

Final Signoff on MLSCN Assigned Assay

This form must be completed and signed by the Screening Center PI and by a Program Director at NIMH or NHGRI to end the Center's responsibility to develop chemical probes for a MLSCN assigned assay. Each assigned assay requires a separate form. A copy of this form will be sent to the assay provider.

Center: NCGC	AID: 445	Grant Number: XO1- MH078957-01	Project Period:
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Last development phase completed Phase IX: SAR-screened purchased analogues (PubCh
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Do you recommend re-screening in the future to find better chemical leads by screening a larger compound library when it becomes available? Yes <input type="checkbox"/> No <input checked="" type="checkbox"/>
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Reason for recommending the closing of this assay project: A probe series that inhibits NF-kappaB activation was identified. The series works in the ABC-DLBCL cell line where a high level of constitutive activity IKK activity is present as well as inducible systems where TNFalpha is used to activate NF-kappaB.

Please complete Section 1 of this form.

Was a chemical probe developed from this project? Yes <input checked="" type="checkbox"/> No <input type="checkbox"/>
If yes, please attach <input type="checkbox"/> and provide the SID: 26752300

Comments from Assay Provider The current series is not selective for lymphoma cell lines and several indications suggest that the targeted activity lies within a common region of the NFkB pathway. Therefore, the current series is not of high interest to our collaborators on this project at NCI who are specifically intereseted in lymphoma targets. Target deconvolution may proceed through collaboration with NFkappaB labs.

Signature of Center Principle Investigator:  _____

Signature of Institute Program Director: _____

Signing date: _____

Closing Progress Report

Assay Name: HTS for Stabilizers of IkappaBalpha

Please be sure to identify the target and include an assay description. Also, include information applicable to the last stage reached before project signoff: dose dependent curves, descriptions of hits, defining activators and inhibitors, and SAR summaries for purchased and/or synthetic analogues.

Stabilizers of $I\kappa B\alpha$ qHTS Report

MLSCN No.: XO1-MH078957-01
Assay title: Identification of $I\kappa B\alpha$ stabilizers in a human lymphoma cell line using a two color luciferase-based cell assay
PI: Douglas Auld, NCGC
Date of assay distributed: June 2006
Date of qHTS report: March 2007
PubChem AID: 445

Summary

Nuclear factor kappa-B (NF-kappa-B) plays an important role in normal B cell development and survival. Diffuse large B cell lymphoma (DLBCL) is the most commonly observed type of non-Hodgkin's lymphoma. Gene expression analysis has identified an activated B cell-like subtype of DLBCL (ABC-DLBCL) which expresses known NF-kappa-B target genes. In ABC-DLBCL cell lines this is due to high constitutive activity of I-kappa-B kinase (IKK), a key regulator of NF-kappa-B. Low molecular weight molecules that inhibit IKK have been shown to be selectively toxic for ABC-DLBCL cell lines. However, targets upstream of IKK have been largely unexplored and so nodes upstream of IKK in the NF-kappa-B pathway remain untested due to the lack of chemical probes.

Introduction and Assay Description:

A cell-sensor assay for I-kappa-B-alpha stabilization was developed with NIH investigator Dr. R. Eric Davis in Dr. Louis M. Staudt's laboratory. The assay uses green (CBG68) and red (CBR) emitting beetle luciferases [Chroma-Glo(TM) developed by Promega Corp.] where the green luciferase is fused to I-kappa-B-alpha and the red luciferase is present in its native state. Both luciferase genes are stably present in the OCI-Ly3 human ABC DLBCL cell line, but their expression is under the control of tetracycline-inducible promoters. Following the addition of doxycycline to induce expression of the luciferases, the green luminescence in control cultures rises only slowly because the CBG fusion partner I-kappa-B-alpha is targeted by IKK activity for rapid degradation. In contrast, green luminescence rises rapidly in the presence of proteasome inhibitors or IKK inhibitors. The rise in red luminescence of CBR is not affected by proteasome or

IKK inhibitors, and serves as normalization for cell number and nonspecific effects. Compounds were screened as a concentration-titration series that ranged from 57 μ M to 0.7 nM.

