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13. ABSTRACT (Maximum 200 Words)
It is important to recruit a cadre of talented investigators whose careers are dedicated to studies of prevention, treatment, and early detection of breast cancer. By investing in young people before they make career choices, and by providing them with first-hand experience in modern breast cancer research (BCR) laboratories, we are finding that several of these young people discover an interest in BCR and are going on to graduate school or medical school and are actively involved with BCR at the next stage of their career. The Summer Undergraduate Research Programs at the Albany Medical College is designed to recruit highly talented undergraduates and expose them to career-defining opportunities. That talented students are being recruited is evident from the diversity of undergraduate schools (39 in number), the quality of the matriculants (average GPA 3.75), and the number of applications (104) received. Students spent 90% of their time in the laboratory of a funded investigator doing authentic, meaningful, mentored BCR. Students also participated in a variety of Enrichment Activities, all focused on breast cancer.

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Introduction

This is an Annual Report for DAMD17-01-1-0121, a training award entitled "Breast Cancer Research Undergraduate Summer Training Program." This training grant is based at the Albany Medical College (AMC) in Albany, NY. The award provides support for 5 students per year; with College supplementation we supported 8 students in the summer of 2003. At the time of report writing, three years of training are complete and recruitment (but not training) of the fourth cohort of students is complete. To comply with the request (in the critiques of the first year's progress report) for cumulative data, information is provided about the first three cohorts (and the report is a little longer than specified).

Body

Relevance: This 2003 Undergraduate Summer Training Program (USTP) was focused on breast cancer research (BCR) in all aspects. Research opportunities focused on breast cancer research were available in 15 laboratories, funded by at least 17 different grants among 14 principle investigators. A broad range of disciplines was available from which undergraduates selected research projects, spanning the areas of peptide chemistry, anti-oncotic pharmaceutical development, cell biology and cell signaling studies, breast cancer prevention trials, and translational and clinical investigations. Enrichment Activities all centered around breast cancer, including career planning discussions, research seminars, literature review training sessions, "Meet the Investigator" sessions, and even sessions for training in scientific ethics. Undergraduates were immersed in a summer of breast cancer study, and will be well prepared to enter a career path that will lead to productive contributions to the efforts to eradicate breast cancer early in this century. Two students from the AMC Summer Research Programs have matriculated in the Graduate Studies Program at Albany Medical College, one of whom is involved actively in Breast Cancer Research. Overall, 12 of 15 program alumni(ae) who have graduated from college have gone on to professional school.

Overview: The USTP at AMC is designed as a 10-week program to provide opportunities for 5 undergraduates per summer. Talented students are being recruited: 104 applications from 39 different colleges, including applications from as far away as California, were received, which provided a competitive applicant pool from which the top 5 candidates were selected. The Albany Medical College supported three additional students, due to the high quality of applicants and demand on the part of mentors. Students spent more than two months in a laboratory doing meaningful, authentic, innovative research on a project specially designed for them, and with the active mentoring of an investigator who was funded and who had sufficient time and inclination to serve as a mentor for an undergraduate student. Funded faculty members were screened by the Program Director for inclusion on the Participating Mentor list based on funding, BCR interests, ability and inclination to serve as a mentor, and past experiences with mentoring undergraduate students. Faculty provided descriptions of BCR opportunities for undergraduates in their laboratory, and these descriptions accompanied the application/recruitment materials so that undergraduate applicants could make choices as to which research topics were of interest to them. Students spent more than 90% of their summer doing research in a laboratory, but also had Enrichment Activities including Safety Training, on-line biomedical information search and retrieval training, training in issues of Responsible Conduct of Research, interactive learning opportunities focusing on an Overview of BCR, seminars to broaden their knowledge of BCR, preparation of their own research presentations, and opportunities to explore BCR career options while at AMC. An important addition for the summer of 2002 was a writing component, which enhanced students' ability to read and write scientific literature. This opportunity was maintained for the 2003 summer and is evaluated as among the best training activities provided. The program provided extensive tracking and evaluation of the Students, of the Faculty, and of the program as a whole so as to make adjustments when necessary. At the suggestion of the reviewers from last years progress report, we have included an appendix with three typical evaluation tools that were used to gather feedback from the participants.

Progress

Task 1 Recruitment
a. Select Participating Mentors

Table 1 – Mentors, Funding Status, and Student Projects 2003

2003 Student	Mentor	Mentor Funding	Student Project Title
William Dowdle	Michelle Lennartz, PhD	5R01AI050821-02	"The Search for PKC-E Binding Partners"
Justin Georgekutty +	Thomas Andersen, PhD	1R01CA102540-01 5R25GM062460-04 1R25GM069249-01	"Therapeutic Effects of a Peptide Derived from Alpha-Fetoprotein on Breast Cancer in a Rat Model"
James Lee	C. Michael DiPersio, PhD	5R01CA084238-05	"Use of RT-PCR and Western Blotting to Access $\alpha 3\beta 1$ Dependent Expression of VEGF and Cell Survival Proteins in Keratinocyte Cell Lines"
Nicole Lemanski	Thomas Andersen, PhD	1R01CA102540-01 5R25GM062460-04 1R25GM069249-01	"Alpha-Fetoprotein Derived Peptide in Non-Toxic in a Mouse Model"
Emily Luidens +	Faith Davis, MD		"Effects of T4 and Estrogen on Resveratrol-induced Apoptosis in Human Cancer Cell Lines"
Ahmed Mousa +	Paul Davis, MD		Studies of Cancer Cells in Culture
Emily Roberge	Mario Canki, PhD	5R01NS040666-04	"Inhibition of HIV-1 Replication in T cells by Extracts from Five Medicinal Plants"
Bharat Yarlagada	J. Andre Melendez, PhD	5K22CA095011-02	"Redox Regulation on MMP-1 Production"

+ Supported by AMC, not by grant funds.

To comply with the request for cumulative data, we also show the information for Year 2 (2002, Table 2) and Year 1 (2001, Table 3).

Table 2 – Mentors, Funding Status, and Student Projects 2002

2002 Student	Mentor	Mentor Funding	Student Project Title
Kelly Fisher	Charles Lowry, PhD	NSF0114040	"Genetic Characterization of the Regulatory Domain of the Mox 4 Transcriptional Factor in C. cerevisiae"
Roland Jacques	Paul Higgins, PhD	2R01GM057242-05	"The Role of Ras in the Signal Transduction Pathway of PAI-1"
Leroy Joseph *	Thomas Andersen, PhD	5R25GM062460-03 DAMD17-01-1-0472	"Optimal Synthesis of Cyclic Peptides that Prevent the Growth of Human Breast Cancer"
Rebekah Klinger +	Thomas Friedrich, PhD	Albany Molecular Research	"Point Mutations in the Nuclear Localization Signal of SV-40 Large T Antigen"
Alicia Strazza	Lisa Petti, PhD	5R29CA73682-03	"Tyrosine to Phenylalanine Mutations at Autophosphorylation Sites Alter the Neu* Receptor's Ability to Transform Human Diploid Fibroblasts"
Gayani Tillekeratne	Paula McKeown-Longo, PhD	5R01CA058626-11 5R01CA069612-07	Ánti-Angiogenic Activity of the First Type III Repeat of Fibronectin"

* Minority Student
+ Supported by AMC, not by grant funds.

Table 3 - Mentors, Funding Status, and Student Projects 2001

2001 Student	Mentor	Mentor Funding	Student Project Title
Kerri Ann Fraterigo	James Bennett, PhD	1R21CA87434-01 Elsa U Pardee Fnd	"The Role of TGF-β in the Growth Regulatory Effects of AFP-derived Peptide"
Jason Laliberte	C. Michael DiPersio, PhD	R01CA84238	"Lack of Integrin $\alpha 3\beta 1$ Correlates to Increased Activation of jun-NH_2-terminal Kinase in Keratinocytes"
Kate Pettrone	Lisa Petti, PhD	5R29CA73682-03	"Determination of Amino Acids in the Transmembrane Domain of the *neu* Receptor Required for its Activation Under Conditions of Overexpression"
Lisa Schoonmaker	J. Andre Melendez, PhD	5K01CA77068-03	"Superoxide Dismutase-Dependent Peroxynitrite Production"
Adam Stallmer	Thomas Andersen, PhD	ANDT01 - New York State DAMD17-01-1-0472	Acylated Lysine Analogs of Anti-Breast Cancer Peptides Retain Chemoprophylactic Effect and Serve as Model Ligands for Affinity Chromatography.
Courtney St. Amour	Michael Fasullo, PhD	5R29CA70105-06	"Mitotic Recombination in Yeast Ku Mutants"

All mentors were assessed as contributing and helpful. Last year, it was hypothesized that the best mentor-student interactions correlate with amount of time the mentor spends with the student. This hypothesis was to be assessed in Year 3 (Summer 2003). However, we need to increase the sample size for this assessment by including the results from the 2004 summer because most students evaluated their mentor-student interactions so highly that the independent variable was virtually a constant. We had good input and good outcomes, so this study will continue by inclusion of this summer's data so as to increase sample size.

b. Develop recruitment materials c. and d.) Distribute materials to colleges

A recruitment poster and application materials were developed and mailed, and application forms were posted on the College web site. These materials were appended in the first year's report and so are not duplicated this year. Since we have such a large number of applications (see Table 4), we conclude that our recruitment is appropriate.

