

Article

**Discovery of Type II inhibitors of TGF-beta-activated kinase 1 (TAK1)
and Mitogen-activated protein kinase kinase kinase kinase 2 (MAP4K2)**

Li Tan, Tyzoon K. Nomanbhoy, Deepak Gurbani, Matthew Patricelli, John Hunter, Jiefei Geng, Lina Herhaus, Jianming Zhang, Eduardo Pauls, Youngjin Ham, Hwan Geun Choi, Ting Xie, Xianming Deng, Sara J Buhrlage, Taebo Sim, Philip Cohen, Gopal Sapkota, Kenneth Dale Westover, and Nathanael S Gray

J. Med. Chem., **Just Accepted Manuscript** • Publication Date (Web): 17 Jul 2014

Downloaded from <http://pubs.acs.org> on July 18, 2014

Just Accepted

"Just Accepted" manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides "Just Accepted" as a free service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. "Just Accepted" manuscripts appear in full in PDF format accompanied by an HTML abstract. "Just Accepted" manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are accessible to all readers and citable by the Digital Object Identifier (DOI®). "Just Accepted" is an optional service offered to authors. Therefore, the "Just Accepted" Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the "Just Accepted" Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these "Just Accepted" manuscripts.

Discovery of Type II inhibitors of TGF-beta-activated kinase 1 (TAK1)
and Mitogen-activated protein kinase kinase kinase kinase 2 (MAP4K2)

Li Tan^{1,2}, Tyzoon Nomanbhoy³, Deepak Gurbani⁴, Matthew Patricelli³,
John Hunter⁴, Jiefei Geng⁵, Lina Herhaus⁶, Jianming Zhang^{1,2}, Eduardo
Pauls⁶, Youngjin Ham^{1,2}, Hwan Geun Choi¹, Ting Xie^{1,2}, Xianming
Deng^{1,2}, Sara J. Buhrlage¹, Taebo Sim⁷, Philip Cohen⁶, Gopal Sapkota⁶,
Kenneth D. Westover⁴, Nathanael S. Gray^{1,2*}

1. Dana Farber Cancer Institute, Harvard Medical School, Boston, MA,
02115, USA

2. Department of Biological Chemistry and Molecular Pharmacology,
Harvard Medical School, Boston, Massachusetts, 02115, USA

3. ActivX Biosciences, La Jolla, CA 92037, USA

4. Departments of Biochemistry and Radiation Oncology, The University
of Texas, Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas,
TX 75390, USA

5. Department of Cell Biology, Harvard Medical School, Boston,
Massachusetts, 02115, USA

6. MRC Protein Phosphorylation and Ubiquitylation Unit, College of Life
Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, UK

7. Chemical Kinomics Research Center, Korea Institute of Science and
Technology, 39-1, Hawolgok-dong, Seongbuk-gu, Seoul, 136-791, Korea

*Correspondence: nathanael_gray@dfci.harvard.edu (NSG)

Abstract

We developed a pharmacophore model for type II inhibitors that was used to guide the construction of a library of kinase inhibitors. Kinome-wide selectivity profiling of the library resulted in the identification of a series of 4-substituted-1*H*-pyrrolo[2,3-*b*]pyridines that exhibited potent inhibitory activity against two mitogen-activated protein kinases (MAPKs), TAK1 (MAP3K7) and MAP4K2, as well as pharmacologically well interrogated kinases such as p38 α (MAPK14) and ABL. Further investigation of the structure activity relationship (SAR) resulted in the identification of potent dual TAK1 and MAP4K2 inhibitors such as **1** (NG25) and **2** as well as MAP4K2 selective inhibitors such as **16** and **17**. Some of these inhibitors possess good pharmacokinetic properties that will enable their use in pharmacological studies *in vivo*. A 2.4-Å co-crystal structure of TAK1 in complex with **1** confirms that the activation loop of TAK1 assumes the DFG-out conformation characteristic of type II inhibitors.

Introduction

There are approximately 518 kinases encoded in the human genome, and they have been demonstrated to play pivotal roles in virtually all aspects of cellular physiology. Dysregulation of kinase activity through

1
2
3 numerous mechanisms results in many pathologies, but therapeutically
4 relevant kinases have been extensively investigated as anti-cancer agents.
5
6 Clinically the most successful kinase inhibitors directly target
7
8 mutationally activated kinases such as BCR-ABL, EML4-ALK, mutant
9
10 EGFR, B-RAF and c-KIT that are bonafide oncogenes in the context of
11
12 tumors that harbor these lesions. Unfortunately, although genome wide
13
14 cancer sequencing efforts have uncovered a large number of kinases that
15
16 are mutated very few have been biologically validated as being singularly
17
18 important to tumor function. One challenge to efficient pharmacological
19
20 validation of new potential kinase targets is the absence of selective
21
22 inhibitors for the majority of kinases. We sought to develop efficient
23
24 kinase inhibitor discovery strategies that will enable the identification of
25
26 inhibitors with sufficient potency and selectivity that they can be used to
27
28 interrogate the functional and therapeutic consequences of inhibiting less
29
30 well characterized kinases.
31
32
33
34
35
36
37
38
39
40

41 The large majority of known small molecule kinase inhibitors target
42
43 the ATP binding site with the kinase assuming a conformation otherwise
44
45 conducive to catalysis (type I inhibitors). A second, broad class of kinase
46
47 inhibitors (type II) binds to the ATP binding pocket in addition to an
48
49 adjacent hydrophobic pocket that is created when the activation loop,
50
51 which contains the conserved DFG motif, is in an 'out' conformation. A
52
53 number of clinically approved inhibitors such as imatinib, nilotinib and
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

sorafenib have been crystallographically proven to be type II inhibitors of kinases such as ABL, c-KIT, B-RAF and p38 kinases.¹⁻⁴ Based upon these crystal structures we developed a pharmacophore model that defined the structural features that are needed to access this type II binding conformation (Figure 1b).⁵ The model posits the need for a ‘head’ heterocyclic motif that occupies the adenosine binding pocket typically making one to three hydrogen bonds to the kinase hinge segment, a linker moiety that traverses the region occupied by the ‘gatekeeper’ residue, a hydrogen bond donor/acceptor motif and a hydrophobic ‘tail’ that occupies the pocket created by the flip of the ‘DFG’ motif of the kinase activation loop (Figure 1b). We constructed a library of potential type II inhibitors based upon this model and performed kinome-wide selectivity profiling in an effort to identify new inhibitors and the kinases that might be susceptible to inhibition by type II inhibitors.^{6, 7} A library of approximately 100 potential type II inhibitors was screened against a panel of over 420 kinases using the KinomeScanTM approach.^{3a} Two compounds to emerge from this effort are **1** (reported as “NG25” in references)^{8, 9} and **2** which possess binding affinity for TAK1 and MAP4K2 (also known as GCK) (Figure 2). These compounds possess a 4-substituted-1*H*-pyrrolo[2,3-*b*]pyridine as the hinge-interacting “head” motif, a 1,3-benzoic acid linker motif inspired by imatinib and a 3-trifluormethylbenzamide ‘tail’ motif inspired by sorafenib/nilotinib.

Herein, we report the syntheses and target identification of two novel type II kinase inhibitors **1** and **2** of two mitogen activated protein kinases: TAK1 (MAP3K7) and MAP4K2 (GCK). While there have been extensive efforts to target a limited number of MAPKs such as A,B,C-RAF (MAP3Ks), MEK1 (MAP2K1), ERK2 (MAPK1), p38 $\alpha,\beta,\delta,\gamma$ (MAPK14,11,13,12) and JNK1,2,3 (MAPK8,9,10), the majority of other MAPKs have not been subjected to significant inhibitor development efforts. The mitogen activated kinases are typically classified as MAP4Ks (6 members), MAP3Ks (21 members), MAP2Ks (7 members) and MAPKs (14 members) for a total of 49 kinases. TAK1 mediates signaling downstream of multiple cytokine receptors and is functionally important in mitogen, immune and inflammatory signaling pathways.^{10, 11} Recently several inhibitors of TAK1 have been reported and characterized with respect to their anti-inflammatory and anti-cancer activity.¹²⁻¹⁵ The biological functions of MAP4K2 are less well elucidated but it may be a regulator of NF- κ B signaling contributing to cancer development for a subset of malignancies,^{16, 17} and its pharmacological inhibition has been reported to reduce the viability of colon cancer cells.¹⁷ MAP4K2 has also been reported to be required to transduce signals from TGF β receptor to p38.¹⁸ Clearly more selective inhibitors of MAP4K2 would be very useful to further elucidate the functions of this kinase. We demonstrate that **1** and **2** and other lead compounds (Figure 2) can inhibit phosphorylation of

1
2
3
4 proteins predicted to be downstream of TAK1 and MAP4K2 such as IKK,
5
6 p38 and JNK at concentrations of less than 100 nM. Further modification
7
8 of **1** and **2** resulted in the identification of compounds such as **16** and **17**
9
10 that exhibit selectivity for MAP4K2 relative to TAK1. Broad kinase
11
12 selectivity profiling using KiNativTM, revealed that a subset of the
13
14 inhibitors could also target additional kinases such as ABL, ARG, p38,
15
16 SRC, CSK, FER, FES and EPH-family kinases suggesting that these
17
18 kinases are also susceptible to type II inhibition providing a wealth of
19
20 potential starting points for further elaborating inhibitors of these kinases.
21
22
23
24
25
26
27

28 Results

29
30
31 The synthesis of lead compound **1** is outlined in Scheme 1. First,
32
33 pyrrolopyridine **27** was coupled with 3-hydroxy-4-methylbenzoic acid
34
35 using K₂CO₃ as base. The resulting intermediate was subjected to
36
37 hydrogenolysis using Pd/C to afford the dechlorinated acid **28** which was
38
39 then reacted with aniline **29** using HATU/DIEA to provide the desired
40
41 amide **30**. The SEM group of **30** was deprotected under sequential acid
42
43 and basic conditions to furnish compound **1**. This four step sequence
44
45 proceeded with high overall yield.
46
47
48
49
50

51 The preparation of compound **2** is outlined in Scheme 2. The iodide
52
53 intermediate **32** was prepared by reacting aniline **29** and acid **31**.
54
55 Meanwhile, a methoxy group was introduced into aldehyde **33** using
56
57
58
59
60

sodium methoxide followed by Wittig olefination to afford the terminal olefin **34**. The SEM protecting group was replaced with a Boc protecting group to provide compound **35** which was coupled with iodide **32** through Heck reaction to furnish compound **36** in good yield and high trans/cis ratio (>20:1). Deprotection of the Boc protecting group using mildly acidic conditions provided compound **2** with retention of the high E/Z ratio in good overall yield.

