Repurposing the FDA-Approved Pinworm Drug Pyrvinium as a Novel Chemotherapeutic Agent for Intestinal Polyposis

Bin Li1,3, Colin A. Flaveny1,3, Camilla Giambelli1, Dennis Liang Fei1, Lu Han1, Brian I. Hang2, Feng Bai1, Xin-Hai Pei1,2, Vania Nose4, Oname Burlingame5, Anthony J. Capobianco1,2, Darren Orton7, Ethan Lee6, David J. Robbins1,2,3*

1 Molecular Oncology Program, Department of Surgery, University of Miami, Miami, Florida, United States of America, 2 Sylvester Comprehensive Cancer Center, University of Miami, Miami, Florida, United States of America, 3 Department of Biochemistry and Molecular Biology, University of Miami, Miami, Florida, United States of America, 4 Department of Pathology, Miller School of Medicine, University of Miami, Miami, Florida, United States of America, 5 Department of Pathology, Jackson Health System, University of Miami, Miami, Florida, United States of America, 6 Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, Tennessee, United States of America, 7 Stemsynergy Therapeutics Inc., Miami, Florida, United States of America

Abstract

Mutations in the WNT-pathway regulator ADENOMATOUS POLYPOSIS COLI (APC) promote aberrant activation of the WNT pathway that is responsible for APC-associated diseases such as Familial Adenomatous Polyposis (FAP) and 85% of spontaneous colorectal cancers (CRC). FAP is characterized by multiple intestinal adenomas, which inexorably result in CRC. Surprisingly, given their common occurrence, there are few effective chemotherapeutic drugs for FAP. Here we show that the FDA-approved, anti-helminthic drug Pyrvinium attenuates the growth of WNT-dependent CRC cells and does so via activation of CK1α. Furthermore, we show that Pyrvinium can function as an in vivo inhibitor of WNT-signaling and polyposis in a mouse model of FAP: APCmice. Oral administration of Pyrvinium, a CK1α agonist, attenuated the levels of WNT-driven biomarkers and inhibited adenoma formation in APCmice. Considering its well-documented safe use for treating enterobiasis in humans, our findings suggest that Pyrvinium could be repurposed for the clinical treatment of APC-associated polyposes.

Citation: Li B, Flaveny CA, Giambelli C, Fei DL, Han L, et al. (2014) Repurposing the FDA-Approved Pinworm Drug Pyrvinium as a Novel Chemotherapeutic Agent for Intestinal Polyposis. PLoS ONE 9(7): e101969. doi:10.1371/journal.pone.0101969

Editor: John P. Lydon, Baylor college of Medicine, United States of America

Received July 18, 2012; Accepted June 13, 2014; Published July 8, 2014

Copyright: © 2014 Li et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by funds from the NIH: SR01-CA-082628, the University of Miami Women’s Cancer Association, and the Sylvester Comprehensive Cancer Center. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: DJR, AJC and EL are founders of StemSynergy Therapeutics Inc. a company commercializing WNT inhibitors, including CK1α agonists. This does not alter the authors’ adherence to all the PLoS ONE policies on sharing data and materials.

* Email: drobbins@med.miami.edu

These authors contributed equally to this work.

Introduction

The WNT-signaling pathway plays a pivotal role in embryonic development, stem cell biology, maintenance of the normal intestinal epithelium, and ultimately as a driver of carcinogenesis. In the absence of WNT activity, steady-state levels of the transcriptional activator β-CATENIN (CTNNB1) are reduced by a destruction complex consisting of ADENOMATOUS POLYPOSIS COLI (APC), GLYCOGEN SYNTHASE-KINASE 3β (GSK3β), CASEIN KINASE-1 (CK1α) and AXIN [1–4]. GSK3β and CK1α phosphorylate CTNNB1 at specific serine and threonine residues leading to its recognition by the F-box protein β-TRCP and eventual proteasomal degradation [1–3, 5]. Upon WNT activation the destruction complex is disassembled, CTNNB1 is stabilized and accumulates in the nucleus where WNT-target gene expression is elevated leading to proliferation and growth [1–3, 5]. WNT-pathway activation is a key factor in the etiology and maintenance of colorectal cancer (CRC), with loss of function mutations in the tumor suppressor APC being the main cause [8–10]. Truncated APC mutants and degradation resistant CTNNB1 point mutations are found in 80% and 10% of all spontaneous CRC cases respectively [10, 11]. In spontaneous CRC, alterations in APC mark the earliest event leading to carcinogenesis whereas mutations in other CRC associated oncogenes including RAS and the tumor suppressor p53 are considered late events [11]. CRC cells are dependent on WNT signaling at the initiation stage of the disease and at later stages when WNT signaling is required to maintain a growth advantage, inhibiting differentiation and promoting stem cell expansion [12–16]. Therefore effective inhibition of activated WNT-signaling is a theoretically viable chemotherapeutic strategy for APC-associated polyposes and associated CRC.

