Check for updates

METHOD ARTICLE

An ELISA DYRK1A non-radioactive assay suitable for the characterization of inhibitors [version 1; peer review: 2 approved]

Yong Liu, Tatyana Adayev, Yu-Wen Hwang

Molecular Biology Department, New York State Institute for Basic Research in Developmental Disabilities, Staten Island, NY, USA

V1 First published: 13 Jan 2017, 6:42
<https://doi.org/10.12688/f1000research.10582.1>

Latest published: 24 Mar 2017, 6:42
<https://doi.org/10.12688/f1000research.10582.2>

Abstract

The DYRK1A (dual specificity tyrosine phosphorylation-regulated kinase 1A) gene encodes a proline-directed Ser/Thr kinase. Elevated expression and/or altered distribution of the kinase have been implicated in the neurological impairments associated with Down syndrome (DS) and Alzheimer's disease (AD). Consequently, DYRK1A inhibition has been of significant interest as a potential strategy for therapeutic intervention of DS and AD. Many classes of novel inhibitors have been described in the past decade. Although non-radioactive methods for analyzing DYRK1A inhibition have been developed, methods employing radioactive tracers are still commonly used for quantitative characterization of DYRK1A inhibitors. Here, we present a non-radioactive ELISA assay based on the detection of DYRK1A-phosphorylated dynamin 1a fragment using a phosphorylation site-specific antibody. The assay was verified by the use of two well-characterized DYRK1A inhibitors, epigallocatechin gallate (EGCG) and harmine. The IC_{50} s for EGCG and harmine determined by the ELISA method were found to be comparable to those previously measured by radioactive tracing methods. Furthermore, we determined the mode of inhibition for EGCG and harmine by a modification of the ELISA assay. This assay confirms the mode of inhibition of EGCG (non-ATP-competitive) and harmine (ATP-competitive), as previously determined. We conclude that the ELISA platform demonstrated here is a viable alternative to the traditional radioactive tracer assays for analyzing DYRK1A inhibitors.

Keywords

non-radioactive kinase assay, EGCG, harmine, inhibitor screening

Open Peer Review

Approval Status

	1	2
version 2 (revision) 24 Mar 2017		
version 1 13 Jan 2017	view	view
1. Walter Becker , Aachen University, Aachen, Germany 2. Stefan Knapp , University of Oxford, Oxford, UK Any reports and responses or comments on the article can be found at the end of the article.		

Corresponding author: Yu-Wen Hwang (yuwen.hwang@csi.cuny.edu)

Competing interests: No competing interests were disclosed.

Grant information: This work is supported by the New York State Office for People with Developmental Disabilities, the parent agency of New York State Institute for Basic Research in Developmental Disabilities. No extramural fund was used to support this research. *The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

Copyright: © 2017 Liu Y *et al.* This is an open access article distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Data associated with the article are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](#) (CC0 1.0 Public domain dedication).

How to cite this article: Liu Y, Adayev T and Hwang YW. **An ELISA DYRK1A non-radioactive assay suitable for the characterization of inhibitors [version 1; peer review: 2 approved]** F1000Research 2017, 6:42 <https://doi.org/10.12688/f1000research.10582.1>

First published: 13 Jan 2017, 6:42 <https://doi.org/10.12688/f1000research.10582.1>

Introduction

The human DYRK1A gene¹ is mapped to a region of chromosome 21 implicated in Down syndrome (DS)². DS, the most common chromosomal abnormality associated with birth defects and developmental disabilities, is caused by full or partial trisomy of chromosome 21³. Almost all DS cases inevitably lead to the development of Alzheimer's disease (AD)-type pathology⁴. Transgenic mice carrying an extra copy of DYRK1A have been shown to exhibit symptoms similar to DS, including brain abnormalities, neurodevelopmental delay, and memory impairments^{5–7}. DYRK1A in the brain displays a distinct structure-specific distribution pattern⁸, and it interacts with an array of factors involved in neuronal development, proliferation, and differentiation⁹. The level of DYRK1A is elevated in a gene dosage-dependent manner in DS, suggesting that the protein not only plays an important role in regulating normal brain functions, but also in the etiology of DS^{8,10}.

DYRK1A has been linked to neurofibrillary degeneration and β -amyloidosis of AD¹¹. DYRK1A was shown to phosphorylate microtubule-associated protein tau at T212 to prime tau for subsequent phosphorylation by GSK-3 β at S308^{12,13}. This inhibits tau's ability to stimulate microtubule assembly and promotes self-aggregation, like abnormally hyperphosphorylated tau in AD brain¹³. DYRK1A was also found to phosphorylate amyloid precursor protein (APP) at T668¹⁴ and prenesilin-1 at T354¹⁵, which correlated with increased cleavage of APP by β and γ secretases^{15,16} respectively, and leads to the formation of neurotoxic β -amyloid peptides (A β). Moreover, A β is shown to be involved in a positive feedback loop for promoting DYRK1A expression, which may further accelerate production of A β ¹⁷.

The collected evidence suggests that DYRK1A is a potential drug target for the treatment of DS and AD. To this end, many classes of DYRK1A inhibitors, both natural and synthetic, have been tested^{18–20}. The potency of such inhibitors has mostly been analyzed using radioactive tracer methods despite the availability of non-radioactive assays^{21,22}. It may be that these methods typically require multiple steps, which is undesirable for screening. Here, we describe a simple ELISA assay for DYRK1A inhibition using dynamin 1a and its phosphorylation site antibody for detection^{23,24}. The ELISA assay has been verified using two known DYRK1A inhibitors and found to be consistent with radioactive tracer methods.

