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Article

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# Design and Synthesis of Novel and Selective Glycine Transporter-1 (GlyT1) Inhibitors with Memory Enhancing Properties

Vincent J. Santora,* Theresa A. Almos, Richard Barido, Jillian Basinger, Chris L. Bellows, Brett C. Bookser, J. Guy Breitenbucher, Nicola J. Broadbent, Clifford Cabebe, Chih-Kun Chai, Mi Chen, Stephine Chow, De Michael Chung, Lindsay Crickard, Anne M. Danks, Graeme C. Freestone, Dany Gitnick, Varsha Gupta, Christine Hoffmaster, Andrew R. Hudson, Alan P. Kaplan, Michael R. Kennedy, Dong Lee, James Limberis, Kiev Ly, Chi Ching Mak, Brittany Masatsugu, Andrew C. Morse, Jim Na, David Neul, John Nikpur, Marco Peters, Robert E. Petroski, Joel Renick, Kristen Sebring, Samantha Sevidal, Ali Tabatabaei, Jenny Wen, Yingzhuo Yan, Zachary W. Yoder, and Douglas Zook

## Abstract

We report here the identification and optimization of a novel series of potent GlyT1 inhibitors. A ligand design campaign that utilized known GlyT1 inhibitors as starting points led to the identification of a novel series of pyrrolo[3,4-c]pyrazoles amides (**21-50**) with good *in vitro* potency. Subsequent optimization of physicochemical and *in vitro* ADME properties produced several compounds with promising pharmacokinetic profiles. *In vivo* inhibition of GlyT1 was demonstrated for select compounds within this series by measuring the elevation of glycine in the cerebrospinal fluid (CSF) of rats after a single oral dose of 10 mg/kg. Ultimately, an optimized lead, compound **46,** demonstrated *in vivo* efficacy in a rat Novel Object

Recognition (NOR) assay after oral dosing at 0.1, 1, and 3 mg/kg.

**Introduction**

The amino acid glycine is both an inhibitory and excitatory neurotransmitter in the mammalian central nervous system (CNS). Glycine acts as an inhibitory neurotransmitter at strychnine sensitive glycine receptor ion channels which are predominantly found in the cerebellum, brainstem and spinal cord (glycine A binding sites).[1,2] Conversely, in areas such as cortex, hippocampus and thalamus, glycine modulates excitatory neurotransmission as an obligatory co-agonist, along with glutamate, of the *N*-methyl-D-aspartate (NMDA) receptor (glycine B binding sites). Synaptic concentrations of glycine are regulated by two sodium-dependent glycine transporters, GlyT1 and GlyT2, which maintain low extracellular glycine concentrations by removing it from synapses via transport into glial cells and neurons. GlyT1 is highly expressed in glial and neuronal cells in forebrain regions of the CNS including the cortex and hippocampus where it acts as an indirect modulator of NMDA function. [1] In addition, GlyT1 is expressed in the retina,[2] as well as outside the CNS in the dorsal root ganglia[3] and in red blood cells.[4] By contrast, GlyT2 is found in the spinal cord, brainstem and cerebellum where it is expressed on presynaptic glycinergic terminals and thought to regulate the function of glycine receptors.

Considerable evidence exists to implicate NMDA receptor-mediated signaling in learning and memory. For example, NMDA receptor activation regulates new synapse formation and long-term memories.[5-8] Synaptic plasticity is dependent on NMDA receptor activation. Specifically, pharmacological block or genetic disruption of NMDA receptor function can inhibit

memory formation and long-term potentiation (LTP), a form of synaptic plasticity.[9-12] In addition, experimental manipulations that enhance NMDA receptor function enhance both memory formation and LTP.[13-16]

Given the obligatory role glycine plays in NMDA activation, it is not surprising that pharmacological manipulation of synaptic glycine levels via GlyT1 inhibition has been an active area of pharmaceutical research. Several GlyT1 antagonists have been described in the literature.[17-21] The first generation of GlyT1 inhibitors were predominantly sarcosine analogs (e.g., **1**[22] and **2**[23]) with initial *in vitro* profiles that appeared promising. However, further investigation showed that many of these sarcosine derivatives produced a range of deleterious side effects including ataxia, lateral recumbency, and respiratory depression.[24,25] These side effects are likely the result of sustained elevation of glycine in caudal regions of the CNS, where GlyT1 and the glycine receptor (Gly A sites) are co-localized and play an important role in control of motor function and respiration.[26,27] More recent efforts to develop GlyT1 inhibitors have focused on non-sarcosine chemotypes. A significant number of these second-generation compounds, exemplified by compounds **3**-**7**,[28-33] have appeared in the literature recently and some of these have demonstrated efficacy in preclinical animal models of cognition, pain, addiction and anxiety.[34,35] In addition, several compounds have advanced into clinical development (including compounds **5**-**7**), as potential treatments for the negative and cognitive symptoms associated with schizophrenia and as treatments for obsessive compulsive disorder (OCD), depression, addiction, and Parkinson's disease.[34, 36-41] To date no GlyT1 inhibitors have received FDA approval.

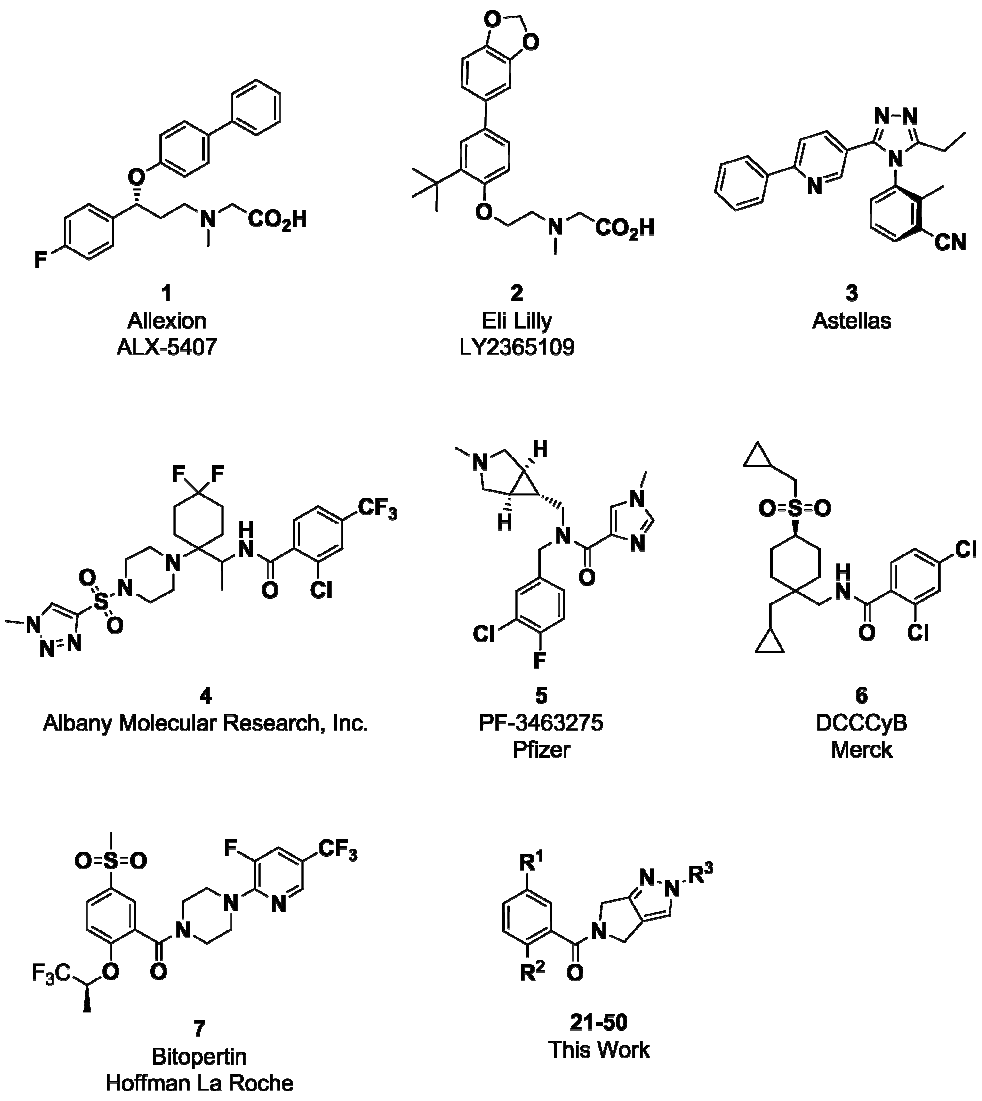
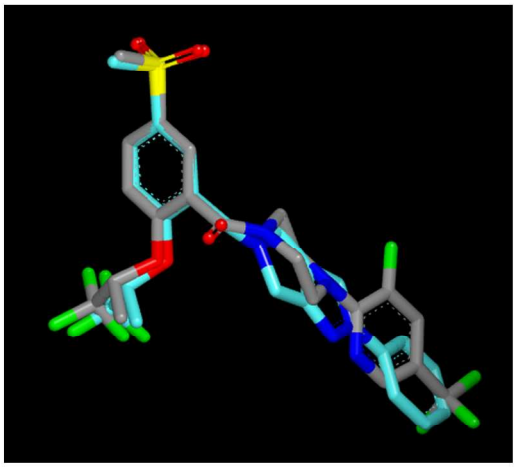
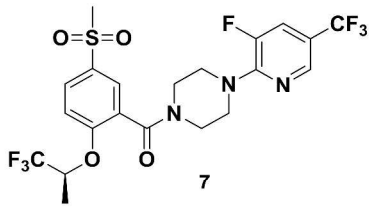



**Figure 1.** Representative GlyT1 inhibitors.

We initiated a program to identify a new series of GlyT1 inhibitors as potential treatments for memory disorders using a ligand design approach based on known chemical matter. In their SAR disclosures Hoffman La Roche identified several potent and selective GlyT1 inhibitors based on a benzoylpiperazine scaffold.[31,42] We speculated that replacing the aryl-piperazine moiety embedded within this chemotype with an aryl-pyrrolopyrazole group could provide additional opportunities for optimization. In particular, based on Rapid Overlay of

Chemical Structures (ROCS)[43] overlays of these chemical structures the aryl ring of the pyrrolopyrazole scaffold extends further, overlaying with the pyridyl $CF_3$ group in compound **7** (Figure 2). We felt that this could allow us to investigate alternate aryl-groups in combination with the pyrrolopyrazole linker.





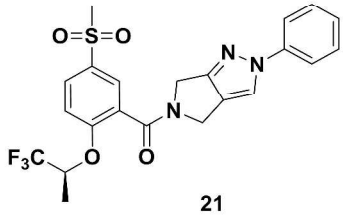
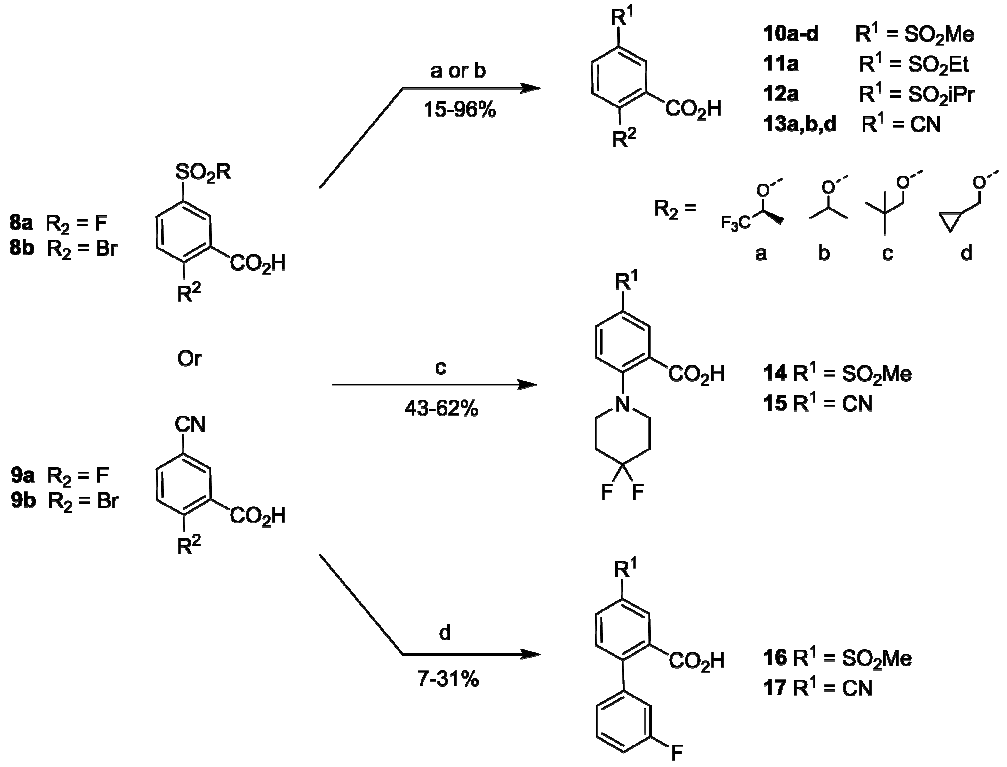




**Figure 2.** ROCS comparison of Roche compound **7** (blue) and aryl-pyrrolopyrazole **21** (grey).

Herein we describe the synthesis and optimization of this pyrrolopyrazole-containing series (**21**-**50**) that led to the identification of several analogs with promising *in vitro* profiles. In addition, we discuss the *in vivo* profiling of select examples from this new series in CNS penetration and CSF glycine elevation studies, leading to the identification of an optimized lead, compound **46**, which had a favorable pharmacokinetic profile and showed *in vivo* efficacy in a rat Novel Object Recognition (NOR) assay.

**Scheme 1.** Synthetic Routes to Intermediates **10-17**.[a]




[a]Reagents and conditions: (a) **8a** or **9a**, iPrOH, NaH, 70 °C; (b) **8a** or **9a**, $R_2OH$, $Cs_2CO_3$, Dioxane, 110 °C; (c) **8a** or **9a**, 4,4-difluoropiperidine, $Cs_2CO_3$, DMF, 120 °C; (d) **8b** or **9b**, 3-F-phenyl boronic acid, $Pd(PPh_3)_4$ or $Pd(dppf)Cl_2$, $Na_2CO_3$, Dioxane/$H_2O$, 80 °C.

