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14. ABSTRACT G-rich oligonucleotides (GROs) are a novel class of non-antisense nucleic acids that exhibit potent antiproliferative effects against malignant cells, including prostate cancer cells. The mechanism of GRO antiproliferative activity depends on their binding to nucleolin protein. Because they work by a novel mechanism (different from antisense oligonucleotides or traditional chemotherapy agents) and are expected to have few side effects, they have promise as new therapeutic agents for the treatment of prostate cancer. The major aims of this study were to test the efficacy of GROs in inhibiting the growth and metastasis of prostate cancer in rodent models, to investigate the mechanism of GROs, and to develop structural models of nucleolin (the primary target of GROs) for the development of new inhibitors. The formulation and delivery of GROs have been optimized in vitro and in vivo. In addition, GROs have demonstrated impressive inhibitory effects against an aggressive hormone-independent tumor (DU145) in mice. In summary, the results strongly support the potential of GROs as novel therapeutic agents for prostate cancer.					
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Final Report for DAMD17-98-1-8583

INTRODUCTION

The proposed research was based on our discovery of novel G-rich oligonucleotides (GROs) that have antiproliferative activity against prostate cancer and other malignant cell lines (1). Previously, we had demonstrated that the biological activity of GROs was correlated with their ability to bind to a multifunctional cellular protein called nucleolin (1). Therefore, we postulated that these oligonucleotides work by a novel non-antisense mechanism that involves nucleolin binding. The major goal of this study was to evaluate the therapeutic potential of GROs by investigating their ability to inhibit tumor growth and metastasis in rodent models of prostate cancer. Experiments to examine the mechanism of GRO activity and to develop novel nucleolin-binding agents were also proposed

STATEMENT OF WORK

The progress on each task outlined in the Statement of Work is detailed below:

- Task 1:* To examine the relationship between levels of nucleolin/GRO binding protein, the rate of cell proliferation, and sensitivity to GRO effects, using a variety of prostate cell lines (*months 1-6*):
- a. establish cell cultures and carry out dose-response studies using GRO29A to determine GI_{50} values for a variety of malignant and immortalized prostate cell lines (*months 1-3*)
 - b. prepare large cell cultures and extract nuclear, cytoplasmic and plasma membrane proteins (*months 4-5*)
 - c. southwestern and western blots of extracts (*month 5*)
 - d. immunofluorescence to examine intracellular and cell surface nucleolin in different cell lines (*month 5-6*)

Progress: In an attempt to compare prostate cancer cells with normal prostate cells, we purchased cell lines CA-HPV-10 and PZ-HPV-7 from ATCC (American Type Culture Collection). These lines were derived by HPV-immortalization of cells from a prostate tumor (CA-HPV-10) or the adjacent non-cancerous cells (PZ-HPV-7) in the same patient. We found that the GRO had similar antiproliferative activity in both cell lines. However, we also found that the PZ-HPV-7 cell line grew very rapidly and was found to be tetraploid, suggesting that it is much more characteristic of transformed cells than of the normal cells from which it was originally derived.

Examination of cell surface nucleolin by immunofluorescence has led to some important data that suggest that nucleolin levels may be higher in the plasma membrane and cytoplasm of cancer cells compared to normal cells. Figure 1A shows that indirect fluorescent staining of non-permeabilized cells using a nucleolin monoclonal antibody indicates high levels of nucleolin in the plasma membrane (some staining is also seen in the cytoplasm) of cancer cells (especially prostate cancer cells, DU145), but undetectable levels in normal skin cells (HS27). This method of staining is much more selective for malignant cells than nuclear nucleolin (Figure 1B), and may represent a novel method to detect malignant cells.

Task 2: To optimize the delivery of oligonucleotides to tumor cells in culture and *in vivo* (*months 1-16*):

- a. obtain or prepare transfection reagents (*months 1-2*)
- b. determine uptake of FITC-labeled GRO29A (*months 2-3*)
- c. determine activity of GRO29A in cultured cells with different delivery methods (*month 4*)
- d. carry out delivery studies in nude mice (9 total) with s.c. DU145 xenografts using internally radiolabeled GRO29A (*months 5-6*)
- e. obtain Dunning rat tumor tissue and establish rat model (6 rats) (*months 9-12*)
- f. carry out delivery studies in rats (9 total) with s.c. tumors (*months 13-16*)

