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# Benzyl prolinate derivatives as novel selective KCC2 blockers

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ABSTRACT

The discovery and optimization of a novel class of selective submicromolar KCC2 blockers is described. Details of synthesis and SAR are given together with ADME properties of selected compounds. A methylsulfone residue on the $R^1$ phenyl group improved the overall general profile of these prolinate derivatives.



KCC2 is a neuronal-specific electroneutral potassium-chloride co-transporter, whose function is to regulate intracellular levels of chloride ions in neurons. It has been shown that, under normal conditions, KCC2 transports $Cl^-$ ions out of neurons, keeping $Cl^-$ concentrations below the thermodynamic equilibrium potential, so that $GABA_A$ channels, when opened, will allow $Cl^-$ to enter neurons, hyperpolarizing them and inhibiting firing.[1,2] KCC2 therefore acts as a modulator of inhibitory neurotransmission, both in the brain and in the spinal cord, hence its growing interest as a new target for neuronal hyperexcitability disorders such as epilepsy or neuropathic pain.[3]

KCC2 belongs to a large family of cation-chloride co-transporters consisting of seven well-characterized members (NCC, NKCC1-2, and KCC1-4) for which the cation (sodium or potassium) transport is always accompanied by the concomitant stoichiometric transport of a chloride ion.[4]

Anticonvulsant properties of loop diuretics such as furosemide **1** were reported in the clinic.[5] It is a known blocker of cation-chloride co-transporters but does not display satisfactory selectivity levels to render it attractive as pharmacological tool to definitively unveil the role of KCC2.

The first KCC2 blockers displaying higher selectivity versus NKCC1 have been recently reported[6] but without detailed SAR and without assessment of the overall druggability profile of the identified compounds. In this Letter, we report the identification of novel potent selective KCC2 blockers displaying suitable properties for their in vivo pharmacological evaluation.

High throughput screening (HTS) of our corporate compound collection using a Rb flux assay[7] on the KCC2 co-transporter led to the identification of benzyl 1-acetyl-2-benzylprolinate (+)-**2** as a hit with an $IC_{50}$ of 0.32 μM. The compound was subsequently found to be inactive in a Rb flux assay[8] on the NKCC1 co-transporter (14% inhibition at 100 μM). This selectivity clearly differentiates compound (+)-**2** from the loop diuretics like furosemide. Compound (+)-**2** is the dextro enantiomer and, interestingly, the levo enantiomer (−)-**2** is about 100-fold less potent on KCC2 ($IC_{50}$ = 50 μM). Unfortunately, the poor metabolic stability ($Cl_{int}$ rat microsomes = 392 μL/min/mg protein) of compound (+)-**2** render its use as an in vivo pharmacological tool problematic. It appeared nevertheless to be a good starting point to identify a compound with an improved profile.

In this Letter, we wish to report structure–activity relationships around our hit compound focusing on the modulation of $R^1$, $R^2$, and $R^3$ (Fig. 1) substituents. Compounds were obtained following the synthetic route depicted in Scheme 1. N-Boc L-proline was reacted with benzyl bromide in the presence of potassium carbonate to form the corresponding benzyl ester. The alkylation step[9] was performed by deprotonation with LiHMDS at −70 °C, followed by addition of various benzyl or alkyl bromides (conditions **c**). Racemization occurred under these conditions and all described compounds are racemic mixtures unless otherwise stated.

