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ORIGINAL ARTICLE

The impact of pyrvinium pamoate on colon cancer cell viability

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Abstract
Purpose The in vitro and in vivo effects of pyrvinium pamoate (PP), a newly identified WNT signaling inhibitor, were evaluated against colon cancer cell lines and primary colon cancer samples.
Experimental design Antiproliferative activity of PP and its effects on protein and RNA levels of WNT targets were evaluated on adenomatous polyposis coli (APC^{mut}) and β-$catenin^{mut}$ cell lines, one WNT^{wt} colon cancer cell line, as well as six primary colon cancer samples with mutant *APC* in vitro. In addition, the effect of PP on the growth of liver metastasis was examined.
Results PP blocked colon cancer cell growth in vitro in a dose-dependent manner with great differences in the inhibitory concentration (IC_{50}), ranging from 0.6×10^{-6} to 65×10^{-6} mol/L for colon cancer cells with mutations in WNT signaling. In addition, PP demonstrated a cytotoxic effect on primary colon cancer samples. A combined cytotoxic effect of PP with 5-fluorouracil (5-FU) was observed for two cell lines. PP decreased messenger RNA (mRNA) and protein levels of known WNT target genes as *c-MYC* and thereby led to the induction of *p21*. PP inhibited the migration of HCT116 colon cancer cells in vitro and decreased tumor growth in vivo after intraportal injection of HCT116 cells in nude mice.

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Conclusions PP displays promising anticancer activity against a broad panel of human colon cancer cell lines, as well as primary colon cancer samples. However, our findings do not demonstrate a predominant cytotoxic effect of PP on colon cancer cells with mutations in WNT signaling.

Keywords Pyrvinium pamoate · Colon cancer cell lines · WNT pathway · Myc

Introduction

Colorectal cancer is the most common gastrointestinal malignancy with approximately 664,000 new cases each year worldwide; 50 % of them are fatal [1]. Currently, standard treatment of colorectal carcinoma is primary surgery and, depending on the tumor stage, additional chemotherapy [2]. Due to the high recurrence rate, new potential targets and drugs are needed to improve patient outcome.

Colorectal cancer emerges over the so-called adenoma carcinoma sequence over several years, acquiring additional mutations during this time [3]. Ninety percent of all colorectal cancers show aberrant activation of the WNT pathway with mutations in the adenomatous polyposis coli (*APC*) gene and β-catenin gene that induce overexpression of WNT target genes [4]. On the one hand, there are several defining mutations in the tumor suppressor *APC* that usually lead to a truncated protein, which is unable to form a complex with *AXIN2* to degrade the transcription factor β-catenin [4]. On the other hand, mutations on the phosphorylation side of β-catenin reduce its degradation. It is generally accepted that mutations in the WNT pathway lead to formation and growth of colorectal cancer [5]. In recent years, notable improvements have been made in the medicinal treatment of patients with colorectal cancer. New cytostatic agents and antibodies targeting epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) have increased median survival to more than 2 years in patients with advanced colorectal carcinoma, almost doubling survival time of the 5-fluorouracil (5-FU) era [6]. Nevertheless, at the stage of metastasis disease, 5-year survival is below 10 % (www.cancer.org). Alternative chemotherapeutic and immunological treatment strategies are needed to further improve survival of patients with metastasized colorectal carcinoma.

Despite the complexity of cancer growth, for some cancers, one or a few genes are important for both maintenance of the malignant phenotype and cell survival, a phenomenon called oncogen addiction [7]. For colorectal carcinoma, the cancer-driven pathway seems to be the WNT signaling pathway, which harbors mutations in more than 90 % of all colorectal cancers [4]. The dependency on mutations in cancer driving pathways allows the development of specific inhibitors, and several efforts have been made in the past years to identify small molecules targeting the WNT pathway [8]. For example, a group of inhibitors was detected that target the poly-ADP-ribosylating enzymes tankyrase 1 and tankyrase 2 and thereby increase β-catenin degradation [9, 10]. Thorne et al. identified pyrvinium pamoate (PP), a well-known antihelmintic drug, as a selective inhibitor of WNT signaling in two ways [11]. On the one hand, PP is supposed to activate casein kinase 1α (CK1α), leading to enhanced β-catenin degradation. One the other hand, PP inhibits *Pygopus* (PYG), a cotranscription factor of β-catenin, and thereby interferes with target gene transcription. PP reduces the survival of three human colon cancer cell lines with mutations in WNT signaling (HCT116, SW480, and SW620) in a dose-dependent manner [11]. Apart from this data, the exact path of action of PP is still under discussion [12].

The aim of the present study was to evaluate the therapeutic effect of PP against a broad panel of human colon cancer cell lines and primary colon cancer samples with mutations in the WNT pathway in vitro and in vivo.

Materials and methods

Colon cancer cells

Human colon cancer cells (Table S1) were routinely cultured in their recommended media supplemented with 10 % (*v*/*v*) heat-inactivated fetal calf serum (FCS), 2 mmol/L glutamine (Invitrogene), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cancer cell lines were obtained from the American Type Culture Collection (www.atcc.org) with exception of HCT116 and HT29 (Leibniz Institute, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), SW620 (European Collection of Cell Cultures, Salisbury, UK), and WiDr (Cell Lines Service, Eppenheim, Germany). Human umbilical vein endothelial cells (HUVEC) and normal human dermal fibroblasts (NHDF) were obtained from PromoCell (Heidelberg, Germany) and cultured according to the manufacturer's recommendations. Primary colon cancer samples (Table S2) were cultured as described elsewhere [13]. For assays, cells were seeded in 96-well flat-bottom plates at 5×10^3 cells per well with 100 μL RPMI 1640 medium supplemented with 1 % (*v*/*v*) FCS, 2 mmol/L glutamine (Invitrogene, Darmstadt, Germany) and antibiotics (100 IU/mL penicillin, and 1 μg/mL streptomycin). The assays were cultured at 37 °C in a humidified atmosphere of 5 % CO_2 in air. The cells were cultured to reach the 50–60 % confluence on the second day when treated with serial dilutions of PP. The assays were cultured at 37 °C in a humidified atmosphere of 5 % CO_2 in air for 3 days.

