Discovery of a Novel Series of Pyrazolo[1,5-α]pyrimidine-Based Phosphodiesterase 2A Inhibitors Structurally Different from N-((1S)-1-(3-Fluoro-4-(trifluoromethoxy)phenyl)-2-methoxyethyl)-7-methoxy-2-oxo-2,3-dihydropyrido[2,3-b]pyrazine-4(1H)-carboxamide (TAK-915), for the Treatment of Cognitive Disorders

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It has been hypothesized that selective inhibition of phosphodiesterase (PDE) 2A could potentially be a novel approach to treat cognitive impairment in neuropsychiatric and neurodegenerative disorders through augmentation of cyclic nucleotide signaling pathways in brain regions associated with learning and memory. Following our earlier work, this article describes a drug design strategy for a new series of lead compounds structurally distinct from our clinical candidate 2 (TAK-915), and subsequent medicinal chemistry efforts to optimize potency, selectivity over other PDE families, and other preclinical properties including in vitro phototoxicity and in vivo rat plasma clearance. These efforts resulted in the discovery of N-((1S)-2-hydroxy-2-methyl-1-(4-(trifluoromethoxy)phenyl)propyl)-6-methyl-5-(3-methyl-1H-1,2,4-triazol-1-yl)pyrazolo[1,5-α]pyrimidine-3-carboxamide (20), which robustly increased 3′,5′-cyclic guanosine monophosphate (cGMP) levels in the rat brain following an oral dose, and moreover, attenuated MK-801-induced episodic memory deficits in a passive avoidance task in rats. These data provide further support to the potential therapeutic utility of PDE2A inhibitors in enhancing cognitive performance.

**Key words** phosphodiesterase 2A; schizophrenia; pyrazolo[1,5-α]pyrimidine; structure-based drug design; phototoxicity; intramolecular hydrogen bond
of clinical candidate 2 (TAK-915) originating from high-throughput screening hit 1 (Fig. 1). Over the course of the discovery of 2, we identified another novel right-hand side (RHS) benzylamine moiety structurally distinct from 2, as exemplified by compound 3 (Fig. 2). Although compound 3 exhibited a good balance of potency and PDE selectivity, it was found to be a P-glycoprotein (P-gp) substrate as evidenced by high multidrug resistance protein 1 (MDR1) efflux ratio, resulting in lower brain exposure. On the other hand, the previously described pyrazolo[1,5-a]pyrimidine series, as represented by compound 4, demonstrated favorable MDR1 efflux ratios, but had suboptimal PDE2A inhibitory activity due to an unoptimized core and RHS benzylamine moieties. We therefore reasoned that replacing the 3,4-dihydropyrimido[2,3-b]pyrazin-2(1H)-one core in 3 with the pyrazolo[1,5-a]pyrimidine core derived from 4 would serve to improve brain penetration by reducing the overall HBD count. Indeed, hybrid compound 5 showed reasonable inhibition of PDE2A with an IC_{50} of 66 nM and no indication of being a P-gp substrate. These promising attributes of 5 as a lead compound encour-
aged us to focus on further optimization, aiming mainly to improve inhibitory activity.

Our previous structure–activity relationship (SAR) study of methyl substitutions on the pyrazolo[1,5-a]pyrimidine core of 1 demonstrated that the 6-methyl substitution provided an approximately 10-fold increase in the PDE2A inhibitory activity (see Fig. 1 for numbering of the substitution positions). The 5-methyl substitution was well tolerated, while the introduction of the methyl group into the 2- or 7-position was detrimental for potency.42) In good agreement with the previous SAR, introduction of the methyl group at the 6-position of compound 5 to give compound 6 (Fig. 3) resulted in a 13-fold boost in potency. An X-ray crystal structure of compound 7 bound to PDE2A revealed an unoccupied and relatively large hydrophobic space around the 5-position of the core (Fig. 4); this finding supported the tolerance for the 5-methyl substitution. These findings provided an avenue for further elaboration at the 5-position (R1 of the structure in Fig. 5) that might lead to potency enhancement. Overall, with the aim of further increasing the inhibitory activity to the levels of our leading clinical candidate 2 (IC50=0.61 nM), our medicinal chemistry effort was focused on variations of substituents at the 5 and 6-positions of the pyrazolo[1,5-a]pyrimidine core. Taking into account synthetic tractability, we initially chose to prepare 5-substituted pyrazolo[1,5-a]pyrimidine analogs with no methyl group at the 6-position (R2=H of the structure in Fig. 5).

Herein, we describe the SAR of 5- and/or 6-substituted pyrazolo[1,5-a]pyrimidine derivatives, culminating in the discovery of highly potent, selective, orally bioavailable, and brain-penetrating PDE2A inhibitors, as represented by N-((1S)-2-hydroxy-2-methyl-1-(4-(trifluoromethoxy)phenyl)propyl)-6-methyl-5-(3-methyl-1H-1,2,4-triazol-1-yl)-pyrazolo[1,5-a]pyrimidine-3-carboxamide (20). This new series successfully overcame in vitro phototoxicity issues identified by the introduction of substituents at the 5-position of the core. In addition, we report an X-ray crystal structure of 20 bound in the PDE2A active site to better understand the origin of high potency and PDE selectivity, as well as in vivo data linking target engagement and pharmacodynamic (PD) effects to relevant in vivo efficacy in a preclinical model of cognitive impairment.

Results and Discussion

Table 1 summarizes the effects of carbon-linked substituents at the 5-position of the pyrazolo[1,5-a]pyrimidine core. The corresponding data for 5 are also included in the table for
comparison. Introducing an isopropyl group at the 5-position (8) had little impact on potency relative to the non-substituted analog 5, while the introduction of a bigger and less lipophilic tetrahydro-2H-pyran group (9) tended to show moderately improved activity. However, compound 9 displayed an unfavorable MDR1 efflux ratio and high in vivo clearance, likely being driven by increased polarity due to the tetrahydro-2H-pyran group, and increased number of methylene moieties.

Table 1. Profiles of Pyrazolo[1,5-a]pyrimidine Derivatives with Substitutions at the 5-Position

| compd | R<sup>1</sup> | PDE2A IC<sub>50</sub> (nm)<sup>a</sup> | PDE selectivity<sup>b</sup> | MDR1<sup>c</sup> | rat in vivo CL (mL/h/kg)<sup>d</sup> | phototox (%)<sup>e</sup> | LogD<sup>f</sup> |
|-------|-------------|------------------|-----------------|-----------|------------------|-----------------|-----------|
| 5     | H           | 66 (41–110)      | >150-fold       | 0.54      | 632              | −0.1            | 2.38      |
| 8     | i-Pr        | 34 (24–49)       | 190-fold        | 0.48      | NT               | −9.2            | 3.30      |
| 9     |             | 10 (5.2–19)      | NT              | 3.6       | 4142             | −6.6            | 2.82      |
| 10    |             | 1.2 (0.80–1.8)   | 2700-fold       | 0.52      | 381              | 102.1           | 3.48      |
| 11    |             | 0.98 (0.61–1.6)  | 3800-fold       | 0.80      | 2361             | 78.9            | 3.38      |
| 12    |             | 0.77 (0.44–1.4)  | 1400-fold       | 0.80      | 4350             | 108.7           | 3.22      |
| 13    |             | 1.7 (1.2–2.5)    | 1300-fold       | 1.2       | 558              | 102.1           | 3.14      |
| 14    |             | 5.4 (3.4–8.7)    | 630-fold        | 0.54      | 353              | 11.0            | 3.29      |
| 15    |             | 1.2 (0.94–1.6)   | 1900-fold       | 5.2       | NT               | −1.0            | 3.08      |
| 16    |             | 7.1 (4.6–11)     | 230-fold        | 2.1       | 637              | 11.8            | 3.42      |
| 17    |             | 5.4 (3.5–8.3)    | 690-fold        | 0.73      | 476              | 7.5<sup>g</sup> | 3.12      |
| 18    |             | 11 (8.8–14)      | 610-fold        | 0.90      | 779              | 3.8             | 3.15      |

<sup>a</sup> IC<sub>50</sub> values (95% confidence intervals given in parentheses) were calculated from percent inhibition data (duplicate, n=1). All values are rounded off to two significant digits.  
<sup>b</sup> Minimum selectivity (rounded off to two significant digits) over other PDEs.  
<sup>c</sup> MDR1 efflux ratios in P-glycoprotein (P-gp)-overexpressing cells.  
<sup>d</sup> Plasma clearance calculated following 0.1 mg/kg, i.v. cassette dosing in rats (non-fasted).  
<sup>e</sup> Values (%) represent the difference between viability of cells exposed to 50 µM of the test compound in the absence of UV irradiation and that in the presence of UV irradiation, where cell viability was measured by intracellular ATP content (%) at 50 µM relative to vehicle control. A larger value is interpreted as a higher risk of phototoxicity.  
<sup>f</sup> LogD values at pH 7.4.  
<sup>g</sup> Precipitates observed.
susceptible to oxidative metabolism, respectively. To address such liabilities of the aliphatic heterocycle, we next sought to add heteroaryl groups. The 2-pyridyl substitution in compound 10 provided not only dramatically improved potency and excellent selectivity over other PDEs, but also a favorable MDR1 efflux ratio and significantly reduced in vivo clearance. In addition, 3- and 4-pyridine derivatives 11 and 12, respectively, retained desirable attributes of the 2-pyridine derivative 10, including inhibitory activity, PDE selectivity and MDR1 efflux ratio; however, they showed increased in vivo clearance probably due to oxidation of the methyl groups adjacent to the pyridine nitrogen atoms and/or N-oxidation of the pyridines. To overcome the potential metabolic liability of the methylpyridine derivatives, the difluoromethyl pyrazole analog 13 was prepared, and it exhibited favorable in vivo clearance coupled with excellent potency and PDE selectivity, and a favorable MDR1 efflux ratio. Unfortunately, however, the series of carbon-linked heteroaryl derivatives 10–13 were all found to possess significant in vitro phototoxicity potential, as evidenced by the large reduction in the viability of cells exposed to the compounds in the presence of UV radiation, relative to that in the absence of UV.\(^a\) Although the incidence of in vivo phototoxicity depends on drug distribution to light-exposed tissues like the skin and eye, it is generally recognized that reducing the in vitro phototoxicity potential of drug candidates during the lead-optimization process is a prudent strategy to minimize the potential risk for in vivo phototoxicity in animals and humans. A phototoxicity liability could impose significant limitations in a drug clinical use, including the requirement to avoid sun exposure by means of sunscreen, protective clothing, and eyewear.\(^{47,48}\) We therefore sought to minimize the in vitro phototoxicity liability observed in this compound series. With the hope of altering the electronic nature of the core, thus lowering the risk of in vitro phototoxicity, nitrogen-linked heteroaryl groups were explored. Installation of a pyrazole at the 5-position of the core to obtain 14, a regioisomer of 13, resulted in moderately decreased potency and PDE selectivity, but a favorable MDR1 efflux ratio and in vivo clearance. Of particular note was the fact that 14 showed greatly improved phototoxic liability. This encouraging result prompted us to synthesize the structurally related imidazole analog 15. The compound exhibited improved potency and PDE selectivity with no appreciable in vitro phototoxicity potential, but was found to be susceptible to P-gp mediated efflux, most likely due to the polar or basic nitrogen atom of the imidazole ring.\(^{44,45}\) In order to attenuate the basicity of the imidazole ring in 15, a two-pronged approach was implemented: first, the methyl group on the imidazole ring was replaced with an electron-withdrawing trifluoromethyl group to give 16, and second, an additional nitrogen atom was incorporated into the imidazole ring to afford two isomeric triazoles 17 and 18. As anticipated, all modifications improved the P-gp efflux ratio, and maintained favorable clearance and in vitro phototoxicity profile, although the result of the phototoxicity

