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TITLE: Beta Catenin in Prostate Cancer Apoptosis

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During the past funding period, we have focused on mapping the cleaved $\beta$-catenin protein following TRAIL-TZD treatment and have determined that this cleaved fragment loss interaction with TCF4, while retaining strong interaction with E-cadherin and $\alpha$-catenin. These indicated (i) a potential mechanism by which TRAIL-TZD antagonizes $\beta$-catenin/TCF4-induced transcription and (ii) suggested that the interaction with E-cadherin and $\alpha$-catenin might be critical for apoptosis induction. To understand these we have recently created six myc-$\beta$-catenin (D/A) mutants (and more are underway) to conclusively map the cleavage site on $\beta$-catenin and to understand its role on apoptosis induction. Our recent results with $\beta$-catenin knockdown and overexpression in AR-positive LNCaP cells suggested that $\beta$-catenin might be promoting a pro-survival axis in these as opposed to AR-negative cells. Studies with two different GSK3$\beta$ inhibitors (AR-A014418 and BIO) showed that GSK3$\beta$ inhibition promotes TRAIL-induced apoptosis in AR-negative DU145 cells and TRAIL-TZD significantly reduces expression of GSK3 $\beta$, GSK3$\alpha$ and Cyclin dependent kinases (CDKs). In addition, this apoptosis pathway seems to involve AMPK, since AMPK-dominant negative (DN) mutant overexpression significantly attenuates TRAIL-TZD-induced apoptosis. The C42 and C42B cells showed significant apoptosis with TRAIL-TZD in vitro and will be utilized in the in vivo xenograft studies to provide critical information regarding the efficacy of TRAIL-TZD and TRAIL-GSK3$\beta$ inhibitor combination in regulating prostate tumor growth.

TRAIL, Troglitazone, beta-catenin, GSK3beta, apoptosis
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Introduction:
Over the last year, we have worked towards completing all the 3 specific aims as was listed in the approved Statement of Work. However, there were some setbacks in the overall studies, since two personnel have left the program last funding period. Since then new personnel has been hired and after an initial delay (covering the training period) we are currently making significant progress and working towards completing the studies within the next year.

Background:
Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer death in males (1), (2). Surgical resection and hormonal therapy (with anti-androgens) are the two major forms of treatment currently available, which is not effective at a late stage (hormone independent forms) of the disease. Designing efficient therapeutic agents that can target both hormone dependent and independent forms of the cancer are thus critically important. Since most of the anticancer therapies limit tumor growth via inducing apoptosis, identification of a novel target for drug induced apoptosis will be helpful for treating resistant forms. One such target is β-catenin, a downstream mediator of Wnt pathway and known to be closely linked with tumorigenesis (3). Overexpression of β-catenin can induce early events of prostate tumorigenesis (4), (5), and contribute to prostate cancer cell growth (6). Activating mutations of β-catenin have also been reported in approximately 5% of human prostate cancers (7), (8). β-catenin can also augment transcriptional function of androgen receptor (AR) (9), (10) (11). Since β-catenin can promote survival via increasing expression of survival related genes (12), (13), decreasing β-catenin expression might be a critical event in activating the apoptotic pathway.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor super-family of death-inducing ligands, has gained increased attention due to its unique capability of inducing apoptotic cell death specifically in cancer cells, without any significant toxicity towards normal cells (14). TRAIL receptors are expressed in both androgen dependent and independent prostate cancer cells, although, some of these cells develop TRAIL resistance (15). Studies by others have revealed that combinatorial treatment with TRAIL and ligands of PPARγ (Peroxisome Proliferator Activated Receptor gamma) can ameliorate TRAIL resistance and induce apoptosis in TRAIL resistant breast cancer cells (16). Despite this information, the detailed mechanism how this drug combination promotes TRAIL sensitivity is still unknown. Identification of downstream molecules regulating this apoptotic pathway is critically required not only to overcome TRAIL resistance, but also to understand the detailed mechanism involved, which can be utilized towards future drug designing. Since TRAIL receptors are expressed in all prostate cancer cells, this therapy might be effective in targeting prostate cancers irrespective of their androgen status.

