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Porcupine and CBP/β-catenin are the most suitable targets for the inhibition of canonical Wnt signaling in colorectal carcinoma cell lines*

Porcupina i CBP/β-katenina – najlepsze cele molekularne do hamowania kanonicznej ścieżki Wnt w komórkach nowotworu jelita grubego

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Aim: The activation of canonical Wnt pathway is etiologically associated with the development of colorectal cancers. There are many possible molecular targets for the therapeutic abrogation of Wnt/β-catenin signaling. The aim of this study was to select the best molecular targets for the attenuation of β-catenin-dependent gene expression in colorectal cancer cell lines.

Material/Methods: An siRNA screen was used for the selection of the best molecular targets for the down-regulation of TCF/LEF-dependent GFP expression in HCT116 cells. The level of the expression of β-catenin target genes was analyzed by qPCR. The effect of the tested chemicals on cell migration, cell cycle and apoptosis was assessed by the wound healing assay, flow cytometric analysis of propidium iodide stained cells and flow cytometric analysis of the activity of caspases-3/7, respectively.

Results: Of the forty three genes which were tested in the screening stage, eight (*KDM6A, KDM1A, PORCN, KDM4C, CARM1, DVL1, CBP, KMT2A*) were selected as most promising. Small molecule inhibitors of these proteins (GSK-J4, GSK-LSD1, IWP-2, ML324, MS049, Dvl-PDZ Domain Inhibitor II, PRI-724, MM-102) were further used. The inhibitors of Porcupine (IWP-2) and CBP (PRI-724) were most effective in the down-regulation of the expression of β-catenin target genes and the induction of apoptosis in HCT116 cells, but showed weaker effects in DLD-1 cells.

Conclusions: The inhibition of CBP and Porcupine is the most effective in attenuating canonical Wnt signaling in colon cancer cells. Future studies should determine which factors affect the sensitivity towards these promising anti-cancer agents.

Keywords: **canonical Wnt signaling • colorectal cancer • Porcupine • CBP**

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INTRODUCTION

Colorectal cancers (CRC) are characterized by high morbidity and mortality rates and novel treatments are necessary to improve therapy outcomes. Among the molecular alterations detected in CRC cells, the aberrations in canonical Wnt signaling are one of the most frequent. It has been estimated that around 90% of all CRC patients show genetic changes affecting Wnt/β-catenin pathway [11]. In most cases, the activation of Wnt signaling is related to mutations in *APC* tumor suppressor gene but mutations in other genes, e.g. *FAT1*, *RNF43* or *CTNNB1*, are also detected. The up-regulation of the activity of canonical Wnt pathway results in increased cell survival, proliferation and migration. These effects are mediated by the transcriptional activation of the expression of key genes associated with tumorigenesis, e.g. *CCND1*, *c-MYC*, *BIRC5* (*survivin*), *MMP-2*, *MMP-7*. The therapeutic effects of the inhibition of canonical Wnt pathway have been shown in preclinical CRC models and several potential molecular targets for its down-regulation have been already identified, including Porcupine, Tankyrase and CBP [13, 15]. However, the complex and multistage nature of canonical Wnt signaling offers many possibilities for its attenuation. The pathway may be blocked at different levels [18, 22], starting with Wnt ligands and proteins responsible for their secretion (Porcupine), Frizzled/LRP receptors, Dishevelled and its regulators (PAR-1, PIP5KIβ, CKIε), through negative regulators of β-catenin inhibitory complex (CIP2A, SET, tankyrase), or proteins which form a transcriptional complex with β-catenin (TCF/LEF transcription factors and other proteins – BCL9, PYGO, XIAP, TNIK). The induction of the expression of genes regulated by β-catenin requires the activity of proteins which are responsible for the epigenetic modification of histones, including histone methyltransferases (CARM1, hSETD1A, MLL1/2, NSD1), histone demethylases (KDM1A, KDM4B/C, KDM6A/B), histone acetyltransferases (CBP) or epigenetic effector proteins (CHD4, TAF3).

The aim of this study was to identify protein targets whose inhibition leads to the most significant decrease in the level of Wnt signaling in colorectal cancer cell lines. The most promising protein targets were selected based on the evaluation of the effects of the siRNA-mediated silencing of Wnt ligands, Porcupine, Frizzled/LRP receptors, Dishevelled, PAR-1, PIP5KIβ, CKIε, CIP2A, SET, tankyrase, TCF/LEF transcription factors, regulatory proteins – BCL9, PYGO, XIAP, TNIK, and epigenetic modifiers – CARM1, hSETD1A, MLL1/2, NSD1, KDM1A, KDM4B/C, KDM6A/B, CBP, CHD4, TAF3 on β-catenin-dependent GFP expression. The inhibition of the selected proteins was further examined functionally using small molecule chemicals. In conclusion, the inhibition of Porcupine and blocking the interaction between β-catenin and CBP were found most effective in attenuating β-catenin-mediated gene expression and in modulating migration and apoptosis in colorectal cancer cells.

MATERIAL AND METHODS

Cell lines and culture

Colorectal carcinoma DLD-1 and HCT116 cell lines were purchased from ECACC (UK), while the immortalized keratinocyte HaCaT cell line was provided by Cell Line Services (Germany). The cells were grown in DMEM (Biowest, France) containing 10% FBS (EURx, Poland) and 1% antibiotics solution (penicillin & streptomycin, Biowest, France) in standard conditions (37°C, 5% CO_2, 95% humidity).

TCF/LEF-dependent reporter assay

In the screening stage, HCT116 cells (2×10^4 per well) were seeded into black 96-well plates and immediately transfected with individual siRNA duplexes (40 nM, Qiagen, Germany) using jetPRIME transfection reagent (Polyplus, France), according to manufacturer's recommendations. Two different siRNA duplexes were used separately for the knockdown of each gene. The list of the genes is presented in Table 1. Non-targeting siRNA was used as a negative control. Moreover, a siRNA molecule which induces cell death was used as a positive control for transfection efficiency evaluation. siRNA targeted against *CTNNB1*, which encodes β-catenin, was used as a positive control of the biological effect on GFP production. After 24 hours, cells were transfected with 150 ng TCF/LEF-dependent reporter or negative control plasmids (Cignal Wnt Reporter Assay Kit, GFP, Qiagen, Germany) using ViaFect transfection reagent (Promega, USA). After another 24 hours, recombinant Wnt3a protein (50 ng, R&D, USA) was added to each well in order to enhance the activity of canonical Wnt pathway (this step was omitted in the case of siRNA duplexes targeting Wnt ligands or Porcupine). The intensity of fluorescence of the accumulated GFP was measured in a plate reader after subsequent 24 hours (ex – 480 nm, em – 515 nm). Five independent experiments were performed for each siRNA.

