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Discovery of GSK8612, a Highly Selective and Potent TBK1 Inhibitor

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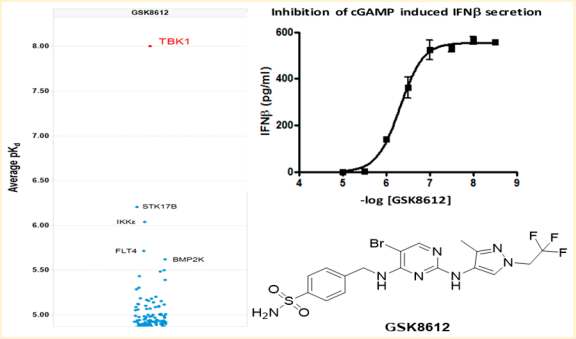
S *Supporting Information*

ABSTRACT: The serine/threonine protein kinase TBK1 (Tank-binding Kinase-1) is a noncanonical member of the IkB kinase (IKK) family. This kinase regulates signaling pathways in innate immunity, oncogenesis, energy homeostasis, autophagy, and neuroinflammation. Herein, we report the discovery and characterization of a novel potent and highly selective TBK1 inhibitor, GSK8612. In cellular assays, this small molecule inhibited toll-like receptor (TLR)3-induced interferon regulatory factor (IRF)3 phosphorylation in Ramos cells and type I interferon (IFN) secretion in primary human mononuclear cells. In THP1 cells, GSK8612 was able to inhibit secretion of interferon beta (IFNβ) in response to dsDNA and cGAMP, the natural ligand for STING. GSK8612 is a TBK1 small molecule inhibitor displaying an excellent selectivity profile and therefore represents an ideal probe to further dissect the biology of TBK1 in models of immunity, neuroinflammation, obesity, or cancer.



KEYWORDS: *TBK1, kinase inhibitor, interferon, inflammation*

TBK1 is a member of the noncanonical IKK family of serine/threonine kinases and a central player in innate immunity.[1,2] Following ligation of TLR3 or TLR4, TBK1 is activated via the adaptor protein TRIF, resulting in the phosphorylation of its target protein IRF3. In a variety of cells including conventional dendritic cells (DC) and macrophages, IRF3 activation subsequently triggers the secretion of IFNβ, an immunomodulatory chemokine.[2−7] Through an autocrine loop via type I IFN receptor (IFNAR) and induction of IRFs and interferon-stimulated genes (ISGs), IFNβ typically primes stimulated cells to produce high levels of IFNα.[8,9]

TLR3 is naturally stimulated by viral double stranded (ds)RNA or by its synthetic mimetic polyinosine−polycytidylic acid (poly(I:C)), a suitable tool to trigger a type I IFN response *in vitro*.[8] Further, TBK1 is activated following dsDNA sensor ligation via the signaling intermediate STING, likewise leading to IRF activation and type I IFN expression.[3,10] In turn, poly(I:C) and IFNα promote STING expression in macrophages and DCs, forming another mechanism of amplifying the IFN-mediated antiviral immune response.[10]

Misregulation of TBK1 activity is associated with autoimmune disorders and has therefore been considered a target for the treatment of (auto)inflammatory diseases.[11−13] This concept is, however, challenged by the observation that TBK1 inhibition may have proinflammatory effects due to its regulatory function in the noncanonical NF-κB pathway.[14−16] More recently, roles for TBK1 in energy homeostasis and tumorigenesis have been revealed, identifying TBK1 as potential target for obesity and cancer.[16,17] Key roles in autophagy and neuroinflammation have also been reported for TBK1.[18] The discovery of TBK1 inhibitors has therefore gained attention from both industrial and academic groups.[19] In this context, the selective degradation of TBK1 using proteolysis targeting chimeras (PROTACs) has been recently explored.[20] Further validation of the effects of TBK1 inhibition in disease-relevant models is required to improve the understanding of the biological function of this key signaling mediator. To this end, the availability of potent and selective chemical probes would greatly facilitate such target validation efforts.[21] We report here the discovery of **GSK8612**, a highly potent and selective inhibitor for TBK1 and its activity in cellular models.

