Supporting Information

Hydroxybenzothiophene ketones are efficient pre-mRNA splicing modulators due to dual inhibition of Dyrk1A and Clk1/4

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1. Biology

Recombinant Clk1 (catalog # PV3315) and Dyrk1B (catalog # PV4649) were purchased from lifetechnologies (Life Technologies GmbH, Darmstadt, Germany). RS domain derived peptide (RS tide) (catalog # 61723) was purchased from AnaSpec (Eurogentec Headquarters, Seraing, Belgium)

Protein expression and purification

Recombinant Dyrk1A and Dyrk2 were prepared according to the protocol previously described.[1]

Kinase Assay

Kinase reactions were performed as earlier described in a reaction buffer containing 50 mM Tris/HCl, pH 7.4, 0.1 mM EGTA, 0.5 mM DTT, 10 mM MgCl2, 100 µM ATP and 0.33 µM, 2 µCi [γ-^{32}ATP] as well as 100 µM Woodtide substrate peptide (KKISGRLSPIMTEQ-NH2) for Dyrk assays and 100 µM RS domain derived peptide (GRSRSRSRSR) for Clk1 assays respectively.[1] Activity of recombinant kinases was 0.1 mU per reaction well and reactions were carried out at 30 °C for 15 min. Results were analyzed using a Fuji FLA-3000 PhosphoImager plate reader. Signals were quantified using AIDA software (Raytest, Version 3.52). For IC_{50} determinations, 8 to 10 different concentrations of each compound were tested in triplicates. IC_{50} curves were plotted and analyzed using Origin Pro 8.6.0. All values shown represent the average of at least two independent determinations. The larger panel of kinases shown in Table S2 was screened by Life Technologies, Paisley, UK (SelectScreen® Kinase Profiling Services).

The Plasmid used to express Dyrk2[1] was a kind gift of Prof. Walter Becker, RWTH Aachen, Germany.

Quantitative real-time (q)PCR assay

To establish a qPCR-based assay for the quantification of splicing modulatory potencies, possible target sequences of differentially spliced transcripts were first validated using reverse transcriptase (RT)-PCR amplication and agarose gel detection. STO cells (derived from mouse embryonic fibroblasts) were chosen as a model since they had been successfully used previously to demonstrate the effects of TG003 on alternative pre-mRNA splicing.[2] For both RT-PCR and qPCR experiments, STO cells were seeded into 6-well plates and grown to confluency in DMEM containing 10 % FCS and antibiotics. The cells were treated with the test compounds or DMSO (0.05% DMSO as final conc. in all wells) in the same medium for 4 h at 37°C in the incubator, then the supernatant was removed, the cells were harvested by trypsinization, transferred to Eppendorf tubes and total RNA isolated without prior freezing using the RNeasy Mini Kit (Qiagen, Cat. No. 74104). 1 µg of total RNA was transcribed to cDNA using the QuantiTect Rev. Transcription Kit (Qiagen, Cat. No. 205311) in combination with random primers, and 20 ng of cDNA (assuming quantitative reverse transcription) were used per PCR. Among several primer pairs tested, the pair targeting the Clk/Sty cDNA as published by Pilch et al.[3] was giving the most consistent results: CLK-For, 5´-GCA TAG TAG CAA GTC CTC TG-3´; CLK-Rev, 5´-TAC TGC TAC ACG TCT ACC TC-3´. Using this primer pair, differential splicing as caused by the effectors was sensitively detected by an increase in the total amplified cDNA which mainly consisted of the 274 bp product including the Clk1/Sty exon2 (cf. Figure S1).[2,3]

The RT-PCR was performed using the “JumpStart REDTaq ReadyMix” reaction mix from Sigma (Cat. No. P0982) using 10µM of each primer. The following cycling conditions were used on an Eppendorf Mastercycler Gradient (Eppendorf AG, Germany): initial denaturation: 94 °C, 2 min; then 35 cycles of denaturation at 94 °C, 30s; annealing at 56 °C, 30s; elongation

at 72 °C, 50 sec. As a control for the amount of cDNA, additional RT-PCRs were performed in parallel under the same conditions, except that β-actin primers of the following sequences were used: β-actin forward: 5´-TGC GTG ACA TTA AGG AGA AG-3´; β-actin reverse: 5´-GTC AGG CAG CTC GTA GCT CT-3´; size of amplified product: 107 bp. Half of the PCR volumes (25µL) were resolved on 1.5% agarose gels and stained using ethidium bromide.

qPCR was performed in a StepOnePlus Real-Time PCR System (Life Technologies) using 20 ng cDNA per reaction with the SYBR green RT-PCR Kit (Peqlab, Cat. No. 07-KK4603-01) according to the manufacturer´s protocol, using the following cycling conditions: initial denaturation: 95 °C, 40 sec, followed by 45 cycles of denaturation at 95 °C, 2 sec, and annealing/extension at 60 °C, 40 sec. Primers were as above. The increase in the amount of specific cDNA corresponding to the alternative Clk/Sty transcripts relative to the samples derived from DMSO-treated cells was calculated after normalization to the β-actin values obtained with the same cDNA samples. To calculate EC_{50} values and the concentration required to induce a 5-fold increase (“$C_{5\text{-fold}}$”), different concentrations of TG003, **16**, **18**, **23**, and **25** were used for the cell treatments, ranging from 0.5 to 15 µM.

