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Inhibition of the Wnt/b-catenin pathway using PNU-74654 reduces tumor growth in in vitro and in vivo models of colorectal cancer

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Declarations of interests

The authors declare that they have no competing interests.

Running title: PNU-74654 /5-FU combination in CRC

Authors' contributions

Forouzan Amerizadeh, Farzad Rahmani, Reyhaneh Moradi-Marjaneh, Seyedeh-Najibeh Nasiri performed the experiments. Forouzan Amerizadeh, Farzad Rahmani conducted the statistical analysis. Seyed Mahdi Hassanian, Elisa Giovannetti, Mina Maftouh, Soodabeh Shahidsales, Mona Joudi-Mashhad, Majid Ghayour-Mobarhan, Majid Khazaei, Amir Avan designed this study. Forouzan Amerizadeh, Amir Avan wrote the first draft of paper, All authors read and approved the final manuscript.

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Abstract

Background: Colorectal-cancer (CRC) is amongst the most lethal-cancers, mainly due to its metastatic spread and drug chemoresistance. Hence there is a need for new approaches to either increase the efficacy of current therapy or introduce new therapies that have greater efficacy. There is increasing evidence that dysregulation of WNT-signaling-pathway plays an essential role in the development and prognosis of CRC. Here we have investigated the therapeutic potential of targeting the WNT/b-catenin pathway using a novel Wnt/b-catenin inhibitor, PNU-74654, in combination with 5-FU in CRC.

Methods: The anti-proliferative-effect of PNU-74654 was evaluated in two-/three-dimensional cell models. The activity of agents on cell growth, migration, invasion, cell cycle and apoptosis was evaluated by MTT, wound healing assay, invasion, FACS, and annexin V staining, respectively. The oxidant/antioxidant levels were also assessed by determining the level of MDA, SOD, as well as using the DCFH-DA assay. We used a xenograft model of CRC to investigate PNU-74654 activity alone and in combination with 5-FU follow by histological staining and biochemical and gene expression analyses by RT-PCR and western blot.

Results: PNU-74654 inhibited cell-growth and synergistically affected the anti-tumor properties of 5-FU via modulation of Cyclin D1 and survivin. This agent inhibited the migration/invasion of colorectal cancer cells via perturbation of E-cadherin. Furthermore, PNU-74654 inhibited the tumor growth, which was more pronounced using the PNU-74654 plus 5-FU combination via induction of reactive oxygen species, down-regulation of SOD and modulation of MCP-1, P53, TNF-α.

Conclusions: Our finding demonstrated that PNU-74654 can target Wnt-pathway, interfere with cell-proliferation, induced-cell death, reduced-migration and interact with 5-FU, supporting further investigations on this therapeutic-approach for colorectal cancer.

Keywords: colorectal cancer; PNU-74654; anti-tumor effect , Wnt pathway, 5-FU combination

Introduction

CRC as one of the four most common causes of cancer-related death, that imposes a heavy economic and social burden (1). Several therapeutic strategies have been implemented for the treatment of CRC, including radiotherapy, surgery, and, most commonly, chemotherapy. Though, the overall survival rate of CRC patients continues to be unsatisfactory, due to side effects, drug resistance and high recurrence rates (2). Among the drugs used to treat colorectal cancer, 5-fluorouracil (5-FU) is one of the most frequently used drugs (3-7). However, 5-FU has limited efficiency, with a low response and high resistance rates in advanced CRC patients (8, 9). These limitations have led to further studies on emerging alternative or combinational drugs, within chemotherapeutic and chemopreventive approaches, intending to enhance anticancer activity while reducing toxicity and resistance.