Progress: The majority of this task was completed. We assessed several methods (including lipids, lipofectin, streptolysin O) for delivering GRO to cultured cells and found that activity was not increased compared to direct addition of the oligonucleotide to the medium. This is in sharp contrast to most antisense oligonucleotides, which are very poorly internalized in the absence of transfection reagents. In light of these results, we carried out more extensive studies on the uptake of fluorescently labeled oligonucleotides. Figure 2A shows that G-rich oligonucleotides that can bind to nucleolin (GRO26B and PS26B) are very efficiently taken up into the nucleus of DU145 prostate cancer cells whereas unstructured, C-rich, and control G-rich oligonucleotides that have no activity (MIX, PS-MIX, CRO, 15B) show very little uptake. We have not yet carried out the experiments to determine distribution of radiolabeled oligonucleotides in animals, because we determined in preliminary experiments that intraperitoneal (i.p.) injection could inhibit xenograft growth, whereas intratumoral injection resulted in local growth inhibition at the injection site but had no effect on overall tumor volume. Therefore, because intravenous injection is an impractical option for studies using large numbers of nude mice, we opted to proceed with the *in vivo* experiments using i.p. injection of GRO. In addition to the studies outlined above, we also compared the stability and antiproliferative activity of GROs with modified backbones in order to determine the optimal oligonucleotide for clinical development (2). These studies, which compare GRO29A (3'-amine modified phosphodiester oligonucleotide) with analogs having phosphodiester (DNA), phosphorothioate (PS), 2'-O-methyl RNA (MR), and mixed backbones, have been published in *Biochemistry*. The key results are summarized below:

1. Antiproliferative activity for analogs with DNA, PS, but not MR backbones
2. Activity of phosphorothioate oligonucleotides is, in part, non-specific
3. All GRO analogs form G-quartets and are stable in biological medium
4. Half-life of DNA analog of GRO29A is >120h in serum-containing medium, compared with < 1 h for non-G-quartet DNA
5. Antiproliferative activity is correlated with nucleolin binding (MR does not bind)
6. Activity depends on recognition of G-quartet grooves (not loops or simple G-quartet motif) and the MR grooves are much shallower than other analogs

In conclusion, the conditions for pursuing preclinical studies were defined, namely using an unmodified DNA analog of GRO29A (GRO26B, 5'-GGTGGTGGTGGTTGTGGTGGTGGTGG) that has been pre-annealed in a buffer containing potassium ions.

- Task 3:* To evaluate the efficacy of GROs in inhibiting prostate cancer growth and metastasis *in vivo* (*months 6-21*):
- determine efficacy in nude mice (approximately 150 total) with DU145, PC-3 and LNCaP xenografts (*months 7-12*)
 - preliminary experiments (12 nude mice) to establish orthotopically implanted PC-3 model (*months 9-11*)
 - determine efficacy in orthotopically implanted mice (24 total) (*months 12-16*)
 - determine efficacy in Dunning rat model (45 Copenhagen rats) (*months 17-21*)

Progress: Part *a.* of this task was completed for DU145 xenografts and has indicated very impressive antitumor activity of GROs *in vivo*. Because of promising results in nude mice, we concentrated our efforts in establishing efficacy in this model, and the orthotopic mouse and Dunning rat experiments were not pursued. In the first experiment, we tested the ability of GRO to inhibit the growth of established xenografts in nude mice. Tumors were established by subcutaneous injection of 10^7 DU145 cells. When tumors were palpable (4 days), mice (6 per group) were treated by i.p. injection of GRO26B resuspended in 100 μ l of phosphate buffered saline (PBS) or with PBS alone as control. The first day of treatment (4 days after tumor inoculation) was designated "day 0". Mice were treated every 48 h (except day 6 after the start of treatment) for a further four doses, and were euthanized on day 14. Figure 3A shows that this treatment significantly inhibited xenograft growth (tumor volume estimated using calipers). However, we were aware that pre-annealing oligonucleotides in potassium containing buffer greatly enhanced GRO activity in cultured cells (compared to GRO in PBS). Because we were concerned about the possibly toxic effects of injecting high concentrations of KCl i.p., we carried out a preliminary experiment to compare oligonucleotides annealed in potassium and sodium buffers. Mice were treated as described, and these experiments showed that pre-annealing in KCl enhanced antitumor activity (Figure 3B) and was not acutely toxic to the mice by this administration route. For the next experiment, we pre-annealed GRO26B or its C-rich analog (CRO) as control in a phosphate buffer containing 100 mM KCl. The phosphorothioate analog of GRO26B (PS-GRO) was also tested. Mice were inoculated with DU145 cells as described and treatment began after the appearance of the tumors. Mice (6 per group) received a total of six doses of oligonucleotide by i.p. injection on days 0, 1, 2, 4, 6, and 8. Figure 3C shows that GRO26B (but not control CRO or PS-GRO) could completely inhibit tumor growth and could cause tumor regression at concentrations equivalent to 1 mg/kg at 5 mg/kg (Figure 3C). Table 1 shows that estimated tumor volume (calculated from the tumor diameters, as measured with calipers) and the standard error of the data on day 14.

