AWARD NUMBER: W81XWH-04-1-0010

TITLE: Treatment of Prostate Cancer with a DBP-MAF-Vitamin D Complex to Target Angiogenesis and Tumorigenesis

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REPORT DATE: February 2008

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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Treatment of Prostate Cancer with a DBP-MAF-Vitamin D Complex to Target Angiogenesis and Tumorigenesis

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14. ABSTRACT The purpose of this study has been to determine the efficacy of combined therapy using vitamin D binding protein-macrophage activating factor (DBP-maf) and vitamin D as therapy for human prostate cancer. We had found that in endothelial tube formation vitamin D and DBP-maf inhibited the tube formation. Both molecules were effective on their own, however the vitamin D showed evidence of toxicity at higher concentration. We here show that the combination of vitamin D, at a level ineffective by itself(10 pM), and DBP-maf at concentrations as low as 100 ng/ml show potent synergistic behavior. We observed that DBP-maf inhibits the expression of urokinase-type plasminogen activator receptor (UPAR), a molecule whose expression has been linked with increased metastasis. We also observed reduced expression of p21 and p27 by DBP-maf but not by the control DBP. The expression of UPAR by DBP-maf may explain its potent activity on tumors.

15. SUBJECT TERMS angiogenesis, prostate cancer, vitamin D, DBP-maf

16. SECURITY CLASSIFICATION OF:

a. REPORT U
b. ABSTRACT U
c. THIS PAGE U

17. LIMITATION OF ABSTRACT

UU

18. NUMBER OF PAGES 20

19a. NAME OF RESPONSIBLE PERSON

USAMRMC

19b. TELEPHONE NUMBER (include area code)

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20
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>5</td>
</tr>
<tr>
<td>Body</td>
<td>5-18</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>19</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>19</td>
</tr>
<tr>
<td>Conclusion</td>
<td>19-21</td>
</tr>
<tr>
<td>Bibliography</td>
<td>21</td>
</tr>
</tbody>
</table>
Introduction

Prostate cancer is the second most common type of cancer among men, second only to skin cancer. According to the American Cancer Society, approximately 186,320 new cases of prostate cancer will be diagnosed in 2008 in the U.S. Treatment options are primarily surgery or radiation in early stages. Standard chemotherapy is not effective in early stages but docetaxel has been found to be effective in advanced stages (Fizazi et al., 2004). Vitamin D has been shown in Phase II clinical trials to be effective in stabilizing disease progression with only mild hypercalcemia (Liu et al., 2003), which is the complicating factor in vitamin D therapy. We proposed that using vitamin D binding protein-macrophage activating factor (DBP-maf) in combination with vitamin D could provide a potent treatment for prostate tumors. Our previous work had shown that DBP-maf was a potent antiangiogenic substance when used in a pancreatic cancer model. Its ability to target tumor beds presented the possibility that, in its role as a vitamin D binding protein, it could target the bound vitamin D to tumor sites. This targeting could increase the amount of vitamin D delivered to the tumor bed and thus might allow a lower overall dose to be used with a reduced risk of hypercalcemia.

Body

Our initial aim was to establish the direct effects of individual and combination therapy of DBP-maf and vitamin D in vitro using endothelial and prostate cancer cells.

**DBP-maf has a biphasic effect on LnCaP cell proliferation** - DBP-maf has been shown to be a potent inhibitor of angiogenesis but its ability to inhibit tumor cell proliferation has not been previously shown. DBP-maf was tested in proliferation assays using the LnCaP parental cell line. As shown in Figure 1, DBP-maf at 1 ug/ml was able to inhibit cell proliferation by approximately 50%. Interestingly, at low doses the effect appears to stimulate proliferation. The control, DBP, showed no ability to either potentiate or inhibit proliferation.

![Figure 1](image_url)

**Figure 1.** Effect of DBP-maf and DBP on LnCaP cell proliferation. LnCaP cells were grown at a density of 1,000 cells/well in 24 well plates, serum-starved overnight, and replaced with medium (Medium 199, pen/strep, 1% fetal bovine serum) +/- DBP-maf or DBP. After 72 hours, cells were quantitated using an acid phosphatase colorimetric assay. Error is +/- s.d.
We next tested proliferation in the LnCaP metastatic line. As shown in Figure 2, DBP-maf inhibited proliferation of the LnCaP metastatic line by greater than 80% while increasing proliferation at low dose. Again the DBP control showed no activity.

