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Putative therapeutic agents for the learning and memory deficits of people with Down syndrome

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Abstract—Mental retardation is the most common and debilitating condition for individuals with Down syndrome (DS). The hyper-activation of DYRK1A by overexpression causes significant learning and memory deficits in DS-model mice. Thus far, no mechanism-based drug has been developed to address this. After a combination of in silico and in vitro screenings, two DYRK1A inhibitors were isolated that are active in a cell-based assay. Further optimization could lead to a novel drug discovery that could address DS learning and memory deficits.

Down syndrome (DS) resulting from the presence of an extra copy of human chromosome 21 is the most common genetic disorder in human, with a frequency of one in 800 live births.[1] Among various phenotypes related to this disorder, mental retardation is a major factor in preventing these patients from leading fully independent lives in their early to middle-age years.[2] Until recently, no mechanism-based drug targeting the responsible gene(s)/protein(s) for the mental retardation has been developed. A few drugs for Alzheimer's disease were tried for DS patients, but the results were controversial.[3] Due to efforts in isolating the gene(s) responsible for DS mental retardation, the DYRK1A gene was isolated.[4] The DYRK1A protein plays a critical role in neurodevelopment and becomes active by autophosphorylation at the Tyr 321 residue. Transgenic mice overexpressing the DYRK1A protein showed significant hippocampal-dependent learning and memory deficits in a kinase activity-dependent manner.[5] In the present study, an initial attempt to discover a mechanism-based drug to treat DS learning and memory deficits by the combination of in vitro and in vivo screenings is reported.

The DYRK1A model was constructed based on the crystal structure of the glycogen synthase kinase-3 beta (GSK-3β) as a template (pdb accession code: 1q3w).[6] In a previous study, Himpel et al. described a structural model of DYRK1A that was built based on the homology of the phosphorylated MAP kinase extracellular signal-regulated kinase 2 (ERK2).[7] They showed that the activation loop of DYRK1A was very similar to the known structure of the activated ERK2. However, the overall sequence identities between ERK2 and DYRK1A are about 29% which is lower than GSK-3β (34%). Therefore, it is considered that the present homology model of DYRK1A is more suitable for the in silico screening study. A 3-dimensional (3D) model structure of the kinase domain of DYRK1A was built by using the HOMOLOGY module of InsightII. It was further refined by using Discover version 2.98 of InsightII.[8] The overall model of the kinase domain of DYRK1A was well conserved as that of GSK-3β. However, the activation loop of DYRK1A is shorter than the template structure of GSK-3β.

For the in silico screening of inhibitors for the DYRK1A autophosphorylation, the ATP binding site of

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DYRK1A was examined. An analysis of the binding model shows that the most important interactions are the hydrogen bondings between the backbones of Glu 239, Leu 240 and the adenine moiety of ATP (Fig. 1). The analysis shows that the hydrophobic interactions of Ile 165, Val 173, Leu 294, Val 306 and the hydrogen bonding interactions of Asn 244 of the DYRK1A side chains are also important. For these reasons, pharmacophore of the DYRK1A ATP binding site that possibly inhibits the catalytic site of DYRK1A was analyzed using PharmoMap^{TM}, IDRTech Inc's in-house software package for structure-based in silico screening.[9] The constructed PharmoMap^{TM} is the feature-based pharmacophore in which the pharmacophoric points are represented by chemical features, such as hydrogen bond acceptors/donors or hydrophobic features. The pharmacophore map was generated using PharmoMap^{TM} for structure-based in silico screening.

The pharmacophore models were employed as search queries to identify DYRK1A catalytic site target inhibitors from a 3D small molecule database, which is commercially available multi-conformer 3D database of 3.6 million compounds (PharmoLib^{TM}). The in silico screening for DYRK1A ATP binding pocket was carried out using the PharmoScan^{TM} system, a structure-based in silico screening tool developed by IDRTech Inc. Compounds that exhibited unfavorable interactions with the binding site or adopted unrealistic conformations were filtered out during the pharmacophore mapping into the ATP binding site of DYRK1A.

Final 182 compounds were selected for an in vitro DYRK1A inhibition assay.[10] Two types of inhibition assays were performed; autophosphorylation of Dyrk1A and phosphorylation of substrate. Dyrk1A activity is dependent on the phosphorylation of the tyrosine 321 residue in the activation loop for activation. And Dyrk1A activity can be inhibited by blocking autophosphorylation.[4] Another way to lower the Dyrk1A activity is to inhibit the kinase activity by blocking the ATP binding site. Results of the inhibition activity are summarized in Table 1.

