Chapter V

Inhibition of Beta-catenin mediated Wnt-signaling using synthetic peptides
Introduction

Beta-catenin is a multifunctional protein and one of the most important members in Wnt signaling pathway. The canonical Wnt signaling pathway plays a critical role in embryonic development, stem cell growth, adult tissue homeostasis and tumorigenesis (Moon \textit{et al.}, 1998; Wodarz \textit{et al.}, 1998; Peifer \textit{et al.}, 2000; Clevers, 2006; Kinzler \textit{et al.}, 1996; Morin \textit{et al.}, 1999; Bienz \textit{et al.}, 2000; Polakis, 2000). Among other things, regulation of canonical Wnt pathway depends on the post translational regulation of the key mediator, beta catenin (Huang \textit{et al.}, 2008). In absence of Wnt signaling, cytoplasmic and nuclear beta-catenin levels are normally maintained at low levels by ubiquitination and subsequent degradation. Degradation of beta-catenin takes place by phosphorylation of its serine residues by glycogen synthase kinase 3 (GSK3). Activation of Wnt signaling leads to the inactivation of destruction complex through unknown mechanism, allowing beta-catenin levels to increase in cytoplasm. The accumulation of beta-catenin in cytoplasm results in its translocation to nucleus, which then leads to the association of beta-catenin with Tcf and BCL9 in nucleus, results the transactivation of downstream Wnt targeted genes. Absence of beta-catenin keeps them repressed (Barker \textit{et al.}, 2006; Hecht \textit{et al.}, 2000). The beta-catenin/Tcf complex acts as the transcriptional co-activator to the promoter (Graham \textit{et al.}, 2002). Deregulation of the canonical Wnt signaling pathway is associated with several cancers (Bienz \textit{et al.}, 2000). Thus, it is important to develop an inhibitor which is able to counteract the deregulation of beta-catenin. Beta-catenin also interacts with a number of transcription regulatory proteins, including BCL9. Beta-catenin/Tcf interaction or Beta-catenin/BCL9 interaction may be important drug
targets for cancer treatment (Hecht et al., 2000; Kramps et al., 2002; Hoffmans et al., 2004).

In this study, we attempt to inhibit the deregulation of beta-catenin in two ways. One way is by blocking the interaction of beta-catenin with Tcf and another is by blocking the interaction of beta-catenin with BCL9 as BCL9 also acts as an important component of the transcription-activation complex of Wnt signaling in the nucleus for transcription of downstream genes. The protein ICAT is an inhibitor of the Wnt signaling pathway that prevents the binding of Tcf to beta-catenin. For blocking of Beta-catenin/BCL9 interaction and Beta-catenin/Tcf4 interaction, mimic peptides of Bcl9 and ICAT proteins were synthesized, respectively. The crystal structure of beta-catenin/ICAT complex reveals the inhibitory mechanism of ICAT (Graham et al., 2002). From the crystal structure it is clear that the helices of armadillo repeats 5-12 of beta-catenin form a platform upon which ICAT binds to beta-catenin in an anti-parallel fashion. ICAT binds to beta-catenin with two discrete modules, an N terminus 3-helix bundle and a C-terminal extended tail (figure 5.1). The 3-helix bundle domain binds to beta-catenin armadillo repeats 10-12 and the tail binds at a region formed by 5-9 repeats of beta-catenin. The 3-helix bundle provides the critical domain for anchoring ICAT to beta-catenin, whereas the tail is necessary for excluding Tcf from beta-catenin. Tcf4 binds to beta-catenin in similar fashion (Sampietro et al., 2006). Tcf4 uses its helical part for anchoring to beta-catenin and then the extended part becomes ready to bind to beta-catenin surface exactly where ICAT-tail binds. Thus, conceptually it is possible to design cancer therapeutics that inhibits beta-catenin-mediated transcriptional activation.
Figure 5.1: Overall Structure of ICAT bound to the armadillo repeat region of Beta-Catenin (Graham et al., 2002).