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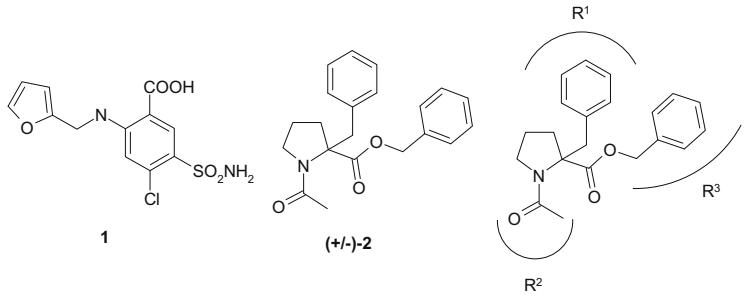
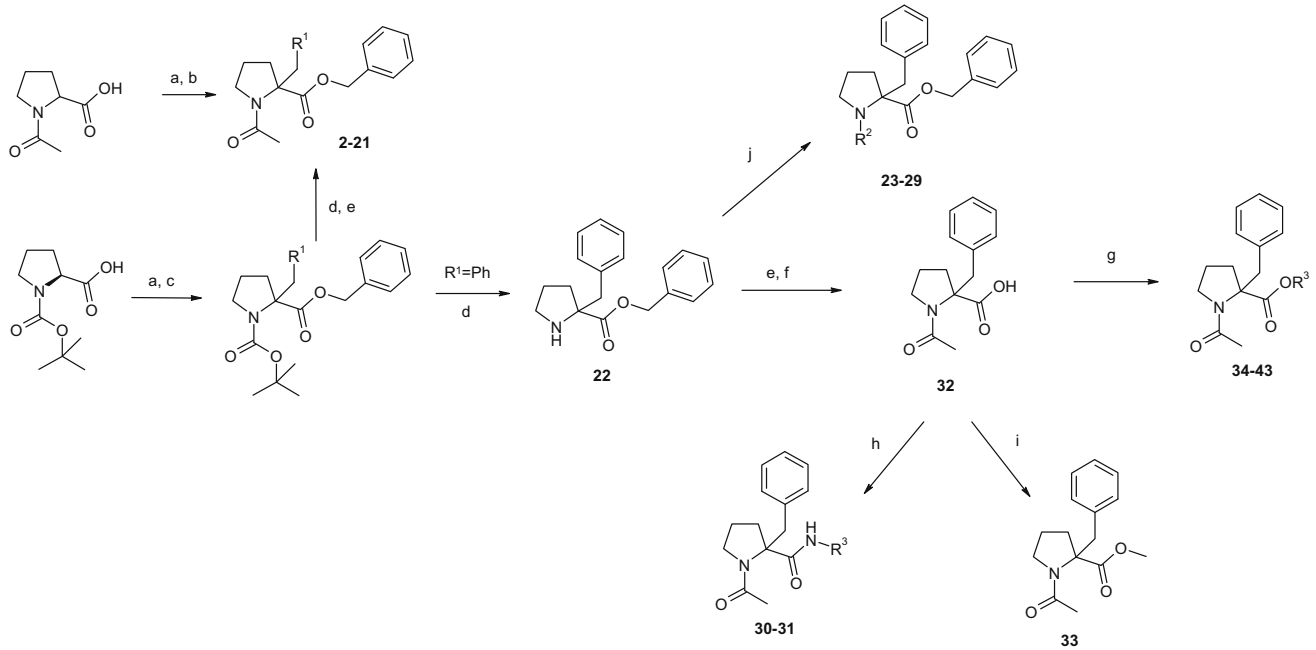



**Figure 1.** Furosemide **1** reference compound, hit compound **2**, and SAR modulations.




**Scheme 1.** Reagents and conditions: (a) benzylbromide (2 equiv), $K_2CO_3$ (2 equiv), KI cat., $CH_3CN$, rt, 80%; (b) *n*-BuLi (1.1 equiv), THF, −70 °C, $R^1CH_2Br$ (1.1 equiv) or EtI (1.1 equiv), 8–30%; (c) LiHMDS (1.2 equiv), THF, −70 °C, $R^1CH_2Br$ (1.3 equiv), 40–60%. (d) TFA, DCM, rt, 100%; (e) $CH_3COCl$ (2 equiv), $Na_2CO_3$ (10 equiv), $CH_2Cl_2/H_2O$ (1:1), rt, 50–100%; (f) NaOH, EtOH, 70 °C, 87%; (g) $R^3Br$ (1 equiv), $K_2CO_3$ (1.1 equiv), KI cat., $CH_3CN$, 50 °C, 25–80%; (h) $BnNH_2$ or BnNHMe (1.2 equiv), EDCI (1.5 equiv), HOBt (1.5 equiv), DIPEA (1.5 equiv), $CH_2Cl_2$, rt, 70–90%; (i) $SOCl_2$, MeOH, rt, 95%; (j) $CH_3SO_2Cl$ (1.2 equiv), NaH (1.2 equiv), THF, 60 °C, 43% or $ClCOOCH_3$ (2 equiv), Pyridine (2 equiv), $CH_2Cl_2$, rt, 65% or EtI (3 equiv), $K_2CO_3$ (1.5 equiv), $CH_3CN$, rt, 40% or EtNCO (1 equiv), $CH_2Cl_2$, rt, 100% or for $R^2$ = RCO: RCOCl (2 equiv), $Na_2CO_3$ (10 equiv), $CH_2Cl_2/H_2O$ (1:1), rt, 56–76%.

