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QSAR analysis of pyrazolidine-3,5-diones derivatives as Dyrk1A inhibitors

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ABSTRACT

Individuals with Down syndrome (DS) suffer from mental retardation. Overexpression and the resulting increased specific activity of Dyrk1A kinase located on chromosome 21 cause a learning and memory deficit in Dyrk1A transgenic mice. To search for therapeutic agents with Dyrk1A inhibition activity, previously we obtained HCD160 as a new hit compound for Dyrk1A inhibition. In the present study, we synthesized 34 HCD160 derivatives to investigate the quantitative structure–activity relationship (QSAR). This analysis could provide important information for novel drug discovery for treatment of DS related learning and memory deficits.

Down syndrome (DS) results from an extra copy of human chromosome 21. With a frequency of one in roughly every 800 births, it is the most common genetic anomaly.[1] In addition to characteristic physical features, individuals with DS show a variety of phenotypes, including mental retardation, low muscle tone, congenital heart defects, gastrointestinal malformations, immune and endocrine system defects, a high incidence of leukemia, and early onset of Alzheimer's disease (AD). Among the phenotypes, mental retardation, such as learning and memory deficit and cognitive decline, is a major factor in preventing DS individuals from leading independent lives in their early to middle-age years.[2] After the third decade of their lives, individuals with DS also develop pathological hallmarks of AD, amyloid plaques and neurofibrillary tangles (NFTs).[3] In the process of searching for genes responsible for these phenotypes, the Dual specificity tyrosine (Y) phosphorylation Regulated Kinase 1A (Dyrk1A) gene was isolated from human chromosome 21.[4] Dyrk1A protein is a serine/threonine kinase known to play a critical role in neurodevelopment, and is activated by autophosphorylation at the Tyr-321 residue.[5] Transgenic mice overexpressing the Dyrk1A protein showed hippocampal-dependent learning and memory deficits.[6] Several recent reports suggested that Dyrk1A may also be involved in the pathological mechanisms of APP and Tau, and may accelerate the formation of amyloid plaques and NFTs which are insoluble deposits of β-amyloid and hyperphosphorylated Tau, respectively, causing the early onset of AD pathogenesis in DS.[7] Thus, the development of therapeutic agents with Dyrk1A inhibition activity will benefit individuals with DS in treating mental retardation and AD.

Harmine and epigallocatechin-3-gallate (EGCG), a major tea compound, were reported as potent Dyrk1A inhibitors in the course of searching for therapeutic agents.[8] Previously, as an initial attempt to develop a mechanism-based drug to treat Dyrk1A associated DS learning and memory deficits, we isolated a synthetic hit compound through a combination of in silico, in vitro, and in cell-based screening.[9] The hit compound, HCD160, appears to be a good starting template for further optimization in order to increase the inhibitory potency against Dyrk1A activity (Fig. 1). In the present study, we further investigated the quantitative structure–activity relationship (QSAR) for 34 pyrazolidine-3,5-diones derivatives of HCD160.

The synthetic strategy was designed to maintain the pyrazolidine-3,5-diones as a core scaffold based on the following observations: (i) the previous result showed that NH and C=O groups of HCD160 bind tightly to the backbones of Glu 239 and Met 240 of Dyrk1A; and (ii) amino acid groups in the ATP binding pocket of Dyrk1A have predominantly negative charges in the surface models as compared with other protein kinases, such as glycogen

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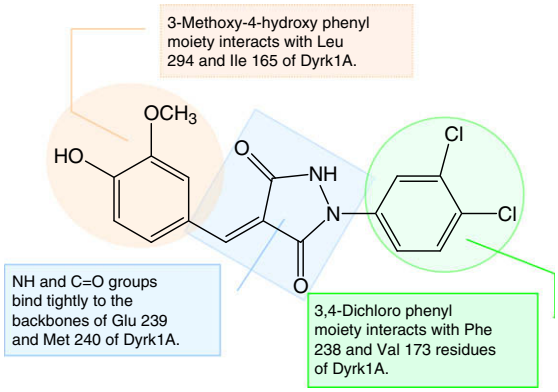


