Antibody masked cytokines as new approach in targeted tumor therapy

DISSERTATION ZUR ERLANGUNG DES DOKTORGRADES DER NATURWISSENSCHAFTEN
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For my Dad

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Table of content

List of figures

List of tables

Abbreviations

$(G_4S)_6$	glutamine serine peptide
ATP	adenosine triphosphate
BSA	bovine serum albumin
CD138	cluster of differentiation 138
CDK	cyclin-dependent kinase
CDRs	complementarity determining regions
CH1 – CH3	constant region heavy chain 1 - 3
CHR	complete hematologic remission
CL	constant region light chain
CML	chronic myelogenous leukemia
CMV	cytomegalovirus
CR	complete remission
DNA	deoxyribonucleic acid
ECM	extra cellular matrix
EDC	1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride
FBN-III	fibronectin domain type iii
FcRn	neonatal fc receptor
FDA	food and drug administration
FRET	förster resonance energy transfer
GAS	interferon gamma activated sites
GTPase	guanosine triphosphate hydrolase
HAI-1	hepatocyte growth factor activator inhibitor-1
HC	heavy chain
HCL	hairy cell leukemia
hCRs	helical cytokine receptors
HCV	hepatitis c virus
HEK293F cells	human embryonic kidney cells
His	histidine
IC50	inhibitory concentration 50%
IFN	interferon

IFNAR1	interferon alpha receptor unit 1
IFNAR2	interferon alpha receptor unit 2
IFNGR1	interferon gamma receptor unit 1
IFNGR2	interferon gamma receptor unit 2
IFNα	interferon alpha
IFNβ	interferon beta
IFNγ	interferon gamma
IgG1	immunoglobulin g type i
IL-2	interleukin-2
ILP	isolated limb perfusion
INFAR	interferon alpha receptor
IRF9	interferon regulatory factor 9
ISG	interferon stimulated genes
ISGF3	interferon stimulated gene factor 3
ISRE	interferon stimulated response elements
JAK	janus activated kinase
KiH	knobs-into-holes
KS	kaposi's sarcoma
LC	light chain
$MgCl_2$	magnesium chloride
MHC-Class I	major histocompatibility complex class i
MIP	macrophage inflammatory protein
MM	multiple myeloma
MMP	matrix metallo proteinase
MTD	maximum tolerable dose
NaCl	sodium chloride
NHS	n-hydroxysuccinimide
NSCLC	non-small cell lung cancer
PBS	phosphate buffered saline
PBST	phosphate buffered saline with tween 20
PR	partial remission
RANKL	receptor activator of nuclear factor-κb
RNA	ribonucleic acid
SPR	surface plasmon resonance

STAT	signal transducers and activators of transcription
TBST	tris-buffered saline containing tween 20
TNF	tumor necrosis factor
TSA	tumor specific antigen
VEGF®	vascular endothelial growth factor (receptor)
VH	variable region heavy chain
VL	variable region light chain

Abstract

Cytokine-based cancer therapies are commonly applied treatment strategies in oncology. A prominent representative of this potent class of anti-tumor agents is interferon alpha. Interferon is well known for its anti-viral and anti-proliferative activities and a potent therapeutic agent in the treatment of multiple myeloma, chronic myeloid leukemia (CML), hairy cell leukemia (HCL) and malignant melanoma. Unfortunately, the application of interferon is always accompanied by severe adverse side effects and shows a strong toxicological profile. In order to overcome these obstacles and generate a more suitable therapeutic agent, a bispecific interferon-antibody fusion protein was designed. By introducing multiple point mutations, the pharmacodynamic potency of the cytokine was significantly improved compared to wild type interferon by increasing its affinity to the receptor IFNAR1. In order to prevent undesired interactions of the fusion protein during circulation in the periphery and consequently decrease toxic side effects, a masking antibody was connected by a linker sequence which led to a temporary neutralization of interferon. Local reactivation of the cytokine at the tumor site was initiated by the efficient cleavage of the dual MMP-9- and matriptase-cleavable linker peptide between masking antibody and interferon with suitable turnover rates. In addition, enrichment of the interferon fusion protein at the tumor site was enforced by using one arm of the antibody for targeting the fusion protein to the multiple myeloma-associated antigen CD138, as successfully confirmed by confocal microscopy. The potent anti-proliferative activity of the cytokine in combination with a striking interferon masking effect by the linked antibody were shown in several cell lines originating from different oncologic indications. The promising approach of temporary inhibition of the cytokine in combination with tumor-selective reactivation of its pharmacodynamic potency holds the potential to overcome current limitations in cytokine-based treatment strategies and makes a broad clinical application of this extraordinary class of biomolecules in oncology feasible.

1. Introduction

1.1 History of Interferon

The story of success of interferon begins early back in 1957 when the scientists Alick Isaacs from the National Institute of Medical Research in London and Jean Lindenmann from the Swiss Academy of Medical Science launched a collaboration to investigate the interference of heat-inactivated influenza viruses on the infectiousness of intact viruses on chorio-allantoic membranes of chicken embryos (Isaacs & Lindenmann, 1957).

Prior to the above mentioned project, Lindenmann worked on red blood cells covered with heat-inactivated virus particles and the processes taking place by challenging those cells with active viruses. To answer the question why the virus particles, which are fixed on the surface of the cells, are able to induce interactions between treated and non-treated cells, Lindenmann decided to cooperate with Alick Isaacs. Due to the experience of Isaacs in the field of electron microscopy both scientists planned to visualize the events taking place between the infected and non-infected cells via a microscope. Unfortunately the experimental set-up turned out to be very challenging and the gained optical data could not be interpreted due to difficulties in discriminating between virus-loaded and empty cells (Pitha, 2007).

In a new attempt the scientists used the Melbourne influenza A strain which was heat-inactivated at 56°C for one hour and following to that, inoculated pieces of chorio-allantoic membranes with the pre-treated virus at 37°C for 24 hours. Following the incubation step, the supernatant was replaced and membranes were washed several times with buffer to remove remaining virus particles from the surface. Afterwards the membranes were transferred into fresh growth medium and challenged with intact and non-modified influenca viruses. To evaluate the reproduction of viruses inside the cells, a haemagglutinin titration series was performed. From this experiment it was seen, that membranes which were pre-treated with heat-inactivated viruses, show a strong resistance against infectious viruses from the same strain. To exclude possible interference of the experiment by remaining virus particles from the pre-treatment step, a new test set-up was carried out (Isaacs & Lindenmann, 1957).

In the final experiment chorio-allantoic membranes were treated with heat-inactivated influenza viruses overnight. Following the incubation, supernatants of the treated membranes were

collected and transferred into test tubes containing fresh membranes which have never been in direct contact with any virus particle. After incubation of the chorio-allantoic membranes in the medium derived from the previous performed virus particle treatment, infectious influenza A viruses were added to challenge the membranes. On the next day the viral replication rate inside the cells of each membrane was determined and it was observed, that the fresh membranes which have never been in direct contact with viral particles before, showed a surprisingly high and significant resistance against the attack of intact influenza viruses mediated by an inhibition of viral replication. A schematic drawing of this experiment can be found in figure 1 (Pitha, 2007).

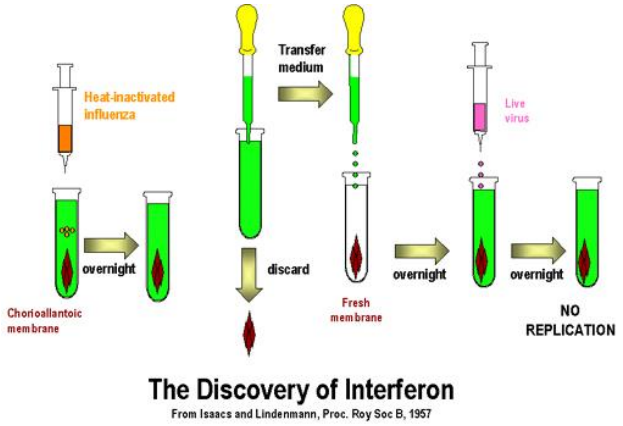


Figure 1: Discovery of interferon mediated viral resistance

Supernatants from chorio-allantoic membranes, pre-treated with heat-inactivated viruses, were transferred to fresh membranes followed by challenging them with intact influenza A viruses. Mediation of viral resistance by an unknown agent contained in the transferred supernatant was determined. (Isaacs & Lindenmann, 1957)

From their experiments, Isaacs and Lindenmann concluded that there must be a kind of interfering agent which mediates viral resistance between treated and non-treated cells without getting into close proximity. Due to this kind of “interference” they named this unknown agent “interferon”. At that moment both scientists had no idea that they had discovered the first cytokine and what tremendous impact this discovery would have on anti-viral and oncological indications in future times (Pitha, 2007).

Jean Lindenmann presented the outcome of the virus induced interference between cells and the discovered mediating agent the first time at a meeting of the Swiss Society for Microbiology in Interlaken in 1957. This was the first time the cytokine interferon became apparent in scientific community and the kick-off for further research in the field of cytokines was initiated (Pitha, 2007).

1.2 Half a century in interferon research

Over the past fifty years the understanding of interferon-induced molecular mechanisms inside mammalian cells and the clinical application of the cytokine led to major break-troughs in anti-viral therapies and the continuous battle against cancer. It quickly became clear that this paracrine and autocrine signaling molecule reveals the potential to revolutionize treatment strategies in infectious diseases and oncological malignancies (Borden et al., 2007).

However, it took a long way from the discovery of interferon in 1957 to its characterization, the understanding of the mode of action and the clinical application of the cytokine. Before promises made at its discovery were able to be kept, more than a decade elapsed until new technologies in molecular biology raised the opportunities to investigate this cytokine in more detail. In the first years of interferon research scientists mainly tried to answer the question about how synthesis of the molecule is induced and the effects that are later on triggered *in vivo*. In the early 60s it was already shown that interferon is able to mediate viral resistance in mice and its expression is not limited to viral infections, but rather can be initialized by other stimulants like microbial products of bacteria, protozoa and virus particles (Borden et al., 2007).

One of the main observations in the first years of interferon research was the discovery of its anti-proliferative activity on tumor cells, shown in several mouse models. In 1969, the group of Ion Gresser inoculated Balb/c and C57/B1$_6$ mice with 2000 – 3000 tumor cells and determined the overall survival rate of untreated mice compared to animals inoculated with interferon. The experiment showed only 3.7% of the untreated mouse population survived more than 22 days. In contrast to that, the treatment of mice with interferon led to an increased survival rate of 98% within the first three weeks and an overall survival of 15% after 60 days. None of the surviving mice showed any signs of remaining tumor mass and all of them stayed progression free (Gresser, Bourali, Lévy, Fontaine-Brouty-Boyé, & Thomas, 1969).

These promising results from early *in vivo* studies enforced the expectations and efforts to apply interferon therapy also for human beings. Already in 1977 the anti-proliferative effects of interferon on human derived tumor cells were able to be confirmed in non-small cell lung cancer (NSCLC) cell lines *in vitro*. In this first set of experiments, the tumor cells were treated with different subtypes of interferon in mono-therapy or combination with chemotherapeutic agents to determine the most effective treatment strategies for following clinical studies (Fujie et al., 2011).

Between 1957 and the late 1970s, the application and study of this cytokine was limited by the low amounts and low purities of material which was isolated from cell cultures stimulated with viral particles. This limitation came to an end in 1980, when striking developments in molecular biology provided the opportunity to produce interferon in a recombinant way. One of the early attempts to produce the cytokine in a recombinant way was performed by the group of Shigekazu Nagata at the institute of molecular biology at the University of Zürich. The scientists isolated the 12S fraction of poly-A RNA, which was coding for interferon, from interferon producing human leukocytes. After back-translation of the RNA by reverse transcriptase, the generated cDNA was cloned in a pBR322 bacterial vector plasmid. As an expression host the *E.coli* strain HB101 was chosen and transfected with plasmids containing the DNA sequence coding for interferon. Resulting to these efforts, a bacterial clone was identified which produced a polypeptide with an interferon-like biological activity in a range of 10.000 IU/g cell mass. In further analyses the previously gained inserts in *E.coli* DNA were isolated and hybridized with poly-A RNA coding for interferon to verify the correct incorporation of the IFN coding gene and to link the observed biological effects to the expression and secretion of interferon (Nagata et al., 1980).

The new acquired possibility to produce interferon in high amounts and with a suitable grade of purity enforced the research in the field of cytokines. From this point of time it was possible to generate sufficient material for animal studies and first clinical trials in humans were launched. Only one year after the initial production of recombinant interferon, first investigations in cancer patients and multiple sclerosis patients were launched with significant treatment effects. In the following years different subtypes of interferon were identified and the mode of action of this class of cytokines was investigated in more detail. From the beginning to the middle of the 80s, more and more intracellular pathways, which are activated by interferon, became clear and first interferon stimulated genes (ISG) were reported (Borden et al., 2007).

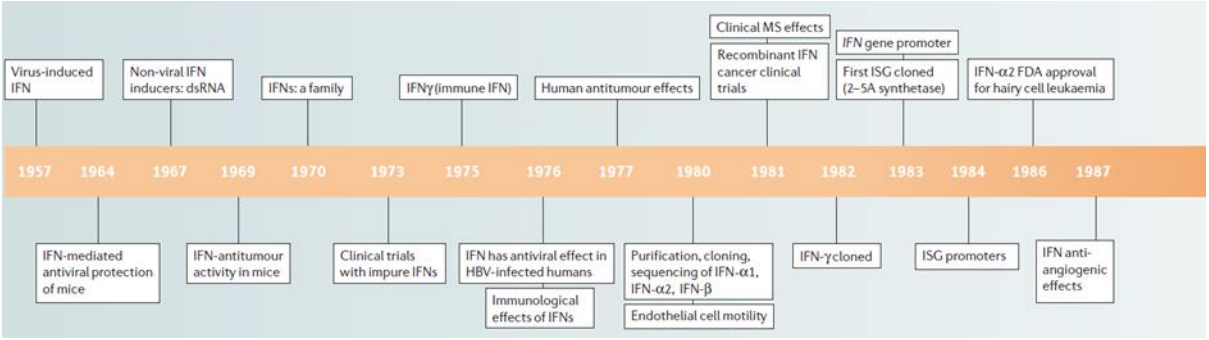
One of the major break-troughs in the history of interferon was the approval of recombinant interferon alpha 2a for clinical application in hairy cell leukemia patients in 1986. This first approval of a cytokine in cancer therapy has led to enormous efforts in pharmaceutical industry to produce high amounts of this new miracle drug. Due to extensive marketing strategies and first beneficial results from the clinic, people became aware of the new drug and a growing need for interferon aroused (Eckart, 2002).

This vital momentum pushed interferon research to a new level and more and more clinical applications of this molecule were investigated in further detail. Due to positive clinical study results and the enforced marketing of interferon by the industry, an interferon hype was initiated

and this new miracle drug was published at the cover page of prestigious newspapers like The New York Times or the German Spiegel. Interferon was considered as the most potent drug against cancer and clinical trials for several oncological indications were launched. At this time in 1990, the Federal Food and Drug Administration (FDA) approved the application of interferon also for anti-viral therapies against Hepatitis C (HCV). Because of the duel functionality of the cytokine against viral invaders and malignant cell proliferation, researchers started to study the exact mode of action of interferon to answer the question about how the pleiotropic effects of this molecule are triggered and which pathways are activated in anti-viral and anti-proliferative action (Borden et al., 2007).

The decade from 1990 to the year 2000 was marked by the investigation of interferon signaling pathways and the identification of the main signal transducing molecules Janus activated kinase (JAK) and the Signal Transducers and Activators of Transcription (STAT). In addition to that, the three dimensional structure of interferon in complex with both receptor units was determined via crystal structure analysis. Following the identification of the intracellular signaling, it became clear that the pleiotropic mode of action of interferon cannot be explained by a single activated downstream cascade. New insights in the mode of action of interferon were disclosed in the end of the 90s by the characterization of hundreds of interferon stimulated genes (ISGs). Due to the activation of only one main downstream signaling pathway by interferon, the pleiotropic action of the cytokine can be possibly traced back to a different transcription profile of the newly discovered interferon-stimulated genes (Borden et al., 2007).

The following illustration summarizes major milestones in interferon history and gives a brief overview about the historical development and scientific efforts in the characterization and clinical application of this miracle drug.



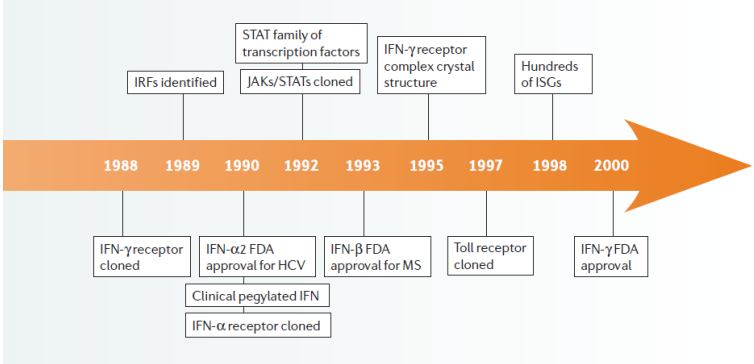


Figure 2: Major milestones and discoveries in fifty years of interferon research

The above shown image summarizes major milestones in interferon research. Starting with the discovery of the cytokine in 1957, early investigations and characterizations of interferon until the end of the 70s and major break-troughs and clinical studies using IFN from 1980 up to 2000. (Borden et al., 2007)

In the past 50 years of interferon research, more than 100.000 scientific papers were published but there are still a lot of open questions which have to be addressed. From the first observation of anti-viral activity of the cytokine to the characterization of a genetic transcription profile, it took a lot of time, effort and money, but further investigations are necessary to identify the exact mode of action, to control its side effect profile and to broaden the clinical application of this cytokine. Perhaps the second half of the interferon century will bring up new technologies to gain deeper insights into the field of interferon research and help to answer questions which have been open for more than fifty years and are still open today.

1.3 Classification of interferon

Since the discovery of interferon by Isaacs and Lindenmann, it became clear that there must be several different kinds of this cytokine which all belong to the same family of interferons. At the early beginning of interferon research, newly found subtypes were categorized according to the cell type they originated from. At these times IFN-α was known as leukocyte interferon and IFN-β as fibroblast IFN. Nowadays natural occurring interferon subtypes are named by their coding gene and the corresponding protein sequence (Bekisz, Schmeisser, Hernandez, Goldman, & Zoon, 2004).

The cytokine family of interferons (IFNs) is divided into two major classes, Type I and Type II interferons. The class of Type I interferon consists of seven different subtypes of this cytokine family: IFN-α, IFN-β, IFN-ω, IFN-κ, IFN-ε, IFN-δ and IFN-τ. Most prominent representatives of this

Type I interferons are IFN-α and IFN-β according to their extensive biological relevance and clinical trials performed with both agents. IFN-τ was only found in ruminants and giraffes but could not be found in human beings. Although it shows biological activity if administered to the human system. [Bekisz 2004] Similar to that, IFN-δ occurs only in pigs and is produced by trophoblasts, which form the outer layer of a blastocyst. In contrast to IFN-τ, IFN-δ does not show any activity in humans (Lefevre, Guillomot, D'Andrea, Battegay, & La Bonnardiere, 1998).

IFN-κ, produced by keratinocytes, and the less well characterized IFN-ε display only limited anti-viral and anti-proliferative activity and seem to be less important in respect to biological functions. From the functional point of view IFN-α with its thirteen subtypes and the single type IFN-β are the major mediators of viral resistance and suppressors of malignant proliferation inside the Type I interferon family. IFN-ω builds up the third potent anti-viral member of this cytokine family and is secreted by viral infected leukocytes. Interferon alpha (IFN-α) consists of thirteen different family members which display a close structural homogeneity. The genetic information for these several subtypes is located in the same region on chromosome 9 in the eukaryotic genome. All types of IFN-α consist of 166 amino acids with interferon alpha 2a being the exception with only 165 due to a deletion of a single amino acid at position 44. These different types of IFN-α show a strong sequential similarity in their peptide code in the range of 75 – 99%. The three dimensional structure of those cytokines consist of 6 alpha-helices and structural integrity is ensured by two disulfide bridges (Bekisz et al., 2004).

The following table summarizes the nomenclature of the thirteen members of the IFN-α family and their corresponding genes.

IFN-α protein subtype	Corresponding IFN-α gene
A	2a
2	2b
B2	8
C	10
D (Val^{114})	1
F	21
G	5
H2	14
I	17
J1	7
K	6
4a	4a
4b	4b
WA	16
1 (Ala^{114})	1

Table 1: Nomenclature of natural occurring IFN-alpha subtypes

Summary of natural occurring interferon alpha subtypes and corresponding nomenclature according their protein and gene name. (Pestka & Meager, 1997)

Compared to the complex classification of Typ I interferons, the family of Type II interferons only consist of a single member termed IFN-γ. IFN-γ is mainly secreted by T-lymphocytes after activation by antigen binding. This Type II interferon owns a significant immune-modulatory capacity and evolves strong anti-viral and anti-tumor activity by the recruitment of macrophages and increased expression of antigen presenting MHC-Class I molecules (Pestka, Krause, & Walter, 2004).

Since 2002 an additional class of interferon-like molecules is known. The members of this family are classified as a new type of interferon because they also possess anti-viral activity and show similarities in the downstream signaling via the Jak-STAT pathway. This so called Type III interferon family contains three different interferon lambdas termed IFN-λ1, IFN-λ2 and IFN-λ3. These three cytokines are also known as IL-29, IL-28A and IL-28B. The genetic information for these types of interferon λ is located at chromosome 19 in contrast to genes for Type I and Type II interferons which are positioned on chromosome 9 (Kotenko et al., 2003).

The previously mentioned interferon subclasses are distinguished from each other not only by their amino acid sequence and the cell type of origin, furthermore they can also bind to different interferon receptors with varying affinities. In case of IFN-α subtypes, the functional unit of the molecule acts in a monomeric fashion whereas IFN-β binds to its receptor in a dimer-like fashion. In contrast to INF-α, IFN-γ builds up a tetrameric structure to bind to its receptor and induce signal transduction. A brief overview about interferon receptors and the intra cellular signaling cascade will be given in the following part of the thesis (Pestka et al., 1983).

1.4 The interferon receptor family and downstream signalling

Each of the previously described interferon types uses its own cell surface receptor complex which is composed out of multiple chains. These three different kinds of cytokine receptors belong to the group of class II helical cytokine receptors (hCRs) and share similar structures and basic elements. The extracellular domain of interferon receptors is build up by tandem domains with a length of up to 100 amino acids. These extracellular regions contain two type III fibronectin domains (FBN-III) which are similar to the constant domain of an antibody. An exception in the number of extracellular domains is represented by the receptor unit 1 (IFNAR1) of the Type I interferon receptor complex which is composed of four tandem domains. The genes coding for Type I and Type II interferon receptors are located close to each other at chromosome 21 and

seem to be evolutionary conserved which indicates a possible functional connection between the receptors and the triggered downstream signaling cascade (de Weerd & Nguyen, 2012).

Despite the heterologous group of Type I interferons and the differences in their peptide sequence, all members of this class signal through the same receptor complex of IFNAR1 and IFNAR2. These two receptor units differ significantly in their affinity for Type I interferons. IFNAR 2 shows high affinity binding to IFN-α or IFN-β in a nM range whereas IFNAR 1 binds in a low affinity mode at µM concentrations. The common form of IFNAR2 is termed IFNAR2c which is composed of an intact intracellular domain connected to the two extracellular tandem domains via a long transmembrane region. IFNAR2c is inevitable for complete signal transduction to induce anti-viral and anti-proliferative activity of interferon. In addition, there are two known splice variants of IFNAR2. IFNAR2b consists of a short transmembrane region without an intracellular domain while IFNAR2a is the soluble form of the receptor lacking the transmembrane and the intracellular domain of IRNAR2c (de Weerd & Nguyen, 2012).

Fully intracellular signaling in case of Type I interferons is engaged after binding of interferon to the receptor unit 2 (IFNAR2) followed by the formation of a ternary complex with IFNAR1. This formation of the IFNAR-complex is common for all members of the Type I interferon family but until these days it is not fully understood how the different biological activity of interferons is mediated via binding to the same receptor units. It was shown that the various binding affinities of the different interferons to their receptors, the stability of the ternary IFNAR complex and the total amount of receptors expressed on the cell surface can influence the expression profile of interferon-stimulated genes (ISGs) and the triggered biological effects (de Weerd & Nguyen, 2012; E. Kalie, Jaitin, Podoplelova, Piehler, & Schreiber, 2008).

The intracellular signal transduction of the IFNAR complex is mediated by the Janus activated kinase 1 (JAK1) located at the intracellular domain of IFNAR2 as well as tyrosine kinase 2 (Tyk2) coupled to IFNAR1. After formation of the ternary complex, JAK1 becomes activated via auto-phosphorylation and triggers the recruitment of STAT (signal transducers and activators of transcription) molecules. Phosphorylation of the receptor-associated kinases induces the activation of the classical Jak/STAT pathway mediated by the phosphorylation of the signal transducers STAT1 and STAT2 which allows STAT homo- or heterodimerization. In case of Type I interferon signaling, the heterodimer composed of phosphorylated STAT1 and STAT2 forms a complex with IRF9 (interferon regulatory factor 9) to build up a transcription factor. This STAT1-STAT2-IRF9 complex, also known as ISGF3 (IFN-stimulated gene factor 3), then translocates into the nucleus and initiates the transcription of interferon-stimulated genes (ISGs) via binding to the

upstream positioned interferon stimulated response elements (ISRE) which act as promoters for ISGs (Platanias, 2005).