Table 1: Estimated volume of tumors after treatment with GRO or control oligonucleotides

Treatment	Tumor volume (mm ³)
5 mg/kg CRO (control)	313 \pm 163
5 mg/kg GRO26B	80 \pm 11
5 mg/kg PS-GRO	280 \pm 124
1 mg/kg GRO26B	86 \pm 27
Buffer	300 \pm 95

We did not observe any significant differences in body weight between different groups of mice, but 3 out of 54 mice died during injection of oligonucleotide (one each from 1 mg/kg PS-GRO, 5 mg/kg PS-GRO, 1 mg/kg GRO26B). This is likely due to the high concentration of KCl and/or the use of bolus injection of oligonucleotide, which is known to have related toxicities. We anticipate that these toxicities can be easily avoided by the use of intravenous infusion in place of bolus injection and by dialysis of GRO prior to administration (the quadruplex is stable once formed and excess K⁺ can be removed).

What is most remarkable about the antitumor activity of GRO26B is that it is observed at such low doses of oligonucleotide. A review of the literature and recent meeting abstracts will reveal that most similar experiments that use antisense oligonucleotides *in vivo* typically require 10-30 mg/kg oligonucleotide for appreciable tumor inhibitory activity (3,4). Certainly, the effective dose here (1 mg/kg) is well below the tolerated dose for other oligonucleotides in humans, and most clinical studies are being carried out using doses of up to 6 mg/kg (5).

Task 4: To evaluate combination GRO-cytotoxic drug therapies for prostate cancer (*months 6-24*):

- a. evaluate efficacy of combination treatments in cultured cells, and optimal dose sequence (*months 6-9*)
- b. examine effects (cell cycle, apoptosis, nucleolin levels) of combination therapies (*months 10-12*)
- c. test synergistic combinations *in vivo* (24 mice) (*months 13-24*)

Progress: Combinations of GRO with cis-platin, taxol, 5-fluorouracil (5-FU), caffeine, vinblastine, mithramycin, and camptothecin have been investigated for their effects against DU145 prostate cancer cells. The results are summarized in Figure 4, and indicate that GRO29A has an additive or synergistic effect with most of the chemotherapy agents tested. These data suggest that GROs would be suitable for clinical use in combination with traditional treatments.

Task 5: To develop homology models of nucleolin and carry out a "virtual screen" of a library of small molecules to identify potential nucleolin inhibitors (*months 1-24*):

- a. optimization of preliminary model (*months 1-5*)
- b. modeling of nucleolin with GRO29A (*months 6-9*)
- c. virtual screen of library of small molecules (*months 6-15*)
- d. *in vitro* testing of identified compounds (*months 12-18*)
- e. preliminary *in vivo* testing of identified compounds in nude mice (*months 19-24*)
- f. rationalization of activity by molecular modeling (*months 16-24*)

Progress: In independent studies (DAMD17-01-1-0067 to Paula Bates), the co-investigator has identified RNA binding domains 1 and 2 (RBD-1,2) of nucleolin as the region that recognizes GROs. Homology models of the nucleolin RNA binding domains 1 and 2 have been successfully created as per Task 5. However, during this grant period the NMR structure of these domains separately and complexed with the nucleolin recognition element, an RNA stem loop structure, were reported (6,7). GRO26B has been computationally docked onto the NMR structure and the orientation is consistent with our structure activity relationship, that is, the loop regions of the GRO are not directly involved in binding to nucleolin. Virtual screening for nucleolin binders was carried out using a library of about 500,000 small molecules. The top 25 "hits" were purchased and tested for their ability to inhibit proliferation of prostate cancer (DU145) and normal skin cells (Hs27). Of the 25 compounds tested, two displayed tumor-selective antiproliferative activity, and one of those demonstrated an ability to compete with a GRO for binding to nucleolin.

