Identification of compounds that potentiate CREB signaling as possible enhancers of long-term memory

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Many studies have implicated the cAMP Response Element Binding (CREB) protein signaling pathway in long-term memory. To identify small molecule enhancers of CREB activation of gene expression, we screened ≈73,000 compounds, each at 7–15 concentrations in a quantitative high-throughput screening (qHTS) format, for activity in cells by assaying CREB mediated β-lactamase reporter gene expression. We identified 1,800 compounds that potentiated CREB mediated gene expression, with potencies as low as 16 nM, comprising 96 structural series. Mechanisms of action were systematically determined, and compounds that affect phosphodiesterase 4, protein kinase A, and cAMP production were identified, as well as compounds that affect CREB signaling via apparently unidentified mechanisms. qHTS folowed by interrogation of pathway targets is an efficient paradigm for lead generation for chemical genomics and drug development.

memory enhancer | phosphodiesterase inhibitor | quantitative high-throughput screening

The Cyclic-AMP Response Element Binding (CREB) protein plays an important role in learning and long-term memory (LTM) formation by coupling neuronal activity with changes in gene expression (1). CREB proteins comprise a family of transcription factors that regulate the transcription of genes through binding to cAMP Responsive Elements (CRE), such as the nucleotide sequence, 5′-TGACGTCA, in DNA. CREB signaling occurs via a multistep pathway in which binding of hormones or neurotransmitters to G protein coupled cell surface receptors activates adenylate cyclase (AC) and catalyzes the production of cAMP. Elevation of cAMP levels leads to activation of protein kinase A (PKA) and translocation of the catalytic subunits of PKA to the nucleus, where they catalyze phosphorylation of CREB at Ser-133, which activates CREB (2, 3). Phosphorylated CREB then binds to CRE nucleotide sequences in DNA as a dimer (4), and recruits cofactors such as CREB-Binding Protein (CBP) and p300 to form a larger transcriptional complex (5), which catalyzes histone acetylation and thereby regulates target genes. Negative regulators of the CREB pathway include phosphodiesterases that degrade cAMP, calcium-calmodulin protein kinase II that catalyzes phosphorylation of CREB at Ser-142, thereby promoting dissociation of the CREB dimer (6, 7), protein phosphatases 1 and 2A catalyze Ser-133 dephosphorylation (8, 9) thereby reducing the binding of CREB to DNA, and histone deacetylase, which catalyzes the removal of acetyl groups from histones.

The role of CREB signaling in long-term memory initially was defined by Kandel and colleagues in *Aplysia* (10). Overexpression of a dominant-negative CREB (11, 12) or a CREB transcriptional repressor (dCREB2b) blocked the formation of LTM in transgenic flies (13). These observations were subsequently extended to mouse models; for example, mutant $CREB^{\alpha\delta}$ mice exhibited impaired long-term memory in both spatial memory and contextual fear-conditioning tasks (14, 15), accompanied by defects in long-term potentiation in hippocampal slices (14). At the same time, small molecule modulators of the CREB signaling pathway were able to improve some measures of memory formation. Inhibition of PKA activity by targeted delivery of a PKA inhibitory peptide to the nucleus resulted in a decrease in CREB phosphorylation and defects in long-term potentiation (16); similarly, the impairment of object recognition long-term memory seen in heterozygous $CBP^{+/-}$ mice was rescued by administrating PDE4 inhibitors, and transgenic overexpression of a dominant active form of CREB partially reversed the late long-term potentiation deficits observed in $CBP^{+/-}$ mice (17, 18). These results suggested that modulators of the CREB pathway may be clinically useful for human disorders of memory and for ameliorating some of the features of Rubinstein-Taybi Syndrome (OMIM 180849), a human genetic disorder caused by mutation in the gene encoding CBP (19).

High-throughput screening (HTS) has developed over the last 2 decades as the principal method of lead identification in drug discovery (20). With advances in molecular biology and genomics, target-based screens, which detect small modulators of proteins, have overtaken traditional phenotypic screens in HTS applications (21). However, the target-based screens may lead to compounds being identified as active that sometimes do not have activity in the physiological environment. Cell signaling, or "pathway" assays offer a potentially attractive assay format intermediate in complexity between isolated molecular target and phenotypic assays, operating in an intact cellular environment but with the readout dependent on signaling through a single pathway, which contains multiple components.

