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Brain-Penetrant, Orally Bioavailable Microtubule-Stabilizing Small Molecules Are Potential Candidate Therapeutics for Alzheimer's Disease and Related Tauopathies

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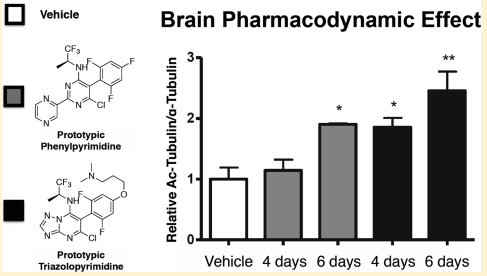
Supporting Information

ABSTRACT: Microtubule (MT) stabilizing drugs hold promise as potential treatments for Alzheimer's disease (AD) and related tauopathies. However, thus far epothilone D has been the only brain-penetrant MT-stabilizer to be evaluated in tau transgenic mice and in AD patients. Furthermore, this natural product exhibits potential deficiencies as a drug candidate, including an intravenous route of administration and the inhibition of the P-glycoprotein (Pgp) transporter. Thus, the identification of alternative CNS-active MT-stabilizing agents that lack these potential limitations is of interest. Toward this objective, we have evaluated representative compounds from known classes of non-naturally occurring MT-stabilizing small molecules. This led to the identification of selected triazolopyrimidines and phenylpyrimidines that are orally bioavailable and brain-penetrant without disruption of Pgp function. Pharmacodynamic studies confirmed that representative compounds from these series enhance MT-stabilization in the brains of wild-type mice. Thus, these classes of MT-stabilizers hold promise for the development of orally active, CNS-directed MT-stabilizing therapies.

Repurposing anti-cancer small molecules for neurodegenerative tauopathies



INTRODUCTION

Neurodegenerative tauopathies, of which Alzheimer's disease (AD) is the most prominent and common example, are characterized by the misfolding and aggregation of the microtubule (MT) associated protein tau.[1] Normally, tau binds to and stabilizes MTs, thereby maintaining the network of MTs essential for axonal transport in neurons.[2] In AD and related diseases, tau becomes hyperphosphorylated, thereby promoting disengagement from MTs and sequestration into characteristic aggregates known as neurofibrillary tangles (NFTs) and neuropil threads.[1] The resulting loss of the normal MT-stabilizing function of tau is believed to perturb the physiological dynamics of MTs. In neuronal axons, MTs are more stable than in other cell types, and hyperdynamic, less organized MTs would likely result in defective axonal transport, with deleterious effects for the affected neurons.[3] Previous studies[4−6] have shown that exogenous MT-stabilizing agents can compensate for loss of tau function in models of tauopathy and thus may provide a viable therapeutic strategy for the treatment of AD and related tauopathies. Indeed, treatment of aged tau transgenic (Tg) mice, which manifest NFT-like inclusions, with low weekly doses of the brain-penetrant MT-stabilizing agent epothilone D (**1**, Figure 1), improved axonal transport, reduced axonal dystrophy, decreased tau neuropathology, and reduced hippocampal neuron loss. Furthermore, these effects mediated by **1** led to a significant improvement of cognitive performance by the tau Tg mice. Compound **1**, however, has thus far been the only brain-penetrant MT-stabilizer to be evaluated in mouse tauopathy models.[6,7] Furthermore, despite the efficacy and safety displayed by **1** in Tg mice, this natural product and many other naturally occurring MT-stabilizing agents or derivatives thereof exhibit potentially significant deficiencies as drug candidates, including a generally preferred intravenous (iv) route of administration and, in the case of **1** and many taxanes, the inhibition of the P-glycoprotein (Pgp)[8] transporter, which upon prolonged dosing could be responsible for undesired central nervous system (CNS) toxicities[9] and/or drug−drug interactions. As a result, the development and evaluation of additional CNS-active MT-stabilizing agents are clearly desirable to identify alternative and potentially improved clinical candidates. In this context, we undertook an evaluation of representative compounds from different classes of non-naturally occurring compounds with reported MT-stabilizing activities (Figure 1). Among these,

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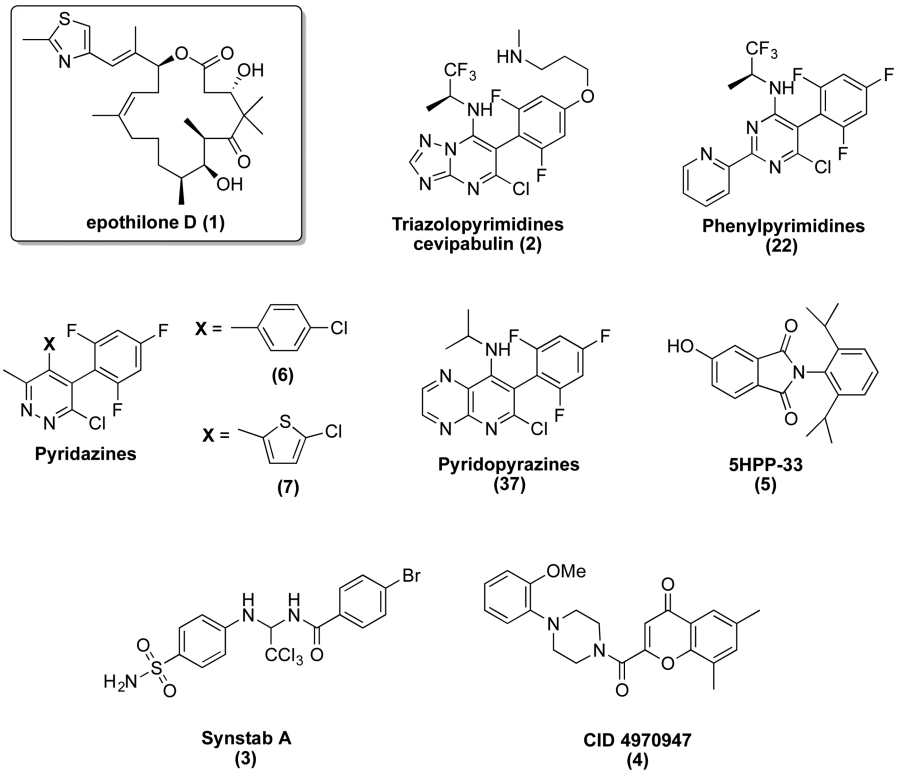

Figure 1. Structures of epothilone D (**1**) and representative examples from different classes of non-naturally occurring compounds with reported MT-stabilizing activity.

particularly attractive were the triazolopyrimidines, as a selected member of this heterocyclic MT-stabilizing series, cevipabulin (**2**), has been reported to exhibit excellent pharmaceutical properties, including oral bioavailability (61%), metabolic stability ($T_{1/2}$ = 13 h in female nude mice), and water solubility (0.89 mg/mL).[10] Although we show here that **2** is not a brain-penetrant compound, other closely related congeners are identified with significant brain penetration and oral bioavailability, as well as MT-stabilizing properties. Finally, we demonstrate that selected examples can elicit a profound pharmacodynamic (PD) response in brain in a manner similar to what was previously reported for **1**.

CHEMISTRY

Compounds **3**[11] and **4**[12] (Figure 1) were commercially available. Phthalimide derivative **5**[13] and pyridazines **6**[14] and **7**[15] (Figure 1) were synthesized as previously described. The synthesis of **2** (Figure 1) and related triazolopyrimidines (**8**–**19**, Scheme 1) and phenylpyrimidines (**20**–**25**, Scheme 1), which included a series of new analogues (**10**, **13**–**17**, **19**, **24**, and **25**), was accomplished following the general synthetic approach depicted in Scheme 1.[10,16] Starting from trifluorophenyl malonate **26**,[17] condensation with either aminotriazole **27** or the appropriate amidine **28** or **29** led to the formation of triazolopyrimidine **30**, or phenylpyrimidines **31** and **32**, respectively, which were directly treated with phosphorus oxychloride to furnish the corresponding dichloro derivatives **33**, **34**, and **35**. Treatment of **33**, **34**, and **35** with the appropriate fluorinated amine then led to triazolopyrimidine **8** and phenylpyrimidines **20**–**23**.

The chemoselectivity of the nucleophilic displacement leading to triazolopyrimidine **8** was confirmed by single crystal X-ray crystallography of azide derivative **36** (Scheme 2).

Next, treatment of the trifluoroaryl compounds **8**, **20**, and **23** with the appropriate amino alcohol or diol side chain in the presence of sodium hydride led selectively to the O-arylated products.[10,16] Finally, acylation of **2** and **11** provided amide derivatives **15**–**17**, while conversion of **12** to the corresponding tosylate, followed by treatment with tetrabutylammonium fluoride, furnished **13**. Attempted displacement of tosylated **12** with 2,2,2-trifluoroethan-1-amine hydrochloride in the presence of Hünig's base, however, resulted in the formation of the chloro derivative **14** rather than the expected compound **10**. The synthesis of **10** was instead achieved by reacting **8** with 3-[(2,2,2-trifluoroethyl)amino]propan-1-ol (Scheme 1).

The synthesis of pyridopyrazine **37** and related analogue **38** was achieved as previously described[18] (Scheme 3). Thus, condensation of pyrazine **39** with 2-(2,4,6-trifluorophenyl)acetyl chloride to form amide **40** followed by base-promoted cyclization to pyridopyrazine **41** and phosphorus oxychloride treatment provided the 6,8-dichloro derivative **42**, which was then reacted with isopropylamine to obtain a separable mixture of pyridopyrazines **37** and **38**, as well as byproduct **43**, whose structure was confirmed by single crystal X-ray crystallography.

