UNIVERSITY OF TROMSØ UIT

FAKULTY OF SCIENCE AND TECHNOLOGY
DEPARTMENT OF CHEMISTRY

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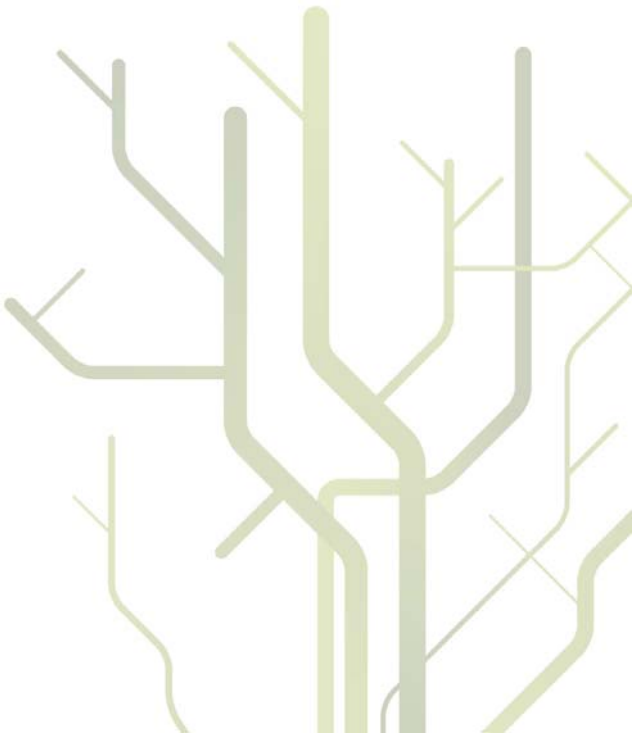
A drug discovery study

Annfrid Sivertsen

A dissertation for the degree of
Philosophiae Doctor

April 2013



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Acknowledgements

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Tromsø, April 2013
Annfrid Sivertsen

Summary

In this study we have explored the structure activity relationship (SAR) of molecules with potential as novel drug classes toward current medical challenges as hypertension, cancer and infections caused by antibiotic resistant bacteria.

Hypertension is an increasing worldwide health problem due to the life styles related with overall growth in the standard of living. Although hypertension is easier to prevent by awareness and active choices regarding risk factors than to cure for the majority of the world population, the number of people affected is still rising. The current hypertension drugs in clinical use are mainly angiotensin converter enzyme (ACE) antagonists and angiotensin receptor blockers (ARBs), which have disadvantages and are not applicable for all patients due to side effects. Direct renin inhibitors (DRIs) have for a long time been an interesting group of potential drugs, as they inhibit the first and rate-limiting step in the signal peptide cascade that regulates blood pressure. However, so far only Aliskiren has reached the market. Renin belongs to the aspartic proteases, a small but significant protease class that is as cumbersome to develop drugs toward, as they are promising as drug targets. It is believed that DRIs would be a superior group of hypertension drugs, as fewer side effects are anticipated and a better control of the regulation feedback system should be achieved. We have found that substituted *trans*-3,4-disubstituted piperidine derivatives containing 1,4- or 1,5-disubstituted 1,2,3-triazoles are potent non-peptidomimetic inhibitors for renin.

The increase in antibiotic resistant pathogenic bacteria is a global health threat, and development of new antimicrobial drugs is a very slow process. History shows that resistance toward new antibiotics is reported shortly after market launch, and documents the additional problem of new drugs not necessary being a solution to previous resistance issues. Unfortunately, to reduce the rate of increasing resistance the worldwide abuse of antibiotics has to come to an end, both as a livestock food supplement and in treatment of infections where

alternative treatment may be used. Antimicrobial and anticancer peptides are based on natural host defense peptides that are a part of the innate immune system of plants, insects and animals. The innate immune system is an ancient firstline of defense predating the adaptive immune system, and similarities have been found within the peptide classes and their regulatory systems in different life forms. The dual properties of antimicrobial activity and cytotoxicity toward cancer cells of modified natural peptides is connected to the mechanisms of peptide-cell interaction. The peptides do not target specific receptors but the cell membrane. The membrane composition of pathogenic bacteria and cancer cells is more negatively charged compared with normal cells and harmless bacteria.

Drugs have to maintain their activity in the complex biological system of a human body, and drug interactions with plasma proteins are known to affect the pharmacokinetics. The major plasma proteins affecting drug distribution are human serum albumin (HSA) and alpha-1 acid glycoprotein (AGP). The binding capacity of these proteins is high and they are complementary in their preference of ligands. We have shown that the peptides used in these studies interact with drug site II of HSA with their hydrophobic moieties, and that this binding affects the activity *in vitro*. In our study with AGP the binding was found to be of similar affinity compared with the HSA interaction, although the binding was hypothesized to be superior based on the ligand preference of the plasma proteins. The CAPs interacted with only a fraction of AGP, and this binding did not affect the peptides´ *in vitro* performance even at the high acute phase concentration of AGP. We suggest that HSA binding should be investigated routinely in antimicrobial and anticancer peptide development studies, as it will likely affect systemic distribution, whereas AGP binding is less likely to influence the activities *in vivo*.

List of papers

The thesis is based on the following papers and manuscripts (I-IV), referred to by their roman numbers in the text.

Paper I

Rianne A. G. Harmsen, Annfrid Sivertsen, Davide Michetti, Bjørn Olav Brandsdal, Leiv K. Sydnes, and Bengt Erik Haug.

Synthesis and docking of novel piperidine renin inhibitors.

Monatshefte für Chemie- Monthly Chemistry, 2013, 144 (4), 479-494.

Paper II

Annfrid Sivertsen, Johan Isaksson, Hanna-Kirsti S. Leiros, Johan Svenson, John Sigurd Svendsen, and Bjørn Olav Brandsdal (2013).

Synthetic antimicrobial peptides bind with their hydrophobic bulk elements to drug site II of human serum albumin.

Submitted to *BMC Structural Biology*

Paper III

Veronika Tørfoss*, Annfrid Sivertsen*, Johan Isaksson, Trude Anderssen, Bjørn Olav Brandsdal, Martina Havelkova, and Morten B. Strøm (2013).

Anticancer potency of small linear and cyclic tetrapeptides and pharmacokinetic investigations of peptide-binding to human serum albumin.

Manuscript in preparation. * The authors have a shared first authorship.

Paper IV

Annfrid Sivertsen, Bjørn Olav Brandsdal, John Sigurd Svendsen, Jeanette Hammer Andersen, and Johan Svenson (2013).

Short cationic antimicrobial peptides bind to human alpha-1 acid glycoprotein with no implications for the *in vitro* bioactivity.

Submitted to *Journal of Molecular Recognition.*

Abbreviations

ACE	angiotensin converter enzyme
ACP	anticancer peptide
AD	Alzheimer´s disease
ADME	absorption, distribution, metabolism, and excretion
AGP	alpha-1 acid glycoprotein, synonymous with orosomucoid
AMP	antimicrobial peptide
APP	amyloid precursor protein
ARA	aldesterone-receptor antagonists
ARB	angiotensin receptor blocker
AT_1	angiotensin II type 1 receptor
AT_2	angiotensin II type 2 receptor
BACE1	β-secretase 1, drug target for Alzheimer´s Disease
Bip	Biphenyl
CAP	synthetic cationic antimicrobial peptide
CL	cardiolipin
DRI	direct renin inhibitors
FA	fatty acid
FA#	fatty acid binding site number #, where # is a number between 1 and 11
FEP	free energy perturbation
GEM	group epitope mapping
GPCRs	G-protein-coupled receptors
HSA	human serum albumin
HTS	high throughput screening
INPHARMA	inter-ligand NOE for pharmacophore mapping
ITC	isothermal titration calorimetry
LF	lactoferrin
LF-B	lactoferrin from bovine
LF-H	lactoferrin from human
Lfcin	lactoferricin,
LfcinB	lactoferricin from bovine
LfcinH	lactoferricin from human
LIE	linear interaction energy
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MIC	minimal inhibitory concentration
ORM	orosomucoid, synonymous with alpha-1 acid glycoprotein
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PM	plasmepsin
PRA	plasma renin activity
PS	phosphatidylserine
Ramos	human Burkitt´s lymphoma
RAS	renin-angiotensin system
rHA	recombinant human albumin
SAMPs	synthetic antimicrobial peptidomimetics, synonymous with CAPs
SAPs	secreted aspartic proteases
SAR	structure-activity relationship
SM	sphingomyelin
STD	saturation transfer difference
Tbt	tri-tert-butyl tryptophan
VPI	vasopeptidase inhibitor
VS	virtual screening
WaterLOGSY	water ligand observation with gradient spectroscopy

Table of Contents

1. Introduction

1.1. The drug discovery process

Drug discovery is a highly interdisciplinary process, incorporating a broad range of fields of expertise, methodologies and approaches in the pioneering of novel drug development. In this thesis the term drug design will be limited to structure-based drug design with focus on receptor-ligand complexes. There are several definitions of what a "drug" is, but the definition adopted in this thesis is "*a single chemical substance marketed for use in human or veterinary medicine, either by prescription or over-the-counter*" [1]. This definition excludes recreation and abused compounds with biological activity, as well as dietary and nutritional substances. Drug discovery and development require large resources, and are time and cost consuming activities. The costs and risk of failure increases as a project approaches the end of the drug discovery and development pipeline, where *in vivo* clinical tests are performed on safety, dose and efficiency levels. The pipeline of drug discovery and development processes, from lead molecule identification to an on-the-market-drug, is illustrated in Figure 1 where the development process of the anti-hypertension drug Aliskiren currently in clinical use is shown.

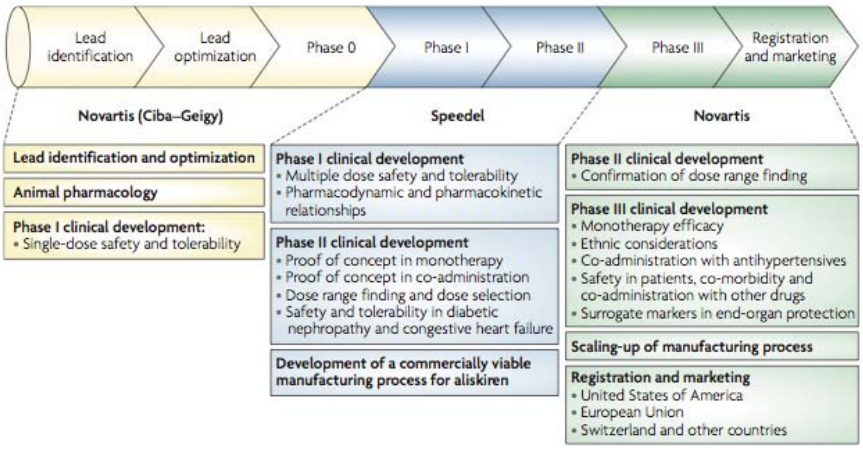

Figure 1. The drug discovery and development pipeline is divided into distinguishable project phases, from the initial lead identification and optimization to the clinical phase III, and registration and marketing processes. The figure illustrates the discovery and development of the anti-hypertension drug Aliskiren. Reprint from Jensen *et al.* 2008 [2] with permission from the Nature Publishing Group.

1.1.1 Strategies in drug discovery for identifying compounds with desired activities

The process from discovering promising compounds to the selection of a clinical candidate can be defined as drug discovery, and the further process from the clinical candidate to the market approved drug as drug development [3]. Identifying and optimizing promising compounds for the discovery process can be done in several ways, and often in a combination of methodologies and approaches. Compounds with desired biological activity for drug discovery may have their origin from sources in nature, either as the active ingredient in traditional medicine, or from bio-prospecting screenings of extracts from biological sources. Another approach in lead identification is based on synthesis of novel compounds. Here the compounds may be synthesized based on the *in silico* virtual screening hypothesis of molecular interactions or by experimental screening approaches. When it comes to experimental identification several paths could be employed: a library design of either a random, a directed design rationally obtaining structure-activity relationship (SAR) information, or fragment based set of compounds can be used. A second strategy is ligand-based design where a compound that is already known to have the desired activity is used as a starting point for SAR elucidation. The third approach takes into account information about the surface of the target target the compound is interacting with and is called structure-based drug design. A fourth method is where interaction is investigated by site-mutations in the target, with a reverse SAR development [1]. Other drug discovery processes, not generally considered in discussions of the topic, are drug repurposing, where new clinical use of already approved drugs is identified, and drug rescue where lead molecules that have failed in reaching clinical trials or in drug efficiency in clinical trials are re-launched in projects with new indications of activity [4].

1.1.2 Non-traditional drug discovery strategies; protein misfolding rescued by molecular chaperones

Drug discovery is not limited to the development of the more traditional agonist and antagonist paradigm, but also includes more recent and innovative approaches as pharmacologic chaperones which rescue protein function in loss-

of-function diseases [5, 6]. A number of disease states have their origin related to improper protein folding, aggregation and mistrafficking after peptide synthesis. This may be caused by point mutations, or other factors such as oxidative stress, protein over-expression, temperature, or activation of signaling pathways involved in folding and qualitative control [5, 6]. Examples of known diseases with loss-of-function proteins are cystic fibrosis, Parkinson, and prion diseases. The overall consequence of the alterations is the retention of proteins in the cellular quality system, which are subsequently unable to be translocated to the appropriate cellular compartments. This can happen even when the protein still retains the same biological activity as the wild type protein. Pharmacologic chaperones act through mechanisms that can stabilize specific conformations of misfolded proteins, modify endogenous molecular chaperones, or reduce aggregation and other unwanted interactions with other unfolded peptide chains [5]. However, not all mutations leading to improper folding events can be rescued by chaperone activity, as seen for G-protein-coupled receptors [7]. In some gain-of-function mutations, the undesirable effects of abnormal enzyme activity are avoided by reversed chaperonin, "ship-wrecking", where the molecular chaperone is destabilization and inducing misfolding, hence leading to removal by the normal cellular system.

Other approaches to drug discovery can be to target protein-protein interaction by developing small-molecule binding partners that interact with hot-spots on the protein-protein binding surface and intervene the original binding state of the complex [8].

1.1.3 Bioavailability of small molecules

The drug candidate oral bioavailability is also of importance in evaluation of lead potential. The famous Lipinski´s rule of 5 predicts the solubility and permeability of a compound based on simple characteristics of the molecule [9]. Dose/potency, solubility and permeability are the three major determinants for acceptable drug absorbance, and have the possibility to compensate for each other *e.g.* if the permeability is low a high solubility may compensate the effect

[9]. The *rule of 5* states that poor absorption or permeation is more likely to happen when there are more than 5 hydrogen bond donors, more than 10 hydrogen bond acceptors, the log P (the logarithm of octanol/water partition coefficient) is above 5, and when the molecular weight is above 500. The exceptions to these criteria are compounds that are substrates for biological transporter molecules, *e.g.* vitamins, antibiotics, antifungals and glycosides [9]. Another more general calculation for oral bioavailability which are independent of molecular size, correlate the reduction in molecular flexibility by bond rotation with polar surface area and the total number of hydrogen bonds [10]. The correlations stated that to ensure satisfactory bioavailability, the count of hydrogen-bond donors and acceptors in total should not exceed 12, the polar surface area should be less than 140 $Å^2$ and the number of rotatable bonds should not exceed 10 [10].

1.1.4 ADME and toxicity

Failure of a promising drug candidate due to pharmacokinetic difficulties and toxicity is not an uncommon event. It is preferable that compounds with poor acivity *in vivo* and undesirable effects are identified and eliminated from further development as early as possible in the research and development process. The ADME (absorption, distribution, metabolism and excretion) and toxicity properties of a drug candidate are therefore thoroughly assessed to minimize elimination in the later and more expensive parts of projects. These properties are often referred to without further explanation in drug discovery literature, but a short definition of each term based on Lin *et al.* 2003 [3] is given below.

Drug absorption is the fraction of un-altered drug that proceeds from the administration site to the systemic circulation. The primary sites of absorption are the gastrointestinal tract and skin. When oral administration is preferred, the drug has to pass through cell membranes, either by passive diffusion, carrier-mediated uptake or active transportation. Problems with poor bioavailability can be: low dissolution, low permeability and gut motility, and un-beneficial ionization states. During distribution, the drug is transferred from the systemic

circulation system to extravascular tissue. Mechanisms that influence drug distribution are: passive diffusion through lipid membranes, carrier mediated transport and protein binding in plasma and tissue. An example of the challenges in drug distribution is the blood-brain-barrier, which drugs with affective targets located in the brain need to pass to reach their bio-phases. Drugs are cleared either via metabolism or directly by excretion. In metabolism the drug is processed into metabolites, either by phase I or phase II category. In phase I, the compounds undergo hydrolysis, reduction and oxidation processes, which introduce polar groups into the molecule. Phase II category acts through conjugation mechanisms of polar moieties to the parent molecule, either acting on phase I metabolites or the drug molecule directly. The phase II metabolites are often more polar than the phase I metabolites. In excretion the drug compound has not been metabolized before clearance. The major clearance organs are the liver and the kidneys. Drug-induced toxicity has several components that need to be investigated: the intrinsic toxicity of the compound itself, the local concentration in particular organs (dose-dependency) and the host defense system´s capability to detoxify and handle any induced injuries. For practical reasons the focus is on minimizing the intrinsic toxicity as much as possible, as it is the easiest part to control. The toxicity can be categorized as genetic toxicity in carcinogens (both mutagenic and non-mutagenic), hepatic toxicity to the liver, or cardiotoxicity. Inhibition of drug clearance and metabolic enzymes can also cause severe toxic reactions. The ideal drug candidate should, in summary, not only have great pharmacological activity regarding potency and selectivity, but also obtain good ADME and toxicity properties [3].

1.1.5 Validation of drug targets and druggability

Drugs have to interact with and modify the activities of macromolecules classified as drug targets, which are associated with certain pathophysiological conditions, and obtain a clinical therapeutic effect. The drug to drug-target interaction should interfere with the signal transduction, receptor signaling or the biochemical equilibrium of the targeted macromolecule [11]. The dynamic of a biological system and induction of imbalance is often required for a drug to

have its desired effect. Examples of how drugs affect and take advantage of the dynamic biological systems are allosteric modulations, freezing of a flexible system in certain conformations, drugs that act upon proliferating cells, prodrug activation into and active substance, and modifications of substrates or co-factors [11].