In our preliminary data, that supported funding of this grant, co-treatment of TRAIL resistant prostate (LNCaP) and liver (Huh-7) cancer cells with a combination of TRAIL and PPARγ ligand Troglitazone (TZD) reduced TRAIL resistance and significantly increased their apoptotic potential. Interestingly, this apoptosis was also associated with a dramatic reduction in the expression of β-catenin protein and a cleavage of β-catenin preceding combinatorial drug-induced apoptosis. Regulation of β-catenin seemed to be independent of the conventional GSK3β-mediated pathway and involved caspase activation. Based on these, in the current application we proposed to study in detail the role of β-catenin and GSK3β in drug-induced
apoptosis of prostate cancer cells (\textit{in vitro}) and prostate cancer xenografts (\textit{in vivo}). The specific aims included: 1) To determine the role of $\beta$-catenin in drug-induced apoptosis of prostate cancer cell lines, 2) To determine the role of GSK3$\beta$ in potentiating drug-induced $\beta$-catenin cleavage and apoptosis and 3) Whether $\beta$-catenin mediates drug-induced apoptosis in prostate xenografts \textit{in vivo}.

\textbf{Body:}

The research accomplishments for the last year along with each task included in the approved Statement of Work are outlined below:

\textbf{Task 1: To determine the role of $\beta$-catenin in drug-induced apoptosis of prostate cancer cell lines (1-36 months)}

\textbf{Subaims 1(i)-1(ii):}

In the previous annual reports (covering the periods from April, 2010 till March 2012), we have provided data on the apoptotic potential of TRAIL-TZD combination in various prostate cancer cell lines including androgen sensitive (LNCaP and 22RV1) and androgen insensitive cells (DU145 and PC3). These also included dose and time course studies with TRAIL and TZD to identify the concentration of either drug and time point for inducing maximal apoptosis. These showed that both androgen sensitive and insensitive cells respond to this apoptotic combination, although it is more potent in the androgen sensitive cells. These studies also revealed a correlation of apoptosis with reduced $\beta$-catenin expression and increased $\beta$-catenin cleavage. Cleavage of $\beta$-catenin was parallel to the degree of apoptosis and was mediated via activation of caspases 3 and 8. Upon induction of apoptosis, this cleaved $\beta$-catenin was enriched in the soluble compartment of the cells, which is in contrast to its localization in the membrane or insoluble compartment of the cells. Additional studies indicated that combination of TRAIL with HDAC inhibitor Valproic acid induced strong apoptotic response associated with $\beta$-catenin cleavage.

The following results were obtained in the past year (covering the periods from April, 2012 till March 2013):

\textit{Characterization of the changes in interaction of cleaved $\beta$-catenin with its binding partners following TRAIL-TZD treatment:}

In order to map the cleaved $\beta$-catenin protein, it is critical to determine its interaction with the normal binding partners change. To understand this, we focused on 2 different binding partners of $\beta$-catenin: E-Cadherin and $\alpha$-Catenin, since in the earlier report we detected that cleaved $\beta$-catenin didn’t show any interaction with TCF4. Immunoprecipitation and Immunoblotting combination studies were designed following treatment of LNCaP cells with TRAIL and TZD. These studies showed a distinct interaction of both full length and cleaved $\beta$-catenin with both E-cadherin and $\alpha$-catenin (Fig 1). In addition these results also showed a dramatic reduction in the expression of E-cadherin as well as $\alpha$-catenin following treatment with TRAIL-TZD. These interesting results indicate that: (i) intact $\beta$-catenin-E-cadherin and $\beta$-catenin-$\alpha$-catenin interaction might be needed for optimal $\beta$-catenin cleavage and apoptosis and (ii) TRAIL-TZD treatment might be completely antagonizing $\beta$-catenin/TCF-mediated transcriptional activity via abolishing $\beta$-catenin’s interaction with TCF4 (shown earlier).
Subaim 1(iii):
Based on our earlier antibody mapping data and in order to map the caspase cleavage site on β-catenin protein, we initially focused on myc-tagged-β-catenin-FL (1-781aa) and the deleted, myc-β-catenin-ΔN (131-781aa) deletion mutant to create cleavage-resistant (D/A) mutants. These included D583A, D624A, D583A/D624A, D751A, D764A, D751A/D764A and were created with both myc-β-catenin–FL (1-781) and myc-β-catenin-ΔN (131-781aa). Even though we initially hypothesized that the deleted β-catenin-ΔN (131-781aa) construct will be better in identifying the cleavage site, we were unable to detect any cleaved fragment with β-catenin antibody when transfected in the LNCaP cells. We thus decided to use β-catenin-FL (1-781aa) and its D/A mutants for the future mapping studies.

