SPAK kinase in normal and maladaptive epithelial ion and water transport

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Introduction: The mammalian SPS1-related proline/alanine-rich serine-threonine kinase SPAK (STK39) coordinates epithelial ion and water transport with environmental inputs, including osmotic stress and inflammation. Extensive research over the last decade has established a central role for SPAK in regulating epithelial Cl^- transport in the distal nephron, colonic crypts, and pancreatic ducts, and has implicated deregulated SPAK signaling in essential hypertension, ulcerative colitis and Crohn's disease, and cystic fibrosis.

Areas covered: We review recent advances in the understanding of SPAK kinase function and regulation in epithelial ion and water transport. We highlight how the SPAK kinase network – which includes its upstream Cl^--sensitive activators, the WNK kinases, and its downstream ion transport targets, the cation-Cl- cotransporters – contribute to human disease. We also discuss the current and future prospects for therapeutically targeting SPAK kinase in the clinic in disorders that feature impaired epithelial function.

Expert opinion: Design of potent of SPAK-WNK interaction inhibitors, SPAK kinase inhibitor or inhibitor that disrupts the activation of SPAK kinase activities by interfering with MO25α/β binding will prove useful to develop new therapeutic strategies for treating essential hypertension, ulcerative colitis and Crohn's disease, and cystic fibrosis.

Keywords: Blood pressure regulation; cation-chloride cotransporters (CCCs); ion homeostasis; kinase inhibitors; signal transduction; SPAK phosphorylation.

1. Introduction

Protein kinases have become one of the most important classes of drug targets in medicine, particularly in the field of oncology [1]. In the past decade, more than 20 different drugs targeting kinases have been approved for clinical use in humans for the treatment of various types of cancer [2]. However, the use of kinase inhibitors in other human diseases, including those with cardiovascular, renal, neurological, and psychiatric phenotypes, have lagged behind despite the existence of promising kinase targets identified by genetic studies in humans and model organisms [2].

SPAK (SPS1-related proline/alanine-rich kinase) and OSR1 (oxidative stress-responsive kinase 1) are closely related protein kinases, which play key roles in regulating cellular ion homeostasis and blood pressure (BP) [3, 4]. SPAK and OSR1 are activated following the phosphorylation of their T-loop residue (SPAK Thr233 and OSR1 Thr185) by one of the four isoforms of the WNK [with no lysine (K) kinase] protein kinase [5, 6]. The activity of SPAK and OSR1 is further enhanced following interaction with the scaffolding protein termed MO25 [7]. The best-characterized SPAK/OSR1 substrates comprise the SLC12A (solute carrier family 12) family of electroneutral CCCs (cation-Cl^- cotransporters) [8-13]. These transporters regulate intracellular chloride concentration critical in controlling BP and cell volume homoeostasis [14, 15]. SPAK/OSR1 protein kinases drive chloride influx by phosphorylation and activating sodium-driven CCC members. These include the NCC (Na-Cl cotransporter) in the distal convoluted tubule of the kidney [11], the NKCC2 (Na-K-2Cl cotransporter 2) in the thick ascending limb (TAL) of the kidney [13] and the ubiquitously expressed NKCC1[8-10]. SPAK/OSR1 also phosphorylate and inhibit potassium-driven CCCs that drive chloride efflux [12], which

comprise four different K-Cl cotransporters (KCC1–KCC4) [15, 16]. This reciprocal regulation of Na^+- and K^+-driven CCCs by SPAK and OSR1 ensures that cellular Cl^- influx and efflux is tightly coordinated [15, 16].

The importance of the WNK signaling pathway is exemplified by its evolutionary conservation from worms to humans and that several Mendelian hypertension disorders in humans are caused by mutations in WNK pathway components [17, 18]. These include various mutations that lead to increased expression of the WNK1 and WNK4 genes causing PHAII [PseudoHypoAldosteronism type II, OMIM [19-24]]. A Gordon-like phenotype is also observed in mice that express a constitutively active SPAK in DCT1. These mice display thiazide-treatable hypertension and hyperkalemia, concurrent with NCC hyperphosphorylation [25]. Conversely, loss-of-function mutations in NCC and NKCC2 cause familial forms of hypotension and hypokalaemia termed Gitelman (OMIM #263800) and Bartter type 1 syndrome (OMIM #601678), respectively [26]. A mutation that ablates the key activating WNK-regulated SPAK/OSR1 phosphorylation site on NCC [T60M[11]] also causes Gitelman's syndrome [27, 28]. Moreover, SPAK-knockout mice [29-31] or knock-in mice expressing a form of SPAK that cannot be activated by WNK kinase isoforms [32] exhibit low BP and are resistant to hypertension when crossed with animals bearing a PHAII-causing knock-in mutation that enhances WNK4 expression [33]. Genome-wide association studies have also identified intronic SNPs within the SPAK gene (STK39) that correlate with increased BP in humans [34]. Two commonly used drugs in medicine to lower high BP also target SPAK sodium-driven CCC substrates, namely thiazide diuretics (such as bendroflumethiazide) that inhibit NCC and the loop diuretics (such as furosemide) that inhibits NKCC2 [35, 36].

