ARNT as a novel anti-fibrotic target in chronic kidney disease

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Conflict-of-interest statement

VHH serves on the scientific advisory board of Akebia Therapeutics, Inc., a company that develops PHD inhibitors for the treatment of anemia.

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Per-Arnt-Sim (PAS) domain proteins are involved in the regulation of cellular responses to environmental stresses such as hypoxia or exposure to polycyclic aromatic hydrocarbons, they furthermore participate in the regulation of circadian rhythm. [1] The therapeutic targeting of PAS domain proteins is likely to impact the clinical practice of Nephrology as multiple compounds that activate hypoxia-inducible factor (HIF) are currently in clinical development for the treatment of renal anemia. [2, 3] HIF, a heterodimeric PAS domain transcription factor, is a central mediator of cellular hypoxia responses and consists of an oxygen-sensitive α-subunit and the constitutively expressed aryl hydrocarbon receptor nuclear translocator (ARNT), also known as HIF-1β.

In a new preclinical study, the research group of Michael Zeisberg at the University of Göttingen has identified the obligatory HIF-α binding partner ARNT as an anti-fibrotic and pro-regenerative inducer of activin-like kinase (ALK) 3 / SMAD signaling. [4] The study suggests that ARNT has therapeutic potential for the treatment of chronic kidney disease (CKD).

What does this important study show?

In their study Tampe and colleagues hypothesized that molecular pathways which mediate the cytoprotective effects of preconditioning regimens can be therapeutically exploited to promote resistance against progressive fibrotic injury. The authors chose a preconditioning regimen that has been shown to protect multiple organs from progressive injury and is based on the widely-used calcineurin inhibitor FK506, also known as tacrolimus. FK506 is currently undergoing clinical evaluation for organ protection in patients with pulmonary arterial hypertension (ClinicalTrials.gov: NCT01647945). [5-7]

Tampe and colleagues demonstrate that oral administration of FK506 at doses of 0.075 and 0.2 mg/kg/day resulted in picomolar non-immunosuppressive blood concentrations and inhibited renal fibrogenesis induced by unilateral ureteral obstruction (UUO). This occurred through activation of the ALK3/SMAD signaling axis and was manifested by a reduction in collagen

accumulation and tubular injury score. BMP receptor ALK3 is known to have anti-fibrotic and pro-regenerative properties and was up-regulated in UUO kidneys following treatment with FK506 but not with cyclosporine A (CsA). [8]

FK506 is a widely prescribed immunosuppressant used in transplantation medicine and for the treatment of autoimmune disorders. It complexes with FK506 binding proteins (FKBP) and prevents activation of nuclear factor of activated T-cells (NFAT) as well as NF-κB pathway-dependent cytokine and immune responses. This occurs mainly through its interaction with FKBP12. [9, 10] Using cell culture-based approaches and animal models, the authors showed that FK506 disrupted the formation of a transcriptional suppressor complex that consists of FKBP12 and Ying Yang 1 (YY1) and inhibits the transcription of *Arnt*. As a consequence, treatment with FK506 raised cellular ARNT levels, which in turn increased *Alk3* transcription. While FK506 treatment also increased the expression of several other transcription factors, only ARNT had effects on *Alk3* mRNA levels.

Although ARNT forms heterodimers with HIF-α or the aryl hydrocarbon receptor (AHR), the study provides evidence that *Alk3* transcription was activated by ARNT homodimers alone and that HIF-α and AHR were not involved in the transcriptional regulation of ALK3. Target-specific manipulations of the FKBP12/YY1/ARNT/ALK3 axis demonstrated that pharmacologic reduction of FKBP12 or YY1 levels using in vivo-morpholinos closely mimicked the protective effects of FK506 treatment, whereas pharmacological reduction of ARNT levels completely abrogated these effects. Taken together, these data provide strong experimental support for a mechanistic model by which FK506 induces renoprotection through a de-repression of *ARNT* transcription, which increases intracellular ARNT levels, subsequently promoting ARNT homodimerization and *ALK3* transcription (Figure 1).

