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Research towards selective inhibition of the CLK3 kinase

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Abstract: The cdc2-like kinases (CLKs), are a family of kinases that attracted recently the interest of scientists due to their significant biological roles, in particular in the regulation of mRNA splicing process. Among the four isoforms of CLKs, the CLK3 is the one for which the biological roles are less well understood, in part because no selective inhibitor of this challenging kinase has been found to date. Based on structural analysis of the CLKs we have identified the lysine 241, present only in CLK3, as an attractive target to design inhibitors with increased affinity towards this kinase as compared to the three others. Based on this, we have been able to transform a molecule (***DB18***) previously established with a low activity on CLK3 into a derivative (**VS-77**) which has now a significant affinity toward this CLK3 kinase (IC_{50} = 0.3μM). Further, since this compound has kept good activities against the other CLKs, **VS-77** can be qualified as a new pan-inhibitor of the CLKs.

Keywords: Kinases • CLK3 • Quinazolines • Cancer • Triazoles • Molecular modelling •

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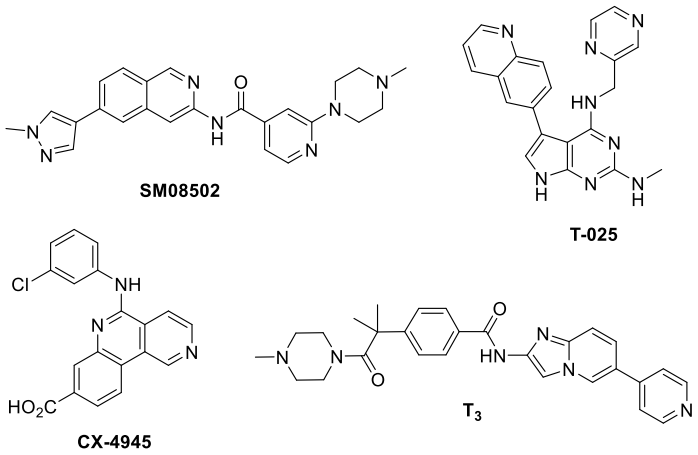
1 Introduction

Human protein kinases are a family comprising nearly 535 phosphotransferases (called the human kimome) involved in specific signaling pathways which regulate cell functions (e.g. metabolism, cell cycle progression, cell adhesion, vascular function and angiogenesis). Therefore, the dysregulation of protein kinase enzymatic activity, induced by genetic alterations as well as overexpression, is implicated in the pathogenesis of numerous deleterious diseases including nervous and inflammatory disorders as well as a number of malignancies [1]. Kinases are also known to be highly druggable by both allosteric and competitive inhibitors. As a consequence, protein kinases have become one of the most important drug targets: between a quarter to a third of the drug discovery efforts worldwide are focused on the discovery of new protein kinase inhibitors. More than 80 FDA-approved drugs that target about two dozen different protein kinases were discovered during the last 25 years and more than 400 orally effective protein kinase inhibitors are in clinical trials worldwide [2]. However, and despite this high interest, the function in human biology of approximately one-third of the kinases members is poorly understood [3]. These enzymes are classified as “Dark Kinases” because of the lack of functional annotations and high-quality molecular probes for functional investigations [4]. Thus, new studies are still required for the discovery of selective inhibitors for each of these kinases in order to better clarify their mechanisms of action and their roles in living systems. It is important to classify the kinases regarding the level of knowledge that scientists have on their physiological and pathological roles. Oprea et al. defined a knowledge-based protein classification that led to the definition of four groups: from the more studied T_{clin} (*clinic*, with approved drug on the market), T_{chem} (*chemistry*, known to bind to small molecules with high potency), T_{bio} (*biology*, kinases that have notably gene ontology leaf term annotations associated with disease and cellular role but lack associations with bioactive molecules) to the less explored T_{dark} (*dark* genome, do not meet any of the criteria for the other classes, see ref [5] for details).

Among the T_{chem} are the members of the cdc2-like kinases (CLKs) family. They have attracted the interest of scientists due to their biological roles in many areas and significant involvement in human diseases [6]. In particular, these CLKs are involved in the regulation of mRNAs splicing with important consequences especially in cancer [7]. There are four isoforms of CLKs (CLK1, CLK2, CLK3 and CLK4) whose structures have been clearly established and are available in PDB.

Among the CLKs family, CLK3 appears presently as the less studied, although its potential role has been proposed not only in cancer but also in other diseases like malaria [8]. Further,

it has been proposed to play also a role in the formation of the central nervous system [9]. The underdevelopment of CLK3 is likely due, at least in part, to the fact that, to the best of our knowledge, no potent and selective inhibitor of this specific kinase has been reported. CLK3 is reported to be a T_{chem} kinase [5], but there are only four molecules which demonstrate significant enzymatic inhibition of CLK3 (from 6.5nM to 110nM, Table 1, [6a]): SM08502, T-025, T3 and CX-4945.



Compound Id	CLK1	CLK2	CLK3	CLK4	DYRK1A	DYRK1B	DYRK2	Refs
SM08502	8	1	22	1	1	1	3	[10]
T-025 *	4.8	0.096	6.5	0.61	0.074	1.5	32	[11]
T3	0.67	15	110	ND	260	230	ND	[12]
CX-4945	3.3	2.9	67	23	14	ND	ND	[13-14]

* Kd values

Table 1. Structures and *in vitro* activities of known CLK3 inhibitors. The IC_{50} values (in nM) are given as numbers. For **T-025,** Kd values (in nM) are specified.

However, these derivatives remain still more potent on the other CLKs than CLK3. Thus, they appear more as potent pan inhibitors which are targeting all CLKs (including CLK3) plus many other kinases. Therefore, they cannot be qualified as bona fide chemical probes for this CLK3 kinase. For the CLKs family members a detailed study has indicated the important role of the residues located N-terminal to the DFG motif (called DFG-1) since, in this particular position, CLK3 has a smaller alanine compared to the others which have bulkier valines. This offers a rationale to explain in particular why most, if not all, known inhibitors have lower affinity for CLK3 [15]. Our approach was different since we wanted to design new molecules which could have now an increase in affinity towards CLK3. Thus, the goal of this paper is to report our first efforts in transforming a compound (***DB18***) which is highly potent and selective inhibitor of

CLK1, -2 and -4, with no activity on CLK3 [16], into a new derivative (**VS-77**) which is now a good pan-inhibitor of the four CLKs. Extensive molecular modelling studies have been used here to design **VS-77** and to propose a model to explain the selectivity observed.

2 Strategy and design of the targets

Our study started by a detailed analysis of the sequence of aminoacids in CLK3 compared to the three other CLKs. There are several differences but one appeared very significant (Figure 1, left part): CLK3 has a polar lysine in position 241 while the other CLKs have nonpolar leucines. Further, examination of the structure of CLK3 shows that this key lysine 241 is very close to the entry of the ATP binding site (Figure 1, right part).

