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Re-exploring the *N*-phenylpicolinamide derivatives to develop $mGlu_4$ ligands with improved affinity and *in vitro* microsomal stability

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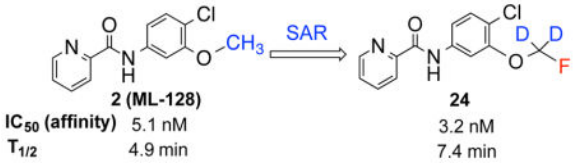
Abstract

In recent years, $mGlu_4$ has received great attention and research effort because of the potential benefits of $mGlu_4$ activation in treating numerous brain disorders, such as Parkinson's disease (PD). Many positive allosteric modulators of $mGlu_4$ have been developed. To better understand the role of $mGlu_4$ in healthy and disease conditions, we are interested in developing an $mGlu_4$ selective radioligand for *in vivo* studies. Thus, we had synthesized and studied [^{11}C]**2** as a PET tracer for $mGlu_4$, which demonstrated some promising features as a PET radioligand as well as the limitation need to be improved. In order to develop an $mGlu_4$ ligand with enhanced affinity and improved metabolic stability, we have modified, synthesized and evaluated a series of new *N*-phenylpicolinamide derivatives. The SAR study has discovered a number of compounds with low nM affinity to $mGlu_4$. The dideuteriumfluoromethoxy modified compound **24** is identified as a very promising $mGlu_4$ ligand, which has demonstrated enhanced affinity, improved *in vitro* microsomal stability, good selectivity and good permeability.

Graphical Abstract



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Supplementary data

Supplementary data (experimental procedures and spectroscopic characterization of all new compounds) associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.bmcl.

Keywords

metabotropic glutamate receptor subtype 4 ($mGlu_4$); positive allosteric modulator (PAM); positron emission tomography (PET); affinity; metabolic stability; structure-affinity relationship (SAR)

L-Glutamate is the most abundant excitatory neurotransmitter in the CNS (Central nerve system) of vertebrates and probably mediates more than 50% of all synapses.[1,2] Two major classes of receptors, mGlu and iGlu, are involved in glutamate signal transfer. The mGlu belong to Class C of the GPCR (G protein-coupled receptor) super family, which are thought to exist as dimers and have a distinct large extracellular N-terminus. This extracellular N-terminal domain contains two hinged globular domains referred as the Venus Flytrap Domain (VFD), which is the orthosteric binding site for the endogenous ligand, L-glutamate.[3] The mGlu can be further divided into three subgroups including eight known receptor subtypes (group I: $mGlu_1$ and $mGlu_5$, group II: $mGlu_2$ and $mGlu_3$, and group III: $mGlu_4$, $mGlu_6$, $mGlu_7$ and $mGlu_8$) based on their structural similarity, ligand specificity, and preferred coupling mechanisms.[4] The mGlu are involved in glutamate signaling in almost every excitatory synapse in CNS, and they have distinctive biodistribution in CNS depending on subtypes and subgroups.[5] In recent years, $mGlu_4$ has received great attention and research effort because of the potential benefits of $mGlu_4$ activation in treating numerous brain disorders, such as Parkinson's disease (PD).[6,7] As a group III mGlu, $mGlu_4$ interacts with the $G_{\alpha i/o}$ subunit of G-protein which negatively couples with adenylate cyclase to inhibit cAMP dependent signal pathways.[8,9] The $mGlu_4$ is expressed at multiple synapses throughout the basal ganglia, mainly localized presynaptically and expressed in the striatum, hippocampus, thalamus, and cerebellum.[4,10,11] Its activation reduces neurotransmitter release, a mechanism implicated in the pathophysiology of PD. The activation of the $mGlu_4$ receptor can be accomplished by two different mechanisms: orthosteric agonists (competing with L-glutamate) or noncompetitive positive allosteric modulators (PAMs). Most orthosteric ligands of $mGlu_4$ made in the past lack clear subtype selectivity and BBB (Blood-brain barrier) penetration, but notable examples exist of selective and brain penetrant orthosteric agonists, such as LSP4-2022.[12,13] Much recent effort has been focused on the development of allosteric modulators, which target the seven-transmembrane spanning domain. In particular, the allosteric modulation of $mGlu_4$ has spurred intense interest after (−)-PHCCC (**1**, N-phenyl-7-(hydroxyimino)-cyclopropa[*b*]chromen-1*a*-carboxamide), a partially selective $mGlu_4$ PAM, was discovered and demonstrated activity in models of neuroprotection and PD. Since then there has been substantial progress in identifying PAMs for $mGlu_4$.[6,14,15] Figure 1 shows some representative $mGlu_4$ PAMs.[6,14,16,17,18,19,20] Subsequent results with PAMs of $mGlu_4$ have further validated the antiparkinsonian activity in animal models of PD,[11,17,21,22,23,24] in which this approach has opened a new avenue for developing nondopaminergic treatments for PD and for identifying a novel disease modifying therapeutics.

