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Award Number: DAMD17-99-1-9104

TITLE: Design, Synthesis and Study of Cell Adhesion Antagonists: Hydroxamate-Based Peptide Inhibitors of avb3 Integrin

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REPORT DATE: July 2000

TYPE OF REPORT: Annual Summary

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

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2000	Annual Summary (1 Jul 99 - 30 Jun 00)

4. TITLE AND SUBTITLE
Design, Synthesis and Study of Cell Adhesion Antagonists: Hydroxamate-Based Peptide Inhibitors of avb3 Integrin

5. FUNDING NUMBERS
DAMD17-99-1-9104

6. AUTHOR(S)
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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)***

Progress report of fellowship recipient describing the synthesis and results of biological testing of a hydroxamate based RGD peptide. The primary cause of death from breast cancer is the progression to metastatic disease. Two biological events that are essential for metastasis are the invasion of tumors to secondary sites and the growth of new blood vessels into the metastatic tumors. Both processes require a cell adhesion receptor called the $\alpha_v\beta_3$ integrin. This receptor is expressed on metastatic breast cancer cells, where it has a role in mediating their migration to secondary sites. There is now great interest in applying small molecule antagonists of $\alpha_v\beta_3$ to prevent tumor progression and metastatic disease. This study is the preliminary test of a novel chemical approach toward the design of antagonists of the $\alpha_v\beta_3$ integrin. So far, the hydroxamic acid derivative of RGDV has been synthesized and tested in both *in vitro* and *in vivo* assays testing the ability of the unnatural peptide to block the binding of vitronectin to $\alpha_v\beta_3$. The hydroxamic acid derivative of RGD does show inhibitory activity but it is not as effective as the natural peptide.

14. SUBJECT TERMS
Breast Cancer, Ph.D. Fellowship, Integrin alpha-v beta-3, Hydroxamic Acid, RGD peptides

15. NUMBER OF PAGES
9

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Chris L. C — July 24, 2000

PI - Signature — Date

Table of Contents

Introduction

Cell contact with the extracellular matrix governs several key aspects in the progression of breast cancer which include two key steps in the progression of breast cancer, tumor invasion and angiogenesis. One adhesion receptor that has been strongly implicated in both processes is the $\alpha_v\beta_3$ integrin. This receptor mediates cell adhesion and migration on a number of extracellular matrix proteins, and is a well-established requirement for both metastasis and angiogenesis. $\alpha_v\beta_3$ binds to the arginine-glycine-aspartic acid (RGD) tripeptide motif in its natural ligands, and small synthetic peptides incorporating this motif interfere with both tumor-associated angiogenesis and tumor metastasis using *in vitro* model systems. There are still several obstacles to overcome if we are to effectively apply antagonists of $\alpha_v\beta_3$ to thwart tumor progression in humans. The objective of this study is to design and synthesize a novel and highly potent antagonist of $\alpha_v\beta_3$ by incorporating our knowledge of the integrin's ligand-binding pocket. A considerable body of evidence indicates $\alpha_v\beta_3$ binds to its ligands via a ternary complex with receptor-bound divalent metal ion (Mg^{2+} or Mn^{2+}). Therefore, I propose to synthesize RGD-like peptides in which the carboxyl group of the key aspartate has been changed to a hydroxamate. I hypothesize that the hydroxamate-based peptides will make a bidentate contact with receptor-bound metal ion, displacing a water molecule within the ion's coordination sphere.

Changes from Proposed Statement of Work.

The attempts to derivatize GRGDSP on solid phase were unsuccessful due to diketopiperazine formation so a less functionalized starting peptide was proposed and synthesized. The decision to derivatization RGDV was made and the synthesis was successful. Enough of the unnatural peptide was made for full characterization and biological testing.

The second change from the statement of work is the iodinated ligand used in the solid phase binding assays, both *in vivo* and *in vitro*. Instead of Fab-9, the optimized antibody, the natural ligand, vitronectin, was used instead.

Training.

I learned solid phase synthesis to make the RGDV peptide and the hydroxamic derivative of the RGDV. I used HPLC purification techniques to purify the peptides and compounds. MALDI-TOF, NMR, and IR were employed to characterize the peptides. I also used *in vitro* and *in vivo* solid phase binding assays to test the efficacy of my compounds in inhibition of $\alpha_v\beta_3$ binding to one of its natural ligands, vitronectin. In the *in vivo* assays, I tested how well my natural and unnatural peptides blocked MDA-MB-435 cancer cell line binding to vitronectin coated on 96-well plates. The skills of synthesis, purification and assaying are pertinent skills in order for me to perform further studies in the chemistry of breast cancer.

Ligand Binding and Inhibition Studies.