2. SPAK kinase

2.1. Discovery and characterization of the SPAK kinase

Ste20/SPS1-related proline/alanine rich kinase (SPAK) was discovered in the late 1990s as an unidentified band recognized by an antibody raised against PARP, the protein gene was cloned and found to be an unknown kinase [37]. The kinase was found to contain an N-terminal kinase domain which showed highest relationship to the Ste20 family of kinases. Furthermore N-terminal 71 amino acids are rich in proline and alanine, consequently Ushiro and coworkers first named the kinase proline-alanine-rich Ste20-related kinase (PASK), however in most subsequent publications the kinase is referred to as SPAK for the mouse isoform [38]. A colon specific splice variant of SPAK has been described, which is slightly shorter than the ubiquitous SPAK due to usage of two alternative splice donors in exon 1 and 7 [39]. Oxidative stress-response kinase-1 (OSR1) was identified in a large scale sequencing effort trying to map tumor suppressors within the human chromosome 3 [40]. OSR1 was named due to its similarity with the Ste20 kinase Ste20/oxidant stress response kinase 1 (SOK1). While the overall sequence identity of human SPAK and OSR1 is 68%, the kinase domains of the two kinases are highly similar and exhibit 88% sequence identity and 96% sequence similarity. Furthermore both kinases have 79% conserved C-terminal (CCT) domain which is unique to SPAK, OSR1 and orthologues of these two kinases. The presence of the unique CCT domain also meant that OSR1 and SPAK were placed in a distinct subfamily (GCK-VI) of the Ste20 kinases in the kinome [41]. Manning et al. placed OSR1 and SPAK in a subfamily called Fray, named after the *Drosophila* orthologue of OSR1 and SPAK [42]. Interestingly these two Fray or GCK-VI kinases evolutionary are not too distant from the WNK kinases.

Both SPAK and OSR1 kinases contain a putative nuclear localization signal and a caspase cleavage site between the kinase domain and the CCT domain. In unstimulated cultured cells full length SPAK exhibits diffuse localization whereas truncated constructs that mimic the caspase-cleaved SPAK targets is located in the nucleus [38, 39, 43]. Immunohistochemical studies of mouse choroid plexus and salivary glands show SPAK localization to be intense where NKCC1 is expressed: at the apical membrane of choroid plexus and basolateral membrane of salivary gland epithelial cells [8, 44]. SPAK overexpressed in Cos-7 cells re-localizes from a diffuse pattern to distinct membrane and vesicular staining patterns upon hypertonic stimulation [45]. Association of SPAK/OSR1 with plasma membrane was also clearly demonstrated by presence of the kinases in exosomes [46].

SPAK mRNA transcripts and protein are found abundantly in brain, salivary gland, pancreas, adrenal gland and testis, and to a lesser degree in heart, lung, kidney, stomach, intestine, ovary, thymus and spleen, and skeletal muscle [37, 38, 44]. OSR1 is more ubiquitously expressed and present in the tissues of the brain, heart, kidney, lung, spleen, testis, liver and skeletal muscle; likely indicative of the more global regulatory actions of OSR1, evidenced by the embryonically lethal constitutive OSR1-KO mouse models previously attempted [4, 32]. The SPAK knockout mouse is viable and shows no adverse behavioral phenotype [47], this may due to the misconstruction of this mouse model, separate studies (Table 1) have shown SPAK knockout mice have low blood pressure [29]. . This tissue specific expression correlates well with the expression patterns of the known substrates of OSR1 and SPAK, namely NCC, NKCC1 and NKCC2 which they directly phosphorylate at conserved key S/T residues to positively regulate transporter activity [5].

There are three different isoforms of SPAK with the full-length isoform (FL-SPAK) being expressed ubiquitously with higher expression in the brain, heart, and testis [32, 44]. **FL-SPAK is also expressed in the thick ascending limb (TAL) and distal convoluted tubules (DCT) of the kidney [30].** SPAK2, the second isoform, lacks the N-terminal PAPA box and a part of the kinase domain, and is also expressed ubiquitously. Kidney-specific SPAK (KS-SPAK) is the third isoform which is expressed mainly in the kidney, as the name suggests. Immunofluorescence studies showed that the FL-SPAK co-localized with NCC **and NKCC2** at the DCT, whereas SPAK2 and KS-SPAK are more abundant in the TAL, the site of NKCC2 expression [30].

