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Prostate cancer is now the second highest cause of cancer death in men in Western society. Early disease is treatable by surgery and radiation, but once late stage disease becomes refractory to hormone removal, patient care is limited to pain management. New treatments are needed. We are using gene therapy, alone and in combination with hormones called cytokines that stimulate the immune system. *The concept is that delivering a cell-killing agent to an accessible tumor, coupled with help from the immune system can promote tumor reduction both at the treatment site and at remote locations.* In this therapy, a gene (a fushion of cytosine dreaminase and uracil phosphoribosyltransferase (DC/UPRT)) is delivered to a cancer cell so that harmless bacterial proteins are made. When a pro-drug, 5 fluorocytosine (5-FC), is then given, cancer cells that make CD/UPRT convert 5-FC to a toxin that kills the original cell and other nearby. This system works in slow growing tumors like prostate cancer. Killing the tumor cells attracts immune cells. We will identify these and use cytokinase to attract more of them into tumors. We will deliver the cytokine gene alone or with the suicide gene because in other studies, combination therapy works better.

14. SUBJECT TERMS
Therapy for prostate cancer metastasis; gene-directed enzyme/prodrug therapy (GDEPT)_mIL-12; mIL-18; cytosine deaminase and uracil phopho-ribosyl transferase (CDUPRT);

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TITLE: Augmentation of a Novel Enzyme/Pro-Drug Gene Therapy "Distant Bystander Effect" to Target Prostate Cancer Metastasis

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Table of Contents

Augmentation of a Novel Enzyme/Pro-Drug Gene Therapy "Distant Bystander Effect" to Target Prostate Cancer Metastasis

INTRODUCTION:

Prostate cancer is now the second highest cause of cancer death in men in Western society. Early disease is treatable by surgery and radiation, but once late stage disease becomes refractory to hormone removal, patient care is limited to pain management. New treatments are needed. The **subject** of this work is a study of gene therapy, used alone and in combination with hormones called cytokines that stimulate the immune system. *The concept is that delivering a cell-killing agent to an accessible tumor, coupled with help from the immune system can promote tumor reduction both at the treatment site and at remote locations.* In this therapy, a gene (a fusion of cytosine deaminase and uracil phosphoribosyltransferase (CDUPRT)) is delivered to a cancer cell so that harmless bacterial proteins are made. When a pro-drug, 5 fluorocytosine (5FC), is then given, cancer cells that make CDUPRT convert 5FC to a toxin that kills the original cell and others nearby. This system works in slow growing tumors like prostate cancer. Killing the tumor cells attracts immune cells. The **scope** of the work involves identification of the immune cells that infiltrate the tumor when gene therapy is used. We will identify these cells and use cytokines to attract more of them into tumors. We will then compare the effects of delivering the cytokine gene alone, the suicide gene alone, or a combination of both into mice that carry a murine prostate cancer cell line, RM1 cells, grown in the prostate. We predict that the combination therapy should interfere with the growth of the cancer cells in the prostate and should also cause a reduction in the number and extent of tumor cells that grow in the lung after introduction into the mice via intravenous injection. This work should pave the way for clinical trials of combination therapy involving suicide gene therapy and cytokine gene therapy given together into the prostate of men with prostate cancer.

BODY:

New cell lines had to be prepared for the studies described below. To test the proof of principle that a combination of gene therapy and immune therapy will target distant as well as local prostate cancer, we decided to perform studies without a delivery vehicle, such as a virus. This would avoid an immune response against the virus. It was therefore necessary, not only to manufacture the plasmids for delivery of the genes of interest, but to use these to prepare stable transfectants from a prostate cancer line derived from a mouse, the RM1 line, kindly provided by Dr T Thompson, Baylor College, Texas. In the first instance, the work was based on our previous studies that showed that the gene, purine nucleoside phosphorylase, could be used for gene-directed enzyme prodrug therapy (GDEPT) directed against prostate cancer (Martiniello *et al.*, 1998; Martiniello-Wilks *et al.*, 2002; Voeks *et al.*, 2002). However, due to problems with intellectual property, we were no longer able to use this gene, and instead have chosen the fusion gene, CDUPRT for the following reasons: CD converts 5 fluorocytosine to 5 fluorouracil, whose metabolites, 5-fluoro-2'-deoxyuridine 5'monophosphate (5FdUMP) and 5-fluorouridine 5'-triphosphate (5FUTP) damage DNA and RNA respectively. The rate-limiting step in the generation of 5FdUMP and 5FUTP is the formation of an intermediary metabolite, 5-fluorouridine mono-phosphate (5FUMP). 5-FUMP is only produced after a series of catalysed enzymatic reactions. This can be circumvented by the ability of UPRT to convert 5FU directly to 5FUMP thereby leading to more efficient production of anti-tumor metabolites, 5FdUMP and 5FUTP (Tiraby *et al.*,

1998). UPRT sensitises cancer cells to low doses of 5FU (Kanai *et al.*, 1998), and when used in conjunction with CD and 5FC in GDEPT, was more effective than CD-GDEPT alone against colon cancer (Koyama *et al.*, 2000; Chung-Faye *et al.*, 2001) and glioma (Adachi *et al.*, 2000) *in vitro* and *in vivo*. There are no reports of this combination (CDUPRT) being used against prostate cancer, making this application novel. Thus *drugs generated by CDUPRT can kill both dividing and non-dividing cells*. This is important in prostate cancer, where the percentage of dividing cells is low. Moreover, *metabolites of 5 fluorocytosine can produce a local bystander effect* (Adachi *et al.*, 2000; Pierrefite-Carle *et al.*, 1999) and finally, *CD-GDEPT has been shown to generate a distant bystander effect* against colon carcinoma of the liver that was largely mediated by natural killer cells (Pierrefite-Carle *et al.*, 1999).

We also decided that it would be preferable to use cells to carry the cytokine genes of interest into the mice, rather than using lipid-plasmid combinations, which would be less efficient. This would allow us to generate a maximum effect, and so achieve a proof of principle more quickly than by using lipid-based transfection *in vivo*.

Trainee-Fellowship: The work was late in starting because of the intellectual property considerations, and the changes that were necessary to the program. Dr Rosetta Martiniello-Wilks started on the program of work in February, 2003 (instead of September, 2002). However, she left the program in August, to take up a permanent position at another Hospital/ University in Sydney. We have now replaced her with Dr Bing Zhang, who will continue the work that has been done, starting in January, 2004. The work below describes 6.5 months of work. It may be necessary to seek a no-cost extension, but we would rather continue the work for the next 12 months before making this request.

Research/Training Please note that Dr Martiniello-Wilks had not previously performed molecular biological work. She received training in the Oncology Research Centre, Prince of Wales Hospital, Department of Clinical Medicine, University of New South Wales; this work is described below.

Reporting: Unfortunately, there was some miscommunication, and we understood that Dr Martiniello-Wilks had submitted her report. We have rectified this situation as soon as we were aware of it, and apologize for the lateness of this report.