Figure 5.2: Overall structure of the beta-catenin/Tcf-4/BCL9 complex. Beta-catenin is represented in green; Tcf-4 in magenta; and BCL9 in red (Sampietro et al., 2006).
Materials and Methods

Materials

Table 5.1: Bacterial Strain used

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>XL1 Blue</td>
<td>endA1 gyrA96 (nalR) thi-1 recA1 relA1 lac glnV44 F’ [:Tn10 proAB+ lacIq Δ (lacZ) M15] hsdR17 (rK mK+)</td>
<td>For general cloning and maintenance of different Plasmids.</td>
</tr>
<tr>
<td>BL21 (DE3)</td>
<td>F– ompT gal dcmlonhsdSB(rB– mB–) λ(DE3 [lacI lacUV5-T7 gene 1 ind1sam7 nin5])</td>
<td>For expression of foreign Genes.</td>
</tr>
</tbody>
</table>

Plasmids used

Following are the vector map of the plasmids used for the cloning of the gene responsible for beta-catenin.
Figure 5.3a: Map of pUC19 plasmid

Figure 5.3b: Multiple cloning site of pUC19 plasmid
Figure 5.4a: Map of pET-28a plasmid

Figure 5.4b: Multiple cloning site of pET-28a plasmid
**Column Materials and Chemicals Used**

Ni-NTA sepharose was purchased from GE Healthcare Bio-sciences AB and Sephadex G-25 was from Amersham Biosciences, GE Healthcare. Kanamycin, DTT, IPTG, and EDTA were purchased from Sigma Chemical Company (St. Louis, MO, USA). Luria Broth, agar powder was purchased from Hi-Media (India). Triton-X 100 was purchased from MP Biomedicals. Anhydrous glycerol, Imidazole, Tris-HCl, NaCl were purchased from J.T. Baker. 4X crystallized acrylamide, DMF, DIPEA, DIPC, HPLC-water, acetonitrile, TFA were purchased from Spectrochem. 5(6)-carboxyfluorescein was purchased from Molecular Probes, Invitrogen. All amino acids, TBTU, HOBt and HATU were purchased from Novabiochem.

**Methods**

**Expression and Purification of Beta-catenin**

For purification of protein, a synthetic gene responsible for beta-catenin (138-686) cloned in pUC19 vector was purchased. Then it was subcloned in the pET28a vector in BamH1/Sal1 cloning site. The pET28a plasmids encoding the protein was transformed in BL21(DE3) cells and the cells were grown in Luria broth containing 50 µg/ml of Kanamycin at 37°C until the O.D₆₀₀ reached 0.5. At O.D₆₀₀ =0.5, the cells were induced with 0.5 mM IPTG at 37°C and shaking was continued for 3 h at that temperature. Then cells were harvested by centrifugation at 6000 rpm, 4°C, and 10 min. The harvested cells were resuspended in lysis buffer (50 mM Tris-HCl buffer, pH 8.0, containing 300 mM NaCl, 2 mM DTT, 10% glycerol, 1% TritonX-100 and 20 mM imidazole) and lysed by sonication. The cell debris was removed by centrifugation at 14000 rpm for 40 min. at 4°C.
Affinity chromatographic purification of the protein was done using Ni-NTA sepharose column equilibrated with the lysis buffer. The column was washed subsequently with a buffer having the same composition as the lysis buffer without TritonX-100 and for increase in imidazole concentration to 50 mM. The protein was eluted from the column with buffer having the same composition as that of the wash buffer but having a concentration of 300 mM imidazole. The purified protein was dialyzed against 50 mM Tris-HCl buffer, pH 8.0 containing 300 mM NaCl, 2 mM DTT, and 10 % glycerol and then stored at -80°C.