Task 2. Selection of Students

Recruitment efforts led to a large number of applicants of very high quality. Table 4 shows that the program was very selective and very attractive. All of our top applicants enrolled in the program in the third year, and the College found funds to add three additional students.

Table 4 – Recruitment of Students

Year	Number of Applications	Number of Acceptances	Number Enrolling
2003	104	8 – Army, Breast Cancer 6 – NIH, Cross Training	8 – ARMY, Breast Cancer (3 students paid from AMC funds) 6 – NIH, Cross Training
2002	81	6 – Army, Breast Cancer 5 - NIH, Cross Training	6 – Army, Breast Cancer (1 student paid from AMC funds) 5 - NIH, Cross Training
2001	90	6 – Army, Breast Cancer 5 – NIH, Cross Training 8 – Volunteers	6 – Army, Breast Cancer 5 – NIH, Cross Training 8 – Volunteers
2000	34	17	16 - All AMC Undergraduate Programs
1999	21	6	6
1998	18	5	5
1997	26	5	5
1996	24	6	5

*Cross Training refers to a similar program for other students at AMC.

Table 5 shows information about the individual matriculants in Year 3 (2003). Table 6 and Table 7 are updates from Year 2 (2002) and Year 1 (2001) cohorts.

Table 5 - Data for BCR Matriculated Students, Summer 2003 (Year 3)
Table 5 shows that the quality of the matriculants was very high.

2003 Student	Undergrad. College	Year Completed at time of Acceptance	Major	GPA at time of Acceptance	Current Status
William Dowdle	Rochester Institute of Technology	Junior	Biotechnology	4.0	Graduated. Working in BCR before applying to professional school
Justin Georgekutty+	Rensselaer Polytechnic Institute	Sophomore	Biology	3.95	Medical School Albany Medical College Fall 2004
James Lee	Michigan State University	Sophomore	Microbiology	3.78	Completing Junior year at Univ. of Michigan
Nicole Lemanski	Hamilton College	Junior	Biology / Art History	3.55	Senior at Hamilton
Emily Luidens +	Hamilton College	Freshman	Biology	3.8	Sophomore at Hamilton
Ahmed Mousa +	Cornell University	Freshman	Biology	3.19	Sophomore year at Cornell
Emily Roberge	SUNY Albany	Sophomore	Biochemistry	3.94	Completing Junior year at SUNYA
Bharat Yarlagada	Rensselaer Polytechnic Institute	Sophomore	Biology	3.77	Medical School Albany Medical College Fall 2004

+ Supported by AMC funds

The average GPA of all matriculants was 3.75 (on a 4.0 scale) for this year. The average GPA of training grant supported students was 3.81

Table 6 – Updated Status of 2002 Cohort (Year 2)

2002 Student	Undergraduate College	Year Completed at time of Acceptance	Major	GPA at time of Acceptance	Current Status
Kelly Fisher	College of Saint Rose	Sophomore	Biology/ Cytotechnology	3.60	Employed Cytotechnology – Albany Medical Center
Roland Jacques	University of Rhode Island	Junior	Microbiology	3.49	Medical School
Leroy Joseph	Cheney University	Junior	Chemistry	3.97	Graduate School at Albany Medical College
Rebekah Klinger	Hartwick College	Junior	Biology	3.59	Graduate School at Colorado State
Alicia Strazza	College of Saint Rose	Junior	Biology	3.85	Graduate School at Tufts University
Gayani Tillekeratne	Massachusetts Institute of Technology	Junior	Biology	4.8 (on a 5.0 scale)	Medical School at Duke University

Table 7 – Updated Status of 2001 Cohort (Year 1)

2001 Student	Undergraduate College	Year Completed At time of Acceptance	Major	GPA at time of Acceptance	Current Status
Kerri Ann Fraterigo	Russell Sage College	Sophomore	Biology	3.98	Medical School
Jason Laliberte	University of Massachusetts at Amherst	Junior	Biology	3.27	Graduate School at UMASS
Kate Pettrone	Williams College	Sophomore	Biology	3.36	Working at Easton Associates, NYC
Lisa Schoonmaker	Siena College	Sophomore	Biology	3.34	Albany Medical College – Breast Cancer Research
Adam Stallmer	Rensselaer Polytechnic Institute	Sophomore	Math/ Science	4.0	Medical School at Syracuse
Courtney St. Amour	Brandeis University	Sophomore	Biology	3.88	Graduate School at Cornell

Task 3. Orientation of Summer Undergraduates
All required training sessions were completed in the first week, and team-building aspects were emphasized. A voluntary mountain hike provided a challenge and an opportunity for students to get to know one another, which helped with interactions throughout the rest of the summer. Other sessions included Laboratory Safety Training, Radioactivity Safety Training, Care and Use of Animals, and Internet-Based Search-and-Retrieval Training. Evaluation tools indicated that all aspects were successful.

Task 4. Research Training
Students participated actively in research for 10 weeks, guided by their mentor, a well-funded BCR investigator. Each student presented the results of their work at the end of the summer in poster or oral format (titles of these presentation are listed in Table 1). Students met weekly with Investigators to learn more about career paths and discuss research relevant to breast cancer.

Two students (Joseph, '02 and Stallmer, '01) coauthored a publication stemming from the research supported by this training grant:

DeFreest LA, Mesfin FB, Joseph L, McLeod DJ, Stallmer A, Reddy S, Balulad S, Jacobson HI, Andersen TT, Bennett JA. (2004) Synthetic peptide derived from alpha-fetoprotein inhibits the growth of human breast cancer: Investigation of the pharmacophore and synthesis optimization. J. Peptide Research, In Press.
A copy of the pre-print is included in the Appendix.

Table 8 – Meet the Investigator Series
Evaluations indicated that students appreciated learning about career choices of these investigators, and about their research.

Week #	Investigator
1	Dr. Michelle Lennartz
2	Dr. C. Michael DiPersio
3	Dr. Livingston VanDeWater
4	Dr. Paul Black
5	Dr. Mario Canki
6	Career Topics – Medical and Graduate
7	Dr. Tara Lindsley
8	Dr. J. Andre Melendez

Table 9 – Responsible Conduct of Research
Students were trained in the Responsible Conduct of Research in accordance with NIH recommendations. Students perceived the sessions as beneficial.

Sessions #	Title of Session
1	Current topics in Scientific Integrity
2	Introduction to Ethical Thinking
3	Workshop on Case Analysis by Moral Reasoning
4	Analytical Skills Workshop
5-8	Student-Led Role Playing and Case Discussion

Overview of Breast Cancer
The Overview of Breast Cancer didactic series included lectures that provided background information on cancer, oncogenes, angiogenesis, and the causes and treatment of breast cancer. Dr. Fassil Mesfin presented a series of sessions encompassing various aspects of breast cancer. The intent of the didactic lectures was to provide students with knowledge to supplement their laboratory research and to engender within the students a passion to pursue breast cancer research as a career.

Students in the BCR program read the following book and participated in detailed discussion of the BCR implications of the book.

Dr. Folkman's War – Angiogenesis and the Struggle to Defeat Cancer
Author: Robert Cooke

Writing Component A writing component was adapted and incorporated into the Breast Cancer Research program for half of the summer of 2002, and for the whole summer of 2003. In this component, students were asked to use their Search and Retrieval skills to identify and read a paper related to Breast Cancer Prevention, and write a 1 or 2 page summary of the work. Students used their literature search skills, read scientific papers, enhanced their writing skills by providing a description appropriate for the scientific disciplines, and became familiar with a broad range of BCR investigations. Students received individualized feedback promptly, and incorporated the suggestions for the next week's assignment. BCR students wrote weekly assignments and made rapid progress in reading and interpreting the scientific literature. The individualized nature of the feedback ensured that each student worked on their own areas of greatest need and this ensured rapid progress. This is, by far, the most successful of all the non-laboratory-based activities, and is of tremendous benefit in preparing students for graduate school.

Table 10 - Career Day
Students were offered an afternoon session in which career options were discussed. Routes to BCR through graduate school and through medical school were outlined. Student evaluations indicated that this was very well received. Students indicated this session should occur earlier in the summer and this was accommodated for 2003. For 2004, this session will occur even earlier, in week 2, so that undergraduates can better distinguish between graduate students and medical students with whom they interact in the labs during the summer.

Career Opportunities Day	Presenter
The Road to Graduate School	Assistant Dean for Graduate Studies
The Paths after Graduate School	Research Professor of Biochemistry
The Road to Medical School	Assistant Dean for Medical School Admissions
Research in Medical School	Assistant Dean for Medical Student Research
Career Paths after Medical School	PGY1 Resident

Students also met individually with the P.I. on several occasions throughout the summer, and career goals were discussed and optimized.

Presentation Preparation - A session was offered to assist students in preparing for the end-of summer presentations.
Dr. Thomas Andersen - Oral Format Presentation
Dr. C. Michael DiPersio - Poster Format Presentation & Writing a Scientific Abstract

Students presented their work (see Table 1 – for titles) before the faculty and students of the College in a Research Day designed especially for the Undergraduate Summer Research Programs at AMC. Students chose to present their results in a poster or oral format. Many students have the opportunity to make presentations at their home campus and were encouraged to select the format that would best suit their needs in upcoming months. Students find this aspect of their training to be as enjoyable as it is challenging.

Evaluation of Program
Aspects of the program were evaluated with multiple, quick, interesting evaluation tools. The intent is to encourage students to respond the evaluation tools they will receive annually for the next decade. Responses to the assessments were made by improving the design of the program, from one year to the next, as well as promptly within a summer.