Figure 1. Schematic representation of the refined docking model of HCD160 with Dyrk1A using PharmoMap™.

synthase kinase-3 beta (GSK-3β), cycline-dependent kinase-2 (CDK-2) and casein kinase-2 (CK-2) (Fig. 2).[9,10] In silico screening for the ATP binding pocket was carried out using the PharmoScan™ system, a structure-based in silico screening tool developed by IDRTech Inc.[11] Modifications of the first series were performed to introduce various substitutions with stronger hydrogen bond interaction than that in the 3-methoxy-4-hydroxyphenyl moiety of HCD160 in the Dyrk1A ATP binding site (Table 1). The second series were modified to obtain various 1,4-disubstituted pyrazolidine-3,5-diones derivatives (Table 2). All the desired derivatives were prepared for testing via an in vitro Dyrk1A inhibition assay as previously described.[9] In order to evaluate the inhibitory potency against Dyrk1A activity, two types of inhibition assays were performed; autophosphorylation of Dyrk1A and kinase reaction on substrate phosphorylation.

The assay results of the first series of 19 HCD160 derivatives are summarized in Table 1. Seven compounds (**2**, **3**, **8**, **13**, **15**, **17** and **18**) show inhibitory activity against Dyrk1A autophosphorylation with IC_{50} values ranging from 0.6 to 20 μM (Table 1). Among them, compound **18**, with IC_{50} values of 0.6 μM, shows roughly fourfold increased inhibitory activity on autophosphorylation relative to that of HCD160, which showed IC_{50} values of 2.5 μM. However, only compounds **2** and **18** showed similar inhibition activity for autophosphorylation and kinase reaction, while other compounds exhibited no inhibition activity for kinase reaction at a concentration of 10 μM. This suggests the operation of different inhibition mechanisms between Dyrk1A autophosphorylation and substrate phosphorylation. Roscovitine, used as an internal control, showed less inhibition activity, with IC_{50} values of 5 μM for autophosphorylation and 63% inhibition of kinase activity.[8] The QSAR values for the second series of 15 HCD160 derivatives with 1,4-disubstituted pyrazolidine-3,5-diones are reported in Table 2. Compounds **21**, **30** and **34** exhibited inhibition activity against Dyrk1A autophosphorylation with IC_{50} values of 0.6, 1.2 and 2.5 μM, respectively, while the kinase activity was inhibited by 44–78%. Among the three compounds, compound **21** showed similar inhibition activity to that of compound **18** on autophosphorylation and kinase reaction. Therefore, we have described the synthetic methods of compounds **18** and **21** in the references and note paragraph.[12]

Most of the compounds tested in the present study showed less potent inhibition activity than that of HCD160. However, the effect of compounds **18** (IC_{50} = 0.6 μM) and **21** (IC_{50} = 0.6 μM) on autophosphorylation were very potent as compared with that of HCD160 (IC_{50} = 2.5 μM). And also, IC_{50} for compounds **18** and **21** on the substrate phosphorylation were 1.25 μM and 6 μM, respectively. Therefore, we further characterized these two compounds **18** and **21** by assessing the specificity of the inhibition and cytotoxicity. To evaluate the specificity, we performed comparative assay for them with other well-characterized kinases (Table 3). Selected kinases included a Dyrk family protein (Dyrk2) and neighboring proteins in the dendrogram constructed by Becker and Joost, such as CLK3 and GSK3β.[13] Other tested proteins represented kinases from the core panel described by Davies et al. and from kinase groups described by Vieth et al.[14] As expected from its close relationship to Dyrk1A, compound **18** showed a strong inhibition (83%) for Dyrk2 while compound **21** was much less inhibitory (21%). For GSK3β we have modeled the active site of Dyrk1A, compounds **18** and **21** showed no inhibition and 24% inhibition, respectively. For other tested kinases, two compounds showed a moderate (less than 30%) inhibition. The CC_{50} values (50% cytotoxic concentration) for compounds **18** and **21** obtained by WST-1 assay are >500 μM and >250 μM, respectively. We also tested if compounds **18** and **21** compete with ATP in the Dyrk1A ATP binding pocket. We observed that the inhibitory activities of these compounds were decreased when the reactions were performed at the increased ATP concentrations, indicating that inhibitors behave as competitors of ATP as we expected from the modeling (data not shown).