EVALUATION OF MICROTUBULE-STABILIZING ACTIVITY

The MT-stabilizing ability of all test compounds was assessed in QBI293 cells (an HEK293 cell derivative) by monitoring for compound-induced increase in acetylated α-tubulin (AcTub). Acetylation of α-tubulin is a post-translational modification that takes place on polymerized tubulin,[8] (i.e., MTs), and as a result, quantification of AcTub provides a convenient method to determine the MT-stabilizing activity of test compounds within the context of a cellular milieu. Previous studies have shown that 50–500 nM concentrations of **1** produce a significant elevation

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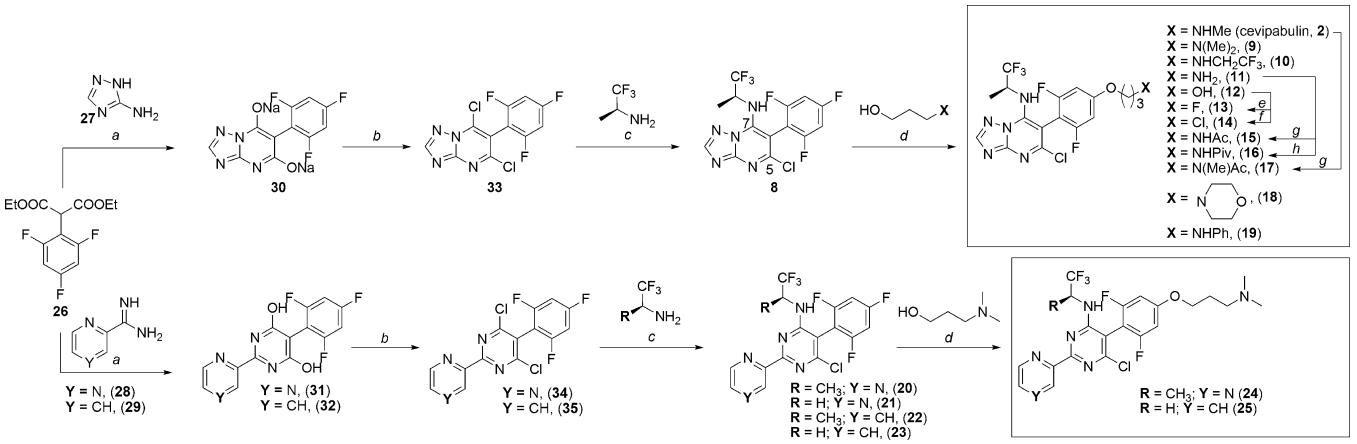
Scheme 1[a]



[a]Reagents and reaction conditions: (a) (i) aminotriazole or the appropriate amidine, tributylamine, 170−180 °C, 2−6 h; (ii) toluene, sodium hydroxide; (b) phosphorus oxychloride, 110−130 °C, 6−16 h, 46−58% over two steps; (c) appropriate amine, diisopropylethylamine, *N*,*N*-dimethylformamide, rt to 90 °C, 1−18 h, 12−50%; (d) appropriate amino alcohol, sodium hydride, dimethylsulfoxide/tetrahydrofuran, 60 °C, 3−12 h, 8−89%; (e) (i) tosyl chloride, triethylamine, dichloromethane, rt, 16 h; (ii) tetrabutylamonium fluoride, acetonitrile, rt, 4 h, 52% over two steps; (f) (i) tosyl chloride, triethylamine, dichloromethane, rt, 16 h; (ii) 2,2,2-trifluoroethan-1-amine hydrochloride, diisopropylethylamine, acetonitrile, 80 °C, 4 h, 86% over two steps; (g) acetic anhydride, triethylamine, *N*,*N*-dimethylformamide, rt, 30 min, 62−90%; (h) pivaloyl chloride, diisopropylethylamine, dichloromethane, rt, 30 min, 38%.

of AcTub levels in cells.[8] To investigate whether a comparable increase could be achieved by the triazolopyrimidines and/or related non-naturally occurring MT-stabilizing heterocyclic compounds, compound **2**, which is believed to be the most potent compound within the triazolopyrimidine series,[10] was compared with **1** in this assay. Interestingly, as shown in Figure 2,

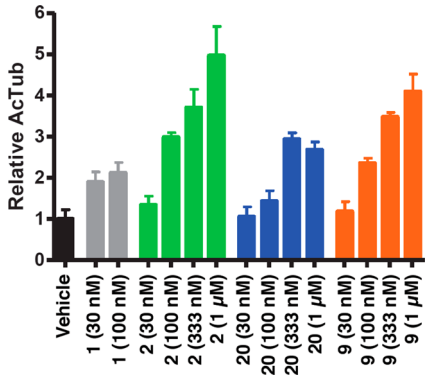
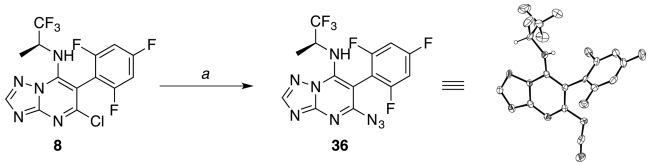


Figure 2. Comparison of activity of representative compounds in the QBI293 cell MT-stabilization (AcTub) assay.

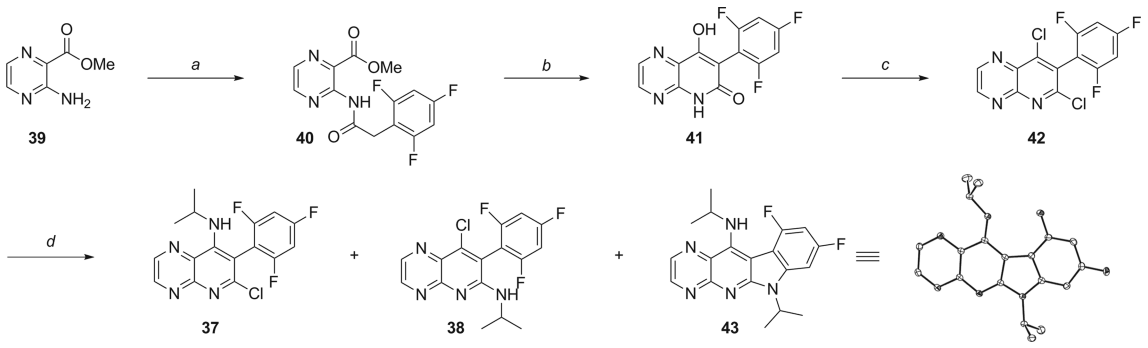
Scheme 2[a]



[a]Reagents and reaction conditions: (a) sodium azide, *N*,*N*-dimethylformamide, 70 °C, 24 h, 47%.

2 elicited a significant increase of cellular AcTub. Moreover, **2** caused an even greater AcTub increase than **1** at 100 nM compound concentration, suggesting that the activity of this triazolopyrimidine may be higher than the natural product, **1**, in this cell-based MT-stabilization assay. In addition, the related triazolopyrimidine, **9**, and the phenylpyrimidine, **20**, were also

Scheme 3[a]



[a]Reagents and reaction conditions: (a) 2-(2,4,6-trifluorophenyl)acetyl chloride, pyridine, dichloromethane, 4-dimethylaminopyridine, rt, 20 h, 44%; (b) potassium carbonate, *N*,*N*-dimethylformamide, 80 °C, 3 h, 88%; (c) phosphorus oxychloride, *N*,*N*-dimethylformamide, 1,2-dichloroethane, 80 °C, 3 h, 80%; (d) isopropylamine, 4-dimethylaminopyridine, potassium carbonate, *N*,*N*-dimethylformamide, rt, 24 h, 40% (**37**), 13% (**38**), 7% (**43**).

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found to elicit a large increase in AcTub in the cellular assay. Again, the increase of cellular AcTub is comparable after treatment with 100 nM **9** or **1**, indicating that both **2** and **9** are of nearly equal potency as the natural product.

To confirm that the observed elevation in AcTub levels caused by **2** was associated with an increase in MT number and/or organization, compound-treated QBI293 cells were examined by AcTub immunofluorescence. As depicted in Figure 3, cells

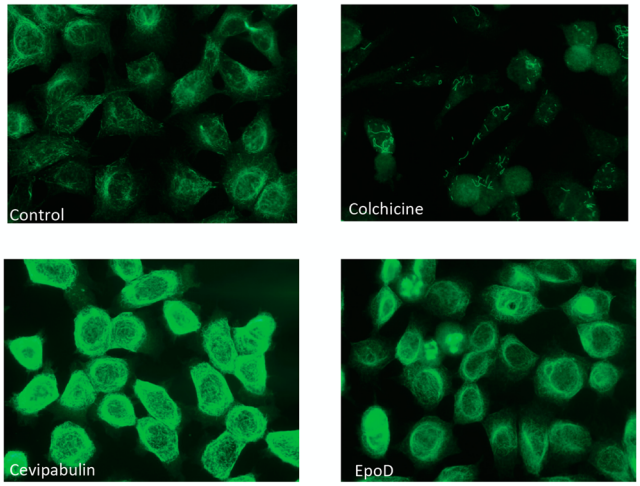

Figure 3. AcTub immunofluorescence of QBI293 cells after 4 h of incubation with no test compound, colchicine (1 μM), **2** (100 nM), or **1** (100 nM).

treated with 100 nM **2** exhibited a large increase in AcTub-positive structures relative to control cells, with the intensity of AcTub staining exceeding that produced by 100 nM **1**, whereas the MT-destabilizing agent, colchicine, led to a significant loss of AcTub staining.

Several additional examples of non-naturally occurring MT-stabilizing agents, including a series of triazolopyrimidines, phenylpyrimidines, pyridazines, pyridopyrazines, and representative compounds from other classes, such as **3**–**5** (Figure 1), underwent testing in the QBI293 AcTub assay at 0.1 or 1 μM (Table 1). Interestingly, a number of test compounds were found to elicit significant increases in cellular AcTub at 1 μM (Table 1), with one additional example (**11**) causing increased AcTub at 0.1 μM, as observed with **2** and **9**. The relative efficacy of each of the active examples in the AcTub cellular assay is also compared to that of 0.1 μM **2** in Table 1.

P-GLYCOPROTEIN (Pgp) INTERACTIONS

Previous bidirectional permeability studies demonstrated that **1** is an inhibitor of the Pgp transporter.[8] Compound **2** and several related congeners have been reported to be cytotoxic against Pgp-expressing cancer cell lines,[10] thus indicating that these compounds may not be Pgp-substrates. However, these cytotoxicity data cannot rule out the possibility that these compounds might be Pgp inhibitors. To determine whether **2** and related analogues inhibit Pgp function, compounds **2**, **9**, and **20** were evaluated in a standard Pgp-interaction assay that monitors the ability of test compounds to disrupt Pgp-mediated transport of a known Pgp substrate, calcein-AM.[19] As shown in Figure 4, both a known Pgp inhibitor (verapamil) and a Pgp substrate (cyclosporine A) affect Pgp activity in this assay, as both compete for calcein-AM binding to Pgp. Notably, unlike **1**, the

Table 1. Efficacy of Compounds in the QBI293 Cell AcTub Assay When Tested at 1 μM[a]

compd	fold-change relative to vehicle control	fold-change relative to 0.1 μM **2**
8	1.3 ± 0.6^{ns}	0.4 ± 0.1
9	9.1 ± 1.7***	1.3 ± 0.2
	(2.0 ± 1.4)*	
10	2.1 ± 1.1*	0.6 ± 0.1
11	26.5 ± 3.0***	3.4 ± 1.3
	(4.2 ± 0.2)**	
12	10.1 ± 5.2***	1.2 ± 0.6
13	1.4 ± 0.7^{ns}	
14	1.2 ± 0.7^{ns}	
15	4.5 ± 2.1***	1.5 ± 0.3
16	2.4 ± 0.9***	0.7 ± 0.1
17	1.3 ± 0.7^{ns}	
18	4.1 ± 1.9***	1.2 ± 0.3
19	1.0 ± 0.6^{ns}	
20	2.7 ± 1.3**	0.9 ± 0.1
21	1.5 ± 0.9^{ns}	
22	4.2 ± 2.1***	0.9 ± 0.1
23	1.6 ± 0.9^{ns}	
24	4.1 ± 2.8*	0.5 ± 0.2
25	0.9 ± 0.7^{ns}	
37	1.0 ± 0.8^{ns}	
38	1.0 ± 0.8^{ns}	
3	1.0 ± 0.2^{ns}	
4	0.9 ± 0.6^{ns}	
5	1.0 ± 0.6^{ns}	
6	1.5 ± 0.8^{ns}	
7	1.3 ± 0.8^{ns}	

[a]Data are expressed as the fold-change relative to vehicle-treated cells and relative to a positive control (0.1 μM **2**). Numbers in parentheses are the relative activity of compounds that showed a significant effect at 0.1 μM. ns = not significant. (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ by two-tailed t-test.

