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Pre-Clinical Evaluation of CCK_2 Antagonist PNB-001 (4-Chloro-5-Hydroxy-1-Phenylethyl-5-Phenyl-1,5-Dihydro-Pyrrol-2-One) Towards The Design For A First-In-Man Clinical Trial

Eric Lattmann[1,*], Jintana Sattayasai[2], Ramesh Narayanan[3], Zikra Ahmed[1], PN Balaram[4] and Pornthip Lattmann[4]

[1]School of Life and Health Sciences, Aston University, Aston Triangle, Birmingham B4 7ET, England
[2]Department of Pharmacology, Faculty of Medicine, Khon Kaen University, 40002 Khon Kaen, Thailand
[3]Department of Medicine, University of Tennessee Health Science Center, Memphis, TN, USA
[4]PNB Vesper Life Science PVT, Cochin, Kerala, India

***Corresponding author:** Dr Eric Lattmann, Aston School of Pharmacy, Aston University, Aston Triangle, Birmingham B4 7ET, England; Tel: +44-(0) 121 204 3980; Fax: +44-(0) 121 359 0733; e-mail: e.lattmann@aston.ac.uk

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Abstract

Study aim: To prepare and evaluate CCK gastrin antagonists PNB-001 for a first in man clinical trial and to provide preclinical formulations, which can be used in later clinical studies.

Methods: In vivo assay in rats, isolated tissue preparations, solid dosage forms, PK analysis to evaluate the anti-inflammatory analgesic PNB-001.

Results: The arylated 5-hydroxy–pyrrol-2-one PNB-001 was prepared in 3 synthetic steps from furfural. The selective CCK_2antagonist (PNB-001, IC_{50}= 22nM) was fully evaluated to design a clinical trial. In vivo evaluation revealed analgesic activity for the gastrin CCK2 antagonist PNB-001 in the tail immersion test at a dose range by IP and oral administration. Plasma concentrations were analysed in rats and in vitro it was shown, that these concentrations, are efficient in vitro using isolated tissue preparations. Solid formulations were prepared and tested active in rats.

Conclusion: From bench side to bed side, the preclinical development for PNB-001 is completed and data formed the foundations for a first in man trial, phase 1.

Keywords: Phenyl-pyrrolone, CCK antagonist, cholecystokinin, gastrin, pharmacokinetics, analgesic, isolated tissue preparations.

Introduction

In terms of cholecystokinin-physiology [1], CCK_8 is the most common peptide hormone, which is extensively found throughout the gastrointestinal tract (GIT) and is also widely distributed through the nervous system [2]. Originally, cholecystokinin was discovered to cause contractions of the gallbladder [3]. It was then rediscovered as pancreozymin, triggering the release of pancreatic enzymes. Finally, it was confirmed that both peptides are identical [4]. Cholecystokinin acts as a neuromodulator as well as gut hormone. CCK-ligands, agonists and antagonists have been extensively investigated as potential drug molecules [5] and here, is was focused on the preclinical evaluation of front runner CCK antagonist PNB-001.

Cholecystokinin antagonists have been extensively investigated as potential drug targets [6]. They were studied as growth inhibitors in certain forms of cancer [7], as anxiolytics [8], in the treatment of schizophrenia [9] and satiety [10]. Cholecystokinin 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.

Asperlicin was the first non-peptidal lead structure from nature [11] and analogues thereof, were studied as CCK ligands [12]. Simplification of this lead structure by Merck led to Devazepide [13], a potent CCK_1 selective cholecystokinin antagonist. Proglumide [14] was the first glutamic acid based agent, marketed as Milid for the treatment of ulcer. The indolyl amide of devazepide was replaced by a urea linkage and Merck's L-365,260 resulted in a CCK2 selective antagonist [15]. Further subsequent SAR optimization led to Zeria's improved Z-360 [16], in which the N-alkyl side chain, the 5- position (cyclohexyl) was optimized for potency and a meta-carboxylic acid on the aryl urea linkage was introduced to enhance water solubility (Figure 1).

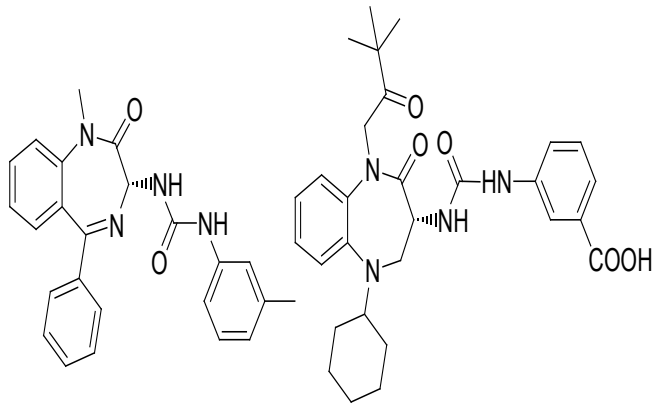


Figure 1: CCK –gastrin antagonists.

Z-360 is a CCK2 –gastrin receptor antagonist and progressed into phase 2 trial with pancreatic cancer [17]. Z-360 is the most recent derivative derived from this original lead structure, with improved selectivity and bioavailability

Molecular pain targets have been reviewed recently [18] and the results are quite disappointing in terms of efficacy and FDA approval rate. Even, this review missed out on CCK antagonists [19] and most importantly on avery positive report, publicised only in form of an abstract [20]. In summary in this study, it was shown that the CCK antagonist devazepidewas found very efficient in pain management at low doses as adjunct to strong opiates in a phase 2 trial. Initial results for CCK antagonists of the pyrrolone scaffold were communicated in the area of cancer therapeutics [21] and GI inflammation [22].

