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THE IN VIVO CHARACTERISATION OF A C-DOMAIN SPECIFIC ACE INHIBITOR

Thesis presented by

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for the Degree of Doctor of Philosophy

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DECLARATION

I, Sarah-Kate Sharp, declare that this thesis is my own, unaided work (except where acknowledgements indicate otherwise). Neither the whole work not part thereof has been, is being, or is to be submitted for any degree or examination at any other university.

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ABSTRACT

The renin-angiotensin system (RAS) is an enzymatic cascade that plays a critical role in the control of blood pressure and the pathophysiology of heart failure. The primary end product of the cascade is the vasoconstrictor peptide angiotensin II (Ang II), produced by the proteolytic activity of angiotensin-converting enzyme (ACE). The ACE protein is a zinc-dependent dipeptidyl carboxypeptidase comprised of two homologous domains termed the C- and N-domain. The C-domain is primarily responsible for the catalytic production of Ang II, while the tetrapeptide acetyl-seryl-aspartyl- lysyl-proline (AcSDKP) is predominantly cleaved by the N-domain, and both domains play a role in the metabolism of vasodilatory peptide bradykinin. In the event of myocardial infarction (MI), cardiac output and blood pressure decreases, resulting in activation of the RAS and an increase in both Ang II production and bradykinin metabolism. While initially compensatory, prolonged RAS activation has been shown to have long-term detrimental effects, and pharmaceutical intervention in the form of ACE inhibition is the first line treatment following an MI event.

The ACE inhibitors currently in clinical use target both domains equally, and it has been suggested that the major side-effects of this drug class are largely attributable to the inhibition of bradykinin breakdown. A novel C-domain selective ACE inhibitor lisinopril-Trp (lisW-S) incorporates a tryptophan moiety into the P2' position of the clinically available ACE inhibitor lisinopril. In the present pre-clinical study, the in vivo efficacy of LisW-S was characterised in a rat model. Initially, ex vivo inhibition assays were established to determine an effective dosage in rat plasma and a plasma concentration of 100 nM was found to be effective for 70% ACE inhibition. Additionally, these assays gave strong evidence of C-domain selective inhibition of rat ACE by LisW-S. Pharmacokinetic studies were then carried out to assess the bioavailability of LisW-S in the rat, and also to determine optimal delivery methods for achieving required dosages. The bioavailability of lisW-S was found to be 3.1%, and an effective dosage was achieved by continuous infusion at a concentration of 30 mg/ml LisW-S. As one primary therapeutic target of a C-domain selective inhibitor would be MI induced heart failure, the effect of LisW-S on RAS peptides was assessed in a rat MI model. Domain-selectivity of LisW-S *in vivo* was investigated by enzyme-linked immunosorbent assay quantification of AcSDKP. Treatment with LisW-S elicited no increase in AcSDKP levels, whilst lisinopril caused a significant increase in serum AcSDKP confirming the *in vivo* domain-selective action of LisW-S. . Quantification of ten Ang peptides in the RAS cascade and three bradykinin breakdown products was achieved with a recently developed

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LC-MS methodology at 1 and 7 days following MI induction in a rat model. Analysis of plasma peptide concentrations and ACE-mediated peptide generation verified the inhibitory potential of lisW-S. A significant 3.4-fold decrease in the Ang II/Ang I ratio relative to that in MI was observed with administration of lisW-S. Interestingly, reduced levels of AngII as a result of LisW-S administration were only observed at day 1, but significantly elevated levels of AngI were observed at both time points.

It should be noted that the limitations of this study include a low product yield of lisW-S as a result of the synthesis process as well as a low bioavailability *in vivo* as established in the pharmacokinetic studies.

The detailed in vivo characterisation of the C-domain selective ACE inhibitor LisW-S reported here establishes it as a promising alternative to current ACE inhibitor therapy and lays the groundwork for further functional analysis.

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Finally, to my fiancé Marnus: I can never thank you enough for your love, your support and your patience – but I will try my utmost.

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ABBREVIATIONS

Abz-FRK(Dnp)P-OH	Non-selective substrate (human ACE)
Abz-LFK(Dnp)-OH	C-domain selective substrate (human ACE)
ACE	Angiotensin-converting enzyme
AcSDKP	N-Acetyl-Ser-Asp-Lys-Pro-COOH
ADH	Antidiuretic hormone
AFU	Arbitrary fluorescent units
Ang	Angiotensin
ANP	Atrial natriuretic peptide
AP	Aminopeptidase
APA	Aminopeptidase A
APN	Aminopeptidase N
ARB	Angiotensin receptor blocker
AT_1R	Angiotensin II type 1 receptor
AT_2R	Angiotensin II type 2 receptor
AT_4R	Angiotensin II type 4 receptor
AUC	Area under the curve
B_1	Bradykinin type 1 receptor
B_2	Bradykinin type 2 receptor
BK	Bradykinin
BQL	Below quantifiable limits
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CHF	Congestive heart failure
CNS	Central nervous sytem
COX	Cyclooxygenase
CVD	Cardiovascular disease
DAP	Diaminopeptidase
DMSO	Dimethyl sulfoxide
DRI	Direct renin inhibitor
ECM	Extracellular matrix
EIA	Enzyme-linked immunosorbent assay
FRET	Fluorescent resonance energy transfer
HCl	Hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic Acid
HPLC	High performance liquid chromatography

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IV	Intravenous
JG	Juxtaglomerular
KCl	Potassium chloride
KKS	Kallikrein-kinin system
KO	Knockout
LAD	Left anterior descending artery
LC-MS	Liquid chromatography - mass spectrometry
LisW-S	The novel ACE inhibitor lisinopril-tryptophan
LV	Left ventricle
Mca-Ala	N-domain selective substrate (human ACE)
Mca-$BK_{(1-8)}$	C-domain selective substrate (human/ rat ACE)
MI	Myocardial Infarction
mRNA	Messenger ribose nucleid acid
MS	Maleate salt
NaCl	Sodium chloride
NEP	Neutral aminopeptidase
NO	Nitric oxide
NOS	Nitric oxide synthase
NSB	Non-specific binding
PGE_2	Prostagalndin E2
PGI_1	Prostaglandin I1
PK	Pharmacokinetics
PRR	(Pro)renin receptor
RAAS	Renin-Angiotensin-Aldosterone system
RAS	Renin-Angiotensin system
ROS	Reactive oxygen species
RXP 407	N-domain specific ACE inhibitor
RXPA380	C-domain specific ACE inhibitor
sACE	Somatic ACE
ST DEV	Standard deviation
tACE	Testis ACE
TFA	Trifluoroacetic acid
TGF	Transforming growth factor
Tris	Tris(hydroxymethyl)aminomethane
WT	Wild-type

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Chapter 1 REVIEW OF THE LITERATURE

1.1 THE RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM AND MYOCARDIAL INFARCTION

The Renin-Angiotensin-Aldosterone system (RAAS) – often referred to as the Renin-Angiotensin system (RAS) - is an enzymatic cascade that plays a critical role in the control of blood pressure, tissue perfusion and volume homeostasis. It is stimulated primarily in response to a systemic loss of blood pressure (hypotension) or volume (hypovolemia) (Reid et al. 1978).

The basic RAS pathway is presented in figure 1.1 and is now textbook knowledge. It is initiated by the release of the enzyme renin from the kidneys. The discovery that renal extracts contained a pressor compound occurred more than 100 years ago, and so renin was named for its origin (Tigerstedt et al.1898). By 1957, the nature of renin's ability to induce hypertension had been successfully clarified by a number of research groups, and the major peptides involved in the mechanism isolated and identified (Basso et al. 2001). It is now known that renin acts proteolytically on the hepatically-derived circulating substrate Angiotensinogen, resulting in the formation of the inactive precursor Angiotensin I (Ang I). Ang I is in turn hydrolysed by Angiotensin Converting Enzyme (ACE) to form the potent vasoconstrictor Angiotensin II (Ang II) (Erdos et al. 1967). The actions of Ang II counteract the stimuli for renin secretion in the first place - namely hypotension and hypovolemia – primarily by causing constriction of the peripheral vascular system. Additionally, secretion of aldosterone is stimulated to increase sodium reabsorption and water retention (Bartter et al. 1956). The system is controlled by a central negative feedback mechanism. Ang II suppresses renin secretion both directly (by its action on renin-producing cells in the kidney) and indirectly (through its hypertensive effects) (DeMello et al. 2009).

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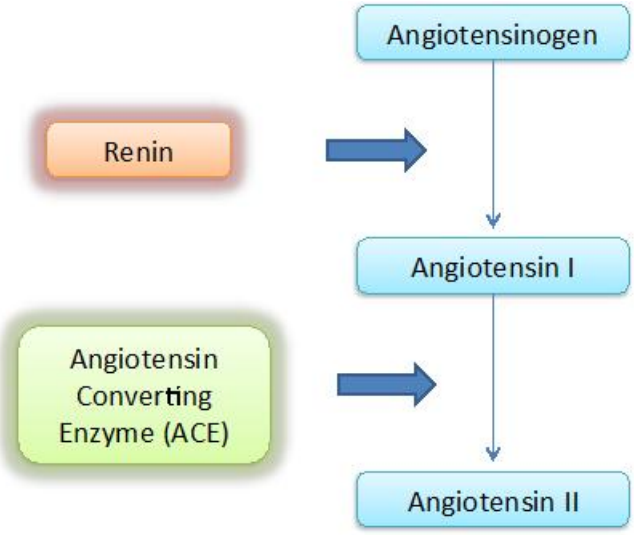

Figure 1.1 The renin angiotensin system pathway

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However, it has recently come to light that this classic view of the RAS is overly simplified and incomplete. It has also been found that the RAS is active not only at a systemic level, but also at both tissue (Paul et al. 2006) and intracellular (Kumar et al. 2007) levels. The classic RAS pathway has thus been modified to include all presently known peptides, pathways and feedback mechanisms. While Ang II is still recognised as the main effector peptide of the RAS cascade, it is by no means exclusively responsible for the physiological effects of RAS activation. The more complete view of the RAS is presented later in figure 1.10.

As a consequence of its primary regulatory role of systemic haemodynamics, the RAS cascade plays a critical role in the pathophysiology of cardiovascular disease (Cohn 2010). Cardiovascular diseases (CVDs) include a number of blood vessel and heart disorders, of which coronary heart disease is one of the most prevalent. According to the World Health Organisation (WHO), approximately one third of deaths globally result from CVDs, and so heart disease is now recognised as one of the leading health problems in the world (WHO 2011). In 2001 an estimated 16 million deaths worldwide were attributed to CVDs, and most alarmingly - and contrary - to previously held views on the prevalence of CVDs, some 13 million of these were estimated to have occurred in low- to middle-income countries, as opposed to just 3 million in higher-income developed countries (Mathers et al. 2006). Indeed, the burden of CVDs has now spread to regions such as China, India and many Sub-Saharan African countries, where the estimates of CVD-related deaths are known to be drastically under-reported due to a lack of accurate data collection (Murray & Lopez 1997). Sub-

Saharan Africa in particular has experienced a dramatic rise in the incidence rate of CVDs and CVD-related deaths, a large proportion of which are HIV-associated heart diseases such as pericardial tuberculosis and cardiomyopathy. Two observational studies have shown that there is a higher rate of CVDs and MIs in HIV-infected patients (Triant et al. 2007) (Lang et al. 2010). This population is further affected by insufficient access to anti-retroviral therapies which preserve immune function and thus reduce the incidence of myopericardial disease, as well as pulmonary hypertension (a known cause of heart disease which will be explored in detail further on) (Ntsekhe & Mayosi 2009; Sliwa et al. 2012).

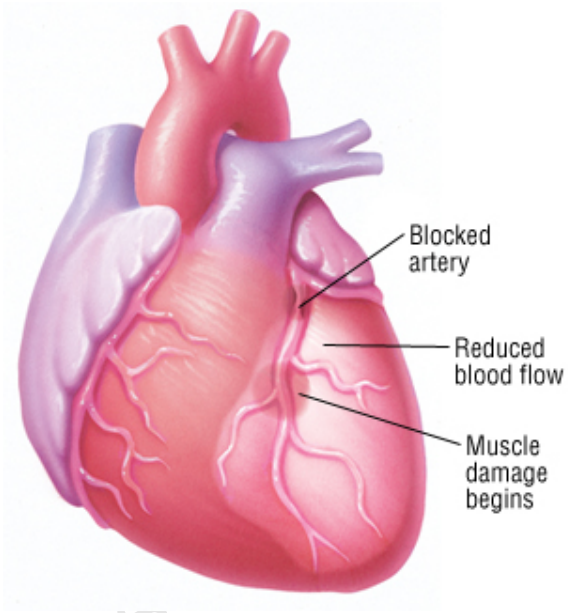

Figure 1.2 Arterial occlusion leads to Myocardial Infarction*(from:http://www.drugs.com/health-guide/heart-attack-myocardial-infarction.html)*

Heart disease is most often preceded by a heart attack or myocardial infarction (MI) event - usually caused by an arterial occlusion (as presented in figure 1.2) - which leads to myocardial ischemia and eventual tissue necrosis; resulting in a significant loss of viable, contractile myocardium (WHO 2011).

Following an MI event, there is an immediate loss of global systolic heart function within the first 24 hours (Francis et al. 2001). This loss is accompanied by the direct activation of a haemostatic compensatory mechanism, which increases left ventricular (LV) pressures in order to sustain cardiac output and peripheral tissue perfusion (Packer 1996). This mechanism is initiated by the activation of the RAS cascade (Rouleau et al. 1993).

Myocardial cell death as a result of MI is largely definitive, as the majority of cardiomyocytes are not self-proliferative (Thygesen et al. 2007). It is well accepted that there is minimal

chance of recovery following an MI event without some form of therapeutic intervention. In the absence of treatment an MI event may occur multiple times or repeatedly over the course of the disease's progression to heart failure. This places increased significance on the treatment and prevention of heart failure post-MI (Soonpaa et al. 1998). The many components of the RAS cascade and the roles they play in the pathophysiology of heart disease will be explored in detail in the next section.

1.2 Myocardial Infarction and progression to Heart Failure

1.2.1 myocardial Remodeling

The critical loss of myocardium that occurs as a result of MI results in immediate activation of the RAS cascade and leads inevitably to a pathophysiological remodeling of the left ventricle (Cohn et al. 2000). Remodeling is a process that begins within hours of injury to the myocardium, and is characterized by a cellular and anatomical response which leads to dilatation of the left ventricle (Cohn et al. 2000; Eaton et al. 1979), wall thinning and hypertrophy (Pfeffer et al. 1991). The consequences of LV remodeling are presented in figure 1.3. Remodeling affects both the infarcted and non-infarcted myocardium, which is recruited to counteract the increase in loading conditions experienced with a loss of contractile function. This remodeling occurs in accordance with the Frank-Starling mechanism, as an increase in left ventricular blood volume through dilatation increases stroke volume, thus preserving the cardiac output. It is regulated by mechanical, neurohormonal and genetic factors (Rubin et al. 1983).

The cellular response to an MI event is inflammatory and is initiated by the ischemia and consequent necrosis experienced by the affected myocardium. This response occurs 3 to 4 days post-MI and is characterised by macrophage and neutrophil infiltration, complement activation and free radical generation (Pfeffer & Braunwald. 1990). The fibrogenic transforming growth factor-beta (TGF-β) is released by both necrotic myocytes and invading macrophages during this inflammatory stage (Bujak et al. 2007). TGF-β plays a role in the phenotypic transformation of cardiac interstitial fibroblasts to myofibroblasts, which specifically express genes for procollagen types 1 and 3 and are thus essential for collagen

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scar formation. In addition, myofibroblasts also play a role in the local formation of Ang II and its receptors in the cardiac tissue (Bujak et al. 2007).

The inflammatory response to MI initiates a process referred to as 'infarct expansion', which involves degradation of the collagen fibers that make up the extracellular matrix (ECM) in the infarcted area and leads to LV wall thinning and chamber dilatation (Olivetti et al. 1990). Degradation of the ECM leads to 'slippage' between myocardial muscle bundles and consequently expands the infarcted area while simultaneously reducing the number of viable cardiomyocytes in the infarct zone (Frangogiannis et al. 2002; Hutchins et al. 1978). During infarct expansion, extensive proliferation of cardiac fibroblasts occurs under the influence of TGF- β. Collagen synthesis in the infarcted myocardium is observed as early as 48 hours post-MI, and eventually results in the formation of a non-contractile collagen scar, which entirely replaces the infarcted myocardium by 28 days post-MI (Warren et al. 1988). The purpose of this scar is redistribution of the increased mechanical stress experienced by the LV wall as a result of post-MI chamber dilatation (Cleutjens, Verluyten, et al. 1995).

Whilst the collagen scar is formed in the infarct zone, cardiomyocytes in the remote, non-infarcted myocardium undergo both compensatory hypertrophy (Hochman at al. 1982) and wall thinning, resulting in further adaptive LV dilatation for the purpose of maintaining cardiac output. However, in the long-term, this dilatation increases LV wall stress according to the Laplace law (Anversa et al. 1985). Increased wall stress in turn stimulates further LV enlargement, which further increases wall stress thus leading to a pattern of continuous LV dilatation and remodeling with long-term detrimental effects on both cardiac function (W Grossman et al. 1975) and mortality (St John Sutton et al. 2003).

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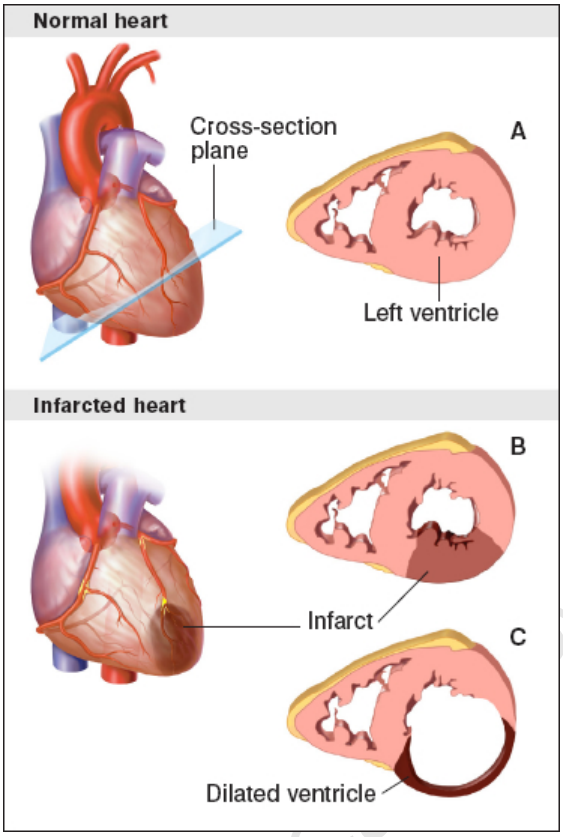

Figure 1.3Post-MI LV remodeling in the heart (*from: http://stemcells.nih.gov/info/Regenerative_Medicine/2006Chapter6.html*)

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1.2.2 The role of Ang II in myocardial remodeling

Ang II directly contributes to post-MI LV remodeling by binding to specific Ang II AT_1 receptors, which are responsible for vasoconstriction and cardiovascular remodeling (Flather et al. 2000). Cardiomyocyte hypertrophy is both mediated and stimulated by Ang II, which is upregulated post-MI as a result of RAS activation (White et al. 1987; Sadoshima et al. 1992). Concurrently, mechanical stretch due to dilatation stimulates the local tissue RAS and Ang II production by cytoplasmic granules in the myocardium (Lindpaintner et al. 1993). Ang II also plays a role in fibrosis and scar formation by promoting expression of the fibrogenic growth factor TGF-β. Upregulation of TGF- β expression occurs as part of the inflammatory response to cardiac injury (Yasuda et al. 2008) and its expression is downregulated by pharmaceutical inhibition of Ang II synthesis (Thompson et al. 1988). Furthermore, Ang II stimulates reactive oxygen species (ROS) formation (Griendling & Ushio-Fukai. 2000), which promotes cardiomyocyte apoptosis in both the infarcted and non-infarcted myocardium

(Peng et al. 2005). Ang II is also a primary stimulant of aldosterone synthesis, which plays a role in promoting cardiac fibrosis (Baldi et al. 2002). In the case of pharmaceutical blockade of Ang II, studies show that cardiac aldosterone nevertheless successfully mediates post-MI LV remodeling, suggesting a significant role for this hormone in the remodeling process that remains to be clarified (Delcayre et al. 2000).

1.3 Treating MI and Preventing Heart Failure

Due to the central role the RAS plays in hypertension and the pathophysiology of heart failure, this system has been the target of pharmacological inhibition since the 1970's (Hall 2003). While the effects of RAS activation are traditionally considered to be compensatory in response to haemodynamic demands, it is well accepted that sustained activation has a negative impact on mortality and morbidity in patients suffering from acute MI and heart failure (Ranadive et al. 1992; White 1998). For this reason, pharmacological curtailment of the RAS and its effects on LV remodeling and blood pressure is critical following an MI event. Post-infarction patients are susceptible to reinfarction, heart failure, development of atherosclerosis and stroke in the absence of therapeutic intervention (Ma et al. 2010). Three classes of RAS intervention drugs have been developed, namely ACE inhibitors, angiotensin receptor blockers (ARB) and direct renin inhibitors (DRI). Their mechanisms of action are presented below in figure 1.4.

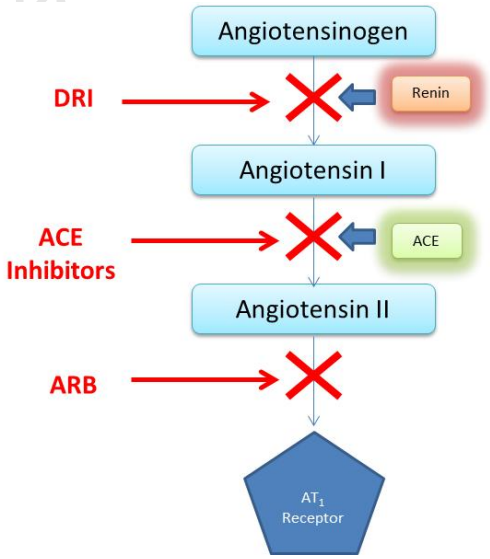

Figure 1.4 Different drugs target different points in the RAS cascade

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ACE inhibitors interrupt the process of Ang II synthesis by preventing hydrolysis of Ang I (Ondetti et al. 1977) (Patchett et al. 1980). ACE inhibitors are prescribed to millions of patients worldwide and have been shown to be effective in the treatment of hypertension, congestive heart failure and renal diseases, as well as dramatically reducing mortality post-MI (Uresin et al. 2007; Yusuf et al. 2000). The beneficial effects of this treatment have been demonstrated to improve clinical outcomes and reduce the risk of myocardial infarction, stroke, heart failure and death (Flather et al. 2000; Isnard et al. 2000). Since the early 1990s large-scale randomized placebo-controlled clinical trials have unequivocally shown that treatment with ACE inhibitors after acute MI produces consistently positive clinical outcomes with reduced mortality (Pfeffer et al. 1992; Køber et al. 1995; Bowling et al. 2012). In addition, ACE inhibitors have been shown to have beneficial effects on atherogenesis, inflammation, endothelial function and cardiac remodeling (DiCarlo et al. 1983; Lonn et al. 1994; O'Keefe et al. 2001).

In contrast to ACE inhibitors, ARBs do not prevent Ang II synthesis, but rather specifically antagonise Ang II AT_1 receptors, thus suppressing this peptide's vasoconstrictive and cellular effects. ARBs are generally used in conjunction with or as a replacement for ACE inhibitors, most often in the event of 'ACE escape' – a condition in which chronic ACE inhibition leads to an eventual rise in Ang II plasma levels up to and beyond initial values (Timmermans et al. 1993; St John Sutton et al. 1997; MacFadyen et al. 1999). As ARBs have no effect on Ang II synthesis, they are effective regardless of the level of Ang II production. ARBs are also often substituted for ACE inhibitors if the patient experiences severe negative side effects, as ARBs do not induce the cough often associated with ACE inhibitor treatment (Lacourcière et al. 1994; Roig et al. 2000). In the case of hypertension, ARBs have been found to be as effective as ACE inhibitors at lowering blood pressure, and have the added benefit of less severe side effects (Benz et al. 1997). However, their impact on LV remodeling is still unclear, although studies have shown that combination ACE inhibitor and ARB therapy does attenuate the remodeling process (Chan et al. 1997; Conlin et al. 2000).

DRIs are the most recent drug class to become commercially available for the treatment of hypertension, post-MI LV dysfunction and heart failure (Tikkanen et al. 1995). The first and only DRI to be approved for clinical use is Aliskiren, an orally-active compound which targets the enzyme renin (Mankad et al. 2001). It has been shown to be effective at lowering blood pressure in short-term studies (Stanton et al. 2003; Wood et al. 2003), and has the advantage of lowering blood pressure even further when used in combination therapy with

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ARBs or ACE inhibitors (Nussberger et al. 2002; Oparil et al. 2007; Weir et al. 2007). Aliskiren has an extremely high specificity for the active site on renin and thus competitively inhibits Ang I generation from Angiotensinigen (Wood et al. 2003). As this compound inhibits renin activity at the top of the biochemical cascade (the 'rate-limiting step'), it decreases production of Ang I and subsequently the pressor Ang II (Nussberger et al. 2002) while increasing plasma renin activity (PRA) – a result of removing the negative feedback mechanism of Ang II on renin production. PRA is a measure of both changing circulatory renin levels as well as plasma angiotensinogen levels. While this is true of all classes of RAS intervention drugs, a review of six large-scale clinical trials with aliskiren found that the reactive increase in PRA was far greater with DRIs and resulted in a considerable offset of this class' positive antihypertensive effects (Sealey & Laragh 2007). This is due to the fact that while PRA is effectively decreased by Aliskiren, plasma renin concentration (PRC) – is elevated to such a degree (up to 500% as a monotherapy (Juerg Nussberger et al. 2005)and up to 1200% in combination with a diuretic (Villamil et al. 2007)) that PRA effectively rises. A large reactive increase in PRA is also seen in treatment with ARBs, but as described earlier, this drug class is able to inhibit the effect of Ang II at receptor level and lower BP. The current opinion is that this drug class does not present a superior treatment option over ACE inhibitors or ARBs.

Despite the development of alternative drug classes for RAS inhibition, ACE inhibition remains the first-line treatment for hypertension, congestive heart failure (CHF) and acute MI (Antman et al. 2004). The haemodynamic effects of ACE inhibition were originally believed to be the major contributor to the positive outcomes seen in patients suffering from acute MI and heart failure. ACE inhibitors primarily induce vasodilation, thus enhancing the cardiac output of the damaged heart by decreasing the loading conditions. More recently however, it has been recognised that ACE inhibitors have properties which enhance cardioprotection independently of their haemodynamic effects on hypertension. Of particular importance are the findings that ACE inhibitors prevent or delay detrimental cardiac remodeling of the LV post-MI These benefits on LV remodeling are generally attributed to two factors: the decrease of ACE-mediated Ang II production (as discussed previously, Ang II contributes negatively towards the remodeling process) and the accumulation of plasma bradykinin levels (a peptide with anti-proliferative and anti-hypertrophic properties). The role of bradykinin will be explored in more detail in a later section.

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1.4 THE RAS IN DETAIL

1.4.1.1 RENIN AND PRORENIN

Following an MI event, the RAS cascade is initiated by the synthesis of the glycoprotein renin, which is formed by the proteolytic cleavage of the precursor enzyme, prorenin. Prorenin is produced renally for the purposes of renin production and it is also secreted constitutively into the systemic circulation. It serves as the precursor for renin production in the kidneys (Agostoni et al. 1999), and although its circulatory levels in the plasma exceed those of renin by up to 100-fold (Hobart et al. 1984), it was until recently believed to have no function of its own. However, discovery of a (pro)renin receptor [(P)RR] which binds both renin and prorenin, highlighted this enzyme's potential function as more than an inactive element of the RAS (Danser et al. 1998). Binding to this receptor induces a non-proteolytic activation of prorenin, as well as increasing the catalytic activity of renin by 4-fold. This observation presents a compelling case for the concept of increased renin activity for the purpose of Ang II production at a cell-surface level (Sealey et al. 1996; Nguyen et al. 1996; Nguyen et al. 2002).

Renin is synthesized from prorenin within the juxtaglomerular (JG) cells of the renal glomerulus of the kidney (Galen et al. 1984). It is stored in the granules of the JG cells and released mainly by exocytosis in response to intracellular signalling pathways (Schweda & Kurtz. 2004). It is the enzyme responsible for the production of Ang I from Angiotensinogen (Leonard T Skeggs et al. 1954). As renin is the major rate-limiting step in the RAS cascade, its release is highly regulated by both local and systemic factors. The negative feedback mechanism controlling renin production and secretion is influenced by a number of inter-related factors as described below. The effects of these factors on renin release are presented in figure 1.5.

1.4.1.2 RENIN RELEASE: SYSTEMIC FACTORS

Activation of the RAS cascade is initiated by renin secretion, which is induced mainly by a decrease in systemic blood pressure or circulating blood volume (both of which are reflected by a decrease in arterial pressure, renal arteriolar pressure and renal perfusion pressure) (Kaneko et al. 1967).

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Arterial circulatory pressures are maintained by two homeostatic inter-related mechanisms: cardiac output and peripheral vascular resistance (Frohlich et al. 1969). Mechanoreceptors located in the left ventricle (Oberg et al. 1972), the carotid sinus (Eckberg 1977), the aortic arch (Heymans 1960) and the renal afferent arterioles (Moss 1982) are responsible for sensing any changes in arterial pressure, and thus play a major role in regulating blood pressure and volume. A decrease in activation of these receptors has four major effects: increased sympathetic nervous activity (Malpas 2010), release of antidiuretic hormone (ADH) (Schrier et al. 1999), stimulation of thirst (Stricker 1968) and activation of the RAS.

In addition to mechanoreceptors, the JG apparatus in the kidney is sensitive to a loss in renal arteriolar and perfusion pressure. The JG cells line the afferent (and sometimes the efferent) arterioles of the glomeruli, which is situated adjacent to the macula densa cells of the distal tubules. The macula densa cells are sensitive to decreases in the sodium chloride (NaCl) concentration of the tubular fluid and an inverse relationship exists between the tubular Cl^- concentration as sensed by the macula densa and renin release (Leonard T Skeggs et al. 1954). A decrease in this concentration is indicative of a decreased glomerular filtration rate as a result of reduced renal perfusion and stimulates release of renin and synthesis of the vasoconstrictor Ang II (see below for mechanism). A drop in renal arteriolar pressure also directly stimulates renin release and activation of the RAS (Frohlich et al. 1969).

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1.4.1.3 Renin release: Extracellular factors

Local factors responsible for regulating the release of renin include a number of hormones, peptides and compounds acting in both a stimulatory and inhibitory capacity by activating intracellular mechanisms. The primary extracellular factors responsible for the regulation of renin release are reviewed below.

1.4.1.3.1 The Prostaglandins

The prostaglandins E_2 and I_1 have been shown to stimulate renin secretion *in vitro* (LA et al. 1971; Johnson et al. 1973; Egan et al. 1983). It is also known that blockade of prostaglandin synthesis reduces plasma renin levels *in vivo* (Friis et al. 2005; Skott et al. 1987).

The formation of prostaglandins is catalyzed by the two cyclooxygenase isoforms COX-1 and COX-2 (Hemler & Lands, 1976). Both forms of the enzyme are expressed in the kidneys and COX-2 in particular is localised in the macula densa (Harris et al. 1994). COX-2 expression has been shown to change in congruence to levels of renin synthesis, providing evidence of a relationship between the two (Traynor et al. 1999; Wolf et al. 1999). In addition, COX-2 expression and the prostaglandins E_2 and I_1 are believed to play a role in the stimulatory effect of decreased NaCl concentrations on renin release. It has been shown *in vitro* that a specific COX-2 blocker (NS-398) prevents the stimulation of renin release by low NaCl in isolated perfused JG cells (Saris et al. 2002). This has also been shown *in vivo*, where COX-2 blockers have the same effect on renin release stimulated by a low NaCl diet or by treatment with the diuretic furosemide (Traynor et al. 1999; Harding et al. 2000).

1.4.1.3.2 Atrial Natriuretic Peptide

Atrial Natriuretic Peptide (ANP) is a hormone that is localized in the myocardial cells of the atria (Cantin et al. 1984). Its release is stimulated by an increase in atrial pressure, and it acts to lower blood pressure by promoting natriuresis (sodium excretion) and inhibiting renin secretion (Edwards et al. 1988). The ANP driven inhibition of NaCl reabsorption can lead to increased macula densa NaCl concentrations, which in turn inhibits the release of renin. (Stichtenoth et al. 1998; Jensen et al. 1996; Friis et al. 2005)

ANP also directly suppresses renin release by stimulating cyclic guanosine monophosphate (cGMP) formation in the JG cells (discussed in more detail below). This particular inhibitory action has been shown both *in vitro* and *in vivo*. (Bolger et al. 1976; Traynor et al. 1999)

1.4.1.3.3 Nitric Oxide

Nitric oxide (NO)-producing cells are located in close proximity to the JG cells and play a stimulatory role in the release of renin. This is evidenced by studies with knockout mice, which have shown that the primary enzyme responsible for NO synthesis - the neuronal NO synthase nNOS - is localized to and expressed by the cells of the macula densa (Wolf et al. 1999; Hartner et al. 1998; Traynor et al. 1999). As with the studies ofCOX-2, expression of nNOS correlates to changes in renin secretion (Harding et al. 2000). *In vitro* and *in vivo* studies provide evidence for a stimulatory role for NO in renin release under tonic conditions,

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although inhibition has also been observed under specific, acute conditions (Kurtz et al. 1998). These effects are believed to be mediated by the stimulation of guanylate cyclase activity in the renin-producing JG cells (Edwards et al. 1988; Stichtenoth et al. 1998; Maack et al. 1984). The dual role of cGMP on renin synthesis is discussed in more detail below.

1.4.1.3.4 ANGIOTENSIN II

As the primary end-product of the RAS cascade, Ang II strongly inhibits renin release as part of a negative feedback loop. It does this both directly via specific receptors (Siragy et al. 2005) and indirectly as a consequence of its systemic haemodynamic effects on arterial and renal arteriolar pressures (Vander 1967).

Renin-releasing JG cells express Ang II AT_1 receptors in abundance (Burnett et al. 1986), and studies show that both Ang II and its receptors are involved in inhibiting stimulatory cyclic adenosine monophosphate (cAMP) activity in renin-producing cells (Obana et al. 1985a; Henrich et al. 1988). Ang II appears to mediate its inhibitory actions by increasing the cytosolic calcium levels in renin-producing cells, which has a strong inhibitory effect on renin release (discussed below) (Mundel et al. 1992; Bachmann et al. 1995).

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1.4.1.4 CELLULAR MECHANISMS THAT REGULATE RENIN RELEASE

1.4.1.4.1 CYCLIC AMP

The primary intracellular stimulant of renin release is cAMP. *In vitro* studies have shown that the hormones responsible for stimulating renin release (such as PGI_1 and PGE_2) from the JG cells do so by increasing intracellular cAMP concentrations in JG cell isolations (Wagner et al. 2000; Bosse et al. 1995; Kurtz et al. 1998; Schmidt et al. 1993). There is also evidence that sympathetic stimulation of renin release via B-adrenoreceptors is facilitated by intracellular cAMP (Kurtz et al. 1998; Jensen et al. 1997; Kurtz et al. 1986a). The exact mechanism through which cAMP triggers the exocytosis of renin is not clear but does involve a protein kinase A-dependent step (Castrop et al 2005).

1.4.1.4.2 CYCLIC GMP

In contrast to the solely stimulatory effects of the activated cAMP pathway, cyclic GMP appears to fulfill a dual role in regards to regulation of renin secretion, both inhibitory (Hautmann et al. 2007; Weinberger et al. 1975) and stimulatory (Friis et al. 1999; Keeton et al. 1980).

In vitro studies with hormones that stimulate cyclic GMP activity (such as natriuretic peptides) show that a slight increase in cGMP levels is responsible for renin stimulation, while higher concentrations of intracellular cGMP appear to inhibit renin release (Henrich et al. 1988; Greenberg et al. 1995; Sayago et al. 2001).

The duality of renin release in response to cGMP concentration is poorly understood. It has most recently been proposed that it may result from the differing affinity of cGMP for two intracellular factors, namely phospodiesterase-3 (PDE-3) and cGMP-dependent protein kinase II (cGKII) (Schweda & Kurtz. 2004). PDE-3 with a higher affinity for cGMP has been shown to be inhibited by cGMP thus decreasing degradation of cAMP and thereby stimulating the release of renin (Beavo 1995). Conversely, cGKII with a lower affinity for cGMP has been shown to directly inhibit renin release. It would appear that NO acts through the cGMP-PDE-3 axis (Kurtz and Wagner 1998) and it might also be surmised that ANP could be acting via cGKII.

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1.4.1.4.3 CALCIUM

Finally, intracellular Ca^{2+} concentrations in the JG cells are considered to be the principal inhibitor of renin secretion (Kurtz 2011). Interestingly, although an increase in Ca^{2+} concentrations triggers exocytosis in most secretory cells in the body, in JG cells a Ca^{2+} increase actually reduces exocytosis, thus inhibiting the release of renin (Beierwaltes 2006; Obana et al. 1985b). This inverse relationship has been termed the “calcium paradox” (Kurtz et al. 1998). As detailed above, ANGII upregulates Ca2+ (as do several other vasoconstrictors) and it is reasoned that this is how it mediates the direct inhibition of renin release.

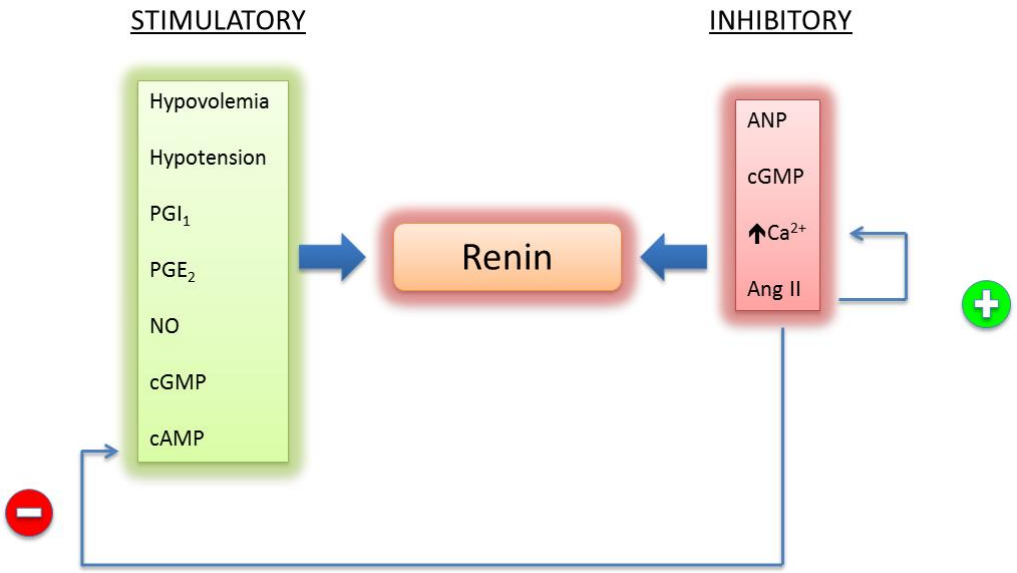

Figure 1.5 The stimulatory and inhibitory factors affecting renin release

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1.4.2 ANGIOTENSINOGEN

Angiotensinogen is a globulin that is produced mainly and constitutively by the liver (Kurtz et al. 1986b). Systemic angiotensinogen plasma levels are thus fairly constant and resistant to acute fluctuations, and serve as the primary substrate of the RAS.

Angiotensinogen messenger RNA (mRNA) expression has also been found in many other tissues, including the brain, heart and adipose tissue (Grünberger et al. 2006; Ortiz-Capisano et al. 2007; Campbell et al. 1987). When the RAS is activated following acute MI, studies show an increase in tissue levels of angiotensinogen mRNA expression in both the kidney and the cardiac ventricular tissue (Sawa et al. 1992; Kelly et al. 1997). Angiotensinogen is cleaved by the enzyme renin to form the decapeptide Angiotensin I.

1.4.3 ANGIOTENSIN I

The production of biologically inactive Ang I is an intermediate step in the cascade towards Ang II production. The formation of a precursor is required as Ang II is an extremely potent peptide with a remarkably short half-life (approximately 30 seconds in human plasma) (Lindpaintner et al. 1993). Ang I production occurs at both systemic and local levels, and

studies have shown that local synthesis accounts for more than 90% of intracellular Ang I in the myocardium (Filippatos et al. 2001).

1.4.4 Angiotensin Converting Enzyme

The ACE protein is a zinc-dependent dipeptidyl carboxypeptidase, meaning it cleaves two amino acids from the carboxyl end of peptides. It is expressed as two isoforms which are both transcribed by a single gene (Soffer et al. 1987). The larger isoform is termed somatic ACE and is predominantly expressed in the vascular endothelium of the pulmonary system (Das et al. 1975). It is also present in the plasma, the vascular endothelium, and the epithelial cells of many other tissues, including the kidney (Soffer et al. 1987; Ehlers et al. 1986). The second isoform is testicular or testis ACE, and is expressed exclusively in male germ cells where it is believed to play a crucial role in male fertility (Danilov et al. 1994; Langford et al. 1993).

Somatic ACE is comprised of two homologous catalytic domains. They are termed the N- and C-terminal domains, classified according to their relative position on the polypeptide chain (Pauls et al. 2003). Each domain is catalytically independent and has a zinc-binding motif essential for enzymatic activity (Soubrier et al. 1988). Notably, the testis ACE isozyme possesses only the C-domain (Ehlers et al. 1989). These two domains are probably the result of an ancient gene duplication, and display 60% sequence homogeneity (Lattion et al. 1989). It appears that both active sites have been evolutionarily conserved over time, which suggest that they each play an essential physiological role in the RAS (Cornell et al. 1995).

Somatic ACE - referred to as ACE in this work - exists in both a soluble, circulating form, and in a physiologically significant membrane-bound form. The soluble form found in plasma is thought to be a reflection of the membrane-bound form's turnover and clearance (Corvol et al. 1995).

ACE is primarily responsible for generating the bioactive vasoconstrictor peptide Ang II by hydrolysis of Ang I. It is also responsible for the hydrolysis and degradation of the vasodilatory peptide bradykinin (Corvol et al. 1995). In this manner, ACE employs a two-pronged approach towards hypotension by both synthesizing vasoconstrictive peptides while metabolising vasodilatory agents.

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1.4.5 ANGIOTENSIN II

As the primary active product of the RAS, Ang II induces vasoconstriction and increases blood pressure in response to hypotension or hypovolemia (Georgiadis et al. 2003). It also increases sodium and fluid retention in the kidneys by acting on both the adrenal cortex to stimulate aldosterone release as well as the posterior pituitary to stimulate the release of Antidiuretic Hormone (ADH) (Reid 1992; Davis 1964; Uhlich et al. 1975). Ang II also counteracts hypovolemia by stimulating the thirst centers in the brain, thus increasing fluid intake and complementing the mechanisms for fluid reabsorption (Brooks et al. 1986).

Ang II mediates these antagonistic effects towards hypotension and hypovolemia by binding to two known plasma membrane receptor sub-types which are responsible for mediating diverse and opposing physiological effects. The Ang II type 1 (AT_1) receptor is responsible for Ang II's primary actions on the cardiovascular system; namely systemic vasoconstriction, sodium retention and cardiac and vascular hypertrophy (Lijnen et al. 2001; Vaughan 2001; Savoia et al. 2011). Interestingly, AT_1R-specific blockade by direct renal infusion results in a highly significant natriuretic response that is not observed under conditions of AT_2R blockade, which demonstrates a principal role for this receptor in the mediation of natriuresis (Bergaya et al. 2004).

In sharp contrast the role of the Ang II type 2 receptor (AT_2) appears to be entirely contradictory to the effects of the AT_1 receptor. It is widely accepted that AT_2 receptors promote apoptosis and inhibit fibroblast proliferation and cardiac hypertrophy (Touyz et al. 2003; Tsutsumi et al. 1999; Tsuzuki et al. 1996). AT_2 receptors also appear to counter-balance the AT_1 receptor-mediated vasoconstrictive effects of Ang II by inducing vasodilation. Evidence for this comes from studies in vascular endothelial cells, which show that vasodilation caused by NO is mediated by activation of the AT_2 receptors (Yamada et al. 1996). Studies have also shown that AT_2 receptor activation leads to production of the vasodilatory peptide bradykinin (Wiemer et al. 1993; Katada et al. 2002). Most notably, AT_2 receptors are capable of binding to AT_1 receptors located on cell surfaces, thus directly antagonising the AT_1 receptor-mediated effects of Ang II (Padia 2006). In addition, expression of the AT_2 receptor is inhibited by Ang II and upregulated by sodium reduction (AbdAlla et al. 2001). These observations regarding the antagonistic nature of the two Ang II receptors strongly suggest the existence of internal feedback mechanisms responsible for regulating the effects of the activated RAS. The systemic effects of AT_1 receptor binding and systemic negative feedback system are presented below in figure 1.6. A more complete view

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of the physiological effects of both AT_1 and AT_2 receptor binding are presented in figure 1.10 (Section 1.7.3)

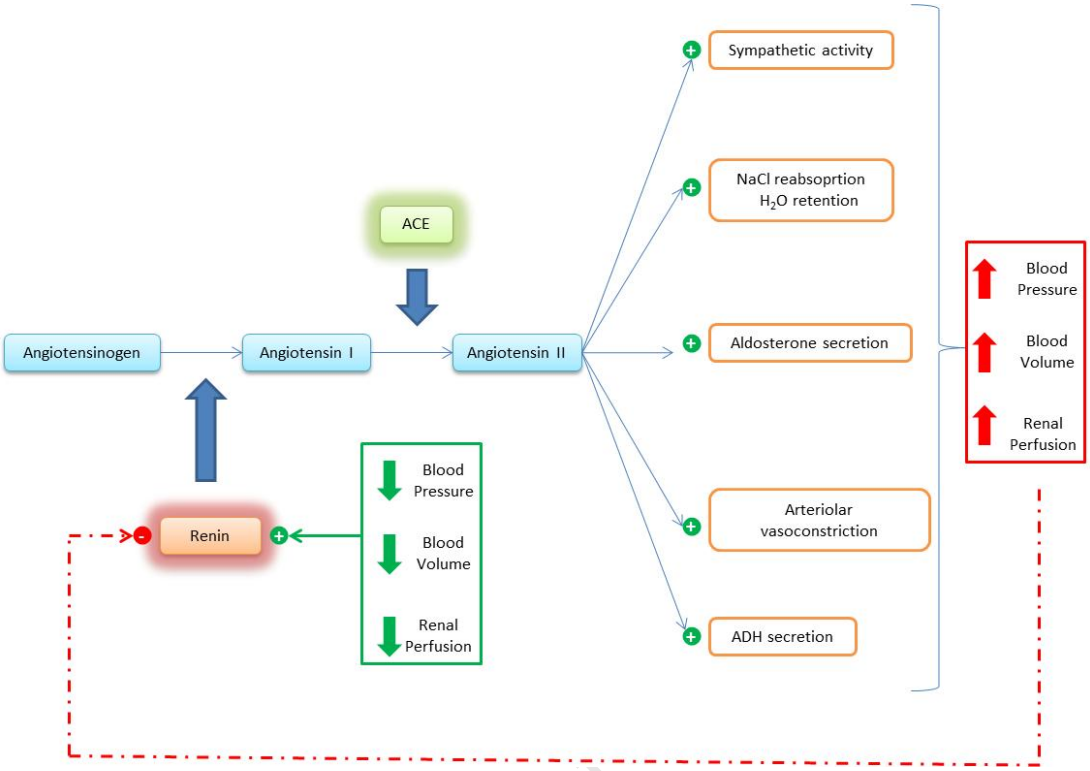

Figure.1.6 The Systemic RAS and its peripheral actions

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1.5 Additional Pathways, Peptides and Enzymes of the RAS

1.5.1 The Alternative Axis

The generation of Ang II in response to hypovolemia and hypotension is the primary function of the RAS. However, upregulation of Ang II production has a number of detrimental effects under pathophysiological conditions, hence the need for pharmacological curtailment. The discovery of an alternative axis in the RAS has opened up a promising new strategy for combating the negative effects of Ang II by enhancing endogenous production of the vasodilatory and cardioprotective heptapeptide Ang (1-7). This so-called 'alternative axis' is presented in figure 1.7.

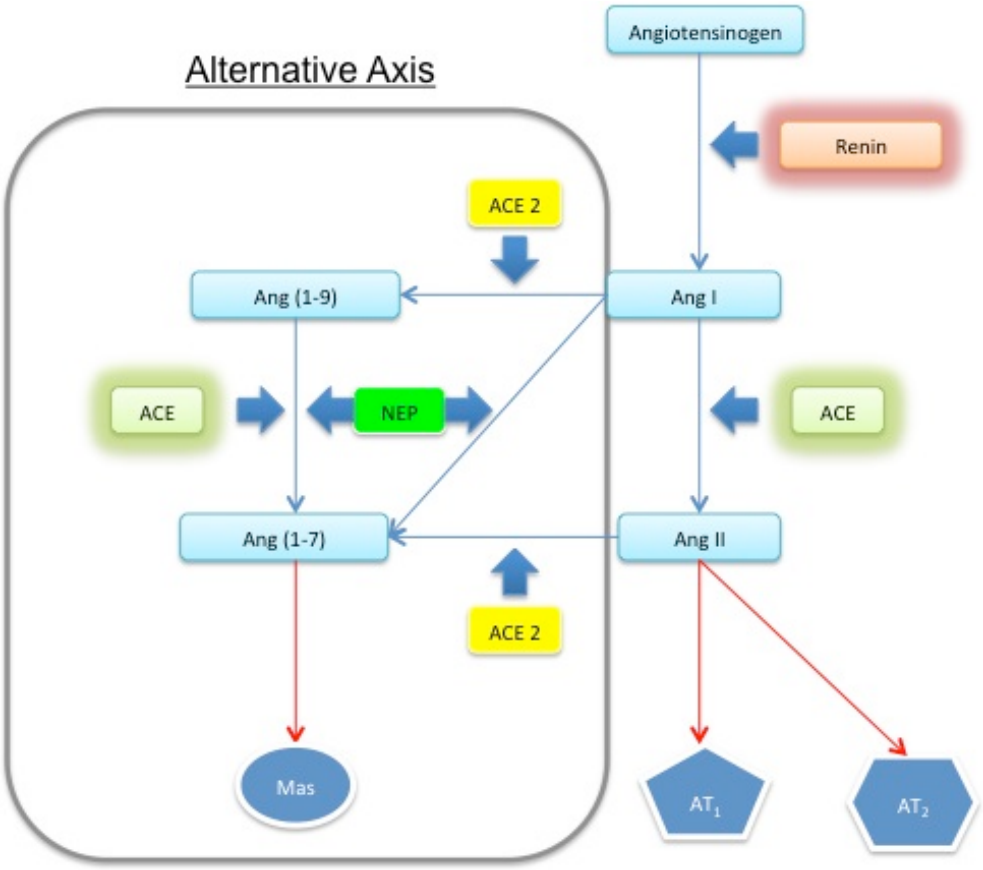

Figure 1.7 Relationship of the alternative Ang (1-7)-producing axis to the Ang II-producing RAS cascade

The existence of an Ang (1-7) generating pathway within the RAS provides further compelling evidence for an internal counter-regulatory mechanism, designed to limit the potent effects of Ang II. The diversity of enzymatic pathways for Ang (1-7) generation (as described below) also supports the concept of an enhanced counter-regulatory mechanism within the RAS (Deddish et al. 1998).

