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Letter

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Gibberellin JRA-003: A Selective Inhibitor of Nuclear Translocation of IKKα

James R. Annand,[1,2] Andrew R. Henderson,[2] Kyle S. Cole,[3] Aaron J. Maurais,[3] Jorge Becerra,[2] Yejun Liu,[2] Eranthie Weerapana,[3] Angela N. Koehler,[4] Anna K. Mapp,[1,2] and Corinna S. Schindler.[*,1,2]

[1]Department of Chemistry, Willard-Henry-Dow Laboratory, University of Michigan, 930 N University Ave., Ann Arbor, MI 48109

[2]Program in Chemical Biology, University of Michigan, 210 Washtenaw Ave, Ann Arbor, MI 48109

[3]Department of Chemistry, Merkert Center, Boston College, 2609 Beacon St., Chestnut Hill, MA 02467

[4]David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, 500 Main St., Cambridge, MA 02139

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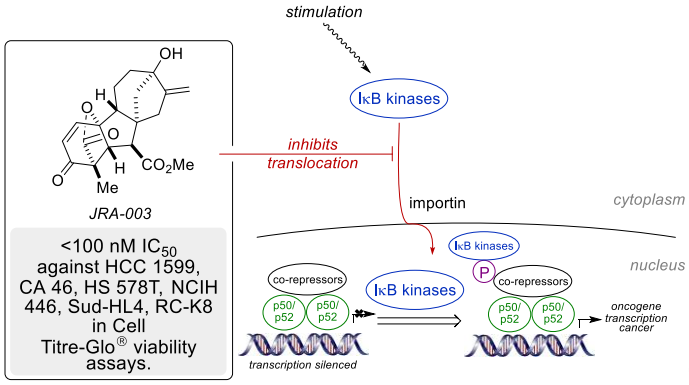
ABSTRACT:



The small molecule gibberellin JRA-003 was identified as an inhibitor of the NF-kB (nuclear kappa-light-chain-enhancer of activated B cells) pathway. Here we find that JRA-003 binds to and significantly inhibits the nuclear translocation of pathway-activating kinases IKKα (IκB kinase alpha) and IKKβ (IκB kinase beta). Analogs of JRA-003 were synthesized and NF-κB-inhibiting gibberellins were found to be cytotoxic in cancer-derived cell lines (HS 578T, HCC 1599, RC-K8, Sud-HL4, CA 46, and NCIH 4466). Not only was JRA-003 identified as the most potent synthetic gibberellin against cancer-derived cell lines, it displayed no cytotoxicity in cells derived from non-cancerous sources (HEK 293T, HS 578BST, HS 888Lu, HS 895Sk, HUVEC). This selectivity suggests a promising approach for the development of new therapeutics.

Chronic inflammation is known to affect all phases of carcinogenesis and targeting inflammation in the tumor microenvironment has been shown to significantly reduce the development, growth and spread of malignancies.[1,2] The NF-κB (nuclear kappa-light-chain-enhancer of activated B cells) signaling pathway is constitutively active in the majority of cancers and considered a critical link between chronic inflammation and tumorigenesis.[3-5] Specifically, NF-κB dysregulation has been implicated in all stages of tumorigenesis including initiation,[6,7] angiogenesis,[8,9] metastasis,[10,11] and tumor survival.[12-14] Though an important target for anti-cancer therapy, the complex regulation of NF-κB activation currently presents significant challenges for the development of new therapeutics. Consequently, selective inhibitors of the NF-κB pathway hold great potential to improve our current understanding of NF-κB's role in carcinogenesis to ultimately design and advance new cancer therapeutics able to selectively target inflammatory pathways for the prevention and treatment of malignancies.[15,16]

NF-κB activity is tightly regulated in healthy cells. Transcriptionally active subunits of NF-κB are bound to inhibitory protein subunits which are phosphorylated by activating kinases (IKKα and IKKβ) and subsequently proteolytically degraded by ubiquitin dependent proteases before active NF-κB can be translocated to the nucleus (Fig. 1).[17] While inhibition of the activating kinases was shown to decrease NF-κB signaling in cellular models, these strategies were unsuccessful in the production of viable therapeutics.[18,19] Specifically, complete inhibition of canonical NF-κB signaling via IKKβ inhibition has been associated with systemic toxicity *in vivo*.[20]