To better understand the role of $mGlu_4$ in healthy and disease conditions, we are interested in developing an $mGlu_4$ selective radioligand for *in vivo* study. As a noninvasive medical imaging technique and a powerful tool in neurological research, positron emission tomography (PET) offers a possibility to visualize and analyze the target receptor expression

under physiological and pathophysiological conditions. PET is being applied more often to detect disease-related biochemical changes before the disease-associated anatomical changes could be found by standard medical imaging modalities. Moreover, PET tracers serve as invaluable biomarkers during the development of potential therapeutic drugs. Thus, extensive research efforts have been directed toward the development of PET radioligands suitable for probing mGlu such as $mGlu_1$ and $mGlu_5$.[15]

Recently, we have reported a carbon-11 labeled PET ligand [^{11}C]**2** (*N*-(4-Chloro-3-[^{11}C]methoxyphenyl)picolinamide)[25], which was based on a reported $mGlu_4$ PAM **2**[16]. In 2009, two research groups at Addex Pharma[26] and Vanderbilt University[16] have independently disclosed a series of small arylamide compounds as a new class of $mGlu_4$ PAMs. Engers et al. found from a high-throughput screening there were a number of small arylamide compounds having $mGlu_4$ PAM activity. They reported the SAR study, *in vitro* pharmacokinetic (PK) parameters and *in vivo* rat PK, which included the SAR results for sixteen *N*-phenylpicolinamide derivatives.[16] Compounds **2** and **3** were the most potent $mGlu_4$ PAMs in this series and showed some potentially suitable properties for PET tracer development, which include: 1) Rapid penetration into rat brain following intraperitoneal injection (T_{max} for brain: 0.5 h); 2) High brain:plasma (B/P) partition coefficients for both compounds (B/P=4.1 for **2** and 9.9 for **3**), in which B/P was determined by $AUC_{0–8h,\ Brain}/AUC_{0–8h,\ Plasma}$; 3) Good *in vitro* potency and efficacy for both human and rat $mGlu_4$ compared to previous reported $mGlu_4$ PAM; 4) Good selectivity over other mGlu subtypes; 5) Compound **2** was the first $mGlu_4$ PAM to demonstrate efficacy in a preclinical rodent model of motor impairments associated with PD.[6] Thus, we had synthesized and studied [^{11}C]**2** as a PET tracers for $mGlu_4$. This compound demonstrated some promising features as a PET radioligand such as the fast uptake into brain and the specific accumulation in $mGlu_4$-rich regions of the brain. However, in comparison to one of the best $mGlu_5$ PET tracer [^{18}F]FPEB (3-[^{18}F]fluoro-5-(2-pyridinylethynyl)benzonitrile)[27,28], [^{11}C]**2** showed the decreased retention time in the brain, which may affect the quality of the imaging. The results indicate that the affinity and metabolic stability of this class of tracers need further optimization. We report here the synthesis and structure-affinity relationship study of new *N*-phenylpicolinamide derivatives to develop $mGlu_4$ ligands with improved affinity and metabolic stability.