1.5.1.1 ANGIOTENSIN (1-7)

Ang (1-7) is present at both systemic and tissue levels, and is found in the brain, heart, liver, kidneys, reproductive organs and the vasculature (Israili et al. 1992).It binds to the G protein-coupled receptor Mas, which is present on endothelial cells (Nussberger et al. 1998). It is this binding that mediates Ang (1-7)'s vasodilatory, anti-proliferative, anti-inflammatory, antithrombotic and antiarrythmogenic effects (Schindler et al. 2007). Studies have also shown elevated Ang (1-7) expression in the border zone of infarcted cardiac tissue - further evidence suggestive of the cardioprotective role played by this peptide under

pathophysiological conditions (Santos et al. 2003). It has furthermore been demonstrated that exogenous administration of this peptide attenuates the development of heart failure in a rat model of MI (Santos et al. 2003). In general, Ang (1-7) plasma levels are extremely low, but during ACE inhibition they are elevated as a consequence of higher Ang I levels, as well as by the inhibition of its own metabolizing agent ACE (Loot et al. 2002). Additionally, studies show that Ang (1-7) is cleaved exclusively by the N-domain of human ACE (Campbell et al. 1993; Deddish et al. 1998)

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1.5.1.2 ACE2

ACE2 is a zinc metalloprotease homologue of ACE which is entirely insensitive to ACE inhibition and shares just 42% of its sequence identity with ACE (Donoghue et al. 2000). Its role in the RAS appears to be counter-regulatory towards Ang II, as it is responsible for the generation of Ang (1-7) either directly from Ang II or indirectly by the intermediate synthesis of Ang (1-9) (Tipnis et al. 2000a).

Expression of ACE2 is not as expansive as that of ACE, although ACE2 mRNA is present throughout all the tissues of the body. Particularly abundant expression has been noted in the venous and arterial endothelial cells, arterial smooth muscle cells, as well as the epithelia of the lung and small intestine (Donoghue et al. 2000). Upregulation of ACE2 expression has recently emerged as a promising therapeutic target for the treatment of MI and heart failure (Donoghue et al. 2000), as it inactivates Ang II by upregulating its conversion to Ang (1-7), thus minimizing the negative effects of Ang II and augmenting the cardioprotective effects associated with Ang (1-7). There is evidence that ACE2 expression is locally upregulated in the ischemic heart following MI (Goulter et al. 2004)and studies with knockout mice reported cardiac dysfunction under physiological conditions in the absence of ACE2 expression (Burrell et al. 2005).

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1.5.1.3 NEP

Neutral endopeptidase (NEP) is also referred to as neprilysin, and is a zinc metallopeptidase with a preference for N-terminal cleavage. It has recently been shown to play a role in the conversion of Ang (1-9) to Ang (1-7), as well as a possible role in the direct synthesis of Ang (1-7) from Ang I (Yamamoto et al. 1992).

1.5.1.4 ANG (1-9)

While the exact role of Ang (1-9) as more than an intermediate peptide in the generation of Ang (1-7) is still unclear, there is evidence that it enhances the actions of the vasodilatory agent bradykinin, thus increasing NO synthesis (Crackower et al. 2002). Recent studies have shown that Ang (1-9) also has cardioprotective anti-hypertrophic effects *in vivo* and *in vitro* (Jackman et al. 2002).

1.6 REVIEW OF ADDITIONAL COMPONENTS OF THE RAS

The primary components of both the Ang II-producing and the counter-regulatory Ang (1-7)-producing pathways of the RAS have been previously described and defined in the current chapter. However, recent studies have shown that our existing view of the RAS must be expanded to include a number of angiotensin metabolites with a range of effects that both augment and attenuate the actions of Ang II. Figure 1.8 illustrates the major peptides of interest in this study, along with the enzymatic pathways responsible for their generation.

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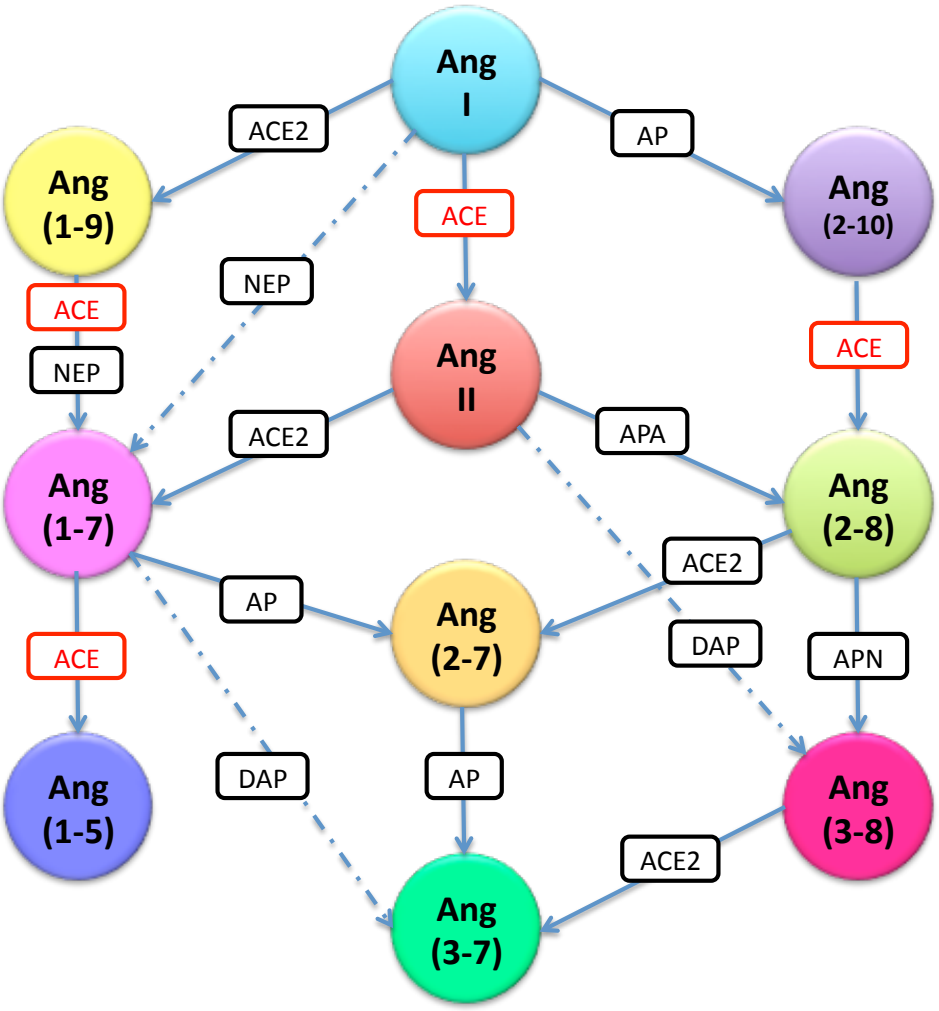


Figure 1.8 The expanded view of the RAS (adapted from (Poglitsch et al. 2012)**)**

1.6.1 Enzymatic pathways

There are a number of enzymes potentially involved in the processing of angiotensinogen-derived peptides in addition to renin, ACE and ACE2. (Aminopeptidase A (APA) cleaves peptides with an N-terminal acidic amino acid, which includes Ang I and Ang II. It therefore converts Ang I to Ang (2-10) (also referred to as Des Asp^{1} Ang I), and Ang II to Ang III (also referred to as Ang (2-8) or Des Asp^{1} Ang II) (Ardaillou et al. 1998).

Aminopeptidase N (APN) hydrolyses peptides with an N-terminal neutral amino acid, facilitating the conversion of Ang III to Ang IV (also referred to as Ang (3-8)) (Sjöström et al. 2000). Dipeptidylaminopeptidase III (DAP) is also active in the RAS, and is responsible for the direct formation of Ang IV from Ang II and Ang (3-7) from Ang (1-7) (Lee et al. 1982).

1.6.2 Angiotensin Peptides

1.6.2.1 Ang (2-10)

Ang (2-10) is a nonapeptide produced by the enzymatic cleavage of Ang I by APA. Studies support an antagonistic role for Ang (2-10) with regards to Ang II, as it has been shown to produce anti-hypertrophic effects on rat cardiomyocytes both *in vivo* (Sim et al. 1998) and *in vitro* (Min et al. 2000). Two separate groups have also reported an anti-inflammatory effect with intravenous or oral administration of Ang (2-10), accompanied by attenuation of infarct size in both a permanent ligation (Rufaihah et al. 2006) and ischemia-reperfusion model (Wen et al. 2004; Wen et al. 2011) in the rat.

1.6.2.2 Ang III

The heptapeptide Ang III is produced either by the enzymatic cleavage of Ang II by APA, or by ACE activity on Ang (2-10) (Campbell 2003).

In the peripheral system, Ang III induces aldosterone secretion from the adrenal glomerulosa by binding to the AT_2 receptor (Yatabe et al. 2011). Recent studies with neonatal cells have shown that Ang III also induces protein synthesis in cardiomyocytes and DNA and collagen synthesis in cardiac fibroblasts (Wang et al. 2010). Some proinflammatory effects have also been observed in rat *in vitro* studies (Ruiz-Ortega et al. 2000).

In the local RAS of the CNS, there is evidence that Ang III plays a highly significant physiological role. In the CNS, Ang II and Ang III display similar affinities for both the AT_1 and AT_2 subtype receptors (Hansen et al. 2000; Marc et al. 2011).

Interestingly, Ang III has been shown to mediate many of the same physiological effects as Ang II via AT_1 receptor binding in the CNS – namely induction of vasoconstriction and vasopressin release (Zini et al. 1996).

Studies employing APA blockers in the CNS have shown that the conversion from Ang II to Ang III is critical for the stimulation of vasopressin release and elevation of BP that is observed with exogenous administration of Ang II (Zini et al. 1996; Zini et al. 1998). In the renal system, similar studies have found that this Ang II to Ang III conversion is required for the AT_2 receptor-mediated natriuretic response observed with exogenous renal infusion of Ang II (Padia et al. 2008).

1.6.2.3 ANG IV

Ang IV is a hexapeptide which is directly generated from Ang III by the hydrolysis of the N-terminal by aminopeptidase N (APN). It is also converted from Ang II by the actions of diaminopeptidases (DAP) – in fact, DAP has a higher substrate affinity for Ang II than that of APN, and this is the preferred pathway of Ang IV synthesis (Lee et al. 1982; Erdös et al. 1990).

Ang IV has a wide range of physiological effects in the local RAS at various tissue sites. In the CNS it has been shown to play a role in learning acquisition and memory recall (Wright et al. 1993; Wright et al. 1995), and these effects are mediated by the angiotensin receptor subtype AT_4 in the hippocampus (Swanson et al. 1992). In the renal system, Ang IV promotes natriuresis (Yoshida et al. 1996), while in the vascular system it has a vasodilatory effect and improves both cerebral (Kramár et al. 1997) and renal blood flow (Swanson et al. 1992).

In addition, there is evidence that Ang IV can promote the synthesis and release of prostaglandins, further enhancing its vasodilatory role in the RAS (Yoshida et al. 1996). Ang IV mediates these effects by binding to an insulin-regulated aminopeptidase receptor (IRAP), which is also assumed to be an AT_4 receptor (Albiston et al. 2001).

1.6.2.4 ANG (3-7)

Ang (3-7) is produced by a variety of pathways and is converted either directly from Ang IV by ACE2, or indirectly from Ang (1-7) and Ang III by the proteolytic actions of ACE2 and aminopeptidases (Neves et al. 1995). It has been reported that this peptide plays a functional role in the local RAS of the CNS, where it has been shown to improve recognition memory in rats (Braszko et al. 1995). It is believed to produce these effects by interaction with either the AT_4 receptors (as is the case with Ang IV) or possibly the AT_1 receptors (Braszko et al. 1994).

1.6.2.5 ANG (1-5)

Forming part of the alternative Ang (1-7)-producing axis of the RAS, Ang (1-5) is produced by the hydrolytic action of ACE on Ang (1-7), and is thus susceptible to ACE inhibition (Yamada et al. 1998). Interestingly, a review of the literature showed conflicting reports as to whether Ang (1-7) was cleaved primarily by the C-domain (Allred, Debra I Diz, Carlos M Ferrario, Mark C Chappell, et al. 2000) or the N-domain (Andrade et al. 1998; Deddish et al. 1998) of ACE. The role of Ang (1-5) as more than an inactive metabolite of the RAS is not known, although Roks *et al*. (1999) did show that this peptide has an inhibitory effect on ACE at extremely high concentrations. However, under ACE inhibition, Ang (1-7) levels are elevated while Ang (1-5) levels are dramatically decreased, thus making this peptide an unlikely candidate for the cardioprotective effects observed with the administration of ACE inhibitors (Chappell et al. 1998).

1.7 Review of Related components of the RAS

1.7.1 Bradykinin

The peptide bradykinin (1-9) (BK (1-9)) is a potent vasodilatory product of the kallikrein-kinin system (KKS) (Silva et al. 1949; Fox et al. 1961). The KKS is a metabolic cascade that produces a number of vasoactive peptides and is an important mediator of pathophysiological conditions of cardiovascular disease (Sharma 2006; Costa-neto et al. 2008). The RAS and the KKS are linked by ACE, which is responsible for the metabolic breakdown of BK (1-9) (Morice et al. 1987). Thus the vasodilatory product of the KKS serves as a physiological counterbalance to the vasoconstrictive product of the RAS (Erdös et al. 1967). Interestingly, it has also been shown that ACE has a higher substrate affinity for bradykinin than that demonstrated for Ang I (Yang et al. 1970).

BK (1-9) is synthesized either directly by the proteolytic cleavage of kininogen by tissue kallikrein, or indirectly by the ACE-mediated cleavage of the intermediate kallidin or lys-bradykinin (Schmaier 2002). Bradykinin is hydrolysed rapidly by the kininases ACE and NEP and has a half-life of less than 30 seconds *in vivo* (Schmaier 2002). Due to this short half-life, bradykinin is synthesized locally at various tissue sites, including the heart and vasculature. The pathway of bradykinin formation and degradation is presented in figure 1.9.

Bradykinin binds to two known G protein-coupled receptors – B_1 and B_2. The B_2 receptors are widely expressed under physiological conditions by various tissues, including endothelial

cells (Benzing et al. 1999), smooth muscle cells (Farmer et al. 1991) and cardiomyocytes (Minshall et al. 1995). The B_1 receptors are only expressed under pathological conditions induced by tissue injury – as is the case with myocardial infarction (Schmaier 2002; Raidoo et al. 1997). B_2 receptors mediate bradykinin's vasodilatory actions primarily by stimulating endothelial NO synthase expression and NO production as well as by generating prostaglandin E_2 (Figueroa et al. 2001). B_1 receptors appear to have the same haemodynamic effects as B_2 receptors, but their complete physiological role is not clearly defined (Foucart et al. 1997; Schremmer-Danninger et al. 1998). Anti-hypertrophic and anti-proliferative effects on cardiomyocytes and fibroblasts have also been attributed to the B_2 receptor (Siragy et al. 1997; Duka et al. 2001). Several studies with B_2 kinin receptor antagonists have shown that bradykinin is responsible for attenuating both left ventricular hypertrophy and progression to heart failure after an MI event (Xu et al. 2005; McAllister et al. 1993). There is evidence that B_2 receptor-binding preserves stores of glycogen and energy-rich phosphates in the myocardium, a possible mechanism for bradykinin's cardioprotective effects during ischemia (Ishigai et al. 1997). Indeed, studies show that kinins are released continuously under ischemic cardiac conditions such as MI (Linz et al. 1992).

The upregulation of kinin release during MI has been shown to be a critical factor in survival rates after coronary artery ligation in a rat model. In addition, pretreatment with a B_2 receptor agonist completely reverses these cardioprotective effects under ischemic conditions. (Wollert et al. 1997; Madeddu et al. 1998) This implies that the B_2 receptors play a crucial role specifically during MI despite the upregulation of B_1 receptor expression.

ACE inhibitors are known to potentiate the effects of bradykinin by preventing or hindering its degradation. Studies with recombinant ACE mutants have unequivocally shown that both the C- and N-domains of ACE metabolise bradykinin with similar kinetic efficiencies (Jaspard et al. 1993). Although NEP also plays a role in metabolizing bradykinin, ACE accounts for 45 – 100% of this activity (depending on the species) (Blais et al. 1997). A quantitative relationship between bradykinin degradation and the potentiating effects of ACE inhibition has been observed in studies with domain-selective ACE inhibitors (B Tom et al. 2001). Bradykinin is equally cleaved by both ACE domains, so blockade of only one domain allows this peptide to be degraded approximately 50% more than that which occurs with full inhibition (Abbas et al. 1999). The uninhibited enzymatic action of ACE leads to the sequential formation of the metabolites BK (1-8), BK (1-7) and BK (1-5) from the BK (1-9) precursor (Campbell 2003).

There is also evidence that bradykinin potentiation by ACE inhibitors could be a result of degradation-independent mechanisms. Co-localization of ACE, AT_1 receptors and B_2 receptors appear to be necessary for the potentiating the effects of ACE inhibition. ACE and B_2 receptors appear to form a heterodimer, thus enhancing the efficiency of B_2 signalling (Abadir et al. 2006).

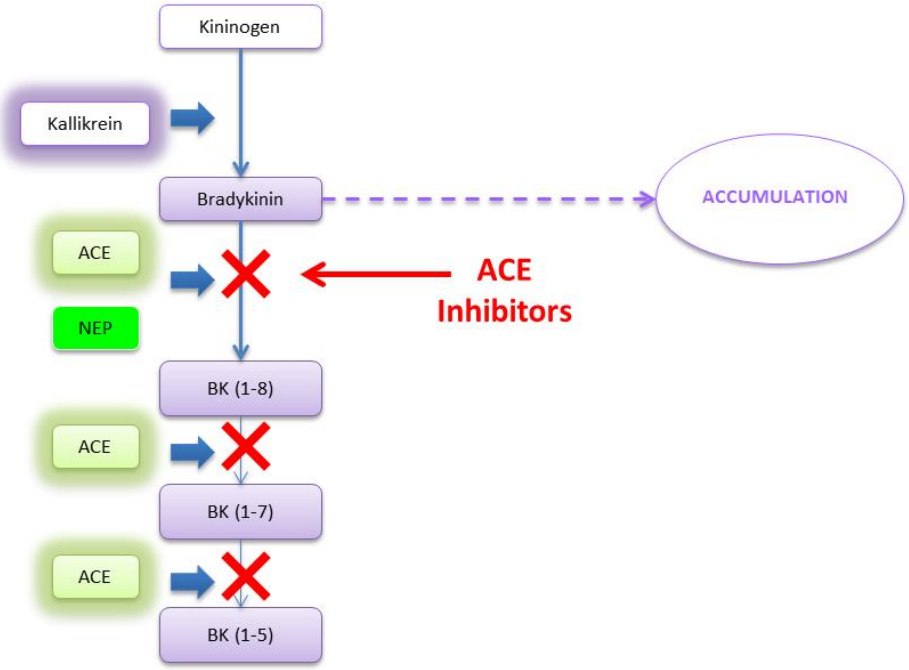


Figure 1.9 Inhibition of bradykinin metabolism by ACE inhibition

1.7.2 CHYMASE

During chronic ACE inhibition, a phenomenon known as 'ACE escape' occurs (Ennezat et al. 2000). In this condition, ACE inhibitors become ineffective at reducing circulating Ang II levels in the long-term, suggesting a role for alternative pathways of Ang II synthesis. It has been suggested that this phenomenon could be attributable to the serine protease chymase, which is responsible for ACE-independent Ang II generation from Ang I in the local cardiovascular system in some species (Urata et al. 1990). Chymase is found mainly in the secretory granules of mast cells – where it is stored until its release into the interstitial space - although it has also been identified in the endothelial cells of human cardiac tissue (Zablocki & Sadoshima. 2011). An increase in bradykinin (as a result of ACE inhibition) may in fact be responsible for chymase elevation, leading to an increase in chymase activity and

maintenance of Ang II levels despite ACE inhibition (Urata et al. 1993). Wei et al. identified a bradykinin-sensitive receptor on mast cells in mice, indicating a role for this peptide in chymase activation and Ang II generation (Urata et al. 1993)

Chymase expression and its role in Ang II generation is highly variable between species (Wei et al. 2010). In humans, dogs (Wei et al. 2010) and hamsters (Miyazaki et al. 2006), chymase has been shown to play a significant role in Ang II generation – indeed, in the human heart, the majority of Ang II generation (> 80%) appears to result from the actions of chymase (Wolny et al. 1997). However, in the rat, mouse and rabbit, no such active Ang II-generating role for chymase has been observed (Abbas et al. 1999).

It should be noted, however, that the recently discovered angiotensin peptide Ang (1-12) serves as a substrate for chymase-mediated Ang II generation in both rat (Abbas et al. 1999) and human (Okunishi et al. 1993) cardiac tissue. However, in rats, administration of a chymase inhibitor does not suppress Ang II synthesis, while treatment with lisinopril does. Furthermore, sequence analysis of rat chymase has clearly demonstrated its inability to form Ang II from the Ang I substrate. Thus, any contribution to Ang II levels by Ang (1-12) chymase-mediated hydrolysis is expected to be negligible (Prosser et al. 2009).

In humans in late-stage heart failure (Ahmad et al. 2011) and in myocardial infarction models in both the hamster (Jin et al. 2001) and the dog (Jin et al. 2004), mast cell density and chymase activity in the infarcted area is significantly upregulated post-MI (as is ACE activity). In a hamster model, administration of chymase inhibitors does not affect plasma Ang II concentrations, but does improve cardiac function and mortality rates (Miyazaki et al. 2006). In a dog model of MI, plasma Ang II levels decrease following administration of a chymase inhibitor (Batlle et al. 2006). It has been suggested that inhibition of chymase activity may have an anti-arrhythmic effect on the infarcted heart, as treatment with a chymase inhibitor reduces the incidence of ventricular arrhythmias in a dog model of MI (Jin et al. 2003). Several studies have demonstrated that stimulation of AT_1 receptors by Ang II has an arrhythmogenic effect (reviewed in (Jin et al. 2004)), thus inhibition of cardiac Ang II generation significantly reduces the incidence of arrhythmia.

In addition to Ang II generation, chymase activates the transforming growth factor-β (TGF-β), which plays a role in cardiac remodeling by increasing fibroblast proliferation and collagen deposition (Jin et al. 2004; Garg et al. 2006). Consequently, despite the absence of chymase-mediated Ang II generation in the rat, chymase inhibition in an MI model in this animal does attenuate fibrosis and improve left ventricular function (Kanemitsu et al. 2006).

1.7.3 N-ACETYL-SER-ASP-LYS-PRO (ACSDKP)

AcSDKP is a tetrapeptide released from the precursor thymosin β4 by the serine peptidase prolyl oligopeptidase (POP) (Vavasin et al. 2004). While studies have shown that AcSDKP has angiogenic, anti-inflammatory and antifibrotic effects (Ocaranza et al. 2010; Rousseau et al. 1995; Wang et al. 2004), its principal function is believed to be as a natural inhibitor of hematopoietic stem cells, preventing proliferation by hindering their entry into the *S* phase of the cell cycle (M Lenfant et al. 1989). Inhibition of AcSDKP may therefore have positive effects on stem cell recruitment under pathophysiological conditions, despite the suppression of other effects – such as fibrosis - that may contribute positively towards cardiac remodeling. It does not form part of the RAS, but as it is cleaved by ACE, endogenous levels are affected by ACE inhibition. Administration of non-selective ACE inhibitors showed that the plasma concentration of AcSDKP increased by 5-fold, confirming that ACE is the major enzyme responsible for degradation of this peptide (Azizi et al. 1996). It has furthermore been shown that AcSDKP is hydrolysed almost exclusively by the N-domain of ACE. (Rousseau et al. 1995).

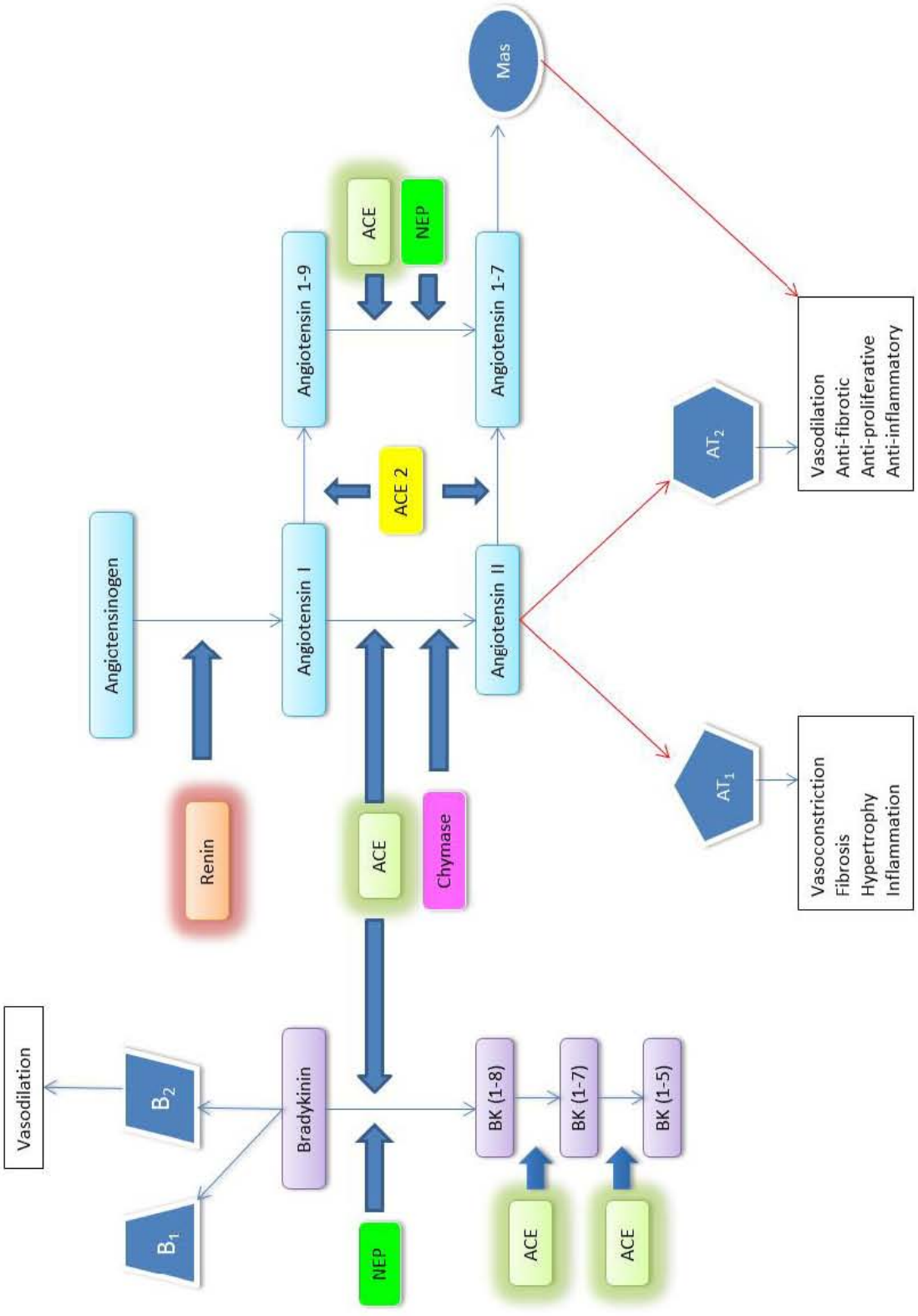


Figure 1.10 The complete RAS pathway: Interactions and Effects

1.8 Development of a novel C-domain selective ACE Inhibitor

1.8.1 Brief History of clinically-available ACE Inhibitors

The first ACE inhibitor was developed in the late 1960s, and was isolated from the venom of the Brazilian arrowhead viper (Bothrops jararaca). It was originally named bradykinin-potentiating peptide (BPP) as it inhibited kininase II, an enzyme responsible for bradykinin degradation. It was later discovered that ACE and kininase II were in fact the same enzyme, and that BPP actually inhibited Ang II production by interrupting the RAS at the ACE level. (Ferreira et al. 1965; Ferreira et al. 1970) (Cushman & Cheung 1971)

Teprotide – a synthetic analogue of BPP – was shown to effectively lower blood pressure in hypertensive patients, as well as demonstrate positive haemodynamic effects in patients suffering from heart failure (Ferreira et al. 1970). This inspired the search for orally active ACE inhibitors, as the teprotide had to be administered parenterally or intravenously, making it an inadequate long-term therapy in the clinical setting (Johnson et al. 1975).

The very first orally active ACE inhibitor was Captopril (Gavras et al. 1978). Commercially released in 1981, Captopril contained a sulfhydryl group, which was eventually found to be responsible for the many side effects experienced by patients (such as skin rashes and proteinuria) (Jaffe 1986). The sulfhydryl group was consequently replaced by a carboxyl group, which conferred the drugs Enalapril and Lisinopril – developed in the 1980s - with improved lipophilicity (Ferreira et al. 1970). Fosinopril, Quinapril, Benazepril and Ramipril followed in the early 1990s, and in the mid-1990s, Perindopril, Moexipril and Trandolapril were released (Sica 2010). The majority of ACE inhibitors on the market contain the carboxyl group, with fosinopril as the only exception with a phosphinyl group as its reactive moiety (Patchett et al. 1980). ACE inhibitors differ in terms of half-lives and routes of elimination, although they are predominantly cleared by the renal system (Ma et al. 2010). Most ACE inhibitors – with the exception of lisinopril and captopril - are administered in a prodrug form (which has enhanced bioavailability when administered orally) which remains inactive form until de-esterified by the liver (Brown et al. 1998).

1.8.2 ADVERSE EFFECTS OF ACE INHIBITORS

In 2010, 168.7 million prescriptions for ACE inhibitors were dispensed by pharmacies in the United States alone (IMS Institute for Healthcare Informatics 2010). Considering the considerable number of patients to whom ACE inhibitor therapy is administered, the incidence and severity of adverse side effects are of serious concern.

ACE inhibitor side-effects are mainly classified as class effects, as they tend to apply to all drugs in the class. Not unexpectedly, the main side effect of ACE inhibition is hypotension. Patients in renin-dependent states are more susceptible to hypotension, and their dosages are carefully monitored (Banerjee et al. 2003). Hyperkalemia is another common side-effect, as ACE is responsible for stimulating aldosterone release from the kidney (Mancini et al. 1996). In addition, deterioration of renal function is often associated with ACE inhibitor treatment. This is caused by the reduction in glomerular filtration rate, which is a direct consequence of Ang II inhibition (Ang II causes vasoconstriction of the efferent arterioles in the kidney, which are responsible for regulating glomerular filtration rates) (Schartl et al. 1994). However, this condition is reversible when ACE inhibitor administration is ceased (Hodsman et al. 1983; Hall et al. 1977).

The most frequent side-effect of ACE inhibitor therapy is a dry, persistent cough, which studies report affects 3 to 35% of patients (Warren et al. 1980; Hall et al. 1981). The exact cause of the cough is still unknown, but may be attributable to elevated levels of bradykinin, prostaglandins and the neuropeptide substance P. Bradykinin and substance P metabolism is dependent on ACE activity, and in the case of inhibition, these peptides accumulate in the lungs (where ACE expression is most prevalent) and upper airways (Irwin et al. 1990; Smyrnios et al. 1995). The only treatment for this cough is complete cessation of ACE inhibitor therapy and substitution with another drug class such as ARBs (Dicpinigaitis 2006).

Finally, drug-induced angioedema is a rare but potentially life-threatening side-effect of ACE inhibition, and is estimated to affect 0.1 to 2.2% of patients on ACE inhibitor therapy (Israili et al. 1992; Brown & Vaughan. 1998). Angioedema involves the

swelling of the dermis, subcutaneous and submucosal tissues, and is characterized by localized swelling of the tongue, lips, mouth, throat and other facial areas (Vleeming et al. 1998; Kostis et al. 2004). The rate of angioedema is highest in the first months following treatment, and should it occur, discontinuation of the drug is essential (Weber et al. 2008). As with the persistent cough experienced by some patients, angioedema has been attributed to the elevated levels of bradykinin observed during ACE inhibition (Banerji et al. 2009; Fox et al. 1996).

1.8.3 Domain-Selective ACE inhibitors

ACE consists of two homologous catalytic domains, each playing different yet essential roles in the metabolism of RAS and KKS peptides. The orally active ACE inhibitors currently in clinical use were all designed before the three-dimensional structure of ACE was successfully elucidated, and thus target both domains relatively equally. The first generation ACE inhibitor Captopril was designed based on the known structure of carboxypeptidase A - an enzyme which was believed to be similar in catalytic activity to ACE (Ondetti et al. 1977). Captopril has since been found to be slightly more selective towards the N-terminal of ACE (Wei & Clauser. 1992). The second generation ACE inhibitors – enalapril and lisinopril – were developed based on the inhibition of the zinc metalloproteinase thermolysin (Patchett et al. 1980) and lisinopril in particular has since been shown to be marginally more C-domain selective (Corradi et al. 2006). However, the modest selectivity of these ACE inhibitors towards either domain has not produced any significant difference in terms of treatment efficacy. Thus the development of ACE inhibitors that exclusively target one of the two domains were required to explore the physiological ramifications of domain-selective inhibition both *in vitro* and *in vivo*. Our current knowledge of the physiological roles of each domain is attributable to both the development of domain-selective ACE inhibitors as well as ACE gene knockout studies in mice (which will be expanded on in sections 1.8.3.1 and 1.8.3.2). The physiological roles of the C- and N-domains of ACE are summarized below in figure 1.11.

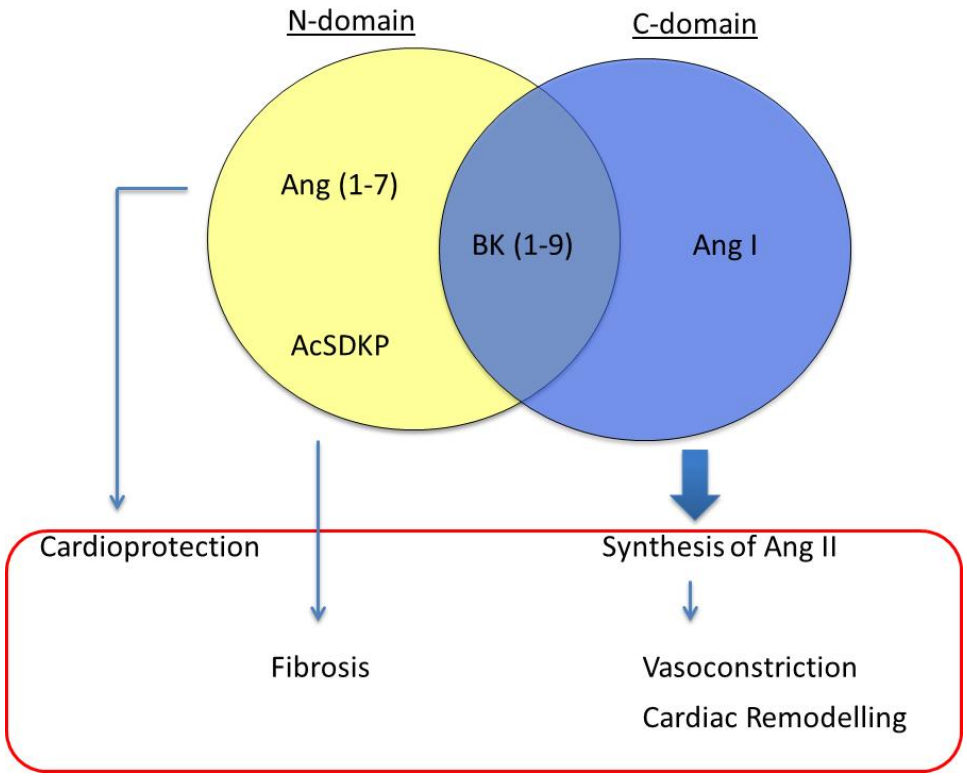


Figure 1.11 The roles of the N- and C-domains of ACE

1.8.3.1 The Role of the N-Domain and RXP 407

The role of the N-domain was the first to be explored with the development of the domain-selective ACE inhibitor RXP 407 (figure 1.13) identified by the group Dive et al. in 1999 (Dive et al. 1999). A phosphinic tetrapeptide, this inhibitor is 1000-fold more selective for the N-terminal than the C-terminal of ACE. This selectivity was attributed to the presence of a C-terminal amide group, an N-terminal acetyl group and an aspartic side chain in the P'_2 position of the inhibitor. The structural design of RXP 407 was found to inhibit binding of the inhibitor to the C-domain of ACE while binding to the N-domain was well-tolerated by the enzyme. As such, the domain-selectivity of RXP 407 is duly ascribed to the antagonistic nature of its structural design towards the C-domain. (Dive et al. 1999)

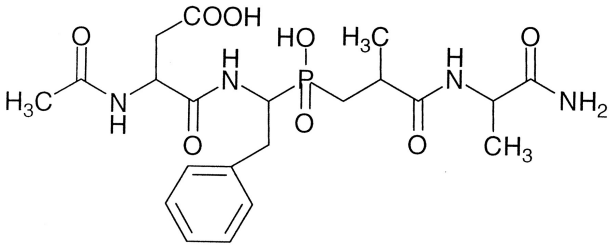


Figure 1.12 The chemical structure of RXP 407 (N-selective ACE inhibitor)

In vitro studies by Dive *et al.* (1999) with RXP 407 showed that the N-domain of ACE was chiefly responsible for metabolizing the peptide AcSDKP (acetyl-Ser-Asp-Lys-Pro-COOH). Later *in vivo* studies with mice demonstrated that selective inhibition with RXP 407 increased plasma AcSDKP levels by up to 6-fold (Junot et al. 2001). Further studies with N-domain knockout (N-KO) mice supported this finding and showed that AcSDKP levels are increased by 7.3-fold in N-KO mice as compared to wild-type (WT) mice (Nchinda et al. 2006). While classic non-selective ACE inhibition blocks AcSDKP degradation and elevates endogenous levels, C domain-selective inhibition should thus have no effect on metabolism of this peptide.

1.8.3.2 The Role of the C-domain and RXP A380

Following the development of RXP 407 came RXPA 380 (figure 1.12), another phosphinic peptide with an affinity 1000-fold higher for the C-domain than the N-domain of ACE (Georgiadis et al. 2003). RXPA 380 is therefore a potent, highly specific C-terminal selective ACE inhibitor. This domain-selectivity was initially attributed to the presence of a pseudo-proline group at P_1' which interacted with the S_1' pocket of ACE, and a tryptophan group at P_2' which interacted with the S_2' pocket of ACE (Tzakos et al. 2005). Clarification on this topic came with the determination of the crystal structure of RXP A380 in complex with tACE (which has an active site homologous to the C-domain of somatic ACE). RXP A380's domain selectivity was explained by the interaction of bulky moieties (in this case tryptophan) at the P_2'

position with residues unique to the C-domain in the S_2 and S_2' ACE subsites (Corradi et al. 2007).

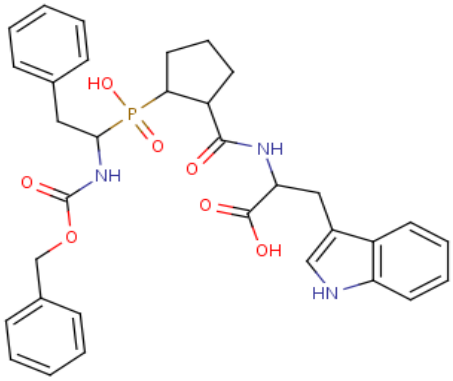


Figure 1.13 The chemical structure of RXP A380 (C-selective ACE inhibitor)

In vitro studies with RXPA 380 originally showed that both domains are responsible for degrading both Ang I and Bradykinin. However, in the case of Ang I *in vivo* studies have shown that the C-domain demonstrates far more efficient cleavage than the N-domain and is thus chiefly responsible for Ang II production and blood pressure regulation (Georgiadis et al. 2003; Junot et al. 2001).

This discrepancy was further confirmed by the group of Bernstein et al., who demonstrated that in ACE N-domain knockout (N-KO) mice with normal blood pressures, plasma renin and Ang II levels were identical to those of wild-type (WT) mice. However, in C-domain knockout (C-KO) mice, while the animals' blood pressures were normal, their plasma renin and Ang II levels were found to be significantly greater - by 2.6-fold and 7-fold respectively - than those of their WT counterparts (Bernstein et al. 2011). This as a consequence of the inefficiency of the N-domain to cleave Ang I and resultant overexpression of both renin – and consequently Ang II – in an effort to maintain normal BP in these animals. This study provides strong evidence for both the inefficiency of the N-domain in terms of Ang II production as well as the different mechanisms by which ACE-mediated Ang I metabolism occurs under *in vivo* and *in vitro* conditions.

Based on the current body of work concerning the differing roles of the two ACE domains, it appears that a C-domain selective ACE inhibitor should have a more desirable clinical profile than non-selective ACE inhibitors. Despite the success of non-selective ACE inhibitors in the treatment of cardiovascular diseases and hypertension, angioedema associated with ACE inhibition is a serious side-effect that can only be remedied by discontinuing ACE inhibitor therapy. Bradykinin accumulation has been implicated in the development of angioedema during both ACE inhibition and dual ACE-NEP inhibition. The opportunity to reduce the effect of dual-domain ACE inhibition on bradykinin metabolism by developing a domain-selective ACE inhibitor presents an attractive option for future, rational drug design. As *in vivo* studies have convincingly demonstrated the critical role of the C-domain in Ang II production and blood pressure control *in vivo*, inhibition of this domain in pathophysiological conditions is essential. Based on our current understanding, a C-domain selective ACE inhibitor should attain an acceptable level of inhibition of Ang II production *in vivo* while simultaneously reducing the effect of ACE inhibition on bradykinin plasma levels, and consequently, the incidence or severity of adverse ACE inhibitor-induced side effects.

1.8.4 DEVELOPMENT OF LISINOPRIL-TRYPTOPHAN: A C-DOMAIN SELECTIVE ACE INHIBITOR

Figure 1.14 Crystal structure of human somatic ACE in complex with the ACE inhibitor lisinopril (Kanemitsu et al. 2006)

The three-dimensional structure of ACE was first elucidated through X-ray crystallography visualisation of human tACE (representing only the C-domain of ACE) in complex with the ACE inhibitor lisinopril (figure 1.14 illustrates the more recent visualisation of lisinopril in complex with somatic ACE) (Natesh et al. 2003). The three-dimensional visualization finally revealed the molecular basis behind the ACE substrate-binding pockets and their interactions with lisinopril, as well as demonstrating that human ACE bears little resemblance to carboxypeptidase A (which was used as the basis for second generation ACE inhibitor drug design). Insights into the significance of the S_1, S_1' and S_2' pockets of ACE in the binding of this complex highlighted their importance for future rational drug design (figure 1.15).

The S_2' subsite was found to be particularly crucial to the potency of lisinopril, as the interaction of this inhibitor's P_2' moiety at this subsite actually increases ACE potency by 25-fold (Patchett et al. 1985).

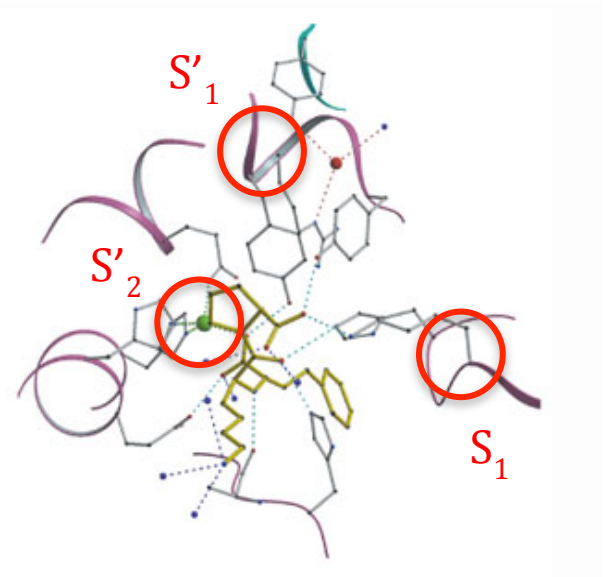


Figure 1.15 Lisinopril (yellow) in complex with human tACE – significant binding subsites are circles and labeled in red.

The Sturrock group extended on the work done by Dive et al. on domain-selective ACE inhibitors by incorporating the key feature of RXP A380 – the tryptophan moiety – into the P_2' position of the clinically available ACE inhibitor lisinopril (Nchinda, Chibale, Redelinghuys, et al. 2006). The chemical structures of lisinopril and its analogue are presented below in figure 1.16. This approach was taken as a C-domain selective ACE inhibitor with the favourable pharmacological profile and efficacy of lisinopril holds significant therapeutic potential. Synthesis of this lisinopril analogue – referred to as lisinopril-tryptophan or lisW – produced a diastereomeric mixture of two isomers requiring purification and separation by HPLC. *In vitro* inhibition assays confirmed the inhibitory potential of these two compounds, and found that they inhibited only the C-domain, although they differed in terms of K_i values. Modeling of the two compounds with both tACE (for the C-domain) and a homologous model of the somatic ACE N-domain were conducted to confirm

observations of C-domain selectivity. The conclusion of this work was that the S-enantiomer was determined to be 100-fold more selective for the C-domain than lisinopril (which has a C-domain-selectivity of 2.6 fold), while the second analogue was found to be just 25-fold more C-domain selective than lisinopril. The more selective LisW-S enantiomer was thus chosen for future ACE inhibitory studies, despite the necessity for a tedious HPLC purification step in the peptide synthesis process.

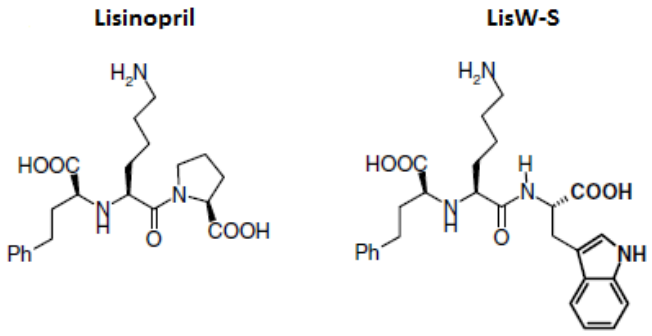


Figure 1.16 The chemical structures of lisinopril and LisW-S

Further studies by the Sturrock group to assess the inhibitory potential of the novel C-domain selective inhibitor led to the successful modeling of lisW-S complexed with tACE. These studies clearly demonstrated the depth of the S'_2 pocket of ACE, which accommodated lisW-S's bulky P'_2 moiety with ease. This modeling also revealed additional binding interactions between lisW-S and C-domain specific S'_2 residues that are not observed in the lisinopril-tACE complex, and could therefore be responsible for the 258-fold greater selectivity demonstrated by lisW-S towards the C-domain (as opposed to the 2.6-fold selectivity of lisinopril for this domain) *in vivo*. This work further supported the implications presented in previous studies evaluating the importance of the deep S'_2 subsite and its interaction with a bulky P'_2 moiety on the conformation and C-domain-selectivity of ACE inhibitors (Georgiadis et al. 2004;

Natesh et al. 2004). Co-crystallization of both lisinopril and the chosen lisW-S enantiomer with tACE also showed the similarity of their conformation when binding to the tACE active site (Corradi et al. 2007). This similarity is presented below in figure 1.17.

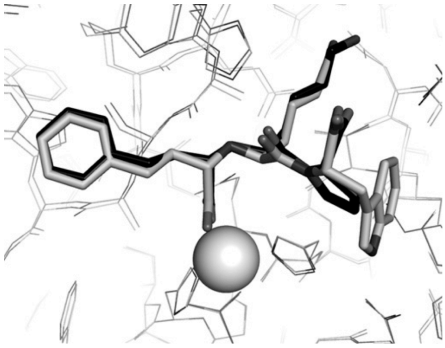


Figure 1.17 LisW-S (in grey) takes a similar conformation to lisinopril (in black) when binding to the active site of tACE. The position of the catalytic Zinc atom is represented by the sphere. (Nchinda et al. 2006)

1.9 AIM

As described in the previous section, this ACE inhibitor has been extensively characterized *in vitro* by the Sturrock group and its domain-specificity both quantified and validated with inhibition assays and modeling of the compound to both the tACE molecule (homologous with the C-domain of ACE) and the molecular recombinant N-domain of ACE. The results of this work showed that lisW-S has the potential to fulfill the role of a successful C-domain specific ACE inhibitor.

Our knowledge of the RAS and the complex interdependency of the cascade on RAS peptide levels as described in this chapter presents an enticing opportunity to apply the C-domain selective inhibitor LisW-S therapeutically in an effort to improve the clinical profile of ACE inhibition. The most common adverse side-effect of non-specific ACE inhibitor treatment is the persistent cough experienced by up to 35% of patients and believed to be attributable to elevated levels of bradykinin. A less common but life-threatening side-effect is angioedema, affecting up to 2.2% of patients. As described in section 1.8.2, there is evidence to suggest that this too is a result of bradykinin accumulation.

Sections 1.8.3.1 and 1.8.3.2 addressed our current knowledge of the physiological roles of each ACE domain and their influence on both RAS and non-RAS peptide levels. The work of Bernstein et al. in domain knockout mice was particularly enlightening with regards to the domain-specific actions of ACE in a physiological model. Indeed, this work reminds us that results *in vitro* do not necessarily predict results *in vivo*, as prior *in vitro* studies suggested that both ACE domains were responsible for metabolizing Ang I and Bradykinin, while Bernstein and others have shown that the C-domain is chiefly responsible for Ang II degradation.

The sum of our knowledge on the roles of the C- and N-domains of ACE are summarized in Figure 1.11. The hypothesis is thus that a C-domain specific ACE inhibitor should largely inhibit Ang I metabolism and subsequent Ang II generation as well as partly inhibit Bradykinin breakdown and the subsequent rise in this peptide's levels as observed with non-selective ACE inhibition. In addition, as the N-domain

remains unaffected, we would expect to see a rise in levels of the cardioprotective peptides Ang (1-7) and AcSDKP – both of which would be expected to have positive effects post-MI. Ang (1-7) forms part of the alternative axis of the RAS (reviewed in section 1.5.1) and elevated levels of this peptide in a pathophysiological setting has the added advantage of providing greater insight into the counter-regulatory effect of this axis on Ang II. The role of Ang (1-7) is briefly reviewed in section 1.5.1 and more extensively discussed in section 4.6.5, while AcSDKP is reviewed in section 1.7.3.

The long-term aim of this research is to establish the potential of a C-domain specific ACE inhibitor as a superior therapeutic option to currently available non-specific ACE inhibitors. To this end, the aim of this work was the initial characterisation of the novel C-domain specific ACE inhibitor LisW-S in an animal model of MI. Given the complexity of the RAS and the range of physiological effects resultant from activation, a strong understanding of the specific effects of lisW-S on relevant RAS peptides *in vivo* is essential. Thus, the main aim of this study was to deliver and reach therapeutic circulatory levels of lisW-S in a rat model of MI and to assess quantitatively the effects of this novel ACE inhibitor on the RAS cascade. Importantly, the availability of highly sensitive assays and techniques for plasma peptide quantification was a major determinant in the design of this study.

The clinical outcomes of this treatment were not measured or considered at this stage. As reviewed in this chapter, ACE inhibitors are responsible for a range of physiological effects, many of which are inter-dependent and thus do not necessarily offer an accurate view of RAS peptide levels.

Three main objectives were pursued in order to achieve this characterization.

In the first objective, enzyme kinetics and inhibition assays were utilized to assess the inhibitory potential and domain-selectivity of the novel ACE inhibitor in an extracellular environment relevant to clinical application – namely plasma.

Once domain-selectivity and effective inhibitory concentrations were established in plasma, the bioavailability and achievable dosages of LisW-S in a rat model were determined in a range of pharmacokinetic studies.

Finally, the *in vivo* domain-selectivity and therapeutic potential of LisW-S in terms of RAS management was evaluated by quantifying the peptide products of the RAS cascade using a novel technique known as RAS-fingerprinting. As a long-tem goal is the determination of efficacy of LisW-S in reducing pathological remodeling post-MI and ACE inhibitors are a frontline treatment post-MI, the impact of LisW-S on RAS peptides (and other relevant peptides) was assessed in a rat MI model.

