Novel, isoform-selective, cholecystokinin A receptor antagonist inhibits colon and pancreatic cancers in preclinical models through novel mechanism of action

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Received November 3, 2015; Accepted December 12, 2015

DOI: 10.3892/or.2016.4588

Abstract. Colon and pancreatic cancers contribute to 90,000 deaths each year in the USA. These cancers lack targeted therapeutics due to heterogeneity of the disease and multiple causative factors. One important factor that contributes to increased colon and pancreatic cancer risk is gastrin. Gastrin mediates its actions through two G-protein coupled receptors (GPCRs): cholecystokinin receptor A (CCK-A) and CCK-B/gastrin receptor. Previous studies have indicated that colon cancer predominantly expresses CCK-A and responds to CCK-A isoform antagonists. However, many CCK-A antagonists have failed in the clinic due to poor pharmacokinetic properties or lack of efficacy. In the present study, we synthesized a library of CCK-A isoform-selective antagonists and tested them in various colon and pancreatic cancer preclinical models. The lead CCK-A isoform, selective antagonist PNB-028, bound to CCK-A at 12 nM with a 60-fold selectivity towards CCK-A over CCK-B. Furthermore, it inhibited the proliferation of CCK-A-expressing colon and pancreatic cancer cells without affecting the proliferation of non-cancerous cells. PNB-028 was also extremely effective in inhibiting the growth of MAC-16 and LoVo colon cancer and MIA PaCa pancreatic cancer xenografts in immune-compromised mice. Genome-wide microarray and kinase-array studies indicate that PNB-028 inhibited oncogenic kinases and angiogenic factors to inhibit the growth of colon cancer xenografts. Safety pharmacology and toxicology studies have indicated that PNB-028 is extremely safe and has a wide safety margin. These studies suggest that targeting CCK-A selectively renders promise to treat colon and pancreatic cancers and that PNB-028 could become the next-generation treatment option.

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Key words: cholecystokinin, CCK-A, CCK-B, gastrin, colon cancer, pancreatic cancer, kinases, G-protein coupled receptor

Introduction

Gastrointestinal cancers that include colon and pancreatic cancers account for over 140,000 new cancer cases and 90,000 deaths each year in the USA (1). These cancers are the second and third leading causes of cancer-related deaths in the USA, respectively. Chemotherapy, radiotherapy, and surgery are the only choices available to treat colon and pancreatic cancers (2-4). Due to failure to diagnose at an early stage, the 5-year survival rate of these cancer patients is much less than that of many other cancers (1,5). Colon and pancreatic cancers lack targeted therapeutics due to heterogeneity of the disease and diverse causative factors, including *Helicobacter pylori* (*H. Pylori*) bacteria (6,7), gut hormones such as gastrin and cholecystokinin (CCK) (8,9), and activation of cyclooxygenase (10,11) and NF-κB pathways (12).

The actions of two gut polypeptide hormones, gastrin and CCK, are mediated by G-protein coupled receptors (GPCRs), CCK-A and gastrin/CCK-B receptors. Since both gastrin and CCK share sequence homology, they bind to both CCK-A and CCK-B receptors (13). Gastrin, a peptide hormone secreted by the parietal cells of the stomach, binds to CCK receptor and elicits its function (14). Upon binding to CCK receptors, gastrin stimulates the release of histamine, which in turn releases hydrochloric acid/gastric acid. In addition, gastrin also plays important roles in cell proliferation and maturation of the GI tract. Gastrin is also produced in excess in gastrinoma or gastric cancers (6,8). Blocking gastrin action by antagonizing CCK receptors will reduce gastric cancer incidence and growth.

Substantial evidence suggests that gastrin plays a pivotal role in stimulating colon cancer growth. It has been identified that gastrin plays an important role in *Heliobacter*-induced stomach cancer (15). More than 50% of the world population infected with *Heliobacter* results in gastric ulcers and stomach cancers. In addition, gastrin also plays an important role in stimulating the *H. pylori*-induced proliferation of gastric

cancer cells. Gastrin increases interleukin isoforms, IL-1β and IL-8 secretion, resulting in inflammatory process and subsequently cancer (16).

Notably, peptide CCK selectively binds to CCK-A, while gastrin binds with equal affinity to both CCK-A and CCK-B receptors (17). CCK-A is over 500-fold more selective towards CCK than gastrin, while CCK-B/gastrin receptor has equal affinity for both CCK and gastrin. This complicates the interpretation of the isoform that is responsible for the gastrin actions in colon cancer. While studies with clinical specimens clearly indicate higher expression of CCK-A in colon and pancreatic cancers, cell and tumor growth studies suggest a role for CCK-A and CCK-B in the proliferative effects of gastrin (13,18). One of the studies compared the antiproliferative effects of non-isoform and isoform-selective CCK inhibitors in HT-29 colon cancer cells (18). While CCK-A isoform-selective inhibitors such as lorglumide and devazipide inhibited the proliferation of HT-29 cells, CCK-B-selective inhibitors such as PD-135158, L-365260 and non-isoform-selective inhibitor such as proglumide had no effect on the proliferation of HT-29 cells. Collectively, tissue-expression and isoform-selective inhibitor studies suggest the importance of CCK-A in the proliferative effects of gastrin in colon cancer. Despite these studies, the role of CCK receptors in pancreatic cancer is unclear.

