University of South Bohemia České Budějovice
Faculty of Science
Department of Medical Biology

Reverse pharmacology: Searching for novel drugs in ticks and testing their effects on host immune cells

Bachelor Thesis in Biological Chemistry

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Annotation

The aim of this work is to use an approach of reverse pharmacology in searching for novel drugs from specific proteins, which are extracted from *Ixodes ricinus* salivary, and have pharmacoactive features that inhibit host haemostasis and modulate host inflammatory and immune responses.

Das Ziel unserer Arbeit ist, die Zuche von Neuartigen Drogen aus speziellen Preoteinen, die aus Speicheldrüse und Midgut des *Ixodes ricinus* Zecke extrahiert sind und Pharmakoaktive Effekte haben, die die Hämostase hemmen und Gastgebers Entzündung und Immunreaktionen modulieren.

Declaration

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Abstract

Saliva of hard ticks (Ixodida) is a rich source of proteins with high pharmacological potential, which insures the attachment of the tick to the host by inhibiting various defense mechanisms. Such proteins can be novel drug candidates and can be used in a drug development. In my thesis, I used the reverse pharmacology/genetics approach to gain an understanding of drug discovery process.

A protein family of *I. ricinus* described as Da-p36 was believed to contain proteins, similar to immunosuppressive Da-p36 protein of *Dermacentor andersoni*. However, after *in silico* analysis, no close similarity was found between these two families. I tried to produce one member of Da-p36 family recombinantly, but I was not successful. In order to further follow reverse genetics approach pipeline, several protease inhibitors, produced in our laboratory, were used to test their activity on platelet aggregation, cell adhesion and T-cell proliferation. Interestingly, only S8k serpin inhibited cell adhesion. Other tested proteins had no significant effects in any experimental model.

List of abbreviation

AAS19 = *Amblyomma americanum* tick serine protease inhibitor 19

AA = Amino acids

ACD = Acid-citrate-dextrose

ADP = Adenosine triphosphate

APC = Allophycocyanin or antigen presenting cell

ATP = Adenosine triphosphate

BPTI = Bovine Pancreatic Trypsin Inhibitor

BSA = Bovine serum albumin

cDNA = Complementary deoxyribonucleic acid

Con-A = Concavalin A

Da-p36 = Dermacentor andersoni protein 36KDa

DNA = Deoxyribonucleic acid

ECM = Extra-cellular matrix

EDTA = Ethylenediaminetetraacetic acid

Factor Xa = Eponym Stuart–Prower factor

FACS buffer = Flow cytometry buffer

FBS = Fetal bovine serum

FITC = Fluorescein isothiocyanate

FRET = Fluorescence resonance energy transfer

GTF = General Transcription Factors

GYF = Proline rich domain binding motif (Glycine-tyrosine-phenylalanine)

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

ICAM-1 = Intercellular adhesion molecule 1

IFN-G = Gamma interferon

IgG = Immunoglobulin G

IRS-2 = *Ixodes ricinus* serpin 2

IxscS-1E1 = *Ixodes scapularis* tick saliva serine protease inhibitor

LACI = Lipoprotein-associated coagulation inhibitor

LC-MS = Liquid chromatography mass spectrometry

MHC I = Major histocompatibility complex 1

MHC II = Major histocompatibility complex 2

mRNA = Messenger ribonucleic acid

NCBI = National center for biotechnology information

NGS = Next-generation sequencing

PCR = Polymerase chain reaction

PBS = Phosphate-buffered saline

PPP = Platelet poor plasma

PRP = Platelet rich plasma

PMA = Phorbol 12-myristate 13-acetate

RGD = Arginine glycin aspartic acid

RHS-1 and 2 = *Rhipicephalus haemaphysaloides* 1 and 2

RHS-1 and 2 = *Rhipicephalus haemaphysaloides* serpin 1 and 2

RPMI1640 = Roswell Park Memorial Institute medium

RNA = Ribonucleic acid

rRNA = Ribosomal ribonucleic acid

TF = Transcription factor

TFPI = Tissue factor pathway inhibitor

TNF = Tumor necrosis factor

tRNA = Transfer ribonucleic acid

TSA = Transcriptome shotgun assembly

TXA = Thromboxane

VCAM-1 = Vascular cell adhesion molecule 1

$\alpha 2\beta 1$ = Integrin alpha2beta 1

$\alpha 2\beta 3$ = Integrin alpha2beta 3

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1 Introduction

1.1 Searching for novel drugs

As our world is full of challenges, drug discovery is one of the important fields in science, which provides modern society with an improved medical care. The search program for new drugs begins with a disease, which has no medical solution or cure available (Hughes et al., 2011), or better solution is needed.

Discovering and developing a new drug from a theoretical idea to a product ready to be used takes around 12 to 15 years (Hughes et al., 2011). Complex processes and several clinical trials must be done to ensure a maximum efficiency with minimum side effects.

The first step of drug development is the formulation of hypothesis of how a certain disease can be cured by a certain molecule, i.e. a physiological mechanism that can be targeted by a drug must be predicted. The second step is the candidate drug identification and selection, where proteins, genes or synthesised chemicals are identified, characterized and tested in preclinical tests for their safe use, clear and specific effect on the disease, good bioavailability and easy accessibility (Hughes et al., 2011). Finally, clinical trials are necessary to test the novel drugs in humans.

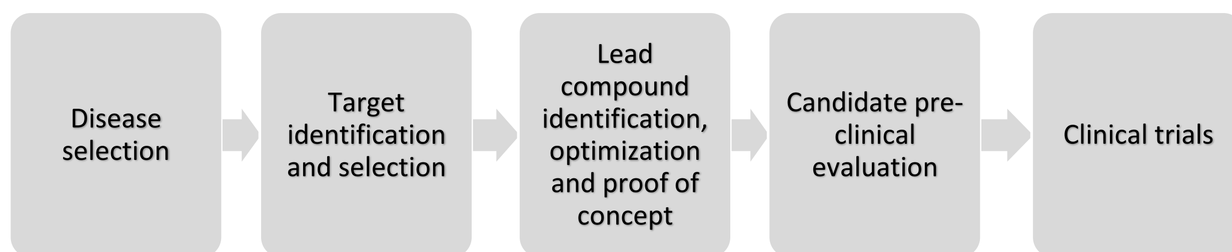


Figure 1: The scheme shows the steps of drug discovery and testing according to WHO/EU requirements

For novel drug discovery there are two main approaches: *classical approach* and *a reverse approach.*

1.1.1 Classical approach

In the history of medicine, a very common and early way of drug discovery was dominated by empirical approach. Potential drugs were discovered and identified by applying them on animal organs or tissues in the absence of fully understanding the mechanism.
With the emergence of synthetic chemistry in pharmaceutical research, the pharmacological evaluation of chemical products for therapeutic purposes became necessary. Many new drugs were discovered by this approach (Takenaka, 2001).
In this classical approach, the research is done primarily in the pharmacology field and starts with the identification of molecules with functional activity. The molecules are tested for understanding their biological role. Subsequently, screening is done to see which drug candidate has a maximum potential. At last, clinical trials are performed and drug candidates are tested at gene level to observe desired mechanism or healing and validate the theory (Takenaka, 2001).

A pharmacological screening involves methods, which systematically test the candidate drugs (new chemical entities or extracts from biological material) in tissue cultures followed by tests in whole animals, mostly rats and mice (Takenaka, 2001). The steps of *classical approach* are shown in figure 2, where the first step is the identification of functional activity of several compounds and subsequently purification. Next, their biological roles are tested and then screened to choose a drug candidate. At last, clinical tries are performed and the molecular mechanism is observed (Takenaka, 2001).

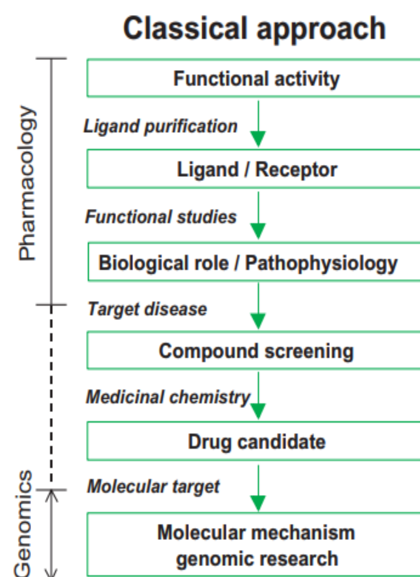


Figure 2: The diagram shows the steps of the classical approach (Takenaka, 2001).

1.1.2 Massive screenings

To discover a single drug and introduce it to the market, novel drug candidate, pre-selected out of millions of compounds, must further fulfil multiple parameters in preclinical and clinical trials before it can be introduced to the market (Wermuth, 1998).
Drug screening relies on the creation of relevant, reproducible, reliable and robust assays. The screening is developed over years to generate faster methods, such as, high-throughput screening, which can test hundreds of thousands of large chemical libraries per day. There are two types of screening strategies, random and focused screenings. Random screening is based on physically testing everything at a large scale. Focused screening is based on computational methods, such as creating three-dimensional structures and characterizations *in silico* (Valler, Green, 2000). Screening methods developed and used today are, NGS sequencing, FRET, flow cytometry, LC-MS/MS and other physicochemical analytical techniques. (Carnero, 2006, Janzen, 2014).

1.1.3 Reverse pharmacology

Scope of reverse pharmacology is the use of predictions, documented clinical experiences and experiential observations for a discovery of a lead compounds, its validation through laboratory studies and experiments, and further preclinical and clinical testing to have a drug candidate.
Reverse genetics employs similar approach as reverse pharmacology - the first step is using experience and traditional knowledge to identify suitable protein or gene targets responsible for certain disease or pathology. Second step, is the identification of gene coding sequence for the protein that could affect target mechanism. Then there is a protein production in a recombinant form and its testing that can result in a proof-of-concept and discovery of underlying mechanisms of its function. Afterwards, efficiency and safety are tested *in vivo* or *in vitro* and the safety is approved in preclinical phase. In the final step, 4 phases of clinical trials follow (Takenaka, 2001).

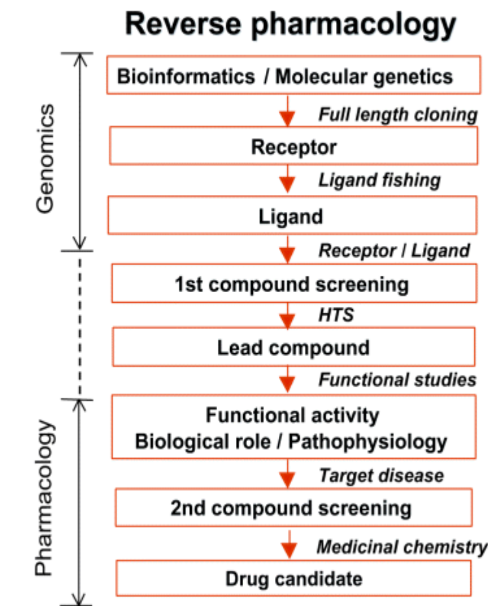


Figure 3: The diagram shows the steps of reverse approach (Takenaka, 2001).

1.2 Reverse pharmacology in ticks

1.2.1 The activity of tick saliva

Ticks are blood-feeding arthropods, known to be vectors of many human and animal diseases. Hard ticks, such as *I. ricinus*, are called slow feeders, because they stay attached to the host for a long period of time. (Siddall, 2004, Sonenshine, Roe, 2014).

