Design and synthesis of a potent inhibitor of class 1 DYRK kinases as a suppressor of adipogenesis

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ABSTRACT

Dysregulation of dual-specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A) has been demonstrated in several pathological conditions, including Alzheimer's disease and cancer progression. It has been recently reported that a gain of function-mutation in the human *DYRK1B* gene exacerbates metabolic syndrome by enhancing obesity. In the previous study, we developed an inhibitor of DYRK family kinases (INDY) and demonstrated that INDY suppresses the pathological phenotypes induced by overexpression of DYRK1A or DYRK1B in cellular and animal models. In this study, we designed and synthesized a novel inhibitor of DYRK family kinases based on the crystal structure of the DYRK1A/INDY complex by replacing the phenol group of INDY with dibenzofuran to produce a derivative, named BINDY. This compound exhibited potent and selective inhibitory activity toward DYRK family kinases in an *in vitro* assay. Furthermore, treatment of 3T3-L1 pre-adipocytes with BINDY hampered adipogenesis by suppressing gene expression of the critical transcription factors PPAR γ and C/EBP α . This study indicates the possibility of BINDY as a potential drug for metabolic syndrome.

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1. Introduction

Protein kinases play crucial roles in multiple physiological processes, including gene expression, signal transduction, and energy metabolism.¹ Perturbation of these processes caused by dysregulation of kinases are tightly linked to many pathological conditions such as neurological disorders, tumorigenesis, diabetes, infectious diseases, and cardiovascular diseases.² Thus, protein kinases have been regarded as potent therapeutic targets.³ Many groups have concentrated efforts towards the development of effective kinase inhibitors:⁴ in fact, approximately one third of all protein targets under investigation in the pharmaceutical industry are protein or lipid kinases.² To date, 30 kinase inhibitors have been approved as clinical drugs by the FDA.²

The dual-specificity tyrosine-phosphorylation-regulated kinase (DYRK) family is a highly conserved group within the CMGC serine/threonine kinases. The DYRK family kinases—DYRK1A, DYRK1B, DYRK2, DYRK3, and DYRK4—are divided into two

classes:⁵ class 1 DYRK family kinases, DYRK1A and DYRK1B, have been regarded as potent drug targets.⁶ DYRK1A is essential for maintaining normal development of the brain, and reduced activity of DYRK1A causes neurological disorders such as microcephaly⁷ and autism spectrum disorders (ASD).⁸ On the other hand, induction of DYRK1A evokes different types of pathogenic symptoms such as hyperphosphorylation of tau protein in Alzheimer's disease,⁹ and cognitive deficits,¹⁰ megakaryoblastic leukemia,¹¹ and hypertrophic cardiomyopathy¹² in Down syndrome. To suppress these pathogenic phenotypes, we attempted to develop a specific inhibitor of DYRK1A, and found that INDY, a benzothiazole derivative, binds to the ATP-binding pocket of DYRK1A.¹³ Administration of INDY suppressed the expression of abnormal neural phenotypes characteristically induced by DYRK1A overexpression in a *Xenopus* developmental model.¹³ Recent study showed that inhibition of DYRK1A by INDY increases human pancreatic beta cell replication,¹⁴ suggesting that INDY might have a

therapeutic potency for diabetes. Enhancing the potency of DYRK1A inhibitors including INDY is important future challenges.

Studies suggest that DYRK1B expression correlates with tumor development,¹⁵ and that the protein is overexpressed in several malignant tissues, including colon cancer,¹⁶ lung cancer,¹⁷ and pancreatic ductal adenocarcinomas.¹⁸ Recently, a gain-of-function mutation of the *DYRK1B* gene was found in three families with juvenile-onset central obesity in southwest Iran,¹⁹ suggesting that DYRK1B enhances adipogenesis. It prompted us to entertain the simple concept that a potent inhibitor of DYRK1B could suppress the symptoms of metabolic syndrome.

In order to obtain a more potent inhibitor of DYRK family kinases, we designed and synthesized a structural derivative of INDY based on the crystal structure of the DYRK1A/INDY complex and examined its inhibitory activity on DYRK1B. The new compound showed a potent inhibitory effect on DYRK1A and DYRK1B, as expected, and hampered adipogenic differentiation of the pre-adipocytic cell line 3T3-L1 by suppressing expression of genes for peroxisome proliferator-activated receptor- γ (PPAR γ) and CCAAT-enhancer binding protein- α (C/EBP α).