In response to a suggestion from reviewers of last year's progress report, we include examples of survey tools that we utilized last summer. We used evaluation tools frequently, and so designed them to be easy, even fun, to complete so as to avoid 'survey fatigue' and to encourage students to continue to complete our post-training annual surveys over the next 10 years. Three examples are included in an Appendix, two of which contain summarized data from all summer undergraduates participating in training during the summer of 2003. One contains the form only, not any data.

Key Research Accomplishments

- Recruited 14 funded investigators; 7 served as BCR mentors
- Received 104 applications
- Recruited 8 highly qualified students; five were supported by the grant, 3 by the College
- Trained students in Research
- Enriched students with a variety of BCR activities
- Recruited two alumni to this graduate school
- Of the 15 students who have graduated from their college, 6 have gone on to graduate school (2 at AMC), 6 to medical school, and 3 are employed (1 in BCR, 1 or more who plan to apply to professional school for 2005).
- Of the 12 students in professional (graduate and medical) school, 4 are involved with breast cancer research.

Conclusion

All short-term objectives were met; all long-term objectives are being met. The program was very successful.

The funding agency did not offer opportunities to compete for continuation of funding of this program, but with careful management of grant funds, and with supplementation from the College, we have made arrangements to provide the Breast Cancer Research program for the summer of 2004. A full cohort of students has been accepted.

Andersen, Thomas T.

Undergraduate Summer Research Program
Albany Medical College
June 19, 2003

Evaluation Tool
MTR-3

In 50 words or less, what is the essence of the science that you heard about today?

Did you enjoy today's "Meet the Researcher" session?

14 1. Yes, I learned a lot of interesting things.
2. It was OK but I would have liked more hardcore data.
3. It was good but I wish we had more discussion about graduate school/medical school/employment.
4. Not really. I had trouble getting interested in this topic.

On a scale of 1 - 5, in your opinion how well was discussion stimulated?

We sat like bumps on logs 1 2 3 4 5 We had a fantastic conversation and/or asked a lot of questions.

2 4 6 2

What was the reason for your above answer about the quality of discussion?

3 1. Although the topic was interesting, we were all too tired to engage in an involved conversation.
2. We wanted to participate more but the research was over our heads.
5 3. The session was too much in the format of a lecture. There was only a brief time for questions/conversation at the end.
7 4. I thought the conversation was pretty good. There was a lot of interaction between the students and the researcher.
5. Other (please describe):

Got comments?

Andersen, Thomas T.

Undergraduate Summer Cross-Training Program
Albany Medical College

Human Genome Lectures Evaluation-1

Summary.
See also comments on each sheet

How is the pace of these lectures?

1. Too fast - ||
2. Too slow
3. About right - 卌 |

What about the content of these lectures?

1. Too basic
2. Too advanced |||
3. About right 卌

How is the delivery of these lectures (circle all that apply)?

The speaker could be *louder*

The speaker should interact more with the students |||

The speaker's eye contact could be better

The speaker could be more **CONFIDENT** ||

The speaker doesn't use enough visual aids

Speaker relies too **HEAVILY** on visual aids |

The speaker's delivery is really pretty good 卌 |

Other (describe):

What is the your level of understanding of these lectures (circle all that apply)?

I know **most** of this stuff already ||||

I know some of this stuff already

What I don't know would be easy enough to capture on my own

I have enough background knowledge to appreciate these lectures ||

I'm learning a lot of new stuff |||

These lectures are **over my head** ||

On a scale from 1-10, how would you describe the content of these human genome lectures?

							||	||||	||		
Needs Improvement	1	2	3	4	5	6	7	8	9	10	**IDEAL**

Please provide comments about how the delivery and content of these lectures could be improved.

Andersen, Thomas T.

Undergraduate Summer Research Program
Albany Medical College
August 1, 2003

Name:________________________________

Evaluation Tool. There are 7 sides to this Evaluation Tool. Your help would be appreciated. Please add your comments, criticisms and suggestions.

1) The program design called for 90% of your time to be in the laboratory and 10% of your time to be in the Enrichment Series. How did that work out?

____I know I spent a lot more than 10% of my time in the Enrichment stuff.
____I guess the proportion worked out as planned.
____I don't know; I never gave it much thought.
____I think we should have had 100% of the time in the laboratory and blown off the Enrichment stuff.
____I think we should have had a little more non-laboratory time, and used it for these purposes:____.

2) Tell me how you really feel about the 8:00 am didactic sessions (Human Genome, Breast Cancer Overview, and Responsible Conduct of Research). (*Check as many as may apply.*)

____I really hated 8:00 am.
____I hated 8:00 am but could live with it.
____I hated it, but at least it left my day free for running my experiments.
____I'd rather have had it at 4:00 pm.
____I'd rather have had it at 9:00 am.
____Actually, it worked pretty well because then I was in the lab by 9:00 am, which probably would not have occurred otherwise.
____It didn't bother me.
____I liked it best at 8:00 am.
____I realized why it was scheduled for 8:00 am, but that didn't guarantee that I'd be awake then.
____Just do away with the didactic sessions entirely.
____Other suggestions:____.

3) Will your summer experience affect your curriculum in the remainder of your undergraduate years? (i.e. Will you take classes or opt out of classes as a result of your experience here?)

____No, my curriculum was set and can't be changed.
____No, I won't make any changes.
____I don't know if I will make changes or not.
____Yes, I will take these classes as a result of this experience:________________
____Yes, I will NOT take these classes as a result of this experience:______________

4) In your research, did you achieve what you hoped to? (*Check as many as may apply.*)

____I hoped to achieve more than I did.
____I achieved more than I thought I would.
____I was pleasantly surprised at how much I did.
____I could have done more if only:__________________________________.
____Other. (Please specify)____.

5) What are your thoughts about the Meet the Researcher series? (*Check as many as may apply.*)

____Do away with them entirely.
____Do away with them, but keep the pizza.
____Keep the MTR sessions as they are.
____Have the Breast Cancer students' and the Cross Training students' MTR together.
____Keep the MTR sessions, but change the time to 4:00 pm so it doesn't interfere with my lab work.
____The MTR sessions were interesting, but not essential.
____At least one of the MTR sessions was informative and well worthwhile.
____At least one of the MTR sessions helped me make a career decision.
____The MTR sessions are probably helpful for recruiting students to come to Albany Medical College.
____The MTR sessions could be improved. Here are some suggestions:
____.

6) How were the social interactions BETWEEN UNDERGRADS?

____We had plenty of opportunities to get to know each other.
____We needed more opportunities to get together with the other undergraduate students.
____We needed more opportunities to get together with the students in the other program (Breast Cancer or Cross Training).
____We needed more PARTIES!
____Oh, forget about that. I didn't come here to get to know other undergrads (whom I will probably never see again anyway). I just came for the science.

7) How were the interactions with the people in your LABORATORY?

____There were no other people in my laboratory.
____I had good (G) or poor (P) interactions with:
 ____My mentor.
 ____A graduate student(s)
 ____A postdoc(s)
 ____A technician(s)
 ____Other (*please specify*):__________________________________.

8) How were the interactions with other people in laboratories *near* where you worked?

____Nobody else even knew I was here.
____They said, "Hi!" to me, but that was about it.
____One or more people were helpful and interactive.
____At least one person from outside of my laboratory was very helpful.
____Comments:______________________________________.

9) Did you interact with people in the department (Center) office (not the Graduate Studies Office, but the Department to which your mentor belongs)?

____My mentor belongs to a department?
____No, not very much.
____I did, but those people were not helpful, not nice, or otherwise not much fun.
____I did, and they were nice and/or helpful.

10) Choose one of this group of statements, or generate your own statement.

____I learned more from the library, didactic, and reading sessions than I did from the laboratory research.
____I learned more from the laboratory experience than from the reading and writing stuff.
____I probably learned more in the lab, but the stuff I learned from the reading is likely to be more generalizable and useful.
____Here's what I think:______________________________________.

11) In relation to your laboratory work: *(Check as many as may apply.)*

____I profited from this, but to be honest, I knew most of this stuff before I got here.
____I learned new techniques, but my laboratory acumen was good enough before I got here.
____I feel a lot more comfortable in laboratory settings than before the summer began.
____I feel as though I could go into most any new laboratory setting and be confident that I could get up to speed in short order.
____This was perhaps the best academic experience of my life, to date.
____Other descriptors: ______________________________________

12) Tell me about the Presentation Workshop: *(Check as many as may apply.)*

____I knew everything I needed before I went in there.
____I picked up a tidbit or two, but it probably wasn't worth the time spent.
____I got several good clues. It helped me a lot.
____It was optimal just as it was.
____It should be expanded to include these things:______________________.

13) In regard to the Writing Component: ***(Check as many as may apply.)***

____I hated this part.
____The WC was okay, but not my favorite.
____I enjoyed finding and reading the science articles.
____I felt I really profited from this series.
____I learned a great deal from this series.
____This series was one of the best parts of the summer.
____This has changed the way I will read about science in the future.
____The feedback from the component was useful.
____The feedback from the writing component would have been more useful if:
____.

14) Tell me about Career Day: ***(Check as many as may apply.)***

____I knew everything I needed for my intended career path before I went in there.
____I picked up a good tidbit here or there, but it probably wasn't worth a whole afternoon.
____I got several good clues. It helped me a lot.
____I wish it had been earlier in the summer so I could have had more time for follow-up.
____It was optimal just as it was.
____It should be expanded to include these things:____________________.