Fig 1: Effect of TRAIL-TZD on the interaction of β-catenin with E-cadherin and α-catenin: LNCaP prostate cancer cells were treated with DMSO or a combination of TRAIL (100ng/ml) and TZD (50µM) for 16hrs. Equal amounts of lysates were then Immunoprecipitated with either E-cadherin antibody or α-catenin antibody (top panels). Each of these Immunoprecipitates was Immunoblotted separately with an antibody against β-catenin to detect Full length (FL) or cleaved (Cl) β-catenin. The bottom panels represent Western Blots of the lysates with the corresponding antibodies.

<table>
<thead>
<tr>
<th>IP</th>
<th>α-catenin</th>
<th>E-cadherin</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>TRAIL+TZD</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Full length → IB: β-catenin
Cleaved →

α-catenin → IB
E-cadherin →

β-catenin
FL → CI → IB
GAPDH →

Fig 2: Effect of overexpression of β-catenin (WT) and D/A mutants in LNCaP cells: LNCaP cells were transiently transfected with β-catenin-WT, D583A or D624A mutant constructs and treated with TRAIL-TZD for 16hours. Equal amounts of protein from each sample were then analyzed by Western Blots.

<table>
<thead>
<tr>
<th>Cell Type (myc-β-catenin)</th>
<th>LNCaP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transfection</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>+</td>
</tr>
<tr>
<td>TRAIL+TZD</td>
<td>-</td>
</tr>
<tr>
<td>D583A</td>
<td>+</td>
</tr>
<tr>
<td>D624A</td>
<td>+</td>
</tr>
<tr>
<td>myc-β-catenin</td>
<td></td>
</tr>
<tr>
<td>β-catenin</td>
<td>FL</td>
</tr>
<tr>
<td>Cl</td>
<td></td>
</tr>
<tr>
<td>myc-(β-catenin)</td>
<td></td>
</tr>
<tr>
<td>PARP</td>
<td>FL</td>
</tr>
<tr>
<td>Cl</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
</tr>
</tbody>
</table>
**Effect of overexpression of β-catenin mutants on TRAIL-TZD-induced apoptosis and β-catenin cleavage:** In order to determine the effect of β-catenin D583A and D624A mutations on β-catenin cleavage and TRAIL-TZD-induced apoptosis, these mutants were transiently transfected in LNCaP cells followed by treatment. The results shown in Fig 2 indicated that these constructs expressed β-catenin protein at the correct sizes (shown by the myc blot). In addition, the D583A mutation seemed to partially inhibit both endogenous β-catenin and PARP cleavage. These suggested that D583A might an important site for β-catenin cleavage by TRAIL-TZD pathway, although additional sites might also be present. In an additional experiment, various D/A constructs created (total 6) were transfected separately in LNCaP cells followed by TRAIL-TZD treatment. This also showed that all the mutants expressed β-catenin protein at the correct sizes (Fig 3, myc blot). However, due to variability in transfection, we were unable to determine conclusively whether D583A or any of the other D/A mutants was resistant to TRAIL-TZD-induced apoptosis. To resolve this, we plan on transfecting the cells with an internal GFP control in the future.

<table>
<thead>
<tr>
<th>Transfection</th>
<th>EV</th>
<th>WT</th>
<th>D583A</th>
<th>D524A</th>
<th>D638A</th>
<th>D634A</th>
<th>D751A</th>
<th>D784A</th>
<th>D751A, D784A</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TRAIL + T</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Fig 3: Effect of overexpression of β-catenin (WT) and D/A mutants in LNCaP cells: LNCaP cells were transiently transfected with Empty vector (EV, lanes 1&2), β-catenin-WT (lanes 3&4), or various D/A mutant constructs (lanes 5-10) and treated with TRAIL-TZD for 16hours. Equal amounts of protein from each sample were then analyzed by Western Blots.

To conclusively identify these cleavage sites, we also analyzed the cleaved product to identify the specific cleavage site using Mass Spectrometry. The initial step of Mass Spectrometry is to obtain a Coomassie-stainable band following Immunoprecipitation of the specific the protein (cleaved β-catenin in this case). There were several obstacles in this initial Immunoprecipitation stage, since we were unable to identify any suitable commercially available β-catenin antibody that can successfully Immunoprecipitate the cleaved β-catenin fragment. As shown in Fig 1, since the cleaved fragment retains interaction with E-Cadherin, we also tried to Immunoprecipitate this with E-cadherin antibody initially, but were unable to enrich this to a Coomassie-stainable band that is a prerequisite for Mass Spec analysis (indicated in the previous funding period). During the current funding period and after several attempts with significant...
enrichment of the lysates containing the cleaved β-catenin fragment, we were successful in detecting the cleaved β-catenin fragment in E-Cadherin IP after Coomassie stain (Fig 4). The cleaved fragment detected at ~70KD (red box) was then sent out for Mass Spectrometric analysis using Bottom up detection system. This detected 3 major cleaved products corresponding to D32, D583 and D764 of beta-catenin. We have already created the D583A and D764A mutants earlier and are currently in the process of creating the D32A single mutant. Based on the results with D32A, we also plan on creating compound mutations with other D/A sites.