APC-associated polyposes include Familial Adenomatous Polyposis (FAP), attenuated FAP and Turcot syndrome are all caused by germline loss of function mutations in the WNT-pathway repressor, APC [8, 9, 11, 17, 18]. These conditions are characterized by the formation of multiple abnormal tissue growths called adenomatous polyps along the inner intestinal walls. Typically FAP patients develop hundreds to thousands of precancerous adenomas at an early age, which if untreated will develop into...
As with most rare diseases, due to the small number of individuals affected, there is reduced incentive within the pharmaceutical industry to develop new drug treatments for $APC$-associated polyposis. Therefore for orphan diseases like FAP, effective drugs often have to be sourced from previously FDA-approved drugs. Current FAP treatment entails colectomy followed by regular screenings, however colectomy does not prevent extra-intestinal tumors, colon-stump tumors and other tumors of the intestines that can occur in FAP patients [19–25]. Few advances have been made in the treatment of pre-symptomatic and post-colectomy patients. Due to their ability to induce apoptosis in cultured CRC cells a number of non-steroidal anti-inflammatories (NSAIDs) have been tested as chemotherapeutic agents for CRC [26–29]. Sulindac and celecoxib are the only FDA approved NSAIDs for treating $APC$-associated polyposis, but these drugs have been used with limited success and prolonged use is accompanied by pernicious cardiovascular and gastrointestinal complications. Neither compound has been shown to reduce tumor size or clinically prevent tumor formation in FAP [26,30,31]. Furthermore, although a number of putative targets for NSAIDs in the intestinal epithelium have been proposed, including $CTNNB1$, $RAS$, $NFkB$ and $PPAR\delta$, in vivo evidence for these targets has been lacking [27,32,33]. There is thus a critical need to identify chemotherapeutic agents for $APC$-associated polyposes that effectively block the pathology of these diseases in vivo.

In both $APC$-associated polyposis and CRC, WNT-activation promotes the transformation of normal colorectal mucosa to adenoma, then subsequent carcinoma following additional somatic mutations. As activated WNT signaling is also required for CRC viability, chemotherapeutic agents that target WNT signaling should have dual utility as inhibitors of polyposis and carcinogenesis in FAP. The FAP mouse model ($APC^{min}$ mice) has been used extensively to assess the efficacy of chemotherapeutic agents for the treatment of FAP and CRC [16,30,34,35]. A number of genes that display elevated expression in $APC^{min}$ mice are also analogously upregulated in cultured CRC cells. Therefore $APC^{min}$ mice are a versatile model for studying the factors influencing the pathology of FAP, and so provide a vital mouse model for gauging the efficacy of novel chemotherapeutic agents for FAP.

We recently demonstrated that the FDA approved anti-helminthic drug Pyrvinium is able to attenuate WNT signaling [36,37], through direct binding to and activation of $CK1\varepsilon$ [36]. Other studies have highlighted the emerging role of $CK1\varepsilon$ in

![Figure 1. Pyrvinium reduces CRC cell viability.](https://example.com/figure1.png)

In both $APC$-associated polyposis and CRC, WNT-activation promotes the transformation of normal colorectal mucosa to adenoma, then subsequent carcinoma following additional somatic mutations. As activated WNT signaling is also required for CRC viability, chemotherapeutic agents that target WNT signaling should have dual utility as inhibitors of polyposis and carcinogenesis in FAP. The FAP mouse model ($APC^{min}$ mice) has been used extensively to assess the efficacy of chemotherapeutic agents for the treatment of FAP and CRC [16,30,34,35]. A number of genes that display elevated expression in $APC^{min}$ mice are also analogously upregulated in cultured CRC cells. Therefore $APC^{min}$ mice are a versatile model for studying the factors influencing the pathology of FAP, and so provide a vital mouse model for gauging the efficacy of novel chemotherapeutic agents for FAP.

We recently demonstrated that the FDA approved anti-helminthic drug Pyrvinium is able to attenuate WNT signaling [36,37], through direct binding to and activation of $CK1\varepsilon$ [36]. Other studies have highlighted the emerging role of $CK1\varepsilon$ in...
regulating intestinal epithelial cell proliferation and inhibiting colorectal cancer progression [39,39]. Further, it has been shown that expression of CK1α inhibits tumor invasion and metastasis [30]. In this study we evaluated the efficacy of Pyrvinium inhibiting WNT-signaling, via activating CK1α, in both CRC cells in vitro and in the intestinal epithelium of APCmin mice. Pyrvinium treatment suppressed intestinal WNT activation and significantly reduced the numbers of intestinal polyps compared to vehicle treated mice. This study demonstrates the potential utility of CK1α activators as WNT-inhibitors in the treatment of WNT-driven diseases like APC-associated polyposis, and provides compelling pre-clinical data to justify repurposing Pyrvinium for FAP patients in future clinical studies.