For the tick to survive, blood must flow constantly to them without any interruption. Vertebrate immune system ensures organism integrity by several mechanisms, such as haemostasis, which includes coagulation, vasoconstriction and platelet aggregation or all branches of immune system. Blood loss, following an injury triggers inflammation, in which leukocytes, plasma proteins, and fluid from blood accumulate at the injured place, eliminate the microbes and facilitate the wound healing. (Ribeiro, Francischetti, 2003).

Primary immunoglobulin and cell mediated immune responses are induced during the first exposure to tick feeding. Ability of an animal to respond to a given immunogen depends upon the genetically defined capacity to process immunogens and present them to immunocompetent T lymphocytes in the context of major histocompatibility complex (MHC) antigens.

Immune system has innate and adaptive branches. The primary defense line is the innate immune system, where neutrophils, macrophages, mast cells, dendritic cells and other type of cells produce cytokines and inflammatory mediators, which move to the injury site to eliminate the pathogens or present the antigens to immunocompetent T lymphocytes. These immune responses are induced during the first exposure to tick feeding (Wikel, 1996).

In order to adapt to blood feeding and resist against host's advanced and complex defense system, tick saliva contains bioactive molecules that secure their attachment and feeding on the host (Islam et al., 2009). Ticks secrete a complex mixture of immunomodulatory and immunosuppressive substances, leading to the prevention of tick death and injury recovery (Wikel, 1996). This complex mixture of substances contains anti-clotting enzymes like Apyrase (inhibits ADP-induced platelet aggregation), enzyme inhibitors like serine proteases (acting as immunomodulator and anticoagulants), immunoglobulin-binding proteins, biogenic amines-binding lipocalins, cytokine expression modulators and many others (Chmelar et al., 2011, Hovius, 2009, Kotál et al., 2015, Ribeiro, Francischetti, 2003, Steen et al., 2006).

1.2.2 Gene to function approach

Many proteins and gene transcripts were explored by transcriptome analysis and reverse genetic approach. (Ribeiro et al., 2006).

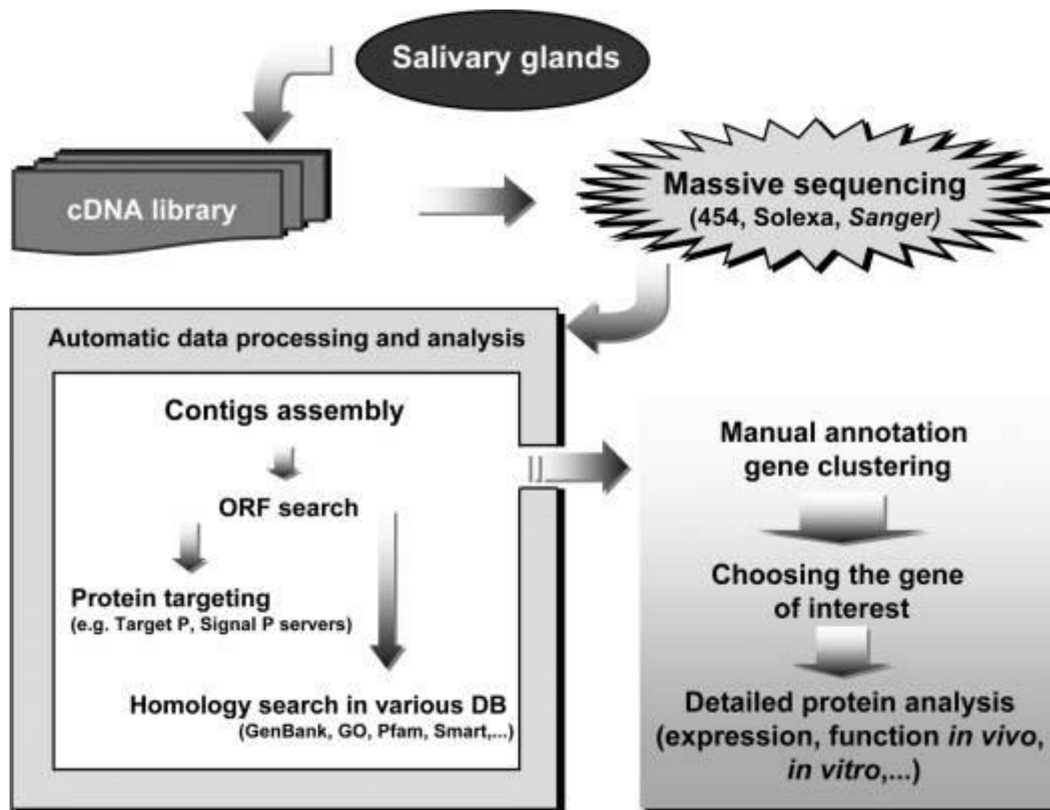


Figure 4: The figure explains the gene-to-function approach to discover the proteins of ticks and the analysis to know their functions. Complementary deoxyribonucleic acid (cDNA) library is created, which contains several sequences. After bioinformatic analysis sequences are elected and further examined manually *in vitro* (Chmelar et al., 2012).

In first works, dealing with sialomes (transcriptomes from salivary glands), the pipeline, described in Figure 4, was used. Tick salivary gland mRNA was isolated, and reverse transcribed to cDNA. Salivary gland cDNA library was constructed. Randomly selected clones were sequenced and compared with gene databases, such as GenBank. All sequence similar in AA sequence or structure, or sequences having same functional domains were aligned, clustered and annotated (Jesus G. Valenzuela et al, 2002).

Nowadays a new high-throughput method - next-generation sequencing (NGS) is being massively used. It has the ability to detect large numbers of novel transcripts due to its capacity to detect even weakly expressed genes by extensive transcriptomic coverage. NGS has thus provided a more complete picture of tick gene expression and its regulation. It has revolutionized the field of genomics and transcriptomics (Pareek et al., 2011, Schwarz et al., 2013).

1.2.3 Pharmacoactive proteins in ticks

At first, some proteins and small chemical compounds were discovered and their activities were identified. These compounds could be divided in several groups:
Small compounds such as adenosine triphosphate (ATP) and prostaglandins, enzymes, inhibitors of proteases, calcium-binding proteins, amine-binding proteins, host cytokine homologs, Immunoglobulin G (IgG) binding proteins, cytokine-binding proteins, neurotoxins, platelet aggregation antagonists, coagulation antagonists and immunomodulators with undisclosed mechanism of action (Chmelař J., 2010).

The largest group of tick saliva proteins are the serine protease inhibitors. These proteins inhibit many important signals and activation reactions. Some of the active compounds in tick saliva, which inhibit coagulation are thrombin inhibitors, Factor Xa inhibitors, Factor VIIa/TF complex inhibitors and contact phase inhibitors. Other groups of tick saliva proteins are the chemokine binding proteins, which prevent the recruitment of leukocytes by inhibiting the chemotactic activity of chemokines. Evasins-1 and Evasins-3 were detected to be inhibiting neutrophils and eosinophils activity and prevent their accumulation. (Déruaz et al., 2008). Immunoglobin-binding proteins protect the tick primarily from ingested host immunoglobins. Other mechanism targeted by immunomodulatory proteins is the cytokines expression, which is achieved by 15kDa salivary gland protein (Salp15). Salp15 binds to membrane of the leukocytes and modulate the cytokines expression at transcription level (Anguita et al., 2002, Steen et al., 2006). This protein also inhibits early CD4+ T cell signalling and activation leading to inhibition of T cell proliferation. (Tomás-Cortázar et al., 2017, Narasimhan et al., 2004).

1.2.4 Protease inhibitors in ticks

Proteases are enzymes that cleave proteins to smaller peptides and amino acids, regulating a range of physiological processes including extracellular digestion, protein degradation, and tissue development (Rawlings, Salvesen, 2012).
Serine protease inhibitors have very important role in the regulation of many proteolysis dependent processes, such as coagulation, platelet aggregation, and complement activation. The homeostasis is kept by an equilibrium between proteases and their inhibitors. Disruption

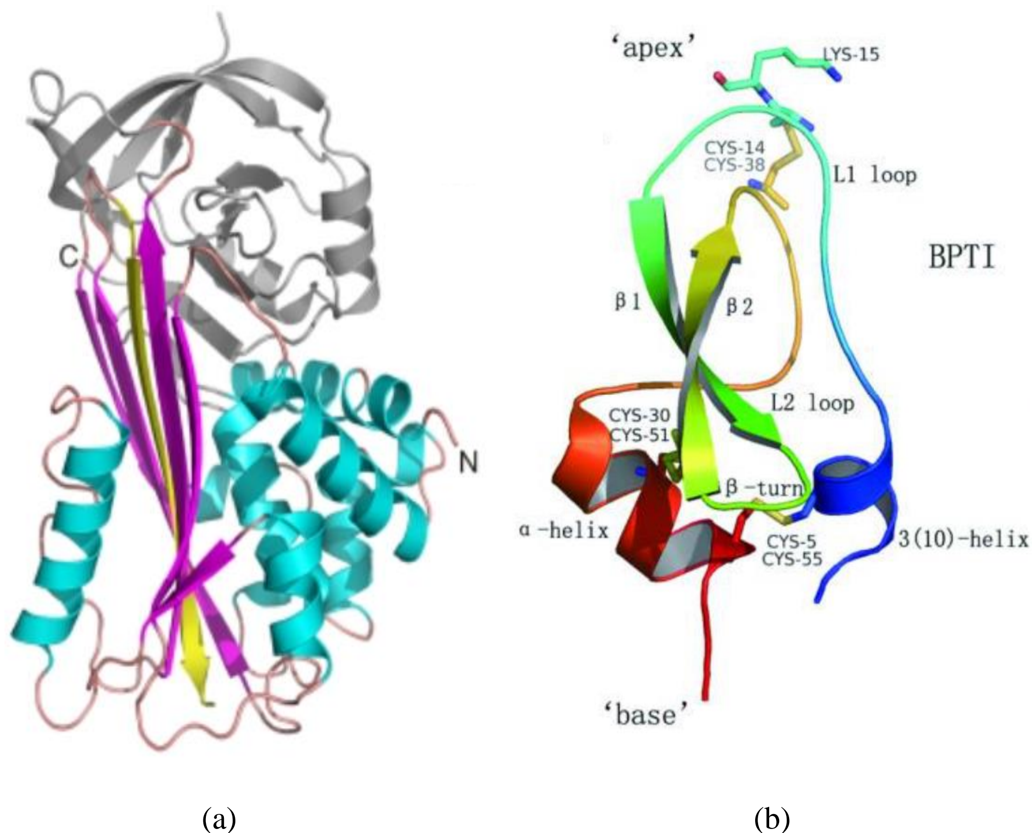
of such an equilibrium by an exogenous inhibitor, e.g. from tick salivary secretion, leads to the development of pathological conditions. Modulating the proteolytic balance in the sites of tick attachment is critical for the tick feeding. One of the important protease inhibitor groups are Kunitz domain-containing proteins. These proteins are serine protease inhibitors and are very abundant in all *Ixodes* species. Their usual targets are serine proteases of the S1 family (Rawlings et al., 2004). Kunitz domain structure consists of two β-strands and one α-helix. Three disulphide bridges stabilize the Kunitz domain structure. The molecular weight of one typical Kunitz domain is about 7 kDa. Many members of this family consist of several Kunitz domains; one, two and five Kunitz domain-containing proteins have been found in ticks. Several Kunitz domain-containing proteins were characterized and demonstrated as anticoagulants with different target proteases (Paesen et al., 2009). One of the Kunitz type protease inhibitors is a tissue factor pathway inhibitor TFPI. TFPI has two different isoforms TFPIα, which contains 3 Kunitz-type inhibitory domains (K1, K2, K3) and a positively charged C terminus and TFPIβ, which contains the K1 and K2 domains of TFPIα, and C terminus encoding for addition of a glycosylphosphatidylinositol anchor (Wood et al., 2014). There are also other Kunitz domains such as Lipoprotein-associated coagulation inhibitor (LACI), which is a protein derived from plasma and inhibits coagulation induced by tissue factor (TF)/factor VIIa (Wun, 1992).

