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14. ABSTRACT In the past twelve months we have demonstrated that bombesin stimulates the androgen receptor preferentially to a proximal androgen response element in the promoter region rather than in the enhancer region, which is primarily stimulated by androgens. We have shown that gastrin-releasing peptide prostate cancer cells have their growth in soft agar inhibited by the specific Src inhibitor AZD0530. This is a dose-dependent response. AZD0530 abolishes the nuclear translocation of the androgen receptor demonstrating specificity. We have also demonstrated that AZD0530 inhibits metastases of gastrin-releasing peptide prostate cancer cells in a SCID mouse model. Finally, we have delineated the downstream signaling cascades of neuroendocrine activation of androgen independent prostate cancer cell growth and have effectively inhibited these using the novel Src kinase inhibitor.					
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DOD Progress Report 2007

Introduction

We believe that androgen withdrawal is an event that initiates a cascade promoting the development of androgen independence through NE progression. To date we know of no adjuvant therapies targeting castration initiated molecular events in clinical practice. As such, we seek to better define these early post-castration molecular events. We ***hypothesize*** that a small population of neuropeptide expressing AI CaP cells generated by castration can support the AI survival and growth of androgen sensitive cells in a paracrine fashion. This concept is a novel one regarding the early propagation of CaP following castration. Secondly, we ***hypothesize*** that neuropeptide mediated non-receptor tyrosine-kinase signaling activates androgen regulated genes both through AR and GRP dependent, and AR and GRP independent mechanisms. Demonstration of this concept establishes the rationale for neuropeptide pathway inhibition as singular and combination therapy at the time of castration.

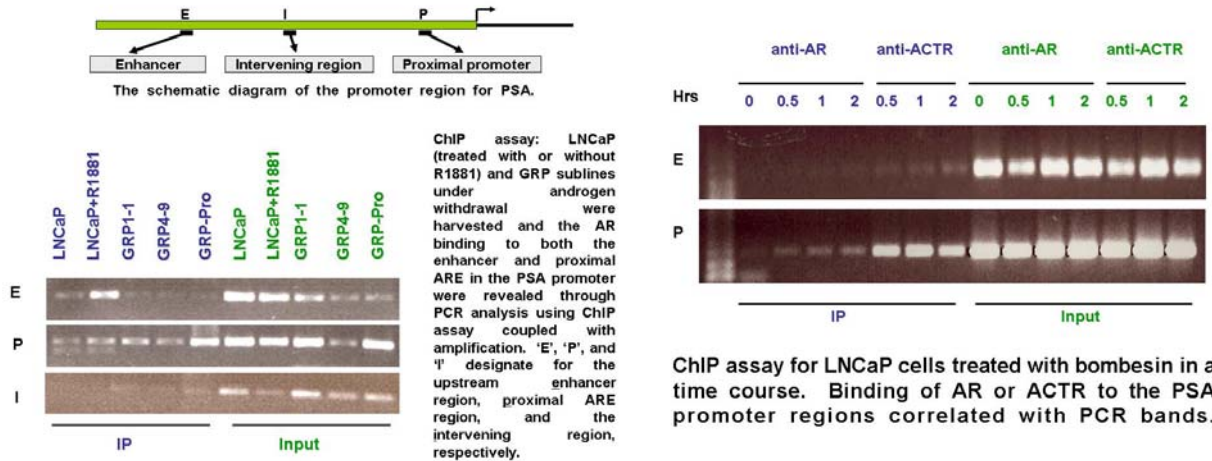
Body

Aim 1. To determine the paracrine effect of NE cells on androgen sensitive CaP cells.

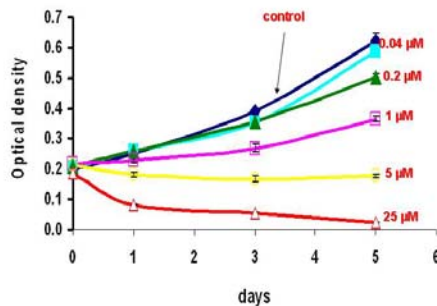
- a. *Determine the in vitro ability for NE cells to support androgen sensitive CaP cell survival and growth (paracrine effect) in androgen-deprived conditions.* Work on this section was replaced by the soft agar assay as results in soft agar are more definitive.
- b. *Determine the paracrine effect in soft agar tumorigenesis.* Work on this section is concluded as reported in the 2006 annual report.
- c. *Determine the paracrine effect on migration in recombinant NE cells.* Work on this section is concluded as reported in the 2006 annual report.
- d. d. *Study the paracrine effect using the in vivo xenograft model with regard to growth and metastasis.* Work on this section is concluded as reported in the 2006 annual report.

