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Heparanase drives the aggressive myeloma phenotype: Preclinical development of a heparanase inhibitor for the treatment of multiple myeloma

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HEPARANASE DRIVES THE AGGRESSIVE MYELOMA PHENOTYPE: PRECLINICAL DEVELOPMENT OF A HEPARANASE INHIBITOR FOR THE TREATMENT OF MULTIPLE MYELOMA

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MOLECULAR AND CELLULAR PATHOLOGY

ABSTRACT

Heparanase, an endoglycosidase which cleaves heparan sulfate chains at specific sites, is rarely expressed in normal tissues but becomes evident in many human cancers. We have previously shown that heparanase promotes myeloma growth and angiogenesis through modulation of the tumor microenvironment. Recognition that heparanase drives the aggressive myeloma phenotype has led to new strategies designed to therapeutically target this enzyme. SST0001, a non-anticoagulant heparin that is 100% N-acetylated and 25% glycol split, was previously described as a potent inhibitor of heparanase activity *in vitro*, and, in limited *in vivo* experiments, SST0001 was identified to have efficacy as an anti-tumor agent in models of myeloma and melanoma. We now report that SST0001 can effectively inhibit myeloma growth *in vivo*, even when confronted with an aggressively growing tumor within human bone. Importantly, we find that SST0001 treatment of tumor bearing animals or myeloma cells *in vitro* causes changes within tumors consistent with the compound's ability to inhibit heparanase. This includes inhibition of expression of HGF, VEGF and MMP-9 accompanied by suppressed angiogenesis. In addition, SST0001 diminishes shedding of syndecan-1, a heparan sulfate proteoglycan previously shown to be a potent promoter of myeloma growth. SST0001 also inhibited the

heparanase-mediated degradation of syndecan-1 heparan sulfate chains thus confirming the anti-heparanase activity of this compound. When used in combination with dexamethasone, a conventional anti-myeloma agent, SST0001 blocked tumor growth *in vivo* presumably through dual targeting of the tumor and its microenvironment. These results provide mechanistic insight into the anti-tumor action of SST0001 and further validate its use as a therapeutic tool for treating multiple myeloma. As the role of heparanase in driving other diseases becomes evident, the impact of a successfully translated heparanase inhibitor will have significant clinical impact, far beyond multiple myeloma and cancer, in improving survival and quality of life for those patients afflicted with diseases exacerbated by this enzyme.

Keywords: heparanase, syndecan-1, multiple myeloma, heparin, tumor microenvironment, angiogenesis

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INTRODUCTION

Multiple Myeloma

Multiple myeloma, the second most prevalent hematologic malignancy in the United States, [1] is a plasma cell dyscrasia which thrives in the bone microenvironment. The plasma cell malignancies comprise several groups of diseases including Waldenström's macroglobulinemia, solitary plasmacytoma and multiple myeloma. These disorders are clonal in origin and are broadly characterized by an accumulation of malignant plasma cells and an overproduction of a specific monoclonal antibody (M-protein) and, in some instances, free immunoglobulin light chain (Bence-Jones protein).[2] Multiple myeloma, the most prevalent plasma cell malignancy, differs from the other diseases in that it is confined predominantly to the bone marrow and causes extensive, multifocal osteolytic lesions.

In 2010 the American Cancer Society estimated that 20,180 individuals will be diagnosed with multiple myeloma and approximately 10,650 people will die from the disease, accounting for nearly 2% of all cancer deaths nationwide.[3] The incidence and mortality rates of myeloma increase with age and sex, with men displaying higher rates than women and can be impacted by race; African-Americans display at least a 2-fold higher incidence than Caucasians.[3] Additional factors contributing to the incidence and mortality of multiple myeloma include obesity,[4] environmental and workplace exposures[5] (e.g. radiation, petroleum industry, Agent Orange[6]) and in some instances, familial aggregation.[7] At any given point there are approximately 70,000 people living with or in remission from myeloma, however, despite the recent successes in development of new therapies, the 5-year relative survival rate remains around 35%.[3]

Pathogenesis

It is now recognized that development of myeloma involves a multistep process beginning with a pre-malignant disorder of plasma cells, either monoclonal gammopathy of undetermined significance (MGUS) or smoldering myeloma (SMM).[8-10] MGUS is characterized by the presence of less than 10% of clonal plasma cells in the bone marrow, serum M protein less than 3 g/dL, and the absence of end-organ disease.[8] SMM differs from MGUS by the presence of greater than 10% of clonal plasma cells in the bone marrow and a serum M protein greater than 3 g/dL but no end organ damage.[8] Several long term studies summarized by the International Myeloma Working Group have identified that the risk of progression to a malignant plasma cell disorder, with myeloma having the highest incidence, is 1 - 1.5% per year in patients with MGUS and 10% per year for the first 5 years following diagnosis of SMM.[9]

The progression of MGUS/SMM into multiple myeloma has been proposed to follow a random, two-hit genetic model of malignancy.[10] The first hit leading to non-malignant clonal proliferation of plasma cells (MGUS/SMM) is believed to be caused via chronic antigen stimulation leading to a primary translocation of the immunoglobulin heavy chain on chromosome14q32[11] and involving several partner chromosome loci, the most common being 11q13, 4p16.3, 6p21, 16q23 and 20q11.[12] It's believed that these initial events drive and support clonal proliferation.[13] The second hit leading to malignant transformation is currently unknown, but several common abnormalities in the plasma cell and the myeloma microenvironment have been identified including secondary translocations and/or chromosome deletions, oncogene activation and silencing of tumor suppressor genes, microenvironmental changes causing induction of angiogenesis,

immune suppression, activation of NFκB, and dysregulation of cytokine and growth factor signaling networks.[14-18]

Myeloma is a devastating malignancy which inflicts a high degree of morbidity; patients present with fatigue, diffuse osteolysis, anemia, hypercalcemia, renal failure and recurrent bacterial infections.[20,21] Of these co-morbidities, osteolytic disease is one of the most incapacitating[19] and accounts for the high incidence of pathologic fractures in myeloma patients.[20] In fact, the extent of lytic disease is a strong prognostic marker for poor outcome.[21] The underlying mechanism of bone loss in myeloma is due to a tumor-induced uncoupling between the bone resorbing cells (osteoclasts) and the bone building cells (osteoblasts).[25-28] This imbalance leads to extensive bone resorption in the absence of new bone formation. Because of the unique interactions between myeloma cells and the bone microenvironment,[21] it's not surprising that this phenomenon, often termed the vicious cycle, is both a consequence and a driving force in the progression of multiple myeloma,[22] as active bone resorption can drive tumor growth and angiogenesis.[31]

Myeloma cells stimulate osteoclastogenesis through several mechanisms: dysregulation of the RANKL:OPG axis, production of osteoclast activating factors, and in some instances, osteomimicry, whereby myeloma cells mimic osteoclasts and are able to degrade bone.[23] Probably the greatest impact on myeloma bone disease is a shift in the balance of the RANKL:OPG ratio.[33] This axis is critical for normal bone homeostasis; RANKL, through interaction with its receptor RANK, stimulates osteoclastogenesis and the activity of such is kept in check by the soluble decoy receptor OPG.[24] However in myeloma, RANKL production is elevated in the bone marrow stroma and can even be expressed by myeloma cells.[25-30] OPG, on the other hand, can be bound by syndecan-1, a

cell surface heparan sulfate proteoglycan highly expressed by myeloma cells, leading to its internalization and degradation.[31] In addition to hijack of the RANKL:OPG axis, myeloma cells can further contribute to bone disease by production of "factors", such as VEGF, HGF and MMP-9, which have been correlated with extent of bone disease;[41,42] the potential for myeloma cells to directly degrade bone may also contribute to the osteolytic phenotype.[43-46]

As well as affecting osteoclastogenesis, myeloma cells have the ability to directly perturb new bone formation. Early in myeloma, osteoblast activity is actually enhanced, but as disease progresses osteoblast function is detrimentally affected.[32] Subsequent studies have revealed multiple mechanisms of myeloma regulation of osteoblast activity, predominantly by direct inhibition of osteoblast differentiation through production of the Wnt signaling inhibitors, DKK1[47] and sFRP-2.[48] Interestingly, recent studies reveal that specific targeting of DKK1 with a neutralizing antibody reversed bone resorption by increasing osteoblast activity.[49,50] The bone anabolic effect of anti-DKK1 therapy was also associated with a reduction in tumor burden, [49,50] further supporting the notion that mature osteoblasts harbor anti-myeloma properties.[33,34]

Diagnosis and Management of Myeloma

The International Myeloma Working Group defines myeloma as a clonal expansion of neoplastic plasma cells producing a monoclonal and/or free immunoglobulin light chain detectable in the serum and/or urine. Myeloma can be further classified based on the type of monoclonal protein produced, with IgG and IgA accounting for more than 70% of cases. Furthermore, ~10% of patients have detectable

levels of immunoglobulin κ or λ light chain in the urine. In some instances, less than 1% of patients with myeloma have no detectable levels of monoclonal protein or free light chain, otherwise known as non-seceretory myeloma. Because levels of monoclonal and/or light chain vary from patient to patient, the most critical criterion for diagnosis of myeloma is the presence of end organ disease (e.g. widespread osteolytic disease, anemia, hypercalcemia, renal impairment, hyperviscosity, amyloidosis or recurrent infections).[8] Diagnosis of multiple myeloma ultimately relies on a battery of laboratory tests including CBC and peripheral blood cytology, clinical chemistry measurements (e.g. β2 microglobulin, CRP, creatinine), skeletal bone survey (e.g. CT/MRI, FDG-PET scan), and bone marrow biopsy to identify myeloma cells by the surface expression of clinical markers such as CD138 (syndecan-1).[8]

Once myeloma is diagnosed patients are staged based on clinical parameters of the International Staging System,[35] recently replacing the Durie-Salmon Staging System,[36] which groups patients into three categories based on serum levels of β2 microglobulin and albumin. Risk stratification using FISH, metaphase spreads and plasma cell labeling index helps to further classify patients as either high risk (25% of patients) or low risk (75% of patients) and can direct caregivers in managing disease in cases of newly diagnosed myeloma.[37]

For newly diagnosed myeloma patients, initial therapy, while not curative, should provide disease control, relief from end organ disease and prevent early death in a manner that is rapid and with limited toxicities. Patients are first identified as either those that are stem cell transplant eligible or those that are ineligible. Patients eligible for transplant, usually determined by age, relative health and personal preference, undergo

induction therapy, which limits the use of alkylating therapies to enhance mobilization and collection of hematopoietic stem cells.[38] Induction therapies typically consist of single agent dexamethasone or combination vincristin/doxorubicin/dexamethasone.[39,40] Recently, clinical trials implementing induction therapy regimens based on the newly approved myeloma agents, bortezomib and thalidomide including its analog lenalidomide, consistently show better response rates, therefore, choice of induction therapy should be tailored to the overall health of each individual patient. Following collection of stem cells, patients undergo autologous stem cell transplantation, and in some instances, a second transplant after recovery (tandem transplant). Tandem transplant has been shown to improve complete response rates in transplant eligible patients.[41] In rare cases, allogenic transplant is conducted when an appropriate HLA-matched sibling donor is available. Those patients ineligible for stem cell transplant are given front line chemotherapy typically consisting of melphalan plus predisone.[42] Recent clinical trial data also supports a role for incorporation of bortezomib or IMiDs into front line therapy.[37] While the advances in front-line therapies have significantly improved response rates, nearly all patients eventually relapse or become refractory to therapy. When considering treatment options for salvage therapy, physicians must take into account patient co-morbidities, length of time from previous therapy, and whether or not the patient is a candidate for stem cell transplant. Many options exist whether conventional therapy, novel agents or a combination of the two are implemented.[43]

The use of biological based therapies designed to target the myeloma tumor microenvironment have improved survival outcomes.[44] These drugs (bortezomib, thalidomide/lenalidomide) appear to exert anti-myeloma activity, in part, by modulating

the interaction of the myeloma cell with the bone marrow microenvironment. For instance, in addition to its ability to induce apoptosis in myeloma cells [45], the proteasome inhibitor bortezomib, has been found to induce osteoblast differentiation resulting in new bone formation and repair of the osteolytic disease accompanied by multiple myeloma.[46-48] The newly approved IMiDs (thalidomide/lenalidomide) have also displayed effects on the tumor microenvironment predominantly by inhibiting angiogenesis through down-regulation of VEGF production from bone marrow endothelial cells.[49] The recent success in microenvironment targeted therapies has provided a solid foundation for future identification and development of novel therapeutic targets designed to "treat" the myeloma microenvironment and improve the outcome in patients with multiple myeloma.

Animal Models of Myeloma

In vivo modeling of human multiple myeloma remains a difficult task; while current models possess certain aspects that resemble human disease, there is not one that meets all criteria. An ideal experimental animal model of multiple myeloma must recapitulate both the clinical features and laboratory anomalies which are characteristic of human disease. Additionally, because of the reliance on *in vivo* animal models for the pre-clinical development of investigational new drugs, there must be a transparency in the effectiveness of myeloma therapy in both animals and humans. Therefore, in order to overcome the limitations of *in vivo* modeling, researchers have developed a number of animal models, displaying both advantages and disadvantages unique to each, which must be taken into account when designing experiments and interpreting results.

Subcutaneous Xenograft Models

Much like solid tumor xenograft models, myeloma cell lines of either human or murine origin, can be injected subcutaneously into immunocompromised [50] (e.g. SCID, SCID/NOD) or wild type BALB/c mice[51] to form a palpable plasmacytoma, with the primary endpoint being tumor volume (depicted in Fig. 1). The subcutaneous myeloma xenograft model represents an inexpensive, reliable model for investigating basic growth characteristics and pre-clinical efficacy of novel anti-myeloma compounds *in vivo*. Major advantages of this model include the ability to easily monitor tumor growth by caliper measurement and the relative ease with which the plasmacytoma can be excised for evaluation by histological and/or molecular profiling. In some instances, subcutaneous myeloma tumors have been shown to spontaneously metastasize to bone, [52] mimicking a clinical feature seen in human patients. Use of this model, in particular, has been predominantly applied to pre-clinical screening of novel anti-myeloma agents, including bortezomib[53] and thalidomide[54], which were recently approved by the FDA for treatment of multiple myeloma.[55,56] Unfortunately, the subcutaneous xenograft model does not display many of the clinical characteristics nor recapitulate the natural microenvironment, including both physical and biochemical interactions of myeloma cells with the bone marrow microenvironment, as observed in human disease. These microenvironment-specific interactions have been shown to play a major role in supporting growth and survival of myeloma cells, as well as, promoting drug resistance,[57] as it has been shown that certain therapies are less effective against myeloma cells growing in the presence of bone marrow stromal cells.[77-80] To this end, one must carefully interpret data generated using this model to assess the anti-myeloma activity of

an investigational drug as it may not translate as well to a more orthotopic model where myeloma cells can interact with the bone microenvironment.

SCID-hu Model

The SCID-hu model of myeloma (depicted in Fig. 1), first described by Urashima, *et al,* [58] was established by implanting human fetal long bones bilaterally in SCID mice. Mice are then rested for 6 to 8 weeks to allow vascularization of human bones. Primary cells from patients or myeloma cell lines are then injected directly into the marrow cavity, which provides a niche for human myeloma cells to interact with the human bone marrow milieu. In this model, engrafted myeloma cells are able to proliferate and preferentially metastasize to and grow within the contralateral, non-injected, human bone, without evidence of tumor growth in any murine tissues including bone. It is important to note that this is the only animal model that will support the growth of primary human myeloma cells[59] and engrafted human bones display bone resorption consistent with human disease.[60]

The major impetus for developing this model was to recreate the interactions between the bone marrow microenvironment and the myeloma cell, which addresses the major limitation of the subcutaneous xenograft model. The rationale for utilizing human fetal long bones, as opposed to direct injection of human myeloma cells into murine bones, is that the biochemical interactions mediated by cytokines, growth factors and cell adhesion molecules may be species-dependent. Therefore, growth of human myeloma cells in murine bone may be less than optimal. For example, myeloma cells readily depend on IL-6 for growth and survival[61] through both autocrine[62] and paracrine[63]

signaling mechanisms. It has been proposed that paracrine production of IL-6 by murine bone marrow stromal cells may be less potent than IL-6 produced by human bone marrow stromal cells leading to a reduced ability of growth and survival of engrafted human myeloma cells.[58]

Key advantages of the SCID-hu model include the ability to establish human myeloma tumors from either primary samples or cell lines within human bone, which allows for the study of the interactions between the bone marrow microenvironment and myeloma cells, the ability to harvest myeloma-bearing bone grafts for downstream analysis, and the presence of osteolytic bone disease in the engrafted tissue. As with any model, the SCID-hu model has its own limitations; quantification of tumor burden relies on bioluminescent imaging or serial measurement of biological markers, such as human monoclonal immunoglobulin in murine serum, lack of diffuse, multifocal lesions as disease is typically confined to implanted bones, and an extended study period, 12 to 14 weeks at a minimum, due to the difficulty in procuring human fetal bones and the length of time for the murine host to vascularize and support the engrafted bone. Currently, there is only one non-profit organization, which is tightly regulated by federal guidelines, that oversees procurement of the fetal bones. In addition, the medical condition and genetic background of the donor is unknown which could potentially affect study outcomes. In light of its limitations, the SCID-hu model of myeloma has provided a species-specific microenvironment that has been key in elucidating the mechanism(s) of myeloma bone disease, and provided a more "realistic" disease model for further testing the efficacy of investigational agents against myeloma cells growing in a microenvironment similar to that in human disease.[64]

Disseminated Model

A common limitation of the subcutaneous xenograft and SCID-hu models is that they are unable to recreate the systemic and multifocal nature of multiple myeloma as it presents in the clinic. To address this issue, the disseminated model (sometimes referred to as experimental metastasis model) was established whereby human myeloma cells are intravenously, and to a lesser extent, intracardiac, injected into immunocompromised mice (depicted in Fig. 1). Some early concerns of this model included the ability of human cells to engraft within the murine bone microenvironment and difficulty in accurately monitoring organotropic localization and quantifying total body tumor burden.

