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WNK Signaling Inhibitors as Potential Antihypertensive Drugs

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Since the discovery of WNK mutations that cause an inherited form of hypertension in humans, there has been increasing interest in targeting WNK signaling as a novel strategy for modulating blood pressure. This notion is now supported by numerous mouse models with impaired WNK signaling that exhibit reduced blood pressure. Biochemical analyses of the various protein components that make up this signaling pathway have identified a number of plausible molecular targets that are amenable to targeting by small molecules. To date, a selection of small-molecule WNK signaling inhibitors have been identified and have shown promise in suppressing the activity of WNK signaling in cells and in animals. In this Minireview, we briefly discuss the WNK signaling pathway and provide an overview of the various druggable targets within this cascade, as well as the different WNK signaling inhibitors discovered to date.

Introduction

Hypertension (high blood pressure) is the most common chronic disorder seen in primary care. It affects more than a billion people worldwide and is a major risk factor for various disabling and fatal diseases such as stroke and heart failure.[1] Although current antihypertensive therapies have been very useful in controlling blood pressure, the emergence of resistance to these drugs, termed resistant hypertension, is being noted as global challenge in treating hypertension.[1] Furthermore, intolerance and allergies to some of the current approved antihypertensive drugs has limited their use. Together, these have contributed to an emerging need for novel antihypertensive drugs.

Among the key signaling pathways that are implicated in the regulation of blood pressure in vivo is the WNK signaling cascade.[2] The first link between this signaling pathway and hypertension came in 2001 when it was discovered that mutations in the genes that encode WNK kinases caused an inherited form of hypertension in humans known as Gordon's syndrome.[3] The first insight into the molecular mechanism by which the WNK serine/threonine protein kinases regulate blood pressure came when it was discovered that they bind and phosphorylate two other serine/threonine protein kinases termed SPAK and OSR1 resulting in their activation (Figure 1).[4] Indeed, WNK kinases phosphorylate SPAK and OSR1 at a highly conserved threonine residue on their T-loops, Thr233 and Thr185, respectively.[4a] Although additional WNK phosphorylation sites on SPAK and OSR1, mainly on their C-terminal domains were also been identified, the function of these phosphorylation sites remains not fully understood.[4a] Upon their phosphorylation by WNK kinases, SPAK and OSR1 subsequently bind to a scaffolding protein termed MO25 resulting in a significant activation of SPAK and OSR1, 80- and 100-fold, respectively.[5] Active SPAK and OSR1 in complex with MO25 then phosphorylate a series of sodium (Na^+), potassium (K^+) and chloride (Cl^-) ion co-transporters, for example, the Na^+-K^+-Cl^- co-transporter (NKCC) 1 and 2, the Na^+-Cl^- co-transporter (NCC) and K^+-Cl^- co-transporter (KCC) (Figure 1).[6] Notably, the phosphorylation of these ion co-transporters by SPAK and OSR1 kinases can lead to either their activation or inhibition and hence influencing electrolyte balance, which is ultimately translated into changes in blood pressure.[2,6]

A more recent link between WNK signaling and hypertension was reported when humans with genetic mutations in two E3 ubiquitin ligases known as KLHL3 and Cul3 had Gordon's syndrome.[7] Subsequent work showed that both KLHL3 and Cul3 regulate the total protein levels of WNK kinases and thus the observed phenotype was a result of impairing WNK signaling.[8] A more detailed account of the WNK-SPAK/OSR1-MO25 signaling pathway can be found in recent reviews by Alessi et al.[6] and Hadchouel et al.[9] The involvement of WNK-SPAK/OSR1-MO25 signaling in regulating blood pressure through the modulation of electrolyte balance in vivo suggested that this signaling pathway could be targeted in the discovery of new antihypertensive drugs. To test this hypothesis, numerous mouse models were generated and these exhibited Gordon's-syndrome-like symptoms.[2] Among the earlier mouse models were SPAK knock-in and knock-out mouse models, which exhibited reduced blood pressure and decreased phosphorylation of SPAK physiological substrates, such as NKCC1.[10]

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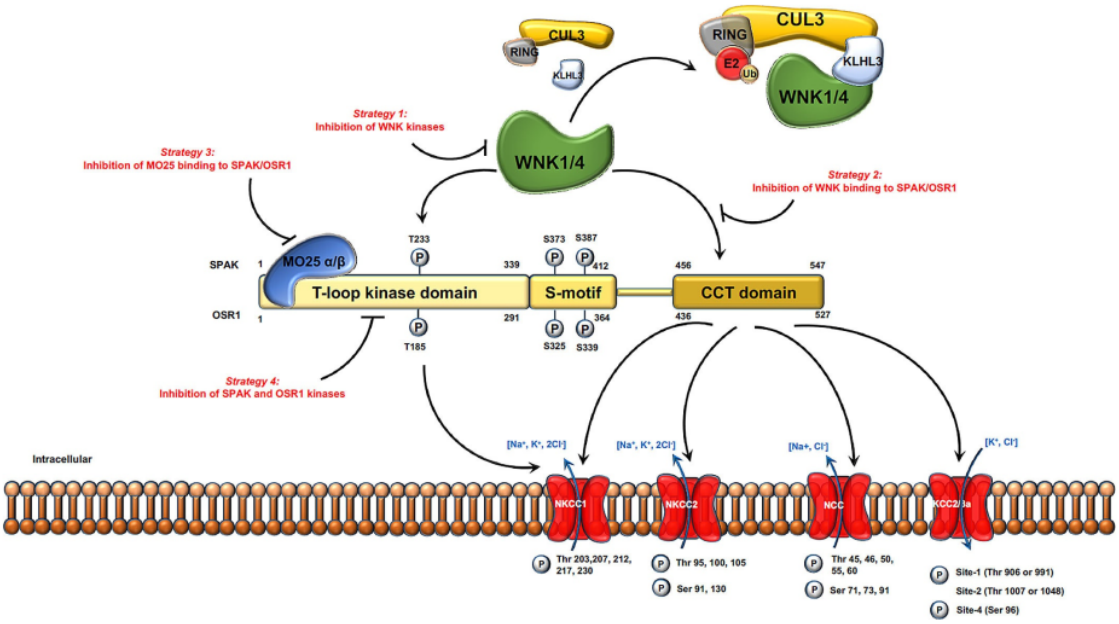

