

AD _____

Award Number: DAMD17-00-1-0088

TITLE: Multivalent Lactulose-amines as Inhibitors of Prostate
Cancer Metastasis

PRINCIPAL INVESTIGATOR: Valerie V. Mossine, Ph.D.

CONTRACTING ORGANIZATION: University of Missouri-Columbia
Columbia, Missouri 65211

REPORT DATE: March 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20021001 125

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE March 2002	3. REPORT TYPE AND DATES COVERED Annual (1 Mar 01 - 28 Feb 02)
---	-------------------------------------	--

4. TITLE AND SUBTITLE Multivalent Lactulose-amines as Inhibitors of Prostate Cancer Metastasis	5. FUNDING NUMBERS DAMD17-00-1-0088
--	---

6. AUTHOR(S)
Valerie V. Mossine, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Missouri-Columbia
Columbia, Missouri 65211

E-Mail: MossineV@missouri.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
The purpose of this research is to develop and test a new unique class of potential antimetastatic agents, lactulose-amines (LAs), which may act through targeting human cancer cell adhesion mechanisms and specifically inhibit metastatic cancer cell aggregation, clonogenic growth and induce apoptosis of target cells. In Year 2, we have developed a synthetic approach to the preparation of various multivalent LAs for biochemical and biological studies. As a result of this work, a general synthetic procedure for multivalent LAs have been developed; a total of 27 mono-, bi-, tri-, tetra-, hexa and octavalent LAs have been prepared and characterized. Several of LAs demonstrated high inhibitory potential against clonogenic growth and proliferation of human prostate carcinoma cell lines *in vitro*. In addition, high affinities to galectin-1, carbohydrate receptor implicated in prostate cancer metastasis, were found for multivalent LAs. Combination of a chosen LA with cancer immunotherapy in a mouse model strongly inhibited tumors *in vivo*.

14. SUBJECT TERMS
Prostate cancer; Prevention and Therapy; Metastasis; Cell Adhesion; Clonogenic Growth; Apoptosis; Chemical Synthesis; B-Galactoside-specific Lectins; Galectins; Antmetastatic Drugs; Chemoprevention

15. NUMBER OF PAGES
15

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT
Unclassified

18. SECURITY CLASSIFICATION OF THIS PAGE
Unclassified

19. SECURITY CLASSIFICATION OF ABSTRACT
Unclassified

20. LIMITATION OF ABSTRACT
Unlimited

Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	14
Reportable Outcomes.....	14
Conclusions.....	14
References.....	15
Appendices.....	15

INTRODUCTION

In this project, we proposed to develop and test a new unique class of potential antimetastatic agents, lactulose-amines (LAs), which may act through targeting human cancer cell adhesion mechanisms and specifically inhibit metastatic cancer cell aggregation, clonogenic growth and induce apoptosis of target cells. In vitro studies on metastatic human prostate cancer DU145 and PC3MLN4 cell lines suggested that LAs may manifest antimetastatic properties against human prostate carcinoma. In addition, LAs displayed high affinity to galectins, a family of β -galactoside receptors expressed by many malignant tumor cell types, including human prostate carcinoma, and believed to mediate cell adhesion and proliferation.

In Year 2, we have developed synthetic approaches to the preparation of various LAs for subsequent biochemical and biological studies. We believe that our research goals which we planned for Year 2 were successfully achieved. These approaches enabled us to synthesize new *mono- and multivalent lactulose-amines*, which have been tailored specifically for targeting suspected mediators of metastatic prostate cancer cell adhesion and proliferation and their biological activity could be verified on human prostate carcinoma cell lines in vitro and, in Year 3 of this Project, on a conventional prostate carcinoma model in vivo.

BODY

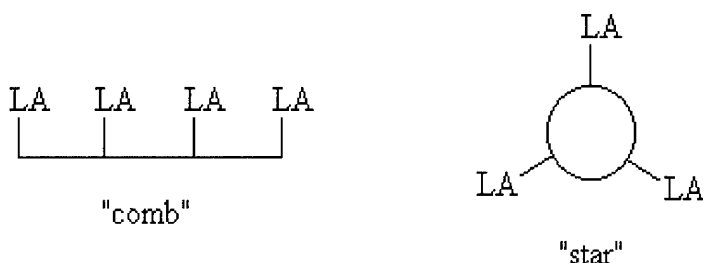
Task 1. Synthesis, purification and characterization of lactulose-amines (LAs)

According to the plan for Year 2, the following results have been achieved:

- prepared 3 LAs specifically designed for attachment to core polyamines
- prepared 4 linear, a branched, and 2 cyclic polyamines
- prepared 6 monovalent, 7 divalent, 8 trivalent, 7 tetravalent, 3 hexavalent and 2 octavalent lactulose-amines using the above and commercial core polyamines

Core polyamines.