2. Results and discussion

2.1. Structural modification of INDY based on the crystal structure of the DYRK1A/INDY complex.

To examine the possibility of structural modification of INDY, we focused on the crystal structure of the DYRK1A/INDY complex, which we determined in a previous study.¹³ In the ATP-binding pocket of DYRK1A, the phenolic hydroxyl group and the carbonyl oxygen of INDY form two hydrogen bonds, with Leu241 and Lys188, respectively (Figure 1A and 1B). Space that is not occupied by INDY can be observed in the transparent view of the ATP-binding pocket of the N-lobe (red dotted circle, Figure 1C). The free space was occupied by the other DYRK1A inhibitor pyrido[2,3-*d*]pyrimidine inhibitor,²⁰ and partly by leucettine L41²¹ (Figure S1), indicating that this space is available for structural modification of INDY. Furthermore, the phenolic hydroxyl group of INDY, a key functional group for binding, is susceptible to sulfate conjugation (Figure S2 and S3 and Table S1). Thus, the phenolic hydroxyl group should be modified with consideration to the free space around INDY. The most salient point of design is that any structural modification should not perturb formation of the two hydrogen bonds. Based on this structural information, we designed a structural derivative of INDY by replacing the phenol structure of INDY with dibenzofuran (Figure 1D). To investigate whether this dibenzofuran derivative fits the ATP-binding pocket of DYRK1A, we performed docking simulation analysis of the derivative structure in the pocket. The simulation showed that the derivative structure could fit the pocket through a binding mode identical to that of INDY, without any steric hindrance (Figure 1E).

2.2. Synthesis of BINDY.

The newly designed benzofuro-fused INDY derivative, which we named BINDY, was successfully synthesized from commercially available 5-methoxy-2-methylbenzothiazole (**1**) in six steps (Scheme 1). Bromination of **1** with *N*-bromosuccinimide gave a regioisomeric mixture of the desired 4-brominated product (**2**) and a 6-brominated by-product in an 88/12 ratio that was chromatographically separated. Suzuki–Miyaura cross-coupling of **2** with 2-chlorophenylboronic acid (**3**)

afforded the biaryl product **4** in high yield. Treatment of **4** with an excess amount of ethyl triflate furnished ammonium salt **5**, which was subsequently transformed into the acetylated product **6**. Demethylation of **6** followed by the formation of benzofuran structure via intramolecular Ullmann condensation²² provided the target compound, BINDY.

Figure 1. Chemical design of the structural derivative of INDY

(A) Crystal structure of the ATP-binding pocket of the DYRK1A/INDY complex (PDB ID: 3ANQ). DYRK1A is shown in ribbon mode. INDY, Lys188, Met240, and Leu241 are shown in stick mode.

(B) Chemical structure of INDY.

(C) Transparent view of the ATP-binding pocket from the N-lobe of the DYRK1A/INDY complex (PDB ID: 3ANQ). The red dotted circle indicates space that is not occupied by INDY.

(D) Chemical structure of BINDY.

(E) Simulation of BINDY docking onto the kinase domain of DYRK1A was performed. This model was built based on the coordinates of the crystal structure of the DYRK1A/INDY complex (PDB ID: 3ANQ).

Scheme 1. Synthesis of BINDY^a

^aReagents and conditions: (a) *N*-bromosuccinimide, CH₂Cl₂, 0 °C to rt, 40 h, 88%; (b) 2-chlorophenylboronic acid (**3**), Pd(PPh₃)₄, Na₂CO₃·H₂O, toluene/EtOH/H₂O (1/1/1), 90 °C, 20 h, 85%; (c) EtOTf, 50 °C, 17 h, 70%; (d) Ac₂O, Et₃N, MeCN, 80 °C, 2.5 h, 80%; (e) BBr₃, CH₂Cl₂, 0 °C to rt, 5 h, quant; (f) Copper(I) thiophene-2-carboxylate (CuTC), DMA, 130 °C, 90 h, 65%.

2.3. BINDY is a potent inhibitor of DYRK family kinases.

To verify the inhibitory potency of BINDY, we performed an *in vitro* kinase assay using recombinant DYRK1A protein and its peptide substrate. The kinase assay revealed that BINDY inhibited the kinase activity of DYRK1A at half-maximal inhibitory concentrations (IC_{50}) of 25.1 nM (Figure 2A). INDY inhibited DYRK1A at an IC_{50} of 112 nM (Figure 2A), indicating that BINDY is a stronger inhibitor than INDY. Furthermore, BINDY also inhibited DYRK1B and DYRK2 at IC_{50} values of 36.0 and 7.94 nM, respectively (Figure 2B and 2C). The IC_{50} values are summarized in Table 1. These results demonstrate that BINDY is a potent inhibitor of DYRK family kinases and that we were able to produce a structural design with the desired efficacy.

Figure 2. BINDY inhibits DYRK family kinases in an *in vitro* kinase assay

BINDY inhibited the *in vitro* catalytic activity of DYRK1A (A), DYRK1B (B), and DYRK2 (C). Recombinant DYRK1A, DYRK1B, and DYRK2 were incubated with the peptide substrate DYRKtide-F in the presence of the indicated concentrations of BINDY and INDY. Both BINDY and INDY inhibited the activity of DYRK1A, DYRK1B, and DYRK2 in a dose-dependent manner. Representative dose-response curves with Hill slopes are shown.

Table 1. IC_{50} values of BINDY and INDY against DYRK/CLK family kinases.

