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13. ABSTRACT *(Maximum 200 words)*

Metastatic cancer cells over-express characteristic variants of the hyaluronic acid (HA) receptors CD44 and RHAMM that mediate cell adhesion, cell motility, and cell proliferation. Rapid uptake and catabolism of HA is common in several breast cancer cell-lines. We have developed a targeted delivery system for controlled release of chemotherapeutic drugs, and we prepared HA-Taxol to test our hypothesis that HA-anti-tumor prodrugs will be more effective therapeutic agents than the unconjugated drugs. Fluorescent HA shows selective uptake, and HA-Taxol shows selective cytooxicity to breast, colon, and ovarian cancer cells in culture; it also shows release of free drug from the HA-prodrug form that is consistent with improved selectivity. Studies are in progress to (1) prepare an HA-brefeldin A prodrug, and (2) evaluate the HA-Taxol in nude mice bearing xenografted human tumors. Chondroitin sulfate will saturate HA uptake by non-target tissues. Animal data are being obtained to support use of radiolabeled HA and HA-Taxol in Phase I clinical trials in terminal diagnosis patients. A second line of research revealed the molecular basis of the RHAMM-HA interaction, and has provided unexpected molecular leads that disrupt the oncogenic transformation of RHAMM-overexpressing cells.

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FOREWORD

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PI - Signature 7/20/99 Date

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A. Introduction

In this project, we are developing an innovative approach to the use of a natural polysaccharide biopolymer, hyaluronic acid (HA), to solubilize, stabilize, and achieve targeted intracellular delivery of anti-cancer agents to tumors and metastases. The coupling of an anti-tumor agent to HA gives a soluble, tumor-targeted drug conjugate. HA receptors are over-expressed on a variety of aggressively growing cancers, and in many cell-types, correlate with increased metastatic potential and with rate of HA uptake and degradation. The use of HA receptor-mediated uptake of a cytotoxic agent to cancerous and invasive cells represents a significant innovation in cell-specific drug targeting, as all commonly-used anti-cancer drugs (e.g., anti-metabolites and DNA-targeted agents) and adjuvant techniques (e.g., radiation therapy) have inadequate specificity for cancerous lesions. Our efforts in the first year have focused on the hydrophobic anti-tumor drug Taxol. Our HA-Taxol adduct has the following properties: (**i**) increased water solubility of inherently hydrophobic drugs; (**ii**) increased stability and longer plateau phase for drug release; (**ii**) enhanced target specificity for transformed and metastatic cells; and (**iv**) increased uptake and liberation of drug in the target tumor cells. Furthermore, the bioconjugates are prepared under mild, aqueous conditions that preserve the molecular size range and structural integrity of HA, a water-soluble and immunoinert, biocompatible, and bioerodable carrier.

B. Body

This section describes research accomplishments associated with the specific tasks outlined in the original IDEA award application.

Task 1. Synthesize HA-anti-tumor drug bioconjugates with four anti-cancer drugs (brefeldin A, taxol, geldanamycin, and camptothecin)

We synthesized and chemically characterized hemisuccinate active ester forms of brefeldin A and taxol. Preparations of chemically-modified geldanamycin, and 9-aminocamptothecin were postponed pending results with the first two drugs. We also prepared hydrazide-functionalized, low molecular weight HA (HA-ADH) with low, medium, and high adipic hydrazide (ADH) loadings, and we employed each of these to make bioconjugates of HA-drug with two or three drug loadings. The purity and molecular size of drug bioconjugates was established by GPC and drug loading was evaluated by UV. (See appendix material for a preprint of this work.) In addition, we synthesized and purified fluorescently-labeled HA with three fluorophores at each of three molecular sizes (12 , 200, and 1,200 kDa), as well as a doubly-labeled HA-Taxol-FITC molecule for simultaneous monitoring of selective cellular uptake and toxicity.

Task 2. Establish analytical methods to monitor enzymatic release of drugs from bioconjugates and quantify metabolites in cell cultures

We conducted *in vitro* tests with commercial esterase and hyaluronidase (HAse) and developed a robust HPLC assay for the quantification of Taxol release. No other Taxol-containing materials (except free Taxol drug) were released from the HA-Taxol bioconjugates. We also investigated the release rates of Taxol from the optimal HA-Taxol preparation using cell culture media (with and without cells) and human serum.

Task 3. Determine efficacy of HA-AT (vs. free AT drugs) in cultured breast cancer and other cell-lines *in vitro*

We obtained and cultured a number of cell-lines: MDA-MB-231, MCF-7, and HBL-100 human breast epithelial cells; HL-60 human leukemia cells,; SK-OV-3 human ovarian cancer cells; HCT-116 human colon cancer cells, and NIH 3T3 mouse fibroblast cells. Optimal HA modification levels and drug loading were established. Extensive studies of dose-response and selective cytotoxicity were performed (see preprint in appendix material) with HA-Taxol, and uptake studies were performed with HA-BODIPY. The uptake of HA-Taxol or HA-BODIPY into cells was blocked by pre-incubation of cells with HA, but not with chondroitin sulfate. A similar result was obtained by flow cytometry using HA-Taxol-FITC.

Task 4. Measure efficacy of bioconjugates (vs. free drugs) using human breast cancer epithelial cell tumor xenografts in nude mice

Protocols to accomplish this task using our optimized HA-Taxol preparation are now being finalized for experiments to be conducted in September-October 1999.

Task 5. Initiate planning for Phase I safety testing of one or two optimal HA bioconjugates in terminal diagnosis human patients with drug- and radiation-refractory metastasis

Preparation of a single high-purity batch of HA-ADH labeled with ^{125}I will provide a preliminary test for safety and localization of HA in human patients pretreated (or not pretreated) with chondroitin sulfate (i.v. or oral). During the next six months, we will be preparing application materials for permission to conduct human trials. In parallel, we will be conducting safety and toxicity trials with rodents and larger animals. Scale-up sterile synthesis of selected HA bioconjugates for human trials would occur following these initial safety trials.

C. Key Research Accomplishments

This section provides a bulleted list of key accomplishments, both those within the initial objectives and tasks and those that arose as promising leads during the pursuit of the originally outlined tasks.

1. Accomplishments based on original tasks

- Eight HA-Taxol preparations were synthesized, purified, and chemically characterized.
- HA-Taxol preparations were evaluated for selective cytotoxicity in six cell-lines.
- Fluorescent HA and fluorescent HA-Taxol were prepared and characterized.
- Cell binding and uptake of fluorescent HA and HA-Taxol was measured by confocal microscopy and flow cytometry.
- Release of Taxol from HA-Taxol in cell cultures was determined by HPLC.
- Protocols were established for *in vivo* testing of HA-taxol in nude mice with xenografted human tumors.
- Brefeldin A was chemically modified as a prodrug for chemical attachment to HA.

2. Accomplishments ancillary to original tasks but leading to a greater understanding of the mechanism of uptake and to novel drug leads

   - The transforming HA receptor RHAMM was expressed in recombinant form and with ^{13}C, ^{15}N labels for structural biological studies using high resolution 2-D and 3-D NMR methods.
   - Rapid and novel HA-binding assays were established.
   - Peptide libraries (phage-display and beads) were prepared and screened for peptides that mimic HA and bind to RHAMM.
   - Peptide leads were synthesized, fluorescently-tagged or biotinylated, and biophysical and functional studies of these peptides were initiated.

D. Reportable Outcomes

1. Publications

   Y. Luo and G.D. Prestwich, "Synthesis and Cytotoxicity of Hyaluronan-Taxol Antitumor Bioconjugates," *Bioconjugate Chem.*, in press (1999).

   G.D. Prestwich, Y. Luo, M.R. Ziebell, K.P. Vercruysse, K.R. Kirker, and J.S. MacMaster, "Chemically-Modified Hyaluronan: New Biomaterials and Probes for Cell Biology," in *New Frontiers in Medical Sciences: Redefining Hyaluronan* Padua, Italy (G. Abatangelo, ed.) Elsevier Science, in press (2000).

   S. Zhang, W.F. Cheung, J. Lu, M.R. Ziebell, S.A. Turley, R. Harrison, D. Zylka, N. Ahn, D. Litchfield, G.D. Prestwich, T. Cruz, and E.A. Turley, " Intracellular RHAMM Isoforms Bind Directly to erk1 and These Interactions are Required for Transformation and for Podosome Formation via the erk Kinase Pathway," *Molec. Cell. Biol.*, submitted (1999).

2. Patents, licenses and disclosures

   G.D. Prestwich, Y. Luo, K.P. Vercruysse, "Method for Preparing and Isolating High-Purity Bioconjugates of Hyaluronic Acid," UUtah disclosure filed March 10, 1998.

   G.D. Prestwich, "Discovery of Peptides That Mimic Hyaluronic Acid," US Provisional Patent Application No. 60/091,758 filed july 6, 1998. Full filing, July 3, 1999.

3. Degrees.

   Michael R. Ziebell, Physiology & Biophysics, The University at Stony Brook, Stony Brook, New York, PhD expected December 1999

4. Leveraged funding.

   The chemical modification technology developed was employed in several on-campus applications which were supported. These include preparation of HA hydrogel films for drug release and wound healing and the development of HA-modified particles for binding cancer cells and discovery of novel HA binding proteins. Other on-campus support was obtained to search for inhibitors of hyaluronidase, an HA-degrading enzyme important in the metastatic spread of cancer cells. In addition, our chemically-modified HA was a prominent part of an application for an NIH consortium project in tissue for developing HA substitutes to treat vocal insufficiency. Such prosthetic applications could be valuable in management of mastectomy or laryngectomy patients.

E. Conclusions

Our results to-date provide an exciting and promising method in the development of a new drug delivery system, and in basic research to identify new drug targets and mechanisms. First, we have successfully demonstrated that HA-Taxol prodrugs are selective and soluble cytotoxic agents against cancer cells, and that their mode of action requires HA receptor mediated uptake by target cells. We expect that *in vivo* experiments in the next year will demonstrate safety and selective toxicity. Second, we have obtained important new knowledge about the structure of RHAMM-HA complexes, and we have identified two kinds of polypeptides that selectively interfere with HA-RHAMM interactions and also affect the ability of RHAMM to cause cell transformation.

F. References

Literature references may be found in the preprints included as appendix materials.

G. Appendices

1. Biosketches of PI and research scientists
   Glenn D. Prestwich
   Koen P. Vercruysse
   Yi Luo
   Michael R. Ziebell

2. G.D. Prestwich, "Discovery of Peptides That Mimic Hyaluronic Acid"
   US Provisional Patent Application No. 60/091,758 filed july 6, 1998.
   Full filing, July 3, 1999.

3. Y. Luo and G.D. Prestwich, "Synthesis and Cytotoxicity of Hyaluronan-Taxol Antitumor Bioconjugates," *Bioconjugate Chem.*, in press (1999).

4. G.D. Prestwich, Y. Luo, M.R. Ziebell, K.P. Vercruysse, K.R. Kirker, and J.S. MacMaster, "Chemically-Modified Hyaluronan: New Biomaterials and Probes for Cell Biology," in *New Frontiers in Medical Sciences: Redefining Hyaluronan* Padua, Italy (G. Abatangelo, ed.) Elsevier Science, in press (2000).

Appendix Materials

1. Biosketches of PI and research scientists
   Glenn D. Prestwich
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BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
GLENN D. PRESTWICH	Presidential Professor and Chair of Medicinal Chemistry

EDUCATION/TRAINING (*Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.*)

INSTITUTION AND LOCATION	DEGREE	YEAR(s)	FIELD OF STUDY
California Institute of Technology, Pasadena, California	1970	1970 (Honors)	Chemistry
Stanford University, Palo Alto, California	Ph.D.	1974	Organic Chemistry

Research and Professional Experience

1974 and 1977: Cornell University, NIH postdoctoral fellowship, Department of Chemistry (Prof. J. Meinwald)
1974-1976: ICIPE, P.O. Box 30772, Nairobi, Kenya; Research Scientist in Chemistry Unit
1977-1982: Assistant Professor of Chemistry, SUNY, Stony Brook, New York
1982-1984: Associate Professor of Chemistry, SUNY, Stony Brook, New York
1984-1996: Professor of Chemistry, SUNY, Stony Brook, New York
1991-1992: Visiting Professor, Departments of Chemistry at Harvard University and at The University of Utah
1992-1996: Professor of Biochemistry and Cellular Biology, SUNY, Stony Brook, New York
1992-1996: Director, Center for Biotechnology, SUNY, Stony Brook, New York
1996-current: Presidential Professor and Chair, Medicinal Chemistry, The University of Utah.
1996-current: Research Professor of Biochemistry; Adjunct Professor of Chemistry and of Bioengineering; Program Leader, Molecular Pharmacology Program, Huntsman Cancer Institute; Director, Center for Cell Signaling, The University of Utah

Research interests: Structures of protein-ligand complexes; phosphoinositide affinity probes; cell signaling; isoprenoid chemical biology; hyaluronic acid biomaterials; molecular olfaction.

Honors: NIH Postdoctoral Fellow, January 1976-June 1977; Fellow of the Alfred P. Sloan Foundation, 1981-1985; Camille and Henry Dreyfus Teacher-Scholar, 1981-1986; Distinguished Research Fellow, Bodega Marine Laboratory, 1989; H.C. Brown Lecturer, Purdue University, 1990; National Institutes of Health Senior Fellowship, 1992; Paul Dawson Biotechnology Award, American Association of Colleges of Pharmacy, 1998.

Publications: G.D. Prestwich is author of over 325 journal research articles and book chapters since 1974. Selected relevant publications on HA and other topics from the last six years are included.

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BIOGRAPHICAL SKETCH

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EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Ghent, Belgium	B.Sc.	1990	Pharmaceutical Sciences
University of Ghent, Belgium	Ph.D.	1995	Pharmaceutical Sciences

Research and Professional Experience

1991: Lab of Galenic Development, Lilly Services S.A., Mont-Saint-Guibert, Belgium
1991-1995: Lab of General Biochemistry and Physical Pharmacy, University of Ghent, Belgium
1995-1996: Postdoctoral Research Associate, Department of Chemistry, SUNY at Stony Brook, Stony Brook, NY
1996-1998: Postdoctoral Research Associate, Department of Medicinal Chemistry, University of Utah, Salt Lake City, Utah.
July 1998-Current: Research Assistant Professor, Department of Medicinal Chemistry, University of Utah, Salt Lake City, Utah.

Publications

Dekeyser, P.M; De Smedt, S.; Vercruysse, K.; Demeester, J.; Lauwers, A. "High-performance size-exclusion chromatography of proteoglycans extracted from bovine articular cartilage" Anal. Chim. Acta, 1993, 279, 123-127.

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Vercruysse, K.P.; Ziebell, M.R. and Prestwich, G.D. "Control of enzymatic digestion of hyaluronan by divalent cations" Carbohydrate. Res, 1999 (in press).

Grants awarded

May 1998 : University of Utah Faculty Research and Creative Grant for "Hyaluronan-fluorescent probe conjugate with quenched fluorescence as substrate for hyaluronidase"
September 1998 : University of Utah Research Foundation Funding Incentive Seed Grant Program for "Development of New, Selective Inhibitors of Hyaluronidase" with Glenn D. Prestwich

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Nanjing University, Nanjing, PR China	BSc	1989	Organic Synthesis
Wuhan University, Wuhan, PR China	MSc	1991	Organic Synthesis
Wuhan University, Wuhan, PR China	Ph.D.	1994	Bioactive Polymers

Research Experience

1994-1996: Postdoctoral Researcher, Department of Chemistry, Beijing University, Beijing, People's Republic of China
1996-1997: Researcher, National Institute of Health Sciences, Tokyo, Japan
1997- current: Postdoctoral Research Associate, Medicinal Chemistry, The University of Utah

Publications: (from a total of 22)

Y. Luo, R.X. Zhou, and C.L. Fan, "Studies on the Magnetic Resonance Contrast Agents: Synthesis and Spin-Lattice Relaxivity of Amino Acids and Oligopeptidyl Derivatives of Gd-DTPA," *Chin. Chem. Lett.*, **6**, 377 (1995).

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BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
MICHAEL R. ZIEBELL	Graduate Research Assistant, The University of Utah

EDUCATION/TRAINING (*Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE	YEAR(s)	FIELD OF STUDY
Earlham College, Richmond, Indiana	BA	1991	Physics
Indiana University, Bloomington		1992-93	Coursework in Chemistry and Biology
The University at Stony Brook, Stony Brook, New York	PhD	PhD (expected 12/99)	Physiology & Biophysics

Publications

G.D. Prestwich, D.M. Marecak, J.F. Marecek, K.P. Vercruysse, and M.R. Ziebell, "Controlled Chemical Modification of Hyaluronic Acid:Synthesis, Applications, and Biodegradation of Hydrazide Derivatives,"*J. Controlled Rel.*, **53**, 93-103 (1998).

G.D. Prestwich, D.M. Marecak, J.F. Marecek, K.P. Vercruysse, and M.R. Ziebell, "Chemical Modification of Hyaluronic Acid for Drug Delivery, Biomaterials, and Biochemical Probes," in *The Chemistry, Biology, and Medical Applications of Hyaluronan and its Derivatives* (T. C. Laurent, ed.), Portland Press, London, pp. 43-65 (1998).

G.D. Prestwich, Y. Luo, M.R. Ziebell, K.P. Vercruysse, K.R. Kirker, and J.S. MacMaster, "Chemically-Modified Hyaluronan: New Biomaterials and Probes for Cell Biology," in *New Frontiers in Medical Sciences: Redefining Hyaluronan* Padua, Italy (G. Abatangelo, ed.) Elsevier Science, in press (2000).

S. Zhang, W.F. Cheung, J. Lu, M.R. Ziebell, S.A. Turley, R. Harrison, D. Zylka, N. Ahn, D. Litchfield, G.D. Prestwich, T. Cruz, and E.A. Turley, " Intracellular RHAMM Isoforms Bind Directly to erk1 and These Interactions are Required for Transformation and for Podosome Formation via the erk Kinase Pathway," *Molec. Cell. Biol.*, submitted (1999).

THE COMMISSIONER OF PATENTS AND TRADEMARKS
WASHINGTON, D.C. 20231

COPY

Sir:

Transmitted herewith for filing is the Utility patent application of:

INVENTORS:	**Prestwich, Ziebell, Luo and Zhao**
FOR:	**Hyaluronic Acid Mimics and Methods Related Thereto**

This application claims priority to Provisional Patent Application US 60/091,758 filed July 6, 1998.

Enclosed are:

___A Verified Statement claiming Small Entity Status
_x_A declaration and power of attorney (unsigned)
___A sequence listing on diskette, paper copy and affidavit
___An Information Disclosure Statement and 1449 form
_x_ _____ total pages of application and ___ claims
_x_A return postcard

_x_The filing fee is calculated as shown below:

FOR	NUMBER FILED	NUMBER EXTRA	RATE	CALCULATIONS
TOTAL CLAIMS	21 -20=	1	x$18 (9) =	$9.00
INDEPENDENT CLAIMS	6 -3=	3	x$78 (39) =	$117.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+$260 = (130)	$0.00
		BASIC FEE (Small Entity)		$380.00
		TOTAL		$506.00

___ A check in the amount of $_____ to cover the filing fee is enclosed.
___ The Commissioner is hereby authorized to charge any additional fees associated with this communication, including patent application filing fees and processing fees under 37 C.F.R. §1.16 and 1.17, or credit any overpayment to **Deposit Account Number 02-0725.**

Respectfully submitted,

Kristine H. Johnson
Registration Number 36, 835

Date: July 3, 1999

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I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

KRISTINE H. JOHNSON
Printed Name

Signature

DECLARATION FOR PATENT APPLICATION AND APPOINTMENT OF ATTORNEY

As a below-named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name; I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention (Design, if applicable) entitled : Hyaluronic Acid Mimics and Methods Related Thereto

the specification of which (check one):

_X_ is attached hereto.
___ was filed on _____, as Application Serial No._____, and was amended on _____ (if applicable).
___ was filed on _____ as International Application (PCT) No. _____, and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with *Title 37, Code of Federal Regulations, § 1.56(a)*. I hereby claim foreign priority benefits under *Title 35, United States Code § 119* of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which the priority is claimed.

PRIOR FOREIGN APPLICATION(S)

NUMBER	COUNTRY	DAY/MONTH/YEAR FILED	PRIORITY CLAIMED
			Yes No
			Yes No

I hereby claim the benefit under *Title 35, United States Code, § 120* of any United States application(s) or PCT international application(s) designating The United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of *Title 35, United States Code, § 112*, I acknowledge the duty to disclose material information as defined in *Title 37, Code of Federal Regulations, § 1.56(a)* which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

APPLICATION NUMBER	FILING DATE	STATUS (Patented, Pending or Abandoned)
60/091,758	July 6, 1998	Pending (Provisional)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine, or imprisonment, or both, under *Section 1001 of Title 18 of the United States Code*, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: I (We) hereby appoint as my (our) attorneys, with full powers of substitution and revocation, to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Kristine H. Johnson, Registration Number 36,835, Jennifer Bales 38,070, and Jean Macheledt 33,956.