**Subaim 1(iv):**

*Role of β-catenin on TRAIL-TZD-induced apoptosis in LNCaP prostate cancer cells:*

*Effect of knockdown:* To understand the role of β-catenin in mediating apoptosis and survival in AR-positive prostate cancer cells more in depth and to see which caspases are affected, β-catenin

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**Fig 4: Detection of cleaved β-catenin fragment following Coomassie stain:**

LNCaP cells were treated with DMSO or TRAIL-TZD as in Fig 3, followed by Immunoprecipitation with an antibody against E-cadherin. The Immunoprecipitates were resolved on SDS-PAGE followed by Coomassie Blue staining. Lane 1 contains protein molecular weight marker. The cleaved β-catenin band at ~70 KD (red box) was sent out for Mass Spec analysis.

<table>
<thead>
<tr>
<th>IP</th>
<th>E-cadherin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Marker</td>
</tr>
<tr>
<td>97KD</td>
<td>β-catenin-FL</td>
</tr>
<tr>
<td>70KD</td>
<td>β-catenin-cleaved</td>
</tr>
<tr>
<td>66KD</td>
<td>IgG</td>
</tr>
</tbody>
</table>

**Fig 5: Effect of β-catenin knockdown on TRAIL-TZD-induced caspase cleavage:** LNCaP cells were transiently transfected with control or β-catenin siRNA for 72hrs followed by TRAIL-TZD treatment for 8hrs (A) or 16hrs (B). The samples were analyzed by Western Blots with the antibodies indicated.
siRNA studies were performed in LNCaP cells. These results showed a significant reduction in endogenous β-catenin expression with β-catenin-siRNA (Fig 5). TRAIL-TZD studies were performed for 8 hrs and 16hrs to see any time-dependent effects of β-catenin knockdown in these cells. These results showed that knockdown of endogenous β-catenin rather increased the cleavage of caspase 3 and 9 as well as PARP, suggesting a prosurvival role of this protein in AR-positive LNCaP cells. These effects were more prominent at 8hrs (A) than at 16hrs (B), suggesting a time dependent effect.

**Effect of overexpression:**
To conclusively establish a prosurvival function of β-catenin in the AR-positive prostate cancer cells, the LNCaP cells were transiently transfected with either empty vector (EV) or β-catenin (WT) vector followed by TRAIL-TZD treatment for 16hrs. Since this apoptosis pathway is dependent on cell density (earlier results), the cells in this experiment were plated at two different densities. The results (Fig 6) indicated that at both cell densities, overexpression of β-catenin antagonized TRAIL-TZD-induced PARP cleavage, indicating again that β-catenin mediates a pro-survival pathway in these cells. The effects of overexpression were modest likely due to the low transfection efficiency of LNCaP cells.

### Fig 6: Effect of β-catenin overexpression on TRAIL-TZD-induced PARP cleavage:
Subconfluent populations of LNCaP cells were transiently transfected with Empty Vector (EV) or myc-β-catenin (WT) followed by TRAIL-TZD treatment for 16 hrs. Western Blots were performed next with the indicated antibodies.

<table>
<thead>
<tr>
<th>Cell density</th>
<th>0.4 x 10^6</th>
<th>0.6 x 10^6</th>
</tr>
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<tbody>
<tr>
<td>Transfection</td>
<td>EV</td>
<td>myc-bcatenin</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>EV</td>
</tr>
<tr>
<td>DMSO</td>
<td>+ - + -</td>
<td>+ - + -</td>
</tr>
<tr>
<td>TRAIL + TZD</td>
<td>- + - +</td>
<td>- + - +</td>
</tr>
<tr>
<td>myc-(β-catenin)</td>
<td>FL</td>
<td>Cl</td>
</tr>
<tr>
<td>PARP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
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**Task 2: To determine the role of GSK3β in potentiating drug-induced β-catenin cleavage and apoptosis (months 1-24)**
In the previous annual reports (covering the periods from April, 2010 till March 2012), we have provided data on the effect of GSK3β inhibitors (subaim 2.i and 2.ii) and (part of subaim 2.iii) on the effect of modulation of GSK3β on TRAIL-TZD-induced apoptosis. In this current funding cycle, we performed studies with additional inhibitors to determine whether GSK3β inhibition also increases TRAIL sensitivity in Androgen Receptor (AR)-negative DU145 cells. We also determined the effect of TRAIL-TZD on several other signaling pathways to obtain a better understanding how it is mediated.

