WNK-SPAK/OSR1-NCC kinase signalling pathway as a novel strategy for the treatment of salt-sensitive hypertension

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Abstract

Hypertension is the most prevalent health condition worldwide, affecting roughly 1 billion people. Gordon's syndrome is a form of secondary hypertension that can arise due to a number of possible mutations in key genes that encode proteins in a pathway containing the with no lysine [K] (WNK) and its downstream target kinases, SPS/Ste20-related proline-alanine-rich kinase (SPAK)l and oxidative stress responsive (OSR)1. This pathway regulates the activity of thiazide-sensitive sodium-chloride cotransporter (NCC), which is responsible for NaCl reabsorption in the distal nephron. Therefore, mutations in genes encoding proteins that regulate the NCC proteins disrupt ion homeostasis and cause hypertension by increasing NaCl reabsorption. Thiazide diuretics are currently the main treatment option for Gordon's syndrome. However, they have a number of side effects and chronic usage can lead to compensatory adaptations in the nephron that counteract their action. Therefore, recent research has focussed on developing novel inhibitory molecules that inhibit components of the WNK-SPAK/OSR1-NCC pathway, thereby reducing NaCl reabsorption and restoring normal blood pressure. This review examines the currently reported molecular inhibitors of the WNK-SPAK/OSR1-NCC pathway and discusses their potential as treatment options for Gordon's syndrome.

Keywords: WNK kinase, SPAK kinase, Sodium-chloride-cotransporter NCC, Chloride (Cl^-) homeostasis, Gordon's hypertension syndrome, Therapeutic target.

HYPERTENSION AND GORDON'S SYNDROME

Hypertension (high blood pressure) is the most widespread chronic health condition globally[1]. Between 1980 and 2008, the total number of hypertension cases in adults rose from 600 million to almost 1 billion [2]. This number has been predicted to rise to 1.56 billion by 2025 [3]. Hypertension is a major risk factor for an array of cardiovascular diseases that can lead to disability and premature mortality[1]. Such diseases include ischaemic heart disease and stroke, which are the two leading causes of death worldwide[4]. This makes hypertension a pressing global health concern and an important area of research.

Gordon's syndrome, also known as Pseudohypoaldosteronism type II (PHA2), or Familial Hyperkalaemic Hypertension (FHHt), is a form of secondary hypertension. It is a rare, inherited form of hypertension that is caused by one of five possible subtypes of mutations mentioned below [5]. The disease shows full penetrance and is characterised by a number of symptoms including hypertension, hyperkalaemia, and metabolic acidosis [6]. The genetic defects that cause Gordon's syndrome lead to greater sodium chloride reabsorption in the distal tubule.

This leads to hypertension and reduced renin levels [6, 7]. Despite the reduced renin secretion, patients display normal or elevated aldosterone levels.

There are five subtypes of Gordon's syndrome (PHA2 A-E), each of which is characterised by a distinct mutation. The first two subtypes, PHA2A and PHA2B, were discovered in 1997 using linkage studies [8]. PHA2A is caused by a mutation in chromosome 1 (1q31-q42). However, the specific gene is yet to be identified. PHA2B is caused by a mutation in *WNK4* (With No Lysine(K)), located in chromosome 17 (17q21.2). PHA2C is caused by a mutation on chromosome 12 (12p13.33). This is a deletion mutation that occurs within *WNK1* [9]. The *WNK4* and *WNK1* genes encode the serine/threonine-protein kinases, WNK4 and WNK1, respectively. Through alternative promoters, the *WNK1* gene generates the long WNK1 (L-WNK1) isoform and kidney-specific WNK1 (KS-WNK1). The L-WNK1 is kinase-active and expressed ubiquitously, whereas KS-WNK1 lacks a functional kinase domain, is expressed in the kidney specifically [10]. These kinases are involved in the regulation of ion reabsorption in the distal renal tubule, via the WNK signalling pathway [11, 12]. PHA2D is caused by a mutation in *KLHL3*, which is located on chromosome 5 (5q31.2) and encodes the protein Kelch-like 3 (KLHL3) [13, 14]. Finally, PHA2E is caused by a mutation in *CUL3*, which is located on chromosome 2 (2q36.2) and encodes the protein Cullin 3 (CUL3) [13]. CUL3/KLHL3 are components of an E3 ubiquitin ligase complex that promotes WNK1/4 degradation [15].

WNK-SPAK-NCC KINASE PATHWAY

RENAL BLOOD PRESSURE REGULATION

The regulation of salt reabsorption (NaCl) in the kidney is critical for the maintenance of blood pressure [16]. Different amounts of NaCl are reabsorbed at various points of the nephron. In the distal convoluted tubule of the nephron, the sodium-chloride-cotransporter (NCC) is responsible for reabsorption of 5-10% of filtered NaCl [17]. NCC is a transmembrane protein, between 1,002 and 1,030 amino acids in length [18]. The NCC protein is encoded by the gene *SLC12A3* (solute carrier family 12 member 3) located on chromosome 16 (16q13) [18]. Other members of the *SLC12* family include potassium-chloride-cotransporters (KCC1-4) and sodium-potassium-chloride-cotransporters (NKCC1-2), both of which are also involved in renal ion homeostasis [19]. While the majority of NaCl is reabsorbed in early segments of the nephron, the activity of NCC is crucial for the fine-tuning of the salt concentration in the extracellular fluid, [18] which affects blood volume and therefore blood pressure.

NCC REGULATION IN THE WNK-SPAK/OSR1-NCC PATHWAY

Two opposing processes occur in the distal nephron in order to control blood pressure; K^+ excretion and NaCl reabsorption. WNK signalling is responsible for regulating this electrolyte

homeostasis and thus blood pressure [20]. L-WNK1, WNK3 and WNK4 are responsible for the regulation of a number of cation-chloride co-transporters [21, 22]. These WNK kinases are expressed in the kidney and can activate NCC, KCC1-3-4, NKCC1 and NKCC2 through a cascade of phosphorylation reactions, involving SPS/Ste20-related proline/alanine-rich kinase (SPAK) and oxidative stress-responsive kinase (OSR1)[23, 24]. SPAK and OSR1 are members of the STE protein kinase family and are responsible for the direct phosphorylation and activation of NCC [25]. Upon activation by WNK kinases, SPAK and OSR1 can phosphorylate and activate the cation-chloride co-transporters (CCCs) [22, 26].