Here, a full biological evaluation of PNB-001 leading to a first in man clinical trial will be analysed and reported with respect to inflammatory pain [23]. It is focussed on the correlation of isolated tissue preparations in rats with in vivo tests in the same species to be transferred in man in a planned phase 1 study.

Materials and Methods

General 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 using a Stuart Scientific (Coventry, UK) apparatus and are uncorrected.

Preparation of 3,4-dichloro-5-phenyl-5H-furan-2-ones, reaction intermediate 1: Synthesis of 3,4-dichloro-5-phenyl-5H-furan-2-one

Dry and powdered aluminium chloride (20g, 0.15 mol) was added slowly to a mixture of mucochloric acid (16.9g, 0.1 mol) and benzene/chlorobenzene (250 ml). The reaction mixture was stirred overnight. It was then poured into a mixture of 100 g ice and 32 ml concentrated hydrochloric acid. The organic layer was separated by separating funnel and washed with 3 x 100 ml 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%; mp: 78-79°C; MS (APCI(+)): 195/197 (M+), 230/232 (M+1) m/z; ^{1}H NMR (CDCl3) 250 MHz: δ = 7.22-7.51 (m, 5H), 5.81 (s, 1H); ^{13}C NMR ($CDCl_3$):165.3, 152.2, 139.8, 130.5, 129.3, 128.5, 127.4, 127.2, 121.2, 83.5; IR (KB_r-disc) υ max: 3445, 3074, 3035, 2959, 2056, 1768, 1630, 1499, 1457 1294, 1224, 1028, 910, 772, 705 cm^{-1}.

Preparation of N-substituted-4-Chloro-5-hydroxy-5-phenyl-1,5-dihydro-pyrrol-2-ones, stage 2 products; general method:

The relevant amine (2.3 times excess) was added to a solution of lactone (0.7 mol) in ether (10 ml) and it was stirred on ice for 30 minutes, allowing to warm up to RT over time.

The resultant mixture was poured into 5 ml of water and was separated by a separating funnel. The organic mixture was washed with water three times. The organic layer was dried over magnesium sulphate and the solvent was removed under vacuum. All compounds gave an oily solid, which were passed through a short silicagel column (80% ether, 20% petrol ether). The resulting fractions were dried from excess solvent under a stream of argon to yield crystals.

4-Chloro-5-(4-chloro-phenyl)-5-hydroxy-1-phenethyl-1,5-dihydro-pyrrol-2-one PNB-001

Yield = 45 %, mp: 145-148 °C; MS (APCI(+)): 348/350/352 (M+) m/z; ^{1}H NMR ($CDCl_3$) 250 MHz: δ= 7.22-7.49 (m, 7H), 7.12-7.18 (m, 2H), 6.13 (s, 1H), 3.68 & 2.64 (m, 2H), 2.88 (m, 2H); 13C NMR ($CDCl_3$) 250 MHz: 167.7, 155.5, 138.8, 135.5, 133.3, 129.1, 128.8, 128,7, 127.7, 126.7, 121.9, 92.3, 42.0, 34.5; IR (KBr-disc) 3421, 3228, 2925, 2848, 2370, 2338, 1684, 1658, 1606, 1461, 1406, 1248, 1190, 1097, 935, 806, 697 cm^{-1}.

Molecular modeling

For target preparation the protein structures, pdb identifier 1HZN for the CCK1 and 1L4T for the CCK_2 –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 CCK_1 / CCK_2 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.

Radioligand cholecystokinin binding assay

CCK_2 and CCK_1 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. [24] Pancreatic membranes were prepared as described by Charpentier et al. [25] 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^{0}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, 1mM 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.

Animal studies

Experiments were conducted in male standard IRC mice obtained from the animal house, Faculty of Medicine, KhonKaen University. Each experimental group consisted of 6 animals and the treatment procedures were approved by the ethical committee, Faculty of Medicine, KhonKaen University (BEA030699).

Mice were intraperitoneal injected with test compound at the volume not more than 0.2 ml/animal. The test molecule was dissolved in DMSO and water was added to give a 5% solution for IP and PO administration. 30 min after drug administration, animals were tested as described in the following sections.

Nociception test

The tail immersion test: The thermal response latency was measured by the tail immersion test. The animals were placed into individual restraining cages leaving the tail hanging freely. The tail was immersed into water at 50°C. The response time, at which the animal reacted by withdrawing its tail from water, was recorded and the cut-off time was 10 sec in order to avoid tissue damage. The base line withdrawal thresholds (BT) were recorded prior to the first injection. Test thresholds (TT) were measured 60 min after the second injection. The test thresholds were expressed as a percentage of Maximal Possible Effect (% MPE) using the equation:% MPE = {(TT-BT) / (45-BT)} x 100; DMSO (5 %), pyrrolone (in 5 % DMSO) was intraperitoneally injected and morphine was administered subcutaneously.

PK analysis

6-8 weekmale 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. 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 150µL 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 (~75µl 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, 5µ) 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.