The application of affinity enrichment based chemoproteomics to the discovery of selective probes for PI3Kγ,[22] mTOR,[23] and TNKS[24] was previously described.[25] Using these

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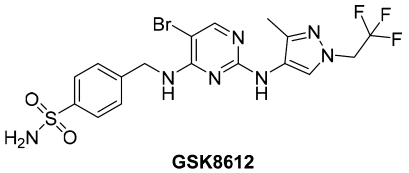
methods, the kinase selectivity of two small molecules used to explore TBK1 biology, **BX795**[26,27] and **MRT67307**,[28,29] was established (Table S1 and Figure S1). **BX795** and **MRT67307** were determined to have an average pK_d of 7.7 and 7.1, respectively, for TBK1 when profiled with kinobeads[30] in a mixture of cell lines and tissue extracts (optimized for a broad protein kinase coverage). However, both molecules show affinity for more than 20 other kinases within a 10-fold window of their TBK1 binding affinity (see Table S1 for full list of kinases). The inhibitor of nuclear factor kappa-B kinase subunit ε (IKKε), a close homologue of TBK1 sharing high sequence identity in the kinase domain, is among these 20 kinases. Of note, both molecules demonstrated highest affinity for AP2-associated protein kinase 1 (AAK1), a protein involved in neuropathic pain.[31] Kinase inhibitors can be classified according to their binding site (catalytic site or allosteric site) and the form of the kinase bound (active or nonactive and Asp-Phe-Gly (DFG) loop in or out).[32] The kinobeads are generated by immobilization of kinase inhibitors that ligate the ATP binding site of kinases. Therefore, molecules that demonstrate competitive binding behavior with the kinobeads are concluded to bind into the ATP site of kinases. In order to investigate to which kinase state these TBK1 inhibitors bind, profiling experiments with the kinobeads were performed using cell extracts with and without enrichment of phosphorylated kinases. The enrichment was achieved by treating Ramos cells with the phosphatase inhibitor Calyculin A before cell lysis. In the Calyculin A extracts, **BX795** and **MRT67307** were determined to have an average pK_d of 7.4 and 7.5, respectively, compared to 6.8 and 6.6 in the control extracts (Table S1 and Figure S2). This shows that **BX795** and **MRT67307** may preferentially bind to the phosphorylated form of TBK1 and the pK_ds determined against this form are within the expected range considering the reported TBK1 inhibition potencies in activity assays.[26,28] Interestingly, in the two different cell lysates, no significant pK_d value changes were observed for other target kinases, such as BMP-2-inducible protein kinase (BMP2K) and serine/threonine kinase 17B (STK17B), which cannot be readily explained (note, AAK1 protein kinase was not detected in Ramos cells).

In an effort to discover new inhibitors of TBK1, the kinase selectivity data of proprietary compounds was interrogated. That revealed a series of 2,4-diaminopyrimidines with good affinity for TBK1, and optimization of this series culminated in the discovery of **GSK8612** (Figure 1). This molecule can be



GSK8612

Figure 1. Structure of TBK1 inhibitor GSK8612.

readily synthesized by two sequential nucleophilic aromatic substitution reactions (Scheme S1; full synthetic details are given in the Supporting Information).

Determination of the physical chemical properties of **GSK8612** (Table 1) revealed that the low log D value translates into aqueous solubility greatly exceeding the determined affinity for TBK1 without being detrimental to its cellular permeability. The low lipophilicity may also contribute to **GSK8612**'s low microsomal clearance in human and rat, and low to medium clearance in mouse. Nonetheless, despite the low log D, **GSK8612** is highly protein bound in mouse, rat, and human blood.

Table 1. Properties of TBK1 Inhibitor GSK8612

assay	value
solubility (CLND)	119 μM
CHROM LogD (pH 7.4)	3.6
artificial membrane permeability	2.1×10^{-5} cm/s
fraction bound in blood (mouse, rat, human)	99.5%, 99.6%, 99.3%
microsomal clearance (mouse, rat, human)	8.5, 2.1, 1.1 mL/min/g tissue
microsomal half-life (mouse, rat, human)	8.5, 34.2, 67.4 min