Peptide synthesis

All the peptides were synthesized on a protein technologies PS3 peptide synthesizer using solid phase Fmoc chemistry. The synthesis was initiated on Rink Amide MBHA resin and the protocol included a capping step with 5% acetic anhydride and 5% 2, 6 dimethylpyridine in DMF after each amino acid coupling. Fmoc-amino acids were activated with TBTU in the presence of HATU and/or HOBt and DIPEA. Peptides were cleaved from the resin, and side-chain protecting groups were removed by incubating with 81% TFA, 5% phenol, 5% thioanisole, 3% water, 2.5% EDT, 2% dimethyl sulfide and 1.5% (by weight) ammonium iodide at 25°C for 3 hrs followed by precipitation in cold diethyl ether. The precipitate was collected by centrifugation, dried, dissolved in 0.1% TFA water and purified by RP-HPLC.
Table 5.2: Peptide derived from ICAT protein and BCL9 protein

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAT-helix</td>
<td>YIQKBRVLBMLRBMSNLTASQQ</td>
</tr>
<tr>
<td>ICAT-helix M25C</td>
<td>YIQKBRVLBCLRBMSNLTASQQ</td>
</tr>
<tr>
<td>ICAT-tail M69C</td>
<td>DVVCASRSESTD</td>
</tr>
<tr>
<td>BCL9+6DR+NLS</td>
<td>PKKKRKV-RRRRRR-</td>
</tr>
<tr>
<td></td>
<td>HRERSLBTLLBIQMLF</td>
</tr>
</tbody>
</table>

Chemical modification of peptides

A small portion of the peptidyl resin was N terminally labeled with 5(6)-carboxyfluorescein reacting 10 times excess dye with HOBr and DIPC at 25°C for 3 hours. Then resin was first washed with 20% piperidine and then with DMF to remove the excess unreacted dye. The labeled peptides were cleaved from resin and purified as described above.

HPLC

The peptides were purified by HPLC on a reverse phase μBondapak C-18 column using 2-80% acetonitrile/water with 0.1% TFA and characterized by MALDI-Tof Mass Spectrometry. The following program or some minor variation of it was used for all the HPLC gradient runs.
Table 5.3: The following table shows the HPLC gradient used for the purification of the peptides

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Water (0.1% TFA) (ml)</th>
<th>Acetonitrile (0.1% TFA) (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0.9</td>
<td>0.1</td>
</tr>
<tr>
<td>10</td>
<td>0.7</td>
<td>0.3</td>
</tr>
<tr>
<td>40</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>45</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>50</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

The peaks were monitored at 215 nm and 230 nm. In case of fluorescein labeled peptides peaks were monitored at 215 nm and 480 nm. In all the cases only major peaks were collected and characterized through mass spectrometry.

**Mass Spectrometry**

Determination of mass was done using MALDI-TOF instrument.

**Fluorometric titrations of ICAT peptides with Beta-catenin**

Binding of ICAT peptides were determined by direct titration of fluorescein-labeled peptides with unlabeled beta-catenin. All the anisotropy measurements were carried out in 50 mM Tris-HCl buffer, pH 8 containing 300 mM NaCl, 2 mM DTT and 10% glycerol at 25°C. All the binding assays were carried out in a Quantamaster 6 (PTI) T-geometry fluorometer fitted with a circulating water bath to maintain the temperature at 25°C. Excitation and emission wavelengths used for each titration were 490 nm and 525 nm, respectively, with a band pass of 5 nm in each channel. The anisotropic data were fitted into a single-site binding
equation, and $K_d$ values for each pair were obtained by fitting them in KyPlot (Koichi Yoshioka, 1997-1999, version 2.0, beta 4) in the following equation.

$$
\frac{(A_\infty - A_0) \cdot ([K_d + [\text{beta-cat}]+[\text{Peptide}]) \cdot \sqrt{([K_d + [\text{beta-cat}]+[\text{Peptide}])^2 - 4([\text{beta-cat}][\text{Peptide}])})}{2.[\text{Peptide}]}
$$

$A_{\text{obs}} = A_0 + \frac{(A_\infty - A_0) \cdot ([K_d + [\text{beta-cat}]+[\text{Peptide}]) \cdot \sqrt{([K_d + [\text{beta-cat}]+[\text{Peptide}])^2 - 4([\text{beta-cat}][\text{Peptide}])})}{2.[\text{Peptide}]}
$

Where, $A_{\text{obs}}$ is the observed anisotropy, $A_0$ is the initial anisotropy, $A_\infty$ is the final limiting anisotropy, [peptide] is the peptide concentration, [beta-cat] is the total protein concentration, and $K_d$ is the dissociation constant. All concentrations were expressed in molar units.