Task 1:
Characterization of the extent of the local bystander effect generated by CDUPRT – GDEPT

a. Development of a stable CDUPRT-RM1 cell line

Objective: To prepare and characterize RM1 cells that stably expressed CDUPRT

Methods and Results:

Plasmid preparation and characterization: CDUPRT was obtained in the pORG-codA::upp plasmid from Invivogen (San Diego, CA, USA). The CodA::upp(CDUPRT) gene was excised from this plasmid using the NcoI and NheI restriction enzyme sites and ligated into complementary sites in the pVITRO2-GFP/LacZ expression plasmid (See Appendix 1, Figure 1). pVITRO2-GFP/LacZ, which also contains the genes for the jelly fish green fluorescent protein, GFP, and for the bacterial enzyme, βgalactosidase, LacZ, that can be used as reporter genes to monitor the progress of the preparation of plasmids, was used as a control vector and cell line throughout these experiments and also provided the backbone plasmid for insertion of all genes of interest. The resulting pVITRO2-GFP/CDUPRT plasmid was characterized for

the presence of the genes of interest by restriction enzyme digestion using NcoI and NheI (Appendix 1, Figure 1).

Transfection of RM1 cells: Once the pVITRO2-GFP/CDUPRT plasmid was grown in sufficient quantity, it was used to transfect RM1 mouse prostate cancer cells (obtained from Dr T Thompson, Baylor College, Texas, USA) to generate stable transfectants. RM1 cells were also transfected with pVITRO2-GFP/LacZ to generate a control cell line. RM1 cells were seeded at $4x10^5$ cell/60mm dish. Twenty-four hours later, these cells were transfected following manufacturer's instructions using a complex formed by combining 15µl Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and 5µg plasmid DNA in 1 mL of serum free, antibiotic free Dulbecco's Minimal Essential Medium (DMEM, Invitrogen). Stable clones were selected and maintained under hygromycin selection at 800µg Hygromycin B per mL of culture medium. Using a FACs sorter, GFP expression was used to sort the high expressers and to eliminate drug-resistant, non-expressing clones (Appendix 1, Figure 2). RM1-GFP/LacZ cells were sorted into high, medium and low GFP expressing populations (Appendix 1, Figure 3).

CDUPRT expression by transfected cells:
(i) In vitro: The stably transfected cells, called RM1-GFP/CDUPRT, were examined to show that the genes of interest were functional. (see section c).

(ii) In vivo: Currently the RM1-GFP/CDUPRT cells are being grown in C57BL/6 immuno-competent male mice to confirm that gene expression occurs *in vivo* and to optimise cell dosage for use in later experiments. Tumors, harvested on 12th Dec, 2003, will be homogenized and the homogenates will be examined for CDUPRT enzymic activity by the spectrophotometric assay (as described in section c).

b. Produce cytokine plasmids.
Objective: To produce stable transfectants of the RM1 line that expressed IL-12 or IL-18.
Methods and Results:
(i) IL-12:
Preparation of plasmids: The murine IL-12 gene was obtained in the pORF-mIL-12 plasmid from Invivogen. The mIL-12 gene was excised from this plasmid using the NcoI and NheI restriction enzyme sites and inserted into the complementary sites in pVITRO2-GFP/LacZ expression plasmid (Appendix 1, Figure 4). The resulting plasmid, pVITRO2-GFP/mIL-12, was characterized for the presence of the genes of interest by restriction digestion (Appendix 1, Figure 4).

Transfection of RM1 cells: In order to examine the expression of the mIL-12 by the plasmid, it was necessary to transfect RM1 mouse prostate cancer cells. Initially, to ensure that RM1 cells did not respond to IL-12 stimulation, the presence or absence of IL-12 receptors on RM1 cells was examined. This was done by FACscan using mIL-12R antibodies (Biotinylated anti-mouse IL-12R, BD Pharmingen, San Diego, CA, USA) (Appendix 1, Figure 5).

Stable transfectants were generated as they could be useful for subsequent work. RM1 cells were seeded at $4x10^5$ cells/60mm dish and after 24h, transfected following manufacturer's instructions using a complex formed by combining combining 15µl Lipofectamine 2000 (Invitrogen) and 5µg plasmid DNA in 1 mL of serum free, antibiotic free DMEM (as described above). Stable clones were selected and maintained under hygromycin B (800µg/mL) selection.

Expression of transgenes: GFP expression by the clones was used to sort the high expressers and to eliminate drug-resistant non-expressing clones (Appendix 1, Figure 6). Low, medium and high GFP expressing populations of cells were selected by FACS sorting (BD FACS VANTAGE XE with VIVA option).
mIL-12 expression is currently being examined by ELISA (BD Pharmingen) and by functional assays using murine CTLL2 cells that proliferate in the presence of mIL-12.

(ii) IL-18
Preparation and characterization of plasmids: The murine IL-18 hybrid gene was obtained in the pCEXV3/hybrid IL-18 plasmid from Dr Isao Hara (Kobe University School of Medicine, Japan). The mIL-18 gene was excised from this plasmid using the EcoRI restriction enzyme sites and inserted into the EcoRI site in the mcs of the pVITRO2-mcs/GFP expression plasmid. The pVITRO2-mcs/GFP plasmid was created by ligating the NheI-AvrII fragment from pVITRO2-GFP/LacZ containing the GFP gene into the NheI and AvrII sites left open by the removal of the corresponding fragment of the pVITRO2-mcs plasmid (Appendix 1, Figure 7). This was characterized for the presence of the genes of interest by restriction digestion using EcoRI and a second digestion using NheI and BamHI (Appendix 1, Figure 7). The gene sequence was confirmed by sequencing (SUPAMAC) using primers specific to the mIL-18 sequence, which were positioned to read through the mIL-18 gene and into the adjacent plasmid sequence.
Forward primer mIL-18 –5' gtaagaggactggctgtgac 3'
Reverse primer mIL-18 – 5' ctccatcttgttgtgtcctg 3'
The sequence is shown in Appendix II.

Transfection of RM1 cells: RM1 cells were analysed for the presence of IL-18 receptors by FACs analysis of immunostained cells using anti-mouse-IL-18 receptor antibody (R&D Systems, Minneapolis, MN, USA). Mouse splenocytes served as a positive control for these experiments. The RM1 cells did not express mIL-18 receptors. RM1 transfectants expressing mIL-18 were generated in the same manner as described above for mIL-12, and are currently under selection using 800μg Hygromycin B per mL of culture medium.

c. Assess the extent and duration of CDUPRT expression in mouse CDUPRT-RM1 prostate tumors by in vitro CDUPRT-GDEPT assay.

Development of in vitro CDUPRT-GDEPT assay:

Two quantitative *in vitro* assays were developed using the RM1-GFP/CDUPRT cells.

1. Cell proliferation assay: This is a qualitative and a quantitative assay, which was optimized to examine the stably transfected RM1-GFP/CDUPRT cells for a) gene-expression and b) functionality of the transgene.

   Rationale: Cells expressing a functional CDUPRT fusion gene would not proliferate in the presence of the prodrug 5FC and the cell proliferation would be inversely proportional to the amounts of the prodrug added.