Reagents

PP salt (≥98 % purity) was obtained from Sigma-Aldrich, Taufkirchen, Germany. Stock solution (10^{-2} mol/L) was prepared with phosphate-buffered saline (PBS; Gibco, Life Technologies GmbH, Darmstadt, Germany) with DMSO (Sigma-Aldrich) with 2 % final concentration and was stored in aliquots at −20 °C. The chemotherapy agent 5-FU was purchased from the local hospital pharmacy and was used at a concentration of 5×10^{-6} mol/L that is in the range, or below, of the inhibitory concentration (IC_{50}) values for 5-FU for the investigated colon cancer cell lines (Table S3).

Measurement of PP cytotoxicity

The cytotoxic activity of PP is indicated by IC_{50} values, which describe the concentration of PP that reduced the number of viable cells by 50 %. The cellular viability was determined after culture with PP for 72 h by crystal violet staining [14] or calcein staining [15]. The measured OD values at a wave length of 570 nm (crystal violet staining) or 515 nm (calcein staining) are directly proportional to the number of viable cells. Data are presented as the mean±standard deviation of hexaplicates and triplicates, respectively for each PP concentration. All experiments were repeated at least once.

Cell migration assay

Serum-starved HCT116 cells were seeded on the bottom of 8-μm pore size transwell inserts (Greiner Bio-One, Frickenhausen, Germany). Transwell inserts were then placed in culture medium supplemented with 5 % FCS in the presence or absence of PP for 6 h after seeding at 37 °C in a standard tissue culture incubator. Invading cells that migrated through the filter pores toward the lower side of the transwell membrane were stained with crystal violet and counted by microscope. At least ten random high-power fields from each of the triplicate membranes were counted [16].

Western blotting

Cultured cells were rinsed three times with ice-cold PBS, harvested, and lysed directly in RIPA buffer (Pierce, Thermo Scientific, Rockford, USA) for immunoblot analysis. Cell debris was removed by centrifugation at 15,000*g* for 10 min at 4 °C. The supernatant was used as total protein lysate. For each sample, 10 μg of total protein lysate was subjected to 10 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblot analysis. Immunoblots were probed with antibodies against c-MYC (Y69, #ab32072; Abcam, Cambridge, UK), β-catenin (#9581; Cell Signaling through New England Biolabs GmbH, Frankfurt am Main, Germany), and β-actin (AC-15, #5441; Sigma-Aldrich, Taufkirchen, Germany). All antibodies were used according to the manufacturer's instructions. The blots were visualized with secondary antibodies (GE Healthcare Life Sciences Europe, Freiburg, Germany) against mouse (NA9310) or rabbit (NA9340) primary antibodies.

RT-qPCR

One million cells were disintegrated in TRIzol reagent (Invitrogen Life Technologies, Darmstadt, Germany), and total RNA was extracted from TRIzol as recommended. RNA integrity was verified using the Experion automated electrophoresis station from Bio-Rad Laboratories Inc. (München, Germany), and the RNA concentration was measured at 260 nm. For first strand complementary DNA (cDNA) synthesis, 1-μg total RNA was employed using the iScript cDNA synthesis kit from Bio-Rad. The cDNA synthesis was performed by heating at 25 °C for 5 min, at 42 °C for 30 min, and at 85 °C for 5 min. Quantitative PCR (qPCR) was performed with MESA Green qPCR Master Mix Kit for SYBR Green containing MeteorTaq hotstart DNA polymerase (Eurogentic GmbH, Köln, Germany). The following primer pairs were used: *MYC* (access no. NM_002467.4), forward CACCAGCA GCGACTCTGA, reverse GATCCAGACTCTGACCTT TTG (102 bp); *p21* (access no. NM_000389.4), forward GGATTCGCCGAGGCACCGAG, reverse GCCGCATG GGTTCTGACGGA (80 bp); *AXIN2* (access no. NM_004655.3), forward CCACACCCTTCTCCAATCC, reverse TGCCAGTTTCTTTGGCTCTT (62 bp); *BCL9* (access no. NM_004326.2), forward TCTCCCAACTTG CCATCAA, reverse GACCTGAAATTCGAGGATTCTG (73 bp); peptidylpropyl isomerase A (*PPIA*; access no. NM_021130.3), forward TGTCCATGGCAAATGCTG GACCC, reverse GCGCTCCATGGCCTCCACAA (140 bp); and *β-actin* (access no. NM_001101), forward CCTTGCCATCCTAAAAGCC, reverse CAC GAAAGC AATGCTATCAC (96 bp). The qPCR reactions were performed on a CFX96 real-time PCR system (Bio-Rad) operated by CFX Manager Software (version 2.0). The cycler protocol was 5 min at 95 °C, 40 cycles with 15 s at 95 °C, 60 s at 60 °C, and 5 min at 72 °C. Gene of interest expression was normalized to the reference genes PPIA and β-actin, and fold expression was calculated with the ΔΔCq method [17]. The two reference genes demonstrated stable expression in all colon cancer cell lines with the lowest *M* values [18] selected from a set of common reference genes. Post-amplification melting curves were checked to exclude primer-dimer artifacts and contamination, and PCR products were sequenced to confirm their accuracy (SeqLab Göttingen GmbH, Göttingen, Germany).

