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Novel IKK Inhibitors: β-Carbolines

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Abstract—Inhibitors of IκB kinase (IKK) have long been sought as specific regulators of NF-κB. A screening effort of the endogenous IKK complex allowed us to identify 5-bromo-6-methoxy-β-carboline as a nonspecific IKK inhibitor. Optimization of this β-carboline natural product derivative resulted in a novel class of selective IKK inhibitors with IC_{50}s in the nanomolar range. In addition, we show that one of these β-carboline analogues inhibits the phosphorylation of IκBα and subsequent activation of NF-κB in whole cells, as well as blocking TNF-α release in LPS-challenged mice.

The transcription factor NF-κB (nuclear factor *kappa* B) mediates the expression of a number of pro-inflammatory cytokines, adhesion molecules, growth factors, and anti-apoptotis survival proteins. NFκB transcriptional activity is normally suppressed in the cytosol via complexation with its natural inhibitor molecule IκB. Various cellular stress and pro-inflammatory stimuli can result in the translocation (and hence activation) of NF-κB into the nucleus via the degradation of IκB. The proteolytic destruction of IκB via the ubiquitin-proteasome pathway is triggered by phosphorylation of IκB at Ser32 and Ser36 by the IκB kinase complex (IKK). As such, inhibitors of IKK may be useful in the treatment of inflammatory diseases as well as in cancer.[1]

Several natural products as well as some NSAIDs have been reported to weakly inhibit IKK. Various natural products (e.g., curcumin,[2] (−)-epigallocatechin-3-gallate,[3] parthenolide[4] and quercetin[5]) are weak non-selective inhibitors of IKK. Some common anti-inflammatory drugs (e.g., sulindac,[6] aspirin,[7] and sulfasalazine[8]) have been found to inhibit IKK at high concentrations. Such weak activity against IKK suggests that their therapeutic modes of action are thought to be via other mechanisms.

We have identified a methylated natural product derivative 5-bromo-6-methoxy-β-carboline (Fig. 1, **1**) as a nonspecific inhibitor of IKK. Compound **1** has an IC_{50} of 1.0 μM against endogenous IKK isolated from HeLa cells and activated with recombinant MEKK1.[9] We further evaluated this natural product derivative against a panel of kinases and found it to be somewhat non-selective (Table 1).

We set out to explore the structure–activity relationship of β-carbolines in order to optimize activity against IKK and to identify key features that could impart potency as well as kinase selectivity. To this end we explored the substitution pattern around the A ring of β-carboline. We synthesized various 6-substituted β-carbolines via Pictet-Spengler reactions of the appropriate tryptamines followed by catalytic oxidation (Scheme 1),[10] halogenation of norharmane (Scheme 2),[11] and cyclization of piperid-2,3-dione-3-arylhydrazones (Scheme 3).[12] Evaluation of these analogues in an IKK ELISA phosphorylation assay[9] clearly shows that electron withdrawing groups at the 6-postion provided the most active compounds (Table 2). Fortuitously, a small amount of byproduct from the chlorination reaction (Scheme 2) was isolated and identified as 6,8-dichloro-β-carboline (Fig. 2, **9**). This analogue proved to be 3-fold

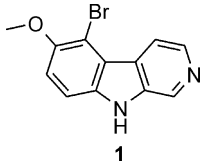

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Figure 1. Initial β-carboline IKK screening hit.

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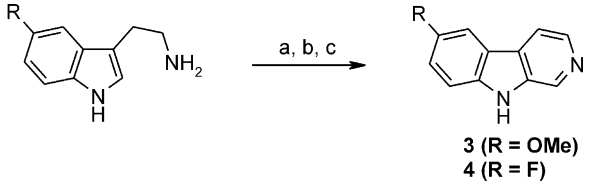
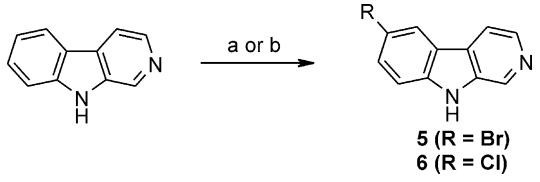
Table 1. Kinase selectivity data for 5-bromo-6-methoxy-β-carboline (**1**)

