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TITLE: Prevention of Post-Radiotherapy Failure in Prostate Cancer by Vitamin D

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14. ABSTRACT Prostate cancer patients receive either surgery or radiation therapy as treatment for cancer. Among patients receiving radiation therapy, nearly 50% have an elevation of PSA within five years of treatment. These patients then receive hormone treatment. In this study, we wish to test the theory that chemopreventive agents, which shoe the ability to prevent or delay the growth of prostate cancer cells in the laboratory, may also prevent or delay the growth of prostate cancer cells in the laboratory, may also prevent or delay the reappearance of prostate cancer in patients who have undergone radiation to treat their prostate cancer. We propose to have prostate cancer patients who have undergone radiation treatment take a non-toxic chemopreventive agent [a synthetic form of vitamin D, 1 ~-hydroxyvitamin D5] for two years and see if their reoccurrence rate can be decreased. Unlike regular vitamin D, D5 does not make calcium in the bloodstream and reach levels that cause serious side effects. Forty patients will participate. They will be randomized to D5 or placebo arms. A biopsy will be done at the end of the study and the tissue will be analyzed for any benefit of D5 in decreasing the recurrence of prostate cancer and also for any differences between the groups in terms of expressed intermediate molecular biomarkers.					
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I. INTRODUCTION

We plan to conduct a phase I/II safety/chemoprevention study to determine whether taking a non-toxic Vitamin D analog, $1\alpha(\text{OH})\text{D}_5$ (D5), can safely delay prostate cancer recurrence when administered after radiation therapy (RT). The newly synthesized analog $1\alpha(\text{OH})\text{D}_5$ (1α -Hydroxy-24-ethyl-cholecalciferol) has shown anti-tumor activity at non-hypercalcemic concentrations in animals. Based on our preliminary research, we believe D5 can be given in effective doses without causing harmful side effects. Forty randomized patients will receive either D5 or placebo, 12-60 months after completion of RT (20 patients/arm). During the study patients will be closely monitored for hypercalcemia as well as other potential toxicities. At the end of the study, subjects will receive final laboratory and clinical evaluations and undergo a prostate biopsy. Study endpoints include differences between study groups in drug tolerance and compliance, toxicity, quality of life, biomarker presence and proportion of patients developing PSA-based biochemical failure or clinical failure. Biopsies will be evaluated for selective markers indicating any benefit of D5 in decreasing the recurrence of prostate cancer and also for any differences between the groups in terms of expressed intermediate molecular biomarkers. Patients will continue to be followed for any clinical recurrences or toxicity as part of their usual cancer care.

II. BODY

2.1. The following are the tasks for this study:

Task		Progress
Task 1	Obtain necessary clinical trial approvals.	Done except FDA approval.
Task 2	Register patients to start the clinical study.	Not yet initiated
Task 3	Following up patients on study.	Not yet initiated
Task 4	Complete the clinical study.	Not yet initiated
Task 5	Follow up patients with Vitamin D treatments.	Not yet initiated

2.2. With regard to Task 1, the following includes work done and accomplishments

Date	Progress
February, 2004	Grant was officially transferred from the University of Illinois at Chicago (UIC) to the University of California, Davis (UCD), a necessary step in allowing us to conduct the study once we obtain IRB approval at UCD and DOD approval.

March, 2004	Completion of Clinical Protocol and Approval by UC Davis IRB. Our principal accomplishment during this period was finalizing the clinical protocol for the study with D5 and securing the approval, with pending minor revision, by the UC Davis IRB for the clinical trial (See Appendix 6 submitted with 2004 Annual Report). On March 8, 2004, the UC Davis IRB met and approved the protocol, pending minor revisions. Revisions (mostly wording) were done and the protocol to resubmitted to the IRB Committee Chair for final approval. The development of the clinical protocol began by taking into account the critique of the protocol made by the UIC Cancer Center Protocol Review Committee in July 2002. While at UIC, Dr. Vijayakumar brought the protocol to about 80% completion. He had set up an Executive Committee to prepare the protocol, and they met several times to design the study. (Minutes were submitted to the DOD previously). Further fine-tuning occurred at UC Davis. In 2003, Dr. Vijayakumar shared the protocol with UCD Radiation Oncology faculty at regular faculty meetings, seeking their input on how to improve the protocol and incorporating their suggestions. Attendees at these meetings were Radiation Oncologists Dr. Allan Chen, Dr. Rachel Chou, Dr. Zelanna Goldberg, Dr. Samir Narayan and Dr. Janice Ryu, and Physicists Dr. Julian Perks, Dr. Robin Stern, and Dr. Claus Yang. In addition, over several months in the fall of 2003, Dr. Vijayakumar consulted extensively with the statistician for the UCD Cancer Center, Dr. Laurel Beckett, to confirm and modify the study design. Dr. Vijayakumar also recruited other investigators for the protocol, especially clinical faculty who will be enrolling patients in the trial, and assembled the rest of his team for the study (Clinical Research Associates, consultants). In October 2003, Dr. Vijayakumar made a presentation to discuss the protocol with several UCD Cancer Center faculty. At the meeting was the director of the Cancer Center, Dr. Ralph deVere White (Urology), as well as Dr. Samir Narayan (Radiation Oncology), Dr. Paul Gummerlock (Hematology & Oncology), Dr. Rajendra Mehta—via speaker phone (Surgical Oncology, UIC), Dr. William Hall (Radiation Oncology), and Phil Boerner (Writer, Radiation Oncology). As a result of this meeting, several important modifications were made to the protocol, including adjusting eligibility criteria, study endpoints, and having a data and safety monitoring committee review the study periodically once it commences.
November, 2003	Before submitting the updated protocol to the UC Davis Cancer Center Scientific Review Committee, Dr. Vijayakumar wanted to have input from the DOD's pre-review. Dr. Vijayakumar received the DOD pre-review of the Vitamin D5 study, and incorporated the valuable suggestions made there into the protocol.

December 2003	Dr. Vijayakumar made a presentation to the UCD Cancer Center Scientific Review Committee and subsequently this committee approved the D5 protocol (see Appendices 1 and 2 submitted with 2004 Annual Report). (This committee's approval is required prior to submitting a protocol to the UCD IRB.) On the advice of this committee, we added a "Treatment Plan" section to the protocol.
February 19, 2004	The D5 protocol was submitted to the UC Davis IRB (the D5 protocol was submitted to the UC Davis IRB (see Appendix 5 submitted with 2004 Annual Report). The protocol was approved, pending minor revision, on March 8, 2004. When we make the minor revision and obtain final IRB approval, we will submit the protocol to the DOD for approval.
October 26, 2004	Updated our Statement of Work (SOW) (see Appendix 1 submitted with 2005 Annual Report).
November 4, 2004	Since the process of required approvals is taking longer than expected, we requested and received a no-cost extension from the DOD for the study, to February 2006 (see Appendix 2 submitted with 2005 Annual Report).
December 6, 2004	Obtained DOD approval for the study (see Appendix 3 submitted with 2005 Annual Report).
December 15, 2004	Obtained UC Davis IRB re-approval for the study, accepting the DOD's changes (see Appendix 4 submitted with 2005 Annual Report).
February 22, 2005	Requested annual renewal of this study with our IRB (see Appendix 5 submitted with 2005 Annual Report).
September 2005-January 2006	Please note Appendix 1, 2006 Annual Report to view papers/publications resulting from scholarly work of Dr. Vijayakumar and his colleagues.
January, 2006	FDA is requiring repeat stability testing of study drug. An India-based company named SaidruSyn has been contracted to do this. This company has a great deal of experience working with the FDA (see Appendix 2, 2006 Annual Report).
February 8, 2006	No-Cost Extension requested (see Appendix 2, 2006 Annual Report).
February 28, 2006	Additional information E-mailed to Wendy Baker to attach to No Cost Extension Request (see Appendix 1, 2006 Annual Report).
March 9, 2006	No-Cost Extension approved for one year. Amendment/Modification #P00004 attached as Appendix 3, 2006 Annual Report.
January 27, 2007	No-Cost Extension approved for 3 months. Amendment attached as Appendix 3, 2007 Final Report

We aggressively pursued FDA approval for the study drug, but have not yet obtained approval (see Appendix 2 for most recent copies of email correspondence regarding FDA approval).

III. KEY RESEARCH ACCOMPLISHMENTS

As this was a clinical study, only findings generated from the clinical trial portion could have been considered key research accomplishments. Since clinical trials never occurred due to lack of approval of the study drug by the FDA, our accomplishments during the grant period include the following:

Laboratory studies:

(A)

Summary

Vitamin D3 (Calcitriol) has been used both alone and in combination with chemotherapeutic agents such as Docetaxel to suppress the growth of prostate tumors. However vitamin D3 has also been shown to upregulate the levels of androgen receptor in prostate tumor cells in culture and in addition has been linked to dose-limiting hypercalcemia. Here we confirm those data indicating that 0.1 μ M vitamin D3 substantially increases the expression of androgen receptor protein, starting 4 days after vitamin treatment. This increase in androgen receptor was linked to a similar increase in PSA. Vitamin D5 reportedly exhibits reduced hypercalcemia in animal models making it a more attractive molecule for therapeutic use. Using doses of vitamin D3 and D5 that were equivalently cytostatic, as determined by an MTT assay, vitamin D5 showed a consistently reduced ability to activate both the androgen receptor and its down stream target, PSA. This indicates that vitamin D5 presents a more useful profile of biological activities for studies tracking prostate growth using PSA as a surrogate marker.

Methods

MTT Assay LNCaP cells were plated in 24-well tissue culture plates at 2×10^4 /well. Cells were allowed to attach overnight and then treated with either control media (RPMI/5% FCS/0.1% Penicillin/Streptomycin), control media supplemented with vitamin D3 (100nM), or control media supplemented with vitamin D5 (10nM – 2 μ M). Media was refreshed every 72 hours. At designated time points, dimethylthiazolyl-2, 5-diphenyltetrazolium bromide (MTT) was added to the culture supernatant and plates incubated for an additional one hour. Cells were then solubilized with DMSO and absorbance assessed as a measure of MTT uptake.

Western analysis LNCaP cells were plated at 2.5×10^6 cells/dish in 60mm tissue culture dishes and allowed to attach overnight. Cells were then treated with either, control media (RPMI/5% FCS/0.1% Penicillin/Streptomycin), control media supplemented with vitamin D3 (100nM), or control media supplemented with vitamin D5 (10nM – 2 μ M). At designated time points, whole cell lysates were collected and protein concentration determined using the Coomassie Plus Protein Assay (Pierce) following manufacturer's instructions. An equal amount of total protein per lane was fractionated by electrophoresis on either a 10% (PSA) or 4-15% (androgen receptor) SDS-polyacrylamide gel. Subsequent to electrophoresis, gels were transferred to a nitrocellulose membrane and immunoblotting was performed using either anti-PSA, anti-AR or anti-actin, and secondary antibodies coupled to horseradish peroxidase. Blots were developed using Pierce West Pico Chemiluminescent blot detection reagent according to manufacturer's instructions and exposed to film.

Results

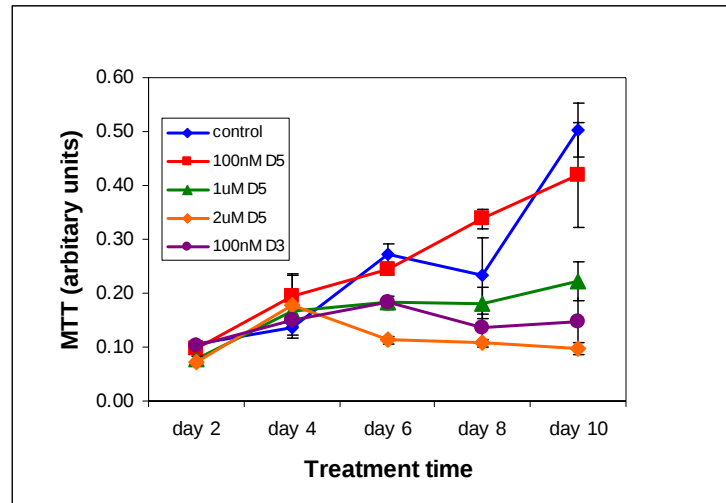


Figure. 1 Anti-proliferative effect of Vitamin D3 and D5. LNCaP prostate cancer cells were exposed to a range (10nM -2 μ M) of Vitamin D5 or 100nM Vitamin D3 for the times shown. Concentrations of Vitamin D5 between 1-2 μ M were found to have an equivalent cytostatic effect as 100 nM Vitamin D3 (other Vitamin D5 concentrations not shown). Thus 1-2 μ M vitamin D5 and 0.1 μ M vitamin D3 were considered of equivalent cytostatic potential.

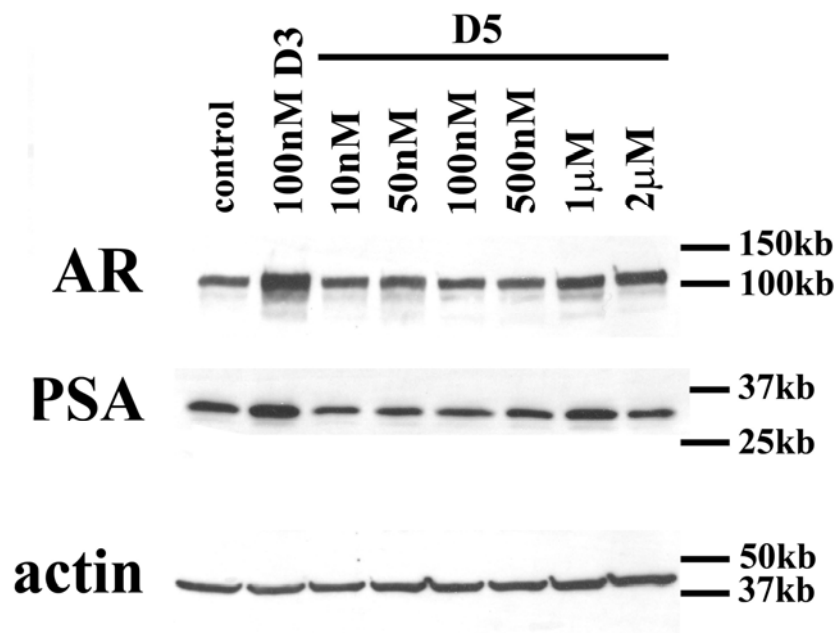


Figure 2.

Androgen Receptor (AR) and PSA protein expression Levels of both androgen receptor and PSA were determined in the LNCaP prostate cancer cell line four days after treatment with Vitamins D3 or D5, at the concentrations shown. At vitamin concentrations that were equally cytostatic, Vitamin D3 treatment was linked to upregulation of both the androgen

Laboratory Studies (continued)

receptor and its transcriptionally regulated target, PSA while cytostatically equivalent concentrations of Vitamin D5 showed minimal effect on the proteins studied.