Both SPAK and OSR1 were shown to be able to autophosphorylate [37, 38, 43]. The crystal structure of the OSR1 kinase domain revealed that the kinase domain assumes a classical bi-lobal kinase fold similar to cyclic AMP-dependent protein kinase (PKA). Furthermore the kinase domain forms a dimer and performs an activation segment exchange, where the two molecules swap α-helix EF [48, 49]. Whether this domain swapping actually occurs in the full length kinases, or whether it is a crystal artefact is still unclear. The kinase domain of OSR1 has however been shown to dimerize when overexpressed [10] and dimerization and domain swapping was shown to facilitate kinase activation [50].

2.2. SPAK as major regulator of CCCs

Biochemical experiments subsequently clarified the molecular mechanism by which the SPAK and OSR1 kinases activated by their upstream kinase WNKs, and to phosphorylate and stimulate

N[K]CC activity [5, 6], or to phosphorylate and inhibit KCC activity [12]. Yeast-2-hybrid experiments have originally demonstrated that a unique 90 amino acid domain, the conserved C-terminal ("CCT") docking domain, of SPAK and OSR1 bind a conserved peptide motif of their downstream targets [8]. The motifs are RFXV/I in the N-terminus of NCC, NKCC1, and NKCC2 [3, 51], RFMV motif in the N-terminus of KCC2A and KCC3A [12, 52]. **However, KCC1 and KCC4 have HFTV or NFTV motif in their N-terminus which did not show interaction with SPAK/OSR1 [8, 53].** The CCT domain in SPAK/OSR1 is also required for the binding and activation of SPAK/OSR1 by the WNKs, which also possess RFXV/I motifs [54]. The structure of this specific CCT domain-peptide interaction was resolved by x-ray crystallography [6]. WNK isoforms, typically WNK1, WNK3 and WNK4, stimulate SPAK/OSR1 kinase activity by phosphorylating a conserved threonine residue (hSPAK Thr233, hOSR1 Thr185) within the SPAK/OSR1 catalytic T-loop motif, and a conserved Ser residue (hSPAK Ser373, hOSR1 Ser325) in the S-motif [32, 55]. Following hypertonic or hypotonic low-Cl^- conditions, WNK isoforms, and hence SPAK/OSR1, are rapidly activated and phosphorylate a cluster of conserved Thr residues in the N-terminal cytoplasmic domain of the N[K]CCs [3]. This mechanism of CCC phosphorylation and activation is conserved for NCC, NKCC1, and NKCC2.

This activation model has been tested and confirmed using both biochemical experiments and functional experiments performed in heterologous expression systems, employing a variety of kinase-dead WNKs and SPAK/OSR1 mutants [4, 11, 51, 55]. A study done in mice showed that the WNK-SPAK/OSR1-NCC signaling cascade in the distal nephron has a circadian rhythm, with phosphorylated levels of NCC, SPAK and OSR1 increasing at the start of the active period

(night for a mouse), while decreasing at the start of the resting period (day) [56]. It has also been shown that OSR1 and SPAK, in the presence of mouse protein-25 (MO25, also called cab39) can form functional homo-dimers and hetero-dimers that are capable of self-activation by transphosphorylation, bypassing the required activation by WNK [48, 50]. MO25 (Cab39) interacts with both SPAK and OSR1 to enhance their catalytic activities over 100-fold [7].

3. Role of SPAK in human physiology and disease

3.1. Targeting SPAK in essential hypertension

One quarter of adults in Western societies have elevated blood pressure (i.e., hypertension), which is a major risk factor for ischemic and hemorrhagic stroke, congestive heart failure, and end stage renal disease [57]. Hypertension is a tremendous burden on the budgets of health care systems worldwide; greater than $130 billion was spent on the treatment of this condition in 2010[57]. While lifestyle changes can modify hypertension, most patients require drugs to lower blood pressure. However, many patients on multi-drug regimens with currently available agents (e.g., hydrochlorothiazides, Ca^{2+} channel blockers, angiotensin converting enzyme inhibitors, loop diuretics, etc.) have poorly controlled disease or suffer from drug side effects, like K^+ wasting. The treatment of hypertension is therefore a current area of unmet clinical need, and the development of more potent agents that harbor fewer side effects is needed.