Methods

Materials

Epigallocatechin gallate (EGCG; #70935) and harmine (#10010324) were obtained from Cayman Chemical. Para-nitrophenyl phosphate (PNPP) tablets and diethanolamine substrate buffer were purchased from Thermo-Fisher Scientific. EGCG and harmine were initially prepared as 50 mM stock in 100% DMSO. Working solutions of EGCG and harmine (0.01 μ M – 3.2 μ M) were prepared from stocks in 2% DMSO by serial dilution. Dynamin 1a pS857-specific mouse mAb 3D3 (RRID: AB_2631263) was prepared as described²⁴. The antibody was partially purified from ascites using Bakerbond ABx resins (#7269-02) before use. Anti-dynamin mAb Hudy-1 (RRID: AB_309677)²⁵ was obtained from EMD Millipore. Alkaline phosphatase (AP) conjugated goat anti-mouse

IgG secondary antibody (#115-055-146) was purchased from Jackson ImmunoResearch Laboratories, Inc.

Kinase and substrate preparation

6xHis tagged rat truncated DYRK1A containing residues 1–497 (HT-497) was used for all assays. This truncation preserves activity of DYRK1A^{26,27}. A bacterial HT-497 expression vector was constructed as follows. The truncated DYRK1A gene was first obtained by PCR from the GST-DYRK1A vector²³ using a pair of primers for producing the DYRK1A fragment with a Cla I site plus a 6XHis tag (cctatcgatgcatcatcatcatcaccatagaggagagacttc) at the start codon and an in-frame termination at codon 498 plus a Xho I site (ggactcgagtcgaaggctgtggacacactgtt), respectively, at 5' and 3' ends. PCR was performed in 50 μ l mixture containing 10 ng template, 0.2 μ g of each primer, 0.2 mM ATP, and PfuUltra (Agilent Technologies), as recommended by the supplier. Amplification was conducted with 20 cycles of the following steps: 94°C, 30-sec, 72°C 90-sec, and 62°C 30-sec. Custom primers were purchased from Integrated DNA Technologies. The resulting amplicon was then cloned into a modified T7 promoter-driven vector pND1²⁸ via the Cla I and Xho I sites, as described²⁹. Proline rich domain (PRD, residues 746–864) of dynamin 1a was also prepared as N-terminal tagged 6xHis fusion protein (HT-PRD) exactly as described above. The PRD fragment was first produced from the semi-synthetic dynamin 1a gene²⁴ by PCR using a pair of primers, (aggatcgatgcatcatcatcatcataacacgaccacgtcagcacg) and (aggctcgagtcagtcgaaggctgtggacacactgtt) for subsequent cloning into expression vector pND1, like HT-497.

Both HT-497 and HT-PRD were expressed and purified using TALON metal affinity resin (Clontech Laboratories) under native conditions as described²⁹. Proteins were quantified by Bradford method³⁰ and stored at -80°C until use.

ELISA assay

Substrate, HT-PRD, was diluted in dilution buffer (25 mM Tris-HCl, pH 7.4 and 100 mM NaCl) to a concentration of 2 ng/ μ l (or higher as in Figure 1 and Figure 2) and used to coat a 96-well plate (BD Falcon #353072) with 100 μ l per well (200 ng/well unless otherwise indicated) at 4°C overnight. Unbound materials were washed away with dilution buffer and wells were blocked with 150 μ l blocking buffer (2% BSA, 1X PBS, and 0.25% Tween 20) at room temperature for 60 min. After blocking, wells were washed extensively with dilution buffer before subjecting to phosphorylation. DYRK1A phosphorylation was performed in wells with 100 μ l reaction mix containing 25 mM HEPES, pH7.4, 100 mM NaCl, 5 mM MgCl₂, 100 μ M ATP (Sigma-Aldrich Chemicals), inhibitor if needed, and 5 ng HT-497 (unless otherwise indicated). Reactions were initiated by adding HT-497 and continued for 30 min (unless otherwise indicated) at 30°C. For time course experiments, reactions were terminated by the addition of 20 mM EDTA at the indicated time points. A set of inhibition experiments typically consists of a no-inhibitor control plus a series of eight inhibitor concentrations (0.001 μ M - 3.2 μ M final). Each point was run in duplicate with DMSO present in all assays at 0.2% final concentration. DMSO, up to 2%, does not affect the potency of EGCG and harmine. HT-PRD phosphorylation was subsequently determined by the sandwich antibody staining protocol, first with 100 μ l mAb

Figure 1. Coating ELISA plate with HT-PRD. Wells were incubated with indicated amounts of HT-PRD (0, 25, 50, 100, 200, 400, and 800 ng/well) at 4°C overnight and the level of coated proteins was then detected with anti-dynamin mAb Hudy-1 by following the sandwich ELISA protocol, as described in Methods (n = 4 for each data point).

Figure 2. Phosphorylation of coated HT-PRD by DYRK1A. Wells were coated with indicated amounts of HT-PRD (0, 25, 50, 100, 200, 400, and 800 ng/well) and then subjected to extensive DYRK1A phosphorylation *in situ* by incubation with 80 ng of HT-497 at 30°C for 60 min. The level of S857 phosphorylation was then detected with 3D3 following the sandwich ELISA protocol, as described in Methods (n = 4 for each data point). Filled circles (●), with kinase; empty circles (○), without kinase.

