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Chemical Biology Strategy Reveals Pathway-Selective Inhibitor of NF-κB Activation Induced by Protein Kinase C

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ABSTRACT Dysregulation of NF-κB activity contributes to many autoimmune and inflammatory diseases. At least nine pathways for NF-κB activation have been identified, most of which converge on the IκB kinases (IKKs). Although IKKs represent logical targets for potential drug discovery, chemical inhibitors of IKKs suppress all known NF-κB activation pathways and thus lack the selectivity required for safe use. A unique NF-κB activation pathway is initiated by protein kinase C (PKC) that is stimulated by antigen receptors and many growth factor receptors. Using a cell-based high-throughput screening (HTS) assay and chemical biology strategy, we identified a 2-aminobenzimidazole compound, CID-2858522, which selectively inhibits the NF-κB pathway induced by PKC, operating downstream of PKC but upstream of IKKβ, without inhibiting other NF-κB activation pathways. In human B cells stimulated through surface immunoglobulin, CID-2858522 inhibited NF-κB DNA-binding activity and expression of endogenous NF-κB-dependent target gene, TRAF1. Altogether, as a selective chemical inhibitor of the NF-κB pathway induced by PKC, CID-2858522 serves as a powerful research tool and may reveal new paths toward therapeutically useful NF-κB inhibitors.

Members of the nuclear factor-κ B (NF-κB) family of transcription factors play crucial roles in the control of many physiological and pathological processes, including host-defense, immune responses, inflammation, and cancer (*1*). In mammals, at least nine pathways leading to NF-κB activation have been elucidated, including (i) a "classical" pathway induced by tumor necrosis factor (TNF) and many TNF-family cytokine receptors, involving degradation of inhibitor of NF-κB-α (IκB-α) and release of p65-50 NF-κB heterodimers (*2*); (ii) an "alternative" pathway activated by selected TNF-family receptors (*e.g.*, CD40, lymphotoxin-β receptor, BAFF receptor) involving p100 NF-κB2 proteolytic processing to generate p52, a preferred heterodimerization partner of NF-κB-family member RelB; (iii) the Toll-like receptor pathway for NF-κB induction, involving TIR domain-containing adapters and IRAK-family protein kinases (*3*); (iv) a pathway activated by exogenous RNA, involving Helicard/Mda5, RIG-I, and mitochondrial protein MAVS, which is of importance for host defenses against viruses (*4*); (v) a DNA-damage pathway involving PIDD, a target of p53 (*5*); (vi) NLR/NOD-family proteins, cytosolic proteins that oligomerize in response to microbial-derived molecules, forming NF-κB-activating protein complexes; (vii) ultraviolet (UV) irradiation and some DNA-damaging drugs, which stimulate NF-κB activation *via* a mechanism involving C-terminal phosphorylation of IκB-α (*6, 7*); (viii) oncogenic fusion proteins composed of portions of cIAP2 and mucosa-associated lymphoid tissue-1 (MALT1), which drive NF-κB activation *via* interactions with TRAF2 and

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TRAF6 (*8*); and (ix) a pathway induced by ligation of B-cell or T-cell antigen receptors, as well as many growth factor receptors, involving a cascade of interacting proteins that includes caspase recruitment domain-containing membrane-associated guanylate kinase protein-1 (CARMA1, Bimp3), Bcl-10, and MALT (Paracaspase), Caspase-8, and other proteins (reviewed in ref *9*). The core event upon which most of these NF-κB activation pathways converge is activation of inhibitor of κB kinases (IKKs), typically composed of a complex of IKK-α, IKK-β, and the scaffold protein, IKK-γ/NEMO (*2*). In all but the "alternative" NF-κB pathway, IKK activation results in phosphorylation of IκB-α, targeting this protein for ubiquitination and proteasome-dependent destruction, thus releasing p65/p50 NF-κB heterodimers from IκB-α in the cytosol and allowing their translocation into the nucleus where they initiate transcription of various target genes.

The NF-κB pathway activated by antigen receptors is critical for acquired (as opposed to innate) immunity, contributing to T- and B-lymphocyte activation, proliferation, survival, and effector functions. Dysregulated NF-κB activation in lymphocytes can contribute to development of autoimmunity, chronic inflammation, and lymphoid malignancy (*9, 10*). The NF-κB activation pathway linked to antigen receptors is initiated by certain PKCs and involves the aforementioned CARMA/Bcl-10/MALT complex. Formation of this complex is stimulated by PKC-mediated phosphorylation of CARMA proteins. Contributions to the PKC-activated NF-κB activation mechanism are also made by Caspase-8, apparently forming heterodimers with c-FLIP and inducing proteolytic processing of c-FLIP (*11*).

In T- and B-cells, this pathway is initiated by protein kinase C (PKC)-θ and PKC-β, respectively, leading ultimately to IKK activation through a mechanism possibly involving lysine 63-linked polyubiquitination of IKK-γ (*12*). In addition to antigen receptors, many growth factor receptors also initiate NF-κB activation *via* stimulation of various PKCs.

Although IKKs represent logical targets for potential drug discovery, chemical inhibitors of IKKs suppress all known NF-κB activation pathways and thus lack the selectivity required to inhibit antigen receptor and growth factor receptor responses without simultaneously interfering with innate immunity and creating broad immunosuppression with considerable risk of infection (*13*). We therefore devised a chemical biology strategy for identification of small molecule chemical probes that selectively inhibit antigen receptor and growth factor receptor-mediated NF-κB activation and describe herein 2-aminobenzimidazole compounds that inhibit at a point between PKCs and IKKs, without blocking other NF-κB activation pathways. These compounds thus provide unique research tools for interrogating the PKC-initiated pathway for NF-κB induction and may represent a starting point for eventually generating pathway-selective drugs with utility for autoimmunity and cancer.

RESULTS AND DISCUSSION

Overview of Screening Strategy and Summary of Results. Our strategy for compound library screening entailed using phorbol ester ([PMA]) and the Ca^{2+}-ionophore ionomycin to achieve PKC activation. For convenience, we used HEK293 epithelial cells, in which it has previously been shown by siRNA-mediated gene silencing and transfection of dominant-negative mutants that PMA/ionomycin-induced NF-κB activation is dependent on CARMA1, Bcl-10, and MALT-1 (*14–17*). HEK293 cells were stably transfected with a luciferase-reporter gene driven by a NF-κB-responsive promoter, and the responsiveness of this integrated promoter to various NF-κB inducing stimuli was confirmed, including PMA/ionomycin and TNF (Supplementary Figure 1). Using these cells, 96- and 384-well plate-based high-throughput screening (HTS) assays were established, with good assay performance characteristics ($Z' > 0.5$) (PubChem AID = 1384) (http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=1384) (http://pubchem.ncbi.nlm.gov/assay/assay.cgi?aid-465&loc=ea_ras). We initially screened 53,280 chemical compounds at an average concentration of 5 μM, of which 519 primary hits were obtained (based on cutoff of 50% inhibition). Of these, 248 were confirmed upon repeated testing (Figure 1). Counter-screening for compounds that inhibit TNFα-induced activation of the reporter gene eliminated 202 compounds, and testing for cytotoxicity of the HEK293 reporter cell line discounted 2 additional compounds, leaving 46 candidates. Fresh stocks of these chemicals were obtained from the vendor, of which 11 showed suppression of PMA-induced NF-κB reporter gene activity. Finally, these 11 candidates were tested in an orthogonal assay in which PMA-induced secretion of interleukin-8 (IL-8) by the HEK293 reporter cell line was measured, thus examining an endogenous NF-κB target gene, leaving only 1 candidate compound,

CID-2858522 (SID-17450324 or ChemBridge-5653914), a substituted 2-aminobenzimidazole (Figure 1; Figure 2, panel A). CID-2858522 also inhibited NF-κB activation induced by another PKC activator, phorbol dibutryate (PDBu), with cellular potency of $\leq$0.1 μM (IC_{50}) using NF-κB reporter gene assays and using assays where secretion of NF-κB-induced cytokine IL-8 was measured (Figure 2, panel E). While inhibiting NF-κB reporter gene activity, CID-2858522 did not interfere with PKC-induced AP-1 or NFAT reporter gene activity in 293 cells (Supplementary Figures 2 and 3), nor did the compound inhibit luciferase (http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=1384) (http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=1269&loc=ea_ras), thus excluding nonspecific activity.