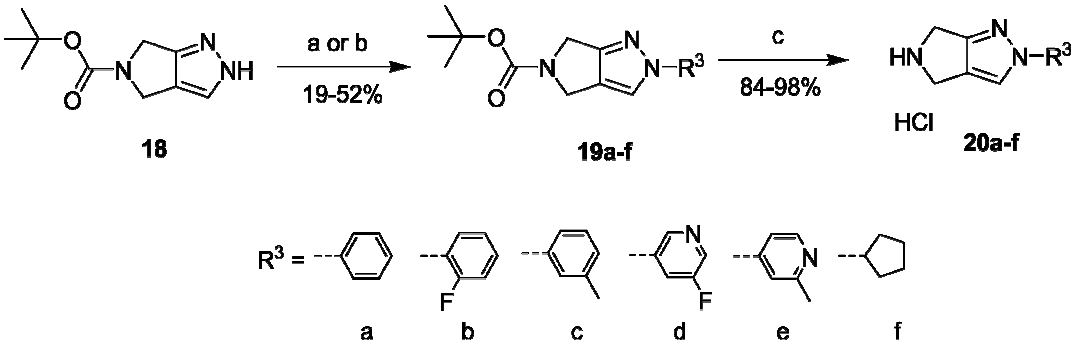
## Chemistry

The new pyrrolopyrazole amides were synthesized using the methods outlined in Schemes 1-3. Carboxylic acid precursors **10-17** were prepared from commercially available acids **8** and **9** using methods similar to those described in the literature (Scheme 1).[31,42] Specifically, 2-fluoro-5-sulfonyl or 2-fluoro-5-nitrile benzoic acids (**8a** or **9a**) were reacted with various alcohols or 4,4-difluoropiperidine under basic conditions to afford the 2-alkoxy substituted derivatives **10-13** and the difluoropiperidine analogs **14** and **15**. Alternatively,

biphenyl carboxylic acids **16** and **17** were prepared from bromides **8b** or **9b**, respectively, by Suzuki coupling with 3-fluoro-phenyl boronic acid.

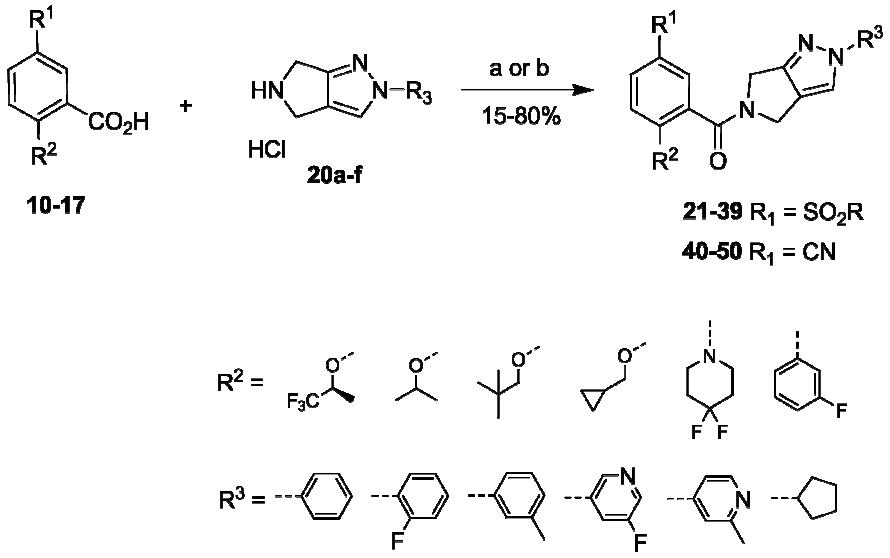
The requisite pyrrolopyrazole intermediates **20a-f** for this study were prepared in two steps. The previously reported *N*-Boc-protected 4,6-dihydropyrrolo[3,4-c]pyrazole (**18**)[44] was first coupled with appropriate aryl halides using either Buchwald-Ullmann conditions[45] or by treating with an alkyl halide under basic conditions. The desired linear regiochemistry was confirmed by NOESY-NMR, which showed the expected interaction between the ortho proton of the aryl group (or CH2 of the cyclopentyl group) and the C-H proton of the pyrazole ring. The intermediate 2-substituted pyrrolopyrazoles (**19a-f**) were then deprotected under acidic conditions to provide pyrrolopyrazoles **20a-f**. The final pyrrolopyrazole amides of this study (**21-50**) were then prepared by amide coupling, either by conversion of acids **10-17** to acid chlorides followed by addition of amines **20a-f**, or by utilizing HATU as the coupling reagent in a standard, one-step process.

**Scheme 2.** Synthetic Routes to Intermediates **20a-f**.[a]




[a]Reagents and conditions: (a) ArI or ArBr, $K_3PO_4$, CuI, (1S, 2S)-(+)-1,2-diaminocyclohexane, THF or Dioxane, 120 °C; (b) Alkyl halide, NaH, DMF; (c) HCl, Dioxane.

**Scheme 3.** Synthetic Routes to Compounds **21-50**.




[a]Reagents and conditions: (a) i. **10-17**, $SOCl_2$. ii. **20a-f**, $Et_3N$, DCM; (b) HATU, $Et_3N$, DMF.

**Structure-Activity Relationships**

Peripheral functionality ($R^1$-$R^3$) for the new series was selected on the basis of knowledge gained from SAR studies with an earlier, in-house series and literature precedent.[31,42,46] *In vitro* profiling of the pyrrolopyrazole amides consisted of extensive testing across a diverse panel of assays. These included *in vitro* GlyT1 and GlyT2 functional potency in HEK cells, as well as rat GlyT1 functional activity in rat cortical cultures. *In vitro* ADMET properties for the new compounds were also assessed for intrinsic clearance in rat and human liver microsomes (RLM and HLM, respectively), inhibition of CYP450 (1A2, 2C9, 2C19, 2D6 and 3A4 isozymes) and hERG channel inhibition as well as MDCK efflux and PAMPA-BBB permeability.[47]

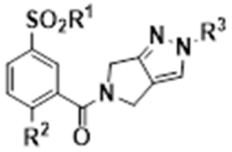
The initial compound **21**, incorporating an unsubstituted ring at the $R^3$-aryl group, exhibited high *in vitro* potency against both rat and human GlyT1 ($IC_{50}$ = 4 and 15 nM, respectively). The compound was also stable in rat and human LMs and showed no hERG channel inhibition at concentrations < 10 μM. Based on these data, the SAR was expanded to include several alkyl sulfones exemplified by compounds **22-39** (Table 1). Initial data were promising, with similar $IC_{50}$'s observed for all compounds against both rat and human forms of GlyT1 and significant *in vitro* potency (hGlyT1 $IC_{50}$ values < 38 nM) observed for all but one compound in the sulfone series (**39**, hGlyT1 $IC_{50}$ = 406 nM). In addition, the new compounds exhibited high selectivity for GlyT1 over GlyT2 (all GlyT2 $IC_{50}$'s > 10 μM) and minimal inhibition of all CYP isozymes tested (all $IC_{50}$'s > 10 μM). Finally, high *in vitro* permeability was observed for most compounds in this series (17 of 19 compounds had PAMPA-BBB permeability values > $5 \times 10^{-6}$ cm/s).

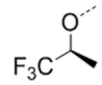
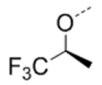
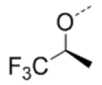
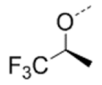
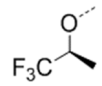
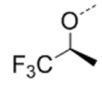
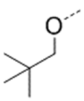
Methyl sulfone analogs with *S*-trifluoromethyl-isopropoxy substituents at $R^2$ served as starting points for scanning the effects of various pyrazole N-substituents ($R^3$) on *in vitro* profiles. Several compounds within this group exhibited unacceptably high efflux ratios in the MDCK assay, which can be indicative of being a substrate of P-glycoprotein (P-gp) or other CNS efflux transport, an undesirable characteristic for CNS drugs.[48] This was particularly true for *N*-phenyl and *N*-cyclopentyl analogs **21** and **26** and pyridyl analogs **24** and **25**. Interestingly, the high efflux observed for *N*-phenyl substituted analogs such as **21** (efflux ratio = 5.9) was significantly improved by installing an o-fluorine substituent on the phenyl ring (e.g., compound **22**, efflux ratio = 2.1). Similar *in vitro* profiles were observed for methyl sulfones with other functionality in the $R^2$ position including alternate alkoxy groups (**27-29**), as well as

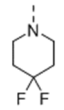
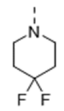
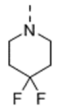
difluoropiperidines (**30-32**) and 3-fluorophenyl substituents (**33-35**). Here again, high efflux ratios were observed for compounds with N-pyridyl substituents at $R^3$. The efflux issue notwithstanding, the methyl sulfone analogs generally exhibited minimal hERG channel inhibition and modest intrinsic clearance in rat liver microsomes, regardless of the structure of $R^2$ and $R^3$ groups. The only exceptions were the neopentyloxy analog **28** and the m-fluorophenyl derivatives **33** and **35**.

Attempts to reduce the MDCK efflux observed for certain methyl sulfones, by increasing the size and lipophilicity of sulfone alkyl groups, were only marginally successful. Ethyl sulfones **36-38** (hGlyT1 $IC_{50}$ = 12-29 nM) largely retained the potency observed with methyl sulfone analogs and their efflux ratios were approximately equal to (**36** *cf*. **22**) or significantly lower than their corresponding methyl sulfone analogs (**37** *cf*. **24** and **38** *cf*. **25**). However, despite these improvements, none of these compounds demonstrated efflux ratios low enough to warrant further testing (*vide infra*). Increasing the steric bulk of the sulfone alkyl group by incorporating an isopropyl sulfone group (compound **39**) considerably lowered the efflux (efflux ratio = 2.2 *cf*. 5.9 for compound **21**), but, unfortunately, this decrease in efflux was accompanied by substantially reduced potency (hGlyT1 $IC_{50}$ = 406 nM).

**Table 1**. Properties of Sulfones Analogs **21-39**.




| Compound | $R^1$ | $R^2$ | $R^3$ | Rat GlyT1 | Human GlyT1 | Rat CLint | Human CLint | hERG | MDCK Permeability[e] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | $IC_{50}$ (nM)[a] | $IC_{50}$ (nM)[a] | (µL/min/mg)[c] | (µL/min/mg)[c] | $IC_{50}$ (µM)[d] | Papp A-B ($x10^{-6}$ cm/s) | Papp B-A ($x10^{-6}$ cm/s) | Efflux Ratio |
| **21** | $CH_3$ |  |  | 4 | 15 | 2.0 | 0.0 | >10 | 10.3 | 61.1 | 5.9 |
| **22** | $CH_3$ |  |  | 5[b] | 8[b] | 1.6 | 0.0 | >10 | 24.6 | 51.7 | 2.1 |
| **23** | $CH_3$ |  |  | 12 | 23 | 0.0 | 0.1 | >10 | 13.6 | 23.5 | 1.7 |
| **24** | $CH_3$ |  |  | 9 | 23 | 0.9 | 0.0 | >10 | 2.7 | 74.4 | 27.3 |
| **25** | $CH_3$ |  |  | 3 | 12 | 0.3 | 1.4 | >10 | 2.1 | 69.5 | 32.5 |
| **26** | $CH_3$ |  |  | 18 | 17 | 6.7 | 0.0 | >10 | 10.9 | 84.0 | 7.7 |
| **27** | $CH_3$ |  |  | 18[b] | 23[b] | 1.5 | 3.3 | >10 | 28.4 | 51.6 | 1.8 |
| **28** | $CH_3$ |  |  | 3[b] | 10[b] | 76.4 | 1.1 | 8.1 | 46.5 | 91.3 | 2.0 |
| **29** | $CH_3$ |  |  | 28[b] | 38[b] | 5.3 | 0.3 | 9.9 | 24.6 | 58.9 | 2.4 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **30** | $CH_3$ |  |  | 12 | 16 | 22.2 | 0.6 | 4.3 | 24.5 | 68.7 | 2.8 |
| **31** | $CH_3$ |  |  | 10 | 10 | 11.3 | 1.7 | 6.7 | 3.8 | 67.1 | 17.8 |
| **32** | $CH_3$ |  |  | 4 | 7 | 3.2 | 2.5 | >10 | 2.8 | 63.7 | 23.1 |
| **33** | $CH_3$ |  |  | 12 | 10 | 56.6 | 21.5 | 4.5 | 26.3 | 72.6 | 2.8 |
| **34** | $CH_3$ |  |  | 10 | 6 | 13.1 | 1.4 | >10 | 10.1 | 85.5 | 8.6 |
| **35** | $CH_3$ |  |  | 2 | 4 | 36.4 | 4.3 | >10 | 6.2 | 97.4 | 15.9 |
| **36** | Et |  |  | 9[b] | 29[b] | 0.0 | 0.9 | >10 | 18.0 | 51.9 | 2.9 |
| **37** | Et |  |  | 8 | 27 | 5.4 | 1.3 | >10 | 5.8 | 99.0 | 17.2 |
| **38** | Et |  |  | 4 | 12 | 2.3 | 5.3 | >10 | 6.1 | 101.0 | 16.8 |
| **39** | i-Pr |  |  | 79[b] | 406[b] | 1.8 | 10.0 | >10 | 56.6 | 123.0 | 2.2 |

[a] *In vitro* GlyT1 inhibition determined using a scintillation proximity assay (SPA). Potency was assessed in inhibiting the uptake of radiolabeled glycine (11-concentration dose response, 2 points per concentration, single determination) in primary rat cortical neurons (rat $IC_{50}$) or HEK cells expressing the human GlyT1c (human $IC_{50}$).

[b] IC50 determined in duplicate or higher (n≥2). Standard deviations (SD) values were typically less than 40%.

[c] Intrinsic *in vitro* clearance determined in rat and human liver microsomes (LM) in the presence of NADPH.

[d] hERG channel inhibition was measured in an electrophysiology assay (QPatch) using Chinese hamster ovary (CHO) cells

stably expressing the hERG potassium channel.

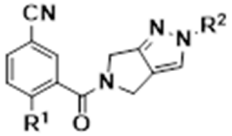
[e]Bi-directional transport determined across MDCK-MDR1 cell monolayers.

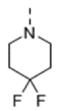
We next examined the *in vitro* profiles of several nitrile analogs (**40-50**) with continued interest in lowering the efflux ratios in this series. As observed for the sulfone analogs, compounds in the nitrile series consistently displayed low activity against GlyT2 (all GlyT2 $IC_{50}$'s > 10 μM), minimal inhibition of various CYP enzymes (all $IC_{50}$'s > 10 μM) and high permeability (PAMPA-BBB > 10 x $10^{-6}$ cm/s for all compounds). However, GlyT1 potency across the nitrile series was generally lower compared to the sulfones, particularly for *N*-2-fluorophenyl and *N*-cycloalkyl analogs (e.g., **40** and **43**), which showed approximately 10-fold lower potency compared to similarly substituted methyl sulfone analogs (**22** and **26**). In addition, a wider range of potencies was observed within subsets of the nitrile series compared to those of the sulfone series. For example, 3-fluoro-5-pyridyl analogs (**41**, **45** and **49**) had hGlyT1 $IC_{50}$ values that ranged from 69 to 383 nM and 3-methyl-4-pyridyl analogs (**42**, **46-48** and **50**) had hGlyT1 $IC_{50}$ values that ranged from 18 to 441 nM, whereas the analogous sulfones had $IC_{50}$ values that were consistently below 12 nM. The loss of potency for some nitrile analogs notwithstanding, several analogs from this class had $IC_{50}$ values in the sub-100 nM range.