Refs: Davis RE, Zhang Y-Q, Southall N, Staudt LM, Austin C, Inglese J, & Auld DS. A cellular assay for I κ B α stabilization using a two-color dual luciferase-based sensor. *Assays Drug Dev. Technologies* 2007;5(1):85-103.

Xie Y, Thomas CJ, Deng S, Liu Y, Zhang Y-Q, Rinderspacher A, Huang W, Gong G, Wyler M, Cayanis E, Aulner N, Többen U, Chung C, Pompou S, Southall N Vidovic D, Schürer S, Mayer T, Branden L, Davis RE, Staudt LM, Inglese J, Austin CP, Landry DW, Smith DH & Auld DS. Identification of N-(quinolin-8-yl)benzenesulfonamides as Agents Capable of Down-Regulating NF κ B Activity within Two Separate High-Throughput Screens of NF κ B Activation. *Bioorganic & Medicinal Chemistry Letters*. 2007, in press, available online 30 October 2007

Protocol:

Cells were seeded in 1536-well plates at 5000 cells/3 μ l in IMDM medium containing 0.5% FBS, w/o phenol red, L-glutamine, 25 mM HEPES, 3 mg/ml sodium bicarbonate. After centrifugation for 1 min. at 1000 rpm, 23 nl of compounds or DMSO were delivered to each well using a pin tool. Then 1 μ l 80ng/ml doxycycline was dispensed into white solid 1536-well plates and the plates were incubated at 37C/5% CO₂ in a cell incubator for 4 hrs. Then 4 μ l Chroma-Glo luminescent substrate mix (Promega) was added to each well. The plate was incubated at R.T. for 10-15 min. The plates were measured on a ViewLux plate reader for green luminescence (540/20 nm filter) and red luminescence (618/8 nm filter). The green and red luminescent signals were corrected for red and green luminescence filter overlap using previously determined filter calibration constants (determined using the procedure outlined in the Promega Technical Manual). The %Activity was determined from the ratio of corrected green/red luminescent values. Green luminescent and ratio %Activity was determined by normalizing to the difference in signal between basal cells (0% Activity) and cells incubated with 10 μ M of the proteasome inhibitor MG-132 (100% Activity). Red luminescent %Activity was normalized to the difference between red luminescence in basal cells (0%Activity) and zero luminescence (-100% Activity).

Concentration-response curves were fitted to the signals arising from the green luminescence (I κ B α -luciferase fusion; I κ B-luc) and the red luminescence (unfused luciferase; luc) as well as the calculated ratio. The concentration-effect curves were then classified based on curve quality (r^2), response magnitude and degree of measured activity. Compounds were then categorized based on their concentration-response curves for all three datasets (I κ B-luc, luc and ratio). Active compounds showed concentration-dependent increases in the I κ B-luc and ratio data with little or no effect on the luc data. Signal Activators showed concentration-dependent increases in both the I κ B-luc and luc data with either no effect or a modest effect on the ratio data. Signal Inhibitors showed concentration-dependent decreases in either the I κ B-luc or luc datasets with either no effect or a concentration-dependent increase in the ratio data. Inactive compounds showed no effect in all three datasets.

Protocol Step Summary

IκBα Cell Sensor Assay			
Sequence	Parameter	Value	Description
1	Reagent	3 μ L	Cells-5000/well (FRD)
2	Cmpd.	20 nL	40 μ M – 0.5 nM
3	Dox.	1 μ L	Induce luciferases
4	Time	4 hr	37 $^{\circ}$ incubation
5	Reagent	4 μ L	Chroma-Glo Detection
6	Time	10 min.	R.T. incubation
7	Detector	540/20 nm and 618/8 nm	PE ViewLux

