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Identification, Synthesis and Evaluation of Substituted Benzofurazans as Inhibitors of CREB-mediated Gene Transcription

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Abstract

Cyclic-AMP response-element binding protein (CREB) is a stimulus-activated transcription factor. Its transcription activity requires its binding with CREB-binding protein (CBP) after CREB is phosphorylated at Ser133. The domains involved for CREB-CBP interaction are kinase-inducible domain (KID) from CREB and KID-interacting domain (KIX) from CBP. Recent studies suggest that CREB is an attractive target for novel cancer therapeutics. To identify novel chemotypes as inhibitors of KIX-KID interaction, we screened the NCI-diversity set of compounds using a split *renilla* luciferase assay and identified 2-[(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)thio]pyridine 1-oxide (compound **1**) was identified as a potent inhibitor of KIX-KID interaction. However, compound **1** was not particularly selective against CREB-mediated gene transcription in living HEK 293T cells. Further structure-activity relationship studies identified 4-aniline substituted nitrobenzofurazans with improved selectivity.

Keywords

CREB; CBP; cancer; inhibitor; screening; selectivity; structure-activity relationships

Cyclic adenosine monophosphate (cAMP) response-element (CRE) binding protein (CREB) is a nuclear transcription factor belonging to a family of basic leucine zipper (bZIP)-containing transcription factors.^{1,2} As a stimulus-activated transcription factor, CREB is activated by a variety of extracellular signals including growth factors through protein serine/threonine kinases.² A key residue in CREB that is phosphorylated in this signaling cascade is Ser133 located in the kinase inducible domain (KID) and the phosphorylated CREB is referred to p-CREB.³ Once phosphorylated, CREB binds mammalian transcription co-activators and histone acetyl transferases, CREB-binding protein (CBP) and its closely related paralog p300, through their KIX (KID-interacting) domain. This complex will recruit additional transcription machinery to initiate CREB-mediated gene transcription activation.⁴

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A variety of protein serine/threonine kinases including protein kinase A (PKA), mitogen activated protein kinase (MAPK), protein kinase B (PKB/Akt), ribosome S6 kinase (p90^{RSK}) can activate CREB through phosphorylation at Ser133.⁵ On the other hand, CREB's transcription activity can be attenuated by protein phosphatases that dephosphorylate Ser133. The phosphatases that are known to remove this phosphate include protein phosphatase 1 (PP1),⁶ protein phosphatase 2A (PP2A)⁷ and phosphatase and tensin homolog (PTEN).⁸ Since most of the CREB kinases are often aberrantly activated while the CREB phosphatases are often inactivated in cancer cells, CREB has been shown to be overexpressed and/or overactivated in both solid and liquid tumor tissues.⁹ As such, CREB has been proposed as a novel target for cancer therapeutics discovery.⁹ Various strategies have been exploited to discover potential small molecule binders for CREB or CBP.⁹⁻¹⁵ Among the ligands discovered to date, naphthol AS-E and its derivatives are the only ones that are known to inhibit CREB-mediated gene transcription in living cells.^{10,14,16,17} Therefore, additional small molecule inhibitors, preferably with different chemotypes, are in great need to further elucidate the roles of CREB in maintaining tumor phenotype.

A critical event for the activation of CREB-dependent gene transcription is the formation of KID-KIX complex between CREB and CBP.⁴ We recently described a split *renilla* luciferase (RLuc) assay to monitor KIX-KID interaction.¹⁰ In this assay, RLuc was split into N-terminal half (RLucN) and C-terminal half (RLucC). KID was fused with RLucN while KIX was fused with RLucC to give fusions KID-RLucN and RLucC-KIX, respectively. On their own, these polypeptides did not present RLuc activity. However, the RLuc activity was specifically restored when KID-RLucN and RLucC-KIX were combined together.¹⁰ Herein we present our studies on the identification of substituted benzofurazans as small molecule inhibitors of KIX-KID interaction and CREB-mediated gene transcription.

To identify novel chemotype(s) as potential inhibitors of KIX-KID interaction, the split RLuc assay¹⁰ was employed to screen the National Cancer Institute (NCI)'s diversity set of ~1,400 compounds (Figure 1), whose structures cover significant variations.^{18,19} The compounds were initially screened at 10 μ M concentration and 2-[(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)thio]pyridine 1-oxide (compound **1**, Figure 1) was consistently shown to efficaciously inhibit KIX-KID interaction. Therefore, this compound was independently synthesized as shown in Scheme 1 for further characterization. S_NAr displacement of chloride from 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl, **2**) by 2-pyridinethiol 1-oxide (**3**) in the presence of K₂CO₃ gave compound **1** in 73% yield.²⁰ The desired *S*-arylation in **1**, instead of the alternative *O*-arylation product **1a**,²¹ was confirmed by its conversion to deoxygenated compound **4** by the action of dimethylthiocarbamoyl chloride (DMTCC) (Scheme 1).²² Compound **4** could also be obtained directly from coupling NBD-Cl (**2**) with 2-pyridinethiol in the presence of K₂CO₃ in 73% yield (Scheme 1).

