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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Abstract.....	4
Introduction.....	5
Body.....	5
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusions.....	8
References.....	8
Appendices.....	8

Abstract.

Retinoids have shown promise for the chemoprevention and treatment of cancer. However, except for the efficient treatment of acute promyelocytic leukemia by all-trans retinoic acid, most natural and synthetic retinoids have failed in clinical trials because of toxicity and limited activity. Novel synthetic retinoid-related molecules represent promising leads for the chemoprevention and treatment of prostate cancer. Our goal is to understand the mechanism of action of these analogs at the molecular level, which seems to be independent of the nuclear retinoid receptors. Using a Genetic Suppressor Elements (GSE) selection approach we aim to identify genes that mediate retinoid function. We have standardized the experimental conditions to transfect GP2-293 packaging cells for optimal virus production and infection of PC3 cells. Cells infected with retroviruses expressing a GSE library have been treated with a lethal dose of MX3350-1 and surviving cells have been collected to rescue GSEs.

Introduction.

Retinoids are natural and synthetic derivatives of vitamin A that bind and activate the nuclear retinoid receptors, RARs and RXRs, to regulate the expression of target genes. Because of their antiproliferative activity, many efforts have been devoted to develop retinoids as cancer preventive and chemotherapeutic agents. However, very few compounds are clinically useful to humans beyond the treatment of skin and dermatological disorders. This is mostly because of the high levels of toxicity observed at effective retinoid doses, which are probably caused by activation of the retinoid receptors. Synthetic derivatives have been obtained that selectively activate a subset of retinoid receptors or lack of transactivation activity, which are expected to show lower toxicity. Of particular interest to our program are the adamantyl containing retinoid-related molecules (RRMs) MX3350-1 and MX781. These compounds induce apoptosis in a variety of cancer cell lines and MX3350-1 is effective in animal models against solid tumors derived from non-small cell lung carcinomas, whereas MX781 is effective in breast cancer xenograft models. These molecules are strong inducers of apoptosis in prostate carcinoma cells independently of p53 status, and therefore represent promising leads for the discovery of novel retinoid-like molecules as chemopreventive agents in prostate cancer.

Body.

The main goal of our research program is to understand the mechanism of action and to identify the targets of synthetic RRM in cancer cells. In this grant we aimed at identifying genes that mediate RRM killing activity in prostate cancer cells. The discovery of genes that sensitize prostate cancer cells to RRM-mediated killing could have tremendous implications in: i) understanding the mechanism of RRM action in prostate cancer and ii) the discovery of novel therapies that could synergize with currently available retinoids in combination therapies. For this purpose we proposed a Genetic Suppressor Elements (GSE) selection approach as a way to identify genes that mediate RRM action in prostate cancer. GSEs are small fragments of DNA produced by random digestion of a cDNA library that function as antisense DNA (when oriented in antisense direction and are able to decrease expression of a target gene) or as dominant negative fragments of a particular gene product (when expressed in sense orientation). We hypothesized that transfection of a GSE library into PC3 cells should generate cellular clones that would be resistant to RRM killing. GSEs isolated from cells surviving a killing dose of RRM would help us identify genes that mediate RRM function.

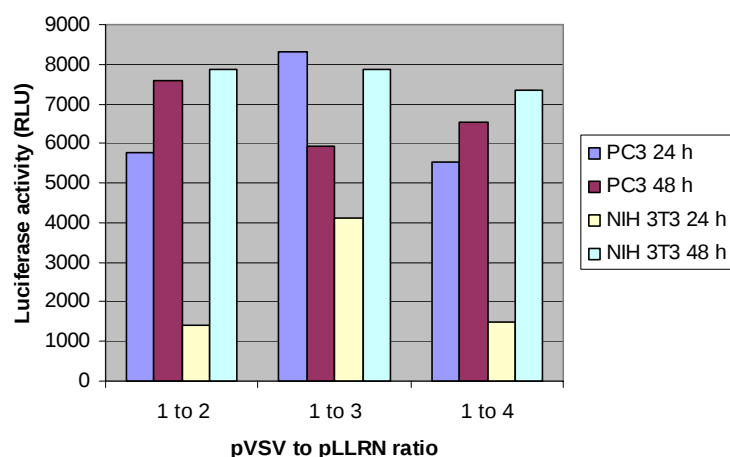
Task 1. Identification of GSEs that confer resistance to RRM-induced cell death (Months 1-18)