Figure 1. A depiction of the WNK-SPAK/OSR1 signaling pathway. The two E3 ubiquitin ligases KLHL3 and Cul3 mediate the ubiquitination of WNK kinases that leads to their degradation. WNK kinase isoforms 1–4 phosphorylate SPAK and OSR1 kinases on their T-loops, Thr233 and Thr185, respectively. Subsequently, SPAK and OSR1 bind the adaptor protein MO25, of which in humans there are two isoforms, α and β. Active SPAK and OSR1 in complex with MO25 phosphorylate a selection of sodium, potassium, and chloride co-transporters at different residues as shown. Such phosphorylation influences the function of these ion co-transporters, resulting in changes in electrolyte balance, which ultimately manifests itself in changes in blood pressure levels. The four different strategies (1–4) that are currently used in the discovery of WNK signaling inhibitors are highlighted. Ub: ubiquitin. CCT: conserved C-terminal.

These mouse models as well as later models provided strong evidence for the targeting of WNK signaling with small molecules in the discovery of new antihypertensive agents. These various SPAK and OSR1 mouse models have recently been reviewed by Murthy et al.[2] Given that the link between WNK signaling and hypertension was reported in 2001 and early knock-in mouse models of this signaling pathway appeared in mid 2006, the discovery of small molecules that target this signaling pathway has been relatively slow. Indeed, the first report of small molecules that inhibit WNK signaling appeared in 2013.[11] Since then, the discoveries of numerous small molecules that inhibit WNK signaling by targeting various molecular targets within the WNK signaling have been reported. Herein, we discuss the different strategies used for inhibiting WNK signaling and the various WNK signaling inhibitors that have been reported to date.

Strategies for Inhibiting WNK Signaling

Considering the WNK signaling pathway illustrated in Figure 1, four different molecular targets for inhibiting this cascade, as discussed below, have been exploited and yielded interesting small molecule WNK signaling inhibitors.

WNK kinase inhibitors

Kinase inhibitors, which are classically divided into ATP-competitive and non-ATP-competitive inhibitors, have been very powerful pharmacological tools and useful agents in treating many diseases.[12] Because the cornerstones of the WNK signaling pathway are the WNK protein kinases, it was only a matter of time until kinase inhibitors of WNKs were reported. However, it took almost 15 years from the discovery of WNK mutation causing hypertension in humans to the first report on the discovery of WNK kinase inhibitors.[13]

WNK ATP-competitive kinase inhibitors

Unlike other protein kinases, WNK kinases are known by the unusual placement of the catalytic lysine residue (Lys233) within their ATP binding site (Figure 2a).[11] The crystal structure of inactive WNK1 Ser382Ala, in which autophosphorylation and activation is prevented by mutation of Ser382 into alanine indicated that the common catalytic lysine's position in strand β3 (subdomain II) in most kinases was occupied by a cysteine residue (Cys250) in WNK1 (Figure 2a,b).[14] The catalytic residue normally binds to the α and β phosphate groups of ATP molecules and contributes along with the glycine-rich loop in stabilizing the ATP molecule in the active site. Moreover, it forms a

