

Probe Report

Title: Discovery of a Highly Selective KCC2 Antagonist

Authors: *Lindsley, Craig, craig.lindsley@vanderbilt.edu; Lewis, Michelle, michelle.lewis@vanderbilt.edu; Weaver, Dave, david.weaver@vanderbilt.edu; Delpire, Eric, eric.delpire@vanderbilt.edu

Assigned Assay Grant #: R21 NS53658

Screening Center Name & PI: Vanderbilt Specialized Screening for GPCRs, Ion Channels and Transporters, David Weaver

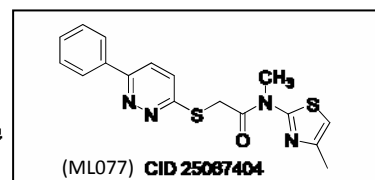
Chemistry Center Name & PI: Vanderbilt Specialized Chemistry Center for Accelerated Probe Development, Craig Lindsley

Assay Submitter & Institution: Eric Delpire, Vanderbilt University

PubChem Summary Bioassay Identifier (AID): 1799

Probe Structure & Characteristics:

***N*-methyl-*N*-(4-methylthiazol-2-yl)-2-(6-phenylpyridazin-3-ylthio)acetamide**



CID	Target Name	IC50/EC50 (nM) [SID, AID]	Anti-target Name(s)	IC50/EC50 (μM) [SID, AID]	Selectivity	Secondary Assay(s) Name: IC50/EC50 (nM) [SID, AID]
25067404 (ML077)	KCC2	537 [SID 56405461, AID 1456, 1793, 1717, 1735, 1738, 1737]	NKCC1	>>50 μM [SID 56405461, AID 1716, 1718, 1713, 1715, 1723, 1714, 1753]	>100-fold	537 nM Rb Flux KCC2 [SID 56405461, AID 1734]

Recommendations for the scientific use of this probe:

This is the most potent, and the only selective KCC2 antagonist ever described. CID 25067404 (ML077) is a highly selective KCC2 antagonist versus NKCC1 and a panel of 68 GPCRs, ion channels and transporters (no inhibition >50% at 10 μM). Thus, this probe can be used *in vitro* to study KCC2 function with confidence.



Specific Aim: To identify small molecule ligands (both agonists and antagonists) of the neuronal K-Cl cotransporter KCC2. Desired ligands would be cell permeable, exhibit submicromolar potency, and show greater than 10 fold selectivity over other other closely related NKCC transporters. This probe report describes a potent, highly selective KCC2 antagonist (CID 25067404 (ML077), SID 56405461) that inhibits KCC2. The probe is 'best in class' providing unprecedented potency and selectivity at KCC2, clean ancillary pharmacology, and soluble in saline (~10 mg/mL).

Significance and Rationale: Cells possess in their plasma membranes proteins that tightly couple the movement of inorganic cations such as Na^+ and K^+ , together with Cl^- . The Slc12 cation-chloride cotransporter family consists of two loop diuretic-sensitive Na-K-2Cl cotransporters (NKCC1 and NKCC2), one thiazide-sensitive Na-Cl cotransporter, and four Na^+ -independent K-Cl cotransporters: KCC1-KCC4. These seven transport mechanisms play key roles in a variety of physiological processes: fluid and salt reabsorption in kidney; fluid and salt secretion in inner ear, Cl-secreting epithelia such as salivary-, sweat-, and lacrimal glands, lung airway epithelium, and intestine; control of neuronal excitability; and control and maintenance of cell volume (for reviews, see (1-3)). Their disruption causes salt wasting disorders (4, 5), alteration of blood pressure (6, 7), hyperexcitability (8, 9), nerve degeneration (10), and alteration of cell proliferation and invasiveness (11).

The cation-chloride cotransporters have received much attention in the past decade for the role they play in the nervous system. In particular KCC2, a neuronal-specific K-Cl cotransporter, has been shown to modulate inhibitory neurotransmission both in the brain and in the spinal cord. By reducing the intracellular Cl^- concentration below its thermodynamic equilibrium potential in central neurons, KCC2, strengthens synaptic inhibition. Several studies have shown that loss of KCC2 function in central neurons results in the development of CNS hyperexcitability (8, 12, 13). Moreover, a paper from Coull and coworkers also showed that disinhibition in the dorsal horn of the spinal cord triggered by peripheral nerve injury was mediated by a significant decrease in KCC2 expression (14). Local blockade or knock-down of spinal KCC2 in intact rats markedly reduced the nociceptive threshold. By linking change in KCC2 function to disruption of Cl^- homeostasis in lamina I neurons, the study clearly linked the loss of KCC2 function to a loss of inhibition in this region of the spinal cord and to an increase in chronic pain. The relationship between KCC2 and nerve or spinal cord injury has been confirmed in subsequent studies that have shown that inflammatory response due to intraplantar injection of formalin (15, 16) or hind paw injection of complete Freund's adjuvant (17), or to loose ligation of the sciatic nerve (18), or contusive spinal cord injury at T9 (19), all led to a down-regulation of cotransporter expression. Altogether, these studies also point to a role of KCC2 in neuropathic pain.

