Discovery and Characterization of Selective and Ligand-Efficient DYRK Inhibitors

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Abstract: Dual specificity tyrosine-regulated kinase 1A (DYRK1A) regulates the proliferation and differentiation of neuronal progenitor cells during brain development. Consequently, DYRK1A has attracted the interest as a target for the treatment of neurodegenerative diseases, including Alzheimer’s disease (AD) and Down’s syndrome. Recently, the inhibition of DYRK1A has been investigated as a potential treatment for diabetes, while DYRK1A’s role as a mediator in the cell cycle has garnered interest in oncologic indications. Structure–activity relationship (SAR) analysis in combination with high-resolution X-ray crystallography leads to a series of pyrazolo[1,5-b]pyridazine inhibitors with excellent ligand efficiencies, good physicochemical properties, and a high degree of selectivity over the kinome. Compound 11 exhibited good permeability and cellular activity without P-glycoprotein liability, extending the utility of 11 in an in vivo setting. These pyrazolo[1,5-b]pyridazines are a viable lead series in the discovery of new therapies for the treatment of diseases linked to DYRK1A function.

Introduction

Therapeutic applications of antagonists of the serine/threonine kinase, dual-specificity tyrosine-regulated kinase 1A (DYRK1A), have been under investigation for two decades. Diseases associated with the abnormal expression of DYRK1A (Alzheimer’s disease (AD), Down’s syndrome, cancer, and diabetes) represent significant unmet medical needs. Targeting the underlying mechanisms that cause these diseases is desirable in the search for novel approaches to treatment. DYRK1A plays a crucial role in neurogenesis and neuronal differentiation during embryonic development.1 DYRK1A is located on the Down’s syndrome critical region (DSCR) of chromosome 21; hence, individuals with Trisomy 21 (Down’s syndrome) express elevated levels of DYRK1A.2 As a result, overexpression of DYRK1A is thought to contribute to cognitive impairments associated with Down’s syndrome.2 AD has been associated with the accumulation of β amyloid plaques and neurofibrillary tangles (NFTs) in the brain.3 Increased DYRK1A activity promotes both NFT formation and aggregation,4 in addition to accelerating the cleavage of β peptides that comprise amyloid plaques.5 The ability of DYRK1A to effect the phosphorylation of nuclear factors of activated T cells (NFAT) and promote the proliferation of β cells has piqued interest in DYRK1A inhibitors as a potential treatment for diabetes.6 Several DYRK1A inhibitors have previously been developed.7 Despite these efforts, few have progressed to in vivo models of disease, largely as a result of limited pharmacokinetics or poor selectivity for DYRK1A. Leucettine L41,8 SM07883 (structure not disclosed),9 GNF2133,10 and compound 2-2c11 represent DYRK1A antagonists that have been investigated in vivo (Figure 1). However, Leucettine 41, SM07883, and GNF2133 potently inhibit other CMGC kinases, so they are more accurately described as dual DYRK1/CLK inhibitors.

Previously, we reported the cheminformatics-based design of a series of selective DYRK1A inhibitors, exemplified by 8b and 6b (Figure 1), which had DYRK1A IC50 values of 76 and 186 nM, respectively.12 Here, we report the structure-guided development of this pyrazolo[1,5-b]pyridazine series, resulting in potent and selective inhibitors of DYRK1A with physical properties compatible with treatment of diseases associated with DYRK1A function. In agreement with our previous conclusions,12 the favorable selectivity profile of the series was achieved by maintaining hydrogen-bonding interactions between the ligand, a consensus water, and amino acid residues Glu239, Leu241, and Lys188, while directing a methyl substituent, or similar moiety, into a shallow lipophilic pocket, exclusive to the hinge of the DYRK and CLK kinases.

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Three regions of 8b were selected for structure−activity relationship (SAR) analysis: (a) the N-Me-aryl substituent, (b) the pyrazolo[1,5-b]pyridazine heterocycle, and (c) the pyrimidine hinge-binding moiety. To maintain and improve upon the selectivity of the series, it was important to preserve the unusual binding mode of the series in the ATP-binding site of DYRK1A. Therefore, a synthetic strategy was devised to access a range of N-alkyl analogues (9−55). The synthesis of key pyrazolo[1,5-b]pyridazine intermediates 6 and 8 followed literature procedures (Scheme 1).12,13 N-Amination of pyridazine was completed with hydroxylamine-O-sulfonic acid. The pyrazolo[1,5-b]pyridazine heterocycle was constructed by reacting 1,3-dipole 2 with 3-butyne-2-one in a Huisgen cycloaddition. Dimethylformamide−dimethylacetamide (DMF−DMA) condensation of ketone 3 gave intermediate 4 in a high yield. Performing a urea melt on 4, followed by dehydration and chlorination with phosphorus(V) oxychloride afforded key intermediate 6. Alternatively, thioether 7 was accessed by treating 4 with thiourea and sodium methoxide, followed by iodomethane. Upon oxidation of 7 with m-CPBA, the key intermediate 8 was isolated.

Compounds 6 and 8 were used as key intermediates for nucleophilic aromatic substitution with assorted primary and secondary amines to afford 9−55 (Scheme 2). N-Alkyl analogues not prepared from commercially available amines were prepared in two steps: initial reaction of 6 or 8 with the primary amine was followed by alkylation with sodium hydride and appropriate haloalkane (Scheme 2).

Aminopyrimidine 9 exhibited marginally weaker binding affinity for DYRK1A than 6b but with superior LE (0.55 vs 0.41) and LLE (5.59 vs 3.55). Compound 10 bound with equal affinity to DYRK1A as 6b, also conferring superior LE (0.56) and LLE (5.75). Compound 11 bound with stronger affinity than 6b, while maintaining favorable lipophilicity (clogP = 1.62), LE (0.55) and LLE (5.50), representing a good starting point for further elaboration. Compound 12 showed similar binding affinity to 11. Overall, maintaining one amino substituent as methyl while increasing the steric bulk of the other amino substituent was tolerated; however, two larger substituents (13−15) led to a reduction in DYRK1A binding affinity, a 10-fold reduction in the case of the matched-molecular pairs 12 and 13, highlighting the importance of an N-methyl substituent for maintaining the binding mode seen in Figure 1.

Advanced DYRK1/CLK inhibitors reported in the literature (Leucettine 41, GNF2133, and compound 2−2c) and previously characterized DYRK1A inhibitors 6b and 8b with DYRK1A IC₅₀ values of 186 and 76 nM, respectively. 6b (shown in yellow) bound to DYRK1A such that the N-methyl group was directed toward a small pocket on the kinase hinge region (PDB 6S1I). Key hydrogen-bonding interactions between 6b, a consensus water, and DYRK1A (Leu241, Glu239, and Lys188) are depicted by black dotted lines. Compound 8b had a selectivity score (S(40)) against DYRK1A of 0.01.12

Figure 1. Advanced DYRK1/CLK inhibitors reported in the literature (Leucettine 41, GNF2133, and compound 2−2c) and previously characterized DYRK1A inhibitors 6b and 8b with DYRK1A IC₅₀ values of 186 and 76 nM, respectively. 6b (shown in yellow) bound to DYRK1A such that the N-methyl group was directed toward a small pocket on the kinase hinge region (PDB 6S1I). Key hydrogen-bonding interactions between 6b, a consensus water, and DYRK1A (Leu241, Glu239, and Lys188) are depicted by black dotted lines. Compound 8b had a selectivity score (S(40)) against DYRK1A of 0.01.
the co-crystal structure of DYRK1A:6b (Figure 1). The longer 4-carbon-chain amino substituent of 18 led to increased binding affinity compared to 17 (Table 1).

Co-crystal structures of DYRK1A bound to 10, 11, and 12 were determined at a high resolution (Figure 2, Table 2). The N-methyl and N-H matched pair, 11 and 10, adopt the same binding mode in DYRK1A, respectively. The selectivity profiles of N-methyl and N-H matched pairs were previously shown to differ significantly, with N-methyl analogues being more selective for DYRK1A.12 The N-methyl groups in 6b, 11, and 12 all occupy the same pocket on the kinase hinge region. The retention of binding mode gave confidence that the selectivity profile of 6b, and by inference, 8b, would be maintained in the chemical series.12

With multiple crystal structures and SAR analysis supporting the existence of a shallow pocket on DYRK1A’s hinge motif that could be occupied by small substituents such as methyl, in silico modeling was used to identify possible replacements. The modeling suggested that a constrained methylene unit would be an appropriate substitution for the methyl group. Therefore, a range of cyclic amine analogues were synthesized to probe the size of the lipophilic pocket (Table 3).

Several cyclic amine analogues exhibited a similar level of binding affinity for DYRK1A as former lead compound 8b but with superior LE.12 All analogues exhibited lower LE than 11, but a small number exhibited comparable levels of binding affinity (22, 24, 28, 30, 31, 33 and 34) and superior LLE (22, 33 and 34). Analogues bearing substituents with smaller ring sizes (4 or 5 carbon atoms) showed, in general, greater DYRK1A binding affinity than analogues with larger rings (>5 carbon atoms), reinforcing the hypothesis that steric bulk is permitted, but limited, close to the hinge of the kinase. The X-ray co-crystal structure of 30 bound to DYRK1A (Figure 2 and Table 2) showed a retention of the binding mode, with a methylene group of the cyclopentyl ring occupying the same pocket as the methyl groups of 6b, 11, and 12, validating the prediction made by molecular modeling. Compound 22 and single R-enantiomers 31 and 34 were the only compounds with binding affinity for DYRK1A (IC50) of less than 100 nM.