One of the known Kunitz domain based inhibitors in ticks is the Bilaris. Bilaris proteins are Ixodes scapularis specific and contain 2 Kunitz domains (Paesen et al., 2009). One of the well-known members of bilaris group is called Ixolaris. It is a protein with 140 amino acids, containing 10 cysteines and 2 Kunitz-like domains. It inhibits factor VIIa (FVIIa)/tissue factor (TF)-induced factor X (FX) activation (Francischetti et al., 2002).
Another Kunitz domain based inhibitor is Haemangin found in *Haemaphysalis longicornis*. This salivary Kunitz inhibitor inhibit different phases of wound healing such as proteolytic degradation of ECM and inhibition of EC proliferation (Islam et al., 2009).
The second large group of serine protease inhibitors found in ticks is the serpin superfamily. Tick serpins are proteins used by the tick to as regulators of haemostasis to facilitate their feeding on host, such as thrombin inhibition, Factor X, Factor IX inhibition and many other inhibitions. To date, there are several known tick serpins, which displayed inhibitory effects on host, such as *Amblyomma americanum* tick serine protease inhibitor 19 (AAS19), *I. ricinus* serpin -2 (IRS-2), *I. scapularis* tick saliva serine protease inhibitor -1E1 (IxscS-1E1),

Rhipicephalus haemaphysaloides serpin -1 and 2 (RHS-1 and 2) and many others (Chmelař et al., 2017).

Two serpins from *I. ricinus* were characterized thus far. IRS-2 targets primarily cathepsin G and mast cell chymase - 2 proinflammatory serine proteases. In higher molar concentrations, it inhibited thrombin and blocked cathepsin G and thrombin-induced platelet aggregation. Its importance is in its dual interference with both inflammation and hemostasis (Chmelar et al., 2011).

IRS-1, S8k serpin (IRS-5, according to unified nomenclature used in our laboratory) and 20314 serpin (IRS-3) were used in our research to test function of serpin superfamily members from tick *I. ricinus*.



(a) (b)

Figure 5: (a) The 3D structure of IRS-2. The magenta part in the centre is the β-sheet and cyan is the surrounding helices. The pale pink part is the loop. N- and C-termini are labelled with N and C (Chmelar et al., 2011). (b) The classical Kunitz/BPTI domain fold. Helices are coloured from blue (N-terminus) to red (C-terminus). The β-sheet structure is in the centre coloured with green. The three conserved disulphide bridges and the P1 residue (Lys15 in BPTI) are shown as an atom-coloured stick

model (Dai et al., 2012).

Other tick salivary proteins, which play an important immunomodulatory role, are the cystatins, inhibitors of cysteine proteases, such as papain and legumain. They have important role in regulating many physiological processes of immune system. Cystatins interfere with antigen processing and presentation, phagocytosis and cytokines expression (Zavasnik-Bergant, 2008). Cystatins target proteases are usually lysosomal cathepsins involved in protein degradation and target the degradation of antigens presented to lymphocytes. Thus, cystatin-type molecules secreted from parasites suppress the host immune response (Jin, Flavell, 2010, Turk et al., 2012, Zavasnik-Bergant, 2008). From this group, cystain G1 was used in my tests.

1.2.5 *Dermacentor andersoni* protein with molecular weight 36 kDa (Da-p36) family

Saliva and salivary glands extracts of *D. andersoni* display several immunomodulatory effects, such as significantly downregulating intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) expressions. It also inhibits the production of tumour necrosis factor (TNF) and interleukin 1-b and the production of gamma interferon (IFN-G) and interleukin 2 (Ramachandra, Wikel, 1992).

The mechanism of feeding by *D. andersoni* ticks is very similar to *I. ricinus*. Both ticks have proteins, which inhibit the inflammation, leading to disabling the host defense for their survival and growth. In *D. andersoni* female ticks, an immunomodulator with the name of Da-p36 has been discovered. It is a soluble protein with a molecular weight of 36 kDa and the Da stands for *D. andersoni*. Da-p36 displays immunomodulatory and immunosuppressive activities. In some research, (Bergman et al., 1998, (Bergman et al., 1995) it was proven that this protein suppresses the *in vitro* proliferation of host T lymphocytes, which was induced by Concavalin A (Con-A), which is a stimulator of many inflammation processes. Da-p36 also reduced production of IFN-γ (Alarcon-Chaidez et al., 2003).
Other homologous genes related to the *D. andersoni*-derived p36 gene were also discovered. Three other proteins where isolated from *R. haemaphysaloides, Ammblyoma variegatum* and *H. longicornis* ticks, and named Ra-p36, Av-p36 and Hl-p36, respectively. They have also a

molecular weight of 36 kDa and immunomodulatory and immunosuppressive effects (Wang et al., 2017).

In one transcriptome project (Schwarz et al., 2013, Kotsyfakis et al., 2015b) from *I. ricinus* tick, 3 groups named Da-p36 like, Da-p36 and Da-p36 cytotoxin were found, which were believed to be related to *D. andersoni*-derived p36 gene. Therefore, we believed that these 3 groups may contain good candidate proteins with immunomodulatory and immunosuppressant activities.

2 Aims

In my thesis, in order to gain experience in many methods and obtain good understanding of drug discovery using reverse pharmacology/genetics approach, following steps were performed:

1- To characterize Da-p36 like protein family *in silico*

2- To clone selected member of this family with possible pharmacological activities into expression vector and express the protein in bacterial expression system (***E. coli***).

3- To prepare the proteins in soluble and active form.

4- To test the protein's activity in suitable *in vitro* immunological assays

3 Material and methods

3.1 Table of materials, chemicals, kits

Table 1: The chemicals and kits used in the project

Name	Description	Source
THP-1 cells	(ATCC® TIB-202™) Human monocytic cell line	ATCC®
RPMI1640 medium		BioSera
HEPES buffer	1 M	BioSera
FBS	Fetal bovine serum	BioSera

Cell culture media	RPMI1640 medium with HEPES, 2 mM stable glutamine, 10% FBS and 1% antibiotics (penicilin-streptomycine).	
BSA	30% Bovine serum albumin	BioSera
PBS	Phosphate-buffered saline	Gibco
PMA	Phorbol 12-myristate 13-acetate (1 mg/ml)	Sigma®
FACS buffer	PBS, 0,1% Na_3N	
ACD (Acid-citrate-dextrose)	25 g trisodium citrate dihydrate 85 mM 14 g citric acid monohydrate 66.6 mM 20 g anhydrous D glucose 111 mM In 1 ml distilled H_2O	
EDTA	Ethylenediaminetetraacetic acid	Sigma®
Cell starvation media	RPMI1640 medium with HEPES, 2 mM stable glutamine, 0,3% BSA and 1% antibiotics (penicilin-streptomycine).	
SYBR® safe DNA Gel Stain	Dye for PCR (10,000x concentration in DMSO	Invitrogen®
Trypan Blue stain 0.5%		SigmaAldrich®
Tyrode's buffer with albumin	NaCl , KCL, $NaHCO_3$, NaH_2PO_4, $MgCl_2.6H_2O$, $CaCl_2.6H_20$, HEPES, 0.35% albumin and anhydrous D glucose	
CellTrace™ CFSE	Cell proliferation kit	ThermoFisher®
cDNA	Ovary, midgut, salivary, nymph	Produced by Jan Kotál and Michail Kotsyfakis
CellTracker™ Green CMFDA	5-chloromethylfluoroscein diacetate (50 µg)	Invitrogen®
Propidium Iodide	(500 mg/ml)	Thermo Fisher
VWR® Cell Stainers		VWR®
Fibronectin	Extracellular matrix molecule	SigmaAldrich®

Laminin	Extracellular matrix molecule from Enelbreth-Holm-Swarm murine sarcoma basement membrane (1 mg/ml)	Sigma®
Collagen	Extracellular matrix molecule from rat tail (1 mg/ml)	Sigma®
Concavalin A	(1 mg/ml)	Sigma Aldrich
Cell proliferation dye eFluor® 670	5 µM Mouse anti-CD4 antibody conjugated with PE fluorescent dye (500x dilution)	eBioscience®
Anti-CD9-FITC	(500x dilution) anti-mouse CD9 antibody conjugated with fluorescein isothiocyanate	eBioscience®
Anti-CD41-APC	(500x dilution) anti-mouse CD41 antibody conjugated with allophycocyanin	eBioscience®
1x RBC Lysis Buffer Solution	Used to lysis the red blood cells	eBioscience®
HBSS	10x with sodium bicarbonate	BioSera
Cell culture Water Pyrogene Free		BioSera
Tick cystatins	cystatin G1	Produced by Jan Kotál and Michail Kotsyfakis
Tick serpins	Elastophilin, two batches of IRS-1, S8k serpin (IRS-5), 20314 serpin (IRS-3)	Produced by Jan Kotál and Michail Kotsyfakis
Tick kunitz	44530 double Kunitz inhibitor	Produced by Jan Kotál and Michail Kotsyfakis
Taq DNA polymerase (recombinant) master mix kit	10x Taq buffer, dNTP, nuclease-free water and Taq DNA polymerase	ThermoFisher
Phusion® High-Fidelity PCR Master Mix with GC Buffer kit	2x phusion master mix and nuclease-free water	ThermoFisher

TrackIt™ 100 bp DNA Ladder	DNA ladder marker	Invitrogen®

Table 2: List of proteins used in our project for the immunoassays.

Protein	Description
Elastophilin	Trypsin Inhibitor-like cysteine-rich domain inhibitor
G1	Cystatin from I. ricinus salivary glands – inhibitor of cysteine proteases
IRS-1a and IRS-1b	Serpins from I. ricinus salivary glands – serine protease inhibitor
IRS-1b buffer	Serpins from I. ricinus salivary glands – serine protease inhibitor in a high buffer salt (detergents)
S8k serpin (IRS-5)	Serpin from I. ricinus salivary glands – serine protease inhibitor
20314 serpin (IRS-3)	Serpin from I. ricinus salivary glands – serine protease inhibitor
44530	Double Kunitz based inhibitor – inhibitor of serine proteases

3.2 Methods

3.2.1 Bioinformatics

All sequences from *I. ricinus* Da-p36 family were collected and compared against Transcriptome Shotgun Assembly (TSA), which is part of NCBI GenBank database (https://www.ncbi.nlm.nih.gov/) by using tblastn algorithm (protein sequences were used as a query and translated nucleotide database was searched). Transcriptome databases, created using next generation sequencing of *I. ricinus* salivary glands, midguts and haemocytes, were used for searching (Kotsyfakis et al., 2015a, Kotsyfakis et al., 2015b, Schwarz et al., 2013).

The similarity threshold was set to E-value 1.0E-20. Highly similar sequences, which were found in the transcriptome of *I. ricinus* and *I. scapularies* were collected. Homologous sequences were aligned by ClustalW multiple alignment algorithm in BioEdit v7.0.5. Various parameters for gap opening and the gap extension penalties were tested (see figure 8 for details). After building an alignment, full-length sequences were chosen for Da-p36 protein families.

Signal peptides were searched using SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP-3.0/) and removed before analyses. Sequences were also manually edited to correct some obvious alignment errors. Mega 7.0.21 software was used to construct phylogenetic trees for the Da-p36 protein family by maximum likelihood model.