In the present study, we synthesized a library of highly potent isoform-selective CCK inhibitors and tested them in various preclinical models of colon and pancreatic cancers. The lead molecule PNB-028 (4-chloro-5-(fluorophenyl)-5-hydroxy-1-isobutyl-1,5-dihydro-2H-pyrrol-2-one) bound potently and selectively to CCK-A at 12 nM with a 63-fold selectivity towards CCK-A. PNB-028 also inhibited CCK-A activity in a low nanomolar concentration. PNB-028 inhibited the proliferation of CCK-A-expressing colon and pancreatic cancers cells without affecting the proliferation of non-cancerous cells. PNB-028 was also extremely effective in inhibiting the growth of MAC-16 and LoVo colon cancer and MIA PaCa pancreatic cancer xenografts in immune-compromised mice. Genome-wide microarray and kinase-array studies indicate a novel mechanism of action for PNB-028 in inhibiting the growth of colon cancer xenograft. Pharmacology and toxicology studies have indicated that PNB-028 is extremely safe and has a wide safety margin. These studies collectively suggest that selectively targeting CCK-A offers promise to treat colon and pancreatic cancers and that PNB-028 may become the next-generation treatment option.

Materials and methods

Reagents. CCK-A and CCK-B antibodies were procured from Abcam (Cambridge, MA, USA). Real-time PCR reagents and TaqMan primers and probes were obtained from Life Technologies (Carlsbad, CA, USA). Phospho-kinase array was purchased from R&D Systems (Minneapolis, MN, USA). Actin antibody was procured from Sigma (St. Louis, MO, USA). Matrigel was obtained from BD Biosciences. Cell culture reagents were purchased from Invitrogen and the American Type Culture Collection (ATCC; Manassas, VA, USA). All other reagents used were analytical grade.

Biological evaluation: $^{[125]}$I-CCK-8 receptor binding assay. CCK-A and CCK-B receptor binding assays were performed with guinea pig cerebral cortex (CCKB) and rat pancreas (CCK-A). Male guinea pig brain tissues were prepared according to the method described by Saita *et al* (19). Pancreatic membranes were prepared as recommended by Charpentier *et al* (20).

Functional CCK assays. The CCK inhibitor lorglumide and PNB-028 were tested in triplicate with sulphated CCK8S as agonist and increasing concentrations of the ligand using a standardized 96-well plate discovery kit from DiscoverRx.

Anti-depression test: Forced swim test. Depression test animal protocols were approved by The Aston University Animal Care and Use Research Committee. The forced swim test was carried out in a glass cylinder filled with water at a temperature of ~25-28˚C. Rats were gently placed into the water and the immobility time was recorded for 5 min. Immobility was defined as the absence of all movement with rats remain floating passively in water with their heads just above the water surface.

Colon and pancreatic cancer xenograft. All xenograft animal protocols were approved by The University of Tennessee Animal Care and Use Research Committee. MAC-16 and MIA PaCa cells were harvested, counted under microscope using trypan blue exclusion and a suspension in growth medium was prepared. LoVo cells were prepared as 1:1 suspension of cells in medium and Matrigel. The cell suspension (5 million cells/mouse) was implanted subcutaneously in NSG mice. Once the tumors reached 100-200 mm^3, the animals were randomized within the respective cell line and treated orally at 50 mg/kg/day. PNB-028 was dissolved in 20% DMSO+80% PEG-300 and administered by oral gavage. Body weight and tumor volumes were measured thrice weekly. Animals were sacrificed (6 h after the last dose) when tumors reached 1,500 mm^3 or when the animals lost over 20% body weight. Tumors were excised, weighed, and stored for further analysis (two parts snap-frozen and stored at -80˚C and one part stored in formalin for histology). Blood was collected, serum separated, and frozen at -80˚C for future analysis.

Cell culture. Colon cancer cells (HT29, Lovo, CaCo2, HCT116, SW480 and SW48) and non-cancerous HEK-293 and COS-1 cells were obtained from ATCC. MAC-16 colon cancer cell line was a kind gift from Dr Jeff Molkentin (Cincinnati Children's Hospital) and Dr Michael J. Tisdale (Aston University, Birmingham, UK). All cells, except LoVo, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). LoVo cells were grown in DMEM:F12 supplemented with 10% FBS.

RNA isolation, microarray analysis and gene expression. RNA was isolated from MAC-16 tumors (n=3/group) using Qiagen RNeasy kit. RNA was quantified using nanodrop and diluted to equal concentrations of 100 ng/μl. RNA quality was determined using Agilent Bioanalyzer and RNA samples with RNA integrity number (RIN) >7 were used for microarray experiments (ST 2.0 mouse array from Affymetrix). Results

from microarray studies were analyzed using Affymetrix software [Transcriptome Analysis Console (TAC)]. Pathways differentially regulated by PNB-028 were identified using Ingenuity Pathway Analysis software.

Human phospho-kinase array. Protein extracts (500 μg/sample) that were prepared from MAC-16 tumors (n=4/group) were incubated with protein kinase array membranes (R&D Systems) and the experiment was conducted according to the manufacturer's protocol. The blots were developed and quantified using ImageJ software and the data were analyzed for statistical significance using JMP Pro 11 software.

Growth assay. Cells were plated at 4,000 cells/well of a 96-well plate in growth medium and treated with a dose response of PNB-028 (1 pM to 10 μM). After 3 days of treatment, cells were stained with sulfrhodamine blue reagent and the optical density, a measure of viable cells, was measured at 535 nm.