1.a. We originally proposed to transfect a GSE library generated from HeLa cells into EcoPack 293 packaging cells. Virus produced by these cells would supposedly express the GSEs and could be used to infect PC3 cells that had been modified to express a murine ecotropic receptor. However, following recommendations from our collaborator and expert in GSE technology, Dr. Igor Roninson, we switched to a Pantropic Retroviral Expression System that uses VSV-G (an envelope glycoprotein of the vesicular stomatitis virus) to mediate viral entry through lipid binding and plasma membrane fusion. Retroviruses produced in GP2-293 packaging cells (a HEK 293-based packaging cell line that stably expresses the viral *gag* and *pol* genes) would be able to infect a wide variety of mammalian and non-mammalian cell lines independently of receptor expression. Using this system we avoid the need for modified PC3 cells and we can target any human or murine cancer cell line.

The Pantropic Retroviral Expression system (BD Biosciences, K1063-1) was purchased, which contained all necessary materials, including GP2-293 packaging cell line and GP-293 Luc packaging cell line (a control cell line used to verify that the pVSV-G is functioning properly and that target cells can be efficiently infected). During the first year of the project, we set up to standardize the optimal conditions for retrovirus generation and infection of PC3 cells. We first standardized the transfection conditions in

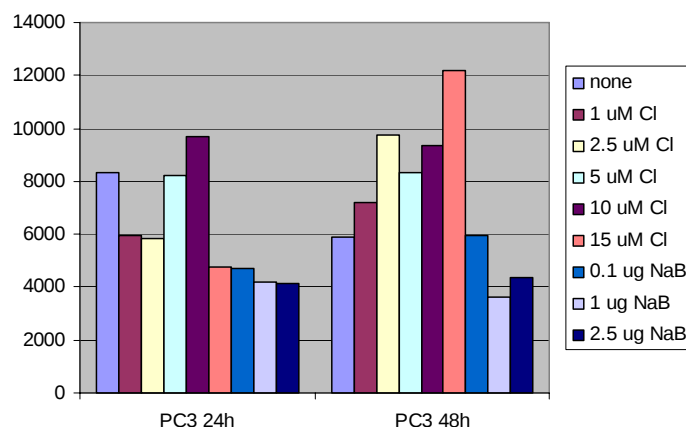
GP2-293 cells using a calcium phosphate protocol with different cell number, DNA concentration, and comparing BBS versus HBS. We transfected 75,000, 100,000, 150,000, or 200,000 cells per well in a 24 well plate with 0.5, 1, or 2.5 μ g of β -galactosidase expression vector. One plate was transfected with BBS at 3% CO₂ and the other with HBS at 6% CO₂. 24 hours after transfection, cells were stained for β -galactosidase activity following standard procedures. We found that 200,000 cells per well and 2.5 μ g of total DNA in BBS were the optimal starting conditions for further standardization (not shown).

Using these conditions as starting point, we then transfected GP2-293 cells with varying ratios of pVSV-G and pLLRN plasmid DNAs. We collected virus-containing supernatant 24 and 48 hours after transfection and used to infect PC3 cells. For comparison, we also infected NIH 3T3 cells. 48 hours post infection, cells were harvested and luciferase activity was measured in cell extracts. Figure 1 shows that