Screened Collection:

qHTS MLSMR: 70,948 compounds
qHTS ECL: 8,379 compounds
qHTS NCGC: 47,101

qHTS Performance Summary

IkBa Assay			
Parameter	Assay opt.	qHTS MLSMR	qHTS NCGC
Dates	6/21/05– 6/29/05	3/21/06–3/29/06, 6/26/06	2/7/07–2/10/07
Compounds (total # tested)	8,379	70,948	47,101
Concentration-Response Titrations (3/sample)	25,137	238,458	148,980
% 7 pt-titrations		95%	100%
Sample-concentration measurements (sample wells)	264,704	1,168,640	757,704
Well measurements (plate wells x channels)	319,488	1,419,264	878,592
Total wells	159,744	709,632	439,296
Plates Screened	104	462	286
Plates Failed QC	0	0	0
Plates Re-run for QC	0	0	0
RZ'	0.74 +/- 0.10	0.59 +/- 0.26	0.46 +/- 0.14
Signal / Background	5.1 +/- 1.4	10.9 +/- 7.0	12.0 +/- 3.5
CV	16 +/- 6	21 +/- 9	26 +/- 18
Total Screening Time			
Screening System		Kalypsys	Kalypsys
Software for stat analysis	Genedata	Genedata	In-house client

qHTS Data Analysis Overview:

Table 1. Curve Classification Criteria for Assay

<i>Curve Class</i>	<i>Description</i>	<i>Efficacy</i>	r^2	<i>Asymptotes</i>	<i>Inflection</i>
1*	Complete response (a) Partial response (b)	> 80% (a) Min** - 80% (b)	≥ 0.9	2	yes
2 [†]	Incomplete curve	> 80% (a) < 80% (b)	> 0.9 (a) < 0.9 (b)	1	yes
3	Single pt activity	> Min	NA	1	no
4	Inactive	NA	NA	0	no

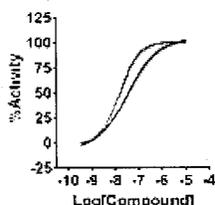
NOTES: *AC₅₀ derived from data; [†]AC₅₀ extrapolated from data; **Min is > 3 SD from the mean activity of the sample field at the highest tested concentration

The qHTS dataset was derived from a cell-based assay employing two reporters. Three concentration-response curves (G', R', and G'/R') were generated for each compound, with complex interdependence.

We found that these cell-based reporters changed similarly in response to certain compounds. Furthermore, a decline in activity at high compound concentrations was observed for some compounds, giving rise to a bell-shaped curve. Therefore, in addition to the obvious category of "Inactive", to properly describe the activity within the current dataset we devised three categories: I κ B α Stabilizers, Signal Activators, and Signal Inhibitors (Figure 1). The I κ B α Stabilizers category contained compounds showing concentration-dependent increases in the G' signal and ratio data with little or no effect on the R' signal. The "Signal Activator" or "Signal Inhibitor" categories contained compounds that increased or decreased the signal of both reporters, respectively, without a major change in G'/R'. We used the curve classification assignments to devise a script that automatically assigned compounds to the four categories based on the concentration-response curves for the G', R', and G'/R datasets (Figure 2).

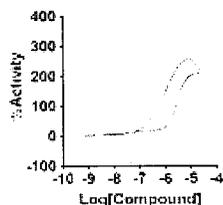
a) I κ B α Stabilizers

Ex. 1



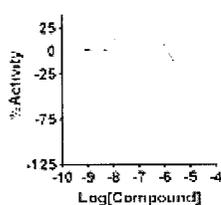
or

Ex. 2



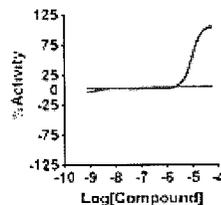
b) Signal Inhibitors

Ex. 1



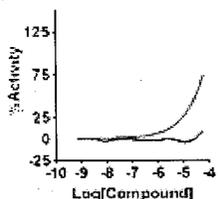
or

Ex. 2



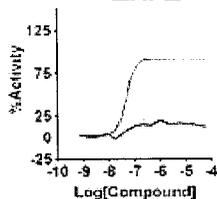
c) Signal Activators

Ex. 1

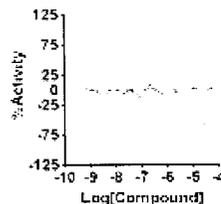


or

Ex. 2



d) Inactives



I κ B α -CBG68, G'
CBR, R'
G'/R'

