



## Aminothienopyridazine inhibitors of tau aggregation: Evaluation of structure–activity relationship leads to selection of candidates with desirable in vivo properties

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### ABSTRACT

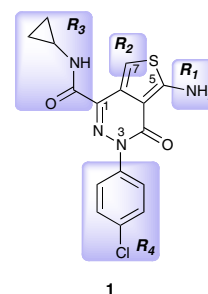
Previous studies demonstrated that members of the aminothienopyridazine (ATPZ) class of tau aggregation inhibitors exhibit a promising combination of in vitro activity as well as favorable pharmacokinetic properties (i.e., brain-penetration and oral bioavailability). Here we report the synthesis and evaluation of several new analogues. These studies indicate that the thienopyridazine core is essential for inhibition of tau fibrillization in vitro, while the choice of the appropriate scaffold decoration is critical to impart desirable ADME-PK properties. Among the active, brain-penetrant ATPZ inhibitors evaluated, 5-amino-N-cyclopropyl-3-(4-fluorophenyl)-4-oxo-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxamide (**43**) was selected to undergo maximum tolerated dose and one-month tolerability testing in mice. The latter studies revealed that this compound is well-tolerated with no notable side-effects at an oral dose of 50 mg/kg/day.

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### 1. Introduction

Alzheimer's disease (AD), which is characterized by the presence of intraneuronal aggregates of tau proteins (e.g., neurofibrillary tangles, or NFTs) and extracellular deposits comprised of Aβ peptide (e.g., senile plaques), remains a challenge for biomedical intervention. Although the exact neuropathological mechanisms underlying this disease remain the focus of intense investigation, several lines of evidence indicate that under pathological conditions tau may act as the mediator of neurodegeneration in response to various upstream events.<sup>1</sup> As a result, in recent years there has been an increasing interest in the development of therapeutic strategies that could target pathological tau.<sup>2</sup> One such strategy calls for the use of small molecules to inhibit the self-assembly of tau into oligomeric/polymeric species, including the well-defined fibrils found within NFTs. Although several classes of tau assembly inhibitors have demonstrated activity in vitro,<sup>3,4</sup> aside from selected cases (e.g., methylene blue<sup>5</sup>), the majority of these inhibitors are unlikely to be considered for in vivo efficacy studies due to potential toxicities and/or limited CNS-uptake.<sup>3</sup>

Recently, we identified a novel class of tau fibrillization inhibitors, the aminothienopyridazines (ATPZs), which exhibit a favorable combination of efficacy in an in vitro tau fibrillization assay, as well as drug-like properties (i.e., no violations of Lipinski's 'rule of five').<sup>6</sup> Furthermore, prototype compounds from this class of inhibitors (e.g., **1**, Fig. 1) have been developed that can reach significant concentrations in the brain of mice after intravenous (i.v.) or oral administration, suggesting that the ATPZs may be amenable to efficacy studies in animal models.<sup>7</sup> However, ATPZ **1**



**Figure 1.** Representative example of a brain-penetrant ATPZ;<sup>7</sup> fragments investigated as part of the SAR studies are marked R<sub>1</sub>–R<sub>4</sub>.

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was found to have limited water solubility (i.e., ~30  $\mu\text{M}$ ). Furthermore, evaluation by equilibrium dialysis revealed that **1** is ~99% bound in brain homogenates, indicating that relatively high doses of the compound would be required to achieve efficacious free drug concentration in the brain.

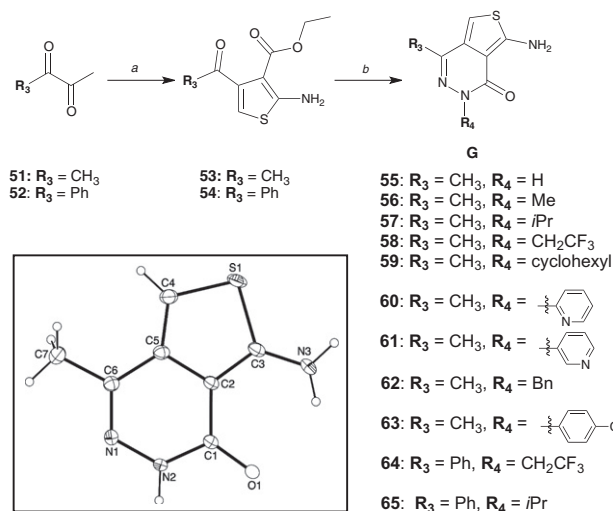
Thus, to circumvent these shortcomings and to identify preferred candidates for future studies, we designed and synthesized a series of new analogues, including a number of closely related congeners and a series of compounds bearing a range of modifications in the ATPZ heterocyclic core (Schemes 1–3).

After synthesis, all test ATPZs were evaluated for aqueous solubility and activity in a heparin-induced tau K18PL fibrillization assay.<sup>6</sup> Analogues that produced full dose-response curves in the fibrillization assay and whose activity was confirmed in a secondary centrifugation assay<sup>6</sup> progressed to an evaluation of pharmacokinetic (PK) properties. Collectively, these studies defined the structure–activity relationship (SAR) of ATPZs and in turn resulted in the identification of selected analogues which exhibit, in addition to excellent brain-penetration and oral bioavailability, more favorable properties compared to **1**, such as higher aqueous solubility and higher unbound fraction in brain homogenates. Among these compounds, ATPZ **43** was evaluated for possible dose-limiting toxicities in wild type mice. The results from PK and toxicity studies suggest that nM free drug levels can be achieved in the brains of mice after oral administration of well-tolerated doses of **43**.

### 1.1. Design and synthesis of test aminothiopyridazine (ATPZ) congeners

Previous SAR studies<sup>6,7</sup> indicated that the  $\text{R}_3$  fragment (Fig. 1) is relatively well tolerant of structural variations, while N-acylations ( $\text{R}_1$ ) and alkylations at C7 ( $\text{R}_2$ ) lead to drastic reduction in the ability of the derived analogues to interfere with tau fibrillization. The SAR of  $\text{R}_4$ , on the other hand, remained incompletely defined. Thus, in addition to a number of congeners of **1** (i.e., compounds of general structure F, Scheme 1), a series of ATPZs bearing modifications at the  $\text{R}_3$  and  $\text{R}_4$  fragments (i.e., analogues of general formula G, Scheme 2), as well as congeners modified at the aminothiophene ring (Scheme 3), were synthesized.

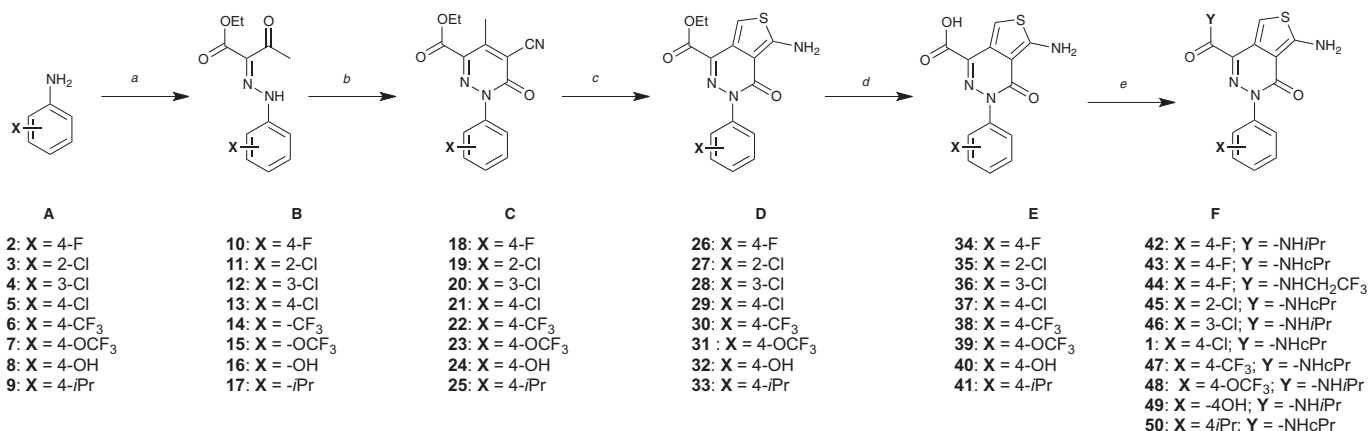
For the synthesis of analogues of general structure F, we employed the reaction sequence highlighted in Scheme 1.<sup>7</sup> Central to this strategy is the Gewald reaction protocol<sup>8,9</sup> to construct the ATPZ heterocyclic core from pyridazines of general structure C. Next, saponification of esters D to the corresponding acids (E) was followed by coupling reactions with the appropriate amine



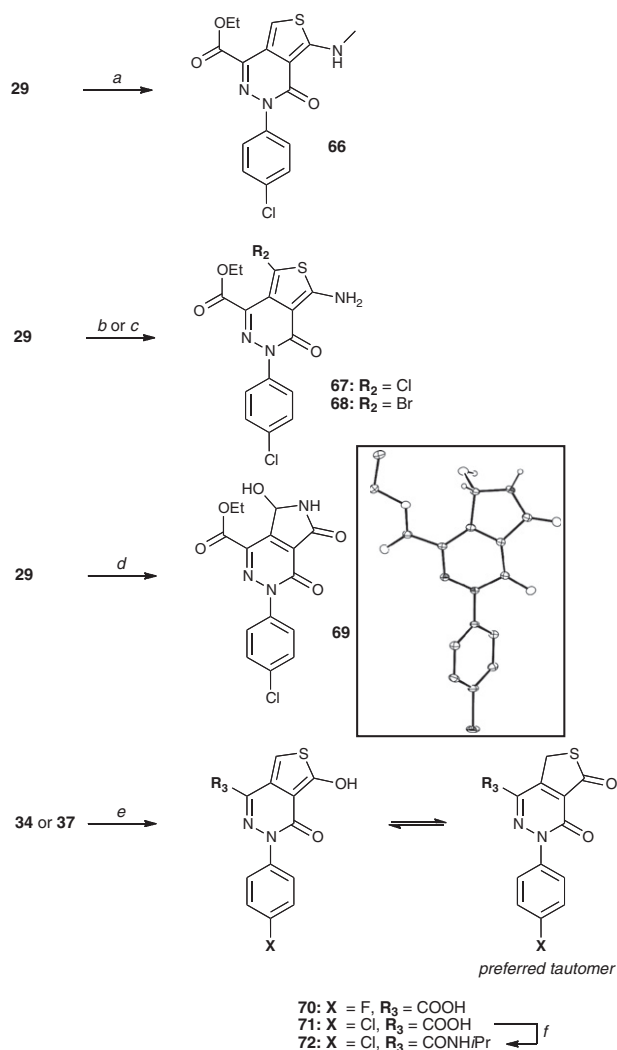
**Scheme 2.** Reagents and conditions: (a) ethyl cyanoacetate,  $\text{S}_8$ , morpholine, ethanol, 150 °C (microwave irradiation), 15 min; (b) appropriate substituted hydrazine, 150 °C (microwave irradiation), 0.5–3 h. The insert shows the X-ray crystal structure of compound **55** (CCDC 853887).

to provide amides of general structure F (Scheme 1). Such an approach proved both effective and reliable for the synthesis of several closely related analogues of **1**. However, as stable diazonium salts can be generally derived from anilines, but not from aliphatic- or heteroaryl-amines, this method could not be exploited to obtain analogues bearing  $\text{R}_4 \neq$  phenyl/substituted phenyl. Thus, to circumvent this limitation, aminothiophene keto-esters **53** and **54** (Scheme 2) were first prepared by subjecting di-ketones **51** and **52** to the Gewald reaction. Next, condensation of **53** and **54** with a series of alkyl-, aryl-, and (hetero)aryl-hydrazines, under microwave irradiation-promoted conditions, provided the corresponding ATPZs (**55–65**) of general structure G (Scheme 2).

