. Mice were treated daily with oral doses of ZKN-0013 (50, 25, or 12.5 mg/kg body weight) or saline for 8 weeks. As animals became moribund or at the end of the study, mice were euthanized; a full necropsy was performed; spleens weighed; the complete gastrointestinal tract was removed for analysis. Whole blood was collected in EDTA tubes, and complete blood counts (CBC) were as performed on a Woodley InSight 5 Diff Retic Haematology Analyzer (Pinmoore Animal Laboratory Services Limited, Cheshire, UK).

Polyp scoring, histopathology, and immunohistochemistry

The excised intestinal tract was flushed with PBS and sliced longitudinally; 10-cm sections were visually inspected; and polyps were enumerated. The mid-section of the small intestines were fixed in 10% formalin; fashioned into Swiss rolls; processed into FFPE blocks; and 4 mm sections were used for analysis. A Tissue-Tek Prisma Plus autostainer (Sakura Finetek USA, Torrance, CA) was used for hematoxylin and eosin (H&E) staining. Sections were scanned with panoramic digital slide scanners at × 40 magnification (3DHISTECH, Pannoramic SCAN, Budapest, Hungary). Analysis and quantification of H&E stained slides were performed by HALO Image Analysis software (Indica Labs, Albuquerque, NM, USA); lesions were identified by nucleus to cytoplasmic staining ratio; lesion area was calculated (percent lesion area = lesion area/small intestine area × 100) [31, 32]. Identification of adenoma regions was performed by a board-certified pathologist. Immunohistochemistry (IHC) was performed on sections from FFPE blocks using the Leica BOND-RXm Automated Stainer (Deer Park, IL, USA), set at the following antigen retrieval conditions: citrate, pH 6.0; 100 °C; 20 min; primary antibodies at 1:100 dilution. Rabbit IgG mAb for β-catenin was obtained from Cell Signaling Technology. A polyclonal anti-rabbit IgG conjugated to horseradish peroxidase was used for detection (Leica Biosystems, Buffalo Grove, IL). Sections were rinsed and counterstained with H&E.

Statistical analysis

GraphPad Prism software version 9 (GraphPad Software, La Jolla, CA) was used for data analysis and the generation of graphs. Mean values are plotted with the standard error of the mean (SEM) indicated by error bars. The unpaired student *t*-test was used to compare the means of the treatment groups to the control group. In survival analysis, the log-rank test was used to determine the significance between the control and treatment groups. *P*-values less than 0.05 were considered significant.

Results

ZKN-0013 growth inhibition of human colon carcinoma cells

Ribosomal targeting antibiotics that promote read through of premature stop codons can inhibit the proliferation of tumor cells harboring nonsense mutations in the APC gene through the down-regulation of β-catenin/c-myc signaling [23, 24, 33]. SW1417 and SW403 human colon carcinoma cells contain a nonsense mutation in the APC gene (R1450* and S1278*, respectively), resulting in a nonfunctional, truncated APC protein [17]. HCT116 human colon carcinoma cells that harbor G13D mutation in KRAS but contain the wild-type APC gene [17] were used as a control cell line. A growth inhibition assay was conducted to evaluate the antiproliferative activity of ZKN-0013 on these 3 tumor cell lines. Tumor cells were cultured in the presence of titrated concentrations of ZKN-0013 or vehicle, and the calculated GI50s for SW1417 and SW403 cells are shown in Table 1 (dose response curves in Supplementary figures). ZKN-0013 had no effect on the growth of HCT116 cells at all the concentrations tested (up to 100 µM).

