Towards the development of small molecule MO25-binders as potential indirect SPAK/OSR1 kinase inhibitors


Abstract: The binding of the scaffolding protein MO25 to SPAK and OSR1 protein kinases, which regulate ion homeostasis, causes up to 100-fold increase in their catalytic activity. Since various animal models showed that the inhibition of SPAK and OSR1 lowers blood pressure, we herein present a novel indirect approach for inhibiting SPAK and OSR1 kinases by targeting their protein partner MO25. To explore this approach, we developed fluorescent polarization assay and used it in screening a small in-house library of 8,400 compounds. This led to the identification of one molecule, HK01, as the first small molecule inhibitor of the MO25-dependent activation of SPAK and OSR1 in vitro. Our data confirms the feasibility of targeting this protein-protein interaction by small molecules and highlights the potential of these molecules to modulate ion co-transporters and thus cellular electrolyte balance.

STE20/SPS1-related proline-alanine-rich protein kinase (SPAK) and oxidative-stress-responsive kinase 1 (OSR1) are two serine/threonine protein kinases that share 68% sequence identity.[1] Although under normal conditions these kinases are inactive, they become activated by osmotic stress via two distinct mechanisms.[2] First, they get phosphorylated in a highly conserved threonine residue in their T-loop motifs, SPAK T233 and OSR1 T185, by a family of protein kinases known as WNKs.[3] The acquired basal activity obtained following WNK-phosphorylation is subsequently further enhanced by 80- to 100-fold through the binding to the mouse protein-25 (MO25), a scaffolding protein (Fig. 1).[4] Two isoforms of MO25 exist in humans, MO25α and β, which are expressed in many tissues mostly in testis, brain, spleen, pancreas and kidney.[4] Both isoforms activate SPAK and OSR1 in equal measures.[4] Once fully activated, SPAK and OSR1 kinases phosphorylate a series of ion co-transporters such as the sodium-potassium-chloride co-transporters 1 and 2 (NKCC1 and NKCC2, respectively).[2]

The involvement of SPAK and OSR1 kinases in regulating ion homeostasis via the phosphorylation of ion co-transporters and their possible roles in the pathogenesis of diseases that involve electrolyte imbalance was confirmed when it was discovered that genetic mutations in the SPAK and OSR1 upstream kinases WNKs cause hypertension in humans (Fig. 1).[5] This role was further emphasized by recent reports which revealed that mutations in the E3 ubiquitin ligases, Cul-3 and KLHL3, which regulate the protein levels of WNK kinases, cause hypertension (Fig. 1).[6] These studies suggested that the inhibition of SPAK and OSR1 kinases would lead to reduced blood pressure. This notion was confirmed by various SPAK knock-in and knock-out mouse models,[7] which collectively indicated that small molecules that inhibit the catalytic activity of SPAK and OSR1 would have the potential to lower blood pressure and represent a new class of antihypertensive drugs.

Figure 1. A general representation of the WNK-SPAK/OSR1-MO25 signaling cascade and its role in the regulation of sodium, potassium and chloride ion co-transporters.