For the *in silico* biochemical characterization of a member from the Da-p36 protein family and comparing it with the Da-p36 of *D. andersoni*, several tools were used, such as: ExPASy ScanProsite tool (https://prosite.expasy.org/scanprosite/), UniProtKB (https://www.uniprot.org/), NetOGlyc 4.0 server, NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/), Conserved Domains Database NCBI (https://www.ncbi.nlm.nih.gov/cdd/).

3.2.2 PCR – amplification of Da-p36

The aim was to clone the selected coding sequence from the *I. ricinus* Da-p36 family into expression vector pET17b, then express it and prepare the selected protein in soluble and active form.

Based on the bioinformatics analysis of the Da-p36 like protein family, three protein family members of Da-p36 like protein family were selected, and for each sequence, expression primers were designed.

The primer design was done by obtaining approximately 20 nucleotides from the beginning and end of the mature sequence (i.e. without signal peptide) and adding restriction site for an enzyme NdeI (CATATG), which is an endonuclease isolated from *Neisseria denitrificans.* Second cloning site was specific for XhoI (CTCGAG), another restriction enzyme. To analyse the primers, OligoAnalyzer 3.1 tool from IDT website (https://eu.idtdna.com/calc/analyzer),

which examines for GC content, melting point, primer-dimers and hairpin formation, was used.

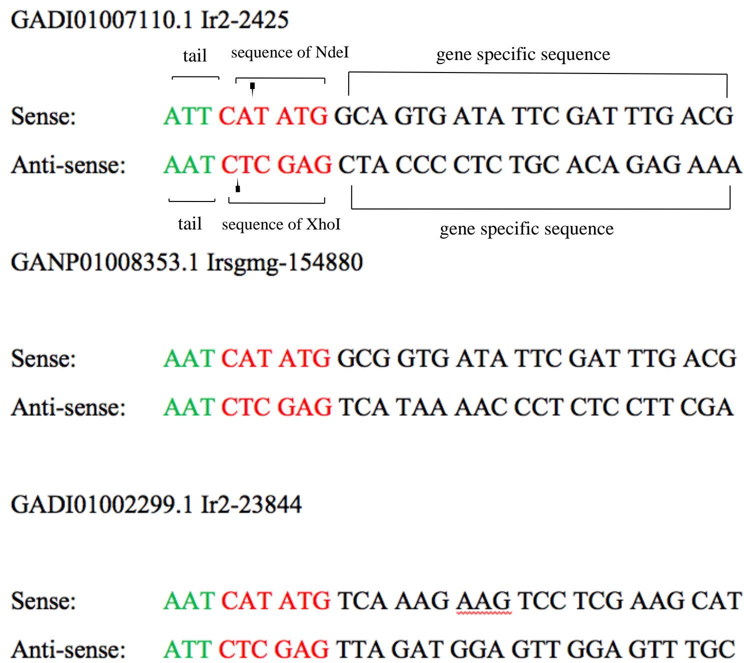


Figure 6: The designed sense and anti-sense of the primers are shown here, including the three parts of the primer: The tail, the restriction site (NdeI or XhoI) and at last the gene specific part.

These primers and enzymes were planned to be used to clone the selected coding sequences into pET17b expression vector. The sense primer is the same as the 5'-3' sequence of the cDNA and the anti-sense is the reverse complement of the cDNA.
For amplification of the Da-p36 cDNAs, 2x Taq DNA polymerase master mix and subsequently Phusion® High-Fidelity PCR Master Mix with GC Buffer kit were used to perform PCR. Both kits were provided by ThermoFisher®. For performing the PCR using Taq DNA polymerase, midgut, ovary, nymph and salivary gland DNAs of *I. ricinus* females were used as templates and mixed with the designed forward and reverse primers. This mixture was prepared in a 0,2ml PCR tube on ice. At first, gradient PCR was performed to find best annealing temperature, using following cycling conditions: the initiation of the denaturation was achieved at temperature of 95˚C for 3 minutes. Denaturation was done at 95˚C for 30 seconds. Annealing gradient between 48˚C and 70˚C for 30 cycles, each cycle for

30 seconds. Extension was done at 72˚C for 1 min. At last the final extension was performed at 72˚C for 10 minutes.

When using Phusion High-Fidelity PCR Master Mix, 2x Phusion master mix, designed reverse and forward primers, DNA template and water were added. The initial denaturation was achieved at 98˚C for 30 seconds. The 30 cycles of denaturation at 98˚C for 10 seconds, annealing at 50,7°C for 30 seconds and extension at 72˚C for 1 minute. Final extension was done at 72˚C for 10 minutes and samples were cooled at 4˚C until further use.

3.2.3 THP-1 cultivation

Human cell line from an acute monocytic leukaemia patient - THP-1 (ATCC® TIB-202™) was used in our experiment to test, whether *I. ricinus* proteins have inhibiting effects on the cell adhesion process.

Frozen THP-1 cells were revived by gentle agitation in a 37˚C warmed water bath. The revived cells were cultured in cell culture medium (see 3.1 for the medium formulation). The cells were cultivated at concentrations between (2 x 10^5) and (8 x 10^5) cells/ml. To keep proper cell culture concentration, cells were passaged every third day – cells were seeded at 2 x 10^5 cells/ml and splitted at 8 x 10^5 cells/ml.

3.2.4 Static adhesion assay

The day before an experiment, 96-well plates were coated overnight at 4°C in humid chamber with molecules of extracellular matrix, concretely fibronectin (10 µg/ml in 50 µL). On the day of the experiment, the coated wells were washed twice with 200 µl of PBS per well. The unspecific binding sites were blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (50 µL) and incubated for one hour in cell culture CO_2 incubator at 37°C with 5% CO_2. After 1 hour of blocking, plate was washed two times with PBS and the wells were filled with PBS until further use. THP-1 cells were harvested from the culture. The cell suspension was centrifuged at 250 g, 22°C for 5 min. The cell pellet was resuspended in 1 ml of pre-warmed RPMI1640 by serological pipette. 1 µl of 1000x concentrated (CellTracker™ Green CMFDA Dye, Invitrogen™) was added, mixed gently and incubated with cells at 37°C for 40 min. Stained cells were washed twice by resuspension in 10 ml of pre-warmed serum free media (RPMI1640 + 0,3% BSA) and centrifugation at 250 g, 22°C, 5

min. After washing, the cells were resuspended in 1 ml of pre-warmed serum free medium. 10 µl of cell suspension was mixed with 40 µl of trypan blue. Cells were counted in Bürker chamber and concentration was calculated according to proper formula. The cell suspension was adjusted to a concentration of 4 x 10^5 cells/ml, 250 µl of cell suspension was added to each 1,5 ml test tube. Samples were divided to negative control, positive control and different treatments with tick proteins (cystatin G1, two batches of IRS-1, S8k serpin (IRS-5), 20314 serpin (IRS-3) and 44530 double Kunitz inhibitor). Inhibitors were added to the cell suspension at final concentration 1 µM and were incubated at 37°C for 1 hour. PBS was removed from the coated plates and cells were divided into 4 wells as quadruplicates for each treatment (2 x 10^4cells per well). To each well, cell adhesion stimulator, Phorbol myristate acetate (PMA) was added to a final concentration of 50 ng/ml, except for negative control wells. Cells with the PMA were incubated for 20 minutes at 37°C, 5% CO_2. At last, 150 µl of PBS was added to each well and the cell adhesion was evaluated by the fluorescence intensity ratio between washed and unwashed wells by using Synergy H1 multi-mode plate reader (BioTek).

3.2.5 T-cell proliferation

3.2.5.1 Splenocyte isolation

Mouse spleen was isolated in serum free media and was placed on ice. Splenocytes were isolated from the mouse spleen by disintegrating the spleen to cells using the cell strainer with 70 µm mesh. Splenocytes were centrifuged for 5 min and 250 g at 20°C. 2 ml of 1x red blood cell lysis buffer solution (eBioscience®) was added to the cells and incubated for 2 minutes in order to remove red blood cells via lysis by hypotonic pressure. 20 ml of PBS solution was added to bring the cells back to isotonic environment. Cells were centrifuged for 5 min and 250 g at 20°C. 3 ml of PBS were added and clumps of neutrophils were removed by using 70 µm cell strainer.

3.2.5.2 *In vitro* T cell proliferation assay

Splenocytes in 3 ml PBS solution were stained intracellularly with 3 µl of 1000x CellTrace™ CFSE Cell Proliferation Kit (Invitrogen™) and were incubated for 10 minutes at 37˚C in CO_2 incubator. Cells were washed with full medium 4 times, resuspended in 1 ml of full medium counted in the Bürker chamber with a dilution factor 25 or 50. Cells were distributed to 96-

well plate (5 x 10^5 cells per well) into three groups (negative control, positive control and different proteins treated group). To experimental groups, different proteins/inhibitors (cystatin G1, Elastophilin and 44530 double Kunitz) were added at concentrations of 3 µM or 6 µM and incubated for 4 hours in CO_2 incubator at 37˚C. After the incubation, Concanavalin A was added to final conc. of 1 µM to the positive control and to the experimental groups with pre-incubated proteins. 200 µl of splenocytes suspension was transferred to 5 ml flow cytometry test tube. 300 µl of PBS solution was added and the cell suspension was centrifuged for 5 min at 400 g. Cells were resuspended in 100 µl of flow cytometry buffer (FACS buffer). CD4+ T-cells were stained with 500x diluted mouse anti-CD4 antibody conjugated with PE fluorescent dye. After adding the antibody, cell suspension was incubated for 15 minutes at 4˚C. To distinguish dead cells, cells were stained with propidium iodide and incubated at 4˚C for 15 minutes. Subsequently, 1 ml of FACS buffer was added and cells samples were centrifuged for 5 min at 400 g. Finally, 300 µl of FACS buffer were added, and the proliferation of CD4+ cells was assessed and analyzed by flow cytometry.
In our project, BD FACSCANTO II™ flow cytometer was used, which consist two lasers for excitation, at 488-nm (blue) and at 633-nm (red). The comparison of divided cells from undivided cells, dead cells from alive cells and singlet cells from the rest was observed and analyzed using FACSDiva Version 6.1.3 (Nguyen et al., 2003).

3.2.6 Platelet aggregation

3.2.6.1 Platelet isolation

Mouse whole blood was collected in Acid-Citrate-Dextrose buffer (ACD) in 1/6 ratio. Diluted blood was centrifuged at 180 g at 22˚C for 10 minutes. Platelet rich plasma (PRP) was diluted further in Tyrode's buffer with Ethylenediaminetetraacetic acid (EDTA) to ratio of 1:3 (blood : buffer). The solution was centrifuged at 250 g at 22 °C for 5 minutes. Platelet poor plasma (PPP) was discarded and pellet with platelets was resuspended as described in following section.

3.2.6.2 Platelet aggregation assay

After isolation, platelets were washed twice and resuspended in 1 ml of Tyrode's buffer with EDTA. Platelets were separated to two halves. Each group was stained with different platelet specific antibody – anti-CD9-FITC and anti-CD41-APC (500x dilution) and stained for 30

min at 4°C. Platelets were washed with 600 µl of Tyrode's buffer without EDTA. After staining, both groups were mixed and incubated for 15 minutes with or without tested proteins. Different proteins (G1, IRS-1, S8k serpin and 20314 serpin) were added at 2 µM concentration to experimental groups and incubated for 30 minutes at 37°C. Afterwards, platelets in tested groups and positive control were stimulated with 100 ng/ml PMA for 20 min. All platelets were washed with FACS buffer and then the platelet aggregation was measured by flow cytometry. I used method described by De Cuyper and colleagues (Cuyper et al., 2013). Briefly, two groups of platelets were stained with single antibody, then the platelets were mixed and activated. In the FACS analysis, aggregated platelets were identified as double positive (figure 7).

