Center Probe Report

Project Title: *N*-(quinolin-8-yl)benzenesulfonamides as Agents Capable of Down-Regulating NFκB Activity within Two Separate High-Throughput Screens of NFκB Activation
Grant Number: 1R01MH076344-01

Screening Center Name:
MLSCN at Columbia University.

Principal Investigator of Screening Center:
James Rothman

Assay Submitter & Institution:
Thomas Mayer (Debby Smith), Columbia University

Assay or Pathway Target: NFκB

Probe PubChem Compound Identifier (CID):

Please provide the following descriptions:

Assay provider information
Specific Aim:
To identify specific compounds that inhibit signaling mediated by NFκB after stimulation of human umbilical vein endothelial cells (HUVEC) by TNFα.

Significance:
Inflammatory disease requires that endothelial cells detect and amplify a pro-inflammatory signal. This results in the adherence, activation, and ultimately transmigration of lymphocytes at the site of damage. Drugs that block this process would significantly alleviate the symptoms of inflammation. This assay detects an early event in this process, the nuclear translocation of the transcription factor, NFκB.

Chronic inflammatory disease is believed to pose a tremendous medical burden in the developed world, not only in terms of patient suffering but also in the cost of treatment and loss of worker productivity. The more common inflammatory diseases include rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, chronic obstructive pulmonary disease and psoriasis. Although each disease has unique aspects regarding the affected tissues and the clinical symptoms, they all share some common biological mechanisms for the establishment and maintenance of the disease state.

A major control point in the initiation of the inflammatory process is at the level of the endothelial cells, lining the blood vessels in the affected tissue. Endothelial cells are of critical importance in detecting the earliest signals broadcast from the damaged tissue and amplifying this signal by attracting and transiently immobilizing lymphocytes. This ultimately allows the responding lymphocytes to transmigrate the vessel wall and invade the damaged tissue to produce a fully fledged inflammatory response.

Rationale:

Some of the major signaling molecules that act on the endothelial cell include cytokines such as tumor necrosis factor alpha (TNFα), interleukin 1 (IL-1), and CD40L. Genotoxic stress is also a known activator of the inflammatory response. Before activation, NFκB exists predominantly in

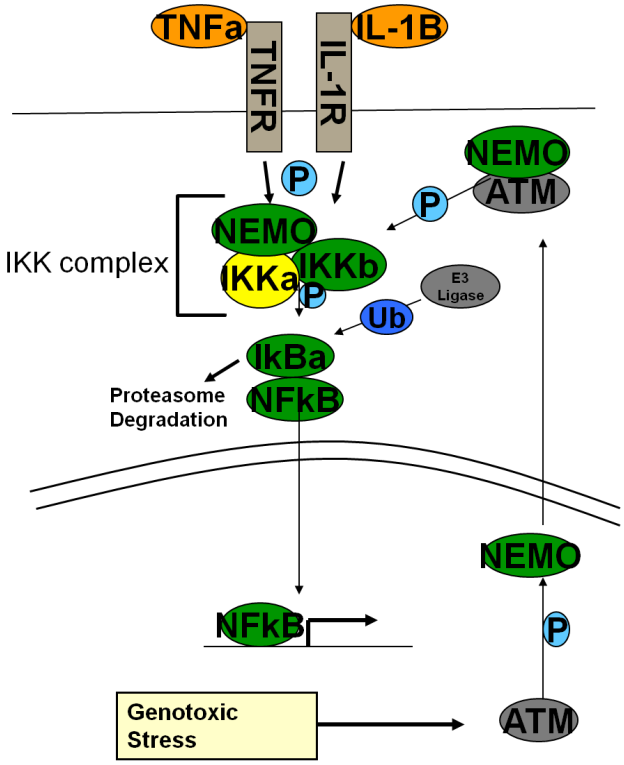


Figure 1: The NFκB pathway can be activated by cytokines such as TNFα and IL-1β and by genotoxic stress.

the cytoplasm as an inactive complex with the inhibitory IκB proteins. Although the earliest signaling events initiated by each cytokine are unique, the pathways soon converge. As shown in Fig. 1, treatment with cytokines or genotoxic stress, activates the IKK complex to phosphorylate IκBα. Phosphorylated IκBα is targeted for ubiquitination and is rapidly degraded by the proteasome, leaving NFκB free to enter the nucleus and initiate transcription of an array of pro-inflammatory proteins. Disruption of the mechanisms that regulate and limit the inflammatory response results in the development of chronic disease.

