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Identification of a selective inhibitor of transforming growth factor β-activated kinase 1 by biosensor-based screening of focused libraries

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Keywords: TAK1, chemogenomic approach, selective Type I inhibitor, biophysical screening

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Abstract

Transforming growth factor-β activated kinase 1 (TAK1), a member of the mitogen-activated protein kinase kinase kinase family, plays an essential role in mediating signals from various pro-inflammatory cytokines and therefore may be a good target for developing anti-inflammation agents. Herein, we report our efforts to identify TAK1 inhibitors with a good selectivity profile with which to initiate medicinal chemistry. Instead of resorting to a high-throughput screening campaign, we performed biosensor-based biophysical screening for a limited number of compounds by taking advantage of existing knowledge on kinase inhibitors. Rather than focusing on one specific inhibition mode, we searched for three different type, Type I (ATP-competitive, DFG-in) and Type II (DFG-out) and Type III binders (non-ATP competitive) in parallel, and succeeded in identifying candidates in all three categories efficiently and rapidly. Finally, the biosensor-based binding kinetics for the active and inactive forms of TAK1 were measured to prioritize the Type I and Type II inhibitors. The effort resulted in the identification of a new TAK1-selective Type I compound with a thienopyrimidine scaffold that served as a good starting point for medicinal chemistry.

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Transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1), a member of the mitogen-activated protein kinase kinase kinase family, was originally discovered as a kinase activated by TGF-β.[1,2] It has since been found that TAK1 mediates many different types of cellular signaling, most notably pro-inflammatory cytokine signaling of tumor necrosis factor-α, interleukin-1, and toll-like receptor ligands, thereby activating downstream kinases such as p38, JNK, and MKK6, as well as the canonical NF-κB pathway. Due to the central role of TAK1 in mediating these signals, interrupting TAK1 activity by a small-molecule inhibitor is considered to be a potentially attractive target for treating inflammatory diseases.[3] In addition, recent findings also suggest it could be a target for anticancer agents.[3] However, because the signaling pathways in which this kinase is involved are highly complex and mutually inter-related, interfering with this kinase can result in adverse effects besides the desired pharmacological effect. Indeed, the suppression of TAK1 by RNAi has suggested that TAK1 would mediate both inflammatory and anti-inflammatory signals, depending on the pathways involved.[3] Thus, TAK1 inhibitors with high kinase selectivity are essential, firstly to assess the therapeutic utility of TAK1, and secondly to develop therapeutic agents once TAK1 has been confirmed as a good target for a specific disease. While several TAK1 inhibitors have been reported, so far none of them has the totally "clean" kinase selectivity profile that is prerequisite for reliably validating TAK1 kinase as a therapeutic target.[3,4]

In this study, we conducted biophysical screening mainly by means of surface plasmon resonance (SPR) to search for TAK1 inhibitors. Biophysical methods (SPR, thermal shift assay, nuclear magnetic resonance spectroscopy) are lower in throughput than biochemical assays, but their simpler formats, which are based on detecting direct binding between test compounds and proteins, make them in general more sensitive and also less prone to false positives.[5] Furthermore, they can also be used for characterizing hits, such as revealing binding kinetics/stoichiometry or mapping the binding sites. Therefore, biophysical screening can be a powerful alternative to conventional high-throughput screening, especially when the number of compounds to be screened can be confined to the order of hundreds to thousands.[6] A kinase target meets just such a condition, since over the past decades a considerable amount of information on inhibitors and protein structures of kinases has accumulated, especially for Type I ATP-competitive inhibitors and Type II inhibitors specific to the inactive form, the so-called "DFG-out conformation".[7] Thus it is possible to pre-select compounds for these types of inhibitors by chemogenomic approaches. However, this approach is not plausible for Type III non–ATP-competitive inhibitors, simply because only a limited number of such inhibitors have been reported.[8] Therefore, it is in general more difficult to identify Type III inhibitors, even though high kinase selectivity can be expected.

