Cholecystokinin-2/gastrin antagonists: 5-hydroxy-5-aryl-pyrrol-2-ones as anti-inflammatory analgesics for the treatment of IBD

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Supplementary Information

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Experimental

1. Materials and Methods

1.1 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 (20g, 0.15 mol) was added slowly to a mixture of mucochloric acid (16.9g, 0.1 mol) and arene, such as benzene, chlorobenzene, fluoro-benzene, anisole and toluene (250 ml). The reaction mixture was stirred overnight. It was then poured into a mixture of 100g 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 ^{o}C; MS (APCI(+)): 195/197 (M+), 230/232 (M+1) m/z
^{1}H NMR ($CDCl_3$) 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 (KBr-disc) υ max: 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% mp: 76-78 ^{o}C; MS (APCI(+)): 227/229/231 (M+1), 262/263/265 (M+) m/z
^{1}H NMR ($CDCl_3$) 250 MHz: δ = 7.48 (m, 2H), 7.35 (m, 2H), 5.91 (s, 1H); ^{13}C NMR ($CDCl_3$) 165.3, 152.0, 136.6, 130.1, 129.6, 128.7, 121.3, 82.9; IR (KBr-disc) υ max: 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%, mp: 74-76 ^{o}C; MS (APCI(+)): 227/229/231 (M+1), 247, 246 (M+) m/z
^{1}H NMR ($CDCl_3$) 250 MHz: 7.48 (m, 2H), 7.34 (m, 2H), 5.91 (s, 1H); ^{13}C NMR ($CDCl_3$) 165.2, 152.0, 136.6, 130.1, 129.6, 128.7, 121.3, 82.8; IR (KBr-disc) υ max: 3451, 3075, 2952, 2051, 1769, 1636, 1497, 1419, 1289, 1231, 1027, 927, 826, 748, 720 cm^{-1}.

3,4-Dichloro-5-(4-methoxy-phenyl)-5H-furan-2-one, Lactone D

Yield = 69%, mp: 76-78 ^{o}C; MS (APCI(+)): 259/258/257 (M+) m/z; ^{1}H NMR ($CDCl_3$) 250 MHz: 7.48 (m, 2H), 7.35 (m, 2H), 5.91 (s, 1H), 3.90 (s, 3H); ^{13}C NMR ($CDCl_3$) 165.3, 152.0, 136.4, 129.1, 129.6, 128.7, 121.3, 82.9, 39.7 ; IR (KBr-disc) υ max: 3451, 3075, 2952, 2051, 1769, 1636, 1497, 1419, 1289, 1231, 1027, 927, 826, 748, 720 cm^{-1}.

3,4-Dichloro-5-(4-methyl-phenyl)-5H-furan-2-one, Lactone E

Yield = 69%, mp: 76-78 ^{o}C; MS (APCI(+)): 259/258/257(M+) m/z; ^{1}H NMR ($CDCl_3$) 250 MHz: δ = 7.49 (m, 2H), 7.34(m, 2H), 5.91 (s, 1H), 2.52 (s, 3H); ^{13}C NMR ($CDCl_3$) 165.3, 152.0, 136.6, 130.1, 129.6, 128.7, 121.3, 82.9, 21.7; IR (KBr-disc) υ max: 3451, 3075, 2952, 2051, 1769, 1636, 1497, 1419, 1289, 1231, 1027, 927, 826, 748, 720 cm^{-1}.

1.2 General Method:

The relevant amine (2.5 times excess) was added to a solution of lactone **A - E** (0.7 mol) in ether (10 ml) and stirred on ice for 30 minutes, allowing to warm up to RT over the time. The resultant mixture was poured into 5 ml water and separated by separating funnel. The 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 column (80% ether, 20% petrol ether). The resulting fractions were dried from excess solvent under vacuum to yield crystals.

4-Chloro-1-cyclopropyl-5-hydroxy-5-phenyl-1,5-dihydro-pyrrol-2-one 1

Yield = 83 %; mp: 177-179 ^{o}C; MS (APCI(+)): 193/195 (M+1), 250/252 (M+) m/z
^{1}H NMR ($CDCl_3$) 250 MHz: δ = 7.41 (m, 5H), 6.09 (s, 1H), 3.50 (m, 1H), 2.18 (m, 1H), 0.95-1.04 & 0.38-0.69 (m, 4H); ^{13}C NMR ($CDCl_3$) 167.4, 154.8, 135.2, 129.2, 128.8, 126.1, 122.2, 93.5, 22.6 , 3.8, 5.1; IR (KBr-disc) υ max: 3416, 3260, 3105, 3011, 2363, 2338, 1671, 1602, 1490, 1450, 1409, 1369, 1256, 1144, 1032, 939, 833, 752, 702 cm^{-1}.

