Inflammatory Signaling by NOD-RIPK2 Is Inhibited by Clinically Relevant Type II Kinase Inhibitors

Highlights
- Discovery of type II kinase inhibitors as highly efficacious for RIPK2 inhibition
- RIPK2 autophosphorylation and ubiquitination are blocked by FDA-approved drugs
- RIPK2 crystal structure reveals an allosteric pocket for improving drug selectivity
- NOD-mediated inflammatory signaling is attenuated without affecting TLRs

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In Brief
Canning et al. report the structure of the diverse kinase RIPK2 and characterize its inhibition by the FDA-approved drugs ponatinib and regorafenib. The inhibitors prevent the autophosphorylation and ubiquitination of RIPK2 upon NOD2 stimulation, and block downstream NF-κB activation and inflammatory signaling.
Inflammatory Signaling by NOD-RIPK2 Is Inhibited by Clinically Relevant Type II Kinase Inhibitors

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SUMMARY

RIPK2 mediates pro-inflammatory signaling from the bacterial sensors NOD1 and NOD2, and is an emerging therapeutic target in autoimmune and inflammatory diseases. We observed that cellular RIPK2 can be potently inhibited by type II inhibitors that displace the kinase activation segment, whereas ATP-competitive type I inhibition was only poorly effective. The most potent RIPK2 inhibitors were the US Food and Drug Administration-approved drugs ponatinib and regorafenib. Their mechanism of action was independent of NOD2 interaction and involved loss of downstream kinase activation as evidenced by lack of RIPK2 autophosphorylation. Notably, these molecules also blocked RIPK2 ubiquitination and, consequently, inflammatory nuclear factor κB signaling. In monocytes, the inhibitors selectively blocked NOD-dependent tumor necrosis factor production without affecting lipopolysaccharide-dependent pathways. We also determined the first crystal structure of RIPK2 bound to ponatinib, and identified an allosteric site for inhibitor development. These results highlight the potential for type II inhibitors to treat indications of RIPK2 activation as well as inflammation-associated cancers.

INTRODUCTION

The nucleotide-binding oligomerization domain-containing proteins NOD1 and NOD2 are cytosolic Nod-like receptor (NLR) family proteins that function in the innate immune system to detect pathogenic bacteria (Philpott et al., 2014). NOD1 is activated upon binding to bacterial peptidoglycan fragments containing diaminopimelic acid (DAP), whereas NOD2 recognizes muramyl dipeptide (MDP) constituents (Chamaillard et al., 2003; Girardin et al., 2003a, 2003b; Inohara et al., 2003). NOD activation induces pro-inflammatory signaling by receptor-interacting protein kinase 2 (RIPK2, also known as RIP2 or RICK), which plays an obligatory and specific role in activation of NOD-dependent, but not Toll-like receptor responses (Park et al., 2007).

Signaling by RIPK2 is dependent on an N-terminal kinase domain with dual Ser/Thr and Tyr kinase activities (Dorsch et al., 2006; Tigno-Aranjuez et al., 2010), as well as a C-terminal caspase activation and recruitment domain (CARD) that mediates CARD-CARD domain assembly with activated NODs (Inohara et al., 1999; Ogura et al., 2001b). Once engaged, RIPK2 is activated by autophosphorylation (Dorsch et al., 2006) and further targeted by XIAP (X-linked inhibitor of apoptosis) and other E3 ligases for non-degradative polyubiquitination (Bertrand et al., 2011; Danggaard et al., 2012; Tao et al., 2009; Tigno-Aranjuez et al., 2013; Yang et al., 2007, 2013). The ubiquitin-conjugated protein subsequently activates the TAK1 and IKK kinases, leading to upregulation of both the mitogen-activated protein kinase and nuclear factor κB (NF-κB) signaling pathways (Kim et al., 2008; Park et al., 2007). In addition, RIPK2 induces an antibacterial autophagic response by signaling between NODs and the autophagy factor ATG16L1 (Cooney et al., 2010; Homer et al., 2012).