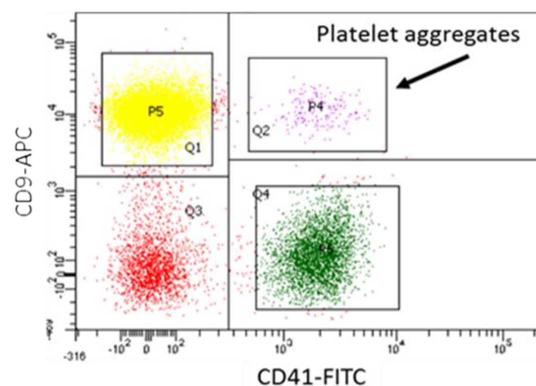


Figure 7: An example of gating strategy used for flow cytometry analysis to screen the platelet aggregation according to De Cuyperet al., 2013. The increase in events in Q2 after platelet aggregation stimulation shows that there was no inhibition. The decrease in events in Q2 shows the inhibition of platelet aggregation (Cuyper et al., 2013). Anti-CD9-FITC and Anti-CD41-APC are antibodies used for staining the platelets. Q1 and Q4 show the different groups of platelets. Q3 shows the unstained plasma.

BD FACSCANTO II™ flow cytometer was used for measurement. Aggregation was analyzed by using FACSDiva Version 6.1.3. The platelet aggregation was detected as double positive in the upper right quadrat due to aggregation of platelets from two differently stained populations.

4 Result

4.1 Da-p36 family description – bioinformatics

Da-p36 protein family is one of the protein families we were interested in as it was believed to be secreted also in *I. ricinus*. Using bioinformatics tools and data mining, all sequences, similar to the Da-p36 protein family sequences in *I. ricinus* were collected and a phylogenetic analysis was performed.

Searching in the transcriptome databases of *I. ricinus*, three protein groups were found. One of the families was called **Da-p36** (10 full length members), being secreted from salivary gland of a nymph at experimental time points of 12 and 24 hours of feeding. The second family was midgut specific and called **Da-p36 like** (21 members) being secreted from the midgut of nymph at 12 and 24 hours of feeding, and from midgut of adult at 12, 24 and 36 hours of feeding. And the last family was called **Cytotoxin Da-p36 like** (42 members), being secreted from the salivary gland of nymph at 12 and 24, and from the salivary gland of adult at 12, 24 and 36 hours of feeding. According to their names, we believed that these families may be similar to the Da-p36 family of *D. andersoni*.

In silico characterization was done using bioinformatic servers and tools mentioned in methods previously, to compare a new member of Da-p36 protein family of *I. ricinus* with the Da-p36 of *D. andersoni.* From the table below, we see that both proteins have signal peptides, N- and O- glycosylation sites and protein kinase C phosphorylation sites but they are at different sites. RGD motif is present only in the new member. Interestingly, both proteins have isoelectric point (pI) at a close range. Different isoelectric points, insure efficient purification of proteins by isoelectric focusing.

Table 3: Comparison between the DA-p35 of *D. andersoni* and Ir2-2425 (member of Da-p36 family of *I. ricinus*).

	Da-p36	Ir2-2425
Signal peptide	1 - 21 AA	1 - 20 AA
Sequence lenght	199 AA	289 AA
Molecular weight	22,84 kDa	33,77 kDa
pI	9,56	9.34
Total number of negative residues (Asp + Glu)	19	39

Total number of positive residues (Arg + Lys)	33	50
RGD motif	none	191 AA
GYF motif	none	182 AA
N-glycosylation sites	5 sites	6 sites
Protein kinase C phosphorylation sites	4 sites	7 sites
O-glycosylation sites	1 site	6 sites

From the alignment (figure 8) we see that all the sequences of the Da-p36 protein family and Av-p36 from *A. variegatum* have RGD motif, which is an attachment site for many adhesive extracellular matrix, blood, and cell surface proteins.
All sequences of the Da-p36 protein family, Rh-36p from *R. haemaphysaloides* and Av-p36 from *A. variegatum* have Glycine-tyrosine-phenylalanine (GYF) motif, which is a proline rich domain binding motif, which facilitates transient intermolecular interactions such as signal transduction, antigen recognition, cell-cell communication and cytoskeletal organization, making it an excellent recognition motif (Srinivasan, Dunker, 2012).

In the partial alignment, two distinctive regions are observed, a high abundance of basic amino acids (positively charged domain) on one site and high abundance of acidic amino acids (negatively charged domain) on another site.

In the phylogenetic tree (figure 9), 4 proteins (Hl-p36, Av-p36, Rh-p36 and Da-p36) were added to see the genetic distance between the Da-p36 family members of *I. ricinus* from the 4 related proteins. From the results, we see that the sequences of Da-p36 protein family, are separated into 2 groups (1 and 2) and each sequence is significantly similar to the others in the same group.
From the sequences in alignment and phylogenetic (figures 8 and 9), we see that the Da-p36 like family from *I. ricinus* is not closely related to Da-p36 of *D. andersoni,* which in turn

showed similarity to Rh-p36, Av-p36 and Hl-p36 from other metastriate ticks. It seems that original Da-p36 protein is specific for metastriates and are not present in prostriate ticks. Some motifs, however, such as RGD and GYF motifs are present also in Av-p36 from *A. variegatum* and Rh-p36 from *R. haemaphysaloides* (Figure 8).

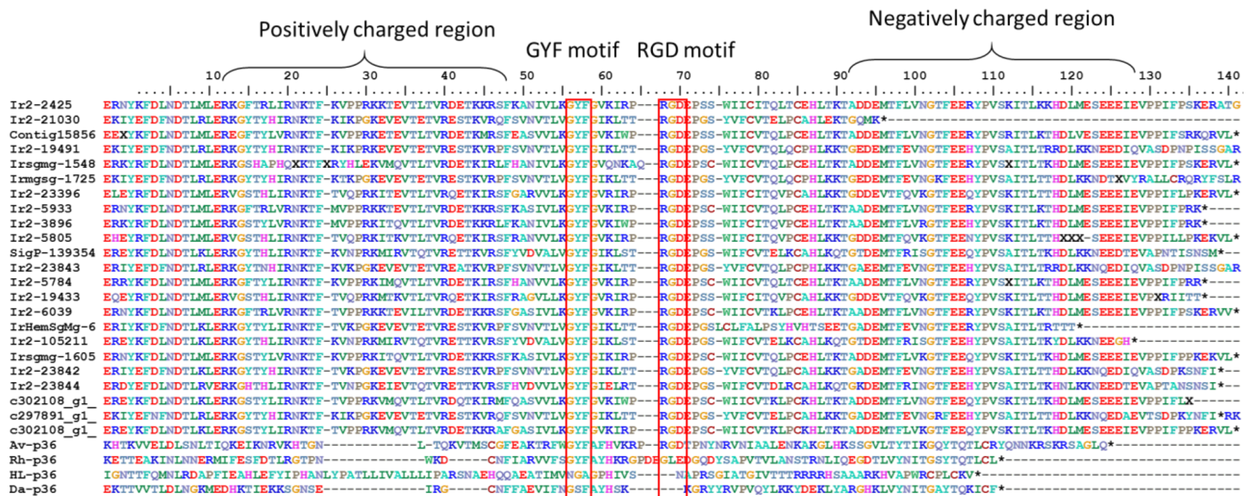


Figure 8: The final ClustalW local multiple alignment of the sequences of Da-p36 protein family using BioEdit v7.0.5 software (Gap opening penalty value = 10 and, gap extension penalty value = 2). The abundancy of positively charged domains are higher on the left site, wherease the abundancy of the negatively charged domains is higher on the left site. All Da-p36 protein family sequences have both RGD and GYF motifs. Av-p36 has both GYF and RGD motifs. Rh-p36 has only GYF.

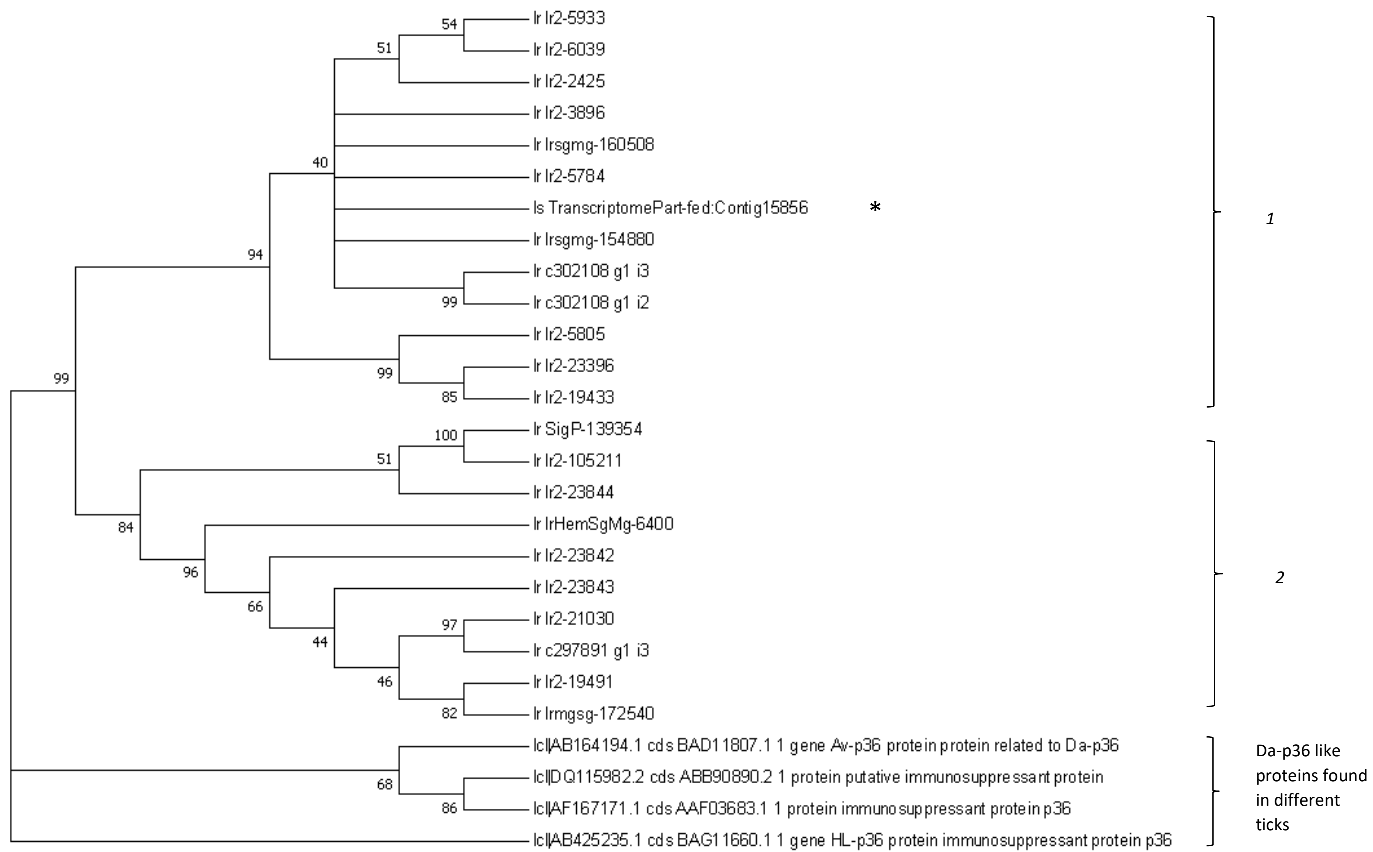


Figure 9: Cladogram of Da-p36 protein family members includes *I.ricinus* and *I. scapularis* obtained from the GenBank and *R. haemaphysaloides, A. variegatum, D. andersoni* and *H. longicornis*. Maximum likelihood algorithm was used. Numbers represent bootstrap support of each clade (1000 rep.). Bootstrap value is higher than 30%. (*) refers to Da-p36 like protein from *I. scapularis* obtained from GenBank. Number 1 and 2 refers to 2 groups of Da-p36 protein family members, which are more closely related in each group.