Chemicals and cell viability assay

Small molecule compounds (GSK-J4, GSK-LSD1, IWP-2, ML324, MS049 (Sigma-Aldrich, USA), Dvl-PDZ Domain Inhibitor II (Calbiochem/Merck, USA), PRI-724, MM-102 (Selleck Chemicals, USA)) were used for the selective inhibition of potential target proteins (KDM6, KDM1A, Porcupine, KDM4, CARM1, Dishevelled, CBP/β-catenin, KMT2A, respectively). The effect of the studied chemi-

Table 1. The list of the genes targeted by siRNA in the screening stage

No	Target gene	Function	No	Target gene	Function
1	PORCN	O-acyltransferase (secretion of Wnt ligands)	23	LEF1	Transcription factor
2	WNT1	Ligand	24	BCL9	Co-activator of β-catenin-dependent transcription
3	WNT2B	Ligand	25	PYGO1	Co-activator of β-catenin-dependent transcription
4	WNT3A	Ligand	26	PYGO2	Co-activator of β-catenin-dependent transcription
5	WNT6	Ligand	27	XIAP	Co-activator of β-catenin-dependent transcription
6	WNT10B	Ligand	28	TNIK	Co-activator of β-catenin-dependent transcription
7	FZD2	Receptor	29	KIAA0101 = p15 (PAF)	Factor promoting silencing of Wnt antagonists
8	FZD4	Receptor	30	KDM1A = LSD1	Histone demethylase (H3K4me1/2, H3K9me2)
9	FZD5	Receptor	31	KDM4B = JMJD2B	Histone demethylase (H3K9me3)
10	LRP5	Co-receptor	32	KDM4C = JMJD2C	Histone demethylase (H3K9me3, H3K36me3)
11	LRP6	Co-receptor	33	KDM6A	Histone demethylase (H3K27me2/3)
12	DVL1	Membrane Wnt signaling activator	34	KDM6B = JMJD3	Histone demethylase (H3K27me2/3)
13	DVL2	Membrane Wnt signaling activator	35	NSD1	Histone methyltransferase (H3K36, H4K20)
14	DVL3	Membrane Wnt signaling activator	36	SETD1A	Histone methyltransferase (H3K4me1/2/3)
15	F2R/PAR-1	Kinase (activation of Dvl)	37	EZH2	Histone methyltransferase (H3K9, H3K27me1/2/3)
16	PIP5KIB	Kinase (activation of LRP)	38	CARM1	Histone methyltransferase (H3R17)
17	CSNK1E	Kinase (activation of LRP)	39	KMT2A = MLL	Histone methyltransferase (H3K4)
18	KIAA1524 = CIP2	Inhibitor of PP2A	40	EHMT2	Histone methyltransferase (H3K9me1/2, K3K27me)
19	SET	Inhibitor of PP2A	41	CREBBP = CBP	(Histone) acetyltransferase
20	TNKS	Antagonist of Axin	42	CHD4	Histone deacetylase
21	TCF1 = TCF7	Transcription factor	43	TAF3	Transcription promoting factor
22	TCF4 = TCF7L2	Transcription factor	44	CTNNB1	Gene encoding β-catenin

cals on the viability of DLD-1, HCT116 and HaCaT cells was assessed by resazurin assay. Cells (1×10^4/well) were seeded into black 96-well plates and fresh medium containing different concentrations of the compounds was added on the following day. Control cells were treated with the vehicle (DMSO). After 24 hours, wells were washed with PBS buffer and fresh medium containing 1 μg/ml resazurin was added. Fluorescence was measured after subsequent 2 hours (ex – 530 nm, em – 590 nm). Two independent experiments were performed with four separate replicates per experiment.

The isolation of RNA and quantitative real-time PCR

The efficiency of gene silencing by selected siRNA duplexes and their effect on the expression of Wnt pathway target genes (*Axin2, MMP7*) was validated by real-time PCR, as previously described [17]. The effect of the small molecule inhibitors on the expression of Wnt pathway target genes (*Axin2, CCND1, c-MYC, MMP7, BIRC5/survivin*) was also studied by qPCR. Cells were seeded into wells of a 24-well plate and either transfected with individual siRNA duplexes

using jetPRIME transfection reagent or treated with the chemicals, as described in previous sections. Total RNA was isolated using Universal RNA Purification Kit (EURx, Poland). RNA samples were subjected to reverse transcription using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Gene expression was analyzed by qPCR using HOT FIREPol EvaGreen qPCR Mix Plus (Solis Biodyne, Estonia) and LightCycler 96 (Roche, Germany). The relative level of transcript was calculated using the ΔΔCt method. The mean expression of two reference genes – *PBGD* and *TBP* was used for data normalization. Two independent experiments were performed.

Preparation of protein extracts and Western blot assay

Cells (2.5×10^5 cells per well) were seeded in 6-well plates and fresh medium containing the indicated concentrations of IWP-2 or PRI-724 was added on the following day. Control cells were treated with DMSO. After 24 hours, protein extracts were prepared by cell lysis using Laemmli buffer, which was immediately followed by protein denaturation by heating at 98°C for 15 minutes. Protein concentration was assessed using the Pierce BCA Protein Assay Kit (Thermo Scientific, USA). The content of Axin2 protein was analyzed by Western blot. Protein extracts (50 μg) were separated on 7.5% SDS-PAGE gels (Bio-Rad, USA) and proteins were transferred onto nitrocellulose membrane. Subsequently, the membranes were blocked with 10% skimmed milk and then incubated with the primary rabbit polyclonal antibody directed against Axin2 (Santa Cruz Biotechnology, USA). The analysis of β-actin content served as a loading control. After washing, the membranes were incubated with alkaline phosphatase-labeled anti-rabbit secondary IgG antibody (Santa Cruz Biotechnology, USA) and stained using the BCIP/NBT AP Conjugate Substrate Kit (Bio-Rad, USA). The determination of the relative intensity of the bands was performed with the Quantity One software and the values were calculated as relative absorbance units (RQ) per mg protein.