KEY RESEARCH ACCOMPLISHMENTS

The following is a list of significant results and achievements:

- Potent inhibition of tumor growth by GRO26B in mouse model of prostate cancer (*Fig. 3*).
- Synergistic or additive effects of GRO with chemotherapy drugs in prostate cancer cells (*Fig. 4*).
- Nucleolin is present at high levels on the surface of cancer cells but not normal cells (*Fig. 1*).
- Identification of two candidate nucleolin inhibitors by virtual screening.

REPORTABLE OUTCOMES

Manuscripts: Research supported, in part, by this grant award was published in the following peer-reviewed manuscripts:

1. Bates PJ, Kahlon JB, Thomas SD, Trent JO and Miller DM. Antiproliferative activity of G-rich oligonucleotides correlates with protein binding. *J. Biol. Chem.* 1999, 274, 26369-26377
2. Xu X, Thomas SD, Burke TJ, Girvan AC, McGregor WM, Trent JO, Miller DM and Bates PJ. Inhibition of DNA replication and induction of S phase cell cycle arrest by G-rich oligonucleotides. *J. Biol. Chem.* 2001, 276, 43221-43230.
3. Dapic V, Bates PJ, Rodger A, Trent JO and Miller DM. Antiproliferative activity of G-rich oligonucleotides backbone and sugar modifications. *Biochemistry* 2002, 41, 3676-3685.

Abstracts and meeting presentations:

Bates PJ, Trent JO, Miller DM, McGregor W, Burke T, Casson L, Castillos T, Dapic V, Girvan A, Hamhouyia F, Khan D, Mi Y, Sharma V, Thomas S, Xu X. Development of antiproliferative agents targeting nucleolin. Presented at the *American Association of Cancer Research Molecular Targets Conference*, Miami, November 2001. Published in *CLINICAL CANCER RESEARCH* 7, 157 Suppl. S.

Yingchang Mi, Shelia D. Thomas, Xiaohua Xu, Lavona Casson, Donald M. Miller, Paula J. Bates. Validation of nucleolin as a novel target for cancer drug discovery. Presented at the *American Association of Cancer Research Annual Meeting*, San Francisco, April 2002.

Patent filed:

Bates PJ, Miller DM, Trent JO, Xu X. A New Method for the Diagnosis and Prognosis of Malignant Diseases.

Awards and Honors:

2001 Founders' Medal from the Southern Society of Clinical Investigators awarded to Donald M. Miller, M.D., Ph.D.

Clinical Trial

A Phase I clinical trial of GRO26B ("AGRO100") for the treatment of refractory solid tumors was initiated in September 2003 at the University of Louisville.

CONCLUSIONS

This study was based on promising preliminary data showing that novel non-antisense G-rich oligonucleotides (GROs) have antiproliferative activity against prostate cancer cells in culture. The potential of these GROs as new therapeutic agents has now been established by our current data showing remarkable activity of the lead GRO (GRO26B) in a mouse model of prostate cancer.

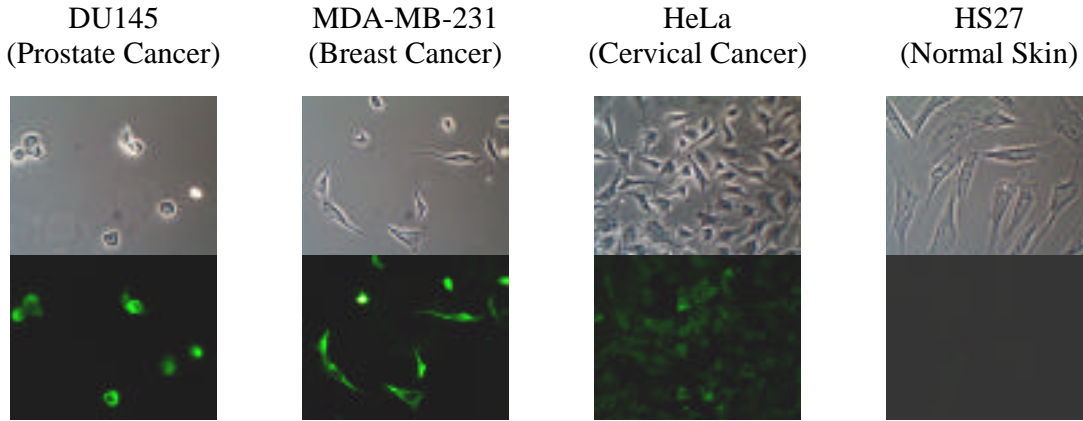
Our discovery that nucleolin is expressed at high levels on the surface of prostate cancer cells, but not on the surface of normal skin cells, confirms our hypothesis that nucleolin is a cancer-selective target for drug discovery. Development of new small molecule nucleolin inhibitors is now in progress based on a molecular model of GRO binding to the relevant domains of nucleolin.

