

Probe Report

Title: B-cell Specific Inhibitors of NF- κ B Activation

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Screening Center Name & PI: Sanford Burnham Center for Chemical Genomics (SBCCG) & John C. Reed (PI)

Chemistry Center Name & PI: *same as above*

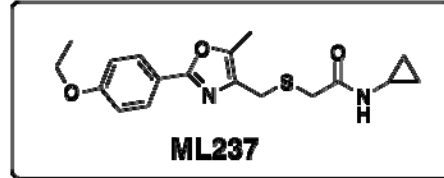
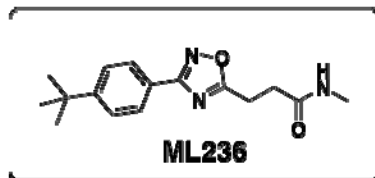
Assay Submitter & Institution: John C. Reed, Sanford-Burnham Medical Research Institute

PubChem Summary Bioassay Identifier (AID): 435012

Abstract. At least six cellular pathways leading to activation of NF κ B-family transcription factors have been identified, participating in host-defense, immunity, inflammation, and cancer. The pathway initiated by Protein Kinase C (PKC) family kinase is critical for signaling downstream of antigen receptors in T-cells and B-cells, as well as many growth factor receptors and some GPCRs that stimulate intracellular Ca²⁺ elevations. This pathway often involves a multi-component protein complex that includes CARMA, Bcl-10, and MALT1. Previously, a potent (~67 nM) and selective probe (**ML029**, Pubchem CID2858522) was identified as an inhibitor of the PKC-initiated NF- κ B pathway after a battery of 12 secondary and counter-screen assays. However, this probe appeared potent only in the original HEK293 reporter cell line and some types of primary lymphocytes, with little potency in several other tumor cell lines. We used HTS assays employing NF- κ B reporter genes in T-cell and B-cell lines for screening the ~330,600 compound MLSMR collection to identify novel pathway-selective inhibitors. We now disclose the discovery and characterization of two novel potent small molecule cell-active NF- κ B inhibitors that are both (a) selective for the NF- κ B pathway induced by PKC activators (phorbol myristic acetate/ionomycin) and (b) selective for B-cells. The first oxadiazole-based probe (**ML236**, PubChem CID665654) is potent (35 nM) in a B-cell line and >400-fold selective over both the NF- κ B activation in T-cells and against TNF α -mediated NF- κ B activation. In comparison, the second oxazole-based probe (**ML237**, PubChem CID4174238) is also potent (208 nM) in a B-cell line and >400 fold selective over both the NF- κ B activation in T-cells and against TNF α -mediated NF- κ B activation. The synthetic methodology, SAR development, and initial pharmacologic characterization are also presented. These probe molecules will be used to define the B-cell specific targets along these pathways and will serve as tools to reveal hitherto unknown components of these signaling pathways. These probes will prove useful to researchers working in immunology, autoimmune diseases, transplantation and allograft rejection, lymphoid malignancies, immune signaling, and general signal transduction.

Probe Structure & Characteristics:

This Center Probe Report describes two novel inhibitors of NF- κ B activation in B-cells, **ML236** (CID665654) and **ML237** (CID4174238). Potency, selectivity and cytotoxicity characteristics are summarized for these probes below.



CID / ML#	Target Name	IC ₅₀ (nM) [SID, AID]	Anti-target Name(s)	IC ₅₀ (μM) (selectivity) [SID, AID]
CID665654 ML236 <i>Oxadiazole</i>	NF- κ B activation in B-cells	34.9 nM SID103061970 AID504485, 504487, 504489	NF- κ B activation in T-cells TNF α -mediated NF- κ B activation Cytotoxicity	>79 μM (>2263X) SID103061970 AID504512, 504514, 504516 53.2 μM (>1524X) SID103061970 AID504506, 504517, 504518 >79 μM SID103061970 AID493251
CID4174238 ML237 <i>oxazole</i>	NF- κ B activation in B-cells	190 nM SID103061890 AID504485, 504487, 504489	NF- κ B activation in T-cells TNF α -mediated NF- κ B activation Cytotoxicity	>79 μM (>379X) SID103061890 AID504512, 504514, 504516 >79 μM (>379X) SID103061890 AID504506, 504517, 504518 >79 μM SID103061890 AID493251

Recommendations for scientific use of the probe:

Regulated control of NF- κ B is critical for the immune, inflammatory, proliferative, anti-apoptotic, and differentiation responses in both T- and B-cells via the ligation of their respective antigen receptors in confluence with a number of other cell specific co-receptor activities. The signaling that leads to the various cell mediated outcomes is complex and the mechanisms by which NF- κ B activation performs specific functions that drive these outcomes is not thoroughly understood. Although many biological tools exist for probing NF- κ B functions, there are few if any small molecule tools that can probe the common non-canonical TCR and BCR signaling pathways leading to NF- κ B activation. This NF- κ B pathway activated downstream of antigen receptors is uniquely required for adaptive immune response, setting it apart from the numerous NF- κ B pathways implicated in innate immunity. Probes that are discovered by the screening for compounds that inhibit TCR and BCR mediated NF- κ B activation will be used to define the specific targets along these pathways and will serve as tools to reveal hitherto unknown components of these pathways. These probes will prove useful to researchers working in immunology, autoimmune diseases, transplantation and allograft rejection, lymphoid malignancies,, immune signaling, and general signal transduction.

1 Introduction

Overall Goal of Project:

This assay was originally assigned to the Scripps Research Institute Molecular Screening Center (TSRI MSC) by the NIH during the MLSCN pilot phase. The Scripps Center completed primary screening (AID: 465) on only 61,609 compounds of the NIH compound collection, as well as dose-response experiments (PubChem AID: 586). Unfortunately, none of the compounds identified from the gene reporter assay suppressed the secretion of IL-8, an endogenous NF- κ B inducible gene product in secondary assays in relevant cell lines. Dr. Reed's lab screened an additional 53,280 from his internal collection and was ultimately able to identify one pathway selective probe molecule (CID2858522-ML029), after a battery of 12 secondary and counter-screen assays. The rationale, assays, flowchart for hit validation, and SAR support to the eventual probes are detailed in the Chemical Probe Report (CPR) that can be accessed from the MLP site: <http://mli.nih.gov/mli/mlp-probes/> or the full pdf can be downloaded from the following URL: https://mli.nih.gov/mli/?dl_id=454. This prior probe, ML029, appears to only be potent in the original HEK293 reporter cell line and shows poor or little potency in HEK293T (containing the SV40 T antigen) or other more relevant cell lines.

As summarized in the CPR for ML029, the previous probe compound inhibits the activity of the NF- κ B luciferase reporter gene in HEK-293 cells stimulated with PMA and Ionomycin with $IC_{50} < 100$ nM. The compound is similarly active using phorbol dibutyrate (PDB) instead of PMA (phorbol myristic acetate) as a PKC agonist. At concentrations up to 4 μ M, the compound is inactive at suppressing NF- κ B luciferase reporter gene activity induced in the same cells (HEK293) by TNF α 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- κ B activation. The probe compound also inhibits PMA-induced secretion of the NF- κ B inducible cytokine IL-8 by HEK293 cells, with $IC_{50} \sim 100$ 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- κ B 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/antiCD28, consistent with partial dependence on NF- κ B for T-cell antigen receptor-mediated induction of the IL-2 gene.

The NIH collection has grown to >325,000 compounds since the original MLSCN screen of 61,609 of the MLSMR. The goal therefore is to identify *additional* compounds that selectively inhibit one of the several known pathways that lead to NF- κ B activation in *relevant* mammalian cells in a T-cell or B-cell specific manner. In the original MLSCN screen a stably transfected reporter epithelial cell line (HEK293) that contains a luciferase gene driven by a NF- κ B responsive promoter was used for compound library screening. The hits identified from that screen were very much less potent when tested in B and T cell lines. Therefore, in this rescreening paradigm, the primary screen(s) will be accomplished with *two separate but related screens* using an NF- κ B-luciferase reporter cloned into a T-cell line (Jurkat) or a B-cell line (697). The data from these *two* separate screens were used to define *three* classes of potent (<1 μ M IC_{50}) cell-type specific small molecule chemical inhibitors of NF- κ B activation: (1) T-cell specific, (2) B-cell specific, and (3) T & B-cell dual specific. These cell-type selective inhibitors are however still to be selective against TNF (>10 X potency), inhibit IL-8 secretion at comparable potencies, and are selective (>10X potency) against representative PKCs (PKC beta and PKC theta that are involved in T and B cell signaling).

As before, at least six cellular pathways leading to activation of NF- κ B-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- κ B activation (Ruland et al. 2001; McAllister-Lucas et al. 2001; Rueffli-Brasse *et al.* 2003; Zhou *et al.* 2004). Counter-screens using activators of other NF- κ B activation pathways identify pathway specific inhibitors, including (1) NF- κ B reporter gene activity or cytokine production induced by Lipopolysaccharide (LPS) in the THP.1 monocyte cell line (TLR4 agonist), (2) by gamma-Tri-DAP and muramyl dipeptide (MDP) in MCF7 breast cancer or HCT116 colon cancer cells (NLR agonists), (3) DNA damaging agent Doxorubicin in Jurkat and 697 cells, and (4) BAFF (a TNF family member that induces a non-canonical, alternative NF- κ B pathway) in 697 cells.

In summary, Dr. Reed's lab seeks potent (<1 μ M IC50) chemical inhibitors of NF- κ B activation that are (1) T-cell specific, (2) B-cell specific, or (3) T & B-cell dual specific, that are selective against TNF and their cognate PKCs (>10 X), and inhibit IL-8 secretion at comparable potencies.

Aims of the project: We propose to use this cell-based HTS assay for screening the NIH's chemical library. The compounds identified using this assay will be useful research tools for analysis of the physiological roles of this NF- κ B activation pathway.

2 Materials and Methods

The details of the primary HTS and additional assays can be found in the "Assay Description" section in the PubChem BioAssay view under the AIDs as listed in **Table 1** below. Additionally the details for the primary HTS are provided in the Appendix at the end of this probe report.

2.1 Assays

Table 1 summarizes the details for the assays that drove this probe project.

Table 1. Summary of Assays and AIDs					
PubChemBioAssay Name	AIDs	Probe Type	Assay Type	Assay Format	Assay Detection & well format
Summary assay for the identification of chemical inhibitors of B-cell specific antigen receptor-induced NF- κ B activation.[Summary]	435025	Inhibitor	Summary	N/A	N/A
uHTS luminescence assay for the identification of chemical inhibitors of B-cell specific antigen receptor-induced NF- κ B activation [Primary Screening]	435022	Inhibitor	Primary	Cell-based	Luminescence & 1536 well
Single concentration confirmation of chemical inhibitors of B-cell specific antigen receptor-induced NF- κ B activation [Primary Screening]	449746	Inhibitor	Confirmatory (Cherry Pick)	Cell-based	Luminescence & 1536 well
Dose response confirmation of uHTS of chemical inhibitors of B-cell specific antigen receptor-induced NF- κ B activation in a 697B cell line using a luminescence assay [Confirmatory]	489023	Inhibitor	Confirmatory (DMSO Dose Response)	Cell-based	Luminescence & 1536 well
SAR Analysis of small molecule inhibitors of B-cell specific antigen receptor-induced NF- κ B activation in a 697B cell line using a luminescence assay	504485; 504508	Inhibitor	Confirmatory (Dry Powder)	Cell-based	Luminescence & 1536 well

[Confirmatory]					
Dose response cytotoxicity of uHTS chemical inhibitors of B-cell specific antigen receptor-induced NF-κB activation in a HEK-293T cell line using a luminescence assay [Confirmatory]	489021	Inhibitor	Secondary (DMSO DR)	Cell-based	Fluorescence & 1536 well
SAR analysis of small molecule cytotoxicity in HEK293 cells for activators of NF-kappaB using a luminescence assay [Confirmatory]	493251; 504528	Inhibitor	Secondary (Dry Powder)	Cell-based	Fluorescence & 1536 well
Dose response counterscreen of uHTS chemical inhibitors of B-cell specific antigen receptor-induced NF-κB activation in a HEK-293T cell line using a luminescence assay [Confirmatory]	489022	Inhibitor	Secondary (DMSO DR)	Cell-based	Luminescence & 1536 well
SAR Analysis of small molecule inhibitors of B-cell specific antigen receptor-induced NF-κB activation in a HEK-293T cell line using a luminescence assay [Confirmatory]	504518	Inhibitor	Secondary (Dry Powder)	Cell-based	Luminescence & 1536 well
Dose Response selectivity of uHTS chemical inhibitors of B-cell specific antigen receptor-induced NF-κB activation in Jurkat cells using a luminescence assay [Confirmatory]	489006	Inhibitor	Secondary (DMSO DR)	Cell-based	Luminescence & 1536 well
SAR Analysis of small molecule inhibitors of B-cell specific antigen receptor-induced NF-κB activation in a Jurkat cell line using a luminescence assay [Confirmatory]	504516; 504515	Inhibitor	Secondary (Dry Powder)	Cell-based	Luminescence & 1536 well

2.2 Probe Chemical Characterization

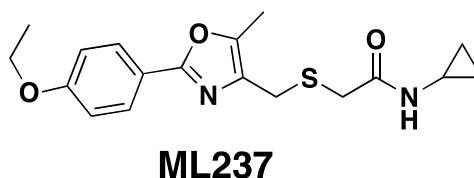
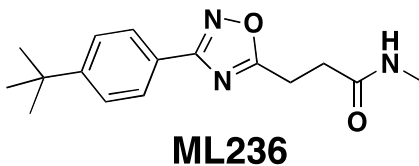
a) Chemical name of probe compound

The IUPAC names of the probes are 3-(3-(4-(*tert*-butyl)phenyl)-1,2,4-oxadiazol-5-yl)-N-methylpropanamide (**ML236**) and N-cyclopropyl-2-(((2-(4-ethoxyphenyl)-5-methyloxazol-4-yl)methyl)thio)acetamide (**ML237**).

