

## The Serpentine Solution

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### Editorial

*Subtle war*

*Circling round*

*Infinite core*

*Sublime door*

*Resolution*

*Eternally bound*

*Extreme creation*

*Absolute Solution*

*One on one*

*Without a sound*

*The game begun*

*The battle won*

*Fatal bite*

*Serpent bound*

*Life and light*

*So bright*

*Alexandra Lucas 2017*

Serpins are serine proteinase inhibitors that regulate central pathways throughout the human body. Many serpin-regulated pathways are essential processes in normal physiological functions. The serpins are a class of ancient proteins, preserved in time from

dinosaur to man and virus to horseshoe crab, representing up to 2-10% of circulating proteins in man. And yet the role of serpins is often overlooked; overlooked as remarkable control mechanisms for crucial pathways in clotting and immune systems, overlooked as biomarkers of diseases such as unstable plaque or even cancers, and overlooked as potential therapeutics in disease. A serpin, as for the 'Seven Percent Solution' in the Sherlock Holmes mysteries, is both an unexpected mystery and a potential source for new therapeutics. With this editorial we will, in brief, review what is known about the molecular mechanism of serpin-mediated protease inhibition, the natural physiological functions of serpins, the role of serpins in genetic disorders, termed serpinopathies, and finally the potential for serpins as therapeutics.

Serpin inhibition is a form of suicide inhibition. In the thrombotic and thrombolytic protease cascades, serpin-mediated regulation ensures a balancing of opposing regulatory steps thus blocking excess protease activation in either direction, as for either excess clot or excess bleeding, herein termed the serpentine solution [1]. This serpentine solution represents an evolutionary adaptation that has proven highly effective. Serpins have proven so effective that many species have developed or borrowed serpin functions or even sequences and adapted these to their own uses. Thus organisms from viruses to bacteria, plants and dinosaurs to horseshoe crabs, and finally mammals, have evolved pathways dependent upon serpin regulation [2]. In fact, serpin-like molecules in horseshoe crabs have been developed as a diagnostic tool for identifying clotting responses in the blood [3].

Serpins have wondrously complex machinery, a truly fascinating mechanism of action. The molecular mechanism of serpin inhibition was discovered through elegant analyses of the crystal structures of serpins and serpin protease suicide complexes performed by Huber et

the Max-Planck-Institut für Biochemie (Martinsried, Germany) who delineated the first serpin crystal structure, as well as Huntington and Carroll at Cambridge University (Cambridge, UK), the statesmen of serpinology in Cambridge, among others [4-7]. Serpins inhibit proteases via a suicide inhibitory mechanism, acting as a bait for target serine proteases, and in some cases also possessing *cross-class* inhibition of other proteases, such as the cysteine proteases [8,9]. Once the reactive center loop (RCL) is cleaved, the cleaved arm springs a trap and drags the targeted protease to the opposite pole of the serpin leaving the peptide arm embedded in the A beta sheet, effectively biting onto the protease enzyme (Figure 1). The RCL is often depicted as a serpentine structure curved or bending up above the serpin protein and exposed as a protease target. The protease and serpin once cleaved remain bound and inactive, forming a fixed inactive complex, a suicide complex [2,4-6]. Thus the cleaved serpin RCL acts as a trap, or perhaps similar to a serpent coiled to strike, where the serpent kills its protease prey swiftly and effectively (Figure 1-the RCL is depicted as a serpent). The serpin can, however, also be cleaved and rendered inactive, as when large snakes swallow predators such as crocodiles.

This mechanism of suicide inhibition implies the use of available serpins in quantities that match the target proteases, which in pathologies may lead to the exhaustion of their supply and therefore a need to increase their production to meet the increased demand and to return to homeostasis. This means that for every protease inhibited by a serpin, there is a necessity for an equivalent number of serpins bound to the inactive complexes. It also becomes evident that in genetic mutations in serpins, there can be a dysfunction of the serpin RCL whereby the RCL of one serpin can insert into the A beta sheet of an adjacent serpin molecule, leading to inactive serpin aggregates [7]. The serpin aggregations form the basis for several known and severe congenital disorders such as alpha 1 anti-trypsin (AAT, SERPIN A1) deficiency that causes emphysema, a severe lung disease. Recent work has also suggested that larger domain swaps can occur between serpins [10].

