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Award Number: W81XWH-12-1-0097

TITLE: "Targeting Alpha5 Beta1 Integrin to Prevent Metastatic Breast Cancer Cell Invasion: PhScN Target Site Definition and Plasma Stability"

PRINCIPAL INVESTIGATOR: Philip Andrews

CONTRACTING ORGANIZATION: Vj g'Tgi gpuu'qh'\j g'University of Michigan
''Cpp'Ctdqt.'O K'6:32;

REPORT DATE: September 2014

TYPE OF REPORT: cppwcn

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

DISTRIBUTION STATEMENT: Approved for Public Release;
                                           Distribution Unlimited

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2014	Annual	1Sep2013-31Aug2014

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Targeting Alpha5 Beta1 Integrin to Prevent Metastatic Breast Cancer Cell Invasion: PhScN Target Site Definition and Plasma Stability	
	5b. GRANT NUMBER
	W81XWH-12-1-0097
	5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)	5d. PROJECT NUMBER
Philip Andrews	
	5e. TASK NUMBER
Email: andrewsp@umich.edu	
	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
The Regents of the University of Michigan Ann Arbor, MI 48109	

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

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

13. SUPPLEMENTARY NOTES

14. ABSTRACT
We proposed to test PHSCN and PhScN derivatives with modifications of the N– or C–termini, and/or cysteine side chain to determine the initial spatial constraints of the binding pocket of the ligand to its target site. This will aid in the selection of crosslinking agents, modifications, tag additions and strategies for determination of the target site(s) performed in Aim 2 and lay the foundation for determining the structure-activity relationships (SARS) of these inhibitors with the target.

15. SUBJECT TERMS- none listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
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INTRODUCTION: It has been suggested that PHSCN inhibits metastatic invasion by forming a covalent, disulfide bond with a cysteine residue on the beta1 (β1) subunit of alpha5 beta1 (α5β1) integrin[1]. However, these studies were performed with purified α5β1 integrin, which also produces evidence of covalent bond formation with the α5 subunit (tandem mass spectroscopy data not shown). Hence, the specificity of the reported interaction between PHSCN and the β1 subunit[1] is suspect. Moreover, the cysteine–rich β1 subunit can heterodimerize with 12 distinct alpha integrin subunits[2], forming integrins that function in many pathways. In contrast, the α5 subunit interacts uniquely with the β1 subunit[2] to induce invasion and support adhesion[3-5]; hence it is a much more desirable target. Our funded research is designed to test definitively the hypothesis that both PHSCN and PhScN peptides inhibit α5β1 integrin fibronectin receptor–mediated invasion through noncovalent interaction with α5β1 receptors of SUM149PT and MDA-MB-231 metastatic human breast cancer cells, and to lay the foundation for detailed structure–activity relationship (SAR) studies for both peptides as inhibitors of α5β1–mediated metastatic breast cancer invasion by precisely defining their target site(s) on α5β1 integrin, and by developing an assay that can be used to evaluate *in vitro* degradation rates in plasma, and to monitor *in vivo* plasma levels.

Objective/Hypothesis: Our preliminary data suggest that the invasion inhibitory potencies of the S–acetylated and the S–methylated PHSCN peptide derivatives are increased by over 1,000–fold, relative to PHSCN. In addition, the PhScN peptide is still more potent: preliminary results suggest that it is 10^4 to 10^5–fold more potent than PHSCN at blocking α5β1–mediated invasion. Thus, we hypothesize that the actual invasion–inhibitory interaction of PHSCN with α5β1 integrin involves a noncovalent interaction with the target site, which is promoted by including D–isomers of histidine and cysteine in the PhScN peptide. Thus, covalent, disulfide bond formation with α5β1 integrin is actually a side reaction that *decreases* potency. Because mammalian proteins do not contain D–amino acids, endoproteinses evolved to cleave between L–amino acids only. Since PhScN does not contain 2 linked L–amino acids, it cannot be degraded by endoproteases, usually present at high levels in tumors and increasing with malignancy[6-8]. Thus, in addition to promoting noncovalent interaction with its α5β1 integrin target, the inclusion of D–amino acids at alternate positions is expected to result in increased PhScN potency as a result of preventing endoproteolytic degradation. Our research is designed to achieve the following specific aims.

Specific Aims: Specific Aim 1: To lay the foundation for determining their structure-activity relationships (SARs) as inhibitors of α5β1–mediated invasion[3-5,9,10], we propose to assay PHSCN and PhScN peptide derivatives, having modifications of N– or C–termini, and/or cysteine side chain. Peptides will be tested in quantitative serum–free invasion assays with naturally occurring basement membranes[3-5,9-15].

Specific Aim 2: To identify the binding site for the PHSCN sequence on α5β1 integrin, which will aid in the development of potent PHSCN/PhScN analogs, biotinylated, S-BPM–derivatized PHSCN and PhScN peptides will be photocrosslinked to SUM149PT and MDA-MB-231 cells. Tagged cells and extracts will be screened by fluorescent antibody staining and Western blotting for biotin and α5β1 integrin. The identity of the BPM–modified subunit and sites(s) of modification will then be determined using tandem mass spectrometry to localize the PHSCN/PhScN binding site(s) on α5β1☐integrin.

Specific Aim 3: To develop a mass spectrometry–based quantitative assay for PHSCN sequence analogs, including PhScN, and use it to evaluate and enhance their stabilities in human and mouse plasma. This assay is crucial for future in vivo studies where bioavailability and clearance rates are to be monitored.