Detailed description of Figure 2:

The strength of the Clk1/Sty mRNA splicing modulation correlates with the potency to inhibit Dyrk1A and Clk1.

After treatment of STO cells with the compounds (concentrations as indicated in the Figure) or DMSO as a control (0.05%), total RNA was isolated and reverse transcribed to cDNA as described above. The RT-PCR amplification was performed as described above, and the products resolved by electrophoresis on 1.8 % agarose gels followed by ethidium bromide staining. Amplification of β-actin was used as a control for the cDNA amounts. To

facilitate the overview, the potencies of the compounds against Dyrk1A and Clk1 are indicated as well (data taken from Tables 2 and 3 in the main part). The more potent inhibitors were grouped to the left, including the reference compound TG003. Treatment with the dual Dyrk1A/Clk1 inhibitors led to a disappearance of all alternative splicing products, promoting the production of only the mature Clk1 mRNA containing exon 2, represented by the 274 bp PCR product (PCR primer positions are indicated by arrows in the scheme to the right). This mRNA splicing isoform encodes for the full-length, active kinase Clk1/Sty, whereas the smaller, alternatively spliced variant (183 bp) lacks exon 2 and is translated to a truncated, catalytically inactive form (Clk1/sty^{T}).[2,4] Some heavier PCR bands (ca. 420 and 510 bp) were also observed; larger products than the expected ones had been detected in previous studies as well, using either the same or other primer pairs encompassing the exon 2 sequence.[2,5] Assumingly, these products derive from incompletely spliced Clk1 transcripts stored in the nucleus,[2,5] but their precise nature remains elusive since the sizes do not fit any combination of the known full-length exon and intron sequences. Duncan et al. stated that the size of their additional larger product was consistent with that of a splicing intermediate derived from a premature transcript, but did not show the corresponding data.[5] It was reported that active Clk1 triggers an intron retention in its own pre-mRNA via phosphorylation of SR proteins, thus creating a pool of Clk1 pre-mRNA retaining specific introns which is stored in the nucleus of tissues and cultured cells.[6] Chemical inhibition of Clk1 by TG003 [2] as well as siRNA-mediated knockdown of mature Clk1/4 mRNA [6] had diminished the amount of the intron-retaining and of the short isoform of Clk1 mRNA by the modulation of splicing, thus producing the mature Clk1 mRNA, in accordance with the results presented here. It was further shown previously that the

modulation of the splicing pattern by TG003 was accompanied by a dephosphorylation of several SR proteins.[7]

As can be seen, **23** was clearly the most potent modulator of Clk1 mRNA splicing in the cells. Compounds which were less active against purified Dyrk1A did not achieve a complete removal of the alternative splicing forms even at 20 µM. Finally, compound **8**, which was in addition less potent against Clk1, even failed to induce the enhanced production of the mature Clk1 mRNA (274 bp PCR product). Results shown are representative of at least two independent experiments; M: 100 bp DNA-ladder from Thermo Scientific (Cat.No. SM1143); relevant bp sizes of the marker are indicated at the right border.

Detailed description of Figure 3:

Modulation of alternative SC35 pre-mRNA splicing by the Dyrk1A/Clk1 inhibitors.

STO cells were treated with compounds or DMSO as indicated on top of the figure, and the Clk1 mRNA analysed by RT-PCR followed by agarose gel electrophoresis as described above.

SC35 is a SR protein which auto-regulates the alternative splicing of its own pre-mRNA via binding to specific splicing enhancers and competing with small nuclear ribonucleoproteins.[8,9] Both its nuclear localization and its splicing activity are regulated by dynamic multi-site phosphorylation and dephosphorylation. Since it was described as a substrate of Dyrk1A and Clk1, analysis of its splicing pattern is suitable to monitor the inhibition of these kinases in intact cells. It was previously shown that treatment of STO cells with the reference compound TG003 modulated the alternative splicing of SC35 to promote the formation of a 1.7 kb splicing isoform.[2,10] This isoform is produced from the SC35 pre-mRNA by skipping the terminal retained intron (denoted “r.I.” in Figure 3) and inclusion of

exon 3.[8,9] The RT-PCR amplification of the characteristic region (see arrows in Figure 3) is predicted to yield a 274 bp product. As can be seen, the potent compounds **23** and **16** induced the strongest increase in the 274 bp product, while this effect was slightly reduced with the reference compound TG003 and weakest with **25**, correlating with their less potent inhibition of Dyrk1A. Due to the high abundance of the 668 bp product, the corresponding decrease of the respective larger splicing isoform cannot be detected. Results shown are representative of at least two independent experiments; M: 100 bp DNA-ladder from Thermo Scientific (Cat.No. SM1143); relevant bp sizes of the marker are indicated at the right border.