At this stage, as 11 possessed favorable LE (0.55) and had been shown to maintain the same binding mode as 6b, it was used as the reference compound/matched-molecular pair for further SAR analysis. The data so far had not identified which of the atoms within the pyrazolo[1,5-b]pyridazine core
contributed to selectivity and binding affinity, and were therefore, essential pharmacophoric features to maintain. The pyrazolo[1,5-b]pyridazine core, although not prevalent in the literature, is not novel and was being explored by others as

Table 1. DYRK1A Binding Affinity of Pyrazolo[1,5-b]pyridazines

| Compound | R   | R'   | DYRK1A pIC50* | clog P | LE  | LLE |
|----------|-----|------|---------------|--------|-----|-----|
| 6b       | Me  | Ph   | 6.73          | 3.18   | 0.41| 3.55|
| 8b       | Me  | m-Toly| 7.10          | 3.69   | 0.41| 3.41|
| 9        | H   | H    | 6.28          | 0.69   | 0.55| 5.59|
| 10       | Me  | H    | 6.74          | 0.99   | 0.56| 5.75|
| 11       | Me  | Me   | 7.12          | 1.62   | 0.55| 5.50|
| 12       | Me  |      | 6.94          | 2.09   | 0.49| 4.85|
| 13       |     |      | 5.92          | 2.86   | 0.38| 3.06|
| 14       | Pr  |      | 6.12          | 3.28   | 0.39| 2.84|
| 15       | Pr  |      | 6.50          | 2.46   | 0.40| 4.04|
| 16       | Me  |      | 6.66          | 1.64   | 0.42| 5.02|
| 17       | Me  |      | 6.64          | 2.82   | 0.40| 3.82|
| 18       | Me  |      | 7.00          | 3.14   | 0.41| 3.86|

*pIC50 in TR-FRET-based ligand-binding displacement assay measured with two technical replicates (n = 1 biological replicate). LE = 1.4(pIC50)/nonhydrogen atoms. LLE = pIC50 − clog P.

Figure 2. Co-crystal structures of pyrazolo[1,5-b]pyridazines (compounds 6b, 10, 11, 12, and 30) and compound 78 (yellow) in complex with DYRK1A. The backbones of the kinase N-lobe, hinge, and C-lobe are colored in gray, pink, and purple, respectively, and polar contacts are indicated by black dotted lines. The conserved water molecule at the back of the pocket is indicated by a red sphere. The electron density of each compound (2Fo−Fc map) is shown contoured at 1.0σ (blue mesh). For clarity, DYRK1A strands β1 and β2 are hidden. (A) Previously identified compound 6b (PDB 6S1I),12 (B) compound 10 (PDB 6S1B), (C) compound 11 (PDB 6S17), (D) compound 12 (PDB 6S14), (E) compound 30 (PDB 6S1J), and (F) compound 78 (PDB 6S1H).
ketone was constructed from commercially available building blocks, then treated, in a similar manner to the pyrazolo[1,5-b]pyridazines (cf. Scheme 1), with DMF–DMA condensation, followed by cyclization with suitable guanidine (Scheme 3B,C).

Alternatively, 4-chloro-N,N-dimethyl-pyrimidin-2-amine (compound 74) was reacted with appropriate boronic heterocycle, either purchased commercially or produced following literature procedures, under Suzuki conditions (Scheme 4).

Compound 60 showed no binding affinity for DYRK1A (Table 4), probably due to unfavorable clashes with the phenylalanine (Phe238) gatekeeper residue of DYRK1A. Substitution in the 6-position resulted in more than a 10-fold drop in DYRK1A binding affinity for 62 and 65. This is likely due to unfavorable clashes between the molecules and the DYRK1A protein surface close to Lys188. Compound 64, bearing a methyl substituent at the 4-position, also showed an approximate 10-fold loss in DYRK1A binding affinity, while the 4-methoxy analogue 66 showed approximately a 2-fold reduction in affinity, despite the potential change in orientation. A methyl substituent in the 5-position (63) was well tolerated.

All bioisosteric replacements of the pyrazolo[1,5-b]-pyridazine motif, and compounds 70–87, designed to interact with, or to displace the structural water molecule, exhibited diminished DYRK1A binding affinity. Potentially, the loss of hydrogen bonding from the water to the inhibitor and to the protein was not compensated for by any other interaction, leading to a reduction in binding affinity. Another possibility is that the phenylalanine gatekeeper obstructs access to the back-pocket of DYRK1A, preventing the ligand from being able to displace the consensus water. Compound 70 showed strong binding affinity for DYRK1A, comparable to 63 and 66 but with better LE (0.53) and LLE (6.18).

The binding affinity and LE of 78 were favorable, despite the change in binding orientation and compound 78 relative to the pyrazolo[1,5-b]-pyridazine series (Figure 2F). Compound 78

Table 2. Data Collection and Refinement Statistics for DYRK1A Co-crystal Structures

| Compound | 10  | 11  | 12  | 30  | 78  |
|----------|-----|-----|-----|-----|-----|
| PDB ID   | 6S1B| 6S17| 6S14| 6S1J| 6S1H|
| space group | C2  | C2  | C2  | C2  | C2  |
| unit cell dimensions (Å) | 101.3, 70.4, 68.7, 90.0, 118.0, 90.0 | 100.9, 70.6, 68.3, 90.0, 117.8, 90.0 | 101.5, 70.3, 68.5, 90.0, 118.0, 90.0 | 99.2, 69.7, 67.4, 90.0, 117.5, 90.0 | 99.6, 69.7, 67.7, 90.0, 117.8, 90.0 |
| Data collection | resolution range (Å) | 30.60–1.30 (1.13–1.30) | 30.46–1.10 (1.13–1.10) | 30.39–1.05 (1.08–1.05) | 30.80–1.41 (1.48–1.41) | 30.11–1.05 (1.08–1.05) |
| unique observations | 103.325 (7306) | 137.242 (3030) | 149.683 (1105) | 75.809 (8676) | 174.657 (6548) |
| average multiplicity | 2.7 (2.0) | 4.0 (2.0) | 2.9 (1.1) | 12.2 (8.8) | 3.0 (1.7) |
| completeness (%) | 98.9 (95.0) | 80.2 (24.3) | 75.9 (7.6) | 96.4 (76.0) | 91.9 (47.0) |
| R MEASURE | 0.04 (0.70) | 0.05 (0.06) | 0.04 (0.61) | 0.20 (2.68) | 0.05 (0.63) |
| mean (|/|/|) | 101.1 (1.1) | 117.1 (1.3) | 15.4 (1.2) | 9.5 (1.8) | 11.1 (1.0) |
| Refinement | resolution range (Å) | 30.60–1.30 | 30.46–1.10 | 30.39–1.05 | 59.80–1.41 | 30.11–1.05 |
| R-value, R MEASURE | 0.16, 0.18 | 0.15, 0.17 | 0.15, 0.16 | 0.18, 0.18 | 0.14, 0.15 |
| r.m.s. deviation from ideal bond length (Å) | 0.012 | 0.022 | 0.013 | 0.013 | 0.006 |
| r.m.s. deviation from ideal bond angle (deg) | 1.15 | 1.62 | 1.22 | 1.28 | 1.03 |
| Ramachandran Outliers | 0.0% 96.7% | 0.0% 96.1% | 0.0% 95.8% | 0.0% 95.8% |

Values within parentheses refer to the highest-resolution shell. Values from Molprobity.
Table 3. DYRK1A Binding Affinity of Pyrazolo[1,5-b]pyridazines

| Compound | R | DYRK1A pIC$_{50}$$^a$ | clog P | LE | LLE |
|----------|---|----------------------|--------|----|-----|
| 19       |   | 6.34                 | 1.51   | 0.47 | 4.83 |
| 20       |   | 6.43                 | 1.99   | 0.43 | 4.35 |
| 21       |   | 6.29                 | 1.08   | 0.40 | 5.21 |
| 22       |   | 7.10                 | 1.28   | 0.45 | 5.82 |
| 23       |   | 5.86                 | 2.03   | 0.41 | 3.83 |
| 24       |   | 6.96                 | 2.45   | 0.46 | 4.51 |
| 25       |   | 6.72                 | 2.73   | 0.43 | 3.99 |
| 26       |   | 6.69                 | 2.39   | 0.45 | 4.30 |
| 27       |   | 6.37                 | 2.77   | 0.41 | 3.60 |
| 28       |   | 6.94                 | 2.39   | 0.46 | 4.55 |
| 29       |   | 6.83                 | 2.39   | 0.46 | 4.44 |
| 30       |   | 6.73                 | 1.77   | 0.45 | 4.96 |
| 31       |   | 7.18                 | 1.77   | 0.48 | 5.41 |
| 32       |   | 6.61                 | 1.59   | 0.40 | 5.02 |
| 33       |   | 6.87                 | 0.78   | 0.46 | 6.09 |
| 34       |   | 7.14                 | 0.78   | 0.48 | 6.36 |
| 35       |   | 6.51                 | 1.85   | 0.43 | 4.66 |
| 36       |   | 5.91                 | 2.74   | 0.36 | 3.17 |
| 37       |   | 5.78                 | 1.21   | 0.35 | 4.57 |
| 38       |   | 6.57                 | 1.66   | 0.38 | 4.91 |

$^a$pIC$_{50}$ in TR-FRET-based ligand-binding displacement assay measured with two technical replicates ($n = 1$ biological replicate). LE = 1.4(pIC$_{50}$) / nonhydrogen atoms. LLE = pIC$_{50}$ – clog P.
could be considered as a distinct series and would potentially serve as an alternative scaffold for DYRK1A inhibition.

