

1) **SPECIFIC AIMS**

The mechanism of blood coagulation is composed of an array of proteolytic reactions that generate fibrin thrombi (1, 2). The pro-anticoagulant activity of the blood-clotting cascade is balanced by several natural anticoagulant mechanisms of the blood vessel wall. The interaction of endothelial cell surface heparan sulfate (HS) with the protease inhibitor, antithrombin (AT), represents one of these crucial control mechanisms. Several lines of evidence indicate that two-specific heparan sulfate biosynthetic enzymes are critical for the synthesis of anticoagulant HS (aHS); heparan sulfate 3-O-sulfotransferase isoform 1 (3-OST-1) (3) and isoform 5 (3-OST-5) (4). These isoforms play important roles in generating the specific and diverse structure of HS that corresponds to HS biological functions. For instance, our recent data suggests that aHS produced by 3-OST-1 and -5 exhibit an approximately 10,000 fold increases in AT-binding compared to the unmodified HS (5). Interestingly, aHS produced by the two enzymes appear to differ in structure since 3-OST-5 generates an entry and membrane fusion receptor for herpes simplex virus type-1 (HSV-1), whereas aHS generated by 3-OST-1 does not (3, 5, 6, 7). In order to further understand the differences in aHS generated by the two enzymes, our goal is to generate small and unique peptides that bind to 3-OST-5 generated aHS, using phage display library screening. Thus, the two specific aims of this proposal are:

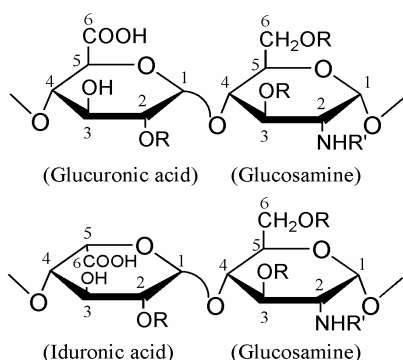
Aim 1. Screening of 12-mer phage display peptide library (PhD-12) against 3-OST-5 modified HS. The working hypothesis is that screening of the peptide library will enable identification and isolation of various peptides that specifically recognize critical features of aHS generated by 3-OST-5. *Since the last submission of this proposal, multiple aHS binding phages have been isolated and encoded peptide sequences have been identified (Table 2).*

Aim 2. Structural and biochemical characterization of the isolated peptides for ability to block HSV-1 entry, gD binding, and AT-binding to aHS. The working hypothesis is that the isolated peptides could provide multiple usages based on ability to interfere with the interactions of aHS with HSV-1 gD and AT. We hypothesize that viral entry blocking and glycoprotein D (gD) binding peptides would bind to aHS generated by 3-OST-5 but not 3-OST-1. However, peptides that block AT binding are expected to bind aHS produced by both 3-OST-1 and 3-OST-5. This new strategy of phage display will increase our understanding of the functional specificity of aHS in relation to its biological functions. In addition, the peptides generated would also be useful for blocking HSV-1 entry (potential anti-HSV agents), as diagnostic tools for aHS expression on different cell types and for purification of high potency anticoagulant aHS from natural sources. Depending on the site of interaction, it is also possible that the peptides could enhance or positively influence the AT and/or gD binding to aHS.

2) **BACKGROUND AND SIGNIFICANCE**

Biosynthesis and biological activities of heparan sulfate (HS). HS is present on the cell surface in the form of heparan sulfate proteoglycans (HSPG), which contain a core protein and polysaccharide side chains (8, 9). These polysaccharides are involved in numerous biological processes, including blood coagulation, angiogenesis, wound healing, viral invasions, embryonic development, regulating tumor growth, and controlling the eating

behavior of mice (10-13). The core protein determines the location of HS in a specific tissue, and may play an important role in enhancing the effects of HS.



R= -H or -SO₃

R'= -SO₃, -H or $-\overset{\text{O}}{\parallel}{\text{C}}-\text{CH}_3$

Figure 1: Disaccharide repeating units of heparan sulfate and heparin. Sulfation (R= -SO₃) at Carbon 6 (known as 6-O-sulfated glucosamine) of glucosamine is common. Sulfation at Carbon-2 of iduronic acid (known as 2-O-sulfated iduronic acid) is common. Sulfation at Carbon-3 of glucosamine (known as 3-O-sulfated glucosamine) is rare. Both N-acetylated (R'=acetyl, GlcNAc) and N-sulfated (R'= -SO₃, GlcNS) are common. N-unsubstituted glucosamine (R' = -H, GlcNH₂) is a low abundant component.

HS belongs to a unique class of macromolecules, known as glycosaminoglycans. Depending on the structures of the repeating disaccharide units, four well studied glycosaminoglycans include: HS, chondroitin sulfate, keratan sulfate and hyaluronic acid. HS is a 1→4 linked glucuronic or iduronic acid and glucosamine residues with various sulfations (Figure 1). Recent reports suggest that the N-unsubstituted glucosamine residue is located in specific positions of a HS polysaccharide chain (14, 15, 16), and is involved in the binding to HSV-1 gD (5-7; 17-20) and enhancing the recycling of glypican-1 (14, 15). 6-O-sulfated glucosamine and 2-O-sulfated iduronic acid are common sulfated monosaccharides, and those residues play critical roles in binding to fibroblast growth factors and growth factor receptors (6, 14, 5). In addition, 6-O-sulfated glucosamine residues are involved in binding L- and P-selectin (14, 15). 3-O-sulfated glucosamine is a rare component (6, 14). The enzymes that produce it or 3-O-sulfotransferases (3OST) are present in six-seven different isoforms (Table 1). It is important to note that 3-OST-1 modified HS binds to AT and has anticoagulant activity, whereas 3-OST-3 modified HS binds to gD and serves as an entry receptor for herpes simplex virus type-1 (HSV-1) (17). Additionally, we have recently demonstrated that a new isoform, 3-OST-5, generates both AT-binding site and a gD receptor that mediates entry and spread of HSV-1 (5, 7).