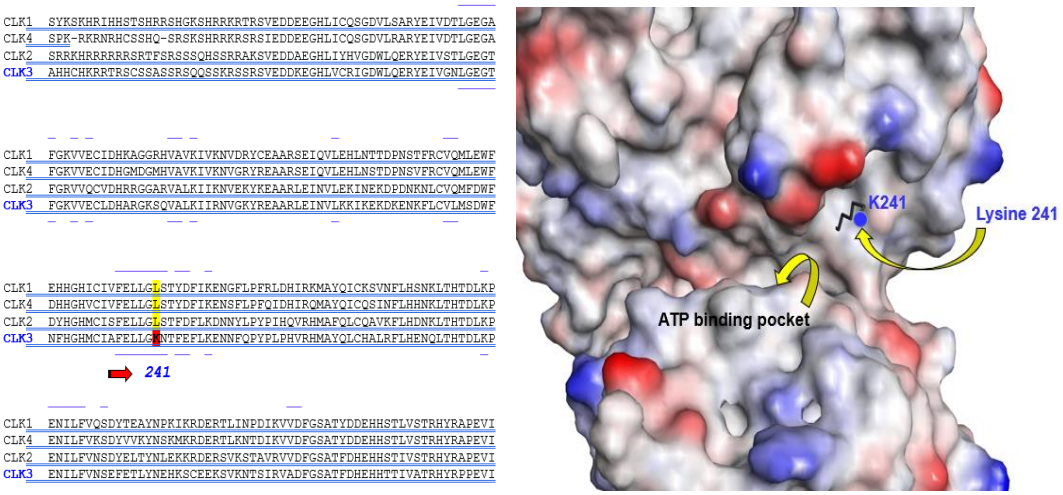


Figure 1. (Left part) Sequence of aminoacids in CLK3 as compared to the three other CLKs; (Right part) Structure of CLK3 highlighting the lysine in position 241 (PDB: 2WU6 [17]).

Therefore, this lysine 241 could be considered as an opportunity to design new molecules with an improved affinity to CLK3 by adding specific interactions with this amino-acid bearing a primary amine in terminal position. Based on this, our strategy was to design new inhibitors with introduction, in their terminal part, of an acid group which could perform an extra hydrogen bond interaction to lysine 241 and therefore could be specific to CLK3. Towards this goal we started from our, previously described ***DB18*** [16], a moderate inhibitor (IC_{50} = 1.28 μM) docked into CLK3 (Figure 2, left part), and we proposed to introduce, through an appropriate linker, an acid group close to this lysine 241 (Figure 2, right part).

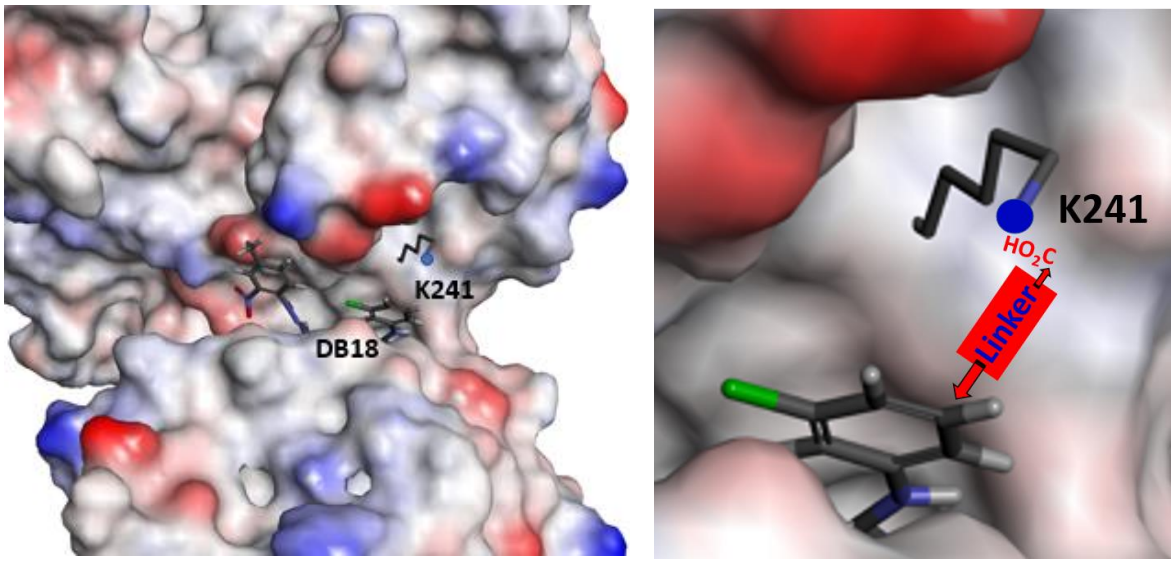


Figure 2. (Left part) Docking of our previous inhibitor (***DB18***) in CLK3 and (Right part) our working hypothesis.

Preliminary studies indicated that a simple aromatic group could be very appropriate as a linker and the acid could be placed in *meta* or *para* positions taking into account the flexible backbone of lysine 241 (Figure 3). Further, in case the docking of these new targets would require a little more flexibility around the basic skeleton, we decided to prepare also the same molecules with hydrogen instead of the chlorine in *meta* position of the anilino group.

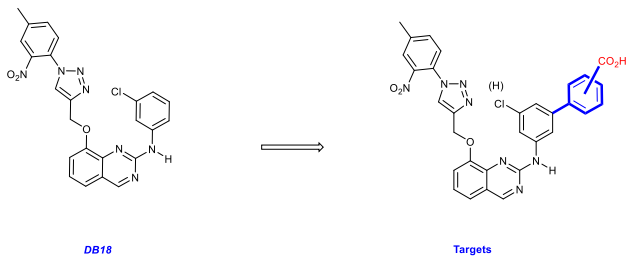


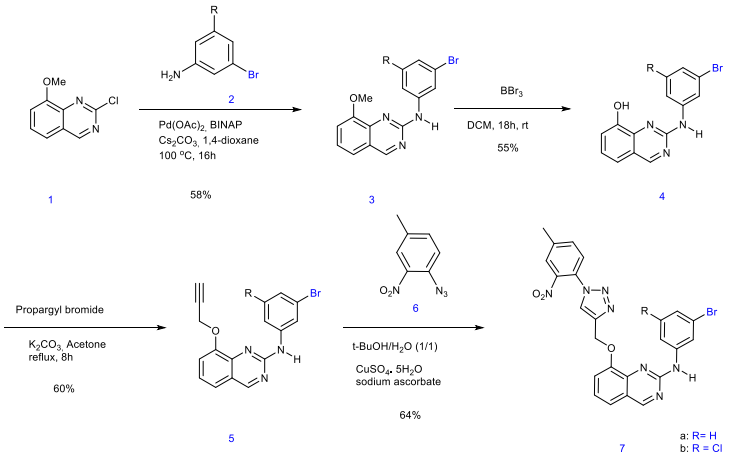
Figure 3. Design of our target molecules