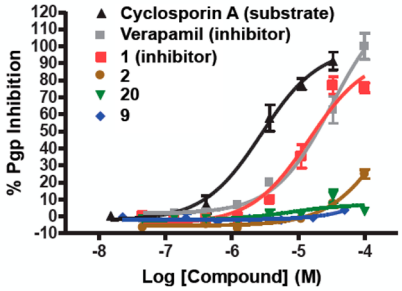

Figure 4. Pgp-interaction assay (calcein-AM). Cyclosporin A is a known Pgp substrate, whereas verapamil and **1** are known Pgp inhibitors.

triazolopyrimidines **2** and **9**, as well as phenylpyrimidine **20**, did not interfere appreciably with Pgp function.

PHARMACOKINETIC (PK) PROPERTIES

A significant hindrance to the development of MT-stabilizing agents for neurodegenerative disease is the relatively poor blood–brain barrier (BBB) penetration of many previously described compounds.[20] To evaluate the brain penetration of active triazolopyrimidines and phenylpyrimidines, the levels of compound in mouse brain and plasma were evaluated 1 h after intraperitoneal (ip) administration of 5 mg/kg test compound.

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The majority of CNS-active drugs typically exhibit brain-to-plasma (B/P) exposure ratios of ≥0.3, although there are exceptions.[21] As summarized in Table 2, compound **2** does not appear to be readily brain-penetrant, as suggested by the $B/P \ll 0.3$. However, several compounds were identified that exhibited $B/P \geq 0.3$, with brain concentrations reaching the low micromolar range for several examples. From these, a representative compound from each of the triazolopyrimidine (**9**) and phenylpyrimidine (**20**) classes were evaluated in a 16 h PK study (Figure 5). These studies revealed that the area under the curve (AUC) B/P ratios for **9** and **20** were 0.37 and 0.72, respectively (Figure 5). Moreover, the elimination half-life ($T_{1/2}$) values in plasma and brain for both compounds were found to be >2 h, with **20** showing particularly long $T_{1/2}$ values.

Table 2. Brain and Plasma Compound Concentration, 1 h after an ip Injection of 5 mg/kg of Test Compound

compd	brain, nM	plasma, nM	B/P
2	92 ± 8	2500 ± 200	0.037 ± 0.003
8	2400 ± 600	2800 ± 200	0.8 ± 0.2
9	1300 ± 200	4900 ± 500	0.27 ± 0.02
10	230 ± 30	160 ± 40	1.5 ± 0.2
11	40 ± 20	1300 ± 70	0.03 ± 0.01
12	78 ± 4	29 ± 5	2.8 ± 0.4
14	160 ± 10	178 ± 5	0.88 ± 0.06
16	170 ± 10	1500 ± 500	0.12 ± 0.03
17	20 ± 10	200 ± 100	0.15 ± 0.03
18	600 ± 200	1200 ± 300	0.49 ± 0.03
19	120 ± 3	75 ± 7	1.6 ± 0.2
20	2900 ± 100	5000 ± 200	0.58 ± 0.01
22	1500 ± 400	4300 ± 900	0.34 ± 0.04
24	860 ± 130	2500 ± 200	0.35 ± 0.06

To more accurately estimate the BBB permeability of these compounds, the relative B/P ratio of free (unbound) compound was estimated after determining the amount of nonspecific binding to plasma proteins and brain tissue by equilibrium dialysis, as previously described.[8] The equilibrium dialysis results revealed that both compounds had high plasma protein binding and brain homogenate binding, with fraction unbound (F_u) values as highlighted in Figure 5. This resulted in estimated free compound B/P ratios of 0.09 ± 0.01 for **9** and 1.7 ± 0.6 for **20**. Thus, these data would suggest that whereas **20** can readily partition across the BBB, the passage of **9** is relatively limited, although an appreciably greater brain exposure is obtained with this compound than with **2** (cf. **2** and **9**, Table 2).

Finally, to ascertain whether these compounds could be orally absorbed, brain and plasma levels of **9** and **20** were determined 2 h after oral administration of the compounds (10 mg/kg). As shown in Figure 6, both compounds were detected at micromolar levels in brain and plasma, confirming that these representative compounds are orally bioavailable.

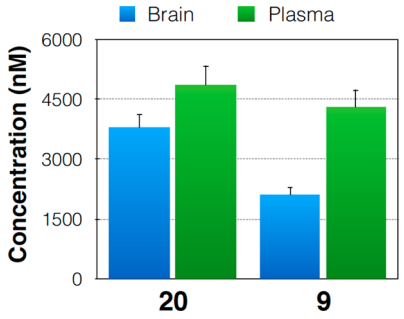

Figure 6. Brain and plasma levels of **9** and **20**, 2 h after oral administration of 10 mg/kg.

EVALUATION OF BRAIN PHARMACODYNAMIC (PD) EFFECTS

Previous studies demonstrated that a single ip injection of **1** (1−6 mg/kg) produces MT-stabilization in the CNS of mice, as revealed by a significant elevation in AcTub levels in brain homogenates after dosing.[4] To investigate whether the representative phenylpyrimidine and triazolopyrimidine examples of orally bioavailable small molecule MT-stabilizing agents could elicit a comparable PD response in the brain, groups of mice (n = 3) received daily doses (1 mg/kg, ip) of **9** or **20** for either 4 or 6 days, followed by a determination of AcTub levels in brain homogenates, as previously described.[8] As shown in Figure 7, both compounds produced a significant PD response, demonstrating that these small heterocyclic compounds can stabilize MTs within the brains of mice. Notably, the compound-induced elevation in brain AcTub levels increased from 4 to 6 days of dosing. Moreover, although **9** shows lesser BBB permeability than **20**, the brain free drug concentrations of the two compounds are predicted to be relatively comparable during the first 2−3 h after dosing based on the total brain concentrations and the observation that the brain F_u value of **9** is ∼3-fold greater than for **20** (see Figure 5). This, coupled with the greater activity of **9** compared to **20** in the cellular assay (Figure 2), likely explains the slightly greater PD effect of **9**. More extensive future studies will be required to determine the minimum doses required to elicit these PD effects, as well as the dosing frequency and time to reach maximal MT stabilization.

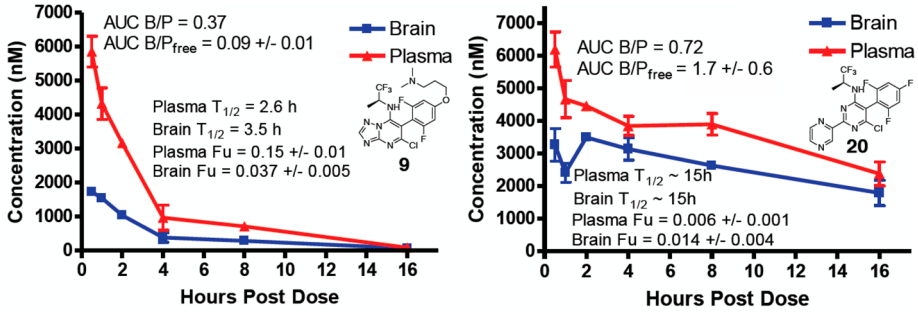

Figure 5. Brain and plasma pharmacokinetics of **9** and **20** after 5 mg/kg ip dosing to CD1 mice.

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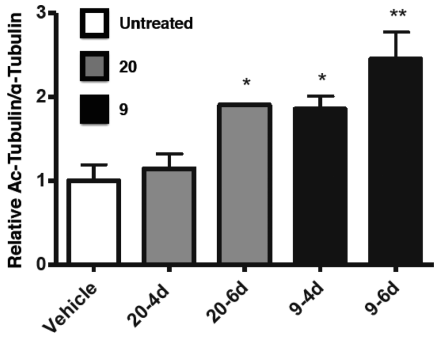

Figure 7. Relative increase in AcTub level in the brain of WT mice that received **9** or **20**, 1 mg/kg daily ip for 4 or 6 days: ∗, $p < 0.05$; ∗∗, $p < 0.01$, as determined by ANOVA with a Dunnett's multiple comparison test.