Chapter 2 INHIBITION ASSAYS

2.1 INTRODUCTION

A series of inhibition assays were performed to assess the C-domain specific inhibitory efficacy of lisW-S *in vitro* in rat plasma. These assays also aided in determining the plasma lisW-S concentration required to obtain an effective inhibitory dosage in the animals for the proposed MI studies.

The assay needed to be optimized for use with rat plasma specifically, as the proposed RAS peptide level studies with lisW-S would be conducted in vivo in a rat model. It was important to validate the C-domain specificity of lisW-S in rat plasma. LisW-S is a known C-domain selective inhibitor of human ACE, and so human serum was used as a control to validate the assays. Although inhibition profiles were obtained by Nchida *et al.* (2006) in their development of lisW-S, these were performed on isolated human recombinant ACE domains in the absence of any plasma or serum.

The work of Jullien *et al*. (Jullien et al. 2006) highlighted the disparity in both ACE inhibitor and substrate specificities between different ACE species. The difference between species' domain selectivity of the substrates Mca-Ala-Ser-Asp-Lys-DpaOH (Mca-Ala) and Mca-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DpaOH (Mca-$BK_{(1-8)}$) were explored in this work. The selectivity of the Mca-Ala for the N-domain was shown to vary significantly between rat and human ACE. Mca-Ala is an N-domain selective substrate for human ACE, but appears to be much more selective for the C-domain in rat ACE. Inhibition profiles of Mca-$BK_{(1-8)}$) showed that while all three species do display C-domain selectivity towards this substrate, rat ACE has substantially the highest substrate affinity (while mouse ACE had the lowest). Differences in the binding affinity of the inhibitor RXP 407 to the C- and N-domains of mouse, rat and human ACE were also observed. RXP 407 is a known N-domain specific inhibitor of human ACE (Dive et al. 1999) and displays a similar profile in the mouse. Binding of

this inhibitor to rat ACE demonstrated a slightly lower affinity for the N-domain as compared to the other two species, but was still N-domain selective.

The conclusion of this work was that there are significant differences between human, mouse and rat ACE in terms of domain selectivity. These distinctions have only recently come to light, and make a compelling case for the need to define domain-selective inhibitor specificity in rat ACE prior to embarking on rat model-based efficacy studies.

2.1.1 Quantification of ACE activity

An *in vitro* technique to measure the catalytic activity of ACE is an essential tool in the development of pharmacological interventions with the potential for more stringent management of the RAS in a clinical setting. The first successful technique was developed in 1971 and played an integral role in the development and synthesis of the first ACE inhibitor, Captopril (Cushman et al. 1971). The concept was based on the hydrolysis of a substrate by ACE and the quantification of the hydrolytic product by spectrophotometry. The development of this spectrophotometric assay facilitated the discovery of the first ACE inhibitory peptides.

Other methods to measure ACE activity in human serum include fluorometric (Friedland et al. 1976; Carmel et al. 1979), radiometric (Rohrbach 1978), radioimmunoassay (Hiwada et al. 1987) and HPLC (Meng & Oparil. 1996). While all of these techniques have had a place in ACE activity research for decades, they do have serious limitations. These include low sensitivity, expense and complexity, as well as the inability to continuously monitor the enzymatic reaction.

Fluorescence resonance energy transfer (FRET) is a highly sensitive fluorometric technique used to measure ACE activity. The process is rapid and samples are assayed directly in the fluorometer in either a cuvette or a plate. Most importantly, measurable fluorescence occurs immediately after substrate hydrolysis, allowing for continuous assessment of ACE activity over a specific period.

2.2.2.1 FLUORESCENCE RESONANCE ENERGY TRANSFER

Fluorescent resonance energy transfer (FRET) is an assay comprising of a fluorescent donor group attached to one amino acid residue in the peptide sequence and a quenching acceptor group attached to another residue in the sequence. Prior to cleavage, excitation of the donor group releases fluorescence which is quenched by the closely situated acceptor group. If cleavage occurs at any point between the donor/ acceptor pair, the donor group is released and is no longer in close proximity to the acceptor. Fluorescent excitation of the donor emits fluorescence which is no longer quenched by the acceptor, and thus can be measured continuously, providing a quantitative measurement of enzyme activity (figure 2.1).

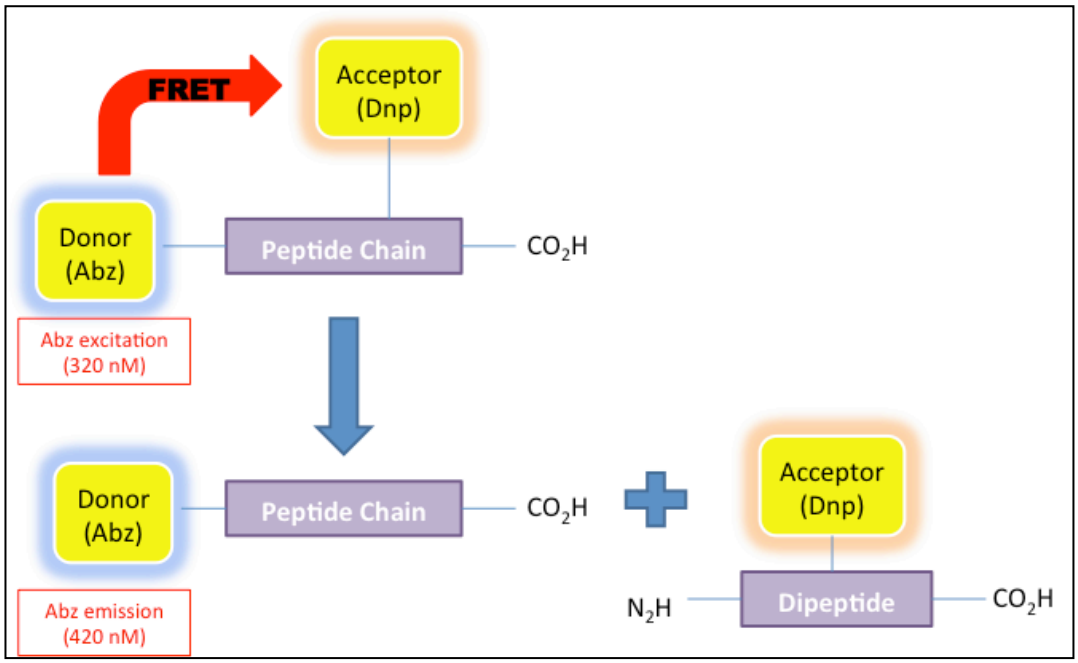


Figure 2.1 FRET is a distance-dependent excited state interaction in which emission of one fluorophore is coupled to the excitation of another. (Adapted from Carmona et al. 2006)

The specific fluorogenic substrates developed for assaying ACE activity were initially based on the bradykinin sequence. Bradykinin was chosen as it has a K_m value for hydrolysis by ACE that is 100 x lower than that of angiotensin I (Dorer et al. 1974) (E G Erdös 1975). The bradykinin analogues were synthesized and the donor/ acceptor pair Abz (*ortho*-aminobenzoic acid) and EDDnp (*N*-[2,4-dinitrophenyl]-ethylenediamine) were added to the N- and C-terminal respectively. These internally quenched bradykinin-related peptides were successfully hydrolysed by ACE and enzyme activity was determined in human plasma (Araujo et al. 1999).

Following on this work, the same group investigated the substrate specificity requirements of the C- and N-domain of ACE (Araujo et al. 2000). This study assayed the activity of two full-length ACE mutants, each with a single functional site – either the C- or N-domain. Two bradykinin-related peptides based on the Abz/ EDDnp donor/ acceptor pair were used as substrates for ACE mutant hydrolysis. The result was the synthesis of the first domain specific bradykinin-related peptide substrates – Abz-GFSPFFQ-EDDnp was found to be preferentially hydrolysed by the C-domain of ACE, while Abz-GFSPFQQ-EDDnp was shown to be N-domain specific. A further advance was the successful synthesis of an AcSDKP internally quenched fluorescent analogue (Abz-SDK(Dnp)P-OH), utilizing the same donor acceptor pair and demonstrated to be highly N-domain specific. The addition of the free C-terminal carboxyl group to this substrate significantly improved the cleavage efficiency by wild-type recombinant human ACE , making it preferable to previously developed bradykinin analogues. Finally, this group also synthesized the peptide Abz-FRK(Dnp)P-OH, and showed it to be an efficiently cleaved, non-specific substrate in the presence of the ACE inhibitor captopril (Figure 2.2) (Araujo et al. 2000).

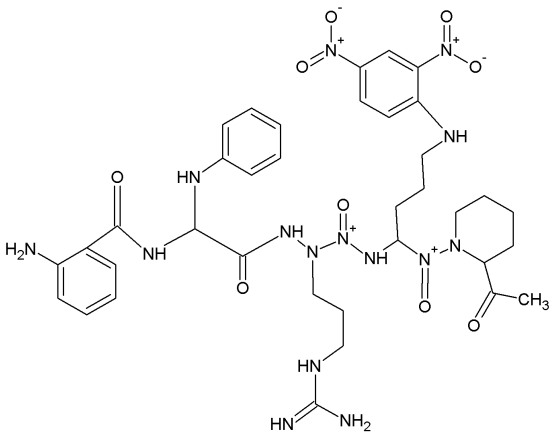


Figure 2.2 Chemical structure of Abz-FRK(Dnp)P-OH

Later, Bersanetti *et al*. made use of positional scanning combinatorial libraries of FRET peptides to assess the effect of the S_3 to S_1' subsites of ACE on domain selectivity. The result was the design and synthesis of the peptide Abz-LFK(Dnp)-OH, which was found to be highly C-domain selective for human ACE (Figure 2.3) (Bersanetti et al. 2004).

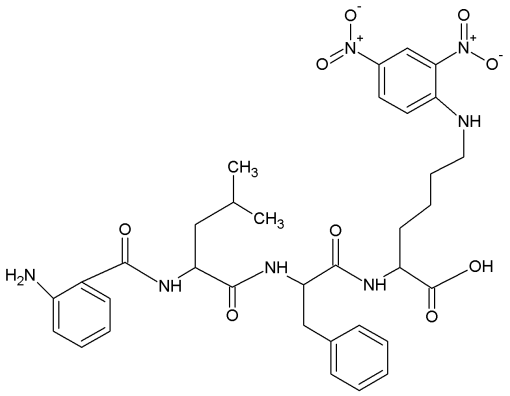


Figure 2.3 Chemical structure of Abz-LFK(Dnp)-OH

In this study, FRET has been employed using both the C- domain specific and non-specific substrates – namely Abz-LFK(Dnp)-OH and Abz-FRK(Dnp)P-OH respectively, to characterise the domain-selectivity of the novel inhibitor lisW-S in rat ACE. Both the non-specific and N-domain specific inhibitors lisinopril and RXP 407 respectively, were used comparatively and as controls throughout these assays.

2.2 METHODS

2.2.1 SUBSTRATES AND INHIBITORS

A panel of inhibitors and FRET substrates were employed to characterise the domain-selectivity of LisW-S in rat ACE. Known domain specificities in human ACE of each inhibitor and peptide are highlighted in the table 2.1.

Table 2-1 Summary of the inhibitors and FRET peptides employed

Substrate	**Inhibitor**	**Specificity in human**	**Specificity in rat**
Abz-LFK(Dnp)-OH		C-domain	Unknown
Abz-FRK(Dnp)P-OH		Non-specific	Unknown
	Lisinopril	Non-specific	Unknown
	LisW-S	C-domain	Unknown
	RXP 407	N-domain	N-domain

2.2.2 FRET PEPTIDE ASSAYS

The powdered fluorogenic substrates Abz-LFK(Dnp)-OH and Abz-FRK(Dnp)P-OH (kindly provided to Prof Sturrock's laboratory by Prof Adriana K. Carmona of the Federal University of São Paulo, Brazil) were prepared to an approximate stock solution of 10 mM in DMSO. Concentrations were confirmed spectrophotometrically at an absorbance of 365 nM and 10 µM working solutions were made up in Tris buffer (50 mM Tris (tris(hydroxymethyl)aminomethane) (pH 7.0), 50 mM NaCl and 10 µM $ZnCl_2$ in ddH_2O). All chemicals were purchased from Sigma-Aldrich (Germany).

Cleavage of these substrates was monitored in a continuous assay in a 96 well microassay plate at ambient temperature (20 – 25^{o}C), under initial rate conditions of less than 10 % hydrolysis, over time (s). Briefly, in triplicate, 25 µL of a 10% human serum or rat plasma sample was added to substrate concentration ranges of 0 – 100 or 0 - 500 nM, to a final volume of 300 µl in Tris buffer. Fluorescence was measured with excitation (Ex) at 320 nm, and Emission (Em) at 420 nm. Arbitrary fluorescent units (AFU) were converted to the units of number of mols of substrate hydrolyzed per ml of plasma per minute (nmol/ ml/ min) using a standard curve (see below). All figures were plotted using GraphPad Prism 5 software.

2.2.3 BLOOD COLLECTION

2.2.3.1 HUMAN SERUM SAMPLES

Whole blood was collected from healthy volunteers by a registered medical practitioner at Groote Schuur Hospital, Cape Town, South Africa. Blood was collected into a sterile tube with no anticoagulant present and allowed to stand for 20 to 30 minutes. Blood samples were centrifuged at 3000 g for 15 minutes (4^{o}C). Serum was collected and transferred to an eppendorf tube and stored at -80^{o}C.

2.2.3.2 RAT PLASMA SAMPLES

All animal experiments were approved by the Animal Research Ethics Committee of the University of Cape Town and were performed in accordance with the National Institutes of Health (NIH, Bethesda, MD) guidelines.

Whole blood was collected from recently sacrificed rats by cardiac puncture. 5 to 10 ml of blood was collected per animal into a 10 ml lithium-heparin coated blood collection tube. The tube was inverted at least 8 times to ensure thorough mixing of the contents. Blood samples were centrifuged at 3000 g for 15 minutes (4^{o}C). Plasma was collected and transferred to an eppendorf tube and stored at -80^{o}C.

2.2.4 Standard Curve

Standard curves of AFU vs. nmol/ml/min were generated for both human serum and rat plasma for the determination of hydrolytic activity of respective rat and human ACE in further assays. Species-specific samples were pooled to ensure the accuracy of the assays and avoid potential variability between plasma and serum samples.

Briefly, a dilution series of 0.3, 0.6, 0.9, 1.2, 1.8, and 2.4 nmol/L was prepared from the 10 µM Abz-LFK(Dnp)-OH stock. In the initial attempt at establishing the standard curve, full hydrolysis was not achieved. As such, all plasma and serum samples were spiked with 2.4 µg tACE to accelerate hydrolysis to completion. The standard curve was calculated by measuring the difference between the basal fluorescence and fluorescence at complete hydrolysis of the standards. To obtain basal fluorescence, 10% dilutions of plasma or serum were incubated with 50 µM lisinopril for 90 minutes to completely inhibit all endogenous ACE activity and effectively provide a ‘zero’ control. Zero samples were added to an equal volume of Tris buffer and plated in triplicate. Standard samples were prepared as a 10% dilution of plasma or serum in 25 mM Tris buffer. These were added to an equal volume of the substrate dilution series and plated in triplicate.

2.2.5 INHIBITION ASSAYS

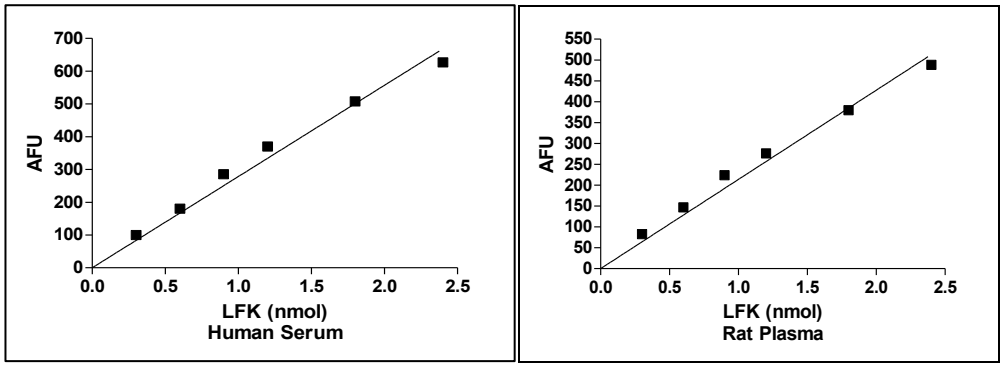
Lisinopril (Zeneca pharmaceuticals, United Kingdom) was made up from powdered form to a 500 nM stock solution in ddH_2O and a dilution series of 0 – 500 nM was prepared. LisW-S and RXP 407 were prepared from powdered form to 100 nM stock solutions in ddH_2O. A dilution series of 0-100 nM was prepared for both inhibitors.

Equal volumes of each inhibitor solution and 10% pooled human serum (n = 5) or pooled rat plasma (n = 6) were incubated for 90 minutes at ambient temperature. 25µl of this complex was added to 275µl of 10 µM substrate in triplicate, and the residual activity was monitored by fluorescence as described in 2.2.2. Substrate concentrations were selected to ensure less than 10% hydrolysis of substrate in order to observe initial rate conditions.

2.3 RESULTS

2.3.1 STANDARD CURVES

The standard curves were plotted as AFU vs. the known Abz-LFK(Dnp)-OH concentration (nmol) as seen in figure 2.4. A slope of 278.4 was obtained for the standard curve with human serum, with a curve fit of $R^2 = 0.9758$ (figure 2.4 (a)). A slope of 213.7 was obtained for the standard curve with rat plasma, with a curve fit of $R^2 = 0.9757$ (figure 2.4 (b)).



(a) **(b)**

Figure 2.4 Standard curves for (a) human serum and (b) rat plasma. Standard deviation is indicated on all plots

2.3.2 LISINOPRIL INHIBITION ASSAYS

The ACE inhibitor lisinopril was used as an experimental control to validate the inhibition assay protocol. The inhibition profile of lisinopril on hydrolysis of the FRET substrate Abz-LFK(Dnp)-OH by somatic ACE in both human serum and rat plasma were determined. As mentioned previously, Abz-LFK(Dnp)-OH has been shown to be preferentially cleaved by the C-domain of human ACE. However, as seen in the work of Jullien *et al (2006)*, this must be validated in rat plasma too as substrate specificities have been shown to differ between species (section 2.1).

AFUs were converted to nmol Abz-LFK(Dnp)-OH hydrolysed/ml/min using the standard curves in figures 4 (a) and 4 (b) and taking the dilution factor of 400 into account. Percent inhibition was calculated by normalising the 0 pM lisinopril reaction values to 0% inhibition and plotted against an x-axis of the log of lisinopril concentration (figures 2.5 and 2.6).

2.3.2.1 LISINOPRIL INHIBITION PROFILE OF ABZ-LFK(DNP)-OH SUBSTRATE IN HUMAN SERUM

The results of this assay (figure 2.5) showed that a maximum of 90% inhibition of human somatic ACE was achieved at a lisinopril concentration of approximately 25 pM (indicated by a red arrow). No inflection point was observed in this profile. 70% inhibition was observed at 2.5 pM (indicated by blue arrow).

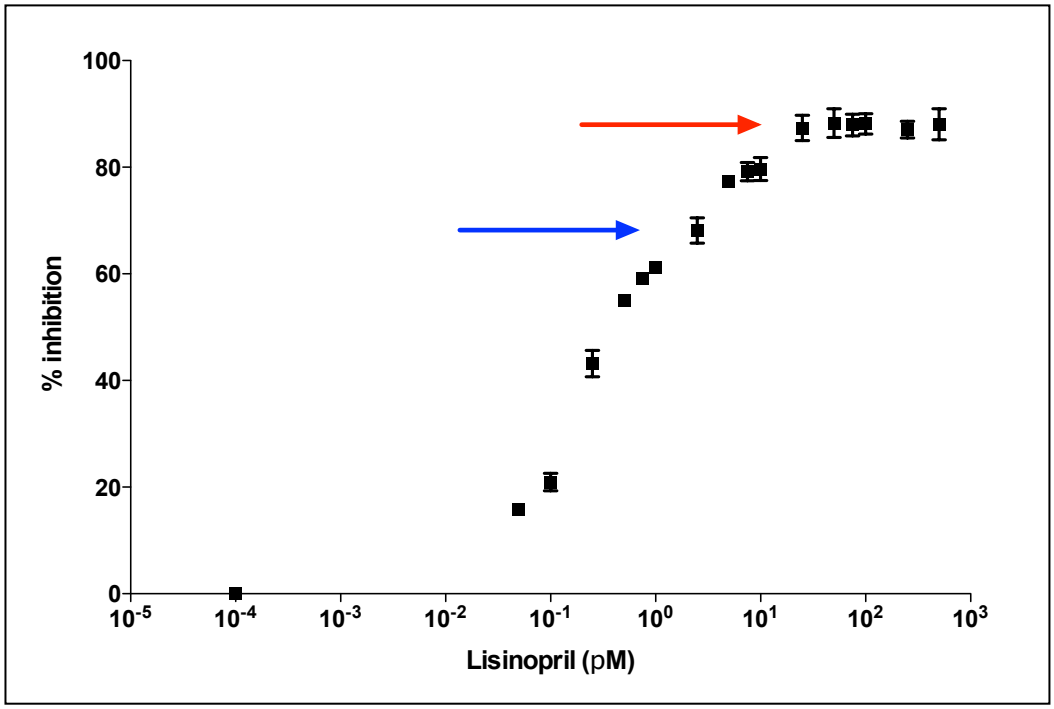


Figure 2.5 Plot of % inhibition of Abz-LFK(Dnp)-OH substrate vs. lisinopril dilution series (pM) in human serum. Red arrow indicates 90% inhibition. Blue arrow indicates 70% inhibition.

2.3.2.2 LISINOPRIL INHIBITION PROFILE OF ABZ-LFK(DNP)-OH SUBSTRATE IN RAT PLASMA

AFUs were converted to nmol Abz-LFK(Dnp)-OH hydrolysed/ml/min using the standard curve in figure 2.4 (b), taking the dilution factor of 400 into account. Percent inhibition was calculated by normalising the 0 pM lisinopril reaction values to 0% inhibition and plotted against an x-axis of the log of lisinopril concentration (figure 2.6). The results of this assay showed that rat somatic ACE was completely inhibited at a lisinopril concentration of approximately 50 pM (indicated by a red arrow), while 70% inhibition was observed at 2.5 pM (indicated by blue arrow)

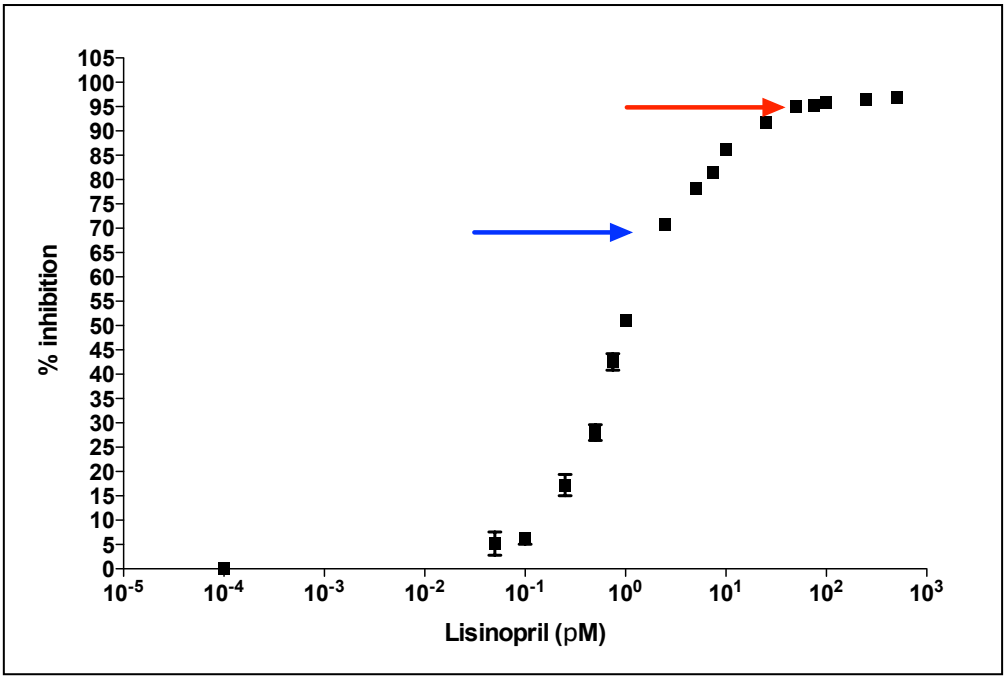


Figure 2.6 Plot of % inhibition of Abz-LFK(Dnp)-OH substrate vs. lisinopril dilution series (pM) in rat plasma. Red arrow indicates 90% inhibition. Blue arrow indicates 70% inhibition.

2.3.3 LISW-S INHIBITION ASSAYS

Inhibition profiles of lisW-S (C-specific for human ACE) were generated using the FRET substrates Abz-LFK(Dnp)-OH and Abz-FRK(Dnp)P-OH to assess the domain-selectivity of this inhibitor on rat somatic ACE. Prior to conducting the Abz-LFK(Dnp)-OH assay in rat plasma, the protocol was validated using human serum.

The FRET substrate Abz-FRK(Dnp)P-OH has previously been shown to display no domain selectivity for human ACE. This substrate was used to further assess the domain-selective behaviour of lisW-S in rat ACE and was not conducted in human serum. AFUs were converted to nmol Abz-LFK(Dnp)-OH/Abz-FRK(Dnp)P-OH

hydrolysed/ml/min using the standard curves in figures 2.4 (a) and 2.4 (b), taking the dilution factor of 400 into account. Percent inhibition was calculated by normalising the 0 nM lisW-S reaction values to 0% inhibition and plotted against an x-axis of the log of lisW-S concentration.

2.3.3.1 LISW-S INHIBITION PROFILE OF ABZ-LFK(DNP)-OH SUBSTRATE IN HUMAN SERUM

The results of this assay (figure 2.7) showed that a maximum inhibition of 80 – 85% was achieved at a lisW-S concentration of 100 nM (indicated by red arrow). A ten-fold higher lisW-S concentration did not increase the percentage inhibition. No inflection was observed on this profile.

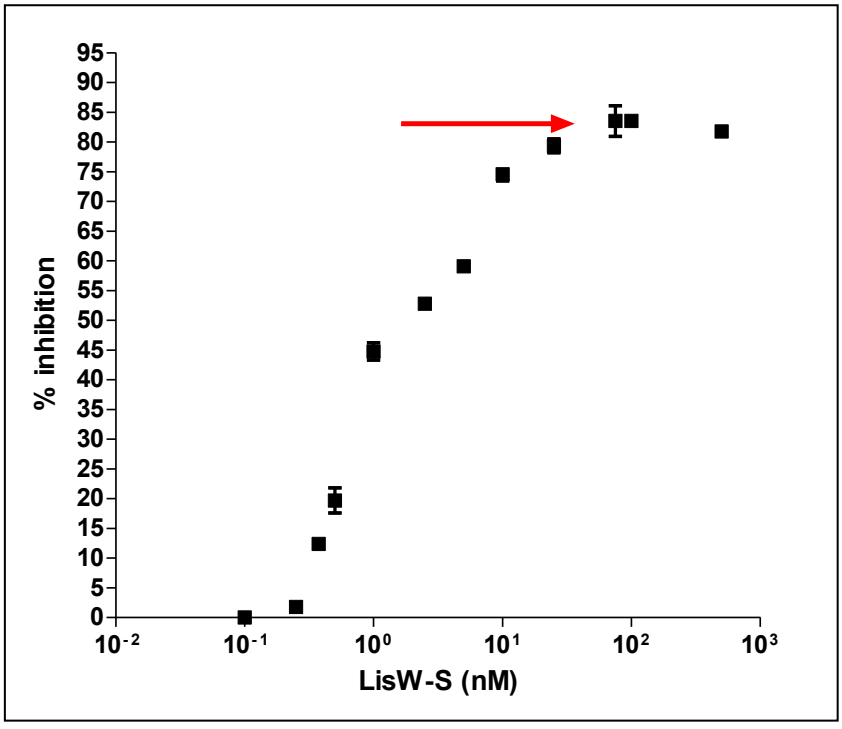


Figure 2.7 Plot of % inhibition of Abz-LFK(Dnp)-OH substrate vs. lisW-S dilution series (nM) in human serum. Red arrow indicates 85% inhibition at 100 nM

2.3.3.2 LisW-S inhibition profile of Abz-LFK(Dnp)-OH substrate in rat plasma

The results of this assay (figure 2.8) showed that rat somatic ACE was completely inhibited (± 90%) at a lisW-S concentration of approximately 5 μM. (indicated by red arrow). 70% inhibition was achieved at a lisW-S concentration of 100 nM (indicated by blue arrow). No point of inflection was observed on this profile.

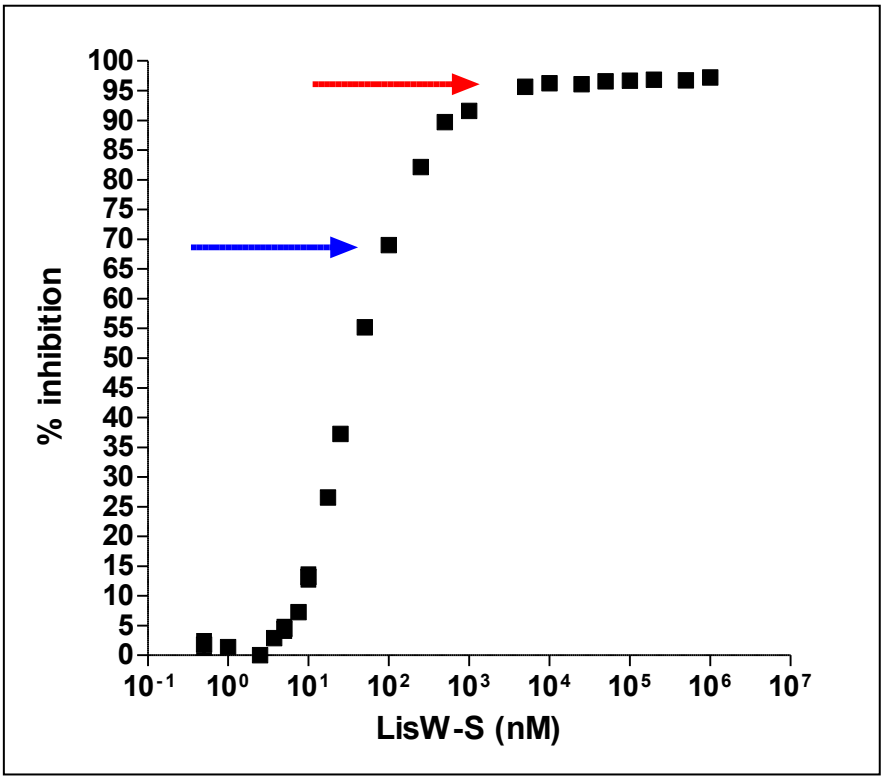


Figure 2.8 Plot of % inhibition of Abz-LFK(Dnp)-OH substrate vs. lisW-S dilution series (nM) in rat plasma. Red arrow indicates 100% inhibition. Blue arrow indicates 70% inhibition at 100 nM.

2.3.3.3 LisW-S inhibition assay of Abz-FRK(Dnp)P-OH substrate in rat plasma

The results of this assay (figure 2.9) demonstrate that hydrolysis of Abz-FRKP(Dnp)P-OH was completely inhibited at approximately 500 µM LisW-S (indicated by red arrow), and a clear point of inflection was observed at approximately 65% inhibition and between 1 and 5 µM lisW-S (indicated by blue arrow).

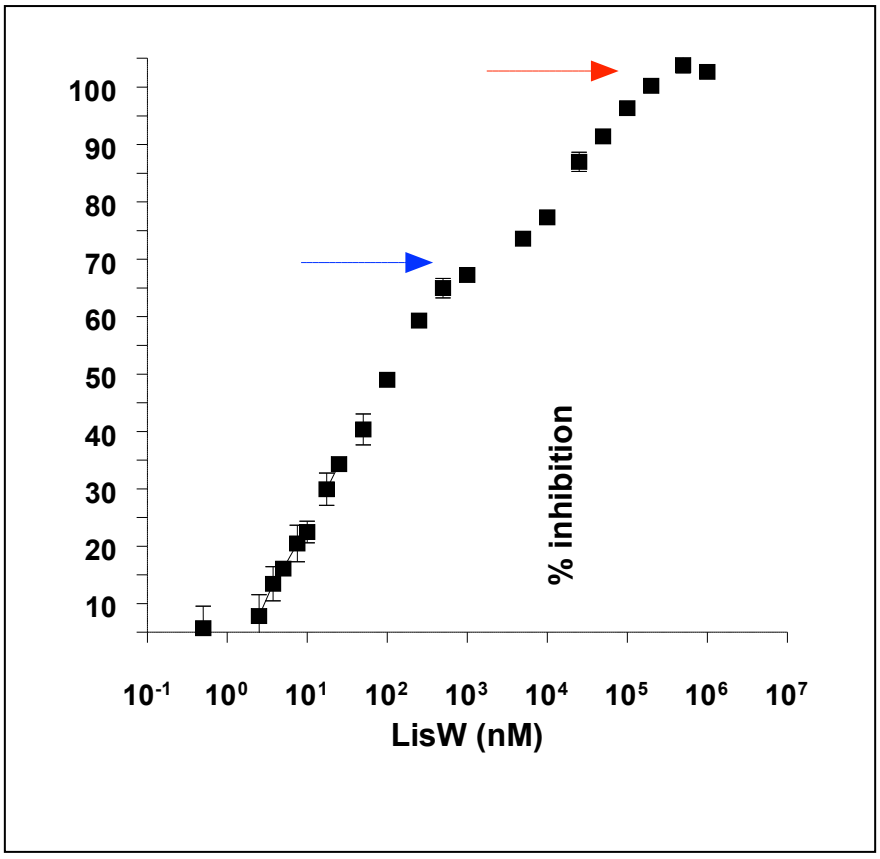


Figure 2.9 Plot of % inhibition of Abz-FRK(Dnp)P-OH substrate vs. lisW-S dilution series (nM) in rat plasma. Red arrow indicates 100% inhibition. Blue arrow indicates point of inflection.

2.3.4 RXP 407 INHIBITION ASSAY OF ABZ-LFK(DNP)-OH SUBSTRATE IN RAT PLASMA

The phosphinic peptide RXP 407 has previously been shown to selectively inhibit the N-domain of both human and rat ACE. To further characterise the domain selectivity of the Abz-LFK(Dnp)-OH substrate on rat ACE, the inhibition profile of hydrolysis of this peptide by RXP 407 was determined and compared to that of lisW-S (figure 2.10).

AFUs were converted to nmol Abz-LFK(Dnp)-OH hydrolysed/ml/min using the standard curves in figures 2.4 (a) and 2.4 (b), taking the dilution factor of 400 into account. Percent inhibition was calculated by normalising the 0 nM lisW-S and RXP 407 reaction values to 0% inhibition and plotted against an x-axis of the log of both lisW-S and RXP 407 concentrations (figure 2.10).

The results clearly show that rat somatic ACE inhibition occurs at a much lower concentration with lisW-S than that seen for RXP 407. For example complete (±90%) inhibition was observed at approximately 5 μM as opposed to 250 μM for LisW-S and RXP407, respectively.

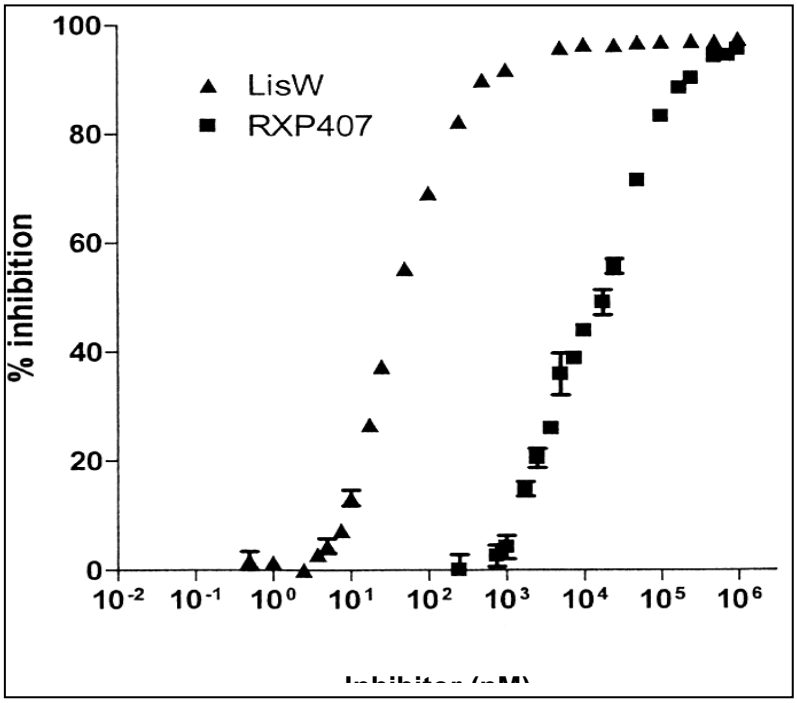


Figure 2.10 Plot of % inhibition of Abz-LFK(Dnp)-OH substrate vs. lisW-S and RXP 407 dilution series (nM) in rat plasma

2.4 DISCUSSION

2.4.1 ASSAY VALIDATION AND OPTIMIZATION

The first aim of this study was to establish the FRET assay protocol for use with rat ACE and lisW-S. Lisinopril and lisW-S inhibition profiles of Abz-LFK(Dnp)-OH hydrolysis were successfully validated with human serum ACE before any studies were done to evaluate their inhibitory effects on rat ACE. The results of these assays on human ACE were also of interest as there is the possibility that ACE will behave differently with regards to inhibitor and substrate specificity between species (Jullien et al. 2006).

The assays required optimization for use with plasma and serum. An initial finding in the first set of assays was that rat plasma and human serum endogenously quench ACE activity dramatically. Attempts to eliminate or reduce the quenching effect observed included adding a protease inhibitor cocktail to all plasma samples, which had no effect, as well as enriching the ACE in plasma samples by passing plasma through an anti-human serum albumin (HSA) column. Unfortunately, this only removed approximately 2% of the total protein in the samples and was therefore not repeated. Maximal ACE activity was observed at a dilution of 10% human serum or rat plasma, and this concentration was therefore used in subsequent assays.

When setting up the standard curve, a problem encountered was that complete hydrolysis was not achieved with human serum or rat plasma samples alone. The decision was therefore made to add purified tACE to the plasma or serum samples to ensure complete hydrolysis of the substrate for generation of the standard curves.

2.4.2 Assessment of domain-selectivity of lisW-S on rat ACE

The second aim of this study was to evaluate the C-domain selectivity of lisW-S in rat plasma ACE *in vitro*.

The lack of an inflection in the inhibition profile obtained for lisW-S on Abz-LFK(Dnp)-OH for rat plasma (figure 2.8) suggests either that lisW-S is not domain-selective and successfully blocks both domains equally, or that Abz-LFK(Dnp)-OH is exclusively cleaved by one domain of rat ACE.

To further investigate the domain specificity of lisW-S on rat ACE, we employed a second FRET substrate, Abz-FRK(Dnp)P-OH, previously shown to be non-domain-

specific for human ACE (figure 2.9). These results demonstrated an inflection at 65% inhibition at between 1 and 5 μM lisW-S. This inflection point represents the complete inhibition of one domain of ACE, while the other domain is still actively hydrolysing Abz-FRK(Dnp)P-OH, and suggests that lisW-S is indeed selective for one domain of rat ACE. However, this assay did not verify the specific domain-selectivity of lisW-S. Of interest was the inflection point at 65% inhibition as opposed to 50%, which would be expected for a non-selective substrate. This suggests that Abz-FRK(Dnp)P-OH may in fact be more domain-selective for rat ACE than non-selective, as is the case for human ACE. It was further noted that the concentration required of LisW-S to achieve the inflection point in rat plasma coincided with that required for maximal inhibition of Abz-LFK(Dnp)-OH. Similarly 45% inhibition of Abz-FRK(Dnp)P-OH which corresponds to approximately 70% inhibition of the domain being inhibited prior to the inflexion point is 100 nM. These correlations also suggest that Abz-LFK(Dnp)-OH is cleaved by the rat ACE C-domain and that this domain is preferentially inhibited by LisW-S.

In order to further elucidate the particular domain-selectivity of lisW-S for rat ACE, we compared the effects of the known N-domain specific ACE inhibitor RXP 407 and lisW-S on the hydrolysis of Abz-LFK(Dnp)-OH (figure 10). The inhibition profile of RXP 407 demonstrated a complete shift to the right indicating that a much higher concentration of this inhibitor was required to block the hydrolysis of Abz-LFK(Dnp)-OH compared to lisW-S. This provides strong evidence suggesting that Abz-LFK(Dnp)-OH is indeed exclusively cleaved by the C-domain of rat ACE, as very high concentrations of the N-selective inhibitor RXP 407 were required to inhibit this hydrolysis. Furthermore, the much lower concentrations of lisW-S required for inhibition of Abz-LFK(Dnp)-OH hydrolysis strongly suggest that it is, in fact, a C-domain selective inhibitor in rat plasma, capable of efficiently blocking hydrolysis of the C-selective substrate at relatively low concentrations.

An inhibition profile of lisW-S on a known N-domain selective rat ACE substrate would have confirmed the C-domain selectivity of the inhibitor. Unfortunately there were no N-domain selective substrates available for assay in this study. There is very

limited information on the domain specificity of substrates for rat ACE, and Jullien et al. (2006) have shown that the domain specificity of substrates in human ACE do not necessarily correlate with that of rat ACE. For example, Mca-Ala is an N-domain selective substrate for human ACE, but a C-selective substrate for rat ACE. Thus the selection of an N-domain specific substrate to further assess the domain selectivity of lisW-S on rat ACE would first need to be carefully validated with known rat ACE domain selective inhibitors such as RXP 407 and RXPA380.

2.4.3 Determination of lisW-S dosage required for effective ACE inhibition

The third and final aim of this study was to determine the concentration of lisW-S required for effective inhibition in rat ACE.

The results of the lisinopril and Abz-LFK(Dnp)-OH inhibition assays showed that a concentration of 25 pM lisinopril successfully inhibited human ACE at a maximum of 90% while 50 pM lisinopril achieved more than 95% inhibition in rat plasma ACE. This suggested that lisinopril is a slightly more effective ACE inhibitor in rats than in humans.

The lisW-S inhibition assays using both rat and human samples and the Abz-LFK(Dnp)-OH substrate demonstrated 70% inhibition at approximately 100 nM lisW-S and this result was confirmed in a duplicate assay (data not shown). Using the molecular weight of lisW-S (494.2 g/mol), the concentration required to inhibit 70% of ACE activity was calculated to be 49.4 ng/ml. As these assays also showed that a substantially larger concentration of 494.2 ng/ml lisW-S would be required to achieve just 20% greater inhibition, it was decided that 70% ACE inhibition would be a reasonable and effective level to aim for. It should be noted that a 1000-fold lower concentration of lisinopril (0.1 nM) achieved complete inhibition in rat ACE, suggesting that lisinopril is a vastly more effective ACE inhibitor than lisW-S of rat ACE in plasma. This was further verified in the inhibition assays conducted with

human serum, where lisinopril was shown to be more effective than lisW-S by approximately 4000-fold. The results of the lisW-S inhibition assays were used for the subsequent pharmacokinetic studies, which were aimed at verifying the administered dose of lisW-S that would be needed to achieve a 100 nM (50 ng/ml) plasma concentration and 70% ACE inhibition *in vivo.*

Chapter 3 PHARMACOKINETICS

3.1 INTRODUCTION

A series of pharmacokinetic (PK) studies were done in order to assess the bioavailability, behaviour and stability of LisW-S *in vivo*. LisW-S was administered by three routes: intravenous (IV) injection, oral gavage and subcutaneous mini-osmotic pump infusion. In addition, studies were conducted with lisinopril to serve as a closely matched control and to evaluate its ACE inhibitory action in a rat model *in vivo*.

The primary purpose of these studies was, first, to assess the bioavailability of lisW-S, and second, to determine whether an *in vivo* dosage could be achieved that allowed for adequate inhibition. It was reasoned from the ex vivo studies detailed in chapter 2 that a plasma concentration of approximately 40-50 ng/ml would be minimally required to achieve significant inhibition. In addition, two routes of administration (oral and pump infusion) were evaluated in order to decide which one provided the best constant dose in the therapeutic range desired for ACE inhibition over a given period of treatment. LisW-S was also converted into a maleate salt in an effort to enhance bioavailability.

3.2 PHARMACOKINETICS OF LISINOPRIL

The main goal of the PK studies was the determination of an optimal and effective dosing regimen for the administration of lisW-S. As lisW-S is closely related to lisinopril, PK studies of lisinopril are discussed below. Lisinopril was developed in the late 1970's, and PK data was first collected on this compound's behaviour in the 1980's. With 30 years worth of research on the subject, the PK behaviour of lisinopril in human subjects is well-established.

In a clinical setting, Lisinopril is administered orally once a day. It has a bioavailability of approximately 25-30% in normal subjects (Ulm et al. 1982; Beermann et al. 1989) although this value varies widely between individual patients. Absorption is not affected by food intake (Mojaverian et al. 1986) and a twice daily dose ensures that plasma concentrations reach a steady state within 24 hours. The plasma half-life for this drug is estimated to be about 12 hours, and peak serum concentrations are reached within 6 to 8 hours. Terminal serum half-life is estimated to be about 40 hours. Lisinopril is not metabolized, but is eliminated completely unchanged by the kidneys. (Ulm et al. 1982; Dickstein 1987; Gomez et al. 1987; Bjorn 1988; Beermann et al. 1989)

Although direct PK studies on lisinopril have not been reported for the rat model, plasma ACE activities in both dogs and rats have been measured in the 24 hours following oral lisinopril administration. Hamlin *et al*. administered lisinopril to healthy dogs at an oral dose of 0.5 mg/kg. ACE activity in plasma samples was analysed by ultraviolet-kinetic techniques. This study found that ACE was inhibited between 1.5 and 3 hours after administration of the ACE inhibitor. However, after the 3 hour time point, ACE activity rapidly increased and it was surmised that plasma lisinopril concentrations were no longer within a therapeutic range. (Hamlin & Nakayama 1998)

Jackson *et al.* measured the ACE activity in rat plasma and tissue homogenates using quantitative radioinhibitor binding. Plasma ACE was acutely inhibited within 2 hours of administration of a 10 mg/kg dose. ACE remained effectively inhibited for a further 6 hours, and only began to rise again after the 8 hour time point. This is somewhat in accordance with human PK studies of lisinopril, which observe a peak plasma concentration – and presumably maximum ACE inhibition – 6 hours after administration. (Jackson et al. 1987)

3.3 METHODS

3.3.1 HPLC PURIFICATION OF LISW-S

Once the lisW-S ACE inhibitor had been synthesized, it required purification on a High Performance Liquid Chromatography (HPLC) column with a low pH trifluoroacetic acid (TFA) buffer.

Lisinopril did not require any purification as it is commercially available and was purchased from Zeneca Pharmaceuticals (United Kingdom).

RP-HPLC purification and separation of the diastereomeric lisW-S mixture was performed using a Jupiter 5U C18 300A, size 250 x 4.60 mm column with gradient elution of t = 0 min (50% A, 50% B) and t = 50 min (40% A, 60% B); solvent A = 0.1% TFA in H_2O (or 10 mM HCl) and solvent B = 0.1% TFA (or 10mM HCl) and 75% CH_3CN in H_2O; a flow rate of 1.0 mL/min and a 215 and 280 nm UV wavelength detection.

(HPLC methods have been adapted from the supplementary material in Nchida *et al. (2006)*. All chemicals were purchased from Sigma-Aldrich (Germany).

3.3.2 SALT FORMATION OF LISW-S

LisW-S was converted to a maleate salt by neutralization in a basic solution to form a lisW-S/MS analogue.

Salt formation is a commonly used and highly effective method of increasing both the solubility and dissolution rates of acidic and basic drug compounds (A.T.M. Serajuddin 2007). It is the most utilized method of improving the solubility of liquid

solutions administered parenterally (Sweetana et al. 1996). The widely-used ACE inhibitor enalapril is also administered in an oral maleate salt form (Davies et al. 1984).

3.3.3 Oral gavage

LisW-S and lisinopril were prepared by weighing out the powder form and dissolving it in ddH_2O to the required concentration. Both lisW-S and lisinopril were administered at a dosage of 5 mg/kg. Animals were weighed immediately prior to gavage, and the dose adjusted accordingly. All animals were administered 1 ml of the ACE inhibitor solution and blood samples were taken at -5 minutes ('zero' time point prior to administration), 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, 12 hours and 24 hours post-administration.

3.3.4 Intravenous injection

The lisW-S solution was made up from the powdered form in ddH_2O to the required volume and concentration and filter-sterilized. Animals were restrained in a custom-built rodent restrainer which granted access to the tail. The injection site was cleaned with an alcohol swab. A minimum volume of 100 µl was injected into the tail vein, although this was adjusted according to weight. Haemostasis at the injection site was ensured by applying pressure to the area with a gauze swab.

3.3.5 Mini-osmotic Pump Implant

3.3.5.1 Pump Selection

A long-term aim is the assessment of lisW-S efficacy in the context of a rat MI model and therefore a pump was chosen that was designed to deliver a solution continuously for 28 days. This pump has a volume of approximately 220 µl and a pump rate of 0.25 µl/hr (Model 2004, Alzet). The solubility of lisW-S also had to be

considered, as the maximum concentration that could be obtained in solution with ddH_2O was 30 mg/ml. The following equation was used:

ko (µg/hr) = Q (µl/hr) x Cd (µg/µl);

Where k_o is the mass delivery rate, Q is the pumping rate of the selected model, and C_d is the concentration of the drug solution. Using this equation, the mass delivery rate was ascertained to be 7.5 µg/ hour or 180 µg/ day.

3.3.5.2 PUMP PREPARATION

Alzet micro-osmotic pumps were prepared under sterile conditions, as per the manufacturer's instructions. The solutions of lisW-S and lisinopril were made up from the powdered form in ddH_2O to the required volume and concentration and filter-sterilized. Pumps were filled as per the manufacturer's instructions. After filling, pumps were transferred to a 50 ml tube filled with 0.9% sterile saline solution and placed in the incubator at 37^oC (5% CO_2) for 48 hours as recommended by the manufacturer. This pre-incubation period ensures that the pumps are fully functional upon subcutaneous implant.

3.3.5.3 ANAESTHESIA AND PREPARATION

The rat was initially anaesthetized by being placed in an induction chamber for a minimum of 2.5 minutes. During the induction phase the vaporizer was set to anaesthetize the animal with 5% Isoflurane, at a flow rate of 1.5% Oxygen. At the 2.5 minute mark, the animal was removed from the chamber, weighed (and body weight recorded), and an area of the back lateral to the spinal column was shaved. The animal was then transferred in a prone position to the pre-warmed operating table (a heating pad maintained the table at approximately 37^oC) and a nose cone was

placed over the snout. Anaesthesia was adjusted and maintained with 1.5 - 2% Isoflurane and 0.3% oxygen for the duration of the procedure.

The animal was secured in a prone position with surgical tape and two front paws and one distal paw were taped down. The remaining paw was left free to monitor depth of anaesthesia during surgical procedure (movement of the paw is indicative of a decrease in anaesthesia depth). The surgical area was then cleaned with an iodine solution. Depth of anaesthesia was checked by non-responsiveness to a tail pinch with forceps.