Scheme 3 highlights the synthesis of analogues modified at the aminothiophene ring, which include examples of N-alkylated derivatives, such as **66**, obtained by treating **29** with methyl iodide in the presence of potassium carbonate, as well as C7-halogenated derivatives (**67** and **68**), obtained from **29** upon treatment with N-chloro- and N-bromo-succinimide, respectively. Interestingly, treatment of **29** with dimethyldioxirane resulted in rapid (~15 min) consumption of the starting material with the formation of the 5,7-dihydroxy-pyrrolopyridazine **69**, whose structure was



**Scheme 1.** Reagents and conditions: (a) (i)  $\text{NaNO}_2$ , 37% HCl, ethanol, water, 0 °C, 20 min; (ii) ethyl acetoacetate, sodium acetate, ethanol, water, 0 °C, 2 h; (b) ethyl cyanoacetate, ammonium acetate, acetic acid, 170 °C (microwave irradiation), 4 min; (c)  $\text{S}_8$ , morpholine, ethanol, 150 °C (microwave irradiation), 15 min; (d)  $\text{LiOH}\cdot\text{H}_2\text{O}$ , tetrahydrofuran, water, rt, 16 h; (e) amine, BOP reagent, *N,N*-diisopropylethylamine, dimethylsulfoxide, rt, 4–16 h.



**Scheme 3.** Reagents and conditions: (a) MeI, K<sub>2</sub>CO<sub>3</sub>, 65 °C, 10 h.; (b) *N*-chlorosuccinimide; (c) *N*-bromosuccinimide; (d) DMSO, acetone, rt, 5 min; (e) 1 N HCl, acetonitrile, 120 °C (microwave irradiation), 30 min; (f) isopropyl amine, BOP reagent, *N,N*-diisopropylethylamine, dimethylsulfoxide, rt, 4 h. The insert shows the X-ray crystal structure of compound **69** (CCDC 853892).

confirmed by single crystal X-ray analysis. Treatment of acids **34** and **37** with hydrochloric acid under microwave-promoted conditions<sup>10</sup> then produced, respectively, thienopyridazines **70** and **71**, in which the amine is replaced by a hydroxyl. Compound **71** was then converted to the corresponding cyclopropyl amide **72**. In agreement with previous reports,<sup>10</sup> the NMR spectra of **70–72** are consistent with the thiolactone tautomeric forms shown in Scheme 3.

## 2. Results

### 2.1. Evaluation of aminothienopyridazine (ATPZ) analogues in the tau fibrillization assay

Initially, each test compound was evaluated for aqueous solubility in the sodium acetate buffer employed in the fibrillization assay.<sup>7</sup> Next, ATPZ analogues were evaluated in vitro for their ability to disrupt the heparin-induced fibrillization of the K18 tau fragment (amino acids 244–369)<sup>11</sup> bearing the P301L<sup>12</sup> mutation found in inherited frontotemporal lobar degeneration (K18PL). The activity of the ATPZs was determined by a thioflavin-T (ThT) fluorescence assay, which monitors the formation of ThT-positive cross-beta fibrils in the fibrillization reaction.<sup>13</sup> In addition, all

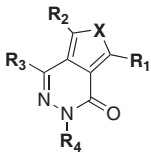
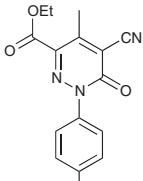
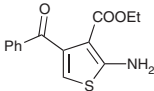
compounds were evaluated in a secondary assay, which consisted of SDS-PAGE analysis of the soluble and insoluble fractions obtained after centrifugation of the ATPZ-treated fibrillizing mixtures (centrifugation assay).<sup>6,13</sup> The results of these experiments, summarized in Table 1, in general reveal a good correlation between the primary and secondary assays, although higher % inhibitions are typically observed in the ThT assay compared to the centrifugation assay. The different % inhibitions seen in the two assays may not be necessarily indicative of some degree of interference of the ATPZ analogues with ThT binding and/or fluorescence, but could be explained considering the differences between the ThT- and centrifugation assay. While the former is dependent on protein-conformation, as it is based on the characteristic red-shift in excitation spectrum of ThT that occurs upon ThT binding to pleated  $\beta$ -sheets found in amyloid fibrils,<sup>14</sup> the latter is based on the total amount of pelletable material, regardless of protein-conformation. Consistent with previous results, the majority of active ATPZs were found to exhibit IC<sub>50</sub> values in the 1–10  $\mu$ M range. Given that the tau concentration in the in vitro assay is kept at 20  $\mu$ M, these results suggest that ATPZ inhibitors may be most effective when present in a ~1:1 ratio with tau protein. Interestingly, the activity data illustrated in Table 1 indicate that all compounds lacking the thienopyridazine heterocycle, such as pyridazine **21**, aminothiophene **54**, and pyrrolopyridazine **69**, were essentially devoid of any activity against K18PL fibrillization as determined by centrifugation assay. Conversely, minimally substituted ATPZs, such as **55** and **56**, as well as variously decorated derivatives, including hydroxythienopyridazines (**70** and **72**), and ATPZs halogenated at C7 (**67** and **68**), all produced near full inhibition in the K18PL fibrillization assay. These results strongly suggest that the thienopyridazine heterocycle is essential for inhibition of tau fibrillization, while the nature of the R<sub>1</sub>–R<sub>4</sub> substituents may play a role in modulating the potency of the active thienopyridazine, possibly by affecting ATPZ-tau interactions and/or the physicochemical properties of the ATPZs. For example, the relatively lower inhibition observed in the centrifugation assay, which is conducted at 50  $\mu$ M compound concentration, with ATPZ esters of structure D relative to the corresponding acids (E) and amides (F) may be attributed in part to the generally lower water solubility of these esters.

### 2.2. Determination of brain-penetration

Among all active tau aggregation inhibitors shown in Table 1, a representative subset having aqueous buffer solubility >50  $\mu$ M were selected for studies consisting of determination of brain and plasma levels 1 h after i.p. administration of 5 mg/Kg (Table 2). For each of the ATPZs found to be brain-penetrant, equilibrium dialysis experiments were also performed to determine the unbound fraction in plasma and brain homogenates (Table 2). These values provide for an estimate of the percentage of drug that is freely available in these tissues. The results from the PK studies suggest that substitution of the amino group with an hydroxyl results in derivatives that are not brain-penetrant (cf., **1** and **72**). Furthermore, the observation that smaller derivatives lacking the R<sub>4</sub> fragment (e.g., **55**) exhibit low plasma levels 1 h after dosing suggests that a phenyl group in R<sub>4</sub>, although not strictly required for biological activity (cf., Table 1), may be an important determinant of metabolic stability and possibly brain-penetration. Notably, among all brain-penetrant ATPZs, **42**, **43** and **44** were found to be able to partition freely between plasma and brain with B/P ratio near unity (Table 2). Equally important, evaluation of non-specific plasma/brain protein binding revealed that the unbound fractions in brain homogenates are ~4.3% (**43**) and 5.0% (**44**), respectively.

Finally, a full PK evaluation of **43** confirms that there is good brain exposure to the compound (Fig. 2). Moreover, the oral bioavailability of **43** was found to be ~60% (see Supplementary data).

**Table 1**  
Activity and Solubility of Test Compounds

Compd number						Solubility in assay buffer	K18PL ThT assay		K18PL centrifugation assay
	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	X	Upper solubility limit (μM)	IC <sub>50</sub> (μM)	Max % inhib.	% Soluble
26	-NH <sub>2</sub>	-H	-COOEt	4-F-Ph	S	>200	1.0 ± 1.11	77	63
<sup>a</sup> 29	-NH <sub>2</sub>	-H	-COOEt	4-Cl-Ph	S	13	4.0 ± 2.99 <sup>b</sup>	71	47
27	-NH <sub>2</sub>	-H	-COOEt	2-Cl-Ph	S	12.8	2.0 ± 1.42 <sup>b</sup>	75	71
28	-NH <sub>2</sub>	-H	-COOEt	3-Cl-Ph	S	32	3.2 ± 2.02 <sup>b</sup>	82	73
30	-NH <sub>2</sub>	-H	-COOEt	4-CF <sub>3</sub> -Ph	S	32	DNC	48	27
31	-NH <sub>2</sub>	-H	-COOEt	4-OCF <sub>3</sub> -Ph	S	12.8	DNC <sup>b</sup>	41	29
32	-NH <sub>2</sub>	-H	-COOEt	4-OH-Ph	S	12.8	5.0 ± 1.29	81	69
33	-NH <sub>2</sub>	-H	-COOEt	4- <i>i</i> Pr-Ph	S	32	DNC <sup>b</sup>	8	17
66	-NHCH <sub>3</sub>	-H	-COOEt	4-Cl-Ph	S	80	DNC	39	51
67	-NH <sub>2</sub>	-Cl	-COOEt	4-Cl-Ph	S	32	2.0 ± 1.53	88	65
68	-NH <sub>2</sub>	-Br	-COOEt	4-Cl-Ph	S	12.8	4.0 ± 1.10	92	62
69	-OH	-OH	-COOEt	4-Cl-Ph	NH	>200	DNC	57	15
34	-NH <sub>2</sub>	-H	-COOH	4-F-Ph	S	>200	2.0 ± 1.27	84	83
<sup>a</sup> 37	-NH <sub>2</sub>	-H	-COOH	4-Cl-Ph	S	>200	5.0 ± 2.15 <sup>b</sup>	84	74
35	-NH <sub>2</sub>	-H	-COOH	2-Cl-Ph	S	>200	3.2 ± 1.58	86	64
36	-NH <sub>2</sub>	-H	-COOH	3-Cl-Ph	S	32	1.6 ± 1.22	92	73
38	-NH <sub>2</sub>	-H	-COOH	4-CF <sub>3</sub> -Ph	S	>200	19.9 ± 1.10	88	83
39	-NH <sub>2</sub>	-H	-COOH	4-OCF <sub>3</sub> -Ph	S	>200	1.6 ± 1.53	92	71
40	-NH <sub>2</sub>	-H	-COOH	4-OH-Ph	S	>200	4.0 ± 1.58 <sup>b</sup>	84	70
70	-OH	-H	-COOH	4-F-Ph	S	>200	2.5 ± 1.26 <sup>b</sup>	92	87
42	-NH <sub>2</sub>	-H	-CONH- <i>i</i> Pr	4-F-Ph	S	>200	1.6 ± 1.39	89	77
43	-NH <sub>2</sub>	-H	-CONH- <i>c</i> Pr	4-F-Ph	S	>200	2.5 ± 2.93 <sup>b</sup>	88	78
44	-NH <sub>2</sub>	-H	-CONH-CH <sub>2</sub> CF <sub>3</sub>	4-F-Ph	S	>200	8.0 ± 1.28	83	75
<sup>a</sup> 1	-NH <sub>2</sub>	-H	-CONH- <i>c</i> Pr	4-Cl-Ph	S	32	8.0 ± 2.48 <sup>b</sup>	83	69
45	-NH <sub>2</sub>	-H	-CONH- <i>c</i> Pr	2-Cl-Ph	S	80	DNC	68	44
46	-NH <sub>2</sub>	-H	-CONH- <i>i</i> Pr	3-Cl-Ph	S	32	3.2 ± 2.02 <sup>b</sup>	82	73
47	-NH <sub>2</sub>	-H	-CONH- <i>c</i> Pr	4-CF <sub>3</sub> -Ph	S	80	6.3 ± 2.28	64	83
48	-NH <sub>2</sub>	-H	-CONH- <i>i</i> Pr	4-OCF <sub>3</sub> -Ph	S	80	15.8 ± 1.95	67	42
49	-NH <sub>2</sub>	-H	-CONH- <i>i</i> Pr	4-OH-Ph	S	>200	15.8 ± 1.38	86	84
50	-NH <sub>2</sub>	-H	-CONH- <i>c</i> Pr	4- <i>i</i> Pr-Ph	S	80	4.0 ± 2.88	82	86
72	-OH	-H	-CONH- <i>i</i> Pr	4-Cl-Ph	S	ND	10.0 ± 1.25	96	85
55	-NH <sub>2</sub>	-H	-CH <sub>3</sub>	-H	S	>200	8.0 ± 1.87 <sup>b</sup>	87	81
56	-NH <sub>2</sub>	-H	-CH <sub>3</sub>	-CH <sub>3</sub>	S	>200	2.5 ± 1.19	82	76
57	-NH <sub>2</sub>	-H	-CH <sub>3</sub>	<i>i</i> Pr	S	80	19.9 ± 1.16	95	17
58	-NH <sub>2</sub>	-H	-CH <sub>3</sub>	CH <sub>2</sub> CF <sub>3</sub>	S	>200	10 ± 1.18 <sup>b</sup>	81	78
59	-NH <sub>2</sub>	-H	-CH <sub>3</sub>	Cyclohexyl	S	>200	31.6 ± 1.12	65	86
60	-NH <sub>2</sub>	-H	-CH <sub>3</sub>	2-Py	S	>200	2.0 ± 1.27	85	78
61	-NH <sub>2</sub>	-H	-CH <sub>3</sub>	3-Py	S	>200	4.0 ± 1.41	74	84
62	-NH <sub>2</sub>	-H	-CH <sub>3</sub>	-Bn	S	80	1.6 ± 1.24	89	73
63	-NH <sub>2</sub>	-H	-CH <sub>3</sub>	4-Cl-Ph	S	80	2.0 ± 1.23	85	70
64	-NH <sub>2</sub>	-H	-Ph	CH <sub>2</sub> CF <sub>3</sub>	S	>200	6.3 ± 1.63 <sup>b</sup>	103	82
65	-NH <sub>2</sub>	-H	-Ph	<i>i</i> Pr	S	>200	19.9 ± 1.63 <sup>b</sup>	89	92
21						12.8	DNC <sup>b</sup>	4.4	21
54						>200	DNC	29	6.5