Note: This report is a duplicate of the original, combined report (Livant and Andrews). The components of this report pertinent to this Co-PI (Andrews) are the portion of Aim 2 dealing with identification of the binding site and Aim 3 (development of the degradation assay).

BODY:

Specific Aim 1
We proposed to test PHSCN and PhScN derivatives with modifications of the N– or C–termini, and/or cysteine side chain to determine the initial spatial constraints of the binding pocket of the ligand to its target site. This will aid in the selection of crosslinking agents, modifications, tag additions and strategies for determination of the target site(s) performed in Aim 2 and lay the foundation for determining the structure-activity relationships (SARS) of these inhibitors with the target.

Selection of Peptides
Table 1 is the list of peptides and their IC50's determined using our *in vitro* invasion assay. Initial focus was on the modification of the cysteine side chain and the C-terminus. Modification of the L-cysteine side chain shows increased potency regardless of the modification, whereas self-derivatization of the D-cysteine had little or no effect on potency.   The only commercially available modification to D-cysteine is S-acetamidomethyl. Comparison of commercially prepared S-acetamidomethyl (S-acm) derivatives of both D and L cysteine of the biotinylated peptides were as expected and are show in Table 1. However, it was determined that modification of cysteine drastically interferes with Kd binding. The S-acm peptide Kd binding values were increase by ~100 fold, with the D-cysteine being more affected than the L-cysteine. This suggests that binding is less efficient with a modified cysteine side chain.

Table 1. Invasion inhibitory potency of PHSCN and PhScN derivatives. Values expressed in Molarity to adjust for molecular weight of modifications. Peptides highlighted in red were performed in year 2. **C**, commercially synthesized; **D,** derivatization in house of the appropriate commercial parent compound; **TBD**, to be determined; **bio**, biotin tag; **MAP**, multiantigenic peptide; **Me**, S–methylated; **OAc**, S–acetylated; **acm**, S–acetamidomethyl; **BPM,** benzophenone-4-maleimide

Table 1	C or D	mw	IC_{50} M	Kd μM	L or D isomer	Modification Region
Ac-PHSCN-NH$_2$	C	598	2.8 E-8		L	parent
Ac-PHSCNGGK-bio	C	1180	4.8 E-9	0.03	L	tail
Ac-PHSC(Me)N-NH$_2$	C	612	5.3 E-13		L	side
Ac-PHSC(OAc)N-NH$_2$	C	640	1.4 E-14		L	side
Ac-PHSC(acm)NGGK-bio	**C**	**1537**	**2.3 E-14**	**5.6**	**L**	**Tail + side**
Ac- PHSC(BPM)GGK-bio	D	1436	TBD		L	Tail + side
Ac-PHSCNGGK-MAP	C	7575	2.0 E-11		L	Tail (MAP)
Ac-PhScN-NH$_2$	C	598	2.2 E-14		D	parent
Ac-PhScNGGK-bio	C	1180	1.3 E-13	0.03	D	tail
Ac-PhSc(BPM)GGK-bio	D	1436	1.2 E-14		D	Tail + side
Ac-PhSc(acm)N-GGK-bio	**C**	**1537**	**0.7 E-14**	**17**	**D**	**Tail + side**
Ac-PhScNGGK-MAP	C	7575	1.7 E-20		D	Tail (MAP)
Ac-PhScNGGK-bio-GGK	**C**	**1480**	**6.9 E-14**	**0.02**	**D**	**Tail + ext**
Ac-KGGPhScNGGK-bio	**C**	**1480**	**2.1 E-14**	**0.02**	**D**	**Head + Tail**

C- and N-Terminus Extended Peptides

The addition of the biotin and the creation of a MAP (multi-antigenic peptide) on the C-terminus and the retention of activity suggest that the "tail" of the peptide has sufficient room for modification. The low Kd value of 0.03 also suggests that the addition of the biotin group to the parent compound has little effect on binding.   Two new peptides Ac-PhScNGGK-bio-GGK and Ac-KGGPhScNGGK-bio were commercially synthesized. These peptides have three additional amino acid residues to either the N or C terminus to introduce an amino group to allow for a new crosslinking site.   The new residues did not affect the invasion inhibitory potency and furthermore were also found to not affect Kd binding (Fig 1 and Table 1. These new peptides will allow for an array of crosslinking reagents to be evaluated in Aim 2.

Figure 1.   Invasion inhibition with C- and N-terminus modified peptide ligands.
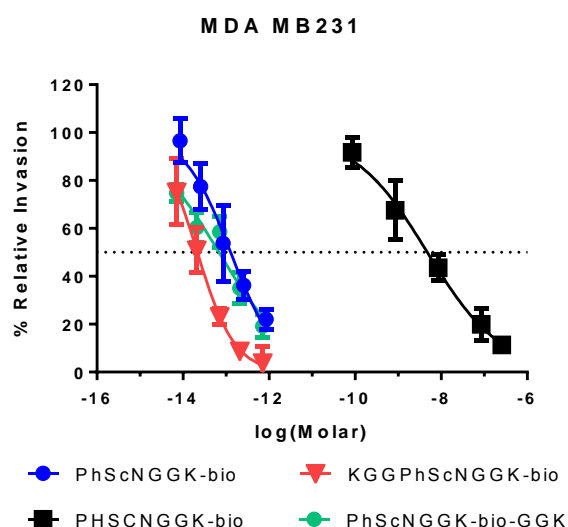
Activity of C- and N-Terminus Extended Peptides Derivatized with Crosslinking Agents
The new peptides (Ac-PhScNGGK-bio-GGK and Ac-KGGPhScNGGK-bio) were self-derivatized with the amine modifying portion of heterobibunctial crosslinkers and tested in the invasion assay at a single dose. Comparison to the underivatized peptide suggests that the addition of the crosslinker to either the N- or C-terminus does not affect invasion potency (Table 2).