Early incarnations of the disseminated model of myeloma utilized the human ARH-77 cell line.[65-68] Mice developed systemic disease comparable to human disease; hind limb paralysis due to spinal cord compression, osteolytic lesions within the vertebrae and skull, and development of extramedullary tumors in the brain, lung, liver and kidney which is sometimes seen in later stages of human disease.[69] It was later discovered that the ARH-77 cell line was in fact not a myeloma, but rather an Epstein-Barr virus infected lymphoblastoid cell line.[70] Following this discovery, the use of the disseminated model has been validated using "true" myeloma cell lines which caused osteolytic lesions, hypercalcemia, hind limb paralysis, and in some instances, extramedullary disease.[71] Additionally, a common feature was noted that regardless of strain of immunocompromised mouse used (e.g. NOG,[72] SCID[73] or SCID/NOD[74]) human myeloma cells were able to engraft and form multifocal lesions with pathological

manifestations similar to human patients, addressing one of the major concerns of these models.

To overcome the early concerns that tracking and monitoring disease progression would be limited by a lack of sensitive detection techniques, researchers have continued to utilize quantification of human immunoglobulin in murine sera to measure tumor burden and established whole body fluorescence[75] and bioluminescence imaging[76] of mice bearing myeloma cells expressing either green fluorescent protein or luciferase construct as an accurate measure for tracking disease localization and tumor burden, even in the case of cell lines that produce very low levels or none at all of serum markers, such as monoclonal immunoglobulin, conventionally used for serial monitoring tumor burden. The introduction of μCT and MRI to measure extent of osteolytic disease has also replaced radiographic estimates of bone resorption at sites of active myeloma. Studies using the disseminated model of myeloma have contributed greatly to the understanding of organotropic homing of myeloma and pre-clinical development of investigational new agents to treat this disease.[71]

Heparanase

Heparanase (HPSE), an endo-β-d-glucuronidase, cleaves heparan sulfate (HS) at specific sites,[77] to orchestrate extracellular matrix and basement membrane remodeling. In addition, heparanase activity can release "stored" heparin-bound molecules and generate HS fragments that are 10-20 sugar residues in length; these fragments have the ability to interact with heparin-binding proteins and modulate their biological activity.[78,79] Originally it was believed that several HS-degrading enzymes existed, however cloning

studies revealed one dominant mammalian heparan sulfate (HS) degrading enzyme.[80-83] Following the identification of heparanase as the sole heparan sulfate degrading enzyme, McKenzie *et al* discovered a heparanase homolog, heparanase-2, which displays ~40% homology,[84] however, while heparanase-2 can interact with both heparin and HS, it lacks enzymatic activity.[85] Heparan sulfate, which exists in large part covalently attached to a core protein constituting a diverse family of proteins known as the heparan sulfate proteoglycans (HSPGs), is a ubiquitous component of the cell surface (e.g. syndecans and glypicans) and extracellular matrix (e.g. perlecan, agrin, collagen XVIII).[86,87] These molecules, through their HS chains and/or core protein, are involved in many biological functions; mediating cell-cell and cell-extracellular matrix interactions, orchestrating assembly of extracellular matrix molecules, serving as a reservoir for heparin-binding factors and regulating growth factor signaling.[88,89] Due to their critical role in cellular physiology, enzymatic remodeling of the HSPGs constitutes a mechanism whereby heparanase can directly regulate both physiological and pathological processes.[90]

Heparanase is expressed as a 543 amino acid pre-pro-enzyme and contains 6 putative N-glycosylation sites. Cleavage of the signal peptide generates a 65 kDa latent pro-enzyme that undergoes pH sensitive, proteolytic processing to generate an active enzyme composed of a 50 kDa subunit non-covalently associated with an 8 kDa peptide.[115] In order to be activated, it is believed that heparanase first undergoes vesicular secretion from the Golgi.[91] Once secreted, latent heparanase interacts with a cell surface receptor, such as the syndecan HSPGs,[117] mannose-6 phosphate receptor or low density lipoprotein receptor-related protein (LRP).[118] These receptors have been further characterized as either high affinity, low abundance (LRP and mannose-6-

phosphate receptor) or low affinity, high abundance (HSPGs).[119] Once bound, heparanase is immediately endocytosed and transferred to the late endosome/lysosome[117] where it is processed, predominantly by cathepsin L, into its active form.[120] The activity of heparanase can be attributed to two conserved glutamic acid residues, Glu^{225} and Glu^{343}, located within the 50 kDa subunit.[92] However, enzymatic activity also requires the 8 kDa subunit which is believed to direct a conformational change within the active site to facilitate HS degradation.[122,123]

Expression of heparanase is primarily restricted to the placenta, lymphoid organs, keratinocytes, osteoblasts and blood platelets[124-128] and appears to be involved in physiological processes such as embryonic implantation, wound/fracture repair, HS recycling, tissue remodeling, immune surveillance and hair growth;[129-133] however, in a variety of pathological conditions such as chronic inflammation,[93] diabetes,[94] and malignancy,[95] heparanase expression is dramatically upregulated and exacerbates the disease condition. This stark difference in expression of heparanase indicates alternative regulation that is both transcriptional and post-translational, and that may contribute to the elevated levels seen in the pathologic state. In addition to proteolytic processing of the latent heparanase protein, multiple mechanisms have been identified that can contribute to heparanase regulation.

Under normal conditions, where expression of heparanase is limited, it is believed that the tumor suppressor p53 inhibits heparanase transcription by directly binding to the heparanase promoter.[96] Once bound, p53 recruits histone deacetylases which further suppress gene transcription by promoting DNA condensation.[138] In tumors, mutational inactivation of p53 can lead to transcription of heparanase which may account for the

high levels of expression during cancer development.[96] In addition to p53, several transcription factors have been implicated in regulation of heparanase gene transcription; Ets and SP1 with basal transcription[95] and EGR1 with inducible transcription.[140,141] Promoter methylation has also been shown to play a major role in regulation of heparanase transcription.[97] High heparanase expression/activity has been found to be correlated with hypomethylation. In contrast, cells with little to no heparanase activity harbored fully methylated alleles; treatment of these cells with 5-azacytidine, a demethylating agent, resulted in increased heparanase expression and metastatic potential.[97,98] In addition to these local regulatory mechanisms, several systemic factors have been identified to induce heparanase gene expression including estrogen,[143,144] TNFα and IFNγ,[99-101] glucose,[102] reactive oxygen species[103] and hypoxia.[102-104] Heparanase promoter sequence analysis revealed the presence of four putative estrogen response elements[143] and two consensus interferon-stimulated response elements.[99] More recently, a mechanism of post-transcriptional regulation of heparanase gene expression has been identified whereby a 185 base pair region within the 3" untranslated region directly regulates heparanase expression.[104] Characterization of this sequence revealed an adenine/uracil-rich consensus element (ARE)[104] which has been previously identified to target mRNAs for rapid degradation.[105] Deletion of this sequence resulted in stabilization of heparanase mRNA, elevated levels of heparanase expression and enhanced enzymatic activity.[104] Taken together, these mechanisms, or a combination of such, may contribute to the enhanced expression of heparanase in the diseased state.

Role in Cancer

The notion that a heparan sulfate degrading enzyme could promote tumor progression (depicted in Fig. 2) became evident in the early 80's where an endoglycosidase was found to drive the metastatic potential of B16 melanoma cells[106] and T-lymphoma.[107] Following the identification and cloning of human heparanase, experimental studies designed to over-express or silence heparanase clearly showed that this enzyme was a key component of tumor progression, where it promotes formation of a vascular network to fuel primary tumor growth and metastatic spread of tumor cells.[95,108] Subsequently, heparanase has been found to be highly expressed in a wide variety of human malignancies and high levels of this enzyme correlate with a poor outcome, including increased microvessel density, lymph node and distant metastasis and a reduced post-operative survival of cancer patients.[95,108]

The first observed function of heparanase in cancer progression was its ability to promote cancer metastasis.[106,109,110] Early experimental studies revealed that the metastatic capability of tumor cells was correlated with heparanase activity.[80,111-113] For example, highly metastatic rat mammary adenocarcinoma cell lines (13762 MAT, DMBA-8A) were found to express higher levels of heparanase when compared to their non-metastatic counterparts.[114] Conversely, Eb lymphoma cells, which lack heparanase activity, display no metastatic capability;[110] introduction of the heparanase cDNA into these cells resulted in high heparanase expression and formation of massive liver metastases and decreased survival *in vivo*.[80] The ability of B16-BL6 melanoma cells to colonize lung, a heparanase-dependent process, was markedly reduced by anti-heparanase siRNA.[115] High heparanase expression was also found to promote

spontaneous metastasis of myeloma cells; subcutaneously formed tumors metastasized to spleen, liver, lung and bone; whereas, in the SCID-hu model, myeloma cells growing in human bone metastasized specifically to a non-injected contralateral bone resulting in extensive bone resorption even when tumor burden was relatively low.[116] Heparanase-transfected MDA-MB-231 breast cancer cells growing in mouse mammary fat pad stimulated bone resorption in the absence of detectable metastases.[117] These studies suggest that heparanase may also act systemically by creating a pre-metastatic niche; stimulation of bone resorption prior to the arrival of cancer cells may lead to the progression of bone homing tumors which thrive at sites of active bone resorption.

The finding that heparanase promotes the angiogenic phenotype is a well accepted observation both clinically and experimentally. High levels of heparanase have been found to correlate with enhanced microvessel density, a surrogate marker of angiogenesis and poor prognosis in multiple myeloma,[118] as well as a variety of solid tumors.[119,120] Overexpression in tumor xenograft models led to enhanced tumor growth and vascularization in a variety of cell lines including, but not limited to, HT29 colon carcinoma,[121] CAG myeloma,[122] MCF7[123] and MDA-MB-231[117] breast carcinoma cells. *In vitro* studies have also demonstrated the ability of heparanase to promote angiogenesis.[124] To date several mechanisms have been identified to explain how heparanase contributes to angiogenesis including enzymatic remodeling of extracellular matrix and release of HS-bound growth factors, activation of signaling cascades, and induction of VEGF expression.[108,125]

Heparan sulfate and heparan sulfate proteoglycans have been well established as playing a role in a variety of steps involved in the process of angiogenesis.[86,126,127] An

early event in the angiogenic process is degradation and remodeling of the subendothelial basement membrane to facilitate migration and invasion of stimulated endothelial cells.[128] It's no surprise that heparanase facilitates this process by degrading heparan sulfate to create a network for new capillary formation by invading endothelial cells. In fact, stimulated endothelial cells have been shown to up-regulate expression of heparanase, presumably to facilitate basement membrane remodeling.[79] Immunohistochemistry of tumor specimens further confirmed this finding by positive heparanase staining within endothelial cell capillaries, but not mature, established blood vessels, indicating that heparanase is involved in the early processes of angiogenesis.[79,129] In addition to remodeling the extracellular matrix, heparanase activity can release stored HS-bound growth factors, such as FGF2[130,131] and VEGF,[132] from the extracellular matrix or cell surface. Once liberated, these factors can further facilitate angiogenesis by creating an "angiogenic gradient" to attract and direct capillary formation. It has also been found that the HS fragments generated by heparanase can stimulate the mitogenic activity of FGF2, and possibly other pro-angiogenic factors.[79]

Outside of its classical enzymatic functions, heparanase can also directly activate a number of intracellular signaling cascades, some of which can be stimulated in the absence of heparan sulfate or heparanase enzymatic activity. Interestingly, signaling through these pathways has been found to be dependent on cell type. Whereas heparanase promotes ERK phosphorylation in hematopoietic cells, heparanase-mediated Akt and Src signaling is primarily seen in firmly attached cells such as those of endothelial or epithelial origin. Heparanase, in an enzyme-activity dependent manner, enhances ERK signaling in multiple myeloma[133] and addition of latent heparanase to

primary T-cells results in activation of this pathway.[100] Endogenous over-expression in cancer cells[134] or addition of recombinant heparanase to primary endothelial cells[124] markedly enhanced Akt phosphorylation in a manner that was found to be independent of HS or enzymatic activity. These results imply that heparanase, through the Akt pathway, may protect tumor cells from apoptosis[135] and can elicit an angiogenic response by a direct effect on the endothelial cell.[108]

Heparanase can also directly contribute to angiogenesis via induction of VEGF; cells engineered to express high levels of heparanase were found to have a significant increase in both VEGF protein and mRNA levels which could be reversed by heparanase-specific siRNA. Investigation into the mechanism of heparanase regulation of VEGF identified Src as a mediator of heparanase-induced VEGF expression.[136] Inhibitors specific for Src blocked heparanase-mediated VEGF production and cell migration indicating that Src may be a critical downstream component of heparanase.

Similar to Akt activation, heparanase induction of Src signaling does not rely on enzymatic activity. Activities independent of heparanase activity have been recently attributed to the identification of the heparanase C-domain.[137] Using 3D modeling software of constitutively active heparanase, this domain was found to be indispensible for secretion and activation of the heparanase enzyme and also mediate non-enzymatic functions of heparanase (e.g. Akt phosphorylation).[137] Recently, a splice variant of human heparanase lacking exon 5 has been detected that lacks enzymatic activity.[138,139] The biological significance of this splice variant remains unclear; however additional splice variants have been predicted *in silico*.[140] Of these, the expression of one identified as T5, which results in a truncated protein lacking enzymatic activity, was found to be

highly expressed in lung carcinoma and chronic myeloid leukemia samples.[140] Subsequently, T5 was found to promote tumor xenograft growth characterized by high microvessel density and enhanced Src phosphorylation, all independent of enzymatic activity.[140] The clinical significance of T5 was further confirmed by analysis of renal cell carcinoma biopsies which revealed expression of heparanase and the heparanase splice variant, T5, in 75% of cases.[140] These findings clearly provide rationale support, both clinical and experimental, for a role of heparanase in driving tumor progression, in part, by promoting angiogenesis and metastasis.[109,110]

Heparanase/Syndecan-1 Axis in Multiple Myeloma

Elevated levels of heparanase have been detected in a variety of human cancers.[125] In most cases high heparanase was detected in about 50% of tumor specimens; the highest incidence being multiple myeloma where enzyme activity was found to be significantly elevated in 86% of patients' bone marrow plasma.[122] Subsequent gene array analysis found high heparanase gene expression in 92% of myeloma patients;[118] confirming the initial finding that heparanase expression and activity are high in multiple myeloma. The elevated level of heparanase in multiple myeloma is correlated with an increase in microvessel density,[122] a surrogate marker of angiogenesis.[141] In addition to myeloma, heparanase is also associated with enhanced angiogenesis in many solid tumors; further corroborating the observation that heparanase drives the angiogenic phenotype. Unique to myeloma, however, is the finding that heparanase is associated with an increased expression and shedding of the heparan sulfate proteoglycan syndecan-1 (CD138).[118,142] Interestingly, the interactions between

syndecan-1 and heparanase have emerged as a driving force in the progression of multiple myeloma (depicted in Fig. 3); where heparanase serves as a "master regulator" of an aggressive myeloma phenotype and contributes to tumor growth, angiogenesis, metastasis and osteolytic disease, in part, by regulating syndecan-1.[95,143,144]

Syndecan-1, a cell surface heparan sulfate proteoglycan, is highly expressed in myeloma and is often used as a clinical marker to distinguish malignant plasma cells in patient samples. Because syndecan-1 is the dominant HSPG expressed by myeloma cells, much work has been done to understand the role of this molecule in multiple myeloma.[143] Early studies revealed that cell surface syndecan-1 has an inhibitory effect on myeloma progression where it promotes adhesion and inhibits invasion *in vitro*;[145] however, syndecan-1 that is shed from the cell surface accumulates in the bone marrow extracellular matrix[146] and elevated levels of shed syndecan-1 detected in the serum of myeloma patients is a predictor of poor patient prognosis.[147] Based on these findings it was hypothesized that once shed from the cell surface, syndecan-1 creates a niche within the bone microenvironment where it drives growth and metastasis of myeloma tumors.[133,145,148] This hypothesis was further confirmed by several *in vivo* studies. Expression of soluble syndecan-1 by myeloma cells led to enhanced tumor growth and metastasis *in vivo*.[148] Conversely, knock down of heparanase[118] or overexpression of a mutated, enzymatically inactive form of heparanase in myeloma cells led to a dramatic reduction of syndecan-1 shedding and limited ability of these cells to form tumor *in vivo*. The critical role of syndecan-1 in supporting myeloma growth *in vivo* has recently been confirmed by knockdown of expression of either syndecan-1 or heparan sulfate.[149,150]

These studies revealed that syndecan-1 is required for growth, angiogenesis and metastasis of myeloma *in vivo*.

Several syndecan sheddases have been previously identified;[151] however, until recently, the mechanism of heparanase-induced syndecan-1 shedding in myeloma was unknown. Because heparanase is an endoglycosidase, it cannot directly "shed" syndecan-1; rather it directly regulates the sheddase responsible for syndecan-1 shedding through activation of the ERK1/2 pathway.[133] Recently, heparanase was found to stimulate expression of matrix metalloproteinase (MMP)-9, a syndecan-1 sheddase.[133] Knockdown of heparanase or overexpression of a mutated, enzymatically inactive form of heparanase led to a dramatic reduction in MMP-9 expression and a subsequent reduction of syndecan-1 shedding.[133] Furthermore, approaches to inhibit MMP-9 (siRNA, neutralizing antibody and chemical inhibitor) led to decreased shedding of syndecan-1.[133]

The ability of heparanase to directly regulate levels of syndecan-1 by controlling MMP-9 induced shedding led to the finding that heparanase can also affect nuclear levels of HS/syndecan-1 in myeloma.[152] Several studies have reported that nuclear HS may play an important role in regulating proliferation, growth factor shuttling, and gene expression.[153-157] More specifically, HS has been shown to inhibit topoisomerase I and histone acetyltransferase (HAT) activity thereby preventing gene transcription.[157,158] In myeloma, high levels of heparanase are correlated with a loss of nuclear syndecan-1, a process that is dependent on enzyme activity.[152] Interestingly, myeloma cells expressing high levels of heparanase have also been found to display enhanced HAT activity, but not expression; indicating that the HS chains of syndecan-1 are important regulators of HAT

activity that can be directly modulated by heparanase (unpublished data, Purushothaman, Ritchie and Sanderson). HAT activity is often upregulated in many pathological settings, including cancer, where it can up-regulate expression of tumor promoting genes.[159] The finding that heparanase directly regulates nuclear syndecan-1 may further shed light into the mechanism of the aggressive myeloma phenotype driven by heparanase.