(B)

Introduction

Carcinoma of the prostate (CaP) is the second leading cause of cancer related deaths among men in the United States. Prostate epithelial cells express the androgen receptor (AR), a transcription factor which regulates the expression of a variety of proteins, including prostate specific antigen (PSA), a serum marker for detection of prostate disease. As prostate epithelial cells are dependent on androgens for growth, the standard treatment for recurrent prostate cancer is androgen withdrawal therapy (AWT). Most recurrent prostate cancer patients initially respond to this treatment, as determined by decreased levels of serum PSA. However, the majority of patients on AWT ultimately progress to an androgen independent state in which AWT has no effect on cancer growth. There is currently no established therapy known to cure androgen-independent prostate cancer (AIPC).

In vivo and *in vitro* studies have demonstrated that the naturally occurring active metabolite of vitamin D, 1,25 dihydroxy D3 (calcitriol), inhibits proliferation and increases differentiation of numerous cancer cell types, including CaP. However, clinical use of calcitriol is severely limited because its anti-tumor activity is achieved at doses that cause hypercalcemia *in vivo*, both in animal models and in human patients as demonstrated by clinical trials. This has led to the development of synthetic analogs of calcitriol that preserve its anti-proliferative and cell-differentiating properties while minimizing or eliminating its toxic profile. One such analog, 1 α (OH)D5, has been successfully tested *in vivo* in a rat model of breast cancer and was demonstrated to inhibit tumor growth but did not induce hypercalcemia at any dose tested (up to 100 nmoles/kg diet). In this study, we evaluate the effects of 1 α (OH)D5 on the growth and biochemical analysis of the androgen dependent LNCaP prostate cancer cell line and its androgen-independent sublines, C4-2SA and LNCaP-AI. LNCaP-AI cells were obtained by long-term culture of LNCaP cells in androgen-reduced media, while C4-2SA cells are a clonal derivative of C4-2 cells, which were derived from LNCaP xenografts in castrated mice.

Cell Culture

LNCaP, LNCaP-AI and C4-2SA cells were maintained in RPMI with phenol red, 5% FBS, and 0.1% penicillin/streptomycin (control media). For experiments, cells were plated with either control media or RPMI media with 5% charcoal stripped FBS and 0.1% penicillin/streptomycin without phenol red (androgen-free media).

Treatment Conditions

Cells were treated with Vitamin D analogs as follows:

- control media or androgen free media
- control media supplemented with 1,25(OH)2D3 (calcitriol, 100nM) or 1 α (OH)D5 (100nM – 2 μ M)
- androgen free media supplemented with 1,25(OH)2D3 (calcitriol, 100nM) or 1 α (OH)D5 (100nM – 2 μ M)

The treatments were replaced every 48-72 hours.

Transfection

For some experiments, cells were transfected with either androgen-receptor specific siRNA (AR-siRNA), vitamin D receptor specific siRNA (VDR-siRNA), or non-specific siRNA (control siRNA) at least 4 hours prior to treatment with vitamin D analogs. At indicated time points, cells were either assessed for proliferation by MTT assay or cell lysates were collected for protein expression analysis by immunoblotting.

MTT Assay

Dimethylthiazolyl-2,5-diphenyltetrazolium bromide (MTT, 5mg/ml in PBS) was added to the media and the cultures were incubated for one hour at 37°C in 5% CO₂. DMSO was then added to dissolve the cells and the absorbance was read at 595nm.

Immunoblotting

Whole cell lysates were collected and analyzed by Coomassie or BCA assay to determine relative protein concentration for equal loading on SDS-PAGE gels and transfer to either PVDF or nitrocellulose membranes. The membranes were probed with indicated primary antibodies and appropriate secondary antibody conjugated to horseradish peroxidase, developed with ECL reagent, and exposed to film.

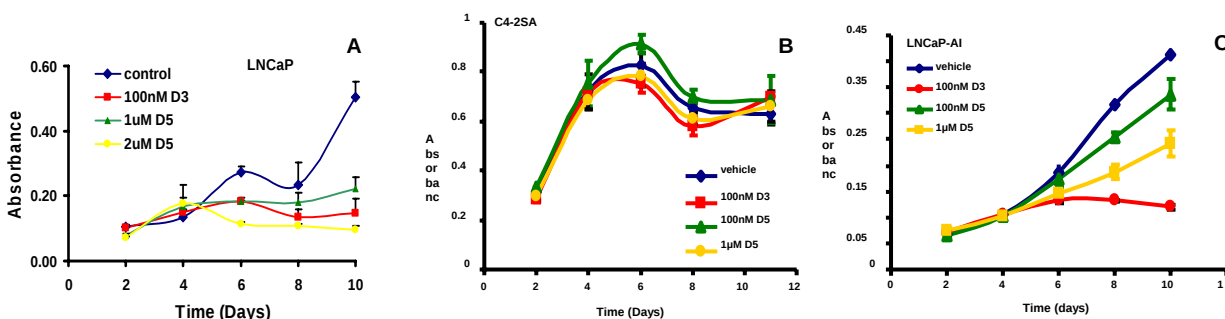


Figure 1: (A) Androgen-dependent LNCaP prostate cancer cells were exposed to either calcitriol (D3) or 1 α (OH)D5 (D5) for the times shown and proliferation estimated by MTT assay. 1-2 μ M 1 α (OH)D5 had an equivalent cytostatic effect as 100nM calcitriol. This is consistent with *in vivo* studies in a breast cancer model with 1 α (OH)D5 in which concentrations 10X greater than calcitriol were required for an equivalent effect on tumor growth and progression. [1] (B-C) Effect of calcitriol and 1 α (OH)D5 on proliferation of androgen-independent prostate cancer cells. Although LNCaP-AI cells were significantly inhibited by 100nM calcitriol and 1 μ M 1 α (OH)D5, neither analog had a significant effect on proliferation in C4-2SA.

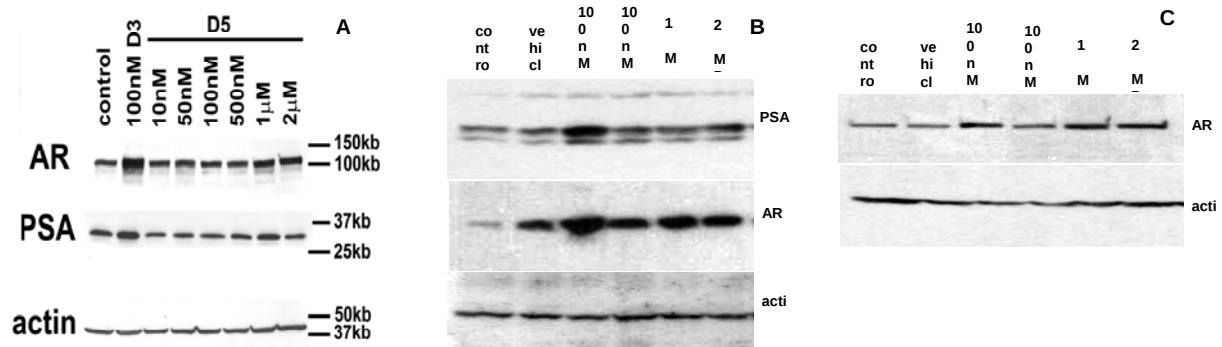


Figure 2: Effect of calcitriol or $1\alpha(\text{OH})\text{D}_5$ on protein expression in prostate cancer cell lines. (A) Levels of androgen receptor (AR) and PSA were assessed by immunoblotting in androgen-dependent LNCaP cells. After 8 days of treatment with either $1\alpha(\text{OH})\text{D}_5$ or calcitriol at the concentrations shown calcitriol has a pronounced effect on expression of the AR. However, $1\alpha(\text{OH})\text{D}_5$ at cytostatically equivalent concentrations (1-2 μM) showed minimal effect. Androgen-independent C4-2SA (B) and LNCaP-AI (C) cells were treated with 100 nM calcitriol or increasing doses of $1\alpha(\text{OH})\text{D}_5$. Calcitriol stimulated AR expression in both cell lines and PSA expression in C4-2SA cells. LNCaP-AI cells do not express PSA, hence this protein was not tested in these cells. In contrast, $1\alpha(\text{OH})\text{D}_5$ had minimal effect on the proteins studied. Levels of β -actin were assessed as loading control.

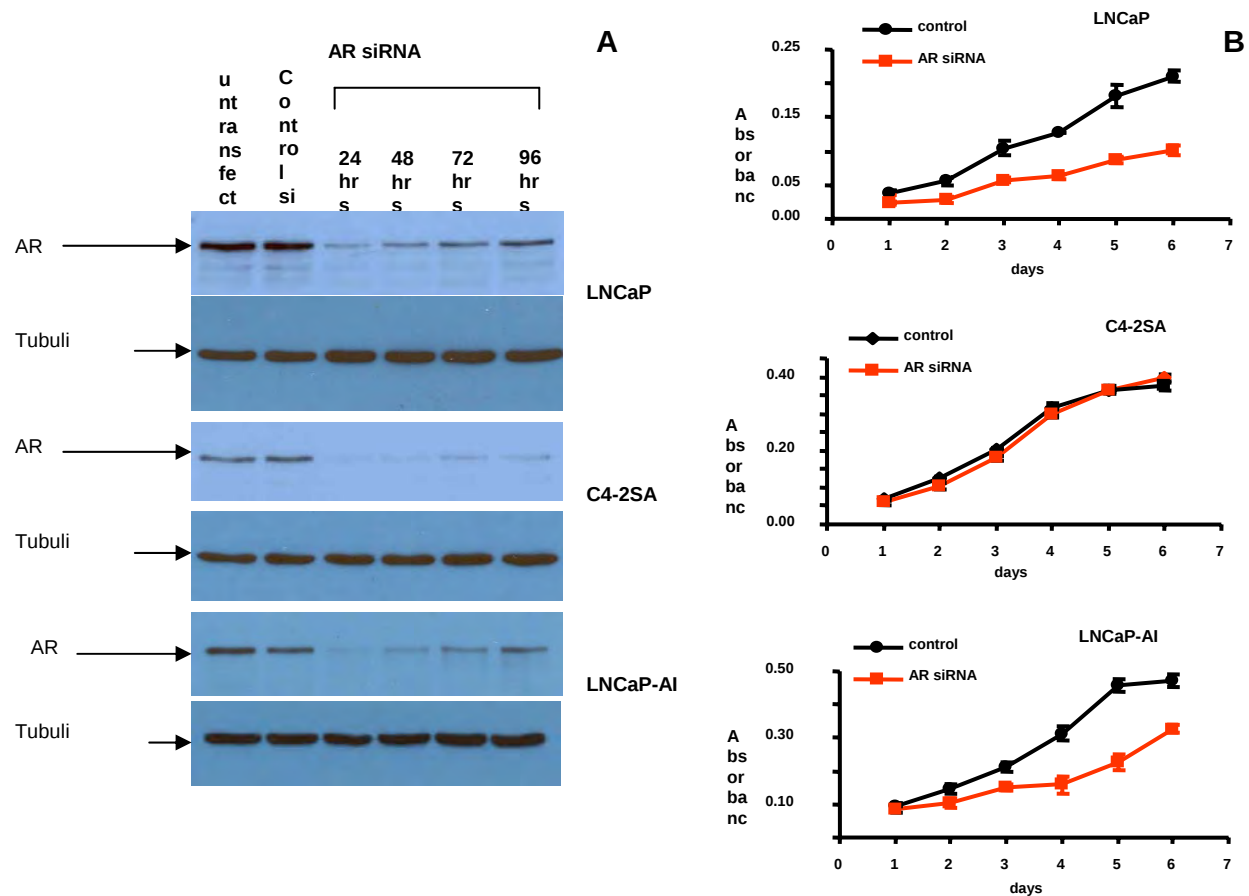


Figure 3: Effect of transfection of AR specific siRNA on protein expression and proliferation in LNCaP, LNCaP-AI, and C4-2SA cell lines. (A) Immunoblotting demonstrates that AR-specific siRNA downregulates expression of the AR in LNCaP, LNCaP-AI, and C4-2 cells. (B) Growth rates of the AR-specific siRNA transfected cells vs. control (non-specific) siRNA were estimated by MTT assay at the time points shown. Reduced expression of the AR resulted in decreased cell proliferation in both LNCaP and LNCaP-AI cells. However, in C4-2SA cells, which were resistant to the growth inhibiting effects of vitamin D, there was no effect of downregulation of the AR on cell growth.

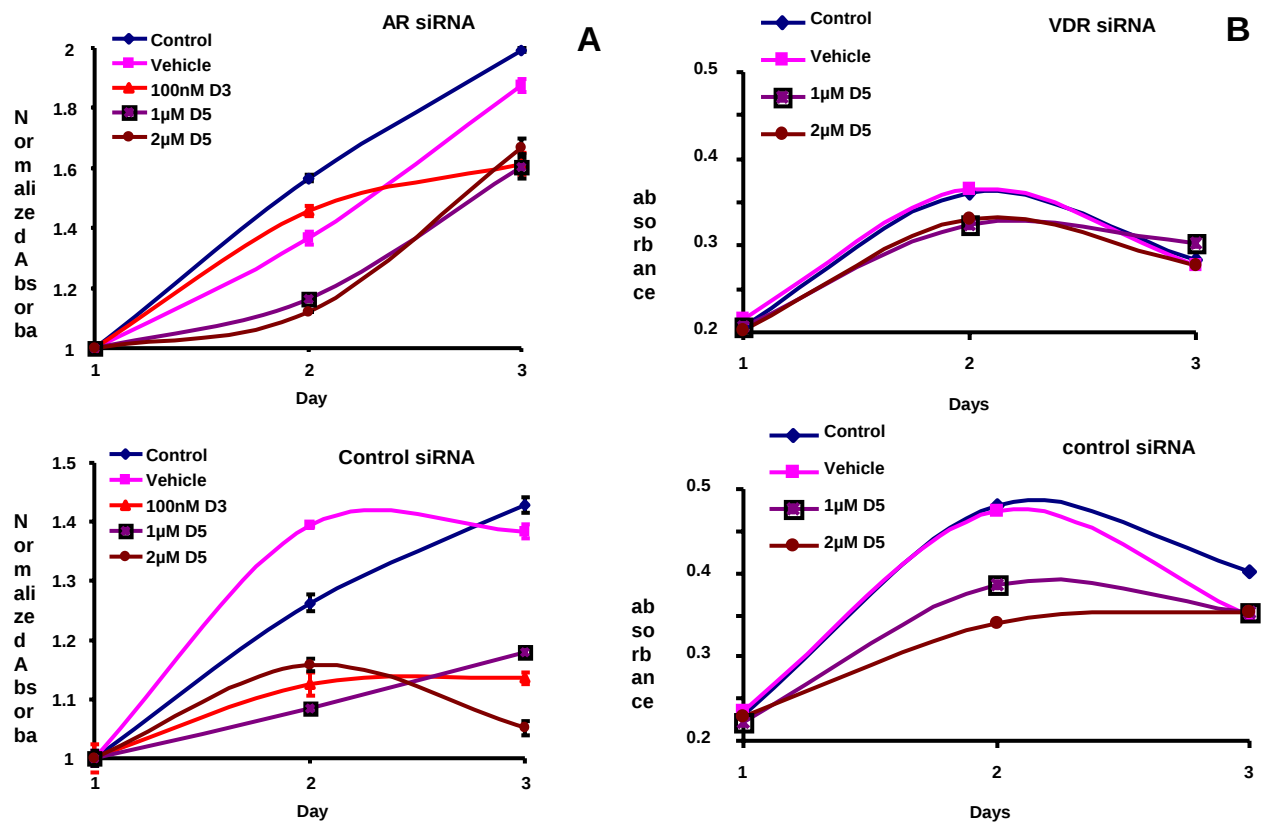


Figure 4: Downregulation of either VDR or AR abrogates the effect of vitamin D analogs. MTT assay showed that either VDR-siRNA (A) or AR-siRNA (B) abrogated the cytostatic effect of calcitriol (D3) or $1\alpha(\text{OH})\text{D5}$ (D5) in androgen-dependent LNCaP cells.