3.3.5.4 Surgical Procedure

A small 1 to 1.5 cm skin incision was made to the right of and at a 90^{o} angle to the spinal column, approximately midway between the front and hind legs. The skin was raised with toothed forceps and a haemostat was gently inserted into the subcutaneous space. The haemostat was used to create a pocket of sufficient size to accommodate the mini-osmotic pump. The pump was then handled sterilely and inserted inside the subcutaneous pocket. The incision was closed with 4-0 silk sutures, and the surgical area was cleaned with iodine solution. An analgesic dose of 0.05 mg/kg buprenorphine was administered by intramuscular injection to the hind leg. The average duration of the procedure was 10 to 15 minutes.

3.3.5.5 Post-Operative Care

The animals were monitored daily for the duration of the study. Water and food intake was monitored, and their weights were recorded once a week. The incision site and sutures were checked for the first three days post-surgery to ensure adequate healing. A single dose of analgesic (0.05 mg/kg buprenorphine) was administered 24 hours post-surgery.

3.3.6 BLOOD SAMPLING

Following administration of lisinopril or lisW-S, blood sampling at specified time intervals was required for periods of 24 hours up to 4 weeks. The same procedure was followed for each blood sample taken.

3.3.6.1 PREPARATION

Animals were placed in a chamber heated to 45^{o}C 5 to 10 minutes prior to sampling in order to aid blood flow following venipuncture of the tail vein.

3.3.6.2 PROCEDURE

As anaesthesia was not recommended for the LC/MS/MS sample analysis, the animals were restrained in a custom-made rodent restrainer which allowed access to the tail vein.

The venipuncture site was cleaned with an alcohol swab. A 26G needle was used to draw a 100ul blood sample into a 1ml syringe. The needle was then removed from the syringe to prevent haemolysis of the blood sample upon injection. The sample was injected into a 2ml lithium-heparin coated blood collection tube. The tube was inverted at least 8 times to ensure thorough mixing and kept at room temperature. Haemostasis at the blood collection site was ensured by applying pressure to the site with a gauze swab.

3.3.6.3 PLASMA COLLECTION

Blood samples were centrifuged at 3000 g for 15 minutes (4^{o} C). Plasma was collected and transferred to an eppendorf tube and stored at -80^{o}C.

3.3.7 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC/MS/MS) ANALYSIS

LisW-S was analyzed using a validated LC/MS/MS assay protocol established for this study. A protein precipitation extraction method using 100 µl plasma and 200 µl methanol was employed to process the samples. The precipitation solvent was spiked with the internal standard Verapamil at a concentration of 14 ng/ ml. Isocratic chromatography was performed on a Phenomenex, Luna 5 µm PFP(2), 100 A, 50 mm × 2 mm analytical column, using acetonitrile, methanol and 0.1% formic acid as a mobile phase (15:15:70), and was delivered at a flow rate of 300 µl/min. An AB Sciex API 4000 mass spectrometer was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transitions of both the protonated molecular ions at m/z 495.0 to the product ions at m/z 84.3 for lisW-S, and the protonated molecular ions at m/z 455.1 to the product ions m/z 165.5 for the internal standard.

The accuracies obtained for lisW-S were between 96.4% and 102.8% at low, medium and high quality control (QC) levels during inter-batch validation. The precision for lisW-S during inter-batch validation was less than 7.6% at low, medium and high QC levels. The calibration range for lisW-S was between 7.04 and 451 ng/ml.

The same protocol was used for lisinopril analysis by LC/MS/MS assay.

3.4 EXPERIMENTS

3.4.1 PILOT PK STUDY: LISW-S AND LISINOPRIL

A pilot study was conducted in order to assess the plasma concentrations of lisW-S and lisinopril that could be achieved *in vivo* following oral gavage. The initial planned experimental procedure was to administer a single dose of lisW-S by oral gavage once every 24 hours, thereby mimicking a clinical setting and a daily dose of ACE inhibitors as prescribed to the patient.

3.4.1.1 EXPERIMENTAL DESIGN

The pilot study comprised a total of 12 rats (n= 6 for the lisinopril group, and n = 6 for the lisW-S group). LisW-S purified by HPLC with TFA buffer was used (LisW-S/TFA). All compounds were administered by oral gavage, at a concentration of 5 mg/kg and a constant volume of 1 ml with dH2O as solvent. All rats were in the weight range of 180 – 250g.

3.4.1.2 RESULTS

3.4.1.2.1 LISINOPRIL

The concentration vs. time profile of lisinopril is presented in figure 3.1. A maximum plasma concentration of 129.1 ± 43.7 ng/ml was measured at the 1 hour time point. At the 4 hour time point this concentration had decreased slightly to 98 ± 43.6 ng/ml, after which the drug concentration decreased steadily to baseline levels (12.8 ± 6.9 ng/ml) at 12 hours.

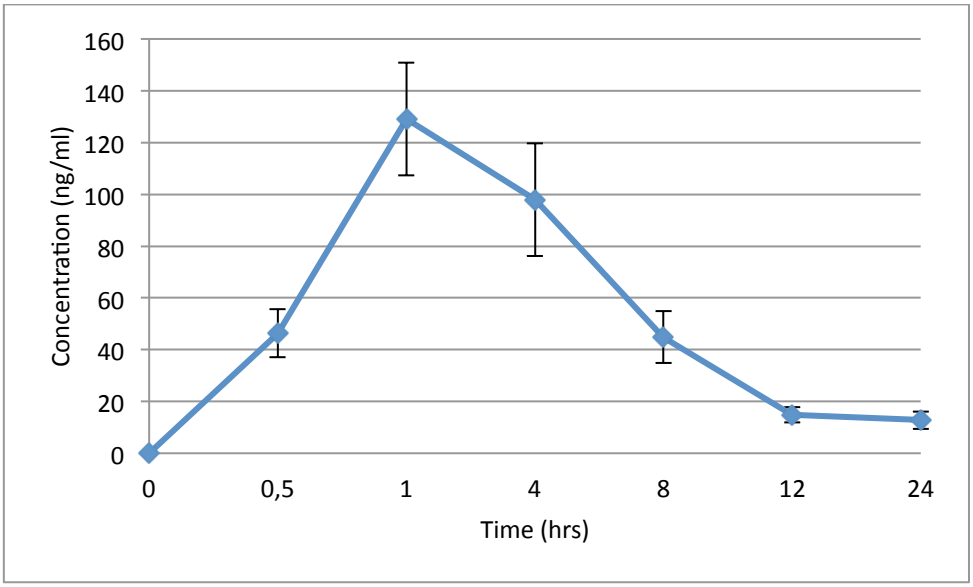


Figure 3.1 Mean concentrations of lisinopril (ng/ml) vs. time (n=6)

Table 3-1 Mean concentrations of lisinopril (ng/ml) at various time points

Time point	0 hr	0.5	1 hr	4hr	8hr	12hr	24hr
Mean Concentration (ng/ml)	0	46.3	129.1	98.0	44.8	14.9	12.8
ST DEV (ng/ml)	0	18.6	43.7	43.6	20.0	6.0	6.9

3.4.1.2.2 LISW-S

As seen in figure 3.2 and table 3.2, LisW-S/TFA reached a maximum concentration of approximately 46.2 ± 11.5 ng/ml by the 1 hour time point, after which a gradual decline to 34.1 ± 15.1 ng/ml was observed until the 4 hour time point. By 8 hours, plasma concentration levels had dropped significantly to 13.1 ± 7.6 ng/ml and by 12 hours they had returned to baseline values.

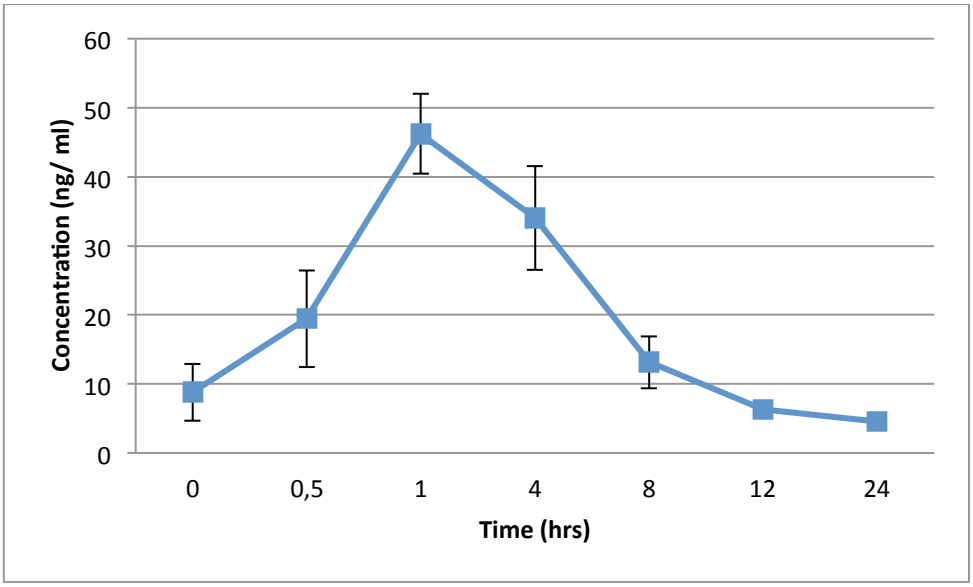


Figure 3.2 Mean concentrations of lisW-S/TFA (ng/ml) vs. time (n=6)

Table 3-2 Mean concentrations of lisW-S/TFA (ng/ml) at various time points

Time point	0 hr	0.5	1 hr	2hr	4hr	8hr	12hr	24hr
Mean Concentration (ng/ ml)	8.8	19.4	46.2	39.5	34.1	13.1	6.4	4.6
ST DEV (ng/ml)	8.2	14.0	11.5	15.2	15.1	7.6	1.9	0.5

3.4.2 LISW-S MALEATE SALT

3.4.2.1 EXPERIMENTAL DESIGN

In an effort to enhance the bioavailability of lisW-S the compound was converted to a maleate salt form (LisW-S/MS). This increased the weight of the compound by 20%, and so all dosages were adjusted accordingly. A total of 5 rats weighing between 180 and 250g were used. LisW-S/MS was administered by oral gavage at a dosage of 5 mg/kg and a constant volume of 1 ml with dH2O as solvent. Blood samples were collected at the following time points: zero (prior to administration), 1 hr, 2 hrs, 4 hrs, 8 hrs, 12 hrs and 24 hrs after pump implant.

3.4.2.2 RESULTS

The results were similar to those obtained in the pilot gavage study with lisW-S/TFA (see figure 3.2). As seen in figure 3.3 and table 3.3, LisW-S/ MS salt reached a peak plasma concentration of 52 ± 24.4 ng/ml by the 2 hour time point, after which levels steadily declined until returning to baseline by the 24 hour time point.

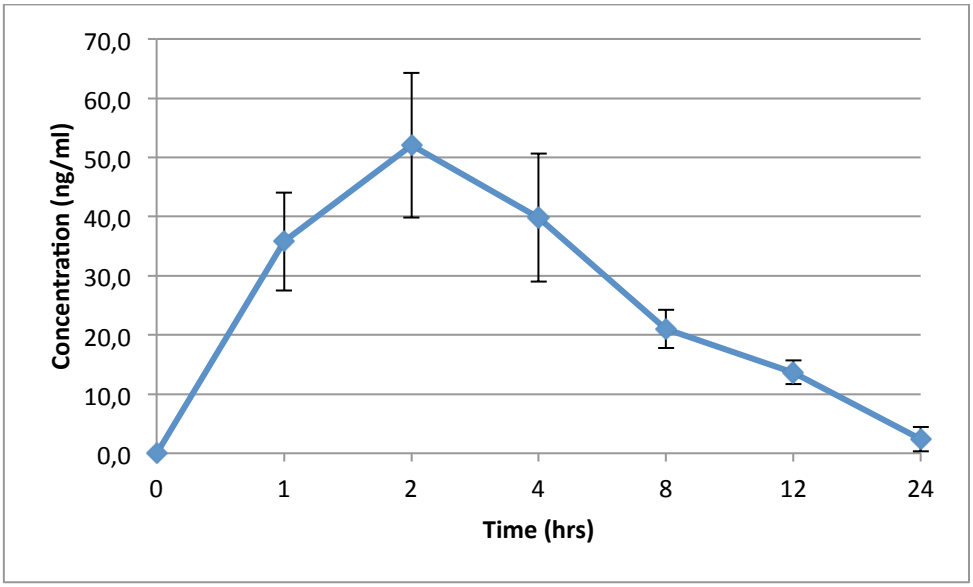


Figure 3.2 Mean concentration of lisW-S/MS (ng/ml) salt dosage vs. time (n=5)

Table 3-3 Mean concentration lisW-S/MS (ng/ml) at various time points

Time point	Zero	1 hr	2hr	4hr	8hr	12hr	24hr
Mean concentration (ng/ ml)	0.0	35.8	52.0	39.8	21.0	13.7	2.4
ST DEV (ng/ml)	0.0	16.5	24.4	21.6	6.4	4.1	4.1

3.4.3 BIOAVAILABILITY EVALUATION: LISW-S

On completion of the pilot study, the next objective was to determine the oral bioavailability of lisW-S. Oral and intravenous (IV) single dose regimens are used to calculate the percentage oral bioavailability of a drug. This was assessed by comparing the dosage and AUC (Area Under the Curve – referring to the curve obtained when plotting plasma drug concentration versus time) obtained with oral

administration against the dosage and AUC obtained with IV administration of lisW-S. Absolute bioavailability is defined as the fraction of the drug absorbed (F), and is calculated as follows:

$$F = \frac{(AUC)oral}{(AUC)IV} \times \frac{(Dose)IV}{(Dose)oral}$$

3.4.3.1 EXPERIMENTAL DESIGN

In order to evaluate bioavailability, two animal experiments were required; one in which lisW-S/ TFA was administered orally, and one in which lisW-S/ TFA was administered intravenously. LC/MS/MS assay of the plasma samples obtained from these two studies enabled us to plot graphs of plasma drug concentration (ng/ ml) vs. time and calculate the AUC for each group in order to obtain a final F value.

3.4.3.1.1 ORAL GROUP

LisW-S/ TFA was administered by oral gavage at a dosage of 5mg/ kg to 20 animals. Blood samples were collected into heparinised tubes at the following time points: 0, 1, 2, 4, 8, 12 and 24 hours after administration. Samples were centrifuged and separated and plasma was stored at -80^{o}C.

3.4.3.1.2 INTRAVENOUS GROUP

LisW-S/ TFA was administered intravenously via the tail vein at a dosage of 1 mg/ kg to 6 animals. Blood samples were collected at the following time points: 0, 5 minutes, 1, 2, 4, 8, 12 and 24 hours after administration.

3.4.3.2 RESULTS

3.4.3.2.1 ORAL GROUP

As seen in figure 3.4 and table 3.4, the results for the oral group showed a mean maximum lisW-S concentration of 47.1 ± 13 ng/ml was reached by the 2 hour time point. By 4 hours, the mean plasma concentration of lisW-S had dropped to almost half of the maximum plasma levels seen (26.5 ± 5 ng/ml). Levels continued to decrease until 12 hours which was the last sample taken from which lisW-S/ TFA levels were detectable.

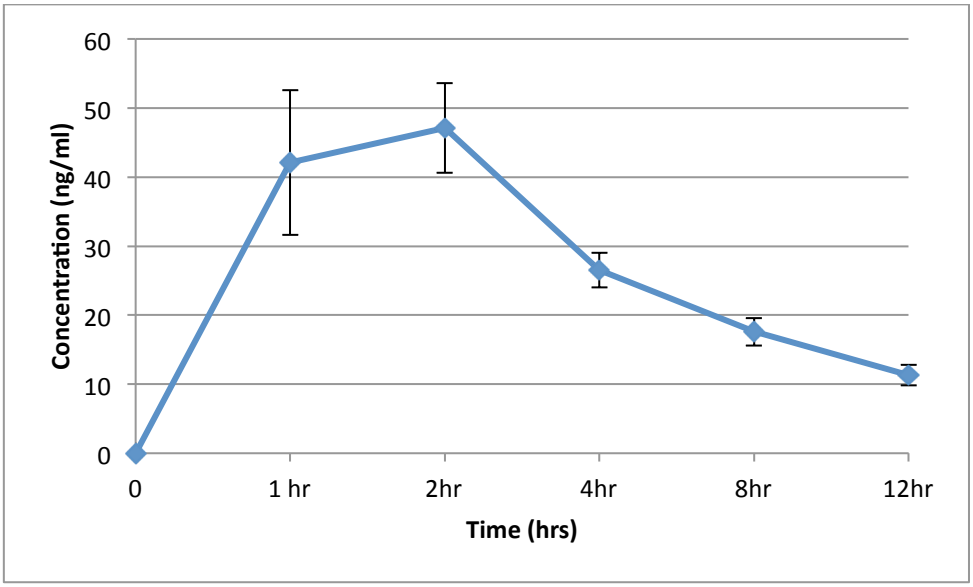


Figure 3.3 Mean concentration of lisW-S/TFA (ng/ml) vs. time - oral administration (n=20)

Table 3-4 Mean concentrations of lisW-S/TFA (ng/ml) at various time points with oral administration (n=20). ND = non-detectable.

Time point	Zero	1 hr	2hr	4hr	8hr	12hr	24hr
Mean Concentration (ng/ ml)	0.0	42.1	47.1	26.5	17.6	11.3	ND
STDEV (ng/ml)	0	21	13	5	4	3	ND

3.4.3.2.2 IV GROUP

The results for the IV group are presented in figure 3.5 and table 3.5 and showed a higher maximum lisW-S/TFA concentration in the plasma than that seen in the oral group – 2660 ± 428 ng/ml immediately following administration. However, levels declined rapidly and reached an effective baseline by the 2 hour time point. By 12 hours lisW-S/TFA was no longer detectable in the plasma samples.

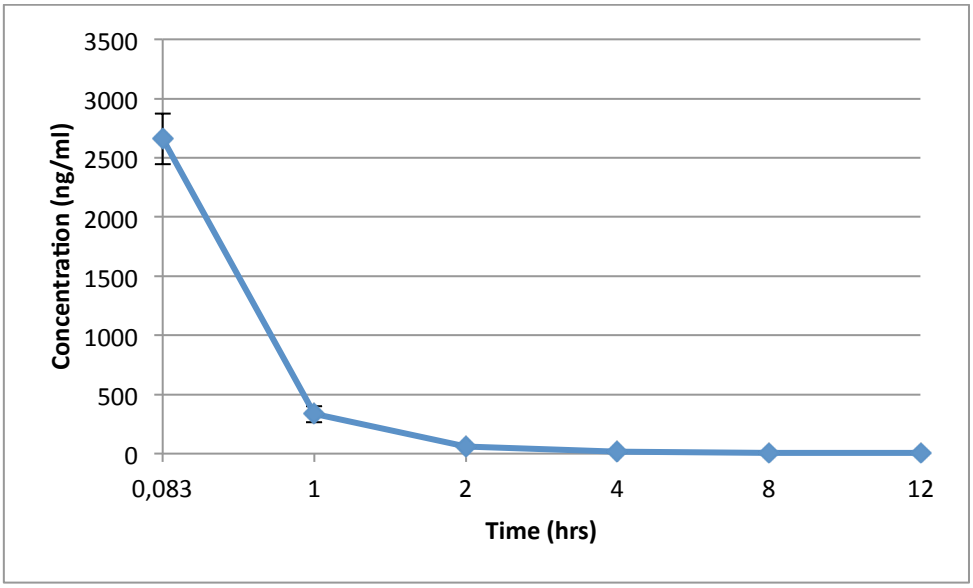


Figure 3.4 Mean concentrations of lisW-S/TFA (ng/ml) vs. time - IV administration (n=6)

Table 3-5 Mean concentrations of lisW-S/ TFA (ng/ml) at various time points with IV administration (n=20). ND = non-detectable.

Time point	0.083 hr	1 hr	2hr	4hr	8hr	12hr	24hr
Mean Concentration (ng/ ml)	2660	334	60.0	18.8	8.08	6.13	ND
ST DEV (ng/ml)	428	138	11	4	2	ND	ND

3.4.3.3 PK MODEL RESULTS

The PK parameters for modeling were derived from the results obtained in the oral group (figure 3.3). Parameters were calculated using WinNonlin software.

3.4.3.3.1 ORAL GROUP

As seen in table 3.6, the mean half-life of lisW-S/ TFA by oral administration was determined to be 6.3 hours. The peak plasma concentration reached after administration (Cmax) was 52 ng/ ml, and the time taken to reach Cmax (Tmax) was approximately 1.6 hours. The mean oral AUC up to 12 hours was 286.2 $ng.h.ml^{-1}$.

Table 3-6 Mean PK parameters for lisW-S – oral administration

	Half-life (h)	**Tmax (h)**	**Cmax (ng/ml)**	**AUC 0-12 ($ng.h.ml^{-1}$)**	**AUC 0-24 ($ng.h.ml^{-1}$)**
Mean Value	6.3	1.6	52.0	286.2	353.7

3.4.3.3.2 IV GROUP

As seen in table 3.7, the mean half-life of lisW-S/TFA by IV administration was determined to be 2.7 hours. The peak plasma concentration reached after administration (Cmax) was 2660 ng/ ml, The mean IV AUC up to 12 hours was 1866.5 $ng.h.ml^{-1}$.

Table 3-7 Mean PK parameters for lisW-S - IV administration

	Half-life (h)	Cmax (ng/ml)	AUC 0-12 ($ng.h.ml^{-1}$)
Mean Value	2.7	2660	1866.5

In conclusion, based on both the oral and IV results, mean bioavailability of lisW-S/TFA was determined to be 3.1%.

3.4.4 PUMP STUDY: LISW-S

The results of the pilot and bioavailability studies showed that plasma clearance of lisW-S/ TFA was relatively fast, with concentrations reaching a peak of approximately 45 – 50 ng/ml around the 2 hr mark at a dose of 5 mg/kg. Plasma clearance appeared complete at approximately 12 hrs, when concentrations returned to basal levels. Were we to follow our initial experimental setup of administration of lisW-S once every 24 hours, our cycle would include 12 hours of non-effective treatment per cycle. Thus it was decided to pursue a second route of administration via subcutaneous mini-osmotic pumps.

Mini-osmotic pumps are a reliable and effective method of delivering compounds at a constant and highly accurate dose.

3.4.4.1 EXPERIMENTAL DESIGN

This study was run for a duration of 4 weeks to assess the stability and delivery of lisW-S in a subcutaneous environment.

A dosage of 15 mg/ ml was administered to a sample size of n = 4 for a duration of 4 weeks. Blood samples were collected at the following time points: zero (prior to administration), 8 hrs, 24 hrs, 48 hrs, 4 days, 1 week, 2 weeks, 3 weeks and 4 weeks after administration. Blood samples were collected at the same time of the day in all instances.

3.4.4.2 RESULTS

The results presented in table 3.8 and figure 3.6 showed an initial peak in plasma concentrations, followed by a significant drop to a steady state condition. The maximum mean concentration obtained was 40.1 ±16.6 ng/ml at the 24 hour time point. After this, plasma concentration levels of lisW-S/TFA decreased to 24.9 ± 5.4 ng/ml after 1 week of administration. A second maximal peak was observed at the 2 week time point, when lisW-S/TFA levels were raised to 32.6 ± 2.9 ng/ml, but this decreased and remained at approximately 20 ng/ml for the remaining 2 weeks of the study.

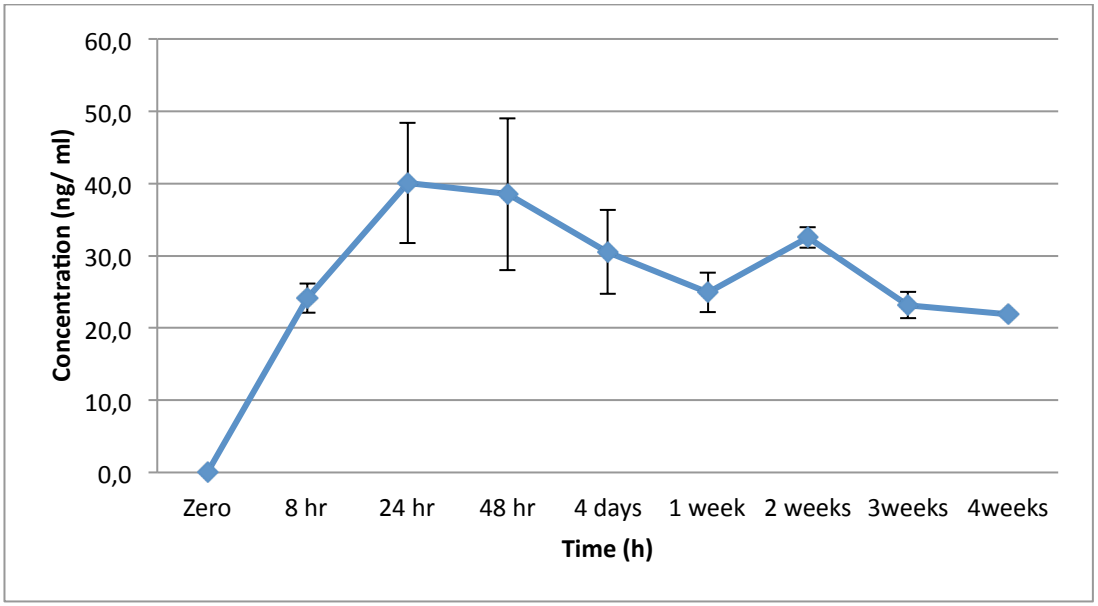


Figure 3.5 Mean concentrations (ng/ml) of 15 mg/ ml lisW-S/ TFA dosage vs. time (n = 4)

Table 3-8 Mean concentrations (ng/ml) of 15 mg/ ml lisW-S/ TFA dosage at various time points

Time point	Zero	8 hr	24 hr	48 hr	4 days	1 week	2 weeks	3 weeks	4 weeks
Mean Concentration (ng/ ml)	0	24.2	40.1	38.5	30.5	24.9	32.6	23.2	21.9
ST DEV (ng/ml)	0	4.1	16.6	21.1	11.6	5.4	2.9	3.7	0.5

3.4.5 PUMP STUDY: LISINOPRIL

3.4.5.1 EXPERIMENTAL DESIGN

The plasma concentration levels of lisinopril were used primarily as a control throughout this work (its ACE inhibitory properties are well-established). As it is an orally administered compound its behaviour under subcutaneous pumping conditions needed to be assessed. Lisinopril was pumped at the same concentration and duration as the 4-week lisW-S/TFA pump study (section 3.4.4).

A dosage of 15 mg/ml was administered to a sample size of n = 3 for a duration of 4 weeks. Blood samples were collected at the following time points: zero (prior to administration), 8 hrs, 24 hrs, 48 hrs, 4 days, 1 week, 2 weeks, 3 weeks and 4 weeks after administration.

3.4.5.2 RESULTS

The results presented in figure 3.5 showed that lisinopril behaved differently to lisW-S under subcutaneous pumping conditions. This was not unexpected due to the higher oral bioavailability of lisinopril. ‘Zero’ samples were measured above 0 ng/ml, possibly due to contamination of the samples. Table 3.7 shows that an initial peak of 51.7 ± 41 ng/ml was seen at the 8 hour time point, after which mean concentrations ranged between 48.2 ± 10.7 ng/ml and 46.3 ± 23.7 ng/ ml for the next 2 weeks. An unexpected maximum peak of 76.7 ± 36.2 ng/ml was observed at the 3 week time point, after which levels returned to a mean concentration of 48.4 ± 25.1 ng/ml by 4 weeks. This is suggestive that the 3 week time point is artefactual and stems from an unknown error that occurred during the quantification of this time point.

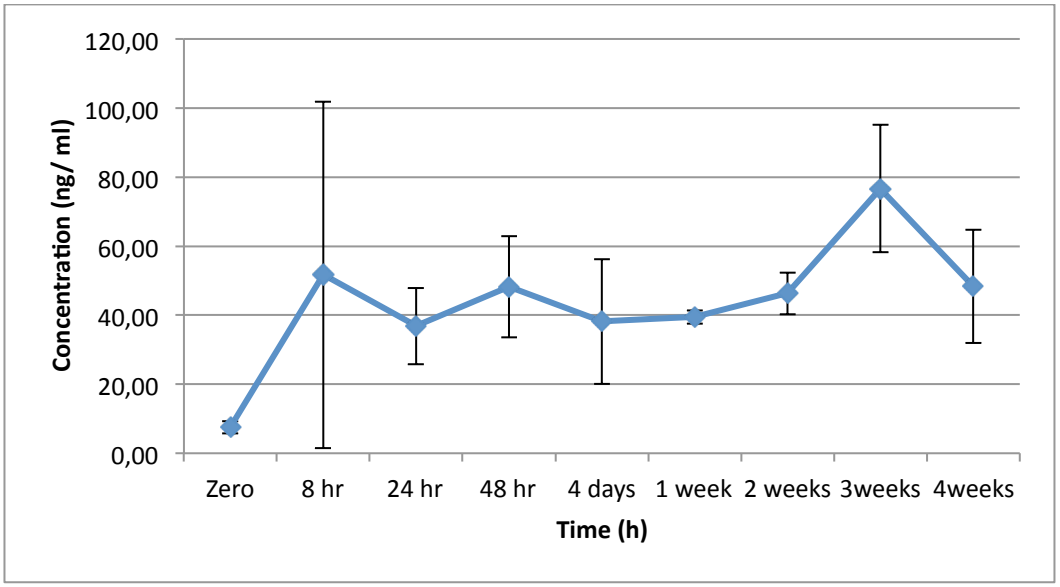


Figure 3.6 Mean concentration (ng/ml) of 15 mg/ ml lisinopril dosage vs. time (n=3)

Table3-9 Mean concentration (ng/ml) of 15 mg/ ml lisinopril dosage at various time points

Time point	Zero	8 hr	24 hr	48 hr	4 days	1 week	2 weeks	3 weeks	4 weeks
Mean Concentration (ng/ ml)	7.5	51.7	36.8	48.2	38.2	39.5	46.3	76.7	48.4
STDEV (ng/ml)	67.3	41.0	132.6	10.7	24.1	19.8	23.7	36.2	25.1

3.5 DISCUSSION

The results of the pilot oral gavage study showed that lisW-S reached a maximum plasma concentration close to the desired therapeutic range of 50 ng/ml by the 1 hour time point, after which levels slowly decreased until reaching a baseline level by 12 hours. A similar profile was observed for lisinopril, thus showing that this behaviour was not exclusive to the lisW-S compound. Based on these results, it was concluded that a once daily dose by oral gavage was not an effective method of drug delivery, and other routes of administration needed to be explored.

This decision was validated by the bioavailability study, which determined the half-life of lisW-S to be approximately 6 hours, and the time to reach peak plasma or serum concentrations to be 1.6 hours with oral dosing. The oral bioavailability of the C-domain selective ACE inhibitor was determined to be just 3.1%. Considering the early stage of development (Veber et al. 2002), this does not exclude lisW-S as a potential therapeutic agent, although it is generally recognised that an oral bioavailability of at least 20% in the rat is a predictor of a compound's success in human trials. While it is difficult to speculate as to the cause, the presence of tryptophan in the compound may be a contributor, as a study by Boyko *et al.* also observed low bioavailability in the pharmacokinetic evaluation of a tryptophan-containing dipeptide (Boyko et al. 2007). It should also be noted that the estimate of bioavailability comes in part from IV administration as presented in Figure 3.4, which is based upon plasma samples drawn from a timepoint of 0.08 hours to 12 hours. Blood drawn at an earlier timepoint may have illustrated a different AUC and thus the bioavailability determined in this study may in fact be underestimated.

In order to compensate for low oral bioavailability, we explored the possibility of intravenous infusion. The results of the IV study demonstrated an initial peak followed by a rapid decline and a return to basal levels at 12 hours. These results provided a further incentive to explore alternative solutions, and resulted in the decision to utilize subcutaneous mini-osmotic pumps for continuous infusion of lisW-S within the therapeutic range. A significantly lower volume of lisW-S is required for

infusion relative to oral dosing (6 mg/kg/day vs. 56 mg/kg/day). A further advantage of these pumps is that the animal undergoes a single, small surgical procedure at the start of the study, thus minimizing long-term stress. The pumps are highly accurate and continuously deliver solutions at a specified rate for a set duration.

The 4-week study infused lisW-S at a concentration of 15 mg/ml and demonstrated that after an initial peak at 24 hours, plasma levels decreased to an approximate steady state of 20 ng/ml. While delivery of the C-domain selective ACE inhibitor was consistent, the 15 mg/ml dosage did not result in a satisfactorily high plasma concentration. A 2-fold higher concentration of 30 mg/ml was therefore considered to be an appropriate dosage for administration by miniosmotic pump infusion, as based on the *in vitro* data reported in Chapter 2, a plasma concentration of 40 ng/ml would achieve 60% ACE inhibition in plasma.

In comparison, Lisinopril infused for 4 weeks at 15 mg/ml did reach the therapeutic target of 50 ng/ml. Considering that this compound has a bioavailability of approximately 25%, this was as expected.

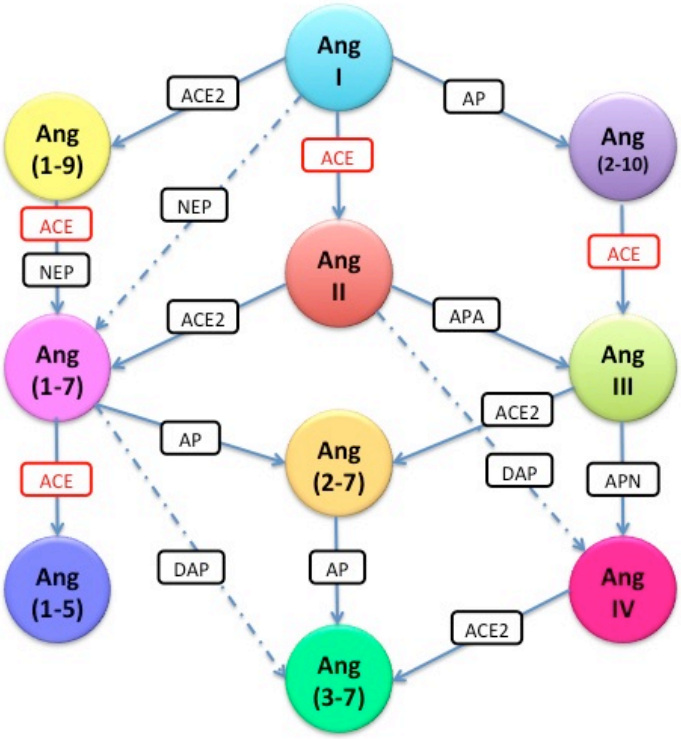
A maleate salt preparation of lisW-S was also evaluated in a preliminary effort to improve bioavailability. Despite the high efficacy of pharmaceutical salts at improving the solubility and oral bioavailability of pharmaceutical compounds (A.T.M. Serajuddin 2007), in this case the lisW-S/MS preparation did not show an improved pharmacokinetic profile over lisW-S/TFA.

Based on the results of the above pharmacokinetic studies, we concluded that the steady state plasma concentrations achieved by infusing lisW-S at a dosage of 1 mg/kg/day would be sufficient to adequately inhibit ACE and have a significant effect on ACE activity in the RAS. Thus, it was decided to administer both lisW-S and lisinopril by miniosmotic pump infusion at a concentration of 30 mg/ml in subsequent studies.

Chapter 4 IN VIVO ANGIOTENSIN PEPTIDE QUANTIFICATION

4.1 INTRODUCTION

Following the assessment of the *ex vivo* efficacy and pharmacokinetics of Lis-W-S, a detailed analysis of RAS peptide levels was carried out in infarcted animals treated with the domain-selective ACE inhibitor in order to determine *in vivo* specificity and efficacy. The components of both the Ang II-producing and the counter-regulatory Ang (1-7)-producing pathways of the RAS have been previously described and defined in Chapter 1. Figure 4.1 is once again presented below for reference purposes. It illustrates the major peptides of interest in this study, along with the enzymatic pathways responsible for their generation.



4.1 The expanded view of the RAS (adapted from (Poglitsch et al. 2012)

4.2 Quantification of RAS Peptides

The endogenous plasma levels of angiotensin peptides are in the low nanomolar range when measured by radioimmunoassay in both humans (A. C. Lawrence et al. 1990) and rats (Campbell et al. 1994). Previously, studies quantifying the effects of ACE inhibition and infarction on plasma peptide levels have been done using enzyme-linked immunosorbent assay (ELISA) kits (Leuschner et al. 2010) and radioimmunoassay combined with HPLC (Campbell et al. 1994). A major drawback of the ELISA method is low-sensitivity, up to 36% cross-reactivity with other angiotensin peptides and time-consuming methodology (Cayman Chemicals 2012).

Radioimmunoassay has greater sensitivity although it too relies on peptide-specific antibodies for identification and is thus also susceptible to cross-reactivity (H. John et al. 2004), especially in the case of RAS peptides with their closely homologous structures. As a result of the drawbacks of these two methods of quantification, and in conjunction with advances in high performance liquid chromatography (LC) - mass spectrometry (MS) instrumentation, LC-MS has recently emerged as the new gold standard for peptide quantification (John et al. 2004). Briefly, LC-MS is used to identify a peptide with high accuracy based on its molecular weight, and LC-MS/MS is used to confirm peptide identification by structure-specific fragmentation (Cui et al. 2007). An internal standard is then used for absolute peptide quantification (Kirkpatrick et al. 2005). Studies using this method have been able to quantify up to four angiotensin peptides in a single LC-MS/MS run (Cui et al. 2007).

More recently a highly sensitive and novel method of LC-MS/MS peptide quantification has been developed by Apeiron Biologics (Vienna, Austria). Referred to as ‘RAS Fingerprinting’, this technique allows for the simultaneous analysis of a full range of known RAS angiotensin-derived peptides from a single low volume plasma sample (Poglitsch et al. 2012). Briefly reviewed in the work by Poglitsch *et al.* (2012), this quantification method has a detection limit of 2 pg/ ml, and incorporates a novel enzyme inhibitor cocktail (patent

pending) which arrests the RAS at the moment of plasma collection - thus preserving the steady state levels of the angiotensin peptides of interest. This novel method was employed in this study for the purposes of detailed analysis of the *in vivo* response of the RAS to both MI and domain-selective and non-selective ACE inhibition. The aim of the present study was to assess the inhibitory potential and the therapeutic potential of the C-domain-selective ACE inhibitor in the context of MI. In addition, this technique allowed for the benchmarking of this novel methodology in a rat model of MI, as well as providing the opportunity to compare this work against other published data. The peptides quantified by the RAS fingerprinting in this study are presented below in Table 4.1.

Table 4-1 Peptides measured by the RAS-fingerprinting method by Apeiron-Biologics

Angiotensin Peptides		**Kinin Peptides**
Ang I (1-10)	Ang (1-9)	BK (1-7)
Ang II (1-8)	Ang (1-7)	BK (1-5)
Ang III (2-8)	Ang (1-5)	
Ang IV (3-8)	Ang (2-7)	
Ang (2-10)	Ang (3-7)	

4.3 Experimental Design

In vivo myocardial infarction studies in the rat model with relevant ACE inhibition were conducted to quantify angiotensin and kinin peptide concentrations in plasma samples by immunoassay and RAS-fingerprinting at 1 and 7 days post-MI. The studies were carried out in infarcted rats as the long-term aim is the evaluation of Lis-WS efficacy as a therapy for the

adverse remodeling that results from MI. Additionally, MI has been reported to elevate levels of a number of key angiotensinogen-derived peptides (Duncan et al. 1997) (Ocaranza et al. 2006). Thus it was reasoned that this would facilitate detection and also allow for a detailed analysis of these critical peptides after an infarction with the newly developed LC-MS/MS technology. Importantly, MI was confirmed histologically in all treatment groups by quantification of infarct size.

4.3.1 Experimental Design : Seven day time point

The first study had a one week time point which - based on a review of the literature - was chosen as the point at which peptide levels after infarction were adequately elevated for quantification (Ocaranza et al. 2006) (Campbell et al. 2008).

A total sample size of 30 male Wistar rats (180 – 220g) were divided into four groups as shown in table 4.2. Each group underwent either sham surgery or induction of myocardial infarction by permanent ligation of the left anterior descending artery (LAD). Immediately following surgery, miniosmotic pumps containing either saline, lisinopril (30 mg/ml) or lisW-S (30 mg/ml) were implanted subcutaneously.

Seven days after surgery, blood was collected by cardiac puncture for peptide quantification. Hearts were explanted and processed histologically to facilitate quantification of infarct size and confirmation of successful induction of MI. AcSDKP and Ang II plasma levels were quantified by EIA assay. RAS-fingerprinting of both angiotensin and kinin plasma peptide levels was done by Apeiron-Biologics. Additionally, PK validation of the plasma samples in the infarcted LisW-S-treated group was done by LC/MS/MS assay to ensure the success of the miniosmotic pumps.

Table 4-2 Animals were divided into four groups for the 7-day study as shown below

Group	Number of animals	Procedure	Treatment
1	6	Sham	Saline
2	8	MI	Saline
3	8	MI	Lisinopril
4	8	MI	LisW-S

4.3.2 Experimental Design: One day time point

The second study was conducted in order to evaluate the short-term effects of C-domain ACE inhibition in an MI model. A total sample size of 12 male Wistar rats (180 – 220g) were divided into two groups as shown in table 4.3. Animals underwent either sham or MI-induction surgery, followed by subcutaneous miniosmotic pump implant. The pumps contained either saline or lisW-S (30 mg/ml) solutions. Animals were sacrificed one day (24 hours) after treatment and blood was collected by cardiac puncture for peptide quantification. RAS-fingerprinting of plasma samples by Apeiron-Biologics was used to quantify both angiotensin and kinin plasma peptide levels.

Table 4-3 Animals were divided into two groups for the one-day study as shown below

Group	Number of animals	Procedure	Treatment
1	6	Sham	Saline

2	6	MI	LisW-S

4.4 METHODS

4.4.1 MYOCARDIAL INFARCTION

4.4.1.1 ANAESTHESIA AND PREPARATION

Rats were initially anaesthetized with isoflurane and were placed in an induction chamber for a minimum of 2.5 minutes. During the induction phase the vaporizer was set to anaesthetize the animal with 5% Isoflurane, at a flow rate of 1.5% Oxygen. After 2.5 minutes of induction the animal was removed from the chamber, weighed (and body weight recorded), and the chest area shaved. The animal was then placed back into the induction chamber under the same conditions for a further 2.5 minutes.

Following complete induction the animal was removed from the chamber and placed on the intubation table at an inclined angle of 45 degrees for intubation. The method of tracheal intubation required the use of an otoscope for laryngoscopic visualization of the vocal cords. A guide wire was then passed through the open vocal cords and into the trachea, followed by a 16 gauge IV catheter. The guide wire was removed and the rat was immediately transferred to the pre-warmed operating table (a heating pad maintained the table at approximately 37 degrees Celsius). The catheter was then attached to the ventilator, and the animal was mechanically ventilated at 5% Isoflurane and 0.3% Oxygen for a further two to three minutes. Following induction and intubation the animal was maintained under anaesthesia with 1.5 - 2% Isoflurane and 0.3% Oxygen. The tidal volume and respiration rate of the ventilator was adjusted for the size of the animal according to the following formulas: tidal volume (Vt, ml) = $6.2 \times M^{1.01}$ (M = animal mass, kg); respiration rate (RR, min^{-1}) = $53.5 \times M^{-0.26}$. The animal was secured in a dorsal recumbent position on the pre-warmed operating table with surgical tape. The two front paws and one distal paw were taped down. The remaining paw was left free to monitor depth of anaesthesia during surgical procedure

(movement of the paw is indicative of a decrease in anaesthesia depth). The surgical area was then cleaned with an iodine solution. Depth of anaesthesia was checked by non-responsiveness to a tail pinch with forceps.

4.4.1.2 SURGICAL PROCEDURE (MI/ SHAM)

A left intercostal thoracotomy was performed to gain access to the heart. A 1 – 1.5 cm incision was made in the area of the fourth intercostal space, at a 90^{o} angle to the sternum. Cotton buds and forceps were used to open the surgical area by separating the dermal layer from the muscles. Cotton buds were used to pull the chest muscles aside by blunt dissection technique and expose the fourth intercostal space (clearly identified by the greater width of the fourth space in comparison to the fifth). Chest muscles were retracted and a pre-heated haemostat was used to gently penetrate the intercostal space, thus opening up the thoracic cavity. A small retractor was used to separate the fifth and sixth ribs in order to clearly visualise the beating heart.

Prior to arterial ligation of the myocardium, the pericardium was removed to enable clear visualisation of the left anterior descending (LAD) artery. The pericardium was removed by grasping the thymus - which tends to cover a large upper portion of the heart – with forceps and lifting it upwards until the membranous pericardium could be clearly seen. The pericardium was then torn and removed from the heart with small, sharp-edged forceps. Once this has been done, the thymus tends to retract away from the heart, exposing more of the myocardium. At this point in a sham operation, the ligation step was skipped. In the MI operations, however, a 6-0 proline suture was passed randomly through the myocardium. This suture was not tied off and was used as a ‘guiding’ suture, as it could be held and used to manipulate the position of the heart in the chest. This manipulation greatly improved the chance of correctly identifying the LAD – which often lies further towards the dorsal side of the heart wall, and so is difficult to see immediately upon opening the chest. Once the LAD was identified, a second 6-0 proline suture was carefully passed through the myocardium and around the artery. This second suture was then tied off, at which point ischemia of the left ventricular wall was clearly observed.

The first ‘guiding’ suture was then pulled out and the retractor removed. Two 4-0 silk intercostal sutures were used to re-approximate the ribs. The first suture was tightened before manual compression of the chest (to remove residual air in the cavity and aid lung re-expansion), after which the second suture was quickly tied off. The chest muscles were also re-approximated with a single 4-0 silk suture. The skin incision was closed with 4-0 silk sutures, and the area was cleaned with iodine. At this point, the animal was removed from the mechanical ventilator. Once spontaneous respiration was achieved, an analgesic dose of buprenorphine (0.05 mg/kg) was administered by intramuscular injection to the hind leg. The catheter was left in place in the trachea to maintain the open airway during recovery. The animal was then placed in a solitary cage to prevent other animals from pulling the sutures out from the incision site. Once consciousness had been regained, the animal was extubated and provided with food and water.

4.4.1.3 Post-Operative Care

The animals were monitored daily for the duration of the study. Their water and food intake was monitored, and their weights recorded. The incision site and sutures were checked for the first three days post-surgery to ensure adequate healing. Analgesia was administered twice daily for 48 hrs after surgery.

4.4.2 Pump implant

Mini-osmotic pumps were prepared and implanted as described in Section 3.3.5.

4.4.3 Blood Collection

Due to the volume of blood required for analysis, blood was collected by cardiac puncture at the experimental endpoint. Animals were anaesthetized with halothane for this procedure. Following blood collection, animals were euthanised by injection of saturated potassium chloride (KCl) solution into the left ventricle.

4.4.3.1 AcSDKP samples for EIA

Blood was collected on ice in heparinised tubes. The ACE inhibitor captopril was immediately added to a final concentration of 10^{-5} M. Blood samples were separated by centrifugation at 1600 g for 20 minutes (4^{o}C). Plasma samples were stored at -80^{o}C.

4.4.3.2 Ang II samples for EIA and RAS peptide fingerprinting

Blood was collected on ice with a 5% enzyme inhibitor cocktail (Apeiron patent pending). Direct communication from Apeiron detailed that suitable QC has been carried out showing complete effectivity of the cocktail in inhibiting peptide hydrolysis in whole blood spiked with all relevant peptides. Blood samples were separated by centrifugation at 3000 g for 20 minutes (4^{o}C). Plasma samples were stored at -80^{o}C.

4.4.4 Quantification of infarct size

4.4.4.1 Explant

Animals were anaesthetized, blood was extracted and hearts were injected with 1 ml saturated potassium chloride to arrest the heart in diastole for histological analysis. Hearts

were explanted, washed and perfused with saline before being transferred to a 4% paraformaldehyde solution for 24 hours before histological processing. **µ**

4.4.4.2 HISTOLOGY

After this fixation period, hearts were cut transversely into 4 equal sections and processed through graded alcohol (Illovo Sugar Ltd., South Africa) and xylene (Saarchem, South Africa) using a Tissue-Tek Rotary Tissue Processor (Sakura Finetek, Japan). Embedded in paraffin wax (Merck, Germany) the sections were cut into 2 - 3 µm slices, picked up on glass slides (Marienfeld GmbH & Co. KG, Germany) and baked on a hot plate at 60 ^{o}C (Kunz Instruments AB, Nynäshamn, Sweden) before being dewaxed through xylene, agitated in absolute alcohol and hydrated in running tap water. Lastly sections were stained with haematoxylin and eosin (H&E) and mounted on glass slides.

4.4.4.3 IMAGE ACQUISITION

Images were acquired by microscopy (Nikon Eclipse 90i, Nikon Corporation, Japan) and captured by digital camera (Nikon DXM-1200C, Nikon Corporation, Japan) . Image analysis for quantification of infarct size was done using the VIS-Visiopharm Integrator system (Visiopharm, Hørsholm, Denmark).

4.4.4.4 IMAGE ANALYSIS

Infarct sizes were acquired and quantified with Visiopharm Integrator Systems (Visiopharm, Hørsholm, Denmark) according to the method of Takagawe *et al*. (Takagawa et al. 2007).

Briefly, the sum of all midline infarct lengths was divided by the sum of all midline circumferences for all sections and expressed as a final percentage.

4.4.5 Enzyme-linked Immunosorbent Assay

4.4.5.1 AcSDKP quantification

The concentration of AcSDKP in the plasma samples was quantified using an AcSDKP enzyme-linked immunosorbent (EIA) assay kit (Cayman Chemicals, France). This was performed according to the manufacturer's instructions. The 96-well microplate is precoated with a mouse anti-rabbit antibody. The assay is based on competitive binding to the coated wells between acetylcholinesterase (AChE) linked to an AcSDKP tracer and free AcSDKP in the plasma sample. Plasma samples were extracted with methanol and evaporated to dryness by vacuum centrifugation prior to assay.

Briefly, wells were washed five times with wash buffer (300 µl/ well). After each wash, the wells were aspirated and any remaining droplets shaken out. Wells for blanking Ellman's reagent were left empty. Reconstituted EIA buffer was added to both non-specific binding (NSB) and maximum binding wells. A range of AcSDKP standards from 0.09 – 12.5 nM and plasma samples were plated in duplicate. The AcSDKP AChE tracer was added to all wells, while the AcDHP antiserum was added to all but the NSB wells.

The plate was then covered and incubated at 4^{o}C for 18 hours. The next day, the plate was washed five times with wash buffer, and any remaining droplets were shaken out. 200 µl of Ellman's reagent was then added to all wells, including the blanks. The plate was covered with aluminium foil and incubated in the dark for 1.5 hrs at ambient temperature. The plate was read continuously at 414 nM on a Benchmark Microplate Reader (Biorad, Japan) as the absorbance of the maximum binding wells increased from 0.2 to 0.8 units.

4.4.5.2 ANG II QUANTIFICATION

The concentration of Ang II in the plasma samples was quantified using an Ang II EIA assay kit (Cayman Chemicals, France). This was performed according the manufacturer's instructions. The 96-well microplate is precoated with a specific anti-Ang II monoclonal antibody. Plasma samples were extracted with phenyl cartridges (Phenomenex/ Strata, USA) and methanol and evaporated to dryness prior to assay.

Briefly, wells were washed five times with wash buffer (300 µl/ well). After each wash, the wells were aspirated and any remaining droplets shaken out. Wells for blanking Ellman's reagent were left empty. Reconstituted EIA buffer was added to NSB wells. Ang II standards in the range of 0.98 – 125 pg/ml and plasma samples were plated in duplicate. The plate was then incubated for 1 hour at ambient temperature with gentle agitation. Gluteraldehyde was added to all wells (excluding blanks) to covalently link Ang II to the plate and incubated for 5 minutes at ambient temperature with gentle agitation. Gluteraldehyde was blocked to permit binding to a monoclonal antibody AChE-labelled tracer by adding borane-trimethylamine and incubating as with the gluteraldehyde step. The plate was washed five times as before and anti-Ang II IgG tracer was added to all wells (excluding blanks). The plate was then covered and incubated overnight at 4^{o}C. The next morning, the plate was washed five times as before. Wash buffer was added to all wells and the plate was incubated for 10 minutes at ambient temperature with gentle agitation. Finally, the plate was washed a further five times as before and Ellman's reagent was added to all wells (including blanks). The plate was covered with aluminium foil and incubated in the dark on an orbital shaker for 1 hour at ambient temperature. The plate was read continuously at 414 nM on a Benchmark Microplate Reader (Biorad, Japan).