REFERENCES

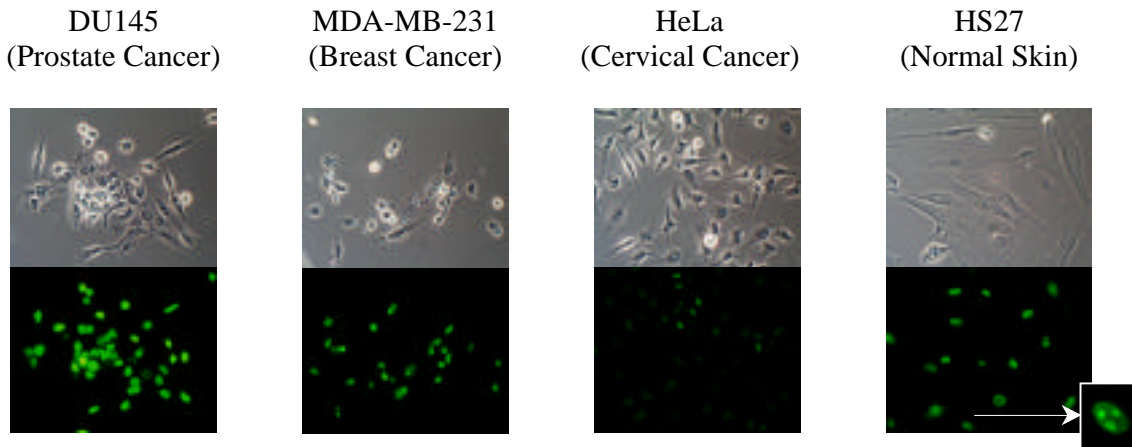
1. Bates PJ, Kahlon JB, Thomas SD, Trent JO, Miller DM. Antiproliferative activity of G-rich oligonucleotides correlates with protein binding (1999), *J. Biol. Chem.* **274**, 26369-26377
2. Dapic V, Bates PJ, Trent JO, Rodger A, Thomas SD, Miller DM. Antiproliferative activity of G-quartet-forming oligonucleotides with backbone and sugar modifications (2002), *Biochemistry* **41**, 3676-3685 (manuscript attached).
3. Green DW, Roh H, Pippin JA, Drebin JA. Beta-catenin antisense treatment decreases beta-catenin expression and tumor growth rate in colon carcinoma xenografts (2001) *J. Surg. Res.* **101**, 16-20
4. Lopes D, Mayer LD. Pharmacokinetics of Bcl-2 antisense oligonucleotide (G3139) combined with doxorubicin in SCID mice bearing human breast cancer solid tumor xenografts (2002), *Cancer Chemother. Pharmacol.* **49**, 57-68
5. Chi KN, Gleave ME, Klasa R, Murray N, Bryce C, Lopes de Menezes DE, D'Aloisio S, Tolcher AW. A phase I dose-finding study of combined treatment with an antisense Bcl-2 oligonucleotide (Genasense) and mitoxantrone in patients with metastatic hormone-refractory prostate cancer (2001), *Clin. Cancer Res.* **7**, 3920-3927
6. Bouvet P, Allain FH, Finger LD, Dieckmann T, Feigon J. Recognition of pre-formed and flexible elements of an RNA stem-loop by nucleolin (2001), *J. Mol. Biol.* **309**, 763-75.
7. Allain FH, Gilbert DE, Bouvet P, Feigon J. Solution structure of the two N-terminal RNA-binding domains of nucleolin and NMR study of the interaction with its RNA target (2000) *J. Mol. Biol.* **303**, 227-41

