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Evaluation of substituted 6-arylquinazolin-4-amines as potent and selective inhibitors of cdc2-like kinases (Clk)

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ABSTRACT

A series of substituted 6-arylquinazolin-4-amines were prepared and analyzed as inhibitors of Clk4. Synthesis, structure–activity relationships and the selectivity of a potent analogue against a panel of 402 kinases are presented. Inhibition of Clk4 by these agents at varied concentrations of assay substrates (ATP and receptor peptide) highly suggests that this chemotype is an ATP competitive inhibitor. Molecular docking provides further evidence that inhibition is the result of binding at the kinase hinge region. Selected compounds represent novel tools capable of potent and selective inhibition of Clk1, Clk4, and Dyrk1A.

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The removal of intron sequences from genes occurs via the actions of the spliceosome, a protein complex that removes intervening sequences at the nuclear pre-mRNA level to afford properly coded mRNA for translation.^{1,2} Many genes produce multiple mRNA isoforms through the actions of alternative splicing and, importantly, numerous human diseases are caused by improper splicing.³ Exogenous manipulation of the spliceosome is theorized to be a powerful means to control gene translation and ultimately correct disease phenotypes via rectification of splicing abnormalities.

The modulation of kinases provides a powerful mechanism to control numerous aspects of cell function and offers the potential for the management of many diseases.⁴ Small molecule inhibitors of kinases (both selective and promiscuous) represent important biochemical tools for basic research and several kinase inhibitors have gained FDA approval as drugs.^{5,6} In 2002, Manning et al. reviewed the human kinome and found 518 putative kinase genes⁷ providing a roadmap to explore the consequences of kinase modulation within cellular biology. The possible control of gene splicing through the modulation of kinase activity represents an intriguing concept.⁸ There are several reports of kinases that alter the function of the spliceosome. Among these is the cdc2-like kinase (Clk) family.⁹ A major target of Clk kinases is the prominent family of serine- and arginine-rich (SR) splicing proteins^{10,11} which are involved in the assembly of the spliceosome and are implicated in

both constitutive and alternative splicing control and selection of splicing sites.^{12,13}

The Clk family contains four characterized isoforms (Clk1, Clk2, Clk3, and Clk4). The Clks are capable of auto-phosphorylation (at serine, threonine, and tyrosine residues) and phosphorylation of exogenous proteins (at serine and threonine residues). Members of the Clk family have been implicated in the regulation of alternative splicing of PKCβII¹⁴, TF¹⁵, Tau,¹⁶ and β-globin¹⁷ pre-mRNA. These studies suggest that small molecule modulation of the Clk family of kinases may represent an important mechanism for the control of mRNA splicing.

Hagiwara and co-workers have reported TG003 (**1**) (Fig. 1) as a small molecule with low-nanomolar IC₅₀ values versus Clk1 and Clk4.¹⁷ Additionally, Clk inhibitors are presented in patent reports from Sirtris Pharmaceuticals (no structures presented) and Chronogen Inc. (a series of substituted quinolines related to structure **2**).^{18,19} The report from Hagiwara and co-workers does not define the selectivity of TG003 beyond the Clk family and four additional kinases (PKA, PKC, SRPK1, and SRPK2) and the patents do not disclose details regarding structure–activity relationships nor selectivity. Thus, a need remains for small molecule probes of this important class of enzymes. Here, we report a novel class of quinazoline small molecules as potent and selective inhibitors of Clk1 and Clk4.

We recently performed a high-throughput screen for small molecule modulators of Lamin A splicing (PubChem AID: 1487); details of this study will be reported elsewhere. Among the actives was a series of substituted quinazolines including **3** (Fig. 1). The

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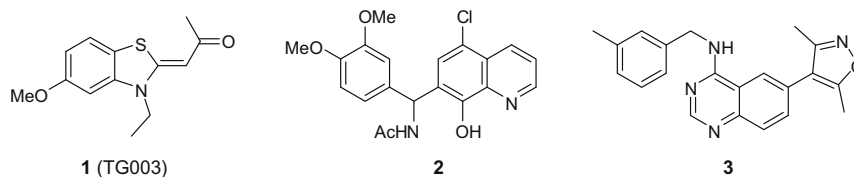


Figure 1. Structures of known Clk inhibitors **1** (TG003), **2** and the lead structure **3**.

previously reported ability of quinazoline small molecules to modulate kinases through competitive inhibition at the ATP binding site suggested potential kinase stimulated influence of splicing. Given the association of the Clk family with splicing, we speculated that **3** might be a Clk inhibitor and this was confirmed via a commercial kinase profile.²⁰ Based on these results, we examined selected in-house compound libraries for Clk4 inhibition via two bioluminescent, luciferase-based assays capable of visualizing substrate (ATP) depletion and product (ADP) formation.²¹ In primary screening, this biochemical assay revealed that **3** (Fig. 1) possessed an IC_{50} value versus Clk4 of 316 nM.