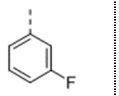
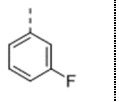
Analogs in the nitrile series generally exhibited modest intrinsic clearance in rat liver microsomes, with the exception of compounds with non-polar pyrazole substituents, such as **40**, **43** and **44**, which were substantially less stable than their corresponding sulfone analogs. These observations, coupled with the significant improvement in MDCK efflux ratios and the lack of any significant hERG channel inhibition observed within the nitrile series, resulted in a

number of analogs with promising *in vitro* profiles (e.g., **41**, **42**, **46**, **48** and **50**).

**Table 2**. *In vitro* Properties of Nitrile Analogs **40**-**50**.



| Compound | $R^1$ | $R^2$ | Rat GlyT1 | Human GlyT1 | Rat CLint | Human CLint | hERG | MDCK Permeability[e] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | $IC_{50}$ (nM)[a] | $IC_{50}$ (nM)[a] | (μL/min/mg)[c] | (μL/min/mg)[c] | $IC_{50}$ (μM)[d] | Papp A-B ($x10^{-6}$ cm/s) | Papp B-A ($x10^{-6}$ cm/s) | Efflux Ratio |
| **40** |  |  | 108 | 78 | 451.3 | 13.5 | >10 | 57.4 | 61.7 | 1.1 |
| **41** |  |  | 82[b] | 69[b] | 7.3 | 0.4 | >10 | 33.8 | 62.9 | 1.9 |
| **42** |  |  | 27 | 18 | 5.7 | 12.5 | 10 | 33.6 | 70.9 | 2.1 |
| **43** |  |  | 331 | 210 | 162 | 10.0 | >10 | 67.8 | 80.8 | 1.2 |
| **44** |  |  | 161 | 105 | 375.8 | 6.3 | >10 | 42.9 | 48.3 | 1.1 |
| **45** |  |  | 183 | 113 | 9.0 | 1.9 | >10 | 42.2 | 59.6 | 1.4 |
| **46** |  |  | 38[b] | 21[b] | 7.2 | 3.7 | 10 | 30.8 | 66.7 | 2.2 |
| **47** |  |  | 690[b] | 441[b] | 7.0 | 22.2 | >10 | 41.0 | 51.6 | 1.3 |
| **48** |  |  | 4 | 20 | 19.4 | 36.9 | 6.9 | 50.4 | 93.0 | 1.8 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **49** |  |  | 619 | 383 | 17.2 | 4.7 | 8.8 | 51.4 | 79.9 | 1.6 |
| **50** |  |  | 78[b] | 55[b] | 16.9 | 10.1 | 10 | 43.0 | 40.4 | 0.9 |

[a] *In vitro* GlyT1 inhibition determined using a scintillation proximity assay (SPA). Potency was assessed in inhibiting the uptake of radiolabeled glycine (11-concentration dose response, 2 points per concentration, single determination) in primary rat cortical neurons (rat $IC_{50}$) or HEK cells expressing the human GlyT1c (human $IC_{50}$).

[b] IC50 determined in duplicate or higher (n≥2). Standard deviations (SD) values were typically less than 40%.

[c] Intrinsic *in vitro* clearance determined in rat and human liver microsomes (LM) in the presence of NADPH.

[d] hERG channel inhibition was measured in an electrophysiology assay (QPatch) using Chinese hamster ovary (CHO) cells stably expressing the hERG potassium channel.

[e] Bi-directional transport determined across MDCK-MDR1 cell monolayers.

**DMPK Profiling and Glycine Elevation Studies**

In order to identify promising compounds for *in vivo* behavioral studies, the CNS penetration properties of four representative compounds (two from the sulfone series and two from the nitrile series) were measured in Sprague−Dawley rats 90 minutes after administration of a single 10 mg/kg oral dose of each compound (Table 3). Sulfone analogs **22** and **29** had similar exposure profiles in terms of plasma concentrations ($C_{plasma}$ = 3093 and 2138 nM, respectively), total brain concentrations ($C_{brain}$ = 687 and 605 nM, respectively) and CSF levels ($C_{CSF}$ = 44 and 26 nM, respectively). In contrast, plasma exposure for nitrile analogs **41** and **46** were higher ($C_{plasma}$ = 4684 and 3237 nM, respectively) and CNS penetration was considerably higher ($C_{brain}$ = 2862 and 1700 nM, and $C_{CSF}$ = 319 and 304 nM, respectively). These results were

somewhat unexpected considering the calculated properties and *in vitro* profiles of the test compounds (Table 3). Specifically, sulfones **22** and **29** showed higher permeability (PAMPA-BBB = 43.1 and 77.9 x $10^{-6}$ cm/s, respectively) compared to nitriles **41** and **46** (PAMPA-BBB = 25.5 and 16.3 x $10^{-6}$ cm/s, respectively) and the calculated topological polar surface area (tPSA) values for sulfones **22** and **29** (tPSA = 81.5 for both compounds) were slightly lower than the nitriles **41** and **46** (tPSA = 84.0 and 85.3, respectively).

In additional experiments, *in vivo* GlyT1 target engagement for these compounds was assessed by measuring changes in glycine concentrations in the CSF of rats in an acute, single dose-response study (Table 3). Specifically, CSF samples from rats were collected 90 minutes after oral administration of 10 mg/kg of test compound, a time point close to $T_{max}$ (data not shown). The CSF glycine concentrations were determined by derivation of glycine with Marfey's reagent[49] and subsequent LC-MS/MS analysis. Interestingly, statistically significant increases in CSF levels of glycine were only observed for one compound in each series (sulfone **22** and nitrile **46**) when compared to vehicle treated animals. These discrepancies are not easily explained given the *in vitro* potency, high structural similarity and similar *in vitro* and CNS penetration profiles shared by each pair of compounds. However, examination of the relationship between free brain concentrations ($C_{u,\ Brain}$) and Rat GlyT1 potency for each compound suggests that significant glycine elevation occurs only when the ratio of $C_{u,\ Brain}$/Rat $IC_{50}$ is greater than 2 (Table 3). In addition, it is possible that these compounds have different binding kinetics profiles (i.e., $k_{on}$ and $k_{off}$) with GlyT1, which could result in different *in vivo* target engagement profiles.[50] To date, the binding kinetics of these analogs have not been explored to confirm if such differences correlate with the levels of glycine elevation observed experimentally.

Of the two compounds that showed *in vivo* elevation of CSF glycine, nitrile **46** emerged as the preferred lead compound by virtue of its superior CNS penetration profile. Subsequent evaluation of its pharmacokinetic profile in rat after a single 1 mg/kg IV or PO dose (Figure 3 and Table 4) revealed that **46** had low systemic clearance (CL = 14.6 min/mL/kg; 23% of liver blood flow) with an elimination half-life of 2.4 h. Compound **46** was rapidly absorbed after oral administration ($T_{max}$ = 0.67 h) with good oral bioavailability (F = 81%). In addition, compound **46** was profiled in a general off-target selectivity panel consisting of 95 targets, including enzymes and receptors, and was found to be highly selective for GlyT1 with < 50% activity when screened at 10 μM against all targets.

**Table 3**. Summary of CNS Penetration, Glycine Elevation, *In vitro* ADME, and Calculated Properties for Compounds **22**, **29**, **41** and **46**[a]

| Compound | Rat $IC_{50}$ | $C_{Plasma}$ | $C_{Brain}$ | $C_{CSF}$ | CSF Glycine | Glycine Elevation | tPSA[f] | cLogP[f] | PAMPA -BBB | Plasma $F_u$ | Brain $F_u$ | $C_{u,\ brain}$ | $C_{u,\ brain}$/ Rat $IC_{50}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | nM | nM[b] | nM[b] | nM[b] | µM[c] | % control[d] | | | $10^{-6}$ cm/s | % unbound[g] | % unbound[g] | nM[h] | |
| **22** | 4 | 3093 | 687 | 44 | 9.5 | 169 ± 29[e] | 81.5 | 2.98 | 43.1 | 5.6 | 1.3 | 8.9 | 2.23 |
| **29** | 28 | 2138 | 605 | 26 | 7.6 | 110 ± 26 | 81.5 | 2.85 | 77.9 | 4.7 | 1.7 | 10.3 | 0.37 |
| **41** | 82 | 4684 | 2862 | 319 | 7.2 | 109 ± 20 | 84 | 2.8 | 25.5 | 10.4 | 3.2 | 91.6 | 1.12 |
| **46** | 36 | 3237 | 1700 | 304 | 11 | 167 ± 18[e] | 85.3 | 2.87 | 16.3 | 11.3 | 6.3 | 107.1 | 2.98 |

[a]Male SD rats (n=5) 90 min after administration of a single 10 mg/kg dose formulated in a mixture of NMP:PEG400:Water (10:50:40)

[b]Standard deviation typically < 35%

[c]Standard deviation typically < 15%

[d]CSF glycine mean % of treatment group vs. vehicle control ± propagated absolute uncertainty of the ratio (test compound glycine conc./vehicle glycine conc.).

[e]Statistical significance, $p < 0.05$ vs vehicle control.

[f]Calculated using Chemdraw 12.

[g]Unbound fraction of compound in rat plasma or brain homogenate determined by rapid equilibrium dialysis.

[h]Calculated according to the formula $C_{u,\ brain} = C_{Brain}$ x Brain $F_u$.

**Figure 3.** Rat plasma concentrations of **46** following either a single 1 mg/kg PO or 1 mg/kg IV administration in 10:50:40 NMP/PEG400/$H_20$.

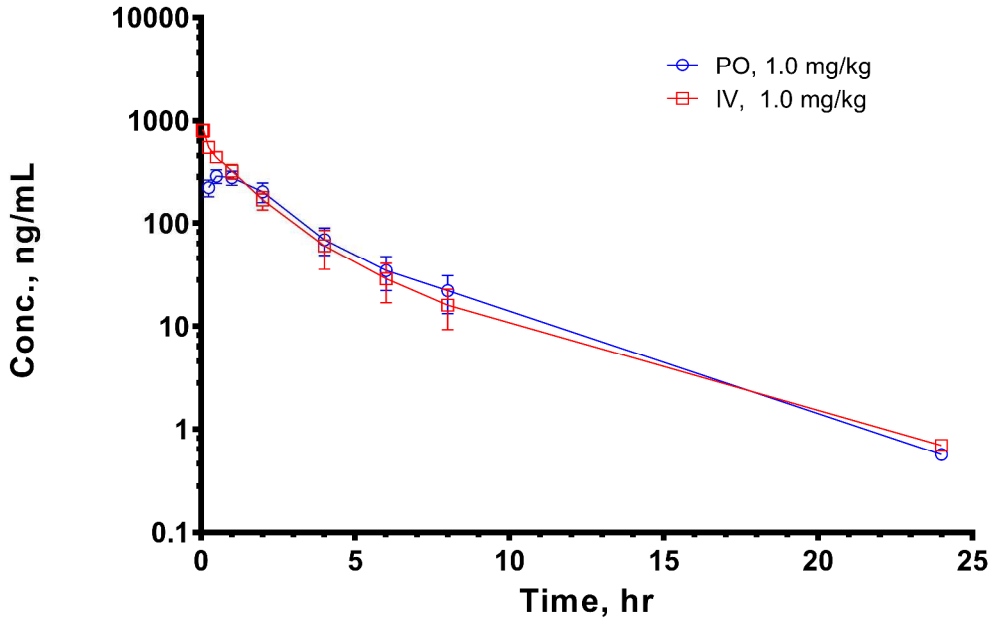



**Table 4.** Rat Pharmacokinetic Parameters of **46** After 1 mg/kg Administration.

| Pharmacokinetic Parameter | Route of Administration[a] | |
|---|---|---|
| | i.v. (1 mg/kg) | p.o. (1 mg/kg) |
| $T_{max}$[b] (hr) | ND | 0.67 |
| $C_{max}$[c] (ng/mL) | ND | 290 |
| $AUC_{inf}$[d] (ng.hr/mL) | 1176 | 958 |
| $t_{1/2}$[e] (hr) | 2.4 | 2.6 |
| $V_{ss}$[f] (L/kg) | 1.83 | ND |
| CL[g] (mL/min/kg) | 14.6 | ND |
| F[h] (%) | - | 81 |

[a]Male SD rats (n = 3) following administration of a single dose formulated in a mixture of NMP:PEG400:Water (10:50:40)

[b]Time of maximum observed concentration of compound in plasma.

[c]Maximum observed concentration of compound in plasma.

[d]Area under the plasma concentration versus time curve from 0 to the last time point compound was quantifiable in plasma.

[e]Apparent half-life of the terminal phase of elimination of compound from plasma.

[f]Volume of distribution at steady state.

[g]Clearance

[h]Bioavailability

**Memory Study**

Given its promising profile, nitrile **46** was evaluated for long term memory enhancement in a NOR test in rat (Figure 4). The NOR task is a widely-used behavioral task to assess visual recognition memory, and is based on an animal's innate preference for novelty.[51] This task consists of a training phase, where rats are presented with two identical objects to explore. Following a delay interval, memory is assessed by presenting rats with a trained object and a novel object. Rats with memory of the previously presented object will preferentially explore the novel object. Rats that receive a memory enhancing drug in conjunction with submaximal training are expected to exhibit improved memory performance similar to that of untreated

animals that have received stronger training (i.e., more exposure to objects during the training phase).

