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INTRODUCTION

The first objective of phase II of this grant is to delinate the regulation of VEGF gene expression by 12-LOX in PCa cells, specifically the involvement of Sp1 and possibly AP2. The second objective of this proposal is to describe the signal transduction pathway by which 12-LOX stimulates VEGF expression. These two objectives are based upon our observations in the Phase I of this grant that that 12-LOX, when overexpressed in human PCa cells, stimulates tumor angiogenesis and growth. The third objective to evaluate the therapeutic potential of 12-LOX inhibitors for PCa treatment.

BODY OF REPORT

PROGRESS

Task 1. Investigate the regulation of VEGF gene expression by 12-LOX, with special focus on the *cis*-elements in VEGF promoter region and the *trans*-factors involved, Months 1 - 12:

This task has been achieved and additional findings were made as summarized as followed:

1). Transcriptional regulation of VEGF expression by 12-LOX

In Phase I of this grant, we observed an increase in VEGF production in 12-LOX transfected PC-3 cells when compared to their vector controls. Here we studied how 12-LOX regulates VEGF gene expression.

To study whether 12-LOX regulates VEGF expression at the transcriptional level, we transfected neo-control cells (neo-α) and 12-LOX transfected PC-3 cells (nL-8 or nL-12) with a VEGF promoter luciferase construct (-1176/+54), along with a lacZ control plasmid to normalize transfection efficiency. As shown in **Figure 1**, there was a more than 10-fold increase in VEGF promoter activity in 12-LOX transfected PC-3 cells as compared with their neo-controls.

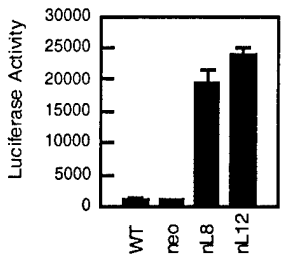

Figure 1. Increased VEGF Promoter Activities in 12-LOX Transfected PC-3 Cells.

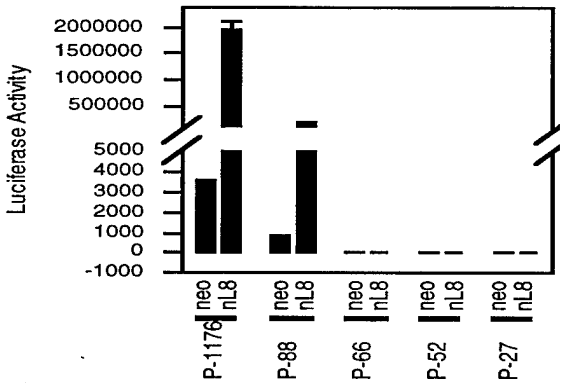

Figure 2. Deletion Analysis of VEGF Promoter Activity.

To determine which region of VEGF promoter is 12-LOX-responsive, we transfected neo-control (neo-α) and 12-LOX transfected PC-3 cells (nL8) with a series of luciferase constructs with different lengths of the VEGF promoter, along with a lacZ control plasmid. As shown in **Figure 2**, deletion of the region between -1176 and -88 significantly reduced, but did not abolish, the increased VEGF promoter activity as observed in nL8. Further deletion of the 23 bp region between -88 and -66 abolished the increased VEGF promoter activity in nL8, indicating the presence of a *cis*-element in this region responsive to 12-LOX. Similar results were obtained with other clones of 12-LOX transfected PC-3 cells (nL2 and nL-12, data not shown).

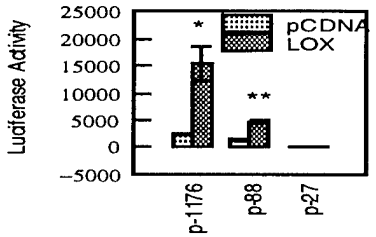

Figure 3. Activation of VEGF Promoter Activity by Co-transfection with a 12-LOX expression construct (LOX). pCDNA, vector control. *, $P < 0.05$; **, $P < 0.01$ when compared to their respective vector control.