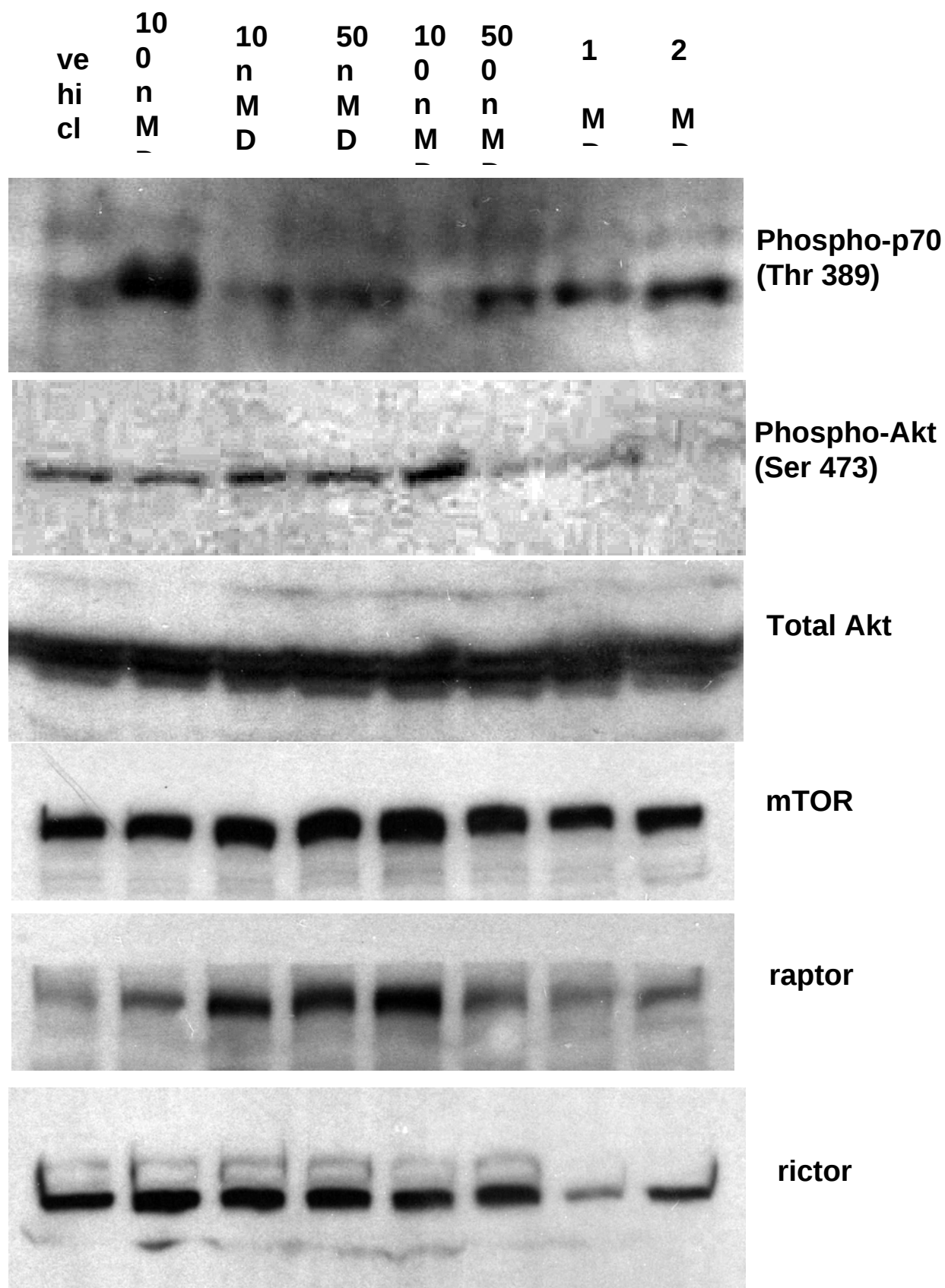


Figure 5: Differential effect of calcitriol and 1 α (OH)D5 on the Akt/mTOR pathway in LNCaP cells by immunoblotting. Panels indicate immunoblotting using various antibodies as indicated. Calcitriol, but not 1 α (OH)D5, caused a significant increase in the phosphorylation levels of p70S6 kinase (upper panel). In contrast, calcitriol did not have a significant effect on Akt phosphorylation, whereas 1 α (OH)D5 inhibited Akt phosphorylation at high concentrations (0.5, 1, 2 μ M) (2nd panel), despite no change in total levels of Akt expression (3rd panel). P70S6 kinase phosphorylation is regulated by mTOR as well as PI3K [21], hence, we also investigated the effect of these hormones on mTOR and its binding partners raptor and rictor [22]. Neither calcitriol nor 1 α (OH)D5 caused any change in mTOR levels (4th panel), whereas 1 α (OH)D5 stimulated raptor expression at low concentrations (5th panel) and inhibited rictor expression at high levels (lowest panel). These results indicate that calcitriol and 1 α (OH)D5 had differential effects on these proteins in LNCaP cells.

IV. REPORTABLE OUTCOMES

See Section 2.2.

V. CONCLUSIONS

We did not initiate the clinical trial portion of this project due to extended delay in acquiring FDA approval for the study. However, a number of accomplishments have been achieved (see Appendix 2 for publications and presentations).

VI. REFERENCES

I. Peer reviewed publications:

2006 Vijayakumar S, Boerner PS, Mehta RR, Packianathan S, Mehta RG, Das Gupta TK. Clinical trials using chemopreventive vitamin D analogs in breast cancer. *Cancer Journal*, 12(6): 445-450.

2005 Vijayakumar S, Mehta RR, Boerner PS, Packianathan S, Mehta RG. Clinical trials involving vitamin D analogs in prostate cancer. *Cancer Journal*, 11(5): 363-73.

2004 Packianathan S, Mehta RR, Mehta RG, Hall W, Boerner PS, Beckett L, Vijayakumar S. *Cancer Journal*, 10(6): 357-367.

II. Scientific Abstracts:

2007 Loreda GA, Moore ME, Moy CV, Mehta RG, Vijayakumar S, Vaughan ATM, Ghosh PM. Vitamins D3 and D5 mediate growth inhibition of androgen-dependent and independent prostate cancer cells by distinct mechanisms of interaction with the androgen receptor. American Association for Cancer Research Annual Meeting, April 17, 2007, Los Angeles, CA.

2006 Loreda GA, Lu XH, Mehta R, Vijayakumar S, Vaughan ATM, Ghosh PM. The low-calcemic vitamin D analog 1-alpha-hydroxyvitamin D5 is anti-proliferative and does not increase androgen receptor expression in prostate cancer cells. American Association for Cancer Research Annual Meeting, April 2, 2006 Washington, DC.

2005 Packianathan S, Vijayakumar S. Intermediate Biomarkers in Male Genitourinary Cancers—Penile Cancer. American Radium Society 87th Annual Meeting, April 30-May 4, Barcelona, Spain (poster).

2004 Vijayakumar S, Meththa R, Mehta R, Hall W, Boerner P, Beckett L. Clinical Trial Design in Chemoprevention Studies: Using a Vitamin D5 Analog Study as an Example. American Radium Society 86th Annual Meeting, May 1-5, Napa Valley, California (poster).

2003 Babbar D, Gandhi M, Mehta RG, Vijayakumar S, Mehta RR. Effects of a 1a-hydroxyvitamin D5 on Prostate Cancer Cells. Proc. Am. Assoc. Cancer Res. 44:1269.

VII. APPENDICES

1. Marillion Pharmaceuticals e-mails: 01/02/2007, 05/16/2007 and 05/21/2007.
2. Papers/publications resulting from scholarly work of Dr. Vijayakumar and his colleagues.
3. Amendment for three-month No-Cost Extension, dated 01/25/2007.
4. Reprint: Vijayakumar S, et al. Clinical trials using chemopreventive vitamin D analogs in breast cancer. Cancer J 2006 Nov-Dec;12(6):445-450.
5. Abstract photocopy: Loreda GA, et al. Vitamins D3 and D5 mediate growth.... AACRA Annual Meeting 2007, Los Angeles, CA.

Appendix 1

From: Zahed Subhan [<mailto:zahed.subhan@marillionpharma.com>]
Sent: Tuesday, January 02, 2007 4:30 PM
To: svijayakum@aol.com
Cc: Mehta Rajendra
Subject: 1a-hydroxy-vitamin D5

Dear Dr Vijayakumar

Further to our recent telephone conversation, I would like to take this opportunity to update you on progress with the Investigational New Drug (IND) application relating to 1a-hydroxy-vitamin D5.

As you may be aware, the IND Sponsor, Professor Tapas Gupta, University of Illinois at Chicago (UIC) submitted a response to the clinical hold imposed by FDA in August of 2005. FDA reverted with additional queries related to Chemistry, Manufacturing and Control (CMC) issues relating to 1a-hydroxy-vitamin D5. UIC was unable to secure timely funding of the analytical studies required to address these CMC issues. Subsequently, 1a-hydroxy-vitamin D5 was licensed to Marillion Pharmaceuticals Inc of Malvern, Pennsylvania.

Marillion Pharmaceuticals has instituted a comprehensive program of analytical method development and validation for both drug substance and drug product. The final clinical trial formulation has been optimized and the first batch of clinical trial supplies prepared and put on stability testing. To date, the cost of "remedial" CMC work has been \$320,000. Marillion hopes to complete the aforementioned studies and submit a response to FDA by February 28th 2007, although this will be contingent on the availability of additional GMP material currently being synthesized by the FDA approved manufacturer.

In summary, I would like to reiterate that every possible effort is being made to enable the clinical hold to be lifted. We are confident that this objective will be achieved shortly and that further evaluation of 1a-hydroxy-vitamin D5 as a potentially valuable new treatment for breast and prostate cancers may be continued at both UIC and UCD.

Please do not hesitate to contact me if I can provide you with any further information.

Kind regards

Dr Zahed Subhan

Zahed Subhan PhD MBA JD
CEO
Marillion Pharmaceuticals Inc
Office: (610) 644-5732
Mobile: (610) 202-5389
Fax: (610) 644 3432
www.marillionpharma.com

(Appendix 1, continued)

"Zahed Subhan"
<**zahed.subhan@marillionpharmma.com**>
05/16/2007 06:12 PM

To "Catherine Metzger" <catherine.metzger@ucdmc.ucdavis.edu>
cc "Srinivasan Vijayakumar"
<srinivasan.vijayakumar@ucdmc.ucdavis.edu>
Subje RE: 1a-hydroxy-vitamin D5
ct

The IND revisions go in May 31st - so we can expect a response from FDA in 30 days following this.

Br

Zahed

-----Original Message-----

From: Catherine Metzger [mailto:catherine.metzger@ucdmc.ucdavis.edu]
Sent: Wednesday, May 16, 2007 6:59 PM
To: Zahed Subhan
Cc: Srinivasan Vijayakumar
Subject: 1a-hydroxy-vitamin D5

Dear Dr. Subhan,

I am working with Dr. Vijayakumar on his final DOD grant report for clinical trials of the D5 analog on which Marillion was doing CMC work. The last e-mail in our file from you about the possible lifting of the clinical hold is dated January 2, 2007. In it, you mentioned that you might be getting a response from the FDA sometime after February 28. Did you ever hear from the FDA?

If you could let either Dr. Vijay or me know the status of the clinical trial supplies, both at Marillion and the FDA response, I would really appreciate it.

Thank you very much for your time.

Sincerely,

Catherine

Catherine Metzger
Senior Writer
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(Appendix 1, continued)

"Zahed Subhan"
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05/21/2007 07:56 AM

To <srinivasan.vijayakumar@ucdmc.ucdavis.edu>
cc

Subj 1-alpha-hydroxy-vitamin D5
ect

Dear Dr Vijayakumar

I am writing to update you on the status of the IND submission for 1-alpha-hydroxy vitamin D5. We have continued to encounter some issues with the capsule formulation – including uniformity and stability. However, these have now been satisfactorily resolved and the IND submission will be sent to FDA on May 31st 2007.

Please do not hesitate to contact me if you have any further questions or queries.

Kind regards

Zahed Subhan

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(Appendix 1, continued)



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19355

May 24th, 2007

Robert Justice, MD, Director
Division of Drug Oncology Products
Center for Drug Evaluation and Research
Food and Drug Administration
5901-B Ammendale Road
Beltsville, MD 20705-1266

Re: IND 56,509 Serial Number 0008
1 α -hydroxyvitamin D5 (MN01) for the treatment of metastatic breast carcinoma
Response to FDA request for information; CMC information amendment

Dear Dr. Justice:

Reference is made to IND 56,509 for 1 α -hydroxyvitamin D5 for the treatment of metastatic breast carcinoma. The 1 α -hydroxyvitamin D5 drug substance and product will hereafter be referred to by the research code MN01.

Reference is also made to a letter from the Division to Dr. Gupta dated September, 7, 2005, regarding chemistry, manufacturing, and controls (CMC) deficiencies and comments for Marillion Pharmaceuticals, Inc.'s (Marillion) investigational drug. Since taking ownership of IND 56,509, Marillion has been working with contract service providers to address the items listed in the Division's letter. This letter is a response to the Division's September 7, 2005 letter. The Division's deficiencies and comments are restated below, followed by Marillion's response.

In addition, an updated CMC section is included in this submission as an attachment. Marillion has secured a fresh batch of MN01 from the drug substance manufacturer, Sai Drusyn Laboratories, developed and qualified necessary analytical methods for assay, related substances, residual solvents, and residual metals for the drug substance, as well as initiated formal stability studies. Marillion has also re-formulated the drug product, manufactured clinical trial supplies, developed and qualified analytical methods for assay, related substances, and dissolution. Formal stability studies of the new drug product are ongoing as described in this amendment.

FDA Comment:

1. Drug Substance manufacturer:

You have indicated that 1 α -hydroxyvitamin D5, with a m.p. of 142-142°C, was manufactured by Sai Drusyn Laboratories, L.B. Nagar, Hyderabad 500035, Andhra Pradesh, India in your resubmission dated 17-Apr-03. A COA of 1 α -hydroxyvitamin D5 from SynQuest, Inc., Chicago, IL was provided in the current resubmission

(Appendix 1, continued)

(Attachment 2). The reported m.p. was 148-150°C. Please clarify if there are drug substance manufacture site and synthesis route changes.

