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Harmin is an ATP-competitive Inhibitor for Dual-Specificity Tyrosine Phosphorylation-Regulated Kinase 1A (Dyrk1A)

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Abstract

Harmin is a β -carboline alkaloid. The compound is a potent inhibitor of dual-specificity tyrosine phosphorylation-regulated kinase 1A (Dyrk1A), a kinase implicated in Down syndrome. In this study, we show that harmin functions as an ATP-competitive inhibitor against Dyrk1A. Our conclusion is supported by kinetic analysis of harmin inhibition as well as by the characterization of a Dyrk1A mutation conferring significant resistance to harmin. The mutation, V306A, is located next to the highly conserved D307 residue in kinases known to coordinate the phosphate groups of ATP through a Mg^{+2} ion. The V306A mutation offers harmin resistance by differentially altering Dyrk1A affinity for harmin and ATP. The V306A mutation causes no apparent alteration to Dyrk1A activity except for the reduction in ATP affinity. This deficiency could be fully compensated by supplying ATP with a concentration in the physiological range. Our results reveal that harmin inhibits Dyrk1A activity by interacting with residues in the ATP-binding pocket and displacing ATP. Our results also suggest that harmin will be a good lead compound for further designing of selective ATP-competitive Dyrk1A inhibitors through exploration of the ATP-binding pocket of Dyrk1A.

Keywords

kinase inhibitors; ATP binding pocket; beta-carbolines; resistant mutants

Introduction

The human dual-specificity tyrosine phosphorylation-regulated kinase 1A (Dyrk1A) gene is mapped to a region of human chromosome 21 generally considered to be crucial for manifesting many phenotypes associated with Down syndrome (DS) [1]. The temporal and spatial distributions of Dyrk1A indicate that the gene is not only required for the early development of the central nervous system [2], but is also involved in maintaining the functions of the adult brain [3,4]. The protein level of Dyrk1A is elevated in proportion to

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the gene-dosage in several regions of DS brains [5]. Several Dyrk1A transgenic mouse lines have been established to mimicking the Dyrk1A gene dosage imbalance in DS. All of these lines express various degrees of learning and cognitive deficits [6-8]. These findings suggest that the aberrant expression of Dyrk1A is a basis for the learning and developmental impairments of DS [1]. Evidence further suggests that the elevated Dyrk1A may also be a risk factor for various neurodegenerative diseases, such as Alzheimer and Parkinson diseases [9]. It appears that Dyrk1A offers a potential target for pharmacological intervention in these diseases.

Dyrk1A (and other members of the DYRK family), together with cyclin-dependent kinases, extracellular signal-regulated kinases (ERK), and casein kinase 2 (CK2), belongs to the CMGC group of protein kinase family [10,11]. Besides the eleven conserved subdomains shared by all protein kinases [12], Dyrk1A contains a few signature motifs unique to the DYRK family [13]. One of the features is the “YXY” motif (residues 319-321), which is considered to be equivalent to the activation loop motif “TXY” of ERK [10]. Phosphorylation of the “TXY” motif is required for full activity of ERK [14]. Recombinant Dyrk1A expressed in *Escherichia coli* and in mammalian cells is highly phosphorylated at Y321 of the “YXY” motif [15,16]. Y321 phosphorylation of Dyrk1A is produced via autophosphorylation [15,17,18] and it has been proposed to be an inceptive event for DYRK activation during synthesis [17]. However, phospho-Y321 could be fully dephosphorylated without affecting the activity of Dyrk1A *in vitro* and moreover, no phospho-tyrosine was regenerated upon extensive incubation with ATP [16]. These results indicate that the role of activation loop phosphorylation in regulating Dyrk1A activity, if any, is different from that in the activity of other kinases, such as ERK [14]. Because of the apparent constitutive activity of Dyrk1A, the only effective means for attenuating Dyrk1A activity is through direct inhibition of active kinase.

