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Bicyclic Cyclohexenones as Inhibitors of NF-κB Signaling

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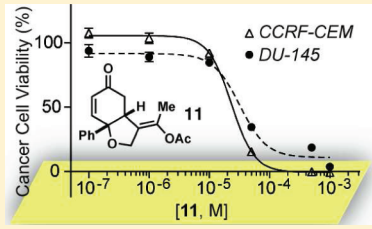
Supporting Information

ABSTRACT: A series of structurally simplified cryptocaryone analogues were synthesized by a facile Pd-catalyzed acetoxylation of alkyne-tethered cyclohexadienones and evaluated as inhibitors of NF-κB signaling. Compounds **10** and **11** were found to possess low micromolar inhibitory properties toward induced NF-κB activity by blocking p50/p65 nuclear protein through a covalent inhibition mechanism. Both compounds were able to inhibit NF-κB-induced IL-8 expression and exhibited antiproliferative activity against two model cancer cell lines. These analogues constitute a promising new scaffold for the development of novel NF-κB inhibitors and anticancer agents.



KEYWORDS: *Natural product analogues, Pd-catalyzed acetoxylation, NF-κB inhibitors, anticancer agents*

Transient induction of the nuclear factor-κB (NF-κB) transcription factors is an essential step in the immune response to pathogens, resulting in the expression of genes associated with cellular proliferation, differentiation, and survival, as well as activating the cellular inflammatory response.[1−3] Equally important is the ability of cells to down-regulate or terminate this activity.[2] Constitutive NF-κB activation has been observed in a spectrum of human cancers, such as acute and chronic myeloid leukemias, prostate, breast, lung, and brain cancers.[4] Chronic inflammation resulting from constitutive NF-κB activation has been strongly implicated in the carcinogenesis of tissues from these organ sites.[5−8] Consequently, the NF-κB signaling pathway has become an important therapeutic target for developing the next-generation of small molecule anticancer agents.[9]

There have been numerous efforts aimed at identifying natural products with activity against the NF-κB pathway.[10−13] Many of these natural products contain reactive moieties (e.g., enones) that can capture nucleophiles and inhibit signaling through a covalent mechanism.[14,15] Drugs that function through a covalent mechanism are widely used in the pharmaceutical industry, and at least 39 FDA-approved medicines can be classified as "covalent drugs".[16] One recently identified natural product NF-κB inhibitor with a reactive enone moiety is cryptocaryone (**1**).[17−19] Researchers at the National Cancer Institute reported that **1** inhibits degradation of the NF-κB repressor protein IκBα in B lymphocytes with high constitutive IKK (IκB kinase complex) activity.[20] Activated IKKs phosphorylate IκB repressor proteins, targeting them for ubiquitination and degradation by the 26S proteosome. Freed p50/p65 (NF-κB) heterodimers can then translocate to the nucleus and activate the expression of NF-κB target genes.[1,8,9] Accordingly, inhibiting the degradation of IκB proteins by small molecules such as cryptocaryone (**1**) is a strategy that can be used to modulate aberrant NF-κB signaling. Due to this promising activity, enantioselective total syntheses of cryptocaryone have been recently described by both Fujioka[21] and Franck.[22]

Our interest in cryptocaryone stems from its structural similarity to a series of compounds we have recently prepared via Pd-catalyzed acetoxylation of alkyne-tethered cyclohexadienones (e.g., **2**→**3**, Figure 1A).[23] While **3** does not have the cinnamoyl side chain present in **1**, we hypothesized that the structurally similar enone moiety that is present in both **1** and **3** may yield similar biological activity profiles for both compounds. Previous studies with sesquiterpene lactones that inhibit NF-κB signaling have revealed covalent Michael adduct formation to exposed cysteine residues on essential NF-κB proteins, thereby eliminating their enzymatic activity and disrupting the signaling pathway.[13] While cryptocaryone's exact protein target(s) and mechanism of binding (e.g., covalent or noncovalent) are unknown at this time, it is possible that it similarly captures accessible cysteine sulfhydryl groups on key protein(s) involved in NF-κB signaling and abolishes their function. If this is the case, then structurally analogous compounds to cryptocaryone (**1**), such as **3**, should also possess NF-κB inhibitory properties. The presence of the fully substituted carbon atom at the γ-position of the enone in **3** does provide some additional steric hindrance that may deter Michael adduct formation in comparison to **1**. Undeterred, we investigated the NF-κB inhibitory activity of several bicyclic enones similar to **3** and we also studied their antiproliferative activities against protypical leukemia and prostate cancer cell lines.

