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Discovery of a Synergistic Inhibitor of cAMP-Response Element Binding Protein (CREB)-Mediated Gene Transcription with 666-15

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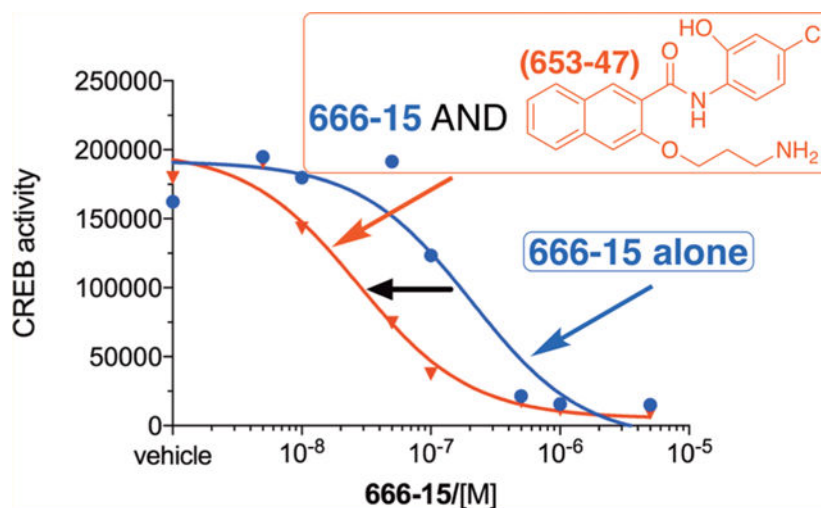
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Abstract

CREB is a transcription factor implicated in the pathogenesis of multiple cancers. Targeting CREB is a promising strategy to develop potential cancer therapeutics. Previously, we identified **666-15** as a potent CREB inhibitor. Herein, we designed an ester prodrug of **666-15** through a long-range *O,N*-acyl transfer reaction for improved aqueous solubility. Unexpectedly, we discovered a small molecule **11** (**653-47**) that can potentiate the CREB inhibitory activity of **666-15** although **653-47** alone does not inhibit CREB.

Graphical Abstract



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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jmedchem.9b01207>.

HPLC traces, CI values, and cell growth inhibition data (PDF)

Molecular formula strings and their associated data (CSV)

The authors declare no competing financial interest.

INTRODUCTION

cAMP-response element binding protein (CREB) is a critical nuclear transcription factor, enabling cells to detect and respond to the extracellular microenvironment through transcriptional regulation.¹ In an unstimulated cell, CREB constitutively binds to the cAMP-response elements (CRE) in the genome in a transcriptionally inactive state. Its transcription activity is activated upon phosphorylation mediated by a number of protein serine/threonine kinases, which are collectively called CREB kinases.¹ Once phosphorylated, CREB can bind to the transcription coactivators, including CREB-binding protein (CBP) and its paralogue p300 through their KIX domain and kinase-inducible domain (KID) in CREB.^{2,3} The known CREB kinases include protein kinase A (PKA), protein kinase B (PKB/Akt), mitogen-activated protein kinases (MAPKs), and ribosomal S6 kinase (pp90^{RSK}). The CREB's transcription activity and phosphorylation status are tightly regulated in normal cells, allowing activation of CREB in a timely and pulsative fashion. The key signaling event to turn off CREB's transcription activity is dephosphorylation. Three phosphatases, including protein phosphatase 1 (PP1),⁴ protein phosphatase 2 (PP2),⁵ and phosphatase and tensin homologue (PTEN),⁶ are known to dephosphorylate CREB and consequently inactivate CREB's transcription activity.

The CREB kinases are often overactivated in cancer cells through either mutation or excessive growth signals. On the other hand, the phosphatases that can dephosphorylate CREB are known as potent tumor suppressors and often inactivated in the cancer cells through both genetic and nongenetic mechanisms.⁷ As a consequence of the positive and negative regulation of CREB, cancer tissues from different organs have been consistently shown to present higher expression levels of CREB and/or phosphorylated CREB than their normal counterparts.⁸ These cancers include acute myeloid leukemia (AML), breast cancer, prostate cancer, nonsmall cell lung cancer, glioblastoma (GBM), and kidney cancer.⁸⁻¹⁰ Very interestingly, the CREB signaling has also been shown to mediate melanoma cell resistance to BRAF(V600E) and MAPK inhibitors as well as the progression of castration-resistant prostate cancer.^{11,12} Therefore, targeting CREB has been pursued as an attractive strategy to develop novel cancer therapies that may ultimately overcome therapy resistance.^{8,13-15}

Recently, we described the identification of **666-15** as a potent and selective inhibitor of CREB-mediated gene transcription (Figure 1).^{13,16} Among a panel of other transcription factors, it was found that **666-15** selectively inhibited CREB-mediated gene transcription.¹⁶ With **666-15** as a chemical tool, we showed that in vivo systemic inhibition of CREB was well-tolerated and produced efficacious anti-breast cancer activity.^{13,16} Others have also shown that **666-15** inhibited AML, GBM, and pancreatic cancer cell growth in different preclinical models.¹⁷⁻¹⁹ Furthermore, recent studies have shown that **666-15** could target the tumor microenvironment to present beneficial anticancer effect. For example, **666-15** was shown to inhibit pro-tumorigenic IL-6 expression from cancer-associated fibroblasts²⁰ and reverse the immunosuppressive effect of myeloid-derived suppressor cells (MDSC),²¹ suggesting the potential of **666-15** to modulate tumor microenvironment and immunotherapy.