Our laboratories have an active interest in identifying new strategies for NF-κB inhibition relying on small molecule inhibitors. Specifically, recent work by one of our groups showed that allogibberic acid (**2**) and gibberellic acid (**3**) selectively bind NF-κB, specifically p50, and inhibit the NF-κB pathway without inhibiting

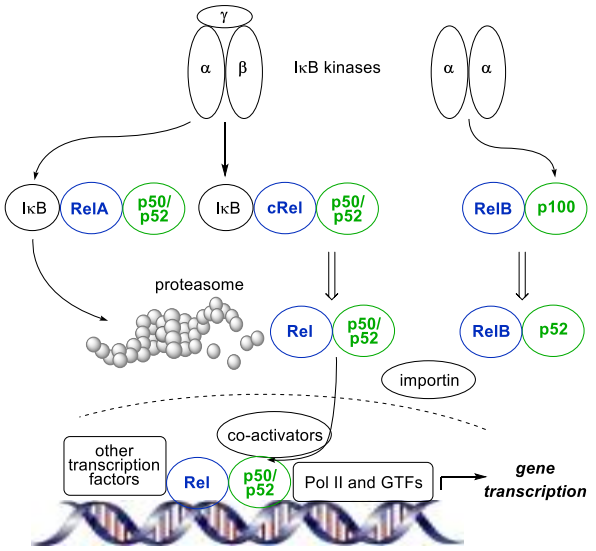


Figure 1. The NF-κB pathway is regulated by inhibitory complexes that keep inactive NF-κB localized in the cytoplasm. Upon phosphorylation by IκB kinases, and proteolytic degradation of inhibitory subunits, active NF-κB is imported into the nucleus to bind to DNA and promote gene expression.

the activation and translocation of NF-κB (Fig. 2).[21] In related studies aimed towards the synthesis of pharbinilic acid (JRA-008), we identified JRA-003 as an active inhibitor of the NF-κB pathway.[22] Herein we report the identification of IKKα and IKKβ as protein targets of JRA-003. Additionally, we show that treatment with JRA-003 significantly inhibits the nuclear translocation of IKKα and IKKβ. We also report the synthesis and evaluation of analogs of JRA-003 as inhibitors of the NF-κB pathway as well as inhibitors of cancer cell viability. Specifically, our studies show that JRA-003 is more than 500-fold selective towards inhibition of lymphoma and breast cancer-derived cell lines than healthy fibroblast derived cell types.

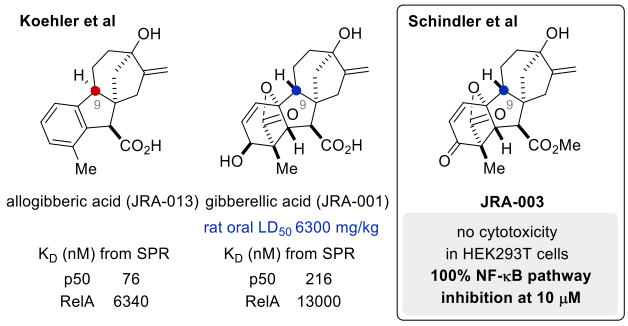


Figure 2. Previously reported modulators of the NF-κB pathway related to the gibberellin family of natural products.[23,24]

JRA-003 is an effective inhibitor of canonical NF-κB signaling upon pathway stimulation by either IL-1β (6.0 μM) or TNFα (2.6 μM) (see Supplemental Information, Fig. S1). To gain more direct insight into the mechanism of action of JRA-003 pull down experiments were performed with the alkyne-tagged analog of JRA-003 (JRA-031) (Fig. 3A). The experiments were conducted with pretreatment of cells with either JRA-003 or the negative control JRA-002 followed by treatment with JRA-031; enrichment was important as many components of the NF-kB pathway are present in low copy numbers.[25] Under these conditions, it is expected that upon pretreatment with JRA-003, but not with JRA-002 or DMSO, specific targets of JRA-003 would be competed away from interacting with the probe molecule, JRA-031. The results obtained are consistent with IKKα and IKKβ as specific targets of JRA-003. Additionally, proteome wide stable isotopic labeling with amino acids in cell culture (SILAC) experiments were performed and only eleven other proteins were found to be targets of JRA-003, none of which are known to be modulators of the NF-κB pathway nor are they known oncogenes (see Supporting Information, Fig. S2). Consistent with these results, direct pulldown of IKKα from IL-1β stimulated HEK 293T cells was observed upon treatment with JRA-031 but not inactive analog JRA-032 (Fig. 3B). Additionally, no loss of binding was observed even with rigorous washing, suggesting that JRA-031 is an irreversible covalent binder of IKK.

