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14. ABSTRACT
The **overall purpose** of this project is to develop targetable water-soluble polymerantiangiogenic drug conjugates for systemic breast cancer therapy. The **rationale** is that by selectively targeting polymer-antiangiogenic drug conjugates to alpha-v-beta-3 receptors on endothelial cells of tumor neovasculature; it is possible to restrict the biodistribution of the antiangiogenic drug to the vascular space, thereby increasing tumor accumulation and subsequent antitumor potency of the drug and decreasing dose-limiting toxicity. In year 1 progress was made in the following areas: i) Synthesis of a series targetable HPMA copolymerantiangiogenic drug conjugates, ii) Physicochemical characterization of the synthesized conjugates. There were challenges in the synthetic strategy for derivatization of the drug to attach to the polymer backbone. Current work is under way during the no cost extension period to optimize the synthetic strategies and to complete the remaining Aims of the grant, i.e., determination of binding and antiangiogenic efficacy of the conjugates on model endothelial cell lines.

15. SUBJECT TERMS
Targeted drug delivery, polymers, antiangiogenic, breast cancer, water-soluble

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INTRODUCTION:

The **overall purpose** of this project is to develop targetable water-soluble polymer-antiangiogenic drug conjugates for systemic breast cancer therapy. The **rationale** is that by selectively targeting polymer-antiangiogenic drug conjugates to alpha-v-beta-3 receptors on endothelial cells of tumor neovasculature, it is possible to restrict the biodistribution of the antiangiogenic drug to the vascular space, thereby increasing tumor accumulation and subsequent antitumor potency of the drug and decreasing dose-limiting toxicity. Three Specific Aims were proposed:

1) To synthesize and physicochemically characterize N-2(hydroxypropyl)methacrylamide (HPMA) copolymer-TNP-470 conjugates with and without the targeting moiety (RGD4C) in the side chains.

2) To evaluate the targeting/binding efficacy of the conjugates on a model endothelial cell line *in vitro*.

3) To evaluate the antiproliferative efficacy of the conjugates in the model endothelial cell lines *in vitro*.

In the first year of this project, progress was made to accomplish Aim 1, i.e. we synthesized and characterized the polymer-antiangiogenic drug conjugates with and without targeting moieties. Current work is underway to accomplish Aims 2 and 3. A one year no-cost extension was requested and approved to complete the remaining proposed work.

BODY:

A) Synthesis of HPMA copolymer-TNP-470 conjugates with and without the targeting moiety (RGD4C) in the side chains:

For this portion of the project the following tasks and subtasks were proposed:

Task 1. Synthesis of the polymer-drug-targeting moiety conjugates

a. Synthesis of monomeric units, and characterization by mass spectrometry.

b. Synthesis of polymer precursors of HPMA copolymers containing reactive functional groups by free-radical precipitation copolymerization and characterization of reactive group content by UV spectrophotometry.

c. Conjugation of TNP-470 and RGD4C to HPMA copolymer precursors by consecutive aminolysis reaction.

d. Purification of the conjugates by size-exclusion chromatography followed by dialysis and lyophilization.

We successfully completed Task 1 and disseminated the results in the annual Era of Hope meeting 2005 in Philadelphia, PA (See Appendix 1 and 2). This progress is summarized below:

The general scheme of synthesis is outlined in Appendix 1. A glycine derivative of Fumagillin (Fumagillol-Glycine, Fu-Gly) was used as a model drug instead of TNP-470 to permit easy attachment of the drug to the polymer backbone. Fumagillin derivatives such as Fu-Gly also have known antiangiogenic effects [Sin et al., 1997] like the proposed drug TNP-470 which is a derivative of the parent compound Fumagillin. The purpose of using Fu-Gly was to demonstrate proof of concept which can then be extended to other model antiangiogenic drugs including TNP-470.