Numerous gene mutations are required for the transition from normal colonic mucosa to carcinoma. Most CRCs harbour mutations of the adenomatous polyposis coli (APC) gene, an important part of the Wnt signalling pathway (10). The Wnt pathway is involved in motility, organ development, morphology, cellular proliferation, and destiny of embryonic stem cells. The pathway is driven by WNT substrate, the employs both canonical and noncanonical pathways. (11). Theese pathways are initiated when WNT ligands bind to a subset of transmembrane receptors, and RTK which induces the activation of downstream proteins (12, 13). In the Wnt canonical pathway, the lack of WNT stimulation leads to proteolysis of b-catenin protein through a group of proteins, accommodating (GSK3b), APC, and axin. In the presence of Wnt ligands, there is an accumulation of b-catenin, which is followed by the upregulation of b-catenin target genes, that include c-Myc, and, cyclinD1. Remarkably, B-catenin within the nucleus acts as a lymphoid activator factor and coactivator of TCF and could mediate gene transcription, affecting several alterations to cell persistence, reproduction, and distinction (11). The association between c-myc, APC, cytoskeleton structure and b-catenin results in APC becoming part of the cell-cell communication and migration systems (14). it has been shown, that when WNT and its downstream effectors, are inhibited, cancer initiation, progression, and invasion are stimulated. Modifications in this pathway have been reported to be involved in the expansion of different tumors that include colorectal, breast, lung, melanoma, and, prostate tumors (15). Up to now, Several studies have revealed that the Wnt pathway is critical to the survival of cancer-initiating cells. Suppression of this pathway appears to lead to a restraint of cancer development in some preclinical cancer models (15).

In this regard, PNU-74654 blocks the contact between β-catenin and TCF and hence inhibits the Wnt/beta-catenin signaling (16). This drug was discovered after in-silico screening and validation using biophysical screening to evaluate protein-protein interactions(16, 17). Our recent data showed an anticancer effect of PNU-74654 on pancreatic and breast cell lines (submitted). In the current study, we explored the anti-cancer effects of PNU-74654 and its combination with 5-FU in in vitro and in vivo models of CRC.

1. Methods and materials

2.1. Drugs and chemicals

PNU and 5-FU were purchased from Cayman (Michigan, USA). PNU-74654 dissolved in DMSO, whilst, 5-FU was obtained and solved in sterile water. DMEM, Penicillin (50 IU/ml) and streptomycin (50 µg/ml), FBS, were procured from Gibco.

2.2. In silico analysis of 5-FU response signature

In order to identify the deregulated pathways between respondent and resistant colon cancer cells to 5-FU (18), the GSE81005 dataset was used from GEO and differentially expressed genes (DEGs) were assessed (adjusted p-value<0.05, -1.5 <Log.fold change<1.5) and extracted using GEO2R. P-value adjusted with the Bonferroni-Hochberg method < 0.05 was set as the threshold. DEGs were then submitted to EnrichR online enrichment tool to identify cellular pathways that are associated with these differentially regulated genes between responsive and resistant colon cancer cells to 5-FU (19, 20). Two drugs may show synergistic effects if they perturb similar pathways and biological processes (20). Thus, to decipher the synergistic mechanism between 5-FU and PNU as a Wnt pathway inhibitor, alterations in the expression of genes in HT29 colon cancer cells treated with 10µM of either PNU or 5FU for 24 hours were extracted from iLINCS (integrative Library of Integrated Cellular Pathway Network Signaling) (21, 22) database (LINCSCP_25515 and LINCSCP_119210 datasets, respectively) and DEGs were extracted (adjusted p-value<0.05, -1.5 <Log.fold change<1.5). Overlapping genes that were differentially perturbed by two agents were extracted and submitted to Enricher. KEGG library was selected for pathway enrichment analyses.

1.3. Cell culture

The CT-26 cell line was procured from ATCC and they were expanded in DMEM comprising 1% streptomycin/penicillin and 10% heat-inactivated FBS. The standard storage conditions of the cells were observed and they were kept at 37 °C in 5% CO_2 (atm) (23).

1.4. Growth suppression studies:

The cell growth inhibitory properties of 5-FU and PNU-74654 both alone and in their use in combination were estimated via MTT test one overnight after treatment. For this purpose, cells were incubated in a 96-well plate and after 24 hours they were treated with various concentrations of PNU-74654 (1-500 μM), and 5-FU (1-500 nM), as well as their admixture at a fixed IC50 dose of 5-FU. After that, the plate was then prepared for the MTT assay, as explained previously (24).