3 Results and Discussion

3.1 Chemical syntheses

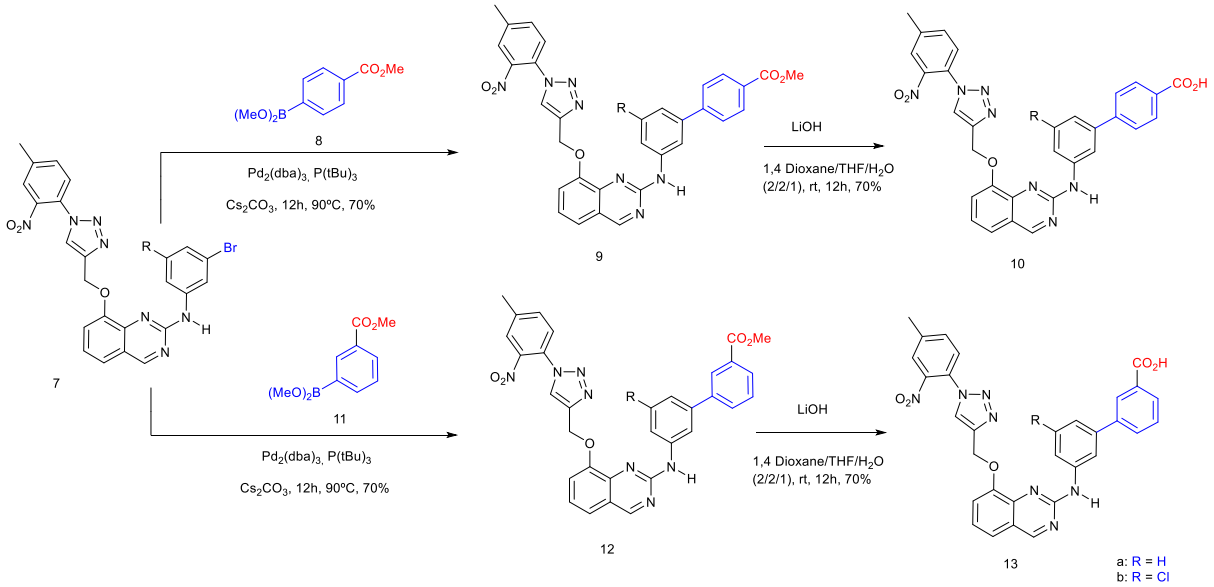
The synthetic strategy is very similar to the one previously developed for the preparation of ***DB18*** and designed analogues (Scheme 1) [16]. It starts from known quinazoline **1** which, on Buchwald-Hartwig-type reaction with 3-bromo-aniline **2a**, gave anilino-quinazoline **3a**. Deprotection of the methoxy group by BBr_3 gave phenol **4a** which was propargylated to

intermediate **5a**. A final click-type reaction [19] with azide **6** gave the first target intermediate **7a**. The second key intermediate **7b** was prepared in a very similar manner, but starting from 2-bromo-3-chloroaniline **2b**.



Scheme 1. Synthesis of the intermediates anilino-2-quinazolines **7a** and **7b.**

Next, we prepared the final targets by Suzuki-type reactions using the aromatic bromides **7**. As indicated before, the acid function designed to interact with the lysine 241 has been introduced both in *para* and *meta* positions of the aromatic linker. Further we have prepared two series of molecules: the ones without chlorine in *meta* position on this linker (series **a**) and the others which kept this chlorine, like in ***DB18*** (series **b**). These syntheses are reported in Scheme 2. A Suzuki-type Pd-catalyzed coupling of **7a** with *p*-carbomethoxybenzoylboronic ester **8** gave **9a** which, after saponification, afforded the first target **10a**. The second molecule **10b** was obtained in the same manner starting from **7b** with the chlorine in *meta* position of the linker. Then, by a similar approach, the targets **13a** and **13b** were prepared by now using as the coupling reagent the *m*-carbomethoxybenzoylboronic ester **11**. All derivatives have spectral and analytical data in agreement with the proposed structures (see Experimental Section and Supplementary Information).



Scheme 2. Synthesis of the targeted anilino-2-quinazolines **10** and **13.**

3.2 Kinase inhibition studies

Our molecules have been submitted first to a primary screening against a panel of eight disease-related kinases: five members of the CMGC (for CDK, MAPK, GSK3 and CLK) group (CDK5/p25, CDK9/CyclinT, GSK3β, CLK1 and DYRK1A), one CAMK (Calmodulin/Calcium regulated kinase PIM1), one CK1 (the Casein Kinase CK1ε) and Haspin as atypical kinase. The results obtained are reported in Table 2. The four acids **10a**, **10b**, **13a**, **13b** demonstrated a significant activity against CLK1 with very low to no action on the other selected kinases. On the other hand, the corresponding esters **9** and **12** were found to be not significantly active against the tested kinases. Indeed, we failed to observed a dose-dependent effect from 1 to 10µM for these compounds (*e.g.* the same level of inhibition was observed when **12a** was tested at 1 or 10µM against CLK1).

Table 2. Primary evaluation of the inhibition of our new quinazolines against a short panel of mammalian kinases.[a]

Compound Id	Concentrations	Hs_CDK5/p25	Hs_CDK9/ CyclinT	Hs_HASPIN	Hs_PIM1	Hs_GSK3β	Hs_CK1ε	Mm_CLK1	Rn_DYRK1A
9a	10 µM	84	95	≥100	90	53	95	58	≥100
	1 µM	70	98	95	≥100	70	78	66	≥100
9b	10 µM	≥100	91	43	96	86	≥100	66	≥100
	1 µM	74	96	65	≥100	90	≥100	60	≥100
10a	10 µM	70	38	92	≥100	32	84	0	84
	1 µM	61	84	93	80	82	93	15	≥100
10b	10 µM	43	66	92	≥100	56	92	8	94
	1 µM	53	99	85	84	92	≥100	38	82
12a	10 µM	48	57	49	72	58	≥100	45	≥100
	1 µM	63	74	51	75	88	≥100	50	≥100
12b	10 µM	92	83	39	98	62	≥100	48	≥100
	1 µM	65	94	47	90	97	≥100	54	≥100
13a	10 µM	9	24	63	≥100	26	≥100	5	60
	1 µM	62	83	79	68	95	≥100	44	92
13b (VS-77)	10 µM	37	47	74	≥100	43	≥100	11	62
	1 µM	92	83	74	88	≥100	≥100	20	92

[a] Residual kinase activities are expressed in % of maximal activity, i.e. measured in the absence of inhibitor but with an equivalent dose of DMSO (solvent of the tested compounds). ATP concentration used in the kinase assays was 10 µM (values are means, n =2). Kinases are from human origin (*Homo sapiens*) except CLK1 (from *Mus musculus)* and DYRK1A (*from Rattus norvegicus)*.

For the hit compounds that showed an inhibitory activity, we next determined their respective IC_{50} against the four mouse CLKs. The results are reported in Table 3 and in SI. For comparison, we have also reported the values obtained earlier with our starting model compound ***DB18*** [16].

Table 3. IC_{50} data for the inhibition of *Mm*_CLK1-4 by the four targeted quinazolines and ***DB18***.[a]

Compound Id	*Mm*_CLK1	*Mm*_CLK2	*Mm*_CLK3	*Mm*_CLK4	*Mm*_CLK3/*Mm*_CLK1,2,4
10a	0,891	0,844	3,732	0,762	4 to 5
10b	0,213	0,118	0,861	0,145	4 to 8
13a	0,68	0,278	1,655	0,428	3 to 4
13b (VS-77)	0,127	0,061	0,303	0,062	3 to 5
DB18[b]	0,011	0,027	1,28	0,02	48 to 116

[a] Kinase activities were measured by radiometric γ^{33}P-ATP assay (Eurofins, UK) using 15 µM ATP. They were calculated from dose-response curves for which each point was measured in duplicate.[b] Data taken from ref [16].