We have modified and synthesized a series of new *N*-phenylpicolinamide derivatives for SAR study, in which the syntheses are shown in Scheme 1 – 3 (see Supplementary data). Three most active known compounds in this series (**2**, **3**, and **10**) were also synthesized and evaluated as the reference compounds for optimization. On the basis of previous SAR results[16], we modified compound **2** at three positions (3- or 4-phenyl, 6-pyrindyl) as illustrated in Figure 2. It is known that the SAR of this series was tight,[6,16] so we started with minor modifications based on the reported data. As shown in Table 1, the modifications include the isosteric replacement of hydrogen by fluorine or deuterium, oxygen by sulfur, methoxy by cyano group and change for different halogen atoms. The radiolabeling strategy was also considered in lead optimization design to generate the facile labeling positions for either C-11 or F-18 tracer.

Since poor BBB permeability and high nonspecific binding (NSB) are among the most frequent causes for failure in CNS PET ligand development, it is necessary to consider some important physicochemical parameter such as MW, ClogP and tPSA at the design stage. It has been recently proposed that more desirable ranges for CNS drugs are ClogP <3, MW < 360 and 40 < tPSA < 90.[29] As shown in Table 1, all compounds except **12** and **22** possess the favorable physicochemical parameters, making them ideal candidates for CNS ligand development.

The lead compounds **2** and **3** were identified as $mGlu_4$ PAMs by using functional assays (calcium mobilization assays for human $mGlu_4$ and thallium flux assays for rat $mGlu_4$) and characterized with EC_{50}, the maximum response and the fold shift values.[16] It is known that the EC_{50} value may not always correlated closely to the affinity value for PAM.[30] It is very important to study the binding affinity for developing PET ligands. Thus, we prepared the tritium-labeled compound **2** ([^{3}H]**2**, *N*-(4-chloro-3-(methoxy-t_3)phenyl)picolinamide) for competitive binding assay.[31] The synthesized compounds were characterized with competitive binding studies using $mGlu_4$ transfected CHO cells by increasing the concentration of test materials from 0.01 nM to 10 μM in presence of 2 nM of [^{3}H]**2**, in which the binding affinities to $mGlu_4$ were described as IC_{50} values (Table 1).[32]

In structure-affinity study, we first evaluated the substitutions at the 4-phenyl position by keeping the 3-methoxy group constant. The 4-phenyl position of *N*-phenylpicolinamide was tolerated with some substitutions as demonstrated in known compounds **6**–**8**, in which compounds **6** and **7** were reported very potent but poor brain penetration.[20] Thus we limited the 4-phenyl substitutions for different halogens. The results show that the 4-chloro substitution give the best affinity, in which the affinity values of **2** and **10–13** are in the following order: Cl < H < F < I < Br. Larger halogen substitutions such as iodine and bromine led to substantial loss in affinity. It was then found that the 3-methylthio group was superior to the 3-methoxy group by comparing compounds **13** and **14**, showing a 2.8 fold enhancement in affinity.

On the other hand, compounds **15**–**17** had been incorporated a fluorine atom at 6-pyrindyl position of *N*-phenylpicolinamide, which can have a relatively facile fluorine-18 labeling. Compared to **2** and **3,** the affinity of **15** and **16** was not significantly reduced.

Next we turned our attention to the 3-phenyl position. It is considered that the metabolic stability was one of major issues for ML-128 (**2**), in which the 3-methoxy group was identified as the soft group. The 3-phenyl position was also very sensitive with substitutions. It was reported a simple change of 3-difluoromethoxy in compound **3** to 3-trifluoromethoxy group imparted a more than 10 fold loss of activity.[16] Our initial effort was directed at 3-cyano substitution, in which ^{11}C-cyanation may be carried out through a palladium-mediated cyanation or the Rousenmund-von Braun reaction.[33] Five 3-cyanophenyl compounds (**18**–**22**) with different 4-phenyl substitutions were evaluated. The results show that the 3-cyano-4-chloro-analog **20** give a similar affinity compared to the 3-methoxy-4-chloro-analog **2**. The affinity values of **18**–**22** are depending on 4-phenyl substitution and in the following order: Cl < F < H < Br < I, which shows different substitution effect compared to 3-methoxy analogs **2** and **10**–**13**. We then replaced 3-methoxy with 3-fluoromethoxy for