Compounds **1** and **2** were screened against a diverse panel of over 420 kinases (DiscoverX, KinomeScan) using an in vitro ATP-site competition binding assay at a concentration of 10 or 1 μM .⁶ These two compounds exhibited relatively selective profiles, and for some kinases known to accommodate a type-II binding mode, e.g., p38 α and ABL, both compounds exhibited potent inhibition. In addition, they also exhibited promising inhibition against several other kinases known to play important roles in cancer-related signaling pathways, but whose functions remain uncharacterized due to a lack of highly selective inhibitors, e.g., TAK1, MAP4K2 and EPHA2. To complement the KinomeScan profiling, both compounds were further profiled at 5 and 0.5 μM concentrations on A375 melanoma cells utilizing the KiNativ technology (ActiveX Biosciences).^{19, 20} This live-cell-treatment approach measures the ability of the compounds to protect a subset of kinases in lysates from labeling with a lysine reactive ATP or ADP -biotin probe. Kinases that exhibited

1
2
3 moderate or high inhibition of labeling by compounds **1** and **2** are listed
4 in Table 1 (see the Supporting Information for full profiling data).
5
6 Consistent with the KinomeScan data, at a concentration of 0.5 μ M, both
7
8 compounds inhibited TAK1, MAP4K2, ZAK, p38 α , SRC and LYN while
9
10 compound **1** also inhibits FER and compound **2** inhibits CSK and
11
12 EPH-family kinases respectively. To confirm the observed binding of **1**
13
14 and **2** to TAK1 and MAP4K2 by the KiNativ approach, we determined
15
16 the IC₅₀ values using biochemical enzyme assays (Invitrogen,
17
18 SelectScreenTM).²¹ Compounds **1** and **2** inhibited TAK1 with IC₅₀s of 149
19
20 nM and 41 nM respectively, and inhibited MAP4K2 with IC₅₀s of 22 nM
21
22 and 98 nM respectively. The *in vitro* IC₅₀s of most other targets from
23
24 KiNativ profiling were also determined and some of them were further
25
26 confirmed while others were not (Table 1). Compound **1** was also
27
28 screened at 0.1 μ M against another panel of over 100 protein kinases
29
30 (International Centre for Kinase Profiling
31
32 <http://www.kinase-screen.mrc.ac.uk/>) and gave similar results (Figure S1);
33
34 the IC₅₀s against TAK1 and MAP4K2 were 15 nM and 17 nM
35
36 respectively in that assay.
37
38
39
40
41
42
43
44
45
46
47
48

49 To further explore the potential of **1** and **2** to be optimized for potency
50 and selectivity against these kinases we synthesized 24 diverse analogs
51 (Table 2). To approach this optimization in a systematic fashion, this
52 chemotype was divided into 3 sections: head, linker and tail, and each of
53
54
55
56
57
58
59
60

these units were varied sequentially. The head unit was altered from pyrrolopyridin-4-yl in **1** to pyrrolopyrimidin-4-yl (e.g., **3**, **13**) and substituted pyrimidin-4-yls (e.g., **6**, **7**); however, analogs of lead compound **2** contained the original head unit. The linker unit was varied through deletion of the methyl group (e.g., **10**, **24**), reversing the amide orientation (**11**, **22**) and altering the alkene in compound **2** via saturation (**14**) or replacement with an ether linkage (**15**). The tail unit contained various hydrophilic groups instead of the 4-ethylpiperazin-1-yl moiety, along with variations of the substitution site from the 4-position to the 3-position. These changes resulted in minor variation to the syntheses with most analogs being prepared via similar routes and in high yields.

All of the prepared analogs were subjected to KiNativ profiling using HUH7 cell lysates which typically detects approximately 150 protein and lipid kinases of interest. This profiling effort revealed that this class of compounds in general provide good multi-targeted scaffolds that can bind to TAK1, MAP4K2, p38 α , Abl, ZAK, CSK, FER, FES, and EPHA2 with varying degrees of potency and selectivity. Herein we primarily focus our discussion on how the structural changes affected the ability of the compounds to inhibit TAK1 and MAP4K2 kinases (Table 3). With compound **1**, we first changed the head from 1*H*-pyrrolo[2,3-*b*]pyridine-4-yl to 7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl (**3**) and 1*H*-pyrazolo[3,4-*d*]pyrimidin-yl (**4**) and found that the activities

against MAP4K2 were maintained, while those against TAK1 were clearly improved. However, the addition of a methyl group at the 6-position of the 7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl moiety (**5**) led to more potent inhibition of MAP4K2 and a decreased potency towards TAK1. We next introduced the monocyclic head units 6-(methylamino)-pyrimidin-4-yl (**6**) and 2-(methylamino)-pyrimidin-4-yl (**7**); **6** exhibited more potent inhibition of MAP4K2 and decreased potency against TAK1, and **7** showed slightly decreased activities. To our surprise, the introduction of a 6,7-dimethoxyquinazolin-4-yl head unit (**8**) yielded potent activity against both kinases while introduction of 4-substituted *N*-methylnicotinamide (**9**) eliminated both TAK1 and MAP4K2 potency. We then varied the linker region and found that deletion of the methyl group (**10**) had little impact on activity; while other modifications only led to loss of activity (**11**). We next investigated the effect of a variety of tail units, and found that all changes led to a decrease in activity against both kinases. However, substitution at the 3-position of the aromatic ring in the tail unit with 4-methyl-1*H*-imidazol-1-yl (**12**) and 4-methylpiperazin-1-yl (**13**) led to only a slight decrease in activity against MAP4K2 but almost complete elimination of TAK1 activity. All analogs of compound **2** generally showed reduced activity against both kinases, with the exception of **14**, which showed similar activity, and **15**, which exhibited a slight decrease in activity against TAK1.

These results indicated that modification of the head unit could improve activity against both kinases, although in most cases more dramatically for MAP4K2 with the exception of **8**. Alteration of the linker unit had little or even negative effect against MAP4K2 and TAK1, while changes in the tail unit always decreased activity against both kinases, although this reduction was small for MAP4K2 but significant for TAK1. This data prompted us to prepare compound **16**, which combined the head of **6**, the tail of **12**, and maintained the original linker of **1**, and exhibited similar inhibitory activity for MAP4K2 compared to **12**. Further modification of the tail of **16** led to **17** which exhibited good biochemical selectivity for inhibiting MAP4K2 relative to TAK1 (37 and 2700 nM IC₅₀s), along with best KiNativ score for MAP4K2 (85% at 1 μ M). The results of KiNativ profiling using HUH7 cell lysates showed that only MAP4K2 and ABL among 220 protein and lipid kinases of interest were inhibited by **17** (Supplemental Table 3), and the inhibition of ABL was rather weak compared with MAP4K2 (49% compared with 85% at 1 μ M). Furthermore, the weak inhibition of p38 α and ZAK of **16** was eliminated with **17** (Table 4). **17** was also subjected to KiNativ live-cell profiling using HUH7 cells and strong inhibition of MAP4K2 (88% at 1 μ M) labeling was observed suggesting that **17** is a potent and relatively selective cellular MAP4K2 inhibitor (Supplemental Table 4).

To further understand the SAR of TAK1/MAP4K2 inhibitors, we

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

solved co-crystal x-ray structures of TAK1 in complex with **1** (PDB 4O91). X-ray diffraction extended to 2.4 Å and the structure was solved using molecular replacement with PDB 2YIY as the initial search model. Examination of the active site revealed strong additional electron density into which **1** was easily modeled (Figure S2A). Final statistics for diffraction and the model are given in Table S1. Interactions with the active site residues are schematically depicted in Figure S2B. In summary, the nitrogens in the pyrrolopyridine moiety form hydrogen bonds with Ala107 in the hinge binding region. The linker amide is stabilized inside the deeper binding pocket by hydrogen bonding with Glu77 and the backbone amide of Asp175. These observations further explain why the reversal of the amide (**11**) resulted in a loss of activity. The nitrogen of the tertiary amine in the piperazinyl tail interacts with carbonyls of Ile153 and His154 on the activation loop (Figure 3c). Compared with the structure of adenosine bound to the active site of TAK1 (PDB 2EVA) which shows DFG in the active, flipped-in conformation (Figure 3b), binding of **1** with TAK1 results in an inactive, DFG-out conformation characteristic of type-II inhibitors (Figure 3a).

51
52
53
54
55
56
57
58
59
60

Simulated docking studies using MAP4K2 as the receptor resulted in a similar overall inhibitor pose relative to the TAK1/**1** co-complex structure. However, the long side chain of Gln80 in the C-helix of TAK1 is located very close to the tail of **1** with only 3.6 Å distance between the

carbonyl of Gln80 side chain and the 5-position of 3-trifluoromethylphenyl group, whereas the shorter side chain of Ile64 in MAP4K2 is not so proximate in the MAP4K2/1 modeling. Substitutions at the 5-position of 3-trifluoromethylphenyl group may be too bulky for the side chain of Gln80 of TAK1, whereas they could possibly interact with the side chains of Ile64 and Gln60 of MAP4K2. This potential interaction might compensate for the loss of interactions with the two carbonyls of Ile133 and His134, providing a rationale for why **12**, **16** and **17** exhibit a preference for binding to MAP4K2 (Figure 3c, d).

Many of our compounds also exhibited potent inhibition of p38 α , the data from KiNativ and enzymatic assays are shown in Table S2. Analogs of **1** that contained the same linker and tail as **1** were all active against p38 α (**3-9**), with **4** being the most potent, and **9** being inactive against TAK1 and MAP4K2. These results indicated that modification of the head unit was tolerated on this scaffold. We were also surprised to find that reversing the amide connectivity of **1** afforded a very selective p38 α inhibitor (**11**). To prepare analogs of compound **2**, we first explored different tail units and found several compounds with potent activity against p38 α (**18-20**), with **19** being the most selective. Modifications of the linker unit, as in compounds **14** and **15**, were also tolerated, and these changes sometimes led to potent activity in compounds with tail units that had previously demonstrated poor activity (i.e., **21**, IC₅₀ < 10 nM).

1
2
3 Similarly, the reversed amide connectivity (**22**) retained potent activity
4 when the methyl of the linker was deleted. In sharp contrast, its analogs
5
6 with the original amide connectivity (**23**, **24**) were inactive against p38 α .
7
8
9

10
11 Many of the compounds exhibited potent binding activity against
12 ABL kinase which is known to be potently inhibited by type II inhibitors
13 such as imatinib and nilotinib. To evaluate whether these new compounds
14
15 could inhibit ABL activity in a cellular context, they were evaluated for
16 their antiproliferative activity against BCR-ABL-dependent Ba/F3 cells
17 (Table S3). To evaluate their potential for non-specific cytotoxicity they
18
19 were also evaluated against parental Ba/F3 cells grown in the presence of
20 interleukin-3. The results demonstrated that many of our compounds
21 selectively inhibited the proliferation of BCR-ABL-dependent Ba/F3
22 cells with EC₅₀ values of less than 400 nM. Several compounds (e.g., **2**,
23
24 **10**) exhibited more than 50% cell growth inhibition at 10 nM, with
25
26 analogs of **2** generally being the most active. These compounds exhibit
27 comparable cellular potency to clinically approved second-generation
28 BCR-ABL inhibitors such as nilotinib and dasatinib. Replacement of the
29 (4-ethylpiperazin-1-yl)methyl tail with a 4-ethylpiperazin-1-yl tail (**25**)
30
31 provided very selective inhibition against ABL (both enzymatic and
32 cellular); more surprising was that elimination of the trifluoromethyl
33 group afforded a very strong inhibitor with high selectivity (**26**).
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55

56 Some of our compounds exhibited potent inhibition of a number of
57
58
59
60

additional kinases (Table S4). In general, analogs of compound **2** were more likely to inhibit ZAK, with **14** being the most active with >96% inhibition at 1 μ M (KiNativ) and an IC_{50} of 72 nM (SelectScreen). Additionally, compound **9**, an analog of **1**, was very selective against ZAK. Only analogs of **2** were found to inhibit CSK, with **14** being the most potent. Compounds **2** and **14** exhibited potent activity against EPHA2, although further modification of their scaffolds led to a severe loss of activity. Surprisingly, we found that only analogs of compound **1** exhibited potency against FES and FER, with the activity of **8** being very strong (93% and 95% inhibition at 1 μ M and IC_{50} s of 51 and 36 nM respectively).

To further validate inhibitory activity against TAK1 and MAP4K2 by these inhibitors, we next measured their ability to block the downstream signals induced by different cytokines. First we checked their effect on NF- κ B signaling in mouse L929 cells following TNF α stimulation, where we found that most inhibitors with strong TAK1 inhibition in previous assays, could effectively reduce the phosphorylation level of IKK α/β at 100 nM concentrations. The degradation of I κ B- α was also effectively inhibited. The reported irreversible TAK1 inhibitor **37** (5Z-7-oxozeaenol)²² was used as positive control and our lead compounds **1** and **2** exhibited comparable cellular potency (Figure 4a). The selective MAP4K2 inhibitors (**12**, **16**, and **17**) and p38 α inhibitors **11**, **38**

(SB203580)²³ and **39** (PH-797804)²⁴ were also tested and demonstrated no effect at the same concentrations (Figure 4b, Figure S3B). The most selective MAP4K2 inhibitor **17** was not very effective even at concentrations up to 1 μ M (Figure S3A), which suggested MAP4K2 does not contribute much to this pathway under these conditions.