The kinase domain of WNK kinases are found in the N terminus. To phosphorylate SPAK and OSR1, L-WNK1 and WNK4 can bind to the conserved C-terminal (CCT) domain of SPAK and OSR1 [11, 27]. SPAK is phosphorylated by WNK on residues Ser373 and Ser387 of the S-motif, and Thr233 of the T-loop. OSR1 is phosphorylated by WNK on residues Ser325 and Ser339 of the S-motif, and Thr185 of the T-loop [28]. The phosphorylation of the S-motif of SPAK/OSR1 has recently been found to be important for their stability under osmotic stress [29]. However, phosphorylation of the T-loop is all that is required for their activation. Once phosphorylation has occurred, SPAK and OSR1 bind to the scaffold protein MO25 (mouse protein 25), which results in a respective 80- and 100-fold activation of SPAK and OSR1 [30]. In complex with MO25, SPAK and OSR1 can subsequently phosphorylate a number of CCCs [31]. For the activation of NCC, the CCT domain of SPAK and OSR1 binds to an RFTI amino acid motif located at its N terminus [27]. Here, SPAK and OSR1 phosphorylate three amino acid residues; Thr46, Thr55 and Thr60 [28]. This activates NCC and allows the cotransport of NaCl from the distal tubule lumen. The SPAK CCT domain knock-in mice, displayed markedly reduced SPAK activity and phosphorylation of NCC at the residues phosphorylated by SPAK [32], as that has been shown in SPAK kinase dead knock-in mice [33]. These animals showed typical features of Gitelman Syndrome with mild hypokalaemia, hypomagnesaemia, hypocalciuria and displayed salt wasting on switching to a low-Na diet [32, 33]. A schematic representation of the WNK-SPAK/OSR1-NCC pathway is shown in **Figure 1**.

REGULATION OF WNK-SPAK/OSR1-NCC VIA CUL3-KLHL3 E3 UBIQUITIN LIGASE COMPLEX

The WNK-SPAK/OSR1-NCC pathway can be downregulated by enhancing the degradation of WNK kinases. This is achieved by the CUL3-KLHL3 E3 ubiquitin ligase complex [34]. This complex includes the proteins KLHL3 and CUL3. KLHL3 is expressed primarily in the distal tubule of the nephron, on the apical membrane, and CUL3 is expressed ubiquitously throughout the nephron [35].

The CUL3-KLHL3 E3 ubiquitin ligase complex stems from a Cullin 3-Ring E3 ubiquitin ligase complex. The Cullin 3-Ring complex contains CUL3 bound to a RING ubiquitin ligase. The

Cullin 3-Ring complex interacts with its substrate via an adaptor protein, KLHL3. The KLHL3 protein contains three domains: a BTB domain in its N terminus; a BACK domain; and a C terminus domain containing Kelch-like repeats in a β-propeller structure [35, 36]. The BTB domain of KHL3 interacts with CUL3. The C terminus domain of KLHL3 binds substrate proteins, allowing KLHL3 to recruit substrates for the Cullin3-based ubiquitin ligase. The RING protein of the Cullin3-based ubiquitin ligase then transfers ubiquitin to the substrate bound to C terminus domain of KLHL3, marking the substrate for proteasome degradation.

L-WNK1 and WNK4 are both substrates of the CUL3-KLHL3 E3 ubiquitin ligase [34, 37]. L-WNK1 and WNK4 contain an acid motif that is the binding site for KLHL3 [9]. Once bound to the ligase, via the C terminus of KLHL3, L-WNK1 and WNK4 can be ubiquitinated by the RING ubiquitin ligase. This ubiquitination targets WNKs for proteasomal degradation [34, 36]. A graphic representation of CUL3-KLHL3 E3 ubiquitin ligase complex interacting with WNKs is shown in **Figure 1**.

WNK-SPAK/OSR1-NCC PATHWAY IN GORDON'S SYNDROME

The Gordon's syndrome-causing mutations affect the function of the WNK-SPAK/OSR1-NCC pathway. This results in disrupted ion homeostasis, thus leading to hypertension. The mutations that occur in the WNK4 and WNK1 kinases give rise to Gordon's syndrome (PHA2B and PHA2C respectively) due to an increase in the abundance of these kinases [38]. In turn, this leads to over-activation of NCC. The mutations that occur in KLHL3 and CUL3 give rise to Gordon's syndrome (PHA2D and PHA2E respectively) due to the insufficient degradation of WNK kinases. This leads to an accumulation of WNKs, thus causing over-activation of NCC. Mutations in KLHL3 and CUL3 occur in 80% of Gordon's syndrome patients [13].

WNK1 mutations are intronic deletions that result in increased expression of L-WNK1 [39]. The expression of L-WNK1 is normally low in the distal tubule. However, this mutation causes increased expression in the distal tubule [39]. As a result, the WNK-SPAK/OSR1-NCC pathway is over-activated and NaCl reabsorption is increased, leading to hypertension.

Mutations in *WNK4* are exonic missense mutations that occur in the C-terminal domain next to the second coiled-coiled motif and a unique amino acid sequence called the acid motif [34, 37]. Mutations in the in the acidic motif of WNK4 markedly impair its binding to KLHL3 [34]. The acid motif, which is present in all the WNK kinases, is the binding site for KLHL3 [9]. Thus, disruption of this binding site hinders the ability of WNK4 to be ubiquitinated by the CUL3-KLHL3 E3 ubiquitin ligase complex. The resulting lack of WNK4 degradation leads to an accumulation of WNK4, which in turn causes NCC phosphorylation and activation. Interestingly, a number of polymorphisms in both *WNK1* [40-42] and *WNK4* [43] have been identified that are not associated with Gordon's syndrome, but are still affiliated with increased

risk of hypertension. This provides a stronger justification for potentially targeting the WNK-SPAK/OSR1-NCC pathway as a treatment option for other types of hypertension.

