Center Probe Report

Project Title: Chemical inhibitors of antigen receptor-induced NF-κB activation

Grant Number: 1 X01 MH077633-01

Screening Center Name: San Diego Center for Chemical Genomics
Principal Investigator of Screening Center: John C. Reed, MD, Ph.D.

Assay Provider & Institution: John C. Reed, MD, Ph.D., Burnham Institute for Medical Research, La Jolla, CA

Assay or Pathway Target: NF-κB activation pathway

Probe PubChem Compound Identifier (SID/CID): CID-2858522, SID: 17450324, SID: 8080245; MLS-0146295 (benzimidazole series).

Assay provider information

Specific Aim:

The stated goal is to identify compounds that selectively inhibit one of the several known pathways that lead to NF-kB activation in mammalian cells. At least six cellular pathways leading to activation of NF-kB-family transcription factors have been identified, participating in host-defense, immunity, inflammation, and cancer. A pathway activated by antigen receptors on T- and B-lymphocytes has been revealed, involving a cascade of participating proteins that includes CARMA1 (BIMP3), Bcl-10, paracaspase (MALT1), TRAF6, and Ubc13. This pathway is initiated by Protein Kinase C-theta, which induces phosphorylation of components of this signaling pathway (reviewed in Thome 2004). Based on experiments using siRNA and dominant-negative mutants, it was determined that treatment of cells with the combination of phorbol ester Phorbol Myristic Acetate (PMA) and Calcium-ionophore Ionomycin triggers this pathway, resulting in NF-kB activation (Ruland et al. 2001; McAllister-Lucas et al. 2001; Rueffli-Brasse et al. 2003; Zhou et al. 2004). Consequently, a stably transfected reporter epithelial cell line that contains a luciferase gene driven by a NF-kB responsive promoter was used for compound library screening. Counter-screens were then performed using activators of other NF-kB activation pathways to identify pathway-specific inhibitors. This probe report describes a compound (PubChem CID-2858522) that suppress NF-kB activity with IC_{50} $\simeq$ 100 nM in HEK293 cells stimulated with phorbol esters (either phorbol myristic acetate [PMA] and phorbol dibutryate [PDB]) but not NF-kB activity

induced by Tumor Necrosis Factor-alpha (TNFa), Lymphotoxin-beta Receptor (LTbR), TNF-family receptor member CD40, Toll-like Receptor 4 (LPS receptor), NLR-family members Nod1 and Nod2, and the DNA damage-inducible protein PIDD in these cells. In addition to the NF-kB reporter gene, the probe compound inhibits PMA-induced secretion of IL-8 by HEK293 cells with IC_{50} ~100 nM. The probe compound does not inhibit Protein Kinase C-beta (PKC-beta) or PKC-theta, and does not inhibit IkB kinase (IKK) in vitro, suggesting a site of action between PKCs and IKKs. The probe compound shows cell type-selectivity for suppression of PMA-induced NF-kB, suggesting that different types of cells vary in their expression or dependence on the targets of the active compounds. The probe compound partially suppresses IL-2 production induced by T-cell antigen receptor-mediated stimulation of the Jurkat T-cell line, a process known to be partially dependent on NF-kB. It does not inhibit NF-kB reporter gene activity or cytokine production induced by Lipopolysaccharide in the THP.1 monocyte cell line (TLR4 agonist), by muramyl dipeptide in MCF7 breast cancer cells (NLR agonist), or by Anti-LymphotoxinB receptor in HeLa cells. Thus, compound CID _2858522 is a pathway-selective and cell type-selective inhibitor of NF-kB activation, which may be useful for discovery of and analysis of targets that regulate components of the NF-kB activation pathway induced by PKC activators.