ZKN-0013 treatment of human colon cancer cells results in decreased levels of nuclear β-catenin and c-myc

Adenomatous polyposis coli is a large, complex protein (312 kDa), which is directly involved in ubiquitin degradation of phosphorylated β-catenin in the cytoplasm [34]. In

Table 1 Effects of ZKN-0013 on human colon carcinoma cell growth. Antiproliferative activity of ZKN-0013 on human colon carcinoma cells. HCT116, SW403, and SW1417 colon carcinoma cells were exposed to titrated concentrations of ZKN-0013, starting at 100 µM, for 48 and 144 h and inhibition of cell proliferation was assessed. No growth inhibition of HCT116 cells was observed at all ZKN-0013 concentrations tested. The concentration of ZKN-0013 that reduced cell proliferation by 50% was 34.8 µM for SW1417 cells and 39.8 µM for SW403 cells

Cell line	ZKN-0013 GI50
HCT116	> 100 µM
SW403	39.8 µM
SW1417	34.8 µM

the absence of APC activity, there is increased translocation of β-catenin to the nucleus. Therefore, the amount of nuclear β-catenin relative to that in the cytoplasm is used as an indicator of the level of functional APC, particularly since there is a lack of reliable, commercially available antibodies for the detection of APC [35, 36]. SW403 and SW1417 cells were treated with ZKN-0013 (50 μM, 48 h), and β-catenin levels were determined by western blot in the nuclear and cytoplasmic extracts. As shown in Fig. 1, the nuclear level of β-catenin was reduced by 40% in response to ZKN-0013 treatment as compared to DMSO-treated cells. This indicates increased levels of functional APC resulting from ZKN-0013-mediated read through of APC nonsense mutations. There was no significant change in the cytoplasmic levels of β-catenin in cells treated with ZKN-0013 as compared to DMSO.

Expression of the c-myc gene is transcriptionally co-regulated by β-catenin [9, 37]. As shown in Fig. 2, ZKN-0013 treatment (50 μM, 48 h) reduced c-myc protein levels by ~ 25% in SW1417 and SW403 cells relative to cells treated with DMSO. These data provide additional evidence that ZKN-0013 treatment leads to increased levels of active APC, leading to a reduction of nuclear levels of β-catenin, which results in decreased expression of c-myc.

ZKN-0013 treatment improves the survival of APC^{min} mice and reduces anemic phenotype

A single-dose mouse study was conducted to determine the pharmacokinetics and exposure of ZKN-0013 in intestinal tissue after oral administration. Male CD-1 mice ($n = 3$) received an oral dose of ZKN-0013 at 100 mg/kg body weight, and drug plasma levels were measured at several time points in a 24 h period. The results are summarized in Table 2. Mean plasma C-max was 1307 ng/ml (±430 SD) which peaked approximately 15 min after dosing. At the end of the 24 h study period, the mean plasma level of ZKN-0013 was 44.7 ng/ml and the mean drug concentration in the jejunum was 24,383 ng/g of tissue. This data demonstrates that ZKN-0013 can achieve intestinal exposure of ~ 35 μM after single, oral dose, where it can affect disease progression. The observed plasma half-life; effective tissue penetration; high tissue-to-plasma concentration ratios are consistent with the pharmacokinetics of macrolide drugs [38].

A well-established model of FAP is the APC^{min} mouse, in which the human APC homolog gene (min) contains a nonsense mutation generating a nonfunctional, truncated protein. APC^{min} mice develop numerous small intestinal adenomas as the primary phenotype and anemia as a secondary, lethal condition, resulting in an average lifespan of