In the kidney, the WNK-SPAK/OSR1-mediated activation of NCC and NKCC2, which together mediate ~25% of renal salt reabsorption, is critical for extracellular volume levels, and this in turn influences blood pressure and electrolyte homeostasis. Of note, NCC is the target of thiazides, and NKCC2 the target of furosemide – these two drugs are some of the most common

agents used in the treatment of hypertension and edematous states in clinical medicine today. The importance of the WNK-SPAK/OSR1-CCC pathway for renal physiology is exemplified most powerfully by human and mouse genetics. Consider: 1) mouse models strongly suggest that gain-of-function mutations in *WNK1* and *WNK4* and SPAK resulting in increased NCC- and NKCC2-activating phosphorylation cause hypertension in humans with PHAII [58-61]; 2) loss-of-function mutations in the upstream regulators of WNK1 and WNK4, *KLHL3* and *CUL3*, also cause PHAII by increasing WNK1 and WNK4 expression due to a failure of protein degradation [21, 23, 24, 62-67]; 3) loss-of-function mutations in *NCC* and *NKCC2* cause *hypotension* in humans with Gitelman's and Bartter's type 1 syndromes, respectively [68, 69]; 4) rare heterozygous mutations in NCC and NKCC2 alter renal NaCl handling and blood pressure variation in the general population, reduce blood pressure, and protect from development of hypertension [70]; 5) a mutation in *NCC* at a residue (Thr60Met) that abolishes the critical WNK-regulated SPAK-OSR1 activating phosphorylation event causes Gitelman's syndrome in Asians [27, 28]; 6) genome-wide association studies of systolic and diastolic blood pressure reveals a strong disease association with common variants of *SPAK* [71, 72]; 7) *SPAK* knock-out mice exhibit reduced NCC activation [29] and knock-in mice expressing SPAK or OSR1 mutants that cannot be activated by WNK kinase isoforms exhibit reduced NCC and NKCC2 activating phosphorylation, hypotension, and are resistant to hypertension when crossed to transgenic knock-in mice bearing a PHAII-causing mutant WNK4 [32, 33, 73]; and 8) in distal nephron cells, WNK4 inhibits epithelia sodium channels (ENaC) [74], decreased ENaC expression compensates the increased NCC activity following inactivation of the kidney-specific isoform of WNK1 and prevents hypertension [75]. **In oocytes, ENaC expression was significantly increased following coexpression of wild-type SPAK and constitutively active**

(T233E)SPAK, but not following coexpression of WNK insensitive (T233A)SPAK or catalytically inactive (D212A)SPAK [76].

Independently generated SPAK-KO [29, 47], kinase inactive SPAK-KI [32] and SPAK-CCT KI mouse models [73] have provided viable animals exhibiting sodium-wasting hypotensive phenotypes similar to Gitelman's syndrome or chronic thiazide use (**Table 1**). These mice have significantly reduced expression of total and phospho-NCC (p-NCC), thus verifying the dominant role of SPAK in DCT regulation of NCC activity *in vivo* [11, 29, 47]. Notably SPAK-KO mice also exhibit an increase in TAL phospho-NKCC2 (p-NKCC2) which cannot be entirely attributed to an increase in phospho-OSR1 (p-OSR1), but rather may be explained by the emergence of a novel theory supporting a role for shorter sequence SPAK isoforms that exert a negative regulatory effect on CCCs reminiscent of the KS-WNK1/L-WNK1 story [30, 31]. Two of these isoforms that have been discovered in the kidney differ from full length SPAK (~60kDa) in predicted molecular weight and kinase activity; the first isoform SPAK2 (~49kDa) is missing part of the N-lobe of the kinase domain and presumed to be kinase impaired, while the second isoform KS-SPAK (~34kDa) is solely kidney specific and kinase inactive as the entire kinase domain is missing [31]. Note that, as an alternative mechanism to the downstream promoter, the role of a protease has also been proposed as a mechanism for producing the short KS-SPAK isoform [77]. As the CCT domain is intact in these isoforms it is presumed that they compete with full length SPAK and OSR1 for RFxV docking sites, thus inhibiting CCC activity. Another distinguishing factor is the differential expression of these isoforms along the nephron; of particular note in the TAL where SPAK2 and KS-SPAK is significantly higher than full length SPAK and also in the DCT where the inverse is true [30]. It was noted in oocyte and HEK-293

experiments that SPAK2 significantly decreased NKCC1 activity and that KS-SPAK attenuates levels of p-NCC, and perhaps in vivo at the TAL this abundance of negatively regulatory SPAK isoforms normally competes with the overwhelmingly OSR1 dominated regulation of NKCC2, while also muting positive SPAK regulation in this region. However, in the DCT full length SPAK is the dominant form expressed and can overcome the inhibitory effects of SPAK2 and KS-SPAK, evidenced by in vitro co-expression of full length SPAK significantly diminishing the inhibitory effects of SPAK2 on NKCC1 activity [30, 31]. Perhaps the most striking find in this newly discovered system of regulation was the presence of an isoform ratio switch in response to extracellular fluid (ECF) depletion; in which a low sodium diet decreased the abundance of KS-SPAK while increasing levels of full length SPAK, promoting sodium retention [31]. It is conceivable that complete SPAK-KO removes this negative competition and leaves OSR1 to increase NKCC2 activity uninhibited, thus accounting for increased pNKCC2 in these models [29, 47] and furthermore explaining the absence of change in NKCC2 activity in SPAK-KI mice, where the ratios of full length SPAK (although mutated), SPAK2, KS-SPAK and OSR1 are maintained [32].