The ideal drug target itself should have a range of combined properties to being considered worthwhile pursuing in the drug discovery pipeline [12]. It should be disease-modifying or proven to have a function in the pathophysiology of a disease, and its modulation should be of minor importance in other diseases or healthy physiological conditions. It should be druggable, or the structure should be available for investigation of its interaction with activity-modifying molecules. The target should also have established assays for measurement of activity which can be readily for used in screening methodologies. It is desirable that the target is restricted to certain bodily compartments due to decrease in off-target adverse effects due to possible different activities in various tissues.

Biomarkers should also be available for monitoring the therapeutic effect resulting from drug administration, and for prediction of potential adverse effects in phenotype models such as knock-down or knock-out animal models. Additionally it is preferable a developed drug for the target should not be in a high-competing situation with endogenous substances. To be druggable, the activity of the macromolecule, protein, peptide or nucleic acid has to be susceptible to modification by interaction with a small-molecule or biologic compound such as antibodies or a recombinant protein [12]. A thorough knowledge of the causative pathophysiological molecular mechanisms as well as target identification are required for drug target validation [12]. Despite the vast diversity and number of macromolecules in the human biological system, only a small selection are targeted by current drugs [11, 13]. Only 218 drug targets, mainly belonging to the hydrolase families (including proteases), the G-protein-coupled receptors (GPCRs), and voltage-gated Ca^{2+} channels of the ion channel family have been exploited [11]. These targets have a distribution of roughly 47% enzymes, 30% GPCRs, 7% ion channels, 4% transporters and 4% nuclear

hormone receptors as shown in Figure 2 [13]. The post-genomic era has made it possible to identify and validate potential drug targets for certain diseases, as validating of targets is possible in animal studies with over-expression or knock-out individuals [14]. Failure in clinical phase II and III trials are in most cases due to the lack of efficacy, which makes the validation of the drug target vital for success in the pharma industry [12]. Additionally, for a drug target to be assessed as worth the investment of developing drugs towards, there must also be a medical and commercial need for the putative novel drug and its activity [12].

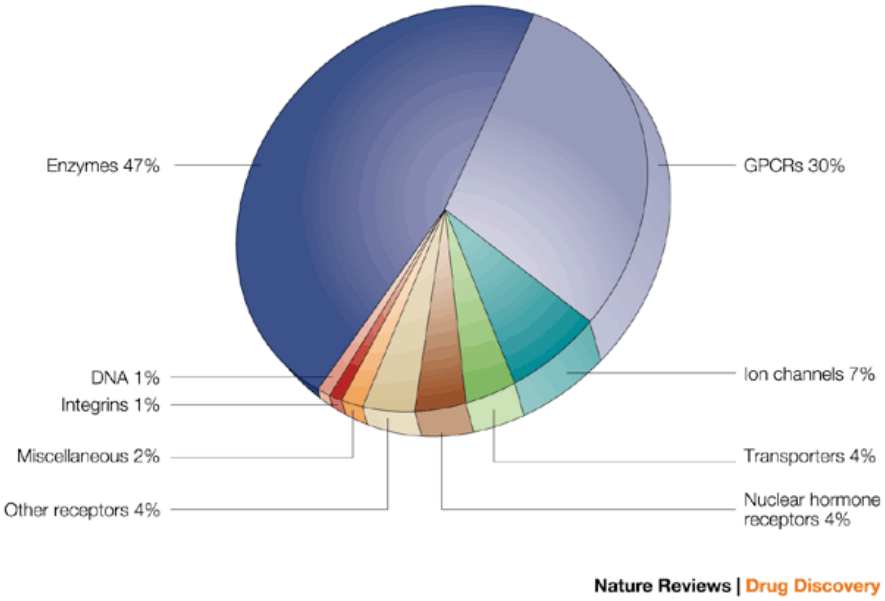

Figure 2. The classification of macromolecule-targeting by current drugs, illustrating that there are limited classes of families that are exploited and considered druggable. Reprint from Hopkins *et al.* 2002 [13] with permission from the Nature Publishing Group.

1.2 Proteases as drug targets

Proteases are proteolytic enzymes that catalyse the hydrolysis of the peptide bond in peptides and proteins [15]. The hydrolysis can either be at the N- or C-terminus of the peptide chain, catalyzed by proteins referred to as exo-peptidases, which are further divided into aminopeptidases or carboxypeptidases respectively. If the cleave of the peptide bond happens in the middle of the peptide chain, the catalyzing protein is an endo-peptidase. Proteolytic cleavage is involved in degradation, digestion and intracellular turnover of proteins, but is also part of signaling pathways. As signaling enzymes, proteases distinguish themselves from other systems with the irreversible nature of their cleavage activity [15]. Although under special conditions proteases have been observed to catalyze the formation of peptide bonds, this requires organic solvent and dehydration conditions that do not correspond to the aqueous environment in biological systems [16]. However, the reverse function of proteases can be exploited for peptide synthesis. The various signaling pathways proteases are involved in span a wide number of the major biological functions of living organisms, as they are a part of cell-cycle progression, cell proliferation and death, DNA replication, tissue remodeling, haemostasis, wound healing and immune responses [15]. Proteases and modified protease activities in disease states are targeted for multiple diseases. For example in cancer, proteases have been found to contribute to all stages of tumor progression [17]. Other diseases where changes in the proteolytic systems are causative are several neurodegenerative disorders, inflammatory, and cardiovascular diseases [18]. Hence, proteases have potential as drug targets, but can also be exploited for diagnostic and as prognosis biomarking purposes [18]. Examples of diseases where the disease-related protease is targeted by drugs are HIV (HIV-protease), hypertension (ACE (angiotensin converter enzyme)) and thrombosis (thrombin) [15].

Proteases are classified based on the key residue they use to catalyze the hydrolysis of the peptide bond. The serine, threonine and cystein proteases bind their substrates covalently, whereas the aspartic, metallo and glutamic proteases

acts through a general acid-base mechanism. These mechanisms are illustrated Figure 3 a) [15]. The human degradome consists of 569 proteases belonging to five protease classes. The most populated classes are the metallo (194), the serine (176), and the cysteine proteases (150), whereas only a minor number belong to the threonine (28) and aspartic (21) classes [18]. Glutamic proteases have so far not been found in mammals [18]. Further distinctions within each class are at family and clan levels, based on similarities in sequence and overall tertiary structure [19]. Figure 3 illustrates general characteristics of proteases as the covalent and general acid-base mechanism, cleavage at endo- or exo-sites of the substrate, and the active site commonly described in S and S´ sub-sites with the corresponding P and P´ interacting side chains of the peptide substrate.

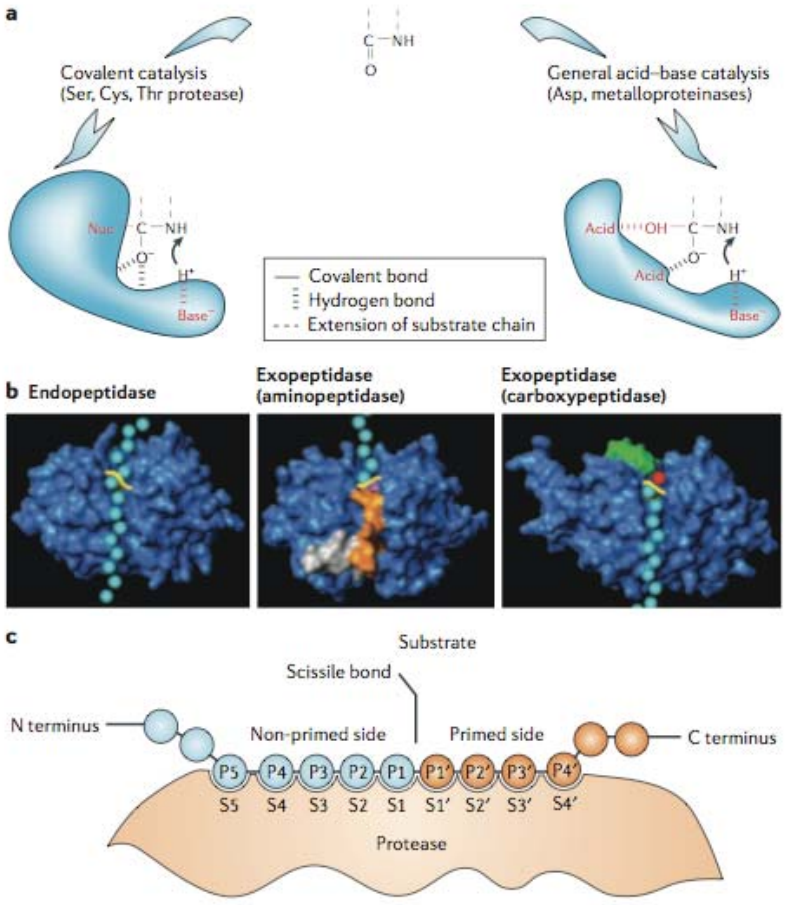

Figure 3. The figure shows the covalent hydrolysis mechanism by the Ser-, Thr- and Cys-proteases, and the general acid-base catalysed mechanism by Asp-, metallo- and Glu-proteases in a). In b) the difference in cleavage mode of exo- and endo-peptidases are shown where the yellow line indicating the cleavage and the peptide sequence as turquoise balls. In c) description of the protease binding sites as S/S´ sub-sites with the corresponding P/P´ substrate residues are illustrated. Reprint from Turk 2006 [15] with permission from the Nature Publishing Group.

1.2.1 Aspartic proteases

Aspartic proteases are one of the minor protease classes and use the general acid-based hydrolysis mechanism. The majority of proteases belong to two families, the A1 family of pepsin-like proteases and the A2 retroviral family [19]. Both families belong to the same clan AA indicating evolutionary conservation. Another smaller clan of aspartic proteases is the AD clan comprised of intramembrane cleaving proteases [20]. Aspartic proteases have been found in mammals, plants, fungi, protozoa and viruses [21]. In humans, members of this protease class are part of the digestive system with non-specificity for peptide substrates as for pepsin and gastricin, but also include proteases with a single substrate such as renin with its substrate angiotensinogen, and β-secretase 1 (BACE1) that hydrolyse the amoloid precursor protein (APP) as its only substrate. The aspartic proteases can be expressed in a single cellular compartment as with cathepsin D in the lysosomes [22], can be secreted, as with pepsin and gastricsin, or can be expressed in specific tissues that restrict their activities, such as BACE1 in the brain [23].

The overall structure of the A1 family of aspartic proteases can be divided into three different topological regions: the N-terminal domain, the C-terminal domain and a six-stranded anti-parallell β-sheet interdomain that connects the two terminal domains as seen in Figure 4 [20]. The A2 family of retroviral aspartic proteases are homodimers, where each chain corresponds to one of the N- or C-terminal domains in the A1 family. The binding site is located in between the two terminal domains in the A1 family, and between the two chains in A2. Each domain/chain contributes with one of the aspartic residues in the catalytic dyad located in the bottom of the binding site, the catalytic dyad is indicated in magenta stick representation in Figure 4. The AA clan has a distinct β-hairpin flap located above the binding site that closes upon substrate binding and in some cases when inhibitors are bound. The catalytic aspartates are positioning and activating a water molecule that plays the role as the nucleophile in the acid-base hydrolysis mechanism.

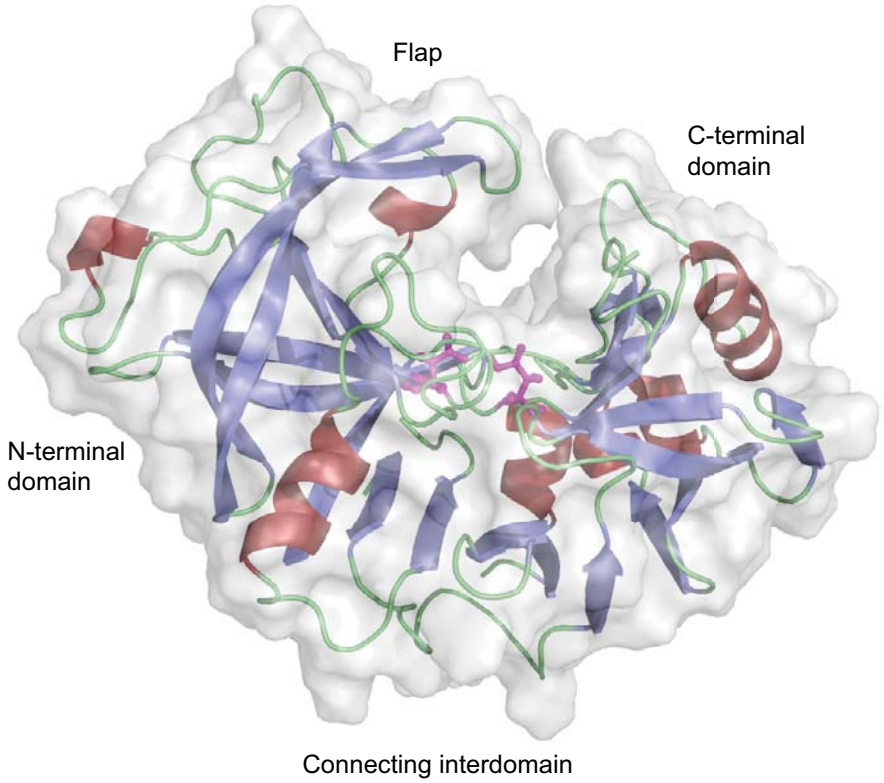

Figure 4. Structural representation of the aspartic protease renin which belongs to the A1 family, AA clan. The characteristic flap in the N-terminal domain is located above the catalytic aspartate-dyad shown as magenta sticks. The binding site and the catalytic dyad are located in the interface between the N- and C-terminal domains. The figure was made from deposited PDB entry 2FS4 [24] by the program PyMol.

1.2.2 The origin and evolution of aspartic proteases, a conserved topology motif

Aspartic proteases have so far been found in both eukaryotes and retroviruses, but not in prokaryotes [25]. This finding along with others has led to speculations that the retroviral aspartic proteases originate from eukaryotes [25]. The eukaryotic proteases, also referred to as the cellular aspartic proteases are about a size of 325 residues and enzymatically active as monomers. In contrast, the aspartic proteases of retroviruses are comprised of two identical monomer chains of 99-125 residues contributing with a single aspartic acid each to the catalytic aspartate-dyad [25]. The genetic economics benefits of decreased genome size may explain the preferred dimer model observed in retroviruses. The aspartic proteases of retroviruses exhibit a two-fold or close to a two-fold

perfect symmetry. For the cellular eukaryotic aspartic proteases there exits twofold symmetry between the N- and C-terminal lobe of the monomer in the secondary and tertiary structure [26]. The signs for homology at the sequence level are however weak and support the theory of a divergent evolutionary process for the two lobes. Also within each lobe there are signs of yet another twofold symmetry by a repeated β-hairpin, loop, α-helix and a second β-hairpin in the topology [27, 28]. This repeat forms a ψ-loop motif of anti-parallel strands connected by a non-succeeding strand in between. This symmetry relationship suggests an ancestral domain that upon gene duplication and fusion formed what corresponds to one of the terminal domains of the eukaryotic aspartic proteases or a single subunit of the retroviral proteases. This fused protein may have formed active dimers from identical subunits before evolved divergently in the eukaryotic aspartic proteases. This last fusion may have been beneficial for making the correct association necessary for the protease activity as the linkers are not crucial for maintaining enzymatic activity. Although the gene duplication is accepted for the C- and N-terminal lobe symmetry relationship, the lack of homology on sequence level for the internal dyad in each lobe has made a dispute if the ψ-motif is strictly a secondary structural hydrophobic core motif and not the result of an evolutionary event [29]. However, the ψ-motif is a rare motif [30], and the connection of the loop evolving independently without duplications is an unlikely evolutionary event. The lack of aspartic proteases in prokaryotes may be explained by the evolutionary process of need for proteases in endocytosis by eukaryotic cells [19].

1.2.3 Diseases with aspartic proteases as validated drug targets

Among the aspartic proteases, several validated drug targets, and more putative targets, with high potential for improvement in medical treatment have been identified [20]. A short presentation of a few of the most promising drug targets are given below for diseases that affects millions and millions of people world wide every year. These diseases are Alzheimer´s disease causing dementia and especially affecting populations of increasing age, hypertension, systemic fungal infections related to immunosuppressed individuals, malaria, and HIV. The only common feature of these diseases, affecting different population groups, and age and geographical distribution, is that they have a single or multiple aspartic proteases contributing to the progress of the disease. However to be noticed, not all of these targets are human aspartic proteases. With the exception of the HIV-protease belonging to the retroviral aspartic proteases, the drug-targets belong to the pepsin-like eukaryotic family A1.

Renin is a highly selective aspartic protease that is involved in the regulatory pathway for blood pressure, and a validated drug target for hypertension. The first activity of renin was reported in 1898 by Tigerstedt and Bergman in observation in animal experiments with kidney extracts [31, 32]. The potential of renin as drug target is expanded on in the subsequent section 1.2.4.

The human β-secretase (BACE1) is a type I transmembrane aspartic protease responsible for the initial cleavage of the amyloid precursor protein (APP) [33]. By further being processed by a γ-secretase, the APP fragments are the origin of the amyloids that aggregate into plaques found as an early observation in the brains of Alzheimer´s disease (AD) patients [33]. The cleavage is highly specific, and mutations in the APP sequence is enhancing the BACE1 activity causing early-on-set familial AD. Knock-out animal models show that BACE1 is the main source of the amyloid fragment formation, and the protein is regarded as a key drug-target for novel AD drug development [34, 35].

Fungi, such as *Candida* species, with *Candida albicans* being the most common pathogen in human, produces a range of secreted aspartic proteases (SAPs) [36,

37]. *Candida* species are a common part of the microflora in humans, and it is only upon immunosuppression of the host that the fungi become pathogenic [36]. These fungi can cause cutanous, mucocutanous and systemic infections, with the latter targeting the inner organs as kidneys, heart and brain in patients with burns, cancer, immunosuppression, and AIDS [37]. The highest systemic infection level correlates with high mortality rates. The SAPs are non-specific proteases that are able to degrade a range of different host proteins as albumin, mucins, and immunoglobulin. The SAPs distinguish themselves structural from other aspartic proteases by having a second, but smaller, flap located above the S3 sub-site. Knock-out fungi strains have shown that SAPs are related to virulence, and that it is the ensemble of SAPs rather than a single protease that are enhancing the pathogenic activity [37]. The SAPs are regarded as potential drug-targets for treatment of fungi, especially as current treatments are facing resistance to current antifungal drugs [36].

