IL-4 ANALOGUES WITH SITE-SPECIFIC CHEMICAL MODIFICATION AS SCREENING TOOLS FOR FOLDAMERS

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TO MY PARENTS
AND GRANDFATHER

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1. Introduction

All living organisms are constantly exposed to potentially harmful substances. There are plenty of ways for the organisms to protect themselves against infectious agents, from viruses to protozoan, for example, by employing physical barriers or chemicals that repel or kill invaders. Vertebrates have a highly advanced protective system based on different cell types. The immune system, which is a complex network of organs, contains a variety of interacting cells that are able to recognize pathogen as a foreign body and neutralize it.

In order to deliver a fast response the vertebrate immunity has non-specific as well as specific components. The so-called innate immunity is important for a first and fast response upon pathogen attack and presents the non-specific component – it does not attack a specific invader but different parts of the innate immunity can act against a different group of pathogens that enter the body. The so-called acquired or specific immunity on the other hand is capable of highly specific and selective immune responses, which, however, requires time to adapt the response to the pathological intruder. To deliver an optimal reaction the immune response must first adapt to the nature of the pathogen and thus two fundamentally different adaptive mechanisms exist. Humoral immunity is based on the action of antigen-recognizing proteins, the antibodies, which are produced by B cells. These antibodies then specifically bind and mark pathogens and prime them for phagocytosis by macrophages and other cells. Due to the nature of antibodies as extracellular proteins this defense is aimed against bacteria and other extracellular pathogens. To fight intracellular pathogens such as viruses another cell-mediated immunity exists. This cell-mediated or cytotoxic response is characterized by the generation of various effector immune cells, such as $CD4^+$ and $CD8^+$ T lymphocytes, which are important for the recognition of the antigen, and macrophages, neutrophils, eosinophils as well as natural killer cells, which are important for the destruction of the antigen-hosting cell. T cells recognize the pathogens that have entered into cells, whereas B cells and antibodies interact with infectious agents that remain outside the cells [Kuby, J., 1994].

Many functions of the T-lymphocytes are controlled by a set of proteins, whose expression, is induced as a result of antigen-stimulated cellular activation. These proteins, termed cytokines act by binding to their high-affinity receptors expressed on target cells there by inducing a defined

signaling cascade within those cells [Paul, W.E. & Sedar, R.A., 1994].

Cytokines can be subdivided into several families, including the interleukins, the interferons, tumor necrosis factor (TNF)-related molecules, immunoglobulin super-family members, and the chemokines, each of which plays a key role in orchestrating cellular interactions [Paul, W.E. & Sedar, R.A., 1994]. New insights into the biochemistry and molecular biology of cytokine actions are a significant contribution to medical knowledge, explaining some well-known and some of the more vague clinical aspects of diseases.

The first step of differentiation of naive T helper cells is initiated by antigen stimulation. T cells are not a uniform group of cells, but rather can be divided into various subsets based on their effector functions and molecular phenotypes. Each of the subsets promote different types of immune response, for example, regulatory T cells are important for the maintenance of immune cells homeostasis, cytotoxic T cells destroy infected or tumor cells, and T helper cells assist the activity of other immune cells. T helper cells are characterized by their cytokine expression profile, which includes IL-2, IFNγ and TNFβ for the subset T_H1, while cells of the T_H2 subset synthesize IL-4, IL-5, IL-6, IL-10 and IL-13 [Romagnani, S., 1991] (Figure 1.1).

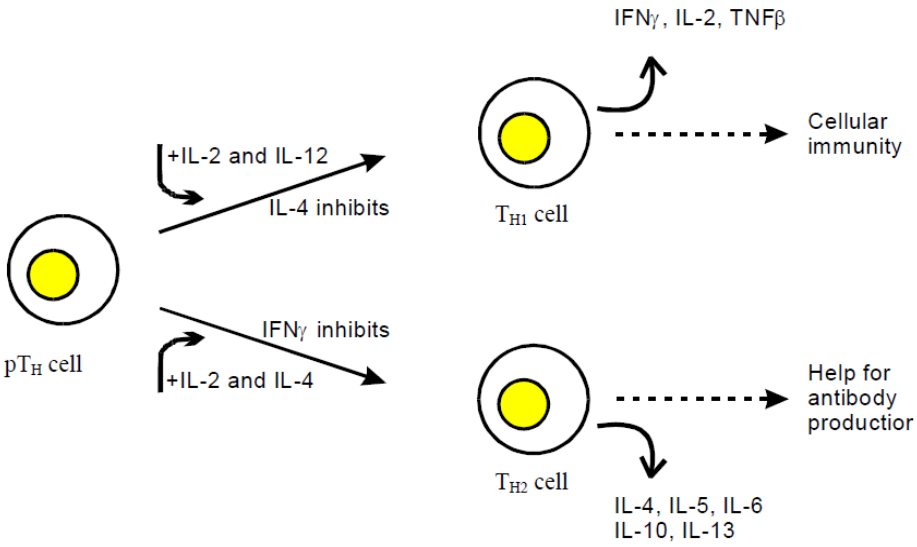


Figure 1.1: The differentiation of T Helper cells into the T_H1 and T_H2 subtypes is controlled by a set of cytokines. The polarization of $CD4^+$ T cells response towards the production of IFNγ and the expression of cellular immunity or towards the production of IL-4 and expression for antibody production [Paul, W.E. & Sedar, R.A., 1994].

1.1. IL-4, structure and biological functions

Interleukin-4 (IL-4) is a pleiotropic cytokine, first described in 1982 and characterized by its ability to stimulate the proliferation of B cells in response to low concentrations of antibodies directed to surface immunoglobulin [Hoffman, R.C. et al., 1995]. IL-4 is produced by TH2 cells (Figure 1.1), basophilic granulocytes and mast cells in response to receptor-mediated activation [Paul, W.E. & Sedar, R.A., 1994].

It plays a key regulatory role in the immune system, by controlling the differentiation of antigen-stimulated naive T cells (also termed TH0 cells) into TH2 subtypes and thus determines the nature of the following humoral immune response [McKenzie A.N., 2000].

Additional functions include induction of the expression of class II major histocompatibility complex molecules (MHC) on resting B cells [Noelle, R. et al., 1984] and enhancing the secretion of IgE and IgG4 immunoglobulins by stimulated human B cells (in murine B cells immunoglobulin expression of the IgE and IgG1 isotypes are enhanced) [Coffman, R.L. et al., 1986; Gascan, H. et al., 1991]. IL-4 also acts on macrophages inhibiting the release of proinflammatory molecules such as TNF, IL-1, IL-8 and other cytokines [Nicola, N. & Hilton, D., 1998].

The structure of IL-4 has been determined by X-ray crystallography and NMR spectroscopy by several groups [Redfield, C. et al., 1991; Powers, R. et al., 1992; Smith, L.J. et al., 1992; Walter, M.R. et al., 1992; Wlodawer, A. et al., 1992; Müller, T. et al., 1994; Müller, T. et al., 1995]. The cytokine displays the characteristic four α-helix bundle structure, with the four helices arranging in an “up-up-down-down” topology. As a consequence the peptide linker elements connecting helix A and B as well as helix C and D are very long [Reinemer, P. et al., 2000] (Figure 1.2).

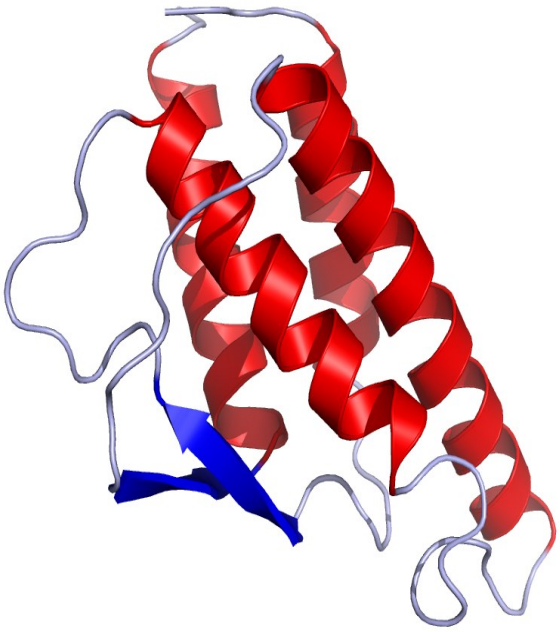


Figure 1.2: Ribbon representation of human IL-4. A helices are depicted in red (α helices αB and αC in the foreground) and orange (α helices αA and αD in the background), β strands in blue, and connecting loops in yellow. [Reinemer, P. et al., 2000].

Human IL-4 contains six cysteine residues forming three disulfide bonds and has thus no unpaired cysteine residue (Figure 1.3). The N- and C- termini of the molecule are linked by a disulfide formed between the residues Cys^{3} and Cys^{127}. The two short loops between helix A and the first β-strand and between helices B and C are reinforced via the disulfide bond between Cys^{24} and Cys^{65}. Similarly the long loop between helices C and D is fixed via a disulfide bond between Cys^{46} located in the middle of helix B and Cys^{99} in the CD loop. The "cross-linking" of flexible elements in the IL-4 structure by these three disulfide bonds also likely contributes to the remarkable overall stability of the IL-4 protein [Walter, M.R. et al., 1992]. Human IL-4 also contains two potential N-glycosylation sites at position 38-40 and 105-107 [Walter, M.R. et al., 1992]. A recent study has shown that Asn38 is the single N-glycosylation site on the human IL-4 protein, and Asn105 has no contribution to the glycosylation [Li, R. et al., 2014].

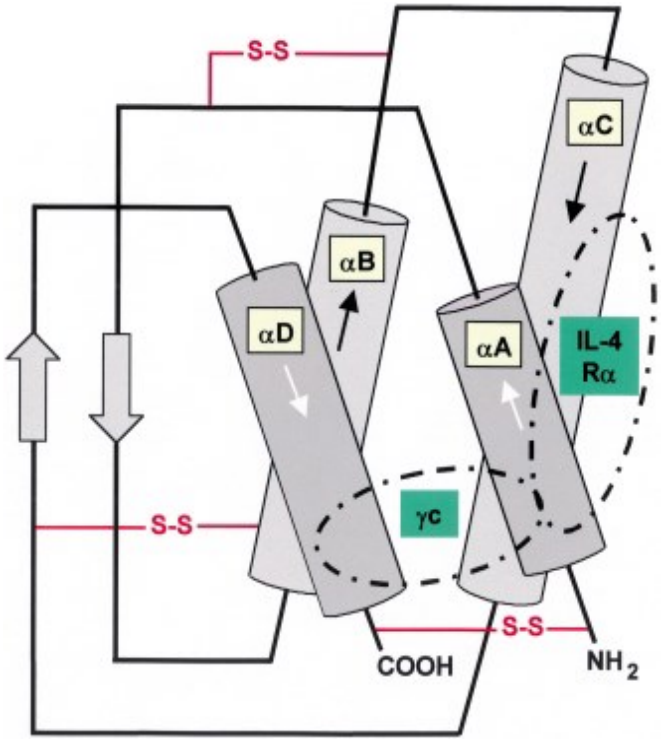


Figure 1.3: Topology of IL-4, secondary structure elements are indicated as well as receptor binding sites for two of the three IL-4 receptors **[**Reinemer, P. et al., 2000**].**

For their proper function the cytokines have to recognize and bind to their cognate receptors. These are cell surface molecules that transduce the binding of the secreted cytokine into a cytoplasmic signal thereby triggering defined biological response in the various immune effector cells [Nicola, N. & Hilton, D., 1998]. IL-4 exerts a cellular response by formation of a heterodimeric receptor assembly, consisting of an IL-4 specific IL-4 receptor α (IL-4Rα), which binds IL-4 with very high-affinity (Kd ~ 100 pM), and a second cytokine receptor, which is either the so-called common γ chain that is shared between IL-2, IL-7, IL-9 and IL-15 or the IL-13 receptor α1 (IL-13Rα1) that IL-4 shares with the functionally related cytokine IL-13. These two assemblies are also known as IL-4 type I (IL-4Rα and γc) and IL-4 type II receptor (IL-4Rα and IL-13Rα1). The IL-4 type I receptor is found on T and B lymphocytes as well as on monocytes [Russell, S.M. et al., 1993]. The IL-4 type II receptor is expressed on non-hematopoietic cells [Jensen, P.L., 2000; Murata, T. et al., 1998; Obiri, N.I. et al., 1995]. The type II receptor is used by both IL-4 and IL-13, whereas the type I is used only by IL-4.

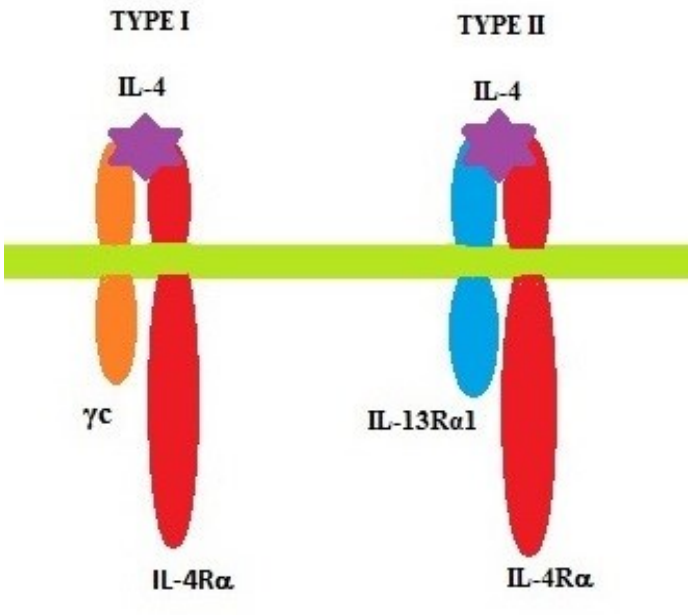


Figure 1.4: Receptor activation by IL-4 occurs via a two-step mechanism. After recruiting IL-4 to the membrane by binding to its high-affinity receptor IL-4Rα, the intermediate complex of IL-4 and IL-4Rα recruits either the γc or IL-13Rα1 receptor [Duschl, A. & Sebald, W., 1996; Müller, T. et al., 2002].

1.2. Ligand: receptor interaction and signal transduction

The epitope of IL-4 determining the high-affinity binding site to the IL-4 receptor α (IL-4Rα) is formed by residues residing on helix A and C with Glu9 and Arg88 being the main binding determinants for the IL-4 - IL-4Rα interaction [Shen, B.J. et al., 1996; Kruse, N. et al., 1993]. The epitope for the binding of IL-4 to γc is composed of the residues Arg121, Tyr124 and Ser125 on helix D of IL-4 [Letzelter, F. et al., 1998; Kruse, N. et al., 1993]. The mechanism of sequential receptor dimerization by two distinct independent binding sites on the ligand was also exploited for the design of antagonistic variants of IL-4. Such protein variants are not capable to recruit the second receptor γc into the receptor complex, since the respective receptor binding epitope (also termed site 2) has been destroyed, however, they still bind to IL-4Rα with high affinity and can thus compete with wild type endogenous IL-4 for binding to this receptor [Kruse, N. et al., 1992].

The ligand-induced dimerization of the cytokine receptors subsequently results in the activation of tyrosine kinases, which then phosphorylate cellular substrates thereby initiating downstream signaling cascades [Miyajima, A. et al., 1992]. Neither the IL-4Rα nor the γc receptor exhibit endogenous kinase activity and therefore receptor-associated kinases are responsible for the initiation of signal transduction. The Janus-family (Jak) tyrosine kinases have been shown to be critically involved in the initiation of signaling through the IL-4 receptor system [Ihle, J.N., 1995; Schindler, C. & Darnell, J.E., 1995, Jiang, H. et al., 2000]. The Janus kinase Jak1 has been proposed to be associated with IL-4Rα, while the kinase Jak3 is in complex bound to the cytoplasmic part of γc [Miyazaki, T. et al., 1994; Nelms, K. et al., 1999; Jiang, H. et al., 2000]. In certain cells, like human colon carcinoma cells, Jak2 associate with IL-4Rα [Murata, T. et al., 1996]. Stimulation of IL-4, results in tyrosine phosphorylation of the associated Janus kinases Jak1 and Jak3 (Figure 1.5). Activation of these IL-4R-associated kinases leads in turn to phosphorylation of defined tyrosine residues in the cytoplasmic domain of the IL-4Rα chain itself, a process that occurs rapidly after IL-4 receptor engagement [Smerz-Bertling, C. & Duschl, A., 1995; Schindler, C. & Darnell, J.E., 1995,].

Jak1 then phosphorylates the Signal Transducer and Activator of Transcription 6 (STAT6), which subsequently dimerizes, migrates to the nucleus, where STAT6 promotes the transcription of target genes, including CD23, MHC class II, ε heavy chain, and IL-4Rα [Ihle, J.N., 1996; Mikita et al., 1996; Bennett, B.L. et al., 1997; Tinnel, S.B. et al., 1998].

Suppressor of cytokine signaling 1, 3 and 5 (SOCS1, SOCS3 and SOCS5) can inhibit the STAT6 activation. SOCS1 and SOCS3 can down-regulate IL-4-induced STAT6 activation by inhibiting Jak1 kinase activity [Haque, S.J. et al., 2000]. SOCS5 binds to a fragment next to the Box-1 motif, inhibiting the binding of Jak1 [Seki, Y. et al., 1999]. In response to IL-4 signaling, Jak1 phosphorylates the 145 kDa Inositol Polyphosphate-5-Phosphatase also termed SHIP followed by positive regulation of cell proliferation [Chatila, T.A., 2004].

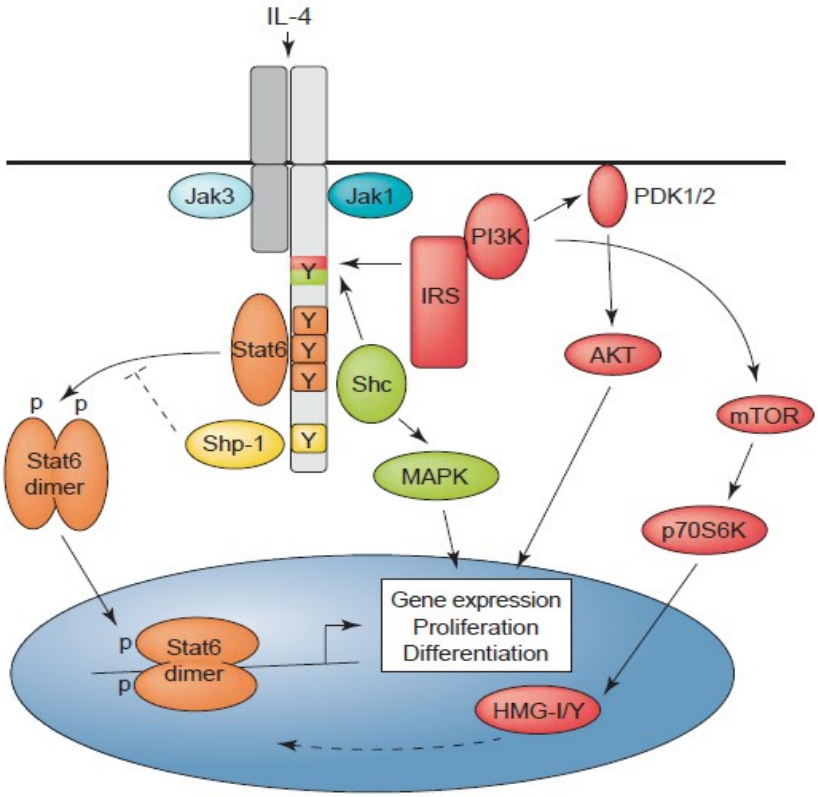


Figure 1.5: Signal transduction through the IL-4R complex [Chatila, T.A., 2004].

Upon IL-4 receptor activation, Jak1 and Jak3 also phosphorylate two adapter molecules, Insulin Receptor Substrate 1 and 2 (IRS-1 and IRS-2), leading to the activation of the Phosphatidylinositol-3-Kinase (PI3K) and the adapter molecule, Grb2. Once activated the PI3K phosphorylates membrane lipids that act as second messenger and are important for the survival of the cell [Franke, T.F. et al., 1997]. As mentioned before, another binding partner of phosphorylated IRS is the adopter molecule Grb2. Grb2 is associated with the guanine nucleotide exchange factor SOS, whose primary function is to catalyze the exchange of GDP in Ras with GTP thereby leading to activation of Ras and hence of the Raf kinase and the MAP kinase as well [Denhardt, D.T. et al., 1996].

1.3. Misguided Immune system

Dysregulation of IL-4 function is strongly associated with type I hypersensitivity reactions, also known as immediate or anaphylactic hypersensitivity. The reaction may involve skin (urticaria and eczema), eyes (conjunctivitis), nasopharynx (rhinitis), bronchopulmonary tissues (asthma) as well as the gastrointestinal tract (gastroenteritis), causing a wide range of symptoms from very mild to lethal. A type I hypersensitivity reaction is characterized by a prompt occurrence of symptoms against the allergens upon exposition. The progression of the allergic disease is shown schematically in Figure 1.6. Briefly, an antigen is absorbed by antigen-presenting cells, subsequently processed, and antigenic/immunogenic determinants are finally presented via an MHC-II complex to T helper cells, which triggers differentiation of a naive T helper cell into a T helper cell of the subtype 2 cell. For Th2 differentiation to occur it is necessary that the T cells are stimulated with IL-4 and IL-2 during their activation, for development of Th1 cells INFγ and IL-2 are the stimulating cytokines. Mast cells and subsets of T cells are considered as a potential source for early production of IL-4. Since Th2 cells and other cytokines also produce IL-4, autocrine amplification and stabilization of the T-helper cell lineage is triggered.

Important for development of allergies and atopic diseases is the class switch of the immunoglobulin type to the class of IgE, which is initiated by the Th2 cytokines IL-4 and IL-13. The resulting allergen-specific IgE antibodies bind to surface Fc receptors on mast cells, basophiles, and eosinophiles and induce their sensitization to the antigen.

Upon a new contact with the allergen, the antibody binding to these cells will trigger an immediate allergic reaction by causing the degranulation of the effector cells. In the case of hay fever it is mainly the mast cells in the mucous membranes of the eyes and the respiratory tract that degranulate thereby releasing compounds, such as histamine, serotonin, and prostaglandins, which finally cause the typical allergy-related symptoms. A variety of additional pathological conditions can be observed in asthma: increased production of mucus in the bronchi, increased reactivity of bronchial smooth muscles, and production of an inflammatory infiltrate in the tissue likely released by eosinophiles. IL-4 and IL-13 furthermore increase the activity of mast cells, basophiles, and eosionphiles, and they also lead to differentiation and hypertrophy of mucus-

-producing goblet cells [Reinemer, P. et al., 2000].

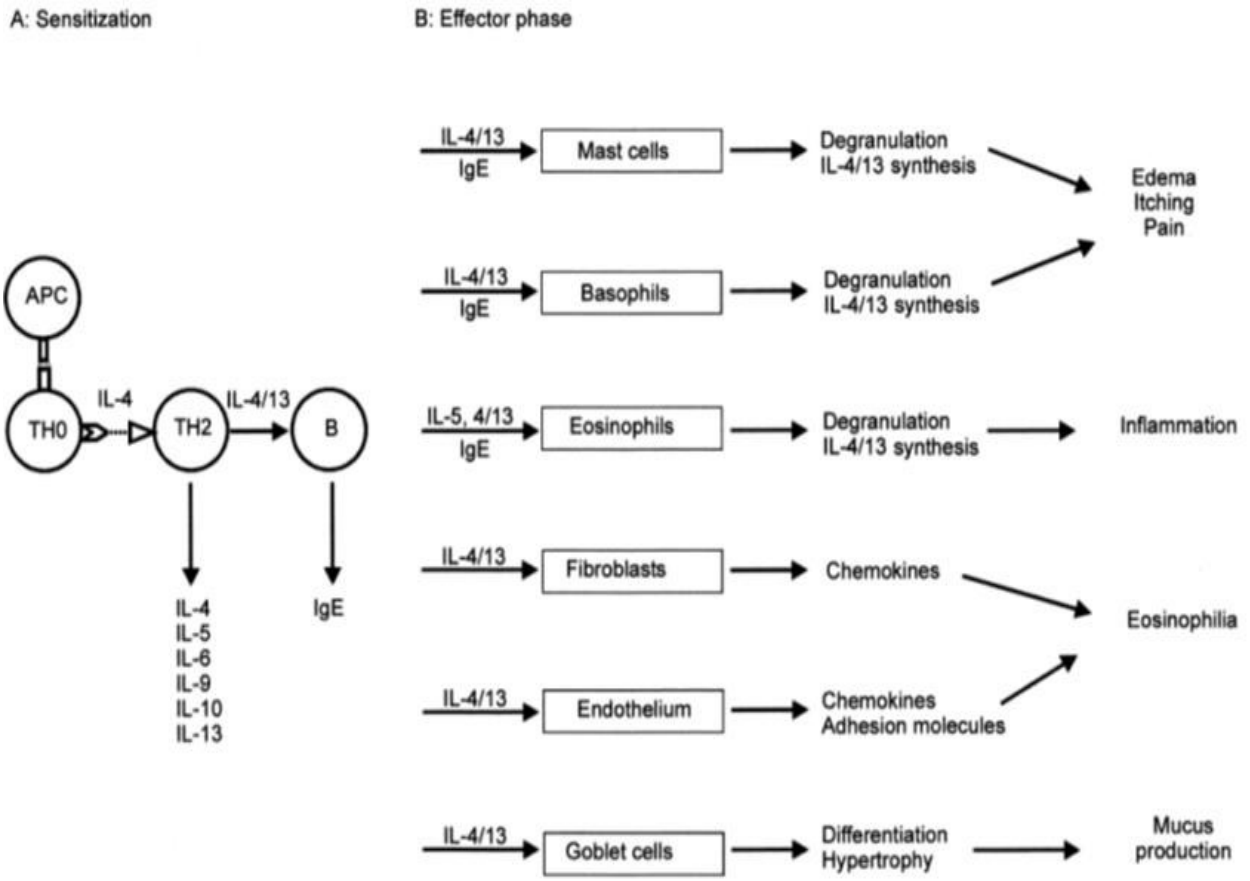


Figure 1.6: Schematic representation of the sensitization (A) and effector phase (B) of the immediate type allergic reaction [Reinemer, P. et al., 2000].

1.4. Drug design and development

Cytokine signaling is important for the growth, maintenance and repair of cells and tissues in our body, and their dysregulation often leads to serious and widespread diseases. Therefore, they represent promising targets for drug design and development [Sebald, et al., 2009]. IL-4 antagonists that can be used in therapy of allergic diseases include various compounds that inhibit the biological activity of IL-4. Hence, molecules such as soluble IL-4 receptor ectodomain, antibodies that bind to and neutralize IL-4 or the IL-4 receptor components, IL-4 muteins competitively bind the IL-4 receptor but do not induce biological responses or molecules that inhibit downstream IL-4 signaling components might be useful tools for such an approach. Several of these routes have been successfully implemented and are currently undergoing testing at different stages to reveal their potential for therapeutic use.

The first trial to target IL-4 for the treatment of asthma made use of a soluble IL-4 receptor α (sIL-4Rα) (Nuvance, Thousand Oaks, California, USA), which can capture endogenous IL-4 thereby blocking the interaction between IL-4 and its naturally occurring cellular receptors [Corren, J., 2011]. There are two studies published for the efficacy of soluble IL-4R (sIL-4R), administrated by nebulization, for treatment of asthma. Nebulized sIL-4R was studied in patients with moderate-to-severe asthma. Both studies, however, did not reveal a major effect in patients with persistent asthma [O'Byrne, P.M., 2006]. But the application of sIL-4R prevented asthma deterioration at the time of inhaled corticosteroid therapy withdrawal [Borish, L.C. et al., 2001; O'Byrne, P.M., 2006; Wang, Z., 1998] and led to a reduction of asthma exacerbations, events that have been associated in some patients with increases in the number of airway eosinophiles.

An alternative strategy to block IL-4 involved the development of a humanized antibody directed against IL-4 (pascolizumab, SB 240683) [Hart, T.K. et al., 2002]. This antibody effectively blocked IL-4 responses in vitro, and showed a favorable in-vivo pharmacokinetic profile in cynomolgus monkeys, but a phase II clinical trial in humans with asthma gave unimpressive results, and further development was discontinued [Long, A., 2009; Tourangeau, L.M., 2011].

A recombinant variant of human IL-4 (Pitrakinra, Aerovant or AER 001, Aerovance, Berkeley, CA, USA), that binds with high affinity to IL-4Rα but does not bind to the other IL-4 receptor components γc or IL-13Rα1, has been developed in the lab of Walter Sebald and is able to block the binding and the activation of endogenous IL-4 and IL-13 to their cellular receptors [Tony, H.P. et al., 1994]. In the first of two small, placebo-controlled trials, subcutaneous administration of pitrakinra was shown to significantly reduce asthma-related adverse events. In the second trial, nebulized pitrakinra also significantly reduced the magnitude of the late asthmatic response [Antoniu, S.A., 2010; Wenzel, S. et al., 2007].

A second approach to inhibit the effects of IL-4 and IL-13, a humanized neutralizing monoclonal antibody was developed directed against IL-4Rα (AMG 317) commonly used by IL-4 and IL-13 [Corren, J. et al., 2010]. A 12-week placebo-controlled dose-ranging study however demonstrated that this agent did not affect asthma symptoms, pulmonary function, or other endpoints [Corren, J., 2011].

In the last decade a novel approach was made in designing new potential drugs for inhibition of protein-protein interactions utilizing foldamers [Gellman, S.H., 1998; Hill, D.J. et al., 2001]. Since production of recombinant proteins and antibodies is expensive and difficult and their oral application still impossible, small synthetic compounds that mimic the protein binding surface and maintain a high binding affinity have come to an interest. In this work we will examine the possibility to use foldamers as new tools for the specific and efficient inhibition of the IL-4-IL-4Rα interaction.

1.5. Foldamers: a novel approach for drug development

Protein-protein interactions are crucial for most biological processes – from intracellular communication to programmed cell death – and therefore represent a large and important class of targets for developing human therapeutic agents [Arkin, M.R. & Wells, J.A., 2004].

Some of the advantages of the therapeutic antibodies are their ability to bind with high affinity and specificity to variety of molecular targets and they tend to be very stable in human serum. But, they suffer from difficulties in manufacture, high costs of goods and the lack of oral bioavailability. Antibodies are not cell-permeable, and antagonism of intracellular protein-protein system has so far been limited [Stockwin, L. & Holmes, S., 2003]. Developing of the small-molecule antagonists has been also difficult for numerous reasons: the problem to of finding a good small-molecule starting point for drug design, the size and the flatness of the surface, the difficulty of distinguishing real from artificial binding, and the size and character of typical small-molecule libraries [Arkin, M.R. & Wells, J.A., 2004].