To date, few WNK-SPAK/OSR1 signalling inhibitors have been reported. These include those that bind directly to WNK kinases[8] or those that bind to SPAK/OSR1 and inhibit downstream WNK-signalling.[9] In this work, we explored the discovery of small molecules that bind to the scaffolding protein MO25 as means of inhibiting its binding to SPAK and OSR1 kinases and consequently inhibiting their significant MO25-dependent activation.
Our pursuit of specific MO25-small molecule binders was inspired by the fact that MO25 binding to SPAK and OSR1 kinases is mediated by two highly conserved tryptophan residues on SPAK (W382 and W384) and OSR1 (W336 and W338).\(^1\) Indeed, we recently solved the crystal structure of MO25 in complex with the WEW motif of SPAK (unpublished data) and this showed that these two tryptophan residues sit in a hydrophilic site on MO25 (Fig. 2A) akin to how the pseudokinase STRADα WEF motif binds to MO25. With this in mind, we developed a fluorescence polarization (FP) assay for measuring the binding of MO25 to a 16-mer WEWpS peptide derived from SPAK and OSR1, which contains the highly conserved tryptophan residues. Briefly, the 16-mer peptide (TEDGDWEWPspSDDEMDEK) derived from human SPAK (aa. 377-392) was synthesised with a N-terminal domain CCPGCCGG motif and was labelled with Lumio® Green. The fluorophore-peptide conjugate was subsequently incubated with increasing concentrations of MO25 and fluorescence polarization was measured to determine the binding affinity, \(K_D = 5 \pm 0.9 \mu M\) (Fig. 2B). We also used a mutated version of the peptide where the tryptophan residues were mutated into alanine (TEDGDAEApSDDDEMDEK) and this did not bind to MO25 confirming the importance of these two tryptophan residues of SPAK and OSR1 in mediating binding to MO25 (Fig. 2B). Given that this assay is compatible with high throughput screening (HTS) technologies, we next employed it in screening 4,000 compounds from our in-house diversity-set library of small molecules to identify hit small molecule MO25-binders.

Prior to running the screen, we first studied the effect of DMSO on the FP signal since the library compounds were already dissolved in DMSO. For this, the FP assay was run in increasing concentrations of DMSO ranging from 0.3% to 10%. A final concentration of 4% DMSO was chosen as 90% of the FP signal was retained (Fig. 2C). Subsequently, we investigated the stability of the FP signal over time. Thus, the assay was read at various times ranging from 20 minutes to 24 h and, impressively, the FP signal was stable over 24 h (Fig. 2D).

Next, we screened 4,000 compounds from the University of Birmingham in-house diversity-set library of small molecules. The compounds were screened at a single final concentration of 20 \(\mu M\) in duplicate. Six compounds were identified from the primary screen as possible MO25-binders since they achieved \(> 30\%\) inhibition of MO25 binding to the 16-mer WEWpS peptide. Further analysis of these compounds indicated that only one compound, HK01 (Fig. 2E, see Supplementary Information), was a true MO25-binder. A prior knowledge search using the structure of HK01 and its reported IDs did not lead to any results. Using the \textit{in vitro} FP competition assay, HK01 was able to inhibit the 16-mer WEWpS peptide binding to MO25, \(IC_{50} = 78 \pm 4 \mu M\) (Fig. 2F). Interestingly, a series of compounds with structural similarity to HK01, especially those with the anthracene moiety, did not show any ability to inhibit the 16-mer WEWpS peptide binding to MO25 at 100 \(\mu M\) (see Supplementary Information). SPR studies showed that the \(K_D\) of HK01 towards wild-type MO25 was 127 \(\pm 6 \mu M\) (see Supplementary Information).

To explore if this inhibition of binding could be translated into inhibition of the catalytic activity of SPAK and OSR1 kinases, we run an \textit{in vitro} kinase assay employing full length OSR1 T185E. We used this OSR1 where the T-loop threonine was mutated into glutamic acid, to mimic phosphorylation, as it is constitutively active.\(^2\) In this assay, full length OSR1 T185E in the presence of MO25 showed that HK01 exerted dose-dependent inhibition of the MO25-dependent activation of OSR1 T185E (Fig. 2G). Although MO25 binding to SPAK and OSR1 kinases is thought to be 1:1, previous studies\(^3\) have established that pronounced activation of OSR1 T185E \textit{in vitro} required five molar access of MO25 and this led us to use high

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concentrations of the MO25 small molecule binder HK01 (Fig. 2G). Interestingly, HK01 was unable to inhibit MST3, a protein kinase that belongs to the same family of kinases as SPAK and OSR1, in vitro even at concentrations up to 500 μM (see Supplementary Information).

To further probe the ability of the hit compound HK01 to bind MO25 and inhibit its binding to SPAK and OSR1 protein kinases, we performed MO25 pull down assay in the presence and absence of HK01. For this, lysates from HEK293 cells, which endogenously express MO25, were incubated with N-terminally biotinylated 16-mer WEWpS peptide in the presence of increasing concentrations of HK01. Following 2 h incubation, streptavidine-biotin-16-mer WEWpS pulldown was performed and the pulled down material underwent blotting for MO25.