Table 1: The Heparan Sulfate D-Glucosaminyl 3-O-Sulfotransferases (3OSTs)

Isoform	Sulfated Residue	Upstream Uronic acid	Tissue Distribution	Known Function(s)
3-OST-1	GlcNS	GlcUA	Heart, brain, lung, kidney	AT binding
3-OST-2	GlcNS	IdoUA 2S/GlcA 2S	Brain	HSV-1 entry*
3-OST-3A	GlcNS	IdoUA 2S	Heart, placenta, lung, liver, kidney	HSV-1 entry
3-OST-3B	GlcNS	IdoUA 2S	Heart, brain, placenta, liver, kidney, Pancrease	HSV-1 entry
3-OST-4	Unknown	Unknown	Brain	HSV-1 entry*
3-OST-5	GlcNS	IdoUA 2S/GlcUA	Skeletal muscle, fetal brain	HSV-1 entry & AT binding
3-OST-6	GlcNS	IdoUA 2S	Kidney, liver	HSV-1 entry

* Unpublished results (Shukla and Spear [11])

Heparan sulfate regulates blood coagulation process. Heparin is the most commonly used anticoagulant drug, despite the facts that numerous side effects are associated with using heparin (8, 14-16). Heparin acts on AT-a plasma protein (MW~58,200), which decreases the activity of thrombin, thus preventing thrombin from cleaving fibrinogen to generate a stable clot (8, 15, 16). Heparin is an exclusive product of mast cells, and is released during degranulation of mast cells (15, 16). Heparin and HS have the same disaccharide repeating units. However,

HS contains lower sulfation level than heparin. It is believed that aHS expressed on the endothelial cell membrane binds to AT to exhibit anticoagulant activity (14, 15). It should be noted that HS and dermatan sulfate also bind to heparin cofactor II, which plays a role in regulating anticoagulant activity (8, 14-16).

The AT-binding heparin contains a structurally defined AT-binding saccharide sequence, a pentasaccharide with the structure of –GlcNS6S-GlcUA-GlcNS3S±6S-IdoUA2A-GlcNS6S- (for structure see figure 2) (14). The AT-binding site is the essential motif to confer anticoagulant activity to heparin. Indeed, a synthetic AT-binding pentasaccharide demonstrated very promising effects on the prevention of deep-vein thrombosis (8, 14-16). It is known that a pentasaccharide without the 3-O-sulfation has a decrease in the binding affinity by 18,000-fold (14, 15). Despite the success in determining the saccharide structure of the AT-binding site of heparin, the knowledge on the structure of the AT-binding site of HS is still limited.

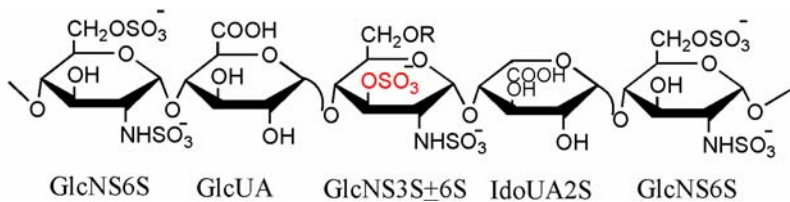


Figure 2: The structure of AT-binding pentasaccharide. The 3-O-sulfation of glucosamine residue (3-O-sulfation is in red) is the critical modification to generate the AT binding site. GlcNS6S, GlcUA, GlcNS3S6S, IdoUA2S and GlcNS6S represent the abbreviation of the individual monosaccharide residue.

3-OSTs add unique specificity to heparan sulfate (HS). Diverse structures in HS are generated by the actions of various 3-OST isoforms. 3-OST-5 generated HS is a unique set of modified HS, which produces both aHS (an anticoagulant HS that binds to AT) and a site that binds to gD—a major envelope glycoprotein that participates during HSV-1 entry. Therefore, 3-OST-5 modified HS is an ideal target for phage display peptide screening, generating unique peptides with multiple usages. Theoretically, some of these peptides could even enhance AT binding to aHS, thus generating agents that can severely retard blood coagulation.

3OST Isoforms	Modification results diverse structure in HS	Modified HS are ligand for		Novel peptides against 3OST-5
		HSV-1 gD	AT	
3OST-1		-	+	<p>3OST-5 modified HS</p>
3OST-3		+	-	
3OST-5		+	+	
3OST-6		+	-	

* 3-O sulfation at different sites on HS chain; 3OST-2 & 4 isoforms are not well studied.

Relevance of the research:

There appears to be an intricate relationship between HSV infection and regulators of anticoagulation. The viral glycoprotein gD and AT appear to bind aHS, which in turn, has a regulatory role in retarding coagulation. Thrombosis disorders continue to be a major cause of morbidity and mortality, resulting in an increased need for anticoagulant therapy (1, 22). Unfortunately, low molecular-weight heparin introduced in the past decade causes several problems, primarily bleeding complications that may prove fatal (23). Therefore, the need for

safer and more effective antithrombotic agents clearly exists. It is well known that large quantity of HS, a highly sulfated polysaccharide, is present on the surface of endothelial cells of the blood vessel walls (21). The HS is involved in monocyte adhesion and anticoagulation (8, 14-16). The binding of endothelial cell surface HS and AT controls the blood coagulation process. It is now known that the AT participates in the regulation of blood clotting in both physiological and pathological stages. For instance, the interaction between HS and AT plays critical roles in anticoagulation as well as in anti-inflammatory process (14-16; 24). Interestingly, specific saccharide sequence generated by 3-OST-5 also binds HSV-1 gD (5, 7). Several reports suggest that herpes infections potentially play a very significant role in atherosclerosis (25-27). It has also been shown that presence of HSV-1 antibodies leads to a two fold increase in the risk of heart attack and death from heart disease (28). Further, it appears that HS and modified HS play a key role in many physiological and pathological processes (5, 7, 29-31). Our goal is to exploit versatile technique of phage display to isolate short peptides against aHS (3-OST-5 modified HS) that affects AT and HSV-1 gD binding sites. The peptide that enhances AT binding to aHS could serve as an anticoagulant agent, on the other hand the peptide that occupies gD binding sites on aHS will interfere with HSV-1 entry and therefore it will be potentially anti-HSV agent. Clearly, the isolated peptides will represent a new class of potentially therapeutic reagents. In addition lack of antibodies against heparan sulfate is a limiting factor in heparan sulfate related research. The peptide isolated during this study is likely to provide useful reagents and information on the structural-functional properties of heparan sulfate.