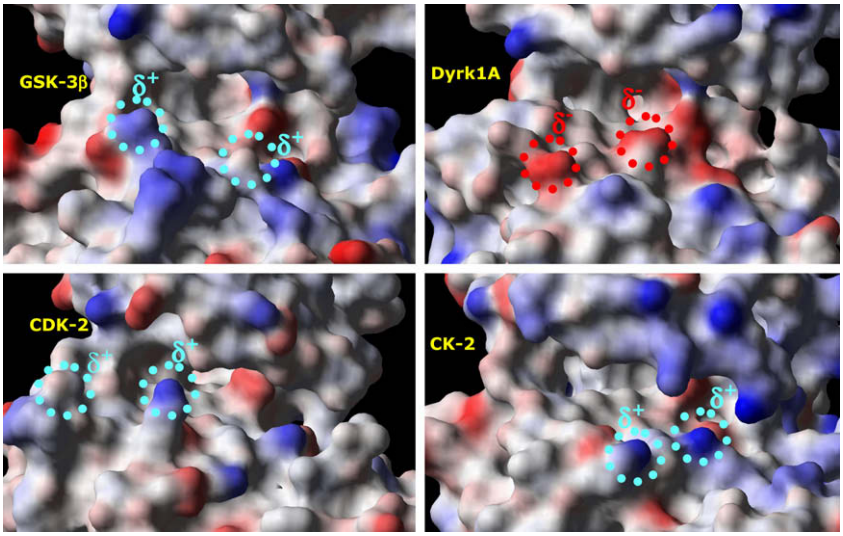
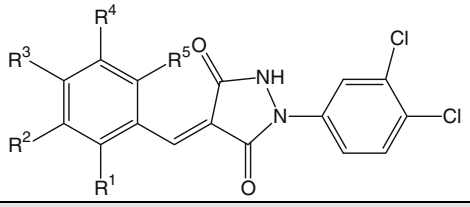


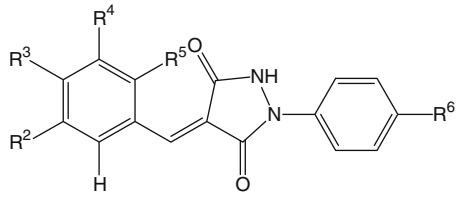
Figure 2. Surface models of the ATP binding sites of GSK-3β, Dyrk1A, CDK-2 and CK-2 (blue: positive charged region and red: negative charged region).

Table 1
Assay results of the first series of 19 HCD160 derivatives



Compounds	R^1	R^2	R^3	R^4	R^5	IC_{50} for autophosphorylation (μM)	% Inhibition for kinase activity at 10 μM (n = 3, mean ± SD)
HCD160	H	H	OH	OMe	H	2.5	82 ± 6
1	H	H	H	H	H	>50	No inhibition
2	H	H	OH	H	H	2.5	73 ± 2
3	H	H	Cl	H	H	20	No inhibition
4	H	H	OMe	H	H	>50	No inhibition
5	H	H	H	OMe	H	>50	No inhibition
6	H	H	H	H	OMe	>50	No inhibition
7	H	H	H	H	NO_2	>50	No inhibition
8	H	H	H	H	Cl	20	No inhibition
9	H	H	H	H	F	>50	No inhibition
10	H	H	OMe	OH	H	>50	No inhibition
11	H	H	OMe	OMe	H	>50	No inhibition
12	H	H	OMe	H	OMe	>50	No inhibition
13	H	OMe	H	OMe	H	20	No inhibition
14	H	HO	H	HO	H	>50	No inhibition
15	H	H	H	Cl	Cl	15	No inhibition
16	Cl	H	H	H	Cl	>50	No inhibition
17	H	H	F	F	H	5	No inhibition
18	H	H	OH	NO_2	H	0.6	76 ± 4
19	H	OMe	OH	OMe	H	>50	No inhibition
Roscovitine						5	63 ± 2