Several compounds have been shown to inhibit Dyrk1A activity. These chemicals include epigallocatechin-3-gallate (EGCG), harmine, roscovitine, purvalanol A, 6-arylquinazolin-4-amines, pyrazolidine-3,5-dione derivatives and others [19-25] [for a review, please see [10]]. In the surveys conducted with an array of 70+ kinases covering different branches [19,20], EGCG and harmine have emerged as the most potent, and perhaps also the most selective, Dyrk1A inhibitors. EGCG is the major flavonoid in tea [26] and it inhibits Dyrk1A with an IC₅₀ value of about 0.3 μ M [19,27]. Like many kinases, Dyrk1A follows a two-substrate reaction mechanism using both ATP (phosphate donor) and the phosphate acceptor as substrates [27]. EGCG functions as a non-competitive inhibitor against both substrates [27]. The mode of EGCG action is consistent with a view that EGCG binds to a distinct site from that of both substrates and inhibits kinase activity. The plant alkaloid harmine (Fig. 1) is a β -carboline derivative [28]. Harmine has been shown to be a more potent Dyrk1A inhibitor than EGCG, with a reported IC₅₀ value of about 0.1 μ M [20]. Harmine appears to inhibit Dyrk1A with a different kinetic mode from that of EGCG [29]. In this study, we characterized the mechanism of harmine inhibition of Dyrk1A by kinetic and mutational approaches.

Materials and Methods

Materials

Harmine, harmine, harmaline, and emodin were purchased from Sigma-Aldrich/Fluka. Tetrabromobenzimidazole (TBB, casein kinase II inhibitor 1) was obtained from EMD Chemicals. [γ -³²P]-ATP (sp act. 6000 Ci/mmol) was procured from PerkinElmer. Dynamin 1-derived Dyrk1A substrate dynatide 3 [30] was obtained from GenScript via custom synthesis. Anti-Dyrk1A mAb 8D9 was produced as previously described [31].

Mutant construction and protein preparation

The glutathione S-transferase (GST) fusion of C-terminal truncated (at residue 497) Dyrk1A (GST-497) and EGCG resistant mutants were described previously [27]. Mutant 303/306 was prepared by cloning the Sac I (codon 210-211) and Sty I (codon 346-347) fragment from mutant R53 into the wild-type (WT) counterpart. Conversely, mutant triple M was prepared by inserting the same Sac I-Sty I fragment from WT into mutant R53. The I303F mutation in mutant R53 accompanied a novel Bsm I site, which was used for following the cloning of mutants 303/306 and Triple M. Single-site mutants I303F and V306A were constructed on the template of GST-497 by oligonucleotide site-directed mutagenesis using the QuickChange II XL kit (Stratagene). All mutants were confirmed by DNA sequencing. The full-length Dyrk1A mammalian expression vectors were constructed in vector pCMV-script similarly as previously described [27]. GST fusion proteins were expressed in *E. coli* strain BL21 by inducing the bacterial culture (at $OD_{600} \sim 1$) with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) either at 37 °C for 3-4 hours (for sensitivity testing) or at 20 °C overnight (for protein production). Fusion proteins were purified by glutathione resin as previously described [32].

Harmine sensitivity testing

The assays were conducted similarly as described for screening EGCG mutants [27] except for replacing EGCG with 2 μ M harmine. Crude Dyrk1A lysate was prepared by lysing IPTG-induced *E. coli* culture in B-Per (Thermo Scientific). The lysate was then used to phosphorylate dynatide 3 in the presence of [γ - 32 P]-ATP with or without harmine, and quantified. Harmine sensitivity was presented as percent of Dyrk1A activity remaining as compared to the no inhibitor control. The data represent the average of at least two independent experiments.

Kinetic analysis and IC₅₀ determination

Kinetic measurements were performed in a 30 μ l reaction mixture consisting of 1X kinase buffer (25 mM HEPES, pH 7.4, 100 mM NaCl, and 5 mM MgCl₂), 10-100 μ M ATP, 2 μ Ci [γ - 32 P]-ATP, 12-96 μ M dynatide 3 [30], 0.2 μ g kinase, and harmine if needed. The amounts of harmine used were 0.1 to 0.4 μ M for WT Dyrk1A and 1 to 8 μ M for mutant V306A. Reactions were initiated by the addition of kinase and were allowed to proceed for 10 minutes at 30 °C. Phosphorylation of dynatide 3 was quantified by the P81 membrane assay similarly to previously described [27]. Apparent K_m and V_{max} (Table 2) were calculated from the double-reciprocal plot as shown in Fig. 2. K_{cat} was calculated as $V_{max}/[Dyrk1A]$. The mode of harmine inhibition was inferred from a series of inhibition plots (Fig. 2). The apparent K_I of harmine inhibition was calculated from the secondary plot either as K_m vs. [harmine] for the competitive mode of inhibition or as V_{max} vs. [harmine] for the non-competitive mode of inhibition [33]. The data reported were obtained from three independent trials.