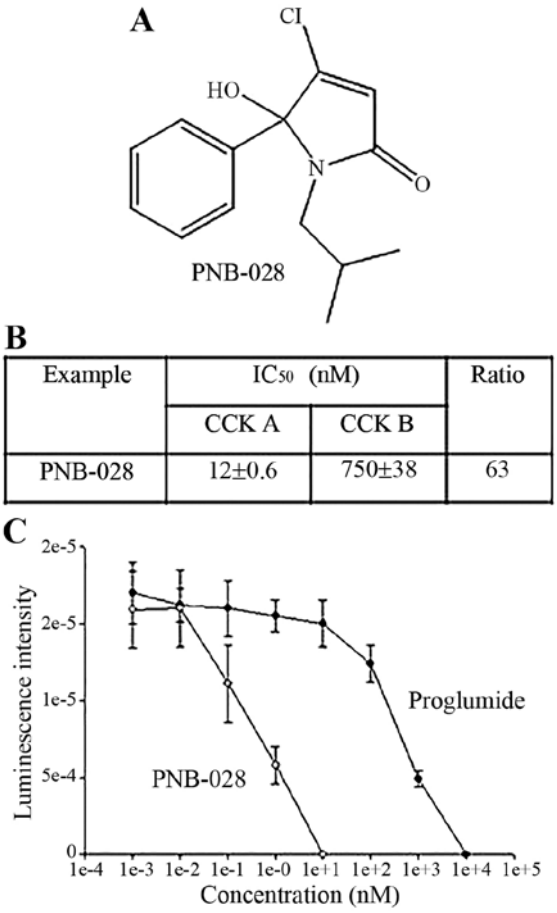
Western blotting. Cells were grown as described above. Protein extracts from cells or tumors were prepared and equal amount was run on a 4-20% SDS-PAGE, transferred to a nylon membrane, and western blotted for CCK-A and CCK-B using respective antibodies. Western blotting with actin antibody was performed as the loading control.

Metabolic stability of PNB028 with rat, dog, and human liver microsomes. PNB-028 was incubated with rat, dog, and human liver microsomes for two different time points and disappearance of test compound was monitored by LC-MS/MS. Briefly, test and reference compounds were incubated with rat, dog or human liver microsomes (100 μl reaction; final drug concentration 1 μM, final DMSO concentration 0.05%, n=2). Incubation was carried out at 37˚C. For 0 min control (100% drug control), after adding the drug, reaction was terminated immediately with 100 μl of acetonitrile containing internal standard (tolbutamide, 500 ng/ml). Separate reactions were set up for 5 and 30 min at 37˚C and reactions were stopped by addition of acetonitrile. Contents were mixed well, and centrifuged at 13,200 rpm (Eppendorf 5810R) for 5 min. Supernatant fraction was collected and subjected to LC-MS/MS analysis. Reference compounds of reported/historical metabolic stability data such as verapamil (high clearance) and atenolol (low clearance) were included in the assay as positive controls.

Metabolic stability was determined as % parent compound remaining (% PCR) using the following formula:

$$\% \text{ PCR} = \frac{\text{(peak area ratio of analysis to internal standard at 30 min)}}{\text{(peak area ratio of analysis to internal standard for 0 min control)}} \times 100$$

Analytical methods. Analysis of samples by LC-MS/MS was carried out using Sciex API 4000 system operated with Nexera™ UHPLC (Shimadzu) as front-end. Samples were separated on a Phenomenex Synergi Polar RP column (75x2.0 mm, 4 μm) using a gradient mode at a flow rate of 1 ml/min. The mobile phase consisted of 0.1% formic acid in Milli-Q water (A) and 0.1% formic acid in acetonitrile (B). MS instrument was operated in positive mode. The multiple reactions monitoring transition of PNB028 was 283.9/210.8 (Q1/Q3) with a de-clustering potential of 40 V, entrance potential 10 V and collision energy of 20 V. The curtain gas (5 V), ion-spray voltage (5,500 V), temperature (500˚C), nebulizer gas (GS1), and auxiliary gas (GS2) were set at 45 and 55 psi, respectively, and the interface heater was on.



Example	IC_{50} (nM)		Ratio
	CCK A	CCK B	
PNB-028	12±0.6	750±38	63

Figure 1. PNB-028 is an isoform-selective CCK receptor antagonist. (A) Structure of PNB-028. (B) CCK-A and CCK-B binding assay. CCK-A (from rat pancreas) or CCK-B (from pig cerebral cortex) was incubated with radioactive CCK peptide alone or in combination with a dose response of PNB-028. Radioactivity, a measure of CCK displacement from the receptor binding pocket, was measured, analyzed and represented as IC_{50} value for PNB-028. (C) PNB-028 inhibits CCK-A effectively. Effect of PNB-028 on the CCK-A activity was evaluated using a standardized kit from DiscoverRx. Proglumide was used as a control. All experiments were performed in triplicate and values are expressed as mean ± SE. CCK, cholecystokinin.

Toxicology and safety pharmacology studies. Toxicology and safety pharmacology studies were performed in accordance with the ICH guidelines in Vimta Labs (Hyderabad, India). The studies were performed in a GLP certified laboratory.

Statistical analysis. Data were statistically analyzed using JMP Pro 11 software. Experiments with only two groups were analyzed by t-test and experiments with more than two groups were analyzed by one way ANOVA.