15) Tell me about the Information Search and Retrieval (ISR) opportunities.

____I was fully cognizant of the ISR capabilities before the training session.
____I did not know of the capabilities of the ISR opportunities until the training session.
____A lot of things whizzed by me in the ISR training session and I found that I needed them later in the summer. Please give an example:
____.
____A lot whizzed by, but I didn't need them later in the summer.
____I used the ISR capabilities later in the summer.
____I feel as though I probably have a pretty good advantage over most undergraduates as a result of all my ISR exercises this summer.
____Some of the things I can do with the various ISR tools are really pretty nifty.
____Other comments:____________________________________.

16) Tell me about the Responsible Conduct of Science (RCS) series: (check as many as may apply)

____Uff Da; this was a waste of time.
____It is probably important, but it was as boring as summer squash.
____It was actually kind of interesting.
____This was cool stuff. I'm glad it was part of the summer.
____I have to admit that there were several topics that I had never appreciated before.
____It was okay, but the 'role-playing' was really pretty uncomfortable.
____I thought the role-playing sessions caused me to be more engaged than I otherwise would have been.
____Actually, I would like to see this series expanded so that we could have more in-depth discussions of some of these topics: ____.

The next several questions refer to your Mentor, defined as the FACULTY member in whose lab you worked. If you were supervised by a post-doc (or somebody else), you may ALSO answer the questions with respect to that person, but use a different color of ink for the TWO sets of answers.

17) How much time did you spend with your mentor:

____Way too little time.
____I could have used a little more time with him/her.
____An appropriate amount of time.
____Too much time.

18) How *valuable* to the progress of your research was the time you spent with your mentor?

____Of no value whatsoever.
____Of very little value.
____Valuable.
____Very Valuable.

19) How comfortable were you talking with your mentor?

____I was very comfortable talking with him/her.
____I was comfortable talking with him/her.
____I was uncomfortable talking with him/her primarily because ____.

20) How accessible was your mentor for <u>unscheduled</u> communication?

____My mentor was inaccessible.
____My mentor was somewhat accessible, but it took some hunting.
____My mentor was always there when I need her/him.

21) To what extent did your mentor teach sound research practices, like establishing significance of research, designing research plans, interpreting results, etc.

____I learned about these practices from my mentor.
____My mentor gave me a clear understanding of these issues.
____My mentor touched on these matters.
____My mentor didn't really stress these practices.

22) Did your mentor improve your grasp of experimental design?

____My mentor did not really teach me much about experimental design.
____I thought I knew all about experimental design, but I learned a few things.
____I didn't really need to learn anything about designing experiments.
____I learned a great deal about designing experiments.
____I learned a great deal about designing experiments, but mostly by observation, not necessarily because my mentor taught it.

23) Has your mentor taught you the value and methodology of record keeping?

____Absolutely. It was stressed repeatedly.
____A little bit.
____No. It was not emphasized.
____No. I already knew how to do a good job of keeping my notebook.
____No. I still don't keep good enough records.

24) How constructive was your mentor's feedback?

____It was very helpful.
____I had to filter out a lot to get to the constructive parts.
____His/her feedback was not at all constructive.

25) Did you feel as though there was someone who you could turn to if you had a problem with your mentor (such as a faculty member or administrator)?

____NO! I was stuck without any help.
____Not really, so I spoke with other students.
____Yes, I found someone, but it wasn't obvious whom I should approach.
____Yes, it was easy to find someone with whom I could talk.
____I did not have any problems with my mentor.

26) Was there a faculty member other than your mentor who kept track of the progress of your research?

____I don't know.
____Yes, there was at least one other faculty member who was aware.
____No.

27) How did your mentor teach you?

____He/She left me to learn by myself.
____We reviewed my objectives, I worked on them and then we discussed results.
____My mentor walked me through everything.
____Other:____.

28) How would you *like* your mentor to teach you?

___He/She should let me learn by myself.
___We should review objectives, I should work on them and we should discuss results.
___My mentor should walk me through everything.
___Other:______________________________________.

29) Tell me more about your mentor: *(Check as many as may apply.)*

___My mentor is the best thing since sliced peaches.
___Well, maybe not that good but he/she was very helpful to me.
___My mentor is all right. I have a lot of respect for him/her.
___I feel comfortable in asking for a letter of recommendation from my mentor.
___I would like to have my mentor come and speak on my campus (in a Biology Department seminar series, for example).
___I would rather have somebody else from the MTR session come and speak on my campus. Please specify whom you would like to see.
____.
___My mentor has a good science program, but I wish I had been in a different laboratory.
___Other descriptors: ________________________________.

30) Please list, and answer, at least one question that you think should have been in this Evaluation Tool, but wasn't.

31) Please provide any additional comments that you think would lead to improvements for the program.

When you are done, please bring this Evaluation Tool, together with your Sign Out sheet, all neatly initialed by the appropriate folks, and give both to Jean in MS-134. She will have a cheesy little gift for you, because we know you'd all like to go back to your campus with the AMC logo.

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Synthetic peptide derived from α-fetoprotein inhibits growth of human breast cancer: investigation of the pharmacophore and synthesis optimization

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Key words: breast cancer; estrogen; α-fetoprotein; head-to-tail cyclization; synthetic peptide

Abstract: A synthetic peptide that inhibits the growth of estrogen receptor positive (ER+) human breast cancers, growing as xenografts in mice, has been reported. The cyclic 9-mer peptide, *cyclo*[EMTOVNOGQ], is derived from α-fetoprotein (AFP), a safe, naturally occurring human protein produced during pregnancy, which itself has anti-estrogenic and anti-breast cancer activity. To determine the pharmacophore of the peptide, a series of analogs was prepared using solid-phase peptide synthesis. Analogs were screened in a 1-day bioassay, which assessed their ability to inhibit the estrogen-stimulated growth of uterus in immature mice. Deletion of glutamic acid, Glu1, abolished activity of the peptide, but glutamine (Gln) or asparagine (Asn) could be substituted for Glu1 without loss of activity. Methionine (Met2) was replaced with lysine (Lys) or tyrosine (Tyr) with retention of activity. Substitution of Lys for Met2 in the cyclic molecule resulted in a compound with activity comparable with the Met2-containing cyclic molecule, but with a greater than twofold increase in purity and corresponding increase in yield. This Lys analog demonstrated anti-breast cancer activity equivalent to that of the original Met-containing peptide. Therefore, Met2 is not essential for biologic activity and substitution of Lys is synthetically advantageous. Threonine (Thr3) is a nonessential site, and can be substituted with serine (Ser), valine (Val), or alanine (Ala) without significant loss of activity. Hydroxyproline (Hyp), substituted in place of the naturally occurring prolines (Pro4, Pro7), allowed retention of activity and increased stability of the peptide during storage. Replacement of the first Pro (Pro4) with Ser maintains the activity of the peptide, but substitution of Ser for the second Pro (Pro7) abolishes the activity of the peptide. This suggests that the imino acid at residue 7 is important for conformation of the peptide, and the backbone atoms are part of the pharmacophore, but Pro4 is not essential.

Valine (Val5) can be substituted only with branched-chain amino acids (isoleucine, leucine or Thr); replacement by D-valine or Ala resulted in loss of biologic activity. Thus, for this site, the bulky branched side chain is essential. Asparagine (Asn6) is essential for activity. Substitution with Gln or aspartic acid (Asp), resulted in reduction of biologic activity. Removal of glycine (Gly8) resulted in a loss of activity but nonconservative substitutions can be made at this site without a loss of activity indicating that it is not part of the pharmacophore. Cyclization of the peptide is facilitated by addition of Gln9, but this residue does not occur in AFP nor is it necessary for activity. Gln9 can be replaced with Asn, resulting in a molecule with similar activity. These data indicate that the pharmacophore of the peptide includes side chains of Val5 and Asn6 and backbone atoms contributed by Thr3, Val5, Asn6, Hyp7 and Gly8. Met2 and Gln9 can be modified or replaced. Glu1 can be replaced with charged amino acids, but is not likely to be part of the binding site of the peptide. The results of this study provide information that will be helpful in the rational modification of *cyclo*[EMTOVNOGQ] to yield peptide analogs and peptidomimetics with advantages in synthesis, pharmacologic properties, and biologic activity.

Abbreviations: AFP, α-fetoprotein; E_2, 17β-estradiol; Fmoc, 9-fluorenylmethyloxycarbonyl; HATU, 1,1,3,3-tetramethyluronium hexafluorophosphate; HPLC, high-pressure liquid chromatography.

Introduction

Small molecule analogs of proteins, intended for pharmaceutical or other uses, are usually intended to mimic a binding site or active site of the protein, and to provide some or all of the function of the parent molecule. After identifying molecules small enough to be synthetically economical and yet large enough to contain information adequate to provide the desired activity, it is often necessary to optimize the newly developed small molecule in terms of several additional parameters, perhaps including conformational flexibility, potency, storage stability or stability in vivo, or even other parameters that arise during the development process, such as targeting to one of several available receptors. Simultaneous optimization of several of these parameters might be achieved through library screening or combinatorial synthesis approaches. Alternatively, and perhaps especially for situations in which unexpected observations contribute importantly to the developmental process, rational design approaches offer unique opportunities to optimize the structure and function of the small molecule protein analog. Furthermore, explorations of the structure–activity relationships during rational or iterative design and synthesis activities would be expected to contribute to the understanding of the biology and mechanism by which the novel analogs function.