DISCUSSION

The rationale for treating AD and related neurodegenerative tauopathies with MT-stabilizing agents is to compensate for the MT dysfunction that likely results from a loss of tau MT-stabilizing function caused by tau hyperphosphorylation and sequestration into inclusions, with a consequent impairment of axonal transport.[22] Initial proof-of-concept for this therapeutic strategy[23] was obtained in 2005, when paclitaxel was found to improve the neurodegenerative phenotype of tau Tg mice that develop brain stem and spinal cord tau pathology.[5] However, paclitaxel, which does not readily cross the BBB, presumably provided benefit in these mice through uptake at peripheral neuromuscular junctions with consequent drug delivery to motor neurons. Recent studies have further validated this therapeutic strategy when it was observed that once-weekly doses of **1** far below levels used in cancer clinical trials were effective in treating both the neuropathology and cognitive deficits of PS19 tau Tg mice,[4,6] which develop age-dependent AD-like tau pathology in the brain.[24] Furthermore, subsequent studies confirmed that **1** was effective in two additional tau Tg animal models,[7] and a clinical trial was subsequently initiated with this agent (referred to as BMS-241027) in AD patients. Collectively, these studies provide important validation that brain-penetrant MT-stabilizing agents may be useful for the treatment of AD and related tauopathies. However, as noted previously, **1** exhibits deficiencies as a drug candidate, including the inhibition of the Pgp transporter and a preferred iv route of administration. Therefore, as part of our efforts toward the identification of alternative and potentially improved clinical candidates, we complemented our ongoing investigations of MT-stabilizing natural products with an evaluation of known non-naturally occurring MT-stabilizing small molecules that are endowed with potentially favorable druglike properties, with the objective of identifying brain-penetrant, orally bioavailable MT-stabilizing candidates. Among these classes of compounds are certain triazolopyrimidines, typified by **2**,[10] as well as some structurally related phenylpyrimidines,[16] pyridopyridazines,[18] and pyridazines.[15] The first examples of these synthetic small molecule MT-stabilizing agents were members of the triazolopyrimidines discovered as antifungal agents.[25] Since then, improved analogues, as well as related scaffolds, have been reported that appear to share similar MT-stabilizing properties, antimitotic activity, and structure–activity relationships.[10,15,16,18,26,27] Interestingly, radioligand binding studies suggested that the mode of action of these heterocyclic MT-stabilizing agents may be rather unique.[28,29] Whereas **2** was found not to compete for the taxane/epothilone binding site on β-tubulin, this compound did affect vinblastine binding to β-tubulin.[28] It is not clear yet whether **2** binds directly at the vinca alkaloid site on β-tubulin or rather at a distinct site that regulates vinblastine binding allosterically.[28] Notably, cytotoxicity studies in cancer cell lines[10] revealed that **2** exhibits potent antimitotic effects that are similar to that observed with classical MT-stabilizing molecules, such as paclitaxel and **1**. Furthermore, **2** was found to be active against Pgp-expressing cancer cell lines, suggesting that the compound is not a substrate for this transporter. Finally, **2** was found to exhibit desirable druglike properties, including good water solubility, oral bioavailability, and metabolic stability.[10] Thus, because of these features of **2**, we evaluated a series of triazolopyrimidines and related heterocycles as potential leads for the development of orally bioavailable, CNS-active MT-stabilizing agents. Interestingly, an evaluation of such molecules in a cell-based assay that employs AcTub as a surrogate marker for compound-induced MT-stabilization revealed that several triazolopyrimidines and phenylpyrimidines produce a significant elevation in AcTub levels that appeared to be comparable to or higher than the response caused by **1**. In contrast, other related heterocycles with reported MT-stabilizing activity, including pyridopyridazine **37**, pyridazines **6** and **7**, and nonrelated compounds **3**–**5**, were found to be essentially inactive in this assay at 1 μM.

Evaluation of representative examples of active triazolopyrimidines and phenylpyrimidines reveals that these small molecules, unlike **1**, do not interfere with Pgp function. Furthermore, although **2** was found to exhibit negligible brain penetration, possibly due to the presence of an ionizable secondary amine in the side chain, relatively simple structural modifications consisting of a removal of the side chain (e.g., **8**, **20**) or substitutions at the terminal amine that reduce the solvation (e.g., **9**) and/or ionization of the amino group in plasma (e.g., **10**, **19**) led to a significant improvement in brain penetration. Although these modifications generally resulted in a moderate attenuation of the MT-stabilizing activity of these compounds relative to **2**, several examples were identified that retained significant MT-stabilizing properties (Table 1). Among these compounds, the representative triazolopyrimidine **9** and phenylpyrimidine **20** underwent further PK analyses, which revealed that both of these compounds achieve meaningful brain exposure after ip or oral administration, although in the case of **9**, correction of the B/P ratio for the unbound fractions in plasma and brain homogenates highlights a limited BBB permeability. Moreover, the elimination $T_{1/2}$ in plasma and brain for both compounds was found to be >2 h, with **20** showing particularly long $T_{1/2}$ values. Importantly, an evaluation of the brain PD activity of these two brain-penetrant, orally bioavailable compounds revealed that relatively low daily dosing of either **9** or **20** resulted in a marked time-dependent elevation in AcTub levels in the brain of WT mice. The extent of AcTub elevation elicited upon repeated dosing of these compounds is comparable to what was previously observed with the natural product, **1**, although the latter required less frequent dosing due to a very prolonged CNS retention.[8] The observation that a marked PD effect could be elicited by relatively low doses of these small heterocycles is clearly promising, and future efficacy studies in tau Tg mice in comparison with **1** will more fully determine the potential of these and related compounds as candidates for the treatment of neurodegenerative tauopathies.

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CONCLUSIONS

Several lines of investigation suggest that CNS-directed MT-stabilizing therapies may be effective in the treatment of neurodegenerative tauopathies, including AD, as well as other neurodegenerative diseases in which axonal transport deficits appear to be caused by hyperdynamic axonal MTs.[30] However, in spite of the large number of MT-stabilizing agents that have been developed to date for cancer chemotherapy, thus far only a few examples have been identified that exhibit the required PK/PD properties to be considered as potential candidates for the treatment of AD and related neurodegenerative diseases.[20] The studies presented herein demonstrate that representative members of the triazolopyrimidine and related phenylpyrimidine MT-stabilizing small molecules exhibit promising PK properties, including oral bioavailability and brain penetration, without significant interference of Pgp function. Furthermore, PD studies confirmed that examples from these series produce an increase in a biomarker of MT-stabilization in the brain of mice. Thus, these classes of MT-stabilizing agents exhibit properties that provide potential advantages over the existing MT-stabilizing natural products. As a result, these compounds may be promising leads for the development of orally active, CNS-directed MT-stabilizing therapies.

EXPERIMENTAL SECTION

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 precoated silica gel plates. Flash chromatography was performed with silica gel 60 (particle size 0.040−0.062 mm) supplied by Silicycle and Sorbent Technologies. TLC spots were detected by viewing under a UV light. Infrared (IR) spectra were recorded on a Jasco model FT/IR-480 Plus spectrometer. Proton (^{1}H) and carbon (^{13}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 Center on either a VG Micromass 70/70H or VG ZAB-E spectrometer. Single-crystal X-ray structure determinations were performed at the University of Pennsylvania with an Enraf Nonius CAD-4 automated diffractometer. Analytical reverse-phased (Sunfire C18; 4.6 mm × 50 mm, 5 mL) high-performance liquid chromatography (HPLC) was performed with a Waters binary gradient module 2525 equipped with Waters 2996 PDA and Waters 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 mL/min, and unless otherwise stated, the purity level was >95%. Preparative reverse-phase HPLC purifications were performed on a Gilson instrument (i.e., Gilson 333 pumps, a 215 liquid handler, 845Z injection module, and PDA detector) employing Waters SunFire preparative C_{18} OBD columns (5 μm, 19 mm × 50 mm or 19 mm × 100 mm). Purifications were carried out employing a linear gradient from 10% to 90% of acetonitrile in water for 15 min with a flow rate of 20 mL/min. Unless otherwise stated, all final compounds were found to be >95% pure as determined by HPLC/MS and NMR.

(*S*)-5-Chloro-6-(2,6-difluoro-4-(3-(methylamino)propoxy)-phenyl)-*N*-(1,1,1-trifluoropropan-2-yl)[1,2,4]triazolo[1,5-*a*]-pyrimidin-7-amine (2). **2** was prepared as described in Zhang et al.[10] Reverse-phase HPLC purification of the crude product afforded the title compound (35% yield). ^{1}H NMR (500 MHz, MeOD) δ 8.48 (s, 1H), 6.84 (d, J = 9.5 Hz, 2H), 5.50 (s, 1H), 4.32−4.06 (m, 2H), 3.23 (t, J = 7.5 Hz, 2H), 2.75 (s, 3H), 2.34−2.12 (m, 2H), 1.43 (d, J = 6.9 Hz, 3H) ppm. ^{13}C NMR (126 MHz, MeOD) δ 163.82 (t, J = 14.3 Hz), 163.65 (dd, J = 247.6, 8.8 Hz), 163.00 (dd, J = 246.7, 9.2 Hz), 162.96, 162.68, 159.51, 156.07, 155.91, 149.28, 126.68 (q, J = 281.7 Hz), 119.35, 117.03, 102.80 (t, J = 21.2 Hz), 100.66 (dd, J = 26.2, 3.3 Hz), 100.29 (dd, J = 26.0, 3.4 Hz), 94.67, 67.48, 52.36 (q, J = 31.6 Hz), 34.01, 27.05, 14.06 ppm. HRMS [ESI]$^+$ calculated for $C_{18}H_{19}N_6OF_5Cl$, 465.1229; found, 465.1230.

2-(2,6-Diisopropylphenyl)-5-hydroxyisoindoline-1,3-dione (5). **5** was prepared as described in Li et al.[13] Reverse-phase HPLC purification of the crude product afforded the title compound. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.89 (s, 1H), 7.80 (d, J = 8.2 Hz, 1H), 7.47 (t, J = 7.8 Hz, 1H), 7.37 (d, J = 2.2 Hz, 1H), 7.31 (d, J = 7.8 Hz, 2H), 7.06 (dd, J = 8.2, 2.2 Hz, 1H), 2.74 (p, J = 6.8 Hz, 2H), 2.16 (s, 1H), 1.18 (dd, J = 6.8, 1.9 Hz, 11H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.70, 168.68, 162.47, 147.35, 134.35, 130.33, 126.71, 126.11, 124.07, 122.94, 121.07, 111.18, 29.35, 23.99, 23.97 ppm. HRMS [ESI]$^+$ calculated for $C_{20}H_{22}NO_3$, 324.1600; found, 324.1563.

5-(4-Chlorophenyl)-6-methyl-4-(2,4,6-trifluorophenyl)-pyridazin-3(2*H*)-one (6). **6** was prepared as described in Manabe et al.[14] Reverse-phase HPLC purification of the crude product afforded the title compound. ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.40 (bs, 1H), 7.34−7.28 (m, 2H), 7.07−7.00 (m, 2H), 6.57 (dd, J = 8.7, 7.2 Hz, 2H), 2.11 (s, 3H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.86, 147.46, 144.87, 135.21, 133.26, 129.08, 129.00, 100.69 (d, J = 25.2 Hz), 20.91 ppm. HRMS [ESI]$^+$ calculated for $C_{17}H_{10}Cl_2F_3N_2$, 369.0173; found, 369.0179.

3-Chloro-5-(5-chlorothiophen-2-yl)-6-methyl-4-(2,4,6-trifluorophenyl)pyridazine (7). **7** was prepared as described in Lamberth et al.[15] Reverse-phase HPLC purification of the crude product afforded the title compound. ^{1}H NMR (500 MHz; $CDCl_3$) δ 6.83 (d, J = 3.8 Hz, 1H), 6.73−6.70 (m, 3H), 2.71 (s, 3H) ppm. HRMS [ESI]$^+$ calculated for $C_{15}H_8Cl_2F_3N_2S$, 374.9737; found, 374.9715.