Together, these data strongly suggest inhibition of the WNK-SPAK/OSR1 pathway might yield a new opportunity to develop improved anti-hypertensives. WNK-SPAK/OSR1 inhibitors are likely to have increased potency over either thiazides or furosemide alone, because they would simultaneously inhibit both NKCC2 and NCC activity. Additionally, WNK-SPAK/OSR1 inhibitors would likely spare K^+ wasting and so may produce robust blood pressure lowering effects without the side effects of hypokalemia that is commonly associated with thiazides and loop diuretics [78]. How can the WNK-SPAK/OSR1 pathway be targeted to treat hypertension?

Table 1 Mouse models in which SPAK have been genetically modified [a]

Gene	Genetic modification	Effect on blood pressure	Expression and activity of NCC	Phenotype	References
SPAK	*SPAK*$^{-/-}$	↓ with a Na^+ depleted diet	↓↓	Hypokalemia when fed a K^+-depleted diet	[30]
	SPAK$^{-/-}$	ND	↓↓	Vasopressin induced NCC phosphorylation	[79]
	SPAK$^{-/-}$	ND	ND	No NKCC2 phosphorylation Decreased NKCC2 mediated Na^+ reabsorption	[80]
	SPAK$^{+/-}$	↓	↓		[29]
	SPAK$^{-/-}$	↓	↓↓	Gitelman syndrome	[29]
	SPAK$^{-/-}$	ND	ND	Na **reabsorption** in the TAL blunted, vasopressin stimulation of NKCC2 intact	[16]
	SPAK$^{T243A/T243A}$	↓	↓↓	Gitelman syndrome	[32]
	SPAK$^{L502A/L502A}$	↓	↓↓	Gitelman syndrome	[73]
	SPAK$^{T243E/S383D}$	asl-sensitive hyper	↑↑	FHHt	[81]
Wnk4-SPAK	*Wnk4*$^{D561A/+}$	↑	↑↑	FHHt	[63]
	Wnk4$^{D561A/+}$*SPAK*$^{T243A/+}$	Partial correction	↑	Partial correction	[33]
	Wnk4$^{D561/+}$ *SPAK*$^{-/-}$	Normal	Normal	None	[82]

a ↑ indicates increase; ↓ indicates decrease. The number of up or down arrows denotes the relative magnitude of increase or decrease. Abbreviations: FHHt, familial hyperkalemic hypertension; ND, not determined.

3.2. Intestine: secretory diarrhea/colitis

The WNK-SPAK pathway has only recently been explored in the regulation of ion transport across secretory epithelia in tissues other than the kidney, such as the skin, pancreas, and intestine. This investigation has stemmed in part from the original observations that, outside the kidney, WNK1 and WNK4 predominantly localized to polarized epithelia, including those lining the lumen of the hepatic biliary ducts, pancreatic ducts, sweat ducts, and colonic crypts [83, 84]. Epithelia in these tissues express channels and transporters that are responsible for transcellular Cl^- and/or HCO_3^- ion movement from the blood, across the epithelial cell basolateral and apical membranes, and into the tissue lumen (e.g., sweat duct, pancreatic duct, or intestinal lumen). In doing so, these secretory epithelial cells therefore produce and maintain the homeostasis of sweat, pancreatic juice, intestinal mucus, and other bodily fluids. So far, the primary transport molecules in these tissues identified as targets of the WNKs-SPAK pathway include the Na^+/HCO_3^- transporter NBCe1 (electrogenic sodium bicarbonate cotransporter 1); the Cl^-/HCO_3^- exchanger family *SLC26A*; and the Cl^- channel CFTR (cystic fibrosis transmembrane conductance regulator) [85-89].