two reasons: first, since both 3-methoxy- and 3-difluoromethoxy-analogs exhibited the activity, fluoromethoxy should be also active; second, it generates a position for fluorine-18 labeling. Fluorine-18 is often the radionuclide of choice for both its physical and nuclear characteristics. Its half-life is long enough to carry out relatively extended imaging protocols when compared to what is possible with carbon-11. This facilitates kinetic studies and high-quality metabolic and plasma analysis. However, fluorine-18 labeling is normally limited to chemical structures already containing a fluorine atom and the possible labeling strategies are limited for the preparation of radiotracers of high specific radioactivity. The result shows that 3-fluoromethoxy compound **23** has an improved affinity (3.2 nM) compared to that (5.1 nM) of **2**, which improves 1.6 fold. However, 3-fluoromethoxy group may not be metabolically stable, since the 3-methoxy and 3-difluoromethoxy groups were metabolically unstable in compounds **2** and **3**. On the other hand, 3-trifluoromethoxy analog of **3** was significantly more stable but lack activity.[16] Hence, we had applied a 3-dideuteriumfluoromethoxy group to replace 3-fluoromethoxy group as shown in compounds **24** and **25**. Deuterium isotope effects have been used to reduce *in vivo* metabolic rates. For example, Zhang et al. reported that a deuterium-substituted analog (with $^{18}FD_2CO$) as a radioligand for peripheral benzodiazepine receptor (PBR) had remarkably prolonged the half-life ($T_{1/2}$) in mice brain.[34] The deuterium substitution may reduce the rate of defluorination initiated by cleavage of the C–H bond without altering the binding affinity to $mGlu_4$. The result shows that the 3-dideuteriumfluoromethoxy modified compounds **24** and **25** have excellent affinity.

On the basis of the affinity of these picolinamide derivatives, we subsequently determined the *in vitro* microsomal stability of the selected compounds that include **2**–**4** and **23**–**24** (Table 2). Compound **4** (ADX88178) is one of a most potent $mGlu_4$ PAM to date and was shown to be orally active in a number of preclinical *in vivo* PD models.[14,22] As Table 2 shows, the dideuteriumfluoromethoxy-compound **24** ($T_{1/2}$ = 7.4 min) is more stable than the corresponding fluoromethoxy-analog **23** ($T_{1/2}$ = 5.8 min) and the methoxy-analog **2**($T_{1/2}$ = 4.9 min). It was reported that the cleaving rate of the C–H bond wasabout 6.7 times faster than that of C–D bond at 25 °C.[34] On the other hand, the half time and the difference of the metabolic rates of the dideuteriumfluoromethoxy analog and the fluoromethoxy analog depended on the level of the enzyme. In developing the PET ligand for PBR, Zhang et al. found that the half time ($T_{1/2}$) in the plasma was 2.575 min for the deuterium-substituted analog (with $^{18}FD_2CO$) and 2.367 min for the non-deuterated analog. However, the half time ($T_{1/2}$) of the deuterium-substituted analog in the brain was >60 min, whereas that of for the non-deuterated analog was only 2.227 min.[34] We anticipate that the difference of the half times in the brain between compounds **24** and **23** as well as **2** could be more significant. Compared to **4**, compound **24** has the same affinity and a similar *in vitro* microsomal stability. It is clear that compound **24** has both enhanced affinity and improved *in vitro* microsomal stability compared to **2**.

The selectivity of compound **24** was also determined among the various mGlu subtypes, in which the functional assays were carried out on $mGlu_1$, $mGlu_2$, $mGlu_5$, $mGlu_6$ and $mGlu_8$. Compound **24** showed little activity against these mGlu (Supporting Information).

