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Evaluation of candidate peptides for the immunisation against angiotensin II

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Zusammenfassung

Arterielle Hypertonie betrifft bis zu 600 Millionen Menschen weltweit. Chronisch erhöhter Blutdruck ist ein großer Risikofaktor für Herz-Kreislauferkrankungen. Das sogenannte Renin-Angiotensin System (RAS) erhält das Gleichgewicht zwischen gefäßverengenden und gefäßerweiternden Effekten auf den Blutkreislauf. Ein wichtiger Bestandteil dieses Systems ist Angiotensin II (Ang II). Dieses aus acht Aminosäuren zusammengesetzte Peptid wirkt gefäßverengend und induziert die Wiederaufnahme von Wasser aus dem Urin ins Blut. Dadurch erhöht Ang II den Blutdruck. Angiotensin (1-7) (Ang 1-7), ein weiteres RAS- Peptid, hingegen hat eine gegensätzliche Wirkung auf das Herz-Kreislaufsystem.

Verschiedene niedermolekulare Substanzen werden eingesetzt, um die Produktion oder die Wirkung von Ang II zu blockieren und somit den Blutdruck zu senken. Diese müssen täglich eingenommen werden und zeigen nur in Kombination Effektivität. Daher ist es ein wichtiges Anliegen, neue effektive Therapien gegen Bluthochdruck und seine fatalen Folgen hervorzubringen. Die Entwicklung eines Impfstoffs gegen Ang II könnte hier einen wesentlichen Beitrag leisten.

Die vorliegende Arbeit ist Teil eines Projekts, das darauf abzielt einen Peptid-Impfstoff zu entwickeln, der es ermöglicht Ang II aus dem System zu entfernen, ohne Ang 1-7 oder seinen Vorläufer, Angiotensin I (Ang I), anzutasten. Hierfür wurde die Immunogenität verschiedener Ang II-Peptid Varianten getestet, sowie deren Fähigkeit überprüft, Seren mit unterschiedlicher Reaktivität zu induzieren. Daten aus verschiedenen ELISA (enzyme linked immunosorbent assay)- Experimenten zeigen, dass Peptid-Varianten, auch Variotope® genannt, selektiert werden konnten, die Antikörper induzieren, welche eine stark reduzierte Reaktivität gegen Ang 1-7 und Ang I aufweisen, das Ang II Peptid jedoch nach wie vor effektiv binden.

Ein zellulärer Assay soll weiters die Überprüfung der inhibitorischen Fähigkeit von Variotop®-induzierten Seren *in vitro* ermöglichen. Der hier entwickelte Assay basiert auf der Expression von Luziferase durch die Aktivierung von NF-κB in HEK293 Zellen. Stimulation der, zusätzlich mit einem Angiotensin II Rezeptor-GFP Konstrukt transient transfektierten, HEK293 Zellen mit Ang II bewirkt einen signifikanten Signal-Anstieg. Die große Differenz zwischen unstimuliertem und stimuliertem Zustand, ermöglicht die Bestimmung der blockierenden Wirkung von Ang II- Antikörpern.

Abstract

Hypertension is the most prevalent risk factor for cardiovascular disease (CVD) and thus constitutes an important worldwide public-health challenge. The renin-angiotensin system (RAS) maintains the balance of contractive and dilutive effects within the vasculature. Angiotensin II (Ang II), an octapeptide, is a potent mediator of vasoconstriction and anti-diuretic effects and thus elevates blood pressure. Angiotensin (1-7) (Ang 1-7), a heptapeptide derived from angiotensin I (Ang I) or Ang II via angiotensin-converting enzyme (ACE) or angiotensin-converting enzyme II (ACE2) respectively is known to counteract these actions. Vaccination therapies targeting a component of the RAS other than Ang II have so far been unsuccessful in achieving target blood pressure or have even induced autoimmune disease. However, a phase II clinical study using Ang II as a target for immunotherapy conducted has been successful in decreasing blood pressure levels in humans.

AFFiRiS AG is developing a peptide-based hypertension vaccine targeting Ang II, while sparing Ang I and Ang 1-7 to maintain its vasodilative potential. Therefore, different candidates of Ang II-derived peptide-variants (Variotopes®) are evaluated for immunogenicity and specificity for Ang II. ELISA results show that it is possible to induce higher antibody titres against Ang II (a measurement for immunogenicity) while reducing the crossreactivity of antibodies to Ang I and Ang 1-7 with Variotopes®. The ability of Variotope®-induced antibodies to block the binding of Ang II to its receptor should be determined with a functional assay based on cell cultures. A luciferase activity assay has been established to measure the effects of Ang II stimulation in HEK293 cells transiently co-transfected with AT_1R-EGFP and NFkB-luciferase expression vectors. The luciferase activity is significantly higher in stimulated than in un-stimulated cells, thus giving enough margin to determine inhibitory properties of antibodies. Furthermore, this signal increase could be reversed with Angiotensin II type I receptor blocker losartan. The established assay can be used to determine the potential correlation between antibody titre, affinity and functionality.

1. Introduction

1.1. Cardiovascular disease (CVD) and hypertension

According to the World Heath Organisation (WHO) approximately 30% of all global deaths are attributed to cardiovascular disease (CVD). Nearly every fifth person over the age of 15 in Austria is affected with high blood pressure [1]. CVD is caused by disorders of the heart and blood vessels and encompasses various manifestations, including myocardial infarction, stroke, heart failure, and chronic kidney disease. Hypertension is the most prevalent risk factor for CVD and thus constitutes an important worldwide public-health challenge. It has been identified as the leading risk factor for mortality and is ranked third as a cause of disability-adjusted life-years. 26.4% of the world's adult population had hypertension in 2000 and international health studies project an increase of 60% in the total number of patients until 2025 [2]. Hypertension, commonly referred to as high blood pressure is defined as chronically elevated blood pressure with a systolic blood pressure above 140 mmHg and/or a diastolic blood pressure above 90 mmHg (see Figure 1 [3]).

Table 1. Classification and Management of Blood Pressure for Adults Aged 18 Years or Older

BP Classification	Systolic BP, mm Hg*		Diastolic BP, mm Hg*	Management*		
				Lifestyle Modification	Initial Drug Therapy	
					Without Compelling Indication	With Compelling Indications†
Normal	<120	and	<80	Encourage		
Prehypertension	120-139	or	80-89	Yes	No antihypertensive drug indicated	Drug(s) for the compelling indications‡
Stage 1 hypertension	140-159	or	90-99	Yes	Thiazide-type diuretics for most; may consider ACE inhibitor, ARB, β-blocker, CCB, or combination	Drug(s) for the compelling indications Other antihypertensive drugs (diuretics, ACE inhibitor, ARB, β-blocker, CCB) as needed
Stage 2 hypertension	≥160	or	≥100	Yes	2-Drug combination for most (usually thiazide-type diuretic and ACE inhibitor or ARB or β-blocker or CCB)§	Drug(s) for the compelling indications Other antihypertensive drugs (diuretics, ACE inhibitor, ARB, β-blocker, CCB) as needed

Abbreviations: ACE, angiotensin-converting enzyme; ARB, angiotensin-receptor blocker; BP, blood pressure; CCB, calcium channel blocker.
*Treatment determined by highest BP category.
†See Table 6.
‡Treat patients with chronic kidney disease or diabetes to BP goal of less than 130/80 mm Hg.
§Initial combined therapy should be used cautiously in those at risk for orthostatic hypotension.

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Figure 1: Classification and Management of Blood Pressure for Adults Aged 18 Years or Older cited from Chobanian AV et al. *JAMA* 2003, 289: 2560-2572: The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure

Lowering the blood pressure is an important strategy to prevent CVD. First steps towards the reduction of blood pressure are changes in life style, targeting primary factors like overweight, high sodium uptake through an unhealthy diet, physical inactivity, high alcohol consumption and smoking. However, treatment of chronic significantly elevated

hypertension requires specific therapies. A key regulator of the blood pressure is the renin-angiotensin system (RAS) which has become an attractive target for therapeutic intervention and treatment of hypertension.

1.2. The renin-angiotensin system (RAS)

The main regulator for vascular tone, fluid and electrolyte homeostasis, and blood pressure is the renin-angiotensin system (RAS). Circulating components of the RAS act as endocrine factors in order to maintain blood pressure and electrolyte as well as fluid balance. In addition to this circulating RAS, the so-called tissue RAS can function locally as paracrine and/or autocrine factor. This enables the local RAS system to cooperate with circulating RAS factors, but also allows it to operate independently of its circulating counterpart.

The RAS pathway is a cascade of protein conversions beginning with the cleavage of angiotensinogen (AGT) by renin. AGT is mainly generated and constitutively secreted into the circulation by hepatocytes. Renin is an aspartyl protease synthesised in and secreted from the granules of juxtaglomerular cells in the kidney. It has high substrate specificity for AGT, cleaving this precursor protein at its N-terminus to produce the inactive peptide angiotensin I (Ang I). The decapeptide Ang I, is rapidly processed by angiotensin-converting enzyme (ACE) to angiotensin II (Ang II) [4]. ACE is a membrane-bound metalloproteinase expressed on the surface of endothelial cells with the highest concentrations found on the vascular epithelium in the lung. Besides ACE, chymase and other enzymes such as tonin and cathepsin have been shown to produce Ang II from Ang I. In contrast to its precursor-peptide, Ang II shows high biological activity. Additionally, other Ang I- and Ang II-derived, functional peptides can be found in the circulation. These are generated by amino-, carboxy- or endopeptidases and include Ang (1-9), Ang (1-7), Ang III (the 2-8 peptide) and Ang IV (the 3-8 peptide).

Ang II, an octapeptide, regulates the body's fluid and sodium balance, the blood pressure, cellular growth and cardiovascular remodelling and is thus considered the main effector of the RAS. The actions of Ang II are predominantly mediated by two G-protein-coupled seven transmembrane receptors, the Ang II type 1 receptor (AT_1R) and the Ang II type 2 receptor (AT_2R). The AT_1 and AT_2 receptor subtypes bind Ang II similarly, but have a different cellular localization, are differentially expressed in diverse tissues and are thought to mediate opposite functions. Most of Ang II hypertensinogenic actions are attributed to the AT_1 receptor (see Figure 2 [5]).

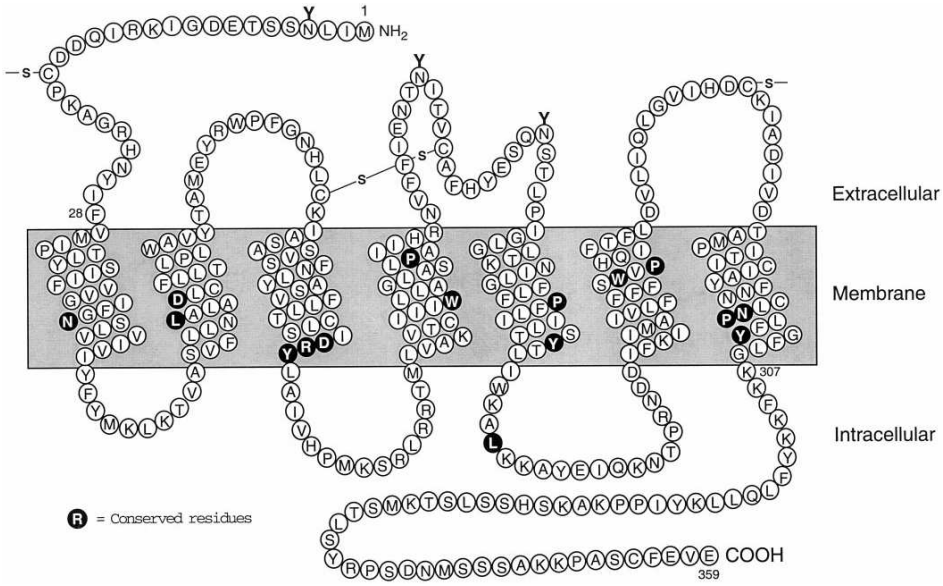


Figure 2: Angiotensin receptor type I

The activation of the AT_1 receptor on vascular smooth muscle cells (VSMC) leads to vasoconstriction throughout the whole body. Thereby, Ang II increases systemic arterial blood pressure and decreases the blood flow. This is also mediated by the induction of fibrosis, cellular growth and migration, and the generation of reactive oxygen species (ROS) by Ang II signalling through AT_1R. In the adrenal cortex, Ang II causes the release of aldosterone which leads to increased re-absorption of sodium and water in the kidneys. Ang II also acts on the central nervous system inducing a feeling of thirst. Additionally, Ang II stimulates the release of Anti Diuretic Hormone (ADH), also called vasopressin. Vasopressin normally acts in cases of dehydration, where it signals the kidney to increase the reuptake of water from the urine and to increase peripheral vascular resistance. Thus it also leads to increased arterial blood pressure [5]. The molecular mechanisms of AT_1R signalling are described in the next part of the introduction.

The AT_2 receptor is located on the X chromosome in humans and rodents and shares only 34% identity with its AT_1R counterpart. Furthermore, the AT_2 receptor is clearly distinct from the AT_1 receptor concerning tissue-specific expression and signalling mechanisms. The AT_1R is expressed in the heart, the kidney, the lung, the aorta, the liver, in testis and ovary, the adrenal and the pituitary gland in the brain. However, it is most abundantly expressed in vascular smooth muscle cells. AT_1R expression is stimulated by glucocorticoids and depends on the activity of the RAS. In most adult tissues, AT_2R is also expressed, though to a much lesser extent than AT_1R. Some fetal tissues, however, express AT_2R at high levels. Levels of AT_1R expression, in contrast, are not age- or development- dependent. AT_2R expression is found to be up-regulated in the non-

pregnant uterus and in pathological circumstances, such as cardiac hypertrophy, myocardial infarction, cardiomyopathy and congestive heart failure. In these pathologic conditions, the AT_1 receptor is significantly down-regulated. The AT_2 receptor also emerges during wound healing of the skin. In the kidney, AT_2R is predominantly expressed in embryonic mesenchymal cells that undergo apoptosis and are replaced by tubular tissue during development. However, the level of AT_2R expression is dramatically decreased in adult tissues. The liver and the pituitary gland are completely devoid of AT_2R. It is not clear how AT_2R expression and activity are regulated, but it is assumed that post-transcriptional and/or translational modulation is involved in these processes [5]. However, the AT_2 receptor lacks internalisation and desensitisation mechanisms and thus is thought to maintain differentiated cells in a quiescent state. In contrast to the effects of AT_1R stimulation, an activated AT_2 receptor suppresses tissue and cellular growth and causes vasodilatation via the release of nitric oxide (NO) [4]. Although, the AT_2R shares the structural features of seven transmembrane domain receptors, it does not modulate cytosolic Ca^{2+} or cyclic AMP, does not seem to be coupled to a heterotrimeric G protein and thus, does not reveal any functional features characteristic for this class of receptors. However, AT_2R might negatively modulate AT_1R-mediated phospholipase C activation, lead to cGMP (or NO) generation and activate protein tyrosine and serine/threonine phosphatases [5]. Furthermore, AT_2R has been found to counteract the mitogenic and the fibrotic action of AT_1R.

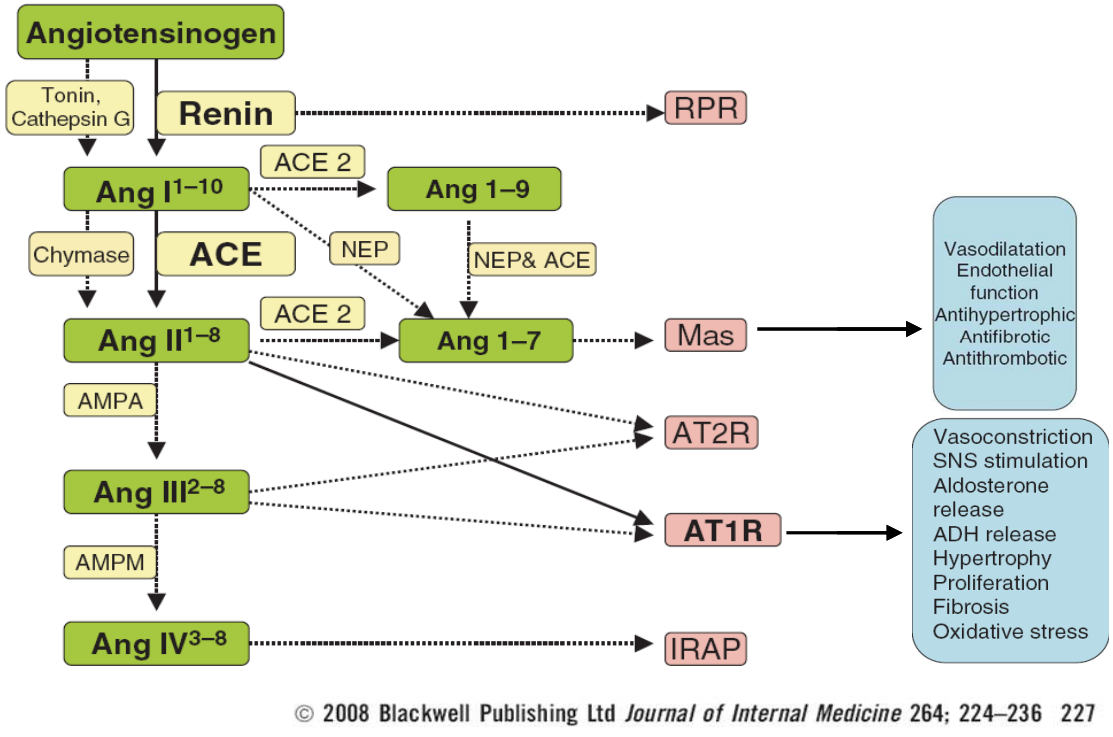


Figure 3: The two arms of the RAS pathway

Ang 1-7 was long thought to lack any biological activity. Recently, a second converting enzyme, the carboxypeptidase, angiotensin-converting enzyme II (ACE2) has been described to act on Ang I as well as Ang II [6] (see Figure 3). ACE inhibitors widely used as drugs against hypertension do not inhibit ACE2, but cause its up-regulation. The disruption of the ACE2 gene leads to severe cardiac contractility defects, increased Ang II levels and the up-regulation of hypoxia-induced genes in humans [4]. ACE2 generates Ang1-9 from Ang I and Ang 1-7 from Ang II. Ang 1-7 can also be produced from Ang I via Ang1-9 by ACE2 and consecutively by ACE, in parallel to the conversion of Ang I to Ang II (see Figure 3). In contrast to Ang II, which elevates blood pressure and appears to be the major mediator of vascular remodelling and inflammation in hypertension, the Ang 1-7 peptide promotes vasodilatation and thus may counterbalance the potentially detrimental actions of Ang II. In addition to a reduction in Ang II levels through Ang 1-7 production, the Ang 1-7 heptapeptide counteracts Ang II by causing vasodilatation, antitropic effects and by interacting with the prostaglandin-bradykinin-NO system. These effects of Ang 1-7 are thought to be mediated by its receptor, the mas oncogen product (MAS) [7]. Like the angiotensin type 1 and type 2 receptors, the Mas receptor belongs to the family of seven transmembrane G-protein coupled receptors too. Many studies show that Ang 1-7 opposes the effects of Ang II indirectly by stimulating NO and prostacyclin release. Interestingly, Sampaio and his group found that Ang 1-7 significantly decreases the Ang II-stimulated activation of Nox-based nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase by Src homology 2-containing inositol phosphatase 2 (SHP-2) phosphorylation in human aortic endothelial cells (HAEC). This shows that Ang 1-7 signalling also directly interplays with Ang II stimulated signalling pathways. In fact, Ang 1-7 has been found to antagonise Ang II-induced activation of protein kinase C and ERK1/2 (extracellular signal related kinases), to inhibit Ang II-stimulated MAP kinase phosphorylation through prostacyclin-mediated production of cAMP and to reduce Ang II-mediated phosphorylation of p38 MAP kinase and jun N-terminal kinase. However, Ang 1-7 alone did not cause any of these effects [7].

Ang III is produced from Ang II by aminopeptidase A and elicits similar effects as Ang II via AT_1 and AT_2 receptors. However, Ang III has a significantly higher clearance rate than Ang II, indicating that the latter plays a more dominant role as an effector of the circulating RAS. Ang IV is generated from Ang III by aminopeptidase M and acts on an insulin-regulated aminopeptidase receptor (IRAP), also known as AT_4R. Its activation leads to renal vasodilatation, hypertrophy and the activation of NF-κB. Several studies suggest that Ang IV is an important regulator of cognition, renal metabolism and cardiovascular damage and is also involved in vascular inflammatory responses [4].

1.3 Ang II type 1 receptor signalling

The AT_1R subtype of Ang II receptors is predominantly expressed in vascular smooth muscle cells. The ligand binding site of this seven transmembrane receptor is located on the extracellular surface of the plasma membrane, while the G-protein binding site faces the cytosol. G-proteins consist of three subunits: α, β and γ. The α-subunit has a guanosine-triphosphate (GTP) binding and GTPase activity. Thereby, activated α-subunits regulate effector molecules such as adenylyl cyclase, and phospholipase C. The signalling reaction is terminated after hydrolysis of GTP to guanosine-diphosphate (GDP). After desensitisation, the receptor is uncoupled from the G-protein, phosphorylated and consequently internalised and recycled [8]. The AT_1 receptor interacts with multiple heterotrimeric G-proteins, including $G_{q/11}$, G_i, G_{12} and G_{13} and thus stimulates the production of second messenger molecules and ROS (reactive oxygen species).

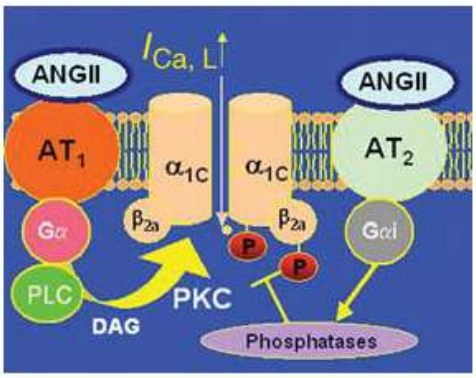


Figure 4: G-protein coupled activation of phospholipase C, formation of diacylglycerate and activation of protein kinase C

Stimulation of the Ang II type I receptor leads to the activation of phospholipase C-β (PLC-β) and the subsequent formation of diacylglycerol (DAG) and inositol triphosphate (IP_3). In cultured rat VSMCs PLC-γ is activated rather than PLC-β. DAG activates protein kinase C (PKC), whereas IP_3 mediates the release of calcium from intracellular stores (smooth endoplasmatic reticulum) (see also Figure 4 [10]).