Materials and Methods

Chemical compound information

Pyrvinium pamoate (Pyrvinium) salt; 6-(Dimethylamino)-2-[2-(2,5-dimethyl-1-phenyl-1H-pyrrol-3-ylthienyl]-1-methyl-4,4'-methylenebis[3-hydroxy-2-naphthalenecarboxylate] (2:1)-quinolinium. (see Fig 1A for structure) (Sigma-Aldrich). The cellular stress inducer Tunicamycin was purchased from Sigma-Aldrich. BKM120 was purchased from Selleck Chemicals.

Cell-culture and cell based assays

All cells were obtained from ATCC except for HCT116 WTKO (in which the WT copy of CTXVB1 has been deleted), which were a gift from Dr. Bert Vogelstein (Johns Hopkins University) [40]. Cells were cultured under standard conditions, 37°C at 5% CO2/95% air. HT29, SW620 and SW480 cells were grown in Dulbecco’s-Minimum Essential Media (D-MEM) and HCT116 cells (which retains the WT copy of CTXVB1) were cultured in Roswell Park Memorial Institute Media (RPMI). All media was supplemented with 10% fetal bovine serum, and 100 IU/ml penicillin/100 μg/ml streptomycin. The cloning and MTT assays were performed as previously described [41,42]. Luciferase activity was determined using Steady-Glo (Promega) according to manufacturer’s guidance. Statistical significance was calculated using Student’s t test, p<0.05 was considered statistically significant and marked with an asterisk.

RT-PCR

For all qPCR experiments 2 μg of purified RNA was converted to cDNA using a cDNA synthesis kit (ABI). cDNA was then subjected to Taqman Real-Time qPCR using the cognate probes, qPCR master mix (Boradv) and PCR conditions as per the manufacturer’s instructions (Invitrogen).

XBP-1 splicing assay

Cells were treated with vehicle control, 100 nM Pyrvinium, 1 μg/ml Tunicamycin or both 100 nM Pyrvinium and 1 μg/ml Tunicamycin for 24 h. Total RNA was isolated and subjected to a semi-quantitative RT-PCR. The PCR product was then resolved using a 3% agarose gel. The size of the PCR product for a full length XBP-1 transcript was ~380 bp and ~350 bp for the active XBP-1S splice variant. The following primers were designed to distinguish between the full length and active short splice variant of XBP-1: XBP-1F: 5’-ccagaggtcgcaaggctgtaaat-3’ and XBP-1R: 5’-gacctgggcccaggtgcctcagaa-3’.

Immunoblotting

Total protein isolated from cells was heat denatured in Laemmli buffer, resolved by SDS-PAGE and transferred to PVDF membrane. These membranes were then probed for specific proteins using the cognate antibodies for CASEIN KINASE 1z (CK1z) (Santa Cruz Biotechnology), CYCLIN D1 (CCND1) (Cell Signaling), PYGOPUS2 (Abcam), CTNNB1 (Cell Signaling), S45 phospho-CTNNB1 (Cell Signaling), GAPDH (Abnova), β-ACTIN (Santa Cruz Biotechnology) and α-TUBULIN (Sigma). Nuclear extraction was performed using the NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce).

Animal model and ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by Institutional Animal Care and Use Committee of the University of Miami (protocol # 2-10-237). All APCmin mice (C57BL/6J-ApcWt 1/2) were obtained from Jackson Laboratories. These mice were housed in sterile ventilated cages, fed a standard diet, and provided water ad libitum. After two weeks acclimation mice were treated with Pyrvinium (25 mg/kg) or DMSO-vehicle control via oral gavage every 48 h or as otherwise specified. Mice were monitored daily for signs of illness, pain, or severe weight loss. All mice were euthanized using CO2 followed by cervical dislocation.

The entire intestinal tract was removed from euthanized mice flushed with PBS then fixed in 10% neutral buffered formalin. The isolated intestines from each mouse were assessed for gross polyp formation using a stereomicroscope. Fixed total intestinal tissue was embedded in paraffin cut into 4 μm thick sections and assessed for neoplasms. In situ TUNEL assays were performed using the DeadEnd colorimetric TUNEL assay as per manufacturer’s instructions (Promega). Intestinal tissue sections were probed for protein expression as per manufacturer’s instructions using anti-CTNNB1 antibody (Cell Signaling). Tissues were isolated from euthanized mice. Total mRNA was isolated from tissues homogenized in Trizol (Invitrogen) and purified using an RNA purification kit (Qiagen).