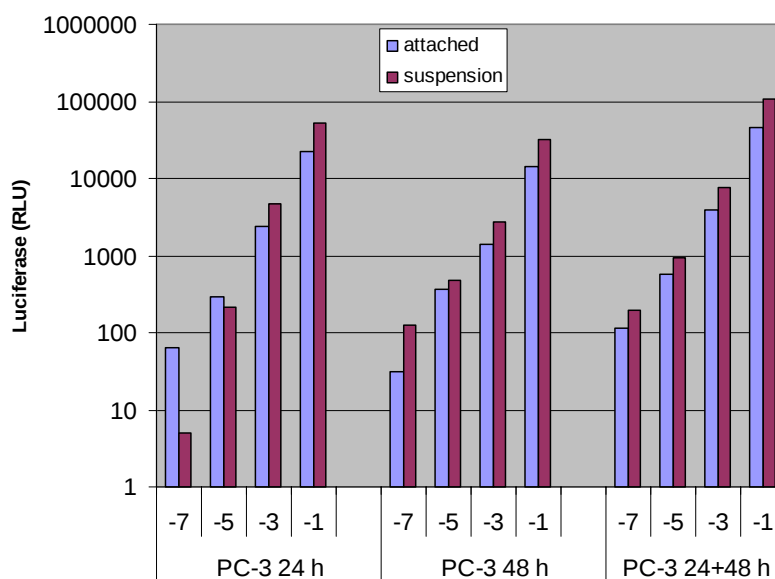


both cell lines were efficiently infected. Virus collected after 24 h of transfection infected PC3 cells more efficiently than NIH 3T3 cells. This demonstrates that PC3 prostate carcinoma cells is a suitable cell model for our purposes. No significant differences were found, although ratios of 1/2 or 1/3 gave enhanced luciferase activity. We repeated the experiment and also collected virus after 72 hours post-transfection and used to infect PC3 cells. We detected significant amount of luciferase activity in these cells infected with virus collected at a later time (not shown).

We next evaluated the effect of increasing concentrations of chloroquine or sodium butyrate, which have been described to enhance transfection efficiencies. Using a 1/3 ratio of pVSV/pLLRN, we transfected GP2-293 cells in the presence or the absence of the indicated concentrations of chloroquine (Cl in μ M) or Na Butyrate (NaB in μ g/ml). 24 and 48 hours after transfection, supernatant was collected and used to infect PC3 cells. Luciferase activity was analyzed 48 hours post-infection. Figure 2 shows that sodium butyrate did not improve transfection efficiency, whereas 10-15 μ M chloroquine enhanced significantly the amount of luciferase activity, especially when virus was collected 48 hours post-transfection.



An independent experiment was set up to estimate the titer of virus production. GP2-293 cells were seeded into 24 well plates and transfected with pVSV-G and pLmGCX vector. 48 and 72 hours post-transfection, virus-containing supernatants were recovered, filtered, and serial dilutions were added onto NIH 3T3 cells seeded in 24 well plates. 24 hours post infection, cells were treated with a lethal dose of neomycin (the library vector contains the neomycin resistance gene to select stable transformants). Two weeks after drug selection, cells were stained with crystal violet to estimate the number of surviving colonies (these will be a measure of virus-infected cells). The estimated virus titer was $\sim 10^6$ pfu/ml. Because this titer was lower than expected and yet was obtained under optimized experimental conditions, we tested the possibility of multiple infections to improve infection efficacies. We also explored whether transfection in suspension was more effective than on attached cells. Thus, cells were trypsinized, counted, and the appropriate amount of cells were incubated with the DNA/CaCl₂ mixture for 20 min at room temperature. Then the cell/DNA mixture was added to the well of a 24 well plate. In parallel, the DNA/CaCl₂ mixture was added onto the same amount of cells previously attached to the bottom of the plate. 24 and 48 hours after transfection, supernatant was collected and used to infect PC3 cells. 4 serial dilutions were used to infect cells. In parallel, one plate of PC3 cells were infected twice with virus collected at 24 and 48 hours post-transfection. To measure the transfection/infection efficiency, we measured luciferase activity 48 hours post-infection. The results shown in figure 3 indicate that transfection with cells in suspension is more effective than on attached cells and, as expected, two consecutive rounds of infection gave significantly higher luciferase activity than individual infections. Note that the y axis is in a logarithmic scale to visualize RLUs at a low virus dilution.