Over the last years, intense efforts have been made to the design, synthesis and structural studies of foldamers – artificial oligomers that adopt a well-defined conformation in solution, mimicking folded structures of biopolymers. Gellman declared in 1998 that any large molecule with a strong tendency to adopt a specific compact conformation is a foldamer [Gellman, S.H., 1998]. In 2001 Moore stated that foldamers can be any oligomer folding into a conformationally ordered state in solution [Hill, D.J. et al., 2001]. These compact structures are stabilized by a number of non-covalent interactions between nonadjacent monomer units.

Interest in these new molecules stems largely from the idea that - due to their defined structures - they may mimic, not only biological structures but also biological functions. There are multiple examples of functional foldamers who can mediate cell penetration [Rueping, M. et al., 2002; Potocky, T.B. et al., 2003], foldamers binding to various targets like RNA [Gelman, M.A. et al., 2003], membranes [Werder, M. et al., 1999; Hamuro, Y. et al., 1999; Porter, E.A. et al., 2000; Liu, D. & DeGrado, W., 2001; Epand, R.F. et al., 2004; Epand, R.F. et al., 2006;], carbohydrates [Choi, S. et al., 2005], and proteins [Gademann, K. et al., 1999; Sadowsky, J.D. et al., 2005; English, E.P. et al., 2006; Kritzer, J.A. et al., 2005; Stephens, O.M. et al., 2005].

Foldamers are generally more stable to enzymatic proteolysis than peptides [Seebach, D. et al., 2004] due to lacking peptide bond backbones and require fewer monomeric units to adopt well-defined secondary structures, thereby fulfilling the promise of designing smaller and more stable versions of peptides. Furthermore, they can be used as stepping stones in the downsizing of peptides to small molecules: the starting peptides had molecular weights in the range of 2,000 to 3,000 Da, those of the β-peptides were 1,000 to 2,000 Da, and the arylamides and phenylalkynyl compounds range from 500 to 1,000 Da [Goodman, C. et al., 2007]. For example, the antimicrobial peptide Magainin has a molecular mass of 2467 Da, and a highly potent phenylalkylnyl-based antimicrobial compound with a molecular weight less than 600 Da that works by the same mechanism as Magainin has been created [Nüsslein, K. et al., 2006].

This new generation of drugs can act as simple as a competitive antagonists by directly competing for a common binding partner, but might also function through a much more subtle mechanism for instance through allosteric inhibition. A variety of synthetic oligomers like, β-peptides and peptoids, aromatic oligoamides and poly-m-phenylene ethynylene have been demonstrated to fold into helical structures. Some biological studies have reported the promising therapeutic potential of this class of molecules, in particular of β-peptides and peptoids. Aromatic oligoamides have advantageous properties such as stability, simple synthesis, and predictable folding, thereby providing possibilities for structure-activity relationships in biological systems and for development of therapeutics [Gillies, E.R. et al., 2007]. Amphipathic helices are promising targets for the exploration of biological activity in synthetic foldamers, as they are involved in a variety of biological functions [Gillies, E.R. et al., 2006].

1.6. Tethering: Fragment-based drug discovery

The drug discovery process normally begins with massive screening of large compound libraries to identify first hits with usually modest affinity (Kd ~ 1 to 10 μM) that then serve as leads for further development. Many of these screening methods rely on inhibition assays, but the small organic molecules are often insoluble or only soluble at low concentrations when on the other hand high concentrations of these compounds may be required due to their inherent low binding

affinity. An even more significant problem is that many of these compounds can act ‘’promiscuously’’ against a wide range of targets, possibly by forming large aggregates [McGovern, S.L. et al., 2002]. Taking into consideration that many factors may interfere with the assay and produce a negative signal that would be misinterpreted as inhibition, fewer artifacts can actually produce a positive signal [Toth, G. et al., 2007]. Besides the traditional screening methods, like SPR, NMR, and MS, an alternative method for fast and reliably screening of compounds has been developed.

Tethering relies on reversible disulfide bond formation between the compound and the protein of interest, thus controlling the region where the selected compound binds to the protein. The method requires relatively small amounts of protein if the tethered protein can be analyzed by MS [Erlanson, D.A. et al., 2004]. Also, tethering provides a possibility to develop on a site-directed basis by choosing the site that is to be targeted and explored. Thereby protein activity can be selectively modulated either by directly targeting active sites and competitive inhibition of this target site or by identifying allosteric sites in proteins and modulating their activity by allosteric regulation [Pargellis, C. et al., 2002; Schindler, T. et al., 2000].

The principle of tethering is demonstrated in Figure 1.7. The target protein must contain a free thiol group (i.e. an unpaired cysteine residue) within or near the target site. This can be accomplished either if the protein has already a naturally occurring cysteine residue or a cysteine residue has to be introduced by site-directed mutagenesis. To allow for unbiased screening of the site the cysteine residue should be within 5 to 10 Å distance of the site of interest and should be surface-exposed to facilities thiol-disulfide exchange [Erlanson, D.A. et al., 2000].

Once the above conditions are met, the target protein is reacted with a library of disulfide-containing compounds under partially reducing conditions. Assuming that the reactivity of the disulfide in each compound is equivalent and assuming there are no non-covalent interaction between the compounds and the protein, at equilibrium the reactant mixture should consist of the protein disulfide-bonded to each compound in the same proportion. However, if one of the fragments of the library has an inherent affinity to the target site in the protein of interest, the reactant mixture will have a higher proportion of this disulfide-linked protein-compound complex.

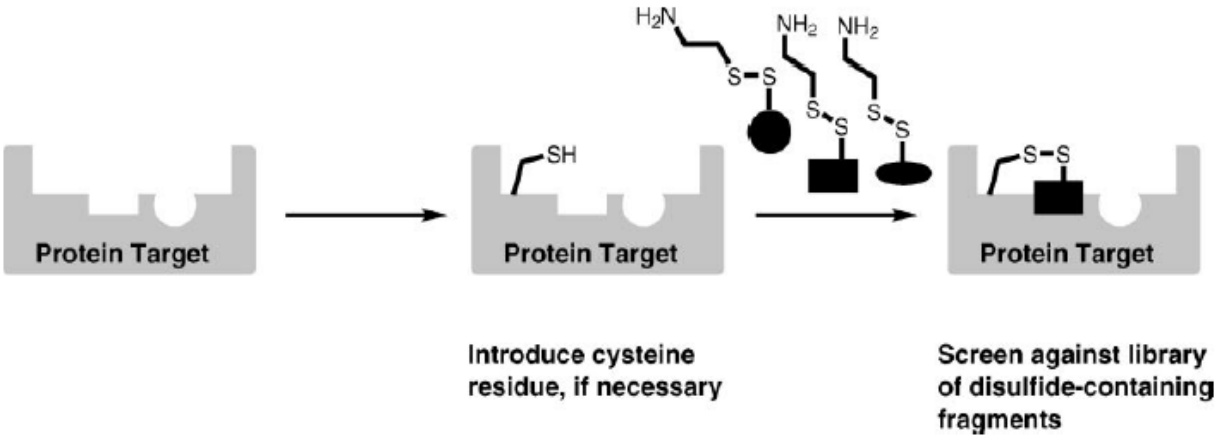


Figure 1.7: Schematic illustration of the tethering approach: a cysteine containing protein is equilibrated with a disulfide-containing library in the presence of reducing agent. Most of the library members will have little or no inherent affinity for the protein, and thus by mass action the equilibrium will lie toward the unmodified protein. If a library member does show inherent affinity for the protein, the equilibrium will shift toward the modified protein [Erlanson, D.A. et al., 2004].

Our target protein IL-4 already contains three disulfide bonds but on the other hand does not have an unpaired cysteine residue. To allow for a thiol-based tethering approach as described above, an additional cysteine residue must be introduced at a defined position for subsequent site-specific chemical modifications.

1.7. AIM OF STUDY

Since IL-4 plays a key role in inflammatory diseases such as allergy and asthma, a strong interest in development of IL-4 inhibitors exists. However the nature of the IL-4 ligand-receptor interaction, which is a prime example of protein-protein interaction difficult to target with small molecule inhibitors, has allowed only development of protein based inhibitors, such as antagonistic protein variants, soluble receptor proteins or neutralizing antibodies.

Protein-protein interactions are difficult targets for small molecules because the contact surfaces on the native protein partners are large and often flat, which makes the search for intermediate affinity binders as starting point tedious. It would therefore be highly valuable to identify alternative sources of antagonists, like peptide-like oligomers with unnatural backbones that can function as agents by blocking a key protein-protein interaction.

We therefore attempted to utilize a new class of inhibitors, foldamers, which can be synthesized and diversified in a peptide-like manner. To introduce this new class into the development of IL-4 inhibitors, we first produced IL-4 muteins, suitable for site-directed modification and tethering. A single cysteine residue will be introduced into IL-4 at strategic locations to allow disulfide bond formation with the thiols added on the foldamers. With these IL-4 variants at hand we then want to tether a set of reference foldamers to IL-4 muteins through disulfide bridges.
Next goal is biological testing of foldamers against the target, where low binding affinities will be detected with appropriate biophysical methods (BIAcore).

2. Materials and methods

2.1. List of abbreviations

Table 2.1: List of abbreviations

%	percentage
˚C	degree Celsius
bp	base pair
CO_2	Carbon dioxide
Da	Dalton
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphates
DTT	Dithiothreitol
E.coli	*Escherichia coli*
EDTA	Ethylendiamintetraacetic acid
Eq., equilib.	Equilibrium
EtBr	Ethidium bromid
EtOH	Ethanol
FCS	Fetal calf serum
Fig.	Figure
g	gram
GuCl	Guanidine hydrochloride
HPLC	High-performance liquid chromatography
IL-x	Interleukin-x
IL-4Rα	Interleukin-4 receptor α
IL-13α1	Interleukin-13 receptor α1

IL-xBP	Interleukin – x binding protein
IL-xR	Interleukin – receptor
Jak	Janus kinase
kbp	kilo base pair
Kd	dissociation equilibrium constant
koff	dissociation rate constant
kon	association rate constant
LB	Luria Broth
L	liter
ln	logarithm to the basis of ‘’e’’
µ	micro-
max.	maximal
M	molar
M	milli-
Min	minute(s)
mRNA	Messenger ribonucleic acid
MWM	Molecular weight marker
n	number of measurements
NK	Naturalkiller (cells)
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
rel.	relative value
RNA	Ribonucleic acid
RP-HPLC	Reversed phase high pressure liquid chromatography

RT	room temperature, reverse transcriptase
RU	resonance units
s	soluble
SDS	Sodium dodecyl sulfate
SE	standard error
sec	second(s)
SPR	Surface plasmon resonance
STAT	Signal transducer and activator of transcription
TB	Terrific Broth
TEMED	N, N, N', N'-tetramethylethylenediamine
TFA	Trifluoroacetic acid
TH	T helper (cells)
TRIS	Tris (-hydroxymethyl)-aminomethane
U	unit
UV	ultraviolet
v/v	volume : volume ratio
Vol	volume
wt	wild type

2.2. Chemicals

All chemicals were purchased from the companies: Amersham, Biorad, Gibco-BRL, Merck, Pharmacia, Roth, or Sigma (Table 2.2). All solutions used in the experiments were made with deionized water (Millipore-Q-System).

Table 2.2: List of chemicals.

Chemical	**Company**	**Quantity**	**Mw [g/mol]**	**Article number**
2-Mercaptoethanol	SIGMA	100 ml	78.13	M7522
Acetonitril	Roth	2.5 l	41.05	8825.2
Ammonium acetate	ROTH	1 kg	77.08	7869.1
L-Arginine monohydrochloride	Roth		210.7	3145.8
Brilliant Blue G	SIGMA	5 g		B0770-5G
Calcium chloride	ALDRICH	5 kg	110.98	22,231.3
CHAPS	Applichem		614.89	
DTT (1,4-Dithiothreitol)	Roth	25 g	154.25	6908.2
EDTA	ROTH	1 kg	372.24	8043.2
Ethanol, Absolut	Baker	2.5 l		8006
Faltenfilter (185 mm)	Hartenstein	100 pieces		
Fractogel EMD SO_3^- 650 (S)	Merck	500 ml		1.1890.0500
GuHCl	ROTH	20 kg	96.53	0037.8
Glutathion; ox.	Roth	5 g	612.64	6378.2
Glutathion; red.	Roth	25 g	307.33	6382.2
Glycerol, p.a.>86%	Roth	5 l		4043.2
Hydrochloride acid (HCl)	Merck	1 l		1.096063.1000

Chemical	Company	Quantity	Mw [g/mol]	Article number
IPTG-β (Isopropyl-β-D-thiogalactopyranoside)	GERBU	100 g	238.30	1043
Isopropanol p.a.	Roth	2.5 l	60.10	6752.4
Kalium acetate	Merck	1 kg	98.15	1.04820.1000
Kalium chloride	Merck	1 kg	74.55	1.04936.1000
Kalium dihydrogenphosphat (KH_2PO_4)	Roth	1 kg	136.09	3904.1
Kanamycin sulfate	ROTH	25 g	582.58	T832.2
Lysosime	ROTH	10 g	14000	8259.2
MOPS	ROTH	1 kg	209.279	6979.3
Natrium hydroxide (NaOH) 2N	Merck	1 l	39.997	1.09136.1000
Natrium hydrochloride (NaCl)	Roth	10 kg	58.44	9265.3
di-Natrium hydrophosphat (Na_2HPO_4)	ROTH	1 kg	156.01	T879.2
Pepton from Casein	ROTH	1 kg		8952.2
Rubidium chloride	SIGMA	50 g	120.9	R-2252
Saccharose	ROTH	5 kg	342.30	4621.2
SP Sepharose Fast Flow	GE Healthcare	300 ml		17-0729-01
Tris	ROTH	5 kg	121.14	5429.2
Tris-HCl	ROTH	5 kg	157.60	9090.4
Trichloracetic acid (TCA) p.a.	Roth	1 kg	163.39	8789.1
Trifluoroacetic acid (TFA)	Merck	100 ml	114.02	1.08262.0100
Triton X-100	Roth	1 kg	647	3051.2
Yeast extract	ROTH	1 kg		2363.2

Chemical	Company	Quantity	Mw [g/mol]	Article number
Whatman membrane filters RC55 (regenerated cellulose) 0.45 µm diameter 50mm	SIGMA			Z612413
ZelluTrans Dialysis tubing	Roth	30 m		E659.1

2.3. Bacterial strains

The following bacterial strains were used (Table 2.3).

Table 2.3: List of E.coli genotypes.

Bacterial strain:	Genotype:
E.coli Rosetta™ (DE3) (Novagen)	F^- ompT $hsdS_B(r_B^- m_B^-)$ gal dcm (DE3) pRARE (Cam^R)
E.coli NovaBlue™ (Novagen)	endA1 hsdR17 (r_{K12}^- m_{K12}^+) supE44 thi-1 recA1 gyrA96 relA1 lac F′[$proA^+B^+$ $lacI^qZ\Delta M15$::Tn10] (Tet^R)

2.4. Cell lines

CHO-S – a cell line, originally established from Chinese hamster ovaries. CHO-S cells were grown in a suspension, obtained from Hanna Haliu, UCB S.A, Celltech, UK.

2.5. Vectors and Oligonucleotides

2.5.1. Expression vector for E.coli

Maps of all plasmids including the expressed proteins are provided in the result section.

pQKA: modified pQE80L vector (Qiagen) for expression in E.coli; T5 promoter, lac operator; Amp^R exchanged with Kan^R.

2.5.2. Expression vector for CHO-S cells

pcDNA3.1 (+): Mammalian expression vector with CMV promoter, replacement of pcDNA3. Differs from pcDNA3.1 in drug resistance; +/- refers to orientation of f1 ori. The MCS is in the forward (+) orientation.

2.5.3. Oligonucleotides

The oligonucleotides, used for cloning and sequencing are presented in Table 2.4.

Table 2.4: (A) Oligonucleotides for IL-4 mutant genesis. The codons mutated are underlined.
(B) Sequencing primers.

(A)

Name	Sequence
5' H74C	5' CACAGCAGTTCTGCAGGCACAAGC 3' -
3' H74C	5' GTGCCTGCAGAACTGCTGTGCAG 3' -
5' Q78C	5' CAGGCACAAGTGTCTGATCCGATTC 3'
3' Q78C	5' CGGATCAGACACTTGTGCCTGTG 3'
5' Q78C	5' CAGGCACAAGTGTCTGATCCGATTC 3' -

Name	Sequence
3' Q78C	5' CGGATCAGACACTTGTGCCTGTG 3' -
5' Q78C	5' CACAGGCACAAGTGTCTGATC 3'
3' Q78C	5' CAGGAATCGGATCAGACACTTGTGC 3'
5' Q78C	5' CACAAGTGTCTGATCCGATTCC 3'
3' Q78C	5' CGGATCAGACACTTGTGCCTGTGGAAC 3'
5' R81C	5' CAAGCAGCTGATCTGTTTCCTG 3'
3' R81C	5' GTTTCAGGAAACAGATCAGCTGC 3'
5' S16C	5' CAAAACTTTGAACTGCCTCACAGAG 3'
3' S16C	5' CTCTGTGAGGCAGTTCAAAGTTTTG 3'
5' I11C	5' CACCTTACAGGAGATCTGTAAAACTTTGAACAGCC 3'
3' I11C	5' GGCTGTTCAAAGTTTTACAGATCTCCTGTAAGGTG 3'
5' I11C	5' GATATCACCTTACAGGAGATCTGTAAAACTTTGAACAGCCTCAC 3'
3' I11C	5' GTGAGGCTGTTCAAAGTTTTACAGATCTCCTGTAAGGTGATATC 3'
5' N38C	5' GCTGCCTCCAAGTGTACAACTGAGAAGG 3'
3' N38C	5' CCTTCTCAGTTGTACACTTGGAGGCAGC 3'
5' N38C	5' CTTTGCTGCCTCCAAGTGTACAACTGAGAAGGAAAC 3'
3' N38C	5' GTTTCCTTCTCAGTTGTACACTTGGAGGCAGCAAAG 3'
5' R121C	5' GGCTAAAGACGATCATGTGTGAGAAATATTCAAAGTG 3'
3' R121C	5' CACTTTGAATATTTCTCACACATGATCGTCTTTAGCC 3'
5' Y124C	5' CATGAGAGAGAAATGTTCAAAGTGTTCGAG 3'
3' Y124C	5' CTCGAACACTTTGAACATTTCTCTCTCATG 3'
5' Y124C	5' CGATCATGAGAGAGAAATGTTCAAAGTGTTCGAGCTG 3'
3' Y124C	5' CAGCTCGAACACTTTGAACATTTCTCTCTCATGATCG 3'

(B)

Name	Sequence
5' QKA for.	5' GTGAGCGGATAACAATTTCACACAG 3'
5' PQE 80L	5' CAACAGGAGTCCAAGCTCAGC 3'

2.6. Restriction enzymes

Restriction enzymes were provided from MBI Fermentas (St. Leon-Rot, Germany) and New England Biolabs (Frankfurt am Main, Germany). If not mentioned otherwise DNA restriction experiments were performed according to manufacturer's protocols.

2.7. Kits

Agarose Gel Extraction:	NucleoSpin Extract II Kit (Macherey-Nagel)
DNA Preparation:	Plasmid Mini Kit (Promega)
	Plasmid Maxi Kit (Qiagen)

2.8. Microbiological methods

2.8.1. Sterilization

Laboratory glassware was sterilized for 6 h at 180˚C using a hot air oven (ST5060, Heraeus). Buffer, medium and plastic vessels were autoclaved for 30 min at 120˚C and 1.2 bar (Varioklav Dampfsterilisator, H+P Labortechnik GmbH). Solutions of thermolabile substances were sterile-filtered via an aseptic syringe filter with a pore diameter of 0.22 µm. Sterile plastic material for cell culture was obtained from Greiner. Before use, dialysis tubes (Zellutrans 3.5 kDa exclusion limit, Roth) were first incubated for 1 h at 60˚C in buffer (20 g/l $NaHCO_3$, 0.4 g/l EDTA), then washed thoroughly with dH_2O and finally stored in 20% ethanol at 4˚C.

2.8.2. Culture media

LB-medium:	10 g/l Bacto-Trypton, 5 g/l Bacto-Yeast Extract, 10 g/l NaCl
(Sambrook et al., 1989)	pH 7.5 with 1 M NaOH adjusted
TB-medium:	13.3 g/l Bacto-Trypton, 26.6 g/l Bacto-Yeast Extract, 4.4 ml/l
(Sambrook et al., 1989)	glycerin
	Before use 10% sterilized 10 x phosphate buffer was added
10x phosphate buffer:	0.17 M KH_2PO_4, 0.72 M K_2HPO_4

Kanamycin solution, 50 mg/ml in 1 M Tris-HCl pH 8.0, must be freshly prepared and added to final concentration of 50 µg/ml.

2.8.3. Culturing of bacteria

Bacteria stored in glycerol stocks were spread on an agar plate and incubated at 37˚C overnight. A single colony was picked, transferred into 2 ml medium and incubated at 37˚C for 12 h. This culture was used for inoculation of larger cultures.

2.8.4. Preparation of competent bacterial cells

Rubidium Chloride Method:

Nova Blue cells from an agar plate or frozen stock were cultivated in 2 ml of LB-medium at 37˚C overnight. From this culture was started 100 ml of a new culture without antibiotics, which is incubated until the cell density at OD_{600} has reached 0.4. The cell suspension is cooled down on ice for 15 min. and centrifuged for 5 min. at 3000-x g. The pellet is resuspended in 40 ml Tfb I buffer and left to stay for 15 min. on ice. After second centrifugation for 5 min., at 3000 x g, the pellet is resuspended in 4 ml Tfb II buffer, and left to stay on ice for another 15 min. The final suspension is aliquoted in chilled Eppendorf tubes (100 µl) and frozen in liquid Nitrogen. The competent cells are stored at -80˚C.

Tfb I buffer: 30 mM potassium acetat
100 mM rubidium chloride
10 mM calcium chloride
50 mM manganese chloride
15% v/v glycerol
Add dH_2O to 200 ml

Adjust the pH 5.8 with 0.1 M acetic acid.
Sterilize by filtration.

Tfb II buffer: 10 mM MOPS
75 mM calcium chloride
10 mM rubidium chloride
15% v/v glycerol
Add dH_2O to 100 ml

Adjust the pH 6.5 with KOH.
Sterilize by filtration.

2.8.5. Transformation of competent E.coli cells

E.coli cells were transformed applying the method of Hanahan, 1983. Competent cells are thawed on ice and 1 µl of a ligation mixture (10-100 ng DNA) is added. The cells are incubated for 30 min. on ice following by a heat shock for 45 sec at 42˚C, 300 µl SOC medium is added and the mixture is cooled immediately on ice for 2-3 min. The cell suspension is incubated on a shaker (150 rpm) at 37 ˚C for 45 min. Then 100 ml of transformed cells are streaked on agarose plates containing kanamycin and incubated overnight at 37˚C.

2.8.6. Storage of bacterial cultures

10 µl bacterial suspension is spread over an agar plate and incubated at 37˚C overnight until colonies appear. Such a plate can be kept 4 to 6 weeks at 4˚C.

For long-term storage, 50 ml of LB-medium are cultivated with single bacterial colony and shaken (150 rpm) at 37˚C until the suspension reached a cell density of OD_{550} of 1.5-1.7 (LKB Novaspec). The cells are harvested by centrifugation at 3000 rpm (Megafuge, T. Heraeus) for 10 min. The cell pellet is resuspended in 2 ml of LB-medium, mixed with 2 ml of sterilized glycerol (87%), and stored in screw-cap glass vials. This culture can be kept at -20˚C for several years.

2.9. Molecular biological methods

2.9.1. Generation of IL-4 ligand

The cDNA encoding for mature human IL-4 (UniProtKB/Swissprot P05112) plus an N-terminal methionine was inserted into the vector pQKA using restriction sites for the endonucleases Xho I and BamHI. Prof. Dr. Walter Sebald provided the expression construct.
For DNA amplification, the plasmid was transformed into the E.coli strain. Transformed bacteria were cultured in LB medium and transferred to agar plates containing the appropriate antibiotics. Mini-preparations of 5 – 10 colonies were performed and the plasmids DNAs were tested for the correct DNA sequence. Positive clones were subjected to DNA maxi preparations, DNA obtained this way was transformed into bacteria of the E. coli strain BLR (DE3) (EMD4Biosciences) and stored as glycerol stocks at -20˚C as described in section 2.8.6.

2.9.2. Ethanol precipitation of DNA

DNA in solutions is precipitated by adding 1/10 volumes of 3 M NaOAc pH 8.0 and 2.5 volumes of ethanol. The mixture is thoroughly mixed, incubated for 30 min. at -20˚C and then centrifuged (15 min., 14000 rpm, JA-14 rotor, Beckman). The DNA pellet is washed with 70% EtOH; the ethanol is removed and dried under vacuum. The pellet is then dissolved in the desired volume sterile dH_2O or TE buffer.

2.9.3. DNA molecular standards

The following DNA molecular weight standards were used throughout this work: GeneRuler™ 1 kb DNA Ladder and GeneRuler™ 100 bp DNA Ladder.

Table 2.5: DNA molecular weight standards

1 kb DNA Ladder			**100 bp DNA Ladder**		
bp	ng/0.5 µg	%	bp	ng/0.5 µg	%
10000	30.0	6.0	1000	45.0	9.0
8000	30.0	6.0	900	45.0	9.0
6000	70.0	14.0	800	45.0	9.0
5000	30.0	6.0	700	45.0	9.0
4000	30.0	6.0	600	45.0	9.0
3500	30.0	6.0	500	115.0	23.0
3000	70.0	14.0	400	40.0	8.0
2500	25.0	5.0	300	40.0	8.0
2000	25.0	5.0	200	40.0	8.0
1500	25.0	5.0	100	40.0	8.0
1000	60.0	12.0	/	/	/
750	25.0	5.0	/	/	/
500	25.0	5.0	/	/	/
250	25.0	5.0	/	/	/

2.9.4. DNA agarose gel electrophoresis

(Hermann et al., 1980)

To analyze PCR reactions and to purify DNA preparations, agarose gel electrophoresis was performed. To do so, 1 µg/ml ethidium bromide is added to 1% (w/v) agarose gel buffered with TAE buffer (see below). DNA samples are mixed with DNA loading buffer (Gel Loading Dye, Blue (6x), NEB) and applied to the gel. According to the grade of DNA separation, a current of 150 mA is applied for approximately 20-40 min. in TAE buffer. DNA bands are visualized using an UV illuminator and recorded using the GenoTools analysis software (Syngene).

TAE buffer: 40 mM Tris-HCl pH 8.0
20 mM Acetic acid
1 mM EDTA

Table 2.6: DNA agarose gel electrophoresis

Length of DNA fragments [kbp]	Agarose concentration in (w/v) %
5-60	0.3
1-20	0.6
0.8-10	0.7
0.4-7	1.0
0.2-4	1.5
0.1-3	2.0

2.9.5. Purification of DNA fragments from agarose gel electrophoresis

DNA fragments were purified from agarose gels using the Nukleotrap™ kit (Macherey-Nagel). Briefly, a preparative agarose gel is run and the DNA fragment of interest is excided under UV light. A slice of 330-350 mg of agarose gel containing the desired DNA is then transferred into an eppendorf tube, and 300 µl of buffer T1 are added per 100 mg of agarose. The mixture is

incubated at 55˚C for 10 min. together with 20 µl of ‘’Nucleotrap’’ glass beads and mixed several times during incubation. The sample is then centrifuged for 30 sec at 13000 rpm (Eppendorf Microcentrifuge) and the supernatant is removed. The glass pellet is consecutively washed twice using 500 µl of T2 buffer and twice with T3 buffer. Then the pellet is dried and the bound DNA is eluted using 50 µl of TE buffer pH 8.0 and incubating the pellet for 10 min. at RT. The glass beads are removed by centrifugation for 1 min. at 13000 rpm (Eppendorf Microcentrifuge). The DNA solution is analyzed on an agarose gel.

2.9.6. Site-directed mutagenesis by PCR

Apparatus: DNA Termocycler (Eppendorf Mastercycler Personal)
10 mM dNTPs
Pfu polymerase
10x Cloned Pfu buffer: 200 mM Tris-HCl pH 8.8, 20 mM $MgSO_4$, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 1% Triton X-100, 1 mg/ml Nuclease-free BSA

Several IL-4 variants were obtained using the two-step mutagenesis protocol. The first step was performed in two different reactions – reaction 1a and reaction 1b. In reaction 1a (Table 2.7) the 5’- primer carries the mutation of interest and a primer annealing at the external 3’- end is used (Table 2.4 oligonucleotides). For reaction 1b the 3’- primer carries the desired mutation and is complementary to the 5’- primer in reaction 1a. The 5’- primer in reaction 1b is external.

Table 2.7: PCR-reaction 1a and reaction 1b

	Reaction 1a	**Reaction 1b**
Template	10-20 ng	10-20 ng
Mutation-carrying primer	5 µM (5’- primer)	5 µM (3’- primer)
External primer	5 µM (3’- primer)	5 µM (5’- primer)
dNTPs	2.5 mM/nucleotide	2.5 mM/nucleotide
10xPfu polymerase buffer	5 µl	5 µl
Pfu polymerase	2.5 U	2.5 U
H_2O	To 50 µl	To 50 µl

The products synthesized from reaction 1a and 1b were analyzed by DNA agarose gel electrophoresis and directly used as a template for the second step of the PCR generated mutagenesis. The recombinant PCR was performed using the two external primers, which were used in the first partial PCR reaction: the 3'- primer from reaction 1a and the 5'- primer from reaction 1b (Table 2.8).

Table 2.8: Second step of the PCR generated mutagenesis

	Reaction 2
Reaction product 1a	1 µl
Reaction product 1b	1 µl
5'- primer (from reaction 1b)	5 µM
3'- primer (from reaction 1a)	5 µM
dNTPs	2.5 mM/nucleotide
10xPfu polumerase buffer	5 µl
Pfu polymerase	2.5 U
H_2O	To 50 µl

All reactions were performed in safe-lock Eppendorf tubes with the following program:

Denaturation:	95˚C	5 min.	
	95˚C	30 sec	
	54˚C	30 sec	Repeat 25 x from step 2 to step 4
	72˚C	2 min.	
Extension:	72˚C	4 min.	
End:	4˚C	∞ min.	

The full-length product, synthesized in the second PCR, contains the desired mutation. After visualization on agarose gel, the DNA fragment was precipitated with EtOH and the DNA pellet was dissolved in 20 µl of H_2O.