In contrast to different Type I interferons, which bind to one common receptor complex, the only Type II interferon IFN- γ interacts with its own cell surface receptors. This Type II receptor complex is composed of two subunits termed IFNGR1 and IFNGR2. Similar to the Type I interferon receptors, the intracellular domains of the receptor are associated with kinases to induce signal transduction. JAK 1 is located at the IFNGR1 subunit while IFNGR2 is associated with the JAK2. Both kinases trigger the phosphorylation of STAT1 molecules which then form homodimers before being translocated into the nucleus. Once in the nucleus, these STAT1 dimers bind to IFN-γ activated sites (GAS) in the genome and induce transcription of certain interferon stimulated genes. The formation of STAT 1 homodimers is not limited to Type II interferon receptor signaling. Phosphorylated STAT 1 molecules activated by Type I interferon signaling can also dimerize and lead to a crosstalk between Type I and Type II signal transduction (Platanias, 2005).

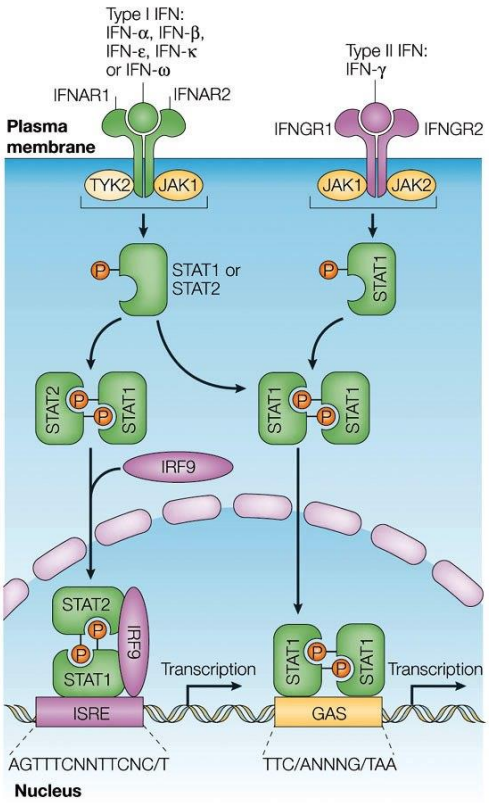


Figure 3: Interferon receptors and associated downstream signaling cascades

Visualization of the downstream signaling cascade of Type I and Type II interferons and their corresponding receptors. Activation of receptor-coupled kinases (JAK1, TYK2), triggers phosphorylation and activation of signal transduction proteins (STAT1 and STAT2). In case of Type I signaling, STAT1 and STAT2 dimerize and form a transcription complex in combination with IRF9. Type II signaling is mediated by phosphorylated STAT1 homodimers. After translocation of the transcription complex to the nucleus, transcription of interferon stimulated genes is initiated. (Platanias, 2005)

In addition to the main downstream signaling of interferons via the Jak/STAT pathway an alternative signaling pathway was identified with possible roles in the pleiotropic biological functions of this class of cytokines (shown in figure 4). In this alternative pathway, activated tyrosine kinase 2, interacting with the intracellular subunit of IFNAR1, triggers the phosphorylation of an adaptor protein termed CRKL. Due to this transient tyrosine phosphorylation of CRKL, the C3G-Rap1 signaling pathway becomes activated, leading to the recruitment of the small GTPase Rap 1. Rap 1 is associated with different functions in cell proliferation, differentiation and cell adhesion. It was shown that this small protein is able to mediate direct suppression of cell growth but in some circumstances can even promote proliferation of cells. Due to this opposed biological functions, it is believed that the mode of action of Rap 1 depends on the kind of stimuli triggering recruitment of C3G-Rap1 signaling. The activation of this alternative signaling cascade was also shown in the treatment of human cell lines by the Type II interferon IFN-γ which indicates a possible significant biological role in the anti-proliferative action of this class of cytokines (Ahmad, Alsayed, Druker, & Platanias, 1997; Alsayed et al., 2000).

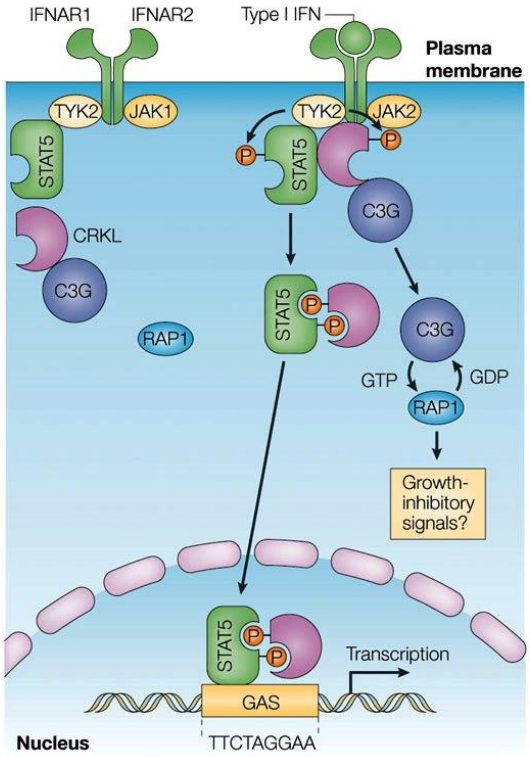


Figure 4: Alternative interferon pathway besides classical JAK-STAT signaling

Alternative signaling cascade of Type I interferons mediated via Crkl protein. Phosphorylation of CRKL triggers the signal transduction via C3G and the activation of RAP1 which strongly interacts in cell cycle control mechanisms. (Platanias, 2005)

1.5 Interferon in oncology practice

Although the discovery of interferon has been early back in 1957, it took more than twenty years to produce this class of cytokines in a recombinant way to obtain sufficient amounts of material for performing clinical investigations. First clinical studies were conducted with crude formulations of cell lysates containing less than 1% interferon. The establishment of cloning procedures in the 1980s raised the opportunity to express large quantities of interferon with high grade purity and enabled the evaluation of the cytostatic, immune-modulating and antiviral activity of interferon *in vivo*. Interferon-based treatment strategies in oncologic and infectious diseases always deal with balancing the pharmacological effect of the cytokine and the prevention of adverse side effects. Due to significant unwanted responses, IFN therapies narrow the optimal treatment ability for physicians and side effects strongly influence patient's quality of life. In oncology research, therefore understanding of the biological mode of action, the choice of suitable clinical indications and the management of the side effect profile and toxicities of Interferon play an important role to exploit the full potential of this class of cytokines (Jonasch & Haluska, 2001).

Clinical indications for interferon alpha in oncology comprises several hematological cancers and solid tumors, including hairy cell leukemia (HCL), chronic myelogenous leukemia (CML), follicular lymphoma, multiple myeloma (MM), AIDS related Kaposi's sarcoma (KS) and malignant melanoma. In addition to the application of interferons in oncology, IFNs play a major role in anti-viral therapy such as chronic hepatitis B and chronic hepatitis C (Rote Liste® 2014). The following section summarizes the main clinical oncological indications with regard to interferon-induced side effects.

The majority of interferons used in the clinic are nowadays produced in recombinant form by expression in eukaryotic cells, followed downstream purification methods ensuring very high purity. For clinical application, prominent interferon preparations are Roferon-A™ (IFN-α2a, Roche) and Intron-A™ (IFN-α2b, Schering-Plough Corporation). Both types of interferon alphas are highly similar in their amino acid sequence and differ just in one position. Besides these two interferon alphas, other interferon subtypes like IFN-β1a (Avonex™, Biogen Inc), an interferon beta type with a serine to cysteine alteration and IFN-β1b (Betaseron™, Berlex), which is expressed in an *Escherichia coli* strain, found their way into the clinic. As an alternative medication to Rofern-A™ and Intron-A™ in chronic hepatitis C treatment, the IFN alfacon-1 (Infergen™, Amgen), an artificial synthesized interferon, based on the sequence comparison of

natural occurring IFN alphas, is approved. The only Type II interferon product on the market (IFN-γ, Actimmune™, Genentech Inc) was approved by the U.S. Food and Drug Administration (FDA) for its immune-modulatory function in the treatment of chronic granulomatous disease (Jonasch & Haluska, 2001).

Interferon alpha therapy in hairy cell leukemia (HCL) was approved in 1986, justified by a multicenter study of 64 patients performed by Harvey M. Golomb et al. in the previous years. In this study, patients were treated with interferon alpha 2b (Intron-A™) subcutaneously at a dosage of 2 x 10^6 U/m^2, three times a week. The outcome of the study displayed complete remission (CR) in three patients (5%), 45 patients (70%) showed up partial response (PR) and nine patients (14%) a minor response. Only three of the 45 patients had no beneficial effect despite interferon treatment. After treatment duration of five month, hemoglobin levels and granulocyte counts re-approached to physiological levels. The evaluation of bone marrow biopsies showed a significant decrease in the median hairy-cell index by half and serious viral infections during treatment were prevented by interferon. Due to promising first responses of interferon treatment, IFN-α2a and IFN-α2b are still applied in HCL therapy with treatment durations usually over two years. Nevertheless later studies showed that patients relapse after therapy and alternative treatment strategies with 2-deoxycoformycin and cladribine in hairy cell leukemia seem to be more beneficial (Golomb et al., 1986; Jonasch & Haluska, 2001).

Hematologic malignancies in particular show a high treatment potential for interferon administration compared to solid tumors. One of these hematological indications represents chronic myelogenous leukemia (CML). Leading clinical evaluations of interferon in CML treatment were performed by the group of *Talpaz et al.* in the early 1980s. They evaluated the influence of interferon alpha 2a (Roferon-A™) on 17 patients suffering from CML, which were positive for the Philadelphia-Chromosome [translocation between chromosome 9 and 22, t(9;22)(q34;q11)]. In this study, interferon alpha 2a was administered daily intramuscularly at a dosage of 5 x 10^6 U/m^2 body surface. Out of 17 patients, 14 showed a response to IFN-α2a treatment. 13 patients displayed a complete hematologic remission (CR) and just one patient a partial remission (PR). The analysis of cytogenetic tests unveiled the suppression of Philadelphia-Chromosome positive cells in 25% of the patients responding to IFN treatment. Besides the promising outcome of the study, physicians had to deal with side effects triggered by interferon which led to treatment interruptions in two patients during the interferon therapy for 9 – 15 month (Talpaz et al., 1986).

A comprehensive study on the anti-proliferative activity of interferon in CML was conducted by the Italian Cooperative Study Group on Chronic Myeloid Leukemia in 1994. To investigate the

potency of interferon compared to conventional chemotherapy, 322 CML patients were treated either with IFN-α2a (Roferon-A™) or a standard chemotherapeutic agent, hydroxyurea or busulfan. In the interferon treated patient population (218 patients), a karyotypic reaction was detectable in 30% compared to only 5% of patients treated with standard chemotherapy (104 patients). The duration from the chronic phase of CML to a more severe blastic phase was significantly prolonged in the interferon treated group (72 months in the IFN-group, 45 month in the chemotherapy group). In addition to the decelerated disease progression, the overall survival of patients in the IFN-group was 21% higher than in the standard therapy group over a period of six years. Many other studies at that time confirmed the beneficial effect of interferon monotherapy compared to cytotoxic agents used in chemotherapeutic treatment of CML (Leukemia, 1994).

During 1980s another effective compound against CML was investigated termed cytarabine (ara-C). Ara-C was shown to efficiently suppress proliferation of malignant myeloid cells, when given via continuous infusion at low doses. The group of *Kantarjian et al.* performed a study on 140 patients positive for Philadelphia-Chromosome in early chronic CML. Interferon was administered subcutaneously in a daily dose of 5 x 10^6 U/m^2 body surface, in combination with a low dose infusion of ara-C at 10 mg/day. Due to the combination of interferon and ara-C a complete hematologic remission (CHR) was achieved in 92% of the treated patient population with a cytogentic response in 74% of responding patients. Despite the promising results of the combination therapy, several adverse events were reported. A significant part of the patients suffered from fatigue accompanied by weight loss, muscle ache, diarrhea and neurologic disorders. According to the outcome of the study, the combination of interferon with low-dose ara-C in the treatment of early-stage CML seemed to be beneficial due to the reduction of the applied interferon doses to a minimal but still effective level and the subsequent prevention of severe side effects (Kantarjian et al., 1999).

Beside the main clinical indications of interferon in malignant hematologic diseases, several kinds of solid tumors can be treated efficiently. One example is the application of interferon in combination with other cytotoxic agents in the treatment of malignant melanoma. For growth inhibition of this aggressive and highly metastatic cancer type, many combination studies including interferon therapies were conducted. In early attempts IFN-alpha was combined with another cytokine, namely interleukin-2 (IL-2). Unfortunately the combination of both immune-modulating agents did not show an additional anti-tumor effect. Other clinical trials in malignant melanoma combining interferon with conventional chemotherapeutics like cisplatin, dacarbacin

and vinblastine showed an increase in treatment efficacy which reached significant levels but were limited due to severe toxicities occurring by overstimulation of the immune system. A more beneficial application of interferon in malignant melanoma therapy was shown in clinical trials with an adjuvant setting, by using IFN as a follow-up therapy after initial chemotherapy. In one of these trials, published by *Kirkwood et al.*in 1994, 287 patients were treated with INFα2b (Intron-A™) following surgical removal of the tumor mass. In the first month after surgery the maximum tolerable dose of interferon 20 x 10^6 U/m^2 per day was administered intravenously. Following the initial high dose treatment, 10 x 10^6 U/m^2 INFα2b were given subcutaneously three times a week over a period of 48 weeks. Summarizing the data generated during this study, performed by the Eastern Cooperative Oncology Group, the relapse-free interval of patients after surgery was prolonged from 1 to 1.7 years due to adjuvant interferon therapy. The overall survival of high risk patients was significantly increased from 2.8 to 3.8 years. The outcome of this study confirmed the potency of interferon in fighting against solid tumors and confirmed interferon as the first molecule in this indication which showed a tremendous impact on the relapse-free period and overall survival rate in malignant melanoma patients classified with high-risk for a relapsing disease (Jonasch & Haluska, 2001; Kirkwood et al., 1996).

Interferon based cancer therapy shows significant effects in many different indications such as hematologic malignancies and solid tumors, e.g. malignant melanoma. Besides the promising activity in chronic myelogenous leukemia (CML) and hairy cell leukemia (HCL) therapy, this class of cytokines shows the potential to efficiently inhibit proliferation of another type of malignant cells of the lymphatic system. Multiple myeloma is one of the currently approved clinical indications (Rote Liste® 2014) for IFN-alpha and provides the capability for further investigations in interferon research due to a high unmet medical need in this field of oncology. According to this, the biology of multiple myeloma and possible interventions in the pathological development of this kind of lymphatic cancer will be explained in more detail in the following section.

1.6 Interferon treatment in multiple myeloma

Multiple myeloma accounts for 13% of all hematologic cancers and is characterized by an uncontrolled clonal expansion of plasma cells in the humoral immune system. The relatively low incidence rate of 5.6 cases in 100.000 persons, with a medium diagnose age of 70 years, should not disguise the fact that multiple myeloma is an incurable type of cancer and demonstrates a

high medical need for effective treatment. Current therapies comprise cytostatic and cytotoxic agents like the proteasome inhibitor bortezomib, the anti-angiogenic and anti-proliferative compounds thalidomide and its derivate lenalidomide. The application of thalidomide (formerly known by the trade name Contergan™) and its derivative lenalidomide in multiple myeloma is one of the last indication these compounds are still applied after severe side effects on newborn children had been detected in times when thalidomide was sold as an over the counter drug for its mild sedative activity. The combination of autologous stem cell transplantation and the earlier mentioned cytotoxic agents increased the overall survival rate of patients suffering from multiple myeloma (30% likelihood for a 10-year survival) (Palumbo & Anderson, 2011).

This kind of hematologic malignancy derives from misguided plasma cells originating from pre-germinal B-cells. Due to a genetic malfunction in terminal differentiation of B-lymphocytes into plasma cells, these cells gain a malignant phenotype. In more than half of the multiple myeloma cells, an incorporation of oncogenes in the genetic locus of the immunoglobulin heavy chain gene on chromosome 14 can be found. Accompanied by the uncontrolled proliferation of a single plasma cell clone, the hematologic system gets flooded with enormous amounts of a type of pathological immunoglobulin which is termed monoclonal gammopathy. In this first stage of the disease, called MGUS (monoclonal gammopathy of undetermined significance), gammopathy can lead to a dysfunction of kidneys by clocking them with large amounts of monoclonal antibodies. Later on during disease progression, further genetic alterations like numerous genetic translocations trigger the activation of known cancer forcing genes like *MYC*, *NRAS* and *KRAS* which are involved in cell cycle control and enable increased proliferation rates. Simultaneously proteins which limit uncontrolled cell cycling like cyclin-dependent kinases inhibitors (CDKN2A and CDKN2C) become silenced. Based on the characterization and progress of genetic alterations, multiple myeloma can be subdivided into three stages, depending on the degree of severity and accompanying symptoms (Palumbo & Anderson, 2011; Smith & Yong, 2013).

In late stage multiple myeloma, large amounts of malignant plasma cells enrich in the bone marrow of the limbs, hip, spinal cord and skull with dramatic consequences. After settling of malignant cells in the bone marrow, myeloma plasma cells bind to bone marrow stromal cells and stimulate the overexpression and release of cytokines like tumor necrosis factor (TNF), macrophage inflammatory protein (MIP) and receptor activator of nuclear factor-κB ligand (RANKL) into the microenvironment. The overstimulation of cells by this cytokine-cocktail triggers bone resorption by transformation of osteoblasts into lytic osteoclasts. As a consequence of the destruction of bones, calcium from the bone marrow is released into the periphery in high

amounts. Hypercalcemia can be found in many multiple myeloma patients and it potentially correlates with the amount of tumor mass. In addition to hypercalcemia the pathologic syndromes in multiple myeloma comprise severe bone pain, a compression of the spinal cord and fractures of bones. The depletion of stem cells and hematologic progenitor cells in the bone marrow also lead to anemia and immune suppression due to the lack of erythrocytes and cells of the adaptive immune system. Because of the lack of physiological cells, bone marrow transplantations represent an important part of treatment strategies in many cases in order to reconstitute an intact cellular microenvironment and to deplete residual multiple myeloma cells in the bone (Oyajobi, 2007).

Until now, there is no pharmacologically active substance known to efficiently cure this kind of disease. Current treatment goals mainly focus on prolonging overall survival of patients and reducing the suffering caused by multiple myeloma to a minimum. Interferon was first used in multiple myeloma treatment in 1979 with promising results, which were confirmed in many randomized follow up studies in combination with standard chemotherapeutic agents. In the last three decades numerous clinical studies in this indication were conducted, using interferon because of its direct anti-proliferative and immune stimulatory mode of action. A meta-analysis of the results of thirty randomized trials in 3948 patients was performed by E. Fritz and H. Ludwig in 2000, summarizing the diverse outcomes of IFN therapies and demonstrating the beneficial effect of interferon chemotherapy on prolongation of relapse free time and overall survival of patients suffering from multiple myeloma. This analysis comprises the results of 17 trials performed with initial interferon chemotherapy treatment and thirteen trials using interferon for maintenance therapy after chemotherapy with proteasome inhibitors or immune stimulator agents (Fritz & Ludwig, 2000).

Meta-analysis of gained data of all investigated clinical trials showed a significant improvement in therapeutic response of patients treated with initial interferon chemotherapy compared to patient groups treated with chemotherapy alone (summarized results are illustrated in figure 5). Interferon administration induced a 6.6% higher response rate, a prolongation of relapse free time up to 4.8 month and increased overall survival of 3.1 month compared to chemotherapy only. In case of interferon used for maintenance treatment after initial therapy with bortezomib or thalidomide, relapse free duration was prolonged for 4.4 month and overall survival for 7 month. Taking into account the beneficial improvement in survival duration of multiple myeloma patients receiving interferon combination therapy, the rationale for interferon based treatment in this oncological indication seems to be validated (Fritz & Ludwig, 2000).

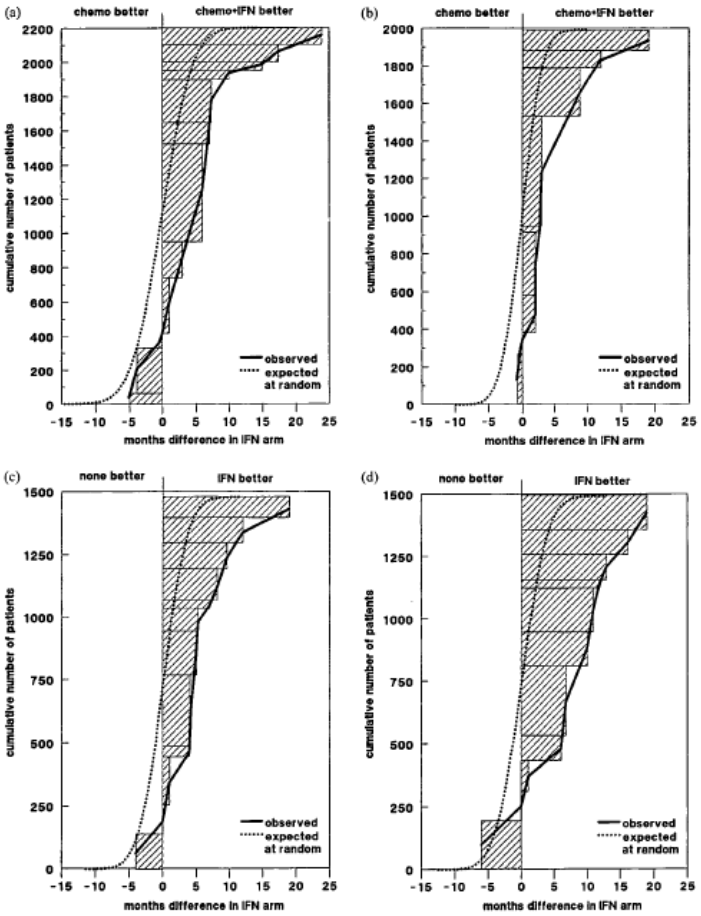


Figure 5: Meta-analysis of thirty clinical trials comparing interferon mono- and combination therapy

Results of a meta-analysis of randomized clinical trials comparing interferon combination therapy and chemotherapy alone. Pictured are the observed values for a) median progression free survival in interferon-chemotherapy, b) median overall survival in interferon-chemotherapy, c) median progression free survival in interferon maintenance treatment and d) median overall survival in interferon maintenance treatment. The solid line indicates observed responses in patients in contrast to expected values calculated by an algorithm (dotted line). (Fritz & Ludwig, 2000)

1.7 Limitations of interferon in cancer therapy

Besides the strong impact of the potent anti-proliferative and immune stimulating activity of interferon in the struggle against cancer, the medical application of this class of cytokines is always accompanied by serious adverse reactions which intensify the suffering of patients during treatment. The side-effect profile of interferon can be divided into four categories: constitutional, hematologic, neuropsychiatric and hepatic. Side-effects triggered by the overstimulation of an immune response can already occur at the beginning of the treatment and become more severe

with prolonged treatment duration. Most of the observed adverse responses to interferon can, to some extent be related to the administered dose of the cytokine and the duration of therapy. From the very beginning of the treatment, patients suffer from flu like symptoms as well as from fevers, rigors and chills. First symptoms can already be observed within the first three to six hours after initial interferon administration (Jonasch & Haluska, 2001).

After initial acute toxicity, side-effects possibly become chronic or even become more severe with increased treatment duration. Fatigue, representing one example of chronic side-effects, can be found in more than 70% of all patients during therapy. The severity of side-effects closely correlates with the applied dose of interferon and contribute to the dose-limiting toxicity in many cases. Anorexia is another major side-effect strongly influencing the health status of patients in more than half of all treated individuals. Until now it is not fully understood, why patients become anorexic and lose a lot of weight which further weakens the physiological condition of patients and most likely increases the development of other unwanted responses (Jonasch & Haluska, 2001).

High levels of interferon in the periphery can cause neuropsychiatric disorders in patients leading to severe mood disorders or even depression. Anxiety syndromes and depressions can become worse during treatment and are most likely dose-related. The following table summarizes observed side-effects in numerous clinical trials with high-level interferon dosages in multiple myeloma therapy (Jonasch & Haluska, 2001).

Study	***Real* [117]**	***Creagan* [96]**	***Kirkwood* [113]**		***Creagan* [116]**		***Sertoli* [159]**		***Creagan* [95]**	***O'Connell* [160]**	
Disease/stage	Kaposi's/HIV	Melanoma IV	Melanoma III		Melanoma III		Melanoma IV		Melanoma IV	NHL, CLL	
Type/dose	IFN-α2a 36 MU IM qd × 4 wks, then tiw	IFN-α2a 50 MU/m² i.m. tiw × 12 wks	IFN-α2b 20 MU/m² IV 5 days/wk × 4 wks, then 10 MU/m² s.c. tiw × 11 mo		IFN-α2a 20 MU/m² i.m. tiw × 12 wks		IFN-α2b 10 MU/m² i.m. tiw until disease progression		IFN-α2a 12 MU/m² i.m. tiw × 12 wks	IFN-α 12 MU/m² i.m. tiw × 8 wks, then weekly if response, and 25 MU/m² tiw × 4 wks if stable disease	
n patients on IFN	34	31	143		131		21		30	20	
Degree of severity	All Grades	Mod-Severe	All Grades	≥Grade III	All Grades	≥Grade III	All Grades	≥Grade III	Mod-Severe	All Grades	Severe
	%	%	%	%	%	%	%	%	%	%	%
Constitutional			97	48							
Fatigue	88	87			89	20	100	43	50	90	10
Anorexia	74	58			55	3	13		47	55	10
Weight loss						13					
Fevers	94				83	24	100	10	80	85	5
Myalgias	74				63	7			27		
Headache	62				44	5				45	0
Hepatic			64	16	N/A	N/A	19	0	0	10	5
Neuropsychiatric			83	28	11	2	5		3		
Depression	21										
Dizziness/vertigo	47										
Confusion		23								15	5
										5	5
Hematologic			64	24	N/A	N/A	29		0		
Thrombocytopenia										35	0
Anemia											
leukopenia										45	0

Figure 6: Side-effect profile of interferon determined during clinical investigations

Observed side-effect profile of randomized clinical trials using interferon based treatment strategies in different oncological indications. Unwanted responses are categorized according to their mode of action and scaled depending on the degree of severity. (Jonasch & Haluska, 2001)

To increase the quality of life of cancer patients treated with interferon, used dosages of the cytokine have to be adapted to a minimum effective level to reduce or even prevent unwanted responses. Due to the severe side-effect profile, interferon in cancer therapy is often replaced by alternative cytotoxic agents or is only administered in case of ineffectiveness of other drugs. Further investigations of the exact mode of action of this anti-proliferative and immune modulating agent probably help to better understand why these diverse side-effects are caused and how interferon therapy can continue to be applied while decreasing the unfavorable interference into patient's quality of life.