Table 2.   Invasion inhibition with C- and N-terminus extension to include an available amine group. Parent peptide was modified with the appropriate crosslinker and converted to molarity to adjust for changes in mass. A single point value was collected and compared to the underivatized peptide at a similar concentration.

	Å arm	Total mw	pM	Ac-PhScNGGK-bio-GGK	Ac-KGGPhScnGGK-bio
No crosslinker	0	1480	0.7	21%	18%
Sulfo-LC-SDA	12.5	1920	~1.6	17%	17%
Sulfo-SANPH	18.2	1972	~1.5	19%	14%
ANB-NOS	7.7	1785	~1.7	15%	6%

Structure-Activity Relationships (SARS) experiments

The importance of the D-amino substitution of cysteine and histidine in Ac-PHSCN-NH2 were also tested (Fig 2). Singly the substitution of either cysteine or histidine imparts a 100- or 1000-fold increase in potency respectively. In combination the substitution of both cysteine and histidine produces the most potent version of the peptide of 100,000-fold increase in potency. It appears that the orientation of the cysteine side chain and histidine ring on the same side is the most efficient. In addition each residue in Ac-PhScN-NH2 was systemically replaced with alanine and tested in the invasion assay (Table 3). Substitution of any amino acid with alanine eliminated the potency of the compound and further suggests that the sequence of the compound is just as important. In addition the D-amino substitution of both the cysteine and histidine produces a sequence of alternating D and L amino acids. The presence of the D-amino acid residues make the compound endoproteinase resistant and increases the stability of the compound.

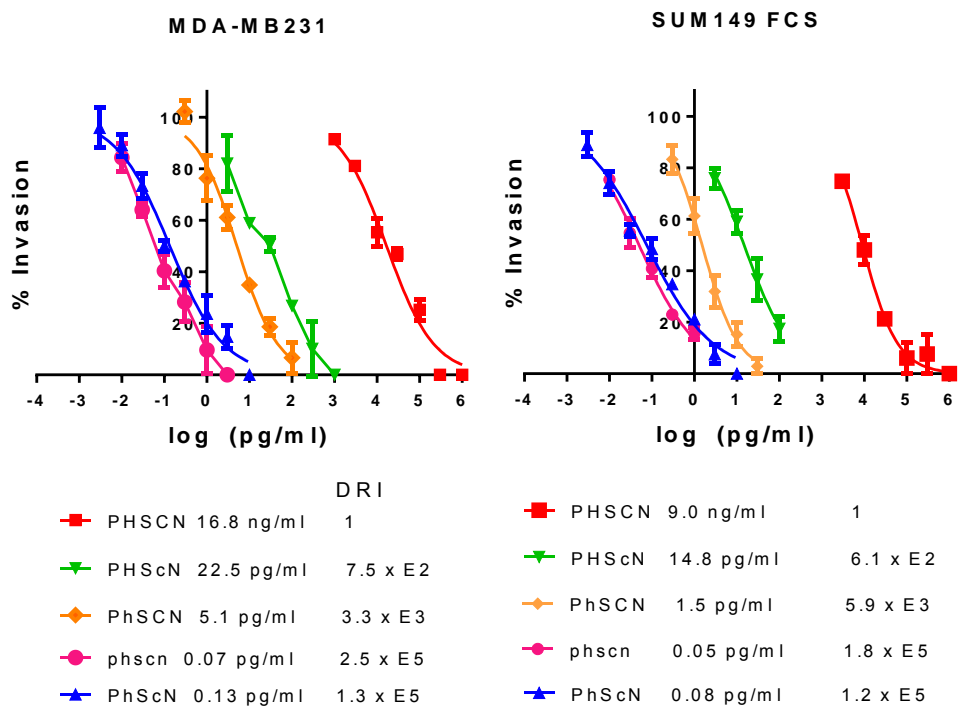**Figure 2.** Invasion inhibition of D-amino substitution of cysteine and or histidine.

	MDA-MB231	DRI		SUM149 FCS	
PHSCN	16.8 ng/ml	1	PHSCN	9.0 ng/ml	1
PHScN	22.5 pg/ml	7.5 x E2	PHScN	14.8 pg/ml	6.1 x E2
PhSCN	5.1 pg/ml	3.3 x E3	PhSCN	1.5 pg/ml	5.9 x E3
phscn	0.07 pg/ml	2.5 x E5	phscn	0.05 pg/ml	1.8 x E5
PhScN	0.13 pg/ml	1.3 x E5	PhScN	0.08 pg/ml	1.2 x E5

Table 3 Invasion inhibition of Alanine substituition of each amino acid in Ac-PhScN-NH2

	Alanine Scan	
	MDA MB211	Sum149PT
1 pg/ml	**Rel. % Inv**	**Rel. % Inv**
PhScN	0%	0%
PhScA	94%	99%
PhSAN	89%	123%
PhAcN	100%	118%
PAScN	95%	112%
AhScN	99%	94%

In addition peptide compounds were tested with selective modifications. The modification rendered the compound inactive and again supports the importance of the overall sequence of the peptide and the spacial constraints of the head and cysteine side chain as shown in Fig 2 and Table 3.