The basis for this screening campaign is an assay to detect nuclear translocation of NFκB in human umbilical vein endothelial cells (HUVEC) after stimulation with TNFα. Blocking the endothelial response at any point in the inflammatory pathway would reduce a localized inflammatory response and would help to alleviate chronic inflammation.
In addition, the NFκB pathway is constitutively activated in some malignant tumors. Thus, inhibitors might also be useful as cancer therapeutics. However, by performing the assay in HUVEC, we are focusing here on inhibitors active in HUVEC that would be relevant to the inflammatory process.

Current "state-of-the-art":

Numerous inhibitors are known to target the NFκB pathway. The most potent inhibitors that have been described block phosphorylation of IκBα by the IKK complex. These include a pyridooxazinone derivative that has an IC_{50} for IKKβ of 4 nM and is effective in A549 cells with an IC_{50} of 60 nM (Murata, 2004). Several thiophene carboxamide derivatives have been described with IC_{50}'s for IKKβ in the low nanomolar range, while cell assays yielded IC_{50}'s of 0.2 – 0.8 μM in PBMN, ca. 0.15 μM in monocytes and 0.1 μM in synovial fibroblasts. None of the compounds mentioned above has been tested in HUVEC. Waelchi et al. (2006) reported a *p*-aminobenzamide with an IC_{50} of 0.3 – 0.5 μM in HUVEC and an *in vitro* IC_{50} for IKKβ of 40 nM. Although we have not identified the specific target of our active inhibitor, we have established that it does not target IKKβ.

Other inhibitors of the NFκB pathway are either less potent or less specific than the IKKβ inhibitors mentioned above. For example, wifatherin, which targets the ubiquitin-dependent proteasome pathway, has an IC_{50} of 0.5 μM in HUVEC but is non-specific as it blocks cell proliferation at the same concentrations. Another compound, Ro106-9920 inhibits the E3 ligase ubiquitination of IκBα with an IC_{50} of 2.3 μM (Swinney, 2002). We have determined the IC_{50} value of this compound to be 3-4 μM for the NFκB nuclear translocation assay in HUVEC (data not shown).

Curiously, two quinazoline derivatives that have been reported to inhibit the NFκB pathway in lymphocytes are not inhibitory in HUVEC. 6-amino-4-phenethylaminoquinazoline has an IC_{50} of 11 nM in Jurkat T-cells but is inactive in HUVEC. Another quinazoline analog, SPC-839, has an IC_{50} of 8 nM in Jurkat cells (Palanki, 2002) and activates NFκB translocation in HUVEC (data not shown).

Screening center information

Assay Implementation and Screening

PubChem Bioassay Name: Cellular assay for TNFα-induced NFκB translocation

List of PubChem bioassay identifiers generated for this screening project (AIDS): 438, 462,

Primary Assay Description as defined in PubChem:
The translocation of NFκB subunit p65 was evaluated in a cell-based high-content primary screen using fluorescent antibody-based detection. Fixed and permeabilized cells were stained with Hoechst to visualize the nuclei and with mouse anti-p65 (Invitrogen) followed by a secondary antibody conjugated to Alexa Fluor 647 (Invitrogen). Compounds affecting VCAM-1 expression were identified by measuring the relative number of cytoplasmic fluorescent grains resulting from VCAM-1 immunostaining in a high-throughput confocal microscope. Fixed and permeabilized cells were stained with Hoechst to visualize the nuclei and with mouse anti-p65 (Invitrogen) followed by a secondary antibody conjugated to Alexa Fluor 647 (Invitrogen). Images were scanned using the In Cell Analyzer 3000 (GE Healthcare) and analyzed using the Nuclear Trafficking module to compute the nuclear to cytoplasmic ratio of p65 staining.

Center Summary of the Primary Screen:
A total of 12,272 compounds were screened in the primary screening campaign. Plates were passed if z' was > 0.4 and signal to base was > 3.0. Using compounds at 2 μM and a 20% inhibition threshold, 60 compounds were scored as active and 15 as inconclusive. All of these were run in the confirmatory dose-response assay, yielding 8 confirmed hits.