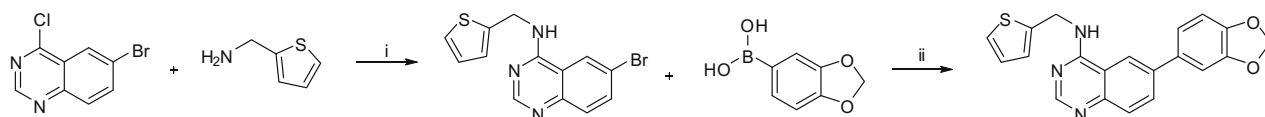
Based upon this result, we examined the SAR for this chemotype versus Clk4. Key to this effort was the synthetic preparation of these agents. In our hands, the most straightforward synthesis began with 6-bromo-4-chloroquinazoline (Scheme 1). Addition of primary and secondary amines (including aliphatic, benzylic, heteroaromatic and substituted anilines) was accomplished in DMF with Hunig's base at room temperature. The resulting 6-bromo-N-substituted-quinazolin-4-amines were then subjected to standard Suzuki–Miyaura couplings with various aryl boronic acids using tetrakis(triphenylphosphine)palladium(0) and sodium carbonate in DMF and heating using microwave irradiation. This general procedure²² resulted in the production of numerous N-substituted-6-arylquinazolin-4-amines including **4** which were purified via reverse-phase HPLC methods prior to testing in biological assays.

The primary phenotypic screen and the follow-up biochemical Clk4 assay were performed on the NIH Molecular Libraries Small Molecule Repository and several internal NCGC compound libraries. Each assay was performed using the quantitative high-throughput screening method whereby each molecular entity is screened in a dose–response format.²³ This screening format provides an unprecedented level of detail with regards to nascent SAR from the primary screening data. The resulting analysis of both of the aforementioned screens provided several clues as to which substitution patterns were likely to result in active compounds. Substitutions at the 4 position of the quinazoline core scaffold were limited to substituted benzylic systems (for instance *m*-tolylmethanamine, pyridin-3-ylmethanamine, and (3-fluorophenyl)methanamine) and various heterocyclic methylamines (for instance 1-(furan-3-yl)-*N*-methylmethanamine, (4-methylthiophen-2-yl)methanamine, and thiophen-2-ylmethanamine). Substitutions at the 6 position of the quinazoline core scaffold were limited to various aryl groups (for instance 3-methoxybenzene, benzo[d][1,3]dioxole, 2,3-dihydrobenzo[b][1,4]dioxine, furan, and 3,5-dimethylisoxazole). As SAR is often not additive, we chose to incorporate all of these substitutions in matrix format in hopes of identifying more potent Clk4 inhibitors and revealing as much SAR as possible. The results of this library are

listed in Figure 2. The original lead **3** was found to possess an IC_{50} value of 282 nM (very similar to the IC_{50} value determined in the primary biochemical screen). Additionally, two novel small molecules were found with potencies of 63 nM including 6-(benzo[d][1,3]dioxol-5-yl)-*N*-(thiophen-2-ylmethyl)quinazolin-4-amine (**4**). The SAR revealed that the benzo[d][1,3]dioxole was consistently favored in the 6 position of the quinazoline ring. The SAR of the amine substitutions was more complex. The thiophen-2-ylmethanamine substitution was typically favored, but several structural combinations favored other moieties at this position.