Table 2. Profiles of Di-substituted Pyrazolo[1,5-a]pyrimidine Derivatives

| compd | R\(^1\) | PDE2A IC\(_{50}\) \(\text{nm}\)\(^a\) | PDE selectivity\(^b\) | MDR1\(^c\) | rat in vivo CL \(\text{mL/h/kg}\)\(^d\) | phototox \(\%\)^\(^e\) | LogD\(^f\) |
|-------|-------|------------------|-----------------|--------|------------------|---------------|--------|
| 6     | H     | 5.1 (4.3–6.1)    | 1600-fold       | 0.53   | 2743             | 4.2           | 2.66   |
| 19    | N     | 0.31 (0.27–0.35) | 5800-fold       | 1.2    | 940              | 13.0\(^e\)    | 3.24   |
| 20    | N     | 0.51 (0.46–0.58) | 5300-fold       | 1.0    | 1169             | 3.7           | 3.42   |

\(a\) IC\(_{50}\) values (95\% confidence intervals given in parentheses) were calculated from percent inhibition data (duplicate, \(n=1\)). All values are rounded off to two significant digits.  
\(b\) Minimum selectivity (rounded off to two significant digits) over other PDEs.  
\(c\) MDR1 efflux ratios in P-glycoprotein (P-gp)-overexpressing cells.  
\(d\) Plasma clearance calculated following 0.1 mg/kg, i.v. cassette dosing in rats (non-fasted).  
\(e\) Values (%) represent the difference between viability of cells exposed to 50 \(\mu\)M of the test compound in the absence of UV irradiation and that in the presence of UV irradiation, where cell viability was measured by intracellular ATP content (%) at 50 \(\mu\)M relative to vehicle control. A larger value is interpreted as a higher risk of phototoxicity.  
\(f\) LogD values at pH 7.4.  

Table 3. Inhibitory Activities of 19 and 20 against Human PDEs\(^a\)

| Compd | PDE1A | PDE2A | PDE3A | PDE4D2 | PDE5A1 | PDE6AB | PDE7B | PDE8A1 | PDE9A2 | PDE10A2 | PDE11A4 |
|-------|-------|-------|-------|--------|--------|--------|-------|--------|--------|---------|---------|
| 19    | 3300  | 0.31  | >10000| 3300   | 8800   | 1800   | >10000| >10000 | >10000 | 1700    | 3000    |
| 20    | 2700  | 0.51  | >10000| 9400   | 9100   | 8000   | >10000| >10000 | >10000 | 3100    | 6100    |

\(a\) IC\(_{50}\) values (\(\mu\)M) were calculated from percent inhibition data (duplicate, \(n=1\)). All values are rounded off to two significant digits.
assay of compound 17 was inconclusive due to its precipitation at the concentration tested, 50 \( \mu M \). Despite these benefits, both compounds showed a concomitant 5- to 10-fold loss in potency, in addition to a decrease in PDE selectivity. Overall, substitutions at the 5-position of the pyrazolo[1,5-\( a \)]pyrimidine core did not result in analogs with aligned potency, PDE selectivity, and other preclinical properties.

In an attempt to rescue insufficient potency and PDE selectivity of compounds 17 and 18, which were otherwise close to meeting our goals, the methyl substitution at the 6-position of the pyrazolo[1,5-\( a \)]pyrimidine core was examined (19, 20 in Table 2), since the 6-methyl substitution was previously shown to significantly enhance both potency and PDE selectivity.\(^{22}\) In good agreement with the previous findings, introducing the methyl group at the 6-position of the pyrazolo[1,5-\( a \)]pyrimidine core of 17 and 18 to provide 19 and 20, respectively, demonstrated a substantial improvement of potency and PDE selectivity (see Table 3 for inhibitory activity profile against each PDE family), without compromising MDR1 efflux ratios and in vitro phototoxicity risks. The phototoxic potential of compound 19 was not accurately assessed due to its low aqueous solubility, with precipitation observed at 50 \( \mu M \) as in the case of 17. It appears certain, however, that its phototoxic potential was considerably lower than those of carbon-linked heteroaryl derivatives such as 10–13. This is because compound 19 showed no signs of phototoxicity liability at 25 \( \mu M \) where no precipitate occurred, while carbon-linked derivatives 10–13 all displayed significant levels of UV-induced cytotoxicity at the same concentration (data not shown). It is also worth noting that plasma clearance of 19 and 20 in rat was significantly improved compared with that of 6, which lacks the substituent at the 5-position. Metabolite identification studies on 6 with human and rat hepatocytes revealed a single metabolite common to both species, and also indicated the 6-methyl group of the core to be the most likely site of oxidative metabolism. Based on these results, it was speculated that the triazole rings in 19 and 20 may contribute to attenuation of oxidative metabolism of the methyl groups through steric shielding effects. In the meantime, further profiling of physicochemical properties of both compounds demonstrated that 19 had extremely poor aqueous solubility that could lead to non-linear oral absorption, while 20 was approximately 10-fold more soluble with high degree of crystallinity similar to 19 (Table 4). These results are consistent with the solubility issue identified in the in vitro phototoxicity assay. Consequently, compound 20 was advanced to further characterization by an X-ray co-crystallization analysis as well as a series of in vivo studies.

The X-ray co-crystal structure of 20 bound to the catalytic domain of the PDE2A enzyme is presented in Fig. 6A and that of 7 in Fig. 6B. Compound 20 retains a number of key interactions observed in the case of the first generation lead 7,\(^{42}\) including (i) a C–H hydrogen bonding interaction of the pyrazolo[1,5-\( a \)]pyrimidine core with the carbonyl oxygen atom of Gln859, (ii) \( \pi-\pi \) and CH–\( \pi \) interactions of the core with the Phe862 and Ile826 located above and below the core, respectively, (iii) van der Waals interactions of the 6-methyl group of the core with a hydrophobic cavity surrounded by Tyr827, Leu858, and Met847, (iv) interactions of the amide carbonyl group with two water molecules, one of which serves as a bridging water molecule to interact with the OH of Tyr655 (these interactions are not shown in the figure for simplicity), and (v) hydrophobic interactions of the RHS trifluoromethoxyphenyl group with the hydrophobic pocket newly formed due to ligand binding. In addition to the above conserved interactions, new ones characteristic of 20 that may greatly contribute to the potency enhancement are observed. The triazole ring incorporated into the 5-position of the core effectively fills the space sandwiched between Ile866 on the top and Met847 on the bottom, thus making favorable van der Waals interactions. It appears that a hydrogen bond between the triazole nitrogen atom with a bridging water molecule and the

**Table 4. Thermodynamic Solubility of Compounds 19 and 20**

| Compd. | Thermodynamic solubility (\( \mu g/mL \)) | Crystallinity (%) |
|--------|------------------------------------------|------------------|
|        | pH 1.2 | pH 6.8 | pH 6.8+GCDC\(^{a}\) |                      |
| 19     | 0.45   | 0.32   | 16     | 89                      |
| 20     | 7      | 4.1    | 140    | 83                      |

\(^{a}\) Solubility in a pH 6.8 solution containing sodium glycochenodeoxycholate (GCDC). This is considered as simulated intestinal fluid (pH 6.8) in the presence of bile acid.

![Fig. 6. (A) X-Ray Crystal Structure of 20 Bound in the PDE2A Catalytic Site (PDB 5VP1) and (B) X-Ray Crystal Structure of 7 Bound in the PDE2A Catalytic Site (PDB 5XKM)](image-url)
backbone carbonyl group of Leu858 may have some positive impacts on the potency. Furthermore, the branched tertiary alcohol moiety would serve to provide greater conformational rigidity to the molecule through not only an intramolecular hydrogen bond between the oxygen atom of the tertiary alcohol and the amide NH, but also restriction of the bond rotation imparted by the gem-dimethyl group, and thereby offer a benefit with reduced entropic cost of binding by biasing the RHS benzylamine moiety toward the bioactive conformation.

The preclinical pharmacokinetic (PK) profiles of compound 20 in rat and mouse are summarized in Table 5. Compound 20 exhibited favorable PK parameters in both rat and mouse, characterized by reduced plasma clearance and moderate oral bioavailability. In addition, the compound demonstrated reasonable brain uptake with a brain to plasma ratio of 0.32 in rat, although it had a high TPSA value (114 Å²) outside the traditional range of CNS drugs. 44,45,49) These results suggested that the intramolecular hydrogen bonds observed in 20 effectively shield the polarity of the molecule, thus improving blood-brain barrier permeability.