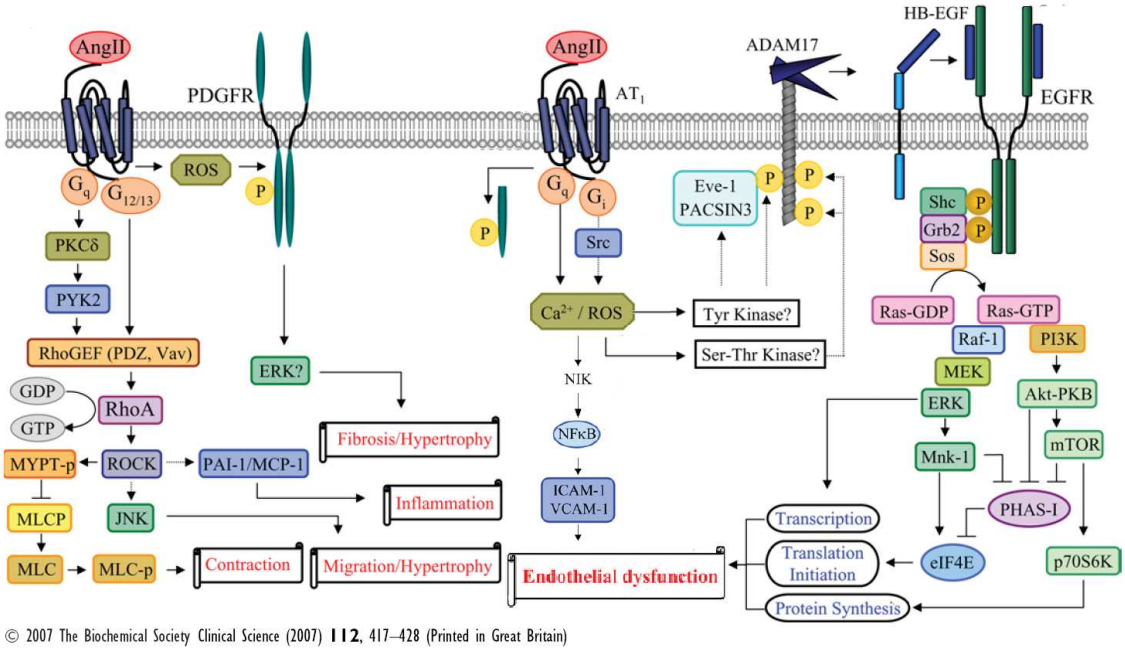


Figure 5: Angiotensin II signalling via the Angiotensin II receptor type I

In addition to this traditionally transcribed pathway, AT_1 receptor stimulation also leads to mitogen-activated protein (MAP) kinase activation in VSMC, glomerulosa cells, renal mesanglial cells, cardiac myocytes and fibroblasts. This is mainly due to the Ang II-mediated transactivation of the endothelial growth factor receptor (EGFR). Ang II-induced EGFR transactivation seems to be Ca^{2+} sensitive and requires the presence of ROS. A, so far unknown, kinase is thought to activate ADAM17 (a disintegrin and metalloproteinase17) that in turn mediates the production of an EGFR ligand, such as heparin-binding EGF (HB-EGF) depending on the cell type. Activated EGF-receptors lead to Shc/Grb2/Sos complex formation via their connected G-proteins. This adaptor-protein complex then activates the monomeric GTP-binding protein, Ras. Ras-GTP interacts with Raf-1 and thus activates kinases of the mitogen-activated protein kinase family, such as MEK-1, MEK-2 and PI3K. Other kinases, such as ERK1, ERK2 and Akt (protein kinase B) are activated downstream of these MAP kinase cascades and in turn activate transcription factors that are responsible for the cellular effects. One of these transcription factors is NF-kB. Ang II induces the phosphorylation of NF-kB through RhoA activation. Rho is another monomeric G-protein activated by a tyrosine kinase downstream of AT_1R stimulation. NF-kB activation subsequently induces cytokine (IL-6, IL-8) and adhesion molecule (ICAM-1, VCAM-1) expression and secretion in vascular endothelial cells. This suggests that the Rho/ROCK (Rho kinase) pathway has a critical role in the Ang II-induced progression of vascular inflammation (see also Figure 5) [9]. A third monomeric G-protein is activated by Ang II, called Rac. Rac is an important component of the NADPH oxidase complex, which in turn produces ROS and is also implicated in pathways

activating c-Jun N-terminal kinases (JNK). JNK are stress-activated phopho-kinases and have been shown to be indispensable for VSMC migration stimulated by Ang II.

Interestingly, experiments with AT_1 receptor point mutants suggest that the activation of MAP kinase and the formation of the second messengers IP_3 and DAG can occur independently through ligand-selective stabilisation of different active states of the receptor [10].

Endothelial AT_1 receptor signalling has been suggested to play a major role in regulating the balance between nitric oxide (NO) and ROS in endothelial cells. NADPH oxidase is a multisubunit enzyme, similar to the neutrophil oxidases that are present in vascular cells, kidney cells, and cells of the central nervous system. Several animal models of experimental hypertension show Nox-enzyme dependent ROS production. Ang II activates NADPH oxidase, and the $O_2^{\cdot -}$ that is subsequently produced, inhibits the endogenous vasodilator NO. Thereby vasoconstriction is promoted, increasing systemic vascular resistance and elevating the blood pressure. Moreover, $O_2^{\cdot -}$ and related ROS might increase renal sodium re-absorption, which would also contribute to hypertension. In addition to its hypertensive effects Ang II also directly promotes inflammation and the development of atherosclerosis. Inflammation occurs through activation of T lymphocytes by Ang II in an NADPH oxidase dependent way. This was shown by abolishing the proliferative effect of Ang II on T cells with the NADPH oxidase inhibitor apocynin [11].

The C-terminal cytoplasmic domain of the AT_1 receptor might be functionally important for AT_1 receptor signalling, desensitisation and receptor internalisation. It associates with non-G-protein signalling molecules and provides a binding site to form homo- and hetero-dimers of the AT_1 receptor. Two AT_1 receptor associated proteins have been identified to show contrary effects on AT_1R signalling upon receptor binding. ATRAP (AT_1-receptor associated protein) is thought to function as a negative regulator by potentiating AT_1 receptor internalisation. Whereas, ARAP1 (type 1 Ang II receptor associated protein 1) might promote AT_1 receptor recycling to the plasma membrane. An ARAP1 overexpressing mouse model suffers from hypertension [9].

Structural analysis of the peptide hormone Ang II by Aumelas and his group in 1985 revealed that the binding of Ang II and related peptides to receptors requires well-defined conformational and dynamical properties of the tyrosine-4 and histidine-6 side chains. Peptides with high agonist activity require the presence of both the phenyl ring and the free carboxyl group at the COOH terminus. However, it seems that the large aromatic residue at position 8 is not directly involved in receptor binding, but mostly the carboxyl group. This has been concluded, because the biological activity of AT_1R agonists is

greatly reduced or destroyed when the carboxyl group is amydated, when its configuration within the peptide is changed or when its position is shifted (such as in Ang I). Modifications in the side chain of the amino acid at position 8, may only affect the orientation of the histidine and tyrosine side chains that are thought to interact with the receptor [12].

1.4. Treatment of hypertension by targeting the RAS

Several drugs are currently available on the market to treat hypertension, including different kinds of diuretics, β-blockers, Ca^{2+}-channel blockers and small molecule inhibitors of diverse components of the RAS. Diuretics reverse the reuptake of salt and water from the urine induced by Ang II in the kidney and thereby also reduce blood pressure. β-blocker act antihypertensive on the sympathetic nervous system that controls heart functions. Ca^{2+} channel blockers reduce the influx of Ca^{2+} and thereby cause vasodilatation. Pharmaceuticals against hypertension that target the RAS encompass renin blockers, ACE inhibitors, angiotensin II receptor blockers (ARBs) and prorenin receptor blockers. ACE inhibitors bind the active site of ACE, thus interfering with the ability of the enzyme to bind and cleave its substrates. Characteristic side effects of ACE inhibitors are dry cough and angioneurotic oedema. ARBs specifically block the vasoconstrictor effects of Ang II by blocking the binding of angiotensin II to the AT_1 receptor.

Although, there is a variety of drugs available, antihypertensive treatment is still inadequately handled. 30% of patients with hypertension are still unaware of the danger of their condition. This is mainly due to the asymptomatic nature of hypertension. Effective treatment requires the motivation of the patient to continue daily drug intake over a long period of time. Because of the vast variety of risk factors and diseases like diabetes or metabolic syndrome that act on high blood pressure conditions, individual diagnosis and prescription of treatments is necessary. Furthermore, two or more medications are generally needed to achieve the goal blood pressure [3]. Thus in addition to inadequate treatment, key factors affecting patient's adherence to treatment, such as the presence of side-effects and concerns over taking long-term medication in the absence of symptoms are major reason for the high percentage of patients with arterial hypertension [13].

1.5. Vaccination approaches to target the RAS

Active immunisation inducing a specific immune response to components of the RAS could simplify antihypertensive treatments. One of the advantages of vaccination over the daily administration of small molecular drugs is that antibodies circulate in the

blood for a longer time span, resulting in a continuous blockade of angiotensin II. Current medications, in contrast, exhibit variations in activity, according to their way of administration that are not ideal for the treatment of a chronic condition, such as hypertension [14]. Ideally a long lasting effect would be achieved with a few injections per year. The ease of this antihypertensive vaccination therapy should encourage better adherence to treatment [13]. Furthermore, hypertensive immunotherapy would reduce drug interactions associated with conventional drug polypharmacy [15]. However, the success of an anti-hypertensive immunotherapy requires the identification of an appropriate target for vaccination, the debarment of the danger of autoimmune damage and the possibility to induce sufficiently large amounts of antibodies.

Facts that speak against the development of a vaccine against a self-antigen involve the inadequate knowledge about the effects of overstimulation of the immune system and the development of autoimmune diseases. Joël Ménard states in his review “A vaccine for hypertension” that high blood pressure is no longer a devastating disorder but a manageable risk factor. Pharmacological treatment of hypertension is widely available, safe and well-tolerated. Thus, the call for a vaccine targeting the RAS might not be justified. Furthermore, Ménard criticises that there are no data from long-term animal studies available that address the effects of a RAS vaccine in salt and water restrictive conditions. In his opinion, the vaccines, developed to treat hypertension so far, are applied in humans too rapidly and thus expose volunteers participating in clinical trials to an unacceptable risk. The booming research on vaccines targeting the RAS and other endogenous systems should thus rather be used to generate a better understanding of the structure and function of these systems and the complexity of immune responses involved in vaccination [16].

The first attempts to actively immunize against components of the RAS were performed by Harry Goldblatt in 1951, using renin as the main target. Renin catalyses the processing of angiotensinogen and thus presents the initial and rate-limiting step in the RAS cascade. Immunization against renin achieved complete inhibition of endogenous plasma renin activity and decrease of blood pressure. However, this attempt has been stopped, because the induced immune reaction caused an organ-specific autoimmune disease in the kidney.

Several other attempts to treat hypertension by vaccination used exogenous or endogenous antibodies raised against the different proteins and peptides of the RAS. Angiotensin converting enzyme was thought to present an attractive target for vaccination, because it is expressed on the endothelial surface and thus is directly exposed to circulating antibodies. On one hand, passive transfer of antibodies raised in rabbits

caused severe autoimmune reactions and death of the vaccinated rabbits. In rats, on the other hand, the same antibodies were able to limit the pressor effect of Ang II and led to a normalization of blood pressure within 24h. Active immunisation of rats with rabbit ACE induced a high specific antibody titre, but was lethal to the animals.

The first approaches to target Ang I or Ang II by active vaccination did not show any effect on the blood pressure (Michel et al., 1989). This could be related to the low affinity of induced antibodies for the peptide or the generation of angiotensin in tissues inaccessible to antibodies. Peptides such as angiotensin are too small for active immunisation and thus have to be coupled to an immunogenic protein carrier [17].

Recently, the potential of Ang I and II to mount antigen-specific and therapeutically effective immune responses against hypertension was re-investigated by several studies. In these approaches the angiotensins were chemically linked to different protein carriers like tetanus toxoid (TT), keyhole limpet haemocyanin (KLH) or diphtheria toxin (DT) and administrated together with aluminium hydroxide (Alum) or other adjuvants. Rats treated with an Ang I-TT conjugate vaccine, showed a significant reduction in the pressor response, when challenged with exogenous Ang I, but not with Ang II. These blood-pressure lowering effects were comparable to those of Angiotensin II type I receptor antagonists (valsartan and frusemide). Although the vaccination with Ang I-TT led to the induction of a high antigen-specific humoral immune response in both experimental animals and healthy human volunteers, the effects on blood pressure could not be seen in humans [15]. This finding was further confirmed in a study by Brown and his group with hypertensive patients who received a 12 amino acid analogue of Ang I covalently linked to KLH and adsorbed to Alum (referred to as PMD3117). The treatment regimen applied in this study was well tolerated and induced antigen-specific antibodies with a half-life of about 100 days. Additionally, PMD3117 vaccination induced changes in renin and aldosterone levels, proving its effect on the RAS. However, vaccination with PMD3117 could not influence the blood pressure of patients compared to the placebo control group [18]. However, a different formulation of this Ang I vaccine which was developed by Protherics [19] and replaced Alum by a new adjuvant, namely Co Vaccine HT™ did show an effect on blood pressure in human healthy volunteers (Figure 6) and also resulted in a 10-fold higher antibody response.

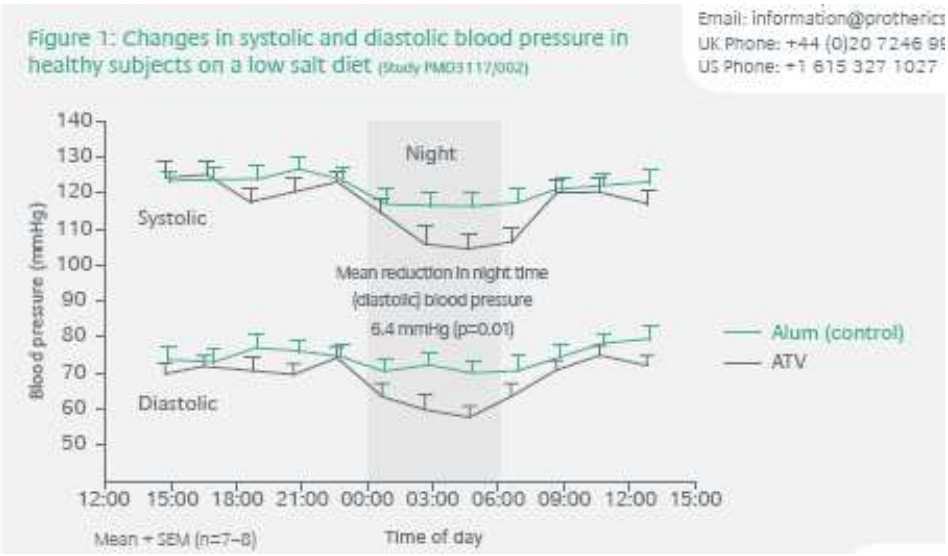


Figure 6: Results of the Proterics study: 18h blood pressure measurements of vaccinated and unvaccinated healthy human volunteers

Immunization against AT_1 receptor is in general complex, because antibodies must act in an antagonistic fashion to block its action. Most anti-AT_1R antibodies have been found to act agonist-like [20]. Furthermore, the accumulation of antibodies on cell surfaces may attract other components of the immune system such as complement system components and can therefore lead to autoimmune reactions. However, a study in young spontaneously hypertensive rats (SHR) immunised against a N-terminal peptide (residues 14- 23) of AT_1R conjugated with bovine gamma globulin (BGG) suspended in Freud's adjuvant found that the development of genetic hypertension could be attenuated in this animal model. When administered intracerebroventricularly, antibodies against the same sequence of AT_1R were shown to antagonise Ang II induced increase of blood pressure. Furthermore, these antibodies also prevented the blood pressure increase in Wistar rats subjected to two-kidney, one-clip procedure. In the early phase of this procedure, circulating Ang II levels are significantly increased. These results might indicate that the antibody competed with circulating Ang II for AT_1R binding or that the AT_1R is inactivated by internalisation upon antibody binding.[21] In another study, SHR were vaccinated against a peptide of the extracellular portion of AT_1R coded ATR12181 conjugated to TT and administered with Freud's adjuvant. Vaccination induced humoral immune responses against peptide ATR12181 as demonstrated by Enzyme-linked immunosorbent assay (ELISA). Furthermore, a significant decrease of blood pressure was observed in the vaccinated group. ATR12181 also significantly attenuated the advance of kidney damages in hypertension. Fibrosis was clearly evident in control rats, but was rarely seen in the ATR12181- vaccinated group. Moreover, any signs of autoimmune damage were not found by microscopic investigation [22].

Other approaches to induce antibodies that are able to block the RAS use Ang II as a target. Vaccination with Ang II derived peptides coupled to virus-like particles (VLP) led to the induction of a high anti-Ang II specific humoral immune response. More importantly, these high antibody titres correlated with a statistically significant reduction of blood pressure in SHR. Based on the knowledge that residues 2, 4 and 8 of the Ang II peptide are involved in its interaction with the AT_1 receptor, a vaccine was designed to induce antibodies that specifically recognise Ang II. To achieve this, a modified Ang II peptide was conjugated via its N-terminus to the virus like particle Qβ. The resulting vaccine is called AngQb. Vaccination of mice with AngQb generated antibodies specific for the carboxy terminus of Ang II, which differs from Ang I and angiotensinogen. AngQb was first tested in mice and its high immunogenicity was further confirmed in rats. Inclusion of Alum, as a mild adjuvant, further increased antibody titres twofold. The antibodies evoked by AngQb were tested for their ability to bind Ang I, Ang II, Ang (2-8) and angiotensinogen. Ang II and Ang (2-8) were most strongly bound, followed by Ang I an order of a magnitude lower. Angiotensinogen could not be recognised with these antibody concentrations [14].

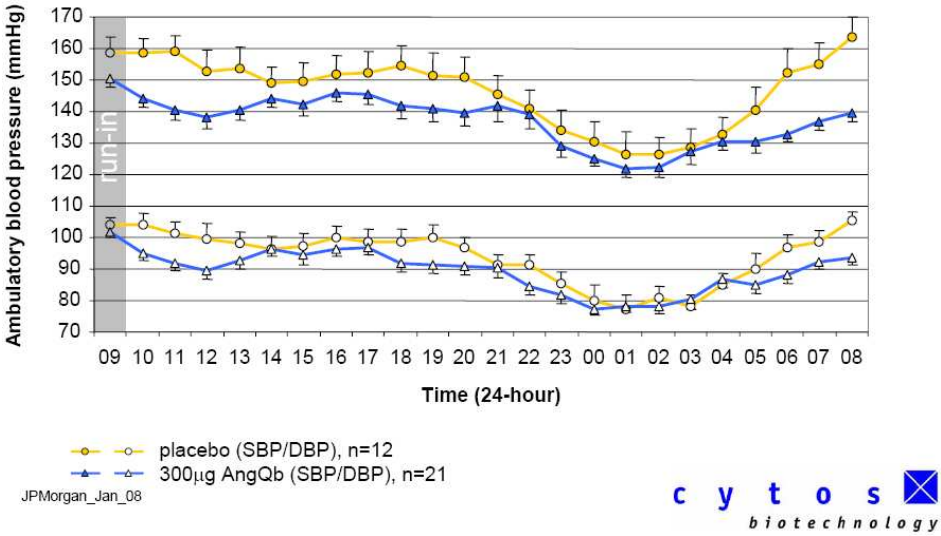


Figure 7: Results from the Cytos study: 24h blood pressure measurement in vaccinated and unvaccinated subjects

Based on these promising results Cytos Biotechnology AG started a combined phase I/IIa study [23]. Here, it could be demonstrated that the vaccine, referred to as CYT006-AngQb, showed no immune complex deposition, was well tolerated and induced a 100% responder rate. This means that vaccination with CYT006-AngQb does not induce severe side-effects and is safe to use. Treatment of hypertensive patients induced a strong and long lasting antibody response and led to a significant reduction of blood pressure in the early morning hours (Figure 7). However, in a second approach differing

only in the vaccination schedule, these data could not be confirmed. Although a higher titre against Ang II was reached in vaccinated subjects, the blood pressure reduction was not significant.

This diploma thesis is part of a project by AFFiRiS AG that aims at the development of a peptide vaccine targeting Ang II. Peptide-based vaccines use short sequences that have low immunogenicity and place a lower risk of inflammation side effects. Therefore, it is not necessary to break self-tolerance, mild adjuvants suffice for efficient vaccination and the risk of autoimmune disease is decreased.

Furthermore, the right selection will create a peptide variant that is different from the target, enough to be recognised as foreign, but similar enough to induce a specific immune reaction. Choosing the right peptide variant enables a vaccine to elicit not only a very specific, but also the right kind (humoral, cellular, cytotoxic, regulative, …) of immune response. Here, the aim is to elicit a humoral response, but with the possibility to induce a long-lasting memory effect via T helper cell activation. This project should identify peptide variants that induce antibodies with an Ang II specific reactivity that excludes Ang I and Ang (1-7), and peptide variants that induce an antibody profile, that is similar to the one induced by the Ang II peptide. The peptide variants described here do not only share common epitopes with the angiotensin II octapeptide but also have high sequence similarities. Therefore, these peptides will be called Ang II-derived peptide variants or Variotope®s.

This novel peptide-based vaccine approach will be developed for the treatment of hypertension and pathological conditions associated with the renin-angiotensin-system. For this purpose AFFiRiS AG selected Ang II-derived peptide variants using in house technologies. These peptides are chemically linked to different protein carriers like tetanus toxoid (TT) or keyhole limped haemocyanin (KLH) and administrated together with Alum as adjuvant. KLH from *Megathura crenulata* was chosen mostly because it is not a common immunogen in man, like TT. The use of common immunogens as peptide carriers is limited because of the risk of epitopic suppression. Epitopic suppression is due to antigenic competition between an existing population of carrier specific B-cell clones, and the newly forming population of peptide-specific B-cell clones. This means, that a subject that has been previously exposed to a specific carrier compound will boost the proliferation of already existing B-cell clones, rather than inducing the activation of new B-cell clones specific for the peptide variant [15].

Variotope® induced sera from animal experiments will be tested for antibody titres, reactivity to the Ang II sequence, specificity for this sequence (compared to Ang I, Ang 1-

7), affinity for Ang II and its derivatives, efficacy in vitro and in vivo, half-life and safety. In this diploma thesis, the selectivity of Ang II-derived peptide induced antibodies will be analysed and their efficiency in blocking Ang II activity in vitro will be determined. Therefore, Variotope®-induced sera will be compared with Ang II peptide-induced sera in ELISA-experiments. Furthermore, a cellular assay to determine the activity of Ang II signalling and to evaluate the inhibitory capacitiy of Variotope®-induced sera on Ang II signalling will be established. This assay could be used to determine the potential correlation between antibody titre, affinity and functionality. Furthermore, this assay could be used to give preliminary data that will form the basis for proof-of-concept studies in spontaneously hypertensive rats. Thus, it provides an additional, cheaper and easier method to establish the functionality of sera.

2. Materials & Methods

2.1. Specificity and selectivity

2.1.1. BSA/KLH-activation

100mg GMBS (N-[g-Maleimidobutyryloxy]succinimide ester; Flucar) are solubilised in 1ml DMSO. 2.5ml of KLH (5.9mg/ml) or BSA (1:10 from 300mg/ml stock) are added to each 0.1ml GMBS in DMSO. This mix is let to react for 30 minutes at 25℃ on a shaker (500rpm). Then, the mixture is centrifuged for 10 minutes at 8000rpm at 4℃ to remove any precipitate. To remove unbound crosslinker the supernatant is purified via PD10 columns (2.5ml/ column). After discarding the first 3ml of flow through, 2.5ml of eluate are collected. The activated-carrier protein solutions are pooled and stored in appropriate aliquots at -80℃. PD10 columns are washed with 25ml of PBS and stored with 0.1% Na-Azide at 4℃. The columns can be reused 3-4 times.

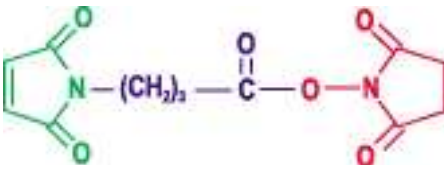


Figure 8: GMBS

The capacity of activated carrier protein is determined by coupling it to a standard peptide. This standard peptide is solubilised at 10mg/ml in 0.2M Na-Phosphat buffer pH 6.8. Different amounts of peptide solution are added to activated BSA (10:1) or activated KLH (2:1). The solutions are let to react for 2h at RT on a shaker. Free SH-groups on uncoupled cysteines are determined by Ellmann Assay: 15µl of each peptide sample are applied to a 96well plate, mixed with 100µl of Ellmann reagent (5,5'-Dithiobis(2-nitrobenzoic acid); Sigma) and the OD at 412nm is measured.

KLH: Sigma; 5.9mg/ml

BSA: Roth; 69g/mol; 8076.3

GMBS: Fluka; 280.24g/mol; CAS 80307-12-6

2.1.2. BSA -coupling

The desired peptide is diluted in 0.2M Na-Phosphate buffer pH 6.8 to give a 500µM peptide solution. 10% DMSO are used to solubilise the peptide if necessary. A t_0 sample of the peptide solution is taken before addition of activated BSA. Then, 10% activated BSA is added to the dissolved peptide. The solution is let to react for 2h at RT on a shaker. The efficiency of the coupling reaction is tested by Ellmann Assay.

2.1.3. ABTS

500mg of ABTS (2,2'- Aznobis-(3-ethylbenzthiazolin-6-sulfonic acid)) are dissolved in 1333ml of 0.1M citric acid. The pH is adjusted to 4.3 with 10M NaOH. Appropriate aliquots are stored at -20℃.

