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Discovery of 4-oxo-6-((pyrimidin-2-ylthio)methyl)-4*H*-pyran-3-yl 4-nitrobenzoate (ML221) as a functional antagonist of the apelin (APJ) receptor

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ABSTRACT

The recently discovered apelin/APJ system has emerged as a critical mediator of cardiovascular homeostasis and is associated with the pathogenesis of cardiovascular disease. A role for apelin/APJ in energy metabolism and gastrointestinal function has also recently emerged. We disclose the discovery and characterization of 4-oxo-6-((pyrimidin-2-ylthio)methyl)-4*H*-pyran-3-yl 4-nitrobenzoate (ML221), a potent APJ functional antagonist in cell-based assays that is >37-fold selective over the closely related angiotensin II type 1 (AT1) receptor. ML221 was derived from an HTS of the ~330,600 compound MLSMR collection. This antagonist showed no significant binding activity against 29 other GPCRs, except to the κ-opioid and benzodiazepinone receptors (<50/<70%I at 10 μM). The synthetic methodology, development of structure–activity relationship (SAR), and initial in vitro pharmacologic characterization are also presented.

Apelin is a circulating peptide hormone, synthesized and secreted by a number of cell types including those of the cardiovascular, endocrine, gastrointestinal and nervous systems. Apelin was recently identified as the endogenous ligand of the APJ, a formerly orphaned G-protein coupled receptor (GPCR) with a similarly broad distribution of expression.[1] The tissue distribution of both apelin and APJ suggests an involvement of this system in a range of physiological functions. Indeed, apelin has been shown to play a role in mediating gastrointestinal function,[2–6] food and water consumption,[7–11] energy metabolism,[12–16] and cardiovascular homeostasis.[17–21] In addition to normal physiological function, apelin has been associated with the pathogenesis of cardiovascular and metabolic diseases including atherosclerosis,[22,23] hypertension,[24–26] heart failure[27,28] and both type 1[29] and type 2 diabetes mellitus.[30,31] Despite this abundance of work, several unanswered questions regarding the role apelin and APJ in normal physiology and pathology remain. Small molecule probes of the apelin/APJ system would advance apelin research significantly. In particular an APJ antagonist would be a useful tool for determining the function and pharmacology of APJ, and ultimately to validate the importance of this system in animal models.

To date, there have been no reports of small molecule antagonists of APJ. Therefore we undertook a high throughput screen of the NIH's small molecule collection (MLSMR) as part of the NIH sponsored Molecular Libraries Program. Approximately 330,600 compounds were tested in the APJ DiscoveRx β-arrestin primary assay that has been described in PubChem (AID 2766),[32] Cheminformatics analysis revealed 1064 hits with activity >50% at a single concentration point of 10 μM. Liquid samples were then ordered through the MLSMR and 948 compounds were received. The compound solutions resupplied by the MLSMR were first confirmed in 10 μM single-point duplicate in the APJ DiscoveRx β-arrestin primary assay. Of these, 622 compounds were confirmed to have at least 50% activity at a 10 μM assay concentration. These were further triaged for direct β-galactosidase inhibition and an additional 237 compounds were eliminated. The remaining 385 confirmed compounds were next tested in dose response in the primary APJ DiscoveRx β-arrestin primary assay to obtain IC_{50} values and these were rank ordered for potency: 67 compounds met probe criteria (IC_{50} = 1–5 μM), 62 additional compounds had IC_{50} = 5–10 μM, and 86 compounds were significantly less potent (IC_{50} = 10–