The following conjugates were successfully synthesized:

HPMA copolymer-(GG-RGD4C)-GFLGG-Fu (Polymer with drug and targeting moiety)
HPMA copolymer-GFLGG-Fu (Polymer with drug but no targeting moiety)
HPMA copolymer-(GG-RGD4C) (Polymer with targeting moiety but no drug)
HPMA homopolymer (Control polymer with no drug or targeting moiety)

Experimental Challenges and Reason for no cost extension:

TNP-470, proposed model antiangiogenic drug is not commercially available. Initial attempts to synthesize TNP-470 from its parent analog Fumagillin were unsuccessful. Fumagillin analogs which retain the active epoxy ring attached to C3 site of the molecule, generally exhibit antiangiogenic activity. This is established in the literature based on the metabolic end products of Fumagillin [Kusaka et al., 1991; Lowther et al., 1998; Cretton-Scott et al., 1996]. Hence we decided to use an alternative Fumagillin analog namely Fu-Gly with an intact epoxy ring and known antiangiogenic activity [Sin et al., 1997]. The purpose is to use this drug merely as a model drug to demonstrate proof of concept.

A second challenge in the current polymer-drug conjugate synthesis is that the presence of Gly in Fu-Gly resulted in a polymer conjugate with the drug attached via a glycylphenylalanylleucylglycylglycine (GFLG**G**) spacer rather than the well established lysosomally degradable (GFLG) (one less glycine) spacer. It is anticipated that the biodegradability and release profile of the drug from the GFLGG spacer might be somewhat different than the GFLG spacer. Current studies are under way to evaluate the synthesized conjugates in vitro to compare their efficacy. As an alternative, work is proposed to resynthesize a polymer conjugate of the drug with a GFLG spacer.

The observed challenges lead us to request a no cost extension on the proposed work to accomplish the alternative synthesis as well as the remaining in vitro evaluation studies.

B) Physiochemical characterization of synthesized conjugates:

For this portion of the project the following tasks and subtasks were proposed:

Task 2. Physicochemical characterization of the conjugates
- a. Drug content measurement by UV spectrophotometry.
- b. Targeting moiety content measurement by amino acid analysis.
- c. Molecular weight and polydispersity measurement by size-exclusion chromatography.

We successfully completed Task 2 and partially disseminated the results in the annual Era of Hope meeting in Philadelphia, PA (See Appendix 1 (Abstract) and 2 (poster)). The complete physicochemical characterization data is tabulated below (Table 1):

Table 1. Physicochemical properties of targetable HPMA-antiangiogenic drug conjugates

Sample	Description	Molar feed ratio			ONp content (mmol/g P)	RGD4C content (mmol/g P)	Fu content (mmol/g P)	Mw (kDa)	Mw/Mn
		HPMA	GG-ONp	GFLGG-Fu					
P1	HPMA	100	0	0	-	-	-	123.0	1.8
P2	HPMA-(GG-RGD4C)-GFLGG-Fu	85	10	5	0.5	0.17	0.28	132.0	1.9
P3	HPMA-(GG-RGD4C)	90	10	0	0.6	0.24	-	38.1	1.4
P4	HPMA-GFLGG-Fu	95	0	5	-	-	0.24	83.5	1.7

Legends:
HPMA= N-(2-hydroxypropyl) methacrylamide, G=Glycine, F=Phenylalanine, L=Leucine, Fu=Fumagillol, M_w=weight average molecular weight, M_n=number average molecular weight, Mw/Mn=polydispersity

The synthesized conjugates were successfully characterized. The contents of drug and targeting moiety were proportional to their molar feed ratios and the data was consistent with report of similar systems in the literature.

C) Work in progress:

Currently work is under way to complete Specific Aims 2 and 3 for which the following tasks have been proposed:

Task 3. In vitro evaluation of targeting efficiency, and antiproliferative activity of the conjugates and concluding the project
a. Endothelial cell adhesion assays to determine binding/targeting affinity of conjugates.
b. Endothelial cell growth inhibition assays to determine the efficacy of the conjugates.
c. Dissemination in a conference, preparation of a manuscript and final report.

Work is also under way to optimize synthetic strategies based on observed challenges as discussed under Section **A**. Current work has been initiated to establish HUVEC cell culture lines for in vitro binding and efficacy evaluations.