The final point of diversification to investigate was the pyrimidine motif while retaining the pyrazolo[1,5-b]pyridazine moiety and the N,N-dimethylamine appendage. Several synthetic strategies were employed to furnish S-substituted pyrimidine analogues. Compounds 95−100 were accessed via Suzuki reaction between pyrazolo[1,5-b]pyridazine boronic ester and appropriate 5-substituted pyrimidine. To construct the pyrazolo[1,5-b]pyridazine boronic ester, 1-aminopyridinium iodide 2 underwent 1,3-dipolar cycloaddition with methyl propiolate. Ester hydrolysis with lithium hydroxide furnished carboxylic acid 90 (Scheme 5A). Facile bromodecarboxylation under mild conditions gave the 3-bromo intermediate 91. Miyaura borylation of 91 furnished the desired boronic ester 92, which was used crude in subsequent Suzuki reactions.

The S-substituted pyrimidine was prepared from either the commercial aminopyrimidine or 2,4-dichloropyrimidine (Scheme 5B,C). Alkylation with sodium hydride and iodo methane gave the required N,N-dimethylamine substituent (Scheme 5B). Alternatively, starting from the 2,4-dichloropyrimidine, the Suzuki reaction was performed first to install the pyrazolo[1,5-b]pyridazine moiety, followed by displacement of the chloride with dimethylamine, in a two-step one-pot reaction (Scheme 5C). After some investigation, it was found that 5-halo analogues 105−107 could be accessed reliably via

Scheme 3. Synthesis of 5,6-Heterocyclic Replacements of the Pyrazolo[1,5-b]pyridazine

Reagents and conditions: (A) (i) Bis(triphenylphosphine)palladium(II) dichloride, copper iodide, TEA, propyne (ca. 5% in THF, ca. 1 mol/L), THF, rt, overnight, 87%; (ii) pyridazin-1-ium-1-amine iodide, DBU, acetonitrile, 50 °C, 16 h, 73%; (iii) m-CPBA, CH2Cl2, rt, overnight, 80%; (iv) 2 M dimethylamine in THF, 110 °C, 2 h, 74%. (B) (i) Hydroxylamine-O-sulfonic acid, KHCO3, KI, H2O, 70 °C, 2 h; (ii) 3-butyne-2-one, KOH, CH2Cl2, rt, overnight; (iii) DMF−DMA, 75 °C, 16 h; (iv) 2-methoxylethanol, potassium carbonate, 1,1-dimethylguanidine sulfate salt, 125 °C, overnight. (C) (i) DMF−DMA, toluene, 90 °C, 2 h—overnight then chloroacetone, EtOH, reflux, overnight; (ii) DMF−DMA, 75 °C, 16 h; (iii) 2-methoxylethanol, potassium carbonate, 1,1-dimethylguanidine sulfate salt, 125 °C, overnight.
electrophilic halogenation of compound 9, followed by alkylation of the free amino group (Scheme 5D).

Triazine 109 and pyridine 111 were prepared from 2,4-dichlorotriazine and 2,6-dibromopyridine, respectively (Scheme 6); nucleophilic aromatic substitution with dimethylamine followed by installation of the pyrazolo[1,5-b]pyridazine motif via Suzuki protocol gave 109 and 111. Dimethylation of building block 112 under the usual conditions followed by Suzuki protocol to install the pyrazolo[1,5-b]pyridazine motif gave compound 113.

Of the pyrimidine motif alterations, only the 5-fluoro analogue, 95, exhibited stronger DYRK1A binding affinity than 11 (Table 5). Increasing the size of the halogen (F → Cl → Br → I) was detrimental to DYRK1A binding affinity for the series. This suggests that when the pyrazolo[1,5-b]pyridazine series binds to DYRK1A, the space around the phenylalanine gatekeeper is restricted, limiting the size of substituent that can be placed favorably in the 5-position. The increased binding affinity observed for compound 95 may be the result of a combination of factors. A favorable interaction may have been established between the lipophilic fluoride atom and the phenylalanine gatekeeper (F−π interaction). A second possibility for the increased binding affinity observed for compound 95 could be the result of a conformational effect. The fluoride at the 5-position of pyrimidine may be acting as a weak hydrogen-bond acceptor with the proton of the 2-position of the pyrazolo[1,5-b]pyridazine, limiting rotation around the Ar-Ar bond, and preorganizing the molecule into an energetically favorable binding conformation. Substituents added to the 6-position of the pyrimidine were not well tolerated, probably as a result of unfavorable clashes between the ligand and the amino acid residues on the hinge region of DYRK1A.

Scaffold hopping from the pyrimidine to all other nitrogenous heterocycles resulted in reduced binding affinity for DYRK1A (Table 6). Removal of the nitrogen atom that serves...
Table 4. DYRK1A Binding Affinity of Pyrazolo[1,5-b]pyridazine Analogues

| Compound | R          | pIC50<sup>a</sup> | clog P | LE  | LLE  |
|----------|------------|-------------------|--------|-----|------|
| 60       |            | 4.51              | 1.75   | 0.33| 2.76 |
| 62       |            | 6.06              | 1.75   | 0.45| 4.31 |
| 63       |            | 6.94              | 2.14   | 0.51| 4.80 |
| 64       |            | 6.14              | 2.14   | 0.45| 4.00 |
| 65       |            | 5.58              | 2.06   | 0.39| 3.52 |
| 66       |            | 6.86              | 1.47   | 0.48| 5.39 |
| 70       |            | 6.80              | 0.62   | 0.53| 6.18 |
| 71       |            | 6.16              | 1.58   | 0.48| 4.58 |
| 72       |            | 4.26              | 1.47   | 0.33| 2.79 |
| 75       |            | < 4               | 2.46   | < 0.3| < 1.54|
| 76       |            | 5.10              | 1.01   | 0.42| 4.09 |
| 77       |            | 5.40              | 2.05   | 0.47| 3.35 |
| 78       |            | 6.87              | 2.12   | 0.53| 4.75 |
| 79       |            | 4.93              | 2.34   | 0.36| 2.59 |
| 80       |            | 5.11              | 2.70   | 0.36| 2.41 |
| 81       |            | 6.05              | 2.40   | 0.47| 3.65 |
| 86       |            | 5.99              | 1.75   | 0.47| 4.24 |
| 87       |            | 6.01              | 1.97   | 0.44| 4.04 |

<sup>a</sup>pIC<sub>50</sub> in TR-FRET-based ligand-binding displacement assay measured with two technical replicates (<i>n</i> = 1 biological replicate). LE = 1.4(pIC<sub>50</sub>)/nonhydrogen atoms. LLE = pIC50 − clog P.
as a hydrogen-bond acceptor to the hinge region of DYRK1A in compound 111 led to an approximately 10-fold reduction in binding affinity. Likewise, removal of the nitrogen atom from the 3-position of the pyrimidine and replacement with C–H (compound 113) also resulted in a 10-fold loss in binding affinity. The diminished binding affinity observed for

Scheme 5. Synthesis of Key Boronic Ester and Pyrazolo[1,5-b]pyridazines

Reagents and conditions: (A) (i) Intermediate 2, potassium carbonate, CH₂Cl₂, rt, 16 h, 36%; (ii) lithium hydroxide, MeOH, rt, 16 h then 1 M HCl, rt, 30 min, 74%; (iii) N-bromosuccinimide, DMF, rt, 3 h, 78%; (iv) bis(pinacolato)diboron, [1,1′-bis(diphenylphosphino)ferrocene]-dichloropalladium(II), complex with CH₂Cl₂, potassium acetate, 1,4-dioxane, 90 °C, 16 h. (B) (i) Sodium hydride, iodomethane, DMF, rt, 16 h; (ii) 92, bis[2-(di-tert-butylphosphanyl)cyclopenta-2,4-dien-1-yl]iron; dichloropalladium, sodium carbonate, acetonitrile/water (10:1), 140 °C overnight. (C) (i) 92, bis[2-(di-tert-butylphosphanyl)cyclopenta-2,4-dien-1-yl]iron; dichloropalladium, potassium carbonate, DMF, 60 °C, 1.5 h; (ii) 2 M dimethylamine in THF, 100 °C, 2 h. (D) (i) NXS, acetonitrile, rt, 2 h; (ii) sodium hydride, iodomethane, THF, rt, overnight.
Scheme 6. Synthesis of Pyrimidine Isosteres

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108  \[\text{Cl}_2\text{N}\]  \\
     (i), (ii)  \[\text{Cl}_2\text{N}\]  \\
   109

110  \[\text{Br}_2\text{N}\]  \\
     (i), (ii)  \[\text{Br}_2\text{N}\]  \\
   111

112  \[\text{Br}\text{N}_2\text{H}_2\]  \\
     (iii), (ii)  \[\text{Br}\text{N}_2\text{H}_2\]  \\
   113
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Reagents and conditions: (i) dimethylamine hydrochloride, N,N-diisopropylethylamine, THF, rt, 3 h; (ii) 92, bis[2-(di-tert-butylphosphanyl)cyclopaenta-2,4-dien-1-yl]iron; dichloropalladium, 2 M potassium carbonate, 1,4-dioxane, 80 °C, 16 h; (iii) iodomethane, sodium hydride, DMF, rt, overnight.