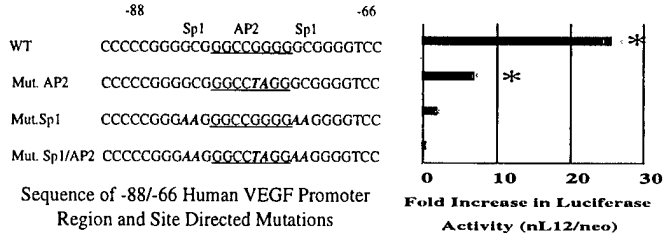

Figure 4. Site-Directed Mutation Analysis of the Role of Sp1 and AP2 Recognition Sequences in VEGF Promoter Activity. Left panel, DNA sequence between -88 and -66 of VEGF promoter. Mutated nucleotides in Sp1 recognition sequences (GC to AA) or AP2 binding site (underlined, GG to TA) are shown in italic. Right panel, fold increase of luciferase activities in nL12 when compared to those in neo-α. *, $P < 0.05$, indicating the significant difference in luciferase activities between neo-α and nL12.

Co-transfection of PC-3 parental cells with both VEGF promoter constructs and a 12-LOX expression construct demonstrated a significant increase in p-1176 and p-88, but not p-27, VEGF promoter activities, as compared with their respective pcDNA3.1 vector control (**Figure 3**). The data suggest that 12-LOX stimulates VEGF promoter activity and the observed increase in VEGF promoter activity in 12-LOX transfected PC-3 cells is not due to possible cloning artifacts associated with a particular transfectant clone. The data also demonstrate that the -88 and -27 promoter region of VEGF gene is responsive to 12-LOX, supporting our previous observation that the 23 bp DNA segment beween -88 and -66 is required for increased VEGF promoter activity in 12-LOX transfected PC-3 cells.

There are one AP2 and two Sp1 binding sites in this 23 bp region. As shown in **Figure 4**, mutation of AP2 decreased, but did not abolish, the stimulation of promoter activity by 12-LOX (from 25 fold to 8 fold increase). In contrast, mutation of two Sp1 binding sites dramatically decreased the stimulation of VEGF promoter activity by 12-LOX in which the increase of VEGF promoter activity in nL12 was no longer statistically significant. Mutation of both AP2 and Sp1 binding sites completely abolished the increased VEGF promoter activity in 12-LOX transfected PC-3 cells. The data suggest that Sp1, and to lesser extent, AP2, are involved in 12-LOX regulation of VEGF promoter activity.

2). Overexpression of the platelet-type 12-LOX in PC-3 cells increases DNA binding and transcriptional activities of NF-κB and promotes IκBα degradation.

In addition to Sp1 and AP2, NF-kB was demonstrated important for VEGF expression. Therefore we tested the effect of overexpression of platelet-type 12-LOX on the activation state of NF-κB in prostate cancer cells. For this study, we have used PC-3 prostate cancer cell line that was stably transfected with platelet-type 12-LOX. Two 12-LOX overexpressing clones (nL-8 and nL-12), a vector only transfected clone (neo), and the native PC-3 cells were used for the experiments. The effect of 12-LOX overexpression on the activation of NF-κB was studied using Electomobility Shift Assays (EMSAs), western blotting for IκBα, and transcriptional activity with luciferase reporter assay.

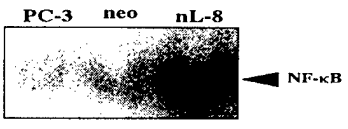

Figure 5: Effect of 12-lipoxygenase overexpression on NF-κB activity:EMSA performed on nuclear extracts of untransfected PC-3, neo, and 12-LOX transfected cells

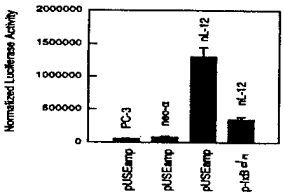

Figure 6: Effect of 12-lipoxygenase overexpression on NF-κB activity: NF-κB-luciferase reporter activities. The cells (PC-3, neo, and nL-12) were transfected with κB-luciferase and LacZ reporters (pUSEamp) and the reporter activities measured. 12-LOX transfected cells were also transiently transfected with a dominant negative mutant IκBα construct (p-IκBdn) in addition to κB-luciferase and LacZ reporters. The normalized data shown is an average of three experiments with standard deviation. LacZ expression was used for normalization.