<sup>a</sup>Previously reported data;<sup>7</sup> DNC = did not calculate due to absence of asymptote; ND = not determined; all IC<sub>50</sub> values were determined through sigmoidal curve fitting of compound concentration-response analyses, with each concentration tested in triplicate. All results are from a single curve fit, except where indicated by<sup>b</sup>, where the mean of 2 or more separate analyses are reported.

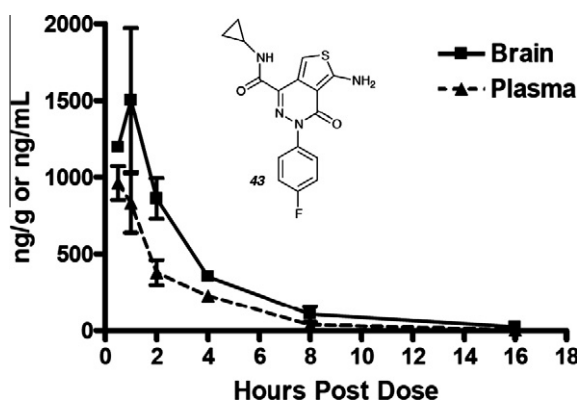
### 2.3. In vivo tolerability evaluation of ATPZ 43

To evaluate potential dose-limiting toxicities of ATPZ **43** in mice, we determined the maximum tolerated dose (MTD) after a single oral administration of the compound. Notably, no overt

signs of toxicity were seen until a dose of **43** of 100 mg/kg, at which point only mild and transient lethargy was observed. Subsequently, **43** was administered in drinking water (containing 3% PEG400 and 0.5% methylcellulose) to normal mice for a 1-month period at a dose equivalent of 50 mg/kg/day (assuming

**Table 2**  
Brain and plasma level of test compounds

Compd	Brain (ng/g)	Plasma (ng/mL)	B/P <sup>a</sup> (total)	Fu <sup>b</sup> (%) brain	Fu <sup>b</sup> (%) plasma	B/P (fu) <sup>c</sup>
1 <sup>e</sup>	1300 ± 110	820 ± 150	1.61 ± 0.28	1.1 ± 0.6	1.9 ± 0.2	0.92 ± 0.16
43	670 ± 100	610 ± 27	1.09 ± 0.15	4.3 ± 0.9	6.9 ± 0.4	0.68 ± 0.09
42	280 ± 410	110 ± 160	2.68 ± 0.47	2.9 ± 0.4	5.7 ± 0.5	1.30 ± 0.23
72	16.3 ± 8.8	4700 ± 1000	0.0035 ± 0.0015	DNC	DNC	—
49	14.1 ± 9.4	150 ± 100	0.098 ± 0.007	DNC	DNC	—
44	637 ± 75	401 ± 14	1.59 ± 0.21	3.2 ± 0.7	2.2 ± 0.2	2.31 ± 0.31
55	BLD <sup>d</sup>	BLD <sup>d</sup>	—	DNC	DNC	—
56	BLD <sup>d</sup>	10 ± 18	—	DNC	DNC	—
60	7.5 ± 5.3	61 ± 20	0.116 ± 0.039	DNC	DNC	—
62	48 ± 18	78 ± 16	0.60 ± 0.12	5.1 ± 1.8	4.0 ± 1.0	0.77 ± 0.15

<sup>a</sup> Brain-to-plasma ratio.<sup>b</sup> Fraction unbound.<sup>c</sup> Ratio of free drug in brain and plasma.<sup>d</sup> Below limit of detection.<sup>e</sup> Previously reported data<sup>7</sup>; DNC = did not calculate due to low B/P.**Figure 2.** Brain and plasma level of **43** after i.p. administration of 5 mg/kg.

5 ml water consumption/day/mouse). The amount of water consumed by the compound-treated mice was normal, and no visible adverse events were noted. Importantly, no differences in body weight gain were observed between the compound- and vehicle-treated (PEG400/methylcellulose) groups ( $n = 5$ /group; 9.7% and 8.2% increases of body weight, respectively). Moreover, there were no differences in organ weights, including heart, liver, kidney, spleen and brain, between the mice that received compound **43** or vehicle only (see [Supplementary data](#)). Similarly, there were no alterations in any blood cell counts in the mice that were administered the ATPZ (see [Supplementary data](#)). An evaluation of brain levels of **43** after 3 days of dosing via drinking water at 50 mg/kg/day revealed total brain levels of  $139 \pm 8$  nM, with a calculated free compound level of  $6.0 \pm 0.4$  nM. Notably, the plasma and brain levels of **43** in mice that received compound in drinking water for 30 days was comparable to the levels in mice that received the compound in water for 3 days. This reveals that repeated dosing of the compound over a prolonged interval did not result in the induction of liver enzymes that might alter the metabolism of the compound.

### 3. Discussion

That ATPZ inhibitors of tau fibrillization hold promise as potential candidates for in vivo testing was suggested by earlier studies from our laboratory.<sup>6</sup> The purpose of this study was to investigate further the SAR of this class of compounds and to identify selected candidates that have solubility, brain-penetration, oral

bioavailability and safety that might be suitable for longer-term in vivo studies. Based on the observation that the  $IC_{50}$  values of all active ATPZs in our in vitro assay are typically in the range of stoichiometric equivalence with tau, it seems plausible that this chemotype may be interfering with the fibrillization reaction by interacting with tau monomers. If this is the case, similar stoichiometric requirement of tau:ATPZ may be expected in vivo for effective inhibition of tau assembly. The intraneuronal tau concentration is estimated to be  $\sim 2$   $\mu$ M. However, under normal circumstances, tau is believed to be almost completely (>99%) bound to microtubules (MTs).<sup>15</sup> Thus, the free intraneuronal tau fraction available to enter the fibrillization cascade may be <20 nM, even under pathological conditions.<sup>4</sup> Although the values of free tau are only estimates, they suggest that candidate ATPZs should preferably achieve free brain levels in this concentration range to be effective if there is indeed a requirement for stoichiometric amounts of the drug. From the results displayed in [Table 1](#), the pharmacophore of ATPZs would appear to be the thienopyridazine heterocycle, while the  $R_1$ – $R_4$  substituents may be important determinants of ADME-PK properties. In addition, our results suggest that the presence of a para-fluorophenyl residue at the N3, a primary amino group at the C5 position, and a carboxylamide moiety at C1 of the thienopyridazine heterocycle, comprise the optimal substitution pattern to achieve the desired combination of activity and ADME-PK properties. Indeed, based on the results from our PK studies, administration of a dose of 50 mg/kg/day of compound **43** to mice in drinking water resulted in total brain compound levels of  $\sim 140$  nM and a predicted brain free drug concentration of 6 nM. Furthermore, since the MTD for compound **43** was found to be >50 mg/kg, it is likely that even higher doses could be utilized during long-term in vivo testing. Taken together, these results indicate that **43** and possibly other closely related congeners, such as **42** and **44**, are suitable candidates for further investigating the in vivo properties and potential biological activity of the ATPZ class of tau fibrillization inhibitors.

### 4. Conclusions

To investigate the SAR of ATPZs and to identify viable candidates for in vivo studies, a series of new analogues were synthesized and evaluated. Among the active, brain-penetrant ATPZ analogues, **43** exhibited good oral bioavailability, as well as acceptable water solubility and non-specific brain tissue binding, such that oral administration of doses lower than the MTD are expected to achieve potentially efficacious free drug concentrations in the mouse brain after oral administration. These results indicate that **43** may be suitable for long-term in vivo testing.

## 5. Experimental

### 5.1. Materials and methods

All solvents were reagent grade. All reagents were purchased from Aldrich or Acros and used as received. Thin layer chromatography (TLC) was performed with 0.25 mm E. Merck pre-coated silica gel plates. Flash chromatography was performed with silica gel 60 (particle size 0.040–0.062 mm) supplied by Silicycle and Sorbent Technologies. Spots were detected by viewing under a UV light. Yields refer to chromatographically and spectroscopically pure compounds. Infrared spectra were recorded on a Jasco Model FT/IR-480 Plus spectrometer. All melting points were obtained on a Thomas-Hoover apparatus. Proton ( $^1\text{H}$ ) and carbon ( $^{13}\text{C}$ ) NMR spectra were recorded on a Bruker AMX-500 spectrometer. Chemical shifts were reported relative to solvents. High-resolution mass spectra were measured at the University of Pennsylvania, Mass Spectrometry Service on a Waters LCT Premier XE LC–MS system. Single-crystal X-ray structure determinations were performed at the University of Pennsylvania. X-ray intensity data were collected on a Bruker APEXII CCD area detector employing graphite-monochromated Mo-K $\alpha$  radiation ( $\lambda = 0.71073 \text{ \AA}$ ) at a temperature of 143(1) K. Analytical reversed-phased (Sunfire™ C18;  $4.6 \times 50 \text{ mm}$ ,  $5 \text{ mL}$ ) high-performance liquid chromatography (HPLC) was performed with a Water binary gradient module 2525 equipped with Waters 2996 PDA and Water micromass ZQ. All samples were analyzed employing a linear gradient from 10% to 90% of acetonitrile in water over 8 min and flow rate of  $1 \text{ mL/min}$ , and unless otherwise stated, the purity level was  $>95\%$ . Preparative reverse phase HPLC purification was performed employing Waters SunFire™ prep C18 OBD™ columns ( $5 \mu\text{m}$   $19 \times 50$  or  $19 \times 100 \text{ mm}$ ). All samples were purified employing a linear gradient from 10% to 90% of acetonitrile in water over 15 min and flow rate of  $20 \text{ mL/min}$ . The preparative HPLC system was equipped with Gilson 333 pumps, a 215 Liquid Handler, 845Z injection module, and UV detector. Unless otherwise stated, all final compounds were found to be  $>95\%$  as determined by HPLC/MS and NMR.

#### 5.1.1. General procedure for the synthesis of hydrazones B

To a solution of the appropriate aniline (39.2 mmol) and 37% hydrochloridric acid (9.93 mL) in water (4.96 mL) and ethanol (4.96 mL), a solution of sodium nitrite (2.93 g, 42.5 mmol) in water (13 mL) was added dropwise at  $0^\circ\text{C}$ . The reaction mixture was stirred at  $0^\circ\text{C}$  for 30 min and then a solution of sodium acetate (12.65 g, 154 mmol) in water (20 mL) and a solution of ethyl acetoacetate (5.10 g, 4.96 mL, 39.2 mmol) in ethanol (10 mL) were added maintaining the temperature below  $5^\circ\text{C}$ . The reaction mixture was stirred at  $0^\circ\text{C}$  for 4 h. Water was then added and the resultant mixture was extracted with ethyl acetate. The combined organic extracts were washed with brine, dried over  $\text{MgSO}_4$ , filtered and concentrated in vacuo. Finally, crystallization from ethanol provided the desired hydrazone B, which was directly used without further purification.