Prior to additional investigations into the SAR and optimization of this chemotype, it was important for us to gain an appreciation of the selectivity of these compounds towards the Clk family of kinases. Quinazoline based small molecules have been reported as potent inhibitors of numerous kinase families. Any small molecule Clk inhibitor that is additionally capable of promiscuous inhibition across the kinome will be of limited value as a tool compound for evaluating Clk biochemistry. To assess the selectivity of this general chemotype we choose to submit a representative reagent (analogue **4**) across a commercial panel of kinases. In 2008, Ambit Biosciences reported a quantitative analysis of 38 known kinase inhibitors across a panel of 317 kinases.²⁴ This commercially available panel contained 402 kinases at the time of submission and is based upon a competition binding assay of kinases fused to a proprietary tag. The data is first recorded as a % of kinase bound to an immobilized ligand in the presence and absence of the test reagent as compared to DMSO. Activities beyond a selected threshold are submitted for K_d determination. In addition to profiling **4** versus this panel, we submitted the reported small molecule Clk1/4 inhibitor TG003 (**1**) to generate a comparison between both agents. The results (tabulated in a dendrogram representation²⁵) are shown in Figure 3 and demonstrate that both agents are remarkably selective. TG003 (**1**) was determined to have K_d 's of 19 nM, 95 nM, and 30 nM versus Clk1, Clk2, and Clk4, respectively. The K_d for TG003 (**1**) versus Clk3 was 3 μ M. It was also found that TG003 had activity versus CSNK1D (150 nM), CSNK1E (300 nM), Dyrk1A (12 nM), Dyrk1B (130 nM), PIM1 (130 nM), PIM3 (280 nM), and Ysk4 (290 nM). The novel quinazoline **4** was found to have K_d 's of 37 nM, 50 nM and 27 nM versus Clk1, Clk4, and Dyrk1A, respectively. The only other locus of relevant activity (below 500 nM) was found for binding to the endothelial growth factor receptor (EGFR) (230 nM).

Based upon the potency and selectivity for **4** we next aimed to understand the binding mechanism of this chemotype at Clk4. We explored the binding modality by examining the inhibitory capacity of **4** in settings that varied both compound and substrate concentrations. The results are shown in Figure 4. The dose–response curve of **4** in the presence of three different ATP



Scheme 1. Reagents and conditions: (i) DIPEA, DMF, rt, 2 h (typical yields: 80–95%); (ii) Pd(PPh₃)₄, Na₂CO₃, DMF, 150 °C (wave), 1 h (typical yields: 50–80%).

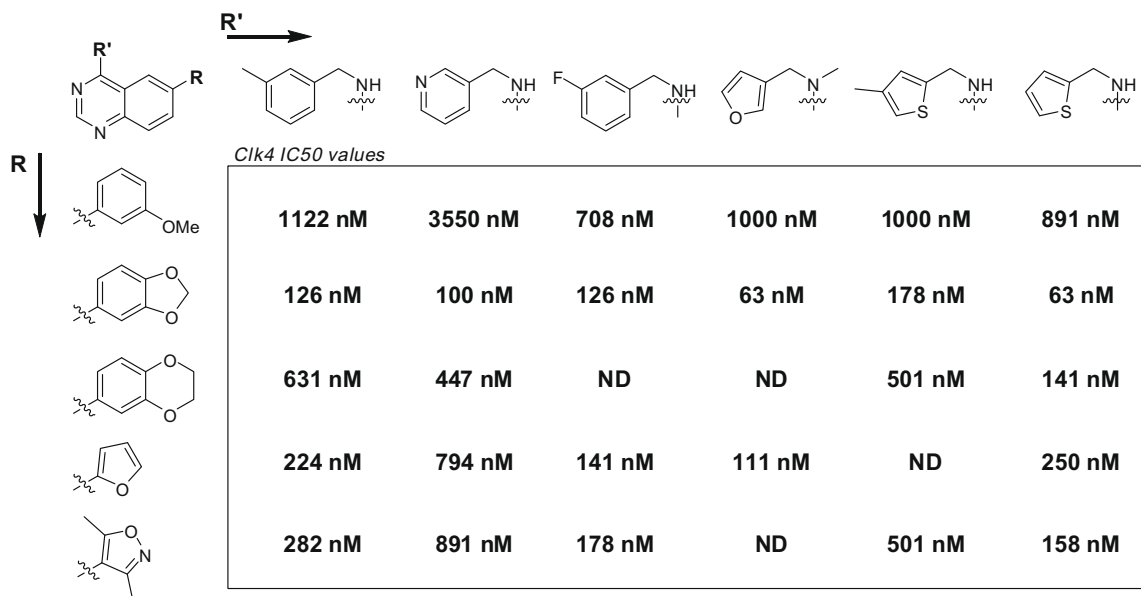


Figure 2. SAR results for matrix library of Clk4 inhibitors. Data represents the results from three separate experiments. All compounds achieved a maximum response greater than 90%.

