ACS Medicinal Chemistry Letters

pubs.acs.org/acsmedchemlett

Letter

In Pursuit of an Allosteric Human Tropomyosin Kinase A (*h*TrkA) Inhibitor for Chronic Pain

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Cite This: *ACS Med. Chem. Lett.* 2021, 12, 1847−1852

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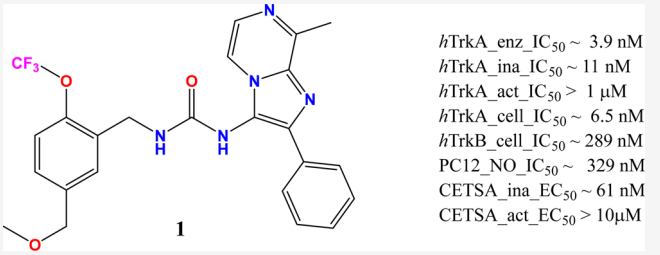
ABSTRACT: Human β-nerve growth factor (β-NGF) and its associated receptor, human tropomyosin receptor kinase A (*h*TrkA), have been demonstrated to be key factors in the perception of pain. However, efficacious small molecule therapies targeting the intracellularly located *h*TrkA kinase have not been explored thoroughly for pain management. Herein, we report the pharmacological properties of a selective *h*TrkA allosteric inhibitor, **1**. **1** was shown to be active against the full length *h*TrkA, showing preferential binding for the inactive kinase, and was confirmed through the X-ray of *h*TrkA···**1** bound complex. **1** was also found to inhibit β-NGF induced neurite outgrowth in rat PC12 cells. Daily oral administration of **1** improved the joint compression threshold of rats injected intra-articularly with monoiodoacetate over a 14-day period. The efficacy of **1** in a relevant chronic pain model of osteoarthritis coupled with *in vitro* confirmation of target mediation makes allosteric *h*TrkA inhibitors potential candidates for modulating pain.



KEYWORDS: *β-NGF, osteoarthritis pain, hTrkA, allosteric, kinase*

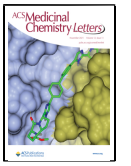
Despite tremendous efforts over the past few decades, uncovering novel mechanistic replacements for non-steroidal anti-inflammatory drugs (NSAIDs) and opioids for the treatment of chronic pain has proved exceedingly difficult. In addition, chronic pain may or may not be associated with ongoing inflammation. New analgesics are in great demand for the myriad chronic pain conditions for which adequate therapies do not exist, making long-term pain management a significant unmet medical need.[1]

Levi-Montalcini discovered nerve growth factor (NGF) as an essential protein for the prenatal growth of sensory and sympathetic nerves.[2−4] Subsequently, it was discovered that in addition to NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) were other factors involved in nerve growth and collectively designated as "neurotrophic factors". The discovery of the receptors of each of these factors followed, with tropomyosin receptor kinase A (TrkA) for NGF displaying high sequence homology to tropomyosin receptor kinase B (TrkB) that binds BDNF and NT-4/5 and tropomyosin receptor kinase C (TrkC) which partners with NT-3.[5,6]

Advances in molecular biology eventually led to the discovery of a genetic mutation in TrkA as the root cause of a congenital insensitivity to pain.[7] Also, it was found that when NGF actions are continuously suppressed by anti-NGF antibodies from the prenatal through the neonatal period, growth of sensory and sympathetic nerves is completely inhibited. However, if NGF is suppressed postnatally, only partial inhibition of nerve growth occurs.[8] In addition, it was discovered that NGF administered to adult rats induces hyperalgesia, confirming NGF as one of the chemical substances that induces pain in adults.[9,10]

Anti-NGF antibody therapies have shown efficacy in preclinical models of pain and in the human clinical setting. However, research into efficacious small molecule therapies has progressed more slowly.[1] The binding of small molecules to the kinase domain of the TrkA receptor can fall into three main categories. Type 1 inhibitors occupy the catalytic adenosine triphosphate (ATP) binding site of an active state conformation of the kinase domain and are competitive with ATP. Type 2 inhibitors occupy the same site but are noncompetitive with respect to ATP, arising from an inactive

Received: September 3, 2021
Accepted: October 21, 2021
Published: October 25, 2021



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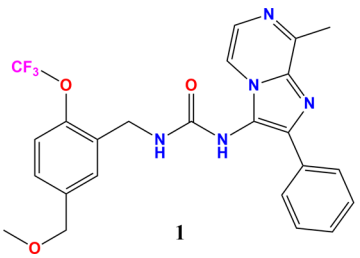
state kinase conformation that restricts access to ATP. Allosteric inhibitors bind to the kinase domain but distal to the ATP site and can effectively bind either kinase conformation.[11] Manipulating the protein conformational versatility and the non-ATP binding pockets can be instrumental in achieving small molecule inhibitor selectivity against Trk subtypes and across the kinome.