(60 min at room temperature) then with 100 μ l AP-linked anti-mouse secondary antibody (60 min at room temperature), followed by colorimetric reaction with 100 μ l PNPP solution. The extent of AP reaction was monitored at $\lambda=405$ nm. For Hudy-1 staining, wells were coated, blocked, and then stained with the antibody (1:3000 dilution) for colorimetric detection as described above.

Dilution factors for both mAb 3D3 and secondary antibody were pre-determined for each batch of antibody to ensure that neither antibody was limiting in the assay. A stock to be determined was serially diluted (from 1000 to 256,000-fold) and each dilution was used together with a non-limiting concentration of the other antibody to assess the level of HT-PRD phosphorylated under standard ELISA reaction conditions without inhibitor (see Results and Discussion). OD₄₀₅ readings were normalized to the 1000-fold dilution and plotted against the dilutions of the testing antibody. Dilutions in the normalized OD₄₀₅ plateau can be used for the assay. We routinely use 1:3000 dilutions for ABx purified 3D3 stock (~1.5 mg/ml) and 1:2000 dilutions of commercial secondary antibody for the assay.

Data analysis

Data transformation, calculation, plotting, curve fitting, and IC₅₀ calculation were performed in KaleidaGraph (http://www.synergy.com/wordpress_650164087; Mac version 4.1). Data was corrected for background (readings from wells with only PNPP) before subsequent manipulations. To determine IC₅₀, the residual DYRK1A activity was first calculated as the ratio to the no-inhibitor control in that set. The resulting residual activity was then plotted against its corresponding inhibitor concentrations in semi-log graph and the plot was fit to the sigmoidal equation, $y = a + (b - a) / (1 + (x/c)^d)$, for IC₅₀ calculation.

ATP competition assay

The standard ELISA protocol was modified to run under conditions allowing a constant inhibitor to compete against varying ATP concentrations in inhibiting DYRK1A. Briefly, a set of competition experiments had four DYRK1A assays in the presence of different ATP concentrations (100, 200, 400, or 800 μ M) with a single fixed concentration of the inhibitor to be tested. An identical set, except without inhibitor, was performed in parallel (no-inhibitor controls). The inhibitor concentration used was roughly twice the IC₅₀ of the inhibitor. All other procedures of the assay are unchanged. Residual kinase activity with the inhibitor at each ATP concentration was first calculated as a percentage of the corresponding no-inhibitor control. The residual kinase activity was subsequently converted to inhibition potency as the difference from 1. The value for each ATP concentration was then normalized to the inhibition potency at 100 μ M ATP and plotted.

Results and discussion

Development of the non-radioactive DYRK1A ELISA assay

We chose to follow the ELISA-based protocol^{31,32} in developing our assay, by immobilizing the substrate followed by kinase phosphorylation in the wells, as this format offers the advantage of a simple, proven design versus other non-radioactive approaches³³. Like many non-radioactive approaches³³, our assay relies on a phospho-specific antibody to differentiate between phosphorylated from un-phosphorylated substrates. The antibody used in the assay, mAb 3D3, was raised against DYRK1A-phosphorylated

Dynatide 3; a peptide derived from the DYRK1A phosphorylation site of dynamin 1a at S857²⁴. 3D3 has been shown to recognize only pS857-dynamin 1a in rat brain extracts upon extensive phosphorylation²⁴.

Dynatide 3, which is used routinely as a substrate to measure DYRK1A activity by the radioisotope/filter binding method^{27,34,35}, was first tested as a substrate. Unfortunately, it failed to produce any signal upon phosphorylating coated Dynatide 3 with DYRK1A, presumably due to a lack of peptide coating. To circumvent this problem, we used a 6X histidine-tagged PRD of dynamin 1a (HT-PRD) as a DYRK1A substrate. This fragment coats wells in a concentration-dependent manner and the amount of immobilized proteins, as revealed by mAb Hudy-1 staining, are proportional to input proteins up to 200 ng/well of HT-PRD (15 pmole/well) (Figure 1).

We then examined whether immobilized HT-PRD is accessible to DYRK1A. Wells coated with varying amounts of HT-PRD were subjected to exhaustive phosphorylation *in situ* with excess DYRK1A (HT-497) (see Methods) for 60 min and probed with excess (non-limiting) mAb 3D3 and secondary antibodies (see below in Figure 5). Phosphorylated immobilized HT-PRD was recognized by 3D3. The 3D3 signal was elevated in response to increasing input of HT-PRD (Figure 2, filled circles) initially, then leveled off, closely resembling the response of substrate coating (Figure 1). As controls, uncoated wells phosphorylated by HT-497 (Figure 1) and coated HT-PRD, processed without HT-497, produced no detectable signals (Figure 2, empty circles). These results indicate that immobilized HT-PRD is phosphorylatable by DYRK1A and that the output of the assay requires DYRK1A phosphorylation.

If a system is to be useful in determining inhibitor potency quantitatively, the output of the system must be solely dependent on DYRK1A activity in a linear fashion. We used a fixed amount of coated HT-PRD (200 ng/well) to identify the proper conditions. The system response to changes of HT-497 was first examined (Figure 3). Our ELISA system produces sufficient signal to be readily distinguished from the noise of no-kinase control, with ~1 ng HT-497 (~17 fmole) phosphorylation at 30°C for 30 min. The output (the equivalent of reaction rate) is elevated accordingly as enzyme concentration increased, but the ratio of elevation to enzyme concentration, in proportion to enzyme, is progressively reduced (Figure 3). This is a typical enzyme concentration-dependent reaction profile when the substrate becomes the limiting factor³⁶. Time-course experiments were subsequently conducted with 5 ng HT-497, as the highest enzyme concentration producing a near-linear enzyme-dependent response. The output was found to be linear with reaction times up to about 75 min (Figure 4). Therefore, we use the following standard conditions [200 ng of substrate, 5 ng HT-497 (0.82 nM), 100 μ M ATP, and 30 min kinase reaction at 30°C] for all subsequent experiments.