Several points have to be noticed from these data:

- These four derivatives demonstrate still a significant inhibition of the CLK1, CLK2 and CLK4 in the low micromolar range and even better (up to 60nM) for **13b** (=**VS-77)**;
- They show also inhibition of CLK3 in the same order of magnitude as for the three other CLKs from 0.3μM for **VS-77** up to 3.7μM for **10a**.
- This translates into a significant improvement in the comparison between action on CLK3 versus the three others (Table 2): for the best compound, **VS-77**, the ratios *Mm*_CLK3 versus the other CLKs are only 3 to 5 while they were 48 to 116 in the initial product ***DB18***.
- These results strongly support our hypothesis that the addition of the terminal acid significantly reinforces the interaction of our new molecules with CLK3, and in particular through the hydrogen bonding with lysine 241.

Finally, in order to complete our studies, we then measured the activity of **VS-77** against the *DYRK 1A*, *DYRK1B* and *DYRK2* kinases and the results are reported in Table 4.

Table 4. IC_{50} data for the inhibition of *Rn*_DYRK1A, *Hs*_DYRK1A, *Hs*_DYRK1B *and* *Hs*_DYRK2 by **VS-77** and ***DB18***.[a]

Compound Id	*Rn*_DYRK1A	*Hs*_DYRK1A	*Hs*_DYRK1B	*Hs*_DYRK2
VS-77	**14.76**	**5.31**	**4.6**	**11.69**
DB18[b]	**>100**	**>100**	**>100**	**>100**

[a] Kinase activities were measured by radiometric γ^{33}P-ATP assay (Eurofins, UK) using 15 μM ATP. They were calculated from dose-ponse curves for which each point was measured in duplicate.[b] Data taken from ref [16].

To our surprise, this new compound **VS-77** exhibited low, but significant, inhibition of these DYRK kinases, contrary to the results obtained earlier with ***DB18***. This point will be discussed in the molecular modelling part below.

3.3 **Molecular modelling studies**

Molecular modeling experiments confirmed that the most potent compound **VS-77** is actually able to form the salt bridge with lysine 241, as we had planned. The acid is located close (3 Ä) to the amino group of this lysine (Fig. 4).

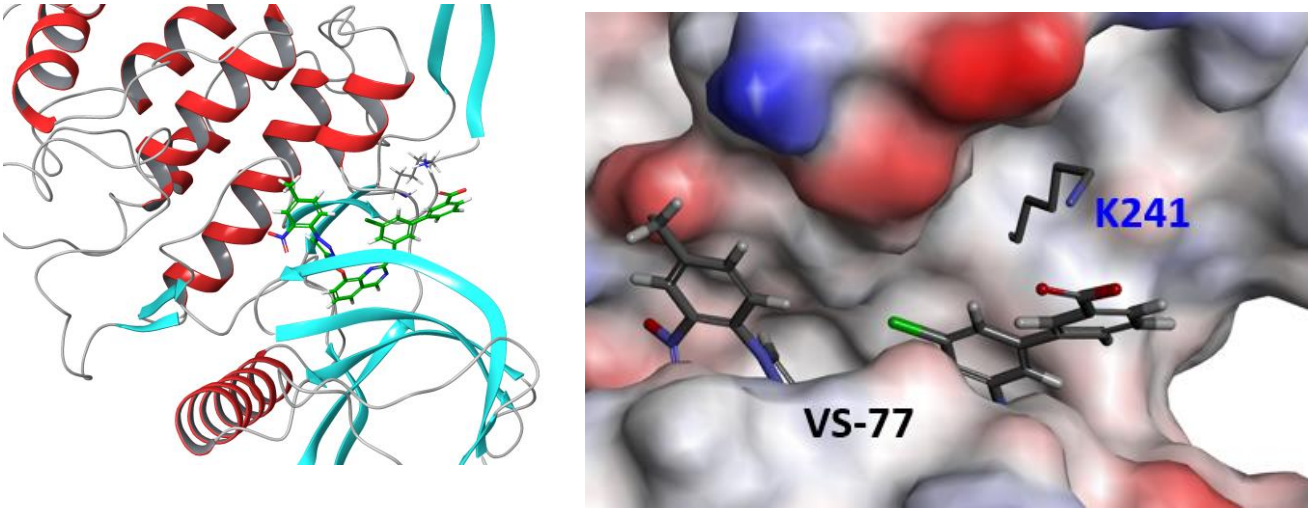


Figure 4. Docking of **VS-77** in CLK3

We continued our analysis to understand the unexpected activity towards the DYRK kinases, using DYRK1A crystal structure (Figure 5). Surprisingly, another leucine (K175) is also located near the acidic group of **VS-77.** This residue is positioned on the opposite lobe of the protein, as compared to K241 of CLK3, but a simple 180° rotation of the benzoic acid allowed the inhibitor to interact with Lys 175. Therefore, this new interaction could explain the higher affinity of **VS-77** toward *Hs*_DYRK1A, as compared to ***DB-18***.

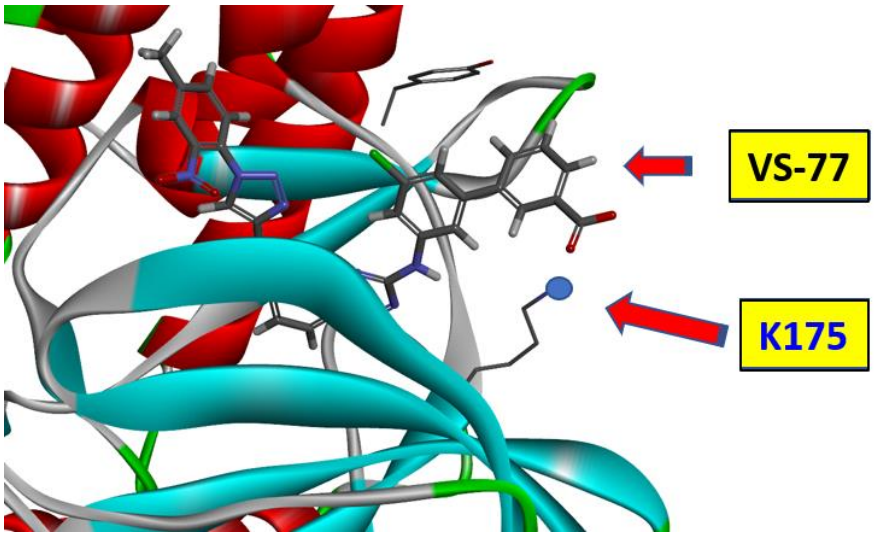


Figure 5. Docking of VS-77 in *Hs*_DYRK1A (PDB : 8T2H [18])

4 Experimental

4.1 Chemical synthesis

General information

All reactions were performed in heat gun-dried round-bottomed flasks under a dry argon or nitrogen atmosphere. Air and moisture-sensitive compounds were introduced via syringes or cannula, using standard inert atmosphere techniques. In addition, the gas stream was passed through glass cylinder filled with P_2O_5 to remove any traces of residual moisture. Reactions were monitored by thin layer chromatography (TLC) using E. Merck silica gel plates and components were visualized by illumination with short wavelength UV light and/or staining (Ninhydrin or basic $KMnO_4$). All aldehydes were distilled right before use. All benzyl bromides and other reagents were used as they were received from commercial suppliers, unless otherwise noted. THF and Et_2O were dried over sodium-benzophenone and distilled prior to use. Anhydrous CH_2Cl_2 was prepared by refluxing in the presence of CaH_2 and distilled right before use unless otherwise noted.