IC₅₀ was determined in the presence of either 50 or 1000 μ M ATP similarly as described previously [27]. The data presented in the Tables were the average of at least three independent trials. [Dynatide 3]-dependent reactions were performed similarly as described above in a 30 μ l reaction mixture consisting of 1X kinase buffer, a series of dynatide 3 with concentrations ranging from 12-96 μ M, 2 μ Ci [γ - 32 P]-ATP, and unlabeled ATP at either 250, 500 or 1000 μ M. The data obtained from three independent trials were averaged and used to fit the Michaelis-Menten equation.

Gli 1-dependent transcription assay

The assay was performed using luciferase as the reporter similarly as described previously, except that harmine was used as the inhibitor instead of EGCG [27]. Briefly, NIH3T3 cells were co-transfected with pcGli1 (Gli1 expression vector), 3' 8xGli-BS-Luc (luciferase reporter vector) [34], pCMV-Dyrk1A (either WT or mutant), and pRL-TK (Renilla luciferase for internal transfection control) for 5 hours at 37 °C. Transfected cells were allowed to recover for 20 hours and then were treated with harmine at various concentrations for an additional 24 hours. Luciferase was quantified using a dual-luciferase reporter assay system (Promega) in a manual Turner Designs luminometer TD20/20 set for dual luciferase reading. The ratio of the two luciferase recordings was used for data analysis. The level of Dyrk1A expression was determined by probing 10 µg of cell lysate prepared for the luciferase assay with anti-Dyrk1A mAb 8D9 in Western blotting as previously described [31].

Results

Inhibition of Dyrk1A by harmine

Widely different efficacies have been reported for harmine inhibition of Dyrk1A [18,20,23,25,29]. In our assay using a truncated wild-type (WT) Dyrk1A (residue 1-497) fused to GST (GST-497) [27] and dynamin 1-derived dynatide 3 as the substrate [30], we obtained an IC₅₀ of 0.075 µM at 50 µM ATP (Table 1, WT). Our value resembles that of Bain, et. al. [20] and Gockler, et. al. [18]. In addition, we detected little difference in harmine sensitivity between the truncated and full-length Dyrk1A (Table 1, full-length). This result suggests that, like EGCG [27], harmine interacts with the first 497 residues of Dyrk1A. The catalysis of Dyrk1A goes through a two-substrate mechanism utilizing both ATP and dynatide 3 as substrates [27]. We then determined the kinetic mode of harmine inhibition. Harmine inhibition experiments with constant dynatide 3 and varying ATP produced a set of lines intersecting at the y-axis in the double-reciprocal plot (Fig. 2). The result confirms that harmine functions as a competitive inhibitor (apparent K_i 0.065 µM, Table 2) against ATP [33] as noted by others previously [29]. In contrast, experiments performed with constant ATP and varying dynatide 3 generated a set of lines intersecting at the x-axis (Fig. 2), suggesting that harmine inhibit dynatide 3 in a non-competitive manner (apparent K_i 0.106 µM, Table 2).

Identification of a harmine resistant Dyrk1A mutant

The competitive nature of harmine against ATP is consistent with the recent structural model showing that the compound interacts with the ATP binding pocket of Dyrk1A [25]. Although the mode of inhibition of EGCG is different from that of harmine, a few mutations located in or around the ATP binding pocket were previously uncovered in EGCG resistant mutants [27]. Therefore, we screened the collection of EGCG mutants for harmine sensitivity by the rapid assay using crude Dyrk1A preparations. We found that, compared to WT Dyrk1A, R53 was the only mutant that displayed significant resistance to harmine (Table 1).

Mutant R53 harbors five mutations. To determine which mutation(s) conferred harmine resistance, the mutations were segregated and tested. We first took the advantage of the two unique restriction sites to separate the five mutations into two groups (see Materials and Methods): one containing the I303F and V306A mutations (303/306) and the other containing the remaining three mutations (Triple M). The harmine sensitivity assay showed that mutant 303/306 displayed similar harmine resistance strength as R53 but not Triple M (Table 1). This result suggests that the I303F and the V306A mutations, either alone or in combination, contributes to harmine resistance in R53. We then constructed individual

I303F and V306A mutants by site-directed mutagenesis and analyzed them. The results confirmed that a single V306A mutation accounts for the full harmine resistance of mutant R53 (Table 1).

