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Virtual Screening and X-ray Crystallography for Human Kallikrein 6 Inhibitors with an Amidinothiophene P1 Group

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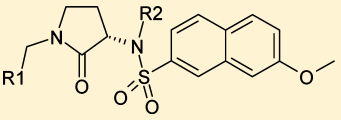
ABSTRACT: A series of compounds with an amidinothiophene P1 group and a pyrrolidinone-sulphonamide scaffold linker was identified as potent inhibitors of human kallikrein 6 by structure-based virtual screening based on the union accessible binding space of serine proteases. As the first series of potent nonmechanism-based hK6 inhibitors, they may be used as tool compounds for target validation. An X-ray structure of a representative compound complexed with hK6, resolved at a resolution of 1.88 Å, revealed that the amidinothiophene moiety bound in the S1 pocket and the pyrrolidinone-sulphonamide linker projected the aromatic tail into the S′ pocket.



KEYWORDS: *serine protease, multiple sclerosis, human kallikrein 6, inhibitor, molecular modeling, virtual screening, X-ray crystallography*

Human kallikreins (hKs, also abbreviated as KLKs for kallikrein genes) encompass a multigene family with 15 members identified to date.[1−3] Belonging to family S1A of Clan PA(S) according to the MEROPS classification, the original pancreatic tissue kallikrein and other kallikrein-related peptidases are trypsin/chymotrypsin-like serine proteases.[4] The first human kallikrein (hK1) was discovered in the 1920s and believed to play a role in lowering blood pressure by releasing bradykinin.[5] Since then, several newer hKs have been identified as potential diagnostic biomarkers for various cancers, such as hKs 2/3 for prostate cancer, hKs 3/6 for breast cancer, and hKs 6/9/10/11 for ovarian cancer.[6−13]

Human kallikrein 6 (hK6), previously known as zyme/protease M/neurosin/myelencephalon specific protease, is abundantly expressed in the central nervous system (CNS).[14,15] It has been reported that hK6 is highly upregulated in the active demyelinating area in human multiple sclerosis (MS) plaques, as well as inflammatory lesions of mouse and marmoset with experimental autoimmune encephalomyelitis (EAE)—the animal model of human MS.[16] It was also reported that immunizing mice with hK6 before or at the time of EAE induction significantly attenuated clinical and histological signs of EAE.[17] Arguably, hK6 enzymatic activity may play an important role in the pathogenesis of MS. Thus, inhibitors of hK6 may have significant therapeutic value in treating inflammatory demyelinating diseases, such as MS.

Unlike other trypsinlike serine proteases, the development of kallikrein inhibitors is rather primitive, especially for hK6. Serpins, accounting for about 10% of human plasma, are the best studied endogenous hK inhibitors.[18−20] More pharmaceutical friendly inhibitors have been developed for hKs 3 and 7. Human kallikrein 3 (hK3), also commonly referred to as prostate specific antigen (PSA), has attracted widespread attention due to its role as both a diagnostic biomarker and a therapeutic target for prostate cancer. Several small organic inhibitors, both peptidic and nonpeptidic, have been reported aiming at further pharmaceutical development.[21−24] So far, to our knowledge, no small organic inhibitor has been reported for hK6 that is suitable for pharmaceutical purposes. The present article describes our effort in identifying hK6 inhibitors that may be further developed as therapeutic agents.

X-ray structures of hK6 were first determined by Blaber's laboratory and became publically available,[25] which made hK6 a good candidate for in silico virtual screening. Figure 1 shows several representative trypsinlike serine proteases superimposed on backbone atoms of residues directly exposed to their substrate-binding sites. As shown in the figure, hK6 (red-colored) has both conserved tertiary folding and a similar substrate-binding site as other trypsinlike serine proteases. It is likely that compounds that bind well with other trypsinlike serine proteases, especially in the conserved area of the substrate binding site, can also bind with hK6 and inhibit its enzymatic activity. The task for our in silico virtual screening was to cover the most relevant chemical space for hK6 with the smallest number of compounds.