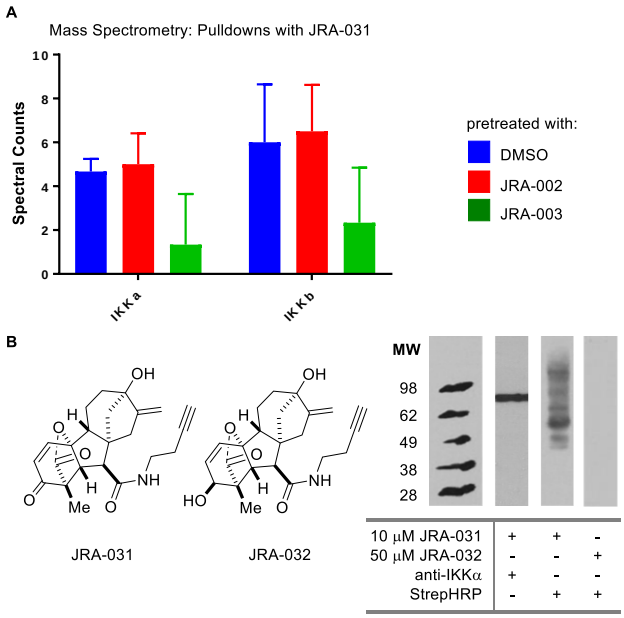


Figure 3. Pulldown experiments using molecular probe JRA-031 and inactive analog JRA-032. **A**. Pulldowns analyzed by mass spectroscopy in TNFα stimulated HEK 293T cells. n = 3 (biological replicates) **B**. Pulldowns analyzed by Western blotting in IL-1β stimulated HeLa cells.

Next, we investigated the localization of NF-κB family members. As IKK kinases are responsible for phosphorylating and degrading IκB, leading to the nuclear translocation of transcriptionally active NF-kB subunits, RelA, RelB, and c-Rel, we anticipated that IKK inhibitors would inhibit the nuclear translocation

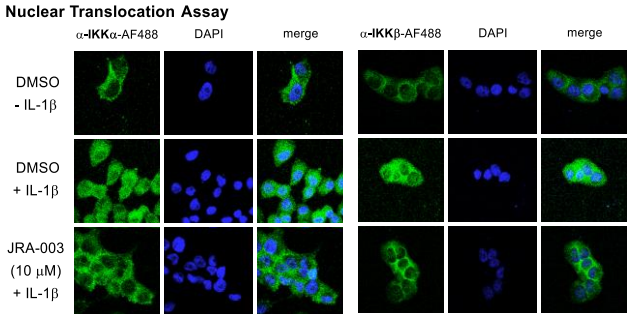


Figure 4. Immunohistochemical staining and confocal microscopy of HeLa cells. Cells were pretreated with DMSO or JRA-003 before stimulation by IL-1β or treatment with vehicle. AF488 tagged antibodies were used to image NF-κB pathway members and DAPI was employed as a nuclear stain.

of Rel.[17] First, we analyzed the cellular compartmentalization of NF-κB family members by immunohistochemical staining and confocal microscopy in HeLa cells that were treated with either

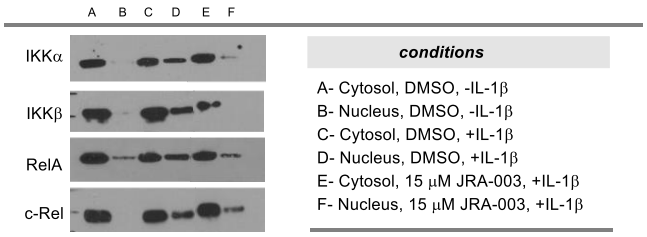


Figure 5. Western blotting analysis in HEK 293T cellular cytosolic and nuclear fractions. Cells were pretreated with vehicle or JRA-003 before stimulation by IL-1β treatment with vehicles. The cells were then lysed, and the nuclei were separated for independent analysis.

