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Discovery of novel small molecule cell type-specific enhancers of NF-κB nuclear translocation

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ABSTRACT

An IKKβ inhibitor reported to block NF-κB transcriptional activities in Jurkat T cells, was found to enhance NF-κB translocation in HUVEC cells. These studies suggested a noncanonical NF-κB signaling pathway independent of IKKβ in HUVEC cells.

Nuclear factor-κB (NF-κB) proteins are a class of 'rapid-acting' transcription factors that regulate the expression of more than 400 target genes and play a pivotal role in several important physiological processes including immune and inflammatory responses.[1] There are five members in mammalian NF-κB family that share a Rel homology domain (p50, p52, c-Rel, RelA/p65 and RelB). Normally, these proteins are sequestered in the cytoplasm in an inactive state by the family of inhibitory proteins IκB. Upon stimulation, IκB proteins are rapidly phosphorylated by an IκB kinase (IKK) and subsequently ubiquitinated by an E3 ligase leading to the proteasomal degradation. Removing the inhibitory protein IκB permits the NF-κB complex to translocate to the nucleus where it activates gene expression. This pathway, with several potential targets for manipulation of NF-κB activities, has emerged as a focal point for intense drug discovery in the areas of cancer, immunology and inflammation, and small molecule regulators are particularly favored for drug development efforts.[2]

Recently, a cell-based assay was designed to identify small-molecule modulators of NF-κB nuclear translocation, a critical event in the NF-κB signaling cascade.[3] Specifically, TNFα-induced translocation of the endogenous p65 subunit of NF-κB in human umbilical vein endothelial cells (HUVEC) was monitored at 20 min post-stimulation by fluorescence labeled anti-p65 antibodies using an automated fluorescence imaging platform. Based on this assay, we performed a high content screening of a 100k library provided by the NIH and successfully identified a class of novel benzenesulfonamides that blocked p65 translocation to the nucleus and thus inhibited the NF-κB pathway.[4] A general synthetic method was also developed for the construction of these interesting compounds.[5] However, compared to inhibitors, specific NF-κB activators are less well studied. Recent findings suggest that activating NF-κB, under certain circumstances, may be useful in cancer therapy,[6] radiation protection[7] and anti-HIV treatment.[8] Herein we describe a series of quinazoline analogues that induce NF-κB translocation, synergistically enhance TNFα's stimulating effect, and thus act as NF-κB activators.

In the validation of the translocation-based assay, we screened a set of known inhibitors that target different elements in the NF-κB pathway such as the proteasome inhibitor MG132 (**1**), IκB phosphorylation inhibitor BAY117082 (**2**), E3 ligase inhibitor Ro106-9920 (**3**), and IKK inhibitors **4**[9] and **5**[10] (Fig. 1).

Most of the agents, as expected, were found to potently inhibit p65 translocation to the nucleus (data not shown). However, surprising results were observed with some IKK inhibitors. The IKK complex, including two major isoforms IKKα and IKKβ, plays a central role in the phosphorylation-initiated NF-κB nuclear translocation. We observed in our assay that specific IKKβ inhibitors did not inhibit this process in HUVEC cells, suggesting that the signal cascade was IKKβ-independent. This non-canonical pathway was fur-

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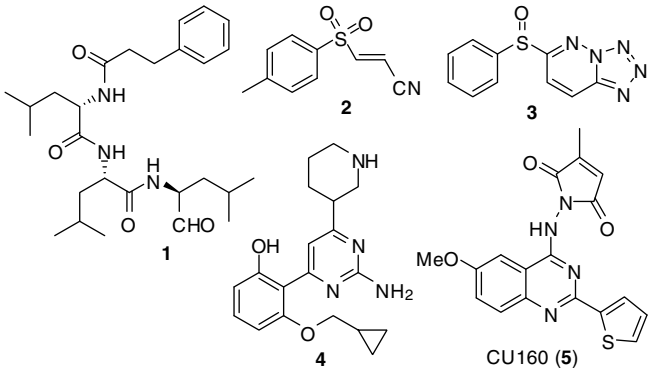


Figure 1. Structures of MG-132 (**1**), BAY 11-7082 (**2**), Ro106-9920 (**3**), and IKK inhibitors **4** and **5**.