Figure 2. Effect of DBP-maf and DBP on LnCaP metastatic cell proliferation. LnCaP metastatic cells were grown at a density of 1,000 cells/well in 24 well plates, serum-starved overnight, and replaced with medium (Medium 199, pen/strep, 1% fetal bovine serum) +/- DBP-maf or DBP. After 72 hours, cells were quantitated using an acid phosphatase colorimetric assay. Error is +/- s.d.

DBP-maf has a biphasic effect on PC3 cell proliferation - We next measured DBP-maf on PC3 cell proliferation and saw a similar profile to that of the LnCaP cells. At 1 ug/ml, DBP-maf inhibited PC3 cell growth by almost 50% while stimulating growth at low doses. DBP had no measurable effect (Figure 3).

Finally, we tested the effect of DBP-maf and DBP on PC3 metastatic cells and observed results consistent with the other cell lines (Figure 4).
Figure 4. Effect of DBP-maf and DBP on PC3 metastatic cell proliferation. PC3 metastatic cells were grown at a density of 1,000 cells/well in 24 well plates, serum-starved overnight, and replaced with medium (Medium 199, pen/strep, 1% fetal bovine serum) +/- DBP-maf or DBP. After 72 hours, cells were quantitated using an acid phosphatase colorimetric assay. Error is +/- s.d.

The combination of DBP-maf and vitamin D had been tested previously in our study to measure any additive or synergistic effects. As shown in Figure 5 from our previously reported work, although each molecule showed inhibition of LnCaP proliferation separately, there appeared to be neither an additive or synergistic effect when used together. The vitamin D concentrations used were a limiting factor because at higher concentrations there was a noticeable toxicity. Our vitamin D dose, therefore, reflected a dose that, in and of itself, had an effect without toxicity and would still allow measurement of additional inhibition by DBP-maf without masking its effect.

As shown in our previous report (Figure 5) we observed a reduction of ~ 40% in the proliferation of LnCaP cells with DBP-maf. Vitamin D showed ~ 60% reduction but there was no observed additive or synergistic effect when the drugs were combined.
Figure 5. DBP-maf and vitamin D inhibit proliferation of LnCaP cells. LnCaP cells were plated at 1,000 cells/well in 24 well plates and incubated overnight at 37°C in Medium 199 (5% FBS). Medium was changed to serum free medium and dbp-maf (5 ug/ml) and/or vitamin D (0.1 nM) was added. Cells were incubated for 72 hours and quantitated using an acid phosphatase colorimetric assay. Results are plotted +/- s.d.

Our previous report showed also that the combination of DBP-maf and vitamin D had a minimal effect on HUVEC proliferation (Figure 6). Again, the vitamin D dose used was below a toxic level and adjusted to allow observable effects by DBP-maf in the combination treatment. These in vitro data suggest a more potent direct effect of DBP-maf on the tumor cells, somewhat surprising considering the potent antiangiogenic effect we have previously observed.

Figure 6. DBP-maf combined with low level vitamin D has a minimal effect on HUVEC proliferation. HUVECs were incubated overnight (15,000 cells/well) in Medium 199(10% FBS). Medium was changed to 5% FBS with 1 ng/ml FGF-2 and incubated for 72 hours with vitamin D (0.1 nM) and DBP-maf. Cells were quantitated using an acid phosphatase colorimetric assay. Results are +/- s.d.

DBP-maf inhibits urokinase-type plasminogen activator receptor expression.- Urokinase-type plasminogen activator receptor (UPAR) has been implicated in several biological processes. Its
elevation in tumor cells has been associated with increased metastasis. Inhibition of the receptor has been shown to induce apoptosis in melanoma cells (Besch et al., 2007). We tested LnCaP and LnCaP metastatic lines for UPAR before and after DBP-maf or DBP treatment. We also measured the effect of our proteins on vitamin D receptor expression. At 1 ug/ml, the same concentration that showed inhibition of proliferation, UPAR expression was reduced at both 24 and 72 hours in the presence of DBP-maf but not DBP(Figure 7 and 8 respectively). Vitamin D receptor expression remained unchanged in all conditions except the 72 hour time point of LnCaP parental cells after control DBP treatment where there was an increase in vitamin D receptor expression.

