RESEARCH ARTICLE

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Dual drug targeting to kill colon cancers

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Abstract

Introduction: Colorectal cancer (CRC) is driven by a small set of oncogenic and tumour suppressor mutations. However, different combinations of mutations often lead to poor tumour responses to individual anticancer drugs. We have investigated the antiproliferative and in vitro cytotoxic activity of pair-wise combinations of inhibitors which target specific signalling pathways in colon cancer cells.

Objectives: To target specific signaling pathways pairwise with inhibitors in order to kill colon cancer cells.

Methods: The effects of different concentrations of two inhibitors on the proliferation and viability of colon cancer cell lines were measured using cell titre glow and cytotoxic assays in 2D and 3D cell micro-cultures. One successful drug combination was used to treat a colon cancer cell line growing as a xenograft in nude mice.

Results: Colon cancer cells in non-adherent cultures were killed more effectively by combinations of pyrvinium pamoate (a Wnt pathway inhibitor) and ABT263 (a pro-apoptotic Bcl-2 family inhibitor) or Ly29004 (a PI3kinase inhibitor). However, in a mouse xenograft model, the formulation and toxicity of the ABT737/PP combination prevent the use of these drugs for treatment of tumours. Fortunately, oral analogues of PP (pyrvinium phosphate, PPh) and ABT737(ABT263) have equivalent activity and can be used for treatment of mice carrying SW620 colorectal cancer xenografts. The PPh/ABT263 induced SW620 tumour cell apoptosis and reduced the rate of SW620 tumour growth.

Conclusion: By combining a Wnt signaling inhibitor (pyrvinium phosphate) and a pro-survival inhibitor (ABT263) colon cancer cells can be killed. Combinations of Wnt signalling inhibitors with an inhibitor of the Bcl pro-survival protein family should be considered for the treatment of patients with precancerous colon adenomas or advanced colorectal cancers with APC mutations.

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†Sadly, Janet Weinstock died on the 29th of May 2021.

1 | INTRODUCTION

The treatment of colorectal cancer has improved considerably over the last decade[1]; while the use of targeted therapies, for example epidermal growth factor receptor inhibitors and antibodies,[2] has improved the progression-free survival for patients with advanced colorectal cancer (CRC),[3] the 5-year survival rates have only improved marginally. The genetics of CRC have been analysed in considerable detail and a small number of oncogenic mutations (APC, TP53, Kr-RAS, PI3K, RNF-43, RSPO fusions, B-RAF, β-catenin, TGF β receptor, SMAD 2,3,4 and the mismatch repair enzymes) drive this cancer[4]; thus, there is sufficient variation caused by different combinations and locations of these mutations to make CRC a genetically diverse disease. Targeting a single mutation may slow the proliferation of CRC cells, and in some cases, may even kill the cells; however, in most cases, the CRC cells will escape targeted therapies such as cetuximab.[5]

We have selected a small panel of CRC cell lines with a range of oncogenic and tumour suppressor mutations and measured the sensitivity of these cells to agents which inhibit a single oncogenic target or signalling pathway which might drive CRC. We have used assays which measure the ability of the drugs to inhibit proliferation or kill the CRC cell lines in vitro. Once we determined the sensitivity of the cell lines to single inhibitors, we tested the ability of pairwise combinations of inhibitors to kill specific CRC genotypes. Depending on the mutational profile we identified dual targeting drug combinations which killed CRC cells in vitro at low concentrations. The most effective combination involved the inhibition of Wnt signalling[6] and the induction of apoptosis by inhibiting the prosurvival protein Bcl-2.[7] Given a significant proportion of CRC are initiated by the loss of APC-function, a Wnt[i]/Bcl-2[i] drug combination was also explored in a mouse xenograft model of colon cancer.

2 | MATERIALS AND METHODS

2.1 | Antibodies and reagents

Antibodies were obtained from BD Transduction Laboratories (mouse monoclonal anti-β-catenin and mouse monoclonal anti-E-Cadherin), Cell Signaling Technology (rabbit polyclonal anti- S33/S37/T41 phospho β-catenin), Sigma-Aldrich (mouse monoclonal anti-β-tubulin), Abcam (rabbit polyclonal anti-Lamin B1), Li-COR Biosciences (IRDye 800CW Goat anti-rabbit; IRDye 800CW Goat anti-mouse). Propidium iodide, Hoechst 33342 for live cell imaging and MTT were purchased from Sigma Aldrich (St. Louis). The LDH cytotoxicity detection kit was purchased from Roche Diagnostics, Mannheim. pyrvinium pamoate (PP) was purchased from USP, Rockville, MD, USA. Pyrvinium phosphate (PPh) was synthesised at the WEHI, Bundoora, VIC, following the protocol published by Yu et al.[8] ABT737 was purchased from SYNTHESIS MED CHEM, South Yarra, VIC, Australia. ABT263 (Navitoclax) was purchased from CAPOT Chemicals, Shanghai. DAPT was purchased from TOCRIS Bioscience, Ellisville. The EGFR small-molecule inhibitor AG1478 mesylate was purchased by from the Institute of Drug Technology (IDT, Boronia). The PI3K inhibitor LY294002 was purchased from Calbiochem (Merck Chemicals, Darmstadt); the SRC- kinase family inhibitor (WEHI-1208800) was synthesised at WEHI, Bundoora according to the Patent Application No. PCT/AU2011/000858. The mechanism of actions and citations for these inhibitors are summarised in Table S2.

The ApopTag Peroxidase In Situ Apoptosis Detection Kit was purchased from Millipore (Billerica)

2.2 | Cell culture

SW620 were a kind gift of Prof. John Mariadason, Ludwig Institute for Cancer Research, Heidelberg, VIC. LIM1899 and LIM2537 colorectal cancer cell lines are available WEHI, Parkville. The cell lines were maintained in RPMI-1640 medium (GIBCO) supplemented with 10% v/v fetal bovine serum (FBS, GIBCO), thioglycerol (10 μM final concentration), insulin (2.5 U/100 ml), hydrocortisone (0.1 mg/100 ml) and antibiotics (penicillin 0.6 g/100 ml and streptomycin 1 g/100 ml), at 37°C and 5% CO_2 in humidified atmosphere. Cells were validated by DNA sequencing.[4]

2.3 | Drug formulations

The stock solutions of the drugs were dissolved in the vehicles suggested by previous reports.[8–11] Briefly, PP was dissolved in 2% DMSO–saline(v/v) (final concentration

0.5 mg/ml); PPh was dissolved in water (final concentration 0.5 mg/ml); ABT737 was dissolved in 30% v/v propylene glycol, 65% v/v D5W (5% w/v dextrose in distilled water), 5% v/v Tween 80 (final concentration 5 mg/ml); ABT263 was dissolved in 30% v/v polyethylene glycol (PEG), 60% v/v Phosal 50 PG (50% w/v phosphatidylcholine in propylene glycol used to enhance solubility and bioavailability of compounds), 10% v/v ethanol (final concentration 5 mg/ml).

2.4 | Mice

Six- to eight-week non-obese diabetic with severe combined immunodeficiency (NOD/SCID) (NODCB17-*Prkdcscid*/ARC) mice were obtained from the Animal Resources Centre (ARC, Perth).

2.5 | MTT assay

Unless otherwise specified, cells were plated at 10^4 cells/well in 100 µl of medium (RPMI-1640 plus FBS 5%) for the assay and incubated overnight at 37°C in 5% CO_2 in a humidified atmosphere. RPMI 1640 with 5% FCS (150 µl/well) with FBS 5% was aliquoted into each well of the 96-well plate and 150 µl/well of the 4× concentration inhibitor was added to the first well of each row to obtain a concentration 2× the one set as the starting concentration of the experiment. A serial twofold dilution was then performed across the plate. After 3–4 days of incubation, MTT (Sigma-Aldrich) 10 µl/well was added to the plates and cells were incubated for 4 h at 37°C following the manufacturer's instructions. Plates were then centrifuged at 1500 rpm (~300g) for 10 min to collect all the cells at the bottom of the wells. Medium was removed carefully and acidified isopropanol (0.04 M HCl in isopropanol) was added at 200 µl/well to solubilise the purple formazan crystals. Plates were shaken on a Vibramax 100 plate shaker (Heidolph Instruments) for 30 min at 450 rpm to speed the solubilisation process. The optical density at 560/690 nm was measured on a Multiskan Ex Spectrophotometer (Thermofisher Scientific).