The mechanistic model proposed by Tampe and colleagues holds up in renal allograft biopsy tissue from patients with comparable renal function and histological injury scores who were treated with either FK506 or CsA. Compared to CsA-treated patients, allografts from FK506-treated

patients were characterized by increased *ARNT* and *ALK3* mRNA expression as well as increased numbers of epithelial cells that expressed phosphorylated SMAD 1/5/8, which is consistent with activation of the ALK3 signaling axis. Furthermore, inverse relationships between *FKBP12/YY1* and *ALK3* mRNA levels were found in renal tissue from patients with different kidney diseases (supplementary data), lending further support to the proposed mechanistic model of ALK3 regulation by the FKBP12/YY1 complex.

Because FK506 acts as a strong immunosuppressant, Tampe and colleagues examined whether GPI-1046, a non-immunosuppressive specific inhibitor of FKBP12, was equally effective in affording renoprotection. Consistent with the proposed mechanistic model of FK506-induced renoprotection, GPI-1046 induced ARNT and ALK3 and afforded cytoprotection not only in UUO kidneys but also in an angiotensin II-induced rodent model of cardiac fibrosis and in carbon tetrachloride-induced liver injury. Because FK506 and GPI-1046 were administered before the onset of UUO-induced renal fibrosis, Tampe and colleagues examined whether GPI-1046 or FK506 retained their renoprotective effects under conditions of already existing renal injury. Although only one time point was examined (treatment started on day 3 post UUO), the authors demonstrated that activation of the ARNT/ALK3 signaling axis had beneficial effects on fibrosis progression even though renal injury was already established.

In summary this novel study from Michael Zeisberg's group establishes that a) FKBP12 and YY1 are negative regulators of ARNT and b) that de-repression of *ARNT* transcription and increased ARNT homodimerization promotes cytoprotective effects through activation of ALK3 signaling (Figure 1). ARNT not only represents a potential target for the treatment of renal fibrosis and prevention of CKD progression but is also of therapeutic relevance for other chronic organ injuries such as cardiac and liver fibrosis.

How does this study compare with prior studies?

Tampe and colleagues identified ARNT as a novel antifibrotic target in a model of obstructive nephropathy. Their study predicts that an increase in epithelial ARNT expression by pharmacologic

means has protective effects in chronic kidney disease. Although strong in vitro and in vivo data provide in-depth mechanistic insights, the study raises multiple concerns that need to be addressed in future investigations.

One limitation of this study is the lack of experimental models that more closely mimic human CKD and its progression. UUO-induced renal injury represents a model of renal fibrogenesis characterized by a complete obstruction of the urinary outflow tract and subsequent rapid destruction of the renal parenchyma. The role of ARNT in CKD progression will need to be investigated in other, more chronic renal disease models, which could include folic acid nephropathy, 5/6 nephrectomy, adenine- or adriamycin-induced nephropathy or genetic CKD models such as Alport's disease.

Other concerns relate to ARNT dimerization. ARNT was initially identified as the factor that is necessary for the nuclear translocation of the ligand-bound aryl hydrocarbon or dioxin receptor (AHR), therefore the name aryl hydrocarbon receptor nuclear translocator. [11] ARNT is not only necessary for AHR nuclear translocation, but also for the generation of the heterodimeric transcription factors HIF-1 and HIF-2, which consist of ARNT and HIF-1α or HIF-2α respectively. [1, 4] Therefore AHR-dependent xenobiotic and HIF-dependent hypoxia responses depend on the presence of ARNT. Several other ARNT binding partners are known. For example the aryl hydrocarbon receptor repressor (AHRR), which modulates the AHR xenobiotic response by competing for ARNT binding. [12] Tampe and colleagues demonstrate that the ARNT homodimer is responsible for the increased expression of ALK3 and thus renoprotection. This does not involve HIF as suggested by the authors' data. Given that ARNT can dimerize with other PAS domain proteins, ARNT homodimerization may be impaired under conditions of HIF activation when HIF-α subunits are stabilized or when ligand-bound AHR is present in the cell. A functional interference between hypoxia and xenobiotic responses is well-documented and competition for ARNT binding may modulate the renoprotective effects afforded by FK506 treatment or FKBP12 inhibition. [13, 14]

Although these theoretical concerns will need to be investigated in vivo, a direct activation of the ALK3/SMAD signaling axis may circumvent these issues. Whereas the study by Tampe and colleagues showed that FK506-mediated renoprotection can be completely abrogated with compound LDN193189, a pharmacologic ALK inhibitor, the identification of other genes that are regulated by the ARNT homodimer would be of interest to the research community.