Results

PNB-028 is a CCK-A-selective antagonist. PNB-028 (Fig. 1A) was selected from a library of structurally-related molecules

A

IC_{50} [nM]	MIA PaCa	AGS	PANC	MAC13
Devazepide	1200	>5000	480	1100
Lorglumide	425±23	289±14	316±21	421±11
PNB-028	154±14	262±18	275±9	536±32

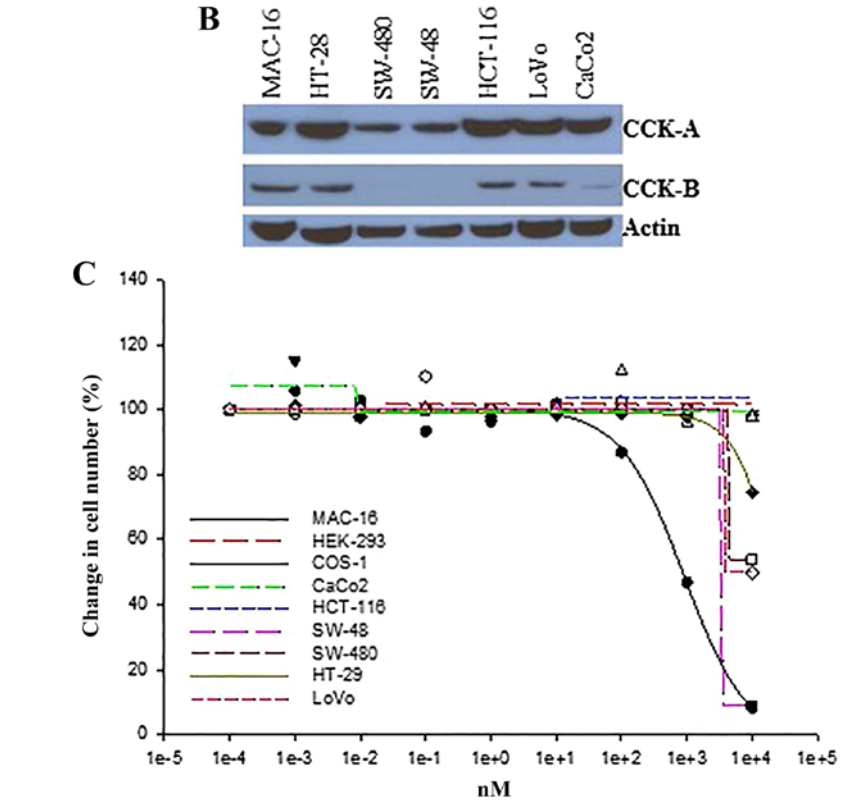


Figure 2. PNB-028 inhibits colon and pancreatic cancer cell proliferation. (A) Cells shown in A were incubated with a dose response of PNB-028, lorglumide and devazepide for 3 days and cell number was measured by WST assay. Values are expressed as IC_{50} value. (B) Expression of CCK-A and CCK-B was evaluated in the indicated colon cancer cells by western blotting. Western blotting for actin was performed as loading control. (C) Colon cancer and non-cancerous cells (HEK-293 and COS-1) were incubated with PNB-028 for 3 days and the number of cells was measured by SRB assay. All experiments were performed in triplicates and the values are expressed as mean ± SE.

based on structure-activity relationship studies. PNB-028 was tested in CCK-A and CCK-B competitive receptor binding assay study using rat pancreas and pig cerebral cortex. As shown in Fig. 1B, PNB-028 bound to CCK-A at 12 nM, but to CCK-B only at 750 nM, demonstrating over 60-fold selectivity for CCK-A. CCK-A and CCK-B functional assays demonstrated that PNB-028 effectively inhibited the CCK-A activity with IC_{50} value comparable to its binding efficacy of 12 nM (Fig. 1C), whereas, proglumide inhibited CCK-A activity with IC_{50} value >1,000 nM. These studies indicate that PNB-028 is a highly potent CCK-A-selective antagonist.

PNB-028 inhibits proliferation of colon and pancreatic cancer cell lines. Effect of PNB-028 on the proliferation of various colon (AGS and MAC13) and pancreatic (MIA PaCa and PANC) cancer cell lines was evaluated. PNB-028 effectively inhibited the proliferation of these cell lines with IC_{50} values in the range of 100-1,000 nM (Fig. 2A), whereas, devazipide and lorglumide inhibited growth of these cells only at concentrations >1,000 and 10,000 nM.

The antiproliferative studies were expanded to additional colon cancer cell lines. Expression of CCK-A and CCK-B in colon cancer cell lines was determined by western blotting. In concordance with clinical specimens (13), colon cancer cell lines used in these experiments expressed CCK-A (Fig. 2B). Although four out of seven cell lines tested positive for CCK-B, CCK-B expression was weaker than that of CCK-A, indicating that CCK-A is the abundant isoform expressed in colon cancer.

Colon cancer cells were incubated with PNB-028 for 3 days and SRB assay was performed. PNB-028 effectively inhibited growth of MAC-16 chemoresistant murine colon cancer cells, SW-48, SW-480 and Lovo (Fig. 2C), while it had no effect on other colon cancer cell lines (HCT-116, HT-28 and CaCo2) and non-cancerous cell lines (HEK-293 and COS-1). Although all colon cancer cell lines expressed CCK-A, it is unclear why PNB-028 inhibited only four cell lines, but not the others.

Drug metabolism and pharmacokinetic (DMPK) properties of PNB-028. We evaluated the effect of PNB-028 on various DMPK properties. It is essential for a drug to possess ideal DMPK properties, to advance to *in vivo* studies and preclinical development. PNB-028 was extremely stable in human (HLM), rat (RLM), and dog (DLM) liver microsomes (Table IA). Liver microsome studies indicated that after 30 min still 99% of PNB-028 in HLM, 43% in RLM and 55% of PNB-028 in DLM remained unmetabolized by respective liver microsomes (Table IA). The data suggest species differences in metabolism with PNB-028 being more stable in HLM than RLM and DLM.