We report here a panel of enhancers of CREB activity discovered by screening >73,000 compounds in a CREB pathway assay. These compounds will serve as probes for known and novel CREB pathway components, will be useful for the study of long-term memory, and potentially lead to new clinical memory enhancers for widely prevalent disorders such as Alzheimer's disease.

Results

Identification of Small-Molecule Enhancers of the CREB Pathway. Approximately 73,000 compounds were screened for CREB enhancer activity using a cell-based CRE-β-lactamase reporter gene assay (Fig. S1) in qHTS mode (22). Because qHTS tests each compound at 7–15 concentrations, compound potencies (EC_{50}s) and efficacies are immediately available after the pri-

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Data deposition: The structures reported in this paper have been deposited in the Pubchem database, http://pubchem.ncbi.nlm.nih.gov (AID: 662, 905, 906, 907, 916).

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Table 1. Potency and number of compounds found that enhance CREB activity

	Number of active compounds in curve classes		
EC_{50} *, μM	1.1	1.2	2.1
0.016–0.10	9	11	0
0.10–1.00	52	65	7
1.00–10.00	224	376	318
10.00–100.00	0	1	737
Total		1,800	

*Concentration for half maximal increase in CREB activity.

mary screen. A total of 1,800 compounds were classified as potentiators of CREB activity having class 1.1, 1.2, and 2.1 curves (see Fig. S2 for curve class definitions) and EC_{50}s <100 μM, yielding a hit rate of 2.5%. The curve classes and potencies of the 1,800 compounds are listed in Table 1. All data produced from this screen have been deposited in the PubChem database (http://pubchem.ncbi.nlm.nih.gov), AID 662. The detailed screen performance was described in *SI Results* (see Figs. S3 and S4) and the most potent compounds identified from this primary screening were listed in Fig. S5.

The information-rich primary results from qHTS allowed us to perform a structure-activity relationship (SAR) analysis on the active compounds. Ninety-six structural series (compounds that share a common substructure or scaffold form a structural series) were identified, 37 of which were selected for follow up studies based on compound selectivity, potency/efficacy ranges, and curve quality. Details on the procedure used for series prioritization can be found in *Materials and Methods*. Fig. 1*A* shows scaffolds of structural series containing compounds with the greatest potencies and highest quality concentration-response curves. These series are of particular interest as the scaffolds have no previously reported pharmacological activity.

A large number of compounds with known pharmacological activities are included in the NCGC screening collection, and many were active in the CREB qHTS (Fig. 1*B*). For example, rolipram (23) and papaverine (24), which are known PDE inhibitors, enhanced CREB activity with EC_{50}s of 200 nM and 1 μM, respectively. Compounds with known activities relevant to CREB signaling aid both in determining mechanisms of action of lead series, and in understanding the types of activities this pathway assay is capable of detecting.

For the follow-up studies, 81 active compounds from 37 prioritized structural series (average 2-3 compounds from each series) were selected and again tested using the original CHO CRE-β-lactamase assay; of the 81 compounds that had been tested in the primary screen, the activities of 80 compounds were confirmed in the repeat assay, yielding a confirmation rate of

