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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**, NSC228155) 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–activityrelationship studies identified 4-aniline substituted nitrobenzofurazans with improved selectivity.

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]

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 assay[10] was employed to screen the National Cancer Institute (NCI)'s diversity set of ~1400 com-

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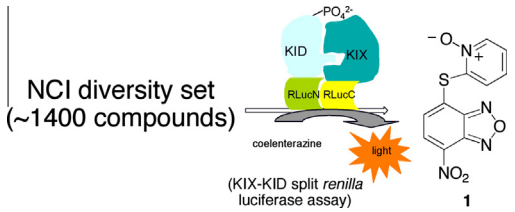
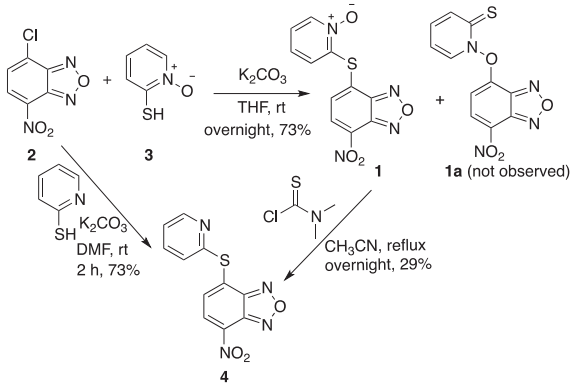


Figure 1. Identification of compound **1** as an inhibitor of KIX–KID interaction from screening the NCI diversity set by KIX–KID split *renilla* luciferase assay.

pounds (Fig. 1), whose structures cover significant variations.[18,19] The compounds were initially screened at 10 μM concentration and 2-[(7-nitrobenzo[*c*][1,2,5]oxadiazol-4-yl)thio]pyridine 1-oxide (compound **1**, NSC228155, Fig. 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 K_2CO_3 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 K_2CO_3 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 IC_{50} of 0.36 μM (Fig. 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 μM). 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 IC_{50} of 2.09 μM. 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



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

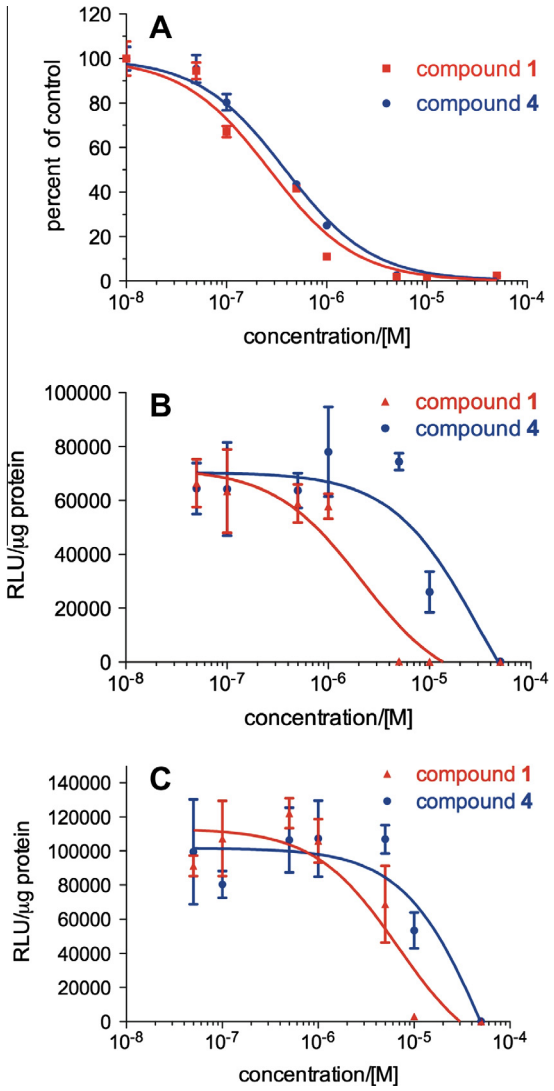


Figure 2. Inhibition of KIX–KID interaction and CREB-dependent gene transcription by **1** and **4**. (A) Inhibition of in vitro KIX–KID interaction. RLuC-KIX and KID-RLucN were combined together in the presence of different concentrations of compounds at 4 °C. The residual RLuc activity was measured 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 μM) 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)/μg 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.

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 IC_{50} of 6.14 μM (Table 1). Although this is about three-fold higher than the IC_{50} of CREB-mediated gene transcription (Fig. 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** (Fig. 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

Table 1
Biological activities of synthesized compounds[a]

Compound	KIX–KID inhibition[b] (IC_{50})	CREB-mediated transcription inhibition[b] (IC_{50})	VP16-CREB-mediated transcription inhibition[b] (IC_{50})
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 IC_{50} 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 IC_{50} was not reached at the highest concentration tested at 50 μM or 100 μM, it is presented as >50 or >100.
[c] The IC_{50} is roughly at the highest concentration tested.
[d] Not determined.

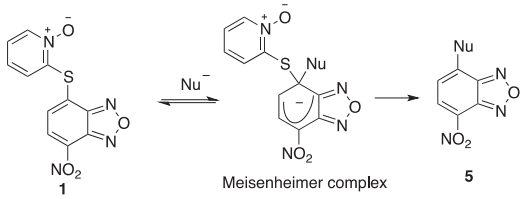
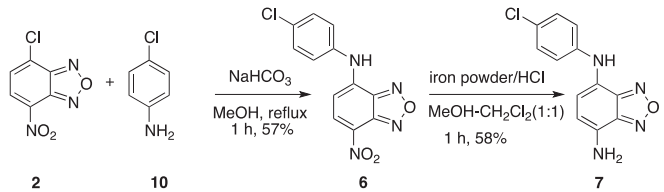


Figure 3. Potential formation of a Meisenheimer complex between compound **1** and cellular nucleophiles.