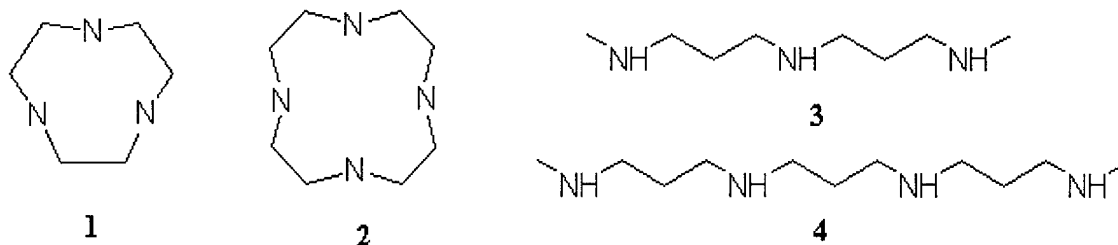
According to the Plan, multiplication of monovalent LAs on core polyamines would include the "comb" and the "star" architectures:



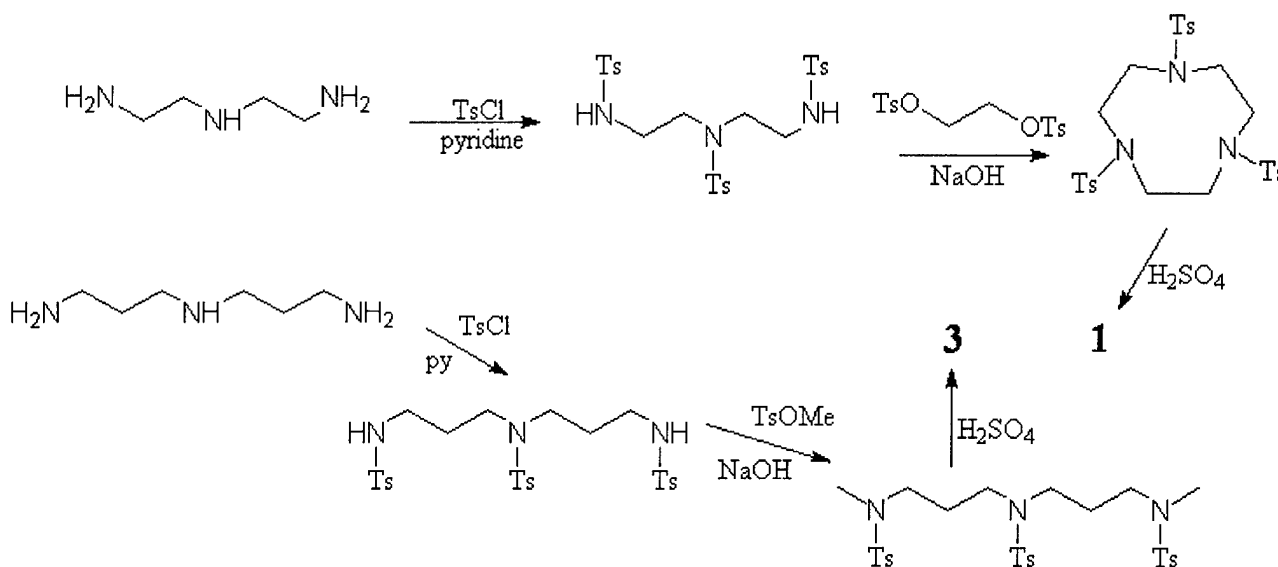
The initial screening of some mono- and divalent LAs for inhibition of clonogenic growth in highly metastatic human prostate carcinoma cell lines was done in Year 1 and revealed at least two structural features common for LAs with better inhibitory potential:

- the LA amino group is connected to an aromatic ring
- the distance between lactulosamino units may be as short as 3 atoms