Formulation studies

Preparation of tablets

The first three Placebo Batches were compressed using the Riva MiniPress tablet press (type MII, Argentina) at a compression force of 21kN. The subsequent Placebo Batches were compressed using a (Specac's Manual Hydraulic Press, UK). Each tablet on the Specac's Handpress was subjected to a similar compression force as the Riva MiniPress (21kN = 2 Tons) for 30 seconds.

Dissolution

The dissolution of both tablets and capsules from Formulation 1 and Formulation 2, in 900 mL of de-ionised water and phosphate buffer (pH 6.8) at (37 °C ± 0.5 °C) was tested using a dissolution apparatus (Erweka DT-600, Germany) operated at a speed of 50 rpm. Samples form the dissolution media were withdrawn at fixed intervals (0 min, 5 min, 15 min, 30 min, 45 min, 60 min, 90 min, 120 min, 150 min, 180 min, 210 min and 240 min), filtered, and then the absorbance of each sample was determined using a UV spectrophotometer (Jenway 6305 spectrophotometer, Bibby Scientific Ltd, UK) at 201 nm. Fresh phosphate buffer or de-ionised water was returned when each sample was withdrawn to maintain sink conditions. Six tablets were tested from each formulation (n=3 in de-ionised water and n=3 in phosphate buffer pH 6.8) and the results were averaged (n= 12 tablets). Similarly, this method was repeated for the capsule dosage form n= 12 capsules).

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% O_2 / 5% CO_2.Tyrode's solution was freshly prepared daily (g/l): NaCl, 8.0; KCl, 0.2; $CaCl_2$, 0.2; $MgSO_4$, 0.1; NaH_2PO_4,0.05; $NaHCO_3$,1.0; Glucose,1.0.The main equipment used was the Radnoti single unit tissue bath system with a chamber capacity of 35ml. Bath aeration with carbogen (O_2 95%, CO_2 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.

General procedure

From the isolated tissue preparation, strips of appropriate length were mounted vertically in organ bath containing Tyrode's solution, under a tension of 1g and allowed to equilibrate for 30 minutes. Agonists, such as CCK_5 and CCK_{8S}were directly applied in the bath and antagonists were pre-incubated for 10min.

Stock solutions of all test compounds including the standard were prepared in DMSO.

CCK_5, penta-gastrin preparations

CCK_5 was dissolved in distilled water to prepare a stock solution of 500μM 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 lorglumidewere added to the organ bath 10 minutes before exposure to the next CCK5 serial concentrations.

DSS induced spontaneous muscle contractions in rat duodenum

A solution of 0.1% DSS dextrane sulfonic acid sodium was freshly prepared in Tyrode solution and it was added to the bath to give 100-300 microM final bath concentration. Within 30 min contractions were induced and stable. The amplitude of these contractions was measured for the test molecules.

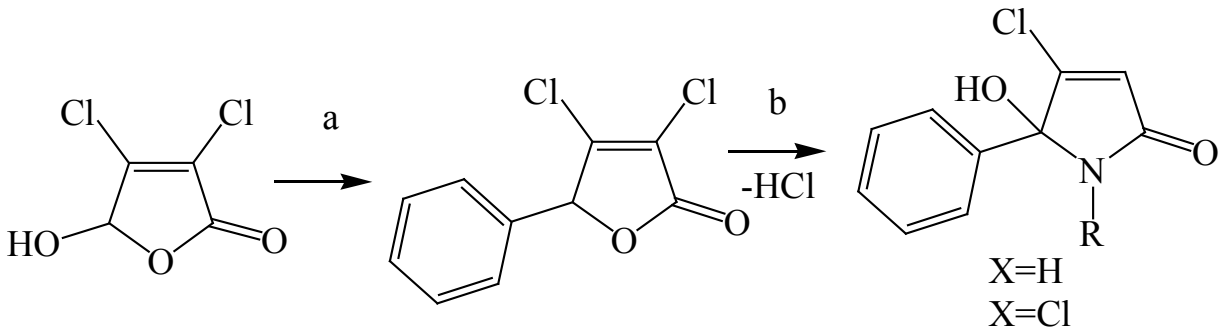
Statistical methods

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

5-arylated dichloro-2(5H)-furanoneintermediates were evaluated previously as anticancer agents [26]. Overall, the desired N-alkylated unsubstituted 5-phenyl pyrrolone PNB-001 was obtained in only a 2 stage process as white crystalline material.The molecule is not present in the ring opened keto form and fully occurred in the 5-membered ring form, as a hydroxy-pyrrolone. The analysis of PNB-001by chiral HPLC showed a 50:50 racemic mixture of both enantiomers in solution in methanol.5-arylated dichloro-2(5H)-furanones were synthesised from mucochloric acid (Scheme 1), which is commercially available from furfural under oxidising conditions with hydrochloric acid. Mucochloric acid was reacted with benzene as reagent and solvent at RT under the development of hydrogen chloride gas. Depending on the scale of the reaction cooling with ice was required. For the small scale synthesis aluminium chloride worked well as Lewis acid. However, during scale up aluminium chloride was replaced by trifluoroborane in THF as the exothermic reaction become problematic on a kg scale.



a) Arene, RT, 10 h, workup: hydrochloric acid; b) amine , excess, ether, RT, 30 min

Scheme 1. Synthesis preparation of lactams from mucochloric acid.

Subsequent reaction of the 5-arylated 3, 4-dichloro-2(5H)-furanone (Stage 1 intermediate) in diethylether with phenylethylamine furnished N-alkylated hydroxyl-pyrrolone (Stage 2 product, PNB-001) in high yield under mild conditions. The general synthetic sequence is outlined in Scheme 1.