4.2 Da-p36 production attempt

After the alignment (Figure 8), three interesting members of Da-p36 protein family were chosen, which have arginine-glycin-aspartic acid (RGD) and glycine-tyrosine-phenylalanine (GYF) domains (Ir2-2425, Ir2-23844 and Irsgmg-154880). Subsequently, expression primers were designed to amplify the sequence coding for the recombinant proteins from the tick cDNA.

After several attemps of amplification using the designed primers, only GADI01007110.1:Ir2-2425 showed an amplification.

After performing several PCR reactions with varying temperatures and various cDNAs of *I. ricinus* (nymph, midgut and salivary gland cDNAs), the outcome of amplification was unfortunately negative, except one sample (Figure 10). However, I was not successful in cloning and I was not able to obtain another positive amplification.

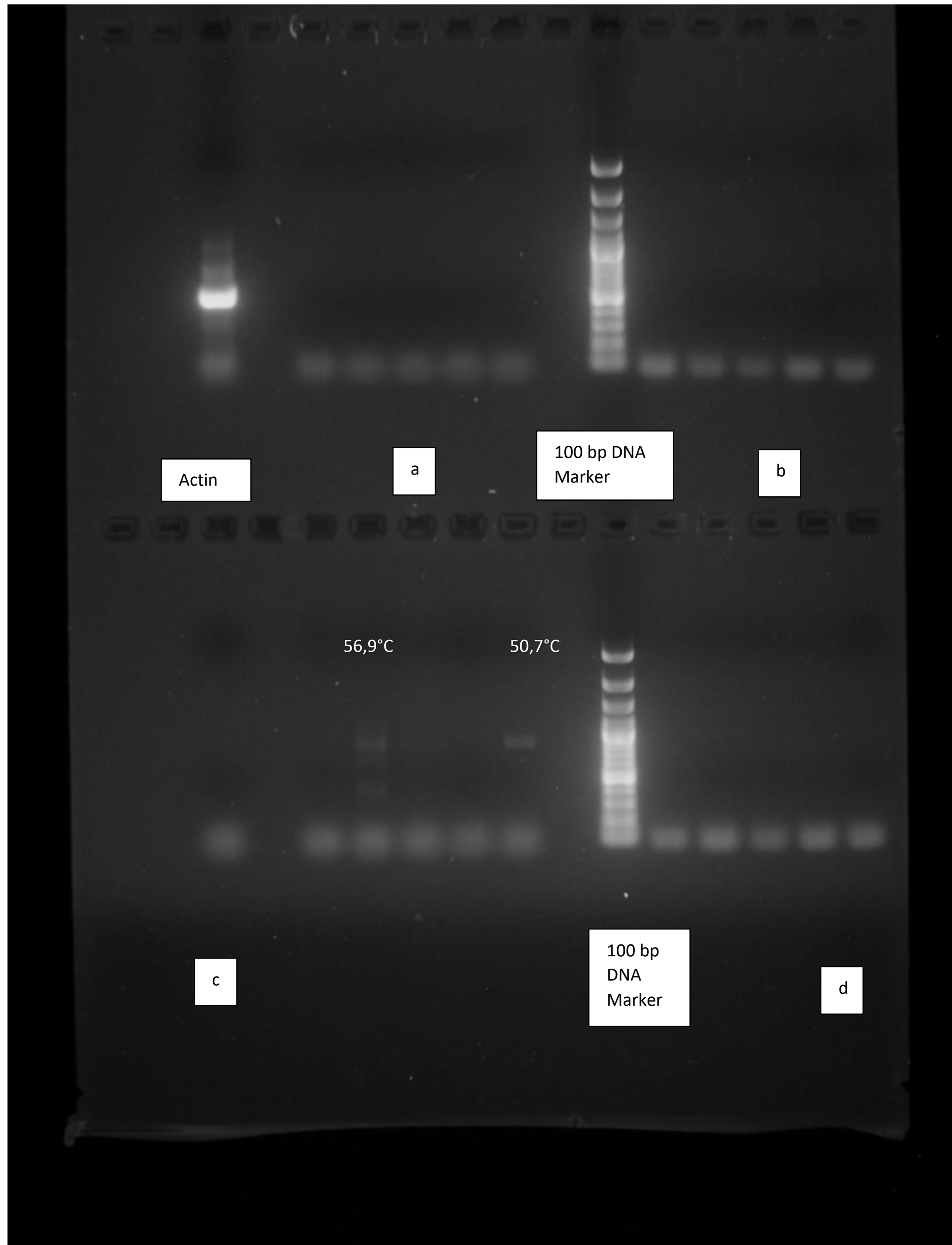


Figure 10: Actin was used as positive control. 1 bp DNA marker was used as reference. (a) No amplification was shown using midgut cDNA of *I. ricinus*. (b) No amplification was

shown using nymph cDNA of *I. ricinus*. (c) We see two amplicons (1000bp) using ovary cDNA of *I. ricinus* at (50,7°C and 56,9°C). (d) No amplification was shown using salivary cDNA of *I. ricinus*.

After several repeats, positive amplification was not reproducible and therefore, I did not sequence it and decide to work further with the other steps. Thus, neither the cloning of selected protein into expression vector nor expressing it in *E. coli* and the production of the recombinant protein could be achieved.

The aim of this project was to walk through all steps of reverse genetic approach from gene to function, to produce recombinant proteins and test it *in vitro* for their inhibitory and immunosuppressive properties. Due to lack of time and to achieve the aim of the project, I decided to stop with attempts to produce recombinant protein and I proceeded to test others, already available, recombinant proteins in our laboratory.

4.3 Immunological tests of recombinant proteins

In order to proceed with the reverse pharmacology/ reverse genetic workflow, I performed several experiments *in vitro* to reveal immunomodulatory and anti-hemostatic effects of tested tick recombinant proteins. The proteins used are described in table 2. I used static cell adhesion, CD4+ T cell proliferation and platelet aggragation.

4.3.1 Cell adhesion

From immunology perspective, cell adhesion is an essential process implicated in leukocyte recruitment to the site of inflammation. Leukocytes that migrate to the injury site need to adhere to the endothelial surface and this is mediated by adhesion molecules expressed on endothelial cells (VCAM1, ICAM1, ICAM2), which interact with counterparts on the leukocytes (β2-integrins), leading to adhesion and migration of the leukocytes through the endothelium to inflammed tissue (Maxwell et al., 2005, Muller, 2003).

Since some proteins of *D. andersoni* and *I. scapularis* have been shown to have immunomodulatory properties and inhibitory effect on cell adhesion (Maxwell et al., 2005), static adhesion assay was performed in our research.
The aim of this experiment was to test cell adhesion inhibition by the tick proteins. THP-1 cells were used, and adhesion was induced by using PMA. The biggest difference between the groups was visible after the second wash and therefore only the results after second wash are shown. The results in figure 11 showed a significant inhibition by S8k serpin protein. The results in figure 12 have shown, that the cell adhesion was inhibited but not significantly by cystatin G1, IRS-1a, IRS-1b buffer and IRS-1b. No inhibition was observed using 23014 serpin and 44530 double kunitz.

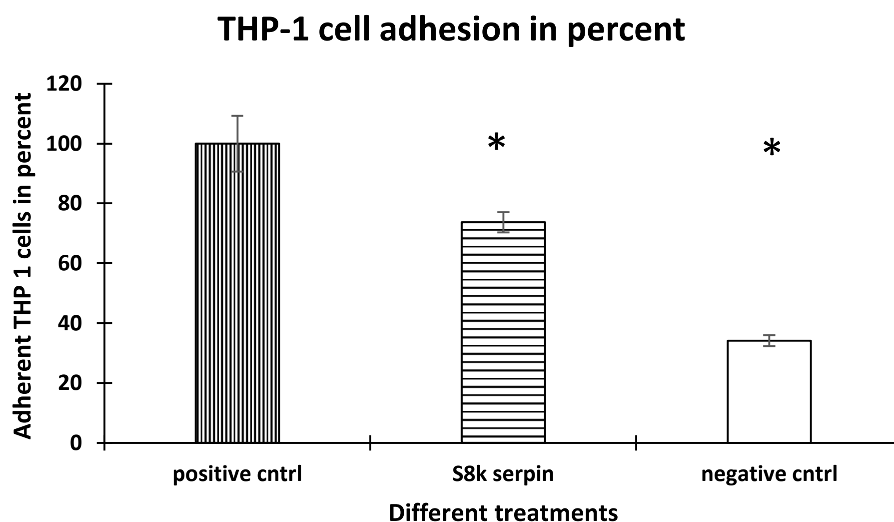


Figure 11: The results shown in the figure are transformed into percent and the average of 3 experiments are taken (N=3). One way Anova and subsequently Dunett's test (Post Hoc test) were performed. The statistical significance of treatments were compared to the positive control group and it is marked (* p-value <0.05). There was a significant difference between the treatments ($F(2,27) = 65.1621$, $p = 0.0001$).

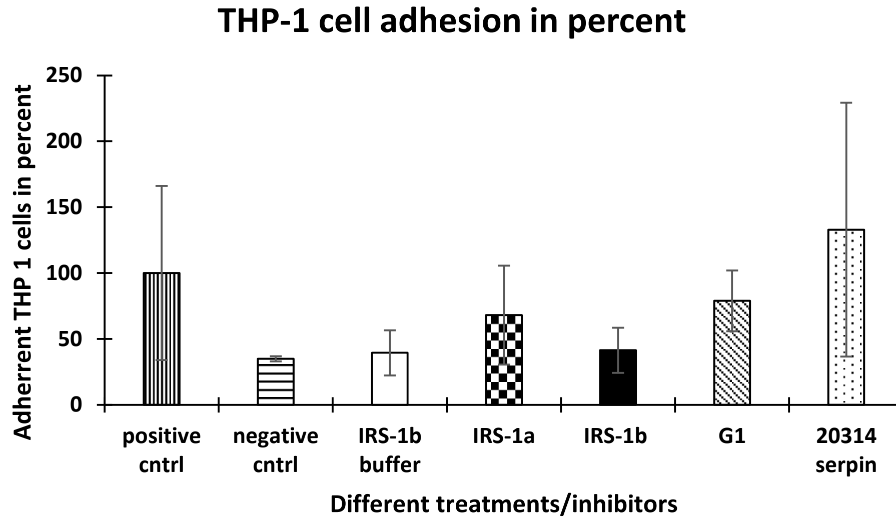


Figure 12: The results shown in the figure are transformed into percent and the average of 2 experiments are taken (N=2). One way Anova and subsequently Dunett's test (Post Hoc test) were performed. The statistical significance of each treatment was compared to the positive control group and it is marked (* p-value <0.05). There was no significant difference between the treatments (F(7,8) = 0.8505, p = 0.5781).

4.4 T-cell proliferation

Bone marrow is the source of thymocytes. Thymocytes migrate to thymus and go through several steps of differentiation and finally they migrate to peripheral lymph nodes as naïve T cells. For the naïve T cells to maturate into effector/memory T cells and proliferate (clonal expansion), they require stimulation and co-stimulation (Vibert, Thomas-Vaslin, 2017). Stimulation is done by antigen-specific signal, which stimulate the T cell receptor (TCR) and the co-stimulation is achieved by signals provided by the interaction between co-stimulatory molecules expressed on the membrane of antigen presenting cell (APC) and the T cell.
In our project, activation of lymphocytes was achieved by Con-A. Con-A is an antigen-independent stimulator, which mimics specific antigen-induced lymphocyte stimulation (Ando et al., 2014).