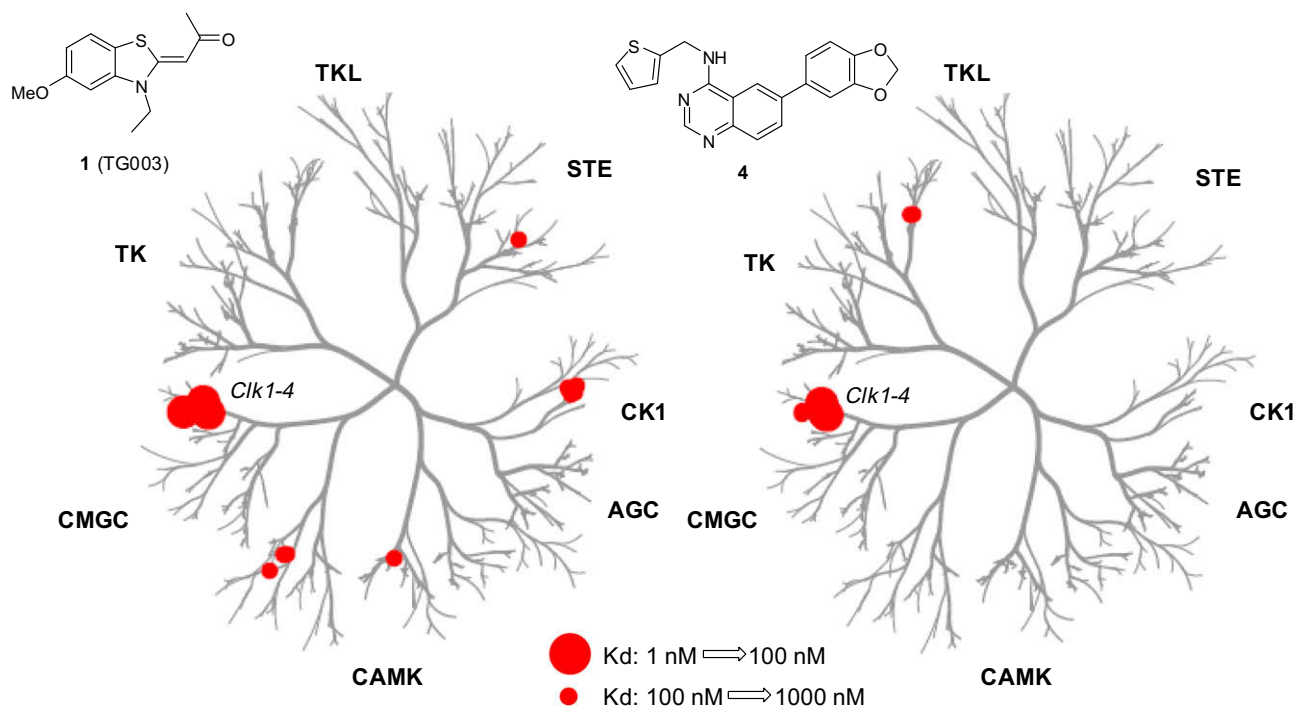


Figure 3. Dendrogram representation of the selectivity profile for kinase binding by TG003 (**1**) and **4** within a panel of 402 kinases. Activity for **1**: Clk1 = 19 nM, Clk2 = 95 nM, Clk3 = 3000 nM, Clk4 = 30 nM, CSNK1D = 150 nM, CSNK1E = 300 nM, CSNK1G2 = 270 nM, CSNK1G3 = 290 nM, Dyrk1A = 12 nM, Dyrk1B = 130 nM, PIM1 = 130 nM, PIM3 = 280 nM, Ysk4 = 290 nM. Activity for **4**: Clk1 = 37 nM, Clk2 = 680 nM, Clk3 = 470 nM, Clk4 = 50 nM, Dyrk1A = 27 nM, Dyrk1B = 430 nM, EGFR = 230 nM. Note: **4** was active (>250 nM) versus numerous EGFR mutants.

concentrations demonstrates a loss in potency when ATP levels rise (Fig. 4A). Conversely, the % activity of **4** in the presence of varying concentrations of the peptide substrate has no effect on the compound potency (Fig. 4B). Additionally, while maintaining **4** at a constant concentration (70 nM), an examination of the dose-response of ATP demonstrated a sharp decline in enzyme inhibition at high ATP concentrations (>200 μ M) while an increase in the dose of the peptide did not effect the potency of **4** (data not shown).