2.6 | LDH cytotoxicity detection assay

To assess the doubling time of each cell line and the optimal number of cells to plate for the experiments, under both adherent and anchorage independent conditions ('hanging drops'), a cell titration experiment was performed for each cell line on cells grown under adherent conditions or organoids formed in hanging drops.

2.7 | LDH assay—adherent conditions

Cells were harvested at 80% confluence after incubation for 5 minutes in a solution containing 0.1% w/v Trypsin in Versene 0.02% w/v. After centrifugation at 1500 rpm for 5 minutes, cells were re-suspended in fresh medium and viability and cell numbers were monitored with vital dye Trypan Blue 0.2% w/v (Trypan Blue 0.4% w/v, Sigma-Aldrich). The starting number of cells for the titration experiments on cells cultured under adherent conditions was set at 2×10^4 cells in 100 µl of culture medium. One hundred microlitres of 5% FBS in RPMI 1640 was aliquoted into each well of a 96-well plate. One hundred microlitres of cells at 2× the set starting number were added to the first rows of the plate and a two fold serial dilution was performed across the plate. At the end of the titration, the volume in each well was 100 µl, with 6 wells of each cell dilution. The last two rows of the 96-well plate were used for the medium alone (background), to be used for the normalisation of the results. For inhibitor studies, cells were plated on 96-well-plates at 5000 cells/well, the plates were incubated overnight at 37°C, 5% CO2 in humidified atmosphere. After 24 h, drugs were diluted to the starting concentration and the respective dilutions of the drugs were then added to the cells across the plate to reach the desired concentration, and plates were incubated for 72 h and processed as per the manufacturer's instructions.

2.8 | LDH assay—anchorage independent conditions

Cells were grown under anchorage independent conditions using the 'hanging drops' method (Robinson et al 2004) adapted to 96-well plates. The starting cell number was set at 10^5 cells per drop (30 µl/drop in RPMI-1640 with FBS 5%). A two fold serial dilution of drugs was then added to the cells.

2.9 | Xenografts

NOD/SCID female and male mice, 9–10 weeks of age, were used for the xenograft experiments. The mice were divided randomly in groups, 8 mice/experimental group, and the mice from each experimental group were placed in two cages, 4 mice per cage. SW620 colorectal cancer cells were harvested by trypsinisation,

TABLE 1 Effects of signalling inhibitors on proliferation and viability of three colorectal cancer cell lines in adherent cultures

CRC cell lines	Proliferation assay (IC$_{50}$) (μM)			Cytotoxicity assay (EC$_{50}$)[a] (μM)		
	SW620	LIM2537	LIM1899	SW620	LIM2537	LIM1899
Inhibitors:Target						
AG1478: EGFR	Inactive[4]	8.9 ± 1.1[3]	2 ± 0[2]	—	>10	4.1 ± 3.2
WEHI-1208800:Src	0.1 ± 0.03[3]	0.08 ± 0.0[3]	0.1 ± 0.0[3]	1.3 ± 0.9	0.23 ± 0.11	0.25 ± 0.05
LY294002:PI3K	>25[2]	22.5 ± 14[3]	7.7 ± 1.1[3]	>40	>40	>40
ABT737:Bcl-2	15 ± 5[3]	14.5 ± 3.5	10–20[3]	10.6 ± 0.7	12.0 ± 3.6	11.6 ± 0.6
Pyrvinium pamoate:Wnt	5.2 ± 0.55[3]	5.9 ± 1.34[3]	4.9 ± 0.6[2]	5.3 ± 1.7	6.3 ± 1.2	3.5 ± 1

[a]Superscripts indicate number of experiments; in each experiment there were three replicates.

washed in serum-free medium and resuspended in PBS at 5×10^7/ml. SW620 cells were inoculated in 100 μl of PBS at 5×10^6 cells/tumour, 2 tumours per mouse, subcutaneously, on the left and right flanks. Mice were inoculated under general anaesthesia with isofluorane. Administration of the drugs was started on the eighth day after cell inoculation. The solutions were administered by gavage (final volume 200 μl). For combination therapy, two drugs were administrated per day with at least a 3-h interval between each other. For the controls, the vehicles were administered without the compounds. Mice were weighed and checked for health problems, and tumour volume measured by caliper in two dimensions, at least twice a week, with the mice under a light general anaesthesia to increase the accuracy of the measurements. The mice were killed after 3–4 weeks of treatment or when the tumour volume had reached ethically unacceptable size. All the tumours were harvested and weighed at the end of the experiment and preserved in 10% v/v buffered formalin for histological analysis. Four mice per group were randomly chosen and the spleens, livers and kidneys from 4 mice/group chosen randomly were preserved in buffered 10% v/v formalin for histological analysis.

2.10 | Histology

The detection of apoptosis in vivo was performed using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore) according to the manufacturer's instructions.

2.11 | Analysis and quantitation of apoptosis

Images of the slides obtained with the ApopTag assay were acquired digitally with the Aperio ScanScope XT (Vista). Twenty fields per image per tumour were chosen arbitrarily within the epithelial tumour tissue, away from the stromal component and from the margins of the sections. Brown-stained cells were manually counted within each field. We used MetaMorph version 7.7.10 (Molecular Devices) to count the total and dead cells fields for each tumour and to calculate the area of positively stained cells as well as the total area. The results were then expressed as apoptotic cells area over the total area.

2.12 | Statistics

Statistical analysis was performed using GraphPad Software-Unpaired *t*-test. Graphs were plotted using GraphPad Prism v.6, Origin or Matplotlib. All in vitro experiments were done in triplicate and each data point was calculated from triplicate wells.

3 | RESULTS

3.1 | Single agent inhibition of proliferation

In order to measure proliferation of cells after the exposure to small-molecule pathway inhibitors and to quantify the relative dependency of the cells on each signalling pathway, three colon cancer cell lines, SW620, LIM1899 and LIM2537 (see cell line genetic characteristics in Table S1) were treated with increasing concentrations of signalling inhibitors: AG1478, WEHI-1208800, LY294002, ABT737, PP or DAPT. The results of these experiments are summarised in Table 1 and Figure S1; we note that DAPT had no effect on the proliferation or killing of any of these cell lines even up to 10 μM, so the results were not included in the table. The EGFR inhibitor AG1478 totally abolished proliferation of LIM1899 cells, but, even at the highest concentrations (>10 μM) it showed only a partial cytostatic effect on LIM2537 (Table 1). SW620 cell

line is also resistant to EGFR inhibition by AG1478; however, this is expected, as these cells do not express the EGF receptor.[12,13] Clearly, the LIM1899 cell line is dependent on the EGFR signalling pathway for proliferation in vitro (Table 1); this result is rather unexpected given that these cells harbour a KRAS mutation, which, in the majority of colorectal tumours, circumvents the EGF signalling pathway inhibition in the clinic.[14]

PP is a recognised inhibitor of Wnt signalling.[15–17] We confirmed that PP (2 μM) inhibited Wnt signalling using a TCF-driven-GFP reporter system in both LIM1899 and SW620 cells (Figure S2). Although PP inhibits Wnt signalling, it has been reported to inhibit other targets.[8] There was no correlation between the genotype of the cell lines tested and their sensitivity to PP: the drug potently inhibited the proliferation of all three cell lines at low concentrations (50% cytostatic effect at around 50 nM, Table 1 and Figure S1), suggesting that Wnt signalling pathway activation is required for proliferation of these cells in vitro, independently of whether they carry an APC or a β-catenin mutation.