PRELIMINARY STUDIES

Isolation and characterization of heparan sulfate 3-O-sulfotransferase isoform 5 (3-OST-5):

Our laboratory has recently cloned and characterized a novel isoform of heparan sulfate 3-O-sulfotransferase, designated as 3-OST-5. The open reading frame of this protein, assigned as 3-OST-5, was amplified from a human placenta cDNA library using specific 5'- and 3'-primers. The isolated 3-OST-5 cDNA contains 1041 bp and the deduced peptide of 346 amino acid residues predicts a type II membrane-bound protein. We discovered that 3-OST-5 synthesizes AT-binding and gD-binding HS and also mediate membrane fusion (5,7). These results serve as the foundation for this proposal.

The activity of 3-OST-5 in assisting herpes simplex virus type-1 (HSV-1) entry and spread:

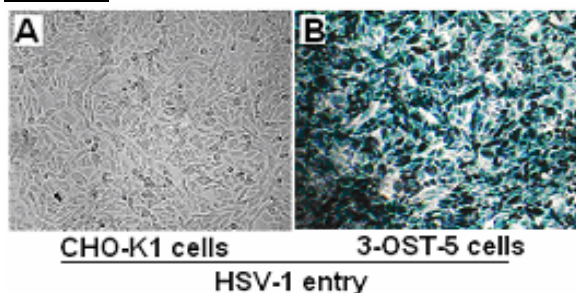


Figure 3. Entry of HSV-1 into wild-type CHO-K1 cells and 3-OST-5 transfected CHO-K1 cells. At 36 hour after transfection, the cells were exposed to recombinant strain of HSV-1 (KOS-gL86) at 100 pfu/cell. Six hour later, the cells were washed, fixed, and incubated with X-gal to identify blue cells. As shown in the figure 3A, the mock transfected cells are resistant to HSV-1 entry (no blue cells) in contrast to 3-OST-5 expressing cells (blue cells) as previously reported (5). Recently we found that 3-OST-5 mediates both HSV-1 entry and viral spread (7).

Identification of a 3-OST-5 and gD-binding 12-mer novel peptide by phage display library screening:

Our initial phage screening against CHO-K1 cell line expressing gD in the lab has shown encouraging results in terms of blocking HSV-1 entry. A gD-binding phage, Ø gDn, was isolated by subtractive panning approach. Ø gDn itself was screened for ability to block entry first and later the outer coat peptide encoded by Ø gDn was synthesized and examined for

entry blocking. As shown in figure 4, the peptide blocks entry of HSV-1 in 3-OS HS cells in a dose dependent fashion. These peptides are being further characterized in our laboratory.

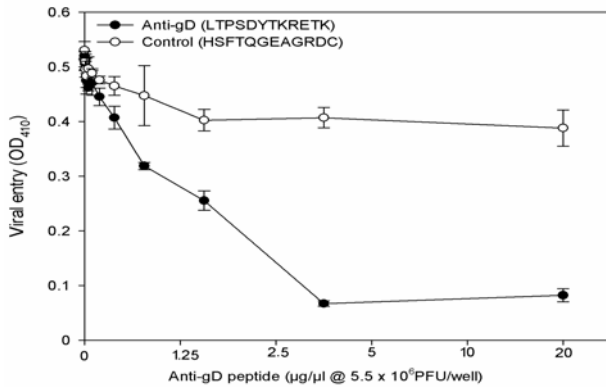


Figure 4. Inhibition of viral entry by anti-gD peptide. An anti-gD peptide was isolated using phage display peptide library screening against gD-expressing CHO-K1 cells. Indicated doses of either anti-gD peptide (LTPSDYTKRETK) or the control peptide (HSFTQGEAGRDC) selected against a streptavidin target were pre-incubated with sucrose density gradient purified HSV-1(KOS)gL86 at 5.5×10^6 PFU/well; 30 min later the virion-peptide mixture was used for infecting 3-OST-3B-expressing CHO-K1 cells. The infection was allowed for 6 h and the cells were then processed for entry using entry assay.

Similarly, phages were isolated against 3-OST-5 expressing CHO-K1 cells. The amino acid sequences of the peptides encoded by the gD and aHS (3-OST-5) binding phages are shown in the Table 2 below. The peptides will be synthesized and characterized as part of this proposal.

Table 2: Amino acid sequence of phage-displayed peptides identified by screening against CHO-K1 cells expressing HSV-1 gD and 3-OST-5.

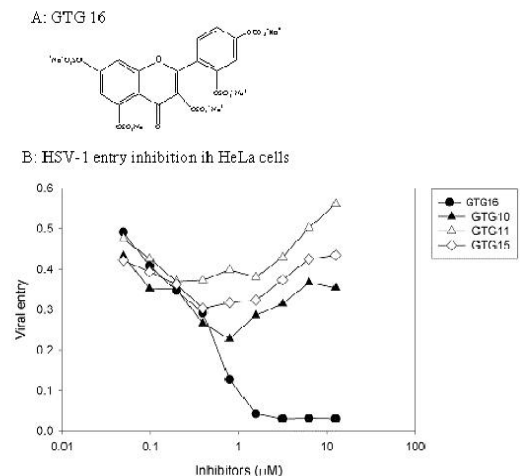
gD-1			3-OST-5		
Peptide	Sequence	Frequency ^b	Peptide	Sequence	Frequency
12pgD1-1 ^a	rlqqlstnrfgv	6	12p3OSHS-1	afnciglsacr	3
12pdD1-2	rlqqlsqtvlgl	1	12p3OSHS-2	ernnyaaaslr	1
12pgD1-3	ehlqtlvtqtsf	1	12p3OSHS-3	rlqqlgkfqflq	1
12pgD1-4	rqqfkkqfghklc	1	12p3OSHS-4	qtlttffqqivlg	1
12pgD1-5	ernnqrnceqqf	1	12p3OSHS-5	rlqqlstnrfgv	1
			12p3OSHS-6	rlqqhfqqivlg	1
			12p3OSHS-7	fnciglsacfrg	1
			12p3OSHS-8	llivsvyqlafe	1
			12p3OSHS-9	plivsvyqlafe	1

^a the prefix 12 designates the number of amino acids in the sequence followed by the peptide number.