Colon cancer xenografts in mice

The in vivo effect of PP on tumor growth was tested in the portal injection model with HCT116 colon cancer cells as described elsewhere [19]. HCT116 cells transfected with luciferase (HCT116Luc^{pos}) were used to quantify intrahepatic tumor burden by in vivo bioluminescence imaging. Athymic NMRI-Foxn1^{nu} (nude) mice, weighing 22–25 g at the time of surgery, were purchased from Janvier, Le Genest-Saint-Isle, Frankreich. For evaluation of PP effectiveness in vivo, mice were randomized 4 days after cell injection into control and treatment groups (seven animals/group). From day 4 to day 36 after tumor cell injection, each mouse in the treatment group received 1 mg/kg pyrvinium daily from Monday to Friday, with a therapy break on Saturday and Sunday. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) i.p. and placed in the NightOWL light-tight chamber (NightOWL, Berthold Technologies, Bad Wildbad, Germany) for imaging for 10 min after they received i.p. 180 mg/kg luciferin (Biosynth, Switzerland). All animal experiments were authorized by the local ethics committee, and mice were treated according to the institutional and European Union guidelines.

Statistical analysis

All graphs and statistical analyses were made using Prism 5 statistical software (GraphPad Software, Inc). IC_{50} values were calculated with nonlinear regression fit to a sigmoidal dose-response curve (variable slope). Comparison of bioluminescence values was made with two-way ANOVA with Bonferroni post hoc tests. One-way ANOVA was performed for comparison between different groups. Differences were considered statistically significant at values of $p<0.05$.

Results

PP affects viability of colon cancer cells

More than 90 % of human colorectal cancers harbor varying mutations in the WNT pathway [4]. Due to this circumstance, the effect of PP on cell viability was examined on six *APC*^{mut} colon cancer cell lines, two β-catenin^{mut} colon cancer cell lines, one WNT^{wt} colon cancer cell line (COLO741), and two nonmalignant cell lines (HUVEC, NHDF); for mutation status, see Table S1. Cell viability was determined following treatment with different concentrations of PP (10^{-10}–10^{-4} mol/L) for 72 h. We found that SW620 (*APC*^{mut}) was extremely sensitive to PP with an IC_{50} value of 0.6×10^{-6} mol/L (Fig. 1). Other colon cancer cell lines with mutant *APC* (HT29, T84) and mutant β-catenin (HCT116, LS174T) demonstrated IC_{50} values for PP between 1.1×10^{-6} and 5.0×10^{-6} mol/L (Fig. S1). PP had less effect on the viability of CX1, HCT15, and WiDr cells with mutant *APC* (IC_{50} between 33×10^{-6} and 65×10^{-6} mol/L). Colo741 and nonmalignant human fibroblasts (NHDF) with no WNT mutation demonstrated increased IC_{50} values of 42×10^{-6} and 54×10^{-6} mol/L. The human endothelial cells (HUVEC) with no WNT mutation were six times less sensitive to PP than NHDF (Table S1).

PP affects viability of primary colon cancer samples

Since established human colon cancer cell lines are cultured for a long time under artificial conditions in vitro, we assessed also the effect of PP on viability of primary colon cancer samples derived from resection specimens with known hotspot mutations in WNT signaling (Table S2) [13]. Cell viability was determined following treatment with different concentrations of PP (10^{-7}–10^{-3} mol/L) for 72 h (Fig. 1). PP decreased cell viability in two of four primary colon cancer samples with mutant *APC* and IC_{50} values between 4.6×10^{-6} and 12×10^{-6} mol/L corresponding to IC_{50} values observed for colon cancer samples with wild-type *APC* (Table S2). Two cell lines with mutant *APC* demonstrated IC_{50} values of 24×10^{-6} and 187×10^{-6} mol/L (Fig. S2, Table S2). As observed for colon cancer cell lines (Table S1), not all primary colon cancer samples with mutant *APC* demonstrated lower IC_{50} values for PP in comparison to primary colon cancer samples with wild-type *APC*.

PP targets WNT signaling in colon cancer cell lines

Independent of the kind of mutation in WNT signaling (*APC*^{mut}, β-catenin^{mut}), an oncogenic mutation leads to the upregulation of WNT target genes. The major WNT target gene in colorectal cancer is the proto-oncogene *c-Myc*. *c-Myc* drives tumorigenesis by upregulation of several cyclins and suppresses the transcription of cyclin-dependent kinase inhibitors such as *p21* [20]. It has been described that PP activates CK1α and thereby leads to degradation of β-catenin in *APC*^{mut} cells [11]. RT-qPCR analysis of *MYC* showed that PP (10^{-7}, 10^{-6} mol/L) reduced messenger RNA (mRNA) expression in a dose-dependent manner in six of seven colon cancer cell lines with mutations in the WNT pathway (Fig. 2). In addition, PP increased the expression of the *c-Myc*-repressed target gene *p21* in four of seven colon cancer cell lines with mutations in WNT signaling. These effects in colon cancer cells with mutations in WNT signaling were not detectable in the WNT^{wt} cell line Colo741 following treatment with PP. The observed effects in Colo741 cells were reversed: *c-Myc* mRNA expression was upregulated and *p21* mRNA expression was not influenced. In addition to the reduced *c-Myc* mRNA expression, the expression of the WNT target