In addition, the permeability values of **2**–**4** and **23** were measured using BBB PAMPA model at pH 7.4, which characterized the rate across the BBB due to passive diffusion. The determined effective permeability (P_e) values are summarized in Table 2, in which the P_e results for internal highly and low permeable standards are 160 for propranolol and <2.8 for atenolol, respectively. This result indicates that compounds **23** and **24** have good BBB permeability. Although high BBB passive permeability does not necessary translate to sufficient unbound drug concentration in the brain because of potential intrinsic clearance and efflux transport, it is beneficial for CNS drug candidates.

In summary, we have modified, synthesized and evaluated a series of new *N*-phenylpicolinamide derivatives. Our research further demonstrated that *N*-phenylpicolinamide is a good template to develop $mGlu_4$ ligands, which has offered extensive SAR results by us and other labs.[16,26] The SAR study has discovered a number of compounds with good affinities (<10 nM) to $mGlu_4$. The dideuteriumfluoromethoxy modified compound **24** is identified as a very promising $mGlu_4$ ligand, which has demonstrated enhanced affinity, improved *in vitro* microsomal stability, good selectivity and good permeability. Compound **24** is considered as an attractive candidate for future labeling with fluorine-18 as an $mGlu_4$ PET tracer. Since a number of compounds have good affinity we are studying their PAM activity to $mGlu_4$ and potential therapeutic applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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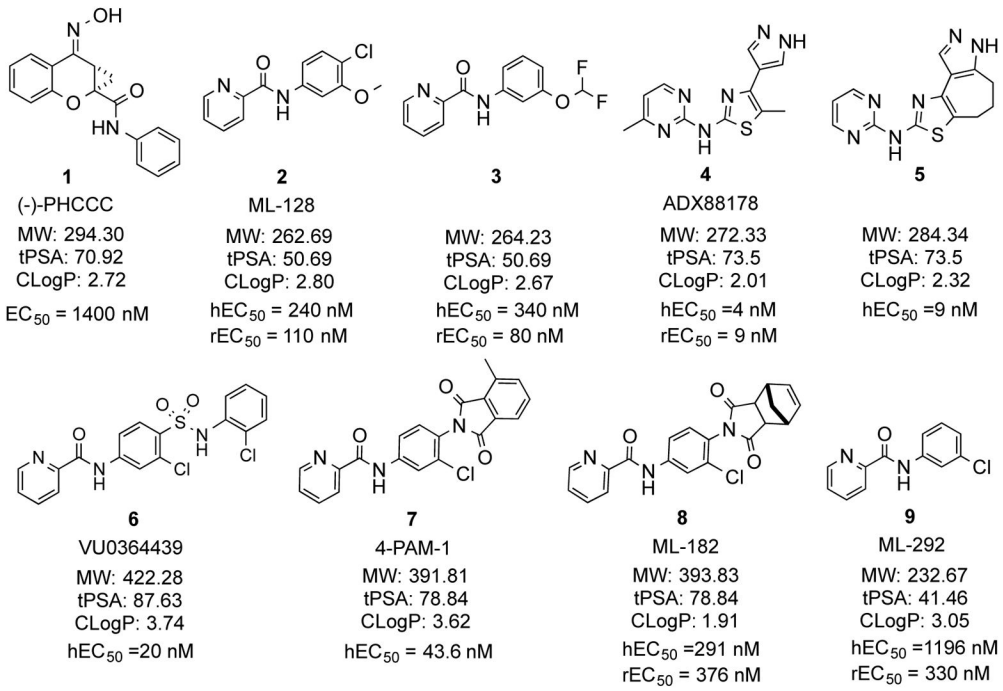


Figure 1.
Some representative $mGlu_4$ PAMs.