**Assessment of cell morphology upon treatment of BCL9+6DR+NLS peptide**

HCT116 and SK-Mel-5 cells were grown in 96-well (0.5x10^4/well) plates in DMEM media supplemented with 10% FBS for 24hrs in a 37°C humidified 5% CO2 incubator. The plated cells were treated with and without (BCL9+6DR+NLS) peptide at different concentration. Each concentration was added as triplicate. The cells were observed with an inverted phase contrast microscope after 1 hr, 4hr and 24 hr (Model: OLYMPUS 1X71 Optical Co. Ltd., Shibuya-ku, Tokyo, Japan) and photographed with the help of a Progres CF cool camera (Jenoptik. Germany).

**MTT assay**

MTT cytotoxicity assays were performed on SK-Mel-5 *in vitro*. Briefly 1x10^4 cells/well were plated in 96-well TC plates. Cells were treated with different doses of the peptides for 24 hr at 37°C in a 5% CO2 incubator. MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) solution was added and
the insoluble derivative formed by cellular dehydrogenase enzymes were solubilized with acidic isopropanol and absorbance was measured at 595 nm by ELISA reader (Lab system Multiscan MS).
Results

Design of peptides

As beta-catenin is one of the most important members in the canonical Wnt signaling pathway and also responsible for transcription through binding with the transcription factor Tcf4 and transcription co-activator BCL9, our target was to disrupt the interaction of beta-catenin with Tcf4 and/or BCL9 using synthetic peptides.

Firstly, we targeted for disruption of beta-catenin-Tcf4 binding by an ICAT mimic peptide. ICAT a protein and an established inhibitor of Tcf4 binding to beta-catenin (Danette et al., 2002; Tago et al., 2000; Tutter et al., 2001). It contains a three-helix bundle region which is responsible for anchoring to beta-catenin and another extended tail which is responsible for exclusion of Tcf4 from beta-catenin.

We have synthesized a mimic peptide of ICAT-helix region guided by the amino acid sequence and the crystal structure (Graham et al., 2002). As ICAT 3-helix region is primarily required for anchoring, we initially targeted this region. We have selected ICAT 3-helix region having residues 15-38 for our peptide synthesis. The sequence of wild type ICAT peptide having residues 15-38 is YIQKVRVLLMLRKMGSNLTSQQ. As this region of the protein is originally helical, a preformed helix may help the binding by overcoming the entropy loss of a disordered peptide. In order to induce alpha-helicity of this peptide even in the unbound state, we have substituted few residues (Q17, V20, L24 and K28) by 2-amino isobutyric acid (Aib) (Banerjee et al., 2002). The sequence of Aib (B) substituted peptide called ICAT-helix was YIBQKBRVLBMLRBGSNLTASQQ. Our hypothesis was that this ICAT-helix peptide would bind to the same surface, where Tcf binds to beta-catenin and
will inhibit the beta-catenin /Tcf binding. So, the above peptide ICAT-helix may inhibit the beta-catenin mediated transcription in Wnt signaling pathway and would act as an inhibitor of cell proliferation. Another peptide has also been synthesized by modification of ICAT-helix for extensive work. For that purpose, the metheonine residue at 25th position was substituted by a cysteine. The sequence of that peptide called ICAT-helixM25C was \textbf{YIBQKBRVLBCLRBMGSNLTAQQ}. For fluorometric titration, peptides were chemically modified by labeling with 5(6)-carboxyfluorescein (cf) at N-terminal of each peptide.

