Perspective

Small molecule inhibition of speckle-type POZ protein-substrate interactions for the treatment of renal cell carcinoma

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Comment on: Guo ZQ, Zheng T, Chen B, *et al*. Small-molecule targeting of E3 ligase adaptor SPOP in kidney cancer. Cancer Cell 2016;30:474-84.

Abstract: Renal cell carcinoma (RCC) is the most common type of kidney cancer and is highly resistant to therapy, clear cell (cc)RCC accounts for 70–75% of cases. Current treatment options include high-dose interleukin-2 (IL-2), and inhibitors of mTOR and HIF-1 downstream signaling. Recently, speckle-type POZ protein (SPOP) has emerged as a promising therapeutic candidate for ccRCC treatment. SPOP is a subunit of the cullin-RING ligase (CRL)-type E3 ligase complex that plays important roles in regulating cell death and proliferation. In 99% of ccRCC tumors, SPOP is overexpressed and mislocalized to the cytoplasm where it acts to lower levels of tumour suppressor genes such as PTEN and DUSP7 by targeting them for ubiquitin-mediated proteasomal degradation. Guo *et al*. have reported the identification of small-molecule inhibitors that block SPOP-substrate interactions, preventing SPOP-mediated ubiquitination and degradation of PTEN and DUSP7, and suppressing the growth of ccRCC cancer cells *in vitro* and tumor growth *in vivo*. These data suggest that therapeutic targeting of SPOP may provide new opportunities for the treatment of patients with ccRCC.

Keywords: Speckle-type POZ protein (SPOP); renal cell carcinoma (RCC); small-molecule inhibitor; E3 ubiquitin ligase; targeted protein degradation

Submitted Nov 13, 2016. Accepted for publication Nov 23, 2016.
doi: 10.21037/tcr.2016.12.60
View this article at: http://dx.doi.org/10.21037/tcr.2016.12.60

Renal cell carcinoma (RCC)

RCC arise from the tubules of the nephron and account for over 90% of kidney cancers. The most common RCC subtypes are clear cell (cc)RCC (70–75% of cases), papillary (p)RCC (10–15% of cases), and chromophobe (ch)RCC (5% of cases). Other less frequent subtypes include oncocytomas and carcinomas of the collecting duct (1). Patients with stage I or II (localized) RCC have a >70% 5-year survival rate following radical or partial nephrectomy, however prognosis is poor in patients with stage III (regional spread) or IV metastatic (m)RCC. mRCC is resistant to chemo- and radio-therapies and virtually incurable (1).

Genome-wide association studies (GWAS) have identified components of the VHL-HIF pathway as major drivers of ccRCC pathogenesis. Under normoxic conditions, hydroxylation of HIF-1α at two conserved proline residues facilitates VHL binding and subsequent cullin-RING ligase (CRL)-type E3 ligase complex-mediated degradation by the ubiquitin-mediated proteasomal pathway. However, under hypoxia, HIF-1α degradation is blocked by the failure of non-hydroxylated HIF-1α to bind VHL, and thus interact with the CRL-type E3 ligase complex. HIF-1α then forms a transcriptional activator complex with HIF-1β, promoting target genes expression relevant to angiogenesis, glycolysis, cell proliferation, invasion and metastasis (2). Loss-of-function VHL mutations are common in ccRCC and lead to hypoxia-independent stabilization of HIF-1α and enhanced expression of downstream target genes including vascular endothelial growth factor (VEGF) A, glucose transporter

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(GLUT) 1, epidermal growth factor receptor (EGFR), and platelet-derived growth factor (PDGF) B (1-4).

Epigenetic regulators are the second most commonly mutated genes in ccRCC, these include PBRM1 (SW1/SNF chromatin remodeling gene), SETD2 (histone H3K36 methyltransferase), and BAP1 (BRCA1-interacting deubiquitinase). It is not clear how mutations in these epigenetic regulators drive the development of ccRCC, but breakdown of genomic stability appears to be involved in the process (1).

The PI3K-AKT-mTOR signaling pathway is a key regulator of cell growth and proliferation and PI3K associated genes are commonly overexpressed or mutated in many types of cancer, including ccRCC. The tumor suppressor, PTEN, acts as a negative upstream regulator of PI3K, and PTEN deficiency and activation of AKT are associated with poor cancer prognosis. The mTOR pathway acts as an upstream translational activator of HIF-1α (1).