To support accurate measurement of IC₅₀, the amounts of antibody, both 3D3 and secondary antibody, must not be limiting. Otherwise, immunostaining will most likely under-report the actual phosphorylation level at lower concentrations of inhibitor, which could skew the IC₅₀ calculation. Therefore, each batch of antibody was titrated to determine the maximal dilution can be used. As shown for

Figure 3. DYRK1A concentration-dependent phosphorylation of coated HT-PRD. Wells were coated with 200 ng/well HT-PRD and then subjected to DYRK1A phosphorylation with varying amounts of HT-497 (1.25, 2.5, 5, 10, 20, 40, and 80 ng) at 30°C for 30 min. The level of S857 phosphorylation was then detected with 3D3 as described in Methods (n = 6 for each data point).

Figure 4. Time-course phosphorylation of coated HT-PRD by DYRK1A. Wells were coated with 200 ng/well of HT-PRD and then subjected to DYRK1A phosphorylation with 5 ng HT-497 at 30°C. The reactions were terminated at the indicated time points (0, 5, 10, 20, 30, 45, 60, 75, and 90 min) by the addition of 20 mM EDTA. The level of S857 phosphorylation was then detected with 3D3 as described (n = 3 for each data point).

Figure 5. 3D3 dilution factor determination. Wells were coated with 200 ng/well HT-PRD and then subjected to phosphorylation with 5 ng HT-497 under the standard reaction conditions. 3D3 to be tested was serially diluted (from 1000 to 256,000x) and used to probe the phosphorylated wells, followed by secondary antibody as described. Normalized OD₄₀₅ was calculated (see Methods) and used for plotting (n = 9 for each data point).

titrating of 3D3, when the antibody is limiting (provided that a non-limiting concentration of secondary antibody is used), the readout will increase upon addition of 3D3 until a plateau indicating saturation (Figure 5). Only dilutions that produce readout in the plateau (non-limiting) region should be used for the assay (Figure 5). Dilution factors for the secondary antibody were similarly determined (Supplementary Figure 1).

Measuring IC₅₀ and the mode of inhibition for DYRK1A inhibitors

We subsequently tested the system by examining two well-characterized inhibitors, EGCG and harmine^{27,35}. A typical inhibition profile conducted by the ELISA method for EGCG (Figure 6) and harmine (Supplementary Figure 2) follows a sigmoidal function. IC₅₀s for EGCG and harmine determined by the ELISA method were $0.215 \pm 0.024 \mu\text{M}$ and $0.107 \pm 0.018 \mu\text{M}$, respectively. These values are comparable to those obtained earlier by us and others with different substrates and protocols, including the radioisotope/filter binding assay, generally regarded as the gold standard for kinase inhibition assays (Table 1)^{22,37–39}. The results obtained from this ELISA assay appear to be as reproducible as any given enzymatic assay. These results confirm that our ELISA platform is a valid system for quantitative characterization of DYRK1A inhibitors.

We then modified the ELISA protocol to run the assays under a single concentration of inhibitor with varying ATP concentrations, to determine whether ATP is competitive against the inhibitor in

question. This allows the efficacy of inhibition to be evaluated with changing ATP. ATP is expected to influence the potency of competitive inhibitors, but not that of non-competitive inhibitors. As shown in Figure 7, harmine loses potency against DYRK1A when ATP is increased from 100 to 800 μM , indicating an ATP-competitive mode, while EGCG potency remains essentially unchanged (non-ATP-competitive). The inhibitory modes for harmine and EGCG revealed by the ELISA assay are the same as previously reported by the radioisotope/filter binding method^{27,35}. This further validates the ELISA assay.

Dataset 1. Raw data for Figures 1–7 and Supplementary Figures 1 and 2 are supplied in KaleidaGraph format (zipped file)

<http://dx.doi.org/10.5256/f1000research.10582.d148787>

As noted, non-radioactive DYRK1A assays have been described^{21,22}. These assays employ similar solution DYRK1A reactions at first stage and then different approaches for measuring the phosphorylated products. One used a phospho-specific antibody to capture products for subsequent immuno/colorimetric detection²¹, while another used a fluorescein-tagged substrate and analyzed products by high performance liquid chromatography/fluorescence detection²². The above methods have been optimized for sensitivity to measure cellular DYRK1A activity. We do not know whether our ELISA method, at the current stage, affords such level of sensitivity. Nevertheless, as we have demonstrated, our ELISA assay

Figure 6. Epigallocatechin gallate (EGCG) inhibition profile. A. EGCG inhibition assays were performed in the presence of serially diluted EGCG (0.001 – 3.2 μM) under the standard reaction conditions. DYRK1A activity at any given EGCG concentration was calculated as a ratio to the activity of the no-inhibitor control and plotted on the Y-axis, versus EGCG concentration. IC_{50} was calculated from the plot after curve fitting, as described in Methods (n = 6 for each data point).

Table 1. List of IC_{50} for epigallocatechin gallate (EGCG) and harmine obtained by different approaches.

