

Irreversible Cholecystikin-1 Receptor Antagonists PNB-028/81: N-isobutyl-5-hydroxy-5-aryl-pyrrol-2-ones as Experimental Therapeutic Agents against Colon and Pancreatic Cancer

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ABSTRACT

A new class of 5-arylated 5-hydroxypyrrolones was derived from mucochloric acid in 2 synthetic steps and the chemical structure was confirmed additionally by x-ray analysis. Using a radiolabelled binding assay, potent CCK1 selective ligands were identified (CCK1: 12nM) and the antagonism was confirmed by using isolated tissue preparations. A series of isobutyl derivatives displayed unsurmountable CCK antagonistic properties. Using electrically induced contractions and CCK induced contractions on isolated rat tissues, an irreversible antagonism was established.

In vitro, using selected cancer cell lines, the viability was measured and IC-50 were obtained in the nanomolar range.

Using allograft models the treatment regimen was further optimised leading to a 48h dosing interval. The downstream analysis revealed inhibition of proliferation analysed via the Ki 67 biomarker. Finally, using xenograft studies in nude mice, two selected pyrrolone derivatives, X=H and X= F a fluorinated analogue (PNB-028), showed a strong inhibition of tumour growth in a human pancreatic cell line (MIAPACA) at 50 mg/kg by oral administration. PK was analysed and the overall conclusion was drawn.

Keywords

Cholecystikin, Gastin, Gallbladder, Schizophrenia, Asperlicin.

Introduction

Cholecystikin (CCK) acts as a neuromodulator in the brain and as a gut hormone in the gastrointestinal tract. CCK-ligands, agonists as well as antagonists [1] have been extensively investigated as potential drug molecules [2]. Originally, cholecystikin was discovered to cause contractions of the gallbladder, it was then rediscovered as pancreozymin, triggering the release of pancreatic enzymes and then, it was confirmed that both peptides are identical [3].

Gastin, closely related to cholecystikin, triggers the release of gastric acid and in vitro in cell lines, gastric cancer is gastrin dependant [4]. Cholecystikin does cause proliferation in colon-

and pancreatic cancer cell lines and therefore, CCK-antagonists were studied as growth factor inhibitors in certain forms of cancer [5]. They were evaluated as anxiolytics [6], in the treatment of schizophrenia [7], satiety [8] and reviewed as anxiolytics [9]. These properties may be beneficial in cancer patients in addition to their anti-neoplastic activity.

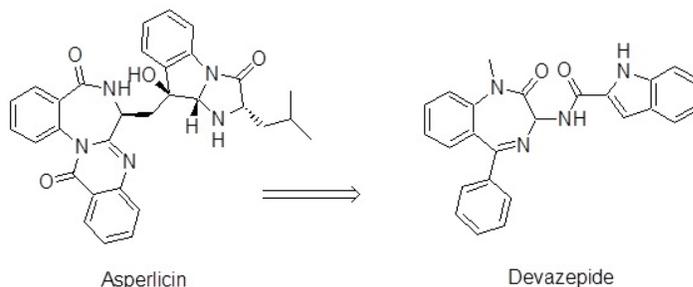


Figure 1: Drug design from natural product Asperlicin, towards fully

synthetic Devazepide, a CCK1 selective antagonist.

Asperlicin (Figure 1) was the first non-peptidal CCK antagonist lead structure from nature and analogues thereof, were studied as CCK ligands [10]. Simplification of the lead structure by Merck led to Devazepide [11], a potent CCK₁ selective cholecystokinin antagonist, containing a 1,4-benzodiazepine template and an indole moiety. Devazepide was tested clinically in 18 patients with advanced cancer [12] without escalating doses and failed to show significant effects due to its poor pharmacokinetic properties.

Z-360 is a CCK₂–gastrin receptor antagonist and progressed into phase 2 trial with pancreatic cancer [14]. Pancreatic cancer (PDA) is an emerging disease and is the fourth leading cause of cancer death worldwide. The prognosis is very poor; the 5-year survival rate is 5%, and the life expectancy of patients with metastatic PDA is approximately 2.8-5.7 months. These poor outcomes are likely due to resistance to chemotherapy and PDA biology. There are very limited options for patients with pancreatic cancer who have failed first line therapy and reflects the unmet need.



Figure 2: Lorglumide CCK standard.

Lorglumide (Figure 2) is 25 times more potent in blocking the cholecystokinin induced contractions of the gallbladder than proglumide, but the activity to block pancreatic amylase secretion is only better by factor 2 [15], indicating a heterogeneity of the CCK1 receptor. Proglumide with mM activity is too non-potent, requiring multi gramme quantities for potential cancer treatment.

Again, having realized the poor pharmacokinetic properties of these agents, a search for a completely novel, smaller template [22] with a molecular weight <350, a log p about 3 and a polar surface area for membrane penetration of less than 100Å, with no urea linkage was initiated.

Here, we identified within a novel chemical class, potent irreversible cholecystokinin antagonists, which translated in vitro and in vivo efficiency into new chemical entities (NCE) for the treatment of colon and pancreatic cancer.

Materials and Methods

Synthesis

The chemicals were obtained from Aldrich (Gillingham, UK) and Lancaster (Lancaster, UK). Atmospheric pressure chemical

ionisation mass spectroscopy (APCI), negative or positive mode, was carried out using a Hewlett-Packard 5989b quadrupole instrument (Vienna, Austria). Proton and Carbon NMR spectra were obtained on a Bruker AC 250 instrument (Follanden, Switzerland), operating at 250 MHz, calibrated with the solvent reference peak or TMS. IR spectra were plotted from KBr discs on a Mattson 300 FTIR Spectrometer. Melting points were recorded from a Stuart Scientific (Coventry, UK) Melting Point and are uncorrected.

Synthesis of 3,4-dichloro-5-phenyl-5H-furan-2-one, Lactone A

Dry and powdered aluminium chloride (20 g, 0.15 mol) was added slowly to a mixture of mucochloric acid (16.9 g, 0.1 mol) and benzene, fluorobenzene or chlorobenzene (250 ml). The reaction mixture was stirred overnight. For work up it was poured into a mixture of 100 g ice and 32 ml concentrated hydrochloric acid. The organic layer was separated by a separating funnel and it was washed with 3 x 100 ml of water. The combined organic layers were dried over magnesium sulphate and the solvent was removed under vacuum. The oily residue was crystallized in n-hexane.