(*S*)-5-Chloro-6-(2,4,6-trifluorophenyl)-*N*-(1,1,1-trifluoropropan-2-yl)[1,2,4]triazolo[1,5-*a*]pyrimidin-7-amine (8). **8** was prepared as described in Zhang et al.[10] Reverse-phase HPLC purification afforded the title compound as a colorless solid (25% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.41 (s, 1H), 6.95−6.89 (m, 2H), 5.88 (d, J = 9.2 Hz, 1H), 4.69 (bm, 1H), 1.43 (d, J = 6.8 Hz, 3H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.55 (dt, J = 255.0, 15.1 Hz), 161.62 (ddd, J = 253.3, 14.9, 8.1 Hz), 161.26 (ddd, J = 251.8, 15.0, 8.2 Hz), 157.91, 155.47, 154.23, 146.04, 124.57 (q, J = 282.0 Hz), 105.46 (td, J = 20.6, 4.8 Hz), 101.74 (td, J = 25.9, 4.2 Hz), 101.36 (td, J = 25.4, 4.5 Hz), 91.54, 51.01 (q, J = 32.2 Hz), 15.18 (q, J = 1.9 Hz) ppm. IR: ν 3393, 3334, 3258, 3112, 2953, 1620 cm^{-1}. HRMS [ESI]$^+$ calculated for $C_{14}H_8N_5F_6Cl$, 396.0445; found, 396.0450.

General Procedure A (Installment of Side Chain). According to reported procedures,[10,16] to a suspension of sodium hydride (60% in mineral oil, 4.0 equiv) in a 2:1 mixture of dimethylsulfoxide and tetrahydrofuran (0.35 M) was added the appropriate amino alcohol (4.0 equiv), and the mixture was heated to 60 °C for 1 h. The resulting yellow turbid solution was treated with a solution of trifluoroarene (1.0 equiv) in a 1:1 mixture dimethylsulfoxide and tetrahydrofuran (0.5 M). The reaction mixture was stirred at 60 °C for 3 h and monitored by LCMS. If the starting material was not fully consumed within 3 h, additional amino alcohol (4.0 equiv) and sodium hydride (4.0 equiv) were added sequentially, and the reaction mixture was heated for 12 h. Following complete consumption of the starting material, the reaction mixture was cooled to room temperature and diluted with water and ethyl acetate. The organic layer was washed with water and brine, and the combined aqueous layers were extracted with ethyl acetate (×3). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated. The crude products were purified by reverse-phase HPLC.

(*S*)-5-Chloro-6-(4-(3-(dimethylamino)propoxy)-2,6-difluoro-phenyl)-*N*-(1,1,1-trifluoropropan-2-yl)[1,2,4]triazolo[1,5-*a*]-pyrimidin-7-amine (9). General procedure A was followed, using 3-dimethylamino-1-propanol and **8**. Reverse-phase HPLC purification afforded the title compound as a colorless, hygroscopic solid (38% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.51 (bs, 1H), 8.18 (s, 1H), 6.89 (dd, J = 4.0, 9.9 Hz, 2H), 5.89−5.83 (m, 1H), 4.10 (t, J = 6.3 Hz, 2H), 2.55 (t, J = 7.6 Hz, 2H), 2.29 (s, 6H), 1.93 (p, J = 6.8 Hz, 2H), 1.37 (d, J = 6.8 Hz, 3H) ppm. ^{13}C NMR (126 MHz, MeOD) δ 170.42, 164.32 (t, J = 14.2 Hz), 163.66 (dd, J = 246.9, 9.2 Hz), 163.01 (dd, J = 246.4, 9.2 Hz), 159.57, 156.12, 155.87, 149.34, 126.71 (q, J = 281.7 Hz), 102.27 (t, J = 21.3 Hz), 100.52 (dd, J = 25.9, 3.3 Hz), 100.18 (dd, J = 25.9, 3.2 Hz), 94.91, 68.32, 52.37 (q, J = 31.8 Hz), 27.77, 14.05 ppm. HRMS [ESI]$^+$ calculated for $C_{19}H_{21}N_6OF_5Cl$, 479.1386; found, 479.1384.

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(*S*)-5-Chloro-6-(2,6-difluoro-4-(3-((2,2,2-trifluoroethyl)-amino)propoxy)phenyl)-*N*-(1,1,1-trifluoropropan-2-yl)[1,2,4]-triazolo[1,5-*a*]pyrimidin-7-amine (10). General procedure A was followed, using 3-((2,2,2-trifluoroethyl)amino)propan-1-ol and **8**. Reverse-phase HPLC purification afforded the title compound (89% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.40 (s, 1H), 6.69−6.65 (m, 2H), 5.92 (d, J = 10.3 Hz, 1H), 4.77 (bs, 1H), 4.15 (t, J = 6.1 Hz, 2H), 3.25 (q, J = 9.4 Hz, 2H), 2.99 (t, J = 6.7 Hz, 2H), 2.04 (quintet, J = 6.4 Hz, 2H), 1.42 (d, J = 6.8 Hz, 3H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) characteristic signals δ 162.39 (t, J = 13.8 Hz), 161.89 (dd, J = 250.3, 8.8 Hz), 161.62 (dd, J = 248.7, 8.7 Hz), 158.71, 155.34, 154.16, 146.19, 124.73 (q, J = 281.9 Hz), 124.57 (q, J = 280 Hz), 101.09 (t, J = 21.1 Hz), 99.52 (dd, J = 25.6, 3.4 Hz), 99.25 (dd, J = 25.6, 3.2 Hz), 92.63, 66.64, 50.96 (q, J = 32.2 Hz), 49.81 (q, J = 32.6, 31.8 Hz), 46.25, 28.20, 15.32 ppm. HRMS [ESI]$^+$ calculated for $C_{19}H_{18}ClF_8N_6O$, 533.1103; found, 533.1199.

(*S*)-6-(4-(3-Aminopropoxy)-2,6-difluorophenyl)-5-chloro-*N*-(1,1,1-trifluoropropan-2-yl)[1,2,4]triazolo[1,5-*a*]pyrimidin-7-amine (11). General procedure A was followed, using 3-aminopropan-1-ol and **8**. Reverse-phase HPLC purification afforded the title compound (22% yield). ^{1}H NMR (500 MHz; DMSO-d_6) δ 8.30 (s, 1H), 8.09 (d, J = 0.3 Hz, 1H), 6.76−6.74 (m, 2H), 5.78 (bs, 1H), 4.13 (t, J = 5.9 Hz, 2H), 3.36 (bs, 2H), 2.98 (t, J = 7.2 Hz, 2H), 2.03 (t, J = 6.6 Hz, 2H), 1.21 (d, J = 6.5 Hz, 3H) ppm. ^{13}C NMR (126 MHz, MeOD) δ 170.43, 163.95 (t, J = 14.2 Hz), 163.67 (dd, J = 247.3, 9.1 Hz), 163.02 (dd, J = 246.7, 9.0 Hz), 159.50, 156.08, 155.90, 149.28, 126.70 (q, J = 281.6 Hz), 102.74 (t, J = 21.3 Hz), 100.62 (dd, J = 26.0, 3.3 Hz), 100.25 (dd, J = 26.0, 3.3 Hz), 94.69, 67.64, 52.37 (q, J = 31.7 Hz), 38.61, 28.97, 14.08 ppm. HRMS [ESI]$^+$ calculated for $C_{17}H_{17}ClF_5N_6$, 451.1073; found, 451.1076.

(*S*)-3-(4-(5-Chloro-7-((1,1,1-trifluoropropan-2-yl)amino)-[1,2,4]triazolo[1,5-*a*]pyrimidin-6-yl)-3,5-difluorophenoxy)-propan-1-ol (12). General procedure A was followed, using propane-1,3-diol and **8**. Reverse-phase HPLC purification afforded the title compound (60% yield). ^{1}H NMR (500 MHz; $CDCl_3$) δ 8.40 (s, 1H), 6.70−6.67 (m, 2H), 5.91 (d, J = 8.7 Hz, 1H), 4.77 (bs, 1H), 4.21 (t, J = 6.1 Hz, 2H), 3.91 (t, J = 5.9 Hz, 2H), 2.18−2.09 (m, 2H), 1.70 (s, 1H), 1.42 (d, J = 6.8 Hz, 3H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ 162.73 (t, J = 13.8 Hz), 161.79 (dd, J = 249.7, 9.2 Hz), 161.53 (dd, J = 248.3, 9.0 Hz), 158.64, 155.44, 154.14, 146.06, 124.67 (q, J = 281.8 Hz), 100.70 (t, J = 21.1 Hz), 99.49 (dd, J = 25.5, 3.3 Hz), 99.21 (dd, J = 25.4, 3.4 Hz), 92.51, 66.28, 59.53, 50.81 (q, J = 32.1 Hz), 31.85, 15.37 ppm. HRMS [ESI]$^+$ calculated for $C_{17}H_{16}ClF_5N_5O_2$, 452.0913; found, 452.0902.

(*S*)-5-Chloro-6-(2,6-difluoro-4-(3-fluoropropoxy)phenyl)-*N*-(1,1,1-trifluoropropan-2-yl)[1,2,4]triazolo[1,5-*a*]pyrimidin-7-amine (13). To a solution of **12** (0.05 g, 0.12 mmol) in anhydrous dichloromethane (1.20 mL) were added tosyl chloride (0.07 g, 0.36 mmol) and triethylamine (0.05 mL, 0.36 mmol). The reaction mixture was stirred at room temperature for 16 h. The reaction was quenched by addition of water, and the resulting mixture was washed with saturated aqueous solution of sodium bicarbonate and brine. The organic layer was then dried over sodium sulfate, filtered, and concentrated. Purification by silica gel column chromatography, using a 1:1 mixture of ethyl acetate/hexanes, provided the tosylated intermediate (0.05 g, 0.07 mmol). To a solution of the latter intermediate (0.03 g, 0.05 mmol) in acetonitrile (0.65 mL) was added tetrabutylammonium fluoride (1 M in tetrahydrofuran, 0.06 mmol). The reaction mixture was stirred at room temperature for 4 h. The reaction mixture was then diluted with dichloromethane and washed with water and brine. The organic layer was dried over sodium sulfate, filtered, and concentrated. Purification by silica gel column chromatography, using a 30% mixture of ethyl acetate in hexanes, afforded the title compound (52% yield). ^{1}H NMR (500 MHz; $CDCl_3$) δ 8.39 (s, 1H), 6.76−6.56 (m, 2H), 5.89 (d, J = 11.0 Hz, 1H), 4.77 (s, 1H), 4.68 (dt, J = 47.0, 5.6 Hz, 2H), 4.18 (t, J = 6.1 Hz, 2H), 2.24 (dt, J = 26.3, 5.8 Hz, 2H), 1.41 (d, J = 6.8 Hz, 3H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ 162.56 (t, J = 13.8 Hz), 161.82 (dd, J = 250.1, 8.8 Hz), 161.56 (dd, J = 248.5, 9.0 Hz), 158.59, 155.48, 154.20, 146.08, 124.69 (q, J = 282.0 Hz), 100.92 (t, J = 20.9 Hz), 99.51 (dd, J = 25.4, 3.3 Hz), 99.21 (dd, J = 25.4, 3.4 Hz), 92.45, 80.35 (d, J = 165.3 Hz), 64.81 (d, J = 4.7 Hz), 50.84 (q, J = 32.1 Hz), 30.24 (d, J = 20.1 Hz), 15.39 (q, J = 1.9, 1.5 Hz) ppm. IR: ν 3340, 2953, 2920, 2851, 1642, 1618 cm^{-1}. HRMS [ESI]$^+$ calculated for $C_{17}H_{15}N_5OF_6Cl$, 454.0864; found, 454.0858.