   Method: Preliminary experiments were done to establish the optimal seeding density of RM1 cells to successfully carry out these experiments. The optimal seeding density was determined to be 10^4 cells/well in a 96 well /plate.

RM1-GFP/CDUPRT cells were seeded at 10^4 cells /well. RM1-GFP/LacZ cells were used as control cells. Twenty four hours post seeding, 5FC (Invivogen) was added to these cells at nine different concentrations starting from 0.1-100 μg/ml. These cells were analysed for viability and proliferation at 24 hours post treatment with the drug. The calorimetric assay is based on the cleavage of tetrazolium salt WST-1 (Roche, Sydney, Australia) to Formazan dye by mitochondrial dehydrogenases in viable cells. Briefly, WST-I was added to the cells and incubated for 48 hours at 37°C in a 5% CO_2 incubator according to the supplier's instructions. The absorbance was measured at 450nm using "Sunrise Touchscreen" microplate reader (TECAN) after shaking the cells for1 minute.

Results: The RM1-CDUPRT cells express a functional CDUPRT gene. The cells showed marked reduction in proliferation especially above 3 ug/ml of 5FC. This effect was not present in the control cells.(Appendix 1, Figure 8).

2. Enzyme activity based on spectrophotometry: This is a modification of the calorimetric assay based on measurement of the enzyme (Cytosine Deaminase, CD) activity in the cell lysates of the transgene expressing cells (Nishiyama *et al.*, 1985). Since the CD and UPRT are fused in our construct and the original fusion cDNA was acquired from a commercial source, measuring the cells for just CD activity was considered to be sufficient for our study. This assay was primarily optimized for use in analyzing the enzyme activity in RM1-GFP/CDUPRT tumors growing in mice. The initial optimization was done using the cultured cells.

   Rationale: CDUPRT expressing cells will have measurable enzyme activity leading to conversion of 5FC to 5FU.

   Method: RM1-GFP/CDUPRT cells were seeded in a T75 flask. RM1-GFP/LacZ cells were used as controls. Cells were harvested 24 hours later followed by lysis by repeat freeze thawing. The cells debris was removed by centrifugation and the supernatants were collected. The protein contents of the supernatants were measured using the BCA protein estimation assay kit (Pierce, Rockford, IL, USA) according to the supplier's instructions (Appendix 1, Figure 9, insert). 3mM 5FC (900 μl in PBS) was added to the remaining supernatant and the reaction mix was incubated at 37°C. 50 μl aliquots were collected at various time intervals (1, 4, 20 and 24 h) and the reaction was stopped at each time point by addition of 950μl of 0.1M HCl. Absorbance was measured at 290 and 255nm using the spectrophotometer (UV 1601, SHIMADZU). Concentration of 5FC was determined as follows:

   5FC(mM)=0.119xA290-0.025xA255.

   The enzymatic unit of CD is nmol of 5FC catalysed per min per mg of protein from whole cell s/n.

   Result: The assay was successfully optimised and showed that RM1-GFP/CDUPRT cells have measurable enzyme activity (Appendix 1, Figure 9). This assay will be useful for analysis of enzyme activity when cells are grown in mice for future *in vivo* experiments. The optimization using the tumors is currently underway (see below).

3. *In vivo* expression of transgene
   In vivo expression of transgene: *In order to confirm that sufficient transgene is expressed to allow its enzymic activity to be measured,* C57BL/6 mice were injected with 2.5 x 10^6 cells RM1-GFP/CDUPRT cells subcutaneously and the tumors were harvested when they reached the size 10x10mm. The tumors were snap frozen and are stored at –80°C until used for analysis. *These tumors will be homogenized and homogenates will be assessed for enzymic activity.* RM1-GFP/LacZ tumors will serve as the controls for this optimization study.

d. Harvest prostate tumors to assess the impact of the GDEPT local bystander effect on RM1 tumor growth.

In order to determine the results of GDEPT, it was first necessary to determine the maximum non-toxic dose of prodrug that could be used. Mice have been injected with 3 different doses of 5FC, every day for 13 days, given intraperitoneally. Any toxic effects will be monitored by assessing liver function and renal function on serum samples and by examining major organs, liver, heart, spleen and kidney by histology for any changes. This experiment is in progress.

Once the dose of prodrug has been established, RM1-CDUPRT cells and RM1 cells will be mixed in different proportions for inoculation of mice, that will be treated with prodrug. The minimal proportion of RM1-GFP/CDUPRT cells required to produce a therapeutic effect will be established.

Task 2: Characterisation of immune cells responsible for the GDEPT distant bystander effect.

This task has not yet been started. However, as we now have the stably transfected cells that express CDUPRT, mIL-12 and mIL-18, respectively, have assays in place to measure CDUPRT expression, as well as both the amount of and functional status of mIL-12 and mIL-18, we are in an excellent position to move ahead with the rest of the experimental program.

KEY RESEARCH ACCOMPLISHMENTS:

- Established stably transfected murine prostate cancer lines from RM1 that express the transgenes and the reporter gene, green fluorescence protein: RM1-GFP/CDUPRT cell line; RM1-GFP/mIL-12 and RM1-GFP/mIL18 cell lines.
- Established and tested assay systems to measure expression of the transgene, CDUPRT *in vitro* and *in vivo*.

REPORTABLE OUTCOMES:

- Establishment of new cell lines derived from RM1: RM1-GFP/CDUPRT; RM1-GFP/mIL-12; RM1-GFP/mIL-18.
- Dr Rosetta Martiniello-Wilks has been appointed as a Senior Hospital Scientist at Royal Prince Alfred Hospital to set up a GLP facility for Gene Therapy trials. She was the successful candidate for this position because she was a DOD Trainee-fellow.

CONCLUSIONS:

At this stage of the work, we do not have sufficient data for writing manuscripts, abstracts or patents. However, we have optimized all of the systems and are well placed to obtain data quickly from the next phase of the work.

REFERENCES:

Adachi Y, Tamiya T, Ichikawa T, Terada K'Y, Ono Y, Matsumoto K, Furata T, Hamada H, Ohmoto T. Experimental gene therapy for brain tumors using adenovirus-mediated transfer of cytosine deaminase gene and uracil phosphoribosyltransferase gene with 5-fluorocytosine. Human Gene Ther 2000;11:77-89.

Chung-Faye GA, Chen JM, Green NK, Burton A, Anderson D, Mautner V, Searle PF, Kerr DJ. In vivo gene therapy for colon cancer using adenovirus-mediated transfer of the fusion gene, cytosine deaminase and uracil phosphoribosyltransferase. Gene Therapy 2001;8:1547-54.

Kanai F, Kawakami T, Hamada H, Sadata A, Yoshida Y, Tanaka T, Ohashi M, Tateishi K, Shiratori Y, Omata M. Adenovirus-mediated transduction of Escherichia coli uracil phosphoribosyltransferase gene sensitises cancer cells to low concentrations of 5-fluorouracil. Cancer Res 1998;58:1946-1951.