Yield = 70%, M.P: 78-79°C; R_f (80% ether / 20% petrol ether) = 0.62; Molecular Weight: 229.1; Molecular Formula: C₁₀H₆C₁₂O₂; MS (APCI(+)): 195/197 (M+), 230/232 (M+1) m/z; ¹H NMR (CDCl₃) 250 MHz: 7.22-7.51 (m, 5H), 5.81 (CH) ppm. ¹³C NMR 165.3, 152.2, 139.8, 130.5, 129.3, 128.5, 127.4, 127.2, 121.2, 83.5 ppm. IR (KBr-disc) 3445, 3074, 3035, 2959, 2056, 1768, 1630, 1499, 1457 1294, 1224, 1028, 910, 772, 705 cm⁻¹.

3,4-Dichloro-5-(4-chloro-phenyl)-5H-furan-2-one, Lactone B

Yield = 69% M.P: 76-78°C; R_f (80% ether / 20% petrol ether) = 0.55; Molecular Weight: 263.5; Molecular Formula: C₁₀H₅C₁₃O₂; MS (APCI(+)): 227/229/231 (M+1), 262/263/265 (M+) m/z; ¹H NMR (CDCl₃) 250 MHz: 7.42-7.55 (m, 2H), 7.28-7.40 (m, 2H), 5.91 (CH) ppm. ¹³C NMR (CDCl₃) 165.3, 152.0, 136.6, 130.1, 129.6, 128.7, 121.3, 82.9 ppm. IR (KBr-disc) 3451, 3075, 2952, 2051, 1769, 1636, 1497, 1419, 1289, 1231, 1027, 927, 826, 748, 720 cm⁻¹.

3,4-Dichloro-5-(4-fluoro-phenyl)-5H-furan-2-one, Lactone C

Yield = 79% M.P: 74-76°C; R_f (80% ether / 20% petrol ether) = 0.53; MS (APCI(+)): 227/229/231 (M+1), 247, 246 (M+) m/z; ¹H NMR (CDCl₃) 250 MHz: 7.42-7.55 (m, 2H), 7.28-7.40 (m, 2H), 5.91 (CH) ppm. ¹³C NMR (CDCl₃) 250 MHz: 165.2, 152.0, 136.6, 130.1, 129.6, 128.7, 121.3, 82.8 ppm. IR (KBr-disc) 3451, 3075, 2952, 2051, 1769, 1636, 1497, 1419, 1289, 1231, 1027, 927, 826, 748, 720 cm⁻¹.

General Method

Isobutylamine in 2.5 times excess was added to a solution of lactone A, B, or C (0.7 mol) in ether (10 ml) and the reaction mixture was stirred on ice for 30 minutes, allowing to warm up to RT over time. The resultant mixture was poured into 15 ml water and the organic phase was separated by a separating funnel. The mixture was subsequently washed with an excess of water for three more times. The organic layer was dried over magnesium sulphate

and the solvent was removed under vacuum. All compounds gave an oily solid on a small scale, which was purified using column chromatography with a mixture of 80% ether / 20% petrol ether to yield crystals after removal of the solvent in vacuum.

4-Chloro-5-hydroxy-1-isobutyl-5-phenyl-1,5-dihydro-pyrrol-2-one PNB-081

Yield = 85 %; MP: 167-169°C; Rf (80% ether / 20% petrol ether) = 0.27; Molecular Weight: 264.7; Molecular Formula: C₁₄H₁₆ClNO₂; MS (APCI(+)): 193/195 (M+1), 266/268 (M+) m/z; ¹H NMR (CDCl₃) 250 MHz: 7.38-7.51 (m, 5H), 6.24 (s, CH), 4.79-4.98 (bs, OH), 3.23-3.32 & 2.18-2.29 (dd, 2H), 1.71-1.90 (m, 1H), 0.76-0.96 (m, 6H) ppm. ¹³C NMR (CDCl₃) 168.5, 155.7, 137.1, 129.2, 128.7, 126.2 (5xArC), 121.7, 93.1, 47.6, 27.5, 20.4 ppm. IR (KBr-disc): 3237, 3114, 2965, 2926, 2881, 2374, 2343, 1675, 1614, 1460, 1416, 1299, 1251, 1202, 1150, 1072, 1027, 878, 758, 696 cm⁻¹.

Crystal data - (sample recrystallised from methanol)

C ₂₈ H ₃₂ Cl ₂ N ₂ O ₄ Mr = 531.46 T = 293(2) K Tabular 0.20 x 0.15 x 0.10 mm Colourless Mo Kα radiation: λ = 0.71073 Å Triclinic P-1 a = 8.3190(13) Å b = 12.614(4) Å c = 13.8106(18) Å α = 93.049(17) ° β = 94.791(12) ° γ = 107.651(19) °	V = 1371.5(5) Å ³ Z = 2 D _x = 1.287 Mg/m ³ D _m not measured R [F ² > 2(F ²)] = 0.0541 wR(F ²) = 0.1165 5136 reflections 331 parameters
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Selected geometric parameters (Å, °)

Cl(1)-C(1)	1.696(4)	Cl(1')-C(1')	1.695(4)
C(1)-C(4)	1.310(5)	C(1')-C(4')	1.322(5)
C(2)-C(5)	1.511(5)	C(2')-C(5')	1.524(5)
C(2)-O(2)	1.410(4)	C(2')-O(2')	1.400(4)
C(3)-O(1)	1.224(5)	C(3')-O(1')	1.237(4)
N(1)-C(11)	1.448(5)	N(1')-C(11')	1.448(5)

4-Chloro-5-(4-fluoro-phenyl)-5-hydroxy-1-isobutyl-1,5-dihydro-pyrrol-2-one PNB-028

Yield = 86%; Melting Point: 158-159°C; Rf (80% ether / 20% petrol ether) = 0.21; Molecular Weight: 284.2; Molecular Formula: C₁₄H₁₅ClFNO₂; MS (APCI(+)): 211/212(M+1), 284/285 (M+) m/z; ¹H NMR (CDCl₃) 250 MHz: 7.30-7.41 (m, 4H), 6.19 (s, 1H), 3.13-3.31 (dd, 1H), 2.49-2.62 (dd, CH₂, J = 8.0 Hz, 1H), 1.69-1.83 (m, 1H), 0.69-0.80 (t, CH₃, J = 4.5 Hz, 6H) ppm. ¹³C NMR (CDCl₃) 163.6, 156.1, 139.0, 134.7, 129.0, 127.2, 122.1, 95.0, 47.1, 27.2, 20.5 ppm IR (KBr-disc) 3425, 3251, 2964, 2924, 2850, 1685, 1406, 1209, 1095, 816, 743 cm⁻¹.