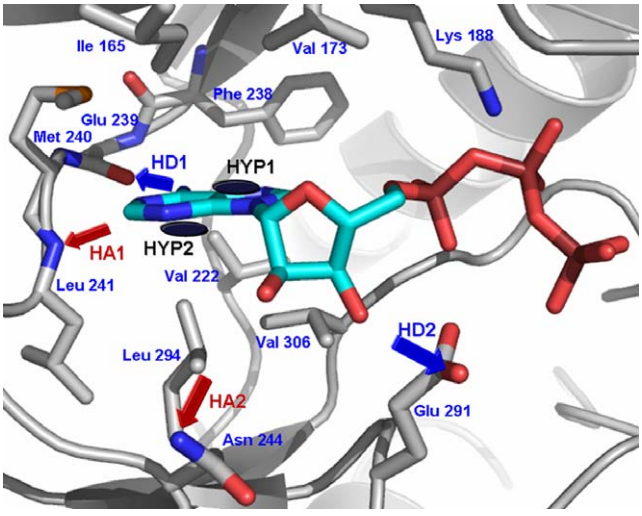


Figure 1. Pharmacophore model of the DYRK1A active site with ATP was generated by PharmoMap^{TM}. The blue and red arrows denote hydrogen bond donors and acceptors, respectively. The black circles denote the hydrophobic interaction sites. HA1 and 2, hydrogen bond acceptors; and HD1 and 2, hydrogen bond donors.

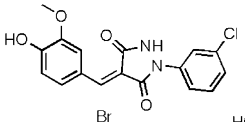
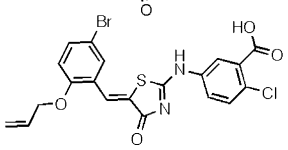
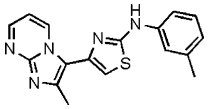
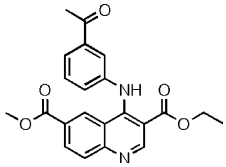
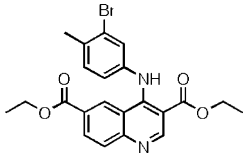
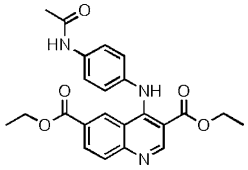
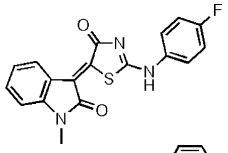
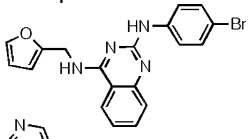
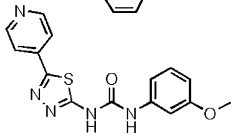
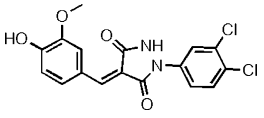
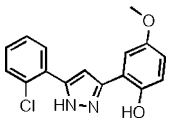
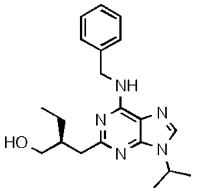
Eleven compounds showed an inhibition activity in the in vitro DYRK1A autophosphorylation assay, with an IC_{50} (concentration for 50% inhibition) ranging from 2.5 to 50 μM. Inhibition by 10 μM roscovitine used as an internal control was 63% which was comparable with the result (85%) reported by Bain et al.[11] Most of the tested compounds in the present study showed the similar inhibition activity in two different assays. However, compounds **2** and **8** exhibited no or little inhibition, while compounds **1** and **4** showed higher inhibition when the phosphorylation of DYRKtide was measured. The difference may come from the different inhibition mechanisms between autophosphorylation (tyrosine kinase) and phosphorylation of serine/threonine as described by Lochhead et al.[12]

A cell-based assay was performed to test if the compounds with inhibition activity from the in vitro assay could go into the cell and inhibit the autophosphorylation of the Dyrk1A protein (Fig. 2).[13] Among the tested compounds, compounds **9** and **10** showed the consistent phosphorylation inhibition activity with IC_{50} of 200 and 100 μM, respectively ($n > 6$, $p < 0.01$). The higher IC_{50} concentrations in the cell-based assay comparing with those needed for the 50% inhibition in vitro may be due to the penetration of compound into cells and/or high concentration of ATP in the cells.

The compound **10** was the most active in in vitro and cell-based assays. The plausible docking model of compound **10** for further optimization was investigated. For the docking model of compound **10**, the affinity module of InsightII was used. The docking protocol was based on the Monte Carlo grid docking methodology. The initial binding model of compound **10** was matched with the pharmacophore map of the DYRK1A catalytic site. All of the default parameters were used. One thousand minimization steps were applied to the final structure, and the active pocket of the receptor was defined as residues 3.5 Å from compound **10**. The potential of the complex was assigned according to the CVFF (consistent valence force field). Non-bonding interactions were used for the group-based approach. Finally, the docked complexes of DYRK1A with compound **10** were selected according to the criteria of the interaction energy and the lower root mean square (RMS) deviation from the feature-based pharmacophore map.