Next we checked the effect of **1** in MEFs and human 293 IL-1R cells stimulated with IL-1 α and found that the phosphorylation level of downstream p105, p38 and JNK1/2 were all inhibited in a dose-dependent manner (Figure 5a-b). Similar results were obtained in mouse RAW cells following LPS stimulation (Figure 5c). We also discovered that compound **1** (1 μ M) blocked signaling following stimulation of human Gen2.2 cells with the TLR7 agonist CL097; analog **S1** (Figure S4) which possessed moderate TAK1 and MAP4K2 inhibitory activity (IC₅₀s of 292 nM and 296 nM, respectively) only partially inhibited signaling. The selective MAP4K2 inhibitor **16** was not able to block CL097 induced signaling (Figure 5d).

It was more challenging to validate the functional cellular consequences of MAP4K2 inhibition since the effector pathways of this kinase are less well characterized. TAK1 mediates much of the IL-1-induced phosphorylation of p38^{10, 25} although recent reports suggest that TAK1 does not mediate TGF β -induced phosphorylation of p38 in TAK1-null MEFs and HaCaT cells.¹⁸ A *siRNA*-based screen targeting all

MAP3Ks individually in HaCaT cells suggested roles for MAP3K4 and MAP3K10 in mediating TGF β -induced phosphorylation of p38.¹⁸ A similar *siRNA* screen targeting MAP4Ks in HaCaT cells demonstrated that depletion of MAP4K2 but not MAP4Ks 1, 3 and 5 resulted in substantial inhibition of both IL-1 and TGF β -induced phosphorylation of p38, while TGF β -induced phosphorylation of SMAD2 was unaffected (Figure 6a). Cumulatively these studies suggest a possible role for MAP4K2 in IL-1 and TGF β -induced phosphorylation of p38. We therefore tested the ability of our selective MAP4K2 inhibitors **16** and **17** to inhibit IL-1 and TGF β induced phosphorylation of p38 MAPK in WT and TAK1-deficient MEFs. Both **16** and **17** caused a dose-dependent inhibition of IL-1 and TGF β -induced p38 MAPK phosphorylation but did not inhibit the TGF β -induced phosphorylation of SMAD2 (Figure 6b-c). Consistent with the primary role of TAK1 in mediating IL-1 signaling, in WT MEFs, **16** and **17** caused ~50% inhibition in IL-1-induced phosphorylation of p38 at 500 nM (Figure 6b-c). However, in TAK1-deficient MEFs, both **16** and **17** resulted in complete inhibition of IL-1-induced phosphorylation of p38 at 500 nM, suggesting that MAP4K2 may mediate the residual IL-1-dependent but TAK1-independent phosphorylation of p38 (Figure 6b-c). The dose-dependent inhibition of TGF β -induced phosphorylation of p38 by **16** and **17** was similar in both WT and TAK1-deficient MEFs, consistent

with the notion that TAK1 does not mediate TGF β -induced phosphorylation of p38 in these cells (Figure 6b-c). Compound **1**, a dual inhibitor of TAK1 and MAP4K2, exhibited similar potency to **16** and **17** while the covalent natural product TAK1 inhibitor **37**, inhibited phosphorylation of both p38 and SMAD2 at very low concentrations regardless of presence of TAK1, consistent with the observation that TAK1 mediates much of the IL-1-induced phosphorylation of p38 and that **37** has several additional kinase targets including MEK1 (Figure S5).

The pharmacokinetic properties of **1-5** were also evaluated following intravenous and oral delivery in mice, respectively. Compounds **1** and **5** demonstrated favorable pharmacokinetic properties, with $T_{1/2}$ values of 2.0 and 2.9 h, AUC values of 1369 and 2136 following a 10 mg/Kg oral dose and %*F* values of 70.2 and 38.6, respectively (Table 5). This suggests that these compounds should be useful for murine efficacy studies.

Discussion

In conclusion, compounds **1** and **2**, along with their analogs, represent two novel ‘type II’ kinase inhibitor scaffolds discovered using a kinome-wide profiling approach and exhibit diverse activity against a limited set of kinase targets. Among these compounds, **1** was especially interesting as a very potent TAK1 inhibitor and has already been used in

some studies,^{8,9} and **2** is also being utilized in cancer studies due to its potent anti-proliferative effects against particular tumor types. Profiling of these type II inhibitors reveals that a subset of the compounds also are capable of potently inhibiting LCK, ABL, p38 α , etc. and this needs to be considered when using them to interrogate TAK1 and MAP4K2 dependent effects. As for application of TAK1 inhibitors, the most commonly used probe nowadays is **37**, which is not a selective inhibitor. **37** strongly inhibits MAP2Ks and MAPKs such as MEK1 (MAP2K1) and ERK1 (MAPK1), and many other targets such as KDR, PDGFR, ZAK, etc. Moreover, the binding to several of these targets is covalent and it is likely that other reactive cysteines can be targeted by this compound intracellularly.²⁶ Compound **1** provides a complementary pharmacological probe of TAK1 relative to **37** due to its distinct chemical structure, non-overlapping off-target pharmacology and reversible mode of inhibition. The co-crystal structure of TAK1/**1** has helped in rationalizing the SAR and will be used to design type-II TAK1 inhibitors in the future. Meanwhile **16** and **17** represent relatively selective MAP4K2 inhibitors, especially compound **17** exhibits impressive selectivity, and excellent potency in cellular assays. **11** is a selective p38 α inhibitor, both **25** and **26** are selective ABL inhibitors. By modification of the tail moiety, these inhibitors (**11**, **17** and **26** etc.) can specifically interact with the allosteric DFG-pockets, which may provide

a means of achieving selectivity amongst otherwise highly homologous kinases. The co-crystal structure of TAK1/1 reveals that Gln80 is proximal (3.6 Å) from the phenyl ring of the benzamide ‘tail’ moiety, which suggests that a unique H-bond may be able to be introduced in this region to gain selectivity for TAK1. Finally, given their wide diversity with respective kinase selectivity, enzymatic and cellular potency and favorable pharmacokinetic parameters, 4-substituted-1*H*-pyrrolo [2,3-*b*] pyridines may represent privileged scaffolds for the development of therapeutic agents targeting various kinases.

Experimental Section

Chemistry. Unless otherwise noted, reagents and solvents were obtained from commercial suppliers and were used without further purification. ¹H NMR spectra were recorded on 400 MHz (Varian 7600 AS) or 600 MHz (Varian Inova), and chemical shifts are reported in parts per million (ppm, δ) downfield from tetramethylsilane (TMS). Coupling constants (*J*) are reported in Hz. Spin multiplicities are described as s (singlet), brs (broad singlet), t (triplet), q (quartet), and m (multiplet). Mass spectra were obtained on a Waters Micromass ZQ instrument. Preparative HPLC was performed on a Waters Symmetry C18 column (19 x 50 mm, 5μM) using a gradient of 5-95% acetonitrile in water containing 0.05% trifluoroacetic acid (TFA) over 8 min (10 min run time) at a flow rate of 30 mL/min.

Purification of compounds was performed with either a Teledyne ISCO combiflash Rf system or a Waters Micromass ZQ preparative system. Purities of assayed compounds were in all cases greater than 95%, as determined by reverse-phase HPLC analysis.

4-methyl-3-((1-((2-(trimethylsilyl)ethoxy)methyl)-1*H*-pyrrolo[2,3-*b*]pyridin-4-yl)oxy)benzoic acid (28). To a stirred solution of **27** (570 mg, 2 mmol) and 3-hydroxy-4-methylbenzoic acid (340 mg, 2.2 mmol) in 13 mL of DMSO was added K₂CO₃ (830 mg, 6 mmol). The reaction mixture was allowed to stand for 5 h at 100 °C, and then cooled to RT. The mixture was acidified with 1N HCl solution and extracted with ethyl acetate, the organic phase was washed with brine and dried over Na₂SO₄, then filtered and evaporated to give a pale yellow liquid (690 mg, 80%). To a stirred solution of this gained liquid (690 mg, 1.6 mmol) in 10 mL of methanol was added Pd/C (10 wt. % loading, 120 mg). The reaction mixture was stirred under hydrogen atmosphere for 2 or 3 days. The reaction was monitored by TLC, more Pd/C might be needed for complete conversion during the period. Then the reaction solution was filtered, concentrated, and purified with column chromatography (hexane : ethyl acetate = 1:1) to give the title compound as a white solid (510 mg, 80%). ¹H NMR (400 MHz, CDCl₃) δ 8.30 (d, *J* = 5.6 Hz, 1H), 8.02 (d, *J* = 8.0 Hz, 1H), 7.90 (s, 1H), 7.48 (d, *J* = 8.0 Hz, 1H), 7.33 (d, *J* = 3.6 Hz, 1H), 6.52 (d, *J* = 3.6, 1H), 6.44 (d, *J* = 5.6, 1H), 5.78 (s, 2H), 3.65 (t, *J* =

8.0 Hz, 2H), 2.37 (s, 3H), 0.99 (t, $J = 8.0$ Hz, 2H), 0.00 (s, 9H). MS (ESI) m/z 399 (M+H)⁺.

***N*-(4-((4-ethylpiperazin-1-yl)methyl)-3-(trifluoromethyl)phenyl)-4-methyl-3-((1-((2-(trimethylsilyl)ethoxy)methyl)-1*H*-pyrrolo[2,3-*b*]pyridin-4-yl)oxy)benzamide (30).** To a stirred solution of **28** (400 mg, 1 mmol) and aniline **29** (430 mg, 1.5 mmol) in 10 mL of dichloromethane was added HATU (570 mg, 1.5 mmol), DMAP (185 mg, 1.5 mmol) and DIEA (520 μ L, 3 mmol). The reaction mixture was allowed to stand for 24 h at room temperature, then diluted with ethyl acetate and washed with water and brine, the organic phase was dried over Na₂SO₄, filtered, then concentrated and purified with column chromatography (dichloromethane : methanol = 20:1) to give the title compound as a colorless oil (600 mg, 90%). ¹H NMR (400 MHz, CDCl₃) δ 8.21 (s, 1H), 8.12 (d, $J = 5.6$ Hz, 1H), 7.91 (d, $J = 8.0$ Hz, 1H), 7.74-7.68 (m, 2H), 7.69 (s, 1H), 7.41 (d, $J = 8.0$ Hz, 1H), 7.22 (d, $J = 3.6$, 1H), 6.37 (d, $J = 3.6$, 1H), 6.33 (d, $J = 5.6$, 1H), 5.64 (s, 2H), 3.61 (s, 2H), 3.55 (t, $J = 8.0$ Hz, 2H), 2.65-2.40 (m, 8H), 2.50 (q, $J = 6.8$ Hz, 2H), 2.28 (s, 3H), 1.24 (t, $J = 6.8$ Hz, 3H), 0.90 (t, $J = 8.0$ Hz, 2H), -0.08 (s, 9H). MS (ESI) m/z 668 (M+H)⁺.

3-((1*H*-pyrrolo[2,3-*b*]pyridin-4-yl)oxy)-*N*-(4-((4-ethylpiperazin-1-yl)methyl)-3-(trifluoromethyl)phenyl)-4-methylbenzamide (1). To a stirred ice-cooled solution of **30** (330 mg, 0.5 mmol) in 5 mL of

dichloromethane was added 1 mL of TFA. The reaction mixture was stirred for 30 min at 0 °C, and then warmed to RT. After 5 h the reaction mixture was concentrated and dried under vacuum, then dissolved in 5 mL of THF, and 5 mL of 1N NaOH water solution was added. The reaction mixture was stirred for 24 h and extracted with ethyl acetate. The combined organic phase was washed with brine and dried with Na₂SO₄, then filtered and concentrated, and purified with column chromatography (dichloromethane : methanol = 10:1) to give the title compound as a white solid (188 mg, 70%). ¹H NMR (400 MHz, DMSO) δ 11.78 (bs, 1H), 10.44 (s, 1H), 8.16 (d, *J* = 2.4 Hz, 1H), 8.09 (d, *J* = 5.6 Hz, 1H), 8.02 (dd, *J* = 8.4, 1.6 Hz, 1H), 7.88 (dd, *J* = 8.0, 2.0 Hz, 1H), 7.78 (d, *J* = 2.0 Hz, 1H), 7.69 (d, *J* = 8.8 Hz, 1H), 7.58 (d, *J* = 8.0 Hz, 1H), 7.38 (dd, *J* = 3.2, 2.8, 1H), 6.32 (d, *J* = 5.6, 1H), 6.21 (dd, *J* = 3.2, 2.0 Hz, 1H), 3.56 (s, 2H), 2.52-2.30 (m, 8H), 2.50 (q, *J* = 7.2 Hz, 2H), 2.24 (s, 3H), 1.00 (t, *J* = 7.2 Hz, 3H). ¹³C NMR (100 MHz, DMSO) δ 164.83, 157.26, 152.90, 151.59, 144.78, 138.50, 134.90, 134.11, 132.48, 132.21, 131.61, 125.31, 125.25, 124.04, 120.62, 117.80, 117.74, 110.13, 101.78, 97.24, 57.81, 52.97, 52.66, 51.94, 16.17, 12.18. MS (ESI) *m/z* 538 (M+H)⁺.