Table 2
Assay results of the second series with 1,4-disubstituted pyrazolidine-3,5-diones derivatives



Compounds	R^2	R^3	R^4	R^5	R^6	IC_{50} for autophosphorylation (μM)	% Inhibition for kinase activity at 10 μM (n = 3, mean ± SD)
20	H	H	H	OMe	CN	40	No inhibition
21	*H*	*OH*	*OMe*	*H*	*CN*	*0.6*	*78 ± 4*
22	H	OMe	OH	H	CN	>50	No inhibition
23	H	H	H	OMe	OMe	>50	No inhibition
24	H	H	OMe	H	OMe	>50	No inhibition
25	H	H	H	F	OMe	>50	No inhibition
26	H	H	Cl	Cl	OMe	>50	No inhibition
27	OMe	H	OMe	H	OMe	>50	No inhibition
28	OH	H	OH	H	OMe	>50	No inhibition
29	H	OMe	OH	H	OMe	>50	No inhibition
30	H	OH	OMe	H	OMe	1.2	55 ± 3
31	OMe	OH	OMe	H	OMe	>50	No inhibition
32	H	H	H	OMe	F	>50	No inhibition
33	H	OMe	OH	H	F	30	No inhibition
34	*H*	*OH*	*OMe*	*H*	*F*	*2.5*	*44 ± 5*

The structural differences between 3-nitro-4-hydroxyphenyl of compound **18** and 4-cyano-phenyl ring of compound **21** have important implications to hydrogen bonding and hydrophobic interaction, respectively in the Dyrk1A ATP binding site. According to molecular modeling and from the present results for compound **18**, the phenyl ring with a *para*-substituted hydroxyl group contributes to a hydrogen bonding interaction with the Asn 244 residue, in addition to hydrophobic interaction of the 3,4-dichloro phenyl moiety with Phe 238 and Val 173 residues (Fig. 3). Hence, the inhibition potency was improved the Dyrk1A inhibition activity by incorporating the more hydrophilic 3-nitro group and thereby reinforcing the hydrogen bonding interaction with the Glu 291 residue. Additionally, the 4-cyano-phenyl group in compound **21** also forms a strong hydro-

Table 3
Inhibition of protein kinases by compounds **18** and **21**

Protein kinase (*K*m)	Compound **18**	Compound **21**
CDK5/p35 (15 μM)	91 ± 3	104 ± 13
CLK3 (90 μM)	75 ± 3	93 ± 3
DYRK2 (15 μM)	17 ± 4	78 ± 6
GSK3β (15 μM)	102 ± 0	76 ± 5
JNK3 (10 μM)	94 ± 7	93 ± 5
MAPK2 (155 μM)	105 ± 1	110 ± 0
PKA (10 μM)	72 ± 4	69 ± 1
PKCα (45 μM)	82 ± 1	77 ± 4

The concentration of the compound is 10 μM in all assays. The *K*m value was used for ATP concentration for each kinase (*K*m value in parenthesis). Results are presented as the kinase activity as a percentage of the control incubations (means of duplicate determinations ± SD). All kinases are originated from human.

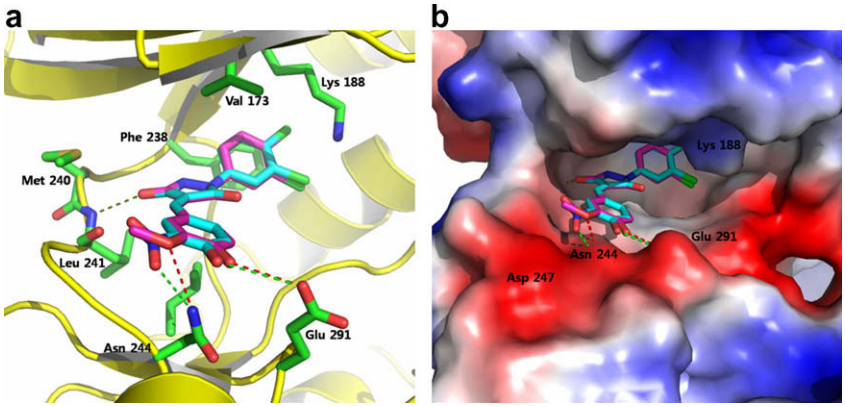


Figure 3. (a) Refined docking and (b) surface binding models of HCD160 and compound **18** with Dyrk1A (blue: positive charged region and red: negative charged region).