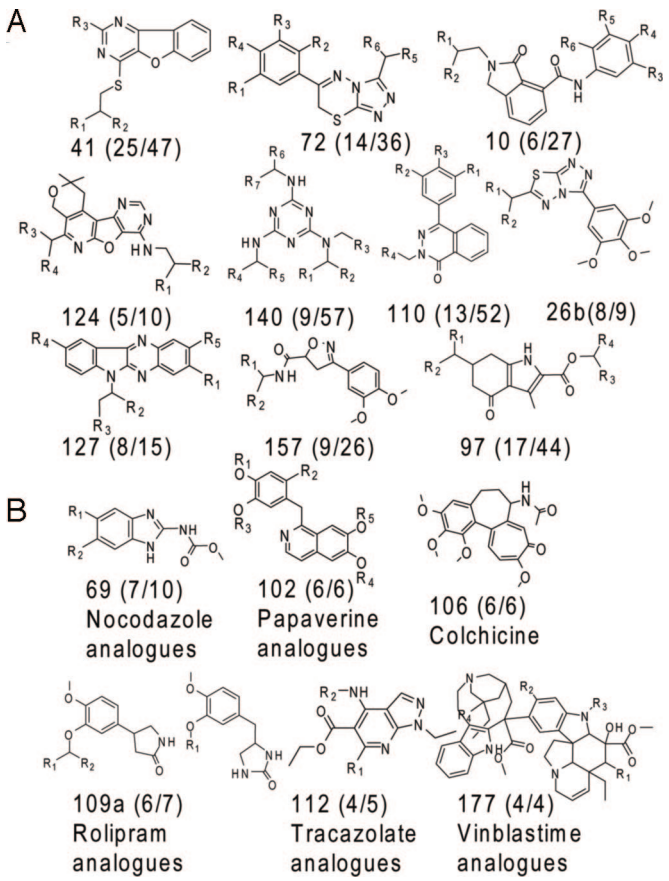

Fig. 1. Scaffolds of enhancers of the CREB signaling pathway. (*A*) Ten of 30 scaffolds we identified are shown. Each scaffold is identified by a number. (*B*) Six scaffolds of compounds with known biological activities are shown. In parentheses are shown the number of active compounds found over the total number of compounds from that scaffold screened.

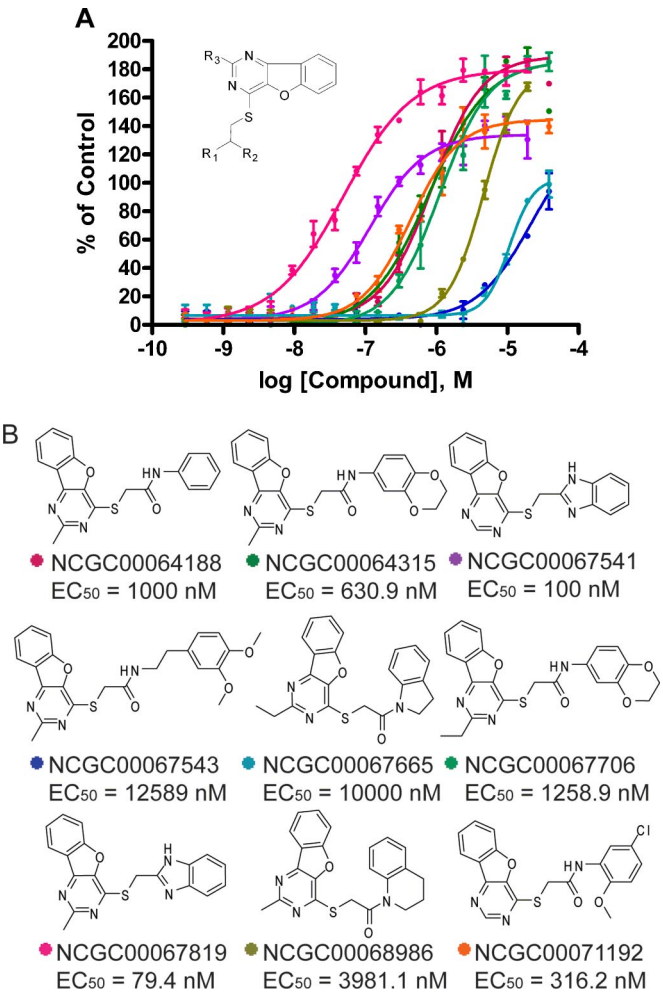

Fig. 2. Confirmation study for compounds from series 41, using the CRE β-lactamase assay. (*A*) Concentration response curves of selected compounds from series 41. (*B*) Structures of these compounds are shown with NCGC compound IDs and EC_{50} values.

Table 2. The potency of compounds in the reporter gene assays and in cAMP and PDE4 assays

Structure (series)	Compound ID	β-Lactamase EC_{50}, μM	Luciferase EC_{50}, μM	cAMP* EC_{50}, μM	PDE4 IC_{50}, μM
10	00084423	7.94	0.63	3.16	10.0
41	00071192	0.32	3.16	1.00	Inactive
41	00067819	0.08	1.26	0.13	10.0
41	00064188	1.00	3.98	1.58	15.8
41	00064315	0.63	3.98	1.26	15.8
41	00067541	0.10	1.26	0.31	25.1
72	00071837	1.99	0.04	3.98	0.03
72	00070065	6.3	1.00	Inactive	7.94
80	00075741	2.51	1.58	3.16	3.16
110	00053417	3.8	0.32	10.0	2.51
26b	00070528	5.01	5.01	7.94	Inactive
124	00065586	6.31	0.40	Inactive	3.16
127	00056237	12.5	5.01	Inactive	1.00
140	00030627	12.5	2.51	Inactive	0.20
109a	00024862	0.20	0.03	Inactive	0.20
102	00015810	1.00	0.50	1.26	7.94
112	00015985	5.01	0.79	15.8	0.31
112	00024590	3.98	3.16	3.98	3.16

*Total cAMP levels were measured in the presence of 100 μM Ro20–1724 and 100 nM NKH477. Names of the last four compounds are: 109a, Rolipram; 102, Papaverine; 112, Tracazolate; 112, Etazolate.