Protozoa species of the *Plasmodium* family are the microorganisms causing malaria, with *Plasmodium falciparum* being associated with the most severe disease prognosis and highest mortality. These parasites have an arsenal of aspartic proteases that are involved in the degradation of hemoglobin as a nutrition source for the parasites in the inter-erythrocytes life-phase [23, 38]. The aspartic proteases of the *Plasmodium* family are named plasmepsins (PM), where PM I and II are involved in the initial degradation of hemoglobin, and other proteases including PM IV and a histo-aspartic protease show cooperative activity in later degradation steps [38]. Other PMs of *Plasmodium falciparum* are also expressed in the inter-erythrocyte life-phase (PM V, IX and X), but are not involved with hemoglobin degradation and are localized outside the food vacuole. Yet other PMs (PM VI, VII and VIII) are not expressed in the inter-erythrocyte life-phase of the protezoa, but may be functional in the insect or the exo-erythrocyte life phase of the organism. Inhibition studies of the PMs which are involved with hemoglobin degradation indicate that the PMs are crucial for normal growth, and hence the organism is exposed to starvation upon lacking the activities of the PMs [38]. The PMs of *Plasmodium falciparum* are pursued as validated drug-targets for malaria in drug discovery projects [39].

The HIV retrovirus is the causative virus for development of AIDS. This virus is dependent on the cleavage of the gag and gag-pol polyprotein precursor by the aspartic protease HIV-protease for assembling of the viral capsule [40]. When the activity of the HIV-protease is blocked, the virus is no longer able to mature into infectious virons, and hence the lifecycle of the virus is arrested. The HIV-protease belongs to the retroviral A2 family of aspartic proteases, and is comprised into a catalytic entity upon dimerization. A range of drug development projects targeting the HIV-protease have successfully been developed into drugs in clinical use [40].

When targeting aspartic proteases for drug development, the selectivity over the two major house-keeping human aspartic proteases, cathepsin D and E, has to be investigated to avoid severe adverse effects [22, 41].

1.2.4 Hypertension and the renin-angiotensin regulatory system

Hypertension, a physiological condition with elevated blood pressure (systolic pressure of >140 mm Hg or diastolic pressure of >90), is one of the major risk factors for the development and increased mortality of cardiovascular diseases [42, 43]. These disorders include coronary heart diseases, stroke, congestive heart failure, chronic kidney disease and end-stage renal disease [42, 43]. Although hypertension belongs to the diseases that are easier to prevent by active and conscious life-style choices than to treat, the number of affected people are rising globally. The increase in people affected is estimated to continue as a result of increase in the population age and the life style associated with an increasing growth in the standard of living [44]. Estimation of individuals in the adult population with hypertension is postulated to affect 1.56 billions in 2025 [44]. Recommended life-style intervention can have major blood-pressure lowering effects, and can be compared with decreases observed by medical treatment. There are six key general interventions which are scientific proven to have a beneficial blood-pressure lowering effect: maintaining a normal body weight, reduce dietary sodium intake, engage in moderate physical activity, limitation in alcohol intake, maintain adequate potassium

dietary intake and have a diet which is rich in fruits and vegetables and reduced content of saturated and total fat [42].

The renin-angiotensin-system (RAS) is a regulatory pathway for blood pressure and determinant of end-organ damage. The regulatory system is illustrated in Figure 5 and described in short below based on Zaman *et al.* 2002 below [45]. The rate-limiting step in the pathway is the cleavage of the angiotensinogen peptide, which is released by the liver, by the aspartic protease renin. Renin is produced and released as inactive prorenin from the kidneys. Prorenin consists of 386 amino acids that upon proteolytic removal of the pro-segmient yield the active form of renin of 340 amino acids [46]. This processing of angiotensinogen by renin takes place in the circulatory system, and yields the in-active decapeptide angiotensin I. Angiotensin I is further processed by the angiotensin converter enzyme (ACE) into the active octapeptide angiotensin II in endothelial cells in the lungs. The angiotensin II main interaction partner is the angiotensin II type 1 receptor (AT_1) which belongs to the GPCRs [47]. The AT_1 is expressed in multiple organs, and upon binding of angiotensin II is causing multiple effects that increases the blood pressure, such as vasoconstriction, and stimulation of the aldesterone synthesis and release. One of the key features in the RAS is the negative feedback system, where the angiotensin II binding to AT_1 inhibit further production and release of renin, the origin of this signal pathway. The angiotensin II type 2 receptor (AT_2) is a second angiotensin II receptor belonging to the GPCRs. AT_2 is differential expressed than AT_1, and mainly observed in fetal tissue where they promote cell differentiation and apoptosis in connection with organ remodeling. The AT_2 is expressed in much lower amount in adults, and is present at inflammation sites, and associated with tissue damage and cellular stress. Inhibition studies show that AT_2 is involved in cell growth and proliferation, tissue repair, apoptosis and protects against ischaemia. Angiotensin I and II can be processed further into even shorter, but less studied peptides and might obtain other biological activities in- or outside the RAS.

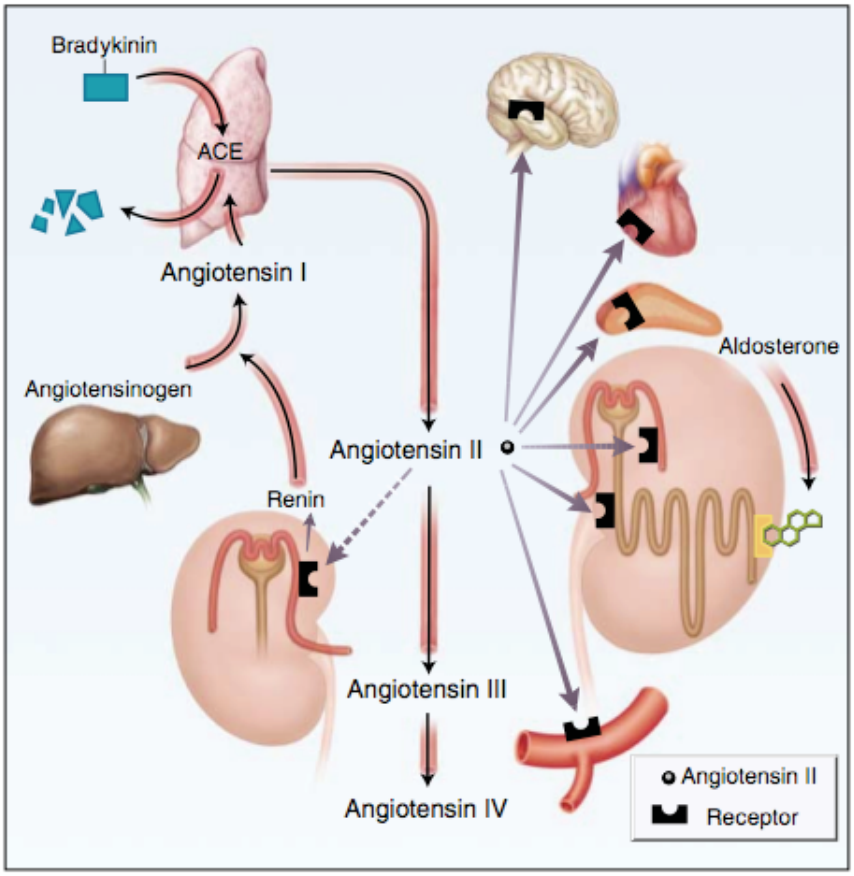

Figure 5. Illustration of the RAS system and organs affected by the signaling pathway, including the proteases involved and the peptide substrates and products. Angiotensin II is indicated as a ball, and the AT_1 receptor as a rectangle with complementary shape. Reproduced with permission from Goodfriend *et al.* 1996 [47], copyright Massachusetts Medical Society.

ACE is also known to have other peptide substrates than angiotensin I, such as bradykinin that induces vasodilation. Angiotensin I can also be processed into angiotensin II by an ACE independent way by other proteases. RAS is typically viewed as a circulating system, but it also has a local tissue component that can operate independent of the vascular system, however this system is regarded as outside the scope of this thesis [48].

1.2.5 Drugs targeting the RAS pathway

Anti-hypertension drugs targeting the RAS regulatory pathway can be classified by which part of the RAS they are modifying. The direct renin inhibitors (DRIs) and β-adrenoceptor blockers which targets renin, the ACE inhibitors, the AT_1 blockers (ARBs), the aldesterone-receptor antagonists (ARAs) and combined ACE and neutral endopeptidase inhibitors called vasopeptidase inhibitors (VPIs) [45]. To date, the most successful classes of anti-hypertension drugs are the ACE inhibitors and the ARBs, which holds protective activities beyond decrease in blood pressure. A short presentation of each drug class is described below.

The development of DRIs has been extensively pursued, as it is a highly eligible target of the RAS. Renin is responsible for the initial and rate limiting step activating the catalytic cascade of peptide modifications of angiotensinogen. The high substrate selectivity of renin, with angiotensinogen as its sole substrate, is limiting the adverse effects of interfere with other bioactive substrates. Targeting renin also ensure a tight control of the negative feedback system of angiotensin II and AT_1. However, problems with bioavailability and lack of *in vivo* efficiency have rendered only a single DRI, Aliskiren, being developed into a clinical drug [2]. Although Aliskiren only possess a bioavailability of 3%, it is a very potent anti-hypertension drug with high end-organ protective properties due to the inhibition of plasma renin activity (PRA). Fewer and less adverse effects are associated with Aliskiren, with the most common being diarrhea and a much lower number of cough incidents than with ACE inhibitors [2]. Alsikiren performs satisfactory in mono-therapy, but when combined with ACE inhibitors or ARBs even higher blood pressure lowering effects are observed without any increase in adverse effects. Hence combination therapy with Aliskiren is a superior treatment regime [2]. The beta-blockers act by inhibiting the β-adrenoceptor, and hence reduce the release of renin into the circulatory system.

The ACE inhibitors have been a highly successful class of drugs by decreasing the amount of angiotensin I converted into angiotensin II. However, as other proteases are capable of performing this cleavage as well, the formation of the bioactive angiotensin II is not fully controlled by ACE inhibition. Increased

expression of ACE is also commonly observed in treatment-regimes with ACE inhibitors. These phenomenons of lack tight regulation when inhibiting ACE are referred to as angiotensin escape. As ACE also process other substrates than angiotensin I, such as bradykinin, this is believed to be the source of adverse effects of these drugs, with the most common being angioedema and dry cough.

ARBs are interacting with the transmembrane part of AT_1, preventing the angiotensin II peptide to bind to the receptor [45, 47]. These inhibitors do not interact with the AT_2 receptor, but are indirectly activating AT_2 as the ARBs causes an increase in the angiotensin II level by also inhibiting the negative feedback system for renin production and release. The ARBs have less adverse effects than the ACE inhibitors, as cough, angioedema and hyperkalaemia appear significantly less frequent. However, due to the inhibition of the feedback system regulating renin, an increase in renin release and the PRA together with the increased angiotensin II level are observed, and unfortunately is causing a higher risk for mortality due to heart attack and accelerates development of renal failure [2]. In treatment with both ACE inhibitors and ARBs, a high percentage of an effect called aldesterone breakthrough has been observed [49, 50]. The decrease in suppression of the aldesterone level in long-term treatment results in a higher risk of end-organ damage in kidneys.

The other classes of anti-hypertension drugs are less abundant, but ARAs are efficient drugs for patients with resistant hypertension to ACE inhibitors and ARBs, but are associated with anti-androgenic adverse effects. The VPIs are showing a superior decrease in blood pressure due to the additional inhibition of neutral endopeptidase to its ACE inhibitory properties. However, angioedema is reported as a significant adverse effect for a high number of patients.

1.3 Host defense peptides

The global threat of increase in pathogenic strains resistant to current antibiotic drugs made the discovery of the host defense peptides into an intriguing expectation of a novel source of antibiotics [51, 52]. Host defense peptides, also called antimicrobial peptides (AMPs), are a part of the innate immune system and first line of defense in plants, insects and animals [53]. The innate system is distinguishable from the acquired immune system by being gene-encoded, and it is not able to acquire or learn with exception of normal evolution [54]. However, the innate immune system do co-function and communicate with the acquired system by distinguish between molecules that are defined as the *non-self* and *self*, a property it does not share with the acquired system [55]. The activation of the innate immune system, including the induction of AMPs, is recognition of common molecular patterns as molecules and receptors shared by microorganisms [55, 56]. Examples of conserved patterns are: the lipopolysaccharides (LPS) of Gram-negative bacteria, the glycolipids of mycobacteria, the lipoteichoic acids (LTA) of Gram-positive bacteria, the mannans in yeasts, and the capsule envelopes of viruses [56]. The great similarities found between regulatory pathways of the innate immune systems of insects and mammals support the hypothesis that this is an ancient first line of defense towards microorganisms [56]. Besides AMPs, the innate immune system also consists of phagocytic and cytotoxic cells, including their effectors and signaling molecules [54].

The AMPs were first discovered in the early 1980s, by the finding of the cecropins in insects by the Boman group [57], and by the defensins in rabbit macrophagesin in vertebrates by the Lehrer group [58]. Later in the decade the magainins in the skin of the African clawed frog, were discovered by the Zasloff group [59]. Common features of AMPs are that they are of <100 amino residues in size, they possess a broad-spectrum antimicrobial activity in the micromolar range, and they have an overall amphipathic molecular nature comprising cationic charge and hydrophobic areas [53]. AMPs may be expressed constitutively in low yield under normal conditions, but can also be induced by

molecular pattern recognition, and through the innate immune system signal pathways. The peptides are in most cases the product of a pro-peptide or protein cleavage by protease processing, yielding the final active AMPs [53]. Some organisms, as *Drosophila*, are also capable of differentiating between different microorganisms and inducing production of the AMPs that is the most efficient response to the threat [56].

The classification system of AMPs is not straight forward as there is a functional, rather than a sequence conservation that characterizes these peptides. However, a classification system based on secondary structural motifs is widely used. The main four classes of this system are α-helical, β-sheet, loop, and disordered random coil without any initial secondary structural motifs, see Figure 6 for representative motifs [60]. Other additional classifications are based on the excess abundance of certain amino acid types, with the common being tryptophan, arginine, lysine, proline, cysteine, glycine or histidine-rich sequences [53, 54, 60, 61].

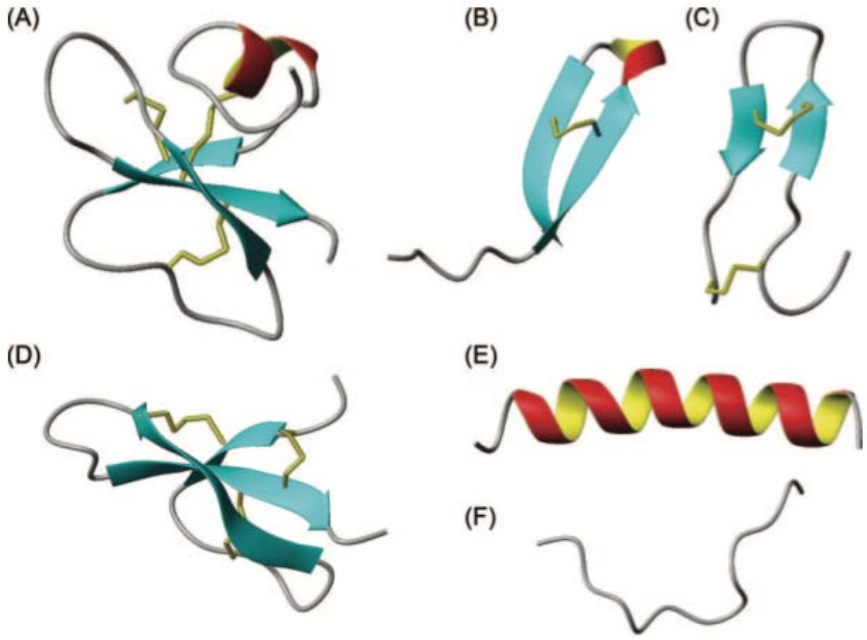

Figure 6. The figure illustrates the commonly used structural classification system of AMPs. In A) mixed structure, in B) looped, the β-hairpin loop in C) and the β-sheet in D), the α-helical as shown in E) and disordered random coil without any secondary structure motifs as in F). Reprint from Jenssen *et al.* 2006 [60] with permission from the American Society for Microbiology.

The knowledge of how AMPs interacts with microorganisms, and which mechanisms are causing the antimicrobial effects are more vaguely understood. For membrane-interacting AMPs a handful of general accepted mechanism hypothesis have been developed, which explains key findings of the antimicrobial activity. The initial interaction with the membrane due to electrostatic attraction between the cationic AMPs and the negatively charged membrane of the microorganism is common for all proposed mechanisms. Eukaryotic cell membranes are comprised of mainly zwitterionic lipids, phosphatidylethanolamine (PE), sphingomyelin (SM) and phophatidylcholine (PC), whereas the cell membrane of bacteria have an overall negatively charged surface due to also being comprised of phosphatidylglycerol (PG), cardiolipin (CL) and phosphatidylserine (PS) [62]. Eukaryotic membranes also benefits from being stabilized by cholesterol, which influence the fluidity of the membrane. However, later steps than the initial attraction of the mechanisms are not fully comprehended. In general, the common accepted mechanisms proposed for AMPs is the barrel-stave model, the carpet mechanism and the toroidal pore formation model. Other mechanisms, as the molecular electroporation and the sinking raft model have also been suggested, although less supported [61]. These generally accepted mechanisms of AMPs activity are shown schematic in Figure 7, A-E.

Microorganisms as bacteria and fungi are also able to produce AMPs, called bacteriocins. The bacteriocins are believed to give the AMP producing microorganism a competitive advantage in their environmental niche [63]. Although exceptions exists, these bacteriocins are generally targeting bacterial receptors unlike the eukaryotic AMPs targeting membranes, which is indicated by their high selectivity and potencies in the pico- and nanomolar range [63]. To avoid affecting themselves, the host-bacteria produces immunity-molecules co-located and regulated with the bacteriocins, or have an efficient efflux-pump system that clears the host for toxic peptides [63]. The high selectivity and potency are viewed as properties that could be taken advantage of in developing novel narrow-specter types of antibiotics [63].