A peptide that inhibits the growth of estrogen receptor positive (ER+) human breast cancers, growing as xenografts in immune-deficient, mice has been synthesized (1). *cyclo*[EMTOVNOGQ] was derived from α-fetoprotein (AFP), a safe, naturally occurring human protein produced during pregnancy, which itself has anti-estrogenic and anti-breast cancer activity (2). The peptide was identified by systematically parsing the AFP molecule until it was demonstrated that the oncostatic activity was localized to an eight amino acid sequence (amino acids 472–479) in domain IIIB of the AFP molecule (1,3,4). Mesfin *et al.* synthesized the linear form of the peptide, EMTPVNPG, and showed that it was the minimal sequence necessary to maintain oncostatic activity against breast cancer cells growing as a monolayer in culture or as a xenograft in immune-deficient mice (4). Substitution of hydroxyproline (Hyp, O) for both prolines resulted in a molecule that showed comparable activity and increased storage stability (1). Head-to-tail cyclization of the molecule to yield *cyclo*[EMTOVNOGQ] was facilitated by addition of Gln to the C-terminus of the octapeptide, and it was demonstrated that this cyclic molecule had an active dose range broader than the linear form of the molecule, and peptide stability and shelf life were not compromised (1). The use of a 'head-to-tail' peptide bond, rather than the more usual disulfide bond, may be advantageous for purposes of shelf life, retention of structure and activity in vitro, and elimination of dimers, trimers, and higher-order aggregates that can sometimes develop when disulfide-bonded peptides are stored or used in conditions that do not carefully control the redox state. The method of cyclization that was used was developed by Kates *et al.* (5,6) and is straightforward and expedient.

Although the anti-breast cancer activity of the linear and cyclic AFP-derived peptides is well-documented, the pharmacophore of the peptide has not been elucidated. Consequently, we designed and synthesized a number of analogs of the linear and cyclized peptides to ascertain which atoms are crucial for activity. Because of the relatively few amino acids to be studied and the availability of interspecies homology data, a rational, rather than a combinatorial, approach to substitution was utilized. Conservative and nonconservative substitutions were made for each amino acid as appropriate. Biologic activity data from the analogs were used to determine the pharmacophore and auxiliary portions of the molecule. Simultaneously, comparisons of synthetic outcomes yielded information that led to improved approaches for synthesizing these peptides. Peptides with key substitutions were produced with high purity, synthetic yield, and biologic activity. The lead analog, *cyclo*[EKTOVNOGN], was demonstrated to inhibit the estrogen-stimulated growth of human breast cancer cells growing as xenografts in immune-deficient mice.

Experimental Procedures

Materials

9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids were obtained from Calbiochem-Novabiochem (San Diego, CA, USA). The amino acids used for synthesis included Fmoc-Glu(OtBu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Hyp(tBu)-OH, Fmoc-L-Val-OH, Fmoc-D-Val-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gly-OH, Fmoc-Met-OH, Fmoc-Ala-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Tyr(tBu)-OH, and the carboxyl-protected amino acids Fmoc-Glu(OAll), and Fmoc-Asp(OAll). Reagents for peptide synthesis including 1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), N-hydroxybenzotriazole (HOBt), Fmoc-PAL-PEG-PS resin, 20% piperidine in dimethylformamide (DMF), and diisopropylethylamine (DIPEA) were obtained from PerSeptive Biosystems, Inc. (Framingham, MA, USA). Trifluoroacetic acid (TFA), 2-propanol, anisole, ethane dithiol, tetrakis triphenylphosphine paladium(0) [$Pd(PPh_3)$], 4-methylmorpholine, DMF, and 17β-estradiol (E_2) were purchased from Sigma-Aldrich (St Louis, MO, USA). Diethyl ether, ethylacetate, acetic acid, chloroform, and sodium tetra ethylenediaminetetraacetic acid (EDTA) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Dulbecco's modified eagle medium (DMEM), crystalline bovine insulin, MEM nonessential amino acids, penicillin/streptomycin, L-glutamine, and trypsin were obtained from GIBCO/BRL (Gaithersburg, MD, USA) and Cosmic Calf serum was purchased from Hyclone (Logan, UT, USA).

Peptide synthesis

Peptides were prepared using Fmoc solid-phase synthesis as previously described (1,4). A Pioneer Peptide Synthesis system (PerSeptive Biosystems, Inc.) was used to assemble the growing peptide chain on Fmoc-PAL-PEG-PS resin beginning with the C-terminus using N^{α}-protected amino acids. Activation of the C-terminus of incoming amino acids was accomplished by treatment with HATU and DIPEA. Following synthesis, linear peptide was washed with isopropanol and cleaved from the resin by incubation for approximately 4 h in 9.5 mL of TFA : anisole : EDT (90 : 2 : 3) per 0.1 mmol of peptide (0.5 g resin) and side-chain protective groups were removed concurrently. Peptide was precipitated by addition of cold (−20 °C) diethyl ether and washed by repeated extraction with diethyl ether and then with ethylacetate/diethyl ether (1.5 : 1). The peptide was dissolved in a volume of deionized water to achieve a concentration of 5–10 mg/mL and lyophilized.

Cyclization of peptides

Cyclization of peptides was accomplished using the method of Kates *et al.* (5,6). An additional residue, Fmoc-Glu(OAll) or Fmoc-Asp(OAll), was included at the C-terminus during synthesis to facilitate this process. This amino acid was coupled to the resin through the γ-carboxylic acid leaving the α-carboxyl protected with the allyl group. N^{α}-Fmoc deprotection allowed for sequential coupling of the remaining amino acids to the N-terminus. Following synthesis, cyclization of the peptide occurred through a three-step process. First, removal of allyl group from the α-carbonyl of Glu9 was initiated by treatment with 15 mL of a catalyst mixture containing 3 equivalents of $Pd(PPh_3)$ in chloroform/acetic acid/4-methylmorpholine (37 : 2 : 1) per gram of resin for 2 h in the dark. The resin was washed three times with 15 mL of DMF. Secondly, removal of the base-labile N^{α}-Fmoc was accomplished by treatment with piperidine (20% in DMF) followed by washing three times with 15 mL DMF. Finally, activation of the newly deprotected α-carboxyl of Glu9 by overnight treatment of the peptide-resin with HATU : HOBt : DIPEA (3 : 3 : 10 equivalents) in about 8 mL DMF per gram of resin, resulted in the formation of an amide bond between Glu9 and the deprotected-terminus. The peptide resin was washed three times with 15 mL of DMF. After each of the three steps, the peptide resin was evaluated for the presence of free amine groups using the Kaiser test (7). Subsequent cleavage of the cyclized peptide from the resin using the method outlined above resulted in the formation of γ-carboxyamido-derivative of Glu9 or Asp9, that is, Gln9 or Asn9, respectively.

Purification of peptides

Linear peptides were purified using a reverse-phase C_{18} Sep-Pak cartridge (Waters, Milford, MA, USA). Briefly, a sample containing peptide of unknown purity was loaded onto a pre-washed cartridge and the sample was sequentially eluted with water, 10, 30, and 60% acetonitrile in water. The fraction containing peptide was then lyophilized. Purity of cyclic peptides was determined after separation of the main component from impurities on a Waters gradient semi-preparative reversed-phase liquid chromatographic system. The fraction containing the main peak was collected and lyophilized. Peptides used for structure–activity relationship analysis were purified prior to use in biologic assays, and those used in biologic assays were at 95% or greater purity. Peptides were evaluated by amino acid analysis and mass spectrometry.

Immature mouse uterine growth assay

The anti-estrotrophic activity of peptide was determined using the immature mouse uterine growth assay as described by Bennett *et al.* (2,3). Administration of 0.5 μg of 17β-estradiol (E_2) i.p. to each mouse has been demonstrated to double the uterine weight in 24 h with a corresponding increase in mitotic figures (2,3,8). Swiss/Webster female mice (13–15-day-old, 6–8 g body weight; Taconic Farms, Germantown, NY, USA) were weighed and distributed into treatment groups typically of five mice each such that groups contained mice of comparable weight ranges. Each group received two sequential i.p. injections spaced 1 h apart. The first injection contained test substance or vehicle control. A dose of 1 μg peptide per mouse was used; this is the dose which has been demonstrated to give the maximal inhibitory response for the linear and cyclic hydroxyproline-containing peptides. The second injection contained 0.5 μg E_2 or vehicle. Twenty-two hours after the second injection, mice were weighed and uteri dissected, trimmed free of mesenteries, and immediately weighed. Uterine weights were normalized to body weight (mg uterine per g of body weight) to compensate for differences between body weights of littermates. The inhibition of estrogen-stimulated uterine growth was calculated from the average values for each group using the following equation:

$$\begin{aligned}\%\ \text{Growth inhibition} &= 100\\ &\times (\text{Full } E_2 \text{ stimulation} - E_2 \text{ stimulation in test group})\\ &/(\text{Full } E_2 \text{ stimulation} - \text{No } E_2 \text{ stimulation})\end{aligned}$$