Although numerous examples of preclinically efficacious small molecules are known, many are pan-Trk inhibitors of the intracellularly located Trk kinase.[12−15] Due to potential hyperphagia associated with inhibiting TrkB[16−18] and propioception-related adverse events with inhibiting TrkC,[19,20] finding inhibitors that exhibit not only kinase selectivity but also selectivity for the Trk isoforms is of potential value for chronically administered analgesics. Subtype-selective, allosteric inhibitors of *h*TrkA kinase are emerging in the literature[21−26] and could serve as potential alternatives to orthosteric inhibitors with improved safety over pan-Trk inhibitors.[27] Although the allosteric inhibitors described in the literature exhibit good potency for TrkA, little has been published on the pharmacology of these inhibitors.

This paper describes the pharmacological profile of a novel benzylic urea lead, **1** [1-(5-(methoxymethyl)-2-(trifluoromethoxy)benzyl)-3-(8-methyl-2-phenylimidazo[1,2-*a*]pyrazin-3-yl)urea], that emerged from the medicinal chemistry lead optimization efforts (Figure 1) at Zoetis. The



*h*TrkA_enz_IC_{50} ~ 3.9 nM
*h*TrkA_ina_IC_{50} ~ 11 nM
*h*TrkA_act_IC_{50} > 1 μM
*h*TrkA_cell_IC_{50} ~ 6.5 nM
*h*TrkB_cell_IC_{50} ~ 289 nM
PC12_NO_IC_{50} ~ 329 nM
CETSA_ina_EC_{50} ~ 61 nM
CETSA_act_EC_{50} > 10μM

Figure 1. Structure and *in vitro* profile of **1**.

effect of **1** on neurite outgrowth (NO) from PC12 cells and its nociceptive effects in a chronic rodent model of osteoarthritis (OA) positions the lead for further evaluation in the discovery and development process.

RESULTS

***In Vitro* Profile of 1.** The chemical series to which **1** belongs was initially identified and evaluated using a high throughput TR-FRET kinase assay that utilizes the inactive form of *h*TrkA enzyme. **1** inhibited *h*TrkA enzymatic phosphorylation with a mean (±SD) IC_{50} of 3.9 (±4.9) nM. To evaluate phosphorylation-state-dependent binding, caliper technology was used to separate and directly quantify substrate phosphorylation for both inactive and active states of *h*TrkA enzyme. The IC_{50} for **1** using the inactive *h*TrkA enzyme was 10.97 (±4.71) nM and showed no activity for the active enzyme up to a maximum of 1 μM screening concentration. Clearly, **1** showed evidence of phosphorylation state binding. In the cell based PathHunter assay, **1** displayed 6.5 (±6.6) nM and 289 (±174) nM IC_{50} values for *h*TrkA and *h*TrkB, respectively, indicating potential selectivity gains against the subfamily member. **1** also exhibited prolonged binding to the inactive state of *h*TrkA kinase relative to **AZ-23**,[28] a known type-1 pan-Trk inhibitor, displaying residence times of 78.25 and 8.05 min, respectively. Conversely, **1** did not bind to the active form of *h*TrkA kinase, displaying a residence time of 1.68 min relative to 111.86 min for **AZ-23**. Finally, **1** produced a dose-dependent inhibition of rat β-NGF-induced neurite outgrowth in PC12 cells[29] with an IC_{50} of 329.4 (±1.8) nM. Additionally, **1** was evaluated via a label-free CETSA assay using PC12 cells[30] and biasing the kinase conformational state by altering the temperature. This assay also revealed **1** to possess EC_{50} ~ 61 nM when screened at 40 °C (inactive state) and EC_{50} > 10 μM at 52 °C (active state) providing support for the target engagement using a full length *h*TrkA with β-NGF stimulation

X-ray Structure of the Kinase Domain *h*TrkA···1 Complex. Crystals of the juxtamembrane (JM) domain including *h*TrkA kinase domain spanning residues 485−795 in complex with **1** were prepared using reported protocols.[30] The *h*TrkA ligand bound complex structure was subsequently solved and refined to 2.06 Å final resolution (PDB code 6PL4). The crystal asymmetric unit contains only one TrkA monomer with the electron density unambiguously defining the ligand orientation in an allosteric binding pocket away from the orthosteric ATP binding site (Figure 2). The overall structure