KEY RESEARCH ACCOMPLISHMENTS:

1) Synthesis of targetable polymer-antiangiogenic drug conjugates for systemic breast cancer therapy

2) Physicochemical characterization of synthesized conjugates

3) Presented this ongoing research in the Era of Hope Meeting (See Appendix 1)

4) Participated in scientific meetings related to the area of controlled drug delivery:
- Controlled Release Society Meeting (CRS-June 2005, Miami, Florida)
- 12th International Symposium on Recent Advances in Drug Delivery Systems (February, 2005, Salt Lake City, Utah)

REPORTABLE OUTCOMES:

1) Abstract and poster presentation in Era of Hope Meeting (2005)

CONCLUSIONS:

In summary progress was made in the following areas:

1) Synthesis of targetable polymer-linked antiangiogenic drug conjugates

2) Physicochemical characterization of targetable polymer-linked antiangiogenic drug conjugates

Currently work is under way to optimize the synthesis of the polymeric conjugates and to complete the in vitro evaluation studies.

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APPENDICES:

Appendix 1: Anjan Nan and Hamidreza Ghandehari. Targetable Polymer-Antiangiogenic Drug Conjugates for Systemic Breast Cancer Therapy. Abstract for the 4th Era of Hope Meeting, Philadelphia, PA, June 8-11, 2005.

Appendix 2: Anjan Nan and Hamidreza Ghandehari. Targetable Polymer-Antiangiogenic Drug Conjugates for Systemic Breast Cancer Therapy. Poster Presentation at the 4th Era of Hope Meeting, Philadelphia, PA, June 8-11, 2005.

Appendix 1: Poster abstract for the 4th Era of Hope Meeting, Philadelphia, PA, June 8-11, 2005.

TARGETABLE POLYMER-ANTIANGIOGENIC DRUG CONJUGATES FOR SYSTEMIC BREAST CANCER THERAPY

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Introduction: Angiogenesis plays a critical role in tumor growth and metastasis. The $\alpha_v\beta_3$ integrin is a unique molecular marker[1] expressed on the surface of neovascular endothelial cells that differentiate them from their mature counterparts. High affinity $\alpha_v\beta_3$ selective ligands, containing the Arg-Gly-Asp (RGD) sequence, have been identified as potential targeting moieties to deliver antiangiogenic drugs to the tumor vasculature[2]. However, unfavorable biodistribution of the small RGD peptides limit their utility[3]. N-(2-hydroxypropyl) methacrylamide (HPMA) copolymers are water-soluble, biocompatible and versatile macromolecular carriers for the targeted delivery of bioactive agents[4]. We describe here the synthesis and characterization of HPMA copolymer-antiangiogenic drug conjugates containing RG**D**4C (a cyclic high affinity binding RGD peptide) for targeting tumor angiogenic vasculature.

Experimental Methods: The comonomers of HPMA, methacyloylglycylphenylalanylleucylglycine-ethylenediamine ($MAGFLGCH_2CH_2NH_2$), methacryloylglycylglycyl-ONp (MAGGONp) were synthesized by adaptation of previously described procedures[5]. TNP-470 (O-(chloroacetylcarbamoyl) fumagillol), a model antiangiogenic drug was derivatized using published procedure[6] from its parent drug Fumagillin. All synthesized comonomers were characterized by standard physicochemical techniques including melting point, mass spectrometry and UV-Vis spectrophotometry.

Results: HPMA (M.P. 67-69 ^{0}C, M+1=144.0), MAGGONp (M.P. 159.9-163.8 ^{0}C, M+1=322.3, ε_{272nm}=9500$M^{-1}cm^{-1}$), $MAGFLGCH_2CH_2NH_2$ (M+1=503, R_f=0.2 (Ethyl Acetate: Methanol: Triethylamine, 72:20:8)) and TNP-470 (M+23=424, R_f=0.3 (Ethyl Acetate: Hexane, 25:75)) were synthesized and characterized.

Conclusion: The comonomer precursors of targetable polymer-antiangiogenic conjugates were successfully synthesized and characterized. Work is under way to copolymerize the monomers followed by attachment of the RG**D**4C targeting peptide, for subsequent in vitro evaluation. The ultimate goal of this research is to develop novel therapeutics that enhances the efficacy and safety of antiangiogenic drugs for the treatment of breast cancer.