^b number of times a particular peptide sequence was identified by sequencing of phage DNA.

Alternative to peptide screening: synthetic inhibitors of 3-OS HS: In collaboration with Dr. Desai at VCU (letter attached) we examined five small poly-sulfated flavanoids or small molecule heparin “mimics” for ability to block HSV-1 entry. Among them GTG16 (Figure 5A) blocked entry in multiple cell-types including HeLa and 3-OST-5-expressing cells (data not shown).

Figure 5. A. Chemical structure of GTG16. B. Viral entry blocking assay. Identical (10^5) units of a recombinant β -galactosidase-expressing HSV-1(KOS) virus were treated for half hour at room temperature with 2-fold serial dilutions of GTG compounds (as indicated in the figure). After the treatment, virus particles were used for infecting HeLa cells in 96-well tissue culture dishes. Six hours later, the cells were permeabilized and assayed for β -galactosidase activity. The values shown (means of triplicate determinations) represent the amount of reaction product detected spectrophotometrically



(OD_{410}) at the plaque forming units (PFU) indicated.

3) **Research Design and Methods:**

A. Choices and Rationale:

My decision to focus on 3-OST-5 modified heparan sulfate (aHS) stems from the following reasons:

- (i) It is becoming increasingly clear that discrete modifications within HS give rise to unique protein binding sequences. In this regard, 3-OST-5 is even more interesting, since the HS modified by this enzyme exhibits the unique property of binding both AT and HSV-1 gD and it also participates in HSV-1 induced cell fusion and viral spread (5,7). Peptides that recognize AT and gD binding motifs would provide useful and very significant structural information on aHS compared to the aHS generated by 3-OST-1.
- (ii) The peptides isolated would be useful for blocking HSV-1 entry, as diagnostic tools for 3-OST-5 expression in various cell types, and also for affinity purifying highly anitcoagulant HS.
- (iii) We have already developed a protocol for isolating phages that recognize HSV-1 gD and aHS generated by 3-OST-5 (32).

B. Critical Materials Available:

- (a) **Plasmids expressing 3-OSTs, and viral envelope proteins.** Our study will require plasmids expressing human 3-OST-1, -2, -3A, -3B, -4, -5, -6 isoforms. These plasmids are all available in my mentor's laboratory.
- (b) **Cell lines.** The pre-screening for the phage library will be initiated with wild-type Chinese hamster ovary (CHO-K1) cells and later with target CHO-K1 cells stably expressing aHS. These cells along with CHO-K1 cell-lines that stably express gD-1, 3-OST-1, 3-OST-3A, 3-OST-3B, and 3-OST-5 are all available in the laboratory. Stocks of naturally susceptible cells (Hep2, HeLa and Vero) are also available.
- (c) **Antibodies and soluble proteins.** Polyclonal antisera and monoclonal antibodies (mAbs) specific for human 3-O-sulfotransferase-3, and many of the HSV envelope glycoproteins are available in my mentor's laboratory. Dr. Jian Liu (University of North Carolina-Chapel Hill, letter attached) has monoclonal antibodies against 3-OST-3s and gD binding octasaccharide that will also be available for us. Soluble forms of HSV-1 gD and AT are also available in our laboratory or through our collaboration.
- (d) **Virus strains.** Characterization of peptides that interfere with HSV-1 gD binding sites on aHS will require recombinant HSV-1 KOS strain for entry assay. This and many other strains are available with us.
- (e) **Instruments, space and required reagents.** Our lab has all the necessary instruments, lab space (1000 sq ft) and reagents for the planned experiments. A 12-mer PhD library from New England Biolabs (Beverly, MA) is available in our lab. A 96 well-plate reader [Molecular Devices, Spectra MAX 190], -80 C freezer for storage of cell and viral stocks, many high and low speed centrifuges, biological safety cabinets, incubators for bacteria, cell and virus culture, florescent microscope (Zeiss Axiovert 200 M), environmental shakers and thermal cycler (PCR). In addition, our Department of Ophthalmology-Visual Sciences has a molecular biology core facility fully equipped with a Beckman automated DNA sequencer. The peptides will be synthesized from the protein biochemistry facility at UIC.

