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PRINCIPAL INVESTIGATOR: Barry D. Nelkin, Ph.D.

CONTRACTING ORGANIZATION: Johns Hopkins University
Baltimore, MD 21205

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Nelkin, Barry, D., Ph.D.

Email: bnelkin@jhmi.edu

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INTRODUCTION: We have recently found that CDK5 is active in prostate cancer cell lines and in almost all human metastatic prostate cancers, and inhibition of CDK5 activity resulted in reduction of spontaneous metastases by 79%. This suggests that CDK5 is a novel potential therapeutic target to limit prostate cancer metastasis. Based on our finding that CDK5 activity is present in prostate cancer and is important for metastasis, we intend to develop CDK5 as a novel therapeutic target. We hypothesized that 1) pharmacological inhibitors of CDK5 can limit or block metastasis, and 2) in established skeletal metastases, inhibition of CDK5 may inhibit tumor growth and sensitize tumor cells to other therapies. Therefore, we proposed to characterize a series of small molecule CDK5 inhibitors for specificity in cell culture, and for their effect on xenograft models of prostate cancer. We also proposed to examine the role of CDK5 activity in growth of prostate cancer metastatic to bone, using PC3 based bioluminescent cell clones, and to explore the potential for CDK5 inhibition to sensitize prostate cancer cells to chemotherapy.

BODY:

1. Cell culture evaluation of hymenialdisine derivatives as CDK5 inhibitors (months 1-8). As reported last year, we have tested the hymenialdesine compounds in vitro, for their effect on cell motility and cell proliferation, using DU145 cells. We selected compound 28p for further development, since 28p has excellent selectivity as a CDK5 inhibitor (Wan et al, 2004). We found that 28p was effective for inhibition of cell motility at a concentration of 2 uM, but not at 0.2 uM, and that it had no effect on cell proliferation at these concentrations. We are now testing the ability of 28p to inhibit cell motility at intermediate concentrations; these experiments will be important for interpretation of our ongoing pharmacokinetic studies of 28p (see below, item 2), which will determine whether 28p can be employed effectively in vivo.

2. In vivo toxicity studies of hymenialdisine derivatives (months 9-11). These studies were temporarily slowed by an unexpected hurdle (now overcome, as described below). Our collaborators at the Novartis Genomics Foundation (GNF), who originally synthesized 28p and identified it as a selective CDK5 inhibitor (Wan et al, 2004), did not have sufficient quantities of 28p for our in vivo studies. Therefore, we had to synthesize the compound here, at the Johns Hopkins Oncology Center Medicinal Chemistry Core Facility. In addition, the final two steps of synthesis had to be modified for scale-up. After some effort, we now have 4.28g of high purity (98%) 28p, which migrates as a single peak by HPLC and LC mass spectrometry (Fig. 1). In collaboration, we have begun pharmacokinetic studies on 28p in mice. These studies are designed to evaluate the achievable plasma concentration, AUC, and half-life of 28p in mice. We intend to examine several routes of administration, including oral, intraperitoneal, and subcutaneous administration. Our very recent initial PK data (which is still proprietary) thus far indicate that oral administration results in significant plasma concentration of the drug, but suggest that improvements in administration will be important for further development.

3. **Identification of protein indicators of CDK5 activity (months 5-16).** As reported last year, we examined a panel of potential CDK5 substrates, based largely on the published substrates of CDK5 in neurons. Unfortunately, phosphorylation of these

substrates was not altered by inhibition of CDK5 in prostate cancer cells, and therefore they were not appropriate biomarkers for CDK5 inhibition in prostate cancer.

In order to develop markers of CDK5 inhibition, we reasoned that a global study of changes in gene expression not only might directly provide markers of CDK5 activity, but that bioinformatics analysis of the gene expression changes might indicate signal transduction pathways altered by CDK5 inhibition. Therefore, we performed an extensive cDNA microarray study of AT6.3 cells expressing dominant negative CDK5 or vector-only control (Strock et al, 2006). Our reasoning appears to have been correct. These data, which were presented at the IMPaCT meeting in Atlanta (Strock et al, 2007), resulted in our identification of reduced phosphorylation of tyrosine 705 of STAT3 as a marker of inhibition of CDK5.