Since proteins of tick saliva, such as serine proteases inhibitors, were proved to inhibit the T cell proliferation (Chmelař et al., 2017), we performed in vitro CD4+ proliferation assay and evaluated by using flow cytometry (BD FACSCANTO II™).

Before the activation with Con-A, different proteins were used for treatement (G1 6 µM, Elastophilin 3 µM, Elastophilin 6 µM, 44530 3 µM and 44530 6 µM). For the measurements, the gating strategy shown in figure 13 was used. Before the analysis of CD4+ cells, gates were set and cells with correct size and molecular weight was selected as P1 (parent cells), then singlets were chosen and the proportion of alive to dead cells were observed as we see in figure 13. After adjusting the gating strategy, final analysis on CD4+ was performed and the divided cells from undivided cells were observed according to figure 14.

To monitor the T cell proliferation, fluorescent dye was used, which enables more specific analysis. Theoretically, as the cells labelled with fluorescence divide and proliferate the fluorescence signal would decrease precisely by ½ and devide equally in the new daughter cells (Lyons, 2000) since the fluorescent dye concentration is higher in the parent cell and it is diluted over progressive cell divisions.

The results in figure 15, show that the CD4+ T-cell proliferation was not significantly inhibited by the proteins G1 6 µM, Elastophilin 3 µM, Elastophilin 6 µM, 44530 3 µM and 44530 6 µM.
The inhibition was detected when cells were not divided (high peak is seen in the not divided section and t no peak in the divided section). No significant inhibition was seen with the tested proteins, although 44530 Kunitz domain inhibitor displayed inhibition effect on T-cell proliferation.

According to our results, we also proved that the period of 3 days incubation with con-A is ideal duration for successful *in vitro* T-cell proliferation.

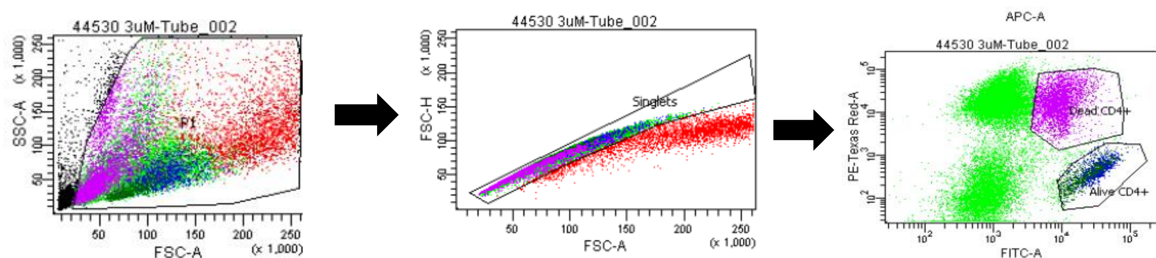


Figure 13: An example of results shown by dot plots by FACSDiva Version 6.1.3. Here we see the gating strategy used to select populations. Black = all events. Red = parent cells. Green = singlets. Blue = alive CD4+. Dark green = not divided cells. Purple = dead cells.

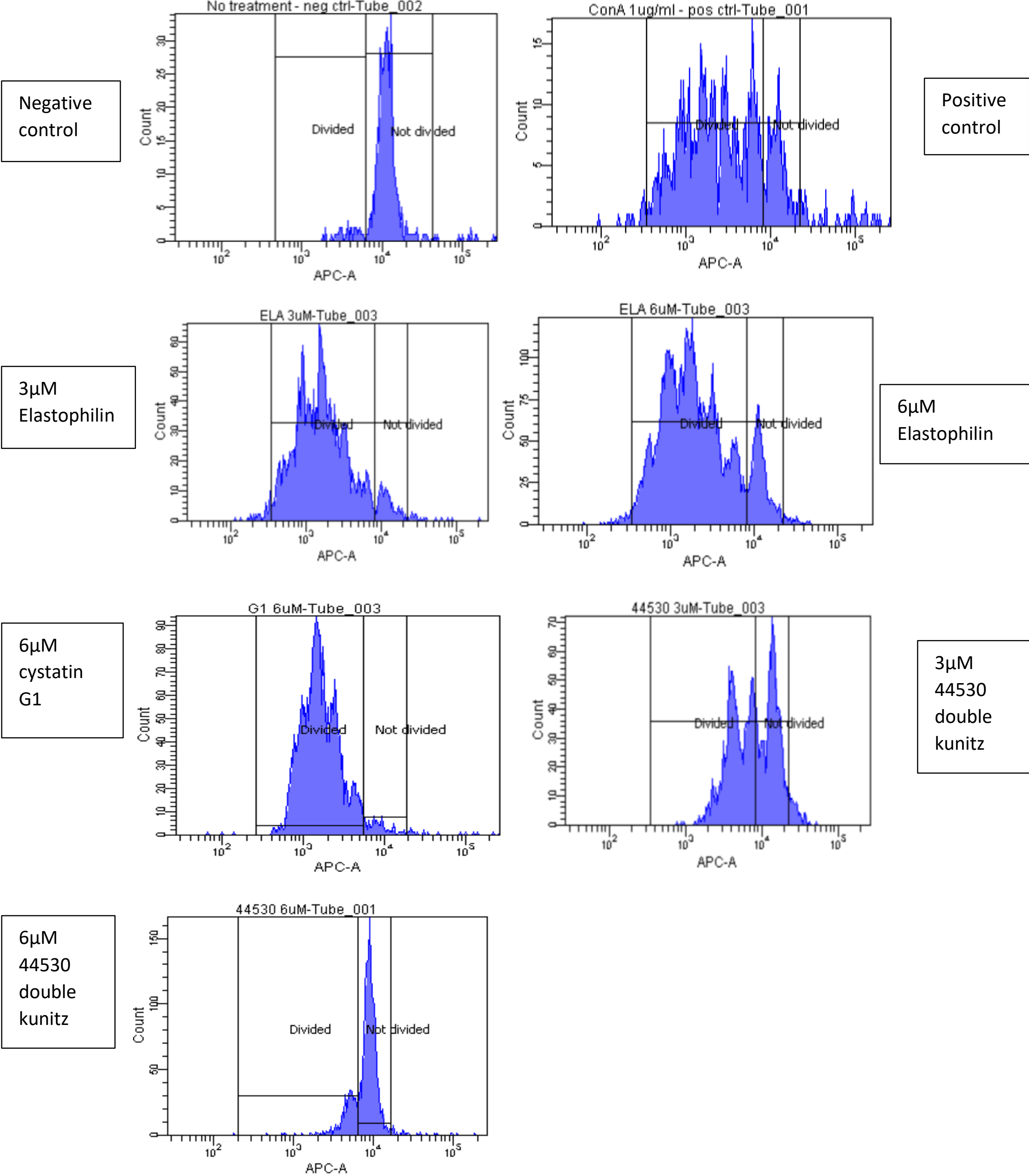
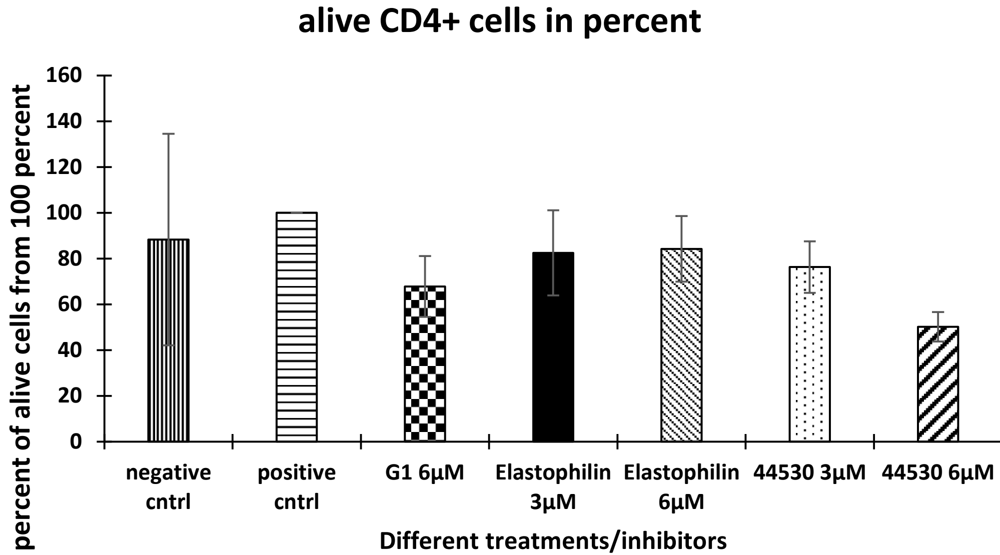
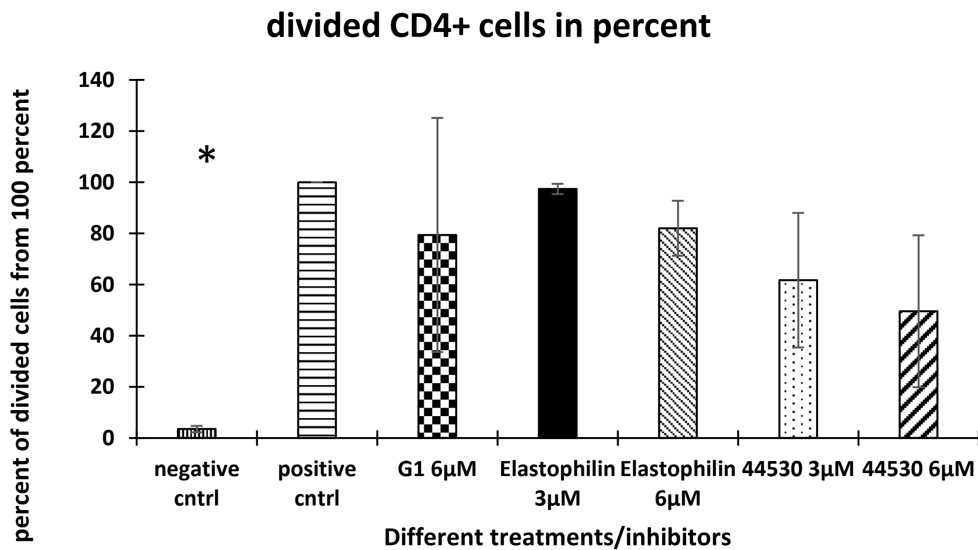


Figure 14: Using FACSDiva Version 6.1.3, The inhibition of T-cell proliferation was evaluated. The figure shows the number of divided and not divided singlet alive CD4+ cells, which are stained.



(a)



(b)

Figure 15: The results shown in the figure were transformed to percents, normalized to positive control (100%) and the average of three experiments were performed for G1 6 µM, Elastophilin 3 µM, Elastophilin 6 µM, 44530 3 µM and 44530 6µM (N=3). (a) The diagram shows the percentage of CD4+ alive cells (b) The diagram shows the percentage of divided (proliferated) T-cells from singlets. One way Anova and subsequently Dunett's test (Post Hoc test) were performed. The statistical significance of each treatment is compared to the positive

control group and it is marked (* p-value <0.05). In the case of alive cells, there was no significant between the treatments ($F(6,14) = 1.1792$, $p = 0.3714$).
In the case of divided cells, there was a significant difference in divided cell numbers between the treatments ($F(6,14) = 4.1561$, $p = 0.0132$).

