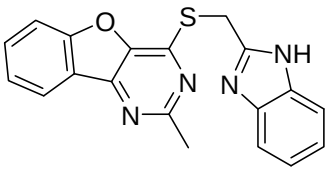
Project: CREB signaling pathway: CRE potentiators

Probe compound: 4-((1H-benzo[d]imidazol-2-yl)methylthio)-2-methylbenzofuro[3,2-d]pyrimidine



SID: 863038
Internal IDs: NCGC00067819

PubChem Bioassay Summary Identifier (AID): 662

SID	MLS	AC50 (μM)	Antitarget	Selectivity*
863038	**MLS000041718**	**0.079**	**NA**	**NA**

Assigned Assay Grant #: MH079867-01

Screening Center Name: NIH Chemical Genomics Center
Principal Investigator of Screening Center: Christopher Austin

Assay Submitter & Institution: Marshall Nirenberg, U.S. National Heart Lung & Blood Institute, NIH

Assay or Pathway Target: The cyclic AMP response element-binding proteins
Link: http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=662

Assay provider information

Specific Aim: Identify small molecular potentiators of the CREB pathway for memory and cognitive disorders using quantitative high throughput screening (qHTS).

Significance: The cyclic response element-binding proteins (CREB) are a class of transcription factors that bind to selected cAMP response element (CRE) segments of DNA and catalyze the transcription of these genetic sequences.[1] CREB has been implicated as an important regulator of long term memory and pharmacological enhancement of the CREB signaling pathway in memory may provide an attractive strategy for development of memory enhancing drugs.[2] Abnormalities in the functioning of CREB signaling has been linked to the development and progression of Huntington's Disease, Tubinstein-Taybi syndrome and has been linked to several cancers in addition to its well characterized role in the activation of neurons during learning with changes in gene expression required for long-term memory formation.

Rationale: Transcription of Cyclic AMP (cAMP) Response Element Binding Protein (CREB) loci have been shown to involve in learning and memory. Some known activators of CREB-dependent expression have been shown to enhance long-term memory in animals. The rationale for studying this system relies on the discovery small molecule activators and inhibitors of this signaling pathway to be used as tools to further characterize the precise roles of CRE up-regulation and down-regulation. In an effort to comprehensively explore the entire signal transduction pathway, a cell based screen was established, optimized and completed. A cell-based assay developed by Invitrogen Corp[3] using a beta-lactamase reporter-gene under control of cAMP responsive element was used to measure the potentiation of CRE signaling. Library compounds were measured for their ability to modulate positively the action of EC10 NKH 477, a water soluble forskolin, on reporter gene activity. Compounds were screened in a titration series in 1536-well format. Positive and negative modulators were identified based on the AC50 values derived from the screening data.

Screening center information

Assay Implementation and Screening

PubChem Bioassay Name: Cell signaling CRE-BLA (Fsk stim)

List of PubChem bioassay identifiers generated for this screening project (AIDs):

AID	Target	Concentration	Bioassay type
662	CREB Pathway	40 µM – 2.4 nM	Primary qHTS
916	CREB Pathway	40 µM – 4.6 pM	CRE-BLA (Fsk stim) confirmation
907	CREB Pathway	40 µM – 4.6 pM	HEK293 CREB Luciferase confirmation
905	N/A	40 µM – 4.6 pM	CRE-BLA CHO cell cytotoxicity (24 hr)
906	N/A	40 µM – 4.6 pM	CRE-BLA CHO cell cytotoxicity (40 hr)

Primary Assay Description as defined in PubChem:

2000 cells/5uL in Opti-MEM medium containing 0.5% dialyzed FBS, 0.1 mM NEAA, 1 mM sodium pyruvate and 10 mM HEPES was dispensed into 1536-well plates and cells were cultured at 37 degrees Celsius overnight. Next day 23 nL of compounds or DMSO were delivered to each well using a pin tool, and 1uL of EC10 level of NKH 477, or EC100 level of IBMX, a PDE inhibitor, in the present of NKH 477, or assay medium was added. And then the plates were incubated at 37 degrees Celsius for 3 hours. 1 uL of CCF4-dye mixture was added to each well. After incubated at room temperature for 2 hours the plates were measured on an EnVision plate reader at Excitation 405nm, Emission1=460nm and Emission 2=530nm. The %Activity was determined from the ratio of 460nm/530nm