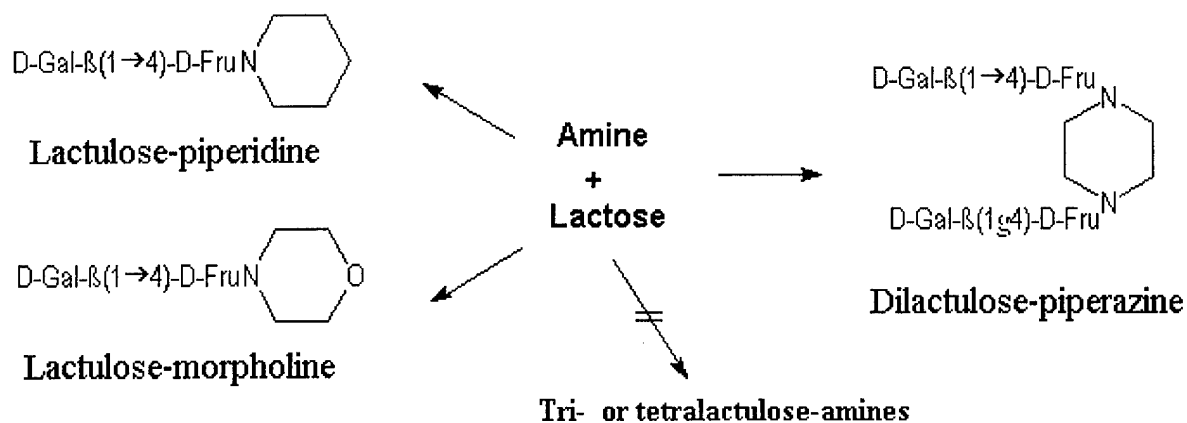
Accordingly, we have considered the following tri- and tetramines for the preparation of polyvalent LAs :



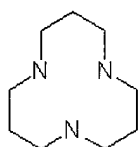
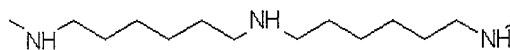
While amine **2** is commercially available at reasonable cost, other amines we have prepared employing the “tosylate” strategy which is much safer than polyamine synthesis methods based on aluminohydride reductions. In addition, intermediates in the “tosylate” method readily crystallized, providing for simple purification procedures and high yields:



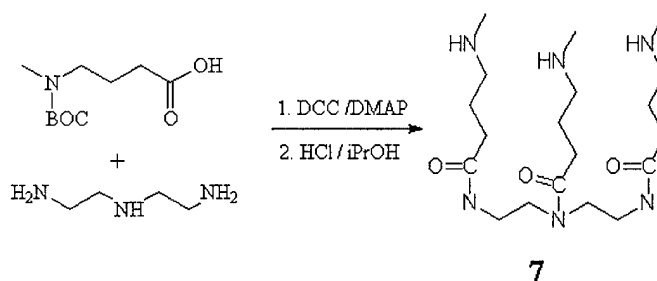
Having at our disposal multigram amounts of both cyclic and linear polyamines, we did attempt to attach lactulose units directly to the secondary amino groups in the polyamines. Our preliminary model syntheses of mono- and divalent LAs derived from cyclic amines morpholine and piperazine went smoothly, with high yields and purity. In contrast, our attempts to obtain tri- and tetralactulose-amines using similar direct reactions of lactulose with amines **1** - **4** were not successful. As a rule, most of starting lactulose in the reaction mixtures remained unreacted while a number of unidentified byproducts and polymers appeared in the reaction mixtures at conditions that favored high yields of LAs in most of our previous syntheses.



Originally, we hypothesized that increase in polyamine ring size or increased distance between amino groups in linear polyamines would make them more flexible and thus more accessible for attachment of lactose. To verify the hypothesis, we have synthesized amines **5** and **6** using similar “tosylate” strategies as in the preparations of **1**, **3**, and **4**.

**5****6**

Still, we could not find proper conditions in the reaction of these amines with lactose, which would lead to corresponding trilactulose-amines. Our latest, and yet unsuccessful, attempt to obtain polyvalent LAs in direct reaction of lactose and a polyamine was done with a branched triamine **7** which we have prepared as an amino acid triamide:



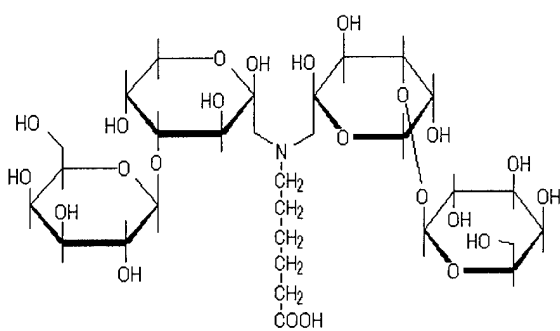
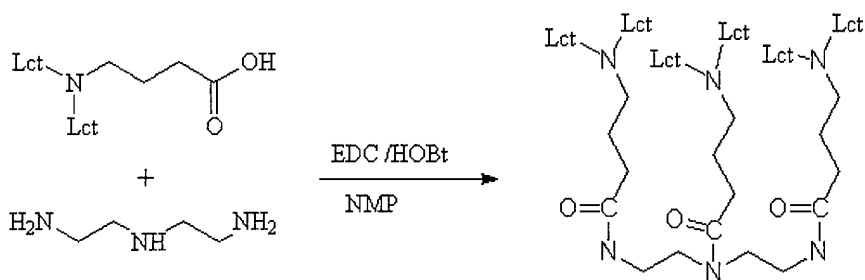
Currently, we have no clear explanation for the failures to obtain poly LAs from the above polyamines and lactose. Note, however, that the common feature of the polyamines we did consider is secondary character of amino groups. While better suited for practical preparations of both cyclic and linear polyamines, these are less reactive as compared to primary amino groups.