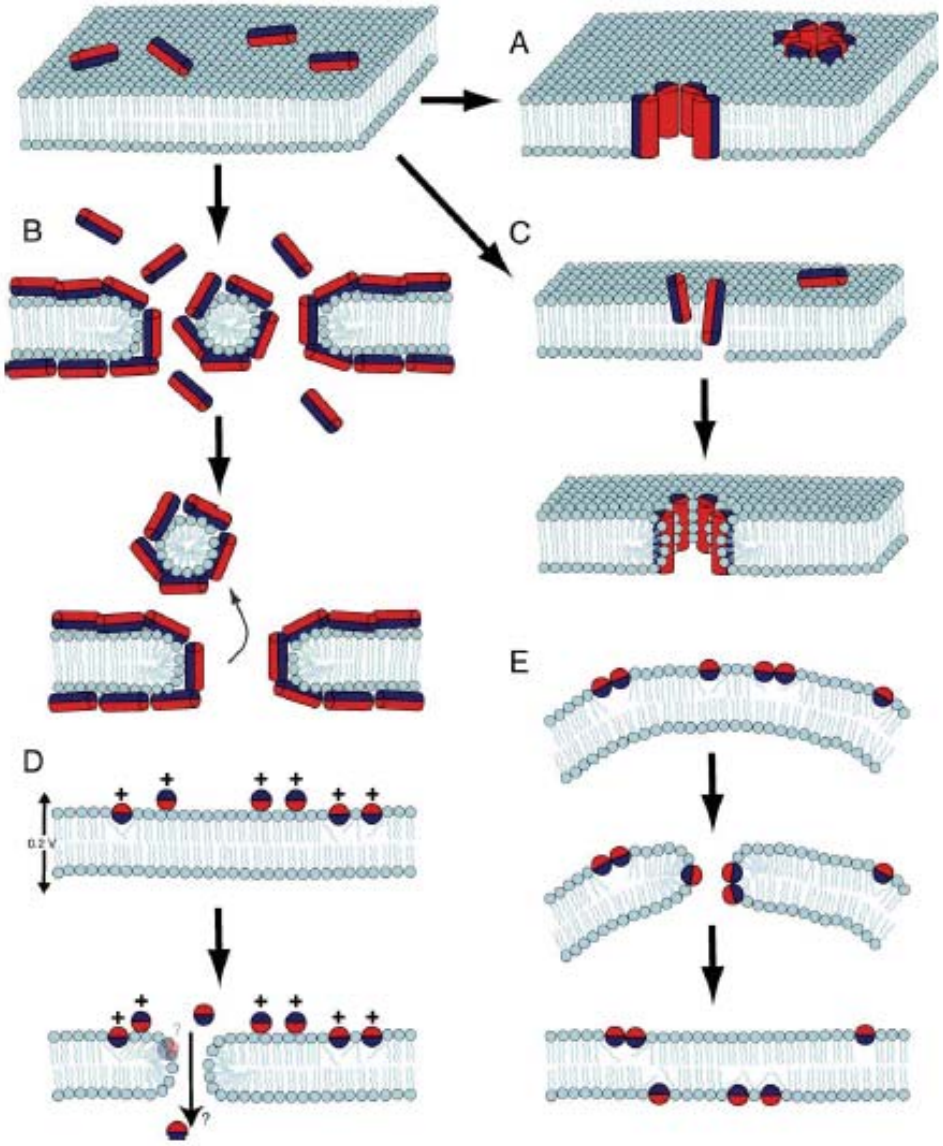

Figure 7. The figure shows several proposed models of mechanisms for membrane-interacting AMPs. The first electrostatic attraction to the membrane is common for all of the proposed mechanisms. The amphipathic property of the AMPs is indicated in red and blue, representing the hydrophilic cationic and the hydrophobic part of the peptide. In A the barrel-stave model is presented, where the peptides span the membrane and forms pores that lead to leakage, in the carpet mechanism in B the peptides destabilize the membrane integrity and possible micelles are formed, and in C the toroidal pore model where pores are formed by both peptide and lipid molecules. In D the electroporation model is presented, where peptides creates an electrical potential difference across the membrane leading to pore formation, and in E the sinking raft model where the peptides form an imbalance in the membrane and local curvature occurs, creating transient pores. Reprint from Chan *et al.* 2006 [61] with permission from Elsevier.

Bactericidal mechanisms of eukaryotic AMPs with membrane distortion mechanisms are believed to be more resistant towards developing resistance compared to receptor-based interactions of clinical small-molecule antibiotics. The tremendous costs related to large alterations of the bacterial membrane are thought to be too expensive, and would rather be of disadvantage than beneficial for the organism [53]. Also believed to restrict the development of resistance *in vivo* is the microorganisms exposure to a cascade of antimicrobial peptides in a natural "AMP-cocktail" and that AMPs may act through multiple mechanisms [64]. However, bacteria which have adapted to the evolutional pressure of AMPs do exist, and a range of strategies for resistance have been found. These strategies are: cell surface modifications by incorporation of positively charged components as D-alanine and lysines, hence reducing the initial attraction to the membrane, inactivation of AMPs by surface- or released compounds, increased protease expression, trigging of up-regulation of protease expression in the host or down-regulation of AMPs, active efflux pumps, and in some cases regulatory networks which can fine-tune the appropriate response to AMP exposure [64, 65]. Other adaptation strategies of bacteria to AMP pressure is applying scavenging strategies of releasing negatively charged bio-molecules as GAGs, polysaccharides, and formation of biofilm [53]. The existence of a broad diversity of strategies for decreasing the effect of AMPs, makes it peculiar why more strains have not been observed as AMPs insensitive. Speculations about AMPs counteracting the evolutional pressure due to low bactericidal potency and low sequence conservation may partially explain the low number of strains with resistant phenotypes [64].

1.3.1 The development of short synthetic antimicrobial peptides from LfcinB.

Lactoferrin (LF) is an 80 kDa iron binding multifunctional glycoprotein of the innate immune system produced in mucosal secretions and neutrophils, with the highest levels found in milk and colostrum [66]. The antimicrobial properties of LF includes the bacteriostatics activity by iron sequestering and bactericidal activity by direct interaction with bacteria surface, but also antiviral, antifungal, antiparasitic, anticarsinogenic, and activity as an immunomodulating agent, as

well as a range of enzymatic activities have been observed [66]. Peptide fragments of LF produced by protease cleavage have been shown to obtain even greater antimicrobial activities [61, 67]. One of these fragments is Lactoferricin (Lfcin), belonging to the ariginine and tryptophan rich class of AMPs. Lfcin is comprised of 25 residue for bovine (LfcinB) and 49 residue for the human counterpart (LfcinH), and the antimicrobial activity is found to be independent of iron sequestering [61]. The antimicrobial activity of LfcinB includes bactericidal activity against both Gram-positive and Gram-negative bacteria, antifungal activity, antiviral and antitumor activity, while LfcinH obtain a less broad antimicrobial activity being mainly bactericidal [61]. While still being a part of LF-B, the fragment that corresponds to LfcinB obtain an α-helix and loop secondary structure that undergoes a hydrophobic collapse to the β-hairpin loop class of AMPs [61]. The same change in secondary structure is not seen for LfcinH, which maintains its secondary structure from its precursor protein. For structural comparison see Figure 8 A-D.

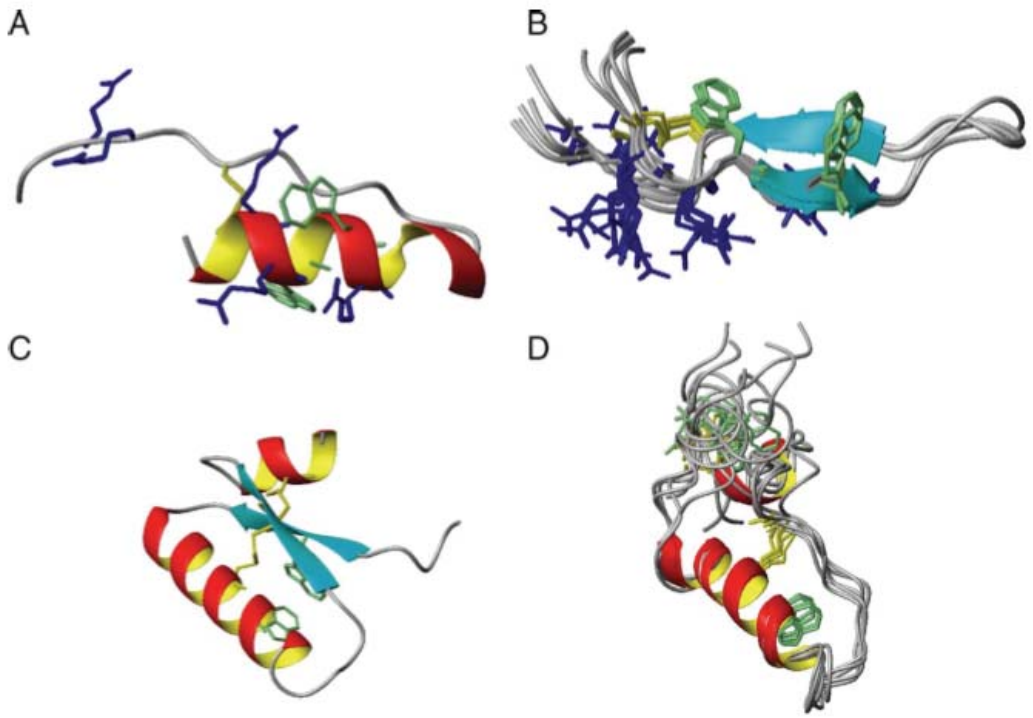

Figure 8. The fraction of LF-B and LF-H that corresponds to LfcinB and LfcinH is shown in A) and C). In B) LfcinB has undergone a transition to its β-loop amphipathic structure, whereas the same transition is not seen for LfcinH which maintains its helical motif shown in D). Adapted from Chan *et al.* 2006 [61], reprint with permission from Elsevier.

Based on truncations, systemic mutations, and synthesis of derivatives, the SAR of Lfcin and LfcinB have been elucidated [68, 69]. Manipulation of size, incorporation of synthetic residues and alterations in the peptides sequences yielded significant shorter active peptides, referred to as short cationic antimicrobial peptides (CAPs) [70, 71]. (CAPs are also being referred to as synthetic antimicrobial peptidomimetics (SAMPs) in the literature.) The pharmacophore of the CAPs was found to be surprisingly small, showing that only two cationic and two hydrophobic elements were necessary for maintaining antibacterial activity [72]. What mechanism the CAPs uses is not entirely clear, but the CAPs have been found to obtain affinity toward bacterial membrane mimics, and to compromise the membrane integrity of bacteria [71, 73]. It is believed that the amphipathic conformation these peptides form in solution, as well as in membrane systems, is important for their membrane destabilizing properties [71, 74]. The incorporation of D-amino acids in CAPs and LfcinB, and reversing the sequence order of LfcinB while still maintaining antimicrobial activity, decreases the probability that receptor interactions are important for the activity of CAPs [72, 74, 75]. However, the small size of the peptides makes it highly unlikely that membrane-spanning mechanisms are valid for CAPs, as the barrel-stave model and the formation of toroidal pores.

The chemical engineering of very short CAPs based on the pharmacophore, resulted in a peptide with satisfactory antimicrobial activity entering a phase II clinical trials for topical treatment against infections by multiresistant bacterial strains [74]. Previous ADME studies of CAPs have been focusing on proteolytic degradation challenges by trypsin and chymotrypsin, and stability in the main organs involved in peptide and drug metabolism and clearance [76-78]. The main pre-systemic challenges for CAPs were found to be degradation in the stomach and duodenum [79]. A follow up on these short half-lives resulted in a significant increase in the protease and organ stability by incorporation of synthetic cationic residues, and provide a sufficient time-span for absorption [76].

The pharmacokinetic studies of CAPs have so far been limited to initial studies of interaction of a selection of peptides with the main plasma protein human serum

albumin (HSA) [71, 80]. The preliminary binding studies showed surprisingly that the CAPs were HSA ligands despite being carrier of multiple cationic charges. The interaction was observed to reduce the antimicrobial activity in the *in vitro* bacterial assays ten-fold [80]. However, further investigations of the binding descriptors for this interaction was not done. One of the most encouraging properties of the CAPs have been their low or non-existing hemolytic activity, indicating a high selectivity for bacteria over eukaryotic cells [71-74, 77, 81-83]. When hemolytic activity was observed for the most hydrophobic CAPs, this was still within an adequate range indicating a very wide therapeutic index. Together with the small sizes, the low cytotoxicity level of CAPs makes them promising peptides for further drug development as novel antibiotics.

1.3.2 Antimicrobial peptides as anticancer agents

The AMPs were found to have an even broader multifunctional range than first expected upon discovery when it was observed that human and rabbit defensins as well as magainins and modified magainins obtained cytotoxic activity toward multiple cancer cell lines *in vitro* and *in vivo* [84-86]. It has been shown that a wide selection of the AMPs exhibit cytotoxic activity towards cancer cells, including cecropins, LL-37, melittin, defensins, lactoferricin, tachyplesin I, and PR-39 to mention a few [87]. Not all AMPs act as anticancer peptides (ACPs), but the ones that do can be separated into two classes; by being cytotoxic toward cancer cells but benign to normal mammalian cells (insect cecropins and magainins) or; showing cytotoxic behavior against both cancer and normal cells (bee venom melittin, insect defensins and human neutrophil defensins) [88]. The selectivity and the toxicity issues of ACPs are poorly understood, and believed to be a combination of physiochemical characteristics rather than the result of a single prominent factor [89]. The two most common mechanisms observed for ACPs are the membranolytic effect on the cell membrane causing necrosis, or interactions with the mitochondrial membrane leading to apoptosis [90]. Although other mechanisms have been observed, as activating of natural killer cells of the immune system, interaction with specific targets, affecting tubulins,

and activation of tumor-suppression agents independent on membrane interaction [90]. ACPs are believed to have an increased affinity to cancer cells due to modifications in the outer membrane compared with normal cells, causing cancer cells to have an overall increase in negatively charged components. Normal mammalian cells have a neutral charged outer leaflet whereas cancer cell lines have been reported to have an increased proportion of PS and alterations in the glycosylation patterns of the mucins [91, 92]. In normal cells the PS are asymmetrically distributed solely on the inside of the membrane, but in tumorigenic cells this phospholipid is found to be 3-7 fold higher on the outer part of the cell membrane [91]. Other factors than membrane composition and modifications that affects selectivity are membrane fluidity as cholesterol content, and the larger surface area of cancer cells due to a higher number of microvilli [87].

Drugs currently used in chemotherapy are primarily acting on intracellular targets of cells with high proliferation rates, with the majority of drugs targeting the DNA replicating process [93]. They can be classified in general as DNA-alkylating agents, antimetabolites, and hormone agonists and antagonsist [94]. The anticancer agents used in chemotherapy are often part of a combination therapy strategy together with radiation and surgery, or as part of drug cocktails, decreasing the rate of resistance development to the treatment [95]. However, resistance and relapses remains a repeating problem in cancer therapies, and multi-resistance mechanisms are not un-common [93, 96]. A major problem with chemotherapy agents in clinical use, are the severe adverse effects caused by lack of differentiating between cancer cells and normal healthy cells with high proliferation rates [95, 97]. This is the main cause of the most associated adverse effects and the corresponding organs affected, in myelosuppression (bone marrow), mucosis (gastrointestinal mucosa) and alopecia (hair follicles) [90]. Later generations of cancer treatments are targeting therapies with monoclonal antibodies or small molecule inhibitors, which act on proliferating cells by interacting with targets that are required for tumor growth [98]. Although less toxic than the more traditional chemotherapy, severe adverse effects and higher costs are limiting these therapies in clinical use [98]. ACPs are hypothesized to

be possible to develop into future superior anticancer agents, which should be able to show increased selectivity for cancer cells and hence reduce the adverse effects. They should also be less prone for the resistance mechanisms correlated with traditional chemotherapy agents, due to targeting the cell and mitochondrial membrane [94]. Another beneficial would be activity toward the quiescent cancer cells, cancer cells in cell cycle arrest and hence not a target for agents which targets high proliferation rates, and ACPs have also been shown to obtain a good synergy with traditional anticancer agents [52, 93].

LF-B and LcinB were found to have ACPs activity, although through different mechanisms, LF-B through iron binding and LfcinB by inhibiting metastasis, angiogenesis, and inducing necrosis [99, 100]. LfcinB were observed to interact with both the cytosolic and the mitochondria membrane and inducing both necrosis and apoptosis, but is in additional capable of producing reactive oxygen compounds and activate the caspase cascade [94, 101, 102]. However, the main mechanism for anticancer activity is though to be destabilization of the mitochondian membrane [102]. Based on the cytotoxic activities of LcinB toward cancer cell lines, derivatives were developed to investigate the potential of LcinB as the origin of potential new ACP drugs [103-106]. In general the repeating drawback in the development of these peptides has been toxicity due to interactions with healthy fibroblasts, however high tolerance toward normal red blood cells is conserved.

Recent findings show that nonapeptide derivatives of LfcinB does not only exhibit anticancer effect upon treatment of tumors in mice, but that they also trigger immunity and hence have a potential as agents for vaccination toward cancer [107, 108]. The ACP used in these studies were administrated directly into tumors by injection and acted through necrosis. At least one was shown to induce immunity by systemic secondary immune responses dependent on both CD4+ and CD8+ T-cell [107]. The established immunity could also be transferred between individuals by transplantation of spleen cells from previous ACP cured mice, and hence the possibility of vaccination toward cancer might be one of the most exciting breakthroughs that have been achieved in cancer treatment

research [107, 108]. The ACP peptide LTX 315 developed by Lytix BioPharma AS have recently been in a phase I dose escalating clinical study in humans for *in situ* vaccination of cancer (conference presentation by Lytix BioPharma AS at the Norwegian Biochemistry Society Contact meeting February 2013, Lillehammer) http://clinicaltrials.gov/show/NCT01058616 .

1.3.3 The development of CAPs and ACPs containing a $\beta^{2,2}$ achiral amino acid

Based on the CAPs pharmacophore for antimicrobial activity even smaller peptides with a lipophilic $\beta^{2,2}$ achiral amino acid (3-amino-2,2-disubstituted propionic acid) incorporated in the sequence have been developed into potent bactericidal agents [109, 110]. These peptides were shown to obtain significant protease stability due to protection by the synthetic lipophilic $\beta^{2,2}$ residue [109]. The hemolytic activities follows the LfcinB based CAPs with low or no hemolytic activity, but when more lipophilic elements were incorporated an increase in the toxicity toward human cell types was observed [109, 110]. Nonetheless, the $\beta^{2,2}$ containing CAPs were calculated to provide for good drug-likeness with indication of absorption, and found to have a medium passive permeability past phospholipid vesicles [110]. However, when tested for *in vitro* clearance it was found that the peptides were substrates for hepatic phase I metabolization by the cytochrome P450 family and experienced significant oxidization [111]. These $\beta^{2,2}$ containing CAPs were however also found to be highly potent agents against a large number of cancer cell lines *in vitro*. In total the peptides where active toward a total of 59 cancer cell lines with highest efficiency toward leukemia, non-small cell lung cancer, colon cancer and melanoma cell lines (IC_{50} of 0.32-0.79 μM toward 12 strains) [112]. The mechanisms for the smallest $\beta^{2,2}$ derivatives were found to be able to differ from peptide to peptide, either by the means of necrosis by destabilization of the cell membrane or apoptosis by being transported intracellular and inducing apoptosis by acting on the mitochondrial membrane [113]. The discrimination in mechanism of cell destruction is however not well understood.