Table 4 Invasion inhibitory potency of selective modification of Ac-PhScN-NH2

Compound 1 pg/ml	% Rel. Inv	Rationale for Modification
Ac-PhScN-NH2	0%	parent
Bu-PhScN-NH2	83%	Head space
Ac-PhScn-NH2	102%	D-amino substitution of Asn
Ac-PhSmN-NH2	97%	D-Cysteine side chain by methionine
Ac-NcPhS-NH2	109%	Scrambled D-amino control

Specific Aim 2

Identification of the binding site for the PHSCN/PhScN sequence on $\alpha5\beta1$ integrin using protein crosslinking techniques and subsequent identification of the protein and peptide location by tandem mass spectrometry.

Crosslinking with BPM derivatized peptides and analysis by Western Blotting

PHSCN binds to activated integrin, specifically the $\alpha5\beta1$ integrin. The exact subunit and location is the goal of this proposal. There are multiple activation states of integrins and they are achieved by a combination of various divalent cations or by specific ligands. Our first attempt in year 1 at crosslinking the peptide to its target protein was to replicate the conditions used for *in vitro* invasion assays. Serum starved cells were stimulated by introduction of serum and were then allowed to incubate with the BPM derivatized peptides, followed by UV crosslinking to activate the BPM tag. To simplify and avoid losses, no washing steps were performed. As shown in Figure 3, the primary biotin labeled band is much smaller than the subunits of $\alpha5\beta1$ integrin and their expected mass assuming full glycosylation of the asparagine in the primary sequence (Table 5).
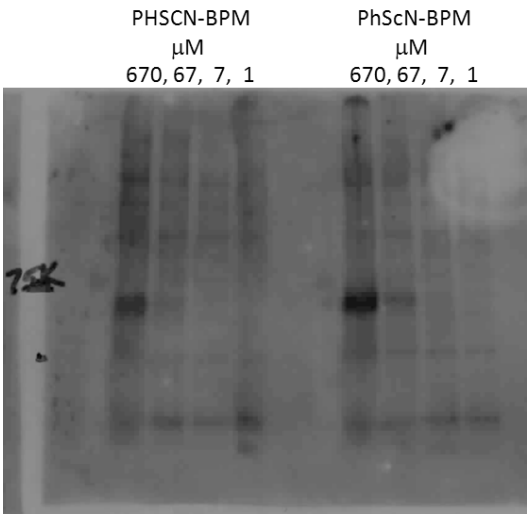

PHSCN-BPM
μM
670, 67, 7, 1

PhScN-BPM
μM
670, 67, 7, 1

Figure 3. SUM149PT cells, serum starved overnight. Cells suspended into medium at 37 C for 45 minutes. 40,000 cells were incubated with various concentrations of BPM derivatized peptides for another 20 min. UV irradiation for 45 minutes at room temp. 7.5% SDS-PAGE blotted with Anti-biotin.
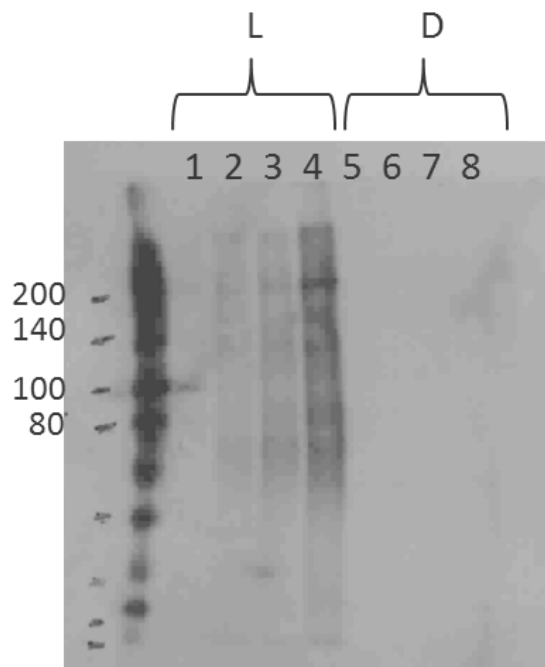
Table 5

Table 2	Da Primary sequence	Da fully glycosylated
$\alpha5$ subunit	114,536	150,964
$\beta1$ subunit	88,465	119,689

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The presence of the major low molecular weight biotin labeled protein suggests that two possible scenarios may have occurred. Either the BPM–peptide has formed an intermolecular crosslink with a nearby protein instead of an intramolecular crosslink within the target protein or the presence of 10% serum introduced a nonspecific binding target. Considering the high concentration of the peptide necessary to observe this low mass complex, the data suggest 1) nonspecific binding and 2) that the BPM peptides have a very high dissociation constant (K_d). To reduce or eliminate nonspecific binding, further attempts at crosslinking were performed after integrin activation by divalent cations. To ascertain whether the modified peptides had altered binding properties because of steric hindrances not detected by the functional *in vitro* invasion assay, the binding constants (K_d) of the relevant compounds were completed in year 2 (Table 1).

Crosslinking after activation with Divalent Cations

The crosslinking experiment, whose results are shown in Figure 3, was repeated using divalent cations to activate integrin and a varying amount of cells to increase signal. As shown in Figure 4 below, the use of divalent cations eliminated the prevalent biotin labeled low molecular weight complex suggesting that this was indeed nonspecific binding to the serum proteins at the high concentrations of peptide required. The presence of bands in the region of interest is promising, however the amount observed suggested that the Kd binding constants of the BPM peptide derivatives were much greater than the underivatized parent. The presence of bands only in the L lanes (L-cysteine) (Fig 4) further suggested that there are more steric hindrances in binding for the D lanes (D-cysteine). These observations were confirmed with the completed binding assays.