2.9.7. Restriction analysis of DNA

The restriction reactions were performed in the presence of the recommended 10 x reaction buffer. For analytical purposes 100-500 ng DNA are digested in a reaction volume of 10 µl, using 1-10 U restriction endonucleases. The reaction mixture is incubated for 1 h at 37˚C, and for analysis, 2-4 µl are examined by agarose gel electrophoresis.

The reaction volume of preparative digestion is dependent on the amount of the used DNA; in our case we used 2.75 µg/ml. It is necessary to consider that the standard enzyme solutions normally contain 50% glycerol. Some restriction endonucleases work non-specifically in the presence of too high glycerol concentrations (‘’star activity’’). Because of this, the glycerol content in the reaction volume should not be more than 10%. Every kind of DNA, independent on the number of restriction sites, was digested with 1-5 U of restriction endonuclease per µg DNA. (1 enzyme unit (U) is defined as the amount of the restriction endonuclease, which is able to digest 1 µg of DNA for 1 h.) The incubation was done – if it is not mentioned otherwise – for 3 h at 37˚C. A small aliquot was examined on an agarose gel. The rest of the DNA was purified by agarose gel electrophoresis, before it was used for ligation reaction.

2.9.8. Ligation of DNA

T4 DNA ligase

10x T4 DNA ligase buffer: 500 mM Tris-HCl pH 7.6, 100 mM $MgCl_2$, 10 mM ATP, 10 mM DTT

A vector: insert molar ration of 1: 3 was determined to be optimal. The total amount of DNA for cloning per 10 µl reaction volume was 200 ng. The reaction mix, containing 1-5 U of T4 DNA ligase is incubated for 30 min. at RT or for 12-16 h at 15˚C. The reaction is then stopped by heating at 65˚C for 10 min. 25% from the ligation mix are loaded on an agarose gel to examine the reaction efficiency. The recombinant plasmid DNA (1 µl of the ligation mixture) is then directly used to transform competent E.coli.

2.9.9. DNA sequencing

DNA sequencing was made by AGOWA GmbH, Berlin, Germany.

DNA Sequencing instrument used:
Applied Biosystems 3730xl and 3700 sequencers

Sample requirements:
200 ng/µl plasmid (10 µl minimum)
5 µM seq. primer QKA Forward

Lalign was used as a sequence analysis program.

2.10. Protein chemical methods

2.10.1. Determination of the protein concentration

The concentration of a protein solution can be measured by spectrophotometry at the absorption spectrum range 250-320 nm (CARY 50 Bio UV-visible Spectrometer, Varion). The absorbance value A at 280 nm was used for calculation. The concentration of IL-4 and IL-4BP per absorbance unit (A_{280}=1) and the path length equal to 1 cm are summarized both in the Table 2.9.

Lambert-Beer law: $E_{280nm} = \varepsilon_{280nm} \cdot c \cdot d = (\varepsilon_{280nm} \cdot c_m \cdot d) \cdot (Mw)^{-1}$

Table 2.9: Molar absorbance factor and concentration of Il-4 and IL-4BP

Protein	**Molar absorbance factor [$mol^{-1}*cm^{-1}$]**	**Concentration per absorbance unit 1 [mg/ml]**
IL-4	8610	1.7
IL-4BP	66930	0.357

2.10.2. Lyophilization of proteins

We divided the purified protein into aliquots of 100 µl each, placed and froze them in an aluminium block at -70˚C overnight. The frozen protein aliquots were then transferred to a Lyophilizator, which was pre-cooled to -55˚C and vacuum pumped to 0.02 bar overnight.

2.10.3. Molecular weight standards for protein samples

The molecular weight of the proteins was estimated by comparison of the protein samples with a protein standard marker, which was loaded in parallel to the samples on a SDS-PAGE. This was used to identify the size of proteins, loaded on the gel. The marker consisted of different molecular eight standards listed in the Table 2.10.

Table 2.10: Molecular weight standards for SDS-PAGE

Protein	**Molecular weight Mw [kDa]**	**Concentration [µg / 5 µl]**
Phosphorylase b	97	0.67
Albumin	66	0.83
Ovalbumin	45	1.47
Carboanhydrase	30	0.83
Trypsin-inhibitor	20.1	0.8
α-lactalbumin	14.4	1.16

2.10.4. SDS – Polyacrylamide gel electrophoresis

(Laemmli, U.K., 1970)

Polyacrilamyde gels were prepared accordingly to a protocol from Laemmli (Laemmli 1970) (see table 2.11). The listed amount is sufficient for two gels. SDS-PAGE was carried out using the Perfect Blue TM Vertical Electrophoresis System (PEQLAB).

Table 2.11: Components of a SDS polyacrylamide gel

Solution	Stacking gel [5%]	Separating gel [12%]
Acrylamide solution	0.5 ml	4.0 ml
4 x lower Tris	-	2.5 ml
4 x upper Tris	1.25 ml	-
ddH_2O	3.2 ml	1.5 ml
Glycerin	-	2.0 ml
TEMED	12 µl	14 µl
40% (w/v) APS	12 µl	14 µl

The separating gel solution is cast between two glass plates which are separated from each other by spacers. To avoid impurities the glass plates are cleaned before with 70% ethanol. 2 cm space from the upper edge of the plates remained free for the stacking gel. The separating gel is covered with a layer of dH_2O, and after polymerization, the dH_2O is poured out, and the stalking gel is filled on the top. A comb for samples is put into the stalking gel. After gel polymerization the gel is inserted into a gel chamber filled with 1 x SDS running buffer.

The protein samples are diluted with SDS sample buffer with a ratio of 1:1 and heated to 95˚C for 5 min. After loading the samples and the protein marker (see 2.10.3) as a control, protein separation is carried out at 40 mA per gel for approximately 50 min. (PHERO-stab. 500, Biotec-Fischer). Finally, proteins are stained either with coomassie blue (> 1 µg protein) or silver staining (< 1 µg protein) (see 2.10.5).

AA/BAA solution:	30% acrylamide 1% N.N methylenebisacrylamide stored at 4˚C after filtration
40 % (w/v) APS:	400 mg APS 1 ml ddH_2O stored at 4˚C
4 x lower Tris:	1.5 M Tris-HCl, pH 8.8 0.4% (w/v) SDS
4 x upper Tris:	0.5 M Tris-HCl, pH 6.8 0.4% (w/v) SDS
5x SDS running buffer:	125 mM Tris-HCl, pH 8.6 0.95 M glycine 0.75% (w/v) SDS
SDS sample buffer:	62.5 mM Tris-HCl, pH 6.8 2% (w/v) SDS 20% glycerin 2% (w/v) bromphenol blue (under reducing conditions: 2% (v/v) β-Mercaptoethanol is added)

2.10.5. Protein staining

Coomasie blue staining

At the end of the gel electrophoresis (see 2.10.4), the stacking gel is removed. The separating gel is incubated for 30 min. in staining solution, destained and treated with 30% methanol for 1 hour. Finally, the gel is packed between two cellophane films, fixed to a frame and dried at room temperature.

Staining solution: 10 g coomassie brilliant blue R25
0.4 l glacial acetic acid
1.6 l methanol
2.0 l dH_2O

Destaining solution: 0.5 l isopropanol
0.5 l glacial acetic acid
4.0 l dH_2O

Silver staining

At the end of the gel electrophoresis (see 2.10.4), the SDS separating gel is put into buffer 1 for 5 min. to fix the protein bands in the gel. Then, the gel is cleansed and incubated in dH_2O for 5 min. Subsequently, it is placed in buffer 2 for 5 min, followed by incubation in buffer 3 for 1 min. After washing three times with dH_2O for 5 sec, the gel is transferred to buffer 4 for 8 min. Thereafter, it is washed thoroughly with dH_2O (five times) and treated with buffer 5 until all protein bands appeared. To stop the staining reaction, the gel is rinsed with 1% glacial acetic acid and washed with dH_2O afterwards. Finally, the gel is treated with 30% methanol for 1 hour, packed in cellophane film, fixed to a frame and dried at room temperature.

Buffer 1: 60 ml 50% (v/v) acetone in dH_2O
1.5 ml 50% (w/v) trichloroacetic acid (TCA) in dH_2O
25 ml formaldehyde
Buffer 2: 60 ml 50% (v/v) acetone in dH_2O
Buffer 3: 100 µl 10% (w/v) sodium-thiosulphate-pentahydrate ($Na_2S_2O_3x5H_2O$) in dH_2O
60 ml dH_2O
Buffer 4: 800 µl 20% (w/v) silver nitrate ($AgNO_3$) in dH_2O (stored in dark)
600 µl formaldehyde
60 ml dH_2O

Buffer 5: 1.2 g sodium carbonate (Na_2CO_3)
25 µl formaldehyde
25 µl 10% (w/v) sodium thiosulphate pentahydrate ($Na_2S_2O_3x5H_2O$) in dH_2O
60 ml dH_2O

2.10.6. Mass spectrometry

Dr. Werner Schmitz determined the molecular weight for all recombinant proteins produced within this work using an Electrospray-Fourier-Transformation-Ion-Cyclotron-Resonance-Mass-Spectometer (ESI-FTICR-MS, Bruker).

2.11. Expression of recombinant proteins in E.coli

2.11.1. Expression of IL-4 and IL-4 muteins in E.coli

The entire production of IL-4 muteins was carried out in the lab of Prof. Dr. Walter Sebald together with Dr. Viswanadham Duppatla. After transformation of the cells and an overnight incubation, an individual clone was picked from the plate and incubated in 200 ml LB medium on a shaker (130 rpm; Cetromat RM, B.Braun) at 37˚C, overnight. This overnight culture was used for a large-scale fermentation.
6-12 shaking flasks containing 800 ml LB medium were inoculated each with 20 ml overnight culture and cultured at 37˚C, on a shaker (130 rpm) until the cell density at OD_{600} nm has reached ~0.6. The protein expression was induced with 1mM IPTG and incubated for another 3-4 h at 37˚C, on a shaker (130 rpm). The cells were harvested via centrifugation at 4˚C (15 min, 6000 rpm, J2-21, Beckman). The pellet was weighed and resuspended in 3x volumes of STE buffer with addition of 1mM DTT and stored in portions at -20˚C.

STE buffer: 10 mM Tris-HCl, pH 8.0
0.325 M sucrose
1 mM EDTA

2.11.2. Denaturation and renaturation of proteins

1.5 mg/m ww Lysozyme, 1 mM DTT and the additional STE buffer is added to the suspension, which is then sonicated with the cell suspension cooled on ice (150W Bandelin Sonopuls HD3200) for 10 min. (30 sec., 30 sec pause) 70% amplitude. The mixture is then centrifuged for 1 h, at 4˚C (12000 rpm, JA-14, Beckman) and the supernatant is discarded. The sonication process is repeated for additional two times, each time followed by centrifugation. The final pellet is resuspended in STE buffer with the addition of 1 mM DTT and 1% Triton X-100, separated in aliquots, centrifuged for 20 min., at 4˚C (14000 rpm, JA-14, Beckman), the weight of the pellet is measured and stored at -20˚C. The IL-4 protein was extracted from the inclusion bodies (IB) using a 20 volumes of GuCl extraction buffer. For refolding of the IL-4, the protein extract was diluted 25-fold with ice cold refolding buffer and kept at 4˚C for three days.
The solution was dialyzed twice against 10 volumes of PBS (150 mM sodium chloride, 10 mM sodium phosphate, pH 7.4) at 4˚C for 12 to 18 h.

GuCl Extraction buffer: 6 M GuCl
(20 volumes) 100 mM Tris-HCl, pH 8.0
add 1 mM DTT (10 volumes per gm wet weight)

Refolding buffer: 100 mM Tris-HCl
1 M Arginine
5 mM EDTA, pH 8.0 adjusted with conc. HCl
Add 5 mM GSSG (glutathione ox.)
Add 2 mM GSH (glutathione red.)

2.12. Expression of IL-4Rα [ECD] in CHO-S cells

2.12.1. Cultivation of CHO-S cells

CHO-S cells were kindly provided by Hanna Haliu, UCB, Celltech, Slough, UK. To prevent any kind of contamination, all experiments dealing with CHO-S cells are performed in sterile conditions. Glass pipettes, plastic materials and solutions are sterilized. Flasks and tubes, containing cells are opened only in a laminar flow hood. CHO-S cells are cultured in serum free CD-CHO Media containing Antimycotic, GlutaMax and HT supplements.
The old medium is exchanged with fresh medium every third day. The culture is incubated at 37˚C, 5% CO_2, 110 rpm.

2.12.2. Viability of CHO-S cells

Trypan blue 0.4% (w/v) solution in PBS

The trypan blue exclusion method is used to count the proportion of viable cells and their concentration in the suspension. Since the exclusion of the stain is a selectively working definition for viability, the dead cells appear as blue spots.
A small aliquot of cells is mixed with trypan blue solution at a ration 1:1 and kept at RT for 1 min. The viability of the CHO-S cells is measured by using of Vi-CELL XR, Beckman Coulter.

2.12.3. Suspension culture

Culture flasks: 25 cm²
75 cm²
175 cm²

CHO-S cells are subcultured when they are ~90% confluent. After the culture is examined for contaminations and cells floating in the medium, the old medium is discarded. A small aliquot of this suspension is stained with trypan blue. The cells are examined and counted. The suspension is diluted and the cells are seeded in a new flask with a density of 10 x 10^4 – 5.0 x 10^5 cells/cm² in a final volume of 5 ml (for 25 cm² flask), 15 ml (for 75 cm² flask) or 35 ml (for 175 cm² flask). A culture prepared with such cell density is ready for the next passage in 2-3 days. The flasks are incubated at 37˚C, 5% CO_2, 110 rpm.

2.12.4. Transfection with method of electroporation

For each 1 L of cells, 2 x 10^9 cells are spinned in conical falcon tubes, for 10 min. (1400 rpm, Megafuge, T. Heraeus). The pellet is resuspended into the Earls Balance Salt Solution EBSS, and transferred into 50 ml tubes, and then spinned again for 10 min. (1400 rpm, Megafuge, T. Heraeus). The supernatant is decanted.
Vector DNA should be above 3 mg/ml, and between 1.8 to 1.9 DNA/RNA ratio.
For transfection to yield 1 L culture, 4 mg DNA, 2 x 10^9 cells are required.

Cell pellet, DNA + x ml EBSS buffer = 9.6 ml Final volume

800 µl of cells/DNA mix are added to each cuvette (12 cuvettes/ 1 L culture). These 12 cuvettes are seeded into 1 L of CD-CHO media and left overnight at 37˚C.
24 hours later, the cells are counted. If the cells are above 2 x 10^6 cells/ml, then they are transferred for incubation at 32˚C.

Electrical parameters for 800 µl cuvette:
1 ms: 9.6 A
10 ms: 0 A
40 ms: 3.2 A
CD-CHO media has Antimycotic/Antibiotics (1:500 dilution), and also GlutaMax and HT supplements.
CHO-S cells are best zapped when cells are in log. phase, at around 2 – 4 x 10^6 cells/ml.

2.13. Purification of recombinant proteins

2.13.1. Purification of IL-4 and IL-4 muteins from E.coli

Buffer A: 25 mM NH_4OAc, pH 5.0
Buffer B: 25 mM NH_4OAc, 0.5 – 1.5 M NaCl, pH 5.0
Ion exchanger: CM-Sepharose fast flow
Column: ø 2.5 cm, l=10 cm, Econo Column™ (BioRad)

The pH of the dialyzed refolding solution was adjusted to 5.0 by adding 0.01 volumes of 4 M ammonium acetate, pH 5.0 and after centrifugation of the slightly turbid solution, the supernatant was loaded on the column together with 10 ml CM-Sepharose FastFlow resin (up to 1 l refolding solution), which was initially equilibrated with 25 mM ammonium acetate, pH 5.0. Flow rate was ca. 300 ml/h at hydrostatic pressure of 1 m. The elution was performed via a step gradient using 2 times 10 ml buffer containing between 0.5 and 1.5 M sodium chloride. The fractions (10 µl from each) were examined on a SDS-PAGE gel. The fractions containing the protein were combined and concentrated via ultrafiltration (cutoff 3 kDa). The protein concentration was measured at 280 nm (spectrophotometer) using buffer A as a blank and was further purified by the use of RP-HPLC.
The described procedure was performed at 4˚C.

2.13.1.1. Protein purification by RP-HPLC

Acetonitrile: Acetonitrile (Roth, HPLC Grade), filtered through a 22 µm filter
TFA: 0.1 % TFA (Merck) in H_2O, filtered through a 22 µm filter
Column: C4 column; 8 mm diameter, 250 mm length; 12.5 ml Vydac
Protein stock buffer: 12.5 ml of 4x upper Tris-HCl, 20 ml of 10 % SDS, 47.5 ml H_2O, 30 ml of glycerol mix

The protein containing fractions, collected during the ion exchange chromatography, were centrifuged at 4˚C, (15 min., 3000 rpm, J2-21 centrifuge, Beckman). The supernatant was injected into a C4 reversed phase HPLC column (Vydac 12.5 ml column volume), equilibrated with 0.1 % trifluoroacetic acid (TFA). The IL-4 was eluted by applying a gradient from 0.1 % TFA to acetonitrile with an increase from 20 to 80 % acetonitrile in 60 min. at a flow rate of 0.8 ml min^{-1}. A major peak was noticed at 25 to 30 % acetonitrile (Table 2.12). The protein concentration of the collected fractions was measured at the absorption spectrum range 250 – 320 nm.

Table 2.12: Acetonitrile gradient and retention time, used for RP-HPLC.

time	**%B 0.1%TFA**	**% C AN**	**Flow ml/min**
0	100	0	1.6
10	80	20	1.6
70	20	80	1.6
80	0	100	1.6
100	0	100	1.6

An aliquot (10 µl) from every fractions was mixed with 30 µl of protein buffer and 5 µl from this solution was analyzed by SDS-polyacrylamide gel electrophoresis. The fractions with the highest amount of purified protein were combined, aliquoted and lyophilized.

2.13.1.2. Chemical reduction of IL-4 muteins

IL-4 muteins that contain an engineered cysteine residue were dissolved in PE7 buffer at 50 mM concentration and incubated with 50, 100, or 150 µM dithiothreitol (DTT) at 20˚C for 24 h. 0.01 volumes of 100 mM TMS(PEG)12 dissolved in DMSO were added for 30 min. The PEGylation reaction was halted by adding 0.02 volumes of 250 mM glutathione (reduced form). After mixing with an equal volume of 2x SDS sample buffer, 5 or 10 µl were submitted to SDS PAGE analysis.

PE7 buffer: 0.1 M potassium phosphate, pH 7.0
2 mM EDTA

2.13.1.3. Reaction with N-ethylmaleimide or TMS (PEG) 12

Reaction at preparative scale was performed at a 3-fold molar excess in PE7 buffer at 20˚C for 30 min. The solution was mixed with 0.1 % TFA to a final volume of 5 ml and loaded on a C4 reversed phase HPLC column (Vydac 214TP54, 250 x 4.6 mm) equilibrated with 0.11 % TFA. The modified protein was purified by applying an acetonitrile gradient running from 20 to 80 % acetonitrile in 60 min. and using a flow rate of 0.8 ml min^{-1}. Protein containing fractions were pooled and freeze-dried. The conjugated IL-4 protein was dissolved in water at concentrations of about 100 µM and stored at -20˚C.

2.13.1.4. Enzymatic reduction of IL-4 cysteine muteins with GRX1 from E.coli

The redox-reaction was started by adding 0.01 volumes of a 300 µM glutaredoxin solution prepared according to the manufacturer's recommendation. The progress of the reaction was monitored by the oxidation of NADPH, which can be measured by the decrease in the extinction at 340 nm. After 30 min., 0.025 volumes of 4 M ammonium acetate pH 5.0 were added and the mixture was immediately loaded on a 1 ml SOURCE 15S column (GE Healthcare) equilibrated with 25 mM ammonium acetate pH 5.0. The IL-4 protein was eluted via a salt gradient increasing from 0.5 M to 1.5 M sodium chloride in 60 min. at a flow rate of 0.5 ml min^{-1}. The protein containing fractions from the ion exchange chromatography step (containing about 0.75 M NaCl) were immediately loaded onto a C4 reversed phase HPLC column (Vydac 214TP54, 250 x 4.6 mm) equilibrated with 0.1 % TFA. The IL-4 protein was eluted via a gradient increasing from 20 to 80 % acetonitrile in 60 min. at a flow rate of 0.8 ml min^{-1}. IL-4 protein containing fractions were pooled, freeze-dried and dissolved in water at a final concentration of about 100 µM.

2.13.2. Purification of IL-4Rα [ECD] from CHO-S cells

2.13.2.1. Ni-NTA purification

Buffer A: 50 mM NaH_2PO_4
300 mM NaCl
20 mM Imidazole
pH 8.0

Buffer B: 50 mM NaH_2PO_4
300 mM NaCl
250 mM Imidazole
pH 8.0

Column size: 7 ml
The sample was concentrated up to 30 ml using Amicon 30K, and then diluted 1:10 into Buffer A. 5 ml Ni-NTA resin (10 ml solution) was washed with Buffer A (~40 ml/wash), for 3 times. The diluted sample was added to the beads and tumble for 2 hours, at 4˚C.
The sample-beads solution was centrifuged at 4˚C, (10 min, 1400 rpm, Megafuge, T. Heraeus) and the flow through FT was collected. The column was packed with the beads and equilibrated with 7 column volumes of Buffer A. The AKTA system was set up for a gradient elution with Buffer B. Fraction size of 3 ml were collected.

2.13.2.2. Buffer exchange

The fractions containing the desired protein were collected. Amicon 10K was equilibrated with PBS, and the collected fractions were applied into the Amicon. The buffer exchange was made with PBS for 3 times at 4˚C (15 ml, 1400 rpm, Megafuge, T. Heraeus).

2.13.2.3. TEV protease cleavage

Buffer A: 50 mM NaH_2PO_4
300 mM NaCl
20 mM Imidazole
pH 8.0

C (TEV) = 4 mg/ml
1:100 TEV was added to the collected fractions and incubated for 2 hours, at room temperature. The Ni-NTA batch was used to remove the entire TEV-tag. The protein sample was concentrated up to V=2 ml.
0.5 ml beads were washed (1 ml solution) with Buffer A. The column was equilibrated for 3 times with Buffer A, and the sample was added. The flow through FT was collected.

2.13.2.4. Desalting of protein sample

Equilibration and elution Buffer: 50 mM $Na_2HPO_4 \times H_2O$
300 mM NaCl
pH 8.0

PD-10 columns were used for desalting of the sample (Amersham Biosciences).
The column was filled with equilibration buffer and allowed for the equilibration buffer to enter the packed bed completely. It was repeated for 4 times (~25 ml equilibration buffer in total) and the flow through was discarded.
Maximum 2.5 ml of sample were added to the column and the sample was left to enter the packed bed completely. The flow through was discarded, and the elution was made with 3.5 ml buffer. The elute fractions were collected.

2.14. Electrospray ionization mass spectrometry analysis (ESI-MS)

ESI-MS was performed using an APEX-II FT-ICR (Bruker Daltonic GmBH, Bremen) equipped with a 7.4 T magnet and an Apollo ESI ion source in positive mode. The proteins were desalted by C4 reversed phase HPLC (see 2.13.1.1) and were dissolved in methanol/water/acetic acid (49.5/49.5/1) to yield a sample concentration between 1 – 5 μM. The sample was injected using a Hamilton syringe at a speed of 2 μL per minute with a capillary voltage of 360 mV. Detection range was set to 300 – 3000 m/z in initial measurements. The detection range was optimized to the signal-containing area. An accumulation of 256 scans was combined at a resolution of 256 K. For evaluation, the mass spectra were deconvolved to the single protonated ion mode using the Bruke Xmas software. The most intense isotope was selected for mass determination.

2.15. Analysis of protein-protein interactions by BIAcore technology

The analysis of kinetic and thermodynamic data of protein-protein interactions between the extracellular domain of IL-4 receptor and its mutated variants with the ligand IL-4 was performed with a BIAcore 2000 (GE Healthcare, Freiburg, Germany). BIAcore 2000 is an instrument, based on the method of surface plasmon resonance (SPR) in order to measure the interactions between the biomolecules in real time [Karlsson, R. & Fealt, A, 1997].

One reactant (IL-4Rα [ECD]) is immobilized on the dextran surface of a sensor chip and a solution containing the binding partner - analyte (IL-4 or IL-4 muteins) flows continuously over the chip.

The sensor chip consists of a glass slide coated with a thin gold film to which is attached, by an inert linker layer, a dextran matrix onto which the mentioned reactant can be immobilized using well-defined chemistry.

The sensor chip forms one wall of a micro-flow cell where its matrix-covered side comes into contact with the solution containing the second reactant. During the interaction, light passing a prism is focused onto the gold surface of the sensor chip through the glass, and reflected light is monitored. Evanescent wave photons produced by the incident polarized light interact with free oscillating electrons (plasmons) in the gold surface. Resonance occurs at a critical angle of the incident light, and light energy is transferred to electrons in the metal film surface, causing a minimum in the reflected light. This angle depends on the refractive index at, or close to the metal surface opposite to where the light is focused. By measuring small changes in refractive index, the instrument monitors the change in mass as a ligand binds to, or dissociates from, its binding partner. Data are presented as sensograms that show the change in resonance units (RU) versus time. For proteins, which have a refractive index increment of approximately 0.18, a signal of 1000 RU is equivalent to a surface concentration of 1 ng/ mm².

The association is monitored when a sample is injected and binding occurs to the immobilized interactant. After sample injection, buffer alone flows over the sensor surface and dissociation parameters can be recorded. At the end of the experiment, the surface can be regenerated using suitable reagents to remove remaining bound analyte without denaturing the immobilized reaction partner and the chip can be used for a new cycle of measurements [Nice, E.C. & Catimel, B. 1999].

2.15.1. Immobilization of proteins by streptavidin-biotin coupling

After streptavidin was covalently immobilized onto the surface of a sensor chip, the biotinylated protein was bound to the prepared matrix.

Sensor chip:	CM5
EDC:	50 mM N-ethyl-N'-(dimethylaminopropyl) carbodiimide
NHS:	200 mM N-hydroxysuccinimide
HBS buffer:	10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005 % surfactant P20

Streptavidin solution: 100 µg/ml streptavidin in 10 mM NaOAc, pH 4.5
Regeneration buffer: 100 mM HOAc, 1 M NaCl, pH 3.0

The immobilization and preparation of a sensor chip was carried out according to the manufacturer's instructions [BIAcore Handbook, 1995]. The immobilization of streptavidin was run automatically following the program shown in Table 2.13.

Table 2.13: Immobilization of streptavidin by amine coupling

Immobilization procedure	
Continuously flow buffer HBS	Flow rate 5 µl/min
EDC/NHS (50 mM/200mM)	35 µl (7 min)
Streptavidin (100 µg/ml)	35 µl (7 min)
1M ethanolamine-HCl	35 µl (7 min)

EZ-Link Maleimide-PEG2-Biotin labelling of 121SH062711

IL-4 121SH062711 Fr. 33/36 102 µM	200 µl	= 300µg = 20 nmol = 50 µM
5xPE7	80 µl	
H_2O	114 µl	
EZ-Link Maleimide-PEG2-Biotin (Thermo Scientific) (10 mM in DMSO; Mr 565,62; 2 mg in 354 µl DMSO)	6 µl	= 60 nmol = 150 µM
	400 µl	

Kept for 30 min. at room temperature, 10 µl 4 M Ammonium acetate pH 5.0 was added, and then 25 mM Ammonium acetate was added to a volume of 2 ml. The sample was concentrated to a volume of 0.1 ml using Centrikon and then diluted with HBS to a volume of 2 ml. The concentrating step was repeated for 2 more times, and UV spectrum at Ab 320 -250 nm was runned.

Biotinylated IL-4Rα [ECD] [Shen, B.J et al., 1996] was immobilized on flow cell 2 and Maleimide-PEG2-Biotin labeled IL-4 121SH was immobilized on flow cell 3. The concentration of biotinylated proteins used for immobilization was usually 0.1 µg/ml.

2.15.2. Measuring and evaluation of protein-protein interactions

A typical sensogram recorded with BIAcore 2000 is characterized by the following three phases:

1. Association phase – when the sample is injected; increases in the signal correspond to binding to the immobilized interactant.
2. The signal reaches a constant plateau when equilibrium between the association and dissociation is reached.
3. Dissociation phase – occurs when buffer alone flows over the sensor surface. The decrease in signal reflects dissociation of analyte from the surface-bound complex.

Kinetic rate constants can be derived from the association and dissociation phases of the sensogram. The height of the plateau represents the thermodynamic affinity of binding.
The program BIAevaluation 2.0 was used to calculate the kinetic and thermodynamic constants. The theoretical equations, which describe the kinetics and equilibrium of interactions in real-time BIA, are listed in Appendix 1.

2.16. Foldamers

2.16.1. Synthesis and purification of foldamers

Foldamers were synthesized by Michael Grotz and Dr. Michael Deligny at UCB Pharma S.A., Belgium. The backbone of the foldamers is made of quinoline moiety, connected with amide groups (procedure developed in I. Huc's lab). Chemically diverse side chains are attached to the 4th carbon of the quinolone ring. The choice of the side chains is decided based on the geometry and chemistry of the interaction site of IL-4 with its receptor protein.

Faced to a very challenging chemistry on solid support, they investigated two successive strategies, which yielded two libraries of respectively 11 and 46 foldamers of six units each. One of the strategies was inspired from strategy described by Baptiste Benoit [Baptiste, B. et al., 2010].

