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LETTER TO THE EDITOR

# Design of stapled α-helical peptides to specifically activate Wnt/β-catenin signaling

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**Dear Editor,**

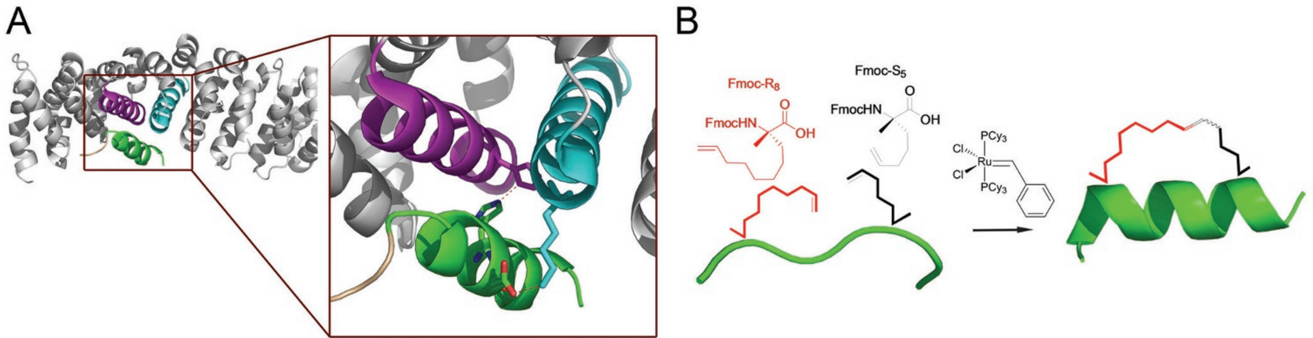
The canonical Wnt/β-catenin signaling pathway orchestrates cell morphology, motility, proliferation and differentiation. This pathway plays important roles in embryogenesis, adult tissue homeostasis and tissue regeneration. Aberrant activation of the Wnt/β-catenin signaling pathway has been implicated in the development of a broad spectrum of tumors, while attenuation of this pathway contributes to a number of human diseases including osteoporosis, neurodegenerative diseases, diabetes and Joubert syndrome [1]. The design of organic compounds that modulate Wnt/β-catenin signaling constitutes an interesting strategy for therapeutic intervention of this key pathway. Additionally, as the Wnt/β-catenin signaling pathway is critical for the maintenance of embryonic stem cells and multiple types of adult stem cells, agonists or antagonists of Wnt/β-catenin signaling may provide useful tools for the studies of stem cell self-renewal and differentiation, and tissue regeneration.

The design of hydrocarbon-stapled peptides was developed by Verdine *et al*. [2] to mimic the α-helix conformation of folded proteins. This method has been demonstrated to efficiently increase the helical propensity and the binding ability of peptides. *In vivo*, stapled peptides can penetrate through cell membranes to attack the intracellular targets and display high protease resistance. Compared with small molecules, stapled peptides have larger surface areas, and therefore can selectively disrupt protein-protein interactions and have been successfully employed to modulate NOTCH and p53 signaling [2].

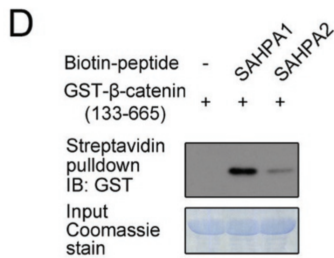
In the canonical Wnt/β-catenin signaling pathway, β-catenin stability is regulated by the destruction complex containing Axin, adenomatous polyposis coli (APC) and glycogen synthase kinase 3β (GSK3β). In the complex, Axin directly interacts with and targets β-catenin for GSK3β-mediated phosphorylation and subsequent ubiquitination and degradation [3]. To design cell-permeable stapled peptides that can activate Wnt/β-catenin signaling with good selectivity, we aim to disrupt the Axin-β-catenin interaction. The crystal structure of Axin reveals that its β-catenin-binding domain (Axin (469-482)) forms a continuous α-helix that fits into a shallow groove of β-catenin formed by the third helices of β-catenin armadillo repeats 3 and 4 [4] (Figure 1A), suggesting that the Axin-β-catenin interaction is suitable for targeting by hydrocarbon-stapled α-helical peptide mimetics. We then synthesized the stapled peptides by incorporation of non-natural amino acids at neighboring positions along one face of the α-helix, followed by ring-closing olefin metathesis (Figure 1B). Two stapled α-helical peptides targeting the Axin-β-catenin complex (SAHPA), SAHPA1 and SAHPA2, were generated (Figure 1C). Circular dichroism analyses revealed that while Axin (469-482) displays 28% α-helical content, SAHPA1 and SAHPA2 have 34% and 57% α-helicity, respectively (Supplementary information, Figure S1).