4.5 Platelet aggregation

Besides having important role in maintaining haemostasis, platelets are also involved in inflammation, infection, host response and even cancer. During a vascular injury, platelets are activated and aggregated by agonists (ADP, thrombine, collagen), forming plugs. Activated platelets release adhesion molecules and pro-inflammatory molecules such as ADP, chemokines, phosphatydilserines and others, which contribute to the coagulation cascade amplification, local inflammatory processes and bridge formation between leukocytes and endothelium by platelet-leukocyte aggregates (Francischetti et al., 2009). Thus, platelet aggregation is very crucial defense mechanism against tick feeding, preventing host vascular injury and bleeding.

For the tick to overcome this defense line, tick saliva contains many molecules, which inhibit platelet aggregation. Tick saliva proteins such as disintegrins, containing RGD motif, can prevent binding of activated platelets to fibrinogen (Francischetti et al., 2009). Other salivary proteins, such as serpins, are also believed to have a crucial role in inhibiting thrombin and other coagulation factors leading to platelet aggregation inhibition (Ibelli et al., 2014).

The aim of this experiment was to evaluate the inhibition of platelet aggregation caused by different proteins. The measurement was done using the BD FACSCANTO II™ flow cytometer.
The results obtained from the flow cytometry show the percentage of aggregates out of total platelet number. We see no difference in platelet aggregation between negative control and positive control after stimulation with PMA. Interestingly, Platelets incubated with G1 and IRS-1 showed slight stimulation of aggregation. A slight inhibition is observed with S8k serpin and 20314 serpin.
Only one experiment was performed and therefore we could not perform any statistical evaluation.

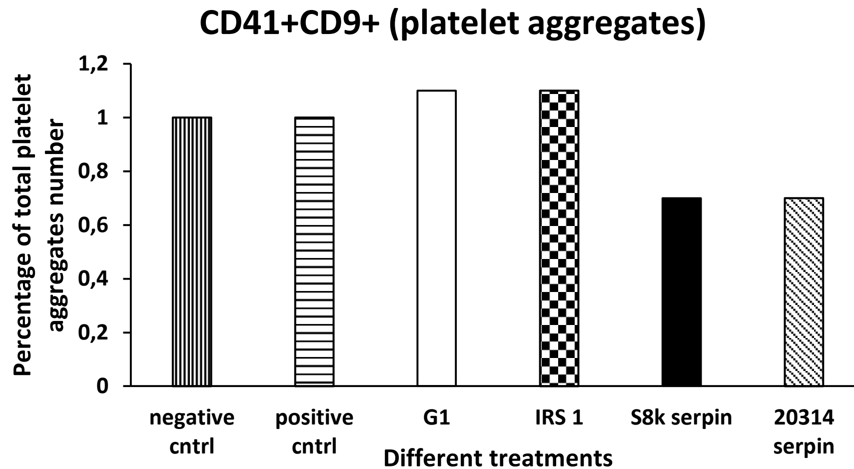


Figure 16: The result shows the percentage of aggregates out of total platelet number.

5 Discussion

5.1 Bioinformatics

Reverse pharmacology/genetics approach is an efficient approach in finding new proteins with pharmacological effects with potential to be future novel drugs. These proteins can be theoretically found in every organism, however, especially blood feeding parasites seem to be very promising organisms, as they must fight against or escape from host defense mechanisms. Therefore, they produce many proteins with anti-hemostatic, anti-inflammatory and immunomodulatory properties. Plenty of different proteins with such features (and many more with unknown function or even structure) were identified and characterized in blood-feeding arthropods, in particular in hard ticks, which stay attached for a long period to the host and must cope with all branches of immune system, including antigen specific one (Chmelar et al., 2012, Francischetti et al., 2009, Kim et al., 2016). In my project, I wanted to get knowledge of all steps of reverse pharmacology approach on the model organism *I. ricinus*, a hard tick and most important vector of several serious human diseases in Europe. To this end, I performed several experiments and analyses that are parts of the reverse pharmacology workflow.

In first transcriptomic work that used NGS (Schwarz et al., 2013), one family was found and named after immunomodulatory Da-p36 protein from the tick *D. andersoni* (Bergman et al., 1995, 1998, Kotsyfakis et al., 2015b). This family was named Da-p36 (Kotsyfakis et al., 2015b, Schwarz et al., 2013) and we thought that it belongs to the same protein family.

Accordingly, we characterized the family *in silico* and found that there is no reasonable similarity, as shown in the alignment in figure 8 and in the phylogenetic tree in figure 9. There were only two motifs – GYF and RGD, present at least in some members of original Da-p36 family, such as Rh-p36 and Av-p36 (Figure 8). The similarity between Da-p36, Rh-p36, Av-p36 and Hl-p36 was observed in our analysis and is in accordance with the literature (Wang et al., 2017).

Also, from table 2 we see that *I. ricinus* Da-p36 proteins do not belong to the same family with original one. There are big differences in glycosylation and phosphorylation sites, molecular weights, RGD motif is not present in most metastriate members of Da-p36 family and also the sequence lengths of the both members differ.

Interestingly, all members of Da-p36 protein family in *I. ricinus* contained RGD motif. RGD motif binds strongly to integrins, thus play a crucial role in cell-cell or cell-ECM interactions, affecting leukocyte migration or tumor metastases spreading (Béress, Béress, 1975) and enhances cell adhesion. Interestingly, this RGD motif can enhance adhesion if it is attached on a surface or inhibit adhesion if it is free in solution. Previously, RGD peptides have been used as anti-thrombotics that act by inhibiting the function of the αIIbβ3 integrin (Ruoslahti, 1996). Therefore, having RGD in secreted and soluble proteins makes them good candidates for testing their adhesion-inhibiting effects either in cell adhesion or in platelet aggregation.

All members of Da-p36 protein family in *I. ricinus* also contained GYF motif. GYF motif is a proline rich domain binding motif. This motif adopts a polyproline type II helical conformation and therefore facilitating sequence-specific recognition without the need of high-affinity interaction. These bindings are weak and specific, insuring rapidly reversible interactions, which are important for cellular communication and signaling functions (Srinivasan, Dunker, 2012).

The aligned sequences have also distinguished sites of differently charged AA domains. Basic and acidic AA side chains. The recognition of these differently charged is important, since charged residues located in protein interaction interfaces are often conserved across the families of homologous proteins and protein complexes (Zhao et al., 2011). Also, proteins can have altered ionization, electrostatic interactions and altered ionic and hydrogen bonds depending on their acidity at a certain pH. This structural alteration leads to a different 3D

structure of the protein and therefore, an altered protein recognition, localization of secreted proteins, specific interaction or inactivation of an enzyme (Gitlin et al., 2006, Zhang et al., 2003).

5.2 Amplification of Da-p36

Amplification of transcripts coding for three proteins of selected members of Da-p36 family of *I. ricinus* was performed using designed primers. Although two of the design primers for certain sequences showed no amplification, one primer, designed for the sequence GADI01007110.1:Ir2-2425 produced amplicons at annealing temperatures 50,7°C and 56,9°C and using cDNA from ovary. However, these amplicons were not reproducible and therefore, I could not clone it and sequence it. The reason of the negative result is assumed to be an error in the primer design, leading to unspecific binding.

Due to short time I could dedicate to the project and the several negative attempts to amplify the desired sequences, I could not continue with cloning of the selected member of Da-p36 family into expression vector and express the protein in bacterial expression system (*E. coli*). And since I was not able to produce chosen protein, I decided to use other *I. ricinus* proteins, already present in our laboratory, to perform next steps of the reverse genetic/pharmacology approach.

With used proteins, belonging to protease inhibitors, we supposed that they could have some inhibitory effect on the host defense mechanism such as platelet aggregation, cell adhesion and T-cell proliferation, because tick protease inhibitors, both serine and cysteine, were shown to have such properties (Bonnet et al., 2018, Chmelař et al., 2017). To test different proteins' activities, I performed several *in vitro* immunological assays, such as cell adhesion, T-cell proliferation and platelet aggregation assays.

5.3 Cell adhesion

In the case of cell adhesion assay, before establishing the assay, different ECM proteins (laminin, fibronectin and collagen) and BSA were used to coat the wells, to achieve the leukocyte adhesion on the ECM protein. After several tests, fibronectin performed as the best

reliable extracellular matrix molecule to coat and therefore, we continued only with fibronectin.

According to previous research, serpin IRS-2 was shown to inhibit inflammation (Chmelař et al., 2017). I wanted to test anti-inflammatory properties of other protease inhibitors (including other serpins) by testing, whether the ability of leukocytes to adhere is affected. In my project, however, the results were mostly negative, However, we see a significant inhibition caused by S8k serpin. According to the result, it is believed that S8k may have similar inhibitory effect to IRS-2. Inhibition was also observed by the IRS-1b but since the inhibition level was almost identical with IRS-1b buffer, the inhibition was caused most likely by some compounds of the buffer, such as detergents. Therefore, IRS-1 has probably no inhibitory effect, similarly to other tested proteins.

5.4 T-cell proliferation

In the case of T-cell proliferation assay, an inhibition of CD4+ cell proliferation was seen using 44530 Kunitz domain inhibitor at a concentration of 6 µM. However, this inhibition was not significant according to statistical analysis. Although Blisnick and colleagues mention that serine protease inhibitors and specially, Kunitz type inhibitors, inhibit cell proliferation (Blisnick et al., 2017), I saw no significant inhibition on T-cell proliferation caused by 44530. I also noticed that the number of alive CD4+ cells have decreased as well as the proliferation. Therefore, the inhibitory effect may be also caused by cytotoxicity of protein 44530.

5.5 Platelet aggregation

Regarding the platelet aggregation, I was trying to establish a new assay that had never been done before in the laboratory and many steps had to be adjusted to obtain reproducible results. In the final assay, two antibodies (anti-CD9-FITC and anti-CD41-APC) were used, according to the protocol described by Cuyper and colleagues (Cuyper et al., 2013). Even after several changes in the assay parameters, the results were not reliable and reproducible. Due to lack of time, I was not able to properly establish a platelet aggregation assay. From one pilot experiment, however, serpins S8k and 20314 seemed to have some effect on platelet aggregation, which resembles the results of Chmelar et al., 2011, where serpin IRS-2 inhibited

thrombin and cathepsin G induced platelet aggregation (Chmelar et al., 2011). Nevertheless, to confirm these results, experiments should be repeated with properly optimized assay.

6 Conclusion

Since tick saliva is a rich source of proteins with high pharmacological potential,
I tried to find and produce new proteins with pharmacological effects in my project, in order to follow the reverse pharmacological/genetic workflow.

I characterized by using bioinformatics tools a Da-p36 like protein family, found in *I. ricinus*. Although a knowledge was obtained from previous study, showing that *I. ricinus* have three protein families similar to Da-p36 protein family, we found that, the new sequences had no close similarity with the original *D. andersoni* Da-p36 protein despite sharing two functional motifs – GYF and RGD.

I intended to produce at least one member of *I. ricinus* Da-p36 family as a recombinant protein, but even after several trials, I was not successful in the recombinant protein production.

To achieve the aim of the project and perform the last steps of reverse pharmacology/genetics, I proceeded to the part of testing, using already present recombinant proteins, which were available in our lab were used. From all experiments only S8k serpin showed significant inhibition effect on cell adhesion. Other tested proteins had no significant effects in any experimental model.

The steps that were followed in my project, are part of one approach that is used to discover novel drugs. Despite I did not have much success in many steps, I walked through almost whole process of reverse pharmacology practically, so the main goal of this bachelor thesis was fulfilled.

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