The NOD2-RIPK2 pathway has attracted special interest due to the role of this signaling node in granulomatous inflammatory diseases, including inflammatory bowel disease (IBD). Such pathologies can arise from either positive or negative dysregulation of the pathway (Caruso et al., 2014; Jostins et al., 2012; Philpott et al., 2014). Genetic variants in NOD2 are the strongest susceptibility factor to Crohn’s disease (Hugot et al., 2001; Jostins et al.,
Crohn’s disease-associated mutations that abrogate NOD2 binding to MDP may induce excessive inflammatory signaling from other pattern recognition receptors, including NOD1 (Couturier-Maillard et al., 2013; Inohara et al., 2003). In contrast, mutations in the second major Crohn’s disease susceptibility factor, ATG16L1, disrupt an inhibitory interaction with NOD2 and consequently increase the activation of RIPK2 (Sorbara et al., 2013). Excessive RIPK2 activation has also been reported in pediatric Crohn’s disease (Negroni et al., 2009). In addition, gain of function in the NOD2-RIPK2 pathway has been linked to Blau syndrome, early-onset sarcoidosis, allergic airway inflammation, and multiple sclerosis (Goh et al., 2013; Jun et al., 2013; Shaw et al., 2011). Overall, these data establish RIPK2 as a key molecule for the understanding of IBD pathogenesis as well as a potential therapeutic target in a wide spectrum of inflammatory and autoimmune diseases.

Importantly, the kinase activity of RIPK2 is essential for its stability and function, offering a promising rationale for small-molecule intervention (Nembrini et al., 2009; Tigno-Aranjuez et al., 2010). To date, all studies of RIPK2 have focused on small molecules of the type I inhibitor class, which bind to the kinase ATP pocket and are ATP competitive. This approach was first validated using SB203580, a pyridinyl imidazole inhibitor of p38, which showed additional inhibition of RIPK2 in vitro and was efficacious in a Crohn’s disease model in mice (Argast et al., 2005; Hellenbach et al., 2005). Further proof of concept was subsequently attained using the clinical epidermal growth factor receptor inhibitor, gefitinib, which also inhibited RIPK2 and improved disease burden in a spontaneous model of Crohn’s disease susceptibility factor, ATG16L1, disrupt an inhibitory interaction with NOD2 and consequently increase the activation of RIPK2 (Sorbara et al., 2013). Excessive RIPK2 activation has also been reported in pediatric Crohn’s disease (Negroni et al., 2009). In addition, gain of function in the NOD2-RIPK2 pathway has been linked to Blau syndrome, early-onset sarcoidosis, allergic airway inflammation, and multiple sclerosis (Goh et al., 2013; Jun et al., 2013; Shaw et al., 2011). Overall, these data establish RIPK2 as a key molecule for the understanding of IBD pathogenesis as well as a potential therapeutic target in a wide spectrum of inflammatory and autoimmune diseases.

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Here, we show that the inhibition of RIPK2 signaling can be improved by two orders of magnitude by using type II inhibitors that alternatively target the inactive “DFG-out” conformation of the kinase domain, including the US Food and Drug Administration (FDA)-approved drugs ponatinib, sorafenib, and regorafenib. Type II binding is confirmed by the first crystal structure of RIPK2 solved in complex with ponatinib, which reveals an allosteric site suitable for the rational design of RIPK2-selective small molecules. The identified clinical inhibitors disrupt RIPK2 activation in monocytes and macrophages to selectively reduce inflammatory signaling from NOD1 and NOD2, but not tumor necrosis factor (TNF) induction from Toll-like receptors. Overall, this work identifies the structural basis to investigate the therapeutic potential of RIPK2 inhibition in inflammatory diseases by defining clinically relevant scaffolds for the development of selective RIPK2 inhibitors.

RESULTS

Identification of Ponatinib as a Potent Inhibitor of RIPK2
To identify inhibitors of human RIPK2, we purified the recombinant kinase domain from Sf9 insect cells and screened it against a library of small-molecule kinase inhibitors using a fluorescence-based thermal shift assay (Niesen et al., 2007). In this assay, the previously reported type I inhibitors gefitinib and LDN-193189 yielded large thermal shift (ΔTm) values of 9.5°C and 12.1°C, consistent with their respective IC50 values of 49 nM (Tigno-Aranjuez et al., 2014) and 25 nM (Vogt et al., 2011). By comparison, the type II inhibitor ponatinib yielded a remarkable ΔTm of 23.1°C and was identified as the most potent hit (Table S1). To further explore type II inhibitors as putative preferred scaffolds for RIPK2, we set out to solve the co-crystal structure of RIPK2 with ponatinib. Protein crystallization was hindered initially by heterogeneous phosphorylation, but was enabled following treatment with lambda phosphatase. Viable crystals were grown in space group P212121 with two molecules in the asymmetric unit. The structure was solved by molecular replacement and refined at 2.75 Å resolution. Crystallographic data collection and refinement statistics are presented in Table S2.