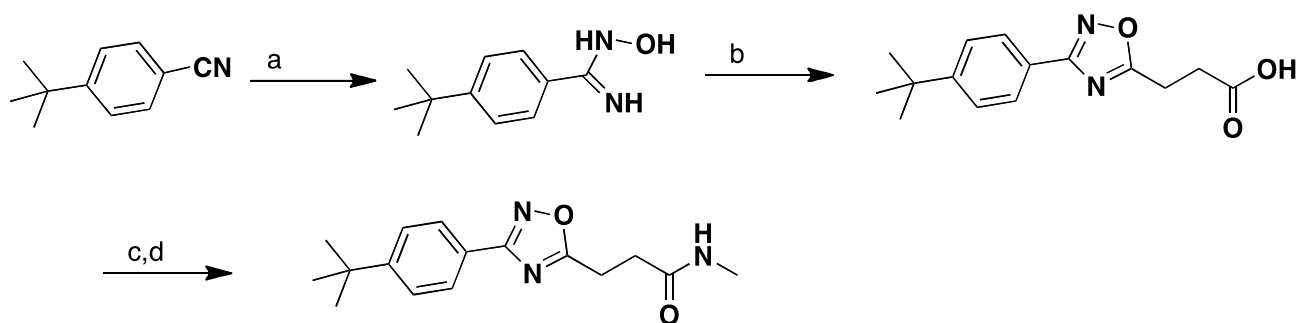
The specific batches prepared, tested and submitted to the MLSMR are archived as SID103061876 corresponding to CID665654 (**ML236**) and SID103061890 corresponding to CID4174238 (**ML237**).

b) Probe chemical structure including stereochemistry if known

The probes **ML236** and **ML237** have no chiral centers

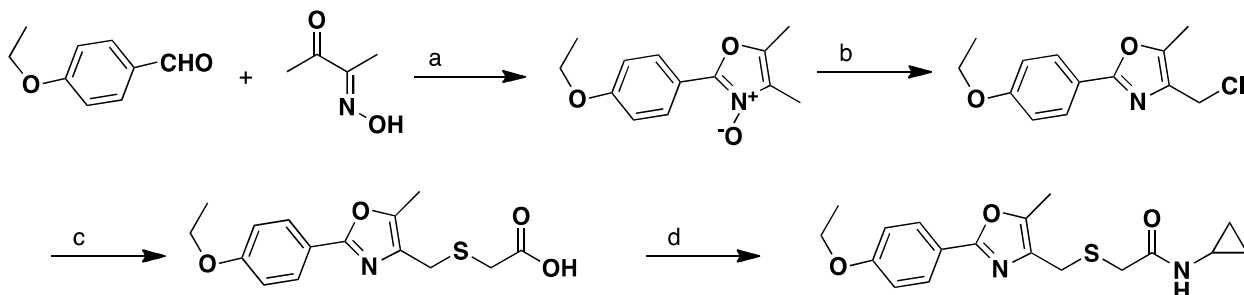


c) **Synthesis and Structural Verification Information of probe SID103061876 corresponding to CID665654 (ML236)**



Scheme 1: Synthesis of **ML236**, conditions: a. Hydroxylamine hydrochloride, triethylamine, methanol, 80 °C, 30 minutes; b. succinic anhydride, DMF, 120 °C, overnight, 47% for two steps; c. thionyl chloride, methylene chloride, 0 °C to room temperature, 3 hours; d. methylamine, methylene chloride, 0 °C to room temperature, overnight, 85% for two steps.

Synthesis and Structural Verification Information of probe SID103061890 corresponding to CID4174238 (ML237).



Scheme 2: Synthesis of **ML237**, conditions: a. acetic acid, hydrochloric acid (aq), 0 °C, 3 hours, 23%; b. POCl₃, chloroform, refluxing, 2 hours, 80%; c. thioglycolic acid, sodium hydroxide, methanol, 65 °C, 2 hours, 79%; d. cyclopropylamine, EDCI, HOBt, diisopropylethylamine, methylene chloride, 0 °C to room temperature, overnight, 80%.

For detailed procedures see Section 2.3. Images of spectral data (¹HNMR, ¹³CNMR, and LC/MS) used to support the structural assignment of **ML236** and **ML237** can be found in Section 6 (Supplementary Information).

d) **If available from a vendor, please provide details.**

Samples of **ML236** and **ML237** synthesized at SBCCG have been deposited in the MLSMR (Bio-Focus DPI) (see Probe Submission **Tables 3a/b** below). **ML236** is available from Ambinter, Cat#: STK86117, ambinter.com; **ML237** is available from Aurora, Cat# K01.550.497, aurorafinechemicals.com.

e) **Solubility and Stability of probe in PBS at room temperature:**

The stability and solubility of **ML236** and **ML237** was investigated in PBS buffer at room temperature (**Figure 1**). **ML236** showed slight instability after 48 hr. in PBS (80% remaining), whereas **ML237** was completely stable (see Figures and Table below)

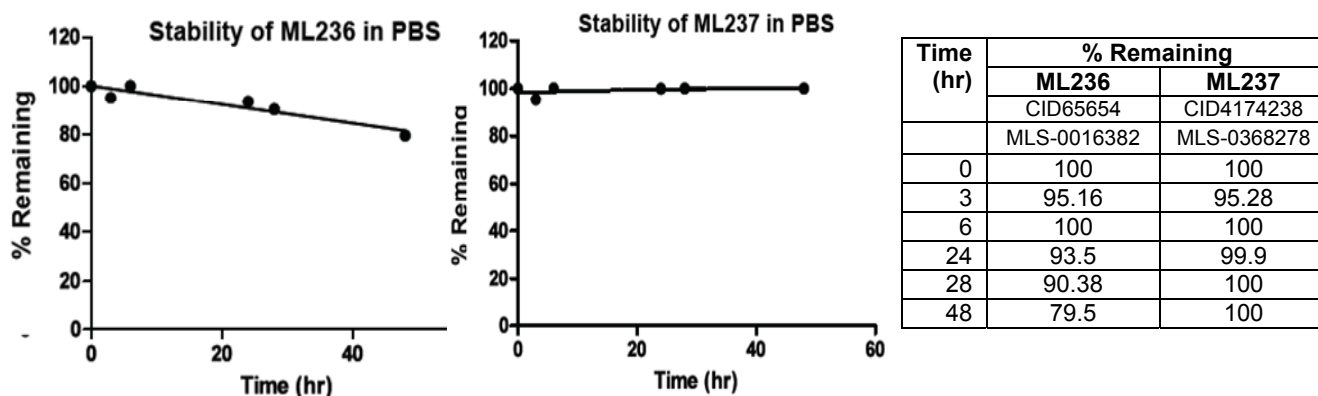


Figure 1: Stability of **ML236** and **ML237** in PBS

f) **A tabulation of calculated and known probe properties**

Table 2. Calculated Probe Properties for ML236 & ML237		
Calculated Property	Value	
	ML236 CID665654	ML237 CID4174238
Molecular Weight [g/mol]	287.3568	346.4439
Molecular Formula	C ₁₆ H ₂₁ N ₃ O ₂	C ₁₈ H ₂₂ N ₂ O ₃ S
XLogP3-AA	3	3.1
H-Bond Donor	1	1
H-Bond Acceptor	4	4
Rotatable Bond Count	5	8
Tautomer Count	6	2
Exact Mass	287.1634	346.1351
Monoisotopic Mass	287.1634	346.1351
Topological Polar Surface Area	68	89.7
Heavy Atom Count	21	24
Formal Charge	0	0
Complexity	346	411
Isotope Atom Count	0	0
Defined Atom StereoCenter Count	0	0
Undefined Atom StereoCenter Count	0	0
Defined Bond StereoCenter Count	0	0
Undefined Bond StereoCenter Count	0	0
Covalently-Bonded Unit Count	1	1

Samples of the probes (>25 mg) and each of five analogs (>20 mg) synthesized at SBCCG were submitted to the MLSMR (**Tables 3a** and **3b**), and 5 mg of the probe was provided to Dr. Reed's lab.

Table 3a. Probe and Analog Submissions to MLSMR (BioFocus DPI) for B-cell Specific Inhibitors of NF- κ BActivation

Probe ML 236-CID665654							
Probe /Analog	MLS_ID (SBCCG)	MLS_ID (MLSMR)	CID	SID	Source (vendor or SBCCG syn)	Amt (mg)	Date ordered/ submitted
Probe ML236	0016382	003448165	665654	103061876	SBCCG syn	46.9	3/23/2011
Analog 1	0013618	003448166	665747	103061878	SBCCG syn	20.4	3/23/2011
Analog 2	0454558	003448167	50898042	110067520	SBCCG syn	22.9	3/23/2011
Analog 3	0454559	003448168	18148563	110067521	SBCCG syn	29.6	3/23/2011
Analog 4	0454562	003448169	50898027	110067523	SBCCG syn	22.9	3/23/2011

Table 3b. Probe and Analog Submissions to MLSMR (BioFocus DPI) for B-cell Specific Inhibitors of NF- κ BActivation

Probe ML 237-CID4174238							
Probe /Analog	MLS_ID (SBCCG)	MLS_ID (MLSMR)	CID	SID	Source (vendor or SBCCG syn)	Amt (mg)	Date ordered/ submitted
Probe ML237	0368278	003448159	4174238	103061890	SBCCG syn	29.2	3/23/2011
Analog 1	0362296	003448160	6622243	103061885	SBCCG syn	23.0	3/23/2011
Analog 2	0052895	003448161	3237327	103061891	SBCCG syn	20.1	3/23/2011
Analog 3	0454588	003448162	50898056	110067550	SBCCG syn	20.8	3/23/2011
Analog 4	0454602	003448163	50898021	110067564	SBCCG syn	23.2	3/23/2011
Analog 5	0454577	003448164	50898054	110067539	SBCCG syn	21.1	3/23/2011
Analog 5	0445868	003448170	49787124	103061879	SBCCG syn	20.5	3/23/2011

2.3 Probe Preparation

ML236 Preparation:

Step 1. A microwave vessel was charged with 4-(*tert*-butyl)benzotrile (1.06 g, 6.66 mmol) and methanol (10 mL). Triethylamine (1.03 mL, 6.66 mmol) and hydroxylamine hydrochloride (460.0 mg, 6.66 mmol) were added. The tube was put in Biotage microwave reactor and was heated at 80 °C for 30 minutes. The mixture was transferred to a round bottom flask and the solvent was removed under reduced pressure. The flask was put under high vacuum for 30 minutes. The solid was dissolved in *N, N'*-dimethylformamide (10 mL). Succinic anhydride (880.0 mg, 6.66 mmol) was added. The mixture was stirred at 120 °C overnight. The solution was cooled down to room temperature. Hydrochloride solution (60 mL, 1N solution) was added. The mixture was extracted with ethyl acetate (3 x 40 mL), washed with brine (60 mL), dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The crude product was titrated with hexane (100 mL) and filtered. The white solid (850.0 mg, 47% for two steps) was used in the next step without further purification. ¹H NMR (400 MHz, CDCl₃) δ 7.97 (d, *J* = 10.5 Hz, 2H), 7.49 (d, *J* = 10.3 Hz, 2H), 3.28 (t, *J* = 7.4 Hz, 2H), 3.05 (t, *J* = 7.4 Hz, 2H), 1.35 (s, 3H). ESI *m/z* 275 [M+H].