**Effect of GSK3β inhibition on TRAIL-TZD-induced apoptosis in DU145 cells:**
To determine whether GSK3β inhibition promotes apoptosis in AR-negative prostate cancer cells, we first determined the effect of GSK3β inhibitor AR-A014418 on TRAIL-TZD-induced apoptosis in DU145 cells. These results shown in Fig 7A indicated that AR-A014418 can sensitize the cells to TRAIL-induced apoptosis even in the absence of TZD (compare lanes 2, 4 and 6). This suggested that AR-A014418-induced GSK3β inhibition promotes apoptosis in various prostate cancer cells and is independent of the AR status. In additional experiments,
DU145 cells were pretreated with a different inhibitor of GSK3β, BIO followed by TRAIL-TZD treatment which also showed that BIO can sensitize towards TRAIL-induced apoptosis (Fig 7B).

**Fig 7:** Effect of GSK3β inhibition on TRAIL-TZD-induced apoptosis in DU145 cells: DU145 cells were treated with the indicated concentrations of TRAIL or TZD alone or in combination in the presence or absence of a pretreatment with 20µM AR-A014418 (A) or 2µM BIO (B). Cells were harvested after 16hrs of treatment in A and after 8hrs of treatment in B and analyzed by Western Blots.

**Effect of TRAIL-TZD on GSK3β and GSK3α pathways in DU145 cells:**
In the previous funding period, we have shown that TRAIL-TZD antagonizes the GSK3β pathway in AR positive LNCaP prostate cancer cells. In order to determine whether this is a generalized event or whether it is dependent on AR expression, we also determined the effect of this combination treatment on GSK pathway in AR negative DU145 cells. These results (Fig 8) showed

**Fig 8:** Effect of TRAIL-TZD on GSK3 α and β pathways in DU145 cells: DU145 cells were treated with DMSO or a combination of TRAIL (25ng/ml) and TZD (50µM) for the indicated amounts of time. The lysates were then analyzed by Western Blots with the antibodies indicated.
a time dependent increase in pGSK3βSer9 levels (lanes 1&2, 3&4) with TRAIL-TZD treatment (suggesting inhibition of the kinase), which corresponds with the induction of apoptosis (see caspase 3 cleavage). In addition, at a later time this combination treatment also showed a reduction of both total GSK3β and GSK3α expressions (see 16 hr and 24 hr treatments). This indicated that TRAIL-TZD can antagonize GSK3β pathway by two mechanisms, first via increasing the inhibitory phosphorylation at Ser9 and second via reducing the total expression. In addition, this antagonism is evident in various prostate cancer cells irrespective of the AR status.

**Effect of TRAIL-TZD on cyclin-dependent kinases (CDKs):**

In order to obtain an in-depth understanding on how TRAIL-TZD might sensitize resistant cancer cells towards apoptosis, it is important to determine how this combination targets various signaling pathways. A close correlation of CDKs in promoting apoptosis resistance in cancer cells have been shown in earlier studies (17), (18). To determine whether TRAIL-TZD combination also targeted CDKs, we determined any change in the expression of these CDKs following this combination treatment. As indicated in Fig 9, TRAIL-TZD dramatically reduced the expression of both CDK1 and CDK2 in both DU145 and LNCaP cells in a time dependent manner. These results are highly novel and indicated the possibility that this combination might be very effective in targeting cancers with high proliferative potential. It will be interesting to elucidate the detailed mechanism by which TRAIL-TZD targets CDKs and whether this regulation is at a transcriptional or post-translational level.

