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Original article

Efficacy of a synthetic lytic peptide in the treatment of prostate cancer

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Abstract

In the last several years, significant effort has been applied to identifying novel agents with effectiveness against prostate cancer. These studies were designed to determine the efficacy of one of these novel compounds, D2A21, in the treatment of an animal model of prostate cancer. Using the Mat-Ly-Lu(MLL) line of the Dunning R-3327 rat prostate adenocarcinoma model, the optimal dose, schedule and route of administration of D2A21 were established. A study involving the G line was used to further support these findings. In addition, hemotoxylin and eosin stained tissue samples were examined to investigate the extent of inhibition of lung metastases in animals injected with MLL cells. When D2A21 was injected intraperitoneally or subcutaneously, MLL and G cell tumor growth was inhibited 50-72% as demonstrated by both tumor volumes and weights. The optimal dosage of 0.179 mg/injection was established and it was determined to be most efficacious when administered five times per week. At this concentration, D2A21 appears to have no significant toxicity. Additionally, D2A21 increased the survival rate from only 25% to 70-75% in animals that were challenged with a large number of tumor cells. The peptide D2A21 is able to significantly inhibit tumor growth in rat models of prostate cancer. In addition, it can inhibit metastases and decrease deaths resulting from metastases in these animals.

Keywords: Peptides; Prostate; Rat; Cancer; Cell membrane

1. Introduction

Osmotically induced membrane disruption is a naturally occurring defense mechanism which is utilized in a number of diverse species [1]. Naturally occurring peptides have been identified which integrate into the membranes of target cells and trigger this lytic reaction. Although the exact mechanism of the membrane interactive function is unknown, the amphipathic secondary structure of these 15–40 amino acid peptides appears to be integral. In addition to their anti-fungal and anti-bacterial activity, these peptides have anti-tumor activity against transformed mammalian cell lines including human lung cancer cell lines [2].

Prostate cancer is the most frequently diagnosed solid tumor in the United States and is the second leading cause of cancer deaths among men [3]. Unfortunately for patients with hormone refractory disease, few treatment options are available. Classical chemotherapeutic agents have not proven to be effective against this disease. In the last several years, significant effort has been applied to identifying novel agents with effectiveness against prostate cancer.

Synthetic peptidyl Membrane Interactive Molecules (MIM^{TM}s) have been developed which, at lower concentrations, appear to mimic their naturally occurring counter-parts but are more active and less toxic. The enhanced activity of these synthetic peptides is the result of selective manipulation of their physical properties effecting charge density, length, amphipathy, hydrophobicity and spatial orientation. One of these peptides, D2A21, has demonstrated significant anti-tumor activity in many solid tumor cell lines [4], most notably in prostate cancer cell lines.

In order to assess the potential efficacy of these peptides in the inhibition of the growth and metastasis of prostate cancer, we utilized the Dunning R-3327 rat model of prostatic adenocarcinoma. This model is one of the most widely utilized animal models of prostate cancer. Since its inception, a

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number of variants have been identified which differ in their androgen sensitivity and metastatic ability [5]. These properties permit the investigation of a variety of critical parameters relating to human prostate cancer. For these studies, we decided to utilize the highly aggressive and metastatic Mat-Ly-Lu (MLL) line, which metastasizes and can quickly result in the death of the animal. When administered subcutaneously, lung and lymph node metastases result; but when intra-cardiac injections are given, bone metastases similar to those identified in human disease are found [6]. Additionally, we investigated the effects of D2A21 on the slower growing, non-metastatic and androgen sensitive Dunning G line. This model represents a less aggressive model of the disease. The goals of these initial studies were to determine the optimal dose, schedule and route of administration effective against prostate tumor growth and metastasis.

2. Material and methods

2.2. Cell culture/preparation

MLL and G cells were grown in RPMI-1640 with L-glutamine (MediaTech, Inc., Herndon, VA) plus 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT), 1% penicillin-streptomycin sulfate (Life Sciences, Inc., Gaithersburg, MD) and 0.1% dexamethasone (Sigma Chemicals, St. Louis, MO). The cells were incubated at 37°C under conditions of 5% CO_2 and 90% humidity. At the start of each study, the cells were trypsinized, centrifuged at 22°C for 5 min at 1300 *g*, and resuspended in Hank's Balanced Salt Solution without magnesium and calcium (Life Sciences, Inc.). The cells were counted and appropriately diluted to a final concentration of 1.25×10^6 cells/ml. This concentration was utilized in all of the studies unless otherwise noted.