ABTS: AppliChem; 548.69g/mol; CAS 30931-67-0

2.1.4. Enzyme linked immunosorbent assay (ELISA)

ELISA methods are generally used as analytic and diagnostic tools to detect the presence of antigens or antibodies in a sample. This is based on the binding activity of antibodies to their antigens that is detected via a conjugated enzyme-based colour reaction.

ELISA (Nunc, Maxisorp) plates are coated with 50µl/ well of coating agent (antigen) at the appropriate concentration (1µM) in coating buffer (0.1M NaHCO3, pH 9.2-9.4) and incubated over night at 4℃ or for 1h at 3 7℃. Unbound coating agent is removed by discarding the supernatant. Free binding sites on the plates are blocked by incubation for 1h at 37℃ with 100µl/ well blocking buffer (1x PBS, 1%BSA). Blocking solution is discarded and 50µl/well diluted sera are added. The titration steps start at a 1:100 fold dilution and continue 1:2 up to a dilution of 1:204800. Sera are diluted with dilution buffer (1xPBS, 0.1%BSA, 0.1%Tween20). The plates are then incubated for 1h at 37℃. Samples are discarded and plates are washed 3 times with washing buffer (1xPBS, 0.1%Tween20). Then, the plates are incubated with 50µl/ well detection antibody (anti-mu IgG (H+L) biotinylated; Southern Biotech.; 1:1000) for 1h at 37℃. After another three washing steps, 50µl/ well Streptavidin-horse radish peroxidase (Roche) is added. The plates are incubated for 30 minutes at 37℃. The su pernatant is fully discarded and the plates are washed 3 times thoroughly. H_2O_2 is diluted 1:1000 fold in ABTS substrate solution (0.68mM ABTS in 0.1M Citric acid pH 4.3). 50µl/ well of this substrate solution is added and the plates are incubated for another 30 minutes at RT. The reactivity of sera with the antigen is determined by measuring the OD at 405nm.

2.2. Cellular Assay

2.2.1. Cell culture

HEK293 (human embryonic kidney) cells are grown in DMEM (Dulbecco´s modified Eagles Medium; Gibco) +Glutamax supplemented with 10% FCS (fetal calf serum; Gibco) and a Penicillin/ Streptavidin antibiotic mix. HEK293 cells may be held in culture for several months.

HUVEC (human umbilical vein endothelial cells) are grown in basal Medium M200 (Cascade Biologics), 2% FCS, 10ng/ml endothelial growth factor (EGF)/ 10µg/ml Heparin and 3ng/ml fibroblast growth factor (FGF) (Low Serum Growth Supplement kit, Cascade Biologics) in 0.25% gelatine coated flasks. HUVEC are primary cells and thus have a limited number of cell cycles. Therefore, HUVEC can only be kept in culture for 2-4 weeks.

The cell number is determined using a light microscope. 0.4% (w/v) trypan blue (Sigma) is added to discriminate between live and dead cells (trypan blue is able to permeate only dead cells). The number of cells per millilitre solution is given by following equation:

$$\text{cells/ ml} = \frac{\text{unstained cells}}{\text{number of counted squares}} \cdot 10^4$$

The percentage of viability of total cells in the solution is calculated as follows:

$$\text{viability [\%]} = 100\% - \left(\frac{100}{\text{unstained cells + blue cells}} \cdot \text{blue cells} \right)$$

2.2.2. Cellular ELISA

15 000 cells/well are seeded in 200µl/well in 96 well plates and incubated over the weekend. On Monday afternoon, the cells are starved by changing the medium to 0.25%FCS. On Tuesday, cells are stimulated at different time points (2-24h) with different concentrations of Ang II and TNFα respectively. The supernatant is collected and stored for detection of soluble ICAM-1 and secreted IL-8. The cells are then fixed with 4% ice cold methanol for 10 minutes and blocked with 5%BSA in 1%PBS (no Tween!). Detection of membrane bound ICAM-1 and VCAM-1 is done with biotinylated ICAM-1 and VCAM-1 antibodies respectively. The detection procedure is the same as for normal ELISA (described in 2.1.4.).

anti- VCAM-1: Chemicon International; CBL206; 0.1mg/ml

mAb IgG1 anti- ICAM-1: R & D Systems; 11c8; 0.1mg/ml

2.2.3. Transformation of E.coli DH5α

Transformation describes the process in which DNA is transferred into a bacterial host strain. Aliquots of transformation ready bacterial cells (DH5α; Invitrogen) are thawed and incubated with 1-10µg of plasmid for 20 minutes on ice. The cells are thermally stressed by heating the cell solution to 42℃ for 45sec. Then, the cells are incubated in 1ml lysogeny broth (LB) medium (LB-Medium Lennox; Carl Roth GmbH; Nr. X964.1) for 1-2h at 37℃ to recover from this heat-shock. This recovery period allows the cells to produce resistance factors encoded by the plasmid. 100-200µl of the cell solution are plated on selective (containing antibiotics, such as Ampicillin (50µg/ml) or Kanamycin (30µg/ml)) LB plates (LB-Agar Lennox; Carl Roth GmbH; Nr. X965.1) and incubated at 37℃ o/n. One colony is selected for a 4ml preculture and incubated for 8h at 37℃ in selective medium. This preculture is then diluted 1:10 in 25ml (for Midiprep) or 100ml (for Maxiprep) selective LB and grown over night. Plasmid DNA is extracted according to QIAgen midi/maxi prep kit protocol. The quality of the DNA is analysed via agarose gel analysis and the quantity of DNA is determined by measuring the absorbance at 260nm. Using following equation, the concentration of DNA is calculated: c[DNA, µg/ml]= A_{260} · 50µg/ml · dilution factor

2.2.4. PCR (Polymerase chain reaction)

Primer for excising the AT_1R cDNA variant 2 from its original expression vector (pCMV6-XL4, Origene):

Forward: 5'-AAAGAATTCATGATTCTCAACTCTTCTACTG-3'
Reverse: 5'-ATATGGATCCTCAACCTCAAAACATGGTGC-3'

Underlined sequences are recognition sites of restriction enzymes introduced for ligation into multiple cloning sites of other vectors (forward: EcoRI; reverse: BamHI). Red highlighted sequences are start- and end-codon of the AGTR1 gene (the stop codon is eliminated, because the PCR product is used to produce a fusion protein).

The high fidelity polymerase (Roche) exponentially amplifies linear DNA strands, initiated by forward and reverse primers that are complementary to a specific part of the template DNA. Following tables describe the composition of the PCR-solution and the PCR thermo-cycler program. The PCR products are purified using QIAquick PCR purification kit.

Reaction Mix:

10x PCR buffer containing 15mM $MgCl_2$	5µl
10mM dNTP Mix	1µl
Forward and reverse primer (3µM stock)	5µl each
DNA template	1-100ng
H_2O	Up to 50µl
high fidelity polymerase (Roche)	0.75µl (=2.6U)

PCR program:

94℃	2 minutes.	initial denaturation
94℃	15sec.	30cycles
55℃	30sec.	
72℃	90sec.	
72℃	7 minutes	final elongation
4℃	∞	storage

2.2.5. Agarose gel electrophoresis

1.2% agarose in 1x TAE (1L 50x stock: 242g Tris Base, 57.1 ml acetic acid, 100ml 0.5M EDTA) is melted in the microwave oven. 5µl (1:10 000) Sybr safe DNA stain (Invitrogen) is added and the gel is casted. DNA samples are loaded, supplied with 5x loading dye (Fermentas). The GeneRuler™ 1kb DNA Ladder from Fermentas is used to estimate the size of the DNA bands (see Figure 9). The gel is run for approximately 30 minutes at 90V in 1x TAE. The resulting bands are evaluated using UV light (Transilluminator M-26, bio Doc-It System).

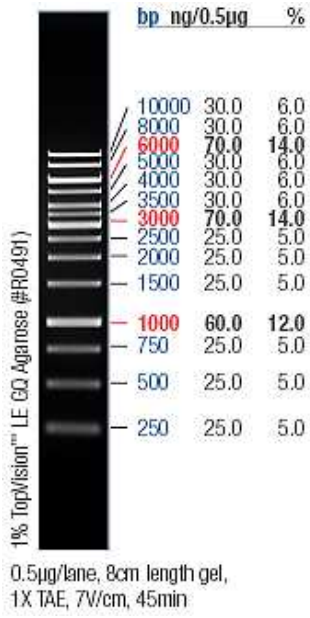


Figure 9: GeneRuler™ 1kb DNA Ladder (Fermentas)

2.2.6. Enzymatic digest

Approximately 1µg DNA is incubated with 1µl of each Enzyme (EcoRI, BamHI; both obtained from Fermentas) in reaction buffer (2x Tango™ or 1x BamHI Buffer; Fermentas) for 1- 5h at 37℃. After the digest, sam ples are loaded on a 1.2% agarose gel and run for approximately 45 minutes at 100V. Samples are extracted from the gel and purified using QIAquick gel extraction kit.

2.2.7. Ligation

The T4 ligase catalyses the generation of phosphodiester bonds between two linear strands of DNA, such as between a vector and an insert. Appropriate amounts of vector (pEGFP-N1) and insert (AT_1R cDNA PCR product) are ligated with 1µl T4 ligase (Fermentas) in 1x reaction buffer (10x Ligation Buffer: 400mM Tris-HCl, 100mM $MgCl_2$, 100mM DDT, 5mM ATP, pH 7.8; Fermentas). The reaction is conducted using a thermocycler programmed to keep 22℃ for 1 hour, th en heat the sample to 65℃ for 10 minutes to inactivate the enzyme and then store the sample at 4℃ until used. Ligated plasmids are transformed into E.coli DH5α (Invitrogen) and grown on selective LB plates (30µg/ml Kanamycin). Transformed E.coli cells are stored at -80℃ in 15% glycerol. The plasmid is isolated using QIAquick Midi/ Maxi plasmid DNA precipitation kit protocol and send to Microsynth AG for sequencing.

Sequencing primers:

AT1R-fw2:	5'-AATGTCGTAACAACTCCGCC-3'
AT1R-fw3:	5'-TGGCTATTGTTCACCCAATG-3'
AT1R-fw4:	5'-AAAAGATATTTTCTCCAGCTTCTAAA-3'
AT1R-fw5:	5'-TATATCATGGCCGACAAGCA-3'
AT1R-rev2:	5'-CCACCAAGCTGTTTCCAAAT-3'
AT1R-rev3:	5'-GAACTTCAGGGTCAGCTTGC-3'
AT1R-rev4:	5'-CAAGTTAACAACAACAATTGCATTC-3'

2.2.8. Transfection of HEK293

Transfection is the process in which DNA is transiently or stably transformed into eukaryotic cells, where it is expressed. For the transfection, $1 \cdot 10^6$ cells/well are seeded on 6-well plates and incubated over night. Then, full DMEM medium is changed to DMEM+ Glutamax without FCS or Pen/Strep.

Two different transfection methods were tested:

2.2.8.1. Calcium Phosphate: 1- 4µg DNA are diluted in H_2O and 9µl/ well $CaCl_2$ to give a total volume of 74µl. This solution is slowly droped into 71µl/ well HeBS buffer (280mM NaCl, 1.5mM Na2HPO4, 50mM Hepes (Sigma H-7006)) while continuously shaking the tube. The transfection mix is added dropwise to the cells. 4h after the transfection, 10% FCS is added to each well. Transfected cells can be harvested 24- 48h after transfection.

2.2.8.2. Lipofectamine Reagent: 1µg DNA is diluted in 100µl medium. Then 10µl Plus Reagent are added and the solution is incubated for 15 minutes at room temperature (RT). 10µl Lipofectamine Reagent is diluted in 100µl medium and this solution is added to the preincubated DNA solution. The Lipofectamine/ DNA solution is incubated for another 15 minutes at RT. The ready mixture is added dropwise to the cells and the plate is shaken softly. Thus transfected cells are incubated for 1- 2h and then FCS is added to gain a 10% FCS solution. Cells can be harvested 24- 48h after transfection.

2.2.9. FACS (Fluorescence activated cell sorting)

Flow cytometry is a technique for counting, examining, and sorting microscopic particles suspended in a stream of fluid. Within the instrument, a laser is directed onto a focused stream of fluid and a number of detectors are positioned at the point where this stream passes through the light beam. Each suspended particle scatters the light in a characteristic way and fluorescent chemicals within the particle or attached to it may be activated to emit light. With this method various types of information about the physical and chemical structure of each individual particle can be obtained. FSC (forward scatter) correlates with the cell volume and SSC (side scatter) depends on the inner complexity of the particle (i.e. shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness).

Transfected HEK293 cells are detached from the plates with 1x trypsin (Sigma) and then counted using trypanblue. $3 \cdot 10^5$- $5 \cdot 10^5$ cells are centrifuged for 2 minutes at 1000rpm and resuspended in 1ml ice cold FACS buffer (1xPBS, 1% BSA, and 0.1% sodium azide) with 1:10000 7-AAD (dead/live staining). 7-AAD intercalates with DNA, which is only accessible in dead cells or cell debris. Cells are measured with the FACS machine (FACScan Flow Cytometer 65; Becton Dickinson GmbH) and analysed using CellQuestPro.

2.2.9.1. FACS staining: Transfected cells are washed with 1xPBS and detached from 6-well plates using cell-dissociation solution (Sigma). Then the cells are counted and split to give $3 \cdot 10^5$ cells/ staining on a V-shaped 96-well plate. Cells are kept on ice at all times. Cells are washed twice by spinning the solution for 2 minutes at 1000rpm and resuspending the pellet in 100µl ice cold FACS buffer. Unspecific IgG reactions are blocked by incubating the cells 10 minutes with 1.1µg/ml Affini Pure goat anti-rat IgG Fc_γ fragment specific antibody (Dianova) on ice. After the first blocking step cells are centrifuged for 2 minutes at 1000rpm and the supernatant is discarded. Unspecific reactions of immunoglobulins, used for detection, to Fc-receptors are blocked by incubating the cells for 5 minutes with 1µg/ml Fc-block (BD Pharmigen; purified rat anti-mouse CD16/CD32) on ice. Again the solution is centrifuged and the supernatant discarded. Then the pellet is resuspended in 100µl FACS buffer and incubated for 30 minutes with the first antibody (anti-AT_1R; Santa Cruz; 1:20). Cells are washed twice with FACS buffer and incubated 30 minutes with 10µg/ml secondary donkey anti-mouse PE-conjugated antibody ($F(ab')_2$ Fragment Donkey Anti-Mouse IgG (H+L); Jackson Immuno Research; 715-116-151) in the dark. Again, the cells are washed and the fluorescence is measured in the FACS machine.

2.2.10. Western Blot

HEK293 cells transfected and untransfected cells are lysed in RIPA buffer (50mM Tris pH7.4, 150mM NaCl, 1% Triton-X, 0.5% Sodium-Deoxychelate, 5mM EDTA) supplemented with a protease inhibitor cocktail tablet (complete mini easypack sample, Roche) for 20 minutes on ice. After centrifugation at 13000 rpm for 10 minutes at 4℃, the supernatant containing protein is collected. These lysates are prepared for gel electrophoresis by mixing them with 5x Sample Buffer (0.2M tris-HCl pH 6.8, 10% w/v SDS, 20% v/v Glycerol, 0.05% w/v Bromophenolblue and 10mM beta-mercapto-ethanol) and boiling them for 2- 3 minutes. at 95℃. The samples are loaded onto a SDS-polyacrylamide gel consisting of a 10% acrylamide running gel (pH 8.8) and a 4% acrylamide stacking gel (pH6.4) and the gel is run in 1x Running Buffer (25mM Tris-HCl, 200mM Glycine, 0.1% w/v SDS) for approximately 1h at 30mA. Proteins within the cell lysates separate according to their mass to charge rate. A prestained protein ladder (Page Ruler Plus; Fermentas) is applied to the gel to enable evaluation of signals obtained by either Coomassie staining or Immunoblotting (Western Blot) of the proteins. The proteins separated within the polyacrylamide gel are then blotted at 4℃ for 1h at 80V or o/n at 30V onto a nitrocellulose membrane. Non-occupied protein binding sites on the membrane are blocked with 5%BSA or 5% milk in 1x TBST (Trizma HCl, NaCl, pH 7.6, 0.1% Tween20) for 1h at RT. Then the membrane is incubated for 2h at RT or o/n at 4℃ in primary

antibody in blocking buffer binding to the protein of interest. After washing 3x 10 minutes with 1x TBST, the membrane is incubated for 45 minutes in secondary antibody in blocking buffer. The membrane is washed again 3 times for 10 minutes each. ECL colour solution (GE lifesciences) is applied and the membrane is exposed to the chemiluminescence-measuring machine for 5- 41 minutes.

antibody		distributor	dilution	blocking buffer
primary α-AT1R	1E10-1A9	sc-81671 Santa Cruz Biotechnology	1:200	5% BSA in 1x TBST
	TONI-1	sc-57036 Santa Cruz Biotechnology	1:200	5% milk in 1x TBST
	N-10	sc-1173 Santa Cruz Biotechnology	1:200	5% milk in 1x TBST
primary α–β-Actin		ab8227 Abcam	1:1000	5% milk in 1x TBST
primary α-GFP		ab6673 Abcam	1:500	5% milk in 1x TBST
secondary	α-mouse	115-035-068 Jackson Immunolab	1:20 000	5% milk in 1x TBST
	α-rabbit	NA934V GE Healthcare	1:10 000	5% milk in 1x TBST
	α-goat	A5420 Sigma	1:2000	5% milk in 1x TBST

2.2.10. Luciferase assay

HEK293 cells are co-transfected with AT_1R-EGFP fusion vector (2µg/ transfection) and NF-κB-luc (2µg/ transfection) expression vector on a 6 well plate or on 60cm^2 Petri-dishes. Approximately 20 hours after the transfection HEK293 cells are detached from the plate using enzyme free cell-dissociation solution (Sigma) and transferred onto a 96-well black VIEW plate (PerkinElmer). There the cells are starved in DMEM without FCS and stimulated with 10ng/ml or 5ng/ml TNFα and 10^{-9}M – 10^{-5}M Ang II for 24h. 10^{-4}M losartan is added 4h before stimulation of cells with Ang II. Sera derived from immunisation of mice with different Ang II peptide variants or anti-Ang II control antibody (0500-0030 Serotec) are applied together with Ang II stimulation. After 24h of stimulation the cell culture supernatant is discarded and the cells are lysed with 100µl/ well Reaction Reagent (Bioassay Systems) containing lysis buffer and luciferase substrate. After 2 minutes, lysis is complete and the chemiluminescence is measured using the GenIOS reader with a gain of 150 and an integration time of 5000 milliseconds.

2.2.11. Fluorescence Microscopy

Different counts of transfected HEK293 cells are applied on adhesion slides (Marienfeld) and air dried for approximately 1h. Then one drop of warm mounting medium (DakoCytomation Glycergel) is added and the slide is covered with a cover slip. Cells are analysed immediately, because the mounting medium is lethal to unfixed cells. Pictures are taken using a fluorescent microscope (60x amplification using an immersol objective).

3. Results

3.1. Specificity for Ang II examined via ELISA

BALB/c mice and Wistar rats were used to test the immunogenicity of the Ang II octapeptide and Ang II derived peptide variants. These peptide variants contain several modifications compared to Ang II. These modifications include changes of chemical properties of the C-terminal end (C-term) or of the amino acid sequence of the peptide. The peptides used for immunisation of mice and rats were bound via their N-terminal end (N-term) to KLH as a carrier protein. An additional cysteine was added to the N-term of Ang II, Ang I, Ang (1-7) and the peptide variants to couple these peptides to the carrier protein. The conjugation to a large protein carrier such as KLH usually increases the immunogenicity of peptides. Furthermore, the C-term of these peptides conjugated to KLH is free to elicit an appropriate immune response. The Ang II C-term is thought to contain the major binding site to the AT_1 receptor. Blockade of Ang II binding to the receptor by antibodies against this site thus might inhibit Ang II function. The peptide-carrier conjugate was formulated with aluminium hydroxide (Alum) and injected subcutaneously into BALB/c mice and Wistar rats. Twelve days after this prime immunisation, Serum1 was taken. Both animal models were boosted for three times in biweekly episodes. Sera2, 3 were taken twelve days after each boost. One week after the third boost, Serum 4 was taken. Another week after this last boost, the animals were sacrificed and Endsera were collected (see Figure 10).

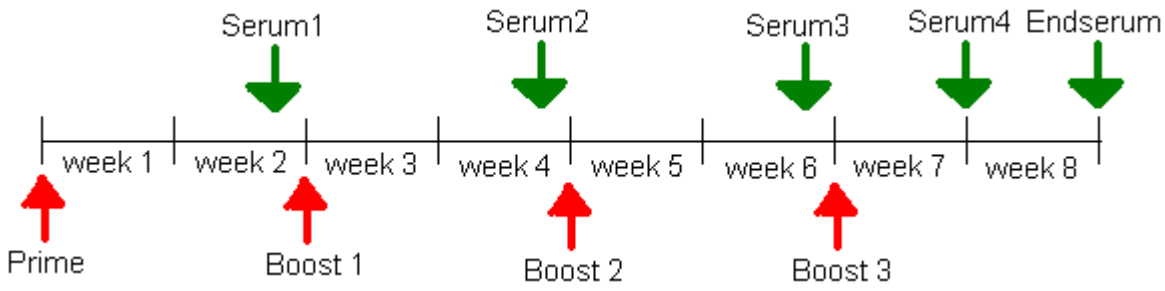


Figure 10: Vaccination Schedule

At first the humoral immune response induced by immunisation of BALB/c mice with Ang II was characterised. For this purpose, total IgG levels against Ang II in mouse sera after immunisation with Ang II were determined by enzyme linked immunosorbent assay (ELISA). The IgG levels against Ang II in mouse sera after immunisation give information about the reactivity of the antibodies in these sera to Ang II. Furthermore, the crossreactivity of these sera generated against Ang II to Ang I, Ang (1-7) and angiotensinogen was also analysed by ELISA.

ELISA experiments are conducted using 1 to 2 dilution steps of each Serum starting with 1:100 down to 1:204800 in twelve steps. Antibody titres are defined as dilution factor at an OD value that equals half of the OD maximum. All titres shown here represent mean values of titres from five animals each. The titres against the original peptide in all graphs represent the mean value from all experiments (approximately 75 mice).

3.1.1. Epitope specificity of Ang II-induced sera changes after multiple vaccination steps

The first experiment examined the anti-Ang II IgG response of BALB/c mice after immunisation with the Ang II-KLH conjugate vaccine. The immunogenicity of different sera (Serum1 to Endserum) of BALB/c mice immunised with Ang II is shown in Figure 11. Titres in Serum1 are very low compared to the other sera. However, the titres increase with each boost vaccination and are at the highest level in the Endserum.

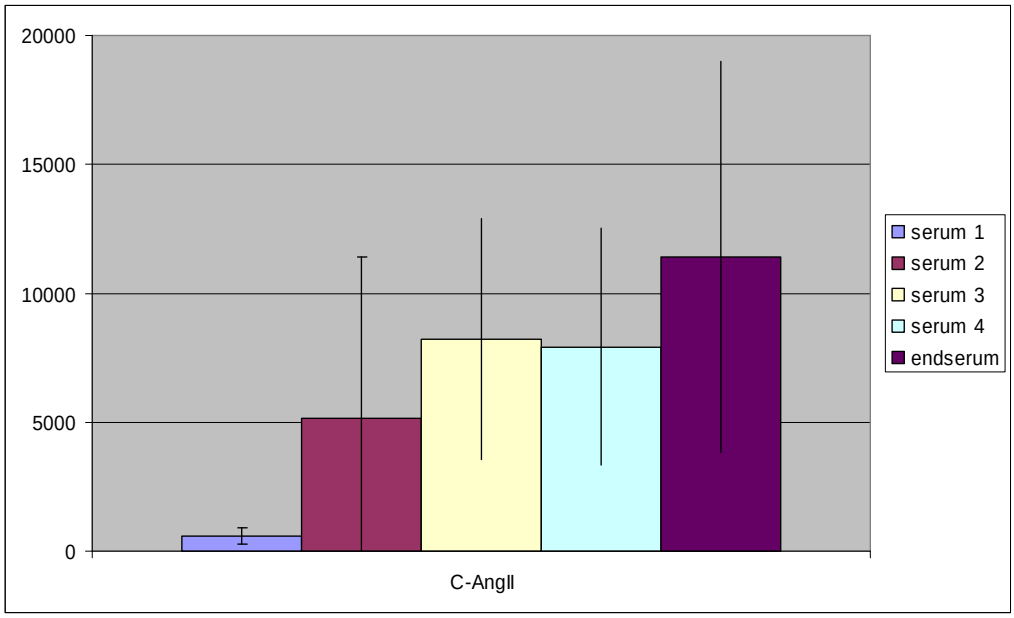


Figure 11: Titres of Serum1 to Endserum of mice immunised with Ang II against Ang II; n = 5; blue bars: Serum1, red bars: Serum2, yellow bars: Serum3, turquoise bars: Serum4, purple bars: Endserum

Ang I and Ang 1-7 differ from Ang II only in their C-terminal end. Thus, an antibody induced against Ang II might also react with Ang I and Ang 1-7. The precursor protein angiotensinogen is abundantly present throughout the body and might have an additional, so far unknown function. Thus, it should not be recognised by antibodies against Ang II. Therefore, the crossreactivity of the same sera derived from Ang II immunisation of BALB/c mice to Ang I, Ang 1-7 and angiotensinogen was examined.