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 $-NH_2$ 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 $NaHCO_3$ (Scheme 2).[20,25] Reduction of $-NO_2$ in **6** with Fe/HCl[26] 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 $NaBH_4$, concomitant nitration and oxidation by HNO_3/H_2SO_4.[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 amination[29] 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

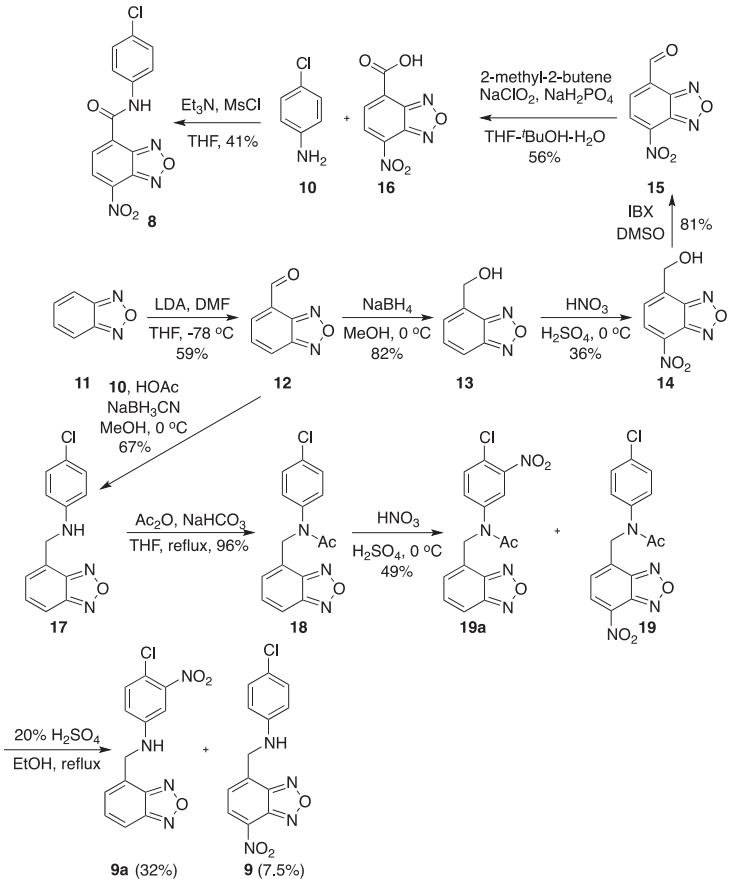


Scheme 2. Synthesis of compounds **6** and **7**.

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% H_2SO_4/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 four-fold to an IC_{50} of 9.42 μM compared to compound **1**. These results suggest that the discordance between in vitro and cellular IC_{50} 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 IC_{50} of 13.17 μM. The 4-chloroaniline-substituted compound **6** was significantly less potent in inhibiting KIX–KID interaction in vitro (IC_{50} = 7.14 μM) than **1**. Its cellular activity (IC_{50} = 13.74 μM) was only ~two-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 μM, the highest concentration tested. Reduction of the $-NO_2$ 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 (IC_{50} = 0.17 μM) compared to **1**. However, no cellular activity was observed (IC_{50} > 50 μM). 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



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

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 (IC_{50} = 0.36 μM), 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.

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20. *Selected characterization data for selected compounds:* (a) Compound **1**: a yellow solid, mp 201–202 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.70 (d, J = 5.7 Hz, 1H), 8.45 (d, J = 3.5 Hz, 1H), 8.05 (d, J = 4.8 Hz, 1H), 7.39 (br s, 1H), 7.25 (s, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 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 $C_{11}H_7N_4O_4S^+$ $(M+H)^+$ 291.01825. Found 291.01830. (b) Compound **4**: a yellow solid, mp 81–82 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.62 (dd, J = 4.7, 1.2 Hz, 1H), 8.40 (d, J = 7.6 Hz, 1H), 7.83 (td, J = 7.7, 1.9 Hz, 1H), 7.66 (d, J = 8.0 Hz, 1H), 7.61 (d,

J = 7.9 Hz, 1H), 7.38 (dd, J = 4.9, 0.9 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 152.10, 151.06, 149.51, 142.65, 138.15, 136.84, 130.49, 127.58, 126.36, 123.86; HRMS (ESI) Calcd for $C_{11}H_7N_4O_3S^+$ $(M+H)^+$ 275.02334. Found 275.02342. (c) Compound **6**: a red solid, mp 156–157 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.04 (s, 1H), 8.54 (d, J = 8.6 Hz, 1H), 7.57 (d, J = 9.1 Hz, 2H), 7.51 (d, J = 9.1 Hz, 2H), 6.76 (d, J = 8.8 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 145.54, 144.64, 142.40, 137.99, 137.35, 130.69, 130.07, 125.94, 124.10, 102.65; HRMS (ESI) Calcd for $C_{12}H_6ClN_4O_3^-$ $(M-H)^-$ 289.01229. Found 289.01255. (d) Compound **8**: an orange solid, mp 229–230 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.97 (s, 1H), 8.82 (d, J = 7.5 Hz, 1H), 8.25 (d, J = 7.5 Hz, 1H), 7.81 (d, J = 8.6 Hz, 2H), 7.49 (d, J = 8.9 Hz, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 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 $C_{13}H_6ClN_4O_4^-$ $(M-H)^-$ 317.00721. Found 317.00777.

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