The mutations in *KLHL3* that cause Gordon's syndrome are predominantly missense mutations [13, 14]. These mutations mainly occur within two locations: The first location is the C terminus domain of KLHL3, which is responsible for binding substrate proteins (i.e. WNKs). The second location is the N terminus BTB domain of KLHL3, which is the binding site for the Cullin 3-Ring E3 ubiquitin ligase complex [13, 14]. Thus, a mutation in either of these locations results in insufficient WNK kinase degradation: The C terminus domain mutation impairs the ability for KLHL3 to bind WNKs, thereby preventing ubiquitination of WNKs. The N terminus domain mutation impairs the ability of KLHL3 to bind the Cullin 3-Ring E3 ubiquitin ligase complex, thus preventing the initial formation of the CUL3-KLHL3 ubiquitin ligase complex.

Mutations in *CUL3* give rise to the most severe Gordon's syndrome phenotype. Compared to the mutations in *WNK1*, *WNK4* and *KLHL3*, the mutations in *CUL3* have been shown to cause significantly more severe metabolic acidosis and hyperkalaemia [13]. Additionally, the onset of hypertension occurs before the age of 18 in 94% of patients. Therefore, potentially developing a treatment that targets CUL3 would be particularly significant [13]. The mutations in *CUL3* that cause Gordon's syndrome are located at sites involved in the splicing of exon 9. As a consequence, exon 9 is skipped during transcription, leading to a 57 amino acid deletion in the CUL3 protein (CUL3-Δ9) [13, 44]. Expression of CUL3-Δ9 results in malfunction of the CUL3-KLHL3 ubiquitin ligase complex, thus impairing the degradation of WNKs [34]. There are a number of mechanisms by which this has been proposed to occur. One suggestion is that there is a reduced availability of wild type CUL3 because it becomes dimerised with CUL3-Δ9 in an unstable structure that cannot ubiquitinate substrate proteins [45]. Another suggestion is that the CUL3 mutation is a gain of function mutation: CUL3 can be activated through a process termed neddylation, which involves the covalent attachment of Nedd8. This process is increased in the mutant CUL3-Δ9 [46]. The increased activation of CUL3-Δ9 allows it to ubiquitinate off-target substrates. KLHL3 has been suggested to be ubiquitinated by CUL3-Δ9 as a result of this gain of function mutation [47]. This would lead to the degradation of KLHL3, and the prevention of CUL3-KLHL3 ubiquitin ligase complex formation [47]. It has also been suggested that this gain of function mutation allows autoubiquitination of CUL3-Δ9. This has been demonstrated in vivo with CUL3-Δ9 transgenic mice that exhibited decreased levels of CUL3-Δ9 expression and normal KLHL3 expression [48]. Wild type CUL3 is also expressed in the vasculature, where expression of CUL3-Δ9 in smooth muscles interferes with the expression and function of wild-type CUL3, consequently affecting blood pressure [49-51].

CURRENT PHARMACOLOGICAL TREATMENTS FOR GORDON'S SYNDROME

Hypertension and metabolic abnormalities in Gordon's syndrome can be controlled pharmacologically using thiazide diuretics [52-54]. Thiazide diuretics can acutely reduce hypertension by decreasing NaCl reabsorption from the distal tubule. This is achieved through the inhibition of NCC [55, 56] which decreases NaCl reabsorption. The mechanism of action of chronic thiazide diuretic use is not fully understood [57].

There are a number of limitations to using thiazide diuretics as a treatment option for hypertension. Diuretics are ineffective as a monotherapy in a portion of hypertensive patients. Furthermore, a study by Morsing and colleagues found adaptations of the distal convoluted tubule of rats after chronic infusion of thiazide diuretics: The membrane density of NCC is increased in response to chronic thiazide use [57]. However, despite the increase in NCC membrane expression, the reabsorption of NaCl is reduced [57]. Thiazide diuretics also have a range of side effects, including; hyperglycaemia, dyslipidaemia, and an increased risk of developing type two diabetes [58, 59]. For these reasons, research into other treatment options for hypertension, including Gordon's syndrome, is becoming increasingly important.

This review examines whether the WNK-SPAK/OSR1-NCC pathway could be an effective target for the treatment of Gordon's syndrome. By altering the activity of various components in this pathway, NCC activation can be prevented, thereby reducing blood pressure. Research has focussed on developing molecules that either inhibit the kinase activity or binding activity of WNKs, SPAK/OSR1, or MO25. Therefore, this review discusses the ability of the currently reported molecules to reduce blood pressure in Gordon's syndrome, and possibly in other forms of hypertension [60].

WNK KINASE INHIBITORS

Due to their integral role in WNK-SPAK/OSR1-NCC pathway, the WNK kinases have been targeted for the treatment of hypertension in Gordon's syndrome. Although homozygous *WNK1* knockout mice have been shown to die during embryonic development [61-63], only one out of these three independent studies investigating heterozygous *WNK1* knockout mice reported mild hypotension [61]. Homozygous *WNK4* knockout mice show mild Gitelman-like symptoms, including hypotension and reduced phosphorylation of NCC [64-66]. This suggests that targeting specific WNK kinases may be beneficial in reducing blood pressure through the reduced activation of NCC. Research has focussed on developing molecules that can bind to the WNK kinases and inhibit their function, thereby preventing their participation in the WNK-SPAK/OSR1-NCC pathway. These molecules can be broadly divided into two groups; orthosteric (ATP competitive) and allosteric (ATP non-competitive). The binding properties of some key WNK inhibitory molecules are presented in **Table 1**.

ORTHOSTERIC WNK KINASE INHIBITORS

The currently reported orthosteric WNK kinase inhibitors take advantage of a unique structural irregularity found in the kinase domain of the WNK kinases. WNK kinases are characterised by the abnormal placement of a catalytic lysine residue (Lys233) in their binding site for ATP [67]. Since this variation is specific to WNK kinases, a high level of selectivity can be achieved for WNK kinase inhibition over other kinases, which do not share this structural configuration. Thus, highly selective inhibitory molecules that exploit the catalytic lysine residue have been generated for the WNK kinases.