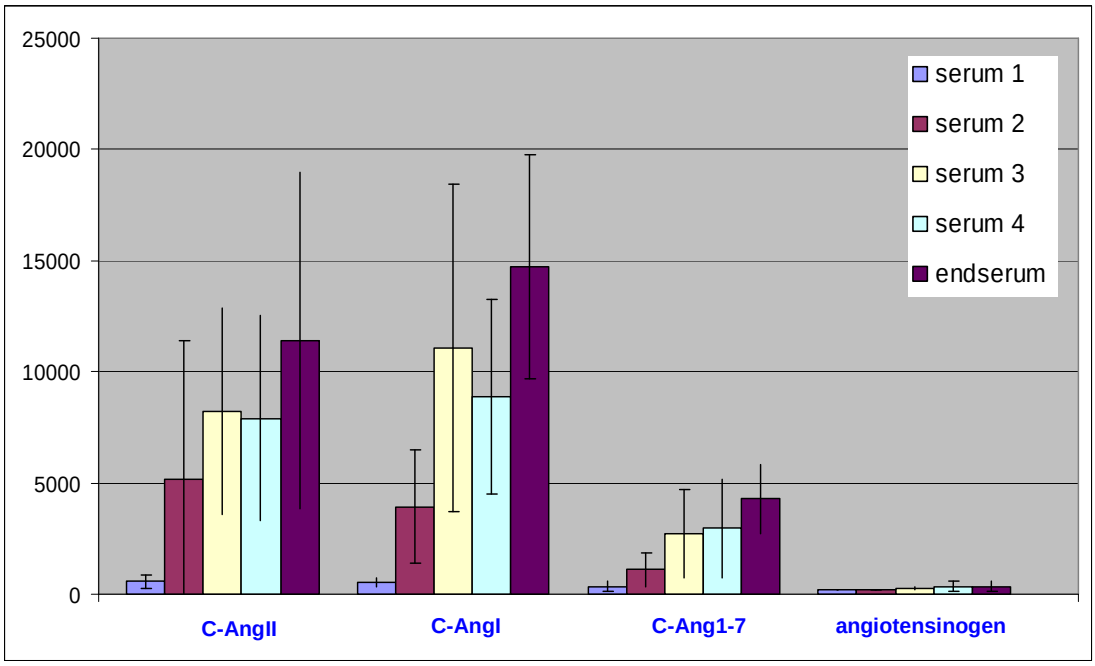


Figure 12: Titres of Serum1 to Endserum of mice immunised with Ang II to Ang II, Ang I, Ang (1-7) and angiotensinogen; n = 5; blue bars: Serum1, red bars: Serum2, yellow bars: Serum3, turquoise bars: Serum4, purple bars: Endserum

As shown in Figure 12 the overall immune response in Serum1 is low. However, in Serum3 to Endserum there are significant differences in the reactivity to Ang II and the crossreactivity to Ang I, Ang 1-7 and angiotensinogen. Ang I has two additional amino acids (histidine and leucine) at its C-term compared to Ang II. Thus, the peptide backbone is continued and the negative charge of the Ang II C-term is lost. In Serum2 the titre against Ang I is lower than the titre against Ang II. However, this changes with additional boost injections. In the Endserum, the crossreactivity to Ang I is even higher, than the reactivity to Ang II. Ang 1-7 consist of the first seven amino acids of Ang II without the phenylalanine residue at the very C-terminal end. The titre against Ang 1-7 in all sera is significantly lower than against the original peptide. In the Endserum the titre of antibodies against Ang 1-7 is about 40% of the titre of antibodies against Ang II. However, the greatest titre difference is seen in Serum2. Here, the level of IgG against Ang 1-7 is only about 5% of the IgG level against Ang II. The specificity of the antibodies in Serum2 seems to depend on the eighth residue. These results indicate that the very C-terminal residue of Ang II might be important for antibody recognition. Angiotensinogen is not recognised from any antibodies in sera of mice immunised with Ang II.

To further examine the specificity of antibodies induced by immunisation of mice with Ang II for the C-terminal end of the octapeptide, the crossreactivity to different peptide variants was examined. These peptide variants contain several modifications in

their amino acid sequence compared to Ang II. The three C-terminal amino acids of the Ang II octapeptide were exchanged to alanine, as a neutral amino acid (C-DRVYIAPF, C-DRVYIHAF, C-DRVYIHPA). These peptides were used to test which residues of Ang II determine the specificity of antibodies against the Ang II C-term. An additional peptide completely lacks the C-terminal end (C-DRVYI). Another one only shares the three very C-terminal amino acids (C-DAAAAHPF) with the original Ang II sequence. This experiment should also examine whether sera taken after early immunisation steps (Serum1 and Serum2) show higher specificity for the Ang II C-terminal end, than sera taken after later immunisation steps (Sera3- Endserum).

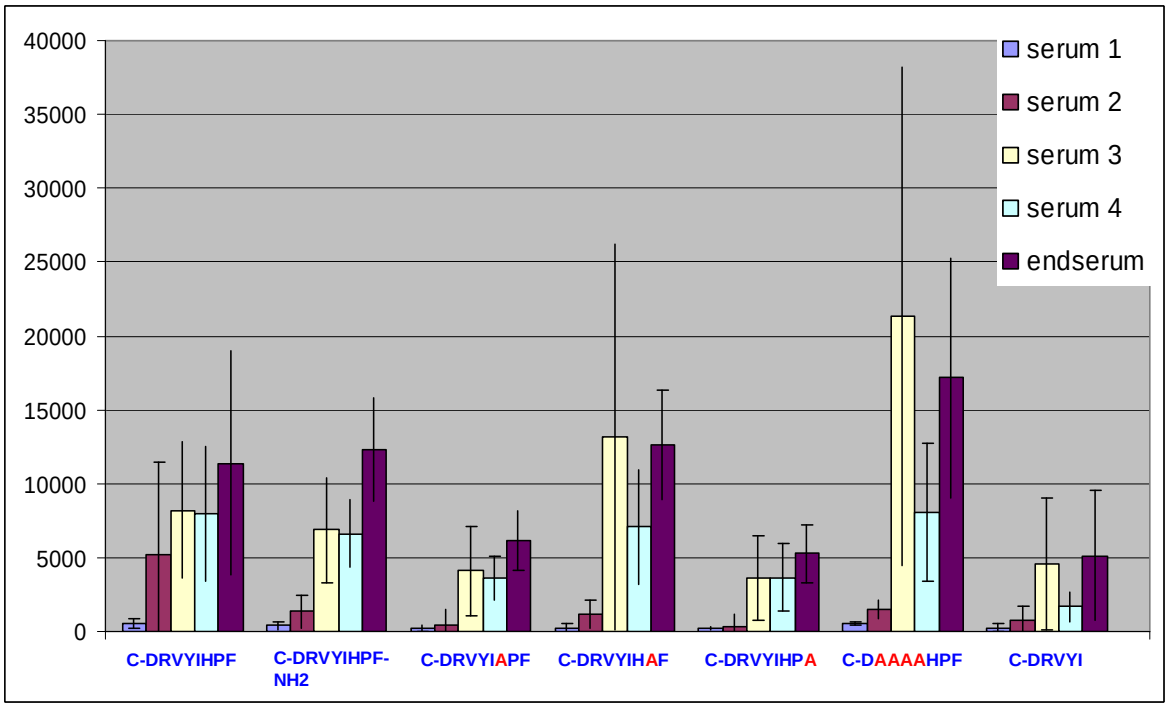


Figure 13: Titres of Serum1 to Endserum of mice immunised with Ang II; n = 5; blue bars: Serum1, red bars: Serum2, yellow bars: Serum3, turquoise bars: Serum4, purple bars: Endserum

Figure 13 shows the development of titres of sera from mice immunized with the original Ang II octapeptide conjugated to KLH via an additional cysteine (C-DRVYIHPF) against several different Ang II derivatives.

Here again, in Serum1 the overall immune response is lower than in following sera. Serum2 shows significant differences in antibody titres against the different peptides in the ELISA. The peptide C-DRVYIHPF-NH_2 differs from Ang II only in the fact that its C-terminal end is amydated. Thus, the negative charge of the terminal carboxy group is lost and an antibody recognising this charge would not bind. The titre of C-DRVYIHPF-NH_2 antibodies in Serum2 is significantly lower than the titre of antibodies against the original

peptide. However, later sera show a similar titre for C-DRVYIHPF-NH_2 as for Ang II, thus the specificity for the negatively charged carboxyl-group might have changed. Peptide variants C-DRVYIAPF, C-DRVYIHAF and C-DRVYIHPA have mutations within the three C-terminal amino acids (HPF) of the Ang II octapeptide. ELISA data show, that the titres against C-DRVYIAPF and C-DRVYIHPA are about 50% reduced compared to the titres against Ang II. The greatest titre difference of antibodies to these test peptides compared to antibodies to Ang II is seen in Serum2. However, the titres against C-DRVYIHAF in Serum3 to Endserum are the same as the titres against the original peptide. Only in Serum2 there is a reduction to 20% of the titres against Ang II. This might indicated that the proline at position 7 is not important for antibodies binding the Ang II C-term. The peptide variant C-DAAAAHPF only shares the first and the last three C-terminal amino acids with the original Ang II sequence. Thus, this peptide variant is used to determine the titres of antibodies against the C-terminal end of Ang II in the different sera. In Serum1 and Serum2 the crossreactivity to C-DAAAAHPF is reduced to 30% of the reactivity to Ang II. In later sera however, the titres increase and reach the same level as titres against Ang II. The peptide variant C-DRVYI completely lacks these last three C-terminal amino acids. Thus, the reaction of sera to this peptide variant should be low, if the C-term is recognised specifically. The titres against C-DRVYI of all sera are significantly reduced, compared to titres against Ang II.

The relation of the reactivity to Ang II and the crossreactivity to Ang I, Ang 1-7, AGT and Ang II derived peptides within the different sera is summarised in Table 1.

Table 1: Titres against Ang II derivatives as percentage of titres against the original peptide

	serum 1	serum 2	serum 3	serum 4	Endserum
C-DRVYIHPF	100	100	100	100	100
C-DRVYIHP	12	4	33	37	38
C-DRVYIHPFHL	12	45	135	112	129
C-DRVYIHPF-NH_2	23	26	84	83	108
Angiotensinogen	12	4	3	4	3
C-DRVYIAPF	12	9	50	46	54
C-DRVYIHAF	12	22	160	90	111
C-DRVYIHPA	12	7	44	46	46
C-DAAAAHPF	30	28	260	102	151
C-DRVYI	15	14	56	21	45

3.1.2. Assessment and selection of peptide variants

The aim of the project is to generate different Ang II derived peptide variants and characterise their immunologic properties. The immunogenicity of these peptide variants, the reactivity of peptide-induced antibodies against Ang II and the crossreactivity of these antibodies to Ang I and Ang 1-7 were analyse by ELISA. These experiments should

examine whether the crossreactivity to Ang I and Ang 1-7 of sera from mice treated with different peptide variants can be varied by modifying the Ang II peptide.

Ideal peptide variants or Variotope®s should have a high immunogenicity and induce sera that show high reactivity to Ang II. However, they should also induce sera that are specific for Ang II and show reduced crossreactivity to Ang I and Ang 1-7. Furthermore, Variotope®s should be able to induce a long lasting humoral response and lead to the development of memory cells. The specificity and duration of the immune response might also depend on the affinity of sera for Ang II and its derivatives. In the following experiments, the effects of mutations within the Ang II octapeptide on the specificity of peptide induced-sera for Ang II, Ang I and Ang 1-7 are analysed.

3.1.2.1. Modifications with a neutral amino acid

The previous experiment shows that the reactivity to Ang II of antibodies induced by immunisation with Ang II differs from the crossreactivity of these antibodies to peptides were single residues of the Ang II octapeptide are exchanged with a neutral amino acid. Thus, the specificity of mouse sera for Ang II, Ang I and Ang 1-7 might be varied by immunisation with peptide variants rather than with Ang II. The next experiment should examine whether immunisation of mice with peptides containing a neutral amino acid at two positions within the Ang II sequence changes the reactivity to Ang II and the crossreactivity to Ang I and Ang 1-7 in the serum.

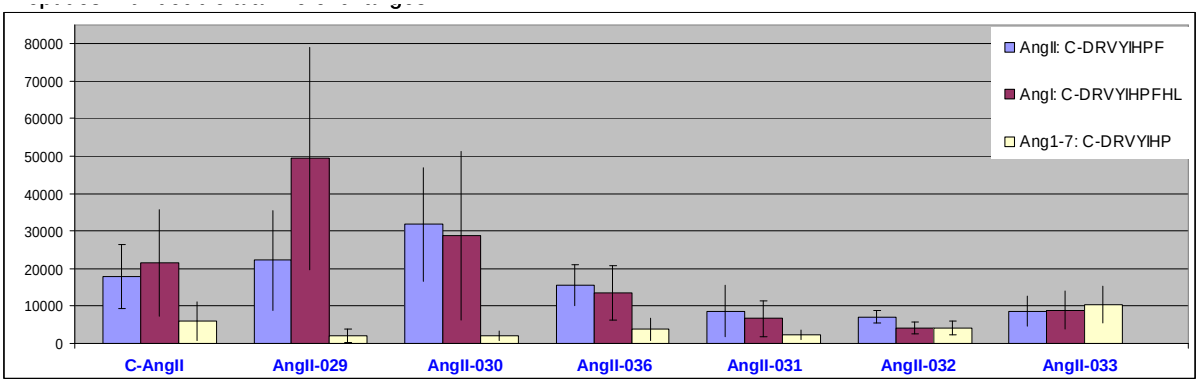


Figure 14: Titres against Ang II, Ang I and Ang 1-7 of Sera of mice immunised with Ang II-derived peptides containing a neutral amino acid at two positions in the Ang II sequence; Endserum; n = 5; blue bars: Ang II, red bars: Ang I, yellow bars: Ang 1-7

Figure 14 shows the titres of Endsera against Ang II (blue), Ang I (red) and Ang 1-7 (yellow) of mice immunised with different Ang II-derived peptides containing two neutral amino acids. In most peptide variant-induced sera the reactivity to Ang II is lower than in sera of mice immunised with Ang II itself. However, three of the six peptide variants shown in Figure 14 (Ang II-029, Ang II-030 and Ang II-036) have the same or even a

higher immunogenicity than the original peptide (C-Ang II), displayed by a higher titre against Ang II. Sera evoked by immunisation with these peptides also recognise Ang I at the same or even a higher level as Ang II. However, the titres against Ang 1-7 are significantly lower in sera obtained by immunisation of mice with these Variotope®s than in sera induced by Ang II. In sera from mice immunised with Ang II-029 or Ang II-030, the titre against Ang 1-7 is only about 6-12% of the titre against Ang II. Ang II-029, Ang II-030 and Ang II-036 all share modifications within the three central amino acids of the Ang II peptide (VYI). As soon as one of the last three C-terminal amino acids of the octapeptide is exchanged to a neutral amino acid, as it is the case in Ang II-032 and Ang II-033, the titres against all three angiotensin peptides (Ang II, Ang I, and Ang 1-7) align.

The next experiment should examine whether the effects on the reactivity of antibodies induced with peptide variants, seen before, can be potentiated when the Variotope®s used for immunisation contain a neutral amino acid at three or four positions within the Ang II sequence.

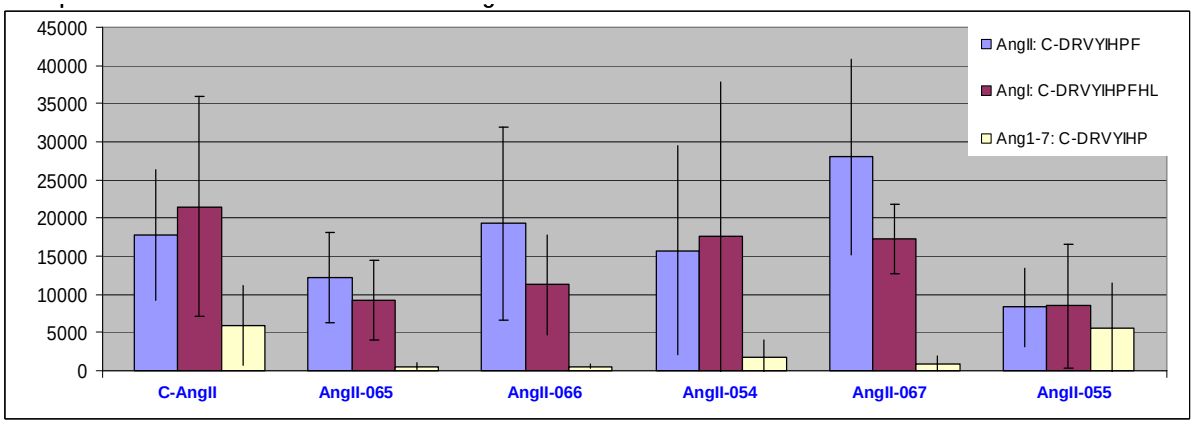


Figure 15: Titres against Ang II, Ang I and Ang 1-7 of Sera of mice immunised with Ang II-derived peptides containing a neutral amino acid at three or four positions in the Ang II sequence; Endserum; n = 5

Figure 15 shows the reactivity of sera to Ang II (blue), Ang I (red) and Ang 1-7 (yellow) evoked by immunisation with Ang II-derived peptides containing a neutral amino acid at three or more positions within the Ang II peptide. Considering the experimental fluctuations of ELISA analysis, the titres of sera induced by most peptide variants shown in Figure 15 (Ang II-054, Ang II-066, Ang II-067) are as high as or higher than of sera induced by Ang II. Also, all peptide variants, except Ang II-055 where one of the three C-terminal amino acids is changed to a neutral amino acid too, show a significant reduction to 3% -11% of the crossreactivity to Ang 1-7 compared to the Ang II immunised group. However, here, especially peptide variants Ang II-066 and Ang II-67 show also a significant reduction of the crossreactivity to Ang I to about 60% of Ang II-induced sera.

These peptide variants contain modifications within the three central amino acids of the Ang II peptide. As already seen in previous experiments, the crossreactivity to Ang 1-7 seems to depend on the three C-terminal amino acids and the crossreactivity to Ang I seems to depend on the three central amino acids of Ang II.

Mice are used as model organisms for a variety of pre-clinical studies. However, data derived from mouse experiments can not always be applied for other species, such as humans. To show that these results are not specific for mice, a similar experiment was done in rats and the rat sera were also analysed for their reactivity to Ang II and their crossreativity to Ang I and Ang 1-7 (Figure 16). This experiment should examine whether the results obtained in mice do also apply for another species.

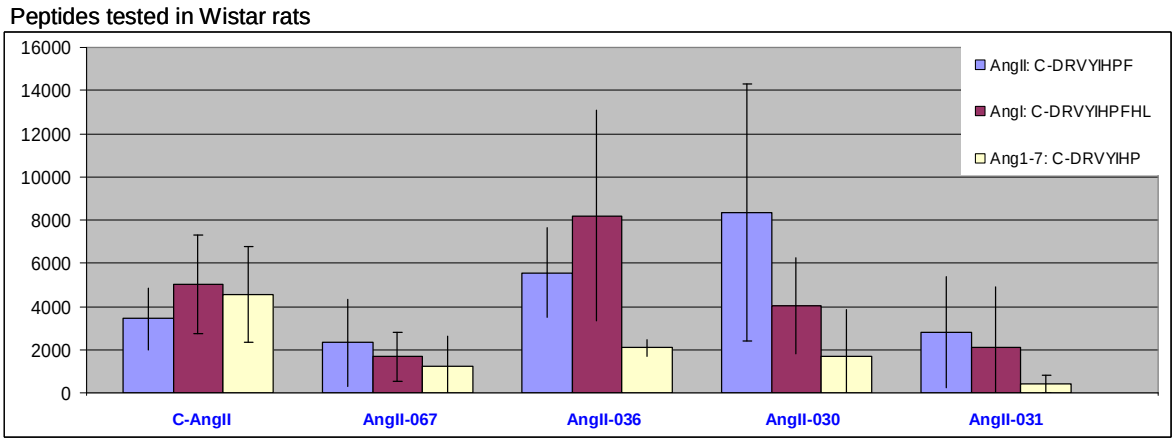


Figure 16: Titres of Sera of rats immunised with Ang II-derived peptides with several alanine exchanges against Ang II, Ang I, Ang 1-7; Serum 4; n = 5

In general, the immunogenicity of Ang II-peptide KLH conjugate vaccine is lower in rats than in mice. In contrast to mice, Ang 1-7 is better recognised by sera derived from rats immunised with Ang II. However, as already seen in mice, titres against Ang II are as high as or higher in most Variotope®-immunised groups compared to the Ang II-immunised group. Peptide variants Ang II-036 and Ang II-030 show a high immunogenicity and antibodies-induced by these peptides have a significantly reduced crossreactivity to Ang 1-7. The IgG levels in Ang II-036 and Ang II-030 induced sera are about 17-50% of the titre against Ang II. Ang II-030 induced sera also show higher selectivity for Ang II, because here, the crossreactivity to Ang I is only 40% of the reactivity to Ang II.

These data indicate that the results obtained in rats are comparable to the results obtained in mice. In both animals, immunisation with peptide variants containing modifications within the three central amino acids of the Ang II sequence change the crossreactivity of sera to Ang I compared to sera induced by Ang II itself.

3.1.2.2. Modifications with chemically distinct amino acids

The chemical properties of the eight amino acids of Ang II might play a role for the conformation of the peptide and the recognition of the peptide by antibodies. By exchanging specific residues with amino acids of a different chemical constitution the conformation of the peptide and the recognition pattern of antibodies can be modified. For the next experiments, Variotope®s were produced that contain substitutions of specific residues of Ang II with amino acids with similar or contrary chemical properties to the original amino acid. These specific modifications were chosen based on preliminary data from previous experiments. It was examined whether the reactivity of peptide induced sera to Ang II and the crossreactivity to Ang I and Ang 1-7 can be modified by changing the chemical properties of the peptide used for immunisation. These experiments were conducted in BALB/c mice.