Table 1. qHTS protocol for CRE-*bla* assays

CRE signaling pathway β-lactamase reporter gene assay protocol			
Step	**Parameter**	**Value**	**Description**
1	Reagent	5 uL	2,000 CRE-bla cells
2	Incubation	overnight	Cells adhere and acclimate
3	Library compound	23 nL	38 uM to 0.5 nM titration series
4	Control compound	23 nL	2 nM to 67 uM titration series (IBMX)
5	Reagent	1 uL	Buffer ± potentiator
6	Incubation	3 hrs	Induce CRE reporter
7	Reagent	1 uL	β-lactamase detection solution
8	Incubation	2.5 hrs	CCF4 uptake, de-esterification and processing
9	Detector	Ex 405/8 nm	Perkin Elmer EnVision

Step	Notes
1	Black clear bottom 1536-well plates, single tip dispense of 2000 cells/well into all wells
2	37 °C, 5% CO_2 incubator
3	Pintool transfer of library to columns 5-48
4	Pintool transfer of controls to columns 1-4
5	Single tip dispense for each reagent, Column 1 and 4, subEC_{50} of agonist- 30 nM NKH 477. Column 2 and 3, buffer only
6	37 °C, 5% CO_2 incubator
7	Single tip dispense of 0.6 uM CCF4-AM, 1 mg/ml Pluronic F127 surfactant, 3.5 % PEG 400, 2.6 % TR40, 2 mM probenecid, 0.1 % DMSO
8	Ambient temperature in the dark
9	460/25 nm and 530/20 nm emission filters

Table 2. CRE qHTS Performance Summary

Parameter	*Value*
System	Kalypsys robot
Compounds (total # tested)	74,000
Samples (total wells)	755,712
% 6 or 7 pt-titrations	90%
% 14 or 15 pt-titrations	10%
Plates Screened	492
Z′	0.60 +/- 0.11
Signal : Background (S:B)	2.35+/- 0.49

Center Summary of the Primary Screen:

Compounds Screened: 69674
Active: 1744
Inactive: 50125
Inconclusive: 18061

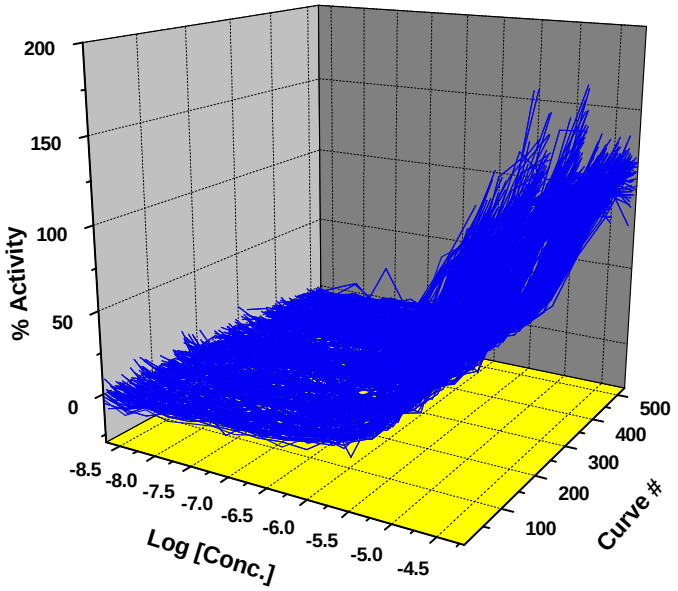


Figure 1a. Intra-plate concentration response titration curves for IBMX in the screening. Each curve was measured in duplicate in column-1 of each assay plate.