Which LAs to multiply?

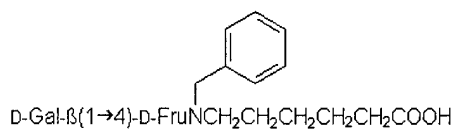
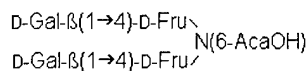
Having proven ineffectiveness of direct lactose/amine reaction for the preparation of multivalent LAs, we have considered the second approach for LA synthesis which we have proposed in Year 1: peptide coupling of pre-synthesized LAs to core architectures (polyamines in this project). This idea had been successfully verified then in preparations of divalent LAs by coupling LAs with spacer arm and linear α,ω -diamines.

When available LAs derived from γ -aminobutyric acid, namely *N,N*-dilactulose- γ -aminobutyric acid and *N*-lactulose-*N*-methyl- γ -aminobutyric acid, were brought into the reaction with polyamines **1**, **2** and diethylenetriamine, the complete coupling did not proceed, with one exception:

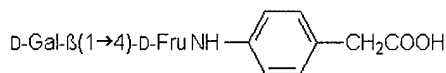
We then investigated whether elongation of the distance between lactulosamino group and carboxylic terminus would lead to improved coupling. Keeping in mind results of biological testing in Year 1, the following LAs with 6-atom spacer arm have been designed and prepared in multigram quantities and of high purity:



Dilactulose-6-aminocaproic acid



Lactulose-*N*-benzyl-6-aminocaproic acid



Lactulose-*p*-aminophenylacetic acid









These compounds represent three major groups of LAs we have been working with in Year 1. An LA derived from aliphatic secondary amine, *Lct-N-Bn-6-Aca*, is also a precursor for LAs of primary amine since benzyl group can be easily removed by catalytic hydrogenolysis. Divalent $(\text{Lct})_2\text{-6-Aca}$ represents a unique group of LA with unusual pattern of tautomerization in solutions. Derivatives of aromatic amines displayed the highest potential in inhibition of

of prostate cancer cells in vitro. Lct-pApa was chosen to carry this type LA in polyvalent LA structures.

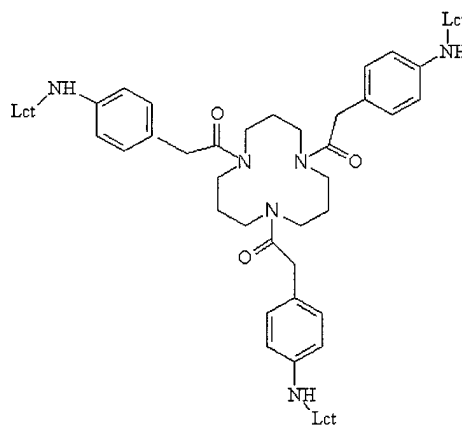
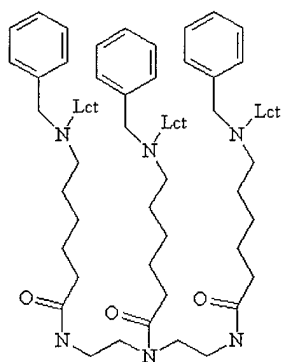
Polyvalent Lactulose-amines

Experiments with coupling of the above three LAs to the core polyamines were successful and a number of LAs with broadly varying number of lactulosamine groups, one through eight, could be prepared in pure form, characterized chromatographically and spectroscopically (MS, NMR). In the Table below, we demonstrate a combinatorial approach that may be useful for future libraries of LAs.

An array of polyvalent LAs we have prepared by combining three LAs with spacer arms and core polyamines. Numbers in cells indicate number of β -galactoside residues on the corresponding synthetic compounds.

Amine \rightarrow	"comb"				"star"			
								
Lct-N-Bn-6-Aca	1	2	3	4	1	2	3	4
Lct-pApa	1	2	3	4	1	2	3	4
(Lct) ₂ -6-Aca	2	4	6	8	2	4	6	8

Below are other representative structures of prepared compounds which are not included in the above table:



In total, 20 LAs with number of lactulosamine groups 3,4,6 and 8 have been prepared and characterized. These compounds, along their mono- and divalent structural analogs, were used in subsequent experiments in Tasks 2 and 3. It is worth to note that this synthetic approach which we have developed for multivalent LA preparation, is suitable for further development of

automated combinatorial chemistry. It provides, in most cases, for pure reaction products which are isolable in high (60-95% recovery) yields by relatively simple work-up. The method also ensured preparation of affinity columns which we will be able to use in planned experiments.