4.4.6 RAS PEPTIDE FINGERPRINTING

LC-MS/MS quantification of angiotensin peptides was performed by Apeiron-Biologics AG. Plasma samples were shipped on dry ice to Vienna, Austria.

Plasma samples were spiked with 100 pg/ml stable isotope-labeled internal standards and processed with a solid phase extraction using Sep-Pak cartridges (Waters, USA) according to the manufacturer's instructions. Efficient stabilization of endogenous angiotensin peptides was assured by addition of stable isotope labeled internal standard peptides.

Following elution and solvent evaporation, samples were reconstituted in 50 µl 50% acetonitrile/ 0.1% formic acid and subjected to LC-MS/MS analysis using a reversed phase analytical column (Luna C18, Phenomenex) The gradient ranged from 10% acetonitrile/ 0.1% formic acid to 70% acetonitrile/ 0.1% formic acid (t = 9 minutes). The eluent was analysed with a QTRAP-4000 mass spectrometer (AB Sciex) operated in the MRM mode using dwell times of 25 milliseconds, at a cone voltage of 4000 volts and a source temperature of 300°C. For each peptide and corresponding internal standard, two separate mass transitions were measured. Angiotensin peptide concentrations were calculated by relating endogenous peptide signals measured to internal standard signals with a signal-to-noise ratio above 10. The quantification limits for individual peptides were found to be between 1 pg/ml and 5 pg/ml in undiluted plasma samples. (Adapted from Poglitsch et al., 2011)

4.4.7 PK VALIDATION OF LISW-S PLASMA CONCENTRATIONS

Separate blood samples were collected and prepared from animals treated with lisW-S as described in Section 3.3.6. In order to ascertain lisW-S plasma concentrations following administration, these samples underwent LC/MS/MS analysis as previously described in Section 3.3.7.

4.4.8 STATISTICAL ANALYSIS

Two-tailed student t-tests were used to assess the differences between two groups. Comparisons between multiple groups were made by means of a one-way ANOVA (Statplus-Pro, AnalystSoft Inc., USA), followed by Fisher's LSD test. Statistical significance was defined as $p < 0.05$. All data are expressed as means ± standard error.

4.5 RESULTS

4.5.1 QUANTIFICATION OF INFARCT SIZE

Seven Days

Prior to performing plasma peptide quantification, myocardial infarction was confirmed by histological staining and image analysis. An example of the images obtained for quantification is presented below in figure 4.2. Infarct sizes are expressed as a percentage of left ventricular midline measurements. Table 4.4 shows the mean infarct sizes and sample sizes for each group (one animal in the lisinopril-treated group died following induction of MI). Substantial infarctions were confirmed in all infarcted animals with a mean value of 34.98 ± 10.38 % and infarct size was not significantly different between groups (p=NS).

Table 4-4 Mean infarct size quantified by image analysis (%)

Group	Mean Infarct Size (%)	Std Error (%)
MI (n = 8)	36,48	15.65
MI/Lisinopril (n = 7)	29,80	4.03
MI/LisW-S (n = 8)	38,24	7.34

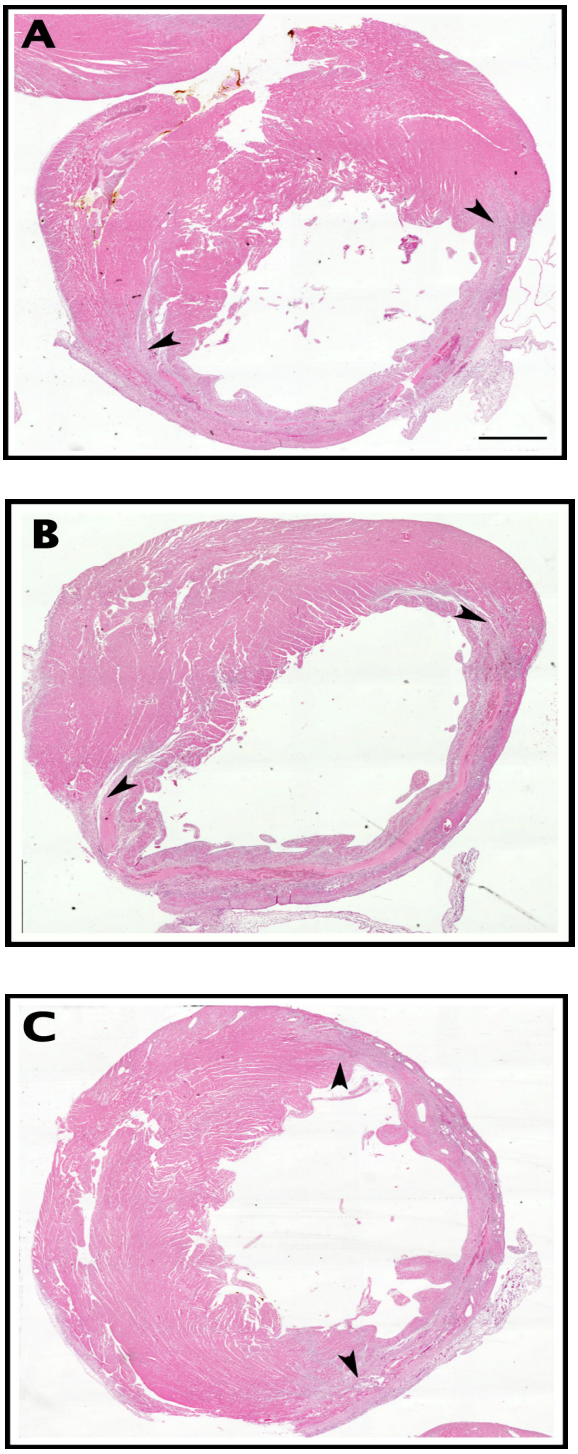


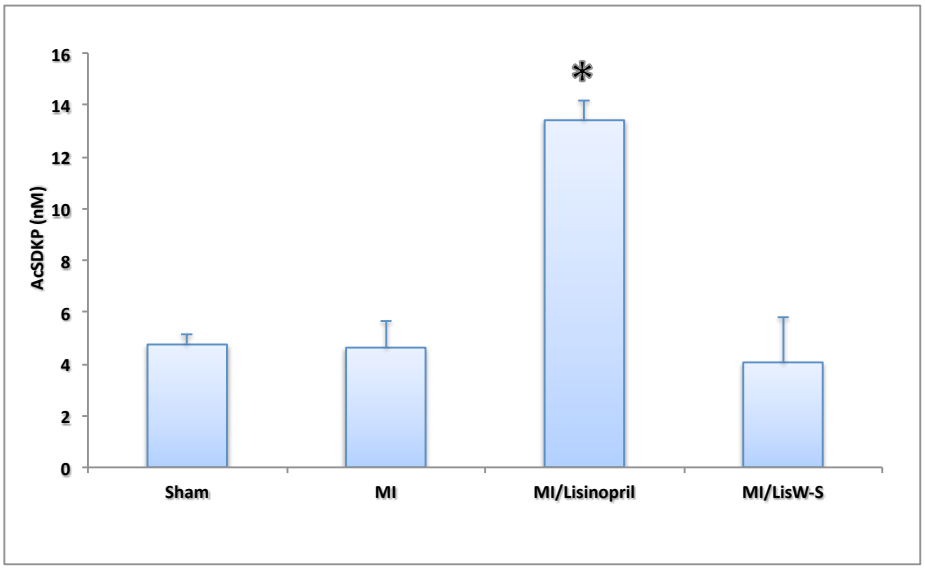
Figure 4.2 H&E staining of representative cardiac tissue sections. Arrows demarcate the infarct zone in A) MI group, B) MI/lisinopril-treated group and C) MI/lisW-S-treated groups. Bar represents 1 mm

4.5.2 ELISA

4.5.2.1 AcSDKP EIA

Seven Days

The results of this assay are presented below in figure 4.3. No significant difference is observed between the untreated sham and MI groups (Sham: 4.75 ± 0.38 nM; MI: 4.62 ± 1.04 nM. p=NS). However, a significant elevation of plasma AcSDKP levels is observed in the lisinopril-treated MI group versus the MI and sham groups (MI/Lisinopril: 13.41 ± 0.77 nM. $p<0.05$ vs. sham and MI) as a result of non-selective ACE inhibition. No significant difference was observed between the sham and MI control groups and the MI/lisinopril-treated group (MI/LisW: 4.05 ± 1.72 nM. p=NS vs. sham and MI). However, mean AcSDKP levels were significantly elevated by more than 3-fold in the MI/lisinopril treated group vs. the MI/lisW-S-treated group ($p<0.05$).



4.3 Mean AcSDKP concentrations (nM) in plasma at 1 week after MI induction as obtained by EIA assay. (* $p<0.05$ vs all groups)

4.5.2.2 ANG II EIA

Seven Days

As shown in figure 4.4, the MI group showed a trend towards an increase by approximately 1.5-fold in plasma Ang II levels when compared to the sham group (MI: 105.39 ± 56.24 pg/ml. p=0.16 vs. sham). The mean plasma concentration of Ang II was significantly reduced by 2.6-fold in the MI/lisinopril-treated group in comparison to the untreated MI group (MI/lisinopril: 41.24 ± 23.36 pg/ml. p<0.01 vs. MI). However, as is clearly seen in figure 4.4, Ang II levels are significantly elevated 1.7 fold relative to the MI group by administration of the novel domain-selective ACE inhibitor lisW-S (MI/lisW-S: 189.25 ± 54.42 pg/ml. p<0.01 vs. MI).

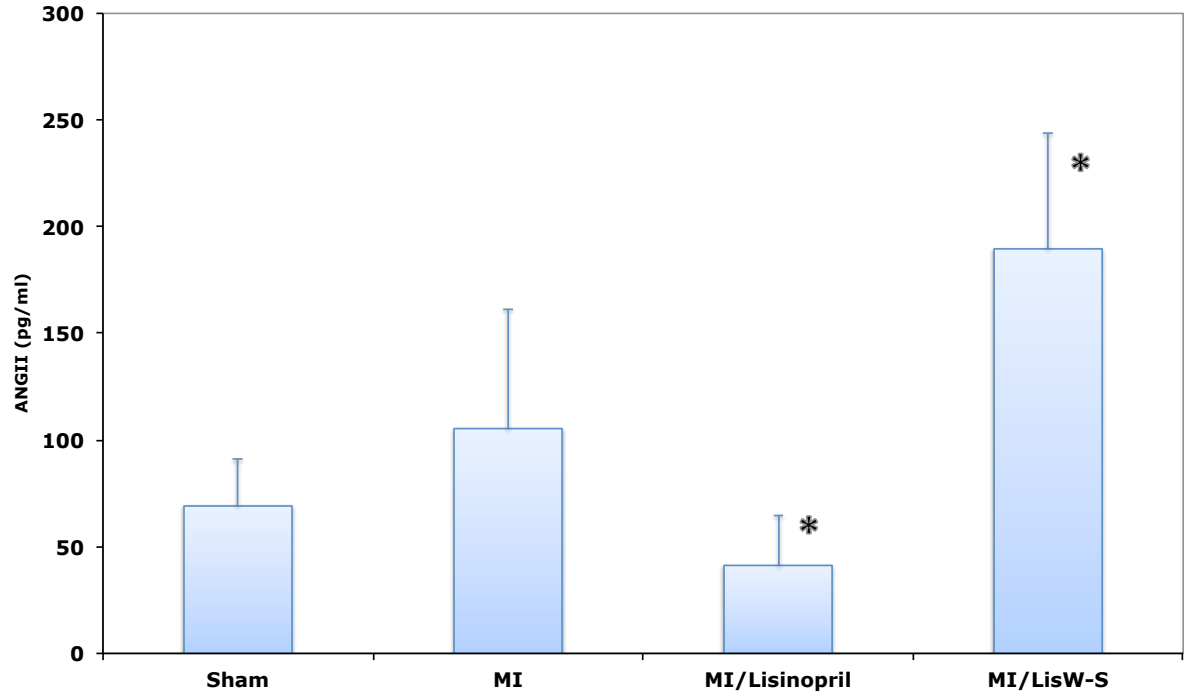


Figure 4.4 Mean Ang II concentrations (pg/ ml) in plasma at 1 week after MI induction as obtained by EIA assay. (* p<0.01 vs. MI)

4.5.3 RAS-FINGERPRINTING

4.5.3.1 OVERVIEW

Due to the significant amount of data points generated – as well as the multi-faceted nature of the relationships between the component peptides of the RAS – the data is presented below in several different formats to facilitate understanding. Figures 4.5 and 4.6 show a graphical interpretation of the flow of angiotensin-derived peptides through the RAS cascade in treated and untreated groups at 7 days post-MI. In addition, Table 4.7 presents the mean concentrations and standard deviation of each peptide analysed at this time point. Figure 4.7 and table 4.8 present the data obtained 1 day post-MI. The sections below include a detailed discussion of every measured peptide in the cascade at both timepoints, as well as comparisons between groups and graphical presentations of peptide levels with relative significance indicated. It should be noted that a significant number of the data points obtained for Ang (1-9) fall below the limit of quantification (BLQ) due to a high signal-to-noise ratio, and so analysis of this peptide and its ACE/NEP-mediated conversion to Ang (1-7) could not be included in this study. In addition, all measurements of Ang (2-7) fell BLQ, and thus have been omitted.

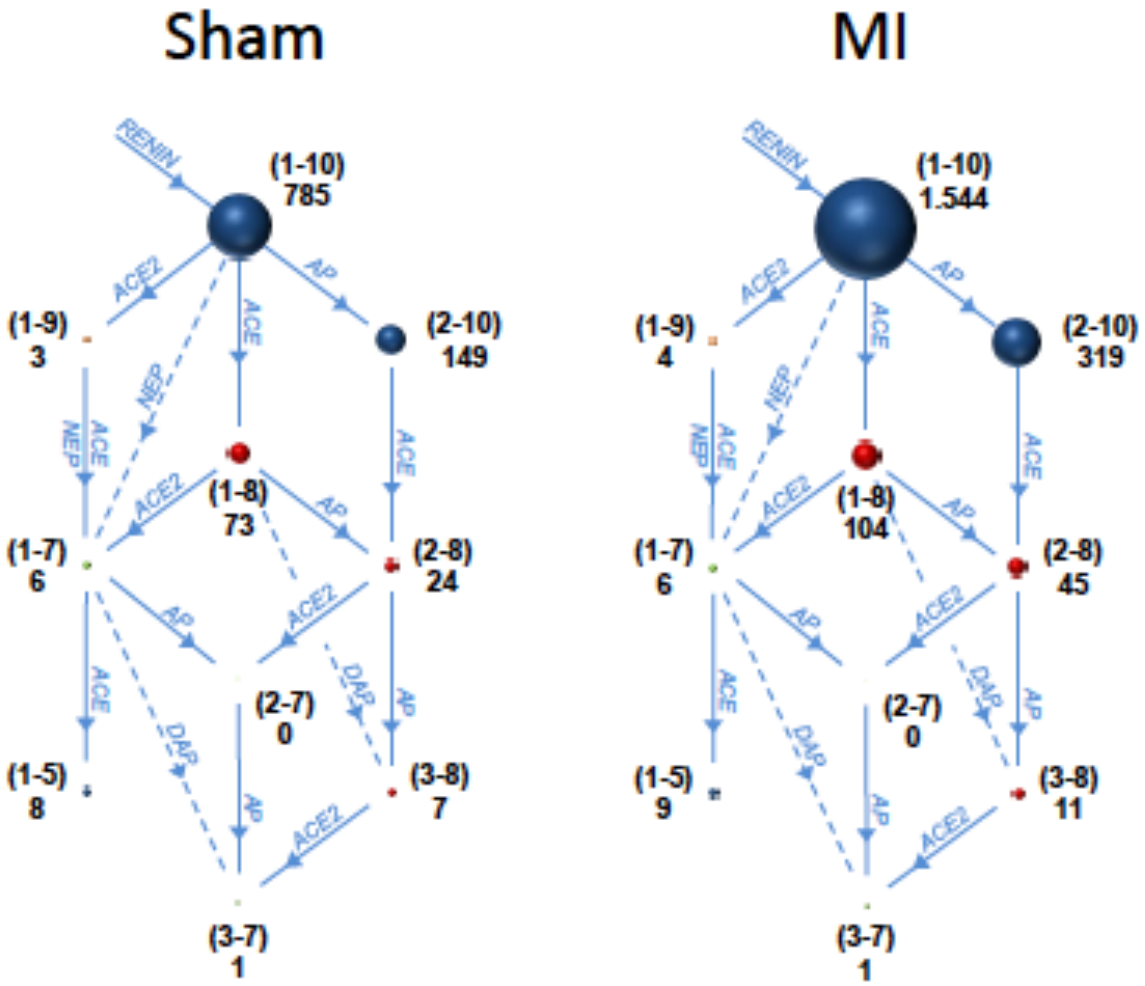


Figure 4.5 RAS-fingerprints of mean angiotensin peptide plasma concentrations for the sham and MI groups at 7 days post-MI. All measurements are in pg/ml, and the size of the representative spheres corresponds to the measured amount of each peptide. Figure provided by Apeiron Biologica.

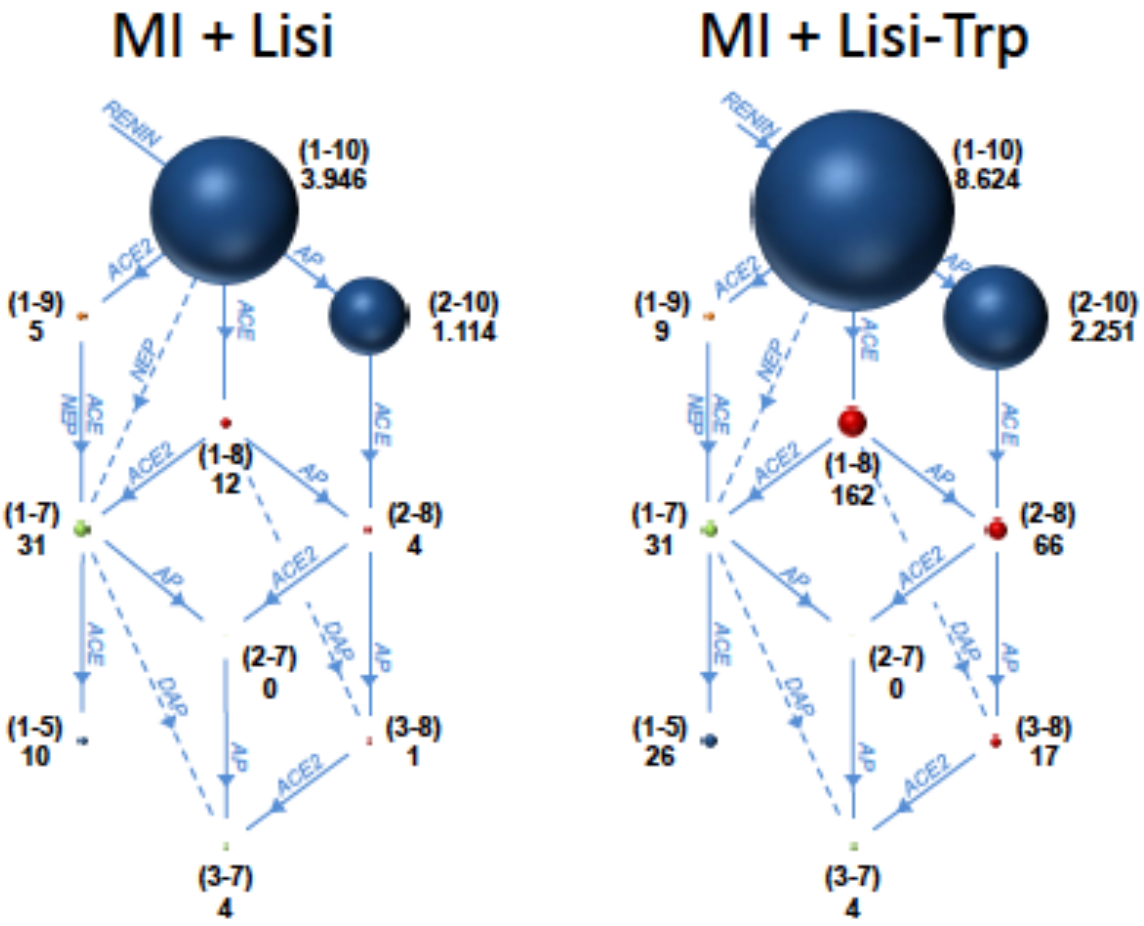


Figure 4.6 RAS-fingerprints of mean angiotensin peptide plasma concentrations for MI/lisinopril and MI/lisW-S groups at 7 days post-MI. All measurements are in pg/ml, and the size of the representative spheres corresponds to the measured amount of each peptide. Figure provided by Apeiron Biologica.

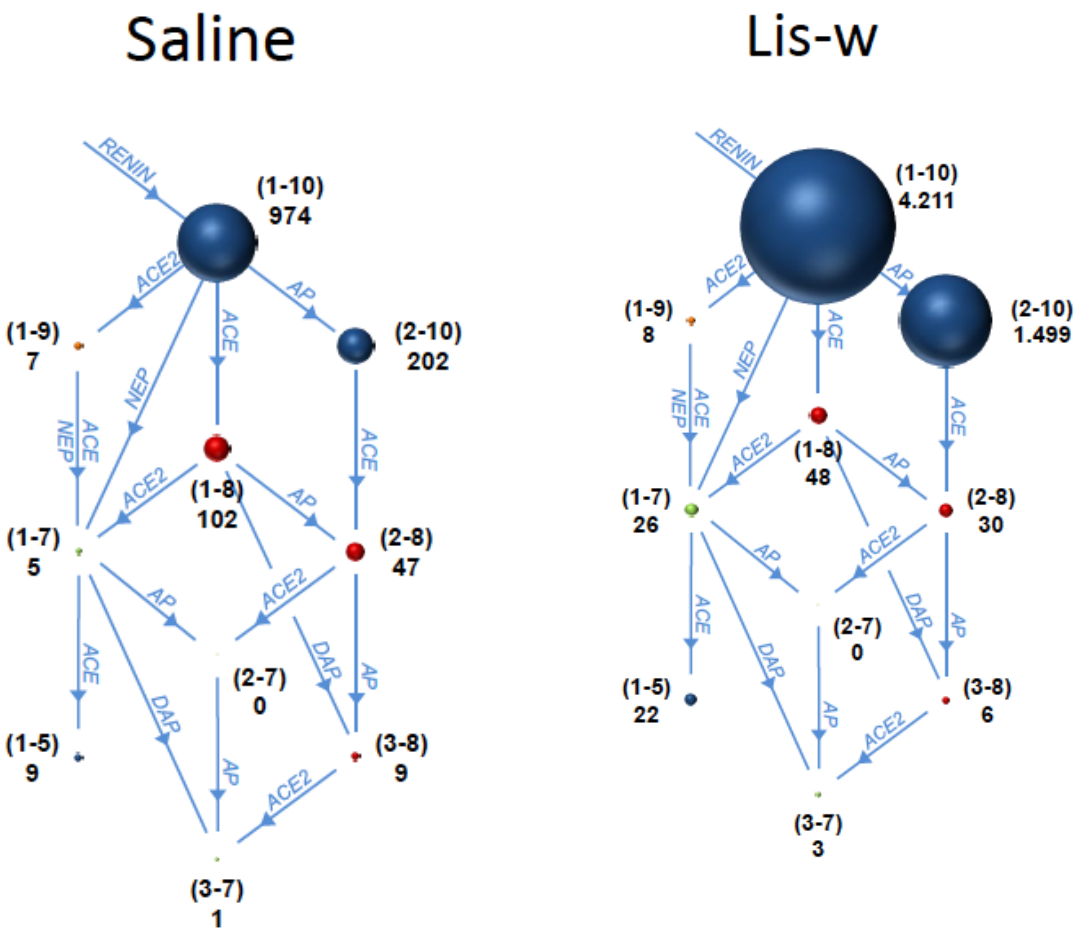


Figure 4.7 RAS-fingerprints of mean angiotensin peptide plasma concentrations for the MI and MI/lisW-S-treated groups at 1 day post-MI. All measurements are in pg/ml, and the size of the representative spheres corresponds to the measured amount of each peptide. Figure provided by Apeiron Biologica.

Table 4-5 Individual and mean peptide plasma concentrations (pg/ml) for all groups at 7 days. ND = non-detectable.

	Sample ID	Ang I (1-10)	Ang II (1-8)	Ang III (2-8)	Ang IV (3-8)	Ang (2-10)	Ang (1-5)	Ang (3-7)
Sham	Mean	785	73	24	7	149	8	ND
	Std Error	149	10	4	1	30	0	ND
MI	Mean	1544	104	45	11	319	9	ND
	Std Error	200	17	9	2	45	1	ND
MI/ lis- inopril	Mean	3946	12	4	1	1114	10	4
	Std Error	277	1	1	0	101	2	0
MI/ lisW-S	Mean	8624	162	66	17	2251	26	4
	Std Error	1103	28	11	3	385	3	1

Table 4-6 Individual and mean peptide plasma concentrations (pg/ml) for groups in the 1-day study

	Sample ID	Ang I (1-10)	Ang II (1-8)	Ang III (2-8)	Ang IV (3-8)	Ang (2-10)	Ang (1-5)	Ang (3-7)
MI	Mean	974	102	47	9	202	9	1
	Std Error	100	15	9	1	18	0	0
MI/ lisW-S	Mean	4211	48	30	6	1499	22	3
	Std Error	929	13	10	2	392	3	1

4.5.3.2 ANALYSIS OF INDIVIDUAL ANGIOTENSIN PEPTIDE DIFFERENCES

4.5.3.2.1 ANG I

Seven Days

Significant differences in Ang I plasma concentrations were observed between all four groups as shown in figure 4.8. An almost 2-fold increase in Ang I peptide levels was observed in the infarcted group relative to sham (MI = 1544.14 ± 20.45 pg/ml; Sham = 784.54 ± 149.29 pg/ml; $p<0.05$) as a result of RAS activation post-MI and upregulation of renin-mediated Ang I synthesis.

A highly significant elevation of 2.6-fold was also observed for the MI/lisinopril-treated group relative to MI (MI/lisinopril = 3946.21 ± 277.13 pg/ml; $p<0.01$).

Most interestingly, Ang I was even more strikingly elevated after treatment with LisW-S (MI/lisW-S = 8624.21 ± 1102.77 pg/ml), demonstrating a 5.6-fold increase relative to MI ($p<0.01$) and a 2.2-fold increase relative to MI/lisinopril ($p<0.01$).

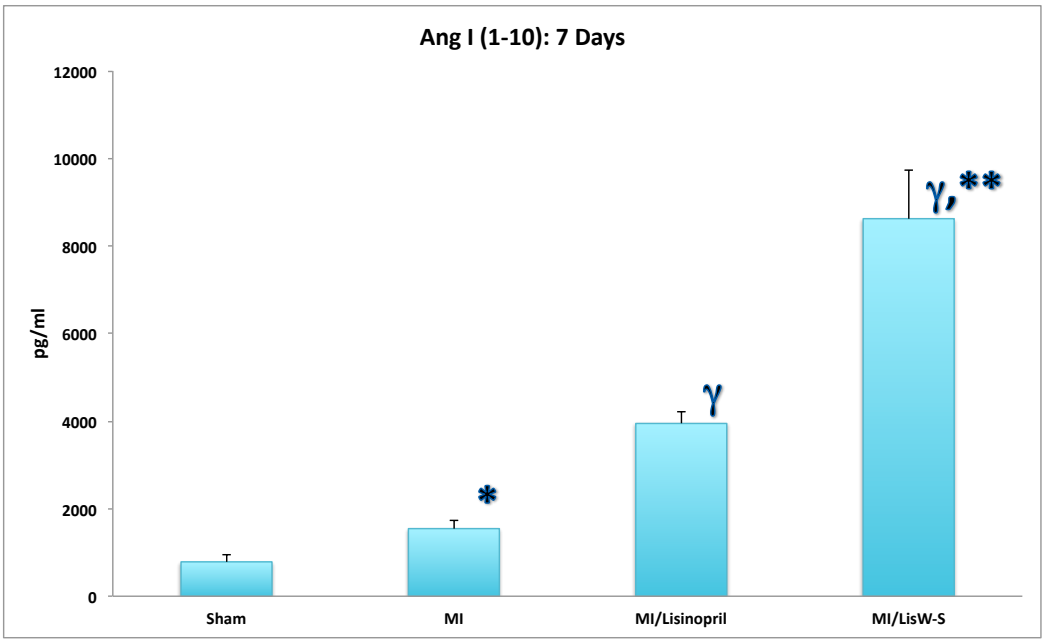


Figure 4.8 Mean Ang I plasma concentrations (pg/ml) at 7 days (* $p<0.05$ vs. sham; γ $p<0.01$ vs. MI; ** $p<0.01$ vs. MI/lisinopril)

One Day

As shown in figure 4.9, a significant 4.3-fold elevation in Ang I was observed in the MI/lisW-S group relative to the untreated MI group when peptide quantification was performed one day post-MI (MI = 974.44 ± 100.41 pg/ml; MI/lisW-S = 4210.99 ± 929.02 pg/ml; $p<0.01$).

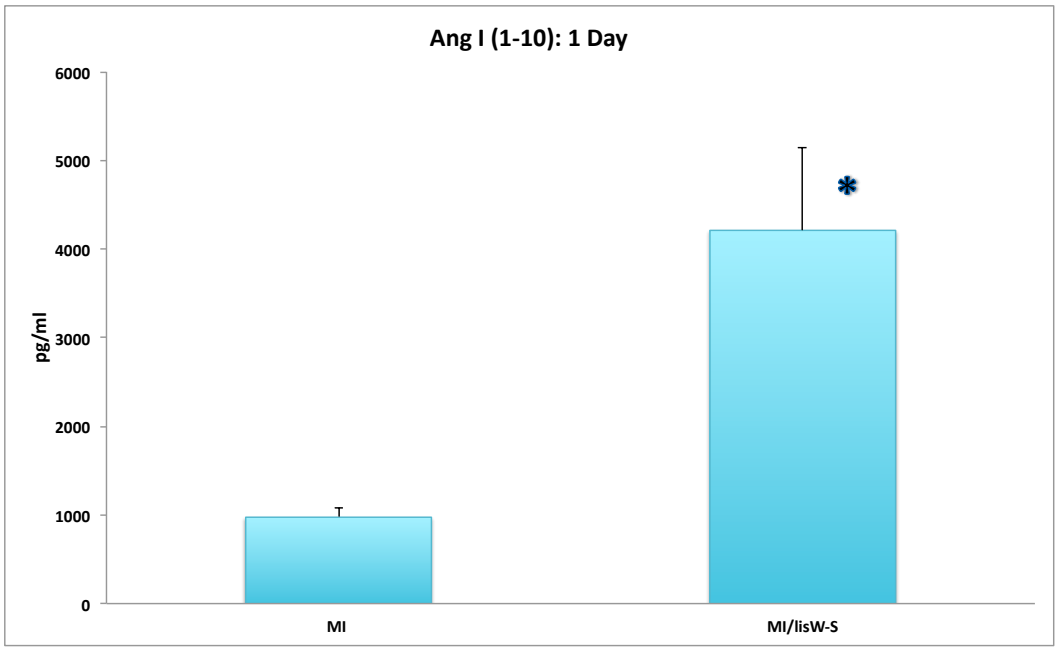


Figure 4.9 Mean Ang I plasma concentrations (pg/ml) at 1 day (*p<0.01 vs. MI)

4.5.3.2.2 ANG II

Seven Days

The plasma concentrations of the vasoconstrictor Ang II at seven days post-MI are presented in figure 4.10. No significant difference was observed between the sham and MI groups (sham = 72.96 ± 9.55 pg/ml; MI = 103.95 ± 47.60; p=NS). The MI/lisinopril-treated group showed a highly significant 8.8-fold decrease relative to MI (MI/lisinopril = 11.75 ± 1.34 pg/ml; p<0.01), while in sharp contrast the MI/lisW-S-treated group exhibited a significant 13.8-fold elevation relative to the MI/lisinopril-treated group, a trend towards a 1.6-fold elevation relative to the untreated MI group and a significant 2.2-fold elevation over

sham (MI/lisW-S = 161.98 ± 28.47 pg/ml; p<0.01 vs. MI/lisinopril; p=NS vs. MI; p<0.05 vs. sham).

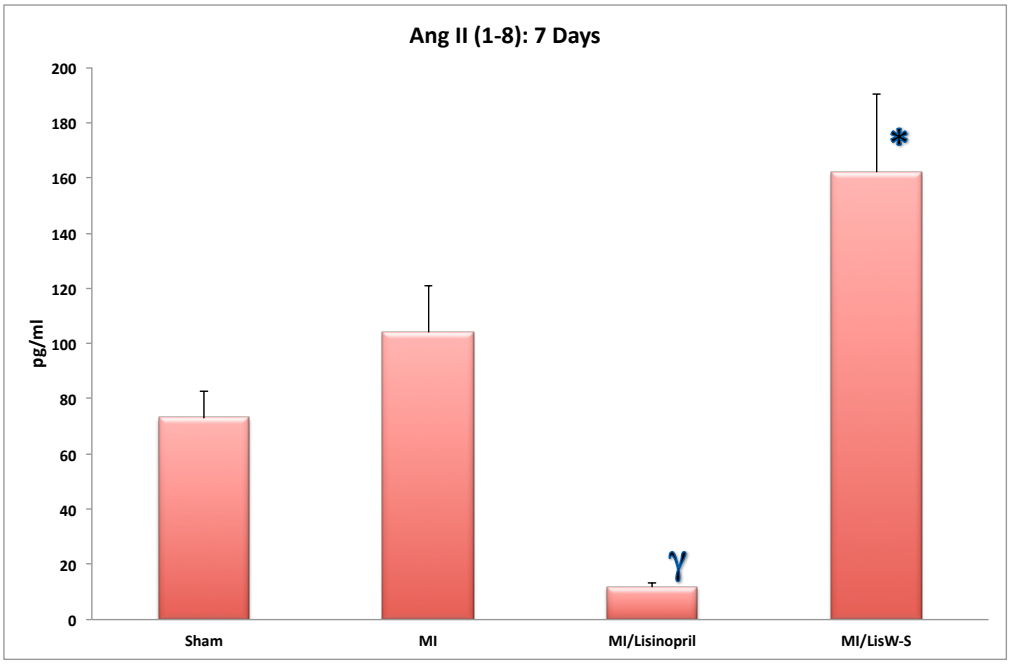


Figure 4.10 Mean Ang II plasma concentrations (pg/ml) at 7 days (γ p<0.01 vs. all groups, *p<0.05 vs. sham)

One Day

Mean plasma concentrations of Ang II one day after MI are presented in figure 4.11. A significant 2.1-fold decrease was observed in the lisW-S-treated group relative to the untreated MI group (MI/ lisW-S = 48.04 ± 12.56 pg/ml; p < 0.05 vs. MI).

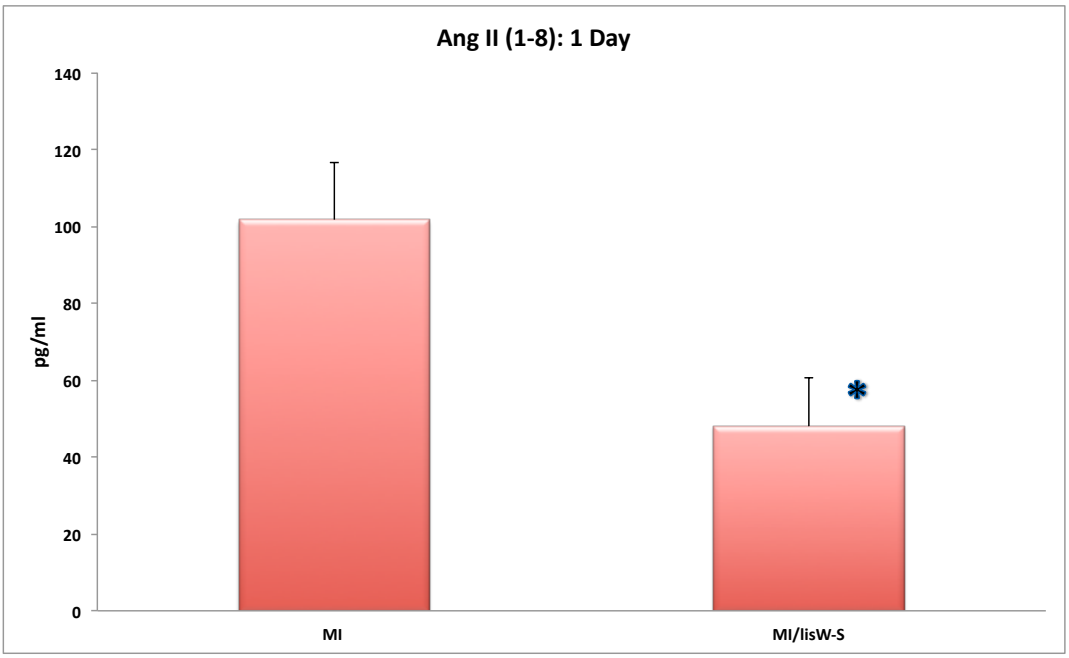


Figure 4.11 Mean Ang II plasma concentrations (pg/ml) at 1 day (*p<0.05 vs. MI)

4.5.3.2.3 ANG II/ ANG I

Seven Days

The ACE-mediated conversion ratios of Ang I to Ang II seven days post-MI are presented below in figure 4.12. The sham group showed a conversion ratio of 0.123 ± 0.01, which was significantly higher than that seen in the MI group (Ang II/Ang I=0.08 ± 0.01; p<0.01 vs. sham). As expected, the MI/lisinopril-treated group demonstrated a dramatic 21.6-fold reduction relative to MI with Ang II/Ang I=0.003 ± 0.001 (p<0.01 vs. MI). A significant 3.4-fold decrease in Ang II/Ang I relative to MI was observed in the MI/lisW-S-treated group, (Ang II/Ang I=0.02 ± 0; p<0.01 vs. MI).

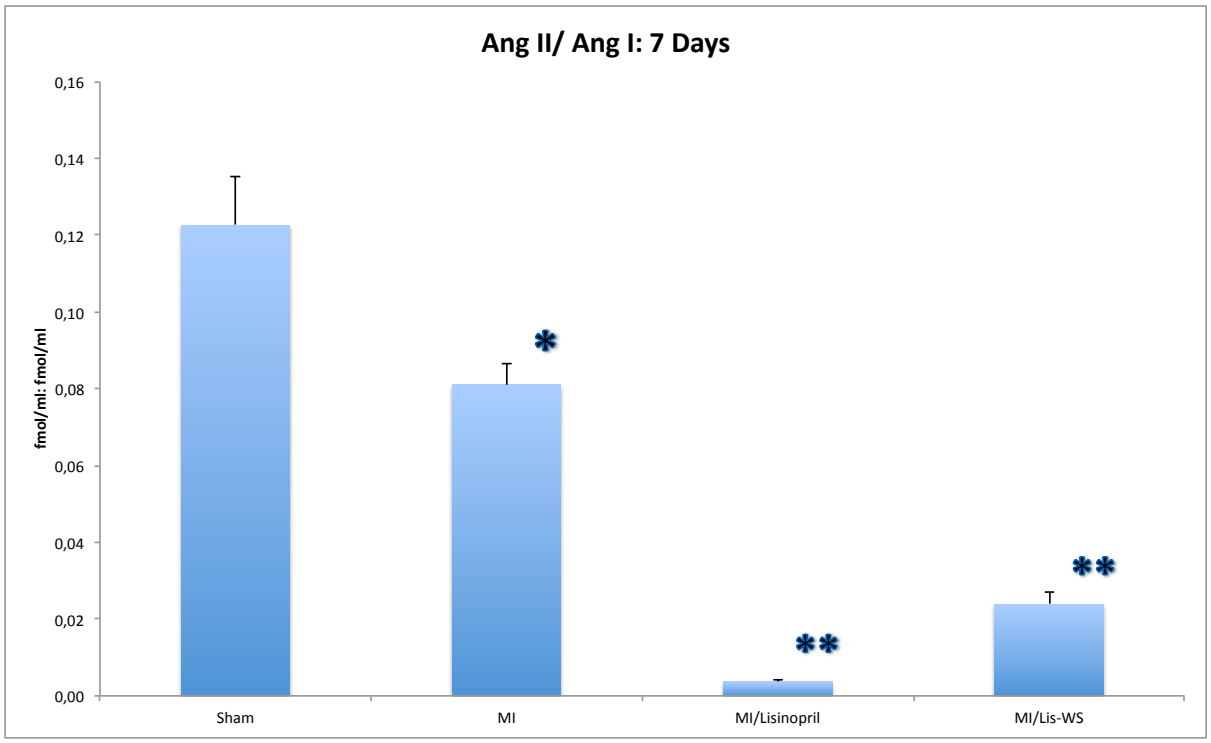


Figure 4.12 Conversion ratios: Ang II/ Ang I at 7 days (*p<0.01 vs. sham; ∗∗ p<0.01 vs. MI)

One Day

The conversion ratios of Ang I to Ang II conversion one day post-MI are presented below in figure 4.13. The untreated infarcted group displayed a conversion ratio of 0.13 ± 0.01; while a highly significant 8-fold decrease was observed in the MI/lisW-S group, with Ang II/ Ang I = 0.02 ± 0 ($p<0.01$).

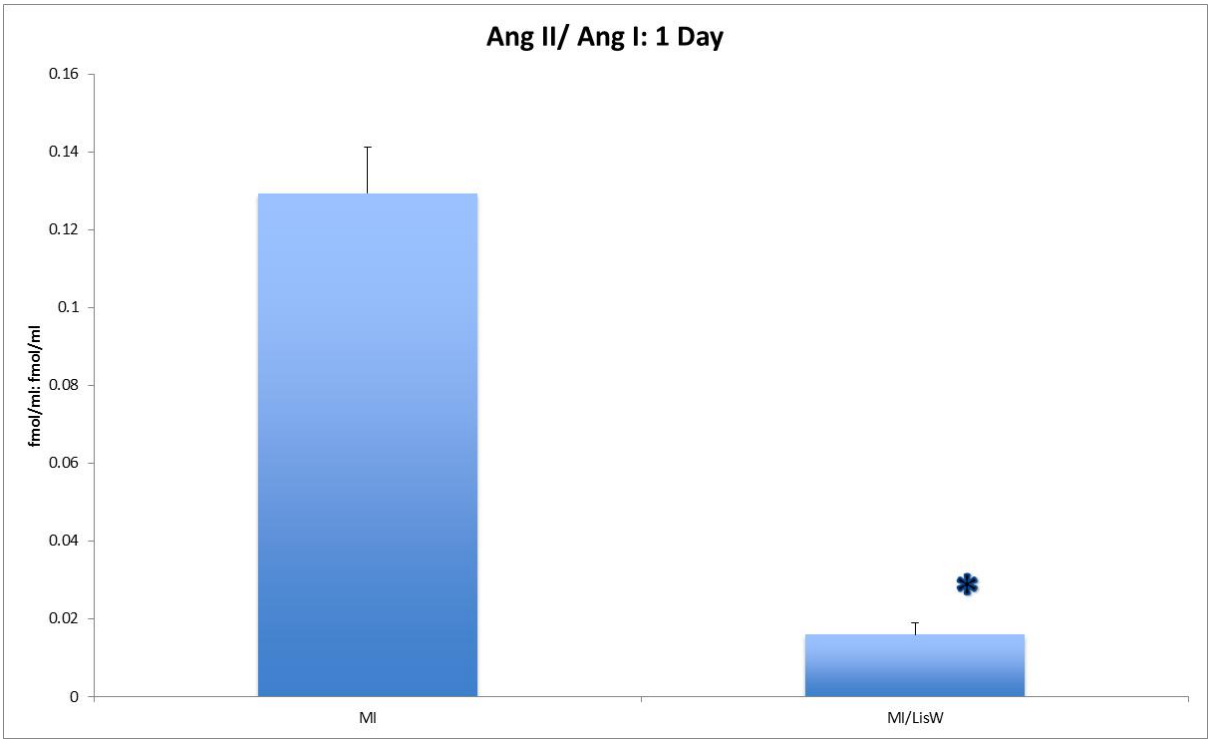


Figure 4.13 Conversion ratios: Ang II/ Ang I at 1 day (*p<0.01 vs. MI)

4.5.3.2.4 ANG (2-10)

Seven Days

Mean plasma concentrations of Ang (2-10) seven days post-MI are presented in figure 4.14. A significant 2.1-fold increase was observed in the MI group relative to the sham (MI = 319.32 ± 45.2 pg/ml; $p<0.05$). The MI/lisinopril-treated group showed a 3.5-fold increase relative to MI (1114.41 ± 100.8 pg/ml; $p<0.01$), while the MI/lisW-S-treated group exhibited a large 7-fold elevation in plasma Ang (2-10) levels relative to MI (2250.55 ± 385.09 pg/ml; $p<0.01$) and a 2-fold increase over the MI/lisinopril-treated group ($p<0.05$).

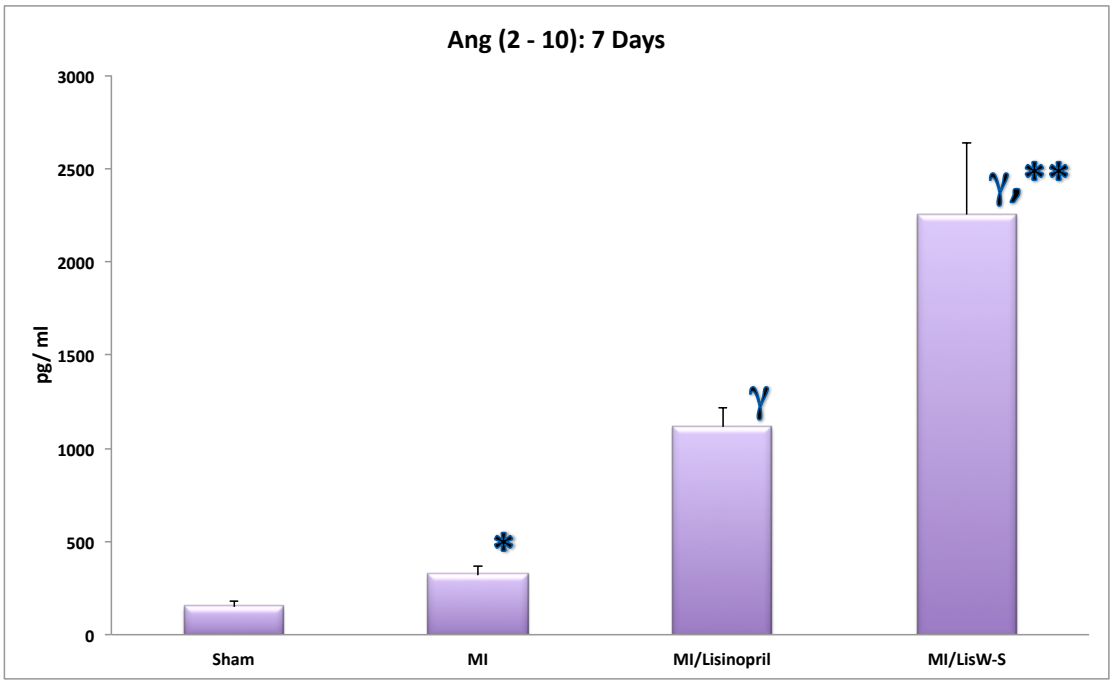


Figure 4.14 Mean Ang (2-10) plasma concentrations (pg/ml) at 7 days (* p<0.05 vs. sham; γ p<0.01 vs. MI; ** p<0.01 vs. MI/lisinopril)

One Day

Mean plasma concentrations of Ang (2-10) one day post-MI are presented in figure 4.15. A highly significant difference was observed between the untreated MI group and the MI/lisW-S-treated group (MI = 201.61 ± 18.16 pg/ml; MI/lisW-S = 1498.86 ± 391.89; p<0.01), as evidenced by a 7.4-fold elevation in peptide levels in the treated group.

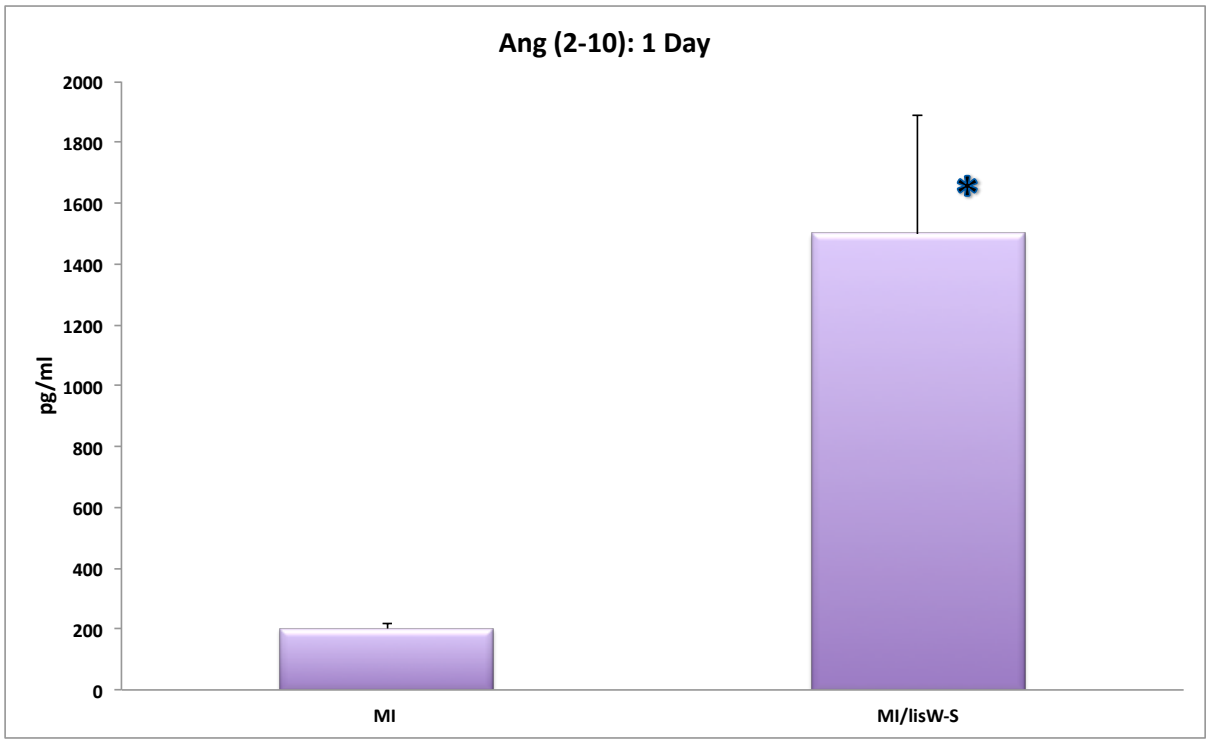


Figure 4.15 Mean Ang (2-10) plasma concentrations (pg/ml) at 1 day (* p<0.01 vs. MI)

4.5.3.2.5 ANG III

Seven Days

Ang III plasma concentrations measured at seven days post-MI are presented in figure 4.16. A 1.9-fold elevation in mean peptide levels was observed in the MI group relative to the sham (MI = 44.87 ± 9.29 pg/ml; Sham = 24.09 ± 4.37 pg/ml; p=NS). A significant 2.8-fold increase was further observed in the MI/lisW-S-treated group relative to the sham (MI/lisW-S = 65.5 ± 10.9 pg/ml; p<0.01)). In contrast, a highly significant 10.1-fold decrease relative to MI and a 14.8-fold decrease relative to MI/lisW-S was observed in peptide levels in the MI/lisinopril-treated group (MI/lisinopril = 4.43 ± 0.66 pg/ml; p<0.01).