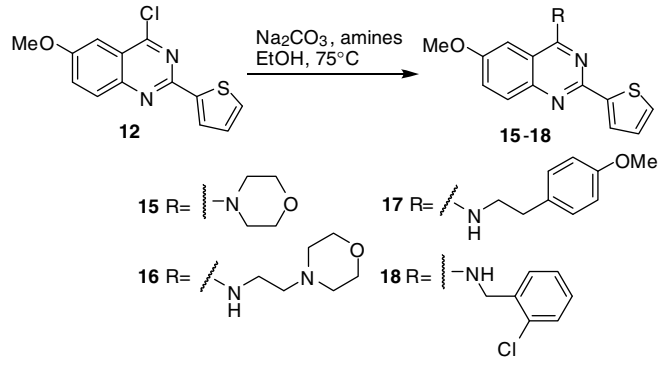
ther confirmed by genetic knockdown of IKKβ with siRNA.[11] More strikingly, CU160 (**5**), a substituted 2-(thiophen-2-yl)quinazoline, which has been reported as a potent IKKβ inhibitor that block NF-κB and AP1-mediated transcriptional activities in Jurkat T cells,[10] was unexpectedly found to potentiate TNFα-induced translocation in our assay. We further demonstrated that CU160 (**5**) alone was sufficient to activate the pathway without TNFα stimulation. These findings have identified CU160 (**5**) as a novel cell type-specific NF-κB activator and provided another example of the complexity and cell type-specificity of the NF-κB pathway.

Since other IKKβ inhibitors tested did not have the same effect, CU160's NF-κB enhancing activity was clearly an off-target effect. For the structure-activity relationship (SAR) study, we developed a convenient synthetic route to CU160 (**5**) and its analogues. As shown in Scheme 1, commercially available 5-methoxy-2-nitro benzoic acid **6** was converted to benzamide **7** by treatment of oxayl chloride and ammonium hydroxide. Palladium-catalyzed hydrogenation of the nitro group afforded amine **8**, which was coupled with chloride **9** to give amide **10**. Cyclization under basic condition followed by the treatment of phosphorus oxychloride converted amide **11** to quinazoline **12**. The chlorine at 4-position of compound **12** was replaced with hydrazine and the resulting hydrazine **13** was further condensed with citraconic anhydride **14** to yield the final product CU160 (**5**).[10]

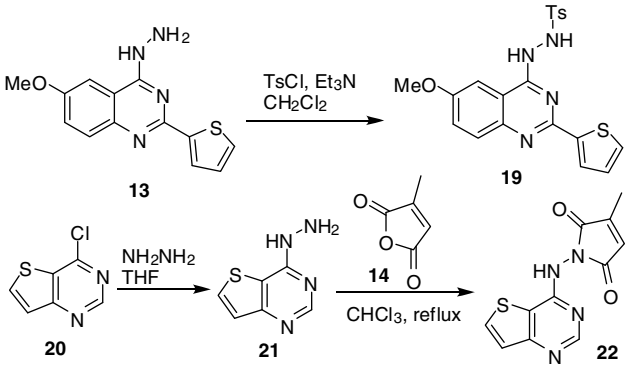
A series of 4-quinazolinamine analogues (**15**–**18**) were conveniently synthesized by condensation of quinazoline **12** with structurally diversified amines as shown in Scheme 2.

The synthesis of two analogues **19** and **22** that alter the pyrrole-2,5-dione ring or the quinazoline ring, respectively, is outlined in Scheme 3. Tosylation of hydrazine **13** afforded **19** in one step. Thienopyrimidine **22** was obtained by conversion of 4-chlorotheino[3,2-*d*]pyrimidine **(20)** to hydrazine **21** followed by condensation with citraconic anhydride **14**.