2.3. Animal studies

Groups of 10–15 syngeneic male Copenhagen rats (Harlan Sprague Dawley, Inc., Indianapolis, IN), weighing 175–200 g each, were injected subcutaneously into the right hind limbs with 2.5×10^5 MLL or G cells. Each group was treated with irrigation grade 0.9% saline (Baxter Healthcare Corporation, Deerfield, IL) or D2A21 (Demegen, Inc., Pittsburgh, PA) diluted in 0.9% saline. The peptide solution was made fresh each day with the exception of the first trial in which the solution was made once each week. D2A21 concentrations ranged from 0.1785 mg to 35.70 mg/ml, administered three or five times each week by intraperitoneal or subcutaneous injections, as noted.

2.4. Measurements

In order to assess the general health of the animals, body weights were measured two or three times each week. At the time of each injection, the overall appearance of each animal was also noted to determine if signs of toxicity (hunched posture, poor grooming, diarrhea, etc.) were evident. Tumor volumes were measured three times each week using Vernier calipers after reaching a minimum size of 10 mm×10 mm. The rats were anesthetized with 250–350 μl of sodium nembutal (Abbott Laboratories, North Chicago, IL) and exsanguinated after the tumors reached 20 mm^3, or approximately 16–18 days after tumor cell injection. The primary tumors and lungs were removed, weighed and stored in 10% neutral-buffered formalin (SurgiPath Medical Industries, Inc., Richmond, IL) for histological examination. Serum was stored at −20°C after whole blood samples were centrifuged at 4°C for 5 min at 3000 *g* for peptide level determination. The protocol for this study was approved by the institutional animal care and use committee of the authors' institution; and all experiments were conducted using the highest standard of humane care in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

2.5. Lung metastases measurements

Rat lungs stored in 10% neutral-buffered formalin were sectioned into 1.5–2.0 mm slices and submitted for standard paraffin embedding. Care was taken such that any grossly evident metastases that were bisected during sectioning were only represented once in the subsequent hematoxylin and eosin stained slides. All of the lung tissue from each rat was embedded for examination. Once embedded, 5-micron sections were placed on glass slides, and the slides were stained with hematoxylin/eosin. The slides were examined by a pathologist blinded to the rats' treatment group, and areas of metastases were delineated. The slides were then analyzed using digital quantitative image analysis. First, the total tissue area (metastases and normal lung tissue) was calculated. Once this was completed for all of the slides from all specimens, each slide was re-examined and areas of metastatic tissue were calculated. Subsequent calculations (i.e. number and percent metastases, etc.) were based on these initial calculations.

2.6. Statistical methods

Statistical analysis of tumor weights, volumes and number of lung metastases was performed using two sample students' *T*-tests assuming unequal variance.

3. Results

In our initial study, D2A21 was administered intraperitoneally three times each week for three weeks beginning on the same day that the MLL cells were injected. Five groups, each consisting of 12–13 rats, received between 0.0357 mg and 3.57 mg of peptide on each injection day. A sixth group of 12 rats served as a control group receiving saline injections three times each week. Due to an error in counting, these animals received more than 1×10^6 cells. An unfortunate side effect from such a large number of tumor cells being injected into the animals was that only 25% (3/12) of the control animals survived during the course of the study due to lung metastases which resulted in death (Fig. 1). How-