Metabolic Stability and Solubility Profiles of Lead Compounds. Several analogues had emerged with superior LE, LLE and binding affinity for DYRK1A compared to the original starting point 6b (LE = 0.41, LLE = 3.55 and pIC\textsubscript{50} = 6.73). These analogues also possessed a lower cLogP, a reduced number of aromatic rings, and were analyzed to determine whether the improvements in physicochemical properties would translate to improvements in metabolic stability and solubility (Table 8), aiming to identify the compounds most suitable for development into in vivo chemical probes.

In general, a reduction in lipophilicity and aromatic ring count led to modest improvements in in vitro metabolic stability and solubility. Larger reductions in lipophilicity, observed for compounds 11, 22, 33, 34, and 70, showed marked improvements in solubility and metabolic stability. It is likely that a number of other factors are affecting the solubility of the series, including π-stacking due to the planarity of the series. In terms of metabolic stability, N-dealkylation is likely to be a major issue, especially for \textit{N}N-dimethylamine analogues, and to some extent cyclic analogues 33 and 34 circumvent this issue. Notably, an increase in polarity led to better solubility and metabolic stability (22, 33, and 34). The addition of substituents onto the pyrazolo[1,5-b]pyridazine motif 66 and substitution of the pyrazolo[1,5-b]pyridazine heterocycle for 70 also appears to improve solubility and metabolic stability.

Selectivity Profiles of Lead Compounds. During inhibitor development selected compounds were analyzed for selectivity against an in-house panel of 48 representative kinases by measuring the change in thermal stabilization of the kinase in the presence and absence of inhibitor (Supporting Information). This data revealed that the series showed generally good selectivity for DYRK1A, DYRK2, CLK1, and Haspin (GSG2). Compound 78 was a notable exception which cross-reacted with several additional kinases including the NAK kinases AAK1 and BMP2K, indicating the importance of the binding mode of the pyrazolo[1,5-b]pyridazine core for selectivity (Figure 2). In contrast to the pyrazolo[1,5-b]pyridazine series, compound 78 appears unable to establish a hydrogen-bonding interaction with the consensus water at the back-pocket of DYRK1A and lacks a substituent, such as a methyl, to occupy the shallow cleft on the hinge of DYRK1A (Figure 2).

The kinome-wide selectivity of key pyrazolo[1,5-b]-pyridazines was further assessed at a 1 μM inhibitor concentration, using the KINOMEscan panel (DiscoverX) of >400 wild-type human kinases (summary Table 9, whole datasets in the Supporting Information). The analysis revealed good selectivity scores (S\textsubscript{50}) for key compounds with common off-targets of the series being the CDC-like kinases (CLKs) and other DYRKs. Crucially, all lead exemplars were selective against the majority of the human kinome and maintained selectivity for DYRK1A and CLKs over CMGC kinases CDK2 and GSK3β, which were known targets of the pyrazolo[1,5-b]pyridazine chemotype and are involved in some of the same mechanistic pathways as DYRK1A.

The selectivity profile of 11 was further investigated by acquiring K\textsubscript{D} values for kinase off-targets identified in the KINOMEscan analysis (Table 10). Compound 11 showed approximately 20-fold selectivity for DYRK1A over DYRK1B, 100-fold selectivity over DYRK2, more than 20-fold selectivity over CLK1 and CLK4 and approximately 70-fold selectivity over CLK2, making 11 one of the most target-specific...
DYRK1A inhibitors reported. Other potential off-targets, such as JAK3, which had been identified in the KINOMEscan analysis were determined to be false positives following dose-response \( K_D \) measurement (Table 10). The only kinase apart from DYRK1A with strong affinity for 11 was the mitotic kinase Haspin. The lead compounds in Table 9 have comparable selectivity profiles to 11 with good physical properties and would serve as advanced starting points for future studies. Our data shows that modifications could be made to the series that result in improved kinome selectivity and more desirable physicochemical properties.

Inhibition of DYRK1A Phosphorylation. To demonstrate the DYRK1A inhibitory activity of the series, compound 11 was profiled in a radiometric kinase assay (Table 5).

**Table 5. DYRK1A Binding Affinity of Pyrazolo[1,5-\(b\)]pyridazines**

| Compound | R | R’ | DYRK1A pIC\(_{50}\) | clog P | LE | LLE |
|----------|---|---|---------------------|-------|----|-----|
| 95       | F | H | 7.24                | 1.77  | 0.53 | 5.47 |
| 96       | OMe | H | 5.93                | 1.47  | 0.42 | 4.46 |
| 97       | H | Cl | 5.87                | 2.45  | 0.43 | 3.42 |
| 98       | H | Me | 4.93                | 1.75  | 0.36 | 3.18 |
| 99       | H | OMe | < 4.00              | 2.06  | < 0.30 | < 1.94 |
| 100      | H |   | 4.72                | 3.00  | 0.31 | 1.72 |
| 102      | CF\(_3\) | H | 4.89                | 2.50  | 0.31 | 2.39 |
| 103      | Me | H | 5.55                | 2.14  | 0.41 | 3.41 |
| 104      | H |   | 5.13                | 1.33  | 0.34 | 3.80 |
| 105      | Cl | H | 6.48                | 2.23  | 0.48 | 4.25 |
| 106      | Br | H | 5.80                | 2.39  | 0.43 | 3.41 |
| 107      | I | H | 5.79                | 2.55  | 0.43 | 3.24 |

*pIC\(_{50}\) in TR-FRET-based ligand-binding displacement assay measured with two technical replicates (\( n = 1 \) biological replicate). LE = 1.4(pIC\(_{50}\))/nonhydrogen atoms. LLE = pIC\(_{50}\) − clog P.

**Table 6. DYRK1A Binding Affinity of Pyrazolo[1,5-\(b\)]pyridazines**

| Compound | A | B | C | DYRK1A pIC\(_{50}\) | clog P | LE | LLE |
|----------|---|---|---|---------------------|-------|----|-----|
| 109      | N | N | N | 6.74                | 1.88  | 0.52 | 4.86 |
| 111      | C | N | C | 6.04                | 2.25  | 0.47 | 3.79 |
| 113      | N | C | C | 6.17                | 1.86  | 0.48 | 4.31 |

*pIC\(_{50}\) in TR-FRET-based ligand-binding displacement assay measured with two technical replicates (\( n = 1 \) biological replicate). LE = 1.4(pIC\(_{50}\))/nonhydrogen atoms. LLE = pIC\(_{50}\) − clog P.

**Table 7. SPR Measurement of Lead Compounds**

| compound | \( k_a \) (\( M^{-1} s^{-1} \)) | \( k_d \) (s\(^{-1} \)) | \( K_D \) (nM) |
|----------|---------------------------------|---------------------|----------------|
| 9        | 7.3 (±0.1) \( \times 10^6 \)     | 0.97 (±0.02)        | 134.1 (±0.5) |
| 10       | 6.0 (±0.1) \( \times 10^6 \)     | 0.33 (±0.1)         | 55.0 (±0.1)  |
| 11       | 2.8 (±0.1) \( \times 10^6 \)     | 0.24 (±0.01)        | 85 (±1)      |
| 12       | 6.3 (±0.2) \( \times 10^6 \)     | 0.57 (±0.01)        | 90.4 (±0.03) |
| 22       | 3.9 (±0.4) \( \times 10^6 \)     | 0.25 (±0.03)        | 64 (±2)      |
| 33       | 2.2 (±0.1) \( \times 10^6 \)     | 0.16 (±0.01)        | 72.8 (±0.3)  |
| 34       | 1.8 (±0.1) \( \times 10^6 \)     | 0.12 (±0.02)        | 65.5 (±0.4)  |
| 95       | 4.7 (±0.1) \( \times 10^6 \)     | 0.06 (±0.01)        | 12.0 (±0.1)  |
| 105      | 1.6 (±0.1) \( \times 10^6 \)     | 1.58 (±0.02)        | 963 (±2)     |
| 109      | 2.9 (±0.1) \( \times 10^6 \)     | 0.44 (±0.01)        | 153.2 (±0.5) |

\( k_a \) and \( k_d \) in SPR measurement (\( n = 1 \) biological replicate).

from DYRK1A with strong affinity for 11 was the mitotic kinase Haspin. The lead compounds in Table 9 have comparable selectivity profiles to 11 with good physical properties and would serve as advanced starting points for future studies. Our data shows that modifications could be made to the series that result in improved kinome selectivity and more desirable physicochemical properties.

**Inhibition of DYRK1A Phosphorylation.** To demonstrate the DYRK1A inhibitory activity of the series, compound 11 was profiled in a radiometric kinase assay (\( ^{33} \)PanQinase...
Activity Assay) provided by Proqinase GMBH. The assay measured the kinase activity of DYRK1A in the presence and absence of an inhibitor at 1 μM concentration. Follow-up IC_{50} profiling confirmed that the phosphorylation activity of DYRK1A was inhibited by compound 11 with an IC_{50} of 220 nM (Table 11).

**Cellular Profiling of Lead Compound 11.** Compound 11 was tested for direct binding to DYRK1A in live cells using a nanoluciferase (nanoLuc) bioluminescence resonance energy transfer (BRET) (NanoBRET, Promega) target engagement assay (Figure 3). The NanoBRET assay measured the ability of compound 11 to displace a fluorescent tracer molecule from the ATP-binding site of DYRK1A protein N-terminally fused with NanoLuc luciferase (NiLuc) in HEK293 cells. In the absence of compound 11, the fusion protein and tracer molecule were in proximity and able to generate a detectable BRET signal. Compound 11 demonstrated acceptable cellular activity, with IC_{50} of 434 nM compared to the enzymatic IC_{50} of 75 nM (Table 1).