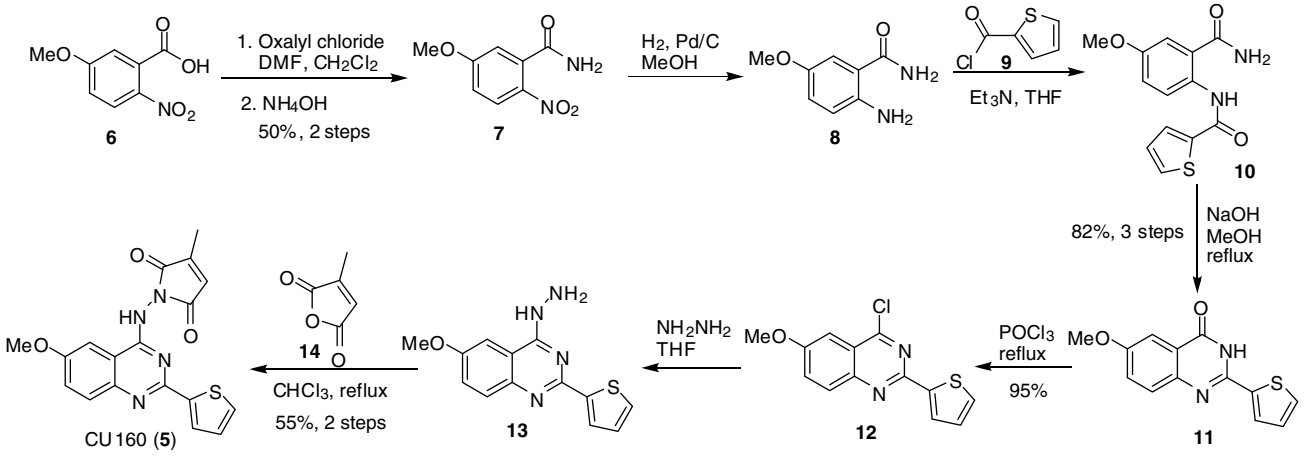
The synthesis of substituted quinazolines **30** and **35,** and reduced pyrrole **31**, is summarized in Scheme 4. One of the nitro groups in the starting material 2,6-dinitrobenzonitrile **23** was replaced with a methoxy group to give compound **24**. Reduction of **24** with Raney-Ni in the presence of hydrazine yielded 2-aminobenzamide **25** in one pot.[12] A similar amidation followed by cyclization transformed **25** to quinazoline **27** in excellent yield. Chlorination of **27** with phosphorus oxychloride at 100 °C provided the product **28**. Interestingly, at 130 °C, the reaction only gave