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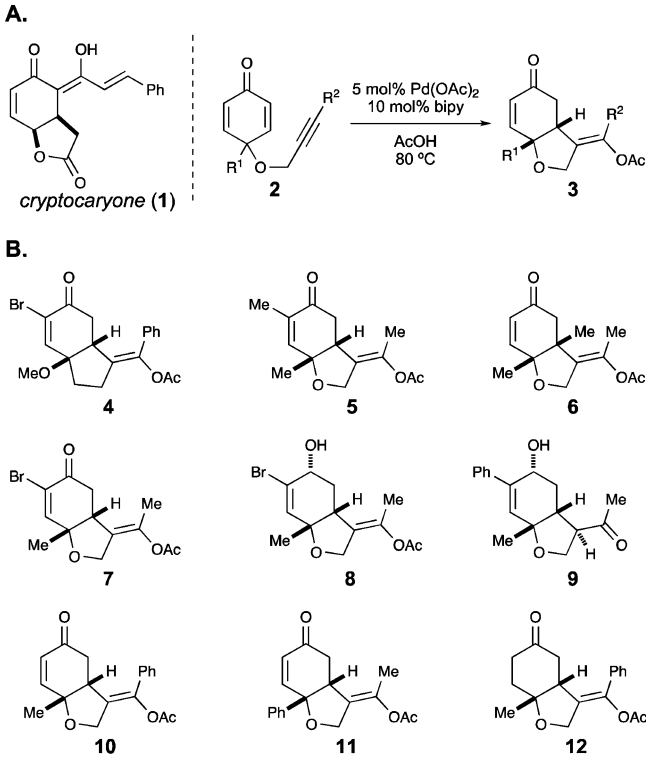

Figure 1. (A) Structure of cryptocaryone (**1**) and methodology for the preparation of the bicyclic enones of interest. (B) Analogues **4**−**12** (racemic) screened for NF-κB inhibitory activity.

The compounds investigated during this study are shown in Figure 1B and were prepared using methods previously described.[23] Compounds **4**−**12** were all prepared as racemic samples. Initial screening of **4**−**11** was carried out by utilizing a standard NF-κB luciferase reporter assay in A549 human lung cancer cells. This cell line bears a stably transfected luciferase reporter construct downstream of six repeats of the consensus NF-κB binding site. Induction of NF-κB signaling is achieved by treatment with TNF-α, and cell permeable small molecules can inhibit this induced activity. We performed a preliminary screen by treating induced cells with 50 μM concentrations of **4**−**11**. This concentration was selected on the basis of cryptocaryone's ability to inhibit the degradation of IκBα at 16 μM.[20] Results from our study found **4** to be poorly soluble under the assay conditions, yielding unreliable values, and compounds **5**−**9** failed to inhibit induced NF-κB activity (Supporting Information Figure S1). Gratifyingly, analogues **10** and **11** both inhibited induced NF-κB activity (Figure 2A and Table 1). At a concentration of 50 μM, both compounds inhibited induced NF-κB activity, with **11** (36% residual NF-κB activity) exhibiting more potent inhibition than **10** (62% residual NF-κB activity) following treatment. The NF-κB inhibitory activity was not attributable to nonspecific cell death, as only 10% (for **10**) and 5% (for **11**) decreases in cell viability were observed following 50 μM treatment with each compound under identical assay conditions (Supporting Information Figure S2). Furthermore, **11** completely inhibited NF-κB activity to noninduced levels at a 100 μM treatment and was more potent than **10** at all concentrations examined.