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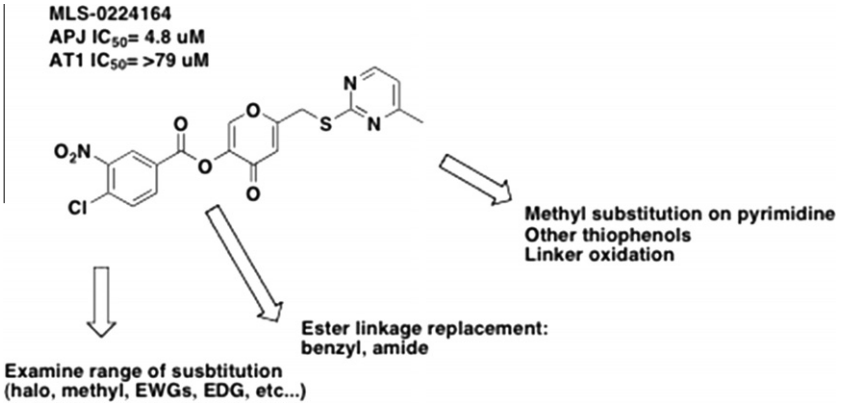
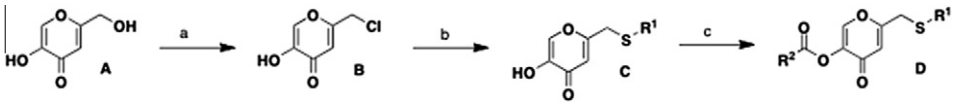


Figure 1. General SAR strategy around Kojic acid scaffold.

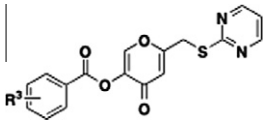


Scheme 1. Synthesis of APJ antagonists, conditions: (a) neat $SOCl_2$ (17 equiv); (b) R^1SH (1 equiv), NaOMe (1 equiv), MeCN; (c) R^2COCl (1.4 equiv), Cs_2CO_3 (1 equiv), MeCN.

20 μM). To eliminate compounds acting through non-specific inhibition of the assay reporter, the activity of the best scaffolds was assessed in a β-galactosidase counterscreen assay. The best scaffolds were also subjected to a counterscreen of the closely related angiotensin II type 1 (AT1) receptor to determine selectivity for APJ. Ultimately, only a single scaffold that was potent and selective against AT1 (MLS-0224164, Fig. 1) was identified.

The general method for preparation of the compounds described herein is shown in the scheme below (Scheme 1). Starting from kojic acid (**A**), chlorination using neat thionyl chloride gave **B**. This was then used to alkylate a thiol to give **C**. Reaction with an acid chloride then provided the desired APJ antagonists **D**. In general yields were reasonable for this sequence and gram scale quantities of materials could be produced.[33]

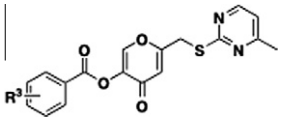
Table 1
SAR analysis of APJ antagonists: Kojic acid scaffold—left region R^3



Entry	R^3	APJ	AT1	SI
1	H	>79	>79	ND
2	3-NO_2-4-Cl	6.20 ± 0.77	>79	>13
3	3-NO_2-4-Me	7.25 ± 0.75	>79	>11
4	3-NO_2	75.70 ± 3.30 (2)	>79	ND
5	4-Cl	54.50 ± 3.12 (2)	>79	ND
6 (ML221)	4-NO_2	1.75 ± 0.19	>79	>45
7	4-CN	6.59 ± 2.87	>79	>12
8	4-CF_3	29.00 ± 4.94 (3)	>79	ND
9	4-Br	18.20 ± 3.36 (7)	>79	ND
10	4-Me	>79	>79	ND
11	4-OMe	>79	>79	ND
12	4-OEt	>79	>79	ND
13	4-OiPr	>79	>79	ND
14	4-OBu	>79	>79	ND
15	4-*t*Bu	>79	>79	ND
16	4-F	>79	>79	ND
17	4-SO_2NMe_2	>79	>79	ND
18	4-$SO_2N(CH_2)_4$	>79	>79	ND
19	3,4-DiMe	>79	>79	ND
20	2-NO_2-5-Cl	>79	>79	ND
21	2-Cl-5-NO_2	51.00 ± 4.07 (4)	60.10 ± 1.35 (3)	ND
22	2-Me-3-NO_2	72.2 (1)	>79	ND
23	4-Ph	>79	>79	ND
24	4-SO_2NEt_2	>79	>79	ND
25	4-$SO_2N(CH_2)_5$	>79	>79	ND
26	4-SO_2morpholine	>79	>79	ND
27	2-NO_2-4,5-diOMe	>79	>79	ND

Potency IC_{50} (μM), Ave. ± SEM (stdv/sqrt (*n*)) (*n* = 4) if number of replicates is different than the default it is noted in parentheses. SI = selectivity index: (IC_{50} AT1)/(IC_{50} APJ).