99% (details including EC_{50}s and curve classes for all 81 compounds are shown in Table S1). Next, these compounds were tested in human embryonic kidney 293 (HEK293) cells using a CRE-luciferase reporter gene assay. Of the 81 compounds tested in the primary screen, the activities of 77 were confirmed in the HEK293 CRE-luciferase assay, giving a concordance rate of 95%.

Two series were of particular interest. Series 41 (benzofuro[3,2-d]pyrimidines) comprised 47 compounds in the library screened, of which 25 were active with EC_{50} values ranging from 79 nM to 13 μM depending on the substituent groups (Fig. 2). The most potent compound identified in the primary screen was compound NCGC00067819, with an EC_{50} of 16 nM in the primary screen and 79 nM in the CHO CRE-β-lactamase confirmation assay. Most compounds from this series were less potent in the follow-up CRE-luciferase assay than in the CRE β-lactamase assay (Table 2) but the rank order of potency was similar. Little pharmacological activity has been reported for compounds in this structural series 41 (25, 26).

Series 72 (3a,6-dihydropyrazolo[3,4-b][1,4]thiazines) also was an unknown scaffold and comprised 36 compounds, of which 14 were active with EC_{50}s from 40 nM to 6.3 μM. In contrast to Series 41, compounds in Series 72 were generally slightly more potent in the CRE-luciferase assay than in the CRE β-lactamase assay (Table 2). The differences in compound potencies in the 2 CRE reporter gene assays may be due to differences in reporter gene constructs, cell lines, and species (Chinese hamster ovary cells vs. human embryonic kidney cells). In general series 72 compounds were more potent PDE4 inhibitors than those from series 41 (see *Inhibition of PDEs*). SAR also was observed for several other structure series; these data are presented in Table S1.

Inhibition of PDEs. CREB activity often is regulated through cAMP, levels which reflect a balance between the synthesis and degradation of intracellular cAMP. PDEs rapidly degrade cAMP. Therefore, inhibition of PDE leads to a decrease in cAMP degradation, which in turn enhances CREB activation. In the primary CRE β-lactamase screen, 13 known PDE inhibitors were identified as active compounds. Among them, papaverine

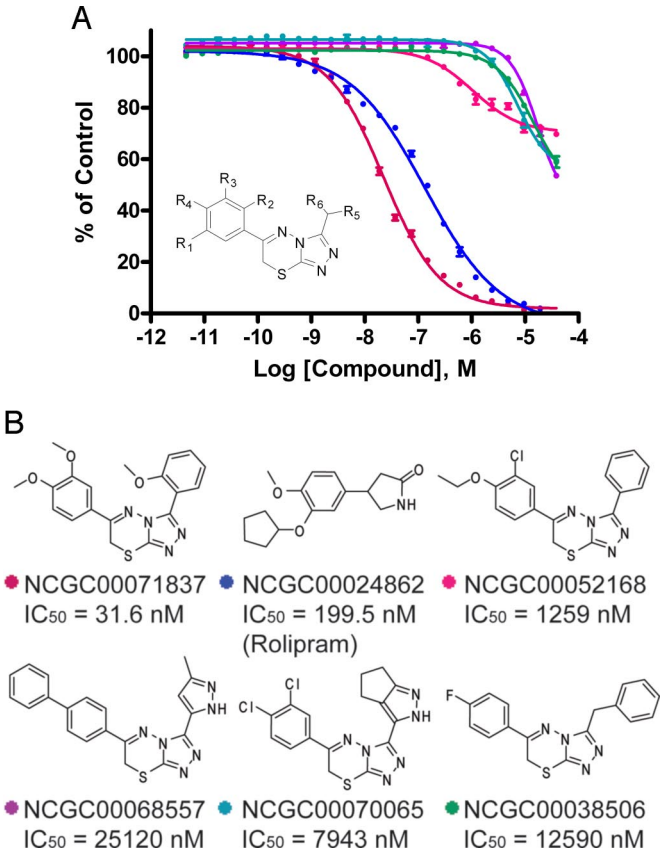

Fig. 3. Compounds from series scaffold 72 in the PDE4 enzyme assay. (*A*) Concentration response curves of selected compounds from series 72. (*B*) Structures of these compounds are shown with NCGC compound IDs and IC50 values. The scaffold for rolipram is 109a.

