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*J Immunol* published online 2 October 2015
http://www.jimmunol.org/content/early/2015/10/02/jimmunol.1500162

Supplementary Material [http://www.jimmunol.org/content/suppl/2015/10/02/jimmunol.1500162.DCSupplemental](http://www.jimmunol.org/content/suppl/2015/10/02/jimmunol.1500162.DCSupplemental)

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Cutting Edge: Inhibiting TBK1 by Compound II Ameliorates Autoimmune Disease in Mice

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TANK-binding kinase 1 (TBK1) is a serine/threonine protein kinase that plays a crucial role in innate immunity. Enhanced TBK1 function is associated with autoimmune diseases and cancer, implicating the potential benefit of therapeutically targeting TBK1. In this article, we examined a recently identified TBK1 inhibitor Compound II on treating autoimmune diseases. We found that Compound II is a potent and specific inhibitor of TBK1-mediated IFN response. Compound II inhibited polyinosinic-polycytidylic acid–induced immune activation in vitro and in vivo. Compound II treatment also ameliorated autoimmune disease phenotypes of Trex1−/− mice, increased mouse survival, and dampened the IFN gene signature in TREX1 mutant patient lymphoblasts. In addition, we found that TBKI gene expression is elevated in systemic lupus erythematosus patient cells, and systemic lupus erythematosus cells with high IFN signature responded well to Compound II treatment. Together, our findings provided critical experimental evidence for inhibiting TBK1 with Compound II as an effective treatment for TREX1-associated autoimmune diseases and potentially other interferonopathies. The Journal of Immunology, 2015, 195: 000–000.

The innate immunity is the first line of defense against invading pathogens. Our cell encodes several pattern recognition receptors that recognize specific components of pathogens and activate appropriate immune responses (1). Many innate immune signaling pathways converge to a key protein TANK-binding kinase 1 (TBK1), which orchestrates the induction of IFN and inflammatory genes that are critical mediators of immune defense (2, 3). Human TBK1 haploinsufficiency is associated with herpes simplex encephalitis (4), demonstrating its importance in antiviral response. Pattern recognition receptors can also recognize self-derivatives that accumulate inappropriately, leading to autoimmune and inflammatory diseases through immune pathways that often depend on TBK1 (5, 6). TBK1 is a member of the IκB kinase (IKK) family and is ubiquitously expressed. In addition to its role in innate immunity, TBK1 also plays an important role in oncogenic transformation through direct regulation of Akt survival signaling (7). Therefore, TBK1 represents an attractive therapeutic target for autoimmune diseases and cancer with underlying hyperactive TBK1 signaling. The in vivo importance of TBK1 in autoimmune disease models has been hampered by the apparent lethality of Tbk1−/− mouse (8, 9). Small molecular inhibitors of TBK1 (e.g., BX795) often lack specificity, thereby limiting their application (5). We have recently identified a 6-aminopyrazolopyrimidine derivative (Compound II) through a biochemical screen of small-molecule inhibitors of TBK1 and demonstrated its effectiveness on limiting cancer cell proliferation (7). In this study, we examined Compound II on treating autoimmune diseases.

Trex1 deficiency causes autoimmune and inflammatory disease phenotypes in mice (10, 11), and we recently determined that the underlying immune activation depends on TBK1 (12). TREX1 mutations in humans are also associated with a spectrum of autoimmune and inflammatory phenotypes, including Aicardi–Goutières syndrome (AGS), familial chilblain lupus, systemic lupus erythematosus (SLE), and retinal vasculopathy with cerebral leukodystrophy (13). Tbk1−/− or Trex1−/− mutant patient cells induce cell-intrinsic activation of IFN-stimulated genes (ISGs) that are critically dependent on TBK1 and a downstream transcriptional factor IRF3 (12). Besides the TREX1 disease model, TBK1 and its associated IFN signaling pathway have also been implicated in the pathogenesis of complex SLE and other autoimmune diseases collectively called interferonopathies (14, 15). No effective treatment is available for these diseases. Thus, there is an urgent need for developing novel therapeutics based on molecular understandings of these diseases.