**5.1.1.1. Ethyl 2-(2-(4-fluorophenyl)hydrazono)-3-oxobutanoate (10).** Yield: 98%, mp:  $81\text{--}83^\circ\text{C}$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.40 (t,  $J = 7.0 \text{ Hz}$ , 3H), 2.59 (s, 3H), 4.34 (q,  $J = 7.2 \text{ Hz}$ , 2H), 7.09 (t,  $J = 8.7 \text{ Hz}$ , 2H), 7.39 (dd,  $J = 9.0$  and  $5.0 \text{ Hz}$ , 2H) ppm.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  14.4, 30.8, 61.1, 116.5 (d,  $J_{\text{CF}}^2 = 22.9 \text{ Hz}$ ), 118.0 (d,  $J_{\text{CF}}^3 = 8.1 \text{ Hz}$ ), 126.1, 138.1, 161.3 (d,  $J_{\text{CF}}^1 = 243.9 \text{ Hz}$ ), 165.0, 197.2 ppm. IR (film):  $\nu$  3125, 1701,  $1620 \text{ cm}^{-1}$ . MS [ESI] $^+$ : Calcd for  $\text{C}_{12}\text{H}_{14}\text{FN}_2\text{O}_3$  253.10. Found 253.25.

**5.1.1.2. Ethyl 2-(2-(2-chlorophenyl)hydrazono)-3-oxobutanoate (11).** See Ref. 16.

**5.1.1.3. Ethyl 2-(2-(3-chlorophenyl)hydrazono)-3-oxobutanoate (12).** Yield: 70%.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.42 (t,  $J = 7.5 \text{ Hz}$ , 3H), 2.60 (s, 3H), 4.36 (q,  $J = 7.2 \text{ Hz}$ , 2H), 7.14 (d,  $J = 8.0 \text{ Hz}$ , 1H), 7.24 (d,  $J = 9.0 \text{ Hz}$ , 2H), 7.30 (t,  $J = 8.2 \text{ Hz}$ , 1H), 7.48 (d,  $J = 3.2 \text{ Hz}$ , 1H) ppm.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  14.4, 30.8, 61.1, 114.6, 116.4, 125.6, 126.8, 130.6, 135.7, 143.0, 164.8, 197.4 ppm. IR:  $\nu$  3345, 1725,  $1686 \text{ cm}^{-1}$ . MS [ESI] $^+$ : Calcd for  $\text{C}_{12}\text{H}_{14}\text{ClN}_2\text{O}_3$  269.07. Found 269.16.

**5.1.1.4. Ethyl 2-(2-(4-chlorophenyl)hydrazono)-3-oxobutanoate (13).** See Ref. 7.

**5.1.1.5. Ethyl 3-oxo-2-(2-(4-(trifluoromethyl)phenyl)hydrazono)butanoate (14).** Yield: 94%;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.39 (t,  $J = 7.1 \text{ Hz}$ , 3H), 2.50 (s, 3H), 4.38 (q,  $J = 7.1 \text{ Hz}$ , 2H), 7.40 (d,  $J = 8.5 \text{ Hz}$ , 2H), 7.62 (t,  $J = 6.6 \text{ Hz}$ , 2H) ppm.

**5.1.1.6. Ethyl 3-oxo-2-(2-(4-(trifluoromethoxy)phenyl)hydrazono)butanoate (15).** Yield: 96%;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.41 (m, 3H), 2.50 (s, 2H), 2.61 (s, 1H), 4.37 (m, 2H), 7.24–7.26 (m, 2H), 7.24–7.26 (m, 1H), 7.34–7.37 (m, 1H), 7.42–7.45 (m, 1H) ppm.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  14.2, 27.0, 61.7, 116.6, 117.5, 122.49, 122.65, 128.0, 140.5, 163.8, 197.4 ppm.

**5.1.1.7. Ethyl 2-(2-(4-hydroxyphenyl)hydrazono)-3-oxobutanoate (16).** Yield: 72%, mp:  $106\text{--}108^\circ\text{C}$ .  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ ):  $\delta$  1.36–1.41 (m, 3H), 2.46 (s, 2H), 2.53 (s, 1H), 4.30–4.38 (m, 2H), 6.84–6.87 (m, 2H), 7.31–7.34 (m, 1H) ppm. MS [ESI] $^+$ : Calcd for  $\text{C}_{12}\text{H}_{15}\text{N}_2\text{O}_4$  251.10. Found 251.23.

**5.1.1.8. (E/Z) Ethyl 2-(2-(4-isopropylphenyl)hydrazono)-3-oxobutanoate (17).** Yield: 94%;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.25–1.27 (m, 12H,  $2 \times \text{CH}(\text{CH}_3)_2$ ), 1.41 (m, 6H,  $2 \times \text{CH}_2\text{CH}_3$ ), 2.49, 2.59 (s, 6H,  $2 \times \text{CH}_3$ ), 2.92 (m, 2H,  $2 \times \text{CH}$ ), 4.32–4.40 (m 4H,  $2 \times \text{CH}_2\text{CH}_3$ ), 7.24–7.30 (m, 6H), 7.36 (d,  $J = 8.5 \text{ Hz}$ , 2H) ppm.

**5.1.1.9. General procedure for the synthesis of pyridazines C.** *Method A:* A mixture of the appropriate hydrazone B (1.0 mmol), ethyl cyanoacetate (0.113 g, 1.0 mmol), and sodium acetate (0.17 g, 2.4 mmol) in acetic acid (3.3 mL) was heated to  $170^\circ\text{C}$  using microwave irradiation for 4 min. After cooling, the precipitate was collected and crystallized in ethanol to give the desired pyridazine C. *Method B:* A mixture of the appropriate hydrazone B (6.29 mmol), ethyl cyanoacetate (1.06 g, 1.0 mL, 9.44 mmol), and 4-aminobutyric acid was heated to  $160^\circ\text{C}$  for 2.5 h. After cooling, the residue was purified by silica gel column chromatography using ethyl acetate–hexanes 1:3 as eluant to provide the desired pyridazine C.

**5.1.1.10. Ethyl 5-cyano-1-(4-fluorophenyl)-4-methyl-6-oxo-1,6-dihydropyridazine-3-carboxylate (18).** Method A. Yield: 55%, mp:  $146\text{--}147^\circ\text{C}$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.40 (t,  $J = 7.0 \text{ Hz}$ , 3H), 2.76 (s, 3H), 4.43 (q,  $J = 7.2 \text{ Hz}$ , 2H), 7.19 (t,  $J = 8.7 \text{ Hz}$ , 2H), 7.63 (dd,  $J = 9.0$  and  $5.0 \text{ Hz}$ , 2H) ppm.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  14.2, 19.3, 62.9, 112.4, 115.9, 116.1 (d,  $J_{\text{CF}}^2 = 23.0 \text{ Hz}$ ), 127.1 (d,  $J_{\text{CF}}^3 = 8.6 \text{ Hz}$ ), 136.1, 137.4, 150.8, 155.9, 162.0, 162.7 (d,  $J_{\text{CF}}^1 = 243.9 \text{ Hz}$ ). IR (film):  $\nu$  3123, 1727, 1683,  $1602 \text{ cm}^{-1}$ .

**5.1.1.11. Ethyl 1-(2-chlorophenyl)-5-cyano-4-methyl-6-oxo-1,6-dihydropyridazine-3-carboxylate (19).** Method B. Yield: 67%, mp:  $93\text{--}95^\circ\text{C}$  (from ethanol).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.38 (t,  $J = 7.1 \text{ Hz}$ , 3H), 2.78 (s, 3H), 4.41 (q,  $J = 7.1 \text{ Hz}$ , 2H), 7.40–7.49 (m, 3H), 7.57 (dd,  $J = 7.9$  and  $1.4 \text{ Hz}$ , 1H) ppm.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  14.3, 19.6, 63.0, 112.3, 115.9, 128.1, 128.8130.8, 131.5, 131.6, 137.6, 137.8, 151.5, 155.4, 162.0 ppm. IR:  $\nu$  2235, 1731,  $1690 \text{ cm}^{-1}$ .

**5.1.1.12. Ethyl 1-(3-chlorophenyl)-5-cyano-4-methyl-6-oxo-1,6-dihydro-1H-pyridazine-3-carboxylate (20).** Method B. Yield: 90%.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.41 (d,  $J$  = 7.0 Hz, 6H), 2.76 (s, 3H), 4.45 (q,  $J$  = 7.1 Hz, 2H), 7.44–7.45 (m, 2H), 7.57–7.59 (m, 1H), 7.67 (s, 1H) ppm.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  14.2, 19.3, 63.0, 112.3, 116.1, 123.3, 125.4, 129.7, 130.1, 134.9, 137.6, 140.8, 150.9, 155.7, 161.9 ppm. IR:  $\nu$  3429, 3221, 1690, 1634  $\text{cm}^{-1}$ . MS [ESI] $^+$ : Calcd for  $\text{C}_{15}\text{H}_{13}\text{ClN}_3\text{O}_3$  318.06. Found 318.08.

**5.1.1.13. Ethyl 5-amino-3-(2-chlorophenyl)-4-oxo-3,4-dihydrothieno[3,4-*d*]pyridazine-1-carboxylate (21).** See Ref. 7.

**5.1.1.14. Ethyl 5-cyano-4-methyl-6-oxo-1-(4-(trifluoromethyl)phenyl)-1,6-dihydropyridazine-3-carboxylate (22).** Method A. Yield: 35%;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.41 (t,  $J$  = 7.1 Hz, 3H), 2.77 (s, 3H), 4.44 (q,  $J$  = 7.1 Hz, 2H), 7.77 (d,  $J$  = 8.6 Hz, 2H), 7.83 (d,  $J$  = 8.6 Hz, 2H) ppm.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  14.2, 19.4, 63.0, 112.3, 116.3, 125.5, 123.7 (q,  $J_{\text{CF}}^1$  = 272.5 Hz), 126.36 (q,  $J_{\text{CF}}^2$  = 3.7 Hz), 131.38 (q,  $J_{\text{CF}}^2$  = 37.8 Hz), 137.9, 142.7, 151.1, 155.7, 161.9 ppm.

**5.1.1.15. Ethyl 5-cyano-4-methyl-6-oxo-1-(4-(trifluoromethoxy)phenyl)-1,6-dihydropyridazine-3-carboxylate (23).** Method B. Yield: 43%;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.41 (t,  $J$  = 7.1 Hz, 3H), 2.77 (s, 3H), 4.44 (q,  $J$  = 7.1 Hz, 2H), 7.36 (d,  $J$  = 8.3 Hz, 2H), 7.72 (d,  $J$  = 9.1 Hz, 2H) ppm.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  14.3, 19.4, 63.0, 112.3, 116.2, 121.5, 126.7, 137.7, 138.3, 150.9, 155.8, 162.0 ppm.

**5.1.1.16. Ethyl 5-cyano-1-(4-hydroxyphenyl)-4-methyl-6-oxo-1,6-dihydropyridazine-3-carboxylate (24).** See Ref. 17.

**5.1.1.17. Ethyl 5-cyano-1-(4-isopropylphenyl)-4-methyl-6-oxo-1,6-dihydropyridazine-3-carboxylate (25).** Method B. Yield: 85%;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.28 (d,  $J$  = 7.0 Hz, 6H), 1.40 (t,  $J$  = 7.0 Hz, 3H), 2.76 (s, 3H), 2.98 (sept,  $J$  = 6.8 Hz, 1H), 4.42 (q,  $J$  = 7.1 Hz, 2H), 7.35 (d,  $J$  = 8.5 Hz, 2H), 7.54 (d,  $J$  = 8.5 Hz, 2H) ppm.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  14.3, 19.3, 24.0, 34.1, 62.8, 112.6, 115.8, 124.9, 127.2, 137.1, 137.9, 150.5, 150.6, 156.0, 162.2 ppm.