(24), IBMX (24), and diprydamole (23) are nonselective PDE inhibitors. Ro20–1724 (23), rolipram (23), and etazolate (27) are PDE4 selective inhibitors. Trequinsin (23) is a PDE3 selective inhibitor, and T-1032 (28) is a PDE5 selective inhibitor. Although many PDE inhibitors are described in the literature as being specific, many have activity against other PDEs, as is the case of trequinisin and T-1032, which are micromolar inhibitors of PDE4 (29, 30) and PDE4 probably is the predominant PDE present in CHO cells.

The 77 compounds confirmed active in the HEK293 CRE luciferase assay were tested in a biochemical assay using recombinant human PDE4; 53 compounds, covering 26 series, inhibited PDE4 with IC_{50}s ranging from 31 nM to 40 μM. NCGC00071837 from series 72 (Fig. 3) was the most potent PDE4 inhibitor with an IC_{50} of 31 nM, which was 6.5-fold more potent than rolipram (series 109a), a known, potent PDE4 inhibitor (23). The IC_{50} values of NCGC00030627 (series 140) and NCGC00065586 (series 124) were 200 nM and 316 nM, respectively, similar to that of rolipram (Table 2). All 3 compounds have structures distinct from rolipram and have no previously reported biological activity.

Effects on Protein Kinase A (PKA). PKA and protein phosphatase (PP) play critical roles in balancing the activation of CREB. PKA, a cAMP-dependent protein kinase, catalyzes the phosphorylation of CREB, resulting in the stimulation of gene transcription. In contrast, nuclear PP attenuates or inhibits cAMP-stimulated gene transcription by dephosphorylating CREB (8, 9) to return activated CREB to the inactive state. To test whether any CREB potentiating compounds identified in

the screen acted on PKA, compounds were tested in a PKA enzyme based assay that measured phosphorylation of a target peptide. 8-bromo-cAMP and 8-(4-chlorophenylthio)-cAMP, which are cAMP analogues, were found to be active in the primary CRE-β-lactamase screen, both with an EC_{50} of 22 μM, although 8-bromo-cAMP was much more potent in the PKA enzyme assay (EC_{50} = 5 nM), which may indicate the degradation of 8-bromo-cAMP in the cells. Of the 81 compounds tested in the PKA enzyme assay, tracazolate was the only compound found to activate PKA, with an EC_{50} of 12.5 μM (Fig. S6*A*). However, tracazolate showed 2–15-fold higher potencies in the cellular assays, with EC_{50} values of 5 μM and 0.8 μM, respectively, in the CRE-β-lactamase assay and CRE-luciferase assay, and a 39-fold higher potency in the PDE4 enzymatic assay (IC_{50} = 0.32 μM, Fig. S6*A*). In the cAMP assay tracazolate also was found to potentiate cAMP production in the presence of EC_{20} of NKH 477, with an EC_{50} of 18.7 μM (Fig. S6*A*). These results indicate that this compound more likely activates CREB through inhibition of PDE than activation of PKA and AC. Nevertheless, it is interesting that tracazolate had multiple effects on the inhibition of PDE and activation of PKA and AC because all of these effects would additively or synergistically enhance CREB activity.

In the CREB pathway, nuclear PP plays a negative feedback role by dephosphorylating CREB. In the primary screen we found that cantharidic acid (EC_{50} = 0.45 μM) and endothall (EC_{50} = 25 μM) were able to potentiate CREB signaling. Cantharidic acid has been reported as an inhibitor of PP2A and PP1 (31) with IC_{50}s of 53 and 562 nM, respectively. Its activity (EC_{50}) in the primary screen of CRE-β-lactamase assay was 450 nM. Endothall, another well-known PP2A inhibitor (31), has a reported activity of 970 nM for PP2A and 5 μM for PP1; its EC_{50} in the CRE β-lactamase assay was 25 μM. Together, these findings demonstrate that the CRE assay enabled the discovery of PP inhibitors and it remains possible that other compounds were identified in this screen via this mechanism.