Figure 1. Characteristic concentration-response curves that defined four categories of activity. Graphs are fits to actual data or lines drawn through the data points for exemplary compounds identified in the qHTS screen. a) I κ B α Stabilizer category, where green luminescence and the ratio data increase. The positive control MG132 is shown for example 1. Red luminescence may decrease at high compound concentrations (example 2). b) Signal Inhibitors category, where both green and red luminescence show inhibitory concentration response curves and inactivity in the ratio dataset (example 1), or an inhibitory concentration-response curve in the red luminescence with an increase in the ratio dataset (example 2). c) Signal Activators category, where red and green luminescence increase to near-equal levels leading to inactivity in the ratio dataset (example 1), or red luminescence increases to a greater degree than the green luminescence (example 2). d) Inactive category, where no concentration dependence was observed in all three datasets.

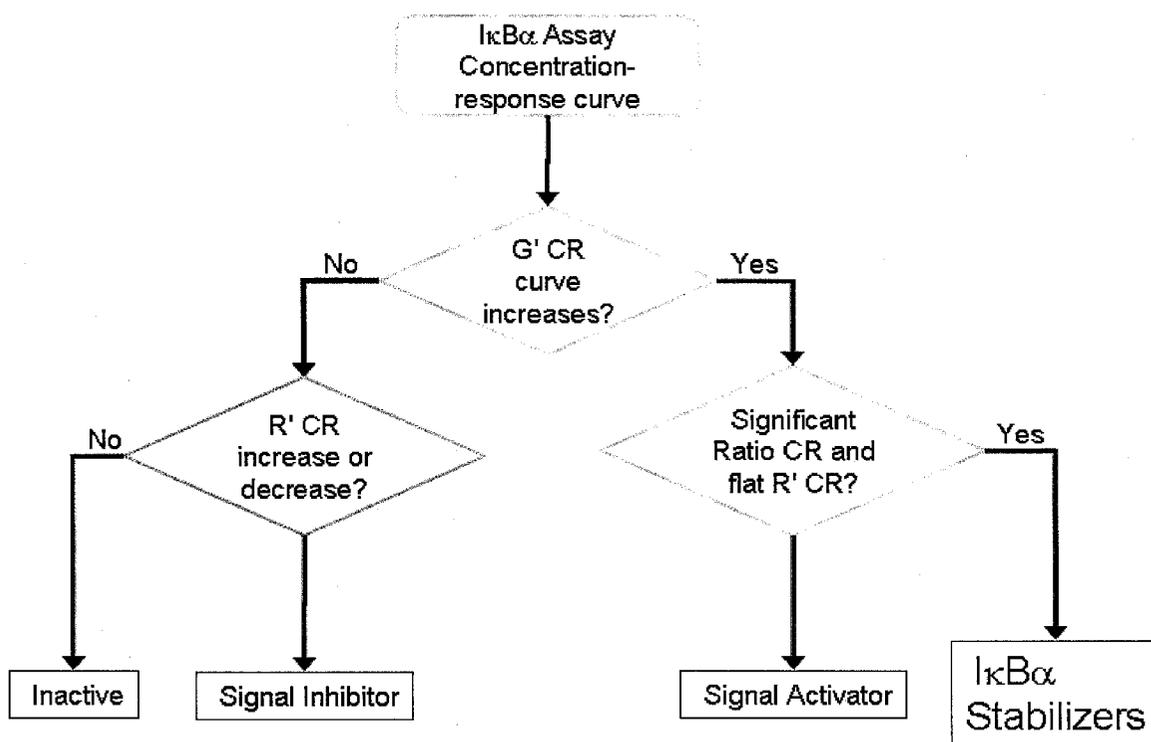
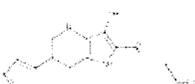
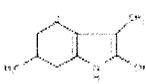


Figure 2. Flow chart for triaging concentration response (CR) curves into Phenotypic Categories. Compounds are triaged using the concentration-response curve classes of the ratio, G' and R' readouts. The first decision point considers whether a significant increase in G' readout has been observed. If it has not, and the R' readout has also been flat, the compound is considered Inactive. If the G' readout did not increase, but a decrease in the R' readout was observed, the compound was categorized as a Signal Inhibitor. If an increase in G' readout was observed, then depending on whether that increase was selectively observed in the G' readout and not also the R' readout, the compound was categorized as a Signal Activator (not selective) or an I κ B α Stabilizer (selective). Compounds that increased the red luminescence without increasing the green luminescence showed curve fits of low confidence and were placed in the Inactive category. Specific criteria for differentiating I κ B α Stabilizers from Signal Activators included either of the following statements being true: 1) No significant change in R' was observed or 2) if R' increased, the ratio was also increasing, and if so, the increase in R' was < 50% of G'.