Structural Features of RIPK2
The structure of RIPK2 exhibits the canonical bilobal kinase fold followed by a 16-residue αJ helix that packs along the side connecting the αD and αE helices (Figure 1A). This C-terminal structural element is common in STE family kinases, but is present additionally in RIPK1-3 (Figure 1B). The bound ponatinib inhibitor occupies the ATP pocket established between the N- and C-terminal lobes of the kinase. As a result, RIPK2 displays an inactive conformation of the catalytic domain characterized by a “DFG-Asp out, αC-Glu in” configuration. The β3 lysine (Lys47) and αC glutamine (Glu66) establish the catalytically relevant salt bridge, whereas the DFG aspartate (Asp164) is flipped away from the active site, rendering the enzyme inactive. Of note, the activation segment helix found in the crystal structures of the two homologous kinases, RIPK1 and RIPK3, is not present in RIPK2, although a significant portion of the activation loop was not visible in the electron density map and not modeled (Figure 1B). Residues from this helix, in particular Ser161, are known to contribute to the binding of RIPK1 to selective small-molecule inhibitors, necrostatins (Xie et al., 2013a). Interestingly, RIPK2 also contains several unusual sequence changes in its catalytic motifs that are not conserved in other RIPKs. The typical HRD triad in the catalytic loop is changed to HHD, while the activation loop APE motif is changed to PPE (Figure 1C). Moreover, the kinase domain of RIPK2 as a whole displays only 33% sequence identity with other proteins in the PDB (namely RIPK1 and RIPK3), indicating its broader diversity.

Oligomerization into multi-protein signaling complexes is a key part of the activation mechanism in the RIPK family. The RIPK2 structure reveals a homodimeric packing arrangement similar to that of RIPK3 (Xie et al., 2013b) and consistent with the dimeric state observed in solution by analytical gel filtration (Figure S1). The protein interface is highly symmetrical, with the two active sites facing in opposite directions and rotated approximately 90° relative to one another (Figure 2). Binding is supported by the αJ helices, which pack against each other in an antiparallel fashion, and form both hydrophobic interactions and a symmetrical pattern of hydrogen bonding between the side chains of Lys310 and Glu299 and the side chains of His159 and Glu157 (Figure 2). Additional contacts are made between the βJ-βJ and βJ-βJ
The Binding Mode of Ponatinib and Other Clinically Relevant Type II Inhibitors

Ponatinib (Figure 3A) has been crystallized previously only in complex with tyrosine kinases. The binding mode in RIPK2 resembles that of DDR1 and KIT with an intact conformation of the $b_1$-$b_2$ hairpin, rather than the collapsed loop of Abl (Figure 3B). The imidazo[1,2-$b$]pyridazine head group establishes a single hydrogen bond to the hinge residue Met98 as well as hydrophobic interactions with Tyr97 and Leu24. The central linker forms two additional hydrogen bonds with the side chain of Glu66 and the main-chain nitrogen of Asp164. The trifluoromethyl group occupies the hydrophobic pocket vacated by the inverted DFG motif, while the protonated methylpiperazine forms an ionic-dipole interaction with the main-chain oxygen atoms of Leu143 and His144, positioned within the $\alpha_D$-$\beta_6$ loop (Figure 3B). Importantly, the structure also reveals opportunities for the development of RIPK2-selective molecules. In particular, the allosteric hydrophobic pocket occupied by the trifluoromethyl group is greatly enlarged in RIPK2 due to the presence of Ala73 in the $\alpha_C$ helix (Figure 3C). Nearly all kinases contain a bulky side chain at this position, such as Leu70 in RIPK1. Thus, larger chemical groups at this site will not only increase potency for RIPK2, but will also sterically restrict inhibitor binding to the wider kinome.