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Appendix 2: Anjan Nan and Hamidreza Ghandehari. Targetable Polymer-Antiangiogenic Drug Conjugates for Systemic Breast Cancer Therapy. Poster Presentation at the 4th Era of Hope Meeting, Philadelphia, PA, June 8-11, 2005.

TARGETABLE POLYMER-ANTIANGIOGENIC DRUG CONJUGATES FOR SYSTEMIC BREAST CANCER THERAPY

Anjan Nan[1,2] and Hamidreza Ghandehari[1,2,3]

[1]Department of Pharmaceutical Sciences, [2]Center for Nanomedicine and Cellular Delivery, [3]Greenebaum Cancer Center, and [4]Bioengineering Program, University of Maryland, Baltimore, Maryland 21201, USA

Introduction

Current breast cancer therapy is limited by dose-limiting toxicity of chemotherapeutic agents and lack of selectivity towards tumor cells.[1] Angiogenic blood vessels have been identified as a target site for therapeutic interventions because of their important role in tumor growth and metastasis.[2] It is established that primary malignant breast tumors exhibit high angiogenic activity.[1]

The $\alpha_v\beta_3$ integrin is a unique, highly specific angiogenic marker[3] that is expressed on the apical side of newly formed capillaries that differentiate them from their mature counterparts. High affinity $\alpha_v\beta_3$ selective ligands, Arg-Gly-Asp (RGD), have been identified by phage display studies.[4] The doubly cyclized peptide, RGD4C (with two disulfide linkages via four cysteine residues) containing a conformationally restrained RGD binds to $\alpha_v\beta_3$ 200 fold more avidly than linear peptides.[5]

The early appearance of angiogenic response of a tumor (relative to tumor growth), the highly selective expression of $\alpha_v\beta_3$-integrins in the neovasculature and the availability of specific $\alpha_v\beta_3$ targeting peptide, RGD4C makes it an ideal choice for developing a tumor angiogenesis-targeting agent.

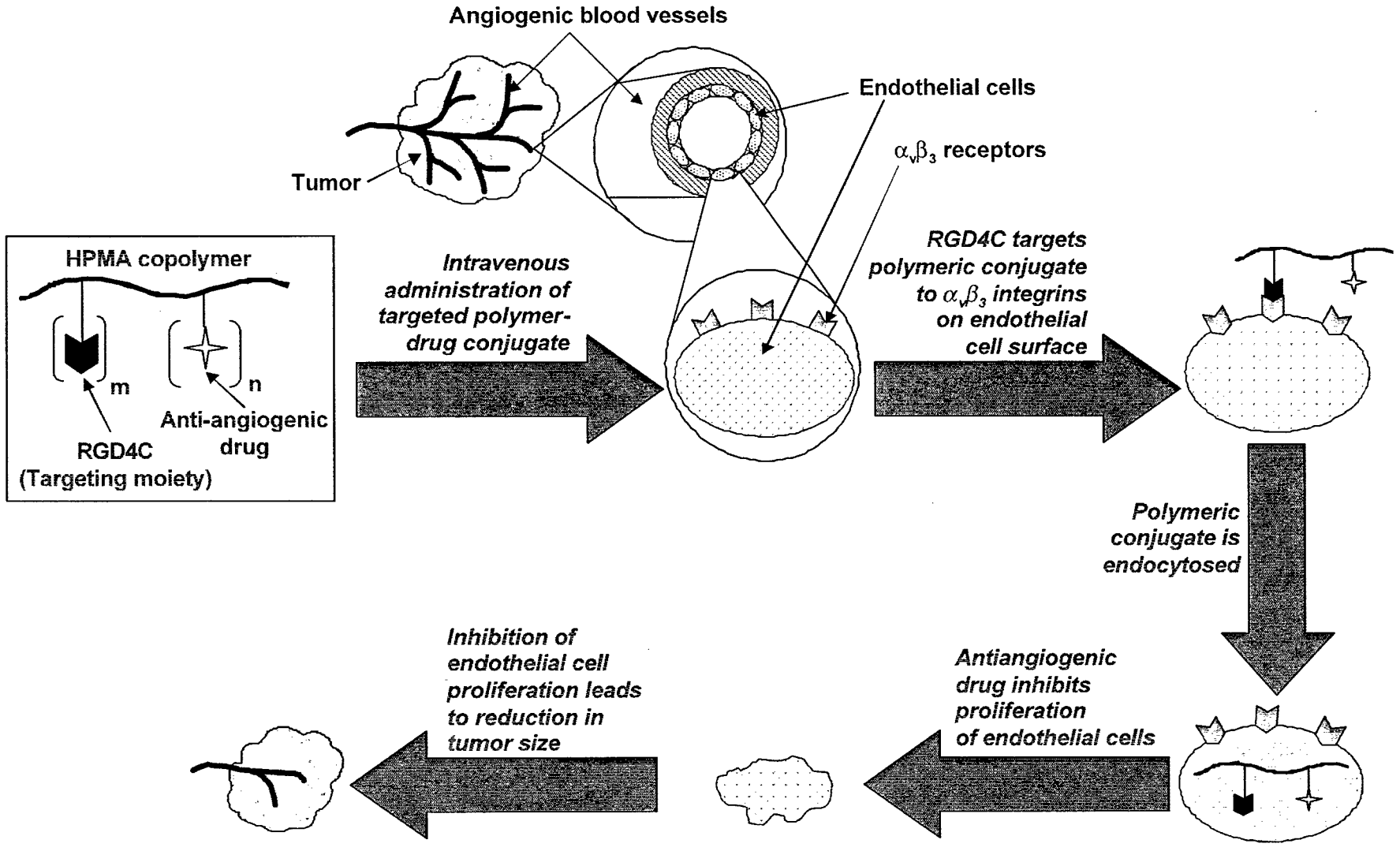
Introduction (cont'd)