In order to balance the difficulty of identifying an inhibitor and the chance of achieving the desired selectivity, we searched for all three types of inhibitors in parallel, using a different set of compounds for each approach. For Type I and Type II inhibitors, we selected compounds from our company library that we already knew exhibited inhibitory activity against some kinases and then assayed them for TAK1-binding activity. For Type III inhibitors, we took a random approach by conducting fragment screening for TAK1 using our own fragment library, because the fragment-based approach allows a wide chemical space to be explored with a relatively small number of compounds.[9] By using these approaches in parallel, we were able to rapidly and efficiently identify compounds that would act as a good starting point for medicinal chemistry to obtain TAK1 inhibitors with a highly selective kinase profile.

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For the biophysical screening, an SPR-based binding assay was developed using a TAK1-TAB1 (TAK1 binding protein) fusion protein that consists of TAK1 kinase domain fused via a linker with the minimal region of TAB1 required for TAK1 binding and activation.[10] TAK1 is known to exert its activity only when it forms a complex with TAB1.[11] The minimally biotinylated TAK1 coupled on the streptavidine-coated surface of a sensor chip showed dose-dependent binding response upon the addition of ATP samples of increasing concentration (Suppl. Fig. S1a, b.). In addition, a known ATP-site covalent binder, 5Z-7-oxozeaenol,[12] showed binding without any apparent dissociation, as expected with covalent binding (Suppl. Fig. S1c.). In the running buffer, a reducing agent, dithiothreitol, was added to offset the rapid loss of activity in the immobilized protein, presumably due to oxidation of a free cysteine, Cys174, present in the ATP pocket (Suppl. Fig. S1d). The Z'-factor[13] was 0.84, when calculated for the repeated injections of 20 μM ATP. The assay is interpreted to be excellent, when the value is larger than 0.5. [13]

For the Type I inhibitor screening, compounds were collected from the in-house library according to two different principles (Fig. 1.). The first set of compounds comprised inhibitors against six kinases that had similar amino acid sequences or for which existing patent information suggested cross-activity against TAK1 kinase. When selecting homologous kinases, we focused on the 39 residues that comprise the ATP binding pocket[14], since no kinases were found with sequence identity higher than 50% when the whole kinase domain was compared. There were 92 kinases with sequence identity ranging from 48% to 64%. Of them, ROCK2, MAPKAPK2, and NEK2 were those for which we had compiled IC_{50} data for a number of in-house compounds. In addition, inhibitors for c-Met, KDR, and Flt-3 were picked out because a patent filed by Vertex claimed a class of compounds that showed cross-reactivity against these three kinases and TAK1[15]; therefore, we considered that our inhibitors against these three kinases might also show binding activity against TAK1 as well. Using the criteria of having a molecular weight of less than 450 and IC_{50} values of less than 10 μM, 170 compounds were collected. The second set of compounds was selected from 968 compounds in our in-house library for which kinase selectivity profile data had been evaluated by KINOME Scan® (DiscoveRX, an in vitro binding panel assay system)[16]. Although at the time of this analysis the number of kinases included in the KINOME Scan® ranged from only 129 to 228, they were distributed well in the human kinome, covering and representing all different families of the kinases (TAK1 was not included in the KINOME Scan®). The selection criteria for the compounds were to have a molecular weight of 300–460 and to show binding inhibition larger than 50% against less than five kinases at a concentration of 1 μM. This selection resulted in 108 compounds. Although it would be challenging to find hits from this set of compounds, any hit found would be a very promising lead candidate with a good kinase selectivity profile.