Aim 2. To evaluate the mechanisms of AR involvement in our NE model.

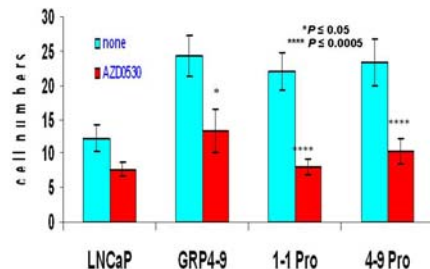
- a. *Testing of inhibition of neuropeptides, signaling molecules and AR inhibitors individually and in combination on soft agar growth of GRP clones and xenograft cells.* The mechanisms of neuropeptide-mediated AR activation were investigated in more details this year. We performed chromatin immunoprecipitation (ChIP) assay and discovered that bombesin-stimulated AR binds preferentially to the proximal ARE site in the promoter region rather than the enhancer region bound by the androgen-stimulated AR. GRP-Pro cells constitutively expressing GRP have the AR occupied on the proximal ARE constantly. This bombesin/GRP-stimulated preferential binding of AR to the proximal site of the PSA promoter is assisted by the AR co-activator ACTR 30 min from addition of bombesin.



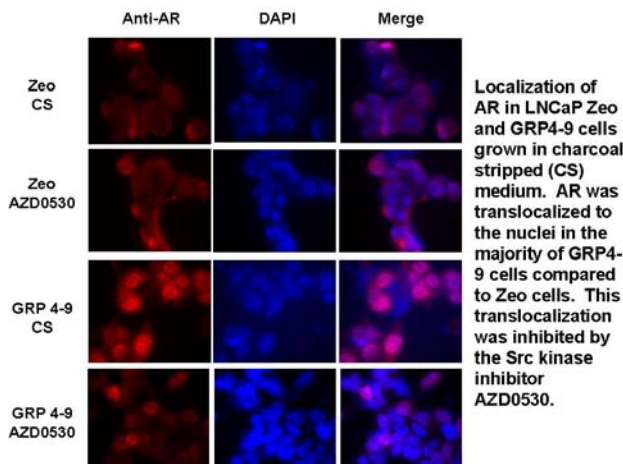
As reported last year, growth of GRP cells in soft agar may be inhibited by the specific Src inhibitor AZD0530. We performed a dose-response growth inhibition curve using GRP-Pro cells grown in CS media and treated with various doses of AZD0530. The IC₅₀ for this inhibition is slightly higher than 1 μ M. The LNCaP GRP cell lines have demonstrated promoted migratory activities than their parental cells. Src kinase inhibitor AZD0530 inhibits the migration assayed by the Boyden chamber assay to the levels similar to the basal activity in the LNCaP cells.



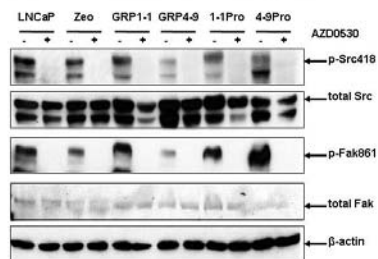
GRP-Pro cells were plated in CS medium with and without the Src inhibitor AZD0530 and their growth was monitored by MTT assay over 7 days. Various concentrations of AZD0530 from 0.04 to 25 μ M were added from day 0. Error bars represented standard error of means.



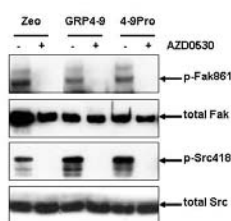
Inhibition of migration of LNCaP-GRP and GRP-Pro cells by AZD0530. Migration assays were carried out in the modified Boyden chamber. Migration assays were performed in a Boyden chamber with 8 mm Nucleopore membrane coated with human plasma fibronectin (50 mg/ml). 2×10^4 LNCaP cells were placed in the upper wells, CS conditioned media with or without 500 nM AZD0530 in the lower wells, and the chamber was incubated at 37°C for 4 hours to allow cell migration. The entire field was counted under a microscope and each experiment was performed in triplicate.