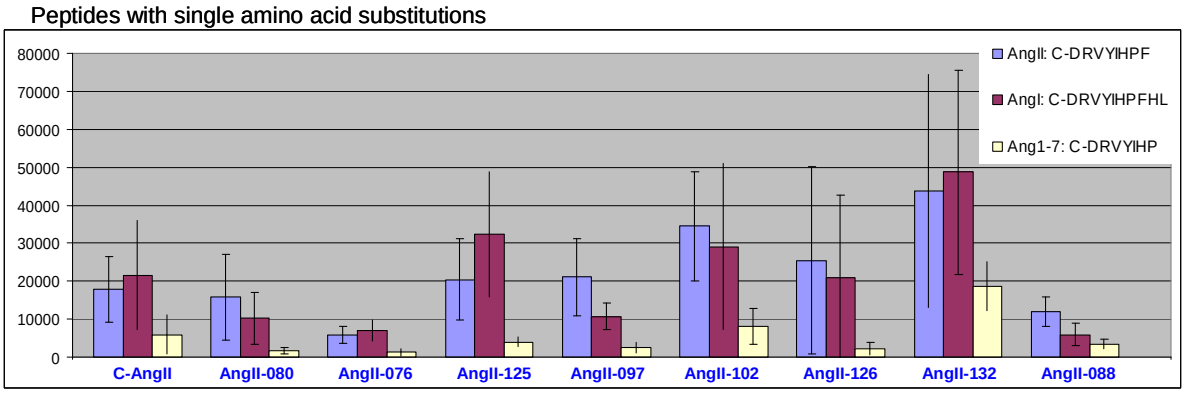


Figure 17: Titres of Sera of mice immunised with Ang II-derived peptides containing single amino acid exchanges on different positions within the octapeptide against Ang II, Ang I and Ang 1-7; Endserum; n = 5

Figure 17 shows titres of sera induced by vaccination with peptides containing single amino acid substitutions. Again, immunisation of mice with most Variotope®s (Ang II-097, Ang II-102, Ang II-125, Ang II-126, Ang II-132) induces a higher titre against Ang II than immunisation with Ang II itself. Most peptide variants also show a significant reduction in the crossreactivity to Ang 1-7 (Ang II-080, Ang II-088, Ang II-097, Ang II-125, Ang II-126) and some even in the crossreactivity to Ang I (Ang II-080, Ang II-088, Ang II-097, Ang II-126). The most dramatic change concerning the crossreactivity to Ang I can be seen in sera derived from immunisation with the peptide variant Ang II-097. However, there are also Variotope®s that, although different from the sequence of the original peptide, behave similar as the original peptide concerning the crossreactivity of induced sera to Ang I and Ang 1-7 (Ang II-076, Ang II-102, Ang II-132). The results from these experiments are summarised in Table 2.

Table 2: Crossreactivity of sera induced by Ang II-derived peptides containing single amino acid exchanges to Ang I and Ang 1-7 as the percentage of their reactivity to Ang II

	C-Ang II	Ang II-080	Ang II-076	Ang II-125	Ang II-097	Ang II-102	Ang II-126	Ang II-132	Ang II-088
Ang II	100	100	100	100	100	100	100	100	100
Ang I	121	65	117	158	51	85	83	111	49
Ang 1-7	33	11	22	19	12	23	9	43	28

The next experiment should examine whether the effects on the reactivity of antibodies induced by Variotope®s containing single amino acid substitutions can be potentiated with Variotope®s containing amino acids with chemical properties that differ from the original amino acids at several positions within the Ang II sequence.

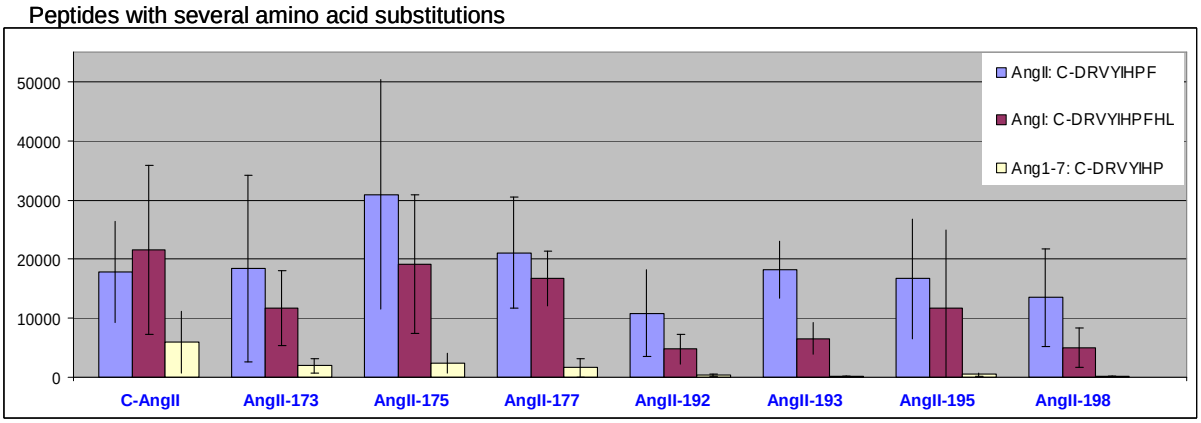


Figure 18: Titres of Sera of mice immunised with Ang II-derived peptides containing multiple amino acid exchanges against Ang II, Ang I and Ang 1-7; Serum 4; n = 5

Figure 18 shows titres of sera induced by vaccination with Ang II-derived peptides containing multiple amino acid substitutions. All Variotope®s depicted here, show good immunogenicity against Ang II compared to sera induced by Ang II-immunisation. Furthermore, the crossreactivity of sera to Ang 1-7 induced by vaccination with any of these peptide variants is significantly reduced to as low as 1% of the reactivity to Ang II (Ang II- 193 and Ang II- 198). Moreover, also the crossreactivity to Ang I is reduced in most of these sera. In mouse sera induced by immunisation with Ang II- 192, Ang II- 193, Ang II- 198 the crossreactivity to Ang I is reduced to 40% of the reactivity to Ang II. These Variotope®s contain five modifications each and do only share two amino acids with the original Ang II sequence. The relation of the reactivity to Ang II and the crossreactivity to its derivatives within the different sera is further outlined in Table 3.

Table 3: Crossreactivity of sera induced by Ang II-derived peptides containing multiple amino acid exchanges to Ang I and Ang 1-7 as the percentage of their reactivity to Ang II

	C-Ang II	Ang II-173	Ang II-175	Ang II-177	Ang II-192	Ang II-193	Ang II-195	Ang II-198
Ang II	100	100	100	100	100	100	100	100
Ang I	121	64	62	80	44	36	70	37
Ang 1-7	33	11	8	8	3	1	3	1

The affinity of most sera described in these ELISA experiments was determined by competition ELISA after Friguet et al 1988 [24] or using a Biacore machine. These data are not shown here. However, the affinity of sera for Ang I and Ang 1-7 is in general reduced, even if the titres against these angiotensin peptides are high. Interestingly only peptides that contain the three central amino acids of the Ang II sequence induce antibodies that show similar affinity for Ang II, Ang I and Ang 1-7 compared to sera induced by Ang II. Peptide variants that contain a neutral amino acid at one of the two C-terminal residues induce sera that show reduced affinity for Ang II and Ang I, whereas the affinity for Ang 1-7 is increased compared to other peptide variant-induced sera.

3.2. Cellular assay (Functional Assays)

A cellular assay should be established to determine the activity of Ang II and to evaluate the inhibitory capacitiy of Variotope®-induced sera on Ang II signalling. This assay will be used to analyse the potential correlation of titre and affinity of Variotope®-induced antibodies and their functionality in blocking Ang II signalling. To establish a reliable in vitro assay for the determination of Ang II activity and for the evaluation of the inhibitory capacity of Variotope® induced sera, the signal measured after Ang II stimulation must differ significantly from the un-stimulated control. This would allow a comfortable margin to see effects of Variotope® induced sera on Ang II signalling.

3.2.1. Cellular ELISA with HUVEC (human umbilical vein endothelial cells)

Human umbilical vein endothelial cells possess functionally active angiotensin II and TNFα receptors. Stimulation via these receptors has been shown to up-regulate cellular adhesion molecules, such as ICAM-1 and VCAM-1 upon stimulation [25]. The amount of ICAM-1 and VCAM-1 molecules expressed on the surface of stimulated HUVECs was determined via cell-based ELISA. The basic parameters for this assay have already been established and the assay was used in house with stimuli other than Ang II. Therefore, it was examined whether Ang II stimulation of HUVEC would give a measurable signal in ICAM-1- or VCAM-1- ELISA experiments. HUVEC cells were grown on a 96 well plate and stimulated with different concentrations of TNFα and Ang II for various lengths of time. TNFα was always used as a positive control. The ICAM-1 or VCAM-1 increase was measured using anti-ICAM-1 or anti-VCAM-1 antibodies on methanol fixed HUVECs.

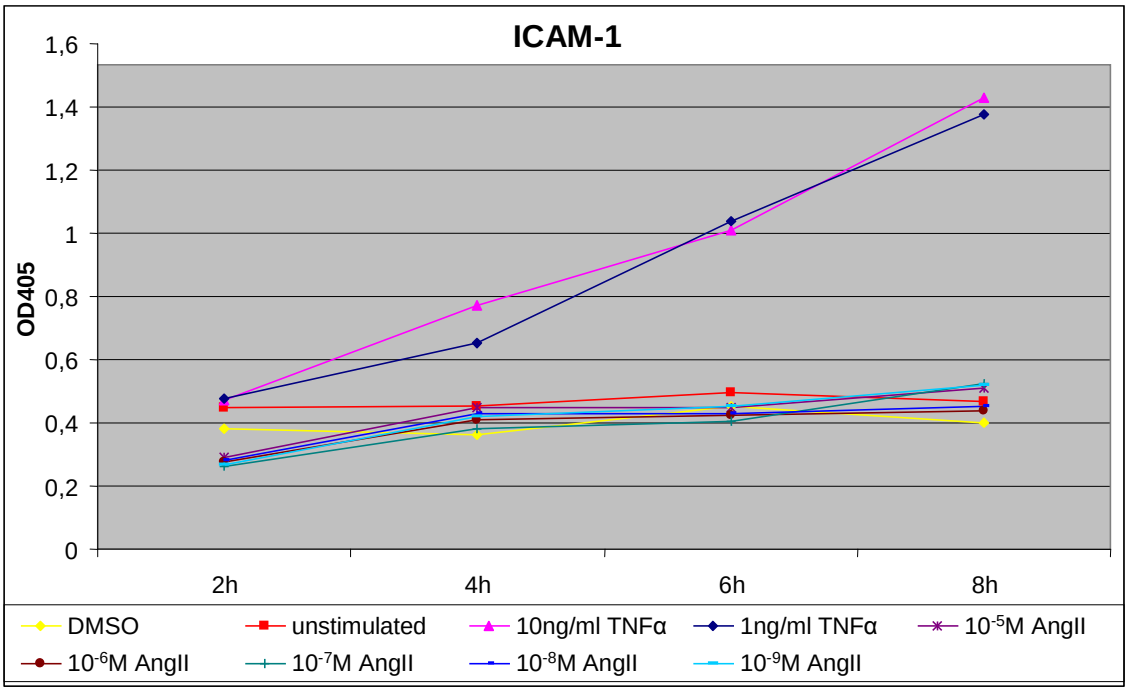


Figure 19: Detection of cell surface ICAM-1 expression via cellular ELISA on fixed HUVECs after 2-8h stimulation with different concentrations of TNFα or Ang II; red: un-stimulated cells; yellow: un-stimulated with DMSO; pink and dark blue: TNFα positive controls; purple, brown, turquoise, dark and light blue: different concentrations of Ang II

As shown in Figure 19, stimulation of HUVEC with 10ng/ml or 1ng/ml TNFα results in a significant increase of ICAM-1 (intracellular cell adhesion molecule) expression on the cell surface. Both concentrations of TNFα used in this experiment increase the ICAM-1 signal continuously as a function of time. The maximum of ICAM-1 expression seems not to be reached after 8h of stimulation with either 10ng/ml or 1ng/ml TNFα. Ang II seems to increase ICAM-1 expression on the cell surface of HUVECs too. However, the ICAM-1 signal is not increased significantly above negative control level. A negative control with 10% DMSO is used, because the 10^{-3}M Ang II stock solution contains 10% DMSO. There seems to be an effect of DMSO on ICAM-1 expression (yellow curve). However, the amount of DMSO is decreased in the Ang II solution with each dilution step and the decrease of ICAM-1 expression caused by DMSO is not significant.

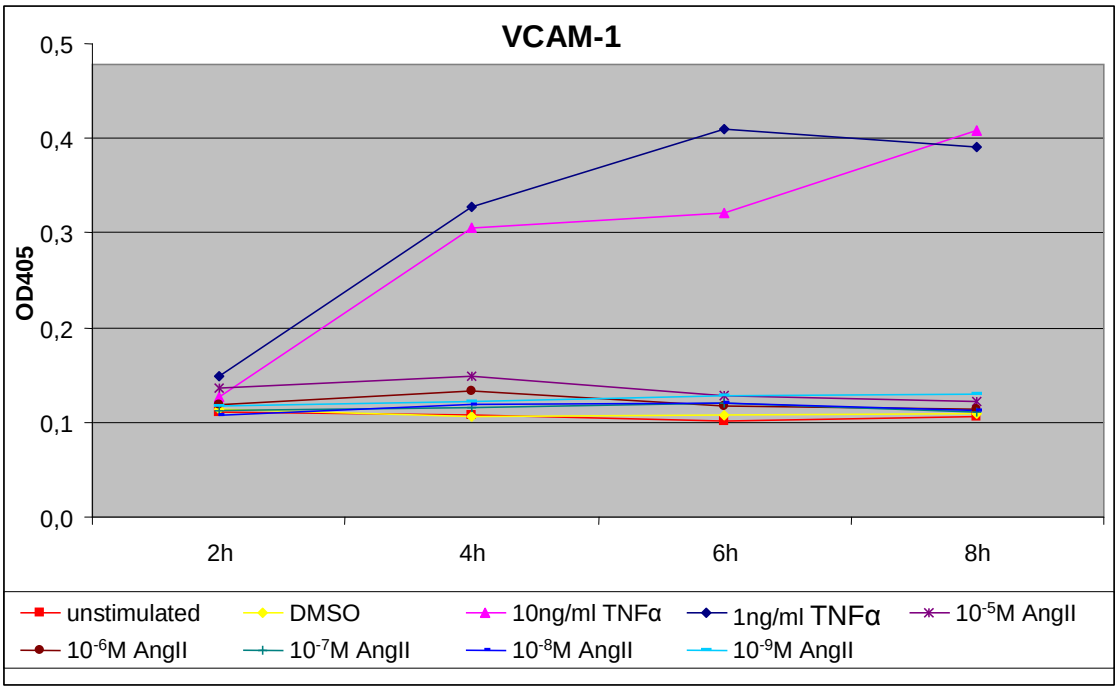


Figure 20: Plasma-membrane bound VCAM-1 detection via ELISA on fixed HUVECs after 2-8h stimulation with different concentrations of TNFα or Ang II; red: un-stimulated cells; yellow: un-stimulated with DMSO; pink and dark blue: TNFα positive controls; purple, brown, turquoise, dark and light blue: different concentrations of Ang II

Figure 20 shows that stimulation of HUVEC with 10ng/ml or 1ng/ml TNFα results in a significant increase of VCAM-1 (vascular cell adhesion molecule) expression on the cell surface. Already after 4h the VCAM-1 signal, measured as the optical density at 405nm is doubled compared to the negative control (in red). The signal increase reaches a plateau after 6h of stimulation with 1ng/ml TNFα. The VCAM-1 expression induced by 10ng/ml TNFα might increase even after 8h of stimulation. However, stimulation of HUVECs with 10^{-5}- 10^{-9}M Ang II did not result in a significant increase of surface bound ICAM-1 or VCAM-1. Although the assay was repeated several times and with different conditions, the ICAM-1 or VCAM-1 upregulation published by Pastore et al. in 2008 could not be reproduced here. Therefore, the cellular ELISA measuring ICAM-1 and VCAM-1 cell surface expression in HUVECs can not be used to determine Ang II signalling activity.

It has been published that soluble ICAM-1 is also secreted by HUVECs upon stimulation with 10^{-6}M Ang II [25]. Therefore, the amount of ICAM-1 molecules in the cell-culture supernatant from previous cell-based ELISA experiments was determined in the next set of experiments. For this purpose a commercially available sandwich ELISA kit for ICAM-1 (R&D Systems) was used. However, not even the positive control (TNFα stimulation) showed any signal increase when the assay was conducted according to the manufacturer's instructions (see Figure 21).

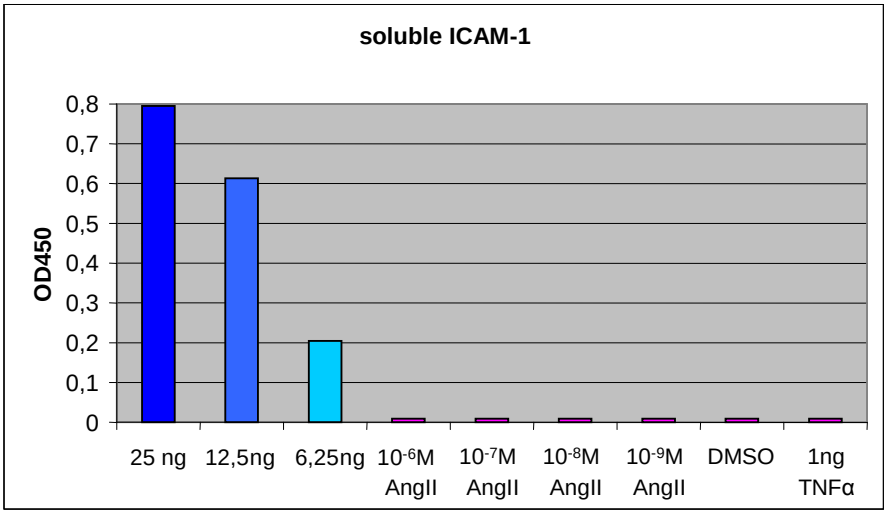


Figure 21: Soluble ICAM-1 measured in supernatants of TNFα and Ang II stimulated HUVEC according to the ICAM-1 ELISA kit protocol and ICAM-1 standards provided with the ICAM-1 ELISA kit (R&D Systems)

As shown in Figure 21, the ICAM-1 standards provided with the ICAM-1 ELISA kit show a signal according to the known concentration of ICAM-1 in each sample. However, none of the HUVEC supernatants induces a measurable signal no matter how the cells were stimulated. To determine the effect of the incubation procedure of the different components of the ICAM-1 ELISA kit, several incubation- steps and –times were tested. To analyse the effect of the cell culture medium the cell culture supernatants were spiked with 50ng ICAM-1 standard solution. Figure 22 shows significant fluctuations between these different procedures.

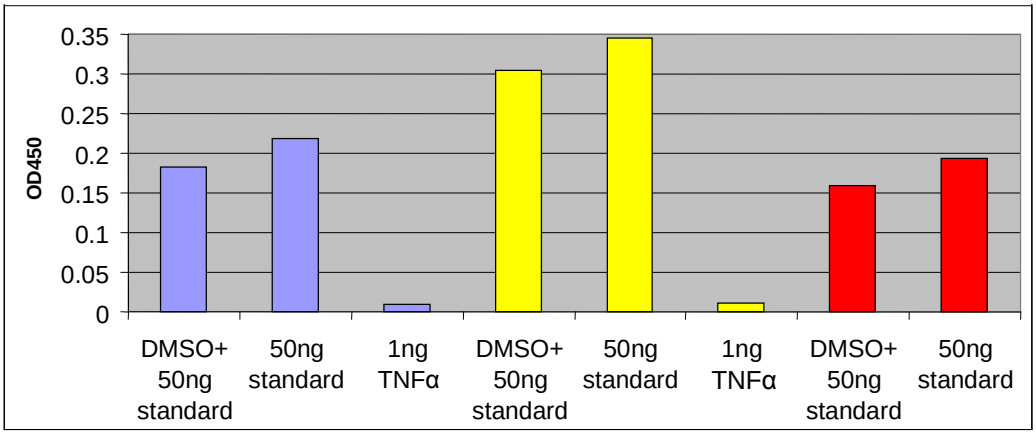


Figure 22: Evaluation of different procedures for detection of soluble ICAM-1 using a ready to use sandwich ELISA kit (R&D Systems) with cell culture supernatants of stimulated HUVECs spiked with 50ng ICAM-1 standard solution. Blue bars: 50µl supernatant was incubated for 1h on antibody coated plates before 50µl of conjugate were added directly to the supernatant. Yellow bars: The supernatant was discarded after the incubation period

and then the conjugated antibody was added to the plates. Red bars: Supernatant and antibody conjugate were added to the plates at the same time without prior incubation period. However, in all three procedures supernatant of DMSO treated cells spiked with 50ng ICAM-1 standard shows a significantly lower signal that 50ng ICAM-1 standard alone. However, the supernatant of cells stimulated with 1ng/ml TNFα does not give any signal in either of these procedures.

The results depicted in Figure 22 show that the presence of DMEM cell culture medium and DMSO in the cell culture supernatant decreases the signal measured with the ICAM-1 ELISA kit (R&D Systems). However, none of the procedures tested in these experiments could increase the ICAM-1 signal measured in cell culture supernatants from TNFα stimulated HUVEC. Therefore, the up-regulation of ICAM-1 secretion upon stimulation of HUVEC can not be used to determine Ang II signalling activity.

Other sources also state that interleukin-8 (IL-8) production is increased upon stimulation of HUVECs with Ang II [35]. Therefore, the supernatants of stimulated HUVECs were also tested for their IL-8 content. TNFα was again used as a positive control. As shown in Figure 23, TNFα stimulated cells secrete IL-8 into the supernatant. There is a slight increase in soluble IL-8 in the supernatant of HUVECs stimulated with 10^{-9}M Ang II. However, here again, Ang II stimulation could not increase the signal significantly beyond control level.

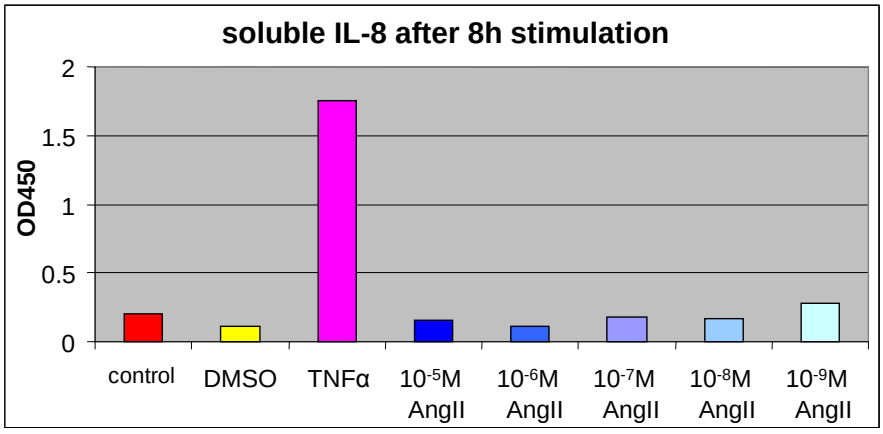


Figure 23: Detection of soluble IL-8 in supernatants of stimulated HUVECs using a ready to use sandwich ELISA kit; supernatants of un-stimulated cells are shown in red, DMSO treated cells in yellow, TNFα stimulated cells in pink, and supernatants of cells stimulated with different concentrations of Ang II in dark to light blue.

The results show that the approach using HUVECs for cell-based ELISA experiments to establish an in vitro assay for the determination of Ang II signalling activity was not successful. Therefore another signal read out had to be found that can be

increased upon stimulation of cells with Ang II. As described in the following chapter, the next approach focuses on the activation of NF-κB in HEK293 cells.

3.2.2. Luciferase assay in HEK293

The scheme of the in vitro assay linking Ang II signalling to a luminescence signal via NF-κB activation and luciferase expression is shown in Figure 24.