The SRC-Family kinases Inhibitor WEHI-1208800 also inhibits the proliferation of all three cell lines within a similar IC_{50} range (100 nM) (Table 1, Figure S1).

The PI3K inhibitor LY294002 partially inhibited the proliferation of LIM1899 at low concentrations and within the range already reported in the literature,[18] whereas it was four times less potent in inhibiting LIM2537 cell line. As was the case for AG1478 (the EGFR small molecule inhibitor), the proliferation of SW620 cell line was resistant to inhibition of PI3K signalling pathway ($IC_{50} \geq 25$ μM) (Table 1 and Figure S1).

At concentrations higher than 10 μM, the BH3-only mimetic ABT737 inhibited LIM2537, LIM1899 and SW620 proliferation to 50% of control (Table 1 and Figure S1).

As well as determining the IC_{50} for the effects of each compound on the cell lines, we assessed the maximum cytostatic effect of each drug on these adherent cell cultures (Figure S3). All the compounds except DAPT induced considerable cytostasis (on at least two of the three cell lines) (Figure S3). Although DAPT can sensitise colon cancer cell lines to cytotoxic agents in vitro,[19,20] even 50μM DAPT did not inhibit proliferation of the three cell lines and was therefore excluded from further testing.

PP is the only drug which completely abolished proliferation of all the three cell lines (Figures S1, S3). The SRC-inhibitor was also very effective in inducing cytostasis of the 3 cell lines: reducing proliferation of SW620 cells by 95%, of LIM2537 by 70% and of LIM1899 by 80%. LIM1899 cells were also 100% responsive to the cytostatic effect of the anti-EGFR small molecule inhibitor AG1478, whereas, as discussed above, SW620 cell line was resistant to EGFR inhibitor treatment (AG1478, Table 1) and the

AG1478 induced only partial inhibition of proliferation in LIM2537 cells.

LIM1899 were the most sensitive cells to the cytostatic inhibition exerted by the PI3K inhibitor LY 294002, whereas SW620 showed only limited response to the inhibitor (Figure S1). LIM2537, on the other hand, responded to LY 294002 with a 70% inhibition of proliferation. Finally, ABT737 displayed around 50% of maximum cytostatic effect on all three cell lines (Figures S1, S3).

3.2 | Cytotoxic effects of signalling inhibitors

While the MTT assay assesses live cell numbers, it does not discriminate between inhibition of proliferation and induction of cell death. We analysed the ability of the drugs used in the proliferation studies to induce death in SW620, LIM1899 and LIM2537 using the LDH cytotoxicity detection and compared the level of killing in cells cultured under both adherent and non-adherent (hanging drop) conditions (Figures 1 and 2, respectively).

Under adherent culture conditions AG1478 (10 μM) only killed 30% of the LIM1899 (Figure 1) and AG1478 did not induce cell death in either LIM2537 cells or SW620 cells. The SRC-inhibitor WEHI1208800 was cytotoxic to SW620 cells (around 30% cytotoxicity at a concentration of 10 μM), but only marginally cytotoxic for LIM2537 cells (around 10% at the same concentration) (Figure 1). This contrasts with its high potency as a cytostatic agent in the proliferation assays (Table 1 and Figure S1). The EC_{50} for WEHI1208800 on SW620 is almost four4 times higher than the EC_{50} for the other two cell lines.

Even at high concentrations (40 μM), the PI3K inhibitor LY294002 does not exhibit any cytotoxic effect on adherent cultures of these cell lines and ABT737 exerts only marginal cytotoxic activity (Figure 1).

PP is by far the most potent cytotoxic drug in our panel, leading to 80% of cell death at 10 μM on SW620 and LIM1899 cells (Figure 1); however, it is not as potent for LIM2537 (maximum cytotoxic effect <30%) and the EC_{50} is higher than for the other cell lines (8μM, see Table 1 and Figure 1). LIM2537 is the only heterozygous APC cell line amongst the three tested, so this result suggests a lower dependence for this cell line on Wnt signalling.

The CRC cell line cytotoxicity responses are different when cells are cultured in an anchorage-independent manner (Figure 2, Figure S4; EC50s are summarised in Table S3). Within the same range of concentrations which are effective in adherent settings, SW620 is the only one of the three cell lines, sensitive to the SRC kinases inhibitor WEHI-1208800 (Figure 2); this drug reaches a maximum induced cell death of about 35%–40% of control (Figure
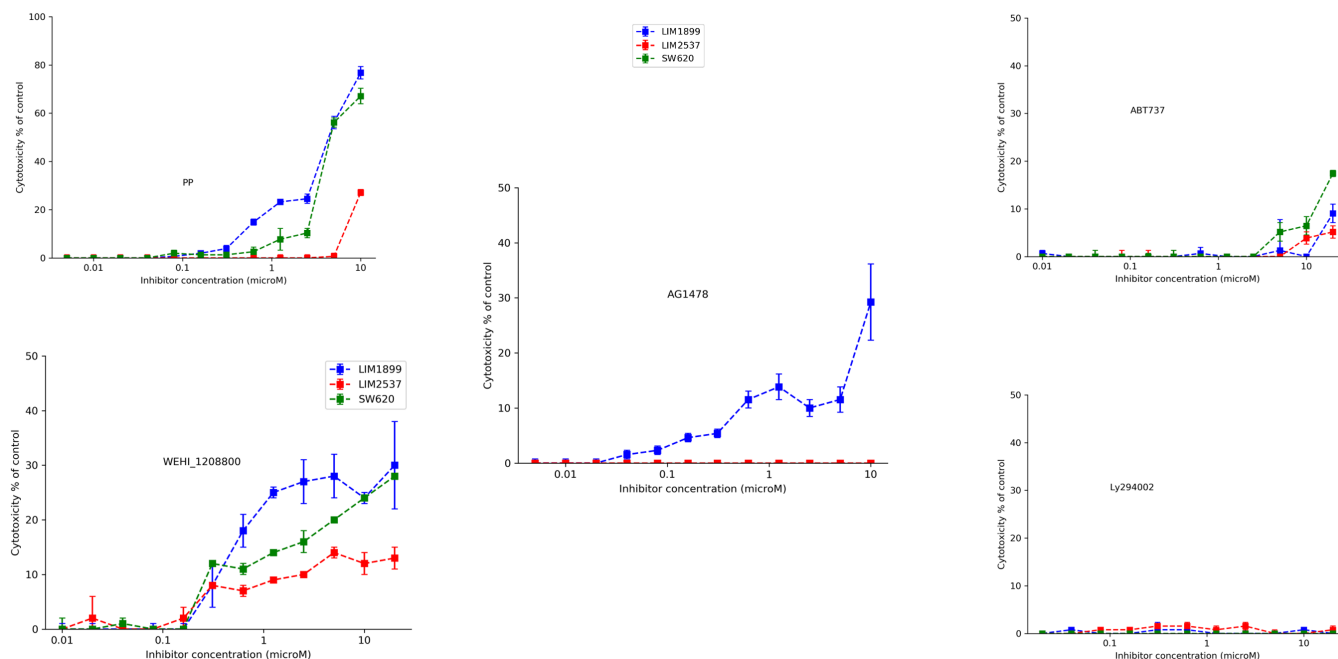
FIGURE 1 Adherent culture cytotoxicity of signalling inhibitors (pyrvinium pamoate PP, Wnt[i]; AG1478, EGFR[i],; WEHI-1208800,src[i];ABT737;bcl2[i]; and Ly294002,PI3K[i]) on three CRC cell lines (LIM1899,LIM2537 and SW620)

S4). Interestingly, the PI3K-inhibitor LY294002, which did not exhibit any cytotoxic effect on any of the cell lines when cultured in monolayers, induced ~40% cell death on the LIM1899 spheroids (Figure 2). The cytotoxic effect of LY294002 is exerted at higher concentrations (from 20 μM onwards), where other cross-reactivities will be more common. These results may indicate a higher dependency on the PI3K pathways under non-adherent conditions, in accord with the observation that PI3K activation can abolish anoikis.[21]

ABT737 was ineffective as a cytotoxic agent in monolayer cultures, but did show some cytotoxicity in non-adherent cultures of SW620 and LIM2537 (Figure 2). LIM1899 cell line remained unresponsive to the treatment with ABT737 (Figure 2). LIM1899 was the only cell line sensitive to the EGFR-inhibitor AG 1478 in the non- adherent cultures. There was a slight shift of the cytotoxicity curve to the right in comparison to the adherent cells; consequently, the EC_{50} values are higher (Table S3). LIM2537 cells did not show any response to AG1478, mirroring the results obtained on the adherent cell cultures.