4-Chloro-5-(4-chloro-phenyl)-5-hydroxy-1-isobutyl-1,5-dihydro-pyrrol-2-one Cl-lactam

Yield = 76%; Melting Point: 155-158°C; Rf (80% ether / 20% petrol ether) = 0.22; Molecular Weight: 300.2; Molecular Formula:

C₁₄H₁₅Cl₂NO₂; MS (APCI(+)): 227/229/231 (M+1), 300/302/304 (M+) m/z; ¹H NMR (CDCl₃) 250 MHz: 7.30-7.41 (m, 4H), 6.19 (s, 1H), 3.13-3.31 (dd, CH₂, J = 8.0 Hz, 1H), 2.49-2.62 (dd, CH₂, J = 8.0 Hz, 1H), 1.69-1.83 (m, CH, J = 5.8 Hz), 0.69-0.80 (t, CH₃, J = 4.5 Hz, 6H) ppm. ¹³C NMR (CDCl₃) 163.3, 156.3, 139.4, 134.8, 129.1, 127.7, 122.3, 95.0, 47.6, 27.6, 20.4 ppm IR (KBr-disc) 3426, 3252, 2964, 2924, 2850, 1684, 1406, 1209, 1095, 817, 743, 703 cm⁻¹.

[¹²⁵I]-CCK-8 Radioligand cholecystokinin binding assay

CCK₂ and CCK₁ receptor binding assays were performed, by using guinea pig cerebral cortex or rat pancreas. Male guinea pig brain tissues were prepared according to the modified method described by Saita et al. Pancreatic membranes were prepared as described by Charpentier et al. Tissues were homogenized in ice cold sucrose (0.32 M, 25 ml) for 15 strokes at 500 rpm and centrifuged at 13000 rpm for 10 minutes. The supernatant was re-centrifuged at 13000 rpm for 20 minutes. The resulting pellet was re-dispersed to the required volume of buffer at 500 rpm and stored in aliquots at 70°C.

Binding was achieved using radioligand [¹²⁵I]-Bolton-Hunter labeled CCK, NEN at 25 pM. The samples were incubated with membranes (0.1 mg/ml) in 20 mM Hepes, 1 mM EGTA, 5 mM MgCl₂, 150 mM NaCl, at pH 6.5 for 2 hrs at RT and then centrifuged at 11000 rpm for 5 minutes. The membrane pellets were washed twice with water and the bound radioactivity was measured in a Packard Cobra Auto-gamma counter (B5005). Binding assays were carried out with L-363, 260 as control.

Isolated tissue preparations

Male Sprague Dawley rats, weighing 200-250g were used and all animal care and experimental protocols adhered to the relevant laws and guidelines of the institution. The animals were housed under standard conditions of temperature (25°C) with unrestricted access to food and water. The animals were sacrificed using cervical dislocation without anaesthesia. From the abdomen of the animals, the duodenum was carefully excised and washed with physiological solution. The mesentery of the tissue was removed and the lumen was gently flushed with Tyrode's solution to clear luminal contents. The prepared isolated tissue was rapidly incubated in Tyrode's solution maintained at 32°C and gassed with 95% O₂ / 5% CO₂. Tyrode's solution was freshly prepared daily (g/l): NaCl, 8.0; KCl, 0.2; CaCl₂, 0.2; MgSO₄, 0.1; NaH₂PO₄, 0.05; NaHCO₃, 1.0; Glucose, 1.0. The main equipment used was the Radnoti single unit tissue bath system with a chamber capacity of 35 ml. Bath aeration with carbogen (O₂ 95%, CO₂ 5%) was maintained at a constant temperature (32°C). The force in grams was measured with an isometric transducer linked to a power lab data acquisition system.

Electrically stimulated muscle contractions

The intramural nerves within the ileal strips were excited by rectangular pulses of 2 ms, 25 mA and a frequency of 0.2 Hz. Transmural stimulation was applied using two platinum electrodes, one placed in the lumen of the ileum and the other outside the tissue.

CCK-5, penta-gastrin and cholecystokinin CCK-8 preparations

CCK-8S was dissolved in distilled water to prepare a stock solution of 500 μ M solution, from which cumulative additions of increasing concentrations (0.1 nM, 1 nM, 5 nM, 10 nM, 20 nM, 30 nM, and 40 nM) were tested to plot a dose response curve. Test molecules and lorglumide were added to the organ bath 10 minutes before exposure to the next CCK-8S serial concentrations. The same protocol was used for pentagastrin, CCK-5.

Molecular modeling

For target preparation the protein structures, pdb identifier 1HZN for the CCK₁ and 1LAT for the CCK₂ –gastrin receptor were downloaded from the protein data bank (www.rcs.org) and docking was performed using Autodock Vina and Hex. After several docking trials for the CCK₁ / CCK₂ receptor the results were analysed and visualized using Chimera and Designer studio 4.5. After visual inspection the results were presented to rationalize drug ligand interactions with the each CCK receptor subtype.

Allograft study

In vivo experiments in mice - assessment of anti-tumour inhibition Pure strain NMRI mice aged between 6 and 8 weeks from our inbred colony were used for transplanting MAC (murine colon cancer) tumours. Animals were fed on RM3E diet (Lillco-England) and water ad libitum. Approximately 2 mm cubes containing 2x 10⁵ cells of MAC 16 tumour fragments were transplanted subcutaneously in the inguinal region via a trocar in a volume of 0.2 ml. Tumour bearing mice were randomised in groups of 7 animals per group and the treatment was started 10 days after transplantation. The test compounds were administered in propylene glycol. The effect of chemotherapy was assessed 20 days after transplantation. Mice were killed after 10 days of drug treatment and the effects were measured by the differences in tumour weight as expressed: % [inhibition] = Treated weight / control weight x 100

The body weight changes were recorded additionally. The procedure was approved by the home office and the bioethics committee of Aston University.