Screening center information

Assay Implementation and Screening

This assay was originally assigned to the Scripps Research Institute Molecular Screening Center by the NIH. The Scripps Center performed primary screening (PubChem AID: 465) of 61,609 compounds from the NIH compound collection, as well as dose-response experiments (PubChem AID: 586). The validated hits with IC_{50} < 3 uM were further characterized by the assay provider, who performed 12 different types of counter-screens to assess pathway selectivity of the compounds. The most potent compound to emerge from the NIH library had IC_{50} ~ 1 uM, in the NF-kB reporter gene assay but failed to suppress in a secondary assay in which an endogenous NF-kB inducible gene encoding IL-8 was measured.

The assay provider wanted to exploit other avenues of probe development for this target. To that end, the San Diego Center for Chemical Genomics acquired the target from the MLSCN Chemistry marketplace. During the development of the assay, the assay provider's laboratory screened 53,280 compounds from the Burnham Institute for Medical Research's small molecule collection. These data have not been submitted to PubChem due to the high variability of the assay during the pilot screens. Despite this variability, 248 of 515 compounds deemed hits were reconfirmed using the same primary screening assay. These compounds were further tested using

12 secondary counter-screening assays to identify pathway-selective inhibitors of NF-κB (see below). Eleven compounds appeared to selectively inhibit the phorbol ester pathway for NF-kB activation that is of interest to the assay provider. From these 11 active compounds, a probe was identified that selectively inhibits in HEK293 cells the NF-kB pathway activated by PMA/Ionomycin with cellular potency of $\leq$ 0.1 uM (IC_{50}) using NF-kB reporter gene assays and also using assays where secretion of NF-kB-induced cytokine IL-8 is measured. The probe also partially inhibits IL-2 production induced by T-cell antigen receptor stimulation of the Jurkat T-cell line.

PubChem Bioassay Name: Primary HTS assay for chemical inhibitors of antigen receptor-induced NF-kappaB activation

List of PubChem bioassay identifiers generated for this screening project (AIDS): 465

List of relevant AIDs that may be used as counter-screen information: AID-411: Luciferase Profiling Assay (NCGC); AID-773: Counter Screen for Luciferase-based Assay Positives (SDCCG).

Primary Assay Description as defined in PubChem:
HEK-293 cells stably transfected with the pUC13-4xNFkB-Luc were cultured in T-175 flasks (Corning part#431080) at 37 degrees Celsius, 5% CO2 and 95% relative humidity.

The growth media consisted of Dulbecco's Modified Eagle's Media (Invitrogen part# 11965-092) supplemented with 10% v/v heat inactivated fetal bovine serum (Hyclone part#SH30088), 1% v/v penicillin-streptomycin mix (Invitrogen part#15140-122) and 1ug/mL puromycin (InvivoGen part#ant-pr-1).

Prior to the assay, cells were suspended to a concentration of 625,000 cells per milliliter in phenol red free Dulbecco's Modified Eagle's Media (Invitrogen part# 21063-029) supplemented with 10% v/v heat inactivated fetal bovine serum (Hyclone part#SH30088), 1% v/v penicillin-streptomycin mix (Invitrogen part#15140-122) and 1ug/mL puromycin (InvivoGen part#ant-pr-1). The assay began by dispensing 4 microliters of cell suspension to each well (i.e. 2,500 cells/well) of a white solid-bottom 1536-well plate (Kalypsys part# K1536 SWST). Plates were then placed in the incubator overnight at 37 degrees Celsius, 5%CO2 and 95% relative humidity. At 24 hours after seeding, cells were treated with 15 nL/well of compounds (3 micromolar, final DMSO concentration of 0.30%). After 1 hour, cells were stimulated with 1 microliter per well of a PBS mix of PMA (100 ng/mL, Calbiochem part#524400) and Ionomycin (50 ng/mL, Calbiochem part#407950). Plates

were subsequently returned to the incubator for 16 hours (37 degrees Celsius, 5%CO_2 and 95% relative humidity).

After the incubation, plates were equilibrated to room temperature for 20 minutes. A luciferase assay was performed by adding 5 microliters per well of the SteadyLite HTS reagent (Perkin Elmer part#6016989). After a 15 minutes incubation time, light emission was measured (30s per plate) with the ViewLux reader (Perkin Elmer).