Probe Optimization

Chemical name of probe compound:

6,6-dioxide 4'-methyl-5*H*-quino[8,7-*c*][1,2]benzothiazene

Draw probe chemical structure and show stereochemistry if known:

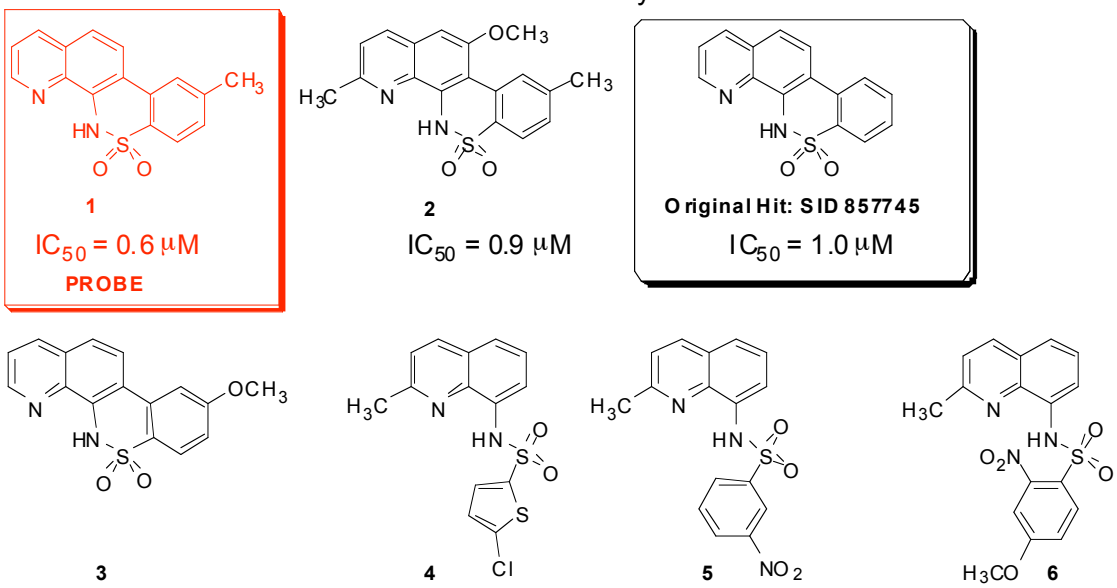


Figure 2: IC_{50} values of our most active compounds.

Describe mode of action for biological activity of probe:

The mode of action is unknown to date. The following proteins have been eliminated as potential targets: Proteasome, ligase, IKK1, IKK2, PI3, E3ligase, TAK1, IRAK1, IRAK4, RIPK2, JNK1α1, JNK2α2, JNK3, MAPK1, MAPK2, PKBα, TBK1, and SAPK2a.

Has this compound been provided to the MLSMR:

Yes

Description of secondary screens used to optimize probe structure:

This screening campaign yielded 8 confirmed hits that were further analyzed using a set of 42 cell-based assays that investigate many of the major signaling pathways in the cell.

