

Phage selection of peptide macrocycles against β -catenin to interfere with Wnt signaling

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Upregulation of β -catenin, the primary mediator of the Wnt signaling pathway, plays an important role in the tumorigenesis of several types of human cancer. Targeting β -catenin to interfere with its ability to serve as a translational co-activator is considered an attractive therapeutic approach. However, the development of inhibitors has been challenging because of the lack of obvious binding pockets for ligands, and because inhibitors should not interfere with other β -catenin functions. Only two ligands with known molecular interactions were developed so far to β -catenin; they are based on stabilized α -helical peptides. Herein, we screened a large combinatorial library of bicyclic peptides by phage display. Binders to different surface regions of β -catenin were identified. The binding site of one group of ligands was mapped to the interaction region of the translational Wnt inhibitor ICAT (inhibitor of β -catenin and Tcf) which is a prime target site on β -catenin for therapeutic intervention, and to which no ligands could be developed before.

β -catenin is a multi-functional protein that plays important roles in cell-cell adhesion at adherens junctions and in the Wnt growth factor signaling pathway.^[1] In its latter role, β -catenin regulates many biological processes, including embryogenesis, development and adult tissue homeostasis. Deregulation of the Wnt pathway is associated with multiple diseases, including various types of cancer.^[2] In the absence of a Wnt signal, β -catenin turnover is regulated by a multicomponent complex in the cytoplasm called the β -catenin-destruction complex, which catalyzes the phosphorylation of β -catenin and thus labels it for ubiquitin-dependent degradation by the proteasome.^[3] In the presence of a Wnt signal, the phosphorylation of β -catenin is inhibited, which leads to its accumulation in the cytosol. Accumulated β -catenin translocates to the nucleus where it interacts with numerous partners including proteins of the Tcf/LEF family, and together they activate the transcription of Wnt responsive genes.^[3] Structurally, β -catenin contains a series of 12 armadillo repeats, each of which is approximately 40 amino acids, flanked by unstructured N- and C-terminal domains.^[4] Around 40 different β -catenin interaction partners have been described.^[1, 4]

Abnormal activation of the Wnt/ β -catenin pathway is caused mostly by loss-of-function mutations in the tumor suppressors adenomatous polyposis coli protein (APC) and axin, or gain-of-function mutations in β -catenin, causing over-proliferation of the cell and the onset of tumorigenesis owing to a constitutive increase in the levels of nuclear β -catenin.^[2] Mutations in proteins of the Wnt pathway are believed to be present in around 20% of all human

cancers, with the majority of colorectal cancers bearing a mutation in the Wnt signaling pathway.^[5] One of the most frequent mutations is found in the APC gene, causing the inherited conditional familial adenomatous polyposis (FAP), which results from the loss of one allele of APC, leading to the formation of a high number of intestinal polyps and an increased predisposition to colorectal cancer.^[5]

Owing to the strong dependence of many tumors on Wnt signaling for growth and survival, inhibition of this pathway is considered an attractive therapeutic approach. Several strategies towards the inhibition of canonical Wnt signaling have been reported in recent years.^[6, 7] Compounds were identified that either inhibit components upstream of the destruction complex, activate the destruction complex to reduce β -catenin levels, or inhibit the transcriptional activator complex. Targeting β -catenin to interfere with its ability to serve as a translational co-activator is considered as a particularly compelling approach because the transcriptional complex is the final effector of canonical Wnt signaling, and because it is downstream of most-cancer associated Wnt pathway mutations. A challenge with β -catenin as target is that it has, in addition to its involvement in Wnt signaling, other critical roles as for example in cadherin binding being important for cell-cell adhesion at adherens junctions.^[8] Several β -catenin binding partners serving in different roles have overlapping binding sites, making it challenging to interfere with only Wnt signaling.^[1, 7, 9] For example a compound that disrupts the β -catenin-Tcf interaction could also interfere with the β -catenin-cadherin and β -catenin-APC interactions, since Tcf, cadherin and APC share the core binding site in the groove formed by the armadillo repeat domain. Another challenge in targeting β -catenin is its rather flat and featureless surface that makes the development of small molecule binders difficult.^[9]