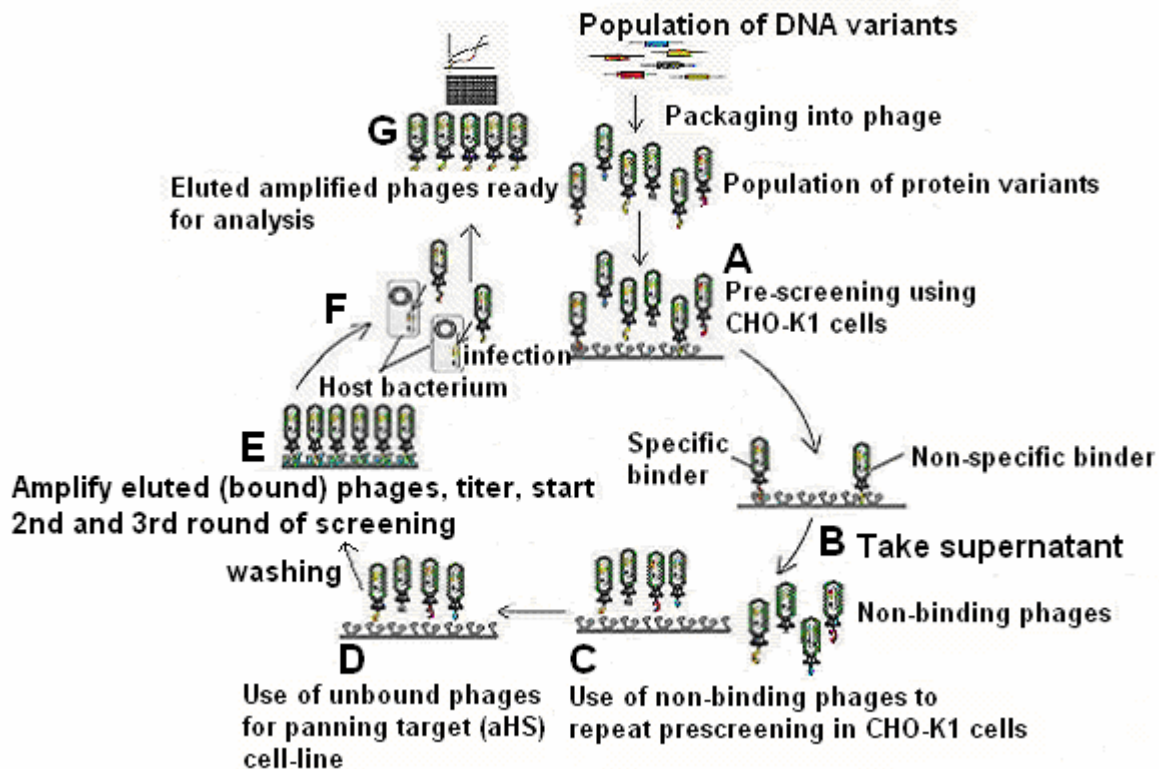
C. Research design and procedure to accomplish the specific aims:

AIM 1: Screening of a phage display library against aHS expressing CHO-K1 cells.

Rationale: In order to aid the studies addressing the significance of aHS, small random peptides that bind aHS will be isolated using phage display (PhD) screening, which is an efficient way of screening more than a billion variant peptides against a target of interest. The aHS binders that also block entry will be useful for assessing the significance of this molecule in cells and tissues. Given the small size of peptides, blocking is expected to be very specific.

Experimental Design and Methods: The technology is an *in vitro* selection technique in which a peptide is genetically fused to a coat protein of a non-lytic bacteriophage (M13) (34). This results in the display of the fused protein on the exterior of the phage virion while the DNA encoding the fusion resides within the virion (35). The physical linkage between the displayed peptide and the DNA encoding it allows screening of greater than a billion variant peptides against the molecule of interest, i.e. aHS produced by 3-OST-5, in our case. Screening of phage display libraries is usually accomplished by an affinity selection (or bio-panning) process during which phage populations are exposed to targets in order to selectively capture binding phage (36). We plan to screen a PhD library using intact cells with aHS expressed in its native form on cell-surface. Since a given cell type will have hundreds or thousands of different receptors, as a result, simple panning against intact cells will likely yield a complex mixture of peptides with no clear consensus. Therefore, to specifically target the library to aHS, subtractive panning will be carried out with cells that do not express aHS (also summarized in the figure below). This will be accomplished by using two cell lines: wild-type CHO-K1 cells and CHO-K1 cells stably expressing aHS or 3-OST-5. The aHS produced by the 3-OST-1 will be used as a control. Under this approach, wild-type CHO-K1 cells will be used for prescreening the library (two times).

Phage Display Technique



An attractive aspect of phage display is that provided appropriate libraries can be obtained, the technique is simple, cheap, rapid to set up, and requires no special equipment.

The process of careful prescreening is a critical step. The overall success of the entire screening process is dependent on this step. Incubating the PhD library once and using the supernatant again to incubate with CHO-K1 cells will achieve the desired results of prescreening. To start the actual screening, the supernatant containing unbound phages will be added to aHS cells. Phages that bind aHS cells will be eluted and amplified by propagation in *E. coli* host strain (ER2738), and taken on to the next round. Two to three rounds of selection will be carried out to select for the specific binders. **Low concentrations of detergent (Tween-20) in the early rounds will result in high eluate titers, and the stringency can be gradually increased with each round by raising the Tween concentration step wise to a maximum of 0.5%. This would allow selection of high affinity binding phages.** For final selection, the eluted phages will be plaque purified on soft-agar plates. One way of selecting phages for further processing would include selection of plaques with clear differences in sizes or morphology; otherwise, a few plaques will be randomly selected. Phages thus isolated from the selected plaques will be individually amplified; DNA will be isolated, and sequenced.

Interpretation of results, potential problems and alternative approaches: Since we have already identified a peptide by phage display screening that binds gD and blocks entry (32), **and recently we have isolated and sequenced some phages that bind specifically to 3-OST-5 expressing CHO-K1 cells (Table 2)**, we are very optimistic that our phage display screening against aHS would also result in identification of several new and potentially very useful peptides. In case, if subtractive panning fails to yield aHS specific peptides, then we plan to use commercially available HS (ICN) and modify it *in vitro* by purified 3-OST-5 [5] for panning. No pre-screening will be required and 2-3 rounds of screening should yield a collection of peptides, which could then be characterized as described above. The only problem with this approach is the potential loss of important epitopes after direct coating onto wells. If that is a concern then we would use streptavidin to coat wells and then add biotinylated aHS. This would restrict any significant epitope loss (however a prescreening against biotin-streptavidin will be required). **To determine the specificity, peptides will be tested against purified aHS (provided by Dr. Jian Liu) and also against aHS expressed on CHO-K1 cells expressing 3-OST-5 and 3-OST-1. In any case, the isolated peptides will have multiple valuable uses in the future.**

AIM 1.2: Characterization of aHS peptides isolated using phase display technology.

Rationale: Peptides that bind aHS may become novel tools for structure-function studies and for defining the significance of aHS in HSV-1 entry and AT binding. The goal of this aim is to test if a subset of aHS binding peptides identified on the previous aim would block gD and/or AT binding to aHS and to determine if AT blocking peptides block interactions with aHS generated by 3-OST-5 and 3-OST-1.