phobic interaction with Phe 238 and Val 173 residues of Dyrk1A. Although, the functional groups on both sides of the pyrazolidine-3,5-diones core scaffold can form a hydrogen bonding interaction in the ATP binding site, they did not show good inhibition activity on kinase reaction relative to that of HCD160.

As shown in Figure 3, the docking model of HCD160 and compound **18** with Dyrk1A indicated that the following interactions are very important in the ATP binding site: the hydrogen bonding of Asn 244 and Glu 291 side chains, the backbone hydrogen bonding interaction of Met 240 residue, polar (hydrophilic) interaction of Lys 188, and the hydrophobic interactions of Phe 238 and Val 173. The Dyrk1A inhibition activity and the specificity of compounds tested in this study seems to be dependent on chemical features such as hydrophobic, hydrophilic, and hydrogen bonding interactions, as the present compounds were selected by using PharmoMap™ for the pharmacophore-based in silico screening.

In the present study, we demonstrated the necessity of the 3-nitro-4-hydroxyphenyl moiety and the *p*-substituted cyanide group in the phenyl ring in both sides of the pyrazolidine-3,5-diones core scaffold, as shown in Figure 3. The presence of those moieties enhanced the Dyrk1A inhibition activity by blocking autophosphorylation of Dyrk1A, as revealed in an in vitro assay. The inhibition assay showed relatively good activity for HCD160 and Roscovitine, which were used as internal controls. Bain et al. reported harmine as a very potent Dyrk1A inhibitor (IC_{50} of 0.08 μM).[8] We performed the substrate phosphorylation assay with harmine using our kinase assay system for comparison. IC_{50} for harmine was 1 μM which was about 12-times higher than that of the reported IC_{50} value. This discrepancy could come from a difference in the Dyrk1A protein used in the assay. We used the 763 amino acid full-length protein while Bain et al. used the C-terminal truncated 1–499 amino acid protein.

In conclusion, the basic pyrazolidine-3,5-diones core presents a good scaffold for the development of novel compounds inhibiting Dyrk1A activity in vitro assay. A more convergent approach and the design of chemical models for further optimization are needed to obtain more potent and specific inhibitors. The pyrazolidine-3,5-diones derivatives could be utilized for the development of novel therapeutic agents for the treatment of DS related learning and memory deficits.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.02.062.

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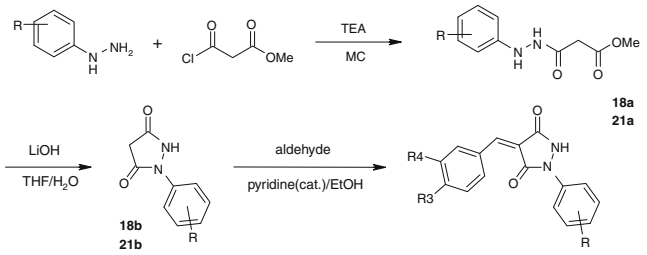
was expressed in *Escherichia coli* and purified with Ni-NTA resin (Qiagen) using its endogenous 13-histidine repeat. Based on the fact that autophosphorylation at Tyrosine residues (e.g., Tyrosine-111, 145, 159 and 321) requires the binding of ATP at the ATP pocket of Dyrk1A, the autophosphorylation assay was developed. The phosphatase treated Dyrk1A (0.8 μg/well) was coated on a 96-well Cova plate (Nalgen) in a phosphate buffered solution containing 3.2 μM *N*-hydrosuccinimide and 3.2 μM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide at 4 °C overnight. Autophosphorylation reaction in the presence or the absence of the testing compound was performed at 25 °C for 1 h in a kinase buffer (25 mM HEPES buffer, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 0.5 mM DTT) containing 10 nM ATP and 0.5 μCi ^{32}P-ATP, followed by liquid scintillation counting. The kinase assay was performed at the conditions that showed the linearity of the reaction. Dyrk1A (0.2 μM) was incubated for 20 min with Dyrktide (50 μM) in a kinase buffer containing 5 μM ATP at 25 °C. The inhibition of the kinase reaction was measured by quantifying the amount of the remaining ATP using Kinase-Glo luminescent reagent (Promega).