Koyama F, Sawada H, Hirao T, Fujii H, Hamada H, Nakano H. Adenoviral-mediated transfer of Escherichia coli uracil phosphoribosyltransferase (UPRT) gene to modulate the sensitivity of the human colon cancer cells to 5-fluorouracil. Eur J Cancer 2000;36:2403-2410.

Martiniello-Wilks R, Garcia-Aragon J, Daja M, Russell P, Both GW, Molloy PL, Lockett LJ, **Russell PJ**. In vivo gene therapy for prostate cancer: preclinical evaluation of two different enzyme-directed prodrug therapy systems delivered by identical adenovirus vectors *Human Gene Therapy*, 1998; 9:1617-1626.

Martiniello-Wilks R, Tsatralis T, Russell P, Brookes DE, Zandvliet D, Lockett LJ, Both GW, Molloy PL and **Russell PJ**. Transcription-targeted gene therapy for androgen-independent prostate cancer. *Cancer Gene Therapy*, 2002;**9**:443-52.

Nishiyama T, Kawamura Y, Kawamoto K, Matsumura H, Yamamoto N, Ito T, Ohyama A, Katsuragi T, Sakai T. Antineoplastic effect in rats of 5-fluorocytosine in combination with cytosine deaminase capsules. Cancer Res 1985;45:1753-1761.

Perrefite-Carle V, Baque P, Gavelli A, Mala M, Chazal M, Gugenheim J, Bourgeon A, Milano G, Staccini P, Rossi B. Cytosine deaminase/5-fluorocytosine-based vaccination against liver tumors: Evidence of distant bystander effect. J Natl Cancer Inst 1999;91:2014-19.

Tiraby M, Cazaux C, Baron M, Drocourt D, Reynes J-P, Tiraby G. Concomitant expression of *E. coli* cytosine deaminase and uracil phosphoribosyltransferase improves the cytotoxicity of 5-fluorocytosine. FEMS Microbiol Letters 1998;167:41-9 .

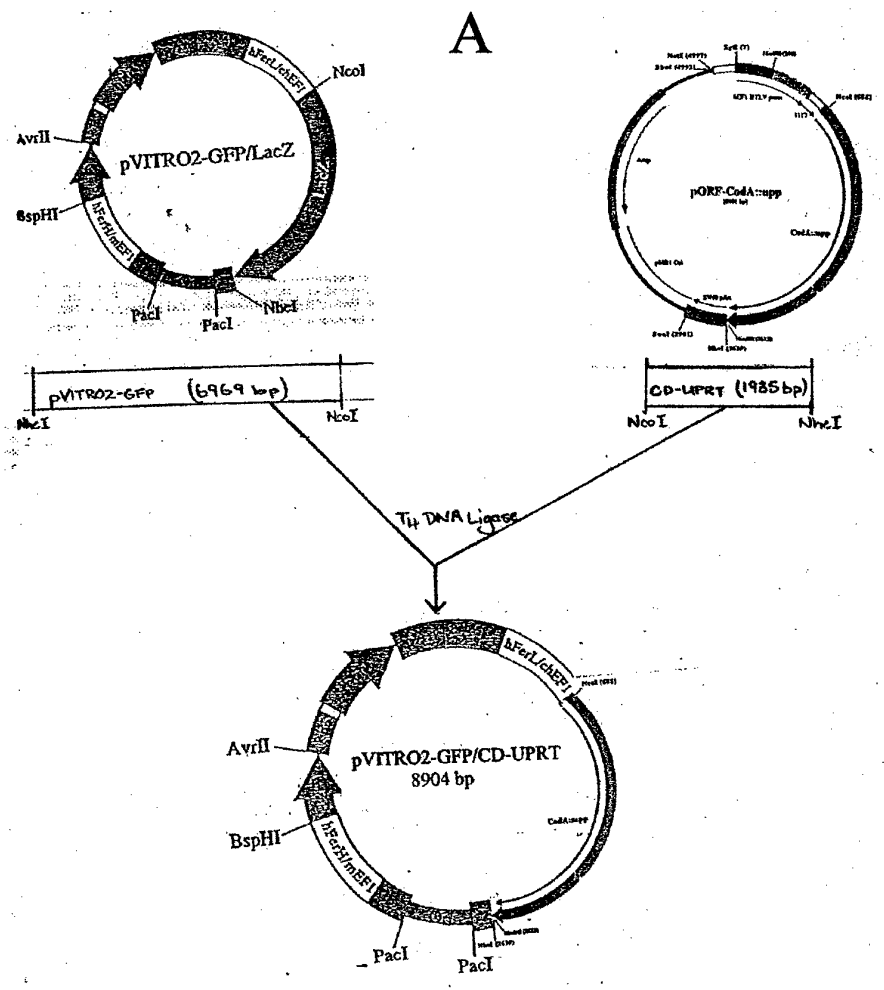
Voeks D, **Martiniello-Wilks R**, Madden V, Smith K, Bennetts E, Both GW, and **Russell PJ**. Gene therapy for prostate cancer delivered by ovine adenovirus and mediated by purine nucleoside phosphorylase and fludarabine in mouse models. *Gene Ther*, 2002; **9**(12): 759-768.

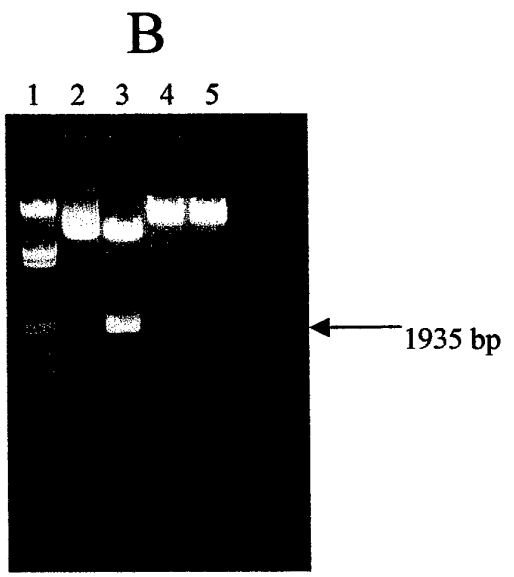
Yoshimura I, Ikegami S, Suzuki S, Tadakuma T, Haykawa M. Adenovirus mediated prostate specific antigen promoter enhanced by the cre-loxp system. J Urol 2002;168:2659-2664.

APPENDICES:

Appendix I contains Figures 1-9

Appendix II contains the sequence of the mIL-18 gene as determined by sequence analysis of the positive clones.





Lane	Content
•	λ DNA EcoRI + HindII marker (1.5µl)
•	Uncut pVITRO2-GFP/CD-UPRT
•	NheI/NcoI digest pVITRO2-GFP/CD-UPRT
•	NcoI digest pVITRO2-GFP/CD-UPRT
•	NheI digest pVITRO2-GFP/CD-UPRT

Figure 1: Panel A: Strategy for constuction of pVITRO2-GFP/CDUPRT. Panel B: Gel image showing restriction digests using NheI/NcoI restriction enzymes. The 1935bp fragment (Lane 3) represents the positive clone containing the CD-UPRT insert.