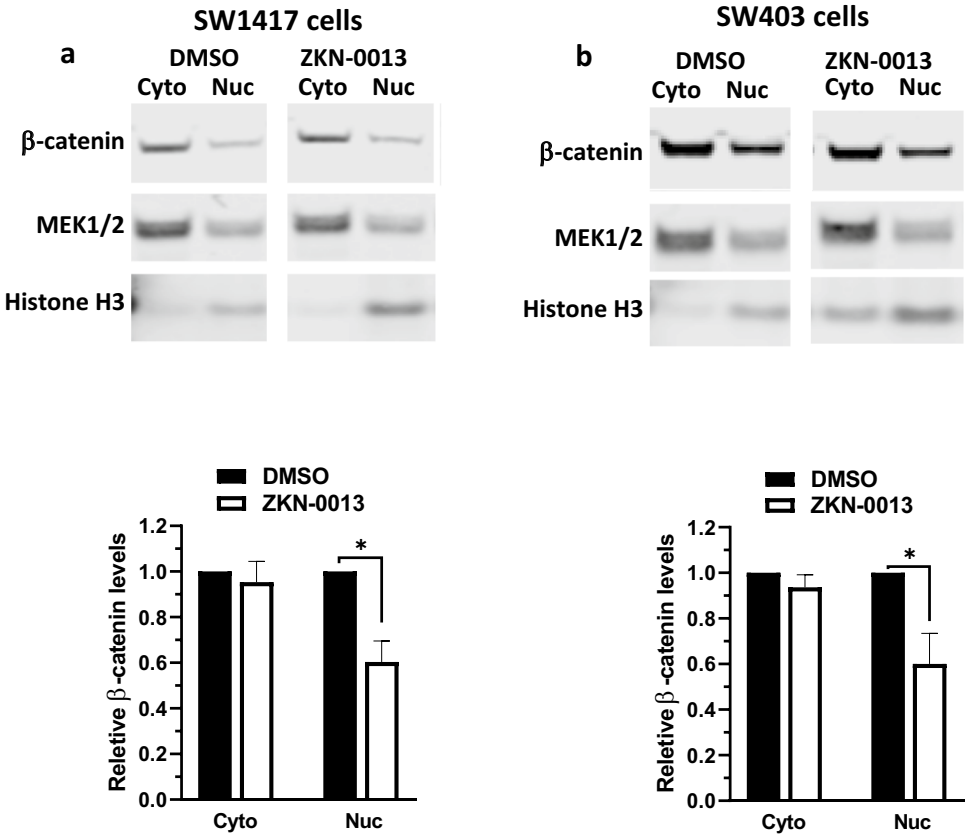

Fig. 1 ZKN-0013 treatment reduces nuclear translocation of b-catenin in colon carcinoma cells harboring a nonsense mutation in the APC gene. Representative western blots showing levels of cytoplasmic (Cyto) and nuclear (Nuc) b-catenin in human **a** SW1417 and **b** SW403 colon cancer cells treated with DMSO or ZKN-0013 at a final concentration of 50 μM for 48 h. Relative b-catenin protein levels in the nucleus are decreased in ZKN-0013-treated colon carcinoma cells. MEK1/2 and Histone H3 were used as loading controls for cytoplasmic and nuclear extracts, respectively ($n = 3$, bars indicate SEM, $^*p < 0.05$)