Secondly, we targeted for disruption of beta-catenin-BCL9 binding. BCL9 is a significant member in Wnt signaling pathway and beta-catenin binding site of BCL9 is distinct from that of most other beta-catenin binding partners. Thus, beta-catenin/BCL9 interaction may be another drug target for the inhibition of Wnt pathway target genes in cancer. From crystal structure it is clear that the conserved HD2 of BCL9 (residue 349-377) is responsible for the binding to the beta-catenin (Sampietro et al., 2006; Kramps et al., 2002) (figure 5.6). This domain forms \( \alpha \)-helix in BCL9 protein. This region of BCL9 interacts with helices 1, 2 and 3 of first armadillo repeats of beta-catenin. We have selected residues 358-374 of BCL9 having the sequence HRERSLQTLRDIQRMLF because this domain is helical in nature and exposed to the surface of beta-catenin. So in order to induce alpha-helicity of this peptide, we have substituted a few residues (Q364, R367, D368 and R371), which are at the opposite face of the beta-catenin interacting site, of BCL9 by 2-amino isobutyric acid (Aib). The Aib substituted peptide sequence is HRERSLBTLBBIQBMLF (B=Aib). For cell penetration, we have added hexa D-arginine tag at the N-terminal of the above sequence (Dhar et al.,...
Further, for localization of the peptide in the nucleus, a nuclear localization sequence (NLS) PKKKRKV (Branden et al., 1999) was coupled at the N-terminal of the above sequence. The fused peptide sequence named as (BCL9+6DR+NLS) was **PKKKRKV-RRRRRR-HRERSL** **BTLBBIQBMLF**. This peptide was used for our work.

**Figure 5.5**: The ICAT helical domain bond to armadillo repeats 10-12 of beta-catenin

**Figure 5.6**: Binding domain of BCL9 with beta-catenin
Table 5.4: sequence and Mass of different peptides

<table>
<thead>
<tr>
<th>Name of peptides</th>
<th>Sequence</th>
<th>Experimental Mass (Da)</th>
<th>Theoretical Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAT-helix+cf</td>
<td>CI-YBQKBRVLBMLRBMGSNLTASQQ</td>
<td>3038</td>
<td>3036</td>
</tr>
<tr>
<td>ICAT-helixM2S+cf</td>
<td>cf-YBQKBRLBCLBMRGSLTASQQ</td>
<td>3075</td>
<td>3079</td>
</tr>
<tr>
<td>BCL9+6DR+ NLS</td>
<td>PKKKRKKV-RRRRR-HRERSLTLBBIQBMLF</td>
<td>3786</td>
<td>3786</td>
</tr>
</tbody>
</table>

The peptides were synthesized using solid-phase peptide synthesis mechanism, purified using reverse phase HPLC and characterized by MALDI-TOF mass spectroscopy. Following figures depict the mass spectra of the peptides.

Figure 5.7: MALDI-TOF Mass spectra of ICAT-helix+cf
Figure 5.8: MALDI-TOF Mass spectra of ICAT-helix M25C+cf

Figure 5.9: MALDI-TOF mass spectra of BCL9+6DR+NLS
Purification of Beta-catenin

Full length beta-catenin protein has total 12 armadillo repeats. Residues 138-686 forms the armadillo repeat domain of beta-catenin. C-terminal part contains a helical region and extended tail region. Armadillo repeat domain containing residues 138-686 was taken into account for our purpose. The plasmid pET28a containing the gene responsible for beta-catenin (138-686) was expressed in BL21 (DE3) cells in presence of Kanamycin and induced using IPTG. The protein was eluted by Ni-NTA sepharose column and characterized by SDS-PAGE gel. The protein was greater than 95% pure. The detail protocol was described in material and methods section. The following figure shows the gel picture of beta-catenin elution.

Figure 5.10: 10% SDS-PAGE gel showing greater than 95% pure beta-catenin. LMW denotes low molecular weight marker. Lane 2: Pellet, Lane 3: Bead, Lane 4: Load flow through, Lane 5: Wash flow through, E1-E9: elution fractions containing pure beta-catenin protein.
Binding of ICAT peptides with beta-catenin