This natural serpin solution developed for the regulation of central and often extraordinarily important pathways and has proven to be a highly effective regulatory mechanism through millions of years of evolution. The regulatory serpins represent a unique solution to controlling these myriad protease pathways, the serpentine solution. Although a serpin suicide inhibition seems perhaps a bit inefficient, requiring one-to-one regulation and a flexible RCL, this mechanism has proven viable. Serpins are so highly effective that this molecular machinery has been adopted by viruses as well as other pathogens. In some poxvirus infections, serpins confer marked survival advantages, blocking host immune responses against viruses at extraordinarily low yatomolar concentrations [11-13].

Serpins regulate the activity of serine proteases in the clotting or thrombotic, and in the clot lysis, or thrombolytic, cascades, preventing excess clotting and end-organ ischemia and also preventing excess bleeding or hemorrhage, respectively [1,14,15]. Serpins also control complement and immune responses, hormone transport and even blood pressure, as well as many other processes such as apoptosis, and neurohormone carriers [16-23]. In disease states such as sepsis, where there is dysregulated bleeding and clotting which is termed disseminated intravascular coagulation (DIC), there is a loss of regulation of the thrombotic and the thrombolytic cascades, both due to loss of protease functions as well as loss of serpin functions [24]. There is also evidence for abnormal serpin expression and function in

cancers [25,26] and in severe inflammatory disorders [24-27]. Regulation of serpin control of many of enzymatic activities in diverse pathologies still needs to be properly addressed and studied. When there are genetic deficiencies of serpins these can cause a profound dysfunction with extensive consequences, termed a serpinopathy.

One of the best known of the serpinopathies is caused by mutations in Alpha 1 anti-trypsin (AAT, SERPINA1), which leads to early and severe emphysema, as well as liver dysfunction. There are reportedly up to 70 or more AAT mutations [28-31]. Other serpinopathies include genetic deficiencies of neuroserpin (NSP, SERPIN I1) and anti-thrombin (AT, SERPINC1) [7,32,33]. For NSP (SERPINI1) mutations this leads to epilepsy and sometimes dementia whereas AT or antiplasmin mutants have disorders in clotting [32-34]. Mutation in the complement inhibitor causes angioedema, a form of excessive immune reaction [33]. In some cases it is reported that the RCL, even in an uncleaved state, can insert into the beta sheet of an adjacent serpin molecule forming inactive protein aggregates [31-36]. These aggregates then can accumulate in the endoplasmic reticulum and cause both serpin deficiency and cellular dysfunction [37-41].

Serpins, as noted, have many central regulatory roles in nature. Serpins regulate the crucial thrombotic and thrombolytic cascades. With genetic deficiency of AT (SERPINC1) there is excess clotting. With malfunction or deficiency of AT (SERPINC1) or PAI-1 (SERPINE1) in sepsis and DIC there can be excess hemorrhaging or thrombosis, testaments to the key roles of serpin modulation in natural clotting pathways. With a separate serpin genetic deficit in neuroserpin (SERPINI1) there is epilepsy and dementia. With genetic mutations in anti-chymotrypsin (SERPINA2) Alzheimer's can develop, and protein aggregates have been identified in the plaques that form in the brain. With deficit in AAT (SERPINA1), as noted above, the connective tissue degrading protease neutrophil elastase is functionally in excess, leading to severe alveolar lung tissue breakdown and emphysema as well as damage and cirrhosis of the liver [29,30]. When C1 inhibitor (SERPING1) is genetically deficient, this serpinopathy causes excess immune responses and angioedema [34]. AAT deficiency is one of the most well understood serpinopathies. The ZZ and MZ AAT mutation alleles cause accelerated emphysema and COPD, which is markedly exacerbated by smoking. Treatment of AAT deficiency with protein augmentation therapy and even gene therapy is also one of the most advanced serpinopathies for treatment.