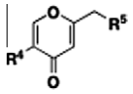
Table 2
SAR analysis of APJ antagonists: Kojic acid scaffold—left region R^3



Entry	R^1	APJ	AT1	SI
28	H	>79	>79	ND
29	3-NO_2-4-Cl	1.75 ± 0.14	>79	>45
30	3-NO_2-4-Me	4.70 ± 0.91 (3)	>79	>17
31	3-NO_2	59.20 ± 4.77 (2)	>79	ND
32	4-Cl	>79	>79	ND
33	4-NO_2	3.11 ± 0.68	>79	>25
		4.32 ± 1.29	>79	>23
34	4-CN	8.51 ± 1.22	>79	>9
		11.50 ± 1.35	>79	>7
35	4-CF_3	20.6 (1)	>79	ND
36	4-Me	>79	>79	ND
37	4-OMe	>79	>79	ND
38	4-F	>79	>79	ND
39	4-$SO_2N(CH_2)_4$	39.90 ± 8.65	>79	ND
40	2-NO_2-5-Cl	58.80 ± 6.30 (2)	>79	ND
41	2-Cl-5-NO_2	74.10 (1)	>79	ND
42	2-Cl	>59	>79	ND
43	3-OMe	>79	>79	ND
44	3-Me	>79	>79	ND
45	3-Cl	>79	>79	ND
46	2-Me	>79	>79	ND
47	4-OCF_3	>79	>79	ND
48	3-CN	>79	>79	ND

Potency IC_{50} (μM), Ave. ± SEM (stdv/sqrt (*n*)) (*n* = 4) if number of replicates is different than the default it is noted in parentheses. SI = selectivity index: (IC_{50} AT1)/(IC_{50} APJ).

Table 3
SAR analysis of APJ antagonists: Kojic acid scaffold—left region R^4 and right region R^5



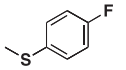
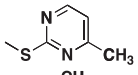
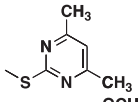
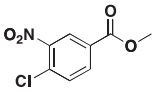
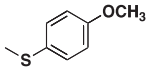
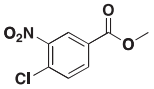
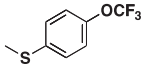
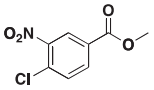
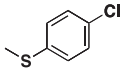
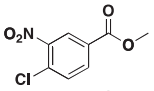
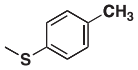
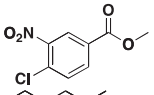
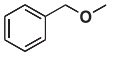
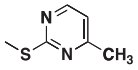
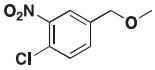
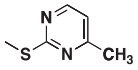
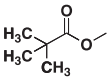
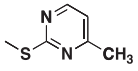
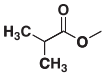
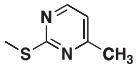
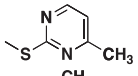
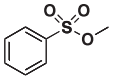
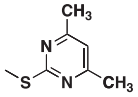
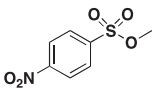
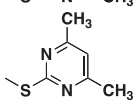
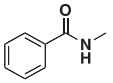
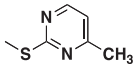
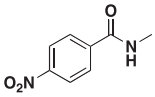
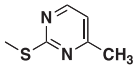
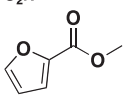
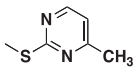
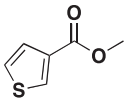
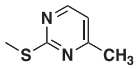
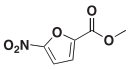
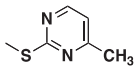
Entry	R^4	R^5	APJ	AT1	SI
49	HO–		>79	>79	ND
50	HO–		>79	>79	ND
51	HO–		>79	>79	ND
52	HO–		>79	>79	ND
53	HO–		>79	>79	ND
54			8.27 ± 1.75	>79	>9
55			24.00 ± 5.45	>79	>3
56			4.53 ± 0.66	>79	>17
67			12.80 ± 0.35	>79	>6
58			9.61 ± 3.02	>79	>8
59			>79	>79	ND
60			>79	>79	ND
61			>79	>79	ND
62			>79	>79	ND
63			>79	>79	ND
64			>79	>79	ND
65			>79	>79	ND
66			>79	>79	ND
67			>79	>79	ND
68			>79	>79	ND