Encouraged by these results, we evaluated compounds **10** and **11** for their cytotoxicity in two standard human cancer cell lines, DU-145 and CCRF-CEM. DU-145 is a model for hormone-independent prostate cancer, and CCRF-CEM is a

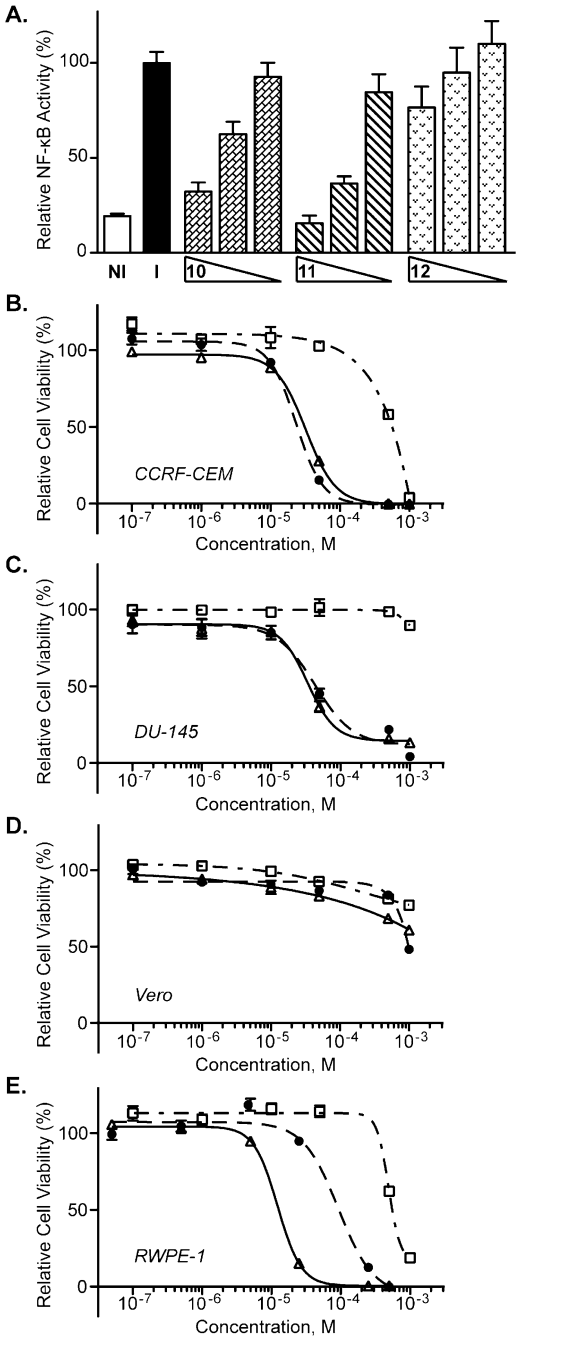

Figure 2. (A) Cellular NF-κB luciferase reporter assay in A549 cells. NI = noninduced control cells, I = cells induced with TNF-α. Compound-treated cells are induced with TNF-α and treated with analogues **10**−**12** at the following concentrations: 100 μM, 50 μM, and 10 μM. (B−E) Cytotoxicity of **10**−**12** against (B) CCRF-CEM, (C) DU-145, (D) Vero, and (E) RWPE-1 cells. Cells were treated with **10** (open triangles), **11** (closed circles), and **12** (open squares) at various concentrations, and cell viability was measured by Alamar Blue staining.

model for childhood T-cell acute lymphoblastic leukemia. DU-145 cells are known to possess constitutive NF-κB activity.[24,25] As shown in Figure 2, **10** and **11** exhibited similar cytotoxicities against DU-145 and CCRF-CEM. Both compounds were slightly more potent against CCRF-CEM cells (IC_{50}: **10**, **11** = 26 μM) than DU-145 cells (IC_{50}: **10** = 34 μM, **11** = 36 μM) in a 48-h cytotoxicity assay (Figure 2B−C and Table 1). The

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Table 1. Results of NF-κB Reporter and Cellular Cytotoxicity Assays Performed with 10−12