A high-throughput screen, conducted by Yamada et al., led to the generation of a potent WNK kinase inhibitor called WNK463 [68]. The X-ray co-crystal structure of this molecule in complex with the kinase-dead mutant of WNK1 (S382A) revealed its unique binding method that arises due to the abnormal placement of the catalytic lysine residue [68]. This provides excellent binding selectivity for the WNK kinases over other kinases. This selectivity for WNK was demonstrated when a high concentration of WNK463 (10 μM) showed >50% inhibition against only two kinases out of 442 that were tested [68]. This suggests that WNK463 will incur fewer unwanted side effects, as fewer off target kinases will be inhibited. *In vitro*, WNK463 inhibited the kinase activity of each of the four WNK kinases (**Table 1**) [68]. Additionally, WNK463 was shown to prevent the WNK-mediated phosphorylation of OSR1, both in a biochemical assay and in human embryonic kidney 923 (HEK293) cells [68]. Oral dosing of WNK463 (1, 3 or 10 mg/kg) in spontaneously hypertensive rats led to a dose dependent reduction in blood pressure, and an increase in urine output with an increase in urinary Na^+ and K^+ excretion [68]. Additionally, in transgenic mice that overexpress human L-WNK1, oral dosing of WNK463 led to reduced blood pressure and a dose dependent decrease in SPAK and OSR1 phosphorylation [68]. These results show that the physiological effects of WNK463 are indeed a result of WNK inhibition.

An issue with such ATP competitive inhibitors is that they must compete with ATP for their binding site on WNKs. This means a molecule with high potency is required in order to overcome the competition from physiological ATP levels. Additionally, since the ATP binding site is highly conserved between the WNK kinases, [69] achieving selectivity between them has proven difficult [68]. This lack of selective inhibition between the WNKs is problematic, as the WNK kinases show various levels of activity in physiological processes other than renal ion homeostasis [27, 70]. It should be noted that the development of WNK463 has been ceased due to issues in preclinical safety, elicited ataxia and breathing difficulties at 1-10 mg/kg doses [71]. This may be due to this lack of selectivity for the WNK family members, resulting in off target effects. For these reasons, molecules that inhibit WNK allosterically have gained traction; they can target less conserved regions of the WNK kinases, possibly allowing them to exhibit selectivity between the WNK family members. Additionally, they do not need to compete with ATP for their binding site on WNKs.

ALLOSTERIC WNK KINASE INHIBITORS

Another high throughput screen was conducted by Yamada et al. in order to identify allosteric WNK kinase inhibitors [72]. 1.2 million compounds were screened at a high concentration (50 μM) so that any weakly binding compounds would still be detected. Initially, the screen identified 8,257 compounds, but further prioritization and modification led to the development of compound 2. Co-crystallisation of compound 2 with L-WNK1 revealed a new allosteric binding pocket that is exclusive to the WNK kinases, thus demonstrating the selectivity for WNK kinases that can be achieved through an allosteric binding site [72]. This selectivity was validated when a high concentration of compound 2 (10 μM) showed >50% inhibition only in L-WNK1, against a panel of 61 other kinases [72]. Additionally, no phosphorylation of OSR1 occurred when exposed to a high concentration of compound 2 (10 μM), again showing selectivity for WNKs [72]. This high level of selectivity for WNKs is vital in developing a kinase inhibitor, given the broad range of physiological processes that other kinases are involved in. *In vitro*, compound 2 inhibited each of the WNK kinases with similar potency (**Table 1**) [72]. In order to test the effects of this inhibition on electrolyte homeostasis, an NKCC1 assay was developed that measured rubidium flux in place of K^+ uptake [72]. Compound 2 inhibited rubidium uptake in a dose dependent manner (IC_{50} = 0.24 μM). Despite the positive *in vitro* results of compound 2, *in vivo* studies were not warranted due to the compound's inadequate pharmacokinetic profile [72].

Further optimisation and refinement of three molecules (compound 1-3) identified by Yamada et al. [72] was conducted in order to develop molecules that could be tested *in vivo* [73]. Various aspects of these molecules were interchanged in order to identify compounds that had improved pharmacokinetic profiles. This led to the development of 9 additional molecules (compounds 4-12) [73]. In a panel of 440 kinases, compound 11 showed inhibition >35% in only four kinases other than L-WNK1, showing the high level of selectivity that can be achieved when utilising a binding site other than the highly conserved ATP binding site [73]. Compound 11 showed allosteric inhibition of the WNK kinases, with L-WNK1 and WNK3 being potently inhibited and WNK2 being moderately inhibited compared to WNK4 (**Table 1**). In HEK293 cells, compound 11 showed a dose dependent inhibition of OSR1 phosphorylation, suggesting its potential to impede the WNK-SPAK/OSR1-NCC pathway [73]. *In vivo* testing of compound 11 in rats revealed a reasonable pharmacokinetic profile, with low oral bioavailability and moderate clearance [73]. Therefore compound 11 was modified, leading to the development of compound 12. This molecule showed an improved pharmacokinetic profile, with lower clearance and a two-fold increase in oral bioavailability [73]. Oral dosing of compound 12 (10, 30 or 100 mg/kg) led to reductions in systolic blood pressure in a dose dependent manner [73]. Additionally, in spontaneously hypertensive rats, ascending oral doses of compound 12 on

successive days led to a dose dependent increase in urine production with increased Na^+ and K^+ excretion [73]. The good pharmacokinetic profile of compound 12 and its efficacy *in vivo* make it a promising candidate for further development as an antihypertensive drug.

In another study, conducted by Pinkas et al., a fluorescence-based thermal shift assay was used to screen 860 kinase inhibitors, in an attempt to identify inhibitors of WNKs [74]. The kinase inhibitor PP121 was the top hit identified in the screen. This inhibitor was originally discovered by Apsel et al [75]. PP121 strongly inhibited L-WNK1 and WNK3 (**Table 1**), indicating its potential as a treatment for Gordon's syndrome [74]. A selectivity profile for PP121 was conducted against a panel of 135 serine/threonine kinases, the same family of kinases from which WNK belongs [75]. At 1 μM, PP121 inhibited only 19 kinases >50%. This suggests that PP121 may not incur many off target effects *in vivo*, since this high concentration of PP121 showed low levels of inhibition in the same family of kinases as WNK. Additionally, another selectivity profile was conducted that included a wider variety of kinases [74]. At 1 μM, PP121 showed inhibition >75% in 29 out of 144 kinases, including L-WNK1. This moderate level of selectivity suggests that further modification of PP121 will be necessary in order to limit off-target effects in a wider range of kinases. The family of kinases that were most affected in this selectivity profile were the tyrosine kinases, suggesting that PP121 may have therapeutic potential as a tyrosine kinase inhibitor. Accordingly, a number of studies have investigated the inhibitory effects of PP121 on various tyrosine kinases [75-77]. The overall moderate level of selectivity for WNK in these selectivity assays suggests that further modification of PP121 as a WNK inhibitor will be necessary in order to limit off-target effects.