In order to assess target engagement, the dose–occupancy relationship of compound 20 was characterized by measuring PDE2A occupancy 2 h after oral administration of five escalating doses: 0.3, 1, 3, 10, and 30 mg/kg. As shown in Fig. 7, compound 20 dose-dependently occupied PDE2A in the frontal cortex, hippocampus, and striatum of rat brain, where the PDE2A enzyme is highly expressed, 13–18) were measured to assess the PD effects of compound 20 (Fig. 8). Consistent with the previous observations, 11,29,42,45,50) compound 20 dose-dependently increased cGMP levels in all selected brain regions at doses as low as 3 mg/kg, corresponding to ca. 37% occupancy levels, while not affecting cAMP levels. These results suggest that oral administration of 20 at doses of 3 mg/kg or above would lead to significant pharmacological effects in preclinical models of cognition. We therefore performed a passive avoidance test in MK-801-treated rats to evaluate the effects of 20 at an oral dose of 3 mg/kg on deficits in episodic memory induced by N-methyl-d-aspartate (NMDA) receptor hypofunction 51,52) (Fig. 9). Subcutaneous administration of MK-801 at 0.1 mg/kg significantly decreased the avoidance time in the retention test, compared to saline treatment (p < 0.01). Pretreatment with 3 mg/kg of 20 significantly attenuated the decrease in the avoidance time induced by MK-801 (p < 0.01). The ability of 20 to reverse deficits in episodic memory produced by MK-801, suggests its potential for the treatment of cognitive deficits seen in a range of psychiatric disorders with impaired glutamatergic neurotransmission.

Chemistry The RHS benzylamine moiety 28 was prepared as outlined in Chart 1. Benzaldehyde 21 was initially converted to the corresponding hydantoin 22 using the Bucherer–Bergs reaction. 23) Hydantoin 22 was then hydrolyzed

Table 5. PK Parameters and $K_p$ Values of Compound 20 in Rats and Mice

| PK Parameter | Rat         | Mouse      |
|--------------|-------------|------------|
| $C_{max}^{(a)}$ (ng/mL) | 58.1        | 181.0      |
| $T_{max}^{(b)}$ (h)     | 2.0         | 0.7        |
| $AUC_{0-\infty}^{(c)}$ (ng·h/mL) | 272.8       | 628.6      |
| $K_{ss}^{(d)}$ (%)      | 31.2        | 48.2       |
| $K_p^{(e)}$             | 0.32        | —          |

(a) Cassette dosing at 0.1 mg/kg, i.v. and 1 mg/kg, p.o. (non-fasted), in an average of 3 rats or mice. b) Total clearance. c) Volume of distribution at steady state. d) Maximum plasma concentration. e) Time of maximum concentration. f) Area under the plasma concentration vs. time curve (0–8h). g) Oral bioavailability. h) Brain-to-plasma ratio at 2h after oral administration of 20 at a dose of 10 mg/kg.

![Fig. 7](image-url). *In Vivo PDE2A Target Occupancy in the Striatum Measured 2h after Oral Administration of 20 in Rats*
under basic conditions to afford amino acid 23. The protection of the amino group of 23 with a Boc group using Schotten–Baumann conditions and subsequent methylation of the carboxylic acid 24 gave methyl ester 25. Treatment of 25 with methyl Grignard reagent, followed by chiral separation provided enantiomers 27a and 27b. Finally, the Boc group of the desired enantiomer 27a was removed under acidic conditions to give RHS benzylamine 28 as a hydrochloride salt.

A series of 5-substituted pyrazolo[1,5-a]pyrimidine derivatives 8–18 were prepared following the procedures depicted in Chart 2. Starting from elaboration of the pyrazolopyrimidine core 30 by the condensation of 5-amino-1H-pyrazole 29 with a mixture of cis- and trans-ethyl 3-ethoxyacrylate under basic conditions, the ethyl ester of 30 was hydrolyzed to afford carboxylic acid 31. Bischlorination of 31 with phosphoryl chloride (POCl₃) gave 32, which was subsequently reacted with RHS benzylamine 28, to furnish 5-chloropyrazolo[1,5-a]pyrimidine 33. Attachment of the substituent at the 5-position of the pyrazolo[1,5-a]pyrimidine core was finally accomplished by the following three approaches: i) Suzuki–Miyaura cross-coupling of the corresponding boronic acids or esters to deliver 11–13; ii) Suzuki–Miyaura cross-coupling followed by catalytic hydrogenation of the olefin moieties to deliver 8 and 9; and iii) nucleophilic displacement with the corresponding azoles to deliver 14–18. However, Suzuki–Miyaura cross-coupling employing 33 to obtain 2-pyridyl derivative 10 was unsuccessful, probably due to the relatively unstable nature of 2-pyridylboron species as well as the presence of polar functionalities such as the amide and the tertially alcohol moieties in 33 that could potentially coordinate with the palladium catalyst. Therefore, attempts were made to introduce the 2-pyridyl group at the early step prior to the amide coupling with the RHS benzylamine moiety. After chlorination of 30 with POCl₃, 5-chloropyrazolo[1,5-a]pyrimidine 36 was successfully coupled with lithium 2-pyridyltriolborate salt 37 via Suzuki–Miyaura cross-coupling catalyzed by palladium(II) acetate and triphenylphosphine (Ph₃P) in the presence of copper(I).
iodide, providing coupled product 38. Ester hydrolysis of 38 and subsequent amide formation with RHS benzylamine 28 furnished the final compound 10.

The synthetic route to access compounds 19 and 20 is highlighted in Chart 3. Aminopyrazole 43 was initially constructed in a two-step sequence of reactions, involving the condensation of tert-butyl 2-cyanoacetate (40) and tert-butoxy bis(dimethylamino) methane (41), followed by reaction with hydrazine. The aminopyrazole 43 was then condensed with 1,3-dicarbonyl related electrophile 46 prepared by bromination of methyl methacrylate (44), prior to treatment with a methanol solution of sodium methoxide to provide pyrazolo[1,5-a]pyrimidine core 47. Regioselective installation of triazole rings at the 5-position of the core was carried out by two different approaches using conditions mild enough to be compatible with the acid-labile tert-butyl ester moiety. One approach involved diphenylphosphoryl azide (DPPA)-mediated azidation prior to the regioselective 1,3-dipolar cycloaddition of the resulting azido group of 48 to the C=C bond of the betainic form of α-formylethylidenetriphenylphosphorane (49) with subsequent spontaneous elimination of triphenylphosphine oxide, providing coupled product 38. Ester hydrolysis of 38 and subsequent amide formation with RHS benzylamine 28 furnished the final compound 10.

In conclusion, a new class of PDE2A inhibitors, structurally different from our clinical candidate TAK-915 were identified by incorporating features from our previously reported PDE2A inhibitor series such as 3 and 4. With an initial goal of enhancing the potency of new lead compound 5, substitutions on the pyrazolo[1,5-a]pyrimidine core, especially at the 5- and 6-positions, were explored on the basis of previous SAR knowledge and crystal structure information of pyrazolo[1,5-a]pyrimidine analog 7. Our optimization efforts resulted in the discovery of N-(1S)-2-hydroxy-2-methyl-1-(4-(trifluoromethoxy)-phenyl)propyl)-6-methyl-5-(3-methyl-1H-1,2,4-triazol-1-yl)-pyrazolo[1,5-a]pyrimidine-3-carboxamide (20), a potent brain-penetrating PDE2A inhibitor highly selective over other PDE families. Introduction of a nitrogen-linked heteroaryl substituent at the 5-position as in 20 was key to alleviating in vitro phototoxicity risk. In addition, despite its high TPSA value (114 Å³) which is beyond the optimal range for CNS drugs, compound 20 exhibited acceptable brain exposure probably due to cloaking of HBD and HBA groups via intramolecular hydrogen bonding. This was also supported by an X-ray crystal structure of 20 bound to PDE2A. In the subse-
quent in vivo studies on 20, we confirmed dose-dependent target engagement in rat brain after oral dosing. Correlated with these results, compound 20 exhibited dose-dependent increases in cGMP levels in the brain and, moreover, improved cognitive function in a rat model of schizophrenia following oral administration at the dose where PD effects were noted. Thus, potent and selective PDE2A inhibitors, such as compound 20, are hypothesized to provide a novel therapeutic approach to treat cognitive dysfunction in a range of neuropsychiatric and neurodegenerative disorders, including schizophrenia and Alzheimer’s disease.