SAR optimisation

The first step was to screen for potent binding affinity and to identify a CCK_1-or CCK_2-selective ligand for subsequent *in vitro* and *in vivo* evaluation. Using radiolabelled iodinated cholecystokinin, inhibition of binding was determined for all test molecules and the IC50 are outlined in Table 1. Lorglumide served as CCK_1 standard and L-365,260 was used as CCK_2 standard.

The introduction of a spacer, a single CH2 group, resulted in a phenyl-ethyl derivative, which represented a highly CCK_2 selective ligand. Lactam PNB-001 is 450 times selective for the CCK_2 / gastrin receptor

Halogenation, the introduction of a para-chlorine atom on the phenyl-position, lactam 20, did not enhance binding affinity any further; possibly due to drug receptor interaction of the phenyl group with a lipophilic cavity within the CCK receptor.

Molecular modelling

The docking of PNB-001 into the CCK_2 receptor is outlined in figure 3 and some key interactions are highlighted-for one final pose of minimal energy (Figure 3).

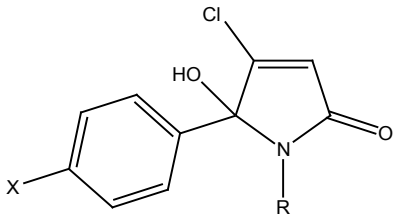
Lactam	X=	R=	CCK_1 [μM]	CCK_2 [μM]
PNB-001	H	Phenylethyl-	>10	0.022 ± 0.002
Lorglumide			0.17±0.01	>10
L-356,260			0.25±0.01	0.003±0.001

Table 1. CCK binding affinity expressed in IC_{50} in micromolar using iodinated hot CCK8 as radioligands with cortex and pancreatic membranes; N=3.

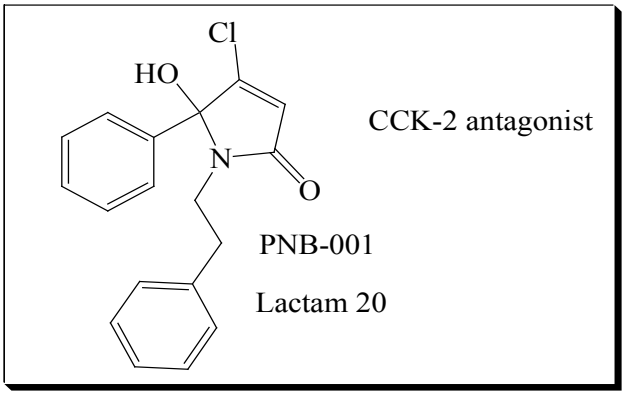


Figure 2. Chemical structure of CCK2 gastrin antagonist PNB-001, a potent anti-inflammatory analgesic.

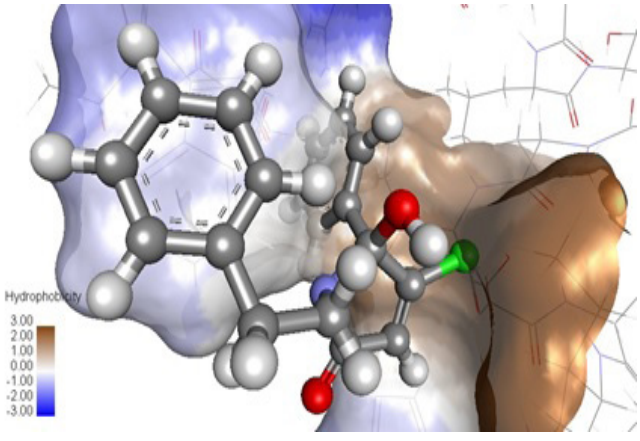


Figure 3. Docking of CCK antagonist PNB-001 into the CCK_2 receptor.

The 5-hydroxy group of the central pyrrolone template interacted via hydrogen binding with the N group of Trp114. The phenyl group of the N-phenylethyl- side chain bound to the aromatic indole system of Trp114 and electron withdrawing groups may enhance these aromatic interactions. The lipophilic pocket allowed principally a wide range of substituents, but only phenyl and not cyclo-hexyl could be realised synthetically. The 5- phenyl group of the pyrrolone template bound via Ile 184 and Leu 133, based on van der Waals interactions and not aromatic interactions. The introduction of electron withdrawing groups, such as halogen atoms, is therefore not enhancing affinity and optimisation on this side is with limited effect. Gaining dual CCK–gastrin antagonistic activity was found beneficial in analgesia potentiation [27] as well as for anxiolytics [28], but here direct analgesic activity and the anti-inflammatory effect is entirely linked with the CCK2 –gastrin receptor.

Pharmacology

Under consideration of failed clinical trials for panic [29], positive pain results and our own results with respect to inflammation, our attention turned to full systematic in vivo evaluation of PNB-001 as anti-inflammatory analgesic. The anti-inflammatory action of the molecule, supported by anti-gastric activity, was tested and re-tested in mice and in rats. Efficacy results in rats are important as the PK analysis is most preferred performed in rats.

In vivo evaluation

CCK antagonists potentiate the analgesia of opiates and usually [30] have no analgesic effect on its own. For Z-360 an interesting weak analgesic effect in the formalin test was observed. Thus, a first evaluation of the analgesic properties of PNB-001 was performed using the formalin test and it was found highly active. Most drug administration in rodents are performed in mice and rats by IP administration. The tail immersion assay is a reliable working assay and it was applied here in rats by PO administration. Aim was to have a confirmed PO dose in rats and then to analyse the C_{max} to guide clinical trials in man.