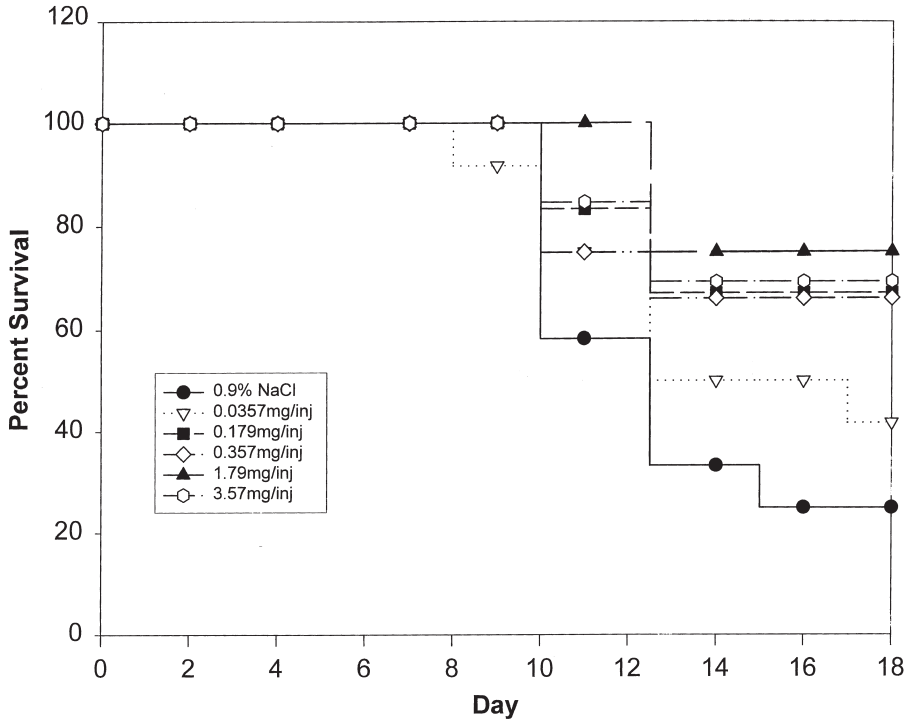

Fig. 1. Survival of animals with metastatic prostate cancer that were administered D2A21 or saline. Male Copenhagen rats were inoculated sc in the right hind limb on day 0 with MLL tumor cells (~1×10^6. Injections of saline (control group) or different concentrations of D2A21 (experimental groups) were administered ip three times each week for three weeks beginning on day 0.

ever, the survival rates of the treatment groups in which the animals were given 0.179 mg or more of D2A21 were significantly increased over the controls with 70–75% (8–9/12) of the animals surviving. Therefore, exposure to doses of 0.179 mg or more of the peptide significantly improved survival by 67%. Since all animals experienced some weight gain, there did not appear to be a substantial toxicity associated with any of the doses utilized (data not shown).

Since no significant toxicities were observed, the dose and frequency with which the peptide was administered was increased. Two treatment regimens were utilized in which the animals were administered D2A21 on three or five times per week schedules. Three groups of ten rats each received between 0.179 mg and 7.14 mg of peptide three times each week. A second set of three groups of ten rats each received between 0.179 mg and 3.57 mg of peptide five times each week. The control group consisted of 13 rats receiving saline injections three times each week. The study was performed as previously described except 2.5×10^5 MLL cells were injected in order to eliminate deaths from metastatic disease during the time course of the study.

Intraperitoneal administration of the peptide five times per week resulted in significantly enhanced anti-tumor activity compared to the three times per week schedule (Fig. 2A). The average tumor weight in the control group was 41.06 g (+/− 4.55 SE) while the average for the treatment group with the smallest tumors was 21.9 g (+/− 2.90 SE) (Fig. 2B). The only group that exhibited any toxicity was the group receiving 7.14 mg of peptide per injection, three times each week. Half of the animals in this group died during the course of the study due to local toxicity. Necropsies performed on several animals in this group indicated peritonitis. For this reason, the administration of D2A21 was discontinued after only ten days. Consequently, we were able to establish a dose of 0.179 mg per injection where significant (50%) antitumor activity was seen without toxicity ($p=0.004$). This dose is forty-fold less than doses where significant toxicity was identified (7.14 mg/injection). The efficacy of this dose was further substantiated in subsequent trials of similar design (data not shown).

After determining the optimal dose and schedule using intraperitoneal injections, studies were performed to determine the efficacy of D2A21 given subcutaneously. Three groups of animals were administered D2A21 subcutaneously into: 1) the right hind limb near the site of tumor cell injection, 2) the left hind limb, or 3) the back of the neck. A control group received saline injections in the right hind limb near the site of tumor cell injection. All animals received 2.5×10^5 MLL cells subcutaneously in the right hind limb at the onset of the study. On average, the animals from the group receiving daily injections in the left hind limb had the smallest tumors after 16 days of treatment; and there was no significant difference in tumor sizes between this group and the back of the neck injection treatment group ($p=0.33$). The tumors in the left hind limb injection treatment group were about 60% smaller (4.97 g +/− 1.54 SE)