To characterize the selectivity of the hit, CID-2858522 was also tested in seven other NF-κB pathways. CID-2858522 did not inhibit the NF-κB activation induced by overexpression of CD40, CD4, NOD1, NOD2, XIAP/TAB, IAP2/MALT1 or induced by either doxorubicin (an inducer of DNA damage) or retinoic acid (an inducer of RIG-1) (Supplementary Figure 4), confirming the specificity of CID-2858522 for the PKC-initiated pathway.

To further investigate the activity and specificity of CID-2858522, we then tested the compound in other cell lines stimulated by various stimuli. CID-2858522 also slightly but reproducibly inhibited IL-2 production in a Jurkat T-cell line (Figure 3) and consistently suppressed proliferation of mouse B-cell splenocytes (Figure 4) induced by anti-IgM but failed to inhibit the NF-κB induced by lipopolysaccharide (LPS) (IL-6 secretion measured in THP.1 cell cultures), anti-Lymphotoxin-β receptor (NF-κB luciferase measured in HeLa cells), γ-Tri-DAP (IL-8 secretion was measured in MCF-7 cell cultures) and MDP (IL-6 measured in THP.1 cell cultures) (Supplementary Figures 5−8).

Testing >250 analogues of CID-2858522 using the HEK293-NF-κB-luc reporter cell line revealed the fundamental structure−activity relationship (SAR) of this compound, in which various moieties within the compound structure were interrogated, resulting in numerous structurally related analogues that lost all activity or had markedly decreased activity and approximately 10 analogues with comparable or equipotent activity, but no analogues with clearly superior activity (data to be published elsewhere).

CID-2858522 Potently and Selectively Inhibits PKC-stimulated NF-κB activity. Compound CID-2858522 is a substituted 2-aminobenzimidazole (Figure 2, panel A). Representative data are provided in Figure 2, contrasting the activity of CID-2858522 with another compound derived from library screening, CID-2998237 (Figure 2, panel A). In the HEK293 cell line used for primary screening, CID-2858522 suppressed NF-κB reporter gene activity in a concentration-dependent manner, with IC_{50} of ~70 nM and with maximum inhibition achieved at 0.25−0.5 μM (Figure 2, panel B). In contrast, this compound did not inhibit TNF-induced NF-κB-reporter gene activity at concentrations as high as 4 μM, thus demonstrating selectivity for the NF-κB pathway activated by PMA/ionomycin (Figure 2, panel B). Cell viability assays indicated that CID-2858522 was not toxic to HEK293 cells at concentrations $\leq$8 μM. Moreover, CID-2858522 also potently inhibited PMA/ionomycin-induced NF-κB reporter gene activity in transient transfection assays, where the NF-κB-luciferase reporter gene activity was measured from an episomal plasmid (not shown), thus excluding an impact of the chromosomal integration site on measured activity. Similar results were obtained with another "hit" compound CID-2998237, though this compound was less potent at suppressing PMA/ionomycin-induced reporter gene activity and had also modest inhibition of TNF-induced NF-κB activity (Figure 2, panel C).

The orthogonal assay for PMA/ionomycin-stimulated NF-κB reporter gene activity in the HEK293 engineered cell line used for primary screening proved to be a key differentiator of true-positive versus false-positive compounds and demonstrated the importance of not relying exclusively on luciferase-based reporter genes. Figure 2, panel D compares CID-2858522 with a false-positive compound, CID-2998237, showing that CID-2858522 inhibited PMA/ionomycin-stimulated IL-8 production in a concentration-dependent manner, with IC_{50} of <0.1 μM and maximum suppression achieved at ~1 μM, whereas CID-2998237 had minimal effect on IL-8 production at concentrations as high as 4 μM. While several compounds derived from library screening demonstrated similar characteristics with respect to suppression of NF-κB reporter-gene activity induced by PMA/ionomycin but not TNF (n = 11 of 114,889 total compounds screened), only CID-2858522 suppressed PMA/ionomycin-induced IL-8 secretion.

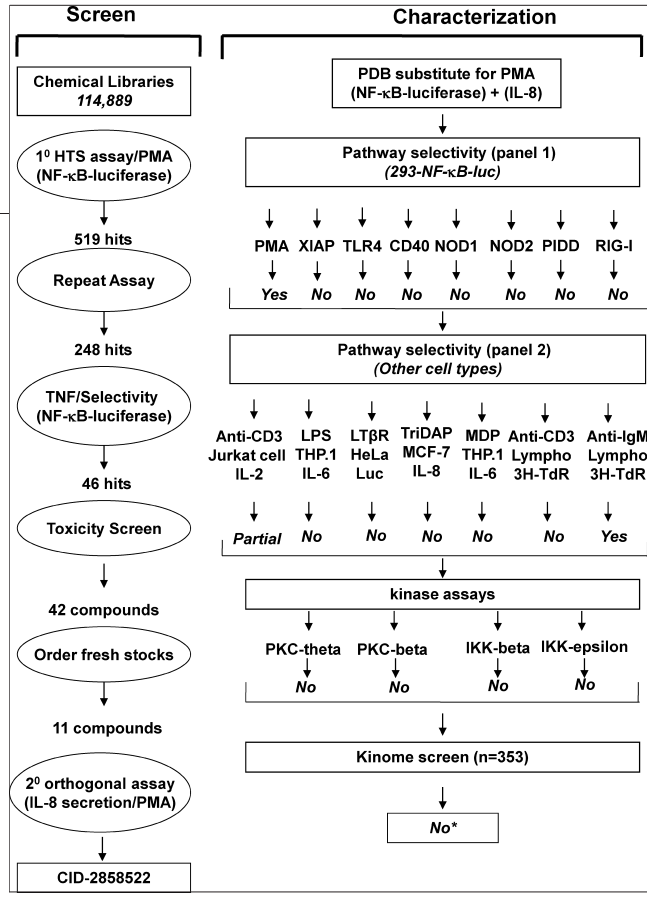

Figure 1. Flowchart of screening and counter-screenings for selective inhibitors of antigen-receptor induced NF-κB pathway. A total of 114,889 compounds were screened. From a screen of 53,280 commercially available compounds (left column), 519 hits were obtained, of which 248 were reconfirmed using the same primary screening assay. Next, the same HEK293-NF-κB reporter gene cell line was stimulated with TNF, leaving 46 compounds that failed to inhibit. Four of these compounds showed ctyotoxic activity, leaving 42 compounds, of which 11 showed activity when fresh stocks were ordered and tested. From these 11 active compounds, one hit, CID-2858522, inhibited PMA/ionomycin-induced production of cytokine IL-8 by the HEK293 cell line (an additional screen of compounds from a 61,609 library provided by the NIH using the same HTS assay resulted in no compounds that fulfilled the desired criteria (http://pubchem.ncbi.nlm.gov/assay/assay.cgi?aid-465&loc=ea_ras)). CID-2858522 was then characterized (right column). PDB was substituted for PMA to confirm suppression of an alternative PKC activator. Pathway selectivity was assessed using panels of cell lines, starting with HEK293 cells in which each of the remaining NF-κB activation pathways was stimulated (see Supplementary Figure 2 for details), showing inhibition only of PMA/ionomycin-induced NF-κB reporter gene activity (panel 1). This was followed by a panel of secondary assays using various cell lines or primary cultured splenocytes (panel 2), measuring various end-points, which included NF-κB luciferase reporter gene activity ("Luc"), cytokine secretion, and [^{3}H]-thymidine (^{3}H-TdR), following stimulation with various inducers of specific upstream activators of NF-κB signaling, including agonists of TCRs (anti-CD3/CD28 for Jurkat, splenocytes), BCRs (anti-IgM for splenocytes), TLRs (LPS for THP.1 monocytes), TNFRs that signal through the "alternative" pathway (LTβR antibody for HeLa cells), and NLRs (γTriDAP for activating NOD1 and muramyl-dipeptide [MDP] for activating NOD2 in McF-7 breast cancer and THP.1 monocytes, respectively). CID-285822 inhibited only anti-CD3/CD28-stimulated and anti-IgM-stimulated splenocytes (see Figures 3 and 4 for details). Various *in vitro* kinase assays were employed, showing no inhibition, followed by a kinome screen using competitive displacement of ATP.