Screening of small molecule libraries led to the identification of several inhibitors of the transcriptional activator complex.^[7] However, the reported small molecule inhibitors have low affinities and/or their exact molecular mechanisms were not identified. Inhibitors with defined binding mechanisms were designed based on α -helices of β -catenin interaction partners. One such peptide developed was a hydrocarbon-stapled α -helical peptide derived from B cell lymphoma 9 (BCL9), a co-activator for β -catenin-mediated transcription.^[10] The peptide dissociated native β -catenin/BCL9 complexes and suppressed Wnt transcription. Another stapled peptide β -catenin antagonist was developed based on an α -helical peptide from axin binding to a surface region within the β -catenin-Tcf binding interface.^[11] The peptide named StAx-35R binds β -catenin with a K_d of 53 nM and suppresses selectively mRNA levels of

canonical Wnt-driven genes. The development of stapled peptide ligands to more surface regions of β -catenin is limited by the requirement of structural information of β -catenin binding partners that bind via α -helices and the availability of proteins binding via α -helices.

In this work, we raised the question if ligands to a wider range of β -catenin surface regions could be developed by screening combinatorial libraries of random cyclic peptides using phage display. We reasoned that such ligands could be instrumental in the search for regions on the surface of β -catenin that can be targeted for blocking transcriptional activation of Wnt genes without interfering with the destruction pathway or other important functions of β -catenin such as cell-cell adhesion. We further reasoned that peptidic ligands fulfilling the latter requirements could potentially be used as templates to engineer cell-permeable macrocycles for therapeutic application. A peptide format that is suited for high-affinity binding is the one of bicyclic peptides. High affinity ligands based on bicyclic peptides were recently developed to a range of different protein targets using a strategy based on phage display.^[12] In this method, peptides are displayed on phage and chemically cyclized by reacting three cysteines, two flanking the peptide and one being in the middle, with reagents containing three thiol-reactive groups. Binders are subsequently isolated in iterative rounds of affinity selection as in conventional phage panning procedures. Bicyclic peptide phage display yields typically binders to several different surface regions of a protein target, and the binding affinities tend to be in the low micromolar to picomolar range.^[12-15]

We prepared phage displaying $> 4 \times 10^9$ different peptides of the format ACX₆CX₆CG^[12] and cyclized the peptides in separate reactions with the three chemical linkers 1,3,5-tris(bromomethyl)benzene (TBMB), 1,3,5-triacryloyl-1,3,5-triazinane (TATA) and N,N',N''-(benzene-1,3,5-triyl)-tris(2-bromoacetamide) (TBAB)^[16] to obtain three libraries with a total diversity exceeding 12 billion peptide macrocycles. β -catenin containing the 12 armadillo repeats but not the flexible N-terminus (amino acids 129-781; Supplementary Figure S1) was biotinylated via amino groups and immobilized on streptavidin- or neutravidin-coated magnetic beads. A careful choice of buffers was required to prevent aggregation of β -catenin during the procedures of β -catenin purification, biotinylation, removal of unreacted biotin and immobilization, to eventually present the target protein on the solid support in its native conformation. The three libraries were panned in parallel against β -catenin. Streptavidin (round 1 and 3) and neutravidin beads (round 2) were used alternatingly to prevent enrichment of binders to these proteins.^[17] The number of phage captured in the affinity selections increased strongly in the second and third round of panning. In the third round, the

number of phage isolated from the three libraries was several orders of magnitude higher than in control reactions in which β -catenin was omitted, indicating that target-specific binders were isolated (Supplementary Table 1).