Nuclear protein extracts of 12-LOX transfected cells (nL-8) showed significant constitutive activation of NF-κB compared to the vector transfected control cells (neo) or the untransfected PC-3 cells (**Figure 5**). This activation was confirmed by the increased transcriptional activity of the luciferase reporter construct in nL-12 cells that were transiently transfected. (**Figure 6**). This increase in transcriptional activity observed in 12-LOX transfected cells was nearly abolished upon co-transfection of a mutant of IκBα that is resistant to proteolytic degradation (**Figure 6**). Activation of NF-κB involves phosphorylation and eventual degradation of IκB protein before NF-κB could bind to DNA. Western blots of whole cell protein extracts from neo, nL-8, and nL-12 cells showed a dramatic decrease in IκBα in nL-8 and nL-12 cells (data not shown). These results strongly suggest that overexpression of 12-LOX induces NF-κB activity by a mechanism involving proteolytic degradation of IκBα.

3). 12(S)-HETE activates NF-κB in PC-3 prostate cancer cells.

The fact that 12(S)-HETE, the stable end product of 12-LOX metabolism of arachidonic acid, mediated the activation of NF-κB was demonstrated by the increased DNA binding activity of NF-κB when native PC-3 cells were treated with 12(S)-HETE (**Figure 7**). The 12(S)-HETE driven increase in NF-κB DNA binding also paralleled the increase in transcriptional activity as determined by the NF-κB-luciferase reporter assay (**Figure 8**).

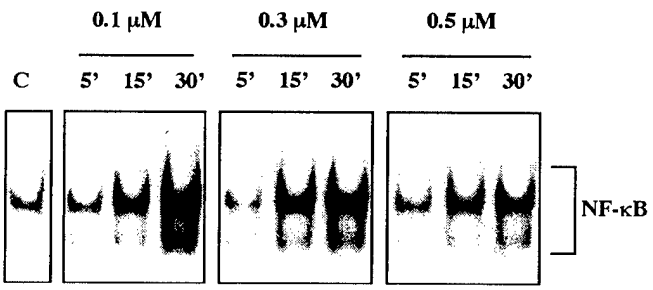

Figure 7: *Effect of 12(S)-HETE on the activation of NF-κB in PC-3 cells:* Time and dose dependent activation of NF-κB DNA binding activity by 12(S)-HETE. EMSA was performed on the nuclear extracts of the treated PC-3 cells. The cells were incubated with serum-free RPMI medium containing the amounts of 12(S)-HETE shown for the indicated time and subjected to EMSA.

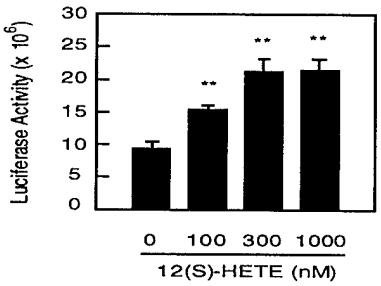

Figure 8: NF-κB-luciferase reporter activities of cells treated with 12(S)-HETE. PC-3 cells were incubated at indicated concentrations for 30 min and luciferase activities measured. **, $p < 0.01$.

Additionally, immunocytochemical analysis of the 12(S)-HETE treated PC-3 cells using antibodies against p65 subunit of NF-κB show a translocation of NF-κB from cytosol to the nucleus (**Figure 9**). Thus, the observations made with 12(S)-HETE corroborate those of 12-LOX overexpressing PC-3 prostate cancer cells.

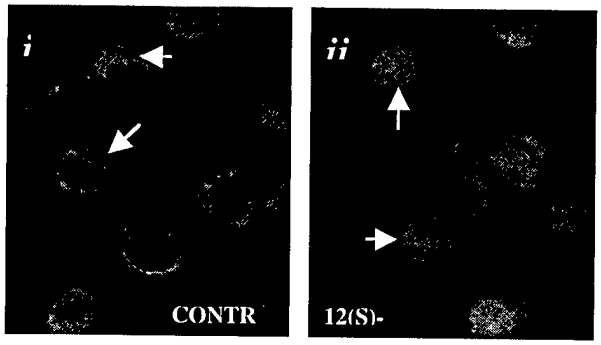

Figure 9: Immunofluorescent localization of NF-κB in PC-3 cells with and without 12(S)-HETE treatment. Cells were treated with 100 nM 12(S)-HETE or buffer for 10 min and immunostained. Staining was predominantly present in the cytoplasm of untreated control cells (*i, arrows*) and nuclear staining increased considerably in 12(S)-HETE treated cells (*ii, arrows*).