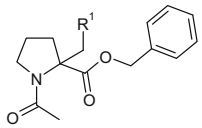
Enantiomers would then be separated by chiral chromatography at the final step if required. After TFA deprotection, the intermediates were acetylated to provide compounds **2–21**. A shorter route was also used starting from *N*-acetyl proline but the yields of the alkylation step were always lower (below 30%) in particular with the less reactive bromides like cyclohexylmethyl bromide or phenethyl bromide (conditions **b**). Compounds **23–29** were obtained by reacting intermediate **22** with various acyl chlorides, sulfonyl chlorides, chloroformates or isocyanates. After acetyl protection and saponification of **22**, the acid **32** was a key intermediate to provide, on one hand, amides **30–31** by peptidic coupling with amines and, on the other hand, esters **34–43** by alkylation with the corresponding bromides.

We investigated the nature of the aromatic group $R^1$ (Table 1). The presence of the phenyl ring is essential for activity on KCC2 as its deletion gave compound **4** which is 100-fold less potent, but it can be replaced by heteroaromatics (thienyl in **7**, pyridyl in **6**) or cycloalkyls (cyclohexyl in **5**) with a minor loss of activity. The increase in the chain length of one methylene provided compound **3** with reduced activity ($IC_{50}$ = 8 μM). We also studied the effects of various substituents on the phenyl ring in $R^1$ (Table 1) by modulating its electron density. Moving a cyano group around the phenyl ring indicates the *ortho* substitution is clearly disfavored (as in **16** and **21**). The *meta* and *para* substitution are both very favorable and can accommodate rather different types of substituents. The most potent compounds are found with bulky groups such as $CF_3$ in *meta* position (compounds **18–20**, $IC_{50}$ from 50 to 80 nM) but those compounds are also amongst the most lipophilic ones (*A*Log *P* > 4.2).

We then modulated the *N*-acetyl moiety (Table 2). Deletions of the acetyl (**22**) or the carbonyl (**23**) groups lead to a complete loss in activity. Increasing the length of the alkyl of one methylene slightly reduces the potency (compound **27**, $IC_{50}$ = 1.2 μM). A cyclopropyl chain is beneficial for activity contrary to a phenyl group. Carbamates, ureas and sulfonamides were also evaluated; they have broadly similar activities to the corresponding amides in the same range of lipophilicity.

Concerning the ester side chain (Table 3), we found that the presence of the phenyl ring is essential for activity (the acid **22** and the methyl ester **23** are inactive). Moreover, this phenyl ring in $R^4$ can not be replaced by a cyclohexyl (**36**) or a pyridyl (**37**). The substitution of this phenyl ring is also clearly detrimental to

**Table 1**
KCC2 activities for compounds **2**–**21**: 2-benzyl modulation

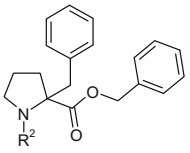


**2-21**

| Compd | $R^1$ | $IC_{50}$ (μM)[a] | *A*Log *P*[b] |
|---|---|---|---|
| **2** | Ph | 0.79 | 3.33 |
| (+)-**2** | Ph | 0.32 | 3.33 |
| (−)-**2** | Ph | 50 | 3.33 |
| **3** | $CH_2Ph$ | 8 | 3.79 |
| **4** | $CH_3$ | 80 | 2.3 |
| **5** | Cyclohexyl | 1 | 4 |
| **6** | 4-Pyridyl | 2 | NA |
| **7** | 3-Thienyl | 0.79 | 2.99 |
| **8** | 4-$CH_3$-Ph | 0.79 | 3.82 |
| **9** | 4-$OCH_3$-Ph | 0.4 | 3.32 |
| **10** | 4-$CF_3$-Ph | 0.63 | 4.28 |
| **11** | 4-F-Ph | 0.20 | 3.54 |
| **12** | 4-Cl-Ph | 0.32 | 4 |
| **13** | 4-($SO_2Me$)-Ph | 1 | 2.86 |
| (+)-**13** | 4-($SO_2Me$)-Ph | 0.40 | 2.86 |
| (−)-**13** | 4-($SO_2Me$)-Ph | 40 | 2.86 |
| **14** | 4-CN-Ph | 0.16 | 3.21 |
| **15** | 3-CN-Ph | 0.32 | 3.21 |
| **16** | 2-CN-Ph | 5 | 3.21 |
| **17** | 3-($SO_2Me$)-Ph | 0.25 | 2.86 |
| **18** | 3-$CF_3$-Ph | 0.08 | 4.28 |
| **19** | (3-$CF_3$, 4-F)-Ph | 0.06 | 4.48 |
| **20** | (3-$CF_3$, 4-$OCH_3$)-Ph | 0.05 | 4.26 |
| **21** | (2-$CF_3$, 4-F)-Ph | 4 | 4.48 |