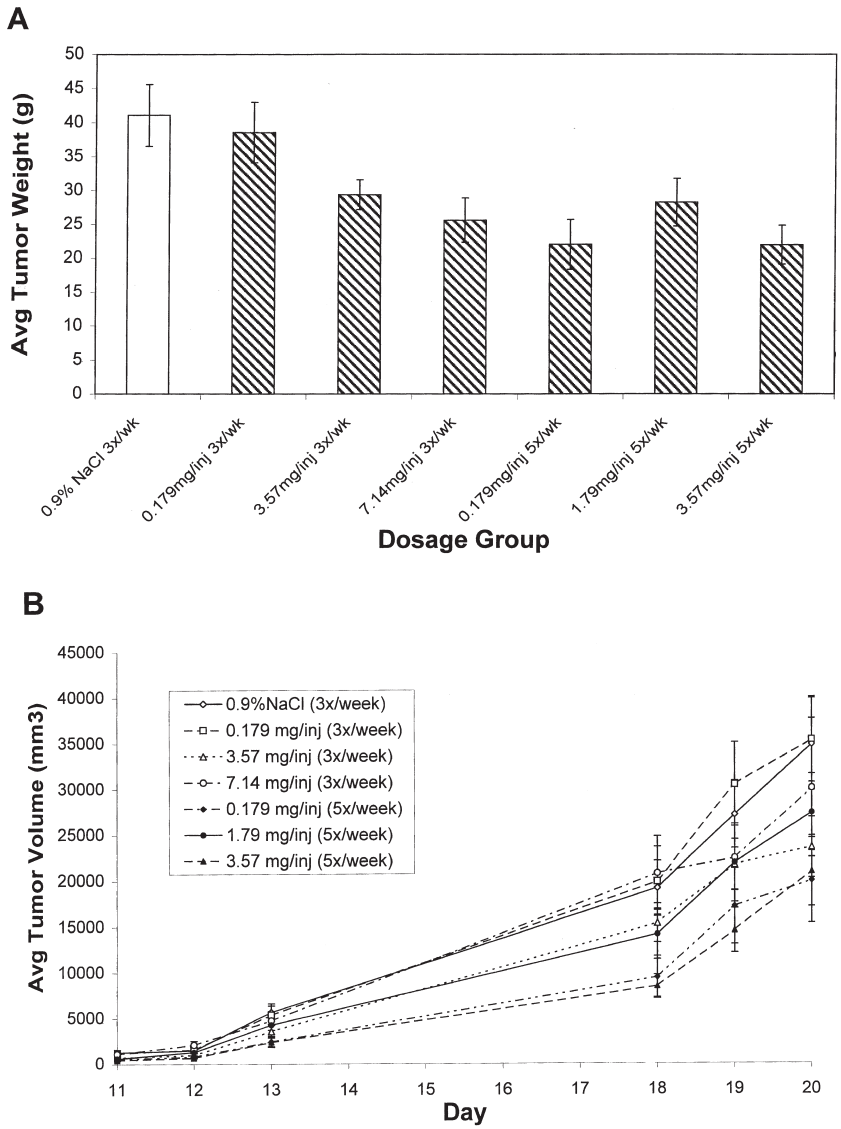

Fig. 2. A and B: Intraperitoneal administration of D2A21 inhibits tumor growth of metastatic prostate cancer. Male Copenhagen rats were inoculated sc in the right hind limb on day 0 with MLL tumor cells (~2.5×10^5). Injections of saline (control group) or different concentrations of D2A21 (experimental groups) were administered ip three or five times each week for three weeks beginning on day 0. B: Average tumor weights (+/− mean SE) obtained at the time of sacrifice. Average tumor volumes (+/− mean SE) measured beginning on day 11.

than those from the control group (12.6 g +/− 1.99 SE) based on average tumor weights (Fig. 3), indicating significant antitumor activity ($p=0.001$). No significant difference in tumor weights was noted between the right hind limb injection group and the control group ($p=0.07$).

After determination of the optimal dose, schedule and route of administration in the aggressive MLL model, animals were injected with the less aggressive G cell line. These cells are androgen sensitive and non-metastatic. Since G cells grow more slowly than the MLL cell line, they may represent a doubling time that more closely represents human cancers. The previously established optimal dosage of D2A21 was administered either intraperitoneally or subcutaneously five times each week for approximately four months beginning on the same day 2.5×10^5 G cells were subcutaneously injected into the right hind limb of the animals. The survival rate of animals in all groups was 100% and no evidence of toxicity due to peptide treatment was observed. While the average tumor weights for both treatment groups were smaller than the control group, the smallest tumors were observed in the group receiving subcutaneous injections of the peptide. This represents a significant ($p=0.037$) reduction in tumor growth compared to the control group. The average tumor volume for the group treated subcutaneously was 71.8% smaller than the average for the control group, which also indicates significant anti-tumor activity ($p=0.019$) (Fig. 4).