Experimental Design and Methods: After confirming the binding to aHS (described above), the peptides will be examined for ability to block the interaction between aHS/gD and aHS/AT. To examine the blocking of interactions, ELISA based binding assay will be used. A 96-well ELISA plate will be coated with aHS (commercially available HS modified *in vitro* by

purified 3-OST-5 has been provided by Dr. Jian Liu, [7]), and incubated with serial dilutions of individual peptides. Fixed amount of either soluble gD or AT will be added to ELISA wells. Secondary reagents (anti-gD and anti-AT antibodies along with horseradish peroxidase conjugated tertiary antibodies) will be used for probing bound gD or AT. **If isolated peptide binds to aHS or gD, the corresponding antibody (anti-AT binding antibody or anti-gD antibody) will provide the positive signal.** Once the peptide that block gD binding are identified, they will then be examined for ability to block HSV-1 entry in a naturally susceptible cell lines available in the lab and other HSV-1 entry receptor (nectin-1, HVEM or 3-OST-5)-expressing stable CHO-K1 cell line. The effective dose (ED_{50}) determinations will be made using a standard viral dose (usually 1PFU/cell) and several dilutions of the peptide as shown for an entry blocking peptide identified using the above procedure (preliminary results section). Peptides that block entry will also be tested for cytotoxicity using ToxiLight non-destructive cytotoxicity bioassay kit, following manufacturer's instructions (Cambrex). Only those peptides that do not cause any significant cytotoxicity will be considered useful for future applications. The peptides that block AT binding will be tested for ability to block the interaction of aHS generated by 3-OST-5 with AT using ELISA (as described above). The aHS/AT interaction blocking peptides will also be examined for ability to interfere with interaction of aHS generated by 3-OST-1 (will be provided by Dr. Liu). **Estimation of binding affinity will be performed using affinity co-electrophoresis [17]. Briefly, HS from commercial sources (ICN Biosciences) will be modified *in vitro* by incubation with purified 3-OST-5 and [35 S] PAPS (as the sulfate donor). Alternatively, metabolically labeled [35 S]aHS will be isolated from 3-OST-5-expressing CHO-K1 cells by tryptic digestion and immuno-purified [17]. Electrophoresis will be performed with dilutions of purified peptide incubated with constant amount of [35 S]aHS. The [35 S] intensity will be plotted against the migration distance through the separation zone to define the distance migrated in the presence or absence of the peptide. As shown before [17], it is expected that the migration of HS will be retarded by the presence of the interacting peptide, with the degree of retardation dependent on the peptide concentrations. From these data the K_D for interaction of HS with the peptide will be calculated by the Scatchard equation. These results will generate information on the affinity for a particular interaction between a peptide of interest and aHS. As an alternative to affinity co-electrophoresis, competition ELISA would be used. If the peptides can compete for gD and/or AT binding to aHS, then it would be a good indication of strong affinity binding to aHS.**

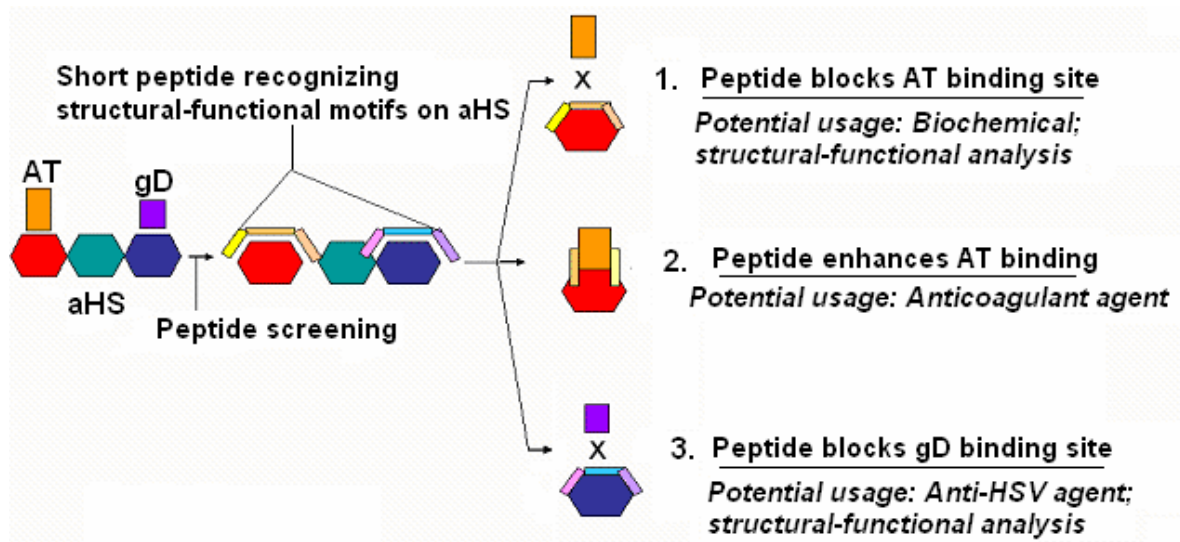
Interpretation of results, potential problems, and alternative approaches:

It is likely that multiple rounds of prescreening and stringent washing conditions would facilitate isolation of high affinity binding peptides. Strong affinity should be evident from the experiments proposed above, or from the ability of the peptide to block entry. Still, if some weak binding peptides were isolated then such peptides would provide the template for designing strong affinity peptides. Since, it is possible that minor variations of amino acids could yield even stronger binding peptides. By the same token, some degree of convergence is expected among the peptides isolated, which in fact, would help determine the consensus sequence required for a particular epitope binding.

After the characterization of peptides as described above, we expect to obtain three sets of peptides. These are (i) aHS binding peptides that block AT binding, (ii) aHS binding peptides that block gD binding, (iii) aHS binding peptides that block both gD and AT binding. It is also possible, although less likely, that we may isolate aHS binding peptides that do not

block either gD or AT-binding. Therefore, depending upon the specificities, the following different combinations of peptides and their usage are discussed below.