Step 2. A round-bottom flask was charged with 3-(3-(4-(tert-butyl)phenyl)-1,2,4-oxadiazol-5-yl)propanoic acid (314.0 mg, 1.24 mmol) and methylene chloride (10.0 mL) at 0 °C under N₂ atmosphere. Thionyl chloride (0.18 mL, 2.48 mmol, 2.0 equiv.) was added dropwise. The resulting solution was stirred for 30 minutes at 0 °C. The mixture was then stirred at room temperature for 3 hour. The solvent was removed under reduced pressure and the flask was put under high vacuum for 30 minutes. The solid was dissolved in methylene chloride (10 mL) at 0 °C under N₂ atmosphere. Methylamine solution (2M solution in tetrahydrofuran, 1.3 mL, 2.48 mmol, 2.0 equiv.) was added dropwise. The mixture was stirred for 1 hour at 0 °C. The solution was diluted with methylene chloride (30 mL) and was transferred to a separation funnel. The mixture was washed with saturated ammonia chloride solution (30 mL). The organic and aqueous layers were separated. The aqueous layer was extracted with methylene chloride (2 x 40 mL). The combined organic layer was washed with brine (30 mL), dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography eluting with 30 -50% ethyl acetate / hexane to afford the desired product as a white solid (302.9 mg, 85% yield): ¹H NMR (400 MHz, CDCl₃) δ 7.98 (d, *J* = 10.5 Hz, 2H), 7.53 (d, *J* = 10.3 Hz, 2H), 5.77 (br, s, 1H), 3.32 (t, *J* = 7.4 Hz, 2H), 2.86 (d, *J* = 4.9 Hz, 3H), 2.79 (t, *J* = 7.4 Hz, 2H), 1.37 (s, 3H). ¹³C (100 MHz, CDCl₃) δ 178.7, 170.9, 168.2, 154.6, 127.2, 125.8, 123.8, 34.9, 32.3, 31.2, 26.5, 22.5. Melting point: 120-122 °C.

ML237 Preparation:

Step 1. A solution of 2,3-butanedione monooxime (5.80 g, 58.3 mmol) and 4-ethoxybenzaldehyde (9.30g, 62.0 mmol) in acetic acid (50 mL) was cooled down to 0 °C and HCl solution (24 mL, 96.0 mmol, 4 M in 1,4 dioxane solution) was added. The mixture was stirred at 0 °C for 3 hour. Diethyl ether (100 mL) was added to the mixture and the resulting slurry was stirred for 1 hour at 0 °C. The white precipitate was filtered and washed with ether (20 mL). The solid was transferred to a beaker and washed with water (100ml) and concentrated ammonia hydroxide solution (12 mL). The mixture was extracted with CHCl₃ (2 x 100 mL), washed with brine (100 mL), dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure to afford 2-(4-ethoxyphenyl)-4,5-dimethyloxazole-3-oxide as a white solid (2.8 g, 23% yield). The unstable compound was used in the next step without further purification.

Step 2. To a solution of 2-(4-ethoxyphenyl)-4,5-dimethyloxazole-3-oxide (2.8g, 12.0 mmol) in anhydrous CHCl₃ (20 ML) was added POCl₃ (1.2 mL, 13.2 mmol) dropwise at room temperature. The mixture was heated to reflux for 2 hour, cooled to room temperature, poured into ice water (100 mL), neutralized with saturated NaHCO₃ solution (100 mL). The mixture was extracted with CHCl₃ (2 x 100 mL), washed with brine and dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The crude product was dissolved in hot hexane (100 mL), decanted from the insoluble dark material and cooled to 0 °C to allow the product to crystallize out. The solid was filtered and dried. Yield: 2.40 g (80%). ¹H NMR (400 MHz, CDCl₃) δ 7.95 (d, *J* = 6.9 Hz, 2H), 6.95 (d, *J* = 8.9 Hz, 2H), 4.11 (q, *J* = 6.9 Hz, 2H), 3.81 (s, 3H), 3.38 (s, 2H), 2.39 (s, 3H), 1.48 (t, *J* = 6.9 Hz, 3H). ESI *m/z* 252 [M+H].

Step 3. A solution of NaOH (770.0 mg, 19.2 mmol) and thioglycolic acid (883.0 mg, 9.6 mmol) in MeOH (30 mL) was stirred at room temperature for 30 minutes. 4-(chloromethyl)-2-(4-ethoxyphenyl)-5-methyloxazole (2.40 g, 9.6 mmol) was added and the mixture was stirred at 65 °C for 2 hour, cooled to room temperature. The solvent was removed under reduced pressure. Hydrochloride solution (100 mL, 1N solution) was added. The mixture was extracted with ethyl acetate (2 x 80 mL), washed with brine and dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The crude product was titrated with hexane (100 mL). The solid was filtered and

dried. Yield: 2.3 g (79%). ¹H NMR (400 MHz, CDCl₃) δ 7.92 (d, *J* = 6.9 Hz, 2H), 6.92 (d, *J* = 8.9 Hz, 2H), ESI *m/z* 308 [M+H].

Step 4. A round-bottom flask was charged with 2-(((2-(4-ethoxyphenyl)-5-methyloxazol-4-yl)methyl)thio)acetic acid (270.0 mg, 0.88 mmol) and methylene chloride (6.0 mL) at 0 °C. *N,N*-Diisopropylethylamine (0.31 mL, 1.76 mmol, 2.0 equiv.) was added followed by addition of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (260.0 mg, 1.32 mmol, 1.50 equiv.) and hydroxybenzotriazole (180.0 mg, 1.32 mmol, 1.50 equiv.). The resulting solution was stirred for 30 minutes at 0 °C. Cyclopropylamine (0.12 mL, 1.76 mmol, 2.0 equiv.) was added and the solution was stirred at room temperature overnight. The solution was diluted with methylene chloride (30 mL) and was transferred to a separation funnel. The mixture was washed with saturated ammonia chloride solution (30 mL). The organic and aqueous layers were separated. The aqueous layer was extracted with methylene chloride (2 x 30 mL). The organic layer was combined and washed with brine (30 mL). The organic layer was dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography eluting with 30 - 50% ethyl acetate / hexane to afford the desired product as a white solid (243.4 mg, 80% yield): ¹H NMR (400 MHz, CDCl₃) δ 7.89 (d, *J* = 6.9 Hz, 2H), 7.45 (br s, 1H), 6.95 (d, *J* = 8.9 Hz, 2H), 4.09 (q, *J* = 6.9 Hz, 2H), 3.58 (s, 2H), 3.13 (s, 2H), 2.63 (m, 1H), 2.33 (s, 3H), 1.42 (t, *J* = 6.9 Hz, 3H), 0.71 (m, 2H), 0.43 (m, 2H). ¹³C (100 MHz, CDCl₃) δ 170.6, 160.9, 160.4, 145.1, 132.1, 127.8, 120.1, 114.8, 63.8, 35.4, 27.6, 22.9, 14.9, 10.3, 6.5. Melting point: 125-126 °C.

3 Results

3.1 Summary of Screening Results

The flowchart on the right summarizes (Figure 2) the screening results for the NF-κB B- and T-Cell Assays. Following primary HTS of approximately 330,000 Molecular Libraries Small Molecules Repository (MLSMR) compounds at 4 μM in the NF-κB B-Cell Assay (AID435022), 1085 initial actives (~0.33% hit rate) were obtained at a 35% activity cut-off. For a cell-based assay, the screen was robust with an average *Z'* of 0.68.

The 1085 compound hits from the screen were cherry-picked in-house and retested in the NF-κB B-Cell assay confirmatory assay (AID449746) at 4 μM. For the single point reconfirmatory assay, a cut-off of 30% inhibition yielded a 63% reconfirm rate resulting in a total of 486 compounds. Fresh stock solution "cherry picks" of the 486 hits were requested from the MLSMR and 470 were received (96.7%) and tested and activities (IC₅₀) were confirmed in full 10-point dose-response

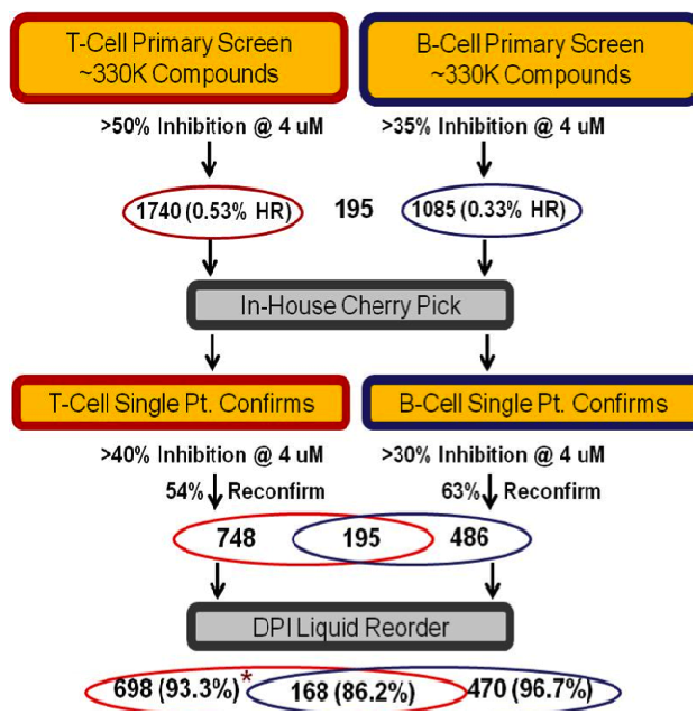
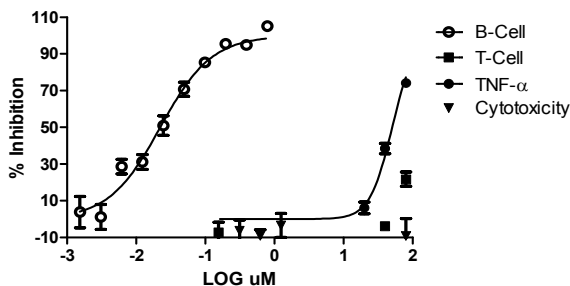


Figure 2. Screening results and Hit validation

titrations (AID489023). In parallel, selectivity was determined against the Jurkat (T-Cell) (AID489006) cell line and against the TNF α modulated pathway (AID489022). Also, the cytotoxicity of the compound hits was evaluated (AID489021). Based on the experimental results, dry powder compounds were selected for follow up studies and additional, commercially available analogues were ordered. From this we identified two series of compounds that were B-cell specific inhibitors of NF- κ B activation: (1) an oxadiazole, CID665654 and (2) an oxazole, CID4174238, that both had <0.5 μ M potency for inhibition of NF- κ B activation in B-cells with > 80 μ M potency for NF- κ B activation in T-cells, and > 80 μ M potency for TNF α mediated NF- κ B activation.

3.2 Dose Response Curves for Probes

Representative Dose Response of ML236 in the NF- κ B B-Cell and T-Cell, TNF- α , and Cytotoxicity Assays



Representative Dose Response of ML237 in the NF- κ B B-Cell and T-Cell, TNF- α , and Cytotoxicity Assays

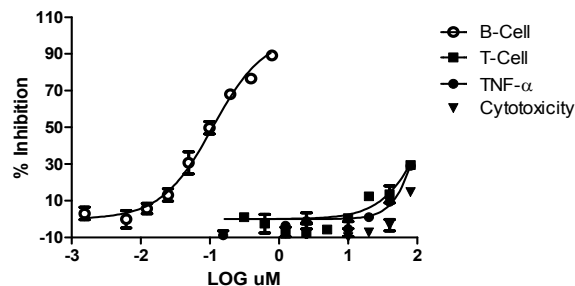


Figure 3: Dose response curves for **ML236** and **ML237**

3.3 Scaffold/Moiety Chemical Liabilities

Neither the oxadiazole and oxazole scaffolds contain no standard chemical liabilities, nor do the lead molecules appear to be frequent hitters. Some drug discovery efforts around similar scaffolds have been reported (6).

3.4 SAR Tables

The general SAR strategy we pursued around the oxadiazole scaffold from the screening hit, CID665654 (entry 7 in **Table 4**) is depicted in **Figure 4**. Efforts were made to expand the SAR around both the aryl group (in green) as well to investigate the SAR of the amide moiety, including examining various esters as well as the parent acid (in blue).