Stimulation and Potentiation of cAMP Production. To detect compounds that directly stimulate cAMP production, intra-and extracellular cAMP were measured in the presence of 100 μM Ro 20–1724 (IC100), a PDE inhibitor, using the LANCE cAMP assay with CHO cells. The concentration of Ro 20–1724 used in the assay was adjusted to obtain almost maximum PDE inhibition. Under these conditions only one compound, NCGC00071837, stimulated cAMP production, with an EC_{50} of 12.6 μM. This result was confirmed in a seperate cAMP assay in HEK 293 cells (data not shown). Rolipram, a known PDE inhibitor, with an IC_{50} value of 200 nM in the confirmation CRE-β-lactamase assay was inactive in the presence of Ro 20–1724. Likewise, all of the other compounds with in vitro confirmed PDE activity were inactive as well. This nearly complete loss of activity among the other confirmed PDE active compounds indicates that NCGC00071837 may stimulate AC or act on targets upstream of AC in addition to PDE inhibition. To find compounds that potentiate the effect of the AC stimulator, NKH-477 on cAMP production, cAMP levels of CHO cells were measured in the presence of an EC_{20} concentration of NKH 477 and an IC_{100} amount of Ro 20–1724. Under these conditions, 44 compounds were found to potentiate the effect of NKH 477 on cAMP production, 31 of which also were inhibitors of PDE4, but 13 compounds did not inhibit PDE4. In addition, 23 compounds that were active against PDE4 did not raise cAMP levels under these conditions. In Table 2 is shown an example of concentration-dependent potentiation of cAMP production by some of these compounds. NCGC00067819, NCGC00071192, NCGC00064188, NCGC00064315, and NCGC00067541 from series 41 were more potent than the other compounds (Table 2). The compounds that potentiated the effect of NKH 477 included some of the known PDE inhibitors such as papaverine (series 102) and etazolate (series 112). Tracazolate (series 112), an analogue of etazolate, also was found to potentiate cAMP production induced by NKH 477.

Effect on Targets Downstream in the Pathway. In the primary screen, nocodazole from series 69 (EC_{50} = 1.00 μM), colchicine (EC_{50} = 5.01 μM) from series 106, and vincristine (EC_{50} = 0.50 μM) from series 177 were found to enhance CREB activation. These compounds bind to tubulin, block cell division at the G_2-M stage, and perhaps affect chromatin remodeling, a process that may cause an increase in gene transcription as a stress response, which may include an increase in CREB mediated chromatin modification. Thus, these compounds might be expected to exhibit CREB potentiation. Consistent with this hypothesis, none of these compounds showed any effect on cAMP production, activation of PKA, or inhibition of PDE4. Eleven compounds, including the above compounds, had no effect in the cAMP, PKA, and PDE assays, although they were positive in both CRE-β-lactamase and CRE-luciferase assays. Of these 11 compounds, NCGC00052491 and 6 other compounds with an unknown mechanism of action can reasonably be expected to act on targets downstream in the CREB pathway, such as activation of CREB binding protein catalyzed histone acetylation or inhibition of histone deacetylation.

Discussion

We have described the discovery of multiple structural series of small molecule compounds that potentiate the CREB signaling pathway, and the identification of the compounds' molecular target via a series of staged deconvolution assays. The compound potencies and efficacies available immediately from the primary screen enabled us to cluster compound structures and analyze compound SAR on a large set of primary actives, resulting in a prioritization of 81 compounds from 37 series for further studies. Overall, 2.5% the compounds screened (1,800 of 73,000) showed activity in the primary screen, which may reflect the sensitivity of the qHTS process (22) and the multiplicity of targets assayed in the CREB signaling pathway screen. The active confirmation rate of 99% in the CRE-β-lactamase assay and 95% confirmation rate in the CRE-luciferase assay, validate the high quality of primary qHTS results.