Cell migration assay

Cell migration was assessed by the wound healing assay. Cells were seeded into 24-well plates and were grown overnight to reach confluency. Then, cell layers were scratched using a 10 μl tip, wells were rinsed with PBS buffer to remove detached cells, and fresh medium containing the tested compounds was added. Representative microscopic photographs (JuLI FL, NanoEntek, South Korea) of scratch areas were taken immediately after the addition of compounds and 24 hours later. Cell migration rate was calculated by the comparison of the change in the surface covered by cells between control (DMSO) and treated cells, as previously described [17].

The analysis of cell cycle distribution

For cell cycle analysis, cells were stained with propidium iodide using The Muse Cell Cycle Kit, and flow cytometric detection was performed with Muse Cell Analyzer (Merck, Germany), according to manufacturer's protocol. Briefly, cells were seeded (10^5 cells per well) in 12-well plates and, on the following day, the medium was replaced with fresh medium containing the indicated concentrations of the analyzed chemicals. Topotecan was used as a positive control. Cells were incubated in the presence of the compounds for 48 hours and subsequently collected by trypsinization. Cells were fixed in cold 70% ethanol and samples were stored at –20°C. Then, fixed cells were collected and stained with propidium iodide and subsequently analyzed by flow cytometry. The experiment was repeated twice. Data were analyzed by Muse Analysis software.

The analysis of apoptosis

The effect on apoptosis was assessed by the combined measurement of the activity of caspases 3 and 7 and the level of staining with 7-AAD, what allowed for the discrimination between early and late apoptotic cells. The analysis was performed by flow cytometry using The Muse Caspase-3/7 Kit and Muse Cell Analyzer (Merck, Germany), according to manufacturer's protocol. Cells were seeded (10^5 cells per well) in 12-well plates and, on the following day, the medium was replaced with fresh medium containing the indicated concentrations of the analyzed chemicals. Topotecan was used as a positive control. Cells were incubated in the presence of the compounds for 48 hours and subsequently collected by trypsinization. Then, cells were incubated in the presence of a labeled DEVD peptide, whose caspase-dependent cleavage released a DNA-binding fluorescent dye. After subsequent staining with 7-AAD, cells were analyzed by flow cytometry. The experiment was repeated twice. Data were analyzed by Muse Analysis software.

Statistical analysis

The Student's t-test was used for the analysis of the significance of the differences between the experimental groups and their respective controls, with $p \leq 0.05$ considered as significant. The analysis was performed using STATISTICA 10 software.

RESULTS

Screening for the best targets for the down-regulation of Wnt signaling

HCT116 cells were transfected individually with siRNAs against each of the studied forty three genes and with the reporter plasmid containing GFP sequence under the control of TCF/LEF transcription factor. The activity of the pathway was enhanced by the addition of Wnt3a. The modulatory effects exerted by individual siRNAs were assessed by comparing the level of GFP fluorescence signal with the level detected for control cells, which were transfected with a non-targeting siRNA molecule (Fig. 1a). The silencing of β-catenin with both siRNA duplexes against *CTNNB1* led to an almost com-