(*S*)-5-Chloro-6-(4-(3-chloropropoxy)-2,6-difluorophenyl)-*N*-(1,1,1-trifluoropropan-2-yl)[1,2,4]triazolo[1,5-*a*]pyrimidin-7-amine (14). To a solution of **12** (0.05 g, 0.12 mmol) in anhydrous dichloromethane (1.20 mL) were added tosyl chloride (0.07 g, 0.36 mmol) and triethylamine (0.05 mL, 0.36 mmol). The reaction mixture was stirred at room temperature for 16 h. The reaction was then quenched by addition of water, and the resulting mixture was washed with saturated aqueous solution of sodium bicarbonate and brine. The organic layer was then dried over sodium sulfate, filtered, and concentrated. Purification by silica gel column chromatography, using a 1:1 mixture of ethyl acetate/hexanes, provided the tosylated intermediate (0.05 g, 0.07 mmol). To a solution of the latter intermediate (0.01 g, 0.02 mmol) in acetonitrile (0.24 mL) were added 2,2,2-trifluoroethan-1-amine hydrochloride (0.01 g, 0.06 mmol) and diisopropylethylamine (0.01 mL, 0.08 mmol). The reaction mixture was stirred at 80 °C for 4 h. The reaction mixture was then diluted with water and ethyl acetate, and the organic layer was washed with brine. The organic layer was dried over sodium sulfate, filtered, and concentrated. Purification by silica gel column chromatography, using a 30% mixture of ethyl acetate in hexanes, afforded the title compound (86% yield). ^{1}H NMR (500 MHz; $CDCl_3$) δ 8.41 (s, 1H), 6.68 (m, 2H), 5.89 (d, J = 9.8 Hz, 1H), 4.79 (bs, 1H), 4.21 (t, J = 5.8 Hz, 2H), 3.79 (t, J = 6.2 Hz, 2H), 2.32 (m, 2H), 1.43 (d, J = 6.8 Hz, 3H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ 162.50 (t, J = 13.7 Hz), 161.84 (dd, J = 250.3, 8.8 Hz), 161.57 (dd, J = 248.8, 9.0 Hz), 158.58, 155.50, 146.11, 124.70 (q, J = 282.0 Hz), 100.99 (t, J = 21.1 Hz), 99.54 (dd, J = 25.5, 3.5 Hz), 99.25 (dd, J = 25.4, 3.4 Hz), 92.45, 65.52, 50.86 (q, J = 32.2 Hz), 41.23, 31.97, 29.92, 15.42 ppm. IR: ν 3341, 2919, 2851, 1617 cm^{-1}. HRMS [ESI]$^+$ calculated for $C_{17}H_{15}N_5OF_5Cl_2$, 470.0568; found, 470.0578.

(*S*)-*N*-(3-(4-(5-Chloro-7-((1,1,1-trifluoropropan-2-yl)amino)-[1,2,4]triazolo[1,5-*a*]pyrimidin-6-yl)-3,5-difluorophenoxy)-propyl)acetamide (15). To a solution of **11** (0.08 g, 0.08 mmol) in *N*,*N*-dimethylformamide (2.52 mL) was added triethylamine (0.01 mL, 0.08 mmol). The reaction mixture was stirred at room temperature for 10 min, and then acetic anhydride (0.01 mL, 0.08 mmol) was added. The reaction mixture was stirred at room temperature for 1 h, and was then diluted with ethyl acetate and washed with water. The organic layer was dried over sodium sulfate, filtered, and concentrated. Purification by silica gel column chromatography, using a 5% mixture of methanol in dichloromethane, afforded the title compound (90% yield). ^{1}H NMR (500 MHz; $CDCl_3$) δ 8.37 (s, 1H), 6.64 (d, J = 8.9 Hz, 2H), 6.11 (s, 1H), 5.98 (d, J = 10.6 Hz, 1H), 4.85 (s, 1H), 4.10 (t, J = 6.1 Hz, 2H), 3.48 (q, J = 6.3 Hz, 2H), 2.08 (t, J = 6.4 Hz, 2H), 2.01 (s, 3H), 1.42 (d, J = 6.8 Hz, 3H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.57, 161.74 (dd, J = 241.2, 8.7 Hz), 161.50 (dd, J = 248.3, 9.4 Hz), 158.46, 155.31, 154.19, 146.17, 124.66 (q, J = 282.4 Hz), 100.77 (t, J = 20.9 Hz), 99.42 (dd, J = 25.6, 3.2 Hz), 99.16 (dd, J = 26.0, 3.3 Hz), 92.54, 67.08, 53.53, 50.86 (q, J = 32.3 Hz), 36.84, 29.24, 23.35, 15.22 ppm. IR: ν 3298, 3103, 2925, 2853, 1642, 1616 cm^{-1}. HRMS [ESI]$^+$ calculated for $C_{19}H_{19}ClF_5N_6O_2$, 493.1178; found, 493.1180.

(*S*)-*N*-(3-(4-(5-Chloro-7-((1,1,1-trifluoropropan-2-yl)amino)-[1,2,4]triazolo[1,5-*a*]pyrimidin-6-yl)-3,5-difluorophenoxy)-propyl)pivalamide (16). To a solution of **11** (0.01 g, 0.02 mmol) in dichloromethane (0.20 mL) were added pivaloyl chloride (3 μL, 0.02 mmol) and diisopropylethylamine (4 μL, 0.02 mmol). The reaction mixture was stirred for 30 min at room temperature, and then it was diluted with dichloromethane and washed with water. The organic layer was dried over sodium sulfate, filtered, and concentrated. Purification by silica gel column chromatography, using a 5% mixture of methanol in dichloromethane, afforded the title compound (38% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.39 (s, 1H), 6.64 (m, 2H), 5.96 (m, 2H), 4.77 (bs, 1H), 4.10 (t, J = 5.9 Hz, 2H), 3.49 (q, J = 6.3 Hz, 2H), 2.07 (m, 2H), 1.41 (d, J = 6.8 Hz, 3H), 1.22 (s, 9H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ 178.92, 162.51 (t, J = 13.8 Hz), 161.86 (dd, J = 250.3, 8.7 Hz), 161.59 (dd, J = 248.1, 8.8 Hz), 158.55, 155.52, 154.20, 146.08, 124.69 (q, J = 281.9 Hz), 100.97 (t, J = 21.0 Hz), 99.46 (dd, J = 25.6, 3.3 Hz), 99.15 (dd, J = 25.5, 3.4 Hz), 92.42, 67.62, 50.85 (d, J = 32.1 Hz) 38.93, 37.27, 29.27, 27.82, 15.40 ppm. IR: ν 3343, 2961, 2931, 2816, 1614, 1581 cm^{-1}.

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HRMS [ESI]$^+$ calculated for $C_{22}H_{25}N_6O_2F_5Cl$, 535.1642; found, 535.1641.

(*S*)-*N*-(3-(4-(5-Chloro-7-((1,1,1-trifluoropropan-2-yl)amino)-[1,2,4]triazolo[1,5-*a*]pyrimidin-6-yl)-3,5-difluorophenoxy)-propyl)-*N*-methylacetamide (17). **17** was prepared as for **15**, starting from **2** (62% yield). ^{1}H NMR (500 MHz, $CDCl_3$) mixture of *E*/*Z* isomers δ 8.39 (apparent d, J = 3.0 Hz, 1H), 6.81−6.54 (m, 2H), 5.92 (d, J = 10.8 Hz, 1H), 4.78 (s, 1H), 4.16−3.96 (m, 2H), 3.57 (m, 2H), 3.06−2.96 (apparent d, 3H), 2.11 (m, 5H), 1.41 (apparent dd, J = 7.0, 1.7 Hz, 3H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) mixture of *E*/*Z* isomers δ 171.07, 170.76, 162.84, 162.76, 162.65, 162.57, 162.54, 162.50, 162.27, 162.17, 162.06, 160.86, 160.78, 160.60, 160.52, 158.57, 158.51, 155.52, 155.48, 154.21, 146.12, 146.05, 128.06, 128.03, 125.82, 125.79, 123.58, 123.55, 121.34, 121.30, 101.47, 101.30, 101.14, 100.92, 100.76, 100.59, 99.62, 99.59, 99.42, 99.39, 99.29, 99.26, 99.19, 99.09, 99.06, 98.98, 92.52, 92.27, 67.13, 65.63, 51.22, 50.97, 50.71, 50.46, 47.37, 44.94, 36.71, 33.33, 32.13, 29.90, 29.86, 29.81, 29.67, 29.57, 29.52, 29.49, 27.77, 27.20, 22.90, 22.14, 21.33, 15.38, 14.34 ppm. IR: ν 3411, 3207, 2923, 2853, 1638, 1616 cm^{-1}. HRMS [ESI]$^+$ calculated for $C_{20}H_{21}ClF_5N_6O_2$, 507.1335; found, 507.1335.

(*S*)-5-Chloro-6-(2,6-difluoro-4-(3-morpholinopropoxy)-phenyl)-*N*-(1,1,1-trifluoropropan-2-yl)[1,2,4]triazolo[1,5-*a*]-pyrimidin-7-amine (18). **18** was prepared as described in Zhang et al.[10] Reverse-phase HPLC purification afforded the title compound (68% yield). ^{1}H NMR (500 MHz; $CDCl_3$) δ 8.39 (s, 1H), 6.67−6.63 (m, 2H), 5.95 (d, J = 10.5 Hz, 1H), 4.81 (bs, 1H), 4.11 (t, J = 6.1 Hz, 2H), 3.82 (t, J = 4.7 Hz, 4H), 2.75 (m, 6H), 2.13 (m, 2H), 1.41 (d, J = 6.8 Hz, 3H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.76, 162.46 (t, J = 13.9 Hz), 161.78 (dd, J = 250.0, 8.9 Hz), 161.50 (dd, J = 248.8, 9.0 Hz), 158.50, 155.39, 154.15, 146.08, 130.76, 124.65 (q, J = 282.1 Hz), 100.82 (t, J = 20.9 Hz), 99.43 (dd, J = 25.5, 3.3 Hz), 99.17 (dd, J = 25.4, 3.3 Hz), 92.47, 66.93, 66.03, 55.08, 53.15, 50.80 (q, J = 32.0 Hz), 25.20, 15.28 ppm. IR: ν 3425, 2958, 2861, 2817, 1640, 1615, 1578 cm^{-1}. HRMS [ESI]$^+$ calculated for $C_{21}H_{23}N_6O_2F_5Cl$, 521.1486; found, 521.1488.