Kinases are attractive and extensively explored drug targets,[33,34] and the 2,4-diaminopyrimidine core is a common structural motif found in many kinase inhibitors; indeed, both **BX795** and **MRT67307** possess this structural feature. The majority of kinase inhibitors that bind in the ATP binding pocket make two conserved hydrogen bonding interactions with the hinge region of the kinase backbone. **GSK8612** was docked into a crystal structure of TBK1 bound to **MRT67307** (PDB 41WQ),[35] and the predicted binding mode is shown in Figure S3. The model predicts that the N^1 of the pyrimidine nitrogen and the NH linking the pyrimidine and pyrazole make the hinge region interactions with the backbone NH and carbonyl of Cys89, respectively. It is further predicted that the bromine sits toward a lipophilic pocket near Met86 and that the pyrazole may bind in two orientations projected toward the solvent interface. The sulfonamide NH_2 is predicted to make two hydrogen bonds, one with the side chain of Asn140 and the second with the side chain of Asp157, helping to explain the high affinity imparted by this moiety. The combination of the substituted pyrazole moiety on the N^2-amine and the benzyl-sulfonamide moiety on the N^4-amine of the pyrimidine conferred **GSK8612** with high TBK1 affinity and good kinase selectivity. This was revealed in the kinobead selectivity profile of **GSK8612** in extracts from a mixture of cell lines and tissue (Figure 2 and Table S1 for a full list of kinases). An average pK_d of 8.0 was determined for **GSK8612** against TBK1. Within 10-fold affinity with respect to TBK1, no off-targets of **GSK8612** could be identified. The highest affinity protein in our study was STK17B with an average pK_d of 6.2, and 100-fold selectivity was determined over the close family member IKKε (average pK_d = 6.0). Binding of **GSK8612** to AAK1, the highest affinity target for both **BX795** and **MRT67307**, showed a pK_d of 5.1, 1000-fold lower than for TBK1. Profiling of **GSK8612** using the lipid kinase affinity matrix in mixed cell extracts revealed that **GSK8612** bound to one lipid kinase, phosphatidylinositol 4-kinase beta (PI4Kβ), though with only an average pK_d of 5.3 (Table S1). The preference of **GSK8612** to bind to activated or nonactivated TBK1 was investigated using extracts from Ramos cells with and without Calyculin A treatment (Table S1 and Figure S2). This revealed that **GSK8612** has lower affinity for phosphorylated TBK1. An average pK_d for TBK1 of 6.8 was determined in the extracts from Calyculin A treated cells compared to 7.7 in the extracts without Calyculin A treatment. In line with the affinity determined in extracts from Calyculin A-activated cells, **GSK8612** inhibited recombinant TBK1 with an average pIC_{50} of 6.8 in a biochemical functional assay.[36] Interestingly, the pK_d of the closest off-targets of **GSK8612**, such as IKKε,

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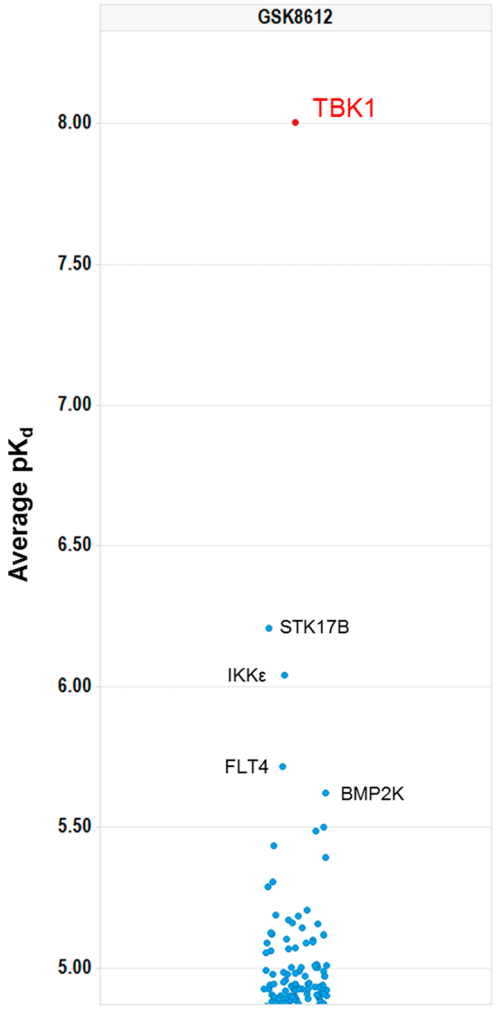