The initial screening set was compiled from compounds synthesized for and shown activity in legacy projects of similar targets. Sanofi-Aventis and its predecessor companies have a long history of medicinal chemistry research in the field of trypsinlike serine proteases, which enriched our in-house compound deck with structures likely to bind with hK6. Those legacy compounds were first clustered on the basis of

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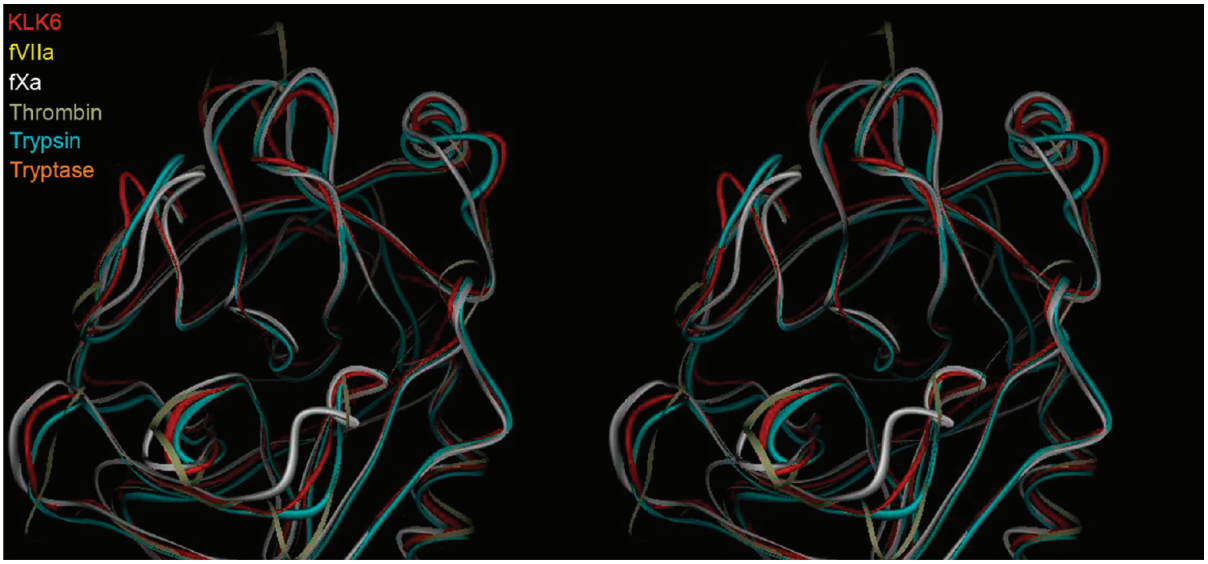

Figure 1. Stereoview of representative trypsinlike serine proteases superimposed on substrate-binding pocket using Discovery Studio from Accelrys with only the α-carbon trace shown.

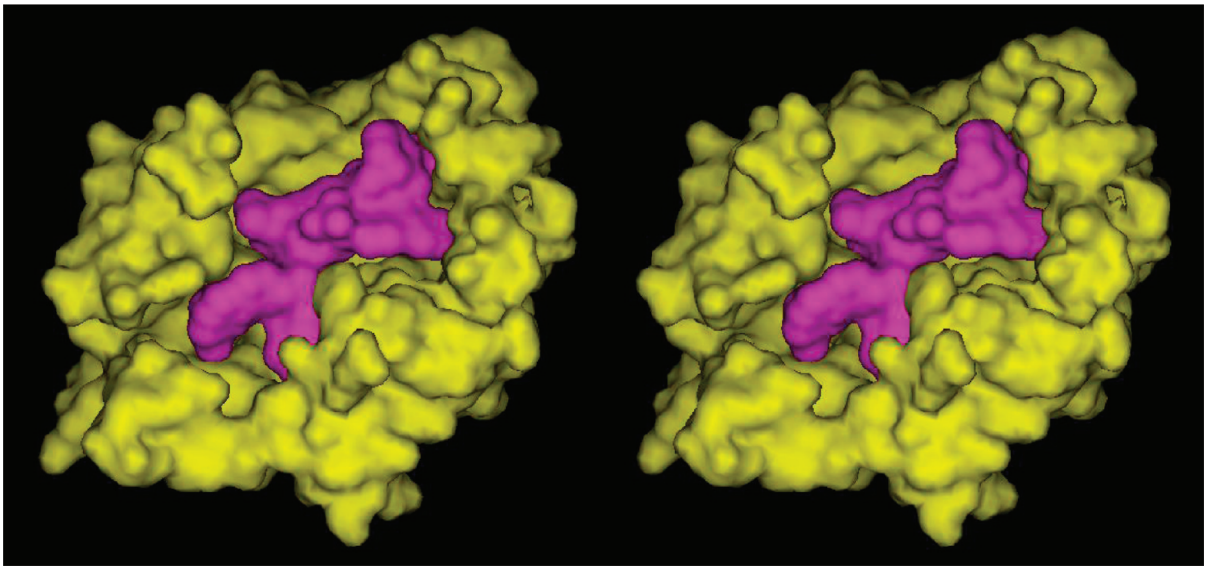

Figure 2. Stereoview of the UABS for representative inhibitors of trypsinlike serine proteases in the substrate-binding site of hK6. The surface of the UABS is colored in magenta, and the one for hK6 is colored in yellow.