Kinase	IC_{50} (nM)	
	BINDY	INDY
DYRK1A	25.1	112
DYRK1B	36.0	82.5
DYRK2	7.94	35.2
CLK1	12.5	12.3
CLK2	124	350

2.4. BINDY is a selective inhibitor of DYRK/CLK family kinases.

Next, we examined the selectivity of BINDY within the human kinome. Potential inhibitory activity against a panel of 304 kinases was assessed with BINDY (1 μ M). Inhibitory activities against kinases are illustrated in the kinase dendrogram in Figure 3, and actual inhibition values are listed in Table S2. The size of each red sphere in Figure 3 represents the inhibitory effect on the corresponding kinase. Fifteen kinases (CHK2, CLK1, DYRK1A, DYRK1B, DYRK2, DYRK3, HIPK1, HIPK2, HIPK3, PIM1, PIM2, PIM3, PKD1, PKD2, PDK3) were inhibited by over 90%, and 7 kinases (FLT3, KIT (D816V), CDK2/CycB1, CDK2/CycA2, CLK2, GSK3 β , HIPK4) were by over 70% (Figure 3, and Table S2). Among the kinases inhibited by over 90%, eight kinases (CLK1, DYRK1A, DYRK1B, DYRK2, DYRK3, HIPK1, HIPK2, HIPK3) belong to the CMGC family, and the rest belong to the CAMK family (Figure 3). This result indicates that BINDY exerts selectivity towards kinases that belong to the CMGC and CAMK kinase groups.

Figure 3. BINDY selectively inhibits DYRK/CLK family kinases

Map of the inhibitory activities of BINDY on a kinase dendrogram. Percentage inhibitions by 1 μ M BINDY were measured for a panel of 304 kinases. Red circles indicate inhibited kinases, where the circle size indicates the percentage inhibition for that kinase.

2.5. BINDY is an effective suppressor of adipogenesis.

Next, we tested the pharmacologic effects of BINDY on adipogenic differentiation of the mesenchymal cell line 3T3-L1. Adipogenic differentiation was initiated by treatment of the cells with insulin, dexamethasone, and 3-isobutyl-1-methylxanthine. To evaluate the extent of adipogenic differentiation, lipid droplets that accumulated were stained with Oil-Red O. Treatment with BINDY significantly decreased the stained area in the culture wells of the cells in a dose-dependent manner (Figure 4A). In the high-magnification images of the stained cells, red-stained lipid droplets appeared in the vehicle-treated well; treatment with BINDY decreased the size and number of

lipid droplets (Figure 4B). In this experiment, no cytotoxicity was observed in cells treated with BINDY (Figure 4B). Thus, BINDY appears to be a potent suppressor of adipogenic differentiation.

Adipogenesis is strictly controlled by critical transcriptional factors such as PPAR γ and C/EBP α .²³ PPAR γ is a master transcriptional regulator of adipogenic differentiation and a canonical target of antidiabetic thiazolidinedione medications prescribed to improve insulin sensitivity.²⁴ C/EBP α orchestrates adipocyte-specific gene expression by binding to regions adjacent to the PPAR γ -binding site on a genome-wide scale.²⁵ Thus, it is reasonable to analyze the expression of PPAR γ and C/EBP α as indicators of adipogenic differentiation.

To investigate whether BINDY affects the expression of these critical factors, we examined protein expression levels of PPAR γ and C/EBP α . Western blot analysis using total cell lysates of differentiated 3T3-L1 cells showed that protein expression levels of PPAR γ and C/EBP α were drastically increased after the initiation of adipogenic differentiation (Figure 4C). Under this condition, treatment with BINDY suppressed the upregulation of these factors (Figure 4C). To determine whether the suppression of protein expression levels by BINDY is due to downregulation of the gene transcription, we prepared total RNAs from the differentiated cells and performed reverse transcription followed by quantitative real-time PCR analysis. Treatment with BINDY suppressed upregulation of *Pparg*, the murine gene coding for PPAR γ , and of *Cebpa*, the murine gene coding for C/EBP α , during adipogenic differentiation (Figure 4D). Thus, BINDY inhibits adipogenic differentiation by suppressing the gene expression of these critical transcriptional factors.

Figure 4. BINDY inhibits adipose differentiation of 3T3-L1 cells

(A) Pre-adipocytic cell line 3T3-L1 was differentiated for 8 days in the presence or absence of the indicated concentrations of BINDY. Adipocyte differentiation was assessed by using Oil-Red O staining for lipid droplets.

(B) High-magnification images of cells in panel (A).

(C) Adipocyte differentiation was assessed by Western blot analysis using antibodies against C/EBP α and PPAR γ . An antibody against β -actin was used as an internal control. 3T3-L1 cells were differentiated for the indicated period in the presence or absence of BINDY (10 μ M), and then collected as total cell lysates.

(D) Adipocyte differentiation was assessed by gene expression using reverse-transcription followed by real-time PCR analysis. Expressions of the C/EBP α gene (*Cebpa*) and PPAR γ gene (*Pparg*) were normalized to that of the cyclophilin A gene (*Cypa*). 3T3-L1 cells were differentiated for the indicated period in the presence or absence of BINDY (10 μ M), and then collected as total RNA. Bar graphs show means $\pm$ SD ($n = 3$).