1.8 Description of the proposed therapeutic attempt in interferon based cancer therapy

To address the limitations of interferon administration in cancer therapy due to the severe side-effect profile, the therapeutic attempt described in the following section of this thesis was developed by Dr. Manfred Schwaiger, Roche Diagnostics GmbH. Clinical applications of interferon in oncology can be broadened in case of the reduction of unwanted responses in patients, while still circulating in the periphery. The use of interferon shows a significant efficacy in several oncological indications but optimal anti-tumor action cannot be reached in many cases because of low maximum-tolerable-doses of the cytokine. The aim of this work is therefore to design a therapeutic molecule that is able to exploit the full anti-proliferative and immune-stimulating potential of interferon in the battle against cancer while at the same time decreasing the severe side-effects that would typically come along with interferon therapy.

One way to reach this goal is the inhibition of undesired interactions of interferon while it still circulates in the periphery. By designing a molecule that remains inactive during circulation, side-effects can be reduced to a minimum level, thus, at the same time enabling the administration of higher dosages of interferon in cancer therapy. In order to gain a temporary inactivation of interferon, it can be bound by an antibody specifically directed against the subtype of the cytokine used for therapy.

By binding of interferon to the complementarity determining regions (CDRs) of the antibody, it becomes masked and will be biologically inactive to a large extend.

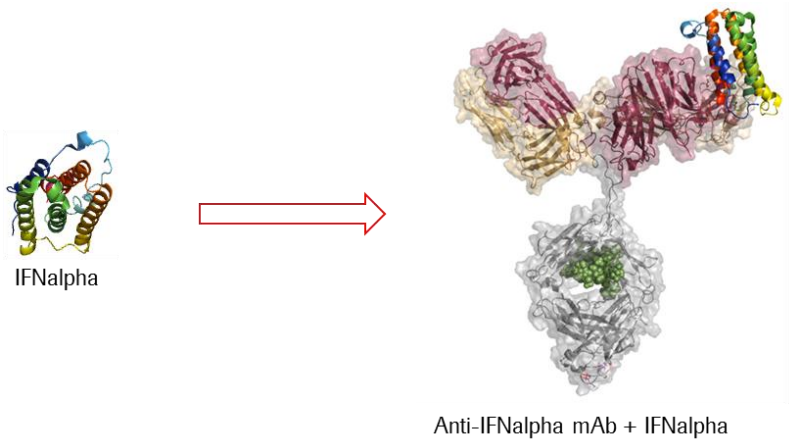


Figure 7: Binding of interferon to masking antibody

Interferon alpha 2a (IFNα2a) is bound by the anti-IFNalpha2a-antibody called 9F3 (Genentech Inc.) Due to binding of the cytokine to the antibody, interferon is masked and mostly biologically inactive during circulation in the periphery.

Depending on the affinity of the antibody against interferon, an equilibration of bound and non-bound interferon with consistently changing situations would be possible. To ensure a stable binding of the cytokine to the masking antibody, an additional peptide linker between both proteins has to be introduced to the fusion protein. Due to the close steric proximity of interferon to the antibody created by a short linker and following the law of mass action, the majority of linked cytokines will be stably bound and masked by the antibodies. To enable a release of the interferon from the antibody at the tumor site, the used peptide linker contains cleavage motifs which are specifically broken up by tumor selective proteases.

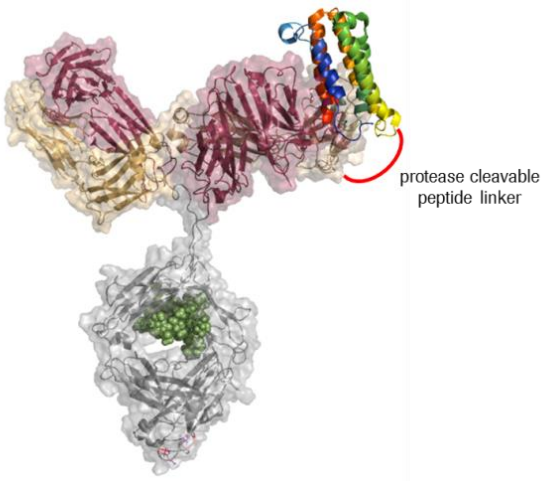


Figure 8: Stabilized binding of interferon to a masking antibody via a protease cleavable linker

To ensure a stable binding of Interferon alpha 2a to its masking antibody, a peptide linker is used to connect both parts of the fusion protein. The used linker contains two cleavage sites for tumor associated proteases which are degraded after arrival of the molecule at the tumor site.

Inhibition of interferon during circulation and tumor selective activation of the cytokine provides major advantages compared to conventional interferon therapy. By ensuring interferon masking by an antibody, another additional attribute can be applied to the proposed fusion protein. State of the art technologies in antibody generation provide the possibility to produce bivalent (two antigen binding arms) and bispecific antibodies which are able to recognize and bind to two different antigens at the same time. With the help of these bispecific molecules, an additional targeting moiety can be introduced to the interferon fusion protein. Using one arm of the antibody with specificity against a tumor-associated antigen, the interferon fusion protein can be targeted to the tumor site and enriched in the tumor microenvironment. As a consequence of the enrichment of the molecule at the tumor site, pharmacodynamic properties of the fusion protein can be increased in combination with a reduction of unwanted responses in the periphery. The following figure summarizes the attributes of the designed bispecific interferon fusion protein.

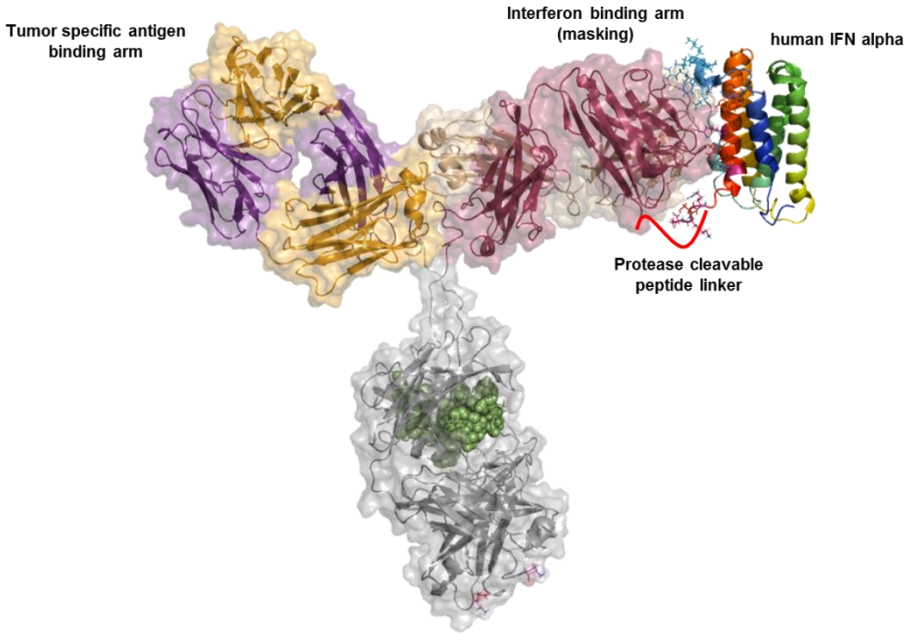


Figure 9: Summary of the proposed bispecific interferon fusion protein

The proposed fusion protein is composed of one antibody arm binding and masking interferon, while the other antibody arm shows binding specificity against a surface protein expressed on tumor cells. Due to this bispecific format, a selective activation of the cytokine parallel to an enrichment of the molecule at the tumor site can be ensured.

For the generation of a bispecific antibody, several techniques, for example Knobs-into-Holes and CrossMab, need to be applied simultaneously and are describe in closer detail in the method section of the thesis. Fusing interferon to a human IgG1 antibody raises an additional improvement of the pharmacokinetic attributes of the cytokine by increasing the half-life of interferon from a view hours up to two weeks which is mediated by the recycling of the carrier antibody via a so-called FcRn-recycling mechanism.

1.9 Proposed mode of action of the interferon fusion protein

After intravenous administration of the interferon fusion protein, the molecule circulates randomly through the body and is distributed to all kind of tissues via the blood stream. Binding of interferon by the antibody inhibits the biological function of the cytokine to a great extent and should also reduce unwanted responses in healthy organs to a minimum. The clearance of interferon from the body via renal exclusion typically occurs very quickly, but because of the increased size of the fusion protein (190 kDa) above the renal exclusion limit of <70 kDa, fast clearance is prevented and enables the molecule to stay in the system much longer. Additional prolongation of half-life is achieved by the recycling mechanism of the antibody inside cells. Human IgG1 antibodies bind to the neonatal Fc-receptor (FcRn) and are recruited to the FcRn-mediated recycling mechanism which leads to endosomal escape of antibodies after unspecific internalization in endothelia cells (Roopenian & Akilesh, 2007).

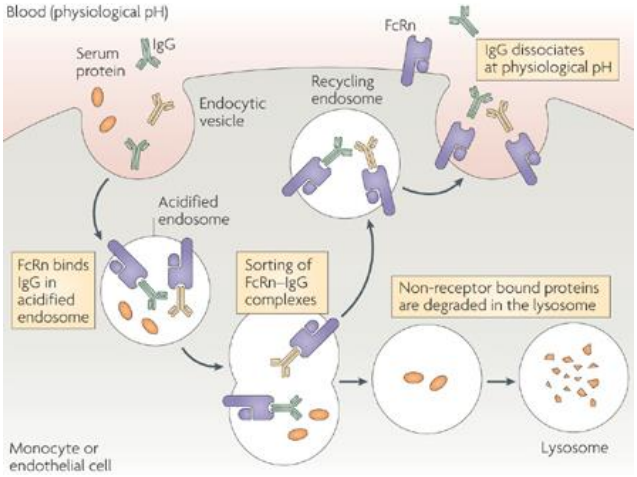


Figure 10: Neonatal Fc-receptor-mediated IgG recycling mechanism

During endocytosis, type G immunoglobulins (IgG) bind to FcRn receptors at an acidic pH inside the endosome. Complexes formed by IgGs and receptors are transported back to the surface of the cell. Endosomal escape and release of the antibody from the FcRn receptor is initiated by the physiological pH level of the blood stream. (Roopenian & Akilesh, 2007)

Fusion proteins which pass by the tumor site get enriched in the tumor microenvironment by binding of the bispecific interferon fusion protein to an antigen expressed by the tumor cells itself (direct targeting), or expressed by tumor-associated supporting cells (indirect targeting) like fibroblasts. The homing of the fusion protein at the pathologic tissue leads to an increased concentration of the pharmacologically active agent at the tumor site and a reduction of circulating interferon in the periphery. Direct targeting strategies would comprise the selection of an antigen expressed on the surface of cancer cells preferentially choosing a non-internalizing target to prevent internalization and degrading of the interferon fusion protein.

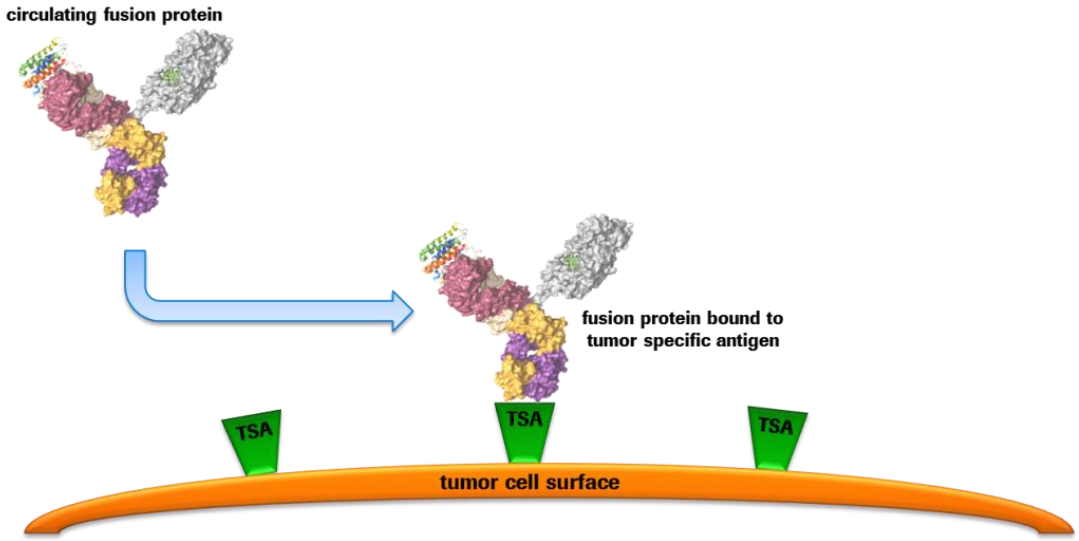


Figure 11: Binding and enrichment of fusion proteins on the surface of tumor cells

Enrichment of an interferon fusion protein on the surface of a tumor cell due to binding of a tumor specific antigen (TSA). Applying a targeting moiety to the fusion protein raises the possibility for an enforced clearance of the cytokine out of the periphery and ensures higher levels of pharmacologically active substance at the site of the tumor.

Following the binding of the interferon fusion protein to the surface of tumor cells, proteases cleave the peptide linker ensuring stable binding of interferon to its masking antibody. This peptide linker contains two specific sequences cleavable by serine proteases which are expressed on the surface of tumor cells or by matrix metalloproteinases secreted in the tumor microenvironment. After cleavage of the stabilizing linker, interferon is enabled to diffuse away from the binding pocket of the antibody and becomes re-activated due to the loss of the masking ability of the interferon binding antibody arm of the fusion protein. Depending on the

concentration of interferon receptors on the tumor cell surface and the concentration of surrounding fusion proteins, an equilibration between antibody bound inactive interferon and released active interferon adjusts at the tumor site. This slow release of the active drug ensures the ability to maintain a stable concentration of interferon at the target site and prevents fast clearance of the cytokine.

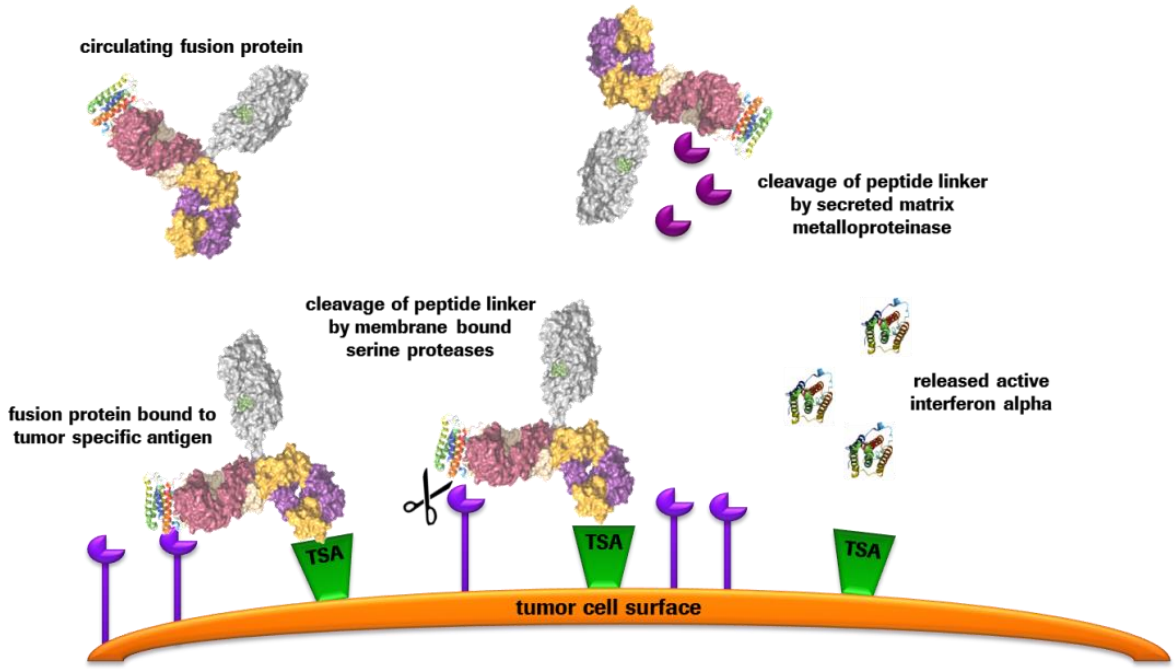


Figure 12: Protease-mediated cleavage of peptide linkers triggering interferon release

Serine-proteases on the tumor surface and matrix metalloproteinases secreted into the tumor microenvironment are able to cleave the used peptide linker which ensures a stable binding of interferon to its masking antibody. After cleavage, an equilibrium between bound and released interferon establishes which ensures a slow release of the cytokine at the tumor.

Re-activated interferon liberated from the fusion protein is able to bind to its receptors IFNAR1 and IFNAR2 expressed on the surface of tumor cells. Triggered by the formation of the ternary signaling complex composed of IFNAR2, interferon and IFNAR1, the downstream signaling cascade via JAK-Stat pathway becomes activated and the expression of more than 300 different interferon stimulated genes is initiated. Upon initiation of the interferon signaling pathways in cells, the activation of pro-apoptotic proteins may lead to the induction of apoptosis of the tumor cells.

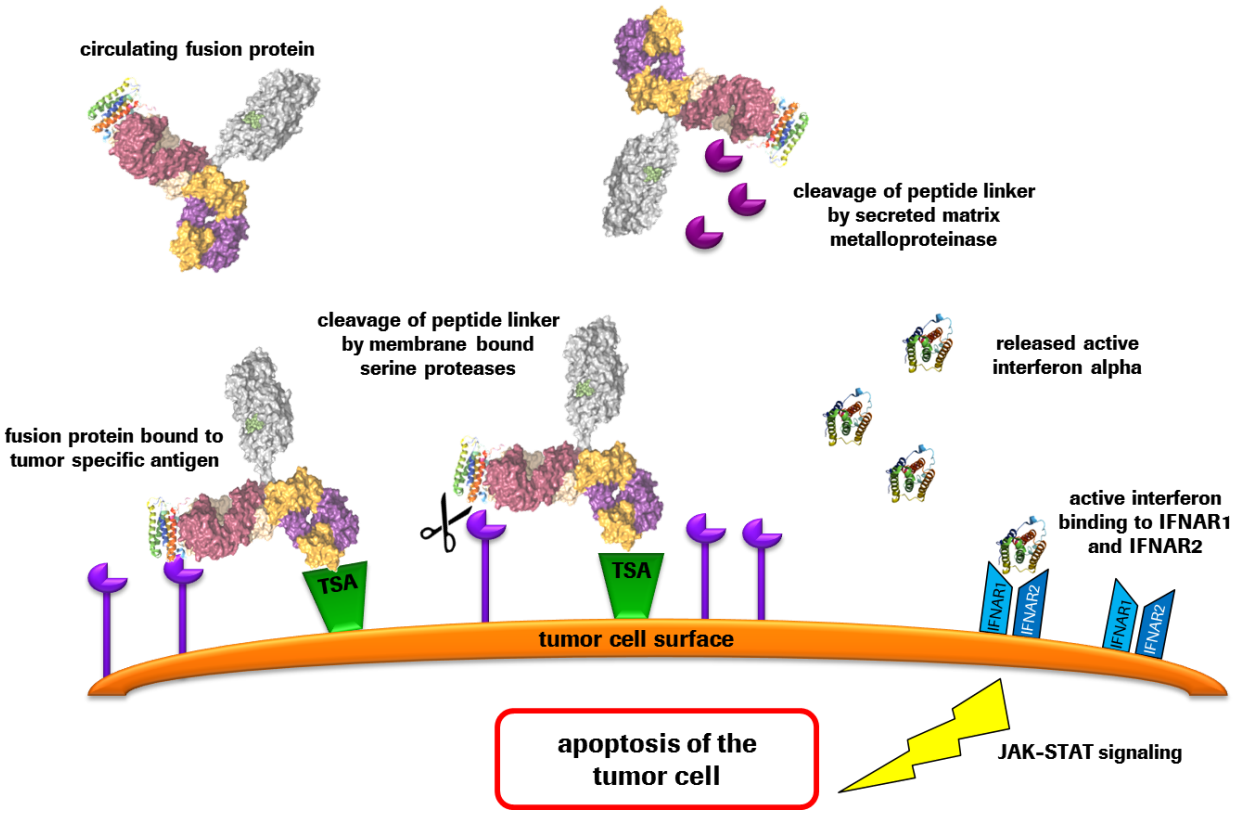


Figure 13: Interferon signaling pathway induces apoptosis induction in tumor cells

Interferon which diffuses away from the antibody after cleavage of the peptide linker is able to bind to its receptors IFNAR1 and IFNAR2 on the surface of the tumor cells. After binding of interferon and the formation of the ternary receptor complex, downstream signaling via JAK-STAT-pathway becomes activated and leads to the induction of apoptosis in the tumor cells.

With this new kind of therapeutic fusion protein, dual-tumor specificity is generated firstly due to the targeting moiety of the second antibody arm and secondly by a tumor selective activation of the cytokine by the linker cleavage of proteases which are expressed on the tumor surface. This concept of a masking and targeting antibody format provides the opportunity to apply different types of cytokines with anti-tumor activity in a much more secured fashion, without losing efficacy of molecule.

The following sections of the thesis describe the attempts to generate and optimize the previously proposed interferon fusion protein and explain in more detail which obstacles had to be overcome to finally obtain a new anti-cancer drug with an improved safety profile.

2. Scientific objectives

The aim of the thesis was to develop a new fusion protein format which enables the inactivation of a cytokine during circulation in the periphery, combined with a tumor specific release and re-activation of the pharmacologically active cytokine at the target site. This could reduce possible side-effects and provide the opportunity to broaden the medical application of cytokines in diverse diseases with a narrow therapeutic window.

To address this purpose, human interferon alpha subtypes were fused to an antibody with an adjustable affinity to an appropriate type of cytokine. Due to the binding of interferon to the complement determining regions of the antibody (CDRs), the cytokine becomes masked and to a large extent biologically inactivated during circulation in the blood stream. To ensure a stable binding between interferon and the antibody, a protease cleavable peptide linker was applied which can then be cleaved by tumor specific proteases. By using new bispecific antibody formats, an additional targeting moiety was introduced to the fusion protein in order to increase the enrichment of the fusion protein at the tumor site paralleled by prolonged residence times of the antibody in the tumor microenvironment. Finally, pharmacological effects may be triggered by the cytokine released from the antibody which binds to its receptors on the tumor cell surface leading to the induction of tumor cell apoptosis.

In this thesis following aspects were addressed:

I. Screening for interferon responsive cell lines to determine possible clinical indications.

II. Masking of human interferon alpha subtypes by binding to anti-IFNalpha2a antibody called 9F3 (Genentech) and modification of binding affinities by targeted point mutations inside the complementarity determining regions of the antibody.

III. Optimization of the protease cleavable peptide linker which ensures stable binding of interferon to the masking antibody. Validation of different cleavage motifs of serine proteases and matrix metalloproteinases.

IV. Investigation of improved anti-proliferative actions of non-masking bivalent interferon fusion proteins and possible explanations for this phenomenon.

V. Improvement of pharmacological parameters of interferon alpha subtypes by modification of binding affinities to receptor unit IFNAR1 leading to increased biological effects.

VI. Manufacturing of a bispecific interferon fusion protein containing a tumor-associated targeting moiety, an optimized protease cleavable linker and mutated interferon for increased anti-proliferative activity.

The results of this thesis will contribute to an improved knowledge in the field of interferon-based treatment strategies in oncology and provide new opportunities to broaden the therapeutic window of this anti-proliferative and immune modulatory cytokine in a wide range of oncological diseases.