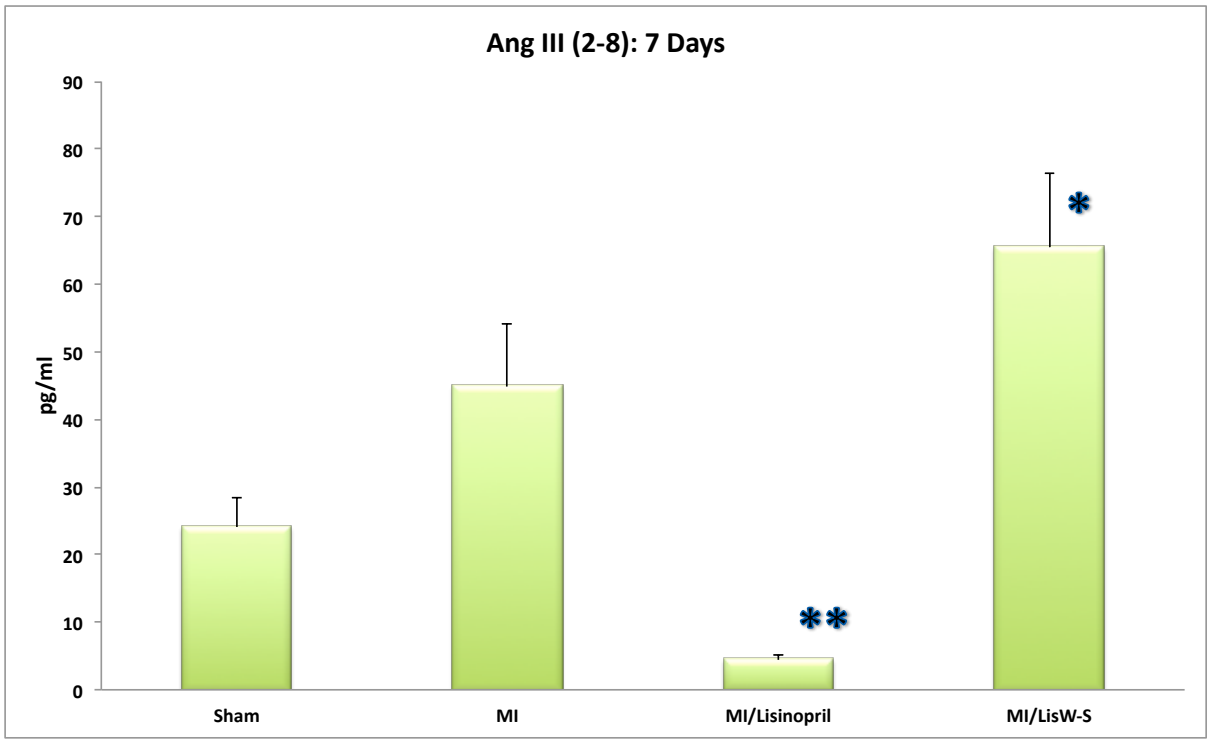


Figure 4.16 Mean Ang III plasma concentrations (pg/ml) at 7 days (* p<0.01 vs. Sham; ** p<0.01 vs. MI and MI/lisW-S)

One Day

As shown in figure 4.17, no significant difference in Ang III plasma concentration was observed between the untreated MI group and the MI/lisW-S-treated group one day post-MI (MI = 46.84 ± 8.89 pg/ml; MI/lisW-S = 29.99 ± 9.79 pg/ml; p=NS).

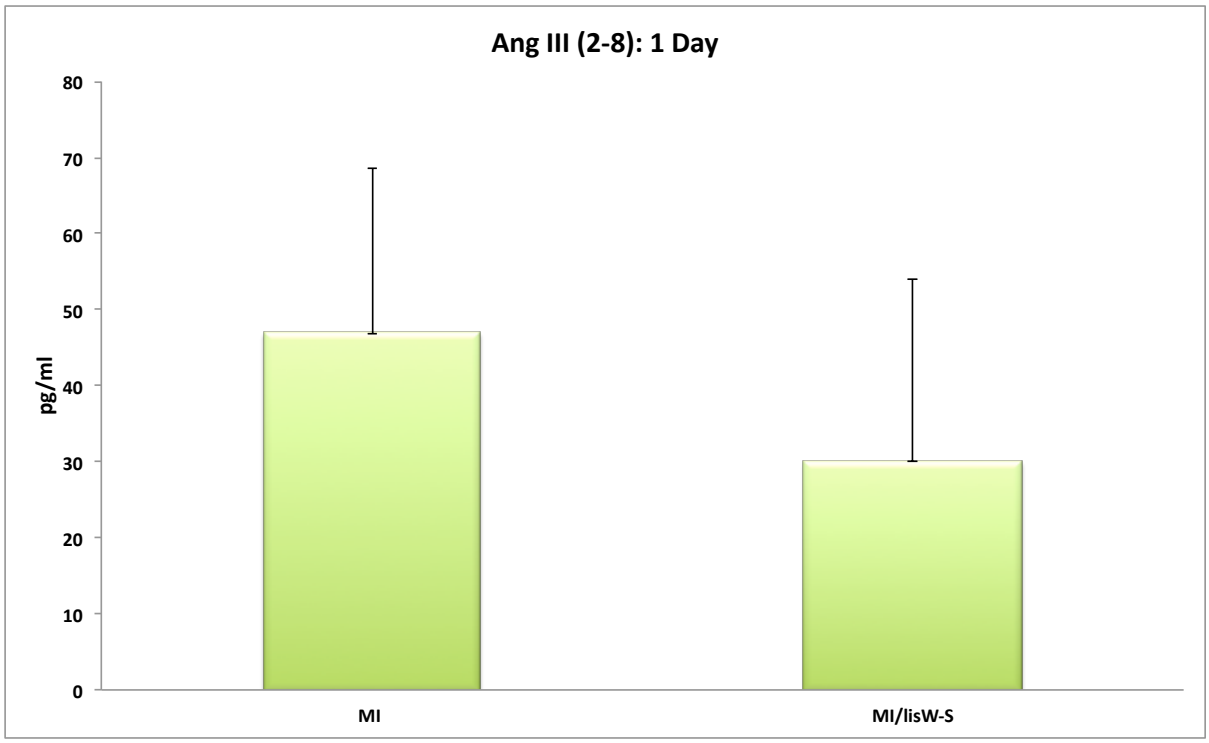


Figure 4.17 Mean Ang III plasma concentrations (pg/ml) at 1 day (p=NS)

4.5.3.2.6 ANG IV

Seven Days

Plasma concentrations for Ang IV measured seven days post-MI are presented in figure 4.18. The results show a similar trend to that observed following quantification of Ang III plasma levels. No significant difference was observed between the sham and MI groups (Sham = 6.55 ± 0.94 pg/ml; MI = 11.11 ± 2.13 pg/ml). However, a significant 2.4-fold elevation was observed in the MI/lisW-S-treated group relative to sham (MI/lisW-S = 16.83 ± 3.34; $p<0.05$), while the 1.5-fold elevation of this group relative to MI was not significant. Conversely, treatment with lisinoprol significantly decreased Ang IV plasma levels by 7.3-fold

relative to sham, 12.1-fold relative to MI and 18.3-fold relative to MI/lisW-S (MI/lisinopril = 0.92 ± 0.18 pg/ml; p<0.01).

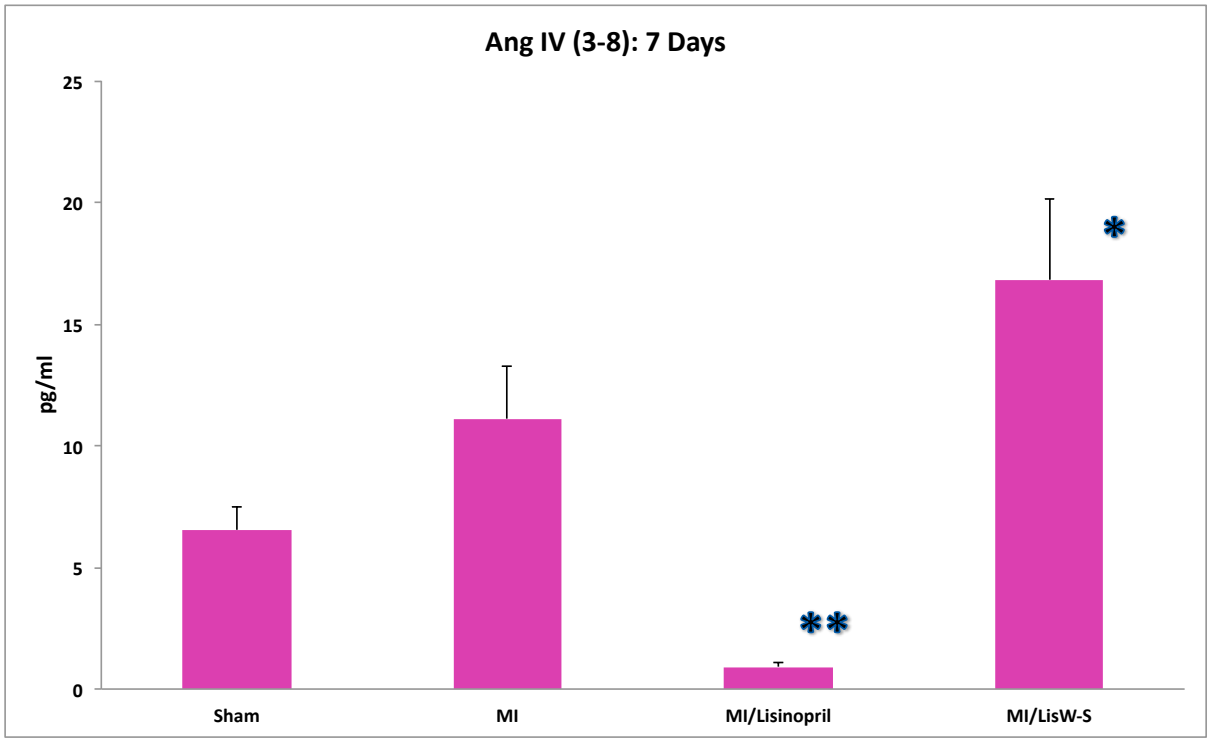


Figure 4.18 Mean Ang IV plasma concentrations (pg/ ml) at 7 days (p<0.01 vs. all groups; *p<0.05 vs. sham)**

One Day

Ang IV plasma concentrations one day post-MI are presented in figure 4.19, and clearly show no significant difference between treated and untreated groups (MI = 8.76 ± 1.19 pg/ml; MI/lisW-S = 5.7 ± 1.61 pg/ml; p=NS).

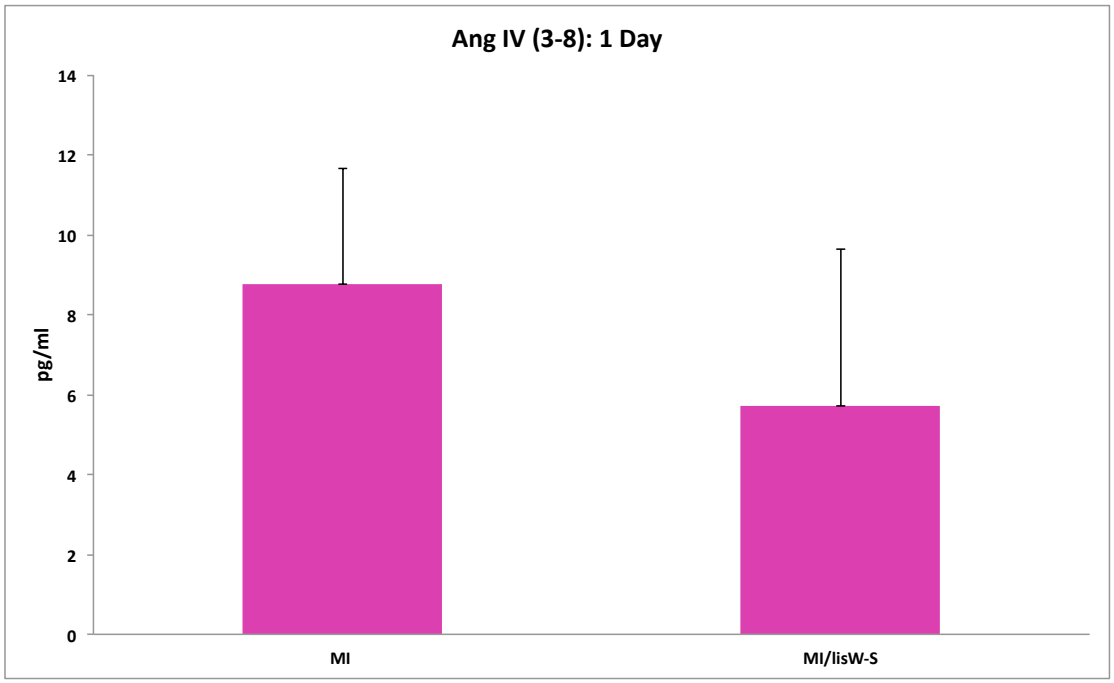


Figure 4.19 Mean Ang IV plasma concentrations (pg/ ml) at 1 day (p=NS)

4.5.3.2.7 ANG (1-7)

Seven Days

As seen in figure 4.20, Ang (1-7) in plasma was at non-detectable levels in the sham and MI groups at seven days post-MI. In the MI/lisinopril and MI/lisW-S-treated groups there were substantial levels of Ang (1-7) and these values were essentially identical (MI/lisinopril = 30.58 ± 3.39 pg/ml; MI/lisW-S = 31.03 ± 4.47 pg/ml ($p>0.05$)).

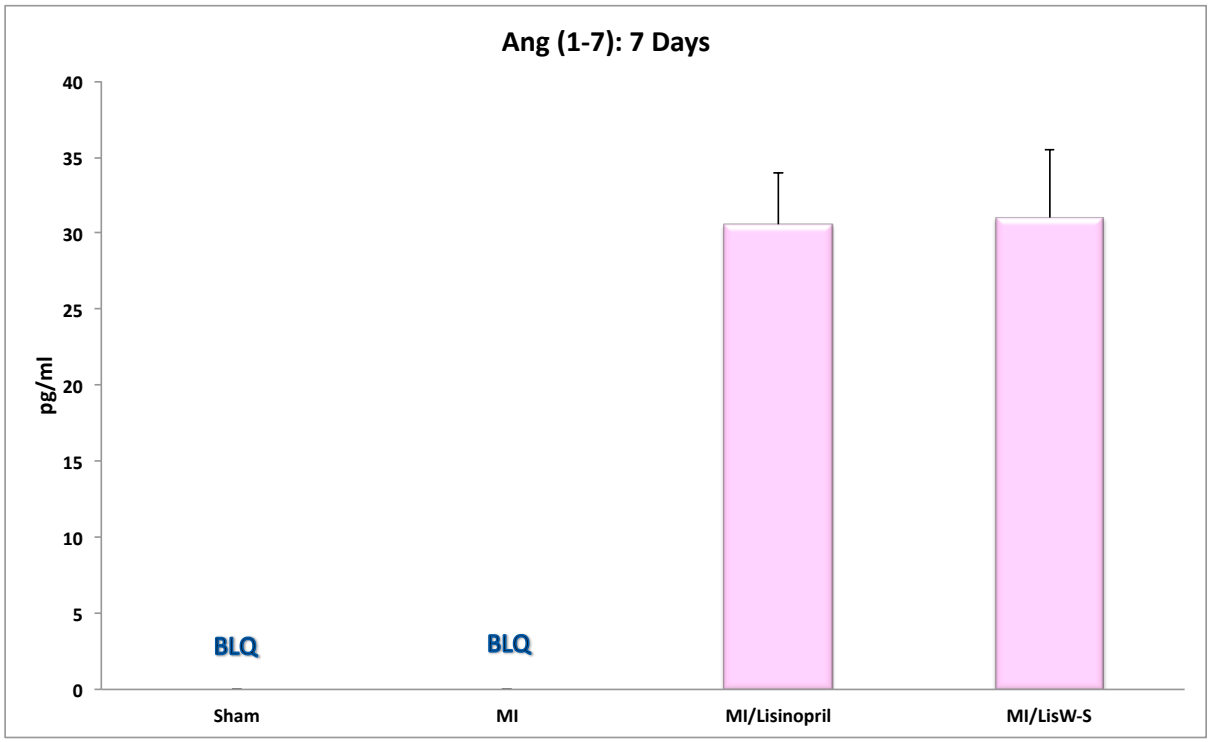


Figure 4.20 Mean Ang (1-7) plasma concentrations (pg/ml) at 7 days; BLQ = Below Limits of Quantification)

One Day

Mean plasma concentrations of Ang (1-7) one day post-MI are presented in figure 4.21. The levels for the two groups assessed in this study are very similar to those seen at seven days. The MI/lisW-S-treated group having appreciable levels (MI/lisW-S = 29.17 ± 8.31 pg/ml) and the MI group having non-detectable levels..

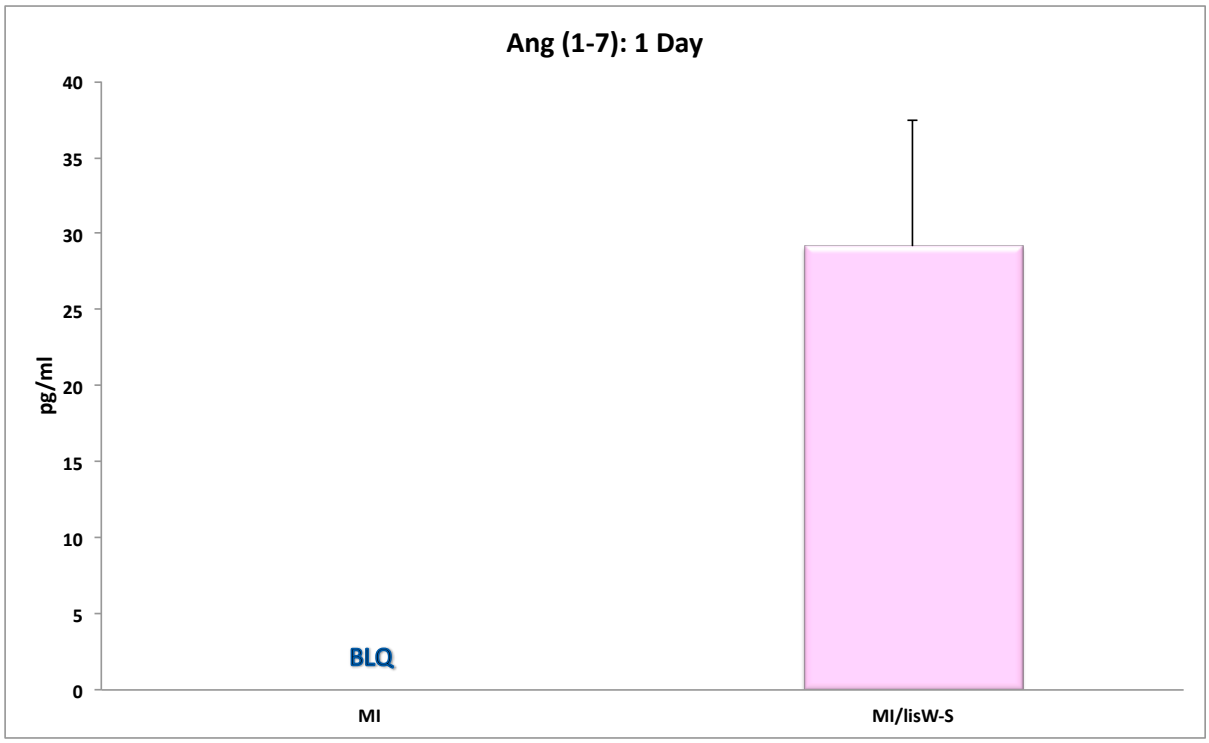


Figure 4.21 Mean Ang (1-7) plasma concentrations (pg/ml) at 1 day

4.5.3.2.8 ANG (1-5)

Seven Days

Figure 4.22 shows the relative mean plasma concentrations of Ang (1-5) at seven days post-MI. No significant difference was observed between the sham, MI and MI/lisinopril-treated groups (Sham = 7.62 ± 0.50 pg/ml; MI = 9.28 ± 1.15 pg/ml; MI/lisinopril = 9.78 ± 1.64 pg/ml). However, the MI/lisW-S-treated group showed a highly significant (MI/lisW-S = 25.95 ± 3.03 pg/ml; $p<0.05$) 2.8-fold elevation in plasma levels of Ang (1-5) relative to the MI group, and a 2.6-fold elevation relative to the MI/lisinopril-treated group.

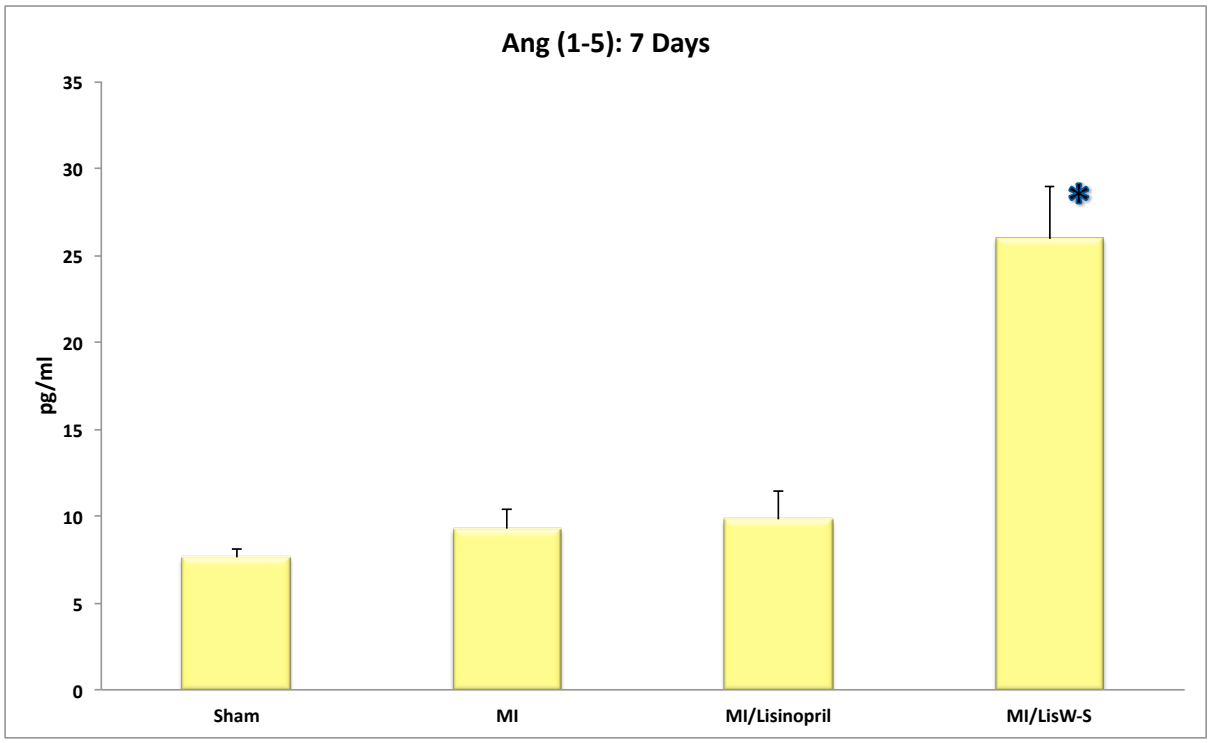


Figure 4.22 Mean Ang (1-5) plasma concentrations (pg/ml) at 7 days (γ p<0.01 vs. all groups)

One Day

Ang (1-5) plasma concentrations measured one day post-MI are presented in figure 4.23. A 2.3-fold elevation was observed in the MI/lisW-S-treated group relative to the untreated MI group (MI/lisW-S = 21.65 ± 3.33 pg/ml; MI = 9.45 ± 0.4 pg/ml; p<0.01).

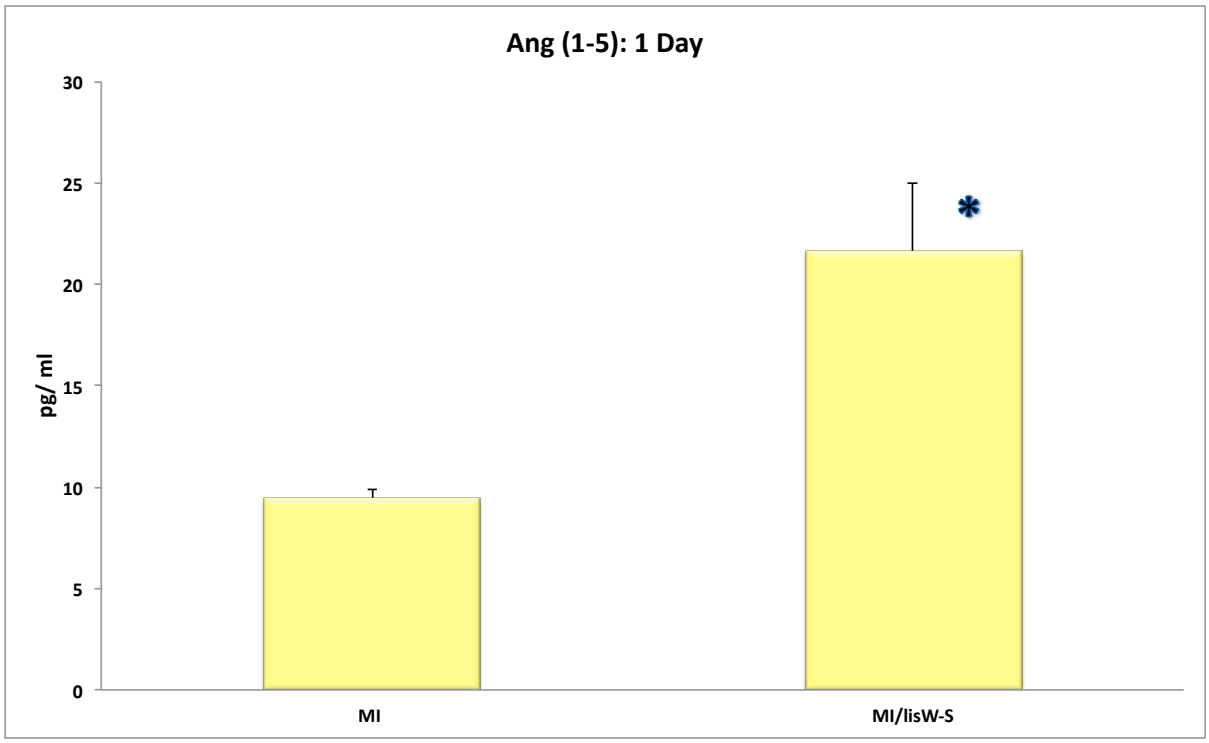


Figure 4.23 Mean Ang (1-5) plasma concentrations (pg/ ml) at 1 day

4.5.3.2.9 ANG (3-7)

Seven Days

Mean plasma concentrations of Ang (3-7) at seven days post-MI are presented in figure 4.24. This peptide was not quantifiable in both the sham and MI groups. Ang (3-7) levels in MI/lisinopril-treated and MI/lisW-S-treated groups were quantifiable (MI/lisinopril = 3.56 ± 0.43 pg/ml; MI/lisW-S = 3.73 ± 0.55 pg/ml) but extremely low.

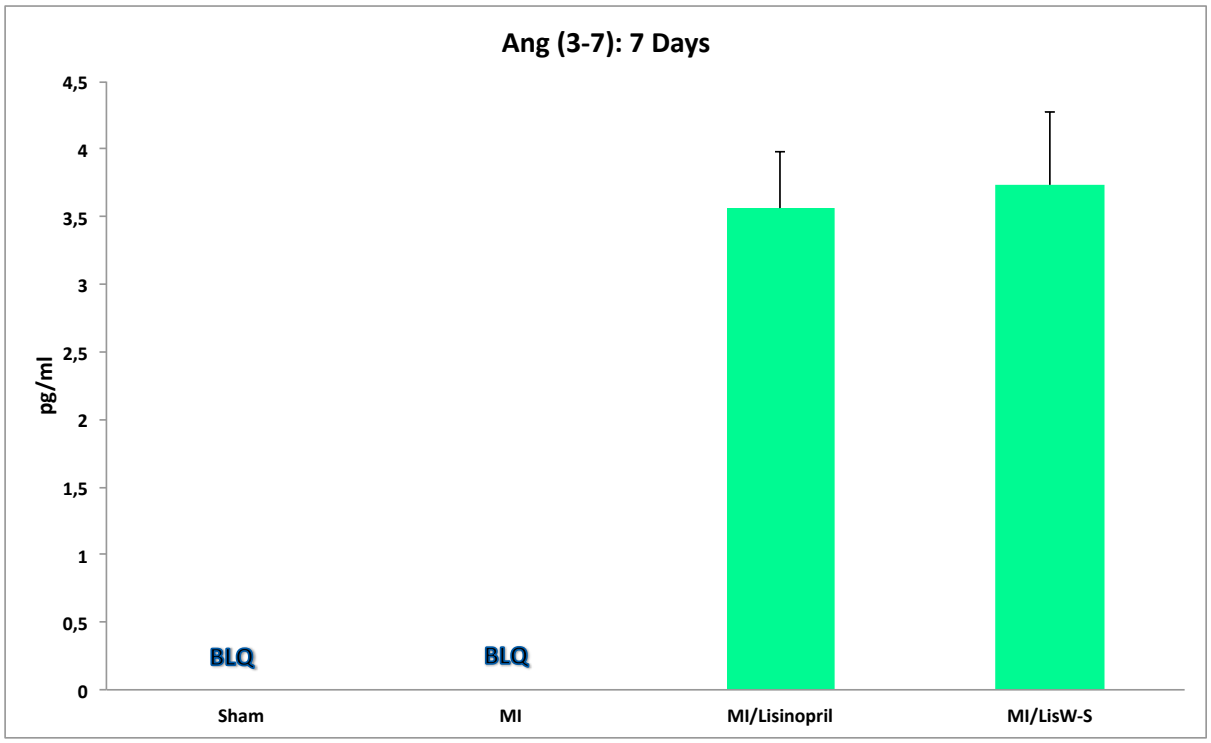


Figure 4.24 Mean Ang (3-7) plasma concentrations (pg/ml) at 7 days

One Day

All measurements performed for this peptide at one day post-MI were below the limits of quantification.

4.5.3.3 QUANTIFICATION OF BRADYKININ METABOLITES

The metabolism of the vasodilator bradykinin (1-9) was reviewed in Chapter 1, and detailed the hydrolysis of BK (1-9) to the products BK (1-7) and BK (1-5) by ACE. The RAS-fingerprinting technique was unable to quantify plasma levels of BK (1-9) itself although it was possible to measure BK (1-9)'s metabolites concurrently with angiotensin peptide analysis for each sample.

4.5.3.3.1 BK (1-7)

Seven Days

The results at seven days post-MI are presented below in figure 4.25. Plasma peptide levels were raised by 1.1-fold in the MI group relative to the sham group (MI = 4113.58 ± 164.71 pg/ml; Sham = 3627.71 ± 131.88 pg/ml; $p<0.05$). BK (1-7) was significantly elevated by approximately 2-fold relative to MI in both the MI/lisinopril and MI/lisW-S-treated groups (MI/lisinopril = 8462.46 ± 770.64 pg/ml; MI/lisW-S = 8403.54 ± 424.57 pg/ml; $p<0.05$ vs. MI and sham).

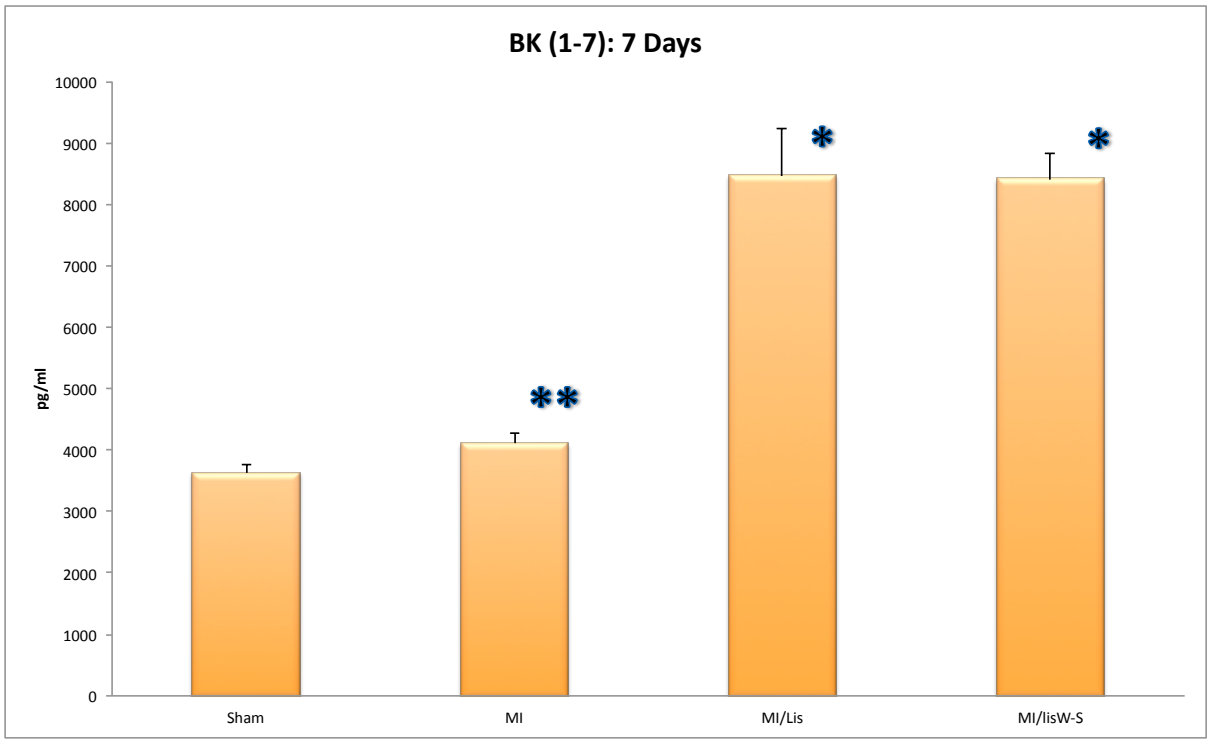


Figure 4.25 Mean BK (1-7) plasma concentrations (pg/ml) at 7 days (*p<0.01 vs. sham and MI, **p<0.05 vs. sham)

One Day

As shown in figure 4.26, plasma peptide levels at one day post-MI were significantly elevated by 1.6-fold in the MI/lisW-S-treated group relative to the untreated MI group (MI/lisW-S = 3377.06 ± 116.14; MI = 5284.76 ± 227.70; p<0.01).

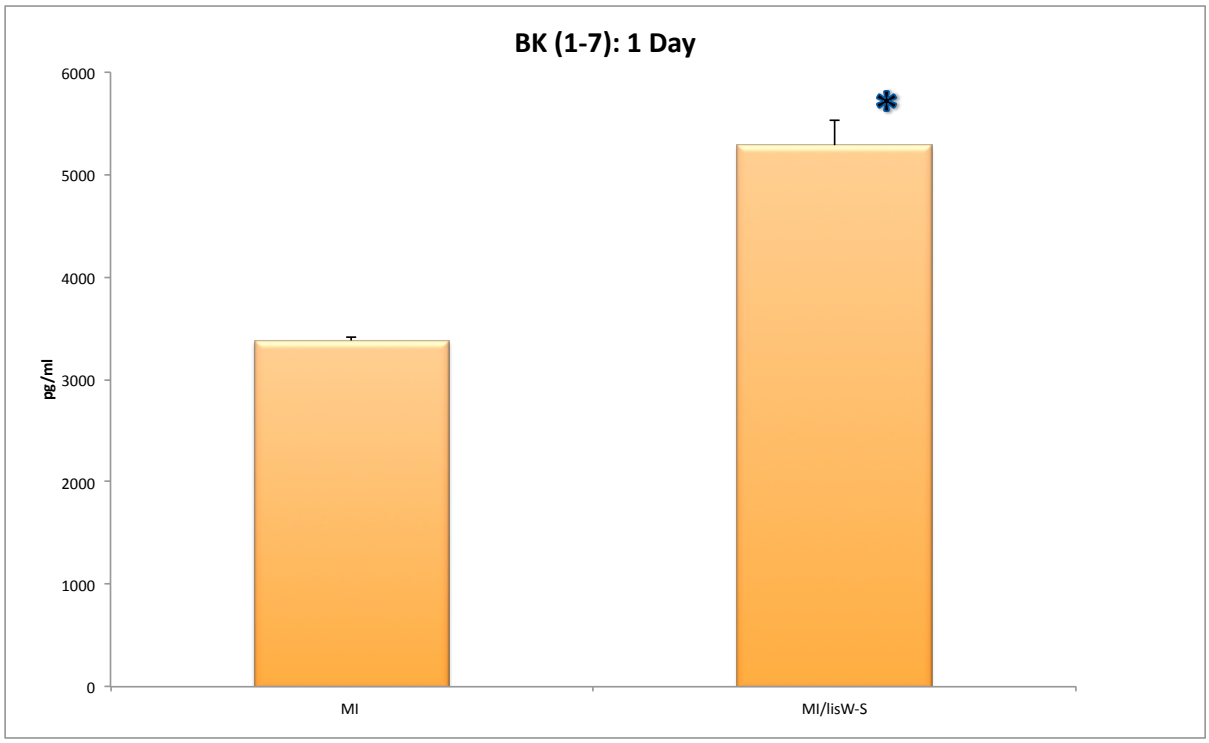


Figure 4.26 Mean BK (1-7) plasma concentrations (pg/ml) at 1 day (*p<0.01)

4.5.3.3.2 BK (1-5)

Seven Days

As shown in figure 4.27, when measured at seven days post-MI BK (1-5) displayed an upward trend with peptide levels significantly raised by 2.5-fold in the MI group relative to the sham group (MI = 1746.36 ± 437.26 pg/ml; Sham = 688.35 ± 62.64 pg/ml; $p<0.05$). Thereafter peptide levels were significantly increased by 2.1-fold in the MI/lisinopril-treated group (MI/lisinopril =3691.85 ± 554.69 pg/ml; $p<0.01$) and 3.4-fold in the MI/lisW-S-treated group (5880.87 ± 486.64 pg/ml; $p<0.01$) relative to MI. A highly significant 1.6-fold increase was observed in the MI/lisW-S-treated group relative to MI/lisinopril ($p<0.01$).

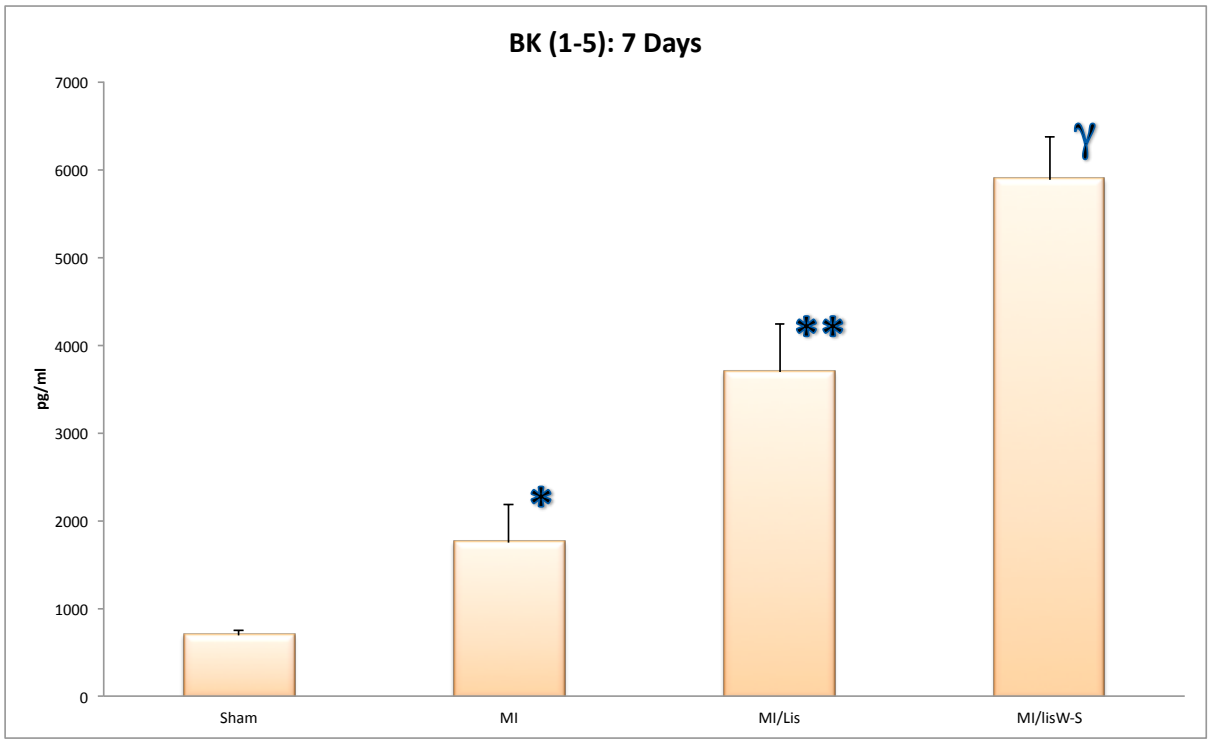


Figure 4.27 Mean BK (1-5) plasma concentrations (pg/ml) at 7 days (*p<0.05 vs. sham, **p<0.01 vs. MI, γ p<0.01 vs. MI/lisinopril)

One Day

BK (1-5) levels at one day post-MI are presented below in figure 4.28. A significant 5-fold elevation in plasma peptide levels was observed in the MI/lisW-S-treated group relative to the MI group (MI = 390.94 ± 38.72 pg/ml; MI/lisW-S = 1988.06 ± 250.60 pg/ml; p<0.01).

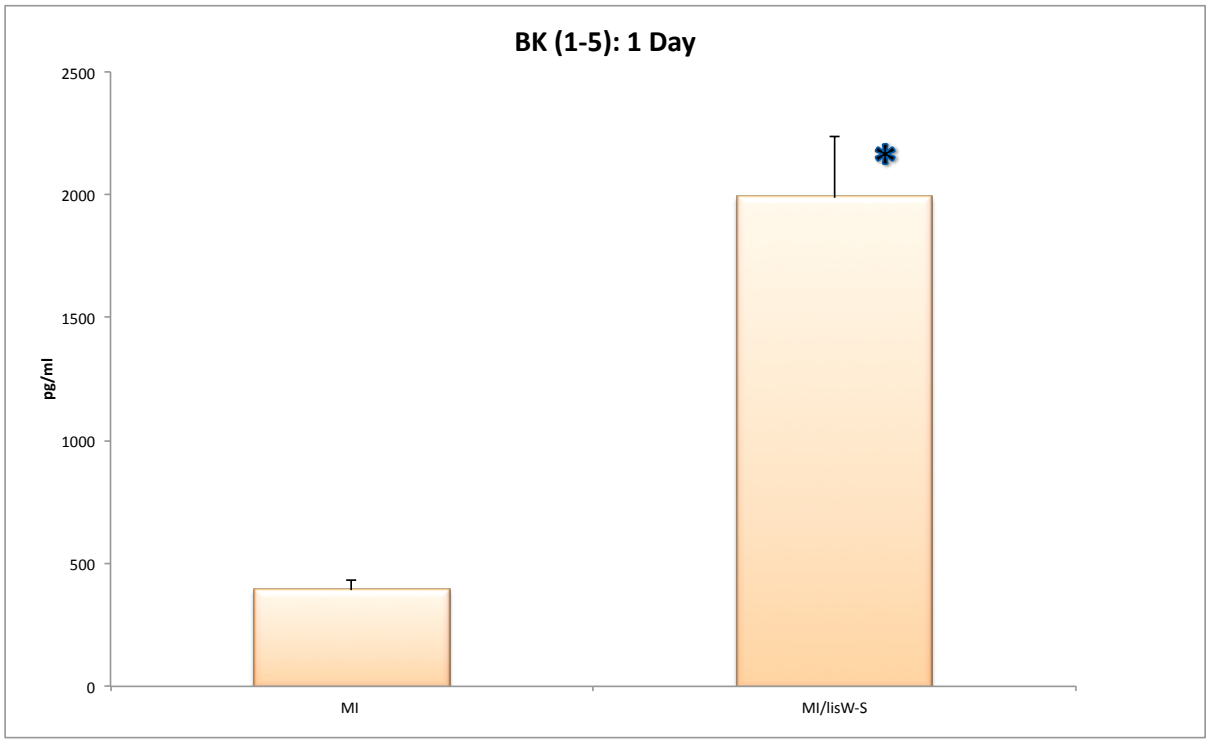


Figure 4.28 Mean BK (1-5) plasma concentrations (pg/ml) at 1 day ($p<0.01$)

4.6 DISCUSSION

RAS fingerprinting and EIA techniques were successfully utilized in this study to determine the effects of domain-selective and non-selective ACE inhibition on plasma peptide levels at two different timepoints post-MI in a rat model of infarction. A discussion of the peptide quantification results previously presented in this chapter now follows; both in their individuality as well as in the context of their larger roles – either within, or in relation to – the

RAS cascade. A summary of the results in this chapter is presented below in figure 4.29 for reference.

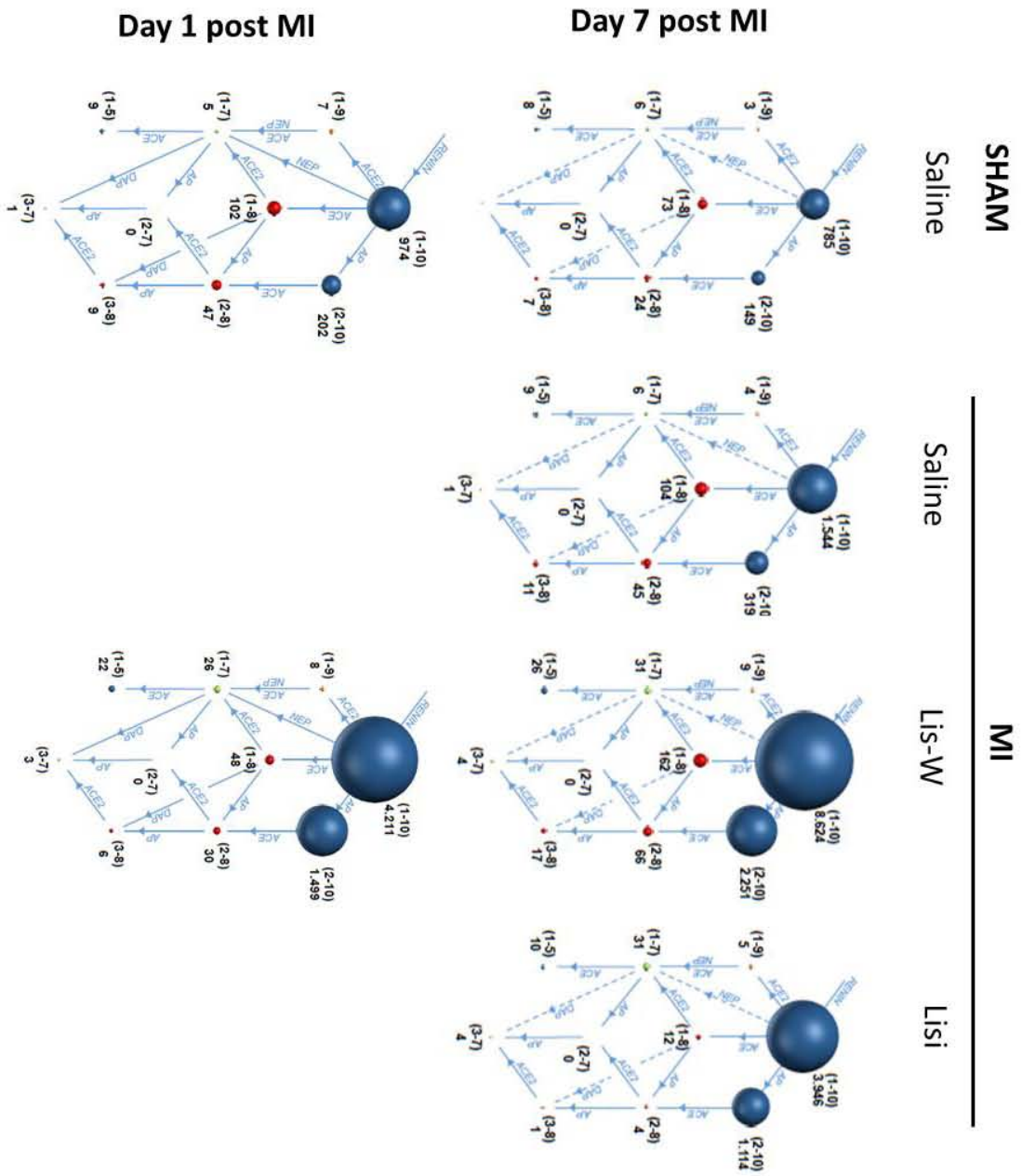


Figure 4.29 Summary of RAS fingerprint results for all treatment groups and timepoints

4.6.1 AcSDKP

AcSDKP is not a product of the RAS system although it is metabolised exclusively by the N-domain of ACE (Rousseau et al.1995). Previous studies in both humans (Azizi et al. 1996) and rats (Cavasin et al. 2004) have demonstrated that administration of a non-selective ACE inhibitor blocks hydrolysis of AcSDKP and results in a 4 to 5-fold elevation of plasma peptide levels (Azizi et al. 1996). There is also evidence that increased levels of this antifibrotic peptide appear to have positive therapeutic effects on the pathophysiology of heart disease in some species. Administration of AcSDKP above endogenous levels was shown to reverse cardiac fibrosis and inflammation following induction of MI in rats – independent of both ACE inhibitor therapy and haemodynamic changes (Yang et al. 2004). Additionally, increased plasma levels of this peptide have been shown to reduce cardiac fibrosis in uninfarcted hypertensive rats (Peng et al. 2003) while reduced basal levels promote cardiac and renal fibrosis in normal rats (Cavasin et al. 2007). The sum of the research thus far points to positive antifibrotic effects above endogenous levels and negative profibrotic effects below basal levels.

The interruption of ACE-mediated AcSDKP metabolism in this study was clearly demonstrated by the 3-fold elevation of this peptide observed in the MI/lisinopril-treated group and measured by EIA. Conversely, no such increase of the non-hydrolysed peptide was observed in the sham, MI or MI/lisW-S-treated groups. The C-domain selective ACE inhibitor lisW-S was predicted to have no effect on endogenous levels due to a lack of interaction with the N-domain (Watermeyer et al. 2010). No change in AcSDKP plasma levels in the MI/lisW-S-treated group was observed relative to either the sham or MI and it is possible to conclude that while elevation of plasma peptide levels – and the subsequent antifibrotic effects – may be inhibited by lisW-S, this treatment should not result in negative profibrotic outcomes as endogenous levels of AcSDKP were not significantly altered.