***N*-(4-((4-ethylpiperazin-1-yl)methyl)-3-(trifluoromethyl)phenyl)-3-iodo-4-methylbenzamide (32).** To a stirred solution of 3-iodo-4-methylbenzoic acid **31** (262 mg, 1 mmol) and aniline **29** (430 mg, 1.5 mmol) in 10 mL of dichloromethane was added HATU (570 mg,

1.5 mmol), DMAP (185 mg, 1.5 mmol) and DIEA (520 μ L, 3 mmol). The reaction mixture was allowed to stand for 24 h at room temperature, then diluted with ethyl acetate and washed with water and brine, the organic phase was dried over Na₂SO₄, filtered, then concentrated and purified with column chromatography (dichloromethane : methanol 20:1) to give the title compound as a yellow solid (480 mg, 90%). ¹H NMR (600 MHz, CDCl₃) δ 8.35 (br, 1H), 8.33 (s, 1H), 7.91 (m, 2H), 7.80 (d, *J* = 8.4 Hz, 1H), 7.63 (d, *J* = 9.0, 1H), 7.32 (d, *J* = 8.4 Hz, 1H), 3.66 (s, 2H), 3.00-2.58 (m, 8H), 2.71 (m, 2H), 2.48 (s, 3H), 1.26 (t, *J* = 7.2 Hz, 3H). MS (ESI) *m/z* 532 (M+H)⁺.

4-methoxy-1-((2-(trimethylsilyl)ethoxy)methyl)-5-vinyl-1*H*-pyrrolo[2,3-*b*]pyridine (34). To a stirred solution of **33** (400 mg, 1.20 mmol) in MeOH (4 mL), was added sodium methoxide (348 mg, 6.43 mmol). The mixture was stirred at 70 °C for 5 h before being cooled to RT and filtered through celite pad. The filtrate was concentrated under reduced pressure and partitioned between ethyl acetate and water, the aqueous layer was extracted with ethyl acetate three times. The combined organic layer was washed with brine, dried over Na₂SO₄, and concentrated *in vacuo*. The residue was used for the next step without purification. To a stirred solution of methyltriphenylphosphonium iodide (923 mg, 2.28 mmol) in THF (10 mL) was added *n*-butyl lithium (2.5 M in hexane, 0.822 mL, 2.056 mmol) at -78 °C. After 30 min, the reaction mixture was

slowly treated with the obtained residue in THF (5 mL) at -78 °C and stirred for 2 h at -78 °C before being quenched with saturated ammonium chloride solution. The reaction mixture was extracted with ethyl acetate three times and the combined organic layer was washed with brine, dried over Na₂SO₄, filtered, then concentrated and purified with column chromatography (hexane : ethyl acetate = 3:1) to give title compound as a colorless oil (208 mg, 57% in 2 steps). ¹H NMR (600 MHz, CDCl₃) δ 8.38 (s, 1H), 7.22 (m, 1H), 6.97 (m, 1H), 6.74 (m, 1H), 5.77-5.50 (m, 1H), 5.25 (m, 1H), 4.33 (s, 3H), 3.55 (m, 2H), 0.90 (m, 2H), -0.06 (s, 9H). MS (ESI) *m/z* 305 (M+H)⁺.

***tert*-butyl 4-methoxy-5-vinyl-1*H*-pyrrolo[2,3-*b*]pyridine-1-carboxylate (35).** To **34** (140 mg, 0.46 mmol) in CH₂Cl₂ (5 mL) was added TFA (0.5 mL) at 0 °C, the reaction mixture was stirred for 30 min at 0 °C, and then warmed to RT. After 5 h, the reaction mixture was concentrated *in vacuo*, then dissolved in 5 mL of THF, and treated with 5 mL of 1N NaOH. The reaction mixture was stirred for 24 h and extracted with ethyl acetate. The combined organic phase was washed with brine and dried with Na₂SO₄, then filtered and concentrated *in vacuo*. The residue was then dissolved in 5 mL of CH₂Cl₂ and treated with Boc₂O (200 mg, 0.92 mmol) and DIEA (240 μL, 1.38 mmol). The reaction mixture was stirred for 24 h and extracted with ethyl acetate, and the combined organic layer was washed with brine, dried over Na₂SO₄, filtered, then concentrated and purified

with column chromatography (hexane : ethyl acetate = 2:1) to give the title compound as a white solid (100 mg, 80% in 2 steps). ^1H NMR (600 MHz, CDCl_3) δ 8.63 (s, 1H), 7.54 (m, 1H), 6.94 (m, 1H), 6.76 (m, 1H), 5.81 (m, 2H), 5.36 (m, 1H), 4.31 (s, 3H), 1.69 (s, 9H). MS (ESI) m/z 275 ($\text{M}+\text{H}$) $^+$.

(*E*)-tert-butyl

**5-(5-(((4-((4-ethylpiperazin-1-yl)methyl)-3-(trifluoromethyl)phe
nyl)carbamoyl)-2-methylstyryl)-4-methoxy-1*H*-pyrrolo[2,3-*b*]pyridin
e-1-carboxylate (36).** To the mixture of **32** (58 mg, 0.110 mmol), **35** (32 mg, 0.116 mmol), DIEA (480 μL , 0.275 mmol), and tri-*t*-butylphosphine (1.0 M in toluene, 11 μL , 0.022 mmol), was added $\text{Pd}_2(\text{dba})_3$ (10 mg, 0.011 mmol) at RT. The reaction mixture was degassed and stirred under Ar atmosphere at 80 $^\circ\text{C}$ for 15 h before being cooled to RT, the filtered and concentrated. The residue was purified with column chromatography (dichloromethane:methanol 20:1) to give the titled compound as a white solid (60 mg, 80%, *E/Z* > 20:1). ^1H NMR (600 MHz, DMSO) δ 8.50 (s, 1H), 8.49 (s, 1H), 8.13 (s, 1H), 7.97 (s, 1H), 7.90 (d, J = 8.4, 1H), 7.71 (d, J = 7.8, 1H), 7.64 (d, (d, J = 8.4, 1H), 7.50 (d, (d, J = 4.2, 1H), 7.30 (d, J = 16.8, 1H), 7.26 (d, J = 16.8, 1H), 7.20 (d, J = 7.8, 1H), 6.71 (d, J = 4.2, 1H), 4.24 (s, 3H), 3.58 (s, 2H), 2.55 (m, 4H), 2.49 (q, J = 7.2, 2H), 2.41 (s, 3H), 2.16 (m, 2H), 1.89 (m, 2H), 1.63 (s, 9H), 1.11 (t, J = 7.2, 3H). MS (ESI) m/z 678 ($\text{M}+\text{H}$) $^+$.

(*E*)-*N*-(4-((4-ethylpiperazin-1-yl)methyl)-3-(trifluoromethyl)phenyl)-3-(2-(4-methoxy-1*H*-pyrrolo[2,3-*b*]pyridin-5-yl)vinyl)-4-methylbenzamide (2). To a solution of **36** (60 mg, 0.088 mmol) in CH₂Cl₂ (2 mL) was added TFA (0.2 mL) at 0 °C, the reaction mixture was stirred at 0 °C for 2.5 h then concentrated and purified with column chromatography (dichloromethane:methanol = 10:1) to give title compound as a white solid (45 mg, 90%, *E/Z* > 20:1). ¹H NMR (600 MHz, DMSO) δ 11.73 (s, 1H), 10.52 (s, 1H), 8.50 (s, 1H), 8.23 (d, *J* = 14.4, 1H), 8.06 (d, *J* = 8.4, 1H), 7.78 (d, *J* = 8.4, 1H), 7.72 (d, *J* = 8.4, 1H), 7.44 (s, 1H), 7.43 (s, 1H), 7.37 (d, *J* = 13.8, 1H), 7.36 (d, *J* = 7.2, 1H), 6.82 (m, 1H), 4.35 (s, 3H), 3.56 (s, 2H), 2.60-2.20 (m, 10H), 2.48 (s, 3H), 0.99 (t, *J* = 7.2, 3H). ¹³C NMR (100 MHz, DMSO) δ 166.14, 157.01, 151.66, 143.64, 139.80, 137.41, 132.78, 132.40, 131.64, 130.89, 127.65, 126.59, 125.35, 124.334, 124.22, 124.07, 117.73, 114.16, 108.40, 100.31, 59.52, 57.91, 53.21, 52.80, 52.01, 20.09, 12.38. MS (ESI) *m/z* 578 (M+H)⁺.

Compounds **3-26** were synthesized with same procedures as **1** and **2**.

37-39 were commercial from Selleckchem.com.

TAK1-TAB1 expression and purification. DNA encoding the TAK1-TAB1 fusion protein (kinase domain residues 31-303 and c-terminal domain residues 468-497) was obtained from GeneScript (GenScript USA Inc. 860 Centennial Ave. Piscataway, NJ 08854, USA). This was cloned into the pFastBac His6 TEV LIC cloning vector (4B)

(Plasmid 30115) from). TAK1-TAB1 fusion protein was expressed in Hi5 insect cells and purified as described previously.^{27, 28}

TAK1-TAB1/1 Crystallization and Structure Determination.

TAK1-TAB1 was concentrated to 10mg/ml and crystallized as reported previously²⁸ with minor modifications. Briefly, the crystals were obtained using the hanging-drop method at 20 °C in 4μl drops by mixing protein with equal volumes of reservoir solution [0.65-0.75M sodium citrate, 0.2M NaCl, 0.1M Tris pH 7.0 and 5mM Adenosine]. The crystals were washed three times in reservoir solution without adenosine. A 10 mM solution of **1** was prepared and crystals were back soaked for overnight. Crystals were frozen for data collection using 20% ethyleneglycol as cryoprotectant. Diffraction data was collected at Argonne Advanced Photon Source (beamline 19-D) and processed with HKL3000.²⁹ The structure was solved by molecular replacement using Phaser,³⁰ using inactive TAK1-TAB1 structures (PDB code 2YIY) as search model. Coot was used for model building³¹ and refinement was carried out using both Phenix version 1.8.4³² and Refmac version 5.8.0049.^{18, 21} Figures were generated by PyMol (The PyMOL Molecular Graphics System, Version 1.6.0.0) and Meastro (Version 1.5.014) from Schrödinger, LLC.

Ba/F3 Cell Proliferation Assay. Compound efficacy against cell proliferation was conducted in 96-well plates. Compounds were added in serial dilutions to cell culture. After 48 hours co-cultured with

compounds, cell viability was measured using CellTiter-Glo (Promega, Wisconsin), and IC₅₀s were determined by XLfit4.0 (IDBS).