Table 8. Metabolic Stability and Solubility

| compound | DYRK1A | HLM | RLM | solubility |
|----------|--------|-----|-----|-----------|
| 6b       | 6.73   | 449.8b | 340.3b | 0.05 |
| 8b       | 7.10   | 75 ± 5 | 783.7 ± 31 | 0.01 |
| 11       | 7.12   | 242 ± 32 | 231 ± 31 | 0.10 |
| 12       | 6.94   | 71 ± 2 | 434 ± 58 | 0.06 |
| 22       | 7.10   | 126 ± 9 | 119 ± 6 | 0.06 |
| 24       | 6.96   | 328 ± 25 | 929 ± 116 | 0.01 |
| 28       | 6.94   | 378 ± 66 | 448 ± 20 | 0.02 |
| 29       | 6.83   | 389 ± 23 | 184 ± 18 | 0.05 |
| 31       | 7.18   | 296 ± 14 | 130 ± 17 | 0.04 |
| 33       | 6.87   | 199b | 48 ± 23 | 1.92 |
| 34       | 7.14   | 40b | 66 ± 24 | 4.37 |
| 63       | 6.94   | 160 ± 66 | 164b | 0.01 |
| 66       | 6.86   | 89 ± 11 | 77 ± 17 | 0.07 |
| 70       | 6.80   | 154 ± 6 | 96 ± 25 | 0.47 |
| 78       | 6.87   | 153.9 ± 39 | 234.2 ± 17 | 0.00 |
| 95       | 7.24   | 697 ± 50 | 983 ± 39 | 0.03 |
| 109      | 6.74   | 93.6 ± 1 | 105.3 ± 7.0 | 0.05 |

“pIC_{50} in TR-FRET-based ligand-binding displacement assay measured with two technical replicates (n = 1 biological replicate). HLM and RLM determinations mean of n = 2 ± standard deviation (unless otherwise stated). aData derived from single experiment. HLM = human liver microsomes, RLM = rat liver microsomes; thermodynamic solubility data was derived from a single experiment at pH 6.8.

Table 9. Selectivity Profiles of Lead Compounds

| compound | DYRK1A | DYRK1B | CLK1 | CLK2 | CLK3 | CDK2 | GSK3β | S score (35) |
|----------|--------|--------|------|------|------|------|-------|--------------|
| 11       | 0.4    | 2.7    | 19   | 7.1  | 9.4  | 54   | 100   | 94           | 0.027 |
| 12       | 34     | 43     | 100  | 79   | 95   | 96   | 100   | 91          | 0.013 |
| 22       | 0.7    | 8.3    | 11   | 33   | 13   | 54   | 100   | 75          | 0.042 |
| 24       | 33     | 81     | 66   | 71   | 61   | 100  | 100   | 92          | 0.004 |
| 28       | 2.4    | 7.2    | 15   | 17   | 16   | 88   | 100   | 77          | 0.050 |
| 29       | 1.5    | 9.1    | 25   | 16   | 15   | 73   | 95    | 100         | 0.035 |
| 31       | 20     | 34     | 39   | 48   | 62   | 96   | 95    | 98          | 0.011 |
| 33       | 1.7    | 18     | 29   | 36   | 72   | 95   | 100   | 100         | 0.027 |
| 34       | 1.3    | 10     | 28   | 47   | 51   | 89   | 86    | 77          | 0.022 |
| 63       | 1.1    | 1.6    | 25   | 15   | 13   | 76   | 89    | 100         | 0.027 |
| 66       | 0.2    | 0.7    | 24   | 7.8  | 12   | 83   | 100   | 100        | 0.022 |
| 70       | 0.7    | 6.2    | 35   | 20   | 31   | 56   | 86    | 77          | 0.022 |
| 95       | 18     | 71     | 55   | 53   | 54   | 84   | 100   | 93          | 0.002 |

% Remaining activity as determined by DiscoverX KINOMEscan experiment. Inhibitor concentration = 1 μM.
Compounds 11 and 95 were profiled in the ClariCELL DYRK1A/Tau cellular phosphorylation assay to demonstrate a functional effect in vitro. The tau phosphorylation activity of DYRK1A was measured in the absence or presence of various concentrations of compound in human embryonic kidney cells (HEK293) transiently transfected with DYRK1A and tau. The activity of 11 was compared to the activity of harmine, a known DYRK1A inhibitor. Table 12 shows that compounds 11 and 95 inhibited tau phosphorylation with IC_{50}’s of 0.59 and 0.25 μM, respectively, and are comparable to harmine (IC_{50} 0.20 μM).

### Table 12. Inhibition of Tau Phosphorylation by DYRK1A by Selected Compounds

| compound | IC_{50} for tau phosphorylation (μM) |
|----------|-------------------------------------|
| 11       | 0.59                                |
| 95       | 0.25                                |
| Harmine  | 0.20                                |

IC_{50} determined in ClariCELL DYRK1A/Tau cellular phosphorylation assay (Cell Assay Innovations, n = 2).

Compound 11 was also progressed to a Madin–Darby canine kidney cells transfected with the human MDR1 gene (MDCK-MDR1) assay to determine whether there would be any issues with P-gp liability or permeability (Table 13). The high central nervous system multiparameter optimization (CNS MPO) score calculated for compound 11 correlated with good levels of permeability and low P-gp efflux, suggesting that 11 is a good starting point for development into a CNS-penetrant DYRK1A chemical probe.

### Table 13. DYRK1A CNS MPO and MDCK-MDR1

| compound | CNS MPO Score | direction = A2B | direction = B2A | efflux ratio (mean P_{app} B2A/mean P_{app} A2B) |
|----------|---------------|-----------------|-----------------|-----------------------------------------------|
| 11       | 6             | 71.4 ± 0.65     | 58.7 ± 1.45     | 0.822                                         |

CNS MPO score calculated using CNS MPO KNIME workflow with ChemAxon nodes. ^6^Data generated by Cyprexot in MDCK-MDR1 assay. Permeability coefficient (P_{app}) calculated across cells in direction: A2B (Apical to Basolateral) and B2A (Basolateral to Apical). Determinations ± standard deviation (mean of n = 2 unless otherwise stated).

In Vivo Pharmacokinetic Profiling of Lead Compound 11. Compound 11 had good physicochemical properties and had demonstrated high levels of enzymatic potency, favorable aqueous solubility, exquisite kinase selectivity, and promising on-target in vitro inhibition. Although compound 11 was not optimized for in vitro clearance, we sought to benchmark 11 as a DYRK1A tool for future optimization.

Compound 11 was dosed intravenously at 1 mg/kg in Sprague–Dawley rats (n = 3) in the fed state. Plasma and brain samples were collected. Consistent with the in vitro findings, high clearance was observed for compound 11 in vivo. As a consequence of the high clearance and low volume of distribution, the half-life of compound 11 is not optimal (Table 14). There were no adverse effects reported in the study and the brain:plasma ratio indicated good brain penetration for compound 11 (total br/pl > 0.35), consistent with what was predicted from the MDCK-MDR1 assay.

### Table 14. In Vivo Pharmacokinetic Study of Compound 11

| PK parameter | compound 11 |
|--------------|-------------|
| half-life (h) | 0.2         |
| T_{1/2} (h)  | 0.03        |
| C_{max} (ng/mL) | 1082.8 |
| AUC_{tot} (h*ng/mL) | 275.3 |
| AUC_{inf} (h*ng/mL) | 276.4 |
| CL (mL/min/kg) | 61.6 |
| V_d | 0.8 |
| MRT (h) | 0.2 |
| C_0 (ng/mL) | 1331.5 |

“Compound 11 was dosed as a suspension of the amorphous solid at 1 mg/kg i.v.

### CONCLUSIONS

Starting from DYRK1A inhibitors previously published by our group (6b and 8b), we were able to design compound 11, and derivatives thereof, that maintained and improved upon the selectivity profiles of 6b and 8b. The binding mode appeared to be the same for compounds 11 and 6b, providing further evidence that this unusual binding mode, and in particular, possession of a motif that occupies a shallow lipophilic pocket close to the hinge of DYRK1A, further stabilized by a hydrogen-bonding interaction between the ligand and consensus water, appears to afford isoformal and kinome-selective DYRK1A inhibitors. Iterative crystallography and structure-based design using the elucidated binding mode of 6b, 11, and other pyrazolo[1,5-b]pyridazine analogues allowed rapid expansion to selective and high-affinity binders of DYRK1A with improved physicochemical properties.

Through focused design to reduce the number of aromatic rings and lipophilicity, compounds such as 11 showed improvements in metabolic stability and solubility relative to 6b. The permeability and on-target cellular activity of 11 were demonstrated. Compound 11 was not a P-gp substrate, giving confidence that 11, and analogues thereof, can be used with confidence as tool compounds to determine the role of DYRK1A. Compound 11 represents a small, highly selective, and ligand-efficient starting point for future drug discovery efforts on DYRK1A in any area of medicine. Further optimization of the in vivo clearance of the series may lead to clinical DYRK1A inhibitors.