2. Chemistry

Chemical starting materials were purchased from Sigma-Aldrich, CombiBlocks or Alfa Aesar and used without further purification. Purity of the compounds was determined using an Agilent 1100 series HPLC system from Agilent Technologies, a GC Trace Ultra from Thermo or a Waters autopurification system from Waters Corporation. The purity of the compounds used in the biological assays was ≥ 95%. Mass spectra (ESI) were measured on an AB Sciex Qtrap2000 from AB Sciex or a Waters 3100 Mass detector from Waters Corporation. Mass spectra (EI) were measured on a DSQ II from Thermo. ^{1}H and ^{13}C NMR spectra were recorded on either a Bruker DRX-500 (^{1}H, 500 MHz; ^{13}C, 126 MHz) instrument at 300 K or on a Bruker Fourier300 (^{1}H, 300 MHz; 13C, ^{75} MHz) NMR spectrometer at 300 K in the deuterated solvents indicated. IR spectra were recorded on a Perkin Elmer Spectrum 100 FT-IR spectrometer, Perkin Elmer, Rodgau, Germany. Flash column chromatography was

performed using silica gel 60 (Merck, 35-70 µm). Reaction/flash monitoring was done by TLC on ALUGRAM SIL G/UV254 (Macherey-Nagel) employing UV detection.

The detailed synthesis of the following compounds is described in Miralinaghi *et al*.[11]

Compound Number	IUPAC name
1	(6-hydroxynaphthalen-2-yl)(3-hydroxyphenyl)methanone
2	(3-hydroxy-4-methylphenyl)(6-hydroxynaphthalen-2-yl)methanone
3	(4-fluoro-3-hydroxyphenyl)(6-hydroxynaphthalen-2-yl)methanone
4	(6-hydroxybenzo[b]thiophen-2-yl)(3-hydroxyphenyl)methanone
5	(6-hydroxybenzofuran-2-yl)(3-hydroxyphenyl)methanone
6	(6-hydroxy-1H-indol-2-yl)(3-hydroxyphenyl)methanone
7	(6-hydroxy-1-methyl-1H-indol-2-yl)(3-hydroxyphenyl)methanone
8	(6-hydroxy-3-methylbenzo[b]thiophen-2-yl)(3-hydroxyphenyl)methanone
9	(6-hydroxy-3-methylbenzofuran-2-yl)(3-hydroxyphenyl)methanone
10	(5-hydroxybenzo[b]thiophen-2-yl)(3-hydroxyphenyl)methanone
11	(5-hydroxybenzofuran-2-yl)(3-hydroxyphenyl)methanone
12	(5-hydroxy-1H-indol-2-yl)(3-hydroxyphenyl)methanone
13	(5-hydroxy-1-methyl-1H-indol-2-yl)(3-hydroxyphenyl)methanone
14	(5-hydroxybenzo[b]thiophen-2-yl)(4-hydroxyphenyl)methanone
15	(5-hydroxybenzofuran-2-yl)(4-hydroxyphenyl)methanone
16	(3-hydroxy-4-methylphenyl)(5-hydroxybenzo[b]thiophen-2-yl)methanone

17	(4-hydroxy-3-methylphenyl)(5-hydroxybenzo[b]thiophen-2-yl)methanone
18	(2-fluoro-5-hydroxyphenyl)(5-hydroxybenzo[b]thiophen-2-yl)methanone
19	(4-fluoro-3-hydroxyphenyl)(5-hydroxybenzo[b]thiophen-2-yl)methanone
20	3-(5-hydroxybenzo[b]thiophene-2-carbonyl)benzonitrile
21	(5-hydroxybenzo[b]thiophen-2-yl)(phenyl)methanone
22	benzo[b]thiophen-2-yl(3-hydroxyphenyl)methanone
24	(5-methoxybenzo[b]thiophen-2-yl)(3-methoxyphenyl)methanone
25	N,5-dimethoxy-N-methylbenzo[b]thiophene-2-carboxamide

Procedure A, general synthesis of methyl benzothiophene-2-carboxylates: A suspension of 2 eq. of potassium carbonate in 20 ml of dry DMF was cooled to 0°C and 1.2 eq. of methyl thioglycolate was carefully added. The solution was stirred for 20 min at 0°C under nitrogen and a solution of 1 eq. of the corresponding 2-fluorobenzaldehyde or 2-fluoro-acetophenone in 10 ml of DMF was added drop-wise. Then, the mixture was heated to 70°C and stirred for 4 hours. The suspension was cooled to room temperature and poured into 60 ml of 10 % citric acid. The aqueous layer was extracted with ethyl acetate (5x) and the combined organic layer was thoroughly washed with small amounts of water and brine (10x), and dried over magnesium sulphate. The solvent was removed in vacuo and the resulting yellow solid was recrystallized in ethanol to give the carboxylic ester in different yields.