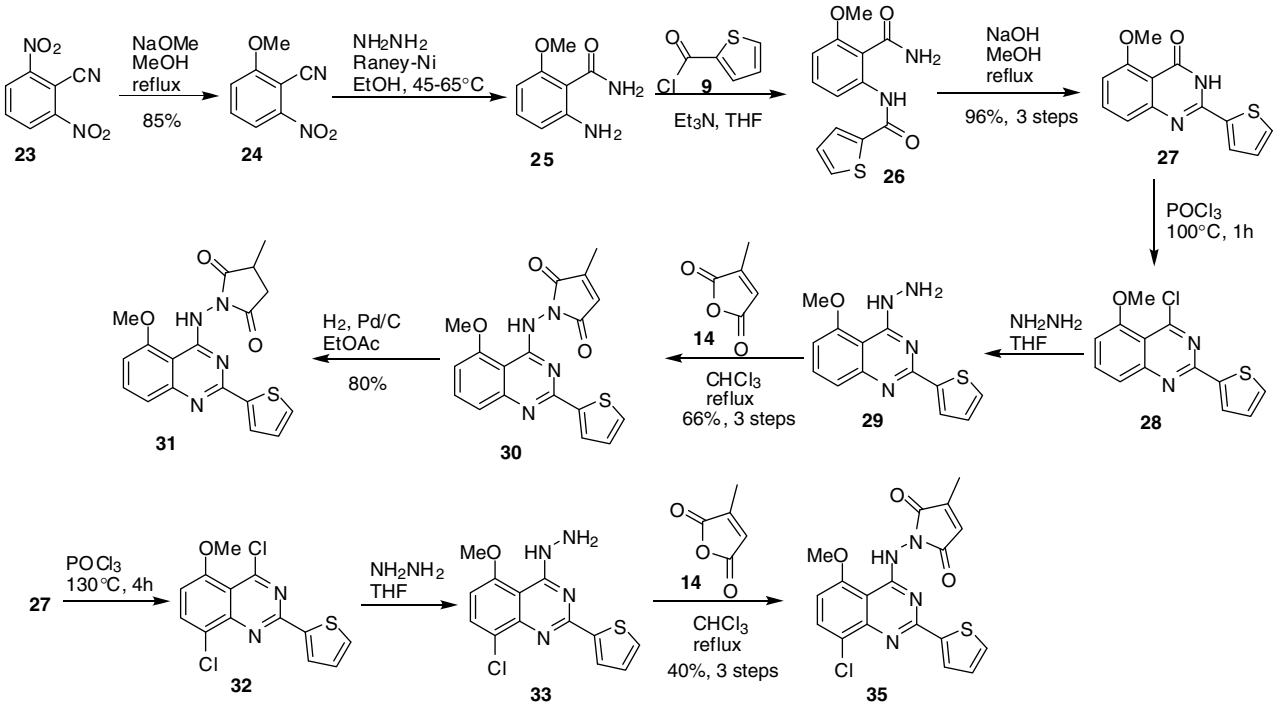
Scheme 2. The synthesis of 4-quinazolinamine analogues **15**–**18**.



Scheme 3. The synthesis of analogues **19** and **22**.



Scheme 1. The synthesis of NF-κB activator CU160 (**5**).



Scheme 4. The synthesis of analogues **30**, **31** and **35**.

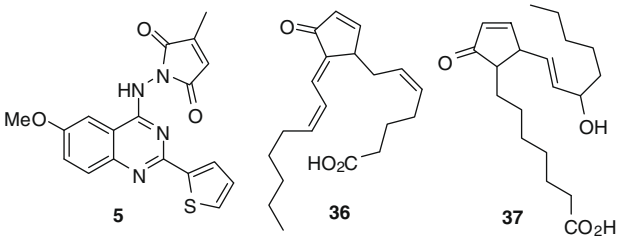


Figure 2. Structures of 15d-PGJ2 (**36**) and PGA1 (**37**).

dichlorinated compound **32** as the single product.[13] Both compounds were converted to the final products **30** and **35** in two steps by a similar procedure described in Scheme 1. The double bond of the citraconic group in **30** was further reduced to give the analog **31**.

All analogues were tested for their ability to interfere with p65 translocation to the nucleus. The preliminary results show that the pyrrole-2,5-dione ring is important for the activity. A variety of replacements all resulted in inactive compounds (**15**–**19**). However, as illustrated by analogue **30**, a position shift of the methoxyl group (5-position to 6) on the quinazoline scaffold significantly improved the potency both in activation and potentiation (see Figs. 3 and 4). Addition of a chlorine para to the 6-methoxyl group led to a further improvement (**35**). Interestingly, simple reduction of the double bond of the pyrroledione group in **30** resulted in a complete loss of activity (**31**). The double bond adjacent to two electron-withdrawing ketones is a very strong Michael acceptor and can serve as an addition site for the thiol groups of cysteine residues. Furthermore, NF-κB proteins are subject to covalent modification by Michael acceptor-containing small molecules such as prostaglandins 15d-PGJ2 (**36**) and PGA1 (**37**) (Fig. 2).[14] Based on these observations, we speculate that CU160 (**5**) and its analogues may modify p65 covalently, decrease its affinity for the inhibitory protein IκB and thereby, promote its nuclear translocation.