Similar results for CID-2858522 were obtained when phorbol dibutryate (PDB) was substituted for PMA (Figure 2, panel E), thus extending the observations to an alternative PKC-activating phorbol ester. The IC_{50} values for suppression of PDB-induced NF-κB reporter gene activity and PDB-induced IL-8 production by HEK293 cells were ~70 and ~100 nM, respectively.

CID-2858522 also suppressed PMA/ionomycin-stimulated NF-κB DNA-binding activity (Figure 2, panel F), as measured by an immunoassay wherein nuclear NF-κB-family proteins were captured on beads displaying oligonucleotides with NF-κB-binding sites and p65 Rel-A was detected using a specific antibody. Suppression was evident at concentrations as low as 60 nM, with IC_{50} of ~125 nM (Figure 2, panel F). Similar results were obtained by electromobility gel-shift assay (EMSA) (Supplementary Figure 9). PMA/ionomycin-induced p65-RelA DNA-binding activity was also inhibited by the PKC inhibitor bisindolylmaleimide I, used here as a control. CID-2858522 did not block p65-DNA binding activity induced by TNF (data not shown), thus demonstrating pathway selectivity.

CID-2858522 Does Not Inhibit Other NF-κB Pathways. Because NF-κB can be activated by at least 9 known pathways, we next triggered each of these pathways in HEK293 cells by either stimulation with appropriate cytokines, transfection with plasmids, or stimulation with various agents that initiate each NF-κB activation pathway (summarized in Figure 1 [right side]; data shown in Supplementary Figures 4–8). The activity of CID-2858522 was compared with an IKK inhibitor, BMS-345541, as a control, relying on the ability of chemical inhibitors of IKKs to block nearly all NF-κB activation pathways. First, we stimulated the TLR-pathway by transfection with a CD4-TLR4 fusion protein, in which the extracellular domain of CD4 is fused with the transmembrane and cytosolic domain of TLR4, and anti-CD4 antibody (rather than the natural ligand, lipopolysaccharide [LPS]) is used to activate TLR4. TLR4 induced robust NF-κB reporter gene activity (>50-fold increase), which was suppressed by IKK inhibitor BMS-345541 but not by CID-2858522 or by the PKC inhibitor bisindolylmaleimide I. Second, the "alternative" NF-κB pathway was stimulated by overexpressing CD40 in HEK293 cells. CD40-induced NF-κB reporter gene activity was potently suppressed by the IKK inhibitor but not by CID-2858522 nor by the PKC inhibitor. Third, the NLR-dependent NF-κB pathway was stimulated by overex-

pressing NOD1 (NLRC1) or NOD2 (NLRC2) in the HEK293-NFκB-luc cells. NOD1 and NOD2 induced 6- to 7-fold increases in NF-κB-luciferase reporter gene activity, which were inhibited by the IKK inhibitor but not by CID-2858522. Fourth, IAP-initiated pathways for NF-κB activation were induced by transfecting 293-NF-κB-luciferase cells with plasmids encoding either cIAP2/MALT oncoprotein or XIAP plus TAB. Whereas an IKK inhibitor effectively suppressed these IAP-driven pathways, CID-2858522 did not. Note that XIAP has recently been implicated in the NF-κB pathway activated by NOD1 and NOD2 (*18*). Fifth, the DNA-damage-inducible pathway for NF-κB activation was triggered by stimulating HEK293-NF-κB-luc cells with doxorubicin, which induced a ~12-fold increase in NF-κB activity in these cells. Again, the IKK inhibitor but not CID-2858522 suppressed NF-κB activity. Finally, the retinoic acid (RA)-inducible pathway involving RIG-I (*19*) was induced by treating cells with all-*trans*-retinoic acid. RA induced a modest ~3-fold increase in NF-κB activity in HEK293 cells, which was significantly suppressed by the IKK inhibitor but not by CID-2858522 (Supplementary Figure 3). Thus, when taken together with the data showing that the "classical" NF-κB pathway activated by TNF was not inhibited by CID-2858522 (Figure 2), the above results demonstrate that CID-2858522 uniquely suppresses the NF-κB pathway initiated by PKC activators.

CID-2858522 Partially Inhibits TCR-Stimulated IL-2 Production by Jurkat T-Cells. In T-cells, the antigen receptor stimulates several signal transduction pathways that converge on the IL-2 gene promoter, including NF-κB, NF-AT, and AP-1 (reviewed in ref 20). To evaluate the effects of CID-2858522 on TCR-initiated, NF-κB-driven events in lymphocytes, we employed Jurkat T-leukemia cells, which have been utilized extensively as a model for studying TCR-signaling leading to IL-2 gene expression (*21*). For these experiments, Jurkat cells were stimulated with either anti-CD3 (to activate the TCR complex) and anti-CD28 (co-stimulator) or with PMA/ionomycin, in the presence or absence of CID-2858522, IKK inhibitor, or PKC inhibitor, and then IL-2 production was measured 24 h later in culture supernatants. Both anti-CD3/CD28 and PMA/ionomycin stimulated marked increases in IL-2 production by Jurkat T-cells, with CD3/CD28 more potent than PMA/ionomycin (Figure 3, panel A). The IKK inhibitor partially suppressed PMA/ionomycin-induced IL-2 production and potently inhibited (~90% suppression) anti-CD3/CD28-induced IL-2 production at