PP was again the most effective cytotoxic drug. PP caused around 70–85% of cell death in LIM1899 and SW620 (Figure 2 and Figure S4). Although there was no change in the EC_{50} for PP on SW620 or LIM2537 cells, there was a significant shift to the left of the cytotoxicity curve for the LIM1899 cells in the non-adherent cultures (EC_{50} for cytotoxicity reduced from 3.5 μM to 1.5 μM) (Figure 2 and Table S3).

3.3 | Sensitivity of colon cancer cells to combinations of targeting drugs in vitro

The drug combinations listed in Table S4 were tested on two of the CRC cell lines, SW620 and LIM1899. The combination of the PI3K inhibitor LY 294002 and Wnt inhibitor PP significantly increased killing when compared to the single drug therapy in LIM1899 cells (Figure 3, Table 2).

Neither the SRC inhibitor (WEHI-1208800) nor the Bcl family inhibitor (ABT737) elicited any increase in cell death or sensitivity to PP (Figure 3) in LIM1899 cells; nor did ABT737 induce any increase the sensitivity of SW620 cells to WEHI 1208800 (Figure S5). Similarly, the SRC inhibitor (WEHI1208800) failed to increase the maximum cytotoxicity or the sensitivity of SW620 to PP (Figure S5). However, PP in combination with either the PI3Kinase inhibitor (LY294002) or the Bcl-2 inhibitor (ABT737) elicits significant increases in sensitivity of SW620 cells to PP, i.e. a sixfold decrease in the EC_{50} compared to PP as a single agent (Figure 4, Table 2). The shift to the left of the curve of cytotoxicity was more pronounced when PP was used together with ABT 737: it appears that ABT737 potentiates the cytotoxic effect of the Wnt inhibitor (PP) starting from concentrations of PP as low as 30 nM.

We investigated the hypothesis that this combination of inhibitors will be more effective on APC- and/or P53-mutated cell lines, whereas it might not induce any cytotoxicity on β-catenin mutated/P53 wild-type cell lines. To test this hypothesis, we examined more colorectal cancer cell lines, including 6 other lines carrying mutations of
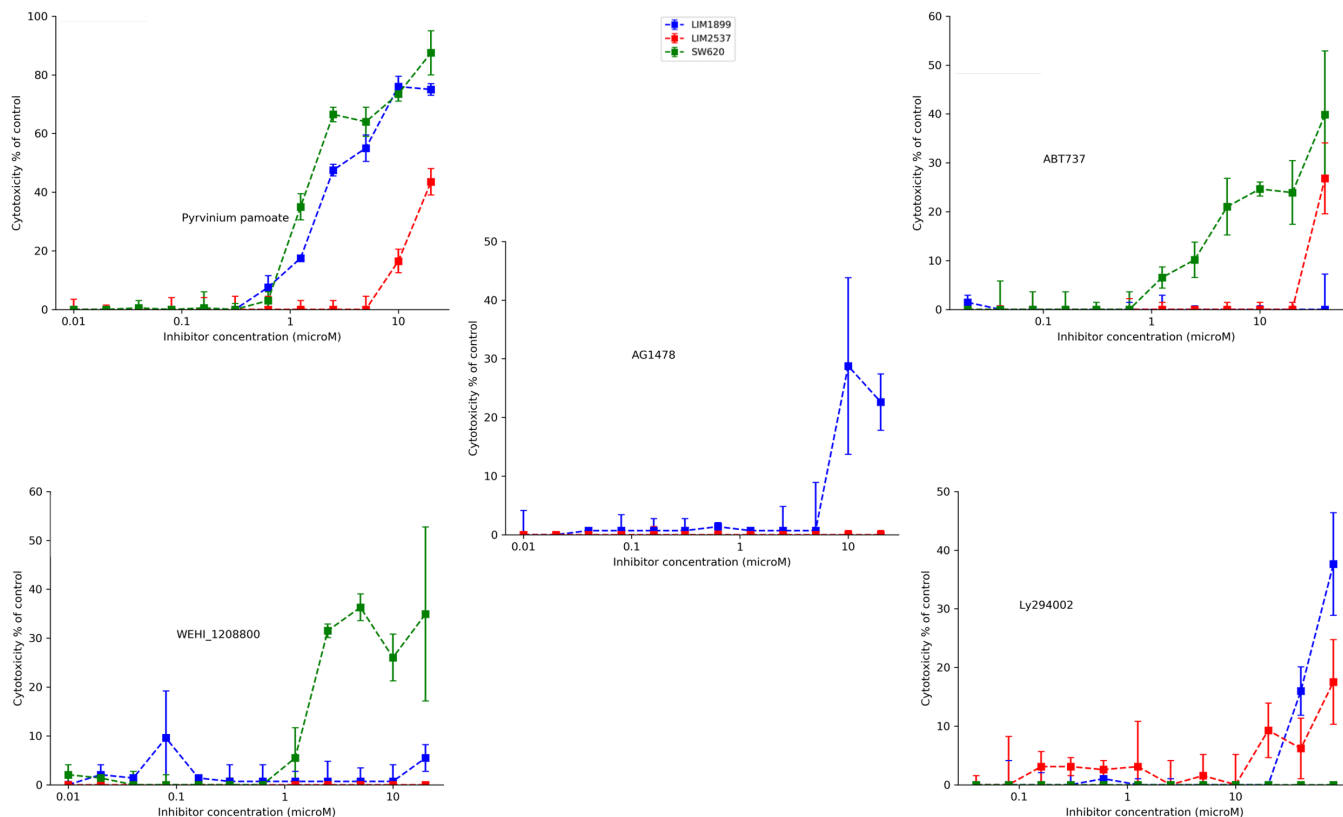
FIGURE 2 Non-adherent culture cytotoxicity of signalling inhibitors (pyrvinium pamoate PP, Wnt[i]; AG1478, EGFR[i]; WEHI-1208800,Src[i];ABT737;Bcl-2[i]; and Ly294002,PI3K[i]) on three CRC cell lines (LIM1899,LIM2537 and SW620)
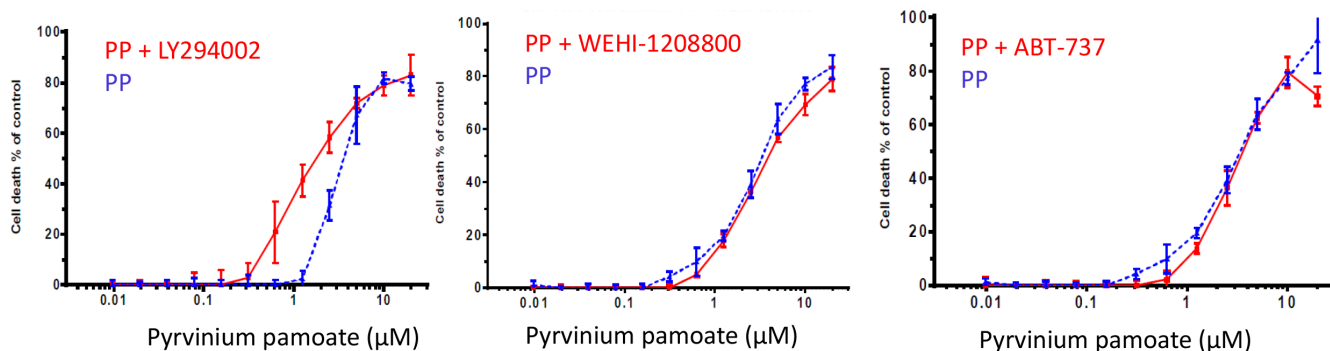