3. Material

3.1 Equipment

Trade name	*Reference number*	*Supplier*
ÄKTAexplorer 100	18-1112-41	GE Healthcare, Munich
ÄKTAFPLC	18-1900-26	GE Healthcare, Munich
Biacore™ T200	28964630 / 1613172	GE Healthcare, Munich
Climo-Shaker ISF1-X	-----	Kuhner, Birsfelden SZ
Eclipse TE2000	-----	Nikon Instruments, Egg SZ
Gel Doc™ XR+ System	170-8195	BIO-RAD Laboratories, Munich
Heraeus BBD 6220 D	51020241	Thermo Scientific, Braunschweig
Heraeus Multifuge X3R	75004515	Thermo Scientific, Braunschweig
iBlot™ 1	794617	Invitrogen, Darmstadt
Infinite® M200 NanoQuant	30016056	Tecan, Mainz
Leica DMIL LED	383902	Leica, Bensheim
Lumi-Imager™ F1	1300161	Roche Diagnostics, Manheim
Microwave 700W	-----	Severin, Sundern
NanoDrop 1000™	H764	Thermo Scientific, Braunschweig
PowerEase® 500	287000154	Invitrogen, Darmstadt
Rocking shaker	7-0056	neoLab, Heidelberg
Sorvall™ RC6 Plus centrifuge	46915	Thermo Scientific, Braunschweig
Thermomixer® comfort	535525758	Eppendorf, Hamburg
ViCell™ XR	AS26138	Beckman Coulter, Krefeld

3.2 Chemicals and reagents

Substance	*Reference number*	*Supplier*
2-Propanol	1.09634.2500	Merck, Darmstadt
Agarose MP	11 388 991 001	Roche Diagnostics, Manheim
Ampicillin	10 835 242 001	Roche Diagnostics, Manheim
ATX Ponceau S red staining solution	09189-1L-F	Sigma-Aldrich, Seelze
Citric acid monohydrate	1.00244.0500	Merck, Darmstadt
Ethanol	8.18760.2500	Merck, Darmstadt
Ethidium bromide solution, 10mg/mL	161-0433	BIO-RAD Laboratories, Munich
Hydrochloric acid 2M	1.09063.1000	Merck, Darmstadt
L-histidine	1.04351.0100	Merck, Darmstadt
Pen Strep	15140-122	Gibco, Life Technologies, Darmstadt
Sodium chloride	1.06444.1000	Merck, Darmstadt
Sodium hydroxide	1.06495.1000	Merck, Darmstadt
Tris (hydroxymethyl) methylamine	T/3710/60	Fisher Scientific, Reinach SZ
Tween 20	11 330 465 001	Roche Diagnostics, Manheim

3.3 Cytokines

Name	*Reference number*	*Supplier*
Human Interferon Alpha A (Alpha 2a)	11100-1	PBL assay science
Human Interferon Alpha H2	11145-1	PBL assay science

(Alpha 14)		
Human Interferon Alpha 2 (Alpha 2b)	11105-1	PBL assay science
Human Interferon Alpha B2 (Alpha 8)	11115-1	PBL assay science
Human Interferon Alpha J1 (Alpha 7)	11160-1	PBL assay science
Human Interferon Alpha 4b (Alpha 4)	11180-1	PBL assay science
Human Interferon Alpha C (Alpha 10)	11120-1	PBL assay science
Human Interferon Alpha D (Alpha 1)	11125-1	PBL assay science
Human Interferon Alpha F (Alpha 21)	11130-1	PBL assay science
Human Interferon Alpha G (Alpha 5)	11135-1	PBL assay science
Human Interferon Alpha I (Alpha 17)	11150-1	PBL assay science
Human Interferon Alpha K (Alpha 6)	11165-1	PBL assay science
Human Interferon Alpha WA (Alpha 16)	11190-1	PBL assay science
Human Interferon Beta 1b	11420-1	PBL assay science

3.4 Consumable supplies

Brand name	***Reference number***	***Supplier***
293fectin™	12347-500	Invitrogen, Life technologies Darmstadt
Dynabeads® Protein A	1000 2D	Novex, Life technologies,

		Darmstadt
Dynabeads® Protein G	1000 4D	Novex, Life technologies, Darmstadt
Eppendorf Research® Plus pipette	3120000	Eppendorf, Hamburg
Eppendorf Safe-Lock tubes	0030120	Eppendorf, Hamburg
epT.I.P.S pipette tips (10 µL, 200 µL, 1000µL)	0030000	Eppendorf, Hamburg
Falcon, serological pipets	13-675	Corning Life Sciences DL, Amsterdam NL
HiLoad™16/600 - Superdex™200pg	28-9893-35	GE Healthcare, Munich
HiTrap™MabSelect Xtra™	28-4082-58	GE Healthcare, Munich
iBlot® Gel Transfer Stacks, PVDF, Regular	IB 401001	Novex, Life technologies, Darmstadt
ImMedia™ Amp Agar	45-0034	Invitrogen, Life technologies Darmstadt
Inoculation spreader	86.1569.001	Sarstedt, Nümbrecht
Lumi-Light WB substrate	12 015 200 001	Roche Diagnostics, Manheim
Microplate 96Well PS F-Bottom	655098	Greiner Bio One, Solingen
MOUSE (virus free) PLASMA (steril) in Lithium Heparin	D523-07	Rockland, Gilberstville US
Nonfat dried milk powder	A 0830,1000	AppliChem, Heidelberg
NuPage® 4-12% Bis-Tris Gel	NP0321 BOX	Novex, Life technologies, Darmstadt
Pipet tips with microcapillary	732-0508	VWR, Ismaning
Precision Plus Protein™ Dual Color Standars	161-0374	BIO-RAD Laboratories, Munich
S.O.C medium	15544-034	Invitrogen, Life technologies Darmstadt
Series S Sensor Chip CM5	BR100530	GE Healthcare, Munich
Steritop™	05/0703-094	Millipore, Billerica US
VacuCap®90 PF filter unit	4628	Pall corporation, Dreieich

3.5 Kits

Brand name	*Reference number*	*Supplier*
Human Ab Capture Kit	BR-1008-39	GE Healthcare, Munich
peqGOLG Gel Extraction Kit	12-2501-02	Peqlab, Erlangen
PureLink™ HiPure Plasmid Megaprep Kit	K2100-08	Invitrogen, Life technologies Darmstadt
Rapid DNA Dephos & Ligation Kit	04 898 125 001	Roche Diagnostics, Manheim

3.6 Detection antibody

Brand name	*Reference number*	*Supplier*
Peroxidase AffiniPure F(ab')2 Fragment Goat Anti-Human IgG, Fcγ Fragment Specific	109-036-098	Jackson Immuno Research, Suffolk UK

3.7 Enzymes

Brand name	*Reference number*	*Supplier*
BamHI-HF®	R3136S	New England BioLabs, Frankfurt am Main
KpnI-HF®	R3142S	New England BioLabs, Frankfurt am Main
XbaI	R0145S	New England BioLabs, Frankfurt am Main

3.8 Buffers

Name	*Reference number*	*Supplier*
HBS-N buffer	BR100369	GE Healthcare, Munich
NuPage® LDS sample buffer	NP0007	Novex, Life technologies, Darmstadt
NuPage® sample reducing agent	NP0009	Novex, Life technologies, Darmstadt
Phosphate buffered saline	20012-019	Gibco, Life Technologies, Darmstadt
TBST buffer	12 014 939 001	Roche Diagnostics, Manheim

3.9 Media and supplements

Name	*Reference number*	*Supplier*
Accutase™ in DPBS, 0.05% EDTA	SCR005	Merck, Darmstadt
Bovine Serum Albumin (BSA) Protease free	P06-139210	Pan-Biotech, Aidenbach
DMEM medium	41966-029	Gibco, Life Technologies, Darmstadt
FreeStyle™ 293 expression medium	12338-018	Gibco, Life Technologies, Darmstadt
Heat inactivated FBS	10500-056	Invitrogen, Life technologies Darmstadt
Leibotwitz's L-15 medium	P04-27500	Pan-Biotech, Aidenbach
McCoy's 5A medium	26600-023	Gibco, Life Technologies, Darmstadt
MEM Eagle medium	P04-08510	Pan-Biotech, Aidenbach

RPMI 1640 medium	P04-18047	Pan-Biotech, Aidenbach
Trypsin-EDTA 0.05%	25300-054	Gibco, Life Technologies, Darmstadt

3.10 Eukaryotic cell lines

All tumor cell lines used in this work were derived from the in-house CELLO cell bank, which was established and characterized by Chugai Pharmaceutical Co., a member of the Roche Group.

3.11 Applied computer software

Software	***Intended use***
Nikon EZ-C1 Version 3.80	Confocal microscope
Biacore T200 Software v2.0	Biacore T200 device
Tecan i-control™ V.3.4.2.0	Infinite® M200 NanoQuant device
Vector NTI Advance® 11.5.0	DNA sequences management
Prism 6 for Windows, Version 6.04	Evaluation and visualization of raw data
UNICORN™ 5.31	Äkta HPLC device

4. Methods

4.1 Antibody design

The manufacturing of a bispecific antibody on the basis of a fully human IgG1 scaffold requires the simultaneous application of several antibody engineering strategies. Due to the complex composition of a bispecific molecule out of four different antibody chains, the correct assembly of the two different heavy and light chains has to be ensured. Without any control of the antibody chain pairing, there are ten different possibilities how any of the four different chains can be paired with each other. As a consequence of an uncontrolled antibody assembly, the achievable yield of the preferred bispecific antibody is dramatically decreased and extensive protein purification steps have to be applied to separate the desired antibody from the mixture of ten different molecules. To overcome these limitations in antibody production and to maximize the accessible product amount, the following antibody engineering strategies were applied for the generation of a bispecific interferon fusion protein.

4.1.1 Knobs-into-holes

The interferon fusion protein proposed in this thesis, is assembled out of two different antibody heavy chains, one heavy chain with specificity for human interferon alpha and another heavy chain specific for a tumor-associated antigen. A method to ensure the formation of heavy chain heterodimers of both types of heavy chains is the “knobs-into-holes” (KiH) technology, published by Ridgway *et al.,* Genentech, Inc.

The concept of this antibody engineering technology is based on the investigations of amino acid interactions by Francis Crick in 1952. Originally, “knobs-into-holes” was developed as a model to describe the interactions and steric conformations of amino acid side chains between α-helices in close proximity. This concept can also be applied to evaluate the interplay of amino acids between both heavy chains within the Fc part of an antibody. First investigations of the α-helices within the Fc part of a human IgG1 antibody via X-ray crystallography were conducted by Johann Deisenhofer in 1981, leading to the finding that dimerization of both heavy chains is mediated by amino acid interactions taking place in the CH3 domains of the Fc part. In contrast to that, glycosylated CH2 domains exclusively interact via carbohydrates. Based on these structural and functional results of amino acid interactions in the CH3 domains, new attempts to enforce heavy

chain heterodimerization via engineering of the amino acid sequence of those domains were launched, which has led to the development of “knobs-into-holes” technology. Using this technique, a “knob” is created by the exchange of a small amino acid in one heavy chain with an amino acid containing a large side chain. Opposite to the created “knob” and within the CH3 domain of the second heavy chain, amino acids with large side chains are replaced by smaller ones to create the “hole”. After dimerization of both heavy chains containing the modifications, the “knob” fits into the “hole” and a stable connection between both heavy chains is forced by an additional introduction of two cysteine below the “knobs-into-holes” region, which form a stable disulfide bond (figure 14) (Deisenhofer, 1981; Ridgway, Presta, & Carter, 1996).

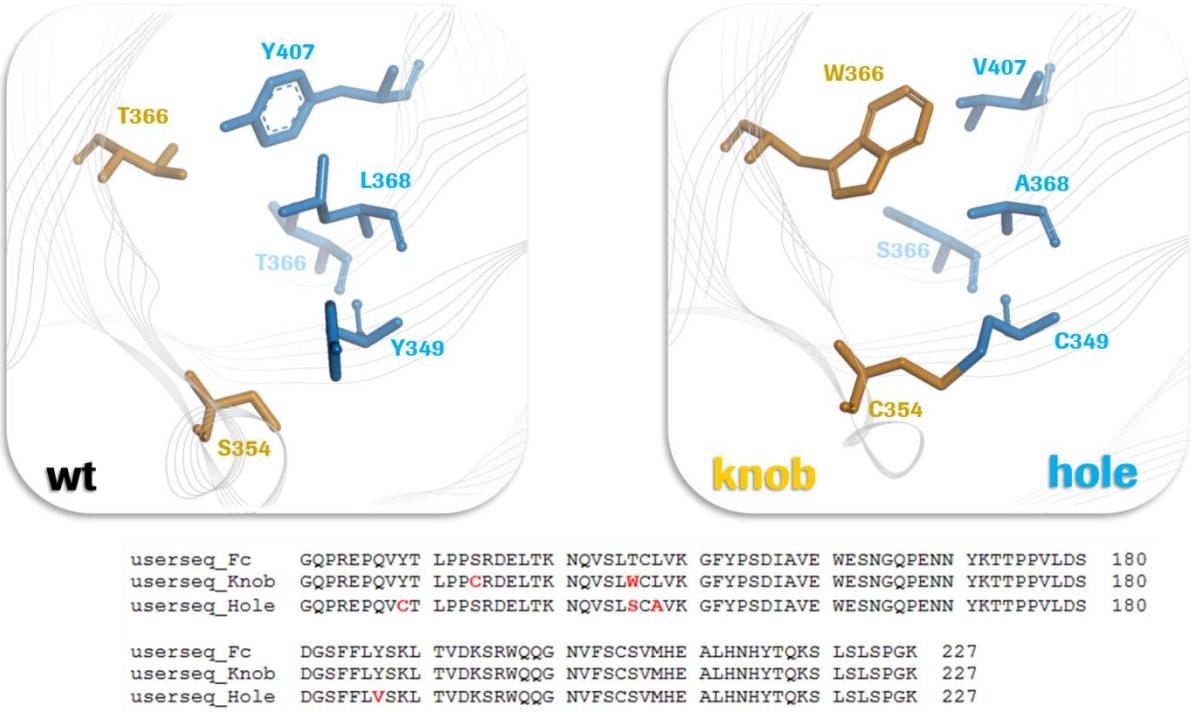


Figure 14: "knobs into holes" modification to enforce heavy chain heterodimerization

A so called “knob” is generated in one CH3 domain of the antibody by the replacement of threonine with tryptophan. The amino acid side chain of tryptophan is build up by a large indole ring which leads to steric hindrances in case of homodimerziation of two heavy chains containing a “knob” modification. On the second CH3 domain of the antibody, amino acids with large side chains are replace by amino acids with steric more favorable residues. By this replacement a “hole” is manufactured in which the “knob” of the corresponding heavy chain fits into. Additional stability of the interaction is enforced by the integration of a cysteine into both heavy chains to build up a stable disulfide bond.

To generate the “knob”, threonine at position 366 within the CH3 domain of the heavy chain is replaced by tryptophan with a large indole side chain. In the CH3 domain of the second heavy chain opposite to the CH3 domain of the first heavy chain, the “hole” is constructed by the replacement of steric unfavorable amino acids (tyrosine at position 407, leucine at position 368 and threonine at position 366) by amino acids with smaller side chains (valine at position 407,

alanine at position 368 and serine at position 366). An additional disulfide bridge is introduced by the incorporation of cysteine at position 354 of the knob - heavy chain and at position 349 of the hole - heavy chain.

The application of this technology for generating bispecific antibodies prevents the formation of knob - knob - homodimers due to steric hindrances and thus, heterodimerization of two different heavy chains is favored. Without any engineering strategies, the four different chains of a bispecific antibody can be combined in ten possible ways. With the help of “knobs - into - holes” and the correct pairing of heavy chains, the number of possible formats is reduced down to four different antibodies which increase the yield of the desirable product.

For the manufacturing of the interferon fusion protein, a “knob” is introduced in the CH3 region of the heavy chain binding to human interferon alpha. Amino acid modifications for the generation of the “hole” are applied in the heavy chain with binding specificity for the tumor-associated antigen CD138.

4.1.2 CrossMab

Even by ensuring heterodimerization of two different heavy chains by KiH technology, the possibility for the formation of four different antibody molecules is still given. To address this problem and reduce the possible outcome to a single desired antibody, another engineering approach has to be applied to force correct light-chain/heavy-chain interactions. In the case of bispecific antibodies, where the main binding affinities are mediated by the complementarity determining regions of the heavy chains, a single type of light chain can be used which can pair with both types of heavy chains. Although, this so called “common light chain” approach is limited by a significant decrease in antigen binding affinity compared to binding mediated by heavy and light chain simultaneously (Schaefer et al., 2011).

To overcome this bottleneck in the production of bispecific IgG antibodies and reduce the various possible combinations of heavy and light chains to a single desired molecule, Wolfgang Schäfer *et al.* combined the “knobs into holes” technology with a new methodology termed “CrossMab” to control additional pairing of two different light chains. By the combination of both antibody engineering techniques, the assembly of a single type of bispecific IgG antibody, made up of four different antibody chains can be ensured and achievable protein yields maximized (Schaefer et al., 2011).

Structure analysis of the molecular architecture of an antibody and investigations of the heterodimerization interfaces of the variable heavy (VH), the variable light (VL), the constant heavy 1 (CH1) and the constant light (CL) domains led to the awareness, that the interfaces between these antibody domains are identical. Due to this finding the team of Schäfer *et al.* developed an engineering approach for crossing over the antibody domains between the heavy and the light chains. With the help of this "CrossMab" technology, VH and VL or CH1 and CL domains can be replaced by each other to generate artificial light and heavy chains which cannot pair with natural unmodified antibody chains anymore. By applying this methodology on one arm of the bispecific antibody, four different antibody chains are generated which can only be paired with the corresponding partner. The following image illustrates the simultaneous application of "knobs into holes" and "CrossMab" to prevent chain fail pairing when generating a bispecific antibody (Schaefer et al., 2011).

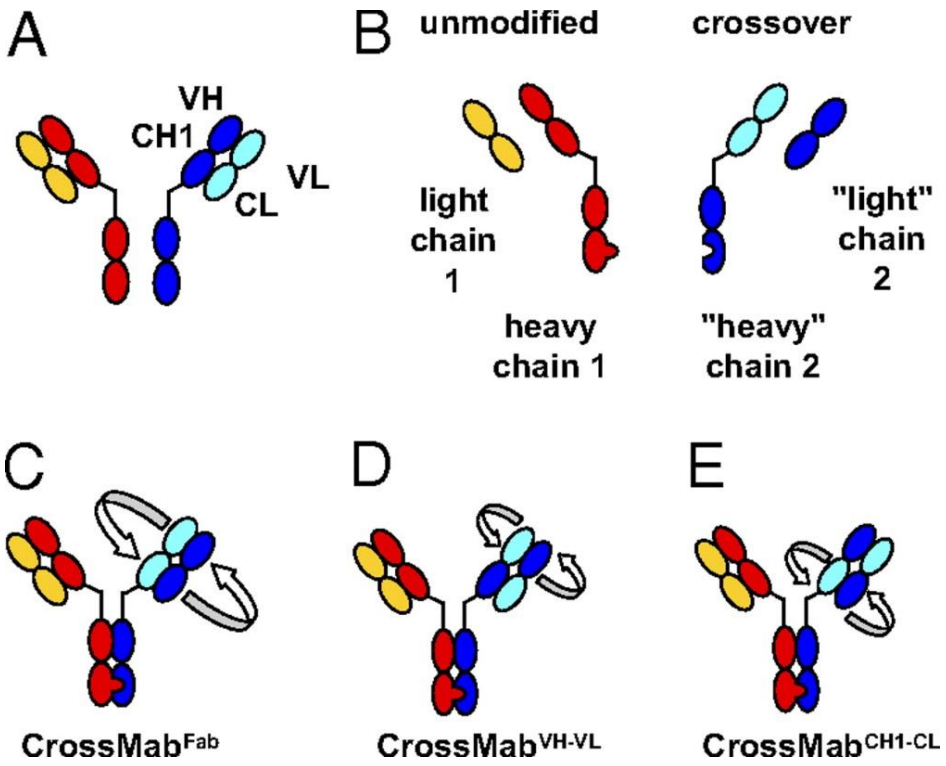


Figure 15: Crossing over of antibody domains

Bispecific antibodies are made up by two different heavy and light chains (A). By applying the "knobs into holes" technology, heterodimerization of two different heavy chains is enforced (B). For the generation of artificial antibody chains, which cannot be paired with wild type chains, CrossMab technology can be utilized by the exchange of the light chain with the VH and CH1 domain of the heavy chain (C), crossing over of variable regions from both antibody chains (D) or by the exchange of CH1 and Ckappa domains (E).

The CrossMab technology is applied for the generation of the interferon fusion protein by crossing over the variable heavy (VH) and variable light (VL) domains of the antibody arm with binding specificity for the tumor-associated antigen CD138. The VL domain of the CD138 antibody arm is attached to the constant domains of the heavy chain in combination with the VH domain fusion to the constant kappa domain of the corresponding light chain (figure 16).

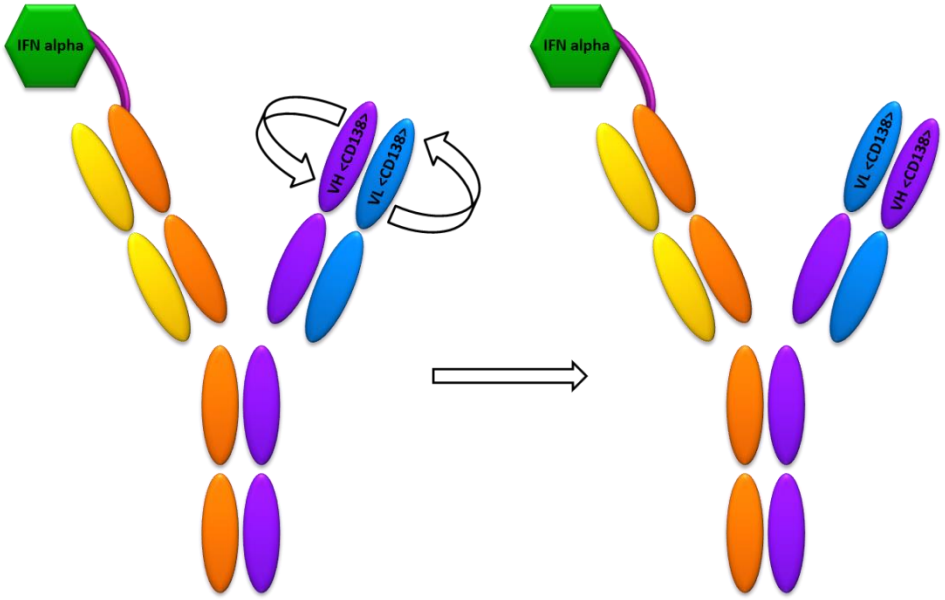


Figure 16: CrossMab technology used in the interferon fusion protein

CrossMab technology is utilized for the production of the interferon fusion protein by crossing over the variable domains of the heavy and light chain of the targeting antibody arm. Due to the artificial generated light chain, composed out of the VH and Ckappa domains, chain miss-pairing between both arms of the bispecific antibody is prevented.

Along with the CrossMab technology used for the modification of the tumor targeting arm of the fusion protein, amino acid exchanges were applied at the interferon binding antibody arm to facilitate pairing of the interferon binding light chain with the interferon binding heavy chain. Natural occurring amino acids with a positive charged side chain in the CH1 domain of the heavy chain are replaced by amino acids with a negative charge (glutamic acid). Additional charge exchanges are performed in the constant kappa domain of the corresponding light chain by replacement of negatively charged amino acids with arginine and lysine containing positively charged residues (shown in figure 17). The conversion of electric charge of the heavy and light chains of the interferon binding antibody arm leads to an additional prevention of miss pairing antibody chains.

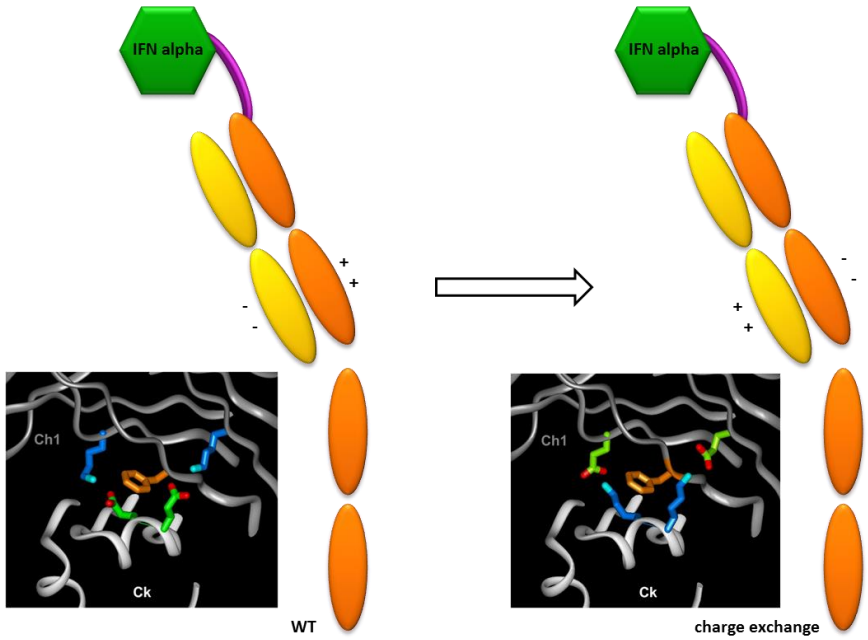


Figure 17: Charge reversal of heavy and light chains

Wild type IgG1 antibodies contain amino acids with positively charged residues in the CH1 domain of the heavy chain and negatively charged amino acids in the constant region of the light chain. To generate a charge reversal for prevention of chain miss-pairing, arginine and lysine in the CH1 domain are replaced by glutamic acid with a negative charge. Additionally to this modification, negatively charged amino acids in the constant kappa domain of the light chain are replaced by arginine and lysine to achieve a charge reversal.

4.1.3 HY-RF mutation

Due to the application of “knobs into holes” technology, it is possible to enforce correct heavy chain heterodimerization by steric hindrances of the “knob”-containing chain with another heavy chain carrying the same point mutation, though there still is a significant likelihood for the formation of hole - hole - homodimers which can pair with each other without any steric unfavorable amino acid residues. To prevent a time consuming protein purification procedure to separate bispecific antibodies from the homodimers, another mutation was applied to the interferon fusion protein. Based on findings from Natsume *et al.* and a patent from the company Regeneron, two point mutations were established in the CH3 domain of the “hole”-heavy chain of the fusion protein. The applied mutations mimic a CH3 domain of a human IgG3 antibody which inherently is not able to bind to protein-A. By introducing two specific point mutations, the modified “hole”- heavy chain is able to bind to a protein-A containing chromatographic column which leads to the separation of hole - hole - homodimers from correctly assembled

heterodimeric antibodies binding to the column via the unmodified “knob”-heavy chain. The following alignment of sequences from a human IgG1 CH3 domain and a human IgG3 CH3 domain demonstrates the applied amino acid exchange from histidine and tyrosine to arginine and phenylalanine to introduce a IgG3 like CH3 domain in the “hole”-heavy chain of the interferon fusion protein (Natsume et al., 2008; Samuel Davis, 2012).