Figure 4. Western blot with Anti-biotin of a 7.5% SDS-PAGE of varying amounts Mn^{2+} activated MDA-MB-231 cells incubated with 50 µM of BPM derivatized peptide. Ac-PHSC(BPM)NGGK-bio(L) and Ac-PhScN(BPM)NGGK-bio(D) denote BPM ligand. The cell quantity varied for each lane ln 1,5 50K; ln 2,6 100K; ln 3,7, 200K and ln 4,8, 500K.

As proposed in the prior progress report, cross-linking with the BPM peptides was continued while the new C- and N- modified peptides were being synthesized and characterized. Attempts to scale the BPM experiments for protein enrichment and identification by MS/MS were unsuccessful and eventually abandoned. Furthermore, as previously stated the binding constants (Table 1) confirmed that modification of the cysteine side chain did indeed perturb the binding constant, more so for D-cysteine than L-cysteine and supported the decision to abandon this approach.

The new extended C- and N-terminus peptides Ac-PhScNGGK-bio-GGK (bio-GGK) and Ac-KGGPhScNGGK-bio (KGG-bio) have been characterized and were found to retain full activity and binding properties (Table 1). The lysine side chain in the extension was first derivatized with the amine reactive region of the heterobifunctional crosslinkers. Samples were tested for invasion inhibitory potency (Table 4) and were found to retain activity after the first derivatization. Cells were incubated with the crosslinked peptides and then exposed to UV light to activate the nonspecific region of the crosslinkers. Different length crosslinkers were tested with both versions of the extended peptides. The results are shown in Fig. 5. The initial assessment is

that the primary band is the same for all the crosslinkers used and that either the bio–GGK version or the KGG-bio version of the peptide can be used. Note: Lanes 1 and 2 are very faint but denote the same bands as seen in lanes 3-6. (not shown). Work is under way to improve the derivatization of the peptides, cleanup and optimization of the photoactivation of the crosslinkers.
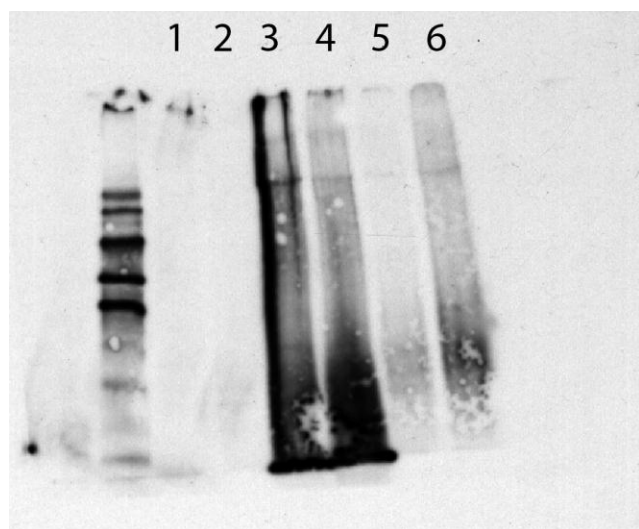

Fig 5.   Photoactivatible crosslinkers with the C- and N-terminus peptides. Western blot by detection with anti-biotin HRP. Lane 0, biotinylated mw markers.

Lane	Peptide	Crosslinker	Length
1	bio-GGK	sulfo-LC-SDA	12.5
2	KGG-bio	sulfo-LC-SDA	12.5
3	bio-GGK	sulfo SANPAH	18.2
4	KGG-bio	sulfo SANPAH	18.2
5	bio-GGK	ANB-NOS	7.7
6	KGG-bio	ANB-NOS	7.7

Our collaborator, Dr. Phil Andrews has developed a cell permeable cross-linker that targets amine groups called DC4 that spans 15 angstoms. It was found to have a minor reaction with histidine and cysteine residues on the shortened parent version of the peptide in year 1. After development of the new extended peptides, the DC4 was retried in year 2 as designed. As seen in Fig 6. The primary band is in the same region of interest as seen with the photoactivatable compounds in Fig 5. The proteins were extracted with 100 mM octyl-β-D-glycopyranoside (OBG) in RIPA buffer and the supernatent enriched by streptavidin agarose. As seen in Fig 6, the majority of the labeled material remained in the pellet. DC4 will react with any free amine and over-crosslinker can occur, trapping proteins in complexes. The presence of biotinylated sample remaining in the pellet indicates that more aggressive solubilization is necessary to extract the sample prior to enrichment; however that must be balanced with compatibility of binding conditions to streptavidin. The alternative is to digest the proteins and then enrich the peptides.   Both methods will be employed.

Figure 6.   Western blot demonstrating crosslinking with DC4 and both the bio–GGK and KGG-bio peptides. The P denotes the pellet sample and the A denotes enrichment of the sample with biotin tag detected by anti-biotin HRP. Lane 1, biotinylated mw markers.