As shown in Fig. 3, in the absence of HK01, endogenous MO25 was pulled down by the 16-mer WEWpS peptide as expected. This binding, however, was abolished by the addition of 100 μM unlabeled 16-mer WEWpS peptide as expected. Interestingly, titration of increasing concentrations of HK01 resulted in the inhibition of MO25 binding to the 16-mer WEWpS peptide in a concentration dependent manner. Notably, western blotting of the flow through material following the streptavidine-biotin pulldown showed higher amounts of MO25 in samples where the MO25 binding to the 16-mer peptide was inhibited by the unlabeled 16-mer WEWpS peptide or HK01 (Fig. 3). This confirms that HK01 directly binds to MO25 and inhibits its binding to the 16-mer WEWpS peptide in HEK293 cell lysates.

**Figure 3. Competition of MO25 binding to the SPAK 16-mer WEWpS peptide by HK01.**

HEK293 cell lysates were treated with either the SPAK 16-mer WEWpS peptide or HK01 at increasing concentrations. This was followed by incubation with the biotinylated version of the SPAK 16-mer WEWpS peptide. The pulled down material underwent blotting for MO25. The flow through from this pulldown experiment was probed for β-catenin as a negative control and MO25.

Given the ability of HK01 to indirectly inhibit OSR1 T185E activity in vitro through the inhibition of its binding to MO25, we next investigated the ability of HK01 to inhibit the phosphorylation of SPAK and OSR1 physiological substrates in cells. In particular, we focused on the inhibition of the sodium, potassium and chloride co-transporter NKCC1, a known in vivo substrate of SPAK and OSR1 kinases.[9] For this experiment, we used HEK293 cells as they endogenously express WNK kinases, SPAK, OSR1, MO25 and NKCC1. The cells were treated with increasing concentrations of HK01 or 10 μM of STOCK1S-50699[9] for 30 minutes followed by hypotonic buffer to activate WNK-SPAK/OSR1 signaling.[11] Subsequently, they were harvested and probed for total SPAK, SPAK pS373, total NKCC1 and NKCC1 pT203, pT207 and pT212. The results show that hypotonic buffer led to a pronounced increase in the phosphorylation of SPAK pS373 and NKCC1 at the T203, T207 and T212 residues indicating the activation of the WNK-SPAK/OSR1 signaling cascade (Fig. 4).

In conclusion, the work presented herein confirms the possibility of targeting the scaffolding protein MO25 with small molecules, which consequently leads to the inhibition of SPAK and OSR1. Indeed, we have shown that compound HK01 can successfully inhibit MO25 binding to SPAK and OSR1 leading to their in vitro inhibition without directly binding to them. Efforts aimed at the structural optimization of HK01 to achieve better binding affinity are currently ongoing and will be reported in the future. Collectively, our work represents a novel strategy in the
discovery of indirect SPAK and OSR1 inhibitors, which have the potential to be developed into new antihypertensive drugs.

Experimental Section

Reagents

Tissue-culture reagents and protein expression materials were purchased from Sigma Aldrich. Peptides were ordered from GLS Peptide Synthesis (China). STOCK15-50699 was purchased from Vitas-M Laboratory, Ltd. ADP-Glo™ Kinase Assay kit was purchased from Promega. LuminoTM Green (FIASH-EDT) was purchased from Carbosynth. HK01 was purchased from ChemBridge (ID:7182449).