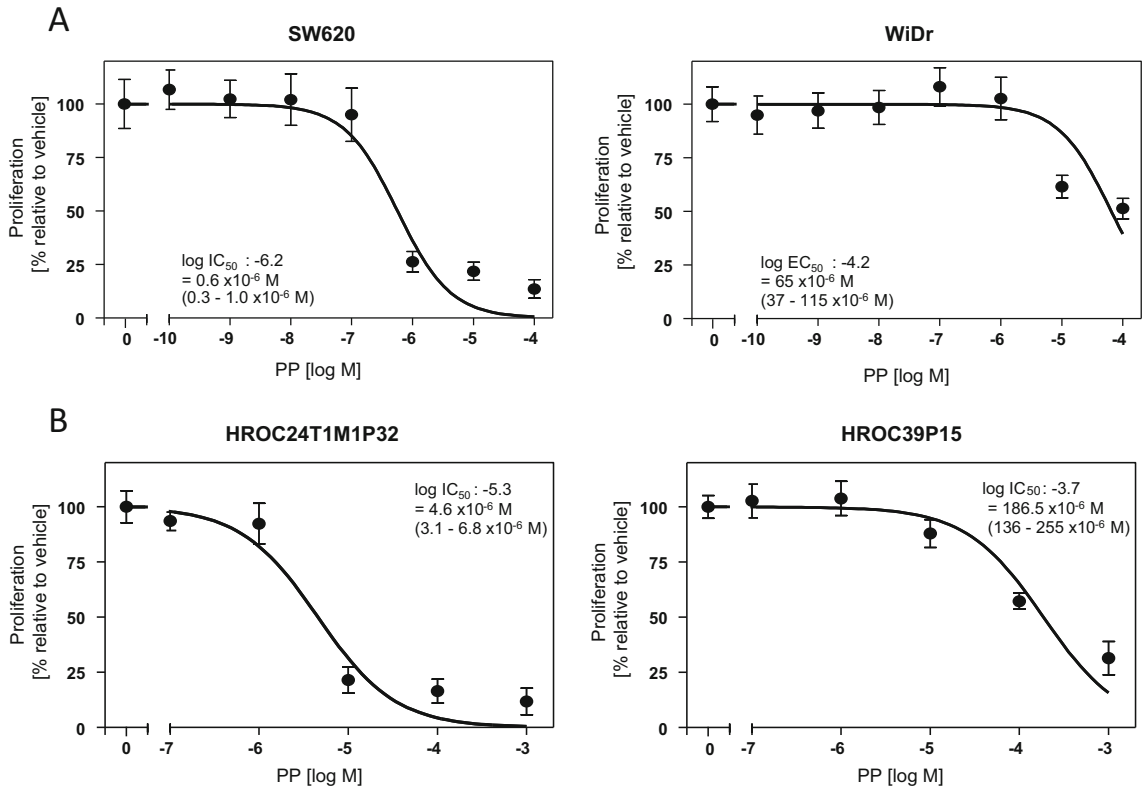

Fig. 1 Sensitivity of different colon cancer cells and primary colon cancer samples to pyrvinium pamoate. Colon cancer cells and primary colon cancer samples were treated for 72 h with the indicated concentrations of pyrvinium in media with low serum (1 %*v*/*v* FCS). Cell viability was subsequently determined by crystal violet staining. **a** Colon cancer cell lines. The results (mean±SD) shown are representative for two to three independent assays performed in hexaplicate. **b** Primary colon cancer samples. Mean±standard deviation is shown (assays performed in triplicate). All graphs were made in Prism 5 (GraphPad Software, Inc) with nonlinear regression fit to a sigmoidal dose-response curve (variable slope). IC_{50} values are listed in Tables S1 and S2. The dose-response data for all analyzed colon cancer cells are presented in Figs. S1 and S2

genes *AXIN2* and *BCL9* is also reduced on mRNA levels after treatment with PP. This is in line with previous results [11]. To check whether the observed PP effect on *MYC* mRNA levels is in accordance with reduced protein expression, cells of the following colon cancer cell lines HCT15, HT29, SW620, and T84 (APC^{mut}), and HCT116 and LS174T (β-$catenin^{mut}$) were treated with different amounts of PP (10^{-8}–10^{-6} mol/L). In all tested cell lines, the treatment with PP led to a reduction of c-Myc protein (Fig. 3). In addition, a reduction or loss of β-catenin on protein level was observed for all tested colon cancer cell lines independent of mutations in WNT signaling (Fig. 3).

PP effects in combination therapy with 5-FU

The standard chemotherapy for colorectal cancer in UICC III is FOLFOX with 5-FU, folinic acid, and oxaliplatin. However, this therapy is limited due to its toxicity and adverse effects. Cell viability was determined after treatment with PP (10^{-7} mol/L), 5-FU (5×10^{-6} mol/L), or both drugs for 24 h. The concentrations of both drugs were in the range of the IC_{50} values for the tested colon cancer cell lines (Tables S1 and S3). The combined treatment of 5-FU with PP significantly increased cell death for HCT116 (β-$catenin^{mut}$) and SW620 (APC^{mut}), but not for Colo741, with no mutation in WNT signaling (Fig. 4). PP did not increase 5-FU cytotoxicity in the other colon cancer cell lines with mutations in WNT signaling (Fig. S3).

PP inhibits migration of colon cancer cells

It is supposed that WNT signaling drives colon cancer metastasis and, therefore, inhibition of WNT signaling inhibitors may decrease cell migration. We observed that PP significantly reduced the migration of HCT116 cells (β-$catenin^{mut}$) in transwell migration chambers in a dose-dependent manner (Fig. 5).

PP induces growth delay of colorectal liver metastases in a xenograft mouse model

The antitumor activity of PP was analyzed in a xenograft model of colorectal liver metastases [19]. All mice were analyzed with bioluminescence 4 days after surgery. The mice with successful intrahepatic tumor take on day 4