Figure 1: Nucleolin Expression on the Surface of Cancer and Normal Cell Lines

A. Surface nucleolin staining.



B. Nuclear nucleolin staining.

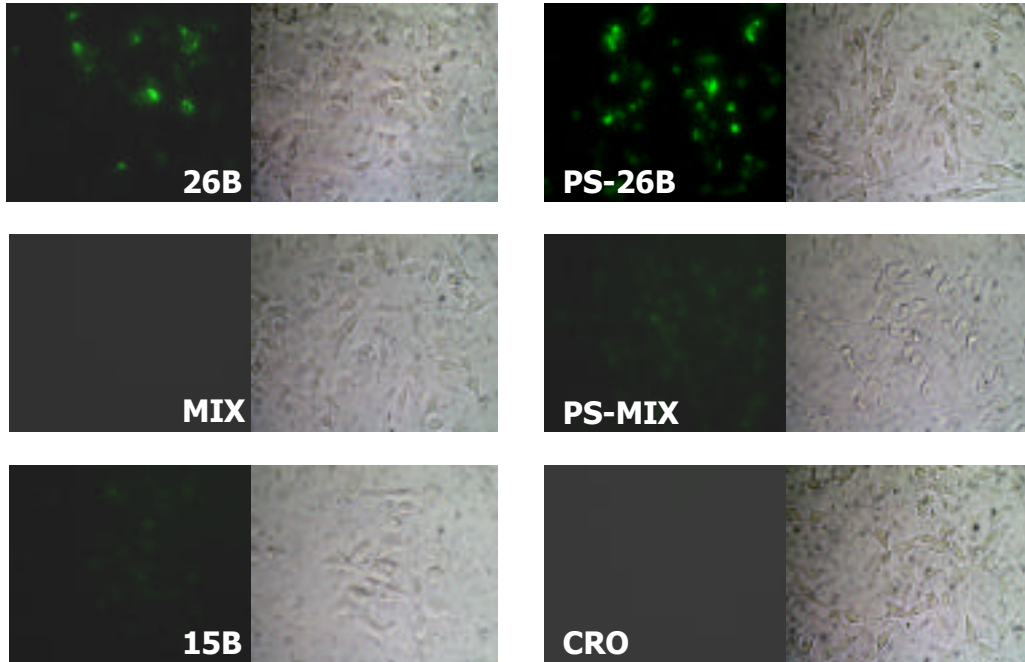


(A) Phase contrast (upper panel) and immunofluorescent (IF) staining (lower panel) of cell lines using nucleolin antibody without permeabilization of cells to show levels of surface nucleolin (some cytoplasmic staining is also detected). Note that surface staining of non-malignant cells (HS27) is negative, whereas cancer cells are strongly stained.

(B) For comparison, phase contrast and IF staining using nucleolin antibody following permeabilization of cells to show levels of nuclear nucleolin. In these preliminary studies, surface/cytoplasmic staining appears to be more tumor-specific than nuclear staining. Magnification shows stained nucleoli in normal HS27 cells.

Figure 2: Efficient Cellular Uptake of GRO26B is Dependent upon G-quartet Formation and Nucleolin Binding

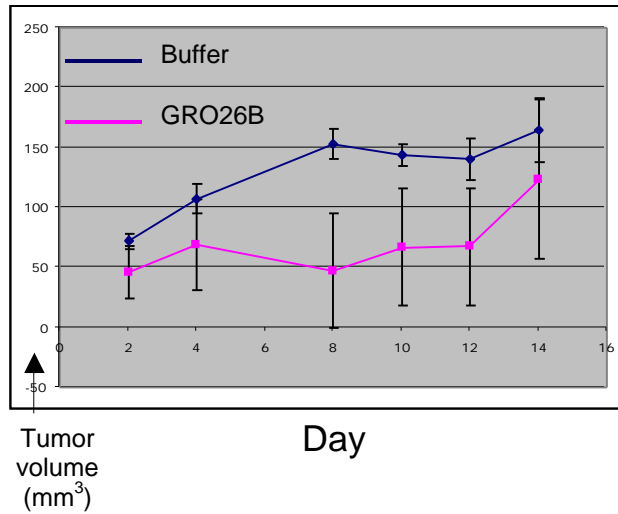
A.



(A) Uptake of FITC-labeled oligonucleotides by DU145 prostate cancer cells. Oligonucleotides (10 μ M) were added directly to culture medium and incubated for 24 h. Active GROs with either phosphodiester or phosphorothioate (26B and PS-26B) are taken up more efficiently than control inactive oligonucleotides.