FIGURE 3 Cytotoxic effects of different concentrations of pyrvinium pamoate (PP) in combination with a fixed concentration of a PI3K inhibitor (LY294002,15 μM), a Src inhibitor (WEHI-1208800, 1 μM) or the pro-apoptotic drug (ABT737, 10 μM) on the LIM1899 colorectal cancer cells growing as colonospheres in hanging drop cultures

either the *APC* tumour suppressor gene or the *CTNNB1* (β-catenin) gene (see Table S5). We treated these cell lines, growing as colonospheres in the hanging drop culture system, with the PP alone and in combination with ABT737 and assessed their responses using the LDH cytotoxicity assay. With the exception of LOVO colorectal cancer cell line, the APC mutant cell lines responded to the treatment

with the PP and ABT737 combination with a shift in the EC_{50} towards the left of the cytotoxicity curve, that is increased sensitivity to PP (Figure 5). As expected, the PP/ABT737 combination induced apoptotic cell death as determined by FACS analysis (Figure S6)

As we found for the β-catenin mutant cell line LIM1899 (see Figure 3), the HCT116 cells, which also have mutant

TABLE 2 EC$_{50}$ for inhibitors as single agents and in combinations on CRC cell lines 3D cultures of colonospheres in hanging drops

Inhibitors (µM) Cell lines	PP	PP + WEHI-1208800	PP + LY294002	PP + ABT-737	WEHI-120880	WEHI-1208800 + ABT-737	AG1478	AG1478 + ABT-737	LY294002	ABT-737
LIM1899	3 ± 0.1	2.7 ± 0.17	1.2 ± 0.06	2.6 ± 0.35			>20	>20	>30	>40
SW620	6.5 ± 0.5	4.3 ± 1.5	1 ± 0.85	1.2 ± 0.7	4.5 ± 3.5	5 ± 2.8			>40	6.6 ± 3.8

Note: EC$_{50}$ are the average of triplicates from three experiments ±SD.

β-catenin, were resistant to the combination of PP and ABT737 (Figure 5). LIM2405 and SW403 cells were 100- and 10-fold more sensitive to the combined treatment of PP plus ABT737 (Figure 5). As in the case of the other responsive cell line SW620, the addition of ABT737 potentiates the cytotoxic activity of PP at low concentrations.

3.4 | Effects of the pyrvinium pamoate/ABT737 drug combination on the growth of SW620 xenografts in mice

Many variables must be considered when switching from cell culture to small animals as experimental models; the process of translating the results obtained in vitro into informative experiments in vivo is often complex. However, two of the drugs used in our in vitro studies PP[10] and ABT737[9] have been used previously in mouse models of disease: ABT737 had been already used in mouse xenografts and reported to be quite effective in slowing the growth of subcutaneous tumours derived from SCLC cell lines (Oltersdorf et al 2005). ABT737 induced a complete regression of the tumours in this SCLC lung cancer model, when injected intraperitoneum at 100 mg/kg/day, in 77% of the cases and significantly slowed tumour growth at a dose of 50 mg/kg/day.[10] PP was used in vivo by Esumi and colleagues to treat subcutaneous human PANC-1 tumour xenografts in nude and NOD/SCID mice[9]. The drug was administered orally at a dose of 100 or 200 µg/mouse/day and reached its maximal effect at 100 µg/day.

Our aim in combining two drugs was to allow lower doses of each drug with equal or more tumour toxicity, thus minimising unwanted side effects at the same time as achieving killing of the tumour cells. With this in mind, we decided to use ABT737 at a submaximal concentration of 50 mg/kg/day and PP at 50 µg/kg/day. The two drugs were formulated for delivery in a mixture of 30% v/v propylene glycol, 5% v/v Tween 80, 65% D5W (5% w/v dextrose in water) and administered intraperitoneum (I.P.) at 50 mg/kg/day 5 days/week. PP, diluted in 2% v/v of DMSO in saline (final volume 200 µl), was administered intragastrically to the mice by gavage at 50 µg/mouse/day, 5 days/week. Administrations of the two drugs were separated by at least 3 h gap to avoid cross reactions. Both drugs were also administered as single agents. Control mice received the vehicles with no drug. ABT737 is difficult to solubilise.[22] Similarly, PP was not soluble in water, heating and stirring were required to keep the compound in solution before administration.

Treatment of the mice carrying the xenografts was started at day 7 after the SW620 cells were inoculated and terminated at day 35 after tumour inoculation. The remaining mice were killed and the tumours collected for histology. Spleen, livers and kidney from 4 mice per
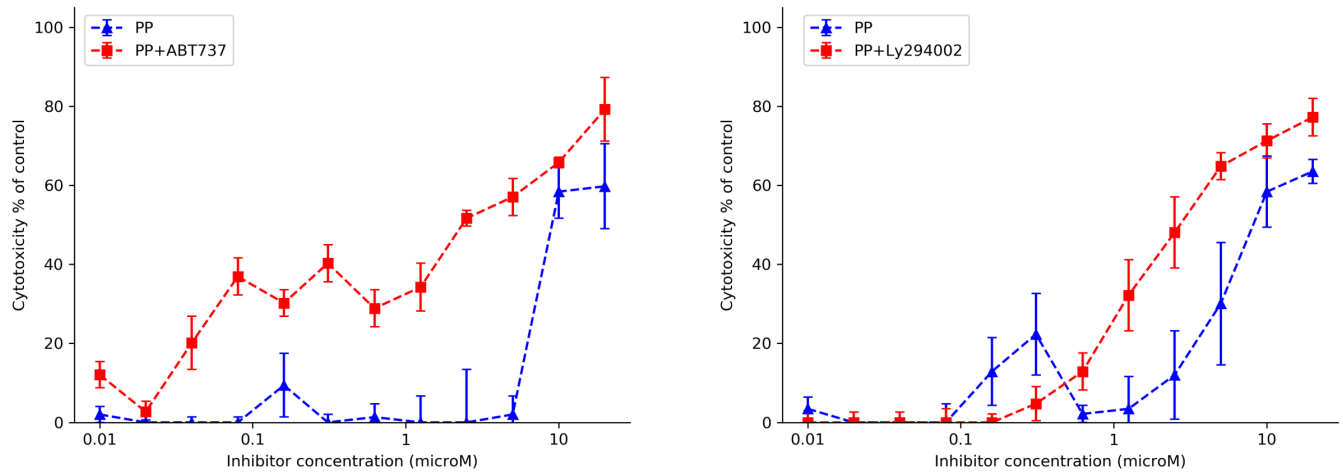
FIGURE 4 Cytotoxic effects of different concentrations of pyrvinium pamoate (PP) in combination with a fixed concentration of the pro-apoptotic drug (ABT737, 10 μM) or a PI3K inhibitor (LY294002, 15 μM) on the SW620 colorectal cancer cells growing as colonospheres in hanging drop cultures
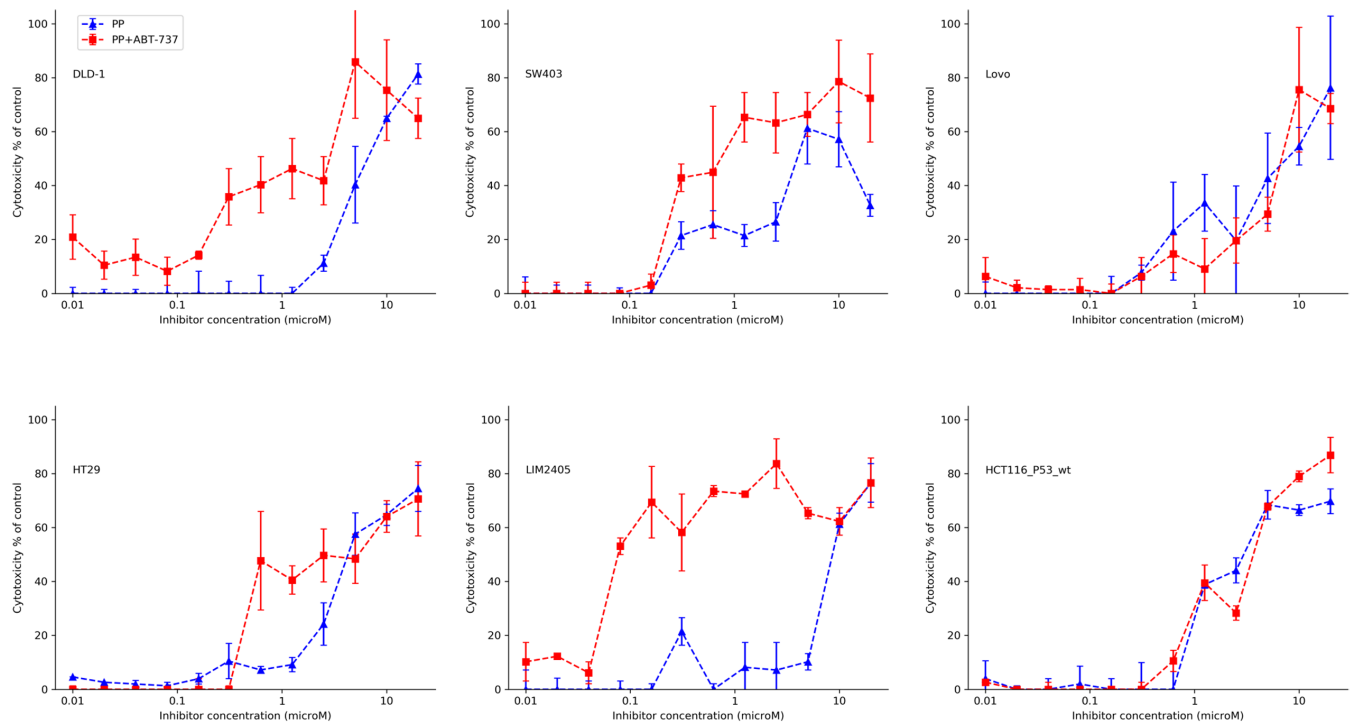