Around 170,000, 69,000 and 84,000 clones isolated from the libraries of peptides cyclized with TBMB, TATA and TBAB respectively, were sequenced. Alignment of the most abundant peptide sequences of each selection with a previously developed software^[18] revealed many different consensus sequences (Figure 1). For peptides cyclized with TBMB, TATA and TBAB, 12, 5 and 4 different consensus sequences were found. The number of identified consensus sequences was comparatively high: bicyclic peptide phage selections against other targets yielded in most cases not more than a few different consensus sequences.^[12-16] Peptides sharing a consensus sequence are likely forming the same molecular interactions with the target and thus binding to the same surface region. Conversely, peptides of different consensus groups form different molecular contacts. Having identified numerous consensus motifs, it was likely that ligands to several different surface regions were isolated. This identification of many binding sites can be explained by the fact that β -catenin has many natural protein binding partners and thus suitable binding faces. Comparison of the consensus sequences with known β -catenin peptide binding motifs identified one striking similarity: the consensus motif 'S^L/VLD' found in TBAB-cyclized peptides (first consensus group in Figure 1, right column) is similar to the tetrapeptide 'SILD' of the axin α -helix (EENPESSILDEHVQRVMK) that forms direct contacts with β -catenin.^[19] The similarity suggested that peptides of this consensus group are binding to the armadillo repeat 3 and 4 through similar interactions as the α -helix of axin.

Representative peptides of the different consensus groups were chemically synthesized. Fluorescein was linked to their N-terminus to allow measurement of binding to β -catenin by fluorescence polarization. Among the 22 peptides that were tested, four were binding with single-digit micromolar K_d s and two showed K_d s between 20 and 30 μ M (Figure 1). The remaining peptides showed no or only weak binding to β -catenin at the highest concentration tested (8.5 μ M). The six bicyclic peptides binding β -catenin with micromolar K_d s were isolated from the peptide libraries cyclized with TBMB (BC1, 2nd consensus group; BC2, 5th consensus group), TATA (BC3, 1st consensus group) and TBAB (BC4, BC5 and BC6, 2nd consensus group).

In order to identify the binding sites of the bicyclic peptides, competition binding experiments were performed using the two natural β -catenin interaction partners axin and ICAT. Axin binds via an α -helix to armadillo repeat 3 and 4 of β -catenin ($K_d = 8.4$ nM). The binding site overlaps with the one of the C-terminal α -helix of Tcf that docks in the groove of armadillo repeats 3 and 4 (Figure 2, surface in red).^[19] A synthetic peptides with the sequence of the α -helix was previously found to bind β -catenin, and its binding affinity could be enhanced by 'stapling' the helix with a hydrocarbon linker.^[11] We thus used such a stabilized helical peptide for the competition binding experiment ($K_d = 8.4$ nM). ICAT is a translational Wnt inhibitor. The 81 amino acid protein binds with its N-terminal helical domain to armadillo repeats 11 and 12 of β -catenin, and with its C-terminal extended region to the groove formed by armadillo repeats 5-10 of β -catenin ($K_d = 3.1$ nM; Figure 2, surface in blue).^[20] For the competition binding experiment, β -catenin at different concentrations (150 nM – 19.2 μ M) was incubated with excess of the axin α -helix (25 μ M) or ICAT (25 μ M). The complexed proteins were added to fluorescence-labeled bicyclic peptides (100 nM) and the fraction of bound peptide quantified by measuring fluorescence polarization (Figure 3). The axin α -helix did not block the interaction of any of the bicyclic peptides with β -catenin. The binding of peptides BC4, BC5 and BC6 was slightly weakened, possibly through conformational changes in β -catenin imposed by the axin helix or through unspecific interactions of the axin peptide that was added at high concentration. Conversely, ICAT competed strongly with the binding of the bicyclic peptides BC1, BC4, BC5 and BC6. Even at the highest concentration of β -catenin, no increase in fluorescence polarization was observed for the four peptides, indicating that ICAT fully blocks their binding. Peptides BC4, BC5 and BC6 share the same consensus sequence, suggesting that they bind to the same epitope. Peptide BC1 belongs to a different consensus group, indicating that it binds β -catenin differently than the other three peptides.

ICAT binds to an extended surface region of β -catenin and many binding sites can be envisioned for the four bicyclic peptides. Structural analysis of β -catenin with bound peptide will be required to identify the exact binding sites of the bicyclic peptides. The finding that the bicyclic peptides bind to the same surface region as ICAT was exciting as ICAT is a native inhibitor of the Tcf- β -catenin interaction that reduces transcription activation. There is a chance that peptide macrocycles binding to the same epitope will inhibit β -catenin mediated transcription too. Given the moderate binding affinities of the bicyclic peptides in the micromolar range, an affinity maturation will be required before the peptides can be tested in cells for their ability to block β -catenin-mediated signaling. Additionally, the peptide might

need to be modified to facilitate their transport into cells, either by conjugating them to a cell penetrating peptide, or by substituting the charged residues and eliminating several polar groups.