Experimental

General Chemistry Information All solvents and reagents were obtained from commercial sources and were used as received. Microwave-assisted reactions were carried out in a single-mode reactor, Biotage Initiator 2.0 or 2.5 microwave synthesizer. Yields were not optimized. All reactions were monitored by TLC analysis on Merck Kieselgel 60 F254 plates or Fuji Silysia NH plates, or LC-MS analysis. LC-MS analysis was performed on a Shimadzu LC-MS system operating in atmospheric pressure chemical ionization (APCI) (+ or −) or electrospray ionization (ESI) (+ or −) ionization mode. Analytes were eluted using a linear gradient with a mobile phase of water/acetonitrile containing 0.05% trifluoroacetic acid (TFA) or 5 mM ammonium acetate and detected at 220 nm. Column chromatography was carried out on silica gel ((Merck Kieselgel 60, 70–230 mesh, Merck) or (Chromatorex NH-DM 1020, 100–200 mesh, Fuji Silysia Chemical, Ltd.)), or on pre-packed Purif-Pack columns (SI or NH, particle size: 60 µm, Fuji Silysia Chemical, Ltd.). Analytical HPLC was performed using a Corona Charged Aerosol Detector or photo diode array detector with a Capcell Pak C18AQ (3.0 mm ID × 50 mm, Shiseido, Japan) or L-column2 ODS (2.0 mm ID × 30 mm, CERI, Japan) column at a temperature of 50°C and a flow rate of 0.5 mL/min. Mobile phases A and B under neutral conditions were a mixture of 50 mmol/L ammonium acetate, water, and acetonitrile (1:8:1, v/v/v) and a mixture of 50 mmol/L ammonium acetate and acetonitrile (1:9, v/v), respectively. The ratio of mobile phase B was increased linearly from 5 to 95% over 3 min, and then maintained at 95% over the next 1 min. Mobile phases A and B under acidic conditions were a mixture of 0.2% formic acid in 10 mmol/L ammonium formate and 0.2% formic acid in acetonitrile, respectively. The ratio of mobile phase B was increased linearly from 14 to 86% over 3 min, and then maintained at 86% over the next 1 min. All final test compounds were purified to >95% chemical purity as measured by analytical HPLC. Elemental analyses were carried out by Takeda Analytical Laboratories, and all results were within ±0.4% of the theoretical values. Melting

Reagents and conditions: (a) neat, r.t., 30 min (taken on crude); (b) NH₂NH₂·H₂O, MeOH, 70°C, overnight, 64%; (c) Br₂, EtOAc, r.t., 16 h, (taken on crude); (d) NaOMe, MeOH, 70°C, 3 h, (taken on crude); (e) C₅H₅CO₂, DMF, 100°C, 16 h, 84%; (f) DPPA, DBU, THF, 60°C, 5 h, 74%; (g) 49, toluene, 80°C, 3 h, then MgCl₂, 60°C, 2 h, 97%; (h) CCl₄, PPh₃, 1,2-dichloroethane, 75–85°C, 4.5 h, 88%; (i) 3-methyl-1H-1,2,4-triazole, K₂CO₃, DMF, 60°C, 2 h, 69%; (j) MsOH, CH₃CN, r.t.–60°C, 5.5 h–overnight, 83–91%; (k) RHS benzylamine 28, EDCI·HCl, HOBT·H₂O, Et₃N, DMF, r.t., 2 h–overnight, 75–82%.

Chart 3. Synthesis of 6-Methyl-5-Substituted Pyrazolo[1,5-a]pyrimidine Derivatives 19 and 20
points were determined on a BÜCHI B-545 melting point apparatus or a DSC1 system (Mettler-Toledo International Inc., Greifensee, Switzerland). Proton nuclear magnetic resonance (1H-NMR) spectra were recorded on a Varian Mercury-300 (300 MHz) or Bruker DPX300 (300 MHz) instrument. All 1H-NMR spectra were consistent with the proposed structures. All proton shifts are given in parts per million (ppm) downfield from tetramethylsilane (δ) as the internal standard in deuterated solvent, and coupling constants (J) are in hertz (Hz). NMR data are reported as follows: chemical shift, integration, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; quint, quintet; m, multiplet; dd, doublet of doublets; td, triplet of doublets; ddd, doublet of doublets; and brs, broad singlet), and coupling constants. Very broad peaks for protons of, for example, hydroxyl and amino groups are not always indicated.

N-(1S)-2-Hydroxy-2-methyl-1-(4-(trifluoromethoxy)phenyl)propyl)-5-(6-methylpyridin-3-yl)pyrazolo[1,5-a]pyrimidine-3-carboxamide (8) A mixture of 34 (38.6 mg, 0.09 mmol) and 5% Pd/C–ethylenediamine complex (10 mg) in MeOH (5 mL) was hydrogenated under balloon pressure at room temperature (r.t.) for 16 h. The catalyst was removed by filtration and the filtrate was concentrated in vacuo. The residue was purified by column chromatography (silica gel, hexane–ethyl acetate, 19 : 1 to 0 : 100) to afford 8 (32.1 mg, 0.07 mmol, 83%) as a white amorphous solid. 1H-NMR (300 MHz, DMSO-d6) δ: 0.99–1.08 (3H, m), 1.27 (3H, s), 1.41 (3H, d, J = 4.5 Hz), 1.43 (3H, d, J = 4.5 Hz), 3.20–3.34 (1H, m), 4.81–5.00 (2H, m), 7.22–7.34 (3H, m), 7.48–7.61 (2H, m), 8.44 (1H, d), 9.07 (1H, d, J = 8.7 Hz), 9.19 (1H, d, J = 7.2 Hz). MS (ESI/APCI) m/z 437.1 [M+H]+. HPLC purity: 98.9%.

N-(1S)-2-Hydroxy-2-methyl-1-(4-(trifluoromethoxy)phenyl)propyl)-5-(tetrahydro-2H-pyran-4-yl)pyrazolo[1,5-a]pyrimidine-3-carboxamide (9) A mixture of 35 (84.4 mg, 0.177 mmol) and Pd/C (en) (15 mg) in MeOH (10 mL) and tetrahydrofuran (THF) (dry) (2 mL) was hydrogenated under balloon pressure at r.t. for 16 h. The catalyst was removed by filtration and the filtrate was concentrated in vacuo. The residue was purified by column chromatography (silica gel, hexane–ethyl acetate, 19:1 to 0:100) to afford 9 (82.9 mg, 0.173 mmol, 98%) as a white amorphous solid. 1H-NMR (300 MHz, DMSO-d6) δ: 1.04 (3H, s), 1.28 (3H, s), 1.86–2.11 (4H, m), 3.13–3.28 (1H, m), 3.45–3.59 (2H, m), 3.97–4.10 (2H, m), 4.88–4.99 (2H, m), 7.24–7.34 (3H, m), 7.50–7.61 (2H, m), 8.45 (1H, s), 9.03 (1H, d, J = 8.7 Hz), 9.21 (1H, d, J = 7.2 Hz). MS (ESI/APCI) m/z 479.1 [M+H]+. HPLC purity: 99.6%.

N-(1S)-2-Hydroxy-2-methyl-1-(4-(trifluoromethoxy)phenyl)propyl)-5-(pyridin-2-yl)pyrazolo[1,5-a]pyrimidine-3-carboxamide (10) A mixture of crude 39 (79.0 mg, 0.329 mmol), 28 (94.0 mg, 0.329 mmol), HATU (163 mg, 0.428 mmol), N,N-diisopropylethylamine (DIEA) (0.172 mL, 0.987 mmol), and N,N-dimethylformamide (DMF) (3 mL) was stirred at 90°C for 30 min and then poured into water. The organic layer was separated, washed with saturated aqueous NaCl, dried over anhydrous Na2SO4 and concentrated in vacuo. The residue was purified by column chromatography (basic silica gel column chromatography followed by silica gel column chromatography). 1H-NMR (300 MHz, DMSO-d6) δ: 1.21 (3H, s), 1.49 (3H, s), 1.96 (1H, s), 5.14 (1H, d, J = 8.3 Hz), 7.14–7.22 (2H, m), 7.44–7.56 (3H, m), 7.88–7.97 (1H, m), 8.26 (1H, d, J = 7.4 Hz), 8.64 (1H, s), 8.75–8.88 (3H, m), 9.25 (1H, d, J = 8.3 Hz). MS (ESI/APCI) m/z 472.1 [M+H]+. HPLC purity: 97.3%.

N-(1S)-2-Hydroxy-2-methyl-1-(4-(trifluoromethoxy)phenyl)propyl)-5-((2-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridine (100 mg, 0.455 mmol), (Amphos)2PdCl2 (21.5 mg, 0.0303 mmol), K2CO3 (62.9 mg, 0.455 mmol), toluene (2 mL) and water (0.2 mL) was heated at 120°C under microwave irradiation for 2.5 h.

To a mixture of crude 33 (130 mg, 0.276 mmol, 84%) was added K2CO3 (62.9 mg, 0.455 mmol), toluene (2 mL) and water (0.2 mL) was heated at 120°C under microwave irradiation for 2.5 h.

To a mixture of crude 33 (100 mg, 0.237 mmol, 78%) was added K2CO3 (62.9 mg, 0.455 mmol), toluene (2 mL) and water (0.2 mL) was heated at 120°C under microwave irradiation for 2.5 h.

To a mixture of crude 33 (130 mg, 0.237 mmol, 78%) was added K2CO3 (62.9 mg, 0.455 mmol), toluene (2 mL) and water (0.2 mL) was heated at 120°C under microwave irradiation for 2.5 h.

To a mixture of crude 33 (130 mg, 0.237 mmol, 78%) was added K2CO3 (62.9 mg, 0.455 mmol), toluene (2 mL) and water (0.2 mL) was heated at 120°C under microwave irradiation for 2.5 h.

To a mixture of crude 33 (130 mg, 0.237 mmol, 78%) was added K2CO3 (62.9 mg, 0.455 mmol), toluene (2 mL) and water (0.2 mL) was heated at 120°C under microwave irradiation for 2.5 h.
and saturated aqueous NaCl, dried over anhydrous Na2SO4 and concentrated in vacuo. The residue was purified by column chromatography (silica gel, hexane–ethyl acetate, 19:1 to 0:100) to afford 14 (102 mg, 0.222 mmol, 75%) as a white solid after trituration with hexane–ethyl acetate (5:1). 1H-NMR (300 MHz, DMSO-d6) δ: 1.02 (3H, s), 1.36 (3H, s), 4.86 (1H, d, J=8.3 Hz), 5.34 (1H, s), 6.75–6.81 (1H, m), 7.23–7.33 (2H, m), 7.48–7.58 (2H, m), 7.76 (1H, d, J=7.5 Hz), 8.05 (1H, d, J=1.5 Hz), 8.46 (1H, s), 9.02–9.14 (2H, m), 9.37 (1H, d, J=7.5 Hz). MS (ESI/APCI) m/z 461.1 [M+H]+. HPLC purity: 100%. mp 241°C.