10. Insight II 2000; Accelrys, Inc., San Diego, CA; http://www.accelrys.com.
11. The IDRTech Inc's in-house program, PharmoMap™, PharmoLib™, and PharmoScan™; http://www.idrtech.com.
12. Synthetic methods of the compounds **18** and **21**.



18 : R=3,4-Cl, R3=OH, R4=NO_2
21 : R=4-CN, R3=OH, R4=OMe

(a) *N*′-(3,4-Dichlorophenylhydrazinocarbonyl)acetic acid methyl ester (**18a**): Et_3N (59.0 mM) was added to a solution of hydrazine (29.5 mM) in dried CH_2Cl_2 (100 ml) at room temperature. This reaction mixture was added drop wise to methyl malonyl chloride (32.4 mM) at 0 °C and then stirred at room temperature for 2 h. After the reaction was completed, mixture was extracted with CH_2Cl_2 and was washed with water and brine. Water layer was extracted with EtOAc. The combined organic layer was dried over $MgSO_4$, filtered and concentrated. The desired product of 4.3 g was obtained as a solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.00 (s, 1H), 8.30 (s, 1H), 7.36 (d, J = 9.0 Hz, 1H), 6.91 (d, J = 3.0 Hz, 1H), 6.70 (dd, J = 13.3 Hz, 1H), 3.66 (s, 3H), 3.35 (d, J = 5.0 Hz, 2H). Compound **21a**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.09 (s, 1H), 8.75 (s, 1H), 7.57 (d, J = 9.0 Hz, 2H), 6.79 (d, J = 11.0 Hz, 2H), 3.67 (s, 3H), 3.38 (d, J = 6.0 Hz, 2H); (b) 1-(3,4-Dichlorophenyl)pyrazolidine-3,5-dione (**18b**): LiOH (0.6 g) was added to a solution of 3 g of N'-(3,4-dichlorophenylhydrazinocarbonyl)acetic acid methyl ester (**18a**) in THF/water (60/20 ml) at room temperature. This reaction mixture was stirred for 1 h. The mixture was evaporated in vacuo, poured into EtOAc and washed with water and brine. The organic layer was dried over $MgSO_4$, filtered and concentrated. The desired pyrazolidinedione product (1.5 g) was obtained as a solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.93 (s, 1H), 7.67 (m, 2H), 3.67 (s, 2H). Compound **22b**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.83–7.87 (m, 4H), 3.69 (s, 2H); (c) 1-(3,4-Dichlorophenyl)-4-(4-hydroxy-3-nitrobenzyliden)pyrazolidine-3,5-dione (**18**): To a solution of pyrazolidinedione (0.2 mM) in ethanol (10 ml) was added 4-hydroxy-3-nitrobenzaldehyde and catalytic amount pyridine. The reaction mixture was refluxed for 10 h. The solid was collected and then washed with EtOAc and dried in vacuo. The desired product was obtained as a pink solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.62–8.66 (m, 1H), 8.02 (d, J = 17.0 Hz, 1H), 7.86 (d, J = 17.0 Hz, 1H), 7.68–7.90 (m, 2H), 7.26 (s, 1H), 7,23 (s, 1H); (d) Compound **21**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.79 (s, 1H), 7.65–8.10 (m, 6H), 6.96 (d, J = 9.0 Hz, 1H), 3.87 (d, J = 6.0 Hz, 3H).

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