	% NF-κB activity[a]			IC_{50} (μM)[b]				
compd	100 μM	50 μM	10 μM	CCRF-CEM	DU-145	Vero	RWPE-1	SI[c]
10	33.9 ± 10.8	61.6 ± 10.7	90.9 ± 13.5	26.4 ± 7.3	33.7 ± 5.8	>500	14.8 ± 2.6	0.44
11	17.3 ± 5.1	36.2 ± 8.2	93.7 ± 15.5	26.4 ± 3.1	35.9 ± 4.8	>500	98.8 ± 15.1	2.75
12	76.1 ± 10.9	94.4 ± 13.1	109.4 ± 12.0	>500	>500	>500	>500	

[a]Percentage (%) NF-κB activity at various doses is shown relative to the induced, DMSO treated control (arbitrarily assigned 100% activity). Mean ± SD values are shown. [b]Cellular cytotoxicity as measured by Alamar Blue staining. IC_{50} values shown are the mean ± SD. [c]The selectivity indices (SI) were calculated by dividing the IC_{50} values obtained for compound-treated RWPE-1 cells by the IC_{50} values obtained for compound-treated DU-145 cells.

observed IC_{50} values for **10** and **11** against DU-145 are approximately 15-fold higher than the reported IC_{50} value for cryptocaryone (IC_{50} = 2.3 μM) in the same cell line.[26]

We next evaluated whether **10** and **11** may exhibit any selectivity toward cancerous versus noncancerous cells. Recently, the enone-containing natural product piperlongumine has been found to possess remarkable selectivity for promoting the death of cancer cells versus normal cells.[27] To determine if **10** and **11** are similarly selective, we measured the cellular cytotoxicities of both compounds against Vero and RWPE-1 cells (Figure 2D-E and Table 1). Vero is a noncancerous kidney epithelial cell line derived from the African green monkey and is widely utilized for screening bioactive candidate molecules for toxicity toward normal cells.[28,29] To our delight, both **10** and **11** exhibited no appreciable toxicity toward Vero cells (IC_{50} values > 500 μM). We then screened both molecules for toxicity against RWPE-1, which is an immortalized human prostate epithelial cell line derived from noncancerous prostate tissue.[30−32] Since **10** and **11** exhibited reasonable potency against DU-145 prostate cancer cells, we deemed that measuring their tissue-specific selectivities for targeting cancerous versus noncancerous prostate cells may yield useful information that could inform future development of this class of molecules as prostate cancer therapeutics. Interestingly, **10** was found to be toxic toward RWPE-1 cells (IC_{50} = 14.8 μM) and exhibited no selectivity toward cancerous DU-145 cells versus noncancerous RWPE-1 cells (selectivity index, SI = 0.44). Conversely, **11** was significantly less toxic than **10** toward normal RWPE-1 cells (IC_{50} = 98.8 μM) and had modest selectivity toward the cancerous DU-145 cell line (SI = 2.75). Consequently, **11** may represent a better lead molecule for future development than constitutional isomer **10**.

To determine if **10** and **11** function through a covalent mechanism of inhibition, the enone moiety of compound **10** was reduced to produce the respective cyclohexanone **12** (Figure 1). If covalent capture of proteins is the mechanism of biological activity for this class of molecules, then **12** should exhibit diminished NK-κB inhibitory activity and be significantly less cytotoxic to cancer cells than enone-containing molecules **10** and **11**. Utilizing the NK-κB luciferase reporter assay, we found that **12** only reduced NF-κB activity by 24% at the highest concentration tested (100 μM), which is a ∼4-fold decrease in potency compared to the case of **11** (Figure 2A and Table 1). Furthermore, **12** exhibited no appreciable toxicity toward any of the cell lines tested (IC_{50} values could not be accurately calculated due to lack of potency). Therefore, the presence of the enone on compounds **10** and **11** is required for biological potency.