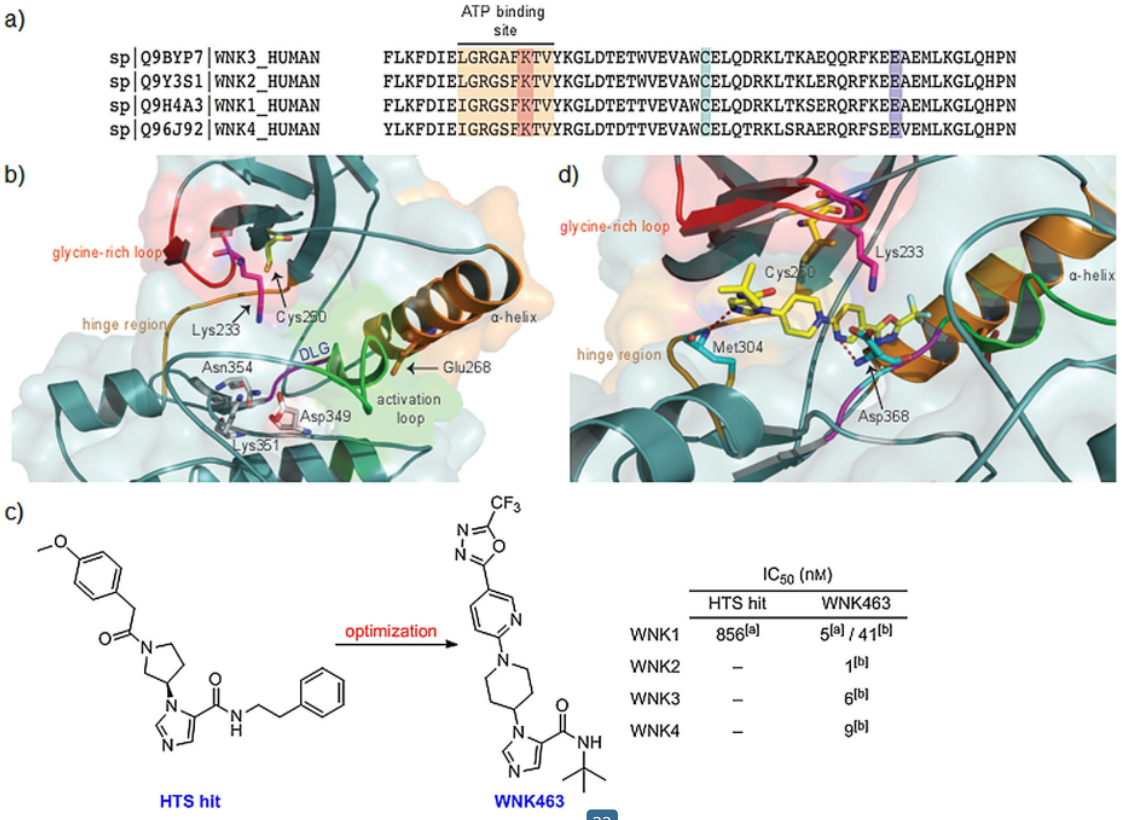

Figure 2. Discovery and development of ATP-competitive WNK kinase inhibitors. a) Sequence alignment of the ATP binding pockets of the four human isoforms of WNK kinases. Residues making up the ATP binding site are highlighted in light orange. The catalytic lysine residue, Lys233 for WNK1 and the corresponding Lys residues for WNK2–4, are highlighted in light pink. Other key residues, namely Cys250 and Glu268 for WNK1 and those of the corresponding WNK isoforms, are highlighted in green and purple, respectively. Human WNK isoform sequences were obtained from www.uniprot.org, and the sequence alignment was conducted using MUSCLE (www.drive5.com/muscle/). b) ATP binding pocket of WNK1 kinase showing the positions of key residues and structural motifs. c) Optimization of the initial HTS hit into WNK463 with the potencies of inhibiting WNK kinase isoforms in vitro. The kinase activity was measured using either MBP[a] or OSR1[b] as a substrate. d) Co-crystal structure of WNK463 with the WNK1 kinase domain (PDB ID: 5DRB). Docking images were produced by AutoDock Vina. Data listed in panel c) were taken from Yamada et al.[13a]

salt bridge with the adjacent glutamate residue in helix C, which contributes to the shape of the ATP binding site. However, in WNK1, the catalytic lysine (Lys233) residue exists in the glycine-rich loop in strand β2 (subdomain I) and is 13 Å away from the glutamate residue (Glu268) in helix C. Therefore, a large cavity in the back of the binding site near a cysteine residue (Cys250) is present.[14]

This unique structural feature was exploited in the targeting of this family of kinases to discover highly selective inhibitors that target their ATP binding site. Indeed, the discovery of the first selective small-molecule pan-WNK kinase inhibitor has recently been reported.[13a] High-throughput screening (HTS) of about 1.2 million compounds from the Novartis compound archive identified an HTS hit (IC_{50} = 856 nM) as a promising inhibitor of WNK1 in vitro (Figure 2c). Further medicinal chemistry efforts and optimization of this hit compound led to the more potent inhibitor WNK463, which showed potent inhibition of the four isoforms of WNK kinases (WNK1 IC_{50} = 5 nM, WNK2 IC_{50} = 1 nM, WNK3 IC_{50} = 6 nM, WNK4 IC_{50} = 9 nM) (Figure 2c). Notably, WNK463 showed potent inhibition of endogenous WNK kinases in HEK293 cells, IC_{50} = 106 nM.[13a]

WNK463 exhibited high selectivity for WNK kinases in that even at a relatively high screening concentration of 10 μM, only two out of the 442 human kinases studied showed inhibition >50%. The co-crystal structure of WNK463 with WNK1 kinase domain WNK463 indicated that it binds to WNK kinases with high affinity regardless of their phosphorylation state. Indeed, the crystal structure of the WNK1 S382A catalytic domain in complex with WNK463 (PDB ID: 5DRB) is generally similar to the one reported for apo-WNK1 Ser382Ala kinase domain. Interestingly, the solved co-crystal structure also revealed that WNK463 has a unique binding way in that it binds to the hinge part of the ATP pocket and extends toward a nearby WNK-specific back pocket through a narrow tunnel

which arises from the nonstandard placement of the catalytic lysine (Lys233) in the glycine-rich loop (Figure 2d).[13a]

Given the impressive in vitro potency and selectivity of the pan-WNK kinase inhibitor WNK463, it was subsequently studied in vivo. Oral dosing of spontaneously hypertensive rats (SHRs) with 1, 3, or 20 mg kg^{-1} led to a dose-dependent decrease in blood pressure and simultaneous increases in heart rate.[13a] Additionally, significant dose-dependent increases in urine output along with urinary sodium and potassium excretion rates were observed.[13a] In transgenic mice overexpressing human WNK1, oral dosing of WNK463 again resulted in a significant decrease in blood pressure in these hypertensive mice.[13a] Notably, kidney lysate samples from these mice showed a dose-dependent decrease in the phosphorylation of SPAK and OSR1, the physiological substrates of WNK kinases.[13a] This provided a strong evidence of the observed decrease in blood pressure was a result of the in vivo inhibition of WNK kinases.