Long-term Memory (LTM) Relevance of PDE Inhibitors. Intracellular cAMP homeostasis is regulated by PDEs. Mammalian PDEs are encoded by 21 genes and are classified into 11 PDE families based on sequence homology, enzymatic properties, and sensitivity to inhibitors (32). Of the 11 families of PDEs identified so far, PDE4, PDE7 and PDE8 are specific for cAMP. The PDE4 family accounts for much of the cAMP-hydrolyzing activity in cells (33). Human PDE4A expression is fairly ubiquitous and relatively high in the brain (32). PDE4 inhibitors are currently under development for the treatment of inflammation and memory disorders (23). The PDE4 inhibitor rolipram has been shown to improve deficits in both long-term potentiation (LTP) and contextual learning in double transgenic mice for the amyloid precursor protein and presenilin-1 in the central nervous system, suggesting that PDE4 inhibitors might be used to treat Alzheimer disease (34). Furthermore, PDE4 inhibition has been suggested as a new approach for the treatment of schizophrenia (35) and the Rubinstein-Taybi syndrome (18). In the primary screen performed here several PDE4 specific inhibitors including Ro20–1724, etazolate, and rolipram were identified, which is consistent with the presence of endogenous PDE4 in CHO cells (36). Of the PDE4 inhibitors identified, NCGC00071837 is the most potent with an IC_{50} of 31 nM, which is 6.5-fold more potent than rolipram (IC_{50} = 200 nM). The structure of NCGC00071837 is distinct from rolipram and other

known PDE4 inhibitors, and therefore represents a chemical starting point for the development of drugs to test the spectrum of central nervous system disorders related to PDE4. Derivatives of NCGC00071837 were synthesized and are reported in ref. 37.

Application of a Pathway Assay to Identify Multiple Mechanisms of CREB Potentiation. The results of various assays suggest that some compounds may act through more than one mechanism to enhance the CREB pathway. NCGC00071837 was shown to be a potent human PDE4 inhibitor based on the PDE4 enzyme assay results (Fig. S6*B*). In follow up studies by measuring cAMP production, however, NCGC00071837 also was found to both directly stimulate (in the absence of NKH 477) and potentiate (in the presence of NKH 477) cAMP production when PDE inhibition was maximized by the addition of Ro 20-1247. NCGC00071837 was unique in this spectrum of activity, and may therefore be more effective in enhancing CREB signaling than compounds with a single mode of action (38).

Cellular signaling pathway screens have the advantage of potentially identifying small molecule probes for multiple targets from one HTS campaign. In addition, compounds with multiple actions can be discovered that would not be found easily in single target screens, because potential additive and/or synergic effects make this type of compound more potent in signaling pathway assays than in single target screens. However, an intensive effort in secondary assays is necessary for effectively characterizing and dissecting the mechanisms of action for these compounds identified from signaling pathway screens. The discovery of multiple-action compounds may provide new opportunities for drug development for LTM disorders by enhancing the CREB signaling pathway.

In summary, we have identified several small molecule potentiator series of the CREB signaling pathway by applying qHTS to a CREB signaling pathway assay. In combination with molecular target follow up studies including cAMP, PKA, and PDE assays, we have characterized the mechanisms of action for some of the compounds discovered via the CREB signaling pathway assay. Although these probes will have immediate application in the study of CREB signaling and LTM, perhaps the most tantalizing compounds are those that potentiate CREB activity through potentially novel members of this pathway, and these compounds are now being intensively studied to discover their molecular target(s). The approach of qHTS, followed by secondary assays covering known pathway targets enables efficient identification of high-quality leads for multiple nodes in a signaling pathway, and is a generally applicable paradigm that promises to accelerate the identification of probes for chemical genomics and drug development.

Materials and Methods

Cell Lines and Culture Conditions. A CHO CRE-β-lactamase cell line from Invitrogen stably expresses a β-lactamase reporter gene under the regulation of a cAMP Response Element (CRE). These cells were cultured in DMEM, 10% dialyzed FBS, 2 mM Glu, 0.1 mM NEAA, 1 mM Na pyruvate, 25 mM Hepes, 1% Pen-Strep, 5 μg/mL of blasticidin at 37 °C in 5% CO_2. HEK293 CRE-luciferase cell line from Promega is a HEK293 derived stable cell line that expresses the rapid response firefly luciferase gene, *luc*2P, under the control of a minimal HSV-TK promoter with CRE. The cells were cultured in DMEM, 10% FBS, 2 mM Glu, 1% pen-strep, 50 μg/mL of hygromycin at 37 °C in 5% CO_2.