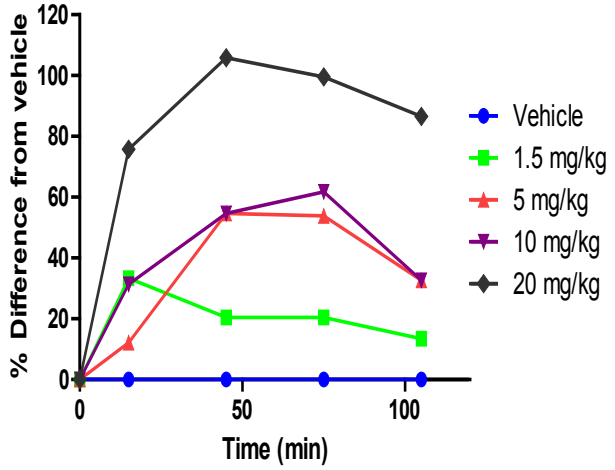


Figure 4. Dose range of PNB-001 in the tail immersion test by PO administration using rats.

The tail immersiontest was performed in rats by oral administration. Rats were used as a second species and the route of administration was changed from IP to PO administration (Figure 4). The % change from the vehicle was assessed and the results are outlined in figure 4. For the 1.5mg/kg dose a 20% change from the vehicle was achieved, which increased dose dependently towards 20mg/kg. A 50% change from the control was observed for 5 and 10mg /kg, while the maximum response was found for 20mg/kg in rats by PO administration after 1h. Rats seem to be loss responsive compared to mice by factor 2, but the active dose range confirmed the oral bioactivity of about 10%, which was obtained in PK analysis *in vivo* in rats. It was found that 0.5mg/kg by IP administration was found equivalent to 5 mg/kg by oral administration.

Activity was achieved in the rat orally in the range from 10 mg/kg and the question was which plasma concentration would be obtained for this dose range in rats.

Pharmacokinetics

As part of the preclinical development, the pharmacokinetics of PNB-001 were fully analysed. In summary PNB-001 showed a good half-life in dog and rat liver microsomes. Protein binding was determined of 92.4% in human plasma and a very high membrane permeability was determined by using the Caco-2 monolayer assay. In terms of regulatory toxicology, for PNB-001 a slight increase of ALT was observed in dogs, but not in rats at doses > 1000 mg/kg and only after 90 days.Here, the solid dosage form of PNB-001 was investigated in vivo using rats.

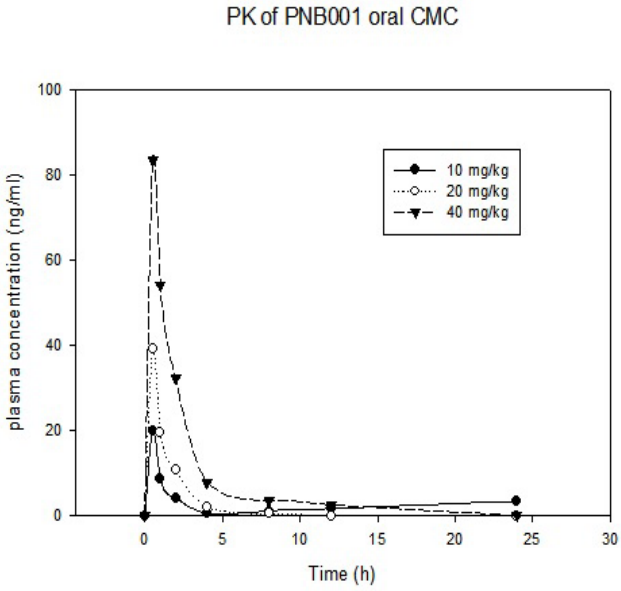


Figure 5a. PK analysis of PNB-001 in rats by oral administration.

Generally plasma concentrations are very low in line with the very high protein binding, which is essential to carry the molecule throughout the body (Figure 5a). 10mg/kg achieved a maximum concentration of 20ng/ml and the increased dose resulted in a dose dependent increase of plasma concentration towards 80ng/ml for the 40mg/kg dose.

For the PK analysis in man it is essential to know, if there is a saturation, no correlation or a linear kinetics and in figure 5b the correlation is outlined (Figure 5b).

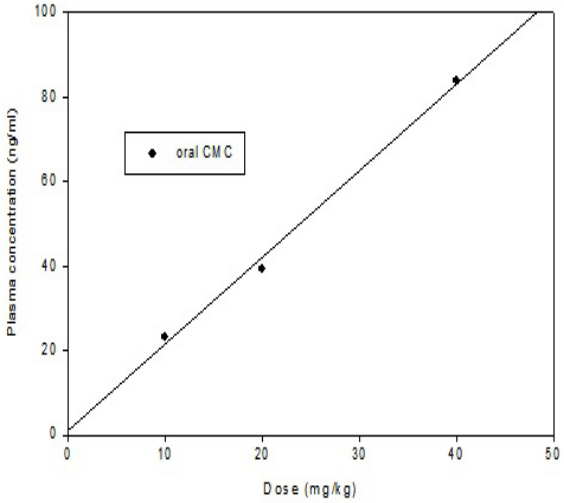


Figure 5b. Linear pharmacokinetics of PNB001 in rats in the active dose range.