LNCaP GRP cells showed translocation of AR into the nuclei in the absence of androgen stimulation (in CS growth media) compared to the mock-transfected LNCaP Zeo cells. Addition of Src kinase inhibitor AZD0530 abolished the AR nuclear translocation as shown in the left. This result suggests that AR is activated through autocrine stimulation of GRP that is dependent of Src

Src and FAK status in cell lysates

Phosphorylation of Src and FAK kinases was inhibited in parental LNCaP, LNCaP-GRP and GRP-Pro cells by AZD0530 as probed by antibodies specific to Tyr(418)-Src and Tyr(861)-FAK, respectively. The ability of AZD0530 to inhibit Src-mediated phosphorylation of FAK kinase was explained by co-immunoprecipitation (co-IP) of FAK and Src kinases with anti-FAK antibody.

CO-IP of Src and FAK

activation. We surveyed the status of Src and FAK in the LNCaP and GRP subclones and found similar levels of phosphorylated Src and FAK kinases. However, when these two kinases were co-immunoprecipitated by anti-FAK antibodies, stronger phospho-Src levels were detected in GRP subclones than their mock control Zeo cells. These findings confirm our hypothesis that in the absence of AR, bombesin/GRP bind to their

receptors, activate Src and FAK kinases in the complex and activate AR through phosphorylation.

b. *Small hairpin RNA (shRNA)-based silencing of NE cells in vitro and in vivo.* We are in the process of designing the shRNA. Once we get the shRNA construct, we will start experiments in this section.

c. *Testing of inhibitory treatments on chimeric tumors in soft agar and in vivo.* We have demonstrated inhibition of paracrine migration. We are presently testing inhibition of chimeric tumor growth and metastasis in vivo.

d. *In vivo testing of inhibitory treatments at different time points.* Since we have identified Src kinase as the key player in neuropeptide-mediated AR activation, we tested the effect of Src kinase inhibitor AZD0530 in vivo with LNCaP GRP-Pro cells. After almost two months of AZD0530 administration to castrated mice injected with LNCaP GRP-Pro cells, we observed a complete inhibition of metastasis by AZD0530. Although inhibition of primary tumor growth was not significant as reported by other researchers working on various cancers, AZD0530 demonstrated potent inhibition on tumor metastasis. None of the treated animals had metastases to regional lymph nodes but both surviving control animals did.

In vivo study: Ten male SCID mice were castrated and orthotopically implanted with 4×10^6 GRP-Pro cells into the prostate. AZD0530 (50 mg/kg) treatment was administered to seven mice (treatment group) while buffer was administered to three (control group) 16 days after surgery. The study was terminated 70 days after injection, mice from both groups were examined for primary tumor growth and metastasis. At the end of study, two remaining control mice both bore tumors and metastasis to lymph nodes, while five out of seven treated mice produced tumors but with NO metastasis.

	Tumor	Tumor weight (g)	Metastasis
Control	3/3 (one died before tumor collection)	1.04 ± 0.34	2/2
Treatment	5/7	0.73 ± 0.29	0/5

Other Research Accomplishments

We have characterized the expression of the NE induced expression of src, FAK and STAT3 in all major prostate cancer cell lines. We have also validated the action of

Src kinase inhibitor AZD0530 through the Src signaling pathway in two androgen-independent prostate cancer cell lines PC-3 and DU-145 by examining the status of phosphorylation of the downstream kinases and substrates. Through this study, we have identified the molecular mechanism of AZD0530. In vivo inhibitions of tumor progression by AZD0530 are also underway. These data are presently being combined for publication submission.

We have determined the downstream signaling cascades from NE activation and delineated the effect of a novel oral src kinase inhibitor AZD0530 at these signaling points. This is presently in preparation for publication.

Key Research Accomplishments

We have demonstrated that Src kinase is the key player in neuropeptide-mediated AR activation. Together with our studies in the chimeric growth of androgen-sensitive and androgen-insensitive cells, we are more confident with our proposed hypothesis. A paracrine effect exists for androgen insensitive CaP cells to support the survival and proliferation and migration of androgen sensitive CaP cells in a castrated environment. We have further delineated the impact of NE differentiation in prostate cancer.