Xenograft study in NSG mice

1 million cells were used per mouse and the test molecule was administered in 20% DMSO + 80% PEG-300. The suspension was vortexed and warmed at 37°C for 5 min to ensure dissolution. MAC 16 mouse chemo resistant colon cancer cells were grown in RPMI medium supplemented with 10% fetal bovine serum (FBS) in 37°C incubator with 5% CO₂. Human cancer cells were grown in DMEM supplemented with 10% FBS in 37°C incubator with 5% CO₂. Cells were grown until 70% confluence before passaging into fresh flasks. For xenograft implantation, above indicated cells were harvested, viable cells determined by trypan blue exclusion, and a cell suspension in growth medium was prepared. The cell suspension in growth medium (100-111/mouse) was implanted subcutaneously in NSG mice. Once tumours reached 100 mm³, the animals were randomized within the respective cell line and treated orally. Body weight and tumour volume were measured

thrice weekly (Mon, Wed, and Fri). Animals were sacrificed (6 hrs after the last dose) when the tumours reached over 1500 mm³ or when the animals lost over 20% body weight.

All experiments were performed in compliance with the relevant laws and institutional guidelines and the institutional bioethics committee has approved the experiments.

PK analysis

6-8 week male rats purchased from Harlan Research Laboratories, North America Registration Number : Syngene-IAEC-412-08-2013 aged 6 to 8 weeks old Identification: They were identified individually with tail marking using permanent marker Acclimatization: At least for one week under laboratory conditions, after veterinary examination. Only animals without any visible signs of illness were used for the study.

Time points for blood sampling (IV dose) were pre-dose, 0.083, 0.25, 0.5, 1, 2, 4,6, 8, & 24 hr post dose (10 time points). Time-points for blood collection (PO dose) were, pre-dose, 0.25, 0.5, 1, 2, 4, 6, 8, & 24 hr post dose (9 time points). At each time point, approximately 150 μ L of blood was collected through jugular vein in labeled tubes containing K2-EDTA as anticoagulant. The tubes were mixed gently and centrifuged at 2500 g for 10 minutes at 4°C. The plasma was separated into labeled polypropylene tubes (~75 μ l of plasma) and stored immediately at -80°C until analysis.

Analysis of samples by LC-MS/MS was done using API Sciex 4000 system operated with Nexera™ UHPLC (Shimadzu) as front-end. Samples were separated on a Phenomenex kinetex C18 (50X2.1 mm, 5 μ) using a gradient mode at a flow rate of 1 ml/min. The mobile phase consisted of 0.1% formic acid in MilliQ water (A) and 0.1% formic acid in acetonitrile (B). MS instrument was operated in positive mode. The multiple reactions monitoring transition of test molecule was 247.9/192.0 (Q1/Q3) with a declustering potential of 70V, entrance potential 10 V, and collision energy of 25 V. The curtain gas (5 V), ion-spray voltage (5500 V), temperature (500°C), nebulizer gas (GS1), and auxiliary gas (GS2) were set at 45 psi & 55 psi respectively, and the interface heater was on.

Statistical methods

The data were expressed as mean + SD and one-way analysis of variance (ANOVA) and supplementary Tukey test for pairwise comparison were tested to determine for any significant difference at p< 0.05.

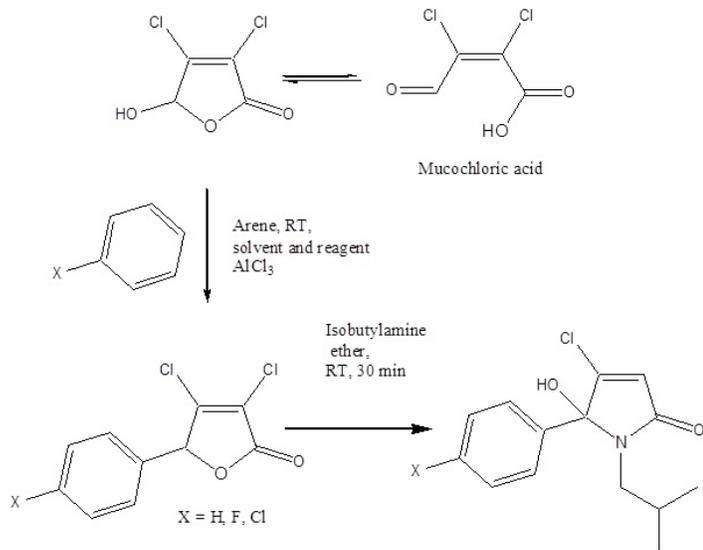
Results and Discussion

Chemistry

The arylated dichloro-2(5H)-furanones (Lactone A, B, C) were synthesised from mucochloric acid (Scheme 1), which is available from furfural under oxidising conditions with hydrochloric acid. Theses arylated intermediates were chemically optimised by us previously and evaluated as anticancer agents [23].

Under mild conditions, using ether as solvent, the arylated

furanones were reacted with isobutylamine, required for best SAR towards the CCK1 receptor (Scheme 1).



Scheme 1: Synthesis of arylated lactams. PNB-028: X=F, PNB-081: X=H.

The experimental details are described in materials and methods and no acyclic ketone, the ring opened form of the hydroxyl-pyrrolone, was observed. On a small scale purification was performed by column chromatography and on a multigramme scale, the isobutyl series was purified by recrystallisation with methanol.

As the gold standard of structure characterisation, the x-ray structure of PNB-081 was determined and is displayed in Figure 3. The molecule is not present in a keto form and fully occurred in the 5-membered ring form, as a hydroxyl – pyrrolone.

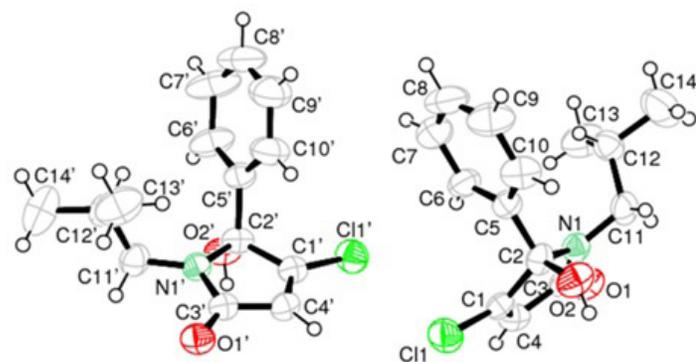


Figure 3: X-ray structure of 4-Chloro-5-hydroxy-1-isobutyl-5-phenyl-1,5-dihydro-pyrrol-2-one, PNB-081 recrystallised from methanol.