In order to determine the influence of administration of

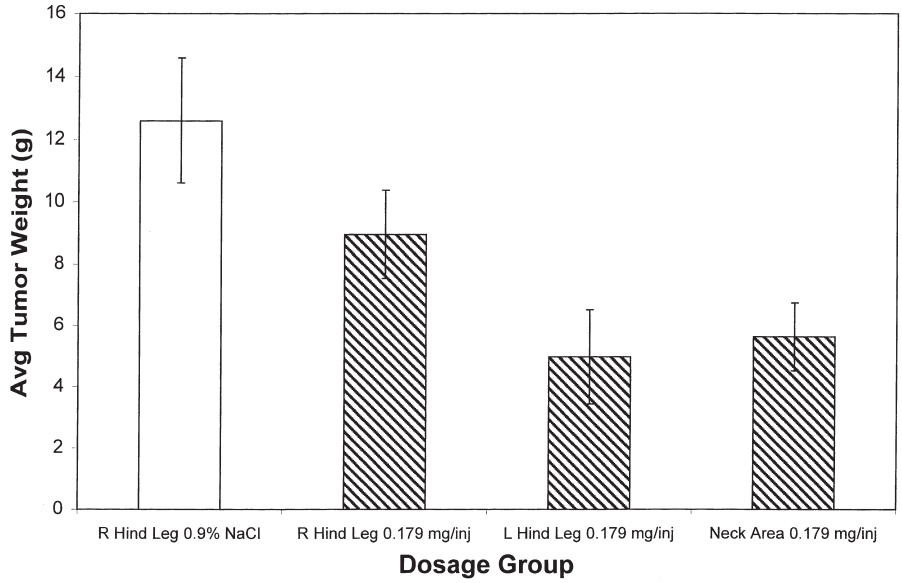

Fig. 3. Influence of subcutaneous injection of D2A21 on the growth of metastatic prostate cancer. Male Copenhagen rats were inoculated sc in the right hind limb on day 0 with MLL tumor cells (~2.5×0^5). Injections of saline (control group) or 0.179 mg D2A21 (experimental groups) were administered sc in the right or left hind limb or the back of the neck five times each week for three weeks beginning on day 0. A: Average tumor weights (+/− mean SE) obtained at the time of sacrifice. B: Average tumor volumes (+/− mean SE) measured beginning on day 11.

D2A21 on lung metastases in this animal model, a quantitative analysis was performed on 12 lungs from animals receiving subcutaneous injections of the peptide and 12 lungs from animals receiving subcutaneous saline injections. These studies were performed by removing the entire lung from the animal and sectioning them. The total lung area was determined utilizing a computer-based digital image analysis system. Grossly evident metastases were identified by the pathologist and their number and area were determined. The values for the area of lung metastases as a percentage of total lung area provide a quantitative description of the size and number of metastases. In these studies, there is a decrease in the extent of lung metastases in the animals receiving peptide injections subcutaneously in the left hind limb compared to animals receiving subcutaneous saline injections in the right hind leg (Fig. 5). The explanation for

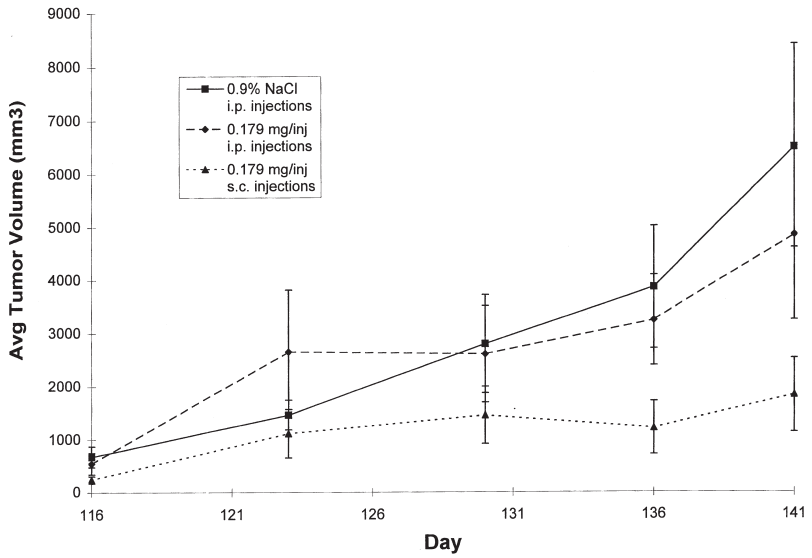

Fig. 4. D2A21 inhibits tumor growth in a non-metastatic androgen-dependent model of prostate cancer. Male Copenhagen rats were inoculated sc in the right hind limb on day 0 with G tumor cells (~2.5×10^5). Injections of saline (control group) or 0.179 mg D2A21/injection (experimental groups) were administered ip or sc five times each week for four months beginning on day 0. Average tumor volumes (+/− mean SE) measured beginning on day 116.