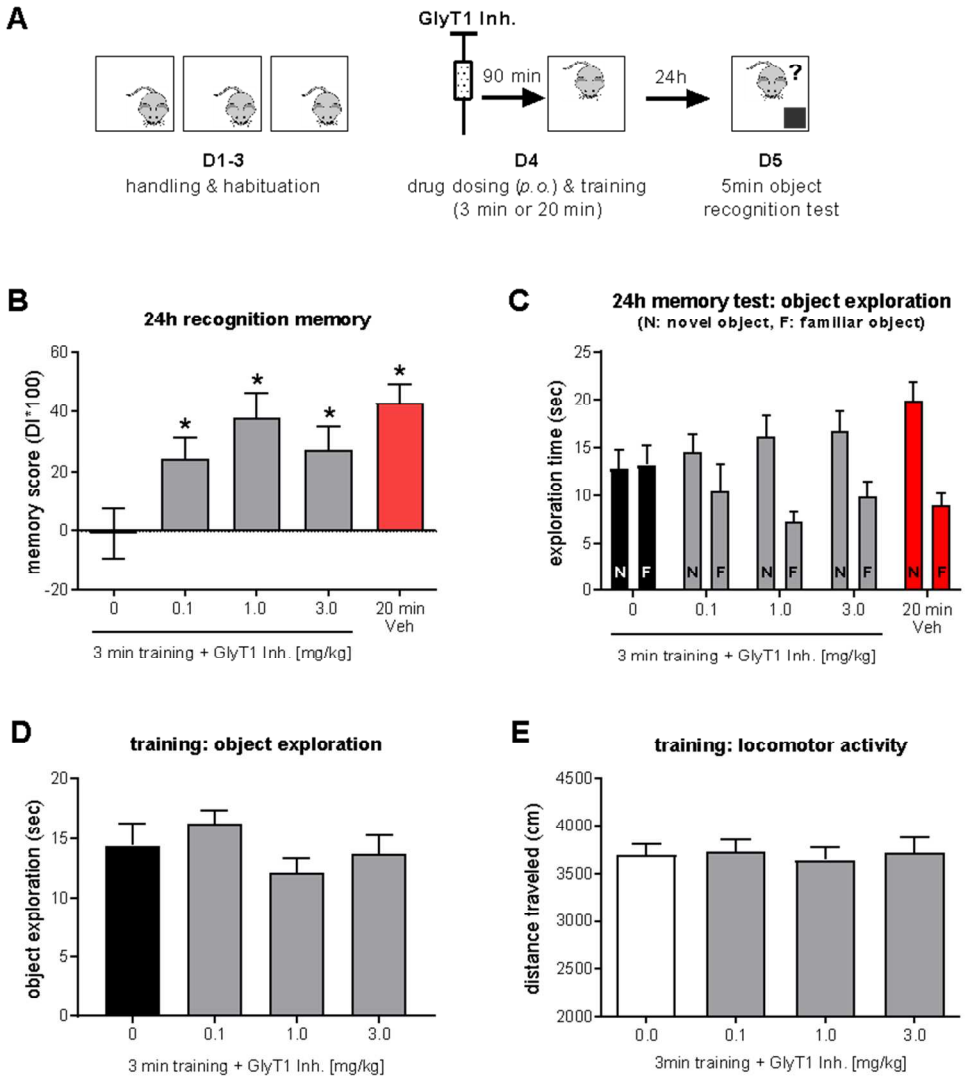



**Figure 4**. Effect of **46** in a rat model of recognition memory. A) Study design, n = 18-24 male, Long-Evans rats/treatment group. B) Effect on 24 h memory retention. Rats treated with 0.1, 1 and 3 mg/kg **46** exhibited significantly higher memory scores than rats treated with vehicle and trained for 3 min. Asterisk = $p < 0.05$ vs. 3 min training + vehicle. C) Exploration of the novel and familiar object during 24 h memory testing. D) Compound **46** did not affect object exploration during training. E) Compound **46** did not affect locomotion during training. The mean ± SEM. are shown.

Rats were dosed orally with compound **46** or vehicle 90 minutes prior to training and then given 3 mins (submaximal memory training) or 20 mins (strong memory training) to explore the arena and objects (Figure 4). Rats treated with compound **46** (0.1, 1 or 3 mg/kg) exhibited enhanced long-term object recognition memory compared to sub-maximally trained vehicle-treated rats at the 24 h retention test ($p < 0.05$, Fig. 4B). Further, the memory scores for the **46**-treated rats were no different from that of vehicle-treated rats that had received strong 20 min training ($p > 0.07$) and these groups also exhibited a significant preference for the object at test (one sample t-test, $p < 0.05$, Fig. 4 B/C). By contrast, sub-maximally trained vehicle-treated rats did not exhibit a preference for the novel object at test ($p = 0.91$). Importantly, analog **46** did not affect object exploration time or the distance traveled during training, indicating that the drug did not alter locomotor activity or motivation to explore the objects (Fig. 4 D/E).

**Conclusions**

Activation of NMDA receptor-mediated signaling plays an important role in learning and memory. Because glycine acts as an obligatory co-agonist of the NMDA receptor, pharmacological enhancement of synaptic glycine concentrations via inhibition of GlyT1 may provide the means to enhance NMDA signaling. We identified and optimized a novel series of GlyT1 inhibitors using a ligand design/scaffold modification strategy starting from known GlyT1 inhibitors. Several compounds from this new series showed promising *in vitro* profiles and selected examples demonstrated good CNS penetration. Further evaluation of these advanced compounds revealed a disparity in their ability to elevate CSF glycine concentrations in rats

following oral dosing at 10 mg/kg.

Analog **46**, which possessed a promising *in vitro* and *in vivo* profile, showed efficacy in a rat NOR test of long-term recognition memory after oral dosing at 0.1, 1 and 3 mg/kg. Unfortunately, subsequent testing of this compound at higher doses revealed a side effect profile that was similar to those of previously reported GlyT1 inhibitors[24,25] and a safety margin we considered insufficient for further development. Taken together, these results suggest that compounds such as **46** can serve as tools to examine GlyT1 inhibitors as potential treatments of cognitive disorders where NMDA receptor hypofunction may play a role. The difficulty in identifying GlyT1 inhibitors with sufficiently wide therapeutic indexes is also highlighted. Further details on compounds from this and related series will be reported in future publications from our laboratories.

**Experimental Section**

**General Methods.** Solvents and reagents were purchased and used without further purification unless otherwise noted. Flash column chromatography (FCC) was performed on Teledyne Isco CombiFlash instruments equipped with silica gel columns. Preparative HPLC purification was performed on a Waters Preparative LCMS system equipped with a Waters XBridge Prep C18 5 µm OBD 19 × 50 mm column. Elution was achieved with increasing concentration of $CH_3CN$ in water and 0.05% TFA added as a modifier. Analytical HPLC was performed on an Acquity UPLC system equipped with a BEH C18 1.7 µM 2.1 × 50 mm column. Elution was achieved with increasing concentrations of $CH_3CN$ in water and 0.1% formic acid added as modifier. NMR was

performed on a Bruker Ultrashield 400 Plus spectrometer. The purity of all compounds tested was determined by LCMS to be > 95%.

**(S)-5-(methylsulfonyl)-2-((1,1,1-trifluoropropan-2-yl)oxy)benzoic acid (10a). General Method A.** To a 100 mL pressure vessel was added 2-fluoro-5-(methylsulfonyl)benzoic acid (4.00 g; 18.33 mmol; 1.00 eq.), (S)-1,1,1-trifluoropropan-2-ol (3.27 mL; 36.66 mmol; 2.00 eq.) and cesium carbonate (11.95 g; 36.66 mmol; 2.00 eq.) in 65 mL of dioxane. The vessel was flushed with $N_2$, sealed and the mixture was heated and stirred at 115 °C for 23 h then cooled to room temperature. The mixture was diluted with 100 mL $H_2O$, acidified with conc. HCl to pH 1 and extracted with EtOAc (2 x 75 mL). The combined organics were dried with $Na_2SO_4$, and concentrated under vacuum to give the title compound as a white solid (5.50 g, 96%) $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 13.26 (br. s., 1H), 8.13 (d, *J* = 2.3 Hz, 1H), 8.03 (dd, *J* = 2.5, 8.8 Hz, 1H), 7.57 (d, *J* = 9.0 Hz, 1H), 5.49 (td, *J* = 6.4, 12.6 Hz, 1H), 3.55 (s, 3H), 3.22 (s, 3H), 1.44 (d, *J* = 6.3 Hz, 3H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 313.1.

**2-Isopropoxy-5-(methylsulfonyl)benzoic acid (10b). General Method B.** To a 500 mL round-bottomed flask was added sodium hydride (60% dispersion in mineral oil, 2.75 g, 68.7 mmol) and isopropanol (100 mL). The mixture was heated to 110 °C and stirred for 10 minutes. Then, the mixture was cooled to 70 °C and 2-fluoro-5-(methyl-sulfonyl)benzoic acid (3.0 g, 13.75 mmol) was added in one portion. Heating and stirring were continued for

7 h. Solvents were removed under reduced pressure and the resulting solids were taken up in water (150 mL). The resulting aqueous mixture was acidified to pH 1 using conc. HCl and extracted with EtOAc (2 X 100 mL). The combined organic layers were dried over sodium sulfate, filtered and evaporated to afford the title compound (2.9 g, 82%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 13.00 (br s, 1H), 8.06 (d, *J* = 2.7 Hz, 1H), 7.95 (dd, *J* = 2.3, 9.0 Hz, 1H), 7.36 (d, *J* = 9.0 Hz, 1H), 4.81 (td, *J* = 6.1, 12.1 Hz, 1H), 3.18 (s, 3H), 1.29 (d, *J* = 6.3 Hz, 6H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 259.0.

**5-(Methylsulfonyl)-2-(neopentyloxy)benzoic acid (10c).** The title compound was prepared using General Method A (0.53 g, 20%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 13.17 - 12.98 (m, 1H), 8.11 (d, *J* = 2.7 Hz, 1H), 7.98 (dd, *J* = 2.3, 8.6 Hz, 1H), 7.31 (d, *J* = 9.0 Hz, 1H), 3.79 (s, 2H), 1.00 (s, 9H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 287.3.

**2-(Cyclopropylmethoxy)-5-(methylsulfonyl)benzoic acid (10d).** The title compound was prepared using General Method A (1.7 g, 68%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ13.07 (br. s., 1H), 8.10 (d, *J* = 2.6 Hz, 1H), 7.99 (dd, *J* = 2.6, 8.8 Hz, 1H), 7.34 (d, *J* =8.9 Hz, 1H), 4.05 (d, *J* = 6.7 Hz, 2H), 3.20 (s, 3H), 1.32 - 1.19 (m, 1H), 0.61 - 0.55 (m, 2H), 0.42 - 0.35 (m, 2H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 271.0.

**(S)-5-(Ethylsulfonyl)-2-((1,1,1-trifluoropropan-2-yl)oxy)benzoic acid (11a).** The title compound was prepared using General Method A (1.4 g 83%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 13.29 (br. s., 1H), 8.11 (d, *J* = 2.4 Hz, 1H), 8.01 (dd, *J* = 2.5, 8.9 Hz, 1H), 7.60 (d, *J* =8.9 Hz, 1H),

5.52 (quin, *J* = 6.4 Hz, 1H), 3.36 - 3.32 (m, 2H), 1.47 (d, *J* = 6.2 Hz, 3H), 1.11 (t, *J* = 7.3 Hz, 3H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 327.0.

**(S)-5-(Isopropylsulfonyl)-2-((1,1,1-trifluoropropan-2-yl)oxy)benzoic acid (12a).** The title compound was prepared using General Method A (0.4 g, 61%). $^1$H NMR (400 MHz, DMSO-d6) δ = 13.30 (br. s., 1H), 8.07 (d, *J* = 2.4 Hz, 1H), 7.98 (dd, *J* = 2.4, 8.8 Hz, 1H), 7.60 (d, *J* = 9.0 Hz, 1H), 5.52 (spt, *J* = 6.4 Hz, 1H), 3.46 (spt, *J* = 6.8 Hz, 1H), 1.48 (d, *J* = 6.4 Hz, 3H), 1.17 (d, *J* = 6.7 Hz, 6H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 341.0.

**(S)-5-Cyano-2-((1,1,1-trifluoropropan-2-yl)oxy)benzoic acid (13a).** The title compound was prepared using General Method A (8.0 g, 84%). $^1$H NMR (400 MHz, CDCl3) δ 8.35 (d, *J* = 2.2 Hz, 1H), 7.84 (dd, *J* = 8.7, 2.2 Hz, 1H), 7.14 (d, *J* = 8.8 Hz, 1H), 4.93 – 4.83 (m, 1H), 1.63 (d, *J* = 6.5 Hz, 3H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 260.2.

**5-Cyano-2-isopropoxybenzoic acid (13b).** The title compound was prepared using General Method B (14.2 g, 76%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 13.00 (br. s., 1H), 7.96 (d, *J* = 2.3 Hz, 1H), 7.89 (dd, *J* = 2.3, 8.6 Hz, 1H), 7.30 (d, *J* = 9.0 Hz, 1H), 4.80 (td, *J* = 5.9, 12.0 Hz, 1H), 1.40 - 1.14 (m, 6H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 206.2.

**5-Cyano-2-(cyclopropylmethoxy)benzoic acid (13d).** The title compound was prepared using General Method A, using DMF as solvent and heating at 120 °C for 30 min in a sealed vial (20 mg, 15%). $^1$H NMR (400 MHz, $CD_3OD$) δ 8.09 (d, *J* = 2.2 Hz, 1H), 7.84 (dd, *J* = 2.2, 8.8 Hz, 1H),

7.27 (d, *J* =8.8 Hz, 1H), 4.07 (d, *J* = 6.7 Hz, 2H), 1.34 (tt, *J* = 1.5, 6.6 Hz, 1H), 0.77 - 0.57 (m, 2H), 0.50 - 0.34 (m, 2H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 218.1.

**2-(4,4-Difluoropiperidin-1-yl)-5-(methylsulfonyl)benzoic acid (14)**. The title compound was prepared using General Method A using 4,4-difluoropiperidine (1.7 g, 62%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 13.40 (br. s., 1H), 8.11 (d, *J* = 2.3 Hz, 1H), 7.90 (dd, *J* = 2.4, 8.8 Hz, 1H), 7.32 (d, *J = 8*.7 Hz, 1H), 3.32 - 3.28 (m, 4H), 3.19 (s, 3H), 2.19 - 2.06 (m, 4H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 320.1

**5-Cyano-2-(4,4-difluoropiperidin-1-yl)benzoic acid (15).** The title compound was prepared using General Method A using 4,4-difluoropiperidine (0.1 g, 43%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.98 (d, *J* = 2.1 Hz, 1H), 7.81 (dd, *J* = 2.2, 8.7 Hz, 1H), 7.24 (d, *J = 8*.8 Hz, 1H), 3.29 (d, *J* = 5.3 Hz, 4H), 2.19 -2.03 (m, 4H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 267.1.

**3'-Fluoro-4-(methylsulfonyl)-[1,1'-biphenyl]-2-carboxylic acid (16). General Method C.** A 100 mL round-bottomed flask, equipped with an argon inlet adaptor, was charged with 2.8 g (10 mmol) of **8b**, 3-fluorophenyl boronic acid (1.5 g, 10.4 mmol), $Pd(PPh_3)_4$ (0.54 g, 0.5 mmol), and $Na_2CO_3$ (2.1 g, 19.8 mmol) followed by 40 mL dioxane and 20 mL water. The reaction mixture was flushed with argon and stirred at 80 $^o$C for 2 days. The mixture was filtered and the filtrate was washed with EtOAc. To the water phase, was added 1N HCl until the solution reached pH 3. Then the mixture was stirred at room temperature for 2 days. The desired compound was filtered as a white solid (0.9 g, 31%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 13.41 (br. s., 1H), 8.31 -

8.23 (m, 1H), 8.13 (dd, *J* = 2.0, 8.1 Hz, 1H), 7.71 (d, *J* = 8.1 Hz, 1H), 7.59 - 7.44 (m, 1H), 7.35 - 7.12 (m, 3H), 3.33 (s, 3H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 295.0.

**4-Cyano-3'-fluoro-[1,1'-biphenyl]-2-carboxylic acid (17).** The title compound was prepared using General Method C using $Pd(dppf)Cl_2$ as catalyst (120 mg, 7%). $^1H$ NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.18 (s, 1H), 8.04 (d, *J* = 8.2 Hz, 1H), 7.62 (d, *J* = 7.8 Hz, 1H), 7.51 - 7.44 (m, 2H), 7.27 - 7.15 (m, 3H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 242.0.