Task 2. Evaluate specificity and affinity of LA binding to prostate cancer galectins

According to the plan for Year 2, the following results has been achieved:

- prepared 3 LAs with spacer arms and 3 affinity media modified with these LAs
- determined affinities of synthetic LAs to galectin-1 using competitive ELISA

Three LA with 6-carbon spacer arms which have been employed for preparation of multivalent lactulose-amines, were also used for affinity media preparation.



Thus, Affi-Gel 102 (Bio-Rad) was mixed with 2-fold excess of each LA, EDC coupling agent added in 2-fold excess, and the reaction was monitored polarimetrically, using the coupling solution, without Affi-Gel, as a reference.

Evaluation of Binding Affinities of Synthetic LAs to Galectin-1.

In these experiments, we intended to investigate whether LAs would compete with a natural Galectin-1 ligand, laminin, for the lectin and how distance between lactulosamine groups in divalent LAs and valency level in polyvalent LAs would influence the affinity of LAs to the lectin.

In a typical experiment, Corning Costar microtiter wells were coated with 0.5 μg /well of mouse sarcoma laminin. Recombinant human galectin-1 (2.5 $\mu\text{g}/\text{ml}$ or 0.17 μM) was diluted in PBS with 0.05% Tween and added to wells (100 μL) coated with the glycoprotein, and galectin-1 binding was detected with anti-galectin-1 rabbit antiserum. Goat Anti-Rabbit Ab conjugated with HRP (Bio-Rad, at 1:1500 dilution) and subsequent treatment with ABTS/ H_2O_2 reagent were used to develop color read with Bio-Tek μQuanta Reader. In binding-inhibition experiments, lactulosamines or control carbohydrates were diluted in PBS-0.05% Tween containing recombinant galectin-1 at indicated concentration.

Inhibition of Galectin-1 binding to laminin by synthetic lactulose-amines. Estimated IC_{50} for Lactulose-amines and representative carbohydrates in competitive ELISA.

Compound	Valency	Lct-Lct distance, atoms	IC_{50} , μM
LctNH ₂	1		100
Lct ₂ -1,3-diaminopropane	2	3	30
Lct ₂ -1,6-diaminohexane	2	6	15
Lct ₂ -1,9-diaminononane	2	9	10
Lct ₂ -1,12-diaminododecane	2	12	8
(Lct- δ Ava) ₂ -1,9-diamine	2	21	20
(Lct- δ Ava) ₂ -1,12-diamine	2	24	40
Lct-pApa-morpholine	1		500
(Lct-pApa) ₂ -piperazine	2	16	150
(Lct-pApa) ₃ -cyclo[2,2,2]	3	16	80
(Lct-pApa) ₄ -cyclo[2,2,2,2]	4	16	80
Lct ₂ Aca- 	2	0	100
(Lct ₂ Aca) ₂ - 	4	0/19	40
(Lct ₂ Aca) ₃ -[3,3]triamine	6	0/19	20
(Lct ₂ Aca) ₄ -[3,3,3]tetramine	8	0/19	10
N-Acetyllactosamine	1		200
β -Thiodigalactoside	1		200
Lactose	1		800

Shown above preliminary data indicate that significant effect on potential of LA in competitive inhibition of Galectin-1 - laminin interaction may be achieved by tuning distance between lactulosamine groups which apparently cross link the lectin molecules.

Task 3. Identify best inhibitors of clonogenic growth of highly metastatic human prostate cancer cells

According to the plan for Year 2, the following results has been achieved:

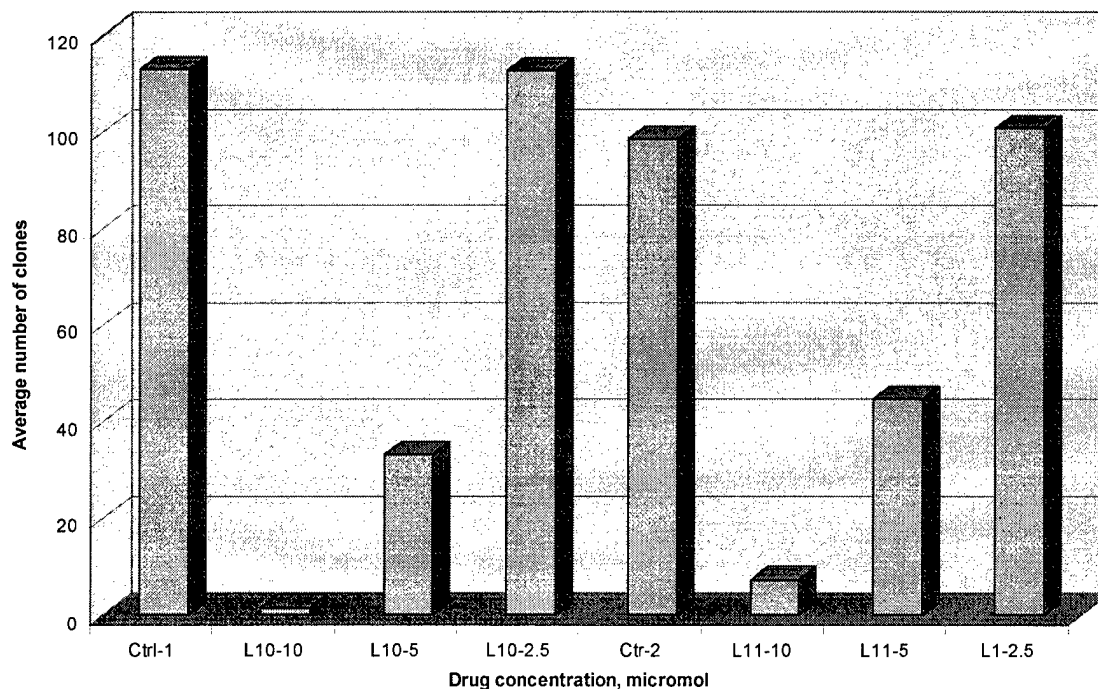
- 2 LAs have been identified as most effective inhibitors of clonogenic growth employing highly metastatic PC3MLN4 and DU145 human cancer cell lines
- inhibition of cell proliferation in monolayers was evaluated for over 70 LAs in experiments with LnCaP, PC3, PC3MLN4 and DU145 human cancer cell lines

Inhibition of clonogenic growth of highly metastatic human prostate carcinoma cell lines PC3MLN4 and DU145 by the first series of synthetic Lactulose-amines has been evaluated in *in vitro* experiments by the Subcontractor (Metastat, Inc., Dr. G.V.Glinsky).

