Towards the Development of Small-Molecule MO25Binders as Potential Indirect SPAK/OSR1 Kinase Inhibitors

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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 ~4,000 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. Although under normal conditions these kinases are inactive, they become activated by osmotic stress via two distinct mechanisms. 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. 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). Two isoforms of MO25 exist in humans, MO25α and β, which are expressed in many tissues mostly in testis, brain, spleen, pancreas and kidney. Both isoforms activate SPAK and OSR1 in equal measures. 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). 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). 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 WK kinases, cause hypertension (Fig. 1). 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, 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.

To date, few WNK-SPAK/OSR1 signalling inhibitors have been reported. These include those that bind directly to WNK kinases or those that bind to SPAK/OSR1 and inhibit downstream WNK-signalling. 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.

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

[Supporting information for this article is given via a link at the end of the document.]
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). Indeed, we recently solved the crystal structure of MO25 in complex with the WEW motif (unpublished data) and this showed that these two tryptophan residues sit in a hydrophobic site on MO25 to bind the pseudokinase STRADα WEF motif. With this in mind, we developed a fluorescence polarization (FP) assay for measuring the binding affinity of MO25 to a 16-mer WEWs peptide derived from SPAK and OSR1, which contains the highly conserved tryptophan residues. Briefly, the 16-mer peptide (TEDGDWEwpsDDMDEK) derived from human SPAK (aa. 377-392) was synthesised with an N-terminal domain CCPGCCGG motif and was labelled with Lumio™ Green. This fluorophore-peptide conjugate was subsequently incubated with increasing concentrations of MO25 and fluorescence polarization was measured to determine the binding affinity, \( k_D \approx 5 \pm 0.9 \text{ \mu M} \) (Fig. 2B). We also used a mutated version of the peptide where the tryptophan residues were replaced with alanine (TEDGDAEpsDDMDEK) 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 form the University of Birmingham in-house diversity-set library of small molecules. The compounds were screened at a single final concentration of 20 μM 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 WEWs 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 in vitro FP competition assay, HK01 was able to inhibit the 16-mer WEWs peptide binding to MO25, \( IC_{50} = 78 \pm 4 \text{ \mu M} \) (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 WEWs peptide binding to MO25 at 100 μM (see Supplementary Information). SPR studies showed that the \( K_D \) of HK01 towards wild-type MO25 was 127 ± 6 μM (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 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. 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 have established that pronounced activation of OSR1 T185E in vitro required five
molar excess of MO25 and this led us to use high 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 WEWP5 peptide in the presence of increasing concentrations of HK01. Following 2 h incubation, streptavidine-biotin-16-mer WEWP5 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 WEWP5 peptide as expected. This binding, however, was abolished by the addition of 100 μM unlabeled 16-mer WEWP5 peptide as expected. Interestingly, titration of increasing concentrations of HK01 resulted in the inhibition of MO25 binding to the 16-mer WEWP5 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 WEWP5 peptide or HK01 (Fig. 3). This confirms that HK01 directly binds to MO25 and inhibits its binding to the 16-mer WEWP5 peptide in HEK293 cell lysates.

![Figure 3. Competition of MO25 binding to the SPAK 16-mer WEWP5 peptide by HK01.](image)

Given the ability of HK01 to directly 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. 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 for 30 minutes followed by hypotonic buffer to activate WNK-SPAK/OSR1 signaling. Subsequently, they were harvested and probed for total SPAK, SPAK pT203, total NKCC1 and NKCC1 pT203, pT207 and pT212. The results show that hypotonic buffer led to a pronounced increase in the phosphorylation of SPAK pT203 and NKCC1 at the T203, T207 and T212 residues indicating the activation of the WNK-SPAK/OSR1 signaling cascade (Fig. 4).