Marillion Response:

The drug substance manufacturer remains Sai Drusyn in Hyderabad, India. The manufacturing process was developed by SynQuest in Chicago, IL. SynQuest manufactured the 1 α -hydroxyvitamin D5 that was used in the nonclinical toxicology studies. The manufacturing process was subsequently transferred to Sai Drusyn for clinical manufacture, as SynQuest did not have the appropriate cGMP controls.

FDA Comment:

2. The release tests provided in the COA are not adequate to assure the quality and purity of the drug substance. The acceptable limits and analytical methods used to support the purity level and impurity profile of the drug substance intended for clinical use should be submitted. We refer you to 21 CFR 312.23 (a)(7)(iv)(a) and Guidance for Industry - Content and Format of Investigation New Drug Applications (IND) for Phase I studies of Drugs, Including Well-characterized, Therapeutic, Biotechnology-derived Products, November, 1995.

Marillion Response:

We agree with the assessment of the COA and have updated the specification accordingly to include an assessment of assay, residual solvents, and residual metal catalysts. Information regarding the updated specification are included in Section 2.4.1 and the analytical methods in Section 2.4.2.

FDA Comment:

3. Batch analysis data of COA of 1 α -hydroxyvitamin D5 intended for clinical use should be submitted for review.

Marillion Response:

Batch results for 1 α -hydroxyvitamin D5 manufactured by Sai Drusyn that is being used for production of clinical trial supplies is provided in Section 2.4.3. The data indicate the batch is suitable for clinical use.

FDA Comment:

4. Data to support the stability of the drug substance for the duration of toxicological studies and the proposed clinical trials should be provided. Container/closure systems and detailed storage conditions including, temperature and tolerance and relative humidity used for the study should be specified.

Marillion Response:

Stability testing of the 1 α -hydroxyvitamin D5 drug substance appears to not have been done properly based on the existing IND documentation. However, Marillion has received retain sample of the 1 α -hydroxyvitamin D5 used in the toxicology studies, and it has a chromatographic purity of 86%, indicating an approximately 10% degradation since the time of manufacture by SynQuest in the year 2000. Details of this analysis are in Section 2.4.3, and the stability protocol for the proposed clinical batch are provided in Section 2.5. As detailed in Section 2.5, Marillion is stability testing the current clinical batch of 1 α -hydroxyvitamin D5 under controlled conditions.

FDA Comment:

5. Acceptable limits and analytical methods used to assure the identity, strength, quality and purity of the drug product capsules should be provided. We refer you to 21 CFR 312.23 (a)(7)(iv)(b) and Guidance for Industry -

Marillion Response:

Marillion has updated the drug product specification to include content uniformity and dissolution. The updated Marillion specification for the clinical drug product is provided in Section 3.4.1. Marillion has also modified the formulation based on short-term stability data that are discussed in Section 3.1.

FDA Comment:

6. Batch analysis data for the drug product capsules of all strengths that will be used in the proposed clinical study should be submitted for review. The release testing on the drug product capsules should be adequate to assure the identity, quality, purity and strength.

Marillion Response:

A COA for the first clinical lot of MN01 capsules manufactured for Marillion is included in Section 3.4.3. The data indicate they are suitable for clinical use.

FDA Comment:

7. Data to support the stability of the drug product for the duration of toxicological studies and the proposed clinical trials should be provided. Container/closure systems and detailed storage conditions including, temperature and tolerance and relative humidity used for the study should be specified.

Marillion Response:

Marillion is stability testing the clinical MN01 drug product, though limited data are available at the time of this submission. Marillion's stability protocol and stability data for the clinical drug product are provided in Section 3.5.

FDA Comment:

8. You reported that for the 5 µg capsules study, using UV detection, the measured amount of was 92% of the initial value after 10 days of storage. By MS/MS detection, the measured amount in the 5 µg capsules was 100% of the initial amount after 10 days of storage. Only Day 0 and Day 159 data were provided (page 005-046). The data, obtained with UV detection, are reproduced below:

Study Day/Date	Day 0 (12/29/04)	Day 159 (6/6/05)
Replicate A (µg)	3.69	6.08
Replicate B (µg)	4.70	4.84
Mean (µg)	4.20	5.46
% of initial	84	109

- a. Please explain why the reproductivity between replicates A and B (3.69 and 4.70 for Day 0, and 6.08 and 4.84 for Day 159) is unacceptably poor.
- b. The great difference between the observed (4.20 µg for Day 0) and the theoretical (5 µg) values are well beyond acceptable experimental errors. Please explain.
- c. The supporting data (2 data points) provided are inadequate. Please specify the time points studied and provide data to demonstrate that the 5 g capsules was 100% of the initial amount after 10 days of storage.

(Appendix 1, continued)

9. Similarity, you reported that for the 35 µg capsules measured by UV detection, no degradation of 1 α-hydroxyvitamin D5 was observed. By MS/MS detection, the measured amount in the 35 µg capsules was 100% of the initial value after 10 days of storage. Your conclusion was that 1 α-hydroxyvitamin D5 appears to be stable when stored at room temperature for up to 10 days. Supporting data provided are inadequate; only Day 0 and Day 159 were included (page 005-049). The data, obtained with UV detection, are reproduced below:

Study Day/Date	Day 0 (12/29/04)	Day 159 (6/6/05)
Replicate A (µg)	42.9	46.0
Replicate B (µg)	39.6	50.7
Mean (µg)	41.3	48.4
% of initial	118	138

- Please explain why the reproductability between replicates A and B between (42.0 and 39.6 for Day 0, and 46.0 and 50.7 for Day 159) is unacceptably poor.
- The great difference between the observed (41.3 µg for Day 0) and the theoretical (35 µg) values is well beyond acceptable experimental errors. Please explain.
- The supporting data (2 data points) provided are inadequate. Please specify the time points studied and provide data to demonstrate that the 5 µg capsules was 100% of the initial amount after 10 days of storage.

Marillion Response:

Rather than try to sort out these old data sets for the 5 and 35 µg capsules, Marillion has contracted Analytical Research Laboratories in Oklahoma to develop and validate a chromatographic assay and purity method for the 1 α-hydroxyvitamin D5 drug substance and drug product. Information regarding the analytical method is provided in the attached CMC section.

FDA Comment:

10. The following comments pertain to the HPLC method used in your studies.
- 1 α-hydroxyvitamin D5 was eluted between 2.3 - 2.9 min (see HPLC traces in pages 005-049 through 005-053) overlapping with corn starch peaks (page 005-054). Your statement: "No interference to the detection of 1 α-hydroxyvitamin D5 peaks was found in the vehicle control samples for either UV or MS/MS detection" (page 005-016) is incorrect. Additionally, you did not determine if the excipient Tenox (added as an antioxidant) is contributing to the interference.
 - Assurance should be provided to show that all of the peaks of interest, including impurities, can be detected and quantitated with accuracy and precision under the prescribed analysis conditions. The limit of detection (LOD) and limit of quantitation (LOQ) of the HPLC method should be determined.
 - Levels of potential degradations products were not monitored.

Marillion Response:

Rather than try to sort out these old data sets, Marillion has contracted Analytical Research Laboratories in Oklahoma to develop and validate a chromatographic assay and purity method for the 1 α-hydroxyvitamin D5 drug substance drug substance and drug product. Information regarding the analytical method is provided in the attached CMC section.

FDA Comment:

11. The following comments pertain to the manufacture of drug product capsules:

(Appendix 1, continued)

- a. You reported that "as a measure of quality assurance, dose uniformity and stability for each batch will be evaluated. From each batch, 10 extra capsules will be prepared. The amount of D5 will be measured in five capsules at the time of dispensing to the patients (i.e., within 24 hours of preparation). The tests for dose uniformity should include tests to assay the content of a 1 α -hydroxyvitamin D5 and a compendial test, such as USP <905> Uniformity of Dosage Test, to determine the total mass of fill in the drug product capsules.
- b. Please provide detailed descriptions for the container/closure systems of the drug capsules intended for the clinical use.

Marillion Response:

Complete CMC information on the Marillion-manufactured MN01 drug product is provided in Section 3 of the attached CMC section.

I hope this information is sufficient to facilitate your review of these issues. Please do not hesitate to call me if you have questions regarding this letter or require additional information.

Very sincerely yours,

Zahed Subhan, Ph.D.
Chief Executive Officer

Attachments: CMC Information Amendment

(Appendix 1, continued)

CHEMISTRY, MANUFACTURING, AND CONTROLS
INFORMATION AMENDMENT

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CHEMISTRY, MANUFACTURING, AND CONTROLS INFORMATION AMENDMENT

1. INTRODUCTION

Since taking ownership of IND 56,509, Marillion Pharmaceuticals, Inc. (Marillion) has been working with contract service providers to address the chemistry, manufacturing, and controls (CMC) deficiencies and comments listed in the Division's letter dated September 7, 2005, that was addressed to the previous IND holder, Dr. Gupta at the University of Chicago. This CMC section provides detailed new information regarding specifications, analytical methods, and stability of the drug substance and the drug product. Marillion has sourced a new batch of MN01 from the manufacturer, Sai Drusyn Laboratories, for clinical production, and batch data are provided herein. CMC information is provided on a new formulation of MN01 (1 α (OH) vitamin D₅) capsules to be used in future Marillion clinical studies. This new formulation was developed as the original formulation developed by the University of Chicago was found to be relatively unstable. Complete CMC information regarding the components and composition, manufacturer, method of manufacture, specification and analytical methods, release results, and stability for this new formulation are provided in Section 3.

2. DRUG SUBSTANCE

This section provides information regarding the drug substance specification, analytical methods, batch results, and stability of the MN01 drug substance.

2.1 Description and Characterization

Marillion has designated the 1 α -hydroxyvitamin D₅ drug substance with the research code "MN01". There are no other changes in this section.

2.2 Manufacturer

The drug substance manufacturer remains Sai Drusyn Laboratories (now Sai Advantium Pharma Limited) in Hyderabad, India. Marillion has contracted with Analytical Research Laboratories, Inc. to perform regulatory release and stability testing of the MN01 drug substance and Quantitative Technologies, Inc. to perform residual metals testing as discussed in Section 2.4. The addresses of these 2 facilities follow.

Analytical Research Laboratories, Inc. (ARL)
840 Research Parkway, Suite 543
Oklahoma City, OK 73104

Quantitative Technologies, Inc. (QTI)
291 RT. 22 East
Salem Industrial Park, Building 5
Whitehouse, NJ 08888

2.3 Method of Manufacture

There is no change to the information provided in the Amendment dated April 21, 2003, Serial No. 001.

(Appendix 1, continued)

2.4 Specification

This section provides updated information for the MN01 drug substance specification, analytical methods, and batch results.

2.4.1 Drug Substance Tests, Methods, and Acceptance Criteria

Marillion has reviewed the Sai Drusyn Laboratories specification for the MN01 drug substance along with the method of manufacture. The Sai Drusyn Laboratories specification includes only description, identification by nuclear magnetic resonance (NMR) and mass, and chromatographic purity. Based on this review, Marillion has updated the specification to include identification by infrared (IR), chromatographic assay, individual and total related substances, residual solvents, and residual heavy metals that are used in the manufacturing process. The Marillion specification for the MN01 drug substance is listed in Table 1. Since current batch sizes are 250 mg each, water content testing is not performed to conserve sample, and it is not possible to perform routine tests such as USP heavy metals or residue on ignition. Marillion will implement more stringent acceptance criteria for related substances and residual metals as more data become available.

Table 1: Marillion Specification for MN01 Drug Substance

Test	Method	Acceptance Criteria
Appearance	Visual	White to light yellow powder
Identification	IR	Sample spectrum corresponds to reference spectrum
Assay	HPLC AMI-827	95.0 to 105.0%
Related substances Single Total	HPLC AMI-827	As reported As reported
Residual solvents Ethyl acetate DMSO Methanol Dichloromethane	GC	5,000 ppm 5,000 ppm 3,000 ppm 600 ppm
Heavy metals Aluminum Selenium Zinc	ICP-MS	NMT 50 ppm NMT 20 ppm NMT 75 ppm

DMSO = dimethylsulfoxide; GC = gas chromatography; HPLC = high performance liquid chromatography; ICP-MS = inductively-coupled plasma—mass spectrometry; IR = infrared; ppm = parts per million

2.4.2 Analytical Methods

ARL has developed and qualified a reversed-phase HPLC (RP-HPLC) method for assay and related substances analysis of the MN01 drug substance along with a gas chromatography (GC) method for residual solvents analysis. QTI has developed and qualified an inductively-coupled plasma—mass spectrometry (ICP-MS) method for residual metals used in the manufacturing process. A brief description of each method follows.

(Appendix 1, continued)

Assay and Related Substances by RP-HPLC

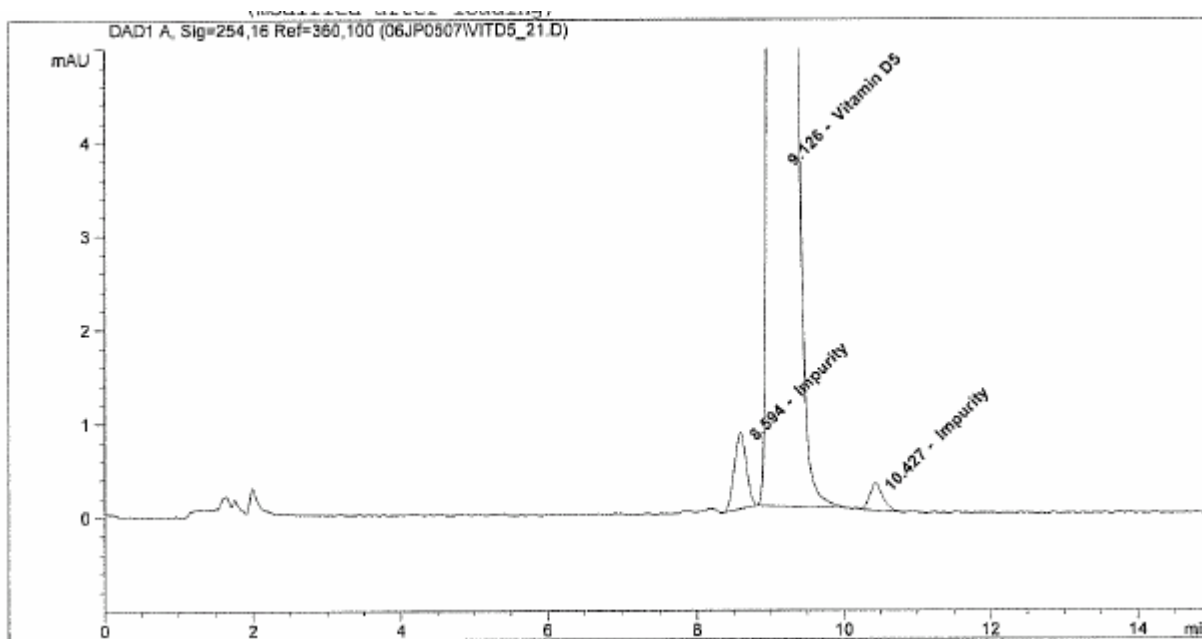
The HPLC method consists of the following.