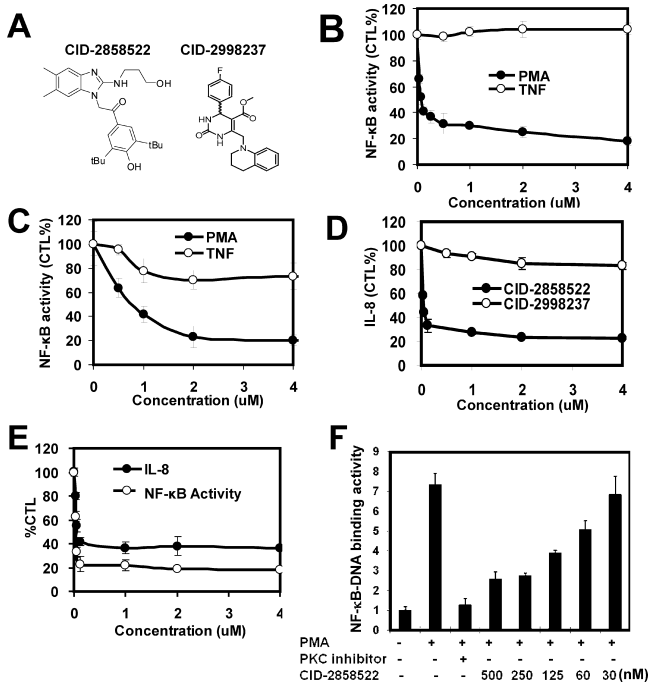

Figure 2. CID-2858522 inhibits NF-κB activation and IL-8 production induced by PKC activators. A) Structures of two hit compounds, CID-2858522 (left) and CID-2998237 (right) are shown. B, C) 293-NF-κB-luc cells were pretreated for 2 h with various concentration of either CID-2858522 (B) or CID-29982387 (C) and then stimulated with TNF (10 ng mL^{-1}) or PMA/ionomycin (10 ng mL^{-1}; 5 ng mL^{-1}) for 16 h. Luciferase activity was measured, and data were expressed as a percentage relative to control treatment with DMSO only (mean ± SD; $n = 3$). D) 293-NF-κB-luc cells were pretreated for 2 h with various concentrations of CID-2858522 or CID-2998237 and then stimulated with PMA/ionomycin for 16 h. IL-8 release into the medium was measured, expressing data as a percentage relative to control cultures treated with DMSO (mean ± SD; $n = 3$). E) 293-NF-κB-luc cells were pretreated with CID-2858522 and then stimulated with PDBu for 16 h. IL-8 production and NF-κB luciferase activity were measured as above. F) 293-NF-κB-luc cells were pretreated for 2 h with various concentrations of CID-2858522 or the PKC inhibitor bisindolylmaleimide I (1 μM) followed by PMA/ionomycin (10 ng mL^{-1} each) for 2 h, and then p65-DNA-binding activity was measured in nuclear extracts (10 μg protein) using an ELISA method, expressing data as fold-increase relative to unstimulated cells (mean ± SD; $n = 3$).

concentrations ≤10 μM (Figure 3, panel B). The PKC inhibitor suppressed IL-2 production by 80−90% in Jurkat cells stimulated with either CD3/CD28 or PMA/ionomycin at concentrations of <0.5 μM (Figure 3, panel C). In contrast, CID-2858522 only partially suppressed IL-2 production by CD3/CD28- and PMA/ionomycin-stimulated Jurkat cells (Figure 3, panel D). The suppres-

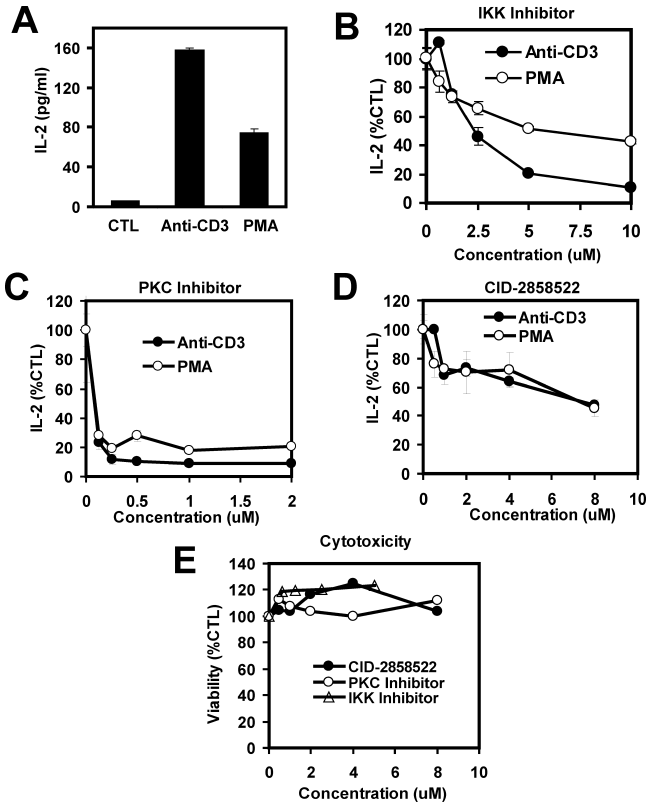

Figure 3. CID-2858522 inhibits IL-2 production induced by anti-CD3/CD28 or PMA/Ionomycin in Jurkat cells. A) Jurkat T-cells were cultured with anti-CD3/anti-CD28/antimouse IgG (6 μg mL^{-1} each) or PMA/ionomysin (10 ng mL^{-1}; 5 ng mL^{-1}) for 24 h, and then IL-2 production in the medium was measured (mean ± SD; *n* = 3). B–D) Jurkat T-cells were pretreated for 2 h with IKK inhibitor, BMS-335541 (B), PKC inhibitor, bisindolylmaleimide I (C), or CID-2858522 (D) and then stimulated with anti-CD3/anti-CD28/antimouse IgG (6 μg mL^{-1} each) or PMA/ionomycin (10 ng mL^{-1}; 5 ng mL^{-1}) for 24 h. IL-2 production in the medium, expressing data as a percentage of control (mean ± SD; *n* = 3). E) Jurkat cells were cultured with CID-2858522, PKC inhibitor, or IKK inhibitor for 24 h, and then cell viability was determined on the basis of ATP levels, expressing data as a percentage relative to control cells cultured with DMSO only (mean ± SD; *n* = 3).

sion of IL-2 production by Jurkat cells by CID-2858522, IKK inhibitor, or PKC inhibitor was not due to cytotoxicity (Figure 3, panel E).

We also assessed effects of CID-2858522 on cytokine production induced by other stimuli. CID-2858522 did not suppress IL-6 production by THP.1 monocytes stimulated with TLR4 agonist LPS, IL-8 production stimulated by NOD1 agonist γ-TriDAP in MCF7 breast cancer cells, or NF-κB luciferase activity induced by anti-lymphotoxin-β receptor antibody in HeLa cells (Supplementary Figures 5–8), all of which involve other NF-κB activation pathways.

CID-2858522 Inhibits Mouse Primary B-Cell Proliferation Induced by Anti-IgM. NF-κB plays roles in antigen receptor-driven lymphocyte proliferation (*10*). We therefore tested the effect of CID-2858522 on mouse lymphocyte proliferation induced by anti-CD3/CD28 or anti-IgM antibodies, measuring [^{3}H]-thymidine incorporation. Anti-CD3/CD28 and anti-IgM significantly induced ~80-fold and ~8-fold increases, respectively, in DNA synthesis in cultures of murine lymphocytes (Figure 4, panel A). The IKK and PKC inhibitors suppressed lymphocyte proliferation in a concentration-dependent manner, inhibiting B-cells (anti-IgM) (IC_{50} ~2 μM for IKK inhibitor; ~0.2 μM for PKC inhibitor) more potently than T-cells (anti-CD3/CD28) (IC_{50} ~4 μM for IKK inhibitor; ~1.5 μM for PKC inhibitor) (Figure 4, panels B and C). In contrast, CID-2858288 inhibited anti-IgM-induced lymphocyte proliferation in a concentration-dependent manner, with an IC_{50} of ~2 μM, while having minimal effect on anti-CD3/CD28, suggesting that the NF-κB inhibitory mechanism of CID-2858288 was more prominent in B-cells than in T-cells. However, because CD3/CD28 stimulation induced stronger proliferative responses than anti-IgM, we cannot exclude a quantitative rather than qualitative explanation for this observation.

To further evaluate the effect of CID-2858522 on antigen receptor signaling in lymphocytes, we examined its effect on leukemia B-cells from patients with chronic lymphocyte leukemia (CLL). Stimulation with biotinylated anti-IgM (cross-linked using streptavidin) resulted in expression of TRAF1 (Figure 4, panel E), an endogenous target of NF-κB (*22*). Adding CID-285252 to cultures of anti-IgM-stimulated CLL cells inhibited TRAF1 induction, in 3 of 3 cases measured at 24 h after stimulation (Figure 4, panel E and Supplementary Figure 10). Levels of actin and TRAF6, which are not regulated by NF-κB, did not show any change (Supplementary Figure 10), thus showing selectivity and confirming equivalent protein loading. As a control, CLL cells were also treated by a structurally related but inactive 2-aminobenzimidazole analogue, MLS-0292123, which did not inhibit PMA/ionomycin-induced NF-κB luciferase activation or IL-8 production in HEK293 cells (Supplementary Figure 11), showing that MLS-0292123 did not inhibit TRAF1 expression (Figure 4, panels E and F and

Supplementary Data). As a positive control, CLL cells were also treated with a PKC inhibitor, bisindolylmaleimide I, which also inhibited TRAF1 expression. The effects of CID-2858522 on the capacity of anti-IgM to induce CLL-cell expression of TRAF1 were not due to cytotoxicity during the time frame analyzed, as confirmed by measuring ATP levels (data not shown). In addition to the indirect evidence of NF-κB activation using TRAF1 expression, CID-2858522 also showed direct suppression on p65-DNA binding activity in human CLL cells induced by anti-IgM, while its inactive analogue, MLS-0292123 did not (Figure 4, panel F). Thus, CID-2858522 inhibited antigen receptor-stimulated NF-κB activation in primary leukemia B cells.