4). NF-κB activation is inhibited by 12-LOX inhibitor. It is conceivable that the observations made with the 12-LOX overexpression system and 12(S)-HETE are mutually independent and follow a different, yet unknown, mechanism. To address this possibility, we used BHPP, a specific inhibitor of 12-LOX, to study the role of the enzymatic activity of 12-LOX in NF-κB activation. DNA binding activity of NF-κB was greatly decreased upon exposure to 20 μM BHPP for 60 min (**Figure 10**). The results show the participation of the 12-LOX enzymatic activity in NF-κB activation and that it is mediated by 12(S)-HETE. Currently we are actively studying the possible involvement of this transcriptional factor in 12-LOX regulation of VEGF expression in human prostate cancer cells.

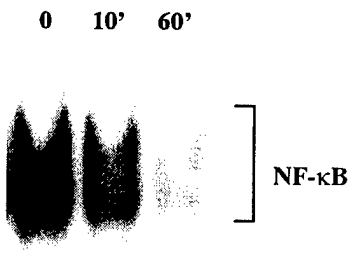

Figure 10. Inhibition of NF-kB activation in 12-LOX transfected PC-3 cells by BHPP, a select 12-LOX inhibitor.

Task 2. Study the signaling pathways involved in 12-LOX regulation of VEGF expression, Months 1-18:

This task has been mostly achieved. Specifically, we found an involvement of PI3 kinase and Akt signaling pathway in 12-LOX stimulated VEGF gene expression.

To study the signaling mechanism from 12-LOX leading to VEGF expression in PC-3 cells, we treated 12-LOX transfected PC-3 cells with PD98059, a Mek inhibitor, and LY294002, a PI3 kinase inhibitor, and studied VEGF expression. As shown in **Figure 11**, LY294002 (20 μM) reduced VEGF expression in both 12-LOX transfected PC-3 cells (nL-8 and nL-12) and neo-controls (neo-α and neo-σ), as well as in PC-3 parental cell line. We also found significant reduction of VEGF expression in DU145 cells by LY294002 data not shown). In contrast, we did not find any effect of PD98059 on VEGF expression in PCa cells (data not shown). The results suggest that PI3 kinase activity is required for VEGF expression in PCa cells as well as in 12-LOX transfected PC-3 cells.

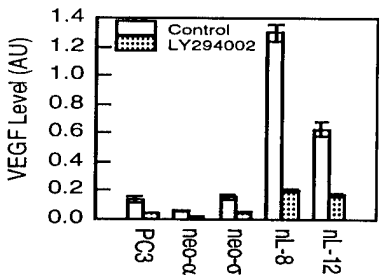

Figure 11. Down-regulation of VEGF Expression by PI3 Kinase Inhibitor LY294002.

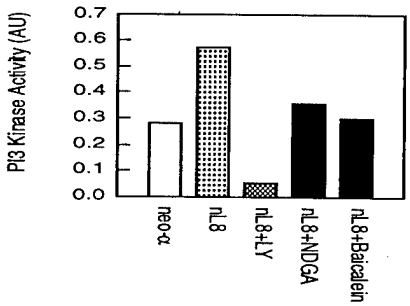

Figure 12. Increased PI3 Kinase Activity in 12-LOX Transfected PC-3 Cells. PI3 kinase activity in p85 immunoprecipitates was measured by incubating with substrate and γ-^{32}P-ATP. Phospholipids were separated by TLC, X-filmed and quantified using a NIH imaging software.

To study whether there is an increase in PI3 kinase activity in 12-LOX transfected PC-3 cells that may mediate the stimulation of VEGF expression, we measured the PI3 kinase activity. As shown in **Figure 12**, there was a 2 fold increase in PI3 kinase activity in 12-LOX transfected PC-3 cells (nL8) and this increase of PI3 kinase was inhibited by pre-treatment of nL8 cells with LOX inhibitors NDGA and baicalein. The data suggest that there is an increase in PI3 kinase activity in 12-LOX transfected PC-3 cells and inhibition of PI3 kinase activities by LY294002 reduced VEGF expression in 12-LOX transfected PC-3 cells and other PCa cells.

We investigated whether the arachidonate product of 12-LOX, 12(S)-HETE, can activate PI3 kinase. We used A431 cells, instead of PC-3 cells, in this study because there is low endogenous level of PI3 kinase activity under serum free condition but readily stimulated in A431 cells. We found 12(S)-HETE activates PI3 kinase /Akt in A431 cells and the activation of PI3 kinase is required for 12(S)-HETE activation of Erk1/2. Currently we are extending the findings obtained using A431 cells into PC-3 cells and prostate cancer cells.