their structures, with each cluster represented by several representative compounds. The number of representative compounds for each cluster is determined by the size of the cluster, the structure−activity relationship (SAR) distribution, and the ADMET profile of the cluster members. The binding mode of each cluster was determined based on either X-ray structures or in silico docking poses of representative compounds on the protein target for which the compound showed the highest activity. A union accessible binding space (UABS) was constructed by combining the binding modes of all cluster representatives, as shown by the magenta-colored space in Figure 2.

The screening set was further filtered based on the overlap of the UABS and the X-ray structure of apo-hK6. As shown in Figure 3, several residues of hK6 intruded significantly into the UABS. Compound clusters associated with parts of the UABS that are intruded by hK6 were removed from the initial screening set. The most significant intrusion is caused by Ile218, which blocked several previously reported scaffold series for both fXa and β-tryptase inhibitors. For instance, as shown in Figure 3 (top), Ile218 had a direct steric collision with the scaffold of Otamixaban, an fXa inhibitor.[26] Similarly, as shown in Figure 3 (bottom), Ile218 prevented the binding of the piperidine ring in its native binding mode for a series of β-tryptase inhibitors from which clinical candidates were selected.[27,28] By removing compounds colliding with hK6 intrusions from the screening set, some false negatives may be introduced because no relaxation of the binding mode was allowed. This protocol, however, can effectively reduce false positives caused by in silico induced fit, which is an important consideration when screening throughput is limited.

The resulting screening set was further screened for undesirable functional groups that included both chemically reactive moieties and enzymatically reactive moieties, which

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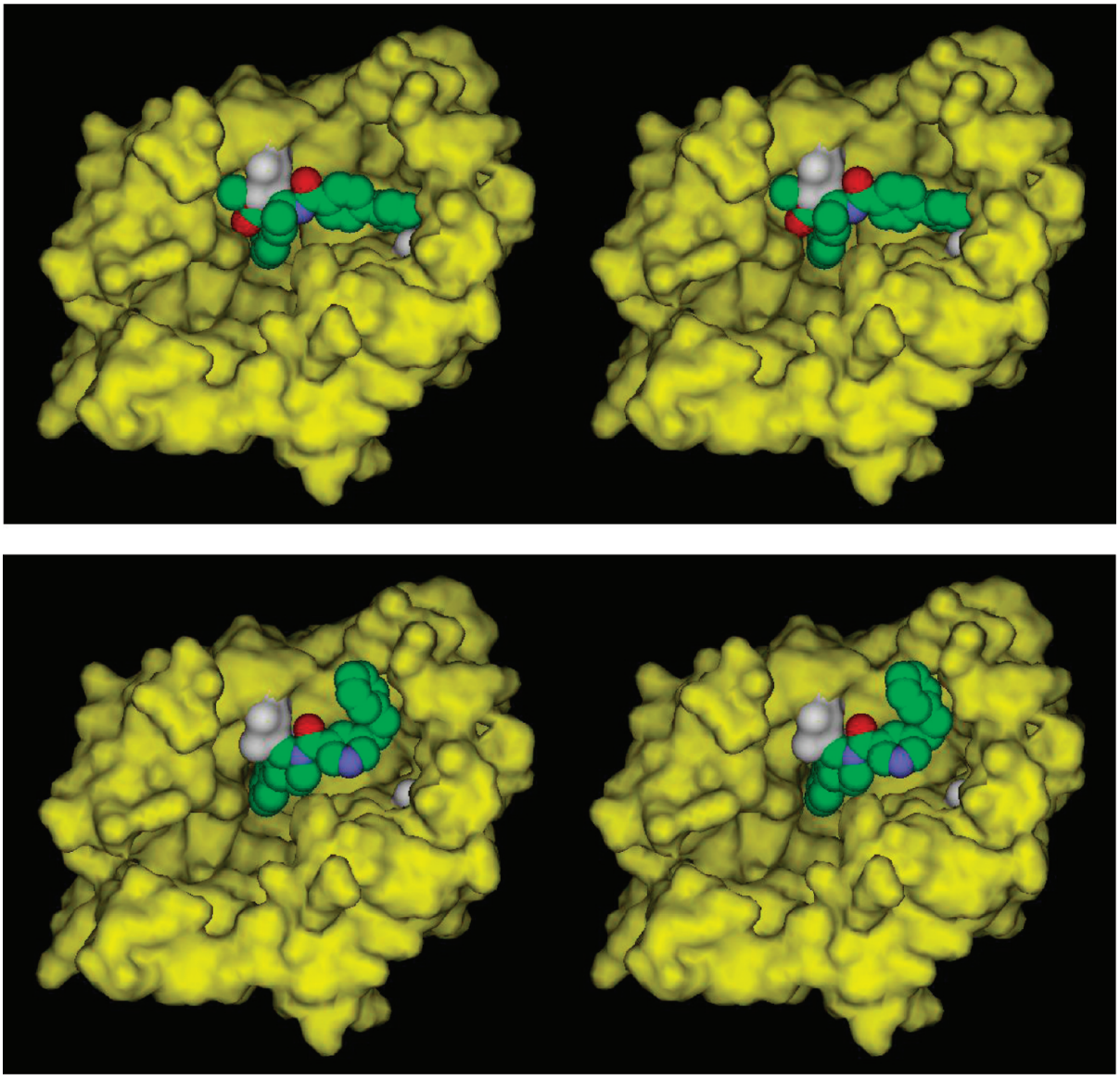