We further characterized the ability of compounds of this class to capture cellular nucleophiles by performing a recently described, NMR-based thiol reactivity assay of small molecules.[33] A solution of **10** in DMSO-d_6 was dissolved in aqueous (D_2O) phosphate buffered saline (PBS), and the vinyl protons were observed by ^{1}H NMR (Figure 3B). Addition of two molar equivalents of cysteamine resulted in the disappearance of the doublets at 6.06 ppm (H_b of **10**) and 6.75 ppm (H_a of **10**) within 5 min, suggesting Michael addition of the cysteamine thiol to the enone of **10** and formation of adduct **13** (Figure 3A). To verify the formation of **13**, the cysteamine reaction was performed again (in H_2O) and the reaction products were analyzed by HPLC (Figure 3C). Analysis of the major reaction product by mass spectrometry confirmed the identity of newly

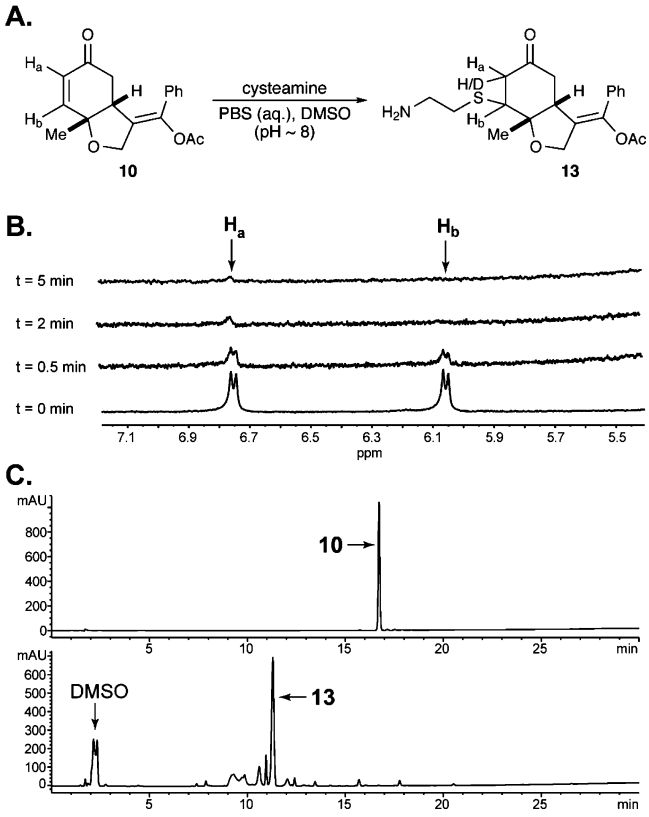

Figure 3. (A) Analogue **10** forms Michael adduct **13** upon reaction with cysteamine in aqueous buffer. The reaction shown in panel A was monitored by ^{1}H NMR spectroscopy and analytical HPLC. (B) ^{1}H NMR analysis of vinyl protons H_a and H_b from **10** before (t = 0 min) and after addition of cysteamine. Reaction was judged to be nearly complete after 5 min. (C) HPLC analysis of **10** before (top chromatogram) and after addition of cysteamine (t = 10 min, bottom chromatogram). Adduct **13** was characterized by mass spectrometry. H/D for **13** is dictated by whether the reaction is performed in D_2O (for NMR analysis, panel B) or H_2O (for HPLC analysis, panel C).

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formed adduct **13**. Together with the biological data for cyclohexanone **12**, these results show that **10** and **11** can capture cellular nucleophiles through their electrophilic enones and that reactivity is required for their inhibitory activities toward NK-κB signaling and cancer cell growth.