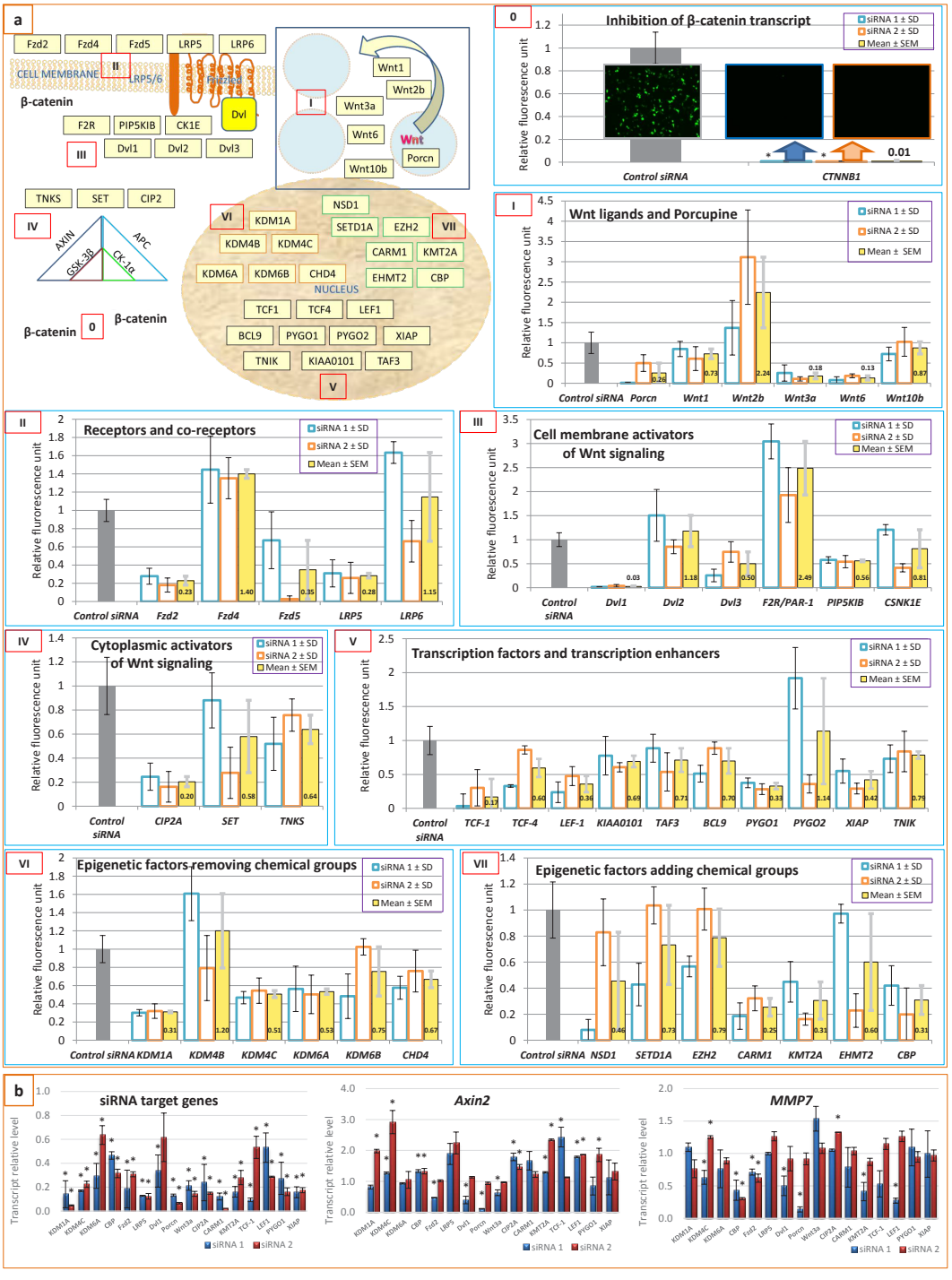

Fig. 1. The results of the siRNA screen for the best targets for the down-regulation of Wnt signaling. **Panel a –** HCT116 cells were transfected with siRNA duplexes targeting genes related to the pathway, which are presented in the diagram. The genes are sub-divided into categories (denoted with roman numerals) related to the stages of Wnt signal transduction. Two different siRNA duplexes were used for the silencing of each target gene. The level of TCF/LEF-dependent expression of GFP was spectrofluorimetrically measured 48 hours after transfection. Five independent replicates were conducted for each siRNA. Cells transfected with non-targeting control siRNA were used for comparison, while the silencing of *CTNNB1* (encoding β-catenin) served as a positive control. The results for control siRNAs, together with exemplary images showing microscopic detection of GFP presence in cells transfected with the reporter plasmid and either non-targeting or anti-*CTNNB1* siRNA, are presented in graph 0. The level of fluorescence in control cells transfected with non-targeting siRNA was expressed as equal 1 and relative fold difference for targeting siRNAs was then calculated. The results of relative GFP level assessment are presented as mean values ±SD independently for each siRNA duplex and mean values ±SEM collectively for two siRNA duplexes targeting each gene. The results are presented separately for each category of target genes (graphs I-VII in panel a). **Panel b –** The results of the qPCR analysis of the efficiency of the silencing of target genes and the modulation of the transcript level of genes transcriptionally regulated by β-catenin (*Axin2*, *MMP7*) by siRNA duplexes. Mean values ±SD from two independent experiments with three replicates per qPCR experiment are shown. Asterisk above bar denotes statistically significant changes, $p \leq 0.05$

plete abrogation of TCF/LEF-dependent GFP production, what indicated that the screening assay setup was suitable for the search of targets for the inhibition of canonical Wnt signaling. Genes whose silencing by both siRNA duplexes led to a significant downregulation of GFP production (by at least 40%) were selected for further investigation (*CARM1, CBP, CIP2A, DVL1, FZD2, KDM1A, KDM4C, KDM6A, KMT2A, LEF1, LRP5, PORCN, PYGO1, TCF1, WNT3A, XIAP*). The efficiency of knockdown of the genes targeted by these selected siRNAs was assessed by qPCR (Fig. 1b). In almost all cases, transfection with siRNA led to effective gene silencing (reduction in target transcript level by at least 60%). Then, the level of expression of genes controlled by β-catenin (*Axin2*, *MMP7*) was measured by qPCR. The level of *Axin2* expression was significantly reduced by siRNA-mediated silencing of *FZD2, DVL1, PORCN, WNT3A*, while the level of *MMP7* expression was significantly reduced by the silencing of *KDM4C, CBP, FZD2, DVL1, PORCN, KMT2A, LEF1*. Based on these results, eight genes were selected (*KDM6A, KDM1A, PORCN, KDM4C, CARM1, DVL1, CBP, KMT2A*), whose silencing was associated with the most significant changes in TCF/LEF-dependent GFP production and/or the level of expression of *Axin2* or *MMP7*.

Cell viability analysis

Small molecule inhibitors (GSK-J4, GSK-LSD1, IWP-2, ML324, MS049, Dvl-PDZ Domain Inhibitor II, PRI-724, MM-102) were used for the inhibition of the activity of the chosen eight protein targets (KDM6, KDM1A, Porcupine, KDM4, CARM1, Dishevelled, CBP/β-catenin, KMT2A, respectively). DLD-1 and HCT116 colorectal cancer cells and HaCaT keratinocytes were grown in the presence of the chemicals for 24 hours and the effect on cell viability was assessed by resazurin assay (Fig. 2). The inhibitors of