Procedure B, general synthesis of benzo[b]thiophene-2-carboxylic acids: The carboxylic acids were released from the corresponding methyl benzo[b]thiophene-2-carboxylates by hydrolysis with potassium hydroxide. Therefore the methyl ester was dissolved in 5 ml of ethanol and 2.5 ml of water was added. Then 4 eq of potassium hydroxide was added and

the mixture was heated to reflux for 2 hours. The solvent was removed under reduced pressure and the resulting potassium salt was dissolved in a small volume of water, cooled to 0°C before conc. HCl was carefully added dropwise to adjust the pH to 1. Then the aqueous layer was extracted with diethyl ether (5x) and the combined organic extracts were dried over magnesium sulphate, filtered off, and concentrated in vacuo. The crude carboxylic acid was recrystallized from ethanol.

Procedure C, general synthesis of Weinreb amides: The corresponding carboxylic acid was dissolved in 5 ml of dichloromethane and cooled to 0°C. To this solution, 1.1 eq. of N,O-dimethylhydroxylamine hydrochloride, 0.1 eq. of dimethylaminopyridine, 1.2 eq. of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimid, and 6 eq. of triethylamine were added successively. After 60 min the solution was allowed to warm to room temperature and was stirred over night at RT. The reaction was quenched by addition of 10 % citric acid and the organic layer was separated. The aqueous layer was neutralized by addition of saturated $NaHCO_3$ and extracted with dichloromethane (4x). The combined organic layer was washed with water and brine, and dried over magnesium sulphate. The solvent was removed in vacuo to give the Weinreb amides as solid in sufficient purity.

Procedure F, Rap Stoermer reaction for the synthesis of intermediate compounds: 1.1 eq. of the corresponding 2-Bromoacetophenone and 2 eq. of Cs_2CO_3 were dissolved in 5 ml of acetonitrile and stirred under nitrogen. After 30 min 1 eq. of a methoxy substituted 2-hydroxybenzaldehyde was added and the mixture was stirred over night at room temperature. After completion of the reaction a precipitate was formed and dissolved in ethyl acetate. The solution was subsequently washed with water and brine. The organic layer was dried over magnesium sulphate, the solvent was evaporated in vacuo, and the

crude product was purified by flash column chromatography eluting with ethyl acetate/hexane 1:3 to give the intermediate compounds in varying yields.

Synthesis of II, methyl 5-methoxybenzo[b]thiophene-2-carboxylate: The title compound was synthesized from 2-fluoro-5-methoxybenzaldehyde using procedure A to yield 2 g (9 mmol, 25 %) of **II** as a light yellow solid. ^{1}H NMR (Acetone-d_6, 500MHz): δ = 8.04 (s, 1 H), 7.88 (d, *J*=9.1 Hz, 1 H), 7.51 (d, *J*=2.2 Hz, 1 H), 7.17 (dd, *J*=8.8, 2.5 Hz, 1 H), 3.91 (s, 3 H), 3.88 ppm (s, 3 H); ^{13}C NMR (Acetone-d_6, 126MHz): δ = 163.5, 159.1, 140.9, 135.4, 135.2, 131.1, 124.4, 119.1, 107.6, 55.9, 52.8 ppm; Purity(UV) > 98 %; t_R:min, ESI-MS $[M+H]^+$: 223.08; calcd. $[M]^+$: 222.04.

Synthesis of III, 5-methoxybenzo[b]thiophene-2-carboxylic acid: The title compound was synthesized from **II** using procedure B to give 0.83 g (3.7 mmol, 68 %) of **III** as a white solid which was directly used for the synthesis of **IV** without further characterization other than NMR. ^{1}H NMR (DMSO-d_6 ,300MHz): d = 13.40 (s, 1 H), 8.01 (s, 1 H), 7.91 (d, *J*=8.9 Hz, 1 H), 7.52 (d, *J*=2.4 Hz, 1 H), 7.15 (dd, *J*=8.8, 2.5 Hz, 1 H), 3.82 ppm (s, 3 H); ^{13}C NMR (DMSO-d_6 ,75MHz): d = 164.0, 157.9, 140.3, 136.1, 134.3, 130.4, 124.2, 118.2, 107.5, 55.8 ppm.