In summary, a range of β -catenin ligands could be isolated from bicyclic peptide phage display libraries. The large number of consensus sequences identified indicates that the peptide macrocycles interact with β -catenin via diverse peptide binding motifs, and bind potentially to various regions of the target protein. Competition experiments with native β -catenin binding partners indeed identified multiple binding sites. Several peptides interfered with ICAT and thus bound to a prime target site on β -catenin for therapeutic intervention. While more work is required to locate the exact binding sites, to affinity mature the binders, and to assess their activity in cells and *in vivo*, the results revealed that β -catenin offers a wide range of binding sites for peptide macrocycles, and encourages to screen larger cyclic peptide diversities to identify even more diverse binders. If binders blocking Wnt signaling without interfering with essential activities of β -catenin can be identified, they might be converted into cyclic peptidomimetics for developing anti-cancer therapeutics.

Materials and Methods

β-catenin expression and purification

Human β -catenin (residues 129-781) was expressed in *E.coli* as a fusion protein containing at the N-terminus a streptavidin binding peptide, a 6x His-tag and a TEV cleavage site (the sequence is provided in the Supplementary Information). The expression vector was transformed into Rosetta blue *E.coli* electrocompetent cells and cells of a single colony were grown in 500 ml TBG media (1.2% w/v tryptone, 2.4% w/v yeast extract, 0.4% v/v glycerol, 17 mM KH_2PO_4 , 72 mM K_2HPO_4) supplemented with kanamycin (50 $\mu\text{g/ml}$) and chloramphenicol (30 $\mu\text{g/ml}$) at 37 °C to an OD_{600} of 0.8. The culture was then cooled down at 21 °C and the protein production induced with 0.1 mM IPTG for 16 hrs. The bacteria were pelleted by centrifugation and resuspended in 25 ml ice cold buffer A (50 mM Tris-HCl, pH 8.5, 15 mM imidazole, 10% v/v glycerol). Bacteria were lysed by sonication on ice and bacterial debris pelleted by centrifugation at 4 °C for 30 min at 4000 rpm. The supernatant was run through a His Trap FF 1 ml column (GE Healthcare Bio-Sciences AB), washed with 15 ml buffer A and eluted via a linear gradient (0-100%) of buffer B (50 mM Tris-HCl, pH 8.5, 150 mM imidazole, 10% glycerol) in 30 ml. The buffer of the eluted protein was exchanged to buffer C (50 mM Tris, pH 8.8, 10 mM NaCl, 10% v/v glycerol). Protein purity was assessed by SDS-PAGE analysis. Aliquots of the protein were stored at -80 °C.

β-catenin biotinylation

For phage selections, the N-terminal streptavidin binding peptide tag of the β -catenin fusion protein was removed with TEV protease as follows. After His-tag purification β -catenin was dialyzed overnight at 4 °C in elution buffer (30 mM imidazole, 10% glycerol, pH 8.3) in the presence of TEV protease in a ration 1:20 w/w TEV protease: β -catenin. The TEV protease carrying a His-Tag was removed by incubating the mixture for 1 hr at 4 °C with Ni-NTA slurry. Ni-NTA slurry was pelleted by centrifugation and the supernatant containing β -catenin was recovered. The buffer of the protein was changed to PBS (137 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4 , 2 mM KH_2PO_4 , pH 7.4) using a PD-10 desalting column (GE Healthcare Bio-Sciences AB). The protein (1 μM) was biotinylated using a 50-fold molar excess (50 μM) of Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific) for 60 min at room temperature. Unreacted Sulfo-NHS-LC-Biotin was removed by gel filtration using a PD-10 desalting column and TNC buffer (50 mM Tris pH 8.8, 150 mM NaCl, 10 mM CaCl_2 , 0.02% NaN_3 , 0.05% Brij 35). The efficiency of the biotinylation reaction was assessed by capturing the protein on 20 μl

streptavidin beads (Dynabeads M-280 Streptavidin, Invitrogen Dynal Biotech AS) and analysis of bound protein by SDS-PAGE.