These phosphorylation sites were suppressed in samples treated with the WNK-signaling inhibitor STOCK1S-50699 as expected. The 16-mer WEWP5 peptide, however, showed no inhibition of NKCC1 or SPAK S373 phosphorylation most likely due to lack of cellular uptake because of its molecular weight of approximately 1800 g/mol. Interestingly, the phosphorylation of NKCC1 at T203, T207 and T212 sites was inhibited by HK01 in concentration-dependent manner with the SPAK/OSR1-mediated phosphorylation of NKCC1 being completely inhibited at high concentrations. Critically, unlike STOCK1S-50699, HK01 had no significant effects on SPAK S373 phosphorylation confirming that it does not inhibit WNK kinases or their binding to SPAK. This reflects the ability of HK01 to inhibit MO25-dependent activation of SPAK and OSR1 kinases. Notably, the HK01 was not toxic as the cells were healthy even at the high concentrations (2 mM) of HK01 and its effects seem to be specific to MO25 binding since there was no effect on the total levels of beta-catenin, a protein unrelated to the WNK-SPAK/OSR1-signalling pathway (Fig. 4).

![Figure 4. Inhibition of SPAK/OSR1 kinases in HEK293 cells by HK01.](image)
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). STOCK1S-M05699 was purchased from Vitas-M Laboratory, Ltd. ADP-Glo™ Kinase Assay kit was purchased from Promega. LumioTM Green (FlAsH-EDT2) 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% (v/v) Nonidet P40, 0.27 M sucrose, 0.1% (v/v) 2-mercaptoethanol, 0.1 mM PMFS, 1 mM benzamidine. Bacterial lysis buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mg/mL triton, 0.27 M sucrose, 0.1 mM PMFS, 1 mM benzamidine, 0.5 mg/ml lysosome, 0.3 mg/ml Dnase and 2 mM 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 lysosome 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-base and 1.92 mM glycine. Transfer buffer: 48 mM Tris-base, 39 mM glycine containing 20% (v/v) methanol. Hypotonic low-chloride buffer: 67.5 mM sodium gluconate, 2.5 mM potassium gluconate, 0.25 mM CaCl2, 0.25 mM MgCl2, 0.5 mM NaH2PO4, 0.5 mM Na2SO4 and 7.5 mM HEPES. SDS sample buffer (4X): 40 % glycerol, 240 mM Tris-HCl (pH 8.6), 8% SDS, 0.04% (w/v) bromophenol blue and 5% of 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**

SPAK phospho-Ser373 [S670B], MO25a [S989A], NKC1 [DU 6146], NKC1 phospho-Thr203+Thr207+Thr212 [S763B] and SPAK [SS51D] antibodies were purchased from DSTT. University of Dundee. SPAK [2281S] and β-Catenin [5962S] were purchased from Cell Signalling Technology, GAPDH antibody [MAS-15738], anti-ship [31480] and anti-rabbit [31460] secondary antibodies conjugated to HRP were purchased from Thermo Scientific.

**Plasmids, protein expression and purification**

pGEX-6-M025alpaha [U2945] DNA plasmid was purchased from the DSTT, University of Dundee and the protein was expressed and purified in-house as described elsewhere.[10] The ORSI T185E protein [DU6231] was purchased from the DSTT, University of Dundee.

**Development of fluorescence polarization assay:**

SGM Perstar plate reader was used to measure the fluorescence polarization, with an excitation and emission wavelengths of 485 nm and 538 nm, respectively. Assays were performed using black, flat bottom Greiner 384-well plates in triplicate with at least twelve data points per curve and data analysis was completed using GraphPad Prism 7.0.

**Peptide labelling and determination of optimum fluorescent probe concentration**

The 16-mer TEGDGWEwpSSDEMDEK peptide derived from human SPAK (aa. 377-392) was synthesised with an N-terminal CCPCCG domain. The lyophilized peptide was initially suspended in 50 mM NH4HCO3 (pH 8). The labelling step was achieved by incubating 10 μM of peptide with 30 μM of FlAsH-EDT2 in 500 μL dialysis buffer in the dark at 4°C for 2 h. The peptides were then dialyzed for 4 h into dialysis buffer using a Mini Dialysis kit with 1 kD cut-off (GE Healthcare) and then for another 12 h with a fresh dialysis buffer. The optimum FlAsH-EDT2 labelled peptide concentration for FP assay was determined to be 10 nM as this concentration exhibited about ten times more fluorescence intensity signal compared to background controls.