![Fig 9: Effect of TRAIL-TZD on Cyclin dependent kinases (CDKs): DU145 (A) and LNCaP (B) cells were treated with DMSO or TRAIL-TZD combination for the indicated periods of time. The samples were then analyzed by Western Blots with the indicated antibodies.](image-url)
**TRAIL-TZD-induced apoptosis involves AMPK:**

Earlier reports have shown that AMP-activated Kinase (AMPK) activation can ameliorate TRAIL resistance and sensitize cells to TRAIL-induced apoptosis (19). Since Troglitazone (TZD) used in our studies can also activate AMPK (20), we hypothesized that TRAIL-TZD-induced apoptosis might be mediated via AMPK pathway. To determine whether AMPK participates in this pathway, we utilized two different prostate cancer cell lines, one that expressed empty vector (C42-EV) and the other that expressed AMPK dominant negative mutant (C42-DN) as described previously (21). TRAIL-TZD studies designed with these two cell types showed a potent induction of apoptosis pathway following TRAIL-TZD addition in C42-EV cells (see cleaved caspases and cleaved PARP), which was antagonized significantly in the C42-DN cells (Fig 10). These results suggested a potential involvement of AMPK in mediating TRAIL-TZD-induced apoptosis. More in-depth studies are currently underway to confirm this and to determine how AMPK might be modulated during this combination treatment.

<table>
<thead>
<tr>
<th>Treatment time (Hrs)</th>
<th>2hrs</th>
<th>4hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TRAIL + TZD</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**Fig 10: Effect of AMPK-Dominant negative (DN) mutant on TRAIL-TZD-induced apoptosis:** C42-prostate cancer cells stably overexpressing Empty vector (C42-EV) or Flag-tagged AMPK-DN mutant (C42-DN) were treated with either DMSO or TRAIL-TZD combination for the indicated amounts of time. The samples were analyzed by Western Blots utilizing the antibodies indicated.

**Task 3: Whether β-catenin mediates drug-induced apoptosis in prostate xenografts in vivo (months 1-36)**

Studies in this section are expected to address the role of TRAIL-TZD combination as well as β-catenin in mediating apoptosis in vivo utilizing a subcutaneous xenograft model. These were initially planned with SCID mice to be injected with LNCaP prostate cancer cells and following approval from Loyola IACUC and ACURO, the xenograft studies were initiated. As indicated in the past funding cycle, due to a very poor tumor take (only 1 out of 5) and a slow tumor growth (> 5 weeks to observe very small palpable tumor) in SCID mice, the initial animal protocol was amended to perform these xenograft studies in nude mice, which is reported to have better tumor take. However, despite the change of mouse strain, no palpable tumors were detected until 8 weeks after injection with LNCaP cells at which time mice were euthanized. This has created a setback in these xenograft studies that were initially planned under this task. Since then, I have
specifically discussed this problem with some of the experts in prostate cancer research during American Association for Cancer Research (AACR) annual meeting of 2012. As suggested by these experts, LNCaP cells in general seems to have very poor tumor take in subcutaneous xenograft models and this can be overcome by using the LNCaP derivative cells LNCaP-C4-2, LNCaP-C4-2B.

Accordingly, we have obtained the C42 and C42B cells and have utilized them \textit{in vitro} to determine how they respond to TRAIL and TZD combination, as was initially planned. TRAIL-TZD studies performed with these cells showed a significant induction of apoptosis in both cell types following incubation with this combination as shown in Fig 11. Based on these results, we have modified our ACORP to include both of these cell types for our xenograft injection. After receiving approval from our institutional IACUC (dated Jan, 2013), we also submitted an amendment of this for ACURO approval to use these different cell types. We have recently received ACURO approval (dated March, 2013) and will be initiating these new xenograft studies with the C42 and C42B cells in nude mice.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|
\hline
\textbf{Treatment Time (Hrs)} & \textbf{8hrs} & \textbf{16hrs} & \textbf{8hrs} & \textbf{16hrs} \\
\hline
\textbf{DMSO} & + & - & + & - & + & - & + & - \\
\textbf{TRAIL + TZD} & - & + & - & + & - & + & - & + \\
\hline
\textbf{Cell Type} & \textbf{C42} & \textbf{C42B} \\
\hline
\end{tabular}
\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig11.png}
\caption{Effect of TRAIL-TZD on the apoptosis of C42 and C42B prostate cancer cells: C42 and C42B cells were treated with DMSO or TRAIL-TZD as in Fig 10 and harvested at the indicated time points. Western Blot analyses were performed with the antibodies indicated.}
\end{figure}
Key Research Accomplishments:
1. Characterization of the status of interaction between TRAIL-TZD-induced cleaved \( \beta \)-catenin, E-cadherin and \( \alpha \)-catenin.
2. Creation of cleavage-resistant (D/A) myc-tagged \( \beta \)-catenin mutants (total 6).
3. Characterization of D/A myc-tagged \( \beta \)-catenin mutants on TRAIL-TZD-induced apoptosis and \( \beta \)-catenin cleavage.
4. Mapping of additional cleavage sites on \( \beta \)-catenin following TRAIL-TZD treatment by Mass Spectrometry.
5. Determining the effect of \( \beta \)-catenin knockdown in mediating apoptotic resistance in AR-positive LNCaP cells in a time dependent manner following TRAIL-TZD stimulation.
6. Determining the effect of \( \beta \)-catenin overexpression on LNCaP cell apoptosis.
7. Determining the effect of various GSK3\( \beta \) inhibitors on increasing TRAIL sensitivity in AR-negative prostate cancer cells.
8. Determining the effect of TRAIL-TZD on GSK3\( \beta \) and GSK3\( \alpha \) pathways in AR-negative prostate cancer cells.
9. Determining the effect of TRAIL-TZD on the expression of cyclin dependent kinases (CDKs) in AR-positive and AR-negative cells.
10. Determining the involvement of AMPK in mediating the apoptosis pathway following TRAIL-TZD treatment.
11. Obtained and characterized the LNCaP derivative cells C4-2 and C4-2B for TRAIL-TZD response in vitro so they can be utilized for in vivo xenograft studies.
12. Obtained Loyola-IACUC and ACURO approval to utilize the C4-2 and C4-2B cells for in vivo studies.