This internal and constant conflict, or balancing, of protease pathways in the blood stream prevents excess clotting and also excess bleeding, a serpin-mediated control of a very vital process. Serpins regulate the thrombotic, clot forming, and thrombolytic, clot lysing protease cascades [1]. These serpins in the circulating blood and adherent along the arterial wall and on platelet surfaces prevent excess clot formation and arterial thrombosis and also prevent excess bleeding after arterial injury. Thus anti-thrombin (SERPINC1) regulates thrombin and the plasminogen activator inhibitors 1, 2 and 3 (SERPINE1) inhibit tissue- and urokinase-type activators (tPA and uPA, respectively) and alpha 2 anti-plasmin (SERPINF2) inhibits plasmin. Plasminogen activator inhibitor-1 (PAI-1, SERPINE1) also regulates connective tissue breakdown via regulation or modulation of plasmin mediated activation of matrix metalloproteinases and prevention of excess elastase or chymotrypsin activation. Thus PAI-1 inhibits tissue and urokinase-type plasminogen activators, tPA and uPA respectively. PAI-1, in addition to preventing excess bleeding, also modifies uPA interaction with the uPA receptor on inflammatory

mononuclear cells, preventing excess inflammation and ongoing damage in some models. uPA and PAI-1 have also both been reported as markers for cancer and metastatic disease and PAI-1 as a marker for inflammatory disease progression [42]. Other serpins such as NSP inhibit tPA which may also have a role in cerebral function or inflammatory responses to tissue injury after cerebral ischemia (Strokes) [43]. Alpha 1-antichymotrypsin, as noted above, is found in amyloid plaques in cerebral specimens from Alzheimer's patients [44]. Other serpins modulate complement activation in immune responses and still other serpins that regulate apoptosis pathways are associated with cancer progression [45].

Serpins are reported to represent up to 2-10% of circulating plasma proteins. This prevalence of serpins in the blood stream illustrates the one-to-one interaction of the serine protease with its serpin inhibitor. Excess activation of the thrombotic or the thrombolytic cascades is seen in sepsis and disseminated coagulopathies, DIC. Serpins when present in adequate amounts prevent the excess clotting and or bleeding. Although the immune mechanisms underlying the origin of bacterial septicemia remain unknown, it seems logical that temporary exhaustion of the supply of serpins also can contribute to the progression of this syndrome when severe and out of control.

Serpins are gradually being recognized as a source for new treatments. Serpins have been targets of drugs designed to modify serpin function for many years, although often not recognized as such. Serpins are also now being developed as treatments for genetic or developmental abnormalities with various approaches such as 1) replacement or supplementation in genetic disorders where serpin functions are modified or replaced through protein or gene therapy, 2) treatment with purified viral and mammalian serpins as therapeutics in disease and 3) treatment with serpin-derived peptides. While considered as potential therapeutics, in many cases these serpin-based agents are in early stages of development.

As noted above, modification of serpin function has been used clinically for many years through heparin treatment. Heparin is a glycosaminoglycan, a polysaccharide that increases AT activity 100 fold. This is the basis for the anticlotting action of heparin when used for heart attacks or unstable coronary syndromes, arrhythmias such as atrial fibrillation when used as a bridge to cardioversion to prevent strokes, or when used to treat deep-vein thrombosis and also pulmonary emboli [12,46]. AT has also been used with some promise, albeit variable, for patients with severe sepsis and DIC [47]. Angiotensinogen has a serpin structure but does not function as a classical serpin. Angiotensinogen is metabolized to the potent native vascular vasomodulators. The angiotensin converting enzyme inhibitors block these vasomotor, hypertension pathways, again representing a drug designed to target serpins at least in part. AAT deficiency in a heterozygous or homozygous AAT deficiency and is now proven to be in part a root cause for advanced early emphysema and COPD in non-smokers, increasing the interest and demand for therapy. Treatment with AAT as either purified protein has proven highly effective and when expressed in viral vectors as gene therapy has great promise. Thus treatment with the AAT serpin protein is already an established clinical therapeutic agent for use in AAT deficiency in emphysema and potentially in other disease states, such as cirrhosis [38-41].