Consistent with the screening results, synthetic compound **1** dose-dependently inhibited KIX-KID interaction as evaluated by the split RLuc assay with an IC₅₀ of 0.36 μ M (Figure 2A and Table 1). Encouraged by its potent *in vitro* activity, we evaluated its cellular activity in inhibiting CREB-mediated gene transcription by a CREB-reporter assay in HEK 293T cells. Therefore, HEK 293T cells were transfected with CRE-RLuc, a plasmid expressing RLuc under the control of three tandem copies of CRE.¹⁰ Then the transfected cells were treated with different concentrations of compound **1** before stimulating the cells with forskolin (10 μ M). The data presented in Figure 2B and Table 1 showed that compound **1** inhibited CREB-mediated gene transcription in living HEK 293T cells with an IC₅₀ of 2.09 μ M. To investigate if the inhibition of the CREB's transcription activity by compound **1** was dependent on KIX-KID interaction, another transcription reporter assay activated by a heterologous transcription activator, VP16-CREB, was performed in HEK 293T cells. VP16-CREB fusion contains full length CREB and the potent transcription activation

domain VP16.^{10,23} Unlike CREB whose transcription activity is dependent on phosphorylation at Ser133, VP16-CREB is a constitutively active transcription activator and its transcription activity is independent of phosphorylation at Ser133.^{10,23} To this end, HEK 293T cells were co-transfected with VP16-CREB and CRE-RLuc. The transfected cells were then treated with increasing concentrations of compound **1**. The results presented in Figure 2C showed that **1** also inhibited VP16-CREB-mediated gene transcription with an IC₅₀ of 6.14 μ M (Table 1). Although this is about 3-fold higher than the IC₅₀ of CREB-mediated gene transcription (Figure 2B), these results suggest that compound **1** is not particularly selective in inhibiting KIX-KID interaction inside the living cells.

We hypothesized that compound **1** might form a Meisenheimer-type complex with cellular nucleophiles (e.g. glutathione and protein cysteine side chain) eventually leading to elimination of 2-pyridinethiol 1-oxide and form a covalent adduct **5** (Figure 3),²⁴ which may account for its limited selectivity. Therefore, the electrophilicity of benzofurazan nucleus and leaving group ability of the appendant aromatic rings were modulated with a goal of trying to enhance the selectivity. This led to the design and synthesis of compounds **4**, **6-9** (Schemes 1-3). The relative leaving group ability follows the order of **1** > **4** > **6** while compound **7** has reduced capability to form a Meisenheimer-type complex due to the electron-donating nature of the -NH₂ group. Compounds **8** and **9** do not have a leaving group and thus cannot form the corresponding adduct **5**.

Compound **6** was synthesized by condensing NBD-Cl (**2**) with 4-chloroaniline (**10**) in the presence of NaHCO₃ (Scheme 2).^{20,25} Reduction of -NO₂ in **6** with Fe/HCl²⁶ gave compound **7** in 58% yield. In order to synthesize compound **8**, we designed the synthesis by coupling a known acid **16**²⁷ with aniline **10**. Acid **16** was reported to be prepared from commercially available benzofurazan (**11**) by a sequence of reactions involving formylation by LDA/DMF, reduction by NaBH₄, concomitant nitration and oxidation by HNO₃/H₂SO₄.²⁷ However, we were only able to obtain alcohol **14** from this series of reactions. The acid **16** was eventually prepared from alcohol **14** via IBX oxidation followed by Pinnick oxidation.²⁸ The desired amide **8** was finally delivered by coupling acid **16** with aniline **10** facilitated by MsCl.²⁰ The synthesis of compound **9** was not so straightforward. Initial attempts to prepare **9** from Borch reductive amination²⁹ between aldehyde **15** and **10** were unsuccessful. We found that the nitro group in **15** had deleterious effects on this reaction because aldehyde **12** could be successfully coupled with aniline **10** to give **17** in 67% yield under the same reaction condition. Direct nitration of **17** only afforded the undesired product with a nitro group incorporated into the aniline ring presumably due to its electron-rich nature. So an acetyl group was introduced on the aniline nitrogen to generate **18** by decreasing its nucleophilicity. Nitrating **18** gave a chromatographically inseparable mixture of **19a** and **19** in a combined 49% yield in a ratio of 69:31 as determined by HPLC. Final hydrolysis of the acetyl group in the mixture of **19a** and **19** with 20% H₂SO₄/EtOH yielded products **9a** and **9**, which could be separated by careful silica gel chromatography (Scheme 3).