In addition to directly regulating protease expression and syndecan-1 shedding, heparanase is also able to stimulate expression of growth factors, such as VEGF[132] and HGF (Ramani et al., submitted for publication) in myeloma. These growth factors are potent stimulators of myeloma growth and angiogenesis[132,160] Once shed into the microenvironment, syndecan-1 can bind HGF [161] and VEGF[132] and form matrix anchored complexes to present these growth factors to their receptors, potentiating myeloma growth and angiogenesis. Alternatively, through its core protein, syndecan-1 can promote integrin activation and drive angiogenesis.[162] In myeloma, shed syndecan-1 has been found to stimulate angiogenesis, in part, by participating in activation of the αvβ3 integrin.[132]

Interestingly, these effects observed to occur downstream of heparanase have been found to rely on heparanase enzymatic activity. Therefore, enzymatic remodeling of the heparan sulfate chains of syndecan-1 is likely a key contributor to the aggressive phenotype imparted by the heparanase/syndecan-1 axis.[144] Treatment of mice bearing myeloma tumors with bacterial heparinase III resulted in a dramatic inhibition of tumor growth.[163] While both heparinase III and heparanase degrade HS chains, their cleavage products are vastly different. Heparinase III functions as a β-eliminase, generating extensively degraded HS fragments, often a mono- or disaccharide,[164] whereas

heparanase degrades HS at specific sites to generate HS fragments of appreciable size.[77] These fragments are able to bind certain growth factors and "present" them to their respective receptors. The unique action of each enzyme reveals distinct outcomes on tumor growth and further highlights the role of the heparanase/syndecan-1 axis and underscores the notion that strategies designed to inhibit the function of heparanase is a valid approach for treating multiple myeloma.

Heparanase inhibitors for cancer therapy

Following the identification of one dominant functional heparanase enzyme and identifying its role in promoting aggressive tumorigenesis, many attempts at developing heparanase inhibitors for cancer therapy have been made. The compounds generated have displayed a wide variety of approaches at targeting this enzyme; ranging from neutralizing antibodies, small molecules, natural products and competitive substrates.[119,120] Several of these inhibitors have been shown to possess anti-tumor and anti-metastatic properties *in vivo*; however, only one has progressed into early clinical trials.[165]

Muparfostat (PI-88, Progen Pharmaceuticals, LTD), a highly sulfated, mono-phosphorylated mannose oligosaccharide mixture isolated from yeast, has been shown to be an effective inhibitor of tumor growth, metastasis and angiogenesis in animal models against a variety of cancers.[121,122] Much of the anti-tumor activity of this compound has been attributed to heparanase inhibition (IC_{50} = 0.98μM);[121] however, PI-88 has also been shown to disrupt the activity of many HS-bound growth factors, such as VEGF-A and FGF2, which further contributes to its tumor inhibitory properties.[121-123] The success of

PI-88 in the pre-clinical setting paved the way for initiating clinical trials; making this compound the first, and subsequent only, heparanase inhibitor to reach the clinic. Unfortunately, while PI-88 displayed substantial anti-heparanase activity and promise as an anti-cancer agent in the pre-clinical setting; clinical development of PI-88 has been hampered due to dose-limited immune mediated thrombocytopenia and poor pharmacokinetic profiling.[166,167] In addition, PI-88 has also been found to possess anti-coagulant activity which may further prevent its success in the clinic.[126,127] Due to these setbacks, clinical trials of PI-88 have been temporarily suspended by Progen.

As an analog of the natural substrate of heparanase, heparin has long been known to possess potent anti-heparanase activity by serving as an alternative substrate.[168,169] Heparin and heparan sulfate (depicted in Fig. 4) are sulfated polysaccharides belonging to the family of glycosaminoglycans and consist of a repeating disaccharide unit composed of a uronic acid (glucuronic or iduronic) alpha 1,4 linked to an amino sugar (N-acetylglucosamine, GlcNac).[170,171] These residues undergo further modifications including epimerization of the uronic acid (glucuronic acid to iduronic acid), N-deacetylation/N-sulfation (GlcNac), 2-O (iduronic acid), 3-O and 6-O sulfation.[171] While both sugars share the same core disaccharide unit, heparin differs from heparan sulfate by the degree of modifications to the sugar residues, displaying a higher degree of sulfation and uronic acid content.[171] Recently, the specific sites of substrate cleavage by heparanase have been identified as the linkage between a non-sulfated glucuronic acid and an N-sulfated glucosamine bearing either a 3-O or 6-0 sulfation with or without a 2-O sulfated glucuronic acid nearby.[77] These compositional differences likely contribute to the limited degradation of heparin by heparanase.

The impact of heparin/low-molecular-weight heparin (LMWH) on cancer was first recognized in several clinical trials using unfractionated heparin and low-molecular-weight heparin (LMWH) in preventing venous thromboembolisms in advanced cancer patients. These studies identified that heparin, in particular the LMWHs, actually prolonged survival.[172-174] Subsequently, several independent, randomized-controlled trials have confirmed the initial finding that LMWH is able to increase survival in patients with cancer,[174-178] with the greatest impact on those patients previously identified as having a better prognosis.[175] It has been suggested that LMWH inhibits tumor growth through one or a combination of several possibly interrelated mechanisms including; sequestration of heparin-binding growth factors thereby limiting their mitogenic potential;[179,180] inhibition of angiogenesis;[181-183] disruption of cancer cell surface selectin-ligand interactions;[184,185] anticoagulation by preventing thrombin generation;[186] and/or inhibition of heparanase enzymatic activity.[170] However, the use of heparin or LMWH as anti-cancer agents would be limited due to the risk of inducing adverse bleeding complications. Fortunately, it is possible to separate the anti-coagulant and anti-heparanase properties of heparin through a series of chemical modifications.[187,188] These non-anticoagulant species of heparin are, therefore, able to be administered at high doses without risk of developing bleeding disorders. Naggi *et al* have recently demonstrated that heparin which is 100% N-acetylated and 25% glycol split (now called SST0001, depicted in Fig. 4) is endowed with properties making it suitable for use as a cancer therapeutic. In addition to its lack of anti-coagulant activity, SST0001 has been characterized as having potent anti-heparanase activity and displays limited ability to potentiate the release of growth factors from the extracellular matrix.[188] In limited animal studies, SST0001 has been shown to

inhibit primary growth of human myeloma tumors[163] and metastasis of murine B16 melanoma cells[189] *in vivo.* These findings clearly provide evidence for non-anticoagulant heparins, such as SST0001, to be suitable heparanase inhibitors for adaptation into the clinic.

Conclusions

The discovery and identification of one functional and dominant heparan sulfate degrading enzyme has led to significant advances in understanding the role of heparanase in normal and pathological settings. The greatest impact of heparanase has been in the context of cancer, where heparanase becomes highly expressed and contributes to tumor growth, angiogenesis and metastasis. In spite of these important discoveries, little progress has been made in developing and adapting a heparanase inhibitor suitable for cancer therapy.

Our finding that heparanase drives myeloma growth, angiogenesis and metastasis through regulation of the heparan sulfate proteoglycan syndecan-1 provides rational, mechanistic evidence that therapeutic targeting of heparanase is a valid approach for myeloma therapy. The present work was undertaken to validate and further develop a novel, heparin-based heparanase inhibitor, SST0001, for the treatment of multiple myeloma. In order to improve the odds for success of translating this compound into the clinic we set out to confirm that SST0001 is able to "hit" its target *in vivo* and provide insight into the anti-myeloma mechanism of action. Studies outlined in chapters 2 and 3 describe the results of extensive *in vivo* testing, validation of target engagement and analysis of the pathways downstream of heparanase activity following treatment with

SST0001. These studies have contributed greatly to the advancement of SST0001 from the bench to the bedside with the objective to enter clinical trials in the near future.

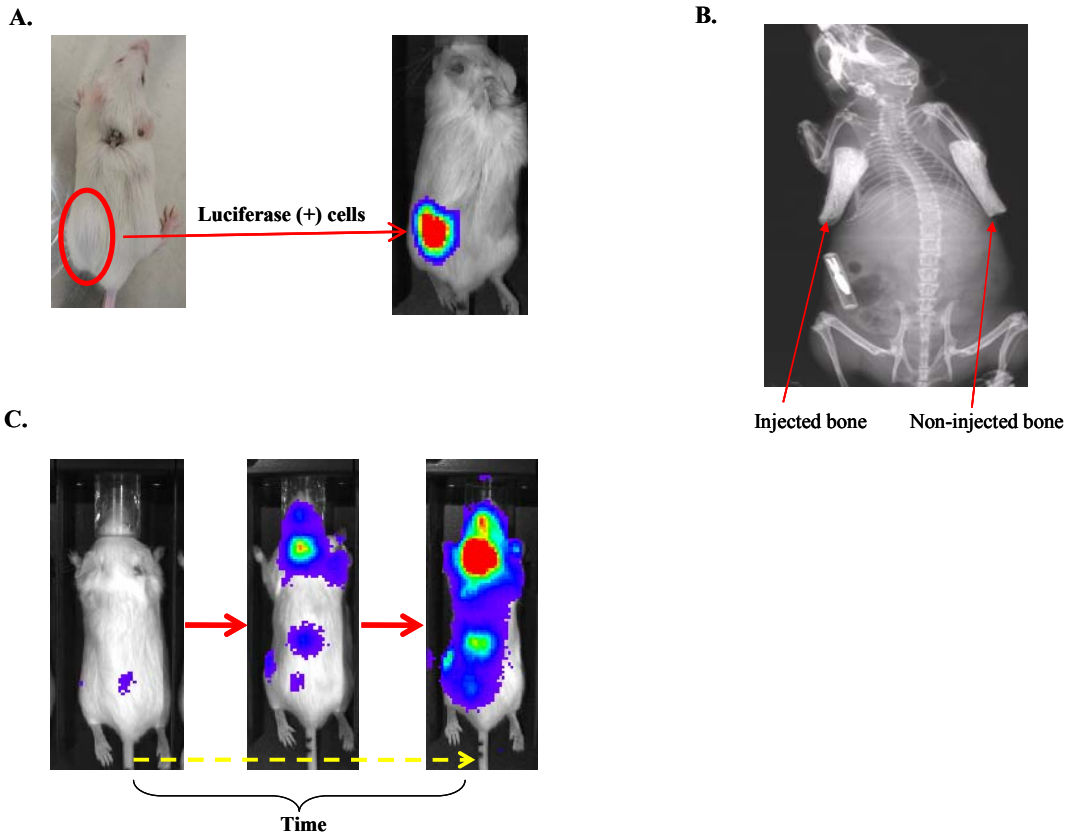

Figure 1. Animal models of myeloma

A. Subcutaneous model of myeloma established by injection of myeloma cells subcutaneously into the flank of mice. Tumors can be tracked by caliper measurement, bioluminescent imaging or serum markers. **B.** The SCID-hu model is established by subcutaneously implanting human fetal long bones into SCID mice. Once vascularized, myeloma cells can be directly injected into the bone marrow cavity and allowed to grow. This model is able to recapitulate the interactions between human bone and human myeloma cells with resultant osteolysis of implanted bones. **C.** The disseminated model of myeloma is initiated by injection of myeloma cells into the blood circulation (tail vein or intracardiac). Tumor growth can be tracked over time with bioluminescent imaging.

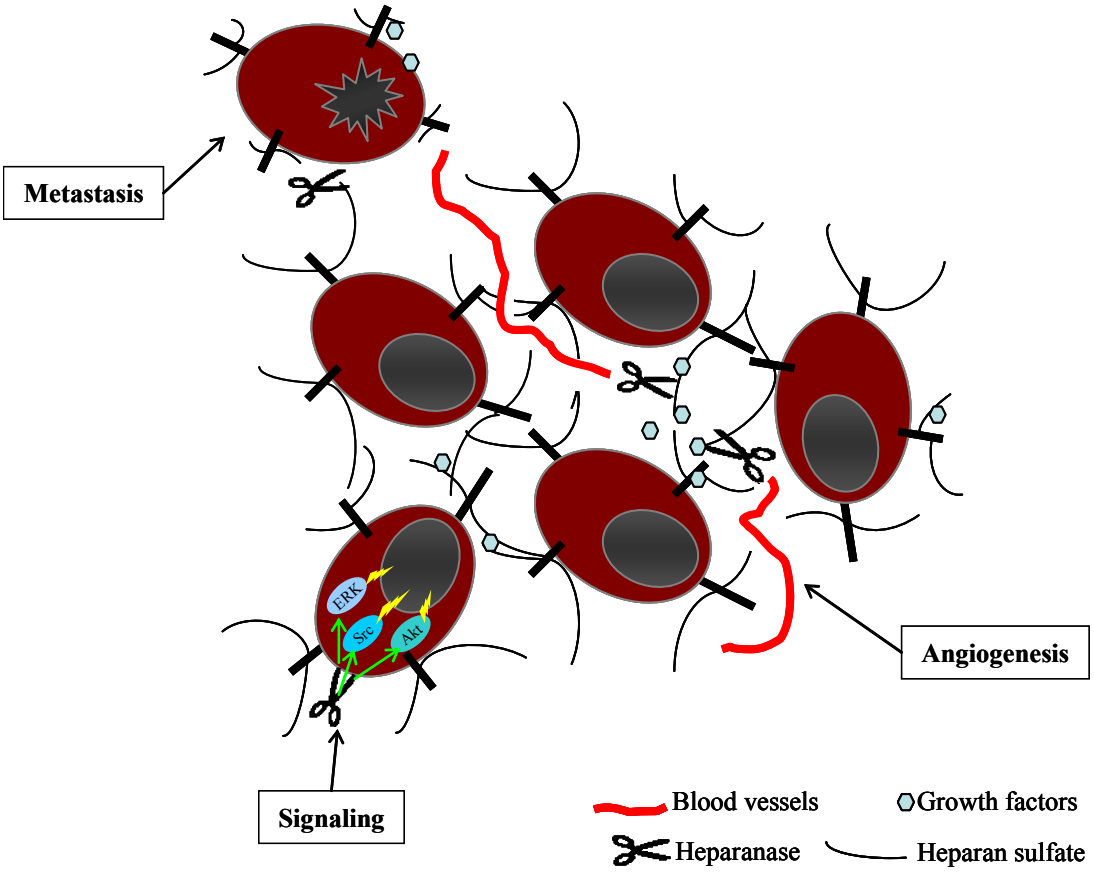

Figure 2. Mode of action of heparanase in cancer

Heparanase contributes to tumor growth, angiogenesis and metastasis by enzymatic-dependent and independent mechanisms. Degradation of heparan sulfate on the cell surface and within the extracellular matrix causes release of heparan sulfate-bound growth factors that stimulate angiogenesis and tumor growth. Disassembly of the basement membrane by heparanase contributes to extravasation of metastatic cells. Heparanase also contributes to cancer progression by activating signaling cascades independent of heparan sulfate and enzymatic activity.

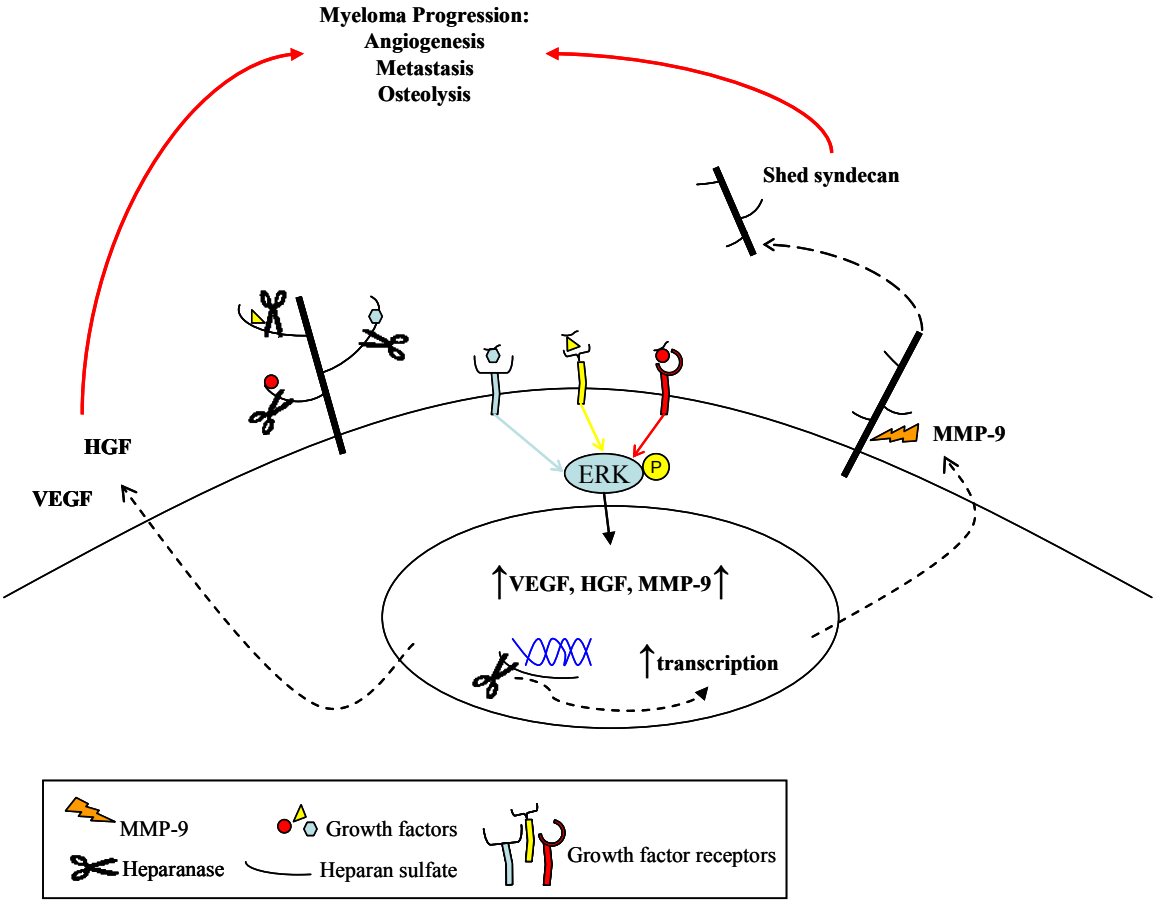

Figure 3. The heparanase/syndecan-1 axis in multiple myeloma

Heparanase acts as a master regulator of an aggressive myeloma phenotype in part by regulating syndecan-1. Myeloma derived heparanase cleaves heparan sulfate chains of syndecan-1 and liberates growth factors that are associated with heparan sulfate fragments. These complexes can interact with their receptors to activate intracellular signaling cascades (e.g. ERK) leading to MMP-9 expression. Activated MMP-9 proteolytically sheds syndecan-1 from the cell surface. Once shed, syndecan-1 accumulates within the tumor microenvironment to create a niche to drive aggressive disease. Within the nucleus, heparanase can fine tune gene transcription by degrading heparan sulfate to promote HAT activity and subsequent gene transcription.