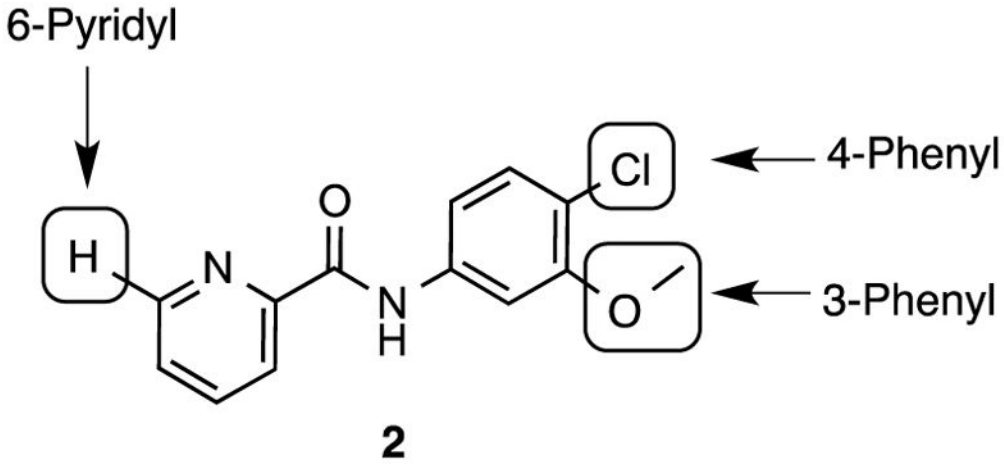
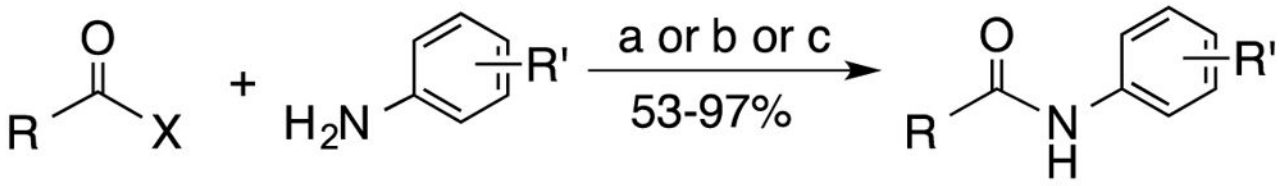


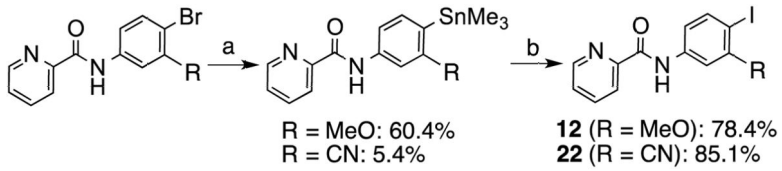
Figure 2.
Modificatons on compound **2**



Scheme 1.

Synthesis of the *N*-phenylpicolinamide derivatives.

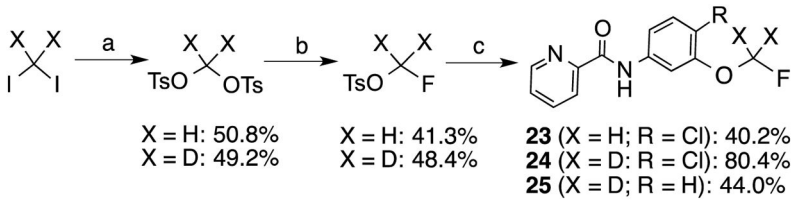
Reagents and conditions: (a) for carboxylic acids, EDC.HCl, HOBt.H_2O, DIPEA, dioxane; (b) for carboxylic acids, 1. thionyl chloride, benzene, reflux for 2 h; 2. TEA, THF, 40 °C, 1 h; (c) for acid chloride, DIPEA, CH_2Cl_2, 4h.



Scheme 2.

Synthesis of the *N*-phenylpicolinamides **12** and **22.**

Reagents and conditions: (a) $(SnMe_3)_2$, $Pd(PPh_3)_4$, Toluene, reflux, 8.5 h; (b) I_2, CH_2Cl_2, 2 h.