Of relevance in this context are ongoing clinical studies in renal anemia, which aim at raising endogenous EPO production and simultaneously improving iron metabolism via pharmacologic HIF activation. [3] Current clinical trials examine HIF activating compounds that specifically inhibit HIF-prolyl-hydroxylases and stabilize HIF-α subunits, which subsequently heterodimerize with ARNT to activate gene transcription (Table 1). It would be important to investigate to what degree renoprotection afforded by either FK506 treatment or specific FKBP12 inhibition is modulated by systemic administration of HIF-prolyl hydroxylase inhibitors, as the binding affinity of HIF-α for ARNT is relatively high. [14]

Pharmacologic disruption of HIF-2α/ARNT heterodimerization is currently in clinical development for the treatment of advanced renal cell cancer and has generated promising results. [15] PT2385 and PT2399 are first-in class selective small molecule inhibitors of HIF-2 that selectively disrupt HIF-2α/ARNT heterodimerization (Table 1). [16, 17] Whether small molecules can be designed that specifically enhance ARNT/ARNT homo-dimerization remains be investigated.

Despite the relatively low plasma levels, renoprotection afforded by FK506 is somewhat counterintuitive to most clinicians as treatment with calcineurin inhibitors is associated with chronic nephrotoxicity, which does not clearly correlate with serum drug levels and may involve genetic factors. [18, 19] Interestingly a landmark study of calcineurin nephrotoxicity reported that the risk of chronic renal failure was higher in liver transplant patients receiving CsA compared to tacrolimus. This would be consistent with Tampe and colleagues' finding that FK506 regulates renoprotective pathways independent of calcineurin inhibition. [20] Despite the small number of renal transplant

patients examined, only FK506- but not CsA-treated allografts were characterized by the predicted relative increase in ARNT and ALK3 levels, which is in support of the mechanistic model proposed by Tampe and colleagues. The clinical or prognostic implications of these findings, however, remain unclear and would require a focused study in a larger group of transplant patients treated with calcineurin inhibitors.

What are the implications for Nephrologists?

Despite the scientific advances made by this preclinical study and their validation in human renal tissue, the clinical translation of the authors' findings is at a very early stage. Additional studies in animal models that more accurately represent human CKD in conjunction with clinical studies in renal patients are needed to firmly establish whether the FKBP12/YY1/ARNT axis represents a robust therapeutic target for the treatment of CKD. Nevertheless, clinicians should make themselves familiar with PAS domain proteins and their signaling pathways, as multiple compounds are now in clinical trials that either activate the HIF hypoxia response for the treatment of renal anemia (promotion of HIF-α/ARNT heterodimer formation) or inhibit HIF-2 for the treatment of advanced renal cell cancer (disruption of HIF-2α/ARNT heterodimer formation).

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Tables and Figure Legends

Table 1: Therapeutic targeting of PAS domain proteins

biochemical intervention	current clinical application	compounds in clinical trials	ref.
HIF activation, promotion of HIF-α/ARNT heterodimer formation	renal anemia	*Advanced clinical development:* Daprodustat, Molidustat, Vadadustat, Roxadustat *Early clinical development:* Desidustat, Enarodustat	2, 3
HIF-2 inhibition, blockade of HIF-2α/ARNT heterodimer formation	metastatic clear cell renal cancer	PT2385 and PT2399	15, 16, 17

Figure 1. The FKBP12/YY1/ARNT/ALK3 axis in renoprotection. Schematic overview of the proposed mechanism underlying FK506-induced renoprotection. FK506 and GPI-1046 disrupt the formation of a transcriptional suppressor complex that inhibits *ARNT* transcription by inhibiting FKBP12. This leads to de-repression of *ARNT* transcription, increased cellular ARNT levels and ARNT homodimerization with subsequent increase in *ALK3* transcription. ALK3 has renoprotective properties. Shown is also the core DNA binding sequence (E-box) for ARNT homodimers found in the *ALK3* promoter region.

Haase, Figure 1