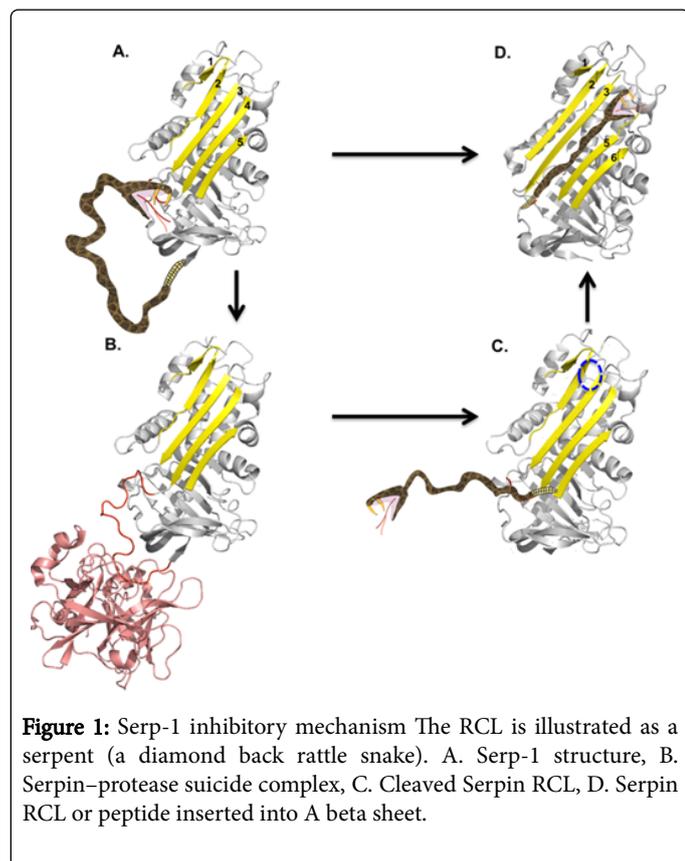
Viruses have evolved the use of serpins, perhaps through adapting changes in mammalian serpins or perhaps even donating their serpins to mammals. One such serpin, Serp-1, is derived from myxomavirus and when deleted from the virus genome turns a lethal viral infection

in European rabbits into a benign infection [11-13]. This demonstrates the marked potency of the Serp-1 gene expression as a protection for the virus against the host immune response. Serpins share approximately 30% homology throughout the clades. The Serp-1 protein inhibits both thrombolytic proteases such as tPA, uPA and plasmin, and also factor X and thrombin in the thrombotic cascade [13]. A related mammalian serpin, neuroserpin (NSP), targets only thrombolytic pathways, tPA and uPA. When compared in rodent models of angioplasty injury and transplant, both the viral serpin, Serp-1, and mammalian derived NSP can reduce local vascular inflammation and plaque growth. Both Serp-1 and NSP are equally effective at reducing vascular plaque and inflammation in a rodent aortic transplant models [48]. Conversely, in the viral sepsis model induced by MHV68 (Mouse herpes virus 68) infection in interferon gamma receptor (IFNGR<sup>-/-</sup>) deficient mice, Serp-1 improves survival whereas NSP is inactive [23]. In this model, as predicted, Serp-1 has a modest effect on bleeding time while NSP markedly reduces bleeding time, paralleling their differing protease target pathways. Serp-1 has also been tested and demonstrated benefit in renal transplant models [49] and in collagen induced arthritis models [50].

Recent systematic studies of the progression of inflammation following a massive white matter trauma in the spinal cord injury (SCI) revealed that severe, destructive and remarkably protracted disease in the rat model may act on the basis of a vicious cycle where damaged myelin acts as a potent immunogen, and macrophages are a main effector and destructor driving the inflammatory response. The white matter inflammation that lasts 12-16 weeks in the rat model of SCI, the most severe inflammatory reaction in the body, appears to get out of control of anti-inflammatory mechanisms that typically inhibit and eliminate the inflammation in any extra-neural tissue within 2-3 weeks, resulting in a quiescent scar [51]. Is the supply of serpins exhausted in the white matter injury and thus a powerful anti-inflammatory mechanism removed? Despite the excessive severity, the white matter inflammation becomes extinguished and infiltration by macrophages is eliminated 12-16 weeks after injury, which indicates a powerful anti-inflammatory mechanism arising in the CNS tissue. These findings raise further inquiry into whether increased levels of serpins participate in this beneficial, neuroprotective process and whether they are part of the attempt of the injured CNS to restore homeostasis. Certainly in the absence of NSP in mice, cerebral arterial occlusion leads to larger stroke sizes suggesting a protective mechanism for NSP in this model. A pilot, 7-day subdural infusion of Serp-1 in the vicinity of the SCI resulted in remarkable inhibition of inflammation seen histologically as inhibition of macrophage infiltration similar to that observed in subdural administration of high dose of dexamethasone, [51]. Administration of dexamethasone however, resulted in severe steroid toxicity in recipient rats while Serp-1 was well tolerated, emphasizing the need to study this protein as a neuroprotective therapy administered chronically, suitable for inhibition and elimination of the destructive inflammation in the white matter injury, not only in spinal cord and brain injuries but also in stroke.