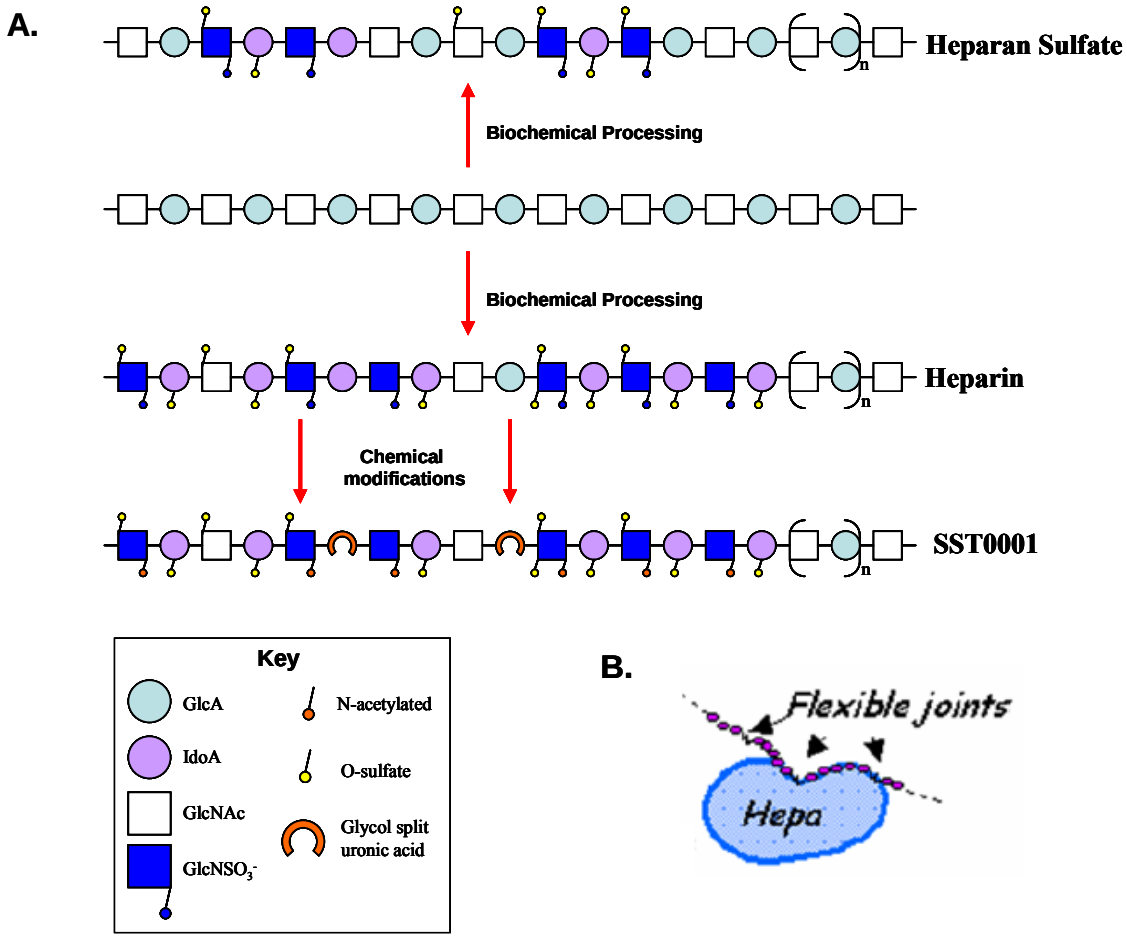

Figure 4. Basic chemical structure of heparan sulfate, heparin and SST0001

A. Biosynthesis of heparin and heparan sulfate begins with a common polysaccharide composed of a repeating GlcA-GlcNAc disaccharide. Biochemical processing including N-deacetylation/N-sulfation of the GlcNAc, epimerization of GlcA to IdoA, 2-O sulfation of IdoA, and 3-O and 6-O sulfation of GlcNS. Chemical processing of heparin to include glycol splitting at 25% of all non-sulfated uronic acid residues and N-desulfation/N-acetylation of all N-sulfated GlcNS residues yields SST0001, a heparin derivative that lacks anti-coagulant activity but retains heparanase inhibitory activity. **B.** Glycol splitting enhances flexibility to facilitate a close interaction and tight binding to heparanase resulting in potent inhibition of enzyme activity.

CHAPTER 2

SST0001, A CHEMICALLY MODIFIED HEPARIN, INHIBITS MYELOMA GROWTH AND ANGIOGENESIS VIA DISRUPTION OF THE HEPARANASE/SYNDECAN-1 AXIS

by

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ABSTRACT

Purpose: Heparanase promotes myeloma growth, dissemination and angiogenesis through modulation of the tumor microenvironment, thus highlighting the potential of therapeutically targeting this enzyme. SST0001, a non-anticoagulant heparin with anti-heparanase activity was examined for its inhibition of myeloma tumor growth in vivo and for its mechanism of action.

Experimental Design: The ability of SST0001 to inhibit growth of myeloma tumors was assessed using multiple animal models and a diverse panel of human and murine myeloma cell lines. To investigate the mechanism of action of SST0001, pharmacodynamic markers of angiogenesis, heparanase activity, and pathways downstream of heparanase were monitored. The potential use of SST0001 as part of a combination therapy was also evaluated *in vivo*.

Results: SST0001 can effectively inhibited myeloma growth *in vivo*, even when confronted with an aggressively growing tumor within human bone. In addition, SST0001 treatment causes changes within tumors consistent with the compound's ability to inhibit heparanase; including down regulation of HGF, VEGF and MMP-9 expression and suppressed angiogenesis. SST0001 also diminishes heparanase-induced shedding of syndecan-1, a heparan sulfate proteoglycan known to be a potent promoter of myeloma growth. SST0001 inhibited the heparanase-mediated degradation of syndecan-1 heparan sulfate chains thus confirming the anti-heparanase activity of this compound. In

combination with dexamethasone, SST0001 blocked tumor growth *in vivo* presumably through dual targeting of the tumor and its microenvironment.

Conclusions: These results provide mechanistic insight into the anti-tumor action of SST0001 and validate its use as a novel therapeutic tool for treating multiple myeloma.

Introduction

Multiple myeloma is the second most prevalent hematologic malignancy in the United States.[1] The emergence of novel, targeted therapeutics (e.g., bortezomib, thalidomide) has greatly improved survival rates in patients with myeloma;[2] however, there is still an unmet need in identifying and developing therapies designed to further prevent the progression of this disease without sacrificing patient quality of life. Recognition that the myeloma tumor microenvironment helps drive the aggressive nature of myeloma has recently led to new strategies for attacking the tumor microenvironment.[3] Our lab and others have shown that heparanase, an endo-β-D-glucuronidase that degrades heparan sulfate (HS) chains of proteoglycans (e.g., syndecan-1) on the cell surface and within the extracellular matrix, is rarely expressed in normal tissues, but becomes highly expressed in a number of human malignancies, including multiple myeloma.[4] Heparanase promotes an aggressive phenotype,[5] in part by synergizing with the heparan sulfate proteoglycan syndecan-1 (CD138) to create a niche within the bone marrow microenvironment further driving myeloma growth and dissemination.[6] Moreover, work in our lab has shown that active heparanase can be detected in the bone marrow of myeloma patients and that the presence of high levels of this enzyme correlates with enhanced angiogenic activity, an important promoter of myeloma growth and progression.[4] In other cancers, enhanced heparanase expression was found to correlate with increased lymph node and distant metastasis, increased microvessel density, and reduced post-operative survival.[7-10] Therefore, therapies designed to disrupt the heparanase/syndecan-1 axis in myeloma will likely prove advantageous in the treatment of this devastating disease.

The finding that heparanase is involved in a wide variety of tumor types and is subsequently linked to the development of pathological processes has led to development of therapeutic strategies to inhibit this enzyme.[11] Of the compounds produced, only one, PI-88, a phosphomannopentose sulfate, has entered clinical trials and has not yet been approved for routine clinical use.[12-14] Heparin has long been known to possess potent anti-heparanase activity.[15] Results from several clinical trials using unfractionated heparin and low-molecular-weight heparin (LMWH) in preventing pulmonary embolism in advanced stage cancer patients indicated that heparin prolonged survival,[16] probably due to a direct effect on the tumor, potentially through inhibition of heparanase enzymatic activity.[17] In fact, a recent retrospective study on the effect of anticoagulation on survival in newly diagnosed myeloma patients revealed a possible beneficial effect of LMWH therapy on outcome in myeloma patients.[18] Additional preclinical studies have further highlighted the potential use of anticoagulant therapy to treat cancer. Defibrotide, an oligonucleotide anticoagulant, was recently shown to possess *in vivo* chemosensitizing properties and inhibited myeloma growth presumably through inhibiting heparanase activity thereby modulating the tumor microenvironment.[19] However, in light of the these findings, use of anti-coagulants such as heparin or LMWH as anti-cancer agents is limited due to the risk of inducing adverse bleeding complications. Fortunately, it is possible to separate the anti-coagulant and anti-heparanase properties of heparin through a series of chemical modifications.[20-22] Non-anticoagulant species of heparin can, therefore, be administered at high doses without risk of causing bleeding disorders. Naggi *et al* [21] identified heparin that is 100% N-acetylated and 25% glycol split (previously designated ^{100}NA,RO-H, now known as SST0001, sigma-tau Research

Switzerland S.A., Mendrisio, CH) and is endowed with properties making it suitable for use as a cancer therapeutic. Notably, N-acetylated glycol split heparins are potent inhibitors of heparanase enzymatic activity that exhibit a markedly decreased ability to release FGF-2 from the extracellular matrix and potentiate its mitogenic activity as compared to unmodified heparin. Moreover, glycol-splitting causes heparin to lose its affinity for anti-thrombin with a resulting loss of anticoagulant activity. Collectively, the combination of high inhibition of heparanase, the low release/potentiation of ECM-bound growth factors and the lack of anticoagulant activity points to N-acetylated, glycol-split heparins as potential anti-angiogenic and anti-metastatic agents.[17] These rationally designed compounds have the potential to be more specific and safer than other heparanase inhibitors.

We now report that SST0001 can effectively inhibit myeloma growth *in vivo*, even when confronted with an aggressively growing tumor within human bone. Importantly, we find that treatment of animals or tumor cells with SST0001 causes changes within tumors consistent with the compound's ability to inhibit heparanase. This included inhibition of expression of HGF, VEGF and MMP-9 accompanied by diminished angiogenesis and decreased shedding of syndecan-1, a heparan sulfate proteoglycan previously shown to be a potent promoter of myeloma growth.[23] Comparison of the molecular size of syndecan-1 from tumor cells treated with or without SST0001 clearly indicates that SST0001 protects the proteoglycan from heparanase-mediated degradation thus confirming the anti-heparanase activity of the compound in cells. In addition to its anti-heparanase activity, SST0001 given in combination with

dexamethasone significantly inhibited myeloma tumor growth *in vivo* providing further rationale for incorporation of SST0001 into the clinic.

MATERIALS AND METHODS

Cell lines and reagents

RPMI-8226, U266 and MPC-11 (all obtained from ATCC); MM.1S and MM.1R (kindly provided by Drs. Nancy Krett and Steven Rosen, Northwestern University). The CAG myeloma cell line was established at the Myeloma Institute for Research and Therapy (Little Rock, AR) as described previously.[24] CAG cells [24] were transfected as previously described [6] with empty vector or vector containing the cDNA for human heparanase to generate heparanase low (HPSE-low) and heparanase high (HPSE-high) cells, respectively. During the course of this study the cell lines were confirmed as myeloma cells by their expression of CD138 and kappa immunoglobulin light chain. Cell lines were cultured in RPMI 1640 growth medium supplemented with 10% fetal bovine serum.

In vivo anti-tumor activity

Subcutaneous model: 6×10^6 MM.1S or 10×10^6 RPMI-8226 or $1x10^6$ MPC-11 or $1x10^6$ CAG cells were injected subcutaneously into the left flank of mice. Ten days after the injection of tumor cells, mice were treated with SST0001 for 28 days at doses of 30 mg/kg/day delivered via Alzet osmotic pumps (Durect Corporation, Cupertino, CA) or 120 mg/kg/day (CAG tumors, delivered by distant subcutaneous injection). After 28 days

of treatment, animals were euthanized, and the wet weights of tumors recorded. Murine sera were collected before, 2 weeks and 4 weeks after treatment with SST0001 was begun. Experiments were carried out using five-to-six week-old male CB.17 scid/scid or Balb/c mice obtained from Harlan-Sprague Dawley (Indianapolis, IN) and were housed and monitored in the animal facility at UAB. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committees of UAB.

Alternatively (studies presented in figure 1b), exponentially growing KMS-11 myeloma cells ($1.0x10^7$/mouse) were injected subcutaneously in the right flank of SCID mice. For RPMI-8226 model, the tumor line was maintained by serial subcutaneous passage of fragments (3x3x3 mm) from growing tumors into healthy mice. Each control or drug-treated group included 7-8 mice. Treatment started at day 1, when tumors were just palpable. SST0001 was dissolved in saline and delivered twice daily via subcutaneous injections of 60 mg/kg, for 22-30 days. Control mice were treated daily in parallel with saline. Growth of the subcutaneous tumor was followed by biweekly measurements of tumor diameters with a Vernier caliper. Tumor weight (TW) was calculated, considering tumor density equal to 1, according to the following formula: tumor weight (mg) = tumor volume (mm^3) = d^2 x D/2, where *d* and *D* are the shortest and the longest diameter, respectively. Experiments were carried out using female SCID mice, 8 to 11 weeks old (Charles River, Calco, Italy). Mice were maintained in laminar flow rooms with constant temperature and humidity. Experimental protocols were approved by the Ethic Committee for Animal Experimentation of the Istituto Nazionale Tumori (Milan, Italy).

SCID-hu model: The SCID-hu model was constructed as previously described.[25] Briefly, human fetal femora were cut into halves (approximately 5×5×10 mm) and implanted

subcutaneously into each SCID mouse. At 6 to 8 weeks after implantation of bone, 10^5 CAG-HPSE cells were injected directly into the marrow cavity of the bone implanted in the SCID-hu host. Twenty days after injection of tumor cells, Alzet osmotic pumps were implanted on the opposite flank of each mouse. Pumps contained either SST0001 (30 mg/kg/day) or saline as a control, and the solution was delivered continuously for 28 days. Murine sera were collected before, 2 weeks and 4 weeks after the treatments. Animals were also imaged before sacrifice on an IVIS-100 system (Xenogen Corporation). Human immunoglobulin κ light chain levels were measured in murine sera to assess whole animal tumor burden. Sera collected during animal studies were stored at −80 °C and analyzed by ELISA (Bethyl Laboratories, Montgomery, TX) in duplicates following manufacturer's protocol.

Combination therapy: 5×10^6 MM.1R cells were injected subcutaneously into the left flank of mice. Ten days after the injection of tumor cells, mice were treated with either saline, SST0001 (60 mg/kg/d via subcutaneous injection), dexamethasone (1 mg/kg/d intraperitoneal) or a combination of SST0001 and dexamethasone for 14 days. After the treatment period, animals were euthanized, and the wet weights of tumors recorded.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections. Briefly, the sections were deparaffinized, dehydrated through a series of graded alcohol washes, followed by antigen retrieval in 10 mM sodium citrate buffer, pH 6.0. The endogenous peroxidase activity was quenched by incubating the sections in 3% H_2O_2 and blocking nonspecific antigen-binding sites with 5% bovine serum albumin in PBS.

The sections were incubated overnight at 4 °C with primary antibodies against human VEGF (Neomarkers), human HGF (R&D Systems), mouse CD34 (Hycult Biotechnology) and human MMP-9 (Chemicon). Primary antibody was omitted for negative control. Following incubation with primary antibody, the sections were washed and incubated in appropriate biotin-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA). Vector ABC solution (Vector Laboratories, Burlingame, CA) was added to enhance sensitivity of detection using diaminobenzidine (Vector) and counterstained with hematoxylin. Immunohistochemistry images were taken using a Nikon microscope with a SPOT camera. Microvessel density was measured using the National Institutes of Health ImageJ software.

Western Blotting

Immunoblot analysis of syndecan-1 (R&D Systems), p-ERK1/2, t-ERK1/2 (Cell Signaling Inc., Beverly, MA) was performed as described. Briefly, protein concentration was determined by BCA assay (Pierce) and equal amounts of protein were separated by electrophoresis on 4–20% Tris-glycine SDS-PAGE gels (Pierce) and transferred onto either nitrocellulose or Nytran+ membrane. After blocking for 1h with TBS containing 0.1% Tween 20 and 5% nonfat dry milk, the blots were exposed to primary antibodies overnight at 4°C, followed by corresponding secondary biotinylated antibodies (Santa Cruz) for 1h at room temperature. The proteins were visualized using enhanced chemiluminescence (Amersham Biosciences).

Quantification of VEGF

CAG HPSE-low and HPSE-high cells treated with either PBS or SST0001 (125 μg/ml) were plated at equal density in serum free RPMI 1640 medium. After 48h, conditioned media were collected and the level of VEGF quantified using ELISA (VEGF; Biosource) following manufacturer's protocol.

Heparanase digestion and shedding of syndecan-1

Recombinant human heparanase was incubated with partially purified syndecan-1 in the presence or absence of SST0001 for 24hrs at 37ºC in heparanase activity buffer containing 50 mM NaCl, 1 mM DTT, 1 mM $CaCl_2$, and 10 mM phosphate citrate buffer, pH 6.0. The effect of SST0001 on inhibition of syndecan-1 heparan sulfate digestion by heparanase was then assessed by immunoblot analysis. To determine the effects of SST0001 treatment on syndecan-1 shedding, medium conditioned for 24 hours by CAG HPSE-low and HPSE-high cells treated with either PBS or SST0001 was collected, and the levels of shed syndecan-1 present in the conditioned medium was determined by ELISA as described.[6]

Gelatin Zymography

To determine levels of active MMP-9, conditioned media from CAG HPSE-low and HPSE-high cells treated with either PBS or SST0001 was subjected to gelatin zymography as previously described.[5] Briefly, equal numbers of cells were plated in serum free media for 48hrs. Media was collected, concentrated using Spin-X UF 30-kD cutoff concentrators (Corning) and equal protein was mixed with non-reducing sample buffer and analyzed by SDS-PAGE using 10% polyacrylamide gels co-polymerized with gelatin following manufacturer's protocol (BioRad). Sites of proteolytic activity were visualized as clear bands against the blue background of Coomassie stained gelatin.