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DATE	*SIGNATURE*

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State or Country *Zip* Utah	*State or Country* *Zip* Utah
DATE	*SIGNATURE*

___ SEE FOLLOWING PAGES FOR ADDITIONAL JOINT INVENTORS

Enclosed for filing herewith is a patent application of :
Glenn D. Prestwich, Ziebell, Luo & Zhao

PATENT APPLICATION
(NEW)
ATTY DKT NO. P-1078

For: Hyaluronic Acid Mimics and Methods Related Thereto

The PTO Stamp hereon acknowledges receipt of :

- x 48 pages of specification and 21 claims
- ___ a check for _____
- ___ a sequence listing on diskette, paper copy and affidavit
- ___ Assignment with PTO
- ___ Priority Documents
- ___ Preliminary Amendment
- x Declaration/Power of Attorney ___Executed _x_Unexecuted
- ___ ___Sheets of Drawings ___Formal ___Informal
- ___ Small Entity Statement ___Ind. ___Sm. Biz ___Non-inv. ___Non-prof.
- ___ IDS/ PTO Form 1449/ References
- X 4 pages of drawings
- ___ _____

Sent via Express Mail mailing label number EL442325922US

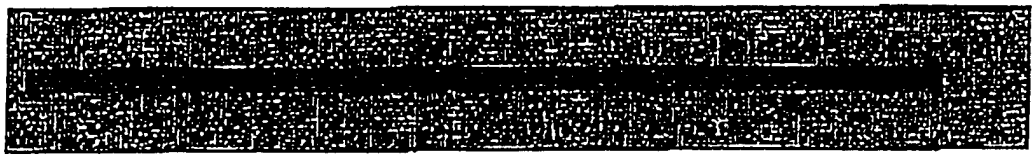
Date of Deposit: July 3, 1999

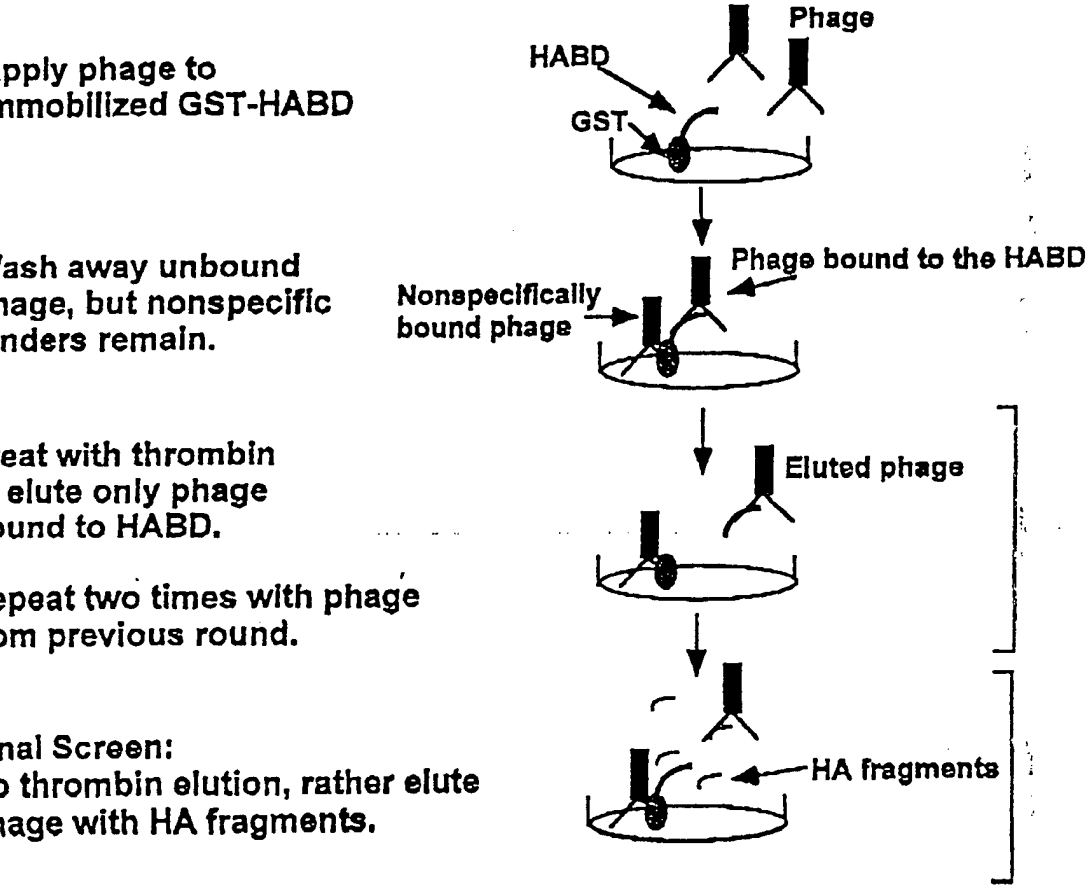
Figure 1

Phage Screened for Peptides that Bind to the HA Binding Domains of RHAMM-1v4

fUSE-5 phage hosting random 15 amino acid peptides were screened against the HA binding domain of RHAMM 1v4.

METHOD





SEQUENCES FOUND

HA2	GVCNADFCWLPAVVV	HA6	HWCLPLLACDTFARA
HA3	SASPSASKLSLMSTV	HA7	YRVYLSVVHNSVLPS
HA4	IPPILPAYTLLGHPR	HA8	PHXRPVVSASSILPV

Scott, J.K. & Smith, G.P., *Science*, 249:386-390 (1990)

Figure 2

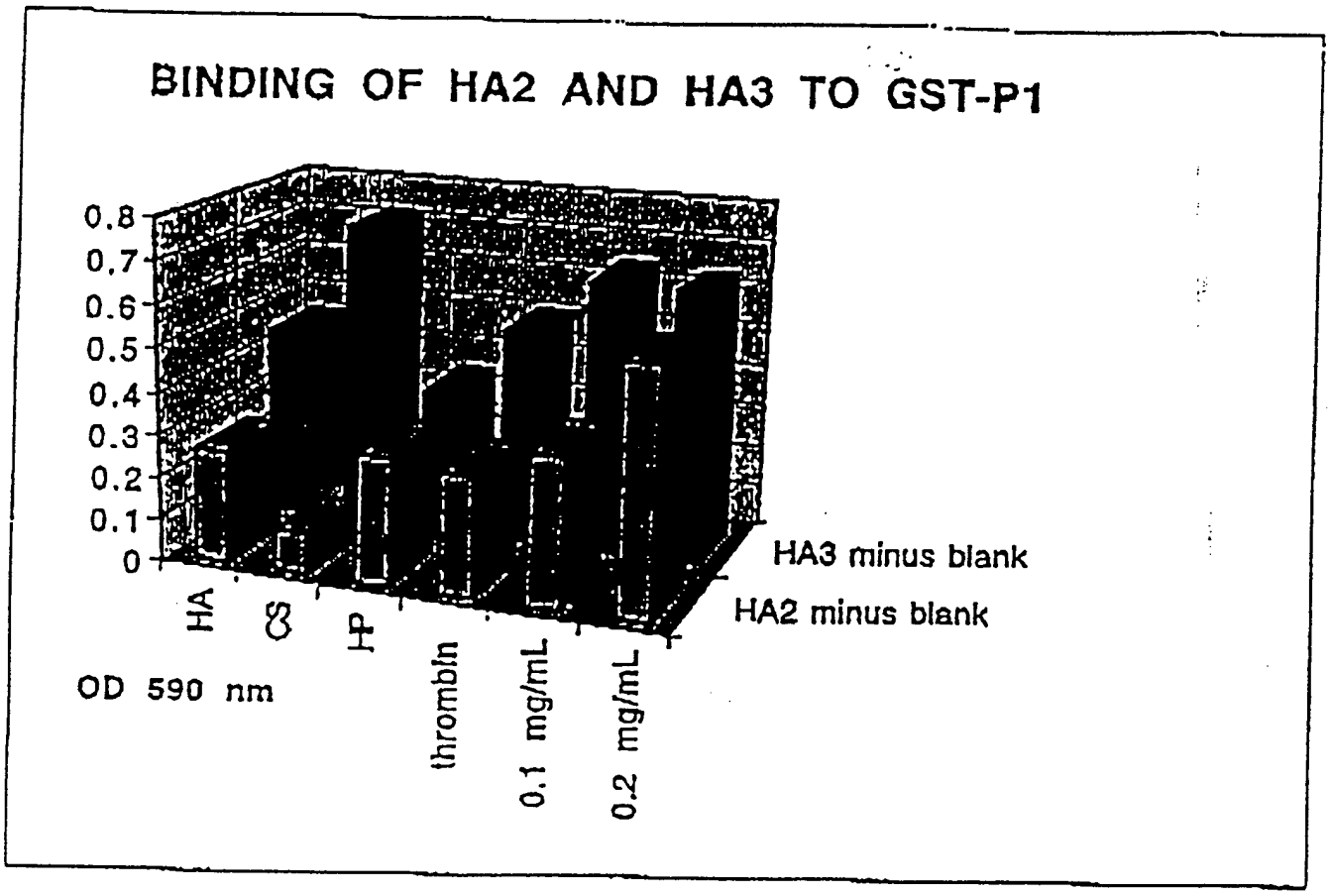


Figure 2. The lanes marked HA, CS, and HP have been preincubated with 1.5 mg/mL hyaluronic acid (HA), chondroitin sulfate (CS) and heparin (HP). These wells contain 0.15 mg/mL HA2 or HA3.

Figure 3

Affinities of Peptides that Bind to the HA Binding Domain

The peptides HA2 and HA3 were synthesized with an N-terminal biotin, and evaluated for their binding properties to the HA binding domains from which they were screened. In a solid phase binding assay, in which GST-P1 is immobilized in 96 well plates the following data was obtained.

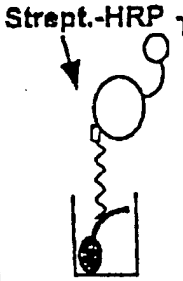
Furthermore, it is noted (data not shown) that HA fragments (20-60 kD) and chondroitin sulfate (60 kD) can compete with the peptides for binding.

GENERAL METHOD

GST-HABD immobilized in a microplate well

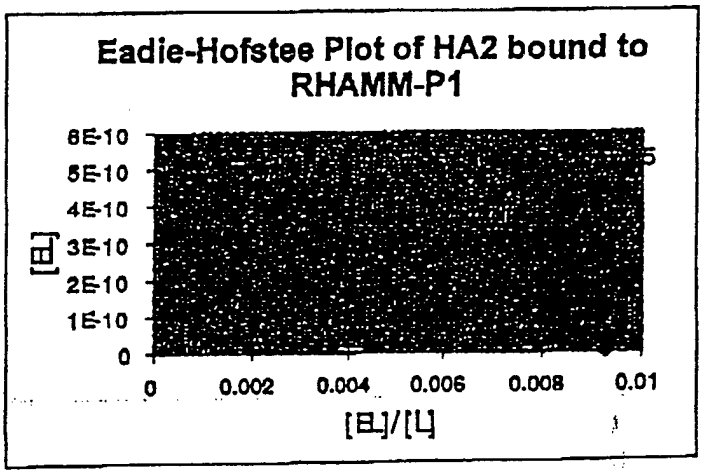
Biotinylated peptide is applied.

Detect bound ligand using streptavidin-HRP



Read plate at 590 nm

$HA2\ K_d = 5x10^{-8}$ $HA3\ K_d = 5x10^{-6}$



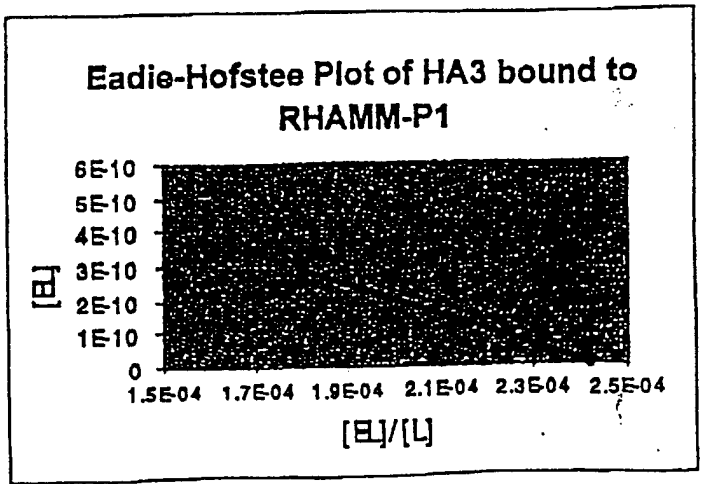


Figure 4

Amino Acid	Abbreviation	
	Three Letter	One Letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic	Asp	D
Asparagine or aspartic	Asx	B
Cysteine	Cys	C
Glycine	Gly	G
Glutamine	Gln	Q
Glutamic	Glu	E
Glutamine or glutamic	Glx	Z
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Application for

United States Letters Patent

of

Glenn D. Prestwich

Michael Ziebell

Bai Luo

Zhan-gong Zhao

for

HYALURONIC ACID MIMICS AND METHODS RELATED THERETO

HYALURONIC ACID MIMICS AND METHODS RELATED THERETO

This application claims priority to U.S. Provisional Patent Application Serial Number 60/091,758, filed July 6, 1998.

FIELD OF THE INVENTION

This invention pertains to the fields of biochemistry, specifically biochemistry related to compounds which interact with hyaluronic acid receptors.

BACKGROUND OF THE INVENTION

Hyaluronic acid (HA) is a large glycoaminoglycan that contains repeating disaccharide units of N-acetyl glucosamine and glucuronic acid. It occurs in the extracellular matrix and on the cell surface. It has been shown, among other things, to promote cell mobility, adhesion, and proliferation. HA has an role in many physiological processes, for example, morphogenesis, wound repair, inflammation, and metastasis. Many of the effects of HA are mediated through cell surface receptors, several of which have been molecularly characterized, namely CD44, RHAMM (Receptor for Hyaluronan Mediated Mobility), and ICAM- I (Intracellular Adhesion Molecule- 1), BEHAB, Link Protein and TSG-6. Binding of the HA ligand to its receptors triggers signal transduction events.

Although considerable information on the structure of the HA-related surface receptors is available, the three-dimensional structure/biochemistry of HA which influences the receptor/HA interaction is not known. The popular hypothesis for receptor-HA interaction is that the HA binding motif is present in sequences of these receptors and is responsible for the binding of these sequences to HA.

Other journal articles which hypothesize motifs that would bind to HA binding domains are:

Day, A. J. (1999) The structure and regulation of hyaluronan-binding proteins. Biochem Soc Trans 27, 115-21.

Knudson, C. B., and Knudson, W. (1993) Hyaluronan-binding proteins in development, tissue homeostasis, and disease. Faseb J 7, 1233-41

Sherman, L., Sleeman, J., Herrlich, P., and Ponta, H. (1994) Hyaluronate receptors: key players in growth, differentiation, migration and tumor progression. Curr Opin Cell Biol 6, 726-33.

Toole, B. P. (1990) Hyaluronan and its binding proteins, the hyaladherins. Curr Opin Cell Biol 2, 839-44.

Ward, A. C., Dowthwaite, G. P., and Pitsillides, A. A. (1999) Hyaluronan in joint cavitation. Biochem Soc Trans 27, 128-35.

Bajorath, J., Greenfield, B., Munro, S. B., Day, A. J., and Aruffo, A. (1998). Identification of CD44 residues important for hyaluronan binding and delineation of the binding site. J Biol Chem 273, 338-43.

Kohda, D., Morton, C. J., Parkar, A. A., Hatanaka, H., Inagaki, F. M., Campbell, I. D., and Day, A. J. (1996). Solution structure of the link module: a hyaluronan-binding domain involved in extracellular matrix stability and cell migration. Cell 86, 767-75.

Maier, R., Wisniewski, H. G., Vilcek, J., and Lotz, M. (1996). TSG-6 expression in human articular chondrocytes. Possible implications in joint inflammation and cartilage degradation. Arthritis Rheum 39, 552-9.

Parkar, A. A., Kahmann, J. D., Howat, S. L., Bayliss, M. T., and Day, A. J. (1998). TSG-6 interacts with hyaluronan and aggrecan in a pH-dependent manner via a common functional element: implications for its regulation in inflamed cartilage. FEBS Lett 428, 171-6.

The following patents discuss modified HAs, none of which are remotely similar to the present HA mimics:

5,874,417 & 5,616,568 Functionalized derivatives of hyaluronic acid

5,652,347 Method for making functionalized derivatives of hyaluronic acid

5,631,241 Pharmaceutical compositions containing hyaluronic acid fractions

5,356,883 & 5,502,081 & 5,356,883 & 5,017,229 & 4,937,270 Water-insoluble derivatives of hyaluronic acid and their methods of preparation and use

5,520,916 Non-woven fabric material comprising hyaluronic acid derivatives and use

5,503,848 Spongy material consisting essentially of hyaluronic acid or its derivatives, and its use in microsurgery

5,202,431 Partial esters of hyaluronic acid

4,636,524 & 4,605,691 & 4,582,865 Cross-linked gels of hyaluronic acid and products containing such gels

The following patents discuss the use of HA for treating various diseases, which uses are also applicable uses of the present HA mimics:

5,914,314 Use of a form of hyaluronic acid and a medicinal agent for reducing rejection of organs transplantation in mammals

5,888,986 & 5,880,108 5,591,724 Method for treating the urinary bladder and associated structures using hyaluronic acid

5,847,002 Compositions, for inhibition, control and regression of angiogenesis, containing hyaluronic acid and NSAID

5,834,444 Hyaluronic acid and salts thereof inhibit arterial restenosis

5,830,882 Compositions containing a form of hyaluronic acid and a medicinal agent for treating acne in mammals and methods for administration of such composition

5,827,834 Method of using hyaluronic acid or its pharmaceutically acceptable salts for the treatment of disease

5,728,391 Hyaluronic acid and its salt for treating skin diseases

5,679,655 Method of treating lesions resulting from genital herpes with hyaluronic acid-urea pharmaceutical compositions

5,674,857 Use of hyaluronic acid to repair ischemia reperfusion damage

5,646,129 Method of using low molecular weight hyaluronic acid for stimulating bone formation

5,639,738 Treatment of basal cell carcinoma and actinic keratosis employing hyaluronic acid and NSAIDs

5,633,003 Use of intratracheally administered hyaluronic acid to ameliorate emphysema

5,631,242 Hyaluronic acid-urea pharmaceutical compositions utilized for treatment of diseases of cutis

5,624,915 & 5,583,120 & 5,583,119 & 5,550,112 & 5,529,987 Hyaluronic acid-urea pharmaceutical compositions and uses

5,614,506 Use of hyaluronic acid and forms to prevent arterial restenosis

5,604,200 Wound therapeutic mixture containing medical grade hyaluronic acid and tissue culture grade plasma-fibronectin in a delivery system that creates a moist environment which simulates in utero healing

5,583,118 Method of treating an anorectal disease using hyaluronic acid-urea pharmaceutical compositions

4,801,619 Hyaluronic acid preparation to be used for treating inflammations of skeletal joints

The following patents have disclosed compositions of matter comprising HA as an ingredient, and which would be useful in making the present compositions claimed, except that the present HA mimics would be substituted for HA:

5,847,002 Compositions, for inhibition, control and regression of angiogenesis, containing hyaluronic acid and NSAID

5,679,655 Method of treating lesions resulting from genital herpes with hyaluronic acid-urea pharmaceutical compositions

The following patents have disclosed other methods of using HA, which other uses would be applicable uses of the present HA mimics:

5,772,982 Method of using hyaluronic acid for the detection, location and diagnosis of tumors

4,804,537 Sperm selection process using a salt of hyaluronic acid

Citation of the above documents is not intended as an admission that any of the foregoing is pertinent prior art. All statements as to the date or representation as to the contents of these documents is based on subjective characterization of information available to the applicant, and does not constitute any admission as to the accuracy of the dates or contents of these documents.

SUMMARY OF THE INVENTION

The present invention provides HA mimics comprising a polypeptide having alternating acidic / non-acidic residues, wherein said mimic binds to the hyaluronic acid binding domain of the receptor for hyaluronan mediated mobility. Those HA mimics wherein said polypeptide is four to 15 residues in length are preferred. More preferred are those HA mimics as described, wherein said residues include at least one dextrorotatory residue. Most preferred are HA mimics comprising an amino acid sequence selected from the group consisting of: SEQ ID NO 1; SEQ ID NO 2; SEQ ID NO 3; SEQ ID NO 4; SEQ ID NO 5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 8; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11; SEQ ID NO 12; SEQ ID NO 13; SEQ ID NO 14; SEQ ID NO 15; SEQ ID NO 16; SEQ ID NO 17; SEQ ID NO 18; SEQ ID NO 19; and SEQ ID NO 20, or a homologue thereof, wherein said homologue binds to the hyaluronic acid binding domain of the receptor for hyaluronan mediated mobility.

In a preferred embodiment of the present invention, the HA mimics comprising the sequence:

$$X\text{-}A/a\text{-}X\text{-}A/a\text{-}X\text{-}a\text{-}X\text{-}a_1$$

wherein (X) can be any amino acid, (A) is L-glutamate or L-aspartate and (a) and (a_1) are D-glutamate or D-aspartate.

Those wherein X is hydrophobic are more preferred and those wherein X is hydrophobic and a_1 is D-glutamate are most preferred.

The present invention also provides nucleic acid compounds comprising a nucleic acid which encodes an amino acid sequence selected from the group consisting of: SEQ ID NO 1; SEQ ID NO 2; SEQ ID NO 3; SEQ ID NO 4; SEQ ID NO 5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 8; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11; SEQ ID NO 12; SEQ ID NO 13; SEQ ID NO 14; SEQ ID NO 15; SEQ ID NO 16; SEQ ID NO 17; SEQ ID NO 18; SEQ ID NO 19; and SEQ ID NO 20; and homologues thereof, wherein said homologue binds to the hyaluronic acid binding domain of the receptor for hyaluronan mediated mobility.