The pharmacology of the cation-chloride cotransporters is dominated by two classes of drugs: the thiazide and loop diuretics (**Figure 1**). Whereas thiazide diuretics target the apical Na-Cl cotransporter, located in the distal convoluted tubule, resulting in decreased salt reabsorption, loop diuretics (furosemide or LASIX and bumetanide or BUMEX), as their names imply, inhibit the apical Na-K-2Cl cotransporter, NKCC2, located in the thick ascending loop of Henle, thereby diminishing kidney salt reabsorption. They also inhibit NKCC1 in the micromolar range in isolated cells, but have little effect on NKCC1 *in situ* due to their binding to albumin in the circulation and consequently their poor access to peripheral tissues. High doses of diuretics have, however, ototoxic effects due to inhibition of NKCC1 in the inner ear (20-22). Furosemide **1** and bumetanide **2** also inhibit K-Cl cotransporter, including KCC2 but at much higher concentrations (500 μM -1 mM) and are pan transporter inhibitors (23-25). There are two compounds that inhibit K-Cl cotransport in the micomolar range: DIDS **3** and DIOA **4**, but their effect is species-specific (26, 27) and

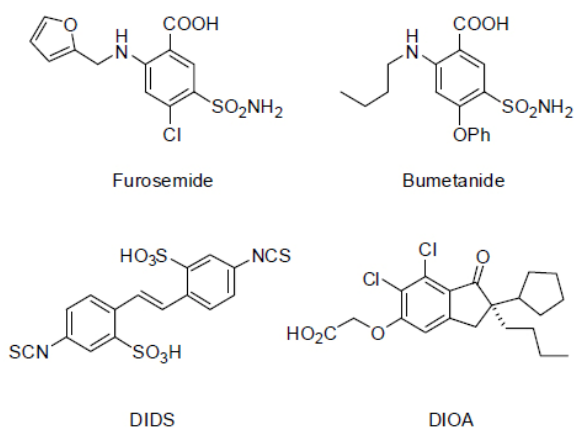


Figure 1. Known KCC2 Antagonists - Unselective and Weak (micromolar to millimolar IC_{50} s).

lacking specificity with regard to other ion transporters and channels (for reviews, see (28, 29)). To identify novel molecules that affect the activity of KCC2, we developed a novel fluorescence-based method appropriate for high throughput screening. Indeed, all traditional methods used to assess cation-chloride cotransporter activity are not adequate for HTS. The method makes use of a fluorescent dye sensitive to thallium, a cation that is transported by cation-chloride cotransporter. Thallium, in combination with dye, was previously used to assay uptakes of K^+ through K^+ channels (30). Using this method, we screened a library of 234,000 compounds with KCC2 as a target, and identified a number of compounds that affect the activity of the cotransporter.

Screening center information

Assay Implementation and Screening

PubChem Bioassay Name: Identification of Novel Modulators of Cl-Dependent Transporter Processes via HTS.

List of PubChem bioassay identifiers generated for this screening project (AIDS):

1456, 1735, 1738, 1717, 1716, 1737, 1734, 1718, 1713, 1715, 1736, 1723, 1714, 1753, 1793.

PubChem Primary Assay Description:

HEK293 cell culture. Wild-type or KCC2-expressing HEK293 cells were grown up to 80-90% confluence in 10-cm dishes containing Dulbecco's modified Eagle's medium/Ham's F-12 (1:1) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), 50 unit/ml penicillin, and 50 pg/ml streptomycin (Invitrogen). KCC2-expressing clones were under puromycin selection (2 μ g/ml, Sigma, St. Louis, MO). Cultures were maintained at 37°C in the presence of 5% CO_2 . Cells were passaged every 3-4 days, using a ratio of 1:10, for a maximum of 16 passages. For fluorescence measurements, the cells were plated in T175 flasks, 4 days prior to the assay.

KCC2 subcloning. The rat KCC2 cDNA (31) inserted in the *Xenopus laevis* oocyte expressing pBF (32) was excised using *SpeI* and *BglII* restriction enzymes and inserted into the mammalian expression vector pIRESpuro2 at the compatible *NheI* and *BamHI* sites (BD Biosciences, San Jose, CA). After transformation into competent *E. coli* DH5 α cells, the DNA was purified (Qiagen, Valencia, CA).

Generation of KCC2 overexpressing clones. HEK293 cells were then plated to reach 60-70% confluence the following day when they were transfected using 7.5 μ g KCC2-pIRESpuro2 cDNA and 11.3 μ l Fugene (Roche Applied Science, Indianapolis, IN). Twenty four hours post-transfection, the cells were subjected to puromycin (2 μ g/ml). After 15 days puromycin selection, the cells were trypsinized, counted, and plated in multiple 96 well plates at \sim 1 cell per well. Using our HTS-compatible functional assay (see below), 48 clones were first selected from 16 x 96-well plates (\sim 1500 clones) and expanded to 24-well plates.