1.5. Examination of pharmacological communication of PNU with 5-FU

Furthermore, to evaluate the pharmacological communication of PNU with 5-FU, it was assessed by investigating the cell growth suppression of 5-Fu and PNU alone with the cell growth suppression of their admixture, by applying the combination index (CI), where CI<0.9, CI=0.9-1.1, and CI>1.1 exposed additive, antagonistic, and, synergistic influences sequentially. Most research has concentrated on synchronous treatment, examining at least six different doses of 5-FU, employing a fixed dose. For additional estimating the interaction between PNU-74654 and 5-FU, the median-drug effect analysis method was employed. Growth suppression of <50% was not regarded as biologically adequate. For each experiment, CI values at fraction affected (FA) of 0.5, 0.75 and 0.9 were averaged and these values were applied to measure the mean between tests [28]. CalcuSyn software version 2.0 was manipulated for the examination of the data.

1.6. Multicellular spheroids formation

At first, cells were seeded at a frequency of 105 cells per well in 100 μl DMEM/F12 in 1.5% agarose coated 96-well plates. The cells were followed for 10 days and the cell growth cytotoxic influences and cell connections were evaluated by the inverted phase contrast Microscope. The volume (V) of the spheroids was calculated as follows: $V= (4/3) \times \pi (D/2)3$ (25).

1.7. migration assay

The inhibition of migratory behaviour was investigated by wound-healing assay, being before reported (26). The cells were exposed to the PNU-74654 and 5-FU at their 5XIC50s concentrations.

1.8. Cell cycle assay

10^6 Cells were seeded and after 24 hours, they were treated with PMU, 5-FU, and, their combination at IC50 concentrations one overnight. The influences on the cell cycle were evaluated after staining of cells using propidium iodide (PI), through using a FACSCalibur flow cytometer (Becton Dickinson, San José, CA). By measuring sub-G1 areas, stimulation of cell death by PNU-74654 was investigated as explained before (27).

1.9. Animal experimentation:

Female inbred BALB/c mice were purchased from Pasteur Institute, and divided into 4 groups of 3 mice as follows: administrated with PNU-74654, 5-FU, and 5-FU/ PNU-74654 admixture, and untreated (i.e, control group). The mice were housed under standard laboratory conditions. CT-26 cells were injected subcutaneously in the flanks of the mice. The treatments were administrated when the tumor grew up to a volume of 100mm3. Every 2 days, the size of the tumors was measured. All animal experiments were supported by the Animal Experimentation Ethics Committee of the University.

1.10. Hematoxylin and eosin staining

Tissue samples were preserved in formaldehyde. After that, they were embedded in paraffin blocks to be sectioned into 5-µm thin slices that were placed onto glass slides and stained using eosin and hematoxylin.

1.11. Estimation of malondialdehyde (MDA)

MDA was applied as an indicator of lipid peroxidation to estimate the effects of PNU-74654 on tissue lipid peroxide concentrations. Briefly, 1 mL of 10% cell homogenate blended with 2 CC of HCL+ TBA+ TCA mixture and kept in a boiling water bath for 45 minutes then, centrifuged for 10 minutes. The amount of MDA can be calculated by absorbance at 535 nm C (M) = A／1.65×105.

1.12. Estimation of total thiol groups (SH)

DTNB reagent was applied to assess total thiol concentrations by evaluating the yellow colour complex reacting with SH groups. Initially, to tissue homogenate, 1 ml of Tris-EDTA buffer (pH = 8.6) was added. Before the absorbance was recorded at 412 nm against Tris-EDTA buffer alone (A1). After adding 20 µl of DTNB reagents to

the solution, and keeping it for 15 minutes at 25° C, the sample absorbance was recorded again (A2). Next time, The absorbance of DTNB was recorded as the blank (B). At the end, thiol amount (mM) was estimated from: (Total thiol concentration (mM) = (A2-A1-B) × 1.07 / 0.05 × 13.6) (28).

1.13. Measurement of superoxide dismutase (SOD) and catalase (CAT) activity

The CAT activity was calculated as described by Aebi et al., while SOD was calculated via the Ransod kit (29), which is based on the hydrolyzation of H2O2, at pH 7.0, and the critical amount of sample and reducing absorbance at 240 nm. The transformation of H2O2 to H2O and O2 in 1 min shows Enzyme response speed.

2.14. ROS quantification by DCFH-DA assay

The effect of PNU-74654 on intracellular ROS production was estimated by employing the DCFH-DA, a nonfluorescent probe, to a green highly fluorescent product, dichlorofluorescein (DCF). With this aim, CT-26 cells were treated with or without PNU, for 6 hours, in a complete medium. The cells were then incubated in a fresh culture medium without FBS plus DCFH-DA at the dose of 10 μM for Half-hour. The fluorescence values were then measured using a fluorimeter after three times washing with PBS. Also, cells were imaged with an inverted fluorescence microscope.