Following confirmation that this chemotype inhibits Clk4 via an ATP competitive mechanism, it was of interest to explore docking of **4** at a Clk kinase. As previously mentioned, quinazoline based small molecules have precedence as kinase inhibitors.²⁶ Among these reagents is the clinically approved drug erlotinib (Tarceva®).²⁷ Erlotinib is currently indicated for treatment of non-small cell lung and pancreatic cancer and its actions are mediated through inhibition of the EGFR tyrosine kinase.²⁸ In 2002, Stomos et al. reported the structure of erlotinib bound to the ATP binding domain of EGFR

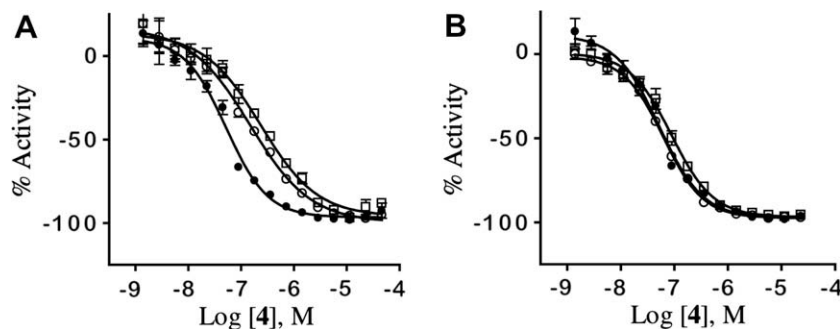


Figure 4. (A) Inhibitory dose–response of **4** versus Clk4 in the presence of three different ATP concentrations [1 μ M (filled circles), 50 μ M (empty circles), 100 μ M (empty squares)]. (B) Inhibitory dose–response of **4** in the presence of three different peptide concentrations [50 μ M (filled circles), 100 μ M (empty circles), 200 μ M (empty squares)].

(PDB code: 1M17).²⁹ We theorized that the relationship of the 4-anilinoquinazoline structure of erlotinib to our newly discovered Clk family inhibitors may provide significant insight into the binding modality and mechanism of action for this class of compounds. There are no published X-ray structures of Clk4. There are structures of Clk1 (PDB code: 1Z57) and Clk3 (PDB code: 2EU9). Clk1 and Clk4 are highly homologous enzymes (>85% sequence identity) while Clk2 and Clk3 also share a high degree of sequence homology (>70% sequence identity). Based upon this, we utilized the X-ray structure of Clk1 as the template to derive a homology model of Clk4 (Fig. 5A) using MOE molecular modeling software.³⁰ Molecular docking was performed on **4** within the ATP binding domain of Clk1 and Clk4 to achieve an optimal binding pose using FRED³¹ (Fig. 5B). In the crystal structure of erlotinib and EGFR, the N1 of the quinazoline heterocycle makes a critical H-bond to an amide NH of the hinge region of the ATP binding pocket. This interaction is mimicked within our docking analysis of **4** at Clk1 and Clk4. The thiophen-2-ylmethanamine moiety is oriented to fill an open pocket formed by the gatekeeper Phe241 (Phe239 in Clk4) while the benzo[d][1,3]dioxole extends toward the solvent exposed face of the hinge region. While this model partially accounts for various aspects of the SAR found to date, more extensive exploration of this binding modality is required to fully understand the potency and remarkable selectivity associated with this chemotype.