Activity of the V306A mutant *in vitro*

We subsequently characterized the kinetic properties of the V306A mutant *in vitro*. Analysis of the inhibition profile revealed that the V306A mutant did not alter the modes of harmine inhibition against ATP and dynatide 3 (see Fig. 2) except that it drastically reduced the inhibition strength (Table 2, apparent K_I 7.02 μ M against ATP and K_I 6.19 μ M against dynatide 3). Under the routine assay conditions employing a constant ATP concentration of 50 μ M, the mutant gave an impression that its activity was compromised. Accordingly, the analysis revealed that the V306A mutation significantly reduced the K_{cat} for dynatide 3 without affecting the K_m (Table 2, measured with 50 μ M ATP). Surprisingly, the same mutation was found to appreciably alter the K_m for ATP but not the K_{cat} (Table 2, measured with 24 μ M dynatide 3). These results suggest that the apparent impaired activity of the V306A mutant is due to insufficient ATP used to sustain the reaction. Indeed, when dynatide 3-dependent reactions were performed at several high concentrations of ATP (250, 500, and 1000 μ M), the reaction rate of the V306A mutant was elevated by the increased ATP and it reached the level of the WT when 1000 μ M ATP was used for the reaction (Fig. 3). The increase in ATP in this range had no apparent effect on the reactivity of WT. As an ATP-competitive inhibitor, an increase in ATP is expected to reduce the efficacy of harmine inhibition. Therefore, we re-determined IC_{50} for harmine at 1000 μ M ATP (Table 1). The IC_{50} values obtained under these conditions were about 2 and 25 μ M, for WT and the V306A mutant, respectively. This alters the difference in harmine sensitivity between WT and mutant V306A from around 70 -fold, at 50 μ M ATP, to approximately 12-fold, at 1000 μ M ATP.

Activity of the V306A mutant in NIH3T3 cells

The activity of the V306A mutant in NIH3T3 cells was then measured using Dyrk1A-driven Gli 1-dependent synthesis of luciferase as the reporter [35]. In this system, the level of reporter output has been shown to be directly proportional to Dyrk1A activity [27,35]. Mutants were reconstructed as full-length clones in the mammalian expression vector pCMV-Script and used for the assay as described [27]. Mutant V306A displayed a similar activity as the WT Dyrk1A in supporting Gli 1-dependent gene expression after normalizing for the level of protein expressed (Fig. 4A). Again, our results confirm that Dyrk1A harboring the V306A mutation is as active as WT Dyrk1A in a high ATP environment. We then compared the sensitivity of luciferase output to harmine in NIH3T3 cells expressing either WT or mutant V306A (Fig. 4B). Since harmine in concentrations of up to 20 μ M has no effect on the activity of luciferase when added to the reaction mixture directly (data not shown), luciferase can be reliably used for reporting Dyrk1A activity in the presence of harmine. Incubating transfected cells with harmine for 24 hours caused a concentration-dependent inhibition of luciferase output promoted by WT Dyrk1A (Fig. 4B). Luciferase output promoted by mutant V306A was significantly more resistant to harmine than output induced by WT Dyrk1A. This result demonstrates that exogenous Dyrk1A V306A mutant could render recipient cells partially resistant to harmine. Moreover, the concentration of harmine required for causing 50% luciferase output reduction roughly corresponds to the IC_{50} of harmine obtained with 1000 μ M ATP (Table 1 and Fig. 4B). It should also be pointed out that harmine up to 20 μ M produced no visible effects on 3T3 cell viability under our assay conditions.

Inhibition of the V306A mutant by harmine analogues and other compounds

To probe the structural requirements for the inhibitor, the efficacy of harmine analogues and other compounds in inhibiting the V306A mutant was subsequently analyzed. Two harmine analogues, harmine and harmaline (Fig. 1), which differ from harmine by a methoxy group and a double bond in the pyrido ring (Table 3), respectively, were selected for testing. Each structural modification reduces the potency of the compounds in inhibiting Dyrk1A (Table 3) and a similar trend was noted previously [19,20]. The V306A mutation also confers significant resistance to both harmine and harmaline; however, the mutation appears to differentially affect the potencies of these two compounds. Comparisons of IC_{50} measurements indicate that the V306A mutation causes similar levels of sensitivity reduction to harmine (76-fold) and harmaline (64-fold) but a much less reduction to harmine (27-fold) (Table 3).