2.15. RT-PCR:

CT26 cells were treated with or without PNU-74654 at 5XIC50. Total RNAs were extracted according to the protocol of the RNXPLOS (CinaColon, Tehran, Iran). Then cDNA was manufactured employing a cDNA Synthesis Kit. Specific primers for E-cadherin, CyclinD1, MCP-1, TNF-α, and P53 (Macrogene Co, Seoul, Korea) were accomplished for Quantitative RT-PCR. Then the cDNA was amplified by the ABI-PRISM Step One device. The data were normalized to GAPDH as described (30).

2.16. Western blot analysis

After treating the cells with or without PNU-74654 at $5XIC_{50,}$ the total protein content was extracted and Western blotting was done as mentioned before (31). Initially, cell protein contents were separated on polyacrylamide gels, and after 24hr, they were transported to a nitrocellulose membrane. After incubation with the primary (rabbit anti-anti-p-70s6k and CyclinD; 1:1000, diluted in the blocking solution; anti-β-actin; 1:10000; all from Abcam, and secondary (goat antirabbit; 1:10000,) antibodies the proteins were distinguished via chemiluminescence.

2.17. Statistical analysis:

All of the tests were repeated at least twice and were conducted in triplicate. Data were analyzed by SPSS v.20 statistical software (IBM, Chicago) and statistical significance was set at $P<0.05$. All data were displayed as mean values±SEM and investigated by Student's t-test or ANOVA followed by Tukey's multiple comparison test (32).

2.18. Predicting molecular structure:

Chem Draw 8.0 was used for predicting molecular structure, while Auto-dock software version 1.5.6 was utilized for the docking process. The geometry was optimized through by Hartree-Fock method with Basic Set 3–21 G. structures were prepared by adding hydrogen atoms and removing non-functional water molecules. The Lamarckian Genetic Algorithm was utilized for the global optimum binding position search. The torsion angles of the inhibitors were detected, hydrogens were added to the macromolecule, bond distances were edited and solvent parameters were inserted into the enzyme 3D structure.

3. Results

3.1. PNU inhibits cell proliferation and migration

The expression profile of sensitive and responsive colon cancer cells to 5-FU showed a statistical difference in 236 genes; of which 122 were upregulated and 114 were downregulated. We next found that the most significant pathways associated with these DEGs were p53, Wnt and FoxO signalling pathways (Figure 1A). Accordingly, a recent study by He et.al (2018) has shown the importance of Wnt signalling in the resistance of colon cancer cells resistance to 5FU. To further determine if the addition of Wnt inhibitors synergizes with 5-FU, we extracted the overlapping genes between the DEGs induced by 5-FU and PNU. The number of 39 genes were found to be similar between the two signatures. Pathway enrichment showed that these similar genes were associated with p53, MAPK, PI3K and VEGF signalling pathways as well as cellular metabolism (Figure 1B). Interestingly, we observed that the expression of many genes such as CDK6, CCNE2, CDK1, and CDC42 within these pathways was reversed by PNU compared to treatment with 5-FU. To further strengthen the hypothesis regarding the synergistic effects of PNU and 5-FU, we identified similar docking sites in beta-catenin for both of these agents (Figure 2C). These results prompted us to launch extensive experimental confirmations regarding the potentiating effects of 5-FU and PNU.

The expression of CyclinD1 was detectable at mRNA and protein levels in 4 CRC cells, SW480, CT26, Caco2 and HT-29 and We found various, ranging among cells 0.225 a.u. in Caco2 to 0.525 a.u. in CT26 cells (Fig. 2A). The mean expression in the cells (0.39±0.14 a.u.) was similar to the median (0.41 a.u.), and ($P<0.01$) than the expression detected in fibroblasts (0.055 a.u.). Remarkably, CyclinD1 gene expression in the 4 CRC cells was highly correlated with protein level (Fig 2A). The selection of CT26 cells seemed to make sense because of their Cyclin D1 high expression.