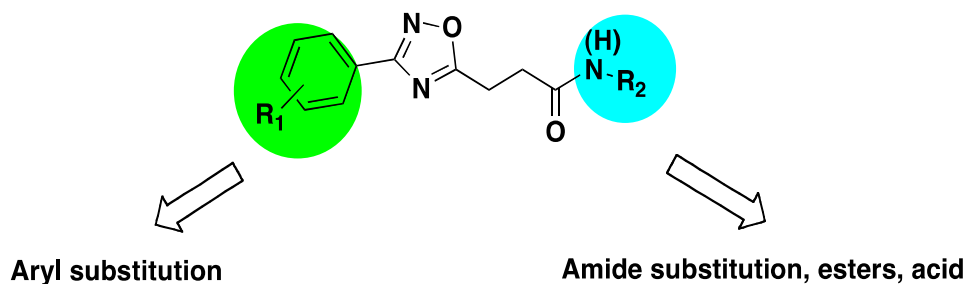


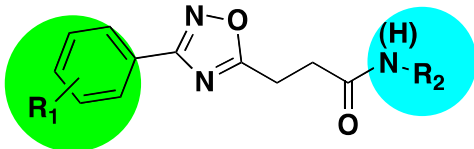
Figure 4: General SAR strategy around oxadiazole scaffold

The results are covered in **Tables 4** and **5** below.

As shown in **Table 4**, oxadiazoles with unsubstituted aryl rings were inactive (entries 1-5). Interestingly, amide substitution was required for selectivity over TNF- α (R₂, in blue). For example, entry 6 (with an -NH₂ group) showed no selectivity, whereas entry 7 (with a methyl amide; a confirmed screening hit) was

both highly potent towards B cells and was also highly selective. After SAR exploration with 61 other analogs (see **Tables 4 and 5**), the initial screening hit, CID665654, was therefore selected as one of our probes, **ML236** (CID665654). Other aliphatic amides were also potent, but as the size of substitution on the amide increased, various measures of selectivity decreased. For example, an ethyl amide (entry 8) was still very selective over TNF- α , but selectivity decreases from propyl (entry 11), to isopropyl (entry 12), cyclopropyl (entry 13), various butyls (entries 19) as well as larger aliphatic groups and all aromatic groups (entries 20-31). Doubly substituted amides, for example dimethyl (entry 9) and diethyl (entry 10) also showed no or poor selectivity.

Activity was also highly sensitive to substitution around the aromatic ring (R_1 , in green). 4-t-butyl substitution appeared optimal (as in the probe, entry 7). For example some loss of activity was observed with 4-i-propyl substitution (entry 37) and 4-n-butyl (entry 42), whereas 4-ethyl (entry 32) substitution led to complete loss of activity as well as various methyl, methoxy, ethoxy, and halo substituted compounds (entries 47-58).

Table 4: SAR Analysis of B-cell Specific Inhibitors of NF- κ B Activation: Oxadiazoles							Potency (μ M)			
							Ave. \pm S.E.M. (stdv/sqrt (n)) (n = 4) if number of replicates is different than the default it is noted in parentheses			
Entry	CID	SID	MLS	S/ P	R_1	R_2	B Cell	T Cell	TNF- α	Cytotox
1	3159503	103061895	0445876	S	H	methyl	>79	>79	>79	>79
		103061904		P			>79	>79	>79	>79
2	3159505	103061892	0445873	S	H	ethyl	>79	>79	>79	>79
3	9210155	110067527	0454567	S	H	c-propyl	>79	>79	>79	>79
4	50898022	110067515	0454500	S	H	s-butyl	>79	>79	>79	>79
5	2025988	110067526	0454565	S	H	t-butyl	>79	>79	>79	>79
6	666941	103061877	0047254	S	4-t-butyl	H	<0.16	0.19 \pm 0.00 (2), <0.16 (2)	0.78 \pm 0.36 (4)	>79
		103061973		P			0.55 (1), <0.16 (3)	0.26 \pm 0.04 (2), <0.16 (2)	0.48 \pm 0.18 (4)	>79
7	665654	103061876	0016382	P	4-t-butyl	methyl	<0.16	>79	66.80 \pm 4.72 (2),	>79
		103061970		S			0.03 \pm 0.01 (4), <0.16 (4)	>79	53.20 \pm 2.57 (2)	>79
8	665747	103061878	0013618	S	4-t-butyl	ethyl	0.03 \pm 0.01 (4), <0.16 (4)	>79	35.80 \pm 4.02 (4)	76.1 (1), >79 (3)
		103061972		P			<0.16	>79	34.00 \pm 5.90 (4)	79.3 (1), >79 (3)
9	665578	103061889	0016303	S	4-t-butyl	dimethyl	41.50 \pm 26.16 (2), >79 (2)	71.30 \pm 3.84 (2), >79 (2)	42.20 \pm 1.73 (2)	>79
		103061969		P			6.56 \pm 3.17 (3), >79 (1)	>79	48.60 \pm 2.20 (2)	>79
10	3760469	103061883	0445871	S	4-t-butyl	diethyl	58.40 (1), >79 (3)	16.40 \pm 4.59 (4)	16.80 \pm 1.65 (3)	54.70 \pm 4.93 (4)
		103061914		P			60.10 (1), >79 (2)	47.80 \pm 4.63 (3)	19.70 \pm 3.95 (4)	51.50 \pm 3.61 (4)
11	17101446	103061908	0445884	P	4-t-butyl	propyl	0.19 \pm 0.02 (2), <0.16 (2)	>79	9.18 \pm 3.96 (4)	>79

12	3150510	103061911	0445887	P	4-t-butyl	i-propyl	0.29 ± 0.05 (3), <0.16 (1)	29.20 ± 13.05 (4)	13.50 ± 3.74 (4)	>79
13	49787124	103061879	0445868	S	4-t-butyl	c-propyl	0.06 ± 0.04 (5), <0.16 (3)	58.70 (1), >79 (3)	12.00 ± 5.43 (3)	>79
14	2982953	103061964	0445943	P	4-t-butyl	allyl	0.19 (1), <0.16 (3)	25.21 ± 24.89 (2)	5.30 ± 2.69 (4)	>79
15	23605402	103061909	0445886	P	4-t-butyl	butyl	14.60 (1), >79 (3)	>79	14.50 ± 4.04 (4)	>79
16	3792005	103061913	0445889	P	4-t-butyl	i-butyl	0.25 (1), <0.16 (3)	2.96 + 2.38 (4)	1.51 ± 0.58 (4)	>79
17	50898048	110067514	0454325	S	4-t-butyl	s-butyl	0.49 ± 0.15 (3), <0.16 (1)	>79	3.00 ± 0.20 (4)	>79
18	1994219	103061910	0445886	P	4-t-butyl	t-butyl	2.50 ± 0.79 (4)	2.77 ± 1.60 (4)	1.18 ± 0.12 (4)	>79
19	50898046	110067516	0454501	P	4-t-butyl	c-butyl	0.41 ± 0.08 (4)	0.94 ± 0.40 (4)	1.18 ± 0.12 (4)	>79
20	2037423	103061963	0445942	P	4-t-butyl	c-pentyl	0.36 ± 0.06 (4)	0.48 ± 0.31 (2), <0.16 (2)	0.78 ± 0.25 (4)	>79
21	1943900	103061912	0445888	P	4-t-butyl	c-hexyl	0.40 ± 0.07 (4)	19.90 ± 13.90 (4)	2.68 ± 1.61 (4)	>79
22	3159504	103061971	0075925	P	4-t-butyl	(tetrahyd rofuranyl) methyl	0.59 ± 0.21 (2), <0.16 (1)	11.00 ± 10.68 (3)	1.54 ± 0.54 (4)	70.90 ± 2.57 (4)
23	1828296	103062016	0252236	P	4-t-butyl	furan-2- ylmetha nyl	0.20 ± 0.01 (3), <0.16 (1)	>79	2.39 ± 1.08 (4)	49.20 ± 5.25 (4)
24	1994282	103061905	0445881	P	4-t-butyl	phenyl	<0.16	0.47 (1), <0.16 (3)	0.24 ± 0.01 (2), <0.16 (1), >79 (1)	>79
25	3159506	103061906	0445882	P	4-t-butyl	2- methoxy phenyl	<0.16	0.66 ± 0.19 (4)	0.48 ± 0.07 (3)	56.00 ± 5.66 (3), >79 (1)
26	1828585	103061915	0445890	P	4-t-butyl	2-ethoxy phenyl	<0.16	1.02 + 0.26 (4)	0.26 ± 0.05 (4)	>79
27	3159507	103061907	0445883	P	4-t-butyl	3- methoxy phenyl	<0.16	2.42 (1), <0.16 (3)	0.24 ± 0.01 (2), <0.16 (1), <0.79 (1)	>79
28	2026055	103061968	0445955	P	4-t-butyl	3-nitro phenyl	<0.16	55.7 (1), >79 (2)	3.73 ± 0.77 (4)	22.10 ± 1.45 (4)
29	1991339	103062017	0354914	P	4-t-butyl	3- pyridinyl	0.16 (1), <0.16 (3)	0.435 (1), <0.16 (2)	0.47 ± 0.04 (3), >80 (1)	20.50 ± 1.12 (4)
30	3159502	103061903	0445880	P	4-t-butyl	Piperidi nyl	30.30 ± 8.05 (4)	12.20 ± 2.52 (3)	17.40 ± 1.02 (4)	39.70 ± 2.96 (4)
31	3159501	103062015	0251479	P	4-t-butyl	Morpho linyl	24.30 (1), >79 (2)	46.70 ± 2.26 (4)	25.00 ± 2.14 (4)	69.20 ± 4.18 (3)
32	18150765	110067532	0454572	S	4-ethyl	methyl	>79	>79	>79	>79
33	50898026	110067524	0454563	S	4-ethyl	ethyl	>79	>79	65.30 ± 0.91 (2), >79 (2)	>79
34	16619151	110067536	0454574	S	4-ethyl	c-propyl	>79	>79	21.10 ± 2.27 (4)	>79
35	50898057	110067534	0454573	S	4-ethyl	s-butyl	>79	>79	14.10 ± 1.19 (4)	>79

36	30780782	110067530	0454571	S	4-ethyl	t-butyl	>79	>79	22.00 ± 2.86 (4)	>79
37	18148563	110067521	0454559	S	4-i-propyl	methyl	0.21 ± 0.04 (4)	>79	56.90 ± 4.17 (4)	>79
38	50898042	110067520	0454558	S	4-i-propyl	ethyl	0.32 ± 0.08 (4)	>79	21.30 ± 1.54 (4)	>79
39	18150898	110067529	0454569	S	4-i-propyl	c-propyl	0.25 ± 0.04 (6), <0.16 (2)	>79	3.11 ± 0.21 (4)	>79
40	46521828	110067522	0454560	S	4-i-propyl	s-butyl	7.96 (1), >79 (2)	>79	3.48 ± 0.18 (4)	>79
41	50898051	110067528	0454568	S	4-i-propyl	t-butyl	26.90 ± 9.00 (4)	15.20 ± 4.02 (4)	1.98 ± 0.09 (4)	>79
42	50898024	110067540	0454578	S	4-n-butyl	methyl	0.14 ± 0.03 (6), <0.16 (2)	>79	1.98 ± 0.09 (4)	>79
43	50898027	110067523	0454562	S	4-n-butyl	ethyl	0.16 ± 0.03 (6), <0.16 (2)	>79	0.78 ± 0.10 (6)	>79
44	50898049	110067544	0454582	S	4-n-butyl	c-propyl	0.21 ± 0.01 (3), <0.16 (1)	1.26 ± 0.55 (3)	0.55 ± 0.05 (4)	>79
45	50898039	110067542	0454580	S	4-n-butyl	s-butyl	0.67 ± 0.11 (4)	0.41 ± 0.09 (4)	0.51 ± 0.05 (4)	>79
46	50898044	110067538	0454576	S	4-n-butyl	t-butyl	2.31 ± 0.30 (4)	1.62 ± 0.57 (4)	0.66 ± 0.03 (4)	>79
47	2023414	103061901	0445878	P	4-methyl	methyl	>79	>79	>79	>79
48	1991416	110067525	0454564	S	3-methyl	ethyl	>79	>79	>79	>79
49	4364818	110067548	0454586	S	2-methyl	ethyl	>79	>79	>79	>79
50	3159497	103061902	0445879	P	4-methoxy	methyl	>79	>79	>79	>79
51	3767515	110067547	0454585	S	3-methoxy	ethyl	>79	>79	>79	>79
52	40012444	103061977	0445959	P	4-ethoxy	methyl	>79	>79	>79	>79
53	30830543	103061887	0445872	S	4-ethoxy	ethyl	>79	>79	>79	>79
54	1988119	103061979	0445961	P	4-fluoro	methyl	>79	>79	>79	>79
55	50898029	110067546	0454584	S	3-fluoro	ethyl	>79	>79	>79	>79
56	2041545	103061916	0445891	P	4-chloro	methyl	>79	>79	>79	>79
57	2016268	110067549	0454587	S	2-chloro	ethyl	>79	>79	>79	>79
58	50898033	110067519	0454557	S	2,6-dichloro	ethyl	>79	>79	>79	>79
* S = Synthesized P = purchased							probe	submitted analog		

Acids and esters in place of amides led to a full loss of selectivity (**Table 5**). For example the acid derivative of ML263 (entry 60) and methyl ester (entry 61) had no selectivity towards TNF- α (R_2 , in blue).