To evaluate the stability of PNB-028 *in vivo*, pharmacokinetic (PK) studies were performed in rats. PNB-028 was administered intravenously (1 mg/kg b.wt.) and orally (20 mg/kg b.wt.) to rats and blood samples were collected at various time points until 24 h. PNB-028 concentration in plasma was measured using appropriate LC-MS/MS method. PNB-028 had a half-life of ~2.5 h with 100% bioavailability (Table IB). The PK result suggests that despite a modest half-life in rat liver microsomes, PNB-028 possesses 100% bioavailability. Considering that PNB-028 is more stable in HLM, we expect it to have longer half-life in the clinic.

Inhibiting drug metabolizing cytochrome (CYP) P-450 enzymes will result in detrimental effects when patients consume more than one drug. CYP inhibition studies were conducted with PNB-028. PNB-028 at 10 μM did not inhibit major CYP enzymes, while the positive control inhibited the CYP enzymes significantly (Table IC).

These results indicate that PNB-028 has favorable DMPK properties and has the potential to advance to *in vivo* studies.

PNB-028 inhibits colon and pancreatic cancer xenografts in NOD SCID γ (NSG) mice. To test the *in vivo* efficacy of PNB-028, colon and pancreatic cancer xenograft studies were performed. Cancer cells were implanted subcutaneously in NSG mice and when tumor volume reached 100-200 mm^3, the animals were randomized and treated with vehicle or 50 mg/kg/day PNB-028 orally. Tumor volume was measured thrice weekly and animals were sacrificed when tumors reached >1,500 mm^3. Vehicle-treated MAC-16 tumors grew ~2,000% in three weeks (Fig. 3A, left panel). MAC-16 is a murine chemoresistant colon tumor that aggressively grew to the extent of causing cachexia in mice. PNB-028 was extremely effective in slowing down the tumor growth significantly providing a tumor growth inhibition (TGI) of ~75%. Tumor growth was completely arrested by PNB-028 for the first two weeks of treatment initiation even before showing signs of growth. Consistent with the tumor volume results, weight of PNB-028-treated tumors were 60-70% lower than the vehicle-treated tumors (Fig. 3A, right panel).

Based on *in vitro* proliferation studies shown in Fig. 2C, LoVo colon cancer cells were chosen as second xenograft model (Fig. 3B). LoVo tumors grew slower than MAC-16 tumors and took almost 3 weeks to grow 600% (Fig. 3B, left panel). Growth of LoVo tumors was also significantly reduced by PNB-028. Tumors weighed at sacrifice demonstrated an ~50% lower weight in PNB-028-treated animals (Fig. 3B, right panel).

Table I. DMPK properties of PNB-028.

A, Stability of PNB-028 in rat (RLM), dog (DLM) and human (HLM) liver microsomes

	Mean % parent remaining after 30 min		
Compound	HLM	RLM	DLM
Verapamil	7.49	2.49	1.42
Atenolol	96.97	106.64	107.77
PNB208	99.66	42.73	55.26

B, The pharmacokinetic (PK) properties of PNB-028 evaluated in rats

Parameters	PO 20 mpk
AUC (0-t) (h*ng/ml)	1136.51±76.86
AUC (0-∞) (h*ng/ml)	1151.78±72.52
Cmax (ng/ml)	527.81±101-19
Tmax (h)	0.38±0.18
$T_{1/2}$ (h)	2.28±1.31
% F	111.32±6.74

C, The effect of PNB-028 on cytochrome p450 (CYP) enzymes.

	% Inhibition		
Compound	CYP2C9	CYP2D6	CYP3A4
Ref.	90.3	95.8	98
PNB-028	5.4	12.1	8.8

PNB-028 possesses optimum drug metabolism and pharmacokinetic (DMPK) properties. A, Stability of PNB-028 in rat (RLM), dog (DLM) and human (HLM) liver microsomes was evaluated by incubating the drug *in vitro* for 30 or 60 min and the percent drug remaining was measured by LC-MS/MS method. B, Pharmacokinetic (PK) properties of PNB-028 were evaluated in rats. Rats were administered with a single dose of 1 mg/kg PNB-028 intravenously and 20 mg/kg PNB-028 orally. Blood was drawn at various time points and the availability of PNB-028 in serum was measured. C, Effect of PNB-028 on cytochrome p450 (CYP) enzymes was measured. Major CYP enzymes, CYP2D6, CYP3A4 and CYP2C9 were incubated with PNB-028 and the effect on enzyme activity was measured. Liver microsome and CYP inhibition studies were performed in triplicates, while the PK study was conducted with n=3 rats.

We tested the efficacy of PNB-028 on the growth of a pancreatic cancer xenograft (Fig. 3C). Cell proliferation studies shown in Fig. 2A indicate that PNB-028 effectively inhibited MIA PaCa pancreatic cancer cell proliferation with IC_{50} value around 150 nM. While vehicle-treated MIA PaCa pancreatic cancer cells implanted subcutaneously grew robustly, PNB-028-treated tumors grew at a slower rate with ~60% TGI (Fig. 3C, left panel). Tumor weights were inhibited by 50% by PNB-028 compared to tumors from vehicle-treated animals.