Despite the promising in vitro and in vivo efficacy and selectivity profile of WNK463, the development of WNK463 into a potential antihypertensive agent was discontinued due to preclinical safety profile issues. A possible explanation for this would be the ubiquitous expression of some WNK kinases, especially WNK1, and the lack of selectivity of WNK463 across the four WNK isoforms, which may be involved in key physiological processes beyond those relating directly to the regulation of blood pressure.

WNK allosteric kinase inhibitors

Beyond ATP-competitive WNK kinase inhibitors, the Novartis group also discovered allosteric WNK kinase inhibitors.[13b] Allosteric modulation of protein kinases can ensure better selectivity profile for the inhibitor by targeting the less conserved regions in the kinase relative to the highly conserved ATP binding pocket. Because there were no structural information available about allosteric sites on WNK kinases, which regulate their catalytic activity, and hence enable the rational design of inhibitors, efforts were directed toward the use of high-throughput screening at high ATP concentrations with the premise of targeting allosteric sites that may exist in other conformational states.

Using a higher ATP concentration (100 μM, twofold above K_M), a single-point screening of the 1.2 million compounds from the Novartis compound archive at 50 μM concentration was first performed.[13b] This resulted in an initial hit list of 8257 compounds (0.8% hit rate), which exhibited >30% inhibition of WNK1. This list was then filtered and sorted using known structural data about kinase inhibitors and in silico approaches to decrease the number of hits to 2298 compounds. Further hit validation through determination of potency and prioritization based on cellular ELISAs narrowed the list of candidates down to HTS hit compound **1** (Figure 3a). This compound was considered a promising starting point as it showed a good selectivity profile for WNK1 kinase across a Novartis in-house 31-kinase panel. Structural optimization of this hit led to compound **2**, which was found to be highly selective inhibitor of WNKs 1–4 with a consistent IC_{50} = 0.570 μM at various ATP concentrations suggesting its binding to an allosteric site in the WNK kinases (Figure 3a).

The crystal structure of compound **2** bound to WNK1 revealed the formation of a novel ligand-induced allosteric pocket proximal to the ATP binding site (Figure 3b).[13b] The binding of compound **2** to WNK1 was found to trigger an outward movement of the αC-helix and stretching of the activation loop from the Apo-WNK1 to form this allosteric site displaying a type III-like allosteric binding mode.

Subsequently, a cellular assay was developed using rubidium as a surrogate for cellular potassium uptake to determine the effects of WNK inhibition by compound **2** on cellular electrolyte handling. In the HT29 cells, compound **2** produced a more potent dose-dependent inhibition of rubidium uptake by the NKCC1 ion co-transporter with an IC_{50} value of 0.24 μM relative to the direct NKCC1 blocker bumetanide, which showed an IC_{50} of 1.54 μM.[13b] Interestingly, compound **2** did not inhibit OSR1 even at a relatively high concentration of 10 μM indicating its selectivity for WNK kinases. Despite the encouraging potency and selectivity of compound **2** (and some of its analogues), the pharmacokinetic (PK) profile of this compound did not support further in vivo efficacy studies. Nevertheless, this compound and its analogues inspired the discovery of better lead compounds that exhibited potent efficacy and selectivity, which together permitted their in vivo testing. Indeed, extensive structure–activity relationship of compound **2** led to the discovery of compound **3** (Figure 3c).[15] This compound exhibited low nanomolar non-ATP-competitive inhibition of WNK kinases in vitro though the compound had high microsomal clearance. Still, compound **3** showed excellent selectivity when tested at 10 μM in a panel of 440 human kinases with few significant off-targets that include Burton's tyrosine kinase (BTK) and feline encephalitis virus related (FER) kinase.[15] Interestingly, compound **3** showed ~1000-fold selectivity for WNK1 vs. WNK4 and 57-fold selectivity for WNK1 vs. WNK2.[15] In vivo data in rats indicated compound **3** to have moderate clearance and low bioavailability. This was hypothesized to be a result of N-demethylation yielding the *N*-methylaminothiazole derivative of **3**, which itself had been noted to be chemically unstable in solution.[15] As means of addressing this, the perdeuteromethyl analogue of compound **3**, referred to as compound **4** (Figure 3c), which was thought to possess better a PK profile, was prepared and studied. In rat PK studies, this compound showed low clearance and a twofold improvement in bioavailability relative to compound **3**.[15]

When single dosed orally in mice overexpressing human WNK1 at 10, 30 and 100 mg kg^{-1}, compound **4** showed dose-dependent decrease in systolic blood pressure in terms of peak and time-weighted average vs. baseline.[15] Also, single ascending oral doses of compound **4** (vehicle, 10, 30 then 100 mg kg^{-1}) given in successive days in SHRs resulted in dose-dependent diuresis, natriuresis and kaliuresis in rats dosed from 10 to 100 mg kg^{-1}.[15]

The discovery of these various small-molecule WNK kinase inhibitors indicates that these protein kinases are amenable to being targeted by small molecules. Indeed, such approach can