Tl-induced fluozin-2 fluorescence increase. The day prior to the experiment, cells were plated in 384-well, black-walled, clear bottom, poly-D-lysine coated plates (Greiner Bio-One, Monroe, NC) at a concentration of 20,000 cells per well using a Multidrop Combi (Thermo Fisher, Waltham, MA). The medium was removed using an ELx405CW cell washer

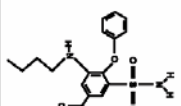
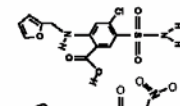
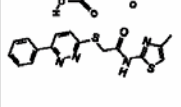
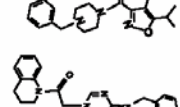
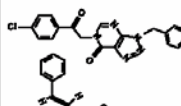
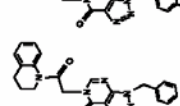
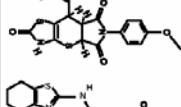
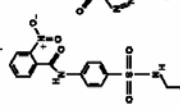
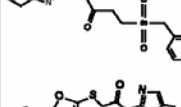
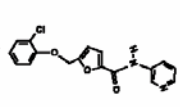
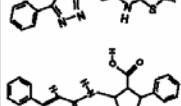
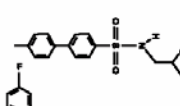
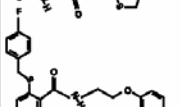
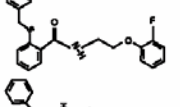
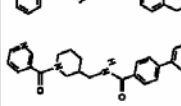
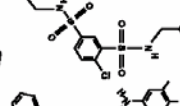
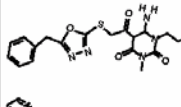
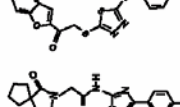
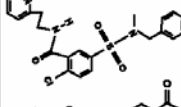
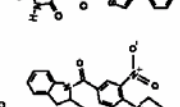
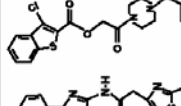
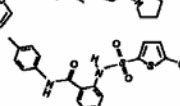
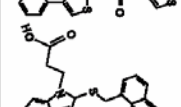
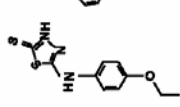
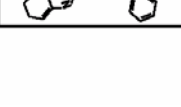
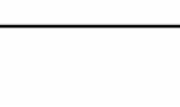


(BioTek, Winooski, VT) and replaced with 20 μ l HBSS + 20 mM HEPES, pH 7.3) and 20 μ l of HBSS + 20 mM HEPES, pH 7.3 containing 2 μ M fluozin-2 dye (Invitrogen) + 0.2 % (w/v) Pluronic F-127 (Invitrogen) using a Multidrop Combi. Cells were incubated with the dye at room temperature for 48 minutes. The wells were then washed thrice with HBSS + 20 mM HEPES (addition of 80 μ l and aspiration to leave \sim 20 μ l) using the ELx405CW. After the washes the cell plate was inserted into a Hamamatsu FDSS 6000 (Bridgewater, NJ) and pre-compound addition fluorescence counts were obtained (5 frames at 1 Hz, excitation 470 \pm 20 nm, emission 540 \pm 30 nm), In parallel, 70 nl/well of compounds from 10 mM (nominal) stocks in DMSO supplied as the Molecular Libraries Small Molecule Repository collection by BioFocus DPI (South San Francisco, CA) were transferred to 384-well polypropylene plates (Greiner) using an Echo 555 (Labcyte, Sunnyvale, CA) and diluted with 35 μ l/well HBSS + 20 mM HEPES' pH 7.3 + 200 μ M ouabain using a Multidrop Combi. Twenty microliters/well of the diluted compounds were then added to the dye-loaded cell plates using a Velocity11 Bravo (Santa Clara, CA) and incubated for 8 min. Next, the compound-treated cell plates were loaded back onto the FDSS 6000. After 10 seconds, 10 μ l/well of 5X thallium stimulus buffer (125mM sodium bicarbonate, 12mM thallium sulfate, 1mM magnesium sulfate, 1.8mM calcium sulfate, 5mM glucose, 10mM HEPES, pH 7.3) was added. All steps of the screening protocol post-compound plating were accomplished using an automated screening system. Plates were moved using a Thermo Fisher F3 robotic arm and all instruments and scheduling were under the control of Thermo Fisher Polara scheduling software version 2.3. After normalizing each well's fluorescence trace by dividing each data point post compound addition by the initial fluorescence values obtained on the FDSS pre-compound addition, activity was measured by obtaining the initial values of the slope of Tl^{+} - stimulated fluorescence increase using linear regression. Hits were selected as compounds that caused a decrease in the slope of \geq 3 standard deviations from the general population of test wells. Hits were reordered from BioFocus DPI and retested in duplicate. Retest positive compounds were further evaluated by testing them at varying concentrations on both the KCC2-expressing cell line and the parental untransfected HEK cells. Compounds displaying selective, concentration dependent activity were selected for further evaluation.