Results

Pyrvinium inhibits CRC cell viability

Pyrvinium (Fig 1A) significantly reduces CRC cell viability and growth as measured by both an MTT-reduction assay and a clonogenic growth assay, each of which quantifies cell viability (Fig 1 and Table 1). In MTT assays Pyrvinium treatment at nanomolar concentrations reduced proliferation of the CRC cell lines (Fig 1B and Table 1). Pyrvinium does not have a general toxicity on cell growth, as its ability to attenuate the proliferation of CRC cells lacking APC can be reduced by re-expressing APC [36]. In addition, Pyrvinium’s ability to attenuate the proliferation of mouse embryonic fibroblasts (MEF) was significantly reduced (IC50~2.5 mM) relative to its effects on CRC cells (Fig 1C). Pyrvinium also potently inhibited CRC cell colony formation (Fig 1D and Table 1; Fig S1). These results show that Pyrvinium can attenuate the growth of transformed intestinal cells harboring an aberrantly activated WNT-signaling pathway. Further, this result demonstrates Pyrvinium’s growth inhibitory effect on CRC cells containing mutant β33, R145 and overexpressed MYC [43], all of which are present within the various CRC cell lines used (Table 1) and commonly found in FAP-associated CRC [44-47].

Pyrvinium attenuates WNT signaling in a CK1α dependent manner

As we have previously suggested that Pyrvinium exerts its biological effects through attenuating WNT signaling activity, we tested Pyrvinium’s ability to inhibit WNT activity in CRC cell
lines expressing a WNT-dependent reporter driving luciferase expression. Pyrvinium was able to reduce luciferase expression in a potent, dose-dependent manner (Fig 2A). Moreover, Pyrvinium also reduced the level of the WNT-dependent protein biomarker in these CRC cells, PYGOPUS2 (Fig 2A). We next examined the expression of a number of endogenous WNT-regulated genes in CRC cell lines. In these cells, Pyrvinium also repressed the expression levels of the WNT-regulated genes AXIN2, DKKOPF-RELATED PROTEIN 1 (DKK1), CYCLIN-D1 (CCND1), CTNNB1 and LEUCINE-RICH-REPEAT-CONTAINING G-PROTEIN RECEPTOR 5 (LGR5) (Fig 2B), which collectively act as biomarkers of WNT-driven tumorigenesis in the intestinal epithelium [3,12–14,44,48–50]. Consistent with the reduced transcription of these WNT biomarkers, Pyrvinium treatment decreased CTNNB1 protein levels in the nucleus of CRC cells (Fig 2C). Pyrvinium also reduced the steady-state protein level of CTNNB1 and  

Table 1. Pyrvinium treated CRC cell lines: summary.

| CRC Cells | MTT Assay IC50 (nM) | Clonogenic Assay IC50 (nM) | PS3 Mutations | Oncogenes Expressed |
|-----------|---------------------|----------------------------|---------------|---------------------|
| HT29      | 135                 | 10                         | G>A (Arg->His) 273 | MYC, RAS, MYB, FOS  |
| SW480     | 47                  | 13                         | G>A (Arg->His) 273/C>T (Pro->Ser) 309 | MYC, RAS, MYB, FOS |
| SW620     | 513                 | 54                         | G>A (Arg->His) 273 | MYC, RAS, MYB, FOS  |
| HCT116    | 87                  | 8                          | WT p53, p21 deficient | MYC, RAS, MYB      |

Figure 2. Pyrvinium inhibits WNT signaling in CRC cells. A. Pyrvinium inhibits WNT reporter gene activity in CRC cells. Cells were treated with Pyrvinium at the indicated concentrations and relative WNT reporter luciferase activity determined. Immunoblots show that Pyrvinium decreases PYGOPUS2 levels in both SW480 and HCT116 WTKO cells in a dose-dependent manner. Error bars indicate ± S.E.M of three experiments. B. Expression of WNT regulated genes AXIN2, DKK1, CCND1 and LGR5 in CRC cell lines treated with 100 nM Pyrvinium or vehicle control was determined using real-time RT-PCR. Error bars indicate ± S.E.M of three experiments. *p<0.05. C. A nuclear or cytosolic fraction was extracted from vehicle or 100 nM Pyrvinium treated SW480 and HT29 cells and CTNNB1 protein levels were determined by immunoblot. D. Immunoblot of the WNT associated biomarkers CTNNB1 and CCND1 in CRC cell lines treated with Pyrvinium or vehicle control. Cells were treated, lysed in protein sample buffer and immunoblotted with the cognate antibodies for CTNNB1, CCND1 and GAPDH control.

doi:10.1371/journal.pone.0101969.t001
CCND1 (Fig 2D). We note that the WNT target genes are not consistently inhibited by Pyrvinium in different CRC cell lines, and that these differences are likely due to the different mutational status of the individual cell lines- in which specific target genes might be regulated by other signaling pathways. Many WNT target genes have also been known to be regulated by multiple signaling pathways (for example, CCND1 is also regulated by Notch [51]). Further, other WNT inhibitors have shown similar variation in distinct CRC cell lines [52]. We also note that the regulation of CCND1 mRNA and protein are differentially regulated by Pyrvinium in two CRC cell lines (Fig2 B & D). This is likely a reflection between differences in steady-state levels of