^{1}H NMR spectra were recorded on Bruker spectrometers at 300 and 400 MHz, and ^{13}C NMR spectra at 75 and 100 MHz, in $CDCl_3$ or DMSO-d_6 using TMS (tetramethylsilane) as an internal standard. Multiplicity was tabulated using standard abbreviations: s for singlet, d for doublet, dd for doublet of doublets, t for triplet, q for quadruplet, ddd for doublet of doublets of doublets and m for multiplet (br means broad). When necessary, in particular in order to have better accuracy on small coupling constants, resolution in ^{1}H NMR was enhanced using Traficante. The synthetic strategy is very similar to the one previously developed for the preparation of ***DB18*** and designed analogues [16]. All triazole compounds were purified by flash column chromatography on neutral alumina unless otherwise noted.

Synthesis of N-(3-bromo-phenyl)-8-methoxyquinazolin-2-amine (3a)

To a stirred solution 2-chloro-8-methoxyquinazoline **1** (500 mg, 2.57 mmol) and 3-bromoaniline **2a** (570 g, 3.35 mmol) in 1,4-dioxane (2 mL) was added BINAP (150 mg, 0.257 mmol), $Pd(OAc)_2$ (28.8 mg, 0.05 mmol) and Cs_2CO_3 (219 mg, 6.75 mmol). Then, the reaction mixture was evacuated under vacuum, back filled with Ar atmosphere and heated to 100 °C for 16 h. The reaction was monitored by TLC and after its completion, the reaction mixture was cooled to rt (room temperature) and then filtered through celite pad. The filtrate was concentrated and to this crude material, ice and water were added and after extraction with ethyl acetate (2 × 100 mL), combined organic extracts were washed with brine (50 mL). Organic layer was dried over anhydrous sodium sulfate and concentrated to dryness. The crude resulting product was purified by chromatography on silica gel eluting with 5–20% ethyl acetate in cyclohexane to give compound **3** (600 mg, 60% yield) as a yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.14 (s, 1H), 8.25 (t, J = 1.9 Hz, 1H), 7.61 (dt, J = 7.5, 1.9 Hz, 1H), 7.46 (s, 1H), 7.39 (dd, J = 8.1, 1.4 Hz, 1H), 7.33 (d, J = 7.6 Hz, 1H), 7.30 – 7.13 (m, 4H), 4.10 (s, 3H).). 13C NMR (75 MHz,

CDCl3) δ 161.7, 156.3, 153.4, 143.6, 141.2, 130.1, 125.1, 124.0, 122.8, 121.7, 121.7, 119.02, 117.2, 112.6, 56.2. HRMS (ESI): m/z calcd for $C_{15}H_{13}N_3O_{79}Br$ $[M + H]^+$: 330.02365, found 330.0230 (1 ppm).

Synthesis of N-(3-bromo-5-chlorophenyl)-8-methoxyquinazolin-2-amine (3b)

% Yield: 1.3 g (58%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.35 (s, 1H), 9.36 (s, 1H), 8.38 (s, 1H), 8.27 (s, 1H), 7.52 (dd, *J*= 1.6 Hz, 7.6 Hz, 1H), 7.40-7.34 (m, 2H), 7.24 (t, *J*= 1.6 Hz, 1H), 3.99 (s, 3H). **^{13}C NMR** (75 MHz, DMSO) δ 162.6, 156.1, 153.5, 143.9, 142.7, 134.5, 124.8, 123.1, 122.5, 121.8, 119.6, 119.6, 117.1, 114.0, 56.5. HRMS (ESI): m/z calcd for $C_{15}H_{12}N_3O^{35}Cl^{79}Br$ $[M + H]^+$: 363.98468, found 363.9846 (0 ppm).

2-((3-bromo-5-chlorophenyl)amino)quinazolin-8-ol (4a)

To a stirred solution of *N*-(3-Bromophenyl)-8-methoxyquinazolin-2-amine **3a** (500 mg, 1.51 mmol) in CH_2Cl_2 (1.5 mL) was added 2.9 mL of BBr_3 in CH_2Cl_2 (1.0 M) at 0 °C, and the reaction mixture was stirred at rt for 18 h. The reaction mixture was poured to a mixture of ice and water and stirred for 2 h at rt. The resulting solid was collected by filtration and washed with water (2 × 40 mL). The obtained pale-yellow solid was dried in a dessicator to give compound **4** (240 mg, 50% yield) as a yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.13 (s, 1H), 8.03 – 7.96 (m, 1H), 7.59 (dt, *J* = 6.7, 2.4 Hz, 1H), 7.48 – 7.21 (m, 8H). HRMS (ESI): m/z calcd for $C_{14}H_{11}N\ O^{79}Br$ $[M + H]^+$: 316.008, found 316.0078 (1 ppm).

2-((3-bromo-5-chlorophenyl)amino)quinazolin-8-ol (4b)

% Yield: 0.7 g (55%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.23 (s, 1H), 9.99 (brs, 1H), 9.31 (s, 1H), 8.33 (t, *J*= 1.6 Hz, 1H), 8.28 (t, *J*= 2.0 Hz, 1H), 7.40 (dd, *J*= 1.2 Hz, 7.6 Hz, 1H), 7.29-7.21 (m, 3H). ^{13}C NMR (75 MHz, DMSO) δ 162.6, 155.7, 151.9, 143.9, 141.8, 134.5, 125.1, 123.1, 122.5, 122.2, 119.5, 118.3, 117.4, 117.1. HRMS (ESI): m/z calcd for $C_{14}H_{10}N_3O^{35}Cl^{79}Br$ $[M + H]^+$: 349.96903, found 349.9691 (0 ppm)

N-(3-bromo-5-chlorophenyl)-8-(prop-2-yn-1-yloxy)quinazolin-2-amine (5a)

To a stirred solution of 2-((3-bromophenyl) amino) quinazolin-8-ol (100 mg, 0.31 mmol) in acetone (2 mL), was added K_2CO_3 (109 mg, 0.791 mmol) and propargyl bromide (56 mg, 0.47 mmol) at rt. The reaction mixture was heated to 40 °C for 8 h. It was then cooled to rt, filtered and concentrated to dryness. Purification by chromatography on silica gel (eluting with EtOAc in cyclohexane) gave compound (2.2 g, 60% yield) as a yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.14 (s, 1H), 8.37 – 8.21 (m, 1H), 7.68 – 7.05 (m, 7H), 5.05 (d, *J* = 2.4 Hz, 2H), 2.58 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (75 MHz, DMSO) δ 162.6, 156.5, 151.2, 143.2, 142.8, 130.8, 124.2, 124.1, 122.2, 121.8, 121.1, 120.8, 117.7, 116.5, 79.2, 57.1, 56.5. HRMS (ESI): m/z calcd for $C_{17}H_{13}N_3O^{79}Br$ $[M + H]^+$: 354.02365, found 354.0240 (1 ppm).