666-15 was derived from optimization of a less potent derivative **1**,¹³ which was initially identified through our discovery of a rapid long-range *O,N*-acyl transfer reaction of the ester compound **2** (Figure 1).¹⁵ One advantage of compound **2** over compound **1** is its high aqueous solubility (>100 mg/mL in ddH₂O).¹⁵ In this report, we took advantage of the fact of high aqueous solubility of **2** and designed a corresponding ester compound **3** as a potential prodrug of **666-15** with improved aqueous solubility. Unexpectedly, compound **3** was found to undergo a different conversion reaction at physiologically relevant pH (7.40). Furthermore, we discovered a novel compound that can potentiate **666-15**'s inhibitory effect against CREB-mediated gene transcription in living cells.

RESULTS AND DISCUSSION

The synthesis of compound **3** is presented in Scheme 1. Building blocks **4** and **5** were prepared as described before.¹³ The ester coupling reaction between phenol **4** and acid **5** was found to be problematic. Under the coupling conditions we used before (BOP, DIPEA, room temperature),¹³ a mixture of compositional isomers **6** and **7** involving acyl group swapping was obtained. These two isomers were only partially separable by conventional silica gel column chromatograph. Other coupling conditions (EDCI, DIPEA; DCC, DIPEA; MsCl, Et₃N) were also attempted, but the results were found to be similar. The formation of the unanticipated alternative ester **7** was likely a result of generation of imide intermediate **8**, although it was not detected from the reaction mixture. The intermediate **8** could be generated either before the formation of **6** or after **6** was formed. Once **8** was produced, it could generate either **6** or **7** through an ester bond formation (Scheme 1). After considerable experimentation involving the modifications of solvents, coupling reagents, and temperature, we found that lowering the reaction temperature from room temperature to 0 °C and shortening the reaction time to 1 h could significantly inhibit the formation of **7**, although complete reaction conversion was not achieved under these conditions. The Boc groups in **6** were then deprotected under acidic conditions (HCl, DCM) to give **3** uneventfully (Supporting Information (SI), Figure S1). As expected, compound **3** was found to be highly soluble in ddH₂O (>100 mg/mL). This is in strong contrast to **666-15**, which is much less soluble in ddH₂O (<0.5 mg/mL).

With the desired compound **3** in hand, we first investigated the possibility of transformation of **3** into **666-15** in the complete cell culture media (Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (FBS)). When **3** was incubated with the complete cell culture media at 37 °C, it was rapidly converted into four new product peaks as assessed by HPLC (Figure 2B). In stark contrast to the structural congener **2**,¹⁵ only a small amount of **666-15** was generated under the reaction condition. To investigate if simple ester hydrolysis was occurring to generate products **9** and **10** (Figure 2A), we compared the main peaks eluted at 10.5 and 11.2 min with standard samples of **9** and **10** by HPLC. However, these peaks did not correspond to either compound **9** or **10** (data not shown).

To identify the products formed from incubating **3** in the complete cell culture media, we treated compound **3** in phosphate-buffered saline (PBS, pH = 7.40) (Scheme 2). An additional Boc protection step was added to facilitate purification of compounds containing free amino groups. After careful silica gel column purification and NMR analyses, we

obtained an inseparable mixture of **13** and **14** in 18% total yield with a ratio of 3:1 as assessed by ^1H NMR. Furthermore, two unexpected products, **15** (47%) and **12** (29%), were formed. The identity of **12** was further confirmed by an independent synthesis from **16**¹³ through Boc deprotection and cyclization (Scheme 2).

With the products identified from Scheme 2 and standard samples from the Boc-deprotected products, we were able to assign the peak eluted at 10.5 min being **12**, 11.2 min being **11**, and the shoulder peak close to **666-15** being **17** (Figure 2B and Scheme 3). On the basis of our previous results with compound **2**,¹⁵ we initially hypothesized that the mechanism of formation of **666-15** from **3** would be through pathway **A** by a long-range *O,N*-acyl transfer reaction (Scheme 3). Although we could not completely rule out this possibility, a more likely scenario is through pathway **B** as shown in Scheme 3. In this mechanism, imide **18** was first formed from **3**. Three possibilities exist for imide **18**. Pathway **a** would generate **11** and **12**, and this route was found to be the major pathway. Minor pathways **b** and **c** would generate **17** and **666-15**, respectively.