**Tert-butyl 2-phenyl-4,6-dihydropyrrolo[3,4-c]pyrazole-5(2H)-carboxylate (19a). General Method D**. To a 50 mL pressure vessel was added *tert*-butyl 4,6-dihydropyrrolo[3,4-c]pyrazole-5(2*H*)-carboxylate (2.0 g, 9.56 mmol) and iodobenzene (2.4 g, 11.95 mmol) in dioxane (20 mL). Nitrogen gas was bubbled through the mixture for 5 minutes. $K_3PO_4$ (4.0 g, 19.1 mmol), CuI (364 mg, 1.9 mmol) and (1*S*, 2*S*)-(+)-1,2-diaminocyclohexane (218 mg, 1.9 mmol) was then added. The reaction vessel was sealed and the mixture stirred at 120 °C for 5 days. The reaction mixture was removed from heating and filtered while still hot. The filtrate was allowed to cool, diluted with EtOAc (250 mL) and washed with concentrated ammonium hydroxide (2 x 100 mL) and brine. The organic layer was dried ($MgSO_4$), filtered and evaporated and the resulting residue was purified by FCC (15% EtOAc/hexanes) to afford **19a** (2.7 g, 35%). $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 7.71 - 7.59 (m, 3H), 7.44 (dd, *J* = 7.6, 8.4 Hz, 2H), 7.34 - 7.27 (m, 1H), 4.64 - 4.42 (m, 4H), 1.52 (s, 9H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 286.1.

**Tert-butyl-2-(2-fluorophenyl)-4,6-dihydropyrrolo[3,4-c]pyrazole-5(2H)-carboxylate (19b).** The title compound was prepared using General Method D (150 mg, 52%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 1.46 (s, 9 H) 4.43 (t, *J* = 9.85 Hz, 4 H) 7.28 - 7.52 (m, 3 H) 7.69 - 7.82 (m, 1 H) 8.00 (dd, *J* = 10.42, 2.26 Hz, 1H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 304.3.

**Tert-butyl-2-(m-tolyl)-4,6-dihydropyrrolo[3,4-c]pyrazole-5(2H)-carboxylate (19c).** The title compound was prepared using General Method D (530 mg, 19%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.24 (d, *J* = 11.0 Hz, 1H), 7.64 - 7.52 (m, 2H), 7.33 (t, *J* = 7.8 Hz, 1H), 7.08 (d, *J* = 7.4 Hz, 1H), 4.43 - 4.36 (m, 4H), 2.36 - 2.33 (m, 3H), 1.44 (s, 9H) ppm. LCMS (ESI) $[M\text{-}COOtBu]^+$ 200.4 (molecular ion was not observed).

**Tert-butyl 2-(5-fluoropyridin-3-yl)-4,6-dihydropyrrolo[3,4-c]pyrazole-5(2H)-carboxylate (19d).** The title compound was prepared using General Method D (320 mg, 31%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.98 (br. s., 1H), 8.51 (d, *J* = 2.7 Hz, 1H), 8.44 (d, *J* = 7.4 Hz, 1H), 8.23 - 8.13 (m, 1H), 4.49 - 4.35 (m, 4H), 1.51- 1.37 (bs, 9H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 305.3.

**Tert-butyl 2-(2-methylpyridin-4-yl)-4,6-dihydropyrrolo[3,4-c] pyrazole-5(2H)-carboxylate (19e).** The title compound was prepared using General Method D (150 mg, 21%). $^1$H NMR (400 MHz, $CDCl_3$) δ 8.51 (d, *J* = 5.9 Hz, 1H), 7.75 (d, *J* = 15.3 Hz, 1H), 7.49 (dd, *J* = 1.8, 5.3 Hz, 1H), 7.36 (dd, *J* = 2.2, 5.7 Hz, 1H), 4.63 - 4.38 (m, 4H), 2.62 (s, 3H), 1.52 (d, *J* =0.8 Hz, 9H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 301.3.

**Tert-butyl 2-cyclopentyl-4,6-dihydropyrrolo[3,4-c]pyrazole-5(2H)-carboxylate (19f).** *To* a 25 mL round-bottom flask was added 60% sodium hydride in mineral oil (0.11 g, 2.87 mmol), anhydrous DMF (5.0 mL), and *tert*-butyl 4,6-dihydropyrrolo[3,4-c]pyrazole-5(2H)-carboxylate (0.5 g, 2.39 mmol). The mixture stirred at room temperature for 1 h. A solution of bromo-cyclopentane (0.53 g, 3.58 mmol) and DMF (7.5 mL) was then added drop-wise to the mixture and the mixture was stirred overnight at room temperature. The crude reaction mixture was quenched with water and then diluted with EtOAc (20 mL), washed with $H_2O$ (3 x 50 mL), dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The crude was purified by FCC (20-30% EtOAc:hexanes) to afford the title compound (0.33 g, 50%). $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 7.16 (d, *J* = 17.6 Hz, 1H), 4.62 (qd, *J* = 7.0, 13.9 Hz, 1H), 4.52 - 4.34 (m, 4H), 2.23 - 2.10 (m, 2H), 2.07 - 1.93 (m, 2H), 1.93 - 1.79 (m, 2H), 1.77 - 1.59 (m, 2H), 1.52 - 1.43 (m, 9H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 278.3.

**2-Phenyl-2,4,5,6-tetrahydropyrrolo[3,4-c]pyrazole (20a). General Method E.** A solution of *tert*-butyl 2-(4-fluorophenyl)-4,6-dihydropyrrolo[3,4-c]pyrazole-5(2H)-carboxylate (7.4 g, 24.4 mmol) in dioxane (60 mL) was treated with 4N HCl/ dioxane solution (61 mL, 244 mmol) and stirred at ambient temperature for 16 hours. Solvents were removed under vacuum and the resulting solids treated with a minimum amount of methanol followed by excess diethyl ether. The title compound was filtered off and dried under reduced pressure to give the title compound white solid. The compound was isolated as the free base by dissolving the crude salt in $CHCl_3$ and washing with 1N NaOH followed by drying under

vacuum (250 mg, 96%). $^1$H NMR (400 MHz, $CDCl_3$) δ 7.60 - 7.71 (m, 3H) 7.41 - 7.51 (m, 2H) 7.24 - 7.33 (m, 2H) 4.00 - 4.25 (m, 4H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 186.1.

**2-(2-Fluorophenyl)-2,4,5,6-tetrahydropyrrolo[3,4-c]pyrazole hydrochloride (20b).** The title compound was prepared using General Method E (1 g, 94%). $^1$H NMR (400 MHz, $CD_3OD$) δ 8.01 (d, *J* = 2.2 Hz, 1H), 7.80 (td, *J* = 7.8, 1.5 Hz, 1H), 7.50 – 7.32 (m, 3H), 4.56 – 4.52 (m, *J* = 3.5 Hz, 4H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 204.3.

**2-(m-Tolyl)-2,4,5,6-tetrahydropyrrolo[3,4-c]pyrazole hydrochloride (20c).** The title compound was prepared using General Method E (6.1 g, 84%). $^1$H NMR (400 MHz, DMSO) δ 10.14 (s, 2H), 8.36 (s, 1H), 7.64 (s, 1H), 7.58 (dd, *J* = 8.0, 1.8 Hz, 1H), 7.38 (t, *J* = 7.8 Hz, 1H), 7.15 (d, *J* = 7.5 Hz, 1H), 4.37 (s, 4H), 2.38 (s, 3H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 200.4.

**2-(5-Fluoropyridin-3-yl)-2,4,5,6-tetrahydropyrrolo[3,4-c]pyrazole (20d).** The title compound was prepared using General Method E. The compound was isolated as the free base by dissolving the crude salt in $CHCl_3$, washing with 1N NaOH followed by drying under vacuum (400 mg, 97%). $^1$H NMR (400 MHz $CD_3OD$) δ 8.91 (s, 1H), 8.44 (s, 1H), 8.295 (s, 1H), 8.08-8.11 (m, 2H), 4.49 (s, 2H), 4.51 (s, 2H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 205.1.

**2-(2-Methylpyridin-4-yl)-2,4,5,6-tetrahydropyrrolo[3,4-c]pyrazole dihydrochloride (20e).** The title compound was prepared using General Method E (12.8 g, 98%). $^1$H NMR (400 MHz,

DEUTERIUM OXIDE) δ 8.58 - 8.52 (m, 1H), 8.35 - 8.29 (m, 1H), 8.09 - 8.06 (m, 1H), 8.05 - 8.01 (m, 1H), 4.62 - 4.50 (m, 4H), 2.81 - 2.66 (m, 3H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 201.4.

**2-Cyclopentyl-2,4,5,6-tetrahydropyrrolo[3,4-c]pyrazole hydrochloride (20f).** The title compound was prepared using General Method E (200 mg, 95%). $^1$H NMR (400 MHz, $CD_3OD$) δ 7.60 (s, 1H), 4.73 (quin, *J* = 7.1 Hz, 1H), 4.43 (s, 2H), 4.40 (s, 2H), 2.25 - 2.12 (m, 2H), 2.05 - 1.95 (m, 2H), 1.94 - 1.83 (m, 2H), 1.80 - 1.68 (m, 2H) ppm. LCMS (ESI) $[M + H]^+$ *m/z* 178.2.

**5-(5-Methanesulfonyl-2-{[(2S)-1,1,1-trifluoropropan-2-yl]oxy}benzoyl)-2-phenyl-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazole (21). General Method F.** To a 10 mL glass vial was added **10a** (40 mg, 0.13 mmol), HATU (58.4 mg, 0.15 mmol), triethylamine (90 μL, 0.06 g, 0.64 mmol) and 34.1 mg (0.15 mmol) of **20a** in 1 mL DMF. The mixture was stirred at room temperature for 5 h then diluted with 1 mL MeOH and purified by preparative HPLC using 5-95% $CH_3CN$-$H_2O$. Pure fractions were combined, diluted with DCM and washed with aqueous $NaHCO_3$. The organic phase was separated and evaporated to give the title compound as an off white solid (27 mg, 44%). $^1$H NMR (400 MHz, CDCl3) δ 8.04 - 7.97 (m, 2H), 7.76 (s, 1H), 7.67 - 7.60 (m, 2H), 7.50 - 7.42 (m, 2H), 7.33 - 7.27 (m, 1H), 7.19 (dd, *J* = 1.6, 8.6 Hz, 1H), 4.90 - 4.78 (m, 3H), 4.53 - 4.36 (m, 2H), 3.07 (d, *J* = 1.2 Hz, 3H), 1.54 (d, *J* = 6.3 Hz, 3H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 480.1200. Found: 480.1194 .

**2-(2-Fluorophenyl)-5-(5-methanesulfonyl-2-{[(2S)-1,1,1-trifluoropropan-2-yl]oxy}benzoyl)-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazole (22). General Method G.** A mixture of acid **10a** (125 mg, 0.40 mmol) in thionyl chloride (500 μL) was heated in a sealed 10 mL glass vial at 70 °C for 45

min. After cooling to room temperature the mixture was evaporated to dryness. The resulting residue was dissolved in EtOAc and evaporated to dryness then dissolved in 2 mL $CH_2Cl_2$. In a second glass vial a solution of pyrazole **20b·HCl** (96 mg, 0.4 mmol) in 2 mL $CH_2Cl_2$ was treated with TEA (226 μL, 1.61 mmol). After stirring at room temperature for 10 minutes the two $CH_2Cl_2$ mixtures were combined and the resulting mixture was stirred at room temperature for 3 h. The reaction mixture was washed with water and the organic phase evaporated to dryness. Purification (HPLC, 30 - 95% gradient ACN-$H_2O$) afforded the title compound as an off white solid (144 mg, 72%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.10 - 7.88 (m, 3H), 7.81 - 7.68 (m, 1H), 7.60 (dd, *J* = 2.0, 9.0 Hz, 1H), 7.50 - 7.28 (m, 3H), 5.60 - 5.50 (m, 1H), 4.67 (d, *J* = 10.2 Hz, 2H), 4.45 - 4.27 (m, 2H), 3.24 (s, 3H), 1.45 - 1.40 (m, 3H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 498.1105 . Found: 498.1096.

**5-(5-Methanesulfonyl-2-{[(2S)-1,1,1-trifluoropropan-2-yl]oxy}benzoyl)-2-(3-methylphenyl)-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazole (23).** The title compound was prepared using General Method G (66 mg, 80%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.36 - 8.20 (m, 2H), 8.00 (dd, *J* = 2.3, 8.6 Hz, 1H), 7.90 (dd, *J* = 2.3, 4.3 Hz, 1H), 7.65 - 7.49 (m, 4H), 7.34 (dt, *J* = 2.0, 7.8 Hz, 1H), 7.09 (d, *J* = 7.8 Hz, 1H), 5.54 (dq, *J* = 3.9, 6.4 Hz, 1H), 4.66 (d, *J* = 5.5 Hz, 2H), 4.39 - 4.30 (m, 2H), 3.24 (s, 3H), 2.35 (d, *J* = 2.7 Hz, 3H), 1.42 (dd, *J* = 2.0, 6.3 Hz, 3H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 494.1356. Found: 494.1352.

**3-Fluoro-5-[5-(5-methanesulfonyl-2-{[(2S)-1,1,1-trifluoropropan-2-yl]oxy}-benzoyl)-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazol-2-yl]pyridine (24).** The title compound was prepared using General Method F (26 mg, 41%). $^1$H NMR (400 MHz, $CDCl_3$) δ 8.77 (d, *J* = 1.6 Hz, 1H), 8.44 - 8.40

(m, 1H), 8.06 - 7.96 (m, 2H), 7.90 - 7.78 (m, 2H), 7.69 (s, 1H), 7.19 (d, *J* = 9.0 Hz, 1H), 4.93 - 4.78 (m, 3H), 4.54 - 4.38 (m, 2H), 3.08 (s, 3H), 1.55 (d, *J* = 6.7 Hz, 3H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 499.1058. Found: 499.1050.

**4-[5-(5-Methanesulfonyl-2-{[(2S)-1,1,1-trifluoropropan-2-yl]oxy}benzoyl)-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazol-2-yl]-2-methylpyridine (25).** The title compound was prepared using General Method F (30 mg, 47%). $^1$H NMR (400 MHz, $CDCl_3$) δ 8.53 (dd, *J* = 2.7, 5.9 Hz, 1H), 8.05 - 7.96 (m, 2H), 7.93 - 7.76 (m, 1H), 7.57 (dd, *J* = 2.0, 18.0 Hz, 1H), 7.48 (ddd, *J* = 2.3, 6.0, 8.1 Hz, 1H), 7.19 (d, *J* = 8.6 Hz, 1H), 4.91 - 4.77 (m, 3H), 4.54 - 4.36 (m, 2H), 3.07 (s, 3H), 2.68 (d, *J* = 3.1 Hz, 3H), 1.54 (d, *J* = 6.3 Hz, 3H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 495.1309. Found: 495.1300.