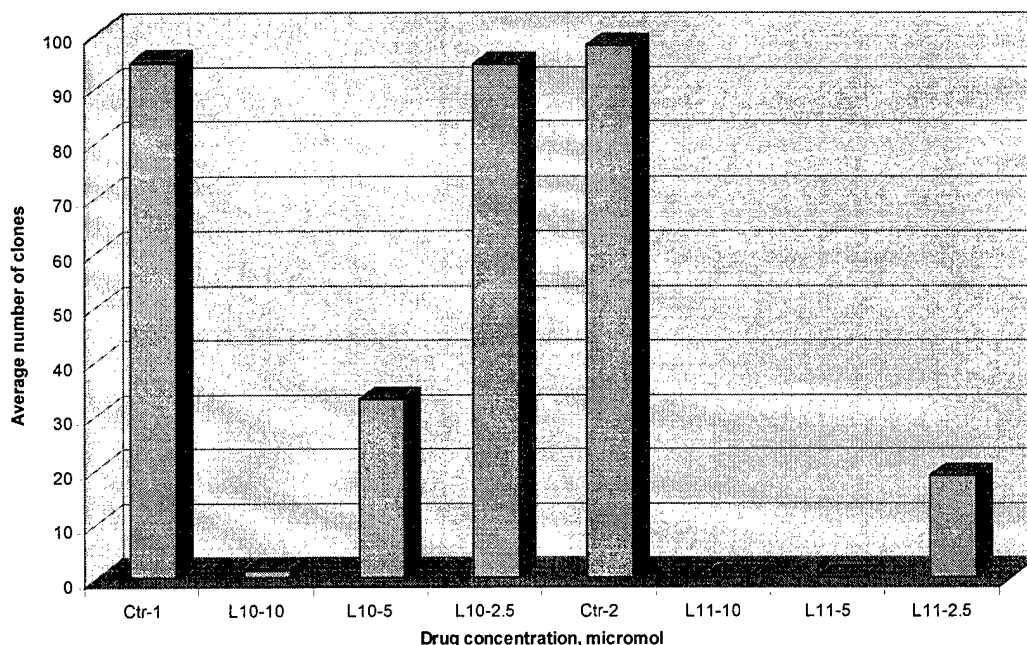
In Vitro Clonogenic Survival Assay

The cells were maintained as monolayer cultures in standard growth medium containing RPMI1640 supplemented with 2 mM L-glutamine, 100 µg/ml gentamicin, and 10% prescreened fetal calf serum (FCS), and incubated in 5% CO₂/95% air at 37°C in a humidified incubator. Tumor cells were harvested non-enzymatically from subconfluent cultures (60-70% confluence) and cell suspensions were gently pipetted to produce a single cell suspension. Only single cell suspensions of greater than 90% viability (determined by Trypan blue dye exclusion) were used for *in vitro* experiments. Cells were resuspended in growth medium and plated at low density in quadruplicate (200 viable cells per well in a 24-well culture plate) without (control samples) and with additional compounds (200 µM) that were tested. Seven days later, the cells were fixed with 1% formaldehyde in PBS, stained with hematoxylin, and colonies of more than 20 cells were scored. Colony forming efficiency (CFE) was then determined as the ratio of the number of colonies/number of cells plated. The surviving fraction of cells was calculated as the ratio of the CFE of the drug-treated culture and the CFE of the nontreated control culture.

Inhibition of clonogenic survival of DU145 human prostate carcinoma cells by synthetic lactulosyl-glycoamines Lac10 and Lac11



Inhibition of clonogenic survival of the PC3MLN4 human prostate carcinoma cells by synthetic lactulosyl-glycoamines Lac10 and Lac11



Inhibition of cell proliferation in monolayers in vitro

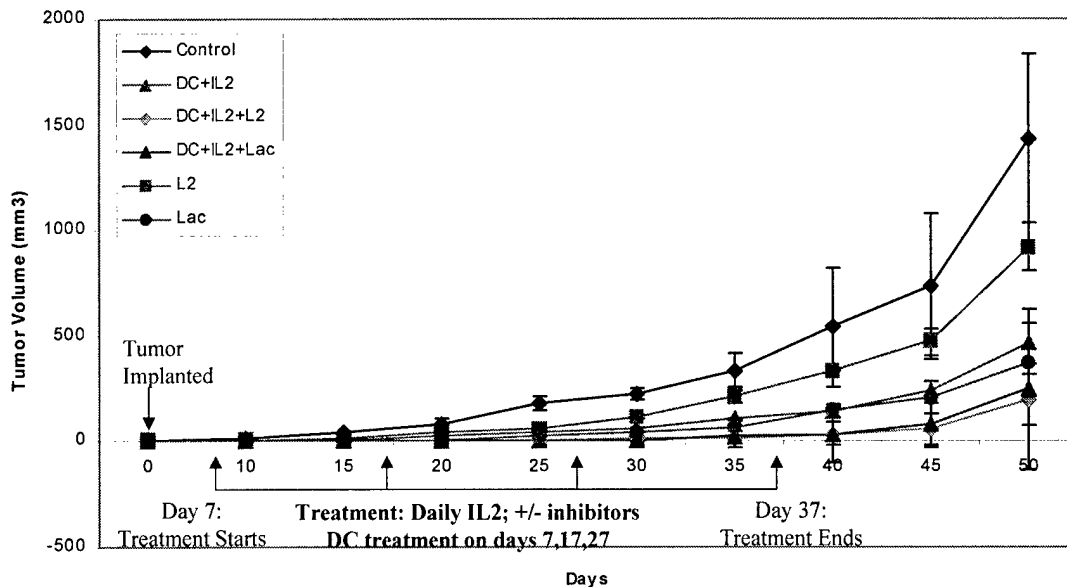
Possible cytotoxic effects of LAs were evaluated in the following experiments: 200 μ L of suspensions containing LnCaP, PC3, PC3MLN4 and DU145 at 10^4 cell/mL concentration in Eagle's MEM supplemented with vitamins, non-essential amino acids, Na pyruvate, L-glutamine and 10% fetal bovine serum, were added to microtiter wells (Corning Costar) and incubated in 5% CO_2 /95% air at 37°C for 1 day. The media were replaced then with solutions of LAs in the same medium for next 2 days, followed by the fresh drug solutions for next 2 days. At the end of the drug exposure period, cells in monolayers were washed with PBS, and fresh medium containing 10% of Alamar Blue (BioSource) or Resazurin (Sigma) added. The plates were incubated at 37°C / 5% CO_2 for next 2 or 4 hours, and fluorescence (530/590 nm) in wells measured by BioTek FLx-800 plate reader.