Reportable Outcomes:
We had an initial set-back in the overall studies, since two personnel have left the program during the last funding period. Since then new personnel has been hired and after an initial delay (covering the training period) we have currently made significant progress in the studies and are working towards publishing the manuscripts within the next year. The following were achieved during this last funding period

Manuscript:
Conclusions:
In conclusion, over the past year we have focused more on mapping the cleavage site on β-catenin protein following incubation of the cells with TRAIL-TZD combination. Extensive Immunoprecipitation and Immunoblotting combination studies have revealed that this cleaved fragment almost completely loses interaction with TCF4, while retaining strong interaction with E-cadherin and α-catenin. These results indicated the possibility that (i) TRAIL-TZD might be completely antagonizing β-catenin/TCF mediated transcriptional activity via abolishing the interaction between cleaved β-catenin and TCF4 and (ii) maintaining the interaction with E-cadherin and α-catenin might be crucial for optimal apoptosis induction. To address these questions, and based on the sizes of the cleaved β-catenin protein, we have created 6 different D/A (Aspartic acid to Alanine) mutants and have initially characterized them to express the correct size protein. In addition, we have also successfully Immunoprecipitated the cleaved β-catenin protein by E-cadherin antibodies and obtained Coomassie-stainable bands and subjected this to Mass Spectrometry. The results of Mass Spectrometry revealed additional cleavage sites on β-catenin and studies are currently underway to create additional D/A mutants based on these. We plan on utilizing all these β-catenin mutants to conclusively map the cleavage site and to elucidate the of β-catenin cleavage on prostate cancer cell apoptosis. We have also obtained interesting information with β-catenin-siRNA and β-catenin overexpression that revealed that in AR-expressing LNCaP cells β-catenin functions as a pro-survival molecule as opposed to the AR-negative DU145 cells. More molecular studies are planned to confirm this, which will reveal important downstream mediators of this pathway. Utilizing AR-negative DU145 cells and with two different GSK3β inhibitors (AR-A014418 and BIO), we have shown that GSK3β inhibition promotes TRAIL sensitivity independent of AR expression. In addition, TRAIL-TZD antagonizes GSK3β and GSK3α pathways in these cells via up-regulating pGSK3βSer9 levels (indicating inhibition), and down-regulating total expression of both GSK3α and GSK3β. Interestingly, this combination also inhibits the expression of cyclin dependent kinases CDK1 and CDK2 and the contribution of these kinases in this apoptosis pathway will be important to investigate in the future. Since Troglitazone used in the combination studies can activate AMPK, we also investigated whether AMPK was involved in mediating this apoptosis by overexpressing AMPK-dominant negative (DN) mutant. These revealed a significant inhibition of the apoptosis pathway in the presence of AMPK-DN, suggesting involvement of AMPK. Studies are currently underway to elucidate the detailed pathway. We have also obtained and characterized the C42 and C42B cells, both of which respond potently to TRAIL-TZD-induced apoptosis. Xenograft studies are planned next with these cells to determine the efficacy of this combination treatment along with GSK3β inhibitors in regulating prostate tumor progression.
References:

Appendices:

See Loyola IACUC approval letter and ACURO approval letter on next page for the ACORP amendment.
January 25, 2013

U.S. Army Medical Research and Materiel Command
Animal Care and Use Review Office
ATTN: MCMR-RPA
504 Scott Street
Fort Detrick, MD 21702-5012
Phone: 301-619-6694
Fax: 301-619-4165
Email: acuro@amedd.army.mil

RE: IACUC APPROVAL
   PI: DR. B. RANA; LU#201974 (2.04) Mouse

To Whom It May Concern,

The Loyola University Chicago, Stritch School of Medicine’s Institutional Animal Care and Use Committee (IACUC) approved the ACORP; DR. B. RANA; LU#201974 (2.04) Mouse (Beta catenin in prostate cancer apoptosis) on January 16, 2013. The protocol has a full board approval until January 16, 2016.