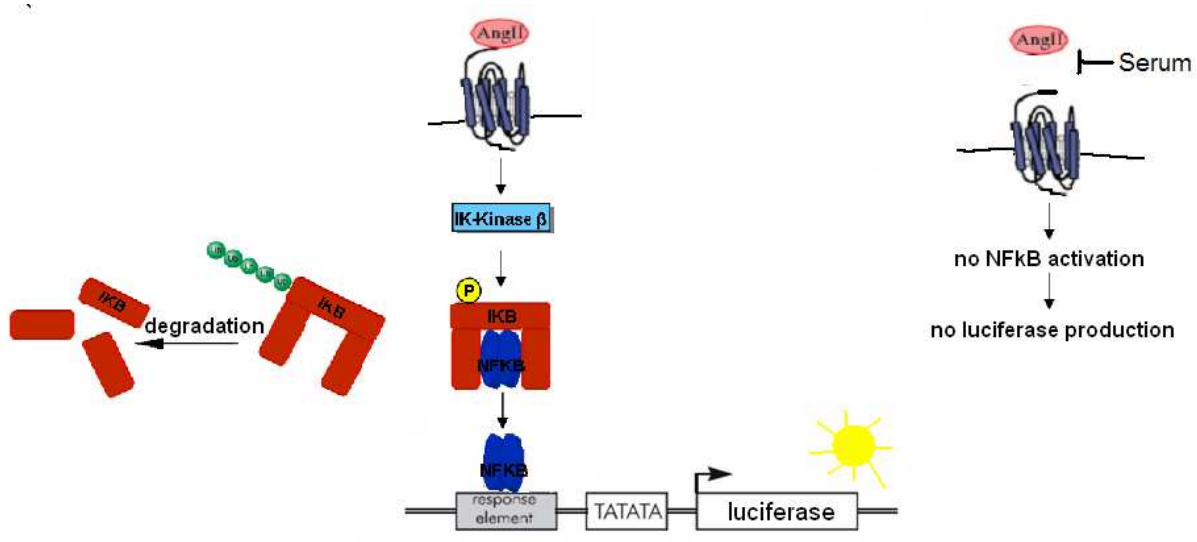


Figure 24: Schematic view of Ang II stimulated NF-κB activation and subsequent luciferase expression via a reporter gene cassette containing several repeats of NF-κB response element

Human embryonic kidney cells (HEK293) are used here, because they are easy to handle in FACS analysis and transfection. Most importantly, HEK293 cells express all the intracellular factors necessary for NF-κB activation. A NF-κB response-element luciferase reporter gene expression vector was used to measure NF-κB activation upon stimulation of HEK293 cells. Unfortunately, these cells do not endogenously express the AT_1 receptor. Therefore, an AT_1R expression vector has to be introduced into the cells to render them susceptible to Ang II stimulation. In order to determine the efficiency of transfection of HEK293 cells with the NF-κB response-element luciferase reporter gene expression vector and the AT_1R expression vector, the AT_1 receptor was fused to EGFP. The green fluorescent signal of positively transfected cells was measured by fluorescence activated cell sorting (FACS).

A CMV (cytomegalo virus) expression vector encoding the human angiotensin II type I receptor cDNA (variant 2) was obtained from Origene. This vector was used as a template for PCR as described in the materials & methods section. Simultaneously to the amplification reaction the coding region of the AT_1R was excised from the vector template.

Primers used here contain recognition sites for the restriction enzymes EcoRI and BamHI at the 5' and 3' ends of the PCR product. These enzymes were chosen, because they cut within the multiple cloning site of most vectors and thus ease future cloning steps. The pEGFP-N1 vector contains an EcoRI and a BamHI recognition site, upstream of and in frame with the EGFP gene and thus allows the production of a C-terminal GFP-fusion protein. The product from the PCR reaction and the pEGFP-N1 expression vector were double-digested with EcoRI and BamHI and relegated together to give an AT_1R-EGFP fusion protein (see Figure 25).

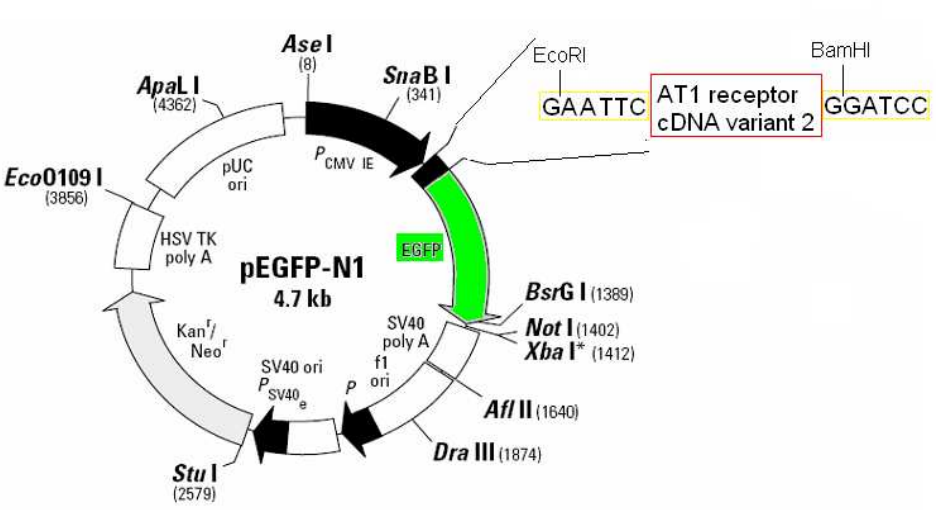


Figure 25: Angiotensin II type I receptor EGFP fusion protein expression vector

The newly generated vector was transformed into E.coli DH5α and bacteria were selected for the successfully relegated pEGFP-N1 vector using 30µg/ml Kanamycin. To analyse the constructs produced by the ligation reaction, 6 different clones were picked for plasmid DNA extraction using the QIAgen Midiprep kit. Isolated plasmid DNA derived from different clones was double digested with EcoRI and BamHI to analyse the success of the plasmid DNA preparation and to determine whether the new vector contains the desired insert. The results were visualised using agarose gel electrophoresis (see Figure 26).

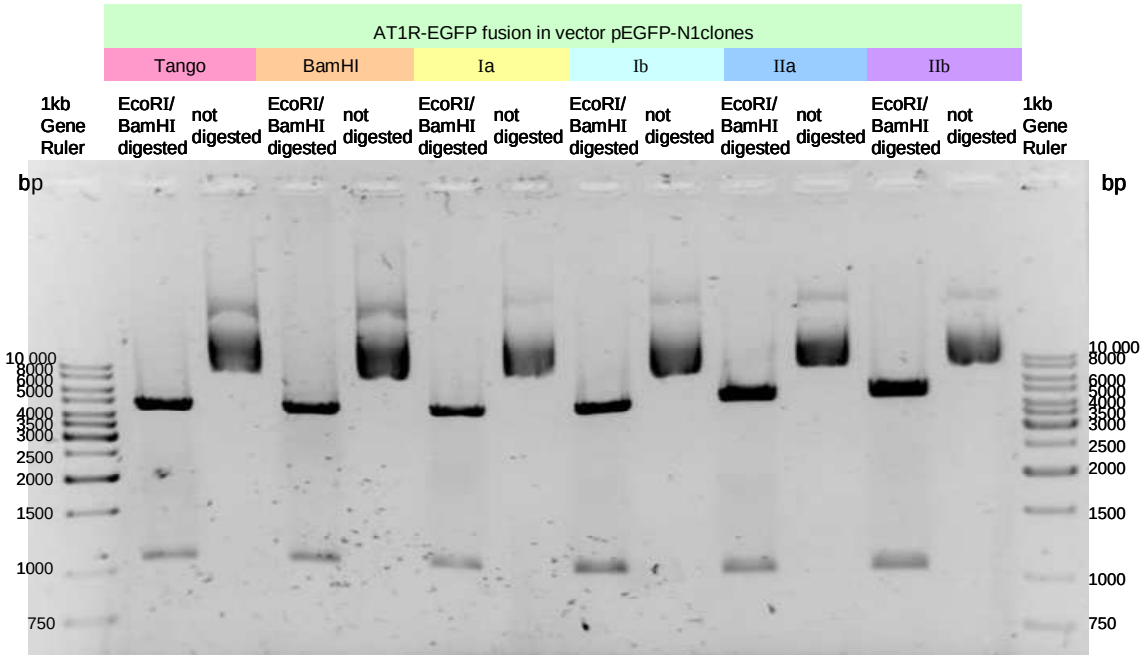
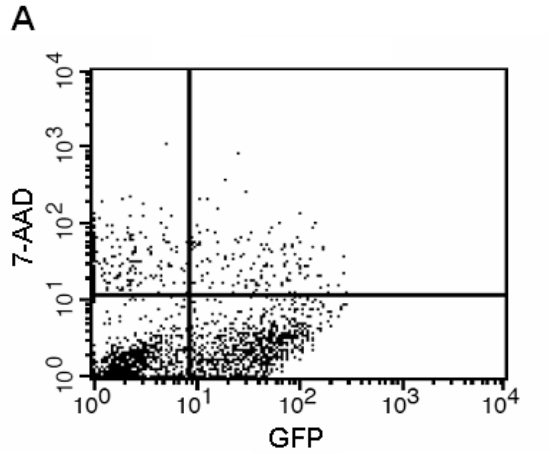


Figure 26: EcoRI/ BamHI double digest of plasmid DNA derived from 6 different *E.coli* clones (Tango, BamHI, Ia, Ib, IIa, IIb) after MidiPrep (QIAgen) on a 1.2% agarose gel

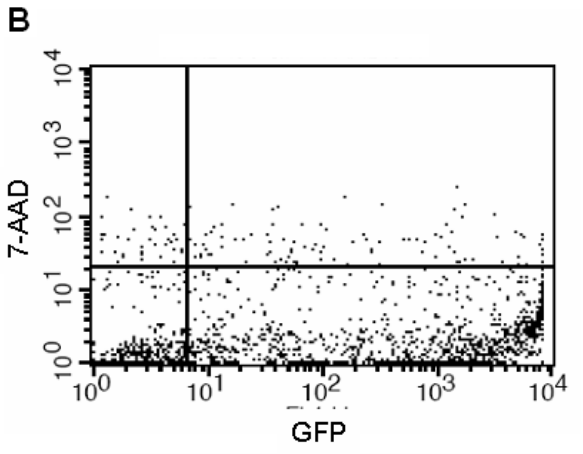
Figure 26 shows that the different plasmid DNA isolations containing AT_1R-EGFP vector are digested into a ~1000bp and a ~5000bp fragment. AT_1R variant 2 cDNA is 1077bp long and the pEGFP-N1 vector without insert has 4.7kb. These results indicate that the ligation was successful and that the isolated plasmids encode AT_1R as well as EGFP.

To further confirm that the correct plasmid construct was isolated from E.coli, a sequence analysis was performed. The results of this analysis are shown in the appendix I. All sequences derived from PCR reaction with different primers are identical with the human AT_1R cDNA variant 2 listed on the NCBI database, except for one nucleotide. This point mutation in position 572 of the AT_1R gene is a wobble base and thus the amino acid at this position of the protein is not changed. These results further confirm that the isolated plasmids encode the AT_1R-EGFP fusion protein.

Next, the expression of this AT_1R-EGFP fusion protein in HEK293 cells transfected with the isolated plasmid was determined via FACS analysis. As shown in Figure 27 tranfected HEK293 cells show green fluorescent activity. This demonstrates that EGFP is expressed and fluorescently active and thus indicates that the AT_1R-EGFP product is recognised as one translation frame.



Quad	Events	% Gated	% Total
UL	922	9.22	9.22
UR	658	6.58	6.58
LL	4200	42.00	42.00
LR	4220	42.20	42.20



Quad	Events	% Gated	% Total
UL	190	2.25	1.90
UR	460	5.45	4.60
LL	1564	18.51	15.64
LR	6234	73.79	62.34

Figure 27: FACS analysis of HEK293 cells transfected with AT_1R-EGFP expression vector clone "tango" (A) and pEGFP-N1 (B)

Figure 27 (A) shows that about 40% of HEK293 cells transfected with the AT_1R-EGFP expression vector have significant green fluorescent activity. This indicates that these cells express EGFP. Figure 27 (B) shows FACS analysis of HEK293 cells transfected with pEGFP-N1 without AT_1R insert. Here, the fluorescent intensity is almost out of scale.

The correct expression of the angiotensin II type I receptor in tranfected HEK293 cells was also determined by Western Blot analysis. Three different anti- AT_1R antibodies (all from Santa-Cruz Biotechnologies) were tested. However, no AT_1R specific signal from HEK293 cell-lysates could be detected with any of these antibodies. An AT_1R membrane isolation (PerkinElmer) and HUVEC lysates were used as positive controls for Western blot analysis of the AT_1R. However, even within these positive controls there was no clearly specific AT_1R signal visible on the nitrocellulose membrane. Therefore, an anti-GFP (Abcam) antibody was used to detect the AT_1R-EGFP fusion protein expressed in transfected cells. The results from the Western Blot analysis using this anti-GFP antibody are shown in Figure 28.

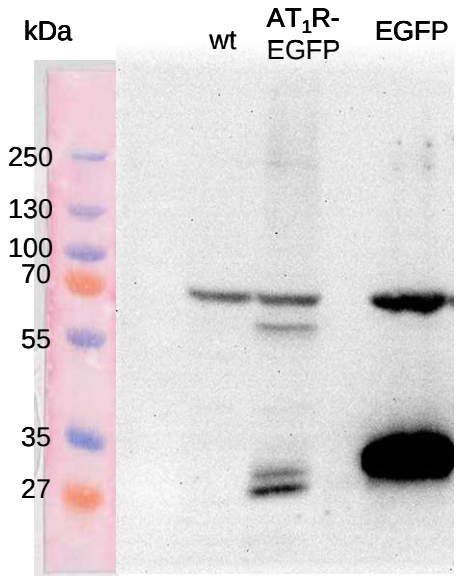


Figure 28: Lysates of un-transfected (wt), AT_1R-EGFP transfected and EGFP transfected HEK293 cells; GFP has a molecular weight of 26.9kDa and the AT_1R-EGFP fusion protein seems to be at a height of ~60kDa (red circle)

The Western Blot of HEK293 cell-lysates after transfection with the empty EGFP vector or with the AT_1R-EGFP encoding vector shows a GFP specific band at ~60kDa that is only present in AT_1R-EGFP transfected cells. A second band recognised by the anti-GFP antibody just above the usual GFP band at 27kDa might derive from a short translational by-product. The band at approximately 70kDa seems not to be GFP-specific, since it also occurs in wild type HEK293 cells.

The AT_1 receptor is a seven transmembrane receptor and should be expressed within the plasma membrane of the cells. HEK293 cells transfected with the AT_1R-EGFP fusion vector were analysed by fluorescence microscopy to determine the cellular localisation of the expressed protein.

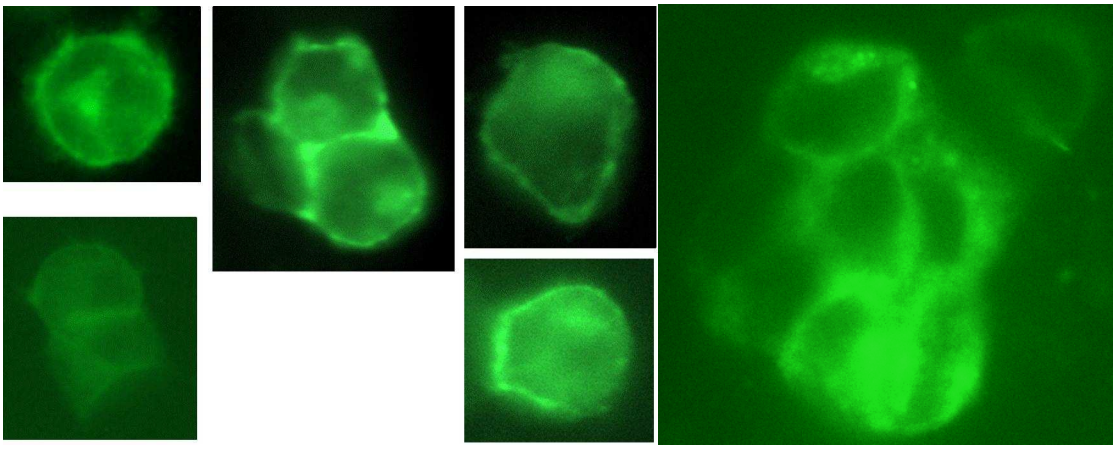


Figure 29: Fluorescent microscopy images of HEK293 cells expressing the AT_1R-EGFP fusion protein

The pictures taken with the fluorescence microscope (Figure 29) show, that the fluorescent activity of transfected HEK293 cells is concentrated in their cell membranes. This further indicates that the AT_1R-EGFP fusion protein is correctly expressed and that the protein is localised within the cell membrane. Therefore, the AT_1 receptor should be susceptible to stimulation with Ang II.

After the integrity of the generated AT_1R-EGFP construct was analysed by these numerated methods, it can be assumed that the receptor should be functionally active. This was assessed in a functional assay linking AT_1R stimulation to the expression of a luciferase reporter gene via NF-κB activation. Ang II is known to activate the transcription factor NF-κB upon receptor binding. Activated NF-κB acts as a transcription factor on gene expression in the nucleus (see Figure 24). Therefore, a reporter cassette consisting of five repeats of the NF-κB response-element upstream of the *Renilla* luciferase gene (NFκB-luc) was inserted into HEK293 cells to work as a reporter gene system. The activation of NF-κB and the subsequent expression of luciferase were used to measure Ang II signalling via chemiluminescent luciferase activity.

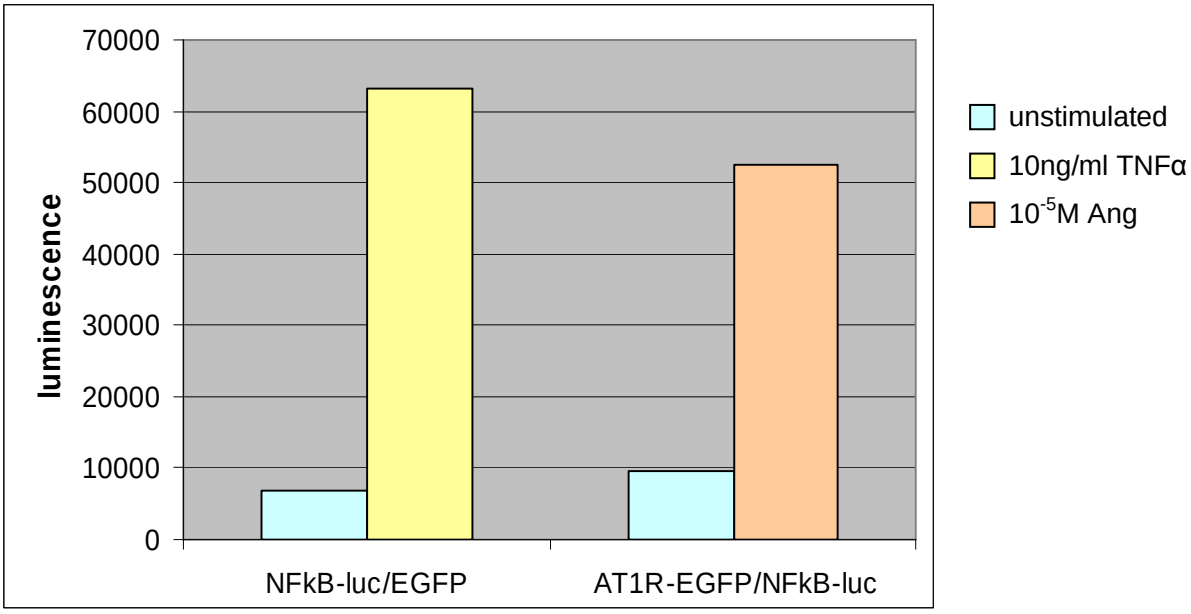


Figure 30: Luminescence of HEK293 cells co-transfected with NFκB-luc and empty EGFP (left) or AT_1R-EGFP and NF-κB-luc (right) after 24h of stimulation with 10ng/ml TNFα or 10^{-5}M Ang II respectively

It can be seen from Figure 30 that the stimulation of HEK293 cells transfected with AT_1R-EGFP and NFκB-luc with Ang II significantly increases the luminescence signal. HEK293 cells transfected with NFκB-luc and the EGFP-N1 vector were used as a reference for NF-κB activity and stimulated with TNFα. Stimulation with TNFα as a positive control significantly increases the luciferase expression through NFκB-luc

activation. These results further indicate that the generated AT_1R-EGFP fusion protein is expressed in the plasma-membrane of transfected cells and is functionally active.

The measured luciferase signal should decrease when cotransfected HEK293 cells are pre-incubated with AT_1 receptor blocker losartan.

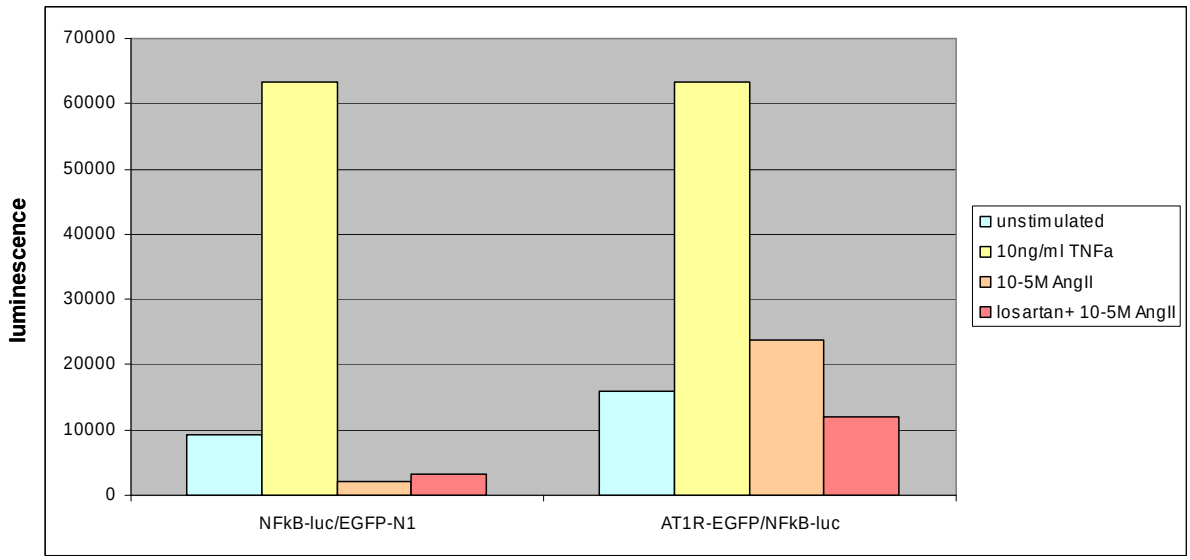


Figure 31: Luminescence of HEK293 cells co-transfected with NFκB-luc and empty EGFP (left) or AT_1R-EGFP and NF-κB-luc (right) after 24h of stimulation with ☐ 10ng/ml TNFα or ☐ 10^{-5}M Ang II respectively or inhibited with ☐ 10^{-7}M losartan before 10^{-5}M Ang II stimulation

Figure 31 shows another luminescence experiment using co-transfected HEK293 cells. Stimulation of HEK293 cells co-transfected with AT_1R-EGFP and NFκB-luc expression vectors with 10^{-5}M Ang II results in a significant increase of luciferase activity. However, in this experiment the luminescence signal of Ang II stimulated cells is lower than in the previous experiment (see Figure 30). Incubation of HEK293 cells co-transfected with AT_1R-EGFP and NFκB-luc expression vectors with receptor blocker losartan prior to stimulation with Ang II, reduces the luciferase activity. HEK293 cells transfected with NFκB-luc and the EGFP-N1 vector were again used as a reference for NF-κB activity. However, neither Ang II nor losartan have any effect on HEK293 cells that are not transfected with the AT_1 receptor construct.

Different concentrations of Ang II and TNFα were used to stimulate co-transfected HEK293 cells in order to determine the minimal amount of stimulus needed to significantly increase the luminescence. Furthermore, losartan should not affect the luciferase expression of HEK293 cells co-transfected with AT_1R-EGFP and NFκB-luc expression vectors stimulated with TNFα.

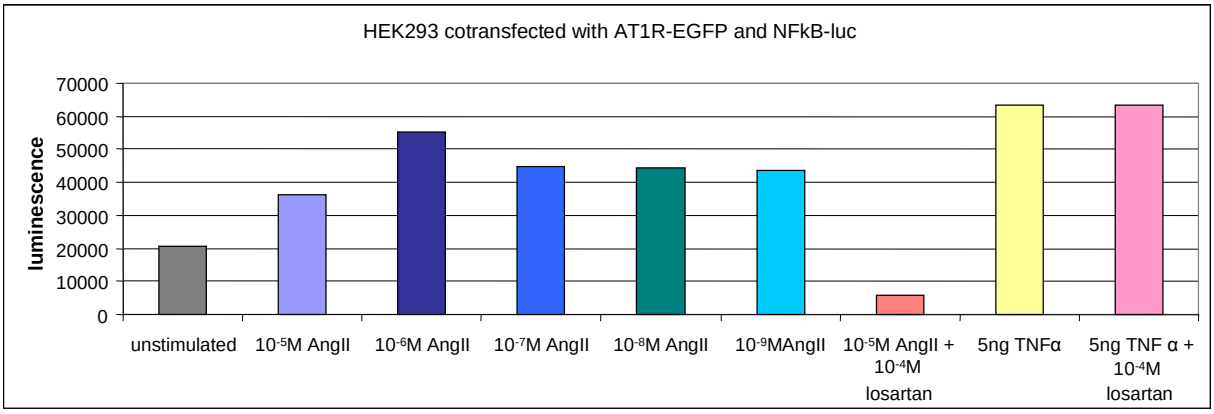


Figure 32: Luminescence of HEK293 cells co-transfected with AT_1R-EGFP and NF-κB-luc after 24h of stimulation with 5ng/ml TNFα or 10^{-5}M- 10^{-9}M Ang II respectively or inhibited with 10^{-4}M losartan before stimulation

Figure 32 shows that HEK293 cells co-transfected with AT_1R-EGFP and NF-κB-luc can be stimulated to express luciferase with different concentrations of Ang II. The highest luminescence signal was measured after stimulation of HEK293 cells with 10^{-6}M Ang II. Interestingly, even the lowest Ang II concentration used here (10^{-9}M Ang II) could significantly stimulate luciferase expression. 10^{-4}M losartan inhibits the activation of AT_1R by Ang II. However, losartan has no effect on HEK293 cells stimulated with TNFα. This further indicates that the increase in luciferase expression upon stimulation of transfected HEk293 cells with Ang II is specific for AT_1R activation and not due to other factors in the medium.