Figure 2. Chemoproteomics based kinase selectivity of **GSK8612** determined using kinobeads in mixed HEK293, K-562, HepG2, and placenta cell extracts and lipid kinobeads in mixed HeLa, Jurkat, and K-562 cell extracts. pK_d values (shown on the *y*-axis) were determined for 285 kinases.

did not show any significant change between Calyculin A treated and control Ramos cells. Therefore, the actual selectivity of **GSK8612** for TBK1 in the cells will depend on the activation state of TBK1. The effects of TBK1 inhibition with **GSK8612** in live cells were then investigated. First, Ramos cells were stimulated with the TLR3 ligand poly(I:C), and the phosphorylation of IRF3 was measured by Western blot. **GSK8612** was able to inhibit phospho-IRF3 with an average pIC_{50} of 6.0 (Figure 3A), confirming effective inhibition of TBK1 kinase activity in live cells. Next, the ability of **GSK8612** to block TBK1-dependent functional responses was evaluated also in primary cells. Type I IFN secretion was measured in poly(I:C)-stimulated human peripheral blood mononuclear cells (PBMCs).[37] Since IFNβ was below the limit of detection of the assay (MSD readout, data not shown) and IFNα could be robustly detected by flow cytometry (CBA assay), the latter was chosen as readout. **GSK8612** inhibited the release of IFNα with a pIC_{50} of 6.1, demonstrating submicromolar potency in primary immune cells (Figure 3B). Of note, IFNα secretion from TLR3-stimulated PBMC is believed to result from a priming effect by IFNβ, a well characterized IFN positive feedback response.[7,9,10] Accordingly, a blocking antibody against IFNAR ablated poly(I:C)-triggered IFNα release in PBMC, confirming IFNα secretion to be a secondary, though biologically relevant downstream effect of TBK1 inhibition (Figure S4). Together, TBK1 inhibition interferes with the activation of the TLR3-IFN axis and disrupts the IFN positive feedback response, resulting in the abrogation of IFNα secretion. It is also well-known that TBK1 propagates biological signaling of IFNβ downstream of DNA sensing by cGAS and STING.[38] To test whether **GSK8612** inhibits this pathway, THP-1 cells were stimulated with dsDNA containing virus (bacmam) or the natural STING ligand cGAMP. **GSK8612** was able to completely inhibit secretion of IFNβ with a measured pIC_{50} of 5.9 and 6.3 for the dsDNA virus and cGAMP stimulated cells, respectively (Figure 4).

In summary, the biological activity of **GSK8612** was demonstrated, resulting in inhibition of IRF3 phosphorylation in Ramos cells, IFNα secretion from human PBMCs, and