Because generation of the potent CREB inhibitor **666-15** from **3** in the complete tissue culture media was not the major pathway (Figure 2B), we expected that compound **3** would not be a potent CREB inhibitor. To test this possibility, we employed a transcription luciferase reporter assay in HEK 293T cells.²² Briefly, the cells were transfected with a CREB *Renilla* luciferase reporter plasmid (CRE-RLuc). This plasmid expresses *Renilla* luciferase under the control of three tandem copies of CRE in the promoter region. After transfection, the cells were treated with different concentrations of compound **3** for 30 min when forskolin (Fsk) was added to stimulate CREB phosphorylation and its subsequent transcription activity. In this assay, **666-15** has an $\text{IC}_{50} \sim 80 \text{ nM}$ (Table 1).¹³ Surprisingly, compound **3** was found to be quite potent, with an $\text{IC}_{50} = 0.25 \pm 0.11 \mu\text{M}$ (Table 1 and Figure 3A) despite the fact that only a small fraction of **3** was converted into **666-15** under these conditions (Figure 2B). This result suggested that other components in the reaction mixture might inhibit CREB-mediated gene transcription. Thus, the major compounds **11** and **12** were separately evaluated for their CREB inhibition potency. Compound **11** was found to be a very weak inhibitor with $\text{IC}_{50} = 26.3 \pm 13.6 \mu\text{M}$, while compound **12** was inactive (Table 1 and Figure 3A). Consistent with its weak activity in inhibiting CREB's transcription, compound **11** did not inhibit KIX-KID interaction as assessed in a biochemical split *Renilla* luciferase complementation assay ($\text{IC}_{50} > 50 \mu\text{M}$).²² The rather weak CREB inhibition activity associated with **11** and **12** suggested other possibilities to account for the potent CREB inhibition activity of **3**. One of the possibilities was that the mixture might have a synergistic activity. To test this possibility, we evaluated the combination of **666-15** with **11** or **12**. As shown in Figure 3B, combination of **666-15** with **12** did not produce further inhibition of CREB-mediated gene transcription. However, when **666-15** was combined with **11**, it significantly potentiated activity of **666-15** in inhibiting CREB-mediated gene transcription. Importantly, the synergistic inhibition occurred at concentrations where **11** alone was not inhibiting CREB-mediated gene transcription (Figures 3A,B). The synergy between **666-15** and **11** was further demonstrated by the low combination indexes (CI) calculated using the Chou-Talalay method (SI, Figure S2 and Table S1), where $\text{CI} > 1.0$ indicates antagonism, $\text{CI} = 1.0$ indicates additive effect, and $\text{CI} <$

1.0 indicates synergism.²³ These results indicated that the potent CREB inhibition activity of **3** is primarily a result of the combination of in situ generated **666-15** and **11**.

To further test that compound **11** could potentiate **666-15**'s CREB inhibitory activity, we evaluated the endogenous CREB target gene expression in HEK 293T cells. Nuclear receptor subfamily 4 group A member 2 (*NR4A2*) is a well-established CREB target gene in HEK 293T cells.²⁴ We have shown that *NR4A2* was robustly stimulated by Fsk, and its expression was suppressed by **666-15**.¹³ Therefore, we investigated the expression level of *NR4A2* in HEK293T cells with the combination of **666-15** and **11** using quantitative reverse transcription polymerase chain reaction (qRT-PCR). As reported previously,¹³ the expression level of *NR4A2* was stimulated by Fsk (Figure 4). When the cells were pretreated with low concentration of **666-15** (50 nM), the expression level of *NR4A2* was not significantly reduced. At higher concentration (100 nM), its transcript level was significantly inhibited. Consistent with the transcription reporter assay results shown in Figure 3B, 5 μ M of compound **11** alone did not inhibit the expression of *NR4A2*. Instead, a minor increase of its expression was observed. However, when low concentration of **666-15** (50 nM) was combined with compound **11** (5 μ M), significant inhibition of *NR4A2* transcription was observed (Figure 4). Together with the transcription reporter assay results shown in Figure 3B, these data support that while compound **11**, which was named as **653-47**, was not active alone, it could significantly potentiate the CREB inhibitory activity of **666-15**.

We previously showed that **666-15** potently inhibited breast cancer cell growth.¹³ Our discovery of **11** as a potentiator to enhance **666-15**'s potency in inhibiting CREB-mediated gene transcription suggested that it might also potentiate **666-15**'s anticancer potential in breast cancer cells. To test this hypothesis, we treated MDA-MB-231 cells with different concentrations of **666-15** along with or without **11** for 72 h. Then the antiproliferative effect was evaluated using MTT assay as described before.¹³ As shown in SI, Figure S3, **666-15** was able to dose-dependently inhibit the cell growth. Compound **11** alone did not appreciably inhibit the cell growth at low concentrations (0.5 or 1.0 μ M). However, when **11** was combined with **666-15**, it further enhanced **666-15**'s antiproliferative effect as indicated by the CI values. The CI values are all less than 1.0, indicating synergism between **666-15** and **11**.

CONCLUSIONS

In this paper, we described our unexpected discovery of a potentiator compound **11** or **653-47** to synergistically inhibit CREB-mediated gene transcription with **666-15**. We initially set out to improve the aqueous solubility of **666-15** by designing an ester prodrug **3**, which was envisioned to undergo a long-range *O,N*-acyl transfer reaction. Our results showed that a modified *O,N*-acyl transfer process through an imide intermediate is a more likely mechanism of formation of **666-15** from the ester prodrug **3** (Scheme 3). Unexpectedly, we discovered compound **653-47** as an enhancer of **666-15**'s activity in inhibiting CREB-mediated gene transcription and breast cancer cell growth even though **653-47** alone does not inhibit these activities. This discovery opens new avenues to further target CREB-mediated gene transcription with small molecules. Because CREB's transcription activity is regulated at multiple levels, including phosphorylation, nuclear

translocation, chromatin modulation, and DNA-binding, this discovery of **653-47** shall also provide a unique tool to further study CREB regulation.

EXPERIMENTAL SECTION

Chemistry: General.