The exocrine gland of the pancreas secretes a pancreatic juice rich in Cl^- and HCO_3^- that also contains enzymes to digest dietary carbohydrates, proteins, and fats. WNK1-SPAK phosphorylation of NBCe1 and CFTR significantly inhibits ductal HCO_3^- secretion by reducing the plasma membrane expression of both NBCe1 and CFTR [88, 90]. Consistent with this, knock-down of several different WNK kinases in pancreatic ducts increases NBCe1 and CFTR-dependent ductal secretion. Interestingly, the NBCe1-B/CFTR activator inositol-1,4,5-trisphosphate (IP(3)) receptor-binding protein released with IP(3) (IRBIT) antagonizes the

effects of the WNKs and SPAK on NBCe1 and CFTR by recruiting PP1 to the complex to dephosphorylate CFTR and NBCe1-B and stimulate their activities [88]. Given that the regulatory modalities in a conserved domain of NBCe1 may be present in CFTR and other transporters like the Slc26a6 sulfate transporter [87], and multiple ion transport proteins in secretory epithelia are regulated by PP1 and/or calcineurin, the WNK-SPAK and IRBIT-PP1 regulatory pathways of Cl^- and HCO_3^- transport may serve to precisely tune the rate of epithelial secretion in response to physiological demands or pathological stimuli in numerous epithelia [86]. The relevance of this pathway for human physiology and disease was recently demonstrated in a large-scale human genetic study. CFTR variants that disrupt the WNK1-SPAK activation are associated with a selective, HCO_3^- defect in CFTR channel function and in turn affects organs that utilize CFTR for bicarbonate secretion (e.g. the pancreas), but do not cause typical CF [91, 92].

The colonic epithelium secretes mucus that is also rich in HCO_3^- and Cl^-. Inflammatory bowel diseases (IBDs), including Crohn's disease and ulcerative colitis, are characterized by impaired immune regulation and epithelial barrier disruption. The mechanisms of the WNK-SPAK pathway in the regulation of colonic transport are less well characterized than in the pancreas. Targeted expression of SPAK has been shown to increase colonic epithelial permeability, and pro-inflammatory cytokines, which are elevated in induced experimental colitis, exacerbate this effect [39, 93]. In contrast, SPAK knockout mice exhibit higher intestinal barrier function and lower cytokine production in induced experimental colitis [94]. The correlated expression of SPAK with colon osmolality and the production of pro-inflammatory cytokines has been linked to SP1 and NF-kB binding sites in the SPAK promoter [95]. These studies highlight the shared

mechanisms and roles of the SPAK in regulating ion homeostasis in different tissues, and have implications for our understanding of CF and IBD, both of which are associated with abnormal epithelial transport. Interestingly, SPAK has also been implicated as potential therapeutic target for the glomerular disorder [96] due to the involvement of NF-κB and p38 MAPK in the nephrogenic effect of SPAK.

4. Strategies of SPAK inhibition

4.1. Inhibition of SPAK kinase catalytic activity

T-loop phosphorylation triggers activation of SPAK, as its mutation to Ala prevents activation [6, 97]. Knock-in mice in which the T-loop Thr residue in SPAK (Thr243) was mutated to Ala to prevent activation by WNK isoforms, and display significantly reduced blood pressure [32]. Therefore, a straightforward approach would be to target SPAK kinase, which is likely to function redundantly in the regulation of NCC and NKCC2, by generating SPAK-specific ATP-competitive kinase inhibitors. A SPAK kinase inhibitor would likely be more efficacious at blood pressure reduction over current agents that target either NCC or NKCC2 alone, since SPAK inhibition would coordinately reduce the activities of both NCC and NKCC2, as well as other less-characterized but no less important substrates of these kinases. **However, Genome-wide association studies of essential hypertension show a strong association with common variants of SPAK [34].** The strategy of targeting the ATP-binding site of the SPAK raises concern regarding the ability to develop sufficiently selective inhibitors that do not suppress other kinases. The development of Closantel, STOCK1S-14279 and Rafoxanide, ATP insensitive inhibitors, has introduced the possibility of developing inhibitors of WNKs signaling by binding to constitutively active or WNK-sensitive SPAK-T233E [98, 99] (**Figure 1**).

4.2. Direct WNK kinase inhibition

An alternative approach would be to target the atypical position of the catalytic lysine residue (Lys^{233} of WNK1) in the WNKs (recall, with no lysine = [K]), which is unique compared with all other proteins in the human kinome. This peculiarity could theoretically be exploited to create WNK-specific ATP-competitive kinase inhibitors. Indeed, Yamada *et al*. exploited these unique structural features to conduct a high throughput screen for inhibitors of WNK1 catalytic activity and discovered the first orally bioavailable pan-WNK kinase inhibitor, WNK463, which exhibits both low nanomolar affinity and high kinase selectivity (**Figure 1**). In spontaneously hypertensive rats, orally administered WNK463 significantly decreased blood pressure, facilitated a brisk diuresis, and reduced the phosphorylation of SPAK and OSR1 [100].