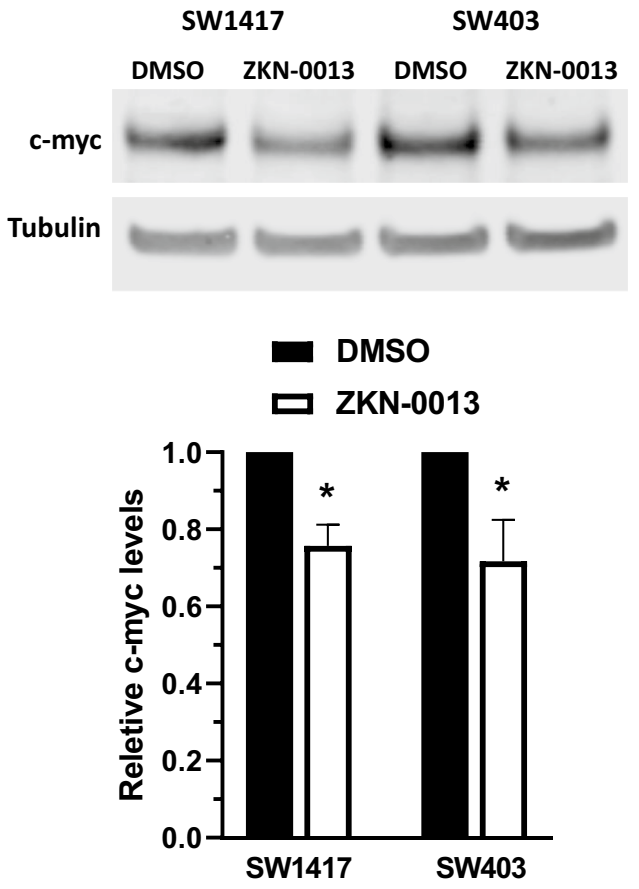

Fig. 2 Decreased protein levels of c-myc in SW403 and SW1417 colon carcinoma cells treated with ZKN-0013. Western blotting results show that colon carcinoma cells treated with 50 μM ZKN-0013 for 48 h have reduced levels of c-myc. Relative c-my protein levels are plotted, and tubulin was used for loading control ($n=3$, bars indicate SEM, $*p<0.05$)

120 days [18–20]. The majority of adenomas are present in APC^{min} mice at 5–8 weeks of age, and the number of adenomas does not significantly increase thereafter [39]. In an efficacy study, 10-week-old APC^{min} mice were treated with daily, oral doses of ZKN-0013 (50 mg/kg body weight) or vehicle saline for a period of 8 weeks. The results are shown in Fig. 3a. During the third week of the study, mice in the vehicle control group began to show severe clinical signs of disease progression and were euthanized. Only 5 vehicle-treated mice (50%) remained in the study at the endpoint. In contrast, all the ZKN-0013-treated mice remained in the study for the full duration. Similar efficacy results were obtained in a repeat study using lower daily doses of ZKN-0013, where 100% survival was achieved at 12.5 mg/kg bw. In this study, ZKN-0013 treatment significantly improved anemia associated with the APC^{min} model as indicated by a ~ 35% reduction of splenomegaly, and increased levels of hemoglobin (~ 45%), packed cell volume (~ 55%), and red blood cell counts (~ 60%) at the doses tested (Fig. 3b). Clinical observations consistent with the APC^{min} model include pale limb extremities and discolored feces, which were reported in all groups, and body weight gain was comparable in the vehicle and treatment groups throughout the course of the study.

Table 2 Pharmacokinetics of ZKN-0013 in male CD-1 mice after oral administration at 100 mg/kg bw. Pharmacokinetic parameters of ZKN-0013 after oral administration at 100 mg/kg bw in CD-1 mice

PK parameter	Mean ± SD ($n=3$)
C_{max} (ng/ml)	1307 ± 430
T_{max} (h)	0.333 ± 0.144
$T_{1/2}$ (h)	6.91 ± 0.328
$AUC_{0\text{-last}}$ (ng·h/ml)	4661 ± 591
$AUC_{0\text{-inf}}$ (ng·h/ml)	5104 ± 548
$C_{24\ hour}$ (ng/mL)	44.7 ± 75
$C_{jejunum}$ (ng/g tissue)	24,383 ± 11,136

ZKN-0013 treatment reduces the number of dysplastic intestinal polyps and lesion area in APC^{min} mice

In the initial efficacy study (ZKN-0013 dosed at 50 mg/kg bw), the entire gastric-intestinal track was removed from moribund mice or at study end, and polyps were manually counted. The total number of visual polyps in the small intestines of mice treated with ZKN-0013 was significantly less than that of control mice, with a 39% reduction in polyp number (Fig. 4a). There were very few polyps detected in the large intestines of the mice from either group, which is characteristic of the model (data not shown). Representative H&E-stained sections from the small intestines of control and ZKN-0013-treated mice that were used for histopathological analysis are shown in Fig. 4b. Total lesion area (polyp, adenoma, and carcinoma) was decreased by 52%, and the area of adenomas was also reduced by 60% in ZKN-0013-treated mice (Fig. 4c). Immunohistochemistry (Fig. 5) revealed prominent nuclear β-catenin staining in numerous epithelial cells in the lesions of saline-treated mice. There were noticeably fewer epithelial cells with nuclear β-catenin staining in the lesions from ZKN-0013-treated mice; numerous epithelial cells in these lesions presented with membrane-associated β-catenin staining; retained their columnar morphology (Fig. 5).