Human breast cancer xenograft assay

An in vivo assay for anti-breast cancer activity was performed using the methodology of Bennett *et al.* (2,9). Confluent MCF-7 human breast cancer cells growing in monolayer in DMEM without phenol red supplemented with 5% bovine calf serum, 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 0.1 mM nonessential amino acids, and 10 ng/mL bovine insulin were released from the flask using trypsin/EDTA (0.25%/0.25%) and 20 million cells were pelleted by centrifugation at 200 ***g*** and then solidified into a fibrin clot by treatment with 10 μL fibrinogen (50 mg/mL) and 10 μL thrombin (50 units/mL). The solid tumor mass was then cut into pieces of approximately 1.5 mm diameter. Each tumor segment was implanted under the kidney capsule of an Institute for Cancer Research (ICR)-severe combined immunodeficient (SCID) male mouse (Taconic Farms) which weighed about 25 g. Estrogen supplementation was accomplished by s.c. placement of a 2 mm silastic tubing implant containing solid E_2 inserted on the day of tumor implantation. Peptide was injected i.p. once daily at a dose of 10 μg/mouse. This is the dose that has been demonstrated to give the maximal inhibitory response for the cyclic hydroxyproline-containing peptide in this assay. Tumor growth was monitored during survival laparotomy at 14 and 20 days after implantation by measurement of the short (d) and long (D) axes of the tumor by using a dissecting microscope equipped with an ocular micrometer. Tumor volumes were calculated assuming the tumor shape to be an ellipsoid of revolution about the long axis (D) using the formula $(\pi/6)d^2D$. Mean tumor volume ± SE was calculated for display in growth curves. All animal care procedures were approved by the Albany Medical College Animal Care and Use Committee.

Homology data

Sequence information was obtained by comparison of the homologous region of AFP for the six species studied using sequence data published in the protein data base of the National Center for Biotechnology Information provided by the National Library of Medicine.

Statistics

Significance of results for the immature mouse uterine growth assay was determined by Dunnett's multivariate comparison using the 8-amino acid, hydroxyproline-containing linear molecule as the control. There were five to 10 replicates per group except for the Met2Lys and control peptide (8-mer Hyp) where there were 28 and 332 replicates, respectively. Results were considered significant at $P < 0.05$. Error is shown as ±standard error of measurement. Significance of differences between groups for the human breast cancer xenograft assay was tested using the one-sided Wilcoxon sum of ranks test. There were five to six mice in each treatment group.

Results

Part I: Identification of the active site of the peptide

To determine which residues were essential to the bioactivity of the 8-amino acid, linear peptide (sequence Glu1-Met2-Thr3-Pro4-Val5-Asn6-Pro7-Gly8), a series of linear analogs was prepared and evaluated using the immature mouse uterine growth assay. It has been documented that there is excellent correlation between the uterine growth assay and the human breast cancer xenograft assay with regard to peptide inhibition of estrogen-stimulated growth (1,3). The uterine growth inhibition assay provided the advantages of faster assay time (1 day vs. 20 days) and substantially lower cost. Hence the xenograft assay was used only for the lead analog. Modifications of peptides included conservative substitutions for each of the amino acids, deletions of the N- and C-termini, and for some amino acids, nonconservative substitutions as well.

Glu1

Replacing the highly conserved Glu1 with Gln or Asn maintained the inhibitory activity of the linear peptide (Fig. 1). Substitution of this residue with Lys or Asp reduced the activity of the molecule, but did not abolish it. However, the nonconservative substitution of Glu1 with Ser resulted in an almost complete loss of the inhibitory activity of the peptide. Deletion of Glu1 ablated the inhibitory activity of the molecule.

Met2

Met2 is not highly conserved across species in AFP (Table 1) and can be replaced with Thr (chimpanzee), Glu (horse), or Ala (mouse, rat). A linear octapeptide was synthesized with a substitution of Lys for Met2. The peptide retained full inhibitory activity (Table 2). Substitution of Tyr for Met2 also maintained the inhibitory activity.

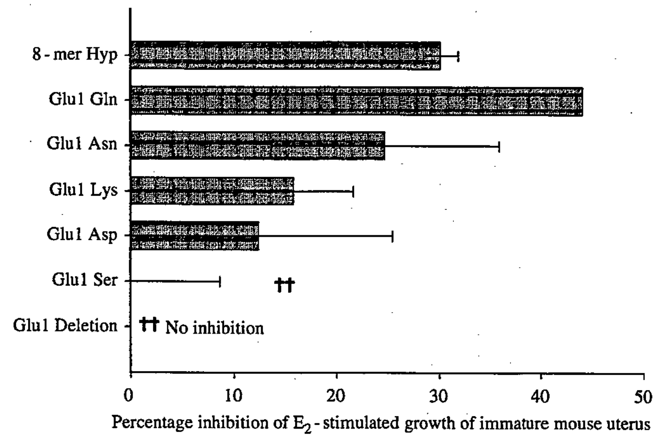

Figure 1. Anti-estrotrophic effect of substitutions for glutamic acid. Glu1 was substituted as shown in 8-amino acid, linear AFP-derived peptide containing substitutions of Hyp (O) for both Pro, EMTOVNOG (8-mer Hyp). Inhibition was measured using the immature mouse uterine growth assay. Peptide at 1 μg per mouse or vehicle control was injected i.p. into immature Swiss mice. One hour later, the mice were injected i.p. with 0.5 μg E_2 or vehicle control. Twenty-two hours after the second injection, mice were killed, the uteri dissected and trimmed free of mesenteries, and weighed. Percent inhibition is calculated as described in Experimental Procedures. 8-mer Hyp showed inhibition of growth of approximately 33% in this assay. $^{††}P < 0.005$ compared with 8-mer Hyp using Dunnett's multivariate comparison.

***Table 1.* Comparison of sequence of AFP and albumin from different species**

Residue number	1	2	3	4	5	6	7	8
Human	E	M	T	*P*	*V*	***N***	P	G
Gorilla	E	M	T	*P*	*V*	***N***	P	G
Chimpanzee	E	T	T	*P*	*V*	***N***	P	G
Horse	E	E	S	*P*	*I*	***N***	P	G
Rat	E	A	N	*P*	*V*	***N***	S	G
Mouse	E	A	S	*P*	*V*	***N***	S	G
Albumin (human)	E	K	T	*P*	*V*	***S***	D	R

Amino acids that are in bold italic are strictly conserved between species and those in italics are semi-conserved. Residues that are in bold are not conserved in AFP across those species studied.

Thr3

Analogs of the peptide with Thr3Val and Thr3Ala substitutions in the 8-mer peptide were prepared and tested (Table 2). Both of these analogs retained the inhibitory activity of the parent molecule. Substitution of Ser for Thr3 resulted in some reduction in inhibitory activity that was not statistically significant.

Pro4,7

The conservation of Pro4 across species (Table 1) suggested that this residue was required for the biologic activity of the

Table 2. **Anti-estrotrophic effect of nonconservative substitutions in AFP-derived linear peptide**

Nonconservative substitutions: analogs of linear peptides		
Peptide/substitution	Sequence	Relative activity
8-mer Hyp	EMTOVNOG	++
Met2	EKTOVNOG	++
	EYTOVNOG	++
Thr3	EMVOVNOG	++
	EMAOVNOG	++
	EMSOVNOG	+
G8-deleted	EMTPVNP	+
Horse	EESPINPG	+
Horse P40,P70	EESOINOG	++
Albumin peptide	EKTPVSDR	–

Percent inhibition was measured using the immature mouse uterine growth assay. Amino acid substitutions of the 8-amino acid, linear peptide containing either Pro or Hyp are shown in bold. ++, 25–40% inhibition; +, 12–25% inhibition; –, less than 5% inhibition.

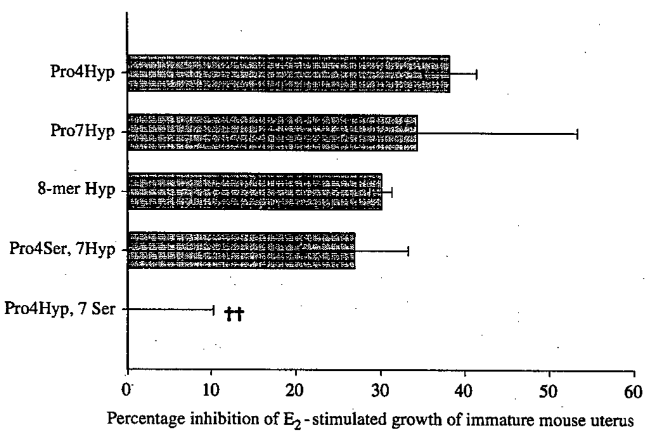

Figure 2. Anti-estrotrophic effect of substitutions for proline in linear AFP-derived peptide. Inhibition was measured using the immature mouse uterine growth assay. ††$P < 0.005$ compared with 8-mer Hyp using Dunnett's multivariate comparison.

peptide; Pro7 is less strictly conserved. As shown in Fig. 2, analogs containing substitutions of hydroxyproline (Hyp) for either Pro4 or Pro7 resulted in retention of inhibitory activity. Similarly, an analog with Hyp substituted for both Pro4 and Pro7 retained inhibition. An analog containing the nonconservative substitution of Ser for Pro4 maintained inhibitory activity. However, substitution of Ser for Pro7 resulted in a complete loss of biologic activity (Fig. 2) although Ser replaces Pro7 in the AFP of rodents (Table 1).