The percent inhibition of each compound was calculated as follows: %inhibition=(1-(test_compound median_positive_control)/(Median_positive_control - Median_negative_control)*100 with positive control: 10 micromolar of the reference inhibitor #5653914 (ChemBridge) and negative control : DMSO only. All data reported were normalized on a per-plate basis.

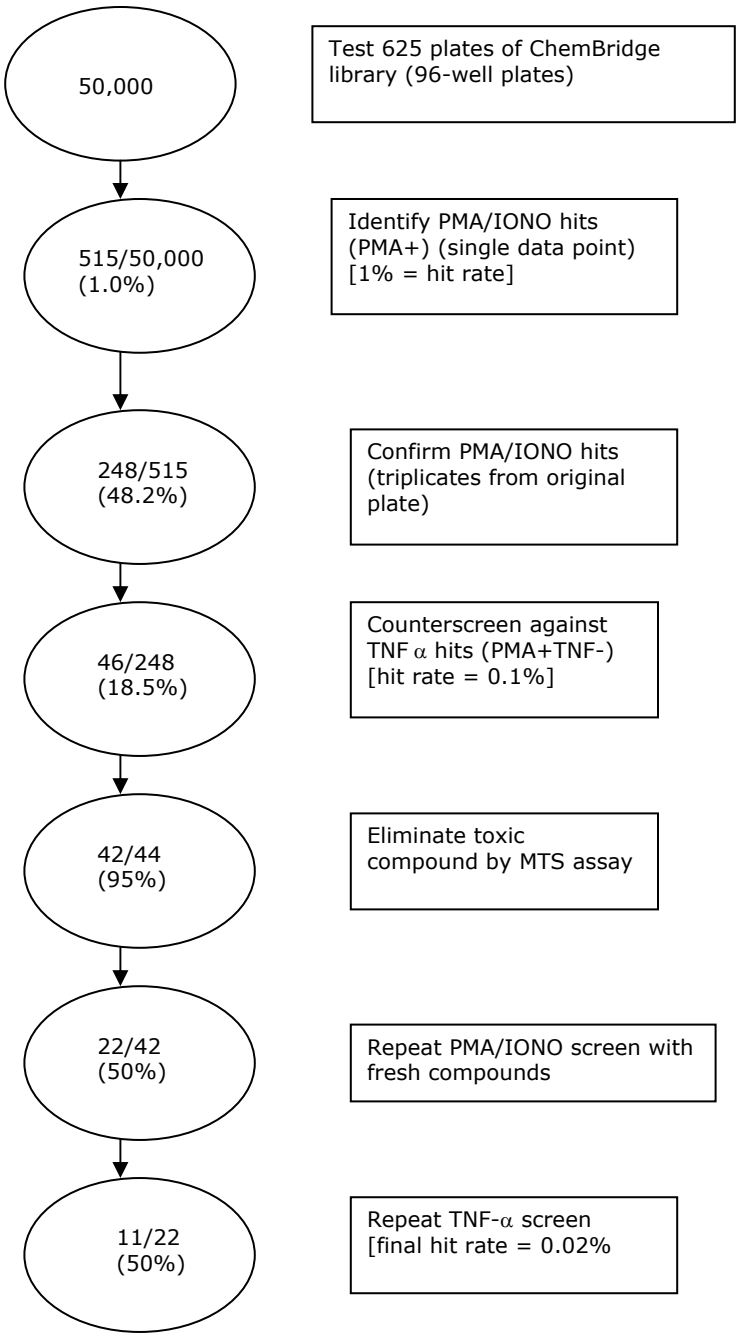
Center Summary of the Primary Screen and confirmatory dose-response studies.