Column:	Agilent SB-C18 250 x 4.6 mm, 3.5 μ m or equivalent
Flow:	1.5 mL/minute
Detector:	UV at 254 nm
Injection volume:	100 μ L for assay 25 μ L for related substances
Mobile phase:	acetonitrile—methanol—water (800:190:10)

The method is isocratic. The current lot of the USP reference standard of calcifediol is used as the analytical standard for the method (there is insufficient MN01 available at this time to properly qualify a MN01 reference preparation). The relative response factor of calcifediol is 1.08 determined as an average of multiple sample preparations. Sample and standards are prepared at 1 μ g/mL for assay, and sample is prepared at 70 μ g/mL for purity analysis.

An example chromatogram of batch 5016/A033-06/XIV/03, the current clinical batch, is provided in Figure 1.

Figure 1: Example Chromatogram of MN01 Batch 5016/A033-06/XIV/03



Accuracy has been assessed at 75, 100, and 125% of the target analyte concentration in triplicate with recoveries of 100.5 to 101.8%. Precision was determined also at 75, 100, and 125% of the target analyte concentration in triplicate with relative standard deviations (RSDs) of 0.21, 0.18, and 0.28%, respectively. The limit of quantitation (LOQ) was found to be 0.090% with a signal to noise ratio of 13.6 and an RSD of 2.56%. The limit of detection (LOD) was found to be 0.024% with a signal to noise ratio of 3.5 and an RSD of 12.56%.

Residual Solvents by GC

Residual solvents are determined by a Hewlett-Packard Series II 5890 System with a flame ionization detector using the following conditions.

(Appendix 1, continued)

Column: Zebtron ZB-624 30 m x 0.25 mm, 6% cyanopropylphenyl or equivalent
He flow: 20 mL/min
Injection: 1 µL
Oven: 35°C for 5 min, ramp 40°C/min to 250°C, hold 250°C 5 min
Injector: 140°C
Detector: 260°C

Standards and samples are prepared in DMSO for analysis.

Residual Metals by ICP-MS

Sample is digested in a mixture of nitric and sulfuric acids at 240°C for 15 minutes. The resulting liquid is diluted to 10 mL with water for analysis. Analysis is performed on a Perkin-Elmer ELAN 9000 ICP-MS. The isotopes of ²⁷Al, ⁷⁸Se, and ⁶⁸Zn are used for analysis. A 20 ppm spike of each metal in ergocalciferol (a related substance surrogate for the MN01 drug substance) is used to verify the system is operating properly during analysis. The LOD for the method has been established as 2 ppm for aluminum, 1 ppm for selenium, and 5 ppm for zinc. Accuracy and recovery have been demonstrated for each metal at both 20 and 200 ppm spikes in ergocalciferol (due to the lack of available MN01 drug substance for such experiments).

2.4.3 Batch Results

The certificate of analysis (CoA) for the current clinical batch 5016/A033-06/XIV/03 of MN01 is provided in Figure 2. The data indicate that the batch is suitable for use in the planned clinical studies. The residual solvents and trace metals were higher than expected, but the levels do not indicate any obvious safety concerns. The solvents DMSO and methanol slightly exceeded specification requirements but given that the International Conference on Harmonisation provides permitted daily exposures (PDEs) of 50 mg and 30 mg, respectively, for these 2 solvents and the fact that the drug will be administered in the low tens of micrograms per day, there is no safety issue. Comparable results for the residual process metals were obtained on a previous sample of the MN01 drug substance obtained from the previous IND holder. Given the low doses proposed for the clinical study, the presence of zinc and aluminum at the current levels does not raise any obvious safety concerns.

Figure 2: CoA for MN01 Clinical Batch 5016/A033-06/XIV/03

Marillion has evaluated the chromatographic purity of the MN01 drug substance batch that was used in the toxicology studies that were performed by the previous IND holder. Comparative chromatographic purity results for the toxicology batch and the new clinical batch are provided in Table 2. This testing was performed by ARL using the newly developed and qualified RP-HPLC method described in Section 2.4.2. The data indicate that the new clinical batch has fewer related substances than the toxicology batch. Two related substances are present in the new clinical batch that were present in the past toxicology batch, but these substances are present at lower levels in the new clinical batch. Marillion assumes that the toxicology batch, manufactured in 2000, has most likely degraded since the time of manufacture. The chromatographic purity reported by the manufacturer (SynQuest) was 96.38% using methodology different from Marillion's.

(Appendix 1, continued)

Table 2: Chromatographic Purity Comparison Between the Past Toxicology and Current Batches of MN01

RRT	Batch Used in Past Toxicology Studies (Manufactured 2000)	MN01 Batch 5016/A033-06/XIV/03
0.68	2.54	ND
0.74	1.44	ND
0.85	8.13	ND
0.94	0.81	0.46
1.14	0.84	0.21
Total	13.76	0.67

2.5 Drug Substance Stability

2.5.1 Drug Substance Stability Protocol

The drug substance stability protocol is listed in Table 3. The bulk MN01 drug substance is being stored frozen at -10 to -20°C. Therefore, an accelerated condition under refrigerated conditions at 2 to 8°C is used. The first clinical batch of MN01 was only 240 mg (though Marillion requested a minimum 1 g batch size), and so it was not possible to put up accelerated stability samples for this particular batch after sampling for release testing and clinical drug product manufacture. However future lots of larger batch scale will be placed on both long-term and accelerated conditions.

Table 3: MN01 Drug Substance Stability Protocol

Test	2 weeks	1 month	3 month	6 month	9 month	12 month
Appearance	x, xx	x, xx	x, xx	x, xx	x	x
Assay	x, xx	x, xx	x, xx	x, xx	x	x
Related Substances	x, xx	x, xx	x, xx	x, xx	x	x

x= -10 to -20°C

xx = 2 to 8°C

2.5.2 Drug Substance Stability Results

The 2-week stability results for the first batch of the MN01 drug substance are listed in Table 4. The results show no change from those obtained at t=0. Again, the batch was too small to allow for accelerated stability testing and so only long-term results for sample stored at -10 to -20°C are available.

Table 4: Drug Substance Stability Results at -10 to -20°C

Test	Acceptance Criteria	T = 0	T = 2 Weeks
Appearance	White to light yellow powder	White powder	White powder
Assay	95.0 to 105.0%	100.2%	
Related substances Individual	As reported	RRT 0.94 0.46% RRT 1.15 0.21%	RRT 0.94 0.42% RRT 1.15 0.16%
Total	As reported	0.67%	0.58%

(Appendix 1, continued)

2.6 Container Closure System

The current container—closure system for the drug substance is a small amber glass vial with a plastic screw cap.

3. DRUG PRODUCT

Marillion contracted with Midwest Institute of Research and Technology (MIRT) to formulate the MN01 drug product. MIRT reviewed the University of Chicago formulation containing corn oil, corn starch, ethanol, and tenox and predicted the drug product would be relatively unstable due to peroxides always present in oils such as corn oil (hence, the need for the antioxidant tenox). MIRT proposed 3 alternate formulations as follows (arbitrary designations were assigned).

Formulation A: Ethanol, corn oil, Tenox, and corn starch (the original formulation)

Formulation B: Ethanol and corn starch

Formulation A3: Ethanol, corn starch, lactose, and dicalcium phosphate

Formulation B4: Ethanol and lactose

Hard gelatin capsules using all 4 formulations were prepared at small scale and subjected to short term stability under ambient, refrigerated (2-8°C), and frozen (-10 to -20°C) conditions. The original formulation A showed significant degradation by 2 weeks (total degradation products over 10% frozen and over 12% ambient). The related formulation B showed almost 7% degradation ambient, but little if any degradation at the other 2 conditions. The novel formulations A3 and B4 showed little if any degradation at any of the 3 storage conditions. Formulation B4 was selected for clinical manufacture as it was the simplest to manufacture and appeared to be comparatively stable.

3.1 Components and Composition

The components and composition of the MN01 capsules for clinical use are listed in Table 5.

Table 5: Components and Composition of the MN01 Capsules

Ingredient	Function	Amount per Capsule
MN01	Active ingredient	5 µg
Lactose, anhydrous, NF	Filler	467 mg
Alcohol, NF	Solvent	-- ¹
Hard gelatin capsules	Capsule	1 each

¹ Alcohol is removed during processing, and so no value is included for the unit formulation.

The current scale of manufacture is approximately 3,000 capsules per lot.

3.2 Manufacturer

The drug product manufacturing facility is:

Midwest Institute of Research and Technology (MIRT)
122 N. Bryant, Suite B4
Edmond OK 73034

(Appendix 1, continued)

Release and stability testing of the drug product is performed by:

Analytical Research Laboratories, Inc.
840 Research Parkway, Suite 543
Oklahoma City, OK 73104

3.3 Method of Manufacture

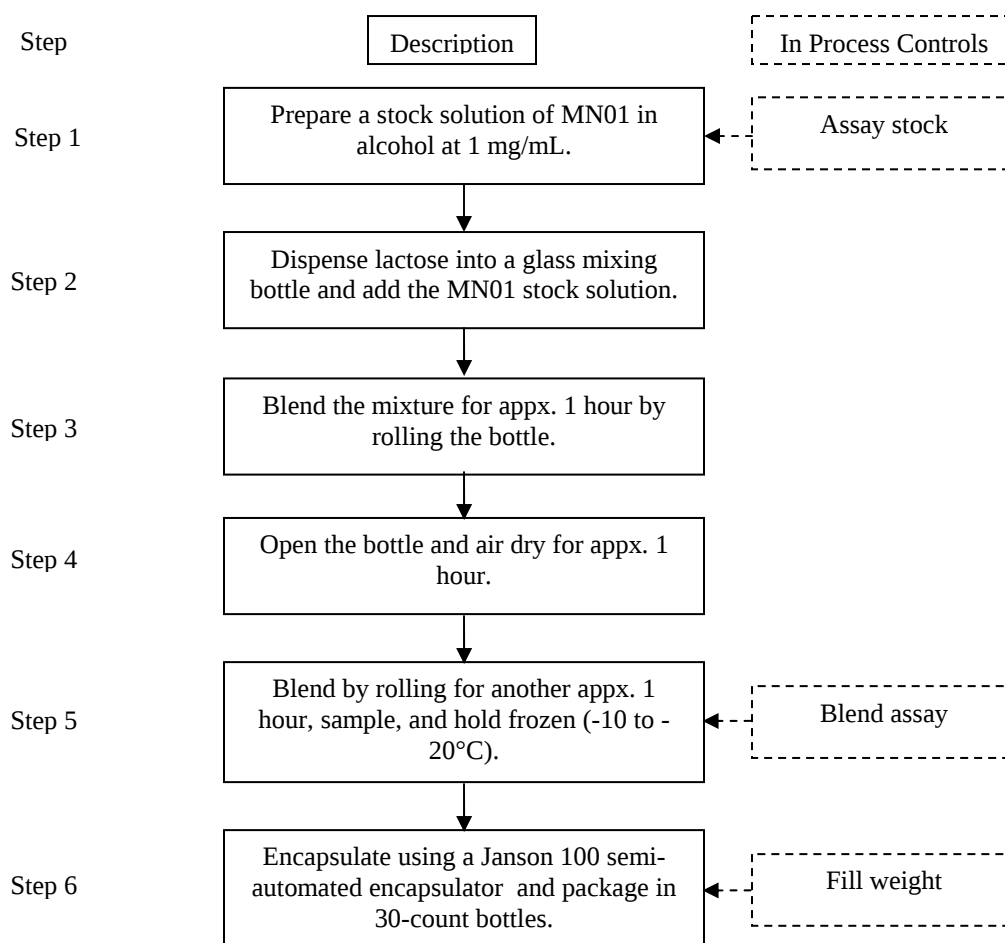
This section provides a detailed flow chart and in process control testing performed during MN01 drug product capsule manufacture. The first lots of the capsules manufactured using the current formulation showed low assay values. A blend stability study indicated no degradation of the drug substance during processing, however low assay values were observed. Initially, disposable plastic bottles were used for the blending steps, and it was found that use of a glass bottle reduced the loss of drug during formulation. The encapsulation step has includes a 5% overage to account for loss of the drug substance observed during the blending step.

3.3.1 Flow Chart

A flow chart for the manufacturing process is shown in Figure 3.

(Appendix 1, continued)

Figure 3: Flow Chart for MN01 Drug Product Capsule Manufacture



3.3.2 In Process Control Tests

In process control testing performed during manufacture of the MN01 capsules is listed in Table 6.

Table 6: In Process Control Testing for Drug Product Manufacture

Step	Test	Requirement
1	Stock solution assay by RP-HPLC	As reported
5	Blend assay	As reported
6	Capsule weights	571 to 631 mg (including capsule shells)

(Appendix 1, continued)

3.4 Drug Product Specification

Marillion has drafted an MN01 drug product specification consistent with the Division's comments to previous IND submissions. The specification now includes an evaluation of related substances, uniformity of dosage units, and dissolution. Information regarding the new analytical methods is provided in Section 3.4.2.

3.4.1 Drug Product Tests, Methods, and Acceptance Criteria

The proposed specification for the MN01 capsules is provided in Table 7. The specification includes appearance, identification, assay, related substances, dissolution, and uniformity of dosage units.