N-((1S)-2-Hydroxy-2-methyl-1-(4-(trifluoromethoxy)phenyl)propyl)-5-(3-methyl-1H-1,2,4-triazol-1-yl)pyrazolo[1,5-alpyrimidine-3-carboxamide (15) The title compound was prepared as a pale yellow solid after trituration with hexane–ethyl acetate (5:1) in 70% yield from 33 and 4-methyl-1H-imidazole using the procedure analogous to that described for the synthesis of 14, except that a preparative HPLC (column: L-Column2 ODS 20 mm ID×150 mm L; mobile phase A: 0.1% TFA in water; mobile phase B: 0.1% TFA in acetonitrile; flow rate: 20 mL/min) was employed in place of silica gel column chromatography. 1H-NMR (300 MHz, DMSO-d6) δ: 0.85 (3H, t, J=7.2 Hz), 1.48 (3H, s), 1.49 (3H, s), 1.74 (1H, s), 2.44 (3H, s), 4.87 (1H, d, J=8.3 Hz), 5.39 (1H, s), 7.23–7.34 (2H, m), 7.48–7.58 (2H, m), 7.93 (1H, d, J=7.5 Hz), 8.55 (1H, s), 8.88 (1H, d, J=0.8 Hz), 9.07 (1H, d, J=8.3 Hz), 9.50 (1H, d, J=7.5 Hz). MS (ESI/APCI) m/z 476.2 [M+H]+. HPLC purity: 99.4%. mp 260°C.

N-((1S)-2-Hydroxy-2-methyl-1-(4-(trifluoromethoxy)phenyl)propyl)-5-(3-methyl-1H-1,2,4-triazol-1-yl)pyrazolo[1,5-alpyrimidine-3-carboxamide (18) The title compound was prepared as a white solid after trituration with disopropyl ether in 35% yield from 33 and 3-methyl-1H,1,2,4-triazole using the procedure analogous to that described for the synthesis of 14, except that basic silica gel was employed in a column chromatography purification in place of silica gel. 1H-NMR (300 MHz, DMSO-d6) δ: 1.02 (3H, brs), 1.36 (3H, s), 2.47 (3H, brs), 4.86 (1H, d, J=8.3 Hz), 5.43 (1H, s), 7.21–7.35 (2H, m), 7.47–7.59 (2H, m), 7.63 (1H, d, J=7.5 Hz), 8.52 (1H, s), 9.06 (1H, d, J=8.1 Hz), 9.46 (1H, d, J=7.5 Hz), 9.55 (1H, s). MS (ESI/APCI) m/z 476.1 [M+H]+. HPLC purity: 100%.

(S)-N-((2-Hydroxy-2-methyl-1-(4-(trifluoromethoxy)phenyl)propyl)-6-methyl-5-(3-methyl-1H-1,2,4-triazol-1-yl)pyrazolo[1,5-alpyrimidine-3-carboxamide (19) To a mixture of 52a (2.81 g, 10.9 mmol) and 28 (3.26 g, 11.4 mmol) in DMF (250 mL) were added HOBt·H2O (2.06 g, 13.1 mmol), EDCI·HCl (2.55 g, 13.1 mmol) and triethylammonium acetate (TEA) (7.65 mL, 54.4 mmol). The mixture was stirred at r.t. overnight and then quenched with water at r.t. The mixture was extracted with EtOAc, washed with saturated NaHCO3 water, and then saturated aqueous NaCl, dried over anhydrous Na2SO4, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, hexane–ethyl acetate, 100:0 to 3:17) to give 19 (3.86 g, 7.89 mmol, 73%) as a white solid. 1H-NMR (300 MHz, CDCl3) δ: 1.17 (3H, s), 1.48 (3H, s), 1.74 (1H, s), 2.50 (3H, d, J=0.8 Hz), 2.84 (3H, d, J=0.8 Hz), 5.05 (1H, d, J=8.3 Hz), 7.17 (2H, d, J=7.9 Hz), 7.38–7.48 (2H, m), 8.60 (1H, s), 8.69–8.82 (3H, m). The obtained product (3.86 g, 7.89 mmol) was dissolved in EtOAc (277 mL) at 50–60°C. To this solution was added hexane (150 mL) at the same temperature and a white solid precipitated. After 2h of stirring at r.t., further hexane (88 mL) was added to the mixture, which was stirred at r.t. for 10 min. The white precipitate was collected by filtration, washed with hexane–ethyl acetate, and dried to give 19 (3.17 g, 6.48 mmol, 82%) as a white solid. 1H-NMR (300 MHz, CDCl3) δ: 1.17 (3H, s), 1.48 (3H, s), 1.74 (1H, s), 2.50 (3H, d, J=0.8 Hz), 2.84 (3H, d, J=0.8 Hz), 5.05 (1H, d, J=8.3 Hz), 7.17 (2H, d, J=8.3 Hz), 7.39–7.48 (2H, m), 8.60 (1H, s), 8.69–8.83 (3H, m). MS (ESI/APCI) m/z 492.0 [M+H]+. HPLC purity: 100%. mp 238–239°C. Anal. Calcd for C27H20F2N6O4: C, 53.99; H, 4.53; F, 11.64; N, 20.03. Found: C, 53.82; H, 4.42; F, 11.64; N, 19.81.

(S)-N-((2-Hydroxy-2-methyl-1-(4-(trifluoromethoxy)phenyl)propyl)-6-methyl-5-(3-methyl-1H-1,2,4-triazol-1-yl)pyrazolo[1,5-alpyrimidine-3-carboxamide (20) To a suspension of 52b (606 mg, 2.35 mmol) in DMF (12 mL) were added 28 (805 mg, 2.82 mmol), EDCI·HCl (540 mg, 2.82 mmol), HOBT·H2O (431 mg, 2.82 mmol), and TEA (0.392 mL, 2.82 mmol) at r.t. The mixture was stirred at r.t. for 2 h. The mixture was poured into water and extracted with EtOAc. The organic layer was separated, washed with water and saturated aqueous NaCl, dried over anhydrous MgSO4, and concentrated in vacuo. The residue (42.7 mg) was crystallized from hexane–ethyl acetate to give 17 (34.4 mg, 0.072 mmol, 7.2%) as a white solid. 1H-NMR (300 MHz, DMSO-d6) δ: 1.04 (3H, s), 1.37 (3H, s), 2.44 (3H, s), 4.87 (1H, d, J=8.3 Hz), 5.39 (1H, s), 7.23–7.34 (2H, m), 7.48–7.58 (2H, m), 7.93 (1H, d, J=7.5 Hz), 8.55 (1H, s), 8.88 (1H, d, J=0.8 Hz), 9.07 (1H, d, J=8.3 Hz), 9.50 (1H, d, J=7.5 Hz). MS (ESI/APCI) m/z 476.2 [M+H]+. HPLC purity: 99.4%. mp 260°C.
(300 MHz, DMSO-d$_6$) δ: 1.00 (3H, s), 1.31 (3H, s), 2.46 (3H, s), 2.64 (3H, d, J=0.8 Hz), 4.87 (1H, d, J=8.0 Hz), 5.32 (1H, s), 7.27 (2H, d, J=8.0 Hz), 7.51 (2H, d, J=8.7 Hz), 8.49 (1H, s), 8.85 (1H, d, J=8.3 Hz), 9.46–9.53 (2H, m). MS (ESI/APCI) m/z 490.3 [M+H]$^+$. HPLC purity: 100%. mp 212°C. Anal. Calc. for C$_{29}$H$_2$_F$_3$N$_2$O$_2$: C, 53.99; H, 4.53; N, 20.03. Found: C, 54.11; H, 4.50; N, 20.04.

2-Amino-2-((trifluoromethoxy)phenyl)acetic Acid (23) The solution of potassium cyanide (19.4 g, 299 mmol) in water (170 mL) at 50°C was added dropwise to a solution of 4-trifluoromethoxy benzaldehyde (21) (34.1 mL, 239 mmol) and ammonium carbonate (62.0 g, 645 mmol) in EtOH (273 mL) and water (109 mL). The mixture was stirred at 60°C for 3 h. After cooling to r.t., EtOH was removed under reduced pressure. To the mixture was added to pH ca. 1 with conc HCl. The solid was filtered off and washed with water. To a solution of potassium hydroxide (66.2 g, 1000 mmol) in water (714 mL) was added 2 M NaOH aqueous solution (714 mL, 290 mmol) at 0°C. The mixture was stirred at 130°C for 4 h and then concentrated to afford 23 (26.9 g, 95.0 mmol, 85%) as a white solid. 1H-NMR (300 MHz, DMSO-d$_6$) δ: 1.05 (3H, s), 1.30–1.47 (12H, m), 4.50 (1H, d, J=6.4 Hz), 5.53 (1H, d, J=8.7 Hz), 7.18 (2H, d, J=7.9 Hz), 7.33 (2H, d, J=8.7 Hz). MS (ESI/APCI) m/z 348.0 [M–H]$^–$.

tert-Butyl ((1S)-2-Hydroxy-2-methyl-1-(4-(trifluoromethoxy)phenyl)propyl)carbamate (27a) Resolution of the enantiomers of 26 was carried out chromatographically using a Chiralpak AD 20 mm ID×250 mm L column (hexane–etherane, 950:50) at 80 mL/min. Resolution of 26 (22.3 g, 63.8 mmol) provided 27a as a white solid (10.2 g, 29.1 mmol, 46%, 91% theoretical) as the first eluting enantiomer. Analytical HPLC analysis carried out on a 4.6 mm ID×250 mm L Chiralpak AD column with the same eluent as above at a flow rate of 1.0 mL/min indicated that 27a was of >99.9% ee. 1H-NMR (300 MHz, CDCl$_3$) δ: 1.05 (3H, s), 1.36 (3H, s), 1.40 (9H, brs), 1.51 (1H, brs), 4.50 (1H, d, J=7.5 Hz), 5.53 (1H, d, J=8.3 Hz), 7.13–7.23 (2H, m), 7.29–7.39 (2H, m).

(1S)-1-Amino-2-methyl-1-(4-((trifluoromethoxy)phenyl)propan-2-ol Hydrochloride (28) A mixture of 27a (10.2 g, 29.3 mmol) and 4 M HCl solution in EtOAc (70.0 mL, 280 mmol) was stirred at r.t. for 1.5 h. The mixture was concentrated in vacuo and the resulting solid was triturated with hexane–dioxane or ether, collected by filtration, rinsed with hexane–dioxane or ether, and then filtered to afford 28 (7.00 g, 24.5 mmol, 84%) as a pale red solid. 1H-NMR (300 MHz, DMSO-d$_6$) δ: 0.98 (3H, s), 1.23 (3H, s), 4.23 (1H, brs), 5.39 (1H, s), 7.37–7.48 (2H, m), 7.63 (2H, d, J=8.7 Hz), 8.46 (3H, brs).