From 10 – 40mg/kg a linear kinetics was obtained for this dose range in rats. Most interestingly, the administration as a solution, compared with the suspension, resulted in a much lowerC_{max}(not shown) and generally a much lower AUC. This finding was ideal, as the planned form of administration in man for phase 1 is not a solution, but also solid dosage form, such as a capsule or a tablet. Based on the body surface area the active 10mg/kg dose in mice would be equivalent to 70 mg dose in man and therefore 50 mg tablets were selected as first in man formulation.

Formulations

Nine different placebo batches were formulated (Table 2) with varying excipient concentrations to deduce the most optimum placebo batch where the drug can be incorporated into. 10% w/w drug loading of PNB001 was incorporated into 500 mg tablets containing various excipients as highlighted in (Table 2).

Capsules with a 50 mg dose of PNB-001 were formulated equally using the same excipients. In the subsequent pharmaceutical analysis a comparison was made to observe differences in dissolution rate between the two formulations. The appearance of the finished product (tablets /left and capsules /right) is shown in figure 6.

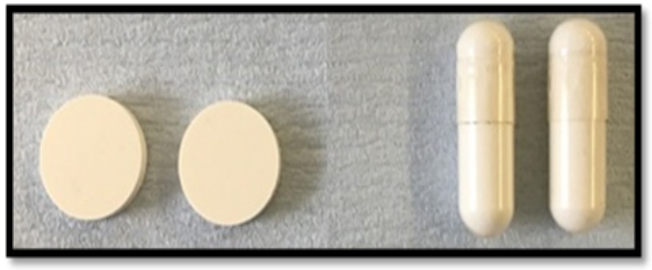


Figure 6. PNB001 50 mg tablets versus 50 mg capsules.

Material	Function	F1(g)	F2(g)
PNB001	Drug	1.6	1.6
Crospovidone	Disintegrant	…	…
Corn Starch	Disintegrant/ binder		
Wheat Starch	Disintegrant/ binder	4.32	4.32
Icing Sugar	Diluent	5.616	4.32
	Diluent/	4.32	5.616
	binder		
	Lubricant	0.144	0.144

Table 2. Various placebo batches and test formulations (F1 and F2) under investigation.

Since magnesium stearate is acknowledged to incur poor mechanical and dissolution properties because of its lubricant and lipophilic nature, only 0.5 to 1% w/w was used [31]. Two test formulations (Figure-1 and Figure-2) for both tablets and capsules were prepared by mixing PNB001 with the optimum placebo batches. Tablets passed disintegration time and content uniformity.

Disintegration time

Placebo Batches showed various disintegration times ranging from (0.8 min ± 3.1 min) to (14.4 min ± 1.6 min). Formulation-1 is a remarkable slow formulation, ideal for a slow release as required in IBD (42.1 min ± 2.8 min). All placebo batches and Formulation-2 passed the disintegration test achieving a disintegration time <15 minutes, which complies with the British Pharmacopeia, 2016 and is ideal for a quick formulation in anti-inflammatory pain (Table 3).

Product	Disintegration (min)
Formulation 1	42.1 ± 2.8
Formulation 2	12.2 ± 0.4

Table 3. Disintegration times of various placebo batches and test formulations under investigation (mean ± SD, n = 3).

The faster disintegration time of Formulation-2 could be accredited to Avicel PH102, which at a higher concentrations, has disintegrant properties when formulated with starch [32].

Dissolution studies

The dissolution profiles of Formulation-1 and Formulation-2 in water or buffer media from either tablets or capsules are shown in (Figure 7). It can be seen that the capsules showed significantly lower dissolution drug release rates compared to the tablets regardless of formulation type (Figure-1 and Figure-2). This was confirmed by the dissolution parameters which are outlined in Table 4.

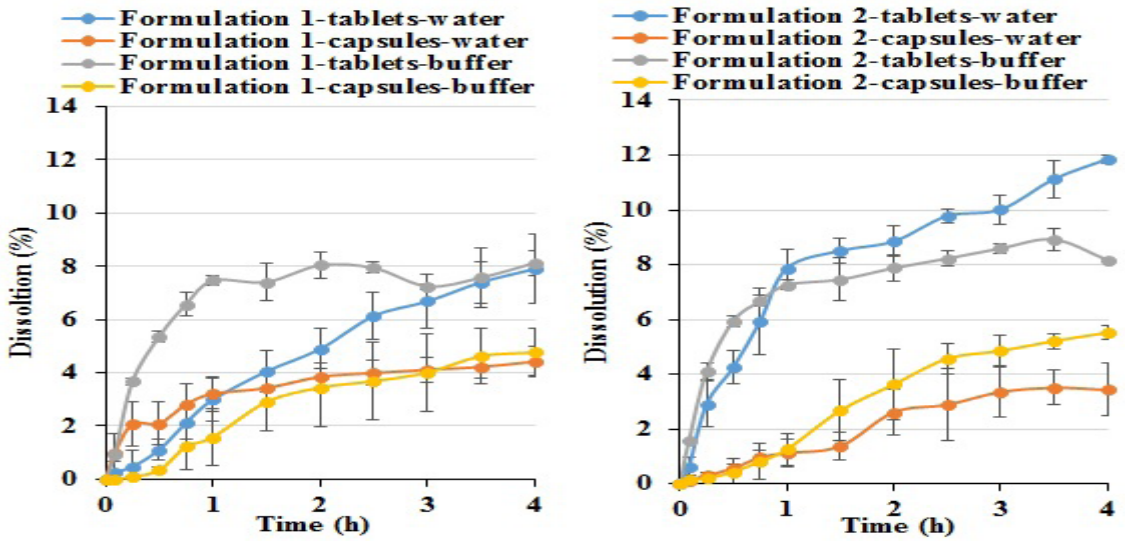


Figure 7. Dissolution profiles of the two formulations under investigation formulated into tablet or capsule solid forms dissolved in either de-ionised water or phosphate buffer (pH 6.8) media.