The class I DYRK family kinases, DYRK1A and DYRK1B, are involved in the regulation of gene transcription. Recently,

DYRK1A has been demonstrated to be preferentially recruited to the proximal promoter region and to phosphorylate the C-terminal domain of RNA polymerase II,²⁶ which is required for transcriptional elongation.²⁷ Vona et al. showed that the TCTCGCGAGA motif is most likely to be the genome sequence responsible for the recruitment of DYRK1A to target promoters.²⁶ DYRK1A interacts with the transcriptional repressor KAISO,²⁸ and KAISO binds to TCTCGCGAGA-containing preadipocyte-specific promoters.²⁹ In contrast to the role of DYRK1A in gene expression, that of DYRK1B has not well documented. However, it has been recently reported that knockdown of *DYRK1B* by shRNAs suppresses the expression of PPAR γ , and that transfection of DYRK1B with the gain-of-function mutation promotes PPAR γ expression and subsequent adipogenic differentiation.¹⁹ Consistent with these observations, BINDY suppressed PPAR γ expression and adipogenic differentiation of 3T3-L1 cells in the present study. These findings indicate that DYRK1B mediates the transcriptional activation of *Pparg* and *Cebpa* in adipogenic differentiation. In contrast to DYRK1B, *Dyrk1A*-overexpressing transgenic mice exhibit a lean phenotype and are resistant to diet-induced obesity.³⁰ Administration of harmine, an inhibitor of DYRK1A, induces *Pparg* expression and promotes adipogenic differentiation.³¹ These results suggest that DYRK1A and DYRK1B may play differential roles on adipogenic differentiation, even though the detailed regulatory mechanisms remain elusive.

DYRK family kinases play important roles on not only adipogenesis, but on cancer progression as well. DYRK1A promotes cell survival through phosphorylation and activation of SIRT1.³² Malinge et al. reported that increased expression of DYRK1A promoted acute megakaryoblastic leukemia (AMKL) in Down syndrome and that harmine suppressed proliferation of AMKL cell lines.¹¹ Many reports have shown that DYRK1B affects cell cycle progression and survival of cancer cells^{18, 33} and that inhibition of DYRK1B has anti-cancer effects.³⁴ Therefore, BINDY may have additional therapeutic potential against cancers.

In this study, we developed a novel synthetic scheme (shown in Scheme 1) to obtain BINDY. This scheme enabled us to synthesize other derivatives of INDY with different functional groups. Introduction of dibenzofuran to the benzothiazole scaffold of INDY increased its inhibitory activity against DYRK1A, DYRK1B, DYRK2, and CLK2. Further rational design around BINDY may thus lead to improvements in the kinase selectivity and bioavailability of INDY derivatives.

3. Conclusion

We successfully developed a potent inhibitor of class 1 DYRK family kinases by structure-based rational design based on INDY. The newly synthesized compound, BINDY, potently inhibited DYRK1B at an IC₅₀ value of 36.0 nM and hampered adipogenic differentiation of the pre-adipocytic cell line 3T3-L1 by suppressing expression of PPAR γ and C/EBP α genes. BINDY may thus have therapeutic potential for the treatment of metabolic syndrome.

4. Experimental

4.1. Docking Simulation.

Simulations of BINDY docking onto DYRK1A were performed by superimposing the common atoms of BINDY on those of INDY in Discovery Studio 3.5 (Accelrys, CA, USA). We selected the crystal structure of the human DYRK1A/INDY complex (PDB ID: 3ANQ) as the starting model for the simulations.

4.2. Chemical Synthesis.

4.2.1. General remarks

Melting points (mp) were measured on an OptiMelt MPA100 automated melting point apparatus (Stanford Research Systems) and are reported uncorrected. IR spectra were obtained by diffuse-reflectance method or single-reflection ATR method on a Shimadzu IRPrestige-21 spectrometer with an attached DRS-8000A or MIRacle™A (ZnSe) single-reflection ATR accessory. Absorption bands of spectra are given in cm^{-1} . ^1H and ^{13}C NMR spectra were obtained with a Bruker AVANCE 500 spectrometer at 500 and 126 MHz. CDCl_3 containing 0.03% tetramethylsilane (TMS; Acros Organics, Cat. No. 36865-1A) and $\text{DMSO}-d_6$ (CIL, cat. no. DLM-10) were used as solvents for obtaining NMR spectra. Chemical shifts (δ) are given in parts per million (ppm) downfield from the TMS peak (δ 0.00 for ^1H NMR in CDCl_3) or the solvent peak (δ 77.0 for ^{13}C NMR in CDCl_3 , and δ 2.49 for ^1H NMR and δ 39.5 for ^{13}C NMR in $\text{DMSO}-d_6$) as an internal reference, along with the coupling constants (J) in hertz (Hz). The abbreviations s, d, t, q, m, and br signify singlet, doublet, triplet, quartet, multiplet, and broad, respectively. High-resolution mass spectra (HRMS) were obtained on a QFT-7 (Varian) Fourier transform ion cyclotron resonance mass spectrometer under positive electrospray ionization (ESI^+) conditions or a JEOL JMS-700V magnetic sector mass spectrometer under electron impact (EI^+) conditions at Molecular Characterization, Collaboration Promotion Unit, RIKEN.