To examine the anti-proliferative action of PNU-74654, the cells were treated by increasing the dose of PNU-74654 (1 μM–1000 μM) for 24 hr. This analysis showed that PNU suppressed cell growth and reduced the IC50 of 5-FU in a dose-dependent approach (Fig. 2A). interestingly, the analysis showed a slight synergism between PNU and 5-FU (Fig.2C), which was confirmed also by bioinformatic analysis for favourable binding configuration between inhibitors and proteins. The latter data shows the interaction of PNU-74654 and 5-FU with b-catenin, which was generated by Orthodox and LigPlot+ software (Fig.2D-E).

The anticancer properties of PNU-74654 were further evaluated in a three-dimensional model of CT26 cells, using spheroid models. These spheroids were exposed to PNU-74654 at 5xIC50 and we observed their disintegration after 9 days, as reported in Figure 2F. As shown in Fig. 2G, PNU-74654 diminished the expression of CyclinD1 and survivin, after one overnight, as detected by RT PCR WB.

In intention to explore the properties of PNU-74654 on cell migration and invasion, scratch mobility and invasion assays were carried out (Fig. 3A-B). After exposure of the cells to PNU-74654 at IC50, a notable decrease in migration was seen after 21 hr (Fig. 3B) ($p<001$), which has correlated with raised expression of E-cadherin mRNA (Fig. 1C). Similarly, the data of invasion assays in cells exposed for 24 hours to PNU-74654 at IC50 values showed a 22% reduction in cell invasion (Fig.3C). Moreover, PNU-74654, 5-FU and PNU-74654 +5-FU reduced the expression of VEGF in the tumor tissue (Fig3E).

3.2. the effect of PNU-74654 on cell cycle

It has been shown that PNU-74654 and its admixture with 5-FU impaired the cell cycle distribution, as reported in Figure 4A-D. After 72 hours, treatment with PNU increased the portions of cells in the G1 phase from 40.2% in the untreated cells to 47.7% in the G1 phase. Hence, PNU blocked the cells in the G1-S boundary. A similar effect was

also detected for its combination with 5-FU, associated with decreased portions of cells in S and G2-M phases, and increased cell mortality (Fig. 4A-D). The examination of the sub-G1 area illustrated indeed that treatment withPNU amended cell mortality compared to untreated cells (Fig4). Additional examination with fluorescence microscopy revealed that cells treated with PNU exhibited apoptotic characteristics with clear cell shrinkage, nuclear fragmentation, consolidation, and fracture of cells into debris, after 24-hour treatment with 5xIC50 of PNU.

3.3. PNU suppresses tumor growth

The CRC in vivo models were created through injection of CT-26 cells into the flank of the mouse, and then these models were treated with PNU-74654 /5-FU/ admixture of PNU-74654 -5-FU (Fig5A). For further study, the histological staining of tumor samples exhibited vascular disturbance as well as substantial RBC extravasation (Fig5B, a), cell necrosis (Fig5B,b), and tumor stroma (Fig5B, c) besides inflammation area (Fig5B, d). We observed that the tumor growth was significantly inhibited in the mice receiving PNU-74654 plus 5-FU, compared to the control group as well as in the groups treated with either PNU-74654 or 5-FU (Fig 5C). Also, the histological staining of tumor tissues illustrated that the combination induced significantly higher tissue necrosis (Fig 5D).

3.4. PNU increases ROS in colon cancer cells

It has been shown that the upregulation of IL-1β, TNF-α, and INF-γ plays a prominent role in inducing cell senescence by increasing ROS (27). Thus, we assessed the influence of the PNU-74654 component on oxidant/antioxidant balance and pro-inflammatory cytokines expression. Our data revealed that PNU enhanced ROS in CRC cells, as detected by intracellular ROS levels measured by DCFH-DA staining at 6 h after treatment with 50, 100 and 200 µm of PNU-74654 (Fig. 6A-B). Also, we evaluated the amounts of thiol, CAT, SOD, and, MDA. The results are shown in Fig 6 A-D. Interestingly, we observed a considerable reduction in SOD activity and increased MDA level in tumor tissue treated with PNU or its combination with 5-FU (Figure 6C-D). In addition, the administration of PNU substantially extended the expression of TNF-α, P53 and MCP-1 (Fig. 6E).