Formulation	Dosage form	Dissolution medium	DE (%)	MDT (min)	MDR (%min-1)	f2
Formulation 1	Tablet	Water	4.65 ± 0.79	1.64 ± 0.07	2.14 ± 0.79	76.2
		Buffer	6.95 ± 0.34	0.58 ± 0.08	4.00 ± 0.07	
	Capsule	Water	3.47 ± 0.56	0.87 ± 0.12	2.29 ± 0.74	91.6
		Buffer	2.88 ± 0.96	1.63 ± 0.41	1.17 ± 0.34	
Formulation 2	Tablet	Water	8.32 ± 0.43	1.19 ± 0.11	4.46 ± 0.41	86.1
		Buffer	7.38 ± 0.26	0.39 ± 0.12	4.40 ± 0.05	
	Capsule	Water	2.15 ± 0.62	1.49 ± 0.28	0.95 ± 0.28	91.1
		Buffer	3.17 ± 0.51	1.71 ± 0.30	1.34 ± 0.05	

Table 4. Dissolution efficiency (DE), mean dissolution time (MDT) and mean dissolution rate (MDR) (mean ± SD, n = 3) for the two formulations under investigation formulated in a tablet or capsule solid form dissolved in either water or buffer (pH 6.8) media.

The dissolution efficiency of Formulation-1 in buffer signifcantly decreased (P< 0.05) from (6.95% ± 0.34%) in the tablet to (2.88% ± 0.96%) in the capsule. The dissolution efficiency of Formulation-1 in water decreased from (4.65% ± 0.79%) in the tablet to (3.47%± 0.56%) in the capsule, although the difference was not significant (P >0.05). Similarily, the dissolution efficiency of Formulation-2 in buffer signifcantly decreased (P< 0.05) from (7.38% ± 0.26%) in the tablet to (3.17% ± 0.51%) in the capsule. The dissolution efficiency of Formulation-2 in water significantly (P< 0.05) decreased from (8.32% ± 0.43%) in the tablet to (2.15%± 0.62%) in the capsule.

Comparable conclusions could be drawn when considering the MDT and the MDR values (Table 4) because the capsule shells were made of HPMC which may have retarded drug release [33]. The exipients used within each formulation were insoluble with the exception of the icing sugar, which could have contributed further to the capsules slow release rate [34].

In the case of the tablet dosage form, the dissolution efficeny of Formulation-1 in buffer was slightly higher than that in the water dissolution media which is attributable to the PNB001's poor solubility in water (Table 4). However, in the case of capsule dosage form, no significant difference (P>0.05) was observed between the dissolution efficieny of Formulation-1 in water and buffer. Similarily, Formulation-2 capsule dosage form showed no significant difference (P>0.05) between the dissolution efficieny in water and buffer. Formulations 1 and 2 regardless of dosage form (i.e. tablet or capsule) or dissolution media (i.e. water or buffer pH 6.8) were not significantly different in their drug release profiles [35]. Direct compression was utilised due to its low manufacturing cost, efficiency and ability to employ hygroscopic and thermolabile drugs [36]. Crospovidone has cost implications on an industrial manufacturing scale, therefore its use is not economically viable [37] and most interestingly interacted with the active ingredient.

The two formulations may open opportunities to create diverse formulations for IBD and pain and even tailed towards the two phases of IBD. A remission phase with (slow release tablets) which will reduce the frequency of dosing and side effects as well as the exacerbation phase with (immediate release tablets) having a rapid onset of action. Whereas magnesium stearate and calcium sulfate may delay the drug release rate, which is of significance if a slow release capsule formulation is desired. As expected, the pharmacokinetic analysis of the original suspension of PNB-001 was identical with the suspension of the formulated tablet/capsule. It was shown clearly, that the presence of sugar and starch products had no effect on the bioavailability of the formulation, which is identical with figure 5a.

Overall, the observed plasma concentration appeared low and we do not exclude active and non-active metabolites. The parent PNB-001 molecule may serve as a concentration marker in the first in man clinical trial.

The overall question was, if the apparently low drug concentration, could achieve efficacy in vitro and this was tested using isolated tissue preparations.

In vitro experiments using isolated tissue preparations

The CCK_2 selective phenyl ethyl derivative PNB-001, occurred a potent binding affinity and the CCK-gastrin -antagonism was studied [38] using penta-gastrin (CCK-5) induced contractions of the rat duodenum. Initially CCK_4 was used, an agent, which trigged panic attacks in patients[39], but *in vitro* CCK_4 has a low solubility and low potency in the micro-molar range. The best CCK2 selective agonist *in vitro* and in humans [40] is CCK-5 and therefore, pentagastrin was used to analyse the agonist or antagonist properties of PNB-001.