Reportable Outcomes

Abstract presentations 2006-2007

1. 2006 Chang, Y-M., Bai, L., Yang, J.C., Kung, H-J., and Evans, C.P. Survey of Src activity and Src-related growth and migration in prostate cancer lines. Proceedings of the American Association for Cancer Research, 47: 2505.
2. 2006 Yang, J.C., Bai, L., Kung, H-J., and Evans, C.P. Androgen-sensitive prostate cancer survival and progression is supported by neuroendocrine prostate cancer cells. Proceedings of the American Urological Association, 175:409.
3. 2006 Evans, C.P., Bai, L., Kung, H-J., and Yang, J.C. Androgen-sensitive prostate cancer survival and progression is supported by neuroendocrine prostate cancer cells. Urological Research Society, Salzburg Austria.

Publications 2006-2007

1. 2007 Yang, J.C., Busby, J.E., OK, J., Borowsky, A.D., Kung, H-J., Evans, C.P. Neuropeptide induced androgen-independent prostate cancer xenograft is mediated through Src tyrosine kinase pathway. Submitted to Cancer Research.
2. 2006 Cambio A.J. and **Evans, C.P.** Minimizing Incontinence During Radical Prostatectomy: Considerations and Evidence. Eur Urol; 50(5):903-13; discussion 913.
3. 2006 Evans, C.P. Editorial Comment on "Penis Conserving Treatment for T1 and T2 Penile Carcinoma: Clinical Implications of a Local Recurrence. Lont, A.P. et al. J. Urol 2006;176:580.
4. 2006 Cambio AJ, **Evans CP.** Outcomes and quality of life issues in the pharmacological management of benign prostatic hyperplasia (BPH). Therapeutics and Clinical Risk Management. In press.
5. 2007 Nelson, E.C., Cambio A.J., Yang, J.C., Ok, J., Lara, P.N., **Evans CP.** Clinical Implications of Neuroendocrine Differentiation in Prostate Cancer. Prostate Cancer and Prostatic Diseases. 2007;10:6-14.

6. 2007 Chang, Y-M., Kung, H-J, **Evans, C.P.** Non-Receptor Tyrosine Kinases in Prostate Cancer. *Neoplasia*. 2007; 9:90-100
7. 2007 Nelson, E.C., Cambio A.J. Yang, J.C., Lara, P., and **Evans, C.P.** Biologic agents as adjunctive therapy for prostate cancer: a rationale for use with androgen deprivation. *Nature Clinical Practice Urology*;4:82-94.
8. 2007 Cambio A.J., Ellison L.M., Chamie, K., deVere White, R.W., and **Evans, C.P.** Cost-Benefit and outcome analysis: effect of prostate biopsy under-grading. *Urology*. 69:1152-6.
9. 2007 Nelson, E.C., **Evans, C.P.**, Mack, P.Cl, deVere White, R.W., Lara, P. Inhibition of Akt pathways in the treatment of prostate cancer. *Prostate Cancer Prostatic Diseases* 2007, in press.
10. 2007 Nelson EC, **Evans CP**, Pan CX, Lara PN. Prostate cancer and markers of bone metabolism: diagnostic, prognostic, and therapeutic implications. *World J Urol*. epub.
11. 2007 **Evans CP**. Editorial Comment on: Long-Term Intravesical Adjuvant Chemotherapy Further Reduces Recurrence Rate Compared with Short-Term Intravesical Chemotherapy and Short-Term Therapy with Bacillus Calmette-Guerin (BCG) in Patients with Non-Muscle-Invasive Bladder Cancer. *Eur Urol*. epub.
12. 2007 Nelson, E.C., **Evans C.P.**, Lara, P.N. Renal cell carcinoma: current status and emerging therapies. *Cancer Treat Rev*. 33:299-313.

Conclusions

We have made headway into understanding the paracrine relationship between neuropeptide expressing, androgen-insensitive CaP cells and their ability to support the proliferation and migration of androgen sensitive CaP cells. Critically, we have identified src kinase as a molecule central to the process. We have been awarded a NIH CTEP phase II trial to study a novel, oral src kinase inhibitor AZD0530 in androgen-insensitive prostate cancer patients based upon our work.

References

None

Appendices

none