[a] Rb influx assay on rKCC2.
[b] Atom-based method to calculate the octanol–water partition coefficient for non-ionisable compounds.[10]

**Table 2**
KCC2 activities for compounds **22**–**29**: N-acyl modulation



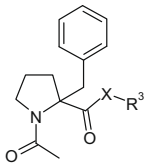
**22-29**

| Compd | $R^2$ | $IC_{50}$ (μM)[a] | *A*Log *P*[b] |
|---|---|---|---|
| **2** | $COCH_3$ | 0.79 | 3.33 |
| **22** | H | >100 | NA |
| **23** | $CH_2CH_3$ | >100 | 4.42 |
| **24** | $SO_2CH_3$ | 1.6 | 3.12 |
| **25** | $CO_2CH_3$ | 0.4 | 3.98 |
| **26** | $CONHCH_2CH_3$ | 2 | 3.68 |
| **27** | $COCH_2CH_3$ | 1.2 | 4 |
| **28** | COcPr | 0.32 | 4.09 |
| **29** | COPh | 4 | 5 |

[a] Rb influx assay on rKCC2.
[b] Atom-based method to calculate the octanol–water partition coefficient for non-ionisable compounds.[10]

the activity, in particular in the ortho position (**38**) or with large substituents (**42**, **43**). The phenethyl ester **35** is 12-fold less active suggesting that the lipophilic pocket is not very deep. The substitution of the benzylic position with a methyl causes a decrease in affinity (compound **34**, $IC_{50}$ = 4 μM). Interestingly, replacing the ester function by amides is detrimental to the KCC2 activity, the NH-benzyl **30** and *N*-methyl-*N*-benzyl **31** analogues displaying $IC_{50}$s of 100 and 32 μM, respectively.

**Table 3**
KCC2 activities for compounds **30**–**43**: ester modulation



**30-43**

| Compd | X | $R^3$ | $IC_{50}$ (μM)[a] | *A*Log *P*[b] |
|---|---|---|---|---|
| **2** | O | $CH_2Ph$ | 0.79 | 3.33 |
| **30** | NH | $CH_2Ph$ | 100 | 2.69 |
| **31** | $NCH_3$ | $CH_2Ph$ | 32 | 2.89 |
| **32** | O | H | >100 | NA |
| **33** | O | $CH_3$ | >100 | 1.75 |
| **34** | O | $CH(CH_3)Ph$ | 4 | 3.71 |
| **35** | O | $CH_2CH_2Ph$ | 10 | 3.65 |
| **36** | O | $CH_2cHex$ | 63 | 3.94 |
| **37** | O | $CH_2$(4-pyridyl) | >100 | NA |
| **38** | O | $CH_2$(2-ClPh) | 6 | 4 |
| **39** | O | $CH_2$(3-ClPh) | 2.5 | 4 |
| **40** | O | $CH_2$(4-ClPh) | 1.3 | 4 |
| **41** | O | $CH_2$(4-$CH_3$Ph) | 0.79 | 3.82 |
| **42** | O | $CH_2$(4-$CF_3$Ph) | 5 | 4.28 |
| **43** | O | $CH_2$(4-($SO_2CH_3$)Ph) | >100 | 2.86 |

[a] Rb influx assay on rKCC2.
[b] Atom-based method to calculate the octanol–water partition coefficient for non-ionisable compounds.[10]

The most potent KCC2 blockers from the benzyl prolinate series were tested for their selectivity versus NKCC1, their aqueous solubility and in vitro metabolic stability. Data are reported in Table 4. They are all much less potent in inhibiting NKCC1 activity than furosemide (**1**). Most compounds are oils and display low solubility. Moreover, compounds are generally extensively metabolized on rat microsomes and hepatocytes. Surprisingly, compound **13** bearing a *para* methylsulfone in $R^2$ displays the best profile with a good aqueous solubility (0.5 mg/mL), a low clearance on rat microsomes (16 μL/min/mg protein), a good permeability on Caco-2 cells (Papps = 310 nm/s) and a low cytochrome P450 isoforms inhibition (<30% inhibition at 50 μM for human Cyps 3A4, 2D6, 2C9, and 2C19). This compound **13** is one of the less lipophilic of the series (*A*Log *P* = 2.86) and the polarity introduced by the methylsulfone[11] can partially explain this overall beneficial effect, in particular, on the metabolic stability. The blockade of a potential metabolic position could also contribute as the *para* CF3 analogue **10** and the *para* CN analogue **14** are also slightly more stable than the original hit **2**. Moreover, the *meta* methylsulfone analogue **17**