Inhibitor IC_{50} (μM)	DYRK1A*	Substrate	[ATP] (μM)	Detection
EGCG				
0.33	1	Woodtide ⁴⁰	100	Radioisotope ³⁷
0.31 (K_i)	1	FAM-Woodtide	100	HPLC/Fluorescence ²²
0.43	2	Dynatide 3 ²⁴	50	Radioisotope ²⁷
0.215 [#]	3	HT-PRD	100	ELISA (this study)
Harmine				
0.08	1	Woodtide	100	Radioisotope ³⁸
0.033	1	DYRKtide ⁴¹	100	Radioisotope ³⁹
0.20 (K_i)	1	FAM-Woodtide	50	HPLC/Fluorescence ²²
0.075	2	Dynatide 3	50	Radioisotope ³⁵
0.107 [#]	3	HT-PRD	100	ELISA (this study)

*DYRK1A used for the assay:

1: GST-DYRK1A (residues 1-499)²⁶

2: GST-DYRK1A (residues 1-497)²⁷

3: HT-DYRK1A (residues 1-497) (this study)

[#]Reported IC_{50} was the average of three independent sets of duplicate assays (n = 6).

Figure 7. ATP competition assay. For each inhibitor (epigallocatechin gallate (EGCG) and harmine), assays were conducted with a single concentration of inhibitor in four different ATP concentrations (100, 200, 400, 800 μ M) and quantified as described in Methods. The inhibitory potency at three other ATP concentrations was calculated as relative to that at 100 μ M ATP and used to plot against ATP concentrations. Inhibitor concentrations used in the assays were 0.4 μ M for EGCG (●) and 0.2 μ M for harmine (■) ($n = 6$ for each data point).

provides sufficient sensitivity for analyzing inhibitor activity with recombinant DYRK1A. Furthermore, because of the standard ELISA protocol, our assay is straightforward to perform with the entire process carried out in a single well. The tools and equipment for adapting this plate-based assay for high throughput automation are widely available, and if necessary, the assay can be refined to further improve the sensitivity. We believe that our assay offers a simple, rapid, and reliable non-radioactive method suitable for replacing the radioactive trace assays in quantifying and screening DYRK1A inhibitors.

Data availability

Dataset 1: Raw data for [Figures 1–7](#) and [Supplementary Figures 1 and 2](#) are supplied in KaleidaGraph format. [10.5256/f1000research.10582.d148787](https://doi.org/10.5256/f1000research.10582.d148787)⁴²

Zipped file, containing the following:

Data for Figure 1. Coating ELISA plate with HT-PRD. Data (OD_{405}) for 0 – 800 ng of coated HT-PRD per well were shown. Background measurements for all [Figures \(1–7 and Supplementary Figure 1 and 2\)](#) were obtained using wells with PNPP only (no coating, no phosphorylation, and no antibodies), which were

performed in parallel with each experimental replicate/triplicate for background correction. The data shown in the files for all [Figures](#) have been corrected using averaged background from each set.

Data for Figure 2. Phosphorylation of coated HT-PRD by DYRK1A. Data (OD_{405}) for 0 – 800 ng coated HT-PRD per well were shown.

Data for Figure 3. DYRK1A concentration-dependent phosphorylation of coated HT-PRD. Data (OD_{405}) for phosphorylation with 0 – 80 ng HT-497 were shown.

Data for Figure 4. Time-course phosphorylation of coated HT-PRD by DYRK1A. Time-course phosphorylation data (OD_{405}) with 0–90 min incubation time were shown.

Data for Figure 5. 3D3 dilution factor determination. Data (OD_{405}) for 3D3 dilution 1:1,000 – 256,000 were shown.

Data for Figure 6. Epigallocatechin gallate (EGCG) inhibition profile. Data (OD_{405}) for EGCG 0 – 3.2 μ M were shown.

Data for Figure 7. ATP competition assay. Data (OD_{405}) for ATP 100 – 800 μ M were shown.

Data for Supplementary Figure 1. Secondary antibody dilution factor determination. Data (OD_{405}) for secondary antibody dilution 1:1,000 – 256,000 were shown.

Data for Supplementary Figure 2. Harmine inhibition profile. Data (OD_{405}) for harmine 0 – 3.2 μ M were shown.

Author contributions

Yong Liu carried out experiments to determine the optimal conditions for running the ELISA assay. He also performed ATP competition measurements.

Tatyana Adayev cloned and expressed the enzyme and the substrate. She demonstrated that mAb 3D3 could stain phosphorylated dynamin in ELISA, which is the prerequisite for the development of the current assay.

Yu-Wen Hwang conceived and designed the ELISA approach. He also carried out the assays for measuring IC_{50} . The manuscript is written by this author.

Competing interests

No competing interests were disclosed.

Grant information

This work is supported by the New York State Office for People with Developmental Disabilities, the parent agency of New York State Institute for Basic Research in Developmental Disabilities. No extramural fund was used to support this research.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

We thank Dr. Kevin Hwang for critical reading of the manuscript.

Supplementary materials

Supplementary Figure 1. Secondary antibody dilution factor determination. Phosphorylated HT-PRD in ELISA wells were prepared and probed with 3D3 (1:3000 dilution) followed by serially diluted secondary antibody (from 1000 to 256,000x) similarly as described in Figure 5. Like 3D3 titering, normalized OD_{405} was calculated for plotting ($n = 6$ for each data point).

[Click here to access the data.](#)

Supplementary Figure 2. Harmine inhibition profile. Harmine inhibition was conducted and analyzed exactly as described in Figure 6 for EGCG ($n = 6$ for each data point).