Loyola University Chicago, Stritch School of Medicine has an Animal Assurance on file with the Public Health Service under #A3117-01 approved through 02/28/2014, a fully AAALAC International accredited institution (certification dated 11/10/2010), and USDA registered/licensed institution under #33-R-0024 through 08/24/2014.

If you have any questions or require additional information, please feel free to contact the IACUC Chair, Dr. Jawed Fareed via the Committee Director, telephone (708) 216 4288; Fax (708) 216-9399.

Sincerely,

Mr. Jamie Caldwell, MBA
Director
Office of Research Services
for the Health Sciences
Loyola University Chicago, Health Sciences Division
Health Sciences Campus
Bldg 120 Suite 400
2160 South First Avenue
Maywood, IL 60153
Phone (708) 216-2636
Fax (708) 216-5881

cc: LU/IACUC File: DR. B. RANA; LU#201974 (2.04) Mouse
March 01, 2013

Director, Office of Research Protections
Animal Care and Use Review Office

Subject: Review of USAMRMC Proposal Number PC093099, Award Number W81XWH-10-1-0195 entitled, "Beta Catenin in Prostate Cancer Apoptosis"

Principal Investigator Basabi Rana
Loyola University Medical Center
Chicago, IL

Dear Dr. Rana:

Reference: (a) DOD Instruction 3216.01, "Use of Animals in DOD Programs"
(b) US Army Regulation 40-33, "The Care and Use of Laboratory Animals in DOD Programs"
(c) Animal Welfare Regulations (CFR Title 9, Chapter 1, Subchapter A, Parts 1-3)

In accordance with the above references, the **rewrite** of protocol PC093099 entitled, "Beta Catenin in Prostate Cancer Apoptosis," IACUC protocol number 201974 is approved by the USAMRMC Animal Care and Use Review Office (ACURO) for the use of mice and will remain so until its modification, expiration or cancellation. This protocol was approved by the Loyola University Chicago, Stritch School of Medicine IACUC.

When updates or changes occur, documentation of the following actions or events must be forwarded immediately to ACURO:

- IACUC-approved modifications, suspensions, and triennial reviews of the protocol (All amendments or modifications to previously authorized animal studies must be reviewed and approved by the ACURO prior to initiation.)
- USDA annual program/facility inspection reports
- Reports to OLAW involving this protocol regarding
  - any serious or continuing noncompliance with the PHS Policy;
  - any serious deviation from the provisions of the Guide for the Care and Use of Laboratory Animals; or
  - any suspension of this activity by the IACUC
- USDA or OLAW regulatory noncompliance evaluations of the animal facility or program
- AAALAC, International status change (gain or loss of accreditation only)
Throughout the life of the award, the awardee is required to submit animal usage data for inclusion in the DOD Annual Report on Animal Use. Please ensure that the following animal usage information is maintained for submission:

- Species used (must be approved by this office)
- Number of each species used
- USDA Pain Category for all animals used

For further assistance, please contact the Director, Animal Care and Use Review Office at (301) 619-2283, FAX (301) 619-4165, or via e-mail: acuro@amedd.army.mil.

NOTE: Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer or Grant Officer can authorize expenditure of funds. It is recommended you contact the appropriate Contract Specialist or Contracting Officer regarding the expenditure of funds for your project.

Sincerely,

James Sheets, DVM, DACLAM
Colonel, US Army
Director, Animal Care and Use Review Office

Copies Furnished:
Mr. Ayi Ayayi, US Army Medical Research Acquisition Activity (USAMRAA)
Dr. Nrusingha Mishra/MCMR-PLF
Dr. Basabi Rana, Loyola University Chicago, Stritch School of Medicine
Dr. Charlene J Repique, Congressionally Directed Medical Research Program (CDMRP)
Dr. Jawed Fareed, Loyola University Medical Center
Mr. Jamie Caldwell, Loyola University Chicago, Stritch School of Medicine