Figure 7. Effect of DBP-maf on UPAR and vitamin D expression on LnCaP and LnCaP metastatic (Ln3) cells. Tumor cells were incubated +/- DBP-maf for 60 minutes, lysed, and immunoblotted with anti-UPAR or anti-vitamin D antibodies. Bands were visualized by chemiluminescence.
Figure 8. Effect of DBP on UPAR and vitamin D expression on LnCaP and LnCaP metastatic (Ln3) cells. Tumor cells were incubated +/- DBP for 60 minutes, lysed, and immunoblotted with anti-UPAR or anti-vitamin D antibodies. Bands were visualized by chemiluminescence.

We then tested both the PC3 and PC3 metastatic lines to determine if the inhibition of UPAR expression was cell-type specific. Unlike the results with LnCaP parental cells, DBP-maf did not affect UPAR expression on the PC3 parental cell line (Figure 9), but showed strong inhibition of UPAR, vitamin D receptor, and p27 at 72 hours in the metastatic line (Figure 9).
Figure 9. Effect of DBP-maf on UPAR, vitamin D, p21, and p27 expression on PC3 and PC3 metastatic (PC3MLn4) cells. Tumor cells were incubated +/- DBP-maf for 60 minutes, lysed, and immunoblotted with anti-UPAR or anti-vitamin D antibodies. Bands were visualized by chemiluminescence.

As shown in Figure 10, DBP had no effect on UPAR, vitamin D receptor, or p27 at either time point. Reduction of both p21 and p27 are interesting, because their increased expression is usually associated with inhibition of the cell cycle and their reduction associated with growth (Caldon et al., 2006). Our proliferation data, however, clearly shows this not to be the case at the DBP-maf concentration used (1 ug/ml). Perhaps the other activities of DBP-maf are potent enough to overcome this reduction of cell cycle proteins.
Figure 10. Effect of DBP on UPAR and vitamin D expression on PC3 and PC3 metastatic (PC3MLn4) cells. Tumor cells were incubated +/- DBP for 60 minutes, lysed, and immunoblotted with anti-UPAR or anti-vitamin D antibodies. Bands were visualized by chemiluminescence.

DBP-maf inhibits migration of LnCaP and PC3 tumor cell lines - Cell migration is an important step in the growth of new blood vessels and also in tumor metastasis. Using a modified Boyden chamber, the ability of DBP-maf to inhibit cell migration was tested. Tumor cell lines were serum starved overnight and placed in the top well of a modified Boyden chamber (150,000/well) in serum-free medium. Medium with 10% fetal bovine serum was added to the bottom wells. As shown in Figure 11, DBP-maf inhibited migration significantly in all tumor cell lines.
Figure 11. Effect of DBP-maf on LnCaP and PC3 cell migration. Cells were plated at a density of 150,000/well in the upper well of a modified Boyden chamber in Medium 199 basal medium +/- DBP-maf. Medium 199 with 10% fetal bovine serum was added to bottom wells and cell were incubated for 6 hours at 37°C. Cells that had not migrated were removed from the upper surface of the membrane by wiping and the cells that had migrated were quantitated using an acid phosphatase colorimetric assay. A- LnCaP parental, B-LnCaP metastatic, C- PC3 parental, D- PC3 metastatic. Error is +/- s.d.

Our previous report had shown that the combination of vitamin D and DBP-maf inhibited endothelial cell migration. DBP-maf at 5 ug/ml inhibited migration by ~ 25% and with the addition of vitamin D inhibited migration by ~ 40% (Figure 12). We have observed more potent inhibition with DBP-maf in our later studies, likely because our proficiency in producing DBP-maf improved over the course of the study.

Figure 12. DBP-maf and vitamin D inhibit endothelial cell migration. Human umbilical vein endothelial cells (150,000/well) were added to the top wells of a modified Boyden chamber +/- DBP-maf (5 ug/ml) with or without vitamin D (.01 nM). Fetal bovine serum(10%) was added to the bottom wells and cells were incubated for 6 hours at 37°C. Cells that did not migrate were removed and migrating cells were quantitated using an acid phosphatase colorimetric assay. Results are plotted +/- s.d.

We have continued throughout the course of this study to test the efficacy of a maf peptide. There is a distinct advantage to using a peptide that can be synthesized as opposed to a protein that must be
enzymatically processed, assuming that the peptide retains the potency of the full length protein. Schneider et al have used a 14 amino acid peptide of DBP-maf to increase bone density that does not contain the vitamin D binding site, but represents the active site exposed by deglycosylation (Schneider et al., 2003). Our previous report showed an effect of the peptide on endothelial cell migration.