Polymeric carriers containing covalently linked RGD peptides can be designed to enhance tumor localization and allow targeted delivery of bioactive agents to selectively kill tumor associated neovasculature. Water soluble N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer-drug conjugates have been extensively studied as biocompatible, hydrophilic and non-immunogenic drug delivery systems[6] which can be tailor made for specific targeting needs.

We propose to enhance the effectiveness of targeting tumor angiogenesis by conjugating antiangiogenic drugs to HPMA copolymers and RGD4C as a targeting moiety. The proposed strategy as depicted in Figure 1 will investigate the potential of active targeting of HPMA-antiangiogenic drug conjugates in the treatment of breast cancer.

In the current work the synthesis and characterization of such polymeric conjugates is described.

Figure 1. Rationale for angiogenesis targeting

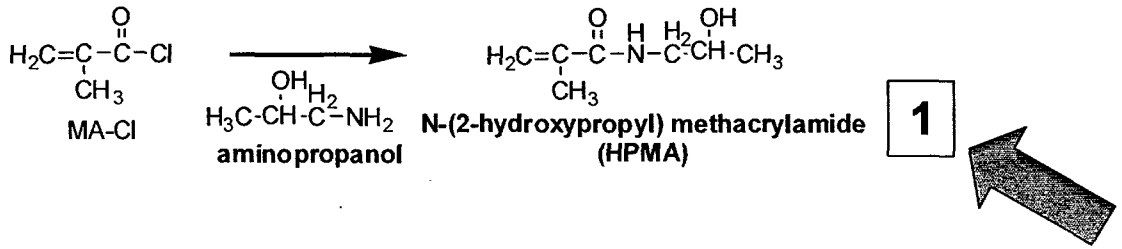


Specific Aims of the Work

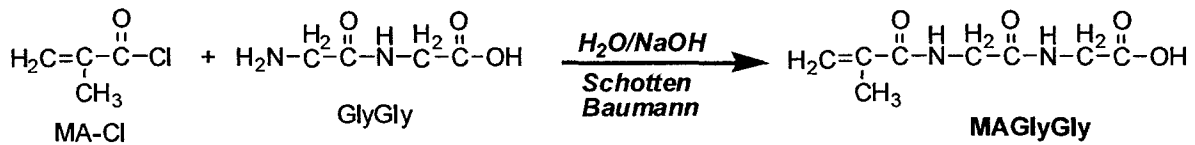
- **Synthesis and characterization of targetable N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer-antiangiogenic drug conjugates**
- **Cyclic RGD4C (KACDC*RGD*CFCG) is used as a model peptide to target $\alpha_v\beta_3$ integrins on the surface of endothelial cells of tumor neovasculature**
- **Fumagillol-Gly, a derivative of Fumagillin is used as a model antiangiogenic drug**
- **The antiangiogenic drug is attached to the polymer backbone via a lysosomally degradable tetrapeptide spacer namely glycylphenylalanylleucylglycine (GFLG)**