Table 5: SAR Analysis of B-cell Specific Inhibitors of NF- κ B Activation: Oxadiazoles

Entry	CID	SID	MLS	S/P	R_1	R_2	Potency (μ M)			
							B Cell	T Cell	TNF- α	Cyto-tox
							Ave. \pm S.E.M. (stdv/sqrt (n)) (n = 4) if number of replicates is different than the default it is noted in parentheses			
59	665968	103061888 screen hit	0016358	S	H	H	>79	>79	>79	>79
60	23008005	103061875	0445867	S	4-t-butyl	H	0.19 (1), <0.16 (3)	0.20 (1), <0.16 (3)	0.29 (1), <0.16 (1)	>79
61	49787126	103061881	0445870	S	4-t-butyl	methyl	<0.16	<0.16	0.22 (1), <0.16 (1)	>79
62	5307115	103061882	0063814	S	4-ethoxy phenyl	H	34.00 \pm 11.49 (3), >79 (1)	9.25 \pm 2.07	10.60 \pm 4.22 (3)	>79

* S = Synthesized P = purchased

A similar SAR strategy was pursued around the oxazole scaffold is depicted in **Figure 5**. In addition to examining the SAR around the aryl ring (in blue) and amide (in red) as in the oxadiazoles, we also examined the effect of core substitution (in green). The results are summarized in **Tables 6 -8** below. In general similar SAR trends were observed compared to the oxadiazoles, although there were some differences as noted below.

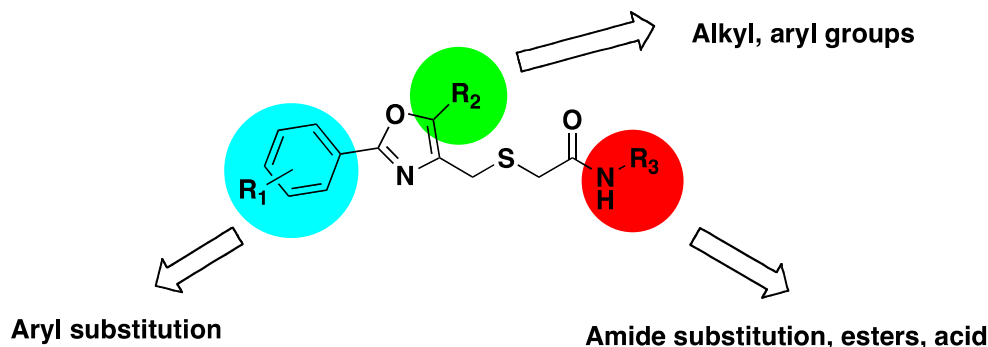


Figure 5: General SAR strategy around oxazole scaffold

As shown in **Table 6**, 4-substitution on the aryl ring (R_1 , in blue) was optimal for activity. An unsubstituted phenyl ring (entry 63) was inactive, but good activity was observed with 4-ethyl (entry 64), 4-thiomethyl (entry 65), and in particular 4-ethoxy (entry 66), which also is the screening hit representative of this oxazole scaffold, was selected as the probe (**ML237**, CID4174238). All other substitutions weakened the potency or selectivity (see **Tables 6, 7** and **8**). For example, the 4-Trifluoromethoxy (entry 70), 4-n-propoxy (entry 73) and 4-n-butoxy (entry 74) were also potent and selective, but smaller groups such as 4-trifluoromethyl (entry 69), 4-methoxy (entry 67) and 4-methyl

(entry 68) were only weakly active. Also, *ortho* and *meta* substituted analogs were inactive, for example 3-ethoxy (entry 71) and 2-ethoxy (entry 72). Lastly, substitution besides methyl at R₂ (in green) led to inactive compounds, for example phenyl substitution (entries 75 and 76).

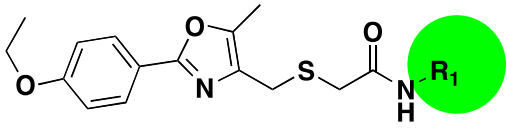
Table 6: SAR Analysis of B-cell Specific Inhibitors of NF-κB Activation: Oxazoles

							Potency (μM) Ave. ± S.E.M. (stdv/sqrt (n)) (n = 4) if number of replicates is different than the default it is noted in parentheses				
Entry	CID	SID	MLS	S/P	R ₁	R ₂	B Cell	T Cell	TNF-α	Cyto-tox	
63	49787127	103061893	0445874	S	phenyl	Me	>79	>79	>79	>79	
64	6622243	103061885 screen hit	0362296	S	4-ethylphenyl	Me	0.48 ± 0.05 (4)	>79	42.20 ± 7.10 (3)	>79	
65	20860198	103061899 screen hit	0361727	P	4-thiomethylphenyl	Me	1.36 ± 0.37 (4)	>79	49.70 ± 4.56 (4)	>79	
66	4174238	103061922 screen hit	0368278	P	4-ethoxyphenyl	Me	0.21 ± 0.03 (3), <0.16 (1)	>79	>79	>79	
		103061890 Probe		S			0.19 ± 0.05 (7), <0.02 (2), <0.16 (1)				
67	44159142	103061880	0445869	S	4-methoxyphenyl	Me	29.2 (1), >79 (3)	>79	>79	>79	
68	6622103	110067517	0454502	S	4-methylphenyl	Me	16.25 ± 3.15 (2), >79 (2)	>79	71.10 ± 2.60 (2), >79 (2)	>79	
69	50898043	110067568	0454606	S	4-trifluoromethylphenyl	Me	15.68 ± 6.26 (3), >79 (1)	>79	24.40 ± 3.60 (4)	>79	
70	50898028	110067569	0454605	S	4-trifluoromethoxyphenyl	Me	2.23 ± 0.70 (4)	>79	27.90 ± 2.27 (4)	>79	
71	50898034	110067543	0454581	S	3-ethoxyphenyl	Me	>79	>79	>79	>79	
72	50898031	110067545	0454583	S	2-ethoxyphenyl	Me	>79	>79	>79	>79	
73	50898056	110067550	0454588	S	4- <i>n</i> -propoxyphenyl	Me	0.09 ± 0.03 (4), <0.16 (4)	>79	31.50 ± 1.38 (4)	>79	
74	50898023	110067551	0454589	S	4- <i>n</i> -butoxyphenyl	Me	0.24 ± 0.01 (4)	>79	11.10 ± 0.68 (4)	>79	
75	50898028	110067569	0454607	S	4-ethoxyphenyl	Ph	>79	54.60 ± 4.52 (2), >79 (2)	>79	>79	
76	50898038	110067560	0454598	S	4-ethylphenyl	Ph	>79	72.80 ± 1.97 (2), >79 (2)	>79	>79	

* S = Synthesized P = purchased

probe submitted analog

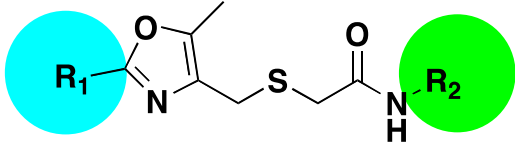
As shown in **Table 7**, amide substitution greatly impacted activity and selectivity, R₁ in green. In general smaller groups such as cyclopropyl (as in entry 66, **ML237**) were optimal. Methyl (entry 98), isopropyl (entry 77), propyl (entry 78), methoxymethyl (entry 81), cyclopropylmethyl (entry 104) and propargyl (entry 106) all gave compounds with good potency and for the most part selectivity. In contrast to the oxadiazole series, an unsubstituted amide (entry 100) was potent and selective. However, both the carboxylic acid (entry 101) and dimethyl amide (entry 99) either lacked selectivity or potency respectively, which was similar to the oxadiazole series. Aromatic substitution or larger groups on the amide also gave compounds that were either not potent or selective (for example entries 83-89, 91-97, 102 and 105) with the exception of furfuryl (entry 90).

Table 7: SAR Analysis of B-cell Specific Inhibitors of NF-κB Activation: Oxazoles									
						<p>Potency (μM) Ave. ± S.E.M. (stdv/sqrt (n)) (n = 4) if number of replicates is different than the default it is noted in parentheses</p>			
Entry	CID	SID	MLS	S/P	R ₁	B Cell	T Cell	TNF-α	Cytotox
77	4174353	103061931	0445904	P	isopropyl	1.24 ± 0.16 (3)	>79	61.70 ± 3.48 (4)	>79
78	4638993	103061919	0445894	P	propyl	1.51 ± 0.28 (4)	>79	74.70 (1), >79 (3)	>79
79	4174343	103061924	0445898	P	<i>n</i> -butyl	8.64 ± 4.42 (3)	>79	38.80 ± 2.06 (4)	75.30 ± 1.05 (2), >79 (2)
80	4638992	103061918	0445893	P	isobutyl	46.90 ± 10.57 (3)	>79	35.70 ± 1.51 (3), >79 (1)	74.60 ± 3.39 (2), >79 (2)
81	4187316	103061937	0445909	P	Methoxy- methyl	0.31 ± 0.11 (9), <0.16 (1)	>79	>79	>79
82	20968920	103061935	0445907	P	3- ethoxypropyl	10.30 ± 2.84 (4)	51.9 (1), >79 (2)	>79	>79
83	4174237	103061921	0445896	P	cyclopentyl	25.70 ± 10.30 (4)	37.60 ± 4.00 (2), >79 (1)	26.40 ± 0.56 (4)	64.20 ± 4.18 (2), >79 (2)
84	4175771	103061936	0445908	P	pyrrolidin-1-yl	>79	>79	46.60 ± 1.68 (4)	>79
85	20968913	103061920	0445895	P	2-(pyrrolidin- 1-yl)ethyl	12.10 ± 2.95 (4)	4.44	28.40 ± 3.05 (4)	>79
86	4187317	103061938	0445910	P	4-methylpi- peridin-1-yl	70.40 ± 3.01 (2), >79 (1)	32.6 (1), >79 (3)	9.88 ± 0.75 (4)	30.00 ± 2.76 (4)
87	4175769	103061934	0445906	P	2,3-dimethyl- cyclohexyl	>79	39.40 ± 14.38 (3)	8.81 ± 1.52 (4)	38.90 ± 1.72 (4)
88	4174347	103061928	0445901	P	2-cyclo- hexenylethyl	36.50 ± 10.91 (3)	9.14 ± 1.92 (4)	17.70 ± 1.27 (4)	42.40 ± 6.95 (4)
89	4175693	103061932	0365210	P	Tetrahydro- furan-2-yl) methyl	7.84 ± 1.64 (4)	>79	67.70 (1), >79 (3)	>79
90	3237327	103061891 screen hit	0052895	P	furfuryl	0.21 ± 0.02 (3)	25.40 ± 1.56 (2), >79 (2)	21.40 ± 4.06 (3)	54.40 ± 4.72 (3), >79 (1)