Buffers

Cells Lysis Buffer: 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% (w/v) Nonidet P40, 0.2 M sucrose, 0.1% (v/v) 2-mercaptoethanol, 0.1 mM PMSF, 1 mM benzamidine. Bacterial lysis buffer: 50 mM Tris-HCl (pH 7.5), 0.15 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.27 M sucrose, 0.1% PMSF, 1 mM benzamidine, 0.5 mg/ml lysozyme, 0.3 mg/ml DNase and 2mM DTT. Buffer A: 50 mM Tris-HCl (pH 7.5), 0.1 mM EGTA and 0.1% (v/v) 2-mercaptoethanol. Normal buffer: bacterial lysis buffer without lysozyme and DNase. High salt buffer: normal buffer with 500 mM NaCl. TBS-T buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.2% (v/v) Tween-20. Running buffer: 34.6 mM SDS, 25 mM Tris-borate and 1.2 mM glycine. Transfer buffer: 48 mM Tris-base, 39 mM glycine containing 20% (v/v) methanol. Hypotonic low-chloride buffer: 67.5 mM sodium glutonate, 2.5 mM potassium glutonate, 0.25 mM CaCl2, 0.25 mM MgCl2, 0.5 mM NaN3, 0.5 mM Na2SO3 and 7.5 mM HEPES. SDS sample buffer (4X): 40 % glycerol, 240 mM Tris-HCl (pH 6.8), 8% SDS, 0.04% (w/v) bromophenol blue and 5% 2-mercaptoethanol. Blocking buffer: 10% (w/v) dried skimmed milk in TBST. Dialysis buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 2 mM DTT. Antibodies

FIASH-EDT-Conjugated peptide, anti-hemoglobin blots were purchased from Cell Signalling Technology, GAPDH antibodies [MAB-15738], anti-sheep [31480] and anti-rabbit [31460] secondary antibodies conjugated to HRP were purchased from Thermo Scientific.

Plasmids, protein expression and purification

pGEX-6-MO25alpha [DU2945] DNA plasmid was purchased from the DSTT, University of Dundee. The OSR1 T185E protein [DU6231] was purchased from the DSTT, University of Dundee.

Streptavidin beads were washed with Buffer A three times. 1.2 mg of HEK293 cells were pre-cleared using the prewashed streptavidin beads then divided into equal amounts using Eppendorf tubes. The tubes were then incubated with 100 µM 16-mer WEPW5 peptide as a positive control or increasing concentrations of HK01 (0.05 mM, 0.1 mM, 0.25 mM, 0.75 mM, 1 mM and 2 mM) for 2 h. 3 µg of the biotinylated 16-mer WEWpS peptide was added to each sample for 10 mins at 4 °C. 20 µl of the washed streptavidin beads slurry was added to each sample and mixed with roller-shaker at 4 °C. Following a spin-down at 3000 rpm, for 10 mins, the supernatant was collected and concentrations were determined using Bradford assay. The beads were washed 3 times more with buffer A. Using 4x SDS, samples were prepared for immunoblotting.

Cell based assays

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20 μg of cell lysates in SDS sample buffer were subjected to electrophoresis on a polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked for 1 h with 10% (w/v) skimmed milk powder in TBS-T. The membranes were then incubated with the relevant primary antibodies overnight. The blots were then washed six times with TBS-T to remove the primary antibody and subsequently incubated with secondary HRP-conjugated antibodies. After repeating the washing steps, the signal was detected with the enhanced chemiluminescence reagent. Immunoblots were developed using a film automatic processor (SRX-101; Konica Minolta Medical), and films were scanned with a 300-dpi resolution on a scanner (PowerLook 1000; UMAX).

**ADP-Glo in vitro kinase assay**

The kinase activity was assayed using ADP-Glo® kinase assay in 96-well plate in triplicate as reported.\[24]\] HK01 was tested at 100 μM and 500 μM final concentrations against GST-OSR1 T185E (0.4 μM final concentration) in the well plate in triplicate as reported.

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HTS screening of a small library of 4,000 compounds led to the identification of one molecule, HK01, as a promising binder to the scaffolding protein MO25. This molecule was able to inhibit MO25-dependent activation of SPAK and OSR1 protein kinases \textit{in vitro} and in cells. Collectively, this confirms that MO25 is amenable to targeting by small molecules and this approach could yield useful SPAK and OSR1 indirect kinase inhibitors.