Discussion

Recent studies have demonstrated that read through of nonsense mutations can be induced by some macrolide antibiotics and aminoglycosides in human cancer cells and partially

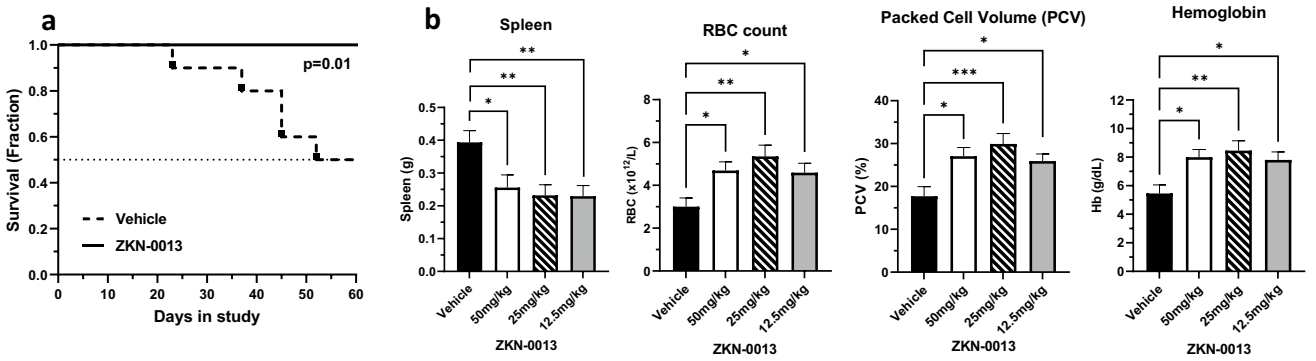

Fig. 3 Long-term survival and decreased anemic phenotype in APC^{min} mice treated with ZKN-0013. **a** Survival of plot of APC^{min} mice treated with vehicle or ZKN-0013 (50 mg/kg bw) showing 100% survival in drug-treated group and 50% in vehicle-treated group ($n=10$). **b** APC^{min} mice treated with ZKN-0013 at doses of 50 mg/kg bw ($n=10$), 25 mg/kg bw ($n=9$), and 12.5 mg/kg bw ($n=10$) show a significant reduction in anemic parameters, as indicated by a reduced spleen weight and elevated levels of hemoglobin, packed cell volume (hematocrit), and red blood cell (RBC) counts as compared to vehicle-treated mice (bars indicate SEM, $*p<0.05$, $**p<0.01$, and $***p<0.001$)

restore the synthesis of functional, full-length proteins such as p53 and APC [23, 24, 40, 41]. As a tumor suppressor, a major role of APC is the formation of a core complex with Axin, Ser/Thr kinases glycogen synthase kinase 3, and casein kinase 1 for the ubiquitination and proteasomal degradation of cytoplasmic β-catenin [34]. Consequently, there is a decrease in nuclear translocation of β-catenin and reduced transcription of β-catenin/wnt pathway genes, including oncogenic c-myc [14, 34]. The APC protein consists of multiple functional domains. The regions between amino acids 1020–1169 and 1342–2075 are essential for β-catenin binding and degradation [8–10]. Nonsense mutations often result in the synthesis of a truncated APC protein lacking these domains, which are unable to mediate β-catenin degradation. Aminoglycoside and macrolide-induced read through typically result in restoration of 2–10% of normal protein levels. It has been reported that this amount of full-length protein is sufficient to restore function of APC [41].