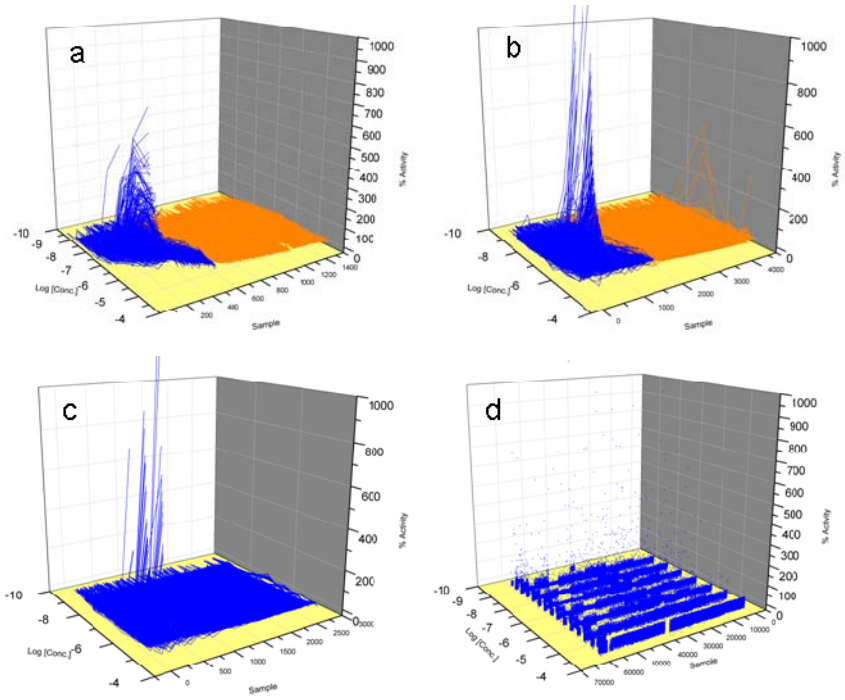
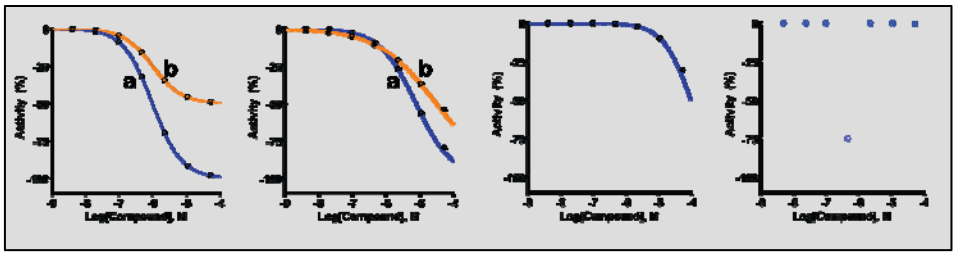


Figure 1b. qHTS curve-fit data from AID 662 binned into curve classifications 1-4 (panels a-c) based classification criteria. Sub-classifications, e.g. 1a vs 1b, are color coded as blue and orange respectively, also see scheme 1 for additional details on qHTS curve classifications.

Identification of Active Clusters: Following the qHTS, the concentration response curves (CRCs) data are subjected to a classification scheme to rank the quality of the CRCs as described in Inglese and co-workers[4] (Figure 1b, see also scheme 1) Briefly, CRCs are placed into four classes. Class 1 contains complete concentration-response curves showing both upper and lower asymptotes and r^2 values > 0.9. Class 2 contains incomplete CRCs lacking the lower asymptote and shows r^2 values greater than 0.9. Class 3 curves are of the lowest confidence because they are defined by a single concentration point where the minimal acceptable activity is set at 3 SD of the mean activity calculated as described above. Finally, class 4 contains compounds that do not show any CRCs and are therefore classified as inactive. Once an active set of compounds was identified, hierarchical agglomerative clustering with a 0.7 Tanimoto cutoff was performed by using Leadscope (Leadscope Inc., Columbus, OH) fingerprints. For each cluster, maximal common substructures (MCS) were extracted, a manual step of MCS trimming was performed to create a list of scaffolds, and any overlapping scaffolds were abridged to a canonical set. Each scaffold was then represented as a precise definition to indicate descriptors such as the number of attachment points or the ring size variability. All filters were then relaxed to include the entire negative (class 4) assay data.



