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Cell Cycle Regulatory Components

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13. ABSTRACT (Maximum 200 Words) The original goal was to target cell cycle regulatory components as a potential therapy. The p16 gene is a tumor suppressor and a CDK inhibitor that is inactivated by gene methylation. The drug 5-aza-2'deoxyctidine (5-Aza-CdR), an inhibitor of DNA methylation, was used for re-expression of a repressed p16. Although p16 re-activation occurred and cell growth was inhibited by 5-Aza-CdR, the effect was not specific to cells with a methylated p16 gene. A mouse model was developed and used to test the efficacy of 5-Aza-CdR. The results were not conclusive because of the inherent toxicity of 5-Aza-CdR. A blood test was developed for the detection of p16 methylation and used to show that the number of patients suitable for a 5-Aza-CdR clinical trial was very low (<3%). These results made a clinical trial with 5-Aza-CdR unrealistic. A phase II clinical trial with a second drug, Bryostatins-1, which regulates the p21 CDK inhibitor, was terminated due to dismal accrual rates even after initial success. Progress was made exploring the Cdc7 protein, which regulates the cell cycle and mutagenesis. Cdc7 may represent a new potential target as it was shown to be up-regulated in many breast cancer cell lines.				
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Introduction

During the past two decades a very large increase in our knowledge of biological mechanisms regulating progression of cells through the cell division cycle has taken place. Together with the development of this knowledge, it has been shown by work in many laboratories that essentially all cancer cells have one or more defects in the components known to regulate cell cycle progression. For example, our completed studies of cell cycle regulatory defects in breast cancer cells, carried out with a grant from the Army Breast Cancer Research Program, showed that loss of expression of the cyclin-dependent kinase inhibitor p16, sometimes accompanied by overexpression of cyclin D1, is a common defect in breast cancer cells. These findings, plus the large amount of work carried out by others, presented a new potential target for cancer chemotherapy. Our proposal to exploit such targets for the chemotherapy of breast cancer was the basis for this Clinical Translational Research Grant. We explored two drugs known or expected to cause changes in the expression of cell cycle regulatory components as potential chemotherapeutic agents in the treatment of late stage breast cancer. Bryostatins, shown by Kraft and coworkers to cause increases in the expression of the cyclin-dependent kinase inhibitor p21, was chosen as an agent to be tested in a phase II clinical trial. 5,6-dihydro-5-azacytidine, a DNA methylation inhibitor with less toxicity than the commonly studied 5-aza-2'-deoxycytidine, was chosen for pre-clinical studies directed towards eventually implementing a phase II clinical trial of that drug. DNA methylase inhibitors have been shown to increase the expression of p16 protein in cells where lack of expression is due to methylation of the p16 gene. Our cell culture studies showed that 5-aza-2'-deoxycytidine was more effective than 5,6-dihydro-5-azacytidine in activating p16. Our animal studies have revealed potential caveats about the use of 5-aza-2'-deoxycytidine in clinical trials. Our human studies have shown that the number of patients who present with methylated p16 is low, so it is not clear if this drug would be useful. Therefore, we analyzed a different cell cycle regulator, the human Cdc7 gene, which we find is overexpressed in many human breast cancer cell lines. A summary of the entire project is reported here.

Body of Report

Relationship to Statement of Work

Tasks 1, 2, and 3. To implement and evaluate a Phase II clinical trial designed to test the chemotherapeutic efficacy of bryostatin 1 in Stage IV breast cancer patients who have failed high dose chemotherapy.

This trial was begun in 1998 and had problems accruing patients. In year 1, only 5 patients entered this phase 2 trial. The initial results with these patients were promising and are described in detail in the year 1 report. The results included measurements of protein kinase C in neutrophils, lymphocyte surface markers and serum bryostatin concentrations to establish pharmacokinetic parameters. Bryostatin was shown to reduce the activity of protein kinase C after 24 hrs. In year 2, the trial was halted because all clinical trials at the UCHSC were under investigation by the FDA. New protocols were written, re-submitted and approved for year 3. However, no patients were accrued even though over 50 patients contacted us from all over the world. None of these patients was acceptable for the trial. Therefore, the trial was terminated by the NCI in the middle of year 4 (March, 2002). The main reason was the dismal accrual rate. Dr. Kraft of this grant is the PI for this trial and he has communicated these results to Dr. Adriene King of AMDEX corp., who is the Human Subjects Protection Specialist for the USAMRMC (see Memo MCMR-RCQ (70-1n).