DMSO or JRA-003 for four hours followed by one hour of pathway stimulation with IL-1β or treatment with vehicle (Fig. 4, see Supporting Information, Fig. S1). Additionally, we further corroborated these data by Western blotting in HEK 293T cytosolic and nuclear fractions in cells that had been similarly pre-treated with DMSO or JRA-003 prior to stimulation with IL-1β or treatment with vehicle (Fig. 5). Significant reduction of nuclear IKKα and IKKβ was observed in these nuclear translocation assays. Nuclear IK kinases are known to have a myriad of targets including co-transcriptional mediators and histones. In particular, the nuclear activity of IKKα has been connected to an upregulation of NF-κB signaling[26] and has been linked to cell cycle regulation and survival in colorectal,[27,28] breast,[29,30] pancreatic,[31] gastric,[32] osteo-sarcoma,[33] and prostate cancers.[34]

These results prompted us to synthesize a small library of gibberellin and allogibberic acid analogs based on a synthetic strategy we had previously developed towards the synthesis of pharbinilic acid,[24] with the goal of identifying structural features necessary for activity. We initially evaluated this compound library in Luciferase reporter gene assays in commercially available HEK 293T cell lines in order to identify the structural features required to inhibit the NF-κB pathway (Fig. 6). JRA-003 was found to be among the most potent against the NF-κB pathway both upon pathway stimulation by TNFα as well as IL-1β (Fig. 6, see Supplemental Information, Fig. S3). Among the active analogs, the stereochemical configuration of H-9, which is historically challenging to control,[24,37-40] plays a significant role in the activity of NF-κB inhibiting gibberellins (Fig. 6, JRA-022 vs. JRA-019). Additionally, only gibberellins bearing electrophilic functionalities were found to be active in the NF-κB pathway. Experiments to determine the contribution of electrophile reactivity are ongoing (See Supplemental Information, Fig. S4).

In ensuing efforts, all gibberellin analogs were subjected to high throughput evaluation (HTE) upon their ability to inhibit the growth of 22 different cell lines derived from cancerous and non-cancerous sources (Table 1 and Supporting Information Table S1). Interestingly, the gibberellin analogs capable of modulating the NF-κB pathway were also found to be active in the HTE Cell Titer Glo® viability assay while non-NF-κB inhibiting gibberellins were found to be inactive even at the highest concentrations tested. We found that highly electrophilic gibberellins, namely JRA-019, JRA-022, and JRA-026, were broadly cytotoxic with single digit micromolar EC_{50}s across several cell lines and significant cytotoxicity at the highest concentrations tested. In contrast, JRA-003 showed unique potency, with EC_{50}s <100 nM against breast cancer derived cell lines (HS 578T, HCC1599), lymphoma derived cell lines (RC-K8, Sud-HL4, CA 46) and a small cell lunch cancer derived cell line (NCIH 446). Importantly, JRA-003 also displayed high selectivity with a >10 μM EC_{50} against all non-cancer derived cell lines as well as several non-inflammatory cancer cell types. Together, this demonstrates that JRA-003 provides more than 500-fold selectivity against inflammatory cancer *in vitro*.

Table 1. High throughput evaluation of synthetic gibberellin analogs.