Regorafenib and sorafenib are two other multi-targeted clinical type II inhibitors that share a phenyl-urea-trifluoromethylphenyl core (Figure 3A). Docking studies suggested that these...
Inhibition of Cellular RIPK2 Activation

The kinase activity of RIPK2 has been shown to mediate the activation of inflammatory signaling by the NOD1/2 family of peptidoglycan receptors. Autophosphorylation of Ser176 in the kinase activation segment has been identified as a specific marker of RIPK2 activation (Dorsch et al., 2006; Nachbur et al., 2015). To confirm inhibition of cellular RIPK2 activation by the inhibitors, we therefore analyzed changes in phospho-Ser176-RIPK2 (p-RIPK2) following stimulation of NOD2-expressing HEK293 cells with L18-MDP ligand (a lipidated form of MDP with enhanced potency). L18-MDP caused a rapid increase in endogenous p-RIPK2 that was inhibited by low nanomolar concentrations of ponatinib, sorafenib, and regorafenib (Figure 4A). Ponatinib displayed the highest activity, completely blocking RIPK2 phosphorylation at 10 nM, followed by regorafenib and sorafenib. This was consistent with the order of activities observed in the in vitro kinase assays. Furthermore, by blocking RIPK2 activation the inhibitors prevented the phosphorylation and subsequent degradation of IkB (Figures 4A and S3), which is required for activation of NF-kB and induction of inflammatory gene expression (Cramer and Muller, 1999). Surprisingly, the type I inhibitor gefitinib showed much lower activity in cells relative to the in vitro assays (Figure 4A). This may partly reflect its ATP-competitive mode of action, with potential loss of activity due to the high concentrations of ATP in cells. None of the molecules significantly inhibited phosphorylation of ERK1/2 or global levels of phosphotyrosine proteins, which served as negative controls (Figure 4A).

To further quantify cellular RIPK2 inhibition, we measured the downstream activation of NF-kB in the same HEKBlue cells, which were also stably transfected with an NF-kB-SEAP reporter. Ponatinib again displayed the most potent, low nanomolar activity (EC50 = 0.8 nM), followed by regorafenib and sorafenib (Figure 4B). By comparison, gefitinib inhibited NF-kB activity with an EC50 value of 7.8 μM, consistent with the p-RIPK2 data. Overall, these data identified ponatinib, sorafenib, and regorafenib as a new class of low nanomolar inhibitors of RIPK2 activation in cells.

Since ponatinib is a potent inhibitor of Abl, we further examined whether Abl may contribute to the inhibition of the NOD2 responses in HEK cells in addition to RIPK2. We found that a different type II inhibitor of Abl, nilotinib, did not inhibit either RIPK2 in vitro (Figure S5A) or the NOD2 response in HEKBlue cells (Figure S5B). Because ponatinib is a large lipophilic molecule, which may cause non-specific effects, we also sought to identify close analogs that would lack activity against RIPK2. We noticed that methyl of the central phenyl ring of ponatinib inserts into the shallow lipophilic pocket formed by aliphatic side chains of Val32, Lys47, Ile93, and Thr95. A bulkier tert-butyl side chain (CS6, Figure 3A) was designed based on this hypothesis and synthesized using previously reported methods (Huang et al., 2010; Najjar et al., 2015). Introduction of the tert-butyl led to the loss of inhibition of both RIPK2 and Abl kinase activity (Figure S5A), providing us with a bulky and lipophilic control molecule. Importantly, CS6 did not inhibit the MDP response in HEKBlue cells (Figure S5B). These data and the lack of cellular activity of nilotinib further confirmed the specific role of RIPK2 inhibition in these assays.

To explore the mechanism of RIPK2 inhibition, we tested the effects of ponatinib on the required interaction with NOD2. For this, we used U2OS cells that inducibly expressed HA-tagged NOD2 and performed HA-immunoprecipitation to recover bound RIPK2. As expected, HA-NOD2 expression was detected only in the presence of doxycycline, and induced
a robust interaction with RIPK2 (Figure 4C) as well as down-
stream NF-κB activation (Figure 4D). Interestingly, the NOD2-
RIPK2 interaction was stably maintained in the presence of
100 nM ponatinib (Figure 4C), whereas NF-κB activation was
completely disabled (Figure 4D). These results indicated that
ponatinib acts to inhibit the activation of the kinase domain of
RIPK2 but does not interfere with the C-terminal CARD domain,
which recognizes NOD2.