Luciferase assays to determine the inhibitory capacity of Ang II- or Variotope®-induced sera were conducted as well. However, the presence of mouse serum in the cell culture supernatant of HEK293 cells seems to have stimulatory effects on NF-κB activation. These effects have to be blocked to study the effect of Ang II specific antibodies on AT_1R stimulation using NF-κB activation as read out. However, the possibilities to use mouse sera in the established lusiferase assay will have to be defined in future experiments.

4. Discussion

4.1. Epitope specificity of Ang II-induced sera changes after multiple vaccination steps

Three of the seven transmembrane domains and the linking extracellular loops of the AT_1 receptor are involved in ligand interactions (see also Figure 33). Binding of Ang II to the AT_1 receptor requires the interaction of the C- terminal phenylalanine side chain of Ang II with the histidine at position 256 of the receptor. This interaction is facilitated by docking the carboxyl-group to the lysine at position 199 of the AT_1 receptor. The interaction at this, so called phenylalanine-switch locus is based on the stacking of the aromatic rings of Phe 8 and His 256. Any modification of these residues that would prevent the planar arrangement of the benzyl side chain above the Pro 7-Phe 8 amide bond has an antagonistic effect on receptor-binding. Interestingly, this loss of function is not consistent with a loss of affinity. Antagonists are known to have higher affinity to the AT_1 receptor binding sites than most agonists. However, the stacking interaction of Phe 8 and His 256 does not induce a large conformational change, but exposes other sites within the receptor for crucial interactions. The asparagine at position 111 within transmembrane helix III of AT_1R is known to interact with the tyrosine at position 4 of the Ang II peptide. This interaction has been found to trigger the major conformational change that efficiently activates the AT_1 receptor [26, 27]. Therefore, any antibody able to block the binding of Ang II and the subsequent activation of AT_1R might have to target the Tyr 4 and the C-terminal Phe 8 of the Ang II peptide.

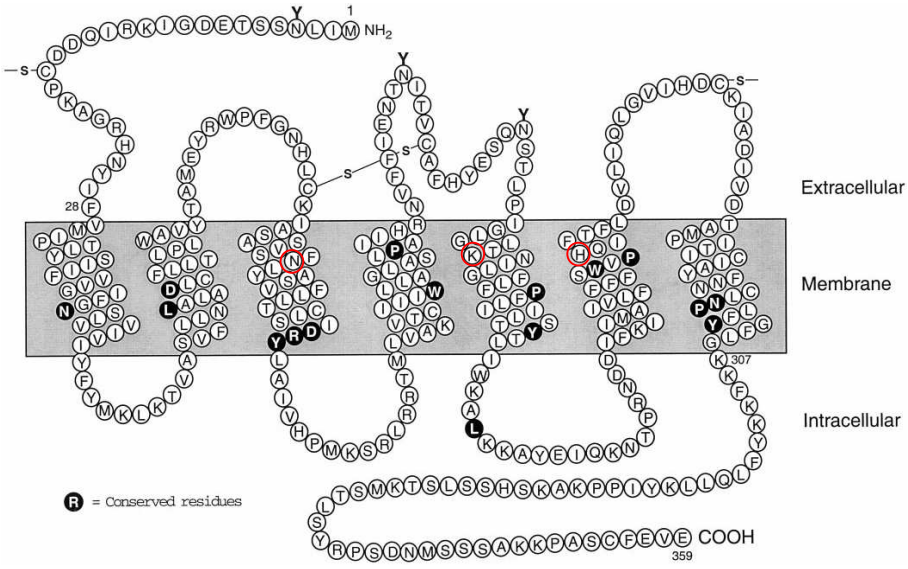


Figure 33: The angiotensin II type I receptor. Red Circles mark residues involved in Ang II binding: N^{111}, K^{199}, H^{256}

The first ELISA experiments described in the results chapter were conducted to find whether the epitope specificity of Ang II-induced sera changes after multiple vaccination steps. The vaccination schedule consisting of one prime vaccination and three boost vaccinations is conducted to reach high antibody titres. Twelve days after each vaccination the serum of the mice is collected. Thus, Serum1, Serum2, Serum3 and the Endserum are obtained. The results show that titres rise from Serum1 to Serum3. However, after the third boost vaccination the titres do generally not increase significantly. One reason for this phenomenon might be that the immune response is directed towards more dominant epitopes of the carrier protein KLH. This shows the down-side of peptide-based vaccines. The low immunogenicity of short sequences places a lower risk of inflammation side effects, but at the same time needs highly immunogenic carrier proteins that might reduce peptide-specific boosting effects.

In Serum2 there is a significant difference in titres against Ang II variant peptides compared to the original Ang II peptide, especially if the C-terminal end of the octapeptide is modified. Any modification of the phenylalanine at position 8, the free carboxyl-group of the 8^{th} amino acid and also the histidine at position 6 in the Ang II peptide results in reduced recognition of the antigenic determinant by Ang II-induced antibodies. This indicates that after the first boost vaccination the major humoral immune response is directed against the C-terminal end of Ang II. Furthermore, it allows us to assume that these residues might play an important role for C-terminal antibody binding.

4.2. Reactivity of Variotope®-induced sera to Ang II and their crossreactivity to Ang I and Ang 1-7

The repertoire of antigenic determinants of the Ang II peptide has been established by Picard et al. in 1986 using inhibition studies and radioimmunoassay. The monoclonal antibodies produced in these studies react to a great variety of antigenic determinants within the octapeptide. These antibodies were generated by immunisation of BALB/c mice with Val^5-Ang II conjugated to KLH, isolation of lymphocytes from the spleens of responding mice and the generation of a hybridoma cell line for each lymphocyte clone. Picard and his group could identify monoclonal antibodies reacting to the C-terminal end (C-term) of the peptide that differ in their specificity for distinct parts of the C-term and in their crossreactivity to Ang I. One group of mAB required the phenylalanine at position 8 as the terminal amino acid, another one reacted rather to the carboxy-terminal moiety than to the side chain of the terminal amino acid and yet another group was also reactive to Ang I, which carries two additional C-terminal amino acids. Additionally, radioimmunoassay showed that antibody binding was dramatically inhibited when the

tyrosine residue at position 4 was modified. Therefore, the C-terminal epitope probably encompasses both amino acids, the phenylalanine at position 8 and the tyrosine at position 4 [28]. A spiral model for isoleucyl-angiotensin II, published in 1962 by Smeby et al., supports this suggestion, because it describes the close apposition of the two aromatic rings of Tyr 4 and Phe 8 [29]. Figure 34 shows the most potential spiral arrangement of the Ang II octapeptide.

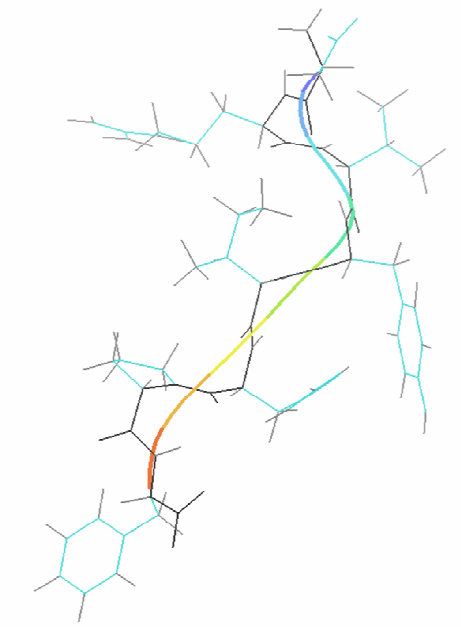


Figure 34: Conformational model for Ang II showing the side chains of all eight residues; from the top: DRVYIHPF

Ang II peptide variants can induce sera that show a significantly increased immunogenicity. In accordance with the results of Picard et al., immunisation with Ang II results in sera containing a variety of antibodies. The results from ELISA experiments presented in this work, show that mouse sera immunised with Ang II contain antibodies that react to different angiotensin peptides with different intensity. Although the oligoclonal antibody mixture in these sera specifically recognises the C-terminal end of the octapeptide, it can not distinguish between Ang II, Ang I or a peptide variant having an amydated carboxyl-group. The aim of the project is to induce oligoclonal antibodies that can distinguish between Ang II, Ang I and Ang 1-7 by immunisation with different Ang II-peptide variants.

Furthermore, the results from ELISA experiments with Variotope®-induced sera indicate that modifications in the central part of the octapeptide affect the selectivity for Ang II, as compared to the crossreactivity to Ang I and Ang 1-7. These central amino acids might play an important role for the affinity of induced antibodies for Ang II, Ang I

and Ang 1-7. Modifications of these central positions of the Ang II sequence might result in structural and conformational changes of the whole Ang II peptide. Especially the tyrosine at position 4 has been previously found to be part of the C-terminal epitope of Ang II. In peptide variants Ang II-066 and Ang II-067 tested in mice, the three middle residues of the Ang II sequence (DRVYIHPF) are exchanged to a neutral amino acid, forming a long sequence without reactive chemical properties. This structure could affect antibody binding, and direct the specificity towards Ang II, while excluding Ang I. These results are consistent in mouse- and rat-experiments. The most successful peptide (Ang II-030) in recognising Ang II while sparing Ang 1-7 and reducing the crossreactivity to Ang I in Wistar rats is also modified within these 3 residues. By specifically choosing exchanges at positions 2-5 (DRVYIHPF) we could achieve a reduction of the crossreactivity to Ang I and almost eliminate the crossreactivity to Ang 1-7. This might mean that middle and N-terminal residues play an important role in directing the antibody response towards the C-term and are not only mere spacers. However, Ang 1-7 is highly recognised when one or more of the last three residues (DRVYIHPF) are changed to alanine, thus directing the antibody respond towards the N-terminal end of the octapeptide. When exchanging one or more residues with various amino acids with similar or contrary chemical properties, we found that there are some Varitope®s that behave similar to the original peptide and some that differ from the original peptide in their recognition pattern to Ang I and Ang 1-7.

4.3. Determination of Ang II signalling by functional cellular assay

A variety of different experiments that define the effects of angiotensin II stimulation in different cell lines has been published. In these publications, mostly cells that endogenously express angiotensin receptors were used. These include vascular smooth muscle cells (VMSC), human umbilical vein cells (HUVEC), rat cardiac fibroblasts and mouse mesanglial cells (MMC). A group around Bin Wang detected the effect of Ang II on vascular smooth muscle cell (VSMC) proliferation via bromodeoxyuridine incorporation and cell cycle distribution. Furthermore, they measured the expression of c-jun and c-fos (cellular markers for proliferation) mRNA in Ang II stimulated VSMC by reverse transcription PCR and Western Blot [30]. Another method to determine the signalling activity of Ang II is to measure the intracellular free Ca^{2+} concentration. This is done using Ca^{2+} sensitive indicators, such as Fluo3-AM or Flura-2 AM, whose emitted fluorescent activity was analysed by a laser scanning confocal microscope [31, 32]. Stimulation of the AT_1R via Ang II activates several intracellular MAP kinases, whose phosphorylation state can also be used as indicator of Ang II signalling. The activation of

MAP kinases such as ERK1/2 or STAT3 is examined by Western Blot using phospho-specific antibodies [32, 33, 34].

The assay developed in this diploma thesis should be feasible, robust, easy to handle, allow medium through-put analysis and give a clear signal to noise ratio. Therefore, we looked for a signal read out that can be changed by Ang II treatment and that can be measured by a method that meets these criteria.

Chae et al. measured the increase of ICAM-1 and VCAM-1 expression in HUVECs after Ang II stimulation by semi-quantitative RT-PCR analysis. Furthermore, they also measured the amount of cell-surface bound ICAM-1 and VCAM-1 molecules on HUVECs fixed on a 96 well plate by ELISA. This is an effective method that can be used for high through-put analysis. Chae et al. published a 1.5 fold increase of both adhesion molecules after Ang II stimulation [32]. Furthermore, a cellular ELISA for the determination of ICAM-1 and VCAM-1 expression in HUVECs was already used in house with another indication. However, in the project presented here, it was not possible to induce a significant difference in the amount of membrane expressed ICAM-1 and VCAM-1 molecules in HUVEC by stimulation with Ang II. A group around L. Pastore published that the secretion of soluble ICAM-1 is also significantly increased upon Ang II stimulation. The attempt to determine the amount of ICAM-1 molecules in the supernatant of stimulated HUVECs using a commercially available ICAM-1 sandwich ELISA kit was not successful. Moreover, the increased secretion of IL-8 after Ang II stimulation published for VSMCs by Kim et al. could not be seen from IL-8 sandwich ELISA of HUVEC-supernatants [35]. Although all experiments have been conducted as described in the respective publications, the results described there could not be reproduced here. The interfering effect of the cell culture medium on the assay read out was not examined more closely, because the approach was stopped to focus on a potentially more successful method.

Like many other G-protein coupled receptors, AT_1R is known to form homo- and heterodimers. The formation of dimers regulates receptor-specific functions, such as ligand binding, signalling, desensitisation and cell surface targeting. Hypertensive patients have been found to show enhanced angiotensin II responsiveness. This might be due to elevated levels of crosslinked AT_1 receptor dimers. An enzyme, called intracellular factor XIIIA transglutaminase crosslinks activated AT_1R homodimers via glutamine at the C-terminal tail of the receptor. Two G-protein coupled receptors can interact with one G-protein. A crosslinked AT_1R dimer may be kinetically favourable for G-protein interaction compared to a dissociable receptor complex, thereby accounting for enhanced activation and signalling. Formation of crosslinked AT_1 receptor dimers requires the activation of the Ang II-AT_1R system and of factor XIIIA [36]. Therefore a signal (such as ICAM-1 or VCAM-1

expression) released by Ang II stimulation might be increased by crosslinking of the angiotensin II type I receptor. This can be achieved by addition of the crosslinking factor XIIIA transglutaminase into the cell medium. An approach stimulating crosslinked receptor dimers in HUVECs could probably be more successful in increasing ICAM-1, VCAM-1 or IL-8 expression.

Other attempts to determine the activation of the AT_1 receptor, measure the activation of NF-κB. This can be done directly via electrophoretic mobility shift assay (EMSA) characterising the binding activity of NF-κB in nuclear extracts to radio-labelled transcription factor consensus oligonucleotides or indirectly by measuring the degradation of IκB via Western Blot [37]. Soehnlein et al. measured ICAM-1, VCAM-1 expression and NF-κB translocation into the nucleus via FACS analysis using fluorescently labelled antibodies to each of the desired proteins [38]. As already mentioned, the measurement of ICAM-1 and VCAM-1 expression was not feasible for the application described here. Therefore we focused on methods to determine the activation of transcription factor NF-κB.

A very elegant experiment conducted by Wolf et al. uses a reporter gene cassette consisting of four tandem copies of the κ enhancer fused to the herpes simplex virus thymidine kinase promoter upstream of a luciferase gene to measure NF-κB activation. They transiently transfected COS7 cells (a kidney fibroblast cell line attained from *Cercopithecus aethiops)* with full-length rabbit AT_1 receptor and pNF-κB-luc. These cells were then stimulated with 10^{-7}M Ang II and the luciferase activity was measured. This assay met the criteria of clear signal to noise ratio, possibility for medium through-put analysis and feasibility. Thus, in the second approach to establish an in vitro functional assay to determine Ang II signalling activity, we tried to reproduce the luciferase assay described by Wolf et al.[39]

In this project, HEK293 cells were chosen, because they are easy to handle and to transfect. Most importantly, HEK293 cells possess all the intracellular signalling factors necessary to activate NF-κB upon an extracellular stimulus. However, HEK293 cells do not endogenously express the angiotensin II type I receptor. Therefore, the cells were transfected with a cyto-megalo virus vector expressing the AT_1 receptor. In order to measure transfection efficiency via FACS the AT_1 receptor was fused to the N-terminal end of EGFP. A fusion protein consisting of the angiotensin II type I receptor and GFP has been shown to be functionally active in a study by Hunyady and his group in 2002 [40]. The construct resulting from the ligation of the AT_1R cDNA variant 2 into the pEGFP-N1 vector conducted in the present work was sequenced. Sequencing data reveal that, except for one point mutation in a wobble base at position 572 of the AT_1R gene, the sequence of

the AT_1 receptor and the EGFP gene are correct and within the same translation frame. Pointmutations in wobble bases usually do not change the amino acid at this position of the gene. This is also the case here, as codons CTT and CTC both encode leucine. The correct expression of the whole AT_1R-EGFP fusion protein was further shown by Western Blot. The GFP specific band detected on the membrane using an anti-GFP antibody is about 10kDa lower than expected from addition of the mass of AT_1R (~40kDa) and EGFP (27kDa). However, AT_1R is a seven transmembrane receptor and these proteins are known to behave differently in SDS-PAGE analysis compared to average protein ladders. In this experiment an anti-GFP antibody was used to detect the expression of the AT_1R-EGFP, because the anti-AT_1R antibodies available in this project did not give a specific signal in Western blot analysis. Fluorescence microscopy of HEK293 cells transfected with the AT_1R-EGFP expression vector showed that the green fluorescent activity of the cells is concentrated on the cell membrane. Thus, we can assume that the receptor is expressed in the cell membrane of transiently transfected HEK293 cells. There, it should be functionally active. Furthermore, cells transfected with the AT_1R-EGFP expression vector showed fluorescent activity in the FACS analysis. However, compared to the fluorescent activity of HEK293 cells transfected with the pEGFP-N1 vector alone, the signal is decreased. This might indicate that the whole fusion protein is expressed and thus GFP activity is obscured. Moreover, stimulation of transfected cells with Ang II induced a significant luciferase activity increase according to NF-κB activation. This signal increase was reversible by incubation of transfected cells with AT_1R inhibitor losartan. This shows that the signal increase is specific for Ang II stimulation and not due to any other stimulatory substance in the cell culture- medium. Losartan even decreased the signal below the background level of unstimulated cells. This might indicate that unspecific activation of NF-κB occurred due to any assay procedures and that this unspecific activation was also reversed by losartan. In summary these data indicate that the AT_1R-EGFP vector was expressed correctly and functionally active in transiently transfected HEK293 cells.

Preliminary experiments determining the inhibitory capacity of Ang II- or Variotope®-induced sera were conducted as well. However, the presence of mouse serum in the cell culture supernatant of HEK293 cells alone, seems to affect NF-κB activation. The stimulatory effects of complete mouse sera have to be blocked to study the effect of antibodies contained in these sera on Ang II-AT_1R binding. It is not clear which component of complete mouse serum stimulates NF-κB activation in HEK293 cells. IgG molecules alone should not activate NF-κB in HEK293 cells, since these cells do not express any Fc-receptors. Thus, Ang II specific antibodies contained in the mouse sera will have to be isolated or immobilised using for example protein A. However, the possibilities to use mouse sera in the established lusiferase assay will have to be defined in future experiments.

Appendix I

Sequence analysis:

Alignment of AT1R-EGFP sequence with sequencing data from AT1R-EGFP tango

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Original–AATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTGGTT  81
Fw2-------------------------------------------------------------------------------------
Fw3-------------------------------------------------------------------------------------
Fw4-------------------------------------------------------------------------------------
Fw5-------------------------------------------------------------------------------------
Rev2-----AATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTGGTT
Rev3-------------------------------------------------------------------------------------
Rev4-------------------------------------------------------------------------------------

Original–TAGTGAACCGTCAGATCCGCTAGCGCTACCGGACTCAGATCTCGAGCTCAAGCTTCGAATTCATGATTCTCAACTCTTCTA 162
Fw2-----------------------------------------------CTCGAGCTCAAGCTTCGAATTCATGATTCTCAACTCTTCTA
Fw3-------------------------------------------------------------------------------------
Fw4-------------------------------------------------------------------------------------
Fw5-------------------------------------------------------------------------------------
Rev2-----TAGTGAACCGTCAGATCCGCTAGCGCTACCGGACTCAGNTCTCGAGCTCAAGCTTCGAATTCATGATTCTCAACTCTTCTA
Rev3-------------------------------------------------------------------------------------
Rev4-------------------------------------------------------------------------------------

Original–CTGAAGATGGTATTAAAAGAATCCAAGATGATTGTCCCAAAGCTGGAAGGCATAATTACATATTTGTCATGATTCCTACTT 243
Fw2------CTGAAGATGGTATTAAAAGAATCCAAGATGATTGTCCCAAAGCTGGAAGGCATAATTACATATTTGTCATGATTCCTACTT
Fw3-------------------------------------------------------------------------------------
Fw4-------------------------------------------------------------------------------------
Fw5-------------------------------------------------------------------------------------
Rev2-----CTGAAGATGGTATTAAAAGAATCCAAGATGATTGTCCCAAAGC-KGAAGGCATAATTACATATTTGTCATGATTCCTACTT
Rev3-------------------------------------------------------------------------------------
Rev4-------------------------------------------------------------------------------------

Original–TATACAGTATCATCTTTGTGGTGGGAATATTTGGAAACAGCTTGGTGGTGATAGTCATTTACTTTTATATGAAGCTGAAGA 324
Fw2------TATACAGTATCATCTTTGTGGTGGGAATATTTGGAAACAGCTTGGTGGTGATAGTCATTTACTTTTATATGAAGCTGAAGA
Fw3-------------------------------------------------------------------------------------
Fw4-------------------------------------------------------------------------------------
Fw5-------------------------------------------------------------------------------------
Rev2-----TATACAGTATC--------------------------------------------------------------------
Rev3-------------------------------------------------------------------------------------
Rev4-------------------------------------------------------------------------------------

Original–CTGTGGCCAGTGTTTTTCTTTTGAATTTAGCACTGGCTGACTTATGCTTTTTACTGACTTTGCCACTATGGGCTGTCTACA 405
Fw2------CTGTGGCCAGTGTTTTTCTTTTGAATTTAGCACTGGCTGACTTATGCTTTTTACTGACTTTGCCACTATGGGCTGTCTACA
Fw3-------------------------------------------------------------------------------------
Fw4-------------------------------------------------------------------------------------
Fw5-------------------------------------------------------------------------------------
Rev2-------------------------------------------------------------------------------------
Rev3-------------------------------------------------------------------------------------
Rev4-------------------------------------------------------------------------------------

Original–CAGCTATGGAATACCGCTGGCCCTTTGGCAATTACCTATGTAAGATTGCTTCAGCCAGCGTCAGTTTCAACCTGTACGCTA 486
Fw2------CAGCTATGGAATACCGCTGGCCCTTTGGCAATTACCTATGTAAGATTGCTTCAGCCAGCGTCAGTTTCAACCTGTACGCTA
Fw3-------------------------------------------------------------------------------------
Fw4-------------------------------------------------------------------------------------
Fw5-------------------------------------------------------------------------------------
Rev2-------------------------------------------------------------------------------------
Rev3-------------------------------------------------------------------------------------
Rev4-------------------------------------------------------------------------------------

Original–GTGTGTTTCTACTCACGTGTCTCAGCATTGATCGATACCTGGCTATTGTTCACCCAATGAAGTCCCGCCTTCGACGCACAA 567
Fw2------GTGTGTTTCTACTCACGTGTCTCAGCATTGATCGATACCTGGCTATTGTTCACCCAATGAAGTCCCGCCTTCGACGCACAA
Fw3-------------------------------------------------------------------------------------
Fw4-------------------------------------------------------------------------------------
Fw5-------------------------------------------------------------------------------------
Rev2-------------------------------------------------------------------------------------
Rev3-------------------------------------------------------------------------------------
Rev4-------------------------------------------------------------------------------------