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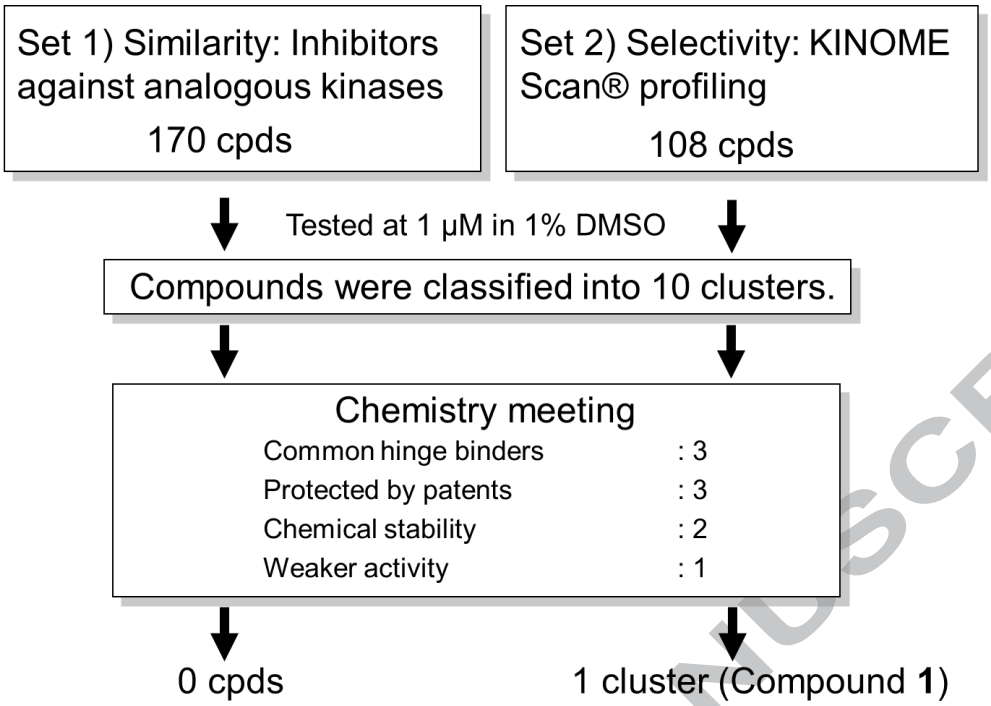

Figure 1. Screening flow for Type I inhibitors

In total, 278 compounds were then subjected to the binding assay using SPR at a concentration of 1 μM. From them, 102 compounds that showed a normalized binding response larger than the noise level were picked out as active candidates and then evaluated by 6- or 7-point dose-response experiments. K_D values were determined for 95 compounds, while the rest were dropped because of a lack of reproducibility or saturation in binding. The hit compounds were classified visually in terms of their chemical structures into 10 clusters. Each cluster was then carefully inspected for binding affinity, drug-likeness, synthetic accessibility, KINOME Scan® profile, and patentability. Eight clusters were immediately deprioritized: three clusters for having typical hinge binding moieties such as 2-aminopyrimidine, indolin-2-one, and 3-aminopyrazole; three clusters for being heavily protected by existing patents; and two for being chemically unfavorable because they possessed a phenol ring or Michael acceptor. The remaining two clusters were both from the second library set and did not have issues with patents or selectivity, but all the compounds in one of the two clusters had only weak affinity, ranging from 1.3 to 9.8 μM. As a result of this inspection, we selected one cluster represented by compound **1**, as a candidate for medicinal chemistry. Compound **1** possesses a thienopyrimidine structure as a core (Fig. 2a), which is a rather rare scaffold for a kinase hinge binder, and showed a moderate binding activity against TAK1 with a K_D value of 59 nM (Fig. 2b, Table 1). An SPR-based competition experiment[17] clearly indicated that the binding response for the mixture of ATP and compound **1** was comparable with the response of the individual compound, confirming that compound **1** indeed competed with ATP for the binding to TAK1, as expected for the Type I inhibitors (Fig. 2c, d). In addition, our in-house kinase panel assay showed no notable inhibition against 33 tested kinases except for Flt-3 kinase (Suppl. Table S1). This result matched the kinase selectivity profile for this compound that was previously measured by KINOME Scan® against 129 kinases, which had shown no inhibitory activity at a concentration of 1 μM except for one kinase, BIKE. However, the TAK1 enzymatic assay for this compound showed only 37% inhibition at 1.0 μM and no higher inhibitory activity was observed at higher concentrations, presumably due to a limited solubility caused by its planar structure. Also, no X-ray analysis was possible for this compound in complex with TAK1

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due to a lack of suitable crystals, which may also be because of low solubility. Indeed, when the solubility of **1** was measured both in fasted state simulated intestinal fluid and in phosphate buffer at pH 6.5, it was found to be less than 3.0 μg/mL.[18] With this low solubility of **1**, the observed excellent kinase selectivity profile should be seen with a caution and needs to be confirmed with a compound with improved solubility. We therefore concluded that further characterization, such as a stability and cell permeability assay, should be conducted on derivatives with better physicochemical properties, so as to reliably evaluate the potential of this class of compound.