Synthesis of IV, N,5-dimethoxy-N-methylbenzo[b]thiophene-2-carboxamide: The title compound was synthesized from **III** using procedure C to give 3.3 g (1.3 mmol, 53 %) of **IV** as colourless needles after recrystallization from ethanol. ^{1}H NMR (Acetone ,500MHz): Shift = 8.13 (d, J=0.6 Hz, 1 H), 7.81 - 7.86 (m, 1 H), 7.49 (d, J=2.5 Hz, 1 H), 7.13 (dd, J=8.8, 2.5 Hz, 1 H), 3.88 (s, 3 H), 3.86 (s, 3 H), 3.36 ppm (s, 3 H); ^{13}C NMR (Acetone ,126MHz): Shift = 162.7, 158.8, 140.5, 136.2, 131.1, 123.9, 118.5, 107.3, 62.3, 55.8, 33.3 ppm; Purity(UV) > 99 %; t_R: 6.95 min, ESI-MS $[M+H]^+$: 252.06; calcd. $[M]^+$: 251.06.

Synthesis of intermediate compound VI, 2-bromo-1-(4-hydroxy-3-methylphenyl)ethan-1-one: The procedure for the synthesis of **VI** was taken from Bakke and co-workers.[12] 5 g (33 mmol) of commercial available 1-(4-hydroxy-3-methylphenyl)ethan-1-one in 100 ml of chloroform was added to a boiling solution of 2 eq. of copper (II) bromide in ethyl acetate. The mixture was refluxed over-night. The progress of the reaction was monitored by TLC. Then, the crude reaction mixture was stirred and warmed over charcoal, filtered off, and the solvent was removed under reduced pressure. The resulting solid was recrystallized from heptane/ethyl acetate 2:1 to yield **VI** as a light purple solid. ^{1}H NMR (DMSO-d_6, 300MHz) Shift = 7.77 - 7.74 (m, 1 H), 7.70 (dd, J = 2.2, 8.4 Hz, 1 H), 6.91 (d, J = 8.4 Hz, 1 H), 5.03 (s, 2 H), 2.16 (s, 3 H); IR (NaCl): (C-Br-stretching 672 cm^{-1}).

Synthesis of intermediate compound VII, 2-mercapto-5-nitrobenzaldehyde: The synthesis was carried out as described by Reinhard and co-workers in 2004.[13] A mixture of 1 g (5.91 mmol) of 2-fluoro-5-nitrobenzaldehyde and 1.1 eq. of sodium sulphide nonahydrate in 15 ml of DMSO were stirred under nitrogen at room temperature. After completion of the reaction the solution was poured into ice water acidified with 2N HCl and extracted with diethyl ethyl ether (3x). The combined organic layer was dried, filtered and concentrated in vacuo to give the crude **VII** as yellow solid which was directly used for the synthesis of **VIII** without further purification.

Synthesis of intermediate compound VIII, (4-hydroxy-3-methylphenyl)(5-nitrobenzo[b]thiophen-2-yl)methanone: **VII** was dissolved in acetonitrile at 0°C and 1 eq. of K_2CO_3 was added. The mixture was stirred for 30 minutes at 0°C and **VI** dissolved in acetonitrile was added drop-wise and the mixture was stirred at room temperature. After 3 hours a precipitate was formed, which was filtered off and washed subsequently with cold acetonitrile and a mixture of water and acetonitrile. The crude product was analyzed by ^{1}H

NMR and then directly used without further characterization for the next step. ^{1}H NMR (300MHz, DMSO-d_6) Shift = 8.99 (d, J = 1.9 Hz, 1 H), 8.40 -8.23 (m, 3 H), 7.80 - 7.64 (m, 2 H), 6.82 (d, J = 8.2 Hz, 1 H), 2.18 (s, 3 H).