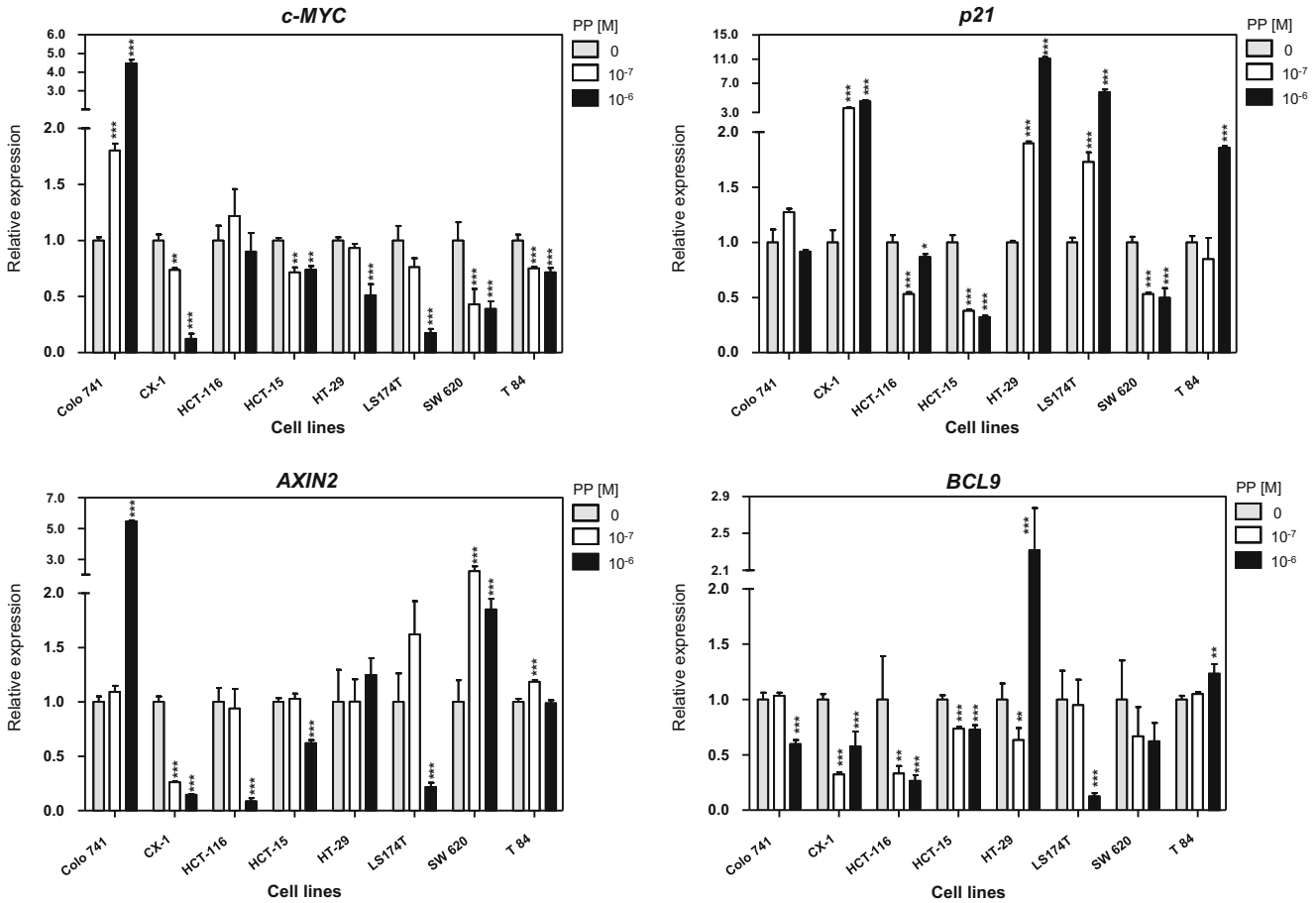

Fig. 2 Pyrvinium pamoate influences expression of *MYC*, *p21*, *AXIN2*, and *BCL9*. **a** For RT-qPCR analysis, cells were treated with increasing concentrations of pyrvinium (10^{-7}, 10^{-6} mol/L) for 48 h. Data are mean± standard deviation of two different assays in triplicate.*$p<0.05$, **$p<0.01$; ***$p<0.001$ compared to control (0 mol/L PP)

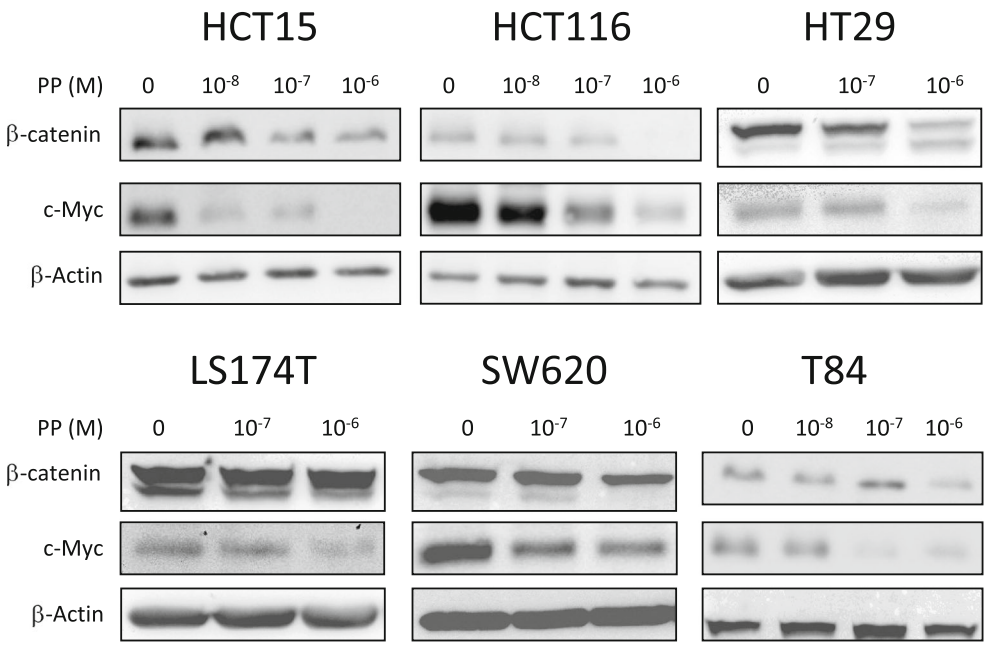

Fig. 3 Pyrvinium pamoate influences expression of c-Myc, and β-catenin. For Western blot analysis, colon cancer cells were treated with increasing concentrations of pyrvinium (10^{-8}–10^{-6} mol/L) for 48 h