SAR optimisation

The initial step of evaluation was to screen for potent binding affinity and to identify a CCK1 selective ligand for the treatment of cholecystinin related conditions. Using radiolabel iodinated cholecystinin, inhibition of binding was determined for all test molecules and the IC₅₀ are outlined in Table 1.

Pyrrolone	X=	R=	CCK-A [μM]	CCK-B [μM]
PNB-081	H	Isobutyl	0.020 ± 0.01	1.2 ± 0.3
PNB-028	F	Isobutyl	0.012 ± 0.01	0.75 ± 0.2
Cl-lactam	Cl	Isobutyl	0.008 ± 0.01	0.4 ± 0.2
Lorglumide	-	-	0.17 ± 0.01	>10

Table 1: CCK binding affinity using radioligands with cortex and pancreatic membranes. IC₅₀ is presented in micromolar; N=3.

The change from a propyl into a butyl group resulted in a manifold increase of activity and the best substituent was found isobutyl, as shown in Figure 4 for the iso-butyl series 9-11. The introduction of a halogen atom resulted in an increase of binding affinity, possibly due to enhanced lipophilicity and the best overall molecule is pyrrolone with an IC₅₀ about 8 nM. The t-butyl analogue was formed in very low yields, same as a pentyl analogue (not included). irreversible inhibition

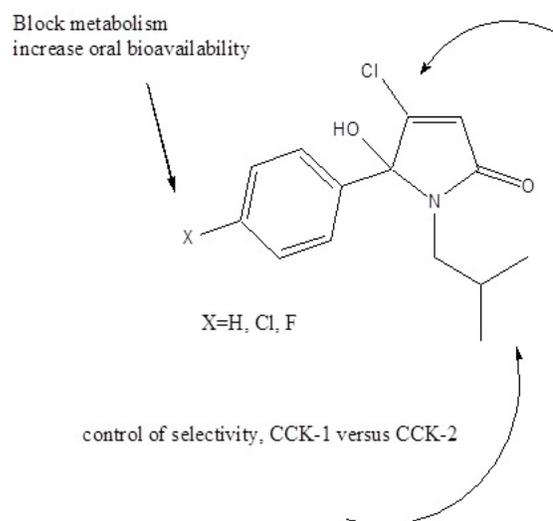


Figure 4: Selected key features of isobutyl-lactams.

Generally, within the isobutyl series the halogen atom on the 5-arylated ring enhanced the binding affinity. The IC₅₀ was enhanced from 20 nM for PNB-081, towards the 10nM range for the halogenated entities. Most importantly the metabolism, such as an expected hydroxylation in the para-phenyl position, would be blocked, resulting in agents with improved pharmacokinetic properties. Under electrophilic catalysis the Michael position should allow the formation of a covalent bond leading to irreversible CCK inhibitors.

Molecular modelling studies were performed to rationalise drug receptor interactions of the Cl-lactam with the CCK1 receptor.

The isobutyl group of the ligand interacted with a hydrophobic cave of the receptor, centred at Ala-14. The carbonyl group in the 2- position bond via hydrogen binding towards the CCK receptor with Arg-9 and the N- atom of the lactam interacted with Glu-17. The 5-hydroxyl group of the lactam ligand displayed interactions with of Asn-6, which are outlined in Figure 5.

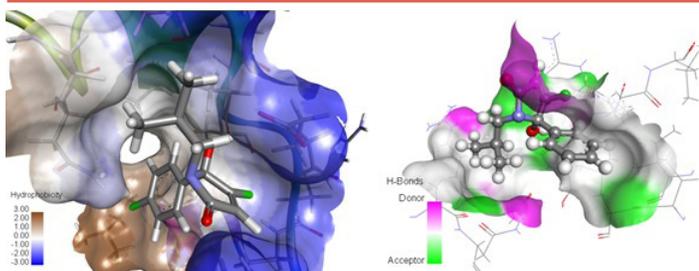


Figure 5: Drug receptor interactions of Cl-lactam with the CCK1 receptor.

The phenyl group has no interaction with tryptophan or phenylalanine and Pi - alkyl interactions of the chloro-phenyl group with Leu-29 and Ile-28 explain the small increase in specific binding. The ligand acted as irreversible inhibitor *in vitro* and the 3rd extracellular loop of the CCK1 receptor with the natural ligand CCK8s binding site, was selected to initiate modelling. It was assumed that irreversible, possibly covalent interaction with this loop of the receptor may deform the site and explain the superiority of the antineoplastic activity of this class of agents compared with competitive inhibitors.

Pharmacology

Opiate agonists, such as morphine and CCK antagonists, such as lorglumide and devazepide reduced electrically induced contractions on the GPI. From the radioligand binding assay the iso-butyl-pyrrolones were identified as the most potent ligands, and the classical isolated tissue preparation served as initial functional assay, confirming the antagonistic properties of these ligands.

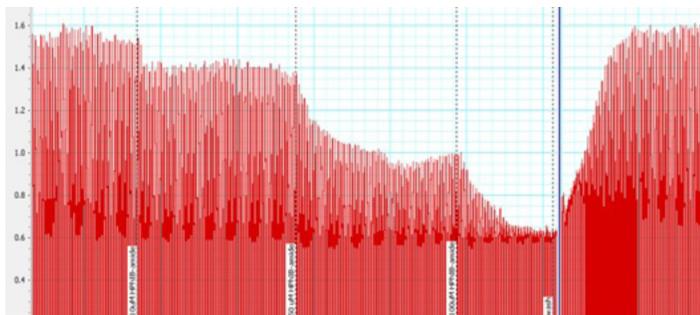


Figure 6: The inhibitory effect of 0.1, 0.5 and 1.0 μM of isobutyl - pyrrolone on electrically stimulated rat duodenum tissue contractions.

Using the isolated rat duodenum preparation, a stable amplitude was generated and a reduction of this amplitude was observed dose dependently for the isobutyl series, which is outlined in figure 6 for the isobutyl lactam. This assay represents a fast and efficient way to screen for CCK antagonists using classical isolated tissue preparations.