4-Chloro-5-(4-chloro-phenyl)-1-cyclopropyl-5-hydroxy-1,5-dihydro-pyrrol -2-one 2

Yield = 72 %; mp: 169-171 ^{o}C; MS (APCI(+)): 227/229/231 (M+1), 284/286/288 (M+) m/z; ^{1}H NMR ($CDCl_3$) 250 MHz: δ = 7.22 (m, 4H), 5.97 (s, 1H), 3.98(bs, 1H), 1.76 (m, 1H), 0.24-0.99 (m, 4H); ^{13}C NMR ($CDCl_3$) 165.8, 155.4, 144.2, 133.7, 129.0, 127.7,

122.2, 91.7, 22.6 , 3.7, 5.2; IR (KBr-disc) υ max: 3433, 3220, 3019, 2935, 2858, 1700, 1675, 1497, 1412, 1251, 1209, 1144, 1089, 1015, 940, 844, 802, 679 cm^{-1}.

4-Chloro-1-cyclopentyl-5-hydroxy-5-phenyl-1,5-dihydro-pyrrol-2-one 3
Yield = 81 %; mp: 180-182 °C; MS (APCI(+)): 193/195 (M+1), 278/280 (M+) m/z; ^1H NMR ($CDCl_3$) 250 MHz: δ = 7.51(m, 5H), 6.08 (s, 1H), 4.87 (bs, 1H), 3.59 (m, 1H), 1.99 (m, 2H), 1.81 (m, 4H), 1.46 (m, 4H); ^{13}C NMR ($CDCl_3$) 167.2, 155.0, 135.2, 129.1, 128.6, 126.5, 122.2, 93.3, 54.3, 30.0, 28.8, 24.5, 24.4; IR (KBr-disc) υ max: 3220, 2961, 2877, 2373, 2341, 1684, 1613, 1448, 1426, 1248, 1199, 1141, 1070, 934, 850, 750, 701 cm^{-1}.

4-Chloro-5-(4-chloro-phenyl)-1-cyclopentyl-5-hydroxy-1,5-dihydro-pyrrol-2-one 4
Yield = 73 %; mp: 157-159 °C; MS (APCI(+)): 227/229/231 (M+1), 312/314/316 (M+) m/z; ^1H NMR ($CDCl_3$) 250 MHz: δ = 7.42 (m, 4H), 6.03 (s, 1H), 4.99(bs, 1H), 3.51-3.62 (m, 1H), 1.97-2.19 (m, 2H), 1.68-1.93 (m, 8H); ^{13}C NMR ($CDCl_3$) 167.1, 154.8, 135.2, 133.9, 128.9, 128.0, 122.3, 93.0, 54.3, 30.0, 28.9, 24.5; IR (KBr-disc) υ max: 3407, 3276, 2968, 2922, 2883, 2379, 2339, 1691, 1491, 1429, 1367, 1249, 1203, 1092, 1013, 932, 843, 787, 709 cm^{-1}.

4-Chloro-1-cyclohexyl-5-hydroxy-5-phenyl-1,5-dihydro-pyrrol-2-one 5
Yield = 57 %; mp: 170-172 °C; MS (APCI(+)): 193/195 (M+1), 292/294 (M+) m/z
^1H NMR ($CDCl_3$) 250 MHz: δ = 7.26-7.61 (m, 5H), 6.08 (s, 1H), 3.77 (bs, 1H), 2.88 (m, 1H), 1.21-2.07 (m, 10H); ^{13}C NMR ($CDCl_3$) 163.9, 153.9, 135.0, 129.25, 128.9, 126.4, 122.9 , 96.0, 53.6, 32.8, 31.1, 29.8, 26.2 , 24.2; IR (KBr-disc) υ max: 3440, 2924, 2858, 2355, 2344, 1641, 1449, 1367, 1250, 1138, 1016, 996,742, 695 cm^{-1}.