Phage display selection

Phage of the 6x6 peptide library^[12] were produced and purified on a 1.5 L scale. Peptides displayed on the phage were reduced with TCEP and the reducing agent removed as previously described.^[17] The phage were split into three tubes to cyclize the peptides with TBMB (20 μ M), TATA (150 μ M) and TBAB (40 μ M) as described previously.^[16] Each of the three bicyclic peptide phage libraries was subjected to affinity selections as described previously.^[17] For each selection, 5 μ g biotinylated human β -catenin immobilized on 50 μ l magnetic streptavidin (1st and 3rd round) or neutravidin beads (2nd round) was used. Neutravidin-coated magnetic beads were used in the second round of selection to prevent enrichment of streptavidin-specific peptides. Eluted phage were incubated with 30 ml of exponentially growing TG1 *E. coli* cells (OD₆₀₀ = 0.4) for 90 min. Bacteria were plated on large 2YT/chloramphenicol plates (30 μ g/ml) and incubated at 37 °C. The cells were harvested the next day with 2YT media and stored in 15% glycerol at -80°C.

Phage DNA sequencing

For Sanger sequencing, cells of single colonies were grown in 5 ml LB media containing chloramphenicol (30 μ g/ml), DNA isolated and sequenced (Macrogen). For high-throughput sequencing, cells of colonies were pooled and DNA isolated. The DNA was processed as described previously^[18] and sequenced by Ion Torrent sequencing. The data was analyzed using software described previously.^[18]

Peptide synthesis

Peptides were synthesized on an Advanced ChemTech 348 Ω peptide synthesizer (Aapptec) by standard Fmoc solid-phase chemistry using Rink Amide AM resin (0.03 mmol scale; GL Biochem) and DMF as solvent. Each amino acid (4 equivalents) was coupled twice (RT, 30 min, 400 rpm) using HBTU/HOBt (4 equivalents each) and DIPEA (6 equivalents). The resin was washed 4 times with DMF after the coupling reaction. Fmoc groups were removed twice using 20% (v/v) piperidine in DMF (RT, 5 min, 400 rpm). The resin was washed 5 times with DMF after deprotection.

N-terminal labeling of the peptides with 5(6)-carboxyfluorescein

5(6)-carboxyfluorescein (Acros organics) was coupled to the N-terminus of peptides before cleavage from the Rink Amide resin. 2 equivalents of 5(6)-carboxyfluorescein (0.06 mmol), 2 equivalents of DCC (0.06 mmol) and 4 equivalents of HOBt (0.12 mmol) were each dissolved in 0.5 ml DMF. The chemicals were added to N-terminal deprotected peptide (0.03 mmol) and mixed at RT 1 hr at 400 rpm. The resin was washed four times with DMF.

Peptide cleavage from the resin

Peptides were deprotected and cleaved from the Rink Amide AM resin by incubation with 5 ml of cleavage solution (90% v/v TFA, 2.5% v/v 1,2-ethanedithiol, 2.5% w/v phenol, 2.5% v/v thioanisole, 2.5% v/v H₂O) for two hrs under shaking. The resin was removed by filtration under vacuum and the peptides precipitated with ice-cold diethyl ether (50 ml), incubated 30 min at -20 °C and pelleted by centrifuging 5 min at 4000 rpm. Diethyl ether was discarded and the process repeated twice using 30 and 20 ml diethyl ether, respectively. The remaining solvent was evaporated at RT.

Peptide cyclization with chemical linkers

The crude peptide (typically around 40 mg) was dissolved in 4.5 ml 50% ACN and 50% H₂O (giving a concentration of around 2 mM). The cyclization linkers TBMB, TBAB and TATA were dissolved in 0.5 ml ACN and added to the peptide. 1.1 equivalents were used for cyclization by TBMB and TBAB, and 2 equivalents for cyclization by TATA. The reaction was started by adding 5 ml of 60 mM NH₄CO₃, pH 8.0. After 1 hr at 30 °C in a water bath, the reaction was stopped by adding 0.5 ml formic acid (98 %). The peptides were lyophilized.