Kinase	% Inhibiton at 25 μM[a]	IC_{50}, μM[a]
IKK	90	1.0
CKII	75	2.0
PKA	10	nd
PKC	65	15

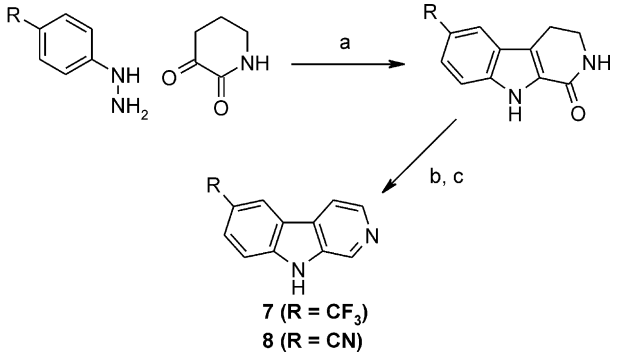
[a]Values are means of three experiments, standard deviation is given in parentheses (nd = not done).



Scheme 1. (a) Glyoxalic acid, pH 6.0 NaOAc buffer EtOAc; (b) aq HCl, reflux; (c) Pd/C, xylene, reflux.



Scheme 2. (a) *N*-Bromosuccinimide, THF, 48 h; (b) *N*-chlorosuccinimide, THF, 48 h.



Scheme 3. (a) Formic acid, microwave, 2 min; (b) BH_3, THF; (c) Pd/C. xylenes, reflux.

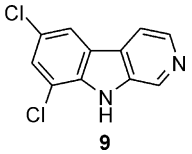
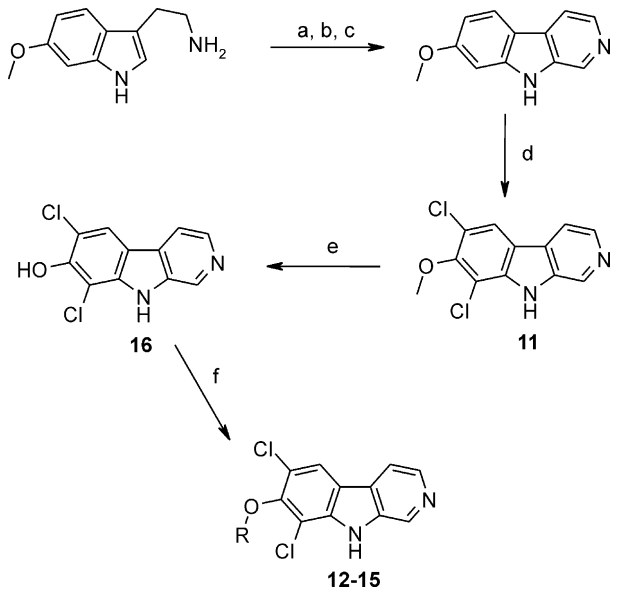


Figure 2. 6,8-Dichloro-β-carboline.

more potent than the monochlorinated analogue **6** and 5-fold more potent than the originally identified β-carboline (**1**). Furthermore, the selectivity for IKK over CKII of this compound had improved to over 20-fold. Unfortunately, compound **9** suffered from poor aqueous solubility and therefore could not be further evaluated in secondary cell assays.[13]



Scheme 4. (a) Glyoxalic acid, pH 4.5–4.8 1 M NaOAc buffer; (b) aq HCl, reflux; (c) Pd/C, xylene, reflux; (d) *N*-chlorosuccinimide, acetic acid; (e) BBr_3, THF; (f) NaH, DMF, electrophile.

Table 2. 6-Substituted-β-carboline analogues

Compd	6-Substituent	IC_{50}, μM[a]
2	–H	15
3	–OMe	4
4	–F	2
5	–Br	0.6
6	–Cl	0.6
7	$–CF_3$	1.1
8	–CN	1.1

[a]Values are means of >3 experiments.