The possible properties of the isolated peptides against aHS



Thus it is clear that these peptides will have multiple uses as to block viral infections, tools to detect expression of various forms of aHS (gD binding and/or AT binding), to affinity purify aHS (obviously this form of aHS will be about 10,000 times more potent in anti-coagulation ability), and also to study structure-function differences between different regions and forms of aHS. These peptides will aid deeply in our understanding of HS produced by not only 3-OST-5 or -1 but also the HS generated by various other 3-OSTs ranging from 3-OST-2 to 3-OST-6. We are very well familiar with all the assays described above; therefore it is unlikely that our peptide screening would not result in useful peptides. As indicated above, screening will be first performed against cell-surface expressed aHS but if that fails then purified aHS will be used for panning. Dr. Jian Liu has already provided us with both forms of aHS. If screening fails against both forms, then small synthetic molecules generated by Dr. Umesh Desai will be tested for ability to block gD binding and AT binding to aHS. Dr. Desai is a formal collaborator of my mentor (Dr. Shukla) and is synthesizing about 20 different small molecules that mimic HS in different aspects. In any case, phage display screening and other assays described in this proposal would allow me to gain experience with some versatile and emerging techniques and also these will enhance my overall knowledge in the field of virology and its significance to cardiovascular/stroke-related science.

b) Tentative sequence for the investigation: Work on AIM 1 has already begun; we have isolated and sequenced 9 phages with aHS (3-OST-5) binding property. Many others were eluted but need sequencing that we hope to finish within the first year of funding. Any additional rounds of panning and sequencing against purified aHS will also be performed during the first year. Based on the sequence, the peptides will be designed and the process of characterization (gD/At binding, HSV-1 entry blocking, cytotoxicity, etc) will be completed within the second year.

c) Statistical analysis: All the data will be analyzed statistically by using Analysis of Variance (ANOVA) and Dunnett Multiple Range Test (DMRT) to define the statistical differences between the values. We will repeat the experiment at least three times and use Sigma-plot version 8.0 to get standard deviation and standard error curves. The data will be plotted in a dose-dependent manner and at single point.

d) Alternative approaches to achieve aims: As an alternate to peptide screening, the small molecule heparin mimics (small poly-sulfated flavanoids) may be screened for ability to block aHS mediated gD and AT binding. Dr. Umesh Desai (VCU; letter attached) and colleagues are developing many such mimics, which will be sent to us for further characterization. The mimics that block entry would be analyzed for ability to block gD binding to aHS and also for any effects on AT binding. Some preliminary data is included with this proposal. Eventually cytotoxic effects of the mimics and/or peptides will also be analyzed.

LITERATURE CITED

1. Desai, U.R. (2004). New antithrombin-based anticoagulants. *Med. Res. Rev.* **24**, 151-181.
2. Olson, S.T., Bjork, I., & Shore, J.D. (1993). Kinetic characterization of heparin-catalyzed and uncatalyzed inhibition of blood coagulation proteinases by antithrombin. *Methods Enzymol.* **222**:525-59.
3. Shworak, N.W., Liu, J., Petros, L.M., Zhang, L., Kobayashi, M., Copeland, N.G., Jenkins, N.A., & Rosenberg, R.D. (1999). Multiple isoforms of heparan sulfate D-glucosaminyl 3-O-sulfotransferase. Isolation, characterization, and expression of human cDNAs and identification of distinct genomic loci. *J. Biol. Chem.* **274**:5170-84.
4. Duncan, M.B., Chen, J., Krise, J.P., & Liu, J. (2004). The biosynthesis of anticoagulant heparan sulfate by the heparan sulfate 3-O-sulfotransferase isoform 5. *Biochim Biophys Acta.* **17**;1671:34-43.
5. Xia, G., Chen, J., Tiwari, V., Ju, W., Li, J., Malmstrom, A., Shukla, D., & Liu, J. (2002). Heparan sulfate 3-O-sulfotransferase isoform 5 generates both an antithrombin-binding site and an entry receptor for herpes simplex virus, type 1. *J. Biol. Chem.* **277**, 37912-37919.
6. Shukla, D., & Spear, P.G. (2001). Herpesviruses and heparan sulfate: an intimate relationship in aid of viral entry. *The J. Clin. Invest.* **108**, 503-510.
7. Tiwari, V., Clement, C., Duncan, M.B., Chen, J., Liu, J., & Shukla, D. (2004). A role for 3-O sulfated heparan sulfate in cell fusion induced by herpes simplex virus type-1. *Journal of General Virology* **85**, 805-809.
8. Esko, J.D., & Lindahl, U. (2001). Molecular diversity of heparan sulfate. *Journal of Clinical Investigation.* **108**, 169-173.
9. Yanagishita, M., & Hascall, V.C. (1992). Cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* **267**: 9451-9454.
10. Kainulainen, V., Wang, H.M., Schick, C., & Bernfield, M. (1998). Syndecans, heparan sulfate proteoglycans, maintain the proteolytic balance of acute wound fluids. *J Biol. Chem.* **273**, 115563-11569.
11. Arikawa-Hirasawa, E., Wantanabe, H., Takami, H., Hassell, J.R., & Yamada, Y. (1999). Perlecan is essential for cartilage and cephalic development. *Nat. Genet.* **23**, 354-358.
12. Lin, X., & Perrimon, N. (1999). Dally cooperates with Drosophila Frizzled 2 to transduce wingless signaling. *Nature* **400**, 281-284.
13. Tsuda, M., Kamimura, K., Nakato, H., Archer, S., Staatz, W., Fox, B., Humphrey, M., Olson, S., Futch, T., Kaluza, V., Siegfried, E., Stam, L., & Selleck, S.B. (1999). The cell-surface proteoglycan Dally regulates wingless signaling in Drosophila. *Nature* **400**, 276-280.
14. Liu, J., & Throp, S.C. (2002). Cell surface heparan sulfate and its roles in assisting viral infections. *Medical Research Reviews.* **22**, 1-25.
15. Esko, J.D., & Selleck, S.B. (2002) Order out of chaos: assembly of ligand binding sites in heparan sulfate. *Annual Review of Biochemistry* **71**, 435-471
16. Nader, H.B., Pinhal, M.A.S., Bau, E.C., Castro, R.A.B., Medeiros, G.F., Chavante, S.F., Leite, E.L., Trindade, E.S., Shinjo, S.K., Rocha, H.A.O., Tersariol, I.L.S., Mendes, A., & Dietrich, C.P., (2001). Development of new heparin-like compounds and other antithrombotic drugs and their interaction with vascular endothelial cells. *Brazilian J Medical and Biological Research* **34**, 699-709.
17. Shukla, D., Liu, J., Blaiklock, P., Shworak, N.W., Bai, X., Esko, J.D., Cohen, G.H., Eisenberg, R.J., Rosenberg, R.D., & Spear, P.G. (1999). A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. *Cell* **99**, 13-22.