All the six newly synthesized compounds were evaluated for their potencies in inhibiting KIX-KID interaction *in vitro* and CREB-mediated gene transcription. For those compounds demonstrating inhibition of CREB's activity, their effects on VP16-CREB-mediated gene transcription in HEK 293T cells were also evaluated. The results are presented in Figure 2 and Table 1. The deoxygeneated compound **4** displayed comparable activity to compound **1** in inhibiting KIX-KID interaction *in vitro*, suggesting the scheme shown in Figure 3 is not a major pathway for the observed *in vitro* inhibition of KIX-KID interaction because thiopyridine 1-oxide is a better leaving group than thiopyridine. On the other hand, the cellular inhibition of CREB-mediated gene transcription by **4** was reduced by about 4-fold to an IC₅₀ of 9.42 μ M compared to compound **1**. These results suggest that the discordance

between *in vitro* and cellular IC₅₀ of compound **1** is not due to its charged nature, which may result in reduced cell permeability as compound **4** is not charged. But its cellular potency is also much weaker than its *in vitro* KIX-KID interaction inhibition potency. Compared to compound **1**, the selectivity of deoxygenated compound **4** was not improved because it inhibited VP16-CREB-mediated gene transcription with an IC₅₀ of 13.17 μ M. The 4-chloroaniline-substituted compound **6** was significantly less potent in inhibiting KIX-KID interaction *in vitro* (IC₅₀ = 7.14 μ M) than **1**. Its cellular activity (IC₅₀ = 13.74 μ M) was only ~2-fold less potent than its *in vitro* activity. More importantly, this compound displayed enhanced selectivity as evidenced by the lack of inhibition of VP16-CREB-mediated transcription activity up to 100 μ M, the highest concentration tested. Reduction of the –NO₂ group in **6** to compound **7** essentially abolished all the activities measured (Table 1). The amide compound **8** exhibited two-fold enhanced activity in inhibiting KIX-KID interaction *in vitro* (IC₅₀ = 0.17 μ M) compared to **1**. However, no cellular activity was observed (IC₅₀ > 50 μ M). This discrepancy might be due to its poor cellular permeability or extracellular inactivation. Insertion of one methylene unit into compound **6** resulted in **9**, which showed reduced *in vitro* and cellular activity. The regioisomer **9a** was inactive in all the assays measured. Collectively, these results suggest that the electrophilic nature of the nitrobenzofurazan nucleus is required for potent KIX-KID interaction inhibition and cellular inhibition of CREB-mediated gene transcription. Furthermore, modulation of leaving group ability of the substituents at 4-position of nitrobenzofurazan can enhance selectivity.

In summary, 2-[(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)thio]pyridine 1-oxide (compound **1**) was identified as a novel inhibitor of KIX-KID interaction from screening the NCI diversity set of compounds using a split RLuc assay. Nitrobenzofurazan derivatives have recently been found to display other significant biological activities including inhibition of hypoxia-inducible factor 2 (HIF-2),³⁰ inhibition of oncogene c-Myc,³¹ and inhibition of aldose reductase.^{32,33} However, the structure-activity relationship is unique in each case, suggesting distinct binding modes with different proteins. Although compound **1** was a potent inhibitor of KIX-KID interaction (IC₅₀ = 0.36 μ M), it was not particularly selective against CREB-mediated gene transcription in HEK 293T cells. Further structure-activity relationship studies showed that 4-aniline substituted compound **6** displayed a higher selectivity index. Therefore, compound **6** represents a novel small molecule inhibitor of CREB-mediated gene transcription that can be further developed into more selective and potent inhibitors for detailed biological evaluations.