To study the biochemical mechanism of NF-κB inhibition by cryptocaryone analogues, we fractionated NF-κB-induced and compound-treated DU-145 and CCRF-CEM cells and then immunoblotted for p65 protein in the nuclear lysate (Figure 4A and Supporting Information Figure S3). Decreased p50/p65 nuclear translocation is expected if **10** and **11** inhibit the degradation of IκB repressor proteins, whereas no change in nuclear p65 levels should be observed if **10** and **11** modulate NF-κB by directly targeting the p50 or p65 protein (e.g., alkylation of NF-κB proteins by the small molecule abolishes DNA-binding capability). Both **10** and **11** completely inhibited nuclear translocation of p65 at a 100 μM treatment in NF-κB-induced DU-145 (Figure 4A) and CCRF-CEM (Supporting Information Figure S3) cell lines. Partial inhibition of p65 nuclear translocation was observed at a 25 μM treatment of **10** and **11** in both cell lines. Immunoblotting of our prepared nuclear fractions for α-tubulin, an abundant cytosolic protein, revealed no detectable signal, which verifies that our nuclear p65 lysate samples are not contaminated with cytosolic p65 (Supporting Information Figure S3). On the basis of these results, we can conclude that cryptocaryone analogues such as **10** and **11** modulate NF-κB activity by inhibiting IκB degradation and p50/p65 heterodimer translocation to the nucleus.

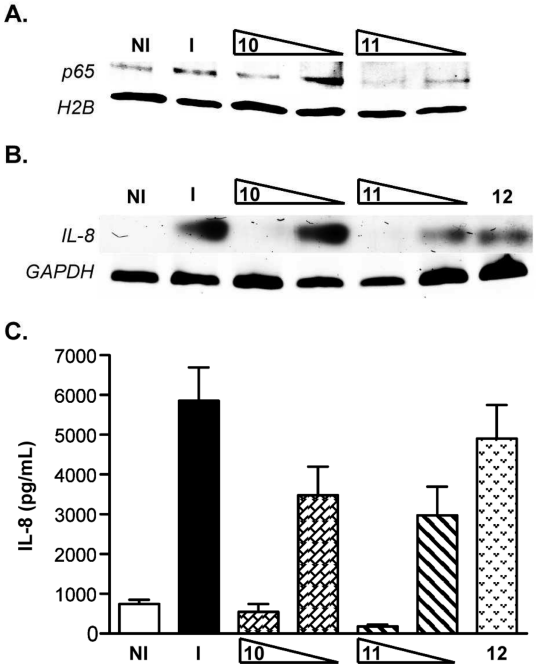

Figure 4. (A) Western blot analysis of p65 and histone H2B proteins from nuclear lysate fractions of compound-treated DU-145 cells. Cells were dosed with either vehicle control (DMSO; NI and I lanes) or analogues **10** or **11** (100 μM and 25 μM), followed by induction of the NF-κB pathway with TNF-α. (B) RT-PCR analysis of IL-8 and GAPDH mRNA from DU-145 cells. (C) Secreted IL-8 protein levels as measured by ELISA. Data shown is mean ± SEM. (B–C) Cells were dosed with either vehicle control (DMSO; NI and I lanes) or analogues **10** or **11** (100 μM and 33 μM) or **12** (100 μM), followed by induction of the NF-κB pathway with TNF-α. (A–C) NI = noninduced cells; I = induced cells.

To further evaluate the ability of our molecules to regulate NF-κB activities in cells, we tested their ability to inhibit the activation of IL-8, a well-known proinflammatory regulator. IL-8 is a chemokine whose gene expression is activated by NF-κB binding to the IL-8 promoter.[34−36] DU-145 cells were treated with **10**–**12**, and NF-κB was induced by addition of TNF-α. Secreted IL-8 levels in cell media were measured by ELISA. Additionally, we further correlated IL-8 protein levels to IL-8 mRNA levels by semiquantitative (traditional) RT-PCR analysis. As expected, analysis of IL-8 mRNA and protein levels revealed significant increases in both following NF-κB induction with TNF-α (Figure 4B–C). Compound **11** was found to inhibit the production of IL-8 mRNA to visibly lower levels than that of **10** at the lowest concentration tested (33 μM). The changes in mRNA levels observed with **10** and **11** were mirrored in the amount of secreted IL-8 protein measured. Compound **10** abolished IL-8 production to noninduced levels, while compound **11** depleted IL-8 protein levels to nearly undetectable levels at the highest concentration tested. Additionally, control compound **12** at 100 μM had an insignificant effect (4,900 pg/mL) on IL-8 levels as compared to **10** and **11** at a 33 μM dose (3,480 pg/mL and 2,970 pg/mL, respectively). These results further support our findings that the formation of Michael adducts is vital to the mechanism of NF-κB pathway inhibition for bicyclic cyclohexenones **10** and **11** and establish the anti-inflammatory features of molecules of this class.