Cell culture and stimulations: TAK1-deficient (TAK1^{-/-}) and corresponding wild type (WT) mouse embryonic fibroblasts (MEFs) were a generous gift from S. Akira (Osaka University, Japan).²⁵ Human keratinocyte (HaCaT) cells, WT and TAK1^{-/-} MEFs were cultured in 10-cm diameter dishes in Dulbecco's Modified Eagle's medium supplemented with 10% Foetal Bovine Serum, 1% penicillin/streptomycin mix and 2 mM L-Glutamine under a humidified atmosphere with 5% CO₂ at 37 °C. In order to knockdown the indicated panels of human MAP4Ks, a pool of four siRNA duplexes designed against each target were purchased from Dharmacon (sold as SMARTpool siRNA) and transfected onto HaCaT cells as described previously.¹⁸ Prior to stimulation with appropriate ligands, cells were cultured in DMEM containing 0.1% FBS for 16h. Inhibitors were added 1 h prior to stimulation with TGFβ (50 pM; 45 min), human IL-1β (1 μg/ml; 10 min) or mouse IL-1α (5 ng/ml; 10 min). Cells were lysed in 0.5 ml ice-cold complete lysis buffer (50 mM Tris-HCl pH7.5, 1 mM EGTA, 1 mM EDTA, 1 % Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5mM sodium pyrophosphate, 0.27 M sucrose, 5 mM β-glycerophosphate, 0.1 % (v/v) 2-mercaptoethanol, 0.5 μM microcystin-LR, 1 tablet per 25 ml of complete protease inhibitor

cocktail). The extracts were cleared by centrifuging at 16,000 g at 4 °C for 10 minutes and processed for Western blot analysis as described previously.¹⁸

Western blot. Cells were harvested in lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, and cocktails of protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO). Cell lysates were clarified by centrifugation for 5 min and the protein concentration of the supernatants was determined using a modified Bradford assay (Bio-Rad, Hercules, CA). For immunoblotting, 20 µg of protein was loaded in each lane, and was separated by SDS-PAGE on 4-12% gradient gels (Invitrogen, Carlsbad, California), transferred to PVDF membranes and detected by immunoblotting with the following primary antibodies. Goat anti-mouse and anti-rabbit secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) conjugated to horseradish peroxidase were used at a 1:3,000 dilution and immunoreactive bands were detected by chemiluminescence (SuperSignal, Pierce, Rockford, IL) and film (Denville Scientific, South plainfield, NJ).

In Vivo Pharmacokinetic Studies. Male Swiss albino mice were dosed via tail vein (intravenous, solution in 20% w/v hydroxy propyl beta cyclodextrin in 25 mM sodium phosphate buffer, dose 1 mg/kg) or via oral gavage (suspensions in 0.5% w/v Na CMC with 0.1% v/v Tween-80

1
2
3 in water). Blood samples were collected at 0, 0.083 (for IV only), 0.25,
4
5
6 0.5, 1, 2, 4, 6 (For PO only), 8, 12, and 24 h for the IV and PO groups.
7
8
9 The blood samples were collected from sets of three mice at each time
10
11 point in labeled micro centrifuge tubes containing K₂EDTA as an
12
13 anticoagulant. Plasma samples were separated by centrifugation and
14
15 stored below -70 °C until bioanalysis. All samples were processed for
16
17 analysis by precipitation using acetonitrile and analyzed with a partially
18
19 validated LC/MS/MS method (LLOQ, 1.138 ng/mL). Pharmacokinetic
20
21 parameters were calculated using the noncompartmental analysis tool of
22
23 WinNonlin Enterprise software (version 5.2).
24
25
26
27
28

29 **Acknowledgements.** This study was supported by research funding
30
31 from NIH grants CA130876-05 (N.S.G.), HG006097-03 (N.S.G) and
32
33 CPRIT R1207 (K.D.W.). We thank Diana Tomchick, Zhe Chen, the staff
34
35 at the structural biology laboratory at UT Southwestern Medical Center
36
37 and at beamline 19ID of Advanced Photon Source for discussions and
38
39 technical assistance with X-ray data collection and processing. Results
40
41 shown in this report are derived from work performed at Argonne
42
43 National Laboratory, Structural Biology Center at the Advanced Photon
44
45 Source. Argonne is operated by UChicago Argonne, LLC, for the U.S.
46
47 Department of Energy, Office of Biological and Environmental Research
48
49 under contract DE-AC02-06CH11357. The Gen2.2 cells used in this
50
51 study were generously provided by Dr. Joel Plumas and Dr. Laurence
52
53
54
55
56
57
58
59
60

Chaperot. We thank Dr. Ying Li for her suggestion and help to this study.

Abbreviations. TAK1, TGF-beta-activated kinase 1; MAP4K2, Mitogen-activated protein kinase kinase kinase 2; GCK, germinal center kinase; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; TGF β , transforming growth factor beta; IKK, kinase of I κ B; I κ B- α , nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; JNK, c-Jun N-terminal kinases; ABL, Abelson murine leukemia viral oncogene homolog 1; ARG, Abelson-related gene; BCR, breakpoint cluster region protein; EPHA2, ephrin type-A receptor 2; SRC, sarcoma; CSK, C-SRC tyrosine kinase; FES, feline sarcoma oncogene; FER, FES related tyrosine kinase; LYN, Tyrosine-protein kinase Lyn; ZAK, Sterile alpha motif and leucine zipper containing kinase AZK; TNF α , tumor necrosis factors alpha; IL-1 α , interleukin 1 alpha; LPS, lipopolysaccharides; TLR7, toll-like receptor 7; siRNA, small interfering RNA; SMAD2, Mothers against decapentaplegic homolog 2; LIC, ligation independent cloning; T_{1/2}, half-life period; AUC, area under concentration-time curve; F, fraction of bioavailability; SAR, structure-activity relationship; TLC, thin-layer chromatography; RT, room temperature; TMS, tetramethylsilane; ppm, parts per million; HPLC, high performance liquid chromatography; Boc, *N*-tert-butoxycarbonyl; DIEA, *N,N*-diisopropylethylamine; DMAP, 4-(dimethylamino)pyridine; DMSO, dimethyl sulfoxide; HATU, O-(7-azabenzotriazol-1-yl)-*N,N,N',N'*

-tetramethyluronium hexafluorophosphate; $\text{Pd}_2(\text{dba})_3$,
tris(dibenzylideneacetone)dipalladium(0), SEM,
2-(trimethylsilyl)ethoxymethyl; TFA, trifluoroacetic acid.

Supporting Information. Figure S1-5; Structures of **37-39** (Figure S6); Table S1-4; Structures and spectral data of **3-26**; Ambit profiling data of **1** and **2**, and ActiveX profiling data of **1** and **2**, **17**. This material is available free of charge via the Internet at <http://pubs.acs.org>. X-ray coordinates and structure factors have been deposited in the Protein Data Bank. PDB accession code: 4O91.

Corresponding Author Information. Phone: 1-617-582-8590;
E-mail: nathanael_gray@dfci.harvard.edu.

Reference:

1. O'Hare, T.; Shakespeare, W. C.; Zhu, X.; Eide, C. A.; Rivera, V. M.; Wang, F.; Adrian, L. T.; Zhou, T.; Huang, W. S.; Xu, Q.; Metcalf, C. A., 3rd; Tyner, J. W.; Loriaux, M. M.; Corbin, A. S.; Wardwell, S.; Ning, Y.; Keats, J. A.; Wang, Y.; Sundaramoorthi, R.; Thomas, M.; Zhou, D.; Snodgrass, J.; Commodore, L.; Sawyer, T. K.; Dalgarno, D. C.; Deininger, M. W.; Druker, B. J.; Clackson, T. AP24534, a pan-BCR-ABL inhibitor for chronic myeloid leukemia, potently inhibits the T315I mutant and overcomes mutation-based resistance. In *Cancer Cell*, 2009/11/03 ed.; 2009; Vol. 16, pp 401-412.
2. Gajiwala, K. S.; Wu, J. C.; Christensen, J.; Deshmukh, G. D.; Diehl,

W.; DiNitto, J. P.; English, J. M.; Greig, M. J.; He, Y. A.; Jacques, S. L.; Lunney, E. A.; McTigue, M.; Molina, D.; Quenzer, T.; Wells, P. A.; Yu, X.; Zhang, Y.; Zou, A.; Emmett, M. R.; Marshall, A. G.; Zhang, H. M.; Demetri, G. D. KIT kinase mutants show unique mechanisms of drug resistance to imatinib and sunitinib in gastrointestinal stromal tumor patients. *Proc Natl Acad Sci U S A* **2009**, 106, 1542-1547.

3. Gould, A. E.; Adams, R.; Adhikari, S.; Aertgeerts, K.; Afroze, R.; Blackburn, C.; Calderwood, E. F.; Chau, R.; Chouitar, J.; Duffey, M. O.; England, D. B.; Farrer, C.; Forsyth, N.; Garcia, K.; Gaulin, J.; Greenspan, P. D.; Guo, R.; Harrison, S. J.; Huang, S. C.; Iartchouk, N.; Janowick, D.; Kim, M. S.; Kulkarni, B.; Langston, S. P.; Liu, J. X.; Ma, L. T.; Menon, S.; Mizutani, H.; Paske, E.; Renou, C. C.; Rezaei, M.; Rowland, R. S.; Sintchak, M. D.; Smith, M. D.; Stroud, S. G.; Tregay, M.; Tian, Y.; Veiby, O. P.; Vos, T. J.; Vyskocil, S.; Williams, J.; Xu, T.; Yang, J. J.; Yano, J.; Zeng, H.; Zhang, D. M.; Zhang, Q.; Galvin, K. M. Design and optimization of potent and orally bioavailable tetrahydronaphthalene Raf inhibitors. *J Med Chem* **2011**, 54, 1836-1846.

4. Simard, J. R.; Getlik, M.; Grutter, C.; Pawar, V.; Wulfert, S.; Rabiller, M.; Rauh, D. Development of a fluorescent-tagged kinase assay system for the detection and characterization of allosteric kinase inhibitors. *J Am Chem Soc* **2009**, 131, 13286-13296.

5. Liu, Y.; Gray, N. S. Rational design of inhibitors that bind to inactive

kinase conformations. *Nat Chem Biol* **2006**, 2, 358-364.

6. Miduturu, C. V.; Deng, X.; Kwiatkowski, N.; Yang, W.; Brault, L.; Filippakopoulos, P.; Chung, E.; Yang, Q.; Schwaller, J.; Knapp, S.; King, R. W.; Lee, J. D.; Herrgard, S.; Zarrinkar, P.; Gray, N. S.

High-throughput kinase profiling: a more efficient approach toward the discovery of new kinase inhibitors. *Chem Biol* **2011**, 18, 868-879.

7. Goldstein, D. M.; Gray, N. S.; Zarrinkar, P. P. High-throughput kinase profiling as a platform for drug discovery. *Nat Rev Drug Discov* **2008**, 7, 391-397.

8. Pauls, E.; Shpiro, N.; Pegg, M.; Young, E. R.; Sorcek, R. J.; Tan, L.; Choi, H. G.; Cohen, P. Essential role for IKKbeta in production of type 1 interferons by plasmacytoid dendritic cells. *J Biol Chem* **2012**, 287, 19216-19228.

9. Dzamko, N.; Inesta-Vaquera, F.; Zhang, J.; Xie, C.; Cai, H.; Arthur, S.; Tan, L.; Choi, H.; Gray, N.; Cohen, P.; Pedrioli, P.; Clark, K.; Alessi, D. R. The IkappaB kinase family phosphorylates the Parkinson's disease kinase LRRK2 at Ser935 and Ser910 during Toll-like receptor signaling. *PLoS One* **2012**, 7, e39132.

10. Sakurai, H. Targeting of TAK1 in inflammatory disorders and cancer. *Trends Pharmacol Sci* **2012**, 33, 522-530.

11. Dai, L.; Aye Thu, C.; Liu, X. Y.; Xi, J.; Cheung, P. C. TAK1, more than just innate immunity. *IUBMB Life* **2012**, 64, 825-834.