### EXPERIMENTAL SECTION

The syntheses of lead compounds (Table 9) are described below. The synthetic procedures for all other compounds are found in the Supporting Information. The purity of all target compounds was confirmed to be >95% by HPLC or 1H NMR.

**General Methods.** All commercial reagents were purchased from Sigma-Aldrich, Alfa Aesar, Apollo Scientific, Fluorochem, or Tokyo Chemical Industry and were of the highest available purity. Unless otherwise stated, chemicals were used as supplied without further purification. Anhydrous solvents were purchased from Acros (AcroSeal) or Sigma-Aldrich (SureSeal) and were stored under nitrogen. Anhydrous solvents and reagents were used as purchased. Thin-layer chromatography (TLC) was carried out using glass plates precoated with Merck silica gel 60 F254. Melting point measurements are recorded on an MPA100 OptiMelt apparatus and are uncorrected. Proton nuclear magnetic resonance spectra were recorded at 500 MHz on a Varian VNMRS 500 MHz spectrometer or at 500 MHz on a Bruker Biospin GmbH 500 MHz spectrometer or at 600 MHz on a Varian VNMRS 600 MHz spectrometer, using residual isotopic solvent (CHCl_{3}, δ_H = 7.27 ppm, DMSO δ_H = 2.50 ppm, MeOH δ_H = 3.31 ppm, DMF δ_H = 2.92) as an internal reference. Chemical shifts
Samples were eluted through a Phenomenex Gemini 5 μm C18 50 mm × 4.6 mm column, using water and acetonitrile acidiﬁed by 0.1% formic acid at 1 mL/min and detected at 254 nm. The gradient employed was a 5 min method 10−90% MeCN over a 4 min gradient, held at 90% MeCN for 1 min, then reequilibrated over 1 min. LCMS-MDAP analytical data was recorded on a Shimadzu Prominence HPLC using a Waters 2695 UPLC detector and a Phenomenex LCQ-ESI MS. Samples were eluted through a Phenomenex Luna 3 μm C18 50 mm × 4.6 mm column, using water and acetonitrile acidiﬁed by 0.1% formic acid at 1 mL/min and detected at 254 nm. The gradient employed was a 30 min method either (A) 5−95% MeCN over a 20 min gradient, held at 95% MeCN for 4 min, then reequilibrated to 30% MeCN over 5 min or (B) 30−95% MeCN over 20 min gradient, held at 95% MeCN for 4 min, then reequilibrated to 30% MeCN over 5 min. Specific Optical Rotations were measured using Schmidt and Haensch UniPol L polarimeter, in cells with a path length of 5 cm. The light source was maintained at 589 nm. The concentration (c) is expressed in g/100 mL (equivalent to g/0.1 dm³). Specific rotations are denoted [α]D^20 and are given in implied units of 10^-1 deg cm^2 g^-1 (T = ambient temperature in °C).

Pyridazin-1-ium-1-amino iodide (2). Aminosulfuric acid (13.10 g, 115.64 mmol) was dissolved in water (25 mL), and the reaction mixture was cooled to 10 °C in an ice bath. Potassium bicarbonate solution (48 mL, 2.4 M, 115.2 mmol) was added to the reaction mixture until it was at pH 5. Pyridazine (5.62 mL, 77.4 mmol) was added in one portion, and the reaction mixture was heated to 70 °C overnight at rt. The solid formed was ﬁltered and triturated with ethanol to afford the title compound, pyridazin-3-yl-prop-2-en-1-one (6). To a pressure vial were added 6-pyrazolo[1,5-b]-pyridazin-3-yl-1H-pyrimidin-2-amine (0.25 mg, 0.35 mmol) and phosphorous(V) oxychloride (2 mL, 21.46 mmol). The vial was sealed and heated to 110 °C for 6 h. The crude reaction mixture was added slowly to a water/ice mix and stirred at rt for 1 h. The brown suspension that formed was ﬁltered and washed with water to afford the title compound, 3-(2-chloropyrimidin-4-yl)pyrazolo[1,5-b]pyridazine, 6, as a brown solid (540 mg, 2.17 mmol, 92% yield). Rf 0.83 (MeOH/CH2Cl2; 1:9); mp 293–295 °C. 1H NMR (500 MHz, DMSO-d6) δH 11.60 (s, 1H), 8.94 (dd, J = 9.0, 1.9 Hz, 1H), 8.89 (s, 1H), 8.61 (dd, J = 4.5, 2.0 Hz, 1H), 7.90 (dd, J = 6.5 Hz, 1H, 1H), 7.50 (dd, J = 9.1, 4.5 Hz, 1H), 6.93 (d, J = 6.6 Hz, 1H); HRMS m/z (ESI+) calculated for C10H6N5O2 + 1 [M + H] is 241.08. 241.0723, found: 241.0721 [M + H]+; LC-MS (ESI+): calculated for C10H6N5O2 + 1 [M + H] is 241.14, found 241.34.

Pyrazolo[1,5-b]pyridazin-3-yl-3-yl-pyrimidin-2-amine (11). To a microwave vial containing 3-(2-chloropyrimidin-4-yl)pyrazolo[1,5-b]pyridazine (80 mg, 0.35 mmol) was added dimethyline (0.03 mL, 0.69 mmol). The reaction mixture was heated at 110 °C for 2 h. The reaction mixture was concentrated under reduced pressure. The residue was puriﬁed using ﬂash silicon column chromatography on an Isco CombiFlash system (12 g silica, elution with 0–50% 20% MeOH in CH2Cl2/CH2Cl2 gradient to afford the title compound, N,N-dimethyl-4-pyrazolo[1,5-b]-pyridazin-3-yl-pyrimidin-2-amine, 11, as an off-white solid (70 mg, 0.26 mmol, 77% yield). Rf 0.68 (MeOH/CH2Cl2; 1:9); mp 241–242 °C. 1H NMR (500 MHz DMSO-d6) δH 8.96 (s, J = 9.0, 1.9 Hz, 1H), 8.84 (s, 1H), 8.61–8.58 (m, 1H), 8.35 (d, J = 5.2 Hz, 1H), 7.74 (dd, J = 9.0, 4.4 Hz, 1H), 7.14 (s, J = 5.2 Hz, 1H), 3.22 (s, 3H); 13C NMR (125 MHz, DMSO-d6) δC 161.9, 159.9, 157.9, 143.9, 134.1, 132.3, 129.1, 118.9, 110.5, 104.6, 37.0; HRMS m/z (ESI+) calculated for C12H12N6 + 1 [M + H] is 249.10. 249.0906, found 249.0917 [M + H]+.
pyrazolo[1,5-b]pyrazidin-3-yl-pyrimidin-2-amine, 12, as an off-white solid (10 mg, 0.04 mmol, 10% yield). Mp 162–164 °C; 1H NMR (500 MHz, DMSO-d$_6$) $\delta$ 9.13–9.09 (m, 1H), 8.86 (s, 1H), 8.60–8.56 (m, 8H), 8.38 (d, $J$ = 5.1 Hz, 1H), 7.46 (dd, $J$ = 9.0, 4.4 Hz, 1H), 7.23 (d, $J$ = 5.1 Hz, 1H), 3.15 (s, 3H), 2.90–2.80 (m, 1H), 0.95–0.89 (m, 2H), 0.70–0.64 (m, 2H). 13C NMR (125 MHz, DMSO-d$_6$) $\delta$ 163.2, 158.8, 157.8, 144.0, 139.7, 132.5, 129.4, 118.9, 110.4, 105.7, 35.7, 31.6, 8.3; HRMS m/z [ESI]$^+$ calculated for C$_{26}$H$_{22}$N$_4$ [M + H]$^+$ is 378.1633, found: 378.1631 [M + H]$^+$. 

1-(4-Pyrazolo[1,5-b]pyridazin-3-yl-pyrimidin-2-yl)-6-oxa-1-azaspiro[3.3]heptane (22). To a reaction vial containing 3-(2-chloropyrimidin-4-yl)pyrazolo[1,5-b]pyridazine (60 mg, 0.26 mmol) in 2-propanol (2 mL) was added 6-oxa-1-azaspiro[3.3]heptane (51 mg, 0.52 mmol). The vial was sealed and heated to 140 °C for 16 h. The reaction mixture was concentrated under reduced pressure. The residue was adsorbed onto solid load material. The crude was purified using flash silica column chromatography on a Biotage system (12 g silica, elution with 10–100% MeOH/water gradient) to afford the title compound, 3-[2-(3S,3S)-3-methylpyrrolidin-1-yl]pyrimidin-4-yl]pyrazolo[1,5-b]pyrazidin-28, as a colorless solid (21 mg, 0.07 mmol, 27% yield). $R_f$ 0.34 (EtOAc/petroleum ether 1:1); $[\alpha]_{D}^22$ = –60.1 (c = 3.3 × 10$^{-4}$ g/mL, CHCl$_3$); mp 213–215 °C; 1H NMR (600 MHz, DMSO-d$_6$) $\delta$ 8.99 (app. d, $J$ = 9.0 Hz, 1H), 8.84 (s, 1H), 8.61–8.57 (m, 1H), 8.33 (d, $J$ = 4.4 Hz, 1H), 7.34 (d, $J$ = 10.4 Hz, 1H), 4.29 (s, 2H), 7.17 (d, $J$ = 5.1 Hz, 1H), 2.36–2.34 (m, 1H), 2.18–2.10 (m, 1H), 1.70–1.54 (m, 1H), 1.13 (s, 3H). 13C NMR (150 MHz, DMSO-d$_6$) $\delta$ 160.0, 159.0, 158.0, 143.9, 139.6, 132.4, 119.0, 110.4, 104.4, 53.4, 46.1, 32.9, 32.6, 17.9; HRMS m/z [ESI]$^+$ calculated for C$_{31}$H$_{33}$N$_6$ [M + H]$^+$ is 515.2387, found: 515.2385 [M + H]$^+$. Chiral HPLC, chiralcel OJ column (20:80 heptane:isopropanol (0.2% v/v NH$_3$), 1.0 mL/min). Retention time = 7.07 min > 99% e.e. 