The NIH library consisted of 61,609 compounds at the time of the primary screen by the Scripps Center. From this screen, 56 hits showed significant inhibition at 3 uM. Of them, 20 compounds were obtained from vendors and studied further for their activity in different assays. Of these, 5 proved to be luciferase inhibitors and 6 compounds did not inhibit PMA/Ionomycin-induced NF-kB activity in HEK-293 cells. Of the remaining active compounds, 9 compounds demonstrated acceptable dose-response activity, with IC_{50}s ranging from 0.25 to 2 uM in the reporter gene assay. Of these active compounds, 6 inhibited NF-kB reporter gene activation induced by TNFa, and were abandoned for purposes of this project. The PubChem AIDs for these pathway non-selective inhibitors are provided above, as these compounds may be of utility for other types of NF-kB studies. Characterization of some of these hits continues. Of the remaining 3 compounds from the NIH library, one compound, CID-2998237, was active against PMA/Ionomycin-stimulated but not TNF-stimulated NF-kB activity with IC_{50} ~ 1 uM. However, this compound failed to inhibit an endogenous NF-kB-inducible gene in assays where HEK293 cells were stimulated with PMA and IL-8 secretion was measured by ELISA. The other two compounds did not inhibit anti-CD3/CD28 induced IL-2 production in Jurkat cells. Thus, ultimately, no viable hits were obtained from the NIH library.

Because the screen of the NIH library did not lead to an acceptable compound, the assay provider requested consideration of alternative compounds identified by screening of an in-house (non-NIH) chemical library. The San Diego Center for Chemical Genomics (at Burnham Institute for Medical Research) accepted transfer of the project. The ChemBridge 50K DiverSet library, the SIGMA LOPAC library, and the Microsource libraries

(50,000, 1280, and 2000 compounds, respectively) were screened for compounds that inhibit PMA/Ionomycin- induced NF-κB activity in stably transfected HEK293 cells. The hit rates for that primary screen were 1% (ChemBridge), 2.8% (SIGMA), and 10.7% (Microsource). These hit rates are the result of one single screen (ChemBridge), 2 independent screens (SIGMA), and 3 independent screens (Microsource). Hits were scored positive if present in at least 1/1 (ChemBridge), 2/2 (SIGMA), and 2/3 (Microsource) experiments. The high hit rate observed for the microsource library may be because this library consists largely of natural products.

In a counter-screen described below, TNFα was substituted for PMA/Ionomycin as a first step toward identification of pathway-specific hits, with the goal of identifying compounds that inhibit NF-κB activation induced by PMA/Ionomycin but not by TNFα. A cell viability assay (MTS) was also used to eliminate toxic compounds. The flow-chart provided shows the respective number of hits for each of the assays performed during the screen of the ChemBridge 50K library. The goal was to identify PMA/Ionomycin hits (PMA+) that do not interfere with the TNFα signaling pathway (TNF-) and that are non toxic (MTS-). The cut-offs for a hit to qualify for further analysis were defined as >50% relative inhibition for PMA/Ionomycin and <30% for TNFα.



Summary of Screening Results*: The flow chart summarizes the strategy applied to identify compounds within the ChemBridge 50K DiverSet set that inhibit NF-κB activity induced by PMA/Ionomycin but not TNFα in HEK293 cells. Shown are the respective numbers of hits for each of the screening step. An important step is in the verification of the identified hits involves using a new batch of the compounds containing fresh lyophilized powder to rule out contamination or degradation of the selected compounds during the initial screen. Both PMA/Ionomycin and TNF screens were repeated to confirm the results shown.*

Probe Optimization

Chemical name of probe compound: 1-(3,5-di-tert-butyl-4-hydroxyphenyl)-2-(2-(3-hydroxypropylamino)-5,6-dimethyl-1H-benzo[d]imidazol-1-yl)ethanone.

Draw probe chemical structure and show stereochemistry if known: see Figure 1.