**Ponatinib Potently Abrogates RIPK2 Ubiquitination and
Induction of Inflammatory Cytokines**

To examine inhibition of RIPK2-induced inflammation in a
more physiological context, we analyzed the receptor sig-
aling pathway in human monocytic THP-1 cells and in mouse
macrophage RAW264.7 cells in response to different PRR
(pattern recognition receptor) ligands and cytokines. Stimula-
tion of NOD2 by MDP leads to rapid ubiquitination of RIPK2
by XIAP and other ubiquitin ligases, a process required
for downstream signaling and transcription of NF-κB target
genes (Damgaard et al., 2012, 2013; Fiil et al., 2013; Yang
et al., 2013). Consistent with the relative potency of the kinase
inhibitors against RIPK2 activity, pre-treatment of THP-1 cells
with 100 nM ponatinib completely blocked L18-MDP-induced
RIPK2 ubiquitination, whereas regorafenib and gefitinib in (E) and (F), experiments were performed in duplicate; error
bars indicate SD values. Kinase activity was measured using the ADPGlo assay. Non-linear
curve fitting to calculate IC50 values was per-
formed using Prism software. See alsoFigure S5.

or no detectable effect (Figure 5A). Importantly, ponatinib interfered with
RIPK2 ubiquitination in a dose-depen-
dent manner. Concentrations as low as
5–10 nM reduced the extent and length
of ubiquitin-modified RIPK2, while
RIPK2 ubiquitination was completely
blocked at concentrations of 25 nM or
higher (Figure 5B). By contrast, ponatinib had no obvious
effects on ubiquitination of the related kinase RIPK1 or IκBα
degradation following treatment of THP-1 cells with TNF
(Figure 5C).

We next analyzed the pattern of inflammatory gene expression
in RAW264.7 macrophage cells. Consistent with the results ob-
tained in THP-1 cells, MDP stimulation led to robust increases in
CCL4, CXCL2, and RANTES mRNA levels that were efficiently in-
hibited by low nanomolar (1–10 nM) concentrations of ponatinib
(Figure 6A). Regorafenib was similarly active at 10–100 nM while
sorafenib was slightly less effective (Figure 6A). Comparable re-
sults were observed following RIPK2 stimulation with the NOD1
agonist, Tri-DAP (Figure 6B). In contrast, ponatinib and the other
inhibitors did not block the mRNA induction by a different class
of PAMPs (pathogen-associated molecular patterns), such as
agonists of Toll-like receptors 2 and 4 (Pam3CSK4 and lipopoly-
saccharide [LPS], respectively) (Figures 6C and 6D). The only
exception was 100 nM ponatinib, which partially attenuated
both the LPS and Pam3CSK4 responses, likely reflecting non-
specific activity of this inhibitor at higher concentrations. Overall,
we were able to observe selective and efficient inhibition
of RIPK2-dependent NOD1/2 responses by both ponatinib and
regorafenib at low nanomolar concentrations and by sorafenib
at ≤100 nM.

Modulation of NOD1/2-RIPK2-XIAP-mediated signaling has
been proposed as a therapeutic approach for inflammatory
disorders (Jun et al., 2013). Therefore, we investigated whether
NOD2 receptor activation in primary human monocytes can be inhibited to an extent similar to that observed for the described epithelial and leukocyte cell lines. Ponatinib, regorafenib, and gefitinib inhibited the production of TNF in peripheral blood-derived monocytes after L18-MDP stimulation (Figure 7A). The most potent inhibition was observed with ponatinib, which completely abrogated NOD2-dependent TNF production at low nanomolar concentrations down to 10 nM (Figure 7B). Regorafenib was similarly effective at 100 nM, whereas a much higher concentration of gefitinib (10 μM) was required for complete inhibition (Figure 7B). TNF production by LPS stimulation was not affected at these inhibitor concentrations (Figures 7A and 7B), and was attenuated only by ponatinib at the much higher concentration of 1 μM (Figure 7B). Similar inhibitor efficacies were observed in the human monocytic cell line THP-1, although baseline response in these cells was low and 10 μM gefitinib also inhibited the LPS pathway (Figure S7).

Overall, the results on primary human monocytes are fully consistent with the earlier data obtained in laboratory cell lines, suggesting that the tested cell lines provide an accurate tool to assess the potency of RIPK2 inhibition. Furthermore, they support the hypothesis that tyrosine kinase inhibitors can be used in primary human cells to selectively target RIPK2.