One of the major issues in the development of WNK inhibitors is the lack of inhibitor molecule specificity between the WNK kinases. For orthosteric WNK inhibitors, this appears to be a limiting factor that cannot be overcome, since the ATP binding site of WNK kinases is so highly conserved [69]. However, recent chemoinformatic analysis has revealed that structural specificities between the WNK isoforms do exist, particularly in the allosteric binding sites [78]. Exploiting these structural differences may be an effective strategy for developing specific WNK kinase inhibitors, and certainly merits further investigation. Compound 12 [73] may be a strong candidate for further development in this manner.

SPAK AND OSR1 INHIBITORS

SPAK and OSR1, the kinases downstream of WNKs in the WNK-SPAK-NCC pathway, have become attractive targets for inhibition. Heterozygous SPAK knockout mice display reduced blood pressure, whilst homozygous SPAK knockout mice exhibit significant electrolyte abnormalities in addition to hypotension [79, 80]. Heterozygous OSR1 knockout mice also exhibit hypotension, and are associated with reduced phosphorylation of SPAK [79]. This information suggests that the inhibition of SPAK or OSR1 may be beneficial in reducing blood pressure in

Gordon's syndrome. The main features of some key SPAK and OSR1 inhibitory molecules are presented in **Table 2**.

A high throughput screen and a drug repositioning strategy, conducted by Kikuchi et al., identified two allosteric SPAK inhibitors; Stock 1S-14279 and Closantel [81]. Stock 1S-14279 was identified in a novel ELISA-based screen of >20,000 small-molecule compounds, whilst Closantel was screened from a library of 840 existing drugs and had therefore already passed several stages of clinical development. Both compounds share a similar chemical structure and both inhibited SPAK independently of ATP concentration, suggesting they are allosteric inhibitors (**Table 2**) [81]. The specificity of these compounds was tested in a panel of 48 kinases [81]. At a concentration of 10 μM, Closantel inhibited 6 kinases >50% and Stock 1S-14279 inhibited 2 kinases >50%, suggesting that these molecules have good selectivity for SPAK. In mouse renal distal tubule–derived (mpkDCT) cells, both compounds exhibited inhibition of NCC phosphorylation in a dose dependent manner [81]. *In vivo* testing in mice revealed that single intraperitoneal injections of Stock 1S-14279 or Closantel resulted in a significant decrease in NCC abundance and phosphorylation after 30 minutes [81]. However by 120 minutes, these effects had reversed. Thus, chronic administration of these compounds was tested. Repeat injections of Stock 1S-14279 were lethal in mice, whilst Closantel had no significant effects on blood pressure or electrolyte levels in the urine. Overall these tests showed promising results *in vitro*, however further pharmacological modification may yield better *in vivo* effects.

AlAmri et al. recently reported that a highly conserved secondary pocket found in the CCT domain of SPAK and OSR1 may be the binding site for Stock1S-14279 and Closantel [82]. Therefore, further testing was conducted *in silico* to identify other inhibitory molecules that bind to this secondary pocket [82]. 1,200 US Food and Drug Administration (FDA) approved compounds were screened with the intension of finding molecules that bind to the secondary pocket in OSR1. Rafoxanide, a structurally similar molecule to Closantel, was one of the best molecules identified in the screen. *In vitro*, it was shown to inhibit constitutively active forms of OSR1 (OSR1 T185E) and SPAK (SPAK T233E) (**Table 2**) [82]. In the presence of MO25, a SPAK/OSR1 activator, Rafoxanide was still able to inhibit OSR1 with a similar potency (**Table 2**) [82]. Additionally, in HEK293 cells, titration of Rafoxanide (1-50 μM) caused dose dependent inhibition of NKCC1 phosphorylation [82]. This suggests that SPAK and OSR1, the upstream kinases of NKCC1, were inhibited. These findings advocate the importance of the CCT domain of SPAK and OSR1 for their kinase activity and highlight the secondary pocket as a good target for inhibitory molecules. *In vivo* testing and compound modification will be important next steps in the development of clinically available SPAK and OSR1 inhibitors.

Given the encouraging results from these *in silico* screening methods, AlAmri et al. recently conducted a high throughput screen of 1,200 FDA approved drugs with the hope of identifying

novel SPAK and OSR1 inhibitors [83]. The drugs were screened at 20 μM and led to the identification of 7 compounds that inhibited OSR1 T185E in a dose dependent manner. Of these, Verteporfin emerged as the most potent OSR1 T185E inhibitor (**Table 2**). In addition, Verteporfin was equally potent in its inhibition of SPAK T233E (**Table 2**). An *in vitro* kinase assay revealed that the inhibition of OSR1 by Verteporfin was not significantly affected by ATP concentration, suggesting that it binds in an ATP independent manner [83]. *In silico* docking of Verteporfin to OSR1 suggested that binding occurs in an allosteric site adjacent to the kinase domain, similarly to the allosteric WNK kinase inhibitors mentioned above [83]. To test the selectivity of Verteporfin for SPAK and OSR1, a screen of 140 kinases was conducted at 1 μM [83]. Other than SPAK and OSR1, eight kinases were inhibited ≥70%. This may be problematic for the future development of Verteporfin as an antihypertensive drug, as the inhibition of these off-target kinases may lead to undesired side effects. The inhibitory effects of Verteporfin were then tested in HEK293 cells [83]. Verteporfin dose-dependently inhibited NKCC1 phosphorylation, suggesting inhibition of SPAK or OSR1. Importantly, this inhibition did not occur in the WNKs phosphorylation site on SPAK and OSR1. This indicates that Verteporfin only inhibits active SPAK and OSR1, rather than inhibiting the activation of SPAK and OSR1 by WNKs. In addition, Verteporfin did not affect the ability of MO25 to activate OSR1 *in vitro* [83]. Although animal testing was not conducted in this study, [83] there have been reports of hypotension in animals being treated with Verteporfin, suggesting that inhibition of SPAK and OSR1 can reduce blood pressure [84].