(*S*)-5-Chloro-6-(2,6-difluoro-4-(3-(phenylamino)propoxy)-phenyl)-*N*-(1,1,1-trifluoropropan-2-yl)[1,2,4]triazolo[1,5-*a*]-pyrimidin-7-amine (19). General procedure A was followed, using 3-(phenylamino)propan-1-ol and **8**. Reverse-phase HPLC purification afforded the title compound (53% yield). ^{1}H NMR (500 MHz; $CDCl_3$) δ 8.40 (s, 1H), 7.20 (m, 2H), 6.73 (m, 1H), 6.67 (m, 4H), 5.89 (d, J = 10.1 Hz, 1H), 4.77 (bs, 1H), 4.16 (t, J = 5.9 Hz, 2H), 3.40 (t, J = 6.6 Hz, 2H), 2.17 (quintet, J = 6.2 Hz, 2H), 1.41 (d, J = 6.8 Hz, 3H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ 162.59 (t, J = 14.0 Hz), 161.77 (dd, J = 250.1, 8.8 Hz), 161.51 (dd, J = 248.4, 8.9 Hz), 158.56, 155.41, 154.11, 148.08, 146.00, 129.51, 124.63 (q, J = 281.4, 280.7 Hz), 117.87, 112.94, 100.77 (t, J = 20.9 Hz), 99.41 (dd, J = 25.6, 3.2 Hz), 99.14 (dd, J = 25.4, 3.5 Hz), 92.43, 67.03, 50.79 (q, J = 32.3 Hz), 40.83, 29.02, 15.33 ppm. IR: ν 3338, 3209, 3113, 3055, 2950, 1614, 1575 cm^{-1}. HRMS [ESI]$^+$ calculated for $C_{23}H_{21}N_6OF_5Cl$, 527.1380; found, 527.1385.

(*S*)-6-Chloro-2-(pyrazin-2-yl)-5-(2,4,6-trifluorophenyl)-*N*-(1,1,1-trifluoropropan-2-yl)pyrimidin-4-amine (20). **20** was prepared as described in Zhang et al.[16] Purification by silica gel chromatography, using gradients of ethyl acetate in hexanes, afforded the title compound (18% yield). ^{1}H NMR (500 MHz; DMSO-d_6) δ 9.57 (d, J = 1.4 Hz, 1H), 8.85−8.83 (m, 2H), 7.70 (d, J = 8.8 Hz, 1H), 7.48−7.42 (m, 2H), 5.52 (m, 1H), 1.35 (d, J = 7.1 Hz, 3H) ppm. ^{13}C NMR (126 MHz, DMSO-d_6) δ 163.59 (dt, J = 248.3, 16.2 Hz), 161.84 (ddd, J = 247.9, 16.2, 9.5 Hz), 161.63, 161.14, 160.48 (ddd, J = 248.2, 16.1, 9.4 Hz), 159.98, 148.51, 146.39, 145.07, 144.59, 125.91 (q, J = 282.8 Hz), 105.42 (td, J = 21.4, 4.6 Hz), 103.73, 101.73 (td, J = 26.8, 3.8 Hz). 101.65 (td, J = 26.7, 3.8 Hz). 47.24 (q, J = 30.6 Hz), 13.14 ppm. IR: ν 3282, 2920, 1638, 1580, 1556 cm^{-1}. HRMS [ESI]$^+$ calculated for $C_{17}H_{11}ClF_6N_5$, 434.0607; found, 434.0616.

6-Chloro-2-(pyrazin-2-yl)-*N*-(2,2,2-trifluoroethyl)-5-(2,4,6-trifluorophenyl)pyrimidin-4-amine (21). **21** was prepared as described in Zhang et al.[16] Purification by silica gel chromatography, using gradients of ethyl acetate in hexanes, afforded the title compound (31% yield). ^{1}H NMR (500 MHz; $CDCl_3$) δ 9.65 (d, J = 1.4 Hz, 1H), 8.79 (dd, J = 2.4, 1.5 Hz, 1H), 8.72 (d, J = 2.4 Hz, 1H), 6.89−6.85 (m, 2H), 5.21−5.18 (m, 1H), 4.37 (qd, J = 8.7, 6.6 Hz, 2H) ppm. IR: ν 3255, 3134, 1695, 1634, 1580, 1557 cm^{-1}. HRMS [ESI]$^+$ calculated for $C_{16}H_9ClF_6N_5$, 420.0451; found, 420.0459.

(*S*)-6-Chloro-2-(pyridin-2-yl)-5-(2,4,6-trifluorophenyl)-*N*-(1,1,1-trifluoropropan-2-yl)pyrimidin-4-amine (22). **22** was prepared as described in Zhang et al.[16] Reverse-phase HPLC purification of the crude product afforded the title compound (50% yield). ^{1}H NMR (500 MHz; $CDCl_3$) δ 8.86 (d, J = 4.6 Hz, 1H), 8.43 (d, J = 7.9 Hz, 1H), 7.90−7.87 (m, 1H), 7.44 (dd, J = 7.2, 5.0 Hz, 1H), 6.92−6.86 (m, 2H), 5.41−5.35 (m, 1H), 4.60 (d, J = 9.3 Hz, 1H), 1.41 (d, J = 7.0 Hz, 3H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.22 (dt, J = 253.7, 15.0 Hz), 162.87, 161.41, 161.11, 160.88 (ddd, J = 252.8, 14.9, 8.9 Hz), 153.24, 149.82, 137.62, 125.69, 125.49 (q, J = 281.8 Hz), 124.29, 105.10 (td, J = 20.5, 20.0, 4.4 Hz), 104.17, 101.82 (td, J = 25.8, 4.1 Hz), 101.68 (td, J = 25.8, 4.1 Hz), 48.22 (q, J = 31.3 Hz), 14.65 (q, J = 1.8 Hz) ppm. HRMS [ESI]$^-$ calculated for $C_{18}H_{10}ClF_6N_4$, 431.0498; found, 431.0508.

6-Chloro-2-(pyridin-2-yl)-*N*-(2,2,2-trifluoroethyl)-5-(2,4,6-trifluorophenyl)pyrimidin-4-amine (23). **23** was prepared as described in Zhang et al.[16] Purification by silica gel chromatography, using gradients of ethyl acetate in hexanes, afforded the title compound (12% yield). ^{1}H NMR (500 MHz; $CDCl_3$) δ 8.83 (d, J = 4.0 Hz, 1H), 8.43 (d, J = 7.9 Hz, 1H), 7.88 (t, J = 7.3 Hz, 1H), 7.44 (t, J = 5.8 Hz, 1H), 6.84−6.81 (m, 2H), 5.04 (t, J = 6.0 Hz, 1H), 4.38 (dd, J = 15.4, 8.0 Hz, 2H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.31 (dt, J = 253.8, 15.1 Hz), 161.59, 161.51, 161.06 (dd, J = 252.8, 9.0 Hz), 160.94 (dd, J = 253.0, 9.0 Hz), 153.37, 149.79, 137.82, 125.77, 124.38 (q, J = 279.3 Hz), 124.37, 124.35, 105.19 (td, J = 21.2, 5.3 Hz), 104.46, 101.75 (td, J = 26.0, 3.4 Hz), 42.72 (q, J = 34.8 Hz) ppm. HRMS [ESI]$^+$ calculated for $C_{17}H_{10}ClF_6N_4$, 419.0498; found, 419.0496.

(*S*)-6-Chloro-5-(4-(3-(dimethylamino)propoxy)-2,6-difluoro-phenyl)-2-(pyrazin-2-yl)-*N*-(1,1,1-trifluoropropan-2-yl)-pyrimidin-4-amine (24). To a solution of **20** (0.03 g, 0.06 mmol) and 3-(dimethylamino)propan-1-ol (0.04 mL, 0.321 mmol) in dimethylsulfoxide (0.32 mL) was added sodium hydride (60% in mineral oil, 0.01 g, 0.321 mmol) at room temperature. The reaction mixture was stirred at 60 °C for 2 h, cooled to room temperature, dissolved in additional dimethylsulfoxide, filtered, and purified by preparative reverse phase HPLC to obtain the title compound as a white solid (8% yield). ^{1}H NMR (500 MHz; DMSO-d_6) δ 9.58 (d, J = 1.2 Hz, 1H), 8.86−8.84 (m, 2H), 7.70 (d, J = 8.8 Hz, 1H), 6.95 (t, J = 10.0 Hz, 2H), 5.53 (dd, J = 15.3, 7.6 Hz, 1H), 4.12 (t, J = 6.2 Hz, 2H), 2.38 (t, J = 7.1 Hz, 2H), 2.17 (s, 6H), 1.90 (quintet, J = 6.7 Hz, 2H), 1.36 (d, J = 7.1 Hz, 3H) ppm. ^{13}C NMR (126 MHz, DMSO-d_6) δ 165.05, 162.01, 161.70 (t, J = 14.4 Hz), 161.07 (dd, J = 244.9, 10.1 Hz), 160.73, 160.72 (dd, J = 244.1, 10.4 Hz), 160.14, 148.65, 146.28, 145.01, 144.57, 125.95 (q, J = 283.1 Hz), 104.77, 100.36 (t, J = 21.7 Hz), 99.37 (dd, J = 25.4, 2.4 Hz), 99.27 (dd, J = 25.5, 2.5 Hz), 66.93, 55.45, 47.21 (q, J = 29.2 Hz), 45.21, 26.69, 13.13 ppm. HRMS [ESI]$^+$ calculated for $C_{22}H_{23}ClF_5N_6O$, 517.1542; found, 517.1536.