N-(3-bromo-phenyl)-8-(prop-2-yn-1-yloxy)quinazolin-2-amine (5b)

% Yield: 0.27 g (49%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.37 (s, 1H), 9.37 (s, 1H), 8.37 (s, 1H), 8.27 (s, 1H), 7.58 (dd, *J*= 2.0 Hz, 7.2 Hz, 1H), 7.43-7.36 (m, 2H), 7.25 (t, *J*= 1.6 Hz, 1H), 5.0 (d, *J*= 2.4 Hz, 2H), 3.66 (t, *J*= 2.4 Hz, 1H). ^{13}C NMR (75 MHz, DMSO) δ 162.7, 156.1, 151.2, 143.8, 142.7, 134.6, 124.5, 123.3, 122.6, 121.9, 120.6, 119.6, 117.2, 116.0, 79.3, 57.0. HRMS (ESI): m/z calcd for $C_{17}\ H_{12}\ N_3\ O\ ^{35}Cl\ ^{79}Br$ $[M + H]^+$: 387.98468, found 387.9847 (0 ppm).

N-(3-bromo-5-chlorophenyl)-8-((1-(4-methyl-2-nitrophenyl)-1H-1,2,3-triazol-4-yl)methoxy)quinazolin-2-amine (7a)

To a solution of $CuSO_4 \cdot 5H_2O$ (6.8 mg, 0.028 mmol), sodium ascorbate (16.6 mg, 0.084 mmol) and $PhCO_2H$ (3.4 mg, 0.028 mmol) in t-BuOH/H_2O (1:2 by v/v, 2.0 mL) was added a mixture of Alkyne **5a** (100 mg, 0.28 mmol) and Aromatic azide **6** (50 mg, 0.28 mmol) at room temperature. The resultant mixture was stirred continuously until the consumption of starting material (1 h). Then CH_2Cl_2 (20 mL) was added to dissolve the crude product. The organic layer was washed with H_2O and brine, and dried over anhydrous Na_2SO_4. Removal of the solvent yielded a residue, which was purified by a short chromatography (silica gel, EtOAc/PE = 1:3) to give 3a (108 mg, 72%) as a light yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.07 (s, 1H), 9.31 (s, 1H), 8.81 (s, 1H), 8.58 (t, *J* = 2.0, 2.0 Hz, 1H), 8.04 (d, *J* = 1.8 Hz, 1H), 7.85 – 7.64 (m, 4H), 7.56 (ddd, *J* = 8.0, 4.7, 1.2 Hz, 3H), 7.37 (t, *J* = 7.9, 7.9 Hz, 1H), 7.16 (t, *J* = 8.1, 8.1 Hz, 1H), 7.10 – 6.99 (m, 1H), 5.45 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 162.6, 156.3, 152.0, 144.2, 143.9, 142.9, 142.7, 142.5, 135.1, 130.7, 127.4, 127.0, 126.4, 126.0, 124.4, 124.1, 122.1, 121.7, 120.9, 120.6, 117.7, 116.0, 62.4, 20.9. HRMS (ESI): m/z calcd for $C_{24}H_{19}N_7O_3{}^{79}Br$ $[M + H]^+$: 532.07327, found 532.0723 (1 ppm).

Synthesis N-(3-bromo-5-chlorophenyl)-8-((1-(4-methyl-2-nitrophenyl)-1H-1,2,3-triazol-4-yl) methoxy) quinazolin-2-amine (7b)

% Yield: 0.25 g (57%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.34 (s, 1H), 9.38 (s, 1H), 8.88 (s, 1H), 8.26 (d, *J*= 7.2 Hz, 2H), 8.08 (s, 1H), 7.79 (d, *J*= 7.2 Hz, 1H), 7.74 (d, *J*= 8.4 Hz, 1H), 7.60 (t, *J*= 6.4 Hz, 2H), 7.42 (t, *J*= 8.0 Hz, 1H), 7.14 (t, *J*= 2.0 Hz, 1H),5.45 (s, 2H), 2.52 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 162.2, 155.6, 151.6, 143.7, 143.3, 142.1, 141.8, 134.6, 134.0, 126.8, 126.5, 125.9, 125.6, 124.3, 122.6, 121.9, 121.4, 119.9, 119.0, 116.5, 115.2, 61.9, 20.4. MS(ESI): m/z calcd for $C_{24}H_{17}{}^{79}Br^{35}ClN_7O_3$, 566.8, found: $[M+H]^+$: 568.1.

Procedure A

Methyl 3'-((8-((1-(4-methyl-2-nitrophenyl)-1H-1,2,3-triazol-4-yl) methoxy) quinazolin-2-yl) amino)-[1,1'-biphenyl]-4-carboxylate (9a)

A Schlenk tube equipped with a magnet stirrer under argon, a solution of N-(3-bromophenyl)-8-((1-(4-methyl-2-nitrophenyl)-1H-1,2,3-triazol-4-yl)methoxy)quinazolin-2-amine (200 mg, 0.375 mmol; in 0.5-0.7 mL of dioxane) and a solution of P(t-Bu)$_3$ (2.2 mg, 0.011 mmol; in 3.0 mL of dioxane) are added to a Schlenk tube charged with $Pd_2(dba)_3$ (5.0 mg, 0.005 mmol), boronic acid (80 mg, 0.45 mmol), Cs_2CO_3 (140 mg, 0.45 mmol) and stirred for 12 h at 80 °C. The reaction mixture was then concentrated and purified by flash chromatography using EtOAc and CH_2Cl_2 to afford **9a** as a yellow compound (130 mg 60 % yield).^{1}H NMR (300 MHz, DMSO-d_6) δ 10.04 (s, 1H), 9.32 (s, 1H), 8.72 (s, 1H), 8.58 (t, *J* = 2.0 Hz, 1H), 8.11 – 7.98 (m, 4H), 7.90 – 7.80 (m, 2H), 7.74 (ddd, *J* = 8.1, 1.9, 0.8 Hz, 1H), 7.63 (d, *J* = 8.1 Hz, 1H), 7.56 (dq, *J* = 8.4, 1.2 Hz, 2H), 7.44 – 7.33 (m, 1H), 7.38 – 7.27 (m, 2H), 5.53 (s, 2H), 3.86 (s, 3H). ^{13}C NMR (75 MHz, DMSO-6_d) δ 166.5, 162.5, 156.8, 151.9, 145.4, 144.2, 144.0, 143.4, 142.4, 141.8, 139.4, 135.1, 130.3, 129.7, 128.9, 127.6, 127.2, 127.1, 126.3, 126.0, 123.9, 121.8, 120.6, 120.4, 119.2, 117.5, 116.4, 62.6, 52.5, 20.9. HRMS (ESI): m/z calcd for $C_{32}H_{26}N_7O_5$ $[M + H]^+$: 588.1989, found 588.1986 (1 ppm).