CID-2858522 Is Not a Potent Protein Kinase C Inhibitor. Protein kinases play critical roles in NF-κB activation. PKCs are proximal kinases in the NF-κB pathways activated by PMA/ionomycin and by T-cell and B-cell antigen receptors, while the IKKs are distal kinases operating in the terminal segments of these and most other NF-κB activation pathways (*23*). We therefore tested whether CID-2858522 inhibits members of these kinase families using *in vitro* kinase assays. For these experiments, we tested PKC-β and PKC-θ (the PKC family members implicated in TCR/BCR signaling) and IKK-β (a component of the IKK complex) and IKK-ε (not shown). At concentrations up to 8 μM, CID-2858522 failed to suppress these kinases, while known PKC and IKK inhibitors and the broad-spectrum kinase inhibitor staurosporin (STS) afforded potent inhibition (Supplementary Figure 12). Thus, CID-2858522 did not directly inhibit PKC-β, PKC-θ, or IKK-β.

Recognizing that *in vitro* kinase assays do not always detect the activity of chemical inhibitors, we also explored the effect of CID-2858522 on endogenous PKC activity by analyzing effects on cellular PKC substrates. For these experiments, 293 cells were preincubated with CID-2858522 or a PKC inhibitor, and then cells were stimulated with PMA and ionomycin for 1 h before preparing cell lysates and analyzing PKC substrates using a phospho-specific antibody. Whereas the PKC inhibitor bisindolmaleimide I suppressed PKC-induced phosphorylation events, our compound CID-2858522 did not (Supplementary Figure 13).

In addition to performing conventional *in vitro* kinase assays for PKCs and IKKs, we also performed a kinome screen using a high-throughput screening method, KINOMEscan, which is an active-site-dependent competition binding assay in which human kinases of interest are fused to a proprietary tag (Ambit).

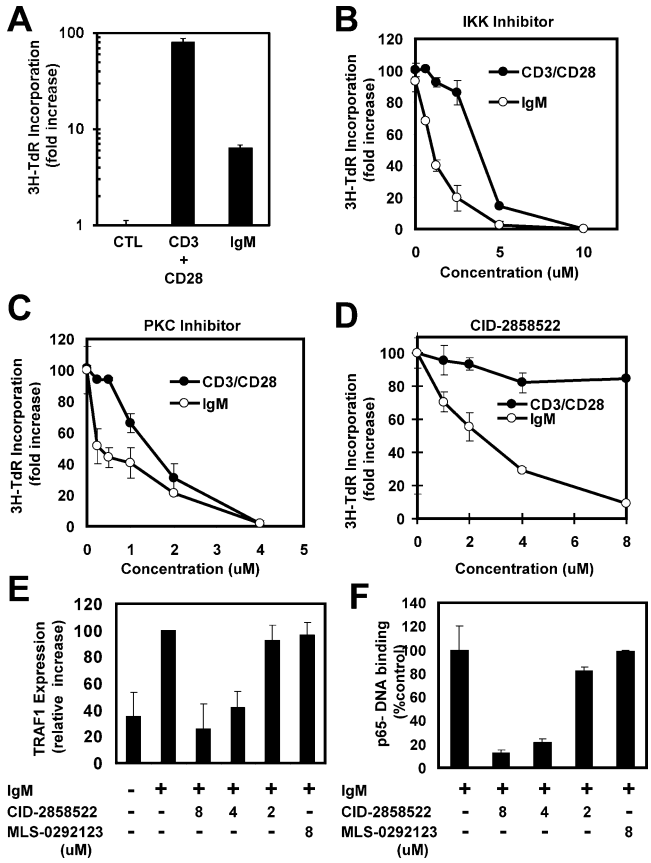

Figure 4. CID-2858522 inhibits anti-IgM-induced NF-κB activation and proliferation of β-lymphocytes. A) Isolated mouse primary splenocytes were cultured with anti-CD3/anti-CD28 (0.3 μg mL^{-1} each) or anti-IgM (3 μg mL^{-1}) for 48 h, then 1 μCi of [^{3}H]-thymidine was added for 12 h, and incorporation into DNA was measured, expressing data as fold increase above unstimulated cells (mean ± SD; $n = 3$). B–D) Primary splenocytes were pretreated for 2 h with IKK inhibitor, BMS-335541 (B), PKC inhibitor, bisindolylmaleimide I (C), or CID-2858522 (D) and then stimulated with anti-CD3/anti-CD28 (0.3 μg mL^{-1}) or anti-IgM (3 μg mL^{-1}) for 48 h. One microcurie of [^{3}H]-thymidine was added for 12 h, and incorporation into DNA was measured, expressing data as percent inhibition relative to control cells treated with DMSO (mean ± SD; $n = 3$). E) Human CLL B cell samples ($n = 3$) were cultured for 12 h with compound CID-2858522 or inactive analogue MLS-0292123 and then stimulated with biotin anti-IgM (10 μg mL^{-1}) for 24 h. Levels of TRAF1 and β-actin were assessed by immunoblotting and quantified by densitometry, and TRAF1 results were reported relative to control cells, after normalization for β-actin (mean ± SD). F) Human CLL B-cells were treated with various concentrations of CID-285822 or its inactive analogue, MLS-0292123, for 12 h followed by biotin-anti-IgM (10 μg mL^{-1}) and avidin (10 μg mL^{-1}) for 2 h. Nuclear extracts were then prepared, and p65-DNA-binding activity was measured (mean ± SD; $n = 3$).