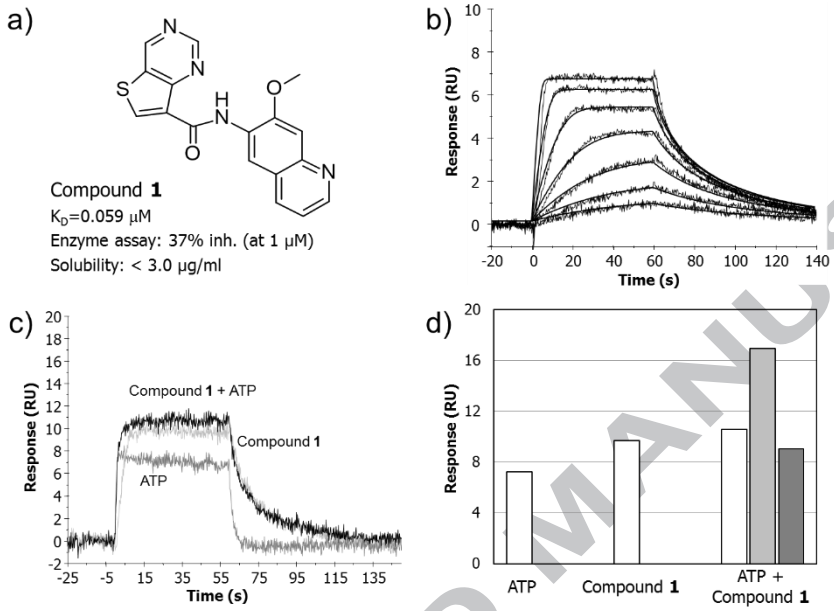

Figure 2. SPR characterization of the binding of compound **1** to TAK1. a) Chemical structure of **1**. b) The interaction of TAK1 with **1** (7 doses, 2-fold dilution series with the highest concentration at 0.5 μM). Theoretical curves were calculated by global fitting of the shown sensorgrams and are overlaid with the experimental curves. c) SPR-based competition experiment for **1**. Sensorgrams corresponding to individual solutions for **1** and ATP are shown in light and dark grey, respectively, and a mixture solution is shown in black. Concentrations of **1** and ATP were 0.5 μM and 20 μM, respectively, both in the individual and mixture solutions. d) Graphical representation of the competition experiment for **1**. White vertical bars represent the experimental responses measured for three different solutions as annotated in the bottom of each bar. Light gray and dark gray bars correspond to calculated signals for expected for non-competitive and competitive bindings, respectively. The signal for the non-competitive binding was calculated as the sum of the individual responses of ATP and **1** solutions, while the value for the competitive binding was determined according to the equation (5) in the reference 16, by using the K_D values in Table 1.

Type II inhibitors are well represented by aryl-urea and aryl-amide types of compounds such as imatinib against Bcr-Abl and BIRB-796 against p38.[19,20] When we embarked on this project, there was no reported Type II inhibitor to TAK1, although such inhibitors have recently been reported by two groups.[21,22] Therefore, in order to assess if TAK1 adopts a DFG-out conformation, we tested the binding activity of representative Type II inhibitors, which included relatively selective and nonselective ones: p38 pyrazole-urea inhibitor BIRB-796, B-Raf inhibitor sorafenib,[23] Bcr-Abl inhibitor imatinib, and an in-house aryl-urea compound, compound **2**, that had been shown to be cross-reactive to many kinases in our in-house kinase panel assay (data not shown). Interestingly, binding was observed for three of these four compounds - BIRB-796, sorafenib, and the in-house compound - and a detailed analysis was conducted for sorafenib and

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2, which resulted in K_D values of 0.60 μM and 0.012 μM, respectively (Fig. 3, Table 1). Remarkably, the determined kinetic parameters indicated that both association and dissociation of the inhibitors were slow, suggesting that conformational change, presumably from DFG-in to DFG-out, was involved in the binding of the inhibitors. Having binding properties with slow dissociation/association has been previously reported as indicative of conformational change for p38 and Type II inhibitors.[24] Finally at a later time, our X-ray structure analysis demonstrated that compound **2** indeed bound to TAK1 in the DFG-out conformation.[25]