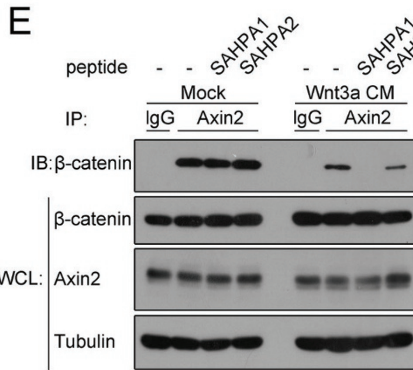
The binding of SAHPAs to β-catenin was investigated using an *in vitro* pull-down assay. Biotin-labeled SAHPAs were immobilized on streptavidin beads to pull down the purified glutathione-S-transferase (GST)-tagged fragment of β-catenin, GST-β-catenin (133-665). Both SAHPAs interacted directly with β-catenin, but SAHPA1 bound much stronger than SAHPA2 (Figure 1D). Quantification with isothermal titration calorimetry revealed that SAHPA1 bound to the purified β-catenin (133-665) with a dissociation constant of ~30.1 μM (Supplementary information, Figure S2).

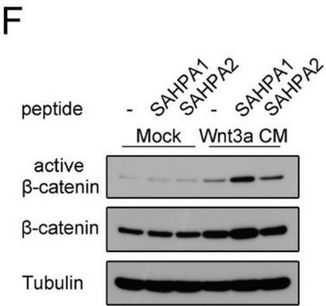
We then assessed cell permeability of the peptides by conjugating fluorescein isothiocyanate (FITC) to SAHPA1. Our data showed that FITC-SAHPA1 efficiently entered into the cytoplasm in Hela cells (Supplementary information, Figure S3). Subsequently we examined the ability of SAHPAs to interfere with the endogenous interaction between Axin and β-catenin in HEK293T cells. After immunoprecipitation of Axin2, the presence of β-catenin was detected by immunobloting. Axin-β-catenin association was greatly diminished by SAHPA1 only in the presence of Wnt3a (Figure 1E). SAHPA2 also had some effects, but its efficiency was much lower. Consistently, SAHPA1 greatly enhanced the effect of Wnt3a on stabilizing the active form of β-catenin (Fig-

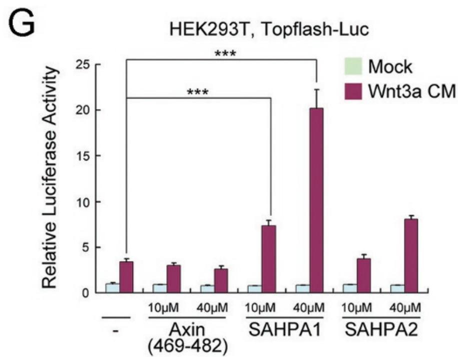


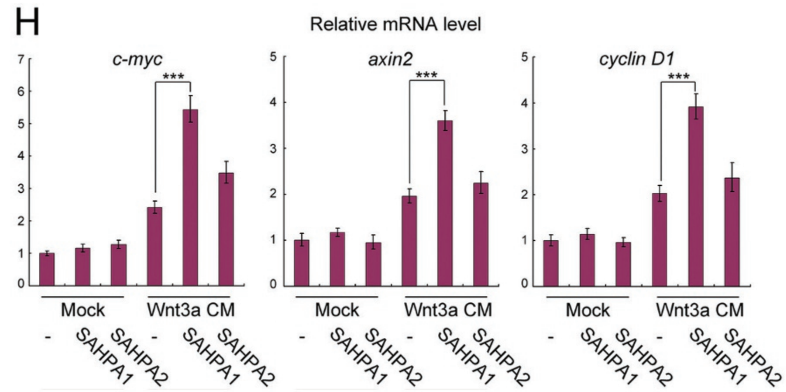

| Compound | Sequence |
|---|---|
| Axin (469-482) | AcPESILDEHVQRVMK-NH2 |
| SAHPA1 | AcPQR8ILDQHVS5RVMK-NH2 |
| SAHPA2 | AcPQSIR8DQHVQRS5MK-NH2 |