**2-Cyclopentyl-5-(5-methanesulfonyl-2-{[(2S)-1,1,1-trifluoropropan-2-yl]oxy}benzoyl)-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazole (26).** The title compound was prepared using General Method F (9 mg, 30%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.03 - 7.92 (m, 1H), 7.86 (dd, *J* = 2.5, 4.9 Hz, 1H), 7.65 - 7.49 (m, 2H), 5.52 (td, *J* = 6.6, 12.7 Hz, 1H), 4.65 (quin, *J* = 7.1 Hz, 2H), 4.54 (s, 2H), 4.35 - 4.08 (m, 3H), 3.22 (s, 3H), 2.14 - 1.94 (m, 3H), 1.86 (br. s., 2H), 1.81 - 1.67 (m, 2H), 1.66 - 1.50 (m, 2H), 1.41 (dd, *J* = 2.2, 6.5 Hz, 3H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 472.1513. Found: 472.1508.

**2-(2-Fluorophenyl)-5-[5-methanesulfonyl-2-(propan-2-yloxy)benzoyl]-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazole (27).** The title compound was prepared using General Method G (164 mg, 77%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.09 - 7.89 (m, 2H), 7.82 - 7.68 (m, 2H), 7.50 - 7.29 (m, 4H), 4.88 - 4.79 (m, 1H), 4.68 (d, *J* = 10.2 Hz, 2H), 4.43 - 4.33 (m, 2H), 3.20 (s, 3H), 1.27 (d, *J* =

5.9 Hz, 6H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 444.1388. Found: 444.1383.

**5-[2-(2,2-Dimethylpropoxy)-5-methanesulfonylbenzoyl]-2-(2-fluorophenyl)-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazole (28).** The title compound was prepared using General Method G (57 mg, 74%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.09 - 7.91 (m, 2H), 7.85 - 7.66 (m, 2H), 7.49 - 7.37 (m, 2H), 7.37 - 7.28 (m, 2H), 4.68 (d, *J* = 9.8 Hz, 2H), 4.43 - 4.33 (m, 2H), 3.81 (d, *J* = 4.7 Hz, 2H), 3.20 (s, 3H), 0.88 (d, *J* = 3.1 Hz, 9H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 472.1701. Found: 472.1695.

**5-[2-(Cyclopropylmethoxy)-5-methanesulfonylbenzoyl]-2-(2-fluorophenyl)-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazole (29).** The title compound was prepared using General Method G (21 mg, 31%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.08 - 7.91 (m, 2H), 7.83 - 7.68 (m, 2H), 7.49 - 7.29 (m, 4H), 4.69 (d, *J* = 9.8 Hz, 2H), 4.49 - 4.36 (m, 2H), 4.06 (dd, *J* = 3.7, 6.8 Hz, 2H), 3.20 (s, 3H), 1.25 - 1.14 (m, 1H), 0.53 - 0.45 (m, 2H), 0.31 - 0.25 (m, 2H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 456.1388. Found: 456.1382.

**4,4-Difluoro-1-{2-[2-(2-fluorophenyl)-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazole-5-carbonyl]-4-methanesulfonylphenyl}piperidine (30).** The title compound was prepared using General Method G (59 mg, 79%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.10 - 7.91 (m, 1H), 7.87 (ddd, *J* = 1.2, 2.3, 8.6 Hz, 1H), 7.80 - 7.66 (m, 2H), 7.50 - 7.29 (m, 4H), 4.72 (d, *J* = 11.3 Hz, 2H), 4.61 - 4.31 (m, 2H), 3.39 - 3.32 (m, 2H), 3.27 (br. s., 2H), 3.19 (d, *J* = 1.2 Hz, 3H), 2.07 - 1.94 (m, 4H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 505.1516. Found: 505.1510.

**3-{5-[2-(4,4-Difluoropiperidin-1-yl)-5-methanesulfonylbenzoyl]-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazol-2-yl}-5-fluoropyridine (31).** The title compound was prepared using General Method

G (42 mg, 56%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.98 (dd, *J* = 1.4, 11.9 Hz, 1H), 8.56 - 8.49 (m, 1H), 8.36 (s, 1H), 8.25 - 8.12 (m, 1H), 7.88 (dd, *J* = 2.5, 8.8 Hz, 1H), 7.75 (dd, *J* = 1.2, 2.3 Hz, 1H), 7.32 (dd, *J* = 1.8, 8.8 Hz, 1H), 4.73 (d, *J* = 11.3 Hz, 2H), 4.46 (br. s., 2H), 3.38 - 3.30 (m, 4H), 3.22 - 3.15 (m, 3H), 2.08 - 1.92 (m, 4H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 506.1468. Found: 506.1464.

**4-{5-[2-(4,4-Difluoropiperidin-1-yl)-5-methanesulfonylbenzoyl]-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazol-2-yl}-2-methylpyridine (32).** The title compound was prepared using General Method G (15 mg, 20%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.55 (s, 0.5H), 8.46 (dd, *J* = 1.6, 5.9 Hz, 1H), 8.37 (s, 0.5H), 7.92 - 7.85 (m, 1H), 7.75 (dd, *J* = 1.6, 2.3 Hz, 1H), 7.69 (dd, *J* = 2.2, 15.8 Hz, 1H), 7.60 (ddd, *J* = 2.0, 5.5, 12.9 Hz, 1H), 7.32 (dd, *J* = 1.0, 8.8 Hz, 1H), 4.72 (d, *J* = 9.8 Hz, 2H), 4.61 - 4.34 (m, 2H), 3.44 - 3.32 (m, 3H), 3.19 (d, *J* = 0.8 Hz, 3H), 2.49 (d, *J* = 1.6 Hz, 3H), 1.99 (t, *J* = 13.3 Hz, 4H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 502.1719. Found: 502.1711.

**2-(2-Fluorophenyl)-5-[2-(3-fluorophenyl)-5-methanesulfonylbenzoyl]-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazole (33).** The title compound was prepared using General Method G (23 mg, 59%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.11 - 7.99 (m, 2H), 7.87 - 7.81 (m, 1H), 7.69 (dtd, *J* = 1.8, 7.9, 19.5 Hz, 1H), 7.55 - 7.20 (m, 8H), 4.53 (d, *J* = 11.7 Hz, 2H), 4.27 - 4.09 (m, 2H), 3.33 (d, *J* = 1.2 Hz, 3H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 480.1188. Found: 480.1184.

**3-Fluoro-5-{5-[2-(3-fluorophenyl)-5-methanesulfonylbenzoyl]-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazol-2-yl}pyridine (34).** The title compound was prepared using General Method G (96 mg, 59%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.93 (d, *J* = 9.4 Hz, 1H), 8.53 - 8.48 (m, 1H), 8.46 - 8.28 (m, 1H), 8.19 - 8.12 (m, 1H), 8.12 - 8.05 (m, 2H), 7.83 (dd, *J* = 4.7, 8.2 Hz, 1H), 7.53 - 7.45 (m, 1H),

7.41 - 7.32 (m, 2H), 7.28 - 7.21 (m, 1H), 4.54 (d, *J* = 10.2 Hz, 2H), 4.19 (d, *J* = 18.8 Hz, 2H), 3.33 (s, 3H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 481.1141. Found: 481.1138.

**4-{5-[2-(3-Fluorophenyl)-5-methanesulfonylbenzoyl]-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazol-2-yl}-2-methylpyridine (35).** The title compound was prepared using General Method G (75 mg, 46%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.49 - 8.40 (m, 1H), 8.35 - 8.09 (m, 1H), 8.09 - 8.04 (m, 1H), 7.85 - 7.78 (m, 1H), 7.65 (d, *J* = 13.3 Hz, 1H), 7.59 - 7.53 (m, 1H), 7.52 - 7.44 (m, 1H), 7.42 - 7.28 (m, 1H), 7.24 (t, *J* = 8.6 Hz, 1H), 4.61 - 4.41 (m, 2H), 4.18 (d, *J* = 17.6 Hz, 2H), 3.32 (d, *J* = 1.6 Hz, 3H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 477.1391. Found: 477.1385.

**5-[5-(Ethanesulfonyl)-2-{[(2S)-1,1,1-trifluoropropan-2-yl]oxy}benzoyl]-2-(2-fluorophenyl)-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazole (36).** The title compound was prepared using General Method G (213 mg, 65%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.09 - 7.96 (m, 1H), 7.95 (dd, *J* = 0.8, 2.3 Hz, 1H), 7.86 (dd, *J* = 2.5, 3.3 Hz, 1H), 7.81 - 7.68 (m, 1H), 7.60 (dd, *J* = 2.0, 9.0 Hz, 1H), 7.50 - 7.29 (m, 3H), 5.54 (td, *J* = 6.4, 12.6 Hz, 1H), 4.67 (d, *J* = 9.8 Hz, 2H), 4.44 - 4.26 (m, 2H), 3.37 - 3.31 (m, 2H), 1.47 - 1.40 (m, 3H), 1.14 - 1.07 (m, 3H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 512.1262. Found: 512.1255.

**3-{5-[5-(Ethanesulfonyl)-2-{[(2S)-1,1,1-trifluoropropan-2-yl]oxy}benzoyl]-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazol-2-yl}-5-fluoropyridine (37).** The title compound was prepared using General Method G (24 mg, 40%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 9.02 - 8.93 (m, 1H), 8.54 - 8.37 (m, 2H), 8.24 - 8.14 (m, 1H), 7.96 (ddd, *J* = 1.4, 2.4, 8.7 Hz, 1H), 7.86 (dd, *J* = 2.3, 3.1 Hz, 1H), 7.60 (dd, *J* = 2.0, 9.0 Hz, 1H), 5.60 - 5.48 (m, 1H), 4.68 (d, *J* = 9.0 Hz, 2H), 4.46 - 4.28 (m, 2H), 3.38 - 3.31 (m, 2H), 1.43 (dd, *J* = 1.6, 6.3 Hz, 3H), 1.11 (t, *J* = 7.4 Hz, 3H) ppm. HRMS (ESI): [M +

H]$^+$ *m/z* calculated: 513.1214. Found: 513.1206.

**4-{5-[5-(Ethanesulfonyl)-2-{[(2S)-1,1,1-trifluoropropan-2-yl]oxy}benzoyl]-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazol-2-yl}-2-methylpyridine (38).** The title compound was prepared using General Method G (26 mg, 44%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.77 - 8.58 (m, 2H), 8.25 - 8.05 (m, 2H), 8.02 - 7.94 (m, 1H), 7.90 - 7.83 (m, 1H), 7.62 (dd, *J* = 2.3, 9.0 Hz, 1H), 5.55 (td, *J* = 6.3, 12.5 Hz, 1H), 4.71 (d, *J* = 12.9 Hz, 2H), 4.50 - 4.31 (m, 2H), 3.32 (q, *J* = 7.3 Hz, 3H), 2.68 (d, *J* = 2.0 Hz, 3H), 1.42 (d, *J* = 6.3 Hz, 3H), 1.11 (dt, *J* = 1.0, 7.3 Hz, 3H) ppm. HRMS (ESI): [M + H]$^+$ *m/z* calculated: 509.1465. Found: 509.1458.

**2-Phenyl-5-[5-(propane-2-sulfonyl)-2-{[(2S)-1,1,1-trifluoropropan-2-yl]oxy}benzoyl]-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazole (39).** The title compound was prepared using General Method F (8 mg, 24%).$^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.39 - 8.22 (m, 1H), 7.93 (ddd, *J* = 1.0, 2.3, 8.8 Hz, 1H), 7.83 (t, *J* = 2.3 Hz, 1H), 7.80 - 7.73 (m, 2H), 7.60 (dd, *J* = 1.6, 9.0 Hz, 1H), 7.51 - 7.43 (m, 2H), 7.32 - 7.24 (m, 1H), 5.60 - 5.48 (m, 1H), 4.66 (d, *J* = 6.7 Hz, 2H), 4.42 - 4.25 (m, 2H), 3.45 (quind, *J* = 6.8, 13.5 Hz, 1H), 1.43 (dd, *J* = 1.6, 6.3 Hz, 3H), 1.16 (d, *J* = 7.0 Hz, 6H) ppm. HRMS (ESI): [M + H]$^+$ *m/z* calculated: 508.1513. Found: 508.1507.

**3-[2-(2-Fluorophenyl)-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazole-5-carbonyl]-4-{[(2S)-1,1,1-trifluoropropan-2-yl]oxy}benzonitrile (40).** The title compound was prepared using General Method F (5 mg, 15%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.10 - 7.93 (m, 2H), 7.89 (dd, *J* = 2.2, 7.6 Hz, 1H), 7.82 - 7.64 (m, 1H), 7.54 (dd, *J* = 2.5, 8.8 Hz, 1H), 7.51 - 7.23 (m, 3H), 5.53 (td, *J* = 6.4, 12.6 Hz, 1H), 4.65 (d, *J* = 10.6 Hz, 2H), 4.48 - 4.19 (m, 2H), 1.41 (d, *J* = 6.3 Hz, 3H) ppm. HRMS (ESI): [M + H]$^+$ *m/z* calculated: 445.1282. Found: 445.1276.

**3-[2-(5-Fluoropyridin-3-yl)-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazole-5-carbonyl]-4-{[(2S)-1,1,1-trifluoropropan-2-yl]oxy}benzonitrile (41).** The title compound was prepared using General Method F (19 mg, 44%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.97 (dd, *J* = 1.6, 10.6 Hz, 1H), 8.55 - 8.32 (m, 2H), 8.19 (tdd, *J* = 2.3, 10.2, 16.4 Hz, 1H), 7.98 (dd, *J* = 2.0, 9.0 Hz, 1H), 7.88 (dd, *J* = 2.0, 7.0 Hz, 1H), 7.54 (dd, *J* = 2.3, 9.0 Hz, 1H), 5.52 (td, *J* = 6.4, 12.6 Hz, 1H), 4.66 (d, *J* = 9.0 Hz, 2H), 4.49 - 4.25 (m, 2H), 1.40 (d, *J* = 6.7 Hz, 3H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 446.1235. Found: 446.1231.

**3-[2-(2-Methylpyridin-4-yl)-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazole-5-carbonyl]-4-{[(2S)-1,1,1-trifluoropropan-2-yl]oxy}benzonitrile (42).** The title compound was prepared using General Method F (13 mg, 9%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.73 - 8.48 (m, 2H), 8.14 - 7.83 (m, 4H), 7.55 (dd, *J* = 2.3, 9.0 Hz, 1H), 5.53 (td, *J* = 6.0, 12.3 Hz, 1H), 4.79 - 4.60 (m, 2H), 4.48 - 4.28 (m, 2H), 2.62 (d, *J* = 2.3 Hz, 3H), 1.40 (d, *J* = 6.3 Hz, 3H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 442.1486. Found: 442.1476.