Also provided are HA mimics comprising a polypeptide comprising double hydrophobic residues, wherein said mimic binds to the hyaluronic acid binding domain of the TSG-6. In particular, provided are HA mimics of which are selected from the group consisting of: GYYFNVAM; WAYNFLVM; TQSLNNHM; WWPFINAY; WWKADMVG; WWPFINAY; MALQLPYY; IIYEEFFV; ISINNRWY; VTPPVYFT; QIRNGWFW; SWWFGPLA; GDWEQILT; PAGFGWNL; NMRFNIEN; and QMTFFDGV, or a homologue thereof, wherein said homologue binds to hyaluronic acid binding domain of the TSG-6. Nucleic acids which encode the above compounds are also part of the present invention.

The present invention also provides methods to affect a hyaluronidase-mediated biological response in a patient in need of such affecting , comprising administering an HA mimic of the present invention.

The present invention also provides methods to inhibit hyaluronidase activity in a patient in need of such inhibition, comprising administering an HA mimic of the present invention.

The present invention also provides methods to bind receptors that bind hyaluronic acid, comprising introducing an HA mimic of the present invention.

The present invention also provides methods to mimic hyaluronic acid in a biological system, comprising introducing an HA mimic of the present invention.

The present invention also provides methods to affect cell signalling associated with hyaluronic acid/hyaluronic acid receptor interactions, comprising introducing an HA mimic of the present invention.

The present invention also provides methods to treat hyaluronic acid-associated disease in a patient in need of such treatment, comprising administering an HA mimic of the present invention. In particular, those methods wherein said disease is selected from the group consisting of: inflammation; tumor angiogenesis; skin disease; bone disease; wound healing; osteoarthritis; rheumatoid arthritis; infectious disease; immune disease; and cardiovascular disease are preferred.

The present invention also provides methods to inhibit metastasis in a patient in need of such inhibition, comprising administering an HA mimic of the present invention.

The present invention also provides methods to inhibit fertilization in a patient in need of such inhibition, comprising administering an HA mimic of the present invention.

The present invention also provides methods to introduce an agent into a cell which binds hyaluronic acid, comprising administering the agent in conjunction with an HA mimic of the present invention.

The present invention also provides methods to isolate peptides that mimic a ligand's binding to a receptor comprising the steps of:

(a) preparing a random library of peptides and binding said library to a bead;

(b) placing the library in contact with the receptor under conditions for binding;

(c) washing off unbound peptides;

(d) contacting the washed bead-bound library with anti-receptor antibody;

(e) detecting and selecting beads having antibody bound thereto;

(f) eluting the antibody;

(g) repeating steps (a) through (f) and then repeating steps (a) through (c), and then incubating the beads in the presence of receptor prior to adding anti-receptor antibody;

(h) detecting and selecting beads that did not bind antibody;

(i) determining the sequence of the bead-bound peptide.

"Protein" means any compound which comprises amino acids, including peptides , polypeptides, fusion proteins, etc.

"in conjunction with" means any physical interaction that results in co-localization.

Moreover, for the purposes of the present invention, the term "a" or "an" entity refers to one or more of that entity; for example, "a protein" or "a nucleic acid molecule" refers to one or more of those compounds or at least one compound. As such, the terms "a" (or "an"), "one or more" and "at least one" can be used interchangeably herein. It is also to be noted that the terms "comprising", "including", and "having" can be used interchangeably. Furthermore, a compound "selected from the group consisting of" refers to one or more of the compounds in the list that follows, including mixtures (i.e., combinations) of two or more of the compounds. According to the present invention, an isolated, or biologically pure, protein or nucleic acid molecule is a compound that has been removed from its natural milieu. As such, "isolated" and "biologically pure" do not necessarily reflect the extent to which the compound has been purified. An isolated compound of the present invention can be obtained from its natural source, can be produced using molecular biology techniques or can be produced by chemical synthesis.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. I shows a scheme for screening a random phage library for binding to HA.

FIG. 2 shows binding of two synthetic peptides to a hyaluronic binding domain.

FIG. 3 shows affinities of peptides that bind to the HA binding domain.

FIG. 4 shows a table describing abbreviations of the amino acids.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides HA mimics comprising a polypeptide having alternating acidic / non-acidic residues, wherein said mimic binds to the hyaluronic acid binding domain of the receptor for hyaluronan mediated mobility. Those HA mimics wherein said polypeptide is four to 15 residues in length are preferred. More preferred are those HA mimics as described, wherein said residues include at least one dextrorotatory residue. Most preferred are HA mimics comprising an amino acid sequence selected from the group consisting of: SEQ ID NO 1; SEQ ID NO 2; SEQ ID NO 3; SEQ ID NO 4; SEQ ID NO 5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 8; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11; SEQ ID NO 12; SEQ ID NO 13; SEQ ID NO 14; SEQ ID NO 15; SEQ ID NO 16; SEQ ID NO 17; SEQ ID NO 18; SEQ ID NO 19; and SEQ ID NO 20, or a homologue thereof, wherein said homologue binds to the hyaluronic acid binding domain of the receptor for hyaluronan mediated mobility.

In a preferred embodiment of the present invention, the HA mimics comprising the sequence:

$$X\text{-}A/a\text{-}X\text{-}A/a\text{-}X\text{-}a\text{-}X\text{-}a_1$$

wherein (X) can be any amino acid, (A) is L-glutamate or L-aspartate and (a) and (a_1) are D-glutamate or D-aspartate.

Those wherein X is hydrophobic are more preferred and those wherein X is hydrophobic and a_1 is D-glutamate are most preferred.

Also provided are HA mimics comprising a polypeptide comprising double hydrophobic residues, wherein said mimic binds to the hyaluronic

acid binding domain of the TSG-6. In particular, provided are HA mimics of which are selected from the group consisting of: GYYFNVAM; WAYNFLVM; TQSLNNHM; WWPFINAY; WWKADMVG; WWPFINAY; MALQLPYY; IIYEEFFV; ISINNRWY; VTPPVYFT; QIRNGWFW; SWWFGPLA; GDWEQILT; PAGFGWNL; NMRFNIEN; and QMTFFDGV, or a homologue thereof, wherein said homologue binds to hyaluronic acid binding domain of the TSG-6. Nucleic acids which encode the above compounds are also part of the present invention.

The present invention also provides nucleic acid compounds comprising a nucleic acid which encodes an amino acid sequence selected from the group consisting of: SEQ ID NO 1; SEQ ID NO 2; SEQ ID NO 3; SEQ ID NO 4; SEQ ID NO 5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 8; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11; SEQ ID NO 12; SEQ ID NO 13; SEQ ID NO 14; SEQ ID NO 15; SEQ ID NO 16; SEQ ID NO 17; SEQ ID NO 18; SEQ ID NO 19; and SEQ ID NO 20; and homologues thereof, wherein said homologue binds to the hyaluronic acid binding domain of the receptor for hyaluronan mediated mobility.

Also provided are compositions of matter comprising an HA mimic of the present invention, in particular, those which comprise a non-steroidal anti-inflammatory drug.

The present invention also provides methods to affect a hyaluronidase-mediated biological response in a patient in need of such affecting , comprising administering an HA mimic of the present invention.

The present invention also provides methods to inhibit hyaluronidase activity in a patient in need of such inhibition, comprising administering an HA mimic of the present invention.

The present invention also provides methods to bind receptors that bind hyaluronic acid, comprising introducing an HA mimic of the present invention.

The present invention also provides methods to mimic hyaluronic acid in a biological system, comprising introducing an HA mimic of the present invention.

The present invention also provides methods to affect cell signalling associated with hyaluronic acid/hyaluronic acid receptor interactions, comprising introducing an HA mimic of the present invention.

The present invention also provides methods to treat hyaluronic acid-associated disease in a patient in need of such treatment, comprising administering an HA mimic of the present invention. In particular, those methods wherein said disease is selected from the group consisting of: inflammation; tumor angiogenesis; skin disease; bone disease; wound healing;osteoarthritis; rheumatoid arthritis; infectious disease; immune disease; and cardiovascular disease are preferred.

The present invention also provides methods to inhibit metastasis in a patient in need of such inhibition, comprising administering an HA mimic of the present invention.

The present invention also provides methods to inhibit fertilization in a patient in need of such inhibition, comprising administering an HA mimic of the present invention.

The present invention also provides methods to introduce an agent into a cell which binds hyaluronic acid, comprising administering the agent in conjunction with an HA mimic of the present invention.

The present invention also provides methods to isolate peptides that mimic a ligand's binding to a receptor comprising the steps of:

(a) preparing a random library of peptides and binding said library to a bead;

(b) placing the library in contact with the receptor under conditions for binding;

(c) washing off unbound peptides;

(d) contacting the washed bead-bound library with anti-receptor antibody;

(e) detecting and selecting beads having antibody bound thereto;

(f) eluting the antibody;

(g) repeating steps (a) through (f) and then repeating steps (a) through (c), and then incubating the beads in the presence of receptor prior to adding anti-receptor antibody;

(h) detecting and selecting beads that did not bind antibody;

(i) determining the sequence of the bead-bound peptide.

HA mimic homologs of the present invention can be produced using techniques known in the art including, but not limited to, direct modifications to the peptide or modifications to the gene encoding the peptide using, for example, classic or recombinant nucleic acid techniques to effect random or targeted mutagenesis.

A HA mimic of the present invention, including a homolog, can be identified in a straight-forward manner by the protein's ability to inhibit an HA target enzyme.

One embodiment of a HA mimic of the present invention is a fusion protein that includes a HA receptor binding domain attached to one or more fusion segments. Suitable fusion segments for use with the present invention include, but are not limited to, segments that can: enhance a protein's stability and/or assist purification of an HA mimic (e.g., by affinity chromatography). A suitable fusion segment can be a domain of any size that has the desired function (e.g., imparts increased stability, imparts increased immunogenicity to a protein, and/or simplifies purification of a protein). Fusion proteins are preferably produced by culturing a recombinant cell transformed with a fusion nucleic acid molecule that encodes a protein including the fusion segment attached to either the carboxyl and/or amino terminal end of an HA receptor binding domain. Preferred fusion segments include a metal binding domain (e.g., a poly-histidine segment); an immunoglobulin binding domain (e.g., Protein A; Protein G; T cell; B cell; Fc receptor or complement protein antibody-binding domains); a sugar binding domain (e.g., a maltose binding domain); a "tag" domain (e.g., at least a portion of ß-galactosidase, a strep tag peptide, other domains that can be purified using compounds that bind to the domain, such as monoclonal antibodies); and/or a linker and enzyme domain (e.g., alkaline phosphatase domain connected to A HA mimic by a linker). More preferred fusion segments include metal binding domains, such as a poly-histidine segment; a maltose binding domain; a strep tag peptide; and a phage T7 S10 peptide.

An HA mimic of the present invention can also be a chimeric molecule comprising an HA mimic and a second molecule. In particular,

there is provided a chimeric molecule that enables the chimeric molecule to be bound to a surface in such a manner that the chimera inhibits an HA-target enzyme in essentially the same manner as an HA mimic that is not bound to a surface. An example of a suitable second molecule includes a portion of an immunoglobulin molecule or another ligand that has a suitable binding partner that can be immobilized on a substrate, e.g., biotin and avidin, or a metal-binding protein and a metal (e.g., His), or a sugar-binding protein and a sugar (e.g., maltose).

Nucleic acid molecules comprising a nucleic acid molecule which encodes the present HA mimics are also provided by the present invention. In particular, there are provided nucleic acids encoding the present HA mimics, wherein said HA mimics comprise, an amino acid sequence selected the group consisting of: SEQ ID NO 1; SEQ ID NO 2; SEQ ID NO 3; SEQ ID NO 4; SEQ ID NO 5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 8; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11; SEQ ID NO 12; SEQ ID NO 13; SEQ ID NO 14; SEQ ID NO 15; SEQ ID NO 16; SEQ ID NO 17; SEQ ID NO 18; SEQ ID NO 19; and SEQ ID NO 20.

The present invention also comprises expression vectors and recombinant cells comprising the present nucleic acid molecules. Also provided are fusion constructs using the present nucleic acid compounds.

One approach is to create fusogenic peptides, in which a short (18-mer) peptide is attached to the effector peptide that causes endocytotic uptake of the peptide into the cell. J. Hawiger, Noninvasive intracellular delivery of functional peptides and proteins, Curr. Opinion Chemical Biology 3, 89-94 (1999) and A. Prochiantz, Getting hydrophilic compounds into cells: lessons from heomeopeptides. Cur. Opin. Neurobiol 6, 629-630 (1996)

Included within the scope of the present invention, with particular regard to the nucleic acids above, are degenerate sequences and homologues. The present invention also includes variants due to laboratory manipulation, such as, but not limited to, variants produced during polymerase chain reaction amplification or site-directed mutagenesis. It is also well known that there is a substantial amount of redundancy in the various codons which code for specific amino acids. Therefore, this invention is also directed to those nucleic acid sequences which contain alternative codons which code for the eventual translation of the identical amino acid. Also included within the scope of this invention are mutations either in the nucleic acid sequence or the translated protein which do not substantially alter the ultimate physical properties of the expressed protein. For example, substitution of valine for leucine, arginine for lysine, or asparagine for glutamine may not cause a change in functionality of the polypeptide.

Knowing the nucleic acid sequences of certain HA mimic nucleic acid molecules of the present invention allows one skilled in the art to, for example, (a) make copies of those nucleic acid molecules, (b) obtain nucleic acid molecules including at least a portion of such nucleic acid molecules (e.g., nucleic acid molecules including full-length genes, full-length coding regions, regulatory control sequences, truncated coding regions), and (c) obtain HA mimic nucleic acid molecules. Such nucleic acid molecules can be obtained in a variety of ways including screening appropriate expression libraries with antibodies of the present invention; traditional cloning techniques using oligonucleotide probes of the present invention to screen appropriate libraries of DNA; and PCR amplification of appropriate libraries or DNA using oligonucleotide primers of the present invention. Preferred libraries to screen or from which to amplify nucleic acid molecules include livestock (cattle, horse, pig) and companion animal (dog and cat) cDNA libraries as well as genomic DNA libraries. Similarly, preferred DNA sources

to screen or from which to amplify nucleic acid molecules include adult cDNA and genomic DNA. Techniques to clone and amplify genes are disclosed, for example, in Sambrook et al., *ibid.*

One embodiment of the present invention includes a recombinant vector, which includes at least one isolated nucleic acid molecule of the present invention, inserted into any vector capable of delivering the nucleic acid molecule into a host cell. Such a vector contains heterologous nucleic acid sequences, that is, nucleic acid sequences that are not naturally found adjacent to nucleic acid molecules of the present invention and that preferably are derived from a species other than the species from which the nucleic acid molecule(s) are derived. The vector can be either RNA or DNA, either prokaryotic or eukaryotic, and typically is a virus or a plasmid. Recombinant vectors can be used in the cloning, sequencing, and/or otherwise manipulation of the HA mimic nucleic acid molecules of the present invention.

One type of recombinant vector, referred to herein as a recombinant molecule, comprises a nucleic acid molecule of the present invention operatively linked to an expression vector. The phrase "operatively linked" refers to insertion of a nucleic acid molecule into an expression vector in a manner such that the molecule is able to be expressed when transformed into a host cell. As used herein, an expression vector is a DNA or RNA vector that is capable of transforming a host cell and of effecting expression of a specified nucleic acid molecule. Preferably, the expression vector is also capable of replicating within the host cell. Expression vectors can be either prokaryotic or eukaryotic, and are typically viruses or plasmids. Expression vectors of the present invention include any vectors that function (i.e., direct gene expression) in recombinant cells of the present invention, including in bacterial, fungal, endoparasite, insect, other animal, and plant cells. Preferred

expression vectors of the present invention can direct gene expression in bacterial, yeast, insect and mammalian cells and more preferably in the cell types disclosed herein.

In particular, expression vectors of the present invention contain regulatory sequences such as transcription control sequences, translation control sequences, origins of replication, and other regulatory sequences that are compatible with the recombinant cell and that control the expression of nucleic acid molecules of the present invention. In particular, recombinant molecules of the present invention include transcription control sequences. Transcription control sequences are sequences which control the initiation, elongation, and termination of transcription. Particularly important transcription control sequences are those which control transcription initiation, such as promoter, enhancer, operator and repressor sequences. Suitable transcription control sequences include any transcription control sequence that can function in at least one of the recombinant cells of the present invention. A variety of such transcription control sequences are known to those skilled in the art. Preferred transcription control sequences include those which function in bacterial, yeast, insect and mammalian cells, such as, but not limited to, *tac*, *lac*, *trp*, *trc*, oxy-pro, omp/lpp, rrnB, bacteriophage lambda (such as lambda p_L and lambda p_R and fusions that include such promoters), bacteriophage T7, T7*lac*, bacteriophage T3, bacteriophage SP6, bacteriophage SP01, metallothionein, alpha-mating factor, *Pichia* alcohol oxidase, alphavirus subgenomic promoters (such as Sindbis virus subgenomic promoters), antibiotic resistance gene, baculovirus, *Heliothis zea* insect virus, vaccinia virus, herpesvirus, raccoon poxvirus, other poxvirus, adenovirus, cytomegalovirus (such as intermediate early promoters), simian virus 40, retrovirus, actin, retroviral long terminal repeat, Rous sarcoma virus, heat shock, phosphate and nitrate transcription control sequences as well as other sequences capable of controlling gene expression in

prokaryotic or eukaryotic cells. Additional suitable transcription control sequences include tissue-specific promoters and enhancers as well as lymphokine-inducible promoters (e.g., promoters inducible by interferons or interleukins). Transcription control sequences of the present invention can also include naturally-occurring transcription control sequences naturally associated with humans. The present invention also comprises expression vectors comprising a nucleic acid molecule described herein.

Recombinant DNA technologies can be used to improve expression of transformed nucleic acid molecules by manipulating, for example, the number of copies of the nucleic acid molecules within a host cell, the efficiency with which those nucleic acid molecules are transcribed, the efficiency with which the resultant transcripts are translated, and the efficiency of post-translational modifications. Recombinant techniques useful for increasing the expression of nucleic acid molecules of the present invention include, but are not limited to, operatively linking nucleic acid molecules to high-copy number plasmids, integration of the nucleic acid molecules into one or more host cell chromosomes, addition of vector stability sequences to plasmids, substitutions or modifications of transcription control signals (e.g., promoters, operators, enhancers), substitutions or modifications of translational control signals (e.g., ribosome binding sites, Shine-Dalgarno sequences), modification of nucleic acid molecules of the present invention to correspond to the codon usage of the host cell, deletion of sequences that destabilize transcripts, and use of control signals that temporally separate recombinant cell growth from recombinant enzyme production during fermentation. The activity of an expressed recombinant protein of the present invention may be improved by fragmenting, modifying, or derivatizing nucleic acid molecules encoding such a protein.

Also provided by the present invention are recombinant cells transformed with a nucleic acid described herein.

Transformation of a nucleic acid molecule into a cell can be accomplished by any method by which a nucleic acid molecule can be inserted into the cell. Transformation techniques include, but are not limited to, transfection, electroporation, microinjection, lipofection, adsorption, and protoplast fusion. A recombinant cell may remain unicellular or may grow into a tissue, organ or a multicellular organism. Transformed nucleic acid molecules of the present invention can remain extrachromosomal or can integrate into one or more sites within a chromosome of the transformed (i.e., recombinant) cell in such a manner that their ability to be expressed is retained.

Suitable host cells to transform include any cell that can be transformed with a nucleic acid molecule of the present invention. Host cells can be either untransformed cells or cells that are already transformed with at least one nucleic acid molecule (e.g., nucleic acid molecules encoding one or more proteins of the present invention and/or other proteins useful in the production of multivalent vaccines). Host cells of the present invention either can be endogenously (i.e., naturally) capable of producing HA mimic of the present invention or can be capable of producing such proteins after being transformed with at least one nucleic acid molecule of the present invention. Host cells of the present invention can be any cell capable of producing at least one protein of the present invention, and include bacterial, fungal (including yeast), other insect, other animal and plant cells. Preferred host cells include bacterial, mycobacterial, yeast, parasite, insect and mammalian cells. More preferred host cells include *Salmonella, Escherichia, Bacillus, Listeria, Saccharomyces, Spodoptera, Mycobacteria, Trichoplusia,* BHK (baby hamster kidney) cells, COS (e.g., COS-7) cells, and Vero cells. Particularly preferred

host cells are *Escherichia coli*, including *E. coli* K-12 derivatives; and insect cell systems which utilize baculovirus.

A recombinant cell is preferably produced by transforming a host cell with one or more recombinant molecules, each comprising one or more nucleic acid molecules of the present invention operatively linked to an expression vector containing one or more transcription control sequences. The phrase "operatively linked" refers to insertion of a nucleic acid molecule into an expression vector in a manner such that the molecule is able to be expressed when transformed into a host cell.

A recombinant cell of the present invention includes any cell transformed with at least one of any nucleic acid molecule of the present invention. Suitable and preferred nucleic acid molecules as well as suitable and preferred recombinant molecules with which to transform cells are disclosed herein.

The translation of the RNA into a peptide or a protein will result in the production of the HA mimic protein which can be identified, for example, by the activity of HA mimic or by immunological reactivity with an anti-HA mimic antibody. In this method, pools of mRNA isolated from HA mimic-producing cells can be analyzed for the presence of an RNA which encodes at least a portion of the HA mimic protein. Further fractionation of the RNA pool can be done to purify the HA mimic RNA from non-HA mimic RNA. The peptide or protein produced by this method may be analyzed to provide amino acid sequences which in turn are used to provide primers for production of HA mimic cDNA, or the RNA used for translation can be analyzed to provide nucleotide sequences encoding HA mimic and produce probes for the production of HA mimic cDNA. These methods are known in the art and can be found in, for example, Sambrook, J., Fritsch, E. F., Maniatis,

T. in *Molecular Cloning: A Laboratory Manual, Second Edition*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 1989.