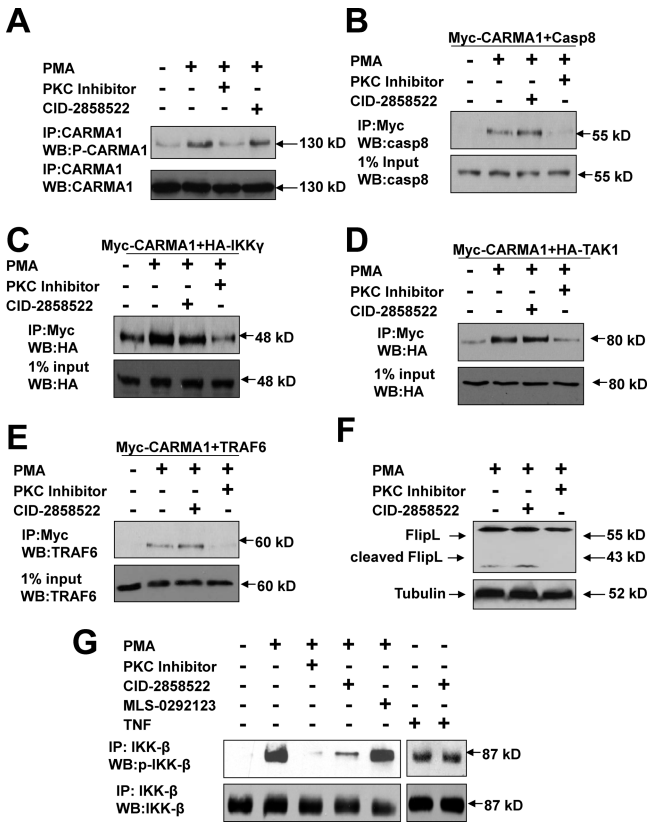

Figure 5. CID-285852 inhibits IKKβ phosphorylation induced by PKC activators. A) HEK293 cells were cultured in 0.5% FBS medium for 24 h and then treated with CID-2858522 (4 μM) or PKC inhibitor, bisindolylmaleimide I (1 μM) for 2 h followed by PMA/ionomycin (10 ng mL^{-1}; 5 ng mL^{-1}) for 2 h. Cell lysates were subjected to immunoprecipitation using anti-CARMA1 antibody and analyzed by immunoblotting with antiphospho-CARMA1 antibody or anti-CARMA1 antibody. B–E) HEK293 cells were transfected with plasmids encoding myc-CARMA1 in combination with plasmids encoding various other proteins including Caspase-8 (cys287ala) (B), HA-IKK-γ (C), HA-TAK1 (D), and TRAF6 (E). After 36 h cells were cultured in 0.5% FBS medium for 12 h and then treated with CID-2858522 (4 μM) or bisindolylmaleimide I (1 μM) for 2 h, followed by PMA/ionomycin treatment (10 ng mL^{-1}; 5 ng mL^{-1}) for 2 h. Cell lysates were immunoprecipated using anti-myc antibody and analyzed by immunoblotting using anti-Caspase8 (B), anti-HA (C, D), or anti-TRAF6 (E) antibodies. F) HEK293 cells were treated with CID-2858522 (4 μM) or PKC inhibitor (1 μM) followed by PMA/ionomycin (10 ng mL^{-1}; 5 ng mL^{-1}) treatment for 2 h. Cell lysates were normalized for protein content and analyzed by immunoblotting using anti-FLIP and anti-α-tubulin antibodies. G) HEK293 cells were cultured in 0.5% FBS medium for 24 h and then treated with CID-2858522 (4 μM) or its inactive analogue, MLS-0292123 (4 μM), or PKC inhibitor (1 μM), followed by treatment for 5 min with PMA/ionomycin (10 ng mL^{-1}) or TNF (10 ng mL^{-1}). Cell lysates were immunoprecipitated using anti-IKK-β and analyzed by immunoblotting using antiphospho-IKK-β antibody or anti-IKK-β antibody (as loading control). Approximate molecular weights of all proteins are indicated in kilodaltons.

The amount of kinase bound to an immobilized, active-site-directed ligand was measured in the presence and absence of the test compound (*24*). Of 353 protein kinases surveyed, CID-2858522 at 10 μM suppressed only 3 protein kinases by more than 50%: Raf (57% inhibition), TLK1 (70% inhibition), and JAK2 (53% inhibition) (Supplementary Table 1), none of which are clearly implicated in NF-κB regulation. Thus, we were unable to find evidence that CID-2858522 inhibits protein kinases previously implicated in regulating NF-κB.

Mapping the Site of Action of CID-2858522 in the Antigen Receptor-Activated NF-κB Pathway. On the basis of these kinase screens, we deduced that CID-2858522 operates somewhere between PKCs and IKK to inhibit the NF-κB pathway involved in signaling by antigen receptors and many growth factor receptors, which is known to include CARMA-family proteins, Bcl-10, MALT, TRAF6 (which binds Ubc13 to induce lysine 63-linked polyubiquitination of IKKγ/NEMO), IKKγ, and Caspase-8 (*9, 12*). To characterize the effects of CID-2858522 on these possible targets of the antigen receptor pathway for NF-κB activation, we first evaluated PMA-induced phosphorylation of Carma1, by phospho-specific antibody immunoblotting, finding no effect of CID-2858522 on this molecular event that initiates formation of the CBM complex (Figure 5, panel A). This result provided further evidence that CID-2858522 does not inhibit PKC in intact cells. Next, we performed co-immunoprecipitation (co-IP) experiments, assessing the interactions of Bcl-10, MALT, TRAF6, IKKγ, and Caspase-8 with either CARMA1 or CARMA3 in transfected HEK293 cells, before and after stimulation with PMA. PMA induced or increased association of CARMA1 or CARMA3 with each of these proteins, which was inhibited by a PKC inhibitor, bisindolylmaleimide I, but not by CID-2858522 (Figure 5, panels B–E and Supplementary Figure 14). Thus, CID-2858522 did not disrupt the formation of CARMA/MALT1/Bcl-10 (CMB) complex induced by PMA/ionomycin in either cells or lysates.

Caspase-8 plays an essential role in antigen receptor-mediated NF-κB activation (*25*). It was recently reported that MALT1 interacts with Caspase-8 and activates this protease upon antigen receptor activation. We confirmed that, in HEK293 cells, caspase-8 activation is required for NF-κB activation, as z-ITED-fmk (a peptidyl caspase-8 inhibitor) or caspase-8 siRNA significantly inhibited NF-κB luciferase activation induced by PMA/ionomycin (data not shown). We then examined

whether CID-2858522 interferes with caspase-8 participation in this pathway. PMA induced caspase-8 recruitment to the CARMA complex in HEK293 cells. The interaction was inhibited by a PKC inhibitor but not by CID-2858522 (Supplementary Figure 15). The caspase-8 p43/41 processing intermediate was generated in HEK293 cells after PMA/ionomycin treatment. Proteolytic processing of caspase-8 was suppressed by a PKC inhibitor but not by CID-2858522 (Supplementary Figure 16). We then assessed the effects of CID-2858522 on PMA-induced proteolytic processing of c-FLIP, a Caspase-8-mediated event recently shown to be required for antigen receptor-mediated NF-κB activation (*11*). Immunoblot analysis of lysates from HEK293 cells following stimulation with PMA/ionomycin showed processing of c-FLIP (Figure 5, panel F), which was completely inhibited by the PKC inhibitor but not affected by CID-2858522. Thus, CID-2858522 failed to inhibit Caspase-8 activation and c-FLIP processing.

Finally, we examined IKK-β phosphorylation (*26*). Phosphorylation of IKK-β was induced by PMA/ionomycin in HEK293 cells and was significantly inhibited by CID-2858522 but not by its inactive analogue, MLS-0292123 (Figure 5, panel G). In contrast, CID-2858522 failed to inhibit TNF-α-induced IKK-β phosphorylation, indicating pathway selectivity. We conclude from these studies that CID-2858522 inhibits PMA/ionomycin-induced NF-κB at a point downstream of CBM complex formation, caspase-8 activation, and c-FLIP processing but upstream of IKK-β phosphorylation.

Chemical inhibitors of NF-κB have been widely sought for potential use as therapeutics for autoimmunity, inflammation, and cancer (*13*). However, the most pharmaceutically tractable of the NF-κB-activating targets, the IKKs, represent a shared component of nearly all known NF-κB activation pathways and thus lack selectivity. In this regard, NF-κB activity is required for innate immunity and host-defense against microorganisms and various viral and bacterial pathogens. In addition to impaired host defense, broad-spectrum suppression of NF-κB pathways may reduce basal NF-κB activity and interfere with the function of NF-κB as a survival factor, leading to potentially toxic side effects. For example, IKK-β knockout mice die at midgestation from uncontrolled liver apoptosis (*27*). In addition to potentially providing for novel therapeutic agents, development of pathway-selective inhibitors could lead to highly useful research tool compounds for interogating which pathways are important for specific cellular responses.