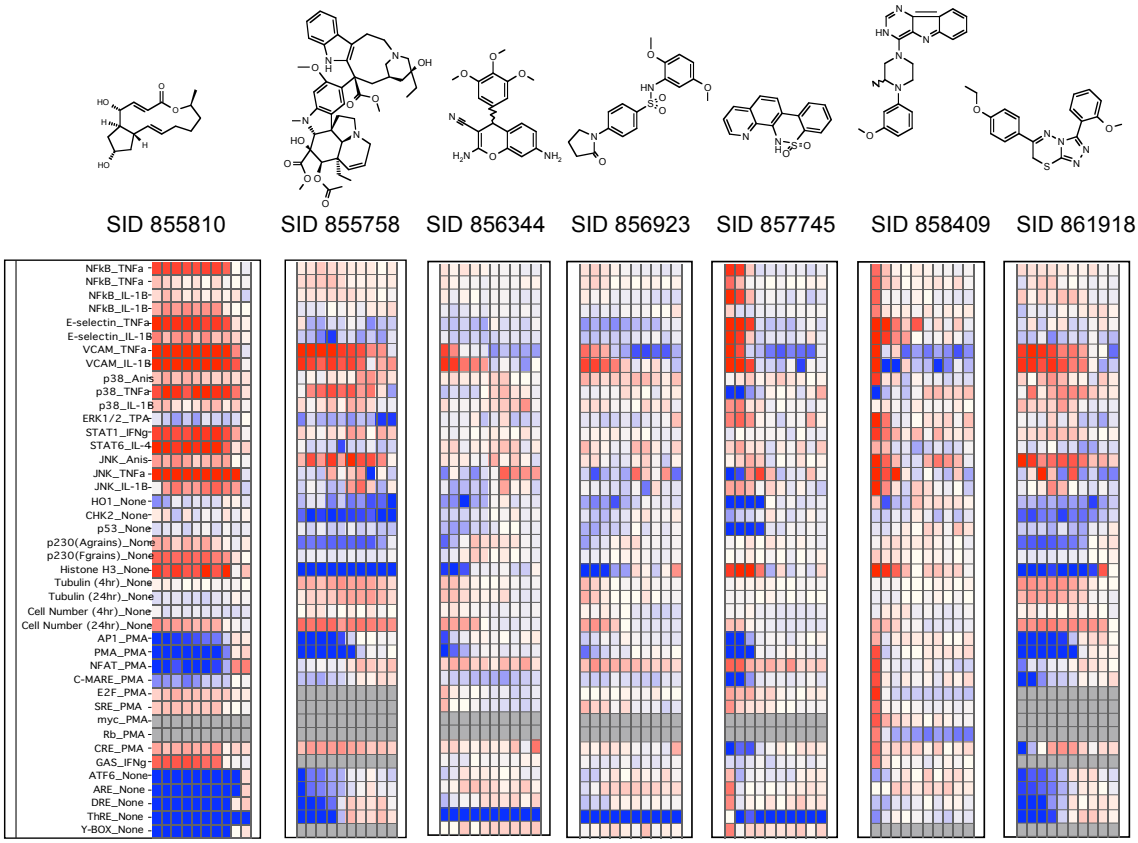


Figure 3: Panel of secondary assays against which we screened our 8 confirmed hits.

This panel of secondary assays was used to evaluate the primary hits from the NFκB screen (**Figure 3**). Based on these results, **SID 857745** and **SID 858409** were chosen as potential leads. Analogs of **SID 858409** did not show good potency in the NFκB assay and this line of investigation was terminated. Analogs of **SID 857745** were run in the NFκB translocation assay in HUVEC stimulated with TNFα or IL-1β in order to confirm and extend the initial result. In addition, the analogs have been evaluated in an Alamar blue cytotoxicity assay.

<u>Center comments on chemistry strategy leading to probe identification:</u>

174 compounds were synthesized and 10 analogs were obtained commercially. A clear structure-activity relationship picture was established (**Figure 4**): a) SO_2 is essential, CO, PO_2 showed no activity; b) NH is essential; c) N is essential; d) Ar is critical; e) The bond can be broken without affecting the activity; f) Ar is critical.

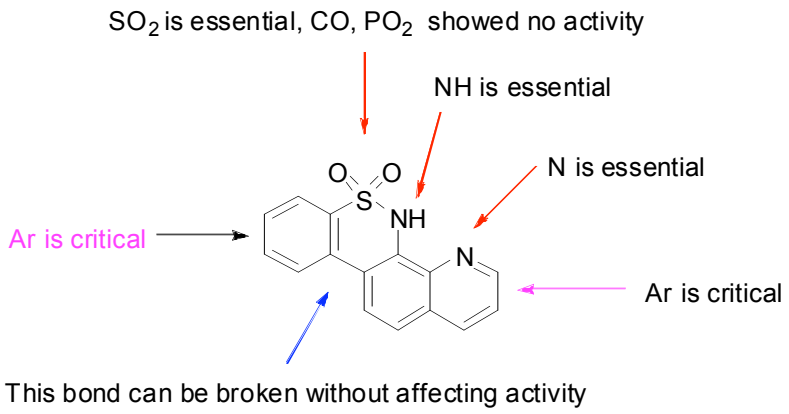
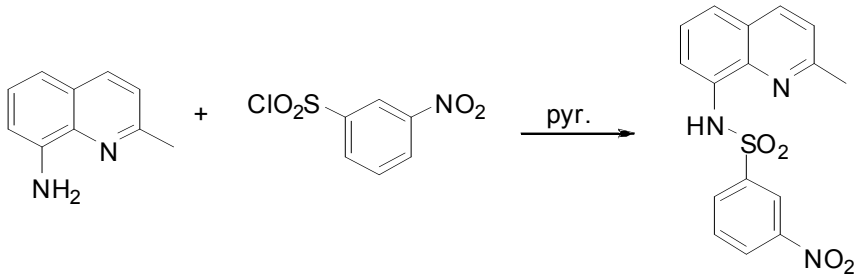


Figure 4. Our SAR picture of SID 857745.