1.b. Once the experimental conditions for cell transfection, virus production, and infection of target cells were established, we were ready for a large-scale GSE selection in PC3 cells. We received 40 micrograms of the library DNA from Dr. Roninson. This is a MCF-7 derived GSE library in the retroviral vector pLmGCX that has been successfully used in his laboratory and offers advantages versus other available GSE libraries. One and perhaps the most important advantage is the high number of individual clones represented in the library: 10^8 . The GSE library was first amplified. We transformed 4 ml of DH5 α competent cells with 1 microgram of DNA (40 parallel transformations with 25 ng of DNA and 100 μ l of cells each). Bacteria was plated into 120 plates (150 mm) containing Amp-LB agar and grown overnight. The bacteria were then collected, amplified in liquid TB for 4 hours, and harvested for plasmid purification. After Qiagen column purification, we obtained about 4 mg of plasmid DNA, which will be used for large scale transfections (enough to transfect 400 150 mm plates with 10 μ g per plate).

1.c. We have performed a large scale transfection in an attempt to isolate GSEs that confer RRM resistance in PC3 cells. 10×10^6 GP2-293 cells per plate (20 plates 150 mm) were mixed with a DNA/CaCl₂ mix containing 10 µg of library DNA (or empty pLmGCX vector for 1 150 plate) and 5 µg of pVSV-G. 20 hours after transfection, the medium containing the DNA precipitates was removed, cells were washed with PBS, and fresh medium was added. We decided to perform 3 consecutive infections of PC3 cells. 24, 48, and 72 hours post transfection, the supernatant was collected, filtered through Millex-HA 0.45 µm filter units (Millipore), and added onto 20 150 mm plates of PC3 cells for infection. Virus supernatant was left for 6 hours, when medium was replaced by fresh DMEM medium containing 10% FBS. 24 hours after the last infection, cells were treated with 6 µM MX3350-1 in medium containing 1% serum for 48 hours, enough to kill 100% of noninfected PC3 cells. Subsequently, cells were grown in the presence of 10% serum for 2 additional weeks. Surviving clones (none in cells infected with empty pLmGCX expressing viruses) have been pooled and subsequent DNA isolation and second round of infection are currently under way.

Although we anticipated that a complete round of GSE selection with two RRMs (MX3350-1 and MX781) could have been finished by the end of year 1, we have run into personnel problems that have delayed the project. A postdoctoral associated was hired in day 1 of the project, who was going to be in charge of this project. Although she was originally enthusiastic about it, she abandoned the lab after 7 months to our surprise, which has caused an undesired delay. A new person has been hired and is now responsible to carry out the proposed experiments. For this reason, the PI will increase his time and effort in the project to accelerate the experiments during the second year, in an attempt to catch up with the original statement of work. No additional funds will be necessary, as we will have a carryover in the budget to cover this increased effort.

Experimental conditions are now well established and we expect no further delays in achieving our goals. GSEs will be isolated from our first round and will be utilized for a second round of selection using MX3350-1 and similar selection experiments of MX781-resistant cells.

Task 2. Selection of GSEs that enhance the killing activity of selective RRMs (months 12-24), We have not begun.

Task 3. Functional validation of selected GSEs (Month 13-36). We have not begun.

Research accomplishments.

- Standardization of transfection conditions, virus production, and infection of PC3 cells.
- Isolation of PC3 cells resistant to cytotoxic doses of MX3350-1.

Reportable Outcomes.

No reportable outcomes are available at this time.

Conclusions.

During our first year, we have standardized the experimental conditions to transfect GP2-293 cells with the GSE library and optimally infect PC3 cells for GSE selection. An initial round of GSE selection is currently underway and we will soon isolate GSEs from PC3 cells that survived a killing dose of MX3350-1 for a second round of selection. Although we are behind in our scheduled plans because of personnel problems, we are confident that we will be able to speed up in the second year to fill the gap.

References: None

Appendix: None