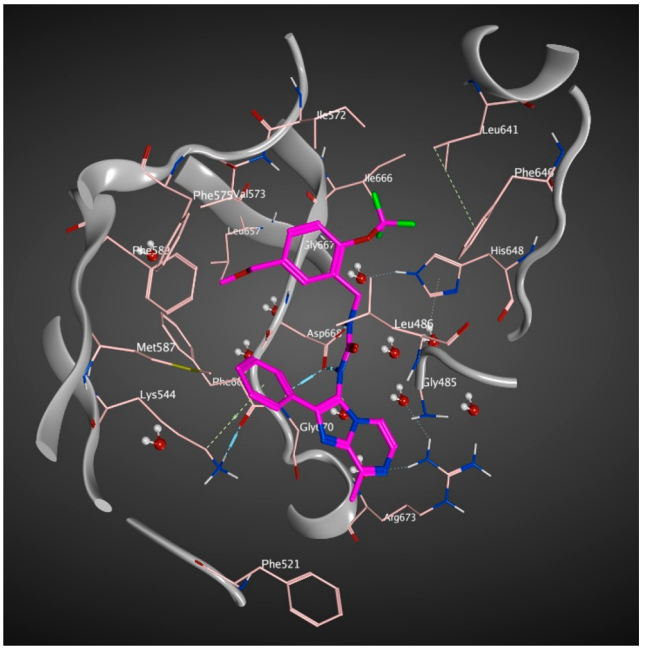


Figure 2. Binding mode of **1** (magenta) bound to extended *h*TrkA kinase domain (PDB code 6PL4).

has two short loops missing between residues 491−499 and 609−611 due to the poor electron density. The protein folding integrity assessed using the Ramachandran plot reveals 93.3% of the amino acids to be in the most favored regions and 6.3% in the additional allowed regions. The completeness of the model, however, was achieved by building the missing loop structures and assigning side chains to residues that could not be clearly defined by the experimental electron density as part of the protein preparation module within the MAESTRO application suite from Schrödinger. This modeled structure was used for all analyses and comparisons.

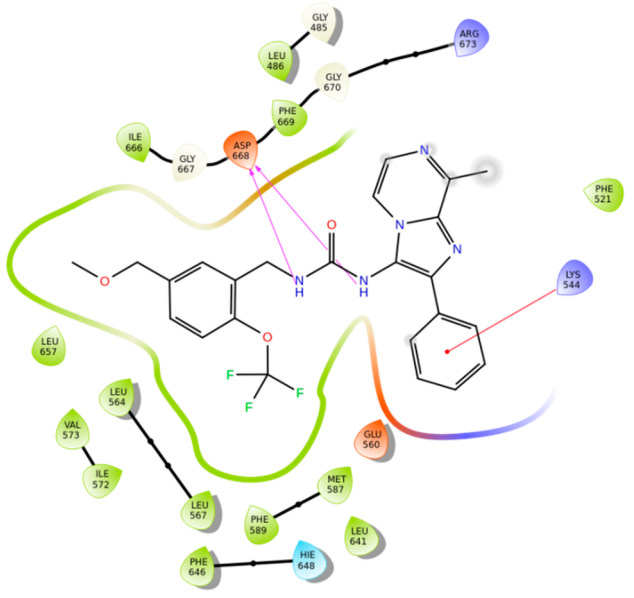
The DFG residues (668−670) side chain orientation and the activation loop (A-loop) unequivocally reveal the kinase to

https://doi.org/10.1021/acsmedchemlett.1c00483
ACS Med. Chem. Lett. 2021, 12, 1847−1852

adapt a DFG-out and α C-helix in conformation, typical for the kinase inactive state.[31] This A-loop conformation partially occludes the orthosteric site, preventing ATP from binding and thereby rendering the enzyme to be catalytically ineffective. In addition, the glycine-rich loop (516−524) is pushed further upward when compared to kinase active state structures. The highly unstructured JM domain and the A-loop residues carve out the allosteric binding site that allows small molecule allosteric inhibitors to bind in an ATP noncompetitive way.[24] The catalytic lysine, K544-E560 salt bridge break-off typically encountered in the enzymatically competent kinase active state also facilitates an additional binding volume used for the ligand occupancy. Superposition of the current *h*TrkA-ligand complex with the reported structure of ATP-competitive inhibitor **AZ-23** (PDB code 4AOJ)[28] bound to *h*TrkA clearly reveals the macromolecular motions of the glycine-rich and A-loop that defines the two unique conformational states of the kinase and removes any ambiguity in defining the kinase conformational state observed via X-ray.