Other types of libraries, as well as libraries constructed from other cells or cell types, may be useful for isolating HA mimic-encoding DNA. Other types of libraries include, but are not limited to, cDNA libraries derived from other mammals or cell lines derived from other mammals, and genomic DNA libraries. Preparation of cDNA libraries can be performed by standard techniques. Well known cDNA library construction techniques can be found in, for example, Sambrook, J., et al., *ibid.*

In one embodiment, an isolated protein of the present invention is produced by culturing a cell capable of expressing the protein under conditions effective to produce the protein, and recovering the protein. A preferred cell to culture is a recombinant cell of the present invention. Effective culture conditions include, but are not limited to, effective media, bioreactor, temperature, pH and oxygen conditions that permit protein production. An effective medium refers to any medium in which a cell is cultured to produce a HA mimic of the present invention. Such a medium typically comprises an aqueous medium having assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. Cells of the present invention can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes, and petri plates. Culturing can be carried out at a temperature, pH and oxygen content appropriate for a recombinant cell. Such culturing conditions are within the expertise of one of ordinary skill in the art.

Depending on the vector and host system used for production, resultant proteins of the present invention may either remain within the recombinant cell; be secreted into the fermentation medium; be secreted into

a space between two cellular membranes, such as the periplasmic space in *E. coli*; or be retained on the outer surface of a cell or viral membrane. The phrase "recovering the protein", as well as similar phrases, refers to collecting the whole fermentation medium containing the protein and need not imply additional steps of separation or purification. Proteins of the present invention can be purified using a variety of standard protein purification techniques, such as, but not limited to, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing and differential solubilization. Proteins of the present invention are preferably retrieved in "substantially pure" form. As used herein, "substantially pure" refers to a purity that allows for the effective use of the protein as a therapeutic composition or diagnostic. A therapeutic composition for animals, for example, should exhibit few impurities.

In addition, a recombinant HA mimic can be separated from other cellular proteins by use of an immunoaffinity column made with monoclonal or polyclonal antibodies specific for the HA mimic, or polypeptide fragments of the HA mimic.

The present invention also includes isolated (i.e., removed from their natural milieu) antibodies that selectively bind to an HA mimic protein of the present invention or a mimetope thereof (i.e., anti-HA mimic antibodies). As used herein, the term "selectively binds to" the HA mimic protein refers to the ability of antibodies of the present invention to preferentially bind to specified proteins and mimetopes thereof of the present invention. Binding can be measured using a variety of methods standard in the art including enzyme immunoassays (e.g., ELISA), immunoblot assays, etc.; see, for example, Sambrook et al., *ibid*. An anti-HA mimic antibody preferably

selectively binds to A HA mimic in such a way as to reduce the activity of that protein. These antibodies may be admixed or conjugated with additional materials, such as cytotic agents or other antibody fragments.

Isolated antibodies of the present invention can include antibodies in a bodily fluid (such as, but not limited to, serum), or antibodies that have been purified to varying degrees. Antibodies of the present invention can be polyclonal or monoclonal. Functional equivalents of such antibodies, such as antibody fragments and genetically-engineered antibodies (including single chain antibodies or chimeric antibodies that can bind to more than one epitope) are also included in the present invention.

A preferred method to produce antibodies of the present invention includes (a) administering to an animal an effective amount of a protein, peptide or mimetope thereof of the present invention to produce the antibodies and (b) recovering the antibodies. In another method, antibodies of the present invention are produced recombinantly using techniques as heretofore disclosed to produce HA mimic proteins of the present invention.

Compositions of the present invention can be administered to any animal having at least one HA mimic-target enzyme that can be inhibited by a therapeutic compound of the present invention or by a protein expressed by a nucleic acid molecule contained in a therapeutic composition. Preferred animals to treat are humans, although other mammals, such as cattle, pigs, sheep, horses, cats, dogs, and other pets, work and/or economic food animals are also within the scope of the present invention.

Therapeutic compositions of the present invention can be formulated in an excipient that the animal to be treated can tolerate. Examples of such excipients include water, saline, Ringer's solution, dextrose solution, Hank's

solution, and other aqueous physiologically balanced salt solutions. Nonaqueous vehicles, such as fixed oils, sesame oil, ethyl oleate, or triglycerides may also be used. Other useful formulations include suspensions containing viscosity enhancing agents, such as sodium carboxymethylcellulose, sorbitol, or dextran. Excipients can also contain minor amounts of additives, such as substances that enhance isotonicity and chemical stability. Examples of buffers include phosphate buffer, bicarbonate buffer and Tris buffer, while examples of preservatives include thimerosal, cresols, formalin and benzyl alcohol. Standard formulations can either be liquid injectables or solids which can be taken up in a suitable liquid as a suspension or solution for injection. Thus, in a non-liquid formulation, the excipient can comprise dextrose, human serum albumin, preservatives, etc., to which sterile water or saline can be added prior to administration.

Administration of the present compounds can be by a variety of routes known to those skilled in the art including, but not limited to, subcutaneous, intradermal, intravenous, intranasal, oral, transdermal, intramuscular routes and other parenteral routes.

In one embodiment of the present invention, a therapeutic composition can include an adjuvant. A preferred adjuvant is a member of the group of non-steroidal antiinflammatory drugs, such as ibuprofen.

In another embodiment of the present invention, a therapeutic composition can include a carrier. Carriers include compounds that increase the half-life of a therapeutic composition in the treated animal. Suitable carriers include, but are not limited to, polymeric controlled release vehicles, biodegradable implants, liposomes, bacteria, viruses, other cells, oils, esters, and glycols.

Another embodiment of the present invention is a controlled release formulation that is capable of slowly releasing a composition of the present invention into an animal. As used herein, a controlled release formulation comprises a composition of the present invention in a controlled release vehicle. Suitable controlled release vehicles include, but are not limited to, biocompatible polymers, other polymeric matrices, capsules, microcapsules, microparticles, bolus preparations, osmotic pumps, diffusion devices, liposomes, lipospheres, and transdermal delivery systems. Other controlled release formulations of the present invention include liquids that, upon administration to an animal, form a solid or a gel *in situ*. Preferred controlled release formulations are biodegradable (i.e., bioerodible).

A preferred controlled release formulation of the present invention is capable of releasing a composition of the present invention into the blood of an animal at a constant rate sufficient to attain therapeutic dose levels of the composition to reduce HA-mediated biological responses in the animal. The therapeutic composition is preferably released over a period of time ranging from about 1 day to about 12 months, and include release over a 2, 3, 4 , 5, 6, 7 day through a 30 day time period.

Acceptable protocols to administer therapeutic compositions of the present invention in an effective manner include individual dose size, number of doses, frequency of dose administration, and mode of administration. Determination of such protocols can be accomplished by those skilled in the art. A suitable single dose is a dose that is capable of protecting (i.e., preventing or treating) an animal from disease when administered one or more times over a suitable time period. The need for additional administrations of a therapeutic composition can be determined by one of skill in the art in accordance with the given condition of a patient.

According to one embodiment, a nucleic acid molecule of the present invention can be administered to an animal in a fashion to enable expression of that nucleic acid molecule into an HA mimic protein in the animal. Nucleic acid molecules can be delivered to an animal in a variety of methods including, but not limited to, (a) administering a naked (i.e., not packaged in a viral coat or cellular membrane) nucleic acid molecule (e.g., as naked DNA molecules, such as is taught, for example in Wolff et al., 1990, *Science 247*, 1465-1468) or (b) administering a nucleic acid molecule packaged as a recombinant virus or as a recombinant cell (i.e., the nucleic acid molecule is delivered by a viral or cellular vehicle).

A naked nucleic acid molecule of the present invention includes a nucleic acid molecule of the present invention and preferably includes a recombinant molecule of the present invention that preferably is replication, or otherwise amplification, competent. A naked nucleic acid of the present invention can comprise one or more nucleic acid molecules of the present invention in the form of, for example, a bicistronic recombinant molecule having, for example one or more internal ribosome entry sites. Preferred naked nucleic acid molecules include at least a portion of a viral genome (i.e., a viral vector). Preferred viral vectors include those based on alphaviruses, poxviruses, adenoviruses, herpesviruses, picornaviruses, and retroviruses, with those based on alphaviruses (such as Sindbis or Semliki virus), species-specific herpesviruses and species-specific poxviruses being particularly preferred. Any suitable transcription control sequence can be used, including those disclosed as suitable for protein production. Particularly preferred transcription control sequence include cytomegalovirus intermediate early (preferably in conjunction with Intron-A), Rous Sarcoma Virus long terminal repeat, and tissue-specific transcription control sequences, as well as transcription control sequences endogenous to viral vectors if viral vectors are used. The incorporation of "strong" poly(A) sequences are also preferred.

Naked nucleic acid molecules of the present invention can be administered by a variety of methods. Suitable delivery methods include, for example, intramuscular injection, subcutaneous injection, intradermal injection, intradermal scarification, particle bombardment, oral application, and nasal application, with intramuscular injection, intradermal injection, intradermal scarification and particle bombardment being preferred. A preferred single dose of a naked DNA molecule ranges from about 1 nanogram (ng) to about 1 milligram (mg), depending on the route of administration and/or method of delivery, as can be determined by those skilled in the art. Examples of administration methods are disclosed, for example, in U.S. Patent No. 5,204,253, by Bruner, et al., issued April 20, 1993, PCT Publication No. W0 95/19799, published July 27, 1995, by McCabe, and PCT Publication No. WO 95/05853, published March 2, 1995, by Carson, et al. Naked DNA molecules of the present invention can be contained in an aqueous excipient (e.g., phosphate buffered saline) and/or with a carrier (e.g., lipid-based vehicles), or it can be bound to microparticles (e.g., gold particles).

A recombinant virus of the present invention includes a recombinant molecule of the present invention that is packaged in a viral coat and that can be expressed in an animal after administration. Preferably, the recombinant molecule is packaging-deficient and/or encodes an attenuated virus. A number of recombinant viruses can be used, including, but not limited to, those based on alphaviruses, poxviruses, adenoviruses, herpesviruses, picornaviruses and retroviruses.

When administered to an animal, a recombinant virus of the present invention infects cells within the recipient animal and directs the production of a protein molecule that is capable of affecting HA-mediated biological responses in the animal. For example, a recombinant virus comprising an

HA mimic nucleic acid molecule of the present invention is administered according to a protocol that results in the animal producing an amount of protein sufficient to affect HA-mediated biological responses. Administration protocols are similar to those described herein for protein-based compositions, with subcutaneous, intramuscular, intranasal and oral administration routes being preferred.

Pharmaceutically useful compositions comprising an HA mimic DNA or an HA mimic protein, may be formulated according to known methods such as by the admixture of a pharmaceutically acceptable carrier, or by modification with additional chemical moieties so as to form a chemical derivative. Examples of such carriers, modifications and methods of formulation may be found in Remington's Pharmaceutical Sciences. To form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the protein or DNA.

The present invention also has the objective of providing suitable topical, oral, systemic and parenteral formulations of the pharmaceutical compounds herein provided. The formulations can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compounds can be formulated for oral administration in the form of tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection. Likewise, they may also be administered intravenously (both bolus and infusion), during angioplasty/catheterization, intraperitoneally, subcutaneously, topically with or without occlusion, or intramuscularly, all using forms well known to those of ordinary skill in the pharmaceutical arts.

An HA mimic of the present invention can be combined with a buffer in which the HA mimic molecule is solubilized, and/or with a carrier. Suitable buffers and carriers are known to those skilled in the art. Examples of suitable buffers include any buffer in which an HA mimic can function to inhibit its target enzyme(s), such as, but not limited to, phosphate buffered saline, water, saline, phosphate buffer, bicarbonate buffer, HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffered saline), TES buffer (Tris-EDTA buffered saline), Tris buffer and TAE buffer (Tris-acetate-EDTA). Examples of carriers include, but are not limited to, polymeric matrices, toxoids, and serum albumins, such as bovine serum albumin.

In the methods of the present invention, the compounds herein described in detail can form the active ingredient, and are typically administered in admixture with suitable pharmaceutical diluents, excipients or carriers (collectively referred to herein as "carrier" materials) suitably selected with respect to the intended form of administration, that is, oral tablets, capsules, elixirs, syrups and the like, and consistent with conventional pharmaceutical practices.

For instance, for oral administration in the form of a tablet or capsule, the active drug component can be combined with an oral, non-toxic pharmaceutically acceptable inert carrier such as ethanol, glycerol, water, and the like. Moreover, when desired or necessary, suitable binders, lubricants, disintegrating agents and coloring agents can also be incorporated into the mixture. Suitable binders include, without limitation, starch, gelatin, natural sugars such as glucose or beta-lactose, corn sweeteners, natural and synthetic gums such as acacia, tragacanth or sodium alginate, carboxymethylcellulose, polyethylene glycol, waxes and the like. Lubricants used in these dosage forms include, without limitation, sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride, and

the like. Disintegrators include, without limitation, starch, methyl cellulose, agar, bentonite, xanthan gum and the like.

For liquid forms the active drug component can be combined in suitably flavored suspending or dispersing agents such as the synthetic and natural gums, for example, tragacanth, acacia, methylcellulose and the like. Other dispersing agents which may be employed include glycerin and the like. For parenteral administration, sterile suspensions and solutions are desired. Isotonic preparations which generally contain suitable preservatives are employed when intravenous administration is desired.

Topical preparations containing the active drug component can be admixed with a variety of carrier materials well known in the art, such as, e.g., alcohols, aloe vera gel, allantoin, glycerine, vitamin A and E oils, mineral oil, PPG2 myristyl propionate, and the like, to form, e.g., alcoholic solutions, topical cleansers, cleansing creams, skin gels, skin lotions formulations. The compounds of the present invention can also be administered in the form of liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles and multilamellar vesicles. Liposomes can be formed from a variety of phospholipids, such as cholesterol, stearylamine or phosphatidylcholines.

The compounds of the present invention may also be coupled with soluble polymers as targetable drug carriers. Such polymers can include polyvinyl-pyrrolidone, pyran copolymer, polyhydroxypropylmethacryl-amidephenol, polyhydroxy-ethylaspartamidephenol, or polyethyleneoxidepolylysine substituted with palmitoyl residues. Furthermore, the compounds of the present invention may be coupled to a class of biodegradable polymers useful in achieving controlled release of a drug, for example, polylactic acid, polyepsilon caprolactone, polyhydroxy

butyric acid, polyorthoesters, polyacetals, polydihydro-pyrans, polycyanoacrylates and cross-linked or amphipathic block copolymers of hydrogels.

Therefore, methods are also provided herein, which utilize the compounds, formulations, compositions and protocols described above. In particular, there are provided methods to antagonize or inhibit HA-target enzymes, comprising administering an HA mimic of the present invention. Preferred methods utilize the preferred and most preferred HA mimics.

The present invention also provides methods to treat and/or reduce the risk of HA-related diseases in a patient in need of such treatment, comprising administering the presently-disclosed HA mimics.

Lastly, the present invention also provides methods for producing the present HA mimics in bodily fluid, comprising: producing a transgenic animal that expresses in bodily fluid a transgene which encodes an HA mimic of the present invention, wherein the HA mimic is secreted into the bodily fluid produced by the transgenic animal; collecting bodily fluid from the transgenic animal, which bodily fluid contains the HA mimic; and isolating the HA mimic from the collected bodily fluid. Preferred are methods wherein the bodily fluid is selected from the group consisting of: milk or urine. Those methods wherein the bodily fluid is milk and the animal is selected from the group consisting of: goat; sheep; and cow are more preferred. Most preferred are methods for producing an HA mimic in goat milk, comprising: producing a transgenic goat that expresses in mammary tissue a transgene which encodes an HA mimic of the present invention, wherein the HA mimic is secreted into the milk produced by the transgenic goat; collecting milk from the transgenic goat which milk contains the HA mimic; and isolating the HA mimic from the collected milk. This aspect of the invention can be

accomplished according to US Patent Serial Number 5,843,705, which patent is hereby incorporated by reference in its entirety.

The present invention also provides methods to identify the ability of a test compound to interfere with the present HA mimic/target enzyme interaction, comprising: contacting the test compound with a protein of the present invention; and determining whether the test compound and said protein interact.

The following examples illustrate the present invention without, however, limiting it. It is to be noted that the Examples include a number of molecular biology, microbiology, immunology and biochemistry techniques considered to be known to those skilled in the art. Disclosure of such techniques can be found, for example, in Sambrook et al., *ibid.*, and related references.

Examples

Example 1 Screening of Phage Library

A completely random phage library expressing a 15-amino acid peptide was used to screen phage that can bind to the HA binding domains. The principle of the screening process relies on selectively eluting phage that bind to the HA binding domains. Three constructs expressing the HA binding domains of RHAMM (Receptor of HA Mediated Motility) were prepared. The first two GST-P1 (70 amino acids) and GST-P6 (68 amino acids are glutathione-S-transferase fusion constructs. The third is a thioredoxin fusion construct (62 amino acids 312c). The HA binding domain portions of these polypeptides are identical except for several amino acids on the N or C terminus. A

portion of the phage screens were performed with 312c as the target peptide while all binding assays used GST-P1. All constructs have a thrombin cleavage site, which was key to the selective elution method used.

To screen phage which selectively bound to the HA binding domains, the following method was used. Purified GST-P6 or 312c (0.1mg/mL) were immobilized on a 30 mm polystyrene petri dish in 50 mM Tris, pH 8.0. The phage library was applied in binding buffer (Tris buffered saline pH 7.5, 0.05% Tween, 0.1% polyvinylpyrolidone) (TBST-PVP). Nonspecific phage were washed off,and the phage bound to the HA binding domains were recovered either by cleaving the HA binding domain from the fusion protein using thrombin (first and second rounds of screening), or eluting with HA (third round of screening), as summarized in FIG. 1. Finally, the peptide encoding DNAs present in the phages were sequenced. Table 1 shows the numbers of phages recovered during the second round of screening.

Table 1

Screen Step	cfU/mL		
	312c	GST-P6	Control
Input	1×10^{10}	1×10^{10}	1×10^{10}
First Wash	7×10^{4}	6×10^{4}	6×10^{4}
Last Wash	8	10	3
40µg/ml HA elution	1160	1320	-
1 mg/mL HA elution	280	160	-
Thrombin cleavage	128	168	20

From the results shown in Table 1, it was concluded that most or all nonspecific phage were washed off. After adding HA, some specific phage were replaced by HA. Therefore, the phage from the HA elution were

considered to have specifically bound to the HA binding domain. The amino acid sequences of eleven peptides (HA1 -HA11; SEQ ID NO: 1 through SEQ ID NO: 11) were determined. Peptides corresponding to HA2-HA5 were synthesized with an amino terminal biotin. The ability of these peptides to bind competitively to GST-P1 was tested. First, GST-P1 was immobilized onto a 96 well plate as described above. After rigorous washing with binding buffer, the biotinylated peptides were applied. Each experiment was performed in quadruplicate. The concentration of peptide applied was titrated such that binding curves could be calculated. The plate was agitated at room temperature for two hours and then washed with binding buffer. Streptavidin alkaline phosphatase (Pharmacia Biotec) was applied in binding buffer at a 1:5000 dilution. This was followed by washing and application of commercially available BCIP/NBT substrate (Sigma). Results are shown in FIG. 2, which shows specific, compatible binding of HA2 and HA3 with GST-P1 L.

Example 2 Screening of One-bead, One-peptide Library

Two bead libraries were constructed in this lab with eight amino acid peptides covalently attached to 100μM Tentagel S (Rapp Polymere GmbH). This method ensures that only one species of peptide is attached to one bead, generally 50nmoles of peptide/bead. One of the bead libraries was designed to be an anionic biased library of 8-mers was designed and prepared on the basis of alternating amino acids with carboxylic acid chains that mimic the 6-carboxylic acids of glucuronic acid (XNXNXNXN; X= any amino acid except Cys, Arg, Lys; N= negatively charged side chains, D-Asp, L-Asp, D-Glu, L-Glu). Both libraries were screened against GST-P1. the method is described as follows:

About 2 ml of beads (approx. 10^5 beads), were prepared in a 5-ml fritted column by sequentially washing and equilibrating in binding buffer. Pure GST-P1 was added to a final concentration of 0.5 ng/ml protein. The beads were incubated with shaking at room temperature for two hours. After vigorous washing in binding buffer, anti-GST-P1 antibody was added (1:2000), the beads were incubated and washed. This was followed by a secondary antibody anti-IgG conjugated to alkaline phosphatase (1:3000). The beads were then washed and placed in a large petri dish. The dish was drained and alkaline phosphatase substrate was added. The substrate was prepared by suspending 50 mg of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in 1 ml of DMF and adding 33 µl of this mixture to 10 ml of reaction buffer (TBS pH 8.5 + 1mM $MgCl_2$) Positive beads in reaction buffer would begin to turn blue/purple after 15 minutes. Positive beads were removed using a pipettor and treated with 6 M guanidium hydrochloride, followed by a series of washes in DMF. This screening process was repeated twice, after which approximately 300 candidate beads remained. A third screen was similar to the process mentioned above except that the beads were preincubated with excess natural ligand, in this case 1 mg/mL of partially degraded HA. The beads then collected were those that did not turn blue/purple, indicating that they bound to the same place as the natural ligand. At least 7 candidates were discovered through this screening process that have unique, but related amino acid sequences. The first such positive bead sequences as the primary structure Phe-Asp-Phe Asp-Ser-Glu-Tyr-Glu (SEQ ID NO: 12).