Task 4. To determine the effects of DHAC and 5aza-2'deoxyctidine on p16 levels and on growth and tumor formation by breast cancer cells.

As described in previous progress reports (years 1-2), DHAC proved to be ineffective as a DNA methylation inhibitor capable of inducing expression of p16 in breast cancer cell lines. Thus, we turned to the more widely studied methylation inhibitor 5aza-2'deoxyctidine (5-Aza-CdR) and revised this task in year 3. We demonstrated that 5-Aza-CdR causes demethylation of p16 DNA and expression of p16 protein in several breast cancer cell lines including T47D-DE and T47D-CO, with concomitant loss of growth of these cells in soft agar (years 3-4). We reported on the effect of the drug 5-

Aza-CdR in nude mice following tumor implantation of breast cancer cells (year 4). Our results also show that 5-Aza-CdR will inhibit the growth of MCF-7 cells in soft agar. In contrast, MCF-7 cells do not have methylated p16 but are deleted for p16. This implies that the effect of 5-Aza-CdR is not specific to p16. Thus, 5-Aza-CdR must be inhibiting the growth of MCF-7 cells by affecting genes other than p16. We confirmed this result by showing that overexpression of p16 arrests T47D cells in G1 of the cell cycle, but that treatment with 5-Aza-CdR does not (years 3-4).

In contrast, our initial animal studies were encouraging in that tumors were smaller in mice treated with 5-Aza-CdR. However, we have seen high toxicity (>80%) for 5-Aza-CdR which has frustrated out attempts to complete these experiments with statistically significant data (years 4-5). All in all, the combination of the lack of specificity and the toxicity do not bode well for the use of 5-Aza-CdR in human patients.

Task 5. To test the effects of combination of DHAC, 5-Aza-CdR and bryostatin 1 on growth and tumor formation by breast cancer cells. Months 24-48.

Again, DHAC was ineffective in vitro so it was not used. Therefore, this task was revised in year 3. The bryostatin 1 trial was closed (See Tasks 1, 2 and 3). 5-Aza-CdR was effective but toxic and not specific (See Task 4).

Task 6. To design a Phase II clinical trial of 5-Aza-CdR.

We proposed this trial as a revised Task in year 3 because of the poor results from the bryostatin trial (see Tasks 1-3 above) and because DHAC was ineffective in vitro (see Task 4 above). We finally decided to recommend against this trial because 5-Aza-CdR is toxic and not specific, which we learned in years 3-5 (See Task 4 above). Thus, we based our decision on our pre-clinical studies in years 3-5 using both breast cancer cells in vitro and in vivo with mouse xenographs.

Task 7. To implement a Phase II clinical trial of 5-Aza-CdR in breast cancer patients who have failed high dose chemotherapy and whose tumors contain methylated p16 DNA.

This was added as a revised Task in year 3, As described in Task 6 above, we recommended against this trial because 5-Aza-CdR is toxic and not specific.

Task 8. To evaluate the outcome of 5-Aza-CdR chemotherapy in terms of effects on tumor response as well as on methylation of tumor p16 DNA, expression of p16, cdk4/cdk6 kinase activity and phosphorylation state of Rb protein.

This revised Task (Year 3) was not accomplished as 5-Aza-CdR is toxic and not specific (See Task4).

Task 9. To develop methodology for determining the p16 methylation status of breast cancer patients by measurements on plasma DNA, and to employ this methodology for the selection of patients for clinical trials of 5-Aza-CdR in breast cancer.

Although we recommended against a 5-Aza-CdR clinical trial, we believe that knowledge of the fraction of breast cancer patients with methylated p16 is still useful to know. Therefore, in year 3, we proposed this revised task. We developed two protocols to evaluate the methylation status of the p16 gene in human samples. These protocols involve a unique assay of methylated p16 DNA done using DNA that is free in the blood of patients. The first protocol #00-848 enables us to examine blood from 100 patients that are known to have metastatic breast cancer. Patients with known metastatic disease have a single sample of blood drawn and the plasma frozen for later analysis. Statistical analysis demonstrates that p16 should be methylated in 30% of the tumors according to the published reports (Herman et al, 1995). This number of samples should give us sufficient numbers of samples to demonstrate whether this blood test is sufficiently sensitive to detect p16 DNA in the blood. The second protocol was designed to enable us to correlate the p16 methylation in samples taken at the time of breast biopsy or surgery with the blood samples. DNA is extracted from the tissue and the blood and then analyzed by sensitive PCR for the methylation of p16.