		103061925		S		0.29 ± 0.02 (4)	>79	25.20 ± 3.67 (4)	53.10 ± 7.30 (4)
91	4174349	103061929	0445902	P	thiophen-2-ylmethyl	0.65 ± 0.18 (3)	>79	24.30 ± 0.87 (4)	34.80 ± 3.56 (4)
92	4174342	103061923	0445897	P	pyridin-3-ylmethyl	2.38 ± 0.38 (4)	72.60 (1), >79 (3)	24.20 ± 3.12 (4)	65.00 ± 4.06 (4)
93	4638990	103061917	0445892	P	benzyl	6.75 ± 2.49 (4)	>79	21.60 ± 0.42 (4)	49.30 ± 6.30 (4)
94	4174346	103061927	0445900	P	4-methylbenzyl	2.77 ± 0.65 (4)	45.90 ± 4.99 (4)	>79	>79
95	4174345	103061926	0445899	P	phenethyl	3.56 ± 1.67 (2)	55.00 ± 13.28 (3), >79 (1)	22.90 ± 1.05 (4)	35.60 ± 3.96 (4)
96	4174351	103061930	0445903	P	4-methylphenethyl	9.49 ± 7.26 (3), <0.16 (1)	54.80 ± 10.74 (3)	40.2 (1), >79 (3)	>79
97	4175766	103061933	0445905	P	4-chlorophenethyl	>79	63.80 (1), >79 (2)	>79	>79
98	50898036	110067558	0454596	S	Me	0.33 ± 0.04 (4)	>79	>79	>79
99	50898058	110067559	0454597	S	dimethyl	>79	>79	78.50 (1), >79 (3)	>79
100	50898050	110067561	0454599	S	H	0.08 ± 0.01 (4), <0.16 (4)	>79	>79	>79
101	20967165	110067562	0454600	S	CO ₂ H (in place of amide)	0.05 ± 0.00 (4), <0.16 (4)	0.03 ± 0.00 (3), <0.16 (3)	0.04 ± 0.01 (4), <0.16 (4)	>79
102	50898025	110067563	0454601	S	t-butyl	60.50 (1), >79 (3)	10.80 ± 2.76 (4)	37.70 ± 1.42 (4)	>79
103	50898021	110067564	0454602	S	cyclobutyl	3.23 ± 0.38 (4)	26.40 ± 2.12 (3)	38.70 ± 3.11 (4)	>79
104	50898055	110067541	0454579	S	Cyclopropylmethyl	1.32 ± 0.28 (4)	20.90 ± 2.25 (4)	55.40 ± 3.63 (4)	>79
105	4175770	110067537	0454575	S	morpholine	>79	>79	64.60 ± 5.80 (4)	>79
106	50898054	110067539	0454577	S	propargyl	0.06 ± 0.01 (4), <0.16 (4)	>79	>79	>79
* S = Synthesized P = purchased						probe	submitted analog		

Lastly, as shown in **Table 8**, we examined a number of combinations of furfuryl at R₂ (in green) with additional aromatic groups (R₁, in blue). However, a similar SAR trend was seen, with only 4-ethoxy (entry 90, above), 4-propoxy (entry 114) and butoxy (entry 115) giving activity, with other groups being only weakly active (entries 107-111). Also as above, the parent carboxylic acids all lacked selectivity (entries 112-113, 116-118).

Table 8: SAR Analysis of B-cell Specific Inhibitors of NF-κB Activation: Oxazoles

							Potency (μM) Ave. ± S.E.M. (stdv/sqrt (n)) (n = 4) <i>if number of replicates is different than the default it is noted in parentheses</i>			
Entry	CID	SID	MLS	S/P	R ₁	R ₂	B Cell	T Cell	TNF-α	Cytotox
107	49787125	103061894	0445875	S	phenyl	furfuryl	>79	>79	>79	>79
108	3240944	103061884 screen hit	0049119	S	4-methoxyphenyl	furfuryl	29.5 (1), >79 (3)	>79	35.80 ± 4.60 (3)	67.10 ± 8.70 (2), >79 (2)
109	16193680	103061886 screen hit	0237348	P	4-ethylphenyl	furfuryl	49.40 ± 14.49	>79	>79	>79
110	16193646	103061898 screen hit	0237244	P	4-methoxyphenyl	n-propyl	>79	>79	42.50 ± 4.04 (4)	>79
111	20860316	110067518	0454503	S	4-methylphenyl	furfuryl	>79	>79	33.60 ± 1.81 (4)	>79
112	50898053	110067565	0454603	S	4-trifluoromethylphenyl	CO ₂ H (in place of amide)	0.97 ± 0.13 (4)	0.52 ± 0.08 (4)	0.38 ± 0.03 (4)	>79
113	50898047	110067566	0454604	S	4-trifluoromethoxyphenyl	CO ₂ H (in place of amide)	0.21 ± 0.02 (7), <0.16 (1)	0.27 ± 0.11 (6), <0.16 (2)	0.11 ± 0.00 (4)	>79
114	50898040	110067552	0454590	S	4-n-propoxyphenyl	furfuryl	0.11 ± 0.02 (5), <0.16 (3)	>79	9.74 ± 0.46 (4)	59.80 ± 0.44 (2), >79 (2)
115	50898045	110067553	0454591	S	4-n-butoxyphenyl	furfuryl	0.26 ± 0.04 (3), <0.16 (4)	>79	13.10 ± 0.73 (4)	>79
116	50898020	110067554	0454592	S	2-ethoxyphenyl	CO ₂ H (in place of amide)	>79	>79	>79	>79
117	50898037	110067555	0454593	S	4-n-propoxyphenyl	CO ₂ H (in place of amide)	0.04 ± 0.01 (4), <0.16 (4)	0.03 ± 0.00 (3), <0.16 (3),	0.03 ± 0.01 (4)	>79
118	50898041	110067556	0454594	S	4-n-butoxyphenyl	CO ₂ H (in place of amide)	0.12 ± 0.02 (7), <0.16 (1)	0.07 ± 0.01 (3), <0.16 (3),	0.04 ± 0.01 (4)	>79

* S = Synthesized P = purchased

3.5 Cellular Activity

All of the primary and selectivity assays are cell based. Specific measures of permeability and toxicity are discussed in section 3.6 below.

3.6 Profiling Assays

The nominated probes were evaluated in a detailed *in vitro* pharmacology screen as shown in **Table 9**.

Table 9: Summary of *in vitro* ADME Properties of B-cell Specific Inhibitors of NF- κ B Activation Probes **ML236** and **ML237**

Probe Probe ML# SBCCG MLS-#	Aqueous Solubility $\mu\text{g/mL}$ <i>$[\mu\text{M}]^a$</i> @pH5.0/6.2/7.4	Aqueous Solubility in 1xPBS ($\mu\text{g/mL}$) <i>$[\mu\text{M}]^a$</i> pH 7.4	PAMPA Pe ($\times 10^{-6}$ cm/s) Donor pH: 5.0/6.2/7.4 Acceptor pH: 7.4	Plasma Protein Binding (% Bound)		Plasma Stability (%Remaining @3hrs) Human/Mouse Plasma: 1x PBS, pH 7.4, 1:1 1x PBS, pH 7.4	Hepatic Microsome Stability Human/ Mouse	Hepatic Toxicity LC ₅₀ (μM)
				Human 1 μM /10 μM	Mouse 1 μM /10 μM			
CID665654 ML236 MLS-0016382	26.4/29.0/29.6 <i>[96.3/106/108]</i>	23.4 <i>[85.4]</i>	751/755/746	94.54/ 90.40	94.13/ 89.95	100/100 87.22/81.41	100/4.97	>50
CID4174238 ML237 MLS-0368278	38.7/45.9/55.3 <i>[114/183/220]</i>	39.5 <i>[157]</i>	513/541/559	96.18/ 74.24	91.17/ 76.00	98.32/94.06 82.02/85.21	22.72/0	>50

^a Solubility also expressed in molar units (μM) as indicated in *italicized [bracketed values]*, in addition to more traditional $\mu\text{g/mL}$ units.

ML236 and **ML237** are moderately soluble in aqueous media at pH 5.0/6.2/7.4.

The PAMPA (Parallel Artificial Membrane Permeability Assay) assay is used as an *in vitro* model of passive, transcellular permeability. An artificial membrane immobilized on a filter is placed between a donor and acceptor compartment. At the start of the test, drug is introduced in the donor compartment. Following the permeation period, the concentration of drug in the donor and acceptor compartments is measured using UV spectroscopy. Consistent with the predicted LogP (see table 3), **ML236** and **ML237** are both moderately permeable in this assay.

Plasma Protein Binding is a measure of a drug's efficiency to bind to the proteins within blood plasma. The less bound a drug is, the more efficiently it can traverse cell membranes or diffuse. Highly plasma protein bound drugs are confined to the vascular space, thereby having a relatively low volume of distribution. In contrast, drugs that remain largely unbound in plasma are generally available for distribution to other organs and tissues. **ML236** and **ML237** show moderate binding to plasma proteins in mouse and human plasma.

Plasma Stability is a measure of the stability of small molecules and peptides in plasma and is an important parameter, which strongly can influence the *in vivo* efficacy of a test compound. Drug candidates are exposed in plasma to enzymatic processes (proteinases, esterases), and they can undergo intramolecular rearrangement or bind irreversibly (covalently) to proteins. Both **ML236** and **ML237** show excellent stability in human and mouse plasma.

The microsomal stability assay is commonly used to rank compounds according to their metabolic stability. This assay addresses the pharmacologic question of how long the parent compound will remain circulating in plasma within the body. **ML236** shows excellent stability in human liver microsomes (100% remaining), but poor stability (<1.0% remaining) in mouse liver homogenates, potentially limiting the utility of this probe in *in vivo* rodent models. **ML237** shows poor stability in both human (23% remaining) and mouse (0% remaining) liver homogenates.

ML236 and **ML237** show no toxicity (LC₅₀ > 50 μM) toward human hepatocytes.

Additional Profiling. The probes, **ML236** and **ML237**, were profiled in the NCI-60 panel as well as a DiscoverX screen of >200 GPCRs, nuclear receptors and kinases. Neither **ML236** nor **ML 237** showed activity in either the NCI-60 panel or at any of the receptors tested with the exception of the EDG2 receptor (81% and 90% inhibition at 10 μ M for **ML236** and **ML237** respectively). They were submitted to the Psychoactive Drug Screening Program (PDSP) at the University of North Carolina (PDSP, Bryan Roth, PI). These probes are fairly "clean". Of the 42 GPCR receptors tested, ML236 has modest activity against D4 (4.1 μ M Ki), and Sigma 1 (8.7 μ M Ki), weak activity against 5HT-2B and GABAA (>10 μ M Ki), while ML237 has weak activity against only M3 and Sigma1 (>10 μ M Ki).

4 Discussion

4.1 Comparison to existing art and how the new probe is an improvement

Previously, a potent (~67 nM) and selective probe (**ML029**, Pubchem CID2858522) was identified as an inhibitor of the PKC-initiated NF- κ B pathway after a battery of 12 secondary and counter-screen assays. However, this probe appeared potent only in the original HEK293 reporter cell line and some types of primary lymphocytes, with little potency in several other tumor cell lines. These previous findings are described in the full Center Probe Report entitled "Chemical inhibitors of antigen receptor-induced NF- κ B" (https://mli.nih.gov/mli/?dl_id=454). There are no patents describing inhibitors of NF- κ B that are B-cell or T-cell specific.

Because of their activity in B-cells but not T-cells, the newly identified probes are superior to the previously reported pathway-selective NF- κ B inhibitors that suppress signaling specifically downstream of PKC without interfering with innate immunity signaling. Cell lineage-selective compounds could serve as powerful research tools for identifying signal transduction components that differ in B-cells versus T-cells. Moreover, compounds that selectively inhibit antigen receptor and PKC-driven NF- κ B activation in B-cells but not T-cells have promise for development of therapeutics that could be targeted to cessation of aberrant antibody responses without interfering with cell-mediated immunity (CMI). For example, in diseases such as rheumatoid arthritis and lupus, autoantibody production drives the pathology of these disorders. Selective suppression of humeral limb (B cell mediated) of the adaptive immune response could potentially reduce autoantibody-mediated pathology, without interfering with T-cell-mediated immunity needed for protection from viruses and cancer. Also, in B-cell malignancies (which are far more common than T-cell malignancies), selective interference with the PKC-initiated pathway for NF- κ B could be used to inhibit the proliferation and survival of malignant B-cells without interfering with T-cell-mediated immune defenses against viruses. For example, activated B-cell (ABC) type non-Hodgkin's lymphomas comprise approximately one-quarter of all lymphomas and have hyperactivity of the PKC-initiated pathway for NF- κ B activation. Components of this NF- κ B pathway, such as MALT1, have been validated by gene knockout and peptide inhibitors to be essential for the growth and survival of these malignant B-cells. Thus, B-cell-selective inhibitors of the PKC-initiated NF- κ B pathway could provide a path forward to development of novel anticancer drugs for some common types of malignant lymphoma, where the therapy would be non-immunosuppressive thus affording a superior therapeutic index compared to currently available therapies.

4.2 Mechanism of Action Studies

The mechanism of action (MOA) studies were undertaken and limited in scope to activities modulating secretion of the cytokines IL-2 and IL-8 in Jurkat T-cells. Studies characterizing effects on the modulation of IL-6 and IL-12 in the 697 pre-B-cell lines are on going and will be the subject of a future publication from Dr. Reed's laboratory.