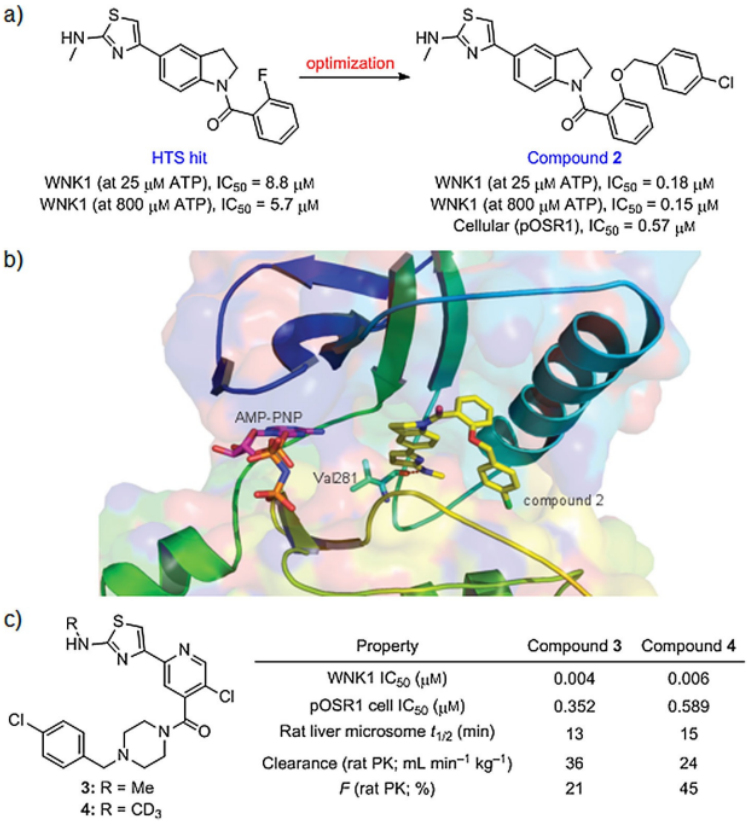

Figure 3. Discovery and optimization of a WNK kinases allosteric inhibitor. A) Chemical structure of the initial HTS hit and the optimized structure compound **2**. The in vitro kinase inhibitory potency of these compounds was measured using an OSR1 peptide as a substrate. Cellular activity was measured by blotting for OSR1 phosphorylation at Ser325, which is a WNK phosphorylation site. Data was taken from Yamada K. et al.[13b] B) Co-crystal structure of compound **2** with WNK1 kinase domain (PDB ID: 5TF9). Image produced with AutoDock Vina. Data listed in panel c) were taken from Yamada et al.[15]

lead to potent and selective WNK kinase inhibitors though achieving selectivity within the four WNK isoforms appears to be a difficult challenge. This may prove to be the bottleneck in the discovery of WNK kinase inhibitors as antihypertensive agents. In fact, the lack of the small molecules' selectivity across the WNK isoforms may have contributed to the observed undesired side effects of these compounds in vivo especially those associated with ATP-competitive WNK kinase inhibitors, for example, WNK463.[13a]

Inhibitors of WNK binding to SPAK/OSR1 kinases

Despite the fact that targeting protein–protein interactions (PPIs) is a stern challenge,[16] this approach is growing exponentially as a modern tool in target-based drug discovery and several PPIs modulators undergo clinical trials as treatments for different diseases.[17] Exploiting this approach, Uchida and colleagues[11] run a screen of 17000 compounds aimed at identifying molecules that inhibit WNK binding to SPAK and OSR1 kinases. This work benefited from earlier studies that identified the highly conserved C-terminal domain of SPAK and OSR1 being responsible for mediating their binding to WNK kinases.[18] In particular the finding that a small tetrapeptide motif RFxV or RFxI that is present in WNK kinases and various ion co-transporters binds SPAK and OSR1 C-terminal domains.[18,19] Inspired by this, Uchida and co-workers[11] labeled with a fluorophore, TAMARA, an 18-mer RFQV peptide derived from WNK4 and used a fluorescent correlation spectroscopy method to measure its binding to GST-tagged C-terminal domain of human SPAK (442–545).

This high-throughput screening exercise resulted in the discovery of the first two WNK-SPAK binding modulators known as STOCK1S-50699 ($IC_{50}=37\ \mu M$, $K_d=32\ \mu M$) and STOCK2S-26016 ($IC_{50}=16\ \mu M$, $K_d=20\ \mu M$) (Figure 4).[11] These compounds possessed different chemical scaffolds and were able to bind to SPAK C-terminal domain and disrupt its binding to WNK1 and WNK4 RFQV peptides in vitro.[11] In cells, these inhibitors were able to inhibit WNK-SPAK/OSR1 phosphorylation of

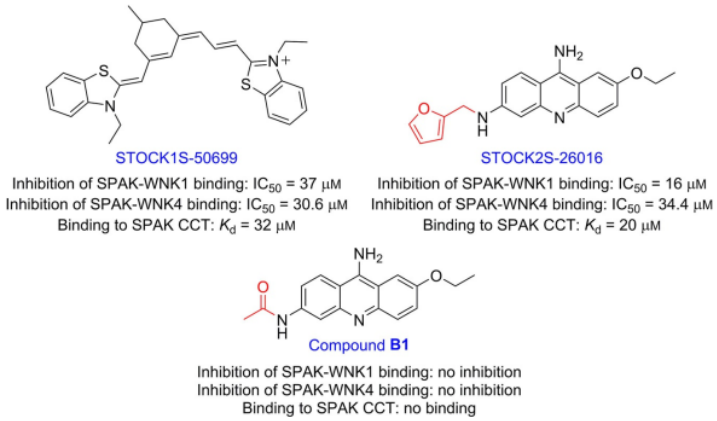

Figure 4. Chemical structures, in vitro inhibitory potency, and binding affinities of reported small molecules that inhibit WNK binding to SPAK/OSR1. Data were taken from Mori et al.[11]

the ion co-transporter NKCC1 at low micromolar concentrations.[11]

Since this report in 2013, STOCK1S-50699 has been widely used in subsequent studies as a WNK signaling inhibitor as compared with STOCK2S-26016, despite its non-drug-like properties. Notably, investigations into the in vitro selectivity of STOCK1S-50699 showed it to have a good selectivity when tested at a single 10 μM concentration against a panel of 150 kinases.[20] Interestingly, the study by Mori et al.[11] revealed a key detail of the structure–activity relationship of STOCK2S-26016, as it was shown that substitution of the flexible methylfuran moiety into a rigid smaller non-hydrophobic moiety, for example, compound **B1** (Figure 4), led to loss of activity.[11] As there is no crystal structure of SPAK or OSR1 C-terminal domain in complex with these inhibitors, it has not been clear why such modification has strong influence on their kinase inhibition properties.