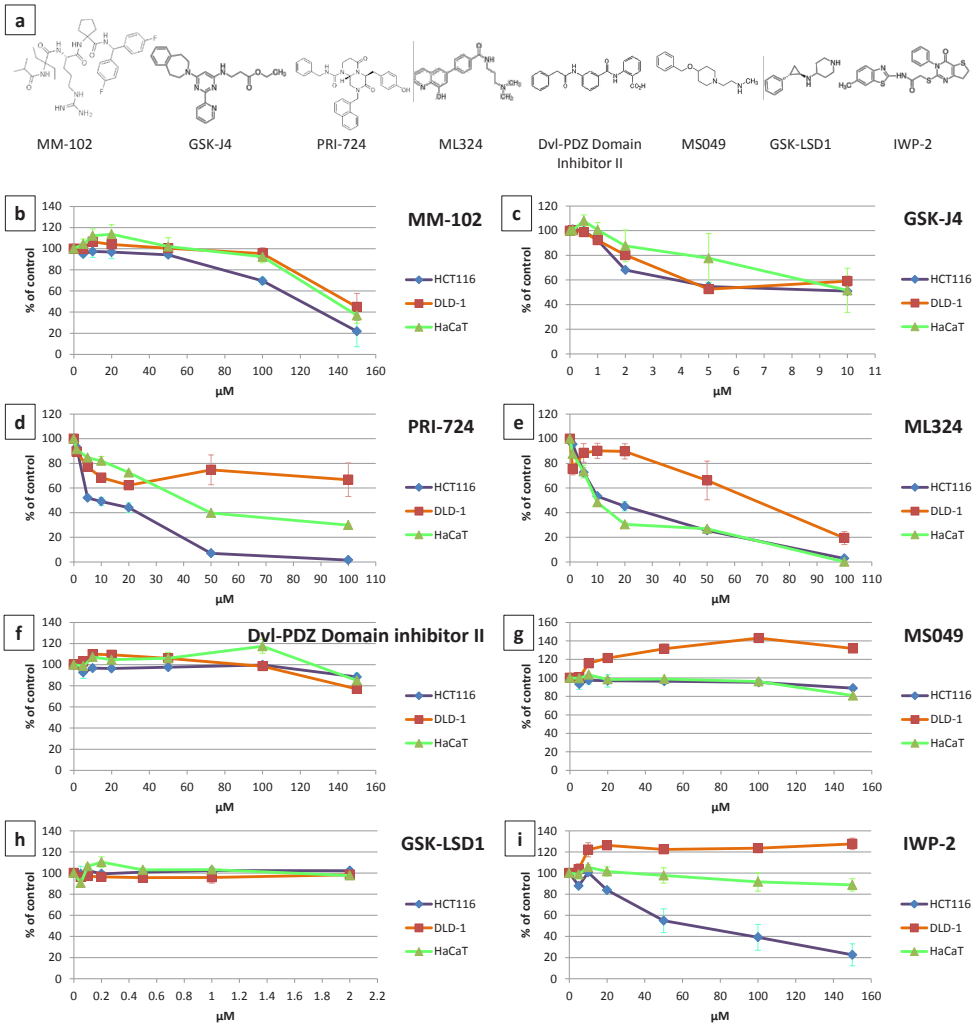

Fig. 2. The results of the analysis of the effect of the studied chemicals on cell viability by resazurin assay. **Panel a –** The chemical structure of the studied compounds. **Panels b–i HCT116,** DLD-1 and HaCaT cells were treated with the compounds at various concentrations for 24 hours. Control cells were treated with vehicle (DMSO). Two independent experiments with four replicates per experiment were performed. Relative changes in cell viability are shown in the graphs as mean values ±SD

Dishevelled and CARM1 were not cytotoxic at concentrations up to 150 µM. MM-102 (inhibitor of KMT2A) showed cytotoxic effects in all three cell lines only at the highest concentration (150 µM). Interestingly, both PRI-724 and IWP-2 (inhibitors of CBP/β-catenin and Porcupine, respectively) showed differential effects in the studied cell lines, leading to strongest reduction in cell viability in HCT116 cells, but only weak reduction in viability (PRI-724) or lack of effects (IWP-2) in DLD-1 cells. Based on these results, subtoxic concentrations of the chemicals were selected for further stages of the study.

The effect of the tested compounds on the expression of β-catenin target genes

In the next step, we evaluated the effect of the small molecule inhibitors on the level of expression of *Axin2, CCND1, c-MYC, MMP7* and *BIRC5/survivin* genes, all of which are transcriptionally regulated by β-catenin. The results are presented in Fig. 3. The changes in the level of expression of *MMP7* were not assessed in DLD-1 cells because of intrinsic lack of expression of this gene in this cell line. IWP-2 led to the strongest reduction in the level

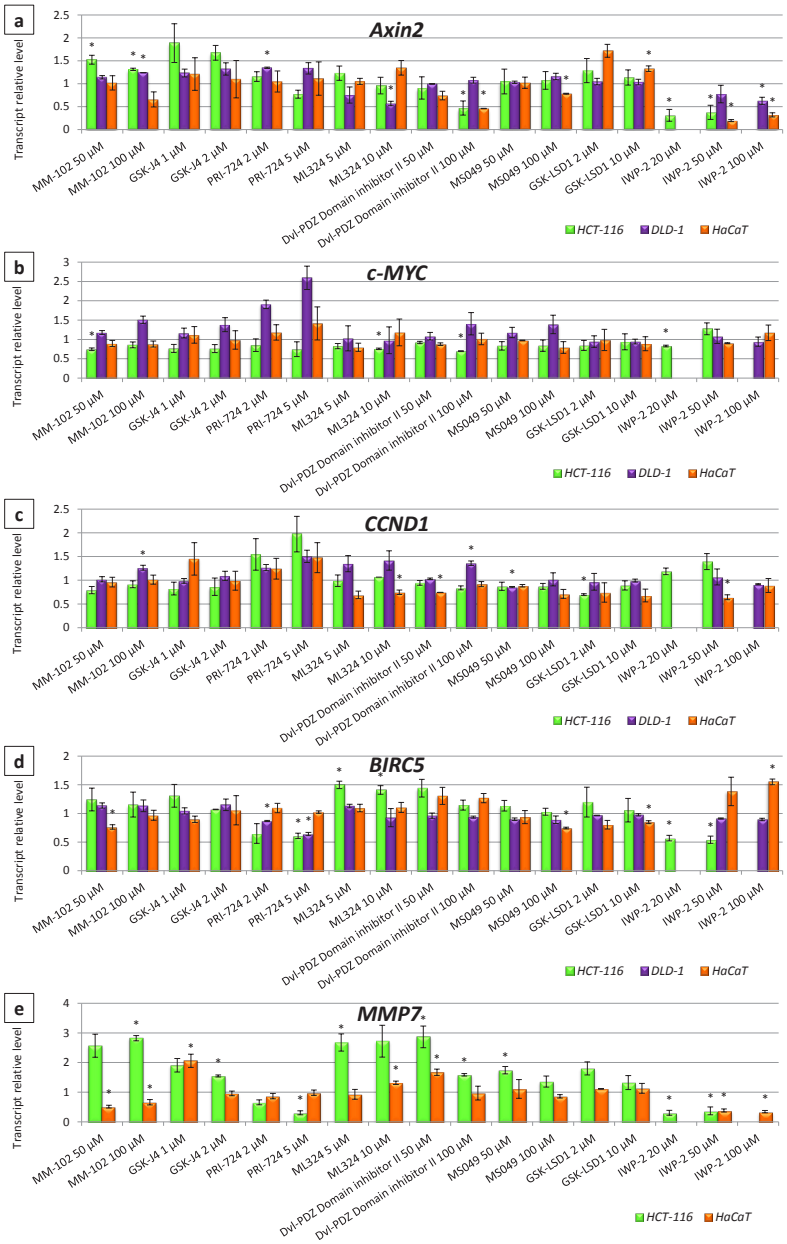

Fig. 3. The effect of the studied chemicals on the relative transcript level of genes transcriptionally regulated by β-catenin in HCT116, DLD-1 and HaCaT cells. Mean values ±SD from two independent experiments with three replicates per experiment are shown. Asterisk above bar denotes statistically significant changes, $p \leq 0.05$