ML236 did not show any inhibitory activity on the secretion of IL-2 and IL-8 in Jurkat T-cells at concentrations shown to inhibit NF- κ B activity in B-cell ($IC_{50}^{B-cells} = 0.16 \mu\text{M}$) but not T-cells ($IC_{50}^{T-cells} = 79.0 \mu\text{M}$). Likewise, **ML237** did not show any inhibitory activity on the secretion of IL-2 and IL-8 in Jurkat T-

cells at concentrations shown to inhibit NF- κ B activity in B-cell ($IC_{50}^{B-cells} = 0.19 \mu\text{M}$) but not T-cells ($IC_{50}^{T-cells} = 79.0 \mu\text{M}$).

4.3 Planned Future Studies

Currently, our immediate future plans are to establish ELISA assays to distinguish the ability of **ML236** and **ML237** to inhibit IL-6 and IL-12 secretion in B-cells. In parallel the activity of the compounds will be tested against primary cultured human lymphocytes and ABC-type lymphoma cell lines. Downstream steps in the signaling transduction cascade initiated by antigen receptors (and PKC) will be interrogated as described for the prior probe compound **ML025** (Shi, R, *et al. ACS Chemical Biology* 2010), including proteolytic processing of Bcl-10 and A20 (substrates of MALT1 protease), formation of the CARMA/Bcl-10/MALT (CBM) complex (assessed by co-immunoprecipitation experiments), phosphorylation of CARMA (determined by phosphospecific antibody immunoblotting), and ubiquitination of TRAF6 and NEMO/IKK-gamma. The goal is to elucidate the step in the pathway where the compound blocks the signaling cascade, thus providing insights into the possible cellular mechanisms of action.

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6 Supplementary Information

6.1 Assay Details:

NF- κ B B-Cell Primary Assay and Dose Response Protocol

Assay materials:

- 1) 697 NF- κ B-Luc cell line obtained from Dr. Reed's lab
- 2) RPMI phenol red (-), Mediatech Car # 17105CV
- 3) Fetal Bovine Serum, Hyclone cat # SH30396.03HI
- 4) L-glutamine, Omega Scientific cat # GS-60
- 5) Na-pyruvate, Sigma cat # S8636-100M
- 6) Penn/Strep, Omega Scientific cat # PS-20
- 7) PMA, Calbiochem cat # 524400
- 8) Ionomycin, Calbiochem cat # 407950
- 9) SteadyGlo, Promega cat # E2550

Day 1 Procedure

- 1) Harvest 697 NF- κ B-Luc at 2,000,000 cells/mL density. Spin, re-suspend in assay media, count.
- 2) Seed 15000 cells/well in 3 μ L/well 697 NF- κ B-Luc to full plate- Aurora 1536 low base white plate # 00029846 plate or non-TC-treated equivalent.
- 3) Plate with Kalypsys dispenser
- 4) Spin down plates @ 500 RPM for 1 min.
- 5) Dispense 10 nL 100% DMSO compounds (col 5-48) & 10nL 100% DMSO controls (col 1-4) to plates with Echo 555 (Labcyte) acoustic dispenser (For dose response assays, the appropriate volume and concentration of 10 mM test compounds is transferred to assay plate to achieve desired final assay concentration and range. Wells are backfilled with DMSO to equalize DMSO concentrations between wells)
- 6) Incubate plate for 1 hour in 37°C 5% CO₂.
- 7) Add 2 μ L PMA/Ionomycin working dilution (6.5 ng/mL PMA/3.25 ng/mL Ionomycin) in assay media to col 3-48 and no agonist media to col 1 and 2 with Kalypsys dispenser.
- 8) Spin down plates @ 1000 RPM for 1 min.
- 9) Lid plates with Kalypsys plate lids.
- 10) Incubate plate overnight (16 hours) in 37°C 5% CO₂ incubator.

Day 2 Procedure

- 1) Remove lid and incubate plate for 10 min in at room temp.
- 2) Add 3 μ L/well SteadyGlo with Kalypsys dispenser
- 3) Spin plates @ 2000 RPM for 2 min, lid plate and incubate for 10 min at room temp.
- 4) Read luminescence on Envision Ultra sensitive Luminescent protocol for white Aurora 1536 low base white plate # 00029846

NF- κ B T-Cell Primary Assay and Dose Response Protocol

Assay materials:

- 1) Jurkat NF- κ B cell line obtained from Dr. Reed's lab
- 2) RPMI phenol red (-), Mediatech Car # 17105CV
- 3) Fetal Bovine Serum, Hyclone cat # SH30396.03HI

- 4) L-glutamine, Omega Scientific cat # GS-60
- 5) Na-pyruvate, Sigma cat # S8636-100M
- 6) Penn/Strep, Omega Scientific cat # PS-20
- 7) PMA, Calbiochem cat # 524400
- 8) Ionomycin, Calbiochem cat # 407950
- 9) SteadyGlo, Promega cat # E2550

Day 1 Procedure

- 1) Harvest Jurkat NF- κ B-Luc at 1000,000 cells/mL density. Spin, resuspend in assay media, count.
- 2) Seed 8000 cells/well in 3 μ L/well to full plate Jurkat NF- κ B -Luc to white Aurora 1536 low base white plate # 00029846 plate or non-TC-treated equivalent.
- 3) Plate with Kalypsys dispenser
- 4) Spin down plates @ 500 RPM for 1 min.
- 5) Dispense 10nl 100% DMSO compounds (col 5-48) & 10 nL 100% DMSO controls (col 1-4) to plates with Echo 555 (Labcyte) acoustic dispenser (For dose response assays, the appropriate volume and concentration of 10 mM test compounds is transferred to assay plate to achieve desired final assay concentration and range. Wells are backfilled with DMSO to equalize DMSO concentrations between wells)
- 6) Incubate plate for 1 hour in 37°C 5% CO₂.
- 7) Add 2 μ L PMA/Ionomycin working dilution (7.5 ng/mL PMA/3.75 ng/mL Ionomycin) in assay media to col 3-48 and no agonist media to col 1 and 2 with Kalypsys dispenser.
- 8) Spin down plates @ 1000 RPM for 1 min.
- 9) Lid plates with Kalypsys plate lids.
- 10) Incubate plate overnight (16 hours) in 37°C 5% CO₂ incubator.

Day 2 Procedure

- 1) Remove lid and incubate plate for 10 min in at room temp.
- 2) Add 3 μ L/well SteadyGlo with Kalypsys dispenser
- 3) Spin plates @ 2000 RPM for 2 min, lid plate and incubate for 10 min at room temp.
- 4) Read luminescence on Envision Ultra sensitive Luminescent protocol for white Aurora 1536 low base white plate # 00029846

TNF- α NF- κ B induction Assay: Dose Response

Assay materials:

- 1) HEK-293-T NF- κ B-Luc cell line obtained from Dr. Reed's lab's laboratory
- 2) Assay Media: DMEM with no phenol red (Gibco 31053 or Hyclone SH30585.02), 2mM L-glutamine (omega sci GS-60 200mM), 1mM Na-pyruvate (Sigma S8636-100ML 100mM), 50IU/ml Penn/Strep(omega scientific PS-20 5000 IU/mL)
- 3) Recombinant Human TNF- α , CF , (R&D Systems 210-TA-010/CF)
- 4) SteadyGlo (Promega E2550)
- 5) Assay plate: 1536-well, TC-treated, black plate (Corning 3893)

Day 1 Procedure

- 1) Harvest HEK-293-T NF- κ B-Luc at 100% confluency
- 2) Make cells suspension at 1X for final seeding density of 6000 cells/well in 5 μ L well volume.
- 3) Add TNF- α to 1X cell suspension at 0.1 ng/mL.

- 4) Dispense 6000 cells/well in 5 μ L of assay media into col. 5-48 of assay plate
- 5) Dispense 5 μ L/well of assay media into col. 1-4 of assay plate
- 6) Spin down plates at 1000 rpm for 1 min in an Eppendorf 5810 centrifuge
- 7) With LabCyte Echo, dispense varying volumes of test compounds in DMSO into assay plate wells (Col. 5-44) to achieve appropriate dose response concentrations and range. Backfill test compound wells with DMSO to equilibrate DMSO concentrations. Transfer equal volumes of DMSO to control wells (Col. 1-4, 45-48). Maximum concentration of DMSO should be 1%.
- 8) Lid Plates. Sandwich 4 plates between 2 lidded 384 plates filled with H₂O
- 9) Wrap plates securely in single layer of Plastic Wrap (Saran Wrap PVDC version).
- 10) Incubate overnight (14 hours) in 37°C 5% CO₂ incubator

Day 2 Procedure

- 1) Add 3 μ L/well SteadyGlo to each well with Multidrop.
- 2) Spin plates at 1000 rpm for 1 minute on Eppendorf 5810 centrifuge
- 3) Read luminescence on Perkin-Elmer EnVision

Cytotoxicity Assay: Dose Response

Assay materials:

- 1) HEK-293-T NF- κ B-Luc cell line obtained from Dr. Reed's lab's laboratory
- 2) Assay Media: DMEM with no phenol red (Gibco 31053 or Hyclone SH30585.02), 2mM L-glutamine (omega sci GS-60 200mM), 1mM Na-pyruvate (Sigma S8636-100ML 100mM), 50IU/ml Penn/Strep(omega scientific PS-20 5000 IU/mL)
- 3) Resazurin (Sigma-Aldrich R7017)
- 4) Assay plate: 1536-well, TC-treated, black plate (Corning 3893)

Day 1 Procedure

- 1) Harvest HEK-293-T NF- κ B-Luc at 100% confluency
- 2) Make cells suspension at 1X for final seeding density of 6000 cells/well in 5 μ L well volume.
- 3) Dispense 6000 cells/well in 5 μ L of assay media into col. 5-48 of assay plate
- 4) Dispense 5 μ L/well of assay media into col. 1-4 of assay plate as positive control.
- 5) Spin down plates at 1000 rpm for 1 min in an Eppendorf 5810 centrifuge
- 6) With LabCyte Echo, dispense varying volumes of test compounds in DMSO into assay plate wells (Col. 5-44) to achieve appropriate dose response concentrations and range. Backfill test compound wells with DMSO to equilibrate DMSO concentrations. Transfer equal volumes of DMSO to control wells (Col. 1-4, 44-48). Maximum concentration of DMSO should be 1%.
- 7) Lid Plates. Sandwich 4 plates between 2 lidded 384 plates filled with H₂O
- 8) Wrap plates securely in single layer of Plastic Wrap (Saran Wrap PVDC version).
- 9) Incubate overnight (14 hours) in 37°C 5% CO₂ incubator