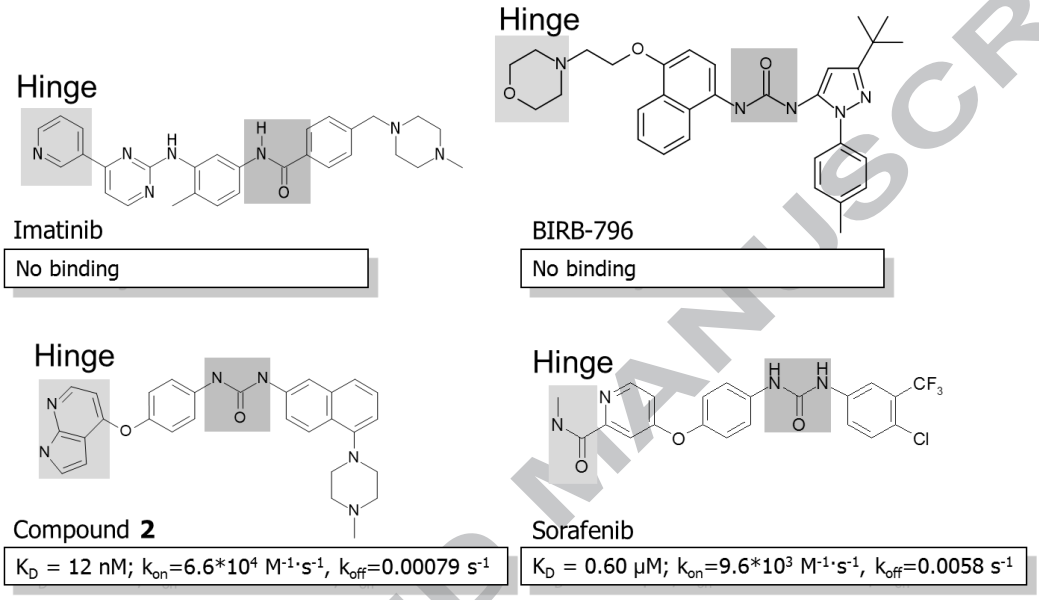

Figure 3. Summary of the binding test on Type II compounds. Chemical structures of imatinib, BIRB-796, compound **2**, and sorafenib are shown, together with kinetic parameters determined for the interaction with TAK1. Hinge- and DFG-binding moieties are highlighted in light and dark grey, respectively.

Table 1. Summary of the kinetic parameters determined for compounds that bind to TAK1 kinase

Compound	TAK1 autophosphorylation	K_D (μM)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})
ATP	Yes	2.8	fast	fast
ADP	No	63	fast	fast
ADP	Yes	4.1	fast	fast
AMP	No	370	fast	fast
AMP	Yes	120	fast	fast
Adenosine	No	34	fast	fast
Adenosine	Yes	4.4	fast	fast
Compound **1**	No	0.15	fast	fast
Compound **1**	Yes	0.059	5.8×10^5	0.034
Sorafenib	No	1.3	1.4×10^4	0.018
Sorafenib	Yes	0.60	9.6×10^3	0.0058
Compound **2**	Yes	0.012	6.6×10^4	0.00079

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In searching for Type III binders, the fragment-based approach was adopted (Fig. 4.). An in-house fragment library consisting of 1951 compounds was screened with SPR by a one-point assay at a compound concentration of 250 μM. To block the ATP binding pocket, adenosine was added at a concentration of 100 μM in the running buffer. We considered that using adenosine rather than other ATP analogues such as ADP for the blocking was advantageous because the smaller molecular size of adenosine would occupy the smallest part of the ATP binding pocket. This would increase the chances of finding fragments that bind to the allosteric site adjacent to the ATP pocket. The binding affinity of adenosine for TAK1 was comparable with that of ADP and ATP, and much higher than that of AMP (Table 1).