Figure 3 shows the refined docking model of compound **10** with DYRK1A. The docking model indicated that NH and C=O groups of the compound **10** tightly bind to the backbones of Glu 239 and Met 240 of DYRK1A, respectively. The 3-methoxy-4-hydroxy phenyl moiety of compound **10** interacts with Leu 294 and Ile 165 residues of DYRK1A, and the 3,4-dichloro phenyl moiety forms a contact with the Phe 238, Val 173, and Val 306 resi-

Table 1. The inhibition of Dyrk1A by selected compounds

Compound	Structure	IC_{50} of autophosphorylation (μM)	% inhibition at 10 μM ($n = 4$, mean ± SD)
1		>50	74 ± 5
2		20	No inhibition
3		>50	25 ± 1
4		50	51 ± 6
5		50	13 ± 2
6		10	52 ± 4
7		50	12 ± 2
8		5	8 ± 2
9		5	41 ± 4
10		2.5	82 ± 6
11		20	43 ± 1
Roscovitine		5	63 ± 2

Inhibition of autophosphorylation is shown as IC_{50}, while inhibition of kinase activity is shown as mean of four determinations ±SD.

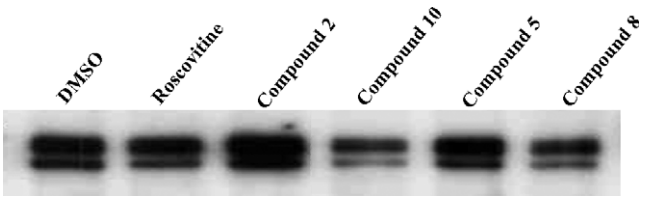


Figure 2. A representative cell-based assay. DMSO (final concentration, 1%) used for compound suspension and roscovitine (100 μM) were a negative and a positive controls, respectively. The treated compound concentration was as follows: compound **2** (200 μM); compound **10** (200 μM); compound **5** (50 μM); compound **8** (100 μM).

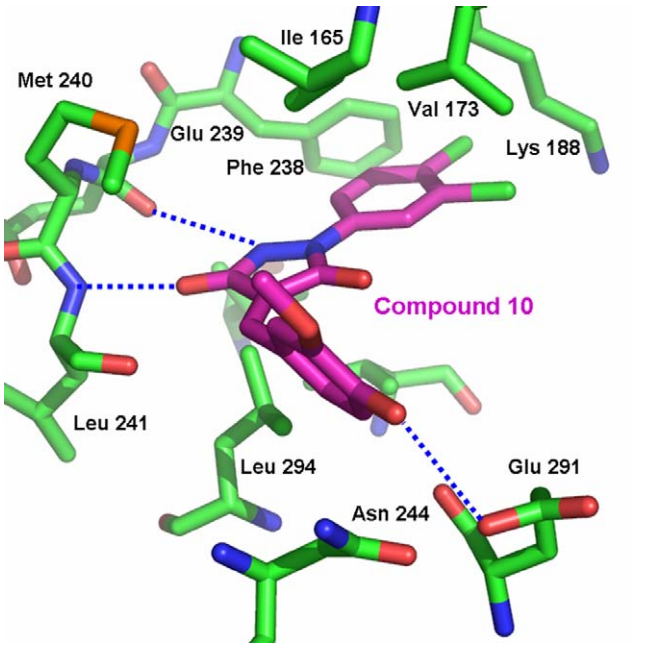


Figure 3. Refined docking model of compound **10** with DYRK1A. The dashed lines denote hydrogen-bonding interactions.

dues. Additionally, compound **10** forms a hydrogen-bonding interaction with the Glu 291 residue.

We also tested the inhibition activity of compounds **9** and **10** for 15 other well-characterized kinases (Table 2).[14] DYRK2 was chosen to represent the DYRK family, and cdc-like kinase 3 (CLK3) was to represent a protein from neighboring branch of the dendrogram constructed by Becker and Joost.[15] GSK3β and CK2 were chosen since they were from the next closest main branch of the dendrogram. The kinase assay for these proteins will indicate how specific the compounds are. The rest of the proteins tested were selected to represent kinases from the core panel described by Davies et al., and from structure–activity relationship-based and sequence-based kinase groups described by Vieth et al.[16] As expected from their close relationship to DYRK1A, inhibition of the tested compounds for CLK3 and DYRK2 was noticeable. Compounds **9** and **10** for CLK3 showed 89% and 64% inhibition at 10 μM, respectively. Compound **10** inhibits DYRK2 by 41%, while compound **9** was less inhibitory (23%). For CK, compound **10** inhibited 49%, while compound **9** showed no inhibition. Most of the remaining protein kinases show the inhibition of less than 30%.