18. Spear, P.G., Eisenberg, R.J., & Cohen, G.H., (2000). Three classes of cell surface receptors for alphaherpesvirus entry. *Virology* **275**, 1-8.
19. Spear, P.G. (1993). Entry of alphaherpesviruses into cells. *Semin Virol.* **4**, 167-180.
20. Spear, P.G., & Longnecker, R. (2003). Herpesvirus entry: an update. *J. Virol.* **77**, 10179-10185.
21. Vogl-Willis, C.A., & Edwards, I.J. (2004). High-glucose-induced structural changes in the heparan sulfate proteoglycan, perlecan, of cultured human aortic endothelial cells. *Biochim Biophys Acta.* **1672**:36-45.
22. Kwaan, H.C., & Samama, M.M. (2004). Anticoagulant drugs: an update. *Expert Rev Cardiovasc Ther.* **2**:511-22.
23. Hirsh, J. (1991). Oral anticoagulant drugs. *N Engl J Med.* **324**:1865-75.
24. Wiedermann, ChJ., & Romisch, J.(2002). The anti-inflammatory actions of antithrombin-a review. *Acta Med Austriaca* **29**, 89-92.
25. Vercellotti, G.M. (2001). Microbes, inflammation and atherosclerosis: will old pathology lessons guide new therapies? *Trans Am Clin Climatol Assoc.* **112**,215-222.
26. Shi, Y., & Tokunaga, O. (2002). Herpesvirus (HSV-1, EBV and CMV) infections in atherosclerotic compared with non- atherosclerotic aortic tissue. *Pathol Int.* **52**, 31-39.
27. Sorlie, P.D., Nieto, F.J., Adam, E., Folsom, A.R., Shahar, E., & Massing, M. (2000). A prospective study of cytomegalovirus, herpes simplex virus 1, and coronary heart disease: the atherosclerosis risk in communities (ARIC) study. *Arch Intern Med.* **160**, 2027-2032.
28. Siscovick, D.S., Schwartz, S.M., Corey, L., Grayston, T., Ashley, R., Wang, S-P., Psaty, B.M., Tracy, R.P., Kuller, L.H., & Kronmal, R.A. (2000). Chlamydia pneumoniae, herpes simplex virus type 1, and cytomegalovirus and incident myocardial infarction and coronary heart disease death in older adults. The cardiovascular health study. *Circulation* Nov **7**: 2335-2340.
29. Spillmann, D. (2001). Heparan sulfate: anchor for viral intruders? *Biochem.* **83**, 811-817.
30. WuDunn, D., & Spear, P.G. (1989). Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *J. Virol.* **63**, 52-58.
31. Shieh, M.T., WuDunn, D., Montgomery, R.I., Esko, J.D., & Spear, P.G. (1992). Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. *J. Cell. Biol.* **116**, 1273-1281.
32. Clement, C., Tiwari, V., & Shukla, D. (2003) Identification of a unique 12-mer peptide that inhibits herpes simplex virus type-1 entry by binding to glycoprotein D using phage display technology. *28th International herpes workshop-2003, Madison, Wisconsin.* Abstract number **4.19**.
33. Scanlan, P.M., Tiwari, V., Bommireddy, S., & Shukla, D. (2003). Cellular expression of gH confers resistance to herpes simplex virus type-1 entry. *Virology* **312**, 14-24.
34. Wang, L-F., & Yu, M. (2004). Epitope identification and discovery using phage display libraries: applications in vaccine development and diagnostics. *Current Drug Targets* **5**: 1-15.
35. Sidhu, S.S., Fairbrother, W.J., & Deshayes, K. (2003). Exploring protein-protein interactions with phage display. *ChemBioChem* **4**: 14-25.
36. Willats, W.G. (2002). Phage display: practicalities and prospects. *Plant Mol Biol.* **50**, 837-854.
37. Shukla, D., Dal Canto, M.C., Rowe, C.L., & Spear, P.G. (2000). Striking similarity of murine nectin-1 α to human nectin-1 α (Hve C) in sequence and activity as a glycoprotein D receptor for alphaherpesvirus entry. *J. Virol.* **74**, 11773-11781.

38. Geraghty, R.J., Jogger, C.R., & Spear, P.G. (2000). Cellular expression of alphaherpesvirus gD interferes with entry of homologous and heterologous alphaherpesviruses by blocking access to a shared gD receptor. *Virology* **268**, 147-158.
39. Montgomery, R.I., Warner, M.S., Lum, B.J., & Spear, P.G. (1996). Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* **87**, 427-436.
40. Liu, J., Shriver, Z., Pope, R.M., Throp S.C., Duncan, M.B., Copeland, R.J., Raska, C.S., Yoshida, K., Eisenberg, R.J., Cohen, G., Linhardt, R.J., & Sasisekharan, R. (2002). Characterization of a heparan sulfate octasaccharide that binds to herpes simplex virus type 1 glycoprotein D. *J. Biol. Chem.* **277**: 33456-33467.
41. Chuang, Y.J., Swanson, R., Raja, S.M., & Olson, S.T. (2001). Heparin enhances the specificity of antithrombin for thrombin and factor Xa independent of the reactive center loop sequence. Evidence for an exosite determinant of factor Xa specificity in heparin-activated antithrombin. *J. Biol. Chem.* **276**: 14961-14971.