Example 3 Peptide Binding Assays

To verify that the peptides obtained from phage screening indeed bound to the HA binding domains of RHAMM, a solid phase 96-well plate assay was employed. 50 µL GST-P1 (0.25 mg/mL) in glutathione elution buffer was

immobilized in wells of a polystyrene 96-well plate (Greiner). The plate is then blocked with 250 μL TBS-T-PVP-40 bovine serum albumin (BSA) (20 mM Tris, pH 7.5, 130 mM NaCl, 0.1% polyvinylpyrolidone-40, 1% BSA) for 2 hr to overnight with shaking. The plate is washed with TBS-T-PVP-40 (no BSA) and if blocking is required, the plate is incubated with excess HA or chondroitin sulfate (CS) for 1 hr prior to the addition of biotinylated ligand. The biotinylated ligand is incubated for 25 min (determined from an initial timecourse). The plate is washed 3 X, and blocked with TBS-T-PVP-40-BSA for 1 hr. Streptavidin HRP is added at a dilution of 0.5 μg /mL in TBS-T-PVP-40. HA is added to the streptavidin solution, since it is known that streptavidin HRP binds nonspecifically to HA and CS, which inherently causes misleading results in the competition experiments. After 40 min incubation, the plate is washed, and the presence of biotinylated peptide is determined using TMB. Colorimetric development is read at 590 nm (Perkin Elmer HTS 7000). All measurements are performed in quadruplicate. The figure on the following page outlines this method.

For the purposes of generating binding data, the color generated from streptavidin HRP is calibrated and used to estimate the total biotinylated molecules immobilized on the plate. Eadie Hofstee analyses were to calculate binding constants.

Example 4 Pepspots assay

Since the biased library contains both D or L isomers of the alternating charged residues it was necessary to decipher which set of natural or unnatural amino acids provided the best binding to GST-RHAMM-P1. For each peptide obtained from sequencing there are 16 possible combinations (44). This peptide library was constructed as an array of spots on cellulose membrane (Pepspots membranes, Jerini Biotools) where each spot contains a

single species of peptide (3-5nmoles/spot). This membrane contained a total of 68 spots representing 6-18, 6-19, 6-20, 6-21 and 4 control spots taken from a negative bead.

To detect binding we incubated the blocked membrane with GST-RHAMM-P1. After washing, the bound protein was semí-dry transferred from the cellulose membrane onto a PVDF membrane. The PVDF membrane was blocked and incubated with anti-GST, followed by anti-goat antibody-horseradish peroxidase conjugated. To detect bound material, a chemilumiscence detection kit was used followed by exposure to film. The developed film was digitized and quantified to evaluate the strength of GST-RHAMM-P1 binding to each well. To verify the results the experiment was repeated several times at various GST-RHAMM-P1 concentrations.

Example 5 Preferred Binding motifs

Depicted below is a summary of the results found from phage and bead screening experiments. The X stands for unknown amino acids that arose from sequencing difficulties.

Random phage libraries

HA1 WPVSLTVCSAVWCPL (SEQ ID NO 1)
HA2 GVCNADFCWLPAVVV (SEQ ID NO 2)
HA3 SASPSASKLSLMSTV (SEQ ID NO 3)
HA4 IPPILPAYTLLGHPR (SEQ ID NO 4)
HA5 YSVYLSVAHNFVLPS (SEQ ID NO 5)
HA6 HWCLPLLACDTFARA (SEQ ID NO 6)

Biased bead peptides

6-18 MDYEPEQE (SEQ ID NO 7)

6-19 YDSEYESE (SEQ ID NO 8)

6-20 FDFDSEYE (SEQ ID NO 9)

6-21 EDQEAXEX (SEQ ID NO 10)

6-22 EDAENXDX (SEQ ID NO 11)

Random bead peptides

R-1 SGRPYKPP (SEQ ID NO 12)

R-2 YXSSNKPG (SEQ ID NO 13)

R-3 EGEWPVYP (SEQ ID NO 14)

R-4 WNYTEAKG (SEQ ID NO 15)

Figure 1 shows the pertinent results from such an experiment.

The phage results show a motif: ILPA (SEQ ID NO 16) or WLPA (SEQ ID NO 17) with the potential for a hydrophobic amino acid to further govern binding. The presence of prolines is enforced by the random bead libraries where motifs as WPVYP (SEQ ID NO 18) and YKPP (SEQ ID NO 19) are seen. It is believed that a four amino acid sequence in the proper conformation is sufficient to regulate binding to the HA binding domains of RHAMM. The biased library showed that hydrophobic amino acids are important in predisposing a peptide to specific binding: YDSEYESE (SEQ ID NO 20). This information was capitalized on by the Pepspots assay exemplified in Example 4.

The results can be summarized in the following way: In all cases unnatural amino acids facilitated binding. In all but one case the binding of completely natural amino acids provided no binding. Further, we note that a C-terminal D-glutamate acid provides optimal binding and the presence of D-glutamate

or D-aspartate as the sixth ensures high binding. From this information we have constructed a model for the best binding peptides in which the pattern of amino acids follows this sequence: X-A/a-X-A/a-X-a-X-a. (X) can be any amino acid and is often hydrophobic, (A) is L-glutamate or L-aspartate and (a) is D-glutamate or D-aspartate.

Example 6 Preferred examples of TSG-6 binding peptides

Tumor necrosis factor (TNF) stimulated gene-6 (TSG-6) is an HA binding protein whose carbohydrate binding domain is a member of the link module consensus family of which CD44 is also a member(Bajorath et al., 1998). TSG-6 is present in higher than normal concentrations at sites of joint and cartilage inflammation (Maier et al., 1996) and is thought to mediate some of the pathological conditions of arthritis. The structure of the HA binding domain was solved using NMR and is the only structure presently available of an HA binding domain (PDB ascension # 1TSG) (Kohda et al., 1996).

Using TSG-6 as a target protein we screened our random library against this protein using the same experimental methods described previously. We performed this experiment at two different pH solutions: pH 5.7 and 7.5. This is because we wanted to compare neutral pH conditions to that of conditions where maximal HA binding occurs (Parkar et al., 1998). The following sequences were found:

pH 5.7
GYYFNVAM
WAYNFLVM
TQSLNNHM
WWPFINAY

WWKADMVG
WWPFINAY
MALQLPYY
IIYEEFFV
ISINNRWY
VTPPVYFT

pH 7.5
QIRNGWFW
SWWFGPLA
GDWEQILT
PAGFGWNL
NMRFNIEN
QMTFFDGV

While these sequences are different from those found in the screening experiment using GST-P1 as the target protein, there are similarities. There is a motif of double hydrophobic amino acids in these peptides.

Although the present invention has been fully described herein, it is to be noted that various changes and modifications are apparent to those skilled in the art. Such changes and modifications are to be understood as included within the scope of the present invention as defined by the appended claims.

WHAT IS CLAIMED IS:

1. An HA mimic comprising a polypeptide comprising alternating acidic / non-acidic residues, wherein said mimic binds to the hyaluronic acid binding domain of the receptor for hyaluronan mediated mobility.

2. An HA mimic of claim 1, wherein said polypeptide is four to 15 residues in length.

3. An HA mimic of claim 2, wherein said residues include at least one dextrorotatory residue.

4. An HA mimic of claim 1, comprising an amino acid sequence selected from the group consisting of: SEQ ID NO 1; SEQ ID NO 2; SEQ ID NO 3; SEQ ID NO 4; SEQ ID NO 5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 8; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11; SEQ ID NO 12; SEQ ID NO 13; SEQ ID NO 14; SEQ ID NO 15; SEQ ID NO 16; SEQ ID NO 17; SEQ ID NO 18; SEQ ID NO 19; and SEQ ID NO 20.

5. An HA mimic of claim 1, comprising a homologue of an amino acid sequence selected from the group consisting of: SEQ ID NO 1; SEQ ID NO 2; SEQ ID NO 3; SEQ ID NO 4; SEQ ID NO 5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 8; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11; SEQ ID NO 12; SEQ ID NO 13; SEQ ID NO 14; SEQ ID NO 15; SEQ ID NO 16; SEQ ID NO 17; SEQ ID NO 18; SEQ ID NO 19; and SEQ ID NO 20, wherein said homologue binds to the hyaluronic acid binding domain of the receptor for hyaluronan mediated mobility.

6. A nucleic acid compound comprising a nucleic acid which encodes an amino acid sequence selected from the group consisting of: SEQ ID NO 1; SEQ ID NO 2; SEQ ID NO 3; SEQ ID NO 4; SEQ ID NO 5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 8; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11; SEQ ID NO 12; SEQ ID NO 13; SEQ ID NO 14; SEQ ID NO 15; SEQ ID NO 16; SEQ ID NO 17; SEQ ID NO 18; SEQ ID NO 19; and SEQ ID NO 20; and a homologue of an amino acid sequence selected from the group consisting of: SEQ ID NO 1; SEQ ID NO 2; SEQ ID NO 3; SEQ ID NO 4; SEQ ID NO 5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 8; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11; SEQ ID NO 12; SEQ ID NO 13; SEQ ID NO 14; SEQ ID NO 15; SEQ ID NO 16; SEQ ID NO 17; SEQ ID NO 18; SEQ ID NO 19; and SEQ ID NO 20, wherein said homologue binds to the hyaluronic acid binding domain of the receptor for hyaluronan mediated mobility.

6a. An HA mimic comprising a polypeptide comprising double hydrophobic residues, wherein said mimic binds to the hyaluronic acid binding domain of the TSG-6.

7. An HA mimic of claim 6a, which is selected from the group consisting of: GYYFNVAM; WAYNFLVM; TQSLNNHM; WWPFINAY; WWKADMVG; WWPFINAY; MALQLPYY; IIYEEFFV; ISINNRWY; VTPPVYFT; QIRNGWFW; SWWFGPLA; GDWEQILT; PAGFGWNL; NMRFNIEN; and QMTFFDGV, or a homologue thereof, wherein said homologue binds to hyaluronic acid binding domain of the TSG-6.

8. A method to affect a hyaluronidase-mediated biological response in a patient in need of such affecting , comprising administering an HA mimic of claim 1.

9. A method to inhibit hyaluronidase activity in a patient in need of such inhibition, comprising administering an HA mimic of claim 1.

10. A method to bind receptors that bind hyaluronic acid, comprising introducing an HA mimic of claim 1.

11. A method to mimic hyaluronic acid in a biological system, comprising introducing an HA mimic of claim 1.

12. A method to affect cell signalling associated with hyaluronic acid/hyaluronic acid receptor interactions, comprising introducing an HA mimic of claim 1.

13. A method to treat hyaluronic acid-associated disease in a patient in need of such treatment, comprising administering an HA mimic of claim 1.

14. A method of claim 13, wherein said disease is selected from the group consisting of: inflammation; tumor angiogenesis; skin disease; bone disease; wound healing;osteoarthritis; rheumatoid arthritis; infectious disease; immune disease; and cardiovascular disease.

15. A method to inhibit metastasis in a patient in need of such inhibition, comprising administering an HA mimic of claim 1.

16. A method to inhibit fertilization in a patient in need of such inhibition, comprising administering an HA mimic of claim 1.

17. A method to introduce an agent into a cell which binds hyaluronic acid, comprising administering the agent in conjunction with an HA mimic of claim 1.

18. A method to isolate peptides that mimic a ligand's binding to a receptor comprising the steps of:

(a) preparing a random library of peptides and binding said library to a bead;

(b) placing the library in contact with the receptor under conditions for binding;

(c) washing off unbound peptides;

(d) contacting the washed bead-bound library with anti-receptor antibody;

(e) detecting and selecting beads having antibody bound thereto;

(f) eluting the antibody;

(g) repeating steps (a) through (f) and then repeating steps (a) through (c), and then incubating the beads in the presence of receptor prior to adding anti-receptor antibody;

(h) detecting and selecting beads that did not bind antibody;

(i) determining the sequence of the bead-bound peptide.

19. An HA mimic comprising the sequence:

$$X\text{-}A/a\text{-}X\text{-}A/a\text{-}X\text{-}a\text{-}X\text{-}a_1$$

wherein (X) can be any amino acid, (A) is L-glutamate or L-aspartate and (a) and (a_1) are D-glutamate or D-aspartate.

20. An HA mimic of claim 19, wherein X is hydrophobic.

21. An HA mimic of claim 20, wherein a_1 is D-glutamate.

ABSTRACT OF THE DISCLOSURE

HA mimics and methods related thereto are disclosed. In particular, mimics with structures determined by virtue of novel methods, and the novel methods are disclosed. The HA mimics are useful for a variety of HA-related uses.

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Synthesis and Selective Cytotoxicity of a Hyaluronic Acid-Antitumor Bioconjugate

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Abstract:

A cell-targeted prodrug was developed for the anti-cancer drug Taxol, using hyaluronic acid (HA) as the drug carrier. HA-Taxol bioconjugates were synthesized by linking the Taxol 2'-OH via a succinate ester to adipic dihydrazide-modified HA (HA-ADH). The coupling of Taxol-NHS ester and HA-ADH provided several HA bioconjugates with different levels of ADH modification and different Taxol loadings. A fluorescent BODIPY-HA was also synthesized to illustrate cell targeting and uptake of chemically modified HA using confocal microscopy. HA-Taxol conjugates showed selective toxicity toward the human cancer cell lines (breast, colon, and ovarian) that are known to overexpress HA receptors, while no toxicity was observed toward a mouse fibroblast cell line at the same concentrations used with the cancer cells. The drug carrier HA-ADH was completely nontoxic. The selective cytotoxicity is consistent with the results from confocal microscopy, which demonstrated that BODIPY-HA only entered the cancer cell lines.

Introduction

Hyaluronic acid (HA)[1] (Figure 1), a linear polysaccharide of alternating D-glucuronic acid (GlcUA) and *N* -acetyl-D-glucosamine (GlcNAc) units, adopts a three-dimensional structure in solution that shows extensive intramolecular hydrogen bonding. This restricts the conformational flexibility of the polymer chains and induces distinctive secondary (helical) and tertiary (coiled coil) interactions (*1*). HA is one of several glycosaminoglycan components of the extracellular matrix (ECM), the synovial fluid of joints, and the scaffolding comprising cartilage (*2*). The remarkable viscoelastic properties of HA and commercial cross-linked derivatives (*3*) account for their usefulness in joint lubrication. HA-protein interactions play crucial roles in cell adhesion, growth, and migration (*4-6*), and HA acts as a signaling molecule in cell motility, in inflammation, wound healing, and cancer metastasis (*7*). The immunoneutrality of HA makes it an excellent building block for the development of novel biocompatible and biodegradable biomaterials used in tissue engineering and drug delivery systems (*8-10*). For example, HA has been employed as both a vehicle and angiostatic agent in cancer therapy (*11-13*).

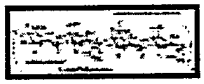

Figure 1 Tetrasaccharide fragment of HA showing the disaccharide repeat units.

The use of biocompatible polymers in the treatment of various ailments has expanded rapidly in the last two decades (14). Moreover, derivatization of such polymers with reporter groups (15) and drugs (16, 17) has emerged as a powerful method for controlling delivery and release of a variety of compounds. Small drug molecules can be linked to the polymer that allows controlled release of the free bioactive group. In general, coupling of antitumor agents to biopolymers provides advantages in drug solubilization, stabilization, localization, and controlled release (18). For example, the linking of a cytotoxic small molecule such as adriamycin to poly(hydroxymethyl)acrylamide (HPMA) gives a new material with improved in vitro tumor retention, a higher therapeutic ratio, avoidance of multidrug resistance (19), and encouraging clinical results. In work with a naturally occurring biocompatible polymer, mitomycin C and epirubicin were coupled to HA by carbodiimide chemistry; the former adduct was selectively taken up by, and toxic to, a lung carcinoma xenograft (20).

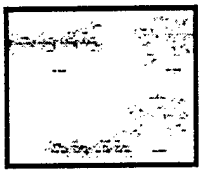

Figure 2 Synthesis of HA-Taxol.

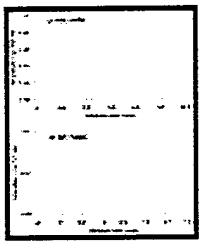

Figure 3 GPC profile. (a) Purified HA-ADH detection at λ = 210 nm. Waters Ultrahydrogel 250, 2000 columns (7.8 mm ID x 30 cm) were used in the analysis. (b) Purified HA-Taxol conjugate detection at λ = 227 nm. Only the Ultrahydrogel 250 column was employed. Eluent was 150 mM, pH 6.5, phosphate buffer/MeOH = 80:20 (v/v); the flow rate was 0.5 mL/min.

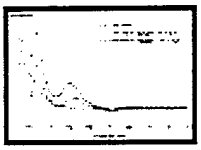

Figure 4 UV spectra of HA-Taxol conjugates.

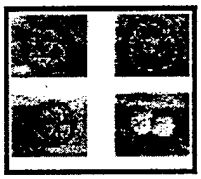

Figure 5 Targeting human breast cancer HBL-100 cells with HA-BODIPY. Panel a fluorescence image shows HA binding to cell surfaces in 3 min. Panels b and c show HA accumulation in the nucleus after 6 and 9 min. In panel d, after 20 min HA, has been completely taken up and fluorescence is dispersed throughout the cells.

Figure 6 Targeting ovarian cancer SK-OV-3 cells with HA-BODIPY. Panel a shows HA uptake by cells in 15 min. Panel b shows HA uptake by cells in 60 min.

HA is overexpressed at sites of tumor attachment (21) to the mesentery and provides a matrix that facilitates invasion (22). HA is an important signal for activating kinase pathways (23, 24) and regulating angiogenesis in tumors (25). Moreover, several types of cellular HA receptors respond to HA as a signal. These include CD44, a family of glycoproteins originally associated with lymphocyte activation; RHAMM, the receptors for HA-mediated cell motility (26, 27); and HARLEC, responsible for receptor-mediated uptake of HA in liver. Using radiolabeled HA analogues and HA-coupled prodrugs, it was possible to selectively target tumor cells and tumor metastases through the use of chondroitin sulfate to block "housekeeping" receptors in the liver without affecting specific HA receptors of tumor cells (28-31). Targeting of anti-cancer agents to tumor cells and tumor metastases can be accomplished by receptor-mediated uptake of bioconjugates of these agents to HA (20). Since HA receptors (CD44, RHAMM) are overexpressed in transformed human breast epithelial cells and other cancers (32), selectivity for cancerous cells is markedly enhanced and overall dosages may be reduced. Moreover, coupling of antitumor agents to biopolymers can provide advantages in drug solubilization, stabilization, localization, and controlled release (18). Our methodology (33) for coupling antitumor agents to HA adds further value by specifically targeting the bioconjugate to aggressively growing cancers that overexpress HA receptors.

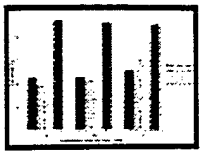

Figure 7 Cytotoxicity of HA-Taxol conjugate with 5% Taxol loading against HBL-100, SK-OV-3, HCT-116, and NIH 3-T-3 cells.

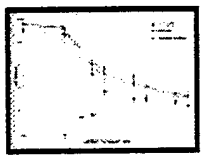

Figure 8 In vitro cytotoxicity of HA-Taxol conjugate with 5% Taxol loading against colon tumor HCT-116 cells.

Paclitaxel (Taxol) (*34*), a diterpenoid originally isolated from the bark of the Pacific yew, *Taxus brevifolia* , is a powerful anti-mitotic agent that acts by promoting tubulin assembly into stable aggregated structures. It binds to microtubules and inhibits their depolymerization into tubulin. Although Taxol has shown tremendous potential as an anti-cancer compound, its use as an anti-cancer drug is compromised by its poor aqueous solubility. One attempt to address this involved preparation of 2'-OH linked water-soluble poly(ethylene glycol) derivatives (*35*). In this paper, we have taken a different approach that uses a prodrug strategy to both increase water solubility and provide cellular targeting. Thus, we selected Taxol as a model anti-cancer drug for covalent attachment to HA that had been modified with adipic dihydrazide (ADH) (*36-38*). The coupling of Taxol-2'-hemisuccinate NHS ester to HA-ADH was optimized to give HA-Taxol conjugates with a variety of ADH and Taxol loadings (Figure 2). Selective in vitro cell cytotoxicity was studied with an untransformed mouse fibroblast cell line (NIH 3-T-3) and with three human cell lines: HCT-116 colon tumor cells, HBL-100 breast cancer cells, and SK-OV-3 ovarian cancer cells. In addition, a fluorescent (BODIPY)-labeled HA bioconjugate was prepared and employed to visualize selective uptake of HA by confocal microscopy.

Materials and Methods

General. Fermentation-derived HA (sodium salt, M_w 1.5 MDa) was provided by Clear Solutions Biotechnology, Inc. (Stony Brook, NY). 1-Ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDCI), ADH, succinic anhydride, diphenylphosphoryl chloride (DPPC), *N* -hydroxysuccinimide, and triethylamine were purchased from Aldrich Chemical Co. (Milwaukee, WI). Bovine testicular hyaluronidase (HAse, 880 units/mg) was obtained from Sigma Chemical Co. (St. Louis, MO). Paclitaxel (Taxol) was purchased from CBI Tech, Inc. (Cambridge, MA). 4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s* -indacene-3-propionic acid, hydrazide (BODIPY-FL hydrazide) was obtained from Molecular Probes, Inc. (Portland, OR). All solvents were of reagent grade or HPLC grade (Fisher Scientific Co., Santa Clara, CA). CH_2Cl_2 and acetonitrile were distilled from CaH_2.