Detailed synthetic pathway for making probe:

Representative Procedure for the Open-chain Analogs:

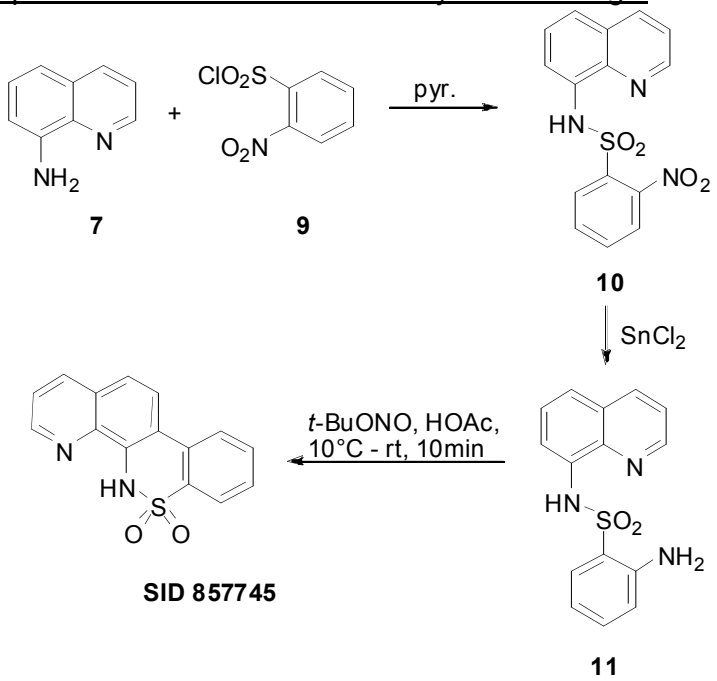


7 **8** **5**

Scheme 1. Synthesis of open-chain analog 5.

To a solution of 8-amino-2-methylquinoline (**7**) (0.16 g, 1.0 mmol) in pyridine (5 ml) was added 3-nitrobenzenesulfonyl chloride (**8**) (0.22 g, 1.0 mmol) at 0 °C. The mixture was slowly warmed up and stirred at room temperature overnight then precipitated with distilled water. The crude product was filtered and recrystallized from ethanol to afford nitro compound **5** (0.24 g, 70%) as red crystals. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.35 (br, 1H), 8.75 (s, 1H), 8.26 (d, 1H), 8.18 (d, 1H), 7.97 (d, 2H), 7.80 (d, 1H), 7.55 (t, 1H), 7.40 (m, 2H), 7.25 (d, 1H), 2.68 (s, 3H).

Representative Procedure for the Cyclized Analogs:



Scheme 2. Synthesis of the original hit SID 857745.

To a solution of 8-aminoquinoline (**7**) (0.14 g, 1.0 mmol) in pyridine (5 ml) was added 2-nitrobenzenesulfonyl chloride (**9**) (0.22 g, 1.0 mmol). The mixture was stirred overnight at room temperature and precipitated with H_2O. The crude product was filtered and recrystallized from ethanol to afford nitrobenzenesulfonamide **10** (0.28 g, 88%) as red crystals. Tin(II) chloride (0.32 g, 1.7 mmol) was added slowly to a suspension of the above-mentioned nitro compound, **10**, (0.20 g, 0.61 mmol) in ethanol (5 ml). The mixture was refluxed for 2 h. After removal of the ethanol, the residue was treated with 1M NaOH. The aqueous solution was extracted with methylene chloride. The combined organic extracts were washed with brine, dried over anhydrous sodium sulfate, and concentrated to yield the amine, **11**, (0.15 g, 83 %) as a white solid. To a solution of the amine, **11**, (80mg, 0.28 mmol) in acetic acid (1 ml) at 10 ºC was added *t*-butyl nitrite (0.05 mL, 0.42 mmol). The reaction was slowly warmed to room temperature over 10 min and quenched with distilled water. The mixture was extracted with ethyl acetate. The combined organic extracts were washed with saturated sodium bicarbonate, dried over anhydrous sodium sulfate, and concentrated. Flash chromatography (EtOAc/CH_2Cl_2 1:10 v/v) gave the product, **SID 857745** (61 mg, 77%) as a slightly yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.75 (s, 1H), 8.10 (d, 1H), 8.05-7.92 (m, 3H), 7.65 (t, 1H), 7.55-7.45 (m, 2H), 7.40(m, 1H); ESI-MS (M^++1): 283.2. (identical to the authentic sample purchased from ChemBridge)

Figure 5 that follows below shows the dose curves for six of the most active compounds. The two most potent compounds, **1** and **2**, had IC_{50} values of 600 nM and 900 nM, respectively (**Figure 2**).