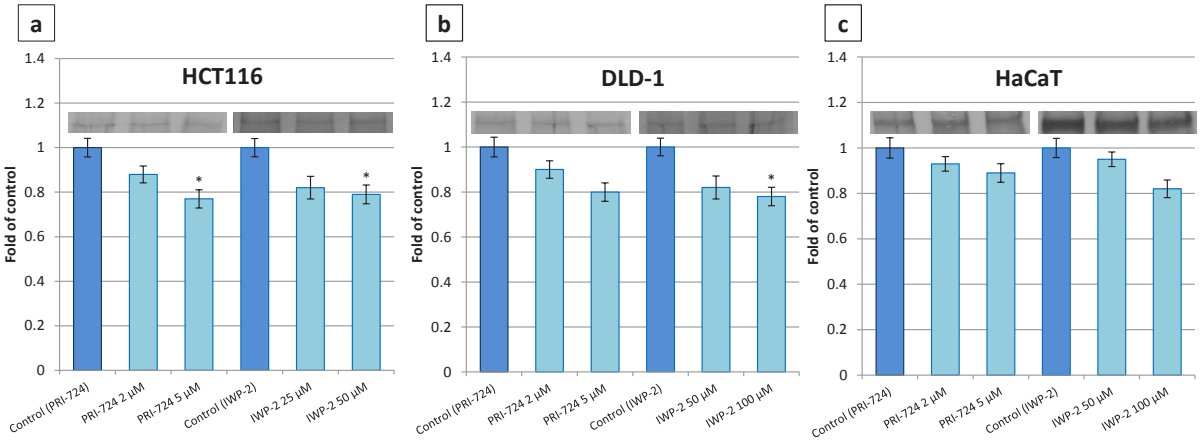

Fig. 4. The effect of PRI-724 and IWP-2 on the level of Axin2 protein in cell extracts after 24 hour incubation. Mean values ±SD from two independent experiments are shown in the graphs. Representative electrophoregrams are presented above corresponding bars. Asterisk above bar denotes statistically significant changes, $p \leq 0.05$

of expression of *Axin2* and *MMP7* in all the tested cell lines. Additionally, IWP-2 reduced the transcript level of *BIRC5* in HCT116 cells while it induced *BIRC5* expression in control HaCaT cells. The inhibitor of Dishevelled reduced the transcript level of *Axin2* in HCT116 and HaCaT cells, whereas ML-324 reduced the level of *Axin2* expression in DLD-1 cells. Both inhibitors also reduced the level of *c-MYC* expression in HCT116 cells and the level of *CCND1* expression in HaCaT cells. Although PRI-724 did not significantly affect the level of expression of *Axin2*, it potently reduced the transcript level of *BIRC5* in both colorectal cancer cell lines and also down-regulated the expression of *MMP7* in HCT116 cells. The other compounds (MM-102, GSK-J4, MS049, GSK-LSD1) did not show any strong activity in cancer cells.

Based on these results, we chose two compounds – IWP-2 and PRI-724, which showed the most promising effects on β-catenin-dependent transcription, for further studies. We assessed the effect of these chemicals on the protein content of Axin2 in the studied cell lines (Fig. 4). Both compounds tended to show slightly stronger reduction

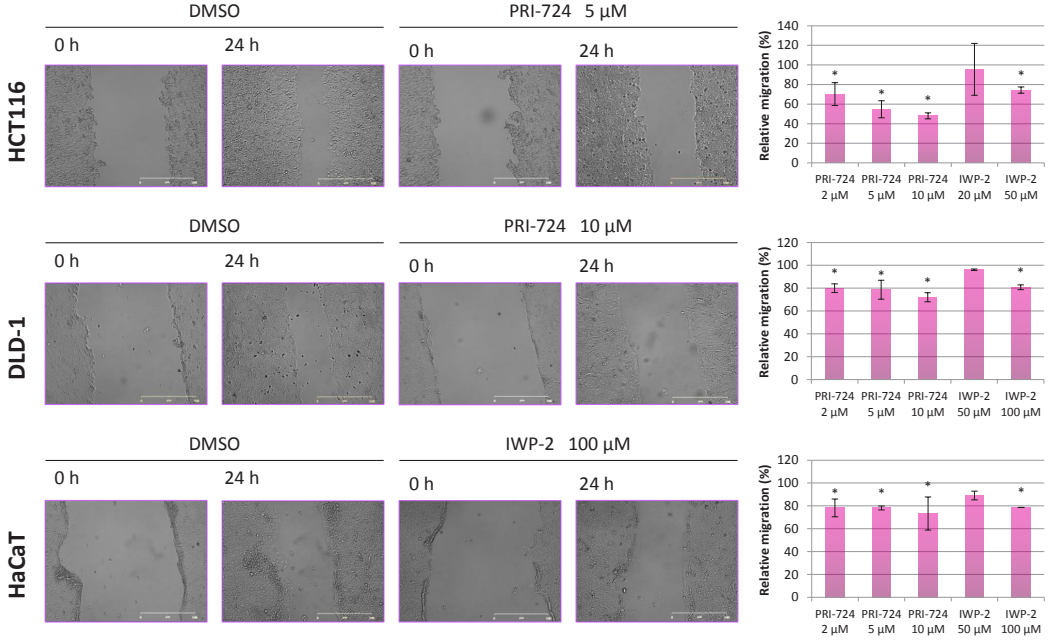

Fig. 5. The effect of PRI-724 and IWP-2 on the relative migration of HCT116, DLD-1 and HaCaT cells. Exemplary microscopic images of the gap, which were taken at the beginning and after 24 hours of incubation with the compounds, are shown on the left. Corresponding graphs show mean values ±SD from two independent experiments with three replicates per experiment. Asterisk above bar denotes statistically significant changes, $p \leq 0.05$