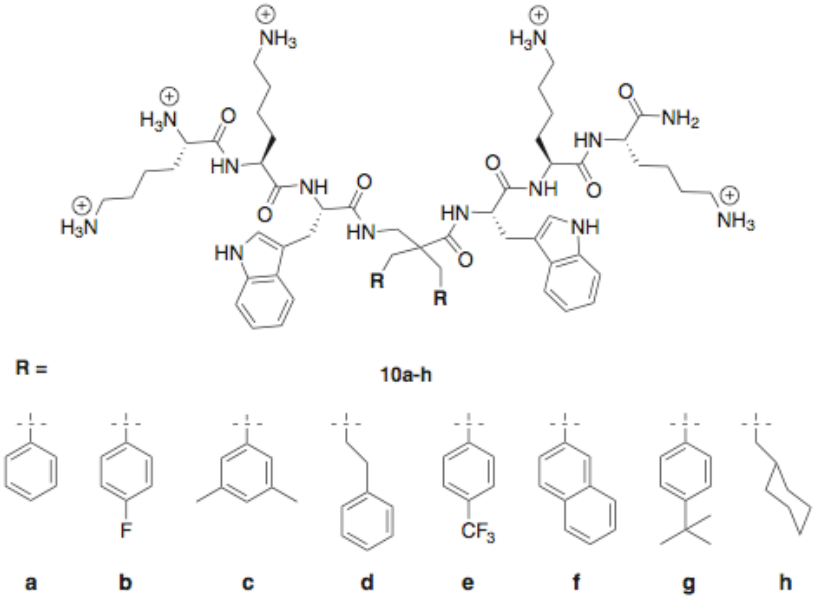

Figure 9. Example of palindromic achiral heptapeptides with cytotoxic activity toward Ramos and A20 cancer cell lines *in vitro*, containing a $\beta^{2,2}$ residue. The various side chains of the $\beta^{2,2}$ amino acid a-h have activities with activities ranging from non-active, 10a, to highly active in 10h. Highest selectivity was obtained for 10e as 10f-g showed increased toxicity toward normal healthy cells. Reprint from Tørfoss *et al.* 2012 [114], with permission from John Wiley and Sons.

The $\beta^{2,2}$ derivatives with cytotoxic activity towards cancer cells have also been explored further in larger peptides up to the size of heptapeptides, see Figure 9 [114, 115]. As cyclization of AMPs have been shown by others to increase the potency, this strategy of structural modification have also been explored for the $\beta^{2,2}$ derivatives [116]. The cyclization of the $\beta^{2,2}$ derivatives did indeed result in higher activities towards cancer cell lines, but also unfortunately to greater toxicity toward normal cells [115]. These linear and cyclic $\beta^{2,2}$ derivatives were found to act through necrosis, and are therefore primary cytotoxic to cancer cells by destabilization of the cell membrane [115]. In MD simulations of the membrane interaction the cyclic variants were shown to penetrate and distort the lipid bilayer to a greater extent than the linear counterpart, see Figure 10 [115]. Although being longer peptides, the $\beta^{2,2}$ amino acid residue was seen to protect against proteases up to at least two adjacent residues on each side [114].

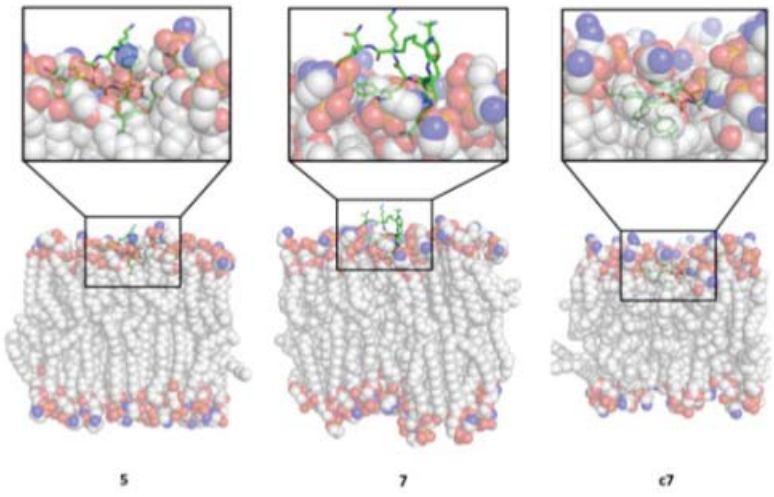

Figure 10. MD simulations of the membrane interaction of the linear heptapeptide **5**, and the linear and cyclic versions of hexapeptide **7** and **c7**, all containing a $\beta^{2,2}$ residue. The depth of the peptide interaction corresponds to the peptides cytotoxic potency to cancer cell lines, IC_{50} **7**>>**5**>**c7**. Reprint from Tørfoss *et al.* 2012 [115], with permission from John Wiley and Sons.

1.4 Plasma protein interaction of small molecules

The investigation of lead molecules interaction with plasma proteins is a standard procedure in drug discovery studies to estimate the pharmacokinetic properties. There are only a couple of plasma proteins that are considered to affect the pharmacokinetics of drugs, but in return these proteins are capable of binding a wide range of exogenous and endogenous molecules. Hence, drugs can be transported either as free compounds or in complexes with proteins in the circulatory system. The main plasma transporter proteins are human serum albumin (HSA) and alpha-1 acid glycoprotein (AGP) with normal physiological concentrations of 40 mg/ml and 0.6-1.2 mg/ml in healthy adults [117]. HSA has a preference for lipophilic and negatively charged ligands, whereas AGP have a preferential of binding lipophilic and positively charged small-molecules, although exceptions to these generalizations exist for both plasma proteins. Both HSA and AGP are acute phase proteins with HSA decreasing by a factor of two and AGP increasing with as much as threefold in concentration (but can also be down-regulated) at certain pathological and physiological conditions [117, 118]. HSA and AGP are being presented in the subsequent sections 1.4.1 and 1.4.2.

A third type of plasma transporter macromolecules are the lipoproteins that can bind and facilitate uptake of highly lipohilic drugs by receptor mediated uptake, hence differ from the other plasma proteins by also increasing bioavailability, see Figure 11 b) [119]. Lipoproteins are not single proteins, but large complexes composed of different lipid types and apolipoproteins, see Figure 11 a) for schematic illustration of composition. They are assigned into four different classes based on composition ratio and particle densities, the chylomicrons, very low, low and high density lipoproteins, and are involved in the endogenous transportation of lipids [119]. Upon disease states or as adverse effect of drugs, the composition can be altered, resulting in conditions called dyslipidaemia. The drug-transportation properties for lipoprotein binding drugs in these conditions may change compared with normal physiological states. Other plasma proteins with impact on drug delivery but with more specific ligand pattern are the globulins that binds steroid drugs [119].

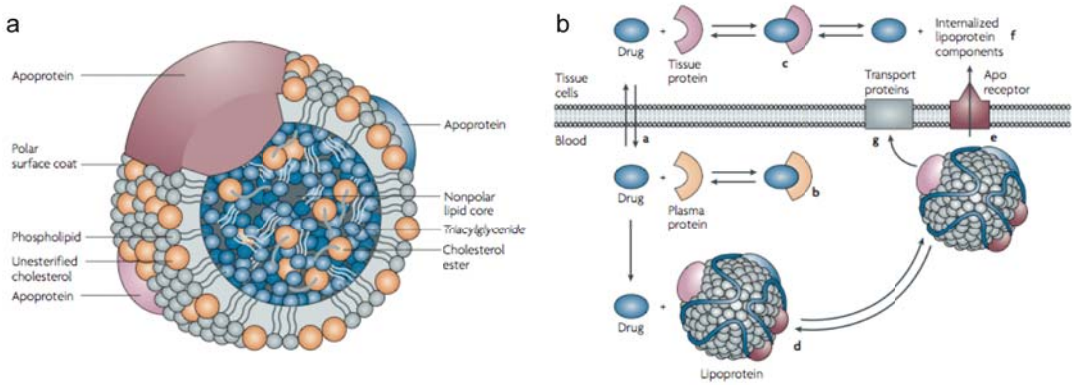

Figure 11. The composition of the lipoproteins is shown in a) as a complex of apolipoproteins, phospholipids, triglyceride, cholesterol and cholesterol esters. In b) the role of lipoproteins is shown as a facilitator for drug uptake through a receptor-mediated mechanism. The passive diffusion through membranes, the binding to tissue proteins, and how binding to plasma proteins can reduce the drug concentration available for membrane diffusion are also illustrated in b). Reprint from Wasan *et al.* 2008 [119], with permission from the Nature Publishing Group.

The general effect of drugs interacting with plasma proteins is the change in the compounds ability to leave the circulation system and enter tissue, and reduction of toxicity, as both volume and clearance of the active compound may be altered. An increase in plasma protein binding corresponds to a decrease in the amount of drug that leaves the circulating system and enter tissue, but also decreases in the clearance rate by modification of the drug concentration and hence it may affect the therapeutic profile [119]. The transferring of *in vitro* observations in shifts assays with plasma proteins into *in vivo* effects should be object for careful interpretation. As plasma protein interactions affects the ratio of free ligand to total ligand concentration, it is the free concentration of ligand at the bio-phase of the therapeutic target that have an impact on the efficiency as a drug [120]. The *in vitro* experiments do not include the highly dynamic and complex systems that a drug faces *in vivo*, and hence the clearance rate and limitations in absorbance may have larger effect than the plasma protein binding. A common effect of this is the increased clearance rate of drugs by metabolism and excretion at higher drug concentrations that counteract the increase in free drug concentration by decreases in the plasma protein binding [120]. If drug distribution occurs by passive diffusion, the free drug concentration in plasma

will be in equilibrium with the effect site, and is a better measure than the total drug concentration for therapeutic response [121].

Highly plasma protein bound drugs with binding fractions of 90-99.9% correlates with a binding constant K_a in the range of 10^5-10^7 M^{-1}, whereas low to moderate binding is considered for binding constants K_a 10^2-10^4 M^{-1} [121]. Changes that affect drug binding of highly bound drugs are the fluctuation in concentration and composition of the plasma proteins, however also competitive effects and displacement by endogenous and exogenous compounds may have impact on the proportion of bound compound [122].

1.4.1 Human serum albumin

Human serum albumin is regarded as the most important plasma protein due to the high physiological vascular concentration of 40 mg/ml, which together with its numerous binding sites makes HSA a high capacity binding protein of endogenous and exogenous small-molecules [117, 123]. Extra-vascular albumin in tissue is however the largest proportion of albumin, counting for 60-70% of the total amount in humans, but with a significant lower concentration than for the plasma albumin [124, 125]. HSA is the transporter of endogenous fatty acids (FA), and each monomer can carry multiple FA at the same time, with normal estimations of bound FA of 0.1-2 mole per mole of HSA. Albumin is a remarkable stabile protein with an average *in vivo* half-life of 19 days in plasma, and being stable over a pH range of 4-9. It is soluble in 40% ethanol and obtain significant thermostability, by tolerating heating at 60°C for up to 10 hours [126]. HSA is not only a transporter for FA and small molecules, but it is also a binder for Cu (II) and Ni (II) by specific binding and an unspecific binder of Ca (II) and Zn (II). Other functions of HSA are maintaining the colloid osmotic pressure and an antioxidant agent in the circulating system, and providing a source of nutrition upon degradation [126, 127]. A more subtle and un-known aspect of HSA is the hydrolytic enzymatic activity, with the most well known being the esterase activity [128].

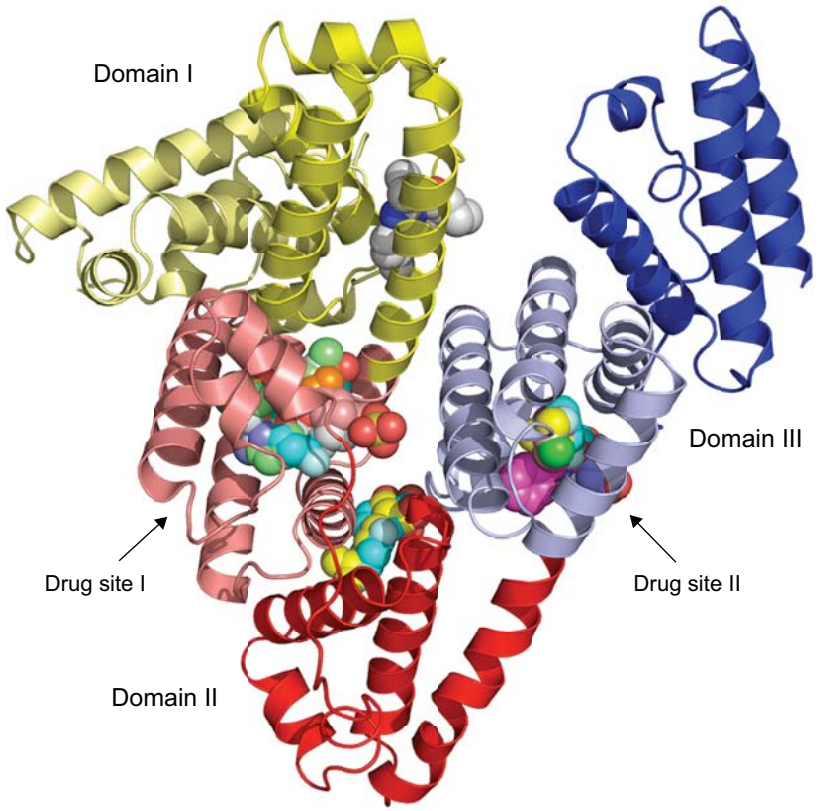

Figure 12. The overall structure of HSA shown in cartoon with color coded domains. Domain I is shown in yellow with IA in pale and IIA in bright yellow, domain II in red with IIA in pale and IIB in bright red, and domain III in blue with IIIA in light and IIIB in bright blue. Drug site I is located in domain IIA (pale red), and drug site II in domain IIIA (light blue) indicated by the clusters of bound small-molecules in these sub-domains. Representation of other binding sites are shown in domain IB and the interface between IIA and IIB. The figure is made from the deposited PDB entry 1BM0 [129], and the ligand coordinates from 2BX8 and 2BX(A-H) [130]. The figure was made in PyMol.

HSA is a 66 kDA globular monomer comprised of 585 residues, and contains 35 cysteins [125]. The protein consists of three structural homologous domains (I-III) containing 10 α-helices each, with each domain comprising the two sub-domains A and B [125]. HSA contains only α-helices secondary structure elements with a total content of 67%, with an overall asymmetric heart shape of approximate size 80x80x30 Å, see Figure 12 for illustration of the structure of HSA [125]. The total of 35 cysteine residues are distributed in 17 disulphide bridges and a single free thiol, which are the responsible residue for covalent ligand binding and minor dimer formation [125]. HSA has also an antioxidant

function in the circulating system due to the free cystein, and is present both at oxidized and reduced forms [122]. The binding of FA is inducing an overall conformational change by rotation of domain I and III, whereas domain II is left unaltered upon FA binding [131]. The FA binding sites are distributed asymmetrically in the different domains, and experimental structures supports 7 to 11 FA binding sites depending on the length of the methylene tail of the fatty acid [131-133]. FA binding has been correlated to both cooperative and competitive effects regarding drug binding [127, 130, 131].

The high binding capacity of HSA for small molecules is due to numerous diverse binding sites distributed over the whole protein [130]. The two best characterized drug binding sites are drug site I located in sub-domain IIA and drug site II in sub-domain IIIA as shown by arrows in Figure 12 [125]. The drug sites I and II are thoroughly studied in Ghuman *et al.* 2005 [130], and a short description is given subsequently. Drug site I lies within the core of the six helices that comprise sub-domain IIA, and forms an apolar pocket with two polar clusters of basic residues. One of the polar clusters located in the middle part of the binding site, and the other at the entrance, but the site also have a large apolar area that causes the ligand preferential of this site to be lipophilic ligands with centrally located negatively charged features or ligands containing negatively charged features on opposite sides of the molecule. The FA binding site FA7 is located in drug site I. Upon FA binding, structural alterations are induced in drug site I due to extensive rearrangement of the hydrogen bond network by the side chains of the binding site residues. Which results in an increased size and alteration of the distribution of polar patches. Drug site II is located in sub-domain IIIA in a topological similar way as drug site I, however different packing arrangements of the two sub-domains leads to differences between the two binding sites. Drug site II is significant smaller than drug site I, and the entrance of the site is to a larger extent exposed to solvent. This site is also containing a single polar patch with basic residues, located at the entrance of the binding site. Drug site II is in general more selective for lipophilic ligands with peripherally located negative features. When FA binds, two of the FA binding sites, FA3 and FA4, are associated with drug site II, resulting in

competition with bound ligands. The methylene tail of FA3 is interacting with the apolar pocket of drug site II, and the carboxyl group of the FA binding in FA4 is interacting with residues in the basic patch of the entrance of the site. FA3 and FA4 are high affinity FA binding sites and affect compounds bound to drug site II, whereas FA7 is a low-affinity site where the bound FA is observed to be displaced upon ligand binding [130].

The great binding capacity and promiscuity of HSA is not uniformly regarded as disadvantageous as the increase in half-life of drug or compounds may be highly desirable in certain cases, such as in medical treatments involving imaging where HSA acts as binding partner for contrast agents [126, 127, 134]. HSA are known to accumulate in solid tumors, and at inflammation sites in patients with active rheumatoid arthritis, and can be used for targeting these sites selectively with albumin-binding drugs [126]. In both instances of accumulation, HSA is covering the increase in demand of nutrition by being degraded at the sites. Other benefits of HSA binding is increased resistant to protease degradation and a lower risk of immunogenic reactions of peptides and proteins as drug agents [126]. Strategies commonly used for taking advantage of the molecular and biological function of HSA are: fusion of the agent or drug of interest to a known high-affinity ligand, or directly fusion to HSA, or as a third strategy encapsulation of active drug in albumin nanoparticles [126]. Examples of these strategies are insulin fused with myristic acid, albumin bound methotrexate and interferon directly fused with albumin [126].