**Figure 3.** Pyrvinium inhibits WNT signaling in a CK1α dependent manner. A. The expression of the WNT target gene: AXIN2 was determined from NIH 3T3 cells expressing control or CK1α-specific shRNAs. Cells were treated with control or WNT3a conditioned media with or without 100 nM Pyrvinium. AXIN2 expression was quantified using real-time RT-PCR. Error bars indicate ± S.E.M of three experiments. *p<0.05. B. Pyrvinium suppressed WNT signaling biomarkers in a CK1α dependent manner. Upper left, CK1α expression was assessed by real-time RT-PCR in control shRNA or CK1α shRNA expressing HCT116 cells. The three other panels show control shRNA or CK1α shRNA infected HCT116 cells treated with or without 100 nM Pyrvinium for 24 hours and AXIN2, DKK1 and LGR5 expression. Error bars indicate ± S.E.M of three experiments. *p<0.05. C. Overexpression of CK1α inhibits WNT reporter activity and decreases steady state levels of PYGOPUS2 in CRC cells. SW480 and HCT116 WTKO cells harboring the TOPflash reporter were transfected with a control plasmid or one expressing HA-CK1α. Immunoblots show decreased PYGOPUS2 levels for SW480 and HCT116 WTKO cells. Error bars indicate ± S.E.M of three experiments. *p<0.05. D. HCT116 stably expressing the indicated shRNA were treated with or without 100 nM Pyrvinium followed by determination of their colony forming capacity. Error bars indicate ± S.E.M of three experiments. *p<0.05. doi:10.1371/journal.pone.0101969.g003
CYCLIND1 mRNA and WNT protein, which is regulated by protein degradation in numerous ways, and as such here serves more as an indicator of decreased proliferation [53].

To establish that Pyrvinium’s inhibition of WNT activity is dependent on CK1α expression, we identified two independent CK1α specific shRNAs capable of reducing CK1α levels (data not shown and Fig S2). Pyrvinium potently repressed the expression of the WNT biomarker AXIN2 in 3T3 cells stimulated with conditioned media containing WNT3a ligand, but did so in a manner that depended on CK1α expression (Fig 3A). We next established HCT116 cells stably expressing a control shRNA or one of these two CK1α specific shRNA (Fig 3B). Pyrvinium failed to suppress three WNT signaling biomarkers (Fig 3B) in cells with reduced CK1α levels, relative to cells expressing a control shRNA. Consistent with Pyrvinium’s ability to enhance CK1α activity, ectopic expression of CK1α attenuated WNT reporter gene activity and reduced PYGOPUS2 levels in CRC cells (Fig 3C).

Pyrvinium also failed to suppress the colony formation in CRC cells with reduced levels of CK1α relative to control CRC cells (Fig 3D), although such colonies were slightly smaller than those expressing control shRNA (see Fig S3). Taken together, these results suggest that Pyrvinium is able to significantly inhibit WNT-driven cell viability in CRC through CK1α.

Figure 4. Inhibition of WNT signaling by Pyrvinium is independent of UPR or AKT inhibition. A. HCT116 cells under normal culture conditions were treated with vehicle control and either 100 nM Pyrvinium, 1 μg/mL Tunicamycin or a combination of 100 nM Pyrvinium and 1 μg/mL Tunicamycin for 24 h. cDNA from these cells was assessed for XBP-1 mRNA splicing using XBP-1 specific probes. XBP-1 full-length transcript produces a 380 bp PCR product, while the XBP-1S shortened splice variant produces a 350 bp PCR product. B. Pyrvinium inhibits WNT signaling at concentrations that does not block AKT activation. HEK 293 cells stably expressing a WNT responsive reporter gene were stimulated with or without WNT3a and the indicated concentrations of Pyrvinium or the pan-PI3K inhibitor BKM120. Error bars indicate ± S.E.M of four experiments. *p<0.05. As a control, we show that while BKM120 is capable of attenuating AKT activation using a phospho-specific antibody to AKT (phospho-AKT (T308)), Pyrvinium does not inhibit AKT activation at concentrations sufficient to inhibit WNT signaling (Upper panels). Lysates from HEK 293 cells treated with indicated drugs were immunoblotted for phospho-AKT (T308) or total AKT, as indicated. doi:10.1371/journal.pone.0101969.g004

CyclinD1 mRNA and WNT protein, which is regulated by protein degradation in numerous ways, and as such here serves more as an indicator of decreased proliferation [53].

To establish that Pyrvinium’s inhibition of WNT activity is dependent on CK1α expression, we identified two independent CK1α specific shRNAs capable of reducing CK1α levels (data not shown and Fig S2). Pyrvinium potently repressed the expression of the WNT biomarker AXIN2 in 3T3 cells stimulated with conditioned media containing WNT3a ligand, but did so in a manner that depended on CK1α expression (Fig 3A). We next established HCT116 cells stably expressing a control shRNA or one of these two CK1α specific shRNA (Fig 3B). Pyrvinium failed to suppress three WNT signaling biomarkers (Fig 3B) in cells with reduced CK1α levels, relative to cells expressing a control shRNA. Consistent with Pyrvinium’s ability to enhance CK1α activity, ectopic expression of CK1α attenuated WNT reporter gene activity and reduced PYGOPUS2 levels in CRC cells (Fig 3C). Pyrvinium also failed to suppress the colony formation in CRC cells with reduced levels of CK1α relative to control CRC cells (Fig 3D), although such colonies were slightly smaller than those expressing control shRNA (see Fig S3). Taken together, these results suggest that Pyrvinium is able to significantly inhibit WNT-driven cell viability in CRC through CK1α.