We speculated that an ether moiety at the 7 position of compound **9** would impart improved solubility due to its hydrophilic nature while providing a handle for exploiting this part of the molecule. To this end, we prepared a small library of analogues as described in Scheme 4. This effort proved fruitful in that it provided intrinsically more soluble IKK inhibitors with greatly improved selectivity and potency (Table 3). The most potent of these, compound **12**, was evaluated against a subset of kinases and found to be >200-fold selective for IKK versus CKII, PKA, and PKC. This study indicated that the protusion of an alkylether moiety of limited size (c.f. compound **12** vs **13**) at the 7-position of 6,8-dichloro-β-carboline results in a more selective and potent IKK inhibitor.

In parallel to the work described above, we explored nitrogen containing substituents at the 8 position in the presence of a 6-chloro group on β-carboline. The nitration of compound **6** with sodium nitrate in trifluoroacetic acid followed by reduction of the 6-chloro-8-nitro-β-carboline (**16**) with $SnCl_2$ ultimately provides 6-chloro-8-amino-β-carboline (**17**) (Scheme 5). Compound **17** served as a key intermediate for generating a library of compounds having amides, sulfonamides, ureas, carbamates, as well as substituted amines at the 8

Table 3. 7-Substituted-6,8-dichloro-β-carboline analogues

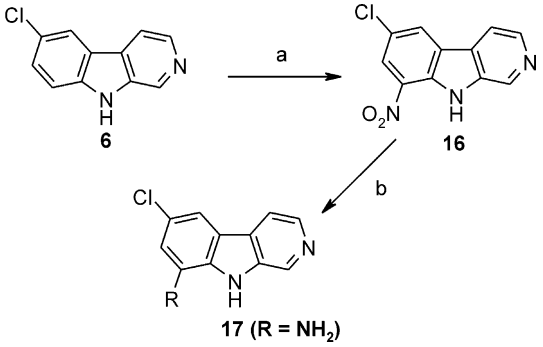
Compd	7-Substituent	IC_{50}, μM[a]
9	–H	0.20
10	–OMe	0.17
11	–OEt	0.14
12	$-OCH_2CH(CH_2CH_2)$	0.08
13	$-OCH_2(C_6H_{11})$	3.0
14	–OC(O)Morpholine	3.2
15	–OH	11

[a]Values are means of >3 experiments.

Table 4. 8-Substituted-6-chloro-β-carboline analogues

Compd	R	IC_{50}, μM[a]
16	$-NO_2$	4
17	$-NH_2$	1.3
18	$-NHCH_3$	1.8
19	$-N(CH_3)_2$	1.8
20	$-NHCH_2Ph$	>20
21	$-NHC(O)CH_3$	0.60
22	$-NHC(O)CH_2CH_2CH_2OH$	>20
23	–NHC(O)Ph	0.70
24	$-NHC(O)CH_2$-2′-pyridyl	3.0
25	–NHC(O)-2′-pyridyl	1.0
26	–NHC(O)-3′-pyridyl	0.10
27	–NHC(O)-4′-pyridyl	0.30
28	–NHC(O)-2′-anisole	>20
29	–NHC(O)-3′-anisole	0.60
30	–NHC(O)-4′-anisole	1.3
31	$-NHS(O)_2CH_3$	8.3
32	$-NHS(O)_2Ph$	>20
33	$-NHC(O)OCH_3$	0.7
34	$-NHC(O)NHCH_3$	>20
35	–NHC(O)morpholine	>20

[a]Values are means of >3 experiments.



Scheme 5. (a) $NaNO_3$, TFA; (b) $SnCl_2$, aq HCl.

position. A subset of the analogues prepared along with their activity against IKK is listed in Table 4. All these analogues with exception of the anilines **17**, **18**, and **19** were found to be inactive (IC_{50}s > 25 μM) against PKA, PKC, and CKII. These results along with those of the ether series described earlier suggest that perhaps the specificity for IKK is conferred by placing appropriate hydrophobic interactions at the 7 or 8 position of β-carboline. Also apparent from the data is the presence of a favorable hydrogen bonding interaction approximately five atoms away from the 8-position (c.f. **20** vs **26**).