## 5.1.2. General procedure for synthesis of aminothienopyridazines D

A mixture of the appropriate pyridazine C (0.31 mmol), sulfur (15 mg, 0.46 mmol), and morpholine (54 mg, 54  $\mu\text{L}$ , 0.62 mmol) in ethanol (1.5 mL) was heated to 150  $^\circ\text{C}$  using microwave irradiation for 15 min. After cooling, the precipitate was collected and purified by silica gel column chromatography using ethyl acetate and hexane 1:3 as eluant to obtain the desired aminothienopyridazine D.

**5.1.2.1. Ethyl 5-amino-3-(4-fluorophenyl)-4-oxo-3,4-dihydrothieno[3,4-*d*]pyridazine-1-carboxylate (26).** Yield: 70%, mp: 178–180  $^\circ\text{C}$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.42 (t,  $J$  = 6.9 Hz, 3H), 4.43 (q,  $J$  = 7.1 Hz, 2H), 6.06 (br s, 2H), 7.13 (t,  $J$  = 8.3 Hz, 2H), 7.22 (s, 1H), 7.54–7.57 (m, 2H) ppm.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  14.4, 62.1, 104.7, 106.2, 115.7 (d,  $J_{\text{CF}}^2$  = 22.8 Hz), 127.9, (d,  $J_{\text{CF}}^3$  = 8.6 Hz), 133.9, 136.7, 159.5, 161.7 (d,  $J_{\text{CF}}^1$  = 269.8 Hz), 163.1 ppm. IR (film):  $\nu$  3423, 3302, 3128, 1718, 1641  $\text{cm}^{-1}$ . MS [ESI] $^+$ : Calcd for  $\text{C}_{15}\text{H}_{13}\text{FN}_3\text{O}_3$  334.07. Found 334.11.

**5.1.2.2. Ethyl 5-amino-3-(2-chlorophenyl)-4-oxo-3,4-dihydrothieno[3,4-*d*]pyridazine-1-carboxylate (27).** Yield: 87%.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.28 (t,  $J$  = 7.0 Hz, 3H), 4.31 (q,  $J$  = 7.0 Hz, 2H), 7.15 (s, 1H), 7.47 (m, 2H), 7.53–7.60 (m, 3H), 7.64 (s, 1H) ppm.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  14.0, 54.9, 61.4, 103.5, 104.1, 126.0, 128.0, 129.7, 130.4, 130.6, 131.7, 133.0, 138.3, 157.9, 162.2, 163.1 ppm. IR:  $\nu$  3425, 3303, 1710, 1648, 1587  $\text{cm}^{-1}$ . HRMS [ESI] $^+$ : Calcd for  $\text{C}_{15}\text{H}_{12}\text{ClN}_3\text{O}_3$  372.0186. Found 372.0190.

**5.1.2.3. Ethyl 5-amino-3-(3-chlorophenyl)-4-oxo-3,4-dihydrothieno[3,4-*d*]pyridazine-1-carboxylate (28).** Yield: 72%.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.44 (t,  $J$  = 7.2 Hz, 3H), 4.45 (q,  $J$  = 7.1 Hz, 2H), 6.20 (br s, 2H), 7.26 (s, 1H), 7.33 (d,  $J$  = 8.0 Hz, 1H), 7.39 (t,  $J$  = 8.1 Hz, 1H), 7.54 (d,  $J$  = 8.1 Hz, 1H), 7.66 (s, 1H) ppm.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  14.4, 62.2, 105.0, 106.7, 124.1, 126.2, 127.0, 127.8, 129.7, 134.1, 134.3, 141.7, 159.4, 161.9, 163.0 ppm. MS [ESI] $^+$ : Calcd for  $\text{C}_{15}\text{H}_{14}\text{N}_3\text{O}_4\text{S}^+$  350.04. Found 350.09.

**5.1.2.4. Ethyl 5-amino-3-(4-chlorophenyl)-4-oxo-3,4-dihydrothieno[3,4-*d*]pyridazine-1-carboxylate (29).** See Ref. 7.

**5.1.2.5. Ethyl 5-amino-4-oxo-3-(4-(trifluoromethyl)phenyl)-3,4-dihydrothieno[3,4-*d*]pyridazine-1-carboxylate (30).** Yield: 90%.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.43 (t,  $J$  = 7.1 Hz, 2H), 4.45 (q,  $J$  = 7.1 Hz, 1H), 7.27 (s, 1H), 7.72 (d,  $J$  = 8.6 Hz, 1H), 7.80 (d,  $J$  = 8.4 Hz, 1H), 7.72 (d,  $J$  = 8.6 Hz, 1H), 7.80 (d,  $J$  = 8.4 Hz, 1H) ppm.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  14.4, 62.2, 100.1, 105.3, 106.7, 123.0, 125.2, 125.85, 125.89, 125.91, 127.0, 129.2, 129.5, 134.4, 143.6, 159.4, 162.0, 162.9 ppm. IR (film):  $\nu$  3438, 3333, 1658, 1604  $\text{cm}^{-1}$ . MS [ESI] $^+$ : Calcd for  $\text{C}_{16}\text{H}_{13}\text{F}_3\text{N}_3\text{O}_3\text{S}^+$  384.06. Found 384.15.

**5.1.2.6. Ethyl 5-amino-4-oxo-3-(4-(trifluoromethoxy)phenyl)-3,4-dihydrothieno[3,4-*d*]pyridazine-1-carboxylate (31).** Yield: 55%.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.43 (t,  $J$  = 7.1 Hz, 2H), 4.45 (q,  $J$  = 7.1 Hz, 2H), 7.26 (d,  $J$  = 6.5 Hz, 1H), 7.31 (d,  $J$  = 8.5 Hz, 2H), 7.67 (d,  $J$  = 2.0 Hz, 2H) ppm.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  14.4, 62.2, 105.1, 119.6, 121.3, 127.1, 127.3, 133.8, 134.1, 139.1, 148.2, 159.4, 161.8, 163.0 ppm. MS [ESI] $^+$ : Calcd for  $\text{C}_{16}\text{H}_{13}\text{F}_3\text{N}_3\text{O}_4\text{S}^+$  400.06. Found 400.07.

**5.1.2.7. Ethyl 5-amino-3-(4-hydroxyphenyl)-4-oxo-3,4-dihydrothieno[3,4-*d*]pyridazine-1-carboxylate (32).** Yield: 46%.  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ ):  $\delta$  1.29 (t,  $J$  = 7.2 Hz, 3H), 4.31 (q,  $J$  = 7.0 Hz, 2H), 6.83 (d,  $J$  = 8.5 Hz, 2H), 7.07 (s, 1H), 7.25 (d,  $J$  = 9.0 Hz, 2H), 7.57 (br s, 2H), 9.68 (br s, 1H) ppm.  $^{13}\text{C}$  NMR ( $\text{DMSO}-d_6$ ):  $\delta$  14.7, 61.9, 103.5, 104.8, 115.5, 126.8, 128.3, 132.8, 157.3, 159.1, 163.1, 163.5. IR (film):  $\nu$  3404, 1639  $\text{cm}^{-1}$ . MS [ESI] $^+$ : Calcd for  $\text{C}_{15}\text{H}_{14}\text{N}_3\text{O}_4\text{S}^+$  332.07. Found 332.04.

**5.1.2.8. Ethyl 5-amino-3-(4-isopropylphenyl)-4-oxo-3,4-dihydrothieno[3,4-*d*]pyridazine-1-carboxylate (33).** Yield: 72%.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.28 (d,  $J$  = 7.0 Hz, 6H), 1.42 (t,  $J$  = 7.5 Hz, 3H), 2.96 (sept,  $J$  = 6.9 Hz, 1H), 4.43 (q,  $J$  = 7.0 Hz, 2H), 6.20 (br s, 2H), 7.23 (s, 1H), 7.31 (d,  $J$  = 8.0 Hz, 2H), 7.48 (d,  $J$  = 8.0 Hz, 2H) ppm.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  14.1, 24.1, 34.0, 62.0, 104.4, 107.2, 126.0, 126.9, 127.4, 133.5, 138.3, 148.6, 159.7, 161.5, 163.2 ppm. IR (film):  $\nu$  3426, 3322, 3179, 1705, 1658, 1601  $\text{cm}^{-1}$ .

## 5.1.3. General procedure for ester hydrolysis

To a solution of the appropriate aminothienopyridazine of general structure D (0.43 mmol) in tetrahydrofuran (3 mL) and water (2 mL), lithium hydroxide monohydrate (0.063 g, 1.29 mmol) was added. The reaction mixture was stirred at rt for 16 h. The reaction was quenched with 1 N hydrochloric acid (pH  $\sim$ 2) and filtered. Purification by reverse phase preparative HPLC afforded the desired acid of general structure E.

**5.1.3.1. 5-Amino-3-(4-fluorophenyl)-4-oxo-3,4-dihydrothieno[3,4-*d*]pyridazine-1-carboxylic acid (34).** Yield: 77%.  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ ):  $\delta$  7.12 (s, 1H), 7.29 (t,  $J$  = 8.7 Hz, 2H), 7.55–7.58 (m, 4H) ppm.  $^{13}\text{C}$  NMR ( $\text{DMSO}-d_6$ ):  $\delta$  104.3, 104.5, 115.8 (d,  $J_{\text{CF}}^2$  = 22.5 Hz), 126.9, 128.9 (d,  $J_{\text{CF}}^3$  = 8.5 Hz), 134.2, 137.7, 159.0, 161.3 (d,  $J_{\text{CF}}^1$  = 242.4 Hz), 163.7, 164.6 ppm. MS [ESI] $^+$ : Calcd for  $\text{C}_{13}\text{H}_9\text{FN}_3\text{O}_3\text{S}^+$  306.03. Found 306.07.

**5.1.3.2. 5-Amino-3-(2-chlorophenyl)-4-oxo-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxylic acid (35).** Yield: 91%. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>): δ 7.17 (s, 1H), 7.50 (m, 2H), 7.55–7.59 (m, 3H), 7.63–7.65 (m, 1H) ppm. <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>): δ 103.7, 104.4, 126.4, 128.0, 129.7, 130.4, 130.7, 131.8, 134.0, 138.4, 158.2, 163.0, 164.0 ppm.

**5.1.3.3. 5-Amino-3-(3-chlorophenyl)-4-oxo-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxylic acid (36).** Yield: 89%. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>): δ 7.13 (s, 1H), 7.44 (d, *J* = 8.4 Hz, 1H), 7.50 (t, *J* = 8.5 Hz, 1H), 7.55 (d, *J* = 8.4 Hz, 1H), 7.65–7.68 (m, 3H) ppm. <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>): δ 104.3, 104.7, 125.2, 126.4, 126.8, 127.6, 130.6, 133.1, 142.6, 148.9, 157.7, 158.9, 164.0 ppm. IR: ν 3439, 3322, 3065 (br, acid band), 1712, 1646, 1590 cm<sup>−1</sup>. MS [ESI]<sup>+</sup>: Calcd for C<sub>13</sub>H<sub>9</sub>ClN<sub>3</sub>O<sub>3</sub>S<sup>+</sup> 322.00. Found 322.10.

**5.1.3.4. 5-Amino-3-(4-chlorophenyl)-4-oxo-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxylic acid (37).** See Ref. 7.

**5.1.3.5. 5-Amino-4-oxo-3-(4-(trifluoromethyl)phenyl)-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxylic acid (38).** Yield: 81%. <sup>1</sup>H NMR (CD<sub>3</sub>OD): δ 7.21 (s, 1H), 7.76 (d, *J* = 8.3 Hz, 2H), 7.86 (d, *J* = 8.3 Hz, 2H) ppm.