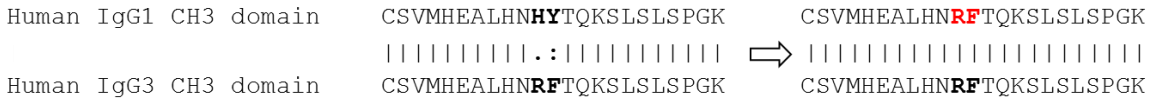


Figure 18: Modification of protein-A binding capacity

The above presented alignment of CH3 domains derived from human IgG1 and IgG3 antibodies explains the protein-A binding moiety and the applied modification of the CH3 domain of the interferon fusion protein. Due to the exchange of histidine and tyrosine to arginine and phenylalanine in the CH3 domain of the targeting antibody arm of the fusion protein, incorrectly assembled bispecific antibodies are not able to bind to protein-A chromatography columns anymore.

4.2 Cloning of expression plasmids

Based on the nucleotide sequence information of human interferon alpha and the anti-interferon antibody 9F3 (Genentech Inc., part of the Roche Group), DNA-Sequences coding for heavy and light chains of the interferon fusion protein were designed in the Vector NTI Advance® 11.5.0 Software (Invitrogen®, part of Life Technologies). Coding DNA sequences were N-terminal flanked by a BamHI restriction site, followed by a signal peptide (MGWSCIILFLVATATGVHS) responsible for extracellular secretion of the fusion protein after protein translation and post-translational modifications. At the C-terminus of each gene, two stop codons (TGA, TAA) were introduced in combination with a XbaI restriction site. Corresponding genes of interest (GOI) were synthesized by GeneArt® (part of Life Technologies) and delivered in a plasmid vector termed 14ACWX.

From the obtained lyophilized vectors, 2.5 µg of each plasmid DNA were dissolved in 25 µL deionized DNA-free water and mixed with 5 µL CutSmart™ Buffer (10x concentration), 15 µL water and 2.5 µL of the DNA restriction enzymes BamHI and XbaI. After an incubation of 30 minutes at 37°C, each sample was prepared for gel electrophoresis by the addition of 5 µL gel loading buffer (10x concentration). Gel electrophoresis of restricted plasmids was performed in a 1% agarose gel at 150V for 90 minutes. Following size separation of the genes and the 14ACWX vectors on the gel, DNA bands were visualized via UV-light at 280 nm and inserts cut out from the

gel. DNA recovery was performed with the peqGOLD Gel Extraction Kit according to the suppliers protocol. Gained insert DNAs coding for each chain of the interferon fusion protein were cloned into the linearized, eukaryotic expression plasmid RB63pUC-SSKHC1 (Roche Diagnostics GmbH), containing a CMV promoter, eukaryotic and prokaryotic origins of replication and an ampicillin resistance gene, according to the protocol of the Rapid DNA Dephos & Ligation Kit (Roche Diagnostics GmbH).

From each of the obtained plasmids, 200 µg of DNA were transformed into 50 µL of XL10-Gold ultracompetent cells (based on the *E.coli* strain K12) and incubated for 20 minutes on ice. After heat shocking the cells at 42°C for two minutes, 300 µL of S.O.C. medium (invitrogen™) were added, followed by an incubation step at 37°C for 45 minutes. Transformed bacteria were selected overnight on ampicillin containing agar plates (ImMedia™ Amp Agar, Invitrogen™) at 37°C. Positively selected clones were picked and transferred into 5mL LB-medium containing 100 µg/mL ampicillin and incubated for six hours in a rolling culture at 37°C. Following incubation, clone suspensions were expanded into 800 mL of ampicillin containing LB-medium and incubated overnight at 37°C. Finally, *E.coli* cultures were harvested via centrifugation at 6000 rpm for 30 minutes at 4°C. After removing the LB supernatant, bacteria pellets were processed according to the protocol of the PureLink® HiPure Plasmid Megaprep Kit (invitrogen™) and obtained plasmid DNA was stored at -20°C until further use.

4.3 Eukaryotic cell transfection and expression of fusion proteins

For the expression of interferon fusion proteins, the human embryonic kidney cell cline HEK293F (invitrogen™) was utilized. Cells were cultivated in FreeStyle™ 293 expression medium at 37°C, 80% humidity and 8.2% CO_2 concentration. For maintenance of the culture, cells were split down to 3*10^5 cells/mL after reaching 2*10^6 cells/mL every three days. On the day before transfection 1*10^6 cells/mL were seeded in 1 L of FreeStyle™ 293 expression medium and cultivated overnight. At the following day, 3.5 mL transfection reagent (293fectin™) were added into 15 mL Opti-MEM®I serum free medium and pre-incubated for five minutes at room temperature before addition of another 15 mL of Opti-MEM®I containing plasmid DNA coding for the desired protein. The four different plasmids containing the genes for heavy and light chains of the interferon fusion protein were applied at an equal ratio up to a total amount of 1.5 µg DNA/mL. After incubation of plasmid DNA and transfection reagent for 20 minutes at room

temperature, the transfection mixture was added dropwise to 1L of HEK293F cell culture under continuous shaking.

Transfected cells were cultivated for one week at 37°C in shaking culture without additional feeding. On day seven after transfection, the cells were harvested via centrifugation at 4750g for 45 minutes at 4°C to separate HEK293F cells and the interferon fusion protein containing supernatant. After sterile filtration, the supernatant was stored at -80°C until further protein purification steps.

4.4 Protein purification

4.4.1 Protein A chromatography

For the separation of correctly assembled bispecific interferon fusion proteins from the cell culture supernatant, protein A chromatography was applied. This type of chromatography was used due to natural occurring binding interactions between protein A and the CH3 domain of human IgG1 antibodies. To ensure endotoxin free protein purification, the Äkta Explorer device (GE health care) was rinsed with 0.5 M sodium hydroxide solution overnight, followed by a pH restoring rinse of the system with phosphate buffered saline (PBS). After installation of a 1mL HiTrap MabSelect Xtra protein A column (GE health care), the fusion protein containing cell culture supernatant was loaded onto the column at a flow rate of 0.9 mL/min overnight. On the following day, proteins bound to the column were washed with 10 mL PBS at a flow rate of 0.5 mL/min to remove remaining residues derived from the supernatant. After washing, bound antibodies were eluted from the protein A column via a pH shift by rinsing with citrate buffer (pH3) at a flow rate of 0.5 mL/min. Fractions containing eluted proteins were collected in 2mL aliquots followed by addition of 200 µL Tris buffer (2M, pH9) to restore a neutral pH value. Aliquots were stored on ice until further purification steps.

4.4.2 Size exclusion chromatography

To exclude undesired dimers and aggregates of interferon fusion proteins from the monomeric fraction, a size exclusion chromatography was performed. Due to varying passing times of molecules with different molecular weights, injected samples were segregated inside the porous

column material and eluted at different time points, depending on their size. For the chromatography, the Äkta FPLC device (GE health care) with an attached HiLoad 16/60 Superdex 200pg (GE health care) was applied. After removal of potential contaminations of the device by endotoxins via overnight rinsing with 0.5M sodium hydroxide, neutral pH was restored by rinsing the device and the enclosed separation column with histidine sodium chloride buffer (His NaCl buffer, pH6). Afterwards five milliliter of the protein A chromatography derived aliquots, containing the total amount of purified fusion proteins, were injected into the sample loop of the device. Size separation was performed by the injection of loaded samples into a continuous flow of His NaCl buffer (pH6) at a flow rate of 1mL/min, passing through the HiLoad 16/60 Superdex 200pg column. Fractions containing aggregates, dimers and monomers of the desired protein were collected manually after leaving the column. Gained fractions of the monomeric protein were pooled and stored in aliquots at -80°C until further analysis and *in-vitro* characterizations of the bispecific molecule.

4.5 Cell viability assay

Evaluation of the direct anti-proliferative activity of the previously proposed interferon fusion protein was conducted via the CellTiter®-Glo luminescence cell viability assay (Promega). In this assay, cell viability is determined via cell lyses followed by the quantitation of total adenosine triphosphate (ATP) concentration with the help of luminescence detection. Therefore the outer wells of a white 96-well plate with clear bottom were filled up with 300 µL PBS/well to prevent boundary effects on cell viability. The remaining 60 wells were seeded with 5000 tumor cell/ well in a final volume of 100 µL medium/well. In case of an adherent tumor cell line, cells were allowed to settle down for four to six hours. Tumor cell lines growing in suspension culture were treated directly after seeding into 96-well plates. To determine dose response curves for the biological activity of fusion proteins, serial dilutions containing 8 steps of 1:10 dilutions were prepared, starting with 10 or 20 nM fusion protein/well down to 0.001 or 0.002 pM, in six replicates. Previously described concentrations of fusion proteins were added in 5 µL of PBS to each well containing 5000 tumor cells. Used seeding and treatment scheme for a representative assay setup can be seen in the following figure.

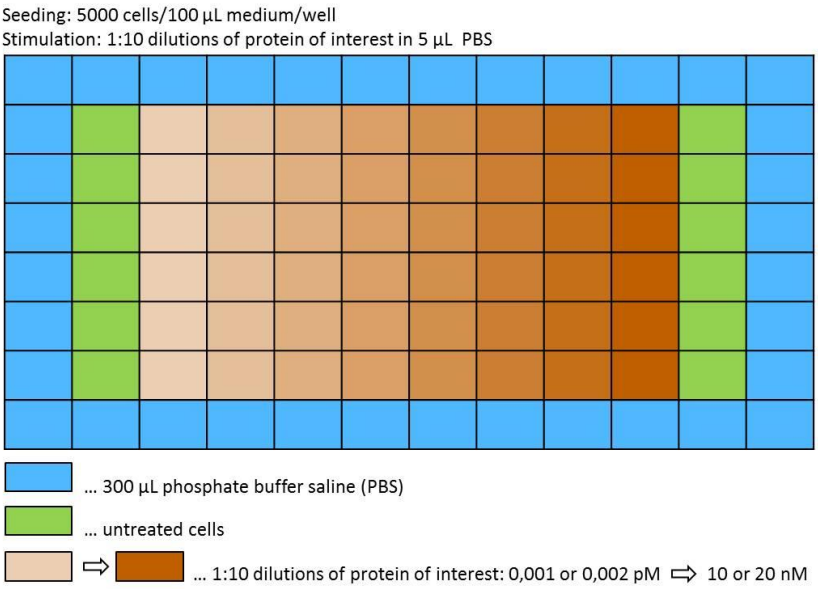


Figure 19: Seeding and treatment scheme for cell viability assays

The outer wells of a 96-well plate (blue) were filled up with PBS to prevent boundary effects. For each experiment 12 wells were seeded with tumor cells without any treatment (green) as a reverence. Serial dilutions of fusion proteins were applied to tumor cells in six replicates (light brown to dark brown).

After addition of the fusion protein containing treatment solution, cells were incubated for 72 hours at 37°C. To evaluate cell viability, the 96 well plate was allowed to cool down to room temperature before adding 100 µL CellTiter®-Glo reagent per well. Following an incubation time of 15 minutes, luminescence signal was then detected via an Infinite® M200 NanoQuant reader (Tecan).

4.6 Plasma stability

To control the stability of the protease cleavable peptide linker, connecting interferon to its masking antibody, plasma stability was evaluated via western blot. Therefore 1 µg of the interferon fusion protein was dissolved in 250 µL steril, virus free mouse plasma containing lithium heparin (Rockland Immunochemicals Inc.) and incubated for 24 hours at 37°C and 300rpm shacking mode. After 23 hours 50 µL of Dynabeads® protein A magnetic beads (Novex®) were added and allowed to catch antibodies dissolved in the mouse plasma for 60 minutes. The magnetic beads were separated from the murine plasma via a magnetic particle separator (SPR) and washed three times with 800 µL PBS. Washed beads were resuspended in 32.5 µL of deionized water and admixed with 12.5 µL NuPAGE® LDS sample buffer and 5 µL NuPAGE®

sample reducing agent. With a heating step at 70°C for 10 minutes, magnetic beads were separated and collected supernatants loaded on a NuPAGE® Novex® 4-12% Bis-Tris gel. Gel separation was performed for 35 minutes at 150V and separated protein bands were transferred onto a PVDF membrane by using the iBlot® western blot system according to the corresponding manual. The blotted membranes were blocked for one hour with 5 % skimmed milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) before further processing. Separated heavy chains of interferon fusion protein were stained by an overnight incubation step of the membrane in skimmed milk containing a 1:1000 dilution of a goat derived anti-human $F(ab')_2$ fragment coupled to peroxidase (Jackson ImmunoResearch Inc.). After removal of the staining solution, the membrane was washed three times with PBS. Luminescence signal was initiated via the addition of 3 mL Lumi-Light western blotting substrate (Roche Diagnostics GmbH) and detected by using a Lumi-Imager™ F1 (Roche Diagnostics GmbH).

4.7 Surface plasmon resonance (SPR – Biacore)

Nowadays many optical sensing methods are applied in pharmaceutical research and one of its most prominent representative is the surface plasmon resonance spectroscopy or shortly SPR. This kind of optical sensing technology detects variations in the refraction index in close proximity to the sensor surface, due to interactions between molecules immobilized on the surface of a chip and other molecules administered in solution to it. For SPR, commonly used chips are coated with a thin gold layer which can be modified by applying an additional layer of long-chained thiols or hydrogel for the covalent immobilization of biological agents which have to be investigated. The application of SPR for the determination of biological interactions provides a major advantage in contrast to alternative optical technologies due to its label-free measurement methodology. A SPR sensorgram generated by the detection of the refraction index delivers information about the kinetic rates between the tested molecules (association and dissociation) and gives additional information about the binding level of one interaction partner to another (figure 21) (Olaru, Bala, Jaffrezic-Renault, & Aboul-Enein, 2015).

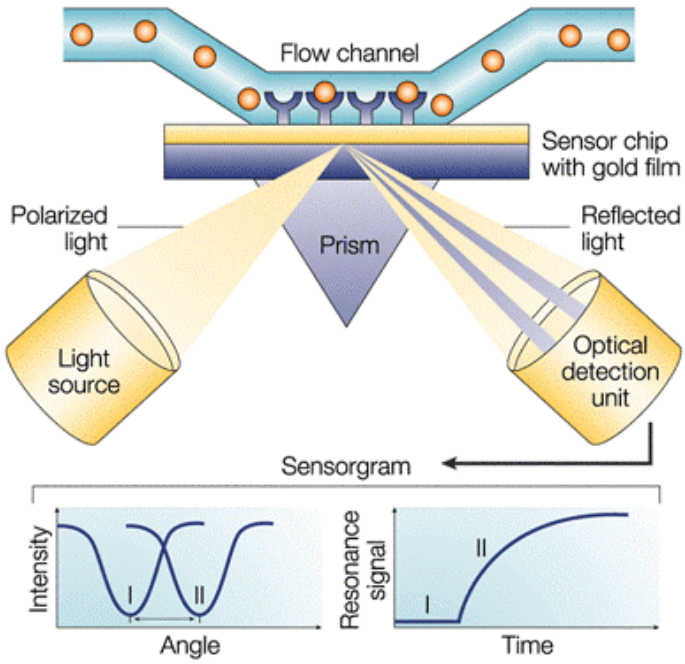


Figure 20: Surface plasmon resonance measurement principle

A SPR effect occurs when a beam of polarized light hits a thin metal film in the interface of two substances with different refractive indices. The SPR device detects variations of oscillating free electrons (surface plasmons) at the surface of the SPR chip which is triggered by the interaction of two binding partners, one immobilized on the chip and another one administered in solution. Due to the caused shift in the angle of reflected light, molecular binding events can be monitored and binding kinetics determined without the need of any labels (Cooper, 2002).

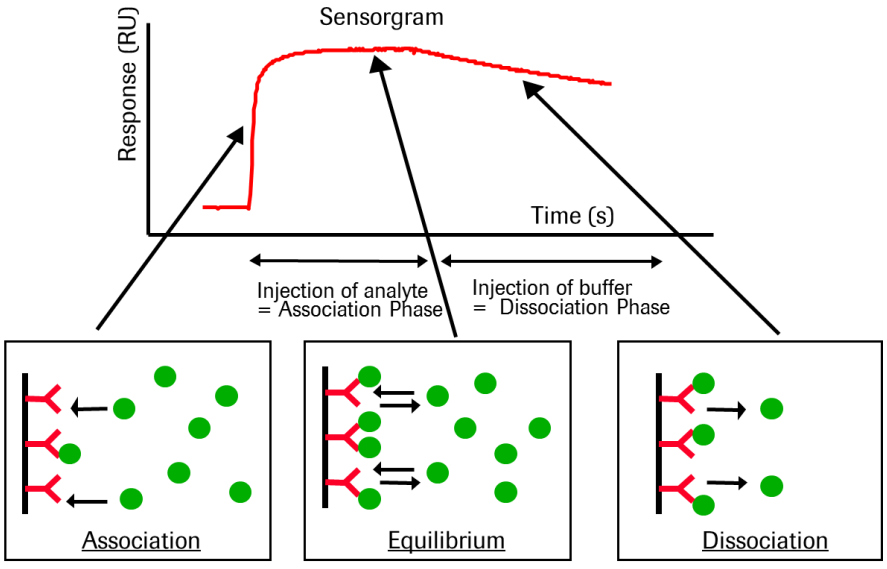


Figure 21: Example and explanation of a SPR senorgram

During the association phase, the SPR effect leads to absorption of light and a variation in the reflection angle over time which is detected in the sensorgram via an increase of the response rate (RU). A constant response is detected via the equilibrium phase of binding interactions followed by a decrease of the response rate during dissociation of both molecular binding partners.

Performed surface plasmon resonance experiments in this thesis were conducted on a Biacore T200 instrument (GE Healthcare). For the covalent immobilization of anti-human Fc catching antibodies (GE Healthcare) diluted in HBS-N coupling buffer (containing 10mM sodium acetate, pH 5), a gold coated CM5 sensor chip was coupled with amine according to the suppliers' recommendation (GE Healthcare) and activated by an EDC/NHS mixture. After loading of the chip with catching antibodies, residual carboxyl groups were inactivated by rinsing with ethanolamine. After the preparation of the chip, three of four champers on the chip were loaded with different interferon fusion proteins via binding of the Fc part of the fusion protein to the immobilized catching antibody. Applied sample dilution buffer was manufactured by the addition of 1 mg/mL bovine serum albumin (BSA) to the running buffer containing PBS admixed with 0.05 % Tween 20 (PBST). Concentrations of the fusion protein were adjusted to reach a total capture level in the range of 30 to 60 RU. After equilibration of the system to 37°C, serial dilutions (0.41 - 300 nM) of the corresponding interaction partner (human interferon alpha or recombinant CD138), dissolved in PBST running buffer, were injected at a flow rate of 50 µL/min with a contact time of 180 seconds. After each injection, the system was regenerated with 30 mM $MgCl_2$. Gained data were recorded and evaluated with the Biacore T200 evaluation software.

4.8 Confocal microscopy

Confocal microscopy represents a powerful technique to achieve high resolution images of tissue and cell structures even in thick specimens. Based on a process called optical sectioning, a focused laser light beam illuminates a diffraction-limited spot inside the sample in contrast to fluorescence microscopy where the whole specimen is illuminated at the same time. Within the limited spot of the sample, fluorescence dyes become excited and emit light. After refocusing this light in the objective image plane and after passing a pinhole, out-of-focus signals are removed and a high resolution image is created without any unwanted background signals. Due to the selective excitation of fluorophores in several optical sections, labelled molecules can be detected in different depths of the specimen without losing signal intensity. A schematic drawing of the light pathway used in confocal microscopy is shown by the following image.

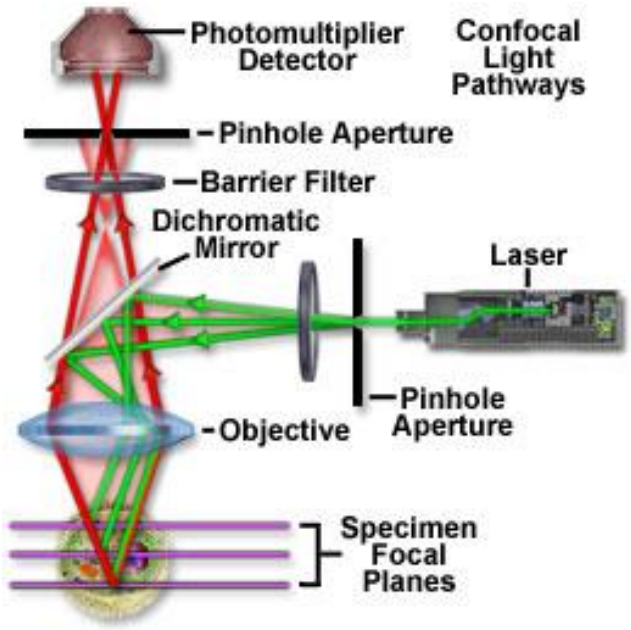


Figure 22: Scheme of the light pathway used in confocal microscopy

Laser light which is reflected by a dichromatic mirror passes the objective lens and leads to the excitation of fluorophores conjugated to the molecule of interest within the specimen. Emitted light from the labels passes a barrier filter and becomes focused via a pinhole before reaching the photomultiplier detector. Due to the pinhole background signals are removed which leads to improved image resolution in contrast to conventional fluorescence microscopy (© Nikon microscopy).

For the evaluation of the targeting moiety of the interferon fusion protein and its internalization rate, the protein was labelled with the fluorophore CF™568 by using the Mix-n-Stain™ antibody labeling kit (Biotium) according to the suppliers' brochure. For this experiment, the CD138 positive multiple myeloma cell lines U266B1, RPMI 8226 and NCI-H929 were used. As a negative control the CD138 low expressing cell lines NCI-H520 and HelaS3 were incubated with labelled fusion proteins. After an overnight incubation of 3*10^3 cells, seeded with standard media in a µ-Slide (ibidi), 5 µg/mL of labeled fusion protein were added to the cells. Images of the cells were taken after 1 h, 3 h, 8 h, 24 h and 48 h incubation time. Confocal microscopy was conducted by using an Eclipse TE2000-E, D-Eclipse C1si LSM microscope (Nikon) with a 4 laser system. For the excitation of labelled fusion proteins, a 561nm laser was applied and emitted fluorescence was collected through a 580-620 nm band pass filter. Gained fluorescence signals were captured and visualized by using the Nikon EZ-C1 V.3.80 software.

5. Results

The following section of the thesis summarizes results from the construction, manufacturing and *in vitro* characterization of the proposed bispecific interferon fusion protein, giving a brief overview of gained experimental data and explaining the rationale for this approach to overcome current bottlenecks in interferon based cancer therapy.

5.1 Screening for interferon responsive cell lines

To evaluate suitable clinical indications for an anti-tumor therapy based on targeted interferon, cell lines originating from several different tissues of the body were screened for their responsiveness to interferon-induced apoptosis. Goal of the performed screening was to determine oncologic indications in which the full potential of the targeted interferon fusion protein can be applied and therapeutic efficacy developed to the maximum. Due to current clinical indications of interferon alpha 2a, the main focus was set on multiple myeloma, soft tissue adenocarcinoma and malignant melanoma (figure 23).

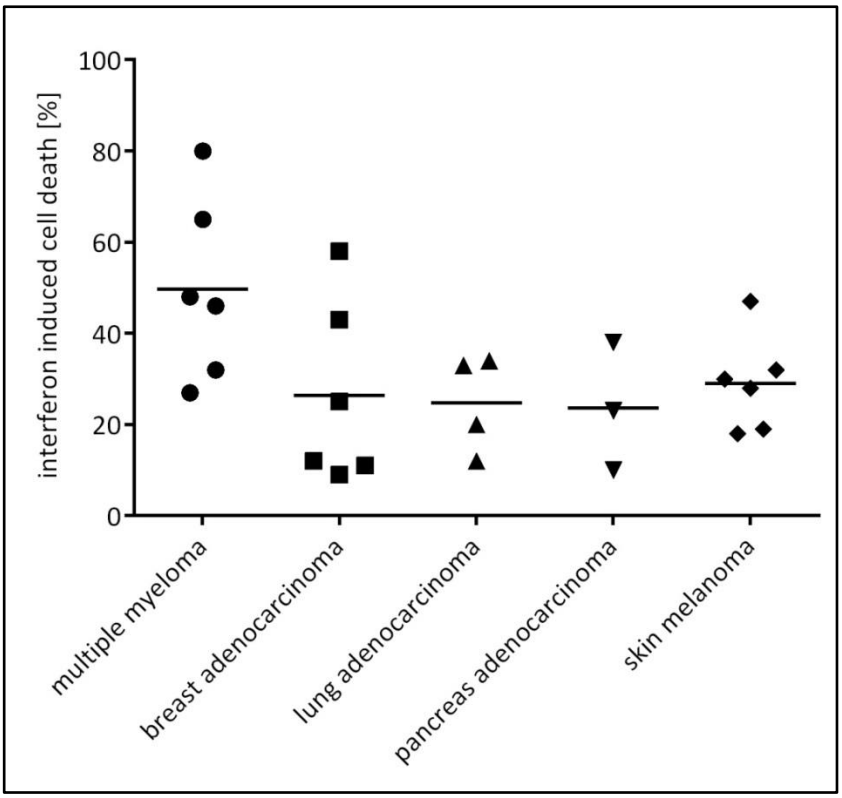


Figure 23: Interferon-induced apoptosis in different cancer cell lines

Cell lines from different oncologic indications were seeded separately in a 96 flat well plate with clear bottom at a density of 5000 cells / well. Cells were treated with a 1:10 serial dilution of interferon alpha 2a (PBL) at a maximum concentration of 10.000 IU for 72 hours. Following incubation, cell viability was determined by the CellTiter-Glo® luminescent assay. Each of the blotted values shows the detected cell death at 10.000 IU interferon/well compared to untreated cells. Average values were calculated on the basis of six replicates. Screenings of additional cell lines of each oncologic indication were performed by Guido Werner, Roche Diagnostics GmbH.