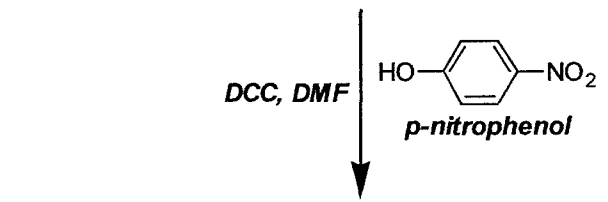
Experiments and Results

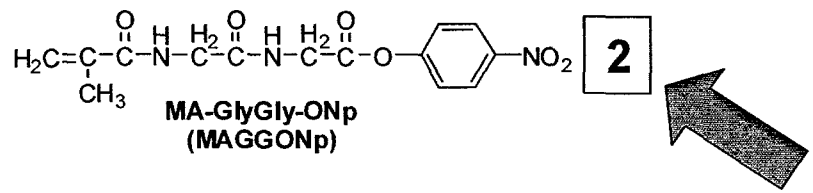
Synthesis of comonomers



This comonomer renders water-solubility and constitutes the polymer backbone

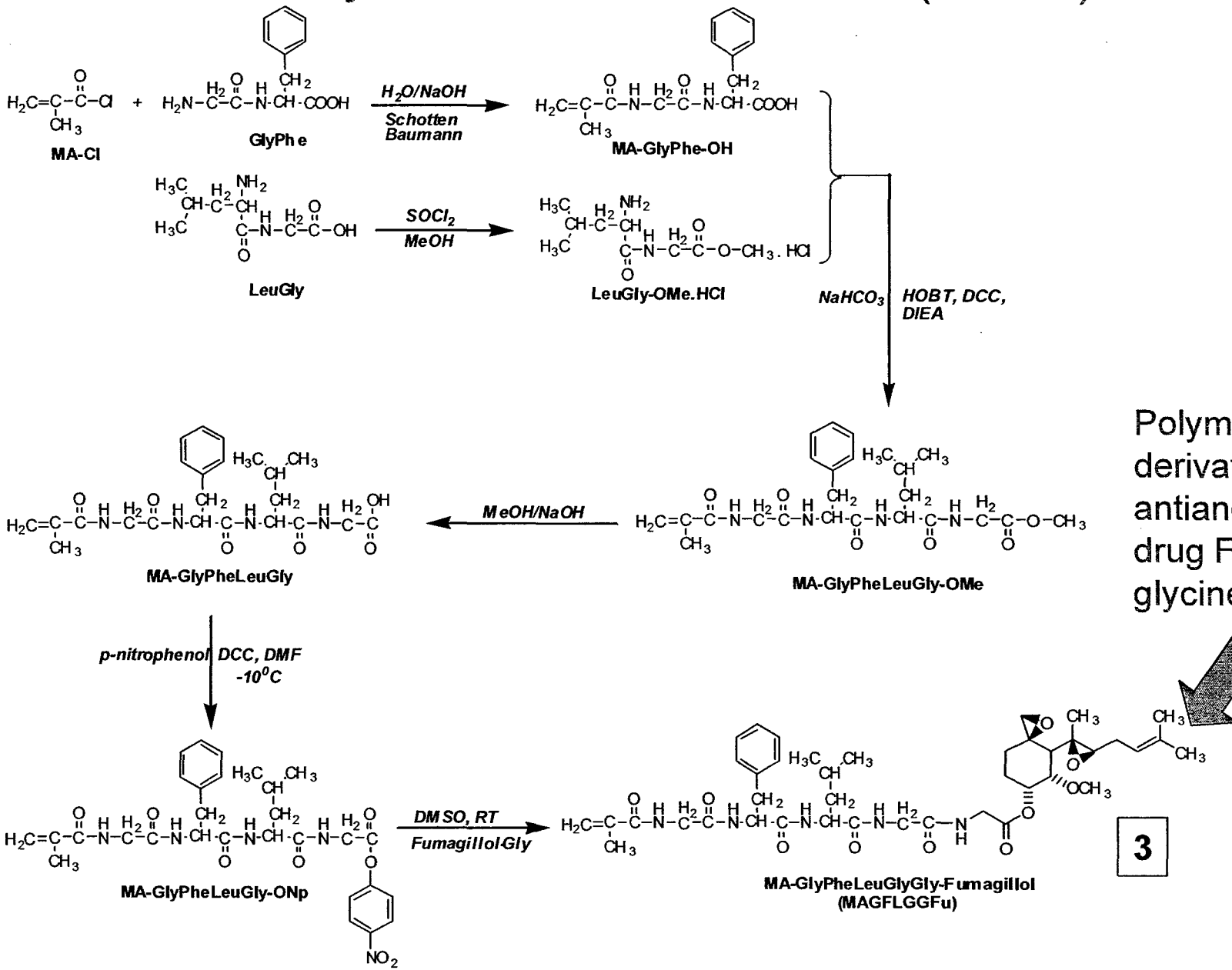






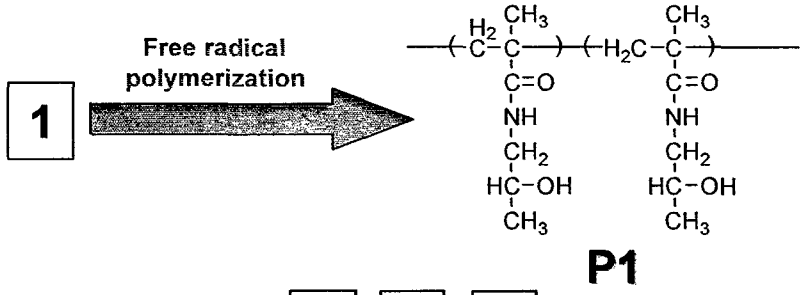
This comonomer is used to attach the targeting moiety (RGD4C)