**5.1.3.6. 5-Amino-4-oxo-3-(4-(trifluoromethoxy)phenyl)-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxylic acid (39).** Yield: 51%. <sup>1</sup>H NMR (CD<sub>3</sub>OD): δ 7.17 (s, 1H), 7.35 (d, *J* = 8.5 Hz, 3H), 7.69 (d, *J* = 8.9 Hz, 3H) ppm. IR (film): ν 3428, 3329, 1725, 1637, 1598 cm<sup>−1</sup>. <sup>13</sup>C NMR (CD<sub>3</sub>OD): δ 102.7, 103.1, 118.1, 119.3, 120.2, 125.0, 126.1, 132.8, 138.1, 146.4, 157.9, 162.4, 162.8 ppm.

**5.1.3.7. 5-Amino-3-(4-hydroxyphenyl)-4-oxo-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxylic acid (40).** Yield: 76%. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>): δ 6.82 (d, *J* = 8.6 Hz, 2H), 7.09 (s, 1H), 7.26 (d, *J* = 8.6 Hz, 2H), 7.55 (s, 2H), 9.63 (s, 1H) ppm. IR (film): ν 3425 (br, acid band), 1725, 1635 cm<sup>−1</sup>.

#### 5.1.4. General procedure for the synthesis of compounds of general structure F

To a mixture of the appropriate acid of general structure E (0.15 mmol), the appropriate amine (0.22 mmol), and benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (0.097 g, 0.22 mmol) in anhydrous dimethylsulfoxide (2 mL), *N,N*-diisopropyl-ethylamine (0.028 g, 38 μL, 0.23 mmol) was added. The reaction mixture was stirred at room temperature for 4 h. The reaction was quenched with water and extracted with ethyl acetate. The combined organic extracts were washed with brine, dried over MgSO<sub>4</sub>, filtered, and concentrated in vacuo. Purification by preparative reverse phase HPLC furnished the desired amide of general structure F.

**5.1.4.1. 5-Amino-3-(4-fluorophenyl)-*N*-isopropyl-4-oxo-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxamide (42).** Yield: 74%. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 1.24 (d, *J* = 7.0 Hz, 6H), 4.17–4.24 (m, 1H), 5.83 (br s, 2H), 6.89 (br s, 1H), 7.15–7.18 (m, 2H), 7.50–7.58 (m, 3H) ppm. <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 22.8, 41.5, 106.6, 106.9, 115.7 (d, *J*<sub>CF</sub><sup>2</sup> = 22.6 Hz), 126.8, 128.0 (d, *J*<sub>CF</sub><sup>3</sup> = 8.5 Hz), 135.5, 136.8, 159.7, 161.5, 161.7 (d, *J*<sub>CF</sub><sup>1</sup> = 245.5 Hz), 162.0 ppm. IR (film): ν 3405, 3301, 1651, 1598 cm<sup>−1</sup>. MS [ESI]<sup>+</sup>: Calcd for C<sub>16</sub>H<sub>16</sub>FN<sub>4</sub>O<sub>2</sub>S<sup>+</sup> 347.10. Found 347.11.

**5.1.4.2. 5-Amino-*N*-cyclopropyl-3-(4-fluorophenyl)-4-oxo-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxamide (43).** Yield: 71%. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>): δ 0.54–0.72 (m, 4H), 2.74–2.81 (m, 1H), 7.20 (s, 1H), 7.23–7.31 (m, 2H), 7.53–7.71 (m, 4H), 8.25 (br s, 1H) ppm. <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>): δ 6.3, 23.2, 104.6, 104.9, 115.5 (d, *J*<sub>CF</sub><sup>2</sup> = 22.2 Hz), 126.6, 128.7 (d, *J*<sub>CF</sub><sup>3</sup> = 9.2 Hz), 136.7, 137.5, 159.0,

161.2 (d, *J*<sub>CF</sub><sup>1</sup> = 246.7 Hz), 163.4, 164.4 ppm. IR (film): ν 3298, 3105 (br band), 1660, 1643, 1587 cm<sup>−1</sup>. MS [ESI]<sup>+</sup>: Calcd for C<sub>16</sub>H<sub>14</sub>FN<sub>4</sub>O<sub>2</sub>S<sup>+</sup> 345.08. Found 345.14.

**5.1.4.3. 5-Amino-3-(4-fluorophenyl)-4-oxo-*N*-(2,2,2-trifluoroethyl)-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxamide (44).** Yield 54%; <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 4.04 (qd, *J* = 8.9, 6.8 Hz, 2H), 6.19 (s, 2H), 7.16–7.19 (m, 2H), 7.38 (t, *J* = 6.4 Hz, 1H), 7.52–7.49 (m, 2H), 7.55 (s, 1H) ppm. <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 40.4, 40.7, 106.5, 106.8, 115.79, 115.97, 123.0, 126.4, 127.92, 127.99, 134.2, 136.48, 136.51, 159.6, 160.9, 161.6, 162.92, 162.97 ppm. HRMS [ESI]<sup>+</sup>: Calcd for C<sub>15</sub>H<sub>9</sub>F<sub>4</sub>N<sub>4</sub>O<sub>2</sub>S<sup>+</sup> 385.0382. Found 385.0397.

**5.1.4.4. 5-Amino-3-(2-chlorophenyl)-*N*-cyclopropyl-4-oxo-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxamide (45).** Yield 62%; <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 0.59–0.62 (m, 2H), 0.82 (dd, *J* = 6.9, 5.4 Hz, 2H), 2.79 (m, 1H), 6.12 (bs, 2H), 7.12 (d, *J* = 4.2 Hz, 1H), 7.46–7.40 (m, 3H), 7.56 (dd, *J* = 5.8, 3.5 Hz, 1H), 7.64 (s, 1H) ppm. <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 6.6, 22.5, 106.8, 107.1, 126.7, 127.7, 130.06, 130.23, 130.5, 132.9, 135.3, 138.2, 159.5, 161.3, 164.3 ppm. HRMS [ESI]<sup>+</sup>: Calcd for C<sub>16</sub>H<sub>13</sub>N<sub>4</sub>O<sub>2</sub>SCl<sup>+</sup> 361.0526. Found 361.0537.

**5.1.4.5. 5-Amino-3-(3-chlorophenyl)-*N*-isopropyl-4-oxo-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxamide (46).** Yield: 53%. <sup>1</sup>H NMR (CD<sub>3</sub>OD): δ 1.27 (d, *J* = 6.6 Hz, 6H), 4.12–4.16 (m, 1H), 7.28 (s, 1H), 7.38 (dt, *J* = 8.0, 0.9 Hz, 1H), 7.46 (t, *J* = 8.0 Hz, 1H), 7.58 (dt, *J* = 8.0, 0.9 Hz, 1H), 7.71 (s, 1H) ppm. <sup>13</sup>C NMR (CD<sub>3</sub>OD): δ 19.7, 40.0, 103.13, 103.28, 122.9, 124.6, 124.9, 125.6, 128.1, 132.2, 135.2, 140.6, 157.9, 161.4, 162.2 ppm. IR (film): ν 3413, 3305, 2971, 1652, 1591 cm<sup>−1</sup>. MS [ESI]<sup>+</sup>: Calcd for C<sub>16</sub>H<sub>16</sub>ClN<sub>4</sub>O<sub>2</sub>S<sup>+</sup> 363.84. Found 363.16.

**5.1.4.6. 5-Amino-3-(4-chlorophenyl)-*N*-cyclopropyl-4-oxo-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxamide (1).** See Ref. 7.

**5.1.4.7. 5-Amino-*N*-cyclopropyl-4-oxo-3-(4-(trifluoromethyl)phenyl)-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxamide (47).** Yield: 53%. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>): δ 0.57–0.60 (m, 2H), 0.66–0.70 (m, 2H), 2.79 (m, 1H), 7.21 (s, 1H), 7.64 (s, 2H), 7.80 (d, *J* = 8.6 Hz, 2H), 7.91 (d, *J* = 8.5 Hz, 2H), 8.32 (d, *J* = 4.1 Hz, 1H) ppm. <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>): δ 6.4, 23.2, 104.3, 105.5, 125.85, 125.88, 125.91, 126.4, 126.8, 137.4, 144.6, 158.9, 164.0, 164.3 ppm. MS [ESI]<sup>+</sup>: Calcd for C<sub>17</sub>H<sub>14</sub>F<sub>3</sub>N<sub>4</sub>O<sub>2</sub>S<sup>+</sup> 395.08. Found 394.98.

**5.1.4.8. 5-Amino-*N*-isopropyl-4-oxo-3-(4-(trifluoromethoxy)phenyl)-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxamide (48).** Yield 50%; <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 1.25 (d, *J* = 6.6 Hz, 6H), 4.18–4.25 (m, 1H), 6.19 (s, 2H), 6.89 (d, *J* = 7.9 Hz, 1H), 7.34 (d, *J* = 8.2 Hz, 2H), 7.60–7.62 (m, 3H) ppm. <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 22.8, 41.6, 106.89, 106.98, 121.4, 126.7, 127.4, 135.8, 139.2, 159.6, 161.5, 161.9 ppm. IR (film): ν 3411, 3281, 3176, 1647 cm<sup>−1</sup>. MS [ESI]<sup>+</sup>: Calcd for C<sub>17</sub>H<sub>16</sub>F<sub>3</sub>N<sub>4</sub>O<sub>3</sub>S<sup>+</sup> 413.09. Found 413.20.

**5.1.4.9. 5-Amino-3-(4-hydroxyphenyl)-*N*-isopropyl-4-oxo-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxamide (49).** Yield: 49%. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>): δ 1.14 (d, *J* = 6.6 Hz, 6H), 4.02–4.07 (m, 1H), 6.82 (d, *J* = 8.8 Hz, 2H), 7.16 (s, 1H), 7.34 (d, *J* = 8.8 Hz, 2H), 7.50 (s, 2H), 7.85 (d, *J* = 8.1 Hz, 1H), 9.61 (s, 1H) ppm. IR (film): ν 3429, 3323, 3269, 2975, 1640, 1507 cm<sup>−1</sup>. MS [ESI]<sup>+</sup>: Calcd for C<sub>16</sub>H<sub>17</sub>N<sub>4</sub>O<sub>3</sub>S<sup>+</sup> 345.10. Found 345.23.

**5.1.4.10. 5-Amino-*N*-cyclopropyl-3-(4-isopropylphenyl)-4-oxo-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxamide (50).** Yield: 55%. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>): δ 0.57–0.60 (m, 2H), 0.65–0.69 (m, 2H), 2.79 (td, *J* = 7.3, 4.0 Hz, 1H), 2.94 (dt, *J* = 13.8,

6.9 Hz, 1H), 7.19 (s, 1H), 7.30 (d,  $J = 8.4$  Hz, 2H), 7.49 (d,  $J = 8.4$  Hz, 2H), 7.54 (s, 2H), 8.20 (d,  $J = 4.3$  Hz, 1H) ppm.  $^{13}\text{C}$  NMR (DMSO- $d_6$ ):  $\delta$  6.3, 23.2, 24.5, 33.8, 104.64, 104.81, 126.58, 126.68, 126.75, 136.5, 139.1, 147.8, 159.1, 163.3, 164.5 ppm. MS [ESI] $^+$ : Calcd for  $\text{C}_{19}\text{H}_{19}\text{N}_4\text{O}_2\text{S}^+$  369.14. Found 369.13.

**5.1.4.11. Ethyl 4-acetyl-2-aminothiophene-3-carboxylate (53).** To a mixture of 2,3-butanedione (20 mg, 20  $\mu\text{L}$ , 0.23 mmol), ethyl cyanoacetate (26 mg, 25  $\mu\text{L}$ , 0.23 mmol), and sulfur (11 mg, 0.35 mmol) in *N,N*-dimethylformamide (0.5 mL) morpholine (40 mg, 40  $\mu\text{L}$ , 0.46 mmol) was added. The reaction mixture was stirred at rt for 16 h. A saturated solution of sodium chloride was added and the resulting mixture was extracted with ethyl acetate. The combined organic extracts were washed with brine, dried over sodium sulfate, filtered and concentrated in vacuo. Crystallization from acetate-hexanes furnished **53**. Yield: 32%, mp: 105–106 °C.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.32 (t,  $J = 7.2$  Hz, 3H), 2.42 (s, 3H), 4.28 (q,  $J = 7.2$  Hz, 2H), 6.07 (br s, 2H), 6.42 (s, 1H) ppm.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  14.2, 30.6, 60.5, 104.6, 109.3, 143.0, 163.7, 164.7, 198.6 ppm. IR (film):  $\nu$  3429, 3324, 1677, 1593  $\text{cm}^{-1}$ . MS [ESI] $^+$ : Calcd for  $\text{C}_9\text{H}_{12}\text{NO}_3\text{S}^+$  214.05. Found 214.22.