Acknowledgments

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20. *Selected characterization data for selected compounds:* (a) *Compound 1*: a yellow solid, m.p. 201–202 °C. ¹H NMR (400 MHz, DMSO-*d*₆) 8.70 (d, *J* = 5.7 Hz, 1 H), 8.45 (d, *J* = 3.5 Hz, 1 H), 8.05 (d, *J* = 4.8 Hz, 1 H), 7.39 (brs, 1 H), 7.25 (s, 2 H); ¹³C NMR (100 MHz, DMSO-*d*₆) 151.20, 146.54, 143.94, 139.05, 137.48, 137.10, 132.31, 128.99, 126.35, 126.24, 124.74; HRMS (ESI) calcd for C₁₁H₇N₄O₄S⁺ (M + H)⁺ 291.01825, found 291.01830. (b) *Compound 4*: a yellow solid, m.p. 81–82 °C. ¹H NMR (400 MHz, CDCl₃) 8.62 (dd, *J* = 4.7, 1.2 Hz, 1 H), 8.40 (d, *J* = 7.6 Hz, 1 H), 7.83 (td, *J* = 7.7, 1.9 Hz, 1 H), 7.66 (d, *J* = 8.0 Hz, 1 H), 7.61 (d, *J* = 7.9 Hz, 1 H), 7.38 (dd, *J* = 4.9, 0.9 Hz, 1 H); ¹³C NMR (100 MHz, CDCl₃) 152.10, 151.06, 149.51, 142.65, 138.15, 136.84, 130.49, 127.58, 126.36, 123.86; HRMS (ESI) calcd for C₁₁H₇N₄O₃S⁺ (M + H)⁺ 275.02334, found 275.02342. (c) *Compound 6*: a red solid, m.p. 156–157 °C. ¹H NMR (400 MHz, DMSO-*d*₆) 11.04 (s, 1 H), 8.54 (d, *J* = 8.6 Hz, 1 H), 7.57 (d, *J* = 9.1 Hz, 2 H), 7.51 (d, *J* = 9.1 Hz, 2 H), 6.76 (d, *J* = 8.8 Hz, 1 H); ¹³C NMR (100 MHz, DMSO-*d*₆) 145.54, 144.64, 142.40, 137.99, 137.35, 130.69, 130.07, 125.94, 124.10, 102.65; HRMS (ESI) calcd for C₁₂H₆ClN₄O₃[−] (M - H)[−] 289.01229, found 289.01255. (d) *Compound 8*: an orange solid, m.p. 229–230 °C. ¹H NMR (400 MHz, DMSO-*d*₆) 10.97 (s, 1 H), 8.82 (d, *J* = 7.5 Hz, 1 H), 8.25 (d, *J* = 7.5 Hz, 1 H), 7.81 (d, *J* = 8.6 Hz, 2 H), 7.49 (d, *J* = 8.9 Hz, 2 H); ¹³C NMR (100 MHz, DMSO-*d*₆) 160.60, 148.27, 143.12, 137.68, 137.18, 131.62, 131.36, 130.55, 128.88, 128.27, 121.80; HRMS (ESI) calcd for C₁₃H₆ClN₄O₄[−] (M - H)[−] 317.00721, found 317.00777.
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Figure 1. Identification of compound **1** as an inhibitor of KIX-KID interaction from screening of the NCI diversity set by KIX-KID split *renilla* luciferase assay.

Figure 2.

Inhibition of KIX-KID interaction and CREB-dependent gene transcription by **1** and **4**. (A) Inhibition of *in vitro* KIX-KID interaction. RLuc-KIX and KID-RLucN were combined together in the presence of different concentrations of compounds at 4 °C. The residual RLuc activity was measured after 20 h of incubation. (B) Inhibition of CREB-dependent gene transcription. HEK 293T cells were transfected with CRE-RLuc and then treated with different concentrations of compounds for 30 min. Then forskolin (10 μ M) was added and incubated for another 5 h. The cells were then lysed and the RLuc activity was measured. The RLuc activity was normalized to protein concentration and expressed as RLU (relative light units)/ μ g protein. (C) Inhibition of VP16-CREB-mediated gene transcription. The experiments were the same as in (B) except the cells were transfected with VP16-CREB and CRE-RLuc and forskolin treatment was omitted.

Figure 3. Potential formation of a Meisenheimer complex between compound **1** and cellular nucleophiles.

Scheme 1.
Synthesis of compounds **1** and **4**.

Scheme 2.
Synthesis of compounds **6** and **7**.

Scheme 3.
Synthesis of compounds **8** and **9**.

Table 1Biological activities of synthesized compounds.^a

compound	KIX-KID inhibition (IC ₅₀) ^b	CREB-mediated transcription inhibition (IC ₅₀) ^b	VP16-CREB-mediated transcription inhibition (IC ₅₀) ^b
1	0.36 ± 0.15	2.09 ± 0.39	6.14 ± 0.88
4	0.41 ± 0.067	9.42 ± 0.47	13.17 ± 4.41
6	7.14 ± 0.57	13.74 ± 3.41	>100
7	~ 50 ^c	~ 50 ^c	>50
8	0.17 ± 0.064	>50	ND ^d
9	13.06 ± 3.69	>50	ND ^d
9a	>50	>50	ND ^d

^a All the stock solutions were prepared in DMSO except **9**, which was dissolved in DMF due to its instability in DMSO.

^b All the IC₅₀ values are shown in μM. The data are presented as mean ± SD of at least two independent experiments performed in duplicates (KIX-KID inhibition assay) or triplicates (reporter assays). If the IC₅₀ was not reached at the highest concentration tested at 50 μM or 100 μM, it is presented as >50 or >100.

^c The IC₅₀ is roughly at the highest concentration tested.

^d Not determined.