⁸⁶Rb assays in HEK293 cells. For rubidium uptake assays, cells were briefly trypsinized from 10-cm dishes and plated for 2 hours on 35-mm dishes coated with poly-L-Lysine (0.1 mg/ml, Sigma). Cells were washed once and pre-incubated for 15 min with 1 ml hyposmotic saline solution containing in mM: 120 NaCl, 5 KCl, 2 CaCl₂, 0.8 MgSO₄, 5 HEPES, 5 glucose, 100 μ M ouabain, 500 μ M N-ethylmaleimide, pH 7.4 with HCl (260-270 mOsm). After the preincubation period, the medium was aspirated and replaced with 1 ml of similar solution without NEM, but containing 1 μ Ci/ml ⁸⁶Rb (Perkin Elmer, Wellesley, MA). After 15 min uptake, the solution was aspirated and the cells were washed thrice with ice-cold solution. Time course experiments have shown that the flux is linear over a 30 min period (not shown). The cells were then lysed for 1 hour with 500 μ l 0.25N NaOH and then neutralized with 250 μ l glacial acetic acid. A 300 μ l aliquot was then added to 5 ml liquid scintillation fluid (Biosafe II, Research Products International, Mt. Prospect, IL) for counting and a 30 μ l aliquot was used for protein assay (Bradford: Biorad, Hercules, CA). A 5 μ l aliquot of the uptake solution was also counted as standard. Uptakes are expressed in pmole K⁺/mg protein/min, and the furosemide-sensitive uptake (KCC mediated flux) is calculated as the difference between the uptake measured in absence of furosemide and the flux obtained in the presence of 2 mM furosemide. Each experimental condition is measured in triplicate.

Western blot. For determination of KCC2 expression, HEK293 cells and clones were grown in 60-mm dishes for 3 days and lysed on ice with 350 μ l buffer containing 150 mM NaCl, 50 mM Tris-Cl, 2 mM EDTA, 0.1% SDS, 1% CHAPS, 0.5% Na-deoxycholate, and cocktail of protease inhibitors (1 tablet/10 ml Roche Applied Science). After 10 min, the cell debris was scraped and the lysate was recovered and placed in a microcentrifuge tube on ice for 30 min. Next, the lysate was spun at 10,000 rpm for 10 min, the supernatant was recovered, and its protein content was quantified using the Bradford assay (Biorad). Proteins (20 μ g/lane) were resolved by 7.5% SDS-PAGE and electroblotted onto polyvinylidene difluoride

(PVDF) membranes (Millipore, Billerica, MA). Membranes were blocked for 2 hours at room temperature with 5% non-fat dry milk in TBST (150 mM NaCl, 10 mM Tris HCl, 0.5% Tween 20 [polyoxyethylene-sorbitan monolaurate]), then incubated overnight at 4°C with a C-terminal polyclonal anti-KCC2 antibody (1:1000) in TBST/5% non-fat dry milk (33). Membranes were washed extensively in TBST, and protein bands were visualized by enhanced chemiluminescence (ECL plus, Amersham Biosciences, Piscataway, NJ).

Supporting Table 1. Inhibition of KCC2 (IC50) and NKCC1 (% at 5 and 50 μ M) with various hit compounds.

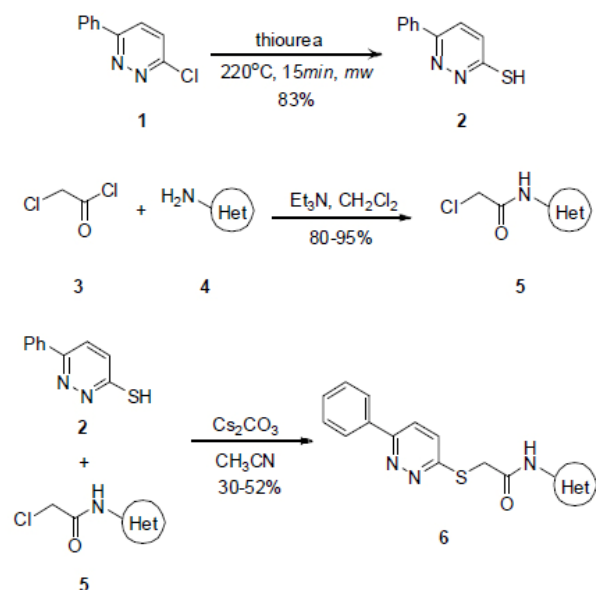
Code	Substance ID	IC50 μ M	% NKCC1 inhibition at 5 μ M	% NKCC1 inhibition at 50 μ M	
D1	furosemide	455			
D2	bumetanide	655			
D3	22411794	1.5	[6.1	15.5]	
D4	7211972	560 nM	[12.9	19.7]	
D5	4245183	1.3	[28.1	27.7]	
D6	7971542	1.2	25.1	40.6	
D7	7970346	6	23.4	33.2	
D8	17512842	300 nM	67.4	90.8	
D9	17504768	5.1	[24.2	31.2]	
D10	4258845	19.7	[35.8	37.1]	
D11	14734981	12.1	N.d.	N.d.	
D12	4259771	9.1	[9.9	16.8]	
D13	24798228	13.4	[21.8	26.6]	
D14	24796400	24	25.2	72.8	
D15	17506378	No	[34.2	45.8]	
D16	17414602	2.2	[45.3	47.9]	
D17	14733862	7.9	13.5	30.5	
D18	24797972	7.7	[31.2	40.3]	
D19	22409652	No	[46.3	53.4]	
D20	3714535	640 nM	[15.4	28.3]	
D21	3714834	2.5	[39.2	43.6]	
D22	24838117	2.9	[44.4	53.4]	
D23	17516589	1.6	22.9	48.5	
D24	3716309	51	33.3	55.4	
D25	22411513	No	[65.1	68.4]	
D26	3714259	79	[35.5	38.1]	
D27	22405981	No	44.9	67.4	
D28	14720512	36	57.5	80.5	