[Click here to access the data.](#)

References

- Becker W, Sippl W: **Activation, regulation, and inhibition of DYRK1A.** *FEBS J.* 2011; **278**(2): 246–256.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Song WJ, Sternberg LR, Kasten-Sportès C, *et al.*: **Isolation of human and murine homologues of the *Drosophila* minibrain gene: human homologue maps to 21q22.2 in the Down syndrome “critical region”.** *Genomics.* 1996; **38**(3): 331–339.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Rahmani Z, Blouin JL, Creau-Goldberg N, *et al.*: **Critical role of the D21S55 region on chromosome 21 in the pathogenesis of Down syndrome.** *Proc Natl Acad Sci U S A.* 1989; **86**(15): 5958–5962.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Wegiel J, Wisniewski HM, Dziwiatkowski J, *et al.*: **Differential susceptibility to neurofibrillary pathology among patients with Down syndrome.** *Dementia.* 1996; **7**(3): 135–141.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Smith DJ, Stevens ME, Sudanagunta SP, *et al.*: **Functional screening of 2 Mb of human chromosome 21q22.2 in transgenic mice implicates *minibrain* in learning defects associated with Down syndrome.** *Nat Genet.* 1997; **16**(1): 28–36.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Branchi I, Bichler Z, Minghetti L, *et al.*: **Transgenic mouse *in vivo* library of human Down syndrome critical region 1: association between *DYRK1A* overexpression, brain development abnormalities, and cell cycle protein alteration.** *J Neuropathol Exp Neurol.* 2004; **63**(5): 429–440.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Ahn KJ, Jeong HK, Choi HS, *et al.*: **DYRK1A BAC transgenic mice show altered synaptic plasticity with learning and memory defects.** *Neurobiol Dis.* 2006; **22**(3): 463–472.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Wegiel J, Kuchna I, Nowicki K, *et al.*: **Cell type- and brain structure-specific patterns of distribution of minibrain kinase in human brain.** *Brain Res.* 2004; **1010**(1–2): 69–80.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Tejedor FJ, Hämmerle B: **MNB/DYRK1A as a multiple regulator of neuronal development.** *FEBS J.* 2011; **278**(2): 223–235.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Dowjat WK, Adayev T, Kuchna I, *et al.*: **Trisomy-driven overexpression of DYRK1A kinase in the brain of subjects with Down syndrome.** *Neurosci Lett.* 2007; **413**(1): 77–81.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Wegiel J, Gong CX, Hwang YW: **The role of DYRK1A in neurodegenerative diseases.** *FEBS J.* 2011; **278**(2): 236–245.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Woods YL, Cohen P, Becker W, *et al.*: **The kinase DYRK phosphorylates protein-synthesis initiation factor eIF2Bepsilon at Ser539 and the microtubule-associated protein tau at Thr212: potential role for DYRK as a glycogen**