Figure 3. Stereoview of fXa and β-tryptase inhibitors in the generic binding site complementary to UABS. The complementary binding site is colored in yellow, the hK intrusion to the generic site is colored in white, and carbon atoms of fXa inhibitor (top, 1KSN.pdb) and β-tryptase inhibitor (bottom, 2BM2.pdb) are colored in green.

eliminated most mechanism-based inhibitors. While the risk of long-term toxicity associated with mechanism-based inhibitors, especially irreversible inhibitors, is still subject to academic debate, many medicinal chemists tend to avoid those inhibitors if other alternatives exist. Therefore, it was decided for the present project that mechanism-based inhibitors with a reactive "warhead" would be excluded from the screening set during the first round of screening and would be included in the second round if no suitable nonmechanism-based inhibitor was identified in the first round.

Enzymatic testing of selected compounds was carried out in a chromogenic assay using hK6 protein expressed in baculovirus infected Sf9 cells. The protein construct consisted of hK6 residues Leu22 to Lys244 with a His6-tag at the N terminus followed by an enterokinase recognition sequence (Asp_4Lys) immediately before Leu22. Because the same protein construct would also be used for crystallography, two mutations were introduced as suggested by Blaber's group,[25,29] with N132Q to block glycosylation and R76Q to minimize autolysis.[25] Because further autolysis was observed at Arg74, an additional mutation, R74G, was introduced, which completely eliminated apparent autolysis. The expressed protein was purified with an Ni-NTA column before being activated with EK (EK light chain, New England Biolabs P8070L) in a ratio of 1:10 000 (EK:hK6) for 2 h at room temperature. The EK/hK6 ratio was optimized so that the interference due to the EK hydrolysis of the substrate was minimal because EK can also hydrolyze the same substrate used in the hK6 assay. The activation buffer was 50 mM Tris-HCl, pH 8, containing 0.15 M NaCl and 10% (v/v) glycerol.

Compounds were assayed in the activation buffer at room temperature in black 96-well plates using the substrate of Boc-Phe-Ser-Arg-AMC (Bachem, I-1400). A SpectraMax GeminiEM fluorescence plate reader from Molecular Devices was used to follow the hydrolysis rate kinetically, using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Whereas Magklara and co-workers reported a K_m of 410 μM for the wild-type hK6 on the same substrate,[30] the K_m value of our triple mutant was measured at 302 μM. A substrate

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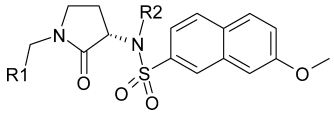
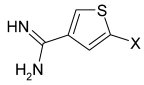
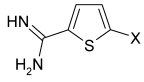
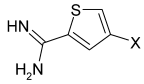
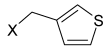
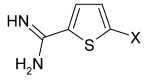
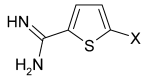
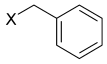
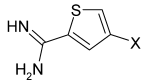
concentration of 400 μM was used for IC_{50} determination. The IC_{50} of an inhibitor was calculated from initial reaction rates in the absence and presence of the inhibitor over a range of concentrations without further correction for [S] relative to K_m.