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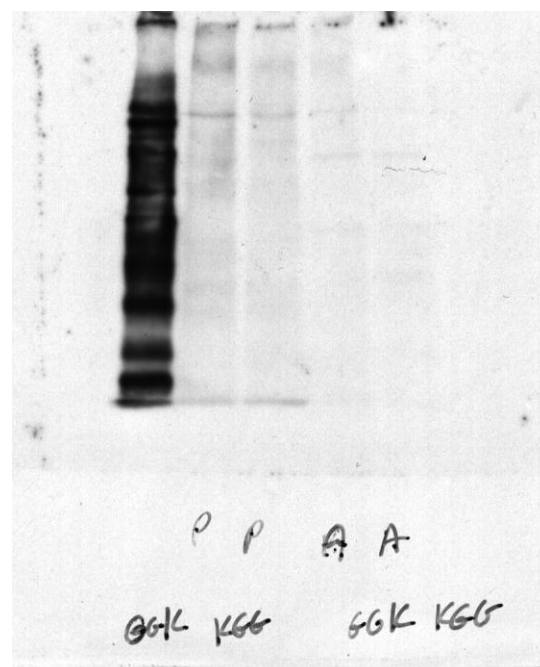
Summary of crosslinking experiments

Our first approach was to use the cysteine side chain modified BPM-derivatized peptides. As seen in Fig 3 and 4, and confirmed by binding assays in year 2 (Table 1), efficient crosslinking via the cysteine was not achieved. It was also necessary to evaluate and determine the conditions necessary to promote integrin activation and efficient binding. In year 2 the extended peptides were characterized and found to be more suitable for crosslinking. As shown in Figs 5 and 6, a dominant labeled protein is present on the western blot and attempts at scaling, enrichment and identification are in progress.

Results:

1. Cysteine side chain derivatized peptides retained favorable activity but the binding properties are unfavorably changed. The characterization of two new peptides Ac-PhScNGGK-bio-GGK and Ac-KGGPhScNGGK-bio allows for additional crosslinkers to be evaluated. These peptides were found to not introduce any binding issues, and the lower Kd and will allow for more efficient binding.   The additional amine group on the new lysine also allows for a considerable array of both homobifunctional amine reagents as well as a selection of heterobifunctional photoactivatable crossslinkers.

2. Activation of integrin by serum introduces additional proteins that can nonspecifically bind to the peptide compound at the high concentrations required to initiate high levels of binding to the active site. Use of divalent cations eliminates the nonspecific binding problem; however the steady state amount of activated integrin is much lower than the amount obtained from serum starvation and subsequent stimulation. The ligand PHSRN consists of the specific sequence in fibronectin that stimulates activation of the $\alpha 5 \beta 1$ integrin. Quantities of this peptide have been obtained and will be used to stimulate activation of integrin after serum starvation

3. The effect of the increased levels of integrin activation from stimulation after serum starvation is relatively short. The maximal effect is at 1 hr after stimulation with either serum or PHSRN and is completely gone by 4 hrs. Serum starvation and stimulation by the ligand PHSRN will be used in combination with divalent cations to promote upregulation of activated $\alpha 5 \beta 1$ integrin.

4. Detection of crosslinking has only been seen on western blots that have been over-developed. Sample enrichment from BPM-peptide experiment using cells at 10^7 have not yielded enough material to analyze. Published papers show that only the activated form of $\alpha 5 \beta 1$ integrin is receptive to the compound. Attempts to increase the amount of activated integrin are being used, but have their limitations. The only remaining option is to continue increasing the amount of cells used and continue enrichment strategies.

5. Assessment of the DC4 and the photoactivatable crosslinkers on western blots show that the extended versions bio–GGK and KGG-bio peptides with any of the four crosslinkers appear to all generate the same crosslinked complex.   The tail region has the least spatial constraint as seen with the MAP-peptide, hence the bio-GGK is the better choice for continued work, effectively reducing the number of combinations by half. Our colleague has had extensive experience with DC4, however the various photoactivatable crosslinkers will each bring their own mass spectrometry challenges.

6. Work is in progress with scaling to 10^8 cells with the DC4 crosslinker and the photoactivatable crosslinkers with the bio–GGK peptide. Enrichment with magnetic beads will be used. It is hopeful that sufficient material will be obtained to allow for masss spectrometry analysis and definition of the target.
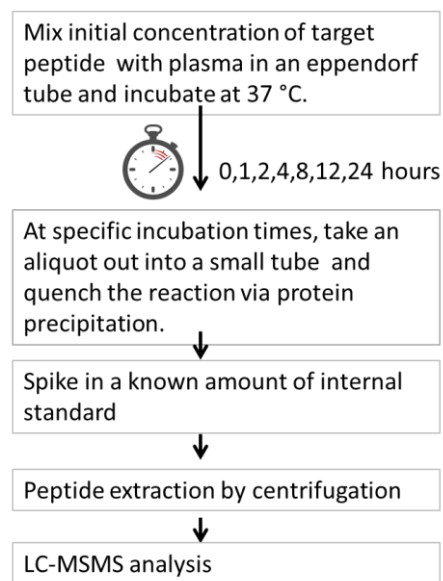
Specific Aim 3

Develop a mass spectrometry–based quantitative assay for PHSCN analogs, including PhScN, and evaluate their stabilities in human and mouse plasma.

Experimental design for quantitative assay of PHSCN analogs

An experimental approach based on mass spectrometric analysis to evaluate the stabilities of PHSCN analogs in human and mouse plasma has been designed as outlined in Scheme 1. A known amount of target peptide is mixed with plasma and incubated at 37 °C for up to 24 hours. At specific incubation time points an aliquot is transferred to a small tube and protease reactions were quenched by adding three volumes of 50% acetonitrile with 0.5%TFA. Precipitated plasma proteins were removed by centrifugations, and a known amount of an internal standard was spiked into the supernatant containing the peptides, in order to increase the accuracy of quantitative measurements of LC-MS by reducing variances associated with sample preparations. MRFA (Met-Arg-Phe-Ala, monoisotopic molecular mass = 523.247.), a stable synthetic peptide with similar molecular mass to the target peptide, Ac-PHSCN-amide (monoisotopic molecular mass = 597.2328), is added into samples prior to LC-MS analysis. Stabilities of target peptides in plasma were evaluated by LC-MS.