Several drugs have been approved for treating patients with ccRCC, including high-dose interleukin-2 (IL-2), anti-programmed cell death protein 1 (PD1) antibody (nivolumab), mTOR inhibitors (everolimus, temsirolimus), and small molecule/antibody inhibitors of the VEGF and PDGF β pathways (axitinib, bevacizumab, cabozantinib, lenvatinib, pazopanib, sunitinib and sorafenib). Although drugs targeting mTOR and VEGF/ PDGF β pathways show better therapeutic responses than conventional IL-2 therapy, it remains important to continue improving patient outcomes by developing new combinations of existing treatments and identifying new targets for drug development.

Targeted protein degradation

The selective promotion of specific protein degradation using degron technology may have therapeutic potential (5,6). In this methodology, target proteins are linked to a destabilization domain (DD) and subsequent exposure to small molecules or light promotes the degradation of the target-DD fusion proteins by the ubiquitin-mediated proteasome pathway (7-12). Although degron technology has many diverse chemical biology applications, the requirement to molecularly engineer target proteins has so far limited the development of therapeutic reagents.

To circumvent the limitations of degron, several groups have developed small molecules, peptides, and proteins that promote the selective degradation of target proteins without prior molecular engineering. Small molecules such as protein-targeting chimeric molecule 1 and phthalimide-conjugated compounds simultaneously bind to both E3 ligase and a target protein, inducing target polyubiquitylation and subsequent degradation by the 26S proteasome (13,14). Similarly, a synthetic death associated protein kinase (DAPK)-binding peptide containing a chaperone-mediated autophagy-targeting motif was shown to promote lysosomal DAPK degradation (15). E3 ligases can also be engineered to selectively bind target proteins, resulting in specific target degradation by the ubiquitin-proteasome pathway (16,17). Synthetic E3 ligases have been developed by the fusion of a target protein-specific nanobody to a truncated form of E3 ligase, in which the substrate-recognition domain was deleted. In the case of the Ab-speckle-type POZ protein (SPOP) synthetic ligase, target proteins were depleted in the cell nucleus, but not in the cytoplasm, and protein degradation occurred more efficiently than treating cells with the corresponding siRNA (17).

Several groups have developed small molecules that prevent interactions between E3 ligase and its substrate, inhibiting protein degradation. The interaction between p53 tumor suppressor and MDM2 E3 ligase for example, has been inhibited by synthetic small molecules (chalcone derivatives and some polycyclic compounds), chlorofusin (a fungal metabolite), and by synthetic peptides (18). Similarly, the small molecule inhibitor MLN4924 prevents the ubiquitin-like polypeptide NEDD8 from activating E3 CRLs (19). CRL-dependent protein degradation is selectively inhibited by MLN4924, leading to the death of human tumor cells (20).

Virtual drug design/screen identified a small molecule that inhibits the interaction between Skp2, a substrate-binding subunit of SCF E3 ligase, and p27, a tumor suppressor that acts as an inhibitor of cell cycle dependent kinase (CDK) (21). Since Skp2 is overexpressed in several cancers, particularly those with poor prognosis and highly metastatic, Skp2 inhibitors could restrict cancer stemness and potentiate sensitivity to chemotherapeutic agents (6).

SPOP and RCC

Ubiquitylation of a specific protein is achieved by the sequential activity of three different classes of enzymes (22,23). Firstly, ubiquitin-activating enzyme (E1) activates the C-terminal carboxyl group of Ub to form a thioester linkage with the active-site cysteine of E1. Next, ubiquitin-conjugating enzyme (E2) transfers the Ub molecule from

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E1 to its own active-site cysteine. Finally, ubiquitin-ligating enzyme (E3) binds to both E2 and the target protein, bringing the target close to the E2 enzyme. Thus, E3 helps E2 transfer Ub from its charged cysteine to the lysine amino group of the target protein. Subsequently, proteins tagged with polyubiquitin chains are degraded by the proteasome (24,25).