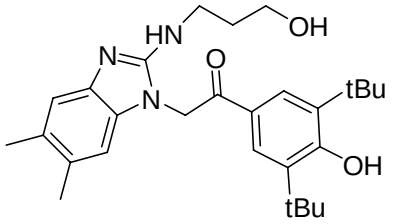


Figure 1. Structure of MLS-0146295 (CID-2858522)

Describe mode of action for biological activity of probe:

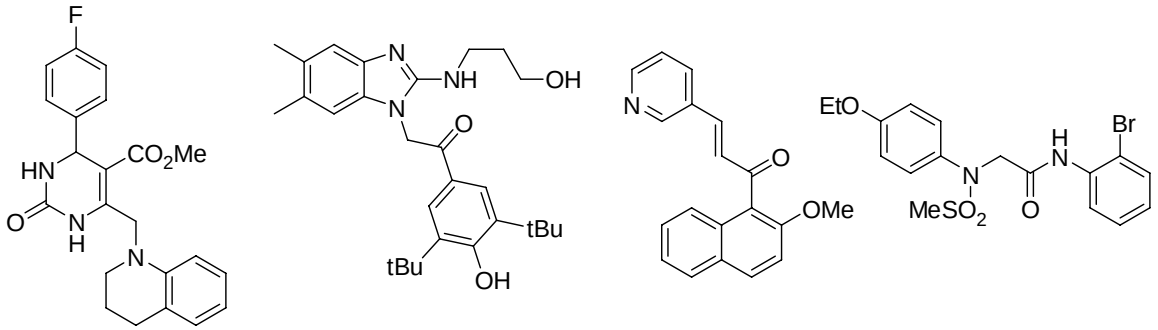
The probe compound inhibits the activity of the NF-kB luciferase reporter gene in HEK-293 cells stimulated with PMA and Ionomycin with $IC_{50} \leq 100$ nM. The compound is similarly active using phorbol dibutryate (PDB) instead of PMA (phorbol myristic acetate) as a PKC agonist. At concentrations up to 4 uM, the compound is inactive at suppressing NF-kB luciferase reporter gene activity induced in the same cells (HEK293) by TNFa or by transfection with an active version of TLR4 (CD4-TLR4 fusion protein; member of Toll-like Receptor family) or by transfection with CD40 (a TNF-family receptor), or by transfection with NOD1, or by transfection with NOD2, or by transfection of PIDD (p53-induced protein with a death domain), a protein playing a critical role in DNA-damage induced NF-kB activation. The probe compound also inhibits PMA-induced secretion of the NF-kB inducible cytokine IL-8 by HEK293 cells, with $IC_{50} \simeq 100$ nM. The compound is not toxic against HEK293, HEK293T, THP.1, Jurkat, MCF7, or HeLa cells. In contrast to HEK293 cells, the probe compound is inactive with respect to suppression of PMA-induced NF-kB reporter gene activity in HEK293T cells, HeLa, MCF7, and PPC-1 cells. The probe compound partially inhibits IL-2 production by Jurkat T-cells stimulated with anti-CD3/anti-CD28, consistent with partial dependence on NF-kB for T-cell antigen receptor-mediated induction of the IL-2 gene.

Has this compound been provided to the MLSMR: Yes.

Commercial vendor information if available for probe or analogs that have been purchased:

The Scripps Center screening led to the identification of a single validated hit CID-2998237 which had an IC_{50} value of 0.7 uM in the NF-kB assay (Figure 2). Follow up on this structure using the "analogue by catalogue" approach was not productive in generating compounds with improved properties compared with the hit. As described above, to identify additional hits from different structural classes with improved potency, we screened the Burnham compound collection consisting of 50K compounds. This led to the identification of three validated hits as shown in Figure 2 (designated

BIMR). One of the hits, the benzimidazole derivative MLS-0146295 (CID-2858522), was a highly potent inhibitor of the NF-kB pathway and we therefore initiated synthetic chemistry on this series (see description below).