Procedure B

3'-((8-((1-(4-methyl-2-nitrophenyl)-1H-1,2,3-triazol-4-yl)methoxy)quinazolin-2-yl)amino)-[1,1'-biphenyl]-4-carboxylic acid (10a)

The compound methyl 3'-((8-((1-(4-methyl-2-nitrophenyl)-1H-1,2,3-triazol-4-yl) methoxy) quinazolin-2-yl) amino)-[1,1'-biphenyl]-4-carboxylate **10a** (30 mg, .051 mol) was added by (3 ml, THF/1-4 Dioxane/ Water 4:4:2). The stirring suspension was cooled to 0 °C and treated with LiOH (10 mg, 0.25 mmol). The mixture was stirred at room temperature until starting material disappeared by TLC analysis (100% EtOAc). the reaction mixture was concentrated, diluted with water and acidified with 2N aqueous HCl (pH = 2). Then the solid formed was filtered washed with CH_2Cl_2 and dried to obtain carboxylic acid **10a** as a yellow solid (20 mg, 70%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.02 (s, 1H), 9.31 (s, 1H), 8.73 (s, 1H), 8.54 (s, 1H), 8.05 (td, *J* = 8.0, 5.9 Hz, 4H), 7.82 (d, *J* = 8.1 Hz, 2H), 7.78 – 7.60 (m, 2H), 7.55 (d, *J* = 7.9 Hz, 2H), 7.42 – 7.24 (m, 3H), 5.53 (s, 2H).). ^{13}C NMR (75 MHz, DMSO) δ 167.8, 162.5, 156.8, 151.8, 144.8, 144.2, 144.0, 143.4, 142.5, 141.8, 139.7, 135.1, 130.7, 130.4, 129.6, 127.7, 127.1, 127.0, 126.4, 126.0, 123.9, 121.7, 120.6, 120.4, 119.0, 117.5, 116.4, 62.5, 20.9. HRMS (ESI): m/z calcd for $C_{31}H_{24}N_7O_5$ [M + H]$^+$: 574.18334, found 574.1837 (1 ppm).

Methyl 3'-((8-((1-(4-methyl-2-nitrophenyl)-1H-1,2,3-triazol-4-yl) methoxy) quinazolin-2-yl) amino)-[1,1'-biphenyl]-4-carboxylate (12a)

Procedure A to afford methyl 3'-((8-((1-(4-methyl-2-nitrophenyl)-1H-1,2,3-triazol-4-yl) methoxy) quinazolin-2-yl) amino)-[1,1'-biphenyl]-4-carboxylate **12a** as a yellow compound (130 mg, 0.222 mmol, 60 % yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.13 (s, 1H), 8.33 (s, 1H), 8.22 (s, 1H), 8.21 (s, 1H), 8.00 (d, *J* = 7.8 Hz, 1H), 7.92 (s, 1H), 7.85 (d, *J* = 8.6 Hz, 3H), 7.69 (s, 1H), 7.50 (d, *J* = 8.2 Hz, 2H), 7.41 (t, *J* = 7.6 Hz, 3H), 7.30 (d, *J* = 11.2 Hz, 4H), 5.62 (s, 2H), 3.95 (s, 3H), 2.53 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.0 161.9, 156.5, 151.9, 144.8, 144.0, 142.0, 141.3, 140.7, 140.3, 134.2, 131.5, 130.7, 129.4, 128.8, 128.5, 128.2, 127.7, 125.8, 124.7, 123.8, 121.9, 121.2, 120.4, 118.5, 117.8, 116.5, 63.7, 52.2, 21.1. HRMS (ESI): m/z calcd for $C_{32}H_{26}N_7O_5$ [M + H]$^+$: 588.1989, found 588.1986 (1 ppm).

3'-((8-((1-(4-methyl-2-nitrophenyl)-1H-1,2,3-triazol-4-yl) methoxy) quinazolin-2-yl)amino)-[1,1'-biphenyl]-3-carboxylic acid (13a)

Procedure B to afford **13a** as a yellow solid (20 mg, 0.0348 mmol, 68%). ^{1}H NMR (300 MHz, DMSO) δ 10.08 (s, 1H), 9.33 (s, 1H), 8.75 (s, 1H), 8.35 (s, 1H), 8.22 (s, 1H), 8.17 (d, *J* = 8.1 Hz, 1H), 8.06 (s, 1H), 7.93 (t, *J* = 8.6 Hz, 2H), 7.75 (d, *J* = 8.2 Hz, 1H), 7.70 – 7.58 (m, 1H), 7.56 (d, *J* = 8.2 Hz, 2H), 7.35 (q, *J* = 7.4 Hz, 2H), 7.27 (d, *J* = 7.7 Hz, 1H), 5.52 (s, 2H); methyl group hidden by DSMO residual peaks. ^{13}C NMR (75 MHz, DMSO) δ 167.7, 156.7, 151.6, 144.2, 144.1, 142.9, 142.4, 141.6, 141.1, 139.9, 135.2, 131.9, 131.4, 129.8, 129.8, 128.7, 127.7, 127.7, 127.1, 126.4, 126.1, 124.1, 120.8, 120.4, 118.8, 117.5, 116.8, 62.8, 20.9, 18.9. HRMS (ESI): m/z calcd $C_{31}H_{24}N_7O_5$ [M + H]$^+$: 574.18334, found 574.1837 (1 ppm).

Methyl-3'-chloro-5'-((8-((1-(4-methyl-2-nitrophenyl)-1H-1,2,3-triazol-4-yl) methoxy)quinazolin-2-yl)amino)-[1,1'-biphenyl]-3-carboxylate (9b)

Procedure A to afford **9b** a cream colour (very light yellow) compound (40 mg, 0,064 mmol, 60 % yield). ^{1}H NMR (300 MHz, DMSO) δ 10.23 (s, 1H), 9.35 (s, 1H), 8.77 (s, 1H), 8.44 (t, *J* = 1.9 Hz, 1H), 8.29 (t, *J* = 1.7 Hz, 1H), 8.04 (dd, *J* = 8.7, 2.0 Hz, 3H), 7.84 (d, *J* = 8.6 Hz, 1H), 7.75 (ddd, *J* = 8.2, 1.9, 0.8 Hz, 1H), 7.65 (d, *J* = 8.1 Hz, 1H), 7.58 (ddd, *J* = 8.2, 4.5, 1.2 Hz, 2H), 7.32 (t, *J* = 1.7 Hz, 1H), 5.74 (s, 0H), 5.51 (s, 2H), 3.86 (s, 3H). ^{13}C NMR (75 MHz, DMSO) δ 166.4, 162.7, 156.4, 152.0, 144.2, 143.9, 143.9, 143.1, 142.9, 142.4, 141.1, 135.1, 134.4, 130.3, 129.4, 127.4, 127.4, 127.0, 126.3, 126.0, 124.5, 121.9, 120.5, 119.6,

117.9, 116.0, 115.8, 62.5, 52.6, 20.9. HRMS (ESI): m/z calcd for $C_{32}H_{25}N_7O_5$ ^{35}Cl $[M + H]^+$: 622.16002, found 622.1598 (0 ppm).