The X-ray diffraction data further reveal a clear binding mode for **1** (Figure 2) with the appropriate binding orientation and unconstrained ligand conformation that maximizes the surrounding residues' interaction in the binding pocket and showcases the contributions of the JM region residues like L486 to the allosteric modulation. It is worth noting that the JM region is crucial as it serves as the SHC[32] phosphotyrosine binding site for protein/protein interaction and the lower sequence identity in this region may also help achieve inhibitor selectivity across the TrkA, -B, and -C subfamily members. On the basis of the standard binding site cutoff distances, the urea NH groups of the ligand effectively H-bond with the carboxylate side chain of D668 (Scheme 1). Apart from this anchoring interaction, the ligand is covered by several hydrophobic residues that strengthen the protein−ligand interaction as reflected by the high binding affinity for *h*TrkA. The binding site also includes several solvent water molecules (Figure 2) captured from the X-ray that seem to be responsible for keeping the protein and binding site intact. However, none of these water molecules are involved in any solvent mediated H-bond between the ligand and the protein.

Scheme 1. . *h*TrkA···1 Binding Interaction Derived from the Solved X-ray Complex[a]



[a]PDB accession code is 6PL4.

Reduction of Pain in Chronic Osteoarthritis Model. On day −14, MIA was injected into the left intra-articular space, producing OA-induced pain, as demonstrated by significant decreases in JCTs in the ipsilateral joints compared to the contralateral joints of the vehicle-treated animals. On day 0, **1** dosed qd produced a significant increase in JCT at 2 h postdose. On day 7, both qd and BID treatments of **1** produced a significant increase in JCTs 5 h postdose. On day 14, qd treatment with **1** produced a significant effect 2 h postdose, and BID treatment produced significant effects at 5 and 8 h postdose (Figure 3). Both treatment groups for **1** showed an increase in pretreatment baselines across the entire study that were significantly different from zero, but vehicle treatment did not produce a similar effect. None of the treatments had any statistically significant effect on the contralateral limb, suggesting any effect was a direct result of altering the pain response induced by intra-articular MIA.

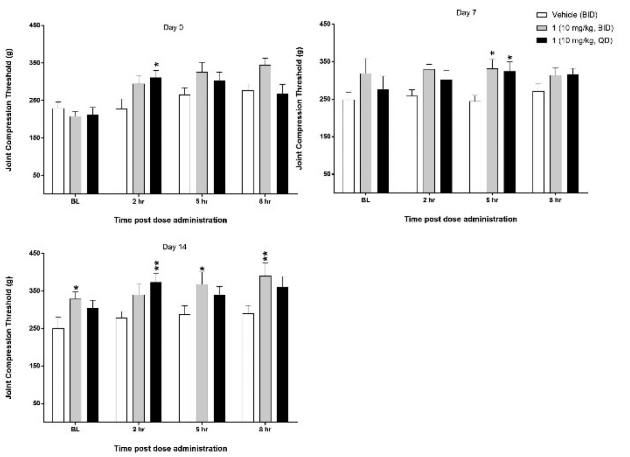


Figure 3. Joint compression threshold (mean ± SEM) over time for qd and BID administration of **1** in a rat MIA model (N = 10 rats/treatment). Animals were injected with MIA on day −14, and treatment with either vehicle or **1** began on day 0 continuing through day 15. Baseline (BL) recordings occurred immediately prior to the AM dose administration. JCTs measured on day 16 occurred 24 h after the last administration of vehicle or **1**. Significance of effects of **1** treatment (qd or BID administration) compared to vehicle-treated animals was calculated at each time point using a two-way repeated-measures ANOVA followed by Tukey's multiple comparisons (*P < 0.05; **P < 0.01).

DISCUSSION

In this Letter, we report on the pharmacological characterization of a *h*TrkA allosteric kinase inhibitor, **1**. The *in vitro* and *in vivo* pharmacological profiles of **1** were evaluated. These studies characterize **1** as a potent, inhibitor of TrkA with selectivity over TrkB.