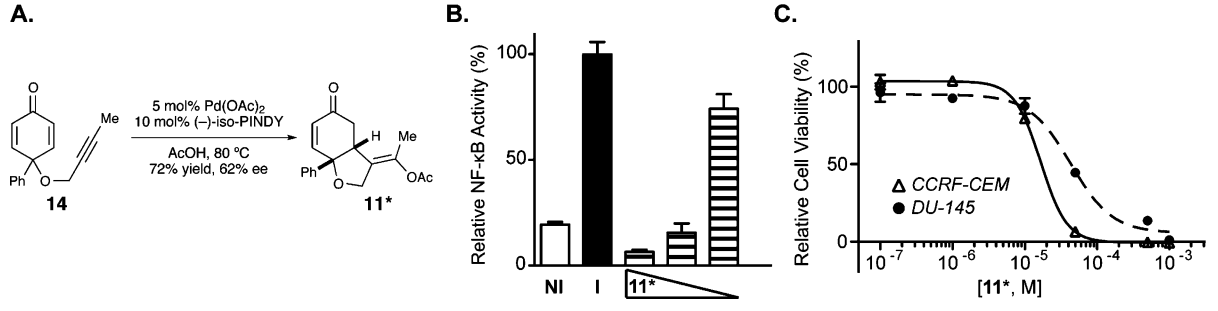

Figure 5. Enhanced potency of enantioenriched **11***. (A) Synthesis of **11***. (B) Cellular NF-κB luciferase reporter assay in A549 cells. NI = noninduced control wells; I = cells induced with TNF-α. Compound-treated cells are induced with TNF-α and treated with analogue **11*** at the following concentrations: 100 μM, 50 μM, and 10 μM. (C) Cytotoxicity of **11*** against CCRF-CEM (open triangles) and DU-145 human prostate cancer cells (closed circles). Cell viability was measured by Alamar Blue staining.

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With another goal of beginning to establish the stereoisomeric preferences of these compounds, we carried out the enantioselective reaction shown in Figure 5. To our delight, the use of the chiral bipyridine ligand (−)-iso-PINDY[37] allowed us to produce enone **11*** in 63% ee from precursor **14**, with the major enantiomer being the one shown (Figure 5A). To determine if enantioenriched **11** (which we have denoted as **11***) possesses enhanced biological potency compared with racemic **11**, we repeated the NF-κB reporter assay and cancer cell cytotoxicity experiments with **11***. Interestingly, **11*** completely abolished induced NF-κB signaling to noninduced levels (Figure 5B), resulting in 18% relative NF-κB activity at 50 μM. This constitutes a 2-fold enhancement in potency compared with the case of racemic **11**. An enhancement in potency with **11*** versus racemic **11** was observed in cytotoxicity assays (Figure 5C) against CCRF-CEM cells (IC_{50} = 19 μM), whereas **11*** was slightly less active toward DU-145 cells (IC_{50} = 46 μM). Unfortunately, (−)-iso-PINDY has not proven to be generally useful for preparing other bicyclic products with high selectivity; therefore, a more thorough study into this effect will have to wait until a more effective ligand is identified.

In conclusion, we have identified a new class of oxygenated bicyclic enones with activity against the NF-κB signaling pathway. While the cytotoxic and NF-κB inhibitory activities of these compounds are modest, they are sufficiently strong to view these compounds as interesting lead compounds for further development. In addition to establishing a more comprehensive structure−activity relationship, we are working to further clarify the exact molecular target(s) of these compounds. All of these results will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures, spectral characterization of new compounds, and experimental procedures for the biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

All authors have given approval to the final version of the manuscript. R.T-A. and A.M.H. synthesized the compounds for this study. D.A.H., J.K.H., and N.B.S. performed the biological assays. J.K.H., A.M.H., and D.A.H. wrote the manuscript.

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Notes

The authors declare no competing financial interest.

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