The ability of the unnatural hydroxamic acid derivative of the RGD peptide to antagonize the binding of $\alpha v\beta 3$ to vitronectin was compared to the ability of natural RGD peptides in purified-receptor binding assays. Radiolabelled vitronectin was used at concentrations of 1 nM and binding was contested with unlabelled peptide and peptide-derivatives. After a 3 hour incubation, unbound vitronectin was removed by washing and

bound vitronectin detected by gamma counting. Two different ion conditions are used in these assays, 1.0 mM Mg2+ and 0.2 mM Mg2+. 1.0 mM Ca2+ was also used but no inhibition by RGD or the hydroxamic acid was seen. The data from this analysis is highly reproducible and all data are expressed as the average of triplicate data points. All experiments were repeated at least five times in each ion.

For the cell-binding assays, MDA-MB-435 breast cancer cell line is used on a polypropylene 96-well plate coated overnight with vitronectin. The cells are added with no inhibitor, RGD inhibitor or a hydroxamic acid derivative inhibitor and allowed to adhere at 37°C for an hour before washing with PBS to remove cells that did not attach. Then lysis buffer is added to each well and the plate is again incubated for an hour at 37°C to open the cell membranes. 1 N NaOH is then added to stop the reaction and facilitate the development of color and the plate is then read at 405 nm on a microplate reader. These cell-adhesion experiments are done with 250 μM or 500 μM final concentrations of inhibitors.

Results.

Synthesis:

Both solid and solution phase synthesis of the peptides and peptide-based hydroxamic acid compounds were explored. Solid-phase peptide synthesis was ultimately used to synthesize RGDV, RGD(HA)V, GRGDSP, and GD(HA)RV. A combination of solid and solution phase synthesis was also explored, with the solution phase synthesis of the Fmoc-D(HA)-OH from Fmoc-D-OtBu and coupling of the unnatural amino acid to the rest of the peptide on solid phase. The method that ultimately led to the desired products was the "reverse coupling" method after selective deprotection of the side chain of aspartic acid. In the "reverse coupling" method, the activated carboxylic acid is on the resin, in this case the side chain of the aspartic acid, and the amine is in solution. In SPPS the peptide is synthesized from the N-terminus to the C-terminus, with excess activated carboxylic acid in solution and the free amine on the resin.

Assay data:

After synthesis of the hydroxamic acid compounds, the inhibitory activity of each is tested in the competition assay described above with iodinated vitronectin and the inhibitor. The inhibitory capacity of the hydroxamic acid derivative of RGD is less than that of the positive control of natural RGD peptides. The negative control, the scrambled RGD hydroxamic acid derivative, does not show any inhibitory activity suggesting that the hydroxamic acid still goes through the same association mechanism as the natural RGD peptide. The IC_{50} of the RGD compounds stayed at 100 nM in both Mg^{2+} and Mn^{2+}, however, the hydroxamic acid derivative seems to show a slight preference for binding in Mn^{2+}. The IC_{50} in Mn^{2+} is 3000 nM, about three times less than the 10,000 nM seen in Mg^{2+}.

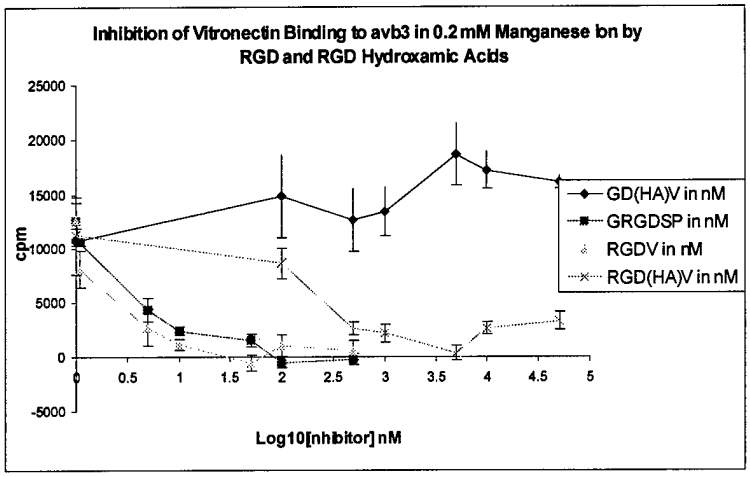

Figure 1 GRGDSP is an optimized natural RGD sequence against vitronectin binding to αvβ3. The hydroxamic acid derivative of RGD does inhibit the binding of vitronectin to αvβ3 but the scrambled hydroxamic acid does not, in the same manner that scrambled RGD peptides do not inhibit the interaction of vitronectin to integrins. This experiment was done in triplicate and the results shown is representative of the results from all experiments.