Conversely, the lack of AcSDKP elevation seen with C-domain selective ACE inhibition could also prove to have an adverse effect on the positive remodeling and associated antifibrotic outcomes observed with non-selective ACE inhibition. It has been shown that

AcSDKP must at least partly mediate the antinflammatory and antifibrotic effects in cardiac and renal tissue observed with ACE inhibition. Studies in normotensive rats treated with an AcSDKP-specific monoclonal antibody have demonstrated that endogenous levels of AcSDKP do not participate in regulating cardiac collagen content, while exogenous administration of AcSDKP achieves the same elevated plasma peptide levels as treatment with a non-selective ACE inhibitor (Peng et al. 2005). Interestingly, these antifibrotic effects have also been observed in rats treated with Ang II to induce hypertension and exogenous AcSDKP despite continued systemic hypertension and resultant aortic hypertrophy (Lin et al. 2008), which further validates a different mechanism of action unrelated to the anti-hypertensive effects of ACE inhibitor therapy, but rather reliant on elevated plasma levels of AcSDKP. The withdrawal of the positive effects of elevated AcSDKP in the presence of non-domain specific ACE inhibitor therapy is therefore a concern in the context of lisW-S as a therapeutic option for MI and warrants further exploration of the implications for post-MI remodeling in future studies. It should be noted that in humans AcSDKP is predominantly found in the plasma and white blood cells (Pradelles et al. 1990), thus presenting the possibility of exogenous administration of AcSDKP as an option in conjunction with lisW-S treatment.

The results of the ACSDKP EIA were thus encouraging as they provided the first *in vivo* data in support of the C-domain specificity of lisW-S, thus reinforcing the findings of the *in vitro* studies in Chapter 2.

4.6.2 Angiotensin I

The results by RAS-fingerprinting showed a significant 2-fold elevation in plasma Ang I levels in the untreated MI group relative to the sham at seven days post-MI. Similar results were observed in a study conducted by the Campbell group in which both plasma renin activity and plasma Ang I levels were measured in a rat model at two and three days post-

infarction (Duncan et al. 1997). The 30 to 40% elevation of plasma Ang I observed in that study was shown to correlate with increases in plasma renin concentrations, thus confirming that elevated Ang I reflects the compensatory response by the RAS to MI. While plasma renin activity was not measured in the present study, based on the literature it is safe to assume the increase observed in the present study reflects this compensatory response.

Interestingly, when the results obtained in pg/ml by RAS-fingerprinting in this study were converted to femtamoles/ml for comparative purposes within the literature, the plasma Ang I concentrations measured in the untreated sham and MI groups at seven days post-MI were approximately 7 to 10-fold higher than those reported by Campbell's group in various studies at the same timepoint (Campbell et al. 1994; Duncan et al. 1996; Duncan et al. 1997). Concurrently, this discrepancy may be attributable to the inhibitor cocktail provided by Apeiron Biologica (described in section 4.6.3.2), which may allow for greater preservation of angiotensin peptides following whole blood collection than the cocktail described by that group (A. M. Duncan et al. 1996) (A. M. Duncan et al. 1997). A methodological argument for overestimation in the present study could be made in the case of inadequate enzyme inhibition following blood collection. As discussed by Campbell *et al.* (Campbell et al. 2004), Ang I generation and consequent Ang II formation by endogenous lysozymes in a tissue sample is a possible outcome of poor sample preparation. To our knowledge the collection methods and Apeiron Biologica's inhibitor cocktail used in this study are more than sufficient for highly accurate peptide quantification by the RAS fingerprinting technique. As the inhibitor cocktail is currently the subject of a pending patent application, data relating to its inhibitory potential can not be given at present. However, Apeiron Biologica has communicated that the cocktail is an effective inhibitor when tested in plasma spiked with reference angiotensin peptides and incubated at 37^{o}C for several hours. In the present study blood samples was collected within a 90 second window into a chilled, heparinized tube containing the inhibitor cocktail and immediately placed on ice prior to snap-freezing within 60 minutes from the time of collection. In addition Apeiron has confirmed that in their own studies the loss of lysozyme-generated Ang II within a sample to the glass or plastic material of the collection tube is minimal. Thus, it appears unlikely that the results presented above are an overestimation of *in vivo* peptide levels at the time of blood collection.

Relative to the MI group, Ang I plasma levels were elevated by 2.6-fold in the MI/lisinopril-treated group. A significant increase in this peptide was anticipated as ACE inhibition prevents the hydrolysis of Ang I and the consequent formation of Ang II (Brown et al. 1998), thus activating the negative feedback mechanisms within the RAS. As described previously, renin secretion is dependent on Ang II plasma levels and stimulation of AT_1 receptors (DeMello et al. 2009). In the case of ACE inhibition, Ang II plasma levels are decreased and receptor stimulation is inhibited (Siragy et al. 2005), leading to upregulation of renin secretion and Ang I generation in an effort to restore endogenous levels of Ang II. The Campbell group has previously assessed the effects of ACE inhibition on angiotensin and bradykinin peptide levels in a rat model of MI. In that study, the ACE inhibitor perindopril was administered at a dose of 2mg/kg/day via the animals' drinking water from 48 hours following infarction for a period of twenty-six days, at which point angiotensin peptides were quantified by radioimmunoassay (Duncan et al. 1996). Perindopril belongs to the same dicarboxylate-containing group of ACE inhibitors as enalapril, ramipril, lisinopril and lisW-S (Brown et al. 1998) and thus those results are comparable to ours (despite differing timepoints). In the present study, a dose of 1 mg/kg/day was administered by miniosmotic pump infusion, resulting in a 2.6-fold increase in Ang I in the MI/lisinopril-treated group relative to the MI group at seven days post-MI. In their study, the Campbell group observed a 6-fold elevation in plasma Ang I levels at twenty-eight days post-MI following oral administration of perindopril.

In another study, that group administered both lisinopril and perindopril to non-infarcted rats for a period of seven days followed by quantification of angiotensin peptide levels by radioimmunoassay (Campbell et al. 1994). That study concluded that perindopril possessed approximately 10-fold the potency of lisinopril with regards to its effects on angiotensin plasma levels, which could possibly account for the differences seen with regards to elevation of Ang I relative to MI between our study and Duncan *et al*. (1996). However, there is no definitive study that has found any one ACE inhibitor to be therapeutically superior over another, although perindopril has been shown to have a slightly higher lipophilicity than lisinopril (Furberg. 2000). A recent randomized study with human subjects measured the effects of the hydrophilic ACE inhibitor lisinopril and the lipophilic ACE inhibitor perindopril on

circulatory Ang II levels and found no discernible difference in their efficacy (Ruzicka et al. 2010). While studies in rats have shown that lipophilic ACE inhibitors prevent adverse cardiac remodeling to a greater degree than hypophilic ACE inhibitors (Ruzicka & Leenen 1995; Ruzicka et al. 1995), this concept has not yet been satisfactorily proven and the observations of the Campbell group in this respect must be carefully considered.

A significant increase in Ang I levels was observed in the MI/lisW-S-treated groups at both one and seven days post-MI relative to both the untreated MI and MI/lisinopril-treated groups. When peptide levels were quantified by RAS-fingerprinting at seven days post-MI, treatment with lisW-S raised Ang I by 5.6-fold relative to MI. At one day post-MI, a 4.3-fold increase in Ang I was observed in the MI/lisW-S-treated group relative to MI. The results obtained for the seven day timepoint are closely comparable to those of the Campbell group's, which showed an approximate 6-fold increase in Ang I with perindopril treatment relative to untreated MI at 28 days (Duncan et al. 1996).

The 2.6-fold increase in the MI/ lisinopril-treated group in this study amounts to less than half the fold increase observed by the Campbell group with perindopril in a similar study. As we believe our sample collection and quantification methods are sound, the difference may be attributable to the timepoints utilized in each study. The Campbell group conducted a long-term study in which angiotensin peptides were measured at 28 days post-MI, a timepoint by which necrotic myocardium has been completely replaced by collagen in the rat myocardium (Cleutjens et al. 1995). Comparatively, at 7 days post-MI, collagen in the myocardium is only microscopically detectable and the heart is in a state of inflammatory flux. The development of heart failure as a consequence of cardiac remodeling is thoroughly reviewed in Section 1.2.

It should be noted however that when the results of the MI/lisW-S-treated group at 7 days are taken together with the significant elevation observed after just 24 hours of treatment in the present study, the results obtained by RAS-fingerprinting strongly suggest that the RAS compensatory mechanism initiated by ACE inhibition is significantly more pronounced

following treatment with lisW-S than lisinopril. There is a possibility that this could be related to the potency of the domain-selective ACE inhibitor as was suggested to be the case with administration of perindopril in the aforementioned Campbell study. Further reasons for this and possible implications will be discussed in the context of the Ang II measurements presented in the next section.

4.6.3 ANGIOTENSIN II

Ang I is hydrolysed by the C-domain of ACE to form the vasoconstrictor Ang II (Erdos et al. 1967). Both non-selective and C-domain selective ACE inhibition may reduce Ang II levels below those of an untreated MI control group. However, it has been reported in the literature that plasma ang II plasma levels may not be reduced due to upregulation of ANG-I levels via the upregulation of renin activity (Yamagishi et al. 1993; Duncan et al. 1996).

In the first instance, the relatively inexpensive and rapid method of EIA was utilized to provide preliminary results with regards to the effects of ACE inhibition on plasma Ang II levels at seven days post-MI. The untreated MI group showed a trend towards an increase relative to the sham group, presumably as a result of RAS activation. The MI/lisinopril-treated group exhibited a significant reduction in plasma Ang II levels, which was as expected based on previous studies in both humans (Dickstein 1987; Gomez et al. 1987) and rats (Forest et al. 2005; Wollert et al. 2010). However, an increase in plasma Ang II levels was measured in the MI/lisW-S-treated group that significantly exceeded the levels measured in the sham, MI and MI/lisinopril-treated groups. The results of this assay compelled us to further explore the effects of lisW-S on the key components of the RAS by utilizing the highly sensitive method of RAS-fingerprinting.

At seven days post-MI, a non-significant increase of approximately 40% was observed in plasma Ang II levels in the MI group relative to the sham. As previously stated, in the sham and MI groups a 7 to 10-fold difference in plasma Ang I levels was observed between the present study and those reported by the Campbell group. Interestingly, the Ang II plasma

levels observed for those treatment groups in the present study differ by only 1.5 to 2-fold from those previously reported by the Campbell group (Campbell et al. 1994; Duncan et al. 1996; Duncan et al. 1997).

In the MI/lisinopril-treated group, Ang II generation was successfully inhibited and Ang II plasma levels significantly reduced by 8.8-fold relative to MI. This clearly demonstrates the high efficacy of lisinopril as an ACE inhibitor under pathophysiological conditions in a rat model. Furthermore, in a study conducted by the Campbell group (Campbell et al. 1994) in which uninfarcted rats were orally administered (via drinking water) a wide range of doses of the ACE inhibitors lisinopril and perindopril, the highest lisinopril dose investigated (0.156 mg/kg/day) did not elicit a decrease in plasma Ang II, while perindopril doses above 1.4 mg/kg/day resulted in a 2.2-fold reduction in plasma Ang II levels relative to untreated control animals. In that study the Campbell group also concluded that lisinopril had approximately one tenth the potency of perindopril. As the present study delivered a lisinopril dose equal to 1 mg/kg/day, it appears that administration by miniosmotic pump infusion is an extremely effective method of drug delivery.

In the MI/lisW-S-treated group, a non-significant trend was observed towards an estimated 50% increase in Ang II levels relative to the untreated MI group seven days post-MI. This was in accordance with the results obtained by EIA quantification. Intriguingly, RAS fingerprinting at one day post-MI showed a contrasting and significant 2.1-fold decrease in plasma Ang II relative to the MI group and in response to treatment with lisW-S. This was despite significantly elevated levels of plasma Ang I at the same time point. In order to explain these results, the efficacy of lisW-S at inhibiting ACE was explored by analysing the ratio of Ang II produced relative to the Ang I substrate. This ratio reflects ACE activity in the plasma and is discussed in the following section.

4.6.4 ANG II/ ANG I

The inhibition of conversion of Ang I substrate to Ang II product is the primary goal of ACE inhibition and thus analysis of the Ang II/ Ang I ratio is useful for gaining a true reflection of the inhibitory activity of both lisinopril and lisW-S *in vivo*. This method of ACE activity quantification was successfully demonstrated by the Campbell group (Campbell et al. 1991) and more recently validated by Claasen at al. (Claassen et al. 2013). One week after infarction, the efficacy of lisinopril in a rat model was clearly demonstrated by a substantial decrease in Ang II/Ang I values relative to the untreated MI group. This result was not unexpected, as lisinopril was administered at a dosage achieving close to 100% ACE inhibition (as described in Chapter 2).

In addition, ACE inhibition by lisW-S was clearly demonstrated at both one and seven days post-infarction, where Ang II production from Ang I was decreased by 8-fold and 3.4-fold respectively relative to MI. These results confirm that significant inhibition was achieved despite the low bioavailability of lisW-S as well as delivery of this compound at a dosage that achieved a maximum of 60% ACE inhibition.

A similar study by the Campbell group was previously described in which both lisinopril and the highly potent ACE inhibitor perindopril were administered to uninfarcted rats for seven days and angiotensin peptide levels quantified by radioimmunoassay (D J Campbell et al. 1994). Interestingly, perindopril administered by drinking water at a dosage of 0.156 mg/kg/day achieved a 10-fold reduction in Ang II/Ang I, close to the inhibition observed at one day post-MI with lisW-S (which was administered at a dosage of 1 mg/kg/day).

However, at the seven day timepoint the inhibitory effect of lisW-S on the Ang II/ Ang I ratio was reduced by approximately 1.5-fold relative to that seen at one day, despite the superior route of administration by miniosmotic pump infusion. This loss of inhibitory efficacy *in vivo* could be attributed to the decrease in lisW-S plasma levels observed one week after subcutaneous miniosmotic pump implant (as described in section 3.4.5). In that instance,

lisW-S plasma concentrations fell by almost 40% relative to peptide plasma levels one day after implant. In addition, reduced efficacy in the MI/lisW-S-treated group seven days post-MI might also be a consequence of the elevated Ang II plasma levels measured at this time point.

A second study by the Campbell group (Duncan et al. 1996) performed peptide quantification twenty-eight days post-MI, and found that orally administered dosages of 0.2 and 2 mg/kg/day perindopril resulted in reduction of the Ang II/Ang I ratio by 7- and 14-fold respectively. It appears that the results obtained in that study one month post-MI with the lowest perindopril dose are most similar to the results found in the present study, one day post-MI and at a lisW-S dose five times that of perindopril.

4.6.5 ANG (1-7) AND THE CARDIOPROTECTIVE AXIS

The cardioprotective peptide Ang (1-7) is formed primarily by the ACE2-mediated hydrolysis of Ang II (Donoghue et al. 2000) although it is also generated directly from Ang I by the hydrolytic action of prolylendopeptidase (PEP), prolylcarboxypeptidase (PCP) (Welches et al. 1991) and the previously described neutralendopeptidase (NEP) (Yamamoto et al. 1992). Both ACE2 and NEP concurrently hydrolyse Ang II, thus fulfilling a counter-regulatory antihypertensive function within the RAS.

Endogenous Ang (1-7) levels are extremely low under pathophysiological conditions in the absence of ACE inhibition (Iyer et al. 1998; Ferrario et al. 2005) and this was clearly demonstrated in the present study whereby the highly sensitive RAS fingerprinting methodology was unable to quantify plasma peptide levels in the untreated MI group at either one or seven days post-MI.

Ang (1-7) is associated with a number of cardioprotective effects as previously described (section 1.5.1.1) and upregulation of this peptide is desirable following an MI event. The upregulatory effect of ACE inhibition on Ang (1-7) levels is well-documented, and this was observed in both the MI/lisinopril and MI/lisW-S-treated groups. It has been shown that the augmentation of circulatory levels of Ang (1-7) that occurs in conjunction with ACE inhibition is most likely a consequence of decreased ACE-mediated peptide metabolism both *in vitro* (Chappell et al. 1998) and *in vivo*, where ACE inhibitor administration was found to significantly extend the half-life of Ang (1-7) and thus endogenous levels in both normotensive and hypertensive rats (Kazuo et al. 1998). In addition, Allred *et al.* showed that ACE was primarily responsible for Ang (1-7) metabolism in the pulmonary tissues in normotensive rats (Allred et al. 2000)

Studies have shown that NEP appears to be largely responsible for the presence of circulatory Ang (1-7) *in vivo*. In a study by Yamamomoto *et al* (1992), both spontaneously hypertensive and control rats were administered the ACE inhibitor enalapril, resulting in elevated levels of Ang I and Ang (1-7) (Yamamoto et al. 1992). However, when enalapril was administered in conjunction with an NEP inhibitor, upregulation of circulatory Ang (1-7) levels was blocked.

It has been previously stated that Ang (1-7) was found to be primarily hydrolysed by the N-domain of human ACE (P. Deddish et al. 1998) and there is evidence to suggest that the opposite may be true for rat ACE. Allred *et al*. (2000) studied the kinetic efficiency of both rat somatic ACE (equipped with both the C- and N-domain active sites) and rat testis ACE (equipped with only one active site equivalent to the C-domain) at metabolizing Ang (1-7) *in vitro* and found them to be identical, suggesting that the C-domain could be primarily responsible for the hydrolytic breakdown of this peptide. *In* vitro studies in the canine model by Chappell *et al*. further suggested that the C-domain of ACE might be predominantly responsible for the metabolism of Ang (1-7) (Chappell et al. 1998). However, a study by Andrade *et al*. delivered conflicting results suggesting that an N-domain fragment of rat ACE was hydrolytically more efficient at metabolizing Ang (1-7) than the full ACE peptide

(Andrade et al. 1998). The concept of species-specific domain-selectivity in both inhibitors and substrates was first introduced in Chapter 2 (Sections 2.1 and 2.2.1) and the domain-specificity of rat ACE remains undetermined.

In the present study, Ang (1-7) levels were clearly upregulated by ACE inhibition and the values obtained by RAS-fingerprinting were identical for both the MI/lisinopril and the Mi/LisW-S-treated groups. However, at both one and seven days post-MI, a significant elevation of Ang (1-5) levels was observed in the MI/lisW-S-treated group relative to the sham, MI and MI/lisinopril-treated groups. As previously described, Ang (1-5) is an ACE-dependent metabolic breakdown product of Ang (1-7) (Yamada et al. 1998). A case could therefore be made for predominantly N-domain ACE-mediated metabolism of Ang (1-7) in the rat, as the C-domain specificity of lisW-S should accede efficient N-domain-mediated hydrolysis of this peptide under ACE inhibitory conditions.

Simultaneously, a lack of conclusive evidence with regards to domain-specificity of the Ang (1-7) substrate in the rat model calls for the exploration of alternative explanations. Elevated levels of Ang II, Ang (2-10), Ang III (2-8), Ang IV (3-8) and Ang (1-5) could be a consequence of peptide 'flow through' in the RAS cascade as a result of the large increase in Ang I substrate in the MI/lisW-S-treated group. The results obtained by RAS-fingerprinting clearly showed significant increases in all of these peptide levels at both one and seven days post-MI following administration of lisW-S. Elevated levels of Ang (2-10) and Ang IV (3-8) could potentially have positive vasodilatory (Swanson et al. 1992; Yoshida et al. 1996), anti-hypertrophic (Sim et al. 1998) and anti-inflammatory (Rufaihah et al. 2006) effects on the cardiovascular system following MI. Ang III (2-8) and Ang II, however, are associated with vasoconstrictive (Hall et al. 1981), profibrotic (Lijnen et al. 2001; Wang et al. 2010) and proinflammatory (Ruiz-Ortega et al. 2000) effects, which are likely to contribute towards the pathology of heart failure following MI.

4.6.6 BRADYKININ

The non-selective ACE inhibitor lisinopril is a known and potent inhibitor of BK (1-9) metabolism and results in an elevation of this kinin in the plasma (Tom et al, 2003). As reviewed in section 1.6.2, the negative side-effects of non-selective ACE inhibitors are attributed to this accumulation of BK (1-9). Thus, it was hypothesized that BK (1-9) plasma levels in the MI/lisW-S-treated group would be approximately 50% lower than those of the MI/lisinopril-treated group due to inhibition of only one ACE domain. Despite the high sensitivity of the RAS fingerprinting technique, BK (1-9) levels could not be quantified in the samples provided by this study. However, it was possible to quantify BK (1-7) and BK (1-5), two principal breakdown products of ACE-mediated BK (1-9) metabolism.

A significant and closely comparable increase in BK (1-7) levels was observed in both ACE inhibitor-treated groups at seven days post-MI, possibly suggesting a similar degree of ACE inhibition. However, analysis of angiotensin peptide quantification by RAS fingerprinting clearly shows that lisW-S is a less effective inhibitor of ACE than lisinopril, and so inhibition of ACE-mediated BK (1-9) metabolism would not be expected to be so closely related between these two treatment groups. However, in previous studies the Campbell group has demonstrated a 'plateau effect' of the ACE inhibitor perindopril on BK (1-9) degradation (D J Campbell et al. 1994) at much lower concentrations of perindopril than those needed to reach a plateau in ACE degradation of Ang I. Thus both inhibitors used in this study may be working at maximal levels for inhibition of BK (1-9) hydrolysis.

However, plasma levels of the terminal degradation product BK (1-5) presented a different picture of BK (1-9) metabolism in all groups in this study. These levels were significantly elevated by almost 2-fold in the MI/LisW-S-treated group relative to that in the MI/lisinopril-treated group. This suggests that despite inhibition of the C-domain, BK (1-9) degradation may still be occurring via the N-domain in the lisW-S treated animals. Clearly data needs to be obtained on the actual levels of BK (1-9) to gain true clarity on this issue.

4.6.7 DOES LISW-S INHIBIT ACE?

The work presented in this chapter offers a large amount of data and permits a thorough investigation of the interplay between the RAS system, myocardial infarction and the ACE inhibitory agents lisinopril and lisW-S. In particular, the results provided by the RAS-fingerprinting technique provide unique insight into the complex mechanisms and reactions of the many counter-regulatory components of the RAS in response to both non-selective and C-domain-selective ACE inhibition under pathophysiological conditions in the rat model.

Activation of the RAS system after an MI event leads to upregulation of renin synthesis and release (Duncan et al. 1997; Ocaranza et al. 2006) – the effects of which are seen throughout the RAS cascade in this group. Although renin plasma levels were not measured in this study, the relationship between the activated RAS and upregulation of renin activity is sound. Upregulation of Ang I is reflected in the elevated levels of peptides further down the cascade – in particular Ang II, Ang (2-10) and Ang III (2-8). RAS activation following MI had no effect on the 'alternative' Ang (1-9)/ Ang (1-7)/ Ang (1-5) axis and did not upregulate the cardioprotective peptide Ang (1-7).

The RAS fingerprint of the clinical ACE inhibitor lisinopril provides entirely novel data on the effects of ACE inhibition on the full RAS cascade in a rat model of myocardial infarction. As expected, a dramatic reduction in Ang II plasma concentrations was observed with ACE inhibition. Due to inhibition of ACE-mediated pathways, the elevation of Ang I is reflected by the NEP-mediated generation of Ang (1-7) and the AP-mediated formation of Ang (2-10). The significant upregulation of Ang (1-7) and Ang (2-10) observed in this group enhances the effects of Ang II inhibition, as both of these peptides display cardioprotective properties that are antagonistic towards those of Ang II.

The main objective of this work was to assess both the C-domain-selectivity and the inhibitory efficacy of the ACE inhibitor lisW-S. To that end, the AcSDKP assay provided confirmation of the C-domain specificity of lisW-S *in vivo.* Furthermore, the 2-fold elevation of bradykinin metabolite (BK (1-5)) measured in this group relative to the MI/lisinopril-treated group suggests that the C-domain of ACE is being effectively inhibited whilst the N-domain is not. In the RAS fingerprint of the MI/lisW-S-treated group at the seven day timepoint, the most striking observation is the extraordinary 5- to 6-fold elevation of Ang I relative to the MI group. Ang II levels in this group are also significantly increased by almost 50% relative to the MI control. However, analysis of the conversion ratios of Ang I to Ang II generation presents strong evidence for a degree of inhibition by this ACE inhibitor.

There is also a possibility that the organ-specific effects of ACE inhibition are far more relevant than those on the circulatory system. The tissue RAS is not dependent on the systemic system and peptide quantification of heart and kidney homogenates in addition to plasma should contribute to our understanding. In support of this, the Campbell group has shown that low doses of the ACE inhibitor perindopril have a much more profound effect on Ang II levels in the renal system than that observed in plasma (Campbell et al. 1994). Interestingly, in their study, the lowest dose of perindopril to elicit an effect on Ang II levels in the kidney also resulted in an increase in plasma Ang I levels. This implies that renal levels of Ang II may be responsible for stimulating systemic renin activity and thus driving circulatory Ang I production. Other studies have indicated that the plasma levels of Ang II may remain unchanged following ACE inhibition despite functional and structural cardiac improvement. The Campbell group showed that while plasma Ang II levels remained unchanged following administration of perindopril in rats, both cardiac Ang II and blood pressure was significantly decreased (Duncan et al. 1996). Yamagishi *et al*. demonstrated similar results in rats with administration of the ACE inhibitor delapril (Yamagishi et al. 1993).

Based on the entirety of the results in the present study, it is tempting to speculate that the C-domain-selective ACE inhibitor lisW-S may have a far more profound effect on renal Ang II levels than that of the non-selective ACE inhibitor lisinopril. However, future studies on the

organ-specific effects of this ACE inhibitor are required before any such conclusions can be drawn.

Chapter 5 CONCLUSIONS

This study described the characterization of a novel C domain-selective ACE inhibitor by means of *in vitro* enzyme kinetics assays, *in vivo* pharmacokinetic studies and *in vivo* peptide quantification. This approach allowed us to examine the effects of C-domain-specific ACE inhibition in a controlled *in vitro* environment as well as in a physiological animal model. In addition, the pharmacokinetics of the novel ACE inhibitor lisW-S was modeled as an initial step towards rational drug design.

The novel ACE inhibitor was previously shown to be highly C domain-selective in both inhibition assays (Nchinda et al. 2006) and analysis of co-crystalization structures with tACE (a germinal form of ACE equivalent to the C-domain of somatic ACE) (Watermeyer et al. 2008). In the present study, the domain-selectivity of lisW-S in both rat plasma and human serum was confirmed using the C-domain selective FRET substrate Abz-LFK(Dnp)-OH and the non-selective FRET substrate Abz-FRK(Dnp)P-OH. The inhibition profiles obtained confirmed domain selectivity, and though C-domain selectivity was not absolutely confirmed, the findings were highly suggestive that C-domain specificity was preserved in rat plasma.

The *in vitro* studies also found that a lisW-S plasma concentration of 50 ng/ml was required to achieve 70% ACE inhibition in the rat. In order to achieve a higher percentage inhibition, the plasma concentration of the novel ACE inhibitor would need to be increased substantially. This would have been logistically difficult to do, owing to the method of lisW-S synthesis which produces a diastereomeric mixture requiring HPLC purification with resultant poor yields. Thus, it was decided that a plasma concentration of 50 ng/ml would produce an acceptable level of inhibition warranting further exploration *in vivo*.

The chief aim of the pharmacokinetic studies was to assess the bioavailability of lisW-S, as well as establishing the administered dosage required to achieve 50 ng/ml

in plasma *in vivo*. The bioavailability of lisW-S was found to be 3.1%, significantly lower than that of the clinical ACE inhibitor lisinopril. Efforts to increase bioavailability by utilizing a maleate salt preparation of lisW-S did not yield any significant improvement despite the general high efficacy of pharmaceutical salts at improving pharmacokinetic profile of pharmaceutical compounds. However, as discussed in section 3.5, there is a possibility that the bioavailability estimate presented in this study has been underestimated and future studies should include a second bioavailability assessment including earlier timpoints following IV administration.

Furthermore, lisW-S plasma concentrations in the desired therapeutic range of 50 ng/ml could not be maintained for longer than 1 hour following oral administration. Thus, an alternative route of infusion by miniosmotic pump was chosen to effectively and continuously deliver the required dosage of the novel ACE inhibitor. Pharmacokinetic studies over a period of four weeks successfully demonstrated the stability of the lisW-S compound in vivo, as well as determining that a 30 mg/ml solution delivered by infusion was the most effective dose for ACE inhibition *in vivo*.

Once the inhibitory potential, domain-selectivity and bioavailability of lisW-S had been successfully established, the effect of the novel ACE inhibitor on the RAS cascade was evaluated by enzyme-linked immunosorbent assay (EIA) and the recently developed LC-MS/MS-based RAS-fingerprinting technology. These studies were conducted in a rat model of MI, as MI-induced heart failure is a major therapeutic target for a C-domain selective ACE inhibitor.

Peptide quantification by EIA confirmed the C-domain-specificity of the novel ACE inhibitor *in vivo* by assessing the plasma concentrations of the haemetopoietic factor AcSDKP, which is metabolised exclusively by the N-domain of ACE. In the case of non-selective ACE inhibition with lisinopril, AcSDKP levels were significantly raised due to suppression of N-domain-mediated hydrolysis. The results of this study showed that AcSDKP levels are unaffected by ACE inhibition with lisW-S, thus indicating the C-domain-specificity of this compound *in vivo* in a rat model.

Unfortunately the implications of withdrawing the positive anti-inflammatory and antifibrotic effects associated with elevated AcSDKP must be considered in future studies.

The RAS-fingerprinting technique successfully quantified ten Ang peptides in the RAS cascade and two bradykinin breakdown products at one and seven days following acute MI in a rat model. Analysis of plasma peptide levels provided a detailed view of the RAS cascade under conditions of both C-domain-selective and non-selective ACE inhibition. Lisinopril was confirmed to be a highly effective inhibitor of ACE, eliciting an 8.8-fold reduction in Ang II levels and a 21.6-fold reduction in the Ang II/Ang I ratio relative to the untreated MI group at seven days post-infarction.

Peptide quantification following administration of lisW-S was conducted at both one and seven days post-MI. A significant 2.1-fold decrease in Ang II levels and an 8-fold decrease in Ang II/Ang I relative to MI was observed twenty-four hours following acute MI. However, at the seven day timepoint, this inhibitory efficacy appeared to be lost as Ang II levels were increased by 1.6-fold relative to MI. The Ang II/Ang I ratio was reduced to 3.4-fold that of MI. These results certainly indicate a degree of ACE inhibition by the C-domain-selective inhibitor, although the efficacy of lisW-S does not appear to be comparable to that of lisinopril. It is surmised that these observations are a result of both significantly elevated Ang I substrate levels by seven days post-MI, as well as a possible decrease in lisW-S plasma levels which was observed one week after miniosmotic pump implant in the pharmacokinetic studies.

Physical limitations in this study included the low product yield of the lisW-S synthesising and purification process as well as the low bioavailability of lisW-S *in vivo*. A principal component of future work therefore needs to address these issues, as such limited bioavailability does not put lisW-S in a therapeutically superior position over any clinically available ACE inhibitors currently in use. The preliminary pharmacokinetic studies presented in this work provide an excellent basis for

additional studies to assess the bioavailability of further lisW-S analogues as the synthesis process is improved. The route of administration chosen in this work has been shown to be superior in the context of animal studies to either oral or intravenous routes and should therefore be maintained. However, in a clinical setting an oral agent would be optimal, thus further emphasising the need to enhance the current bioavailability profile of the C-domain selective ACE inhibitor.

While it has been shown that lisW-S behaves as a C-domain selective ACE inhibitor both *in vitro* and *in vivo*, the significance of this to the RAS response has not been fully explored and requires further investigation. Based on the results obtained in this study, a number of suggestions can be made with regards to recommended aims for future studies.

A dramatic upregulation of the Ang I substrate was observed after treatment with lisW-S, and a thorough review of the literature has not provided a clear reason for this extreme response to domain-selective ACE inhibition. Concurrently, several ACE inhibitor studies have reported a positive therapeutic effect on cardiac function and blood pressure in the absence of quantifiable changes in plasma angiotensin peptide concentrations. Assessment of the therapeutic benefits of lisW-S independent of peptide quantification is thus highly recommended. As the animal model for *in vivo* work has now been established, future studies should aim to evaluate the effects of domain-specific ACE inhibition on systolic blood pressure, cardiac function and myocardial remodeling. Dose response studies and a critical evaluation of peptide levels at further time points throughout the development of heart failure would provide critical insight into the effects of lisW-S on the RAS.

Additionally, the elevation of Ang (1-5) observed following administration of lisW-S is suggestive of N-domain ACE-mediated metabolism of its precursor Ang (1-7). While not conclusive, this provides further evidence of the C-domain specificity of this ACE inhibitor *in vivo*, and warrants further exploration.

The theoretical basis for the rational drug design of the C-domain selective ACE inhibitor does appear to be sound, and prior to disregarding this compound and its potential efficacy as a therapeutic option in the context of MI, the physiological effects of the *in vivo* peptide results presented in this work must be thoroughly investigated. Of particular interest is the influence of endogenous AcSDKP levels in the presence of ACE inhibition on cardiac remodeling and the inflammatory process post-MI. Also of interest is the upregulation of the cardioprotective petide Ang (1-7) in conjunction with the vasodilatory, anti-inflammatory and anti-hypertrophic peptides Ang (2-10) and Ang (3-8). Conversely, the elevation of these peptides was observed to occur in conjunction with an unexpected increase in both Ang I and Ang II, and a physiological profile of this rare imbalance in the RAS will provide greater insight into the counter-regulatory mechanisms involved in the cascade. Peptide levels in renal and cardiac tissues must also be explored and their impact on the systemic RAS independently evaluated.

Importantly, the results of BK (1-5) quantification demonstrated a significant increase in the presence of lisW-S relative to lisinopril, a novel result that suggests BK (1-9) degradation may be mediated by the N-domain of ACE. Future studies should prioritize the effective quantification of BK (1-9) under conditions of C-domain specific ACE inhibition in order to validate these findings.

In conclusion, the thorough *in* vitro and *in* vivo characterisation of the C-domain selective ACE inhibitor reported in this study provides an excellent basis for future studies and establishes lisW-S as a promising alternative to current ACE inhibitor therapy.

REFERENCES

Abadir, P.M. et al., 2006. Angiotensin II Type 2 Receptor–Bradykinin B2 Receptor Functional Heterodimerization. *Hypertension* , 48 (2), pp.316–322.

Abbas, S.A., Sharma, J.N. & Yusof, A.P., 1999. The effect of bradykinin and its antagonist on survival time after coronary artery occlusion in hypertensive rats. *Immunopharmacology*, 44(1-2), pp.93–98.

Agostoni, A. et al., 1999. Angioedema due to angiotensin-converting enzyme inhibitors. *Immunopharmacology*, 44(1-2), pp.21–25.

Ahmad, S. et al., 2011. Chymase-Dependent Generation of Angiotensin II from Angiotensin-(1-12) in Human Atrial Tissue. *PLoS ONE*, 6(12), p.e28501.

Albiston, A. et al., 2001. Evidence that the angiotensin IV (AT(4)) receptor is the enzyme insulin-regulated aminopeptidase. *Journal of Biological Chemistry*, 276, pp.48623–48626.

Allred, A.J. et al., 2000. Pathways for angiotensin-(1—7) metabolism in pulmonary and renal tissues. *American Journal of Physiology - Renal Physiology*, 279 (5), pp.F841–F850.

Andrade, M. et al., 1998. Purification and character- ization of angiotensin I-converting enzymes from mesangial cells in culture. *Journal of Hypertension*, 16, pp.2063–2074.

Andújar-Sánchez, M., Cámara-Artigas, A. & Jara-Pérez, V., 2004. A calorimetric study of the binding of lisinopril, enalaprilat and captopril to angiotensin-converting enzyme. *Biophysical Chemistry*, 111(2), pp.183–189.

Antman, E.M. et al., 2004. ACC/AHA Guidelines for the Management of Patients With ST-Elevation Myocardial Infarction—Executive Summary. A Report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *Journal of the American College of Cardiology*, 44(3), pp.671–719.

Anversa, P. et al., 1985. Left ventricular failure induced by myocardial infarction. I. Myocyte hypertrophy. *American journal of physiology*, 248(6, Pt 2), pp.H876–882.

Araujo, M.C. et al., 1999. Internally quenched fluorogenic substrates for angiotensin I-converting enzyme. *Journal of Hypertension*, 17(5), pp.665–672.

Araujo, M.C. et al., 2000. Peptidase specificity characterization of C- and N-terminal catalytic sites of angiotensin I-converting enzyme. *Biochemistry*, 39(29), pp.8519–25.

Ardaillou, R. & Chansel, D., 1998. Synthesis and effects of active fragments of angiotensin II. *Kidney International*, 52, pp.1458–1468.

Atif Abbas, S. et al., 1999. Effect of bradykinin and its antagonist on survival time after coronary artery occlusion in rats. *General Pharmacology*, 33(3), pp.243–7.

Azizi, M. et al., 1996. Acute angiotensin-converting enzyme inhibition increases the plasma level of the natural stem cell regulator N-acetyl-seryl-aspartyl-lysyl-proline. J Clin Inves. *Journal of Clinical Investigation*, 97, pp.839–844.

Bachmann, S, Bosse, H.M. & Mundel, P., 1995. Topography of nitric oxide synthesis by localizing constitutive NO synthases in mammalian kidney . *American Journal of Physiology - Renal Physiology* , 268 (5), pp.F885–F898.

Baldi, A. et al., 2002. Apoptosis and post-infarction left ventricular remodeling. *Journal of Molecular and Celular Cardiology*, 34, pp.165–174.

Banerjee, A. et al., 2003. Evolving Rationale for Angiotensin-Converting Enzyme Inhibition in Chronic Heart Failure. *Mount Sinai Journal of Medicine*, 70(4), pp.225–231.

Banerji, A. & Sheffer, A.L., 2009. The spectrum of chronic angioedema. *Allergy and Asthma Proceedings*, 30(1), pp.11–6.

Bartter, F.C. et al., 1956. The regulation of aldosterone secretion in man: the role of fluid volume. *The Journal of Clinical Investigation*, 35(11), pp.1306–1315.

Basso, N. & Terragno, N., 2001. History about the discovery of the Renin-Angiotensin System. *Hypertension*, 38(6), pp.1246–1249.

Batlle, M. et al., 2006. Increased expression of the renin-angiotensin system and mast cell density but not of angiotensin-converting enzyme II in late stages of human heart failure. *Journal of Heart and Lung Transplant*, 25, pp.1117–1125.

Beierwaltes, W.H., 2006. cGMP stimulates renin secretion in vivo by inhibiting phosphodiesterase-3 . *American Journal of Physiology - Renal Physiology*, 290 (6), pp.F1376–F1381.

Benz, J. et al., 1997. Valsartan, a new angiotensin II receptor antagonist: a double-blind study comparing the incidence of cough with lisinopril and hydrochlorothiazide. *Journal of Clinical Pharmacology*, 37(2), pp.101–7.

Benzing, T. et al., 1999. Angiotensin-Converting Enzyme Inhibitor Ramiprilat Interferes With the Sequestration of the B2 Kinin Receptor Within the Plasma Membrane of Native Endothelial Cells. *Circulation*, 99 (15), pp.2034–2040.

Bernstein, K.E. et al., 2011. Different in vivo functions of the two catalytic domains of angiotensin-converting enzyme (ACE). *Current Opinion in Pharmacology*, 11(2), pp.105–111.

Bersanetti, P. et al., 2004. Positional-scanning combinatorial libraries of fluorescence resonance energy transfer peptides for defining substrate specificity of the angiotensin I-converting enzyme and development of selective C-domain substrates. *Biochemistry*, 43(50), pp.15729–36.

Biollaz, J. et al., 1982. Antihypertensive therapy with MK 421: angiotensin II-renin relationships to evaluate efficacy of converting enzyme blockade. *Journal of Cardiovascular Pharmacology*, 4(6), pp.966–72.

Blais, C. et al., 1997. Contribution of angiotensin-converting enzyme to the cardiac metabolism of bradykinin: an interspecies study. *The American Journal of Physiology*, 273(5, Pt 2), pp.H2263–71.

Bolger, P.M. et al., 1976. Effect of prostaglandin synthesis on renal function and renin in the dog. *Nature*, 259(5540), pp.244–245.

Bosse, H.M. et al., 1995. Parallel regulation of constitutive NO synthase and renin at JGA of rat kidney under various stimuli. *American Journal of Physiology - Renal Physiology* , 269 (6), pp.F793–F805.

Bowling, C.B. et al., 2012. Effects of enalapril in systolic heart failure patients with and without chronic kidney disease: Insights from the SOLVD Treatment trial. *International Journal of Cardiology*. Epub ahead of print. PMID: 22257685

Boyko, S.S. et al., 2007. Experimental Study of the Pharmacokinetics of a Tryptophan-Containing Dipeptide GB-115. , 144(3), pp.312–313.

Braszko, J et al., 1994. Effect of losartan on the memory enhancing activity of angiotensin II and angiotensin II (3-7). *Polish Journal of Pharmacology*, 4, pp.263–264.

Braszko, J.J., Kulakowska, A. & Wisniewski, K., 1995. Angiotensin II and its 3-7 fragment improve recognition but not spatial memory in rats. *Brain Research Bulletin*, 37, pp.627–631.

Brooks, V.L., Keil, L.C. & Reid, I.A., 1986. Role of the renin-angiotensin system in the control of vasopressin secretion in conscious dogs . *Circulation Research*, 58 (6), pp.829–838.

Brown, N. & Vaughan, D., 1998. Angiotensin-converting enzyme inhibitors. *Circulation*, 97, pp.1411–1420.

Bujak, M. & Frangogiannis, N.G., 2007. The role of TGF-β signaling in myocardial infarction and cardiac remodeling. *Cardiovascular Research*, 74 (2), pp.184–195.

Burnett, J.C., Opgenorth, T.J. & Granger, J.P., 1986. The renal action of atrial natriuretic peptide during control of glomerular filtration. *Kidney international*, 30(1), pp.16–19.

Burrell, L.M. et al., 2005. Myocardial infarction increases ACE2 expression in rat and humans. *European Heart Journal*, 26(4), pp.369–375.

Campbell, D.J. et al., 1991. Differential regulation of angiotensin peptide levels in plasma and kidney of the rat. *Hypertension*, 18(6), pp.763–73.

Campbell, D.J., Kladis, A. & Duncan, A.M., 1994. Effects of converting enzyme inhibitors on angiotensin and bradykinin peptides. *Hypertension*, 23 (4), pp.439–449.

Campbell, D.J. & Habener, J.F., 1987. Cellular localization of angiotensinogen gene expression in brown adipose tissue and mesentery: quantification of messenger ribonucleic acid abundance using hybridization in situ. *Endocrinology*, 121(5), pp.1616–1626.

Campbell, D.J. & Kladis, A., 1994. Effects of converting enzyme inhibitors on angiotensin and bradykinin peptides. *Hypertension*, pp.439–449.

Campbell, D.J. et al., 2004. Effect of reduced angiotensin-converting enzyme gene expression and angiotensin-converting enzyme inhibition on angiotensin and bradykinin peptide levels in mice. *Hypertension*, 43 (4), pp.854–859.

Campbell, D.J., 2003. The renin–angiotensin and the kallikrein–kinin systems. *International Journal of Biochemistry*, 35, pp.784–791.

Cantin, M. et al., 1984. Immunocytochemical localization of atrial natriuretic factor in the heart and salivary glands. *Histochemistry and Cell Biology*, 80(2), pp.113–127.

Carmona, A.K. et al., 2006. A continuous fluorescence resonance energy transfer angiotensin I-converting enzyme assay. *Nature Protocols*, 1(4), pp.1971–1976.

Cavasin, M.A. et al., 2007. Decreased endogenous levels of Ac-SDKP promote organ fibrosis . *Hypertension*, 50 (1), pp.130–136.

Cavasin, M.A. et al., 2004. Prolyl oligopeptidase is involved in release of the antifibrotic peptide Ac-SDKP. *Hypertension*, 43, pp.1140–1145.

Cayman, 2012. Cayman Chemicals EIA Kit Ang II Product Information available at: http://www.caymanchem.com/app/template/Product.vm/catalog/589301.

Chan, P. et al., 1997. Double-blind comparison of losartan, lisinopril, and metolazone in elderly hypertensive patients with previous angiotensin-converting enzyme inhibitor-induced cough. *Journal of Clinical Pharmacology*, 37(3), pp.253–7.

Chappell, M.C. et al., 1998. Metabolism of angiotensin-(1-7) by angiotensin-converting enzyme. *Hypertension*, 31, pp.362–367.

Claassen, K. et al., 2013. A detailed physiologically-based model to simulate the pharmacokinetics and hormonal pharmacodynamics of enalapril on the circulating endocrine renin-angiotensin-aldosterone system . *Frontiers in Physiology*, Epub ahead of print, PMID: 23404365.

Cleutjens, J.P. et al., 1995. Collagen remodeling after myocardial infarction in the rat heart. *The American Journal of Pathology*, 147(2), pp.325–338.

Cohn, J., 2010. Role of the renin-angiotensin system in cardiovascular disease. *Cardiovascular Drugs and Therapy*, 24(4), pp.341–344.

Cohn, J., Ferrari, R. & Sharpe, N., 2000. Cardiac remodelling—concepts and clinical implications: a consensus paper from an international forum on cardiac remodelling. *Journal of the American College of Cardiology*, 35, pp.569–582.

Conlin, P.R. et al., 2000. Angiotensin II antagonists for hypertension: are there differences in efficacy? *American Journal of Hypertension*, 13(4), pp.418–426.

Cornell, M.J. et al., 1995. Cloning and expression of an evolutionary conserved single-domain angiotensin converting enzyme from drosophila melanogaster . *Journal of Biological Chemistry*, 270 (23), pp.13613–13619.

Corradi, H.R. et al., 2006. Crystal structure of the N domain of human somatic angiotensin I-converting enzyme provides a structural basis for domain-specific inhibitor design. *Journal of molecular biology*, 357(3), pp.964–74.

Corradi, H.R. et al., 2007. The structure of testis angiotensin-converting enzyme in complex with the C domain-specific inhibitor RXPA380. *Biochemistry*, 46(18), pp.5473–5478.

Corvol, P. et al., 1995. Recent advances in knowledge of the structure and function of the angiotensin I converting enzyme. *Journal of Hypertension*, 13(3), S3-S10.

Cui, L., Nithipatikom, K. & Campbell, W., 2007. Simultaneous analysis of angiotensin peptides by LC-MS and LC- MS/MS: metabolism by bovine adrenal endothelial cells. *Analytical Biochemistry*, 369(1), pp.27–33.

Cushman, D.W. & Cheung, H.S., 1971. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochemical Pharmacology*, 20(7), pp.1637–1648.

Danilov, S. et al., 1994. Structure-function analysis of angiotensin I-converting enzyme using monoclonal antibodies. Selective inhibition of the amino-terminal active site. *Journal of Biological Chemistry*, 269 (43), pp.26806–26814.

Danser, A.H. et al., 1998. Determinants of interindividual variation of renin and prorenin concentrations: evidence for a sexual dimorphism of (pro)renin levels in humans. *Journal of hypertension*, 16(6), pp.853–862.

Davies, R.O. et al., 1984. An overview of the clinical pharmacology of enalapril. *British Journal of Clinical Pharmacology*, 18, pp.215S – 229S.

Davis, J.O., 1964. Aldosterone and Angiotensin. *The Journal of the American Medical Association*, 188 (12), pp.1062–1068.

Deddish, P. et al., 1998. N-domain specific substrate and C-domain inhibitors of angiotensin converting enzyme. *Hypertension*, 31, pp.912-917.

Delcayre, C. et al., 2000. Cardiac aldosterone production and ventricular remodeling. *Kidney International*, 57, pp.1346–1351.

DeMello, W.C. & Frohlich, E.D., eds. 2009. Renin Angiotensin System and Cardiovascular Disease, New York, Humana Press.

Dendorfer, A. et al., 1997. Intravascular and interstitial degradation of bradykinin in isolated perfused rat heart. *British Journal of Pharmacology*, 122(6), pp.1179–1187.

Deschepper, C.F. & Reudelhuber, T.L., 1990. Rat angiotensinogen is secreted only constitutively when transfected into AtT-20 cells. *Hypertension*, 16 (2), pp.147–153.

DiCarlo, L. et al., 1983. Enalapril: a new angiotensin-converting enzyme inhibitor in chronic heart failure: acute and chronic hemodynamic evaluations. *Journal of the American College ofCcardiology,* 2 (5), pp. 865-871.

Dickstein, K., 1987. Hemodynamic, hormonal, and pharmacokinetic aspects of treatment with lisinopril in congestive heart failure. *Journal of Cardiovascular Pharmacology*, 9(3), pp.S73 – S81.

Dicpinigaitis, P. V., 2006. Angiotensin-converting enzyme inhibitor-induced cough: ACCP evidence-based clinical practice guidelines. *Chest*, 129(1), p.S169–S173.

Dive, V. et al., 1999. RXP 407, a phosphinic peptide, is a potent inhibitor of angiotensin I converting enzyme able to differentiate between its two active sites. *Proceedings of the National Academy of Sciences of the United States of America*, 96(8), pp.4330–5.

Donoghue, M. et al., 2000. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin (1-9). *Circulation Research*, 87(5), pp.E1–9.

Dorer, F.E. et al., 1974. Hydrolysis of bradykinin by angiotensin-converting enzyme. *Circulation Research*, 34(6), pp.824–7.

Duka, I. et al., 2001. Vasoactive potential of the b(1) bradykinin receptor in normotension and hypertension. *Circulation Research*, 88(3), pp.275–81.

Duncan, A.M. et al., 1997. Angiotensin and bradykinin peptides in rats with myocardial infarction. *Journal of Cardiac Failure*, 3(1), pp.41–52.

Duncan, A.M. et al., 1996. Effects of angiotensin-converting enzyme inhibition on angiotensin and bradykinin peptides in rats with myocardial infarction. *Journal of Cardiovascular Pharmacology*, 28(6), pp.746–754.

Eaton, L.W. et al., 1979. Regional cardiac dilatation after acute myocardial infarction: recognition by two-dimensional echocardiography. *The New England Journal of Medicine*, 300(2), pp.57–62.

Eckberg, D.L., 1977. Adaptation of the human carotid baroreceptor-cardiac reflex. *The Journal of Physiology*, 269 (3), pp.579–589.

Edwards, B.S. et al., 1988. Atrial stretch, not pressure, is the principal determinant controlling the acute release of atrial natriuretic factor . *Circulation Research* , 62 (2), pp.191–195.

Egan, B.M. et al., 1983. Role of cardiovascular receptors on the neural regulation of renin release in normal men. *Hypertension* , 5 (5), pp.779–786.

Ehlers, M.R.W., Maeder, D.L. & Kirsch, R.E., 1986. Rapid affinity chromatographic purification of human lung and kidney angiotensin-converting enzyme with the novel N-carboxyalkyl dipeptide inhibitor N-[1(S)-carboxy-5-aminopentyl]glycylglycine. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 883(2), pp.361–372.

Ehlers, M.R. et al., 1989. Molecular cloning of human testicular angiotensin-converting enzyme: the testis isozyme is identical to the C-terminal half of endothelial angiotensin-converting enzyme. *Proceedings of the National Academy of Sciences* , 86 (20), pp.7741–7745.

Ennezat, P. V et al., 2000. Therapeutic implications of escape from angiotensin-converting enzyme inhibition in patients with chronic heart failure. *Current Cardiology Reports*, 2(3), pp.258–262.

Erdös, E.G. & Yang, H.Y., 1967. An enzyme in microsomal fraction of kidney that inactivates bradykinin. *Life Sciences*, 6(6), pp.569–74.

Erdös, E.G., 1975. Angiotensin I converting enzyme. *Circulation Research*, 36(2), pp.247–55.

Erdös, E.G. & Skidgel, R.A., 1990. Renal metabolism of angiotensin I and II. *Kidney International. Supplement*, 30, pp.S24–S277.

Farmer, S.G., Ensor, J.E. & Burch, R.M., 1991. Evidence that cultured airway smooth muscle cells contain bradykinin B2 and B3 receptors. *American Journal of Respiratory Cell and Molecular Biology*, 4(3), p.273.

Ferrario, C.M. et al., 2005. Effects of renin-angiotensin system blockade on renal angiotensin-(1-7) forming enzymes and receptors. *Kidney International*, 68(5), pp.2189–2196.

Ferreira, S.H. et al., 1970. Activity of various fractions of bradykinin potentiating factor against angiotensin I converting enzyme. *Nature*, 225(5230), pp.379–380.