Peptide purification by reverse phase HPLC

Modified peptide powder was resuspended in 1 ml DMSO, 2 ml ACN containing 0.1% TFA and 7 ml H₂O containing 0.1% TFA, and purified on a preparative C18 column (Vydac C18 TP1022 250 × 22 mm, 10 μm) using a linear gradient of solvent B (99.9% v/v ACN and 0.1% v/v TFA) over solvent A (99.9% v/v H₂O, 0.1% v/v TFA) (28 min, 10% to 50%, 20 ml/min flow rate). Fractions containing the desired product were identified by ESI-MS analysis and lyophilized.

Fluorescence polarization binding assay

β -catenin was serially diluted (2-fold, starting from 57.6 μ M) in buffer D (50 mM Tris, pH 8.8, 10 mM NaCl, 10% v/v glycerol, 0.01% Tween-20). 5 μ l of each dilution were transferred to wells of a black Corning 384 flat-bottom polystyrene microtiter plate. 5 μ l of buffer D were added to each well. 5 μ l of fluorescein-labeled peptide in buffer D (300 nM) were added to each well and incubated for 5 min. Anisotropy of each solution was measured in a multiwell plate reader (Infinite[®] 200 PRO, Tecan) using a 485 nm excitation filter and a 535 nm emission filter. For the competition experiments, the same dilutions of β -catenin were prepared in a 384 microtiter plate (5 μ l per well). 5 μ l of either 75 μ M axin α -helix (fAx-4-CDCB) or 75 μ M ICAT (ATGen, Seongnam-si) in buffer D were added to each well. After 5 min incubation, 5 μ l of 300 nM fluorescein-labeled peptide in buffer D were added and anisotropy measured as described above.

Keywords

β -catenin, bicyclic peptide, cyclic peptide, phage display, protein-protein interaction, Wnt signaling