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Received for publication February 2, 2015. Accepted for publication September 8, 2015. This work was supported by the National Institutes of Health (Grants AI098569 and AR067135 to N.Y.) and the Alliance for Lupus Foundation (to N.Y.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: AGS, Aicardi–Goutières syndrome; AST, aspartate aminotransferase; BMDM, bone marrow–derived macrophage; IKK, IκB kinase; ISG, IFN-stimulated gene; poly(I:C), polyinosinic-polycytidylic acid; qRT-PCR, quantitative RT-PCR; SLE, systemic lupus erythematosus; TBK1, TANK-binding kinase 1.

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Materials and Methods

Cells and mice

Trex1−/− mice and TREX1-R114H patient lymphoblasts were described previously (12). SLE cell lines were obtained from Oklahoma Medical Research Foundation. Leukocyte subsets microarray analysis of healthy control and SLEs were described by Becker et al. (14). Cells were maintained in DMEM or RPMI 1640 with 10% (v/v) heat-inactivated FCS, 2 mM L-glutamine, 10 mM HEPES, and 1 mM sodium pyruvate (complete DMEM) with the addition of 100 U/ml penicillin, 100 mg/ml streptomycin and cultured at 37°C with 5% CO2. Bone marrow–derived macrophages (BMDMs) were generated as described previously (12). Experiments in WT mice with Compound II and polyinosinic-polycytidylic acid [poly(I:C)] were done by i.p. injections of DMSO or Compound II (10 mg/kg) for 3 d followed by one i.p. injection of pol(I:C) (300 µg in 100 µl vol of PBS) and isolated peritoneal cells after 2 h. Experiments with Trex1−/− mice were started when the mice were 4 wk old. Trex1−/− mice were treated with DMSO or Compound II (i.p. injections 3 d/wk) for 7 wk. Stocks of DMSO and Compound II were further diluted in PBS to obtain 100 µl total volume per injection for each mouse. Mouse serum and tissues were harvested after indicated weeks of treatment. Mouse tissues were fixed in 4% PFA followed by standard H&E staining for histology analysis. Experiments involving human and mouse materials were approved by the Institutional Animal Care and Use Committee and Institutional Review Board of University of Texas Southwestern Medical Center.

Reagents and Abs

TRI Reagent (Invitrogen) was used for RNA isolation. Compound II was chemically synthesized (7). Abs used in this study include anti–phospho–IRF-3 (Ser396; Cell Signaling), anti–HMG1 (Abcam), anti–Tubulin (Sigma), anti–phospho–TBK1 (Cell Signaling), and anti–Phospho–NF–κB (Cell Signaling); secondary Abs (GE Healthcare) were used for immunoblot analysis according to standard protocols.

Quantitative PCR array, microarray, and serum analysis

Quantitative PCR array analysis of immune gene profiles was performed as in Hasan et al. (12) using custom-ordered PCR array plates containing primer sets pre aliquoted (Bio-Rad). Each primer set was validated by Bio-Rad and in-house. Leukocyte subsets from healthy control and SLE subjects were analyzed by microarray (14). Mouse serum asparagus aminotransferase (AST) level was measured by Vitros 250 at Mouse Metabolic and Phenotyping Core (University of Texas Southwestern Medical Center). Autoantibody arrays were performed as described previously (10).

Statistical methods

Data are presented as the mean ± SEM. GraphPad Prism 6 was used for statistical analysis. Statistical tests performed are indicated in the figure legends: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Results and Discussion

Compound II is a potent and specific inhibitor of TBK1-mediated IFN response

To evaluate the effect of Compound II in TBK1-mediated IFN response, we first compared Compound II with another commonly used TBK1 inhibitor, BX795. We pretreated RAW264.7 (a mouse macrophage cell line) with either drug at a range of concentration (1 nM to 10 µM) followed by poly(I:C) transfection to induce IFN response. Both drugs inhibited poly(I:C)-stimulated IRF3 activation (measured by Ifnb and Ifit1 mRNA levels), but not NF-κB activation (measured by Tnfα mRNA level), suggesting that Compound II specifically targets the TBK1/IRF3 pathway (Fig. 1A, Supplemental Fig. 1A). Compound II also appeared to be significantly more potent, with a more gradual dose response and lower IC50 (20 nM) compared with BX795 (IC50 of 1 µM). We also found that BX795 at high doses was toxic to RAW264.7 cells, whereas Compound II did not appear to be toxic across the entire dose range (Supplemental Fig. 1B).