Statistical analysis

Experiments were repeated a minimum of 3 times. Comparisons were analyzed by Student *t* test or one-way ANOVA using GraphPad Prism. *P* values less than 0.05 were considered statistically significant. For combination therapy studies, the effect of drug combination was determined using the GLM procedure. All data are mean plus or minus standard error of the mean (SEM).

RESULTS

SST0001 is a potent inhibitor of myeloma growth *in vivo*

In limited studies, we and others have reported that SST0001 inhibits the *in vivo* subcutaneous growth of CAG myeloma cells [23] and the experimental metastasis of B16-BL6 melanoma cells.[26] To further validate SST0001 as a potential myeloma therapeutic we examined the efficacy of SST0001 against growth of several myeloma cell lines using two different *in vivo* models (human MM.1S and RPMI-8226 cell lines in SCID mice and the murine MPC-11 cell line in syngeneic Balb/c mice). Ten days after injection of cells, mice bearing established subcutaneous tumors were treated for 28 days with SST0001 (30 mg/kg/day delivered by Alzet pump). This resulted in significant inhibition of myeloma tumor growth. Average weights of MM.1S, RPMI-8226 and MPC-11 tumors were reduced 50%, 56% and 61%, respectively, as compared to controls (Fig. 1A). In a separate line of experiments, KMS-11 cells or actively growing RPMI-8226 tumor fragments were passaged subcutaneously into tumor free animals. Treatment began on day one, when tumors were just palpable. In contrast to delivery by Alzet pump, in these experiments SST0001 was delivered by subcutaneous injection of 60 mg/kg of the compound, twice a day. Treatment of mice bearing KMS-11 (Fig. 1B) and RPMI 8226 (Fig. 1C) tumors resulted in nearly complete inhibition of tumor growth (98% and 99%, respectively) as compared to controls (Fig. 1B).

In order to more rigorously examine the therapeutic utility of SST0001 we employed the SCID-hu animal model. This model facilitates growth of myeloma tumors within the human bone marrow thus accurately mimicking the human disease.[25] CAG human myeloma cells expressing high levels of heparanase (HPSE-high cells) were injected directly into human bones engrafted in SCID mice. The tumors were allowed to establish and grow for 20 days, followed by treatment of the tumor-bearing mice for 28 days with saline or SST0001 (30 mg/kg/day delivered by Alzet pump). SST0001 significantly inhibited the growth of HPSE-high tumors within the bone as determined by bioluminescent imaging (Fig. 1D, right) and quantification of levels of human immunoglobulin κ light-chain present within the serum of tumor-bearing mice (Fig. 1D, left). We did not observe any adverse side effect in any of these in vivo animal models, even at doses as high as 120 mg/kg/day. Pathological evaluation of liver, lung spleen and kidney of SST0001 treated mice displayed no altered morphology as compared to mice treated with saline, indicating no adverse toxicities. These data demonstrate that SST0001 is a potent inhibitor of myeloma growth *in vivo* and the anti-tumor effect of SST0001 is not cell line specific.

SST0001 inhibits angiogenesis *in vivo*

Heparanase is known to enhance angiogenesis and we previously reported that high levels of heparanase correlate with an increase in bone marrow microvessel density (MVD) in myeloma patients and in animal models of myeloma.[4] Therefore, to determine if the heparanase inhibitor SST0001 was interfering with tumor angiogenesis, we measured the MVD in tumors derived from CAG and RPMI-8226 myeloma cells

using anti-CD34 immunohistochemistry, a surrogate marker of angiogenesis in myeloma.[27] Treatment of animals with SST0001 significantly reduced the numbers of CD34+ vessels in these tumors as compared to control animals treated with saline (Fig. 2A). We have recently discovered that in myeloma cells, heparanase enhances expression of HGF (Ramani, Yang and Sanderson, unpublished observation) and VEGF,[28] two factors important for myeloma growth and angiogenesis,[29,30] Therefore, we assessed whether treatment of tumor-bearing animals with SST0001 would inhibit expression of HGF and VEGF and contribute to inhibition of tumor growth and angiogenesis. Immunohistochemical analysis revealed a dramatic decrease in the intra-tumoral levels of both HGF and VEGF in HPSE-high tumors treated with SST0001 as compared to saline-treated controls (Fig. 2B). Moreover, treatment of HPSE-high cells *in vitro* with SST0001 resulted in a significant reduction in the level of VEGF secreted into the medium (Fig. 2C). These results point to a novel mechanism of action for SST0001 whereby it blocks heparanase activity leading to diminished HGF and VEGF production, two factors regulated by heparanase,[28] and inhibition of myeloma growth and angiogenesis.

Disruption of the heparanase/syndecan-1 axis in myeloma by SST0001

Therapeutic targeting of the heparanase/syndecan-1 axis, which drives an aggressive myeloma phenotype through regulation of multiple pathways, represents a unique opportunity to strategically inhibit myeloma growth.[31] SST0001 has been previously identified as an inhibitor of heparanase enzymatic activity in cell-free assays;[21] however, the ability of SST0001 to inhibit heparanase activity in a tumor cell

has not been demonstrated. To better establish the therapeutic potential and provide insight into the mechanism of action of this compound, we examined whether treatment of cells making high levels of heparanase with SST0001 would affect the size of the syndecan-1 heparan sulfate proteoglycan being expressed by those cells. Syndecan-1, the predominant heparan sulfate proteoglycan on myeloma cells,[32,33] runs as a broad smear when analyzed by western blotting due to the molecular heterogeneity in the size and number of heparan sulfate chains attached to the syndecan-1 core protein. When heparanase levels are elevated in the CAG cells (HPSE-high cells), syndecan-1 resolves as a lower molecular weight smear than in control cells due to clipping of the heparan sulfate chains by the heparanase enzyme.[6] Therefore, we hypothesized that treatment of HPSE-high cells with SST0001 would inhibit heparanase activity and the syndecan-1 size would not be reduced. To test this in a cell-free system, partially purified syndecan-1 was incubated with recombinant heparanase in the presence of SST0001 in a buffer that promotes heparanase activity. Western blotting for syndecan-1 revealed that SST0001 inhibited heparanase digestion of heparan sulfate as reflected by the high molecular weight smear as compared to syndecan-1 digested with heparanase in the absence of SST0001 (Fig. 3A). Next, we treated HPSE-high cells with SST0001 for 24 hrs and then assessed the molecular weight of syndecan-1 to determine if SST0001 was able to inhibit heparanase activity in those cells. Western blotting of cell extracts revealed that as the concentration of SST0001 was increased, the molecular size of the syndecan-1 smear increased thus indicating that SST001 effectively blocked the cellular heparanase enzyme activity (Fig. 3B).

Heparanase enzyme activity upregulates expression of MMP-9 which cleaves syndecan-1 causing its shedding from the cell surface;[5] this shed syndecan-1 promotes angiogenesis, growth and metastasis of myeloma tumors.[6,28,34,35] To determine if shed syndecan-1 was diminished by treating cells with SST0001, we quantified by ELISA the amount of shed syndecan-1 in conditioned medium from HPSE-high cells treated with saline or SST0001. Treatment with SST0001 significantly reduced the amount of shed syndecan-1 in the conditioned medium of HPSE-high cells as compared to saline-treated HPSE-high cells (Fig. 3C). Western blotting of shed syndecan-1 in conditioned medium further confirmed that SST0001 diminished shedding of syndecan-1 and inhibited heparanase digestion of heparan sulfate as indicated by the loss of the lower molecular weight portion of the syndecan-1 smear (Fig. 3D).

Because of the link between MMP-9 expression and syndecan-1 shedding, we assessed levels of MMP-9 expression in tumors formed by HPSE-high cells. Results demonstrate markedly reduced MMP-9 expression in SST0001 treated tumor as compared to control tumors (Fig. 4A). In addition, treatment of cells with SST0001 *in vitro* revealed that MMP-9 proteolytic activity is diminished (Fig. 4B). In myeloma cells, heparanase stimulates MMP-9 expression through activation of the ERK 1/2 MAPK pathway.[5] Treatment of HPSE-high cells with SST0001 *in vitro* resulted in a dose-dependent reduction in ERK phosphorylation (Fig. 4C). These findings further illustrate that SST0001 impacts the downstream targets of heparanase activity on tumor cells *in vitro* and *in vivo*.

SST0001 in combination with dexamethasone

potently inhibits myeloma growth in vivo

The emergence of novel myeloma therapeutics (*e.g.*, bortezomib, thalidomide) that functions through pathways different than conventional cytotoxic drugs, has greatly impacted myeloma therapy.[36] Recent clinical studies have demonstrated that these drugs can significantly improve therapeutic outcomes when combined with common myeloma drugs such as dexamethasone and other cytotoxic agents.[37] Therefore, we sought to determine if SST0001, which acts by inhibiting heparanase and appears to target the tumor microenvironment, would be effective when used in combination with dexamethasone. SST0001 and dexamethasone combination therapy was tested against subcutaneous myeloma tumor growth in SCID mice (using human MM.1R myeloma cells) and in Balb/c mice (using murine MPC-11 myeloma cells) thereby representing drug-resistant and immuno-competent models of myeloma, respectively. In both settings, the combination therapy significantly inhibited tumor growth more effectively than single agent therapy alone. In the drug-resistant MM.1R model, combination therapy inhibited tumor growth by 80% when compared to saline-treated controls, whereas, dexamethasone and SST0001 single agent therapy modestly inhibited tumor growth, 26% and 12% respectively (Fig. 5A). In the syngeneic model, combination therapy inhibited tumor growth 97% when compared to saline-treated controls, whereas, dexamethasone and SST0001 single agent therapy only resulted in 80% and 61% inhibition of tumor growth, respectively (Fig. 5B). In both cases, assessment of the combination of SST0001 and dexamethasone revealed an additive effect in inhibiting myeloma tumor growth;

warranting more in-depth experiments to fully assess the potential of SST0001 as part of a combination therapy regimen.

DISCUSSION

The present study demonstrates that the heparanase inhibitor SST0001 disrupts the myeloma tumor microenvironment resulting in diminished tumor growth. The compound was highly efficacious against human myeloma tumors growing in mice, including tumors that were established and growing within human bones. Pharmacodynamic studies demonstrated that SST0001 effectively targets heparanase and its downstream effects *in vivo* including inhibition of tumor angiogenesis, reduction in levels of HGF, VEGF and MMP-9 and diminished shedding of syndecan-1 (Fig 6). Together these effects of SST0001 dramatically blunt the normally aggressive phenotype of myeloma driven by the heparanase/syndecan-1 axis thus establishing the potential of this compound for myeloma therapy.

The results also indicate that SST0001 effectively blocked activity of the target enzyme. The anti-heparanase activity of SST0001 was originally determined using an assay that measures heparanase-mediated release of radiolabeled heparan sulfate from extracellular matrix.[21] However, this assay does not assess the ability of SST0001 to block heparanase activity in living cells. We assessed the anti-heparanase activity of SST0001 on myeloma cells by examining the size of the heparan sulfate proteoglycan syndecan-1 from cells grown in the presence or absence of SST0001 and found that in the presence of the inhibitor, the molecular size of syndecan-1 was higher than that in untreated cells. This result demonstrates that trimming of heparan sulfate by heparanase

produced by the cells was blocked by SST0001 thus indicating that heparanase activity was inhibited.

The downstream effects of heparanase including enhanced angiogenesis, growth factor levels and shed syndecan-1[4-6,28] were all diminished following treatment with SST0001, effects that were likely due to the enzyme inhibitory activity of the compound. Heparanase has been closely associated with increased tumor angiogenesis in a number of cancers.[38] We previously demonstrated that myeloma patients with high heparanase enzyme activity within their tumors exhibit enhanced angiogenesis as compared to patients with low levels of enzyme activity.[4] This study also demonstrated a relationship between heparanase and myeloma angiogenesis in animal models of myeloma. Thus, observation in the present work that SST0001 inhibits angiogenesis is entirely consistent with it being an inhibitor of heparanase. Moreover, the mechanism behind the reduction in angiogenesis in tumors from animals treated with SST0001 is likely driven, at least in part, by reduced levels of HGF, VEGF and MMP-9, all factors that are known to contribute to the angiogenic phenotype of myeloma.[28,30,39] Heparanase is known to up-regulate VEGF expression in HEK293, MDA-MB-435 breast carcinoma and rat C6 glioma cells as well as in myeloma cells.[5,40] HGF working in concert with syndecan-1 has also been shown as critical for myeloma growth and angiogenesis,[29,41] as circulating and bone marrow levels of HGF are significantly elevated in myeloma patients and the blood levels of HGF correlate with bone marrow MVD.[42]

MMP-9 can also contribute to angiogenesis by promoting the release of VEGF from tumor cells.[43] Although relatively little is known about the role of MMP-9 in myeloma, our lab discovered that MMP-9 expression is upregulated by heparanase and

that MMP-9 increases shedding of syndecan-1 from the surface of myeloma cells.[5] Our finding that treatment of animals with SST0001 diminishes intra-tumoral levels of MMP-9 and that this results in decreased cellular syndecan-1 shedding also points to SST0001 as a potent *in vivo* inhibitor of heparanase.

Disruption of the heparanase/syndecan-1 axis by SST0001 with reduction in syndecan-1 shedding likely has multiple effects on tumor progression. For example, high levels of shed syndecan-1 correlate with poor prognosis in myeloma patients and when shed syndecan-1 is increased in animal models of myeloma, tumor growth, metastasis and progression are dramatically enhanced.[35] Once shed into the microenvironment, syndecan-1 can complex with heparin-binding growth factors, such as FGF2,[44] HGF [45] and VEGF,[28] and serves to present these growth factors to their receptors, potentiating myeloma growth and angiogenesis. Alternatively, through its core protein, syndecan-1 can promote integrin activation and drive angiogenesis,[46] In myeloma, shed syndecan-1 stimulates angiogenesis, in part, by participating in activation of the αvβ3 integrin.[28] The critical role of syndecan-1 in supporting myeloma growth *in vivo* has recently been confirmed by knockdown of expression of either syndecan-1 or heparan sulfate.[47,48] Thus, the effect of SST0001 on inhibiting syndecan-1 shedding may contribute significantly to its anti-tumor effects.

Although use of SST0001 as a single agent effectively dampened tumor growth, the combination of dexamethasone and SST0001 was much more potent. Because we showed that this combination was effective against a dexamethasone-resistant cell line (MM.1R), it is possible that SST0001 may help overcome tumor resistance to dexamethasone and other chemotherapeutic agents. Additionally, inclusion of SST0001

to a therapeutic regimen might facilitate lowering the dose of chemotherapeutic drugs thereby lessening the side-effects of treatment.

The data presented here confirms that SST0001, a modified non-anticoagulant heparin, is an effective heparanase inhibitor *in vivo* against myeloma exhibiting no obvious side effects at the concentrations used in this study. Its strong effect as an anti-angiogenic agent and its ability to diminish shedding of syndecan-1 demonstrate that SST0001 has dramatic impact on the myeloma microenvironment resulting in diminished tumor progression. SST0001 is now completing its preclinical development with the objective to enter into clinical testing soon. These further investigations of SST0001 and similar compounds will determine their potential as therapeutic agents against myeloma and other heparanase-expressing cancers.

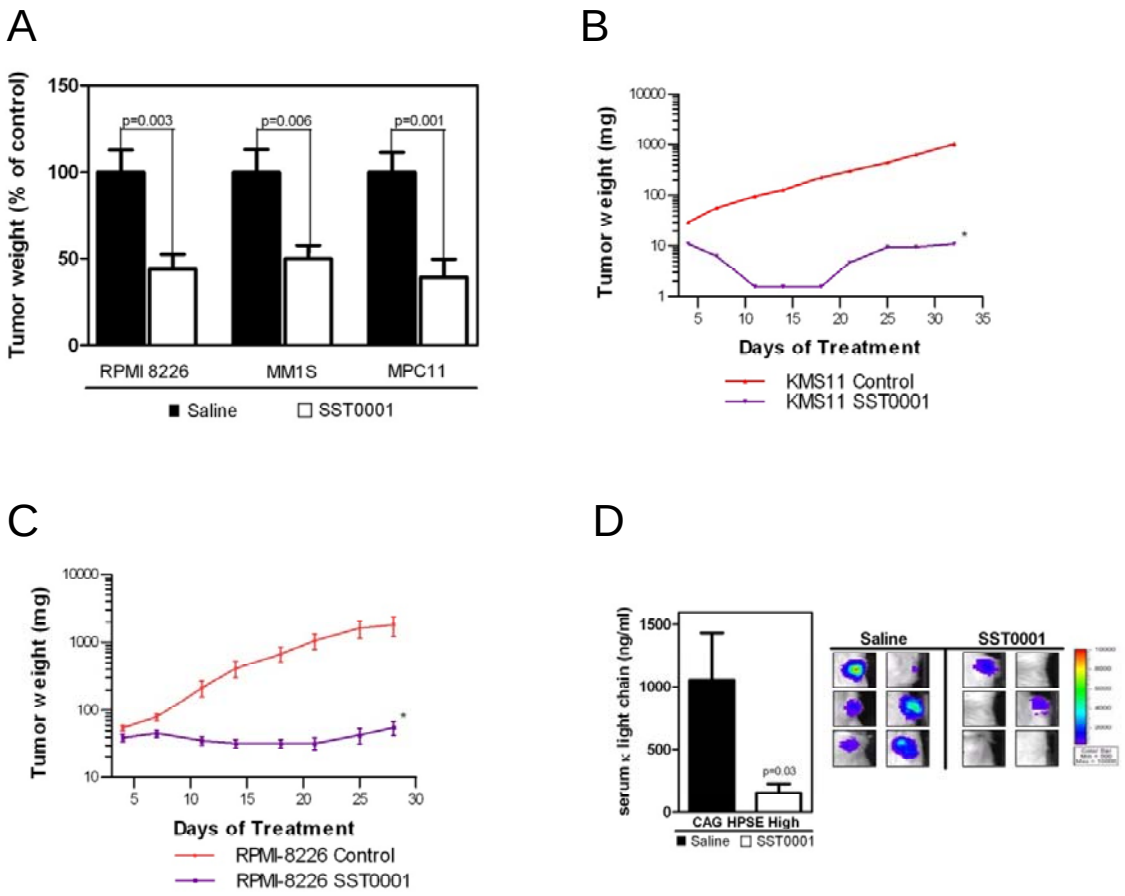

Figure 1. SST0001 is a potent inhibitor of myeloma growth *in vivo.* Treatment of mice bearing either subcutaneous or intra-osseous myeloma tumors were treated with saline or SST0001. **A.** SST0001 (30 mg/kg/d, 28 days), delivered by Alzet osmotic pumps inhibited subcutaneous tumor growth in the SCID (RPMI-8226 or MM.1S cells) and syngeneic (MPC-11 cells) models of myeloma. **B-C**. Growth of KMS-11 (cells injected subcutaneously) or RPMI-8226 (tumor fragments implanted subcutaneously) myeloma tumors was inhibited by twice daily subcutaneous injection of SST0001 (120 mg/kg/d, total daily dose) for 22-30 days. *$p<0.005$ vs controls, by Student's *t* test. **D**. Quantification of human kappa immunoglobulin light chain in murine sera (left panel) and bioluminescent imaging (right panel) were used to determine tumor burden in the SCID-hu model of myeloma; mice receiving SST0001 (30 mg/kg/day via Alzet pump) displayed significantly lower tumor burden than control mice as assessed by both measures.