Table 7: Specification for the MN01 Drug Product

Test	Method	Acceptance Criteria
Appearance	Visual	White to off white fill inside white opaque hard gelatin capsules
Identification	HPLC AMI-827	Sample retention time consistent with reference
Assay	HPLC AMI-827	90.0 to 110.0%
Related substances Single largest Total	HPLC AMI-827	NMT 1.0% NMT 2.5%
Dissolution	USP<711> HPLC AMI-830	70% Q in 60 minutes
Uniformity of dosage units	USP<905> HPLC AMI-827	Complies with current USP

NMT = not more than

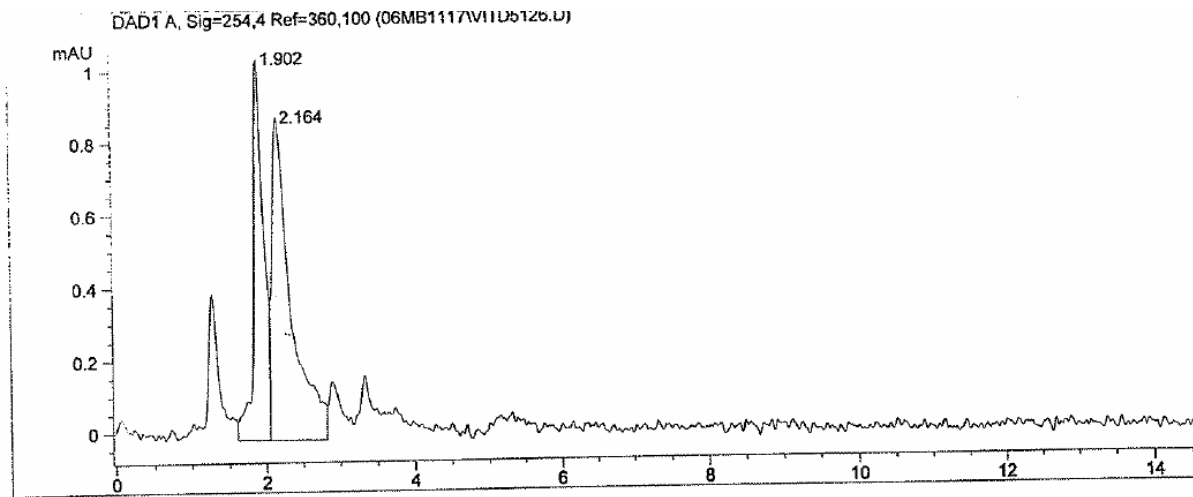
3.4.2 Analytical Methods

The same RP-HPLC system described for assay and related substances in Section 2.4.2 is used for identification, assay, related substances, and uniformity of dosage units testing for the MN01 drug product, differing only in sample preparation. For assay analysis, the contents of 5 capsules are dissolved in 25 mL of mobile phase. For related substances analysis, the contents of 4 capsules are brought up in 5 mL of mobile phase. For uniformity of dosage units testing, the contents of 1 capsule are brought up in 5 mL of mobile phase.

In response to the Division's comment number 10 from the letter dated September 7, 2005, this method has been qualified for selectivity, accuracy, precision, and limits of quantitation and detection. In the Marillion formulation, the only inactive ingredient is lactose. A representative lactose control chromatogram is provided in Figure 4.

(Appendix 1, continued)

Figure 4: Representative Lactose Control Chromatogram



=====
External Standard Report
=====

Sorted By : Signal
Calib. Data Modified : Wednesday, November 22, 2006 10:15:00 AM
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 A, Sig=254,4 Ref=360,100

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ug/mL]	Grp	Name
9.097	-	-	-	-	-	Vitamin D5

Totals : 0.00000

Dissolution:

The dissolution method is as follows.

Apparatus: USP apparatus II, paddles
Media: 0.5% Tween 20 in water
Agitation rate: 100 rpm
Media volume: 50 mL
Sampling time: 60 minutes

Dissolution samples are analyzed using an RP-HPLC method comparable to that used for drug substance and drug product analysis except that a gradient is used to keep the run time short (6 minutes). Tween 20 was selected as the surfactant for the dissolution media as other surfactants, such as sodium dodecyl sulfate caused precipitation when mixed with the HPLC buffer. The level of 0.5% was selected as this was sufficient to fully dissolve the MN01 in the capsules within 60 minutes.

(Appendix 1, continued)

3.4.3 Lot Results

The CoA for the first clinical lot of the MN01 drug product intended for clinical use is provided in Figure 5. In particular, the assay value is very close to the target of 5 µg and the related substances are very comparable with those reported for the corresponding drug substance batch in Section 2.4.3. The data indicate the drug product is suitable for clinical use.

Figure 5: CoA for MN01 Capsule Lot D5.5.050907

3.5 Drug Product Stability

3.5.1 Drug Product Stability Protocol

The drug product stability protocol is listed in Table 8. At this time, insufficient data for the current formulation are available to warrant a refrigerated storage condition, and so the long-term storage condition has been set at -10 to -20°C. Accelerated stability testing is being performed at 2 to 8°C.

Table 8: Stability Protocol for the MN01 Drug Product

Test	2 weeks	1 month	3 month	6 month	9 month	12 month	18 month	24 month
Appearance	x, xx	x, xx	x, xx	x, xx	x	x	x	x
Assay	x, xx	x, xx	x, xx	x, xx	x	x	x	x
Related substances	x, xx	x, xx	x, xx	x, xx	x	x	x	x
Dissolution	x, xx	x, xx	x, xx	x, xx	x	x	x	x

x = -20 to -10°C

xx = 2 to 8°C

3.5.2 Drug Product Stability Results

The clinical lot of MN01 capsules D5.5050907 are on stability but no results are available at this time. A development lot, manufactured using the original plastic mixing vessel, was tested for stability through 2 weeks are reported in lieu of the clinical lot. The development lot, D5.5.04\$07, showed low assay results but the 2 week stability results showed no change in assay, no degradation products, and no change in appearance or dissolution rate. These results are fully consistent with the formulation development stability results discussed in Section 3. The results for the long-term samples stored at -10 to -20°C are listed in Table 9 and the results for accelerated samples stored at 2 to 8°C are listed in Table 10.

Table 9: Two Week Stability Results at -10 to -20°C

Test	T = 0	T = 2 Weeks
Appearance	White fill inside white opaque hard gelatin capsules	White fill inside white opaque hard gelatin capsules
Assay		
Degradation products	None detected	None detected
Dissolution		

(Appendix 1, continued)

Table 10: Two Week Stability Results at 2 to 8°C

Test	T = 0	T = 2 Weeks
Appearance	White fill inside white opaque hard gelatin capsules	White fill inside white opaque hard gelatin capsules
Assay		
Degradation products	None detected	None detected
Dissolution		

Marillion will continue to monitor the stability of the clinical lot of the MN01 capsules according to the protocol listed in Table 8. The results of the stability studies will be reported in the Annual Reports under this IND. Any lot of capsules that fails to meet acceptance criteria during the course of stability testing will be withdrawn from clinical use.

3.6 Container Closure System

The container—closure system for the MN01 clinical capsules is a translucent-yellow, HPDE bottle with a child resistant plastic cap. Each bottle contains 30 capsules and the same samples to be used for the clinical studies are on stability as discussed in Section 3.5.

5 PLACEBO

There is no placebo for the proposed clinical trials.

6 LABELING

An example label for the clinical trial supplies is provided in Figure 6.

Figure 6: Clinical Label for the MN01 Capsule Bottles

MN01 Capsules, 5 µg each Contents 30 capsules	
Lot No. \$\$\$	Date of Mfg: DD/MM/YY
Store Frozen (-10 to -20°C)	
Caution: New Drug – Limited by Federal Law to Investigational Use Only	
Manufactured for: Marillion Pharmaceuticals, Inc., 1811 Page Place Malvern, PA 19355	

Appendix 2

Papers, Presentations and Publications

Please note: The following resulted from the work of Dr. Vijayakumar and his colleagues during the grant period.

I. Peer-reviewed papers:

1. 2006 Vijayakumar S, Boerner PS, Mehta RR, Packianathan S, Mehta RG, Das Gupta TK. Clinical trials using chemopreventive vitamin D analogs in breast cancer. *Cancer Journal*, 12(6): 445-450.

2. 2005 Vijayakumar S, Mehta RR, Boerner PS, Packianathan S, Mehta RG. Clinical trials involving vitamin D analogs in prostate cancer. *Cancer Journal*, 11(5): 363-73.

3. 2004 Packianathan S, Mehta RR, Mehta RG, Hall W, Boerner PS, Beckett L, Vijayakumar S. Designing a randomized phase I/II prostate cancer chemoprevention trial using 1alpha-hydroxy-24-ethyl-cholecalciferol, an analogue of vitamin D3. *Cancer Journal*, 10(6): 357-367.

II. Scientific Abstracts:

1. 2007 Loredó GA, Moore ME, Moy CV, Mehta RG, Vijayakumar S, Vaughan ATM, Ghosh PM. Vitamins D3 and D5 mediate growth inhibition of androgen-dependent and independent prostate cancer cells by distinct mechanisms of interaction with the androgen receptor. American Association for Cancer Research Annual Meeting, April 17, 2007, Los Angeles, CA.

2. 2006 Loredó GA, Lu XH, Mehta R, Vijayakumar S, Vaughan ATM, Ghosh PM. The low-calcemic vitamin D analog 1-alpha-hydroxyvitamin D5 is anti-proliferative and does not increase androgen receptor expression in prostate cancer cells. American Association for Cancer Research Annual Meeting, April 2, 2006 Washington, DC.

2. 2005 Packianathan S, Vijayakumar S. Intermediate Biomarkers in Male Genitourinary Cancers—Penile Cancer. American Radium Society 87th Annual Meeting, April 30-May 4, Barcelona, Spain (poster).

3. 2004 Vijayakumar S, Mehta R, Mehta R, Hall W, Boerner P, Beckett L. Clinical Trial Design in Chemoprevention Studies: Using a Vitamin D5 Analog Study as an Example. American Radium Society 86th Annual Meeting, May 1-5, Napa Valley, California (poster).

4. 2003 Babbar D, Gandhi M, Mehta RG, Vijayakumar S, Mehta RR. Effects of a 1a-hydroxyvitamin D5 on Prostate Cancer Cells. *Proc. Am. Assoc. Cancer Res.* 44:1269.

III. Lectures:

1. Vijayakumar, Srinivasan. Prostate Cancer Clinical Trials with Vitamin D. CeDAR Symposium, Boston, MA, Sept, 2005.

AMENDMENT OF SOLICITATION/MODIFICATION OF CONTRACT		1. CONTRACT ID CODE S	PAGE OF PAGES 1 2
2. AMENDMENT/MODIFICATION NO.	3. EFFECTIVE DATE 25-Jan-2007	4. REQUISITION/PURCHASE REQ. NO. FORM95FC010148-000	5. PROJECT NO (If applicable)
6. ISSUED BY USA MED RESEARCH ACQ ACTIVITY 870 CHANDLER ST FORT DETRICK MD 21702-5014	CODE WB1XWH	7. ADMINISTERED BY (If other than item 6) USA MED RESEARCH ACQ ACTIVITY ATTN: WENDY BAKER WENDY.BAKER@AMEDD.ARMY.MIL FORT DETRICK MD 21702-5014	CODE WB1XWH
8. NAME AND ADDRESS OF CONTRACTOR (No., Street, County, State and Zip Code) UNIVERSITY OF CALIFORNIA DAVIS ONE SHIELDS AVENUE 110 EVERSON HALL DAVIS CA 95618-0871		9A. AMENDMENT OF SOLICITATION NO.	
		9B. DATED (SEE ITEM 11)	
		X 10A. MOD. OF CONTRACT/ORDER NO. DAMD17-02-1-0070	
		X 10B. DATED (SEE ITEM 13) 19-Feb-2002	
CODE 1C8G4		FACILITY CODE	
11. THIS ITEM ONLY APPLIES TO AMENDMENTS OF SOLICITATIONS			
☐ The above numbered solicitation is amended as set forth in Item 14. The hour and date specified for receipt of offer ☐ is extended, ☐ is not extended. Offer must acknowledge receipt of this amendment prior to the hour and date specified in the solicitation or as amended by one of the following methods: (a) By completing Items 8 and 15, and returning _____ copies of the amendment; (b) By acknowledging receipt of this amendment on each copy of the offer submitted; or (c) By separate letter or telegram which includes a reference to the solicitation and amendment numbers. FAILURE OF YOUR ACKNOWLEDGMENT TO BE RECEIVED AT THE PLACE DESIGNATED FOR THE RECEIPT OF OFFERS PRIOR TO THE HOUR AND DATE SPECIFIED MAY RESULT IN REJECTION OF YOUR OFFER. If by virtue of this amendment you desire to change an offer already submitted, such change may be made by telegram or letter, provided each telegram or letter makes reference to the solicitation and this amendment, and is received prior to the opening hour and date specified.			
12. ACCOUNTING AND APPROPRIATION DATA (If required)			
13. THIS ITEM APPLIES ONLY TO MODIFICATIONS OF CONTRACT ORDERS IT MODIFIES THE CONTRACT/ORDER NO. AS DESCRIBED IN ITEM 14.			
A. THIS CHANGE ORDER IS ISSUED PURSUANT TO: (Specify authority) THE CHANGES SET FORTH IN ITEM 14 ARE MADE IN THE CONTRACT ORDER NO. IN ITEM 10A.			
B. THE ABOVE NUMBERED CONTRACT/ORDER IS MODIFIED TO REFLECT THE ADMINISTRATIVE CHANGES (such as changes in paying office, appropriation date, etc.) SET FORTH IN ITEM 14, PURSUANT TO THE AUTHORITY OF FAR 43.103(B).			
X C. THIS SUPPLEMENTAL AGREEMENT IS ENTERED INTO PURSUANT TO AUTHORITY OF: Article 16 "Amendment of Grant"			
D. OTHER (Specify type of modification and authority)			
E. IMPORTANT: Contractor ☐ is not, ☒ is required to sign this document and return <u>1</u> copies to the issuing office.			
14. DESCRIPTION OF AMENDMENT/MODIFICATION (Organized by UCF section headings, including solicitation/contract subject matter where feasible.) Modification Control Number: wcockerh071841 1. The purpose of this modification is to officially extend the period of performance to read as shown. This is being done at no additional cost to the Government and in accordance with the recipient's request dated 19 January 2007. This extension is being granted to provide the Principal Investigator one final attempt to obtain the necessary FDA approval of the IND. Pursuant to mutual agreement and the recipient's email dated 19 January 2007, if this approval is not obtained by 31 May 2007, closeout procedures will begin and all unspent funding shall be returned to the DoD. Additionally, the PI shall submit a monthly update email to Wendy Baker, Contract Specialist and Dr. Nusingha Mishra, Grants Officer's Representative. 2. All other terms and conditions remain unchanged.			
Except as provided herein, all terms and conditions of the document referenced in Item 9A or 10A, as heretofore changed, remains unchanged and in full force and effect.			
15A. NAME AND TITLE OF SIGNER (Type or print) Ahmad Hakim-Elahi, Ph.D., J.D. Director of Sponsored Programs		16A. NAME AND TITLE OF CONTRACTING OFFICER (Type or print)	
15B. CONTRACT OR OFFER NO.		16B. UNITED STATES OF AMERICA	
15C. DATE SIGNED APR 24 2007		16C. DATE SIGNED 4/25/07	
(Signature of person authorized to sign)		(Signature of Contracting Officer)	

EXCEPTION TO SF 30
APPROVED BY OIRM 11.K4

30-105-04

STANDARD FORM 30 (Rev. 10-83)
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Page 2 of 2

SECTION SF 30 BLOCK 14 CONTINUATION PAGE

SUMMARY OF CHANGES

SECTION F - DELIVERIES OR PERFORMANCE

The following Delivery Schedule item for SUBCLIN 0006AB has been changed from:

DELIVERY DATE	QUANTITY	SHIP TO ADDRESS	UIC
15-DEC-2004	8	N/A FOB: Destination	

To:

DELIVERY DATE	QUANTITY	SHIP TO ADDRESS	UIC
31-MAY-2007	8	N/A FOB: Destination	

The following Delivery Schedule item for CLIN 0008 has been changed from:

DELIVERY DATE	QUANTITY	SHIP TO ADDRESS	UIC
28-FEB-2007	10	N/A FOB: Destination	

To:

DELIVERY DATE	QUANTITY	SHIP TO ADDRESS	UIC
31-MAY-2007	10	N/A FOB: Destination	

(End of Summary of Changes)

Clinical Trials Using Chemopreventive Vitamin D Analogs in Breast Cancer

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ABSTRACT

This article comprehensively reviews the clinical trials and considers the future directions of the use of vitamin D and its analogs in the treatment or chemoprevention of breast cancer. Chemopreventive treatment strategies strive to delay the onset of certain cancers, prevent the progression of malignant disease after diagnosis, or delay the advent of recurrence after curative treatment. We first summarize the epidemiological evidence that led to the hypothesis that vitamin D may have an anti-cancer activity. Vitamin D shows great potential as a therapy for breast cancer; however, its use in clinical trials has been hindered by the induction of hypercalcemia at a concentration required to suppress cancer cell proliferation. This has led to the development of less calcemic analogs of vitamin D. We review the clinical trials with breast cancer patients using vitamin D analogs. (*Cancer J* 2006;12:445-450)

KEY WORDS

Breast cancer, vitamin D, chemoprevention

SEARCH STRATEGY AND SELECTION CRITERIA

Data for this review were identified by searches of PubMed, the Cochrane Library, Biosis, and references from relevant articles, using the search terms "vitamin D," "breast cancer," "chemoprevention," and "vitamin D analog." Abstracts from recent international meetings were also reviewed but were included only when they were the only known reference to the clinical trial or the research mentioned. Only papers published in English were included.