Treatment of tumor cell lines originating from different oncologic indications exhibited a strong variability in the efficacy of interferon-induced cell death. Response rates ranged from 10 % up to 85 % cell death after three days of stimulation with interferon alpha 2a. Moderate responses were seen in soft tissue carcinoma like breast adenocarcinoma (26.3 %) and lung adenocarcinoma (24.7 %). Promising results from the triple negative breast cancer cell lines MDA-MB468 (58 % cell death) and MDA-MB231 (43 % cell death) were not able to be confirmed for other tumor cell lines derived from the same indication. Treatment of malignant melanoma cell lines led to a slightly improved average response rate (29 %) compared to soft tissue adenocarcinomas. Most efficient induction of cell death was monitored in multiple myeloma cell lines with an average response rate of 49.6 %. Prominent representatives from this class of cancer type are the cell lines U266B1 (80 % interferon-induced cell death) and RPMI 8226 (65 % interferon-induced cell death). Based on the previously described findings, additional screening assays were performed with ten

different multiple myeloma cell lines to evaluate the suitability of this indication for further characterizations and possible applications of a targeted bispecific interferon fusion protein (figure 24). Multiple myeloma cell lines were derived from the in house CELLO cell bank which was established and characterized by Chugai, a member of the Roche Group based in Tokyo, Japan.

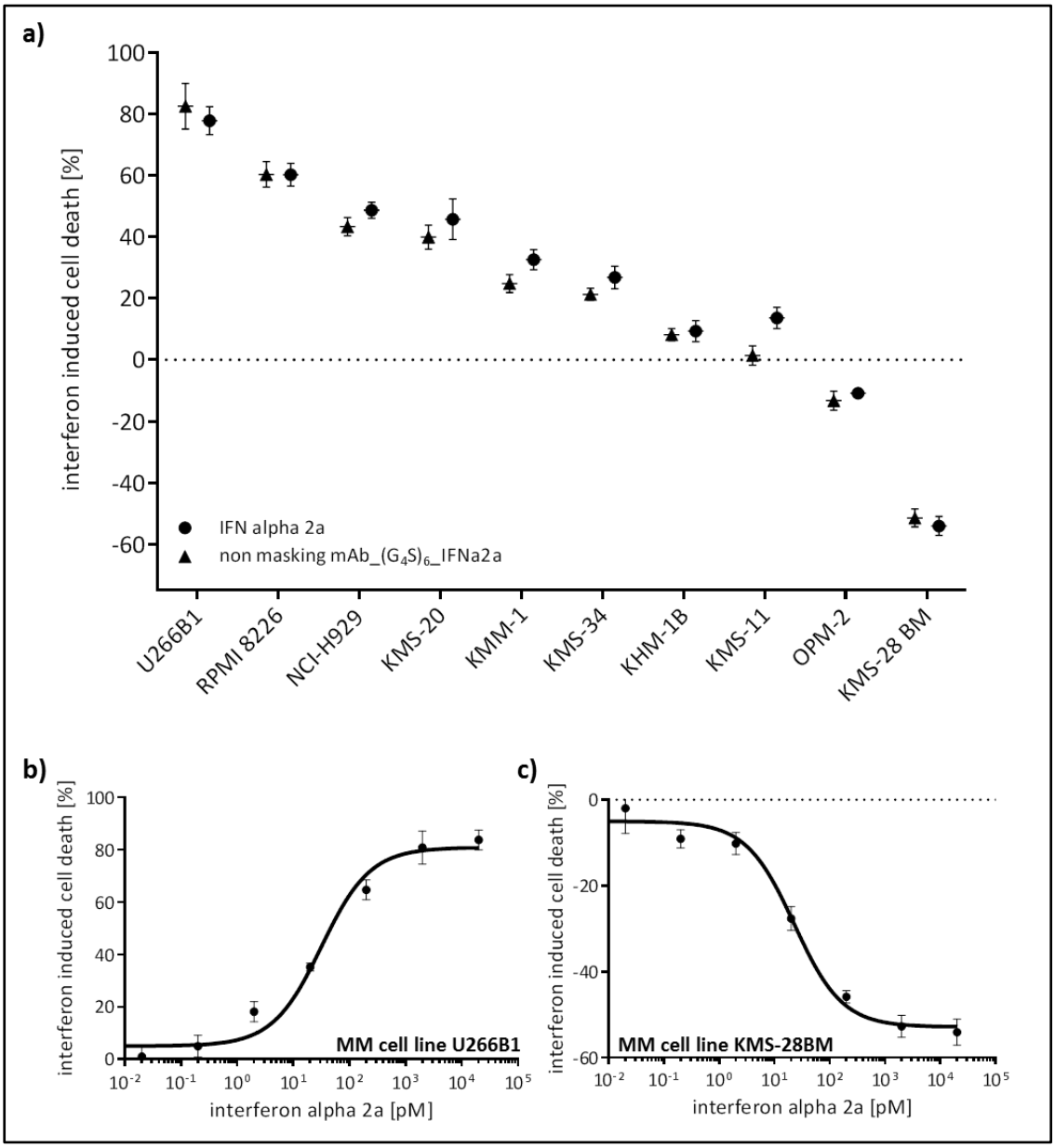


Figure 24: Interferon-induced cell death in multiple myeloma cell lines

Cell viability assay of multiple myeloma cell lines obtained from the CELLO cell bank were seeded separately in a 96 flat well plate with clear bottom at a density of 5000 cells / well. After 72 hours cell viability was determined by a CellTiter-Glo® luminescence assay. Blotted response rates were calculated in comparison to untreated cells. Standard deviations for each data set were determined based on six replicates. **a)** Cells were treated with a 1:10 serial dilution of 20 µM interferon alpha 2a (dots) or 10 nM of a non-masking antibody fused with IFN alpha 2a (triangle). **b)** Dose response curve of the multiple myeloma cell line U266B1 treated with a serial dilution of human interferon alpha 2a. **c)** Dose response curve showing growth stimulation triggered by IFNalpha2a in KMS-28BM. Half of the shown cell viability assays were generated in close collaboration with Guido Werner, Roche Diagnostics GmbH.

Treatment of multiple myeloma cell lines with interferon alpha 2a or a non-masking fusion protein containing IFN alpha 2a resulted in a diverse set of cell viability responses. Six of ten evaluated cancer cell lines (NCI-H929, KMS-20, KMM-1, KMS-34, KHM-1B and KMS-11) demonstrated moderate induction of cell death up to 50 % in the presence of interferon alpha 2a or the corresponding antibody fusion protein. In contrast to the common anti-proliferative effect of interferon alpha in multiple myeloma, the tumor cell lines OPM-2 and KMS-28 BM showed increased cell growth rates (OPM-2: 10-13 %; KMS-28 BM: 51-54 %) in the presence of the cytokine or the fusion protein. Most efficient induction of cell death by interferon-mediated apoptosis was monitored in the cell line U266B1 (82 % cell death) and RPMI 8226 (60 % cell death). Evaluated interferon responsive multiple myeloma cell lines showed similar dose response rates in the range of 0.1 pM up to 10 nM with differences in the maximum achieved induction of cell death.

5.2 Anti-proliferative effect of Type I interferons

Initial interferon activity evaluations and cell viability assays were performed with interferon alpha 2a due to the in-house production of IFN alpha 2a (Roferon™) at the company Roche Diagnostics GmbH and approved oncologic indications for this type of cytokine. The family of Type I interferons consists of 13 naturally occurring IFN alpha subtypes with differences in their anti-proliferative potency. To evaluate the anti-proliferative activity of each IFN subtype and determine the most suitable cytokine for an antibody fusion protein, all 13 wild type interferon alphas, an artificial murine and human cross-reactive IFN (universal IFN) and IFN beta 1b were tested in a cell viability assay on the multiple myeloma cell line U266B1 (figure 25).

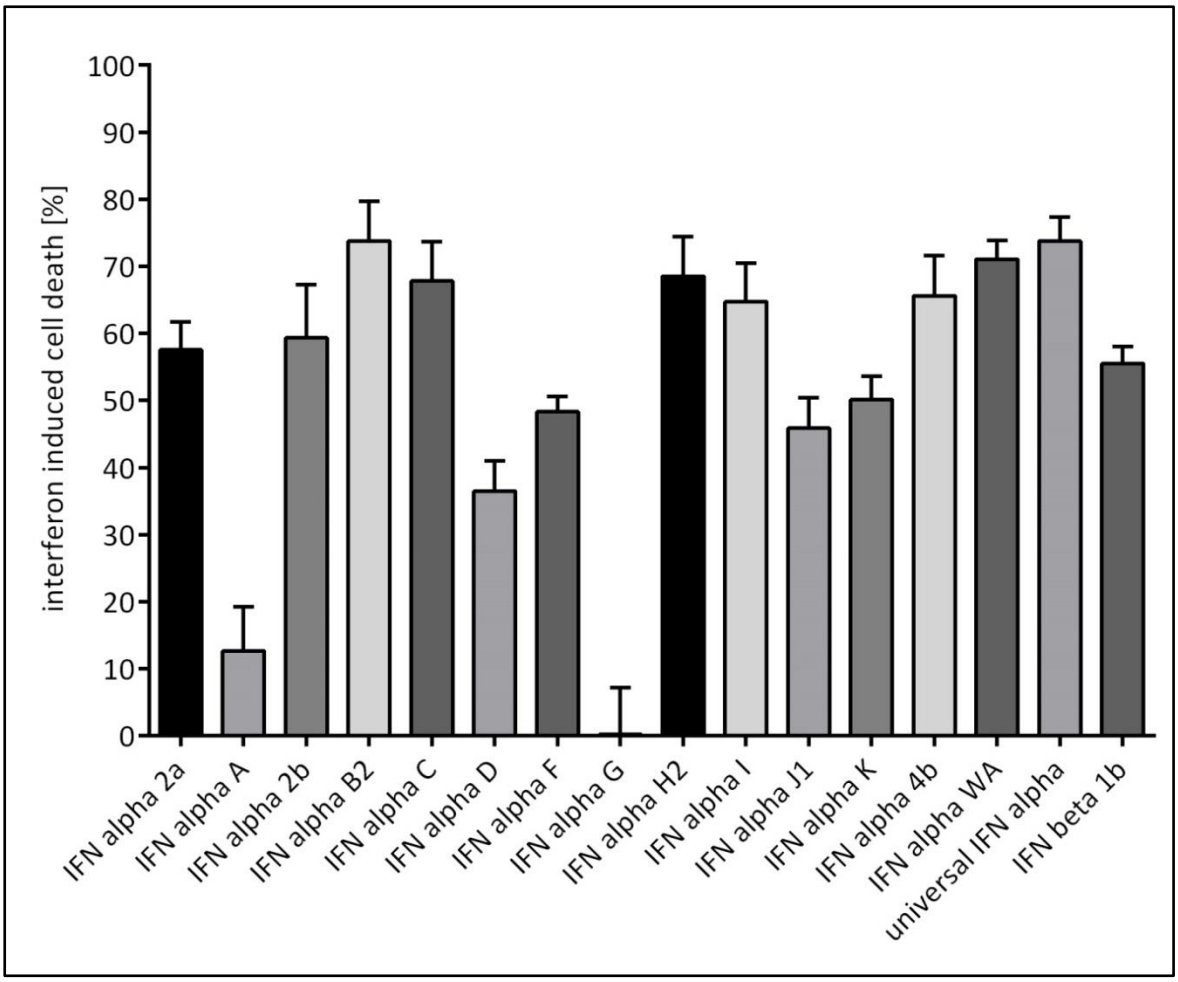


Figure 25: Efficacy screening of human IFN alpha subtypes

Cells from the multiple myeloma cell line U266B1 were seeded separately in a 96 flat well plate with clear bottom at a density of 5000 cells / well and treated with a 1:10 serial dilution of human IFN alpha 2a with a maximum concentration of 10.000 IU. Cell viability was determined by a CellTiter-Glo® luminescence assay after 72 hours. Maximum achieved growth inhibition values, compared to untreated cells, are shown in the graph. Standard deviations were calculated on the basis of six replicates. Except treatment of cells with IFN alpha 2a, IFN alpha H2 and universal IFN alpha, screenings were performed in collaboration with Guido Werner, Roche Diagnostics GmbH.

Treatment of U266B1 multiple myeloma cells with members of the interferon alpha family resulted in various anti-proliferative response rates, in the range of 12.6 % to 73.8 % cell death. Despite the overall high response rate of U266B1 to interferon alpha subtypes, treatment with IFN alpha A (12.6 % cell death) and IFN alpha G (no clear response) showed low or no detectable induction of cell death. Compared to the clinical approved treatment of multiple myeloma with interferon alpha 2a, additional members of Typ I interferon alphas showed enhanced anti-proliferative activity. Especially treatment of U266B1 cells with 10.000 IU of IFNαB2 (73.8 %), IFNαH2 (68.6 %), universal type IFN alpha (73.8 %) and IFNαWA (71.1 %) resulted in an increased pro-apoptotic effect compared to IFNα2a (57.6 %). As a result of the performed cell viability assays, potent candidates for interferon antibody fusion proteins were identified, like IFNαH2 and universal type IFN.

5.3 Improvement of pharmacodynamics parameters of interferon

Previously described cell viability assays revealed differences in the anti-proliferative potency of natural occurring interferon alpha subtypes on cancer cells. It is known that all members of the Typ I interferon family bind to a common receptor (IFNAR) on the surface of cells and mediate their biological effect via the same downstream signaling cascade. According to findings from Piehler *et al*, differences in the anti-proliferative activity of interferon subtypes can probably be explained by varying affinities of interferon alphas to the interferon receptor unit 1 (IFNAR1) (Piehler, Thomas, Garcia, & Schreiber, 2012). To investigate the correlation between the affinity of interferon alpha to IFNAR1, a 3D modelling approach was performed and biological effects shown by *in vitro* assays.

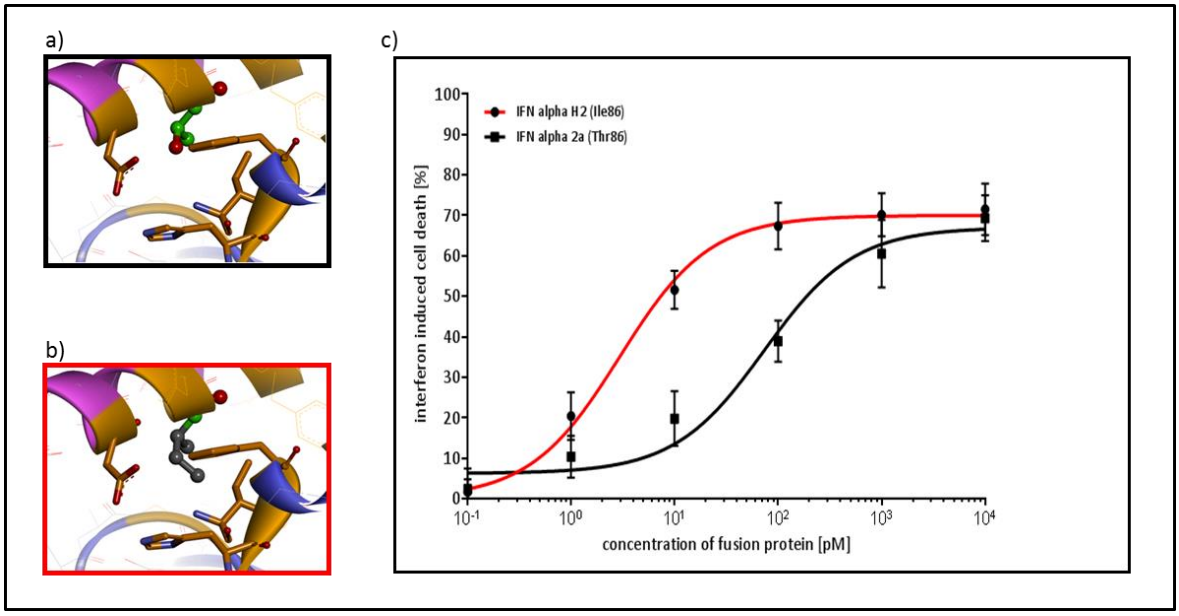


Figure 26: Receptor affinity-mediated increase of anti-proliferative effects

A 3D computer modelling approach reveals relevant amino acid interactions between human IFN alpha 2a and its corresponding receptor unit IFNAR1 **(a)**. Interferon alpha H2 displays an exchange of threonine to isoleucine at position 86 compared to interferon alpha 2a **(b)**. Cell viability assays of human IFN alpha 2a and human IFN alpha H2 on the multiple myeloma cell line U266B1 **(c)**. Dose response curves were determined by treatment of 5000 cells / well in a 96 flat well plate with serial dilutions of interferon, containing a maximum concentration of 10 nM. Standard deviations were calculated based on six replicates. Differences in the anti-proliferative response were confirmed in several tumor cell lines of different oncologic indications, like breast adenocarcinoma, pancreatic carcinoma and ovarian cancer (data not shown). 3 D modelling of biomolecules was conducted by Dr. Guy Georges, Roche Diagnostics GmbH.

Affinity-mediating amino acids in the binding region of interferon alpha to the interferon receptor unit 1 were identified via a 3D modelling approach based on open source crystallographic data. In

case of interferon alpha 2a, threonine at position 86 of the molecule was identified as key player in the interaction between the cytokine and its receptor. In contrast to IFN alpha 2a, interferon alpha H2 possesses an exchange of threonine to isoleucine at position 86 (Thr 86 Ile). A cell viability assay performed with the multiple myeloma cell line U266B1 revealed major differences in the anti-proliferative potency of both IFN alpha subtypes. Significant differences were seen in the half maximum inhibitory concentration (IC50) of the cytokines. Based on the evaluated dose response curves, an IC50 value of 146.6 pM for IFN alpha 2a and 5.87 pM for IFN alpha H2 was determined.

The company Teva GmbH presented data of an interferon efficacy study on multiple myeloma patient-derived bone marrow at the 6th World ADC Summit, San Diego 2014 (figure 27).

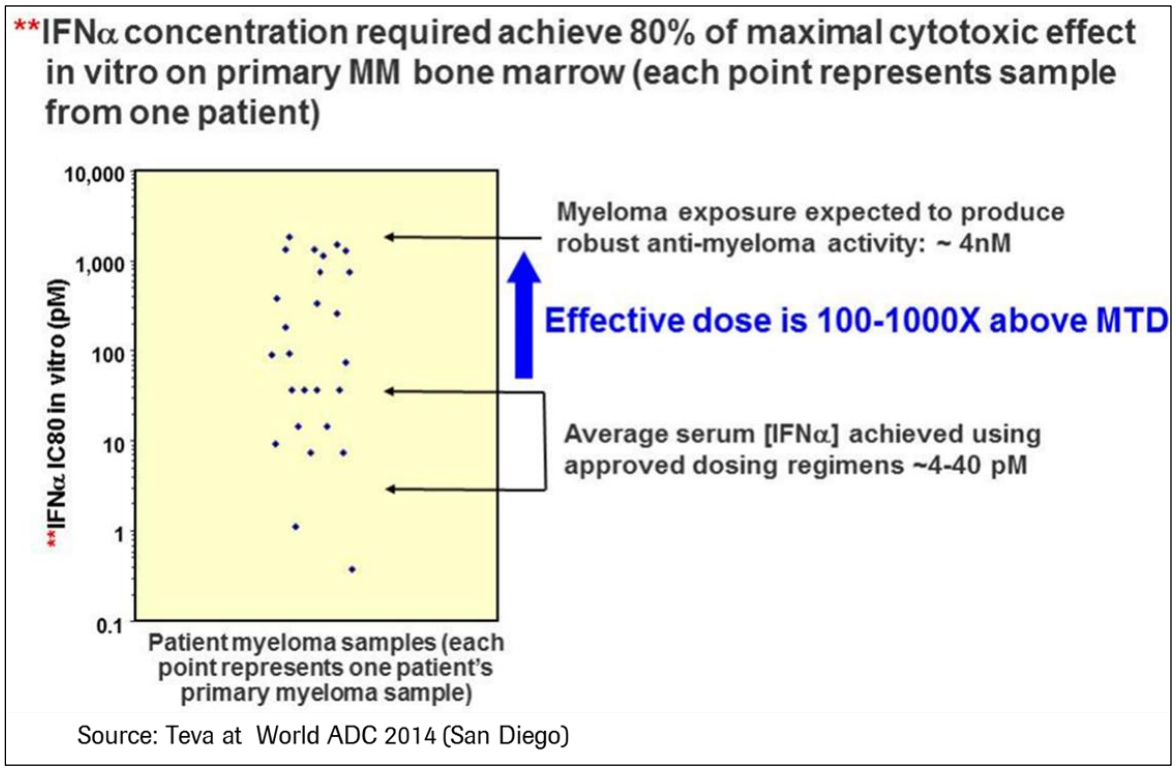


Figure 27: IFN efficacy study in MM patient-derived bone marrow

Presented data showed that effective treatment of multiple myeloma patients with interferon alpha would require IFN doses above the maximum tolerable dose of the cytokine. One way to overcome this bottleneck in interferon-based treatment strategies is the improvement of the pharmacodynamics potency of interferon. To address this task, previously gained knowledge about the differences in the anti-proliferative potency of IFN alpha 2a and IFN alpha H2 was used

to further investigate the interaction between interferon and its receptor. Based on the identification of the important receptor interaction capability of threonine at position 86 of interferon alpha, further point mutations were determined for a potential enhancement of this effect (figure 28).

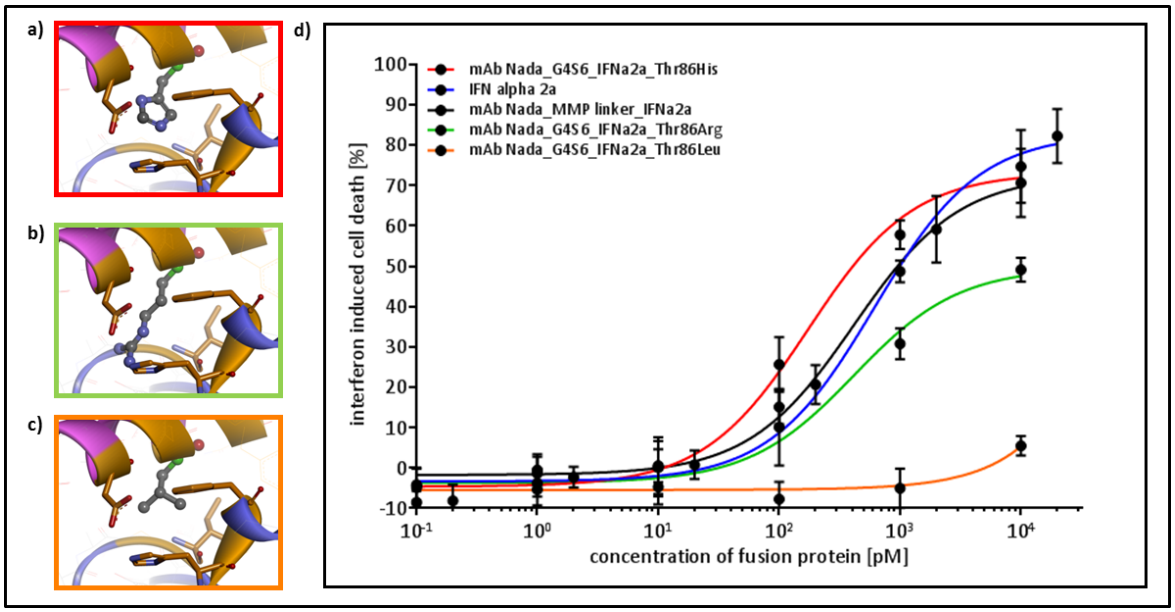


Figure 28: Affinity maturation within the IFN/IFNAR2 interaction domain

Threonine at position 86 of wild type human interferon alpha 2a was replaced by point-mutations to histidine **(a),** arginine **(b)** and leucine **(c)**. Antibody fusion proteins containing point mutated interferon were tested in a 1:10 serial dilution, starting at 10 nM, on the multiple myeloma cell line U266B1 **(d)**. Cell viability was determined by a CellTiter-Glo® luminescence assay after 72 hours and standard deviations calculated based on six replicates. 3 D modelling of biomolecules was conducted by Dr. Guy Georges, Roche Diagnostics GmbH.

Table 2: IC 50 values of IFN point mutations

IFN alpha 2a variations	IC 50 [pM]
wild type	415.0
Thr 86 Leu mutation	~ 7.276e+006
Thr 86 Arg mutation	415.6
Thr 86 His mutation	173.0

Interferon fusion proteins containing interferon alpha 2a with an exchange of threonine at position 86 to arginine (green dose response curve) and leucine (orange dose response curve) showed a decreased anti-proliferative potency compared to an unmodified variant (black dose response curve). Replacement of threonine at position 86 to histidine has led to a significant improvement of the pharmacodynamic potency of the fusion protein (red dose response curve) (figure 28). Due to the exchange of threonine 86 to histidine, it was possible to reduce the IC50 value of the wild type IFN fusion protein (415 pM) by more than half (173 pM).

5.3.1 Combined mutations for maximum IFNAR1 affinity

Similar improvements in the modifications of the interaction between interferon alpha and the corresponding receptor unit 1 were achieved by the group of Gideon Schreiber at the Weizmann Institute, Israel (Eyal Kalie, Jaitin, Abramovich, & Schreiber, 2007). The outcome of a randomized mutagenesis of interferon and affinity screening against IFNAR1 has led to the discovery of three point mutations termed YNS mutation which mediate an enhanced affinity of interferon to IFNAR1 and trigger an increased anti-proliferative effect of the cytokine. Due to these findings it was tried to validate this data in-house and generate an interferon fusion protein containing IFN with YNS mutations. To investigate the potential improvement of the pharmacologic potency of interferon by applying the previously proposed Thr 86 His mutation in combination with YNS mutations, additional fusion proteins were generated and tested on the ovarian cancer cell line OVCAR-3 (figure 29).