A Glass Contour solvent purification system was used to purify all the anhydrous solvents to be used for reactions. Melting points were determined in capillary tubes using Mel-Temp and are uncorrected. All ^1H and ^{13}C NMR spectra were obtained in a Bruker Avance 400 MHz spectrometer using CDCl_3 or $\text{DMSO}-d_6$ as the solvent, and the chemical shifts of the residual CHCl_3 (δ 7.24) or DMSO (δ 2.50) were taken as reference. Chemical shifts (δ) are reported in parts per million (ppm), and the signals are described as brs (broad singlet), d (doublet), dd (doublet of doublet), td (triplet of doublet), m (multiplet), q (quartet), s (singlet), and t (triplet). Coupling constants (J values) are given in Hz. Silica gel flash chromatography was performed using 230–400 mesh silica gel (EMD). All reactions were monitored using thin-layer chromatography (TLC) on silica gel plates (EMD). Yields were of purified compounds. All final compounds for biological evaluations were confirmed to be of >95% purity based on reverse phase HPLC (Waters, Milford, MA) analysis using an XBridge C18 column (4.6 mm \times 150 mm) and detected at 254 nm. The mobile phases for HPLC are water and acetonitrile, both of which contained 0.1% TFA. The mass spectra were obtained from a Thermo Electron LTQ-Orbitrap Discovery high resolution mass spectrometer (Thermo Scientific) with electrospray operated either in positive or negative mode.

2-(3-(2-Aminoethoxy)-2-naphthamido)-5-chlorophenyl 3-(3-Aminopropoxy)-2-naphthoate Dihydrochloride (3).—An HCl solution in Et_2O (2 M, 3 mL) was added to a stirred solution of **6** (75 mg, 0.095 mmol) in DCM (3 mL) at room temperature. The resulting mixture was stirred at room temperature for 4 h. The solvent was removed and the residue was treated with acetone and the precipitate was collected by filtration to give product **3** (8 mg, 13%) as a white solid; mp 216–217 °C. ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 10.18 (s, 1 H), 8.75 (s, 1 H), 8.24 (brs, 3 H), 8.09 (s, 1 H), 8.01–7.78 (m, 8 H), 7.66–7.60 (m, 3 H), 7.58–7.45 (m, 4 H), 7.40 (t, $J = 7.6$ Hz, 1 H), 7.35 (t, $J = 7.7$ Hz, 1 H), 4.28 (t, $J = 5.4$ Hz, 2 H), 4.23 (t, $J = 5.4$ Hz, 2 H), 3.21 (q, $J = 5.8$ Hz, 2 H), 3.01 (q, $J = 5.8$ Hz, 2 H), 2.03 (quintet, $J = 6.0$ Hz, 2 H). HRESIMS calcd for $\text{C}_{33}\text{H}_{31}\text{ClN}_3\text{O}_5$ $[\text{M} + \text{H}]^+$ 584.1947, found 584.1943.

2-(3-(2-((tert-Butoxycarbonyl)amino)ethoxy)-2-naphthamido)-5-chlorophenyl 3-(3-((tert-Butoxycarbonyl)amino)propoxy)-2-naphthoate (6).—To a stirred solution of **5**¹⁵ (173 mg, 0.5 mmol) in dichloromethane (10 mL) was added BOP (221 mg, 0.5 mmol) and DIPEA (90 μL , 0.5 mmol). The reaction mixture was stirred at room temperature for 5 min. Then the reaction mixture was cooled down to 0 °C. Compound **4**¹³ (229 mg, 0.5 mmol) and another portion of DIPEA (120 μL , 0.65 mmol) were added sequentially. The resulting mixture was stirred for 1 h at 0 °C. The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography, eluting with dichloromethane–ethyl acetate (20:1) to give the crude product **6**, which was

further purified by the Biotage purification system, eluting with hexanes–ethyl acetate (20:1 to 4:1) to give pure product **6** (75 mg, 19%) and some less pure product (170 mg, 43%). ¹H NMR (400 MHz, CDCl₃) δ 9.81 (s, 1 H), 8.74 (s, 1 H), 8.58 (s, 1 H), 8.41 (d, *J* = 8.8 Hz, 1 H), 7.87 (d, *J* = 8.2 Hz, 1 H), 7.81 (d, *J* = 8.2 Hz, 1 H), 7.76 (d, *J* = 8.2 Hz, 1 H), 7.67 (d, *J* = 8.2 Hz, 1 H), 7.59 (t, *J* = 7.6 Hz, 1 H), 7.51 (t, *J* = 7.5 Hz, 1 H), 7.44–7.37 (m, 3 H), 7.34 (dd, *J* = 8.8, 2.3 Hz, 1 H), 7.24 (s, 1 H), 7.16 (s, 1 H), 5.40 (brs, 1 H), 4.73 (brs, 1 H), 4.12 (t, *J* = 7.1 Hz, 2 H), 3.91 (t, *J* = 5.4 Hz, 2 H), 3.33–3.07 (m, 4 H), 1.89 (brs, 2 H), 1.35 (s, 9 H), 1.33 (s, 9 H). ¹³C NMR (101 MHz, CDCl₃) δ 163.67, 163.47, 156.28, 155.88, 155.05, 153.27, 142.16, 136.73, 135.79, 134.20, 129.69, 129.62, 129.58, 129.10, 128.98, 128.68, 128.35, 127.33, 126.70, 126.60, 126.38, 125.13, 125.03, 124.84, 122.73, 108.42, 107.92, 79.62, 78.88, 68.33, 67.55, 39.37, 38.30, 29.05, 28.36, 28.27.