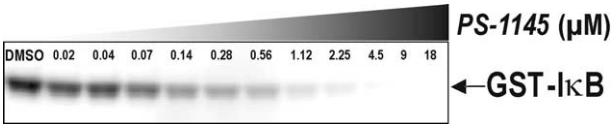


Figure 3. Inhibition of immuno-precipitated IKK complex isolated from TNFα-induced HeLa cells with varying concentration of compound **26** (PS-1145).[19]

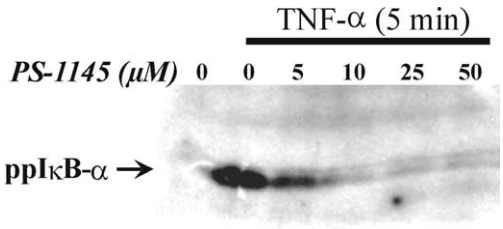


Figure 4. Western blot analysis of lysates from compound **26** (PS-1145) treated HeLa cells.[14]

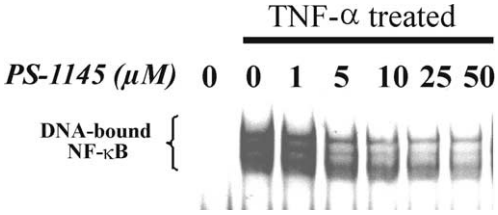


Figure 5. Electromobility shift assay (EMSA) of lysates from of compound **26** (PS-1145) treated HeLa cells.[15]

The remarkable activity of the simply decorated β-carboline compound **26** (also referred to as PS-1145) prompted us to evaluate its biological activity in whole cell assays. To demonstrate that compound **26** inhibits the relevant kinase in cells, we chose to perform an immuno-precipitation kinase assay from TNF-α induced HeLa cell lysates,[19] rather than using recombinant IKK1 or IKK2 (Fig. 3). Quantitation of the ^{32}P-radiolabelled GST-IκB[5-55] substrate bands showed that this compound inhibits endogenous IKK complex with an IC_{50} of 150 nM, comparable to that of the MEKK1-activated enzyme assay. Compound **26** was then tested for its ability to block the phosphorylation of IκB-α in HeLa cells following TNF-α stimulation for 5 min. Immunoblot analysis of whole cell lysates showed a dose-dependent inhibition of phosphorylated IκB-α when compared to vehicle control (Fig. 4).[14] We then evaluated the effect of compound **26** on NF-κB activation by measuring DNA binding activity after TNF-α treatment in the same HeLa cells. Similar to the inhibition of endogenous IκB-α phosphorylation, EMSA results (Fig. 5) showed a dose-dependent inhibition of NF-κB activation by compound **26** with $EC_{50} = 5$ μM.[15] Consistent with the inhibition of NF-κB activation, compound **26** also blocks the transcription of ICAM-1, a known NF-κB target gene, in HUVEC primary cultures (data not shown).[16] With this data set in hand, we next examined the ability of compound **26** to block the production of TNF-α in lipopolysaccharide (LPS) treated mice. To this end, compound **26** was dosed orally (50 mg/kg) in mice 1 h prior to LPS

challenge. At 5-h post-LPS challenge, plasma was collected and assayed for systemic levels of TNF-α. This single oral dose of **26** resulted in a 60% reduction of TNF-α versus vehicle control animals.[17] Taken together, we conclude that compound **26** can effectively inhibit IKK and its downstream signaling events in cells and in vivo.

In summary, a new lead series was discovered based on our initial identification of natural product derivative **1** as an IKK inhibitor. We identified an optimal substitution pattern on the A ring of β-carboline that results in potent and selective inhibitors of IKK. We have demonstrated the ability of a selective IKK inhibitor to block the activation of NF-κB in whole cells, exemplified with **26**. Compound **26** (PS-1145) will be further examined as a potential treatment of various cancers[18] and inflammatory diseases.