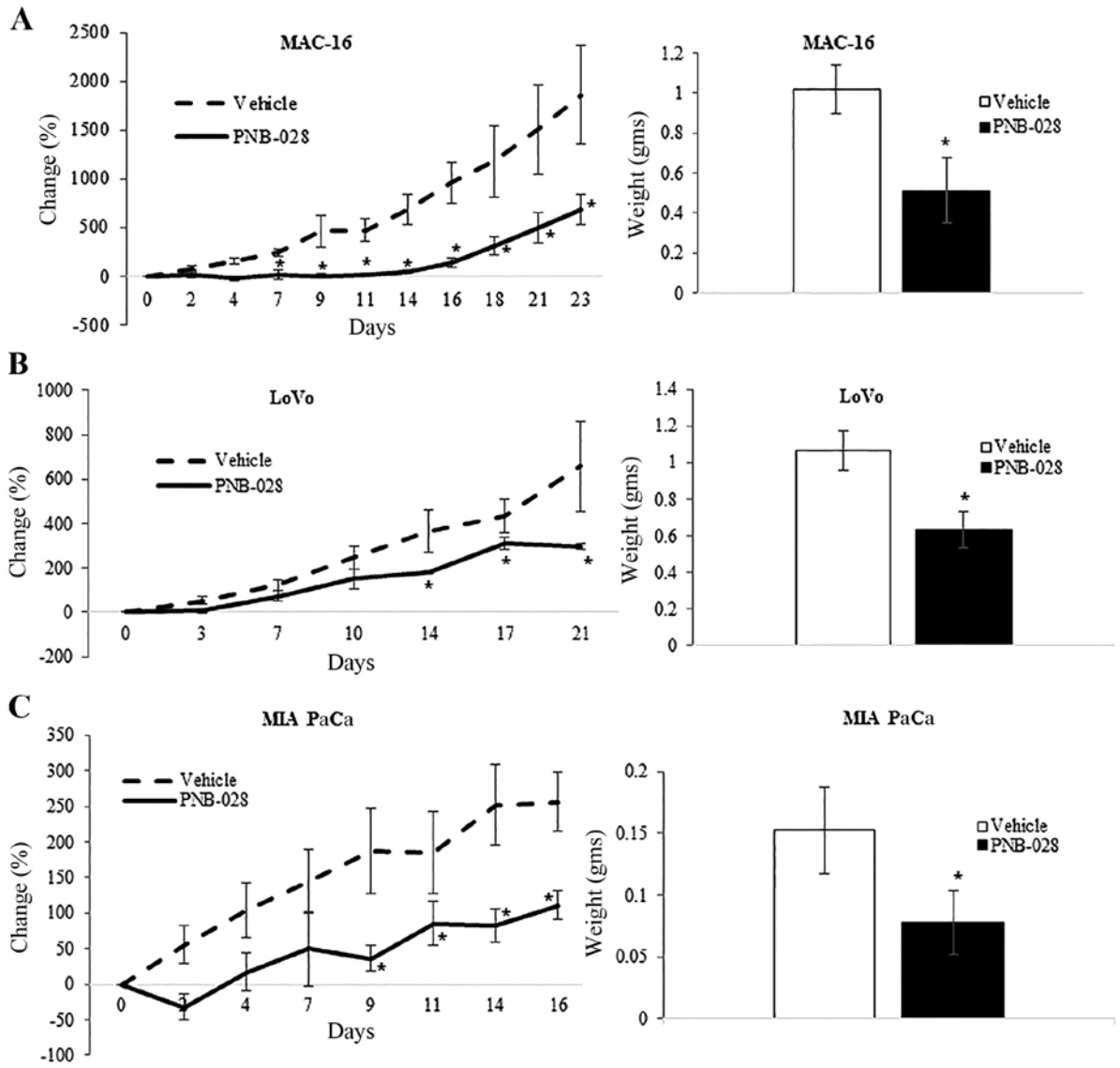


Figure 3. PNB-028 effectively inhibits colon and pancreatic cancer xenografts. Colon cancer [MAC-16 (A) and Lovo (B)] and pancreatic cancer (MIA PaCa) (C) cells were implanted subcutaneously on the flanks of NSG mice. Once the tumors reached 100-200 mm^3, the animals were randomized and treated with vehicle or PNB-028 (50 mg/kg/day p.o.). Tumor volume was measured thrice weekly. Tumor weights were recorded at sacrifice (right panel) and tumors were stored at -80˚C for further analysis. Values are expressed as mean ± SE. $^*p<0.05$, statistical significance.

PNB-028 inhibits oncogenic kinases in MAC-16 tumor xenografts. GPCR membrane receptors elicit their physiological effects through second messengers and kinases (21). To understand PNB-028's mechanism of action, various molecular studies were performed. Protein extracted from MAC-16 xenograft tumors (n=4/group) were subjected to phospho-kinase array to determine the effect of PNB-028 on kinase activity. Table II contains the list of kinases significantly altered by PNB-028 in MAC-16 tumors. Except for p53 and STAT3, all other kinases shown in Table II were inhibited significantly by PNB-028 compared to vehicle-treated controls. Several important oncogenic kinases, including src, yes, FAK and JNK were significantly inhibited by PNB-028. These results suggest that PNB-028 blocks oncogenic signaling pathways required by CCK-A to promote growth of colon and pancreatic cancers.

PNB-028 modulates expression of genes involved in cancer. To determine the effect of PNB-028 on gene expression, microarray studies were conducted with RNA isolated from vehicle- or PNB-028-treated MAC-16 tumors (n=3/group). PNB-028 significantly regulated over 1,400 genes compared to vehicle-treated samples (data not shown) with ~450 genes upregulated and 1,100 genes downregulated by 1.5-fold or greater. The list also contains 42 differentially regulated miRNAs, with 20 of them upregulated and 22 downregulated. Some of the microRNAs required for colon cancer growth (miR-34a, miR-194, miR-301b and miR-192) were differentially regulated by PNB-028 (22-24).