**3-{2-Cyclopentyl-F-pyrrolo[3,4-c]pyrazole-5-carbonyl}-4-{[(2S)-1,1,1-trifluoropropan-2-yl]oxy}benzonitrile (43).** The title compound was prepared using General Method F (20 mg, 62%). $^1$H NMR (400 MHz, CDCl3) δ 7.71 (ddd, *J* = 2.3, 4.1, 8.8 Hz, 1H), 7.65 (d, *J* = 2.0 Hz, 1H), 7.17 - 7.04 (m, 2H), 4.87 - 4.67 (m, 3H), 4.62 (dt, *J* = 2.7, 7.2 Hz, 1H), 4.47 - 4.19 (m, 2H), 2.27 - 2.09 (m, 2H), 2.07 - 1.94 (m, 2H), 1.94 - 1.81 (m, 2H), 1.78 - 1.61 (m, 2H), 1.51 (d, *J* = 6.3 Hz, 3H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 419.1690. Found: 419.1685.

**3-{2-Phenyl-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazole-5-carbonyl}-4-(propan-2-yloxy)-benzonitrile (44).** The title compound was prepared using General Method F (24 mg, 66%). $^1$H NMR (400

MHz, CDCl3) δ 7.80 - 7.28 (m, 8H), 7.03 (d, *J* = 8.6 Hz, 1H), 4.85 (d, *J* = 19.6 Hz, 2H), 4.75 - 4.60 (m, 1H), 4.45 (d, *J* = 12.9 Hz, 2H), 1.36 (d, *J* = 5.9 Hz, 6H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 373.1659. Found: 373.1653.

**3-[2-(5-Fluoropyridin-3-yl)-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazole-5-carbonyl]-4-(propan-2-yloxy)benzonitrile (45).** The title compound was prepared using General Method F (13 mg, 34%). $^1$H NMR (400 MHz, CDCl3) δ 8.75 (d, *J* = 5.5 Hz, 1H), 8.41 (br. s., 1H), 7.90 - 7.73 (m, 2H), 7.74 - 7.57 (m, 2H), 7.03 (d, *J* = 8.6 Hz, 1H), 4.85 (d, *J* = 14.9 Hz, 2H), 4.70 (qd, *J* = 5.8, 11.5 Hz, 1H), 4.47 (d, *J* = 11.3 Hz, 2H), 1.44 - 1.29 (m, 6H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 392.1518. Found: 392.1511.

**3-[2-(2-Methylpyridin-4-yl)-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazole-5-carbonyl]-4-(propan-2-yloxy)benzonitrile (46).** The title compound was prepared using General Method F (125 mg, 44%). $^1$H NMR (400 MHz, CDCl3) δ 8.54 (d, *J* = 5.9 Hz, 1H), 7.96 - 7.76 (m, 1H), 7.74 - 7.58 (m, 2H), 7.59 - 7.41 (m, 2H), 7.04 (dd, *J* = 1.2, 8.6 Hz, 1H), 4.85 (d, *J* = 15.3 Hz, 2H), 4.76 - 4.62 (m, 1H), 4.47 (d, *J* = 11.0 Hz, 2H), 2.68 (d, *J* = 7.0 Hz, 3H), 1.36 (dd, *J* = 2.0, 6.3 Hz, 6H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 388.1768. Found: 388.1762.

**4-(Cyclopropylmethoxy)-3-[2-(2-methylpyridin-4-yl)-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazole-5-carbonyl]benzonitrile (47).** The title compound was prepared using General Method F (6 mg, 26%). $^1$H NMR (400 MHz, CDCl3) δ 8.54 (dd, *J* = 2.2, 5.7 Hz, 1H), 7.89 - 7.72 (m, 1H), 7.71 - 7.63 (m, 2H), 7.58 - 7.46 (m, 1H), 7.40 (d, *J* = 5.5 Hz, 1H), 7.06 - 6.99 (m, 1H), 4.87 (d, *J* = 16.0 Hz, 2H), 4.51 (d, *J* = 12.1 Hz, 2H), 3.98 (dd, *J* = 2.0, 7.0 Hz, 2H), 2.66 (s, 3H), 1.32 - 1.12 (m, 1H), 0.70 - 0.45 (m, 2H), 0.41 - 0.19 (m, 2H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 400.1768. Found:

400.1762.

**4-(4,4-Difluoropiperidin-1-yl)-3-[2-(2-methylpyridin-4-yl)-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazole-5-carbonyl]benzonitrile (48).** The title compound was prepared using General Method F (12 mg, 6%). $^1$H NMR (400 MHz, $CDCl_3$) δ 8.55 (dd, *J* = 1.6, 5.9 Hz, 1H), 7.92 - 7.71 (m, 1H), 7.70 - 7.59 (m, 2H), 7.55 - 7.37 (m, 2H), 7.10 (dd, *J* = 3.9, 8.6 Hz, 1H), 5.04 - 4.20 (m, 4H), 3.35 (br. s., 4H), 2.67 (br. s., 3H), 2.14 - 1.91 (m, 4H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 449.1896. Found: 449.1892.

**4-(3-Fluorophenyl)-3-[2-(5-fluoropyridin-3-yl)-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazole-5-carbonyl]benzonitrile (49).** The title compound was prepared using General Method G (15 mg, 33%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.98 - 8.85 (m, 1H), 8.50 (d, *J* = 2.3 Hz, 1H), 8.47 - 8.29 (m, 1H), 8.14 (tdd, *J* = 2.4, 10.4, 16.8 Hz, 1H), 8.08 - 7.99 (m, 2H), 7.80 - 7.68 (m, 1H), 7.54 - 7.42 (m, 1H), 7.39 - 7.28 (m, 2H), 7.28 - 7.19 (m, 1H), 4.52 (d, *J* = 11.7 Hz, 2H), 4.23 (d, *J* = 13.7 Hz, 2H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 428.1318. Found: 428.1312.

**4-(3-Fluorophenyl)-3-[2-(2-methylpyridin-4-yl)-2H,4H,5H,6H-pyrrolo[3,4-c]pyrazole-5-carbonyl]benzonitrile (50).** The title compound was prepared using General Method F (15 mg, 65%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.47 - 8.32 (m, 2H), 8.07 - 8.01 (m, 2H), 7.77 - 7.72 (m, 1H), 7.65 (dd, *J* = 2.2, 15.5 Hz, 1H), 7.56 (ddd, *J* = 2.0, 5.6, 12.0 Hz, 1H), 7.51 - 7.44 (m, 1H), 7.37 - 7.29 (m, 2H), 7.27 - 7.18 (m, 1H), 4.51 (d, *J* = 11.3 Hz, 2H), 4.22 (d, *J* = 14.1 Hz, 2H), 2.48 (s, 3H) ppm. HRMS (ESI): $[M + H]^+$ *m/z* calculated: 424.1568. Found: 424.1560.

**GlyT1 Inhibition Uptake Assay.** A scintillation proximity assay (SPA) was used to measure the uptake of [$^{14}$C]-glycine in HEK293 cells stably expressing human GlyT1c and in cultured primary

rat cortical neurons.[52]

**Human GlyT1c Assay.** HEK293 cells stably expressing hGlyT1c were plated onto 96-well Cytostar plates at a density of $4.5x10^4$ cells per well in 100 μL of growth media (DMEM containing 1x penicillin/streptomycin and 10% fetal bovine serum) and incubated overnight in a 37 $^oC$, 10% $CO_2$ incubator. The following day stock compounds, 10 mM solution in DMSO, were serially diluted in DMSO and 2X compound solutions were prepared by diluting compound again (1:100) in HBSS. Growth media was removed from the plate and 30 μL of 2X compound solution was added. Immediately after, 30 μL of 15 μM [$^{14}C$] glycine in HBSS was added and plates were sealed and allowed to incubate at room temperature for 2 hours. Plates were then read on a MicroBeta plate counter (Perkin Elmer). Dose-response data (11-concentration dose response, 2 data points per concentration) for tested compounds were analyzed and curves were fit using a four-parameter logistic fit to determine $IC_{50}$ values.

**Human GlyT2 Assay.** GlyT2 inhibition was measured in HEK293 cells stably expressing GlyT2 for each compound at a single concentration of 10 μM using the methods analogous to those described above for in the GlyT1 assay.

**Rat Primary Cortical Neuron Assay.** Primary rat cortical neurons were harvested from rat E18 pups and plated onto poly-D-lysine-coated 96-well Cytostar plates at a density of $3.5x10^4$ cells per well in 100 μL of astrocyte media (MEM media with 20 mM glucose, 1x penicillin/streptomycin, and 10% fetal bovine serum) and incubated at 37$^oC$ in a 5% $CO_2$ environment. Twenty-four hours later, media was replaced with 200 μL of neuronal media (BME media with 20 mM glucose, 1 mM sodium pyruvate, 2 mM GlutaMAX, 1x

penicillin/streptomycin, 1% horse serum, and B27 supplement) and the cells were cultured for an additional 5 days in a 37$^{o}$C, 5% $CO_2$ incubator. The following day, stock compounds in 10 mM DMSO were serially diluted in DMSO and 2X compound solutions were prepared by diluting compound again (1:100) in HBSS. Growth media was removed from the plate and 30 μL of 2X compound solution was added. Immediately after, 30 μL of 25 μM [$^{14}$C] glycine in HBSS supplemented with 5 mM L-alanine and 5 mM HEPES was added and plates were sealed and allowed to incubate at room temperature for 2 hours. Plates were then read on a MicroBeta plate counter (Perkin Elmer). Dose-response data (11-point dose response, 2 data points per concentration) for tested compounds were analyzed and curves were fit using a four-parameter logistic fit to determine $IC_{50}$ values.

**Metabolic Stability in the Presence of Rat or Human Liver Microsomes.** Test compound was incubated at a final concentration of 1 μM in a reaction mixture containing rat liver microsomes of 0.5 mg protein/mL in tris buffer at 37 °C. The reaction was started by addition of NADPH (2 mM). Aliquots of the incubation mixture were removed and quenched with three volumes of cold ACN containing internal standard, indomethacin (0.5 μM) at 0, 5, 15, and 30 min for RLM and at 0, 15, 30, and 60 min for HLM. The samples were vortex mixed and centrifuged at 4000 rpm for 10 min at 4 °C; and the supernatant was transferred to a 384-well plate followed by dilution with same volume of distilled water. Five microliters of the samples were analyzed using a Shimadzu HPLC system (Kyoto, Japan) equipped with a reverse-phase column (Thermo Hypersil GOLD 3μm Drop-In guard cartridges, 10 × 2.1 mm). Mobile phase consisted of 0.1% formic acid in water and of 0.1% formic acid in ACN with a flow rate of 0.7 mL/min. Eluent was directed to an API4000 triple quadruple mass spectrometer (AB Sciex, Framingham, MA)

equipped with a turbo electrospray interface. Multiple reaction monitoring (MRM) transition in positive ion mode was used. Integration of the sample peaks was performed using AB Sciex Analyst and DiscoveryQuant software; peak area ratio of the analyte to the internal standard was determined accordingly. *In vitro* intrinsic clearance (Clint) was calculated by dividing the first-order degradation rate constant (Kdeg) by the concentration of microsomal protein used in the incubation. Kdeg was the slope using least-squares fit to the curve of percent test compound remaining vs time (0 to 30 min for RLM and 0 to 60 min for HLM).

**hERG Potassium Channel Inhibition.** The hERG potassium current was recorded from a CHO cell line stably expressing the hERG channel (Kv11.1) cell line. hERG tail currents were recorded using a QPatch® automated patch clamp instrument in standard extracellular and intracellular recording solutions. The voltage protocol was: 1) Vstep from -80 mV to -50 mV for 0.5 sec to determine holding current, 2) Vstep from –50 mV to +10 mV for 2 sec to activate HERG current, 3) Vstep to -50 mV for 2 sec to remove voltage-dependent inactivation and elicit the HERG tail current and 4) return to the holding potential of -80 mV. The voltage waveform was repeated every 12 sec. The baseline HERG tail current was recorded for at least 5-10 min in drug-free external recording. After a stable baseline HERG tail current was established, four concentrations of test compound(s) were cumulatively applied (10 nM, 100 nM, 1 μM, 10 μM) and the hERG current was measured at the end of a 5 min application of each drug concentration. The hERG current at each test concentration was normalized to the pre-drug baseline and concentration-response curves were generated.

**MDCK-MDR1 Cell Permeability and Efflux Transport**. Efflux was determined across monolayers

of Madin Darby Canine Kidney (MDCK) cells transfected with the human Multidrug Resistance 1 gene (MDR1 or P-gp) (Netherlands Cancer Institute, Amsterdam, The Netherlands). Cell monolayers were grown for 3 days on trans well cell culture devices (Millipore, Billerica, MA) at a density equal to 1.1 x $10^6$ cells/mL. Bi-directional transport of each test compound was assessed using a final concentration equal to 5µM dissolved in Hanks Balanced Salt Solution (HBSS) (Gibco, Grand Island, NY). Permeability experiments were conducted at 37°C, 5% $CO_2$ with compounds dosed either on the apical (for A to B determinations) or the basolateral (for B to A) compartment with donor samples collected at time zero and donor and receiver samples collected at 60 minutes incubation. Samples were collected from the MDCK-MDR1 cell monolayers and appropriately diluted with blank HBSS. The final step of sample preparation was the addition of 0.1 % formic acid in $CH_3CN$ containing buspirone as the internal standard (I.S.). Sample plates were analyzed by high pressure liquid chromatography tandem mass spectrometry (LC-MS/MS) to determine the amount of parent compound in each chamber. Each well was monitored for the presence of the test compound, buspirone and atenolol (negative control). Time 0 samples were collected in order to obtain initial concentration measurements and peak area ratios were used to calculate the permeability of the test compounds. All the High Performance Liquid Chromatography-Tandem Quadrupole Mass Spectrometry (LC-MS/MS) experiments for quantitative analysis were performed using a triple quadrupole mass spectrometer (API4000, AB Sciex, Framingham, MA) coupled with a Shimadzu HPLC system (Shimadzu Scientific Instruments, Columbia, MD). The Shimadzu LC system consisted of a pump, column and column heater (40°C), autosampler, and a vacuum degasser/mobile phase tray. Mobile phase A was water containing 0.1 % formic acid and mobile

phase B was $CH_3CN$ containing 0.1 % formic acid. All samples were analyzed in Multiple Reaction Monitoring (MRM) positive ion mode. A turbo spray ion source was used on the API4000 mass spectrometer. The reverse-phase column was a Phenomenex Luna C18 100A column 50 × 2 mm, 5 µm (Phenomenex, Torrance, CA). Integration of the sample peaks was performed using AB Sciex Analyst software; peak area ratio of the analyte to the internal standard was determined accordingly. Apparent permeability coefficients ($P_{app}$) were determined for each compound in both the A to B and B to A directions. Efflux ratios were calculated using the ratio of $P_{app}$ B → A to $P_{app}$ A → B.