One series of inhibitors identified during this round of enzymatic testing is the chemotype with an amidinothiophene P1 group (P1 refers to the group bound in the S1 pocket of trypsinlike serine proteases) and a pyrrolidinone-sulphonamide scaffold linker, as shown in Table 1. The SAR indicates that the

Table 1. IC_{50} Values of hK6 Inhibitors with an Amidinothiophene P1 Group and a Pyrrolidinone-Sulphonamide Scaffold Linker



	R1	R2	IC_{50} (μM)
1		-H	> 30
2		-H	7.75
3			2.9
4		-CH_3	8.2
5			1.5
6		-CH_3	> 30

amidino moiety, the R1 group as shown in Table 1, prefers a "pseudo-*para*" configuration relative to the pyrrolidinone scaffold. While the sulfur atom on the thiophene ring can be at all three possible positions, 2-amidino-thiophene connected to the scaffold at the 5-position is more preferred. For example, compounds **2** and **4** are more potent than compounds **1** and **6**, respectively. An aromatic substitution at the R2 position is also strongly preferred for strong hK6 activity. As shown in Table 1, when R2 is unsubstituted, such as compounds **1** and **2**, the potency is much lower than those with aromatic substitutions, such as compounds **3** and **5**. Similarly, a simple methyl group at the R2 position is not adequate. For instance, compounds **4** and **6** are less potent than their aromatic counterparts **5** and **3**, respectively.

The hK6 protein produced for enzymatic assay was further purified for crystallographic use through gel filtration chromatography to >95% purity as assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and concentrated to 11.5 mg/mL in buffer consisting of 20 mM Tris, pH 8.5, and 150 mM NaCl. Crystals were grown using the hang-drop method by mixing 1 μL of protein solution with 1 μL of well solution consisting of 0.20 M trimethylamine *N*-oxide, 0.1 M Tris-HCl, pH 8.5, and 20% PEG MME 2000 in the presence of 10 mM benzamidine. Compound **3** was soaked into hK6-benzamidine crystals prior to freezing for data collection. The structure was solved by the molecular replacement method using a model based on the Protein Data Bank entry 1LO6. The structure was refined using CNX-2005,[31] with crystallographic statistics summarized in Table 2. The coordinates of

Table 2. Crystallographic Statistics for hK6 Complexed with Compound 3 (Values in Parentheses Correspond to the Highest Resolution Shell)

data collection	
beamline	ESRF−ID21
wavelength (Å)	0.933
space group	$P2_12_12_1$
cell parameters (Å)	a = 43.938, b = 47.909, c = 107.553
resolution (Å)	40−1.88 (1.95−1.88)
redundancy	2.9 (2.8)
completeness (%)	97.9 (97.2)
$I/\sigma I$	17.7 (3.7)
R_{merge} (%)	5.8 (26.8)
refinement	
resolution (Å)	30−1.88
no. of reflections	17947
percentage of R_{free}	5
R_{work}/R_{free} (%)	19.3/22.3
rms deviations	
bond lengths	0.004
bond angles	1.3
Ramachandran plot (%)	
preferred region	97.2
allowed region	2.8
outlier	0

hK6−compound **3** complex structure have been deposited with Protein Data Bank under an accession code of 3VFE.

The overall structure of hK6 complexed with compound **3** is highly similar to those of other trypsinlike serine proteases, which is consistent with the findings by Bernett and co-workers.[25] Each complete unit of hK6/inhibitor complex includes two β-barrels, each of which has six antiparallel β-strands connected by α-helices and loops. Each hK6 has one substrate binding site, which is situated at the junction of the two β-barrels.