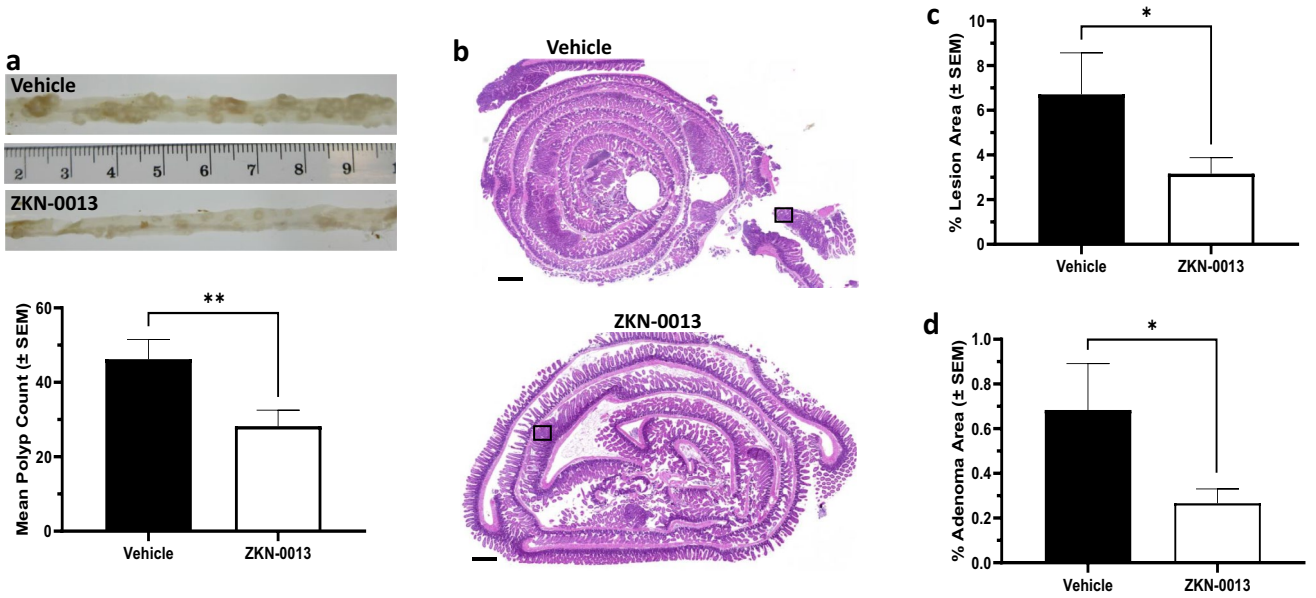

Fig. 4 Reduced number of intestinal polyps and dyplasic lesions in APC^{min} mice treated with ZKN-0013. **a** Gross pathology of the small intestines of APC^{min} mice treated with vehicle or with ZKN-0013 (50 mg/kg bw) showing a lower number of visual polyps and graphical representation of reduced polyp burden in the small intestines of ZKN-0013 treated mice (combined counts from duodenum, jejunum, and ileum, $**p<0.01$). **b** Histological sections of the small intestines from vehicle- and ZKN-0013- (50 mg/kg bw) treated mice showing lesion areas with dense nuclear staining (H&E, 2×, and bar = 500 μM) and quantified the percentage of **c** lesion and **d** adenoma areas in small intestines (in **c** and **d**, error bars indicate SEM, $*p<0.05$)

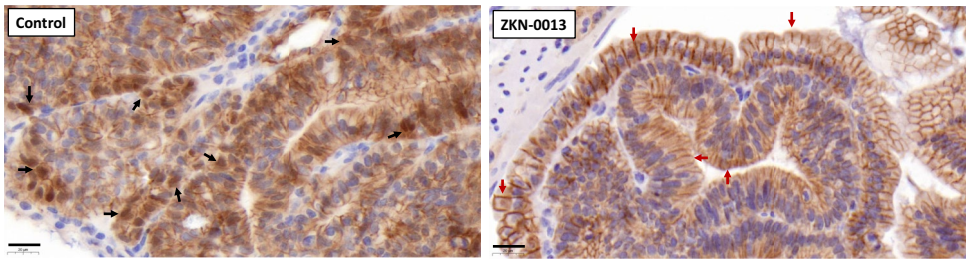

Fig. 5 Epithelial cells in the small intestinal lesions from ZKN-0013-treated mice have reduced nuclear levels of b-catenin. Immunohistochemistry for b-catenin in lesion areas in Fig. 4B (squares) shows prominent nuclear staining in epithelial cells from control mice (black arrows). In ZKN-0013 treated mice, b-catenin staining is decreased in the nucleus of the epithelial cells and is more pronounced in the cytoplasm with localization to the cell membrane (red arrows) bar = 20 μM