In humans, there are two E1 genes, about 40 E2s, and more than 600 E3s (26,27). Thus, the fate of most intracellular proteins is generally determined by temporal and spatial expression patterns, intracellular localization, and substrate specificities of E3 ligases (26,28). There are around 30 HECT domain E3 ligases, in which active-site cysteine forms an Ub-thioester intermediate during the transfer of Ub from E2 to substrate (29,30). The vast majority of E3 ligases belong to the group of RING and RING-related E3s that serve as a scaffold to bring E2 and substrate together, enabling the direct transfer of Ub from E2 to substrate (22,28). CRLs form a prominent subclass of RING-type E3 ligases, they consist of cullin (CUL) isoforms, RING-BOX (RBX)-containing proteins, and various adaptor-substrate recognition proteins that bind to a variety of substrates for ubiquitylation (31,32). NEDD8-activating enzyme (NAE) inhibitor studies suggest that at least 20% of all proteasome-mediated protein degradation is CRL-dependent (20).

Structural analysis indicates that all CRLs share a common elongated molecular architecture: CUL is bound to RBX at the C-terminal domain, and to an adaptor protein at the N-terminal domain. Interaction with target proteins usually occurs via a separate substrate-recognition protein that binds to the adaptor protein, the exception being CUL3, where both substrate-recognition and adaptor proteins are merged in a single "broad complex-tramtrack-bric-a-brac" (BTB)-domain-containing polypeptide (33-35).

SPOP is a subunit of the CRL-type E3 ligase complex, containing domains for substrate-recognition (MATH) and CUL3-binding (BTB-3-box) in single polypeptide (34). In normal cells, SPOP is localized in the nucleus through a nuclear localization signal at its C-terminus (36). The BTB domain is involved in homo- or hetrodimerization with other BTB-containing proteins, which is necessary for its E3 ligase activity (17,34,37). The *C. elegans* SPOP ortholog, MEL-26, degrades MEI-1 to promote the meiotic-to-mitotic transition (38), and in *Drosophila*, SPOP degrades *Cubitus interruptus* (Gli transcription factor) and *Puckered* (JNK phosphatase) to regulate Hedgehog (Hh) and tumor necrosis factor (TNF) pathways (39,40). In addition to its conserved role in Hh and TNF pathways, human SPOP plays important roles in cell death and proliferation, as well as epigenetic regulation by degrading several proteins including death domain-associated protein (Daxx) (41), the Polycomb group protein BMI1 (42), the variant histone MACROH2A (42), the proto-oncogene DEK (43), tripartite motif-containing (TRIM) 24 (44), and Nuclear receptor coactivator (NCOA) 3 (45). Wild-type SPOP also appears to enhance homology-directed DNA repair (HDR) presumably by degrading an unidentified substrate (46). Thus, when SPOP is mutated, non-homologous end joining predominates, resulting in more genomic errors and rearrangements (47).

SPOP is mutated in 8% to 14% of prostate and endometrial cancers; in prostate cancer, the mutations are confined to the substrate-binding MATH domain, suggesting that the mutations affect its interaction with substrates (48,49). An ubiquitylome analysis measuring the interactions between the mutated SPOP proteins and their interacting proteins showed that SPOP mutations found in prostate cancer caused a dominant negative effect (repressing the function of the wild-type SPOP), and did not result in a gain-of-function effect (increasing the binding affinity for the same substrates), nor a neomorphic effect (binding to new substrates) (37). Since SPOP homodimer, not monomer, ubiquitylated substrates (17,34), SPOP mutations that form heterozygous dimers with the wild-type allele are able to decrease the ubiquitylation and subsequent degradation of substrates in a dominant-negative manner.

Tissue microarray screening has shown that SPOP is overexpressed, without mutation, in kidney cancer (85%, 17/20), uterus/endometrial cancer (71%, 10/14), and testis/germ cell cancer (90%, 18/20) (40). In corresponding normal tissues, SPOP was expressed very weakly or not at all. When expression levels were measured in different types of RCC, most ccRCC cells overexpressed SPOP protein (179 positive and 1 negative), but penetrance was low in other types of RCC cells (40), suggesting that overexpressed SPOP could drive the pathogenic development of ccRCC. In normal kidney cells and non-ccRCC cell lines, SPOP is predominantly localized within the nucleus, however in ccRCC, SPOP accumulates predominantly in the cytosol (50). Cytoplasmic SPOP appears to promote tumorigenesis by degrading DAXX, Gli2, and other targets including the tumor suppressors, PTEN and DUSP7 (an ERK-specific MAPK phosphatase that acts as a negative regulator of the ERK pathway) (50). Under hypoxic conditions, HIF proteins directly activate SPOP transcription and SPOP