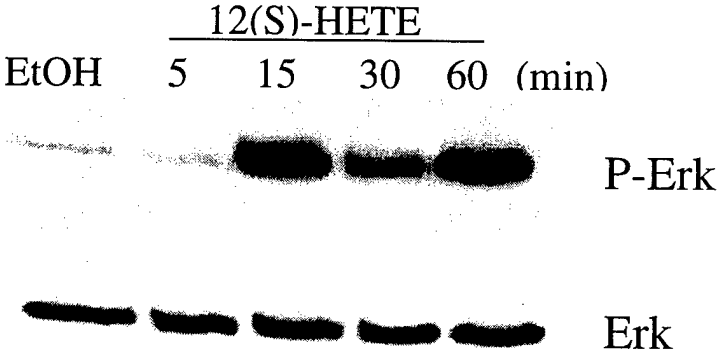

Figure 13. Activation of p42/44 MAP kinase by 12(S)-HETE in LNCaP cells. LNCaP cells were serum starved overnight and treated with 300 nM 12(S)-HETE for indicated times. Activation of p42/44 MAP kinase was indicated by the levels of phosphorylated form of Erk (upper panel), in comparison with the level of total Erk protein (bottom panel). As shown in the figure, the activation of p42/44 MAP kinase by12(S)-HETE is evident with 15 minutes of treatment.

Task 3. Evaluate the efficacy of 12-LOX inhibitors against angiogenesis induced by PCa cells using Matrigel implantation model, Months 1 -9:

In Phase I of this grant, we found that 12-LOX functions as an "angiogenic switch," governing PCa angiogenesis and growth. The findings suggest that 12-LOX may be a promising target for developing anti-angiogenesis therapy, at least for those patients whose PCa is 12-LOX positive. Previously, Dr. Honn, in collaboration with Dr. Carl Johnson (Department of Chemistry, Wayne State University) synthesized several classes of hydroxamic acid derivatives, using rational drug design for inhibition of platelet-type 12-LOX (US Patent No. 5,234,933). Compounds were initially screened for inhibition of platelet-type 12-LOX and a select group of them later tested for selectivity. The first lead compound was BMD188 which inhibits 12-LOX with IC_{50} of 3 μM. BMD188 was initially chosen for study because of its ease of synthesis in bulk, its stability and preliminary pharmacokinetic data indicates a circulating half-life in mouse of 50 hours. To demonstrate the plausibility of using 12-LOX inhibitors to reduce PCa angiogenesis and tumor growth, we first tested the effect of BMD188 on angiogenesis induced by 12-LOX transfected PC-3 cells in Matrigel plug assay. Athymic mice were injected with 2 X 10^6 12-LOX transfected PC-3 cells suspended in Matrigel. After 24, mice were injected (i.p.) with 20, 60 or 100 mg BMD188 per kg of mouse weight every other day for a total of 4 treatments. A dose dependent inhibition of angiogenesis was observed (**Figure 14**).

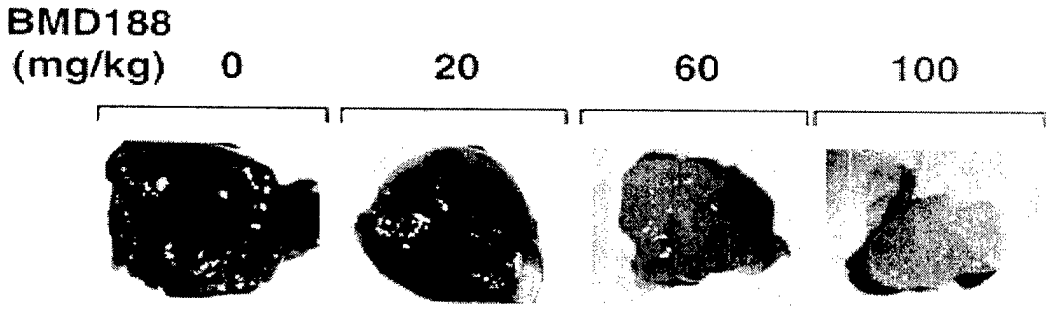

Figure 14. Inhibition of PCa cell induced angiogenesis by BMD188.