CID-2998237	MLS-0146295 (BIMR)	BIMR	BIMR
NF-κB: IC_{50} = 0.7 μM	NF-κB: IC_{50} = 0.07 μM	NF-κB: IC_{50} = 1.5 μM	NF-κB: IC_{50} = 1.0 μM
Analogues tested: 12	Analogues tested: 8	Analogues tested: 11	Analogues tested: 3

Figure 2. Structure and potency of NF-κB hits and the number of purchased analogues from commercial vendors tested in the initial phase of evaluation.

Description of secondary screens used to optimize probe structure:

In total, 26 secondary screens have been used to date to characterize the Probe compound, CID-2858522. The compound is active with IC_{50} ~100 nM as an inhibitor of NF-kB reporter gene activity in HEK293 cells (assay #1) and as an inhibitor of PMA-induced IL-8 production by HEK293 cells (assay #2). The compound similarly inhibits NF-kB reporter gene activity induced by PDB in HEK293 cells (assay #3). The compound is also partially active as an inhibitor of IL-2 production by Jurkat T-cells stimulated with anti-CD3 plus anti-CD28 antibodies (assay #4), suppressing activity by about half at concentrations < 1 uM, implying that the T cell antigen receptor pathways leading to IL-2 gene induction are partially dependent on the target of the probe compound in this T-cell line. The compound is not toxic against HEK293 (assay #5), HEK293T (assay #6), Jurkat (assay #7), THP.1 (assay #8), MCF7 (assay #9), or HeLa (assay #10) cells. The compound does not inhibit purified luciferase (assay #11), PKC-beta (assay #12), PKC-theta (assay #13), or IKK-beta (assay #14) in vitro at concentrations up to 4 uM. In HEK293 cells, the probe compound does not inhibit NF-kB reporter gene activity induced by TNFa (assay #15) or by transfection with plasmids encoding CD4-TLR4 (assay #16), CD40 (assay #17), NOD1 (assay #18), NOD2 (assay#19) or PIDD (assay#20) at concentrations up to 4 uM. The compound does not inhibit NF-kB reporter gene activity induced by PMA in HeLa (assay #21), HEK293T (assay #22), MCF7 (assay #23), or PPC-1 (assay #24) cells at concentrations up to 4 uM. The compound does not

inhibit NF-kB reporter gene activity in THP.1 monocytes stimulated with LPS (assay #25) at concentrations up to 4 uM, or by NOD1 agonist muramyl dipeptide (MDP) in MCF7 breast cancer cells (assay #26), at concentrations up to 4 uM. Thus, probe compound CID-2858522 is a pathway-selective and cell type-selective inhibitor of NF-kB activity.

Center comments on chemistry strategy leading to probe identification:

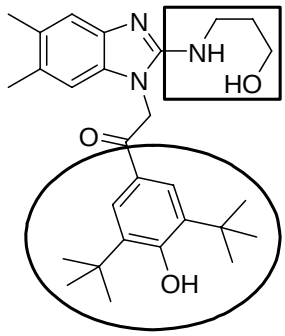


Figure 3. SAR analysis of MLS-0146295

A library of 200 compounds was synthesized according to the procedures illustrated in Schemes 1 and 2 below and the compounds were compared with MLS-0146295 in the bioassays (see Table 1). Two main areas of interest were examined for the SAR (see rectangle and oval in Figure 3).

Compounds were synthesized by replacement of the aminopropanol group, in red, by heterocyclic amines, (i.e., pyrrolidine and piperidine analogs) but these analogs showed minimal activity, (i.e., IC_{50} values > 8 uM) in bioassays. In addition, analogs with changes in the phenyl ring, (in blue), also gave IC_{50} values > 8 uM in the bioassays used. Based on these results, the SAR around MLS-0146295 appears to be very steep. Notably, the amine side chain (circled in red) apparently is required to be a secondary amine as tertiary amines in this part of the molecule significantly decrease potency. Replacement of the methyl substituents on the benzimidazole with H, as in MLS-0111683, appears to have little effect on potency (Table 1). Furthermore, replacement of the phenolic OH with methoxy provides a compound (MLS-0202041) that retains potency compared with the lead structure.