**Ethyl 5-Hydroxyprazolo[1,5-a]pyrimidine-3-carboxylate (30)** To a mixture of ethyl 3-amino-1H-pyrazole-4-carboxylate (29) (30.0 g, 193 mmol) and ethyl 3-ethoxy-2-propenoate (cis- and trans-mixture, 41.9 mL, 290 mmol) in DMF (387 mL) was added cesium carbonate (113 g, 348 mmol). The mixture was stirred at 100°C for 2 h, diluted with water and then acidified to pH ca. 5 with AcOH. The resulting solid was filtered by filtration, washed with water and dried to afford 30 (36.4 g, 176 mmol, 91%) as a beige solid. 1H-NMR (300 MHz, DMSO-d$_6$) δ: 1.28 (3H, t, J=7.1 Hz), 4.28 (2H, q, J=7.1 Hz), 6.15 (1H, d, J=7.9 Hz), 8.13 (1H, s), 8.57 (1H, d, J=7.9 Hz), 11.73 (1H, brs).

5-Hydroxyprazolo[1,5-a]pyrimidine-3-carboxylic Acid (31) To a solution of 30 (20.8 g, 100 mmol) in THF (223 mL) and EtOH (111 mL) was added 2 M NaOH aqueous solution (201 mL, 401 mmol). The mixture was stirred at 30°C overnight. The mixture was evaporated under reduced pressure to remove solvents and then neutralized with 2 M HCl aqueous solution (201 mL, 401 mmol). The resulting solid was collected by filtration, rinsed with water/EtOH and dried to give 31 (17.7 g, 99 mmol, 98%) as a beige solid. 1H-NMR (300 MHz, DMSO-d$_6$) δ: 6.14 (1H, d, J=7.9 Hz), 8.09 (1H, s), 8.56 (1H, d, J=7.9 Hz).

5-Chloropyrazolo[1,5-a]pyrimidine-3-carboxyl Chloride (32) To cooled (0°C) POCl$_3$ (71.0 mL, 762 mmol) were added 31 (5.00 g, 27.9 mmol) and DIEA (16.1 mL, 92.1 mmol). The mixture was stirred at 130°C for 4 h and then concentrated in vacuo. The residue was diluted with toluene and water at 0°C, and then extracted with EtOAc. The organic layer was separated, washed with saturated NaCl, dried over anhydrous MgSO$_4$, filtered through a column of silica gel pad (eluted with ethyl acetate) and concentrated in vacuo. The resulting solid was triturated with heptane, collected by filtration, washed with heptane and dried to give 32.
To a cooled (0°C) mixture of 32 (1.48 g, 6.85 mmol) and CH₃CN (30 mL) were added 28 (1.96 g, 6.85 mmol) and DIEA (3.58 mL, 20.6 mmol), and the mixture was stirred at 0°C to r.t. for 16 h. The mixture was poured into water and extracted with EtOAc. The organic layer was separated, washed with saturated aqueous NaCl, dried over anhydrous Na₂SO₄, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, hexane–ethyl acetate, 100:0 to 0:100) to afford 33 (2.27 g, 5.29 mmol, 77%) as white amorphous solids. ¹H-NMR (300 MHz, CDCl₃) δ: 1.19 (3H, s), 1.40 (3H, s), 1.88 (1H, s), 5.12 (1H, d, J=8.7 Hz), 6.99 (1H, d, J=7.2 Hz), 7.17–7.23 (2H, m), 7.45–7.53 (2H, m), 8.56 (1H, d, J=8.7 Hz), 8.62 (1H, s), 8.67 (1H, d, J=7.2 Hz). MS (ESI/APCI) m/z 429.1 [M+H]^+.

Ethyl 5-(Pyridin-2-yl)pyrazolo[1,5-a]pyrimidine-3-carboxylate (38) A mixture of ethyl 36 (300 mg, 1.33 mmol), lithium 4-phenylcyclopentadienylbis(dimethylamino) methane (34 mg, 0.436 mmol), 4 M NaOH aqueous solution (0.363 mL) and EtOH (3.5 mL) was stirred at 60°C for 2 h. After being cooled to 0°C, the mixture was acidified with 6M HCl aqueous solution to give crude 38 (70.9 mg, 0.329 mmol, 75%) as a pale yellow solid. This was used in the next reaction without further purification.

tert-Butyl 2-Cyano-3-(dimethylamino)acrylate (42) A mixture of tert-butyl 2-cyanoacetate (40) (24.3 g, 172 mmol) and tert-butoxy bis(dimethylamino)methane (41) (35.5 mL, 172 mmol) was stirred at r.t. for 30 min. The mixture was evaporated to give 42 as a pale yellow solid. This was used in the next reaction without further purification.

tert-Butyl 3-Amino-1H-pyrazole-4-carboxylate (43) A mixture of 42 (33.8 g, 172 mmol) and hydrazine monohydrate (8.39 mL, 172 mmol) in MeOH (344 mL) was stirred at 70°C overnight. After cooling to r.t., the mixture was poured into water at r.t. and extracted with EtOAc. The organic layer was separated, washed with saturated aqueous NaCl, dried over anhydrous MgSO₄ and concentrated in vacuo. The residue was crystallized from diisopropyl ether to give 43 (20.0 g, 109 mmol, 64%) as a pale orange solid. ¹H-NMR (300 MHz, CDCl₃) δ: 1.47 (3H, t, J=7.1 Hz), 4.46 (2H, q, J=7.1 Hz), 7.40–7.47 (1H, m), 7.86–7.95 (1H, m), 8.25 (1H, d, J=7.4 Hz), 8.59 (1H, s), 8.71–8.76 (2H, m), 8.80 (1H, d, J=7.4 Hz). MS (ESI/APCI) m/z 269.1 [M+H]^+.
in vacuo to afford 45 (152 g, 584 mmol, 97%) as a colorless oil. 1H-NMR (300 MHz, DMSO-d_6) δ: 1.95 (3H, s), 3.76 (3H, s), 4.08–4.14 (1H, m), 4.16–4.22 (1H, m).

**Methyl 3,3-Dimethoxy-2-methylpropanoate (46)** A suspension of sodium methoxide (28% MeOH solution, 226 g, 1.17 mol) in MeOH (234 mL) was heated to 70°C and then a solution of 45 (152 g, 584 mmol) in MeOH (20 mL) was added rapidly. The mixture was stirred at 70°C for 3 h. After cooling to r.t., the mixture was filtered and washed with MeOH, and then the filtrate was concentrated in vacuo. The residue was partitioned between EtO and water. The organic layer was washed with water and saturated aqueous NaCl, dried over anhydrous MgSO_4, filtered, and concentrated in vacuo to give 46 (54.5 g, 336 mmol, 58%) as a pale yellow oil. This was used in the next reaction without further purification. 1H-NMR (300 MHz, CDCl_3) δ: 1.17 (3H, d, J=7.2 Hz), 2.71–2.85 (1H, m), 3.35 (3H, s), 3.38 (3H, s), 3.70 (3H, s), 4.50 (1H, d, J=7.6 Hz).

**tert-Butyl 5-Hydroxy-6-methylpyrazolo[1,5-a]pyrimidine-3-carboxylate (47)** To a mixture of 43 (20.0 g, 109 mmol) and 46 (26.6 g, 164 mmol) in DMF (219 mL) was added cesium carbonate (64.2 g, 197 mmol). The mixture was stirred and added MgCl_2 (4.54 g, 47.7 mmol). After 15 min, to the mixture was added MgCl_2 (3.03 g, 31.8 mmol). The mixture was stirred at 80°C for 3 h. To the mixture was added DBU (4.52 mL, 30.0 mmol) and DPPA (5.17 mL, 24.0 mmol) in THF (100 mL) was added 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (4.03 g, 20.0 mmol). The mixture was stirred at r.t. overnight. The starting material remained on TLC and further methanesulfonic acid (0.555 mL, 8.40 mmol) was added. The mixture was stirred at r.t. for 1 h and then neutralized with 1 M NaOH aqueous solution at 0°C. The organic solvent was evaporated under reduced pressure and then diluted with water. The resulting solid was collected by filtration, washed with water and then hexane, and dried to give 52a (1.98 g, 7.67 mmol, 91%) as a white solid. 1H-NMR (300 MHz, DMSO-d_6) δ: 2.40 (3H, s), 2.52 (3H, d, J=0.8 Hz), 8.50 (1H, d, J=1.1 Hz), 8.62 (1H, s), 9.50 (1H, d, J=1.1 Hz), 12.52 (1H, brs). MS (ESI/APCI) m/z 259.2 [M+H]^+.

**tert-Butyl 5-Chloro-6-methylpyrazolo[1,5-a]pyrimidine-3-carboxylate (50)** Carbon tetrachloride (9.73 mL, 100 mmol) was added to a solution of PPh_3 (27.1 g, 100 mmol) in 1,2-dichloroethane (223 mL) at r.t. The mixture was stirred at the same temperature under N2 for 30 min. To this mixture was added a suspension of 47 (5.0 g, 20.1 mmol) in 1,2-dichloroethane (111 mL) at r.t. and the resulting mixture was stirred under N2 at 75–85°C for 4.5 h, and then concentrated in vacuo. The residue was partitioned between EtOAc and water. The phases were separated and the aqueous phase was extracted with EtOAc. The combined organic phases were washed with saturated aqueous NaCl, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, hexane–ethyl acetate, 100:0 to 7:3) to give 50 (4.75 g, 17.7 mmol, 88%) as a white solid. 1H-NMR (300 MHz, CDCl_3) δ: 1.62 (9H, s), 2.43 (3H, d, J=0.9 Hz), 8.40 (1H, s), 8.52 (1H, d, J=0.9 Hz).