Numerous ATP-competitive inhibitors developed for CK2 are also effective against Dyrk1A [21,36]. The corresponding residue to V306 in Dyrk1A in CK2, I174, has been shown to contribute to the binding of many competitive inhibitors as revealed by structural as well as by mutational analyses [21,36,37]. Therefore, we evaluated the effect of the V306A mutation on the inhibition of Dyrk1A by two ATP-competitive inhibitors, emodin and tetrabromobenzimidazole (TBB) (Fig. 1). Both compounds were found to inhibit Dyrk1A (Table 3) and CK2 (IC_{50} 0.89-5.9 μ M and 0.5-0.9 μ M for emodin and TBB, respectively) [21,37] with comparable strengths. Like the I174A mutation in CK2, the V306A mutation was also found to significantly reduce the inhibition potency of emodin and TBB (Table 3).

Discussion

We employed two lines of investigation in this study to examine the mechanism of harmine inhibition. First, kinetic analysis shows that the mode of harmine inhibition is competitive when measured against ATP (phosphate donor) (Fig. 2). Second, the V306A mutation, located in the ATP-binding pocket, could simultaneously but differentially reduce Dyrk1A's affinities to ATP and harmine (Tables 1 and 2). This findings coupled with the structural model of Dyrk1A/harmine [25] and offer convincing evidence to support the conclusion that harmine inhibits Dyrk1A by competing for access to the ATP-binding pocket of the kinase with ATP. On the other hand, harmine competes against the other substrate, dynatide 3 (phosphate acceptor), in a non-competitive mode (Fig. 2). This observation is consistent with the view that the phosphate donor and acceptor often occupy distinct domains in kinases [14,38]. This is clearly the case for Dyrk1A as revealed by the X-ray structure of the kinase (PDB: 2WO6) complexed with an indazole inhibitor (D15) and a peptide phosphate acceptor [39].

V306 is located just ahead of the conserved DFG motif (kinase subdomain VII, residues 307-309 in Dyrk1A) of the kinase [10,12]. The motif is situated at the beginning of the activation segment and the conserved Asp residue is known to coordinate the phosphate moiety of ATP through a Mg^{2+} ion [14]. A perturbation in this region would significantly disrupt ATP binding. Therefore, it is not surprising that even a conserved change (Val to Ala) at residue 306 causes a drastic reduction in ATP affinity (Table 2 and Fig. 3). However, the physical change of the substitution appears to be too small to create additional structural alterations in the kinase. This is evident from superimposing the structure of WT Dyrk1A (PDB: 2WO6 chain a) [39] with that of the V306A mutant, produced by mutagenesis followed by energy minimization with the program Swiss-PdbViewer [40]. No apparent change was observed. More importantly, the V306A mutant could function like the WT counterpart as long as sufficient ATP is provided (Figs. 2 and 3). Therefore, it is clear that the V306A mutation only locally affects the ATP-binding pocket, but not other functions of Dyrk1A.

The model of Dyrk1A/harmine (PDB: 3ANR) places the harmine molecule within the ATP-binding pocket in an orientation such that the pyrido nitrogen and the methoxy oxygen of harmine interact via hydrogen bonds to the ϵ -amino group of the highly conserved K188 and the backbone NH of L241 in the hinge region, respectively [25]. Apparently, the interactions involving the pyrido nitrogen and the methoxy oxygen of harmine are critical for maintaining high binding affinity to the ligand. Either the loss of the methoxy group, as in harmine, or an increase of torsional constraint in the pyrido ring, as in harmaline, causes a significant reduction in affinity (Table 3) [19,20]. Additional residues that may be involved in ligand binding include A186, E203, V222, F238, E239, L294, V306, and D307 [13,41]. All of these and corresponding residues in other kinases are well recognized in binding ATP [14,42]. V306 is located in a position that could bind harmine through hydrophobic interactions. This residue clearly contributes to the binding affinity of harmine as shortening the side chain by replacing valine with alanine reduces the affinity by two orders of magnitude (Table 2). The orientation of harmine in the published model also dictates that the V306 residue is more likely to interact with the pyrido moiety than with the indolyl ring methoxy group of the bound harmine molecule because of the distance [25]. The combination of mutant V306A and harmine analogues (Table 3) offers the opportunity for testing the model of Dyrk1A/harmine. A loss of methoxy group, as with harmine versus harmine, was found to significantly alter the ratio of inhibitor sensitivity between WT and the V306A mutant, but no such change occurred with a loss of torsional constraint in the pyrido ring, as with harmaline versus harmine (Table 3). Our data imply that the V306 residue is interacting with harmine through the area at or near the methoxy group rather than through the pyrido ring, if all three compounds bind Dyrk1A in a similar fashion. This would require harmine to be situated in the binding site in an orientation opposite (about 180° rotation on the plane) to that shown by others [10,25]. Docking simulations suggest that harmine can dock into Dyrk1A in opposite orientations with roughly equal stability.