- synthase kinase 3-priming kinase. *Biochem J.* 2001; **355**(Pt 3): 609–615.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
13. Liu F, Liang Z, Wegiel J, *et al.*: Overexpression of Dyrk1A contributes to neurofibrillary degeneration in Down syndrome. *FASEB J.* 2008; **22**(9): 3224–3233.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 14. Ryoo SR, Cho HJ, Lee HW, *et al.*: Dual-specificity tyrosine(Y)-phosphorylation regulated kinase 1A-mediated phosphorylation of amyloid precursor protein: evidence for a functional link between Down syndrome and Alzheimer's disease. *J Neurochem.* 2008; **104**(5): 1333–44.
[PubMed Abstract](#) | [Publisher Full Text](#)
 15. Ryu YS, Park SY, Jung MS, *et al.*: Dyrk1A-mediated phosphorylation of Presenilin 1: a functional link between Down syndrome and Alzheimer's disease. *J Neurochem.* 2010; **115**(3): 574–584.
[PubMed Abstract](#) | [Publisher Full Text](#)
 16. Lee MS, Kao SC, Lemere CA, *et al.*: APP processing is regulated by cytoplasmic phosphorylation. *J Cell Biol.* 2003; **163**(1): 83–95.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 17. Kimura R, Kamino K, Yamamoto M, *et al.*: The DYRK1A gene, encoded in chromosome 21 Down syndrome critical region, bridges between beta-amyloid production and tau phosphorylation in Alzheimer disease. *Hum Mol Genet.* 2007; **16**(1): 15–23.
[PubMed Abstract](#) | [Publisher Full Text](#)
 18. Becker W, Soppa U, Tejedor FJ: DYRK1A: a potential drug target for multiple Down syndrome neuropathologies. *CNS Neurol Disord Drug Targets.* 2014; **13**(1): 26–33.
[PubMed Abstract](#) | [Publisher Full Text](#)
 19. Duchon A, Herault Y: DYRK1A, a Dosage-Sensitive Gene Involved in Neurodevelopmental Disorders, Is a Target for Drug Development in Down Syndrome. *Front Behav Neurosci.* 2016; **10**: 104.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 20. Totani S, Giordanetto F, Medda F: DYRK1A inhibition as potential treatment for Alzheimer's disease. *Future Med Chem.* 2016; **8**(6): 681–696.
[PubMed Abstract](#) | [Publisher Full Text](#)
 21. Lilienthal E, Kolanowski K, Becker W: Development of a sensitive non-radioactive protein kinase assay and its application for detecting DYRK activity in *Xenopus laevis* oocytes. *BMC Biochem.* 2010; **11**: 20.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 22. Bui LC, Tabouy L, Busi F, *et al.*: A high-performance liquid chromatography assay for Dyrk1a, a Down syndrome-associated kinase. *Anal Biochem.* 2014; **449**: 172–178.
[PubMed Abstract](#) | [Publisher Full Text](#)
 23. Chen-Hwang MC, Chen HR, Elzinga M, *et al.*: Dynamin is a minibrain kinase/dual specificity Yak1-related kinase 1A substrate. *J Biol Chem.* 2002; **277**(20): 17597–17604.
[PubMed Abstract](#) | [Publisher Full Text](#)
 24. Huang Y, Chen-Hwang MC, Dolios G, *et al.*: Mnb/Dyrk1A phosphorylation regulates the interaction of dynamin 1 with SH3 domain-containing proteins. *Biochemistry.* 2004; **43**(31): 10173–10185.
[PubMed Abstract](#) | [Publisher Full Text](#)
 25. Warnock DE, Terlecky LJ, Schmid SL: Dynamin GTPase is stimulated by crosslinking through the C-terminal proline-rich domain. *EMBO J.* 1995; **14**(7): 1322–1328.
[PubMed Abstract](#) | [Free Full Text](#)
 26. Himpel S, Panzer P, Eirnbter K, *et al.*: Identification of the autophosphorylation sites and characterization of their effects in the protein kinase DYRK1A. *Biochem J.* 2001; **359**(Pt 3): 497–505.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 27. Adayev T, Chen-Hwang MC, Murakami N, *et al.*: Kinetic properties of a MNB/DYRK1A mutant suitable for the elucidation of biochemical pathways. *Biochemistry.* 2006; **45**(39): 12011–12019.
[PubMed Abstract](#) | [Publisher Full Text](#)
 28. Cunningham PR, Weitzmann CJ, Nurse K, *et al.*: Site-specific mutation of the conserved m⁶Am⁶A residues of *E. coli* 16S ribosomal RNA. Effects on ribosome function and activity of the KsgA methyltransferase. *Biochim Biophys Acta.* 1990; **1050**(1–3): 18–26.
[PubMed Abstract](#)
 29. Hwang YW, Zhong JM, Pouillet P, *et al.*: Inhibition of SDC25 C-domain-induced guanine-nucleotide exchange by guanine ring binding domain mutants of v-H-ras. *J Biol Chem.* 1993; **268**(33): 24692–24698.
[PubMed Abstract](#)
 30. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; **72**(1–2): 248–254.
[PubMed Abstract](#) | [Publisher Full Text](#)
 31. Yano T, Taura C, Shibata M, *et al.*: A monoclonal antibody to the phosphorylated form of glial fibrillary acidic protein: application to a non-radioactive method for measuring protein kinase activities. *Biochem Biophys Res Commun.* 1991; **175**(3): 1144–1151.
[PubMed Abstract](#) | [Publisher Full Text](#)
 32. Farley K, Mett H, McGlynn E, *et al.*: Development of solid-phase enzyme-linked immunosorbent assays for the determination of epidermal growth factor receptor and pp60^{c-src} tyrosine protein kinase activity. *Anal Biochem.* 1992; **203**(1): 151–157.
[PubMed Abstract](#) | [Publisher Full Text](#)
 33. Wang Y, Ma H: Protein kinase profiling assays: a technology review. *Drug Discov Today Technol.* 2015; **18**: 1–8.
[PubMed Abstract](#) | [Publisher Full Text](#)
 34. Adayev T, Chen-Hwang MC, Murakami N, *et al.*: Dual-specificity tyrosine phosphorylation-regulated kinase 1A does not require tyrosine phosphorylation for activity *in vitro*. *Biochemistry.* 2007; **46**(25): 7614–7624.
[PubMed Abstract](#) | [Publisher Full Text](#)
 35. Adayev T, Wegiel J, Hwang YW: Harmine is an ATP-competitive inhibitor for dual-specificity tyrosine phosphorylation-regulated kinase 1A (Dyrk1A). *Arch Biochem Biophys.* 2011; **507**(2): 212–218.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 36. Segel IH: *Enzyme Kinetics, Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems.* John Wiley & Sons, Inc. 1975.
[Reference Source](#)
 37. Bain J, McLauchlan H, Elliott M, *et al.*: The specificities of protein kinase inhibitors: an update. *Biochem J.* 2003; **371**(Pt 1): 199–204.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 38. Bain J, Plater L, Elliott M, *et al.*: The selectivity of protein kinase inhibitors: a further update. *Biochem J.* 2007; **408**(3): 297–315.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 39. Göckler N, Jofre G, Papadopoulos C, *et al.*: Harmine specifically inhibits protein kinase DYRK1A and interferes with neurite formation. *FEBS J.* 2009; **276**(21): 6324–6337.
[PubMed Abstract](#) | [Publisher Full Text](#)
 40. Woods YL, Rena G, Morrice N, *et al.*: The kinase DYRK1A phosphorylates the transcription factor FKHR at Ser329 *in vitro*, a novel *in vivo* phosphorylation site. *Biochem J.* 2001; **355**(Pt 3): 597–607.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 41. Himpel S, Tegge W, Frank R, *et al.*: Specificity determinants of substrate recognition by the protein kinase DYRK1A. *J Biol Chem.* 2000; **275**(4): 2431–2438.
[PubMed Abstract](#) | [Publisher Full Text](#)
 42. Liu Y, Adayev T, Hwang YW: Dataset 1 in: An ELISA DYRK1A non-radioactive assay suitable for the characterization of inhibitors. *F1000Research.* 2017.
[Data Source](#)

Open Peer Review

Current Peer Review Status:

Version 1

Reviewer Report 26 January 2017

<https://doi.org/10.5256/f1000research.11404.r19700>

© 2017 Knapp S. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Stefan Knapp

Nuffield Department of Medicine, Structural Genomics Consortium, University of Oxford, Oxford, UK

I approve the article by Liu et al.