Analytical Instrumentation. All ^1H NMR spectral data were obtained using an NR-200 FT-NMR spectrometer at 200 MHz (IBM Instruments Inc.). UV-Vis spectra were recorded on a Hewlett-Packard 8453 UV-Vis diode array spectrophotometer (Palo Alto, CA). Gel permeation chromatography (GPC) analysis was carried out on the following system: Waters 515 HPLC pump, Waters 410 differential refractometer, Waters 486 tunable absorbance detector, Waters Ultrahydrogel 250 and 2000 columns (7.8 mm ID x 30 cm) (Milford, MA). The GPC eluent was 150 mM phosphate buffer, pH 6.5:MeOH = 80:20 (v/v), and the flow rate was 0.5 mL/min. The system was calibrated with HA standards supplied by Dr. S. Gustafson (University of Uppsala, Sweden), and thus the molecular sizes of each HA oligosaccharide and the corresponding bioconjugates were calculated from the standard samples. Confocal fluorescence images of HA-BODIPY binding and uptake by cells were recorded using a Zeiss Microscope (Carl Zeiss, Inc., Germany). Coulter Counter was from Coulter Electronics, Inc. (Hialeah, FL). Cell viability was determined by thiazoyl blue (MTT) dye uptake protocols at 540 nm, which was recorded on a Bio-Rad M-450 microplate reader (Hercules, CA).

Synthesis of Taxol-NHS Ester. To a stirred solution of 270 mg of Taxol and 38 mg (1.2 equiv) of succinic anhydride in 13 mL of CH_2Cl_2 at room temperature was added 36 μL (10-fold molar excess) of dry pyridine. The reaction mixture was stirred for 3 days at room temperature and then concentrated in vacuo. The residue was dissolved in 5 mL of CH_2Cl_2, and Taxol-2'-hemisuccinate was purified on silica gel (wash with hexane; elute with ethyl acetate) to give 258 mg of product (86%). FAB-MS calcd for $C_{51}H_{55}NO_{17}$: 953.99. Found: 954.4 (MH^+).

Next, *N* -hydroxysuccinimido diphenyl phosphate (SDPP) was prepared from 10 mmol of diphenylphosphoryl chloride, 10 mmol of *N* -hydroxysuccinimide, and 10 mmol of triethylamine in 6 mL of CH_2Cl_2 as previously described (*39*). Crude SDPP was triturated with ether, dissolved in ethyl acetate, washed (2 x 10 mL H_2O), dried ($MgSO_4$), and concentrated in vacuo to give SDPP with mp 89-90 °C (85%). To a solution of 150 mg of Taxol-hemisuccinate and 82 mg (1.5 equiv) of SDPP in 5 mL of acetonitrile was added with 88 μL (4 equiv) of triethylamine. The reaction was stirred for 6 h at room temperature, and then concentrated in vacuo. The residue was dissolved in 5 mL of ethyl acetate and 2 mL of hexane and purified on silica gel. SDPP gave superior yields in less time and under milder conditions than did any carbodiimide coupling reagent. The purified Taxol-NHS ester was dried for 24 h in vacuo at room temperature to give 134 mg (80%). FAB-MS calcd for $C_{55}H_{58}N_2O_{19}$: 1051.07. Found: 1051.5 (MH^+).

Preparation of Low Molecular Weight (LMW) HA. To a solution of 2.0 g of high molecular mass HA (1.5 MDa) in pH 6.5 PBS buffer (4 mg/mL) was added hyaluronidase (HAse) (10 units/mg of HA). The degradation was carried out at 37 °C, 190 rpm stirring for 1 h, then 95 °C for 20 min. Dialysis tubing (M_w cutoff 3 500 Da) was prepared by soaking the membrane in water at room temperature for 3-4 h and subsequently rinsing it with water. The solution was dialyzed against water for 4 days using this washed membrane tubing. The retained material was filtered through a 0.2 μm cellulose acetate membrane (Corning) and lyophilized to give 1.12 g of LMW HA (56%).

Adipic Dihydrazido-Functionalized HA (HA-ADH). HA-ADH was prepared as described previously (*37* , *38* , *40*). In a representative example, LMW HA (50 mg) was dissolved in water to give a concentration of 4 mg/mL, and then a 5-fold excess of ADH was added into the solution. The pH of the reaction mixture was adjusted to 4.75 by addition of 0.1 N HCl. Next, 1 equiv of EDCI was added in solid form. The pH of the reaction mixture was maintained at 4.75 by addition of 0.1 N HCl. The reaction was quenched by addition of 0.1 N NaOH to adjust the pH of reaction mixture to 7.0. The reaction mixture was then transferred to pretreated dialysis tubing (Mw cutoff 3 500) and dialyzed exhaustively against 100 mM NaCl, then 25% EtOH/H_2O and finally H_2O. The solution was then filtered through 0.2 μm cellulose acetate membrane, flash frozen, and lyophilized. The purity of HA-ADH was measured by GPC. The substitution degree of ADH was determined by the ratio of methylene hydrogens to acetyl methyl protons as measured by ^1H NMR. In this example, 37 mg of HA-ADH was obtained with an 18% loading based on available carboxylates modified.

HA-Taxol. Several loadings were prepared following a general protocol (Figure 2). In a representative example, HA-ADH with 18% ADH loading (10 mg, 4.4 μmole hydrazide) was dissolved in 3 mM, pH 6.5, phosphate buffer to give a concentration of 1 mg/mL. To this mixture was added Taxol-NHS ester dissolved in sufficient DMF (DMF:H_2O = 2:1, v/v) to give a homogeneous solution, and the reaction mixture was stirred at room temperature for 24 h. The reaction mixture was dialyzed successively against 50% acetone/H_2O and water (membrane tubing, Mw cutoff 3 500). The solution was then filtered through a 0.2 μm membrane and then lyophilized. The purity of HA-Taxol conjugate was measured by GPC analysis. Taxol loading was determined by UV absorbance (λ_{max} = 227 nm, $\varepsilon = 2.8 \times 10^4$) in 80:20 CH_3CN:H_2O.

BODIPY-FL-Labeled HA. In a representative reaction, 100 mg of LMW HA was dissolved in 10 mL of H_2O, and 10 mL of an acetone solution (0.8 mg/mL) of BODIPY-FL hydrazide was added. The pH was adjusted to 4.75 with 0.1 N HCl, and then 51.5 mg (3 molar equiv) of EDCI was added in solid form into the mixture. The reaction was stirred overnight at room temperature at pH 4.75. The HA-BODIPY was purified by dialysis against H_2O, and its purity was characterized by GPC with detection at 210 nm (HA) and 502 nm (BODIPY).

In Vitro Cell Culture Cytotoxicity. The cytotoxicity of HA-Taxol conjugates was determined using a 96-well plate format in quadruplicate with increasing doses: 0.001, 0.01, 0.1, 0.5, 1, 5, 10, 50, and 100 μg/mL. Each well contained approximately 20 000 cells in 200 μL of cell culture media. Cells were cultured in the following media: HBL-100 cells, high glucose D-MEM (Dulbecco's Modified Eagle Medium) + 10% FBS (fetal bovine serum) + 1% sodium pyruvate; SK-OV-3 cells, D-MEM/F12 + 10% FBS; HCT-116 cells, α-MEM (Minimal Essential Medium, Eagle) + 10% FBS; NIH 3-T-3 cells, high glucose D-MEM + 10% FBS.

HA-Taxol conjugates and HA-ADH control were added as stock solutions in DMSO:H_2O = 1:1 (v/v); free Taxol was added as a DMSO stock solution. A 2 μL aliquot of the stock solution was added to each well of the cell culture plate. Cells were incubated at 37 °C for 3 days with the test material, and cell viability was determined using MTT dye uptake by reading plates at 540 nm. Response was graded as percent live cells compared to untreated controls (*41*).

Results

Degradation and Modification of HA. LMW HA was generated by degradation of high molecular mass HA (1.5 MDa) with testicular HAse. This enzyme degrades HA to generate a series of even-numbered HA oligosaccharides with the *N* -acetylglucosamine moiety at the reducing terminus (*42*). Thus, incubation of a solution of high molecular mass HA in pH 6.5 PBS buffer with testicular HAse at 37 °C provided partially degraded HA. Small fragments, HA oligosaccharides, and buffer salts were removed by dialysis against H_2O (four changes per day). The final LMW HA product was lyophilized, and an aliquot was analyzed by GPC analysis: M_n = 3883, M_w = 11 199, and molecular dispersity (DP) = 2.88. This corresponds to an average of 28 disaccharide repeats/LMW HA molecule. This preparation is quite reproducible as time, temperature, and concentrations of HA and HAse are controlled.

The use of the mild and versatile hydrazide method for preparation of chemically modified HA derivatives (*36*, *37*) allows attachment of reporter molecules, drugs, cross-linkers, and any combination of the above moieties to HA (*10*, *33*, *40*). Covalent attachment of ADH to the carboxylic acid groups of HA provides a controlled loading of pendant hydrazide functionalities arrayed along the hyaluronate backbone, used herein for the attachment of an antitumor agent. We selected LMW HA for modification in this study for four main reasons. First, it was possible to perform reproducible chemical modifications and to monitor the extent of modification by simple proton NMR methods. Second, LMW HA can be cleared from the body via ultrafiltration by the kidney. Third, the LMW HA bioconjugates were expected to provide a readily injectable nonviscous solution at concentrations up to 10 mg/mL, and the LMW materials should suffer minimal further degradation in plasma and would be rapidly taken up by cells. Finally, with LMW HA bioconjugates, we were confident that future efforts to cross-link the material into a hydrogel could also be easily controlled.

Thus, LMW HA (4 mg/mL) was mixed with ADH at several concentrations (Table 1). The pH of the reaction mixture was adjusted to 4.75, and then different quantities of solid EDCI were added in solid form to initiate the reaction. An increase in pH was observed immediately corresponding to proton uptake in the coupling reaction. The pH of the reaction mixture was maintained at 4.75 by addition of 0.1 N HCl. Preliminary studies had provided guidelines as to ratios of HA:ADH:EDCI suitable to achieve a given percentage modification of the glucuronate functions of HA. The reaction was stopped by addition of 0.1 N NaOH to adjust the pH of reaction mixture to 7.0. The HA-ADH was purified by sequential dialysis against 100 mM NaCl, 25% EtOH/H_2O, and H_2O. The dialyzed solutions were filtered through a 0.2 μm membrane, and then lyophilized to give HA-ADH in yields ranging 50-70%.

The purity and molecular size distribution of the HA-ADH was measured by GPC. The narrow single-peak GPC profile detected by UV (210 nm) (Figure 3a) and RI indicated that both large and small impurities had been completely removed. It is essential to establish this fact rigorously to ensure that all subsequent molecules added also become covalently attached, rather than remaining noncovalently associated. In addition, the GPC results showed that there was virtually no molecular weight decrease due to further HA degradation or increase due to bifunctional cross-linking during the modification reaction. The loading of ADH on the polymer backbone was determined by ^1H NMR spectroscopy studies with D_2O as a solvent. The degree of substitution could be calculated by integration of the ADH methylene signals using the methyl resonance (δ = 1.95-2.00 ppm) of the acetamido moiety of the GlcNAc residues of HA as an internal standard (*37*). Thus, ^1H NMR integration confirmed that different ADH loadings occurred with different ratios of reactants and for different reaction times. The relationship between the degree of substitution of ADH and the reaction conditions, i.e., molar ratio of HA:ADH:EDCI, and reaction time was optimized. The results are shown in Table 1. Importantly, the degree of ADH substitution on HA was influenced primarily by the HA:EDCI ratio, with the amount of excess ADH varying from 5 to 25-fold having little effect. The carbodiimide quantity is thus the controlling factor for determining ADH loading on HA.

Taxol (paclitaxel) is a taxane natural product that promotes polymerization of tubulin and stabilizes the structure of intracellular microtubules. This process has the effect of inhibiting the normal dynamics reorganization of the microtubules, which is necessary for interphase and mitotic functions. Because of the problems in administering emulsified forms of this water-insoluble drug, Taxol was selected as the model anti-cancer drug in our study. Taxol was first converted to its 2'-hemisuccinate derivative by standard methods (*43*), and its structure was confirmed spectroscopically. Second, the activated Taxol-NHS ester was prepared by coupling with SDPP. Taxol-NHS was then coupled to HA-ADH in 3 mM phosphate buffer at pH 6.50 using DMF as a cosolvent to maintain a homogeneous solution. The purification of HA-Taxol bioconjugate was carried out by dialysis of the reaction mixture against 50% acetone/H_2O. The purity of the HA-Taxol conjugate was determined by GPC analysis, monitoring absorbance at 227 nm (Figure 3b) and RI. The single symmetrical GPC peak showed that no free Taxol or other small molecular impurities remained in this preparation. The Taxol was quantified by UV absorbance at 227 nm (Figure 4) in 80% acetonitrile:H_2O. To obtain the optimal modification on HA for anti-cancer ability, HA-Taxol conjugates with different Taxol loading were synthesized. Table 2 shows the optimization of Taxol loading and the molar ratio of HA-ADH to Taxol-NHS during the conjugation reaction. These data demonstrate that the molar ratio of ADH to Taxol-NHS during the grafting reaction is critical in determining the Taxol loading of the bioconjugate.

Fluorescently labeled HA has been prepared with several chemistries and used in other studies of receptor-mediated uptake. Most recently, RHAMM-mediated uptake and trafficking of HA by transformed fibroblasts (*44*) was observed with Texas Red-HA. Previously, fluorescein-HA was employed to study HA uptake in a variety of systems, e.g., cells expressing CD44 variants (*21* , *45-48*), uptake by tumor cells for correlation with metastatic potential (*49* , *50*), internalization by chondrocytes (*51*), and as a measure of liver endothelial cell function (*52*). In this study and in the Texas Red study, hydrazide derivatives of the dyes were used to form covalent bishydrazide linkages to the HA carboxylic acid functions under the mild all-aqueous conditions employed for other hydrazide modifications of HA (*33*).

To study the binding ability of HA to tumor cells and uptake by cells, a variety of fluorescently labeled HA derivatives were prepared. Of those evaluated for use with cancer cells, we obtained the best results with the BODIPY fluorophore (Y. Luo, M. R. Ziebell, unpublished results). Thus, BODIPY-FL hydrazide was coupled to LMW HA using EDCI as the condensing agent to give HA-BODIPY, which was purified by dialysis against H_2O; the loading of GPC-homogeneous HA-BODIPY was determined spectrophotometrically to be 1.8% (based on available glucuronates).

Cell-Based Assays for Uptake and Toxicity. An aliquot (2 μL) of a 1.5 mg/mL aqueous stock solution HA-BODIPY was added to 100 μL cell culture media with tumor cells cultured on cover slips. Confocal images of HA-BODIPY uptake by HBL-100 cells can be seen in Figure 5. Initially, the HA-BODIPY can be seen on cell membrane; over the course of several minutes, it is taken up into the cell and then gradually begins to accumulate in the nucleus. After 20 min, cells showed HA-BODIPY in most compartments. Uptake of HA-BODIPY into SK-OV-3 cells occurred with a similar appearance and time course (Figure 6). These data suggest that HA binds readily to tumor cell surface and is rapidly taken up via HA receptor-mediated pathways. This supports the notion that HA should be a good targeting polymer for selective delivery of anti-cancer drugs to tumor cells. Similar results were observed independently with HA-Texas Red uptake by transformed fibroblasts (*44*). Importantly, nontransformed cells, such as the NIH 3-T-3 fibroblasts, did not show this binding and rapid uptake of HA-BODIPY.

Next, the cytotoxicity of HA-Taxol conjugates was measured using a 96-well plate format in quadruplicate with increasing doses from 0.001 to 100 μg/mL. The cytotoxicity of HA-Taxol conjugates was studied by using the MTT assay to identify cells still active in respiration (*41*). HA-Taxol conjugates showed effective cytotoxicity against SK-OV-3, HBL-100, and HCT-116 cell lines, while no cytotoxicity against NIH 3-T-3 cells was observed at concentrations up to 10 μg/mL of Taxol equivalents (Figure 7). These results confirm the selective toxicity of HA-Taxol toward different cells lines, and the known overexpression of CD44 by HBL-100 (*53*) and SK-OV-3 cells (*54*) suggests that this selective toxicity is due to receptor-mediated binding and uptake of the HA-Taxol bioconjugate.

The HA-BODIPY binding and uptake results support the hypothesis that selective toxicity of HA-Taxol is due to receptor-mediated events. This was further explored by investigating the relative toxicity of polymer-bound and free Taxol, as well as examining the effect of the polymeric carrier itself on the toxicity of the drug. Thus, Figure 8 summarizes the cytotoxicity data for HA-Taxol (5% Taxol loading) with HCT-116 colon cancer cells. The

bioconjugate showed increased cytotoxicity, e.g., lower IC_{50}, relative to free Taxol or free Taxol mixed with the HA-ADH carrier. In a further control experiment, we had established that HA-ADH alone elicited no detectable change in cell viability at a concentration 10 times higher than the maximal concentration of HA-Taxol conjugate used. These data support the notion that the increased cytotoxicity of HA-Taxol conjugates requires cellular uptake of the complex followed by hydrolytic release of the active Taxol by cleavage of the labile 2' ester linkage.

The in vitro cytotoxicity results of HA-Taxol conjugates with different modifications against different cell lines are shown in Table 3. For the least-modified HA (9% ADH modification), higher cytotoxicity was observed as Taxol loading increased. However, the cytotoxicity of highly modified HA actually decreased at the highest Taxol loading. Apparently, high loading of Taxol decreased the solubility of HA-Taxol conjugate, masked the HA receptor recognition elements of HA, caused aggregation of the polymeric conjugate, and thus limited the toxicity of the conjugate relative to that of free drug. Clearly, the cytotoxicity of HA-Taxol conjugates depends on a balance between minimal HA modification and maximal Taxol loading.

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Abbreviations: α-MEM, Minimal Essential Medium, Eagle; ADH, adipic dihydrazide; BODIPY-FL, 4,4-difluoro-5,7-dimethyl-4-bora-3a, 4a-diaza-*s* -indacene-3-propionic acid; D-MEM, Dulbecco's Modified Eagle Medium; DPPC, diphenylphosphoryl chloride; ECM, extracellular matrix; EDCI, 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide; FBS, fetal bovine serum; GlcNAc, *N* -acetyl-D-glucosamine; GlcUA, D-glucuronic acid; GPC, gel permeation chromatography; HA, hyaluronic acid; HA-ADH, adipic dihydrazide-modified HA; HAse, hyaluronidase; HMPA, poly(hydroxymethyl)acrylamide; LMW, low molecular weight; MTT, thiazoyl blue; SDPP, *N* -hydroxysuccinimido diphenyl phosphate.

Table 1. Optimization of ADH Modification of HA and the Reaction Condition

molar ratio of HA:ADH:EDCI	reaction time (min)	ADH loading (%)
	5	28.3
	10	31.2
	15	31.4
1:40:4	30	32.2
	60	38.6
	120	45.7
	240[a]	44.0
1:5:0.5	15	9.0
1:5:1	15	17.5
1:10:1	15	18.0
1:20:1	15	19.0
1:10:0.2	180	19.3

$(HA)_x(HA\text{-}ADH)_y(HA\text{-}ADH\text{-}Taxol)_z$					
	composition of HA-Taxol conjugates				
prepar- ation	HA (x) (%)	HA-ADH (y) (%)	HA-ADH-Taxol (z) (%)	solubility in H_2O	ADH%: Taxol-NHS[a]
A. ADH loading = 9%[b]					
1	91	7.8	1.2	yes	9:5
2	91	7.7	1.3	yes	9:9
3	91	3.8	5.2	yes	9:18
B. ADH loading = 18%					
4	82	16.4	1.6	yes	18:5
5	82	16.1	1.9	yes	18:10
6	82	15.8	2.2	yes	18:15
7	82	3.1	14.9	partially[c]	18:36
C. ADH loading = 45%					
8	55	30	15	no	45:90

[a] The molar ratio used in the grafting reaction of HA-ADH and Taxol-NHS that resulted in the composition of the HA-Taxol conjugates.[b] Total ADH loading for modified HA = $y + z$.[c] Limited solubility; only soluble below 0.1

	IC_{50} (μg/mL)[d]					
	HBL-100[e]		SK-OV-3[e]		HCT-116[e]	
HA-Taxol preparations	conjugate[b]	Taxol equivalents[c]	conjugate	Taxol equivalent	conjugate	Taxol equivalent
1	48.0 (118 μM)	1.21 (1.42 nM)	21.5 (53 μM)	0.54 (0.64 nM)	7.20 (17.8 μM)	0.18 (0.21 nM)
3	16.4 (37 μM)	1.65 (1.93 nM)	8.0 (18.2 μM)	0.80 (0.94 nM)	0.52 (1.2 μM)	0.052 (0.061 nM)
7	6.8 (12.4 μM)	1.58 (1.85 nM)	0.37 (0.68 μM)	0.086 (0.10 nM)	0.11 (0.20 μM)	0.026 (0.030 nM)
8[a]	68.0 (115 μM)	1.48 (17.3 nM)	7.2 (17.3 nM)	1.56 (1.83 nM)	2.15 (3.65 μM)	0.47 (0.55 nM)

[a] Dissolved in 3:1 DMSO/H_2O.[b] The data in these columns show the IC_{50} of HA-Taxol conjugates against the tumor cell line.[c] The data in these columns are calculated as the Taxol equivalents present in the HA-Taxol bioconjugate using the molar ratios in Table 2. This calculation allows comparison of conjugated and free Taxol.[d] The IC_{50} value is the molarity at which 50% of tumor cell death was observed after 72 h under standard tissue culture conditions.[e] Cell type.