Ingenuity pathway analysis identified cAMP signaling as the top canonical pathway overrepresented in the data set (Table III). As expected cancer is the top disease represented in the data set with 476 genes belonging to cancer were differentially regulated by PNB-028. Various genes involved in cancer progression such as mug1, FLG2, CDHRS, wnt16 and others were the top ranked genes regulated by PNB-028 (25-27).

Table II. The kinases activated by PNB-028 in MAC-16 xenografts.

	Vehicle		PNB-028		
Kinase	Avg	SE	Avg	SE	P-value
Ref.	1	0	1	0	
JNK1/2/3	0.308248	0.01989	0.09294	0.010537	0.0016
EGFR	0.241026	0.027824	0.105439	0.01653	0.022
MSK1/2	0.542699	0.040771	0.311258	0.012877	0.009
p53	0.025831	0.003003	0.039743	0.002691	0.013
HSP27	0.185137	0.013716	0.055009	0.005738	0.008
Src	0.274963	0.027102	0.092557	0.006062	0.00236
Yes	0.382946	0.032932	0.14205	0.010512	0.0019
STAT3	0.027749	0.005336	0.040122	0.003246	0.0426
Chk-2	0.371529	0.02679	0.143098	0.013097	0.00134
FAK	0.281422	0.021302	0.098282	0.015685	0.00195
PDGF Rb	0.205851	0.019172	0.065606	0.014887	0.00374
PRAS40	0.541283	0.035194	0.23595	0.010209	0.00098

PNB-028 significantly inhibits kinase activity. Effect of PNB-028 on kinase phosphorylation was evaluated by incubating protein extracts obtained from vehicle- or PNB-028-treated MAC-16 tumors (n=4) from the xenograft study on phosphor-kinase array. Arrays were processed according to the manufacturer's protocol. Only kinases that are significantly regulated by PNB-028 are expressed in the present study.

Table III. Microarray ingenuity pathway analysis.

A, Top canonical pathways

Top canonical pathway	P-value
cAMP signaling	1.32e-4
Transcriptional regulatory network	8.06e-4
GPCR signaling	5.54e-3

B, Molecular and cellular functions

Molecular and cellular functions	No. molecules
Cell signaling	27
Nucleic acid metabolism	22
Cell death and survival	29

C, Disease

Diseases	No. molecules
Cancer	476
Organismal injury	480
Neurological disease	90

PNB-028 alters expression of genes. RNA was extracted from vehicle- or PNB-028-treated MAC-16 xenograft tumors (n=3) and were subjected to microarray analysis (mouse ST2.0 array). Pathways significantly altered by PNB-028 were identified using ingenuity pathway analysis software and represented as A, top canonical pathways; B, molecular and cellular functions; and C, disease.

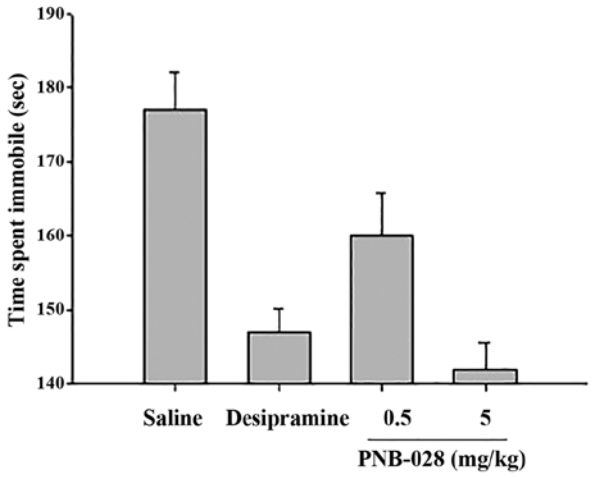


Figure 4. PNB-028 alleviates depression. PNB-028 (0.5 or 5 mg/kg) or desipramine was administered to rats (n=5) and their effect on depression was evaluated using the forced-swim test.

PNB-028 reduces depression and anxiety. Depression and anxiety are serious cancer-associated symptoms (28). CCK peptides alter neurological functions to promote depression and anxiety (29). CCK inhibitors have been demonstrated to alleviate depression and this property combined with anti-oncogenic potential will provide additional benefits. We tested PNB-028 in a rat model of depression (Fig. 4). PNB-028 at 0.5 and 5 mg/kg significantly reduced depression compared to saline-treated animals and the efficacy was comparable to the positive control desipramine.

PNB-028 is safe and well tolerated. As PNB-028 exhibited excellent efficacy in colon and pancreatic cancer xenografts, it

Table IV. Toxicology and safety pharmacology results.

Study	Species	Dose (mg/kg/day p.o.)	Adverse effect
28 days toxicology	Rat	50,100,200	No adverse effects or mortality
Motor coordination safety pharmacology	Rat	50,80	No difference from vehicle in latency of fall in roto-rod motor coordination experiment
Gastrointestinal safety pharmacology	Rat	50,80	No change in charcoal intestinal transit study
7 days DRF	Rat	100,200,400	No treatment-related adverse events or mortality
14 days DRF	Dog	50,200	No treatment-related adverse events of mortality
Ames test	Bacteria	39-5,000 (μg/plate)	Negative response at all concentrations
i.v. acute toxicology	Mice	15,30,60	2 males and 2 females died at 60 mg/kg dose 15 and 30 mg/kg did not have any adverse events
28 day toxicology	Rat	50, 100	No adverse effects of mortality
Chromosomal aberration	Rat	200, 1,000	No chromosomal aberration

Toxicology and safety pharmacology studies with PNB-028 were performed in accordance with ICH guidelines in GLP-accredited facility.