Breast cancer, the strongest risk factors for which include gender, age, and country of birth, continues to be a significant source of morbidity and mortality for women. Other primary risk factors for breast cancer are related to the female reproductive cycle and include age at menarche, nulliparity, age at first birth and duration of lactation, and age at menopause. Additional risk factors include exogenous estrogens, radiation exposure, alcohol consumption, and higher income and educational level.¹ Interestingly, location of residence has also been cited as a risk factor for breast cancer, which combines the two previously cited risk factors of radiation exposure and country of birth.² In the United States, the American Cancer Society estimates that 211,240 women are likely to be diagnosed with breast cancer in 2005 and 40,410 will die from their disease, making it the cancer with the greatest incidence in the United States and the second highest mortality, after lung cancer.³

Chemoprevention is an intervention in the carcinogenic process, possibly by a synthetic compound, which blocks, arrests, or reverses the progression of cancer.^{4,5} Age is the most significant risk factor for many cancers, and awareness of this fact is a driving force behind research in cancer chemoprevention. With life expectancy continuing to rise in the general population, the incidence of breast cancer is likely to increase in the coming years. A large proportion of

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women diagnosed with this disease can expect to experience significant morbidity during the course of their illness and the associated treatments. Chemopreventive treatment strategies strive to delay the onset of certain cancers, or prevent the progression of malignant disease after diagnosis, or delay the advent of recurrence after curative treatment. Initiatives using safe chemopreventive agents that are directed toward these tasks would be greatly welcome and are likely to have a major impact on women's health. Initial patient recruitment for breast cancer chemoprevention trials, however, is likely to be focused on patient groups with the specific high-risk factors alluded to earlier.

Vitamin D deficiency is common in the elderly.⁷ Aging also lowers the ultraviolet radiation-mediated production of cholecalciferol in the skin. Moreover, estrogen deficiency, which primarily affects postmenopausal women, decreases the metabolic activation of vitamin D, as well as the expression of the vitamin D receptor (VDR).⁸ VDRs are known to be expressed in a variety of cancer cells. Specific VDR polymorphisms can increase susceptibility to breast cancer, and women with certain genotypic variations may also be burdened with a more aggressive form of the disease, especially if the cancer metastasizes.⁹ In addition, vitamin D deficiency per se may contribute to the incidence and mortality of breast cancer, and vitamin D deficiency prevention may thus be possible through increased sunlight exposure, improved diet, and supplemental vitamin D. Several studies measuring solar radiation have supported its beneficial role in breast and other cancers through its mediation of vitamin D synthesis, providing support for the hypothesis that vitamin D may provide some degree of protection against cancer. Epidemiologists estimate that perhaps 30% to 60% of all cancers could be avoided by modifications in diet,¹⁰ and vitamin D is ingested in the diet, as well as synthesized through skin exposure to solar radiation. One potential chemopreventive agent for breast cancer that is currently being developed at our institutions is $1\alpha(\text{OH})\text{D}_3$, or vitamin D_3 , a synthetic analog of vitamin D. The effects of this analog will be investigated in two clinical trials, one involving breast cancer patients and the other with prostate cancer patients.⁶

VITAMIN D

Vitamin D was discovered by Edward Mellanby in 1919 in his experiments using dogs that were exclusively raised indoors, without exposure to sunlight or ultraviolet light.¹¹ Subsequently, E.V. McCollum was able to differentiate between the fat soluble vitamins

A and D.¹² Vitamin D is a steroid hormone that has been shown to have antiproliferative and anti-tumor properties, making it a strong candidate for chemoprevention in breast or other malignancies. However, the usefulness of vitamin D in pharmacologic doses or over long periods of time has been limited because it can cause life-threatening hypercalcemia. For this reason, many new analogs that demonstrate less calcemic activity than vitamin D have been developed and some of these are being tested in phase I and phase II trials. Several recent reviews have also addressed the anti-cancer effects of vitamin D on breast cancer cells.^{13,14}

A paper by Bertone-Johnson et al has suggested that vitamin D may be modestly beneficial for management of breast disease.¹⁵ These researchers examined the relationship between stored plasma levels of 25-hydroxyvitamin D [$25(\text{OH})\text{D}$] and 1,25-dihydroxyvitamin D [$1,25(\text{OH})_2\text{D}$] and risk of breast cancer in a case-control study nested within the Nurses' Health Study cohort. Breast cancer cases had a lower mean serum $25(\text{OH})\text{D}$ level in comparison to matched controls. This association was stronger in women aged 60 years and older.

VITAMIN D AND CANCER RISK

That adequate vitamin D intake may prevent the development of certain diseases—such as rickets, osteoporosis, tuberculosis, and even specific types of cancer—has been well documented.¹⁶⁻²⁰ The initial evidence suggesting an association between vitamin D and cancer protection was primarily epidemiologic in nature. Peller, for instance, observed that in occupations and environments where skin cancer rates were higher, the rates for other cancers were lower.²¹ Subsequently, Apperly also reported that populations living farther from the equator had higher overall cancer death rates compared to those living closer to the equator, suggesting that increased sun exposure—and with it increased synthesis of vitamin D—led to decreased cancer-associated mortality.²²

Historically, breast cancer mortality rates among American women have varied geographically and longitudinally, with the highest mortality occurring in the Northeast and the lowest mortality being reported in the South,^{2,19,23,24} suggesting that solar radiation, which leads to vitamin D synthesis, might be protective against breast cancer.²⁵ Breast cancer mortality is also increased in cities compared to rural areas,² apparently because people living in urban areas may receive less sunlight exposure than those in rural areas at the same latitude, owing to air pollution. For instance, an analysis of data from a national cohort

NHANES I Epidemiologic Follow-up Study found that among women living in areas of high solar radiation, sunlight exposure, and adequate dietary vitamin D intake were associated with a 25% to 65% reduction in breast cancer risk.²⁴ Gorham et al also have shown statistically significant positive associations between acid haze air pollution, which blocks ultraviolet-B light, and age-adjusted breast and colon cancer mortality rates in a study covering 20 Canadian cities.¹⁷ They hypothesized that the populations in such cities with high levels of acid haze may have been encumbered with vitamin D deficiencies. In addition, a similar ecological study in the former Soviet Union by Gorham et al also found a pattern of increased breast cancer incidence in those regions experiencing low sunlight levels.²⁶

These geographic variations in which breast cancer mortality is inversely proportional to the intensity of the local sunlight have also been duplicated in the United States.¹⁹ More recent studies have found that exposure to sunlight was inversely associated with mortality from breast cancer,²⁷ as was UV-BH radiation exposure per se.²⁸ Other investigations have also suggested this epidemiologic link between vitamin D and breast cancer.²⁹⁻³¹ The most likely mechanism by which sunlight exposure could inhibit the development of breast cancer is through the production of vitamin D. Casual exposure to sunlight remains one of the primary sources of vitamin D for women in the United States, and is, along with diet, a modifiable lifestyle factor.

A few studies contradict these findings. For example, Hiatt et al identified no relationship between elevated prediagnostic serum levels of $1,25(\text{OH})_2\text{D}$ and the later diagnosis of breast cancer. However, the serum levels of vitamin D in this study were obtained an average of 15 years prior to the actual diagnosis of cancer, leaving unanswered the question whether elevated vitamin D could have a protective effect at a time closer to the clinically evident breast cancer.³²

A single Canadian case control study evaluating dietary histories also did not identify an association between low vitamin D consumption and breast cancer development in women.³³ Indeed, breast cancer patients were found to have had a higher consumption of vitamin D than comparable controls. This study, however, did not consider the sunlight exposure-induced synthesis of vitamin D in these subjects.

Another study, examining incidence of breast cancer rather than mortality, also found little evidence of regional variation in breast cancer incidence rates.³⁴ Sturgeon et al, however, argued that the historically higher breast cancer mortality rates reported in the North are declining. Women in the Northeast are now

experiencing a faster rate of decline in breast cancer mortality than their counterparts in the South, especially in specific groups such as black women of all ages and white women aged 20–49 years.²³

Likewise, a study in Norway did not identify a negative association between cancer incidence and mortality and geographical latitude.³⁵ However, the investigators did point out that cases of breast, colon, and prostate cancer diagnosed in the summer and fall—the seasons when serum levels of vitamin D₃ are expected to be the highest—had a significantly better prognosis relative to the cases diagnosed during the winter months. Thus, vitamin D may have a beneficial effect on cancer-specific mortality, and supplemental vitamin D intake may improve cancer-related outcomes.

CLINICAL TRIALS WITH VITAMIN D OR ITS ANALOGS

There have been only a few breast cancer clinical trials with vitamin D or one of its analogs; these are reported in Table 1. In contrast to prostate cancer, such investigations in clinical trials are not as advanced (see Vijayakumar et al for a summary of clinical trials with prostate cancer patients and vitamin D analogs³⁶).

To the best of our knowledge, the first study involved the use of topically applied calcipotriol. This vitamin D analog, also known as compound MC903, was used in the treatment of advanced breast cancer.³⁷ Treatment was administered to 19 patients with locally advanced or cutaneous metastatic breast cancer, with selected cancer nodules receiving the topically applied calcipotriol in doses of 100 micrograms daily. Five patients had to be withdrawn from the study before completion of the treatment; two of them because they developed hypercalcemia. The response rate too was low, with improvements noted in only 3 of the 14 patients who completed the 6 weeks of treatment (these 3 showed a 50% reduction in the bidimensional diameter of treated lesions). Of the remaining 14 patients, 5 unfortunately experienced progression of their disease, 5 reportedly had no change in their disease, and one had only a minimal response. Vitamin D receptors (VDR) were identifiable in the breast cancer cells of 7 patients, including all 4 who had had some response to the topical treatment. These data with calcipotriol suggested that this vitamin D analog may function through a mechanism involving the VDR.

Gulliford et al conducted a phase I trial to evaluate the maximum tolerated dose of another vitamin D analog, EB 1089 (Seocalcitol), in 36 patients with

TABLE 1 Studies with Breast Cancer Patients and Vitamin D Analog Therapy

Study	No. of Patients	Therapy	Dose/Frequency	Duration
Bower 1991 ³⁷	19	Calcipotriol ointment	100 µg, QD	6 weeks
Gulliford 1998 ⁴⁵	36	EB 1089	0.15–17.0 µg/m ² QD	1.5–33.5 weeks (10–234 days)
The Women's Health Initiative Study Group 1998 ³⁹	45,000 women without breast cancer	Calcium and vitamin D ₃	1000 mg elemental calcium + 400 IU vitamin D ₃ QD	8 years (to be completed in 2007)
Das Gupta and Salti (planned study, 2006)	42	D ₅	5–35 µg, QD	12 weeks

advanced breast ($N = 25$) or colorectal ($N = 11$) cancers. EB 1089 is a newly synthesized vitamin D analog that is much more potent in regulating cell growth and differentiation than cholecalciferol ($1\alpha,25(\text{OH})_2\text{D}_3$), has a lower tendency to induce hypercalcemia, and can induce apoptosis in some types of cancer cells.³⁸ All patients received the EB 1089 solution for 5 consecutive days per protocol, and it was continued as compassionate treatment beyond that time in 21 cases for 10–234 days. The first 11 patients enrolled had also received a single dose one week before starting the schedule of protocol doses. The treatment doses used started at $0.15 \mu\text{g}/\text{m}^2$ body surface area daily and were gradually increased to a maximum of $17.0 \mu\text{g}/\text{m}^2$ daily.

All patients receiving the maximum dose suffered from hypercalcemic toxicity. This study identified the optimal dose of EB 1089 to be $7.0 \mu\text{g}/\text{m}^2$ daily. Six of the patients receiving compassionate treatment for more than 90 days showed stabilization of their disease. EB 1089 was found to be much less calcemic than $1\alpha,25(\text{OH})_2\text{D}_3$. Eleven patients in the protocol treatment phase experienced hypercalcemia, with 4 showing severe hypercalcemia at doses of 0.45, 12.4, and $17 \mu\text{g}/\text{m}^2$. During the compassionate treatment phase, 10 patients experienced hypercalcemia, 6 of them severely. However, this study did not demonstrate any anti-tumor effect, as determined by an objective reduction in tumor volume, although six patients showed stabilization of their disease for over three months. Clinical trials evaluating the effectiveness of EB 1089 was then carried out in other cancer types as well.³⁸

The Women's Health Initiative (WHI) Clinical Trial and Observational Study also included a vitamin D supplementation arm. Supplementation was primarily hypothesized to prevent hip and other fractures, and secondarily to prevent colorectal and breast cancer.³⁹ The WHI was established by the National Institutes of Health (NIH) in 1991 and the study involves over 161,000 postmenopausal women aged 50–79, who were enrolled in the study at 40 nationwide clinical centers between 1993 and 1998.

As indicated, one of the hypotheses being tested in the vitamin D arm of the WHI study is that women who receive calcium and vitamin D supplements will benefit with a lower risk of breast cancer than women receiving a placebo. This large-scale trial of a breast cancer chemopreventive agent is a 1:1 randomized double-blind trial using 1000 mg elemental calcium plus 400 international units (IU) of vitamin D₃ daily, versus a placebo. Participants take two pills per day. The planned completion date of the WHI study is 2007 and it is projected to enroll 45,000 women in the calcium and vitamin D supplementation arm. The complete findings of this study are eagerly awaited.

Results published in 2006 from the WHI study found that vitamin D and calcium were not protective against colon cancer in women.⁴⁰ A study finding such as this raises major questions regarding the cancer-vitamin D connection and requires reevaluation of the ongoing work in the area of vitamin D and cancer prevention and therapy. However, several factors in the study may have contributed to the finding that vitamin D had no significant beneficial effect on colon cancer.