Discussion

The present study shows that inhibition of the Wnt/b-catenin pathway using PNU-74654 reduces tumor growth and increases apoptosis in colorectal cancer (Fig 7). Wnt signalling is vital for the abnormal proliferation of many malignancies and has a crucial role in the inhibition of cell death. This pathway is upregulated in the primary stages

of CRC (33). Wang et al. showed that TGFβ induced factor homeobox 1 encourages the development and metastasis of CRC by stimulating the Wnt/β pathway (34, 35). Another study explored the effect of a GSK-3β suppression and WNT/β-catenin motivation, 6-Bromo-indirubin-3'-oxime, in CRC cells. The result showed that treatment with the BIO agent causes a reduction in the activity of GSK-3β, consequently augmentation and transportation of β-catenin to the nucleus in CRC cells, which can thereby stimulate both drug resistance (36). Similarly, Shin et al. showed the suppression of the β-Catenin-TCF4 Interplay contains CRC extension In vitro and In vivo (37), while Chikazawa et al. described that extinguished of β-catenin causes promotion in the cell sensitiveness to irinotecan and paclitaxel n CRC models (38). Of note, a recent study has also shown, that barring Wnt action can be used as a way to convert the drug sensitivity of CD133-positive cells to 5-FU (39).

Cyclin D1 is one of the chief downstream targets of the Wnt pathway. A large number of malignancies have displayed overexpression of this protein, including breast, oesophagus, bladder, lung and pancreatic cancer(40-44). Upregulation of cyclin D1 is also a well-recognized feature of CRC. Biliran et al. suggested that overexpression of cyclin D1 could be involved in the chemoresistance of pancreatic cancer cells due to the role of cyclin D1 in stimulating cell proliferation (43). Therefore, we attempted to further confirm the effects of the inhibitory activity of PNU on this pathway in CRC cells by assessing the protein levels of cyclin D1 (Fig. 7A). Here we demonstrated that PNU suppressed cyclin D1 expression in CRC in vitro and in vivo models. In addition, we explored the crosstalk between Wnt and mTOR Pathways in CRC cells, by assessing the phosphorylation of the 70S6K protein. Keeping with our result, a study evaluated the key role of the expression of mTOR / AKT /PI3K pathway proteins p-70S6K, p-AKT, and, p-mTOR, in the viability of breast cancer treated with NVP-BEZ235.thay also found that p-70S6K, p-AKT, and, p-mTOR were notably inhibited by NVP-BEZ235. So NVP-BEZ235 could suppress tumor growth and promote autophagy and apoptosis in breast cancer cells (45). Moreover, to explore the anti-inflammatory influence of PNU, the oxidant effect of PNU in colorectal tumor samples was investigated by measuring total thiol, CAT, SOD activity, and MDA amount in samples (31). Growing evidence displayed that oxidative stress in the tumor microenvironment has a dual role in the expansion and treatment of cancers (46). Chang et al. have reported that oxidative stress was improved in CRC subjects in comparison with the control population (47). Likewise, some data have described that an indicator of oxidative stress, like lipid peroxide amount, is higher in tumor tissue in

comparison with normal samples (48, 49). Our results show that PNU could inhibit SOD activity in tumor homogenate, so it could intensify the oxidative stress in the tumor microenvironment by the augmentation of ROS. This can result in increased induction of apoptosis in CRC cells. This hypothesis is further supported by a novel study, presenting that apoptosis and cell cycle arrest can be caused via the augmentation of oxidative stress levels in SW620 cells (50). Furthermore, we previously demonstrated that a high ROS promotes colorectal cancer cells' sensitivity to 5-FU. Consequently, as reported in the present study, PNU may have a significant role in CRC treatment by growing oxidant levels of ROS and making the cell more sensitive to chemotherapy drugs. To the additional examination of the mechanisms bearing anticancer influences of PNU, the p53 expression was matured. p53 protein as effectively limits tumor progress, principally via governing various genes required in the stimulation of apoptosis, senescence, and cell cycle constraint as a tumor suppressor. According to the perturbation of the cell cycle, treatment with PNU could lead to a notable increase in the p53 expression. Mentioned evidence supports the thesis that the anti-proliferative properties of PNU, which begins cell cycle interruption in the G1 phase, are reasonably facilitated by the overexpression of p53. Finally, to better understand the inflammatory mechanisms involved in the anti-tumor properties of PNU, the level of the pro-inflammatory cytokines such as TNF-α and MCP-1 were examined. Our results showed that PNU extended the pro-inflammatory cytokines, TNF-α and MCP-1 expression in CRC cells.