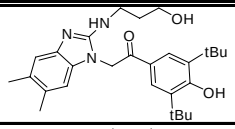
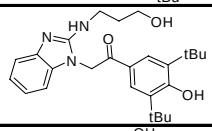
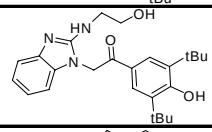
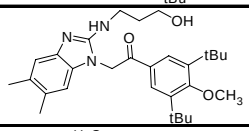
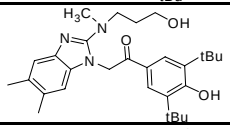
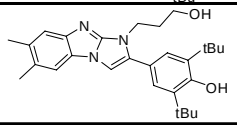
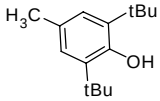
		NF-kB
Compound #	**Structure**	**IC_{50} (uM)**
MLS-0146295		0.07
MLS-0111683		0.10
MLS-0111673		0.30
MLS-0202041		0.10
MLS-0146299		inactive
MLS-0146296		inactive
MLS-0146297		inactive

Table 1. Data on selected analogues of NF-κB hit MLS-0146295.

Chemical resynthesis of MLS-0146295 was performed to compare an authentic sample with the commercially purchased compound in the bioassay used. The synthesis of

MLS-0146295 (shown in Scheme 2) gives two major products, the open chain form, see Figure 3 and a cyclized product shown in Figure 4.

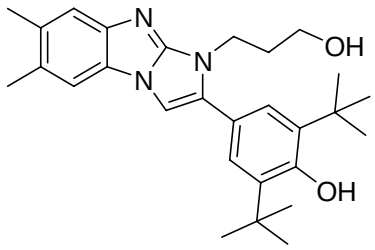
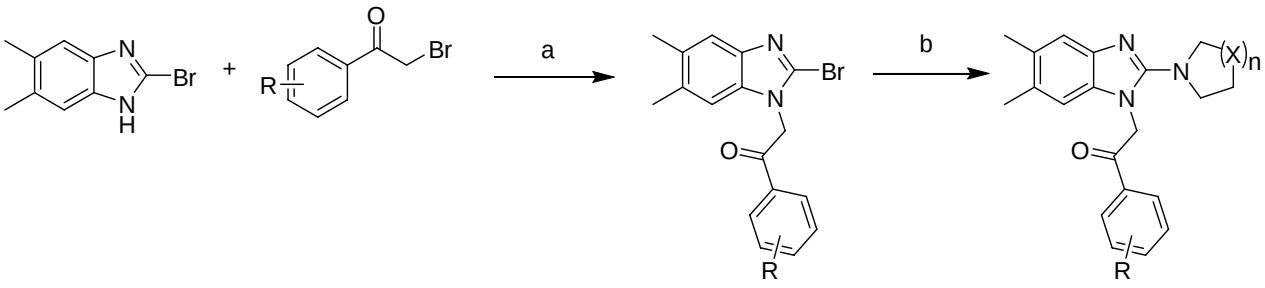


Figure 4. Cyclized product isolated during synthesis of MLS-0146295.

Both the cyclic and open chain products along the route to the synthesis of MLS-0146295 were tested in parallel to determine: 1) the potency of each compound, 2) to verify that the structure of the purchased compound was identical to that of the synthetic compound and 3) to examine the chemical stability of MLS-0146295 towards cyclization. The resynthesized MLS-0146295 was equipotent with the purchased compound; but the cyclized compound was only weakly active. To examine these results in greater detail, the N-methyl analog of MLS-0146295 was synthesized (MLS-0146299, see Table 1) but it too was found to be weakly active presumably because a tertiary amine is not accepted at this position for full biological potency.