Serp-1 has been tested in a small Phase IIa 48-patient, randomized, dose escalating blinded clinical trial in unstable coronary syndromes with coronary stent implant. Serp-1 was tested as three daily dose infusions in 7 sites in Canada and the US and demonstrated safety with a MACE of 0% at the maximum dose used and also efficacy in reducing early markers for myocardial damage, reducing CK-MB and TN levels [52]. In prior Phase I trials Serp-1 was found to be safe with no excess bleeding, clotting nor infection and neutralizing antibodies

were minimal. In the acute coronary syndromes with stent implant, Serp-1 treatment at the highest dose tested significantly reduced serum markers of Troponin I (TNI) and Creatinine kinase MB (CKMB) with no major adverse events reported. Serp-1 did not, however, demonstrate reduced plaque burden at follow up as measured by IVUS at follow-up angiography.



Other cross-class serpins, such as Serp-2 and CrmA, also derived from myxoma and cow-poxviruses respectively, inhibit the apoptotic serine protease, granzyme B and the cysteine proteases caspase-1 in the inflammasome pathways and the caspase-8 in the apoptotic pathways [8]. In rodent model studies Serp-2 but not CrmA demonstrated marked improvement in aortic transplant models and more recently in liver reperfusion injury (Chen et al. unpublished data).

It is generally reported that for true serpin mouse-trap or serpent-like functions, the near complete 80-90% conserved serpin structure is necessary as for heparan cofactor II [53]. However, in contrast, as for cleavage of angiotensinogen to active blood pressure modulating peptides or for the anti-viral functions of other serpins, there are other notable reports of active serpin peptides. Serpin peptides have been derived from AAT, PAI-1 and HCII with apparent beneficial function in endotoxemia and HIV infections [54-57]. In our ongoing work we recently developed peptides derived from the RCL of the serpins, Serp-1 and NSP. The peptides were developed predicted upon cleaved metabolites Serp-1 or NSP RCL sequences. The concept was that these metabolites may naturally extend serpin functions. Several RCL peptides were developed based upon predicted cleavage sites and did demonstrate reduced inflammation in mouse aortic transplants [58]. The Serp-1 RCL-derived S-7 peptide also significantly improved survival in the MHV68 infected IFN $\gamma$ <sup>-/-</sup> mouse viral sepsis and

vasculitis model (the S-7 solution). Modified S-7 peptides have been designed by Cloud Pharmaceuticals in a collaborative venture and are currently under investigation for efficacy.

Thus in summary, serpins represent a key regulatory mechanism in clotting immune and neurological pathways. While serpin modification has been used through heparin treatment and even treatment with ACEI for hypertension and heart failure for many years, actual serpin treatments for AAT deficiency and sepsis as well as serpin treatment for genetic serpinopathic disorders are under investigation. Undoubtedly, as for all new drugs, there will be unexpected benefits and also unexpected risks, as was true for the seven percent cocaine solutions once introduced as a new drug in the 1800s. However, virus-derived serpins and peptides represent a new class of therapeutics, the serpentine solution, which has shown great promise. While in early development, these new serpin-based therapeutics have the promise and extraordinary mystery of a Sherlock Holmes mystery. We would suggest that serpins, as a new class of therapeutic, now require rigorous and careful testing to fully assess their true potential benefits.

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