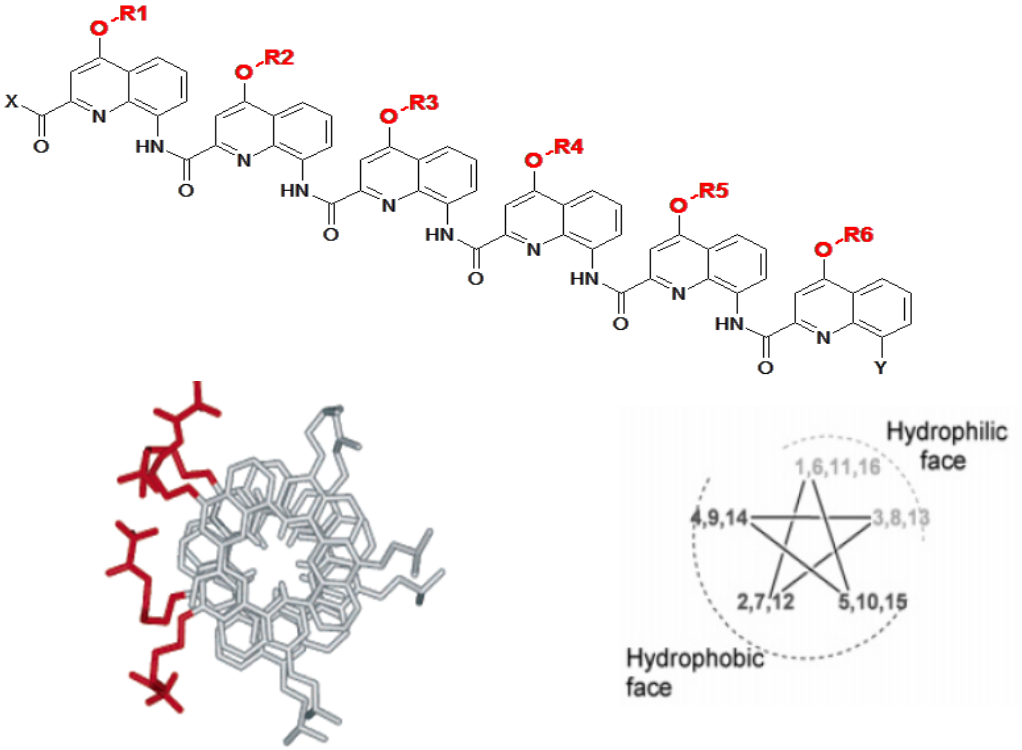


Figure 2.1: Different representations of helical oligomers of quinolines showing the side chains spread out around the helical structure.

The side chains are chosen to mimic the typical interaction of natural amino acids (hydrophobic, HBA, HBD, ionic) and to allow acceptable solubility of such folded structures. The best compromise for the length of these foldamers is fixed to 6 units, meaning more than one turn (Figure 2.2).

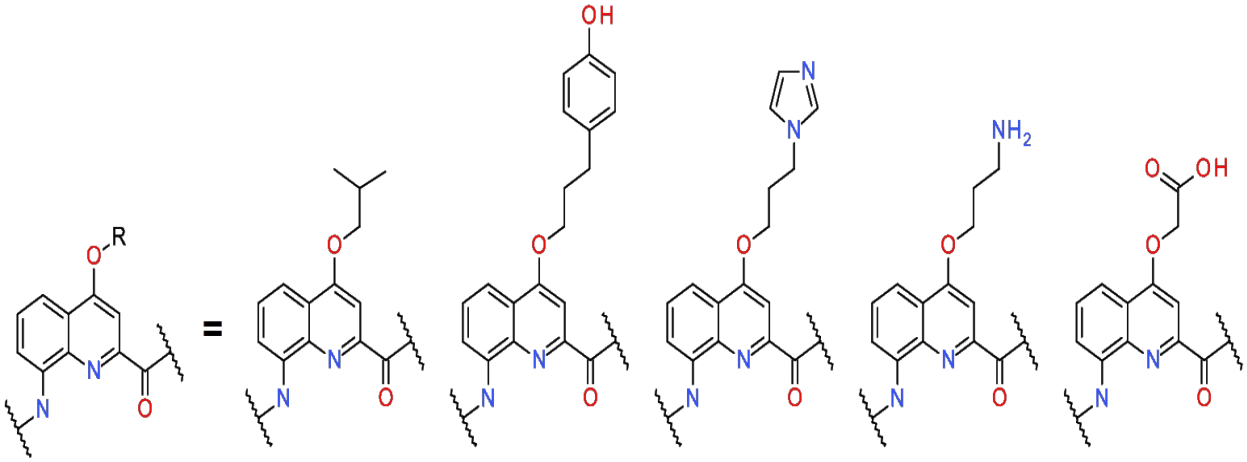


Figure 2.2: Different side chains to mimic the typical interaction of natural amino acids and enhance the solubility of the foldamers.

Resins are evaluated in terms of functionalities, linkers, nature of the polymer, final functions on the foldamer. In the first strategy, Sieber resin gave access to amides after cleavage. On the second one, the most straightforward low loaded Wang resin appeared to be the best compromise (Figure 2.3).

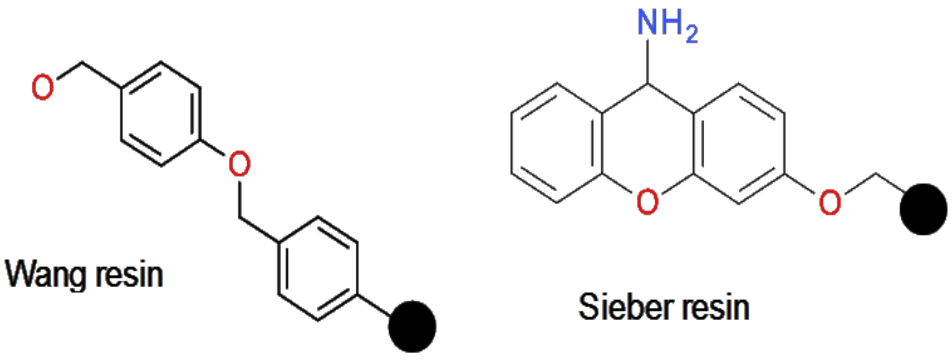


Figure 2.3: Resins involved in synthesis of the foldamers.

The typical procedure for quinolone synthesis is performed on a multigram scale.

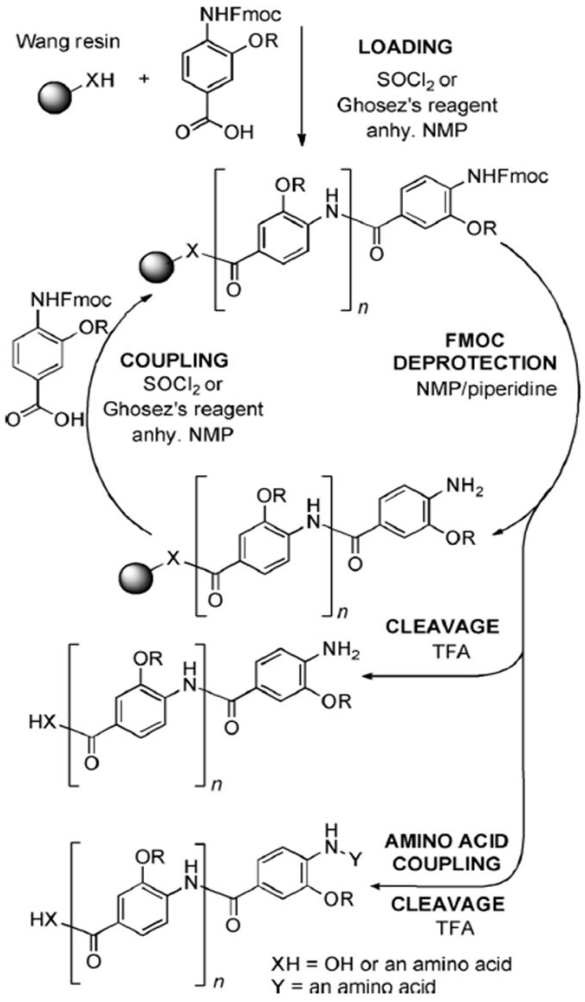


Figure 2.4: Scheme of a solid-phase synthesis protocol.

The library was synthesized in a Trident External Agitation-Thermal unit with an Automatic solvent wash module. This equipment offered a full control of temperature, agitation and solvent/reagent delivery in cassette holding up to 48 glass-made reaction vessels. This equipment enabled coupling steps of the whole library in one cassette, at stable temperature (typically 50˚C) in controlled atmosphere.

Reverse phase acid LC/MS was used for purification of foldamers and HMRS and NMR characterized them.

3. Results

3.1. Preparation of IL-4 and IL-4 mutant variants

It was the main goal of this project to generate IL-4 analogues disulfide-bonded to a foldamer at the binding sites for the IL-4Rα. As a second goal IL-4 analogues should be prepared with site-directed chemical modification at the binding epitope for the γc receptor chain in order to disrupt receptor signaling. To this end, IL-4 muteins were produced with cysteine substitution at position at the receptor binding epitope, for the IL-4Rα and γc chains (as shown in the space-filling model of IL-4 in Figure 3.1.

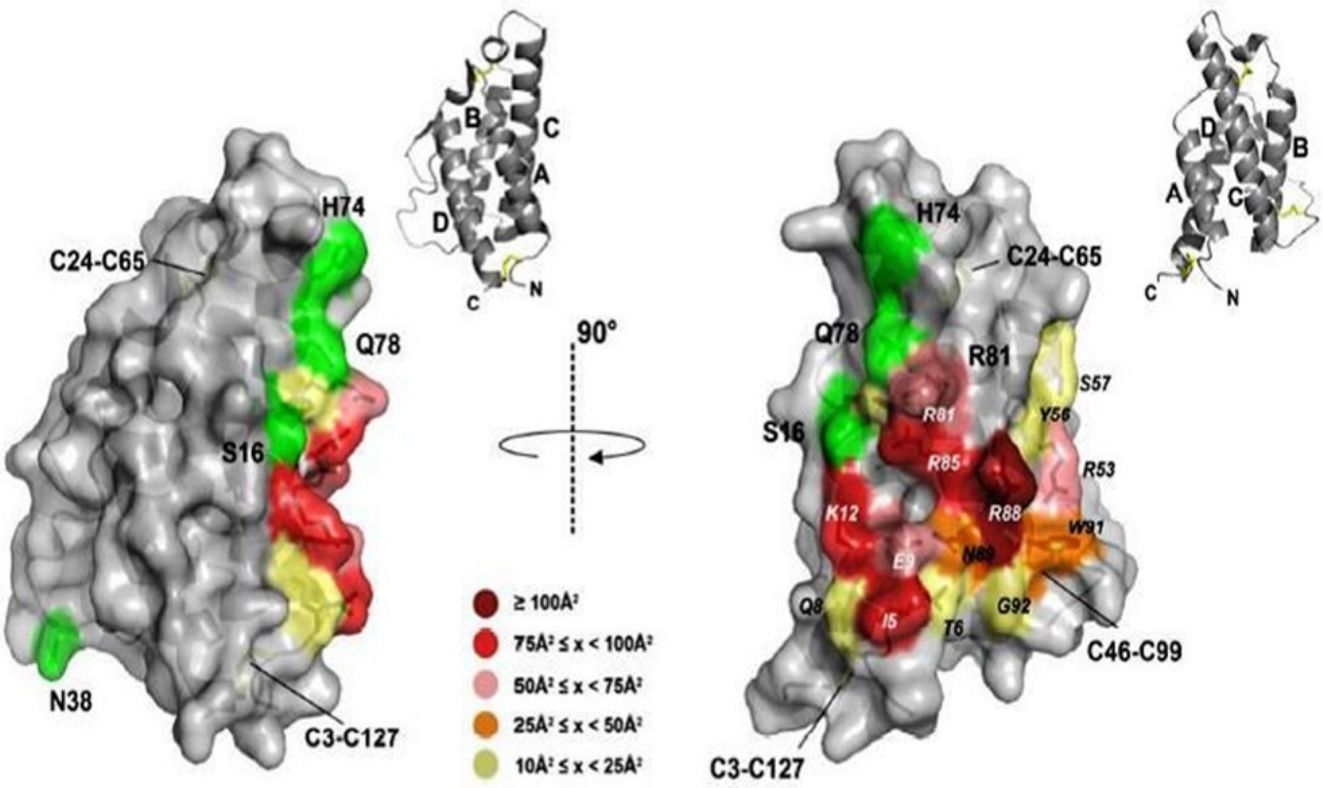


Figure 3.1: Sites S16, N38, Q78, and R81 chosen for engineering an unpaired cysteine residue into human IL-4 are indicated in green. The area of IL-4 surface residues buried in the contact with IL-4Rα (PDB entry 1IAR, chain A) (marked in italic) are color coded as indicated in the displayed scheme. Small ribbon plot panels on the upper right indicate the orientation of IL-4. Positions of the native disulfide bonds are marked.

The sites were selected on the basis of molecular modeling of possible Foldamer / IL-4 complexes (Figure 3.2).

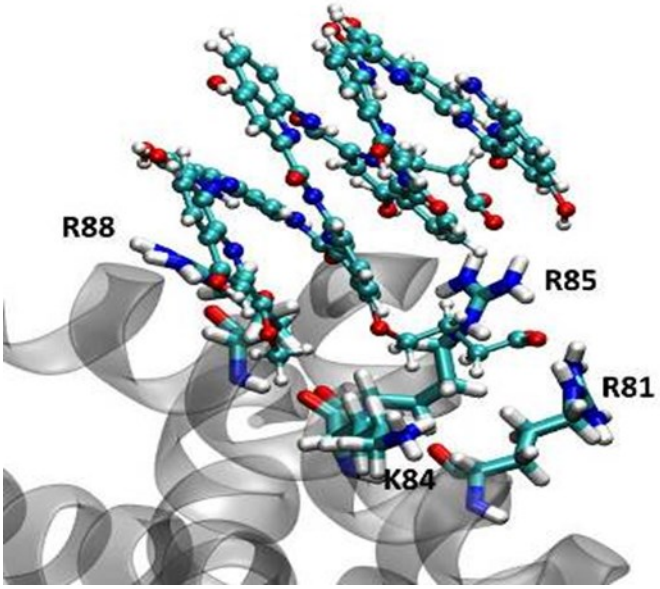


Figure 3.2: IL-4 interacting with Quinoline-Based Foldamer as obtained from the MD simulation after 10ns (data obtained from Chayan Acharya, Michel Laguerre, University of Bordeaux).

Asn38 is located far away from receptor binding epitope responsible to bind to IL-4Rα, therefore N38C has been prepared and used as a control. Asn38 is glycosylated in native IL-4. Two sets of IL-4 variants were prepared. Set I variants were prepared for the IL-4Rα chain. The second set of IL-4 variants was prepared with cysteine residues introduced in the IL-4 binding epitope for the γc receptor chain. The objective was to explore in how far the specific chemical modification can disrupt protein – protein interactions. All variants of IL-4, which were constructed by specific substitution of a single amino acid, are shown in Table 3.1.

Table 3.1: IL-4 variants (Set I and Set II) designed by site-specific mutagenesis.

IL-4 variants for IL-4Rα chain (Set I)	IL-4 variants for γc chain (Set II)
Ser 16	Ile 11
His 74	Arg 121
Gln 78	Tyr 124
Arg 81	
Asn 38	

3.1.1. Cloning of IL-4 mutant variants (set I and set II)

The complete nucleotide and amino acid sequence of the human IL-4 was obtained from P05112 UniProtKB/Swiss-Prot. To allow bacterial expression, a methionine residue was added at the N-terminal end. The possible mutant variants are outlined (Figure 3.3).

```
115     atgcacaagtgcgatatcaccttacaggagatcatcaaaactttgaacagcctcacagag
         M  H  K  C  D  I  T  L  Q  E  I  I  K  T  L  N  S  L  T  E

175     cagaagactctgtgcaccgagttgaccgtaacagacatctttgctgcctccaagaacaca
         Q  K  T  L  C  T  E  L  T  V  T  D  I  F  A  A  S  K  N  T

235     actgagaaggaaaccttctgcagggctgcgactgtgctccggcagttctacagccaccat
         T  E  K  E  T  F  C  R  A  A  T  V  L  R  Q  F  Y  S  H  H

295     gagaaggacactcgctgcctgggtgcgactgcacagcagttcacaggcacaagcagctg
         E  K  D  T  R  C  L  G  A  T  A  Q  Q  F  H  R  H  K  Q  L

355     atccgattcctgaaacggctcgacaggaacctctggggcctggcgggcttgaattcctgt
         I  R  F  L  K  R  L  D  R  N  L  W  G  L  A  G  L  N  S  C

415     cctgtgaaggaagccaaccagagtacgttggaaaacttcttggaaaggctaaagacgatc
         P  V  K  E  A  N  Q  S  T  L  E  N  F  L  E  R  L  K  T  I

475     atgagagagaaatattcaaagtgttcgagctgataa
         M  R  E  K  Y  S  K  C  S  S  -  -
```

Figure 3.3: Protein/DNA sequence for wild type human IL-4.

For expression in bacteria, the cDNA of human IL-4 was inserted into the expression vector QKA using Xho I and BamHI restriction sites (Figure 3.4).

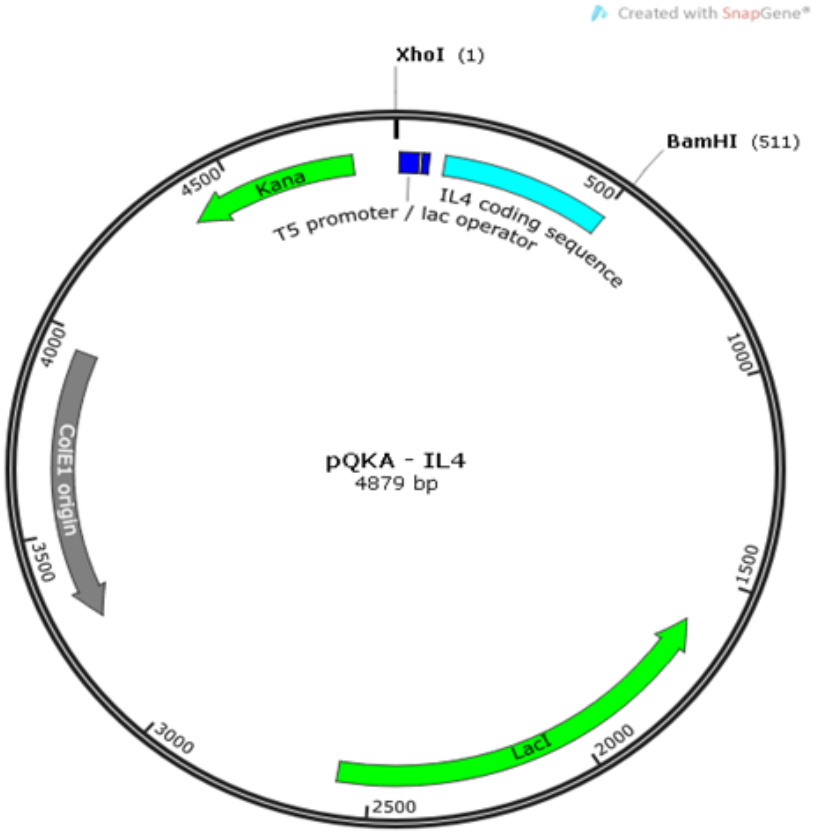


Fig.3.4: Schematic diagram of expression vector pQKA-IL4.

3.1.2. Mutagenesis

Some of the IL-4 variants were obtained by usage of Polymerase Chain Reaction (PCR), which relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers containing the desired mutation, along with a DNA polymerase are key components to enable selective and repeated amplification. During this reaction, a product of ~5000 bp was synthesized (Figure 3.5 A).

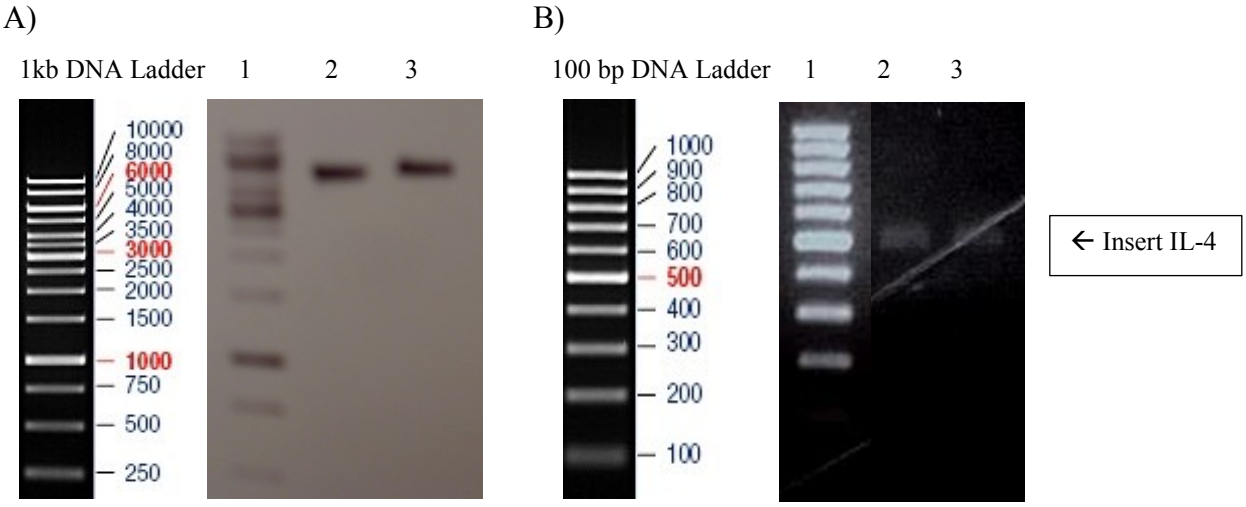


Figure 3.5: PCR product (A) lane 1 - 1kb DNA Ladder (see Table 2.4); lane 2 and 3 - pQKA-IL4 ~5000 bp; (B) lane 1 - 100kb DNA Ladder (see Table 2.4); lane 2 and 3 - XhO I / BamHI digestion – insert IL-4 ~500bp

The PCR reaction product was then transformed into Nova Blue™ cells. Plasmid DNAs from few bacterial colonies representing all the variants were isolated for analytical application. The intact circular plasmids were digested with restriction enzymes XhoI and BamHI to confirm the presence of the insert, and all analyzed plasmid samples after separation on an agarose gel, showed the ~500 bp fragment representing the human IL-4 cDNA (Figure 3.5 B). In addition, the exact DNA sequence and the presence of the mutation were verified by DNA sequencing with QKA forward primer.

3.1.3. Expression of IL-4 variants

For protein expression, the QKA-IL4 plasmid was transformed into E.coli Rosetta™ (DE3) cells, which were cultivated until reaching early logarithm phase with OD_{600} of ~0.6 and then induced by adding 1 mM IPTG to exponentially growing cells. Then, cells were shaken for another 4 h at 37˚C and finally harvested. Protein expression was controlled via SDS-PAGE (Figure 3.6).
After the induction, the gel displayed a strong band at about 14 kDa. The yield was approximately 3 g per liter culture medium (for summary: see table 3.2.)

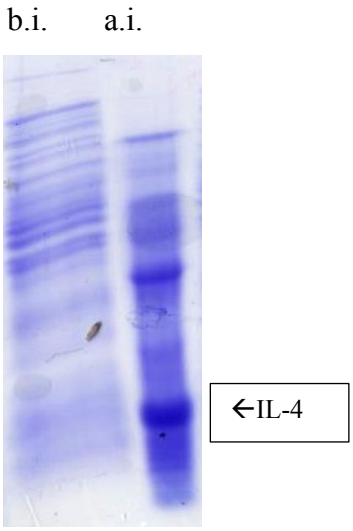


Figure 3.6: expression SDS-PAGE gel, noninuced-induced SDS-PAGE for control of IL-4 induction. The cell lysate before (b.I.) and after 4 hours induction (a.I.).

Table 3.2: Total yields of IL-4 and IL-4 variants after different purification steps.

	Wet weight of cells after fermentation [g/L medium]	Wet weight of IB after sonication [g/g cells]	CM Sepharose [mg/g IB]	HPLC [mg/mg CM Sepharose product]
IL-4 wt	3.1	1.2-2.4	19-28	7.2-8.1
H74C	3.3	1.6-3.2	19.9-70	11-20
Q78C	2.8	1.5-2.5	31.5-44	8.2-12.1
R81C	3.8	1.1-2.2	30	10.4

3.1.4. Cell disruption and isolation of IL-4 inclusion bodies

Recombinant expressed proteins were mostly deposited in E.coli cells in an insoluble form called inclusion bodies. Therefore, cells were sonicated and centrifuged to separate the inclusion bodies containing IL-4 and IL-4 variants from the rest of the soluble proteins and impurities. The cell disruption and the sedimentation and resuspension of the inclusion bodies were repeated for 3 times in total. After several washing steps with STE buffer, in the last washing step 1% Triton X-100 was added. Subsequently, IL-4 and IL-4 variants were extracted from inclusion bodies using 6 M GuCl, pH 8.0. To reduce the disulfide bonds between cysteine residues and to accomplish the protein denaturation, 0.1 % DTT was added. Most part of the IL-4 and IL-4 variants could be

solubilized and were recovered on the supernatant. A small part remained in the cell pellet despite varying extraction conditions (Figure 3.7).

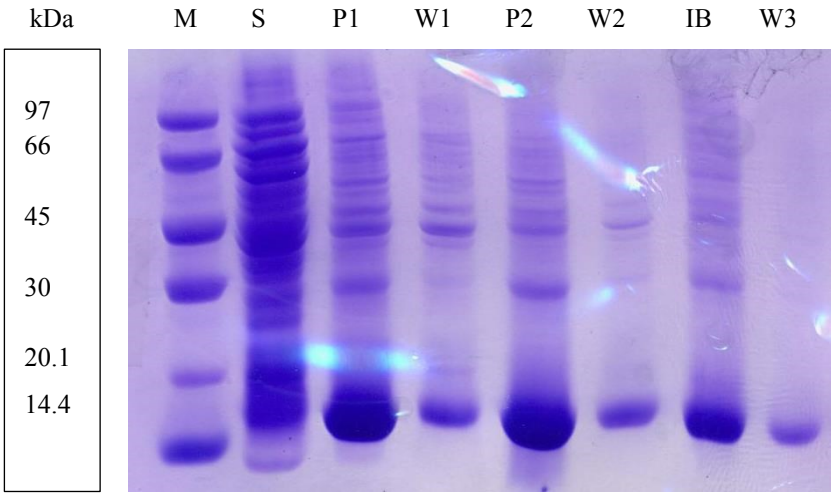


Figure 3.7: Cell disruption and formation of inclusion bodies – SDS-PAGE with protein marker (M), supernatant (S), pellet (P), wash 1 (W1), pellet 1 (P1), wash 2 (W2), inclusion bodies (IB), wash 3 (W3).

3.1.5. Refolding of IL-4 and IL-4 variants

For refolding of the IL-4 and IL-4 variants, the clarified protein extract was diluted 25-fold with ice cold refolding buffer, yielding a protein concentration of 50-100 µg/ml, and kept at 4˚C for three days. The original refolding buffer contained 1.2 M GuCl. Later on, a buffer containing 1 M arginine [Baynes, B.M. et al., 2005; Ishibashi, M. et al., 2005], was used. The main advantage of the arginine refolding buffer was that the IL-4 proteins could be refolded at much higher concentrations. 2 mM reduced and 5 mM oxidized glutathione were also added to the refolding buffer. The solution was dialyzed twice against 10 volumes of PBS at 4˚C for 12 to 18 h (see method 2.11.2).

3.1.6. Purification of IL-4 and IL-4 variants

Because the pI of human IL-4 is 10.5 [Callard, R. & Gearing A., 1994], the refolded proteins were applied to CM-Sepharose matrix at pH 5.0. During this cation-exchange chromatography, the acidic and neutral contaminating proteins, could not bind to the column and therefore the IL-4 and IL-4 variants were effectively separated. The bound proteins were eluted using a salt gradient from 0.5 M NaCl to 2 M NaCl. Elution fractions containing the IL-4 and IL-4 variants were identified by means of SDS-PAGE analysis (see Figure 3.10. Lane 1; 5; and 9 belong to non-reducted 16GS; 38GS; and IL-4 wt respectively. Lane 2; 6; and 10 belong to reducted 16GS; 38GS; and IL-4 wt respectively). These fractions were combined and concentrated.

Further, the protein purification was accomplished by reversed phase HPLC. Based on hydrophobic interactions, the protein bound to the chromatography matrix and eluted with increasing concentration of acetonitrile (Figure 3.8). The IL-4 variants were eluted at around 40% of acetonitrile. All variants were successfully purified, suggesting that neither the protein refolding neither the protein stability were disrupted by the individual mutations. The collected fractions during HPLC were monitored by SDS-PAGE, which revealed high purity of the obtained protein. The highly purified proteins were divided into aliquots lyophilized and in this way stored for the following experiments.

A) B)

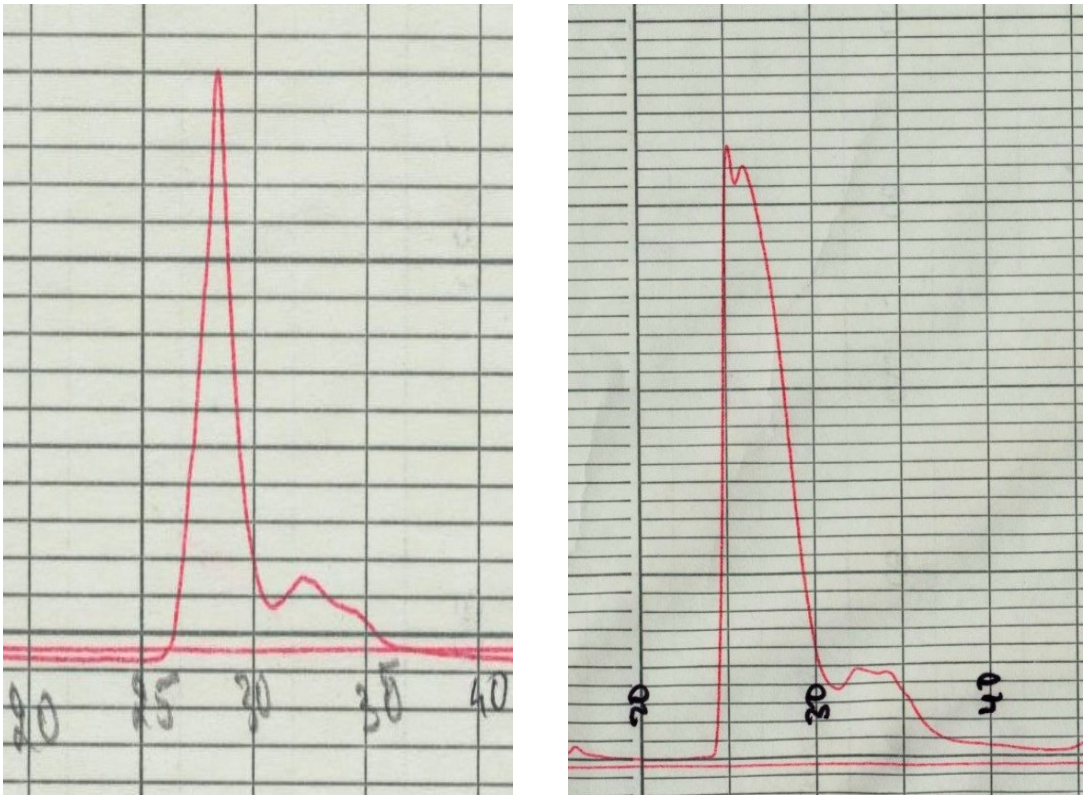


Figure 3.8: RP-HPLC purification step of IL-4 variants. A) Elution chart of IL-4 wt B) Elution chart of variant H74C.