3,4-Dihydronaphtho[2,3-f][1,4]oxazepin-5(2H)-one (12).—Compound **16** (103 mg, 0.3 mmol) was treated with TFA (1 mL) for 1 h at room temperature. TFA was removed under reduced pressure, and the residue was redissolved in MeOH (4 mL) and DIPEA (1 mL). The mixture was heated under reflux for overnight. The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography, eluting with dichloromethane–methanol (20:1) to give product **12** (25 mg, 39%) as a yellow solid; mp 128–129 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.39 (s, 1 H), 7.91 (d, *J* = 8.2 Hz, 1 H), 7.78 (d, *J* = 8.3 Hz, 1 H), 7.56–7.51 (m, 1 H), 7.50 (s, 1 H), 7.45 (m, 1 H), 7.07 (s, 1 H), 4.39 (t, *J* = 5.4 Hz, 2 H), 3.48 (q, *J* = 5.6 Hz, 2 H). ¹³C NMR (101 MHz, CDCl₃) δ 171.14, 151.27, 136.06, 132.05, 130.11, 128.93, 128.09, 127.50, 126.81, 125.55, 118.36, 73.79, 40.50. HRESIMS calcd for C₁₃H₁₂NO₂ [M + H]⁺ 214.0863, found 214.0858.

Biology.

General.: HEK293T cells were from ATCC and MDA-MB-231 cells were from the Development Therapeutics Program at the National Cancer Institute. The cells were routinely cultured in DMEM (Life Technologies) with 10% fetal bovine serum (FBS, Hyclone) in a humidified incubator with 5% CO₂ at 37 °C. The cells were confirmed to be mycoplasma negative and authenticated through STR profiling. pCRE-RLuc was reported before.²² All the compounds for biological testing were dissolved in DMSO as stock solutions except compound **3**, which was dissolved in DMF because compound **3** was found to be unstable in DMSO for long-term storage. Compounds **11** and **17** were reported previously.¹³

Inhibition of CREB-Mediated Gene Transcription.: HEK293T cells in a well of 6-well plate were transfected with a plasmid (1 μg) encoding *Renilla* luciferase under the control of three copies of CRE (pCRE-RLuc) using Lipofectamine²⁰⁰⁰ (Life Technologies) for 3 h. Then the cells were replated into a 96-well plate, and the cells were allowed to attach to the bottom of the plate for overnight. The cells were treated with different concentrations of the compounds for 30 min, when Fsk (10 μM) was added to the cells. The cells were further incubated for 5 h at 37 °C. The media were removed and the cells were lysed in 30 μL of 1× *Renilla* luciferase lysis buffer (Promega) and the *Renilla* luciferase activity was measured using the *Renilla* luciferase assay system (Promega). The protein concentration in each well was determined using the Protein Assay Dye Reagent Concentrate (Biorad). The luciferase

activity in each well was normalized to the protein concentration and expressed as relative luciferase unit (RLU)/ μg protein. The IC_{50} was calculated in Prism 5.0 using the nonlinear regression analysis.

Conversion of Ester 3 in DMEM.: Compound **3** ($200 \mu\text{M}$) was incubated with complete tissue culture media (DMEM with 10% FBS) at 37°C for various time periods. An aliquot of $10 \mu\text{L}$ was taken at a given time point and mixed with acetonitrile ($90 \mu\text{L}$). The mixture was centrifuged at $14000\times$ rpm for 5 min at room temperature in a tabletop centrifuge to precipitate proteins. The supernatant was collected and analyzed by HPLC eluting with a linear of water and acetonitrile, both of which contained 0.1% TFA.

Inhibition of Endogenous CREB Target Gene Expression.: HEK 293T cells in 6-well plates were treated with different concentrations of **666-15** along with or without **11** for 1 h. Then the cells were stimulated with Fsk ($10 \mu\text{M}$) for 45 min. The total RNA was isolated using NucleoSpin RNA isolation kit (Takara Bio). Following RNA isolation, the first strand cDNA was synthesized using a PrimeScript first strand cDNA synthesis kit (Takara Bio) with random hexamers as the primers. Quantitative PCR reactions were performed with TB Green Advantage qPCR Premix (Takara Bio). The relative mRNA level was determined with C_t method using *HPRT* as a reference gene. Primers used for qRT-PCR are available upon request.

Cell Growth Inhibition Assay.: The cells were plated in 96-well plates at 1000 cells/well and allowed to attach to the bottom of the plate for overnight. Then the cells were treated with different concentrations of compounds for 72 h at 37°C . At the end of treatment, the viable cells were determined by incubating cells with MTT reagent (0.5 mg/mL) in the complete tissue culture media for 3 h. Then the media were removed, and the formed purple formazan was dissolved in DMSO for quantification by absorbance at 570 nm using a SpectraMax i3 plate reader (Molecular Devices). The percent of growth is defined as $100 \times (A_{\text{treated}} - A_{\text{initial}})/(A_{\text{control}} - A_{\text{initial}})$, where A_{treated} represents absorbance in wells treated with a compound, A_{initial} represents the absorbance at time 0, and A_{control} denotes media-treated cells.