1.4.2 Human alpha-1 acid glycoprotein

The other major plasma protein affecting the pharmacokinetics of drugs is human alpha-1 acid glycoprotein (synonymous with orosomucoid (ORM) in literature). Although by comparison of the normal physiological concentration of AGP of 0.6-1.2 mg/ml to HSA of 40 mg/ml, AGP is considerably less abundant in the circulating system and it also contain a single binding site. Nonetheless, AGP is a high affinity, low capacity plasma protein binder with a wide range of endogenous and exogenous compounds [117, 118]. AGP belongs to the lipocalin

family, is of weight 41-43 kDa and is heavily glycosylated with a carbohydrate content around 45% [135]. The overall high sialic acid content of 12% leads to the low pI of 2.8-3.8 [136]. AGP is comprised of 183 residues forming an eight-stranded β-barrel flanked by α-helices, and with five glycosylation sites and two disulphide bridges, see Figure 13 [137]. The barrel is closed in one end by the N-terminal part of the sequence. Three of the glycosylation sites are located underneath the barrel, whereas the last two are in the proximity of the binding pocket entrance, and may alter the ligand preferential by glycosylation state and pattern [137].

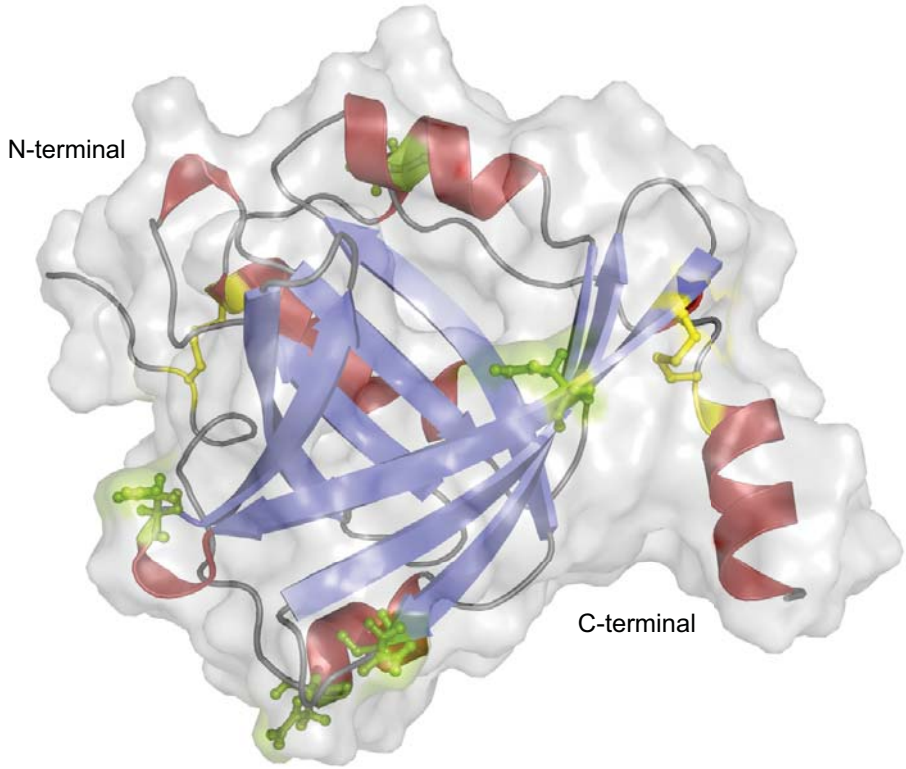

Figure 13. The overall structure of AGP phenotype F1*S shows the β-barrel flanked by α-helices. The glycosylation sites are indicated in green sticks, and the disulphide bridges stabilizing the N- and C-terminal in yellow. The binding site pocket entrance is located at the top of the barrel. The figure is made from deposited PDB entry 3KQ0 from Schönfeld *et al.* 2008 [137] in PyMol.

The physiological role of AGP is not fully understood, but it is a transporter of basic and lipophilic compounds, as well as an anti-inflammatory and immunomodulating protein [138]. Examples of activities are the inhibition of

neutrophils, inhibition of platelets aggregation, protection against toxins, induction and enhanced secretion of cytokines, stimulation of fibroblast proliferation in wound healing, and act on the clearance of pathogenic bacteria in blood [118, 138]. The glycosylation pattern and changes in branching can differ upon physiological conditions and modifies the immunomodulating activity of AGP [136, 138].

AGP is being expressed as three major phenotypes, the F1, the S, and the A phenotype that are encoded by the gene AGP-A (phenoptype F1 and S) and AGP B/AGP B (phenotype A) respectively [139, 140]. In acute-phase it is the AGP-A gene that is activated, and hence the increase in AGP concentration is due to expression of phenotype F1 and S [140]. Phenotype F1 and S differ by a single point mutation in the two allelic variant of the AGP-A gene, mutation Q20R respectively, and are commonly referred to collective as F1*S [141]. The F1*S and A phenotype differ by a minimum of 21/20 sequence substitutions [142]. These substitutions have been shown to induce ligand selectivity for some compounds to the different phenotypes, whereas other ligands show the same affinity [143, 144]. The binding of drugs to AGP and phenotype specific binding has been shown to affect clearance rates in patients [143]. The ratio of F1, S and A is approximately 40%, 30% and 30% respectively in commercial available AGP, however large inter-individual differences in phenotype pattern are common and is not necessary reflecting this ratio [145]. Also ethnicity has been seen to affect the AGP expression pattern, with Caucasians showing the highest level of AGP [118, 146]. Imatinib is one of the drugs that show phenotype selective with high affinity for the F1*S phenotype, where the AGP binding have shown to have impact on the therapy response [147, 148]. Altogether, the phenotype variation and multiple glycosylation states make AGP a very heterogeneous plasma protein.

The binding site of AGP is located inside the β-barrel, and is comprised of the three sub-pockets I, II and III in the F1*S phenotype, but only two sub-pockets for the A form, I and II respectively, due to conformational changes in the side chains of crucial sub-pocket III residues [137, 149]. However, the overall fold of

phenotype F1*S and A are close to identical with only minor rotation of a single α-helix [149]. The binding pocket of the F1*S phenotype is of size 9-12 Å in diameter and with a depth of approximately 18 Å [137]. Sub-pocket I is a large and apolar pocket located in the middle of the AGP binding site, and is flanked on each side by the negatively charged and smaller sub-pockets II and III [137]. The sequence substitutions induce alteration both in the entrance area and inside the binding site pocket, making it more narrow and changing polarity pattern, see Figure 14 [149].

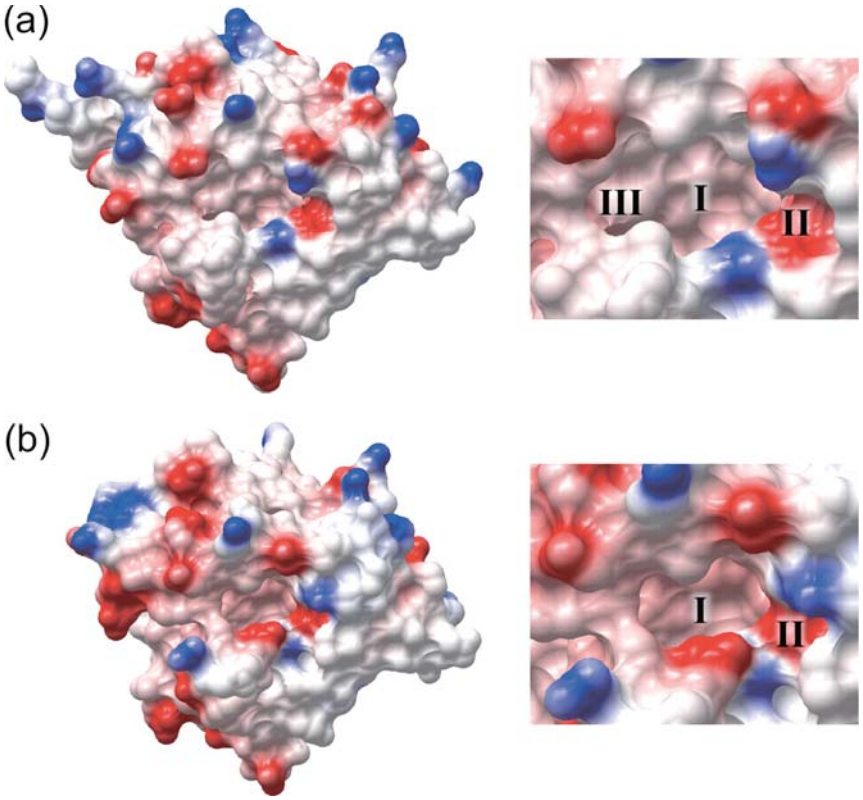

Figure 14. The figure shows the comparison of the phenotype F1*S and A of AGP, in a) for phenotype F1*S and in b) for phenotype A respectively. Both the pattern of polar residues and shape of the binding pocket are altered, with the binding site of phenotype A lacking sub-pocket III due to changes in side chain conformations of sub-site III residues. The binding site is located in the middle of the figures, with the binding site section viewed to the right for closer inspection. The electrostatic surface potential is shown for both phenotypes. Reprint from Nishi *et al.* 2011 [149], with permission from the American Society for Biochemistry and Molecular Biology.

1.5 Methods for studying small molecule-protein interactions

There are a number of both experimental and theoretical approaches for studying non-covalent small-molecules protein interactions. Where each methodology has advantages, drawbacks and artifacts in the resulting data, and differences in areas of utilization. High throughput methods may be able to process large screenings at low costs on the expense of the accuracy in the resulting data, and low throughput methods may provide highly thrust-worthy data but have high computational costs or the need of large amount of high quality materials. Most studies combine a selection of methodologies that are suitable and available for the end-point requirements and the cost limit of the project. Corresponding theoretical and experimental methods can be linked to each step of ligand-protein interaction studies, for example virtual screening (VS) and high throughput screening (HTS) for compound library mining, molecular docking and crystallization or NMR co-complexes studies for detailed structural interaction information, molecular dynamics simulation (MD) and linear interaction energy (LIE) calculations and microcalorimetry study to investigate the thermodynamic of the binding, to mention a few. However, each project will require a set of methodologies that are appropriate for its means, hence experimental HTS will be required for screening bio-prospecting compound libraries and VS for mining virtual libraries for novel ligand scaffolds with affinity to novel drug targets. A short presentation of commonly used theoretical and experimental methodologies is given in section 1.5.1 and 1.5.2 respectively, but detailed descriptions are considered outside the scope of this thesis.

1.5.1 Theoretical methods for studying ligand-protein interactions

VS is a powerful high throughput theoretical method for data mining of large ligand-libraries, with the purpose of filtering the libraries down to a manageable size of prioritized compounds [150]. The filters can be selected for identifying similar compounds as known ligands based on 2D or 3D searches (ligand-based), or to be complementary to a binding site structure in high throughput molecular docking (structure-based). VS allows for searches through huge compound

libraries with the drawback in poorly estimated docking scores and with low reliable of the compounds ranking orders, hence producing a high number of false positives. These drawbacks can be reduced by applying a second and more accurate method for assessing the resulting VS data. The more accurate methods can be molecular docking with more descriptors incorporated, or simulation approaches as free energy perturbation (FEP) or LIE calculations [150]. The extensive sampling needed for FEP and LIE is usually done with either MD or Monte Carlo simulations. Adding more descriptors and working on a single system at the time decreases the throughput dramatic and adds to the computational costs. Iterative rounds of filtering may be appropriate before entering the most resource demanding methodologies. Other theoretical approaches are the development of a pharmacophore, the ensemble of features of steric and electronic descriptors that is necessary traits for a successful ligand interacting with a specific target [151]. Pharmacophores may be ligand-based or structure-based and be used beneficially with a range of other modeling methodologies as VS, MD simulations, molecular docking and hot spot analysis [151].

1.5.2 Experimental methodologies for ligand interaction studies

In the experimental approaches HTS using a range of biophysical or biological assays may be applied for searching vast ligand libraries for active compounds [152]. As with the theoretical approaches, the hits discovered in HTS step are processed by more accurate means to check for false positives and more detailed information. These can be either biophysical or biological assays as cell and enzymatic assays, isothermal titration calorimetry (ITC), surface plasmon resonance (SPR) or structural studies within crystallization or NMR methodologies [152, 153]. The cellular and enzymatic assays apply in general a reporter signal, which can either be colorimetric, fluorescence, or bioluminescence that is detectable and corresponds to the desired activity. SPR is used to investigate the kinetics and measures the on and off rates of compounds that bind to an immobilized binding partner. An electric field is generated near the metal surface by the plasmon, and changes in this field can be

measured as a change in the refractive index. The alteration in refractive index is due to that the shape of the curve and the angular position are sensitive to the dielectric medium near metal surfaces, and a binding will represent such a change, see Figure 15 [154]. ITC methodologies are presented in the subsequent section 1.5.3.

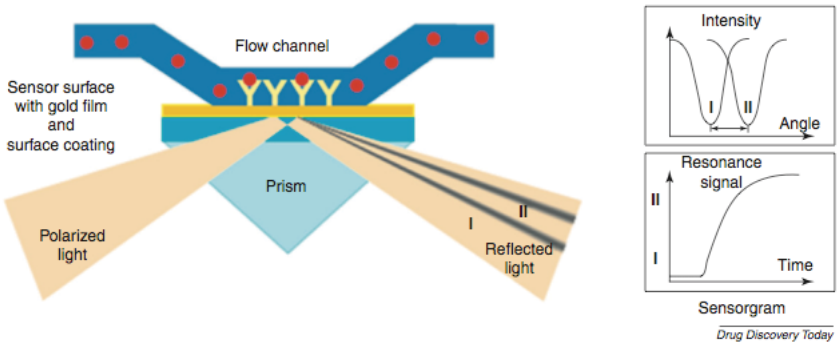

Figure 15. The figure shows the principle behind a SPR sensor, with the immobilized target protein and the ligand being flushed past in a microfluidic flow channel. The plasmon is generated when the incident light is absorbed, and a decrease in the reflected light is observed. The plasmon generates an electric field close to the metal surface that is sensitive to changes in the chemical environment close to the metal surface, and affects the conditions of the light coupling with the plasmon. This can be seen as a change in the angle or wavelength, and that the light are being absorbed rather than reflected. Reprint from Nunez *et al.* 2011 [153], with permission from Elsevier.

A range of NMR methodologies can be applied in powerful mapping of ligand-protein interactions. The general idea behind these methods is that a signal can be transferred from- or via the protein to ligands that are bound. The water-ligand observation with gradient spectroscopy (WaterLOGSY) exploits that selective magnetization of bulk water is transferred through the protein to the bound ligand, which retains the signal after dissociation and can be detected [155, 156]. WaterLOGSY is useful for screening purposes of compound-cocktails as well as qualitatively estimating binding constants by titration or competitive experiments [156]. The pathways of transferring the magnetization from bulk water through the protein, and at last to the ligand are shown in Figure 16. These pathways include water molecules in the ligand-protein interface and chemical exchange of protons with carboxyl, amino, hydroxyl, imidazole, guanidinium and amide groups of the protein.

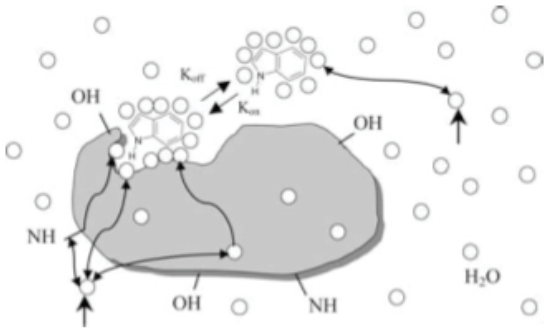

Figure 16. Water molecules represented by circles are being magnetized indicated by straight arrows and transferred via the protein to the ligand in the WaterLOGSY method. The different pathways the signal can be transferred to the ligand are indicated in curved arrows. Reprint from Dalvit *et al.* 2001 [156], with permission from Springer.

Another NMR based approach is the inter-ligand NOE for pharmacophore mapping (INPHARMA), where the signal is transferred between competing ligands for the same binding site, and can be used to determine binding modes of a novel ligand if the reference compounds interaction mode is already know [157]. Saturation transfer difference (STD) is a third NMR approach, where the protein is selective magnetized and the signal is transferred to protons of the ligand that are in close contact with the protein. Hence STD gives an indication of which groups of the ligand that are interacting with the protein and is the source of group epitope mapping (GEM) of the ligand [158].

Crystal structures of ligand-protein complexes are in general looked upon as the benchmark of interaction studies, where the 3D coordination of the binding partners can be determined at atomic levels, yielding high-resolution information. However, the dynamic aspect of the interactions is lost in crystallography methods. The two crystallographic methods of obtaining complexes are the co-complex experiments where the ligand is added to the protein prior to crystallization, and the soak method where protein is first crystallized and the ligand is soaked into the crystal via water channels. However, these methods acquire crystallizable proteins and robust crystals for the soaking method. The artifact of disadvantageous crystal packing and the lack if dynamic information are drawbacks, but dynamic information may be rescued if combined with MD simulations, FEP, or ITC.

1.5.3 Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) is an experimental microcalorimetry method, which maps the complete thermodynamic profile of a binding interaction. In short the instrumentation relies on a signal from a reference feedback power based on the difference in temperatures between an inert reference cell and a sample cell where a binding partner is titrated into its interaction partner [159]. The formation of a ligand-protein complex will be linked to a change in the free energy ΔG between the unbound and bound state of the protein and ligand, either causing a release or absorbance of energy measured as a change in temperature. This causes either a decrease or an increase in the applied feedback power of the sample cell. The sample and reference cells are kept isolated in an adiabatic jacket to avoid influence by external heat sources, see Figure 17 for schematic representation of the instrument.