3'-chloro-5'-((8-((1-(4-methyl-2-nitrophenyl)-1H-1,2,3-triazol-4-yl)methoxy)quinazolin-2-yl)amino)-[1,1'-biphenyl]-4-carboxylic acid (10b)

Procedure B to obtain carboxylic acid **10b** as a yellow solid (10 mg, 60% yield). ^1H NMR (400 MHz, DMSO) δ 10.25 (s, 1H), 9.35 (s, 1H), 8.80 (s, 1H), 8.47 (s, 1H), 8.25 (s, 1H), 8.10 – 7.96 (m, 3H), 7.81 (d, *J* = 8.1 Hz, 2H), 7.75 (dd, *J* = 8.2, 1.7 Hz, 1H), 7.66 (d, *J* = 8.1 Hz, 1H), 7.59 (dd, *J* = 7.9, 3.9 Hz, 2H), 7.39 (t, *J* = 7.8 Hz, 1H), 7.30 (s, 1H), 5.50 (s, 2H). ^{13}C NMR (101 MHz, DMSO) δ 166.5, 162.7, 156.4, 152.0, 144.2, 143.9, 143.1, 143.0, 142.4, 141.3, 139.9, 135.1, 134.5, 131.9, 130.9, 130.1, 129.1, 127.5, 127.4, 127.1, 126.2, 126.0, 124.4, 121.9, 120.5, 119.4, 117.5, 116.1, 115.7, 62.6, 52.8, 20.9. HRMS (ESI): m/z calcd for $C_{31}H_{21}N_7O_5$ ^{35}Cl $[M + H]^-$: 606.12982, found 606.1299 (0 ppm) (1 ppm).

Methyl-3'-chloro-5'-((8-((1-(4-methyl-2-nitrophenyl)-1H-1,2,3-triazol-4-yl)methoxy)quinazolin-2-yl)amino)-[1,1'-biphenyl]-3-carboxylate (12b)

Procedure A to afford methyl 3'-chloro-5'-((8-((1-(4-methyl-2-nitrophenyl)-1H-1,2,3-triazol-4-yl)methoxy)quinazolin-2-yl)amino)-[1,1'-biphenyl]-3-carboxylate a cream colour (very light yellow) compound **12b** (40 mg, 60 % yield). ^1H NMR (400 MHz, DMSO) δ 10.26 (s, 1H), 9.37 (s, 1H), 8.80 (s, 1H), 8.58 (t, *J* = 1.9 Hz, 1H), 8.29 – 8.04 (m, 3H), 7.97 (ddt, *J* = 8.9, 3.7, 1.2 Hz, 2H), 7.77 (ddd, *J* = 8.1, 1.9, 0.8 Hz, 1H), 7.72 – 7.48 (m, 4H), 7.44 – 7.34 (m, 1H), 7.29 (t, *J* = 1.8 Hz, 1H), 5.51 (s, 2H), 3.90 (s, 3H), 2.55 – 2.35 (m, 5H). ^{13}C NMR (101 MHz, DMSO) δ 166.5, 162.7, 156.4, 152.0, 144.2, 143.9, 143.1, 143.0, 142.4, 141.3, 139.9, 135.1, 134.5, 131.9, 130.9, 130.1, 129.1, 127.5, 127.4, 127.1, 126.2, 126.0, 124.4, 121.9, 120.5, 119.4, 117.5, 116.1, 115.7, 62.6, 52.8, 20.9. HRMS (ESI): m/z calcd for $C_{32}H_{25}N_7O_5{}^{35}Cl$ $[M + H]^+$: 622.16002, found 622.1598 (0 ppm).

3'-chloro-5'-((8-((1-(4-methyl-2-nitrophenyl)-1H-1,2,3-triazol-4-yl)methoxy)quinazolin-2-yl)amino)-[1,1'-biphenyl]-3-carboxylic acid (13b)

Procedure B to obtain carboxylic acid **13b** as a yellow solid (10 mg, 70%). ^1H NMR (400 MHz, DMSO) δ 10.24 (s, 1H), 9.36 (s, 1H), 8.79 (s, 1H), 8.59 (d, *J* = 2.1 Hz, 1H), 8.21 (t, *J* = 1.9 Hz, 1H), 8.14 – 8.03 (m, 2H), 8.02 – 7.90 (m, 2H), 7.77 (dd, *J* = 8.4, 1.9 Hz, 1H), 7.69 (d, *J* = 8.1 Hz, 1H), 7.66 – 7.55 (m, 3H), 7.40 (t, *J* = 7.9 Hz, 1H), 7.27 (t, *J* = 1.8 Hz, 1H), 5.50 (s, 2H), 3.59 – 3.55 (m, 4H), 2.57 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ 167.8, 162.7, 156.4, 152.0, 144.2, 143.9, 143.1, 143.0, 142.4, 141.5, 139.7, 135.1, 134.5, 132.3, 131.3, 129.9, 129.3, 127.7, 127.4, 127.0, 126.2, 126.1, 124.5, 121.8, 120.5, 119.3, 117.4, 116.0, 115.7, 62.5, 20.9. m/z calcd for $C_{31}H_{21}N_7O_5{}^{35}Cl$ $[M-H]^-$: 606.12982, 606.1290 (1 ppm).

4.2 Kinase inhibition studies

Kinase enzymatic activities were assayed using both luminescent ADP detection assay (ADP-Glo™ assay kit, Promega, Madison, WI) or radiometric kinase assay. These assays were performed using the protocols described in our previous publication [16].

4.3 Molecular modelling studies

These studies were performed using the methods described in our previous publication [16].

Crystal structures of were used for docking: CLK3: (PDB:2WU6 https://doi.org/10.2210/pdb2WU6/pdb, [17]) and DYRKs: (PDB:8T2H https://doi.org/10.2210/pdb8T2H/pdb [18]).

Conclusions

In the present work, we disclosed a new strategy to improve the affinity of ligands towards the CLK3 kinase. It is based on the Lysine 241 which is present only in CLK3, while the three others have apolar leucines in this position. Thus, by grafting an extra benzoic acid on a molecule (***DB18***), previously established as having low activity on CLK3, we have been able to transform this compound into a derivative (**VS-77**) which has now a significant affinity toward this kinase (IC_{50} = 0.3μM). Further, this compound has kept good activities against the other CLKs and therefore it can be qualified as a new pan-inhibitor of the CLKs. On the other hand, surprisingly this modification of structure has also transformed this **VS-77** into a weak inhibitor of the DYRK kinases, while ***DB-18*** was absolutely inactive on later kinases. Analysis by molecular modelling allowed us to suggest that this change could be due, in the same way, to the interaction of the newly introduced acid with the primary amino group of the leucine 175 present in DYRK kinases. These results, highlighting the potential role of the lysine 241, pave the way towards the long-term goal of getting more potent and selective inhibitors of this understudied CLK3 kinase.

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Sample availability: Sample of the compounds are available from authors.

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