FIGURE 5 Cytotoxic effects of different concentrations of pyrvinium pamoate (PP) in combination with a fixed concentration of the pro-apoptotic drug (ABT737, 10 μM) on six colorectal cancer cell lines (DLD-1,SW403,Lovo,HT29,LIM2405 and HCT116_P53_wt) growing as colonospheres in hanging drop cultures

group were also collected for histology. The mice treated with the PP/ABT737 combination started to lose weight from day 10 onwards (Figure S7A). We supplemented the mouse food with a combination of Sustagen and normal food at a ratio of 30:70, starting from day 13. Even with this addition, the mice treated with PP/ABT737 continued to lose weight. The average weight loss within the

group in comparison to control was 15%, however, one mouse from the combination group needed to be killed for excessive weight loss (>20%); 2 mice from the same group were killed after showing multiple signs of stress and sickness; 3 mice from the combination group died before the completion of the experiment. Unfortunately, the high general toxicity of the combination therapy,

indicated by the body weight loss and the number of deaths in the combination group (75% of deaths within the group), together with the peritoneal precipitation of the ABT737 (Figure S7B), indicated it was not safe to use ABT737 under the dose and administration regime chosen for this experiment. This finding was unexpected as it has not been reported in the literature before. Moreover, an excessive weight loss may affect the overall metabolism of the mouse, with a catabolic status that would affect the growth of the tumours as well, when the tumour cells are deprived of the nutrients they need to keep multiplying. These facts heavily impacted on the overall significance of the in vivo model, underlining the need for finding good alternatives to the drug formulations and dosage currently in use, while maintaining the same inhibitory combination of targeted therapeutics (Wnt inhibitor and BH3-only mimetic) which achieved such promising results in vitro.

3.5 | Replacement of pyrvinium pamoate and ABT737 with orally available analogues

Fortunately oral analogues of both PP (PPh[8]) and ABT737 (ABT263,[11]) were available. ABT263 has been shown to be highly effective in inducing regression of SCLC and ALL cell lines tumours in mouse xenografts as well as potentiating the anti-tumour activity of chemotherapeutic regimes already in use in B-cell malignancies (Tse et al 2008). PPh was synthesised from PP in our laboratory, following the

process detailed by Yu and colleagues.[8] We tested the efficacy of PPh and ABT263 in vitro on SW620 cells grown in the hanging drop culture system. PPh and ABT263 were individually even more potent than PP and ABT737, respectively (Figure 6). We also tested the PPh/ABT263 combination on another CRC cell line (LIM2405) and again, the combination was more effective than the single agents at killing these CRC cells in the hanging drop cultures (Figure S8).

3.6 | Effects of the pyrvinium phosphate/ABT263 treatment on the growth of SW620 xenografts in mice

The treatments with vehicle, PPh, ABT263 or the combined PPH/ABT263 were started 7 days after inoculating the tumour cells. The mice were inspected and weighed twice a week for the duration of the treatment. The tumour growth was measured with calipers while the mice were under a light general sedation. ABT263 (50 mg/kg/day) diluted in a mixture of PEG 30% v/v, Phosal 50 PG 60% v/v and ethanol 10% was administered intragastrically by gavage (total volume of each dose was 200 μl), 5 days/week. No problems were encountered in the preparation or administration of the ABT263 solution. PPh (5 mg/kg/day) diluted in water was administered intragastrically by gavage in a maximum volume of 200 μl for 5 days/week. To avoid direct interactions between the drugs and to allow the mice to recover from the gavage, the two drugs were administered separately (at least 3 h
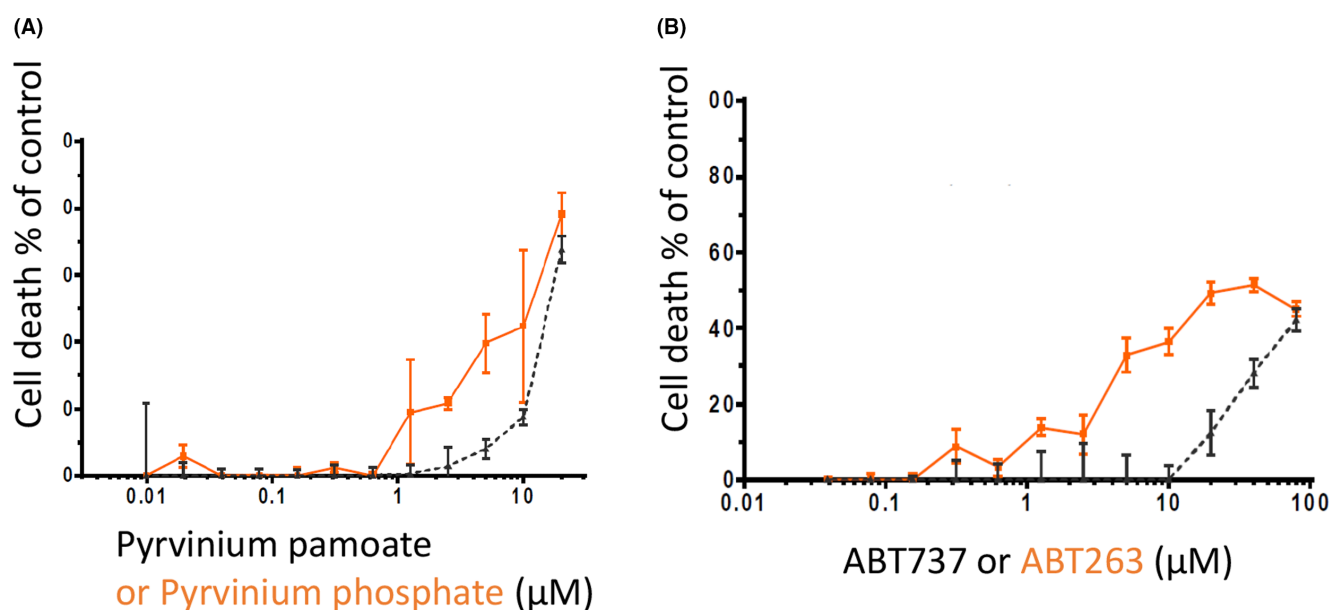

FIGURE 6 Cytotoxic effects of different concentrations of (A) pyrvinium pamoate and pyrvinium phosphate; (B) ABT737 and ABT263 on the SW620 CRC cell line growing as colonospheres in 3D-hanging drop cultures

apart). The experiment was repeated twice, the first time with NOD/SCID female mice, the second with NOD/SCID male mice. Each treatment group contained 8–10 mice. Treatment was continued for 23 days for the female mice and for 22 days for the male (Figure 7).