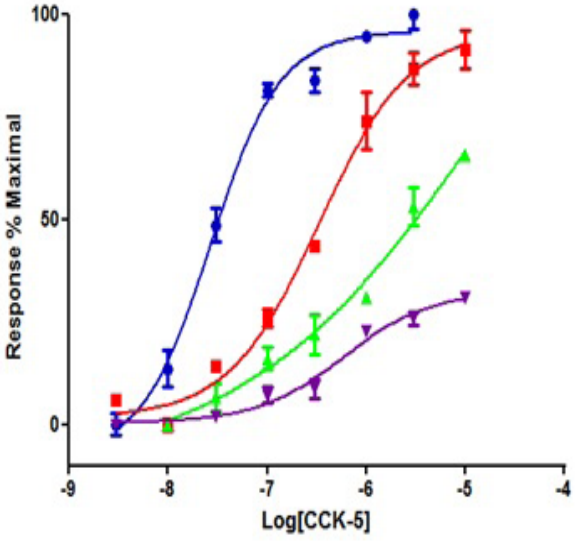


Figure 8: Responses to CCK-5 in the absence and presence of PNB-001, using the rat duodenum assay; CCK-5, CCK-5 +10 nM PNB-001, CCK-5 +30 nMPNB-001, CCK-5 + 100 nM PNB-001; N = 2 for each data point.

The concentration response curve of penta-gastrin, CCK-5, was recorded and shifted to the right by a nanomolar concentration of PNB-001, thus confirming the antagonistic properties [41] of this ligand (Figure 8). 100 nM of PNB-001 with 30 min of incubation time fully blocked the CCK4 and CCK5 induced contractions. For the 5 min incubation cycle, 10 nM of PNB-001 shifted the CCK-5 concentration response curve to the right and from 30 nM onwards, the antagonist showed a reduced maximum response (Figure 8). At 100nM concentrations PNB-001 acted as non-competative antagonist.

100nM are equivalent to 30 microg/l = 30ng/ml and this is the maximum plasma concentration, which was analysed in vivo in rats for an oral dose of 15mg/kg PNB-001. So, in principal a good correlation, but other underlying biological mechanisms may contribute additionally to the anti-inflammatory activity.

Therefore, not a CCK based assay, but a direct anti-inflammatory in vitro assay was performed, again using isolated rat tissue. Rats were again used for pharmacokinetic analysis to minimise possible species specific differences.

In vitro, spontaneous contractions correlate with inflammation and anti-inflammatory steroids such as dexamethasone reduced spontaneous contractions of the duodenum [42].

DSS, dextran sulfonic acid sodium, is a standard agent to induce inflammation and it was used here in vitro using isolated organ preparations of the duodenum.

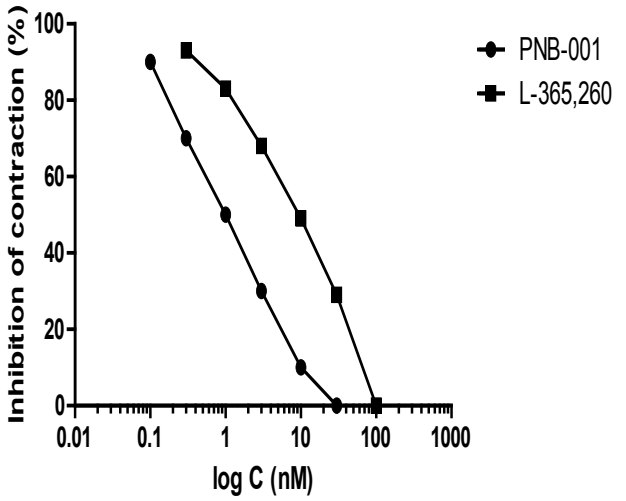


Figure 9: The inhibitory concentration-response curves of PNB-001 and L-365,260 (standard) for DSS stimulated contractions using the rat duodenum.

L-365,260 was applied as CCK2 standard together with the highly CCK2 selective antagonist, PNB-001 under the same conditions. The concentration response curves, showing how these agents inhibit contractions a very low nanomolar concentrations, are outlined in Figure 9.

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An IC_{50} of 1 nM was determined for CCK_2 antagonist PNB-001 (Figure 9). The Merck CCK2 standard occurred a ten times higher IC_{50}. The IC50 found in vitro for the anti-inflammatory assay was 100 times lower than the IC_{50} or the concentration required to shut down CCK related activity and therefore, other pathways may be important in the activity of PNB-001. In this second direct anti-inflammatory assay the 10nM concentration fully blocked the measured inflammation response. 10 nM is equivalent to only 3ng/ml, a concentration, which is achieved from a dose of 2-3mg/kg PNB-001 onwards.

Conclusion

The target molecule PNB-001 was synthesised in only 2 steps from one readily available starting material and will potentially deliver an affordable anti-inflammatory medicine. Solid formulations, such as tablets and capsules giving suspensions, are preferred over solutions in terms of PK and patient adherence. The plasma concentrations are low, but efficient in terms of anti-inflammatory activity in isolated rat-preparations in vitro. Other mechanisms and metabolites may contribute to the actions of PNB-001. PNB-001, a highly potent and selective gastrin antagonist, is reaching phase 1 clinical trial in May 2018 as anti-inflammatory analgesic.

Acknowledgements

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The animal studies were performed in compliance with relevant laws and institutional guidelines. The treatment procedures were approved by the ethical committee, Faculty of Medicine, KhonKaen University (BEA030699).

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