The clinical administration of Trk inhibitors has been associated with potential liabilities that have limited their use. It has been noted that pan-Trk inhibition leads to ataxia and hyperphagia.[17,20] Isoform selectivity was measured through *in vitro* functional assay against TrkB and kinase conformation selectivity through binding kinetics using SPR. With a preference for binding the inactive state of TrkA kinase as

https://doi.org/10.1021/acsmedchemlett.1c00483
ACS Med. Chem. Lett. 2021, 12, 1847−1852

evidenced from the biophysical and label-free CETSA assays, coupled with the observed selectivity in the functional assay, **1** was suggested to be a novel, allosteric inhibitor of *h*TrkA. The *h*TrkA···**1** extended kinase domain X-ray complex confirmed the inactive state kinase conformation and binding to the distal non-ATP site.

In addition to the binding and selectivity characteristics, **1** proved to be effective at inhibiting NO in rat PC12 cells. It has been hypothesized that growing and damaged peripheral nerves display sensitization and are associated with increased pain sensation.[33,34] In OA, articular cartilage becomes vascularized and these new vessels may be associated with new sensory nerves.[35−37] Likewise, patients with chronic lower back pain due to degenerative disks have demonstrated that the damaged disks release a combination of factors, including NGF, leading to neurite sprouting and increased calcitonin gene related-peptide (CGRP) expression, which is strongly associated with pain.[38] Therefore, it is thought that effectively arresting neurite outgrowth via inhibition of the action of NGF during OA is a possible mechanism for the relief of perceived pain.[39,40]

The combination of *in vivo* methods and *in vitro* biochemical and biophysical techniques in engineered cells used to characterize **1** represents the most thorough characterization of an allosteric TrkA inhibitor reported to date for pain indication. Preliminary immunohistochemistry of the DRGs and spinal cord explants performed to comprehend the expression and internalization of the TrkA receptor in the rat MIA model also provides evidence for receptor internalization in the efficacy model. Interestingly, **1** appeared to display some degree of analgesia in rats beyond the dosing period of 15 days, continuing into the final 16th day time point. Although not validated experimentally, one possible mechanism for this action is through the modulation of CGRP release. Synthesis and release of substance P (SP) and CGRP are known to occur as a result of NGF stimulation, and this excessive release may lead to neurogenic inflammation. CGRP potentiates SP release at the spinal cord level and delays its degradation, thus augmenting the response of nociceptive dorsal horn neurons to repetitive type IV/C fiber stimulation.[41] Others have reported elevated levels of CGRP as well as activated peptidergic afferent C fibers in the spinal cord of rats in MIA models of OA pain.[42]

It is also worth noting that **1** produced statistically significant reduction in JCT on day 0 at the 8 h time point. This is interesting, as it is generally accepted that there is an NSAID-sensitive and NSAID-insensitive phase of the MIA model. The NSAID-sensitive phase occurs early after MIA injection (within 14 days) and is responsive to treatment with NSAIDs, most likely due to an inflammatory component of the model. The fact that **1** produced a statistically significant effect during this NSAID-sensitive phase suggests that this effect may be due to an anti-inflammatory effect of **1**.[23,43,44]

In conclusion, **1** is a potent and selective *h*TrkA inhibitor. The selectivity of this molecule is likely to afford a much wider margin of safety over known pan-Trk inhibitors. **1** was able to effectively inhibit neurite outgrowth in rat PC12 cells which has been demonstrated to have high translatability to human dorsal root ganglion.[45] Robust efficacy in a rat MIA model of OA has been shown to be translatable to field efficacy for this pathway.[46] The combined efficacy in a translatable rodent model and potency in the NO assay make **1** a potential candidate for human use in the relief of pain associated with OA.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.1c00483.

Complete experimental information for the chemistry, *in vitro* biochemical and biophysical assays, and *in vivo* pharmacological assay, along with the table for the X-ray data (PDF)

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Author Contributions

†G.S. and B.D. contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): Authors are past or present employees of Zoetis and have financial interest in the company.

■ ACKNOWLEDGMENTS

The authors thank Nicole Roush, Kevin Esch, Phyllis Malpas, Catherine Rugg, Jim Messamore, Jennifer Paquette, Robert Sturm, Leslie Kuiper, Roger Martin, Ann Janssen, and Graeme Bainbridge for technical support. G.S. also thanks Stefan Steinbacher from Proteros Biostructures GmbH, Germany, for structural biology, and Arthur Wittwer and Jeffrey Hirsch from Confluence Discovery Technologies for the caliper assays.

■ ABBREVIATIONS

qd, quarter in die; BID, bis in die; JCT, joint compression threshold; SPR, surface plasmon resonance; CETSA, cellular thermal shift assay; DRG, dorsal root ganglion

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https://doi.org/10.1021/acsmedchemlett.1c00483
ACS Med. Chem. Lett. 2021, 12, 1847−1852