An interesting aspect of these agents is the inhibitory activity of this chemotype versus Dyrk1A. The Dyrk1A [dual specificity tyrosine (Y)-phosphorylation-regulated kinase 1A] gene is located on chromosome 21 and is known to be highly expressed in CNS tissues. Dyrk1A knock-out mice are embryonic lethal and transgenic mice overexpressing Dyrk1A display learning and memory deficiencies.^{32,33} The Dyrk1A gene is located on the Down's syndrome (DS) critical region of chromosome 21 and trisomy-driven overexpression in DS patients has been demonstrated.³⁴ Further evidence has been presented that hyperphosphorylation of Tau by Dyrk1A is a causative factor in the early onset of Alzheimer disease in DS patients.³⁵ To date, a class of pyrazolidine-3,5-diones discovered via in silico screening have been reported as Dyrk1A inhibitors.^{36,37} The most potent of these agents were demonstrated to have an IC₅₀ value of 0.6 μ M and selectivity versus a panel of 15 selected kinases highly suggested that these agents were fairly promiscuous. The binding of **4** to Dyrk1A with a potency of 27 nM suggests that **4** and related 6-arylquinazolin-4-amines may represent important new tool compounds for exploration of Dyrk1A biochemistry. We have confirmed that **4** and related analogues are potent inhibitors of Dyrk1A (data not shown). Interestingly, Dyrk1A has been implicated as an important modulator of pre-mRNA splicing via several molecular interactions including the phosphorylation of the SR protein cyclin L2.³⁸

The fact that both **4** and TG003 were highly selective for the Clk family and Dyrk1A leads to questions regarding the relationship

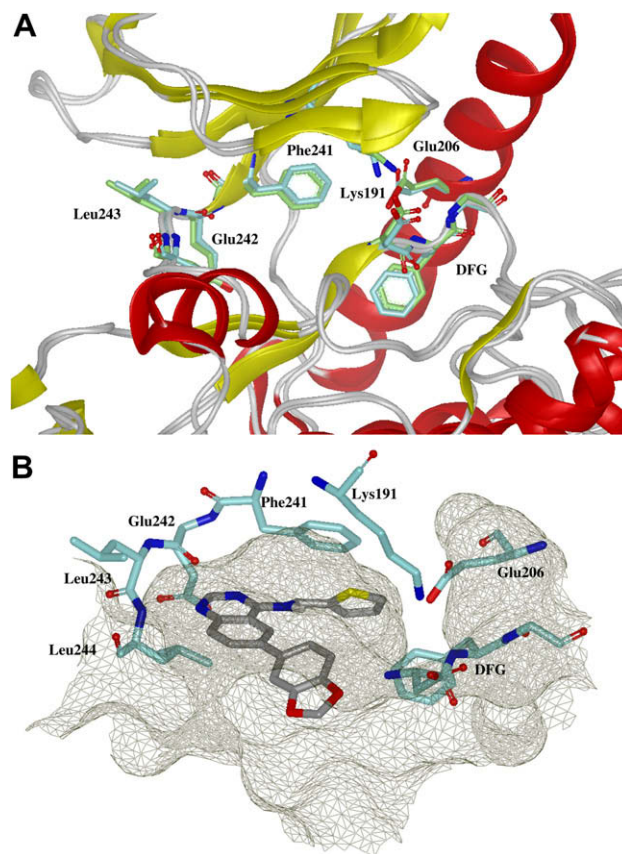


Figure 5. (A) Ribbon representation of the catalytic clefts in the Clk1 and Clk4 kinase domain. Clk4 kinase domain is a homology model derived from the X-ray structure of Clk1 (PDB code: 1Z57). Protein kinase structural elements are labeled and the key residues are colored: Clk1 in cyan, Clk4 in green. This figure was prepared with the program VIDA (OpenEye Scientific Software). (B) Docking model of **4** in the Clk1 catalytic cleft. The binding pocket is depicted by molecular surface in mesh grey. This figure was prepared with the program VIDA (OpenEye Scientific Software).

between these two enzyme classes. Clk and Dyrk are both members of the CMCG branch of the kinome, however, Dyrk1A and Clk1 are only 32.8% homologous. A sequence comparison is provided in Figure 6. While each kinase retains several key amino acids residues that seem to be fundamental to forming the ATP binding domain (including Glu206, Lys191 and matched hydrophobic residues at positions 243 and 244) there are significant differences that likely confer divergent structural aspects between the Clks and Dyrk1A. A concerted effort to correlate compound SAR at each enzyme will be required to better understand the relationship between these kinases.