Summary of compounds in the I κ B α Stabilizers:

NCGC ID (best potency)	Representative cmpds	Scaffold
NCGC00070544-01 (0.7 μ M)		
NCGC00018053-01 (1 μ M)		
NCGC00059731-01 (4 μ M)		
NCGC00083505-01 (5 μ M)		
NCGC00042730-01 (4 μ M)		
NCGC00058326-01 (30 μ M)		

The chemical series highlighted in yellow was pursued and nominated as chemical probes for inhibition of NF κ B activation.

A series *N*-(quinolin-8-yl)benzenesulfonamides as Agents Capable of Down-Regulating NFκB Activity

NIH Chemical Genomics Center/Columbia Univ.

December 2007

PubChem AIDs: 445, 438

A series of *N*-(quinolin-8-yl)benzenesulfonamides capable of suppressing the NFκB pathway was identified from two high-throughput screens run at two centers of the NIH Molecular Libraries Initiative. These small molecules were confirmed in both primary and secondary assays of NFκB activation and expanded upon through analogue synthesis. The series exhibited potencies in the cell-based assays as low as 0.6 μM, and several indications suggest that the targeted activity lies within a common region of the NFκB pathway.

Reference: Xie et al., Identification of *N*-(quinolin-8-yl)benzenesulfonamides as agents capable of down-regulating NFκB activity within two separate high-throughput screens of NFκB activation. *Bioorganic & Med. Chem. Lett.* doi:10.1016/j.bmcl.2007.10.100.

Bioassay data for the probe series is summarized in Tables 1 and 2 below.

Table 1. SAR surrounding the *N*-(quinolin-8-yl)benzenesulfonamides

Control compound #				<i>IκBα</i> stabilization EC ₅₀ (μM) and efficacy*	<i>IκBα</i> stabilization EC ₅₀ (μM) ratio**	<i>translocation of NF-κB</i> IC ₅₀ (μM)***	<i>NF-κB</i> bla IC ₅₀ (μM)	Cytotoxicity IC ₅₀ (μM)
1				1.0, 100%	5.2, 100%	ND	0.6	>10
2				NA	NA	3.0	ND	ND
3				Inactive	Inactive	Inactive	Inactive	Inactive
Analogue #	R	R'	R''					
4	phenyl	H	H	17, 350%	20	>10	8.0	Inactive
6	4-nitrophenyl	H	H	6.5, 560%	17	1.5	5.0	Inactive
7	2-nitrophenyl	H	H	11, 620%	12	3.2	10	Inactive
8	2-nitrophenyl	H	Me	6.8, 200%	8.0	3.8	6.0	Inactive
9	3-nitrophenyl	H	Me	6.0, 110%	13	0.9	1.0	>57
10	4-methyl-2-nitrophenyl	H	H	6.8, 275%	10	2.1	6.0	>57
11	2-methyl-5-nitrophenyl	H	H	1.0, 18%	>10	1.6	1.8	>57
12	2-nitro-4-(trifluoromethyl)phenyl	H	H	3.6, 200%	20	3.7	1.3	>57
13	2-nitro-4-(trifluoromethyl)phenyl	H	Me	10, 50%	3.6	1.1	13	>57
14	4-methyl-2-nitrophenyl	OMe	H	6.8, 60%	7.6	2.1	4.0	>57
15	4-methoxy-2-nitrophenyl	H	Me	ND	ND	1.0	ND	ND
16	2-methyl-5-nitrophenyl	H	Me	>20	>57	Inactive	>57	Inactive
17	4-methyl-2-nitrophenyl	OMe	Me	>10	>57	Inactive	Inactive	>57
18	4-methylphenyl	OMe	Me	20, 150%	20	>10	>57	>57
19	2-aminophenyl	H	H	11, 300%	8.5	2.0	13.5	>57
20	2-aminophenyl	H	Me	8.0, 250%	8.0	2.8	5.5	Inactive
21	2-amino-4-methylphenyl	OMe	H	4.0, 90%	4.0	1.0	2.0	>57
22	2-amino-4-methylphenyl	OMe	Me	9.4, 23%	3.0	Inactive	>57	Inactive
23	thiophen-2-yl	H	H	5.3, 250%	9.0	3.6	7.0	Inactive
24	5-chlorothiophen-2-yl	H	H	2.7, 190%	2.7	1.3	1.0	>57
25	5-bromothiophen-2-yl	H	H	5.9, 170%	6.8	1.1	3.4	Inactive
26	5-chlorothiophen-2-yl	H	Me	1.4, 60%	2.1	1.0	1.3	>10 μM
27	5-bromothiophen-2-yl	H	Me	6.1, 22%	6.4	1.4	5.0	>57