Table 3 (*continued*)

Entry	R^4	R^5	APJ	AT1	SI
69			>79	>79	ND
70			>79	>79	ND
71			>79	>79	ND
72			3.21 ± 0.46	>79	>25

Potency IC_{50} (μM), Ave. ± SEM (stdv/sqrt (*n*)) (*n* = 4) if number of replicates is different than the default it is noted in parentheses. SI = selectivity index: (IC_{50} AT1)/(IC_{50} APJ).

Table 4
ADMET properties for ML221

Compound characteristic	
Aqueous solubility pH 7.4	25.5 μg/mL
Plasma protein binding (mouse)	99.1%
Permeability (PAMPA) pH 7.4	271.0×10^{-6} cm/s
Hepatic microsome Stability (mouse)	4.9%
Plasma stability	42.2%
Cytotoxicity	>50 μM

Figure 2. Representative dose response curve for ML221. The compound antagonized Ap13-mediated activation of APJ in a concentration-dependent manner in both a cAMP assay (■), and a β-arrestin recruitment assay (●). Data plotted are the mean ± SEM% inhibition of Ap13. Curves represent the best fit of a four parameter logistic generated using GraphPad Prism5.

The general SAR strategy we pursued around the kojic acid scaffold is depicted in Figure 1. The structure represented by the screening hit MLS-0224164 was the only scaffold that exhibited selectivity over the AT-1 receptor. Efforts immediately focused on replacing the undesirable 3-nitro-4-chloro motif due to the inherent electrophilic nature, subsequent exploration of alternatives to the ester linkage to address potential hydrolytic instability, and substitution/replacement of the thiopyrimidine moiety. The results are summarized in the four tables below (Tables 1–4). The first three tables cover changes around the benzoate group (in green) with either unsubstituted, monomethyl, or dimethyl thiopyrimidine groups.

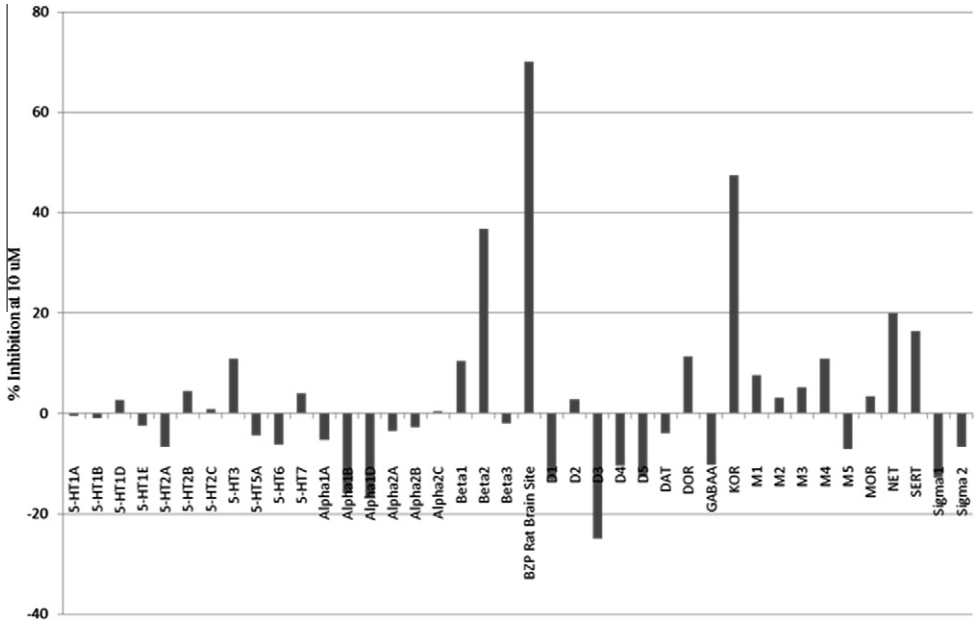


Figure 3. GPCR profiling panel for **ML221**.