Scheme 1: Example classification scheme for assignment of resulting curve-fit data into classes. Curves fitting the following classification criteria are then used to establish SAR: Class 1 curves display two asymptotes, an inflection point, and r2 ≥0.9; subclasses 1a vs. 1b are differentiated by full (>80%) vs. partial (≤ 80%) response. Class 2 curves display a single left-hand asymptote and inflection point; subclasses 2a and 2b are differentiated by a max response and r2, >80% and >0.9 or <80% and <0.9, respectively. Class 3 curves have a single left-hand asymptote, no inflection point, and a response >3SD the mean activity of the sample field. Class 4 defines those samples showing no activity across the concentration range.

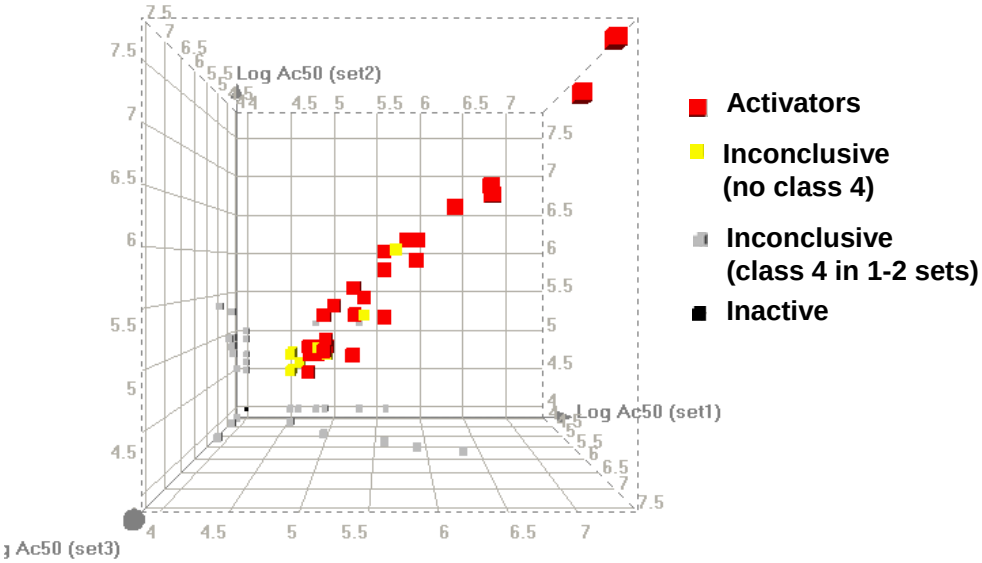


Figure 2. qHTS reproducibility of β-lactamase reporter gene assay. LOPAC library was screened independently in CRE-bla CHO cells on three separate times. Linear correlations of AC50s from 48compounds with curve classes 1-3 in three independent screening yielded an average r2 = 0.94±0.02 (0.95, run 1 vs run 2; 0.94, run 1 vs run 3; and 0.92, run 2 vs run 3). 47 compounds were inactive in one or two runs and are colored gray in the figure. 1185 compounds were inactive in all three runs.

Lead Identification:

Activity summary for CRE qHTS:

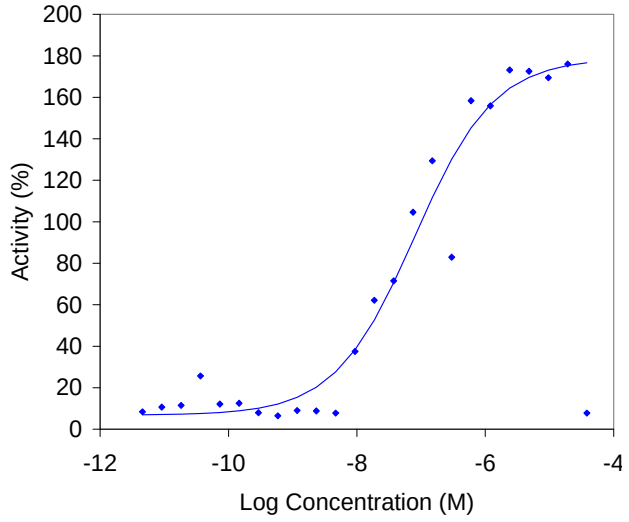
Table 3. Potency (AC50) distribution of active compounds in qHTS screening