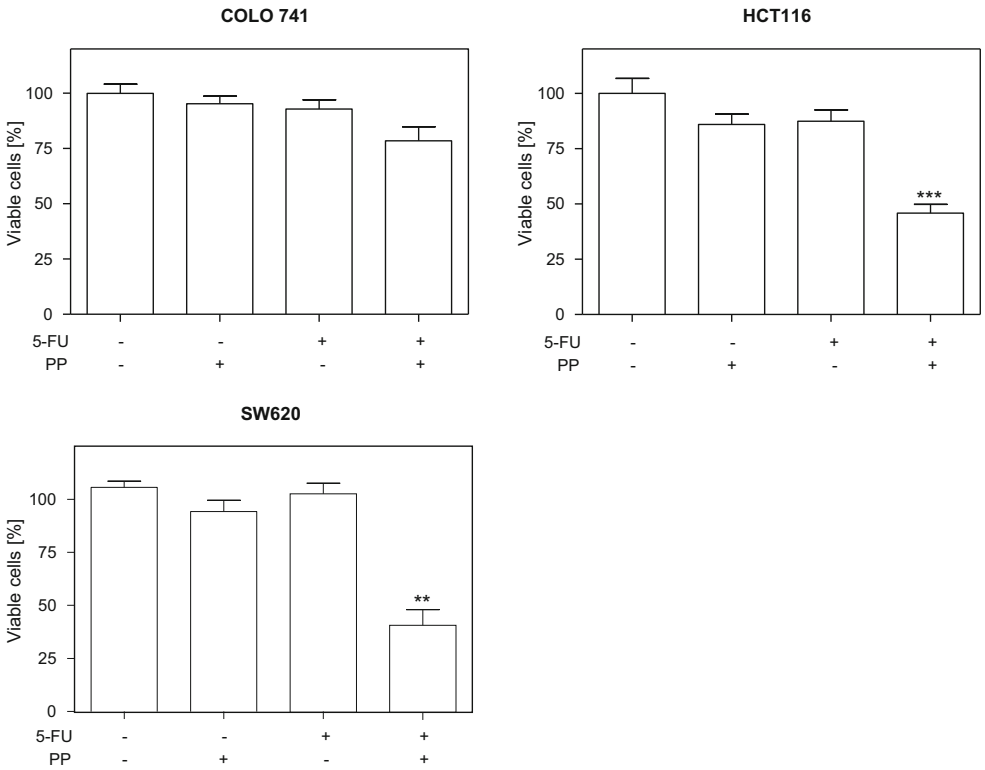

Fig. 4 Pyrvinium pamoate potentiates the cytotoxic effect of 5-FU on colon cancer cells. Colon cancer cells were treated with a combination of PP (10^{-7} mol/L) and 5-FU (5×10^{-6} mol/L) for 24 h in growth media with low serum (1 % (*v*/*v*) FCS). Low-dose pyrvinium (10^{-7} mol/L) increased the cytotoxic effect of 5-FU for HCT116 (β-catenin^{mut}) and SW620 (*APC*^{mut}) but not for Colo741 (WNT^{wt}). $^{**}p<0.01$; $^{***}p<0.001$ compared to monotherapies. Further results for the other cell lines are presented in Fig. S3; the IC_{50} values for 5-FU are shown in Table S3

were divided into treatment and control groups (n=7). PP significantly reduced liver metastasis growth between days 23 and 36, in contrast to continuous tumor growth in control mice (Fig. 6a–c). A daily dosage of 1 mg/kg was well tolerated by the mice with a stable body mass increase (Fig. 6d).

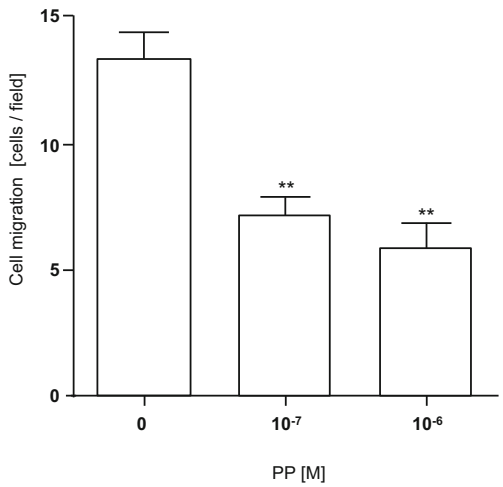

Fig. 5 Pyrvinium pamoate inhibits colon cancer cell migration. HCT116 cell migration was assayed with 8-µm pore size transwell migration chambers in culture medium supplemented with 5 % FCS in the presence or absence of PP (10^{-6} and 10^{-7} mol/L) for 6 h after seeding at 37 °C in a standard tissue culture incubator. $^{**}p<0.01$ compared to control (0 mol/L PP). Shown is mean±standard deviation

Discussion

In the present study, we analyzed the therapeutic potential of PP, a new WNT signaling inhibitor, against a broad panel of colon cancer cell lines. PP is an antihelmintic drug with reported anticancer effects that influences cell metabolism on transcriptional and protein levels [11, 21–26].

Esumi and colleagues were the first to describe a cytotoxic effect for PP in vitro and in vivo, especially under glucose starvation [21]. The authors also observed reduced phosphorylation of Akt^{S473} in line with another report [12]. Tomitsuka proposed that PP disrupts energy metabolism by interfering with the NADH-fumarate reductase system [22, 23]. Harada et al. showed that PP impairs the function of mitochondrial NADH-coenzyme Q oxidoreductase (complex I) and thereby inhibits STAT3 activation, leading to suppression of cancer cell growth [24]. Yu et al. showed that PP targets the unfolded protein response (UPR) induced by glucose deprivation and hypoxia [25]. PP suppresses the transcriptional activation of the two URP genes encoding the glucose-regulated protein 78 (GRP78) and GRP94. In contrast, the overexpression of GRP78 can partially rescue tumor cells from PP-mediated inhibition of genes regulating URP in vitro and in vivo. Deng et al. described that PP promotes cancer cell death by targeting autophagy [26].