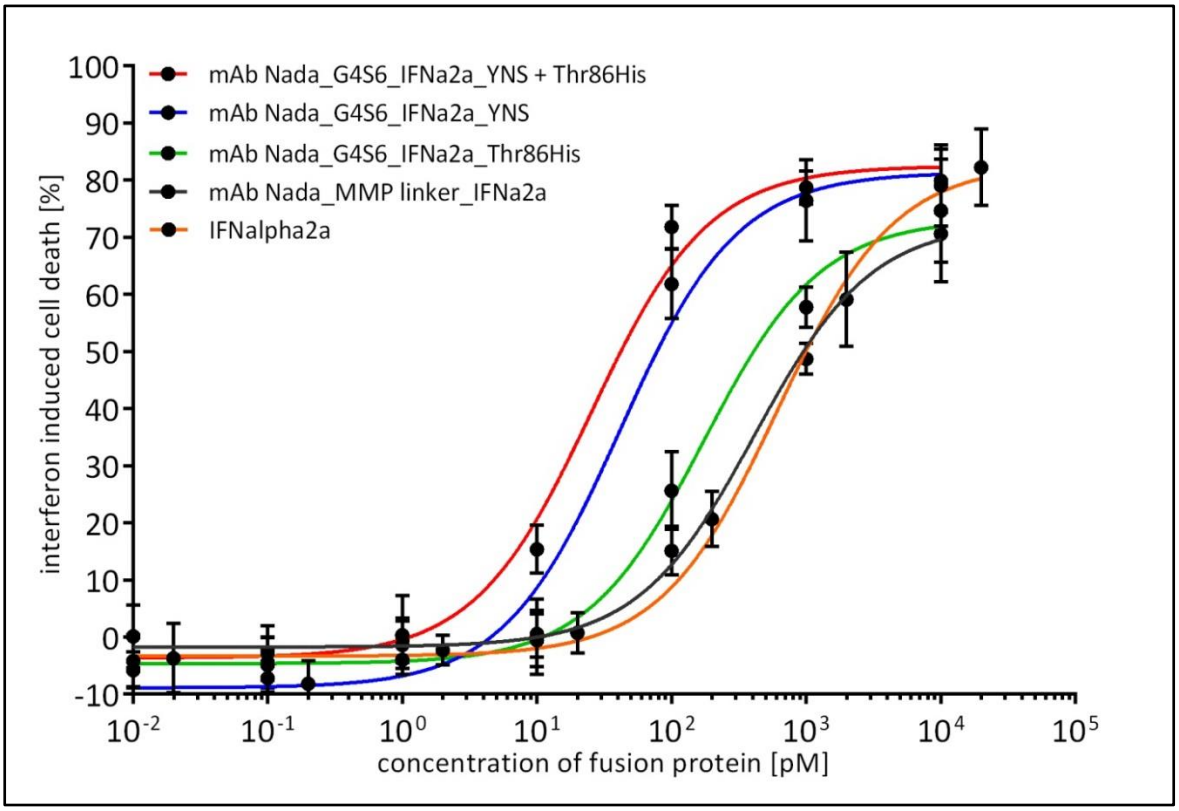


Figure 29: Stepwise improvement of the pharmacodynamic efficacy of interferon alpha

Induction of cell death was monitored on the interferon sensitive ovarian cancer cell line OVCAR-3 after stimulation with serial dilutions of interferon alpha 2a (orange curve) [20nM], an antibody fusion protein including wild type IFN alpha 2a (**black line**) [10nM], and fusion proteins with IFN alpha 2a containing a Thr86His mutation (**green line**) [10nM], a YNS mutation (**blue line**) [10nM] published by Schreiber *et al.* and a combination of Thr86His and YNS mutations (**red line**) [10nM]. Cell line response was evaluated by a CellTiter-Glo® luminescence assay after 72 hours. Each treatment was performed and values calculated based on six replicates.

Table 3: Potency of IFN alpha 2a mutations

Anti-proliferative agent	IC 50 [pM]
IFN alpha 2a, wild type	613.2
Fusion protein containing IFN alpha 2a, wild type	415.0
Fusion protein containing IFN alpha 2a, Thr 86 His mutation	173.0
Fusion protein containing IFN alpha 2a, YNS mutation	41.43
Fusion protein containing IFN alpha 2a, Thr 86 His + YNS mutation	24.24

Treatment of OVCAR-3 cells with interferon alpha 2a and the corresponding interferon fusion protein resulted in similar anti-proliferate effects. Application of a Thr 86 His mutation in interferon alpha has led to an improvement of the pharmacological potency of the fusion protein by a factor of 2.4 (green curve). A significant shift in the potency of the fusion protein was achieved by the implementation of YNS mutations into to cytokine (blue curve). Compared to the interferon fusion protein containing wild type interferon alpha 2a, a tenfold reduction in the IC50 value (415 pM to 41.43 pM) was determined. Although YNS mutations trigger a potent anti-proliferative effect, the combination of YNS mutations and the investigated Thr 86 His mutations showed the most effective induction of cell death in the ovarian cancer cell line OVCAR-3.

A significant improvement of the anti-proliferative action by the implementation of the Thr 86 His and the YNS mutations was shown in fusion proteins containing interferon alpha 2a. Previously presented data (figure 26) revealed an enhanced potency of wild type interferon alpha H2 compared to IFN alpha 2a. To evaluate the effect of the found point mutations on a more potent interferon alpha subtype, fusion proteins were generated containing interferon alpha H2 and variants with the above mentioned point mutations. It was tried to enhance the potency of IFN alpha H2 to generate an artificial interferon alpha which exhibits maximized anti-proliferative efficacy to achieve high response rates in oncologic indications at minimal serum levels (figure 30).

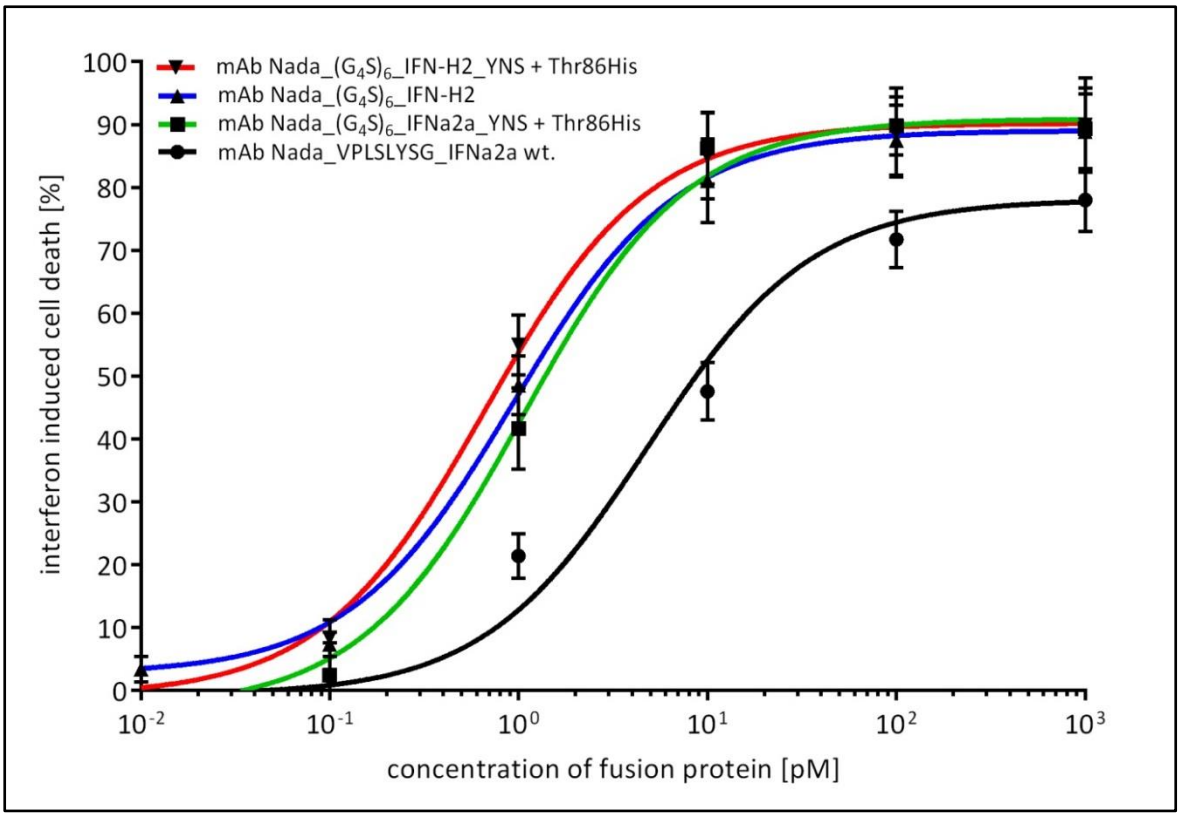


Figure 30: Potency enhancing mutations in IFN alpha 2a compared to IFN alpha H2

Affinity increasing point mutations, YNS and Thr86His were implemented in fusion proteins containing human IFN alpha 2a or IFN alpha H2. Multiple myeloma cell line U266B1 was seeded at a density of 5000 cells / well in a 96 flat well plate and treated with serial dilutions of both wild type interferons [20nM] and the corresponding fusion proteins [10nM] containing modified interferon variants. After 72 hours cell viability was determined by performing a CellTiter-Glo® luminescence assay. Blotted values display the difference in cell viability compared to untreated cells. Standard deviations were evaluated based on six replicates.

Table 4: IC50 values of IFN alpha H2 mutants

Interferon fusion protein	IC 50 [pM]
Fusion protein containing IFN alpha 2a, wild type	4.81
Fusion protein containing IFN alpha 2a, Thr 86 His + YNS mutation	1.07
Fusion protein containing IFN alpha H2, wild type	0.94
Fusion protein containing IFN alpha H2, Thr 86 His + YNS mutation	0.67

The implementation of Thr 86 His and YNS mutations in interferon alpha 2a has triggered a remarkable shift in the potency of the cytokine (green curve) compared to wild type interferon alpha 2a (black curve). An interferon fusion protein containing wild type IFN alpha H2 (blue curve) displayed a similar anti-proliferative potency than fusion proteins with IFN alpha 2a with affinity enhancing mutations. Modification of IFN alpha H2 by applying Thr 86 His and YNS mutations (red curve) has led to a slight improvement in the apoptosis induction capability of the fusion protein (figure 30). According to these results it was possible to improve the potency of wild type IFN alpha 2a by specific point mutations to a similar level than IFN alpha H2. Additional modifications

of the affinity of IFN alpha H2 against IFNAR1 resulted only in minor improvements of the anti-proliferative action compared to wild type IFN alpha H2. The comparison of IC50 values of the tested interferon variants (table 4) revealed fusion proteins containing IFN alpha H2 with Thr 86 His and YNS mutations as the most potent anti-proliferative constructs.

To compare the previously presented potency (figure 30) of the interferon fusion protein with conventional treatment strategies in oncology, the multiple myeloma cell line U266B1 was treated with several cytotoxic or cytostatic compounds as well as with the interferon fusion protein containing point mutated IFN alpha 2a (figure 31). The used cell line is known for its robustness and high resistance against chemotherapeutic agents. Due to the characteristics of this U226B1 cancer cells and the target indication for interferon fusion proteins, this multiple myeloma cell line was chosen for the performed comparison.

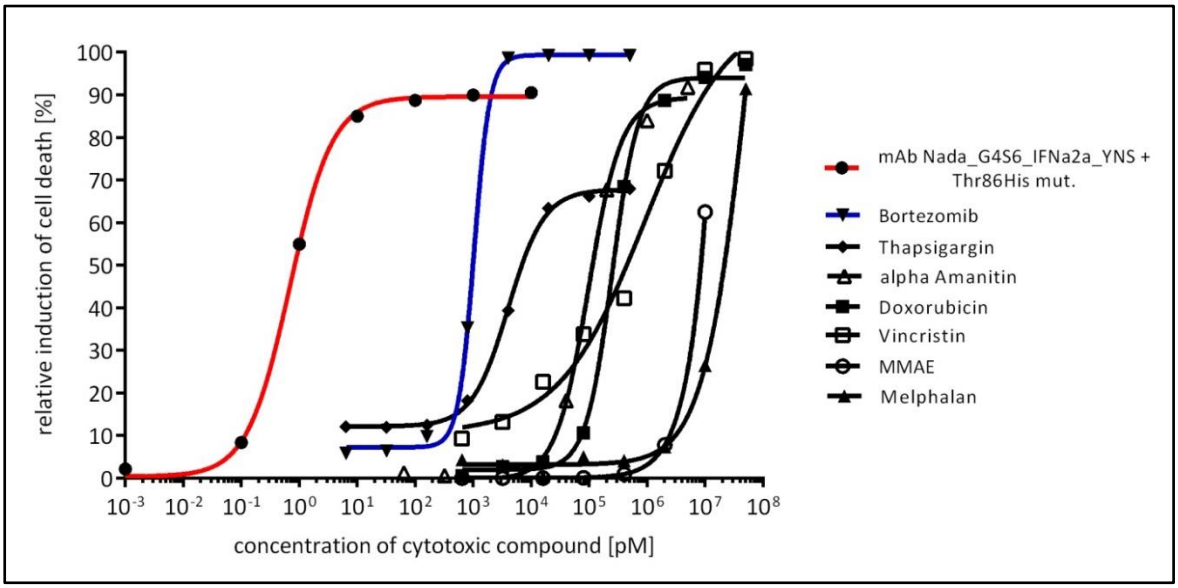


Figure 31: Outstanding IFN potency compared to standard of care treatment

Cell viability was determined after treatment of the multiple myeloma cell line U266B1 with standard of care proteasome inhibitor bortezomib, various cytostatic agents and the targeted interferon fusion protein. Serial dilutions of each inhibitor were administered to 5000 cells / well for 72 hours. Final read out was performed by a CellTiter-Glo® luminescence assay. Treatment of U266B1 cells with cytotoxic agents was performed by Guido Werner, Roche Diagnostics GmbH due to safety reasons.

Table 5: Potency of cytostatic compounds

Cytostatic compound	IC 50 [pM]
Targeted interferon fusion protein	0.69
Bortezomib	1.03e+003
Thapsigargin	4.0e+003
α-Amanitin	9.7e+004
Doxorubicin	2.47e+005
Vincristin	9.14e+005
Melphalan	6.27e+007
MMAE	2,84e+008

Treatment of U266B1 suspension cells with the DNA alkylating compound Melphalan and the anti-mytotic drug MMAE resulted in weak responses with IC50 values in micromolar range. Moderate anti-proliferative effects were determined via the inhibition of the multiple myeloma cell line with serial dilutions of the anti-mytotic drug Vincristin, the DNA intercalating agent Doxorubicin and the DNA alkylating compound Melphalan in a nanomolar IC50 range. Although the sarco/endoplasmatic reticulum Ca^{2+} ATPase inhibitor Thapsigargin showed a potent anti-proliferative acivity in a low nanomolar range, apoptosis was merely incuced in 67.76 % of the treated cells. Treament of U266B1 cells with the standard of care therapy in multiple myeloma, Bortezomib (proteasome inhibitor), resulted in a 99.35 % killing rate at an IC50 value of 1.03 µM. In contrast to Bortezomib, the application of serial dilutions of the point mutated interferon fusion protein has triggered an apoptosis induction of 89.56 % with an IC50 value of 0.69 pM. Due to the anti-proliferative actions of Bortezomib and the IFN fusion protein, the antibody cytokine fusion protein showed an improvement in therapeutic potency by more than a factor of 1000 compared to standard of care treatment.

5.4 Masking of interferon

The following part of the thesis deals with attempts to temporary inhibit the activity of interferon by masking the cytokine with an antibody. Interferon therapy in oncologic indications is accompanied by unspecific interactions in the body, leading to severe side effects (Jonasch & Haluska, 2001) and a reduction in the patient's quality of life. Due to these unwanted interactions, maximum tolerable serum concentrations of interferon are often below the necessary therapeutic level. To address this limitation in interferon based cancer therapy and

possibly broaden the clinical applicability in oncology, a masking attempt of the cytokine was investigated.

To bind interferon and temporary inactivate the cytokine, the anti-interferon alpha 2a antibody termed 9F3 (Genentech Inc.) was used to generate an interferon fusion protein. Due to a high affinity of the 9F3 antibody to IFN alpha 2a in a range of 25 pM, the antibody seemed to be suitable for an efficient masking and inactivation of the cytokine. The proposed mode of action of the antibody interferon fusion protein requires a masking of the cytokine during circulation in the periphery and a release and reactivation of interferon after cleavage of the peptide linker at the tumor site. Because of the very high affinity of the 9F3 antibody to IFN alpha 2a, it was possible to establish an efficient masking but without the possibility of a release of the cytokine. To enable the diffusion of interferon away from the antibody after cleavage of the peptide linker, it was necessary to reduce the affinity of the antibody to a level at which sufficient inactivation of the cytokine takes place in combination with a possible release of it at the target site.

For the reduction of the affinity of the 9F3 antibody, a 3D modelling approach was used to determine amino acids in the CDR region in the light and heavy chain of the antibody which mainly mediate binding of the antibody to interferon alpha 2a. As a result of this investigation, two amino acids in the heavy chain and three amino acids in the light chain were identified to be key players in the interaction between the antibody and the cytokine. Specific point mutations of the previously described position to amino acids with non-reactive side chains were performed to achieve a reduced affinity of the antibody against IFN alpha 2a with simultaneous maintaining of its masking capability. Efficacy of the performed affinity modulating mutations were evaluated in the multiple myeloma cell line U266B1 (figure 32).

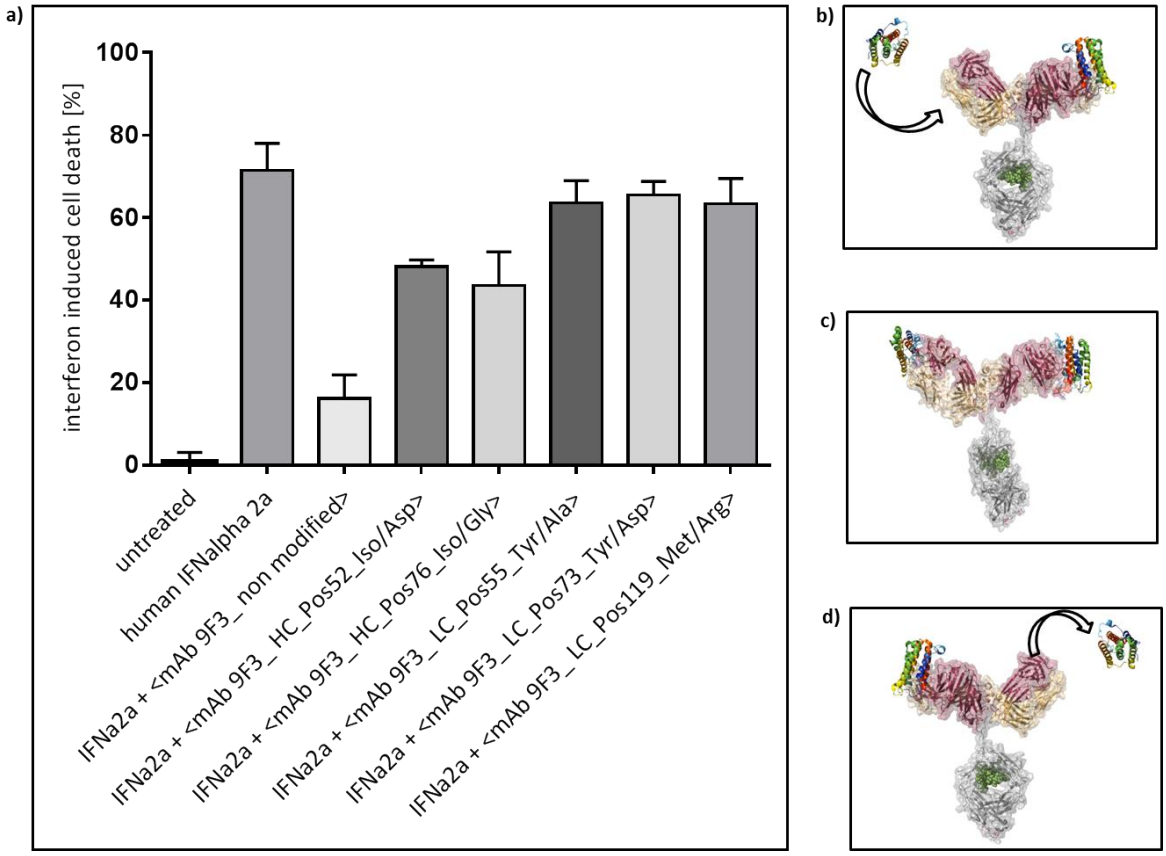


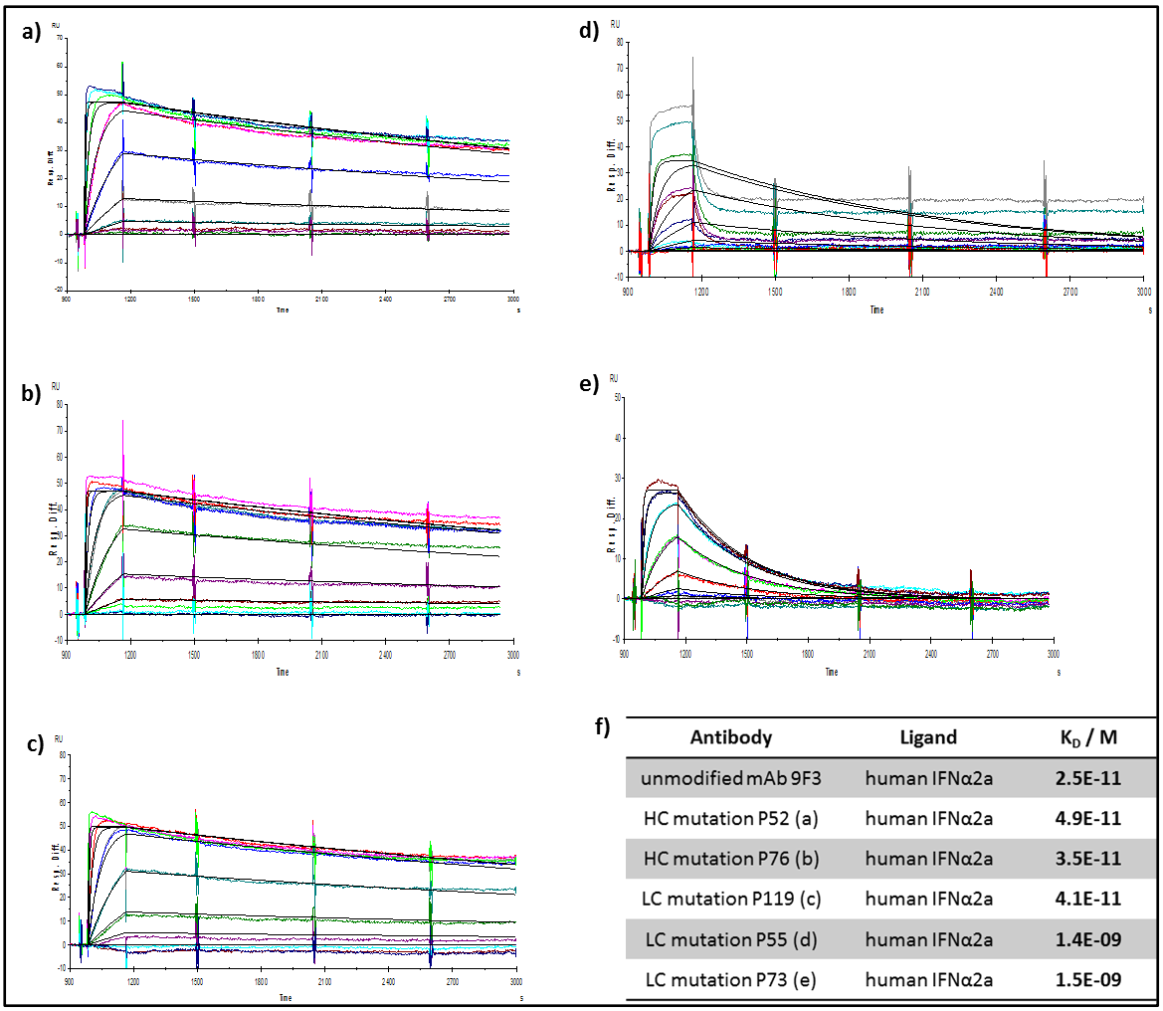
Figure 32: Masking and temporary inactivation of interferon

a) Evaluation of the masking ability of the anti-IFN alpha 2a antibody 9F3 and its modified versions. The multiple myeloma cell line U266B1 was treated either with human interferon alpha 2a [10.000 IU/mL] as single agent or in combination with inactivating antibodies, containing specific point mutations for modified masking abilities. A ratio of IFN : masking antibody of 2 : 1 was used. Masking efficiency was determined by the detection of interferon activity in combination with its masking antibody compared to cells treated with IFN as monotherapy by a CellTiter-Glo® luminescence assay. Blotted values and calculated standard deviations are based on six replicates. **b)** Binding of Interferon alpha 2a to its masking antibody 9F3. **c)** Masking and temporary inactivation of the cytokine by the antibody. **d)** Release and reactivation of interferon due to reduced antibody affinity. 3 D modelling of biomolecules was conducted by Dr. Guy Georges, Roche Diagnostics GmbH.