Pyrvinium has specificity for CK1α in CRC cells

We have previously shown that Pyrvinium binds directly, with high affinity, to CK1α to enhance its kinase activity, consistent with the results shown above. However, other groups have suggested alternate mechanisms underlying Pyrvinium’s anticancer properties. One study suggested that Pyrvinium can inhibit CRC cell growth via inhibition of the unfolded protein response (UPR), which is often utilized by tumor cells for survival under low nutrient conditions [54]. Under normal nutrient conditions, 100 nM Pyrvinium was still able to inhibit CRC cell proliferation (Fig 1B) without influencing X-BOX BINDING PROTEIN (XBP-1) splicing (Fig 4A), a process commonly upregulated upon response to activation of UPR (such as that induced by Tunicamycin). Therefore, the Pyrvinium mediated repression of cell viability
observed in CRC cells was not dependent on UPR repression. More recently, it has been suggested that Pyrvinium attenuates WNT activity by blocking activation of AKT, preventing GSK3β phosphorylation and its subsequent inactivation, which results in the phosphorylation and de-stabilization of β-CATENIN [55]. However, our data suggests that Pyrvinium at concentrations capable of attenuating WNT signaling do not block AKT activation (Figure 4B). Consistent with these latter findings, we recently determined Pyrvinium’s activity using Ambit’s scanMAX kinase profiling service. Utilizing 442 purified kinases (the largest commercially available kinase panel), the scanMAX kinase screen failed to detect inhibition of any of the kinases tested (data not shown). Significantly, this panel contained AKT1-3 isoforms and all known PI 3-kinase isoforms. Furthermore, consistent with Pyrvinium acting through CK1α, we show that the addition of Pyrvinium to CRC cells results in the time-dependent phosphorylation of a known CK1α substrate (Fig 4C). These latter results are consistent with Pyrvinium-mediated WNT inhibition acting via activation of CK1α to reduce CRC cell viability (Fig 1B and D).

Pyrvinium inhibits intestinal adenomatous polyp formation in APCmin mice

As Pyrvinium proved to be an efficient suppressor of WNT-regulated gene expression and CRC cell growth, we next determined Pyrvinium’s ability to attenuate the pathological phenotype characteristic of FAP: adenomatous polyp formation. To accomplish this we treated APCmin mice via oral gavage with 25 mg/kg Pyrvinium, or vehicle control, once every 48 h for 10 weeks. Pyrvinium treatment significantly inhibited polyp formation in APCmin mice (Fig 5A). Consistent with this attenuation occurring through attenuation of WNT signaling, mice chronically treated with Pyrvinium exhibited a 50% reduction in AXIN2 and LGR5 expression in the colon epithelium (Fig 5B) relative to vehicle treated mice. This effect was achieved at a dose of Pyrvinium that did not produce any overt systemic toxicity, as evidenced by no reduction in body weight in treated mice over the course of the study (Fig 5C). Previous studies have shown that mice exhibit no toxicity at doses up to 60 mg/kg Pyrvinium (p.o) [56–59], above the effective dose used here. When assessed by in situ TUNEL assay Pyrvinium treated mice also displayed more regions of apoptotic DNA fragmentation relative to that of vehicle treated mice.
mice (Fig 5D). Pyrvinium treatment also inhibited the nuclear localization of CTNNB1 in the intestines of APCmut mice (Fig 5D). Thus, Pyrvinium can efficiently disrupt intestinal hyperplasia in vivo through targeted inhibition of WNT signaling with high efficacy and limited toxicity.

Discussion

The WNT-signaling pathway has a well-documented, important role in FAP and eventual CRC. Our study demonstrates that targeted WNT-pathway inactivation may be an effective strategy for treating APC-mutant driven intestinal neoplasms. Utilizing the FAP mouse model APCmut mice, we tested whether or not targeted inhibition of WNT activity using Pyrvinium could decrease polyp numbers in vivo. We demonstrated that Pyrvinium is a potent inhibitor of cultured CRC cell growth and viability and has efficacy in vivo when orally administered to APCmut mice. Pyrvinium is an FDA-approved drug and has been proven safe for use in humans at doses as high as 35 mg/kg without any toxic effects. Therefore, Pyrvinium is a prime candidate for rapid transition to clinical trials as a chemotherapeutic agent for treatment of polyposis in FAP patients. Intrinsically to this discovery is the broader implication that Pyrvinium and related WNT-inhibitors may comprise a new spectrum of potent FAP drugs.