Center Summary of Screen: The screen was conducted in multiple 384-well plates containing HEK293 cells over-expressing KCC2. For each plate, 4 columns were dedicated to controls: 2 columns (32 wells) using standard HBSS conditions with ouabain and 2 columns using HBSS + 2 mM bumetanide. These wells allowed for internal quality assessment. Each of the remaining 320 wells was dedicated to one compound tested at a concentration of nominally 10 μ M. Utilizing 733 plates, we screened 234,560 compounds with an average Z' value of 0.686 ± 0.090 , indicating both high fidelity and good separation between the two control signals (plus and minus bumetanide). Compounds that decreased or stimulated fluorescence signals more than three standard deviations from the test compound population measured on a per-plate basis were counted as primary hits. The screen yielded 4,933 hits (2%) with 1880 compounds reducing and 2954 compounds increasing the fluorescence signal, respectively. We only identified antagonists.

After elimination of duplicates, a secondary screen was performed with 3,695 compounds. Each compound was tested in duplicate at a concentration of 10 μ M using both KCC2-overexpressing cells and naïve HEK293 cells. With each cell line, the secondary screen was performed in the presence and absence of ouabain, and 45% of the compounds (2,224) verified. We identified 465 compounds that were positive in KCC2 over-expressing cells, but not in naïve HEK293 cells, irrespective of the presence or absence of ouabain. Each of the 465 compounds was then tested at various concentrations (ranging from 0.5 nM to 30 μ M) in triplicate and \sim 76% of them displayed a typical dose dependence.

Based on their potency and structure, we selected 26 compounds that inhibited thallium-induced fluorescence increase and tested them on KCC2 and NKCC1 functions using ^{86}Rb uptakes in HEK293 cells. For KCC2, we used N-ethylmaleimide pre-treatment in KCC2 over-expressing HEK293 cells. The alkylating agent not only stimulates K-Cl cotransport, but abrogates the function of the native Na-K-2Cl cotransporter. As seen in Figure 3, furosemide inhibits 93% of the uptake at a concentration of 2 mM and had no effect at a concentration below 30 μ M. For NKCC1, we used a hyperosmotic solution to stimulate the transporter in naïve HEK293 cells. ^{86}Rb uptake was 95% inhibited by a low dose (20 μ M) of bumetanide. Table 1 lists the efficacy of each of the 26 compounds on KCC2 and NKCC1 function. As seen in the table, some compounds like D4 inhibit KCC2 with a measured IC_{50} in the submicromolar range while minimally affecting NKCC1. In contrast, other compounds like D8 acted equally well on KCC2 and NKCC1.

Of the confirmed hits, D4 (KCC2 IC_{50} = 560 nM) proved to be the most drug-like lead for probe development, and displayed selectivity versus NKCC1 (\sim 19.7% inhibition at 50 μ M) – already best in class and meets probe criteria for a KCC2 antagonist. We then