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protein accumulates in the cytosol (50). Since hypoxic stress and HIFs play important roles in a wide range of tumors, the mechanisms permitting targeted overexpression and mislocalization of SPOP in particular cell types, such as RCC, endometrial and germ cell tumor cells should be the focus of future research.

The work of Guo *et al*. is based on a virtual drug design/screening and has led to the development of small molecules that inhibit the interaction between SPOP E3 ligase and tumor suppressors, including PTEN and DUSP7 (51). Using a computational screen that combined pharmacophore modeling, molecular docking, and chemical scaffold diversification, 109 small-molecule candidates predicted to inhibit the interaction between SPOP and a peptide containing a SPOP binding consensus (SBC) were identified. From these candidates, the small molecules 6a (initial-hit compound, K_D: 62 μM) and 6b (lead compound after synthetic optimization, K_D: 35 μM) were confirmed to physically interact with SPOP and PTEN *in vitro* and inhibit the proliferation of the A498 ccRCC cell line.

Several assays were performed to assess the suitability of compound 6b as a potential therapeutic reagent. Circular dichroism (CD) spectra analysis suggests that 6b does not dramatically perturb the structure of SPOP, and HPLC analysis has confirmed its purity.

Dynamic light scattering showed that 6b is highly soluble in cellular assay media, does not aggregate to form particles, is not itself fluorescent, has excellent cellular permeability, and accumulates rapidly inside cells. Compound 6b directly binds to SPOP *in vitro*, as indicated by surface plasmon resonance and NMR techniques, and *in vivo* cellular thermal shift assay. Binding to SPOP inhibits not only ubiquitylation and subsequent degradation of the tumor suppressors, PTEN and DUSP7, but also the proliferation of ccRCC cell lines and primary human ccRCC cells. Evidence supporting the specific targeting of SPOP in ccRCC by 6b is provided by experiments using ccRCC lines treated with shRNA to reduce SPOP expression; in these modified cells 6b does not inhibit cellular proliferation. Finally, 6b inhibits the growth of A498 tumor cell xenografts in nude mice, without histological changes in multiple organs except kidney. Combined with the very low level of toxicity to mice (toxicity was not observed by daily treatment of 120 mg/kg for 6 days), 6b appears to be a promising therapeutic reagent for treating patients with ccRCC (51).

Ubiquitin-mediated protein degradation and modification pathways play major regulatory roles in maintaining genome integrity, gene expression, and various cellular processes including cell cycle, death, differentiation, proliferation, and signaling (27,28). E3 ligases are implicated in a number of disease pathologies, making them attractive therapeutic targets (52-55). Ubiquitin-mediated pathways are regulated by the selective binding of over 600 E3 ligases to a variety of intracellular proteins. Of over 600 E3 ligases in the human genome, physiological roles are understood for only a small number. Thus, as shown in the case of SPOP, systematic efforts are necessary to identify E3 ligase target proteins, determine their expression patterns in normal and pathological cells and tissues, and correlate their distribution with that of target proteins, providing opportunities to develop new targeted therapeutics.

Acknowledgments

Funding: This work was supported by the SGER Program through the NRF by the Ministry of Education (grant number NRF-2015R1D1A1A02060346); Basic Science Research Program through the NRF by the Ministry of Education (grant number NRF-2015R1D1A3A01015641); the 2014 Research Grant from Kangwon National University to BJ Hwang.

Footnote

Provenance and Peer Review: This article was commissioned and reviewed by the Section Editor Peng Zhang (Department of Urology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China).

Conflicts of Interest: Both authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2016.12.60). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Cite this article as: Hwang BJ, Kee Y. Small molecule inhibition of speckle-type POZ protein-substrate interactions for the treatment of renal cell carcinoma. Transl Cancer Res 2016;5(Suppl 7):S1509-S1514. doi: 10.21037/tcr.2016.12.60

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