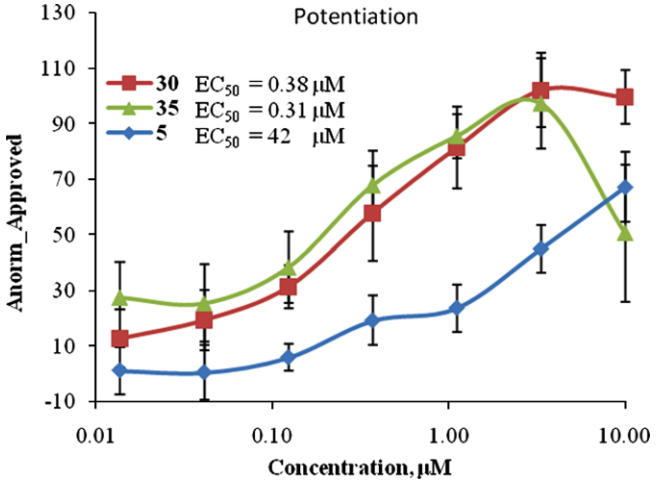


Figure 3. Potentiation of TNF-induced NF-B nuclear translocation by compounds **30**, **35** and **5**. HUVEC cells were obtained from Cambrex as frozen stocks. Cells were seeded and incubated with increasing concentrations of compounds. Cells were then stimulated with TNF (200 ng/ml) for 20 min. Translocation of NF-B was monitored by immunostaining for the p65 subunit. To detect distribution of the transcription factor, cells were fixed and permeabilized and then incubated with a monoclonal antibody against p65 and secondary antimouse IgG antibodies coupled to Alexa Fluor 647.

In summary, utilizing a cell-based assay, we studied an early but critical translocation event in the NF-κB pathway in HUVEC cells with a variety of small molecule probes. These studies revealed a noncanonical NF-κB signaling that was independent to IKKβ. More importantly, we serendipitously discovered a class of novel cell type-specific NF-κB activators. SAR studies suggested that these compounds might work through covalent modification of NF-κB proteins. The detailed mechanism is currently under investigation.

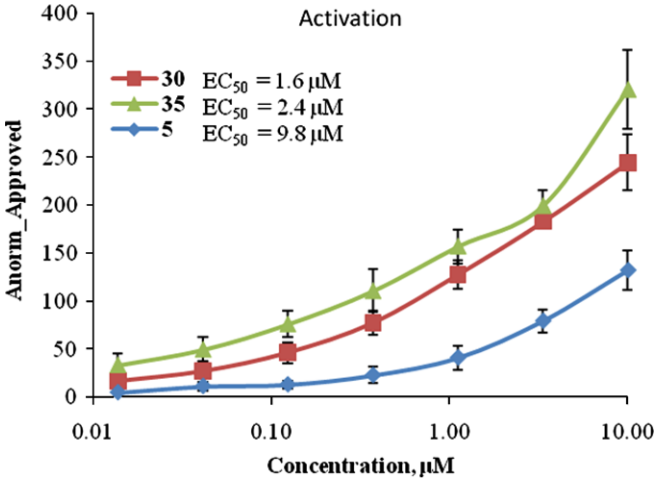


Figure 4. Activation of NF-B nuclear translocation by compounds **30**, **35** and **5**. HUVEC cells were incubated with increasing concentrations of compounds. Without stimulation, distribution of p65 was measured as described in Figure 3.

Acknowledgment

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13. Compound **35**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.53 (s, 1H), 7.91 (dd, J = 1.2, 3.6 Hz, 1H), 7.63 (d, J = 8.4 Hz, 1H), 7.40 (dd, J = 1.2, 5.1 Hz, 1H), 7.08 (dd, J = 3.6, 4.8 Hz, 1H), 6.62 (m, 1H), 6.46 (d, J = 8.4 Hz, 1H), 3.75 (s, 3H), 2.26 (d, J = 2.1 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.3, 169.2, 159.1, 157.3, 155.1, 149.1, 145.6, 143.8, 133.4, 130.2, 129.5, 128.3, 127.1, 124.2, 105.3, 105.2, 56.6, 12.1; ESI m/z $[M+H]^+$ 401.
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