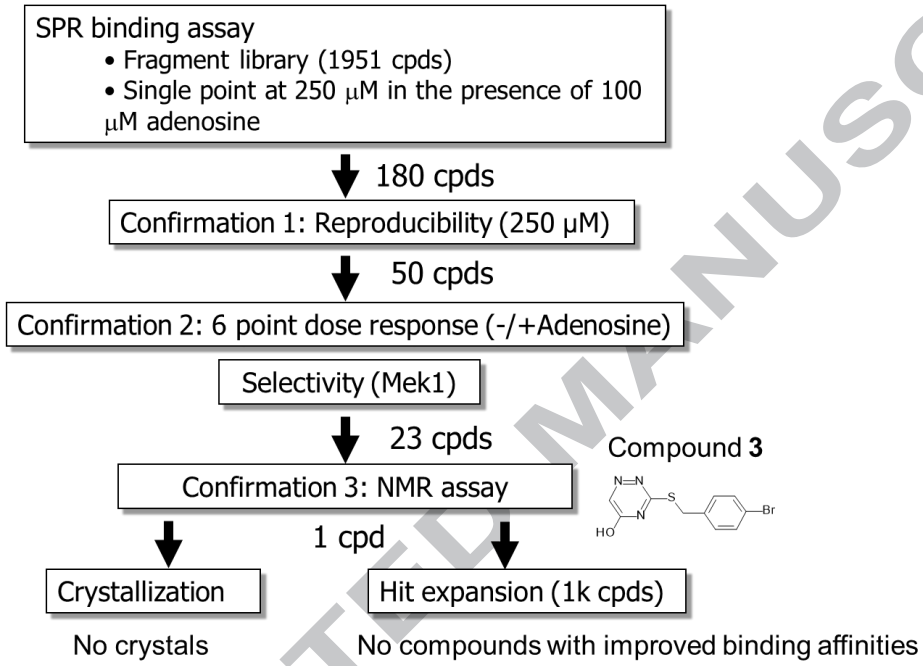

Figure 4. Schematic drawing of the workflow for Type III fragment screening. The chemical structure of compound **3** is also shown.

One hundred and eighty fragments that showed a binding response larger than 2 RU normalized by MW100 were selected for further evaluation. For the selected fragments, three selection filters were applied to pick out "true" allosteric binders. First, the reproducibility of fragment binding was evaluated again at a concentration of 250 μM. The shape of the binding response was also critically checked at this stage.[26] In total 50 compounds then proceeded to the next evaluation. As a second step, a seven-point dose response was measured for these selected compounds in the presence and the absence of adenosine, so that fragments could be ranked according to their K_D values and could also be confirmed as non-competitive binders against adenosine. For allosteric binders binding response should not change, regardless of the presence of adenosine (Suppl. Fig. S4). In addition, to assess the binding specificity of the selected fragments for TAK1, the binding to active mutant of MEK1 kinase was monitored simultaneously.[27] 14 compounds showed even greater binding to MEK1 and no dose-dependent response was observed for 13 compounds. For the remaining 23 fragments, dose-dependent and also higher or comparable binding response to TAK1 was observed. Also, the size of the response was not significantly affected by the presence of adenosine. However, none of these 23 fragments showed saturation in binding, which could suggest that either the identified hits were binding nonspecifically or binding

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to a secondary site might come into play at higher concentrations (Suppl. Fig. S2).