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Figures

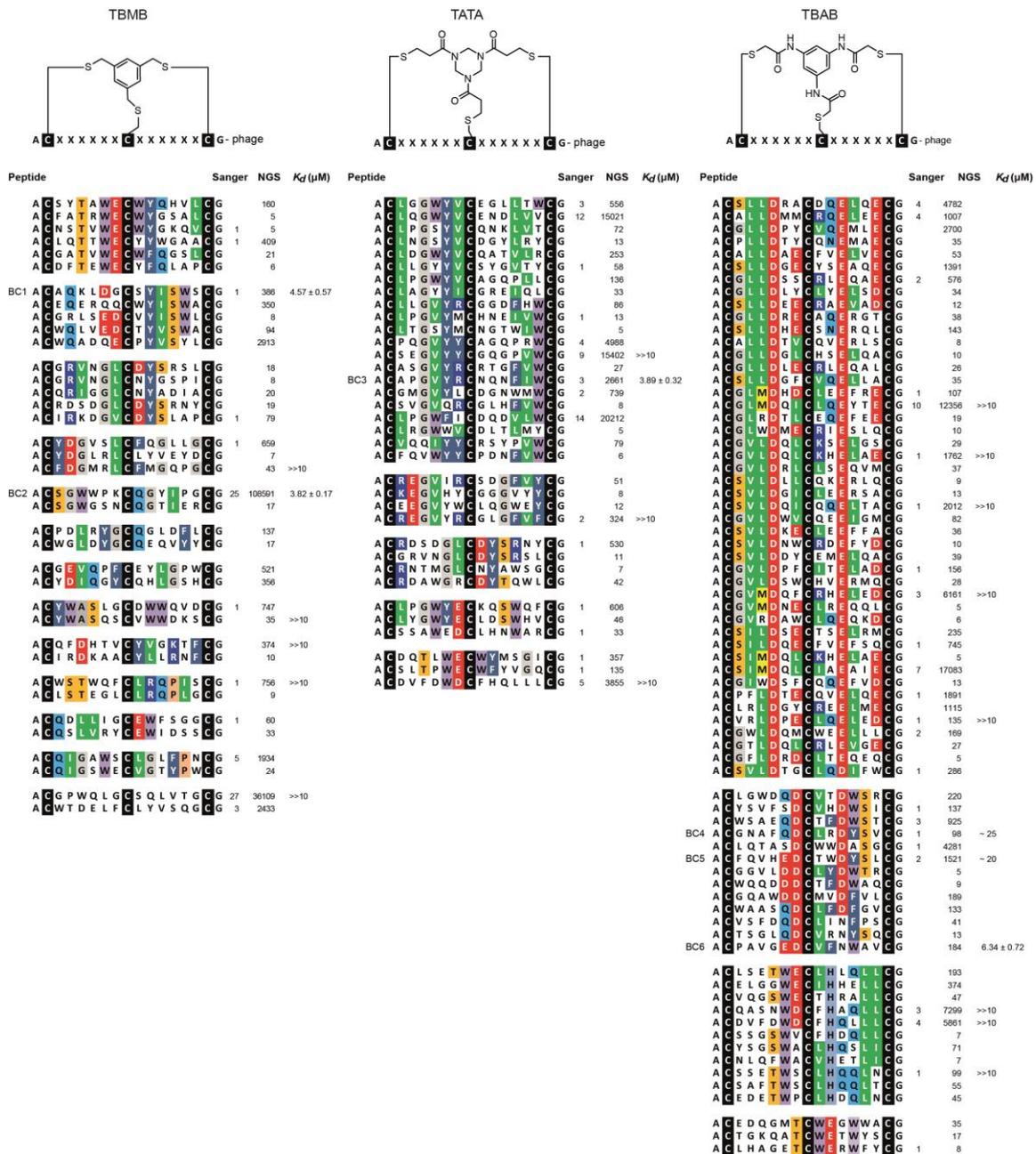


Figure 1. Sequences of phage-selected bicyclic peptides. Peptides of the 6x6 phage display library were cyclized with TBMB, TATA and TBAB to obtain the indicated bicyclic peptide format. 'X' indicates any of the 20 natural amino acids. Peptides sharing similar sequences are aligned in groups and amino acid similarities highlighted in color. The abundance of the peptide identified by Sanger sequencing and Ion Torrent next generation sequencing (NGS) is indicated. K_d s were determined for peptide labeled with fluorescein at the N-terminus. Standard deviations are indicated.

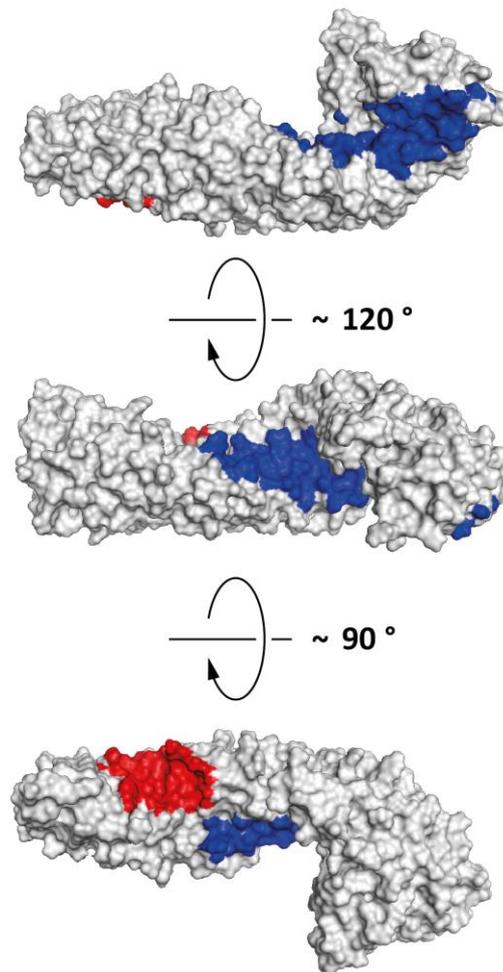


Figure 2. Surface of β -catenin structure. The binding interfaces between β -catenin and the axin α -helix (red), and between β -catenin and ICAT (blue) are indicated.