We also analyzed phosphorylation of TBK1 substrates to gain more molecular insights on the mechanism of inhibition. Poly(I:C) transfection activates both IRF3 and NF-κB pathways in RAW264.7 cells as indicated by robust phosphorylation of TBK1, IRF3, and NF-κB. Compound II completely inhibited TBK1 autophosphorylation (17) and significantly delayed IRF3 phosphorylation without affecting either total protein level (Fig. 1B). In contrast, BX795 (at the same dose as Compound II, 1 µM) only partially inhibited IRF3 phosphorylation and had no effect on TBK1 autophosphorylation. Neither drug inhibited NF-κB phosphorylation. These data suggest that Compound II is a potent and specific inhibitor of TBK1-mediated IFN response.

Compound II inhibits poly(I:C)-induced immune activation in vitro and in vivo

We next examined whether Compound II also inhibits immune activation of TBK1 in BMDMs or mice stimulated with poly(I:C) through the TLR pathway. Poly(I:C) activates TLR3, which signals through TBK1 and IRF3 to activate IFN and inflammatory cytokines. We pretreated BMDMs with DMSO or Compound II overnight, then stimulated with poly(I:C) for 2 h and measured IRF3 phosphorylation as a marker for TBK1 activation. Compound II dramatically reduced poly(I:C)-induced IRF3 phosphorylation (Fig. 2A). We also treated wild type C57BL/6 mice with DMSO or Compound II by i.p. injections for 3 d, followed by poly(I:C) stimulation (i.p) for 2 h, and measured immune activation in peritoneal macrophages. Compound II significantly reduced poly(I:C)-induced immune gene activation and IRF3 phosphorylation compared with DMSO control (Fig. 2B, 2C). These data demonstrate that Compound II is a potent inhibitor of immune activation of TBK1 in vitro and in vivo.

Compound II ameliorates autoimmune disease phenotypes of Trex1−/− mice and TREX1-mutant patient cells

We previously showed that Trex1 deficiency induces cell-intrinsic immune activation that depends on TBK1 and IRF3 (12). We then asked whether Compound II could suppress the immune gene signature in Trex1−/− cells. We treated wild type and Trex1−/− cells with DMSO or Compound II and measured immune gene activation by quantitative RT-PCR (qRT-PCR). Ifit1 mRNA expression is elevated 90-fold in Trex1−/− cells compared with wild type cells, and Compound II reduced Ifit1 mRNA level in Trex1−/− cells in a dose-dependent manner (Fig. 2D). Many other ISGs were also elevated in Trex1−/− cells compared with wild type cells (but not IFN or inflammatory genes), and the entire ISG signature was completely suppressed by Compound II (Fig. 2E). TREX1 patient lymphoblasts carrying R114H mutation (associated with both AGS and SLE) display a strong ISG signature similar to that of Trex1−/− mouse cells (12). We next treated TREX1-R114H patient lymphoblasts and a healthy control with DMSO or Compound II and measured expression of two ISGs, CXCL10 and RSAD2 (Supplemental Fig. 1C). Both ISGs were significantly reduced after Compound II treatment, suggesting that inhibiting TBK1 by Compound II could be therapeutically useful for treating TREX1 patients with elevated ISG signature.