A further proposed course of PP action is the interference with WNT signaling, identified by Thorne et al. [11]. The authors showed that PP has two targets in the WNT signaling cascade. On the one hand, PP enhances the activity of CK1α

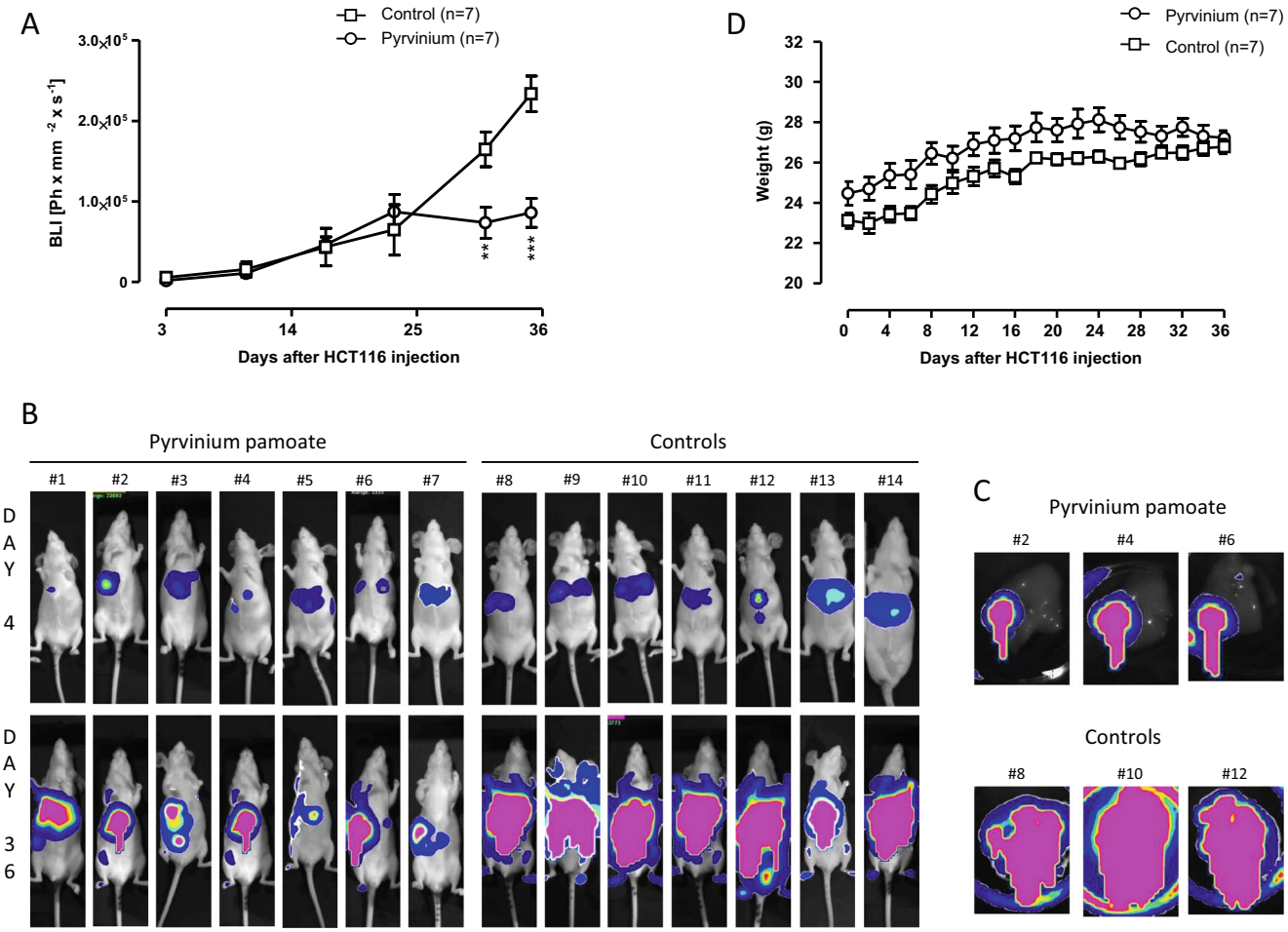

Fig. 6 Pyrvinium pamoate delays intrahepatic colorectal tumor growth in vivo. For monitoring pyrvinium efficacy on intrahepatic tumor growth, HCT116Luc^{pos} cells (2.5×10^6) were injected intraportally into nude mice. From day 4 to day 36 after tumor cell injection, mice were treated with intraperitoneal injections of pyrvinium (daily 1 mg/kg from Monday to Friday with a therapy break on Saturday and Sunday). Tumor growth was noninvasively monitored and quantified weekly by bioluminescence (BLI). **a** BLI data are plotted as mean with error bars (SEM). $^{**}p<0.01$; $^{***}p<0.001$. **b** BLI images of mice from control and treatment groups are shown ($n=7$ animals). **c** Ex vivo BLI images from livers of three representative mice from treatment and control groups at the end of experiment on day 36. **d** Stable body mass increase of pyrvinium treated and untreated mice. The differences between both groups are not significant

and thereby forces β-catenin phosphorylation and its degradation. On the other hand, PP inhibits the co-transcription factor *Pygopus* and thereby is also effective against β-catenin-mutated cells. In 2013, Venerando et al. published that PP is not a "bona fida" activator of CK1 but leads to an activation of GSK3 by downregulation of Akt/PKB and, thus, regulates WNT signaling [12]. According to the literature, the activation of GSK3β can lead to an enhanced turnover of *c-Myc* itself by phosphorylating *c-Myc* at threonine 58 and thereby priming it for degradation [27]. In the present paper, we did not explore the effect of PP on Akt signaling, and therefore, we cannot rule out that this is also a possibility of regulating WNT signaling. But in line with data of Thorne and Venerando [11, 12], we observed a reduction in total β-catenin levels. The more important effect of PP for our research was the reduction of *c-Myc*, which is reached with both postulated courses of action of PP.