We continued to test the peptide with our tumor lines to determine whether the peptide was capable of inhibiting tumor cell migration as well. As shown in Figure 13, the peptide inhibited migration of all four tumor lines at fairly low concentration (25 ng/ml). The inhibition ranged from 50-75% but increasing the concentration did not enhance the inhibition.

![Graphs showing absorption at 405 nm for different concentrations of MAF Peptide.](image)

**Figure 13.** Effect of DBP-maf peptide on LnCaP and PC3 cell migration. Cells were plated at a density of 150,000/well in the upper well of a modified Boyden chamber in Medium 199 basal medium +/- DBP-maf peptide. Medium 199 with 10% fetal bovine serum was added to bottom wells and cell were incubated for 6 hours at 37° C. Cells that had not migrated were removed from the upper surface of the membrane by wiping and the cells that had migrated were quantitated using an acid phosphatase colorimetric assay. A- LnCaP parental, B- LnCaP metastatic, C- PC3 parental, D- PC3 metastatic. Error is +/- s.d.
Proliferation assays in our previous report using the peptide had shown no significant effect on the proliferation of parental LnCaP cells. Assays using the LnCaP metastatic cells also showed no significant effect on proliferation by the peptide (Figure 14).

**Preparation of DBP-maf**

Due to the unfortunate and untimely death of our collaborator, Narasimha Swamy, whose role as an expert in this work was to supply vitamin D binding protein-macrophage activating factor (DBP-maf) for our assays, we were required to generate the protein in our own laboratory. There is no commercial source for this protein. Our efforts have been successful but at a significant loss of time and effort. We have found after much trial and error that the source of enzymes is crucial to ensure DBP-maf activity. Frankly, many sources of these enzymes are of poor quality. We have chosen Sigma neuraminidase and Roche (1,3)β-D-galactosidase for optimal activity.

We have refined the process of DBP-maf generation as follows:

**Coupling of (1-3)β-D-galactosidase to cyanogen bromide-activated agarose**

Note: Type VI-A neuraminidase-agarose (*clostridium perfringens*) was purchased from Sigma.

1. Cyanogen bromide-activated beads (0.5 g) were washed with 1 mM HCl (3X, 10 ml per wash) using suction filtration.
2. The beads were then washed with DDI water and resuspended in 2 ml of coupling buffer (0.1 M HNaCO₃, 0.5 M NaCl, pH 8.3).
3. (1-3)β-D-galactosidase (1000 units, Roche) was added to the beads/coupling buffer and then shaken with an over and under mixer for 2 hours at room temperature.
4. The beads were then washed with coupling buffer and resuspended in blocking buffer (coupling buffer plus 0.2 M glycine). The suspension was shaken overnight at 4 °C.
5. The beads were then washed with coupling buffer followed by 0.1 M sodium acetate, 0.5 M NaCl, pH 4.0, and the washes were repeated for a total of four times. Finally, the beads were washed with a final wash of coupling buffer.
6. The beads were then spun down and resuspended in 1.0 M NaCl.

**Determination of activity (1-3)β-D-galactosidase-agarose beads**
1. A volume of 50 μl of the bead suspension was added to 0.95 ml of assay buffer/chromogenic substrate (PBS, 3 mM 2-nitrophenyl-β-D-galactopyranoside, 10 mM MgCl₂, 0.1 mM β-mercaptoethanol), and the suspension was shaken at room temperature for 15 min.
2. The reaction was stopped with 33 μl of 1 M sodium carbonate, and the absorbance was measured at 405 nm.
3. Activity was expressed as units/ml of bead suspension, where 1 unit is defined as the number of μmoles of p-nitrophenol formed per minute. A molar absorbity of 18380 L/mole cm, and a path length of 0.25 cm corresponding to a 200 μl volume in a 96 well plate were used to calculate the concentration of p-nitrophenol.

**Synthesis of DBP MAF**
1. DBP was added to a final concentration of 0.05 mg/ml in PBS pH 6.0, 10 mM MgCl₂, with 0.007 U of (1-3) β-D-galactosidase-agarose and 0.004 U of neuraminidase-agarose, and shaken at room temperature for 4 hours. The total reaction volume was 1 ml.
2. Beads were then spun down and removed. DBP MAF was stored in aliquots at -20 °C.