Synthesis of comonomers (cont'd)

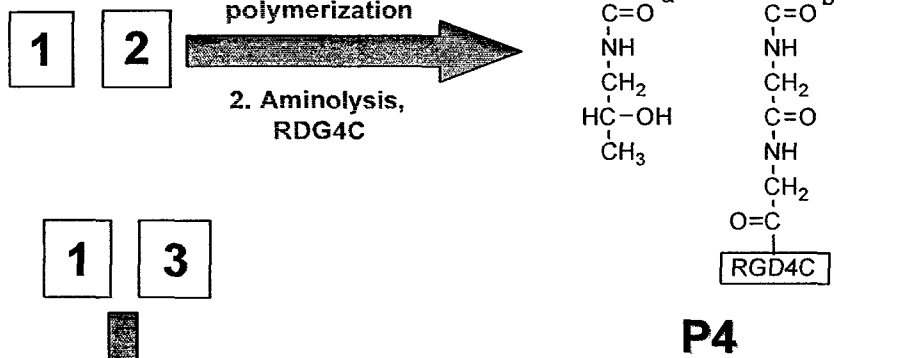


Polymerizable derivative of antiangiogenic drug Fumagillol·glycine

Synthesis of polymer-drug-targeting moiety conjugates



P1



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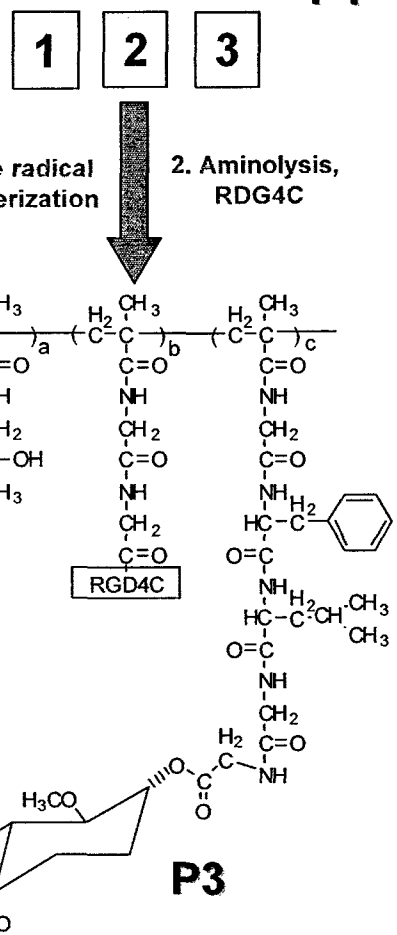