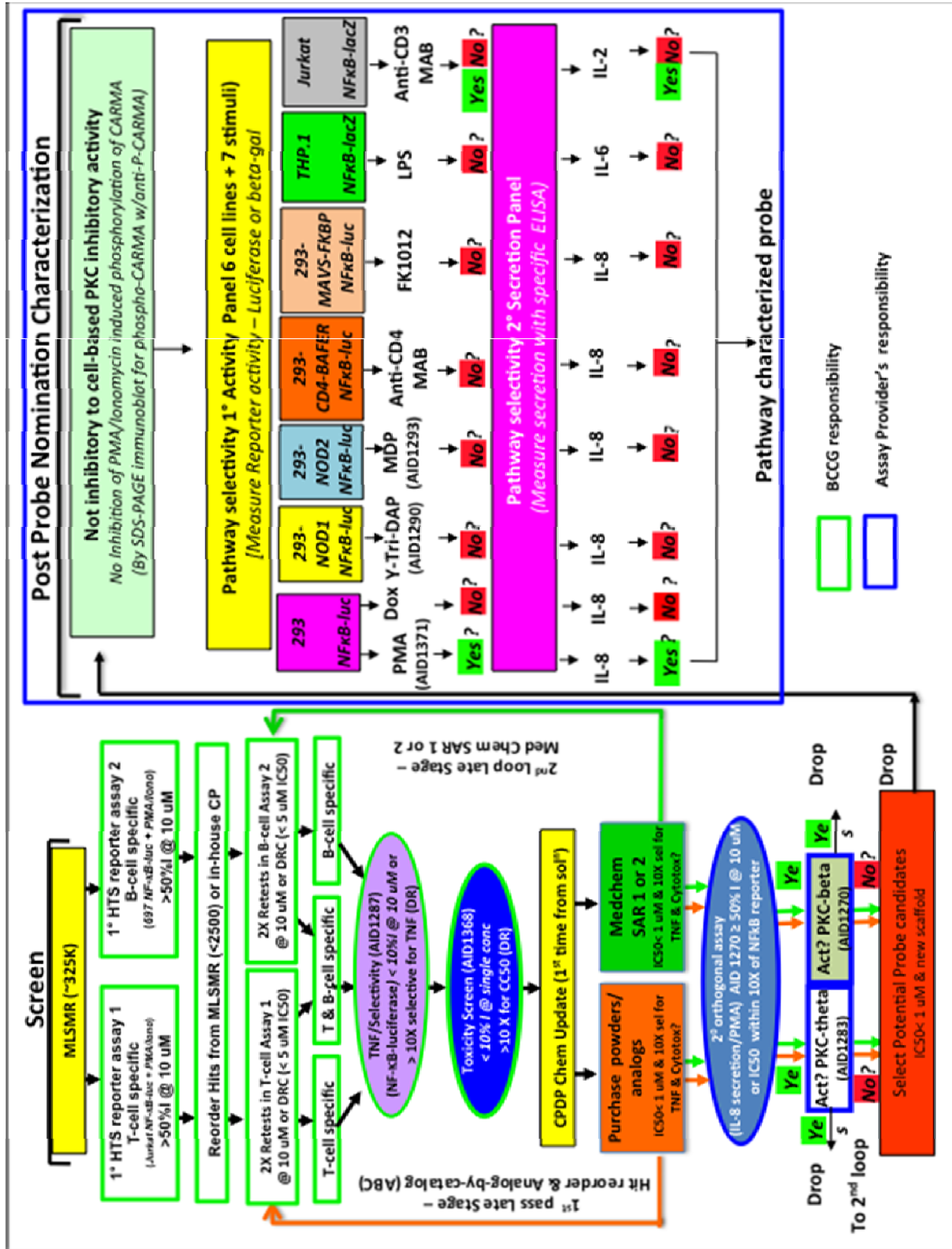
Day 2 Procedure

- 1) Dispense 20nL of 2mM Resazurin in DMSO (FAC is 8 μ M) with LabCyte Echo to all wells of assay plate
- 2) Lid Plates. Sandwich 4 plates between 2 lidded 384 plates filled with H₂O
- 3) Wrap plates securely in single layer of Plastic Wrap (Saran Wrap PVDC version).
- 4) Incubate for 1 hour in 37°C 5% CO₂ incubator
- 5) Top read Fluorescence Intensity (Ex. 531, Em. 595nm) on Perkin-Elmer EnVision

6.2 Critical Path Flowchart for NF-κB B-cell Inhibitors

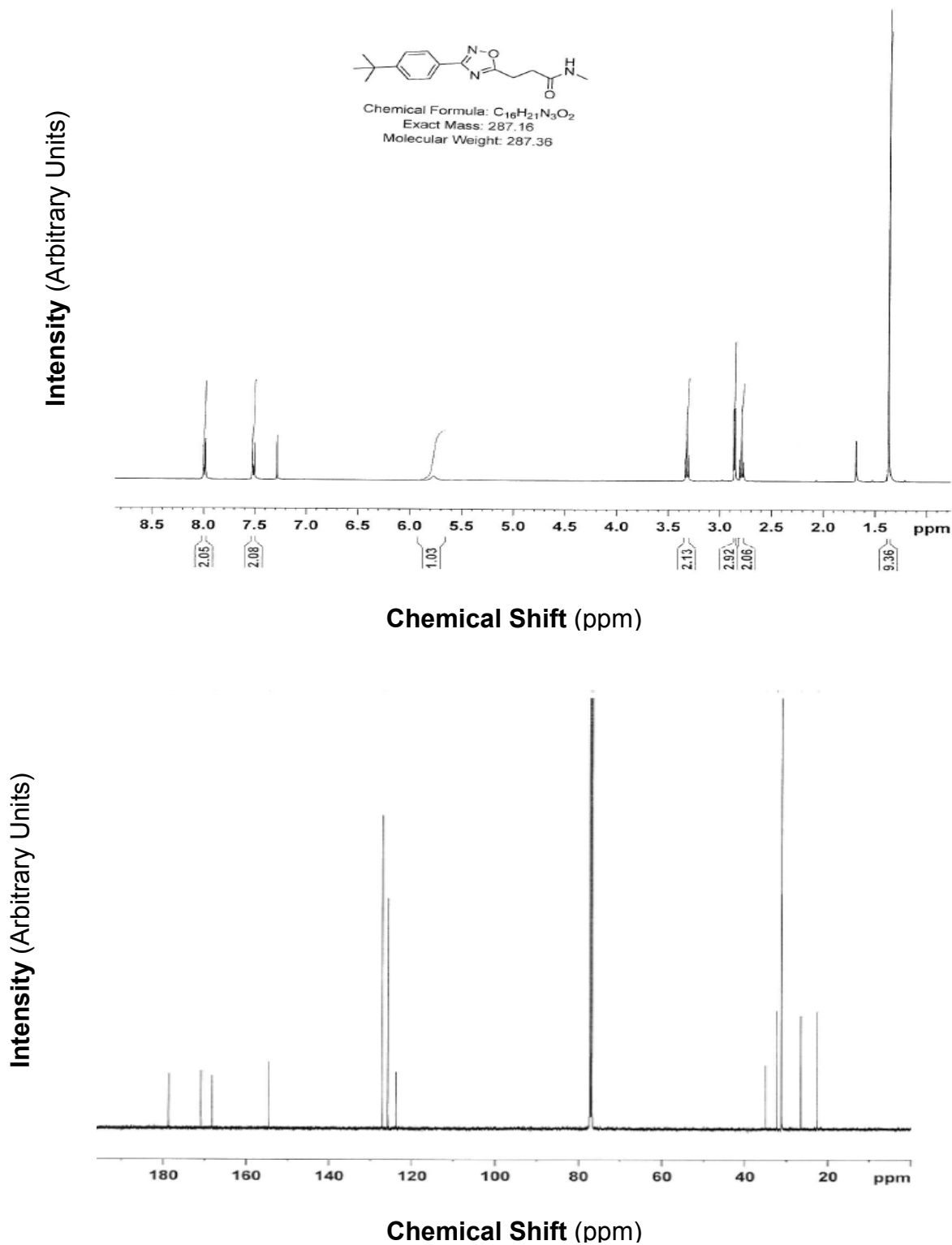
The critical path for the selection of the probe and intended further studies for the probe are shown below. The actual assay was conducted as comparative screen for each cell type and the differential activities were used to define whether a compound was B-, T- or B & T-cell dual selective.

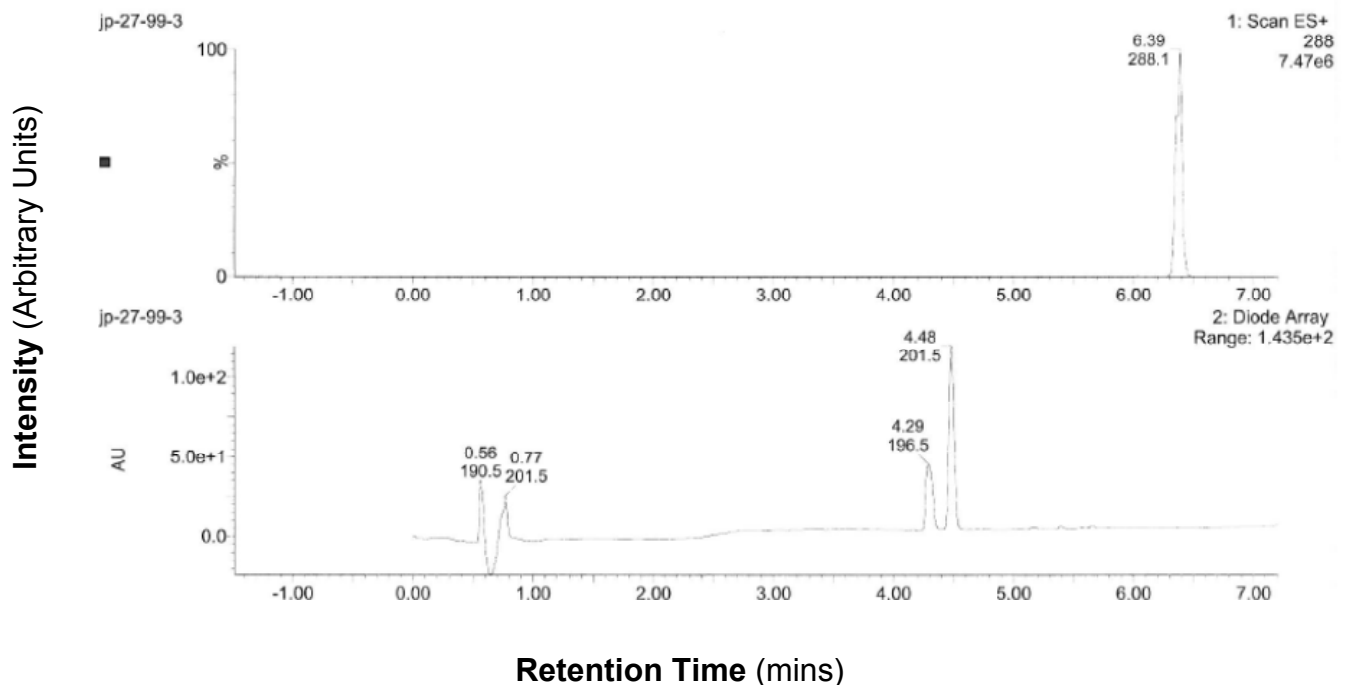
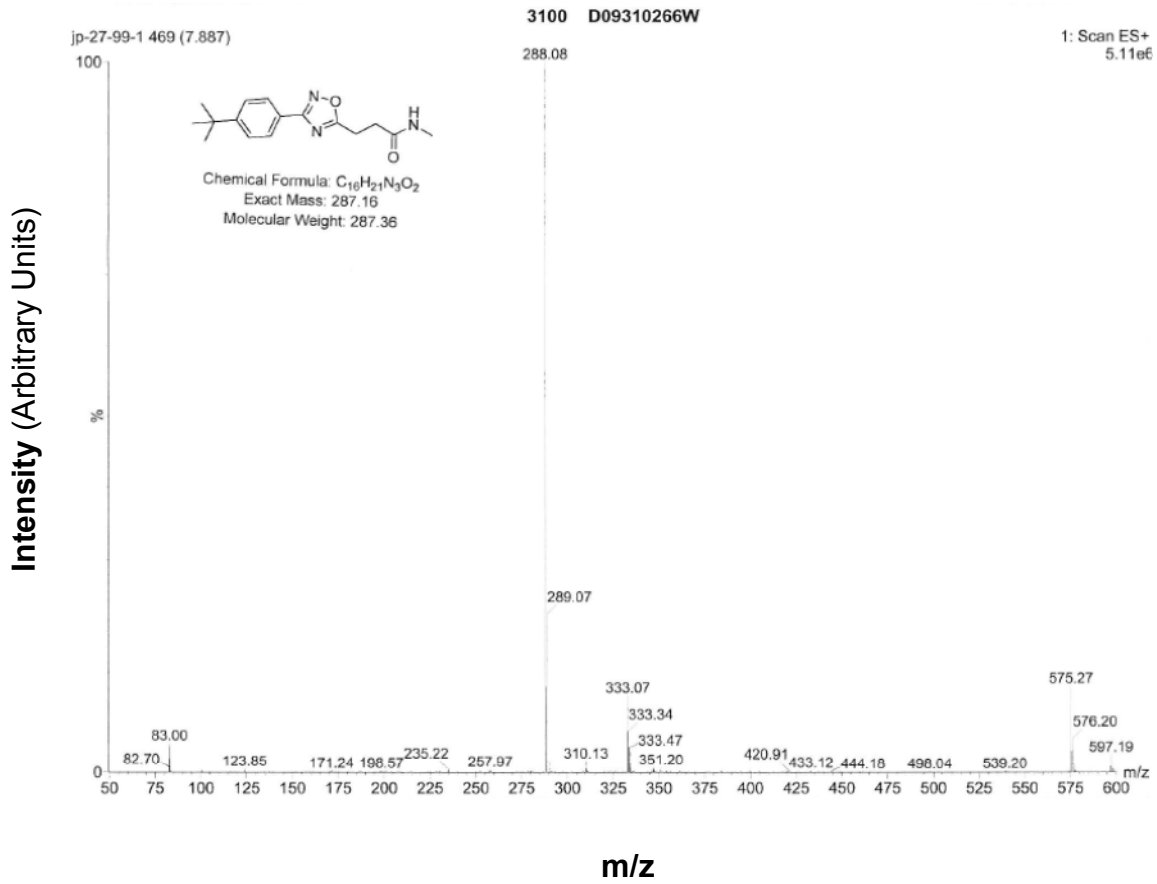
Critical Path Flowchart for NF-κB B-cell Inhibitors



6.3 Supplementary Information for Probe Characterization.

^1H & ^{13}C NMR Spectra and LC/MS Data for **ML236**





^1H & ^{13}C NMR Spectra and LC/MS Data for **ML237**