4.3. Experimental procedures and analytical data for compounds

4-Bromo-5-methoxy-2-methylbenzo[d]thiazole (2)

Under an argon atmosphere, *N*-bromosuccinimide (NBS) (6.05 g, 34.0 mmol) was added in portions to a solution of 5-methoxy-2-methylbenzo[d]thiazole (1) (commercial, 5.54 g, 30.9 mmol) in dichloromethane (60 mL, dehydrated) at 0 °C. After the suspension was gradually warmed to room temperature over 40 h, an aqueous solution of $\text{Na}_2\text{S}_2\text{O}_3$ was added to the mixture, and then organic compounds were extracted with dichloromethane. The combined organic extracts were dried over Na_2SO_4 and filtered, and then the filtrate was concentrated under reduced pressure. The residue was purified by a YAMAZEN Smart Flash EPCLC W-Prep 2XY system (*n*-hexane/EtOAc ratio of 93/7 to 72/28) to yield 4-bromo-5-methoxy-2-methylbenzo[d]thiazole (2) (7.02 g, 27.2 mmol, 88.0%) as a colorless solid. A byproduct, presumably regioisomeric 6-brominated compound 2' as judged based on ^1H NMR analysis, was also produced (ca. 12%). TLC desired product 2: R_f = 0.35 (*n*-hexane/EtOAc ratio = 5/1); byproduct 2': R_f = 0.20 (*n*-hexane/EtOAc = 5/1); mp 117–118 °C; IR (cm^{-1}) 2986, 1526, 1460, 1435, 1400, 1277, 1219, 1175, 1155, 1101, 1070, 1003, 787, 748, 741, 650; ^1H NMR (500 MHz, CDCl_3) δ 7.68 (d, J = 9.0 Hz, 1H, aromatic), 7.02 (d, J = 9.0 Hz, 1H, aromatic), 3.98 (s, 3H, OCH_3), 2.87 (s, 3H, ArCH_3); ^{13}C NMR (126 MHz, CDCl_3) δ 169.7, 154.9, 153.1, 128.2, 120.3, 110.5, 104.7, 57.2, 20.5; HRMS (EI^+) m/z 256.9502 (256.9505 calcd for $\text{C}_9\text{H}_8\text{BrNOS}^+$, $[\text{M}]^+$).

4-(2-Chlorophenyl)-5-methoxy-2-methylbenzo[d]thiazole (4)

Under an argon atmosphere, a solution of 4-bromo-5-methoxy-2-methylbenzo[d]thiazole (2) (516 mg, 2.00 mmol), (2-chlorophenyl)boronic acid (3) (375 mg, 2.40 mmol), tetrakis(triphenylphosphine)palladium (116 mg, 0.100 mmol), and sodium carbonate monohydrate (424 mg, 3.42 mmol) in a 1/1/1 mixture of toluene/EtOH/ H_2O (7.0 mL each) was stirred

with heating at 90 °C (bath temperature) for 20 h. After cooling to room temperature, the mixture was passed through a florisil pad (Kanto Chemical Co., 75–150 μm) using ethyl acetate as an eluent, and the eluted solution was concentrated under reduced pressure. The residue was purified by a YAMAZEN Smart Flash EPCLC W-Prep 2XY system (*n*-hexane/EtOAc ratios of 90/10 to 70/30) to afford 4-(2-chlorophenyl)-5-methoxy-2-methylbenzo[d]thiazole (4) (490 mg, 1.69 mmol, 84.5%) as a colorless solid.

TLC R_f = 0.35 (*n*-hexane/EtOAc = 5/1); mp 91–92 °C; IR (KBr, cm^{-1}) 3404, 3057, 2937, 1459, 1395, 1275, 1216, 1100, 749, 642; ^1H NMR (500 MHz, CDCl_3) δ 7.79 (d, J = 8.5 Hz, 1H, aromatic), 7.53–7.51 (m, 1H, aromatic), 7.39–7.33 (m, 3H, aromatic), 7.11 (d, J = 8.5 Hz, 1H, aromatic), 3.83 (s, 3H, OCH_3), 2.74 (s, 3H, ArCH_3); ^{13}C NMR (126 MHz, CDCl_3) δ 168.2, 155.5, 153.3, 134.8, 134.4, 132.4, 129.4, 128.9, 128.2, 126.4, 121.8, 121.2, 110.0, 56.8, 20.5; HRMS (EI^+) m/z 289.0325 (289.0323 calcd for $\text{C}_{15}\text{H}_{12}\text{ClNOS}^+$, $[\text{M}]^+$).