DISCUSSION

To date, all reported RIPK2 inhibitors have been ATP-competitive type I molecules, such as the clinical drug gefitinib. Importantly, we observed that the cellular activity of this type I inhibitor was vastly outperformed by the identified type II inhibitors, despite their comparable potencies in the in vitro kinase assay (e.g., comparing regorafenib and gefitinib). Multiple factors could contribute to these differences. For example, the endogenous full-length RIPK2 may display a higher affinity for ATP than the isolated kinase domain, resulting in greater competition for type I binders. Alternatively, the DFG-out conformation might represent a preferred conformation in cells, or perhaps form a dominant-negative species. Indeed, activation loop phosphorylation in JAK2 is incompatible with the binding of type II inhibitors, but permissible with inhibitors of the type I class (Andraos et al., 2012). While the mechanistic basis for these differences remains to be further elucidated, these data clearly identify type II inhibitors as the most efficacious molecular class for targeting RIPK2.

The potency of the type II inhibitors also affords new tools with which to investigate the molecular mechanisms of RIPK2 signaling and the effects of kinase inhibition. We found that ponatinib blocked the activation of the kinase domain of RIPK2 without affecting the C-terminal CARD domain and its engagement of NOD2. A similar breakdown in RIPK2 activation has been reported for a NOD1 variant containing the rare Asn43Ser polymorphism (Mayle et al., 2014). However, in the absence of structural information there is little understanding of how the CARD domain status of RIPK2 is communicated to the kinase domain. Potentially, new stimulatory interactions could be formed or inhibitory interactions broken. All the tested inhibitors were able to block the phosphorylation of the kinase activation loop (Ser176), which is a known marker for RIPK2 activation. Activated RIPK2 is also targeted by multiple E3 ligases for polyubiquitination by mechanisms that are...
yet to be structurally characterized. Unexpectedly, we found that ponatinib could inhibit this modification completely, whereas type I inhibitors have previously only caused a delay in ubiquitination (Nachbur et al., 2015). This result suggests that RIPK2 binding to E3 ligases is more strictly regulated than previously imagined. Thus, it will be interesting to decipher the precise mechanism of E3 recruitment in future work. We also observed excellent selectivity of the inhibitors toward MDP-dependent signaling relative to LPS-dependent pathways. This result is noteworthy given the known promiscuity of ponatinib within the kinome (Zhao et al., 2014). It also further supports the specificity of RIPK2 for NOD-dependent signaling (Park et al., 2007).

In a separate study, we have found that ponatinib is also an efficient inhibitor of RIPK1 and RIPK3 kinase activity in necrosis (Najjar et al., 2015), making this molecule the only known pan-RIPK inhibitor. The current work using ponatinib and TNF stimulation, in line with previous reports, shows that RIPK1 kinase activity is dispensable for its ubiquitination as well as for the downstream degradation of $\text{i}B\text{Bz}$ (Lee et al., 2004). Thus, the experiments with ponatinib reveal important differences in the mechanisms of RIPK1 and RIPK2. Nonetheless, the ability of ponatinib to simultaneously target multiple RIPKs may be of interest for investigating inflammatory disorders. By contrast, inhibition of RIPK1/RIPK3 was not observed with sorafenib or regorafenib (data not shown). These two inhibitors lack the methylpiiperazine group that is present in ponatinib. The activation segments in RIPK1/RIPK3 also show a high propensity to form a short $\alpha$ helix that is not observed in the RIPK2 structure. Additional co-structures and SAR may be required to understand the selectivity differences that must exist between the DFG-out (DLG in RIPK1) pockets of these kinases. Importantly, ponatinib, regorafenib, and sorafenib are FDA-approved medications that are used clinically against various forms of cancer. Inhibition of RIPK2 represents a novel off-target activity, although microbiota-driven inflammation has emerged as a potentially important player in tumorigenesis (Elinav et al., 2013; Saxena and Yeretssian, 2014). IBD is also a known risk factor for colorectal cancer (Sebastian et al., 2014). Nonetheless, the broad kinase selectivity of these drugs currently prohibits their use in chronic inflammatory conditions. In particular, ponatinib can cause serious adverse events including vascular thrombosis (Cortes et al., 2013). Sorafenib and regorafenib are better tolerated and are used clinically at low-micromolar doses that are higher than those used in our study (Moore et al., 2005; Mross et al., 2012). These molecules are therefore potentially interesting tools with which to further explore RIPK2 function in pre-clinical models of colitis and other inflammatory conditions.