Using a chemical biology strategy, we devised chemical library screens for inhibitors that selectively inhibit the NF-κB activation pathway induced by PKCs. This pathway is uniquely involved in acquired immunity (rather than innate immunity) and has been linked to numerous autoimmune diseases and some types of lymphomas and lymphocytic leukemia (*28*). NF-κB is also induced *via* PKC by many growth factor receptors. In this reagard, PKC hyperactivity has been associated with some solid tumors (*29*), and thus the pathway interrogated here may also be relevant to a variety of malignancies. The NF-κB activation pathway linked to PKCs is known to involve proteins unique to this pathway among the nine known NF-κB activation pathways, namely, CARMA (Bimp)-family proteins, Bcl-10, and MALT (reviewed in ref 12). Upon phosphorylation of CARMA1 by PKC in the context of antigen receptor signaling, these proteins form a complex, which recruits TRAF6, an E3 ligase that binds Ubc13, resulting in lysine 63-linked poly ubiquitination of IKK-γ/NEMO, resulting in IKK activation (*30*). Caspase-8 is also recruited, resulting in proteolytic processing of c-FLIP, an event required for antigen receptor-induced activation of NF-κB (*11*). The components of this complex required for IKK activation may not be completely known, and an active complex has not been reconstituted *in vitro* using purified components, thus making biochemical screens difficult. For this reason, a cell-based strategy for chemical library screening was the only practical option.

Using HEK293 cells containing an NF-κB-driven reporter gene stimulated by PMA/ionomycin, followed by an orthogonal screen in which we measured levels of the protein product of an endogenous NF-κB target gene (*e.g.*, IL-8) secreted by these same cells, we screened 114,889 compounds, finding only one that had the desired properties, namely, CID-2858522. This substituted 2-aminobenzimidazole compound potently inhibits NF-κB reporter gene activity and IL-8 production induced by PKC activators in HEK293 cells, with $IC_{50} < 0.1$ μM, while failing to inhibit NF-κB reporter gene activation by agonists of the other NF-κB activation pathways (Figure 1). CID-2858522 also suppressed anti-IgM-stimulated proliferation of murine B-lymphocytes, as expected for an antagonist of the NF-κB activation pathway activated by B-cell antigen receptors. Because CID

2858522 inhibits NF-κB activation induced by phorbol esters and antigen receptors, it cannot be argued that the compound somehow interferes with uptake of PMA or other PKC-activating phorbol esters. Also, CID-2858522 did not inhibit PKC-mediated phosphorylation of various endogenous substrates in intact cells, arguing against a direct or indirect inhibitory effect on PKCs.

The observation that CID-2858522 only partially suppressed CD3/CD28- or PMA/ionomysin-induced production of IL-2 by Jurkat T-cells is consistent with the fact that NF-κB is only one of several transcriptional regulators of the IL-2 gene, which also include NF-κB, NFAT, and AP-1 (*20*). We documented that CID 2858522 inhibited NF-κB while failing to suppress AP-1 or NFAT reporter gene activity induced by PKC. Furthermore, given that a variety of NF-κB-activating cytokines were elaborated upon stimulation of cultured lymphocytes with antibodies cross-linking CD3 (TCR) or surface IgM (BCR), it is perhaps not surprising that CID-2858522 only partially suppressed anti-IgM-induced proliferation of primary B-cells and had little effect on anti-CD3/CD28-induced T-cell proliferation. In contrast, an IKK inhibitor essentially completely suppressed lymphocyte proliferation at concentrations of ~5 μM, consistent with its ability to neutralize nearly all known NF-κB activation pathways. CID-2858522 also inhibited anti-IgM-induced expression of the endogenous NF-κB target gene, TRAF1, in CLL B-cells. In this regard, the TRAF1 gene promoter contains four NF-κB target sites and a TATA-box, but essentially no other recognizable transcriptional elements (*22*), thus making it a good surrogate marker of NF-κB activity in primary cells.

Although the mechanisms involved in antigen receptor-mediated NF-κB activation (upstream of PKC activation) in T-cells and B-cells are distinct, the downstream events following PKC activation share great similarity. Knockout mice models showed that CARMA1, Bcl-10, and MALT1 are required for antigen receptor-induced NF-κB activation and proliferation of both T-cells and B-cells (*16, 31*). However, CARMA1 mutant mice exhibited normal T- but impaired B-cell development (*32*), and MALT1 deficiency has only mild effects on B-cell activation MALT1 (*33*), indicating that the signal transduction apparati by which antigen receptors stimulate NF-κB downstream of PKC activation in T-cells versus B-cells are not identical. In this regard, it is also possible that antigen receptors and other upstream activators of PKCs induce NF-κB activation by more than one pathway, with CID-2858522 inhibiting only one of them. In this regard, it will be interesting to explore whether various lymphocyte subsets differ in their reliance on the NF-κB-activation pathways targeted by CID-2858522.

The mechanism by which CID-2858522 suppresses PKC-induced NF-κB activity remains to be determined. We mapped at least one site of action of this compound downstream of PKCs and upstream of IKK-β. PKCs induce phosphorylation of CARMA1, an event that was not inhibited by CID-2858522. This compound also inhibited neither PMA-induced recruitment of Bcl-10, MALT, TRAF6, Caspase-8, or IKKγ to CARMA1/CARMA3 nor caspase-8 or FLIP proteolytic processing. The active compound, however, selectively inhibited IKK-β phosphorylation induced by PkC activators but not TNFα, suggesting that CID-2858522 acts upstream of IKK-β. However, we cannot exclude the possibility that CID-2858522 has more than one site of action within the PKC-driven pathway for NF-κB activation, including acting at steps downstream of IKKβ.

The CARMA family proteins include three members in mammals, each of which contain a N-terminal CARD domain followed by a coiled-coil domain, a PDZ domain, a SH3 domain, and a C-terminal guanylate kinase-like (GUK) domain (*34–36*). Predominantly expressed in spleen, thymus, and peripheral blood leukocytes (PBL), CARMA1 has been definitively implicated in antigen receptor signaling. In contrast, CARMA3 is expressed in broad range of tissues but not in spleen, thymus, or PBL (*37*), and CARMA2 is expressed only in placenta. Suppression of selected members of the CARMA family could provide another plausible explanation for partial inhibition by CID-2858522 of events such as IL-2 production by CD3/CD28- or PMA/ionomycin-stimulated Jurkat cells.

In summary, using a chemical biology approach, we have identified the first selective chemical inhibitor of the PKC-initiated NF-κB activation pathway. This compound and its active analogues provide novel research tools for elucidating the role of this NF-κB pathway in cellular responses and may pave the way for future therapeutic applications of specific inhibitors of selective pathways involved in pathogenic activation of NF-κB.

METHODS

Reagents. Phorbol myristic acetate (PMA), ionomycin, muramyl dipeptide (MDP), retinoic acid (RA), doxorubicin, and γ-TriDAP were from Sigma-Aldrich (St. Louis, MO). Phorbol dibutyrate (PDBu), PKC inhibitor (bisindolylmaleimide I), and IKK inhibitor (BMS-345541) were from Calbiochem (Gibbstown, NJ). Antimouse-CD3, antimouse-CD28, and antimouse-IgM were obtained from Biomeda (Foster City, CA). Antihuman CD3, antihuman CD28, and antimouse-IgG antibody were from R&D Systems (Minneapolis, MN). Antihuman TRAF6 antibody has been described (*38*).

Plasmids encoding HA-IKK-γ (*39*), XIAP (*40*), HA-TAK1, TAB1 (*41*), CD4-TLR4 (*42*), CD40 (*43*), NOD1, NOD2 (*44*), cIAP1/MALT (*45*), Caspase-8 and Caspase-8 (C360S) (*46*), and TRAF6 (*47*) have been previously described. Myc-CARMA1 and CARMA3 were gifts from Dr, Xin Lin (University of Texas, M. D. Anderson Cancer Center).

Cell Engineering. HEK293 cells were co-transfected with pUC13-4xNFκB-luc and p-TK-puromycin-resistance plasmids. Stable clones were selected by culture in Dulbecco's Modified Eagle's Media (InVitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone) and 1% v/v penicillin-streptomycin (InVitrogen) containing 1 μg mL^{-1} puromycin. Individual clones were tested for responsiveness to PMA/ionomycin- and to TNF-induced NF-κB reporter gene activity, and a clone was selected for HTS.