12. Buglio, D.; Palakurthi, S.; Byth, K.; Vega, F.; Toader, D.; Saeh, J.; Neelapu, S. S.; Younes, A. Essential role of TAK1 in regulating mantle cell lymphoma survival. *Blood* **2012**, 120, 347-355.
13. Hornberger, K. R.; Chen, X.; Crew, A. P.; Kleinberg, A.; Ma, L.; Mulvihill, M. J.; Wang, J.; Wilde, V. L.; Albertella, M.; Bittner, M.; Cooke, A.; Kadhim, S.; Kahler, J.; Maresca, P.; May, E.; Meyn, P.; Romashko, D.; Tokar, B.; Turton, R. Discovery of 7-aminofuro[2,3-c]pyridine inhibitors of TAK1: Optimization of kinase selectivity and pharmacokinetics. *Bioorg Med Chem Lett* **2013**, 23, 4511-4516.
14. Hornberger, K. R.; Berger, D. M.; Crew, A. P.; Dong, H.; Kleinberg, A.; Li, A. H.; Medeiros, M. R.; Mulvihill, M. J.; Siu, K.; Tarrant, J.; Wang, J.; Weng, F.; Wilde, V. L.; Albertella, M.; Bittner, M.; Cooke, A.; Gray, M. J.; Maresca, P.; May, E.; Meyn, P.; Peick, W., Jr.; Romashko, D.; Tanowitz, M.; Tokar, B. Discovery and optimization of 7-aminofuro[2,3-c]pyridine inhibitors of TAK1. *Bioorg Med Chem Lett* **2013**, 23, 4517-4522.
15. Kilty, I.; Green, M. P.; Bell, A. S.; Brown, D. G.; Dodd, P. G.; Hewson, C.; Hughes, S. J.; Phillips, C.; Ryckmans, T.; Smith, R. T.; van Hoorn, W. P.; Cohen, P.; Jones, L. H. TAK 1 Inhibition in the DFG -Out Conformation. *Chem Biol Drug Des* **2013**.
16. Ivanov, V. N.; Kehrl, J. H.; Ronai, Z. Role of TRAF2/GCK in

- melanoma sensitivity to UV-induced apoptosis. *Oncogene* **2000**, 19, 933-942.
17. Zhang, T.; Inesta-Vaquera, F.; Niepel, M.; Zhang, J.; Ficarro, S. B.; Machleidt, T.; Xie, T.; Marto, J. A.; Kim, N.; Sim, T.; Laughlin, J. D.; Park, H.; LoGrasso, P. V.; Patricelli, M.; Nomanbhoy, T. K.; Sorger, P. K.; Alessi, D. R.; Gray, N. S. Discovery of potent and selective covalent inhibitors of JNK. *Chem Biol* **2012**, 19, 140-154.
18. Sapkota, G. P. The TGFbeta-induced phosphorylation and activation of p38 mitogen-activated protein kinase is mediated by MAP3K4 and MAP3K10 but not TAK1. *Open Biol* **2013**, 3, 130067.
19. Patricelli, M. P.; Szardenings, A. K.; Liyanage, M.; Nomanbhoy, T. K.; Wu, M.; Weissig, H.; Aban, A.; Chun, D.; Tanner, S.; Kozarich, J. W. Functional interrogation of the kinome using nucleotide acyl phosphates. *Biochemistry* **2007**, 46, 350-358.
20. Patricelli, M. P.; Nomanbhoy, T. K.; Wu, J.; Brown, H.; Zhou, D.; Zhang, J.; Jagannathan, S.; Aban, A.; Okerberg, E.; Herring, C.; Nordin, B.; Weissig, H.; Yang, Q.; Lee, J. D.; Gray, N. S.; Kozarich, J. W. In situ kinase profiling reveals functionally relevant properties of native kinases. *Chem Biol* **2011**, 18, 699-710.
21. Lebakken, C. S.; Riddle, S. M.; Singh, U.; Frazee, W. J.; Eliason, H. C.; Gao, Y.; Reichling, L. J.; Marks, B. D.; Vogel, K. W. Development and applications of a broad-coverage, TR-FRET-based kinase binding

assay platform. *J Biomol Screen* **2009**, 14, 924-935.

22. Ninomiya-Tsuji, J.; Kajino, T.; Ono, K.; Ohtomo, T.; Matsumoto, M.; Shiina, M.; Mihara, M.; Tsuchiya, M.; Matsumoto, K. A resorcylic acid lactone, 5Z-7-oxozeaenol, prevents inflammation by inhibiting the catalytic activity of TAK1 MAPK kinase kinase. *J Biol Chem* **2003**, 278, 18485-18490.

23. Arana-Argaez, V. E.; Delgado-Rizo, V.; Pizano-Martinez, O. E.; Martinez-Garcia, E. A.; Martin-Marquez, B. T.; Munoz-Gomez, A.; Petri, M. H.; Armendariz-Borunda, J.; Espinosa-Ramirez, G.; Zuniga-Tamayo, D. A.; Herrera-Esparza, R.; Vazquez-Del Mercado, M. Inhibitors of MAPK pathway ERK1/2 or p38 prevent the IL-1 {beta}-induced up-regulation of SRP72 autoantigen in Jurkat cells. *J Biol Chem* **2010**, 285, 32824-32833.

24. Pereira, L.; Igea, A.; Canovas, B.; Dolado, I.; Nebreda, A. R. Inhibition of p38 MAPK sensitizes tumour cells to cisplatin-induced apoptosis mediated by reactive oxygen species and JNK. *EMBO Mol Med* **2013**, 5, 1759-1774.

25. Sato, S.; Sanjo, H.; Takeda, K.; Ninomiya-Tsuji, J.; Yamamoto, M.; Kawai, T.; Matsumoto, K.; Takeuchi, O.; Akira, S. Essential function for the kinase TAK1 in innate and adaptive immune responses. *Nat Immunol* **2005**, 6, 1087-1095.

26. Schirmer, A.; Kennedy, J.; Murli, S.; Reid, R.; Santi, D. V. Targeted

- covalent inactivation of protein kinases by resorcylic acid lactone polyketides. *Proc Natl Acad Sci U S A* **2006**, 103, 4234-4239.
27. Brown, K.; Vial, S. C.; Dedi, N.; Long, J. M.; Dunster, N. J.; Cheetham, G. M. Structural basis for the interaction of TAK1 kinase with its activating protein TAB1. *J Mol Biol* **2005**, 354, 1013-1020.
28. Wu, J.; Powell, F.; Larsen, N. A.; Lai, Z.; Byth, K. F.; Read, J.; Gu, R. F.; Roth, M.; Toader, D.; Saeh, J. C.; Chen, H. Mechanism and in vitro pharmacology of TAK1 inhibition by (5Z)-7-Oxozeaenol. *ACS Chem Biol* **2013**, 8, 643-650.
29. Minor, W.; Cymborowski, M.; Otwinowski, Z.; Chruszcz, M. HKL-3000: the integration of data reduction and structure solution--from diffraction images to an initial model in minutes. *Acta Crystallogr D Biol Crystallogr* **2006**, 62, 859-866.
30. Collaborative Computational Project, N. The CCP4 suite: programs for protein crystallography. *Acta Crystallogr D Biol Crystallogr* **1994**, 50, 760-763.
31. Emsley, P.; Cowtan, K. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* **2004**, 60, 2126-2132.
32. Adams, P. D.; Afonine, P. V.; Bunkoczi, G.; Chen, V. B.; Davis, I. W.; Echols, N.; Headd, J. J.; Hung, L. W.; Kapral, G. J.; Grosse-Kunstleve, R. W.; McCoy, A. J.; Moriarty, N. W.; Oeffner, R.; Read, R. J.; Richardson, D. C.; Richardson, J. S.; Terwilliger, T. C.;

1
2
3
4 Zwart, P. H. PHENIX: a comprehensive Python-based system for
5
6 macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr*
7
8 **2010**, 66, 213-221.
9

Figure Legends:

10
11
12
13
14
15
16
17
18
19
20
21 Figure 1. A general pharmacophore model for the rational design of type
22
23 II inhibitors. (a) Examples of known type-II inhibitors, which can be
24
25 divided into a 'type I' head (black) attached to a 'type II' tail (blue). (b) A
26
27 schematic representation of the rational design of new type II kinase
28
29 inhibitors. A, hydrogen bond acceptor; D, hydrogen bond donor; HRB,
30
31 hinge-region binding; HM, hydrophobic motif.
32
33
34
35
36
37
38

39 Figure 2. Chemical structures of 'lead' compounds.
40
41
42
43

44 Figure 3. Compound **1** is a Type II inhibitor. (a) Binding of **1** to the active
45
46 site of TAK1-TAB1 results in the DFG-out conformation characterized
47
48 by Type II inhibitors. (b) The structure of adenosine bound to the active
49
50 site of TAK1-TAB1 (PDBID 2EVA) is provided for comparison and
51
52 shows the DFG-in conformation. The DFG motif is highlighted in red. (c)
53
54 Key interactions of **1** with TAK1. (d) Molecular model of the binding
55
56
57
58
59
60

mode of MAP4K2 with **1**.

Figure 4. Evaluation of the ability of compounds to block TNF α -induced phosphorylation of IKK α/β and stabilize I κ B- α in L929 cells. L929 cells were pre-treated with indicated: (a) TAK1/MAP4K2 inhibitors (**1**, **2**, **3**, **4**, **5**, **7**, **10**) and covalent TAK1 inhibitor (**37**) (b) more selective MAP4K2 inhibitors (**12**, **16**, **17**, **11**) and p38 inhibitor (**38**) at a concentration of 100 nM for 30 min, and then TNF α was added and incubated for 5 min. Samples were collected and subjected to Western blot. Tubulin was used as a loading control.

Figure 5. Evaluation of the ability of compounds to inhibit IL-1 α , LPS or CL097-induced phosphorylation of p105, p38 and JNK in several cell types. (a)-(c) 293 IL-1R, MEFs and RAW cells were pre-treated with **1** at different concentrations for 1 hour then stimulated with IL-1 α or LPS for 30 min. Samples were collected and subjected to Western blot for phosphorylation of p105, p38, JNK1/2 and total p38. (d) Gen2.2 cells were pre-treated with **1**, **S1** and **16** at 1 μ M for 1 hour then stimulated with CL097 for 30 min, Samples were collected and subjected to Western blot.

Figure 6. Evaluation of signaling following depletion of MAP4Ks by siRNA and evaluation of the ability of selective MAP4K2 inhibitors **16** and **17** to inhibit signaling in wild-type and TAK1-null MEF cells. (a) HaCaT cells were transfected with pools of 4 different siRNAs targeted against the indicated MAP4Ks. 48h-post transfection cells were left untreated or treated with TGF β or IL-1 β prior to lysis and Western blot. (b) Wild type and TAK1-null MEFs cells were pre-treated with selective MAP4K2 inhibitor compound **16** and **17** for 1 hour, followed by stimulation with TGF β or IL-1 α . Samples were collected and subjected to Western blot.

Figure 1.

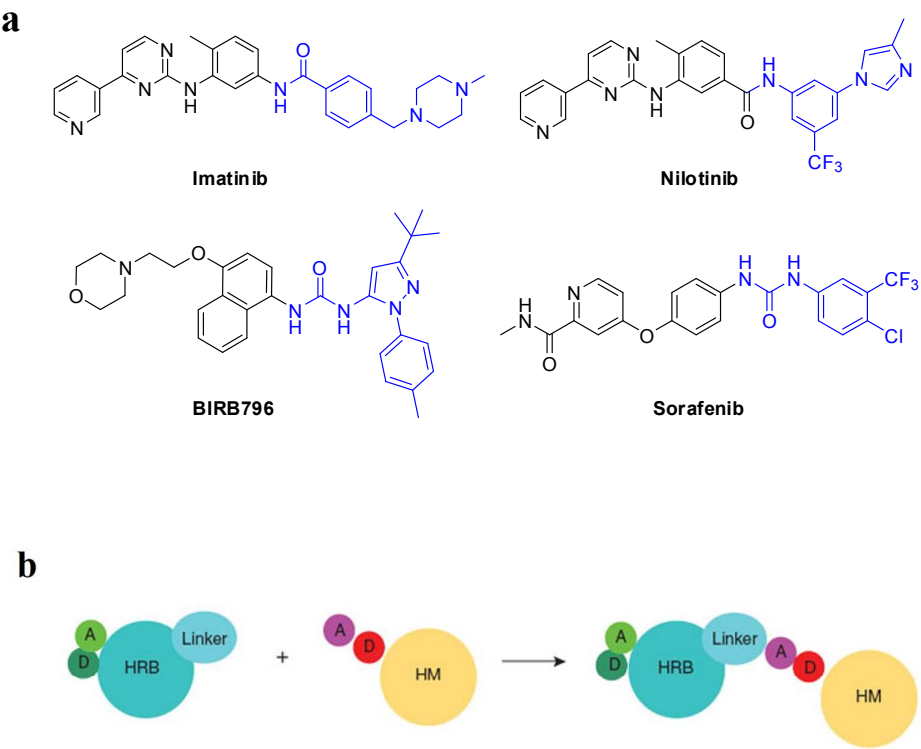


Figure 2.