To examine whether the synthetic peptides were capable of inhibiting the binding of cellular Tcf4 with Beta-catenin, first the peptides were studied for their ability to bind beta-catenin, *in vitro*. For this purpose, fluorescence anisotropy titrations were done. Fluorescein labeled peptides were titrated with increasing concentrations of the unlabeled protein. We got the dissociation constants (Kₐ) of the complexes at nanomolar range. Figure 5.11 shows the binding isotherms of the titrations.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Dissociation constant (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAT-Helix+cf/ Beta-Catenin</td>
<td>((188\pm38)\times10^{-9})M</td>
</tr>
<tr>
<td>ICAT-Helix M25C+cf/ Beta-Catenin</td>
<td>((96.5\pm24)\times10^{-9})M</td>
</tr>
</tbody>
</table>
Figure 5.11: Binding isotherm of ICAT-helix and ICAT-helixM25C with beta-catenin respectively. 5 nM 5(6)-carboxyfluorescein labeled peptide was incubated with increasing concentrations of beta-catenin and anisotropy was measured at each point. The excitation and emission wavelengths used for titration were 490 nm and 525 nm respectively, with a band pass of 5 nm in each channel. The titrations were carried out in 50mM Tris-HCl buffer pH 8 containing 300mM NaCl, 2mM DTT and 10% glycerol at 25°C. The plots are the average of three titrations.
Biological assay using BCL9+6DR+NLS peptide

To study the inhibition effect in cellular model, we took the help of BCL9+6DR+NLS peptide. We primarily did the viability assay (Figure 5.12) to check the anti-proliferative effect of the peptide on SKMel-5 cell. Figure 5.12 shows the plot of cell viability as determined by MTT assay. Percentage (%) viability indicates the number of live cells remain with time and increasing concentration of peptide. After treatment of above peptide at different time and doses, morphological changes (Figure 5.13 - 5.16) have been monitored on SKMel-5 and HCT-116/- cell line. Both SKMel-5 and HCT-116/- cell-lines were Wnt active, thus those were used to carry on our experiments.

Figure 5.12: MTT assay of SK-MEL-5 cells after treatment with BCL9+6DR+NLS
Figure 5.13: Morphology of SK-MEL-5 cell after treatment with BCL9+6DR+NLS peptide after 4 h at different concentrations
Figure 5.14: Morphology of SK-MEL-5 cell after treatment with 20µM BCL9+6DR+NLS peptide at different time.
Figure 5.15: Morphology of HCT-116/- cell after treatment with BCL9+6DR+NLS peptide after 4 h at different concentrations
Figure 5.16: Morphology of HCT-116-/- cell after treatment with 20µM BCL9+6DR+NLS peptide at different time

Figure 5.13 - 5.16 clearly indicate the effect of BCL9+6DR+NLS peptide on both SKMel-5 and HCT-116-/- cell-lines by changing the morphology of the cell with different doses of the peptide.
Discussion

The canonical Wnt pathway plays critical roles in embryonic development, stem cell growth and tumorigenesis. As beta-catenin is the key player in the Wnt signaling pathway, inhibition of beta-catenin mediated transcriptional activation may be a good way to design cancer therapeutics. Here we have chosen two ways. One is through the inhibition of beta-catenin/Tcf interaction and another is by inhibition of beta-catenin/BCL9 interaction. For inhibition of beta-catenin/Tcf interaction we took ICAT-mimic peptides ICAT-helix and ICAT-helixM25C. They bind to beta-catenin having dissociation constant 188×10^{-9}M and 95×10^{-9}M respectively i.e. in nanomolar range. The binding affinity of synthetic peptides to beta-catenin is strong. BCL9 also acts as important components in Wnt signaling, so, the interacting domain of BCL9 which binds to beta-catenin has been targeted for drug designing. We have investigated the cytotoxicity effect of BCL9+6DR+NLS peptide on the SK-Mel-5 (S100B and p53 status is wild-type) cell lines. The IC_{50} value appeared to be around 12 µM. Results (5.13-5.16) show the morphological changes of the different cell lines at different concentrations and at different time. The peptidomimetics designed and developed against beta-catenin here demonstrated specificity as it initiates cell death in SK-Mel-5 and HCT116/- cells within 1h. Thus, the peptidomimetics which are designed from ICAT and BCL9 will be good starting point for developing drugs against cancer.
References