In an attempt to discover novel pathways and cellular functions controlled by CDK5 activity in prostate cancer, we analyzed two Dunning AT6.3 vector-only control cell clones and three AT6.3 CDK5 DN clones using Affymetrix Rat 230.2 GeneChips. Each line was grown in duplicate, and separate mRNA isolations and hybridizations were performed following standard Affymetrix protocols, providing a total of 10 arrays. The data were analyzed using the R/Bioconductor package (Gentleman et al, 2004). Normalization and background correction were performed using RMA (Irizarry et al, 2003), and statistical tests were done using SAM (Tusher et al, 2001) in the MEV tool (Saeed et al, 2006) and Gene Set Enrichment Analysis (GSEA) (Subramanian et al, 2005) in the R tool using ranking based on the t-statistic. GSEA p-values were adjusted for multiple testing using the Benjamini-Hochberg correction procedure (Klipper-Aurbach et al, 1995), and a cutoff value of alpha = 0.05 defined significant enrichment terms. Using SAM with a false discovery rate of 1%, 86 probesets were detected as upregulated in CDK5 DN lines relative to control lines, while 119 probesets were detected as downregulated. These mapped to 76 unique UniGene clusters and 35 known genes for upregulated probesets, and 94 unique UniGene clusters and 38 known genes for downregulated probesets. GSEA analysis showed gene ontology enhancement in 13 biological process terms. However, eliminating general metabolic categories left only two terms, “interphase” and “interphase of mitotic cell cycle”. GSEA analysis of KEGG pathways showed no significant enhancement following correction for multiple testing.

CDK5 activity affects IL-6/STAT3 signaling: from in silico to in vitro. We were most interested to find both IL6 and IL6st (IL-6 signal transducer – an IL-6 specific subunit of the gp130 cytokine receptor) in the downregulated gene list in DN lines (1.6 fold for IL6st; 3.5 fold for IL6, confirmed by qRT-PCR). In order to explore whether there might be a change in IL-6 signaling in the AT6.3 CDK5dn cells, we looked for modulation of expression of other genes in the IL-6 pathway, and found apparent enrichment of these genes (Table 1). We also used the NetPath database (http://www.netpath.org). NetPath contains a manually curated IL-6 pathway, which we downloaded in PSI-MI format and loaded into Cytoscape (Shannon et al. 2003). We used NetPath to retrieve a curated list of genes known to be regulated by IL-6 signaling (see Table 1). Since NetPath curates human pathways, we needed to assume for this preliminary work that the corresponding rat genes would be similarly affected by IL-6 signaling, although this is at best an approximation. Using these 52 upregulated genes and 20 downregulated genes, we performed GSEA on this single pathway. We used the absolute value of the signed statistic in order to treat downregulated genes with significantly lower expression similar

to upregulated genes in GSEA. Despite the limited genomic coverage provided by NetPath, and the necessary assumption of equivalence between human and rat downstream responders, we obtained a p value of 0.08 for a change in IL-6 pathway activity in DN lines vs. controls (see Figure 2). We also manually rearranged the protein nodes to clarify the potential connections in this pathway, presented in Figure 3.

This finding of inhibition of the IL-6 pathway has potentially important implications both for the biology of prostate cancer, and for the development of biomarkers for CDK5 inhibition. IL-6 signaling appears to be important in prostate cancer. Increased IL-6 levels have been shown in metastatic prostate cancer, and correlate with worse prognosis (Nakashima et al, 2000). An IL-6 autocrine loop has been demonstrated in prostate cancer cell lines, and blockade of this signal resulted in decreased growth (Chung et al, 1999). IL-6 signaling also has an important role in tumor immune response (Wang et al, 2004). Follow up studies in the cell lines confirmed that inhibition of Cdk5 result in lower levels of phosphorylation of STAT3 at Y705 (Fig. 4); this site is phosphorylated by JAK in response to IL-6 signaling, resulting in STAT3 activation. Thus, phosphorylation of STAT3-pY705 serves as a biomarker for CDK5 inhibition in prostate cancer cells. These studies suggest that CDK5 inhibition may interfere with prostate cancer cell signaling pathways; this finding could indicate potential sensitization to specific therapies.

4. Time course studies of CDK5 inhibition in vivo (months 12-15). Not done – pending completion of PK studies (above, item 2)

5. Evaluation of effect of hymenialdisine derivatives on spontaneous metastasis (months 17-24). Not done- pending completion of PK studies (above, item 2) and in vivo time course (item 4)

6. Development and characterization of AT6.3luc vector-only, AT6.3luc dnCDK5, PC3luc vector-only, PC3luc dnCDK5 cell clones (months 5-12). We reported last year that we had developed PC3 cells expressing luciferase and either dominant negative CDK5, or a vector-only control. Unfortunately, these PC3luc-based cells were not tumorigenic in nude mice. Therefore, we have had to begin once again, to develop a PC3 based cell that can be imaged and in which CDK5 is genetically inhibited. This time, we have used a lentiviral vector to stably knock down CDK5 expression in PC3 cells. The lentivirus expresses either CDK5 shRNA, of a control shRNA, as well as GFP and puromycin resistance. These cells were selected for puromycin resistance, fluorescence sorted for expression of GFP, and assessed for CDK5 expression by Western blotting. The knockdown in the CDK5 shRNA cells was essentially complete. We are now examining the tumorigenicity of these cells both subcutaneously and intratibially, as described below.