4-Chloro-1-(phenyl)-5-hydroxy-5-phenyl-1,5-dihydro-pyrrol-2-one 6
Yield = 48 %; mp: 168-171 °C; MS (APCI(+)): 193/195 (M+1), 314/316 (M+) m/z
^1H NMR ($CDCl_3$) 250 MHz: δ = 7.46 (m, 5H), 7.34 (m, 5H), 6.38 (s, 1H), 3.68 (bs, 1H);
^{13}C NMR ($CDCl_3$) 168.9, 159.7, 136.9, 135.1, 132.4, 129.9, 129.0, 126.9, 123.0, 122.3, 122.2, 93.5; IR (KBr-disc) υ max: 3517, 3357, 3114, 2840, 2674, 2361, 2342, 1678, 1607, 1464, 1412, 1361, 1208, 1138, 1071, 988, 755, 700 cm^{-1}.

1-Benzyl-4-chloro-5-hydroxy-5-phenyl-1,5-dihydro-pyrrol-2-one 7
Yield = 71 %; mp: 165-167 °C; MS (APCI(+)): 193/195 (M+1), 300/302 (M+) m/z
^1H NMR ($CDCl_3$) 250 MHz: δ = 7.36 (m, 5H), 7.24 (m, 5H), 6.08 (s, 1H), 4.69 (m, 2H), 3.62 (bs, 1H); ^{13}C NMR ($CDCl_3$) 167.9, 155.9, 137.6, 134.4, 129.3, 128.7, 128.4, 128.4, 127.3, 127.1, 126.4, 93.2, 43.4; IR (KBr-disc) υ max: 3446, 3279, 3098, 2931, 2850, 2374, 2334, 1684, 1611, 1456, 1413, 1349, 1276, 1205, 1128, 1051, 696 cm^{-1}.

1-Benzyl-4-chloro-5-(4-chloro-phenyl)-5-hydroxy-1,5-dihydro-pyrrol-2-one 8
Yield = 59 %; mp: 149-152 °C; MS (APCI(+)): 227/229/231 (M+1),334/336/338 (M+) m/z; ^1H NMR ($CDCl_3$) 250 MHz: δ = 7.33 (m, 4H), 7.16 (m, 5H), 6.09 (s, 1H), 4.60 (m, 2H), ^{13}C NMR ($CDCl_3$) 167.6 , 155.4 , 137.5 , 135.3 , 133.2 , 129.1, 129.0 , 128.9, 128.6, 128.4 , 127.9, 127.4, 121.9, 92.6, 43.2; IR (KBr-disc) υ max: 3442, 2931, 2849, 2365, 2339, 1674, 1616, 1492, 1406, 1349 1272, 1199, 1094, 1018, 817, 699 cm^{-1}.

1-Benzyl-4-chloro-5-(4-fluoro-phenyl)-5-hydroxy-1,5-dihydro-pyrrol-2-one 9
Yield = 69 %; mp: 143-145 °C; ^1H NMR ($CDCl_3$) 250 MHz: δ = 7.32 (m, 4H), 7.16 (m, 5H), 6.09 (s, 1H), 4.55(d, 1H), 3.89 (d, 1H); ^{13}C NMR ($CDCl_3$) 167.6 , 155.4 , 137.5, 135.3 , 133.2 , 129.1 , 129.0 , 128.9 , 128.6 , 128.4 , 127.9 , 127.4, 121.9 , 92.6 , 43.2; IR (KBr-disc) υ max: 3440, 2928, 2848, 2361, 1676, 1614, 1490, 1404, 1348 1272, 1199, 1094, 817, 697 cm^{-1}.

1-Benzyl-4-chloro-5-(4-methoxy-phenyl)-5-hydroxy-1,5-dihydro-pyrrol-2-one 10
Yield = 39 %; mp: 129-132 °C; ^1H NMR ($CDCl_3$) 250 MHz: δ = 7.32 (m, 4H), 7.06 (m, 5H), 6.09 (s, 1H), 4.55(d, 1H), 3.90 (s, 3H) 3.89(d, 1H); ^{13}C NMR ($CDCl_3$) 167.6, 155.4, 137.5 , 135.3, 133.2, 129.1, 129.0, 128.9, 128.6, 128.4, 127.9, 127.4, 121.9, 92.6, 43.2, 39.1; IR (KBr-disc) υ max: 3442, 2931, 2849, 2365, 2339, 1674, 1616, 1492, 1406, 1349 1272, 1199, 1094, 1018, 817, 699 cm^{-1}.