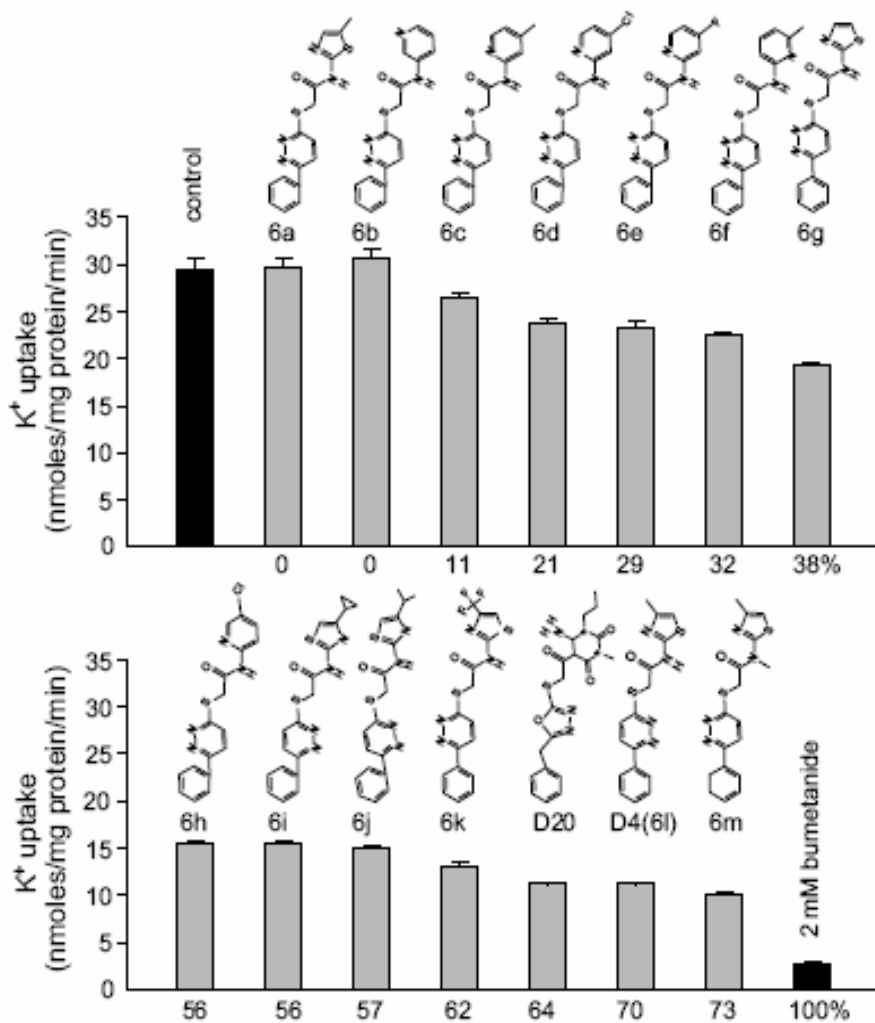


Scheme 1. Library synthesis of analogs of D4 (CID 24814385).

resynthesized D4, in a library format, along with 12 new analogs. As shown in Scheme 1, commercial 3-chloro-6-phenylpyridazine **1** was exposed to thiourea under microwave irradiation (220°C, 15 min) to deliver 6-phenylpyridazine-3-thiol **2** in 83% yield.

Alpha-chloroacetyl chloride **4** was then treated with 12 different heterocyclic amines **3** to deliver alkylation substrates **5** in yields ranging from 80-95%. Alkylation of **2** with **5** employing CsCO_3 in CH_3CN provides analogs **6** in yields ranging from 30-52%. This library was then screened for its ability to inhibit KCC2 function at 1 mM with 2 mM bumetanide as a positive control (Figure 2). This series displayed robust, tractable SAR. The parent HTS hit D4 (CID 7211972) repeated, displaying 70% inhibition of KCC2 at 1 μ M dose. Other thiazole songeners, with substitution in the 4-position (**6i** (CID 7211972) and **6k** (CID 25067401)), or

unsubstituted (**6g** (CID 25067400)) displayed activity (38-57% at 1 μ M). Interestingly, if the methyl substituent was moved to the 5-position, as in **6a** (CID 25067400), all KCC2 inhibitory activity was lost. Pyridines, known thiazole mimetics, were weak to moderate KCC2 antagonists. In order to afford a more favorable ancillary profile, we also prepared within the library a tertiary amide analog of D4/**6l** (CID 7211972), **6m** (CID 25067404 (ML077)), which displayed good inhibition of KCC2 (73% at 1 μ M).



At this point, **6l** (CID 7211972) and **6m** (CID 25067404 (ML077)) were the leading probe candidates. As shown in Figure 3, **6m** provided an IC₅₀ of 537 nM for inhibiting KCC2 - >3-orders of magnitude improvement over bumetanide (KCC2

Figure 2 SAR of analogs of D4 (CID 7211972).

IC₅₀ = 655 μ M). Moreover, **6m** was found to be highly selective for KCC2 versus NKCC1 (IC₅₀ >>50 μ M). The original hit, **6l** (CID 24814385) afforded similar results.

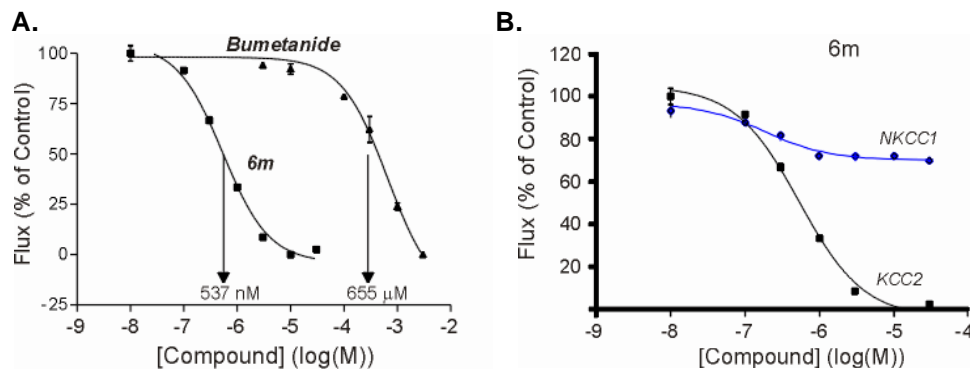


Figure 3. A) KCC2 CRC for **6m** and bumetanide; B) Selectivity. CRCs for **6m** on KCC2 and NKCC1.