7. **Evaluation of the effect of CDK5 inhibition on skeletal growth of prostate cancer cells (months 13-24).** As discussed in section 6, above, our initial attempts to examine the effect of CDK5 inhibition on growth of prostate cancer cells in the bone microenvironment were unsuccessful, requiring two rounds of re-engineering of the prostate cancer cells. We now have constructed PC3 cells stably expressing a CDK5 shRNA and GFP, and control cells expressing GFP. We have implanted these cells intratibially in nude mice, and we will be able to evaluate their growth shortly.

8. **Evaluation of the effect of CDK5 inhibition on sensitivity of prostate cancer cells to chemotherapy (months 25-36).** Not done

KEY RESEARCH ACCOMPLISHMENTS:

- Preliminary identification of a selective CDK5 inhibitor
- Development of PC3luc cells expressing dnCDK5 or CDK5 shRNA
- Gene expression profiling and bioinformatics based identification of the IL-6/STAT3 signal transduction pathway as a target of CDK5 inhibition, and STAT3-pY705 as a biomarker of CDK5 inhibition.

REPORTABLE OUTCOMES: PC3luc dnCDK5 and PC3luc vector only cells; PC3 CDK5 shRNA GFP and PC3 control vector GFP cells; available from the investigators for collaborative projects.

Abstract: Strock, CJ, Pandey, N, Ochs, MJ, and Nelkin, BD. Consequences of Inhibition of CDK5 in Prostate Cancer Cells. IMPaCT meeting (Atlanta, Ga, September 5-8, 2007), abstract P27-19, pp. 261-2.

CONCLUSION: We have shown that CDK5 activity is required for metastasis in prostate cancer cells. The identification of a selective CDK5 inhibitor will allow us to explore the potential of CDK5 inhibition by small molecule therapeutics to limit metastasis. In addition, the development of the PC3 cells expressing CDK5 shRNA will allow us to test our hypothesis that inhibition of CDK5 will inhibit the ability of prostate cancer cells to grow
in the bone microenvironment, the metastatic site that is most lethal in this disease. Our identification of the IL-6/STAT3 signal transduction pathway as a target of CDK5 inhibition suggests potential strategies for employing CDK5 inhibition in therapy for prostate cancer.

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APPENDIX:

Abstract

Strock, CJ, Pandey, N, Ochs, MJ, and Nelkin, BD. Consequences of Inhibition of CDK5 in Prostate Cancer Cells. IMPaCT meeting (Atlanta, Ga, September 5-8, 2007), abstract P27-19, pp. 261-2.

Consequences of Inhibition of CDK5 in Prostate Cancer Cells

Barry D. Hobbs, Christopher J. Strock, Neha Pandey, and Michael J. Ocke.
Johns Hopkins University School of Medicine, Baltimore, MD 21231.

Abstract

[illegible]

Background

CDK5

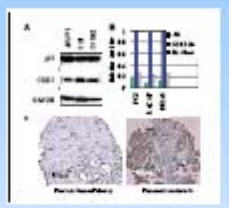
- First identified by its close structural homology to the mitotic cdks. But CDK5 is not a cyclin dependent kinase, and has no known direct cell cycle function.
- Cytoplasmic and is activated by p35 kinase.
- Association with a non-cyclin regulatory subunit absolutely required for activity.
- Ubiquitously expressed, but most only known to be active in post-mitotic neuronal precursors, malignant melanoma, and keratinocytes. Role in tissue-specific expression of regulatory subunits.