**tert-Butyl 6-Methyl-5-(3-methyl-1H-1,2,4-triazol-1-yl)-pyrazolo[1,5-a]pyrimidine-3-carboxylate (51b)** To a solution of 50 (1.08 g, 4.03 mmol) in DMF (20 mL) was added 3-methyl-1H-1,2,4-triazole (0.469 g, 5.65 mmol) and K_2CO_3 (0.781 g, 5.65 mmol) at r.t. The mixture was stirred at r.t. overnight and warmed to 60°C for 2 h. After cooling to r.t., water was added to the mixture. The solid was collected by filtration, washed with water, and dried to give 51b (0.870 g, 2.77 mmol, 74%) as a pale green solid. 1H-NMR (300 MHz, DMSO-d_6) δ: 1.51–1.65 (9H, m), 2.12–2.55 (3H, m), 8.38–8.49 (1H, m), 8.85–9.11 (1H, m). MS (ESI/APCI) m/z 275.1 [M+H]^+.

**tert-Butyl 6-Methyl-5-(3-methyl-1H-1,2,4-triazol-1-yl)-pyrazolo[1,5-a]pyrimidine-3-carboxylic Acid (52b)** To a suspension of 51b (0.87 g, 2.77 mmol) in CH_3CN (14 mL) was added MsOH (1.26 mL, 19.4 mmol) at 0°C. After being stirred at r.t. for 5 h, the mixture was warmed to 60°C for 30 min. One molar NaOH aqueous solution (19.4 mL, 19.4 mmol) was added there to give 52b (0.606 g, 2.35 mmol, 85%) as a tan solid. 1H-NMR (300 MHz, DMSO-d_6) δ: 2.42 (3H, s), 2.48–2.53 (3H, m), 8.60 (1H, s), 9.09 (1H, s), 9.44 (1H, d, J=1.1 Hz), 12.49 (1H, brs). MS (ESI/APCI) m/z 259.1 [M+H]^+.

**Enzyme Assay Protocol**

Preparation of Human PDE

Human PDE1A, 3A, 4D2, 5A1, 7B, 8A1, 9A2, and 11A4 enzymes were purchased from BPS Bioscience. Human PDE6AB enzyme was purchased from SB Drug Discovery. Human PDE2A3 full-length gene was transduced into SF9 cells, and the enzyme was purified by His-tag affinity column and gel filtration. Human PDE10A2 was generated from...
COS-7 cells that had been transfected with the full-length gene. The enzymes were stored at −70°C until use.

PDE2A3 Enzyme Inhibitory Assay

PDE activity was measured using an SPA (Scintillation Proximity Assay) (GE Healthcare). To evaluate the inhibitory activity of a compound, 10 µL of serially diluted compounds were incubated with 20 µL of PDE enzyme (final concentration 0.023 nM) in assay buffer (50 mM N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (HEPES)–NaOH, 8.3 mM MgCl₂, 1.7 mM ethylene glycol bis(2-aminoethyl)ether-N,N,N′,N′-tetraacetic acid (EGTA), and 0.1% bovine serum albumin (BSA) (pH 7.4)) for 30 min at r.t. Final concentration of dimethyl sulfoxide (DMSO) in the reaction solution was 1%. Compounds were tested in duplicate in 96-well half-area plates (Corning) or 384-well OptiPlates (PerkinElmer, Inc.). We used an 8 concentration serial dilution dose response ranging from 100 µM to 10 µM compound concentrations. To start the reaction, 10 µL of substrate [³²H] cGMP (final concentration 77 nM, PerkinElmer, Inc.) were added to a total volume of 40 µL. After 60 min at r.t., 20 µL of 20 mg/mL yttrium silicate SPA beads containing zinc sulfate were added to terminate the PDE reaction. After resting undisturbed for an additional 60 min, the assay plates were counted in a scintillation counter (PerkinElmer, Inc.) to allow calculation of the inhibition rate.

The IC₅₀ value was obtained by fitting the results to the following 4 Parameter Logistic Equation:

\[ y = A + \frac{(B - A)}{1 + (10^{C - 4 \times D})} \]

where \(A\) is the minimum value, \(B\) is the maximum value, \(C\) is the \(\text{IC}_{50}\) value, and \(D\) is the slope factor.

Human PDE Enzyme Assay

PDE activities were measured using an SPA (GE Healthcare). To evaluate the inhibitory activity, 10 µL of serially diluted compounds were incubated with 20 µL of PDE enzymes (except for PDE1A) in assay buffer (50 mM HEPES–NaOH, 8.3 mM MgCl₂, 1.7 mM EGTA, and 0.1% BSA (pH 7.4)) for 30 min at r.t. The PDE1A enzyme assay was performed in a different assay buffer (50 mM Tris–HCl, 8.3 mM MgCl₂, 0.2 mM CaCl₂, 0.1% BSA, and 30 mM Calmodulin (pH 7.5)). The final concentration of DMSO in the assay was 1%. Compounds were tested in duplicate in 96-well half-area plates (Corning). We used an 4 concentration serial dilution dose response ranging from 10 µM to 10 nM compound concentrations. To start the reaction, 10 µL of substrate [³²H] cGMP (final concentration 77 nM, PerkinElmer, Inc.) for PDE1A, 5A1, 6A, 9A2, 10A2, and 11A4 or [³²H] cAMP (final concentration 14.7 nM, PerkinElmer, Inc.) for PDE3A, 4D2, 7B, and 8A1) were added for a final assay volume of 40 µL. After 60 min incubation at r.t., 20 µL of 20 mg/mL yttrium silicate SPA beads containing ZnSO₄ were added to terminate the PDE reaction. After resting undisturbed for more than 120 min, the assay plate was counted in a scintillation counter (PerkinElmer, Inc.) to allow calculation of the inhibition rate.

Transcellular Transport Study Using a Transporter-Expression System

Human MDR1-expressing LLC-PK1 cells were cultured with minor modifications to the method reported previously. The transcellular transport study was performed as reported previously. In brief, the cells were grown for 7 d in HTS Transwell 96-well permeable support (pore size 0.4 µm, 0.143 cm² surface area) with polyethylene terephthalate membrane (Corning Life Sciences, Lowell, MA, U.S.A.) at a density of 1.125×10⁵ cells/well. The cells were preincubated with M199 at 37°C for 30 min. Subsequently, transcellular transport was initiated by the addition of M199 either to apical compartments (75 µL) or to basolateral compartments (250 µL) containing 10 µM digoxin, 200 µM lucifer yellow (as a marker for the tightness of the monolayer), and 10 µM test compounds. The assay was terminated by the removal of each assay plate after 2 h. Aliquots (25 µL) from the opposite compartments were mixed with CH₃CN containing alprenolol and diclofenac as internal standards, and then centrifuged. The compound concentrations in the supernatant were measured by LC-MS/MS. The apparent permeability (P_app) of test compounds in the receiver wells was determined and the efflux ratio (ER) for the MDR1 membrane permeability test was calculated using the following equation:

\[ ER = \frac{P_{\text{app, A to B}}}{P_{\text{app, A to B}}} \]

where \(P_{\text{app, A to B}}\) is the apical-to-basal passive permeability-surface area product and \(P_{\text{app, B to A}}\) is the basal-to-apical passive permeability-surface area product.

Phototoxicity Test

Phototoxicity assay was carried out as described in the OECD guideline No. 432 with some modifications for a high-throughput screening. BALB/c 3T3 cells were cultured at 37°C under 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 50 IU/mL penicillin and 50 µg/mL streptomycin. Cells were seeded at 2.5×10⁴ cells/well in 384-well white plates, and cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 IU/mL penicillin, and 50 µg/mL streptomycin for 1 d. Two 384-well plates per test compound (50 µM) in Earle’s Balanced Salt Solution (EBSS) supplemented with 1 mM HEPES were preincubated for 1 h. One of the two plates was irradiated (+UV) for 60 min with 1.4–1.7 mW/cm² (5–6 J/cm²), whereas the other plate was kept in the dark (−UV). In both plates the treatment medium was replaced with the culture medium and after another 24 h of culture, the cell viability was determined by measuring the cellular ATP content. The cellular ATP content was measured using the Celltiter-Glo® assay kit (Promega) following the manufacturer’s instruction. ATP content was calculated as follows. ATP content (% of control)=(relative light unit (RLU) of test compound/RLU of 1% DMSO)×100.

Estimation of LogD at pH 7.4

LogD₇.₄, which is the partition coefficient of the compounds between 1-octanol and aqueous buffer at pH 7.4, was measured using a chromatographic procedure based on a published method. The instruments utilized were a Waters Alliance 2795 HPLC system and a 2996 UV-Vis detector (Milford, MA, U.S.A.).

Thermodynamic Solubility Measurement Using the Shake-Flask Method

The measurement of thermodynamic solubility was carried out as previously described. Briefly, the drug substances were weighed into Thomson filter vials (Chrom Tech, Inc., Minnesota, U.S.A.). JP1 (pH 1.2), JP2 (pH 6.8), and JP2-containing 20 mM GCDC were added to the vials. The vials were incubated at 37°C for 18 h and the resulting suspensions were filtered by compressing the vials. The drug concentration of the filtrates was determined using a UHPLC system.