Acknowledgements

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9. Briefly, HeLa S3 cells were lysed in buffer A (50 mM Tris–HCl pH 7.6, 0.5 mM EDTA, 1 mM DTT, 5 mM benzamide, 1 mM PMSF, 1 mM NaF, 10 mM 2-glycerophosphate) and clarified by ultracentrifugation at 100,000×*g* for 1 h. (S100 fraction). S100 was concentrated by 40% ammonium sulfate precipitation on ice for 1 h, resuspended in minimal buffer A, and. 2-mL sample was chromatographed using a 120-mL Superose-6 (Amersham-Pharmacia Biotech) pre-equilibrated with buffer B (A + 50 mM NaCl). Fractions between 600-900 kDa were collected, pooled, and loaded onto a 1-mL Mono-Q (Pharmacia) pre-equilibrated in buffer B. Using step-gradients, fractions containing IKK complex were eluted at 300-mM NaCl using buffer C (A + 1 M NaCl). Partially purified IKK was then pre-activated with a poly-his tagged kinase domain of MEKK1 expressed and purified from sf9 baculovirus (Lee, F. S.; Peters, R. T.; Dang, L. C.; Maniatis, T. *Proc. Natl. Acad. Sci. U.S.A. (United States)* **1998**, *95*, 9319). IKK kinase activity was then assessed using 25 μM of a biotinylated IκB-α peptide (RHDSGLDSMKD, $K_{m,peptide} = 30$ μM; $K_{m,ATP} = 10$ μM) and 50 μM MgATP as substrates in buffer D (50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 10 mM 2-glycerophosphate, 2 μM Microcystin-LR, 1 mM DTT, 0.05% BSA). The reaction was quenched with excess 20 mM EDTA, and detected using a specific [Ser^{32}]-phosphoantibodies in an ELISA format with appropriate standard curves for quantitation.
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14. Hela cells were stimulated with 10 ng/mL of TNF-α (R&D Systems) for 5 min in the presence of varying concentration of compound **26**. Cytoplasmic cell lysate (50 μg) was fractionated on Tris-glycine-buffered 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane (Amersham, Cleveland, OH) for WB analysis. Membranes were probed with anti-phosphoantibodies against Serine32 of IκBα (Cell Signaling, #9241). Immunoreactive proteins were detected by chemiluminescence.
15. As were performed as described in Fried, E. M. S.; Crothers, M. D. M. *Nucl. Acids Res*. **1981**, *9*, 6505.
16. ICAM-1 expression assay was performed as described in Read, M. A.; Neish, A. S.; Luscinskas, F. W.; Palombella, V. J.; Maniatis, T.; Collins, T. *Immunity* **1995**, *2*, 493.
17. Female BALB/c mice were dosed orally with compound **26** at a volume of 5 mL/kg. Lipopolysaccharide (*E. coli* Serotype 0127:B8, Sigma) was administered intraperiteneally 30 min later. After 1.5 h mice were euthanized via CO2 asphyxiation and blood collected by cardiac puncture into sodium heparin-coated eppendorf tubes. Blood samples were placed on ice and spun down at 6600 *g* for 10 min at 4 °C. Plasma was collected from each blood sample and assayed for TNF levels using the ELISA kit (BioSource International).
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19. Immuno-precipitation kinase assay was performed with 100 μg of anti-IKKγ antibodies (Santa Cruz Biotech, #sc-8330) incubated with 45 mg of cell lysates and 100 μL of Protein-A Trisacryl beads (Pierce, Rockford IL) for 4 hrs at 4 °C with rocking. Lysates were prepared by treating HeLa cells with TNF-α (20 ng/mL, R&D Systems) for 5 min. at 5×10^6 cell density. Cells were washed in cold PBS and lysed in IP buffer (50 mM Tris pH 7.5, 20 mM PNPP, 20 mM 2-glycerophosphate, 10% glycerol, 1% NP-40, 1 mM EDTA, Promega Protease inhibitor cocktail tablet). Protein-A beads were washed 5× with IP buffer and 2× with kinase buffer (50 mM HEPES pH 7.5, 10% glycerol, 10 mM $MgCl_2$, 10 mM 2-glycerophosphate). Beads were then aliquoted into 50 μL reaction volumes containing varying concentration of inhibitor for 30 min at 30 °C with shaking. Reactions were initiated by adding 4 μg of baterial expressed GST-IκB[5-55] and 25 μM MgATP containing 5 μCi of γ^{32}P-ATP for 30 min at 30 °C, then quenched with SDS sample buffer and quantitated by 10% SDS-PAGE gel and phosphorimaging.