4.3. Inhibiting the WNK-SPAK interaction

As hypertension is a chronic largely asymptomatic condition it will be important to develop WNK or SPAK inhibitors that are sufficiently selective that do not cause intolerable side effects by inhibiting other signaling components. The strategy of targeting the ATP binding site of the SPAK or WNK, raises concern whether it will be possible to develop sufficiently selective inhibitors that do not suppress other kinases. The development of STOCK1S-50699 has introduced the possibility of developing inhibitors of SPAK signaling which target the CCT domain rather than the kinase domain (**Figure 1**). Crystallographic analysis demonstrates that the CCT domain adopts a unique fold not found in other proteins which possesses a pocket which forms a network of interactions with the conserved RFXV/I residues on WNKs and substrates [101]. A compound that binds to this structurally distinct CCT domain pocket and thus blocks

RFXI/V binding motif, could be expected to display highly selectivity and not interfere with other signaling pathways.

Recently, Mori et al. utilized high-throughput screening of > 17,000 chemical compounds with fluorescent correlation spectroscopy and discovered inhibitors that disrupt the WNK(RFXV/I)-SPAK/OSR1(CCT) interaction which resulted in the identification of the aforementioned STOCK1S-50699 as well as a distinct compound termed STOCK2S-26016 [102]. We have confirmed that in vitro both compounds potently suppress CCT domain binding to RFXV motifs, but that in cellular studies we observed that only STOCK1S-50699 but not STOCK2S-26016 suppressed SPAK/OSR1 and NKCC1 phosphorylation induced by hypotonic low chloride conditions [12, 52]. Consistent with STOCK1S-50699 and STOCK2S-26016 being selective, they did not inhibit the activity of 139 different protein kinases tested [102]. Further experiments are required to study the pharmacokinetics and pharmacodynamics of STOCK1S-50699 to establish whether it could be deployed in live animals experiments. **Ishigami-Yuasa et al. further applied screening their chemical library for WNK-SPAK binding inhibitors, and discovered novel inhibitors of this signal cascade from the 9-aminoacridine lead compound 1 [103]. Acridine derivatives were synthesized, such as several acridine-3-amide and 3-urea derivatives, show certain inhibition of the phosphorylation of NCC with doses of 10-20 mg/kg in mouse [103].** These initial studies offer encouragement that targeting the CCT domain could lead to the development of a novel class drugs that would be effective at lowering blood pressure. Given the phenotypes of human and mice with similar alterations in the WNK-SPAK pathway, a drug that suppressed SPAK might elicit particular potent anti-hypertensive effects due to its ability of suppressing renal NaCl reabsorption in a more coordinated and balanced

manner than thiazide or loop diuretics, which only suppress activity of NCC (thiazide) or NKCC2 (loop diuretics) individually, while concurrently sparing renal K^+ wasting – a common side effect of these diuretics. Also intriguing is suggestion that WNK-SPAK inhibition may elicit anti-hypertensive effects via a decrease in NKCC1-mediated vasoconstriction in blood vessels [29], though this hypothesis needs to be further explored. Such an action would offer synergistic effects on both renal and extra-renal targets for blood pressure reduction.

4.4. Inhibition of MO25, a key SPAK/OSR1 regulator

In addition, the closely related isoforms of the MO25α and MO25β scaffolding proteins operate as critical regulators of SPAK and OSR1 as well as a number of STE20 family protein kinases (e.g. MST and STRAD isoforms) [7, 104]. Therefore compounds that disrupt the activation of SPAK/OSR1 kinase activities by interfering with MO25α/β binding could potentially represent a strategy for lowering blood pressure. To explore this approach, Kadri et al. developed a fluorescent polarization assay and used it in screening of a small in-house library of ~4000 compounds. This led to the identification of one compound-HK01-as the first small-molecule inhibitor of the MO25-dependent activation of SPAK and OSR1 *in vitro* [105] (**Figure 1**). This data confirm the feasibility of targeting this protein-protein interaction by small-molecule compounds and highlights their potential to modulate ion co-transporters and thus cellular electrolyte balance.