Nevertheless, in a recent study,[21] derivatives of STOCK2S-26016 with modifications mostly in the 2-furanylmethylamino as well as the ethoxy and amino positions has revealed some interesting structure–activity relationship. Indeed, a series of 21 compounds were synthesized, of which six exhibited better inhibitory potency of WNK binding to SPAK/OSR1 than the parent compound STOCK2S-26016 (Figure 5).[21]

First, the substitution of the methylfuran side group of STOCK2S-26016 to a *para*-substituted benzamidine moiety was tolerated and resulted in potent inhibitors of SPAK/OSR1 binding to WNK (for example, analogues **1** and **2**, Figure 5).[21] In particular, electron-withdrawing groups gave the most potent inhibitors. Similarly, introducing a (4-cyanophenyl)urea functionality instead of the methylfuran group was also tolerated and led to potent inhibitors of SPAK/OSR1 binding to WNK.[21] The switching of the side chain ethoxy group of the parent compound STOCK2S-26016 into a methoxy or an unmasked hydroxy group did not seem to alter the biological activity too much, for example, analogues **5** and **6** (Figure 5), as the compounds were similar to analogues **1** and **2**.[21] However, the most striking structure–activity relationship from this study was observed with analogues **3** and **4**. Indeed, replacement of the methylfuran side group of STOCK2S-26016 with 4-cyanobenzamidine functionality and the side amino group to either a hydrogen of a phenoxy group led to a loss of biological activity. As one of the analogues (structure not shown), which had only one change from the parent compound, a 4-cyanobenzamidine functionality instead of the methylfuran group, showed good inhibition of SPAK/OSR1 binding to WNK (IC_{50} = 6.9 μM), the lack of activity observed with analogues **3** and **4** could be attributed to the replacement of the side ammine group by other functionalities that are either bulkier or do not act as hydrogen bond acceptors. This suggests that the amine group is forming key interactions with an amino acid residue on SPAK CCT domain. As there is no co-crystal structure of any of these compounds with SPAK or OSR1 CCT domain, the identity of this amino acid residue remains unknown.

Although the binding of these compounds by-design is the (primary) pocket where the RFQV peptide from the upstream WNK kinases dock (Figure 6),[22] it was recently suggested that these compounds may also bind an adjacent pocket termed the secondary pocket.[23]

This observation was made when STOCK1S-50699 was able to inhibit constitutively active full-length OSR1 Thr185Glu in vitro, IC_{50} = 40.25 μM.[23] Molecular docking studies supported the preference of STOCK1S-50699 binding to the secondary pocket as opposed to the primary pocket of OSR1 C-terminal domain.[23] Interestingly, the binding of the 18-mer RFQV peptide derived from WNK4 to SPAK and OSR1 was not affected by STOCK1S-50699 supporting the notion that this binds to the secondary pocket of SPAK and OSR1 kinases and hence may act as an allosteric inhibitor of these two important protein kinases.[23] Such suggestion needs to be confirmed by a crystal structure of SPAK or OSR1 C-terminal domain in complex with this compound though this remains unreported.

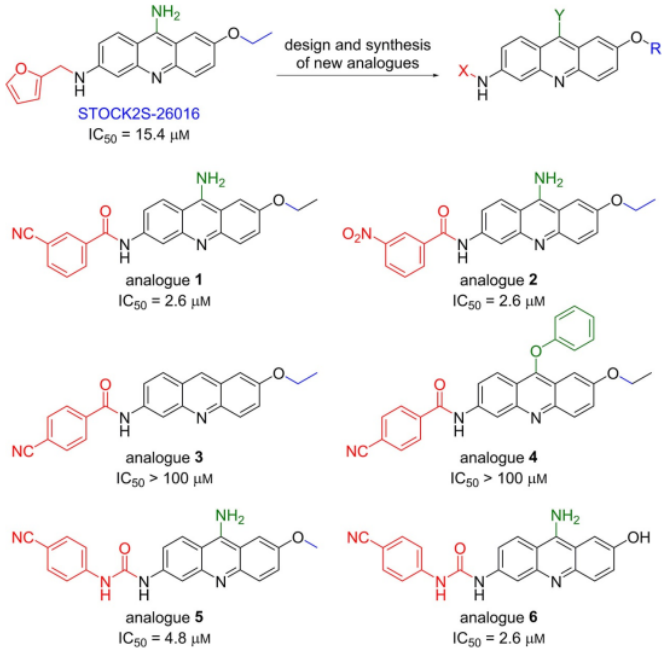

Figure 5. Chemical structures of STOCK2S-26016 and its analogues as well as their potency of inhibiting WNK binding to SPAK/OSR1 in vitro. Data were taken from Ishigami-Yuasa et al.[21]