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CHEMICALLY-MODIFIED HYALURONAN:

NEW BIOMATERIALS AND PROBES FOR CELL BIOLOGY

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ABSTRACT

A mild, controllable modification of hyaluronic acid (HA) has been developed in which monovalent, divalent, or polyvalent hydrazides can be covalently attached to HA to give functionalized derivatives with a high degree of synthetic versatility. First, HA can be covalently modified to produce drug delivery systems and novel hydrogel biomaterials with a variety of desired physical and chemical properties. Specific applications include localizable hydrogels for release of anti-inflammatory agents, materials for tissue engineering and prevention of post-surgical adhesions, novel grafted copolymers for drug delivery, tumor-targeted anti-cancer drugs, and techniques for coating surfaces of polymeric and metal medical devices. Second, basic cell biological research on the changes in the location of HA and HA binding proteins (e.g., CD44 and RHAMM) can be demonstrated using cellular probes. Our laboratories have developed versatile routes to HA-fluors (fluorescein, Texas Red, BODIPY), HA-nanogold, and HA-biotin with controllable levels of modification on the carboxylate groups. Third, HA can be modified to provide biochemical probes for developing new hyaluronidase (HAse) assays and for the discovery of new HA binding proteins. Examples of each of these three current research areas will be presented.

Key words: drug delivery, hydrazide, hyaluronidase, binding proteins

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INTRODUCTION

We have developed a versatile method for chemical modification of hyaluronic acid (hyaluronan, HA) in which monovalent, divalent, or polyvalent hydrazides can be covalently attached to HA to give functionalized derivatives with many subsequent uses [1-3]. Covalent modifications can alter the chemical and biomechanical properties in ways that permit production of drug delivery systems and novel hydrogel biomaterials [4]. Specific applications described herein include tumor-targeted anti-cancer drugs, hydrogels for localized release of anti-inflammatory or other therapeutic agents, preparing scaffold materials for tissue engineering and producing slowly bioresorbable films for prevention of post-surgical adhesions. In addition, HA fragments can be incorporated either pre- or post-polymerization to provide novel receptor-targeted grafted copolymers for drug delivery. Several techniques for covalently attaching HA onto the surfaces of polymeric or metal medical devices have also been developed [5, 6].

HA can be modified to provide biochemical probes for developing new hyaluronidase (HAse) assays and for the discovery of new HA binding proteins (HABPs) [6]. These probes can be used to understand the effects of HA on cell physiology, and the uptake, transport, and signaling functions of HA in cells [7]. For example, changes in the location and abundance of both HA and HABPs such as CD44 and RHAMM can be demonstrated using cellular probes.

CURRENT RESEARCH AREAS

Five areas of current research at The University of Utah are summarized in this overview: (**i**) tumor-targeted drug delivery of Taxol®; (**ii**) swellable HA hydrogel biomaterial for wound healing, adhesion management, and drug delivery; (**iii**) biophysical and biochemical studies of HA-receptor interactions, including novel binding assays and ligands; (**iv**) surface modification chemistry; and (**v**) assays for HAse that permit screening for new inhibitors.

1. **Tumor-targeted Drug Delivery**. The uses of chemically-modified HA for drug delivery have been recently reviewed [5, 8]. A fluorescent BODIPY-HA was synthesized to illustrate cell targeting and uptake of chemically-modified HA using confocal microscopy [9]. Next, a cell-targeted prodrug was developed for the anti-cancer drug Taxol®, using HA as the drug carrier [9]. HA-Taxol® bioconjugates were synthesized by linking the Taxol® 2'-OH via a succinate ester to adipic dihydrazide-modified HA (HA-ADH) (Figure 1A). The coupling of Taxol®-NHS ester and HA-ADH provided several HA bioconjugates with different levels of ADH modification and different Taxol® loadings. HA-Taxol® conjugates showed selective toxicity towards the human cancer cell-lines (breast, colon, and ovarian) that are known to over-express HA receptors, while no toxicity was observed towards a mouse fibroblast cell line at the same concentrations used with the human tumor cells. The drug carrier HA-ADH was completely non-toxic. The selective cytotoxicity is consistent with the results from confocal microscopy, which demonstrated that BODIPY-HA entered only the tumor cells.

a. Selective HA-BODIPY uptake by tumor cells. HA-BODIPY was used to probe the selectivity of HA targeting to tumor cells [9]. HA-BODIPY binding and uptake by tumor cells such as human breast cancer cells HBL-100, ovarian cancer cells SK-OV-3 and colon tumor cells HCT-116, were studied by laser confocal microscopy. The fluorescence images indicated that HA bound readily to tumor cell surfaces and was rapidly taken up via HA receptor-mediated pathways. This supports the notion that HA should be a good targeting polymer for selective delivery of anti-cancer drugs to tumor cells. Similar results were observed independently with HA-Texas Red uptake by transformed fibroblasts [10]. Importantly, non-transformed cells, such as the NIH 3T3 mouse fibroblasts, did not show this binding and rapid uptake of HA-BODIPY. The HA-BODIPY binding and uptake results support the hypothesis that selective toxicity of HA-Taxol® is due to receptor-mediated events.

b. Selective toxicity of HA-Taxol®. The in vitro cytotoxicity of HA-Taxol® conjugates was studied by using the MTT assay to identify cells still active in respiration [11]. HA-Taxol® conjugates showed toxicity to SK-OV-3, HBL-100 and HCT-116 cell-lines, while no toxicity was observed towards a mouse fibroblast cell line NIH 3T3 at the same concentrations used with the cancer cells (Figure 1B). The selective cytotoxicity is consistent with the results from confocal microscopy, which demonstrated that BODIPY-HA only entered the cancer cells. In addition, the efficacy of HA-Taxol® conjugate could be blocked by pre-incubation of the cells with a 20-fold excess of HA. Together with the known overexpression of CD44 by HBL-100 [12] and SK-OV-3 cells [13] , these data suggest that the selective cytotoxicity is due to receptor-mediated binding and uptake of the HA-Taxol® bioconjugate. The bioconjugate showed higher potency, e.g., lower IC_{50}, relative to free Taxol® or free Taxol® mixed with the HA-ADH carrier [9]. In a further control experiment, we established that the drug carrier HA-ADH alone was completely non-toxic.

Taxol release studies from HA-Taxol® were carried out in cell culture media with and without cells, with HAse and esterase, and human plasma. Figure 1C shows the data for the effects of media and human plasma. HPLC analysis suggested that only active Taxol® was released, corresponding to cleavage of the labile 2' ester linkage; no Taxol® hemisuccinate was detected. The bioconjugate was stable in cell culture media, and the presence of HAse did not affect the Taxol® release rate. Taxol® release rate is significantly faster in the presence of added esterase. These data support the notion that the increased cytotoxicity of HA-Taxol® requires cellular uptake of the complex followed by hydrolytic release of the active Taxol® by cleavage of the labile 2' ester linkage.

c. Fluorescence-activated cell sorting (FACS) with FITC-HA-Taxol®. Laser flow cytometry was used to investigate the interaction of the HA-Taxol® conjugate with tumor cells using fluorescein-labeled HA-Taxol® as the fluorescent probe. It was found that FITC-HA-Taxol® bound to the cell surface and was taken up rapidly by tumor cells (HBL-100, SK-OV-3, HCT-116). Binding and uptake of this dual-modified HA could be blocked by pre-incubation with excess of HA, while no binding and uptake was detected in fibroblasts. In addition, the binding and uptake of FITC-HA-Taxol® by different cell-lines was also evaluated by confocal microscopy, giving results analogous to those obtained with HA-BODIPY. The selective cytotoxicity of HA-Taxol® is thus clearly due to receptor-mediated uptake followed by hydrolytic release of the active Taxol® via cleavage of the labile 2' ester linkage.

2. **Swellable HA Hydrogels**. Hydrogels have received significant attention as delivery vehicles. These materials can be engineered to be tissue compatible and to be permeable to different solutes [14]. HA hydrogels can in principle be completely bioresorbable materials and have been studied for over two several decades [15, 16]. Mirroring the new sol-gel injectable drug delivery system [17], a novel fast gelling and fast swelling HA hydrogel film was developed as a potential drug delivery system. The new HA film is biocompatible and biodegradable and is produced from HA-ADH and a bioinert crosslinker. An in vitro drug release device was evaluated and drug release was initially studied by using dyes (acridine orange, amaranth and fast green FCF), followed by examination of release rates of several therapeutic agents (hydrocortisone, dexamethasone, indomethacin, gentamicin, pilocarpine, and diclofenac). It was found that the new HA film could maintain a slow release rate for certain drugs, such as acridine orange, dexamethasone and gentamicin. Differential scanning calorimetric analysis suggested that a polymer-drug interaction exists between the HA hydrogel and could account for the slow release. In particular, prolonged delivery of anti-inflammatory or anti-infective drugs suggested the utility of this novel HA film as a wound dressing material.

The functionalized HA-ADH derivative was crosslinked by a macromolecular crosslinker to give an interpenetrating network hydrogel. The hydrogel could be prepared under extremely mild conditions, e.g., in water, phosphate-buffered saline (PBS), or in cell culture media at room temperature. The gelling process began immediately and was essentially complete in minutes. A solvent-casting method was used to obtain HA hydrogel films, which were then dried in air at 37 °C for 24 h.

The swelling extent of HA hydrogel films was investigated using both kinetic and equilibrium swelling studies (Figure 2). For the kinetic studies HA films were cut into small disks and dyed with acridine orange to facilitate visualization. Next, the diameters of dried disks were measured using a microscope. Then, a buffer solution was added to the film and the diameter was measured at various times. A similar procedure was followed for the equilibrium studies; however, the diameter of the film was measured only after 24 h of equilibration in a buffer solution at 37 °C. The swelling ratio, Q, was calculated as indicated below.

$$\left(\frac{Diameter_{t=x}}{Diameter_{t=0}}\right)^3 = Q$$

where: $Diameter_{t=x}$ = Diameter of disk at time interval x

$Diameter_{t=0}$ = Diameter of dry disk

Figure 2B shows the results of kinetic studies, while Figure 2A shows the actual films as seen under the microscope at times 0, 4, and 600 sec. These data indicate that the films swell quickly and reach and equilibrium size in less than a minute. The rapid swelling of these proprietary films will be exploited for a variety of in vivo applications in human medicine.

3. Surface Modification Chemistry

a. Plasma etching. Surfaces of polypropylene (PP), polystyrene (PS) and polytetrafluroethylene (PTFE)were activated with Ar and NH_3 plasmas to aminate the polymer surface [18]. Aminated surfaces were then reacted with HA using three modification conditions. Results showed that ammonia plasma treated polymers were more reactive toward HA attachment. Of the three chemistries tried, condensation of the aminated surface with succinic anhydride followed by coupling of the newly-formed carboxylic acid group with HA-ADH gave the most effective and reproducible HA attachment. HA coatings were evaluated by spectroscopic and physicochemical methods. HA-modified plastic surfaces were quite hydrophilic, as determined by measuring the water contact angle, and should exhibit selectivity in cell attachment and growth.

b. Controlled chemical modifications of particles. Three types of HA-modified particulate materials (HAMPs) have been produced: (**i**) affinity resins based on cross-linked agarose, (**ii**) superparamagnetic polystyrene (PS) beads, and (**iii**) controlled-pore glass (CPG). In each case, ADH-modified HA was covalently coupled to chemically-activated residues on the surface. The affinity resins were prepared by coupling HA-ADH to NHS ester-activated Affigel. This affinity matrix has been employed for purification of native and recombinant HABPs. The HA-modified magnetic beads and CPG were prepared by oxidative cleavage of glycol-modified surfaces to give surface aldehyde functionalities. Coupling of HA-ADH to the particle surface resulted in hydrazone linkages, and the extent and location of coupling was monitored in two ways (Figure 3). First, fluorescence microscopy was employed to detect coupling of fluoresceinylated HA-ADH (rather than nonfluorescent HA-ADH). In addition, the presence of HA on these HAMPs was also checked functionally by testing their ability to bind to the HA binding domain (HABD) of receptor for HA-mediated motility (RHAMM), described in more detail in Section 4 below. For this detection strategy, Texas Red was conjugated to a GST fusion protein of the 61 amino acid recombinant RHAMM-P1 peptide to give a novel non-immunological reagent for the detection of HA on surfaces. HAMPs will be employed to isolate HABPs in automated high throughput screens, and in an in vitro process to selectively remove cells expressing high affinity cell-surface HABPs.

4. Biophysical and Biochemical Studies

a. Biochemical probes and surfaces. Versatile routes to HA-fluors (including fluorescein, Texas Red, and BODIPY), HA-nanogold, and HA-biotin with controllable levels of modification on the carboxylate groups [10, 19] have been developed. In addition, the preparation of HAMPs, such as HA affinity resins on cross-linked dextran (> 300 μm), CPG (100 μm), and superparamagnetic beads (4 μm) was summarized above.

b. Structural studies of RHAMM. The RHAMM is a cell surface HA receptor found on fibroblasts and certain tumor cell-lines that modulates cell function through binding extracellular matrix components [20]. Certain isoforms of this receptor have been identified as important in intracellular signaling and extracellular binding [21]. High-resolution multi-dimensional NMR is being used to solve the structure of the HABD of RHAMM, and this will be the first predominately helical HABD to be studied this way. A solution structure of the mostly beta TGS-6 link module has been previously determined by NMR [22, 23]. A RHAMM polypeptide that contains the two base-rich HABDs was expressed and purified. From circular dichroism and NMR experiments, it appeared that the domain was alpha helical. A complete structure may provide a model for how the two domains selectively bind HA and which amino acid side chains account for the high binding affinity. Molecular modeling suggests the hypothesis that a long narrow groove is formed by a helix-loop-helix structure in which the substrate (e.g., an HA octasaccharide) would be flanked on each side by one of the two HABDs.

c. Peptide mimics of HA. HABPs control cell function and are implicated in cancer, arthritis, adhesion, and wound healing [7, 24] One medicinal chemistry approach to developing new antagonists and agonists that mimic HA in binding to HABPs involves the use of combinatorial libraries of peptides, either synthetic or from phage display. The first library was a random set of 15 amino acid polypeptides encoded in the fUSE-5 phage [25]. The next libraries were eight amino acid, "one-bead, one-peptide" libraries synthesized on 100 μm PS beads [26]. Each of these libraries was screened against our RHAMM construct expressed as a GST fusion protein (GST-RHAMM-P1). To detect peptides that bound solely to the HABD, we included a competition step in each of our screens in which HA was first incubated with GST-RHAMM-P1; this preincubation was followed by addition of peptides. In this negative selection step, the peptides that did not bind in this final step were selected as those molecules that exhibited exclusive interaction with the HABD. Phage-displayed peptides are summarized in the Table 1, and the affinity of independently-synthesized peptides for RHAMM-P1 has been assessed using the assay described below. Interestingly, these peptides interfere with the interaction of RHAMM with erk1 [27]. Although not shown, the bead-derived peptides include an abundance of aromatic and acidic residues. Several recognizable motifs repeat in the twenty peptides sequenced, and the inclusion of unnatural amino acids resulted in novel peptides with nanomolar affinity.

Table 1. Phage-displayed peptides that interact with RHAMM

HA1	WPVSLTVCSAVWCPL
HA2	GVCNADFCWLPAVVV
HA3	SASPSASKLSLMSTV
HA4	IPPILPAYTLLGHPR
HA5	YSVYLSVAHNFVLPS
HA6	HWCLPLLACDTFARA

We conclude that a series of hydrophobic amino acids are important in binding, and that the amino acid motif may have quite different characteristics from HA itself. Current efforts focus on identification of peptides that bind uniquely to either RHAMM or to TSG-6 link module.

d. Rapid binding assays in microtiter plates. In order to verify the binding selectivity and affinity of these peptides, we developed a series of binding assays that allow us to monitor the binding and find constants to describe the interactions. One such method is in a 96-well plate format in which the target protein, GST-RHAMM-P1, is immobilized and biotin-labeled peptides are incubated in the wells. Streptavidin conjugated horseradish peroxidase is then added followed by a chromogenic substrate. Figure 4A illustrates the basic method, and Figure 4B shows the determination of a 21 nM binding constant for the binding of the HA2 peptide to RHAMM-P1. Figure 4C illustrates the relative affinity and relative HA-displacement of binding for four of the phage-derived peptides.

e. Fluorescence polarization (FP) assays. To compare to solution phase binding we used FP, which measures the degree of anisotropic change of a fluorescent probe [28]. As the complex forms, the anisotropy increases, which in turn is a marker for peptide binding. In these experiments, fluorescently-labeled peptides were titrated with increasing amounts of the recombinant RHAMM-P1. Preliminary data (not shown) allow estimation of the K_d and provided data consistent with the microplate assay.

5. Hyaluronidase Assays

a. Effects of metals on HAse. The enzymatic degradation of HA by testicular HAse, hyaluronate 4-glucanohydrolase, has an absolute requirement for the inclusion of mono- or divalent cations in the reaction mixture. We tested the effects of metal salts on the enzymatic degradation of HA by HAse by preincubating the metal salts with either HA or with the HAse prior to the enzymatic reaction [29]. The digestion occurred more slowly in the presence of monovalent cations compared to the divalent cations. Most divalent cations activated HAse with equal potency, except for Cu^{2+}. Compared to the digestion in the presence of the other divalent salts, Cu^{2+} suppressed the degradation of HA; however, compared to the digestion in the absence of any salt, Cu^{2+} still activated the digestion of HA by HAse. When HA was preincubated with NaCl or when HAse was incubated with salts like $CaCl_2$, $CoCl_2$, $ZnCl_2$ or $CuCl_2$, surprisingly no effects on the enzymatic activity could be observed (except for $CuCl_2$). The combined results suggested that the activating effect of the cations occurs through an activation of HA rather than an activation of HAse. That is, the addition of the cations to HA may change its conformation such that more endoglucanase sites are exposed, thus facilitating the hydrolysis by the enzyme.

These experiments suggested that any metal-chelating compound might inhibit the degradation of HA by HAse. As predicted, preincubation of EGTA-Na_4 with HA/$CaCl_2$ inhibited the degradation of HA by HAse. No inhibition was observed when EGTA-Na_4 was preincubated with HAse prior to the digestion. Thus, judicious selection of HAse assay parameters is critical for the discovery of novel, selective HAse inhibitors and not mere metal-chelating compounds. Total enzymatic digestion of HA in the presence of varying concentrations of Ca^{2+} showed a concentration-dependent regulation of the size of the oligosaccharide end products. These oligosaccharides were fractionated to monodisperse species using anion-exchange perfusion chromatography and their size and purity were confirmed using MALDI-TOF analysis.

b. Fluorescence-based assays for HAse. In most mammals, HAse is found on the acrosomal membrane of spermatozoa and plays a major role in the passage of the spermatozoa towards the oocyte [30] . The enzyme is also present in most animal venoms and several bacterial species produce HAse, enhancing their virulence. Tumors are often enriched in HAse activity compared to normal tissues. This production of HAse can affect the further development of the tumor, e.g., by generating small, angiogenic oligosaccharide fragments from HA polymers present in the extracellular matrix [31]. Thus, inhibitors of the enzyme could have potential as non-hormonal contraceptive agents or as novel anti-angiogenic compounds. In view of the importance of HAse and its inhibitors, there is a need for a simple, rapid, and sensitive assay to evaluate the HAse activity present in any sample or to search for inhibitors of this enzyme.

Methods currently employed often lack the necessary sensitivity, selectivity or versatility to perform all these tasks. We are developing a fluorescence-dequenching assay using a fluorescently-labeled HA biconjugate as a substrate for HAse (Figure 5A). Using the hydrazide methodology, HA was modified with FITC and with Texas Red. This gave a doubly-labeled HA substrate in which the fluorescence of FITC was partially quenched by the presence of nearby Texas Red fluorophores. Digestion of this substrate by HAse separates FITC from the Texas Red-containing regions, resulting in an increased fluorescence that can be monitored as a function of the reaction time. Figure 5B illustrates such an increase in FITC fluorescence when HA conjugated with FITC and Texas Red is incubated with two different amounts of HAse compared to the incubation with buffer alone. This proprietary assay can be performed in a 96-well format yielding a high-throughput screening assay for HAse activity and/or inhibition.

CONCLUSIONS

Modification of HA using adipic dihydrazide (ADH) permits preparation of a wide variety of biomaterials and biochemical probes. In this overview, we showed five examples. First, selective toxicity of anti-cancer drugs coupled to HA to tumor cells illustrated an important method for solubilization and cell-targeted intracellular delivery of known anti-tumor drugs. This method can significantly improve the therapeutic ratio. Second, swellable HA hydrogel films with slow release properties for both small molecules and macromolecular drugs allow penetration of a combined wound dressing - drug delivery system that is bioresorbable. Third, attachment of HA to surfaces provides materials for cell sorting and protein panning to identify new HABPs. Fourth, interactions of HABPs with HA and HA-mimetic peptides offers insight into cell biology and structural biology of HABP-HA complexes. Finally, doubly-labeled HA (using two different covalently-attached fluors) creates a novel assay for detection of HAse activity and thus for discovery of new HAse inhibitors.