Synthesis of compound 11a, (5-aminobenzo[b]thiophen-2-yl)(4-hydroxy-3-methylphenyl)methanone: The procedure for the synthesis of **11a** was earlier described by Dey and co-workers in 2012.[14] 3 eq. of $FeSO_4$ x $7H_2O$ and 0.24 eq. of citric were dissolve in 100 ml of water and purged with nitrogen. Then 5 eq. of $NaBH_4$ was quickly added and the resulting suspension was stirred for 5 min at room temperature. Stirring was stopped and the water was carefully decanted. The iron on the bottom of the flask was washed twice with degassed water suspended in 10 ml of water again. Then **VIII** was added and the suspension was stirred at room temperature. The reaction was monitored by TLC. After completion of the reaction, the mixture filtered and the filtrate was extracted with ethyl acetate (4x). The combined organic layer was dried over magnesium sulphate, and the solvent was removed under reduced pressure. The crude product was recrystallized from ethanol to give 3.6 g (12.6 mmol, 85 %) of **11a**. ^{1}H NMR (DMSO-d_6 ,500MHz): δ = 10.32 (s, 1 H), 7.79 (s, 1 H), 7.63 - 7.70 (m, 3 H), 7.09 (d, *J*=2.1 Hz, 1 H), 6.95 (d, *J*=8.2 Hz, 1 H), 6.90 (dd, *J*=8.5, 2.1 Hz, 1 H), 5.24 (br. s., 2 H), 2.21 ppm (s, 3 H); ^{13}C NMR (DMSO-d_6 ,126MHz): δ = 187.7, 160.6, 147.1, 143.1, 141.0, 132.7, 131.3, 130.2, 129.8, 128.8, 124.9, 123.3, 118.5, 114.8, 108.3, 16.4 ppm; Purity (UV) : 90 %; t_R: 10.58 min, ESI-MS $[M+H]^+$: 284.21; calcd. $[M]^+$: 283.07.

Synthesis of compound 11, (4-hydroxy-3-methylphenyl)(5-hydroxybenzo[b]thiophen-2-yl)methanone: The procedure for the synthesis of **11** was earlier described by Martin-Smith and co-workers in 1956.[15] 0.5 g (1.8 mmol) of **11a** were refluxed in 100 ml of a 40 % (w/v) $NaHSO_3$ solution for 48 hours. The reaction was stopped by diluting the mixture with 200 ml

of water, and an excess of sodium hydroxide was added. The mixture was heated to reflux for an hour. The solution was allowed to cool to room temperature and the resulting precipitate was filtered off. The filtrate was acidified using concentrated HCl and the newly formed precipitate was collected and recrystallized from ethanol to yield 0.14 g (0.49 mmol, 28 %) of **11** as a white solid. ^{1}H NMR (DMSO-d_6, 300MHz): δ = 10.54 (br. s., 2 H), 8.07 - 8.25 (m, 2 H), 7.97 (br. s., 1 H), 7.61 - 7.81 (m, 2 H), 7.47 (d, *J*=8.4 Hz, 1 H), 7.02 (d, *J*=7.8 Hz, 1 H), 2.21 ppm (s, 3 H); ^{13}C NMR (DMSO-d_6, 75MHz): δ = 187.4, 161.1, 145.1, 140.1, 139.6, 132.8, 131.7, 131.5, 130.0, 128.1, 125.0, 124.6, 122.4, 119.5, 115.0, 16.4 ppm; Purity (UV) 95 %; t_R: 10.48 min, ESI-MS $[M+H]^+$: 285.10; calcd. $[M]^+$: 284.05.

Synthesis of intermediate compound IX, methyl 5-nitrobenzo[b]thiophene-2-carboxylate: The title compound was synthesized from 2-fluoro-5-nitrobenzaldehyde according to procedure A to yield 14 g (59 mmol, 99 %) of **IX** as a white solid. After confirmation of the identity by NMR, the compound was directly used without further characterisation in the next step. ^{1}H NMR (DMSO-d_6, 300MHz) δ = 9.00 - 8.96 (m, 1 H), 8.43 (s, 1 H), 8.38 - 8.33 (m, 1 H), 8.33 - 8.27 (m, 1 H), 3.92 (s, 3 H) ppm; ^{13}C NMR (DMSO-d_6, 75MHz) δ = 162.3, 147.3, 146.0, 138.9, 136.7, 132.1, 125.0, 122.1, 121.5, 53.5 ppm.

Synthesis of intermediate compound X, methyl 5-aminobenzo[b]thiophene-2-carboxylate: The procedure for the synthesis of **11a** was earlier described by Dey and co-workers in 2012.[14] 3 eq. of $FeSO_4$ x7H_2O and 0.24 eq. of citric were dissolve in 100 ml of water and purged with nitrogen. Then 5 eq. of $NaBH_4$ was quickly added and the resulting suspension was stirred for 5 min at room temperature. Stirring was stopped and the water was carefully decanted. The iron on the bottom of the flask was washed twice with degassed water suspended in 10 ml of water again. Then **IX** was added and the suspension was stirred at room temperature. The reaction was monitored by TLC. After completion of the reaction,

the mixture filtered and the filtrate was extracted with ethyl acetate (4x). The combined organic layer was dried over magnesium sulphate, and the solvent was removed under reduced pressure. The crude product was recrystallized from ethanol to give 5.35 g (25.8 mmol, 86 %) of **X** as a white solid. ^{1}H NMR (DMSO-d_6, 300MHz) δ = 7.92 (s, 1 H), 7.65 (d, J = 8.6 Hz, 1 H), 7.06 (br. s., 1 H), 6.89 (d, J = 8.8 Hz, 1 H), 5.26 (br. s., 2 H), 3.85 (s, 3 H) ppm; ^{13}C NMR (DMSO-d_6, 75MHz) δ = 163.2, 147.3, 140.4, 132.7, 130.6, 130.0, 123.4, 118.4, 107.8, 52.9 ppm.