3-[2-(3R,3S)-3-methylpyrrolidin-1-yl]pyrimidin-4-yl]pyrazolo[1,5-b]pyridazine (31). To a microwave vial containing 3-(2-chloropyrimidin-4-yl]pyrazolo[1,5-b]pyrazidin-28 (0.07 mmol) in 2-propanol (1 mL) was added R-(-)3-fluoropyrrolidin-1-yl]pyrimidin-4-yl]pyrazolo[1,5-b]pyrazidin-31, as a cream solid (70 mg, 0.23 mmol, 80% yield). $R_f$ 0.24 (EtOAc/petroleum ether 1:1); $[\alpha]_{D}^22$ = +60.1 (c = 3.67 × 10$^{-4}$ g/mL, CHCl$_3$); mp 228–230 °C; 1H NMR (600 MHz, DMSO-d$_6$) $\delta$ 8.97 (app. d, $J$ = 9.0 Hz, 1H), 8.82 (s, 1H), 8.59–8.55 (m, 1H), 8.34 (d, $J$ = 5.1 Hz, 1H), 7.43 (dd, $J$ = 9.2, 4.4 Hz, 1H), 7.16 (d, $J$ = 5.1 Hz, 1H), 5.47 (d, $J$ = 53.3 Hz, 1H), 4.05–3.46 (m, 4H, 2.37–2.12 (m, 2H). 13C NMR (150 MHz, DMSO-d$_6$) $\delta$ 160.0, 159.1, 158.1, 143.9, 139.7, 132.5, 129.4, 119.0, 110.2, 105.1, 93.2 (d, $J$ = 170.5 Hz), 53.2 (d, $J$ = 22.5 Hz), 44.2, 31.6 (d, $J$ = 22.0 Hz), HRMS m/z [ESI]$^+$ calculated for C$_{27}$H$_{25}$F$_2$N$_6$ [M + H]$^+$ is 425.1858, found: 425.1856 [M + H]$^+$. Chiral HPLC, Lux A2 column (50:50 isopropanol:CO$_2$ (0.2% v/v NH$_3$), 4.0 mL/min). Retention time = 2.04 min > 99% e.e.
(3S)-1-(4-pyrazolo[1,5-b]pyridazin-3-yl-pyrimidin-2-yl)pyrrolidin-3-amine (34). To a microwave vial containing a suspension of 3-[(2-chloropyrimidin-4-yl)pyrazolo[1,5-b]pyridazine (60 mg, 0.26 mmol) in 2-propanol (2 mL) was added (3S)-1-(4-pyrazolo[1,5-b]pyridazin-3-yl-pyrimidin-2-yl)pyrrolidin-3-amine, 34, as a pale yellow solid (32 mg, 0.11 mmol, 42% yield). Rf = 0.09 (MeOH/CH2Cl2 1:9); [α]25D = +89.6 (c = 6.7 × 10−4 g/mL, CHCl3); decomposed > 210 °C; 1H NMR (600 MHz, DMSO-d6) δ 8.99 (app. dd, J = 9.1 Hz, 1H), 8.82 (s, 1H), 8.60–8.56 (m, 1H), 8.32 (d, J = 5.1 Hz, 1H), 7.45 (dd, J = d = 9.1, 4.4 Hz, 1H), 7.11 (dd, J = d = 5.1 Hz, 1H), 3.92–2.90 (m, 5H), 2.30 (m, 4H); 13C NMR (150 MHz, DMSO-d6) δc 160.1, 158.9, 158.0, 143.9, 139.6, 132.4, 129.3, 118.8, 110.4, 104.4, 54.9, 50.7, 44.9, 34.1; HRMS m/z (ESI−) calculated for C14H16N7 [M + H]+ is 282.1462, found: 282.1455 [M + H]+; LCMS (MDAP): Rf = 8.1 min, >95% (method A); LC-MS (ESI): calculated for C14H16N7 [M + H]+ is 282.1353, found: 255.1350 [M + H]+; LCMS (MDAP): 11.6 min, >95% (method A); LC-MS (ESI): calculated for C14H16N7 [M + H]+ is 255.1, found: 255.9 [M + H]+.

(3R)-1-(4-pyrazolo[1,5-b]pyridazin-3-yl-pyrimidin-2-yl)pyrrolidin-3-amine (34). To a microwave vial containing a suspension of 3-[(2-chloropyrimidin-4-yl)pyrazolo[1,5-b]pyridazine (60 mg, 0.26 mmol) in 2-propanol (2 mL) was added (R)-pyrrolidin-3-amine (22 mg, 0.26 mmol). The vial was sealed, and the reaction mixture was heated to 140 °C for 16 h. The reaction mixture was concentrated under reduced pressure. The crude was purified using flash silica column chromatography on an Isco CombiFlash system (12 g silica, elution with 0–50% MeOH/CH2Cl2/CH3Cl gradient) to afford the title compound. Desired fractions were combined and concentrated under reduced pressure. The crude was purified by flash reversed-phase column chromatography on a Biotage system (12 g silica, elution with 100% MeOH/water gradient) to afford the title compound, (3R)-1-(4-pyrazolo[1,5-b]pyridazin-3-yl-pyrimidin-2-yl)pyrrolidin-3-amine, 34, as a pale yellow solid (32 mg, 0.11 mmol, 42% yield). Rf = 0.09 (MeOH/CH2Cl2 1:9); [α]25D = +89.6 (c = 6.7 × 10−4 g/mL, CHCl3); decomposed > 210 °C; 1H NMR (600 MHz, DMSO-d6) δ 8.99 (app. dd, J = 9.1 Hz, 1H), 8.82 (s, 1H), 8.60–8.56 (m, 1H), 8.32 (d, J = 5.1 Hz, 1H), 7.45 (dd, J = d = 9.1, 4.4 Hz, 1H), 7.11 (dd, J = d = 5.1 Hz, 1H), 3.92–2.90 (m, 5H), 2.30 (m, 4H); 13C NMR (150 MHz, DMSO-d6) δc 160.1, 158.9, 158.0, 143.9, 139.6, 132.4, 129.3, 118.8, 110.4, 104.4, 54.9, 50.7, 44.9, 34.1; HRMS m/z (ESI−) calculated for C14H16N7 [M + H]+ is 282.1462, found: 282.1455 [M + H]+; LCMS (MDAP): Rf = 8.1 min, >95% (method A); LC-MS (ESI): calculated for C14H16N7 [M + H]+ is 282.1353, found: 255.1350 [M + H]+; LCMS (MDAP): 11.6 min, >95% (method A); LC-MS (ESI): calculated for C14H16N7 [M + H]+ is 255.1, found: 255.9 [M + H]+.

1-(4-Methoxypyrazolo[1,5-b]pyridazin-3-yl)-N,N-dimethylpyrimidin-2-amine (66). To a solution of 4-methoxypyrazolo[1,5-b]pyridazin-3-yl-N,N-dimethylpyrimidin-2-amine, 66, as a white solid (12 mg, 0.04 mmol, 8% yield). Rf = 0.41 (EtOAc); mp 180–182 °C; 1H NMR (600 MHz, DMSO-d6) δ 8.54 (s, 1H), 8.42 (d, J = 5.6 Hz, 1H), 8.34 (d, J = d = 5.1 Hz, 1H), 7.13 (d, J = 5.1 Hz, 1H), 6.88 (d, J = 5.6 Hz, 1H), 4.08 (s, 3H), 3.18 (s, 6H); 13C NMR (150 MHz, DMSO-d6) δc 161.1, 158.3, 157.9, 157.7, 144.0, 139.8, 126.5, 112.6, 108.2, 96.9, 56.8, 36.6; HRMS m/z (ESI−) calculated for C13H15N6 [M + H]+ is 255.1353, found: 255.1350 [M + H]+; LCMS (MDAP): 11.6 min, >95% (method A); LC-MS (ESI): calculated for C13H15N6 [M + H]+ is 255.1, found: 255.9 [M + H]+.

1-Imidazo[1,2-ajpyrimidin-3-ylthetanone (68a). Pyrimidin-2-amine (1 g, 10.52 mmol) was dissolved in toluene (70 mL), and N,N-dimethylformamide dimethyl acetel (3 mL, 22.58 mmol) was
added. The reaction mixture was heated to 90 °C for 2 h. The reaction mixture was concentrated under reduced pressure. The crude was dissolved in EtOH (10 mL) and chloroacetone (1.6 mL, 20.1 mmol) was added to the mixture. The reaction mixture was heated to reflux and stirred overnight. The reaction mixture was concentrated under reduced pressure. The crude was purified using flash silica column chromatography on an isocratic flash silica column (24 g silica, elution with 0–50% MeOH in CH2Cl2/CH3Cl gradient) to afford 1-imidazo[1,2-a]pyrimidin-3-yl-thiophen-3-ylacetone (360 mg, 1.73 mmol, 78% yield). Rf = 0.10 (EtOAc); 1H NMR (600 MHz, DMSO-d6) δ 9.79 (dd, J = 6.8, 2.1 Hz, 1H), 8.83 (dd, J = 4.2, 2.1 Hz, 1H), 7.85 (s, 1H), 7.40 (dd, J = 6.8, 4.2 Hz, 1H), 2.58 (s, 3H); LC-MS (ESI): calculated for C11H13N4O+ [M + H]+ is 217.1, found 216.13 [M + H]+.