Independent of the exact course of action, PP is supposed to inhibit downstream activation of WNT target genes. More than 90 % of colorectal carcinoma display aberrant activation of WNT signaling important for development and progression of colorectal carcinoma [4]. This is mainly due to a truncating mutation of *APC* that disrupts the β-catenin destruction complex and, to a lesser extent, mutations in the β-catenin gene [4]. The concept of oncogen addiction is based on shutting down the driving oncogen [28]. For colorectal carcinoma, the WNT pathway appears to be the optimal target for anticancer agents. However, currently, no FDA-approved drugs that regulate WNT signaling are available. The results of this study show that PP can inhibit cell growth of human colon cancer cell lines and are in line with a previous report [29]. It is conspicuous that published data are generally based on the analysis of one or two cell lines, whereas we analyzed a broad panel of human colon cancer cell lines and primary colon

cancer samples. This may be the reason why we did not observe clear differences of PP-mediated cytotoxicity against colon cancer cell lines with and without mutations in WNT signaling. In addition, we demonstrated a cytotoxic effect of PP on primary colorectal cancer specimens for the first time. Again, the cytotoxic effect of PP was independent of mutations in WNT signaling. For the primary colon cancer samples, only hot spot mutations in *APC* were sequenced. Therefore, we cannot exclude that the primary colon cancer samples with APC^{wt} still harbor a point mutation in β-catenin or outside the sequenced hotspots in *APC*. Nevertheless, it seems to be a fact that colon cancer samples with known mutations in *APC* can display high relative resistance to PP treatment.

We compared the four colon cancer cell lines SW620, HCT116, LS174T, and T84, which are extremely sensitive to PP ($IC_{50} \leq 3.4 \times 10^{-6}$ mol/L), with the four colon cancer cell lines HCT15, WiDr, COLO741, and CX1, which are more resistant to PP (IC_{50} between 33 and 65×10^{-6} mol/L) and found that both groups of cell lines show significant differences in the expression of the two genes stanniocalcin 2 (STC2) and FDCP 6 homolog (DEF6). For this analysis, we used the interactive web tool GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r). GEO2R allows users to compare two or more groups of samples to identify genes that are differentially expressed. For both groups of cell lines, we found a log FC value (log 2-fold change) of 1.343 (2.5-fold change) and an adjusted *p* value of 0.0137 for STC2, a log FC value of 0.983 (2-fold change) and an adjusted *p* value of 0.0137 for DEF6. In a further study, we will focus our attention on the expression of STC2 and DEF6 as a possible predictive marker for the outcome of PP treatment.

The Cancer Genome Atlas Network demonstrated that deregulated *c-Myc* expression is a hallmark of virtually all colorectal carcinoma, independent of the set of specific mutations that are present in each tumor. Furthermore, a mouse model of colon cancer driven by loss of *APC* shows that colon tumor formation depends on continuous *c-Myc* expression [5]. As *c-Myc* itself cannot be inhibited and WNT signaling is an important *c-Myc* driving pathway in colorectal carcinoma [4], shutting down the *c-Myc* inducing pathway may provide therapeutic options for treating colorectal carcinoma. With RT-qPCR analysis, we were able to demonstrate a sufficient downregulation of *c-Myc* in most of the tested WNT-mutated colon cancer cell lines. In addition, the two well-defined WNT target genes *AXIN2* and *BCL9* are downregulated in a wide range of WNT-mutated colon cancer cell lines. *c-Myc* induces the suppression of the cell cycle inhibitor *p21* as one important step in the development of colorectal carcinoma, and vice versa, a downregulation of *c-Myc* triggers the expression of *p21* [20]. Consistent with these data, the authors of the present study show that the PP-induced suppression of *c-Myc* leads to a reactivation of *p21* on mRNA level. One major driving effect of PP seems to be influencing *c-Myc* expression in colon cancer cells in vitro. In addition, PP affected tumor growth in vivo with no obvious side effects in PP-treated mice.

In several cancer types, the use of chemotherapeutics is limited due to toxicity in higher dosage. One way to avoid dose-dependent side effects is the combination of anticancer drugs. Several reports showed that PP can act synergistically with established anticancer agents. For example, Harada et al. described a synergistic effect of PP in combination with dexamethasone [24], and Yu et al. reported the successful combination of PP with doxorubicin, whereas both monotherapies had no effect [25]. In line with these results, we were able to confirm a combined cytotoxic effect of PP with 5-FU, the standard chemotherapy agent for colorectal carcinoma. In addition to data of Thorne et al. [11], the combined cytotoxic effect of PP and 5-FU was only observed for the two cell lines HCT116 ($\beta\text{-catenin}^{mut}$) and SW620 ($APC^{mut}$). The combination of PP and 5-FU failed on all other colon cell lines with mutations in WNT signaling tested in the present study.

In summary, in the present study, we showed that PP influence a downregulation of WNT/β-catenin signaling and demonstrate an antitumor effect against a large panel of WNT-mutated colon cancer cell lines and primary colon cancer samples in vitro. In addition, we presented data on the effect of PP in vivo. Overall, we were not able to show a predominant anticancer effect of PP for colon cancer cells and primary colon cancer samples harboring WNT mutations.

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Author contributions Conceived and designed the experiments: AW, AT, FK, CTG, ML, CO; performed the experiments: AW, FWU, MH, BM, ML, CO. Analyzed the data: AW, FWU, MH, BM, AT, CO; contributed reagents/materials/analysis tools: AW, FWU, MH, BM, ML, CTG, FK; wrote the paper: AW, CTG, FK, AT, CO.

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