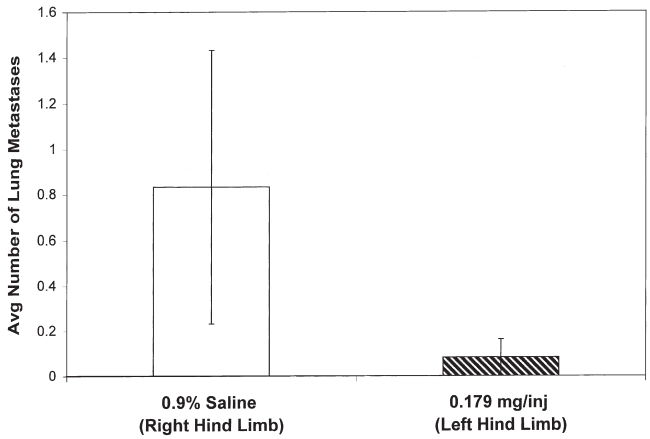

Fig. 5. D2A21 reduces the number of prostate cancer lung metastases. Sections of lung from male Copenhagen rats inoculated sc in the right hind limb with MLL tumor cells and administered sc saline injections in the right hind limb or sc peptide injections in the left hind limb were stained with hematoxylin/eosin. The slides were analyzed by quantitative digital image analysis to determine the number of metastases. Average number of lung metastases (+/− mean SE).

not observing these same effects in animals receiving peptide injections in the same area as the tumor may be related to the differences in the vasculature of this region. We are currently investigating this finding.

4. Conclusions

Prostate cancer is the second leading cause of cancer deaths among men in the United States. The current treatment options include radical prostatectomy, external beam irradiation, hormonal ablation therapy, chemotherapy and watchful waiting. While treatments may exist for localized disease, few are available for those men with advanced androgen independent disease. There clearly is a need for better treatment options for patients with advanced disease. D2A21 has shown the potential to prevent metastasis and decrease the growth rate of prostate tumor cells.

The studies described herein demonstrate that the peptidyl MIM™ compound D2A21 is able to significantly reduce the tumor growth of the highly aggressive rat prostate cancer cell line, MLL. This cell line has a doubling time that is much faster than that seen in human cancers and, if left untreated, is lethal in 3–4 weeks. Models by definition are, at best, approximations of disease and this metastatic model provides a system in which to test novel agents with significant potential against prostate cancer. In addition, D2A21 significantly reduced the growth of G cell tumors, which more closely resemble the growth rates of human cancers.

The results described here provide evidence that peptidyl mimetic compounds such as D2A21 may provide a novel area of cancer therapeutics. D2A21 can be administered through a variety of sites including subcutaneous and intraperitoneal injections. This peptide has little or no toxicity with the only significant adverse reaction being noted in animals that received very high concentrations of the peptide. When these toxicities were observed, they were only local in nature. In addition to the anti-tumor activity, we have noted that, in animals with substantial metastatic tumor burden, D2A21 provides a survival advantage. This is clearly a significant finding especially when considering the very aggressive nature of this model system.

It is apparent from these studies that D2A21 has a preferential effect on tumor cells. Studies are underway to further investigate the mechanisms of the anti-tumor and anti-metastatic activities of this peptide. One proposed mechanism for the differential effect between normal and tumor cells are differences in cytoskeletal and membrane organization. It has been well established that alterations in cellular and membrane structure are frequently altered in cancer cells. These differences may result in a propensity for cancer cells to be more susceptible to this peptide's action than normal cells. While these are lytic peptides, it is possible that they may have additional mechanisms of action when administered systemically. Furthermore, since the mechanism of action is dramatically different from that of classical chemotherapeutic agents, they may function synergistically and, therefore, combination therapies are also being evaluated. Finally, we are refining a method for detecting the peptide in serum using HPLC and an ELISA assay. Overall, these studies suggest that peptidyl Membrane Interactive Molecules are an exciting new avenue in prostate cancer therapeutics.

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