Mix initial concentration of target peptide with plasma in an eppendorf tube and incubate at 37 °C.

0,1,2,4,8,12,24 hours

At specific incubation times, take an aliquot out into a small tube and quench the reaction via protein precipitation.

Spike in a known amount of internal standard

Peptide extraction by centrifugation

LC-MSMS analysis

Scheme 1. Experimental workflow

Mass spectrometry

The LTQ-Orbitrap XL(Thermo Finnegan) coupled to a nanoLC(Exigent) and an autosampler in the Andrews laboratory provides high mass resolution and accuracy for analytes separated on a capillary column at a flow rate of 200 nL/min, which assures confident identification of target molecules. Extracted peptides from plasma are reconstituted in 0.1% TFA and loaded into a C18 trap by autosampler and washed for 6 min at 5 microL/min with 0.1% TFA in water delivered by the LC loading pumps. Trapped peptides were introduced by switching valve into a capillary column (75 micro-m x 15 cm) custom-packed with C18 resin (3 micron) and separated over 60 min gradient at a flow rate of 200 nL/min. Analytes were introduced into the LTQ-Orbitrap via an electrospray device (Triversa Nanomate, Advion). MS and MSMS data were acquired in data-dependent mode for ions within 400-800 m/z. Figure 7 shows the total ion chromatogram (TIC) of LC-MSMS analysis of PHSCN and the internal standard MRFA.
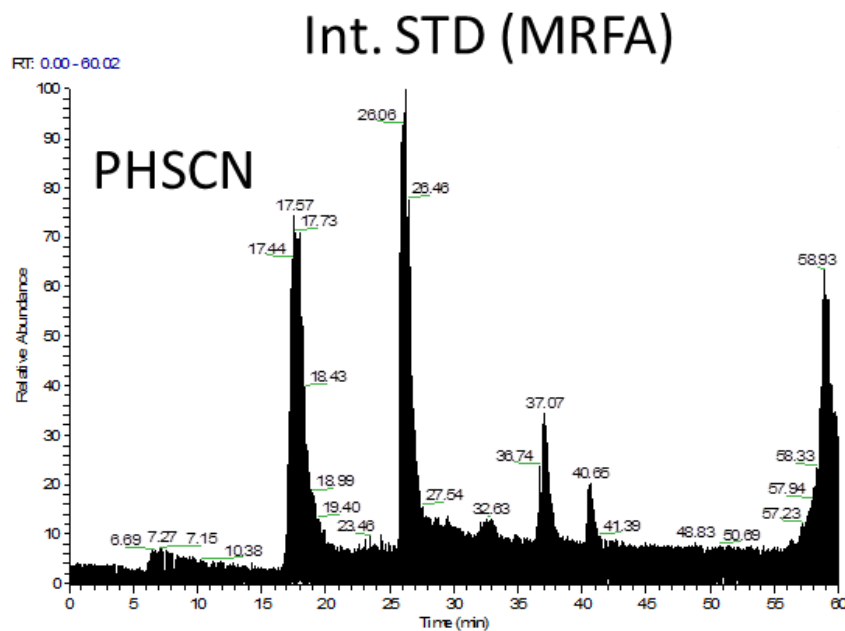
Figure 7. TIC from an LC-MSMS of PHSCN and MRFA mixture.

Quantitative assay of target peptides

Peptide quantification can be achieved by ion intensities in MS or by MSMS spectral counts in data-dependent acquisition mode. These two approaches are well established label-free quantification methods for peptides and proteins by LC-MSMS, which can be readily applied to a bioanalytical assay of therapeutic peptides. We are currently testing a new assay method, an MRM-like analysis via data-independent acquisition mode in the LTQ-Orbirap, in which ion packets within a mass range of 5 or 10 Da accumulated for a set time are co-fragmented. One or multiple fragment ions specific to the target peptide can be monitored in MSMS based on highly accurate precursor ion mass and LC retention time. This MRM-like approach can provide a highly selective and sensitive bioanalytical assay under conditions of high chromatographic interference.

Study of collision–induced dissociation (CID) fragmentation of Ac-PHSCN-NH$_s$ in online LC-LTQ-Orbitrap