**5.1.4.12. Ethyl 2-amino-4-benzoylthiophene-3-carboxylate (54).** A mixture of the 1-phenylpropane-1,2-dione (500 mg, 0.45 mL, 3.37 mmol), sulfur (162 mg, 5.05 mmol), ethyl cyanoacetate (382 mg, 0.36 mL, 3.37 mmol), and triethylamine (682 mg, 940 mL, 6.00 mmol) in *N,N*-dimethylformamide (10 mL) was stirred at rt for 12 h. The reaction was quenched with water and extracted with ethyl acetate. The combined organic extracts were washed with brine, dried over sodium sulfate, filtered and concentrated in vacuo. Purification by silica gel column chromatography using ethyl acetate-hexanes 2:3 as eluant provided **54**. Yield: 95%, mp: 103–105 °C.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  0.78 (t,  $J = 7.2$  Hz, 3H), 3.90 (q,  $J = 7.2$  Hz, 2H), 5.95 (br s, 2H), 6.44 (s, 1H), 7.42 (t,  $J = 7.7$  Hz, 2H), 7.53 (t,  $J = 7.2$  Hz, 1H), 7.84 (d,  $J = 8.5$  Hz, 2H) ppm.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  13.5, 60.1, 106.0, 109.3, 128.4, 129.4, 133.0, 138.0, 140.0, 163.5, 164.7, 193.0 ppm. IR (film):  $\nu$  3431, 3327, 1667, 1595  $\text{cm}^{-1}$ .

#### 5.1.5. General procedure for the synthesis of compounds of general structure G

A mixture of the appropriate aminothiophene keto-ester (**53** or **54**, 0.23 mmol), the appropriate hydrazine (0.23 mmol) in ethanol was stirred for 0.5–3 h at 150 °C (microwave irradiation). After cooling, the solvent was removed and the residue was purified by either preparative reverse phase HPLC, or silica gel column chromatography using gradients of ethyl acetate in hexanes 1:3 as eluent, to give the desired compound of general structure G.

**5.1.5.1. 7-Amino-4-methylthieno[3,4-*d*]pyridazin-1(2H)-one (55).** Yield: 77%.  $^1\text{H}$  NMR (DMSO- $d_6$ ):  $\delta$  2.18 (s, 3H), 6.62 (s, 1H), 7.26 (br s, 2H), 11.04 (br s, 1H) ppm.  $^{13}\text{C}$  NMR (DMSO- $d_6$ ):  $\delta$  31.3, 101.4, 104.9, 31.8, 141.5, 160.9, 162.0 ppm. IR:  $\nu$  3422, 3260, 1642  $\text{cm}^{-1}$ . MS [ESI] $^+$ : Calcd for  $\text{C}_7\text{H}_8\text{N}_3\text{OS}^+$  182.04. Found 182.20.

**5.1.5.2. 7-Amino-2,4-dimethylthieno[3,4-*d*]pyridazin-1(2H)-one (56).** Yield: 79%, mp: 158–160 °C.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  2.29 (s, 3H), 3.57 (s, 3H), 6.13 (br s, 2H), 6.36 (s, 1H) ppm.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  18.4, 36.6, 100.9, 107.3, 132.3, 141.5, 159.9, 160.1 ppm. IR:  $\nu$  3386, 3266, 1604  $\text{cm}^{-1}$ . MS [ESI] $^+$ : Calcd for  $\text{C}_8\text{H}_{10}\text{N}_3\text{OS}^+$  196.05. Found 196.17.

**5.1.5.3. 7-Amino-2-isopropyl-4-methylthieno[3,4-*d*]pyridazin-1(2H)-one (57).** Yield: 59%.  $^1\text{H}$  NMR (DMSO- $d_6$ ):  $\delta$  1.20 (d,  $J = 6.7$  Hz, 6H), 2.22 (s, 3H), 5.01 (7,  $J = 6.7$  Hz, 1H), 6.61 (s, 1H), 7.31 (s, 2H) ppm.  $^{13}\text{C}$  NMR (DMSO- $d_6$ ):  $\delta$  19.0, 21.5, 45.2, 100.8,

105.0, 131.4, 141.2, 159.0, 162.0 ppm. MS [ESI] $^+$ : Calcd for  $\text{C}_{10}\text{H}_{14}\text{N}_3\text{OS}^+$  224.08. Found 224.00.

**5.1.5.4. 7-Amino-4-methyl-2-(2,2,2-trifluoroethyl)thieno[3,4-*d*]pyridazin-1(2H)-one (58).** Yield: 47%.  $^1\text{H}$  NMR (DMSO- $d_6$ ):  $\delta$  2.23 (s, 3H), 4.65 (q,  $J = 9.2$  Hz, 2H), 6.75 (s, 1H), 7.46 (s, 2H) ppm.  $^{13}\text{C}$  NMR (DMSO- $d_6$ ):  $\delta$  18.5, 48.13 (d,  $J_{\text{CF}} = 33.1$  Hz), 103.0, 103.4, 131.0, 142.9, 159.5, 163.2 ppm. HRMS [ESI] $^-$ : Calcd for  $\text{C}_9\text{H}_7\text{N}_3\text{OF}_3\text{S}^-$  262.0262. Found 262.0272. X-ray crystal structure shown in [Supplementary data](#).

**5.1.5.5. 7-Amino-2-cyclohexyl-4-methylthieno[3,4-*d*]pyridazin-1(2H)-one (59).** Yield: 64%.  $^1\text{H}$  NMR (DMSO- $d_6$ ):  $\delta$  1.10–1.18 (m, 1H), 1.34 (qt,  $J = 13.1$ , 3.4 Hz, 2H), 1.61–1.73 (m, 6H), 1.79 (d,  $J = 13.3$  Hz, 2H), 2.22 (s, 3H), 4.58 (m, 1H), 6.61 (s, 1H), 7.32 (s, 2H) ppm.  $^{13}\text{C}$  NMR (DMSO- $d_6$ ):  $\delta$  18.9, 25.7, 26.0, 31.5, 53.0, 100.7, 105.0, 131.3, 140.9, 159.0, 162.1 ppm. MS [ESI] $^+$ : Calcd for  $\text{C}_{13}\text{H}_{18}\text{N}_3\text{OS}^+$  264.12. Found 264.07.

**5.1.5.6. 7-Amino-4-methyl-2-(pyridin-2-yl)thieno[3,4-*d*]pyridazin-1(2H)-one (60).** Yield: 53%, mp: 227–229 °C.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 2.41 (s, 3H), 6.02 (br s, 2H), 6.46 (s, 1H), 7.29 (t,  $J = 8.5$  Hz, 1H), 7.66 (d,  $J = 8.0$  Hz, 1H), 7.83 (t,  $J = 8.5$  Hz, 1H), 8.66 (d,  $J = 4.0$  Hz, 1H) ppm.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  18.7, 102.2, 106.5, 121.5, 122.5, 131.7, 138.0, 143.3, 148.9, 153.0, 159.8, 161.8 ppm. IR:  $\nu$  3415, 3113, 1637, 1594  $\text{cm}^{-1}$ . MS [ESI] $^+$ : Calcd for  $\text{C}_{12}\text{H}_{11}\text{N}_4\text{OS}^+$  259.06. Found 259.11.

**5.1.5.7. 7-Amino-4-methyl-2-(pyridin-3-yl)thieno[3,4-*d*]pyridazin-1(2H)-one (61).** Yield: 43%.  $^1\text{H}$  NMR (DMSO- $d_6$ ):  $\delta$  2.29 (s, 3H), 6.80 (s, 1H), 7.45–7.52 (m, 3H), 7.95 (dd,  $J = 8.1$ , 1.3 Hz, 1H), 8.46 (d,  $J = 0.7$  Hz, 1H), 8.78 (d,  $J = 0.8$  Hz, 1H) ppm.  $^{13}\text{C}$  NMR (DMSO- $d_6$ ):  $\delta$  18.7, 102.9, 103.9, 123.9, 131.0, 133.2, 143.5, 146.9, 147.4, 149.9, 159.2, 163.7 ppm. MS [ESI] $^+$ : Calcd for  $\text{C}_{12}\text{H}_{11}\text{N}_4\text{OS}^+$  259.06. Found 259.05.

**5.1.5.8. 7-Amino-2-benzyl-4-methylthieno[3,4-*d*]pyridazin-1(2H)-one (62).** Yield: 97%, mp: 123–125 °C.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 2.30 (s, 3H), 5.17 (s, 2H), 5.99 (br s, 2H), 6.35 (s, 1H), 7.25 (t,  $J = 8.5$  Hz, 1H), 7.32 (t,  $J = 8.2$  Hz, 2H), 7.41 (d,  $J = 8.5$  Hz, 2H) ppm.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  18.5, 52.2, 101.0, 107.3, 127.4, 128.2, 128.6, 132.3, 138.1, 141.9, 159.9, 160.3 ppm. IR (film):  $\nu$  3397, 3112, 1600  $\text{cm}^{-1}$ . MS [ESI] $^+$ : Calcd for  $\text{C}_{14}\text{H}_{14}\text{N}_3\text{OS}^+$  272.09. Found 272.17.

**5.1.5.9. 7-Amino-2-(4-chlorophenyl)-4-methylthieno[3,4-*d*]pyridazin-1(2H)-one (63).** Yield: 73%.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  2.38 (s, 3H), 6.09 (br s, 2H), 6.47 (s, 1H), 7.40 (d,  $J = 9.0$  Hz, 2H), 7.56 (d,  $J = 9.0$  Hz, 2H) ppm.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  18.6, 101.7, 107.2, 127.1, 128.8, 132.1, 132.5, 139.8, 142.7, 159.6, 162.3 ppm. IR:  $\nu$  3411, 3329, 1633, 1590  $\text{cm}^{-1}$ . MS [ESI] $^+$ : Calcd for  $\text{C}_{13}\text{H}_{11}\text{ClN}_3\text{OS}^+$  292.03. Found 292.12.

**5.1.5.10. 7-Amino-4-phenyl-2-(2,2,2-trifluoroethyl)thieno[3,4-*d*]pyridazin-1(2H)-one (64).** Yield: 46%.  $^1\text{H}$  NMR (DMSO- $d_6$ ):  $\delta$  4.77 (q,  $J = 9.2$  Hz, 2H), 6.69 (s, 1H), 7.51–7.53 (m, 3H), 7.59 (s, 2H), 7.67–7.69 (m, 2H) ppm.  $^{13}\text{C}$  NMR (DMSO- $d_6$ ):  $\delta$  48.2, 48.5, 103.8, 104.3, 128.4, 128.9, 129.3, 130.1, 135.4, 144.5, 159.2, 163.6 ppm. HRMS [ESI] $^+$ : Calcd for  $\text{C}_{14}\text{H}_{11}\text{N}_3\text{OF}_3\text{S}^+$  326.0575. Found 326.0571.

**5.1.5.11. 7-Amino-2-isopropyl-4-phenylthieno[3,4-*d*]pyridazin-1(2H)-one (65).** Yield: 36%.  $^1\text{H}$  NMR (DMSO- $d_6$ ):  $\delta$  1.27 (d,  $J = 6.6$  Hz, 6H), 5.11 (dt,  $J = 13.3$ , 6.6 Hz, 1H), 6.62 (s, 1H), 7.51–7.45 (m, 5H), 7.71 (dd,  $J = 7.9$ , 1.5 Hz, 2H) ppm.  $^{13}\text{C}$  NMR (DMSO- $d_6$ ):  $\delta$  21.6, 45.8, 101.9, 105.4, 128.3, 129.13, 129.21, 129.6, 136.5, 142.5, 158.8, 162.5 ppm. MS [ESI] $^+$ : Calcd for  $\text{C}_{15}\text{H}_{16}\text{N}_3\text{OS}^+$  286.10. Found 285.96.