Therefore, so as to confirm whether the fragments were specific allosteric binders or not, we performed NMR experiments as a final filter, according to the method described previously.[28] As the outline in Fig. 5 shows, the method exploits the paramagnetic effect of spin-labeled adenine (SL-Ad), which enhances relaxation of test fragments as manifested by weaker 1D ^{1}H NMR signals of the fragments only when they bind to TAK1 with SL-Ad simultaneously in the vicinity of the adenosine binding site. A drawback of using this selection filter is that this will eliminate the binders that interact with TAK1 at sites remote to the ATP binding site, since the paramagnetic effect of SL-Ad reaches only 15-20 Å. However, we argued that ensuring the binding specificity of the hit fragments was more important than collecting all the possible binders regardless of their binding specificities. Remarkably, out of 23 tested fragments, one fragment, compound **3**, showed the expected result in this evaluation, as outlined in Fig. 5a. In this experiment, it is important to ensure the binding specificity of SL-Ad since it potentially interacts with several different sites of the target protein. This point was confirmed by a displacement experiment, in which recovery of the NMR signal intensities was monitored upon the addition of 5Z-7-oxozeaenol, which binds tightly to the ATP site. The binding mode of compound **3** could not be elucidated from the NMR or SPR experiments and the NMR experiments demonstrated it interacted with the site close to the adenine binding site. The docking study conducted for **3** using TAK1 in the c-helix out conformation (PDB code: 5GJG), which was considered to be appropriate for this class of binders,[8] suggested that the compound could be well accommodated in the location close to but distinct from the adenosine binding site (Suppl. Fig. S3). An absolute confirmation of the specific binding of **3** can be done only by the X-ray analysis of the complex between **3** and TAK1, and also such structure was absolutely essential for the follow-up chemistry; however, none of the efforts to obtain suitable crystals for analysis were successful. An alternative approach is to collect analogues and/or derivatives of **3** and to test them for their binding activity to TAK1 protein. Approximately 1000 compounds were collected from the in-house library by running a similarity search for compound **3**. However, none of the compounds showed binding activity better than **3** (data not shown). Therefore, even though the Type III hit fragment had a high potential to achieve the desired high selectivity over other kinases, we decided not to immediately follow this compound by medicinal chemistry. Instead, we proceeded to further characterize the binding kinetics of Type I and Type II hit compounds by taking advantage of SPR technology to prioritize them.

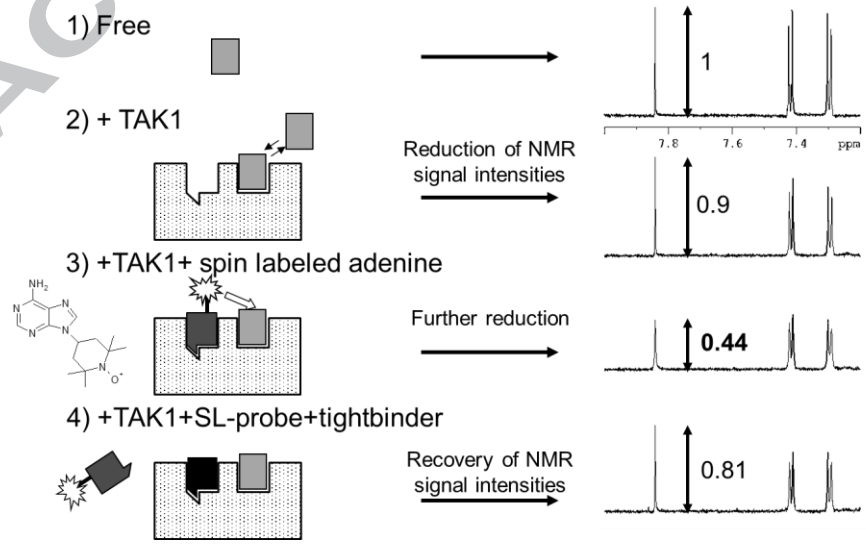

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Figure 5. NMR experiments to evaluate the binding site of a hit fragment, compound **3**. Experimental detail is described in the main text. The chemical structure of spin-labeled adenine with TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) is shown.