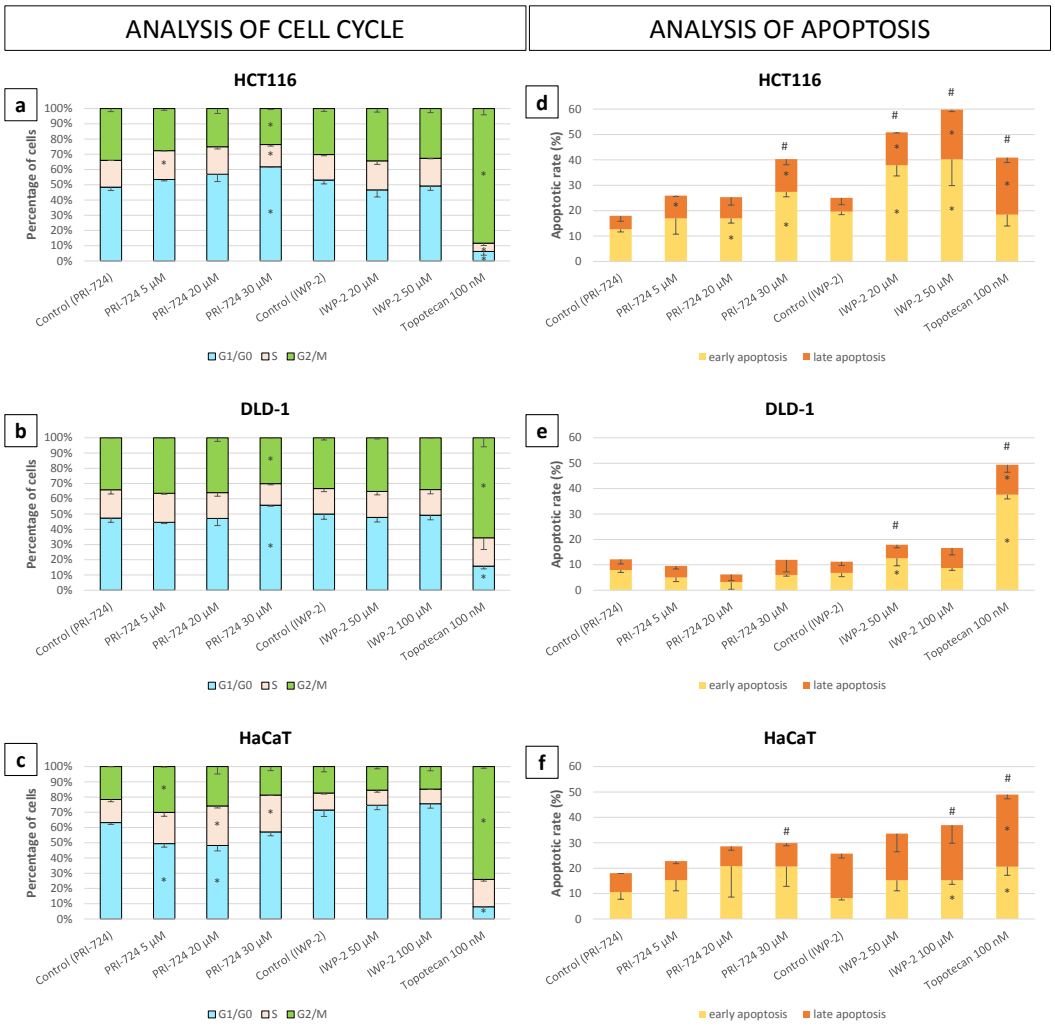

Fig. 6. The effect of PRI-724 and IWP-2 on cell cycle distribution (panels a-c) and apoptosis (panels d-f) after 48 hour incubation in HCT116, DLD-1 and HaCaT cells. Cells treated with topotecan were used as a positive control. Mean values ±SD from two independent experiments are shown in the graphs. Asterisk denotes statistically significant changes, $p \leq 0.05$. Hash above bar denotes statistically significant changes in total apoptotic rate, $p \leq 0.05$

in Axin2 content in cancer cells than in control HaCaT cells, but these differences in response between cell lines were not statistically significant.

The effect of PRI-724 and IWP-2 on cell migration, cell cycle and apoptosis

The modulation of canonical Wnt signaling affects cell migration capacity, therefore we assessed by the wound healing assay whether such effects can by exerted by the treatment of cells with IWP-2 or PRI-724 (Fig. 5). HCT116 cells responded dose-dependently to low concentrations of PRI-724 with significant reduction in cell migration. On the other hand, this compound showed weaker effects in DLD-1 and HaCaT cells. IWP-2 showed statistically significant but moderate reduction in cell migration in all three studied cell lines.

In the last step, we evaluated whether the chemicals alter cell cycle distribution or affect apoptosis in the cell lines (Fig. 6). Topotecan was used as a positive control leading to cell cycle arrest in G2/M and strong induction of apoptosis. Neither of the two inhibitors showed strong effects on cell cycle progression, although PRI-724 led to statistically significant, but weak, increase in the percentage of cells in the S phase and the reduction in the percentage of cells in G1 phase in HaCaT keratinocytes. In contrast, this compound tended to increase the number of cells in G1 phase and decrease the number of cells in G2/M phase in HCT116 colorectal cancer cells. Importantly, HCT116 cells were sensitive to the induction of apoptosis by both inhibitors and IWP-2 was more potent in the induction of apoptosis in this cell line. Neither of the two inhibitors was able to induce apoptotic response in DLD-1 cells. On the other hand, HaCaT cells showed weak to moderate induction of apoptosis by high concentrations of both PRI-724 and IWP-2.

DISCUSSION

The dysregulation of Wnt signaling significantly contributes not only to the loss of control over the cell cycle and to the impairment of apoptosis, which lead to increased cell survival, but also to the enhancement of cell migration and, importantly, to the propagation of cancer stem cells. There is high prevalence of the aberrations of canonical Wnt signaling in colorectal cancers, what makes this pathway a good therapeutic target. The complexity of this multi-stage pathway offers many possibilities for its down-regulation. The aim of this study was to identify the proteins which can be considered as suitable molecular targets for Wnt pathway inhibition and to evaluate the effects of their abrogation by siRNA or small molecular inhibitors on β-catenin-dependent transcription. In the initial siRNA-based screen, which tested over forty genes, a group of sixteen genes was selected based on the effective down-regulation of the expression of GFP. Among these proteins, a few are related to the initiation of signal transduction. Unsurprisingly, both the maturation of Wnt ligands mediated by the catalytic activity of Porcupine and the action of some of the Wnt ligands appeared as good inhibitory strategies. In this regard, the silencing of Wnt3a and Wnt6 resulted in decreased production of GFP, while the silencing of Wnt2b led to opposite effects. In fact, the relevance of Wnt2b for canonical Wnt pathway has remained ambiguous [19], however our results suggest that this protein acts antagonistically towards Wnt/β-catenin pathway. Also, the inhibition of several proteins associated with cell membrane receptors for Wnt ligands (Fzd2, LRP5, Dvl1) showed high efficacy. Moreover, the silencing of CIP2A resulted in strong reduction in GFP level, however this protein is not easily druggable. Nevertheless, the CIP2A/PP2A system seems to be important for the cytoplasmic regulation of the activation of β-catenin.