The second protocol #00-849 enables us to correlate the p16 methylation in samples taken at the time of breast biopsy or surgery with the blood samples. DNA is extracted from the tissue and the blood and then analyzed by sensitive PCR for the methylation of p16. We found only one out of 31 breast cancer patients with a methylated p16 allele (years 3-4).

We re-wrote both protocols after being contacted in March of 2002 by Dr. Adriene King of AMDEX corp., who is the Human Subjects Protection Specialist for the USAMRMC. We were contacted by Dr. Robin Ditmer at USAMRMC in October of 2002 that our two revised protocols had been approved. We received 10 more samples from October of 2002-February of 2003. Unfortunately, none of them contained a methylated p16 allele. This, we have only detected 1/41 (2.4%) methylated p16 alleles, which makes this biomarker unusable in the clinic.

Task 10. To measure the level of Cdc7 protein in human breast cancer cell lines and in breast cancer patient samples.

We proposed this revised task in year 5. We had found that 11/12 breast cancer cell lines overexpress Cdc7 protein (reported in year 4). In contrast, no overexpression was seen in the normal epithelial cell lines MCF-10A and MCF-12A. We found a correlation between Cdc7 overexpression and a de-regulated G1 to S phase in these cell lines (year4). We believe Cdc7 represents an important biomarker in cancer as shown by previous results from my laboratory (Hess et al., 1998; Scalfani, 2000). Cdc7 is known to effect the regulation of both DNA replication and mutagenesis, which are both altered in cancer

cells (Sclafani, 2000). Our hypothesis is that Cdc7 protein levels could be predictive of poor prognosis in breast cancer. This new Task was approved in March of 2003. We spent the remaining time producing and purifying chicken and rabbit anti-Cdc7 antibodies. We used the antibodies to show that Cdc7 is overexpressed in many cancer cell lines (Fig. 1 in Appendix). We also constructed a vector that overexpresses an epitope-tagged Cdc7 protein and tested it in breast cancer cell lines. We used vector pcDNA 3.1 His (Invitrogen), which produces a tagged (6XHis and Xpress epitopes) Cdc7 (Fig. 2 in Appendix). You can select for this vector with G418. This vector helped us to characterize the specificity of our antibodies. In addition, this vector will be useful for defining the molecular role of Cdc7 in human carcinogenesis.

Key Research Accomplishments

- We have shown that the inhibitory effects of 5-AzaCdr on breast cancer cells are not specific to cell lines containing a methylated p16 gene.
- We have developed a model that will allow us to study human breast cancer tumors in nude mice. We have used this model system to test the efficacy of methylation inhibitors such as 5-Aza-CdR. Our results show that 5-Aza-CdR is toxic in mice and we recommend against using it in patients.
- We have found that methylated p16 sequences are readily detectable in DNA isolated from plasma of patients. However, a low percentage (<3%) of patients present with this defect.
- We have found that Cdc7 protein represents a potential biomarker in breast cancer in that a majority of breast cancer lines have overexpressed Cdc7 protein. We show a high level of positive correlation of Cdc7 overexpression with deregulation of the G1 to S phase transition of the cell cycle.
- We have produced a recombinant vector that expresses an epitope-tagged Cdc7 for use in human breast cancer cell lines. This vector will be useful for defining the molecular role of Cdc7 in human carcinogenesis.

Reportable Outcomes

Langan, T.A., Todd, M.C., Siriwardana, S., Johnson, Sclafani, R.A. and Kraft, A.S. Breast Cancer Chemotherapy Targeted Towards Cell Cycle Regulatory Components. Era of Hope Meeting,, Dept. of Defense Breast Cancer Program, Atlanta, GA June 8-11, 2000 (Enclosed in Appendix).

Conclusions

Our previous studies of the effects of 5Aza-Cdr on p16 gene methylation and p16 protein expression in breast cancer cell lines have shown that this drug effectively blocks p16 gene methylation and induces p16 protein production. 5-Aza-Cdr also blocks anchorage-independent growth of breast cancer cell lines, indicating that treatment with this drug abrogates the tumorigenic properties of these cells. However, control experiments employing breast cancer cell lines with deleted p16 genes also are inhibited by 5-Aza-Cdr, indicating that effects on gene expression other than the induction of p16 may contribute to this effect.

We have added to our previous data and have showed that our animal model for re-activation of p16 by 5-Aza-Cdr shows high toxicity. We believe that these combined results raise serious concerns about this type of chemotherapy and we recommend against it.