3.2. Mass spectrometry

Surprisingly, the mass spectrometry of the purified IL-4 variants (Figure 3.9) showed one major protein species with a molecular weight consistent with the IL-4 mutein protein plus one glutathione moiety lacking the two hydrogen atoms of the two thiol groups. Only wild type IL-4 was observed as an unmodified protein with a molecular weight 14 kDa. Thus, in the muteins the glutathione was disulfide bonded to the engineered cysteine residue, and few different approaches were made to remove the IL-4 conjugated glutathione.

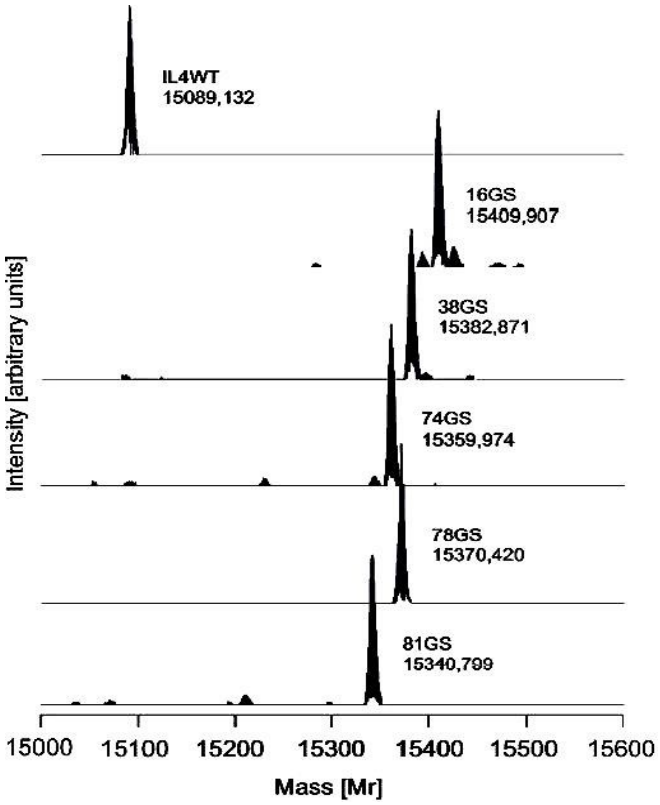


Figure 3.9: Mass spectrometry of the purified glutathionylated IL-4 proteins (wild type IL-4 (IL4wt), 16GS, 38GS, 74GS, 78GS, and 81GS). The molecular masses deduced from the deconvoluted spectra are consistent with the molecular weights of the respective IL-4 mutein plus one glutathione moiety added and two hydrogens subtracted (derived from the oxidation of the two thiol groups).

3.3. Glutathione modification of IL-4 variants

3.3.1. Chemical reduction of glutathione modified IL-4 analogues

Trials to remove the IL-4 conjugated glutathione with various chemical compounds and without destroying the IL-4 protein were not successful.

In a set of experiments the IL-4 muteins 16GS and 38GS were incubated with DTT at molar ratios of 1:1 (50 µM : 50 µM), 1:2 (50 µM : 100 µM) and 1:3 (50 µM : 150 µM) and free thiol groups were identified by MA-PEGylation and SDS-PAGE analysis (Figure 3.10).

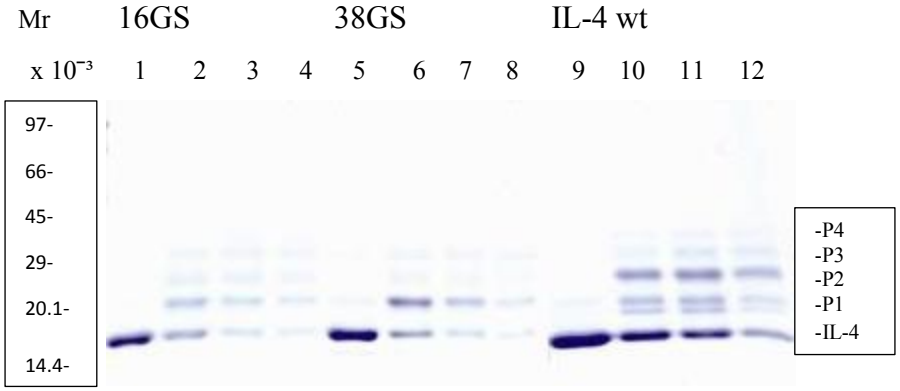


Figure 3.10: Chemical reduction of 50 µM IL-4 proteins. 16GS (lanes 1-4), 38GS (lanes 5-8), and wild type IL-4 (lanes 9-12) with DTT at concentration of 50 µM (lanes 2, 6, and 10), 100 µM (lanes 3, 7, and 11), and 150 µM (lanes 4, 8, and 12). Controls without DTT are shown in lanes 1, 5, and 9. After chemical reduction proteins were maleimide-PEGylated and analysed by SDS-PAGE on 12% gels. P1 – mono-pegylated IL-4. P2, P3, and P4 – multi-pegylated IL-4. The same aliquot on the reaction mixture containing 1.9 µg IL-4 proteins was loaded on each lane.

Upon reaction with DTT, a reduction of multiple disulfide bonds was observed, indicating that the engineered cysteine was reduced in parallel with the native disulfide bonds and mono- and di-PEGylated proteins could be detected. Upon reduction, the solution of the IL-4 proteins became turbid. This aggregated material was not dissolved by the SDS sample buffer and therefore was not recovered in the stained gels. Higher percentage of mono-PEGylated protein

38GS could be noticed compared to the 16GS mutein, suggesting a higher reactivity of the glutathionylated Cys38. The dose-dependent reduction of 74GS, 78GS, and 81GS with DTT showed that multi-PEGylated species have formed beside the monopegylated mutein even at the lowest DTT concentrations of 50 μM. Also some non-reduced protein remained.

Second set of experiment was made, where wild type IL-4 was reduced with a 3-fold molar excess of DTT and subsequently modified by N-ethylmaleimide (NEM). Di-NEM IL-4 was isolated as a major product of the reaction. The NEM label was distributed among several cysteines of the IL-4 protein involved in several of the native disulfide bonds, which suggested that there is no disulfide bond which is more sensitive to DTT reduction than the others.

3.3.2. Enzymatic deglutathionylation of IL-4 Cysteine muteins with glutaredoxin

Finally, an IL-4 cysteine mutant protein with a free thiol group was observed by using an E.coli glutaredoxin-1 assay, which allowed observing the deglutathionylation by the decline in the extinction at 340 nm as shown in Figure 3.11. When using buffer with pH 8.0 the protein solution became turbid, possibly because of the aggregation of the reduced IL-4 mutein. This problem was solved, by using a buffer with pH 7.0 and low concentrations of reduced glutathione (0.5 mM). The rate of glutaredoxin-mediated deglutathionylation differed among different cysteine muteins. The rate of reduction was fast for 38GS and 74GS and slow for 16GS, 78GS, or 81GS. The early decline of the reduction rate seen for 38GS and 74GS could be fitted to a monoexponential decrease with a half-life of 18 sec (38GS) and 55 sec (74GS), and for the others is 10 min. (16GS, 81GS) and 20 min. (78GS). The differences in the reaction rate of the enzymatic reduction pointed to a different accessibility and/or mobility of the S-S conjugated glutathione in the different IL-4 muteins.

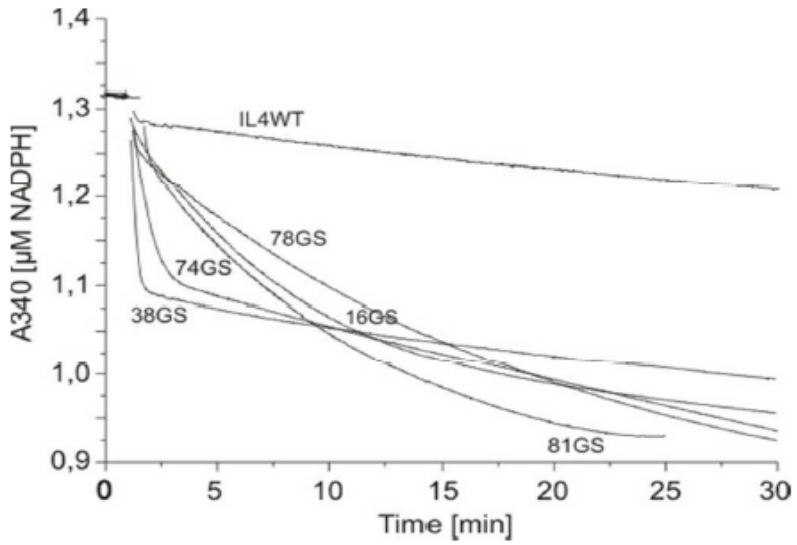


Figure 3.11: Enzymatic deglutathionylation of IL-4 proteins using the enzyme glutaredoxin (E. coli GRX-1). The NADPH-dependent reduction of GS-modified IL-4 cysteine muteins was recorded after addition of glutaredoxin by the decline in the extinction at 340 nm through the conversion of NADPH to NADP+.

The very slow reduction of the wild type IL-4 showed that even glutaredoxin mediated some reduction of the native disulfide bonds. This possibility was explored by determent of free thiol groups at different time points of the reaction by MA-PEGylation. Reaction products obtained after 2.5 to 30 min. were analyzed by SDS PAGE (Figure 3.12). The mono-PEGylated IL-4 H74C reached peak levels already after 2.5 to 5 min. and the amount of this product then declined at the expense of small amounts of tri- and multi-PEGylated species. The mono-PEGylated R81C accumulated until 20 to 30 min. reaction time, and towards the later time points, multi-PEGylated species became apparent as minor bands. This shows that there some unspecific reduction of the native disulfide bonds, and the reaction conditions should be optimized for each cysteine mutein to minimize incomplete and unspecific reduction. Even after 30 min. reaction time still a small amount of unmodified mutein remained.

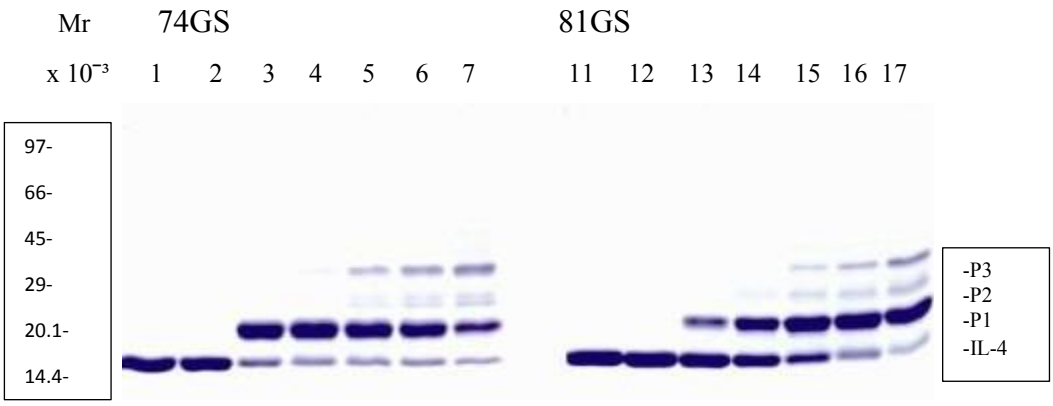


Figure 3.12: Reaction kinetics of GRX-mediated reduction of 74GS (left) and 81GS (right) are monitored by maleimide-PEGylation at 0, 2.5, 5, 10, 15, 20, and 30 min time points and subsequent SDS-PAGE analysis.

The enzymatically reduced mutant proteins could be purified using RP-HPLC with a final yield of 40 to 60%. SDS-PAGE analysis in the reduced and non-reduced gels showed that enzymatically reduced proteins occurred for more than 95% in the monomeric form. Small and variable amounts of dimeric protein could be observed for some muteins (Fig.3.13). Further, the enzymatically reduced proteins were chemically modified with N-ethylmaleimide, or TMS(PEG)12, for possible enhance of the properties and application of proteins.

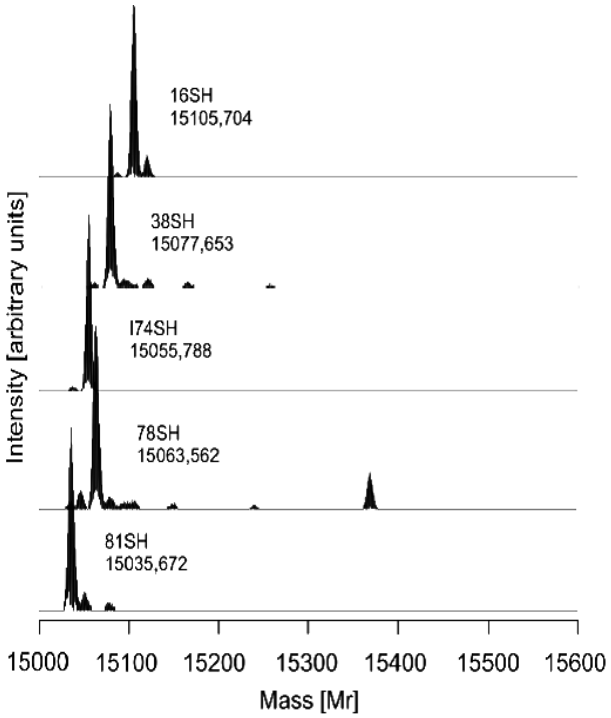


Figure 3.13: Mass spectrometry analysis of the enzymatically (glutaredoxin) reduced IL-4 muteins.

3.3.3. Reaction with N-ethylmaleimide or TMS(PEG)12

The conjugation of the reduced IL-4 cysteine analogues with N-ethylmaleimide at pH 7.0 is highly selective. Only the mono-NEM conjugate of the IL-4 mutein proteins could be detected by mass spectrometry analysis. Di- or multi-NEM conjugates and non-conjugated IL-4 proteins were absent.

After reaction with 2.363 Da MA-PEG mono-PEGylated analogues could be purified which were contaminated with only minor amounts of protein migrating during SDS-PAGE at the position of the unmodified protein (Figure 3.14). It was not very different when the proteins were analysed under reducing and non-reducing conditions rendering it unlikely that the lack of PEGylation resulted from disulfide-mediated dimer formation of the reduced proteins. A small amount of di-PEGylated protein was seen with the IL-4 H74C mutein possibly due to too strong reduction.

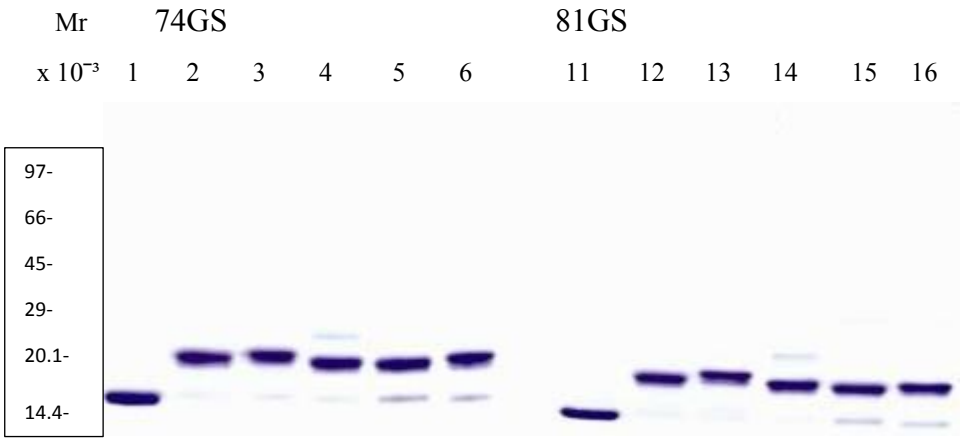


Figure 3.14: SDS-PAGE analysis of PEGylated IL-4 cysteine muteins under reducing (lanes 2-6) and non-reducing (lanes 12-16) conditions. Lanes 1,11: wild type IL-4; 2,12: 16PEG; 3,13: 38PEG; 4,14: 74PEG; 5,15: 78PEG; 6,16: 81PEG.

The following experiments will show the effect on IL-4Rα [ECD] binding of cysteine substitution and of the site specific modification by glutathione, N-ethylmaleimide (NEM), or a branched 2.36 kDa poly(ethylene glycol) (PEG).

3.4. Preparation of IL-4Rα [ECD]

3.4.1. Cloning of IL-4Rα [ECD]

The IL-4Rα [ECD] could be used to analyze if the IL-4 muteins retain their structural integrity and bind as IL-4 wild type to the receptor. A cDNA comprising residues 1-207, extracellular domain [ECD] was inserted into vector pcDNA3.1 (+). The cDNA encoding the extracellular domain of IL-4Rα [ECD] was mutated by recombinant PCR to substitute Cysteine at position 182 to Alanin (C182A), to avoid forming of any intermolecular disulphide bonds.
Different tags were fused to IL-4Rα [ECD] for better expression and easy protein purification. 5 constructs were kindly provided by Benedicte Danis, UCB, S.A. Braine L'Alleoud, Belgium.

1. pcDNA3.1(+)_Nterm+IL-4R-SNAP-TEV-hscFc (MBG909)
2. pcDNA3.1(+)_Nterm+IL-4R-SNAP-TEV-6His (MBG910)
3. pcDNA3.1(+)_Nterm+IL-4R-TEV-SNAP-6His (MBG911)
4. pcDNA3.1(+)_Nterm+IL-4R-TEV-6His-SNAP (MBG912)
5. pcDNA3.1(+)_Nterm+IL-4R (MBG913)

All 5 constructs were expressed in mammalian CHO-S cells, but only two of them, MBG 910 and MBG 911 were purified, mainly because of the easiness to capture 6His-tag by Ni NTA affinity chromatography.
The mature part of the nucleotide and amino acid sequence of the extracellular domain of human IL-4Rα [ECD] was obtained from P24394 UniProtKB/Swiss-Prot (Figure. 3.15).

```
60    gccaccatggggtggctttgctctgggctcctgttccctgtgagctgcctggtcctgctg
       A  T  M  G  W  L  C  S  G  L  L  F  P  V  S  C  L  V  L  L
120   caggtggcaagctctgggaacatgaaggtcttgcaggagcccacctgcgtctccgactac
       Q  V  A  S  S  G  N  M  K  V  L  Q  E  P  T  C  V  S  D  Y
180   atgagcatctctacttgcgagtggaagatgaatggtcccaccaattgcagcaccgagctc
       M  S  I  S  T  C  E  W  K  M  N  G  P  T  N  C  S  T  E  L
240   cgcctgttgtaccagctggtttttctgctctccgaagcccacacgtgtgtccctgagaac
       R  L  L  Y  Q  L  V  F  L  L  S  E  A  H  T  C  V  P  E  N
300   aacggaggcgcggggtgcgtgtgccacctgctcatggatgacgtggtcagtgcggataac
       N  G  G  A  G  C  V  C  H  L  L  M  D  D  V  V  S  A  D  N
360   tatacactggacctgtgggctgggcagcagctgctgtggaagggctccttcaagcccagc
       Y  T  L  D  L  W  A  G  Q  Q  L  L  W  K  G  S  F  K  P  S
420   gagcatgtgaaacccagggccccaggaaacctgacagttcacaccaatgtctccgacact
       E  H  V  K  P  R  A  P  G  N  L  T  V  H  T  N  V  S  D  T
480   ctgctgctgacctggagcaacccgtatccccctgacaattacctgtataatcatctcacc
       L  L  L  T  W  S  N  P  Y  P  P  D  N  Y  L  Y  N  H  L  T
540   tatgcagtcaacatttggagtgaaaacgacccggcagatttcagaatctataacgtgacc
       Y  A  V  N  I  W  S  E  N  D  P  A  D  F  R  I  Y  N  V  T
600   tacctagaaccctccctccgcatcgcagccagcaccctgaagtctgggatttcctacagg
       Y  L  E  P  S  L  R  I  A  A  S  T  L  K  S  G  I  S  Y  R
660   gcacgggtgagggcctgggctcaggcctataacaccacctggagtgagtggagccccagc
       A  R  V  R  A  W  A  Q  A  Y  N  T  T  W  S  E  W  S  P  S
702   accaagtggcacaactcctacagggagcccttcgagcagcactag
       T  K  W  H  N  S  Y  R  E  P  F  E  Q  H  -
```

Figure 3.15: Protein/DNA sequence for extracellular domain of human IL-4Rα [ECD].

For mammalian expression, the cDNA of extracellular domain of IL-4Rα [ECD] was inserted into the expression vector pcDNA3.1 (+) using KpnI I and XhO I restriction sites (Figure 3.16).

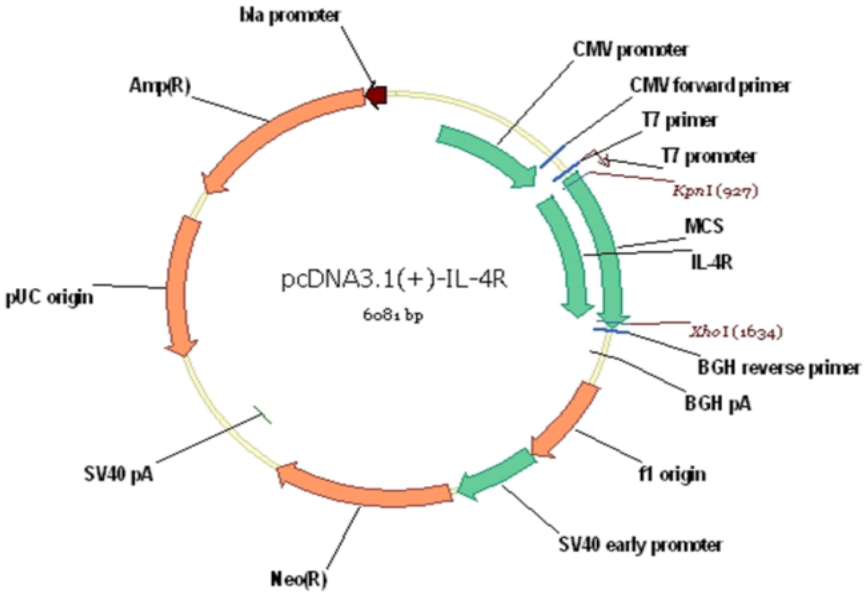


Fig.3.16: Schematic diagram of expression vector pcDNA3.1 (+)-IL4R.

For mammalian expression, the cDNA of extracellular domain of IL-4Rα [ECD] together with TEV-SNAP-6His tag was inserted into the expression vector pcDNA3.1 (+) using KpnI I and XhO I restriction sites (Figure 3.17).

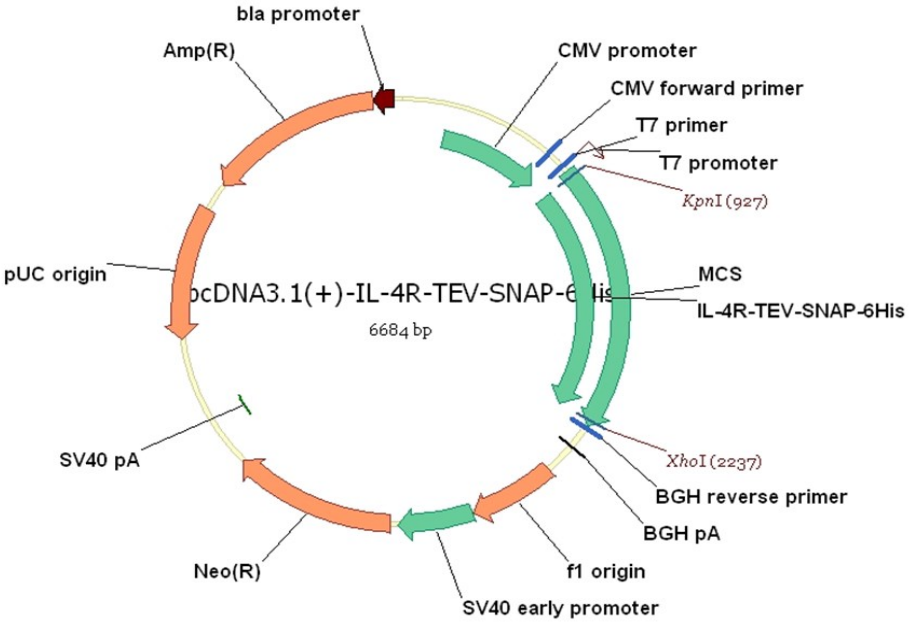


Fig.3.17: Schematic diagram of expression vector pcDNA3.1 (+)-IL4R-TEV-SNAP-6His.

3.4.2. Expression of IL-4Rα [ECD] constructs

The constructs were transfected into CHO-S cells in serum free CD-CHO media, by means of electroporation.

A time course experiment was performed, by harvesting cell and media at 5, 9, and 12 days post-transfection to optimize the expression of the target protein.

200 ml culture with cell density 2 x 10^9 was spinned for 10 min., 4˚C, 1400 rpm. The pellet was resuspended in ca. 20 ml Earls Balanced Salt Solution EBSS, transferred into a 50 ml tube, and spinned at 4˚C (10 min., 1400 rpm, Megafuge, T. Heraeus). The supernatant was discarded, and the pellet was kept the entire time at 4˚C.

For 200 ml culture, we need 0.8 mg DNA. The DNA was added to the cell pellet, with the addition of small amounts of EBSS up to final V=2.4 ml.

3 cuvettes were needed for 200 ml culture. 800 µl of cells/DNA mix was added to each cuvette, zapped and seeded into 200 ml of CD-CHO media, which contained 2 ml GlutaMAX and 0.4 ml Actinomycin (1:500). The culture was left at 37˚C, for 12 days.

After harvesting the 200 ml culture at 4˚C (15 min., 1500 rpm, Megafuge, T. Heraeus) the supernatant was kept.

Equal amounts of protein sample were used for SDS-PAGE electrophoresis to detect the expression level of the IL-4R constructs (Figure 3.18).

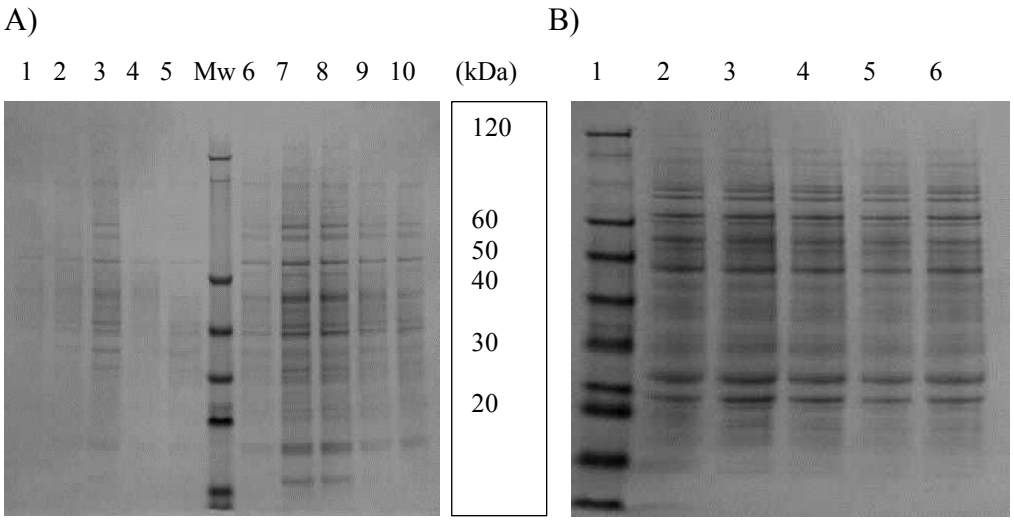


Figure 3.18 A) expression SDS-PAGE gel: 1-5 after 5th day of cell culture, 1- MBG909; 2- MBG910; 3- MBG911; 4- MBG912; 5- MBG913; 6-10 after 9th day of cell culture, 6- MBG909; 7- MBG910; 8- MBG911; 9- MBG912; 10- MBG913:

B) expression SDS-PAGE gel after 12th day of cell culture, 1- Mw NuPAGE®MES 2- MBG909; 3-MBG910; 4- MBG911; 5- MBG912; 6- MBG913.

SDS-PAGE gels showed no visible expression bands, due to or low expression level or/and due to glycosylation (see Figure 3.21, for glycosylation, lane 7-12; not sharp bands). Ni-NTA purification was made only on MBG 910 and MBG 911.

3.4.3. Ni-NTA purification

The 12th day cell culture was centrifuged at 4˚C (15 min., 1400, Megafuge, T. Heraeus) and the supernatant was kept and concentrated up to V= ~30 ml, Amicon 30K. The concentrated sample was then diluted 1:10 with Buffer A, and 300 ml of sample was mixed with 5 ml Ni2+ beads (10 ml solution) previously washed 3x with PBS. The mixed was tumbled for 2 hours, at 4˚C, and afterwards centrifuged and protein-bead mixture was loaded on the column. Gradient elution was performed, with 100 mM and 250 mM Imidazole, where the entire protein was eluted with only 100 mM Imidazole (Figure 3.19; 3.20; 3.21).

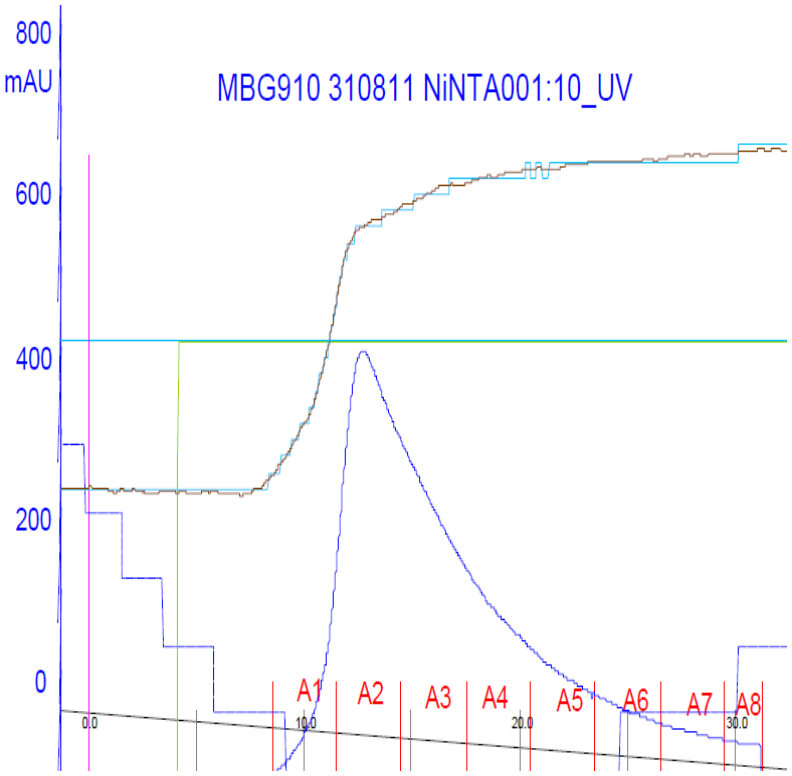


Figure 3.19: Elution chart of MBG 910. Protein elution was done with 100 mM Imidazole.