1-Benzyl-4-chloro-5-(4-methyl-phenyl)-5-hydroxy-1,5-dihydro-pyrrol-2-one 11
Yield = 69 %; mp: 149-152 °C; ^1H NMR ($CDCl_3$) 250 MHz: δ = 7.32 (m, 4H), 7.06-7.25 (m, 5H), 6.09 (s,1H), 4.55 (m, 2H), 2.10 (s, 3H); ^{13}C NMR ($CDCl_3$)167.6, 155.4, 137.5, 135.3, 133.2, 129.1, 129.0, 128.9, 128.6, 128.4, 127.9, 127.4, 121.9, 92.6 , 43.2 21.1; IR (KBr-disc) υ max: 3442, 2931, 2849, 2365, 2339, 1674, 1616, 1492, 1406, 1349 1272, 1199, 1094, 1018, 817, 699 cm^{-1}.

(major): 4-Chloro-5-hydroxy-5-phenyl-1-((*S*)-(-)-1-phenyl-ethyl)-1,5-dihydro-pyrrol-2-one 12
Yield = 66 %; mp: 162-164 °C; MS (APCI(+)): 193/195 (M+1), 314/316 (M+) m/z
^1H NMR ($CDCl_3$) 250 MHz: δ = 7.44(m, 7H), 7.08-7.25 (m, 3H), 5.96 (s, 1H), 4.16 (m, 1H), 3.37 (bs, 1H), 1.49 (m, 3H); ^{13}C NMR ($CDCl_3$) 167.3, 154.3, 142.5, 134.7, 129.4, 128.7, 128.4, 127.7, 127.3, 126.4, 123.0, 93.8, 53.5, 18.8; IR (KBr-disc) υ max: 3241, 2983, 2932, 2863, 2366, 2347, 1686, 1661, 1614, 1494, 1456, 1425, 1356, 1258, 1202, 1025, 931, 855, 755, 692 cm^{-1}.

(minor): 4-Chloro-5-hydroxy-5-phenyl-1-((*S*)-(-)-1-phenyl-ethyl)-1,5-dihydro-pyrrol-2-one 13
Yield = 4 %; MS (APCI(+)): 193/195 (M+1), 314/316 (M+) m/z; ^{1}H NMR ($CDCl_3$) 250 MHz: δ = 7.29-7.53 (m, 7H), 6.95-7.21 (m, 3H), 6.08 (s, 1H), 4.78 (m, 1H), 2.71 (bs, 1H), 1.63 (m, 3H).

4-Chloro-5-hydroxy-1-phenethyl-5-phenyl-1,5-dihydro-pyrrol-2-one 14
Yield = 89 %; mp: 155-158 ^{o}C; MS (APCI(+)): 193/195 (M+1), 314/316 (M+) m/z
^{1}H NMR ($CDCl_3$) 250 MHz: δ = 7.09-7.53 (m, 10H), 6.20 (s, 1H), 3.74 (m, 1H), 2.88-3.29 (m, 3H), 2.65 (m, 1H); ^{13}C NMR ($CDCl_3$) 168.0, 155.7, 139.0, 134.6, 129.4, 128.85, 128.84, 128.6, 126.6, 126.2 , 121.8 , 92.7, 41.9 , 34.6; IR (KBr-disc) υ max: 3433, 3246, 2929, 2366, 2334, 1681, 1658, 1607, 1455, 1406, 1251, 1151, 1128, 1066, 931, 753, 699 cm^{-1}.

4-Chloro-5-(4-chloro-phenyl)-5-hydroxy-1-phenethyl-1,5-dihydro-pyrrol-2-one 15
Yield = 45 %, mp: 145-148 ^{o}C; MS (APCI(+)): 227/229/231 (M+1), 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), 3.68-3.79 & 2.64-2.77 (m, 2H), 2.88-3.29 (m, 2H); ^{13}C 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) υ max: 3421, 3228, 2925, 2848, 2370, 2338, 1684, 1658, 1606, 1461, 1406, 1248, 1190, 1097, 935, 806, 697 cm^{-1}.

2. Pharmacology

2.1 $^{[125]}$I-CCK-8 Radioligand cholecystokinin binding assay

CCK receptor binding assays were performed by using guinea pig cerebral cortex (CCK_2) or rat pancreas (CCK_1). Male guinea pig brain tissues were prepared according to the modified method described by Saita et al[1]. Pancreatic membranes were prepared as described by Charpentier et al[2].
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 ^{125}I-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 $MgCl_2$, 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.

2.2 Isolated tissue preparations[3]

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 ileum was carefully excised at a site 15cm away from the ileocaecal junction and washed with physiological solution. The mesentery of the ileum 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^{0}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[4]
From the isolated tissue preparation, GI 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 were directly applied to the bath and antagonists were pre-incubated for about 10 min. Stock solutions were prepared in DMSO.