These experiments were done with over 70 synthetic lactulose-amines at starting highest concentration of 1 mM, along with two conventional chemotherapeutic drugs, Taxol and Etoposide as controls. The obtained data confirmed high cytotoxicity for the two most potent inhibitors of the clonogenic growth ($\text{IC}_{50} < 60\mu\text{M}$ and $100\mu\text{M}$ correspondingly for L10 and L11). In total, however, only 8 LAs had significant inhibitory effect in all four cell lines at concentrations 1mM and below. This is in contrast to much higher percentage of LAs which work as inhibitors of the clonogenic growth at submillimolar concentrations and may reflect the importance of cell adhesion molecules for survival in small clones versus monolayers.

Task 4. Determine antimetastatic properties of selected LAs *in vivo*

When submitting the proposal for this project, we theorized that galectins may play an integral part in the cancer process, from early to late stages: 1) immunosuppressive properties of galectin-1 have been reported in autoimmune diseases, 2) tumor-promoting activities of galectin-3 are well known, and 3) anti-apoptotic activity of intracellular galectin-4 has been noted. These observations warrant search for galectin inhibitors, which could be critical in disrupting the cancer process.

As we have demonstrated earlier *in vitro*, one of the first investigated divalent LAs, dilactulose-hexamethylenediamine (L2hmda), not only inhibits binding of galectin-1 to its extracellular physiological ligands 90K/MAC-2BP and laminin in ELISA assays, but more importantly, L2hmda blocks the immunosuppressive activity of galectin-1 on T-cells. To demonstrate the *in vivo* relevance of these observations, we (in collaboration with Dr. Margaret Huflejt, Sidney Kimmel Cancer Center, San Diego, CA) tested L2hmda for its anti-tumor activity in Her-2/*neu* transgenic mice. These mice spontaneously develop human-like lobular carcinomas, which are notoriously difficult to inhibit. Despite the fact that these mice are heavily immunosuppressed, this galectin inhibitor markedly reduced the growth of tumors when used alone, and nearly completely eradicated tumors when used in conjunction with dendritic cell-based immunotherapy:



Dilactulose-hexamethylenediamine (L2) enhances anti-tumor effect of dendritic cell therapy in *neu* mice.

Neu mice were inoculated on day 0 with 10^6 N202.1A cells. On day 7, second group of animals was immunized with 10^6 DC-pulsed with apoptotic N202.1A cells (dendritic cell therapy). Animals in the third group received only L2 treatment (100 μ l of 100 mM sterile solution). Animals in the fourth group received both dendritic cell therapy and L2hmda treatment. Animals treated with the dendritic cell therapy received also i.p. injections of rIL-2 (10^4 IU/injection) from day 10 to day 17. Four animals were included per group. (Lac – uncharged precursor of L2).

KEY RESEARCH ACCOMPLISHMENTS

- a general synthetic procedure for multivalent lactulose-amines have been developed
- using this procedure, a total of 20 tri-, tetra-, hexa-, and octavalent lactulose-amines have been prepared and characterized
- agarose gels modified with 3 lactulose-amines have been prepared for affinity column experiments
- two of prepared lactulose-amines demonstrated high inhibitory potential (IC_{50} 2 - 5 μ M) in a *in vitro* human prostate carcinoma clonogenic growth assay
- inhibition of *in vitro* human prostate cancer cell proliferation in monolayer was evaluated for 82 lactulose-amines
- binding affinities of some synthetic lactulose-amines to galectin -1 have been evaluated in a competitive (laminin) ELISA
- an effectiveness of a combination cancer immunotherapy with a divalent lactulose-amine has been demonstrated in *in vivo* mouse model

REPORTABLE OUTCOMES

None

CONCLUSIONS

We are pleased to conclude that synthetic approaches we have been developing during **Years 1 and 2** as well as predictions in regard with anticlonogenic potential and affinity to galectins of lactulose-amines have proven to be valid, at least for the set of LAs prepared so far.

Such synthetic approaches open a way for development of a new class of stable neoglycoconjugates with desired number of specific glycoepitopes: β -galactoside residues. These compounds could be prepared, as we have demonstrated, inexpensively, in multigram quantities, or as libraries for combinatorial chemistry studies.

Demonstration of strong anticlonogenic potential by some of the newly prepared lactulose-amines makes these compounds potential drug candidates and justifies their further research valid not only within this project, but also makes them attractive for similar trials on other types of cancer.

Unusually high affinities to galectins of di- and polyvalent lactulose-amines tested so far surpass those displayed by natural glycoligands. This observation could make di- and multivalent LAs very attractive molecular probes for glycobiology of galectines which is a quickly expanding topic of biomedical research both in oncology and such areas as immunology or neuroscience.

Our studies are the first to show that simple glyco-conjugates can inhibit tumor growth *in vivo*, with

a potential future role in cancer prevention

REFERENCES None

APPENDICES None