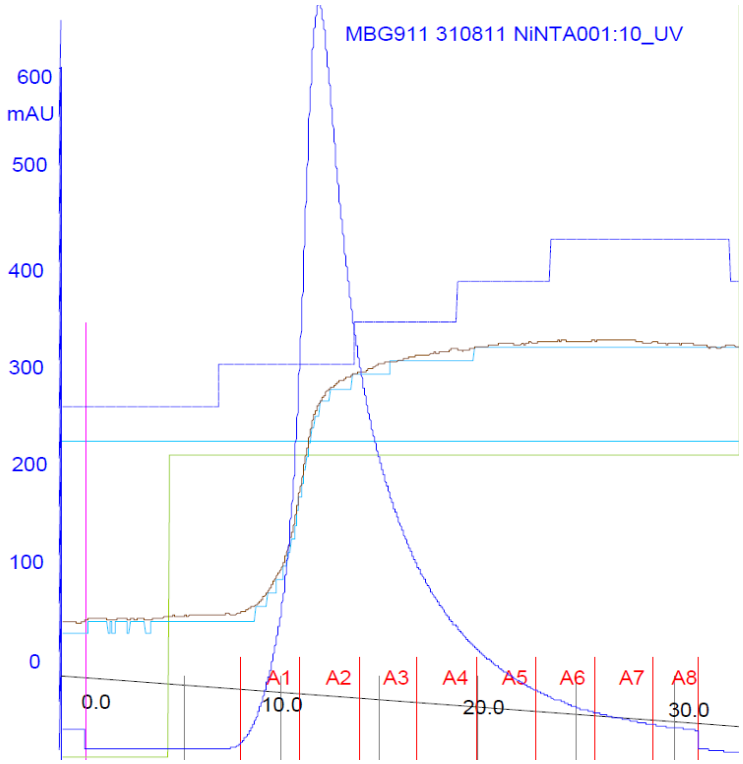


Figure 3.20: Elution chart of MBG 911. Protein elution was done with 100 mM Imidazole.

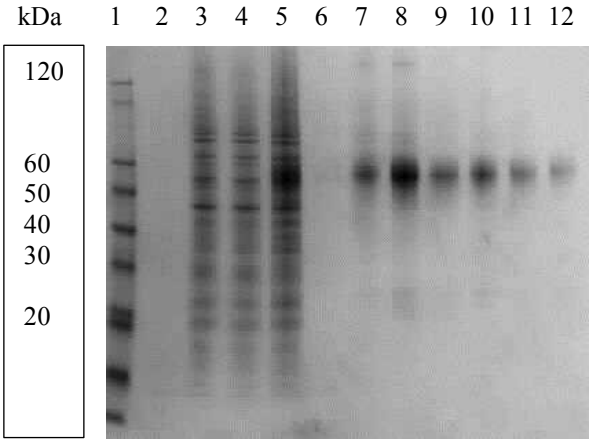


Figure 3.21 : SDS-PAGE gel, MBG911 Ni-NTA purification : 1 – Mw, NuPAGE®MES; 2- Filtrate; 3- diluted Load; 4- FT; 5- conc. Load; 6- Fr.A1; 7- Fr.A2; 8- Fr.A3; 9- Fr.A4; 10- Fr.A5; 11- Fr.A6; 12- Fr.A7.

The fractions containing the protein MBG 911 (A1-A7) were collected and concentrated up to V=2.5 ml and TEV-cleaved (1:100) for 2 hours, to remove the TEV-SNAP-6His tag and buffer-exchanged for removal of traces of imidazole (Figure 3.22).

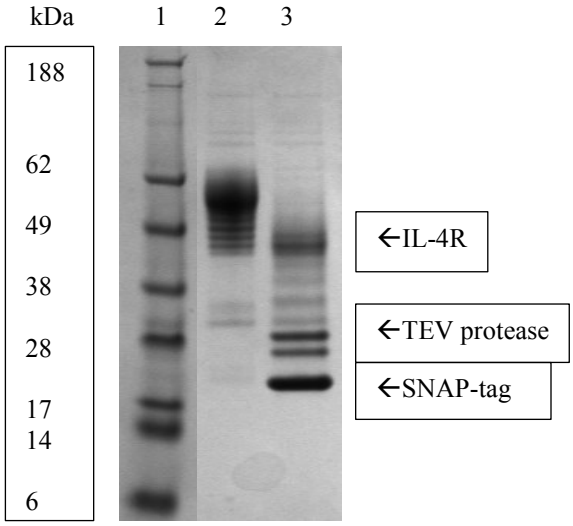


Figure 3.22: SDS-PAGE of TEV-cleaved MBG911: 1- Mw NuPAGE ®MES; 2- MBG911; 3- TEV-cleaved MBG911.

Mw SNAP-tag = 19.4 kDa ; Mw TEV = 27 kDa

Only MBG911 was TEV cleaved. The yield was approximately 2.0 – 3.0 mg from a starting 200 ml CHO-S culture (Table 3.3).

Table.3.3: Total yields after different purification steps.

IL-4R construct	Ni-NTA purification step (mg)	TEV-cleaved and desalting step (mg)
MBG910	2.5 – 3.0	/
MBG911	3.0 – 3.8	2.5 - 3.5

Purified IL-4Rα [ECD] was used for biothynilation and further BIAcore experiments for binding affinity to IL-4 and foldamers.

3.5. Foldamers

Initial modeling work focused on analyzing the key interactions between IL-4 and the IL-4Rα [ECD], and the design of quinoline foldamer structures that could mimic the interacting residues of the IL-4 (See Figure 3.2).

These foldamers have a very well-defined structure that lends itself to the rational design of substituents and the production of focused combinatorial libraries of foldamers capable of interacting with the IL4/IL-4R binding epitope. They are also large enough to block a protein-protein interaction, a feat that is not possible with small molecules.

The two libraries were produced in UCB Pharma S.A., Brussels, Belgium by Michael Grotz, PhD Student, under supervision of Dr. Michael Deligny.

❖ Braine I Library (62-68; 74-77)

Foldamer Structure:

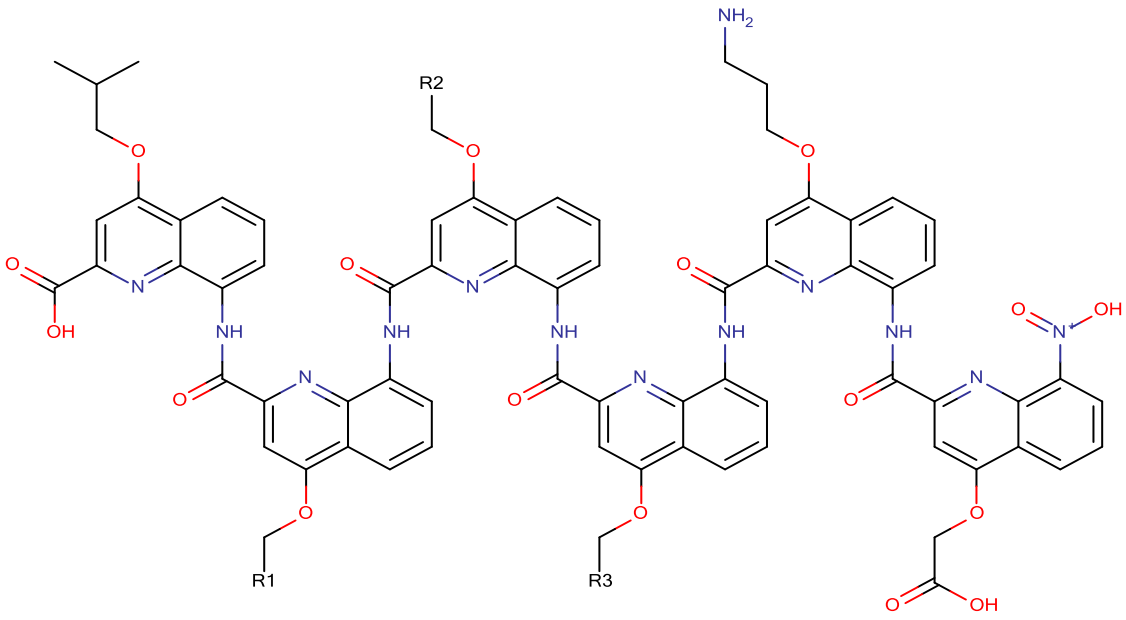
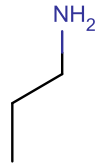
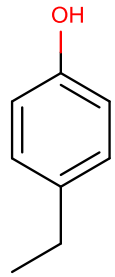
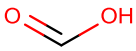
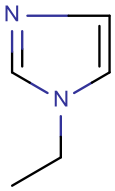


Figure 3.23: Structure of the foldamers.

Different radical groups were used to enhance the foldamer solubility (Table 3.4).

Table 3.4: Radical groups used for the design of foldamers.

Radical	Structure	short
Ethylamine		EA
Ethyl phenol		EP
Formic acid		FA
Ethyl imidazole		EI

Foldamer	R1	R2	R3	Molecular weight
62	EA	FA	FA	1509.4443
63	EA	FA	EP	1585.5120
64	EA	EP	EA	1584.5466
65	EA	EP	FA	1585.5120
66	EA	EA	EA	1507.5491
67	EA	EA	FA	1508.4967
68	EA	EA	EP	1584.5644
74	EA	EI	EP	1635.5753
75	EA	EI	EA	1558.5600
76	EA	FA	EA	1508.4967
77	EA	EP	EP	1661.5797

❖ Braine II Library

Foldamer structure:

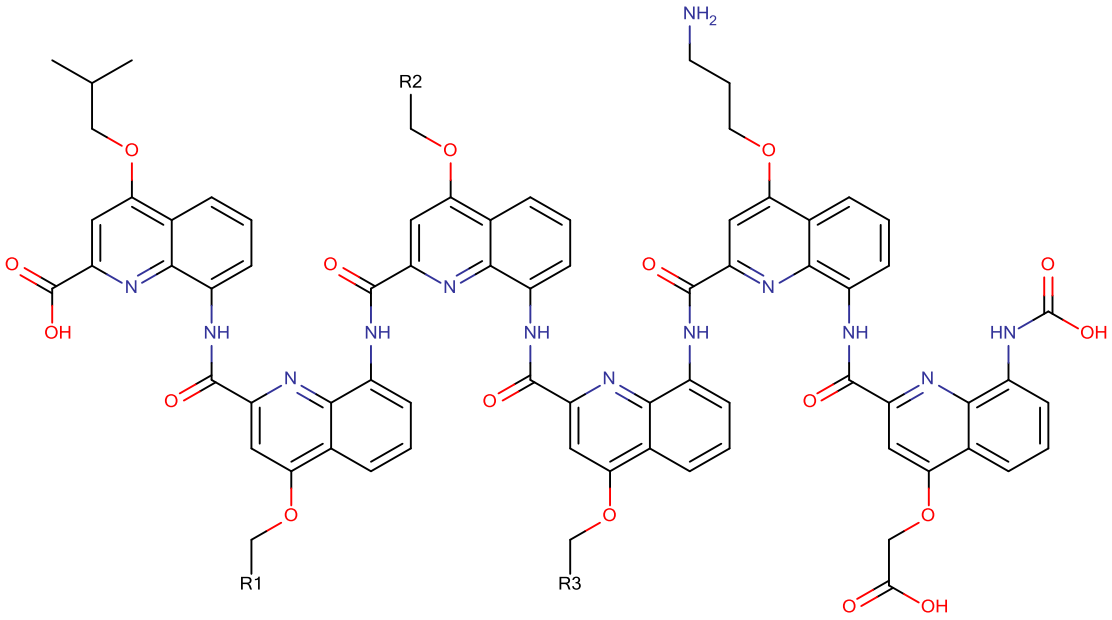
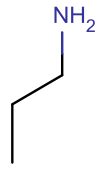
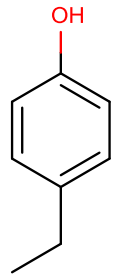
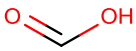
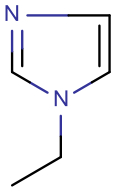
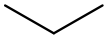


Figure 3.24: The structure of foldamer.

Different radical groups were used to enhance the foldamer solubility (Table 3.5).

Table 3.5: Radical groups used for the design of foldamers.

Radical	Structure	short
Ethylamine		EA

Radical	Structure	short
Ethyl phenol		EP
Formic acid		FA
Ethyl imidazole		EI
Propane		P

Foldamer	**R1**	**R2**	**R3**	**Molecular weight**
A1	P	EP	EA	1595.67
A2	P	EP	P	1594.68
A3	P	EA	EA	1518.58
A4	P	EA	P	1517.60
A5	P	FA	EA	1519.53
A6	P	FA	P	1518.54
B1	P	P	EA	1517.60
B2	P	P	P	1516.61
B3	P	EP	EP	1672.75
B4	P	EP	FA	1596.61
B5	P	EA	EP	1595.67
B6	P	EA	FA	1519.53
C1	P	FA	EP	1596.61
C2	P	FA	FA	1520.47
C3	P	P	EP	1594.68
C4	P	P	FA	1518.54
C5	FA	EP	EA	1597.60
C6	FA	EP	P	1596.61
D1	FA	EA	EA	1520.51
D2	FA	EA	P	1519.53
D3	FA	FA	EA	1521.46
D4	FA	FA	P	1520.47
D5	FA	P	EA	1519.53
D6	FA	P	P	1518.54

Foldamer	R1	R2	R3	Molecular weight
E1	FA	EP	EP	1674.68
E2	FA	EP	FA	1598.54
E3	FA	EA	EP	1597.60
E4	FA	EA	FA	1521.46
E5	FA	FA	EP	1598.54
E6	FA	FA	FA	1522.40
F1	FA	P	EP	1596.61
F2	FA	P	FA	1520.47
F3	EP	EP	EA	1673.73
F4	EP	EP	P	1672.75
F5	EP	EA	EA	1896.65
F6	EP	EA	P	1595.67
G1	EP	FA	EA	1597.60
G2	EP	FA	P	1596.61
G3	EP	P	EA	1595.67
G4	EP	P	P	1594.58
G5	EP	EP	EP	1750.82
H1	EP	EP	FA	1674.68
H2	EP	EA	EP	1673.73
H3	EP	EA	FA	1597.60
H4	EP	FA	EA	1674.68
H5	EP	FA	FA	1598.54

Both libraries, Braine I and II, were examined on their ability for direct and indirect binding to IL-4 and in disrupting the IL-4 / IL-4Rα interaction.

3.6. BIAcore interaction analysis of ligand receptor interplay

The purified IL-4 and its variants were used to determine both, the kinetic and affinity binding constants characterizing the interaction with the ligand. This analysis was carried out to check for the roles of the mutated amino acids in modulating the affinity and kinetics of binding.
A biosensor technology that relies upon surface plasmon resonance to measure changes in the index of refraction at the surface where the binding occurs was explored to evaluate the kinetic parameters of the interaction. The evaluation methods are described in 2.15.2.

3.6.1. Immobilization of the biotinylated IL-4R alpha [ECD] and IL-4 on the sensor chip

Streptavidin-biotin coupling was chosen to immobilize IL-4Rα [ECD] on the sensor chip. Earlier experiments proved that the method offered a higher ligand binding capacity of the chip, considering the certain amount of immobilized receptor [Shen, B.J. et al., 1996]. Because of the nonspecific nature of the amine coupling technique and stereo obstacle, we used the streptavidin-biotin coupling as a standard immobilization method in this study. First, the four cells of a sensor chip CM5 were coated with streptavidin employing the amine coupling procedure as described in 2.15.1 (Figure 3.25). The matrix of flow cells 2 and 3 was loaded separately with respectively IL-4Rα [ECD] and IL-4 121SH, which earlier were biotinylated (Figure 3.26 and Figure 3.27). Nothing was loaded on flow cell 1. It was used to record a background sensogram that was subtracted during the evaluation from the sample sensograms in flow cells 2 and 3.

The receptor was immobilized on the chip until a density of ca. 1000 RU. Since the streptavidin-biotin interaction has an extremely high affinity (~10^{15} M^{-1}) [BIAcore Handbook, 1995] the chip could be repeatedly regenerated.

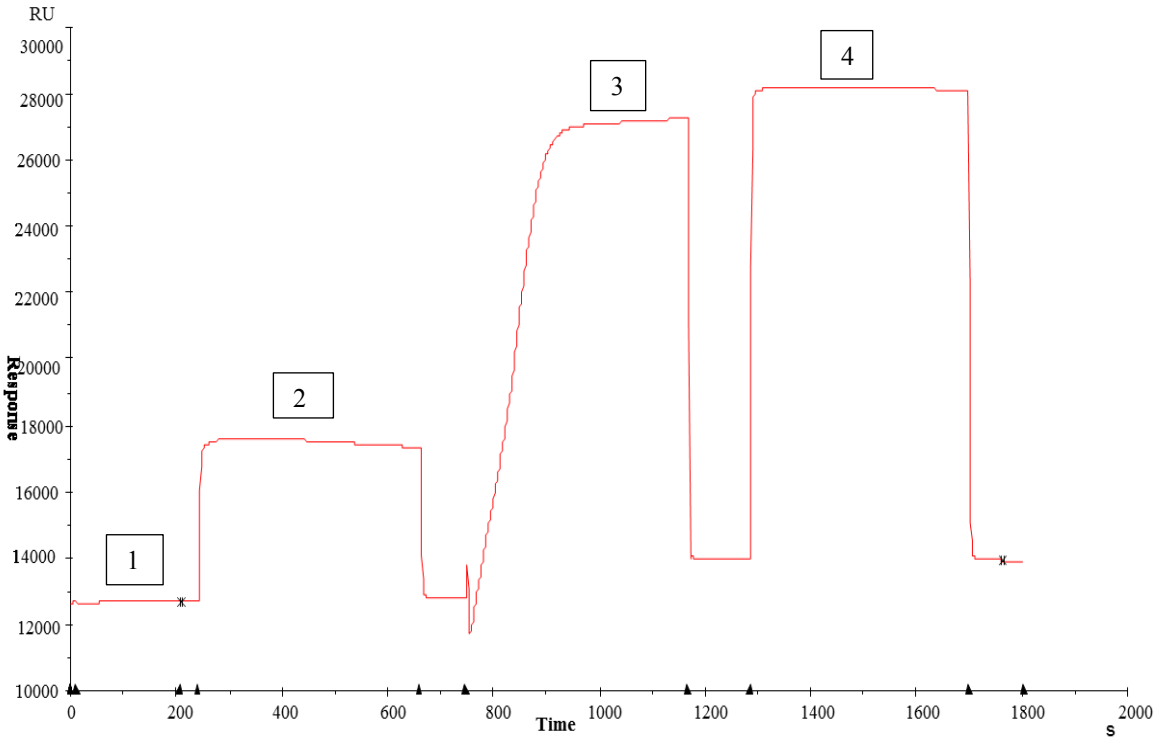


Figure 3.25: Sensogram showing the immobilization of streptavidin on a sensor chip CM5 by using a standard amine coupling. (1) Baseline signal for the unmodified sensor chip treated with continuous flow buffer HBS (5 µl/min). (2) Injection of 35 µl of NHS/EDC for 7 min to activate the dextran matrix. (3) 7 min injection of 35 µl of streptavidin (50 µg/ml). (4) Injection of 35 µl of ethanolamine hydrochloride (1 M) to deactivate the matrix and remove non-covalently bound streptavidin.

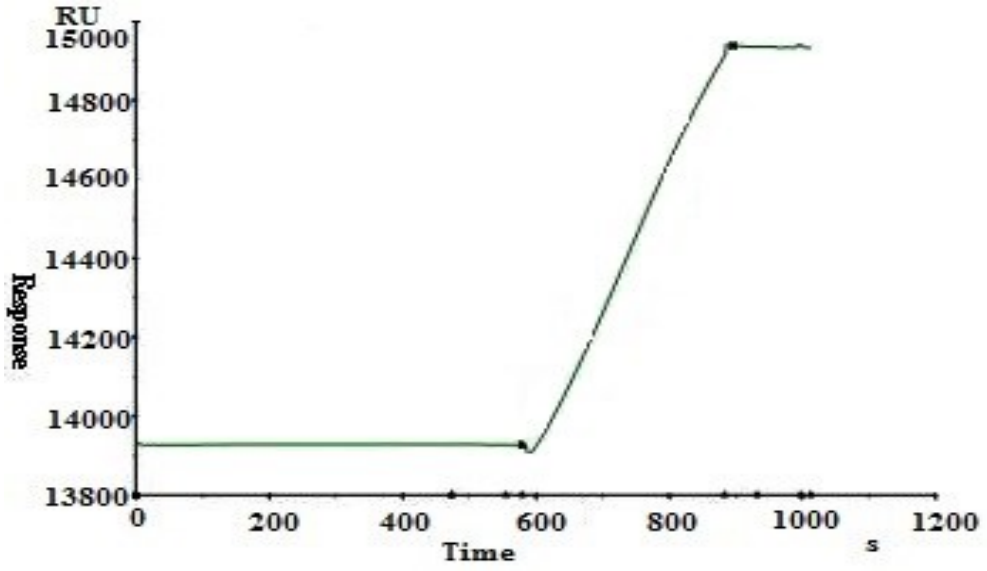


Figure 3.26: Immobilization of a biotinylated IL-4Rα [ECD] on a streptavidin chip [Shen, B.J. et al., 1996]. The protein was manually injected into the chip and the injection was stopped at 1000 RU.

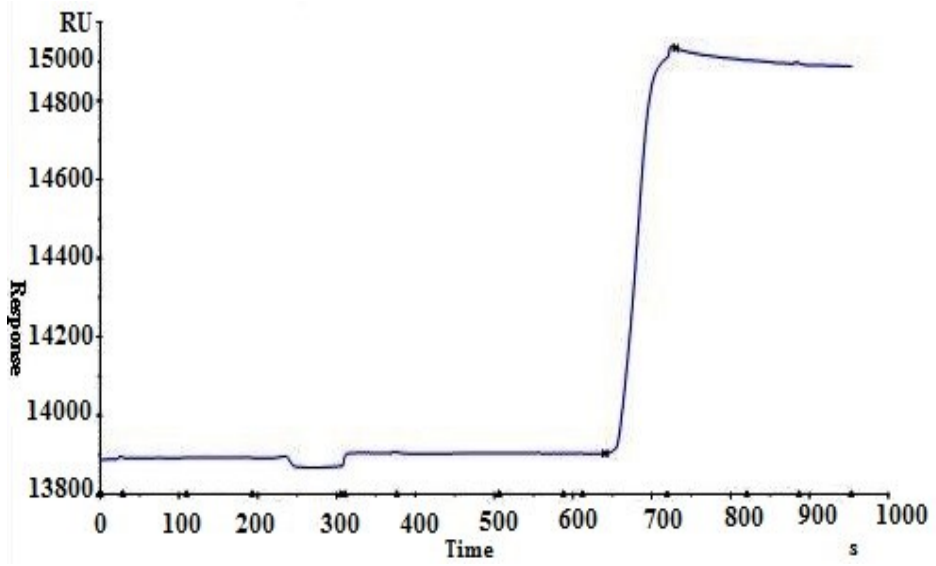


Figure 3.27: Immobilization of MA-PEG biotinylated IL-4 121SH on a streptavidin chip. The protein was manually injected into the chip and the injection was stopped at 1000 RU.

3.6.2. Kinetics of the interaction of IL-4, IL-4 variants and IL-4Rα [ECD]

Although the kinetics of binding of IL-4 to IL-4Rα [ECD] was investigated in an earlier study [Shen, B.J. et al., 1996], similar measurement was performed again as a control basis to estimate the changes in binding affinity of the mutated variants. Initial experiment was performed with 20 nM IL-4 in 0.15 M HBS buffer (Figure 3.28). The sensogram showed the typical model of extremely fast association and slow dissociation, observed earlier.

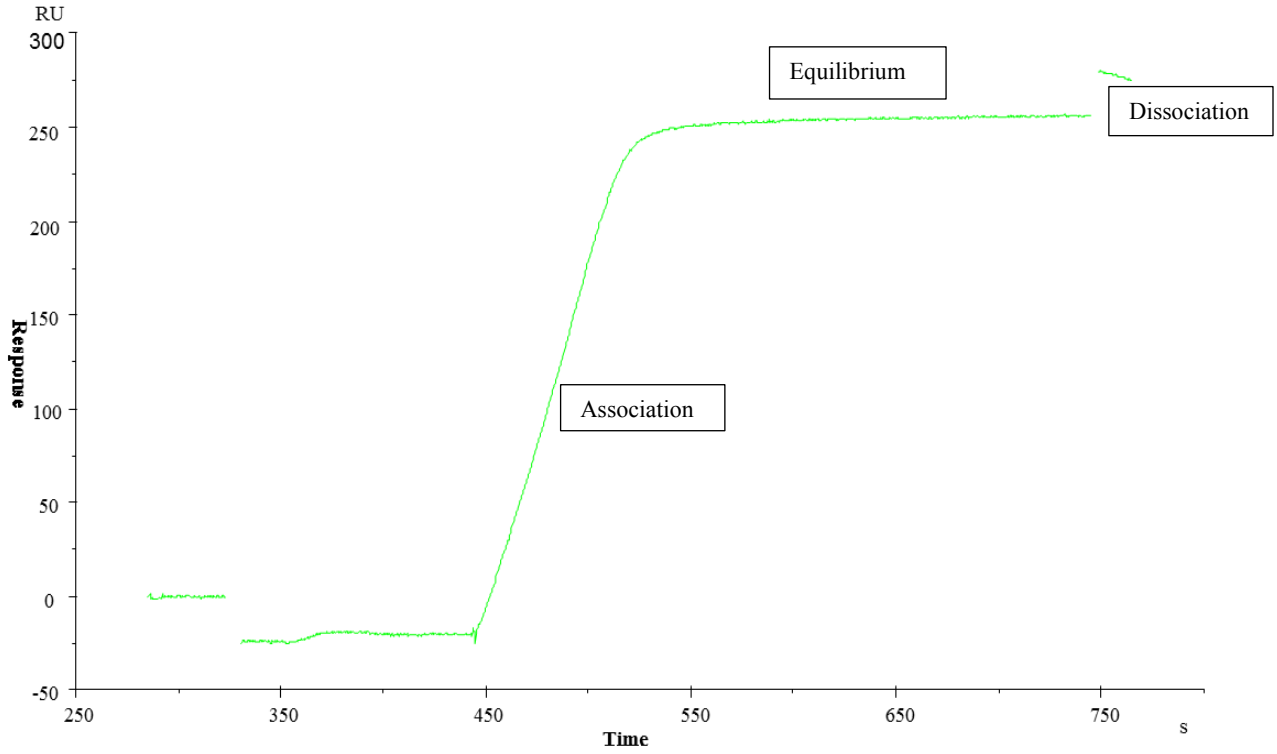
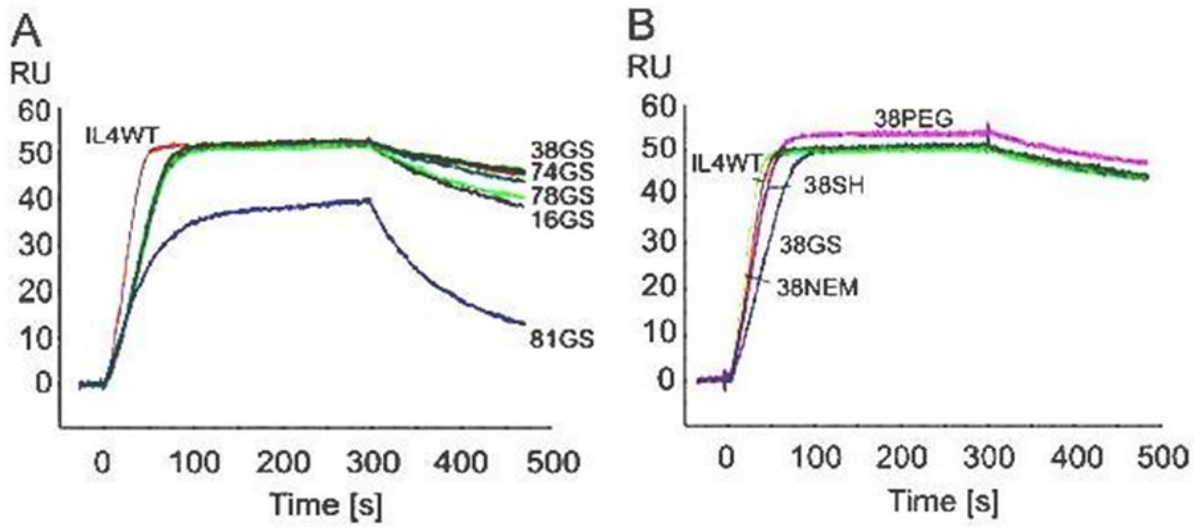


Figure 3.28: Overlay of sensogram representing the binding of 20 nM IL-4 to immobilized IL-4Rα [ECD]. The sensogram shows: Association of analyte IL-4 with ligand IL-4Rα [ECD] during sample injection; Equilibrium or steady state during sample injection, where the rate of analyte binding is balanced by dissociation from the complex; and Dissociation of analyte IL-4 from the surface during buffer flow.

3.6.3. Kinetics of the binding of IL-4 variants to IL-4Rα [ECD]

The introduced cysteine in the IL-4 molecule nearly did not affect the IL-4 receptor binding in the 16SH, 38SH, and 78SH analogues. The 5-fold decrease in affinity observed with 81SH resulted mainly from an increased dissociation rate constant k_d. More than 2-fold lower on-rate constant determined for 74SH was possible as a result from a partial inactivation due to over-reduction. 16NEM, 38NEM, and 74NEM as well didn't affect the receptor binding affinity. With 78NEM and 81NEM the affinity was similarly decreased as with the free thiol analogues. PEGylation of the thiol group resulted in 2- to 3-fold decrease in affinity for IL-4Rα with all analogues, except 81PEG which showed strongly reduced affinity for the receptor due to a slightly increased dissociation rate and strongly reduced association rate indicating some sterical hindrance effect of the peptide or the PEG moiety attached to the thiol group in close proximity of the receptor binding epitope (Table 3.6).