**Table 4**
NKCC1 activity, solubility and in vitro metabolic stability of selected compounds

| Compd | Inhibition% NKCC1[a] | Solubility[b] | $Cl_{int}$ on rat microsomes[c] | $Cl_{int}$ on rat hepatocytes[d] |
|---|---|---|---|---|
| **1** | 100 | nt | nt | nt |
| **2** | 44 | 0.002 | 385 | 28 |
| **5** | 39 | 0.003[e] | 382 | nt |
| **9** | 59 | 0.01[e] | 333 | 23 |
| **10** | 53 | 0.002[e] | 128 | 20 |
| **13** | 35 | 0.5 | 16 | 12 |
| **14** | 31 | 0.02 | 123 | 31 |
| **17** | 25 | nt | 107 | 24 |
| **19** | 24 | 0.0005 | 356 | 20 |
| **24** | <20 | 0.001[e] | 400 | 23 |

[a] Rb flux assay on NKCC1 transporter at 100 μM.
[b] In mg/mL in PBS buffer at pH = 7.4.
[c] In μL/min/mg protein.
[d] In μL/min/$10^6$ cells.
[e] Samples obtained as oils; nt = not tested.

**Table 5**
Compared in vivo rat PK profile of hit and optimized compound

| Compd | $t_{1/2}$[a] (h) | Cl[a] (mL/min/kg) | AUC[b] (ng h/mL) | $C_{max}$[b] (ng/mL) | *F* (%) |
|---|---|---|---|---|---|
| 2 | 2.4 | 61 | 8.6 | 11.9 | 0.5 |
| 13 | 0.3 | 26 | 726 | 457 | 18 |

Compounds dosed at [a]1 mg/kg iv and [b]6 mg/kg po in male Wistar rats.

does not display similar low clearance such as **13**. Compound **13** was also tested in an extended receptogram of more than 50 CNS receptors, channels or enzymes and no activity was observed at 10 μM. Enantiomers of **13** were also prepared and a 100-fold difference in potency was found as for the hit compound **2** (compare (+)-**13** and its enantiomer (−)-**13**).

With its improved properties, compound **13** was investigated further and the in vivo PK profile was evaluated in rat (Table 5). Compound **13** showed in rat a lower clearance, higher plasma exposures and an improved oral bioavailability compared to compound **2**. Plasma and brain exposure were also evaluated in NMRI mice in order to assess its potential as tool for in vivo pharmacology evaluation. At 5 minutes after ip dosing at 43.3 mg/kg, compound **13** reached free brain levels close to 6 μM, which was sixfold higher than the in vitro $IC_{50}$ for KCC2 and then sufficient to be evaluated in an in vivo model of epilepsy, the audiogenic mouse seizure test.[12] Compound **13** is inactive in this test up to 75 mg/kg (ip injection 5 min before stimulation). Thus, the blockade of KCC2 co-transporter doesn't protect against sound-induced seizure in the audiogenic mouse. This observation may be consistent with the fact that furosemide shows limited efficacy in this model only at doses exceeding 100 mg/kg.[14] Furthermore, furosemide displays anti-epileptiform activity in vitro only at mM concentrations[13], which are much higher than its $IC_{50s}$ for KCC2 and NKCC1, 25 μM and 3 μM, respectively. Thus, anticonvulsant activity of furosemide appears to be model dependent[15] and difficult to link specifically with KCC2 inhibition.

In conclusion, we describe the discovery of a novel class of selective submicromolar KCC2 blockers: the benzyl *N*-acyl 2-benzylprolinate derivatives. SAR modulations allowed for the identification of an analogue **13** with drug-like properties and good brain exposure in mice. This compound was found inactive in an in vivo model of seizures, which could argue against the concept of using KCC2 blockers for the treatment of epilepsy. However, further characterization of compound **13** will be performed in other seizure models, which may yield more conclusive results regarding the anticonvulsant potential of KCC2 inhibition.

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8. The NKCC1 assay was developed on a MDCK (Madin Darby Canine Kidney) cell line expressing an endogenous canine NKCC1 isoform. The NKCC1 co-transporter is activated by incubating the cells during 2 h in a $Cl^-$ free medium (which induces phosphorylation of the co-transporter) and $Rb^+$ influx is stimulated during 45 min by adding 20 mM RbCl to the extracellular solution. The amount of intracellular $Rb^+$ is determined by atomic absorption spectroscopy.
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