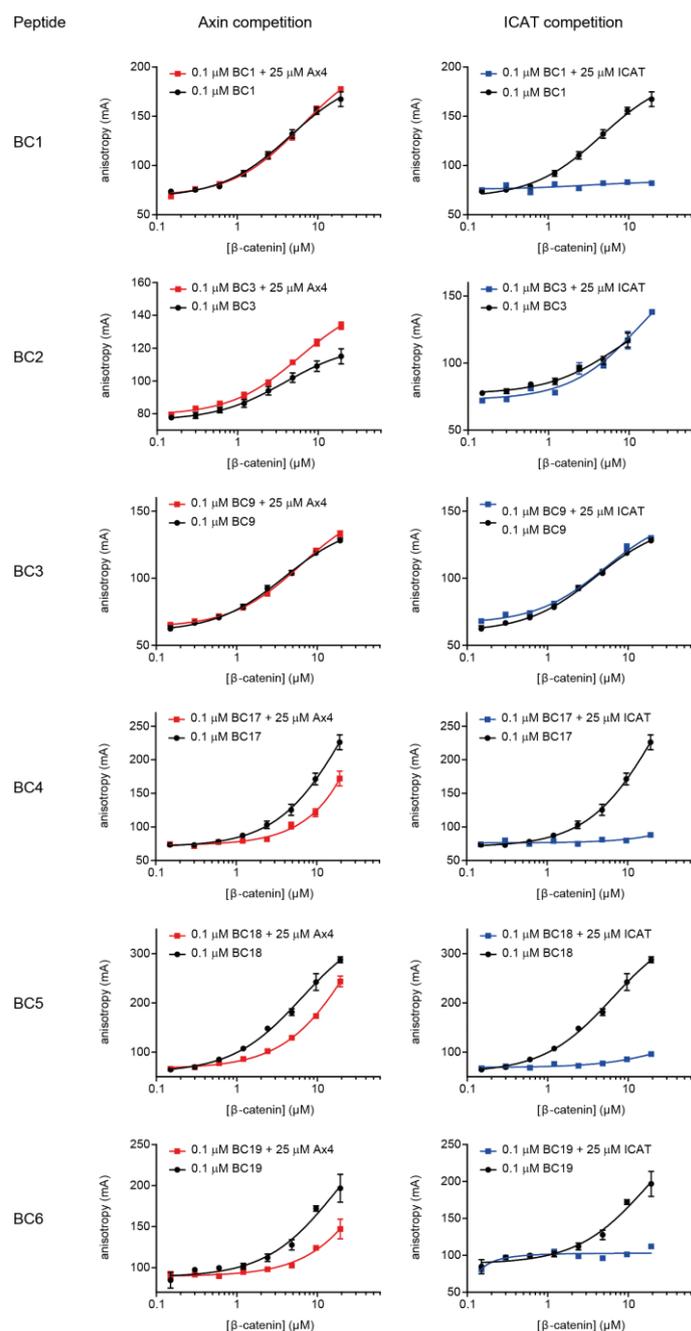
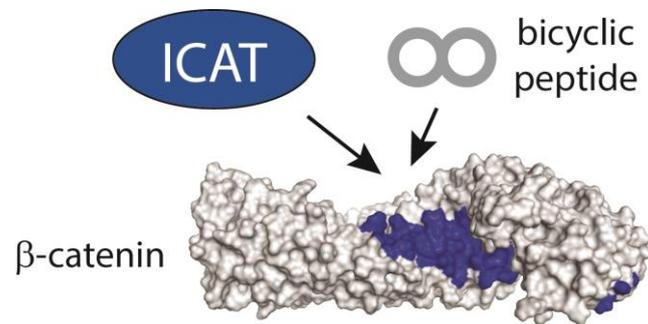


Figure 3. Competition of bicyclic peptides and natural protein ligands for binding to β -catenin. Bicyclic peptides labeled with fluorescein ($0.1 \mu\text{M}$) were incubated with increasing concentration of β -catenin and the extent of binding quantified by fluorescence polarization (black curves). The binding assay was repeated in presence of axin α -helix ($25 \mu\text{M}$, red curves) or ICAT ($25 \mu\text{M}$, blue curves). Average values and standard deviations of three measurements are shown.

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Bicyclic peptides binding to different surface regions of β -catenin were developed by phage display. Several of the ligands compete with binding of ICAT and thus bound to the prime target site on β -catenin for therapeutic intervention.