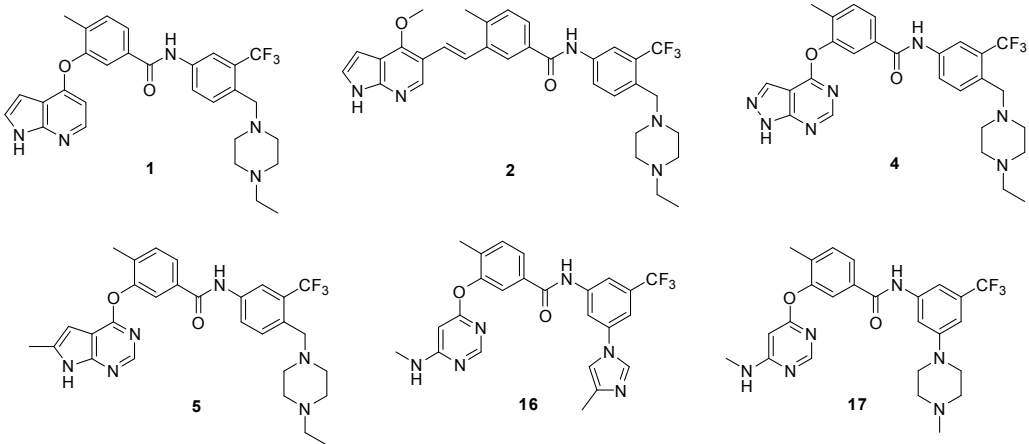


Figure 3.

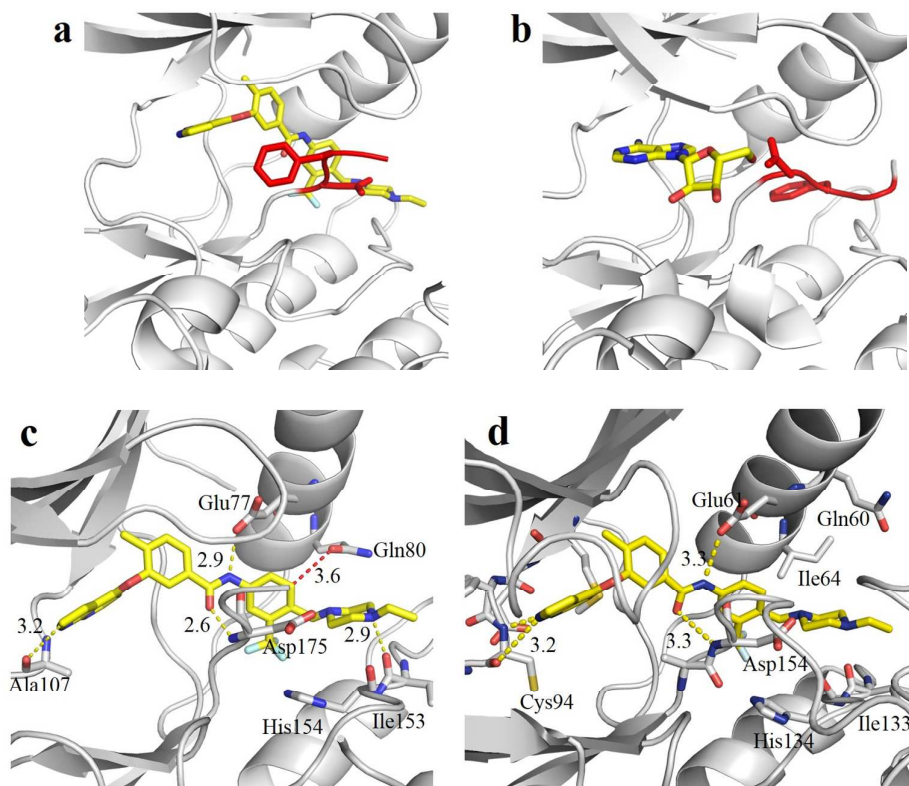


Figure 4.

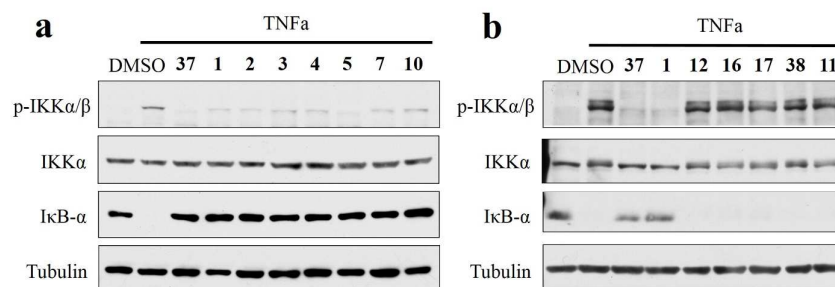


Figure 5.

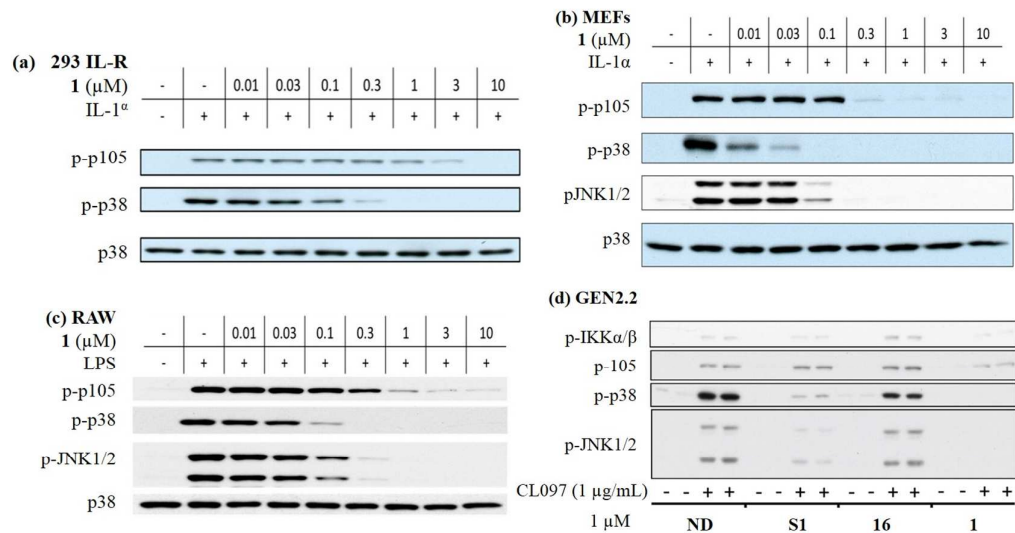
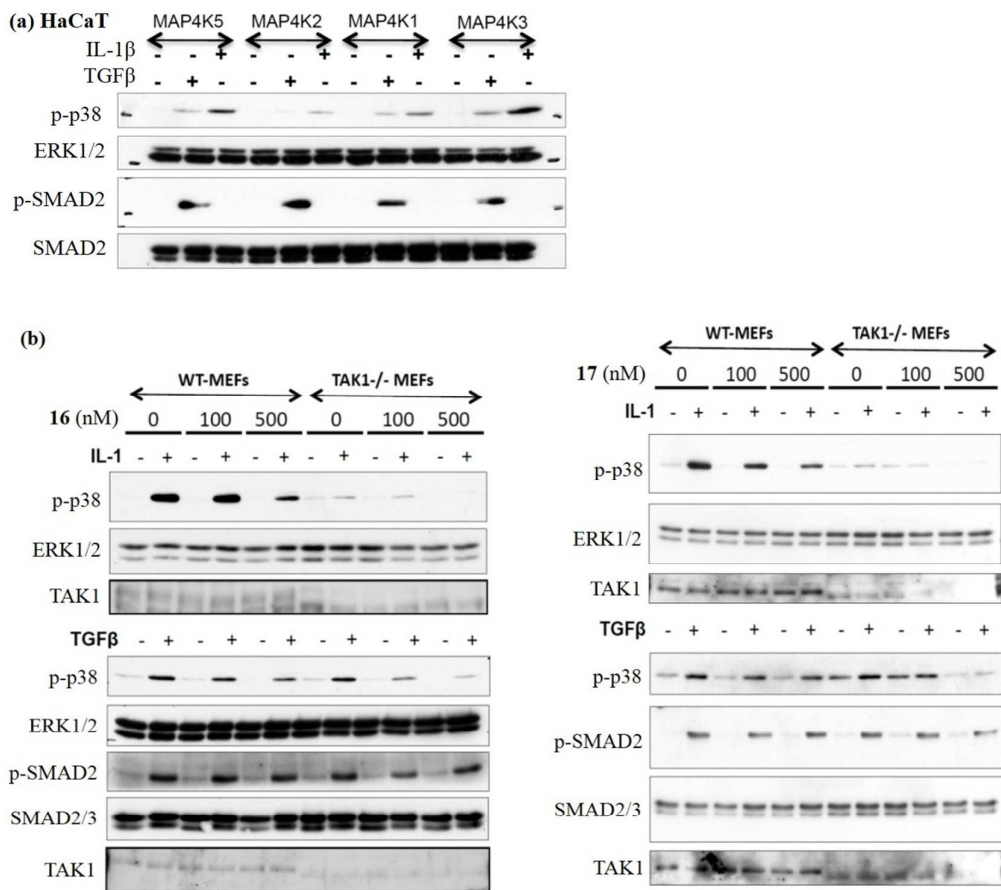
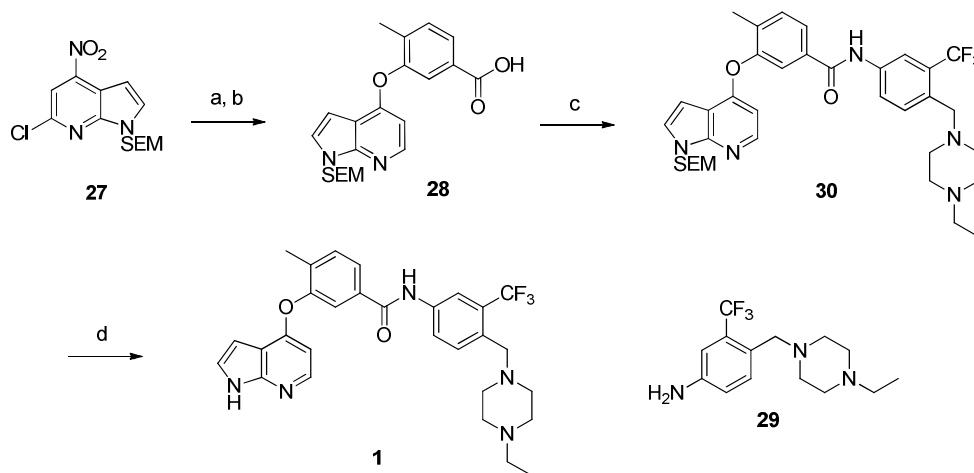


Figure 6.



Scheme 1. Synthesis of 3-((1*H*-pyrrolo[2,3-*b*]pyridin-4-yl)oxy)-*N*-(4-((4-ethyl-

piperazin-1-yl)methyl)-3-(trifluoromethyl)phenyl)-4-methylbenzamide **1**.