5. Expert opinion

The importance of coordinating cellular Cl^- influx and efflux in renal epithelia and neurons is well known [106, 107]. The finding that SPAK/OSR1 kinases phosphorylate and thereby trigger

activation of the Na^+-driven, Cl^- influx CCCs (NKCC1, NKCC2 and NCC) and also phosphorylate and inhibit K^+-driven, Cl^- efflux CCCs (KCC1, KCC2, KCC3 and KCC4) helps explain how the CCCs are normally reciprocally and coordinately controlled to achieve homeostasis in multiple tissues. This coordinated and potent mechanism, with opposite effects on the main Cl^- influx and Cl^- efflux mediators involved in cellular Cl^- homeostasis, is of obvious interest to drug development. The WNK-SPAK-CCC pathway is critically important for normal human physiology, and humans and mice with mutations in this pathway have illustrated the potential effects of targeting this pathway for therapeutic benefit in human diseases. The current data suggest that this mechanism is most specifically and powerfully druggable by the targeting of 1) WNK catalytic lysine residue, 2) the CCT domain within SPAK, which interferes with WNK kinase activation, 3) SPAK with inhibitors able to bind to constitutively active or WNK-sensitive SPAK-T223E and 4) MO25 interacts with SPAK. A disease most obviously amenable to inhibition of the WNK-SPAK/ OSR1 pathway would include essential hypertension, one of the most common diseases of the industrialized world. In addition, given the recent enthusiasm for the discovery of KCC2 activators to enhance neuronal Cl^- extrusion in diseases featuring GABAergic disinhibition, exploring the effects of WNK-SPAK/OSR1 inhibition in seizures, neuropathic pain, spasticity, and other diseases featuring neuronal excitability seems like a very compelling idea. SPAK inhibition enhances cellular Cl^- extrusion by concurrently inhibiting NKCC1-mediated Cl^- influx via NKCC1 and activating KCC-mediated Cl^- efflux via the KCCs Therefore, targeting SPAK kinase might also prevent inhibition of feedback on other CCCs or molecules that aim to equilibrate ion gradients, offering a coordinated, multivalent, and sustained effect.

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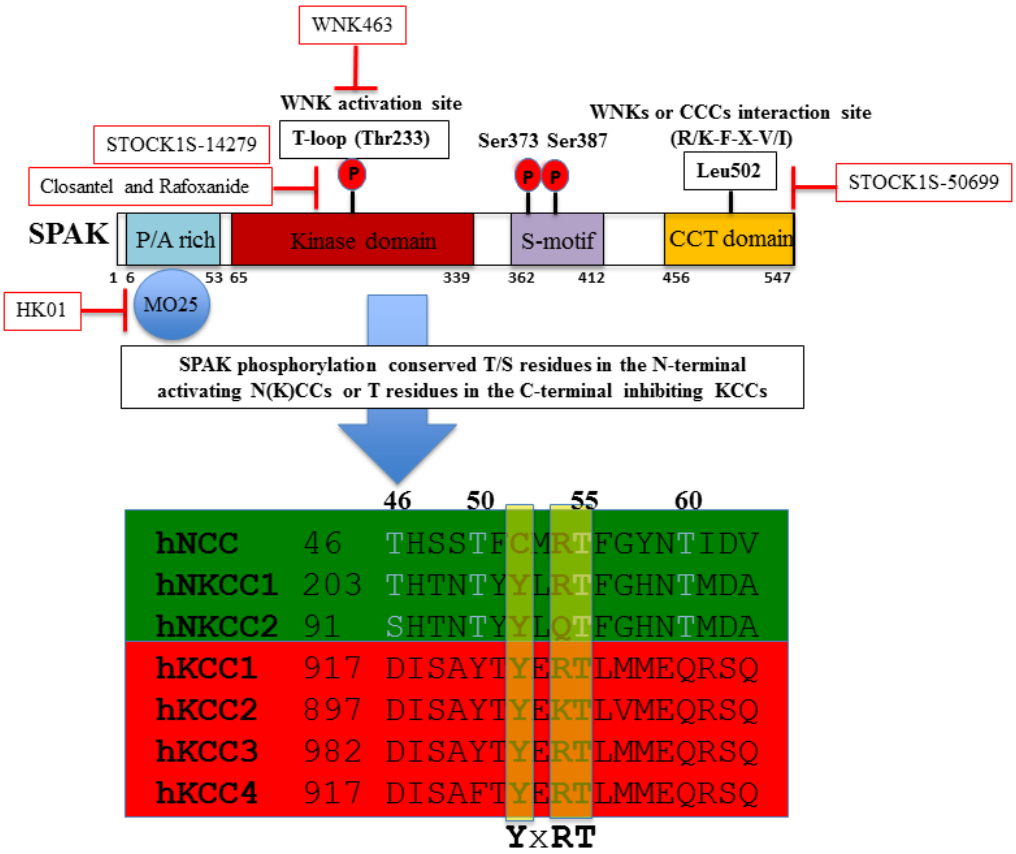

Figure 1. The domain structure of SPAK and the phosphorylation target sites on NCC, NKCC1, NKCC2 and KCCs. OSR1 differs from SPAK in lacking the P/A rich (PAPA) domain.

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