Ac-PHSCN-NH$_2$ introduced into the mass spectrometer via a nanoESI device (Triversa Nanomate) was predominantly observed as singly protonated form at 598.2328 m/z. However, in online LC-MS, the peptide was observed in doubly charged dimeric forms at 597.23 m/z and 589.23 m/z. The peptide dimer ions were characterized by CID that revealed disulfide-bond formation between two peptides that was catalyzed on C18 LC, as shown in a CID spectrum of 597.23 m/z. This is a potential source of interference with the assay that can be easily addressed by incorporating reduction and alkylation of the Cys residues into the protocol prior to LCMSMS.
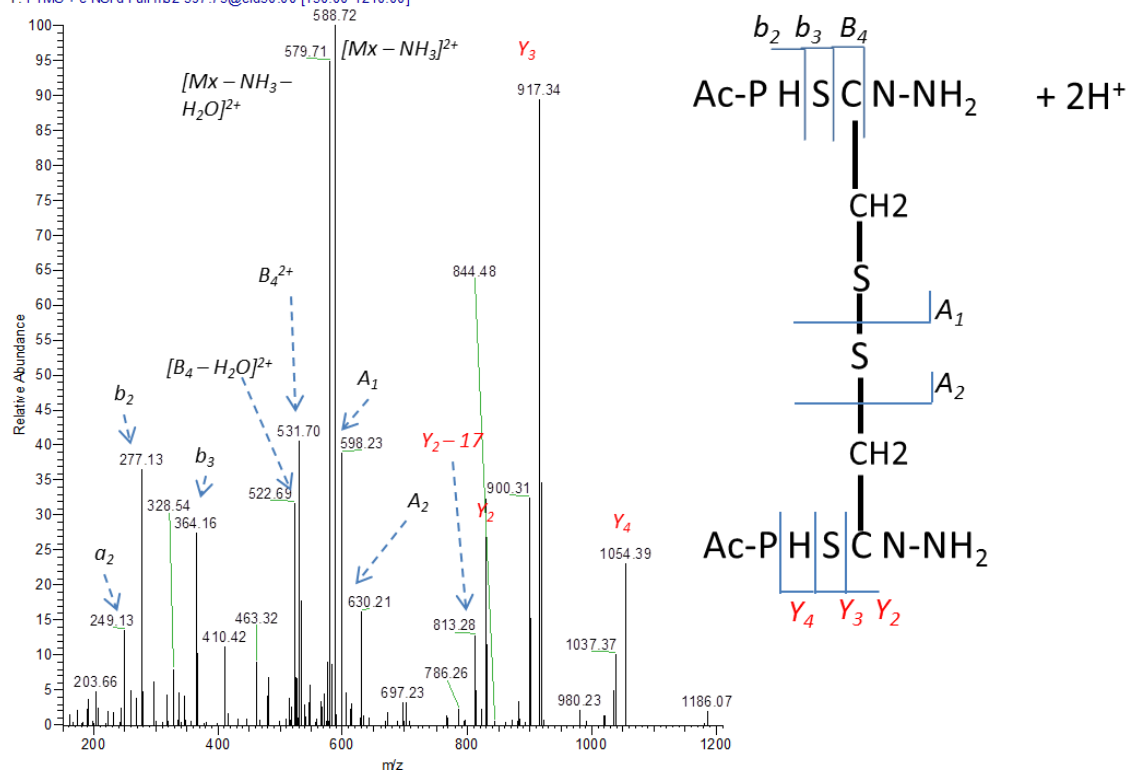
Figure 8. CID fragmentation of doubly–charged ion at 597.73 m/z (the second isotope to 597.23 m/z). Fragment ions B4, A2, Y2,Y3, and Y4 containing S-S cross-linked peptides confirm disulfide bond formation between two thiols of cysteine.

KEY RESEARCH ACCOMPLISHMENTS:

1. Determination of invasion–inhibitory potencies for a total of 12 PHSCN and PhScN derivatives, targeting α5β1 integrin–mediated invasion of naturally serum–free basement membranes by 2 metastatic human breast cancer cell lines, SUM149PT and MDA-MB-231. Demonstration that covalent S–modification by several different moieties increases invasion–inhibitory potency by 100,000– to 1,000,000–fold, and that a 1,000,000–fold increase in potency is also obtained by substiution of D–His and D–Cys. These results demonstrate that the reason for the greatly increased invasion–inhibitory potency of PhScN is the suppression of covalent disulfide bond formation between the PHSCN sequence and its integrin target, and hence they are a key step in the determination of the PhScN target site on α5β1 integrin receptors of breast cancer cells.

2. Determination of invasion–inhibitory potencies of PHSCN and PhScN peptides, containing BPM– derivatized cysteine or D–cysteine. The greatly increased invasion–inhibitory potencies of the BPM– modified PHSCN and PhScN peptides also confirm the noncovalent nature of invasion inhibition by the PhScN peptide.
   Research accomplishments 1 and 2 complete Specific Aim 1 of the SOW.

3. Crosslinking with BPM–derivatized peptides, after integrin α5β1 activation with divalent cations, and analysis by Western blotting. Successful crosslinking with biotin labeled peptides, modified either at the cysteine or on the C–terminus, is anticipated to pave the way for protein enrichment and binding site identification by MS/MS.

4. Determination of binding constants for PHSCNGGK-Bio and PhScNGGK-Bio on MDA-MB-231 and

SUM149PT cells, and demonstration of their similarity provides the basis for acquiring information on any steric hindrances introduced by modification of the ligands not detected in functional invasion assays.

5. Demonstration that PHSCNGGK-Bio and PhScNGGK-Bio compete for the same binding site on SUM149PT and MDA-MB-231 cells. The results of these binding assays, in conjunction with the functional invasion inhibition results shown in Table 1, will allow a more complete profile of the peptide ligand properties and their functional effects on $\alpha5\beta1$ integrin–mediated invasion. Moreover, these results provide key support of the hypothesis that the increased potency of the PhScN peptide results from suppression of covalent, disulfide bond formation with the integrin target.

   Research accomplishments 3, 4, and 5 provide key support towards accomplishing Specific Aim 2 of the SOW.

6. Demonstration of CID fragmentation of Ac-PHSCN-NH$_2$ in online LC-LTQ-Orbitrap indicates that development of a mass spectrometry–based quantitative assay for PHSCN analogs, including PhScN, for the purpose of evaluating their stabilities in human and mouse plasma (Specific Aim 3) is underway.

REPORTABLE OUTCOMES: None to date.

CONCLUSION: The PhScN peptide is a highly potent inhibitor of $\alpha5\beta1$ integrin–mediated, serum–induced invasion because it suppresses covalent, disulfide bond formation with its target. Moreover, results of competition assays show that it targets the same region of $\alpha5\beta1$ integrin as the PHSCN peptide parent. A mass spectrometry-based assay is a viable approach to degradation analysis.

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