Finally, this work also suggests that more selective compounds may be derived by targeting the expanded allosteric pocket identified in the RIPK2 structure. For example, the trifluoromethyl group could be replaced with a larger substitute, such as methoxyethyl, isopropyl, or isopropoxy. All of these groups have the potential to extend further into the RIPK2 pocket without dramatic changes in hydrogen bonding or lipophilicity. In addition, changes to the hinge-binding “head” group could be considered by comparison with RIPK2-selective type I inhibitors such as WEHI-345 (Nachbur et al., 2015). The recently identified macrocycles OD36 and OD38 would, however, form steric clashes in a type II binding mode (Tigno-Aranjuez et al., 2014). The toolbox of new compounds will form valuable reagents for...
further investigation of the complexity of RIPK2 regulation in both normal signaling and pathobiology.

SIGNIFICANCE

Clinical kinase inhibitors have been utilized almost exclusively in oncology. The recent approval of JAK inhibitors for the treatment of inflammatory conditions, in particular rheumatoid arthritis, has demonstrated the potential of this drug class to target other indications. RIPK2 is one emerging therapeutic target in inflammation strongly supported by genetic evidence of activating NOD2 mutations in the monogenic autoinflammatory disease Blau syndrome, characterized by early-onset granulomatous arthritis, uveitis, and dermatitis.

To date, pre-clinical validation studies for inflammatory conditions have largely focused on the clinical inhibitor gefitinib, which binds to kinases in their active conformation. Here, we show that RIPK2 is highly amenable to type II inhibition, which affords dramatic improvements in cellular potency. Furthermore, the most potent molecules, ponatinib, regorafenib, and sorafenib, extend the available inhibitor activities from micromolar to subnanomolar, allowing fine-tuning insights into RIPK2 regulation. In particular, binding of ponatinib to the kinase domain is sufficient to block all ubiquitination on RIPK2, and demonstrates the requirement for this modification for the downstream destruction of IκBα, in contrast to the requirements of RIPK1. In addition, regorafenib offers selectivity for RIPK2 over RIPK1/3 as well as low nanomolar potency to alleviate some “off-target” effects. Further scaffold improvements to overcome such liabilities are also suggested by the presented RIPK2 structure. Overall, this work identifies advanced tools to investigate the functional role of RIPK2 in control of the intestinal microbiota as well as clinically relevant scaffolds to explore the therapeutic potential of RIPK2 inhibition in inflammatory diseases.

EXPERIMENTAL PROCEDURES

Cells and Reagents
Detailed information on reagents, qPCR, and immunoblotting is provided in the Supplemental Experimental Procedures.

Purification of RIPK2
Human RIPK2 (Uniprot: O43353, residues 8–317) was expressed in Sf9 insect cells, and purified by nickel affinity and size-exclusion chromatography. Detailed information is provided in the Supplemental Experimental Procedures.

Crystallization and Structure Determination
RIPK2 was concentrated to 3.7 mg/ml. Crystals with ponatinib were grown in sitting drops using a reservoir solution containing 0.1 M ammonium citrate and 16% (w/v) polyethylene glycol 3350. Diffraction data were collected on Diamond Light Source beamline I04. Detailed information on structure determination is provided in the Supplemental Experimental Procedures.

Thermal Shift Assay
RIPK2 protein at 2 μM concentration was mixed with inhibitor compounds at 10 μM and a 1:1,000 dilution of SyproOrange fluorescent dye (Invitrogen).
MDP)

\[ \text{[control (DMSO, L18-MDP)]} \]

inhibition: \[ \% = (1 - \frac{C_0}{C_1}) \times 100 \]

Values of empty media were subtracted from all samples. Absorbance at 620 nM was determined at the end of the incubation using a Victor3V plate reader (PerkinElmer). Procedures.

HEKBlue cells (1 x 10⁵ cells/ml) were suspended in QUANTI-Blue detection medium and seeded into 96-well plates (100 µl/well). Cells were treated with small-molecule inhibitors and 1 µg/ml L18-MDP for 6–10 hr. Absorbance at 620 nM was determined at the end of the incubation using a Victor3V plate reader (PerkinElmer). Values of empty media were subtracted from all samples. Resulting specific signal values were used to calculate inhibition: \[ \% = (1 - \frac{C_0}{C_1}) \times 100 \].

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2015.07.017.

**AUTHOR CONTRIBUTIONS**

A.N.B., A.D., M.G.-H., and H.H.U. designed the research; P.C., Q.R., T.S., H.H.U., M.G.-H., and A.D. wrote the paper.

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