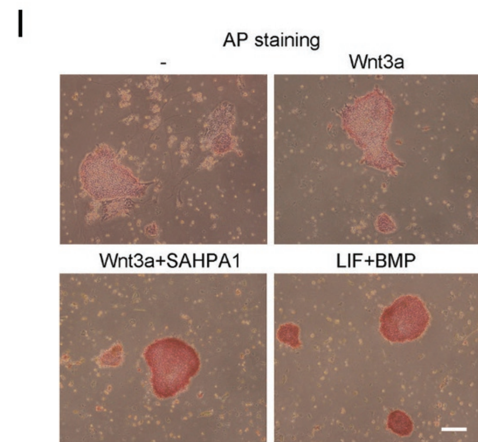


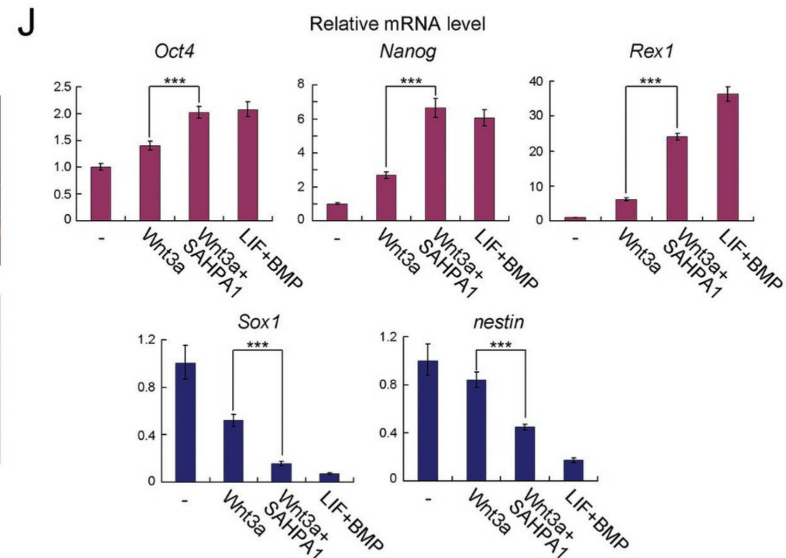



**Figure 1** Design of stapled α-helical peptides to activate Wnt/β-catenin signaling. **(A)** The crystal structure of the β-catenin-Axin complex with highlights of Axin (469-482) (green) and the shallow groove of β-catenin (purple and blue). **(B)** Schematic of peptide stapling. Two non-natural alkenyl amino acids (R8 and S5) are incorporated at two positions in the peptide chain and then cross-linked by ring-closing olefin metathesis. **(C)** Sequences of Axin (469-482)-derived SAHPAs. **(D)** Biotinylated-peptides-bound streptavidin beads were employed to pull down bacterially-expressed recombinant GST-β-catenin (133-665) fragment. The precipitants were immunoblotted with anti-GST antibody, and GST-β-catenin (133-665) inputs were shown by Coomassie staining. **(E)** Coimmunoprecipitation of endogenous β-catenin with Axin2 in the presence of SAHPAs (40 μM). **(F)** HEK293T cells were treated with Wnt3a conditioned medium (CM) and SAHPAs (40 μM) for 12 h and then harvested for immunoblotting. **(G)** HEK293T cells transfected with Topflash-luciferase were treated with Wnt3a CM and SAHPAs at the indicated concentrations for 12 h and then harvested for luciferase measurement. **(H)** Expression analyses of Wnt/β-catenin target genes by quantitative RT-PCR in HEK293T cells treated with Wnt3a CM and SAHPAs (40 μM) for 12 h. **(I)** R1 mESCs were cultured in N2B27 medium supplemented with LIF (10 ng/ml), BMP4 (10 ng/ml), Wnt3a (10 ng/ml), or SAHPA1 (40 μM) as indicated for 5 days and subjected to AP staining. Scale bars: 50 μm. **(J)** R1 cells were cultured in the conditions as described in **I** before being harvested for qRT-PCR. The data in **G**, **H** and **J** represent mean ± SD ($n$ = 3). *** indicates $P < 0.001$.

ure 1F). Together, these experiments demonstrate that SAHPA1 binds to β-catenin and efficiently disrupts the endogenous Axin-β-catenin complex in the presence of Wnt3a.

To explore the effects of SAHPAs on Wnt/β-catenin-dependent transcriptional activity, we performed the Topflash reporter assay in which the firefly luciferase is transcriptionally activated by β-catenin [5]. Consistent with the data above, SAHPA1 treatment greatly enhanced the reporter expression in a dose-dependent manner in the presence of Wnt3a (Figure 1G). SAHPA2 also promoted Wnt3a-induced reporter expression albeit less effectively than SAHPA1. Consistently, quantitative RT-PCR revealed that SAHPA1 increased the Wnt3a-stimulated expression of *c-myc*, *axin2* and *cyclin D1*, the classic Wnt/β-catenin target genes [6] (Figure 1H). In contrast, the unstapled fragment of Axin (469-482) had no enhancing effect. As controls, SAHPA1 did not affect other tested signaling pathway reporters (c-Jun-responsive AP-1 reporter, TGF-β/Smad-responsive CAGA reporter, BMP4-responsive BRE reporter, Notch/RBP-Jκ-responsive pGa981-6 reporter, TNFα-responsive NF-κB reporter and PKA-responsive CRE reporter) (Supplementary information, Figure S4), indicating the specific effect of SAHPA1 on Wnt/β-catenin signaling. These data together suggest that SAHPA1 efficiently activates Wnt/β-catenin signaling in a ligand-dependent manner.