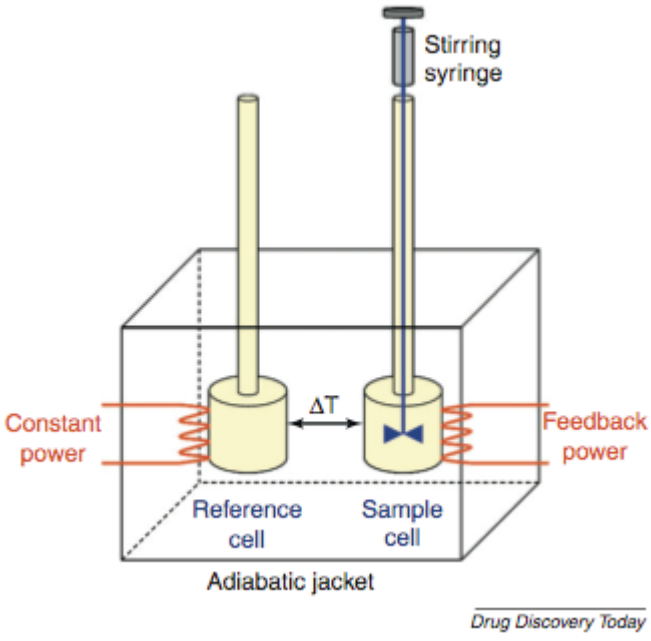

Figure 17. Schematic representation of the reference and sample cell located in the adiabatic jacket of an ITC instrument. A constant power is applied to the reference cell, and a feedback power to the sample cell. The heat corresponding to a binding interaction is indirectly measured in the differential of the temperature between the sample and reference cells as a decrease in the feedback power for an exothermic reaction and as an increase in the reference power for an endothermic reaction. Reprint from Nunez *et al.* 2011 [153], with permission from Elsevier.

By integration of the peak area when power is plotted against time, followed by non-linear regression and the known ligand and macromolecule concentrations, the entire profile of the enthalpy ($\Delta H°$), the stoichiometry (n) and the affinity (K_a) can be determined from a single multi-titration experiment [159]. However, the heat measured will be a combination of all reactions taking place in the sample cell, with contributions form the binding interaction itself, the dilution heat of the ligand, the dilution heat of the macromolecule and the heat from the mixing process. The dilution of the macromolecule and the heat originated by stirring is regarded as insignificant as the increase of volume by the titrations is small compared to the total volume, and that mixing heat is regarded as a constant contribution. This renders the main contributions to the measured heat to be the binding interaction itself and the dilution process of the ligand. To remove the dilution heat of the ligand, control experiments of ligand titrated into buffer is performed and subtracted from the total heat signal.

The enthalpy is a measure of changes in total hydrogen bonds, and dependent on the total net gain and loss of number of bonds, and the strengths of these bonds. The entropy contributions are dependent on the release of water molecules from the binding surfaces, whereas entropy penalties are created by restrictions in conformations [159]. The stoichiometry requires that both the ligand and the macromolecule concentration are known, and it is calculated based on the depletion of total ligand upon the macromolecule being gradually saturated by each titration. The asymmetry in macromolecule and ligand concentration and saturation pattern also forms the basis of the estimation of $\Delta H°$. The multi-titration experiments described above are most commonly associated with ITC, but other areas of use are single titration experiments and kinetics experiments, that can be performed with the same instrumentation but requires different experimental setup [160]. Additional areas of application for ITC are quality control of protein preparation and activity of construct variations, validation of the biological activity of structurally derived complex information and proton transferring in interaction systems [159].

The thermodynamic profile can add valuable information in which compounds to pursue in lead optimization, by being applied as guidance in selection processeses of which of the binding ligands that have the highest potential in being further developed [161]. If only assessed by binding affinities, the differences in profile will in general be masked by enthalpy-entropy compensation, keeping the binding constants at the same range [162]. To obtain higher affinities, either the enthalpic or the entropic contributions to the interaction have to be increased. Added entropy is often acquired through the addition of hydrophobic elements, but problems with solubility and bioavailability are commonly encountered when this strategy is applied. The increase in the enthalpic contribution by incorporated polar groups is more difficult to obtain as both enthalpy penalty from desolvation of polar groups and entropy penalty from desolvation and conformation changes arises [162]. In small-molecule protein interaction studies it has been found that enthalpy driven interactions are preferred to pursue for further optimization, due to increased selectivity and potential of being developed into higher affinity leads [161].

2 Aims of the study

The field of drug discovery was dominated by serendipity until the middle of the 20th century. The knowledge of cause of disease and effect of drugs were very limited as detailed information about biology systems at molecular level was not available. The emerging of scientific fields as molecular and structural biology opened an era of previously in-conceivable possibilities in the development of drugs. Molecular biology made it possible to validate and produce drug targets through work at the genetic level, as cloning and production of hypothetical drug targets, and the creation of transgenic animals. Structural biology explained why drug targets failed to conform in the biologic system, and how drug molecules can modify the activity of such targets upon binding. The latter part is better known as the structure-activity relationship (SAR). Bioactive compounds in drug discovery projects may have their origin from bio-prospecting or be novel synthetic compounds developed by chemists, or in a combination be synthetic modified compounds originated from isolated natural products. The pharmacokinetics of drug candidates, the evaluation of possible challenges regarding drug administration, distribution, metabolism, excretion (ADME) and toxicity, has to be studied carefully before intended for used in humans. The aims in this study is summarized below:

1. Develop novel synthetic substituted piperidines with inhibitory activity toward the aspartic protease renin, which is a part of the renin-angiotensin-system controlling blood pressure in human.
2. Investigate the interaction of short cationic antimicrobial peptides (CAPs) with the plasma protein human serum albumin (HSA) in detail, including identification of binding site and suggested binding mode.
3. Study the effect of HSA binding on the potency of cytotoxic peptides with activity towards cancer cell lines, and investigate the interaction determinants in detail.
4. Elucidate whether CAPs would bind to the second major plasma protein alpha-1 acid glycoprotein (AGP), and investigate if this binding will affect the *in vitro* bactericidal potency.

3 Summary of papers

3.1 Paper I

Synthesis and docking of novel piperidine renin inhibitors.

Rianne A. G. Harmsen, Annfrid Sivertsen, Davide Michetti, Bjørn Olav Brandsdal, Leiv K. Sydnes and Bengt Erik Haug.

In this study a library of 15 novel 4-triazolyl substituted piperidine derivatives was synthesized and tested for inhibitory activity against renin, a drug target in the renin-angiotensin system (RAS). Renin belongs to the class of aspartic proteases, and is sole responsible for the cleavage of the rate-limiting step in the signal peptide cascade which regulate blood pressure in humans. Selectivity regarding other aspartic proteases was investigated by measuring inhibitory activity toward BACE1, which is a drug target for Alzheimer´s disease. To assist further development of these compounds a molecular docking protocol was established to assess the binding affinities *in silico*. The potency of the substituted piperidines was tested at 5 μM concentration for renin and 300 μM for BACE1 in endpoint FRET assays. All of the substituted piperidines showed inhibitory potency against renin with selectivity over BACE1. The inhibitory activity of the 1,5-disubstituted 1,2,3-triazole piperidine derivates showed highest inhibition of renin, and performed significantly better than the corresponding 1,4-disubstituted analogues. The IC_{50} for the most active compound (**9ga**) was found to be in the mid nM range as a racemic mixture. The modeled binding conformations suggest that the binding is stereochemistry selective for the *RR*-enantiomers, and that the compounds explore the S1 and S3 sub-sites of the binding pocket through their piperidine substituents.

3.2 Paper II

Synthetic antimicrobial peptides bind with their hydrophobic bulk elements to drug site II of human serum albumin

Annfrid Sivertsen, Johan Isaksson, Hanna-Kirsti S. Leiros, Johan Svenson, John Sigurd Svendsen, and Bjørn Olav Brandsdal.

Human serum albumin (HSA) is regarded as the main transporter protein in plasma due to its great promiscuity and binding capacity regarding small molecules, and its high concentration. HSA is known to have a ligand specificity for negatively charged and neutral lipophilic compounds, although exceptions to this generalization do exist. We have determined the binding affinity of a selection of short synthetic cationic antimicrobial peptides (CAPs) to be in the lower μM range by isothermal titration calorimetry (ITC), in agreement with previously results published by our group. By competitive ITC and WaterLOGSY NMR experiments we conclude that the interaction take place at the well-known drug site II located in sub-domain IIIA of HSA. We have mapped the interaction in more detail using NMR WaterLOGSY, INPHARMA and saturation transfer difference (STD) experiments. The NMR results unambiguously show that the peptides interact with their hydrophobic moieties, and that the binding is independent of the multiple cationic residues. To provide a visual binding model for the interaction, molecular docking was performed. The docking results support the experimental data, including the hypothesis of multiple binding modes of the peptides through interaction by several hydrophobic moieties to drug site II. The pharmacophore developed for the activity of CAPs requires multiple hydrophobic moieties to ensure bactericidal potency. We suggest that binding to HSA should be taken into careful consideration in future antimicrobial peptide studies, as the distribution will be highly affected by the interaction.

3.3 Paper III

Anticancer potency of small linear and cyclic tetrapeptides and pharmacokinetic investigations of peptide-binding to human serum albumin.

Veronika Tørfoss [*], Annfrid Sivertsen [*], Johan Isaksson, Trude Anderssen, Bjørn-Olav Brandsdal, Martina Havelkova, and Morten B. Strøm.

[*] The authors have a shared first authorship

In this study the cytotoxic potency of linear and cyclic tetra-peptides containing an achiral $\beta^{2,2}$-residue is reported. The cytotoxic effect of the peptides was tested in *in vitro* MTT assays for Burkitt´s lymphoma (Ramos cells), but also for healthy human lung fibroblast cells (MRC-5), and haemolytic activity of human red blood cells (RBC). A selection of these tetra-peptides and previously published hexa-peptides was investigated for possible interactions with the main blood transporter protein human serum albumin (HSA). The potency and selectivity of the cytotoxic peptides followed the same trends as reported for larger peptides, with a delicate balance in the lipophilicity-to-charge ratio. Applying isothermal titration calorimetry (ITC) we found that the peptides bound to HSA with a K_d of 22-28 μM, disregarding differences in size, linear or cyclic forms, or variation in the $\beta^{2,2}$-residue side chain. By addressing the main drug binding sites of HSA using a competitive ITC design, we determined the primary binding site to be drug site II. The interaction was studied in more detail using saturation transfer difference (STD) NMR, establishing a group epitope mapping (GEM) of the cytotoxic peptides. The hydrophobic moieties were found to be sole responsible for the interaction with drug site II, and hence the multiple cationic lysine residues did not contribute to the binding. *In silico* molecular docking suggested that it was only the outer part of drug site II that was accessible for the peptides due to size exclusion, and that multiple binding orientations were possible. When HSA was added in the cellular MTT assay for Ramos cells, a significant decrease in the cytotoxic potency was observed. Based on our findings, the necessity to investigate HSA binding for further development of peptides with cytotoxic activity toward cancer cells is emphasized.

3.4 Paper IV

Short cationic antimicrobial peptides bind to human alpha-1 acid glycoprotein with no implications for the *in vitro* bioactivity.

Annfrid Sivertsen, Bjørn Olav Brandsdal, John Sigurd Svendsen, Jeanette Hammer Andersen, and Johan Svenson

Plasma protein interactions with drugs may be beneficial or disadvantageous regarding pharmacokinetic properties. Previous studies by our group have illustrated the adverse effect of albumin binding on the *in vitro* activity of short cationic antimicrobial peptides (CAPs). The aim of this study was to investigate whether these CAPs would interact with the second major plasma protein, the alpha-1 acid glycoprotein (AGP), which is known to have a preference for positively charged and neutral lipophilic ligands. Due to this preferential, CAPs were expected to be custom ligands for AGP, and the binding was anticipated to exceed the affinity observed for albumin. Surprisingly, we found that the binding strengths were in the same order for both plasma proteins, but when included in cellular assays AGP did not interfere with the bactericidal potency of CAPs. AGP was included in the assays at the normal physiological concentration, and additionally at the increased acute phase concentration for selected active CAPs. The low stoichiometric ratio measured with isothermal titration calorimetry (ITC) indicated that only a fraction of the total AGP concentration interacts with the peptides. This suggests a possibility for phenotype selectivity towards either the F1*S or A form of AGP. To study the possible selectivity in more detail molecular docking was performed with both phenotypes. The results suggest that the majority of the peptides were able to align the hydrophobic C-terminal capping group in sub-pocket I, and have an additional electrostatic interaction in sub-pocket II of AGP. Other favorable interactions were observed in sub-pocket III by the second hydrophobic moiety for a selection of the peptides. A second major conformation was observed for CAPs which contained a biphenyl (Bip) side chain. Here the Bip would interact with sub-pocket I, but in these conformations the additional binding in sub-pocket II was not seen. No conclusive phenotype selectivity was observed for either AGP forms, and further investigation will be necessary for explaining the low binding stoichiometry.

4 General discussion

4.1 *Trans*-3,4-disubstituted piperidine derivatives as non-peptidomimetic inhibitors for renin

For a long time the search for lead compounds in DRI development was based on peptidic and peptidomimetic molecules. As this direction did not result in clinical candidates reaching the market due to poor bioavailability and high production expenses, the attention was transferred to smaller non-peptidomimetic compounds. The introduction of 3,4-disubstituted piperidines as part of a renin inhibitory scaffold was successfully identified in the late 1990s, with the role of the piperidine nitrogen as an anchoring segment to the catalytic aspartate-dyad in the active site [163-165]. Further exploration of substituent building blocks have been conducted by several research groups leading to even more potent series of substituted piperidines, with activities in the lower nano- and pico-molar range [166-170]. Unfortunately, the high inhibitory potencies of these series have been coinciding with either low bioavailability, problems with metabolism, or off-target interactions with CYP 3A4 of the cytochrome P450 family, as well as inhibition of hERG. These very undesirable effects led to the termination of entire series of highly potent compounds [166-170]. However, a few promising lead molecules with satisfactorily profiles within potency, off-target interactions, metabolism and bioavailability properties were identified from these series for further drug development [167, 170].

4.1.1 Enantiomer selectivity of the piperidine derivatives favoring the *3R,4R isomer*

The potencies of the *trans*-3,4-disubstituted piperidine derivatives, containing a 1,4- or 1,5-disubstituted 1,2,3-triazole group, evaluated in this thesis have considerable lower potencies in the micro-molar range toward renin compared with the compound series referred to in the previous section. The project is although in its preliminary development phase, and the potential of this class of 3,4-disubstituted piperidines have not yet been fully investigated. The most promising compound identified in Paper I, **9ga**, obtained an IC_{50} of 631 nM for the racemic mixture, and exhibits though inhibitory potency in the mid nano-

molar range. It has been shown in several studies that enantiomers of piperidine derivatives differ in potency, hence stereochemical specificity is important for renin inhibitory properties [163, 166]. The stereochemistry selectivity of the *3R,4S* over the *3S,4R* isomer have been measured to vary with as much as 400 fold in buffer (0.22 nM *3R,4S* versus 92 nM *3S,4R*) and about 200 fold in plasma (12.3 nM versus 2615 nM) for a tested substituted piperidine derivative, whereas a second compound was reported with a 150 fold (0.24 nM vs 35 nM) difference in potency in buffer [166]. Differences in inhibitory activity for *3S,4S* and *3R,4R* enatiomers of 3,4-disubstituted piperidines have also been reported, and resembles the stereochemistry of the racemic library of piperidine derivatives presented in Paper I. The selectivity for this system have been reported to be around 45 fold (IC_{50} as 26 μM versus 1200 μM, and the racemic mixture with an IC_{50} of 50 μM) in favor of the *3R,4R* eneantiomer [163]. The selectivity for the *3R,4R* isomer was also observed visually in the crystal structure of the renin-piperidine derivative complex for this ligand. In our molecular docking data, indications of stronger binding affinities for the *3R,4R* enantiomers were observed, as the docking scores for this enantiomer were the only ones that corresponded with the experimentally measured affinities. Therefore, it is expected that the two enantiomers should have different inhibitory activities if separated and individually tested in the enzymatic assay. The estimation of such variation in inhibitory activity for the tested compounds is highly speculative. However, a calculation with the most modest ratio difference of 45 fold in activity for the **9ga** enantiomers in Paper I yields the estimated values of: IC_{50} of 330 for the *3R,4R* isomer, IC_{50} of 14940 nM for the *3S,4S* isomer based on the IC_{50} racemic value of 631 nM, applying the ratio for the racemic mixture and enantiomers in [163]. But the numbers may differ significant from this rough estimation when tested.

4.1.2 Selectivity and possible off-target interactions with other aspartic proteases

The compounds were shown to possess selectivity for renin over a second aspartate protease, BACE1, and hence indicate selectivity within the class of asparatate proteases for our novel piperidine derivatives. However, substituted piperidines have also been identified and developed as BACE1 ligands, with the

most active compounds reported with inhibitory activities in the lower nano-molar range [171, 172], and also been patented as ligands to plasmepsins with activities in the upper nano-molar range [39]. In our results we observe a clear selectivity for renin over BACE1, with no compound showing inhibitory activity above 50% at the tested concentration well above the range of renin inhibitory concentrations. This result indicates that our compounds at least obtain partially selective within the class of aspartate proteases. If the project is developed into improved compounds with higher affinities, the selectivity property and possible off-target implications should be tested against the relevant housekeeping aspartic proteases Cathepsin D and E [22, 41].

4.1.3 Potential of exploring the chemical space of the piperidine substituents

The main focus in the design of our novel *trans*-3,4-disubstituted piperidine serie has been in the exploration of the 1,2,3-triazol di-substitution pattern at the 4-position. Less attention has been paid to the variation in the group at the 3-position of the piperidine, where only the size of the aromatic substituents and the linker group has been varied between ether or ester. For further progress, it is recommended to explore a larger variety of functional groups in the 3-position for evaluating the potential of the project. Also the structural element attached in the meta-position of the benzyl in the 1,5-disubstituted 1,2,3-triazol could be explored further to investigate whether the inhibitory effect could be increased.

4.1.4 Other possible off-target interactions

Although off-target interactions within the aspartate protease family may be regarded as satisfactory, the CYP 3A4 and hERG inhibition, but also the poor bioavailability, observed for close to all 3,4-disubstituted piperidine series developed so far is disturbing. If the project results in more potent compounds, these off-target interactions are key aspects that have to be investigated and considered for future design strategies. Nonetheless, it is encouraging that promising lead molecules has been produced successfully by other groups by exhaustive exploration of the piperidine substitution pattern.