As an anti-helminthic drug Pyrvinium has limited absorption into the bloodstream when orally administered [57,60]. This limited bioavailability restricts the use of Pyrvinium as a treatment for CRC metastases and other extra-intestinal FAP tumors. This also may partially explain the inability of Pyrvinium to effectively inhibit tumor xenograft growth observed by other groups [54]. Conversely, since Pyrvinium only exerts its effect on the intended target tissue: the intestinal epithelium, there should be little if any off-target effects of such treatment, as seen with the documented pernicious cardiovascular effects associated with NSAIDs. Similar WNT-inhibitors that are more efficiently absorbed into the bloodstream could be developed and tested against extra-intestinal FAP-associated tumors. Although Pyrvinium itself has not been demonstrated to directly cause DNA damage [56,57], it substantially enhanced the efficacy of the chemotherapy drugs 5-Flourouracil, Irinotecan and Oxaliplatin [54] (data not shown). These factors make Pyrvinium a promising candidate for both FAP and CRC chemoprevention. Importantly, the use of Pyrvinium to treat FAP patients is expected to lack the cardiovascular and gastrointestinal side effects of NSAIDs.

CK1z expression is required for Pyrvinium to inhibit WNT-driven target gene expression and clonogenicity, consistent with Pyrvinium acting through CK1z to inhibit WNT signaling [36]. Interestingly, Pyrvinium appears to inhibit WNT signaling by increasing the phosphorylation of a number of factors, including ones downstream of CTNNB1 like PIGOPUS [36]. Recent studies in CK1z conditional-knockout mice showed that loss of CK1z protein resulted in robust WNT-activation and enhanced intestinal epithelial cell proliferation [39]. Abrogated CK1z expression, after loss of p53 expression, resulted in a more invasive CRC phenotype than with p53 loss alone [39]. In addition, CK1z expression was suppressed in melanoma metastases and reduced the number of melanoma metastases when ectopically expressed in vivo [38]. In this and other studies CK1z expression and activation in CRC cells inhibited WNT-driven luciferase reporter and endogenous WNT-regulated gene expression (Fig 2A; Fig 3C). It has also been shown that p53-mediated repression of WNT-activity requires CK1z kinase activity [39]. Together these results suggest that CK1z may have a pivotal anti-tumorigenic role in the intestinal epithelium in vivo. Whether targeted activation of CK1z is an effective strategy for CRC treatment in vivo has yet to be demonstrated and requires further study.

Supporting Information

Figure S1 Clonogenic assay of CRC cell lines. Representative images from Fig 1D. (TIF)

Figure S2 Knockdown efficiency of CK1z shRNA in NIH 3T3 cells. A. Cultured NIH 3T3 cells were infected with viruses containing either control or CK1z shRNAs. Cells were lysed 72 h after infection and CK1z and β-ACTIN protein detected using the cognate primary antibodies. (TIF)

Figure S3 Clonogenic activity of HCT116 cells is CK1z dependent. Representative images from Fig 3D. (TIF)

Acknowledgments

We would like to thank Karoline Briegel, Theresa Zimmers and members of the Robbins, Capobianco and Lee laboratories for providing insight during discussions regarding this manuscript.

Author Contributions

Conceived and designed the experiments: BL CAF DJR. Performed the experiments: BL CAF CG LH BIH. Analyzed the data: BL CAF EL DO DLF FB XHP OB VN AJC. Contributed reagents/materials/analysis tools: EL. Wrote the paper: BL CAF DJR.

References

1. Rubinfield B, Souza B, Albert I, Muller O, Chamberlain SH, et al. (1993) Association of the APC gene product with beta-catenin. Science 262: 1731–1734.

2. Miller JR, Moon RT (1996) Signal transduction through beta-catenin and specification of cell fate during embryogenesis. Genes & Development 10: 2527–2539.

3. Hart MJ, de los Santos R, Albert IN, Rubinfeld B, Polakis P (1998) Downregulation of beta-catenin by human Axin and its association with the APC tumor suppressor, beta-catenin and GSK3 beta. Curr Biol 8: 573–581.

4. van Noort M (2002) Wnt Signaling Controls the Phosphorylation Status of beta- Catenin. Journal of Biological Chemistry 277: 17981–17985.

5. Orford K, Crockett C, Jensen JP, Weissman AM, Byers SW (1997) Sertine phosphorylation-regulated ubiquitination and degradation of beta-catenin. J Biol Chem 272: 24735–24738.

6. Liu C, Kato Y, Zhang Z, Do VM, Younker BA, et al. (1999) beta-Tryp couples beta-catenin phosphorylation-degradation and regulates Xenopus axis forma- tion. Proc Natl Acad Sci U A S A 96: 6273–6278.

7. Yamamoto H, Kishida N, Kishida M, Ikeda S, Takada S, et al. (1999) Phosphorylation of axin, a Wnt signal negative regulator, by glycogen synthase kinase-β beta regulates its stability. J Biol Chem 274: 10681–10684.