Statistics.: The statistics was computed using student *t*-test in Microsoft Excel, and $P < 0.05$ was considered significant. The CI values were calculated using Chou–Talalay method implemented in CompuSyn program.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

AML	acute myeloid leukemia
CRE	cAMP-response element
CREB	cAMP-response element binding protein
CBP	CREB-binding protein
Fsk	forskolin
GBM	glioblastoma
HPLC	high-performance liquid chromatography
MAPK	mitogen-activated protein kinase
NR4A2	nuclear receptor subfamily 4 group A member 2
PBS	phosphate buffered saline
PKA	protein kinase A
PKB	protein kinase B
RSK	ribosomal S6 kinase
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PTEN	phosphatase and tensin homologue
qRT-PCR	quantitative reverse transcription polymerase chain reaction
SD	standard deviation
SEM	standard error of the mean

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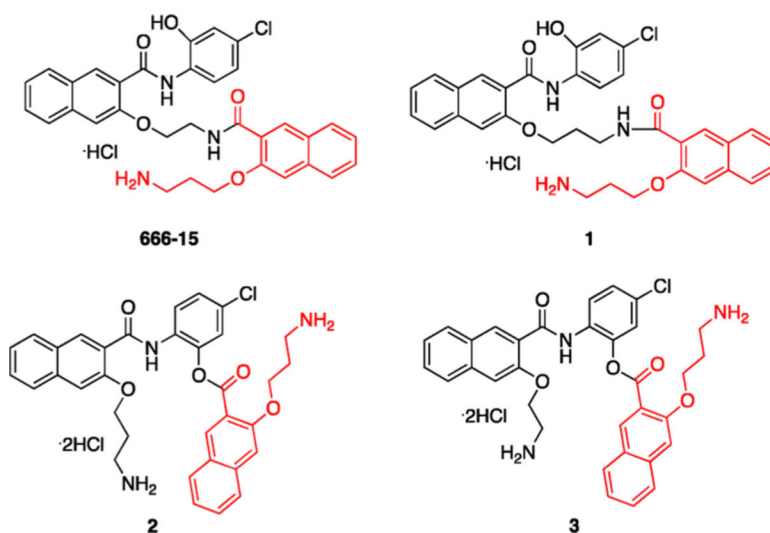


Figure 1. Chemical structures of CREB inhibitors **666-15**, **1**, **2**, and designed potential ester prodrug **3**. The migrating acyl group is highlighted in red.

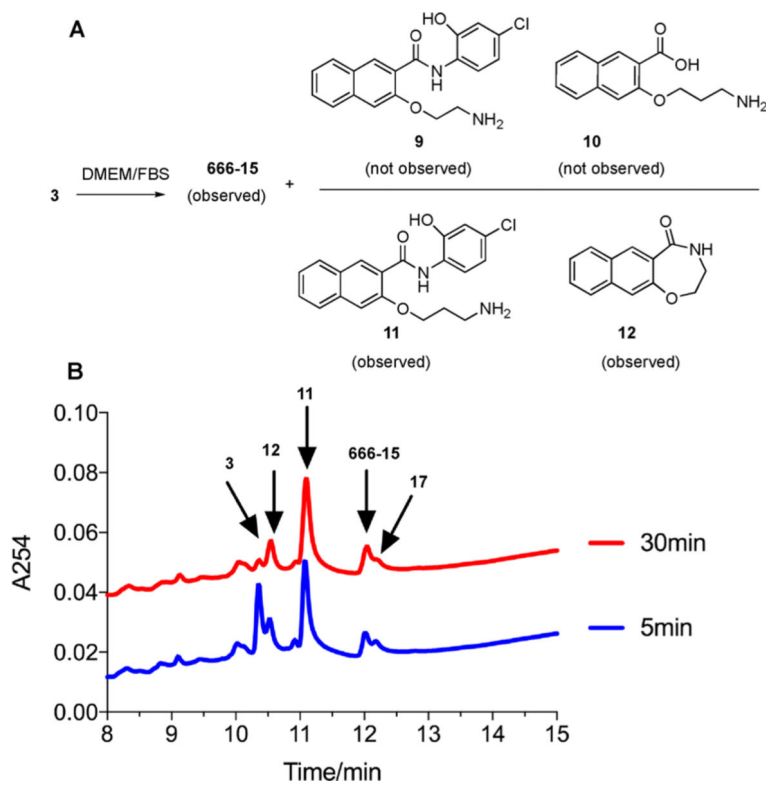


Figure 2. Compound **3** was converted into **666-15**, **11**, and **12** in the complete cell culture media. (A) Reaction conversion of **3** in complete cell culture media to generate a mixture of **666-15**, **11**, and **12**. (B) HPLC chromatograms of compound **3** incubated in complete cell culture media for 5 and 30 min.