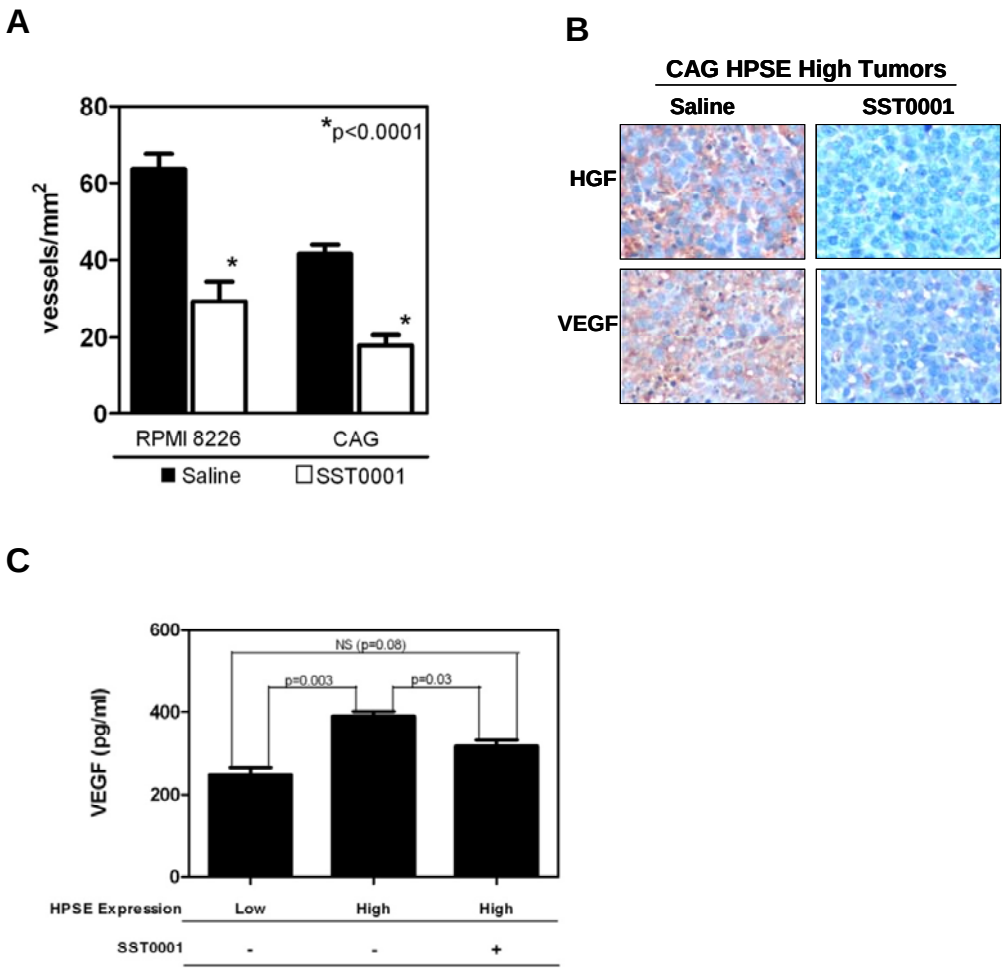

Figure 2. SST0001 inhibits angiogenesis and HGF and VEGF expression *in vivo*. A. SCID mice bearing subcutaneous myeloma tumors formed by RPMI-8226 or CAG cells were treated with SST0001 or saline for 28 days. After treatment, microvessel density was quantified in sections of the tumor tissue using anti-CD34 immunohistochemical analysis. SST0001 significantly inhibited tumor vascularity. **B.** Mice bearing subcutaneous tumors formed by HPSE-high cells were treated with either saline or SST0001 (30 mg/kg/d, delivered by Alzet osmotic pumps) for 28 days. Following euthanasia of animals, the tumors were removed and subjected to immunohistochemical analysis of HGF and VEGF. In tumors from animals treated with SST0001, the intra-tumoral levels of both HGF and VEGF were reduced dramatically as compared to animals treated with saline. **C.** Levels of VEGF in the conditioned medium from HPSE-low or HPSE-high CAG cells treated with saline or 125 µg/ml SST0001 (6.75µM) were measured by ELISA. SST0001 treatment significantly decreased the accumulation of VEGF in HPSE-high cells (n=3 for each group).

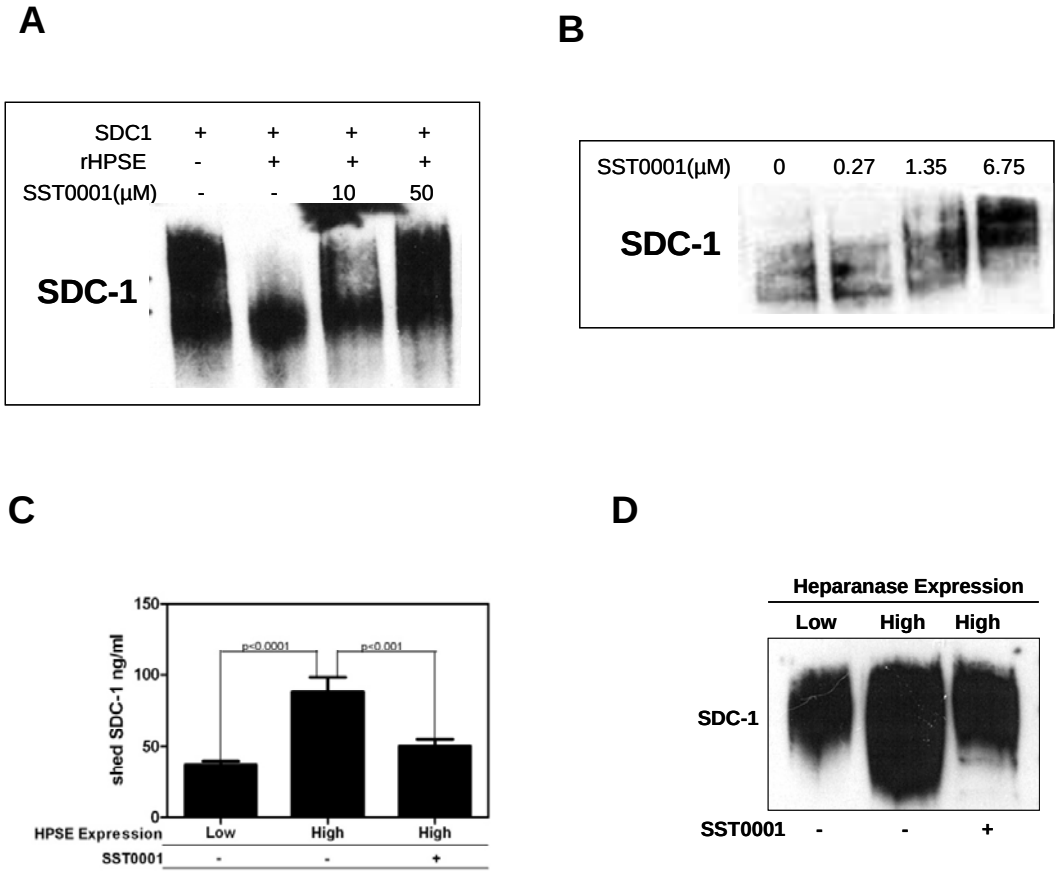

Figure 3. SST0001 inhibits heparanase activity in tumor cells expressing high levels of the enzyme. A. In a cell free system, SST0001 blocked heparanase-mediated digestion of the heparan sulfate chains of partially purified syndecan-1(SDC1). The proteoglycan was incubated with recombinant heparanase (rHPSE) in the presence or absence of SST0001 followed by western blotting. Note that in the presence of SST0001, the molecular size of syndecan-1 is larger than in the absence of the inhibitor. **B.** HPSE-high cells were treated overnight with increasing concentration of SST0001; the cells were extracted and analyzed by western blotting. SST0001 inhibited heparanase digestion of the heparan sulfate chains of syndecan-1, resulting in the high molecular weight form of syndecan-1. **C.** The amount of syndecan-1 shed into the conditioned medium of HPSE-low or HPSE-high cells treated with SST0001 (125 μg/mL; 6.75μM) or saline was quantified by ELISA. SST0001 significantly inhibited shedding of syndecan-1 from HPSE-high cells (n=3 for each group). **D.** Conditioned medium from HPSE-low cells or HPSE-high cells treated with SST0001 (6.75μM) or saline was subjected to western blot analysis of syndecan-1. Results confirm that levels of shed syndecan-1 are reduced following treatment of HPSE-high cells with SST0001. Note also that the molecular size of shed syndecan-1 in conditioned medium from HPSE-high cells treated with SST0001 is also larger than that found in untreated cells, again confirming the ability of the compound to block the activity of heparanase.

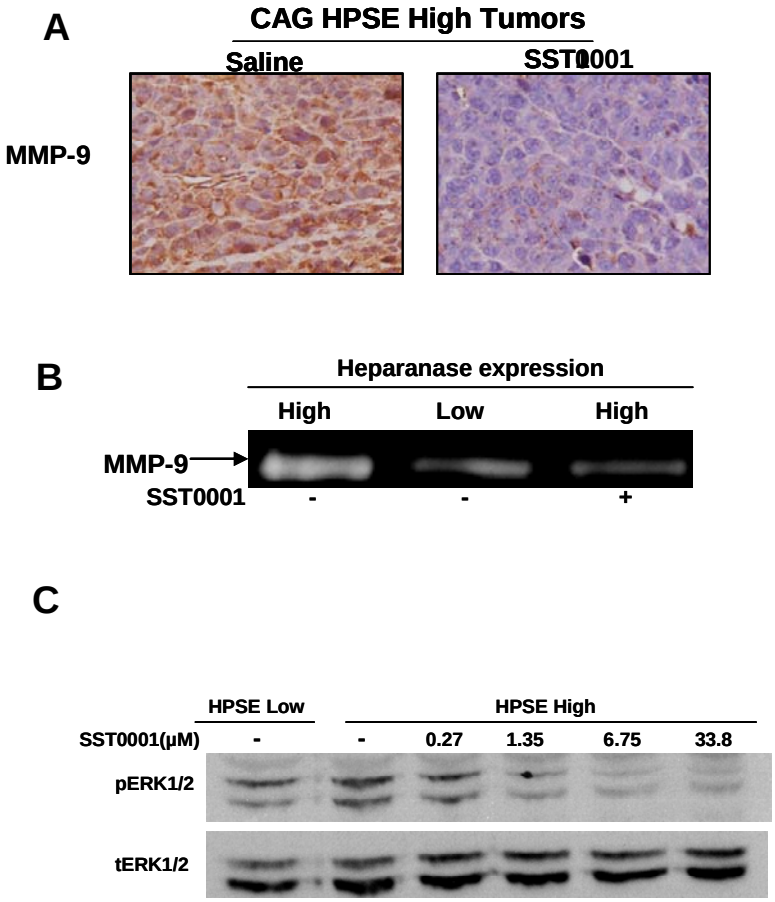

Figure 4. SST0001 blocks heparanase-mediated MMP-9 expression and ERK signaling. A. Mice bearing tumors formed by HPSE-high cells injected subcutaneously were treated with SST0001 or saline. Tumors were excised and immunohistochemical analysis for MMP-9 revealed diminished levels of expression within SST0001 treated tumors as compared to tumors from animals treated with saline. **B.** Conditioned media from HPSE-low cells or HPSE-high cells treated with SST0001 (125μg/ml; 6.75μM) or saline were subjected to gelatin zymography. HPSE-high cells treated with SST0001 had significantly reduced levels of MMP-9 activity in their medium as compared to cells treated with saline. **C.** ERK signaling was assessed by western blotting of extracts from HPSE-low and HPSE-high cells treated with increasing concentrations of SST0001 or saline by western blotting for phosphorylated ERK 1/2. Heparanase inhibition with SST0001 caused a dose-dependent reduction in ERK signaling.

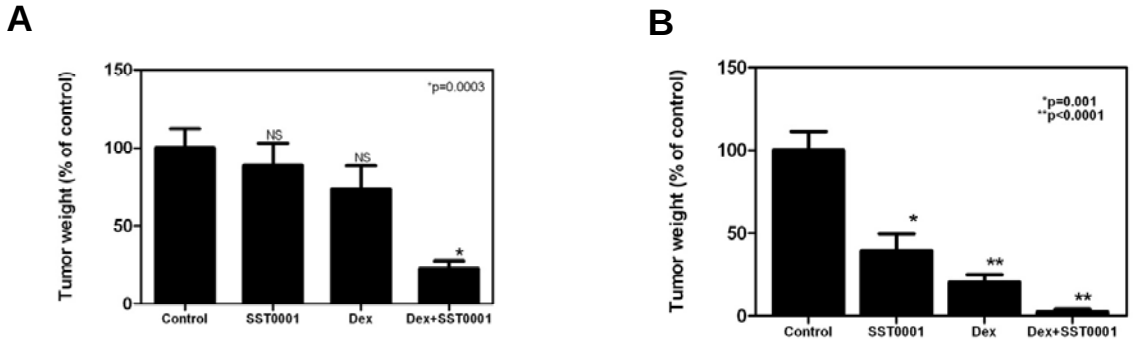

Figure 5. SST0001 in combination with dexamethasone is a potent inhibitor of myeloma tumor growth *in vivo*. A. Dexamethasone resistant MM.1R tumors were established subcutaneously in SCID mice and mice were injected with either saline, a low dose of SST0001 (60 mg/kg/day, as compared to 120 mg/kg/day used for studies in Fig. 1B), dexamethasone (1 mg/kg/day) or SST0001+dexamethasone for 14 days. Mice receiving combination therapy had significantly smaller tumors compared to control mice (n=8 for each group). **B.** Murine MPC-11 tumors were established in syngeneic BALB/c mice. Mice were then treated with either saline, SST0001 (60 mg/kg/day), dexamethasone (1 mg/kg/day) or SST0001+dexamethasone for 14 days. At the doses utilized, combination therapy potently inhibited tumor growth and was significantly more effective than single agent therapies when compared to saline treated controls (n=6-10 for each group). In both experiments, the effect of the drug combination was additive as determined using the GLM procedure.

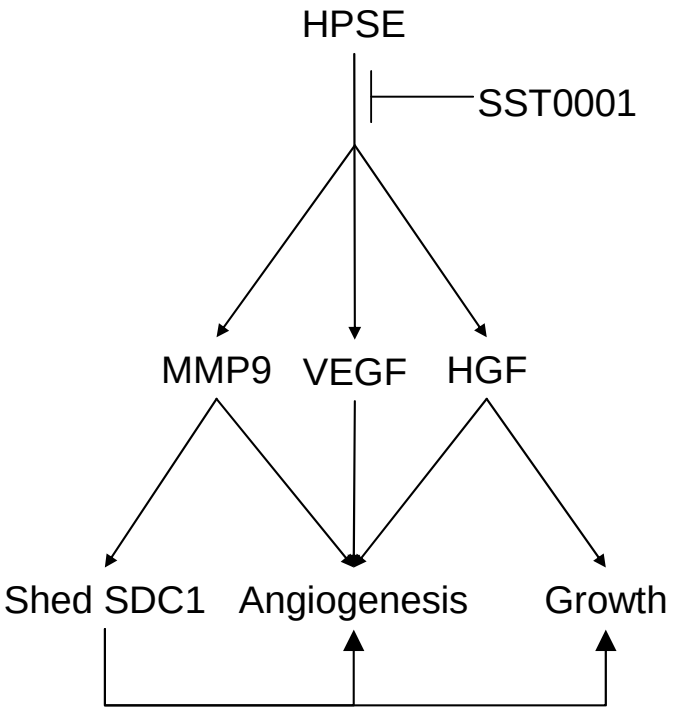

Figure 6. SST0001 inhibits heparanase and its downstream effectors to block myeloma growth and angiogenesis. Heparanase activity enhances expression of VEGF, HGF and MMP-9 and stimulates shedding of syndecan-1, thereby fueling an aggressive myeloma phenotype. Through inhibition of enzyme activity, SST0001 shuts down these multiple pathways that together stimulate myeloma tumor growth.

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CHAPTER 3

SUPPLEMENTAL DATA

SUPPLEMENTAL MATERIALS AND METHODS

Cell lines

U266 and RPMI-8226 cells were obtained from the American Type Culture Collection (Manassas, VA). The CAG myeloma cell line[190] was transfected with empty vector or vector containing the cDNA for human heparanase to generate heparanase low (HPSE-low) and heparanase high (HPSE-high) cells, respectively and have been previously reported.[142] The human osteosarcoma cell line, Saos-2 was provided generously by Dr. Majd Zayzafoon, University of Alabama at Birmingham. All the myeloma cell lines were cultured in RPMI- 1640 growth medium supplemented with 10% fetal bovine serum (FBS). Saos-2 was cultured in DMEM medium supplemented with 10% FBS.