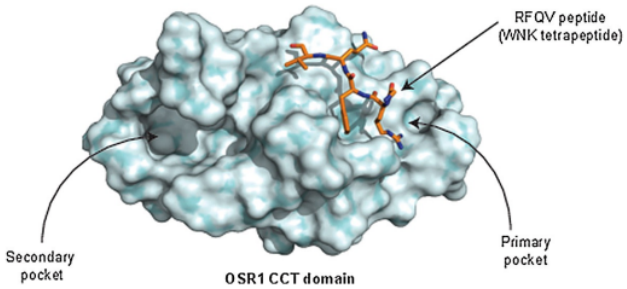

Figure 6. Crystal structure of OSR1 CCT showing the primary pocket in which the RFQV tetrapeptide from WNK kinases bind and the secondary pocket, which is thought to bind small-molecule allosteric inhibitors of SPAK and OSR1 kinases. The image was produced by AutoDock Vina using the OSR1 CCT crystal structure (PDB ID: 2V3S).

Inhibitors of SPAK/OSR1 kinases

As various SPAK mouse models have indicated the inhibition of this kinase as a viable strategy in lowering blood pressure,[10] it has (along with its closely related kinase OSR1) been an attractive target in the discovery of new antihypertensive agents. Although the crystal structure of SPAK is yet to be reported, the crystal structures of OSR1 C-terminal domain and OSR1 kinase domain were reported in 2007[22] and 2008[24], respectively. Despite such information that could aid rational drug design, the first SPAK and OSR1 kinase inhibitors were only reported in 2015.[25] For this, the Uchida lab developed an ELISA assay for measuring the phosphorylation of human NKCC2 (residues 1–174) by SPAK.[25] This assay was subsequently used in screening a library of small molecules that consists of >20000 compounds in addition to a small library of FDA-approved drugs (840 compounds). This led to the identification of two compounds namely STOCK1S-14279 (IC_{50} = 0.26 μM, K_d = 0.77 μM) and Closantel (IC_{50} = 0.7 μM) (Figure 7).[25]

In vitro, both molecules bind to SPAK and inhibit its catalytic activity in the sub-micromolar concentration.[25] A profiling study using the RapidKinase48 panel revealed that they inhibited some serine/threonine kinases at 10 μM.[25] However, in previous studies the IC_{50} values measured for these compounds were far lower, showing that both compounds at therapeutic concentrations had the ability to be highly specific for SPAK inhibition exclusively. Interestingly, these two compounds, STOCK1S-14279 and Closantel, are structurally related and inhibit SPAK kinase activity in an ATP-independent manner, suggesting that they may act as allosteric inhibitors rather than being ATP-competitive inhibitors.[25] In mpkDCT and MOVAS cells, these compounds elicited a dose-dependent inhibition of total and phospho-NCC and NKCC1. In addition, these compounds had no effects on the separate kinase MAPK, even at high concentrations showing that their inhibition is specific to SPAK.

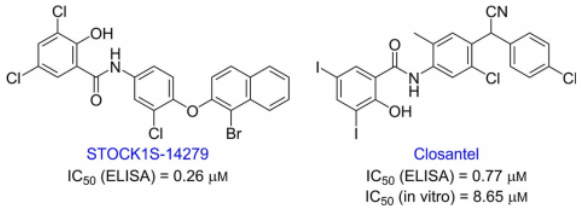

Figure 7. Chemical structures of STOCK1S-14279 and Closantel, two SPAK and OSR1 kinase inhibitors, as well as their SPAK/OSR1 inhibitory potencies. Data were taken from Kikuchi et al.[25] and AlAmri et al.[23]

In mice, acute administration of Closantel and STOCK1S-14279 led to a rapid drop in blood pressure and heart rate within minutes post administration and significant decrease in the phosphorylation of NCC in the kidney and NKCC1 in the aorta.[25] However, this effect was short and fully recovered 120 minutes after administration. This suggests that this observed effect was possibly due to the vasodilation effects of SPAK inhibition rather than the diuretic effects. Unlike Closantel, repeated injections of STOCK1S-14279 were lethal.[25] Consequently, prolonged administration of Closantel caused a decrease in the phosphorylation of NKCC2, suggesting that it can inhibit not only SPAK, but also OSR1.[25] Moreover, by day 7, Closantel did not have any effects in decreasing blood pressure and no major differences in serum electrolytes were noticed.[25] This may be because Closantel was only be effective in hypertensive animals and further investigation is therefore needed to validate this.

Although the study by Kikuchi et al.[25] indicates that Closantel and STOCK1S-14279 are allosteric inhibitors of SPAK and OSR1 kinases, their exact binding site remained elusive. Recently, we reported[23] that these may also bind the secondary pocket of SPAK and OSR1 akin to STOCK1S-50699 (Figure 6). This finding was supported by molecular docking studies and pulldown experiments. To test this hypothesis of these compounds binding to the secondary pocket of SPAK and OSR1, Mehellou and colleagues[23] performed in silico screening to identify small molecules that bind the secondary pocket of OSR1. This resulted in the identification of Rafoxanide, a structurally related compound to Closantel (Figure 8). Indeed, this agent was able to inhibit OSR1 Thr185Glu (T185E) in vitro, IC_{50} = 8.18 μM, in an ATP-dependent manner.[23] Rafoxanide was also to inhibit endogenous SPAK and OSR1 in cells at concentrations ≤ 15 μM. To support the idea of its binding to the secondary pocket, Rafoxanide was unable to abolish the binding of the 18-mer RFQV peptide, which binds the primary pocket to endogenous SPAK.[23] Akin to STOCK1S-50699, a crystal structure of these agents in complex with SPAK or OSR1 C-terminal domain or full-length would be needed to map the exact binding site of these compounds.