4-(2-Chlorophenyl)-3-ethyl-5-methoxy-2-methylbenzo[d]thiazol-3-ium triflate (5)

Under an argon atmosphere, ethyl triflate (freshly opened, 2.0 mL) was added to 4-(2-chlorophenyl)-5-methoxy-2-methylbenzo[d]thiazole (4) (661 mg, 2.28 mmol) at room temperature, and the mixture was stirred with heating at 50 °C (bath temperature) for 17 h. After the reaction mixture was cooled to room temperature, the precipitate that formed was collected by filtration using a funnel. The collected solid was washed on the funnel with *n*-hexane and then dried under reduced pressure to afford 4-(2-chlorophenyl)-3-ethyl-5-methoxy-2-methylbenzo[d]thiazol-3-ium triflate (5) (744 mg, 1.59 mmol, 69.7%) as an orangish solid, which was used in the next step without further purification.

mp 164–165 °C; IR (KBr, cm^{-1}) 3404, 3057, 2937, 1459, 1395, 1275, 1216, 1100, 749, 642; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 8.52 (d, J = 9.0 Hz, 1H, aromatic), 7.74–7.67 (m, 2H, aromatic), 7.61–7.50 (m, 3H, aromatic), 4.15–4.06 (m, 1H, $\text{NCH}_{\text{gem-AA}}\text{CH}_3$), 3.98–3.90 (m, 1H, $\text{NCH}_{\text{gem-AA}}\text{CH}_3$), 3.81 (s, 3H, OCH_3), 3.12 (s, 3H, ArCH_3) 0.99 (dd, J = 7.0, 7.0 Hz, 3H, CH_2CH_3); ^{13}C NMR (126 MHz, $\text{DMSO}-d_6$) δ 179.0, 158.2, 138.6, 133.9, 132.7, 131.2, 130.7, 129.6, 127.5, 125.8, 121.9, 120.7 (q, J = 324 Hz), 116.1, 113.4, 57.2, 45.6, 17.3, 13.1; HRMS (ESI^+) m/z 318.0713 (318.0714 calcd for $\text{C}_{17}\text{H}_{17}\text{ClNOS}^+$, $[\text{M}]^+$).

(Z)-1-(4-(2-Chlorophenyl)-3-ethyl-5-methoxybenzo[d]thiazol-2(3H)-ylidene)propan-2-one (6)

Under an argon atmosphere, triethylamine (0.56 mL, 4.0 mmol) and acetic anhydride (0.28 mL, 3.0 μmol) were added to a solution of 4-(2-chlorophenyl)-3-ethyl-5-methoxy-2-methylbenzo[d]thiazol-3-ium triflate (5) (468 mg, 1.00 mmol) in acetonitrile (10 mL, dehydrated) at room temperature, and the mixture was stirred with heating at 80 °C (bath temperature) for 2.5 h. After the mixture cooled to room temperature, water (ca. 5 mL) was added and the resulting organic compounds were extracted with dichloromethane (3 mL $\times$ 4). The combined organic extracts were dried over Na_2SO_4 , filtered, and then the filtrate was concentrated under reduced pressure. The residue was purified by a YAMAZEN Smart Flash EPCLC W-Prep 2XY system (*n*-hexane/EtOAc = 58/42–37/63) to afford (Z)-1-(4-(2-chlorophenyl)-3-ethyl-5-methoxybenzo[d]thiazol-2(3H)-ylidene)propan-2-one (6) (288 mg, 0.800 mmol, 80.0%) as a pale yellow solid.

TLC R_f = 0.30 (*n*-hexane/EtOAc ratio = 1/1); mp 198–200 °C; IR (KBr, cm^{-1}) 2935, 2839, 1458, 1424, 1194, 1091, 1044, 969, 765; ^1H NMR (500 MHz, CDCl_3) δ 7.55–7.50 (m, 2H, aromatic), 7.42–7.31 (m, 3H, aromatic), 6.84 (d, J = 9.0 Hz, 1H, aromatic),

5.78 (s, 1H, olefinic), 3.72 (s, 3H, OCH₃), 3.65–3.46 (m, 2H, NCH₂CH₃), 2.21 (s, 3H, C(O)CH₃), 0.93 (dd, *J* = 7.0, 7.0 Hz, 3H, CH₂CH₃); ¹³C NMR (126 MHz, CDCl₃) δ 191.1, 161.6, 156.8, 137.5, 135.3, 134.0, 132.2, 129.6, 129.4, 126.6, 122.3, 120.3, 112.9, 106.3, 90.7, 56.7, 41.9, 29.1, 11.9; HRMS (EI⁺) *m/z* 359.0742 (359.0741 calcd for C₁₉H₁₈ClNO₂S⁺, [M]⁺).

(Z)-1-(4-(2-Chlorophenyl)-3-ethyl-5-hydroxybenzo[d]thiazol-2(3H)-ylidene)propan-2-one (7)

Under an argon atmosphere, boron tribromide (1.0 M solution in dichloromethane, 1.20 mL, 1.20 mmol) was added to a solution of (Z)-1-(4-(2-chlorophenyl)-3-ethyl-5-methoxybenzo[d]thiazol-2(3H)-ylidene)propan-2-one (**6**) (144 mg, 0.400 mmol) in dichloromethane (4.0 mL, dehydrated) at 0 °C. After warming to room temperature, the mixture was stirred for 5 h at the same temperature. Water (*ca.* 5 mL) was added to the mixture, and organic compounds were then extracted with dichloromethane containing a small amount of MeOH to make the organic layer homogeneous (*ca.* 3 mL × 4). The combined organic extracts were dried over Na₂SO₄, filtered, and then the filtrate was concentrated under reduced pressure. The residue was purified by silica-gel column chromatography (eluent: *n*-hexane/EtOAc/MeOH at 100/100/1 ratio) to afford (Z)-1-(4-(2-chlorophenyl)-3-ethyl-5-hydroxybenzo[d]thiazol-2(3H)-ylidene)propan-2-one (**7**) (138 mg, 0.399 mmol, 99.8%) as a yellow solid.