4.2 Implications of albumin binding of antimicrobial and cytotoxic peptides

Albumin binding have in a previous study been found to interfere with the *in vitro* bactericidal activity of short cationic antimicrobial peptides (CAPs) [80]. The binding strengths in the micro-molar range were not expected as the ligand preferential of HSA is for negatively charged and lipophilic compounds. Also the CAPs characteristic as carrier of multiple cationic charges is thus contrary to what is considered custom HSA ligands. Together with the larger size compared with small drug-like molecules that are commonly reported as albumin ligands, the CAPs were not considered strong albumin binding candidates. Nonetheless, although the binding and the *in vitro* effect of albumin interaction was determined in this previous study, detailed knowledge of the interaction determinants of the CAPs, being predisposed as poor albumin ligands, was not well understood.

4.2.1 The effect of plasma protein binding on the pharmacokinetics of peptides

The antimicrobial activity of CAPs have been considered satisfactory for a member of this peptide class, LTX 109 (**CAP 4** in Paper II), to enter clinical phase II trials for topical administration against infections by multi-resistant bacteria [74]. The restriction in areas of utilization lies thus in the limitation of ADME and toxicity properties of the CAPs, and not in the antimicrobial potency. Hence the investigation of plasma protein interaction is required for estimating possible interference in the therapeutic effect if transported in the systemic distribution system. Plasma protein interaction may not solely be considered as a negative effect, as with the decrease in the concentration of the free circulating active compound, the systemic half-life will increase as *in vivo* clearance rates are reduced. The incorporation of HSA binding tags into peptides has also been examined as an approach for systematically increasing the circulating half-life for potentially use in therapeutic peptides [134]. In general, all plasma protein-binding drugs are being shielded from metabolism and clearance, but for peptide and protein based drugs an additional advantage is protection from degradation by proteases. As the dynamics of biological systems is not incorporated, the

compensation of the reduction in activity by the longer half-life in plasma makes adverse effects observed for *in vitro* plasma protein binding not directly transferrable into *in vivo* settings [120]. Increased solubility in plasma is also a favorable effect for compounds that are highly hydrophobic, although solubility issues do not apply for the CAPs used in Paper II.

4.2.2 The CAPs binding mode hypothesis with drug site II of HSA

In Paper II, the interaction of CAPs to HSA has been thoroughly explored by a range of experimental techniques, including ITC and NMR. In addition molecular modeling-approaches have been applied in forming the final binding mode hypothesis. The binding was found to take place at the known drug site II in sub-domain IIIA of human albumin by observation of competition with a drug site II ligand. The interaction was shown to be solely dependent on the hydrophobic moieties in the peptides by coinciding results from multiple methodologies. However, only a single hydrophobic moiety of the CAPs molecular structure was interacting with the binding site at a time, but multiple binding modes were observed. This indicates that the CAPs can bind with both of the hydrophobic moieties comprised in the molecular structure, and that two binding modes exist for each peptide. An exception to this promiscuity was observed in the docking experiments with the bulky tri-tert-butyl tryptophan (Tbt) side chain present in the sequence, replacing an un-subtituted indole group of tryptophan as seen for **CAP 4** and **CAP 2** in Paper II respectively. For **CAP 4** (Tbt side chain), the only binding mode observed in the docking data was by interaction with the benzyl C-terminal capping group. This may be a size exclusion mechanism that can be further explored and utilized if decreased HSA affinity is desired in future projects.

4.2.3 How the CAPs circumvent their own un-favorable HSA ligand profile

The identification of drug site II as the CAPs binding site among the numerous sites of HSA was highly unexpected, with its preferential for peripherally negatively charged ligands. In comparison with the other major small-molecule binding site of HSA, drug site I located in sub-domain IIA, drug site II is both smaller in size and less dynamic than its counterpart due to different packing

environment of otherwise topological similar sub-domains [130]. The CAPs are interacting with drug site II by one of the aromatic hydrophobic groups aligned into the binding site, while the rest of the peptide is exclusively located outside the binding site. The solvent states of the cationic arginines of the CAPs are considered similar in the free and HSA bound state of the CAPs, and are therefore neither contributing nor opposing the interaction energetically.

4.2.4 HSA interaction of cytotoxic peptides, similarities and differences with CAP binding

The even larger peptides with cytotoxic activity toward cancer cells of the lengths of tetra- and hexamers in Paper III, were observed to be HSA ligands with similar binding modes as the CAPs in Paper II. Also for the cytotoxic peptides, a single hydrophobic moiety was observed to interact with the outer part of drug site II at a time, while the majority of the peptide was located in the solvent phase. Compared with the CAPs binding mode, the interaction of the cytotoxic peptides was located further out in the binding site, most likely due to the increase in size from tri-peptides to tetra- and hexapeptides, decreasing the overall flexibility of the peptide backbone. This reduction in flexibility would make it difficult for the hydrophobic binding group to be aligned deeper into drug site II. The interaction with HSA was not observed to change with peptide length or within linear or cyclic versions of the same sequences. Neither a variation in the achiral $\beta^{2,2}$-residue was found to have any impact on the interaction with HSA. Similar with the CAPs, the cytotoxic peptides were not found to be restricted to a certain hydrophobic element for forming the interaction, but were able to utilize either one of the $\beta^{2,2}$-residue side chains or the tryptophan residue as their anchoring element. When HSA was added in the MTT *in vitro* assay with Ramos cells, the cytotoxic activity for all of the tested peptides was significant reduced. The decrease in activity was observed even if less than half of the normal physiological concentration of HSA was added in the assay media. The reduction in potency was observed to be 3-14 fold, with the highest affected being the cyclic peptides.

4.2.5 Possible *in vivo* implications of HSA binding for the two peptide classes

The vast number of drugs currently in clinical use that exhibits greater than 98% binding to plasma proteins shows that interacting with plasma proteins are not equivalent to being excluded as therapeutic agents with distribution by the circulatory system [120]. Although the peptide binding modes for both the CAPs and the cytotoxic peptides showed great similarities, the *in vivo* implications for systemic distribution may differ for the two peptide classes. If assumed that the expected increase in systemic half-life does not compensate for the reduction in activity, the *in vitro* experiments indicate that the systemic distribution will be similar affected by albumin binding by a decrease in activity. However, to what extend the therapeutic activity will be affected by plasma protein binding also rely on the therapeutic indices of the compounds. As a too low concentration than necessary for therapeutic usage of antibiotic drugs would in general enhance the probability of evolving resistance, resistance to CAPs have so far not been reported [74]. Other factor that should be included is the toxicity level of CAPs, and if an increase in concentration due to the decreased acute phase concentration of HSA would be problematic due to metabolism, excretion, or off-target interactions. The activity and selectivity properties of the cytotoxic peptides have been shown to be in a delicate balance, as the highly active peptides are observed to loose their selectivity properties for cancer cells over normal healthy cells [114, 115]. This implies that there is a larger probability for encountering toxicity issues for these cytotoxic peptides *in vivo*. One way of evading non-selectivity and toxicity is to administrate the peptides directly into the tumors by injection rather than intravenous or oral treatment. This will however limit the therapeutic use to cancer forms to tumors that are accessible for such injections. Applying this administration strategy, the albumin binding may be beneficial as it would ensure absorbance of leakages and protect neighboring tumor tissue from being harmed by the toxic activity of the peptides.

4.2.6 The potential of cytotoxic peptides as anticancer agents; treatment, immunity and vaccination

As the cytotoxic peptides in Paper III are found to kill cancer cells by necrosis, immunity toward the same cancer type as the eradicated tumor may be triggered as seen in mice study with other peptides with necrotic activity [107, 108, 115]. This immunity which is observed in peptide-treated rodents appear as a sharp contradiction with previous knowledge of relapses based on intrinsic cancer cell phenotype resistance, and evolving resistance to chemotherapy treatment regimes [93]. An additional beneficial of eradicating cancer cell by a membrane based mechanism, is the possibility to be effective on quiescent cancer cells, the "cancer stem cells", that due to cell cycle arrest is not proliferating, and hence is not targeted by either conventional or targeting chemotherapy agents [93]. As with the antimicrobial peptides with mechanisms that acts on the bacterial cell membrane, the extensive measures for altering the membrane composition to obtain resistance has to be acquired by cancer cells. The majority of cancer drugs affect intracellular targets, and some of the resistance mechanisms involved with these types of resistance, as the multi-drug efflux pumps, are not expected to affect agents with mechanisms that destabilize membrane integrity. Although, by no means should resistance toward these peptides be regarded as infeasible, as intrinsic resistance phenotypes with down-regulation of negatively charged membrane components may exist as it does for microorganism phenotypes with insensitive to AMPs. Tumors may also be comprised of heterogeneous assembles of phenotypes, including insensitive cancer cell types.

4.3 Binding of CAPs to alpha-1 acid glycoprotein

The CAPs peptide class, as positively charged compounds with hydrophobic elements, was prior to experimental testing thought to be superior AGP ligands compared with HSA binding, as they fit the ligand preferential profile for this plasma protein. However when tested, we observed similar binding affinities toward AGP as found for HSA. More surprisingly when included in the *in vitro* assays, AGP was observed to have no significant effect on the bactericidal activity, even at the enhanced acute phase mimicking concentration where AGP was increased threefold.

4.3.1 Low stoichiometric ratios indicate phenotype specific binding to AGP

The lack of implication for the *in vitro* bioactivity was identified as the low stochiometric ratio of the binding, an indication that only a fraction of AGP was binding to the CAPs. The normal physiological amount of AGP in individuals is expressed as a mixture of different phenotypes, with the major ones being the F1*S and A forms, but others do exist. The structural differences of the forms can be summarized with the A phenotype binding pocket being narrower by closing off the sub-pocket III, as well as changes in the pattern of polar areas compared with the F1*S [149]. The CAPs were found to form beneficial interactions with the sub-pockets I, II and III of the AGP binding site in the molecular docking results for F1*S, and with sub-pockets I and II of the A form. The hydrophobic interactions with sub-pocket I was observed to be crucial for maintaining AGP binding for the CAPs, and was found for both phenotypes. Either the aromatic C-terminal capping group or the Bip side chain of the hydrophobic residue was seen to form this interaction. The additional electrostatic interaction in sub-site II between a cationic residue and Glu64 was not found to be sufficient to retain AGP affinity if the capping group was missing, as observed for **CAP 1**. If the interaction in sub-pocket I was provided by the Bip side chain the additional electrostatic in sub-pocket II was not present, but formed sufficient interaction to maintain the affinity toward AGP as seen for **CAP 6** in Paper IV, which does not contain a benzyl capping group.

4.3.2 AGP binding is proposed to be insignificant for the *in vivo* activity of CAPs

There are not many detailed structural studies reported for AGP binding, and only a few experimental ligand-AGP complexes structures are available [137, 149]. So far, all of the available co-complexes have contained considerable smaller molecules than the CAPs. Although the energetic and docked modes of known small drug-like compounds have been thoroughly explored by others, AGP ligands that utilize all of the sub-site I, II and III at the same time have not been reported before [173]. The modeled binding modes of CAPs in the AGP binding pocket showed excellent complementary to the binding site, and thus the low stoichiometry is not explained by the resulting modeled binding modes. The docking results did not provide a clear indication of selectivity towards either of the F1*S or A phenotype of AGP, and thus the possible phenotype selectivity cannot be conclusively determined before experimental tested. However, our study presented in Paper IV clearly indicates that AGP binding of CAPs is expected to be insignificant *in vivo*, and that the main plasma protein that is expected to affect the *in vivo* activity of CAPs is HSA.

5 Concluding remarks

In this study, SAR knowledge has been applied in multiple drug discovery projects to ensure further progress. Novel compounds have been developed as direct renin inhibitors, representing an early phase of a drug discovery project, and the pharmacokinetic properties of two different peptide classes, one class with antimicrobial activity and a second class with cytotoxic activity toward cancer cells have been investigated. In the latter projects the focus has moved toward ADME and toxicity properties of the peptides, hence representing later stages in already well-established drug development projects.

In Paper I we showed that novel *trans*-3,4-disubstituated piperidines containing a 1,2,3-triazole were potent inhibitors for the hypertension target renin, with selectivity over a second aspartic protease BACE1. The 1,5-disubstituted 1,2,3-triazols were found to form more active compounds than their 1,4-disubstituted 1,2,3-triazol analogues. Molecular docking results indicate that the piperidine substituents explore the S1 and S3 sub-sites of the renin binding site. The most active compound **9ga** exhibited an IC_{50} of 631 nM for the racemic mixture. With the docking results suggesting a stronger affinity for the enantiomer with 3*R*,4*R* stereochemistry, higher inhibitory activities should be anticipated if the enantiomers were tested individually. However, to bring the project forward a range of initiatives are required as the piperidine derivatives presented in Paper I are not as potent as 3,4-disubstituated piperidines reported by other research groups. One of the first steps that should be initiated is the separation of the enantiomers, to verify if the activities of the isomers correspond to the differences in interaction observed in the molecular docking. The inhibitory activity of the active enantiomer, and not the racemic mixture, will give a more correct indication of the potential of these compounds as renin inhibitors. The enantiomer identification will also make it possible to produce a pharmacophore as starting point for further exploration of the chemical space of the piperidine substituents. Secondly, the binding hypothesis from the molecular modeling would benefit from verification by experimental techniques, as crystallization complex studies or NMR interaction studies. If a crystal structure of the complex

within respectable resolution and occupancy of the small molecule is obtained, significant information will be acquired. Key structural information of the system would include the exact positioning of the piperidine and the catalytic aspartate-dyad relative to each other, and the conformation of the flexible flap located above the active site of renin. There is also a possibility of identifying the stronger-binding enantiomer if a high quality structure is obtained and the stereochemistry of the bound isomer can be visually investigated. The available experimental complex would also form a validated starting point for further molecular modeling studies. The modeling output from this experimental structure should lead to more reliable and trustworthy *in silico* results, guiding the exploration of substituent patterns. If more potent piperidine derivatives of this class are synthesized, some of the severe problems other research groups have experienced with piperidine-based ligands should be addressed. This includes low bioavailability, the reported off-target interactions of inhibition of CYP 3A4 of the cytochrome P450 family and hERG, as well as selectivity over the housekeeping aspartic proteases Cathepsin D and E.

In Paper II-IV the interaction of two different peptide classes with plasma proteins have been investigated, for short cationic antimicrobial peptides (CAPs) and for peptides with cytotoxic activity toward cancer cells. In Paper II we found that CAPs interacted with drug site II of human serum albumin (HSA) with their hydrophobic moieties. The binding and binding site was validated by multiple experimental techniques including ITC and NMR, and further explored by molecular modeling. As hydrophobic elements are a part of the pharmacophore of CAPs, we suggest that HSA binding is close to inevitable and should be viewed as one of the pharmacokinetic properties of these peptides. The bactericidal activity of this peptide class is considered satisfactorily for clinical use, as LTX 109 (**CAP 4** in Paper II) has been in phase II clinical trials with topical administration for infections of multiresistant bacterial strains. The potential for further progress and expansion in areas of utilization lies though in the limitation in ADME and toxicity properties, with the main challenges being stability towards proteases, and toxicity issues. However, CAPs interaction with HSA are thought to decrease the *in vivo* activity, but also increase the circulation

time by protection toward protease degradation, metabolism and excretion. The main question is whether the necessary dosage concentration, regardless of administration route of oral or intravenous nature, will be in conflict with the toxicity level of CAPs. If the therapeutic index allows for it, HSA binding might not affect the potential for clinical usage requiring systemic distribution. Although, if reduction in HSA binding is desired, the development of increasingly larger and bulkier hydrophobic groups are expected to decrease the interaction with drug site II, as indicated for the Tbt side chain in **CAP 4**. However, this would most likely also decrease the selectivity for bacterial cell membranes over human cells, and increase the toxicity of the peptides.

Substantial larger peptides with an achiral $\beta^{2,2}$ amino acid incorporated with cytotoxic activity toward cancer cells were found to interact with drug site II of HSA in Paper III. No differences were seen between the HSA binding of tetra- and hexamers, or between the linear and cyclic forms, or with variation of $\beta^{2,2}$ amino acid side chains. The cytotoxic activity and selectivity determinants were found to be a delicate balance of the lipophilicity-to-charge ratio of the peptides as observed in previously reported studies. The *in vitro* potency of the cytotoxic peptides was observed to decrease when HSA was present in the assays, indicating that a negative effect in the activity should be anticipated due to albumin binding *in vivo.* The details of the interaction show a great promiscuity in which of the hydrophobic group the peptides uses to interact with drug site II. As with CAPs, albumin binding is expected to increase the circulation time by preventing degradation, metabolism and excretion. However, the cytotoxic nature of these peptides, and the relative low selectivity for cancer cells over healthy human cell types, will most likely give rise to a too small therapeutic index for utilizing systemic distribution as administration strategy. Off-target interactions are a general problem in cancer treatment, and are the cause of the harsh adverse effects experienced by patients receiving chemotherapeutic treatment. A possible advantage of albumin interaction can however be utilized if the peptides are administrated directly into tumors by injection. In this setting the absorbance by extravascular albumin would decrease and prevent damages to local healthy tissue surrounding the tumor.

In Paper IV the interaction to the second major plasma protein alpha-1 acid glycoprotein (AGP) was studied for the CAPs peptide class. The CAPs were found to bind a fraction of AGP with a similar strength as they interact with HSA. The *in vitro* effect of AGP binding in the antimicrobial activity was nonetheless shown to be insignificant, both at the normal physiological concentration and at the increased acute phase mimicking level. Molecular modeling with the different phenotype forms of AGP indicate two main binding conformations dependent on the nature of the side chain of the hydrophobic residue and the C-terminal capping group, where the interaction with sub-pocket I was shown to be the crucial binding determinant. Although the modeling showed some indication of phenotype selectivity, further studies are needed to conclusively explain the low stochiometric binding ratios observed. This study clearly indicates that AGP binding should not be considered relevant for decreasing the CAPs activities *in vivo* compared with the considerable larger effect of HSA binding observed in previous studies. Together with the significant lower physiological concentrations compared with HSA, it clearly indicates that the main plasma protein regarding *in vivo* effects is HSA. However, the AGP binding is of academic interest as the CAPs are exploiting the AGP binding pocket to a maximum, compared with the interaction with considerable smaller drug-like molecules investigated by others. For conclusively determine if phenotype selectivity is the reason for the low stochiometric ratios observed in the ITC data, affinity chromatography can be used to separate the F*S and A forms of AGP with subsequent binding investigation. Experimental structural studies of a complex could be performed either with crystallography or NMR methodologies for verification of the suggested binding mode. However, as the results of the present study clearly indicate that AGP binding is not expected to have any influence on the therapeutic activity of CAPs, it is difficult to justify more detailed studies of this interaction.

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Paper I

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Paper IV

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