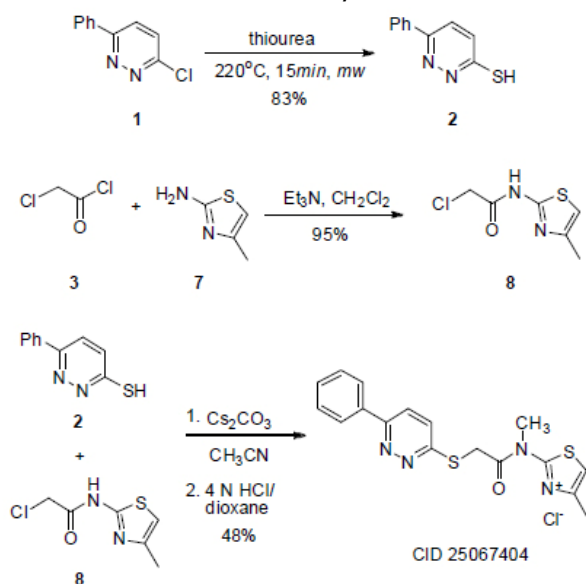
At this point, the Lead Profiling Screen (68 GPCRs, ion channels and transporters) from MDS Pharma was performed on the two probe candidates (CID 7211972 and CID 25067404 (ML077)) to attempt to distinguish which would be promoted to probe status. CID 25067404 (ML077) (SID

56405461) possessed superior ancillary profile to CID 25010774 (**Figure 4**), with *NO* activities >50% at 10 μ M, and was thus declared an MLPCN probe. CID 7211972

Targ	Species	% Inhibition (10 μ M) CID 25067404	Targ	Species	% Inhibition (10 μ M) CID 25067404
Adenosine A1	human	31	Histamine H ₃	human	7
Adenosine A2	human	38	Imidazoline I ₂ Central	rat	6
Adenosine A3	human	38	Interleukin IL-1	mouse	6
Adrenergic α_{1A}	rat	33	Leukotriene, Cysteinyl CysLT ₁	human	10
Adrenergic α_{1B}	rat	3	Melatonin MT ₁	human	7
Adrenergic α_{1D}	human	16	Muscarinic M ₁	human	2
Adrenergic α_{2A}	human	-7	Muscarinic M ₂	human	-9
Adrenergic β_1	human	10	Muscarinic M ₃	human	5
Adrenergic β_2	human	9	Neuropeptide Y Y ₁	human	3
Androgen (testosterone)AR	rat	4	Neuropeptide Y Y ₂	human	9
Bradykinin B ₁	human	6	Nicotinic Acetylcholine	human	3
Bradykinin B ₂	human	2	Nicotinic Acetylcholine 1 α , Bungarotoxin	human	1
Calcium channel L-type, benzothiazepine	rat	10	Opiate δ (OP1, DOP)	human	20
Calcium channel L-type, dihydropyridine	rat	-2	Opiate κ (OP2, KOP)	human	-11
Calcium channel N-type	rat	-4	Opiate μ (OP3, MOP)	human	-3
Dopamine D ₁	human	8	Phorbol Ester	mouse	24
Dopamine D _{2S}	human	-3	Platelet Activating Factor (PAF)	human	4
Dopamine D ₃	human	-1	Potassium Channel [K _{ATP}]	hamster	10
Dopamine D _{4.2}	human	-12	Potassium Channel hERG	human	10
Endothelin ET _A	human	0	Prostanoid EP ₄	human	-4
Endothelin ET _B	human	-3	Purinergic P _{2X}	rabbit	-18
Epidermal Growth Factor (EGF)	human	-1	Purinergic P _{2Y}	rat	8
Estrogen ER α	human	10	Rolipram	rat	3
G protein-coupled receptor GPR103	human	12	Serotonin (5-Hydroxytryptamine) 5-HT _{1A}	human	15
GABA _A Flunitrazepam, central	rat	25	Serotonin (5-Hydroxytryptamine) 5-HT ₃	human	-7
GABA _A Muscimol, central	rat	1	Sigma σ_1	human	-5
GABA _{B1A}	human	1	Sigma σ_2	rat	15
Glucocorticoid	human	7	Sodium Channel, Site 2	rat	0
Glutamate, Kainate	rat	11	Tachykinin NK ₁	human	-10
Glutamate, NMDA, Agonism	rat	33	Thyroid Hormone	rat	3
Glutamate, NMDA, Glycine	rat	6	Transporter, Dopamine (DAT)	human	28
Glutamate, NMDA, Phencyclidine	rat	2	Transporter, GABA	rat	0
Histamine H ₁	human	7	Transporter, Norepinephrine (NET)	human	34
Histamine H ₂	human	7	Transporter, Serotonin (5-Hydroxytryptamine) (SERT)	human	2