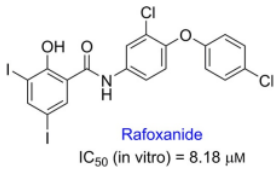

Figure 8. Chemical structure and in vitro OSR1 inhibitory activity of Rafoxanide, an allosteric SPAK/OSR1 kinase inhibitor. Data were taken from AlAmri et al.[23]

Inhibitors of SPAK/OSR1 binding to MO25

Because the basal kinase activity of SPAK and OSR1 is significantly increased by 80- to 100-fold following the binding to the adaptor protein MO25,[5] this protein–protein interaction represents an interesting target for the inhibition of SPAK and OSR1 kinases. Admittedly, such approach would only result in the inhibition of the MO25-dependent activation of SPAK and OSR1 while their basal kinase activity would be retained. However, this may be favorable in the clinical indication being targeted—hypertension—as a tuning of blood pressure is what is desired, rather than robust reduction, which may lead to hypotension.

Targeting this protein–protein interaction has been pursued by the discovery of small molecules that bind MO25 and hence inhibit its ability to bind and activate SPAK and OSR1 kinases. For this, a fluorescence polarization that employed a 16-mer peptide, which contains a WEW motif derived from SPAK that is known to mediate the binding to MO25,[5] was developed.[26] This assay was used in screening a library of ~4000 compounds and this led to the identification of HK01 as a binder of MO25 (K_d = 127 ± 6 μM) (Figure 9).[26]

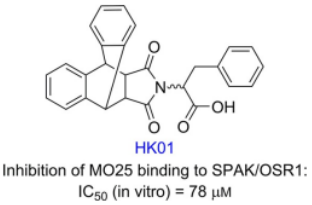

Figure 9. Chemical structure of HK01, a small-molecule MO25 binder. Data were taken from Kadri et al.[26]

Notably, this compound was able to inhibit the 16-mer WEW peptide binding to MO25 in vitro (IC_{50} = 78 ± 4 μM).[26] Further characterization showed that HK01 was able to inhibit the MO25 dependent activation of OSR1 Thr185Glu in vitro and in cells.[26]

Although HK01 lacked potency, it provided a compelling evidence that the scaffolding protein MO25 is amenable to targeting by small molecules and such binders can lead to the indirect inhibition of SPAK and OSR1 kinases in vitro and in cells.

Conclusions and Outlook

The discovery of WNK genetic mutations in humans causing an inherited form of hypertension inspired the decoding of the WNK signaling pathway. Such endeavor has led to the identification of a series of molecular targets within the WNK-SPAK/OSR1 signaling cascade that could be targeted for discovering new agents that lower blood pressure. Indeed, numerous compounds that inhibit some of these kinases or protein–protein interactions within this signaling pathway have been reported. The most advanced of these are the WNK kinase inhibitors. However, achieving exquisite selectivity across the four human WNK isoforms has so far proved to be difficult especially for ATP-competitive WNK inhibitors. This may explain the undesired adverse effects observed with WNK463 that did not support its further development. Pursuing WNK kinase inhibition via the discovery of allosteric inhibitors maybe more promising than the ATP-competitive inhibitors. This is due to the fact that achieving high potency and excellent PK profiles with non-ATP-competitive WNK inhibitors has been shown to be possible and even good selectivity profiles across the four different WNK isoforms has been achieved as it was the case with compound **4** (Figure 3c). Nevertheless, the challenges arising from achieving excellent selectivity across the four human WNK kinase isoforms may shift the focus onto SPAK and OSR1 kinases, as animal models have supported their targeting for lowering blood pressure and achieving selectivity toward one or two of these enzymes may not be as difficult as achieving selectivity across the four WNK isoforms. To date, no selective SPAK and OSR1 ATP-competitive inhibitors have been reported, but many allosteric inhibitors have emerged. These largely act by binding to SPAK and OSR1 highly conserved CCT domains and as a result act as allosteric inhibitors or prevent SPAK and OSR1 binding to WNK kinases. Admittedly, the potency of these compounds is yet to reach the levels achieved by the reported WNK kinase inhibitors. Hence, there is an opportunity for the discovery of SPAK/OSR1 inhibitors that potently and selectively inhibit SPAK/OSR1 kinases activity and have excellent drug-like properties. Additionally, the promise of selectively targeting MO25 and inhibiting its binding and hence activation of SPAK and OSR1 kinases is yet to be fully explored. This approach may yield potent indirect SPAK and OSR1 kinase inhibitors, but the off-target effects of such molecules remains unclear especially with regard to their impact on LKB1:STRAD[27] signaling of which MO25 is an integral component.

Given that WNK-SPAK/OSR1 signaling controls electrolyte balance in vivo and this expands beyond regulating blood pressure, for example, controlling neuronal hyper-excitability[28] and cell volume,[29] the impact of WNK signaling inhibitors on these physiological processes remains largely unexplored. Such studies would offer an insight into the possible side effects of WNK signaling inhibitors and may provide new indications that could be treated with this class of molecules.

In conclusion, there is no doubt that a significant progress toward the discovery of WNK signaling inhibitors as new antihypertensive drugs has been achieved. With more structural biology and medicinal chemistry focus on this important signaling cascade, there will be new WNK signaling kinase inhibitors being discovered in the future, some of which would hopefully be developed into novel, effective and safe drugs for the treatment of hypertension.

Conflict of interest

The authors declare no conflict of interest.

Keywords: hypertension · inhibition · kinases · SPAK · WNK

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