Val5

Conservative substitutions for Val5 included Ala, Leu, and Ile, each of which has hydrophobic character. Substitution

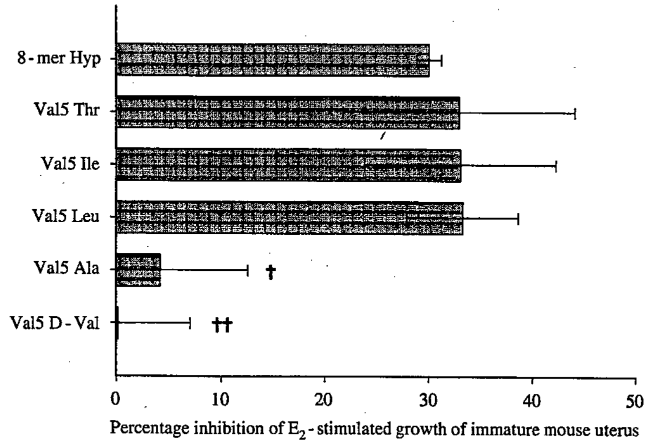

Figure 3. Anti-estrotrophic effect of substitutions for valine in linear AFP-derived peptide. Inhibition was measured using the immature mouse uterine growth assay. †$P < 0.01$; ††$P < 0.005$.

of Val5 with Leu, Ile, or Thr maintained biologic activity (Fig. 3). However, replacement of Val5 with Ala resulted in a nearly complete loss of inhibitory activity, and replacement of Val5 with D-Val completely abrogated the activity of the analog.

Asn6

Peptide analogs were synthesized with conservative substitutions for Asn6 and analyzed for inhibitory activity using the immature mouse uterine growth assay. Replacement of Asn6 with Gln resulted in a significant loss of inhibitory activity (Fig. 4). Substitution with the carboxylic acid derivative of this residue, Asp, also resulted in a reduction in activity.

Gly8

Glycine has been noted as essential for the linear molecule because deletion resulted in a loss of inhibitory activity (4).

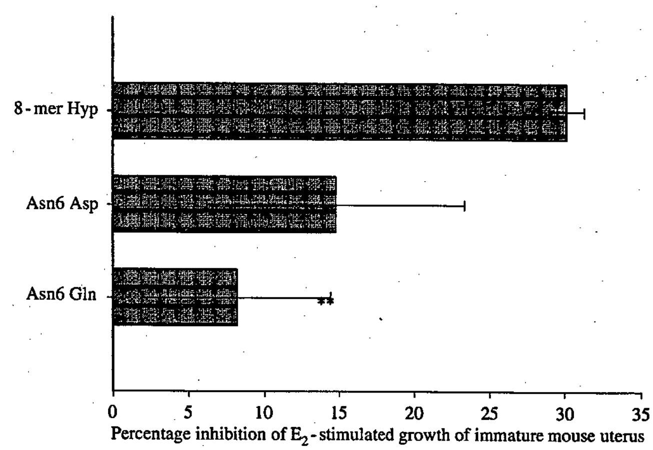

Figure 4. Anti-estrotrophic effect of substitutions for asparagine in linear AFP-derived peptide. Inhibition was measured using the immature mouse uterine growth assay. **$P < 0.05$.

This is consistent with its conservation across species. However, replacing Gly8 with Asn resulted in a peptide that retained full activity (data not shown).

Albumin peptide

AFP is a member of the albumin family of proteins and has a 39% homology of primary structure with albumin (10). This similarity is greatest in the third domain where the AFP-derived peptides were identified. A synthetic peptide with the sequence of human albumin, EKTPVSDR (Table 1), was prepared and analyzed using the immature mouse uterine growth assay (Table 2). This molecule differs from the AFP-derived linear peptide by substitutions of Lys for Met2, Ser for Asn6, Asp for Pro7, and Arg for Gly8. The synthetic albumin peptide had no inhibitory activity.

Part II: Comparison of activity, yield, and purity of cyclic analogs of peptide

Mesfin *et al.* (1) had shown previously that the linear peptide could be cyclized and that the resultant molecule, *cyclo*[EMTOVNOGQ], was anti-estrotrophic and that cyclization of the molecule broadened the effective dose range. Cyclization of the molecule was facilitated by addition of a Gln to the C-terminus of the molecule, and, in the linear form, this addition did not alter biologic activity (1). As the data in Table 2 indicated that Lys could be substituted for Met2 without loss of biologic activity, we added either Gln or Asn to the C-terminus of the Met2Lys analog and cyclized it. These analogs had inhibitory activity comparable with that of the cyclized molecules containing methionine. However, when the purity of the Lys-containing molecules was evaluated by HPLC, the chromatograms showed the presence of predominantly one peak. The cyclic Met-containing peptides chromatographed as multiple peaks and required further purification by semi-preparative HPLC. This additional step in processing of the peptides resulted in a significant loss of material. The cyclic peptide containing the Met2Lys substitution increased the purity of the preparation 2.4-fold from 39 to 94% for the Gln-containing analog and fourfold from 24 to 95% for the molecule cyclized using Asn (Table 3). As a consequence, the yield of the peptide was increased compared with the cyclic molecule containing Met.

The cyclized peptide containing the Met2Lys substitution was evaluated for its ability to inhibit the growth of ER+ human breast cancer cells growing as xenografts under the kidney capsule in immune-deficient mice. As shown in Fig. 5, growth of MCF-7 tumors was dependent on estrogen for growth: the presence of E_2 resulted in a threefold increase in tumor volume after 20 days in comparison with the control without E_2. Administration of 10 μg *cyclo*[EKTOVNOGN] daily to animals concurrently treated with E_2 prevented tumor growth over this same period.

Table 3. **Activity, purity, and yield of cyclic analogs of AFP-derived peptide**

Analog name	Activity (% inhibition)	Purity (%)	Yield (%)
cyclo[EMTOVNOGQ]	29	39	56
cyclo[EMTOVNOGN]	23	24	62
cyclo[EKTOVNOGQ]	24	94	67
cyclo[EKTOVNOGN]	32	95	72

Cyclization of the 9-amino acid linear peptides was performed as described in Experimental Procedures. Biologic activity was measured in the immature mouse uterine growth assay after final purification of the peptides. Purity was determined as area percent by HPLC prior to purification. Yield was calculated as amount of peptide recovered after lyophilization without further purification by HPLC.

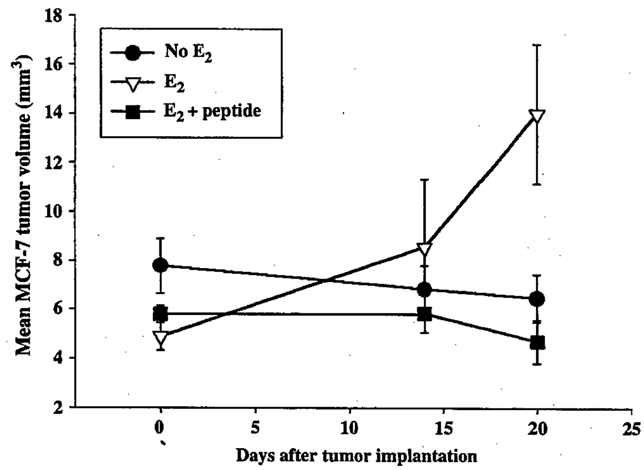

Figure 5. Anti-estrotrophic activity of *cyclo*[EKTOVNOGN] against MCF-7 human breast cancer xenografts. There were five to six mice per treatment group. The assay is described in Experimental Procedures. E_2 was provided by implantation of a silastic tube containing solid E_2 inserted subcutaneously on day 0. Peptide was given once daily at 10 μg/mouse. Peptide significantly inhibited the estrogen-dependent growth of the tumor. $^{**}P < 0.05$.

Discussion

Rationale for investigation of the pharmacophore of AFP-derived peptides

It had been demonstrated previously that both the linear and cyclic AFP-derived peptides were capable of inhibiting the growth of estrogen-dependent breast cancers growing as xenografts in immune-deficient mice (1,3). Furthermore, it had been shown that these peptides inhibit ER+ tumors that had been made resistant to tamoxifen (3). Although the mechanism of the oncostatic action of the peptides has not been elucidated, it is known that, unlike tamoxifen or other selective estrogen receptor modulators, the peptides do not compete with estrogen for the ligand-binding domain of the estrogen receptor (3). Rather, it is likely that the peptides mediate their activity by interacting with a cell surface-binding protein, as does their parent molecule AFP, although this receptor has not been fully characterized (11–15). As the receptor for the peptides is unknown, we chose to evaluate the structure–activity relationship of the peptides employing rational design techniques to determine the key amino acids required for maximal biologic activity. Two important biologic endpoints, namely inhibition of estrogen-stimulated growth of uterus and of breast cancer were employed as measures of biologic activity. A combinatorial approach to analog development was not chosen because: (a) comparative sequence data for the oncostatic region of AFP were available for six mammalian species (Table 1), suggesting which residues were essential for activity based on conservation of those residues. Although the oncostatic potential of peptides derived from the AFPs of each of the six species shown in Table 1 was not tested, comparison of the sequences of these peptides served to guide our choices for individual amino acid substitutions when designing analogs; (b) relatively few amino acids (eight) comprise the minimal sequence necessary for anti-estrotrophic activity in AFP-derived peptides; and (c) data generated while determining the minimal sequence and optimizing the stability of the peptides (1,4) suggested key residues that were required for activity or stability in storage and other residues which could be replaced without loss of biologic activity. The unexpected observation that cyclic molecules provided a wider dose–response curve (1) suggested the importance of maintaining the residues necessary for the cyclization process. Thus, the rational approach seemed more appropriate for these studies.