Tumour xenografts from SW620 cells were established in both male and female cohorts and mice were treated with PPh and ABT263 alone and in combination (Figure 7). Female and male mice from the combination group and male mice treated with PPh as a single agent started showing body weight loss from day 10 and day 15, respectively (Figure S9). Further weight loss was prevented by the dietary supplement (Sustagen). The average weight was less than 10% for both male and female mice, however, one mouse from the PPh group was killed for excessive weight loss. We noted that the female cohort treated with PPh and ABT263 showed 'matted fur' whereas mice treated with single agents did not. No other adverse health effects were observed.

Tumours in the PPh/ABT263 combination groups for both male and female cohorts exhibited slower growth rates in comparison to the controls and the single drug groups (Figure 7). In the female mice the tumour volume reached a plateau in the combination group on day 20. There was no difference between the growth of the SW620 tumours in the control group and the growth of the tumours in mice treated with PPh. The ABT263 as a single agent decreased tumour growth in the female mice ($p < 0.05$) (Figure 7), but had no effect on the tumour growth in the male mice.

Histological analysis of the tumours showed that the vast majority were mucinous, with some tumours containing significant amounts of fluid. There were no signs of direct or indirect toxicity or damage to other organs and the tissue collected for histology looked normal by microscopy, with no detectable differences between treated and untreated samples as evidenced by H&E staining (Figure 8A–D).To assess whether the PPh/ABT263 treatment exerts in vivo anti-tumour activity through induction of apoptosis, the levels of apoptosis in SW620 xenograft tumours were assessed using immunohistochemical staining with ApopTag.[23,24] To exclude stromal tissue from the analysis, we stained two consecutive sections for each tumour, one with hematoxylin eosin, which allows a clear distinction between stroma and glandular epithelium, and the other for apoptosis (Figure 8A–D). The percentage of apoptotic cells was close to zero in tumour sections derived from untreated and PPh-treated mice (Figure 8A,B). ABT263 treated tumours displayed only a slightly higher number of apoptotic cells in comparison to the control and PPh-treated mice (Figure 8C). While the frequency of apoptotic cells appeared to be higher in tissue from the PPh/ABT263

combination (Figures 8D,E), there was no statistical difference when compared to the single agent treated or the control mice ($p < 0.25$).

4 | DISCUSSION

The colorectal cancer cell lines we tested respond to one or more single agent targeted treatments in both adherent cell cultures and the non-adherent hanging-drop culture system. The potent inhibition of LIM1899 cells by PP was unexpected: this cell line is characterised by the presence of a point mutation in β-catenin which constitutively activates the Wnt pathway and renders these cells resistant to the extracellular Wnt-inhibitor Dickkopf-1 (DKK-1).[18] If PP exerts its only inhibitory function through CK-1α, the mutation which alters β-catenin at the Ser45 residue should render the cells resistant to inhibition of the Wnt pathway by PP. The inhibition of LIM1899 by PP strongly suggests that the inhibitory activity of the compound is either exerted on components of the Wnt signalling pathway downstream of β-catenin, or that the drug acts through GSK3 activation, as suggested by Venerando et al.[15] or that the drug affects cell viability independently of the Wnt pathway.

Our data indicate that the colon cell lines have a strong dependency on the SRC signalling pathway for proliferation in vitro; however, as for most kinase inhibitors, cross-reactivity with other kinases[25] mean that the SRC specificity is only a potential indication of its importance as a therapeutic target[26].

There are only a small number of studies on the effect of BH-3 only inhibitors on colorectal cancer cell lines in vitro,[27,28] but there is preliminary evidence of an increased response to chemotherapy when ABT737 is administered in a combination regimen for the treatment of other cancers.[29] It must be noted that although ABT737 inhibits Bcl2, it also inhibits BclXL another member in this prosurvival family, so further studies with more specific inhibitors (e.g. ABT199 which targets Bcl2 more selectively[30]) would be needed to clarify if one or both of these proteins need to be inhibited to induce cytotoxicity.

From our initial results on the drug combinations studies, it was clear that the combination of the Wnt pathway inhibitor (PP) and the Bcl-2 inhibitor (ABT737) killed SW620 effectively, with sensitisation of the cells to PP at very low concentrations in comparison to when the drug was used as single agent (Figure 4). This same combination was ineffective on LIM1899 (Figure 3). SW620 carries an inactivating mutation of APC with loss of heterozygosity, whereas LIM1899 carries an activating β-catenin mutation. Moreover, SW620 cells are P53 mutant, whereas LIM1899 cells are P53 wild-type. These
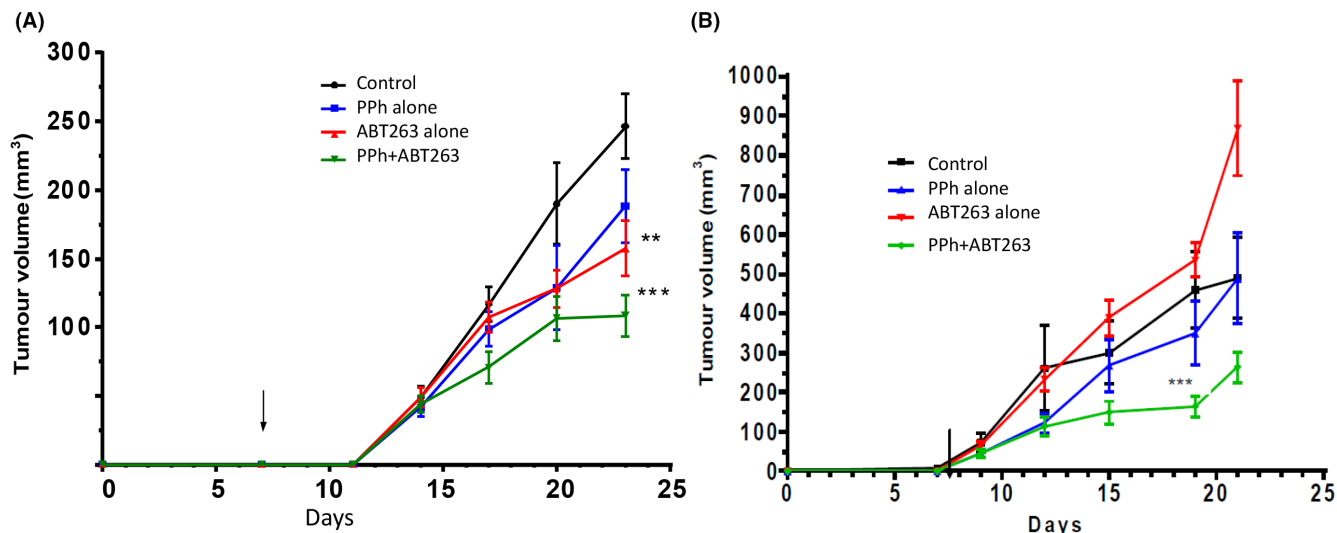
FIGURE 7 Effects of the Wnt inhibitor pyrvinium phosphate (PPh) and the pro-apoptotic Bcl-2 inhibitor ABT263 on the growth of SW620 tumor xenografts in (A) female mice; (B) male mice. The arrow shows the beginning of the treatment (day 7). The tumour volumes were measured twice a week, with the mice under general sedation. Each point represents the average of the volume of all the tumours within a group. Error bars = SEM, ***$p < 0.01$, **$p < 0.05$
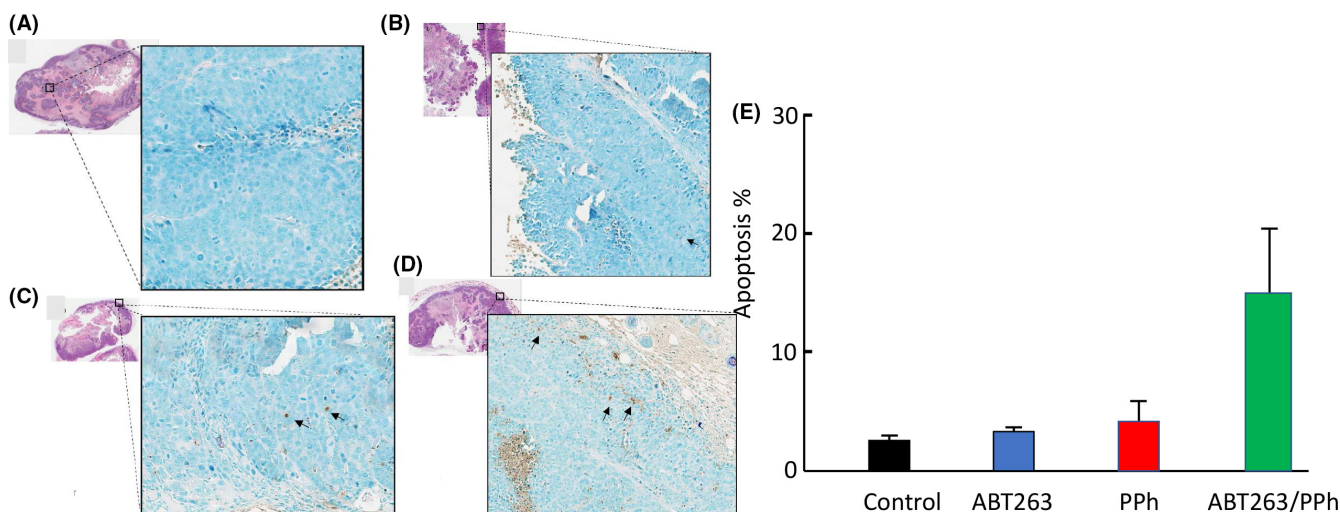