Figure 3: Activity of GRO26B against in a nude mouse model of human prostate cancer (DU145 xenografts)

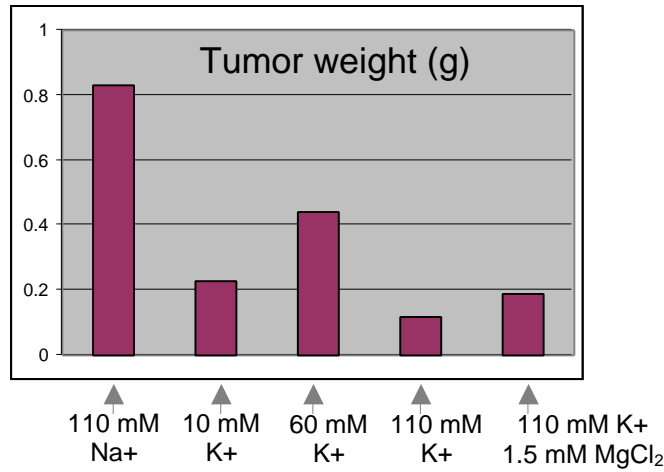
A.



(A) Tumor volume in mice treated by injection (i.p.) of 2 mg/kg GRO26B in PBS or buffer alone (PBS). Treatment began when tumors were palpable (marked as day 0) and mice were injected again on days 2, 4, 8, 10, and 12.

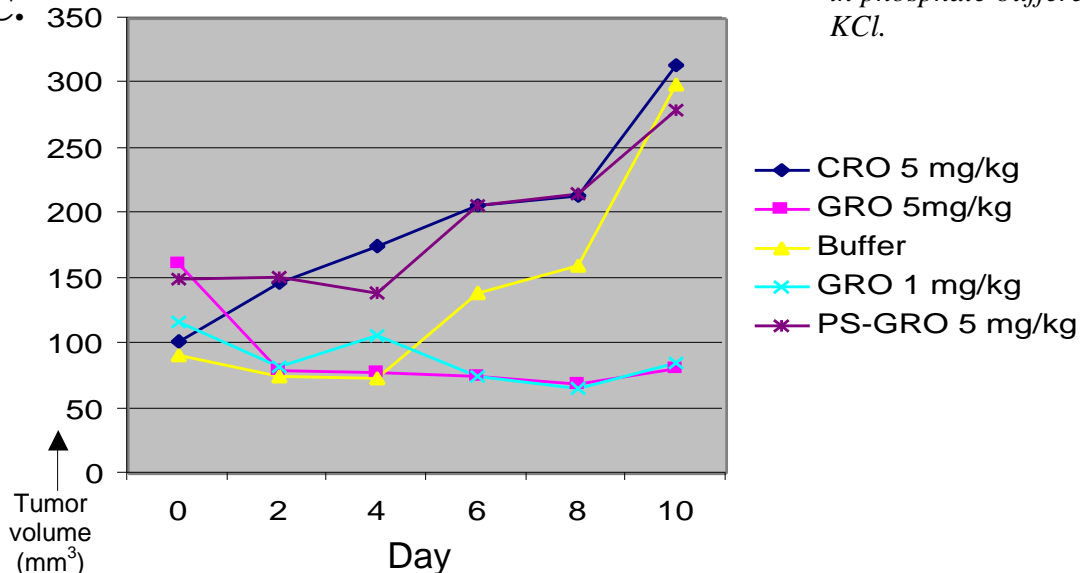
(B) Weight of excised tumors in mice treated as described above, except that GRO26B was pre-annealed in buffers containing 110 mM Na⁺ (PBS), or in phosphate buffers containing K⁺ at the concentrations shown. Each dose was equivalent to 1.5 mg/kg of GRO26B.

B.



(C) Average tumor volume in mice treated with GRO26B or its phosphorothioate analog or C-rich analog as control. Treatment began when tumors were palpable (marked as day 0) Mice received further injections on days 1, 2, 4, 6, 8. All oligos were pre-annealed in phosphate-buffered 0.1 M KCl.

C.