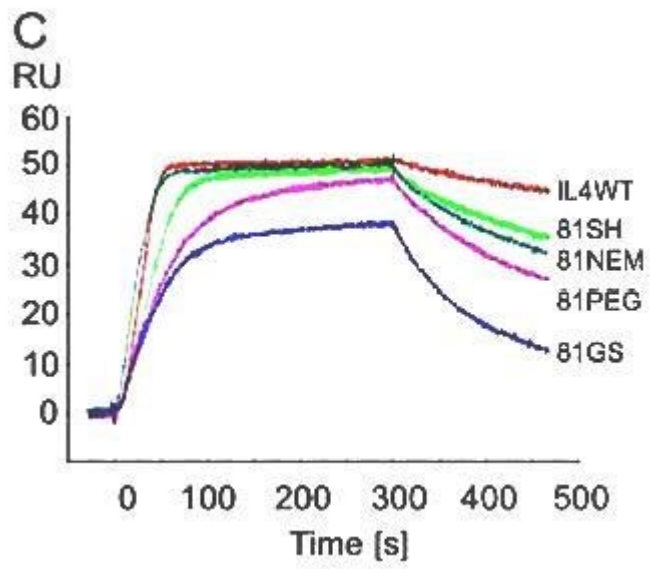


Figure 3.29: Surface Plasmon resonance (SPR) analysis of the interaction of IL-4 proteins with IL-4Rα ectodomain which was immobilized on a CM5 biosensor chip. Perfusion with IL-4 protein at a concentration of 10 nM starts at time point 0s for a duration of 300s after which the biosensor is again perfused with buffer to measure the dissociation started at time 300s (A) Interaction of IL-4 wt and glutathionylated IL-4 muteins (16GS, 38GS, 74GS, 78GS, 81GS), (B) IL-4 wt and modified IL-4 S38C analogues (38GS, 38SH, 38NEM, 38PEG); (C) IL-4 wt and modified IL-4 R81C analogues (81GS, 81SH, 81NEM, 81PEG). Calculation of the kinetic rate constants and equilibrium binding constants are shown in table 3.6.

Table 3.6: Kinetic Parameters Deduced from SPR Analysis for the IL-4Rα [ECD] Interaction of IL-4 Analogues. The rate constants for dissociation (kd) and association (ka) were derived from sensograms as shown in Figure 3.29.

IL-4 proteins	*k*d [s^{-1}] x 10^{+3}	*k*a [M^{-1} s^{-1}] x 10^{-6}	KD (=kd/ka) [PM]
IL-4 wt	1.5	19.0	78
16GS	2.9	8.4	350
16SH	1.3	20.0	67
16NEM	1.4	16.0	70
16PEG	1.1	7.0	160
IL-4 wt	1.3	15.0	90
38GS	1.3	8.0	170
38SH	1.5	11.0	130
38NEM	1.6	15.0	110
38PEG	1.6	10.0	160
IL-4 wt	1.7	13.0	130
74GS	1.4	7.8	170
74SH	1.3	5.0	270
74NEM	1.9	13.0	140
74PEG	1.5	8.1	180
IL-4 wt	1.2	14.0	80
81GS	10.0	1.8	5700
81SH	2.9	7.9	400
81NEM	4.1	9.1	450
81PEG	4.2	1.6	2600

3.7. BIAcore analysis of ligand : receptor interactions in the presence of foldamers

The kinetics of the interaction between the both IL-4 wild type and IL-4Rα [ECD] in the presence of foldamers was analyzed by using biosensor technology. Biotinylated IL-4Rα [ECD] and MA-PEG-Biotin IL-4 121SH wild type were immobilized on the streptavidin-coated chip with a density of 1000 RU [see 3.6.1]. Each foldamer was dissolved in 100% DMSO, with final concentration 10 mM. Initial experiment was made by using 1% DMSO / 0.15 M HBS buffer, but the results showed that foldamers were sticking to the chip. Even after the washing step with $MgCl_2$, some of the foldamer was still left and was affecting the results of the next subsequent experiment (data not shown). 25 mM CHAPS was added to the 0.15 M HBS buffer, to remove the traces of the remaining foldamer.

Another experiment was made, to check if 1% DMSO and 25 mM CHAPS are affecting the binding capacity of IL-4 to IL-4Rα. Experiments were performed with different IL-4 concentration (Figure 3.30). The sensograms showed that the IL-4 / IL-4Rα interaction was not affected (see Figure 3.28 for comparison).

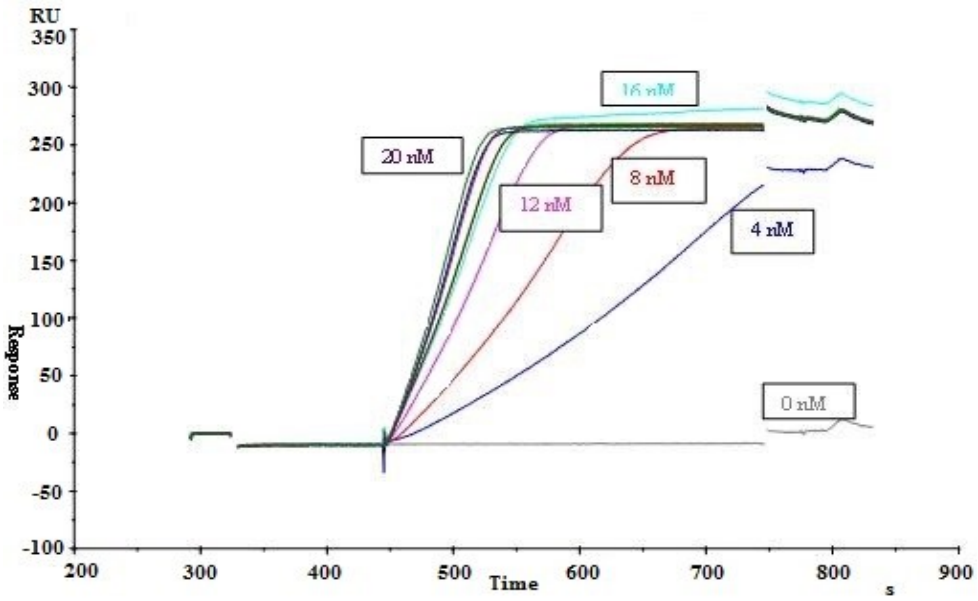


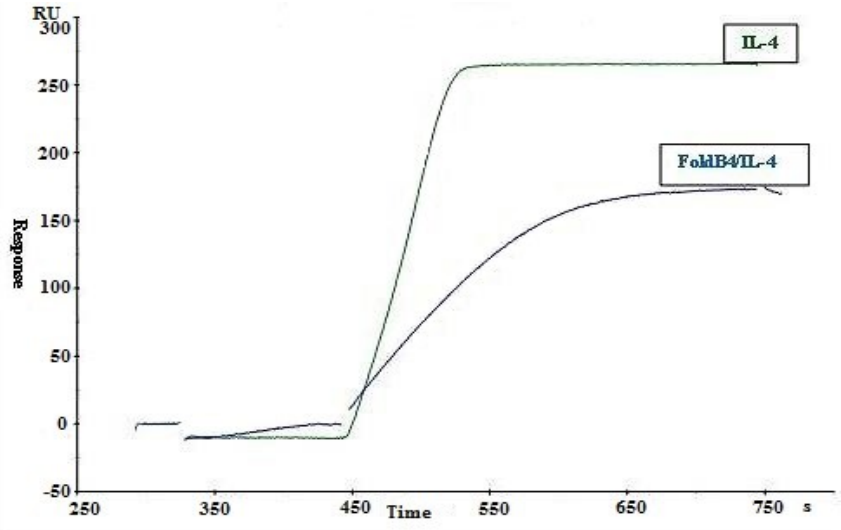
Figure 3.30: Overlay of sensograms showing the binding of IL-4 to the immobilized IL-4Ralpha ECD. IL-4 concentrations range of 0 nM, 4 nM, 8 nM, 16 nM, and 20 nM were applied. IL-4 is in 1% DMSO; 25 mM CHAPS; 0.15 M HBS buffer.

Both libraries, Braine I and Braine II went under direct and indirect method of binding of foldamers to IL-4. Through all further experiments, 1% DMSO; 25 mM CHAPS; 0.15 M HBS buffer was used.

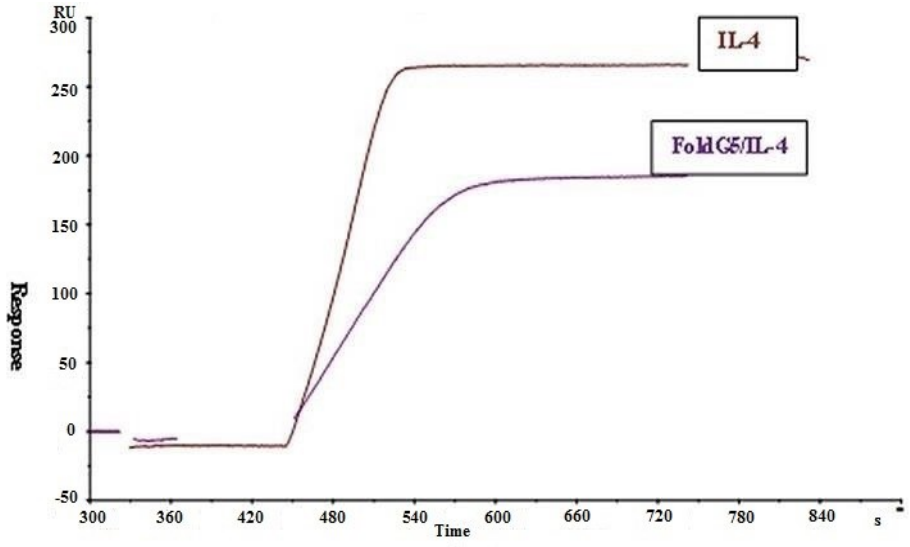
3.7.1. Indirect method

Indirect method of foldamer binding to IL-4, through the IL-4Rα [ECD] was made to Braine I and Braine II foldamer libraries. The results showed that few of the foldamers inhibited the IL-4 / IL-4Rα interaction. Some of the examples are shown in Figure 3.31, and the association rate constant *k* summarized in Table 3.7.

A)



B)



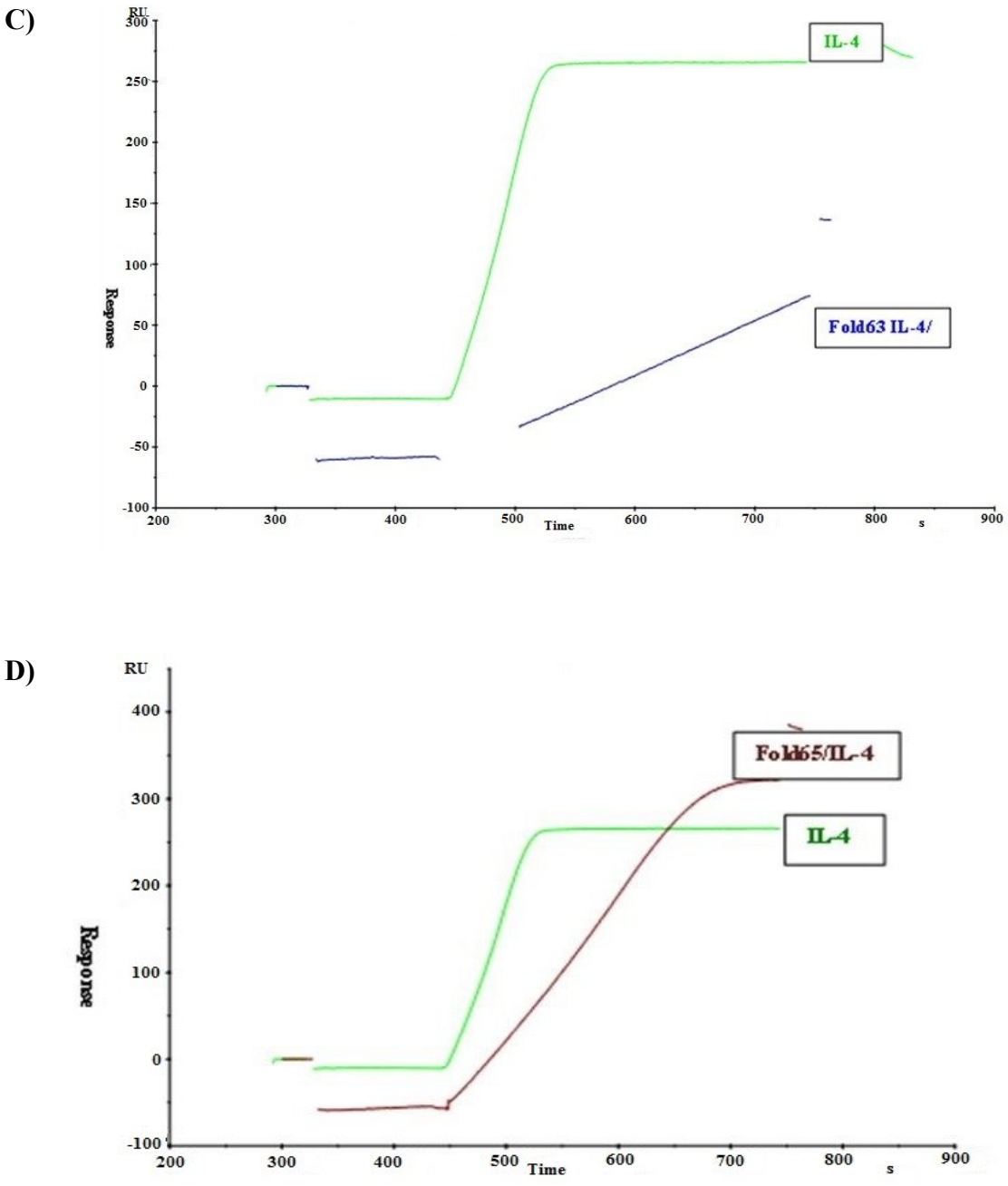


Figure 3.31: Sensograms of potential inhibitors of IL-4/IL-4Rα interaction. Braine II Library: (A) Foldamer B4; (B) Foldamer G5; Braine I Library: (C) Foldamer 63; (D) Foldamer 65;

Table 3.7: Comparison of the association rate constant *k* between 20nM IL-4 in 1% DMSO; 25mM CHAPS; 0.15M HBS buffer; and 20nM IL-4/100µM Foldamer in 25mM CHAPS; 0.15 M HBS.

sample	$k[M^{-1} s^{-1}]$
IL-4 wt	3.416
IL-4/FoldB4	1.154
IL-4/FoldG5	1.577
IL-4/Fold63	0.36
IL-4/Fold65	1.08

3.7.2. Direct method

BIAcore analysis of 100 µM Foldamer showed very low binding to biotinylated IL-4Rα [ECD] and MA-PEG-Biotin IL-4 121SH (Figure 3.32 and 3.33).

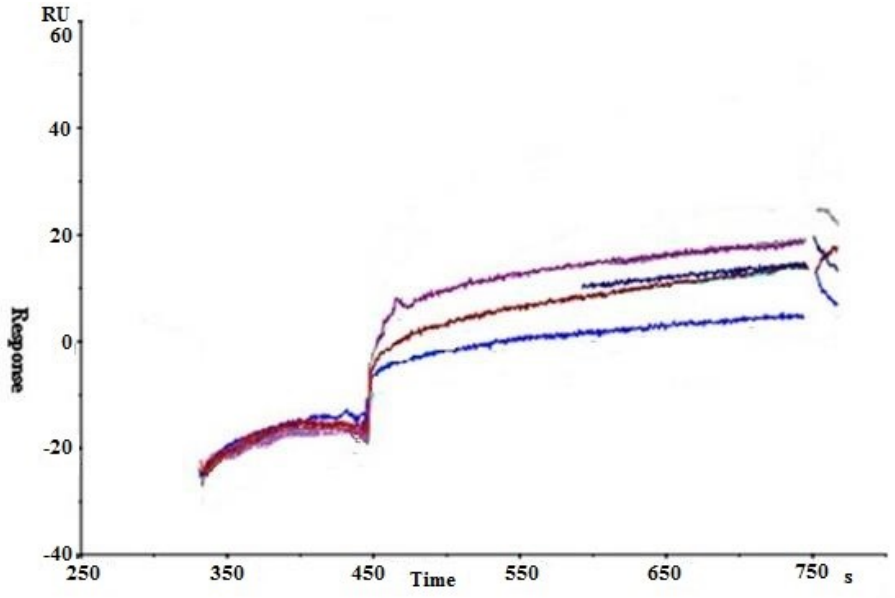


Figure 3.32: Low binding of 100 µM Foldamer to IL-4Rα [ECD].

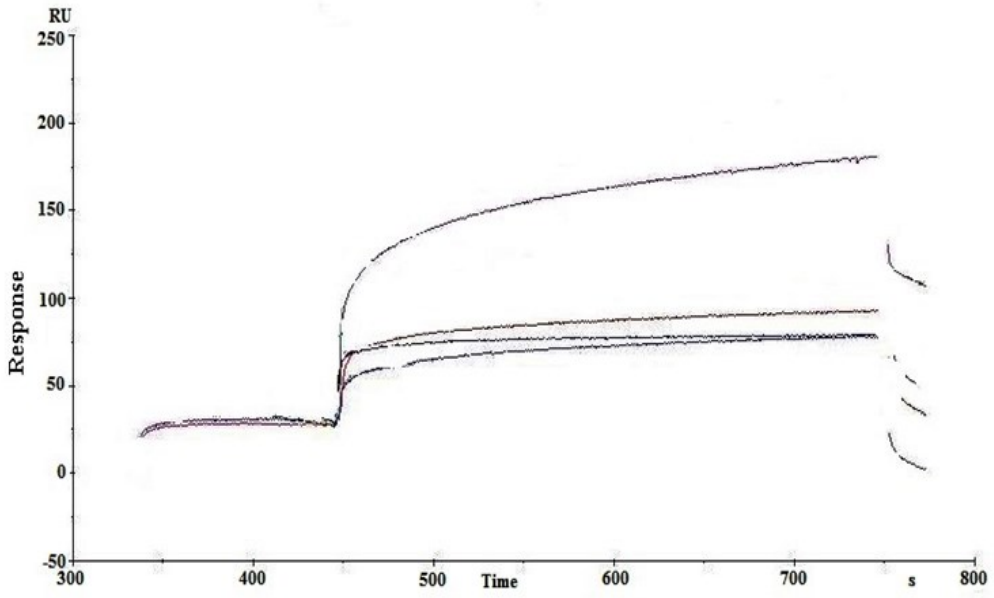


Figure 3.33: Low binding affinity of 100 µM Foldamer to MA-PEG-Biotin IL-4 121SH.

Werner Schmitz performed a mass spectrum for some of the inhibiting foldamers in order to check for their purity and exact molecular weight. The mass spectrum results showed, that all of the tested foldamers from library Braine II have large differences in their molecular weight from the results that were obtained in UCB Pharma S.A., Brussels, Belgium, as well in some cases, the desired compound was not at all present (Table 3.8) (Figure 3.34).

Table 3.8: Comparison between the MS data received from UCB Pharma A.S., Brussels and MS made by Werner Schmitz.

Foldamer	Monoisotopic Mass [Da]			
	(including H+)			
	UCB Pharma S.A, Brussels	MS Werner Schmitz	Difference [Da]	Max Peak [Da]
A6	1518,54	----	----	1596,556
B1	1517,60	----	----	1597,553
B4	1596,61	1596,382	-0,171	1520,492
E3	1597,60	1597,523	-0,026	1277.425
F1	1596,61	1598,494	1,941	1520,487
G5	1750,82	1519,543	-0,042	1521,485
H3	1597,60	1597,566	0,017	1521,488

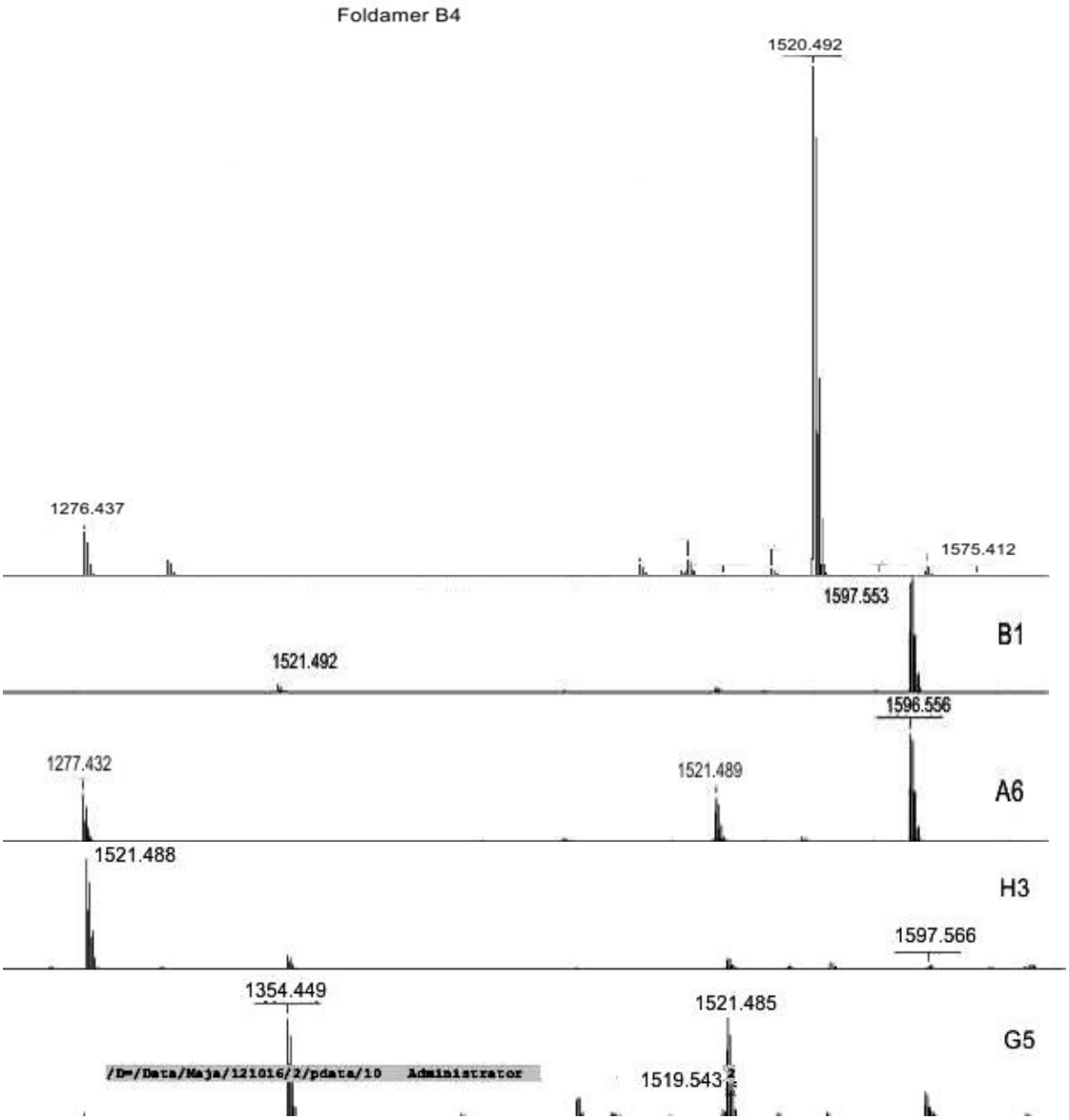


Figure 3.34: Mass spectrum data from Werner Schmitz. Foldamer B4 has a main peak 1520.492, Mw measured by Werner Schmitz, but no presence at all of Mw 1596.61, measured in UCB Pharma; Foldmare B1 – not present at all with Mw 1517.60; Foldamer A6 – not present at all with Mw 1518.54; Foldamer H3 – minor peak with Mw 1597.566; Foldamer G5 – minor peak with Mw 1519.543.

Thus, the study with the Braine II foldamer library was discontinued.

For a difference from the Braine II library, the molecular weight of the foldamers from Braine I library were exactly the same as the molecular weight from the MS data made by Werner Schmitz. Two of the foldamers, 63 and 65 showed an inhibition of the IL-4/IL-4Rα reaction. The MS data showed a main peak of the desired compound, as well as many other minor peaks with different molecular weight, due to the low purity level (Figure 3.35). To avoid any non-specific binding, further purification was performed.

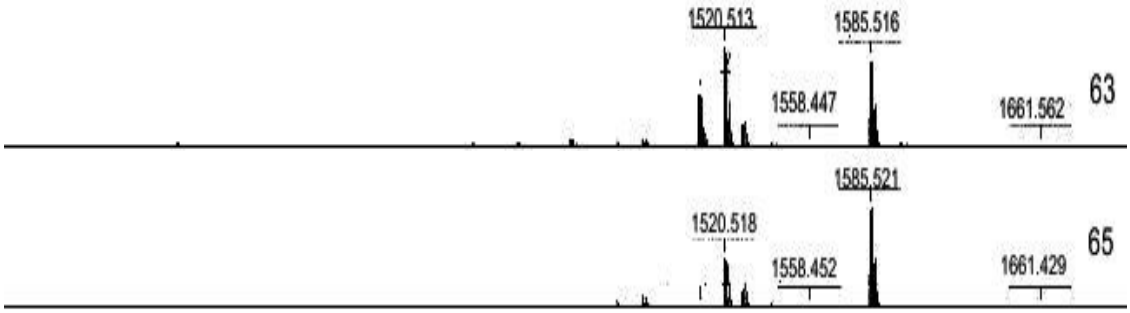


Figure 3.35: Mass spectrum for foldamer 63 and 65 showing a peak of the desired compound, Mw 1558.447 and Mw 1585.521 respectively, as well minor peaks of impurities.

The purification of foldamer 63 and 65 was accomplished by reversed phase HPLC on RP-18.
Column: Nucleosil 120-5 C18 SP250/10
250 mm long, 10 mm diameter, 5 µm particle

Buffer A: 10 mM $(NH_4)OAc$ in 0.1% Formic acid
Buffer B: Acetonitrile/Water (90/10)
Detection: Spectrophotometer – Extinction of 328 nm (Figure 3.36)
Flow rate: 0.5 ml/min
Fractions: fractions at 2 min. (fraction = 1 ml)

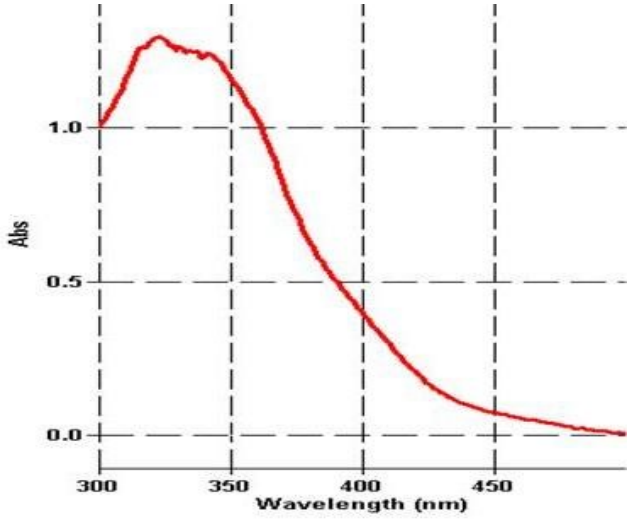


Figure 3.36: An absorption spectra of Foldamer 63. All of the foldamers from Braine I and II library showed same absorption spectra.

Table 3.9: Acetonitrile gradient and retention time, used for RP-HPLC

Time [min]	%A	%B
0 – 5	30	70
5 – 40	Linear rise to	95
40 – 55	5	95
55 - 60	30	70

Sample: 30 µl 10 mM Foldamer in DMSO is first mixed with 120 µl Buffer B, and then diluted with another 100 µl Buffer A.

After addition of Buffer A, a yellow precipitate occurred, and the analysis of the precipitate on MS showed a mass of 1520.5 Da. The samples were centrifuged and the supernatant was applied to HPLC.

The elution was according photometer (Figure 3.37).

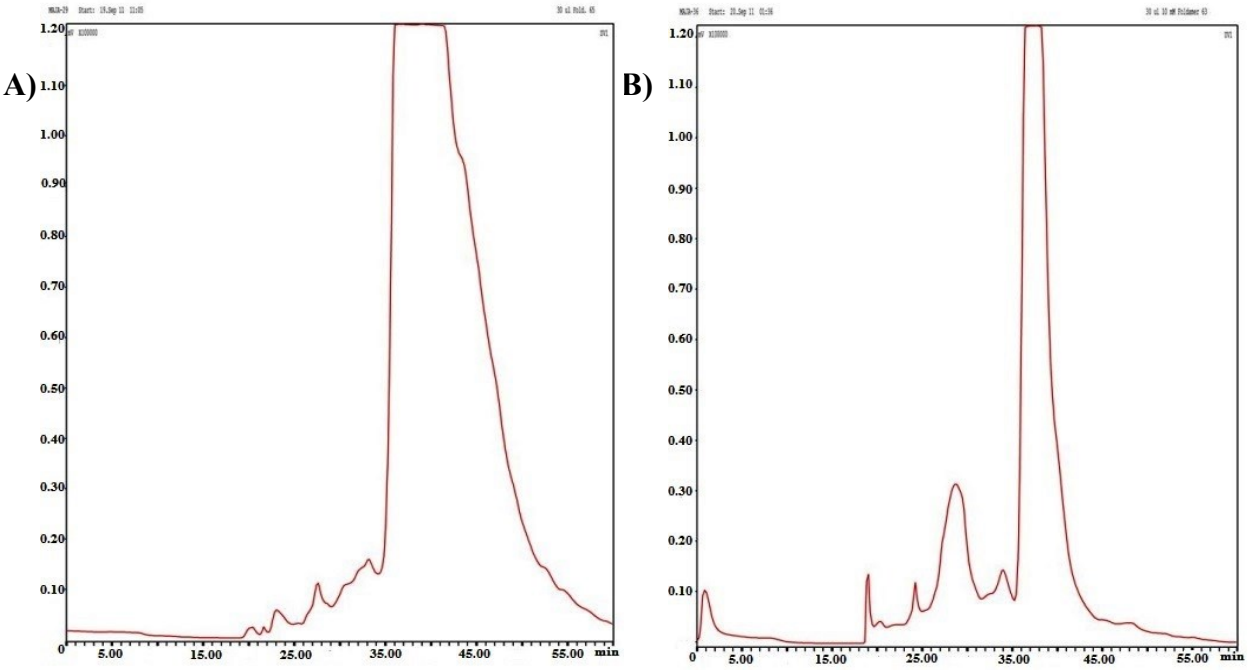
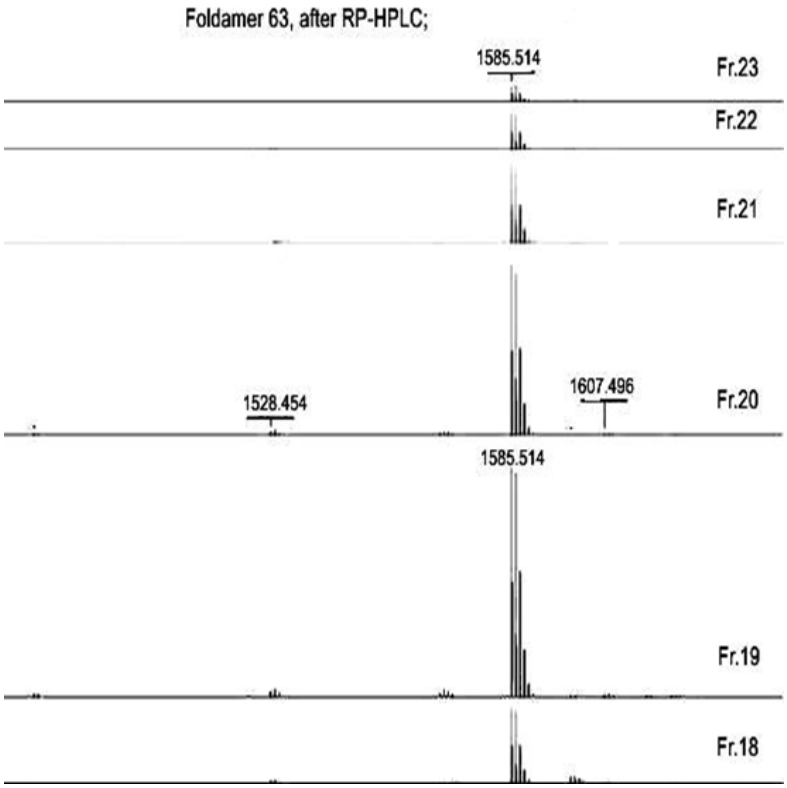


Figure 3.37: Elution chart from RP-HPLC purification of (A) Foldamer 65 and (B) Foldamer 63.