CDK5 Functions

Neuronal migration
Neurite outgrowth
Cell adhesion
Membrane organization
Synaptic structure and plasticity
Neuronal degeneration
Apoptosis

(Dhavan Nikolic, Trends in Cell Biology 16:1, 2006)

Results



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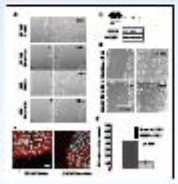

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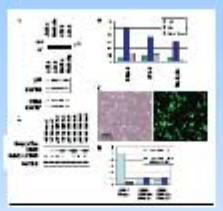

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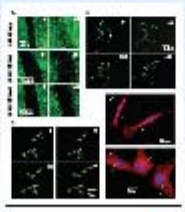

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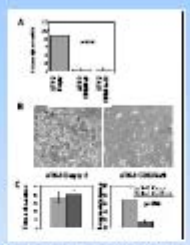

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CDK5 activity affects IL-3/STAT3 signaling: How to affect it in vivo

[illegible]

Results

[illegible]

[illegible]

Conclusion

[illegible]

Reference

[illegible]

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SUPPORTING DATA:

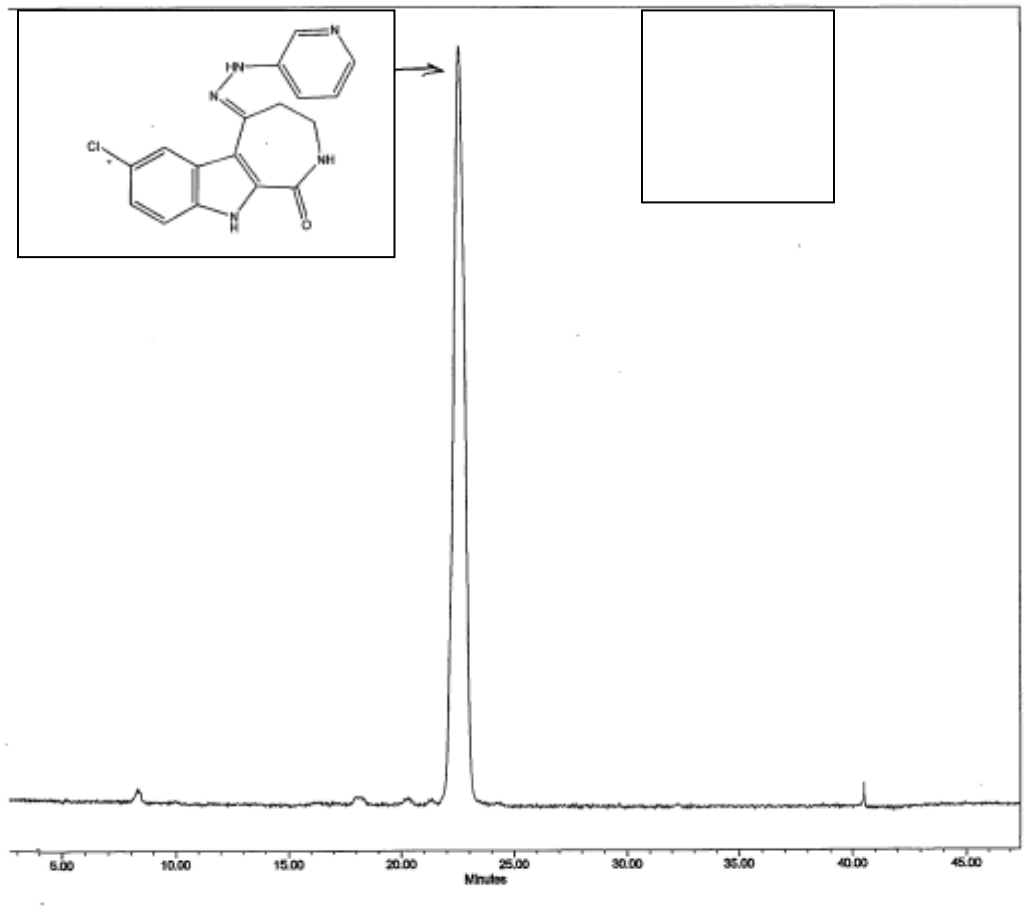

Figure 1. Structure of 7-chloro-5-(pyridine-3-yl-hydrazono)-3,4,5,10-tetrahydro-2H-azepino[3,4-b]indol-1-one (compound 28p; insert) and HPLC analysis of scaled up synthesis.

Fig. 2. **The distribution of the t-statistic for GSEA enrichment analysis of the IL6 pathway**. The black lines represent genes that are regulated by IL6 based on the NetPath database, white represents all other genes, with a total of 13781 unique genes plotted. The index is the ordered rank of genes based on the t-statistic, and the top horizontal indicates the range of genes plotted.