Compounds. Chemical libraries were screened using the cellular NF-κB luciferase reporter assay, including a ChemBridge library (San Diego, CA) having 50,000 compounds, Microsource Spectrum collection (Gaylordsville, CT) having 2,000 compounds, the LOPAC library (Sigma) having 1,280 compounds, and the NIH library having 61,609 compounds at the time of screening.

HTS. NF-κB-luciferase-expressing HEK293 cells were seeded at 10^5 per well in white 96-well plates (Greiner Bio-One) in 90 μL of DMEM and incubated overnight. Then, 10 μL of compound-containing solution was added to each well (final 1.5 μg mL^{-1} in 1% DMSO) using a liquid handler (Biomek FX; Beckman Coulter). After 2 h of incubation, cells were stimulated using 11 μL of a PMA (final 100 ng mL^{-1}; Calbiochem) and ionomycin (final 50 ng mL^{-1}; Calbiochem) in DMEM. Cell plates were incubated for 16 h before media was removed and 40 μL of 0.5x passive lysis buffer (Promega Corp.) was added to cell plates. Plates were allowed to stand at RT for at least 15 min before adding 40 μL of 0.125x luciferin substrate (Promega Corp.) to each well. Plates were analyzed within 30 s with a Criterion Analyst using the luminescence method (0.1 s read/well).

Counter Screening and Secondary Assays. To counter-screen for inhibitors of the TNF pathway, HEK293-NFκB-luc cells were seeded at 10^5 cells per well in 90 uL medium in white 96-well plates (Greiner Bio-One) and cultured overnight, before adding compounds (5 μL in medium) to cells. After 2 h of incubation, 5 μL of TNF (200 ng mL^{-1}) (R&D Systems) was added (final concentration 10 ng mL^{-1}), and cells were incubated for 16 h. Luciferase activity was measured using a Britelite kit (Perkin-Elmer). To counter-screen for inhibitors of NF-κB induced by TLR4, CD40, NOD1, NOD2, cIAP2/MALT, or XIAP/TAB, the 293-NF-κB-luc cells cultured in 96-well plates as above were pretreated with compounds for 2 h and then transfected using Lipofectamine 2000 with various plasmids including pcDNA3 ("empty vector" control) or plasmids encoding CD4-TLR4, CD40, NOD1, NOD2, cIAP2/MALT, XIAP/TAB, using 0.2 uL of transfection reagent containing 100 ng DNA per well. Cells were cultured in medium containing CID-2858522 or other compounds, and luciferase activity was measured 48 h later. Alternatively, 293-NF-κB-luciferase cells were cultured with 16 μM all-*trans*-retinoic acid for 48 h or 2 μM doxorubicin for 48 h before measuring luciferase reporter gene activity.

The counter-screen for inhibitors of luciferase was performed in 96-well white plates (Greiner Bio-One) containing 45 μL per well of ATPlite solution and luciferase (Perkin-Elmer). Compounds diluted in 5 μL of phosphate-buffered saline (PBS) were added at 8 μM final concentration. Reactions were then initiated by addition of 50 μL of 160 nM ATP (Sigma) in PBS, and luciferase activity was measured 2 h later using a luminometer (LJL Biosystems, Sunnyvale, CA).

Cell Viability Assay. Cell viability was estimated on the basis of cellular ATP levels, measured using an ATPlite kit (Perkin-Elmer). Cells at a density of 10^5 mL^{-1} were seeded at 90 μL per well in 96-well white plates and cultured overnight. Compounds were added (5 μL in medium) to wells, and cells were cultured for 16 h, Finally, 50 μL of ATPlite solution was added to each well, and luminescence activity was read using a luminometer (LJL Biosystems, Sunnyvale, CA).

Lymphokine Measurements. Human IL-2 or IL-8 levels in culture medium were measured by enzyme-linked immunoadsorbant assays (ELISAs), using BD OptEIA ELISAs (BD Biosciences, San Diego, CA), according to the manufacturer's protocol, using 96-well ELISA plates (BD Biosciences) and measuring absorbance within 30 min of initiating reactions using a SpectraMax 190 spectrophotometer (Molecular Devices).

NF-κB DNA-Binding Activity Assays. Nuclear extracts were prepared from 10 cm^2 plates of confluent cells using a kit (Active Motif, Carsbad, CA). The total protein content of nuclear fractions was quantified by the Bradford method, followed by storage at −80 °C. NF-κB DNA-binding activity was measured in nuclear extracts (10 μg protein) using an immunoassay method (TransAM Kit [Active Motif]) employing 96-well plates coated with double-strand oligodeoxynucleotides containing NF-κB consensus binding site (5′-GGGACTTTCC-3′) and anti-p65 antibody, which was detected by secondary horseradish peroxidase (HRP)-conjugated antibody, using a colorimetric substrate with absorbance read at 450 nm within 5 min using a spectrophotometer, SpectraMax M5 (Molecular Devices).

EMSAs. EMSA assays were performed using LightShift Chemiluminescent EMSA Kit (Thermo Scientific, Rockford, IL) following the manufacturer's protocol. Briefly, nuclear extracts normalized for protein concentration (1 mg mL^{-1}) were incubated with EMSA binding buffer, poly dI-dC, and BSA for 20 min on ice. Then 2 μL of biotin-NF-κB probe was added and incubated at RT for 30 min. The samples were then resolved by gel electrophoresis using 4−20% precast nondenaturing polyacrylamide gels in 0.5X TBE (Bio-Rad, Hercules, CA) and then transferred to positively charged nylon membranes (Amersham Biosciences UK limited, Little Chalfont, United Kingdom). The DNA was cross-linked to membranes using UV Stratalinker 2400 (Stratagene, La Jolla, CA). The biotin-NF-κB oligo was detected using a Chemiluminescent Nucleic Acid Detection Module Kit (Thermo Scientific), with exposure to X-ray film.

Lymphocyte Proliferation Assay. Splenocytes were isolated from normal Balb/c mice, and red blood cells were removed using a mouse erythrocyte lysis kit (R&D Systems). Splenocytes were suspended in RMPI-1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin, and 1 mM L-glutamine. Cells were diluted into 2×10^6 cells mL^{-1}, and 200 μL was seeded in round-bottom 96-well plates (Greiner Bio-One) and incubated at 37 °C in 5% CO2 and 95% relative humidity. Cells were pretreated with compounds or DMSO diluted in medium for 2 h and then treated with 0.3 μg mL^{-1} anti-CD3/anti-CD28 or 3 μg mL^{-1} anti-IgM antibodies for 48 h, prior to adding 1 μCi [^{3}H]-thymidine (MP Biomedical, Solon, OH) for 12 h. Cells were transferred to fiberglass filters (Wallac, Turku, Finland) using a FilterMate Harvester (Perkin-Elmer) and dried, and [^{3}H]-incorporation into DNA

was quantified by scintillation counting (Betaplate Scint, Perkin-Elmer) and a MicroBetaTrilux LCS and luminescence counter (Perkin-Elmer).

Chronic Lymphocytic Leukemia (CLL) Cell Culture. Peripheral blood mononuclear cells from CLL patients were obtained under IRB approval from whole blood by Ficoll density gradient centrifugation and cultured with RPMI 1640 Medium supplemented with 10% FBS and antibiotics. Cells were ≥90% leukemia B-cells, as assessed *via* flow cytometry.

***In Vitro* Kinase Assays.** PKC-β, PKC-θ, and IKK-β *in vitro* kinase assays were performed using the HTScan Kinase Assay Kit (Cell Signaling, Danvers, MA) according to manufacturer's protocols. A panel of >300 kinases was screened by Ambit, Inc. (*24*).

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Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

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