Compound **1**, identified from the Type I chemogenomic approach, had high potential of achieving good specificity against TAK1 but had issues with physicochemical properties that needed to be addressed by chemistry. This drawback made the Type II inhibitors seem more attractive. Also, it could be argued, somewhat controversially, that Type II inhibitors are generally more specific than Type I inhibitors in terms of kinase selectivity.[29,30] This idea is grounded in the knowledge that, first, the amino acid residues of the binding site are less conserved for Type II than for Type I since, in the DFG-out conformation, the DFG motif is flipped over and the residues that are not directly involved in ATP binding, are exposed to the surface of the protein. Moreover, depending upon the bound inhibitors, the activation loop can be fixed in largely different orientations from the DFG-in form. Secondly, Type II compounds are suggested to preferentially bind to the inactive form of the kinases where the activation loop is not phosphorylated and therefore, the loop would adopt the DFG-out conformation more easily. Consequently, the binding of Type II compounds to the kinases would shift the equilibrium of the kinases between the unphosphorylated inactive and phosphorylated active states toward the inactive one, which should be another advantage of Type II to suppress kinase activity without having to compete with a high concentration of intracellular ATP.[31] To evaluate whether this argument held true for the identified TAK1 Type II inhibitor or not, we took advantage of the direct binding platform of the SPR-based assay to determine the K_D values of compound **1** and sorafenib for TAK1 with and without autophosphorylation. The autophosphorylation activity for Thr187 of TAK1[32] was reported to be conserved for the TAK1-TAB1 fusion protein employed in this study and this was confirmed for our protein (Suppl. Fig. S4.).[10] Thus, we immobilized TAK1 on two separate flow cells of a sensor chip, one with and one without autophosphorylation, and evaluated the binding affinities of three ATP analogues, namely, adenosine, AMP, and ADP, to the two different forms of TAK1. The comparable size of Z'-factor (0.88) with the autophosphorylated TAK1, was confirmed for the non-autophosphorylated TAK1 surface, when evaluated by the repeated injections of 50 μM adenosine. As expected, all three compounds showed higher affinity for the autophosphorylated TAK1 (Suppl. Fig. S5, Table 1). Then, we proceeded to evaluate the binding of compound **1** and sorafenib. Interestingly, both compound **1** and sorafenib showed approximately three-fold higher affinity for the active phosphorylated TAK1 (Suppl. Fig. S6, Table 1). This was somewhat surprising since, as the discussion above makes clear, a lower affinity of sorafenib for the active TAK1 was expected. The results indicated that TAK1 was able to adopt the DFG-out conformation in the autophosphorylated active form. Indeed, in our X-ray structure of the complex between Type II inhibitor **2** and the inactive form of TAK1 (PDB code: 5GJD), [25] the part of the activation loop, Asp181-Lys190, that follows the DFG motif and includes the autophosphorylation site of Thr187, is invisible (data not shown), which suggests that the loop remains flexible in complex with a Type II inhibitor, and that the activation loop of TAK1 can adopt the DFG-out conformation also in the autophosphorylated form. With these observations, we concluded that the identified Type II inhibitors offered no advantage in terms of obtaining high kinase specificity. Thus, we focused our chemistry efforts on compound **1** to explore for a simplified structural scaffold that had better synthetic accessibility and for improved solubility arising from the non-planar structure of the compound. These efforts, which have been reported in a separate paper, were guided by X-ray structure analysis and resulted in a compound with much improved potency (with IC_{50} of 11 nM) and physiochemical properties that, importantly, showed excellent kinase selectivity against

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TAK1.[4]

In summary, so as to identify specific and selective TAK1 binders for the starting chemistry in lead generation, we took three approaches focusing on Type I, Type II, and Type III inhibitors, by fully exploiting the pre-existing internal and external information. Biophysical analysis, mainly the SPR-based binding assay, of a set of selected compounds resulted in compounds that interact with TAK1 being identified from all three approaches. Finally, compound **1**, a Type I inhibitor, was selected as the starting point for chemistry efforts for lead generation. We hereby demonstrated that for a kinase inhibitor project in which rich sources of accumulated information both on structure and inhibitors is to hand, we were effectively able to search for different types of inhibitors in parallel and then efficiently select the best candidate from the three approaches. This method can generally be applied to any kinase inhibitor program where the relevant information is available.

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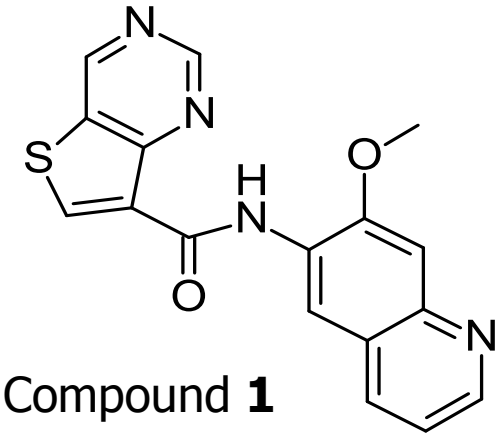
Type I: ATP competitive
Focused library

Type II: DFG-out conformation specific
Known inhibitors

Type III: Allosteric
Fragment-based approach

Biophysical screening (SPR, NMR)



Compound **1**

(Type I: ATP competitive)

K_D=0.059 μM