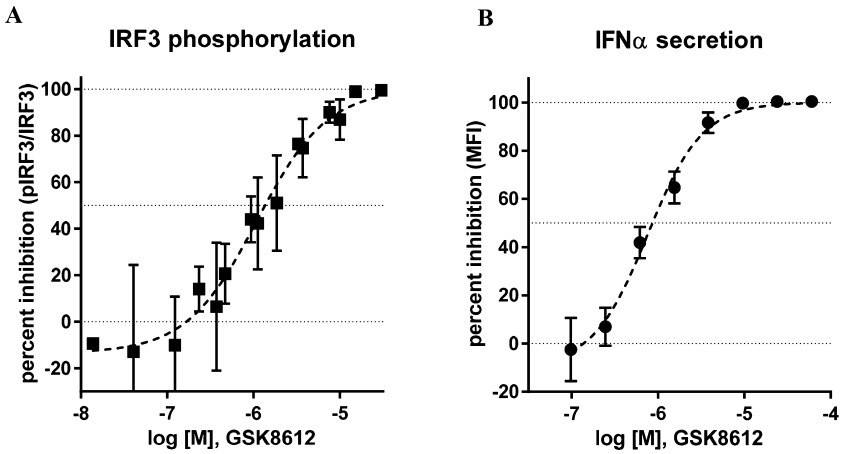

Figure 3. Cell-based activity of **GSK8612**. (A) **GSK8612** inhibits IRF3 phosphorylation in Ramos cells. Western blot analysis reveals inhibition of IRF3 phosphorylation (Ser396) by **GSK8612** in poly(I:C)-stimulated Ramos cells with an average pIC_{50} = 6.0 (n = 5). Western blot densitometry data for pIRF3 normalized to total IRF3 are displayed as percentage of maximal inhibition. (B) GSK8612 inhibits IFNα secretion in human PBMC with an average pIC_{50} = 6.1 (n = 3). IFNα was measured in supernatants of poly(I:C)-stimulated human PBMCs at 16 h by FACS-based Cytometric Bead Array (CBA) assay. Percent of maximal inhibition was derived from normalizing mean fluorescence intensity (MFI) data to vehicle-treated, poly(I:C)-stimulated samples (0% inhibition).

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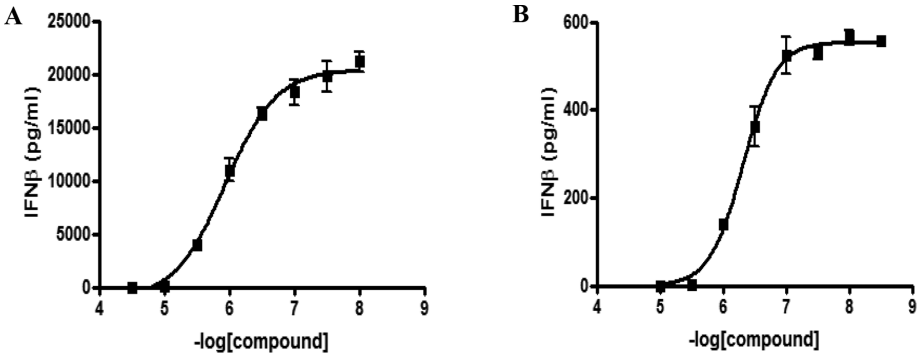

Figure 4. GSK8612 inhibits secretion of IFNβ from THP-1 cells. **GSK8612** inhibits secretion of IFNβ (pg/mL) in THP-1 cells stimulated with (A) dsDNA-containing virus (Baculovirus) with a pIC_{50} = 5.9 (n = 3) or (B) 60 μg/mL cGAMP with a pIC_{50} = 6.3 (n = 3).

IFNβ secretion from THP-1 cells with low micromolar potency. **GSK8612** is a highly selective TBK1 inhibitor, thus representing an ideal tool to further dissect the physiological roles of TBK1 in biological models of immunity, neuroinflammation, obesity, and cancer.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.9b00027.

Synthesis and characterization of **GSK8612**, protocols for selectivity profiling, cellular assays and in vitro physchem properties determination (PDF) Figures on kinase selectivity of **BX795** and **MRT67307,** docking studies and inhibition of IFNα secretion by IFNAR-blocking antibody (PDF)

List of proteins identified and pK_d determined in kinase-binding profiling (XLSX)

Coordinates of docking models (PDB, PDB)

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Author Contributions

$^{\perp}$These authors contributed equally to manuscript. K.S., J.P., D.P., V.K., and G.S.P. contributed to the design, execution, and analysis of the cellular experiments; N.Z., C.R., and B.D. contributed to the design, execution, and analysis of the proteomic experiments; A.P.G. executed and analyzed computation experiments; D.W.T. and A.J.W. contributed to the chemical design and synthesis; M.M. led the chemistry efforts; J.M.R. and M.B. contributed to data interpretation; D.W.T., M.M., D.P., N.Z., A.J.W., G.S.P., and G.B. wrote the manuscript; G.B. designed and supervised the study. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): The authors are employees or former employees of Cellzome GmbH and GlaxoSmithKline, and the company funded the work.

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■ ABBREVIATIONS

IKK, inhibitor of nuclear factor kappa-B kinase; IFNα, interferon-alpha; IFNβ, interferon-beta; IFNAR, interferon-α/β receptor; IL-12, interleukin 12; IRF3, interferon regulatory factor 3; ISG, interferon stimulated gene; poly(I:C), polyinosinic−polycytidylic acid; TBK1, TANK-binding kinase 1; TLR, toll-like receptor

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