Cholecystokinin, CCK8s, induced contractions of the guinea pig gall bladder and this second tissue based assay was applied to reconfirm with this standard preparation the antagonistic properties of the potent CCK ligands.

CCK-8s induced contractions of the gall bladder and these contractions were reduced dose dependently, which is outlined for the PNB molecule in figure 7. The concentration - response curve

was calculated based on the percentage response to 10^{-5} M ACh for a better comparison. Increased concentrations of the antagonist were added to the bath cumulatively and a shift of the curve to the right was observed.

The function of the halogen atom is to enhance binding affinity/ increase lipophilicity and most importantly, to block metabolism of the molecules in the para - phenyl position.

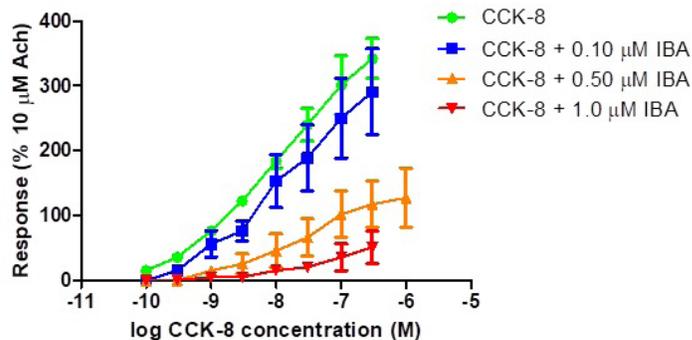


Figure 7: Mean cumulative concentration–response curve for CCK-8s in the absence and presence of 0.1, 0.5 and 1.0 μM lactam determined for the isolated guinea pig gallbladder (Internal standard: ACh $10 \mu\text{M}$ = 100 % contraction).

It appeared that the effect of the antagonist in the gallbladder assay is insurmountable and an irreversible inhibitor is ideal for the development of antineoplastic agents. Reactive chlorine in the 4-position may be suitable for covalent drug receptor interactions, explaining an irreversible antagonism for the isobutyl- pyrrolone series.

The antineoplastic properties of selected NCE and standard CCK antagonists, such as L-365,260 (CCK₂), devazepide and lorglumide (CCK₁) was subsequently investigated, using a range of CCK associated cancer cell lines.

In vitro cancer cell based testing

CCK antagonists are associated with an array of therapeutic applications, but the focus of our research programme was to provide a non-toxic orally available CCK antagonist for the treatment of cancers.

In vitro tests - Cytotoxicity assays

In vitro tests were originally performed using a cell count assay, as the aim was to inhibit proliferation and not to optimise cytotoxicity, but the results were identical in the methylthiazolyltetrazolium (MTT) assay, possible due to induced apoptosis. It was screened for an inhibitor of viability in certain CCK related cancers cell lines using the MTT assay.

MIA PACA: Miapaca is a human pancreatic carcinoma cell line and selected data are outlined in Table 2. The isobutyl pyrrolone was found 20 times better in terms of IC₅₀ concentrations than lorglumide. Devazepide, which gave an IC₅₀ about 1 μM showed agonistic activity (unpublished results). Lorglumide, a CCK₁

antagonist, was acting on the gallbladder CCK₁ receptor and not the pancreatic CCK receptor. None of the known CCK antagonists, devazepide or lorglumide proved to be clinically useful, which is in line with our results.

PANC: The PANC cell line is a human pancreatic cell line. PNB-081 and PNB-028 are many times better than devazepide / lorglumide on this selected pancreatic cell line. Cancer treatment is combination therapy, even for hormone dependent cancers. For this pancreatic cell line the synergistic effects of MK-329 = devazepide in conjunction with cisplatin were reported [24] and studies to investigate PNB-028 with cis - platin and 5-FU are ongoing.

Overall, the isobutyl pyrrolones were not significantly different from each other and PNB-81 as well as the halogenated fluorinated PNB-028 were tested subsequently *in vivo* in xenograft models. *In vitro* in cell based assays they were found of equal selective toxicity towards 2 human pancreatic and 2 colon cancer cell lines. The cytotoxicity results for CCK related cell lines are outlined in table 2 with lorglumide and devazepide as CCK₁ standard and L-365,260 as CCK₂ standard.

IC ₅₀ [nM]	MIA PACA	PANC	MAC13	MAC16
L-365,260	>5000	>1000	>5000	>5000
Devazepide	1200	480 ± 32	1100	1000
Lorglumide	425 ± 23	316 ± 21	421 ± 11	861 ± 22
PNB-081	15 ± 4	27 ± 9	536 ± 32	96 ± 25
PNB-028	11 ± 4	18 ± 6	508 ± 35	76 ± 20
Cl-lactam	13 ± 4	22 ± 9	526 ± 34	86 ± 23

Table 2: Cytotoxicity assay, IC₅₀ of selected examples against a variety of GI and brain cell lines. IC₅₀ values are based on inhibition of viability in the MTT assay in various cell lines.

MAC 16: MAC 13 and MAC 16 cancers are derived from the colon of the mouse and MAC 16 is of particular interest, as it is resistant to alkylating agents.

For PNB-081, an IC₅₀ about 100 nM was determined for the MAC 16 cell line and the fluorinated analogue PNB-028 was slightly more potent. Most interestingly, no other CCK antagonist, such as L-365,260, devazepide or lorglumide was found active for this cell line, which is, when transplanted into mice lethal within weeks. Therefore, it may be concluded, that the unsurmountable irreversible properties of the antagonists are key to colon cancer.

Subsequently, this MAC 16 tumour was studied initially in mice to analyse, if the *in vitro* results can be translated into relevant anti-tumour activity.

Animal studies in mice

Cholecystokinin is known to mediate cancer progression and metastasis, which were antagonised by sufficient concentrations of devazepide [27]. One animal in vehicle-treated group died during the course of the study, which is typical for MAC 16 induced

cancer, showing the lethality of this selected cancer model.

Allograft colon cancer *in vivo* study

MAC 16 tumours developed quickly within 6 days after implantation and grew robustly. Once the tumours reached 100 mm³, the animals were divided into the various treatment groups and treated with vehicle or the fluorinated PNB-028. Iso-butylated pyrrolone inhibited the tumour growth significantly from the day treatment was initiated and maintained tumour inhibitory activity until sacrifice. At sacrifice, interestingly in some animals growths was not only inhibited, but tumours shrank after reaching a maximum volume. Most interestingly, while MAC 16 tumours in the vehicle-treated group metastasized to lungs, tumours in drug-treated groups did not metastasize to lungs.