In vivo experimental metastasis model

3 x 10^6 CAG HPSE-high or HPSE-low cells expressing firefly luciferase were injected intravenously via lateral tail vein. Tumor progression was monitored by weekly bioluminescent imaging on an IVIS-100 system (Xenogen Corporation). For the early treatment model, SST0001 or saline was administered during injection of HPSE-high and HPSE-low cells and treatment continued daily for 14 days at a dose of 60 mg/kg/d subcutaneously. For the delayed treatment model, injected myeloma cells were allowed to establish disseminated disease for one week followed by 14 days of treatment with SST0001 (60 mg/kg/d) or saline subcutaneously. Tumor burden was determined by quantification of human immunoglobulin kappa light chain in murine serum by ELISA (Bethyl Laboratories). For each group n = 9-10.

MTT Proliferation Assay

The effect of SST0001 on proliferation of myeloma cells *in vitro* was determined using CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega). CAG HPSE-high, CAG HPSE-low, U266 and RPMI-8226 myeloma cells were plated at a density of 1 x 10^4 cells in 100 µl of complete growth media per well in a 96 well plate in the presence of increasing concentrations of SST0001. Viable cell number for each cell line was determined at time 0, 24, 48 and 72 hrs. Cell number is directly related to the conversion of the tetrazolium salt into a formazan product as detected by absorbance at 570nm. The effect of SST0001 was determined by comparing the absorbance at each time point to non-treated controls.

Immunohistochemistry analysis

Formalin-fixed, paraffin-embedded tissue of saline or SST0001 treated tumors formed from CAG HPSE-high cells was used for immunohistochemical staining. Briefly, sections were deparaffinized and hydrated through a series of xylene and graded-alcohol washes, followed by antigen retrieval in 10 mM sodium citrate buffer, pH 6.0. Endogenous peroxidase activity was quenched by incubating the sections in 3% H_2O_2 and blocking non-specific antigen binding sites with 5% bovine serum albumin (BSA) in PBS. Sections were incubated overnight at 4^{o}C with primary antibody against human ki-67 (Santa Cruz Biotechnology), or cleaved caspase 3 (Cell Signaling). After washing with PBS, sections were incubated in appropriate biotin-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA). Antibody complexes were visualized using 3, 3' diaminobenzidine substrate (Vector Laboratories). All slides were counterstained with

Gill's formulation #2 hematoxylin. Photographic images were taken using a Nikon microscope equipped with a SPOT camera.

HGF ELISA

CAG HPSE-low and HPSE-high cells treated with either saline or SST0001 (125 μg/ml) were plated at equal density in serum free RPMI 1640 medium. After 24 hours, conditioned media were collected and the level of HGF quantified using ELISA (R&D Systems) following manufacturer's protocol.

Cell Based HGF Activity Assay

Cells from the osteosarcoma cell line Saos-2 were seeded in 24-well plates at 5×10^4 cells per well in complete growth medium. After overnight incubation, the medium was removed and the monolayer was washed once with sterile PBS before treatments. For determining the activity of HGF in the presence of SST0001, 1ml of serum-free Saos-2 media containing 10 ng recombinant HGF +/- SST0001 (125 μg/ml) was added to Saos-2 monolayer and incubated for 24 hours. At the end of incubation, conditioned media was collected, centrifuged to remove cell debris and the levels of IL-11 was determined using Human IL-11 DuoSet ELISA (R&D systems) as per manufacturer's instructions. The experiment was repeated three times and assayed in duplicate.

SUPPLEMENTAL RESULTS

The work previously described in chapter 2 demonstrates that SST0001 blocks heparanase activity leading to disruption of MMP-9 dependent syndecan-1 shedding, decreased bioavailability of the angiogenic growth factors, HGF and VEGF, and inhibition of angiogenesis resulting in diminished myeloma growth. The following set of experiments were designed to (i) further examine the therapeutic potential of SST0001 in a model of disseminated disease, (ii) demonstrate that SST0001 acts in a "cytostatic" rather than cytotoxic manner, and (iii) understand how SST0001 regulates HGF bioavailability.

SST0001 inhibits heparanase mediated metastasis of myeloma *in vivo*

The impact of heparanase in driving metastasis is a widely accepted phenomenon.[95] Previously, heparanase has been found to promote the spontaneous metastasis of myeloma cells *in vivo*; [116] however, the classification of myeloma as a metastatic disease in the clinical setting remains a controversial topic. Despite the controversy, the experimental metastasis model is a widely utilized animal model of myeloma because it recreates the systemic and multifocal nature of the disease. Previously, SST0001 has been shown to inhibit metastasis of B16-BL6 mouse melanoma cells in a heparanase dependent manner.[189] To examine if heparanase inhibition could prevent myeloma metastatic disease, CAG HPSE-high and HPSE-low cells were injected into the lateral tail vein of SCID mice. Two treatment modalities were utilized, early and delayed. The early treatment arm was designed to determine if SST0001 could block the

colonization and growth of myeloma at secondary sites, whereas, the delayed treatment arm was designed to determine the effectiveness of SST0001 in treating established, disseminated disease. For each cell line, early and delayed treatment groups were compared to saline-treated controls. SST0001 treatment was initiated at the same time or one week following injection of myeloma cells for the early and delayed treatment groups, respectively, and continued for 14 days.

In the early model, SST0001 was able to significantly inhibit growth of both CAG HPSE-high (Fig. 1A, $p=0.003$) and HPSE-low cells (Fig. 1B, $p=0.002$) when compared to saline treated controls as determined by bioluminescent imaging and quantification of serum immunoglobulin light chain. Interestingly, SST0001 appeared to be more effective at inhibiting growth of HPSE-high cells than HPSE-low cells, 7-fold versus 2.5-fold reduction in overall tumor burden. In the delayed treatment model, SST0001 was less effective at blocking myeloma growth; tumor burden, though lowered, was not significantly different from non-treated controls for both cell lines. The limited ability of SST0001 in the delayed model could be due to the high number and aggressive nature of the cells used for this study. Overall, these results indicate that SST0001 may better benefit those patients with high levels of heparanase and who have been diagnosed early in the disease setting.

SST0001 does not significantly affect myeloma proliferation or apoptosis

In order to increase the odds of success for translating SST0001 into the clinic a thorough understanding of how SST0001 inhibits tumor growth is necessary. Chapter 2 highlights that SST0001 functions via inhibition of heparanase-mediated angiogenesis leading to suppressed myeloma growth *in vivo*. In addition, heparanase does not affect proliferation of myeloma cells *in vitro,*[116] rather it drives tumor growth *in vivo* by modulation of the myeloma microenvironment.[143,144] Based on these findings it is proposed that SST0001 functions as a cytostatic rather than a cytotoxic agent. To confirm this hypothesis, the ability of SST0001 to affect growth and proliferation of myeloma was assessed *in vitro* and *in vivo*. Proliferation of myeloma cells *in vitro* was not affected by the addition of SST0001 (Fig. 2A). *In vivo*, treatment of mice bearing subcutaneous tumors formed by CAG HPSE-high myeloma cells with SST0001 resulted in no significant changes in proliferation (ki67) or apoptosis (cleaved caspase-3) as determined by IHC analysis; however, a trend was noticed which suggests that SST0001 may inhibit proliferation and stimulate apoptosis of myeloma cells *in vivo* (Fig. 2B). These findings warrant further investigation into the role of SST0001 in regulating proliferation and apoptosis in myeloma and suggest that the anti-angiogenic effects of SST0001 may indirectly promote apoptosis of myeloma cells by disrupting endothelial cell-myeloma cell interactions.

SST0001 inhibits bioavailability but not expression of heparanase-induced HGF

Heparanase regulation of growth factors expression can occur in an enzyme activity dependent or independent manner. Previously, all the actions of heparanase in

myeloma were found to require enzyme activity; however, it was recently discovered that heparanase can stimulate HGF expression in an enzyme activity independent manner (Ramani et al., submitted for publication). Myeloma cells expressing a mutated, enzymatically inactive form of heparanase express high levels of HGF, comparable to that of cells expressing active enzyme. Interestingly, mice bearing tumors formed by CAG HPSE-high myeloma cells displayed a reduced intra-tumoral level of HGF following treatment with SST0001 as detected by IHC analysis (Ch. 2), suggesting that SST0001 is able to regulate HGF in a heparanase-independent manner. Because heparanase regulation of HGF does not depend on enzyme activity, understanding how SST0001 alters HGF bioavailability is important in deciphering how SST0001 inhibits myeloma tumor growth.

As a derivative of heparin, SST0001 retains many of the same biochemical properties of its parent compound, including the ability to interact with molecules containing heparin binding domains. For example, heparin can facilitate the release of matrix bound FGF2 and participate in the dimerization and interaction of FGF2 with FGF receptor.[191] Chemically modified heparins that are glycol split have also been shown to bind FGF2 and VEGF with similar affinity to unmodified heparin.[192,193] On the other hand, it has been shown that SST0001 can block the mitogenic activity of FGF2, presumably through sequestration of FGF2 and blocking its interaction with FGF receptor.[188]

To determine how SST0001 regulates HGF, conditioned media from CAG HPSE-low and CAG HPSE-high cells treated with saline or SST0001 was assayed by ELISA to determine the effect of SST0001 on HGF bioavailability. Surprisingly, CAG HPSE-high

cells treated with SST0001 resulted in a marked increase in HGF detected in the conditioned media (Fig.3). This finding is opposite to the effect of SST0001 *in vivo* where treatment results in a dramatic decrease of HGF staining in tumor samples (Ch. 2, Fig. 2B). The *in vitro* observation could be explained by the ability of SST0001 to interact with HGF through its heparin-binding domain. Once bound, SST0001 could render HGF inactive leading to significant accumulation *in vitro*; whereas *in vivo*, binding of SST0001 to HGF could prevent its retention within the tumor microenvironment.

To test whether SST0001 could inhibit HGF, an established, cell based HGF activity assay was utilized.[194] In the presence of HGF, Saos-2 osteosarcoma cells up-regulate expression and secretion of IL-11. Therefore, the activity of HGF can be determined by measuring the amount of IL-11 in Saos-2 conditioned media. As expected, SST0001 significantly inhibited HGF-dependent IL-11 expression by Saos-2 cells (Fig. 4). While these results identify a potential heparanase-independent function of SST0001, it is important to note that while HGF expression can be driven by heparanase expression and not activity, the biological activity of HGF in myeloma is potentiated by shed syndecan-1.[195] Therefore, the ability of SST0001 to inhibit syndecan-1 shedding, and subsequently activity of HGF, highlights a dual mode of SST0001 regulation of HGF in myeloma.

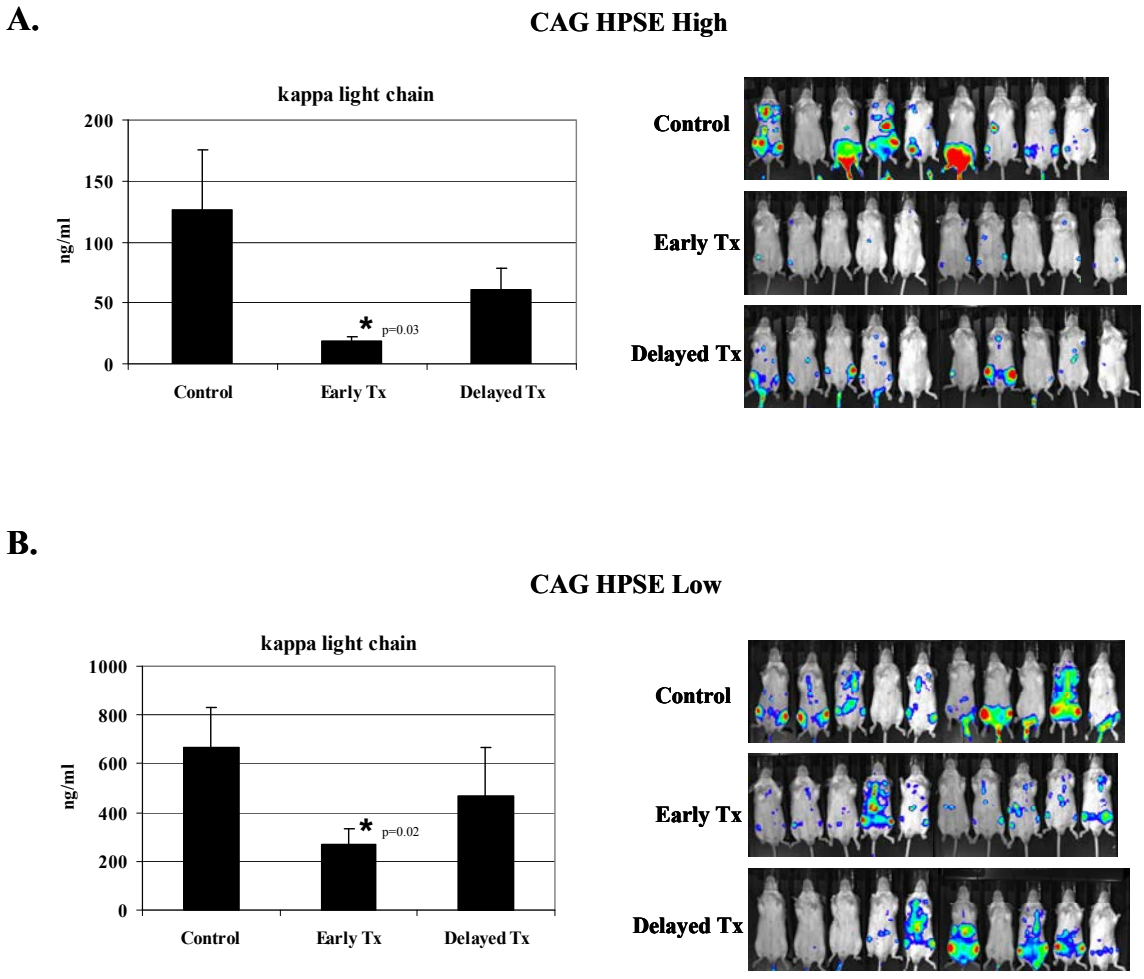
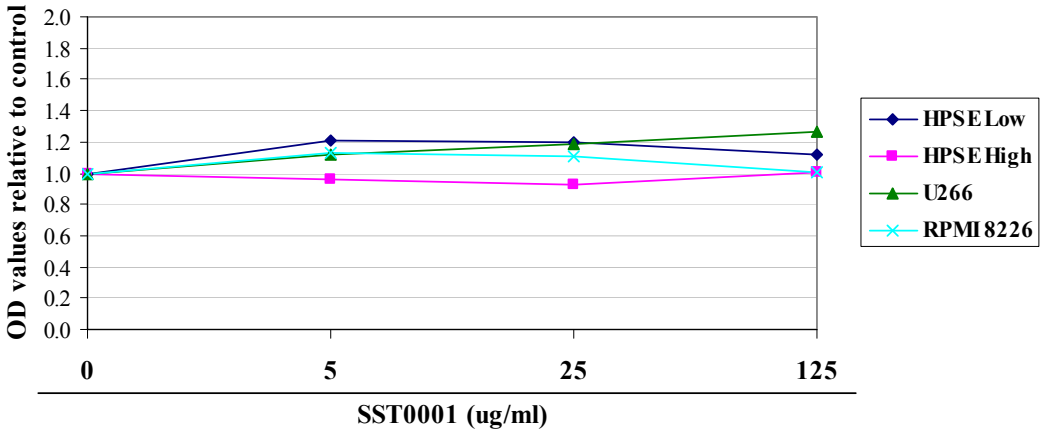


Figure 1. SST0001 inhibits heparanase mediated metastasis of myeloma *in vivo*. CAG HPSE-High and HPSE-low myeloma cells were injected via tail vein into SCID mice ($3x10^6$ cells/mouse). The ability of SST0001 in blocking dissemination of myeloma cells (early Tx) and established, disseminated disease (delayed Tx) was assessed. **A.** SST0001, administered at a daily dose of 60 mg/kg/d, significantly inhibited growth of CAG HPSE-High cells (p=0.03) when treatment was initiated at the time of injection (early). Delaying treatment by one week only resulted in a modest, but not significant, reduction in tumor burden. **B.** SST0001, administered at a daily dose of 60 mg/kg/d, significantly inhibited growth of CAG HPSE-Low cells (p=0.02) when treatment was initiated at the time of injection (early). Delayed treatment did not significantly impact tumor growth. Early treatment of SST0001 was more effective against HPSE-high expressing cells than HPSE-low expressing cells (7-fold versus 2.5-fold reduction in tumor burden).

A.



B.

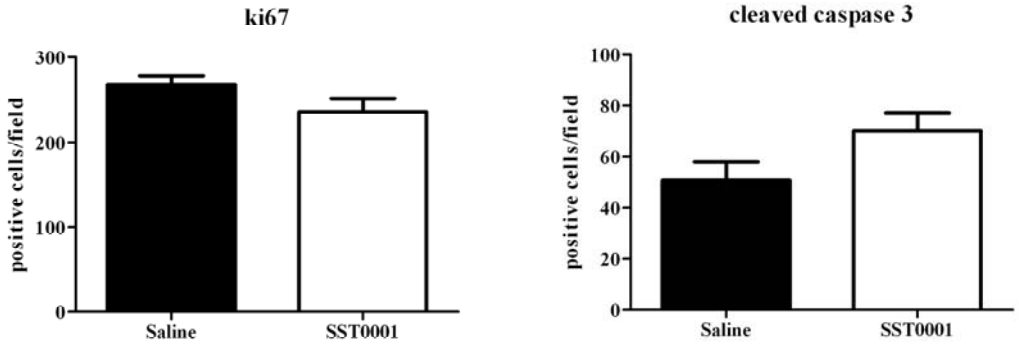


Figure 2. SST0001 does not significantly affect myeloma proliferation or apoptosis. **A.** The effect of SST0001 on myeloma proliferation was assessed *in vitro* by MTT assay. Equal numbers of CAG HPSE-high, CAG HPSE-low, U266 and RPMI 8226 myeloma cells were seeded in 96 well plates in complete growth media in the presence of increasing concentrations of SST0001 for 72 hrs. SST0001 did not affect proliferation of myeloma cells *in vitro*. Data is presented as the OD_{570nm} value blanked against time zero and relative to non-treated control for each cell line. **B.** Subcutaneous CAG HPSE-high tumors were established in SCID mice. Ten days following injection of myeloma cells, mice were treated for 10 days with either saline or SST0001 (30 mg/kg/d) by Alzet osmotic pumps. The effect of SST0001 on proliferation (ki67) and apoptosis (cleaved capsase-3) *in vivo* was measured by IHC analysis of paraffin embedded tumor sections (n=3 for each group). SST0001 did not significantly affect proliferation (p=0.1) or apoptosis (p=0.07).