Although the physical size of the ATP-binding pocket in CK2 is predicted to be smaller than that of Dyrk1A due to bulky side chains in the pocket (for example, I66, M163, and I174 for CK2 vs. A186, L294, and V306 for Dyrk1A), Dyrk1A and CK2 appear to share structural similarity in the ATP-binding site. The fact that either emodin or TBB can inhibit both CK2 and Dyrk1A with a comparable strength suggests that these two kinases use similar interactions in binding to emodin or TBB. The side chain of CK2 I174, the equivalent of Dyrk1A V306, is part of the hydrophobic surface in the ATP-binding pocket, which contributes to the majority of the binding strength of TBB and may be of emodin as well [43]. The shortening of the hydrophobic side chain in the I174A mutant causes a significant reduction in the potency of emodin (5.1 - 7.3-fold) and TBB (2.8-fold) in inhibiting CK2 [21,37] and a similar mutation at the corresponding position of Dyrk1A (V306A) also bring about appreciable reductions in the inhibition potency of emodin and TBB (Table 3). This observation provides further support for the conclusion that either TBB or emodin binds to Dyrk1A in a mode similar to that of CK2 [44].

In addition to being an effective Dyrk1A inhibitor, harmine is an even more potent inhibitor of monoamine oxidase [45], which limits *in vivo* applications of harmine as a Dyrk1A inhibitor. Molecular docking shows that harmine has many degrees of freedom in the ATP-binding pocket of Dyrk1A and could adapt different poses with similar docking free energies. This property could be further exploited to achieve selective inhibition of Dyrk1A among different targets. INDY, a benzothiazole compound recently found to inhibit Dyrk1A but not monoamine oxidase [25], appears to utilize this principle.

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Abbreviations

CK2	casein kinase 2
DS	Down syndrome
Dyrk1A	dual-specificity tyrosine phosphorylation-regulated kinase 1A
EGCG	epigallocatechin gallate
ERK	extracellular signal-regulated kinase
GST	glutathione S-transferase
IPTG	isopropyl β -D-1-thiogalactopyranoside
TBB	tetrabromobenzimidazole
WT	wild-type

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Fig. 1.
Harmine derivatives, emodin, and TBB.

Fig. 2. Inhibition of Dyrk1A by harmine

A. Competition of harmine against ATP. Harmine and ATP competition was performed with varied ATP (10-100 μM) and harmine (0 - 0.4 μM) at a constant dynatide 3 (24 μM) as described in the Materials and Methods. The initial rate (V) obtained from three independent trials was averaged and plotted as $1/V$ vs $1/[\text{ATP}]$. K_I was calculated from the secondary plot of apparent K_m vs. [harmine]. **B. Competition of harmine against dynatide 3.** The reaction was performed with varied dynatide 3 (12-96 μM) and harmine (0 - 0.4 μM) at a constant ATP (50 μM) similarly as described above. The initial rate (V) obtained from three independent trials was averaged and was plotted as $1/V$ vs $1/[\text{dynatide 3}]$. K_I was calculated from the secondary plot of apparent $V_{\max}$ vs. [harmine]. Harmine concentrations used in the reactions: (●) 0, (■) 0.1 μM , (◆) 0.2 μM , (▲) 0.4 μM .

Fig. 3. Activity of the V306A mutant with high concentrations of ATP *in vitro*

Dynatide 3 concentration-dependent reactions were performed with 6-96 μM peptide in the presence of a constant ATP as described in Materials and Methods. Data (reaction rate) from three independent assays were averaged and used for plotting against [dynatide 3] followed by curve fitting using the Michaelis-Menten equation ($v = V_{\max} * [\text{dynatide 3}] / (K_m + [\text{dynatide 3}])$). WT with 250 μM (□), 500 μM (not shown), and 1000 μM (○) ATP. The V306A mutant with 250 μM (■), 500 μM (◆), and 1000 μM (●) ATP.