compound	Cell Titre Glo® EC_{50} (nM)											
	HEK 293T embrionic kidney	**HS 578BST** fibroblast	**HS 888Lu** fibroblast	**HS 895Sk** fibroblast	**PSN-1** pancreatic adenocarcinoma	**HS 895.T** melanoma	**HS 578T** breast carcinoma	**HCC 1599** breast carcinoma	**RC-K8** lymphoma	**Sud-HL4** lymphoma	**CA 46** Burkitt's lymphoma	**NCIH 446** small cell lung cancer
JRA-001	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
JRA-002	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
JRA-003	N.S.	N.S.	N.S.	N.S.	N.S.	9200	<40	150	<40	<40	<40	61
JRA-004	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
JRA-005	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
JRA-006	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
JRA-007	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
JRA-008	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
JRA-009	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
JRA-010	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
JRA-011	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
JRA-012	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
JRA-013	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
JRA-014	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
JRA-015	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
JRA-017	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
JRA-018	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
JRA-019	N.S.	N.S.	N.S.	9500	N.S.	N.S.	4800	1100	1900	690	460	2900
JRA-021	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
JRA-022	2900	10,000	N.S.	N.S.	N.S.	N.S.	5200	1400	970	620	2500	2100
JRA-023	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
JRA-026	7100	N.S.	N.S.	N.S.	N.S.	N.S.	5200	830	72	<40	880	<40
JRA-027	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
JRA-028	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
JRA-029	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	5500	4300	3000	3700	5900

Cell Titre Glo® viability assay was conducted by HTE. Data reported as average of n =2. N.S. = Not Significant (IC_{50} >10,000 nM).

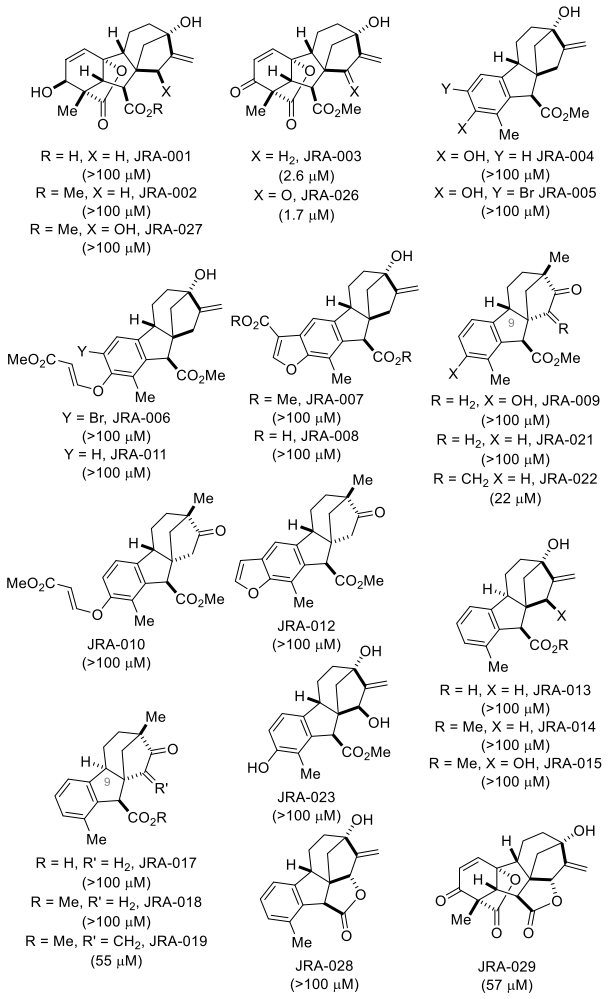


Figure 6. Structures of synthetic gibberellins with their EC_{50} in a commercially available NF-κB driven Luciferase assay in TNFα stimulated HEK 293T cells.

In conclusion, these results suggest that JRA-003 directly acts on IKKα and ultimately prevents it from entering the nucleus, representing a new approach toward inhibition of the NF-κB pathway. Further efforts to identify a specific site and manner of binding are ongoing areas of research within our research programs. Both the SILAC proteomics and the HTE cell viability assay suggest that JRA-003 is selective in its biological activity. Finally, the selectivity observed in the cell viability assay demonstrates that small molecules with the ability to affect the localization of IKKα can provide a promising avenue for the discovery and development of new therapeutics.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Supplemental figures and tables, experimental conditions, and synthesis and characterization of all compounds (PDF)

AUTHOR INFORMATION

Corresponding Author

* Tel: 764-763-6853 E-mail: corinnas@umich.edu

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

NF-κB, nuclear kappa-light-chain-enhancer of activated B cells; IKKα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor kinase, alpha; IKKβ, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor kinase, beta; DMSO, dimethyl sulfoxide.

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