DYRK1A has developed into an interesting pharmacological target. The assay described in this paper offers an interesting alternative to previously published assays.

For assay validations the authors made however a poor choice. Epigallocatechin gallate (EGCG) has been identified as a DYRK1 inhibitor but this compound is highly reactive and not a meaningful inhibitor for assay validation (even though there might be clinical benefits using this natural product due to the broad spectrum of published possible cellular activities). Recently the diverse problem using promiscuous, reactive and chemically unstable inhibitors (PAINS) has been highlighted in the literature. Certainly EGCG should not be used as a control compound in any assay.

However, the data is well presented (antibody source should be described) and a good addition to the kinase assay repertoire that can be used studying DYRK1A inhibitors.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 24 Mar 2017

Yu-Wen Hwang, New York State Institute for Basic Research in Developmental Disabilities, Staten Island, USA

We thank Dr. Knapp for the comments.

We agree with Dr. Knapp that EGCG is known to be unstable and is reactive toward many cellular targets. Clearly, EGCG is not a suitable reagent for validating biological assays. However, in a defined *in vitro* system consisting of only DYRK1A, substrate, ATP, and buffer, like our ELISA-based assay, EGCG is a well-behaved small molecule. Its property is predictable and can be reliably measured under such conditions as has been done by us and others. In addition, EGCG inhibits DYRK1A through a non-ATP-competitive mechanism. Pairing EGCG with ATP-competitive inhibitors such as harmine provides different prospects for assessing the ELISA assay.

The antibody used in the study, 3D3, has been deposited with the Developmental Studies Hybridoma Bank of the University of Iowa.

Competing Interests: No competing interests.

Reviewer Report 18 January 2017

<https://doi.org/10.5256/f1000research.11404.r19325>

© 2017 Becker W. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Walter Becker

Institute of Pharmacology and Toxicology, Aachen University, Aachen, Germany

Liu and coworkers have developed an ELISA-based assay for the protein kinase DYRK1A and show that their assay compares well with the traditional radioactive assays in the analysis of DYRK1A inhibitors. As correctly pointed out the authors, a previous ELISA-based DYRK1A assay developed in my own lab had been optimized for sensitive detection of endogenous DYRK kinases, while the new ELISA assay is more straightforward and provides a simple and rapid method for analyzing inhibitor activity with recombinant DYRK1A. The inhibitions curves with the DYRK1A inhibitors EGCG and harmine support the conclusion “that the ELISA platform demonstrated here is a viable alternative to the traditional radioactive tracer assays for analyzing DYRK1A inhibitors”.

This methods article clearly represents a substantial modification of an existing procedure. The study is well designed, the methods and the analysis of the results are appropriately described and the conclusions are justified on the basis of the results. In summary, this is sound and useful study that will be valuable for other researchers in the field.

Recommendations

- In the 2nd paragraph the introduction, presenilin is misspelled.
- I appreciate the identification of the used antibodies in the method section by their accession numbers in the antibody registry. Direct links to this registry would be helpful for

the reader.

- For the convenience of the reader, I suggest to include the harmine inhibition curve (supplemental Fig. 2) in the main text as a second panel in Fig. 6.
- The final statement of the manuscript suggests the use their assay for the screening of DYRK1A inhibitors. It may be worth to validate this application by determining the Z'-factor of the assay (according to Zhang et al. 1999¹).
- The availability of plasmids and antibodies should be indicated. Are the plasmids available from the authors or from Addgene? Will the authors make the hybridoma clone for the pSer857 3D3 antibody available or can the antibody be commercially purchased?
- It must be stated in the figure legends whether the error bars show SD or SEM. I suggest to show SD, which provides the reader a measure for the experimental error.
- The raw data for the figures are provided in the KaleidaGraph format and were not accessible to me. Is it possible to submit as Excel or PDF?
- The authors may consider to include the term "kinase" in the title to enhance visibility of their article to readers not aware of DYRK1A.

References

1. Zhang JH, Chung TD, Oldenburg KR: A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen*. 1999; **4** (2): 67-73 [PubMed Abstract](#)

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 24 Mar 2017

Yu-Wen Hwang, New York State Institute for Basic Research in Developmental Disabilities, Staten Island, USA

We thank Dr. Becker for comments and suggestions. We have revised the article and addressed most of the questions. Here is the summary of changes.

- The typo is corrected.
- Direct link to antibody registry is added.
- Supplementary Figure 2 for harmine inhibition is now incorporated in the main text as Figure 6B.
- The Z'-factor for the ELISA assay has been estimated. The results are shown in the Supplementary Table.
- We have deposited the vectors (pHT-497 and pHT-PRD) and antibody 3D3 with

Addgene and the Developmental Studies Hybridoma Bank of the University of Iowa, respectively.

- The error bars are SEM. This is now indicated in the revised article.
- The raw data in Excel format have been submitted.
- The term “kinase” is added to the title.

Competing Interests: No competing interests.

The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com

F1000Research