Fig. 4. Activity and harmine sensitivity of the V306A mutant in NIH 3T3 cells

A. Kinase activity. Dyrk1A activity in 3T3 cells was measured by using Gli 1-dependent transcription of luciferase as the reporter as described in Materials and Methods. The reaction was driven either by empty pCMV (*vector*), wild-type (*WT*) or mutant V306A (*306*) Dyrk1A. After correcting for the internal control, the luciferase output was normalized to the reactions promoted by empty pCMV (set as 1) before plotting. The data represent the average ($\pm$ standard error) of four independent trials. The level of Dyrk1A expression (insert) in transfected cells was detected as described in Materials and Methods. A typical result was shown. **B. Harmine sensitivity.** WT and mutant V306A-driven luciferase synthesis was set up similarly as described above. After transfection, cells were incubated for 20 hours to allow Dyrk1A expression before treating with the indicated concentrations of harmine for 24-hour until luciferase determination. The luciferase activity at each harmine concentration was calculated as the percent of activity relative to each respective luciferase output for the no harmine control (set as 100%). The data represent the average ($\pm$ standard error) of four independent trials. WT, open column and mutant V306A, solid column.

Table 1**Harmine Inhibition of Dyrk1A Mutants**

Mutant	Mutation Sites ^a	Harmine Sensitivity Assay ^b	IC ₅₀ (μM) ^c	
		Activity Remaining (%)	50 μM ATP	1000 μM ATP
WT	Not applicable	9.6 ± 5.2	0.075 ± 0.022	2.03 ± 0.41
R20	D143E; L224M; P380T	8.3 ± 3.5	ND	ND
R21	K465R	20.1 ± 8.0	ND	ND
R34	W403R; Y415F; L457I	6.5 ± 3.6	ND	ND
R53	A32E; I101V; I303F; V306A; A364D	85.3 ± 12.7	5.02 ± 1.65	ND
Triple M	A32E; I101V; A364D	9.5 ± 4.4	ND	ND
303/306	I303F; V306A	80.8 ± 14.1	6.34 ± 1.15	ND
303	I303F	ND	0.091 ± 0.023	ND
306	V306A	ND	5.71 ± 0.98	25.63 ± 4.38
WT, full-length	Not applicable	ND	0.079 ± 0.016	ND

ND: not done.

^a Mutation sites of EGCG mutants were as described previously [27].

^b Harmine inhibition was conducted using IPTG-induced crude Dyrk1A lysate (all in the GST-497 format) in the presence of 2 μM harmine as described in Materials and Methods and reported as “Activity Remaining” (% of control) for each Dyrk1A.

^c IC₅₀ was determined using purified GST fusion truncated Dyrk1A (GST-497) unless indicated otherwise.

Table 2

Kinetic Parameters of the V306A Mutant

Kinase	Apparent K_m (μM)		Apparent K_{cat} (s^{-1})		Apparent K_i (μM)	
	ATP ^a	Dynatide 3 ^b	ATP ^a	Dynatide 3 ^b	ATP ^a	Dynatide 3 ^b
WT	59.12 $\pm$ 9.18	40.61 $\pm$ 5.40	0.365 $\pm$ 0.058	0.297 $\pm$ 0.046	0.065 $\pm$ 0.016	0.106 $\pm$ 0.019
V306A	649.3 $\pm$ 89.79	46.57 $\pm$ 8.22	0.348 $\pm$ 0.053	0.072 $\pm$ 0.012	7.02 $\pm$ 1.37	6.19 $\pm$ 1.82

^a measured with 24 μM dynatide 3.

^b measured with 50 μM ATP.

Table 3

Inhibition of WT and V306A mutant by harmine analogues and other compounds

Compound	IC ₅₀ (μM)		Sensitivity Change ^a (fold)
	WT	V306A	
Harmine (Table 1)	0.075 ± 0.022	5.71 ± 0.98	76.1
Harmaline	4.59 ± 0.46	293.2 ± 46.58	63.8
Harmane	1.82 ± 0.40	49.84 ± 2.34	27.4
Emodin	0.67 ± 0.08	6.64 ± 0.93	9.9
TBB	1.08 ± 0.15	22.33 ± 3.63	20.7

IC₅₀ was determined using purified GST-497 with 50 μM ATP.^aRatio of V306A IC₅₀/WT IC₅₀.