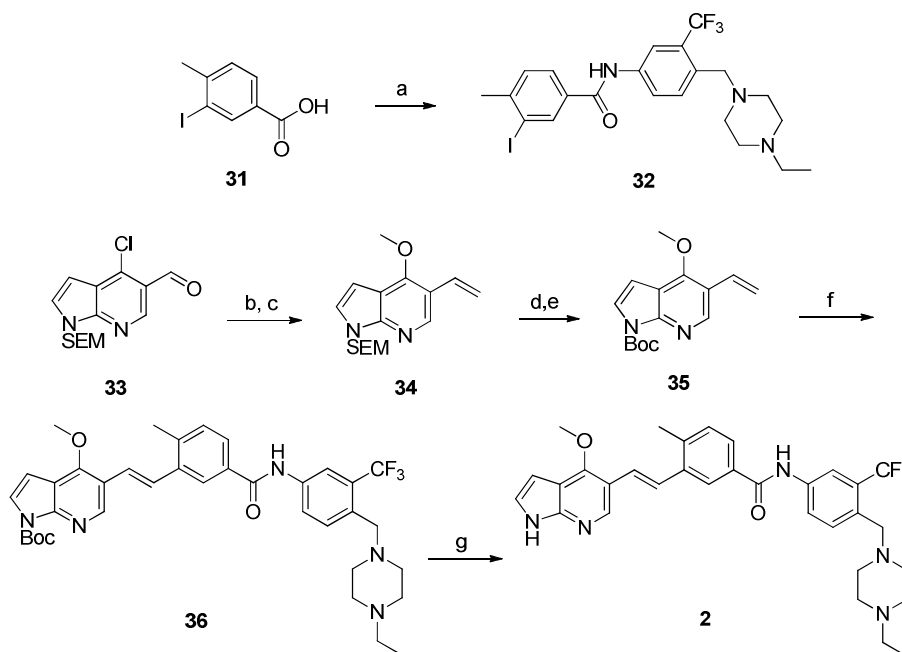
^aReagents and conditions: (a) 3-hydroxy-4-methylbenzoic acid, K₂CO₃, DMSO, 100 °C;

(b) Pd/C, MeOH; (c) **29**, HATU, DMAP, DIEA, CH₂Cl₂; (d) i) TFA, CH₂Cl₂, ii) LiOH,

H₂O/THF.

Scheme 2. Synthesis of (*E*)-*N*-(4-((4-ethylpiperazin-1-yl)methyl)-3-(trifluoromethyl)

phenyl)-3-(2-(4-methoxy-1*H*-pyrrolo[2,3-*b*]pyridin-5-yl)vinyl)-4-methylbenzamide **2**.



^aReagents and conditions: (a) **29**, HATU, DMAP, DIEA, CH₂Cl₂; (b) NaOMe, MeOH, 60

°C; (c) MePPh₃I, n-BuLi, THF, -78 °C to RT; (d) i) TFA, DCM, ii) LiOH, H₂O/THF; (e) Boc₂O, DMAP, CH₂Cl₂; (f) **32**, Pd₂(dba)₃, P(*t*-Bu)₃, DIEA, 80 °C; (g) TFA/CH₂Cl₂, 0 °C.

Table 1. Kinase hits for **1** and **2**.

Kinase Family	Kinase	Labeling Site*	KiNativ** Inhibition (%)				Enzymatic***	
			1		2		1	2
			5 uM	0.5 uM	5 uM	0.5 uM	IC50 (nM)	
STE	MAP4K2	Lys1	> 99.5	> 99.5	96.9	94.2	21.7	98
	MAP4K2	Lys2	94	91.1	96.6	98.0		
	LOK	Lys1	82.3	71.7	>97	60.7		
	ZC1,ZC2,ZC3	Lys2	92.7	70.8	89.8	48.1	3250	9750
	ZC1/HGK	Lys1	86.4	75.6	>88	26.3		
	ZC2/TNIK	Lys1	>95	77.9	>95	74.9		
	KHS1	Lys1	61.3	24.3	85.9	53.7	1200	
TKL	TAK1	Lys2	>98	97.1	97.5	88.5	149	41
	ZAK	Lys1	98.1	93	>99	98.9	698	469
	BRAF	Lys2	66.8	22.5	61.5	58.7	>10000	>10000
	RAF1	Lys2	83.8	79.2	90.3	82.6	7590	3310
CMCG	JNK1,JNK2,JNK3	Lys2	90.3	73.7	76.9	40.6	>10000	>10000
	p38α	Lys2	21.9	20.5	52.4	59.5	102	238
	p38α	Other	53.3	51.9	83.3	69.2		
	p38d/g	Lys2	>94	86.2	52.7	25.2		
	PCTAIRE2	Lys2	87	54.6	27.8	29.6		
TK	ABL,ARG	ACT	> 98	> 98	> 98	> 98	75.2	51.5
	ABL,ARG	Lys1	86.8	85.5	> 90	> 90		
	CSK	ACT	57.7	-14.9	>98	94	56.4	80.9
	EphA2	ACT	57.7	27.9	>99	96.8	773	77
	EphB2	ACT	21.6	-2.2	90.9	55.5	672	75
	EphB4	ACT	-4.2	39.2	>90	78.1	999	54
	FER	Lys1	98.1	87	80.3	30	82.3	
	FER	ACT	96.8	88	74	-10		
	LYN	Lys1	>95	90.5	>95	>95	12.9	29.5
	SRC	Lys1	>92	77.2	>92	72.5	113	204
FYN,S,RC,YES	ACT	89.6	36.5	>99	72.6			
CAMK	CAMK1a	Lys1	91.8	69.7	31.9	37		

* ACT = Activation loop; Lys1 = Conserved Lysine 1; Lys2 = Conserved Lysine 2;
Other = Labeling of residue outside of the protein kinase domain, possibly not in ATP binding site.
** A375 live cell were treated with **1** and **2** in 5 μM and 0.5 μM, lysed and probe-labeled.
*** The enzymatic assays were using the SelectScreen™ Kinase Profiling Service.

Table 2. Structures of all compounds.

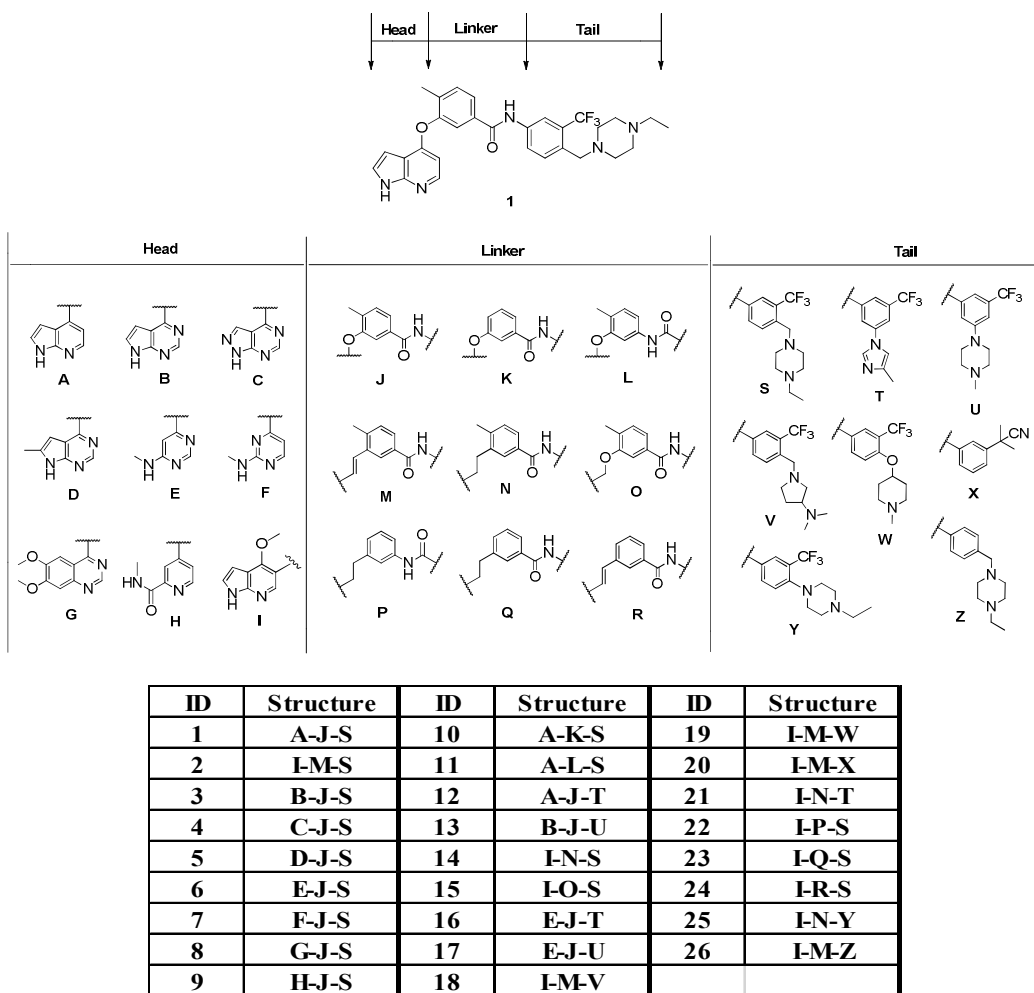


Table 3. SAR for TAK1 and MAP4K2.

ID	Kinativ *		Enzymatic	
	Inhibition (%)		IC50 (nM)	
	TAK1	MAP4K2	TAK1	MAP4K2
1	97.1	>99.5	149	21.7
2	88.5	94.2	41	98
3	77.7	>86	44.9	19.8
4	86.5	89.9	75.5	43.8
5	57.7	>94	41.1	18.2
6	78.5	91.3	364	30.6
7	77.7	>85	165	26.5
8	83.7	93.2	129	30.5
10	75.6	>80	119	23.8
12	-1.5	72	4490	30.1
13	29.3	73.6	465	177
14	74.3	84.3	217	20.6
15	59.6	89.4	150	49.4
16	29.3	79.7	>1110	129
17	7	85.3	2700	37.2

* **1** and **2** were profiled at 0.5 μ M on A375 live cell, while all others were at 1.0 μ M on HUH7 lysate.

Table 4. Kinase selectivity of **16** and **17**.

Kinase	Labeling Site*	Inhibition (%)**	
		16	17
ABL,ARG	Lys 1	51.8	48.4
ABL,ARG	ACT	59.7	49.4
BRAF	Lys2	-12.5	-1.4
CaMK1a	Lys 1	5	8.8
CSK	ACT	-22.5	0.4
CSK	ACT	-2.8	26.9
CSK	ACT	0.3	7.9
CSK	ACT	-14.2	7.9
EphA2	ACT	25.2	25.1
EphB4	ACT	1.2	0.2
FER	Lys 1	18.2	10.6
FES	Lys 1	20	11.2
FYN, SRC, YES	ACT	-55.8	22
JAK1_domain2	ACT	4.9	-20.7
JNK1, JNK2, JNK3	Lys2	18.4	7
MAP4K2	Lys 1	79.7	85.3
TAK1	Lys2	29.3	7
p38a	Lys2	50.2	30.2
p38a	Other	13.4	27.1
p38d,p38g	Lys2	5.5	12.8
RAF1	Lys2	-13.3	23.2
SRC	Lys 1	-2.8	18.1
ZAK	Lys 1	43.6	31.8
ZC1/HGK	Lys 1	-27	0.3
ZC1/ZC2/ZC3	Lys2	24.1	2.7
ZC2/TNIK	Lys 1	-12.6	11.9

* ACT = Activation loop; Lys1 = Conserved Lysine 1; Lys2 = Conserved Lysine 2;
Other = Labeling of residue outside of the protein kinase domain, possibly not in ATP binding

site.

* **16** and **17** were profiled at 1.0 μM on HUH7 lysate.

Table 5. Pharmacokinetic parameters of **1**, **2** and analogs.

cmpd	route	dose (mg/kg)	T_{max} (h)	C_{max} (ng/mL)	AUC_{last} (h*ng/mL)	$T_{1/2}$ (h)	CL (mL/min/kg)	V_{ss} (L/kg)	F (%)
1	IV	1	-	396	195	2.03	80.8	11.9	-
	PO	10	0.5	316	1369	-	-	-	70.2
2	IV	1	-	47.1	403	4.66	55.4	14.2	-
	PO	10	4.0	163	295	-	-	-	13.7
3	IV	1	-	230.9	236.6	2.33	65.8	9.5	-
	PO	10	2.0	133.6	516.2	-	-	-	21.8
4	IV	1	-	280.3	167.9	0.87	99.2	6.1	-
	PO	10	0.5	61.3	186.2	-	-	-	11.1
5	IV	1	-	317	554	2.94	26.5	5.42	-
	PO	10	4.0	233	2136	-	-	-	38.6

Table of Contents graphic.