TLC *R*_f = 0.20 (*n*-hexane/EtOAc ratio = 1/1, broad spot); mp 215–218 °C (dec); IR (KBr, cm⁻¹) 3118, 1473, 1420, 1287, 991, 814, 765; ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.62 (s, 1H, ArOH), 7.59 (d, *J* = 8.0 Hz, 1H, aromatic), 7.54 (d, *J* = 8.5 Hz, 1H, aromatic), 7.48–7.40 (m, 3H, aromatic), 6.80 (d, *J* = 8.5 Hz, 1H, aromatic), 5.93 (s, 1H, olefinic), 3.63–3.55 (m, 1H, NCH_{AA'}-GemCH₃), 3.46–3.38 (m, 1H, NCH_{AA'}-GemCH₃), 2.05 (s, 3H, C(O)CH₃), 0.81 (dd, *J* = 7.0, 7.0 Hz, 3H, CH₂CH₃); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 189.9, 160.4, 155.2, 137.4, 134.7, 134.4, 133.2, 130.4, 129.5, 127.5, 122.9, 116.9, 111.5, 111.0, 90.7, 41.7, 29.2, 12.1; HRMS (EI⁺) *m/z* 345.0590 (345.0585 calcd for C₁₈H₁₆ClNO₂S⁺, [M]⁺).

(Z)-1-(1-Ethylbenzo[2,3]benzofuro[4,5-*d*]thiazol-2(1H)-ylidene)propan-2-one (BINDY)

Under an argon atmosphere, a solution of (Z)-1-(4-(2-chlorophenyl)-3-ethyl-5-hydroxybenzo[d]thiazol-2(3H)-ylidene)propan-2-one (**7**) (72.2 mg, 0.209 mmol) and copper(I) thiophene-2-carboxylate (CuTC) (62.4 mg, 0.327 mmol) in *N,N*-dimethylacetamide (DMA) (dehydrated, 2.5 mL) was stirred with heating at 130 °C (bath temperature) for 90 h. After the reaction mixture cooled to room temperature, 0.1 M aqueous solution of HCl (3 mL) and dichloromethane (1 mL) were added, and the separated organic layer was washed repeatedly with water (3 mL × 4) to remove DMA. The organic layer was dried over Na₂SO₄, filtered, and then the filtrate was concentrated under reduced pressure. The residue was purified by silica-gel column chromatography (eluent: *n*-hexane/EtOAc = 1/1) to afford (Z)-1-(1-ethylbenzo[2,3]benzofuro[4,5-*d*]thiazol-2(1H)-ylidene)propan-2-one (BINDY) (41.8 mg, 0.135 μmol, 64.6%) as a light-brown solid.

TLC *R*_f = 0.30 (*n*-hexane/EtOAc ratio = 1/1); mp 190–192 °C; IR (KBr, cm⁻¹) 3058, 2987, 2931, 1346, 1203, 1011, 741, 647, 542; ¹H NMR (500 MHz, CDCl₃) δ 8.07 (d, *J* = 8.5 Hz, 1H, aromatic), 7.67–7.64 (m, 2H, aromatic), 7.52 (dd, *J* = 8.0, 1.5 Hz, 1H, aromatic), 7.46–7.39 (m, 2H, aromatic), 6.05 (s, 1H, olefinic), 4.69 (q, *J* = 7.0 Hz, 2H, NCH₂CH₃), 2.30 (s, 3H, C(O)CH₃), 1.75 (t, *J* = 7.0 Hz, 3H, CH₂CH₃); ¹³C NMR (126 MHz, CDCl₃) δ 191.1, 160.7, 156.7, 156.1, 135.2, 127.2, 123.3, 122.6, 121.4,

121.3, 121.1, 112.4, 109.3, 107.0, 90.2, 43.7, 29.1, 14.4; HRMS (EI⁺) *m/z* 309.0824 (309.0818 calcd for C₁₈H₁₅NO₂S⁺, [M]⁺).

4.4. Biological Materials.

Penicillin–streptomycin mixed solution and 1 mol/L-M HEPES buffer solution were purchased from Nacalai Tesque (Kyoto, Japan). Fetal bovine serum, human insulin solution, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma-Aldrich (MO, USA). Antibodies against PPARγ (D69), C/EBPα (D56F10), and β-actin (13E5) were purchased from Cell Signaling Technology (MA, USA).

4.5. Mass Spectrometry Analysis of the Metabolite of INDY.

Mass spectra were acquired using a XEVO Q-TOF MS System (Waters Japan, Tokyo, Japan) coupled with an online Acquity UPLC[®] System (Waters, Japan) using an ODS Acquity UPLC BEH C18 column (2.1 × 50 mm, Waters Japan). The mobile phases consisted of water (phase A) and acetonitrile (phase B). The metabolite of INDY was separated with a gradient mobile phase at a flow rate of 0.2 mL/min. The following gradient was used: 0–1 min, 0% B; 1–16 min, 0–80% B. The metabolite of INDY was eluted at 5.6 min.