Synthesis of intermediate compound XI, 5-hydroxybenzo[b]thiophene-2-carboxylic acid: : The procedure for the synthesis of **XI** was earlier described by Martin-Smith and co-workers in 1956.[15] 3 g (14.5 mmol) of **X** were refluxed in 150 ml of a 40 % (w/v) $NaHSO_3$ solution for 48 hours. The reaction was stopped by diluting the mixture with 230 ml of water, and an excess of sodium hydroxide was added. The mixture was heated to reflux for an hour. The solution was allowed to cool to room temperature and the resulting precipitate was filtered off. The filtrate was acidified using concentrated HCl and the newly formed precipitate was collected and recrystallized from ethanol to yield 1.6 g (8.25 mmol, 57 %) of **XI** as colourless needles. ^{1}H NMR (DMSO-d_6, 300MHz) δ = 9.91 - 9.38 (m, 2 H), 8.00 (s, 1 H), 7.87 (d, J = 8.6 Hz, 1 H), 7.48 (d, J = 2.0 Hz, 1 H), 7.16 (dd, J = 2.1, 8.7 Hz, 1 H) ppm; ^{13}C NMR (DMSO-d_6, 75MHz) δ = 164.0, 140.1, 139.4, 136.0, 135.0, 130.2, 124.1, 120.1, 113 ppm; Purity (UV) > 95 %, t_R : 2.75 min; ESI-MS $[M+H]^+$: 194.04; calcd. $[M]^+$: 194.00.

Synthesis of compound 16, 1-(5-hydroxy-1-benzothiophen-2-yl)ethan-1-one: The title compound was synthesized starting from **XI** using the procedure described by Rubottom and co-workers in 1983.[16] Therefore **XI** was dissolved in dry THF and cooled to -78°C. Then 8 eq. of methyl lithium was added dropwise and the reaction was stirred for 30 min at -78°C, before it was allowed to warm to room temperature. The mixture was stirred for another 30

min at RT, before 27 eq. of TMSCl were added. Then a solution of 1N HCl was carefully added and the aqueous layer was extracted with ethyl acetate (4x). The combined organic layer was dried over magnesium sulphate and the solvent was removed in vacuo. The crude product was purified on a waters autopurification system to give compound **16** as yellow solid. ^{1}H NMR (Methanol-d_4, 300 MHz) δ = 8.02 (br. s., 1H), 7.71 (d, *J* = 7.82 Hz, 1H), 7.31 (br. s., 1H), 7.05 (d, *J* = 7.45 Hz, 1H), 2.65 (br. s., 3H) ppm ; ^{13}C NMR (Methanol-d_4, 75 MHz,) δ = 193.3, 155.4, 144.2, 140.7, 134.2, 130.2, 123.2, 118.3, 109.5, 25.3 ppm; Purity (UV): > 95 %; t_R: 7.96 min; ESI-MS $[M-H]^-$: 191.0; calcd. $[M]^+$: 192.02.

3. Tables

Table S1. Potencies of the reference compound TG003 in different studies depending on the experimental conditions, to facilitate a comparison of our compound´s potencies with those reported in the cited studies.

	Reference compound	**TG003**	
	IC_{50}(Dyrk1A)/nM	**IC_{50}(Clk1)/nM**	**Selectivity factor (SF)**
This study	830	170	4.9
Fedorov et al.[17]	156	49	3.2
Mott et al.[18]	12[a]	19[a]	0.6
Ogawa et al.[19]/ Muraki et al.[2]	930[b]	20[b]	46[b]

4. [a] K_d values for TG003 on Dyrk1A and Clk1
5. [b] IC_{50} values were not provided in the same study. There is a discrepancy between the assay conditions used in the two papers of the group

Table S2: Inhibitory potency of naphthalene–based inhibitors

Compound	R	IC_{50} [µM]/ % inhibition @5µM[a]				
		Dyrk1A	Dyrk1B	Dyrk2	Clk1	Ck2α
1	H	2.9	2.3	30 %	1.9	19 %
2	Me	2.9	3.1	23 %	1	11 %
3	F	7.6	0.9	17 %	1	14 %