Powder X-Ray Diffraction (PXRD) and Crystallinity
**Calculation** PXRD patterns were collected using a RINT Ultima IV powder X-ray diffractometer (Rigaku Corp., Tokyo, Japan) according to previously described conditions. The peak intensities of the crystalline (Ic) and non-crystalline (Ia) fractions were integrated from the baseline collection according to Herman's method. The crystallinity was calculated using the following equation with an autocrystallinity calculation software (Rigaku).

\[
\text{Crystallinity} = \frac{Ic}{Ic + Ia} \times 100
\]

**Protein Expression and Purification** The PDE2A catalytic domain (578–919) was cloned into a pFastBac vector, for expression in SF9 cells, utilizing an N-terminal 6× polyhistidine tag containing a TEV cleavage site. Large scale production of recombinant protein was carried out in SF9 cells. The pellet from 10L of baculovirus infected SF9 cells was resuspended in 600 mL lysis buffer containing 25 mM Tris pH 7.6, 1 mM NaCl, 20 mM imidazole, 5% glycerol, and 3 Roche Complete Protease Inhibitor tablets. The cell suspension was homogenized with the Polytron PT-3100, centrifuged for 1 h at 13000 rpm (JA-14 rotor), and the clarified supernatant was brought to 800 mL with lysis buffer before batch binding with 10 mL of ProBond Ni resin (Invitrogen) for 2 h at 4°C, rolling. The beads were collected by low speed centrifugation (3500 rpm with JS-4.2 rotor), loaded into a gravity column, and washed slowly overnight with 2L of wash buffer containing 25 mM Tris pH 7.6, 1 mM NaCl, 20 mM imidazole, 5% glycerol. The following day the protein was eluted with buffer containing 25 mM Tris pH 7.9, 50 mM NaCl, 250 mM imidazole, 10% glycerol. The 1.5 mL sample eluted from the Nickel capture step was brought to 9 mL with Mono Q buffer A containing 25 mM Tris pH 7.9, and 10% glycerol. After the full sample volume was bound to the Mono Q column, a salt gradient was applied from 0x NaCl to ca. 800 mM NaCl in 40 mL. Fractions corresponding to the unphosphorylated protein (identified by MS with MW=40178 Da) were pooled for further purification by size-exclusion chromatography on a Superdex 200 column equilibrated in 1×TBS pH 7.4, 0.5 mM DTT, 1 mM EDTA, 10% glycerol. Peak SEC fractions were collected and concentrated to 12 mg/mL for crystallization.

**Crystallization and Structure Determination** Crystals suitable for data collection were first grown using the vapor diffusion method in hanging drops at r.t. by adding 0.5 µL protein solution with 1 mM IBMX (1-methyl-3-(2-methylpropyl)-2,3,6,7-tetrahydro-1H-purine-2,6-dione) and 0.5 µL reservoir solution (30% PEG 3350, 0.1 mM Tris pH 7.5, and 0.2 mM MgCl2). PDE2A IBMX crystals were soaked in a drop containing 25 mM Tris pH 7.6, 1 M NaCl, 20 mM imidazole, 5% glycerol. After the full sample volume was bound to the Mono Q column, a salt gradient was applied from 0x NaCl to ca. 800 mM NaCl in 40 mL. Fractions corresponding to the unphosphorylated protein (identified by MS with MW=40178 Da) were pooled for further purification by size-exclusion chromatography on a Superdex 200 column equilibrated in 1×TBS pH 7.4, 0.5 mM DTT, 1 mM EDTA, 10% glycerol. Peak SEC fractions were collected and concentrated to 12 mg/mL for crystallization.

**Brain and Plasma Concentration in Rats** Compound 20 was administered orally to Long–Evans rats (male, non-fasted, 7-week old) at 10 mg/kg. Blood and whole brain samples were collected 2 h after oral administration. The brain samples were centrifuged to obtain the plasma fraction. The brain samples were homogenized in saline to obtain the brain homogenate. Compound concentrations were measured in aliquots of rat plasma and brain, which were mixed well with acetonitrile containing an internal standard and then centrifuged. The supernatants were diluted with solvents for LC-MS/MS analysis (mobile phase A: 10 mM ammonium formate–formic acid (100:0.2, v/v), mobile phase B: acetonitrile–formic acid (100:0.2, v/v)). The diluted solutions were injected into an LC-MS/MS (APIS5000, AB Sciex, Foster City, CA, U.S.A.) equipped with a Shimadzu Shim-pack XR-ODS column (2.2 µm packing particle size, 2.0 mm ID×30 mm L) maintained at 50°C. The chromatographic separation was performed using gradient elution at a flow rate of 0.7 mL/min. The LC time program was as follows: Mobile phase B was held at 5% for 0.1 min, and increased linearly to 95% in 0.1 min. After maintaining B at 95% for another 0.8 min, it was decreased to 5% in 0.01 min, followed by re-equilibration for 0.59 min. The total cycle time for one injection was 1.6 min. Compound 20 was detected using multiple reaction monitoring mode and the transition m/z 490.01→240.96. Analyst® software (version 1.4.2) was used for data acquisition and processing.

**In Vivo Occupancy Study** In vivo target occupancy study
of compound 20 was conducted using LC-MS/MS. Compound 20 was suspended in 0.5% (w/v) methylcellulose in distilled water, and PF-05270430 (0.1 mg/mL/kg) was dissolved in dimethylacetamide and 1,3-butaneisol (1:1). Sprague-Dawley (SD) rats were pretreated with vehicle (p.o., n=6) or compound 20 (0.3, 1, 3, 10, 30 mg/kg, p.o., n=4 in each group) 2h before sampling. PF-05270430 (0.1 mg/mL/kg) was administered by bolus intravenous injection via lateral tail vein 30 min before sampling. Rats were decapitated, and blood and brain samples (striatum as target tissue and cerebellum as reference tissue) were collected. Brain samples were weighed and saline was added (20% (w/v)), followed by homogenization with Lysing Matrix I beads (MP Biomedicals). Homogenized samples were stored at −30°C until quantification of tracer (PF-05270430) matrix I beads (MP Biomedicals). Homogenized samples were added (20% (w/v)), followed by homogenization with Lysing Matrix I beads (MP Biomedicals). Homogenized samples were stored at −30°C until quantification of tracer (PF-05270430) using LC-MS/MS. The supernatants were diluted with solvents for LC-MS/MS analysis (mobile phase A: 10 mM ammonium formate–formic acid (100:0.2, v/v), mobile phase B: acetonitrile–formic acid (100:0.2, v/v)). The diluted solutions were injected into an LC-MS/MS (API5000, AB Sciex) equipped with a Shimadzu Shim-pack XR-ODS (2.2 μm packing particle size, 2.0 mm ID×30 mm L) maintained at 50°C. The chromatographic separation was performed using gradient elution at a flow rate of 0.7 mL/min. The LC time program was as follows: Mobile phase B was held at 5% for 0.1 min, and increased linearly to 95% in 0.1 min. After maintaining B at 95% for another 0.8 min, it was decreased to 5% in 0.01 min, followed by re-equilibration for 0.59 min. The total cycle time for one injection was 1.6 min. PF-05270430 was detected using multiple reaction monitoring mode and the transition m/z 432→386. Analyst™ software (version 1.4.2) was used for data acquisition and processing. Specific tracer binding (B_g) in the striatum was represented as the difference between the tracer concentration in striatum and that in cerebellum. PDE2A occupancy was calculated using the following equation: Occupancy (%)=(B_{SP.bas}−B_{SP.drug})/B_{SP.bas}×100, where B_{SP.bas} and B_{SP.drug} are the concentrations at baseline (vehicle treatment) and at drug treatment, respectively. Curve fitting of the saturation curve was carried out by nonlinear regression using GraphPad Prism 5.02 (GraphPad Software, Inc., San Diego, CA, U.S.A.).

Measurement of Cyclic Nucleotide Contents in Rat Brain Animals

Five-week-old male Long–Evans rats were purchased from Japan SLI, Inc. (Japan). The rats were housed in groups of 3/cage in a light-controlled room (12h light/dark cycles with lights on at 07:00). Food and water were provided ad libitum. After a one week acclimation period, the six-week-old rats were used for experiments.

Measurements

Compound 20 was suspended in 0.5% (w/v) methylcellulose in distilled water, and was administered in a volume of 2 mL/kg body weight for rats. Rats were administered orally with either vehicle or compound 20 (1, 3, 10 mg/kg) after >1 h of habituation. A microwave fixation system (Muromachi Kikai, Tokyo, Japan) was used to sacrifice unanesthetized rats by exposure of the head to the microwave beam at 2 h after administration of 20. Brain tissues were isolated and then homogenized in 0.5 mol/L HCl, followed by centrifugation. The concentration of cyclic nucleotides in the supernatant was measured using a cyclic AMP EIA kit or cyclic GMP EIA kit (Cayman Chemical, U.S.A.) following the manufacturer’s instructions. Values were expressed as pmol/mg tissue weight.

Step-through Passive Avoidance Task

This task was performed as previously described with some modifications. This experiment was conducted in 7–8-week-old male Long–Evans rats. The apparatus (Brainscience idea, Osaka, Japan) consisted of an illuminated chamber (25×10×25 cm) connected to a dark chamber (30×30×30 cm) by a sliding door (8×8 cm). On the training day, each animal was subjected to a single pre-training trial 4–6 h before the acquisition trial. The rat was placed in the light chamber, and the sliding door was opened 30 s later. As soon as the rat entered the dark chamber with all four paws, the door was closed. The rat was then allowed to remain in the dark chamber for 30 s before being returned to its home cage. In the acquisition trial, the rat was placed in the light chamber, and the sliding door was opened. The time required for the rat to enter the dark chamber was then recorded. As soon as the rat entered the dark chamber, the door was closed, and an electric shock (0.5 mA, 3 s) was delivered from the floor grid. The rat was then returned to its home cage. The retention test was conducted 24 h later. The rat was again placed in the light chamber with the sliding door. After 30 s, the door was opened and the latency for the rat to cross over into the dark compartment was recorded. If the animal did not enter the dark chamber within 300 s, the retention test was terminated, and the animal was given a ceiling score of 300 s. Vehicle or compound 20 (3 mg/kg) was administered p.o. 2 h prior, and saline or MK-801 (0.1 mg/kg) was administered s.c. 30 min prior to the acquisition trial. The statistical significance of differences between group latency scores was determined by Wilcoxon’s test with significance set at p<0.05.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Materials

The online version of this article contains supplementary materials. Synthesis of (+)-di-(p-tolouyl)-d-tartaric acid salt (S2) of free base of 28 suitable for X-ray crystallography. Synthesis of pyrazolo[1,5-a]pyrimidine derivatives 5 and 6. Full details of the X-ray structure analysis of S2. Molecular formula strings.

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