**Fluorescence polarization binding and stability studies**

10 nM of FlAsH-EDT2-labelled peptide (TEDDGWEwpSSDEMDEK or TEDDGDEpSSDEMDEK) was mixed with MO25 at various concentrations (0.005-50 μM) in dialysis buffer in a final volume of 30 μL. The plates were incubated at RT and readings were taken at various time points.[12]

**DMSO tolerance**

Increasing concentrations of DMSO (%—10% of assay volume) were added to the reaction mixture containing 10 nM of the FlAsH-EDT2 labelled 16-mer peptide (TEDDGWEwpSSDEMDEK) and 5μM MO25 protein. The reaction mixture was incubated at RT for 2 h and then fluorescence polarization measurements were taken.

**Fluorescence polarization competition studies**

Competitive binding experiments were performed in a total of 25 μL dialysis buffer. Fluorescent peptide and MO25 were used at final concentrations of 10 nM and 5 μM respectively and DMSO was added to all wells and kept at final concentration of 4%. 0.05% of Tween-20 was used in the FP assay to prevent aggregation. For each assay, blank controls contained the fluorescent peptide only; negative controls contained the fluorescent peptide and MO25 while the 16-mer WEPwS was added to the fluorescent peptide and MO25 for positive controls. The effect of the competitors on the disruption of the interaction is expressed as % of control. This latter (% of control) for each dose of competitor was calculated using the following equation:

\[
\%\text{ of control} = \frac{\text{recorded FP signal competitor} - \text{recorded FP signal blank}}{\text{recorded FP signal control} - \text{recorded FP signal blank}} \times 100
\]

Where recorded FP signal blank is the average measured FP signal from fluorescent peptide only wells, the recorded FP signal control is the average FP signal from wells containing MO25 and fluorescent peptide in the absence of inhibitors, the recorded FP signal competitor is the FP signal from wells containing MO25 and fluorescent peptide in the presence of competitors. Dose-dependent experiments to determine the IC50 for HK01 were performed in the same manner using at least 12 concentrations in 2-fold serial dilutions from 1000 μM. After 2 h incubation at room temperature, the fluorescence polarization measurements were taken as described above.

**Assay performance**

Z’ factor was used to evaluate the assay quality and was calculated to be 0.7 indicating the robustness of the assay.[12] This reported Z’ factor is the average of 3 experiments of 16 replicates performed on different days. It was determined based on the following equation:

\[
Z' = 1 - \frac{3(\sigma_{positive} + \sigma_{negative})}{|\mu_{positive} - \mu_{negative}|}
\]

Where \( \sigma \) and \( \mu \) are the standard deviations and means of the negative (FlAsH-EDT2-labelled peptide bound to MO25 in 4% DMSO) and positive (FlAsH-EDT2-labelled peptide bound to MO25 in the presence of SPAK 16-mer WEPwS peptide) controls respectively.

**Pull-down assay**

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 WEPwS 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 WEPwS 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**

HEK-293 cells were cultured using 6-well plates in DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 units/mL penicillin and 0.1 mg/mL streptomycin at 37°C in a humidified 5% CO2 incubator. Cells were incubated with STOCK1S-M05699 (10 μM), SPAK 16-mer WEPwS peptide (100 μM) or HK01 at increasing concentrations (0.1 mM, 0.25 mM, 0.5 mM, 0.75 mM, 1 mM and 2 mM) for 30 minutes then stimulated with low chloride hypotonic buffer to activate WKI signalling for 30 minutes. Cells were then lysed with 200 μL lysis buffer and
centrifuged at 12,000 g at 4 °C for 10 minutes and the supernatant was collected.

**Immunoblotting**

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-dp1 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.[4] HK01 was tested at 100 µM and 500 µM final concentrations against GST-OSR1 T185E (0.4 µM final concentration) in the presence and absence of 5x MSO concentration. Kinase reactions were performed for 40 minutes at 30 °C with gentle agitation in 25 µL reaction buffer consisting of 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 0.1% (v/v) 2-mercaptoethanol, 0.1 mM ATP and employing 0.25 mM CATCHide (RRHYYDYTHNTYLYRTFCHNTR). The plate was then developed according to the manufacturer's protocol.[5] The plate was subsequently read on a BMG FLUOstar Omega plate reader. The final DMSO concentration was 4%.

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**Keywords:** MO25 • Scaffolding • Inhibitor • SPAK • OSR1

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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.

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