We next treated Trex1−/− mice with Compound II and examined its effect on autoimmune disease phenotypes associated with Trex1 deficiency. Trex1−/− mice develop autoantibodies, splenomegaly, and liver dysfunction, and the average survival of these mice is 8–10 wk (10, 11). We first
treated Trex1−/− mice i.p. with DMSO or Compound II (three injections per week, 10 mg/kg per injection) and found that overall survival of Trex1−/− mice was significantly improved after 7 wk of Compound II treatment compared with DMSO controls (Fig. 3A). Compound II–treated Trex1−/− mice also have reduced AST level in the serum (indicating improved liver function) and reduced number of splenocytes and spleen size compared with DMSO-treated Trex1−/− mice (Fig. 3B, 3C). We also analyzed autoantibodies in the serum using an autoantigen microarray that contains 95 autoantigens that are commonly found in a variety of autoimmune diseases (16). We found that Trex1−/− mice produce a wide array of autoantibodies compared with wild type littermates and Compound II treatment significantly reduced most of the autoantibodies in Trex1−/− mice (Fig. 3D, 3E). Other phenotypes of Trex1−/− mice, such as diminished hair and reduced mobility, were also dramatically improved after Compound II treatment (data not shown). Trex1−/− mice develop severe systemic inflammation in multiple organs and succumb to disease mostly caused by inflammatory myocarditis (10, 11).

We found that Compound II–treated Trex1−/− mouse heart showed reduced inflammation and pathology compared with DMSO-treated or untreated Trex1−/− heart, although inflammation in the liver did not appear to improve at the point of our analysis (Supplemental Fig. 2). These histological findings are consistent with the partial rescue we have observed, and suggest that some of the tissue damage caused by the inborn genetic defect may not be completely reversible by blocking TBK1-mediated IFN signaling, or it may require an earlier and higher dose treatment. Collectively, our data suggest that targeting TBK1 by Compound II is an effective treatment at ameliorating autoimmune disease phenotypes associated with Trex1 deficiency.

Compound II reduces immune gene signature in SLE patient cells

To further explore the potential application of Compound II in complex autoimmune diseases such as SLE, we first analyzed the expression of TBK1 and other kinases in the IkB family (i.e., IKKα, IKKβ, and IKKe) in SLE and healthy control primary leukocyte subsets. Three leukocyte populations, CD4+CD3+ (T cells), CD19+CD3− (B cells), and CD33+CD3− (myeloid cells), were sorted from PBMCs and analyzed by microarray (14). We found that TBK1 expression...
was consistently elevated in SLE patient cells compared with healthy controls (Fig. 4A), and the elevation is significant in CD4+ and CD33+ populations. Despite that IKK family kinases often act in similar signaling pathways, we did not observe elevated expression of other IKK genes (IKKA, IKKB, and IKKE) in SLEs compared with healthy controls, suggesting that TBK1 or the underlying signaling pathway(s) may be uniquely associated with SLE (data not shown).

To examine whether Compound II is effective at reducing IFN signature in SLE patient cells, we selected several SLE lymphoblast cell lines with low, intermediate, and high ISG signature. We did not observe elevated expression of IFN genes likely due to immortalization of these cell lines. SLE cells with intermediate and high ISG signature responded well to Compound II treatment as indicated by reduced CXCL10 mRNA expression (Fig. 4B). Together, our results identify TBK1 as a potential target in SLE and demonstrate that inhibiting TBK1 by Compound II could be potentially useful for treating SLEs with high IFN signature.

In conclusion, our data demonstrated that inhibiting TBK1 by Compound II is an effective treatment option for a variety of autoimmune diseases with elevated IFN signature. Elevated TBK1 expression or function has been associated with many important human immune disorders and cancer (5–7, 14, 18). Thus, TBK1 represents an attractive therapeutic target, and a potent and specific inhibitor of TBK1 could have broad applications. Several small-molecule inhibitors of TBK1 including Compound II are in various stages of development and clinical trials for treating cancer. These drugs are potentially useful for repurposing to treat autoimmune diseases. Prolonged inhibition of TBK1 may increase the possibility of viral infections; thus, such treatments should proceed with caution or be used in combination with other antiviral therapies. Our findings provided important experimental evidence and a rational for using TBK1 inhibitors such as Compound II for treating autoimmune diseases such as AGS, SLE, and potentially other interferonopathies, many of which are life threatening and currently lack effective treatment.
Acknowledgments
We thank Rolf Brekken and members of the Yan Laboratory for helpful
discussions.

Disclosures
The authors have no financial conflicts of interest.

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