Although these findings are promising, there are a number of limitations to targeting SPAK and OSR1. NCC and NKCC1 become activated when phosphorylated by SPAK and OSR1, whereas KCCs are inhibited when phosphorylated by SPAK and OSR1 [85]. Neuronal excitability of GABAergic neurons is partly mediated by Cl^- efflux by KCCs [86]. Thus, the inhibition of SPAK/OSR1 may prevent the phosphorylation and inhibition of these transporters, leading to undesirable effects in the central nervous system. This effect has been observed with the inhibition of WNK3, which is an upstream kinase of SPAK and OSR1 [87], suggesting that a similar phenotype may occur with SPAK and OSR1 inhibition. Another potential limitation is that the kidneys employ adaptive mechanisms to counteract physiological change. Although SPAK-null mice display hypotension, compensatory mechanisms for NaCl reabsorption have been observed in SPAK knockout mice [88]. These adaptations may explain why the hypotension observed is relatively mild in comparison to the predicted effects of loss of NCC function. Such mechanisms include the distal nephron modelling and activation of the paracrine signalling system to activate salt transport pathways in the distal nephron and ultimately enhance NaCl reabsorption.

PREVENTION OF SPAK AND OSR1 PROTEIN-PROTEIN INTERACTIONS

A number of molecules have been identified that target protein-protein interactions between SPAK/OSR1 and other components of the WNK-SPAK/OSR1-NCC pathway. Some of these molecules are involved in the inhibition of SPAK/OSR1 binding to WNK, whilst the others inhibit MO25 from binding to SPAK/OSR1. By preventing these interactions, the activity of the WNK-SPAK/OSR1-NCC pathway can be reduced, thereby leading to a decrease in blood pressure. The main features of some key molecules that inhibit SPAK and OSR1 binding are presented in **Table 3**.

INHIBITION OF SPAK/OSR1 BINDING TO WNK

A screen of 16,902 compounds, conducted by Mori et al., led to the identification of 10 potential SPAK binding inhibitor compounds [89]. Two of these compounds, Stock 1S-50699 and Stock 2S-26016, showed the greatest inhibition of SPAK binding to WNK4 out of the compounds that were screened (**Table 3**). Both compounds were shown to bind to the CCT domain of SPAK, thereby preventing interactions with WNK [89]. Stock 1S-50699 exhibited good drug properties, such as slow binding and dissociation, compared to Stock 2S-26016, suggesting that it may have greater potential to be developed into an antihypertensive drug [89]. To test the specificity of these compounds to SPAK, two negative controls were conducted [89]. Firstly, at high concentration (200 μM), neither molecule inhibited mitogen-activated protein kinase, a closely related kinase that is not involved in the WNK-SPAK-NCC pathway. Secondly, a non-inhibitory analogue of Stock 2S-26016 had no effect on the phosphorylation of SPAK and NCC, showing that the inhibitory activity of Stock 2S-26016 was not due to non-specific effects. Dosing of both compounds in mpkDCT cells (25, 50, 100, and 200 μM) inhibited the phosphorylation of SPAK and NCC in a dose dependent manner [89]. These *in vitro* and *in vivo* tests are encouraging, so further testing in animal models will be beneficial for their development.

In a recent study, conducted by Zhang et al., a novel SPAK binding inhibitor named ZT-1a was developed [71]. Pharmacophores from Closantel, Rafoxanide, and STOCK1S-14279 were combined in order to create this specific SPAK inhibitor; from Closantel, the 2-(4-amino-2-chloro-5-methylphenyl)-2-(4-chlorophenyl)acetonitrile moiety was used, and from STOCK1S-14279 and Rafoxanide, the chloro-substituted 2-hydroxybenzoic acid was used. To test the selectivity of ZT-1a for SPAK, a selectivity profile was conducted at 10 μM ZT-1a [71]. Inhibition ≥50% was achieved in 6 out of the 140 kinases that were tested, suggesting a high level of selectivity for SPAK. *In vitro*, ZT-1a reduced the kinase activity of SPAK in a dose dependent manner [71]. Interestingly, the potency of this inhibition was only marginally reduced in the presence of the SPAK activator, MO25 (**Table 3**). ZT-1a was shown to inhibit SPAK independently of ATP concentration, suggesting an allosteric binding site [71]. A SPAK antibody pull-down assay, using HEK293 cell lysates, was conducted to determine the inhibitory action

of ZT-1a [71]. Co-immunoprecipitation of SPAK with L-WNK1 was reduced significantly, in a dose dependent manner, suggesting that ZT-1a inhibits the binding of SPAK and WNKs. *In vivo* testing in mice revealed that although there was no mortality in naive mice treated with ZT1-a and Closantel, all ischaemic stroke mice died within 2 days while the ZT-1a treated mice prolonged median survival. 30 minutes after intraperitoneal injection of ZT-1a, the phosphorylation of NCC (pThr46/50/55/60) was significantly reduced, in a dose-dependent manner. Similar effects were seen in other cation-chloride co-transporters, suggesting a potential role for ZT-1a in other areas of physiology, such as neuronal signalling [71]. This could be a limitation as ZT-1a may interfere with other SPAK regulatory activity, notably the SPAK regulation of GABA signalling via KCC. Unlike the NCC, phosphorylation of KCC inactivates the co-transporter. However, the elevated selectivity for SPAK, achieved by combining the functional moieties of Closantel, Rafoxanide, and STOCK1S-14279 coupled with the prolonged median survival observed in mice, could make ZT-1a a promising therapy option for Gordon's syndrome. Although, the results from mouse studies demonstrate effective inhibition of NCC, further *in vivo* testing will be required to assess the effects of ZT-1a on blood pressure.