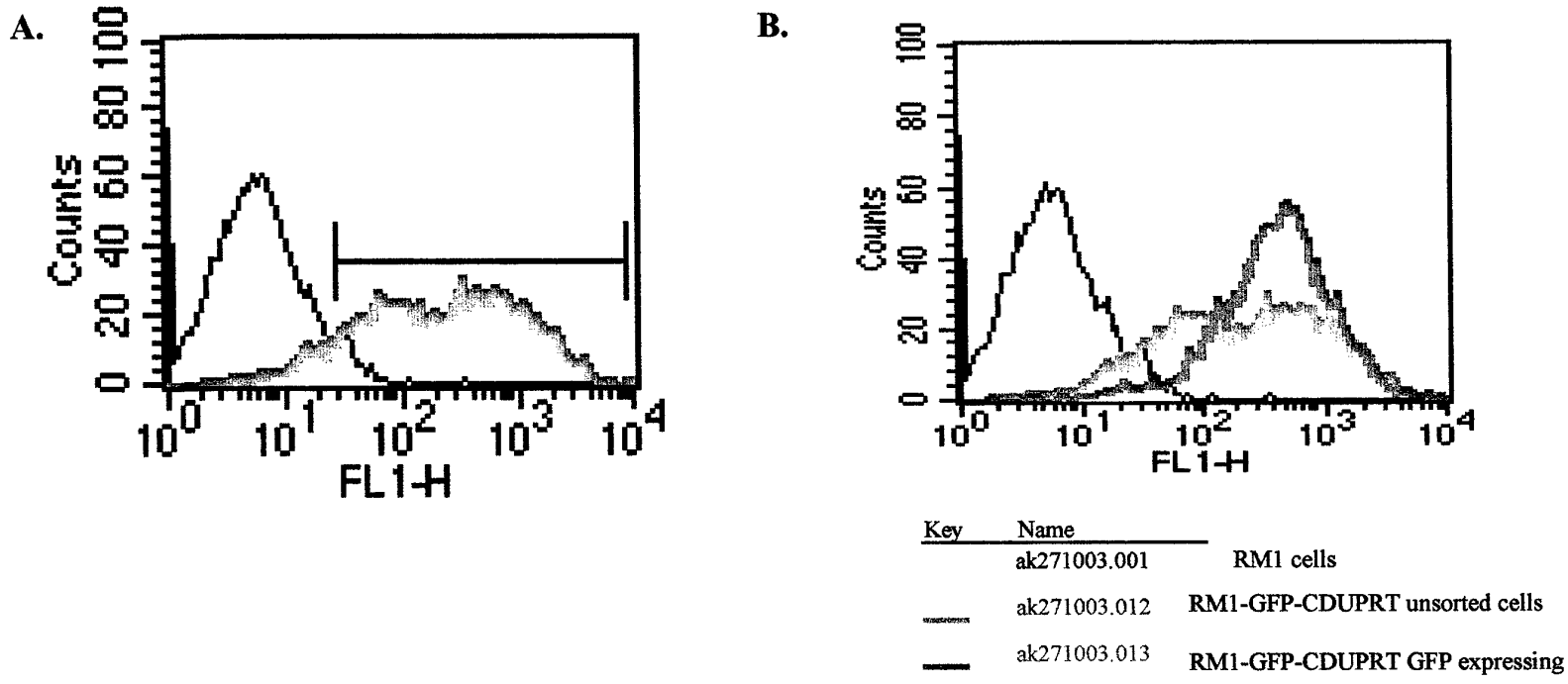


Figure 2: Sorting of RM-1-GFP/CDUPRT cells using a FACS sorter.
Panel A: Unsorted cells , Panel B:Cells sorted into GFP expressing cells.
This was to ensure seperation of hygromycin resistant non GFP expressing cells from GFP expressing cell population.

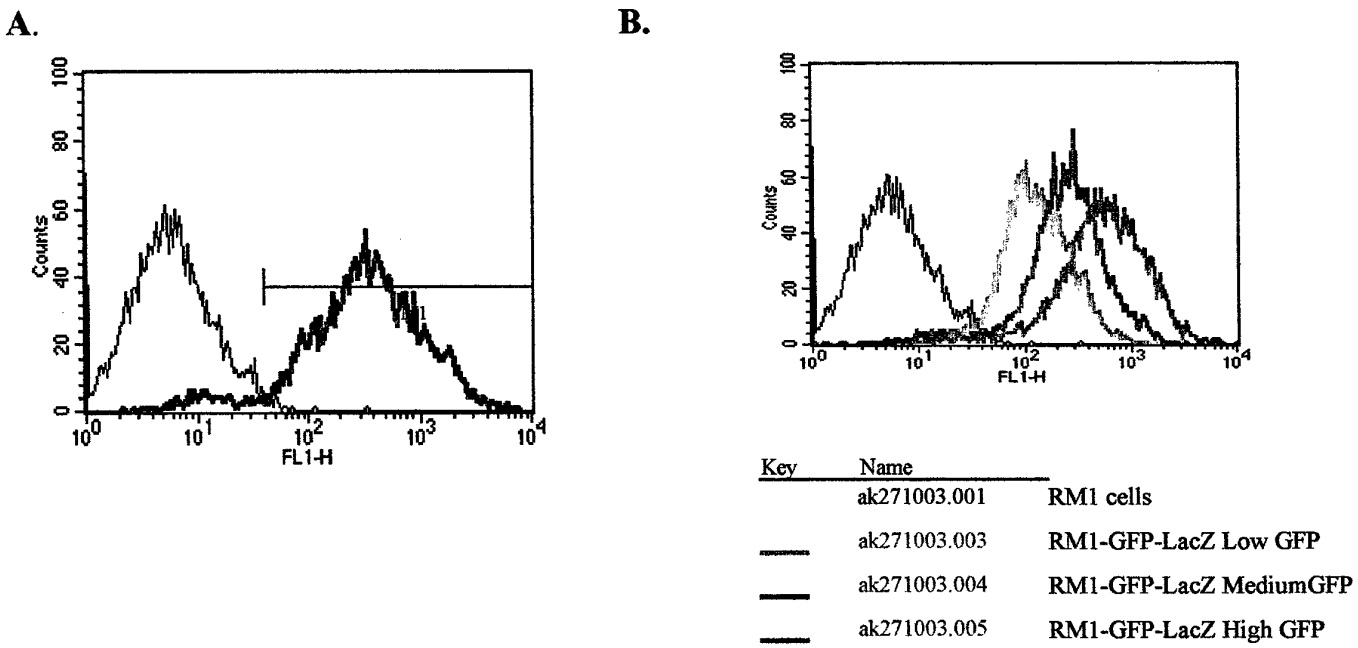
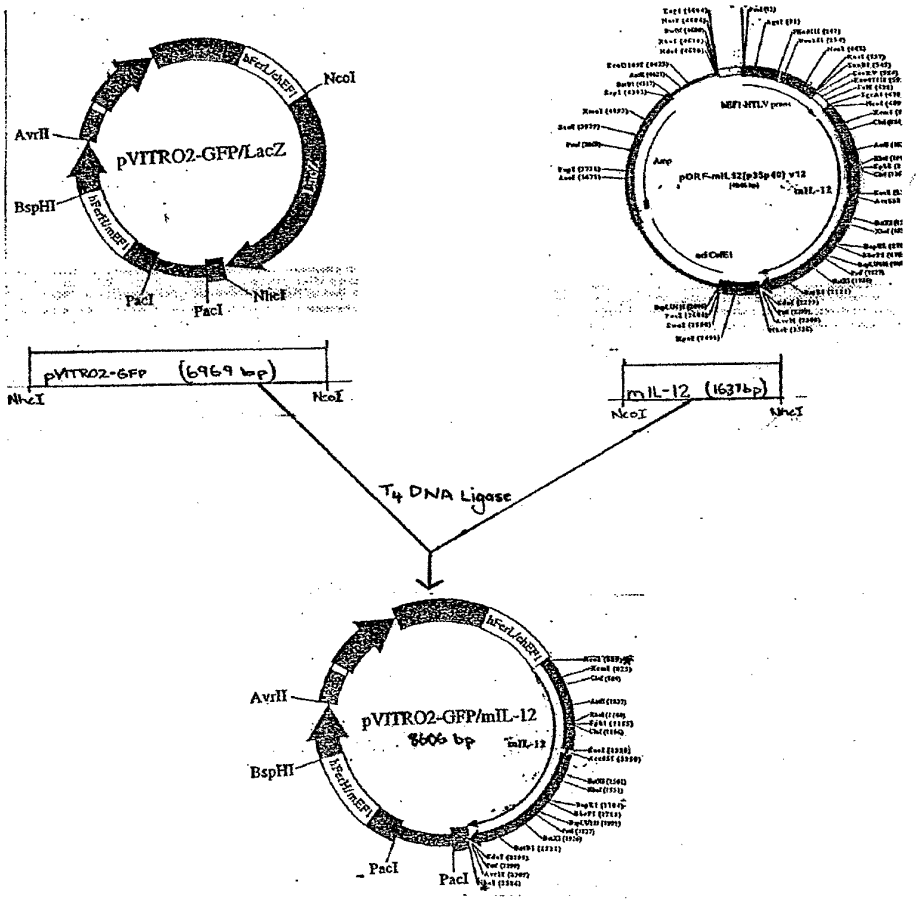