**BBB PAMPA Permeability Assay.** The determination of permeability was performed using an Evolution instrument (Pion Inc., Billerica, MA) combined with a Tecan Freedom Evo Workstation (Tecan Group Ltd. Mannedorf, Switzerland) robotic liquid handling system. The liquid handling draws 8 µL of 10 mM DMSO stock of new chemical entities and mixes it into 800 µL of an aqueous universal PRIZMA buffer solution at pH 7.4 (Pion Inc., Billerica, MA) so that the final sample concentration is 100 µM in buffer solutions. The DMSO concentration was kept at 1.0% (v/v) in the final buffer solutions. 200 µL of the 100 µM NCE was dispensed to a 96-well microtiter plate "sandwich" (pION, PN 110212, preloaded with magnetic stirrers) of the donor side and 200 µL of Brain Sink Buffer (Pion Inc., Billerica, MA) to the receiver solutions contained a surfactant mixture ("lipophilic sink") to mimic tissue binding. Vigorous stirring was employed in the assay, with stirring speed set to produce an ABL thickness of about 60 µm, to minimize the ABL contribution to the measured permeability. The PAMPA sandwich was assembled and allowed to incubate for 60 min in a controlled-environment chamber (pION Gut-Box, PN 110205) with a built-in magnetic stirring mechanism. Both the donor and receiver wells were

assayed for the amount of material present, by comparison with the UV spectrum (210−500 nm) obtained from a reference standard. Permeability values were corrected for membrane retention.

**CYP Inhibition Assay.** In Vitro incubation to determine CYP inhibition of 5 CYPs (1A2, 2C9, 2C19, 2D6, and 3A4) were performed by using a semi-automated procedure on a Hamilton Microlab Nimbus Personal Pipetting Workstation (Hamilton Robotics HQ, Reno, NV). Incubations from frozen pooled human liver microsomes from 200 donors (Xenotech, Lenexa, KS), 50 µl was added to each well containing 0.1 mg/ml final in 50 mM Potasium Phosphate buffer (pH 7.4) and spiked with test compound solution (100 µL) 10 µM final, known specific CYP inhibitors (10 µM Furafylline 1A2, 10 µM Sulphenazole 2C9, 20 µM Tranylcypromine 2C19, 1 µM Quinidine 2D6, 1 µM Ketoconazole) or vehicle control. 50 µl of NADPH-generating system (all final concentration; 0.6 mM MgCl2, 0.2 mM EDTA, 1 mM Glucose-6-Phosphate, 0.2 mM $NADP^+$, and 0.2 units/ml Glucose-6-phosphate dehydrogenase). To start the reaction 50 µl of known CYP specific substrate solution was added to each plate (50 µM Phenacetin 1A2, 4 µM Diclofenac 2C9, 35 µM S-Mephenytoin 2C19, 5 µM Dextromethorphan 2D6, and 3 µM Midazolam 3A4/5). Samples were incubated in a shaking water bath at 37°C for 10 minutes for all CYPs except 3A4 (7 minutes). The reaction was stopped by adding 300 µL stop solution containing an internal standard of 40 nM diazepam in acetonitrile. Samples were vortexed and centrifuged at 3500 rpm for 10 minutes. 150 µl of supernatant was transferred into a 96-deep well containing 150 µl of 0.3% Formic Acid in milli-q water and analyzed using LC-MS/MS. Five µL of the samples were analyzed using a Shimadzu HPLC system (Kyoto, Japan) equipped with a reversed-phase column (Thermo Hypersil GOLD 3um Drop-In guard cartridges, 10 x 2.1 mm).

Mobile phase was consisted of 0.1% formic acid in water and of 0.1% formic acid in ACN with a flow rate of 0.7 mL/min. Eluent was directed to an API5500 triple quadruple mass spectrometer (AB Sciex, Framingham, MA) equipped with a turbo electrospray interface. Multiple Reaction Monitoring (MRM) transition in positive ion mode was used for formation of metabolites (Acetaminophin 1A2, 4-hydroxydiclofenac 2C9, 4-hydroxymephnetoin 2C19, Dextrorphan 2D6, 1-hydroxymidazolam 3A4/5) Integration of the sample peaks was performed using AB Sciex Analyst software; peak area ratio of the analyte to the internal standard was determined accordingly. For each test compound or positive control sample, % Inhibition = $((S_{vehicle}-S_{compound})/S_{Vehicle})x\ 100$. Where $S_{vehicle}$ is the average area ratio from the vehicle samples and $S_{compound}$ is the area ratio from the test compounds or positive controls.

**Plasma Protein Binding and Brain Tissue Binding Assay.** The free fraction of compounds in the rat plasma and brain homogenate were determined using a 96-well Rapid Equilibrium Dialysis (RED) device with MWCO 8000 RED inserts (Thermo Fisher Scientific, Waltham, MA). Rat plasma and brain were purchased from Bioreclamation IVT (Baltimore, MD) and thawed on the day of experiment. Rat brain was homogenized with BupH™ phosphate buffered saline, 0.1 M, pH 7.2 (Thermo Scientific), to a final 1:6 brain/buffer using a FastPrep-24. Plasma and brain homogenate were spiked with each tested compound (final concentration of 1.0 µM) and triplicate 300 µL was loaded to designated RED device donor chambers followed by addition of 500 µL of PBS buffer to the corresponding receiver chambers. Dialysis against the PBS buffer was started by shaking the sealed RED device at 125 RPM using an orbital microplate shaker in an incubation oven (Panasonic, Newark, NJ) at 37 °C for 6 hours. Post-dialysis donor (plasma or brain homogenate) and receiver (dialysate) samples along with the standard curve samples

(0.005 -1 µM in plasma or brain homogenate) were prepared in a similar method to become mixed matrix samples. In brief, 20 µL of plasma/ brain homogenate standards or plasma/ brain homogenate donor samples were added into 100 µL of phosphate buffered saline; and 100 µL dialysate receiver samples were added into 20 µL of control matrix fluid, so that the ratio of the final volume of the buffer to the plasma or brain homogenate was the same. The mixed matrix samples were then mixed with 300 µL of protein precipitation solution containing diazepam as internal standard in $CH_3CN$ (250 nM) followed by vortex mixing for approximately 2 min and centrifugation for 10 minutes at 2830 G-force. After mixing the supernatant (150 µL) with the same volume of water containing 0.1% formic acid, the final samples were injected and analyzed by LC-MS/MS system using a Sciex QTrap 5500 mass spectrometer (AB sciex, Framingham, MA) coupled with Shimadzu LC-20 UPLC system (Shimadzu Scientific Instruments, Columbia, MD). The samples were eluted using a Luna C18 100A 50 × 2 mm, 5 µm column (Phenomenex, Torrance, CA) at a flow rate of 0.6 mL/min (mobile phase A was water containing 0.1 % formic acid and mobile phase B was $CH_3CN$ containing 0.1 % formic acid). Quantification for the analyte was calculated based on the analyte to internal standard peak area ratios plotted against its concentration using a linear regression with $1/x^2$ weighting.

The unbound fraction in plasma and in diluted brain homogenate are calculated based on the following equation: $f_{u,}= C_r/ C_d$, where $C_r$ and $C_d$ are the RED post-dialysis receiver and donor concentrations with mixed-matrix dilution factor corrected, respectively. The unbound fraction in undiluted brain homogenate was corrected for dilution factor according to the following equation (Kalvass and Maurer, 2002): Undiluted $f_u = (1/D) / (((1/f_{u,apparent}) -1) +1/D)$, where D =

dilution factor in brain homogenate, and $f_{u,apparent}$ is the measured free fraction of diluted brain homogenate.

**Rat Pharmacokinetics Determination**. Pharmacokinetics properties in male SD rats (200−250 g) were determined following intravenous (i.v.) and oral (p.o.) administration. For i.v. dosing (N = 3, 1 mg/kg), rats were catheterized in the jugular and femoral vein. For p.o. dosing (N = 3, 10 mg/kg) rats were catheterized in the femoral vein. Test article was formulated in NMP/PEG400/water (10:50:40). Rats were not fasted during this study. Blood was sampled at 0.033, 0.083, 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h following i.v. and at 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h following p.o. dosing. Plasma was isolated by centrifugation, and all samples were frozen at −80 °C. Calibration standards were prepared by the addition of known concentrations of test article to blank rat plasma to provide a calibration range of 0.5 ng/mL-2000 ng/mL. Fifty microliter plasma samples or calibration standard was added to 250 µL of internal standard solution in $CH_3CN$. Samples were vortex mixed and centrifuged at 12000 rpm for 5 min at 4 °C. Supernatant (100 µL) was transferred to labeled autosampler vials containing 300 µL of mobile phase ($CH_3CN$/water 10:90 with 0.1% formic acid), vortex mixed, and analyzed by LC-MS/MS. A bioanalytical method was developed for the quantification of test article in rat plasma. Method development and sample analysis were conducted using a Waters Quattro Premier LC-MS/MS system equipped with a Waters Acquity UPLC system. Five microliters of the samples were analyzed using a Waters Acquity UPLC system equipped with a C 18 reverse-phase column (Phenomenex Kinetex C18, 1.7um, 2.1 × 50.0 mm). Mobile phase consisted of 0.1% formic acid in water and 0.1% formic acid in $CH_3CN$ with a flow rate of 0.6 mL/min. Eluent was directed to a

Waters Quattro Premier mass spectrometer equipped with a turbo electrospray interface. Multiple reaction monitoring (MRM) transition in positive ion mode was used.

**Rat CSF Glycine Determination.** Glycine levels in rat cerebrospinal fluid (CSF) were determined following oral administration of the test article at 10 mg/kg in male Sprague-dawley (CD) rats (300−400 g, n = 5) obtained from Charles River (Hollister, CA). Food was provided ad libitum. Test articles were formulated in NMP/PEG400/water (10:50:40) and dosed at 10 mL/kg. A vehicle only control group (n = 5) was included in every study to establish a CSF glycine baseline level for comparison to the treatment groups. Samples were collected at 90 min; prior to collection, animals were anesthetized (confirmed with toe-pinch) and approximately 100µL of CSF was collected with a butterfly catheter (Exel $27G^{3/4}$ needle attached to the PVC catheter containing DEHP). A LoBind microcentrifuge tube (2 mL, Eppendorf) was used for CSF sample collection and storage. All samples were frozen at −80 °C until analysis. On the day of analysis fresh glycine calibration standards and QC samples were prepared in artificial CSF (Harvard Apparatus, Holliston, MA) by addition of known concentrations of glycine (>99%, Sigma) to provide a calibration range of 400 - 20,000 µmol/L. 50 µL of CSF, calibration standards and quality control samples were aliquoted into conical glass tubes (2 mL, Flex-tier 96 well, Analytical Sales and Service) to which 10 µL of 10 µM $^{13}C_2$-$^{15}N$-Glycine (>98%, Aldrich) internal standard solution was added followed by dry-down under a stream of nitrogen at 45˚C. 50 µL of 2,4-dinitro-5-fluorphenyl (Marfey's reagent) at 0.5% w/v acetone (99.8% Extra Dry, Acros Organics) was then added to the dry tubes followed by 100µL of 0.125 M sodium tetraborate decahydrate (>99.5%, Sigma). After sealing, the samples were placed in a water bath at 45˚C and mixed gently for 30 min; the amine derivatization reaction was stopped by addition of 25

µL of 4 M HCl with mixing. The quenched derivatization mixture was then diluted using 1.2 mL of 4 mM ammonium formate, pH 4.6, and transferred into a 96 well polypropylene plate (2 mL, True taper, Analytical Sales and Service). Sample analysis was conducted using a Sciex QTRAP 5500 liquid chromatography mass spectrometer (LC-MS) system equipped with a Shimadzu autosampler and HPLC system. Mobile phase A consisted of 4 mM ammonium formate, pH 4.6, and mobile phase B was acetonitrile. 10 µL of processed sample was introduced to a Waters Atlantis dC18 reverse-phase column (3.0 µm, 3.9 × 150 mm) using 10-90% B gradient elution at a flow rate of 0.4 mL/min with a 16 min runtime. The eluent was directed to the mass spectrometer equipped with a turbo electrospray interface operated in negative ion mode. The analyte and internal standard were detected with selected ion monitoring. Analyst software (v 1.5.2) was used for LC-MS data processing using a linear regression and 1/x2 weighting. Statistical analysis of determined glycine concentrations included 1-way ANOVA with multiple comparisons between treatment groups and vehicle control (GraphPad Prism).

**Novel Object Recognition Training and Testing.** Male, Long-Evans rats (n = 24/group; 330-380 g) were obtained from Envigo (Livermore, CA). Rats were habituated to handling and an empty test arena for 7 min on each of 3 consecutive days. On the training day, the drug was administered p.o. in a vehicle of 10:50:40 NMP/PEG400/H2O at a volume of 1 mL/kg. Ninety minutes after dosing, each rat was placed in the test arena, which now contained two identical objects located centrally in the arena. Rats were given either 3 min (to induce a weak memory) or 20 min (to induce a strong memory) to explore the arena and objects. Memory retention was tested 24 h after training. Rats were placed back in the arena with one “familiar” (previously trained) and one “novel” object and given 5 min to explore. The spatial position of objects

left−right position) and which object was novel (ball or cube) was counterbalanced across subjects. Objects and arenas were cleaned with diluted quaternary ammonium disinfectant (5ml Coverage Plus NPD®: 1L $H_2O$; Steris Corporation, OH) between trials to remove rat feces and urine. To determine memory performance, an object-discrimination index (DI) was calculated as [novel exploration − familiar exploration]/ [total exploration]. Rats were excluded from the analysis if total exploration during training or test was less than 5 s. Locomotor activity and object exploration during training was also recorded. Data were analyzed by ANOVA with least-squares means planned comparisons (Student's t-test). All data are presented as the mean ± SEM.

**Statement on Animal Research**

All studies involving animals were conducted in compliance with protocols approved by the Dart NeuroScience LLC., Institutional Animal Care and Use Committee, and followed the guidance of the National Research Council Guide for the Care and Use of Laboratory Animals Studies (2011).

**Supporting Information**

Molecular formula strings (SMILES data, CSV file).

Off-target selectivity panel for compound **46** (PDF).

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**Notes**

The authors declare no competing financial interest.

**Abbreviations Used**

ADMET, absorption, distribution, metabolism, elimination and toxicology; br s, broad signal; CYP450, cytochrome P450; hERG, human ether-a-go-go-related gene; HLM, human liver microsomes; LTP, long-term potentiation; MDCK, Madin-Darby canine kidney; NOR, novel object recognition; PAMPA-BBB, parallel artificial membrane permeability assay - blood brain barrier; RLM, rat liver microsomes

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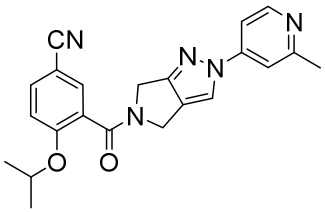
**Table of Contents Graphic**




**Compound 46**
rGlyT1 $IC_{50}$ = 38 nM
$t_{1/2}$ = 2.4 h
F = 81%