Ferreira, S. H. & Rocha e Silva, M., 1965. Potentiation of bradykinin and eledoisin by BPF (bradykinin potentiating factor) from Bothrops jararaca venom. *Experientia*, 21 (6), pp. 347-349.

Ferreira, S.H., Bartelt, D.C. & Greene, L.J., 1970. Isolation of bradykinin-potentiating peptides from Bothrops jararaca venom. *Biochemistry*, 9(13), pp.2583–2593.

Figueroa, C.D. et al., 2001. Differential distribution of bradykinin B2 receptors in the rat and human cardiovascular system. *Hypertension*, 37 (1), pp.110–120.

Filippatos, G. et al., 2001. Regulation of apoptosis by angiotensin II in the heart and lungs (Review). *International Journal of Molecular Medicine*, 7(3), pp.273–280.

Flather, M.D. et al., 2000. Long-term ACE-inhibitor therapy in patients with heart failure or left-ventricular dysfunction: a systematic overview of data from individual patients. *The Lancet*, 355(9215), pp.1575–1581.

Forest, W. & Carolina, N., 2005. Effects of renin-angiotensin system blockade on renal angiotensin-(1-7) forming enzymes and receptors. *Kidney International*, 68, pp.2189–2196.

Foucart, S. et al., 1997. Modulation of noradrenaline release by B1 and B2 kinin receptors during metabolic anoxia in the rat isolated atria. *Canadian Journal of Physiology and Pharmacology*, 75(6), pp.639–45.

Fox, A.J. et al., 1996. Bradykinin-evoked sensitization of airway sensory nerves: a mechanism for ACE-inhibitor cough. *Nature Medicine*, 2(7), pp.814–7.

Fox, R.H. et al., 1961. Bradykinin as a vasodilator in man. *The Journal of Physiology*, 157, pp.589–602.

Francis, J. et al., 2001. Progression of heart failure after myocardial infarction in the rat. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, 281(5), pp.R1734–R1745.

Frangogiannis, N.G., Smith, C.W. & Entman, M.L., 2002. The inflammatory response in myocardial infarction . *Cardiovascular Research* , 53 (1), pp.31–47.

Friedland, J. & Silverstein, E., 1976. A sensitive fluorimetric assay for serum angiotensin-converting enzyme. *American Journal of Clinical Pathology*, 66(2), pp.416–24.

Friis, U.G. et al., 1999. Direct demonstration of exocytosis and endocytosis in single mouse juxtaglomerular cells . *Circulation Research* , 84 (8), pp.929–936.

Friis, U.G. et al., 2005. Prostaglandin E2, EP2 and EP4 receptor activation mediates cAMP-dependent hyperpolarization and exocytosis of renin in juxtaglomerular cells . *American Journal of Physiology - Renal Physiology* , 289 (5), pp.F989–F997.

Frohlich, E.D., Tarazi, R.C. & Dustan, H.P., 1969. Re-examination of the hemodynamics of hypertension. *The American Journal of the Medical Sciences*, 257(1), pp.9-23.

Fuchs, S. et al., 2008. Angiotensin-converting enzyme C-terminal catalytic domain is the main site of angiotensin I cleavage in vivo. *Hypertension*, 51, pp.267–274.

Furberg, C.D., 2000. Class effects and evidence-based medicine. *Clinical Cardiology*, 23, pp.IV15–IV19.

Galen, F.X. et al., 1984. Renin biosynthesis by human tumoral juxtaglomerular cells. Evidences for a renin precursor. *The Journal of Clinical Investigation*, 73(4), pp.1144–1155.

Garg, S. et al., 2006. Role of angiotensin receptor blockers in the prevention and treatment of arrhythmias. *Journal of Cardiology*, 97(6), pp.921-925

Gavras, H. et al., 1978. Angiotensin converting enzyme inhibition in patients with congestive heart failure. *Circulation*, 58 (5), pp.770–776.

Georgiadis, D. et al., 2003. Roles of the two active sites of somatic angiotensin-converting enzyme in the cleavage of angiotensin I and bradykinin. *Circulation Research*, 93(2), pp.148-154.

Georgiadis, D. et al., 2004. Structural determinants of RXPA380, a potent and highly selective inhibitor of the angiotensin-converting enzyme C-domain. *Biochemistry*, 43(25), pp.8048–54.

Gomez, H.J., Cirillo, V.J. & Moncloa, F., 1987. The clinical pharmacology of lisinopril. *Journal of Cardiovascular Pharmacology*, 9(3), pp.S27 – S34.

Goulter, A. et al., 2004. ACE2 gene expression is up-regulated in the human failing heart. *BMC Medicine*, 2(1), p.19.

Greenberg, S.G. et al., 1995. Effect of nitric oxide on renin secretion. I. Studies in isolated juxtaglomerular granular cells . *American Journal of Physiology - Renal Physiology* , 268 (5), pp.F948–F952.

Griendling, K.K. & Ushio-Fukai, M., 2000. Reactive oxygen species as mediators of angiotensin II signaling. *Regulatory Peptides*, 91(1–3), pp.21–27.

Grossman, W., Jones, D. & McLaurin, L.P., 1975. Wall stress and patterns of hypertrophy in the human left ventricle. *The Journal of Clinical Investigation*, 56(1), pp.56–64.

Grünberger, C. et al., 2006. The Calcium Paradoxon of Renin Release. *Circulation Research*, 99 (11), pp.1197–1206.

Hall, J.E. et al., 1977. Control of glomerular filtration rate by renin-angiotensin system. *American Journal of Physiology - Renal Physiology*, 233 (5), pp.F366–F372.

Hall, J.E. et al., 1981. Control of glomerular filtration rate by circulating angiotensin II. *The American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, 241(3), pp.R190–197.

Hall, J.E., 2003. Historical perspective of the renin-angiotensin system. *Molecular Biotechnology*, 24(1), pp.27–39.

Hamlin, R.L. & Nakayama, T., 1998. Comparison of some pharmacokinetic parameters of 5 angiotensin-converting enzyme inhibitors in normal beagles. *Journal of Veterinary Internal Medicine*, pp.93–95.

Hamming, I. et al., 2004. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *The Journal of Pathology*, 203(2), pp.631–7.

Hansen, J.L. et al., 2000. Functional reconstitution of the angiotensin II type 2 receptor and G(i) activation. *Circulation Research*, 87(9), pp.753–759.

Harding, P., Carretero, O.A. & Beierwaltes, W.H., 2000. Chronic cyclooxygenase-2 inhibition blunts low sodium-stimulated renin without changing renal haemodynamics. *Journal of Hypertension*, 18(8), pp.1107–1113.

Harris, R. et al., 1994. Cyclooxygenase-2 is associated with the macula densa of rat kidney and increases with salt restriction. *Journal of Clinical Investigation*, 94(6), pp.2504–2510.

Hartner, A., Goppelt-Struebe, M. & Hilgers, K.F., 1998. Coordinate expression of cyclooxygenase-2 and renin in the rat kidney in renovascular hypertension. *Hypertension* , 31 (1), pp.201–205.

Hautmann, M. et al., 2007. Pituitary adenylate cyclase–activating polypeptide stimulates renin secretion via activation of PAC1 receptors. *Journal of the American Society of Nephrology*, 18 (4), pp.1150–1156.

Hemler, M. & Lands, W., 1976. Purification of the cyclooxygenase that forms prostaglandins. Demonstration of two forms of iron in the holoenzyme. *The Journal of Biological Chemistry*, 251(18), pp.5575–5579.

Henrich, W.L. et al., 1988. Guanosine 3',5'-cyclic monophosphate as a mediator of inhibition of renin release. *American Journal of Physiology - Renal Physiology* , 255 (3), pp.F474–F478.

Heymans, C., 1960. Reflexogenic areas of the cardiovascular system. *Perspectives in Biology and Medicine*, 3, pp.409–417.

Hiwada, K. et al., 1987. Direct radioimmunoassay of angiotensin-converting enzyme in sera from patients with pulmonary diseases. *Lung*, 165(1), pp.27–35.

Hobart, P.M. et al., 1984. Human renin gene: structure and sequence analysis. *Proceedings of the National Academy of Sciences* , 81 (16), pp.5026–5030.

Hochman, J.S. & Bulkley, B.H., 1982. Expansion of acute myocardial infarction: an experimental study. *Circulation* , 65 (7), pp.1446–1450.

Hodsman, G.P. et al., 1983. Factors related to first dose hypotensive effect of captopril: prediction and treatment. *British Medical Journal (Clinical Research ed.)*, 286(6368), pp.832–834.

Hubert, C. et al., 1991. Structure of the angiotensin I-converting enzyme gene. Two alternate promoters correspond to evolutionary steps of a duplicated gene. *Journal of Biological Chemistry* , 266 (23), pp.15377–15383.

Huentelman, M.J. et al., 2005. Protection from angiotensin II-induced cardiac hypertrophy and fibrosis by systemic lentiviral delivery of ACE2 in rats. *Experimental Physiology*, 90(5), pp.783–790.

Hutchins, G.M. & Bulkley, B.H., 1978. Infarct expansion versus extension: two different complications of acute myocardial infarction. *The American Journal of Cardiology*, 41(7), pp.1127–32.

IMS: Institute for Healthcare Informatics, 2010. The Use of Medicines in the United States: Review of 2010.

Irwin, R.S., Curley, F.J. & French, C.L., 1990. Chronic cough: The spectrum and frequency of causes, key components of the diagnostic evaluation, and outcome of specific therapy. *The American Review of Respiratory Disease*, 141(3), pp.640–647.

Ishigai, Y. et al., 1997. Role of bradykinin-NO pathway in prevention of cardiac hypertrophy by ACE inhibitor in rat cardiomyocytes. *The American Journal of Physiology*, 273(6, Pt 2), pp.H2659–H2663.

Isnard, R. et al., 2000. Prognostic value of neurohormonal activation and cardiopulmonary exercise testing in patients with chronic heart failure. *The American Journal of Cardiology*, 86(4), pp.417–421.

Israili, Z.H. & Hall, W.D., 1992. Cough and angioneurotic edema associated with angiotensin-converting enzyme inhibitor therapy: A review of the literature and pathophysiology. *Annals of Internal Medicine*, 117(3), pp.234–42.

Iyer, S.N. et al., 1998. Vasodepressor actions of angiotensin-(1–7) unmasked during combined treatment with lisinopril and losartan. *Hypertension*, 31(2), pp.699–705.

Jackman, H.L. et al., 2002. Angiotensin (1-9) and (1-7) release in human heart. *Hypertension* , 39 (5), pp.976–981.

Jaffe, I.A., 1986. Adverse effects profile of sulfhydryl compounds in man. *The American Journal of Medicine*, 80(3), pp.471–476.

Jaspard, E., Wei, L. & Alhenc-Gelas, F., 1993. Differences in the properties and enzymatic specificities of the two active sites of angiotensin I-converting enzyme (kininase II). Studies with bradykinin and other natural peptides. *The Journal of Biological Chemistry*, 268(13), pp.9496–9503.

Jensen, B.L., Schmid, C. & Kurtz, A., 1996. Prostaglandins stimulate renin secretion and renin mRNA in mouse renal juxtaglomerular cells. *American Journal of Physiology - Renal Physiology* , 271 (3), pp.F659–F669.

Jensen, B.L., Krämer, B.K. & Kurtz, A., 1997. Adrenomedullin stimulates renin release and renin mRNA in mouse juxtaglomerular granular cells . *Hypertension* , 29 (5), pp.1148–1155.

Jin, D. et al., 2001. Possible roles of cardiac chymase after myocardial infarction in hamster hearts. *The Japanese Journal of Pharmacology*, 86(2), pp.203–214.

Jin, D. et al., 2003. Impact of chymase inhibitor on cardiac function and survival after myocardial infarction. *Cardiovascular Research*, 60, pp.413–420.

Jin, D. et al., 2004. An antiarrhythmic effect of a chymase inhibitor after myocardial infarction. *Journal of Pharmacology and Experimental Therapeutics*, 309(2), pp.490–497.

John, H. et al., 2004. Analytical procedures for quantification of peptides in pharmaceutical research by liquid chromatography–mass spectrometry. *Analytical and Bioanalytical Chemistry*, 378(4), pp.883–897.

Johnson, J.A. & Davis, J.O., 1973. Angiotensin II: Important Role in the Maintenance of Arterial Blood Pressure . *Science* , 179 (4076), pp.906–907.

Johnson, J.G. et al., 1975. Treatment of patients with severe hypertension by inhibition of angiotensin-converting enzyme. *Clinical Science and Molecular Medicine,* 2, pp.S53–S56.

Jullien, N.D. et al., 2006. Combined use of selective inhibitors and fluorogenic substrates to study the specificity of somatic wild-type angiotensin-converting enzyme RXP407. *FEBS Journal*, 273, pp.1772–1781.

Junot, C. et al., 2001. RXP 407, a Selective Inhibitor of the N-Domain of Angiotensin I-Converting Enzyme , Blocks in Vivo the Degradation of Hemoregulatory Peptide Acetyl-Ser-Asp-Lys-Pro with No Effect on Angiotensin I Hydrolysis. *The Journal of Pharmacology and Experimental Therapeutics*, 297(2), pp.606–611.

Kaneko, Y. et al., 1967. Renin release during acute reduction of arterial pressure in normotensive subjects and patients with renovascular hypertension. *The Journal of Clinical Investigation*, 46(5), pp.705–716.

Katada, J. & Majima, M., 2002. AT(2) receptor-dependent vasodilation is mediated by activation of vascular kinin generation under flow conditions. *British Journal of Pharmacology*, 136(4), pp.484–91.

Van Kats, J. et al., 1998. Angiotensin production by the heart: a quantitative study in pigs with the use of radiolabeled angiotensin infusions. *Circulation*, 98(1), pp.73–81.

Keeton, T.K. & Campbell, W.B., 1980. The pharmacologic alteration of renin release. *Pharmacological Reviews*, 32(2), pp.81–227.

Kelly, M.P. et al., 1997. Tissue expression of components of the renin-angiotensin system in experimental post-infarction heart failure in rats: effects of heart failure and angiotensin-converting enzyme inhibitor treatment. *Clinical Science (London, England : 1979)*, 92(5), pp.455–65.

Kirkpatrick, D.S., Gerber, S.A. & Gygi, S.P., 2005. The absolute quantification strategy: a general procedure for the quantification of proteins and post-translational modifications. *Methods*, 35(3), pp.265–273.

Køber, L. et al., 1995. A Clinical trial of the angiotensin-converting–enzyme inhibitor trandolapril in patients with left ventricular dysfunction after myocardial infarction. *New England Journal of Medicine*, 333(25), pp.1670–1676.

Kostis, J.B. et al., 2004. Omapatrilat and enalapril in patients with hypertension: the Omapatrilat Cardiovascular Treatment vs. Enalapril (OCTAVE) trial. *American Journal of Hypertension*, 17(2), pp.103–11.

Kramár, E.A., Harding, J.W. & Wright, J.W., 1997. Angiotensin II- and IV-induced changes in cerebral blood flow. Roles of AT1, AT2, and AT4 receptor subtypes. *Regulatory Peptides*, 68(2), pp.131–8.

Kumar, R., Singh, V.P. & Baker, K.M., 2007. The intracellular renin–angiotensin system: a new paradigm. *Trends in Endocrinology & Metabolism*, 18(5), pp.208–214.

Kurtz, A. et al., 1986. Atrial natriuretic peptide inhibits renin release from juxtaglomerular cells by a cGMP-mediated process. *Proceedings of the National Academy of Sciences*, 83 (13), pp.4769–4773.

Kurtz, A. & Wagner, C., 1998. Role of nitric oxide in the control of renin secretion. *The American Journal of Physiology*, 275(6, Pt 2), pp.F849–F862.

Kurtz, A. et al., 1998. Stimulation of renin secretion by NO donors is related to the cAMP pathway. *American Journal of Physiology - Renal Physiology* , 274 (4), pp.F709–F717.

Kurtz, A., 2011. Renin release: sites, mechanisms, and control. *Annual Review of Physiology*, 73, pp.377–399.

Brennan, L.A. et al., 1971. Influence of right and left atrial receptors on plasma concentrations of ADH and renin . *American Journal of Physiology*, 221 (1), pp.273–278.

Lacourcière, Y. et al., 1994. Effects of modulators of the renin-angiotensin-aldosterone system on cough. Losartan Cough Study Group. *Journal of Hypertension*, 12(12), pp.1387–1393.

Lang, S. et al., 2010. Increased risk of myocardial infarction in HIV-infected patients in France, relative to the general population. *AIDS*, 24(8), pp.1228-1230.

Langford, K.G. et al., 1993. Regulated expression of testis angiotensin-converting enzyme during spermatogenesis in mice. *Biology of Reproduction*, 48 (6), pp.1210–1218.

Lattion, A.L. et al., 1989. The testicular transcript of the angiotensin I-converting enzyme encodes for the ancestral, non-duplicated form of the enzyme. *FEBS Letters*, 252(1-2), pp.99–104.

Lawrence, A.C. et al., 1990. An alternative strategy for the radioimmunoassay of angiotensin peptides using amino-terminal-directed antisera: measurement of eight angiotensin peptides in human plasma. *Journal of Hypertension*, 8(8), pp.715–724.

Lee, C. & Snyder, S., 1982. Dipeptidylaminopeptidase III of rat brain. Selective affinity for enkephalin and angiotensin. *Journal of Biological Chemistry*, 257, pp.12043–12050.

Lenfant, M. et al., 1989. Inhibitor of hematopoietic pluripotent stem cell proliferation: purification and determination of its structure. *Proceedings of the National Academy of Sciences.*, 86, pp.779–782.

Leuschner, F. et al., 2010. Angiotensin-Converting Enzyme Inhibition Prevents the Release of Monocytes From Their Splenic Reservoir in Mice. *Circulation Research*, 107(11), pp.1364-1373.

Lijnen, P.J., Petrov, V.V. & Fagard, R.H., 2001. Angiotensin II-induced stimulation of collagen secretion and production in cardiac fibroblasts is mediated via angiotensin II subtype 1 receptors. *Journal of the Renin-Angiotensin-Aldosterone system*, 2(2), pp.117–22.

Lin, C.X. et al., 2008. Prevention of aortic fibrosis by N-acetyl-seryl-aspartyl-lysyl-proline in angiotensin II-induced hypertension. *American Journal of Physiology - Heart and Circulatory Physiology*, 295 (3), pp.H1253–H1261.

Lindpaintner, K. et al., 1993. Selective activation of cardiac angiotensinogen gene expression in post-infarction ventricular remodeling in the rat. *Journal of Molecular and Cellular Cardiology*, 25(2), pp.133–143.

Lonn, E.M. et al., 1994. Emerging role of angiotensin-converting enzyme inhibitors in cardiac and vascular protection. *Circulation* , 90 (4), pp.2056–2069.

Loot, A.E. et al., 2002. Angiotensin-(1-7) attenuates the development of heart failure after myocardial infarction in rats. *Circulation*, 105(13), pp.1548–50.

Lynch, K.R., Hawelu-Johnson, C.L. & Guyenet, P.G., 1987. Localization of brain angiotensinogen mRNA by hybridization histochemistry. *Molecular Brain Research*, 2(2), pp.149–158.

Ma, T.K. et al., 2010. Renin-angiotensin-aldosterone system blockade for cardiovascular diseases: current status. *British Journal of Pharmacology*, 160(6), pp.1273–92.

Maack, T. et al., 1984. Effects of auriculin (atrial natriuretic factor) on blood pressure, renal function, and the renin-aldosterone system in dogs. *The American Journal of Medicine*, 77(6), pp.1069–1075.

MacFadyen, R.J. et al., 1999. How often are angiotensin II and aldosterone concentrations raised during chronic ACE inhibitor treatment in cardiac failure? *Heart (British Cardiac Society)*, 82(1), pp.57–61.

Malpas, S.C., 2010. Sympathetic Nervous System Overactivity and Its Role in the Development of Cardiovascular Disease. *Physiological Reviews* , 90 (2), pp.513–557.

Mancini, G.B. et al., 1996. Angiotensin-converting Enzyme inhibition with quinapril improves endothelial vasomotor dysfunction in patients with coronary artery disease: The TREND (Trial on Reversing Endothelial Dysfunction) Study. *Circulation*, 94 (3), pp.258–265.

Mankad, S. et al., 2001. Combined Angiotensin II Receptor Antagonism and Angiotensin-Converting Enzyme Inhibition Further Attenuates Postinfarction Left Ventricular Remodeling. *Circulation* , 103 (23), pp.2845–2850.

Marc, Y. & Llorens-Cortes, C., 2011. The role of the brain renin-angiotensin system in hypertension: implications for new treatment. *Progress in Neurobiology*, 95, pp.89–103.

Marcic, B. et al., 2000. Replacement of the transmembrane anchor in angiotensin I-converting enzyme (ACE) with a glycosylphosphatidylinositol tail affects activation of the B2 bradykinin receptor by ACE inhibitors. *The Journal of Biological chemistry*, 275(21), pp.16110–8.

Mathers, C., Lopez, A. & Murray, C., 2006. The burden of disease and mortality by condition: data, methods, and results for 2001. *In: Lopez AD, Mathers CD, Ezzati M, et al., editors. Global Burden of Disease and Risk Factors. Washington (DC): World Bank*.

Meng, Q.C. & Oparil, S., 1996. Purification and assay methods for angiotensin-converting enzyme. *Journal of Chromatography. A*, 743(1), pp.105–22.

Min, L., Sim, M.K. & Xu, X.G., 2000. Effects of des-aspartate-angiotensin I on angiotensin II-induced incorporation of phenylalanine and thymidine in cultured rat cardiomyocytes and aortic smooth muscle cells. *Regulatory Peptides*, 95, pp.93–97.

Minshall, R.D. et al., 1995. Characterization of bradykinin B2 receptors in adult myocardium and neonatal rat cardiomyocytes. *Circulation Research*, 76(5), pp.773–80.

Miyazaki, Mizuo et al., 2006. Pathological roles of angiotensin II produced by mast cell chymase and the effects of chymase inhibition in animal models. *Pharmacology & Therapeutics*, 112, pp.668–676.

Morice, A.H. et al., 1987. Angiotensin-converting enzyme and the cough reflex. *The Lancet*, 330(8568), pp.1116–1118.

Moss, N.G., 1982. Renal function and renal afferent and efferent nerve activity . *American Journal of Physiology - Renal Physiology*, 243 (5), pp.F425–F433.

Mundel, P. et al., 1992. Expression of nitric oxide synthase in kidney macula densa cells. *Kidney International*, 42(4), pp.1017–1019.

Murray, C.J. & Lopez, A.D., 1997. Mortality by cause for eight regions of the world: global burden of disease study. *The Lancet* , 349(9061), pp.1269–1276.

Natesh, R. et al., 2004. Structural details on the binding of antihypertensive drugs captopril and enalaprilat to human testicular angiotensin I-converting enzyme. *Biochemistry*, 43(27), pp.8718–8724.

Natesh, R., Schwager, S. & Sturrock, E.D., 2003. Crystal structure of the human enzyme – lisinopril complex. *Nature*, 1429(1995), pp.1427–1429.

Nchinda, A.T., Chibale, K., Redelinghuys, P., et al., 2006a. Synthesis and molecular modeling of a lisinopril-tryptophan analogue inhibitor of angiotensin I-converting enzyme. *Bioorganic & Medicinal Chemistry Letters*, 16(17), pp.4616–4619.

Nchinda, A.T., Chibale, K. & Sturrock, Edward D, 2006b. Synthesis of novel keto-ACE analogues as domain-selective angiotensin I-converting enzyme inhibitors. *Bioorganic & Medicinal Chemistry Letters*, 16 (17), pp.4612–4615.

Neves, L.A. et al., 1995. Metabolism of angiotensin I in isolated rat hearts: effect of angiotensin converting enzyme inhibitors. *Biochemical Pharmacology*, 50(9), pp.1451–1459.

Nguyen, G et al., 1996. Specific receptor binding of renin on human mesangial cells in culture increases plasminogen activator inhibitor-1 antigen. *Kidney International*, 50(6), pp.1897–903.

Nguyen, G. et al., 2002. Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. *The Journal of Clinical Investigation*, 109(11), pp.1417–27.

Ntsekhe, M. & Mayosi, B.M., 2009. Cardiac manifestations of HIV infection: an African perspective. *Nature Clinical Practice Cardiovascular Medecine*, 6(2), pp.120–127.

Nussberger, J. et al., 1998. Plasma bradykinin in angio-oedema. *Lancet*, 351(9117), pp.1693–7.

Nussberger, J. et al., 2002. Angiotensin II suppression in humans by the orally active renin inhibitor Aliskiren (SPP100): comparison with enalapril. *Hypertension*, 39(1), pp.E1–8.

Nussberger, J. et al., 2005. P-624: Changes in plasma renin match the antihypertensive effects of aliskiren in patients with hypertension: placebo/irbesartan-controlled trial with the orally active renin inhibitor aliskiren. *American Journal of Hypertension* , 18 (S4), p.234A–234A.

O'Keefe, J.H. et al., 2001. Should an angiotensin-converting enzyme inhibitor be standard therapy for patients with atherosclerotic disease? *Journal of the American College of Cardiology*, 37(1), pp.1–8.

Obana, K. et al., 1985. Synthetic rat atrial natriuretic factor inhibits in vitro and in vivo renin secretion in rats. *Endocrinology*, 117(3), pp.1282–4.

Oberg, B. & Thorén, P., 1972. Studies on left ventricular receptors, signalling in non-medullated vagal afferents. *Acta physiologica Scandinavica*, 85(2), pp.145–63.

Ocaranza, M.P. et al., 2010. Angiotensin-(1-9) regulates cardiac hypertrophy in vivo and in vitro. *Journal of Hypertension*, 28(5), pp.1054-1064.

Ocaranza, María Paz et al., 2006. Enalapril Attenuates Downregulation of Angiotensin-Converting Enzyme 2 in the Late Phase of Ventricular Dysfunction in Myocardial Infarcted Rat. *Hypertension*, 48(4), pp.572-578.

Okunishi, H. et al., 1993. Marked species difference in the vascular angiotensin II-forming pathways: humans versus rodents. *Japanese Journal of Pharmacology*, 62, pp.207–210.

Olivetti, G. et al., 1990. Side-to-side slippage of myocytes participates in ventricular wall remodeling acutely after myocardial infarction in rats. *Circulation Research* , 67 (1), pp.23–34.

Ondetti, M.A., Rubin, B. & Cushman, D.W., 1977. Design of specific inhibitors of angiotensin-converting enzyme: new class of orally active antihypertensive agents. *Science (New York, N.Y.)*, 196(4288), pp.441–444.

Oparil, S. et al., 2007. Efficacy and safety of combined use of aliskiren and valsartan in patients with hypertension: a randomised, double-blind trial. *The Lancet*, 370(9583), pp.221–229.

Ortiz-Capisano, M.C. et al., 2007. Decreased intracellular calcium stimulates renin release via calcium-Inhibitable adenylyl cyclase . *Hypertension* , 49 (1), pp.162–169.

Packer, M, 1996. New concepts in the pathophysiology of heart failure: beneficial and deleterious interaction of endogenous haemodynamic and neurohormonal mechanisms. *Journal of Internal Medicine*, 239(4), pp.327–333.

Padia, S.H. et al., 2008. Conversion of Renal Angiotensin II to Angiotensin III Is Critical for AT2 Receptor–Mediated Natriuresis In Rats. *Hypertension* , 51 (2), pp.460–465.

Patchett, A.A. et al., 1980. A new class of angiotensin-converting enzyme inhibitors. *Nature*, 288(5788), pp.280–283.

Paul, M., Mehr, A.L. & Kreutz, R., 2006. Physiology of Local Renin-Angiotensin Systems. *Physiological Reviews*, pp.747– 803.

Peng, H. et al., 2003. Ac-SDKP reverses cardiac fibrosis in rats with renovascular hypertension. *Hypertension*, 42(6), pp.1164–1170.

Peng, H. et al., 2005. Angiotensin-converting enzyme inhibitors: a new mechanism of action. *Circulation*, 112, pp.2436–2445.

Pfeffer, M.A. & Braunwald, E., 1990. Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. *Circulation*, 81 (4), pp.1161–1172.

Pfeffer, J.M. et al., 1991. Progressive ventricular remodeling in rat with myocardial infarction . *American Journal of Physiology - Heart and Circulatory Physiology*, 260 (5), pp.H1406–H1414.

Pfeffer, M.Aa et al., 1992. Effect of captopril on mortality and morbidity in patients with left ventricular dysfunction after myocardial infarction. *New England Journal of Medicine*, 327(10), pp.669–677.

Poglitsch, M. et al., 2012. Recombinant expression and characterization of human and murine ACE2: Species- specific activation of the alternative Renin-Angiotensin-System. *International Journal of Hypertension*, 2012, p.428950.

Pradelles, P. et al., 1990. Negative regulator of pluripotent hematopoietic stem cell proliferation in human white blood cells and plasma as analysed by enzyme immunoassay. *Biochemical and Biophysical Research Communications*, 170(3), pp.986–993.

Prosser, H.C. et al., 2009. Cardiac chymase converts rat proAngiotensin-12 (PA12) to angiotensin II : effects of PA12 upon cardiac haemodynamics. *Cardiovascular Research*, 82, pp.40–50.

Raidoo, D.M. et al., 1997. Kinin receptors in human vascular tissue: their role in atheromatous disease. *Immunopharmacology*, 36(2-3), pp.153–60.

Ranadive, S.A., Chen, A.X. & Serajuddin, Abu T.M., 1992. Relative lipophilicities and structural-pharmacological considerations of various angiotensin-converting enzyme (ACE) Inhibitors. *Pharmaceutical Research*, 9(11), pp.1480–1486.

Rasoul, S. et al., 2004. Antifibrotic effect of Ac-SDKP and angiotensin-converting enzyme inhibition in hypertension. *Journal of Hypertension*, 22(3), pp.593-603.

Reid, I.A., 1992. Interactions between ANG II, sympathetic nervous system, and baroreceptor reflexes in regulation of blood pressure. *American Journal of Physiology - Endocrinology And Metabolism* , 262 (6), pp.E763–E778.

Reid, I.A., Morris, B.J. & Ganong, W.F., 1978. The Renin-Angiotensin System. *Annual Review of Physiology*, 40(1), pp.377–410.

Rocha E Silva, M., Beraldo, W.T. & Rosenfeld, G., 1949. Bradykinin, a hypotensive and smooth muscle stimulating factor released from plasma globulin by snake venoms and by trypsin. *The American Journal of Physiology*, 156(2), pp.261–273.

Rohrbach, M.S., 1978. [Glycine-1-14C]hippuryl-L-histidyl-L-leucine: a substrate for the radiochemical assay of angiotensin converting enzyme. *Analytical Biochemistry*, 84(1), pp.272–276.

Roig, E. et al., 2000. Clinical implications of increased plasma angiotensin II despite ACE inhibitor therapy in patients with congestive heart failure. *European Heart Journal*, 21(1), pp.53–77.

Rouleau, JL et al., 1993. Activation of neurohumoral systems in postinfarction left ventricular dysfunction. *Journal of the American College of Cardiology*, 22(2), pp.390–398.

Rousseau, Anne et al., 1995. The hemoregulatory peptide N-Acetyl-Ser-Asp-Lys-Pro is a natural and specific substrate of the N-terminal active site of human angiotensin-converting enzyme. *Journal of Biological Chemistry*, 270(8), pp.3656–3661.

Rubin, S.A., Fishbein, M.C. & Swan, H.J., 1983. Compensatory hypertrophy in the heart after myocardial infarction in the rat. *Journal of the American College of Cardiology*, 1(6), pp.1435–1441.

Rufaihah, A.J. et al., 2006. Cardioprotective effect of des-Aspartate-angiotensin-I (DAA-I) on cytokine gene expression profile in ligation model of myocardial infarction. *Life Sciences*, 78(12), pp.1341–51.

Ruiz-Ortega, M., Lorenzo, O. & Egido, J., 2000. Angiotensin III increases MCP-1 and activates NF-kappaB and AP-1 in cultured mesangial and mononuclear cells. *Kidney Internationa*, 57, pp.2285–2298.

Ruzicka, M. & Leenen, F.H., 1995. Relevance of blockade of cardiac and circulatory angiotensin-converting enzyme for the prevention of volume overload–induced cardiac hypertrophy. *Circulation*, 91 (1), pp.16–19.

Ruzicka, M., Skarda, V. & Leenen, F.H., 1995. Effects of ACE inhibitors on circulating versus cardiac angiotensin II in volume overload-induced cardiac hypertrophy in rats. *Circulation*, 92 (12), pp.3568–3573.

Ruzicka, M. et al., 2010. Effects of ACE Inhibitors on cardiac angiotensin II and aldosterone in humans: “Relevance of lipophilicity and affinity for ACE”. *American Journal of Hypertension* , 23 (11), pp.1179–1182.

Sadoshima, J et al., 1992. Molecular characterization of the stretch-induced adaptation of cultured cardiac cells. An in vitro model of load-induced cardiac hypertrophy. *The Journal of Biological Chemistry*, 267(15), pp.10551–60.

Sampaio, W.O. et al., 2007a. Angiotensin-(1-7) counterregulates angiotensin II signaling in human endothelial cells. *Hypertension*, 50 (6), pp.1093–1098.

Sampaio, W.O. et al., 2007b. Angiotensin-(1-7) through receptor Mas mediates endothelial nitric oxide synthase activation via Akt-dependent pathways. *Hypertension* , 49 (1), pp.185–192.

Santos, R.A.S. et al., 2003. Angiotensin-(1–7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proceedings of the National Academy of Sciences* , 100 (14), pp.8258–8263.

Saris, J.J. et al., 2002. Prorenin-induced myocyte proliferation. *Hypertension* , 39 (2), pp.573–577.

Sawa, H. et al., 1992. Expression of the angiotensinogen gene and localization of its protein in the human heart . *Circulation*, 86 (1), pp.138–146.

Sayago, C.M. & Beierwaltes, W.H., 2001. Nitric oxide synthase and cGMP-mediated stimulation of renin secretion. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* , 281 (4), pp.R1146–R1151.

Schartl, M. et al., 1994. Remodeling of myocardium and arteries by chronic angiotensin converting enzyme inhibition in hypertensive patients. *Journal of Hypertension,* 12(4), pp.S37-S42

Schindler, C. et al., 2007. Role of the vasodilator peptide angiotensin-(1-7) in cardiovascular drug therapy. *Vascular Health and Risk Management*, 3(1), pp.125–137.

Schmaier, A.H., 2002. The plasma kallikrein-kinin system counterbalances the renin-angiotensin system. *The Journal of Clinical Investigation*, 109(8), pp.1007–1009.

Schmidt, H.H., Lohmann, S.M. & Walter, U., 1993. The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. *Biochimica et Biophysica Acta*, 1178(2), pp.153–175.

Schremmer-Danninger, E. et al., 1998. B1 bradykinin receptors and carboxypeptidase M are both upregulated in the aorta of pigs after LPS infusion. *Biochemical and Biophysical Research Communications*, 243(1), pp.246–252.

Schrier, R.W. & Abraham, W.T., 1999. Hormones and hemodynamics in heart failure. *New England Journal of Medicine*, 341(8), pp.577–585.

Schweda, F. & Kurtz, A., 2004. Cellular mechanism of renin release. *Acta Physiologica Scandinavica*, pp.383–390.

Sealey, J.E. et al., 1996. Specific prorenin/renin binding (ProBP) identification and characterization of a novel membrane site. *American Journal of Hypertension*, 9(5), pp.491–495.

Sealey, J.E. & Laragh, J.H., 2007. Aliskiren, the first Renin inhibitor for treating hypertension: reactive renin secretion may limit its effectiveness. *American Journal of Hypertension*, 20(5), pp.587–597.

Serajuddin, A.T.M., 2007. Salt formation to improve drug solubility. *Advanced Drug Delivery Reviews*, (59), pp.603 – 616.

Sharma, J.N., 2006. Role of tissue kallikrein–kininogen–kinin pathways in the cardiovascular system. *Archives of Medical Research*, 37, pp.299–306.

Sica, D., 2010. The evolution of renin-angiotensin blockade: angiotensin-converting enzyme inhibitors as the starting point. *Current Hypertension Reports*, 12(2), pp.67–73.

Sim, M.K. & Min, L., 1998. Effects of des-Asp-angiotensin I on experimentally-induced cardiac hypertrophy in rats. *International Journal of Cardiology*, 63, pp.223–227.

Siragy, H.M., Jaffa, A.A. & Margolius, H.S., 1997. Bradykinin B2 receptor modulates renal prostaglandin E2 and nitric oxide. *Hypertension*, 29 (3), pp.757–762.

Siragy et al., 2005. Angiotensin subtype-2 receptors inhibit renin biosynthesis and Angiotensin II formation. *Hypertension*, 45, pp.133–137.

Sjöström, H., Norén, O. & Olsen, J., 2000. Structure and function of aminopeptidase N. *Advances in Experimental Medicine and Biology*, 477, pp.25–34.

Skeggs, L.T. et al., 1954. The existence of two forms of hypertensin. *The Journal of Experimental Medicine*, 99(3), pp.275–282.

Skott, O. & Briggs, J.P., 1987. Direct demonstration of macula densa-mediated renin secretion. *Science*, 237 (4822), pp.1618–1620.

Sliwa, K. et al., 2012. Contribution of the human immunodeficiency virus/acquired immunodeficiency syndrome epidemic to de novo presentations of heart disease in the Heart of Soweto Study cohort. *European Heart Journal*, 33 (7), pp.866–874.

Smyrnios, N.A., Irwin, R.S. & Curley, F.J., 1995. Chronic cough with a history of excessive sputum production. The spectrum and frequency of causes, key components of the diagnostic evaluation, and outcome of specific therapy. *Chest*, 108(4), pp.991–997.

Soffer, R.L. et al., 1987. Pulmonary and testicular angiotensin-converting isoenzymes. *Clinical and Experimental Hypertension*, A9(2-3), pp.229–234.

Soonpaa, M.H. & Field, L.J., 1998. Survey of studies examining mammalian cardiomyocyte DNA synthesis . *Circulation Research*, 83 (1), pp.15–26.

Soubrier, F. et al., 1988. Two putative active centers in human angiotensin I-converting enzyme revealed by molecular cloning. *Proceedings of the National Academy of Sciences of the United States of America*, 85(24), pp.9386–90.

St John Sutton, M. et al., 1997. Cardiovascular death and left ventricular remodeling two years after myocardial infarction: baseline predictors and impact of long-term use of captopril: information from the Survival and Ventricular Enlargement (SAVE) trial. *Circulation*, 96(10), pp.3294–9.

St John Sutton, M. et al., 2003. Left ventricular remodeling and ventricular arrhythmias after myocardial infarction. *Circulation*, 107, pp.2577–2582.

Stanton, A. et al., 2003. Blood pressure lowering in essential hypertension with an oral renin inhibitor, Aliskiren. *Hypertension*, 42 (6), pp.1137–1143.

Stichtenoth, D.O., Wagner, B & Frölich, J.C., 1998. Effect of selective inhibition of the inducible cyclooxygenase on renin release in healthy volunteers. *Journal of Investigative Medicine : the Official Publication of the American Federation for Clinical Research*, 46(6), pp.290–296.

Stricker, E.M., 1968. Some physiological and motivational properties of the hypovolemic stimulus for thirst. *Physiology & Behavior*, 3(3), pp.379–385.

Swanson, G.N. et al., 1992. Discovery of a distinct binding site for angiotensin II (3-8), a putative angiotensin IV receptor. *Regulatory Peptides*, 40(3), pp.409–19.

Sweetana, S. & Akers, M.J., 1996. Solubility principles and practices for parenteral dosage form development. *PDA Journal of Pharmaceutical Science and Technology*, 50, pp.330 – 342.

Takagawa, J. et al., 2007. Myocardial infarct size measurement in the mouse chronic infarction model : comparison of area- and length-based approaches. *Journal of Applied Physiology*, pp.2104–2111.

Thompson, N. et al., 1988. Transforming growth factor beta-1 in acute myocardial infarction in rats. *Growth Factors*, 1, pp.91–99.

Thygesen, K., Alpert, J.S. & White, H.D., 2007. Universal definition of myocardial infarction. *Journal of the American College of Cardiology*, 50(22), pp.2173–95.

Tigerstedt, R. & Bergman, P., 1898. Niere und Kreislauf. *Skand Arch Physiol*, 8, pp.223–271.

Tikkanen, I., Omvik, P. & Jensen, H.A., 1995. Comparison of the angiotensin II antagonist losartan with the angiotensin converting enzyme inhibitor enalapril in patients with essential hypertension. *Journal of Hypertension*, 13(11), pp.1343–51.

Timmermans, P.B. et al., 1993. Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacological Reviews*, 45(2), pp.205–251.

Tipnis, S. et al., 2000. A human homolog of angiotensin-converting enzyme: cloning and functional expression as a captopril-insensitive carboxypeptidase. *Journal of Biological Chemistry*, 275, pp.33238–33243.

Tom, B. et al., 2001. Bradykinin potentiation by angiotensin-(1-7) and ACE inhibitors correlates with ACE C- and N-domain blockade. *Hypertension*, 38(1), pp.95–99.

Tom, B., Dendorfer, A. & Danser, A.H., 2003. Bradykinin , angiotensin-(1–7), and ACE inhibitors : how do they interact ? *International Journal of Biochemistry*, 35, pp.792–801.

Touyz, R.M., Tabet, F. & Schiffrin, E.L., 2003. Redox-dependent signalling by angiotensin II and vascular remodelling in hypertension. *Clinical and Experimental Pharmacology & Physiology*, 30(11), pp.860–866.

Traynor, T.R. et al., 1999. Inhibition of macula densa-stimulated renin secretion by pharmacological blockade of cyclooxygenase-2. *American Journal of Physiology - Renal Physiology*, 277 (5), pp.F706–F710.

Triant, V.A. et al., 2007. Increased acute myocardial infarction rates and cardiovascular risk factors among patients with human immunodeficiency virus disease. *Journal of Clinical Endocrinology & Metabolism* , 92 (7), pp.2506–2512.

Tsutsumi, Y. et al., 1999. Angiotensin II type 2 receptor overexpression activates the vascular kinin system and causes vasodilation. *The Journal of Clinical Investigation*, 104(7), pp.925–935.

Tsuzuki, S. et al., 1996. Angiotensin II type 2 receptor inhibits cell proliferation and activates tyrosine phosphatase. *Hypertension*, 28 (5), pp.916–918.

Tzakos, A.G. & Gerothanassis, I.P., 2005. Domain-selective ligand-binding modes and atomic level pharmacophore refinement in angiotensin I converting enzyme (ACE) inhibitors. *Chembiochem : a European Journal of Chemical Biology*, 6(6), pp.1089–103.

Uhlich, E. et al., 1975. Angiotensin stimulated AVP-release in humans. *Journal of Molecular Medicine*, 53(4), pp.177–180.

Urata, H et al., 1990. Identification of a highly specific chymase as the major angiotensin II-forming enzyme in the human heart. *The Journal of Biological Chemistry*, 265(36), pp.22348–57.

Urata, Hidenori et al., 1993. Cellular localization and regional distribution of an angiotensin II-forming chymase in the heart. *Journal of Clinical Investigation*, 91(4), pp.1269–1281.

Uresin, Y. et al., 2007. Efficacy and safety of the direct renin inhibitor aliskiren and ramipril alone or in combination in patients with diabetes and hypertension. *JRAAS: Journal of the Renin-Angiotensin-Aldosterone system*, 8(4), pp.190–8.

Vander, A., 1967. Control of renin release. *Physiological Reviews*, 47(3), pp.359–382.

Vaughan, D.E., 2001. Angiotensin, fibrinolysis, and vascular homeostasis. *The American Journal of Cardiology*, 87(8A), p.18C–24C.

Vavasin, M. et al., 2004. Prolyl oligopeptidase is involved in release of the antifibrotic peptide Ac-SDKP. *Hypertension*, 43, pp.1140–1145.

Veber, D.F. et al., 2002. Molecular properties that influence the oral bioavailability of drug candidates. *Journal of Medicinal Chemistry,* 45(12), pp.2615–2623.

Vickers, C. et al., 2002. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *The Journal of Biological Chemistry*, 277(17), pp.14838–43.

Villamil, A. et al., 2007. Renin inhibition with aliskiren provides additive antihypertensive efficacy when used in combination with hydrochlorothiazide. *Journal of Hypertension*, 25(1), pp.217-226.

Vleeming, W. et al., 1998. ACE inhibitor-induced angioedema. Incidence, prevention and management. *Drug Safety*, 18(3), pp.171–188.

Wagner, C. et al., 2000. Regulation of renin gene expression in kidneys of eNOS- and nNOS-deficient mice. *Pflügers Archiv: European Journal of Physiology*, 439(5), pp.567–572.

Wang, D. et al., 2004. N-acetyl-seryl-aspartyl-lysyl-proline stimulates angiogenesis in vitro and in vivo . *American Journal of Physiology - Heart and Circulatory Physiology* , 287 (5), pp.H2099–H2105.

Wang, H.X. et al., 2010. Effects of angiotensin III on protein, DNA, and collagen synthesis of neonatal cardiomyocytes and cardiac fibroblasts in vitro. *Journal of Cardiovascular Pharmacology and Therapeutics*, 15, pp.393-402.

Warren, S.E. et al., 1988. Time course of left ventricular dilation after myocardial infarction: influence of infarct-related artery and success of coronary thrombolysis. *Journal of the American College of Cardiology*, 11(1), pp.12–9.

Warren, S.E. & O'Connor, D.T., 1980. Hyperkalemia resulting from captopril administration. *JAMA : the Journal of the American Medical Association*, 244(22), pp.2551–2552.

Watermeyer, J.M. et al., 2008. Probing the basis of domain-dependent inhibition using novel ketone inhibitors of angiotensin-converting enzyme. *Biochemistry*, 47(22), pp.5942–5950.

Watermeyer, J.M. et al., 2010. Characterization of domain-selective inhibitor binding in angiotensin-converting enzyme using a novel derivative of lisinopril. *The Biochemical Journal*, 428(1), pp.67–74.

Weber, M.A. & Messerli, F.H., 2008. Angiotensin-Converting Enzyme Inhibitors and Angioedema. *Hypertension* , 51 (6), pp.1465–1467.

Wei, C.C. et al., 2010. Mast cell chymase limits the cardiac efficacy of Ang I-converting enzyme inhibitor therapy in rodents. *Journal of Clinical Investigation*, 120(4), pp.1229–39.

Wei, L. et al., 1991. The two homologous domains of human angiotensin I-converting enzyme are both catalytically active. *Journal of Biological Chemistry*, 266 (14), pp.9002–9008.

Wei, L. & Clauser, E., 1992. The Two Homologous Domains of Human Angiotensin I-converting Enzyme Interact Differently with Competitive Inhibitors *. *Biochemistry*, 267(19), pp.13398–13405.

Weinberger, M.H., Aoi, W. & Henry, D.P., 1975. Direct effect of beta-adrenergic stimulation on renin release by the rat kidney slice in vitro. *Circulation Research*, 37 (3), pp.318–324.

Weir, M.R. et al., 2007. Antihypertensive efficacy, safety, and tolerability of the oral direct renin inhibitor aliskiren in patients with hypertension: a pooled analysis. *JASH : Journal of the American Society of Hypertension*, 1(4), pp.264–277.

Welches, W.R. et al., 1991. Evidence that prolyl endopeptidase participates in the processing of brain angiotensin. *Journal of Hypertension*, 9(7), p.631.

Wen, Q. & Sim, M.-K., 2011. Effects of des-aspartate-angiotensin I on myocardial ischemia-reperfusion injury in rats. *European Journal of Pharmacology*, 658(2-3), pp.193–9.

Wen, Q., Sim, Meng-kwoon & Tang, F., 2004. Reduction of infarct size by orally administered des-aspartate-angiotensin I in the ischemic reperfused rat heart. *Regulatory Peptides*, 120, pp.149–153.

White, C., 1998. Pharmacologic, pharmacokinetic, and therapeutic differences among ACE inhibitors. *Pharmacotherapy*, 18(3), pp.588-599.

White, H. et al., 1987. Left ventricular end-systolic volume as the major determinant of survival after recovery frommyocardial infarction. *Circulation*, 76, pp.44–51.

WHO, 2011. Cardiovascular diseases (CVDs) WHO Fact Sheet.

Wiemer, G. et al., 1993. The possible role of angiotensin II subtype AT2 receptors in endothelial cells and isolated ischemic rat hearts. *Journal of Hypertension*, 11(5), pp.S234-S235.

Wolf, K. et al., 1999. Inhibition of the renin-angiotensin system upregulates cyclooxygenase-2 expression in the macula densa. *Hypertension*, 34 (3), pp.503–507.

Wollert, K.C. et al., 2010. Survival after myocardial infarction in the rat. Role of tissue angiotensin-converting enzyme inhibition. *Circulation, 90(5), pp.2457-2467.*

Wolny, A. et al., 1997. Functional and biochemical analysis of angiotensin II-forming pathways in the human heart. *Circulation Research*, 80(2), pp.219–227.

Wood, J., Maibaum, J. & Rahuel, J., 2003. Structure-based design of aliskiren, a novel orally effective renin inhibitor. *Biochemical and Biophysical Research Communications*, 308, pp.698–705.

Wright, J.W. et al., 1993. Angiotensin II(3-8) (ANG IV) hippocampal binding: potential role in the facilitation of memory. *Brain Research Bulletin*, 32(5), pp.497–502.

Wright, J.W. et al., 1995. The angiotensin IV system: functional implications. *Frontiers in Neuroendocrinology*, 16(1), pp.23–52.

Xu, J. et al., 2005. Role of the B 1 kinin receptor in the regulation of cardiac function and remodeling after myocardial infarction. *Hypertension*, 45(4), pp.747-753.

Yamada, K. et al., 1998. Converting enzyme determines plasma clearance of angiotensin-(1–7). *Hypertension*, 32, 496-502.

Yamada, T., Horiuchi, M. & Dzau, V.J., 1996. Angiotensin II type 2 receptor mediates programmed cell death . *Proceedings of the National Academy of Sciences* , 93 (1), pp.156–160.

Yamagishi, H. et al., 1993. Contribution of cardiac renin-angiotensin system to ventricular remodelling in myocardial-infarcted rats. *Journal of Molecular and Cellular Cardiology*, 25(11), p.1369.

Yamamoto, K. et al., 1992. In vivo metabolism of angiotensin I by neutral endopeptidase (EC 3.4.24.11) in spontaneously hypertensive rats. *Hypertension*, 19(6, Pt 2), pp.692–696.

Yang, F. et al., 2004. Ac-SDKP reverses inflammation and fibrosis in rats with heart failure after myocardial infarction. *Hypertension* , 43 (2), pp.229–236.

Yasuda, N. et al., 2008. Conformational switch of angiotensin II type 1 receptor underlying mechanical stress-induced activation. *EMBO Reports*, 9(2), pp.179–186.

Yatabe, J. et al., 2011. Angiotensin III stimulates aldosterone secretion from adrenal gland partially via angiotensin II type 2 receptor but not angiotensin II type 1 receptor. *Endocrinology*, 152, pp.1582–1588.

Yoshida, M. et al., 1996. Modulation by nitric oxide and prostaglandin of the renal vascular response to angiotensin II (3-8). *British Journal of Pharmacology*, 117(5), pp.885–90.

Yusuf, S. et al., 2000. Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. *The New England Journal of Medicine*, 342(3), pp.145–53.

Zablocki, D. & Sadoshima, J. 2011. Knocking Out Angiotensin II in the Heart. *Current Hypertension Reports*, 13(2), pp.129–135.

Zini, S. et al., 1996. Identification of metabolic pathways of brain angiotensin II and III using specific aminopeptidase inhibitors: predominant role of angiotensin III in the control of vasopressin release. *Proceedings of the National Academy of Sciences USA*, 93, pp.11968–11973.

Zini, S. et al., 1998. Inhibition of vasopressinergic neurons by central injection of a specific aminopeptidase A inhibitor. *Neuroreport*, 9, pp.825–828.