Our development of a protocol for detecting methylated p16 sequences in plasma DNA from breast cancer patients provides a potential non-invasive procedure for determining the p16 methylation status of breast cancer patients. We have used this procedure to demonstrate that the amount of late stage breast cancer patients with a methylated p16 gene is much lower (<3%) than expected according the literature (30%).

We obtained IRB for the Bryostatins-1 phase 2 clinical trial. However, this trial was terminated by the NCI in March of 2002. The main reason was the dismal accrual rate.

"So What" Section

Our results have provided important caveats to cancer chemotherapy in which treatment with a methylation inhibitor, 5-Aza-Cdr, is proposed to activate a repressed, methylated tumor suppressor gene. A combination of a lack of specificity and toxicity makes this type of chemotherapy untenable. Furthermore, our clinical studies have shown that the number of patients with this type of lesion may be much lower than originally thought.

We hope that our new studies with Cdc7 protein may represent a potential biomarker in breast cancer, be important for patient prognosis and may represent a potential target for therapy.

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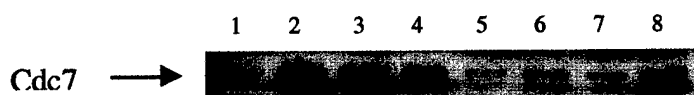
Personnel

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APPENDIX COVER SHEET

Appendix

Figure 1: Immunoblot of Cdc7 protein levels in different normal and cancer cell lines. Extracts from different cell lines were subjected to SDS-PAGE and then transferred to membranes and probed with polyclonal anti-Cdc7 antibody. Re-probing the membrane with anti-tubulin showed equal protein loading (not shown).



<u>Lane number</u>	<u>Cell Line</u>	<u>Tissue-Type</u>
1	MCF12A	Breast, Normal
2	ZR75.1	Breast Cancer
3	MCF7	Breast Cancer
4	HCC38	Head and Neck Cancer
5	DU145	Prostate Cancer
6	JGA.1	Prostate Cancer
7	LNCaP	Prostate Cancer
8	PPC-1	Prostate Cancer

Figure 2: Immunoblot of Human Cdc7 protein in breast cancer cells. ZR75.1 cells were transfected with liposomes with either pcDNA3.1 vector or pcDNA3.1-Cdc7. Extracts were subjected to SDS-PAGE and then transferred to membranes and probed with polyclonal anti-Cdc7 antibody. Upper band is His-tagged Cdc7 and lower band is endogenous Cdc7 protein.



BREAST CANCER CHEMOTHERAPY TARGETED TOWARDS CELL CYCLE REGULATORY COMPONENTS.

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The majority of cancer cells exhibit one or more defects in the components which regulate progression of cells through the cell division cycle. Correction of these defects has been demonstrated in some cases to abolish the transformed properties of cancer cells, suggesting a potential approach to cancer chemotherapy. We have investigated chemotherapeutic agents for use in late stage breast cancer. The first 5, 6-dihydro-5-azacytidine (DHAC), and DNA methylation inhibitor, has been tested for its potential to reduce unregulated growth in cultures breast cancer cell lines which fail to express the cyclin-dependent kinase inhibitor protein p16 due to p16 gene methylation. DHAC was found to significantly affect the growth and cell cycle distribution of cultured T47-D breast cancer cells. However, this was not accompanied by detectable expression of p16 protein in these cells, suggesting that expression levels promoted by the drug may be very low. We are currently employing a related methylation inhibitor, 5-aza-2'-deoxycytidine, in attempts to optimize condition for promotion of detectable p16 expression, since high levels of p16 have the potential to produce greater reductions in cell growth as well as in tumorigenic properties. We have found that 5-aza-2'-deoxycytidine causes significant expression of p16 in T47-D cells as well as readily detectable demethylation of the p16 gene. We are currently determining the effect of this expression on growth in soft agar and tumor formation in nude mice of T47-D cells. The second drug studies, bryostatin-1, promotes expression of another type of cyclin-dependent kinase inhibitor, p21. We have implemented a phase II clinical trial of bryostatin-1 in patients with advanced-stage breast cancer, and accrued five patients to this study. However, no positive responses have been observed to date. Bryostatin-1 does modulate the activity of protein kinase C, another target of its action, in treated patients as shown by measurements in peripheral blood mononuclear cells. These studies will provide information on the usefulness of drugs which correct defects in cell cycle regulatory control as cancer chemotherapeutic agents.

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