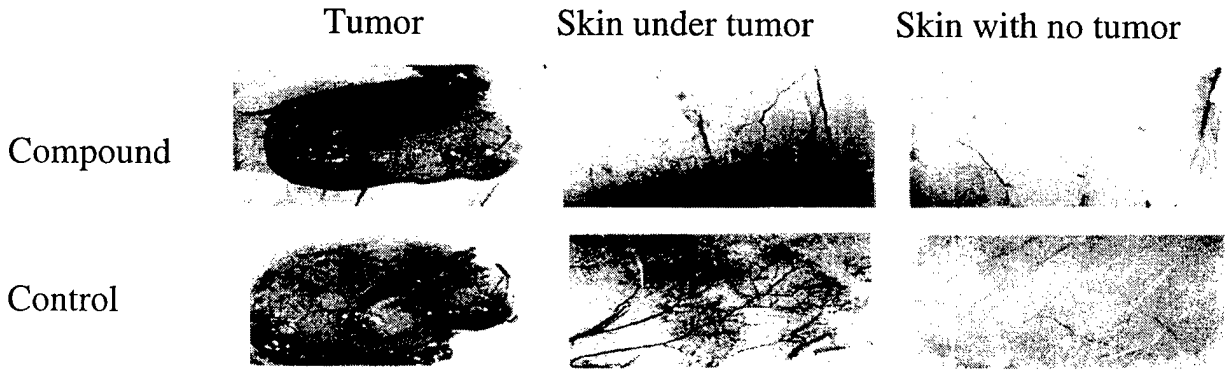

Figure 15. Inhibition of tumor angiogenesis by systematic administration of BMD188 (50 mg/Kg/day)

Using a SCID-human bone model (Nemeth et al., 1999), we studied whether BMD188 can inhibit the growth of tumors in human bone environment. As shown in **Figure 16**, at 50 or 100 mg BMD188 per kg of mouse weight, significant reduction in tumor growth was observed. Considerable reduction in angiogenesis

on the surface of tumor or on the skin around tumors also was observed. The results raise the exciting possibility that inhibition of 12-LOX is a novel approach to inhibit tumor angiogenesis and curb prostate tumor growth (**Figure 15**).

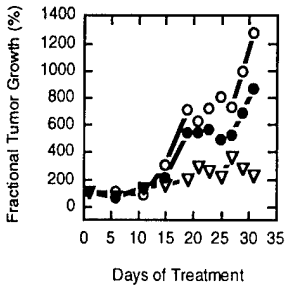

Figure 16. Inhibition of Tumor Growth in SCID-Hu Bone by BMD188. Open circle, Solvent control; Filled circle, BMD188 50mg/Kg mouse; Open triangle, BMD188, 100mg/Kg mouse.

We also find HA215 inhibit bFGF- and VEGF-induced endothelial cell migration. Further study is under way to study whether HA215 inhibits tumor angiogenesis and growth (**Figure 17**).