For the mass spectrum analysis, 5 µl aliquots of the main peak fractions were diluted with 50ul MeOH, and then with 45 µl MeOH/H_2O/AcOH (49.5/49.5/1).

Dilution 1:20 for the MS analyzes (Figure 3.38).

A)



B)

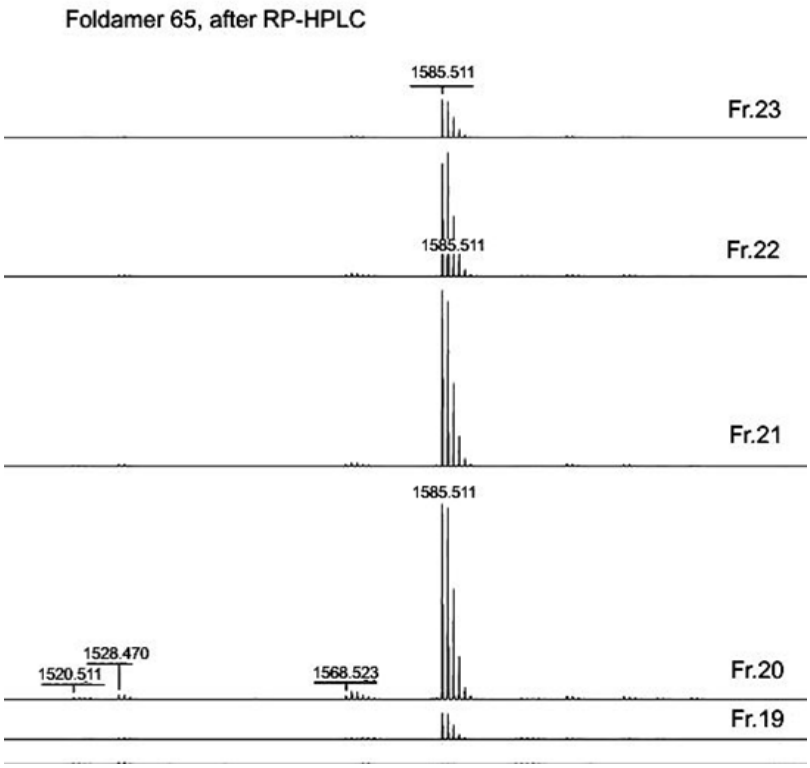
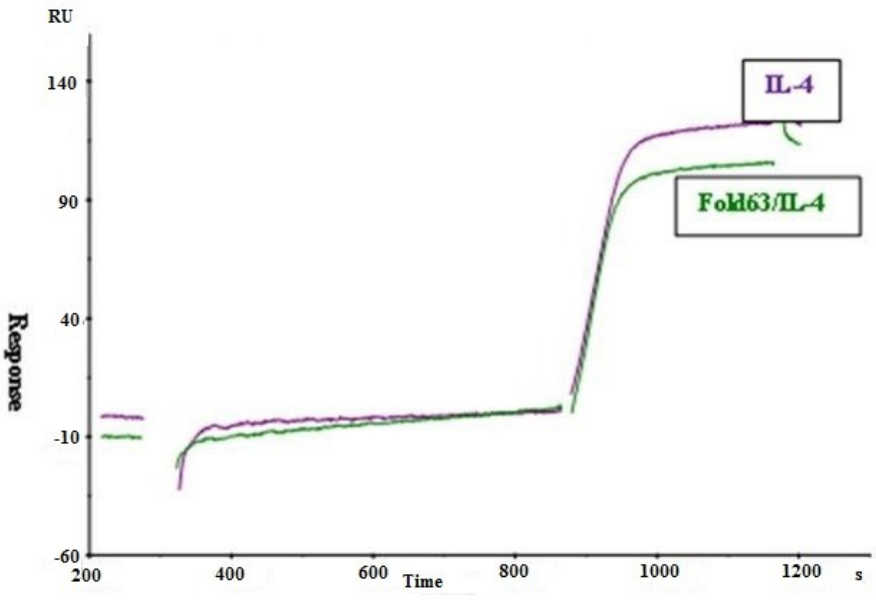


Figure 3.36: Mass spectrum of purified fractions of (A) Foldamer 63 and (B) Foldamer 65.

MS data showed clean fractions of the desired foldamers 63 and 65.

All the fractions of the purified foldamers were combined and dissolved into total of 100 µl DMSO (100%), and biosensor analysis of the purified foldamers was performed (Figure 3.39).

A)



B)

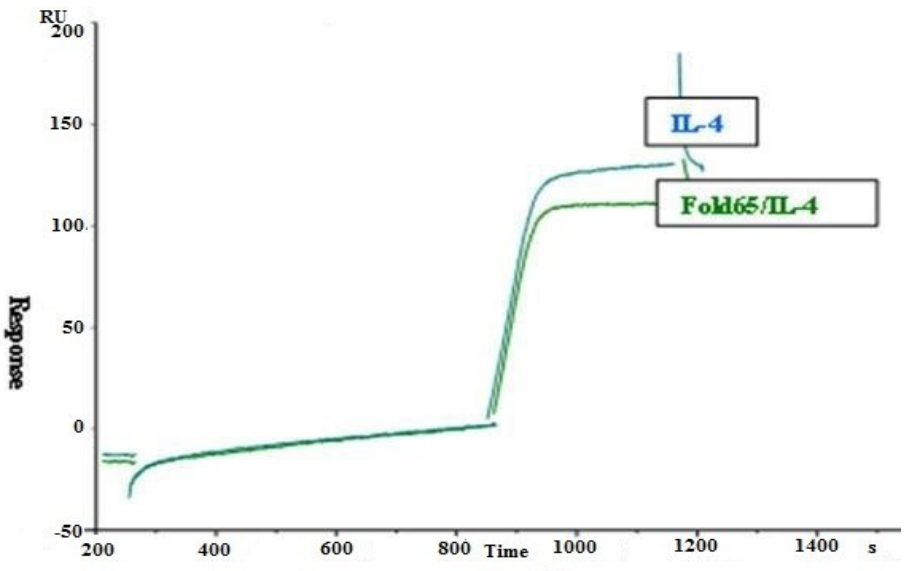


Figure 3.39: BIAcore analysis of a purified (A) Foldamer 63 and (B) Foldamer 65.

Table 3.10: Comparison of the association rate constant *k* between 20 nM IL-4 in 0.15 M HBS; 25 mM CHOPS; 1% DMSO and 20 nM IL-4/100 µM Foldamer in 0.15 M HBS; 25 mM CHOPS, after purification.

Sample	$k[M^{-1} s^{-1}]$
IL-4 wt	1.645
Fold63/IL-4	1.623
Fold65/Il-4	1.599

The BIAevaluation data showed that the purified products do not inhibit the IL-4/IL-4Rα interaction, as showed prior the purification, leading us to conclude that the previous inhibition was due to some non-specific binding of the impurities (Table 3.10).

4. Discussion

Asthma is one of the most common chronic inflammatory conditions affecting the airways. IL-4 and IL-13 are the hormones that are considered crucial for development and maintenance of airway hyper responsiveness. Therefore, strong interest and medical need are associated with the development of inhibitors of IL-4 signaling. Small molecule inhibitors of IL-4 or its receptor seem not to have been identified so far or have not been published yet. Only large biomolecules, i.e. proteins such as monoclonal antibodies or IL-4 muteins have been obtained, which modify or attenuate the IL-4 / IL-4Rα interaction thereby interfering with IL-4 activities.

The cytokine IL-4 contains three disulfide bonds but no unpaired cysteine residue, which makes it an attractive target for introducing a single unpaired cysteine residue at defined positions for subsequent site-specific chemical modification. Protein tethering has been proposed in particular to employ single cysteine muteins for the screening of low-affinity binding molecules – such as the newly defined class of foldamers.

The primary objective of this study was to generate IL-4 muteins containing an unpaired cysteine residue and characterize their applicability for post-translational protein modification usually referred to as Chemical Biology.

The second objective was to exploit the use of the newly aromatic amide foldamers for disrupting the protein-protein interaction between IL-4 and its receptor.

4.1. Production of IL-4 and IL-4 muteins for chemical modification

Cysteine residues were introduced at IL-4 positions localized near the binding epitope for the IL-4Rα chain as known from structural data (Hage T. et., al. 1999; Mueller, T.D. et al., 2002). The recombinant proteins were expressed in insoluble form in *E. coli* and the protein was extracted and solubilized from the inclusion bodies using the chaotropic reagent guanidinium chloride.

For the refolding of IL-4 and muteins thereof, new conditions were tested and optimized. Finally the refolding step containing 1.2 M guanidinium chloride was substituted by applying L-arginine at a concentration of 1 M as it gave consistently higher yields of refolded IL-4. In addition a redox couple comprising 2 mM reduced and 5 mM oxidized glutathione was also added to the refolding buffer, because in the absence of any thiol compound or redox couple the refolding yield particularly for the muteins containing an unpaired cysteine was poor. In this way, we manage to get natively folded IL-4 muteins in good yields at about 2-3 mg per wet weight of E.coli cells. IL-4 wt and IL-4 muteins were successfully purified by using a cation exchange chromatography employing CM Sepharose FastFlow resin, followed by reversed phase HPLC purification using a C4 reversed phase column. All IL-4 mutant proteins eluted at an acetonitrile concentration of 25-30%.

Subsequent mass spectrometry analysis of the purified proteins revealed that the thiol group of the additional unpaired cysteine was only partially free and to the most part disulfide bonded to the reducing agent. The three intramolecular native bonds were however always correctly formed. For wild type IL-4, only a single species with the correct molecular weight of the unmodified protein could be observed. This suggests that in the IL-4 muteins a glutathione moiety is disulfide bonded to the engineered cysteine residue.

4.2. Chemical and enzymatic reduction of glutathione-modified IL-4 analogues (set I)

Disulfide formation has been shown to be essential for many proteins such as the bovine pancreatic trypsin inhibitor (BPTI), to adopt their native fold [Creighton, T.E., 1974; Creighton, T.E., 1997]. There are basically two types of functional disulfides; forming either catalytic or allosteric bonds. The catalytic bonds are found in the active sites of enzymes that mediate thiol/disulfide exchange in other proteins, the oxidoreductases. . Allosteric disulfide bonds control the function of the protein in which they reside by mediating a conformational change when they are reduced or oxidized. Their actions are often linked in that the redox state of

allosteric disulfides is usually controlled by the catalytic disulfides of the oxidoreductases [Hogg, P.J., 2003; Hogg, P.J., 2009; Schmidt, B. et al., 2006].

In various cases, disulfide reduction led to protein unfolding, indicating that disulfides are often required for maintenance of the native conformation. Consistently, the disulfide bridges in IL-4 play a critical role in maintaining the thermodynamic stability and core packing of the helix bundle [Vaz, D.C., et al., 2006]. In several proteins, e.g. GSF, erythropoietin, BMP-2, immunoglobulin fragments, and others the native disulfide bonds seem to be protected from the environment and redox reaction and are not affected by the incubation with a molar excess of strong reducing agents like DTT and TCEP (US patent 7855275). Unfortunately, this is not the case with human IL-4. Chemical reduction of IL-4 using various chemical reducing agents led to fast cleavage of both the native and the engineered (mixed) disulfide in parallel. Both disulfide species, the native and the mixed engineered, exhibited a comparable reactivity under a variety of conditions impeding site-specific reaction conditions.

Of the three disulfide bonds of IL-4 some disulfides might contribute only little to conformational stability. As a consequence the redox potential might be increased. Functional analysis showed indeed that two of the three native disulfide bonds of IL-4 (Cys^{3} - Cys^{127}; Cys^{24} - Cys^{65}) can be disrupted mutationally by a substitution of the cysteine with threonine while preserving the biological activity whereas the disulfide bond Cys^{46} - Cys^{99} has appeared to be crucial for structure and/or function of IL-4 [Kruse, N. et al., 1991]. Structure analyses of IL-4 have provided different conformations with different accessibility for the three disulfide bonds, depending whether the structure was determined by NMR or by crystallography. A comparison of X-ray and NMR structures [Smith, L.J. et al., 1994] has shown that the disulfide Cys^{3} - Cys^{127} seems highly accessible in the IL4 crystal structure, and the disulfide bond between Cys^{46} - Cys^{99} seems highly accessible in the NMR protein structure determined in solution. A large exposed surface area of a disulfide has been shown to greatly accelerate its chemical reduction by DTT [Narayan, M. et al., 2004; Vaz, D.C., et al., 2006]. Disulfides that need global unfolding (relatively larger free energy) to be exposed are buried inside the protein, for a difference from those disulfides that can be exposed by local unfolding (relatively small free energy required, which results in more rapid reduction of the cystines) tend to be on the surface [Narayan, M. et al., 2004]. Thus a high mobility/flexibility might diminish the stability of a disulfide bond. A comparison of the temperature factors of the backbone and side chain atoms of the six cysteine

residues in wild type IL-4 (PDB entry 2B8U), shows however only little variation [Kraich, M., et al., 2006]. In summary, all available evidence suggests that the three disulfide bonds of IL-4 are placed in a very similar structural environment and exhibit a similar accessibility not different of that of the engineered and surface-exposed cysteine residue. Preliminary data further suggest that during reduction of IL-4 using dithiothreitol no prefential pathway for reductive unfolding exists. After reduction with 3-fold molar excess of DTT and subsequent modification with N-ethylmaleinimid, no preferential labeling of a particular cysteine pair that would normally form a native disulfide bond could be detected in the di-NEM IL-4 species.

The present study thus offers a different approach to prepare IL-4 single cysteine muteins to obtain a protein comprising a non-conjugated free thiol group. The IL-4 protein was refolded under conditions facilitating the formation of the native disulfide bonds together with an almost complete formation of a mixed disulfide conjugation of the additional engineered cysteine residue with the cysteine-containing tripeptide glutathione (GS). The protectin group glutathione was then enzymatically removed with high specificity by means of the redox enzyme glutaredoxin [Hu. J. et al., 2010]. The IL-4 mutein having a free non-conjugated cysteine thiol group could then be easily isolated with good yield.

The rate of enzymatic reduction depends very much on the accessibility of the mixed disulfide and thus on the position of the engineered cysteine residue. The highest rate has been observed for 38GS. Asn38 occurring in the wildtype protein is usually N-glycosylated under physiological conditions or in recombinant protein derived from eukaryotic sources indicating that the side chain is readily accessible from the bulk phase. The side chain atoms of Asn38 have high temperature factors in the crystal structure and are disordered in the NMR solution structure indicating that they are probably highly flexible [Smith, L.J. et al., 1994]. 74GS could be also reduced at a high rate and only about 4-times slower than 38GS.

Therefore the reaction time to obtain the majority of the protein with the engineered cysteine in in its reduced form had to be optimized separately for each cysteine mutein. The study has shown that a reaction time of 30 min was too short when used for the mutein 78GS, but under the same condition the reaction time was too long for the mutein 74GS, which was ‘’over-reduced’’ as seen from contaminants of IL-4 protein with the native disulfide bonds being partially reduced.

4.3. Site-specific modification of IL-4 muteins

Site-specific modification offers a chemical method to study the interaction of IL-4 with its IL-4Rα receptor. The cysteine substitution yielded a similar effect on receptor binding as the previously described alanine substitution [LaPorte, S.L. et al., 2008], but the conjugation with the tripeptide glutathione or MA-PEG resulted in additional effects. N-ethylmaleimide conjugation did not produce major changes in affinity compared to the free thiol form possibly due to the small size of the ethanolamine moiety. Conjugated glutathione had the same or in some cases and even larger effect than conjugated branched poly(ethylenglycol) (branched PEG, MW 2360 Da) when employed at positions near to or within the receptor binding epitope suggesting that the both sterical requirements and charge are important factors influencing receptor binding.

When comparing the positions of the engineered cysteines using the structure of IL-4 bound to the IL-4Rα ectodomain, the affinity of these mutants to the receptor declined with the distance of the engineered cysteine from the IL-4Rα binding epitope. A glutathione moiety at position 38 and 74 caused less than a 2-fold decrease in the affinity. Glutathione modification at position 16 and 78, which are at the periphery of the epitope, led to a 2- to 3-fold increased K_D. A glutathione attached to the cysteine introduced at position 81, which presents a minor binding determinant Arg81 for the interaction with IL-4Rα and which is partially buried in the contact forming a salt bridge to Asp66 in the receptor [Hage, T. et al., 1999], resulted in an about 50-fold reduction of its binding affinity to IL-4Rα. In a previous mutational analysis a removal of one positive charge in IL-4 resulted in a 1.3 to 2-fold reduced association rate [Wang, J. et al., 1997]. Thus the additional negative charge introduced by the glutathione in the mixed disulfide might account for the 2-fold reduced association rate. The glutathione tripeptide attached to the cysteine at position 81 in 81GS will also likely impose some steric hindrance thereby altering the rate of dissociation and association.

Previously, it has been shown that two mutations in human IL-4 are required to disrupt γc as well as IL-13α1 receptor binding. Only the IL-4DM double mutein with the substitutions R121D and Y124D was an efficient inhibitor (antagonist) of IL-4 as well as of IL-13 dependent responses

[Tony, H.P. et al., 1994]. An IL-4 single mutein with the substitution R121E was however shown to be a selective agonist stimulating immune cells via interacting with γc but to be inactive in eliciting inflammatory responses due to a loss of interaction with IL-13α1 [Shen, B.-J., et al., 1996]. Further results [Duppatla, V. et al., 2014] indicate that a single chemical modification at position 121 of IL-4 can disrupt γc as well as IL-13α1 binding and consequently the resulting analogues can inhibit IL-4 and IL-13 dependent responses in immunological and inflammatory cells. Thus, IL-4 analogues with modification at position 121 have antagonist properties similar to the IL-4DM double mutein.

Disrupting the protein-protein interactions using site-directed chemical modification offers opportunities that are not possible with genetic modification. For instance, PEGylation has showed to slow down the renal clearance of the protein leading to prolonged half-life, reduced dosage frequency, enhanced solubility, stability and resistance from proteolitic degradation [Fishburn, C.S., 2008]. PEGylation has shown an effect on immunogenicity, by suppressing and delaying the immune response [Katre, N.V., 1990; Marshall, D. et al., 1996]. It is unquestionable that site-specific chemical modification will play a pivotal role in pharmaceutical science and technology.

4.4. Foldamers

In pharmaceutical applications, foldamers may play an important role in the future as a novel class of drug scaffolds with tailored molecular shape and surface. This raises the need for more versatile frameworks with the aim of designing foldamers that are capable to bind to any given surface, and part of this study was devoted to biological testing of foldamers against the target, IL-4 and/or IL-4Rα. Surface plasmon resonance was used to provide us with the information about the binding affinity and binding specificity for non-covalent IL-4 / Foldamer and IL-4Rα / Foldamer interaction.

Because of their great properties, like their simple synthesis, high stability of their folded structures, the predictability of their architecture, and their tendency to crystallize, aromatic oligoamids are becoming an important class of foldamers [Guichard, G. & Huc. I., 2011]

Aromatic foldamers were synthesized by Michael Grotz and Dr. Michael Deligny (as described in chapter 2.16). They used the solid phase synthesis as a commonly used technique to prepare different kind of foldamers, to speed up oligomer preparation and allow the introduction of various side chains.

During our experiments, we encountered few difficulties with both of the foldamer libraries. One of the difficulties was poor solubility of the foldamer compounds. Some of the foldamers have crystalized once dissolved in 100% DMSO, other have resulted in a turbid solution.

Other problem that came out after the MS data obtained by Werner Schmitz, was that none of the examined samples from Braine Library II, contained the foldamer with desired molecular weight. All further experiments with this library were stopped.

The MS data also revealed that all the foldamers from Braine Library I have high percent of impurities. Although few of the foldamers showed an inhibition of IL-4/IL-4Rα interaction, after the purification step they could not inhibit the interaction, suggesting to a non-specific binding. Beside the peak of the desired foldamer, one of the dominant peaks in each of the sample had a molecular weight of 1520 Da, most likely due to not entirely washed out Wang resin, but that has not been confirmed.

4.5. Summary

The cytokine Interleukin-4 (IL-4) plays a crucial role in the pathophysiology and progression of asthma and other atopic diseases. Its activities are signaled into the cells upon binding to and signaling through a shared receptor complex composed of the subunits IL-4Rα and common γc. Another cytokine, Interleukin-13 shares many functions with IL-4. This can be explained by the fact that both, IL-4 and IL-13, can signal via a shared receptor complex comprising the IL-4R□ and the IL-13R□1 subunit.

Therefore, the IL-4Rα receptor subunit has become a highly promising drug target, since it mediates IL-4 and IL-13 responses and blocking IL-4Rα will abrogate IL-4 as well as IL-13 effector functions. Currently, an IL-4 based mutein (Pitrakinra), acting as a dual IL-4/IL-13 receptor antagonist is in clinical development.

This work describes the generation and production of biologically active IL-4 muteins, which contain a single additional engineered cysteine. The introduction of a free thiol group allows site-specific chemical modification. The muteins were expressed in E. coli in insoluble form, refolded and purified. The thiol group of the mutein was protected as mixed disulfide with the tripeptide glutathione.

A first attempt to chemically reduce the engineered cysteine residue failed, because the three native disulfide bonds of IL-4 exhibit a similar reactivity and chemical reduction of the native disulfide resulted in full deactivation and precipitation of the IL-4 protein. Therefore, an enzymatic approach was developed which specifically reduces the mixed disulfide bonds with an attached glutathion moiety and thus leaves the native structurally essential disulfide bonds unaltered. For optimization, four different IL-4 cysteine muteins with four cysteine residues introduced at positions close to the IL-4Rα binding site were tested and their reduction rates by glutaredoxin was determined. The enzymatic reduction occured at different rates for all four muteins indicating that accessibility is an important influence and must be determined individually for each mutant protein. After optimization of the pH value and particularly the reaction time, all muteins could be prepared with the engineered thiol group being released in reasonable yield. The proteins exhibiting the free thiol group were then modified by

N-ethylmaleimide (NEM) or maleimido-PEG. The effects of these modifications at different positions on binding to IL-4Rα were measured employing SPR biosensor technology.

In the second project of this study, foldamers, which represent a new class of stable, compactly folded biomolecules and can specifically interact with proteins and nucleic acids, were examined to identify their potential as new drugs to interfere with IL-4 activities.
Fragment-based drug discovery offers great promise for providing new starting points for drug discovery and facilitates the lead optimization. As foldamers equipped with a thiol-group for tethering could not to be produced; only the effect of foldamers present in a synthesized foldamer library on the binding to IL-4Rα could be tested. Two libraries containing different foldamers based on aromatic amide were synthesized by Michael Grotz and Dr. Michael Deligny and tested in our lab for their capability to disrupt the ligand-receptor interaction of IL-4 and its receptor IL-4Rα [ECD] using surface plasmon resonance technology. None of the studied foldamers could specifically inhibit the IL-4/IL-4Rα interaction. Some foldamers showed non-specific binding.

The study presented here shows the design and production of a potentially new type of IL-4 antagonists, which employ site-specific chemical modification to exert their antagonistic function.

4.6. Zusammenfassung

Das Zytokin Interleukin-4 (IL-4) spielt eine entscheidende Rolle in der Entstehung und Pathophysiologie von Asthma und anderen atopischen Krankheiten. Seine Aktivitäten können in die Zelle durch die Bindung an einen Rezeptorkomplex übertragen werden, welcher aus den Untereinheiten IL-4Rα und γ_c besteht. Interleukin-13 (IL-13), ein verwandtes Zytokin, und IL-4 besitzen viele gemeinsame Funktionen. Das kann dadurch erklärt werden, dass IL-4 wie auch IL-13 ihre Signale über einen gemeinsamen Rezeptorkomplex übertragen können, der aus der IL-4Rα und der IL-13Rα1 Untereinheit besteht.
Die IL-4Rα Untereinheit ist ein vielversprechendes Zielmolekül für die Entwicklung von Pharmaka, da sie IL-4 und IL-13 Reaktionen vermittelt. Durch Blockieren von IL-4Rα werden die Aktivitäten von IL-4 sowie IL-13 unterdrückt. Ein IL-4 basiertes Doppelmutein (Pitrakinra), welches als Gegenspieler zu IL-4 und IL-13 Rezeptoren fungiert, befindet sich derzeit in der klinischen Entwicklung.

In dieser Arbeit wird die Bildung und Produktion von biologisch aktiven IL-4 Muteinen mit einem einzelnen zusätzlich eingefügten Cysteinrest beschrieben. Die Einführung einer freien Thiol-Gruppe ermöglicht ortsspezifische chemische Modifizierungen. Ein „Tethering“ Ansatz sollte dann auch eine sehr schwach Bindung von thiol-reaktiven Verbindungen an IL-4 messbar machen. Die Muteine wurden in unlöslicher Form in *E. coli* exprimiert, zurückgefaltet und auf gereinigt. Dabei wurde die Thiolgruppe des Muteins als Disulfid mit dem Tripeptid Glutathion geschützt.
Erste Versuche gezielt den eingeführten Cysteinrest selektiv chemisch zu reduzieren schlugen fehl, da die drei proteineigenen Disulfidbrücken von IL-4 eine ähnliche Reaktivität zeigten, und die Reduktion zur vollständigen Desaktivierung und Fällung des IL-4 Proteins führte. Daher wurde ein enzymatischer Ansatz entwickelt, der gezielt die Disulfidbrücke zum Glutathionrest reduziert und die proteineigenen strukturell essentiellen Disulfidbrücken unverändert lässt.

Zur Optimierung wurden vier verschiedene IL-4 Cystein-Muteine mit Cysteinresten an verschiedenen Positionen nahe der IL-4Rα Bindungsstelle getestet und die Reduktionsgeschwindigkeit in Gegenwart von Glutaredoxin bestimmt. Die enzymatische Reduktion verlief für alle vier Muteine mit verschiedenen Geschwindigkeiten. Dies deutet darauf hin, dass die Zugänglichkeit der Disulfidgruppe einen wichtigen Einfluss besitzt. Die Reduktionsbedingungen mussten daher für jedes Mutein neu bestimmt werden. Nach Optimierung des pH Wertes und insbesondere der Reaktionszeit konnten alle Muteine mit einer freien Thiolgruppe in angemessener Ausbeute erhalten werden.
Die Proteine mit jeweils einer freien Thiolgruppe wurden daraufhin mit N-Ethylmaleinimid (NEM) oder Maleimido-PEG modifiziert. Die Effekte der Modifizierung an verschiedenen Positionen des IL-4 auf die Bindung an IL-4Rα wurden mit Hilfe der SPR-Spektroskopie (Oberflächen Plasmon Resonanz Spektroskopie) gemessen.

Im zweiten Teil dieser Arbeit wurde die Interaktion von Foldameren mit der IL-4Ra Rezeptorkette untersucht. Foldamere stellen eine neue Klasse von stabilen, kompakt gefalteten Biomolekülen dar, die möglicherweise spezifisch mit Proteinen und Nukleinsäuren wechselwirken können. Es sollten Vorversuche durchgeführt werden um zu sondieren, ob aus Foldameren Hemmstoffe für IL-4 und IL-13 entwickelt werden können. Da Foldamere mit einer Thiolgruppe zur Anbindung (Tethering) an IL-4 nicht hergestellt werden konnten, wurden zunächst nur nichtreaktive Foldamare aus einer synthetisierten Foldamer-Bibliothek getestet.
Zwei Bibliotheken mit verschiedenen auf aromatischen Amiden basierenden Foldameren wurden von Michael Grotz und Dr. Michael Deligny synthetisiert und von mir mit Hilfe der SPR Spektroskopie auf ihre Fähigkeit getestet, die Ligand-Rezeptor Wechselwirkung von IL-4 und derIL-4Rα Rezeptoruntereinheit zu unterbinden. Keines der untersuchten Foldamere konnte die IL-4/IL-4Rα Wechselwirkung spezifisch hemmen. Einige Foldamere zeigten eine unspezifische Bindung.

Die hier dargestellten Studien zeigen das Design und die Herstellung eines potentiell neuen Typs von Gegenspieler zu IL-4, welcher ortsspezifische chemische Modifikationen ausnutzt um seine antagonistische Funktion zu erfüllen.

5. References

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6. Publication list

1. Duppatla, V., **Gjorgjevikj, M.,** Schmitz, W., Hermanns, M.H., Schäfer, C.M., Kottmair, M., Müller, T. & Sebald, W. (2014) **IL**-4 analogues with site-specific chemical modification at position 121 inhibit IL-4 and IL-13 biological activities. *Bioconjug. Chem.* 31;25(1):52-62.

2. Duppatla, V., **Gjorgjevikj, M.,** Schmitz, W., Kottmair, M., Müller, T. & Sebald, W. (2012) Enzymatic Deglutathionylation to Generate Interleukin-4 Cysteine Muteins with Free Thiol. *Bioconjug. Chem.*, 23 (7), pp 1396–1405

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Erklärung

Erklärungen gemäß § 4 Absatz 3 der Promotionsordnung der Fakultät für Biologie der Bayerischen Julius-Maximilians-Universität Würzburg vom 15. März 1999:

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und dabei keine anderen als die hier angegebenen Quellen und Hilfsmittel verwendet habe.

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