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Harmine is an ATP-competitive inhibitor for dual-specificity tyrosine phosphorylation-regulated kinase 1A (Dyrk1A)

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ABSTRACT

Harmine 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 harmine functions as an ATP-competitive inhibitor against Dyrk1A. Our conclusion is supported by kinetic analysis of harmine inhibition as well as by the characterization of a Dyrk1A mutation conferring significant resistance to harmine. 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 harmine resistance by differentially altering Dyrk1A affinity for harmine 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 harmine inhibits Dyrk1A activity by interacting with residues in the ATP-binding pocket and displacing ATP. Our results also suggest that harmine will be a good lead compound for further designing of selective ATP-competitive Dyrk1A inhibitors through exploration of the ATP-binding pocket of Dyrk1A.

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] [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 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 11 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

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[1] *Abbreviations used:* 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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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 IC_{50} value of about 0.3 μM [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 IC_{50} value of about 0.1 μM [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.

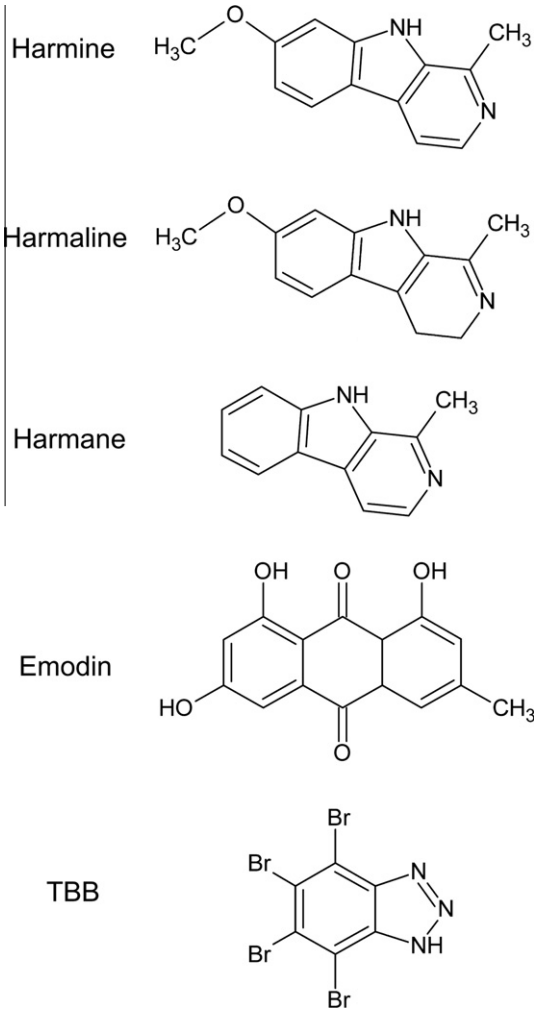

Fig. 1. Harmine derivatives, emodin, and TBB.

Materials and methods

Materials

Harmine, harmane, harmaline, and emodin were purchased from Sigma–Aldrich/Fluka. Tetrabromobenzimidazole (TBB, casein kinase II inhibitor 1) was obtained from EMD Chemicals. [γ-^{32}P]-ATP (sp act. 6000 Ci/mmole) 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 h (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 μM 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 IC_{50} determination

Kinetic measurements were performed in a 30 μl reaction mixture consisting of 1× kinase buffer (25 mM HEPES, pH 7.4, 100 mM NaCl, and 5 mM $MgCl_2$), 10–100 μM ATP, 2 μCi [γ-^{32}P]-ATP, 12–96 μM dynatide 3 [30], 0.2 μg kinase, and harmine if needed. The amounts of harmine used were 0.1–0.4 μM for WT Dyrk1A and 1–8 μM for mutant V306A. Reactions were initiated by the addition of kinase and were allowed to proceed for 10 min 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