6-Chloro-5-(4-(3-(dimethylamino)propoxy)-2,6-difluoro-phenyl)-2-(pyridin-2-yl)-*N*-(2,2,2-trifluoroethyl)pyrimidin-4-amine (25). **25** was prepared as for **24** from **23** and 3-(dimethylamino)-propan-1-ol (10% yield). ^{1}H NMR (500 MHz; MeOD) δ 8.72 (dd, J = 4.7, 0.7 Hz, 1H), 8.54−8.52 (m, 1H), 8.03 (td, J = 7.8, 1.7 Hz, 1H), 7.58 (ddd, J = 7.5, 4.9, 1.0 Hz, 1H), 6.79 (d, J = 9.3 Hz, 2H), 4.38 (q, J = 9.1 Hz, 2H), 4.14 (t, J = 6.0 Hz, 2H), 2.73 (t, J = 7.6 Hz, 2H), 2.45 (s, 6H), 2.07 (dd, J = 13.9, 7.3 Hz, 2H) ppm. ^{13}C NMR (126 MHz, MeOD) δ 170.36, 164.16, 163.70 (t, J = 14.2 Hz), 163.67, 162.82 (dd, J = 246.7, 10.0 Hz), 161.93, 154.88, 150.44, 139.20, 127.18, 126.34 (q, J = 279.2 Hz), 125.50, 106.57, 102.34 (t, J = 21.6 Hz), 100.48−100.17 (m), 100.56−100.17 (m), 68.10, 57.15, 45.20, 42.91 (q, J = 34.6 Hz), 27.54 ppm. HRMS [ESI]$^+$ calculated for $C_{22}H_{22}ClF_5N_5O$, 502.1433; found, 502.1425.

5,7-Dichloro-6-(2,4,6-trifluorophenyl)[1,2,4]triazolo[1,5-*a*]-pyrimidine (33). Following a reported procedure,[10] a slurry of 2-(2,4,6-trifluorophenyl)malonate (1.19 g, 4.10 mmol) and aminotriazole (0.36 g, 4.31 mmol) in tributylamine (1.03 mL) was heated to 170 °C for 2 h. The resulting homogeneous brown mixture was cooled to 130 °C, and toluene (4.00 mL) was added before cooling to 50 °C. A solution of sodium hydroxide (0.36 mL, 50% aqueous) was added, and the precipitated solids were collected by vacuum filtration, washed with

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cold toluene, and dried to afford the bis-sodium salt of **30** as a beige powder (1.24 g, 3.80 mmol, 93% yield).

A mixture of bis-sodium **30** (0.30 g, 0.9 mmol) and phosphorus oxychloride (1.50 mL, 16 mmol) was heated to 130 °C for 6 h. The reaction mixture was cooled to room temperature and was carefully quenched with H_2O (2 mL). This aqueous mixture was extracted with ethyl acetate (3 × 5 mL), and the combined organic layers were dried ($MgSO_4$), filtered, and concentrated to give the title compound as an orange oil that was used without further purification and without full characterization (50% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.62 (s, 1H), 6.94−6.89 (m, 2H) ppm. MS (ESI^+) 319.00 [M + H^+].

4,6-Dichloro-2-(pyrazin-2-yl)-5-(2,4,6-trifluorophenyl)-pyrimidine (34). **34** was prepared as described in Zhang et al.[16] Purification by silica gel chromatography, using gradients of ethyl acetate in hexanes, afforded the title compound that was used without full characterization (32% yield). ^{1}H NMR (500 MHz; $CDCl_3$) δ 9.73 (s, 1H), 8.84 (s, 1H), 8.77 (s, 1H), 6.88−6.84 (m, 2H) ppm.

4,6-Dichloro-2-(pyridin-2-yl)-5-(2,4,6-trifluorophenyl)-pyrimidine (35). **35** was prepared as **34** from picolinimidamide hydrochloride and 2-(2,4,6-trifluorophenyl)malonate (58% yield). The title compound was used without full characterization. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.16 (d, J = 5.4 Hz, 1H), 9.00−8.98 (m, 1H), 8.78 (td, J = 7.9, 1.2 Hz, 1H), 8.36 (t, J = 6.7 Hz, 1H), 6.88 (dd, J = 8.4, 7.5 Hz, 2H) ppm.

(*S*)-5-Azido-6-(2,4,6-trifluorophenyl)-*N*-(1,1,1-trifluoropropan-2-yl)[1,2,4]triazolo[1,5-*a*]pyrimidin-7-amine (36). To a solution of **8** (0.013 g, 0.032 mmol) in *N*,*N*-dimethylformamide (1 mL) under nitrogen was added sodium azide (0.002 g, 0.032 mmol). The reaction mixture was heated at 70 °C for 24 h. The reaction mixture was cooled to room temperature and then concentrated. After addition of water (5 mL), the mixture was extracted with ethyl acetate (3 × 5 mL) and the combined organic layers were washed with water (5 mL), dried ($MgSO_4$), filtered, and concentrated. Purification by silica gel preparative thin layer chromatography (hexanes/ethyl acetate 7/3) afforded the title compound as a brown solid (47% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.33 (s, 1H), 6.87 (dtd, J = 8.9, 6.5, 2.0 Hz, 2H), 5.92 (d, J = 10.9 Hz, 1H), 4.50 (s, 1H), 1.41 (d, J = 6.9 Hz, 3H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.34 (dt, J = 254.4, 15.3 Hz), 161.59 (ddd, J = 252.9, 14.9, 8.3 Hz), 161.26 (ddd, J = 250.7, 14.8, 8.3 Hz), 160.01, 154.79, 154.44, 145.64, 124.65 (q, J = 282.0 Hz), 103.99 (td, J = 21.1, 20.5, 4.5 Hz), 101.63 (td, J = 25.8, 4.2 Hz), 101.12 (td, J = 25.9, 4.1 Hz), 83.28, 50.69 (q, J = 32.1 Hz), 15.32 ppm. IR: ν 2918, 2852, 2136, 1624 cm^{-1}. MS (ESI^+) calculated for $C_{14}H_9F_6N_8$ [M+H^+], 403.27; found, 403.12.

6-Chloro-*N*-isopropyl-7-(2,4,6-trifluorophenyl)pyrido[2,3-*b*]-pyrazin-8-amine (37). **37** was prepared as described in Crowley et al.[18] Purification by silica gel preparative thin layer chromatography using a 1:1 mixture of ethyl acetate in hexanes as eluent furnished the title compound, as well as **38** and byproduct **43**. C-8 alkylated pyridopyrazine **37**[18]: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.99 (d, J = 1.9 Hz, 1H), 8.66 (d, J = 1.8 Hz, 1H), 6.93 (bs, 1H), 6.83 (dd, J = 8.6, 7.6 Hz, 2H), 3.36 (m, 1H), 1.11 (d, J = 6.3 Hz, 6H) ppm. HRMS [ESI]$^+$ calculated for $C_{16}H_{13}ClF_3N_4$, 353.0781; found, 353.0793. **38**[18]: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.84 (d, J = 2.0 Hz, 1H), 8.63 (d, J = 2.0 Hz, 1H), 6.93 (dd, J = 8.6, 6.9 Hz, 2H), 4.72−4.60 (m, 1H), 4.54 (s, 1H), 1.24 (d, J = 6.5 Hz, 6H) ppm. HRMS [ESI]$^+$ calculated for $C_{16}H_{13}ClF_3N_4$, 353.0781; found, 353.0782. Byproduct **43**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.84 (d, J = 1.7 Hz, 1H), 8.60 (d, J = 1.7 Hz, 1H), 7.13 (dd, J = 9.5, 2.1 Hz, 1H), 6.77 (ddd, J = 12.7, 9.4, 2.1 Hz, 1H), 5.76 (s, 1H), 5.61−5.42 (m, 1H), 1.68 (d, J = 7.1 Hz, 6H), 1.38 (d, J = 6.3 Hz, 6H) ppm. HRMS [ESI]$^+$ calculated for $C_{19}H_{20}F_2N_5$, 356.1687; found, 356.1688.

Acetyl α-Tubulin (AcTub) Assay. Compound-induced changes in QBI293 cell AcTub levels were determined as previously described.[8,31]

P-Glycoprotein (Pgp) Assay. The ability of test compounds to affect the Pgp transporter was assessed in NCI/ADR-RES cells (ATCC), which express the human Pgp. The NCI/ADR-RES cells were plated at 35 000 cells/well into 96-well flat-bottomed clear plates in 0.1 mL of minimal essential medium supplemented with 10% fetal bovine serum, 50 units/mL penicillin, and 50 μg/mL streptomycin. After overnight growth at 37 °C in 5% CO_2, test compounds, a known Pgp substrate (cyclosporin A), or known Pgp inhibitors (verapamil and epoD) that were dissolved in dimethylsulfoxide and diluted in PBS were added in triplicate (50 μL volume) over a range of concentrations such that the final dimethylsulfoxide concentration was 0.25%. Vehicle control wells were also included. After incubation for ∼20 min at 37 °C in 5% CO_2, Calcein AM was added to a final concentration of 0.25 nM (50 μL at a dimethylsulfoxide concentration of 0.2%) and allowed to incubate an additional 25 min at 37 °C in 5% CO_2. The medium was then removed, and the cells were washed twice with 0.2 mL of cold PBS, followed by analysis of calcein fluorescence with excitation at 485 nm and emission at 530 nm. Data were fit with a nonlinear regression sigmoidal dose−response, with the buffer control defining no inhibition and the average signal obtained with 0.1 mM verapamil defining 100% inhibition.

Determination of Plasma and Brain Drug Concentrations. Test compounds were administered to 2-month old CD1 mice (20 g average body weight) under a protocol approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Whole brain hemispheres, obtained from mice euthanized according to approved protocols, were homogenized in 10 mM ammonium acetate, pH 5.7 (1:2, 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 4500*g* at 4 °C. Aliquots (50 μL) of brain homogenate or plasma were mixed with 0.2 mL of acetonitrile, centrifuged at 15 000*g*, and the resulting supernatants were used for subsequent LC−MS/MS analysis. The LC−MS/MS system comprised an Aquity UPLC instrument and a TQ MS instrument that was controlled using MassLynx software (Waters Corporation, Milford, MA, USA). 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 water with 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 MS instrument 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 auto tune utility. Standard curves were generated for each compound from brain homogenate and plasma samples that had compound added at concentrations ranging from 0.005 to 5 μM and extracted as above. Drug half-life was calculated using the elimination rate constant determined from the slope of a line plotted through natural log plasma concentration versus time for the 4, 8, and 16 h time points using GraphPad Prism. Area under the brain or plasma concentration curve from 0.5 to 16 h was calculated using the trapezoid rule with GraphPad Prism.

Determination of Compound Unbound Fractions in Plasma and Brain. The assessment of nonspecific compound binding to plasma proteins and brain homogenates was as previously described.[8]

■ ASSOCIATED CONTENT

Supporting Information

NMR spectra of test compounds and X-ray crystallographic information and structures for compounds **23**, **36**, and **43**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

AcTub, acetylated α-tubulin; AD, Alzheimer's disease; AUC, area under the curve; BBB, blood–brain barrier; *B*/*P*, brain-to-plasma ratio; CNS, central nervous system; FTD, frontotemporal dementia; ip, intraperitoneal; iv, intravenous; MT, microtubule; NFT, neurofibrillary tangle; PD, pharmacodynamic; Pgp, P-glycoprotein; PK, pharmacokinetic; Tg, transgenic

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