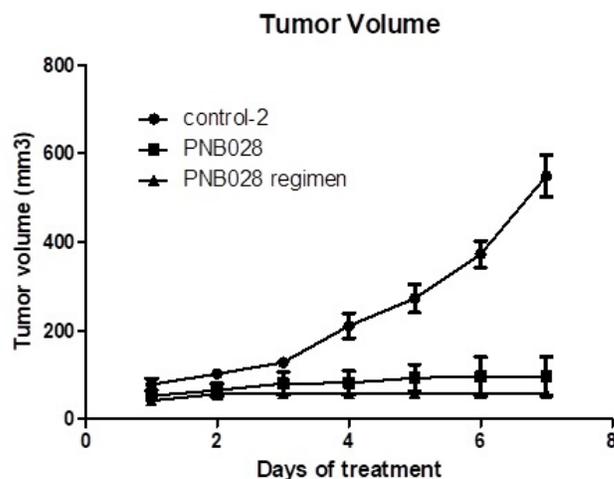


Figure 8a: Tumour volume in mice for the treatment with PNB-028 over the period of 7 days.

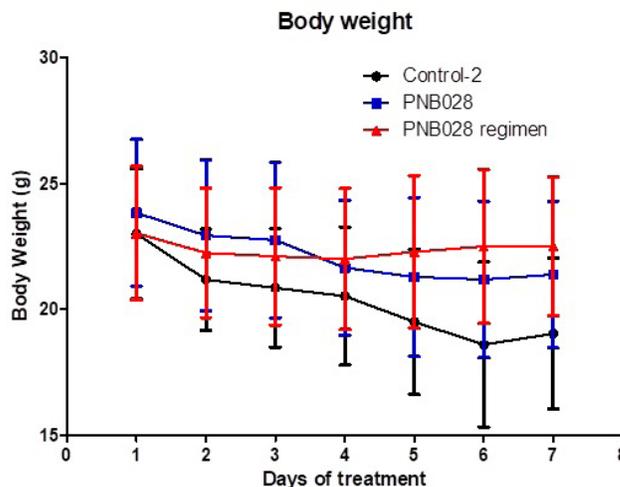


Figure 8b: Body weight loss during PNB-028 treatment in mice over the period of 7 days.

The control showed nearly exponential growths for this aggressive tumour. The treatment group by oral administration at optimum level (100 mg/kg) showed strong inhibition of tumour growths and the regiment study with bi-daily instead of daily dosing intervals (24hr-48hr) with a calculated daily dose of 50 mg/kg showed

enhanced activity and reduced toxicity (Figure 8a) The analysis of body weight showed an expected loss of 20%, which lead to termination at day 7 in the control group and for the 48h regimen even an increase of body weight was found (Figure 8b).

Cholecystokinin is known to mediate cancer progression and metastasis, which were antagonised by sufficient concentrations of devazepide [27] in vivo in mice, but translation failed clinically as inhibition was reversible with devazepide and the receptor occupancy was also insufficient. Colon cancer is generally linked with the CCK₁ receptor and only under certain conditions it is associated with the CCK₂ receptor [28]. Colucci tested only one 1 nM concentration of devazepide, which stimulated growths in the study and by our own observations [12]. Overall for the MAC 16 cell line proliferation was blocked *in vitro* and *in vivo* by PNB-028. Here, the link CCK₁ receptor and colon cancer was confirmed experimentally.

Efforts to elucidate the underlying anticancer action were made and no significant apoptosis and no influence on angiogenesis by angiogenesis marker CD34, was observed. Proliferation, analysed by proliferation marker KI67, was performed and the results were outlined in figure 9.

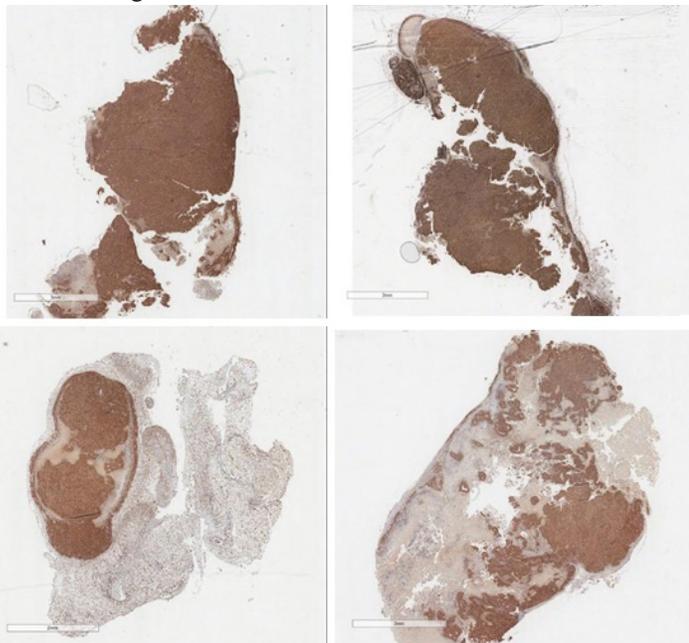


Figure 9: Histology. Ki67 staining: top control, bottom PNB-028 treated 50 mg/kg by PO administration.

For the minimal dose of 50 mg/kg in mice, the tissue was stained (TOP) and in the treatment group large white areas of proliferation free tissue was seen (figure 9). These findings are particularly important for the understanding of the standard colon cancer biomarker CEA. Dell death, as a result of apoptosis caused by Pt agents and antimetabolite 5-FU resulted in an increase of biomarker CEA, followed by a decline from week 4 onwards in man. The cell lines differ in CCK expression and the selected

pancreatic cancer cell line in vivo has some expression of the cholecystokinin receptor.

MIAPACA tumour xenograft study

MIAPACA tumours developed quickly after implantation and grew modestly, though not as robustly as MAC 16 tumours. Once the tumours reached 100 mm³, the animals were divided into various treatment groups and were treated with vehicle or respective drugs. Though vehicle-treated MIAPACA tumours grew from 100 mm³ to over 750 mm³ within 3 weeks, pyrrolone treated tumours grew slower.

Both agents inhibited tumour growth significantly from the day treatment was initiated and maintained the growth inhibitory properties until sacrifice (Figure 10).