Wnt/β-catenin signaling has been shown to play important roles in the maintenance of self-renewal and prevention of differentiation of mouse embryonic stem cells (mESCs) [7, 8]. We further investigated the effects of SAHPA1 on fate determination of R1 mESCs. R1 cells can be maintained in N2B27 medium containing LIF and BMP4, and withdrawal of these growth factors drives mESCs to neural differentiation [9]. Although Wnt3a alone could slightly reverse the neural differentiation process, Wnt3a plus SAHPA1 efficiently promoted self-renewal and inhibited differentiation of mESCs, as shown by colony morphology and alkaline phosphatase (AP) staining (Figure 1I). This observation was further supported by examining the expression of the pluripotency markers – *Pou5f1* (*Oct4*), *Nanog*, and *Rex1*, and the neural markers – *Sox1* and *nestin* [10]. Upon the withdrawal of LIF and BMP4, the expression of these pluripotency markers was significantly decreased and the expression of the neural markers was significantly increased (Figure 1J). SAHPA1 greatly enhanced the activities of Wnt3 to activate the pluripotency markers and to repress the neural markers. These results indicate that super activation of Wnt/β-catenin signaling by SAHPA1 plus Wnt3 could replace LIF and BMP4 to maintain mESC self-renewal.

By far, most of the Wnt/β-catenin signaling agonists function through inhibition of GSK3β [7, 11]. However, as GSK3β also plays important roles in metabolism and in the regulations of numerous signaling pathways [12], such agonists could produce unwanted side effects. Therefore, Wnt/β-catenin agonists with high specificity would be appreciated for potential medical applications. Indeed, Gwak *et al*. [13] recently reported the small molecule SKL2001, which can activate Wnt/β-catenin signaling even without Wnt treatment by disrupting the Axin-β-catenin interaction. Still, the specificity of SKL2001 waits to be tested. Due to the high binding specificity of stapled peptides, our approach can achieve high specificity as shown for SAHPA1 to activate the Wnt/β-catenin pathway without affecting other tested pathways.

The potential carcinogenic effect of universal upregulation of Wnt/β-catenin signailing greatly limits the clinical application of Wnt/β-catenin agonists. Our results reveal that the activation of Wnt/β-catenin signaling by SAHPA1 is ligand-dependent. In the absence of Wnt3a, SAHPA1 had no obvious effect. However, even with low levels of Wnt3a, SAHPA1 greatly amplified Wnt/β-catenin signaling. The ligand-dependent feature of SAHPA1 enables the activation of Wnt/β-catenin signaling in the tissues where Wnt is present, while not in the tissues without Wnt ligands, achieving another level of

specificity.

During the submission of our paper, Grossmann *et al*. [14] reported that a hydrocarbon-stapled peptide (fStAx-35R) inhibited Wnt/β-catenin signaling by interfering with β-catenin-TCF interaction. To better discuss the difference between these two peptides, we compared their effects on Wnt/β-catenin signaling in HEK293T and colon cancer SW480 cells. fStAx-35R inhibited Wnt/β-catenin signaling in both cell lines. However, SAHPA1 enhanced Wnt/β-catenin signaling in HEK293T, but had no effect in SW480 cells, in which β-catenin is hyperactivated due to APC mutation and Wnt ligand has little effect (data not shown). Furthermore, SAHPA1 is exclusively localized in the cytoplasm of Hela cells (Supplementary information, Figure S3) and SW480 cells (data not shown), suggesting that the opposite effects of these two peptides could be due to their distinct subcellular localization (SAHPA1 in the cytoplasm and fStAx-35R in the nucleus [14]).

In summary, we reported here a stapled α-helical peptide that targets the Axin-β-catenin interaction and activates Wnt/β-catenin signaling with a high selectivity. We have also demonstrated that this stapled peptide together with Wnt ligand could maintain the self-renewal of mouse embryonic stem cells in the absence of LIF and BMP. Our study provides a new strategy to modulate the Wnt/β-catenin pathway, and the highly specific agonist has potential applications in biomedical research and the treatment of Wnt/β-catenin signaling-defective diseases.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)