Drug	Drug → GRO	GRO → Drug
cis-platin	Slight synergy (A)	No effect
taxol	No effect	Additive effect (B)
5-FU	Additive (C)	Synergy (D)
caffeine	Slight synergy (E)	No effect
vinblastine	No effect	No effect
mithramycin	Synergy (F)	Synergy (G)
camptothecin	No effect	Additive (H)

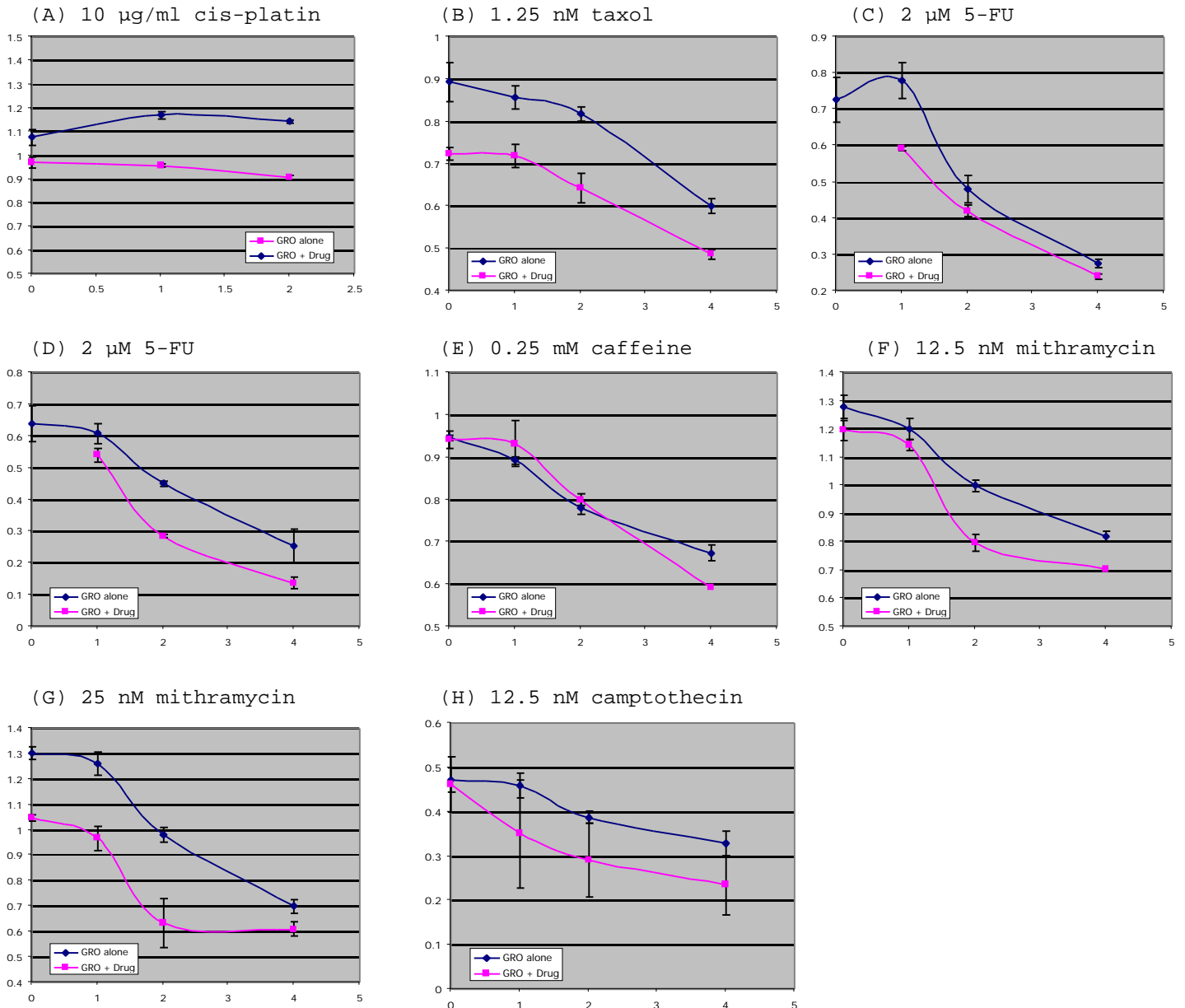


Figure 4: Effect of GRO29A with various chemotherapy and other agents on the proliferation of DU145 prostate cancer cells. Cells were plated in 96 well plates (2,500 cells/well) and incubated for 4 h at 37°C to allow adherence, after which the first agent was added. Cells were incubated for a further 24 h and the second agent was then added. On the sixth day after plating cell viability was assayed by the MTT assay (1). In the graphs, the X-axis is concentration of GRO29A (μM), and the Y-axis is A₅₉₅ (relative cell number).