Figure 3: Sorting of RM-1-GFP/LacZ cells.
Panel A: Unsorted cells , Panel B:Cells sorted into low, medium and high GFP expressing cells.
Cells were seperated into three different populations on the basis of level of GFP expression.

A



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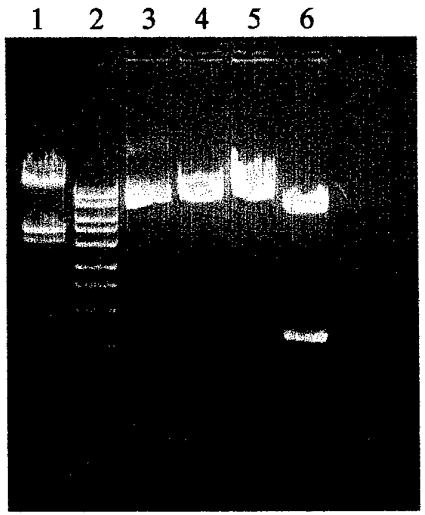
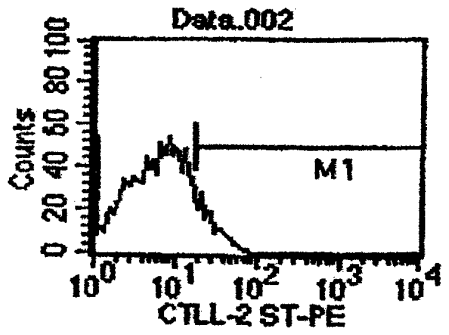


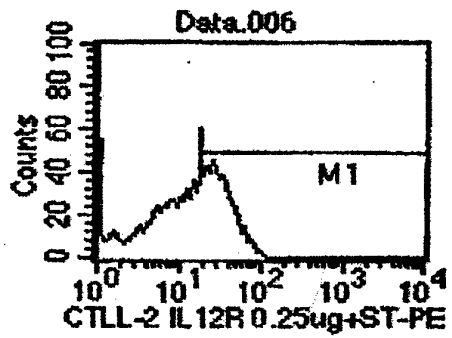
Lane	Content
•	λ DNA EcoRI + HindII marker (2μl)
•	MBI Mass Ruler DNA ladder, High Range
•	Uncut pVITRO2-GFP/ mIL-12
•	NheI digest pVITRO2-GFP/mIL-12
•	NcoI digest pVITRO2-GFP/mIL-12
•	NheI/NcoI digest pVITRO2-GFP/mIL-12

Figure 4. Panel A: Strategy for construction of pVITRO2-GFP/mIL-12; Panel B: Gel image representing NheI/NcoI digestion to screen for positive clone. The 1637bp fragment (Lane 6) represents the mIL-12 insert.

A.



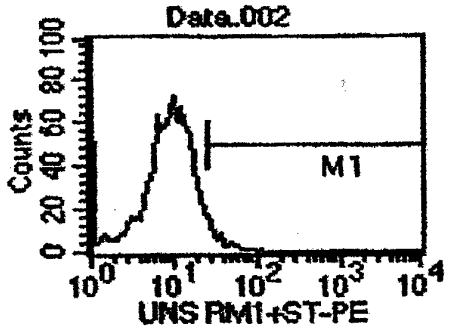
Secondary antibody alone



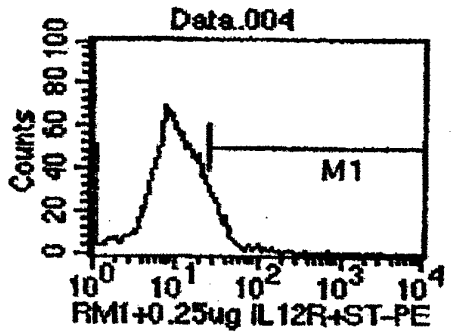
Anti-IL12-receptor antibody

mCTLL2 cells

B.



Secondary antibody alone

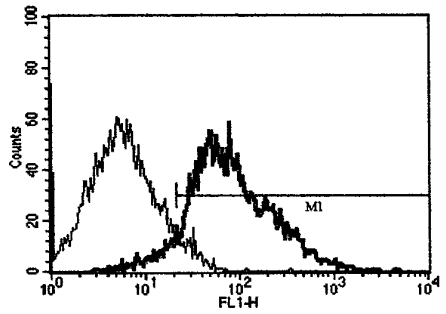


Anti-IL12-receptor antibody

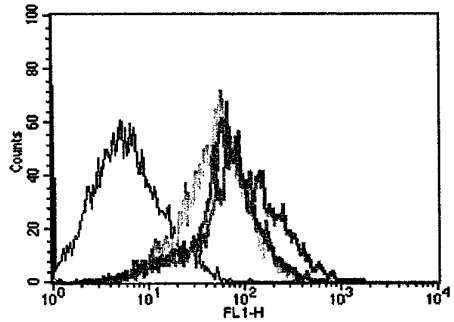
mRM1 cells

Figure 5: RM1 cells do not have mIL12 receptors.
Murine RM1 and Murine CTLL2 cells were stained with anti-mIL12 receptor antibody and analysed using FACs; Panel A shows that 25% of the mCTLL2 cells stained positive for mIL12. These cells were used to serve as a positive control.
Panel B: Shows mRM1 cells stained negative for mIL12 receptors.