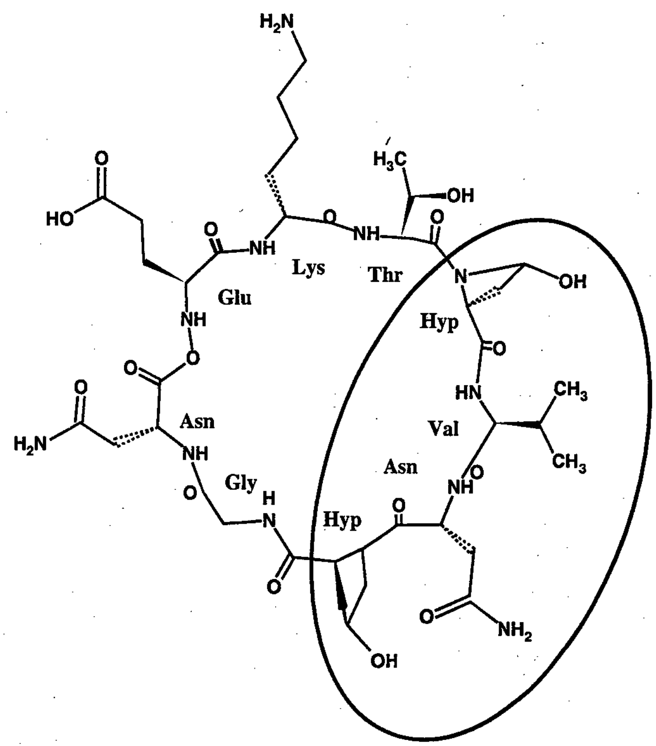

Figure 6. Sequence and structure of *cyclo*[EKTOVNOGN]. Structure of the cyclic nonapeptide. Amino acids are labeled adjacent to their side chains and the pharmacophore is circled.

Investigation of the pharmacophore

Cyclization of the peptide as previously described (1) constrains the molecule, causing the peptide to exist largely as a planar macrocycle (Fig. 6). The region of the cyclic molecule near the ring-closing peptide bond (the Gln to Glu link) is probably not the pharmacophore, as that peptide bond is artificial and does not exist in the parent protein. As such, it is logical to assume that the portion of the molecule diametrically across from that Gln to Glu peptide bond, that is, the 'middle' of the linear peptide, ought to be the pharmacophore. As a first approximation, the region may be postulated to consist of the amino acids between the two proline residues (Fig. 6).

In the six species evaluated, Val5 is found at this site for all species except equine where the highly conserved Ile is found in place of Val5 (Table 1). Consistent with the presence of Ile in equine AFP, synthetic analogs with Val5 substitutions, which retained the hydrophobic and branched character, such as Ile and Leu, retained biologic activity. However, replacement of Val5 with the smaller, nonbranched Ala resulted in a significant loss of inhibitory activity, suggesting that the branched structure of the side chain at this site was required to maintain the activity of the peptide. To test this hypothesis, Thr was substituted for Val5, a substitution that conserves the branched nature, but is more hydrophilic than Val. This analog retained anti-estrotrophic activity suggesting that the branched nature of the residue at this position is necessary for maximal activity. Interestingly, substitution of Val5 with its enantiomer, D-Val, completely ablated the activity and suggested that the specific orientation of the Val side chain or of the backbone participates in the binding of the pharmacophore to its target.

Asn6 is strictly conserved across those species evaluated and even conservative substitutions of Asp or Gln for Asn6 resulted in a loss of inhibitory activity. This strongly suggests that Asn6 is an essential residue of the active site of this molecule, which correlates with what was expected based on the interspecies homology.

As shown in Fig. 2, Hyp can be substituted for either or both proline residues without a loss of biologic activity and it has been demonstrated that Hyp increases the storage stability of the AFP-derived peptides (1). An interspecies comparison of Pro4 and Pro7 indicates that Pro4 is highly conserved, while Pro7 is substituted by Ser in rodents. To examine the requirement for proline at these sites, analogs were prepared which replaced Pro4 or Pro7 with Ser. The inhibitory activity of the molecule was retained when Pro4 was substituted, but completely abolished when Ser replaced Pro7. Thus, the imino acid at residue 7 is important for conformation of the peptide, and the backbone atoms at this site are part of the pharmacophore. The substitution of Ser for Pro4 suggests that Pro4 is not essential.

The pharmacophore of the peptide seems to lie between the two proline residues and includes the side chains of valine and asparagine, and the backbone atoms contributed by valine, asparagine, and Pro7. This region is highly conserved among mammalian species and conservative substitutions in this area led to diminution or loss of biologic activity.

Amino acids distal to the pharmacophore contribute to the conformational stability of the peptide

Glu1 is highly retained across species and this initially suggested that it may be important to the activity of the molecule. Conservative substitutions in the linear, 8-amino acid peptide of Gln or Asn showed no significant loss of activity (Fig. 1). Replacing this residue with Lys, which retains a charge at this site, or the highly conservative substitution of Asp showed some loss of activity, but it was not significant. However, replacing Glu1 with Ser causes a loss of biologic activity and deletion of Glu1 completely ablated the inhibitory activity of the molecule. As substitutions in the linear molecule, which affect both the charge at this site and the size of the side chain resulted in analogs, which retained their biologic activity, it is unlikely that Glu1 is part of the binding site of this peptide but rather it more likely plays a role in maintaining the conformational stability of the molecule.

Replacing Thr3 with Val, which is more hydrophobic but retains the branched nature of the side chain, maintained the inhibitory activity of the linear molecule (Table 2). The nonconservative substitution of Ala also maintained this activity. Although there was some loss of activity when Ser is substituted, this loss was not significant (data not shown), and considered with the nonconservative Val and Ala substitutions, it can be concluded that the side chain of this residue is not required for biologic activity.

The highly conserved nature of the Gly8 residue suggested that this residue may be required (Table 1). Deletion of Gly8 resulted in a reduction in the biologic activity of the molecule, but did not abolish it (Table 2). Furthermore, the nonconservative substitution of Asn for Gly8 resulted in a molecule that retained full activity. This indicates that Gly8 is not part of the active site of this peptide.

Met2 shows no interspecies homology for the six species evaluated (Table 1) and synthetic analogs in which Met2 was replaced by either tyrosine or lysine in the linear peptide showed no loss of biologic activity. Taken together, the data indicate that Glu1, Met2, Thr3, and Gly8 are not part of the pharmacophore, but it is likely that they contribute to the conformational stability of the molecule because they can be nonconservatively substituted without significant loss of activity. They probably function to maintain the linear molecule in a conformation that is conducive to binding to its receptor. The energy-minimized structure of the 8-amino acid Met-containing peptide has been previously published (4) and this molecule shows the potential to form a horseshoe shaped structure.

The substitution of Lys for Met2 was especially advantageous. Molecular modeling (not shown) indicated that the 8-amino acid molecule containing this substitution maintained the horseshoe structure seen previously with the methionine-containing molecule (4). Cyclization of the linear peptide containing the Met2Lys substitution (Fig. 6) produced a peptide that was far superior in synthetic outcomes with increased purity (Table 3). The optimized analog, *cyclo*[EKTOVNOGN] not only had anti-uterotrophic activity (Table 3) but also had oncostatic activity in that it inhibited the estrogen-stimulated growth of human breast cancer cells growing as xenografts in immune-deficient mice in a dose dependent manner consistent with that of *cyclo*[EMTOVNOGQ] (Fig. 5) (1).

The cyclic peptide is derived from AFP which itself has anti-estrogenic and anti-breast cancer activity. Sonnenschein *et al.* (16) have demonstrated that the growth of an estrogen-dependent breast cancer is inhibited in rats bearing an AFP-secreting hepatoma. Tumor growth inhibition was not found when these investigators used an estrogen-independent breast cancer. Furthermore, Jacobson *et al.* (17) and Richardson *et al.* (18) have shown that elevated levels of AFP during pregnancy are associated with subsequent reduction in the lifetime risk for breast cancer. Jacobson and Janerich (19) have hypothesized that this reduction should be caused by a diminution in estrogen-dependent breast cancers. Bennett *et al.* (2,3) have shown that AFP purified from a human hepatoma culture and then injected into tumor-bearing immune-deficient mice stopped the growth of ER+, but not ER−, human breast cancer xenografts in these mice. Although AFP has demonstrated oncostatic activity (2), it has limited clinical usefulness. It has been shown to aggregate (20) and commercial preparations do not maintain biologic activity. The AFP used for the studies of Bennett *et al.* (2) utilized AFP isolated and purified from a hepatoma culture and the purification procedure is not trivial. Peptides produced using solid-phase Fmoc synthesis can be produced in high yield and purity in relatively large amounts, and the AFP-derived peptides have shown consistent activity over prolonged storage (1). Generation of a small, stable, biologically active peptide such as described here, and earlier (1,3,4), may suffice for investigations of the mechanism by which the peptide, and of AFP itself, stops the growth of tumor xenografts and exerts its anti-estrotrophic activity. Further, this peptide may be adequate for preclinical investigations as an oncostatic agent, especially if the peptide can be shown to have appropriate pharmacokinetics and low toxicity. At the very least, this cyclic peptide can serve as a model on which to develop peptidomimetics.

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