[a][ATP] = 100 µM

Table S3: Inhibitory potencies of 6-hydroxybenzoheterocyclic inhibitors

Compound	X	IC_{50} [µM]]/ % inhibition @5µM[a]				
		Dyrk1A	Dyrk1B	Dyrk2	Clk1	Ck2α
4	S	4.5	4	49 %	1	15 %
5	O	2.4	3.8	34 %	1.4	16 %
6	NH	20	45 %	15 %	54 %	24 %
7	NMe	36	-	24 %	35 %	8 %
		Dyrk1A	Dyrk1B	Dyrk2	Clk1	Ck2α
8	S	1.3	1.6	51 %	1	17 %
9	O	2.3	2.8	39 %	1.4	34 %

[a][ATP] = 100 µM

Table S4. 17-β HSD1 and -2 inhibition by compounds **16**, **23**, and **25**

Compound	% 17-β HSD1 inhibition	% 17-β HSD2 inhibition
16	IC_{50} = 195 nM	IC_{50} = 1200 nM
23	33 % @ 5µM	11 % @ 5µM
25	0 % @ 5µM	7 % @ 5µM

Table S5. Comparison of potencies dependent on the ATP concentration in the cell free assay for **16** and harmine in different studies.

	ATP concentration [µM][1]	16	Harmine
		IC_{50}[nM]	
This study	100	170	100
	10	83	39
Ogawa et al.[19]	10	-	350
Göckler et al.[20]	100	-	33
Bain et al.[21]	50	-	80
Tahtouh et al.[22]	15	-	34

[1] One obvious factor which strongly influences the measured potencies of ATP-competitive inhibitors under cell-free conditions is the ATP concentration. This explains, at least in part, the apparently lower potencies obtained with our standard assay conditions (100 µM ATP), as can be seen from the reference compound harmine, a well-established, potent ATP competitive inhibitor of Dyrk1A. However, working at ATP concentrations under the Km value of the kinase for ATP (37 µM [19]- 59 µM[23]) leads to a strong over-estimation of the potencies and, concomitantly, to a large drop of activities when changing to cell-based assays.

Table S6. Selectivity of compounds **16** and **23** against a panel of 31 selected protein kinases[a] covering all kinase families from the kinome tree.

Kinase Family	Kinase	16	23
		% inhibition at 5 µM[b]	
CMGC	Dyrk1A	96	95
	Dyrk1B	99	98
	Dyrk2	50	46
	DYRK3	12	23
	DYRK4	11	16
	Ck2α	10	9
	Clk1	98	99
	CLK2	67	85
	CLK3	16	43
	CLK4	90	97
	HIPK1	10	1
	GSK3β	3	16
STE	MST4	5	9
AGC	PKCβ	-8	19
CAMK	PIM1	44	59
	PIM2	10	23
	CaMKI	-2	1
	STK17A (DRAK1)	47	65
	PKD2	6	32
	MYLK2 (skMLCK)	19	36
	MLCK (MLCK2)	53	49
	MYLK (MLCK)	15	12
Other	Haspin	69	83
CK1	CSNK1D (CK1 delta)	-1	16
	CSNK1G2 (CK1 gamma 2)	3	26
	CSNK1G3 (CK1 gamma 3)	13	12
	CSNK1E (CK1 epsilon)	8	15
TKL	MLK1	10	-3
TK	EGFR	20	-3
	ROS	9	4
	TrkB	-8	-18

[a] the screening list was especially composed to include all kinases that were frequently reported as off-targets for diverse chemical classes of Dyrk inhibitors.[22,24–27] Hence, the low hit rate besides the main targets Dyrk1A/1B and Clk1/4 (basically limited to Haspin for **23**) suggests a very high degree of selectivity. In comparison, reference compound TG003 also inhibited CSNK1D and CSNK1G2 (IC_{50}= 150 and 270 nM, respectively)[18]. [b] All screenings were performed in the presence of 100 µM ATP.

Table S7. IC_{50} values (nM) of **16** and **23** against Dyrk1A, Clk1, and the off-target Haspin.

	16	**23**
Dyrk1A	230	230
Dyrk1B	170	104
Clk1	52	64
Haspin	2500	800

Table S8. Compounds **16** and **23** exhibit lower off-target activity than TG003

	% inhibition		
Kinase	**TG003**[a] (at 1 µM)	**16**[b] (at 5 µM)	**23**[b] (at 5 µM)
Dyrk2	95	50	46
Haspin	83	69 (IC_{50} 2.5 µM)	83 (IC_{50} 0.8 µM)
CLK2	80	67	85
Pim1	75	44	59
CK1δ	71	0	16

[a] Top 5 off-target kinases identified for TG003 by Gao et al. in a panel of 234 kinases.[28] These all belong to the list of kinases frequently reported as off-targets for diverse chemical classes of Dyrk inhibitors (cf. Table S6), and our compounds showed a significantly reduced inhibition compared with TG003 (please note that the screening concentrations differed); [b] values taken from Tables S6 and S7 for easier comparison.

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