Table 1 depicts the SAR of the scaffold with an unsubstituted thiopyrimidine. In general, we found that an electron-withdrawing group on the benzoate was necessary for APJ activity. Interestingly, while 3-nitro substitution was tolerated with a 4-chloro or methyl group (entries 2 and 3), an analog with only 3-nitro substitution was only weakly active (entry 4). Singly substituted analogs with electron-withdrawing groups in the 4-position were the most active with the exception of sulfonamides (for example, entry 17). The, 4-nitro analog, ML221, (entry 6) was the most potent of this series and was selected for further characterization. The 4-nitro group could be replaced with a 4-cyano group (entry 7), albeit with a 4-fold loss in potency. Unsubstituted benzoates (entry 1) as well as a broad range of other para-substituted benzoates were all inactive or weakly active (entries 5 and 8–29), with the exception of 4-bromobenzoate analog (entry 9) and 4-trifluoromethyl analog (entry 8). Similar trends were seen with monomethyl substituted pyrimides (Table 2) as well as 4,6-dimethylpyrimidines (data not shown). In both cases potency was typically lower.

In addition, an expanded range of substituents was explored around the scaffold (Table 3). Early evaluation of MLS-0224164 (entry 29) found the ester bond to be readily hydrolyzed in aqueous acetonitrile to produce the truncated analog depicted as entry 52 (data not shown). To confirm the observed activity of MLS-0224164 was due to the intact molecule and not the product of hydrolysis, the compounds represented by entries 49–53 were prepared and found to be inactive. Interestingly, replacing the thiopyrimidine with simple thiophenols led to a number of active analogs, indicating that a range of groups is tolerated in that region. In particular a 4-chlorothiophenol (entry 56) and unsubstituted thiophenol (entry 61) were both <10 μM. Attempts to replace the ester linkage of the benzoate were less successful. A simple benzyl linkage (entry 60) was inactive, as were a range of aliphatic esters (entries 61–63). Sulfonates were also inactive (entries 64 and 65) as were amides (entries 66 and 67). A range of heteroaryl esters also showed no activity (entries 68–71). Lastly, oxidation of the sulfur of the thiopyrimidine to a sulfone gave a compound that was of comparable activity (entry 72 as compared to entry 29 in Table 2), although we did not further pursue this finding due to the potential reactivity of the sulfone-pyrimidine motif.

Antagonism of apelin-13-mediated activation of APJ by ML221 was assessed using two complimentary assays of APJ function; inhibition of cAMP and recruitment of β-arrestin. Increasing concentrations of ML221 antagonized a fixed concentration of Ap13 (EC_{80} = 10 nM) in both assays, with a calculated IC_{50} equal to 0.70 μM in the cAMP assay, and 1.75 μM in the β-arrestin assay (Fig. 2).

The drug-like and ADME/T properties of ML221 were evaluated in a detailed in vitro pharmacology panel (Table 4). ML221 is poorly soluble in aqueous media at pH 7.4. We note that the aqueous solubility obtained at physiological pH is 14-fold higher than the obtained potency of the probe. In a PAMPA permeability assay, ML221 exhibits moderate permeability. ML221 displays moderate plasma and poor microsomal stability, as it is rapidly metabolized in both human and mouse liver homogenates (4.2% and 4.9% remaining at 60 min). Neither the plasma nor the microsomal stability assay results are surprising given the ester linkage in this probe. Ultimately this limits the utility of this probe to in vitro studies or apelin receptor or in vivo studies using acute intravenous doses to avoid metabolism. Lastly, ML221 shows no toxicity (>50 μM) toward human hepatocytes.

Profiling against other GPCRs: ML221 was submitted to the Psychoactive Drug Screening Program (PDSP) at the University of North Carolina and the data against a GPCR binding assay panel is shown in Figure 3. Overall the compound shows a relatively clean binding profile, with the only significant activity at the kappa opioid and the benzodiazepinone receptors.

In conclusion, we have discovered the first reported APJ antagonist, ML221, which represents a selective tool compound to further explore the function of the apelin/APJ system. ML221 displays limited cross reactivity against a range of GPCRs. Current efforts to optimize this scaffold to explore the in vivo effects of APJ antagonists are underway.