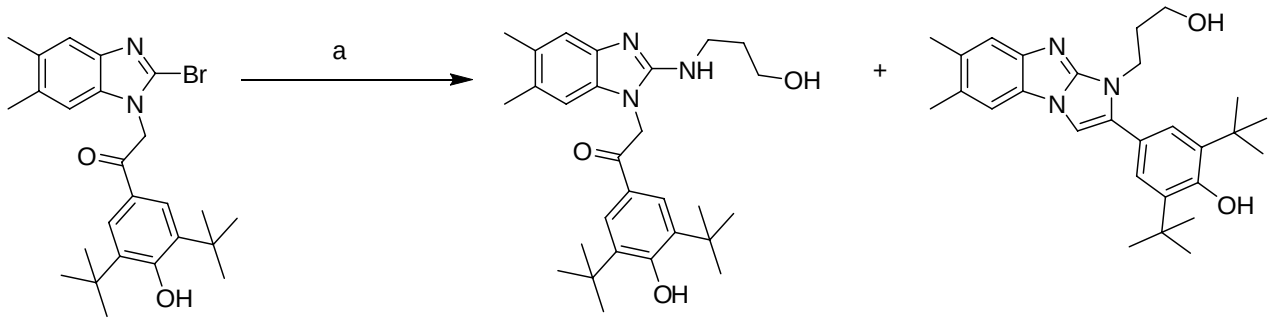
Based on all of the analogs synthesized and tested, MLS-0146295 (CID-2858522) was selected as a probe for NF-κB. The results show the secondary amine on the aminopropanol side chain requires a hydrogen atom for full biological activity (i.e., possibly to aid in H-bond donation function). In addition, the aromatic ring of the acetophenone moiety apparently requires some aliphatic and polar groups to help in binding or recognition and appears to prefer the di-tertbutyl group. Interestingly, the known antioxidant MLS-0146297 (see Table 1) was inactive in the bioassay, suggesting that the mechanism of action of the lead structure is more complex than simple redox. The Probe was profiled in the panel of Ambit assays and was inactive against all 353 kinases. It was profiled in 30 secondary assays and confirmed to be highly pathway-specific. The compound has also been sent to other Centers within the network for additional profiling in secondary cell-based assays that measure activity of cellular pathways that may or may not involve NF-κB.

Reduced to practice detailed synthetic pathway for making probe:



1 **2** **3** **4**

Scheme 1. Reagents and conditions: (a) K_2CO_3, THF, rt; (b) cyclohexylamine or cyclopentylamine, 110°C, 4 h.



5 **6** **7**

Scheme 2. Reagents and conditions: (a) 3-aminopropanol, 110°C, 4 hr, two products isolated (6 and 7).

Known probe properties:

A summary of the properties of CID-2858522 is provided in the table below**.**

Table 2. Activity on different stimuli inducing NF-kB activation in 293 cells

Stimuli	PMA/Iono	PMA/Iono	PDB	PDB	TNF	CD4-TLR4	CD40	NOD1	NOD2	PIDD
Readout	Luc.	IL-8	Luc.	IL-8	Luc.	Luc.	Luc.	Luc.	Luc.	Luc.
Cell type	293	293	293	293	293	293	293	293	293	293
Result	+	+	+	+	-	-	-	-	-	-

(Luc. = luciferase)

Table 3. Activity on other cell types

Stimuli	Anti-CD3/CD28	LPS	Anti-LTbR	MDP	PMA	PMA	PMA	PMA
Readout	IL-2	IL-8	Luc.	IL-8	Luc.	Luc.	Luc.	Luc.
Cell type	Jurkat	THP-1	HeLa	MCF-7	HeLa	293T	MCF-7	PPC-1
Result	+	-	-	-	-	-	-	-

Table 4. Activity using in vitro kinase assays

Stimuli	PKC-beta	PKC-theta	IKK-beta
Readout	kinase assay	kinase assay	Kinase assay
Cell type	in vitro	in vitro	In vitro
Result	-	-	-

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

The solubility of the probe compound CID-2858522 in DMSO is excellent at concentrations of at least 50 mM. No noticeable precipitation formed when mixed with culture medium, PBS or water. At concentrations exceeding 50 mM, the compound in DMSO shows slight light brown color.

Bibliography

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