It would be ideal to assess read through activity by quantitating full-length APC protein levels. However, detection of small amounts of a large protein such as APC by traditional antibody-based methods is challenging. Recently, the sensitivity and specificity of commercially available anti-APC antibodies have come into question; therefore, we quantified the relative levels of cytoplasmic and nuclear β-catenin as a measure of functional activity of APC protein [35]. Our results from western blot analysis and immunohistochemistry provide evidence that ZKN-0013 treatment of human colon carcinoma cells and adenomatous epithelial cells in APC^{min} mice, both of which contain a nonsense mutation in the APC gene, causes a reduction of nuclear β-catenin. Furthermore, in the colon carcinoma cells, this decrease of nuclear β-catenin results in a subsequent reduction of c-myc protein levels and inhibition of cell proliferation. This is consistent with other studies in which macrolide and aminoglycoside antibiotic treatment of cultured colorectal cancer cells containing APC nonsense mutations resulted in decreased cell growth and nuclear levels of β-catenin [23, 24, 33]. In these studies, tylosin treatment of SW1417 colon carcinoma cells promoted a degree of read through of the nonsense mutation, leading to the synthesis of a low level of full-length, active APC protein [23]. In studies aimed at assessing aminoglycoside-mediated read through of several APC nonsense mutations, Floquet et al. utilized a luciferase-based reporter vector to measure the interaction between active APC and β-catenin and showed that the L360X stop codon was most susceptible to gentamicin-mediated read through [41]. It was noted that this indirect approach alleviated the potential difficulties in detecting low levels of APC protein, which may be sufficient for the biological activity but not detectable by western blotting [41]. It is possible that restoration of even 1% of normal protein function may be adequate to lessen the severity of diseases associated with APC dysfunction.

In certain disease animal models that are associated with inactive APC, such as the APC^{min} mouse or human colon carcinoma tumor xenografts, suppression of premature translation termination of APC mRNA has shown to ameliorate disease in terms of reduced intestinal polyps and tumor growth inhibition [23, 24]. The results of our in vivo studies using the APC^{min} model are similar in that we show treatment with the novel macrolide compound, ZKN-0013, reduced the number of intestinal polyps, and the neoplastic appearance of intestinal lesions. Moreover, 100% of the APC^{min} mice treated with ZKN-0013 survived the 8-week study with no overt clinical signs of disease. In the control group, adverse clinical observations were noted by the third week of the study, and only 50% of the vehicle-treated mice remained at the end of the study. Additionally, ZKN-0013 treatment reduced the number of visual polyps in the small intestines by 39%, which is a major contributing factor to anemia and the survival of the treated APC^{min} mice [20]. In a subsequent dose–response study, efficacy was observed at all doses of ZKN-0013 tested in APC^{min} mice. Even at the lowest daily dose of 12.5 mg/kg bw, anemia was alleviated, as demonstrated by a 43% increase in hemoglobin levels and a 41% reduction of splenomegaly, contributing to long-term survival. It is important to note that our in vivo studies utilized older APC^{min} mice at an age where intestinal adenomas are preexisting, therefore providing pertinent support for the therapeutic use of ZKN-0013 for the treatment of FAP. In addition to its function in the nucleus, β-catenin is also an essential part of the adherens junction at the membrane, where it binds to E-cadherin [42]. Dysregulation of the Wnt pathway, leading to increased nuclear localization of β-catenin, also results in reduced cell–cell adhesion and cell loss from the villus [42]. As shown in Fig. 5, numerous epithelial cells in the adenomas of ZKN-0013 treated mice possess traditional columnar morphology and present with β-catenin staining aligned with the cell membrane. We have also observed elevated levels of e-cadherin associated with epithelial cell membranes of the adenomas of ZKN-0013-treated mice (data not shown). Collectively, ZKN-0013 treatment may enhance differentiation and inhibit or reverse disease progression, as suggested by increased levels of membrane-associated β-catenin and e-cadherin where they form adherens junctions [43].