A.

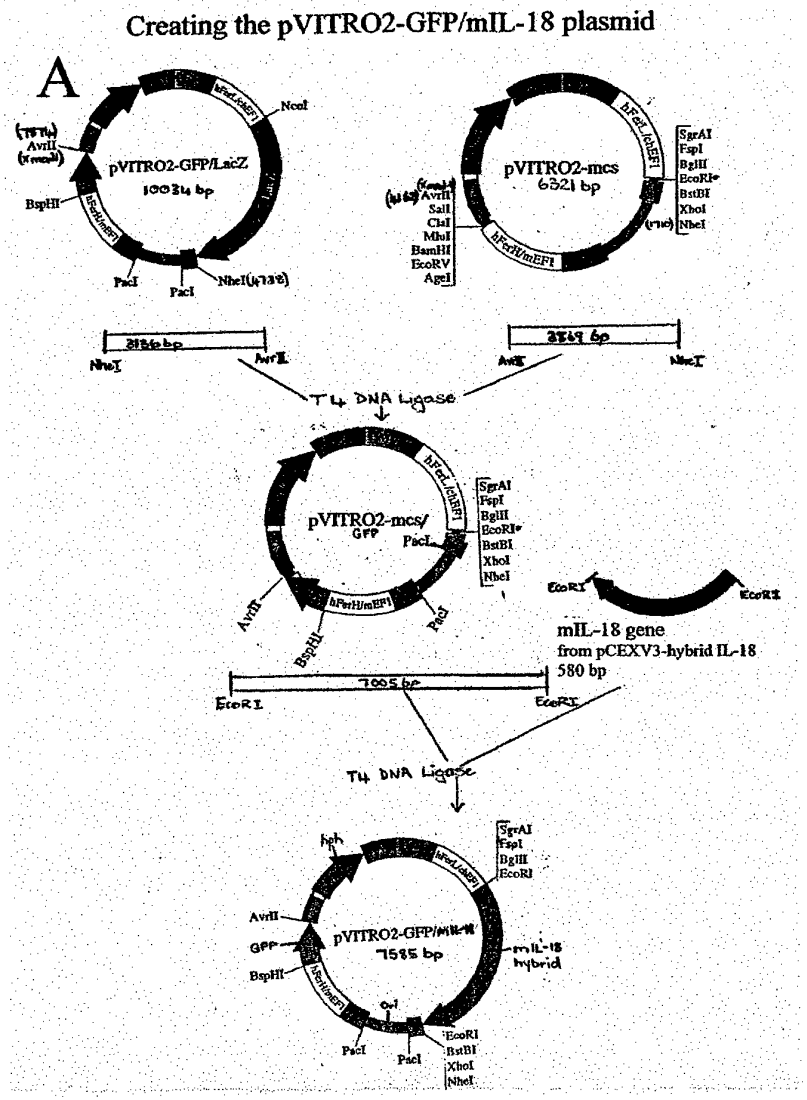


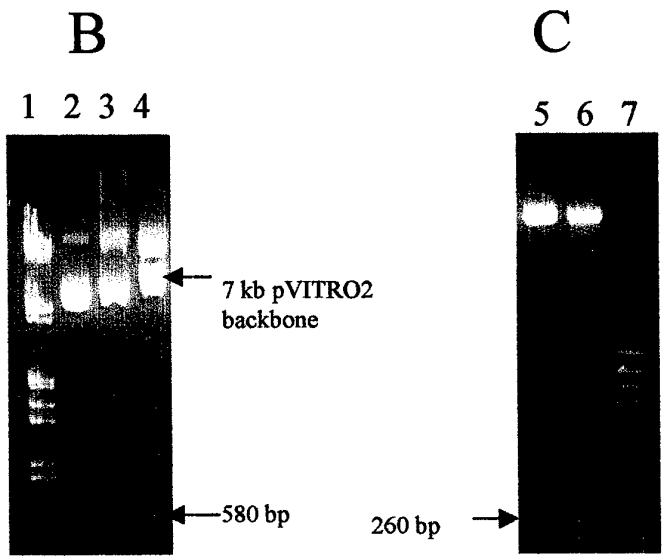
B.



Key	Name	
	ak271003.001	RM1 cells
	ak271003.007	RM1-IL12 low GFP
	ak271003.008	RM1-IL12 medium GFP
	ak271003.009	RM1-IL12 lhigh GFP

Figure 6: Sorting of RM-1-GFP/mIL12 cells..
Panel A: Unsorted cells , Panel B:Cells sorted into low, medium and high expressing cells.
Cells were separated into three different populations on the basis of level of GFP expression.





Lane	Content
•	λ DNA EcoRI + HindII marker (3µl)
•	Uncut pVITRO2-GFP/mIL-18
•	EcoRI digest Insert negative clone
•	EcoRI digest pVITRO2-GFP/mIL-18
•	NheI/BamHI digest pVITRO2-GFP/mIL-18
•	NheI/BamHI digest Insert negative clone
•	DNA Ladder Low Range (MBI Fermentas)

Figure 7: Panel A:Strategy for construction of pVITRO2-GFP-mIL-18; Panels B and C are gel images that represent restriction digests using EcoI and NheI/BamHI respectively to screen for a positive clone. In Panel B, the 580bp fragment represents the entire mIL-18 insert, and in panel C, the 260 bp fragment (Lane 5) represents the correct orientation of the insert.

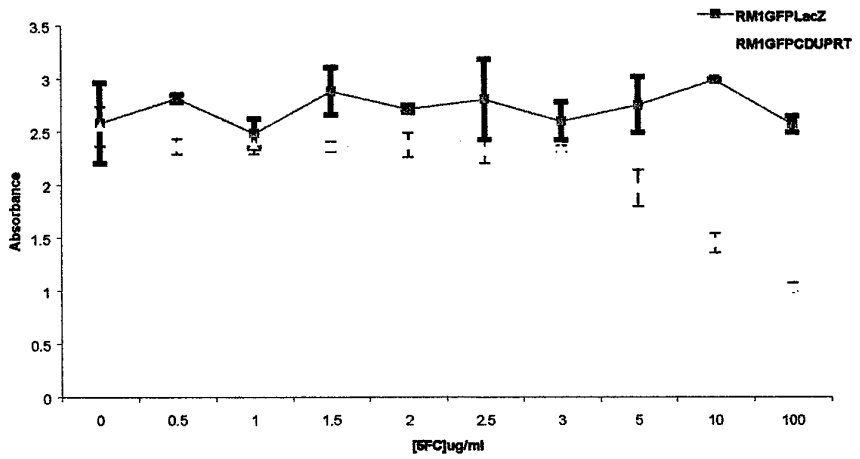


Figure 8: Proliferation of RM-1-GFP-CDUPRT and RM-1-GFP-LacZ cells in response to treatment with different doses of 5FC. RM-1-GFP-CDUPRT cells showed a marked decrease in viability at doses higher than 3 microgram/ml of 5FC.