Figure 4. MDS Pharma Lead Profiling Screen of 68 GPCRs, ion channels and transporters against CID 25067404 (ML077) (SID 56405641) at a concentration of 10 μ M.

possessed 5 significant activities (>50% inhibition at 10 μ M) and numerous activities in the 40-50% range of inhibition at 10 μ M at other K⁺ and Ca²⁺ ion channels and transporters. The old medicinal chemistry 'trick' of dialing out ancillary ion channel activity with a tertiary amide analog proved successful. CID 25067404 (ML077) has the following IUPAC nomenclature: *N*-methyl-*N*-(4-methylthiazol-2-yl)-2-(6-phenylpyridazin-3-ylthio)acetamide. Scheme 2 highlights the optimized route to prepare CID 25067404 (ML077) as the mono-HCl salt in 82.5% overall yield. All of the reagents are commercially available from Aldrich



Scheme 2 Synthesis of KCC2 antagonist probe CID 25067404 (6m).

Chemical company. A Mixture of 3-chloro-6-phenylpyridazine **1** (1.5g, 7.87mmol) and thiourea (4g) was irradiated at 220°C for 15 min by microwave. The cooled solution was diluted with water to remove excess amount of thiourea. The product was filtered, washed with water, dried over in vacuo, and recrystallized from methanol to afford a brown solid 6-phenylpyridazine-3-thiol **2** (1.23g, 83%). ¹H NMR (DMSO, 400 MHz) δ (ppm) 7.93-7.89 (m, 3H), 7.87 (d, *J* = 9.6 Hz, 2 H), 7.70 (d, *J* = 9.6 Hz, 2 H), 7.54-7.48 (m, 3 H); ¹³C NMR (DMSO, 100 MHz) δ (ppm) 179.23, 149.86, 141.78, 134.09, 130.55, 129.47, 126.38, 125.44; LCMS, single peak, 2.46 min, *m/e*, 189.04 (*M*+1). To a solution of 2-chloroacetyl chloride (3.6 mL, 43.8mmol) in dichloromethane (145mL) was added triethylamine (6.1mL, 43.8mmol) at

0 °C. The reaction mixture was stirred for 30min at 0 °C, quenched with saturated NH₄Cl (50mL), and extracted with dichloromethane (3 x 40mL). The combined extracts were dried (MgSO₄) and concentrated *in vacuo* to give 2-chloro-*N*-methyl-*N*-(4-methylthiazol-2-yl)acetamide **8** (7.90g, 95%). The mixture of 6-phenylpyridazine-3-thiol **2** (365mg, 1.92mmol) and 2-chloro-*N*-methyl-*N*-(4-methylthiazol-2-yl)acetamide **8** (300mg, 1.60mmol) in acetonitrile (5mL) was treated with cesium carbonate (782mg, 2.40mmol) at room temperature for 3h, then quenched with water (10mL), and extracted ethylacetate (3 x 10mL). The combined extracts were dried with MgSO₄ and concentrated *in vacuo* affording crude product which was purified by flash column chromatography on silica gel (Hexane/Ethyl acetate = 7/3) to provide brown solid *N*-methyl-*N*-(4-methylthiazol-2-yl)-2-(6-phenylpyridazin-3-ylthio)acetamide, CID 25064704. ¹H NMR (DMSO, 400 MHz) δ (ppm) 8.08 (dd, *J* = 9.2, 7.6 Hz, 3 H), 7.83 (d, *J* = 9.2 Hz, 1 H), 7.53-7.50 (m, 3 H), 6.82 (s, 1H), 4.75 (s, 2 H), 3.81 (s, 3 H), 2.29 (s, 3 H); ¹³C NMR (CDCl₃, 100 MHz) δ (ppm) 167.86, 160.18, 156.08, 146.50, 136.00, 130.29, 129.39, 126.89, 126.73, 124.44, 110.15, 35.27, 34.43, 17.55; LCMS, single peak, 3.13 min, m/e, 357.78 (M+1).

We then calculated a number of *in silico* parameters for *N*-methyl-*N*-(4-methylthiazol-2-yl)-2-(6-phenylpyridazin-3-ylthio)acetamide, CID 25067404 (ML077) using TRIPOS ADME/TOX software. The KCC2 probe had the following profile: MW, 356.5; hydrogen bond donors, 0; hydrogen bond acceptors, 5; clogP, 1.8; XlogP, 2.5; total polar surface area, 58.9. The KCC2 antagonist probe, CID 25067404 (ML077), had no Rule of 5 violations and possessed a favorable calculated logP value, in agreement with known CNS agents.

The KCC2 antagonist probe, CID 25067404 (ML077), as the mono-HCl salt, possessed the following measured solubility profile: >50 mM in pure DMSO, ~10 mg/mL in saline.

The combination of KCC2 potency, selectivity versus NKCC1, broad panel selectivity, favorable calculated physiochemical properties and observed solubility profile, provides a best in class KCC2 antagonist probe which can be used *in vitro* to study KCC2 function with confidence.

MLS#s: MLS002279954, MLS002279955

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