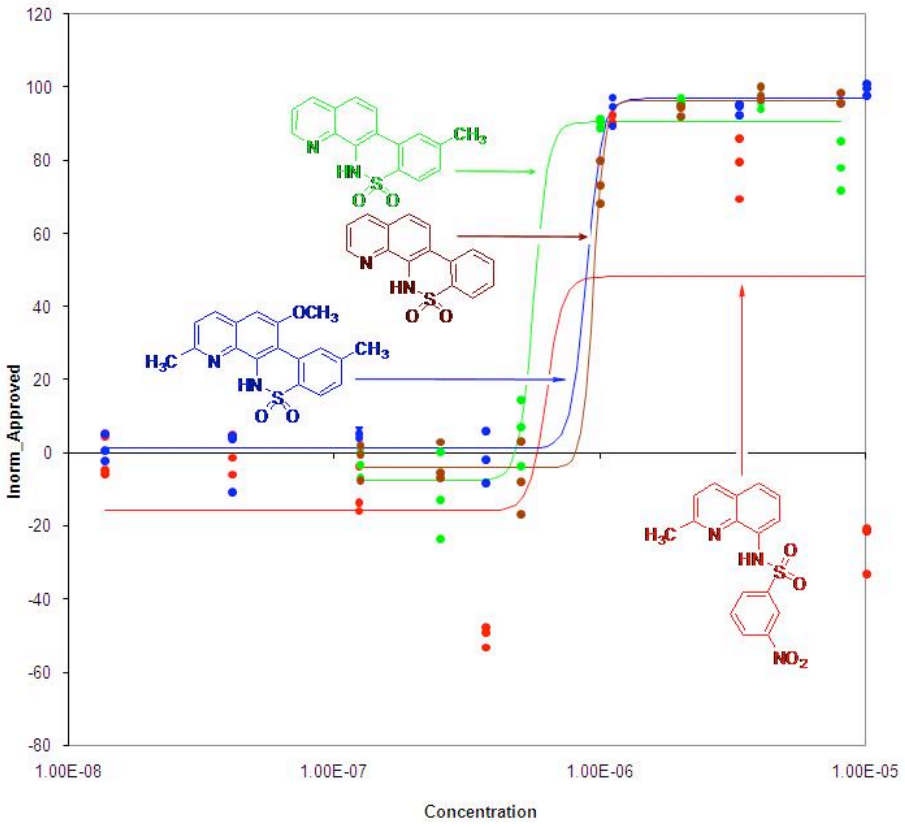


Figure 5. Dose curves for our most active compounds.

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

Appendices

Bibliography

Baxter, A. et al. Hit-to-lead studies: the discovery of potent, orally active, thiophenecarboxamide IKK-2 inhibitors. Bioorg. Med. Chem. Lett. 14, 2817-2822 (2004).

Mohan, R. et al., Withaferin A is a potent inhibitor of angiogenesis. Angiogen. 7, 115-122 (2004).

Murata, T. et al., Synthesis and structure-activity relationships of novel IKK-b inhibitors. Part 3: Orally active anti-inflammatory agents. Bioorg. Med. Chem. Lett. 14, 4019-4022 (2004).

Palanki, M.S. et al. Structure-activity relationship studies of ethyl 2-[(3-methyl-2,5-dioxo(3-pyrrolinyl)amino]-4-(trifluoromethyl)pyrimidine-5-carboxylate; an inhibitor of AP-1 and NF-kB mediated gene expression. Bioorg. Med. Chem. Lett. 12, 2573-2577 (2002).

Podolin, P.L. et al., Attenuation of murine collagen-induced arthritis by a novel, potent selective small molecule inhibitor of IkB Kinase 2, TPCA-1 (2-[(Aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide), occurs via reduction of proinflammatory cytokines and antigen-induced T cell proliferation. J. Pharm. Exp. Ther. 312, 373-381 (2005).

Swinney, D.C. et al., A small molecule ubiquitination inhibitor blocks NF-kB-dependent cytokine expression in cells and rats. J. Biol. Chem. 277, 23573-23581 (2002).

Tobe, M. et al., Discovery of Quinazolines as a noved structural class of potent inhibitors of NF-kB activation. Bioorg. Med. Chem. 11, 383-391 (2003).

Wailchli, R., et al. Design and preparation of 2-benzamido-pyrimidines as inhibitors of IKK. Bioorg. Med. Chem. Lett.16. 108-122 (2006).