CCK-5 stimulated contractions of the isolated rat duodenum
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. Test molecules and standard were added to the organ bath 10 minutes before exposure to the next CCK-5 serial concentrations.

DSS induced spontaneous muscle contractions in rat colon / 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.

2.3 In vivo rats

Indomethacin induced ulceration

Male Sprague Dawley rats, 150-200 g body weight, were housed in *polypropylene* cages and maintained at 27±2°, relative humidity 65±10% and 12 h light/dark cycles. The animals were given standard rodent chow. The animals were quarantined for three days before the initiation of the experiment. The animals were randomly divided into five groups, containing four animals in each. The control group received 1 ml of vehicle containing 15% DMSO+35% polyethylene glycol+50% water.

All remaining groups received 10 mg/kg indomethacin (solubilized in 100% alcohol and then diluted with 5% w/v sodium bicarbonate solution) on 4^{th} and 5^{th} day subcutaneously alone or in combination with treatments. All standard and test compounds were treated continuously for 5 days.

Groups and treatments

Group	Treatment
1	Vehicle
2	Indomethacin 10 mg/kg (sc)
3	Indomethacin + 5 mg/kg PNB-001 p.o
4	Indomethacin 10 mg/kg (sc) + 20 mg/kg PNB-001 p.o
5	Indomethacin 10 mg/kg (sc)+ 10 mg/kg prednisolone p.o.

At the end of the treatment (on 5^{th} day) animals were anaesthetized with ketamine (100 mg/kg, i.m.) 24 h after the last treatment. The animals were sacrificed by cervical dislocation and dissected to remove GIT (duodenum to anus). The dissected intestinal tissues were used to evaluate the following parameters.

Weight of the tissues

The intestinal tissues, 5 cm proximal duodenum, 10 cm distal jejunum, 10 cm proximal ileum, whole caecum and 5 cm proximal colon were cleaned with saline, blotted dry, placed on aluminium foil and weighed on an electric balance.

Scoring of the animal intestinal tissues

The intestinal tissues, 5 cm proximal duodenum, 10 cm distal jejunum, 10 cm proximal ileum, whole caecum and 5 cm proximal colon were isolated and were scored visually for inflammation based on macroscopic features.

Macroscopic inflammation scores were assigned based on clinical features using an arbitrary scale ranging from 0-8.

Score	Description
0	No visible change
1	Hyperemia at sites
2	Loss of mucosal integrity
3	Lesions having diameter 1 mm or less
4	Lesions having diameter 2 mm or less (no<5)
5	Lesions having diameter 2 mm or less (no 5-10)
6	Lesions having diameter 2 mm or less (no>10)
7	Lesions having diameter more than 2 mm (no<5)
8	Lesions having diameter More than 2 mm (no 5-10)
9	Lesions having diameter more than 2 mm (no>10)

2.4 Nociception tests

Formalin test
The rats were placed in a plastic behavior cage (30x30x30 cm) and a mirror was mounted at 45* angle below the transparent floor to allow an unobstructed view. 0.05 ml of 1% formalin was injected subcutaneously into the dorsal surface of the right hind paw. Subsequently behavior responses were recorded by the number of licks / bites in the early phase (0-5 min) and late phase (20-25 min) in all groups. PNB-001 was injected 30 min prior to the formalin injection.

The hot plate test
Mice were placed on a hot plate that was thermostatically maintained at 50^{o}C. A Plexiglass box was used to confine the animal to the hot plate. The reaction time of each animal (either paw licking or jumping) was considered a pain response. The latency to reaction was recorded. For prevention of heat injury, the cut-off time of the test was 30 sec.

3. Molecular modeling
For target preparation the protein structures, pdb identifier 1HZN for the CCK_1 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_2 receptor the results were analysed and visualized using Chimera and Designer studio 4.5. After visual inspection and scores, results were presented to understand drug ligand interaction with the CCK_2 receptor.

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

Animal studies ethics
On behalf of all authors I state that all experiments were performed in compliance with the relevant laws and institutional guidelines.
Animal studies (RD-09/102014-4482403) were performed and approved by the bioethics committee at the University of Tennessee Health Science Center, Cancer Research Building 19, Manassas, Memphis, TN 38103 and no human subjects were involved in this study.
Experiments were conducted in male standard IRC mice obtained from the animal house, Faculty of Medicine, Khon Kaen University. The treatment procedures were approved by the ethical committee, Faculty of Medicine, Khon Kaen University (BEA030699).

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