Compound **3** binds in the substrate-binding site of hK6 in a binding mode similar to many trypsinlike serine proteases. As shown in Figure 4, the amidinothiophene group binds in the S1 pocket of hK6 with the amidino group forming an H-bonding network with the carboxylic side chain of Asp189 and the backbone carbonyl oxygen of Asn217. The thiophene ring interacts with the hydrophobic side chain of Ile218, which is part of a unique insertion distinguishing kallikreins from many other trypsinlike serine proteases, such as fXa and β-tryptase. The pyrrolidinone group is situated adjacent to Ser195 and His57 of the catalytic center with the ketone oxygen H-bonding with the backbone NHs of Gly193, Asp194, and Ser195 in the "oxyanion hole". Oxygen atoms of the sulphonamide group form an H-bonding network with the side chain NH_2 of Gln192, which anchors the binding mode and projects the naphthalene tail further into the S2′ pocket, where the 7-methoxy-naphthalene tail displays hydrophobic interactions with Gln192, Phe151, and Leu40. In addition to constraining the conformational flexibility of the naphthalene ring, the

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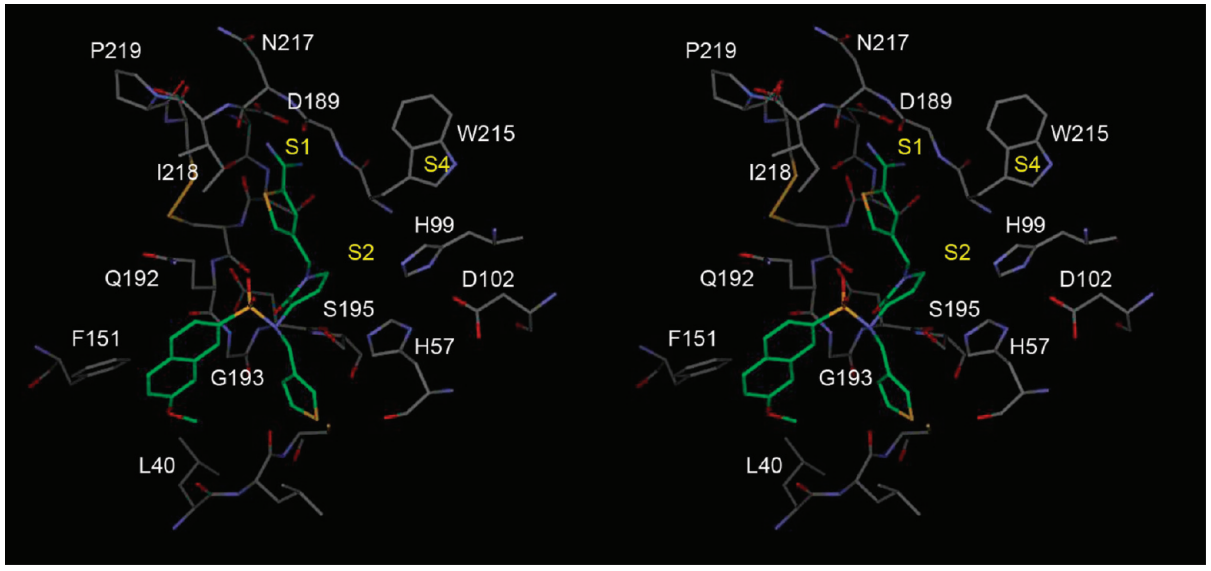

Figure 4. Stereoview of compound **3** in the hK6 substrate binding site, where carbon atoms of compound **3** are colored in green and those of hK6 are colored in gray. The coordinates of this complex have been deposited in the Protein Data Bank (PDB code: 3VFE).

thiophen-3-yl methyl group connected to the sulphonamide further contributes to the overall binding affinity through hydrophobic interactions with both His57 of the catalytic triad and Leu40 in the S1′ pocket. The X-ray structure of this complex supports the SAR observation that an aromatic substitution at the R2 position, as shown in Table 1, contributes significantly to the overall binding affinity.

In conclusion, a series of compounds with an amidinothiophene P1 group and a pyrrolidinone-sulphonamide scaffold has been identified as possible binders for hK6 through an in silico virtual screening. Their inhibitory activity for hK6 was confirmed through an enzymatic assay. One representative compound was soaked into hK6 crystal with its structure determined at a resolution of 1.88 Å. The X-ray structure indicated that the amidinothiophene of the inhibitor bound in the S1 pocket and the pyrrolidinone-sulphonamide linker bound adjacent to the catalytic center, where the aromatic tail further extended into the S1′ and S2′ pockets. As the first nonmechanism-based small organic inhibitor for hK6, its identification is beneficial for further inhibitor-based target validation. As the first reported X-ray structure complexed with such inhibitor, it may be used as a roadmap to guide future inhibitor design and optimization.

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Notes

The authors declare no competing financial interest.

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