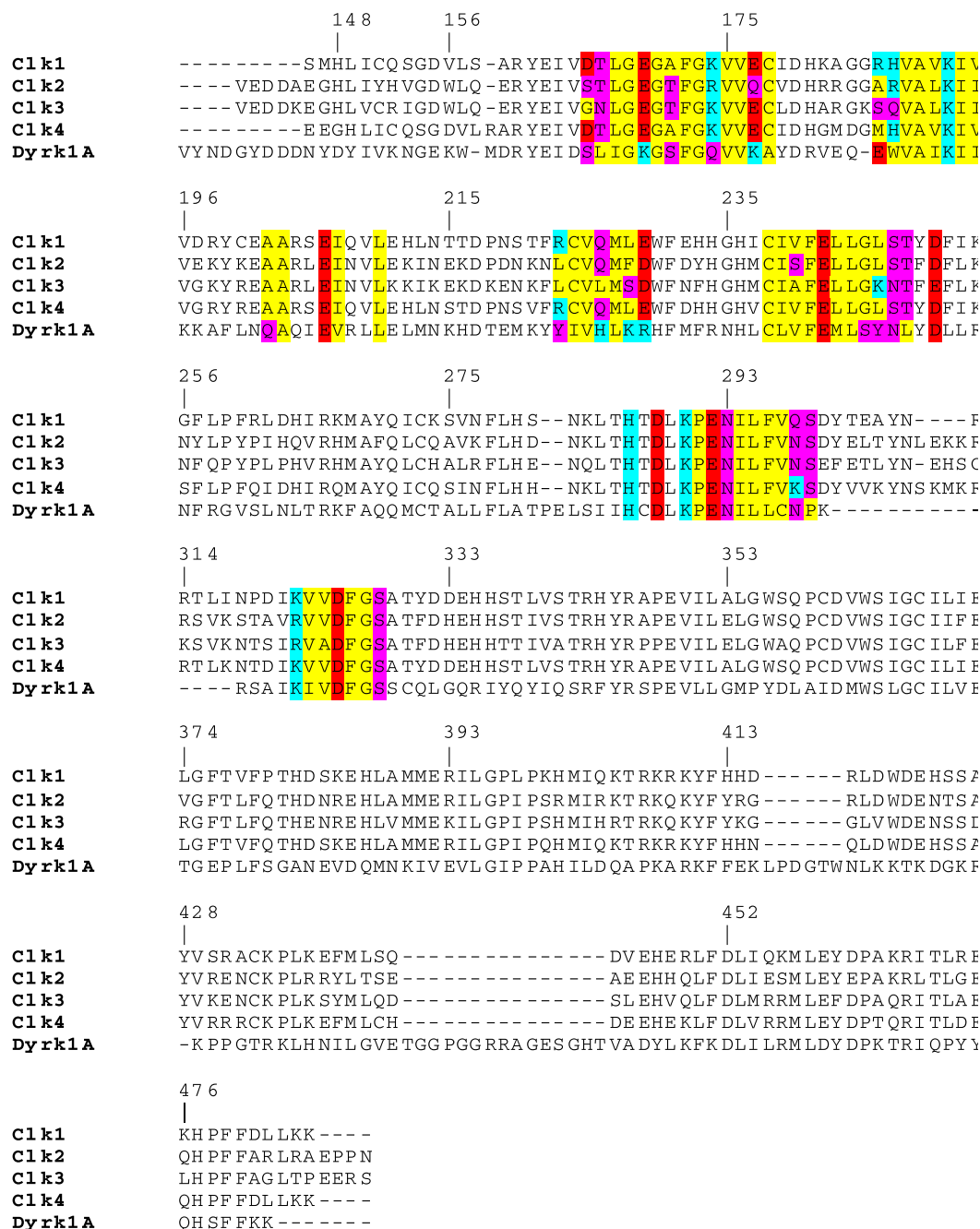


Figure 6. Multiple sequence alignment of the catalytic domain of protein kinase for all four human Clk isozymes (Clk1, Clk2, Clk3, and Clk4) and human Dyrk1A. The amino acid residues that are within 10 Å of the ATP binding site are highlighted: red for negatively charged, cyan for positively charged, yellow for hydrophobic and purple for hydrophilic. The numbering of amino acid residues is taken from Clk1 crystal structure (PDB code: 1Z57). Multiple sequence alignment was prepared by MOE molecular modeling software.