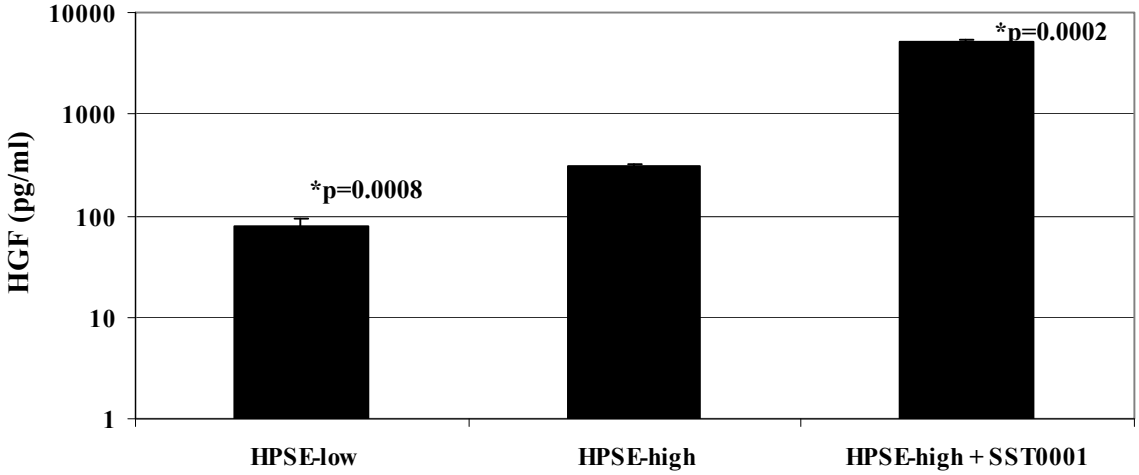


Figure 3. SST0001 causes HGF accumulation *in vitro*. CAG HPSE-low, CAG HPSE-high and CAG HPSE-high cells in the presence of 125 µg/ml of SST0001 were seeded in serum free media at equal density in a 12 well plate and incubated at 37ºC. 24 hrs later, conditioned media was collected and the amount of HGF was quantified by ELISA. CAG HPSE-high cells secreted significantly higher levels of HGF than CAG cells expressing low levels of heparanase (p=0.0008). In the presences of SST0001, the amount of HGF detected in the conditioned media of CAG HPSE-high cells was significantly increased when compared to non-treated CAG HPSE-high cells (p=0.0002).

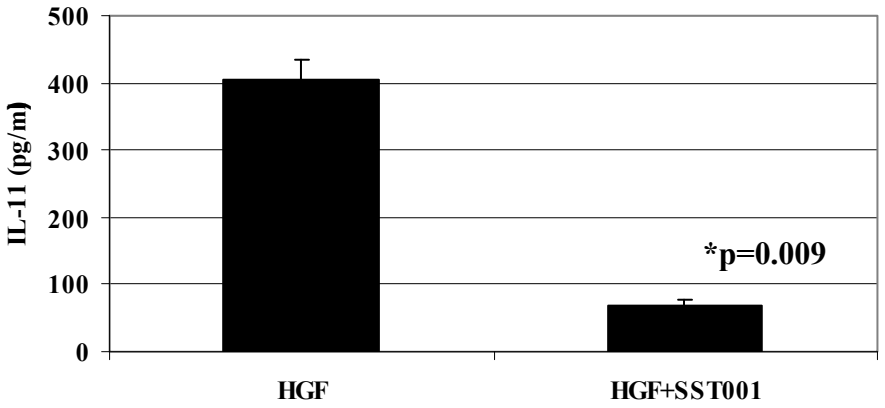


Figure 4. SST0001 inhibits HGF-stimulation of IL-11 production. Saos-2 osteosarcoma cells were seeded in 24-well plates at 5×10^4 cells per well in complete growth medium. After overnight incubation, the medium was removed and the monolayer was washed once with sterile PBS before treatments. For determining the activity of HGF in the presence of SST0001, 1ml of serum-free Saos-2 media containing 10 ng recombinant HGF or HGF + SST0001 (125 μg) was added to Saos-2 monolayer and incubated for 24 hours. At the end of incubation cell culture media was collected, centrifuged to remove cell debris and the level of IL-11 determined by ELISA. SST0001 significantly inhibited HGF stimulation of IL-11 production by Saos-2 cells (p=0.009) Experiment was repeated three times, assayed in duplicate and blanked against non-treated Saos-2 cells. Addition of SST0001 alone had no significant effect on IL-11 secretion.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Heparanase, the only known mammalian heparan sulfate degrading enzyme, is rarely expressed in normal tissues, but becomes evident in a variety of pathological situations including cancer where it is associated with enhanced angiogenesis and metastasis. Virtually all human malignancies that have been screened express high levels of heparanase, with multiple myeloma displaying the highest incidence of heparanase up-regulation. Of the myeloma patient samples that were screened, ~90% revealed increased heparanase expression and activity which was further correlated with enhanced angiogenesis and poor outcome.[118,122] Clinically, heparanase is associated with driving tumor growth, angiogenesis and the development of metastatic disease through enzyme-dependent and independent mechanisms; however, the majority of these effects in myeloma occur through enzymatic remodeling of extracellular matrix and cell surface heparan sulfate.[196] In myeloma, much of the growth promoting effects of heparanase can be attributed to regulation of the cell surface heparan sulfate proteoglycan syndecan-1, the dominant heparan sulfate proteoglycan expressed by malignant plasma cells. Heparanase-induced MMP-9 expression drives syndecan-1 shedding,[133] such that soluble syndecan-1, serving as a biological mediator downstream of heparanase, accumulates within the myeloma microenvironment to create a niche supporting aggressive myeloma growth.[142] Therefore, heparanase can be considered as a regulator of the aggressive myeloma phenotype and through shed syndecan-1, drives tumor growth, angiogenesis, metastasis and osteolysis. Based on the impact of heparanase in myeloma, therapies designed to specifically inhibit this enzyme will significantly impact disease progression. The overall goal of the work described earlier was to confirm the potential of anti-

heparanase therapy for multiple myeloma and provide mechanistic insight into the mode of action of SST0001, a novel heparin-based inhibitor of heparanase enzymatic activity.

For the first time, we show conclusive evidence that anti-heparanase therapy is a valid approach for treating multiple myeloma. Early conceptual studies employing myeloma cells with siRNA heparanase knockdown or myeloma cells expressing mutant heparanase lacking enzymatic activity revealed that active enzyme is required for robust tumor growth *in vivo*, but not expansion *in vitro*, as both cell lines thrive in culture but are severely limited in their ability to form tumors in animals (Sanderson Lab, unpublished data). These preliminary studies suggested that active heparanase was necessary for myeloma growth *in vivo*; therefore, therapies designed to inhibit the enzymatic activity of heparanase could significantly impact heparanase-driven myeloma growth. In fact, SST0001 was highly effective in blocking myeloma growth *in vivo*; including the subcutaneous and disseminated models, as well as, the SCID-hu model which provides for an aggressive human myeloma tumor growing within its natural bone microenvironment. These studies overwhelmingly confirm previous, proof-of-principle studies which demonstrated the potential of SST0001 in perturbing primary tumor formation and heparanase-dependent metastatic growth of myeloma and B16-BL6 melanoma tumors, respectively.[163,189]

It has been discovered that therapies designed to disrupt the interactions between myeloma cells and the tumor microenvironment can greatly impact outcome in patients with multiple myeloma.[197] The newly approved immunomodulatory drugs (IMiDs) have been found to function as anti-angiogenic agents, independent of their immunomodulatory function. In fact, thalidomide was the first angiogenesis inhibitor to

demonstrate clinical efficacy in myeloma.[198,199] Thalidomide disrupts angiogenesis by preventing endothelial cell migration via inhibiting stromal and myeloma cell-derived secretion of VEGF, FGF2 and TNFα. and PI3K/Akt signaling.[49,200-202] Moreover, lenalidomide, a second generation IMiD, was found to antagonize VEGF production in the bone marrow milieu and block microvessel formation through disruption of adherens junction formation and inhibition of the hypoxia-mediated angiogenic cascade.[203,204] The success of thalidomide and its analogues have paved the way for anti-angiogenic therapy for multiple myeloma. Recently, pre-clinical studies with sorafenib, a dual MAPK/VEGF receptor inhibitor currently approved for the treatment of hepatocellular and metastatic renal cell carcinoma, was shown to possess significant anti-myeloma activity by disrupting the interactions between myeloma cells and the bone microenvironment culminating in loss of the ability of myeloma cells to fuel angiogenesis.[205] The anti-angiogenic properties of this particular compound were found to indirectly decrease myeloma cell proliferation and induce apoptosis by disrupting the exchanges between the tumor and its microenvironment.

Studies undertaken to provide mechanistic insight into the anti-myeloma action of SST0001 revealed that this compound functions in an angiostatic manner by disrupting the tumor microenvironment, rather than a direct cytotoxic effect on the myeloma cell. In myeloma, heparanase drives angiogenesis through a multi-step process. In addition to the classical pathway of basement membrane remodeling to "clear the way" for invading endothelial cells, heparanase appears to flip the angiogenic switch in myeloma through several mechanisms, including up-regulation of growth factors (e.g., VEGF, HGF), activation of proteases and shedding of syndecan-1 (Ch.1, Fig. 3). Ultimately, the

presence of high intra-tumoral levels of angiogenic growth factors, MMP-9 and soluble syndecan-1, all driven by heparanase, fuels angiogenesis, tumor growth and disseminated disease in multiple myeloma. By inhibiting heparanase with SST0001, these downstream angiogenic events which depend on heparanase activity are also suppressed; thus, SST0001 functions as an indirect anti-angiogenic agent by inhibiting tumor-induced pro-angiogenic stimuli.

Despite the promise of anti-angiogenic therapy in multiple myeloma, recent publications suggest that angiogenesis inhibitors may actually accelerate malignant progression and tumor metastasis.[206,207] Yamashiro *et al* reported that sustained VEGF blockade resulted in accumulation of VEGF in the tumor microenvironment and persistent VEGF receptor-2 activation.[208] Initially, VEGF trap caused vascular collapse and regression of hepatoblastoma xenograft tumors, but under sustained therapy, tumors recovered. Interestingly, the authors noticed a surprising increase in tumor-derived perlecan, a heparan sulfate proteoglycan, and up-regulation of endothelial derived heparanase. They postulated that perlecan, through its heparan sulfate chains, functioned to store the VEGF within the tumor microenvironment and endothelial heparanase, by degrading the heparan sulfate of perlecan, mediated the release of VEGF resulting in persistent VEGF receptor-2 activation and endothelial cell survival.[208] In a mouse model of pancreatic neuroendocrine cancer (RIP1-Tag2 mice), anti-VEGF therapy resulted in significant reduction of primary tumor growth; although smaller, treated tumors displayed invasive characteristics and in some instances, mice were found to have a higher incidence of liver micro-metastases.[209] Similar to theses effects, specific tumor cell deletion of VEGF gene in the RIP1-Tag2 mouse model and VEGF blockade in a

mouse model of glioblastoma resulted in suppressed tumor growth and angiogenesis, but remaining tumors possessed the similar invasive phenotype as seen in the sunitinib treated RIP1-Tag2 model.[209] Furthermore, short-term VEGF blockade inhibited orthotopic growth of human breast cancer (MDA-MB-231), melanoma (MeWo) and murine B16-BL6 melanoma cells, but accelerated their dissemination and reduced survival in experimental metastasis models.[210] An interesting observation that can be made from these studies is that direct anti-angiogenic therapy (i.e. those that exert their effects on the endothelium independent of tumor or stromal derived angiogenic stimuli[211]) while effective at reducing primary tumor growth, induces tumor hypoxia and malignant progression, an event possibly mediated by heparanase.[208]

Hypoxia-tolerant tumor cell clones might become refractory to anti-angiogenic therapy by circumventing the classical VEGF-angiogenesis pathway and subsequently escape the hypoxic microenvironment by inducing expression of alternative pro-angiogenic and metastatic genes that facilitate their adaptation, evasion and ultimate survival.[212,213] For example, Pennacchietti *et al.* discovered that hypoxia stimulates tumor cell invasion and metastasis in a manner that is mediated by HGF,[214] a growth factor that is also regulated by the heparanase/syndecan-1 axis in myeloma. In the presence of angiostatic agents, regions of intra-tumoral hypoxia are generated due to regression of tumor vasculature. To adapt, HIF1α, the biological mediator of the cellular response to hypoxia, complexes with HIF1β and other co-activators where it can then bind DNA at hypoxia response elements and stimulate transcription of genes involved in the hypoxia-induced evasive mechanisms.[215-218] In addition to being involved in driving the angiogenic and metastatic phenotype, heparanase may also be involved in adaptation

and metastasis in response to angiogenesis inhibitors, as evidence by up-regulation of endothelial heparanase following sustained VEGF trap.[208] Furthermore, hypoxia has been shown to modulate heparanase by inducing its expression, activity and enhancing heparanase-dependent tumor cell invasion.[219-222] These findings have clinical relevance, as heparanase has been correlated with HIF1α in both pancreatic and gallbladder adenocarcinomas, where they are associated with poor prognosis and distant metastasis.[223,224]

Recently, I discovered that expression of heparanase may promote adaptation to hypoxia. Myeloma cells with elevated heparanase expression showed a significant increase in nuclear accumulation of HIF1α in response to hypoxia, a process dependent on heparanase enzymatic activity in multiple myeloma. Moreover, we have discovered that heparanase regulates histone acetyl transferase (HAT) activity and these enzymes (e.g. CBP/p300, p/CAF) are important co-activators of HIF1α mediated gene transcription.[225,226] Based on these findings, it is plausible that heparanase and hypoxia may synergize to drive metastasis and tumor progression in response to direct angiogenesis inhibitors. Therefore, inclusion of anti-heparanase therapy, such as SST0001 (an indirect angiogenesis inhibitor), may be an important tool in preventing the metastatic dissemination of tumor cells following treatment with direct angiogenesis inhibitors. This is further supported by the ability of SST0001 to not only inhibit primary tumor growth, but limit the formation and progression of myeloma in a model of experimental metastasis (Ch.3, Fig. 1)

While SST0001 is able to inhibit heparanase enzymatic activity and thus myeloma tumor growth, several questions remain to be answered that will ultimately

enable successful development and translation of a heparanase inhibitor into the clinic. Currently, the active component of SST0001 is unknown and may only represent a small fraction of the total oligosaccharide composition of this compound. Building on the pre-clinical success of SST0001, our goal now is to maximize the potential of oligosaccharide inhibitors of heparanase by developing second generation compounds that possess more favorable pharmacokinetic and pharmacodynamic properties. This is important because the serum half-life of SST0001 is only 30 minutes.[189] Therefore, identifying the optimal saccharide composition and structural requirements necessary for enzyme inhibition will enable development of more specific heparanase inhibitors that will ultimately possess properties making them more potent that SST0001. To accomplish this, structure/function studies of heparanase and molecular modeling of the interactions between heparanase and the heparan sulfate substrate (i.e. the structural features and sulfation patterns of heparan sulfate that facilitate recognition by the enzyme) will smooth the progress of rational design of second generation carbohydrate-based heparanase inhibitors and in parallel, neutralizing antibodies targeted against the specific amino acid residues of heparanase involved in the interactions with heparan sulfate.

The recent success of microenvironment targeting agents in multiple myeloma and the ability of SST0001 to significantly reduce tumor burden, with minimal, if any, toxicities as a single agent *in vivo* has paved the way for incorporation of this compound into the clinic. Due to its unique mode of action, SST0001 would likely have multiple uses in the treatment of multiple myeloma. A lack of any observable toxicity suggests that this compound may be of importance in the setting of induction therapy, where the ultimate goal is tumor shrinkage with preservation of the healthy hematopoietic stem cell

compartment prior to autologous stem cell transplant. In addition, SST0001 displayed combination effect with dexamethasone in reducing tumor burden and overcoming drug resistance *in vivo*. A common feature with myeloma is the high rate of relapse and incidence of developing drug resistance; therefore, SST0001 may be an important tool in the setting of salvage therapy and could potentially have an impact in managing end-stage disease while preserving quality of life as much as possible.

In summary, my work has confirmed anti-heparanase therapy as an effective treatment modality for multiple myeloma, provided necessary evidence of target engagement and insight into the mechanism of action of SST001. These data validate SST0001 as an inhibitor of heparanase *in vivo* leading to disruption of the heparanase/syndecan-1 axis; a critical mediator of the aggressive myeloma phenotype. Through disruption of the activity of heparanase, SST0001 is able to abrogate myeloma progression via an indirect angiostatic mechanism of action without evidence of promoting malignancy and metastatic progression; an important finding that differentiates SST0001 from direct-acting angiogenesis inhibitors which have been associated with potentially exacerbating the metastatic phenotype. As the role of heparanase in driving other diseases becomes evident, the impact of a successfully translated heparanase inhibitor will have significant clinical impact, far beyond multiple myeloma and cancer, in improving survival and quality of life for those patients afflicted with diseases mediated by this enzyme.

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APPENDIX

IACUC APPROVAL FORMS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: April 15, 2010

TO: Sanderson, Ralph
SHEL 814 2182
6-6226

FROM: Judith A. Kapp
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Heparanase Regulation of Myeloma Metastasis: Mechanism and Therapy
Sponsor: NIH
Animal Project Number: 100408732

On April 15, 2010, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	C	168

Animal use is scheduled for review one year from April 2010. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 100408732 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
CH19 Suite 403
933 19th Street South
205.934.7692
FAX 205.934.1188

Mailing Address:
CH19 Suite 403
1530 3RD AVE S
BIRMINGHAM AL 35294-0019

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: June 11, 2010

TO: Sanderson, Ralph
SHEL 814 2182
6-6226

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Heparanase Regulation of Tumor-Host Interactions in Myeloma and Breast Cancer
Sponsor: NIH
Animal Project Number: 100608479

On June 11, 2010, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	B	85
Mice	A	50

Animal use is scheduled for review one year from June 2010. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 100608479 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

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