The siRNA screen pointed to many nuclear proteins (LEF1, TCF1, CBP, CARM1, KMT2A, KDM1A, KDM4C, KDM6A, PYGO1, XIAP) as potentially good targets for the inhibition of canonical Wnt signaling. Indeed, once β-catenin is activated, it translocates to the nucleus where it displaces the Groucho/TLE co-repressor from its complex with the TCF/LEF transcription factors and a final transcriptional complex with BCL9 and PYGO proteins can be formed. This leads to the induction of the expression of β-catenin target genes. XIAP monoubiquitylates Groucho inhibitory protein and thus disrupts its binding to TCF/LEF [8]. Importantly, the β-catenin-mediated enhancement of gene transcription requires the introduction of changes in the profile of histone marks, what requires the activity of epigenetic modifiers, such as histone methyltransferases, histone demethylases or histone acetyltransferases. The β-catenin-driven activation of the expression of genes associated with cell proliferation requires the presence of CBP acetyltransferase [20]. Moreover, the interaction of β-catenin with both CBP and KMT2A/MLL histone methyltransferase, which catalyzes the trimethylation of H3K4, was necessary for the regulation of stem cell-associated genes in tumor propagating cells [23]. Also, the depletion of CARM1 significantly reduced the expression of β-catenin-dependent genes in colorectal cancer cells [16]. Additionally, the MLL methyltransferase interacts with the KDM4B (JMJD2B) demethylase, which erases H3K9me3, and thus facilitates the expression of β-catenin target genes. The activity of KDM4B was necessary for β-catenin-driven expression of genes associated with epithelial to mesenchymal transition in gastric cells [26]. The inhibition of KDM4B attenuated the nuclear accumulation of β-catenin and decreased its transcriptional activity. Similarly, the down-regulation of KDM4B reduced the expression of β-catenin target genes, such as *c-MYC* or *CCND1*, while its over-expression supported β-catenin-mediated gene transcription in colorectal cancer cells [3, 4]. Interestingly, our screening stage has shown that the inhibition of KDM4C has greater capacity for the down-regulation of β-catenin-dependent transcription than the inhibition of KDM4B. It has been shown that KDM4C stimulates the growth of colon cancer cells [12] and mediates sphere-forming capacity in colorectal cancer cells by affecting Wnt and Notch signaling pathways [25]. KDM1A/LSD1 has also been shown to stimulate Wnt signaling in colorectal cancer cells by attenuating the level of DKK1 pathway antagonist [10].

In the next step, we narrowed down the set of potential targets to the most promising eight proteins (Porcupine, Dishevelled, KDM1A, KDM4, KDM6, CBP/β-catenin, CARM1, KMT2A), for which selective small molecule inhibitors were available. We used these chemicals to further verify the effect on gene expression. All the compounds showed at least weak down-regulation of at least one of the five tested β-catenin target genes, however the inhibitors of KMT2A/MLL, KDM1A, KDM6 and CARM1 (MM-102, GSK-LSD1, GSK-J4 and MS049, respectively) were least effective. With the exception of GSK-LSD1, the effect of these compounds on the activation of canonical Wnt signaling has not been studied, so far. GSK-LSD1 was found to down-regulate the Wnt/β-catenin pathway in myoepithelial oral tumor cells [1]. On the other hand, a different KDM1A inhibitor, CBB1003, decreased the transcriptional activity of β-catenin in colorectal cancer cells. This, however, required the use of a very high concentration (250 μM) of the chemical [9]. The inhibitor of KDM4, ML324, showed moderate down-regulation of gene expression in some cases, similarly to the inhibitor of Dishevelled. The inhibitor of the PDZ domain of Dishevelled proteins has been shown to inhibit canonical Wnt signaling in prostate cancer cells [7].

The inhibitors of Porcupine (IWP-2) and of the interaction between CBP and β-catenin (PRI-724) were the most potent when it comes to the down-regulation of the expression of β-catenin target genes in the studied cell lines. The pharmacological inhibition of these proteins has already been shown to possess anti-cancer effects in previous studies. Unfortunately, the compounds did not show selectivity towards the studied colon cancer cells

and they affected HaCaT keratinocytes as well. Both of these inhibitors significantly reduced the viability of HCT116 cells, while leaving DLD-1 cells largely unaffected. This cell line was generally more resistant to the action of pharmacological agents. It has to be noted that the activity of canonical Wnt pathway is around 15 times higher in DLD-1 than in HCT116 cells [2]. PRI-724 weakly affected the cell cycle while IWP-2 did not show any effect on cell cycle distribution. In a recent study, the inhibition of Porcupine was found not to impact the cell cycle [5]. On the other hand, the compounds showed significant induction of apoptosis in HCT116, but not in DLD-1, cells. This may be associated, at least in part, with the inhibition of the expression of anti-apoptotic survivin. PRI-724 was also more potent in reducing cell migration, especially in HCT116 cells. All these results show, that while IWP-2 more potently affected β-catenin-dependent gene transcription, PRI-724 was more effective in functional assays. This may also suggest that the pharmacological effects of PRI-724 are partly Wnt-independent. In this regard, ICG-001, of which PRI-724 is a derivative, has been shown to act via Wnt-independent mechanisms in glioma cells [24]. On the other hand, a difference in the profile of gene expression changes was observed between pancreatic and colon cancer cells and Wnt signaling was significantly affected by ICG-001 treatment only in the latter [6], suggesting that Wnt-independent mechanisms are more probable in cells characterized by lower level of activation of Wnt/β-catenin signaling. Additionally, ICG-001 reduced β-catenin-mediated gene expression in colorectal cancer cells, but these effects did not correlate with cytotoxic effects [2]. Another derivative of ICG-001, IC-2, also reduced the transcriptional activity of β-catenin in DLD-1 cells [21]. All these data suggest that CBP is a very promising target for the therapy of colorectal cancers. In fact, PRI-724 is already tested in second phase trials in patients with solid tumors [20]. The two colon cancer cell lines, which were used in this study, show a difference in the mechanism of the activation of Wnt signaling. HCT116 cells bear a heterozygotic mutation in *CTNNB1*, while DLD-1 cells show the inactivation of *APC*, a component of the β-catenin destruction complex. This may explain the lack of response of DLD-1 to the treatment with IWP-2, the inhibitor of Porcupine, since the downstream activation of the pathway cannot be affected by Wnt ligands, unless they would act antagonistically via non-canonical pathways. IWP-2 reduced Wnt signaling in gastric cancer cells [14] similarly as in HCT116 cells in this study. However, this compound is not likely to be effective in cells showing downstream activation of this pathway. Since the up-regulation of canonical Wnt signaling is related to *APC* mutation in most colorectal patients, it suggests that this inhibitor may be potentially useful only in a narrow subgroup of CRC patients.

Altogether, we have shown that the inhibition of CBP and Porcupine is the most effective in down-regulating canonical Wnt signaling in colon cancer cells. Future studies should determine the exact factors affecting the sensitivity towards these promising pharmacological anti-cancer agents.

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