**DBP-maf disrupts endothelial cell tube formation** - A well published and often used measure of antiangiogenic molecules has been their potential to disrupt the spontaneous organization of endothelial cells into tubes when cells are plated on MATRIGEL™, a matrix of the Engelbreth-Holm Swarm mouse tumor. This assay involves both migration of cells and organization into endothelial tubes that have three dimensional structure. Our previous report had shown that DBP-maf effectively disrupted endothelial cell tube formation and that the peptide, albeit at much higher concentration (1 ug/ml, 25 ug/ml respectively).

In our current study, when vitamin D was added alone, there was little effect on tube formation even at 1 nM. The combination, however, of vitamin D at 10 pM and DBP-maf at 100 ng/ml showed significant disruption of tube formation (Figure 15). When the vitamin D concentration was increased to 100 pM the effect was even more pronounced, suggesting strong synergy between the two molecules.
Figure 15. DBP-maf inhibits endothelial cell tube formation. HUVEC were plated at a density of 100,000 cells/well in 24 well plates in Medium 199 with 10% FBS +/- DBP-maf and +/- vitamin D at indicated concentrations. Cells were incubated at 37° C for 12 hours then photographed under a microscope (4x).

Our results have shown that DBP-maf is able to inhibit the migration of LnCaP cells. We hypothesized that this inhibition involved changes in the actin cytoskeleton. This possibility was investigated using a phalloidin antibody and included in our previous report. LnCaP and LnCaP LN3 cells were plated on tissue culture slides and probed with an anti-phalloidin antibody. No significant differences were observed between the treated and untreated cells. The LnCaP cells, however, have a very compact cell body with the nucleus comprising most of the cell area. This made it difficult to observe subtle changes in the cytoskeleton.
Figure 16. DBP-maf effect on LnCaP actin filament formation. LnCaP cells were plated on slides and treated for 4 hours with or without DBP-maf (A, control; B, 1 ng/ml; C, 10 ng/ml; D, 100 ng/ml). An anti-phalloidin antibody was applied and a secondary antibody that was FITC conjugated.

Figure 17. DBP-maf effect on LnCaPLn3 actin filament formation. LnCaPLn3 cells were plated on slides and treated for 4 hours with or without DBP-maf (A, control; B, 1 ng/ml; C, 10 ng/ml; D, 100 ng/ml). An anti-phalloidin antibody was applied and a secondary antibody that was FITC conjugated.

HUVEC cells, whose tube formation was inhibited by DBP-maf, showed no obvious differences between treated and untreated cells.

Figure 18. DBP-maf effect on LnCaPLn3 actin filament formation. LnCaPLn3 cells were plated on slides and treated for 4 hours with or without DBP-maf (A, control; B, 1 ng/ml; C, 10 ng/ml; D, 100 ng/ml). An anti-phalloidin antibody was applied and a secondary antibody that was FITC conjugated.
Key Research Accomplishments

- Demonstrated the ability of DBP-maf to inhibit proliferation of LnCaP parental, LnCaP metastatic, PC3 parental and PC3 metastatic cell lines
- Demonstrated the ability of DBP-maf to inhibit the migration of LnCaP and PC3 parental and metastatic tumor lines
- Demonstrated the ability of DBP-maf to disrupt endothelial cell tube formation
- Demonstrated synergy between DBP-maf and vitamin D in disruption of endothelial cell tube formation
- Demonstrated the ability of DBP-maf to inhibit the expression of urokinase plasminogen activated receptor (UPAR) whose increased expression is linked to metastasis
- Demonstrated the ability of DBP-maf to inhibit expression of p21 and p2, both linked to apoptosis, in PC3 cells
- Demonstrated the potential of using a DBP-maf peptide in lieu of the full protein to facilitate synthesis

Reportable Outcomes

Based on the ideas and concepts brought out in this study, the P.I. was able to apply and be awarded an NIH R01 (HL086644-01). Kalvin Gregory, a postdoc funded by this work, is a candidate for several faculty positions. Eva Bach, as a result of the training she received under this award was admitted to the Ph.D. program of the Integrated Biomedical Sciences Program at the University of Kentucky. We are currently writing a manuscript for publication based on the results of this study.