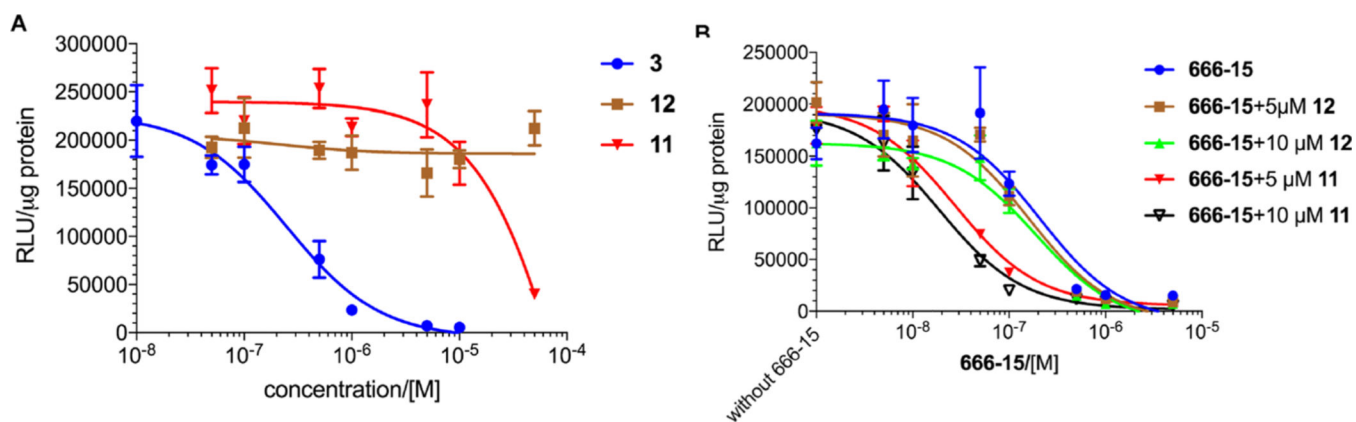


Figure 3.

Inhibition of CREB-mediated gene transcription. HEK 293T cells were transfected with CRE-RLuc. Then the cells were treated with increasing concentrations of individual compounds (A) or drug combinations (B) for 30 min before the addition of Fsk ($10 \mu\text{M}$) for 5 h. The *Renilla* luciferase activity was normalized to the protein concentration in each well and expressed as relative luciferase unit (RLU)/ μg protein. The errors are SEM from one representative experiment in triplicates.

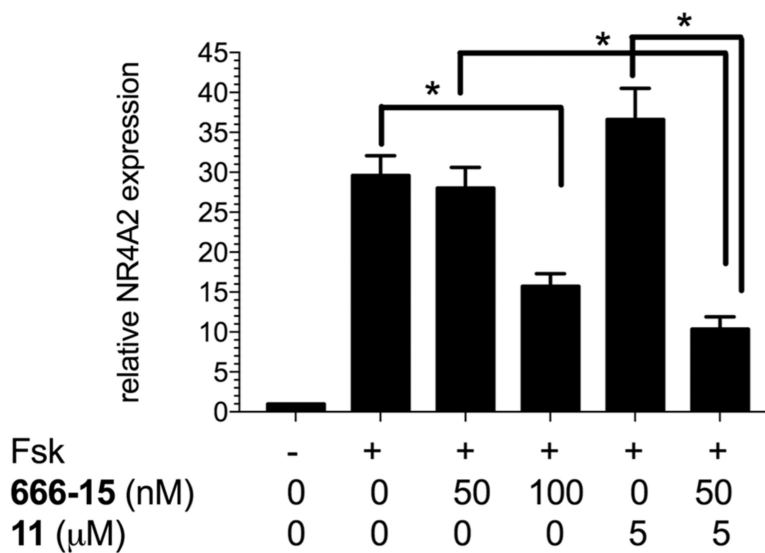
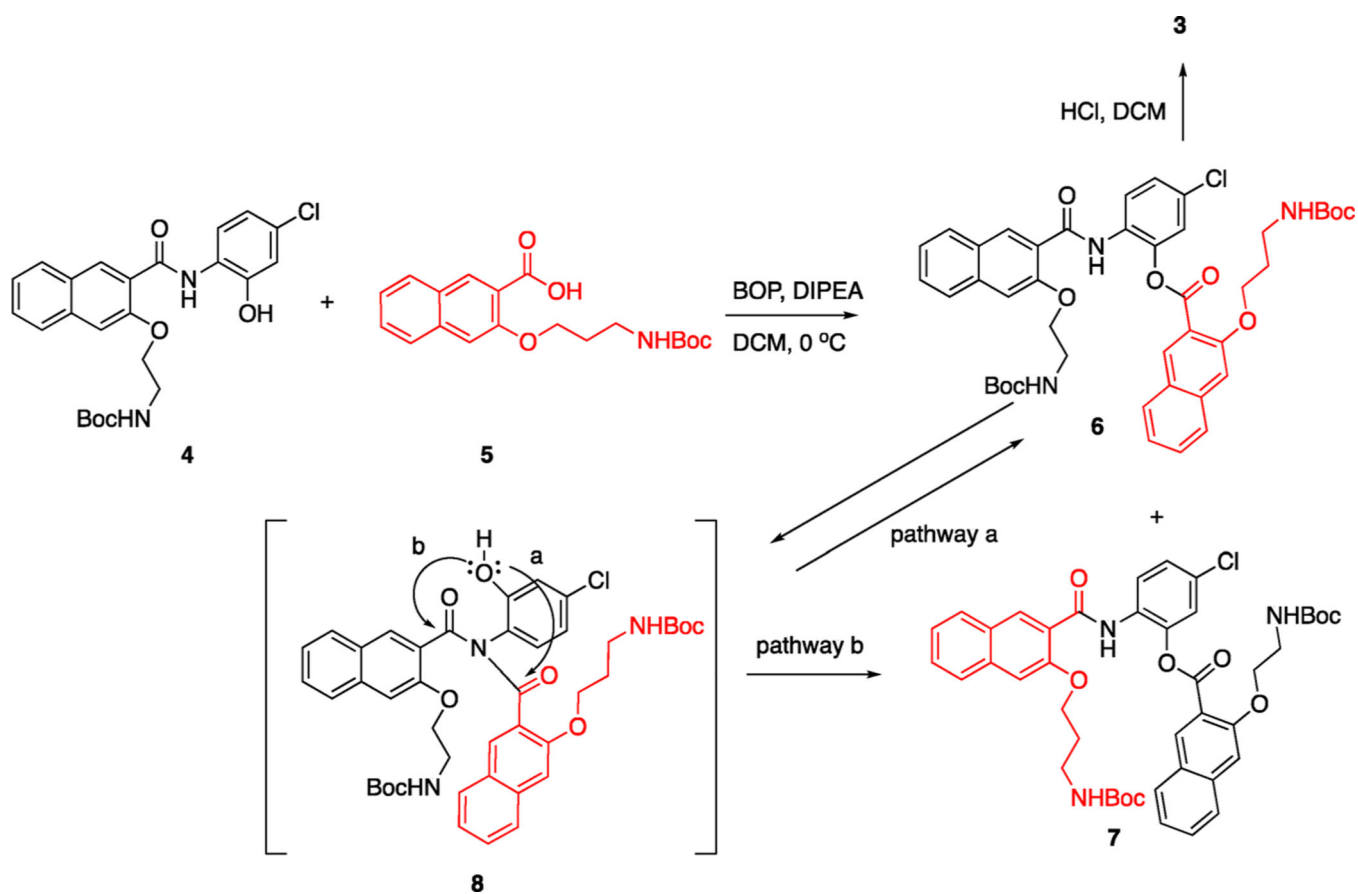
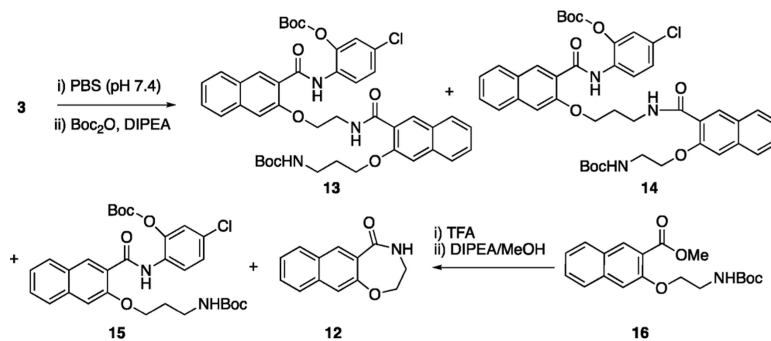