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FIGURE LEGENDS

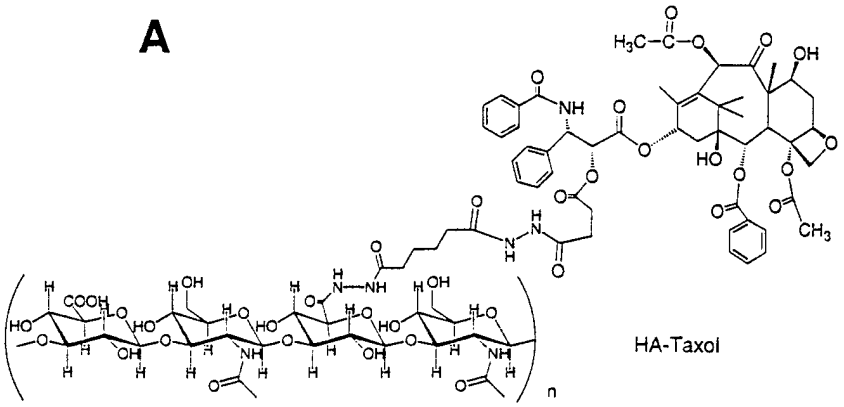
Figure 1. Tumor-targeting of Taxol® with HA. **Panel A**: structure of HA-Taxol®; **Panel B**: selective toxicity of HA-Taxol® to three human cancer cell lines and nontoxicity to mouse fibroblasts; **Panel C**: time course of Taxol® release from HA-Taxol®.

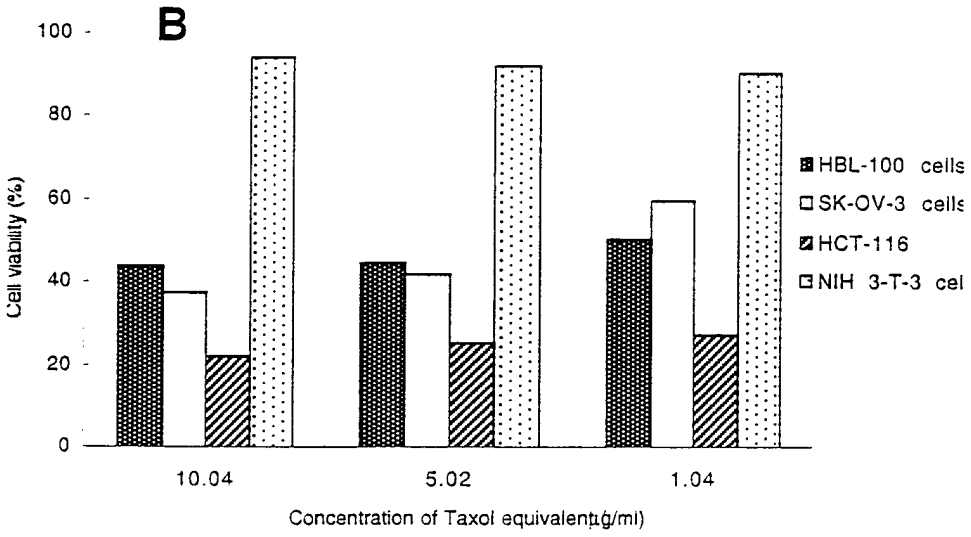
Figure 2. Swellable HA hydrogel films. **Panel A**: photographs of an acridine orange-stained HA hydrogel film swelling in PBS. From left to right: dry HA film, film after 4 sec in PBS, film after 600 sec in PBS; **Panel B**: swelling kinetics of HA hydrogel film in PBS.

Figure 3. Surface modification chemistry. The detection of HA covalently coupled through hydrazone linkages to CPG can be accomplished with direct fluorescent detection of the binding of a Texas Red-labeled recombinant GST-RHAMM-P1 polypeptide.

Figure 4. Binding of HA mimetic peptides to GST-RHAMM-P1-coated microtiter plates. **Panel A**: schematic of assay; **Panel B**: Eadie Hofstee plot of data for binding of biotinylated HA2; **Panel C**,: relative affinity of binding of four biotinylated phage-derived synthetic peptides showing competition by excess HA.

Figure 5. Fluorescence-dequenching assay for HAse. **Panel A**: schematic of assay; **Panel B**: data from digestion of HA labeled with FITC and Texas Red by HAse (0, 1.25 or 12.5 mUnits) in phosphate buffer (pH = 6.4) containing 150 mM NaCl in wells of a 96-well microplate. The fluorescence of FITC (excitation 490 nm, emission 535 nm) was monitored as a function of the reaction time; each data point is the average of three measurements.


A
HA-Taxol
n


B
Cell viability (%)
100
80
60
40
20
0
10.04
5.02
1.04
Concentration of Taxol equivalent(μg/ml)
HBL-100 cells
SK-OV-3 cells
HCT-116
NIH 3-T-3 cel

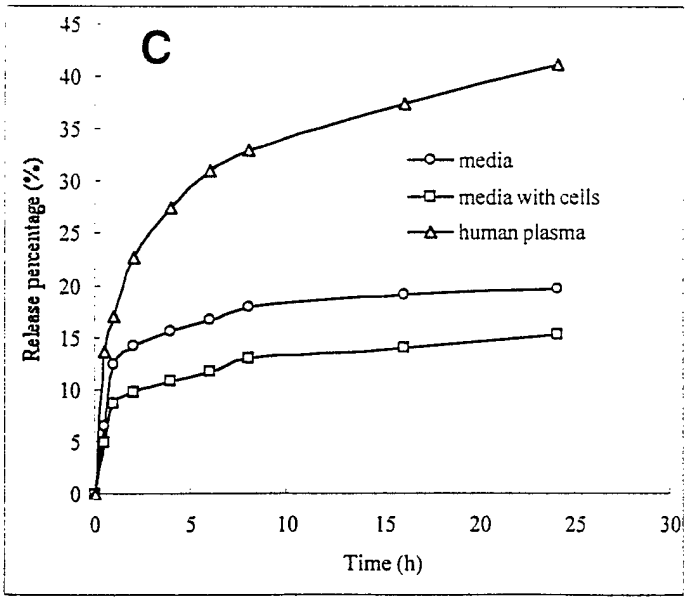
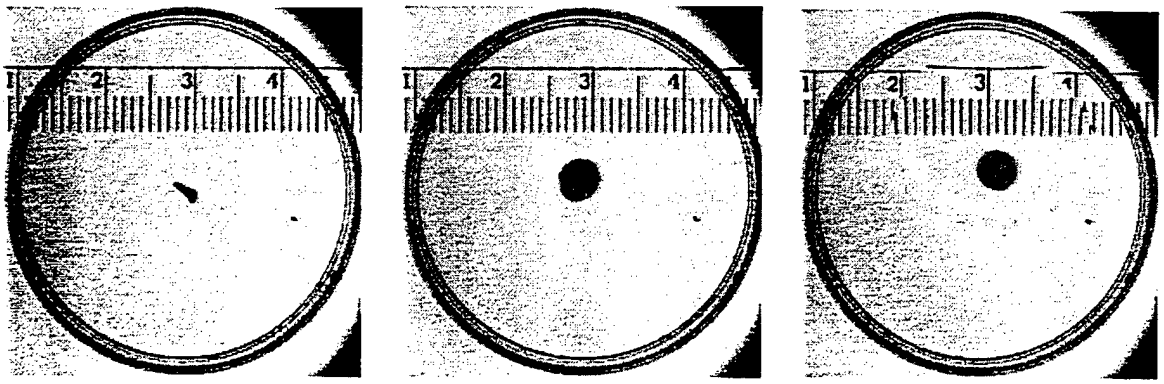

C
Release percentage (%)
45
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
Time (h)
media
media with cells
human plasma

Figure 1

A



B

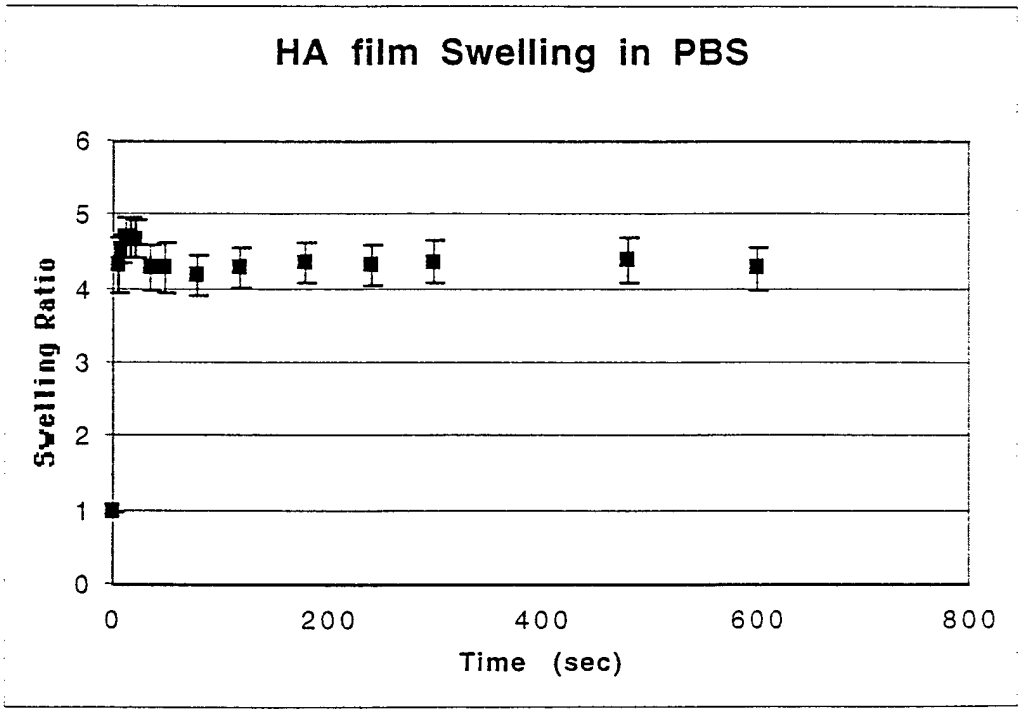

HA film Swelling in PBS
Swelling Ratio
0
1
2
3
4
5
6
0
200
400
600
800
Time (sec)

Figure 2

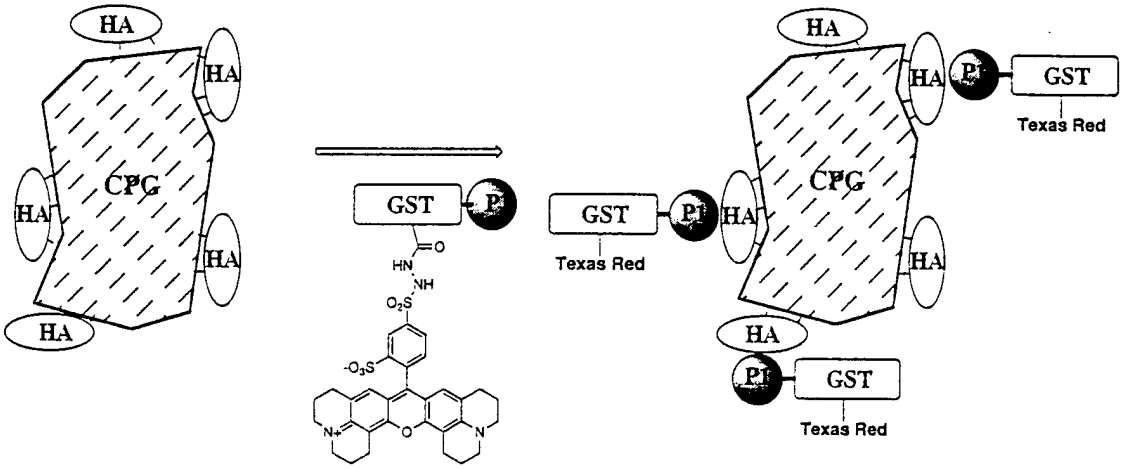
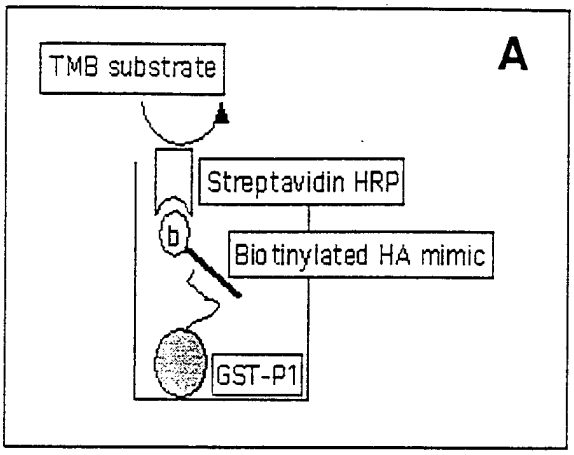
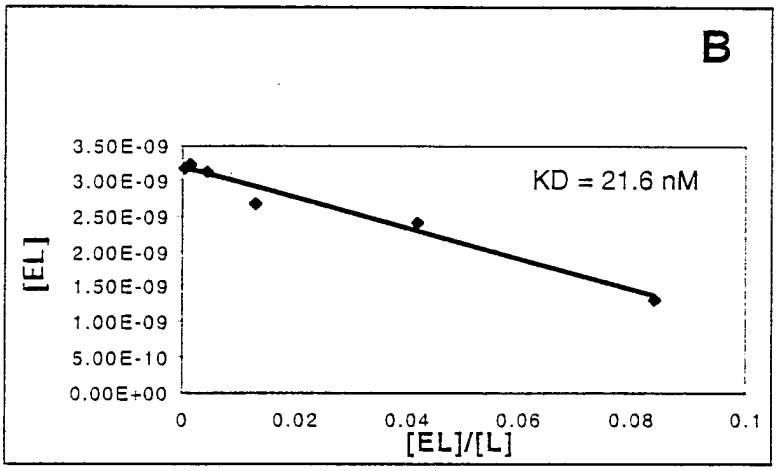

HA
HA
HA
CPG
HA
HA
GST
P
HN
NH
O_3S
$-O_3S$
N+
O
N
GST
P
Texas Red
HA
HA
P
GST
Texas Red
CPG
HA
HA
HA
P
GST
Texas Red

Figure 3


A
TMB substrate
Streptavidin HRP
b
Biotinylated HA mimic
GST-P1


B
KD = 21.6 nM
[EL]
3.50E-09
3.00E-09
2.50E-09
2.00E-09
1.50E-09
1.00E-09
5.00E-10
0.00E+00
0
0.02
0.04
0.06
0.08
0.1
[EL]/[L]

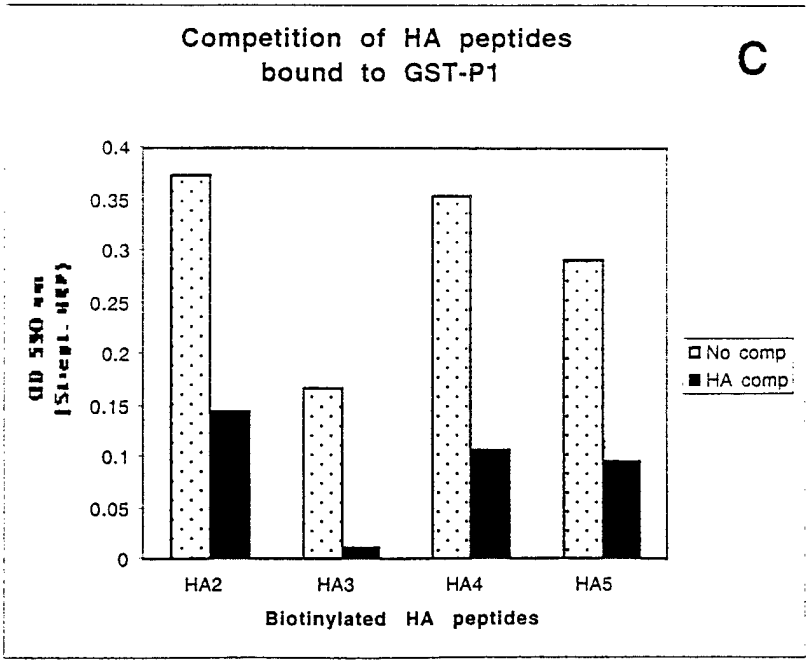
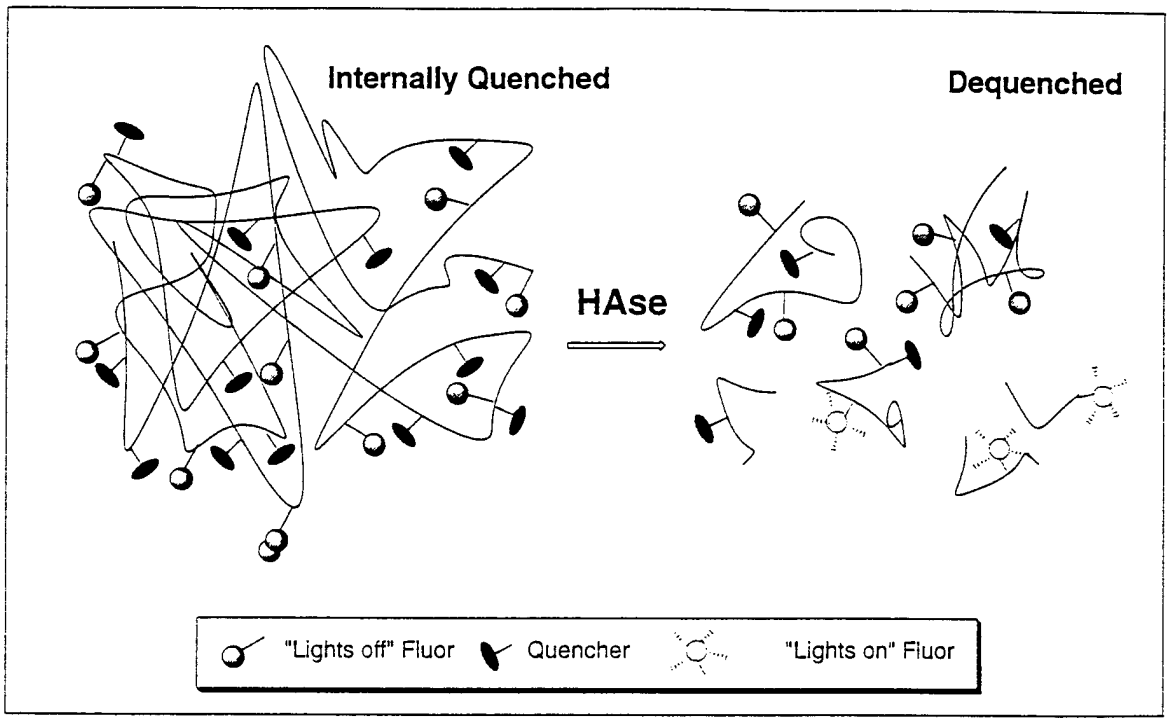

Competition of HA peptides bound to GST-P1
C
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
No comp
HA comp
HA2
HA3
HA4
HA5
Biotinylated HA peptides

Figure 4

A


Internally Quenched
Dequenched
HAse
"Lights off" Fluor
Quencher
"Lights on" Fluor

B

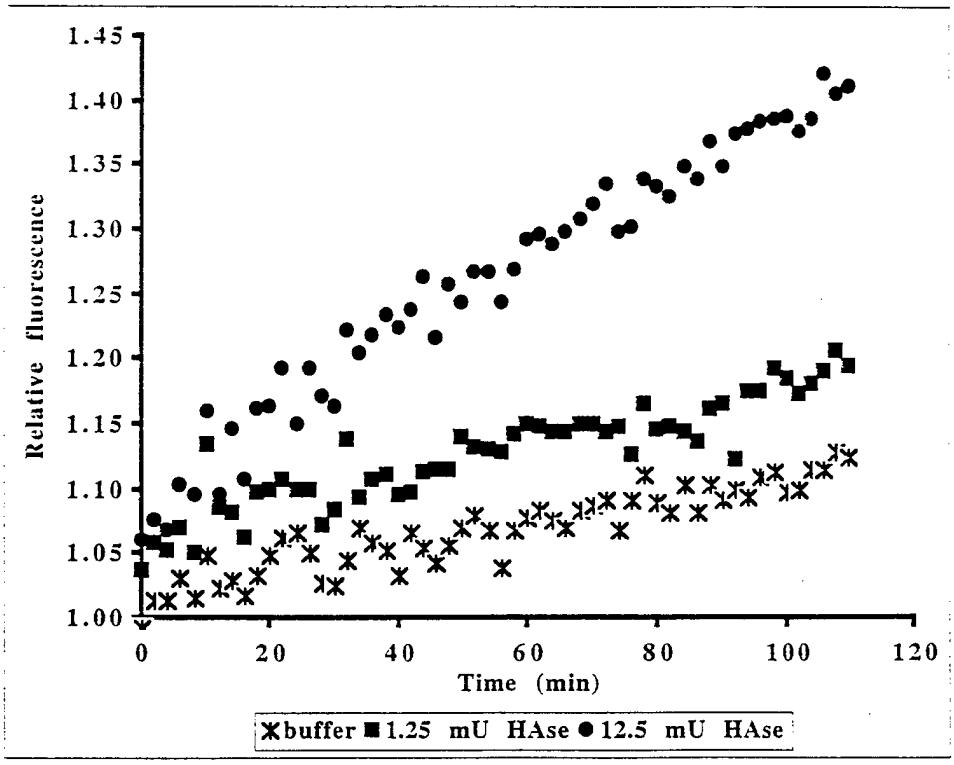

Relative fluorescence
1.45
1.40
1.35
1.30
1.25
1.20
1.15
1.10
1.05
1.00
0
20
40
60
80
100
120
Time (min)
buffer
1.25 mU HAse
12.5 mU HAse

Figure 5