INHIBITION OF MO25 BINDING TO SPAK/OSR1

To prevent the MO25-dependent activation of SPAK and OSR1, research into binding inhibitors of MO25 has been conducted. This approach would only inhibit MO25-mediated activation of SPAK/OSR1, therefore basal kinase activity would not be affected. This mild approach may in fact be beneficial, given the strong phenotypic effects of SPAK/OSR1 knockout mice [79, 80]. A study by Kadri et al. developed a fluorescent polarisation assay to screen a library of 4,000 compounds [90]. The assay measured the binding of MO25 to a peptide that is derived from SPAK/OSR1 and contains two highly conserved tryptophan residues. Alteration of the tryptophan residues prevented MO25 binding, thus confirming the importance of these residues in SPAK/OSR1 and MO25 interactions [90]. This assay may be useful for further identification of MO25 binding inhibitors. The screen led to the identification of HK01.The selectivity of HK01 for MO25 was demonstrated when a high concentration of HK01 (500 μM) did not inhibit the protein kinase MST3, which belongs to the same family of kinases as SPAK and OSR1 [90]. *In vitro*, HK01 reduced MO25-mediated activation of OSR1 T185E in a dose dependent manner [90]. Additionally, HK01 was shown to inhibit MO25 from binding the SPAK/OSR1 derived peptide, suggesting it would also inhibit SPAK/OSR1 (**Table 3**) [90]. This was confirmed *in vitro* using HEK293 cells, which exhibited a decrease in SPAK/OSR1-mediated phosphorylation of NKCC1 as HK01 dose increased [90]. Overall, this study has revealed a novel method of WNK-SPAK-NCC pathway inhibition, demonstrated by reduced SPAK/OSR1 activity that was caused by inhibition of MO25. This indirect inhibition of

SPAK/OSR1 could form a promising new class of antihypertensive drugs for Gordon's syndrome and other forms of hypertension.

CUL3 AND KLHL3 STABILISATION

Although no CUL3 or KLHL3 stabilising molecules have been reported, they have been suggested as a potential treatment option for Gordon's syndrome. Stabilising CUL3 or KLHL3 could result in improved degradation of WNK kinases, thereby inhibiting the WNK-SPAK/OSR1-NCC pathway and lowering blood pressure in Gordon's syndrome. An issue with targeting CUL3 and KLHL3 is that developing molecules that can stabilise or enhance their function would be more challenging than developing the WNK, SPAK, OSR1 and MO25 inhibitory molecules discussed above. For instance, the Gordon's syndrome subtype, PHA2D, is caused by mutations in two locations in the KLHL3 protein [13, 14]. This means that to treat this subtype of Gordon's syndrome, molecules would need to be developed that can counteract either of these mutations. In comparison, inhibitory molecules only need to prevent the activity of their target, rather than restore function. Although patients of Gordon's syndrome are effectively treated with hydrochlorothiazide, research to identify compounds that could reduce blood pressure will benefit patients with hypertension in the general population.

Although mutations in CUL3 cause the most severe form of Gordon's syndrome [13], CUL3 is probably not the most suitable target for blood pressure reduction. Both activation and inhibition of CUL3 can lead to increased WNK kinase signalling: Over-activation might lead to off target- or self-ubiquitylation, as demonstrated by CUL3-Δ9 [47] , whilst inhibition of CUL3 would prevent degradation of WNK kinases, again leading to increased activation of the WNK-SPAK/OSR1-NCC pathway. An alternate treatment option for PHA2E (the Gordon's syndrome subtype that gives rise to CUL3-Δ9) [13, 44], may be to inhibit the process of neddylation, which is responsible for CUL3 activation [45].

CONCLUSION

Inhibition of various components of the WNK-SPAK/OSR1-NCC pathway have been demonstrated to be effective targets for reducing blood pressure in Gordon's syndrome and potentially other forms of hypertension. A number of molecular inhibitors have been developed, however it is clear that further modification and *in vivo* testing is required before clinical trials can begin. One of the main issues with thiazide diuretic usage as a treatment for Gordon's syndrome is that chronic usage can lead to adaptation in the nephron. It still remains to be seen whether similar effects will occur with chronic usage of the newly developed WNK pathway inhibitors discussed in this review.

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ADDITIONAL INFORMATION

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FIGURES AND TABLES

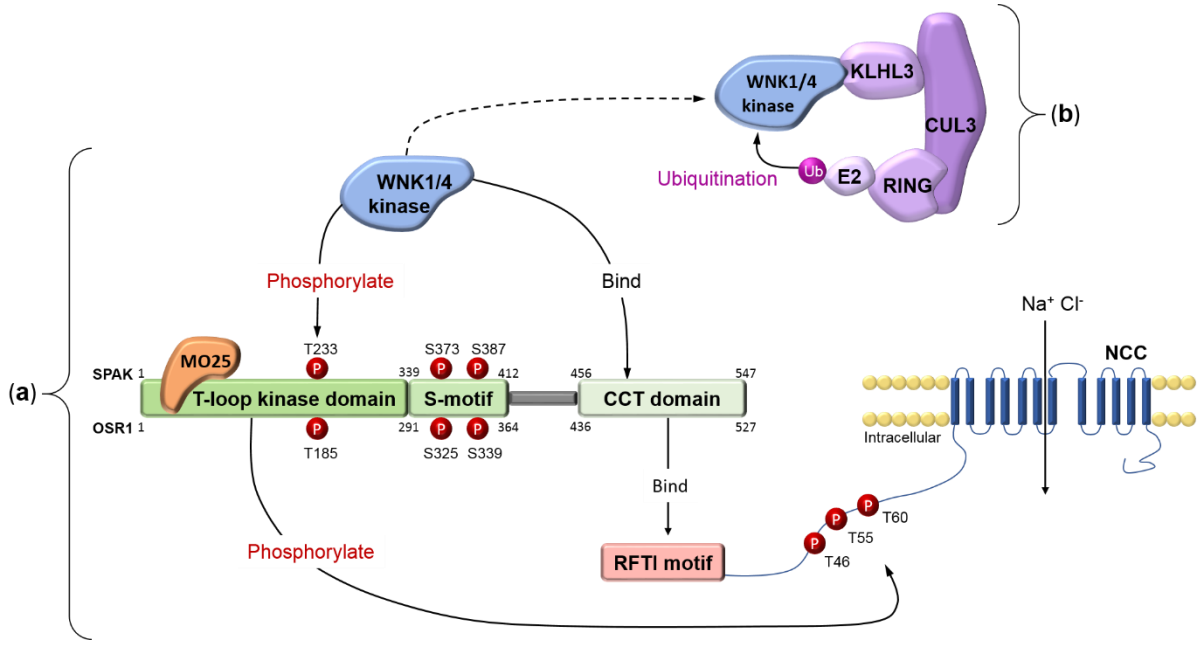


Fig. 1 Schematic representation of the WNK-SPAK-NCC pathway and WNK ubiquitination by the CUL3-KLHL3 E3 ubiquitin ligase complex.