8. Kinzler K, Nilbert M, Su L, Vogelstein B, Bryan T, et al. (1991) Identification of Fap Locus Genes From Chromosome-5q21. Science 253: 661–665.

9. Nakamura Y, Nishishio I, Kinzler K, Vogelstein B, Miyoshi Y, et al. (1991) Mutations of the adenomatous polyposis coli gene in familial polyposis coli patients and sporadic colonic tumors. Princess Takamatsu Symp 22: 285–292.

10. Canningham C, Dunlop MG (1996) Molecular genetic basis of colorectal cancer susceptibility. Br J Surg 83: 321–329.

11. Panduro Cerda A, Lima Gonzalez G, Villalobos JI (1993) Molecular genetics of human colorectal carcinogenesis and its potential correlation with beta-catenin. Rev Invest Clin 45: 493–504.

12. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, et al. (2007) Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 449: 1003–1007.

13. Fun X, Wu HY, Yu HP, Zhou Q, Zhang YF, et al. (2010) Expression of Lgr5 in human colorectal carcinogenesis and its potential correlation with beta-catenin. Int J Colorectal Dts 25: 583–590.
14. Kanwar SS, Yu Y, Nautiyal J, Patel BB, Majumdar AP (2010) The Wnt/beta-catenin pathway regulates growth and maintenance of colomnones. Mol Cancer 9: 212.

15. Lee KH, Li M, Michalowski AM, Zhang X, Liao H, et al. (2010) A genomewide study identifies the Wnt signaling pathway as a major target of p53 in murine embryonic stem cells. Proc Natl Acad Sci U S A 107: 69–74.

16. Paoni NF, Feldman MW, Gutierrez LS, Ploplis VA, Castellino FJ (2003) Transcriptional profiling of the transition from normal intestinal epithelia to adenomas and carcinomas in the ApcMin/+ mouse. Physiol Genomics 12: 228–235.

17. Groden J, Gelbert L, Thibertis A, Nelson L, Robertson M, et al. (1998) Molecular biological background of FAP and HNPCC, and treatment strategies of both diseases depend upon genetic information. Nihon Geka Gakkai Zasshi 99: 336–344.

18. Wallace MH, Phillips KK (1998) Upper gastrointestinal disease in patients with familial adenomatous polyposis. Br J Surg 85: 742–750.

19. Wallace MH, Phillips KK (1999) Preventative strategies for periampullary tumours in FAP. Ann Oncol 10 Suppl 4: 201–203.

20. Okuno S (2006) The enigma of desmoid tumors. Curr Treat Options Oncol 7: 430–443.

21. Baba S (1996) Small molecule inhibition of Wnt signaling through activation of casein kinase 1alpha. Nat Chem Biol 6: 829–836.

22. Ambroze WL Jr, Orangio GR, Lucas G (1998) Use of NSAIDs for the chemoprevention of colorectal cancer. J Assoc Acad Minor Phys 10: 68–76.

23. Keller JJ, Giardiello FM (2003) Chemoprevention strategies using NSAIDs and COX-2 inhibitors. Cancer Biol Ther 2: S140–149.

24. Spirio L, Olschwang S, Groden J, Gelbert L, Thliveris A, Nelson L, Robertson M, et al. (1993) Mutational analysis of patients with adenomatous polyposis: identical inactivating mutations in unrelated individuals. Am J Hum Genet 52: 263–272.

25. Mahmoud NN, Dannenberg AJ, Mestre J, Bilinski RT, Churchill MR, et al. (1995) Sulindac and quantification of aberrant crypt foci in the colon of Min mice—a murine model of familial adenomatous polyposis. Proc Natl Acad Sci U S A 92: 8265–8270.

26. Okuno S (2006) The enigma of desmoid tumors. Curr Treat Options Oncol 7: 430–443.

27. Herendeen JM, Lindley C (2003) Preventative strategies for periampullary tumors in FAP. Ann Oncol 10 Suppl 4: 201–203.

28. Groden J, Gelbert L, Thibertis A, Nelson L, Robertson M, et al. (1993) Alleles of the APC gene: an attenuated form of familial polyposis. Cell 75: 951–957.

29. Rice PL, Kelloff J, Sullivan H, Driggers LJ, Beard KS, et al. (2003) Sulindac and quantification of aberrant crypt foci in the colon of Min mice—a murine model of familial adenomatous polyposis. Proc Natl Acad Sci U S A 92: 8265–8270.

30. Lee KH, Li M, Michalowski AM, Zhang X, Liao H, et al. (2010) A genomewide study identifies the Wnt signaling pathway as a major target of p53 in murine embryonic stem cells. Proc Natl Acad Sci U S A 107: 69–74.

31. Thorne CA, Hanson AJ, Schneider J, Tahinci E, Orton D, et al. (2010) Pyrvinium is a potent small molecule WNT inhibitor for treatment of FAP.