Scheme 3.

Synthesis of the *N*-(3-fluoromethoxyphenyl)picolinamides **23 – 25.**

Reagents and conditions: (a) Ag(OTs), MeCN, reflux, overnight; (b) CsF, $HO(CH_2O)_6H$, reflux, 3.5 h; (c) *N*-(4-R-3-hydroxyphenyl)picolinamide, K_2CO_3, 40–50 °C, 3 days.

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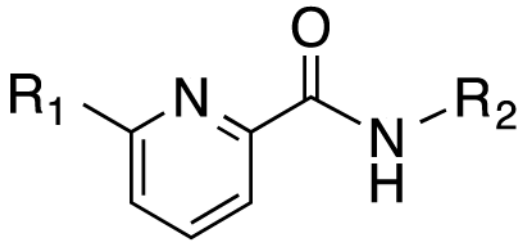
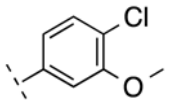
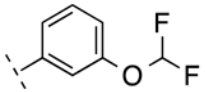
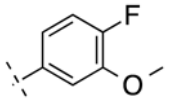
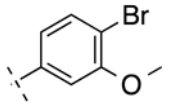
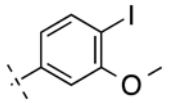
Table 1

SAR of *N*-phenylpicolinamide derivatives.



Compd	R_1	R_2	MW	CLogP	tPSA	Affinity IC_{50} (nM)	Log(IC_{50} ± SE)
2	H		262.69	2.80	50.69	5.1	−8.29 ± 0.09
3	H		264.23	2.67	50.69	4.6	−8.34 ± 0.08
10	H		246.24	2.26	50.69	31.6	−7.50 ± 0.09
11	H		307.15	2.95	50.69	322	−6.49 ± 0.08
12	H		354.15	3.16	50.69	146	−6.84 ± 0.10

Compd	R_1	R_2	MW	CLogP	tPSA	Affinity IC_{50} (nM)	Log(IC_{50} ± SE)
13	H		228.25	2.22	50.69	13.7	−7.86 ± 0.10
14	H		244.31	2.81	41.46	4.9	−8.31 ± 0.07
15	F		280.68	2.96	50.69	7.3	−8.13 ± 0.11
16	F		282.22	2.85	50.69	6.7	−8.18 ± 0.10
17	F		264.23	2.42	50.69	89.2	−7.05 ± 0.10
18	H		223.23	1.89	65.25	10.4	−7.98 ± 0.05

Compd	R_1	R_2	MW	CLogP	tPSA	Affinity IC_{50} (nM)	Log(IC_{50} ± SE)
19	H		241.23	2.06	65.25	7.4	−8.13 ± 0.04
20	H		257.68	2.50	65.25	5.3	−8.28 ± 0.04
21	H		302.13	2.78	65.25	47	−7.33 ± 0.08
22	H		349.13	3.04	65.25	172	−6.77 ± 0.08
23	H		280.68	2.97	50.69	3.2	−8.49 ± 0.07
24	H		282.70	2.97	50.69	3.2	−8.49 ± 0.11

Compd	R_1	R_2	MW	CLogP	tPSA	Affinity IC_{50} (nM)	Log(IC_{50} ± SE)
25	H		248.25	2.39	50.69	3.7	−8.43 ± 0.03

Table 2

In Vitro properties of the selected compounds.

Compound	Affinity IC_{50} (nM) (n=3)	κ^a	SEM(κ) (n=2)	$T_{1/2}$ (min)	Avg. P_e (10^{-6} cm/s)
2	5.1	0.141	0.010	4.9	256
3	4.6	0.132	0.008	5.2	214
4	3.2	0.099	0.005	7.0	257
23	3.2	0.120	0.010	5.8	272
24	3.2	0.093	0.005	7.4	

[a]The decay constant that is slope of log concentration vs time profile ($T_{1/2}$=Ln2/κ).