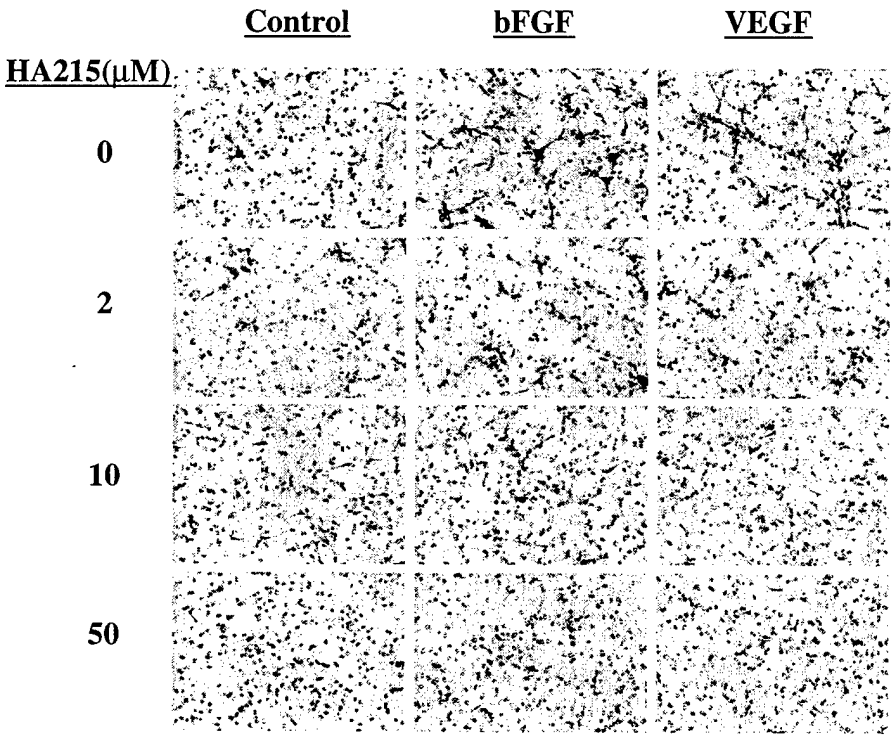

Figure 17. Dose-dependent inhibition of bFGF- and VEGF-induced endothelial cell migration by HA215.

CONCLUSIONS:

The research progress made in the first year of Phase II of Award# DAMD17-98-1-8502 indicates that 12-LOX increases VEGF expression in human prostate cancer cells in a PI3 kinase/Akt dependent pathway. We also found 12-LOX and its arachidonate product, 12(S)-HETE, activate NF-κB transcriptional activity and p42/44 MAP kinase activity in human prostate cancer cells. The link between 12-LOX, a free fatty acid metabolizing enzyme, and VEGF, a putative angiogenic factor, is both novel and exciting, providing significant insights into our understanding of the regulation of VEGF expression during PCa progression. The study also found the transcriptional regulation of VEGF expression by 12-LOX and by its lipid product, 12(S)-HETE. The study also demonstrated the plausibility of using 12-LOX inhibitors such as BMD188 to inhibit prostate tumor angiogenesis and growth.

REPORTABLE OUTCOMES

- Research article published.
  Szekeres, C.K., Trikha, M., Nie, D., and Honn, K.V. 2000. Eicosanoid 12(S)-HETE activates phosphatidylinostol 3-kinase. Biochem. Biophys. Res. Commun. 275: 690 – 695.
- Research article published.
  Timar, J., Raso, E., Dome, B., Li, L., Grignon, D., Nie, D., Honn, K.V., and Hagmann, W. 2000. Expression, subcellular localization and putative function of platele-type 12-lipoxygenase in human prostate cancer cell lines of different metastatic potential. Int. J. Cancer 87: 37 – 43.
- Research article published.
  Nie, D., K. Tang, C. Diglio and K. V. Honn. 2000. Eicosanoid regulation of angiogenesis: Role of endothelial arachidonate 12-lipoxygenase. Blood 95: 2304 - 2311.
- Research article published.
  Tang, K., Finley, R.L. Jr, Nie, D., and Honn, K.V. 2000. Identification of 12-lipoxygenase interaction with cellular proteins by yeast two-hybrid screening. Biochemistry 39: 3185 – 3191.
- Abstract published.
  Nie, D., J. A. Nemeth, M.L. Cher, Y. Chen, U. Barrosso, and K.V. Honn. 1999. Inhibition of prostate cancer cells by a novel 12-lipoxygenase inhibitor in a human orthotopic bone metastasis model. Proc. Amer. Assoc. Cancer Res. 40: 126.
- Abstract published.
  Nie, D., Y. Tang, K. and K.V. Honn. 2001. Signaling pathways in VEGF expression in human prostate cancer cells. Proc. Amer. Assoc. Cancer Res. 42: 940.
- Abstract.published.
  Nie, D., Y. Chen, K. Tang, J. Milanini, G. Pages, D. Grignon, and K.V. Honn. 2000. Arachidonate 12-lipoxygenase stimulates VEGF expression in human prostate cancer cells. Proc. Amer. Assoc. Cancer Res. 41: 792.
- Presentation.
  Nie, D., Y. Chen, K. Tang, G.G. Hillman, D. Grignon, and K.V. Honn. "Arachidonate 12-lipoxygenase stimulates angiogenesis by up-regulation of vascular endothelial growth factor expression." Oral Presentation Selected by the 6th International Conference on Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation and Related Diseases, Boston, MA, Sept. 12 -15, 1999.
- Development of animal models: Yes.
  We developed a formula for administering BMD188 for study of inhibition of PCa cell induced angiogenesis in animal model.