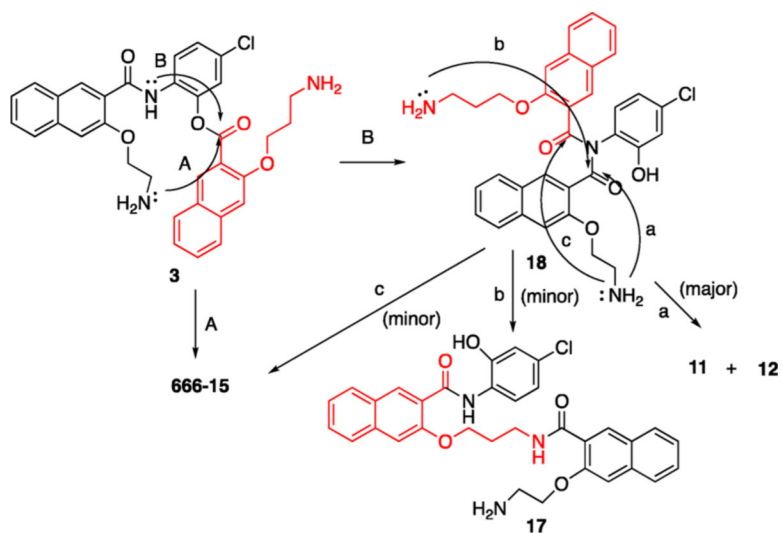
Figure 4. Synergistic inhibition of CREB-mediated gene transcription in HEK 293T cells by **666-15** and **11**. HEK 293T cells were treated with **666-15** and **11** for 1 h, when the cells were stimulated with Fsk (10 μ M) for 45 min. Then the cells were subjected to qRT-PCR analysis after the total RNA was isolated using *HPRT* as a reference gene. The errors are SEM of two independent experiments performed in triplicates. * $P < 0.05$ by Student *t*-test.



Scheme 1. Synthesis of Ester Compound 3



Scheme 2. Reaction Conversion of 3 in PBS



Scheme 3. Proposed Mechanistic Pathways of Conversion of 3 into 666-15, 11, 12, and 17 in Physiologically Relevant Buffers

Table 1.

CREB Inhibitory Activity of Different Compounds

compd	CREB inhibition (IC ₅₀ , μ M) ^a
666-15	0.081 \pm 0.04
3	0.25 \pm 0.11
11	26.3 \pm 13.6
12	>50

^aThe IC₅₀ refers to the concentration needed to inhibit 50% of the CREB-mediated gene transcription in HEK 293T cells using a transcription *Renilla* luciferase reporter assay. The data are presented as mean \pm SD of at least two independent experiments, which were performed in triplicate.