Original–TGCTTGTAGCCAAAGTCACCTGCATCATCATTTGGCTGCTGGCAGGCTTGGCCAGTTTGCCAGCTATAATCCATCGAAATG 648
Fw2------TGCTTGTAGCCAAAGTCACCTGCATCATCATTTGGCTGCTGGCAGGCTTGGCCAGTTTGCCAGCTATAATCCATCGAAATG
Fw3-----------------------------------TTTGGCTGCTGGCAGGCTTGGCCAGTTTGCC_GCTATAATCCATCGAAATG
Fw4-------------------------------------------------------------------------------------
Fw5-------------------------------------------------------------------------------------
Rev2-------------------------------------------------------------------------------------
Rev3-------------------------------TCATTTGGCTGCTGGCAGGCTTGGCCAGTTTGCCAGCTATAATCCATCGAAATG
Rev4-------------------------------------------------------------------------------------

Original–TATTTTTCATTGAGAACACCAATATTACAGTTTGTGCTTTCCATTATGAGTCCCAAAATTCAACCCTCCCGATAGGGCTGG 729
Fw2------TATTTTTCATTGAGAACACCAATATTACAGTTTGTGCTTTCCATTATGAGTCCCAAAATTCAACCCTTCCGATAGGGCTGG
Fw3------TATTTTTC_TTGAGAACACCAATATTACAGTTTGTGCTTTCCATTATGAGTCCCAAAATTCAACCCTTCCGATAGGGCTGG
Fw4-------------------------------------------------------------------------------------
Fw5-------------------------------------------------------------------------------------
Rev2-------------------------------------------------------------------------------------
Rev3-----TATTTTTCATTGAGAACACCAATATTACAGTTTGTGCTTTCCATTATGAGTCCCAAAATTCAACCCTTCCGATAGGGCTGG
Rev4-------------------------------------------------------------------------------------
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Original-GCCTGACCAAAAATATACTGGGTTTCCTGTTTCCTTTTCTGATCATTCTTACAAGTTATACTCTTATTTGGAAGGCCCTAA 810
Fw2------GCCTGACCAAAAATATACTGGGTTTCCTGTTTCCTTTTCTGATCATTCTTACAAGTTATACTCTTATTTGGAAGGNCCTAA
Fw3------GCCTGACCAAAAATATACTGGGTTTCCTGTTTCCTTTTCTGATCATTCTTACAAGTTATACTCTTATTTGGAAGGCCCTAA
Fw4----------------------------------------------------------------------------------------
Fw5----------------------------------------------------------------------------------------
Rev2---------------------------------------------------------------------------------------
Rev3-----GCCTGACCAAAAATATACTGGGTTTCCTGTTTCCTTTTCTGATCATTCTTACAAGTTATACTCTTATTTGGAAGGCCCTAA
Rev4----------------------------------------------------------------------------------------

Original-AGAAGGCTTATGAAATTCAGAAGAACAAACCAAGAAATGATGATATTTTTAAGATAATTATGGCAATTGTGCTTTTCTTTT 891
Fw2------AGAANGCTTATGAAATTCAGAAGAACAAACCAAGAAATGATGATATTTTTAAGATA------------------------
Fw3------AGAAGGCTTATGAAATTCAGAAGAACAAACCAAGAAATGATGATATTTTTAAGATAATTATGGCAATTGTGCTTTTCTTTT
Fw4----------------------------------------------------------------------------------------
Fw5----------------------------------------------------------------------------------------
Rev2---------------------------------------------------------------------------------------
Rev3-----AGAAGGCTTATGAAATTCAGAAGAACAAACCAAGAAATGATGATATTTTTAAGATAATTATGGCAATTGTGCTTTTCTTTT
Rev4----------------------------------------------------------------------------------------

Original-TCTTTTCCTGGATTCCCCACCAAATATTCACTTTTCTGGATGTATTGATTCAACTAGGCATCATACGTGACTGTAGAATTG 972
Fw2----------------------------------------------------------------------------------------
Fw3------TCTTTTCCTGGATTCCCCACCAAATATTCACTTTTCTGGATGTATTGATTCAACTAGGCATCATACGTGACTGTAGAATTG
Fw4----------------------------------------------------------------------------------------
Fw5----------------------------------------------------------------------------------------
Rev2---------------------------------------------------------------------------------------
Rev3-----TCTTTTCCTGGATTCCCCACCAAATATTCACTTTTCTGGATGTATTGATTCAACTAGGCATCATACGTGACTGTAGAATTG
Rev4----------------------------------------------------------------------------------------

Original-CAGATATTGTGGACACGGCCATGCCTATCACCATTTGTATAGCTTATTTTAACAATTGCCTGAATCCTCTTTTTTATGGCT1053
Fw2----------------------------------------------------------------------------------------
Fw3------CAGATATTGTGGACACGGCCATGCCTATCACCATTTGTATAGCTTATTTTAACAATTGCCTGAATCCTCTTTTTTATGGCT
Fw4----------------------------------------------------------------------------------------
Fw5----------------------------------------------------------------------------------------
Rev2---------------------------------------------------------------------------------------
Rev3-----CAGATATTGTGGACACGGCCATGCCTATCACCATTTGTATAGCTTATTTTAACAATTGCCTGAATCCTCTTTTTTATGGCT
Rev4----------------------------------------------------------------------------------------

Original-TTCTGGGGAAAAAATTTAAAAGATATTTTCTCCAGCTTCTAAAATATATTCCCCCAAAAGCCAAATCCCACTCAAACCTTT1134
Fw2----------------------------------------------------------------------------------------
Fw3------TTCTGGGGAAAAAATTTAAAAGATATTTTCTCCAGCTTCTAAAATATATTCCCCCAAAAGCCAAATCCCACTCAAACCTTT
Fw4----------------------------------------------------------------------------------------
Fw5----------------------------------------------------------------------------------------
Rev2---------------------------------------------------------------------------------------
Rev3-----TTCTGGGGAAAAAATTTAAAAGATATTTTCTCCAGCTTCTAAAATATATTCCCCCAAAAGCCAAATCCCACTCAAACCTTT
Rev4----------------------------------------------------------------------------------------

Original-CAACAAAAATGAGCACGCTTTCCTACCGCCCCTCAGATAATGTAAGCTCATCCACCAAGAAGCCTGCACCATGTTTTGAGG1215
Fw2----------------------------------------------------------------------------------------
Fw3------CAACAAAAATGAGCACGCTTTCCTACCGCCCCTCAGATAATGTAAGCTCATCCACCAAGAAGCCTGCACCATGTTTTGAGG
Fw4---------CAAAAATGAGCACGCTTTCCTACCGCCCCTCAGATAATGTAAGCTCATCCACCAAGAAGCCTGCACCATGTTTTGAGG
Fw5----------------------------------------------------------------------------------------
Rev2---------------------------------------------------------------------------------------
Rev3-----CAACAAAAATGAGCACGCTTTCCTACCGCCCCTCAGATAATGTAAGCTCATCCACCAAGAAGCCTGCACCATGTTTTGAGG
Rev4----------------------------------------------------------------------------------------

Original-TTGAGGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGG1296
Fw2----------------------------------------------------------------------------------------
Fw3------TTGAGGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGG
Fw4------TTGAGGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGG
Fw5----------------------------------------------------------------------------------------
Rev2---------------------------------------------------------------------------------------
Rev3-----TTGAGGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGC---
Rev4------TGAGGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGG

Original-ACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGT1377
Fw2----------------------------------------------------------------------------------------
Fw3------ACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGT
Fw4------ACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGT
Fw5----------------------------------------------------------------------------------------
Rev2---------------------------------------------------------------------------------------
Rev3---------------------------------------------------------------------------------------
Rev4-----ACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGT

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Fw2----------------------------------------------------------------------------------------
Fw3------TCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCG------------------------------------
Fw4------TCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCC
Fw5----------------------------------------------------------------------------------------
Rev2---------------------------------------------------------------------------------------
Rev3---------------------------------------------------------------------------------------
Rev4-----TCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACNTACGGCGTGCAGTGCTTCAGCC

Original-GCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCT1539
Fw2----------------------------------------------------------------------------------------
Fw3----------------------------------------------------------------------------------------
Fw4------GCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCT
Fw5----------------------------------------------------------------------------------------
Rev2---------------------------------------------------------------------------------------
Rev3----------------------------------------------------------------------------------------
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Rev4-----GCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCT
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Original–TCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGG1620
Fw2-------------------------------------------------------------------------------------
Fw3-------------------------------------------------------------------------------------
Fw4------TCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGG
Fw5-------------------------------------------------------------------------------------
Rev2-------------------------------------------------------------------------------------
Rev3-------------------------------------------------------------------------------------
Rev4-----TCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGG

Original–GCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGG1701
Fw2-------------------------------------------------------------------------------------
Fw3-------------------------------------------------------------------------------------
Fw4------GCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGG
Fw5-------------------------------------------------------------------------------------
Rev2-------------------------------------------------------------------------------------
Rev3-------------------------------------------------------------------------------------
Rev4-----GCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGG

Original–CCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACC1782
Fw2-------------------------------------------------------------------------------------
Fw3-------------------------------------------------------------------------------------
Fw4------CCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACC
Fw5-----------------------------------------------------CAACATCGAGGACGGCAGCGTGCAGCTCGCCGACC
Rev2-------------------------------------------------------------------------------------
Rev3-------------------------------------------------------------------------------------
Rev4-----CCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACC

Original–ACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGA1863
Fw2-------------------------------------------------------------------------------------
Fw3-------------------------------------------------------------------------------------
Fw4------ACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGA
Fw5------ACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGA
Rev2-------------------------------------------------------------------------------------
Rev3-------------------------------------------------------------------------------------
Rev4-----ACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGA

Original–GCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACG1944
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Fw3-------------------------------------------------------------------------------------
Fw4------GCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCG-------------------------
Fw5------GCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACG
Rev2-------------------------------------------------------------------------------------
Rev3-------------------------------------------------------------------------------------
Rev4-----GCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACG

Original–AGCTGTACAAGTAAAGCGGCCGCGACTCTAGATCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAA2025
Fw2-------------------------------------------------------------------------------------
Fw3-------------------------------------------------------------------------------------
Fw4-------------------------------------------------------------------------------------
Fw5------AGCTGTACAAGTAAAGCGGCCGCGACTCTAGATCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAA
Rev2-------------------------------------------------------------------------------------
Rev3-------------------------------------------------------------------------------------
Rev4-----AGCTGTACAAGTAAAGCGGCCGCGACTCTAGATCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAA

Original–CCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTG 2086
Fw2-----------------------------------------------------------------
Fw3-----------------------------------------------------------------
Fw4-----------------------------------------------------------------
Fw5------CCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTG
Rev2----------------------------------------------------------------
Rev3----------------------------------------------------------------
Rev4-----CCTCCCACACCTCCC------
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Appendix I: The original sequence of the AGTR1 cDNA variant 2, used as a reference here, was obtained from the NCBI nucleotide database (NM_009585.3). The sequence of the pEGFP-N1 vector can be found at the Addgene vector database (www.addgene.org).

Forward primer sequences are highlighted in grey; Reverse primer sequences are highlighted in dark green; Start and stop codons of the AT_1R and the EGFP gene are highlighted in bright green; Restriction enzyme recognition sites are highlighted in yellow and the point mutation in the wobble base at position 572 of the AT_1R gene is highlighted in red. Red underlined letters are positions that could not be clearly identified.

Appendix II: Abbreviations

7-AAD	7-aminoactinomycin D
aa	amino acid
ABTS	2,2'-Aznobis-(3-ethylbenzthiazolin-6-sulfonic acid)
ACE	angiotensin converting enzyme
ADAM17	a disintegrin and metalloproteinase 17
ADH	antidiuretic hormone
AGT	angiotensinogen
Akt	protein kinase B
Alum	aluminium hydroxide
Ang I	angiotensin I
Ang II	angiotensin II
Ang 1-7	angiotensin (1-7)
Ang1-9	angiotensin (1-9)
Ang III	angiotensin III
Ang IV	angiotensin IV
ARAP1	type 1 Ang II receptor associated protein 1
ARB	angiotensin II receptor blockers
Asn	asparagine, N
AT_1R	angiotensin II type I receptor
AT_2R	angiotensin II type II receptor
ATRAP	AT_1-receptor associated protein
BGG	Bovine Gamma Globulin
BP	Blood Pressure
BSA	Bovine Serum Albumin
cAMP	cyclic adenosine monophosphate
cDNA	complementary desoxyribunucleic acid
CMV	cytomegalo virus
C-term	Carboxy-terminal end
CVD	cardiovascular disease
DAG	diacylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulfoxid
DNA	desoxyribunucleic acid
dNTP	desoxyribonucleotide triphosphate
DT	Diphtheria Toxin
ECL	enhanced luminol-based chemi-luminescent substrate

EDTA	Ethylene Diamine Tetraacetic Acid
EGF	Epidermal Growth Factor
EGFP	Enhanced Green Fluorescent Protein
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked Immunosorbent Assay
ERK2	extracellular signal related kinases
FACS	Fluorescent-Activated Cell Sorting
FCS	Foetal Calf Serum
FGF	Fibroblast Growth Factor
FSC-H	forward scatter
GDP	guanosine diphosphate
GFP	Green Fluorescent Protein
GMBS	Maleimidobutyryloxy-Succinimide ester
GST	Glutathione S-Transferase
GTP	guanosine triphosphate
HAEC	Human Aortic Endothelial Cell
HB-EGF	Heparin-Binding EGF
HEK	Human Embryonic Kidney
Hepes	2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonsäure
His	histidine, H
HUVEC	Human Umbilical Vein Endothelial Cell
ICAM-1	Intracellular Adhesion Molecule-1
IgG	immunoglobulin G
IL	interleukin
IP_3	inositol triphosphate
IRAP	Insulin-Regulated Aminopeptidase Receptor; AT_4R
JNK	c-Jun N-terminal kinases
KLH	Keyhole Limpet Hemocyanin
LB	Lysogeny Broth
Lys	lysine, K
MAP	Mitogen-Activated Protein
MAS	mas oncogen product
MCS	Multiple Cloning Site
MEK	MAP/ ERK kinase
MMC	Mouse Mesanglial Cell
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid

NADPH	nox-base nicotinamide adenosine dinucleotide phosphate
NF-κB	Nuclear Factor κB
NFκB-luc	NF-κB response element luciferase gene cassette
NO	Nitric Oxide
N-term	Amino-terminal end
OD	Optical Density
PAGE	Poyacrylamide Gel-Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Phe	phenylalanine, F
PI3K	Phosphoinositid-3 Kinase
PKC	Protein Kinase C
Pro	proline, P
Raf-1	v-raf oncogen murine leukemia viral oncogene homolog 1
Ras	rat sarcoma
RAS	Renin-Angiotensin System
ROCK	Rho kinase
ROS	Reactive Oxygen Species
RT	Room Temperature
SDS	Sodium Dodecyl Sulfate
SHP-2	Src homology 2-containing inositol phosphatase 2
SSC-H	side scatter
TAE	Tris Acetic acid EDTA
TBST	Tris Buffered Saline Tween20
TEMED	N,N,N',N'-Tetramethylethylenediamide
TNFα	Tumour Necrosis Factor α
TT	Tetanus Toxoid
Tyr	tyrosine, Y
UV	Ultra Violet
Val	valine, V
VCAM-1	Vascular Cell Adhesion Molecule-1
VSMC	Vascular Smooth Muscle Cell
WHO	World Health Organisation

Curriculum vitae

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Special skills: basic computer skills, skiing instructor allowed accompanying school skiing trips, driving licence, chemical and biological laboratory skills, fluent in English and German

Education:

May 2003	School leaving examination at "Gymnasium Sacré- Coeur" Vienna
Oct 1st 2003	Start of academic studies of Molecular Biology in Vienna
July 2005	Internship with the Institute of Parasitology of the Medical University of Vienna with Dr. Julia Walochnik: *"ITS1 sequence variabilities correlate with 18s rDNA sequence types in the genus Acanthamoeba"* sterile work with Acanthamoeba cultures; DNA isolation, sequencing and phylogenetic analysis using online databases and analysis programs
July 2006	Practical with Ao. Univ. Prof. Dr. Timothy Skern at the Institute of Biochemistry of the Medical University of Vienna: *"Defining residues involved in human rhinovirus 2A protease substrate recognition"* plasmid restriction & ligation, cloning in *E.coli*, in vitro transcription, in vitro translation, PAGE

Sept. 2006- June 2007	Studentship of the European student exchange program ERASMUS at the University of Glasgow, Scotland
October 15th- December 15th 2006	Practical at the Division of Infection and Immunity at the University of Glasgow with Professor Sylke Muller: "Expression, purification and activity-assay of lipoate protein ligase A (LplA) from *Escherichia coli* and *Plasmodium falciparum."* WB, SDS-PAGE, protein expression, colorimetric activity assay
July 1st- August 31st 2007	Internship at Böhringer-Ingelheim in Vienna: sterile work with tumour cell cultures, 2D & 3D proliferation assay, kinase activity assay
October 2008- June 2009	Diploma project with AFFiRiS AG: *„Evaluation of candidate peptides for the immunization against angiotensin II"* ELISA, SDS-PAGE, WB, generation of a GFP-fusion protein, establishment of a cellular assay for the determination of receptor functionality/ signalling Mode of Action using luciferase activity

References

[1] Statistik Austria, Gesundheitsbefragung 2006/ 2007

[2] Kearney PM et al. *Lancet* 2005, 365: 217–223: Global burden of hypertension: analysis of worldwide data

[3] Chobanian AV et al. *JAMA* 2003, 289: 2560-2572: The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure

[4] Fyhrquist F et al. *Journal of Internal Medicine* 2008, 264: 224-236: Renin-Angiotensin system revisited

[5] de Gasparo M et al. *Pharmacological Review* 2000, 52: 415-472: International Union of Pharmakology XXIII. The Angiotensin II Receptors

[6] Danilczyk U et al. *Circulation Research* 2006, 98: 463-471: Angiotensin-Converting Enzyme II in the Heart and the Kidney

[7] Sampaio WO et al. *Hypertension* 2007, 50: 1093-1098: Angiotensin-(1-7) Counterregulates Angiotensin Signalling in Human Endothelial Cells

[8] Daniels D et al. *Experimental Physiology* 2007, 92: 523-527: Angiotensin II receptor signalling

[9] Higuchi S et al. *Clinical Science* 2007, 112: 417-428: Angiotensin II signal transduction through the AT_1 receptor: novel insights into mechanisms and pathophysiology

[10] Goette A & Lendeckel U *Europace* 2008, 10: 238-241: Electrophysiological effects of Angiotensin II. PartI: signal transduction and basic electrophysiological mechanisms

[11] Guzik T et al. *JEM* 2007, 204: 2449-2460: Role of the T cell in the genesis of Angiotensin II-induced hypertension and vascular dysfunction

[12] Aumelas A et al. *Biochemistry* 1985, 82: 1881-1885: Studies on Angiotensin II and analogs:Impact of substitution in position 8 on conformation and activity

[13] Tissot AC et al. *Lancet* 2008, 371: 821-827: Effect of immunisation against Angiotensin II with CYT006-AngQb on ambulatory blood pressure: a double-blind, randomised, placebo-controlled phase IIa study

[14] Ambühl PM et al. *Journal of Hypertension* 2007, 25: 63-72: A vaccine for hypertension based on virus-like particles: preclinical efficacy and phase I safety and immunogenicity

[15] Downham MR et al. *Journal of Clinical Pharmacology* 2003, 56: 505-512: Evaluation of two carrier protein-Angiotensin I conjugate vaccines to assess their future potential to control high blood pressure (hypertension) in man

[16] Ménard J *Journal of Hypertension* 2007, 25: 41-46: A vaccine for hypertension

[17] Michel JB et al. *Americam Heart Journal* 1989, 117: 756-766: Immunologic approaches to blockade of the renin-Angiotensin system:A review

[18] Brown MJ et al. *Clinical Science* 2004, 107: 167-173: Randomised double-blind placebo-controlled study of Angiotensin immunotherapeutic vaccine (PMD3117) in hypertensive subjects

[19] Protherics/BGT

[20] Wang B et al. *Heart Vessels* 2005, 20: 153-158: Arterial structural changes in rats immunised by AT_1-receptor peptide

[21] Zelezna B et al. *Physiological Research* 1999, 48: 259-265: Influence of Active Immunisation against Angiotensin AT_1 or AT_2 Receptor on Hypertension Development in Young and Adult SHR

[22] Zhu F et al. *Cellular & Molecular Immunology* 2006, 3: 107-114: Target Organ Protection from a Novel Angiotensin II Receptor (AT1) Vaccine ATR12181 in Spontaneously Hypertensive Rat

[23] www.cytos.com

[24] Friguet B et al. *Journal of Immunological Methods* 1985, 77: 305-319: Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay

[25] Pastore L et al. *Circulation* 1999, 100: 1646-1652: Angiotensin II Stimulates Intrercellular Ashesion Molecule-1 (ICAM-1) Expression by Human Vascular Endothelial Cells and Increases Soluble ICAM-1 Release In Vivo

[26] Noda K et al. *Journal of Biological Chemistry* 1995, 270: 28511-28514: Interaction of Phe^{8} of Angiotensin II and Lys^{199} and His^{256} of the AT_1 Receptor in Agonist Activation

[27] Miura S et al. *Journal of Biological Chemistry* 1999,274: 7103-7110: Role of Aromaticity of Agonist Switches of Angiotensin II in the Activation of the AT_1 Receptor

[28] Picard C et al. *Immunology* 1986, 57: 19-24: Epitope diversity of Angiotensin II analysed with monoclonal antibodies

[29] Smeby RR et al. *Biochimica et Biophysica Acta* 1962, 58: 550: A proposed conformation of isoleucyl-Angiotensin II

[30] Wang B et al. *Heart Vessels* 2005, 20: 153-158: Arterial structural changes in rats immunized by AT_1-receptor peptide

[31] Wang Y et al. *Biochemical and Biophysical Research Communications* 2007, 364: 118-123: $[Ca^{2+}]_i$ and PKC-α are involved in the inhibitory effects of Ib, a novel nonpeptide AngiotensinII subtype AT1 receptor antagonist, on AngiotensinII-induced vascular contraction in vitro

[32] Gifford SM et al. *Journal of Endocrinology* 2004, 182: 485-499: Functional characterisation of HUVEC-CS: Ca2+ signaling, ERK1/2 activation, mitogenesis and vasodilator production

[33] van Eickels M et al. *Naunyn-Schmiedeberg's archives of pharmacology* 1999, 359: 394-399: Angiotensin converting enzyme inhibitors block mitogenic signalling pathways in rat cardiac fibroblasts

[34] Chae et al. *Cellular Physiology and Biochemistry* 2007, 20: 859-866: Epigallocatechin-3-O-Gallate Inhibits the Angiotensin II-Induced Adhesion Molecule Expression in Human Umbilical Vein Endothelial Cell Via Inhibition of MAPK Pathways

[35] Kim HY et al. *Hypertension Research* 2008, 31: 515-523: Upregulation of Interleukin-8/ CXCL8 in Vascular Smooth Muscle Cells from Spontaneously Hypertensive Rats

[36] AbdAlla S et al. *Cell* 2004, 119: 343-354: Factor XIIIA Transglutaminase Crosslinks AT1 Receptor Dimers of Monocytes at the Onset of Atherosclerosis

[37] Guo RW et al. *Peptides* 2006, 27: 3269-3275: Angiotensin II induces NF-κB activation in HUVEC via the p38MAPK pathway

[38] Soehnlein O et al. *Journal of Vascular Research* 2005, 42: 399-407: ACE Inhibition Lowers Angiotensin-II-Induced Monocyte Adhesion to HUVEC by Reduction of p56 Translocation and AT1 Expression

[39] Wolf G et al. *Journal of the American Society of Nephrology* 2006, 17: 1585-1593: Angiotensin II Upregulates Toll-Like Receptor 4 on Mesanglial Cells

[40] Hunyady L et al. *Journal of Cell Biology* 2002, 157: 1211-1222: Differential PI-3 kinase dependence of early and late phase of recycling of the internalized AT_1 Angiotensin receptor