Our results provide a novel vision into the antiproliferative activity of PNU-74654 in CRC, via targeting the Wnt pathway, inducing apoptosis, preventing of the cell-proliferation, and migration, encouraging further investigation of this new therapeutic strategy for the treatment of CRC.

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Figure legends

Figure 1. Analysis of pathways leading to resistance of colon cancer cells to 5-FU and the synergistic effects of Wnt inhibitors with 5-FU. A) Comparison of responsive versus resistant expression profiles of HCT-8 colon cancer cell lines from GSE dataset and pathway enrichment analysis to cluster resistance-associated genes. Three pathways including p53, wnt and FoxO were among the significant (adjusted p-value <0.05) pathways. B) Analysis of gene expression changes following treatment of cells with 10 um 5-FU or PNU as Wnt inhibitor and determine the pathways associated with overlapping genes between the two drugs. C) Docking analysis: The plots generated by autodock and LigPlot+ software shows the interaction between β-catenin and (D) PNU or (E) 5-FU. Hydrogen binding pockets of b-catenin and PNU (green line) or 5-FU and Hydrophobic interactions (red line).

Figure 2. **PNU-74654 suppresses cell proliferation**. (A) expression of CyclinD1 at mRNA and protein level in 4 CRC cells, SW480, CT26, Caco2 and HT-29, as detected by RT-PCR and Western blot, respectively. (B) Growing inhibitory properties of PNU-74654, 5-FU and their combination after 24 hours. On the X-axis the drug concentrations for the combination are referred to as 5-FU, (C) Mean CI of the PNU-74654/5-FU combination. (D) Influence of PNU-74654 on the spheroids cells; cell connections were evaluated by the inverted phase contrast Microscope. The volume (V) of the spheroids was calculated as follows: V= (4/3) ×π (D/2)3 (25); the sized scale bars by Microscope was 10x (E) expression of CyclinD1 and survivin before and after treatment with PNU-74654. Expression of p70S6k and Cyclin D1 at protein levels as detected by Western blot. Columns, mean values obtained from two independent experiments;

Figure 3. Effects of PNU-74654 on cell invasion and migration. (A) Results of the wound-healing assay in CT-26 cells (insert: representative picture at 24 hours). Cells were exposed to PNU-74654 at 5xIC50 values. the sized scale bars by Microscope was 10x (B) Results of invasion experiment in the CRC cells exposed for 24 hours to PNU-74654 at 5xIC50 values p< 0.001; (D) Modulation of E-cadherin levels after 24 hours exposure as determined by q-RT-PCR. (E) Modulation of VEGF levels in tumor tissue exposure to PNU-74654, 5-FU and their combination, as determined by ELISA.

Figure 4. Cell cycle perturbation and apoptosis index. (A-C) Effects of PNU-74654, plus 5-FU on cell cycle compared to control (A) considering PNU-74654 alone (B) and in combination with 5-FU (C) after 24hr. Columns, mean values obtained from three independent experiments;

Figure 5. PNU-74654 inhibits tumor growth. (A) Experimental design of the study in the in vivo setting; (B) Histological staining of tumor tissues by H&E (the sized scale bars by Microscope was ×10). Tumor tissues revealed more vascular density and RBC extravasation (a), inflammation (b), necrosis (c) and tumor stroma (d). (C) Tumor size in the xenograft mouse model treated with PNU-74654, 5-FU, and their combination. (D) Histological staining of tumor tissue samples by H&E (the sized scale bars by Microscope was 10X and 40X).

Figure 6. PNU-74654 increases oxidative stress. (A-B) ROS production after PNU-74654 treatment was measured in CT-26 using an oxidation-activated fluorescent dye and was quantified using a fluorimeter (the sized scale bars by Microscope was 10X).. (C) SOD activity and (D) MDA activity were calculated in tumor tissues. € Expression of TNF-α, MCP-1 and p53 at mRNA levels as detected by RT-PCR. *Significantly different from controls.

Figure 7. Schematic representation of the molecular mechanisms involved in the effects of PNU-74654 on the Wnt pathway.