CRE β-Lactamase Reporter Gene Assay. CHO CRE-β-lactamase cells were seeded in 1536-well assay plates at a density of 2000 cells per well in 5 μL of assay medium with 1% dialyzed FBS. After culturing overnight, 23 nL of compound in DMSO or DMSO alone was added to each well with a pin-tool station (Kalypsys) followed by addition of either 1 μL of NKH477 or assay medium in each well. The plates were incubated at 37 °C for 3 h followed by addition of 1 μL of fluorescent detection mixture (CCF4, Invitrogen). The plates were incubated at room temperature (RT) for 2 h, and fluorescence intensities were measured with an Envision plate reader (PerkinElmer) at an excitation of 405 nm and emission at both 460 and 530 nm.

qHTS and SAR Analysis. Approximately 73,000 compounds were screened each at 7 or more concentrations ranging from 0.0024 μM to 38 μM. Compounds from qHTS were classified into 4 major classes (curve class 1–4, see Fig. S2) based on quality of curve fit and efficacy using criteria published in ref. 22. We have the highest confidence in class 1.1, 1.2, or 2.1 concentration curves, and have less confidence in class 2.2 and 3 curves. Class 4 compounds that show no concentration response are inactive compounds. Active compounds with curve classes 1.1, 1.2 or 2.1 were selected to further analyze compound structure-activity relationships (SAR) using Leadscope software, which yielded 180 clusters from the active compounds. Maximal common substructures were extracted from each cluster containing at least 3 active compounds, which was used to search the entire compound library to find all, including inactive, analogs, yielding 96 structure-activity relationships (SAR) series. A set of criteria was applied to exclude series containing potentially fluorescent compounds, and series with low potencies or efficacies, resulting in 37 prioritized series.

CRE Luciferase Reporter Gene Assay. HEK293 CRE-luciferase cells were seeded in 1536-well assay plates at a density of 2500 cells per well in 5 μL of assay medium with 1% FBS. After culturing overnight, 23 nL of compound in DMSO or DMSO alone was added into each well, followed by addition of 1 μL of NKH477 (final concentration, 200 nM) or media alone in the assay plates. After 4 h of incubation at 37 °C, 6 μL per well of Bright-Glo luciferase (Promega) detection mix was added and incubated at RT for 10 min. Luminescence intensity was measured by a ViewLux plate reader (PerkinElmer).

cAMP Assay. Total cAMP levels (intracellular plus extracellular) were measured using LANCE cAMP kits (PerkinElmer). The assay is based on competition between a Europium-labeled cAMP tracer complex and total cAMP for binding sites on antibodies labeled with a dye. The energy emitted from the Eu-chelate is transferred to the antibody, generating a time resolved fluorescent resonant energy transfer signal at 665 nm. CHO cells were seeded in 1536-well assay plates at a density of 2500 cells per well in 5 μL of assay medium with 1% FBS. After culturing overnight, 23 nL of compound in DMSO or DMSO alone was added to the assay plates, followed by addition of 1 μL of cAMP antibody with either Ro 20–1724 (final concentration, 100 μM) or NKH 477 (final concentration, 100 nM) plus Ro 20–1724 into each well. After the plates were incubated at 37 °C for 20 min, 1 μL of detection reagent was added into each well. The plates were incubated for 3 h at RT and measured in a ViewLux plate reader using 340 excitation and emission at both 647 and 665 nm.

Protein Kinase A (PKA) Assay. PKA activity was measured using commercial available HTRF KinEASE kits (Cisbio International). Details of assay protocol are given in *SI Text*.

Phosphodiesterase 4 Assays. PDE4 activity was measured using IMAP technology (Molecular Devices, CA). Briefly, 2 μL per well of human PDE4A1A (BPS Bioscience,) mixture (0.05 ng/μL PDE4A1A, 10 mM Tris pH 7.2, 0.1% BSA, 10 mM MgCl2, 1 mM DTT, and 0.05% NaN3, final concentration) was dispensed into in 1536-well assay plates. After centrifugation for 30 s, 23 nL per well of compound in DMSO or DMSO alone was added to each well. After incubation for 5 min at RT, 2 μL per well of cAMP (final concentration, 100 nM) was dispensed for a final assay volume of 4 μL per well. The plates were centrifuged at 163 $\times$ *g* for 30 s, incubated for 40 min at RT, and followed by addition of 4 μL of IMAP binding reagent. After 2 h of incubation at RT, the fluorescence polarization (FP) signal (excitation at 485 nm and emission at 530 nm) was measured on an EnVision plate reader.

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