FIGURE 8 Detecting apoptotic cells (ApopTag IHC) in the SW620 tumour xenografts treated with (A) Vehicle, (B) Pyrvinium phosphate (PPh), (C) ABT263 or (D) PPh + ABT263. A total of 64 sections were stained using the Apoptag peroxidase Apoptosis Detection Kit (2 sections/tumour, 2 tumours/mouse, 4 mice/treatment group). Twenty fields per section were chosen at random within the glandular epithelium (excluding stromal component and necrotic/infiltrating inflammatory tissue). The apoptotic cells (brown) and the total number of cells were counted within each field and the percentage of apoptosis calculated per field. The average percentage of apoptosis was then calculated for each section and the results averaged per treatment group. The graph shows the average total percentage of apoptosis in treatment each group expressed as mean ± SD. The apparent increase in apoptosis induced by the combination treatment was not statistically significant in comparison to control ($p = 0.25$). Metamorph and GraphPad were used to perform the statistical analysis of the results: an unpaired t-test was performed to calculate the p values

differences could be very relevant to the drug sensitivity, as PP is postulated to act by preventing β- catenin activation,[31,32] while the loss of P53 function may upregulate the pro-life role Bcl-2.[33] It is also interesting to note that ABT263 appears to kill senescent cells preferentially[34,35] and that inhibition of the wnt pathway can trigger cell senescence[36–39]; so the synergistic cytotoxicity of the combination may be related to the pyrvinium induced senescent.

In the clinic, colon cancer patients[40] receiving targeted therapies usually relapse and die as a result of their cancer.[41] Although the adherent cell proliferation was blocked by the EGFR, Wnt and src inhibitors, the PI3K inhibitor was only active in the non-adherent cultures on two of the

cell lines and the src inhibitor was only active on two of the three cell lines in the non-adherent cultures. This suggests that integrin signalling is likely to modify responses to anti-cancer targeting drugs. Some consideration should be given to modulating integrin signalling[42,43] when trying to optimise the use of anti-cancer drugs for treating advanced cancers. One of our cell lines (LIM1899) was resistant to the pro-apoptotic drug ABT737. Now that there are several options for inhibiting the prosurvival pathways, other BH3 inhibitors should be tested for activity on colorectal cancer cell.[27,44]

In our experiments, PP was the most consistent and potent cytotoxic agent for the three cell lines, suggesting that inhibition of Wnt signalling should be a key component option for treating advanced cancers. In five CRC cell lines with APC mutations, treatment with PP was enhanced by the presence of the pro-apoptotic agent ABT737. For example, SW620 cells are sixfold more sensitive to PP in the presence of ABT737 or the PI3K inhibitor (LY294002).[45] In cells with *CTNNB*1 mutations, the cells were killed by PP, but there was no increased potency in the presence of the pro-apoptotic drug. Our results suggest, genetic screening[46] and or profiling for the expression levels of the Bcl-2 family members[40] will be helpful in predicting which patients might be the best responders to the dual Wnt inhibitor/pro-apoptotic drug treatment.

Despite successful reports which use either ABT737[47] or PP in mouse models[48], we found the formulation and injection leads to precipitation in the peritoneum, rendering these agents unsuitable for use in vivo. Fortunately, soluble analogues of both ABT737 (ABT263[11]) and PPh[8] were readily available and appeared to work just as well in vitro. By themselves neither PPh nor ABT263 reduced SW620 tumour growth; however, in combination, PPh with ABT263 showed low toxicity and was effective for reducing the growth of SW620 tumour xenografts. Where patients are suffering with advanced colorectal cancers and carrying *APC* mutations consideration should be given to dual drug treatments which target Wnt signalling and enhance apoptosis. It would be interesting to know which of the pro-apoptotic drugs is the best sensitiser for PPh and whether more specific/potent Wnt inhibitors might be even more effective. Both PPh and ABT263 can be administered orally (as in our experiments), but other routes of administrations should be tested. In our experiments, we have used cancer cell lines and mouse xenografts; in the next steps, the anticancer potential of the PPh/ABT263 combination could be tested in orthoptic colon cancer cell line grafts[49] , patient-derived colon cancer organoids,[50] mouse models of colon cancer[51] or patient-derived tumour xenografts.[52] Given the increased potency of PPh in the presence of a pro-apoptotic drug, it is conceivable that a dual drug treatment (Wnt inhibitor plus a pro-apoptotic drug) could also kill colon adenoma stem cells (most of which have APC mutations[53]) and thus act as a chemoprevention strategy for reducing the incidence of colorectal cancer.

CONFLICT OF INTEREST

The authors certify that they have NO affiliations with or involvement in any organisation or entity with any financial interest in the subject matter or materials discussed in this manuscript.

AUTHORS' CONTRIBUTIONS

SPC, FW, JW performed the experiments. SPC, FW, GL and AWB guided the design and interpretation of the experiments and wrote the first drafts of the manuscript. SPC, FW, MCF and AWB analysed and interpreted the data and contributed to the critical evaluation of the manuscript. All authors contributed to the final text of the manuscript.

ETHICS

All animal procedures were approved and carried out in accordance with the Animal Ethics Committees of the Ludwig Institute for Cancer Research, Melbourne Branch or WEHI.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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