In conclusion, we report a novel class of Clk inhibitors based upon a core 6-arylquinazolin-4-amine scaffold. Selected agents were screened versus Clk4 to gain an appreciation of this chemotypes SAR and selected agents were found to inhibit this enzyme with potencies below 100 nM. One agent (analogue **4**) was profiled against a panel of over 400 kinases and found to be remarkably selective for Clk1, Clk4, and Dyrk1A. The only other reported inhibitor of the Clk family [TG003 (**1**)] was also profiled and found to bind selectively to Clk1, Clk2, Clk4, and Dyrk1A. Analysis of the mechanism of action highly suggests that this chemotype inhibits Clk4 via competition with ATP binding. Molecular modeling also suggests that **4** and related agents inhibit the Clk isozymes through

binding at the ATP binding domain. These agents provide useful tools for the study of Clk1, Clk4, and Dyrk1A and their respective roles in pre-mRNA splicing. Efforts to expand on the SAR of this chemotype in hopes of finding small molecules with divergent SAR for each isozyme of the Clk family and Dyrk1A are currently underway.

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- Procedure for the preparation of 6-bromo-N-(thiophen-2-ylmethyl)quinazolin-4-amine*: To a solution of 6-bromo-4-chloroquinazoline (0.3 g, 1.23 mmol) in DMF (8 mL) were added thiophen-2-ylmethanamine (0.139 g, 1.23 mmol) and Hunig's base (0.21 mL, 1.23 mmol). The reaction mixture was stirred at rt for 2 h. Upon completion, the reaction mixture was diluted with EtOAc (100 mL) and washed with 10% KHSO₄ (25 mL) and three times with 3 N LiCl (3 × 30 mL). The organic layer was extracted, dried on MgSO₄, filtered, and concentrated in vacuo. The residue was purified directly on silica column. Gradient elution with ethyl acetate (15–75%) in hexanes provided the title compound as a colorless solid: yield (0.39 g, 1.22 mmol, 99%). *Procedure for the preparation of 6-(benzo[d][1,3]dioxol-5-yl)-N-(thiophen-2-ylmethyl)quinazolin-4-amine (4)*: To a solution of 6-bromo-N-(thiophen-2-ylmethyl)quinazolin-4-amine (0.1 g, 0.31 mmol) in DMF (3 mL) were added benzo[d][1,3]dioxol-5-ylboronic acid (0.078 g, 0.47 mmol), sodium carbonate (0.066 g, 0.63 mmol), and tetrakis(triphenylphosphine)palladium(0) (0.036 g, 0.03 mmol). The reaction mixture was heated in a Biotage Initiator[®] microwave at 150 °C for 1 h. Upon completion, the reaction mixture was diluted with EtOAc (75 mL) and washed with NaHCO₃ (50 mL), three times with 3 N LiCl (3 × 30 mL), and brine (30 mL). The organic layer was collected, filtered through a pad of Celite, and concentrated in vacuo. The residue was purified directly on silica column. Gradient elution with ethyl acetate (5–65%) in hexanes provided **4** as a colorless solid: yield (0.065 g, 0.18 mmol, 58 %). ¹H NMR (DMSO-*d*₆) δ 4.97 (d, *J* = 5.53 Hz, 2H), 6.09 (s, 2H), 6.92–7.01 (m, 1H), 7.02–7.18 (m, 2H), 7.29–7.41 (m, 2H), 7.44 (s, 1H), 7.73 (d, *J* = 8.56 Hz, 1H), 8.08 (d, *J* = 8.46 Hz, 1H), 8.51 (d, *J* = 4.70 Hz, 2H), 8.99 (d, *J* = 4.89 Hz, 1H); ¹³C NMR (DMSO-*d*₆) δ 54.88, 101.28, 107.14, 108.70, 115.04, 119.52, 120.54, 125.11, 125.79, 126.54, 127.99, 131.04, 133.21, 137.02, 142.18, 147.14, 148.11, 148.26, 154.75, 159.05. HRMS (ESI) *m/z* 362.0957 (M+H)⁺ (C₂₀H₁₆N₃O₂S requires 362.0958). Purity determination via LC–MS was performed using an Agilent Diode Array Detector using a 3 min gradient of 4–100% acetonitrile (containing 0.025% trifluoroacetic acid) in water (containing 0.05% trifluoroacetic acid) with a 4.5 min run time at a flow rate of 1 mL/min and a 7 min gradient of 4–100% acetonitrile (containing 0.025% trifluoroacetic acid) in water (containing 0.05% trifluoroacetic acid) with an 8 min run time at a flow rate of 1 mL/min. Three minutes gradient retention time = 3.050; 7 min gradient retention time = 4.531 min. Purity was determined to be >95% in both methods.
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