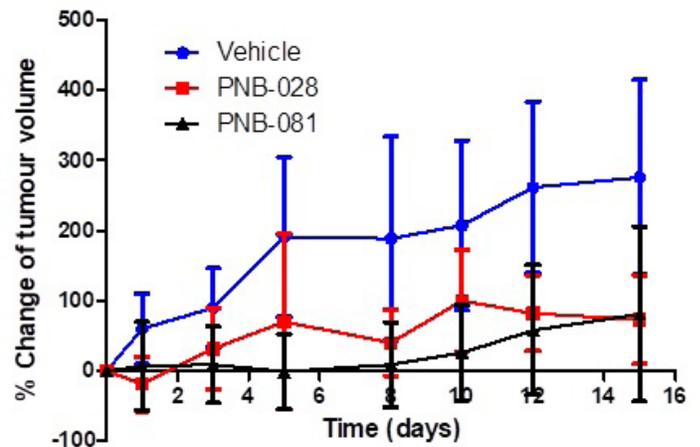


Figure 10: Effect of PNB molecules on MIAPACA pancreatic cancer tumour growth.

Overall, it was clearly shown, that colon and pancreatic cancers response to CCK₁ antagonists and not CCK₂ antagonists [29]. We agree, it is unlikely that, gastrin is more important in pancreatic cancer than cholecystokinin. PNB-028 was clearly more efficient in colon cancer, based on the MAC16 murine colon cancer cell line. For pancreatic cancer the results are even more interesting when the oral bioavailability is considered. It may be wise to say, that it is pancreozymin, via the pancreatic cholecystokinin receptor, that promotes cancer or possibly the CCK-C receptor. Smith clearly stated the role of the CCK-C receptor in pancreatic cancer [30] and reviewed the cholecystokinin receptor as molecular target in treating pancreatic cancer [31].

If it is the CCK-C receptor, then this molecular target would antagonist best in the treatment of cancer as the CCK1 receptor is required for the physiological role of cholecystokinin. Blocking of the contractions of the gall bladder leads to gallstones in the long term and reductions of bile flow will result generally in liver toxicity.

Pharmakinetetic analysis

For lactam PNB-081 an oral bioavailability was determined as 24% in rats for the 10mg/kg dose. PNB-028 had 98% oral bioavailability

and represents the CCK1 antagonist with the highest known bioavailability of all published molecules. The analysis of the plasma concentration in rats was analysed for PNB-081 (24%) and is outlined in figure 11.

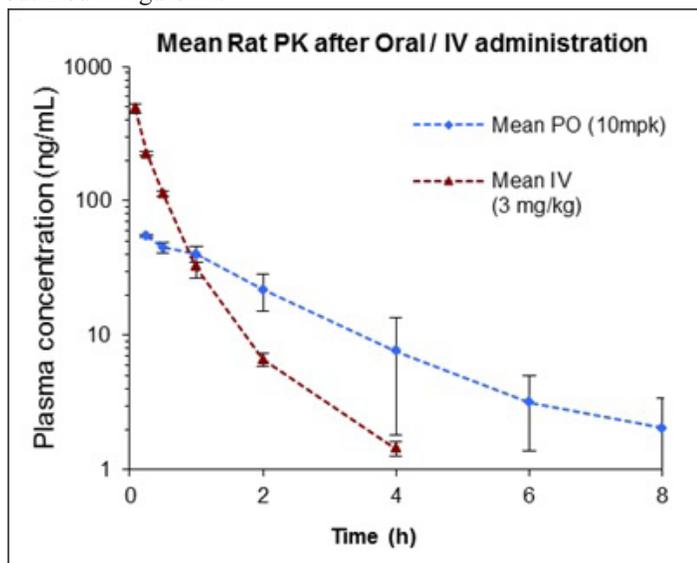


Figure 11: Kinetics of PNB-081.

The isobutyl group, as aliphatic side chain, is inert towards metabolism and the bioavailability for PNB-081 is medium with 24%. After the introduction of a fluorine atom in PNB-028 a nearly full oral bioavailability was achieved. This CCK antagonist occurred the best bioavailability compared to present competitors and will enable us to achieve plasma concentrations to shut down CCK mediated cancer growths pathways.

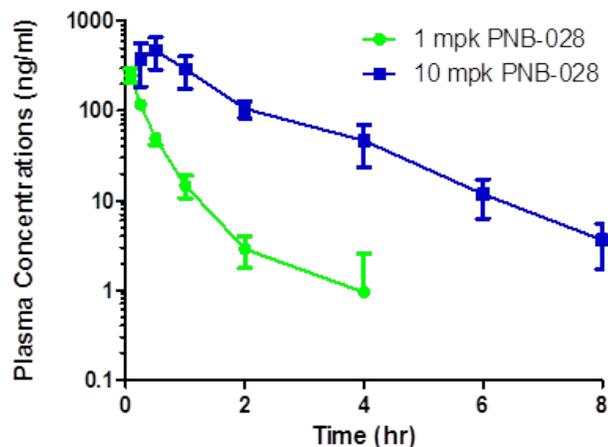


Figure 12: Kinetics of PNB-028, bioavailability.

PNB-028 was best agent in murine colon cancer models and the results for pancreatic cancer under the consideration of plasma require further analysis. In nude mice both agents are not significantly different, but apparently PNB-081 is giving higher inhibitions. Considering the 4 times higher bioavailability of PNB-028 over PNB-081 this effect should be achieved by only a quarter of the dose. So, based on a bell shaped dose response curve known

for CCK antagonists, a dose reduction may have given the same or even better effect, similar to the effect observed in MAC16 models.

Conclusion

This novel arylated hydroxyl-pyrrolone template was synthesised in only 2 synthetic steps in high overall yields from readily available starting material.

For the potent CCK1 ligands, a full non-competitive, possible irreversible CCK antagonism was confirmed classically, using isolated tissue preparations, which is ideal for an antineoplastic agent. For this novel template, it was found, that CCK antagonism translated into anti-cancer activity within the GI system and the known CNS activity of CCK antagonists may be of additional therapeutic benefit in treating cancer-associated diseases.

The isobutyl series stopped the growths of colon- and pancreatic cancer models. A full pre-clinical development programme was completed for PNB-028 and IND documents were submitted to launch a combined first in man clinical trial in patients at Lambda in India.

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