In eukaryotic cells, macrolide antibiotics target the proximal region of the nascent peptide exit tunnel on the large ribosomal subunit, interfering with the peptidyl transferase center and slowing down the synthesis of the nascent polypeptide. This stalling allows for the insertion of a near-cognate tRNA amino acid at the premature termination codon site and continued translation [44, 45]. The traditional macrolide antibiotics that have demonstrated read through activity are not suitable for chronic dosing that would be required to treat FAP patients. Prolonged use of these traditional macrolide antibiotics has the potential to result in cardiac and liver toxicity. These toxicities have been linked to the macrolide-mediated inhibition of the hERG potassium channel in cardiomyocytes and the bile salt export pump (BSEP) in hepatocytes [28–30, 46–48]. We have developed and optimized a novel macrolide, ZKN-0013, with increased specificity for the mammalian ribosome and minimal off-target inhibition of hERG or BSEP activity (data not shown). Moreover, our pharmacokinetic data demonstrate that ZKN-0013 can penetrate and effectively accumulate in intestinal tissue after a single oral dose. This pharmacological profile with improved potency, minimal off-target activity, and adequate target tissue exposure supports the application of ZKN-0013 for the chronic treatment of FAP patients.

The results of our studies demonstrate that macrolide ZKN-0013 can suppress premature termination of protein translation induced by nonsense mutations in the APC gene, resulting in the restoration of active APC protein. In vitro, ZKN-0013 treatment restored APC function in human colon carcinoma cells with known nonsense mutations in the APC gene. This was demonstrated by decreased levels of β-catenin in the nucleus of SW403 and SW1417 colon carcinoma cells treated with ZKN-0013, indicative of APC-mediated degradation of cytosolic β-catenin. Additionally, protein levels of c-myc, a downstream target of the β-catenin/wnt-pathway, was decreased in SW1417 and SW403 cell treated with ZKN-0013. These results provide strong evidence that ZKN-0013 promotes read through of nonsense codons, restoring functional APC protein. In vivo efficacy of ZKN-0013 was demonstrated in the APC^{min} model using animals of age where polyps are already present. ZKN-0013 treatment significantly reduced the number of intestinal polyps and adenomatous tissue in these mice, alleviated the anemia associated with the model, leading to improved survival. Moreover, immuno-pathology showed numerous epithelial cells in the lesions from the ZKN-0013 treated mice had reduced levels of nuclear β-catenin; an increase of cell membrane-associated β-catenin, and possessed a more differentiated morphology. Taken together, our findings provide preclinical support to evaluate the therapeutic value of long-term use of ZKN-0013 for the treatment of FAP patients.

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Author contribution All authors contributed to the study's design and experimental procedures. S.S. and R.C. designed and synthesized ZKN-0013; S.A. and S.P. conducted the cell proliferation and western blot experiments and analysis; E.T. provided oversight of the in vitro studies; M.C., V.M., and V.B. critically reviewed the manuscript; V.B. designed and managed the APC^{min} studies; V.B., V.M., and M.G. analyzed in vivo data and histopathology; M.G. wrote the manuscript. All authors approved the final manuscript.

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Data availability The data used in this study for analysis and presented in the results are available upon reasonable request.

Declarations

Ethics approvals Pharmacokinetic studies were performed at WuXi AppTec Laboratory Testing Division (Cranbury, NJ), which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and compliant with the Office of Laboratory Animal Welfare (OLAW) Assurance to Conduct Public Health Service (PHS)-funded studies. Efficacy studies were conducted at the Crown Biosciences, Loughborough, UK, in compliance with the UK Animals Scientific Procedures Act 1986 (ASPA) and in line with Directive 2010/63/EU of the European Parliament and the Council of September 22, 2010, on the protection of animals used for scientific purposes.

Competing interests All authors are employees and shareholders of Eloxx Pharmaceuticals.

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