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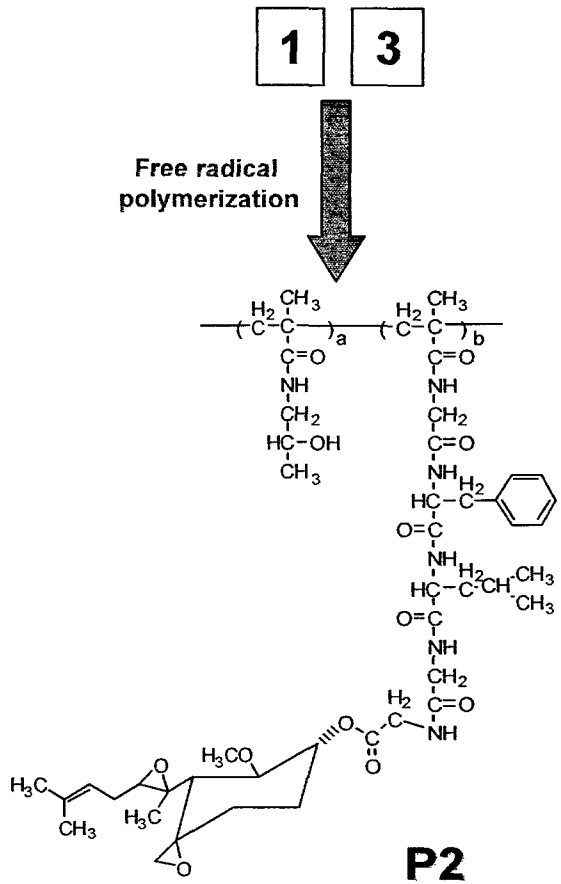

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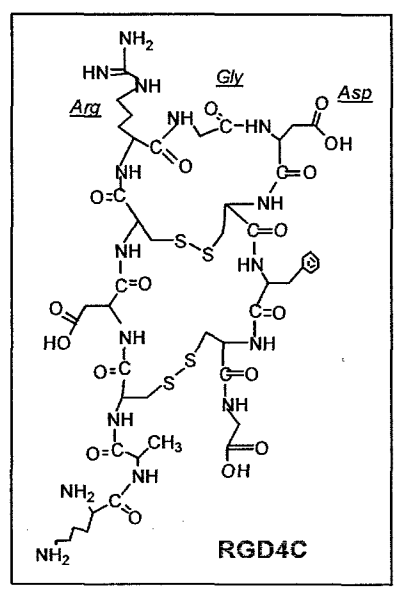

Table 1. Physicochemical characterization

Sample	Feed comonomer composition (mole%)			ONp content (mmol/g)*	Drug content (mmol/g)	Peptide content (mmol/g)	Mw (kDa)**	Mw/ Mn***
	HPMA	MAGGONp	MAGFLGGFu					
P1	100	0	0	-	-	-	123.0	1.8
P2	95	0	5	-	ND	-	83.5	1.7
P3	85	10	5	0.5	ND	ND	132.0	1.9
P4	90	10	0	0.6	-	ND	38.1	1.4

*Polymer precursor (ONp content measured by UV spectrophotometry)
**Weight average molecular weight (measured by size exclusion chromatography)
***Polydispersity (measured by size-exclusion chromatography)
ND = Not determined

Observations and conclusions

- **HPMA antiangiogenic drug conjugates successfully synthesized and characterized**
- **Content of reactive ONp groups in the polymer precursors were consistent with reports in the literature on similar systems**
- **Targetable conjugate (P3) showed high molecular weight and polydispersity suggesting inter and intra molecular association of RGD4C with the polymeric network**
- **Work is under way to characterize the drug and peptide content by amino acid analysis**

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