Table 2. Inhibition of protein kinases by compounds **9** and **10**

Protein kinase	Compound **9**	Compound **10**
Abl	96 ± 4	94 ± 2
CaMKII	98 ± 2	72 ± 3
CDK2/cyclinA	96 ± 1	90 ± 6
CDK5/p35	104 ± 7	99 ± 3
CK2	110 ± 2	61 ± 2
CLK3	11 ± 1	36 ± 2
DYRK2	77 ± 0	59 ± 2
GSK3β	47 ± 1	69 ± 0
JNK3	98 ± 2	86 ± 3
Lck	113 ± 2	105 ± 1
MAPK2	138 ± 1	122 ± 5
PKA	104 ± 3	107 ± 7
PKCα	83 ± 0	78 ± 1
PRAK	69 ± 1	71 ± 6
SAPK2α	101 ± 4	112 ± 2

The concentrations for compound and ATP used are 10 and 100 μM, respectively, in all assays. 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 except rat CaMKII.

Bain et al. showed that 10 μM roscovitine inhibited the DYRK1A activity by 85%, while it did not inhibit the activity of GSK3β, indicating that 3D structure of ATP binding sites or inhibition mechanisms of DYRK1A and GSK3β could be quite different. Thus, it might be better to consider other proteins as a Dyrk1A structural model in stead of GSK3β for further optimization of the compounds. In addition since the inhibition by roscovitine for more than 25 other tested kinases was not detectable or very low, consideration of the structure of roscovitine would be useful to design the DYRK1A-specific inhibitor for further optimization.

In conclusion, utilizing a combination of in silico, in vitro, and cell-based screenings, a novel compound inhibiting the DYRK1A activation was isolated. Thus far, no drug development has been reported; although further study and optimization are needed, it may be possible to treat the learning and memory deficits of those with DS by intervening in the hyper-activity of DYRK1A resulting from the overexpression.

Acknowledgments

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9. <http://www.idrtech.com/>; The IDRTech Inc's in-house program, PharmoMap™, PharmoLib™ and PharmoScan™, will be published elsewhere in the near future.
10. In vitro assay. The full-length (763 amino acid) mouse *Dyrk1A* cDNA was cloned into pET-25b (Novagen). After IPTG induction in *Escherichia coli* BL21(DE3) codon plus RIL (Stratagene), the Dyrk1A protein was purified with Ni–NTA resin (Qiagen) using its endogenous 13-histidine repeat. For autophosphorylation assay, the purified Dyrk1A (100 μg) was treated with lambda phosphatase (4,000 unit; Bio-Rad, cat. no. P0753L) for 2 h at 30 °C. Then Dyrk1A (0.8 μg/well) was coated on a 96-well Cova plate (Nalgen, cat. no. 244105) in a phosphate-buffered solution (PBS) containing 3.2 μM *N*-hydrosuccinimide and 3.2 μM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide at 4 °C overnight, followed by washing with PBS. Autophosphorylation in the presence or the absence of compound (in final 1% DMSO concentration) was performed at room temperature (RT, 21–22 °C) for 1 h in a kinase buffer (25 mM HEPES buffer, 5 mM $MnCl_2$, 5 mM $MgCl_2$, and 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 by incubating DYRKtide (50 μM) with Dyrk1A (0.2 μM) in a kinase buffer containing 5 μM ATP at RT for 20 min. The inhibition of the kinase reaction was measured by quantifying the amount of the remaining ATP in the reaction using kinase-glo luminescent reagent as described (Promega, V-6711).
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13. Cell-based assay. An anti-Dyrk1A antibody for immunoprecipitation was raised by immunizing rabbit with the C-terminal Dyrk1A peptide as previously described (Okui et al., 1999). The Dyrk1A-specific antibody was further purified from the serum on an immunogen-peptide coupled Affi-gel 15 affinity column (Bio-Rad, cat. no. 153-6051) by eluting the bound antibody with 0.1 M glycine, pH 2.5. The Dyrk1A overexpressing stable HEK293 cell line was established by transfecting the linearized pcDNA3.1a-Dyrk1A plasmid with Lipofectamine Plus and by selection of neomycin-resistant cells. The stable cells were subcultured (8×10^5 cells/well, 6-well plate) for 70–80% confluency next day and treated with the compounds (at 50% cytotoxic concentration) for 4 h. Cells were lysed with a RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, and 0.1% SDS) containing 0.5 mM Na_3VO_4 and protease inhibitor (Sigma, cat. no. P8425), and cell lysate (350 μg) was incubated with Dyrk1A antibody (2.5 μg) at 4 °C overnight in a RIPA buffer with Na_3VO_4 and protease inhibitor. After the addition of protein A beads (PIERCE, cat. no. 20333), the captured Dyrk1A-antibody complex was subjected to 8% SDS–PAGE and Western analysis for phospho-Tyr detection by the PY99 antibody (Santa Cruz, cat. no. sc-7020).
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