Conclusions

We had previously published that DBP-maf was a potent inhibitor of human pancreatic cancer in a mouse model (Kisker et al., 2003) based on its antiangiogenic and anti-tumor properties. In this study we attempted to exploit the ability of DBP-maf to bind vitamin D as a strategy to deliver two strong inhibitors to the tumor bed. Although vitamin D has been shown to inhibit prostate tumor growth, the doses required to be effective cause the dangerous side effect of hypercalcemia. The ability of DBP-maf to inhibit pancreatic tumor growth at very low concentration suggested that the protein was targeting the tumor bed. If DBP-maf could target the tumor bed while associated to vitamin D, then perhaps a much lower, but targeted systemic dose of vitamin D could be effective without the side effects.

Although we were not able to complete our in vivo work as outlined in our objectives, we were able to make significant progress toward the understanding of potential mechanisms by which DBP-maf is effective. Our finding that DBP-maf inhibits UPAR expression is a potentially important finding since the increased expression of UPAR has been shown to correlate with greater metastatic potential.

The ability to inhibit tumor growth is important, but the ability to inhibit metastasis is equally or more important. Patients die primarily from metastasis, not from local tumors. The ability to inhibit the metastatic process would be a potent tool in cancer treatment. Also, since there has been no evidence of toxicity this type of treatment might eliminate the side effects seen with traditional chemotherapy.
We have shown the ability of DBP-maf to inhibit the proliferation of tumor cells. This finding increases the value of DBP-maf treatment in cancer because it directly affects the tumor compartment. Interestingly, the treatment of tumor cells with low dose DBP-maf appeared to stimulate cell proliferation but by the 1 ug/ml dose potent inhibition was observed. The control molecule, DBP, had no effect on proliferation. Although the inhibition of endothelial cell proliferation by DBP-maf was weak, it inhibits vessel formation in other ways to successfully disrupt angiogenesis.

The demonstrated ability of DBP-maf to inhibit cell migration may be important in its potential to inhibit metastasis. In both angiogenesis and tumorogenesis cell migration is a crucial step in the process. We demonstrated the ability of DBP-maf to potently inhibit the migration of both endothelial cells and tumor cells. At 1 ug/ml in all tumor lines, DBP-maf demonstrated a potent inhibition of cell migration. In one case, the LnCaP parental line, lower doses of DBP-maf increased its inhibitory activity. Perhaps the combination of activity in proliferation and migration undermines the local and metastatic growth.

Using the endothelial tube formation assay we were able to demonstrate the ability of DBP-maf to disrupt tube formation, an obligate step in vessel growth. In addition, the combination of DBP-maf and vitamin D allowed a level of inhibition not seen with DBP-maf alone at higher concentrations. Vitamin D alone seemed to have no significant effect. This synergy is valuable evidence of the effect of both molecules when used in combination.

Our greatest obstacle in this study was the generation of DBP-maf. The initial commitment to produce DBP-maf was made by Narasimha Swamy of Brown University. His untimely death left us with no source for the molecule, since only one or two groups were actively studying the protein. There were no commercial products available and, although the preparation was fairly straightforward in theory, in practice it was very troublesome. Our increased proficiency in making active DBP-maf had little to do with altering the protocol, but a great deal to do with finding a suitable source for our reagents. Most of the commercial sources of our enzymes proved to be unsuitable for the task. Our present capability to produce active DBP-maf is very good. This complication did slow our progress considerably. In spite of this, however, we have made potentially important findings that we will continue to pursue. Our demonstrations of synergy using vitamin D and DBP-maf are evidence that the idea of combination therapy is a sound one.

So what

The search for effective and safe treatments for cancer is a continuous process. As effective as chemotherapy is in some forms of cancer, it has had limited success with prostate cancer. Only recently has the use of docetaxel been recommende for prostate cancer and only for patients with metastatic or progressive local tumors(Fizazi et al., 2004). There is a clear need for therapy that is effective in all stages of prostate cancer.

DBP-maf is a molecule that has therapeutic potential in prostate cancer treatment. The parent protein, DBP, is present at high concentration in blood but has none of the antiangiogenic or antitumor activity that we have reported for DBP-maf. DBP is abundant and easily purified and, although the conversion to DBP-maf can be challenging in terms of high volume production, the
benefits could be many. In addition we explored the potential of a DBP-maf peptide, whose synthesis would be simple and inexpensive. Although the peptide did not exhibit similar potency as DBP-maf, it showed strong activity in inhibition of migration of tumor cells and moderate activity in tube formation. Perhaps a variation of this peptide could enhance the inhibitory activity.

Bibliography

Personnel

Michael Fannon, Ph.D.-, P.I.
Kalvin Gregory, Ph.D. –postdoc
Eva Bach- student

References


Appendices

N/A