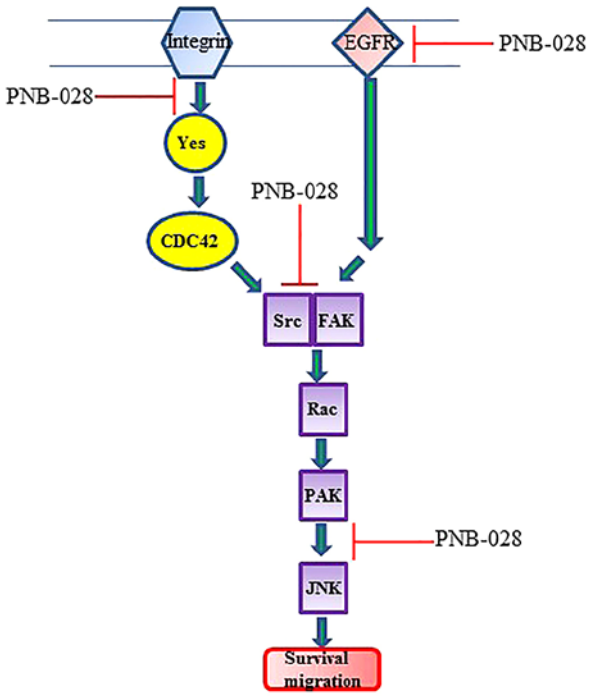


Figure 5. Model depicting actions of PNB-028.

was advanced to toxicology and safety pharmacology studies conducted in accordance with ICH guidelines. PNB-028 tested for up to 28 days exhibited impeccable safety with no adverse events (Table IV). PNB-028 at doses as high as 400 mg/kg was safe. Genotoxic studies also demonstrated lack of effects.

Collectively these studies demonstrate that PNB-028 has the potential to become the next-generation colon and pancreatic cancer targeting therapeutic.

Discussion

In the present study we showed, using preclinical models, that a novel and safe CCK-A-selective antagonist can effectively treat colon and pancreatic cancers in preclinical models. Although other CCK inhibitors have been used in the past to treat gastrointestinal ulcers (proglumide, devazepide and lorglumide), pain in combination with opioids (proglumide), anorexia (devazepide), and irritable bowel syndrome (lorglumide), PNB-028 is the first to show activity on both colon and pancreatic cancers and on depression (30-32). Development of other CCK antagonists L-365,260, L-369,293, YF-476, RP-69758, LY-288,513 and PD-145,942 was halted due to poor oral bioavailability (33). Even the PK properties of widely used CCK antagonist proglumide is not optimum. PNB-028 may be the first in the CCK antagonist class to have high oral bioavailability in preclinical species and have significantly better stability in human liver microsomes. PNB-028 possesses all desirable drug-like properties to advance to clinic. Although previous clinical trial with a CCK antagonist, MK-329, in pancreatic cancer patients failed to provide benefit, PNB-028 may offer a breakthrough for gastrointestinal cancers that have no targeted therapeutics (2).

PNB-028 effectively inhibited growth of MAC-16 chemoresistant aggressively growing murine colon cancer allograft (Fig. 3A) (34). This suggests that inhibiting CCK-A may benefit patients relapsed from chemotherapy. Similarly, PNB-028 slowed the growth of another colon cancer cell line xenograft (Fig. 3B) and a pancreatic cancer xenograft (Fig. 3C). These growth inhibition results were obtained without affecting body weight of animals or inhibiting growth of normal cell lines (Fig. 2C). Gross pathological observation of MAC-16 tumors indicated that while vehicle-treated tumors were highly vascular, PNB-028-treated tumors were not vascular. In order to understand the effect of PNB-028 on angiogenic factors, microarray data was probed for growth factors that promote angiogenesis. Fibroblast growth factor (FGF)-2 and -4 were inhibited significantly by PNB-028. Literature evidences suggest that FGF is a highly potent angiogenic growth factor with FGF-2 being more potent than even vascular endothelial growth factor (VEGF) (35). Another important growth factor responsible for angiogenesis and cancer cell proliferation, FGF-4, was also significantly inhibited by PNB-028 (36).

PNB-028 selectively inhibited kinases and cell signaling pathways that are responsible for cancer cell proliferation (Table II). Pathway analysis of the kinase-array data indicated that PNB-028 inhibits integrin- and EGF-signaling pathways. Both integrin and EGFR pathways activate src-FAK association resulting in activation of RAC, PAK, JNK, MSK 1 and 2, resulting in increased proliferation and survival (Fig. 5) (37). Activation of FAK and src by integrin and EGFR family results in increased proliferation, migration and cell survival. While most of these oncogenic kinases were activated, phosphorylation of tumor suppressor protein p53 was increased by PNB-028. This suggests that although the predominant mechanism for anti-tumorigenic effect of PNB-028 is to inhibit proliferative signals, it also positively affect tumor suppressors to provide additive effects.

Unlike many oncogenic targets, CCK is a pleotropic therapeutic target that promotes the development of multiple diseases. Many of these diseases, including pain, depression, and anxiety, could be considered cancer-associated diseases. Inhibiting CCK will not only reduce the growth of colon and pancreatic cancers, but will also alleviate depression, anxiety and pain. On the contrary inhibiting therapeutic targets such as estrogen receptor α (ER-α) will reduce breast cancer cell proliferation, while promoting osteoporosis and hot flashes. This demonstrates that the pleotropic role is unique to only certain therapeutic targets such as CCK, while others have opposing role in different tissues.

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