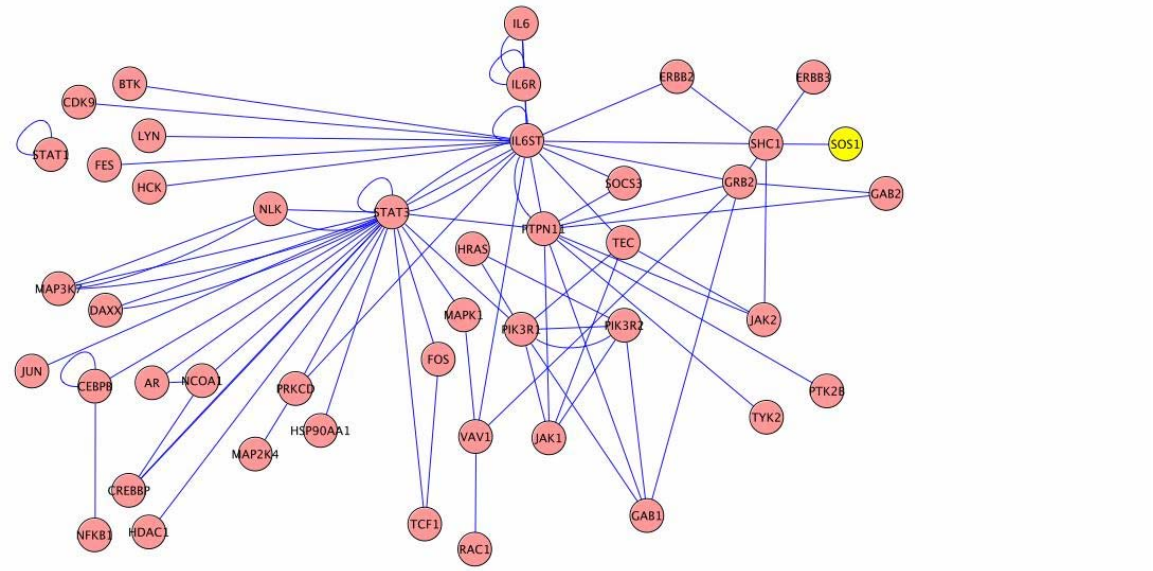

Figure 3: The NetPath curated IL6 signaling pathway as represented in Cytoscape.

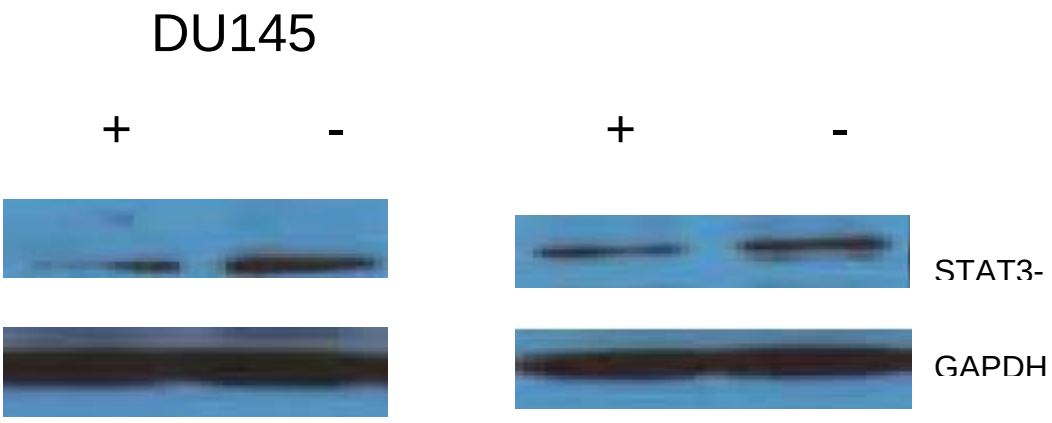

Figure 4. STAT3 activation is decreased by CDK5 inhibition. Prostate cancer cell lines DU145 and AT6.3 were treated with roscovitine for 48 hr. Phosphorylation of STAT3 tyrosine 705, indicative of STAT3 activation, was examined by Western blotting, using a STAT3-pY705 phosphospecific antibody (Cell Signaling Technology #9131). GAPDH was assessed as a loading control. Inhibition of CDK5 by roscovitine treatment resulted in lower levels of phosphorylated STAT3.

Table 1

Examples of Genes in the IL-6 Pathway Regulated by CDK5 Inhibition in AT6.3 Cells

Gene	Name	Fold Change
Il6	IL-6	-3.5
Il6st	IL-6 receptor subunit	-1.6
Cp	Ceruloplasmin	-3.4
Rgs7	Regulator of G-protein signaling 7	-2.7
Orm1	Orosomucoid	-1.6
Mmp10	Matrix metalloproteinase 10	-2.5
Sgk	Serum/glucocorticoid regulated kinase	1.5
Lcn2	Lipocalin 2	-4.9
B2m	Beta-2-microglobulin	-1.5
Wars	Tryptophanyl-tRNA synthetase	-1.8