After the solid phase binding assay with purified proteins, a system of *in vivo* testing for the activity of the hydroxamic acids is used. In *in vivo* systems the hydroxamic acid derivative of RGD is also active in inhibiting the binding of a cell-line that over-expresses αvβ3, the MDA-MB-435 cells, but at a concentration that is one order of magnitude higher than the RGD peptides. The scrambled RGD hydroxamic acid shows slightly greater than total binding and the RGD hydroxamic acid shows approximately 40% of total binding. RGDV and GRGDSP show less than 20% of total binding that is consistent with the data seen before in the purified *in vitro* system.

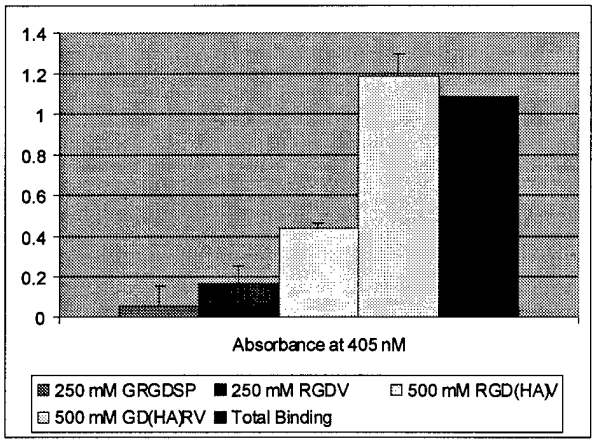

Figure 2 Cell binding assay with the MDA-MC-435 breast cancer cell line to vitronectin indicates the hydroxamic acid is also effective in an in vivo *assay. The positive controls of GRGDSP, RGDV inhibit cell binding to less than 20% of total binding and the RGD(HA)V to approximately 40%. The negative control of the scrambled RGD(HA) shows no inhibition of cell binding to vitronectin.*

Discussion

The data in Figure 4 show comparative inhibition between the RGD peptides and the hydroxamic acids in a purified receptor assay. GRGDSP is the best inhibitor of vitronectin binding to $\alpha_v\beta_3$ followed by

RGDV. These positive controls take the binding of iodinated vitronectin to $\alpha_v\beta_3$ to background at less than 100 nM. Inhibiting at a higher concentration than the natural RGD inhibitors is RGD(HA)V, showing that hydroxamic acid derived RGD do bind to $\alpha_v\beta_3$ but it does not bring the binding to background until 50,000 nM . The negative control, GD(HA)RV, does not show any inhibition of $\alpha_v\beta_3$ binding to vitronectin; the level of vitronectin binding remains at total binding, or binding of the two proteins with no inhibitors present. The data from the purified receptor assay prompted us to explore an *in vivo* assay with a cell line that over-expresses $\alpha_v\beta_3$; the cell line chosen was the MDA-MC-435 cell line.

The *in vivo* assay of the inhibition of breast cancer cell line MDA-MC-435 binding to vitronectin shows the in vivo data is consistent with the purified receptor assays of vitronectin binding to $\alpha_v\beta_3$. The most effective inhibitors are still the RGD peptides followed by the RGD derived hydroxamic acid inhibitor. The negative control hydroxamic acid, GD(HA)RV, shows it is not the presence of hydroxamic acid that causes the inhibition but is the combination of the amino acid sequence RG and the hydroxamate modified D that is responsible for the binding.

Conclusion

The hydroxamic acid derived RGD does not bind with stronger affinity to $\alpha_v\beta_3$ when compared to the natural RGD peptides. The inhibition is based on a combination of the amino acid sequence RGD and the hydroxamic acid side chain on D because GD(HA)RV, the scrambled RGD(HA) does not show inhibtion of vitronectin binding to $\alpha_v\beta_3$ in either the *in vitro* or the *in vivo* assay. The conclusion of this study is hydroxamic acids interact differently with the active site of $\alpha_v\beta_3$, perhaps in a bidentate fashion. Crystallographic studies of the interaction of RGD(HA)V with I-domains are underway and further structural information should become available in the near future.

Research Accomplishments

Design, Synthesis and Study of Cell Adhesion Antagonists: Hydroxamate-Based Peptide Inhibitors of the $\alpha_v\beta_3$ Integrin

Task 1. Synthesize RGD*(hydroxamate)V, Months 1-2

a. Use traditional SPPS to synthesize peptide with unnatural side-chain
b. Purify the RGD(hydroxamate)V with HPLC
c. Characterize the purified fractions by NMR and mass spectrometry

Task 2. Test RGD*(hydroxamate)V, Months 3-13

a. Use RGD*(hydroxamate)V in competitive solid phase binding assay with purified $\alpha_v\beta_3$ and iodinated vitronectin
b. Compare RGD*(hydroxamate)V solid phase binding assay data with GRGDSP and RGDV as inhibitor
c. Change ion conditions of binding assays and test for difference in affinity

Reportable Outcomes

1. A manuscript is being prepared for submission to a peer-reviewed journal with the findings stated in the summary.