(**a**) WNK-SPAK-NCC pathway: L-WNK1 or WNK4 binds to the CCT domain of SPAK/OSR1 and phosphorylates its T-loop kinase domain. SPAK is phosphorylated at Thr233 and OSR1 is phosphorylated at Thr185. The scaffold protein MO25 binds to phosphorylated SPAK/OSR1, activating it further. The CCT domain of activated SPAK/OSR1 binds to the RFTI motif of NCC. The T-loop kinase domain of SPAK/OSR1 phosphorylates NCC at three amino acid residues: Thr46, Thr55, and Thr60. This activates NCC, allowing the cotransport of NaCl from the tubule lumen. (**b**) WNK ubiquitination by the CUL3-KLHL3 E3 ubiquitin ligase complex: L-WNK1 or WNK4 is bound to the CUL3-KLHL3 E3 ubiquitin ligase complex via the C terminus domain of KLHL3. RING-box protein 1 is bound to the BTB domain of CUL3 and recruits an E2 ubiquitin conjugating enzyme that ubiquitinates L-WNK1 or WNK4. This targets WNK1/4 for proteasomal degradation, thereby reducing the amount of available L-WNK1 or WNK4.

Table 1. Binding Properties of WNK Kinase Inhibitors

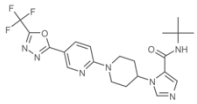
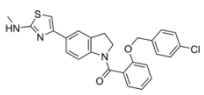
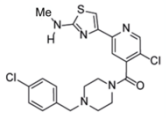
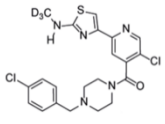
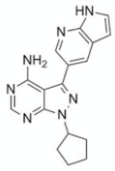
Molecule	Chemical structure	Binding target (IC_{50})	Selectivity for target	Reference
WNK463 (orthosteric)		L-WNK1 (IC_{50} = 5nM) WNK2 (IC_{50} = 1nM) WNK3 (IC_{50} = 6nM) WNK4 (IC_{50} = 9nM)	>50% inhibition in 2/442 kinases (at 10 μM)	[68]
Compound 2 (allosteric)		L-WNK1 (IC_{50} = 64nM) WNK2 (IC_{50} = 50nM) WNK3 (IC_{50} = 337nM) WNK4 (IC_{50} = 187nM)	>50% inhibition only in WNK1 out of 61 other kinases (at 10 μM)	[72]
Compound 11 (allosteric)		L-WNK1 (IC_{50} = 13nM) WNK2 (IC_{50} = 747nM) WNK3 (IC_{50} = 8nM) WNK4 (possibly activated)	>35% inhibition in 4/420 kinases (at 10 μM)	[73]
Compound 12 (allosteric)		Not conducted. Expected to be similar to compound 11 due to the minor structural differences between them		[73]
PP121 (allosteric)		L-WNK1 (IC_{50} = 160nM) WNK3 (IC_{50} = 215nM)	>50% inhibition in 19/135 serine/threonine kinases (at 1 μM). >75% inhibition in 29/144 kinases (at 1 μM).	[74, 75]

Table 2. Binding Properties of SPAK and OSR1 Inhibitors

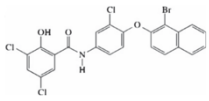
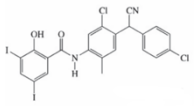
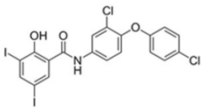
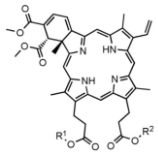
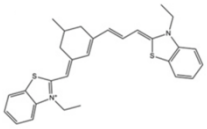
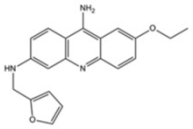
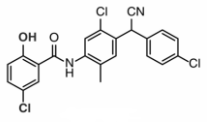
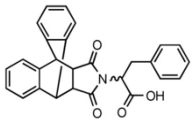
Molecule	Chemical structure	Binding target (IC_{50})	Selectivity for target	Reference
Stock 1S-14279 (allosteric)		SPAK (IC_{50} = 260 nM)	>50% inhibition in 2/48 kinases (at 10 μM)	[81]
Closantel (allosteric)		SPAK (IC_{50} = 770 nM)	>50% inhibition in 6/48 kinases (at 10 μM)	[81]
Rafoxanide (allosteric)		SPAK (T233E) (IC_{50} = 1,303 nM) OSR1 (T185E) (IC_{50} = 818 nM) OSR1 + MO25 (IC_{50} = 1,391 nM)	Not reported. Likely to be similar to Closantel, due to structural similarity	[82]
Verteporfin (allosteric)		SPAK (T233E) (IC_{50} = 330 nM) OSR1 (T185E) (IC_{50} = 207 nM)	≥70% inhibition of 8/140 kinases (at 1 μM)	[83]

Table 3. Binding Properties of SPAK and OSR1 Binding Inhibitors

Molecule	Chemical structure	Binding target (IC_{50})	Selectivity for target	Reference
Stock 1S-50699		SPAK (IC_{50} = 37,000 nM)	MAPK not inhibited at 200 μM	[89]
Stock 2S-26016		SPAK (IC_{50} = 16,000 nM)	MAPK not inhibited at 200 μM. Non-inhibitory analogue of Stock 2S-26016 did not affect SPAK and NCC phosphorylation	[89]
ZT-1a (allosteric)		SPAK (IC_{50} = 40,500 nM) SPAK + MO25 (IC_{50} = 41,300 nM)	≥50% inhibition of 6/140 kinases (at 10 μM)	[71]
HK01		MO25 (IC_{50} = 78,000 ± 4,000 nM)	MST3 not inhibited at 500 μM	[90]