**5.1.5.12. Ethyl 3-(4-chlorophenyl)-5-(methylamino)-4-oxo-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxylate (66).**

A mixture of **29**, potassium carbonate and iodometane in acetonitrile was stirred at 70 °C for 10 h. The reaction was quenched by addition of water and then extracted with ethyl acetate. The combined organic extracts were washed with brine, dried over MgSO<sub>4</sub>, filtered and concentrated in vacuo. Purification by silica gel column chromatography using ethyl acetate/hexanes 1:3 as eluant provided **68**. Yield 27%; <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 1.43 (t, *J* = 7.0 Hz, 3H), 3.07 (d, *J* = 5.5 Hz, 3H), 4.44 (q, *J* = 7.0 Hz, 2H), 7.19 (s, 1H), 7.42 (d, *J* = 9.0 Hz, 2H), 7.54 (bs, 1H), 7.58 (d, *J* = 9.0 Hz, 2H) ppm. <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 14.39, 33.99, 62.05, 102.83, 104.32, 127.07, 127.60, 128.82, 133.07, 134.08, 139.34, 159.39, 163.15, 165.50 ppm. IR (film): ν 3370, 2923, 1718, 1653, 1567 cm<sup>-1</sup>. MS [ESI]<sup>+</sup>: Calcd for C<sub>16</sub>H<sub>15</sub>ClN<sub>3</sub>O<sub>3</sub>S<sup>+</sup> 364.05. Found 364.07.

**5.1.5.13. Ethyl 5-amino-7-chloro-3-(4-chlorophenyl)-4-oxo-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxylate (67).**

A mixture **30** (30 mg, 0.086 mmol) and *N*-chlorosuccinimide (11 mg, 0.086 mmol) in anhydrous *N,N*-dimethylformamide (0.5 mL) was heated to 80 °C for 30 minutes. After cooling, the reaction was quenched with water and extracted with ethyl acetate. The combined organic extracts were washed with brine, dried over sodium sulfate, filtered and concentrated in vacuo. Purification by preparative reverse phase HPLC furnished **50**. Yield: 79%. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 1.42 (t, *J* = 7.5 Hz, 3H), 4.45 (q, *J* = 7.2 Hz, 2H), 6.25 (br s, 2H), 7.40 (d, *J* = 9.0 Hz, 2H), 7.51 ppm (d, *J* = 9.0 Hz, 2H). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 14.1, 62.8, 105.7, 106.7, 122.7, 127.1, 128.9, 133.3, 136.2, 138.7, 158.5, 159.1, 162.9 ppm. IR (film): ν 3435, 3324, 1725, 1656, 1594 cm<sup>-1</sup>. MS [ESI]<sup>+</sup>: Calcd for C<sub>15</sub>H<sub>12</sub>Cl<sub>2</sub>N<sub>3</sub>O<sub>3</sub>S<sup>+</sup> 384.00. Found 384.01.

**5.1.5.14. Ethyl 5-amino-7-bromo-3-(4-chlorophenyl)-4-oxo-3,4-dihydrothieno[3,4-d]pyridazine-1-carboxylate (68).**

Prepared as **67** from **29** and *N*-bromosuccinimide. Yield: 75%. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 1.43 (t, *J* = 7.1 Hz, 3H), 4.46 (q, *J* = 7.2 Hz, 2H), 6.34 (br s, 2H), 7.40 (d, *J* = 8.5 Hz, 2H), 7.52 (d, *J* = 9.0 Hz, 2H) ppm. IR: ν 3425, 3317, 1732, 1656, 1589 cm<sup>-1</sup>. MS [ESI]<sup>+</sup>: Calcd for C<sub>15</sub>H<sub>12</sub>BrClN<sub>3</sub>O<sub>3</sub>S<sup>+</sup> 427.95. Found 427.73.

**5.1.5.15. Ethyl 2-(4-chlorophenyl)-5-hydroxy-1,7-dioxo-2,5,6,7-tetrahydro-1H-pyrrolo[3,4-d]pyridazine-4-carboxylate (69).**

A solution of **29** in acetone (2 mL) was added at room temperature with a freshly prepared solution of dimethyldioxirane in acetone (0.07–0.09 M solution prepared as described in Organic Syntheses, Coll. Vol 9, p.288 (1998); Vol 74, p.91 (1997)). The reaction was monitored by TLC and after ~20 minutes the starting material was completely consumed. The reaction mixture was then evaporated to dryness and the resulting material purified by preparative HPLC obtaining **71**. Yield: 20%. <sup>1</sup>H NMR tautomeric mixture (DMSO-*d*<sub>6</sub>): δ 1.31 (t, *J* = 7.1 Hz, 3H), 3.30 (d, *J* = 11.8 Hz, 1H), 4.33–4.40 (m, 2H), 6.12 (d, *J* = 7.1 Hz, 1H), 6.65 (d, *J* = 8.6 Hz, 0.5H), 7.60–7.65 (m, 4H), 9.22 (s, 0.5H) ppm. IR (film): ν 3359, 2923, 2852, 1734 cm<sup>-1</sup>. HRMS [ESI]<sup>+</sup>: Calcd for C<sub>15</sub>H<sub>12</sub>ClN<sub>3</sub>NaO<sub>5</sub><sup>+</sup> 372.0363. Found 372.0374. See X-ray crystal structure report in the [Supplementary data](#).

**5.1.5.16. 3-(4-Fluorophenyl)-4,5-dioxo-3,4,5,7-tetrahydrothieno[3,4-d]pyridazine-1-carboxylic acid (70).**

<sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>): δ 4.93 (s, 2H), 7.39–7.43 (m, 2H), 7.62–7.64 (m, 2H) ppm. MS [ESI]<sup>+</sup>: Calcd for C<sub>13</sub>H<sub>8</sub>FN<sub>2</sub>O<sub>4</sub>S<sup>+</sup> 307.02. Found 306.97.

**5.1.5.17. 3-(4-Chlorophenyl)-4,5-dioxo-3,4,5,7-tetrahydrothieno[3,4-d]pyridazine-1-carboxylic acid (71).**

To a solution of **41** (50 mg, 0.15 mmol) in acetonitrile (1 mL) 1 N hydrochloric acid (1.5 mL) was added. The reaction mixture was stirred at 130 °C (microwave irradiation) for 30 minutes. Water was then added

and the desired product was filtered and washed with water. Yield: 58%. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>): δ 4.93 (s, 2H), 7.61–7.66 (m, 4H) ppm.

**5.1.5.18. 3-(4-Chlorophenyl)-*N*-isopropyl-4,5-dioxo-3,4,5,7-tetrahydrothieno[3,4-d]pyridazine-1-carboxamide (72).**

Prepared following the general procedure described for the synthesis of compounds of general structure F. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>): δ 1.17 (d, *J* = 6.6 Hz, 6H), 4.07–4.13 (m, 1H), 4.96 (s, 2H), 7.62–7.65 (m, 2H), 7.70–7.72 (m, 2H), 8.36 (d, *J* = 8.2 Hz, 1H) ppm. <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>): δ 22.6, 35.1, 41.6, 114.4, 128.8, 129.4, 131.1, 133.9, 136.8, 155.3, 157.5, 161.2 ppm.

**5.1.6. Pharmacokinetic studies**

Test compounds were administered to 2-month old female B6C3F<sub>1</sub> mice (20–25 g average body weight). Typical studies involved i.p. injection of test ATPZ at a dose of 5 mg/kg, followed by sacrifice 1 h later. For full PK analysis of compound **43**, mice (*n* = 3/time point) received i.p. injections at a dose of 5 mg/kg, followed by sacrifice at times ranging from 0.5–16 h post-dosing. Perfused whole brain hemispheres, obtained from mice euthanized according to protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee, were homogenized in 10 mM ammonium acetate, pH 5.7 (50% w/v), using a hand-held sonic homogenizer. Plasma was obtained from blood that was collected into a 1.5 mL tube containing 0.5 M EDTA solution and subjected to centrifugation for 10 min at 4500g at 4 °C. Aliquots (50 μL) of brain homogenate or plasma were mixed with 0.2 mL of acetonitrile and centrifuged at 15000g, and the resulting supernatants were used for subsequent LC–MS/MS analysis. The LC–MS/MS system comprised an Aquity UPLC and a TQ MS that was controlled using MassLynx software (Waters Corporation, Milford, MA). Compounds were detected using multiple reaction monitoring (MRM) of their specific collision-induced ion transitions. Samples (5 μL) were separated on an Aquity BEH C18 column (1.7 μm, 2.1 mm × 50 mm) at 35 °C. Operation was in positive electrospray ionization mode, with mobile phase A of 0.1% (v/v) formic acid and mobile phase B of acetonitrile with 0.1% (v/v) formic acid at a flow rate of 0.6 mL/min using a gradient from 5% to 95% B over 2 min, followed by wash and re-equilibration steps. The mass spectrometer was operated with a desolvation temperature of 450 °C and a source temperature of 150 °C. Desolvation and source nitrogen gas flows were 900 and 50 L/h, respectively. Source and MS/MS voltages were optimized for each compound using the MassLynx autotune utility. Standard curves were generated for each compound from brain homogenate and plasma samples and extracted as above. Peak areas were plotted against concentration and a linear regression curve was used to quantify the unknowns. In all cases, the tissue-derived sample peak areas fell within the linear portion of standard curves that were prepared and analyzed concurrently with the samples.

**5.1.7. Determination of unbound fraction in plasma and brain homogenates**

The unbound fractions of drug in mouse plasma and brain were determined in vitro using rapid equilibrium dialysis (Pierce Biotechnology, Rockford, IL). Test compounds were mixed at a concentration of 1 μM with mouse plasma or brain homogenate that was diluted threefold with 100 mM sodium phosphate, pH 7.4. Triplicate 100 μL aliquots were placed in sample chambers and dialyzed against 300 μL of 100 mM sodium phosphate, pH 7.4, for 5 h at 37 °C with shaking at 100 rpm. After incubation, 20 μL aliquots were removed from each side of the dialysis membrane and an equal volume of plasma, brain homogenate, or buffer added in order to create identical matrix compositions. Compounds were subsequently extracted with 120 μL acetonitrile, vortexed and centrifuged at 15,000g for 15 min. Supernatants were analyzed

by LC–MS/MS. Unbound fraction ( $f_u$ ) was calculated as the ratio of the buffer chamber response to the sample chamber response. Brain unbound fraction was calculated using the following equation, adjusting for tissue dilution (D).

$$\text{Undiluted } f_u = (1/D) / [(1/f_{u,\text{measured}}) - 1 + (1/D)]$$

### 5.1.8. Mouse tolerability studies

To determine the maximum tolerated dose of ATPZ **43**, 2-month old female B6C3F1 mice ( $n = 5$ ) were administered compound by oral gavage (in 0.5% methylcellulose) starting at 1 mg/kg. The mice were carefully monitored for the next 48 h for signs of ataxia, lethargy, piloerection, salivation or other unusual behaviors. If no signs of intolerance were observed, dosing was increased in gradual steps (2–3 fold increases) until a noticeable change occurred in at least 2 mice. For the 1-month tolerability testing of compound **43**, groups of 2-month old female B6C3F1 mice ( $n = 5$ ) were administered compound in drinking water containing 3% PEG400 and 0.5% methylcellulose. Control mice received the vehicle only. Water consumption was monitored, and normal water intake was observed (~5 ml/mouse/day). At the completion of dosing, body weights were determined. The mice then underwent perfusion and euthanization as described above, and organs weights were recorded and blood was collected for complete blood cell counts. Samples of plasma and brain tissue were also kept for LC–MS/MS analysis of the levels of compound **43**.

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### Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmc.2012.05.027>.

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