Potency range (μM)	Number of compounds in curve classes		
	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	1800		

Description of secondary screens used to optimize probe structure:

Confirmation studies: Approximately 100 compounds were selected and retested in the original CHO CRE-β-lactamase assay. The confirmation rate was 95% compared to primary qHTS. Next, these compounds were tested in HEK293 CRE-luciferase reporter gene assay, an alternative CRE reporter gene assay in human HEK293 cells. The concordance rate is 80% between these two CRE reporter gene assays. Among these compounds, NCGC00067819-01 (SID:863038, Fig 1) is most potent compound with AC50 of 79nM in confirmation study and 16 nM in primary screening.

Figure 3. Concentration response curve of SID: 863038 in confirmation study



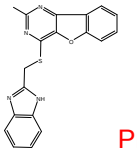
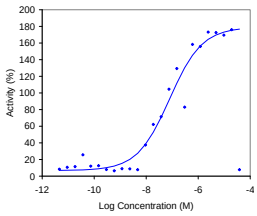
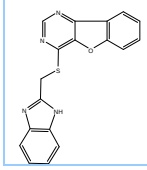
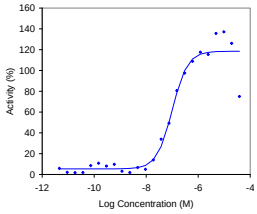
Known probe properties:

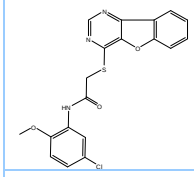
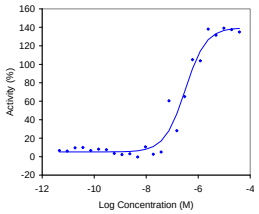
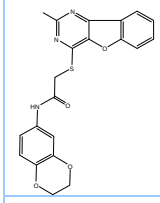
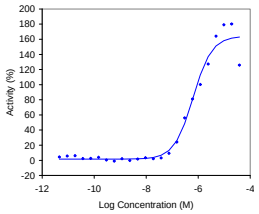
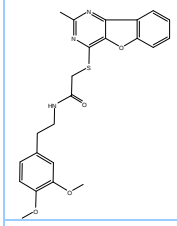
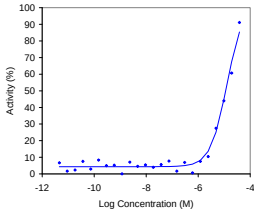
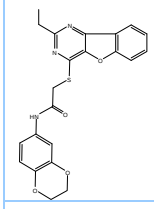
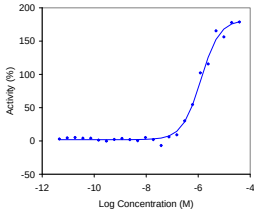
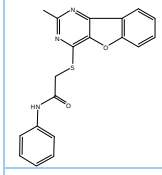
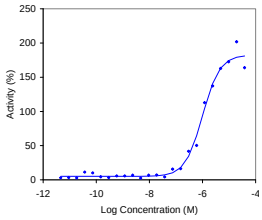
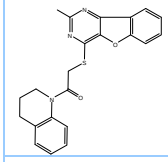
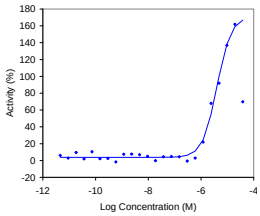
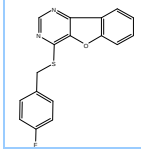
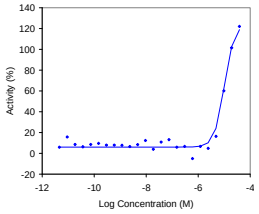
Center summary of probe properties (solubility, absorbance/fluorescence, reactivity, toxicity, etc.) and recommendations for the scientific use of probe as research tool:

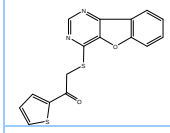
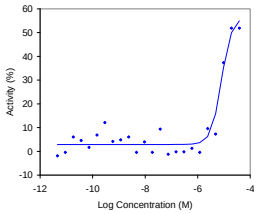
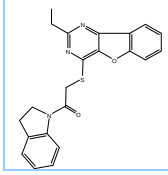
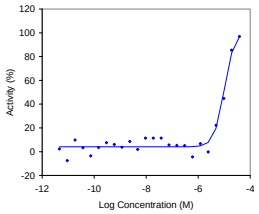
Molecular Weight	346.40566 [g/mol]
Molecular Formula	$C_{19}H_{14}N_4OS$
XLogP	3.1
H-Bond Donor	1
H-Bond Acceptor	5
Rotatable Bond Count	3
Exact Mass	346.088832
MonoIsotopic Mass	346.088832
Topological Polar Surface Area	67.6
Heavy Atom Count	25
Formal Charge	0
Complexity	480
Isotope Atom Count	0
Defined Atom StereoCenter Count	0
Undefined Atom StereoCenter Count	0
Defined Bond StereoCenter Count	0
Undefined Bond StereoCenter Count	0
Covalently-Bonded Unit Count	1

Appendices

Representative active and inactive analogs from the qHTS

Structure	NCGC ID Supplier ID PubChem SID	qHTS AC50 (M)	Follow Up AC50 (M) (BLA)	Follow Up AC50 (M) (Luciferase)	MW	Purity (%)	cLog P (calculated)	Follow Up Curve (BLA)
 Probe	NCGC00067819-01 InterBioScreen: STOCK4S-66236 863038	1.58E-08	7.94E-08	1.26E-06	346.4	100	4.08	
	NCGC00067541-01 InterBioScreen: STOCK4S-36931 862436	1.00E-07	1.00E-07	1.26E-06	332.4	100	3.87	

	NCGC00071192-01 InterBioScreen: STOCK4S-61804 863033	1.00E-07	3.16E-07	3.16E-06	399.9	100	3.78	
	NCGC00064315-01 InterBioScreen: STOCK4S-40852 862310	2.51E-07	6.31E-07	3.98E-06	407.4	100	3.65	
	NCGC00067543-01 InterBioScreen: STOCK4S-94418 863939	2.51E-07	1.26E-05	1.26E-05	437.5	100	3.69	
	NCGC00067706-01 InterBioScreen: STOCK4S-94981 863981	3.98E-07	1.26E-06	3.98E-06	421.5	100	4.22	
	NCGC00064188-01 InterBioScreen: STOCK4S-63156 863123	5.01E-07	1.00E-06	3.98E-06	349.4	100	3.77	
	NCGC00068986-01 InterBioScreen: STOCK4S-51288 862673	5.01E-07	3.98E-06	5.01E-06	389.5	100	4.25	
	NCGC00072646-01 InterBioScreen: STOCK4S-45764 862477	1.00E-06	5.01E-06	1.26E-05	310.3	100	4.4	

	NCGC00070477-01 InterBioScreen: STOCK4S-68921 863211	5.01E-06	7.94E-06	1.58E-06	326.4	100	3.72	
	NCGC00067665-01 InterBioScreen: STOCK4S-95898 864040	>5.7E-05	1.00E-05	1.26E-05	389.5	95.1	4.29	

Bibliography:

(1) Carlezon, W.A., Jr. et al. (2005) The many faces of CREB. Trends Neurosci 28 (8), 436-445

(2) Josselyn, S.A. and Nguyen, P.V. (2005) CREB, synapses and memory disorders: past progress and future challenges. Curr Drug Targets CNS Neurol Disord 4 (5), 481-497

(3) Huang, Z. et al. (2001) The next generation of PDE4 inhibitors. Curr Opin Chem Biol 5 (4), 432-438

(4) Inglese, J. *et al.* Quantitative high-throughput screening: A titration-based approach that efficiently identifies biological activities in large chemical libraries. *Proc Natl Acad Sci U S A* **103**, 11473-11478 (2006).