4.6. Cell Culture and Adipocyte Differentiation.

3T3-L1 cells were maintained in low-glucose Dulbecco's modified Eagle's medium (Nacalai Tesque) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10 mM HEPES (standard medium). Adipogenic differentiation was induced by treatment of 2-day-postconfluent 3T3-L1 cells with insulin (10 μg/mL), 1 μM dexamethasone, and 0.5 mM IBMX in standard medium (initiation step, day 0). At day 2, the medium was replaced by insulin (10 μg/mL) in standard medium (progression step). Starting at day 4, the medium was changed to standard medium every 2 days until the cells became mature adipocytes. Cells were maintained and allowed to differentiate at 37 °C in 5% CO₂.

4.7. Oil-Red Staining.

Differentiated 3T3-L1 cells on day 8 were fixed with 4% formaldehyde for 10 min. After immersion in 60% isopropanol for 1 min, the fixed cells were stained with Oil-Red O solution (1.8 mg in 60% isopropanol) for 20 min.

4.8. Quantitative Real-Time PCR.

Total RNAs were extracted using TRIzol reagent (Life Technologies, MD, USA). Isolated RNAs were purified using RNeasy Mini Kit with RNase-Free DNase set (QIAGEN, Hilden, Germany). Reverse transcription was performed with High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Life Technologies) according to the manufacturer's instructions. Real-time PCR was performed with FastStart Universal SYBR Green Master (Roche, Switzerland) using cyclophilin A as an internal control. Fluorescent detection and data analyses were done using Step One Plus Real-Time PCR system (Applied Biosystems). Primer sequences for real-time PCR were as follows: PPARγ, forward 5'-CCATTCTGGCCACCAAC-3', reverse 5'-AATGCGAGTGGTCTTCCATCA-3'; C/EBPα, forward 5'-GCGGGCAAAGCCAAGAA-3', reverse 5'-GCGTCCCCGCCGTACC-3'; cyclophilin A, forward 5'-

TTTGTACTTGCAGGCGCATTTT-3', reverse 5'-
GCACGCTCTCCTGAGCTACAG-3'.

4.9. In Vitro Kinase Assay.

Detailed information on the assay conditions is available on the website of Carna Biosciences (<http://www.carnabio.com/english/index.html>; Kobe, Japan). In brief, full-length human recombinant kinases were expressed as amino-terminal GST-fusion proteins by using a baculovirus expression system and purified by glutathione-sepharose chromatography. The GenBank accession numbers of DYRK1A, DYRK1B, and DYRK2 are NP_001387.2, NP_004705.1, and NP_003574.1, respectively. Kinase activities were evaluated by off-chip mobility shift assay after reaction of 1 μ M of the substrate DYRKtide-F with ATP (25 μ M for DYRK1A, 50 μ M for DYRK1B, and 10 μ M for DYRK2). For each compound, a DMSO solution was diluted in assay buffer to yield a final concentration of 1% DMSO. After incubation for 1 h at room temperature, substrate phosphorylation was analyzed by electrophoretic separation of the substrate and products using a Caliper LC3000 platform (Caliper Life Sciences, Mountain View, CA, USA). The phosphorylated product (P)/(P + substrate) ratio was calculated at each concentration of each small molecule, and the percent inhibition was expressed relative to that of a corresponding control assay in the absence of the small molecule. Staurosporine was used as reference inhibitor in each kinase assay. The IC₅₀ of each compound was calculated by interpolation on a log-concentration-response curve fitted with a four-parameter logistic equation.

The effects of 1 μ M BINDY against 304 kinases (listed in Table S2) were analyzed by using the QuickScout screening assist Mobility Shift Assay or with an immobilized metal ion affinity-based fluorescence polarization screening express kit with an ATP concentration at K_m value or 1 mM. All kinase assays were carried out at Carna Biosciences. The inhibitory map was constructed using Kinome Render.³⁵

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4.10. Statistical Analysis.

Statistical analysis of experimental data was performed by Mann-Whitney tests. Results are reported as means $\pm$ SD with p values (*p < 0.01). Data were fitted to a four-parameter logistic curve (variable slope) for Hill slope determination, from which IC₅₀ and EC₅₀ values were calculated, using Prism 6.0 (GraphPad Software, San Diego, CA, USA).

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Abbreviations used

CHK: CSK (C-terminal Src kinase)-homologous kinase
CLK: Cdc2-like kinase
HIPK: Homeodomain-interacting protein kinase
PIM: Proviral integration of moloney murine leukemia virus
PKD: Protein kinase D
FLT: Fms-like tyrosine kinase
CDK: Cyclin-dependent kinase
GSK3 β : Glycogen synthase kinase-3 β

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Design and synthesis of a potent inhibitor of class 1 DYRK kinases as a suppressor of adipogenesis

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