Acknowledgments

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hensive centers of the NIH Molecular Libraries Probe Production Centers Network (MLPCN).

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32. Cells (angiotensin II receptor-like 1 (AGTRL-1) cell line (DiscoveRx, Cat# 93-0250C2)) were seeded at 1000 cell/well (1536 plate, Corning) in 4 μL and grown overnight (16–18 h) at 37 °C, 5% CO_2, 100% humidity, then 60 nL of either DMSO control or 2 mM stock test compounds in DMSO were transferred to each well, followed by 2 μL of 30 nM Apelin-13 to negative control and test compound wells, and 2 μL of assay media (F12 nutrient mix HAMs supplemented with 10% hi-FBS, 1X penicillin/streptomycin) to positive control wells. This yielded a final concentration of test compound of 20 μM and 1% final DMSO. Assay was incubated for 90 min at room temperature, and then developed with 3 μL of detection reagent (PathHunter Detection Reagents (DiscoveRx, Cat# 93-0001)) for 60 min and luminescence read on a Perkin Elmer ViewLux.
33. For the synthesis of ML221: Step 1: A mixture of 5-hydroxy-2-(hydroxymethyl)-4*H*-pyran-4-one (Kojic acid) (0.55 g, 3.87 mmol) was dissolved in thionyl chloride (5 mL, 68.5 mmol) and was stirred at ambient temperature for 3 h. Excess reagent was removed in vacuo to provide 0.61 g. (98%) of 2-(chloromethyl)-5-hydroxy-4*H*-pyran-4-one as an off-white solid. ^{1}H NMR. (500 MHz, DMSO-d_6): δ (ppm) 8.13 (s, 1H), 6.57 (s, 1H), 4.66 (s, 2H). Step 2: A mixture of pyrimidine-2-thiol (161 mg, 1.433 mmol) in 2 ml methanol was treated with sodium methoxide solution (310 mg, 1.433 mmol) and stirred until dissolved. Acetonitrile (10 mL) was added followed by 2-(chloromethyl)-5-hydroxy-4*H*-pyran-4-one (230 mg, 1.433 mmol) and the mixture was stirred at ambient temperature for 3 h at which time analysis by LC/MS indicated the reaction to be complete. The solvent was removed in vacuo to provide 406 mg (96%) of a yellow solid containing crude 5-hydroxy-2-((pyrimidin-2-ylthio)methyl)-4*H*-pyran-4-one and an equimolar amount of sodium chloride which was used without further purification. ^{1}H NMR. (500 MHz, $CDCl_3$): δ (ppm) 8.52 (d, 2H, J = 4.9 Hz), 7.80 (s, 1H), 7.02 (t, 1H, J = 4.8 Hz), 6.63 (s, 1H), 4.23 (s, 2H). Step 3: A mixture of 5-hydroxy-2-((pyrimidin-2-ylthio)methyl)-4*H*-pyran-4-one (200 mg, 0.847 mmol), cesium carbonate (276 mg, 0.847 mmol), and 4-nitrobenzoyl chloride (220 mg, 1.185 mmol) in acetonitrile (8 mL) was stirred at ambient temperature overnight. The solvent was removed in vacuo to provide a pale yellow solid, which was partitioned with approximately 20 ml of 1:1 ethyl acetate and water. The desired product remained insoluble in the biphase and was collected by filtration. The solid was dried in vacuo to yield 202 mg (62%) as a tan solid. ^{1}H NMR. (500 MHz, DMSO-d_6): δ (ppm) 8.69 (d, 2H, J = 4.8 Hz), 8.68 (s, 1H), 8.40 (d, 2H, J = 8.8 Hz), 8.29 (d, 2H, J = 8.8 Hz), 7.29 (t, 1H, J = 4.9 Hz), 6.65 (s, 1H), 4.45 (s, 2H). ^{13}C NMR. (125 MHz, DMSO-d_6): δ (ppm) 171.2, 168.8, 165.9, 161.7, 158.1, 150.8, 149.9, 140.3, 133.0, 131.4, 124.2, 118.0, 114.6, 31.2.