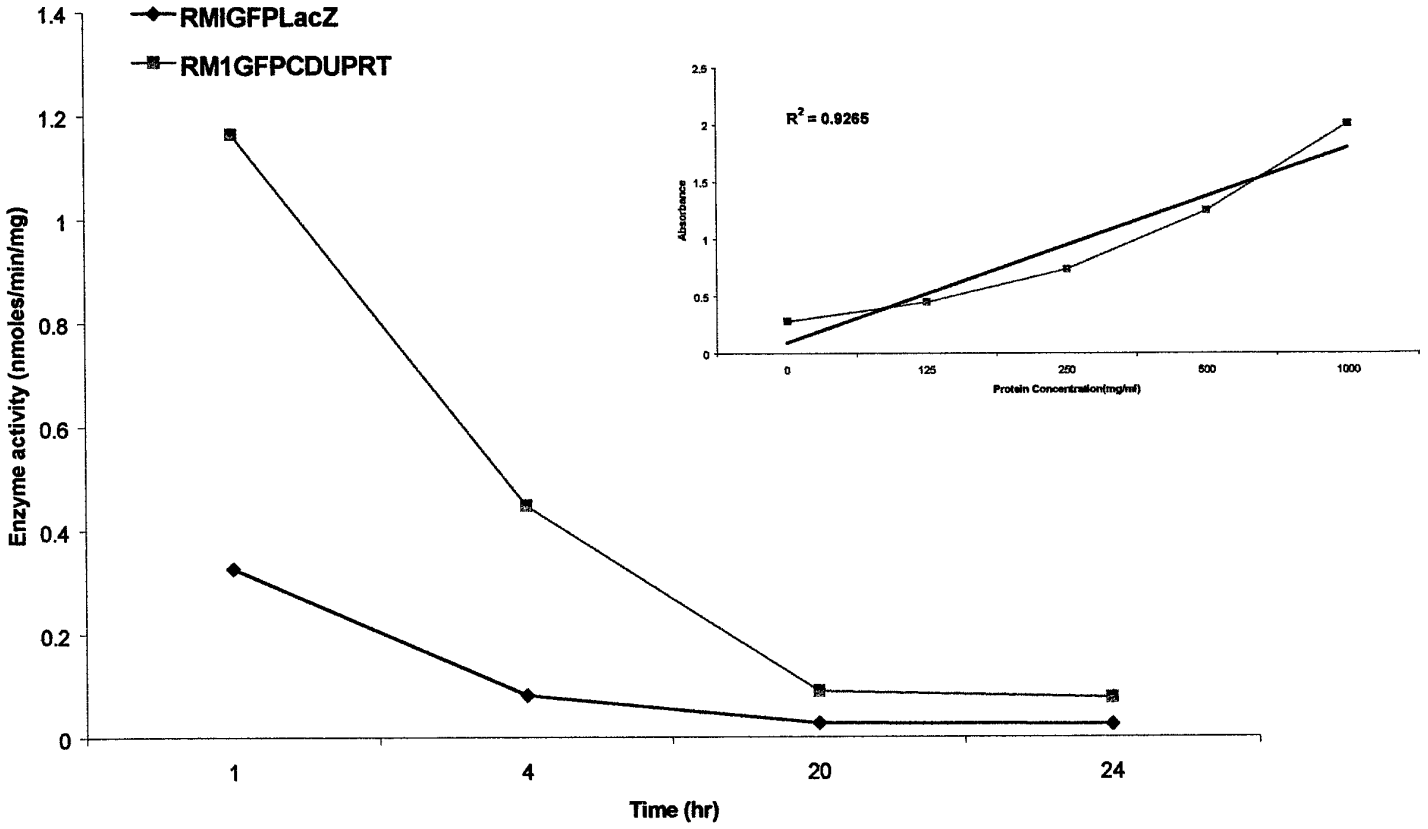


Figure 9: CD-UPRT enzyme activity in RM-1-GFP-CDUPRT cells.
The Graph shown in the inset is the standard curve generated using the albumin standards as controls. This was used to calculate the protein concentration of the cell lysates used . The RM-1-GFP-LacZ cells showed some basal non-specific activity.

APPENDIX II:

Sequence of Hybrid mIL-18 gene in pVITRO2-GFP/mIL-18

TTTTNGGGCCGCGGGGGCGACGGGNCCNTGCGTCCCAGCGCACATGTTCG
GCGAGGCGGGCCTGCGAGCGCGGCCACCGAGAATCGGACGGGGGTAGTCT
CAAACTGGCCGGCCTGCTCTGTGCCTGGCCTCGCGCCGCCGTGTATCGCC
CCGCCCTGGGCGGCAAGGCTGGCCCGGTCGGCACCAGTTGCGTGAGCGGA
AAGATGGCCGCTTCCCGGCCCTGCTGCAGGGAGCTCAAAATGGAGGACGC
GGCGCCCGGGAGAGCGGGCGGGTGAGTCACCCACACAAAGGAAAAGGGCC
TTTCCTTCCTCATCCGTCGCTTCATGTGACTCCACGGAGTACCGGGCGCC
GTCCAGGCACCTCGATTAGTTGTCGAGCTTTTGGAGTACGTCGTCTTTAG
GTTGGGGGGAGGGGTTTTATGCGATGGAGTTTCCCCACACTGAGTGGGTG
GAGACTGAAGAGTTAGGCCAGCTTGGCACTTGATGTAATTCTCCTTGGAA
TTTGCCCTTTTTGAGTTTGGATCTTGCCTCATTCTCAAGCCTCAGACAGT
GGTTCAAAGTTTTTTTCTTCCATTTCAGGTGTCGTGAAAACTACCCCTAA
CCGGCGTGCGCAAGATCTGAATTCGGCTTACACCAGCCTGGCTTCCATCA
TGAACAACAGGTGGATCCTCCACGCTGCGTTCCTGCTGTGCTTCTCCACC
ACAGCCCTCTCCAACTTTGGCCGACTTCACTGTACAACCGCAGTAATACG
GAATATAAATGACCAAGTTCTCTTCGTTGACAAAAGACAGCCTGTGTTCG
AGGATATGACTGATATTGATCAAAGTGCCAGTGAACCCCAGACCAGACTG
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GCTTTCAAACTCATTCTGAAAAAAAAGGATGAAAATGGGGATAAATCTGT
AATGTTCACTCTCACTAACTTACATCAAAGT**TAG**GTGGGGAGG.