(E)-3-(Dimethylamino)-1-imidazo[1,2-a]pyrimidin-3-yl-prop-2-en-1-one (69a). To 1-imidazo[1,2-a]pyrimidin-3-ylthiophen-3-ylacetone (100 mg, 0.62 mmol) was added NaN3 dimethylformamide dimethyl acetal (4 mL, 30.11 mmol). The reaction mixture was heated to 75 °C for 16 h. The reaction mixture was concentrated under reduced pressure to afford the title compound, (E)-3-(dimethylamino)-1-imidazo[1,2-a]pyrimidin-3-yl-prop-2-en-1-one, 69a, as a red solid (150 mg, 0.62 mmol, 99% yield) plus impurities. Rf = 0.23 (MeOH/CH2Cl2, 5:95); 1H NMR (600 MHz, CDCl3) δ 10.05 (dd, J = 6.9, 2.1 Hz, 1H), 8.66 (dd, J = 4.1, 2.1 Hz, 1H), 8.35 (s, 1H), 7.79 (dd, J = 12.3 Hz, 1H), 7.03 (dd, J = 6.9, 4.1 Hz, 1H), 5.66 (d, J = 12.3 Hz, 1H), 3.28–2.76 (m, 6H), 1.91–1.58 (m, 2H); LC-MS (ESI): calculated for C13H14N5O+ [M + H]+ is 239.1, found 239.2 [M + H]+.

To a solution of (100 mg, 0.46 mmol) in 2-methoxyethanol (35 mg, 0.46 mmol) were added 1,1-dimethylguanidine and stirred overnight. The reaction mixture was concentrated under reduced pressure. The crude was purified using flash silica column chromatography on an isocratic flash silica column (12 g silica, elution with 0–60% EtOAc/petroleum ether gradient) to afford 3-bromopyrazolo[1,5-b]pyridazine, 91, as a white crystalline solid (360 mg, 1.73 mmol, 78% yield). Rf = 0.74 (EtOAc/petroleum ether 1:1); mp 102 °C. 1H NMR (600 MHz, DMSO-d6) δ 8.52 (d, J = 4.4, 1.8 Hz, 1H), 8.28 (s, 1H), 8.20 (dd, J = 9.0, 1.8 Hz, 1H), 7.31 (dd, J = 9.0, 4.4 Hz, 1H); LC-MS (ESI): calculated for C3H5BrN4+ [M + H]+ is 198.0 and [M + 2]+ is 200.0, found 198.0 [M+H]+ and 200.0 [M+2]+.

3-Bromopyrazolo[1,5-b]pyridazine (91). To a solution of 3-bromopyrazolo[1,5-b]pyridazine (360 mg, 2.21 mmol) in DMF (4 mL) was added N-bromosuccinimide (790 mg, 4.41 mmol), and the reaction was stirred at rt for 3 h. The reaction mixture was concentrated under reduced pressure. The crude was purified using flash silica column chromatography on an isocratic flash silica column (12 g silica, elution with 0–50% EtOAc/petroleum ether gradient) to afford 3-bromopyrazolo[1,5-b]pyridazine, 91, as a white solid (250 mg, 0.62 mmol, 36% yield). Rf = 0.10 (EtOAc/petroleum ether 1:1); decomposed > 295 °C. 1H NMR (500 MHz, DMSO-d6) δ 12.83 (s, 1H), 8.63 (dd, J = 4.4, 2.1 Hz, 1H), 8.54–8.44 (m, 2H), 7.50 (dd, J = 9.1, 4.4 Hz, 1H); LC-MS (ESI): calculated for C13H13N5O+ [M + H]+ is 164.0, found 164.1 [M+H]+.

Pyrazolo[1,5-b]pyridazine-3-carboxylic acid (90). To a solution of methyl pyrazolo[1,5-b]pyridazine-3-carboxylate (350 mg, 1.98 mmol) in MeOH (2.5 mL) was added lithium hydroxide (135 mg, 3.22 mmol) in water (2.5 mL). The reaction mixture was stirred at rt for 16 h. The reaction mixture was concentrated under reduced pressure. Water (10 mL) was added to the residue, and the resulting solution was cooled in an ice bath. HCl (10 mL, 1M) was added, and the mixture was stirred at rt for 30 min. A suspension immediately formed and was filtered and washed with water. The resulting solid was dried under reduced pressure to afford the title compound, pyrazolo[1,5-b]pyridazine-3-carboxylic acid, 90, as a white solid (250 mg, 1.46 mmol, 74% yield). Rf = 0 (EtOAc/petroleum ether 1:1); mp 119 °C. 1H NMR (500 MHz, DMSO-d6) δ 8.12 (s, 1H), 7.50–7.48 (m, 2H), 7.40–7.33 (m, 4H), 7.22 (t, J = 6.9 Hz, 2H), 7.00 (dd, J = 6.9, 3.8 Hz, 1H), 3.86 (s, 3H); LC-MS (ESI): calculated for C8H7N4O5+ [M + H]+ is 241.1, found 241.7 [M+H]+.
solid 4-chloro-5-fluoro-N,N-dimethyl-pyrimidin-2-amine (80 mg, 0.21 mmol). The reaction mixture was heated to 140 °C overnight. The reaction mixture was concentrated under reduced pressure. The crude was purified using flash silica column chromatography on an Isco Combiflash system (12 g silica, elution with 0%–60% EtOAc/petroleum ether gradient). Desired fractions were concentrated under reduced pressure. The crude was purified using flash reversed-phase column chromatography on a Biogate system (12 g silica, elution with 10–100% MeOH/water gradient) to afford the title compound, 5-fluoro-N,N-dimethyl-4-pyrazolo[1,5-b]pyridazin-3-yl-pyrimidin-2-amine, 95, as an off-white solid (50 mg, 0.18 mmol, 57% yield). Rf 0.57 (EtOAc/petroleum ether 1:1); mp 181–183 °C; 1H NMR (600 MHz, CDCl3) δH 9.00 (dd, J = 9.0, 2.0 Hz, 1H), 8.66 (d, J = 3.1 Hz, 1H), 8.41 (dd, J = 4.4, 2.0 Hz, 1H), 8.23 (d, J = 3.2 Hz, 1H), 7.19 (dd, J = 9.0, 4.4 Hz, 1H), 3.25 (s, 6H);13C NMR (150 MHz, CDCl3) δC 159.2, 148.6 (d, J = 16.2 Hz), 134.4, 129.7, 118.0, 107.1, 38.2; HRMS m/z (ESI+) calculated for C12H11FN6Na [M + Na]+ is 281.0921, found: 281.0918 [M + Na]+; LCMS (MDAP): 19.6 min, >95% (method A); LC-MS (ESI): calculated for C12H12FN6 [M + Na]+ is 259.1, found 259.9 [M + H]+.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c01115.

- DSF analysis of selected compounds (XLSX)
- KINOMÉscan analysis of compounds in Table 9 (XLSX)
- SMILES (CSV)

Materials and methods for synthetic chemistry, protein expression, purification, crystallization, data collection, and structure determination; characterization of compounds (PDF)

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**Author Contributions**

S.H.H. was involved in the conception and design, acquisition of data, analysis and interpretation of data, and drafting and revising of the article. F.S. was involved in the conception and design, acquisition of data, analysis and interpretation of data, and drafting and revising of the article. J.B., O.F., P.H.G., and R.R.d.S. were involved in the acquisition, analysis, and interpretation of data. M.T.H was involved in the acquisition of data. S.R. and A.A.-K. were involved in the acquisition of data. L.H.N was involved in the acquisition of data and revising of the article. D.S.W. was involved in the analysis and interpretation of data. J.M.E was involved in the conception of the project, analysis and interpretation of data, and drafting and revising of the article. S.E.W. was involved in the conception of the project, analysis and interpretation of data, and revising of the article. All authors approved the final version to be published.

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**Notes**

The authors declare no competing financial interest.

PDB X-ray structures: PDB 6S1B (compound 11); PDB 6S17 (compound 10); PDB 6S14 (compound 12); PDB 6S1J (compound 30); PDB 6S1H (compound 78). The authors released the atomic coordinates on June 26, 2019.

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ABBREVIATIONS USED

BBB, blood–brain barrier; CDK, cyclin-dependent kinase; CLK, CDC-like kinases; CMGC, including cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAP kinases), glycogen synthase kinases (GSK) and CDK-like kinases; CNS MPO, central nervous system multiparameter degeneration optimization; DYRK, dual-specificity tyrosine phosphorylation-regulated kinase; GSK, glycogen synthase kinases; HBA, hydrogen-bond acceptor; HLM, human liver microsomes; LE, ligand efficiency; LLE, lipophilic ligand efficiency; MDCK-MDR1, Madin–Darby canine kidney cells transfected with the human MDR1 gene; MMP, matched-molecular pair; PDB, Protein Databank; P-gp, permeability glycoprotein 1; PK, pharmacokinetics; RLM, rat liver microsomes; SAR, structure–activity relationship; SBDD, structure-based drug design

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