First, at enrollment, the participants had mean total calcium (1151 mg) and vitamin D (367 IU) intakes that were twice the national average. Second, with about 40% of the study population not complying completely with the study medication regimen, there may have been insufficient numbers of study participants to demonstrate any beneficial effect on colorectal cancer. Third, the basic calcium and vitamin D doses used—1000 mg of elemental calcium and 400 IU of vitamin D₃—may have been insufficient to provide protection against cancer, as conceded by the authors. Fourth, the intervention period of seven years may have been too short to demonstrate an effect, given that colon cancer takes 10 to 20 years to appear.

Because the effects of vitamin D and calcium are target organ specific, a study not demonstrating a benefit from calcium and vitamin D supplementation on colorectal cancer does not definitively rule out the possibility that such supplementation may provide

protection against cancers in other organs. This study also only evaluated a single drug regimen for colorectal cancer and leaves open the possibility that other formulations and doses, targeted toward other cancers or even colorectal cancer, may have different results. For example, in experimental carcinogenesis models, vitamin D has no effect on lung cancer, whereas it suppresses development of breast and prostate cancer.

VITAMIN D₃

The first evaluation of D₃ as a chemopreventive agent for breast cancer will be conducted in our upcoming clinical trial. At the University of Illinois at Chicago (UIC) we have carefully designed a combined Phase I/II clinical trial to evaluate the safety and efficacy of 1 α (OH)D₃ in patients with metastatic breast cancer. This safety/chemoprevention study, in addition to finding the maximum tolerated dose (MTD) for D₃, will monitor the clinical response as evaluated by decreases in measurable disease determined by physical examination, radiographic studies, and/or nuclear medicine scans.

The breast cancer trial with D₃ at UIC is a companion trial to another that will soon be conducted with D₃ and prostate cancer patients.⁶ There are many similarities between breast and prostate cancer, which both respond to vitamin D. The latter trial will also be a phase I/II safety/chemoprevention study to determine whether 1 α (OH)D₃ can safely delay prostate cancer recurrence when administered after definitive radiation therapy (RT).

In addition to epidemiologic and ecological studies, many animal studies have pointed to the possibility that vitamin D may be an effective chemopreventive agent against breast cancer. There are a number of good reviews on these topics.⁴¹⁻⁴⁴ These animal studies, which are the first steps in the process that a new chemopreventive agent must undergo, include preclinical studies in *in vitro* and *in vivo* animal experiments, followed by phase I, II, and III clinical trials for toxicity and efficacy.

CONCLUSION

Vitamin D and its analogs such as 1 α (OH)D₃ may have a role to play in chemoprevention of breast cancer. Although a modest to moderate degree of toxicity may be acceptable in the context of chemoprevention to prevent disease recurrence, only minimal toxicity would be acceptable in the use of chemoprevention of primary breast cancer in healthy women. Thus, a strong focus on evaluating the known and potential side effect profiles of chemopreventive agents is urgently needed. Funding agencies involved in cancer

research, such as the National Cancer Institute, must demonstrate strong leadership initiatives to identify and evaluate new chemopreventive agents and strategies to both deepen our understanding of cancer, and reduce its morbidity and mortality. Foundations and organizations involved in cancer education also play a strong role in increasing public and patient awareness of the importance of the research process in advancing the therapeutic armamentarium against cancer. Through well designed preclinical and clinical studies, we believe that efficacious chemopreventive agents can be identified and used to prevent primary and recurrent breast cancer.

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Vitamins D3 and D5 mediate growth inhibition of androgen-dependent and independent prostate cancer cells by distinct mechanisms of interaction with the androgen receptor

Appendix 5

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Introduction

Carcinoma of the prostate (CaP) is the second leading cause of cancer related deaths among men in the United States. Prostate epithelial cells express the androgen receptor (AR), a transcription factor which regulates the expression of a variety of proteins, including prostate specific antigen (PSA), a serum marker for detection of prostate disease. As prostate epithelial cells are dependent on androgens for growth, the standard treatment for recurrent prostate cancer is androgen withdrawal therapy (AWT). Most recurrent prostate cancer patients initially respond to this treatment, as determined by decreased levels of serum PSA. However, the majority of patients on AWT ultimately progress to an androgen independent state in which AWT has no effect on cancer growth. There is currently no established therapy known to cure androgen-independent prostate cancer (AIPC).

In vivo and *in vitro* studies have demonstrated that the naturally occurring active metabolite of vitamin D, 1,25 dihydroxy D3 (calcitriol), inhibits proliferation and increases differentiation of numerous cancer cell types, including CaP. However, clinical use of calcitriol is severely limited because its anti-tumor activity is achieved at doses that cause hypercalcemia *in vivo*, both in animal models and in human patients as demonstrated by clinical trials. This has led to the development of synthetic analogs of calcitriol that preserve its anti-proliferative and cell-differentiating properties while minimizing or eliminating its toxic profile. One such analog, 1 α (OH)D5, has been successfully tested *in vivo* in a rat model of breast cancer and was demonstrated to inhibit tumor growth but did not induce hypercalcemia at any dose tested (up to 100 nmoles/kg diet). In this study, we evaluate the effects of 1 α (OH)D5 on the growth and biochemical analysis of the androgen dependent LNCaP prostate cancer cell line and its androgen-independent sublines, C4-2SA and LNCaP-AI. LNCaP-AI cells were obtained by long-term culture of LNCaP cells in androgen-reduced media, while C4-2SA cells are a clonal derivative of C4-2 cells, which were derived from LNCaP xenografts in castrated mice.

Materials and Methods

Cell Culture

LNCaP, LNCaP-AI and C4-2SA cells were maintained in RPMI with phenol red, 5% FBS, and 0.1% penicillin/streptomycin (control media). For experiments, cells were plated with either control media or RPMI media with 5% charcoal stripped FBS and 0.1% penicillin/streptomycin without phenol red (androgen-free media).

Treatment Conditions

Cells were treated with Vitamin D analogs as follows:

- control media or androgen free media
- control media supplemented with 1,25(OH)₂D3 (calcitriol, 100nM) or 1 α (OH)D5 (100nM – 2 μ M)

- androgen free media supplemented with 1,25(OH)₂D3 (calcitriol, 100nM) or 1 α (OH)D5 (100nM – 2 μ M)

The treatments were replaced every 48-72 hours.

Transfection

For some experiments, cells were transfected with either androgen-receptor specific siRNA (AR-siRNA), vitamin D receptor specific siRNA (VDR-siRNA), or non-specific siRNA (control siRNA) at least 4 hours prior to treatment with vitamin D analogs. At indicated time points, cells were either assessed for proliferation by MTT assay or cell lysates were collected for protein expression analysis by immunoblotting.

MTT Assay

Dimethylthiazolyl-2,5-diphenyltetrazolium bromide (MTT, 5mg/ml in PBS) was added to the media and the cultures were incubated for one hour at 37°C in 5% CO₂. DMSO was then added to dissolve the cells and the absorbance was read at 595nm.

Immunoblotting

Whole cell lysates were collected and analyzed by Coomassie or BCA assay to determine relative protein concentration for equal loading on SDS-PAGE gels and transfer to either PVDF or nitrocellulose membranes. The membranes were probed with indicated primary antibodies and appropriate secondary antibody conjugated to horseradish peroxidase, developed with ECL reagent, and exposed to film.

Results

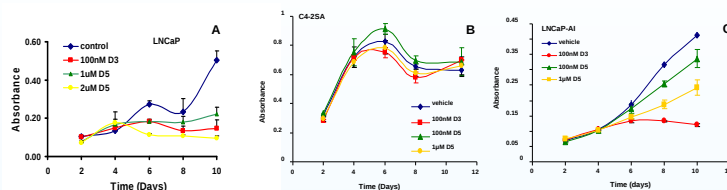


Figure 1: (A) Androgen-dependent LNCaP prostate cancer cells were exposed to either calcitriol (D3) or 1 α (OH)D5 (D5) for the times shown and proliferation estimated by MTT assay. 1-2 μ M 1 α (OH)D5 had an equivalent cytostatic effect as 100nM calcitriol. This is consistent with *in vivo* studies in a breast cancer model with 1 α (OH)D5 in which concentrations 10X greater than calcitriol were required for an equivalent effect on tumor growth and progression. [1] (B-C) Effect of calcitriol and 1 α (OH)D5 on proliferation of androgen-independent prostate cancer cells. Although LNCaP-AI cells were significantly inhibited by 100nM calcitriol and 1 μ M 1 α (OH)D5, neither analog had a significant effect on proliferation in C4-2SA cells.

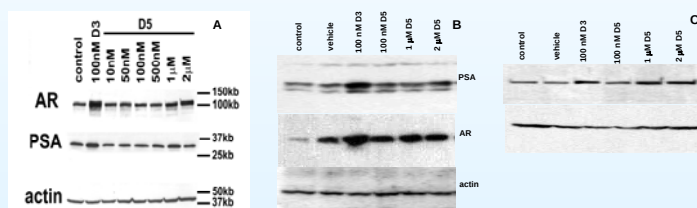


Figure 2: Effect of calcitriol or 1 α (OH)D5 on protein expression in prostate cancer cell lines. (A) Levels of androgen receptor (AR) and PSA were assessed by immunoblotting in androgen-dependent LNCaP cells. After 8 days of treatment with either 1 α (OH)D5 or calcitriol at the concentrations shown calcitriol has a pronounced effect on expression of the AR. However, 1 α (OH)D5 at cytostatically equivalent concentrations (1-2 μ M) showed minimal effect. Androgen-independent C4-2SA (B) and LNCaP-AI (C) cells were treated with 100 nM calcitriol or increasing doses of 1 α (OH)D5. Calcitriol stimulated AR expression in both cell lines and PSA expression in C4-2SA cells. LNCaP-AI cells do not express PSA, hence this protein was not tested in these cells. In contrast, 1 α (OH)D5 had minimal effect on the proteins studied. Levels of β -actin were assessed as loading control.

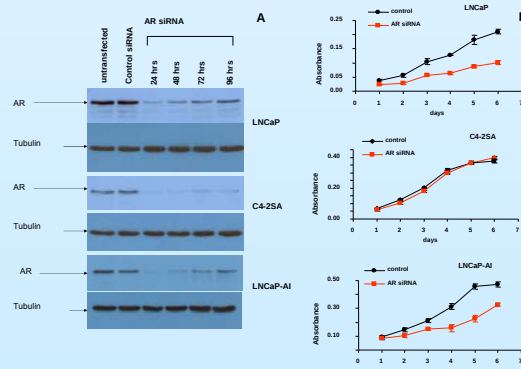


Figure 3: Effect of tranfection of AR specific siRNA on protein expression and proliferation in LNCaP, LNCaP-AI, and C4-2SA cell lines. (A) Immunoblotting demonstrates that AR-specific siRNA downregulates expression of the AR in LNCaP, LNCaP-AI, and C4-2 cells. (B) Growth rates of the AR-specific siRNA transfected cells vs. control (non-specific) siRNA were estimated by MTT assay at the time points shown. Reduced expression of the AR resulted in decreased cell proliferation in both LNCaP and LNCaP-AI cells. However, in C4-2SA cells, which were resistant to the growth inhibiting effects of vitamin D, there was no effect of downregulation of the AR on cell growth.

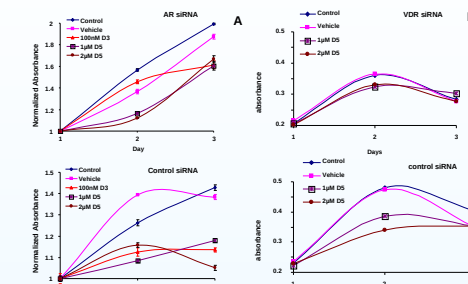


Figure 4: Downregulation of either VDR or AR abrogates the effect of vitamin D analogs. MTT assay showed that either VDR-siRNA (A) or AR-siRNA (B) abrogated the cytostatic effect of calcitriol (D3) or 1 α (OH)D5 (D5) in androgen-dependent LNCaP cells.

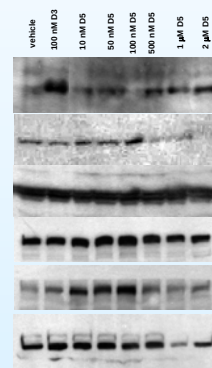


Figure 5: Differential effect of calcitriol and 1 α (OH)D5 on the Akt/mTOR pathway in LNCaP cells by immunoblotting. Panels indicate immunoblotting using various antibodies as indicated. Calcitriol, but not 1 α (OH)D5, caused a significant increase in the phosphorylation levels of p70S6 kinase (upper panel). In contrast, calcitriol did not have a significant effect on Akt phosphorylation, whereas 1 α (OH)D5 inhibited Akt phosphorylation at high concentrations (0.5, 1, 2 μ M) (2nd panel), despite no change in total levels of Akt expression (3rd panel). P70S6 kinase phosphorylation is regulated by mTOR as well as PI3K [21], hence, we also investigated the effect of these hormones on mTOR and its binding partners raptor and rictor [22]. Neither calcitriol nor 1 α (OH)D5 caused any change in mTOR levels (4th panel) and inhibited rictor expression at high levels (lowest panel). These results indicate that calcitriol and 1 α (OH)D5 had differential effects on these proteins in LNCaP cells.

Discussion

- 1 α (OH)D5 at 1-2 μ M, similar to calcitriol at 100 nM, inhibited growth of both androgen-dependent LNCaP cells and its androgen-independent subline LNCaP-AI, but had little effect on another androgen-independent subline C4-2SA.
 - LNCaP-AI cells, despite being androgen-independent, are AR dependent, similar to LNCaP cells, whereas C4-2SA cells are both androgen-independent and AR independent.
 - The effects of both calcitriol and 1 α (OH)D5 are abrogated by either VDR-specific or AR-specific siRNA.
 - AR dependent cells respond to calcitriol and 1 α (OH)D5 whereas AR-independent cells do not.
- Conclusion 1: These data indicate that not only the VDR, but also the AR, mediates the effects of both calcitriol and 1 α (OH)D5 on prostate cancer cells.

- Calcitriol substantially increased the expression of the AR and its transcriptional target PSA, whereas 1 α (OH)D5 at cytostatically equivalent concentrations (1, 2 μ M) showed minimal effect on these proteins.
 - Calcitriol, but not 1 α (OH)D5, induces p70S6 kinase phosphorylation in both androgen-dependent and -independent prostate cancer cells.
 - 1 α (OH)D5, but not 100 nM calcitriol, decreases the level of Akt phosphorylation.
- Conclusion 2: We previously showed that growth of LNCaP cells and its androgen-independent sublines are mediated by Akt [2]. Hence, our results suggest that the effects of calcitriol on cell proliferation and AR expression are mediated by p70S6 kinase phosphorylation, while the effects of 1 α (OH)D5 on proliferation are mediated by downregulation of Akt phosphorylation.

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