* EC₅₀ values from the *IκBα* stabilization assay shown for the green luminescence reporter along with the %efficacy. ** EC₅₀ values from the ratio of the green and red luminescent values for the *IκBα* stabilization. Data are averages from two to three experiments where each experiment consisted of concentration-titration for each compound performed and duplicate and fitting concentration-response curves to the response after the bioassay. NA = not applicable, the compound only showed a strong inhibitory response in the original qHTS (IC₅₀ = 2.5 μM, 95% inhibition) in the non-specific (red luminescence) dataset. ***IC₅₀ values are derived from curve-fitting to data from a single experiment performed in triplicate. ND = not determined. All compounds showed >90% efficacy in the translocation assay except compounds 15 (78%) and 27 (75%). The cytotoxicity assay was performed in OCI-Ly3 cells using a 4 hr endpoint.

Table 2. SAR surrounding the C7-locked N-(quinolin-8-yl)benzenesulfonamides

Analogue #	R	R'	R''	R'''	<i>IκBα</i> stabilization	<i>IκBα</i> stabilization	<i>translocation of NF-κB</i>	<i>NF-κB</i> bla	<i>Cytotoxicity</i>
					EC ₅₀ (μM) and efficacy*	EC ₅₀ (μM) ratio**	IC ₅₀ (μM)***	IC ₅₀ (μM)	IC ₅₀ (μM)
5	H	H	H	H	2.2, 120%	2.6	1.0	3.4	Inactive
28	H	H	H	OH	ND	ND	Inactive	ND	ND
29	H	H	H	OMe	1.3, 20%	1.4	1.0	1.3	Inactive
30	H	H	H	Me	0.5, 60%	0.8	0.6	0.8	>57
31	H	H	H	CF ₃	0.7, 32%	1.0	1.0	1.2	Inactive
32	H	H	OMe	Me	1.3, 34%	1.6	1.2	2.0	Inactive
33	H	H	H	F	4.1, 122%	12	1.7	1.8	Inactive
34	H	H	H	Cl	1.1, 66%	11	1.1	1.9	Inactive
35	H	OMe	H	H	ND	ND	2.5	ND	ND
36	H	Me	H	H	7.0, 32%	10	ND	8.1	Inactive
37	Me	H	OMe	Me	1.4, 300%	2.3	0.9	1.1	>57
38	Me	H	H	OH	8.0, 120%	11	10	6.7	>57
39	Me	H	H	OMe	3.7, 200%	3.4	2.5	3.2	>57
40	Me	H	H	CF ₃	8.9, 700%	7.2	1.2	3.6	Inactive

* EC₅₀ values from the *IκB* stabilization assay shown for the green luminescence reporter along with the %efficacy. ** EC₅₀ values from the ratio of the green and red luminescent values for the *IκBα* stabilization. Data are averages from two to three experiments where each experiment consisted of concentration-titration for each compound performed and duplicate and fitting concentration-response curves to the response after the bioassay. ***IC₅₀ values are derived from curve-fitting to data from a single experiment performed in triplicate. ND = not determined. All compounds showed >90% efficacy in the translocation assay. The cytotoxicity assay was performed in OCI-Ly3 cells using a 4 hr endpoint.