In the performed assay, treatment of U266B1 with wild type interferon alpha 2a without any masking antibody has led to a reduction of cell viability of 71.37 % compared to untreated cells. Addition of anti-IFN alpha 2a antibody 9F3 in a half molar ratio decreased the anti-proliferative effect of IFN alpha 2a down to a level of 16.18 % due to a strong masking (neutralizing) of the cytokine by the antibody. An exchange of isoleucine to asparagine at position 52 in the heavy chain of the 9F3 antibody triggered a reduced masking capability of the antibody, leading to a 48.17 % interferon mediated induction of cell death. Similar effects were seen by point mutating isoleucine to glycine at position 76 of the heavy chain with a cell killing response of 43.53 %. Mutations in the complementarity determining region of the light chain showed unwanted effects on the masking capabilities of the antibody compared to point mutations in the heavy chain of the 9F3 antibody. The exchanges LC_Pos55_Tyr/Ala (63.53 %), LC_Pos73_Tyr/Asp (65.49 %) and

LC_Pos119_Met/Arg (63.39 %) in the light chain of the 9F3 antibody triggered a significant loss of affinity of the antibody against interferon alpha 2a which resulted in poor or only partial inhibition of the cytokine. Due to the insufficient masking capability of antibodies with point mutations in the variable domain of the light chain, modifications in the heavy chain of 9F3 were preferred to achieve efficient masking of interferon and a possible release of the cytokine at the tumor site at the same time.

Previously described point mutations in the binding region of the anti IFN alpha 2a antibody 9F3 were additionally evaluated via surface plasmon resonance spectroscopy to determine the affinity modulating effect of each attempt (figure 33).



Antibody	Ligand	K_D / M
unmodified mAb 9F3	human IFNα2a	2.5E-11
HC mutation P52 (a)	human IFNα2a	4.9E-11
HC mutation P76 (b)	human IFNα2a	3.5E-11
LC mutation P119 (c)	human IFNα2a	4.1E-11
LC mutation P55 (d)	human IFNα2a	1.4E-09
LC mutation P73 (e)	human IFNα2a	1.5E-09

Figure 33: Adaptation of antibody masking intensity

Binding affinities of modified anti-IFN alpha 2a antibodies were determined by using a Biacore T200 device. Anti-human IgG1 catching antibodies were immobilized on a CM5 gold coated sensor chip by amine coupling. Modified anti-IFN alpha 2a antibodies were loaded on three of the four chambers of the sensor chip. Human interferon alpha 2a was applied to the system in PBS running buffer containing 0.05% Tween, at a flow rate of 50 µL / min and a contact time of 180 seconds. After each washing step, the system was regenerated with 30 mM MgCl. Gained data were recorded and visualized by using the Biacore T200 evaluation software. Presented data were generated under the guidance of Ute Jucknischke, Roche Diagnostics GmbH.

The unmodified anti IFN alpha 2a antibody 9F3, developed by Genentech Inc., exhibits an affinity to human wild type interferon alpha 2a in a range of 25 pM (data were provided by Genentech Inc.). Point mutations at position 52 (a) and position 76 (b) in the heavy chain of the antibody have triggered a moderate decrease of the affinity to a level of 49 pM (a) and 35 pM (b). Although an exchange of methionine to arginine at position 119 in the light chain of the antibody has just slightly decreased its affinity down to 41 pM, masking capability of the antibody was completely lost (figure 32). An exchange of tyrosine at position 55 to alanine in the variable domain of the light chain has led to a reduction of the antibody affinity down to 1.4 nM. Similar effects were seen for another amino acid exchange at position 73 of the light chain. The replacement of tyrosine by asparagine reduced the affinity of the 9F3 antibody to human interferon alpha 2a to 1.5 nM and triggered a significant loss of masking capability.

The intended use of the antibody 9F3 was to prevent increased levels of interferon alpha 2a in the body of patients caused by inflammatory diseases. Due to this planned clinical indication, the affinity of the antibody was adjusted specifically to interferon alpha 2a. To determine the binding capability of the antibody to other natural occurring interferon alpha subtypes and to evaluate its masking effect on several members of this family, a screening assay was performed on the multiple myeloma cell line U266B1 with interferon alpha subtypes as monotherapy or in combination with the anti IFN alpha 2a antibody 9F3 (figure 34).

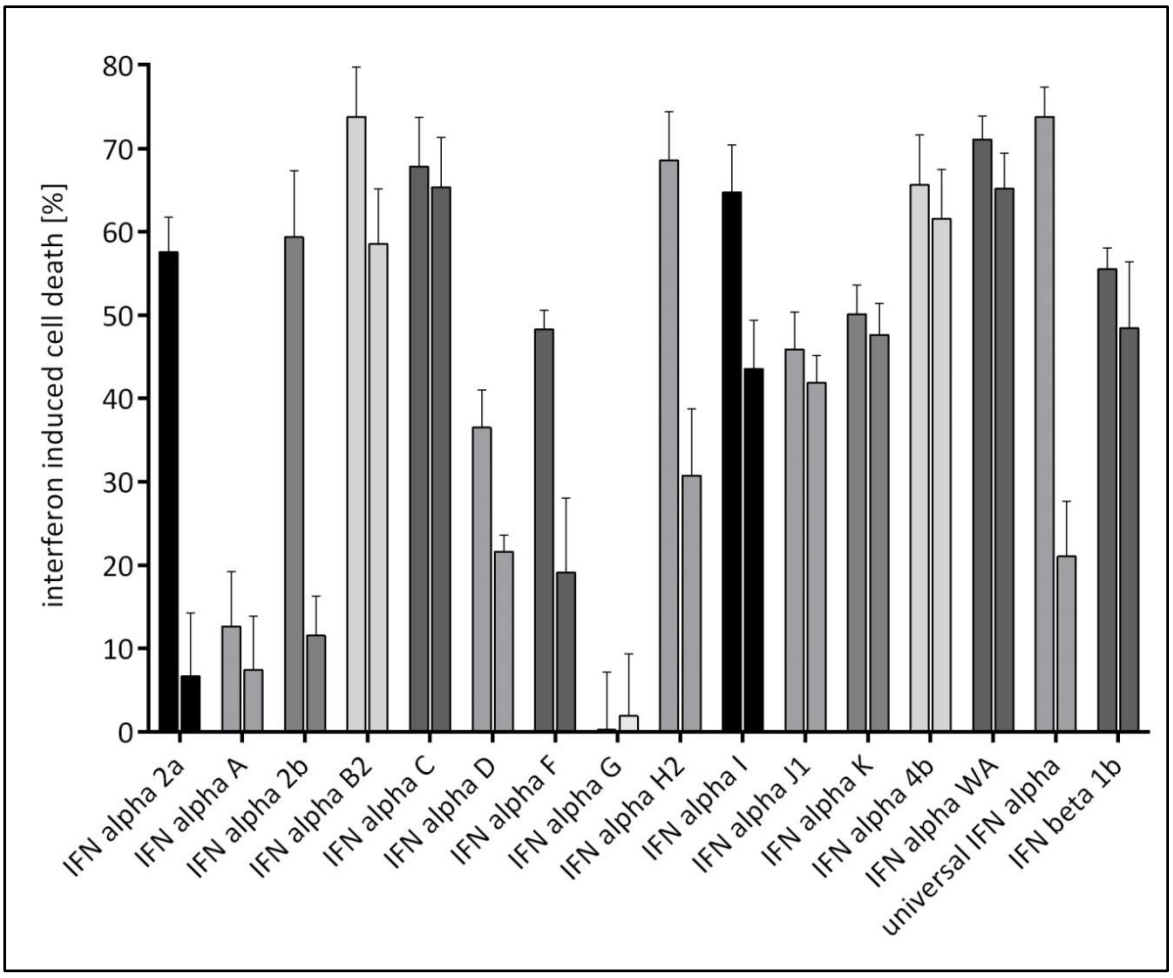


Figure 34: Antibody-induced inactivation of IFN alpha subtypes

The multiple myeloma cell line U266B1 was treated with thirteen natural occurring IFN alpha subtypes at 10.000 IU / mL in combination with the anti-IFN alpha 2a antibody 9F3 in a ration of IFN : antibody of 2:1. Masking efficiency was determined by a CellTiter-Glo® luminescence assay. Masking effects were evaluated by the detection of interferon activity in combination with the 9F3 antibody compared to cells treated with IFN as monotherapy. Blotted values and calculated standard deviations are based on three replicates. Except treatment of cells with IFN alpha 2a, IFN alpha H2 and universal IFN alpha, screenings were performed in collaboration with Guido Werner, Roche Diagnostics GmbH.

Treatment of multiple myeloma cells with different interferon alpha subtypes resulted in various levels of anti-proliferative responses. Interferon alpha 2a as monotherapy has led to an inhibition of cell growth by 57 %. After addition of the antibody 9F3, the major part of the cytokine was bound and pharmacologically inactivated which reduced the inhibitory effect of interferon down to 6.7 %. A similar strong inactivation of the cytokine after addition of the 9F3 antibody was seen for IFN alpha 2b. In contrast to the strong binding capability of the antibody 9F3 to IFN alpha 2a and IFN alpha 2b, no significant inhibitory effects were determined for IFN alpha J1, IFN alpha K, IFN alpha 4b, IFN alpha C and IFN alpha WA. Moderate cytokine binding and inhibition of the anti-proliferative effect by the antibody 9F3 was evaluated for IFN alpha D, IFN alpha F, IFN alpha I and universal IFN alpha. The stimulation of U266B1 cells with interferon alpha H2 showed a reduction

in cell growth of 68.6 % compared to untreated cells. After addition of the antibody 9F3, the anti-proliferative potency of IFN alpha H2 was reduced down to 30.7 %. It was seen the anti IFN alpha 2a antibody 9F3 exhibits a moderate binding affinity to interferon alpha H2 and no further point mutations were necessary to adjust the antibody affinity down to a level for efficient masking and at the same time maintain a possible release of the cytokine.

Structure analysis and 3D modelling approaches revealed the three-dimensional structure and antigen binding interface of the 9F3 antibody. To evaluate the intensity and the biological effect of the masking capability of the 9F3 antibody, the binding ability of the antibody was abolished by removing any amino acids with an antigen binding mediating side chain. The generated antibody, incapable to bind any antigen, was called a “Nada”- variant (figure 35).

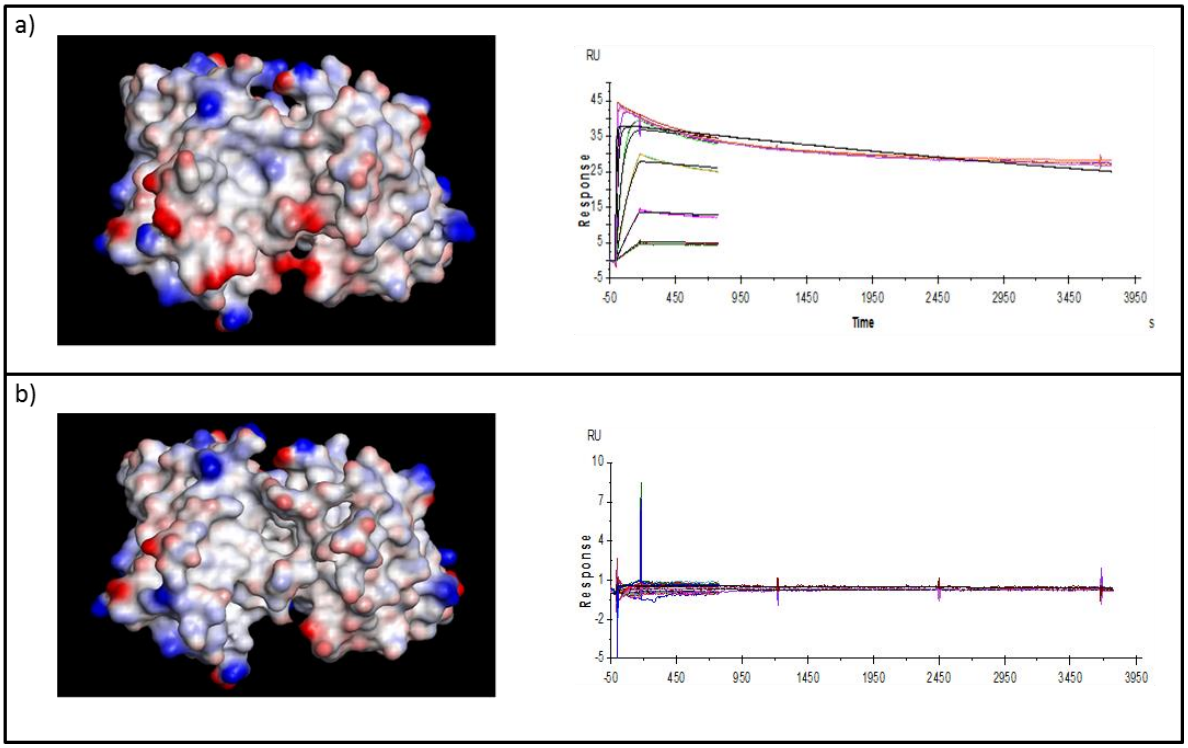


Figure 35: CDR burnishing of the anti IFN alpha 2a antibody 9F3

Three dimensional modelling of the CDR regions of the anti-IFN alpha 2a antibody 9F3 **(a)** and corresponding binding kinetics to interferon alpha 2a. “Nadazeptized” antibody containing multiple modifications of amino acids in the CDR region of the 9F3 antibody to prevent recognition and binding of an antigen **(b)**. Binding kinetics were evaluated via a Biacore T200 device as previously described. 3 D modelling of biomolecules was conducted by Dr. Guy Georges, Roche Diagnostics GmbH. Results from SPR measurements were generated under the guidance of Ute Jucknischke, Roche Diagnostics GmbH.

The three dimensional structure of the human anti-interferon alpha 2a antibody 9F3 revealed essential amino acids with side chains responsible for interacting with interferon alpha 2a. The evaluation of the antibody affinity to its antigen by surface plasmon resonance spectroscopy displayed binding events at a concentration of 25 pM. The exchange of amino acids responsible for antigen binding to amino acids with non-reactive side chains in the CDR domains of the VH and VL domains of the 9F3 antibody triggered a complete loss of the antigen binding capability of the newly generated “Nada-antibody”. Surface plasmon resonance spectroscopy showed no detectable binding of interferon alpha 2a by the immobilized Nada-antibody. Due to this loss of affinity, the Nada-antibody was used in *in vitro* assays as a negative control with no masking capabilities.

5.5 Effects of a bivalent non-masking anti-interferon antibody

Interferon fusion proteins containing the non-masking Nada antibody and two molecules of interferon alpha H2 were manufactured and evaluated on the multiple myeloma cell line U266B1. The experiment showed that interferon fused to a non-masking antibody exhibits a more potent anti-proliferative effect on U266B1 cells than unfused interferon alpha H2 at an equimolar ratio. This effect was additionally confirmed in other multiple myeloma cell lines like RPMI 8226 and NCI-H929. To investigate whether the improvement of the anti-proliferative efficacy of the unmasked fused interferon was derived from a clustering of IFN receptors, interferon fusion proteins based on the non-masking Nada-antibody were pre-incubated with anti-human Fc secondary antibodies to crosslink the interferon fusion proteins. After this pre-incubation, the multiple myeloma cell line U266B1 was treated with the crosslinked antibodies in serial dilutions for 72 hours (figure 36).

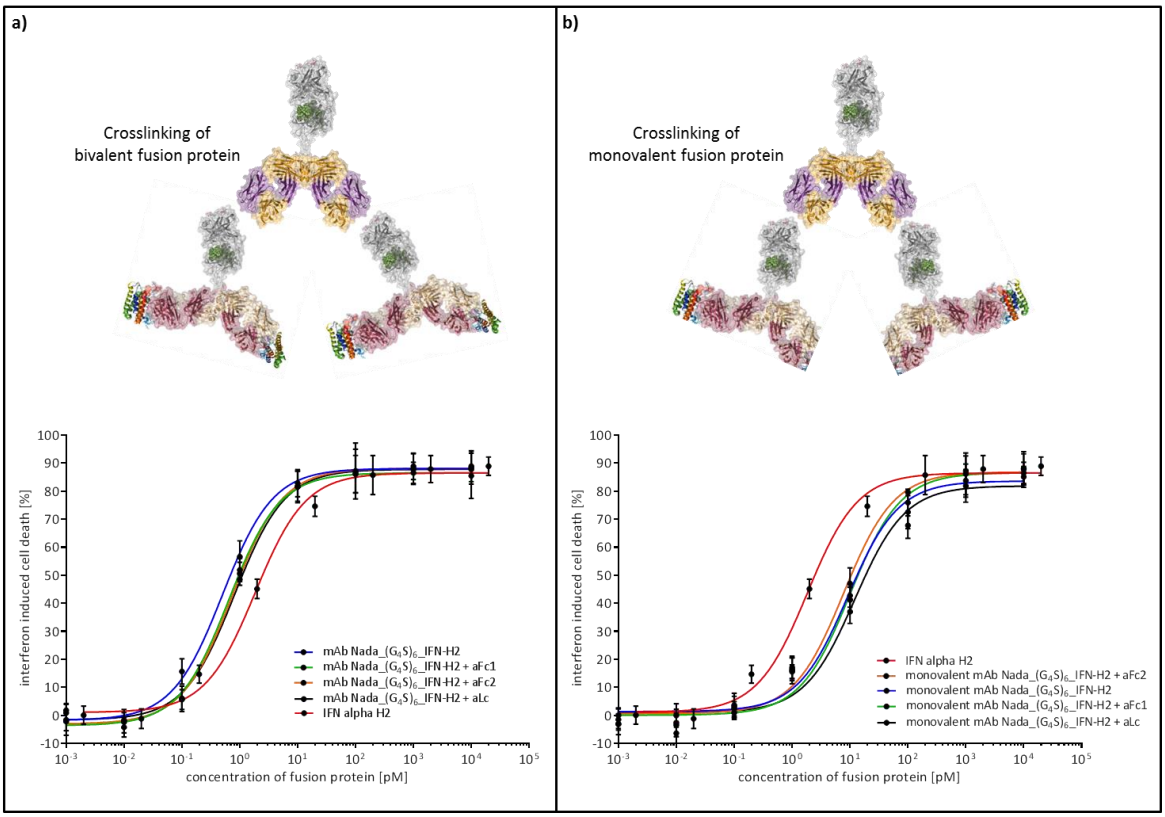


Figure 36: IFN receptor clustering to explain improved anti-proliferative efficacy

Cell viability assay of the multiple myeloma cell line U266B1. Bivalent IFN fusion proteins **(a)** and monovalent IFN fusion proteins **(b)** were applied in serial dilutions to 5000 cells / well in combination with crosslinking secondary antibodies, binding to human IgG1 Fc part **(green, orange line)** or to human IgG1 kappa domain **(black line)**. Crosslinking antibodies were pre-incubated with IFN fusion proteins in a ratio of 1:2 for one hour before treatment of the cells. Cell viability was evaluated after 72 hours by using a CellTiter-Glo® luminescence assay. Blotted values and calculated standard deviations are based on six replicates. 3 D modelling of biomolecules was conducted by Dr. Guy Georges, Roche Diagnostics GmbH.

Treatment of U266B1 cells with non-masking bivalent interferon fusion proteins showed an enhanced anti-proliferative potency (IC50 = 0.51 pM) compared to unfused interferon alpha H2 (IC50 = 1.85 pM). However pre-incubation of interferon fusion proteins with two different anti-human Fc antibodies or an anti-human LC antibody slightly reduced the anti-proliferative action of interferon fusion proteins but still demonstrated an increased inhibitory effect on all growth compared to free interferon alpha H2. In contrast to the synergistic effect of bivalent interferon fusion proteins, no beneficial effects were seen in case of monovalent fusion proteins. Equimolar ratios of interferon alpha H2 (IC50 = 1.85 pM) and a monovalent interferon fusion protein (IC50 = 9.19 pM) displayed significant differences in the anti-proliferative activity of both stimulants. The application of secondary antibodies to crosslink the monovalent interferon fusion proteins did not show any improvement in the potency of the monovalent antibody. Due to these results a potential explanation for the synergistic effect of bivalent non-masking interferon fusion proteins by the crosslinking of IFN receptors on the cell surface was not able to be confirmed. Improved

pharmacologic activities of Nada-IFN fusion proteins were not seen in tumor cell lines originating from solid tumors (data not shown).

5.6 Screening for tumor specific proteases

A key aspect of the proposed interferon fusion protein approach is the selective release of the cytokine at the tumor site. Due to the masking effect of the used antibody, interferon stays inactivated as long as it is stably fused to the antibody by a peptide linker. After cleavage of the linker, interferon is able to diffuse away from the antibody and becomes reactivated at the tumor site. To ensure a specific release of the cytokine only in the tumor microenvironment, a peptide linker is required which contains a selective cleavage motif for tumor associated proteases. During circulation of the fusion protein in the periphery, the peptide linker has to be stable to ensure masking and inactivation of interferon for the prevention of unwanted interaction of the cytokine in off-target tissue.

The following section of the thesis summarizes the approach to identify suitable cleavage motifs inside the peptide linker which show a high grade of stability in plasma and suitable turn-over rates by tumor associated proteases.

5.6.1 Peptide linkers specific for matrix metalloproteinases

Matrix metalloporteinases (MMPs) play an import role for the degradation of the extracellular matrix of tumors which facilitates the spread of tumors to other tissues and enforces metastasization. Due to an overexpression of several subtypes of matrix metalloproteinases in cancerous tissue, different substrates and cleavage motifs for MMPs were investigated to be incorporated in the peptide linker, connecting interferon to its masking antibody. Results from literature search and patent applications have led to a selection of different amino acid sequences which are preferentially cleaved by MMP-9 and MMP-2. These two prominent representatives of the matrix metalloproteinase family show high similarities for their substrates and are expressed at high levels in many tumor types.

The amount of enzymatically active MMP-9 and MMP-2 in several tumor lysates was determined by zymograms (figure 37, a)) in order to identify suitable *in vitro* models. Sequences for peptide linkers specifically cleaved by MMP-9 and MMP-2 were selected by a literature search (figure 37, c)) and incorporated in interferon fusion proteins containing a masking anti-IFN antibody. Manufactured interferon fusion proteins were tested on the multiple myeloma cell line U266B1 in comparison with a non-cleavable $(G_4S)_6$ peptide linker (figure 37, b)).

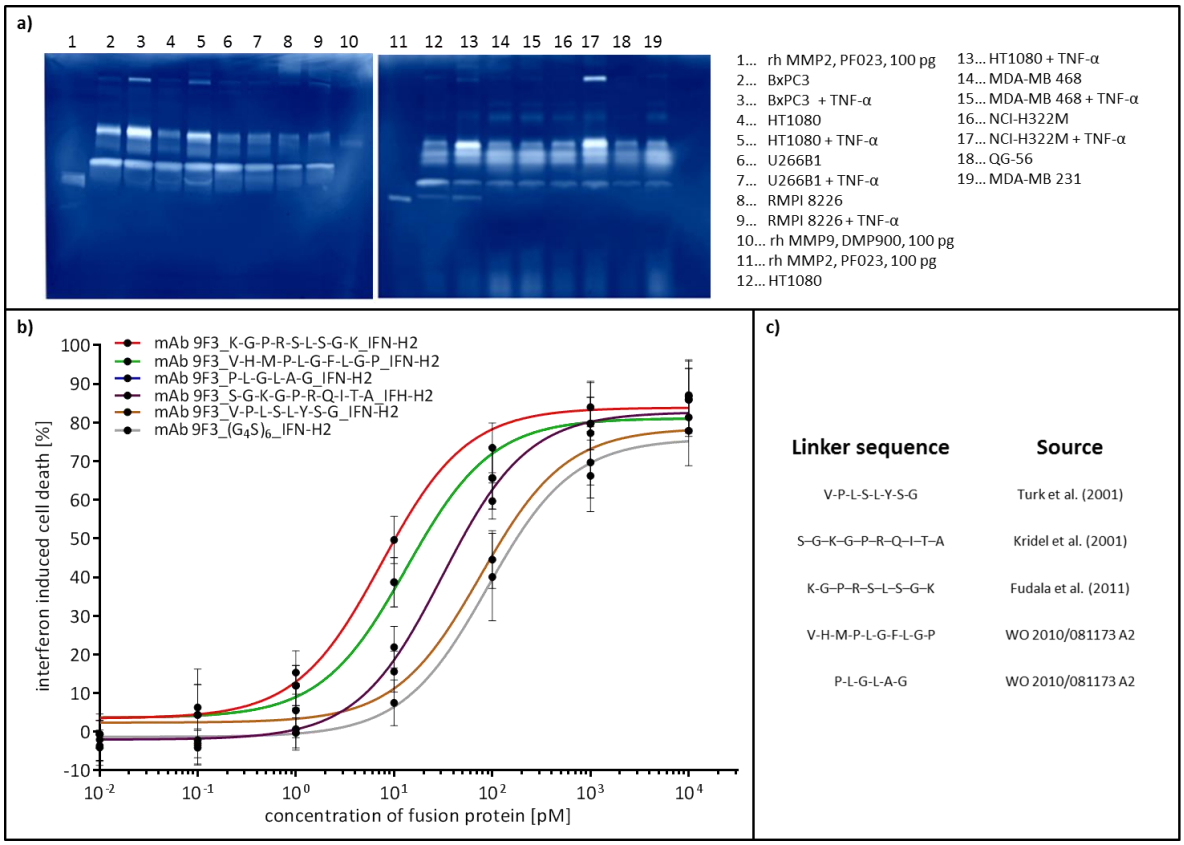


Figure 37: Matrix metalloproteinase cleavable peptide linker

Enzymatic activities of MMP- 9 and MMP-2 were determined for several IFN alpha treated and untreated tumor cell line lysates by using zymography technology **(a)**. Peptide linker sequences found in literature **(c)** were incorporated in IFN fusion proteins and evaluated on the multiple myeloma cell line U266B1 **(b)**. Cells were seeded at a density of 5000 cells / well and treated with serial dilutions of interferon fusion proteins [10nM] for 72 hours. Final readout was performed by a CellTiter-Glo® luminescence assay. Blotted values and calculated standard deviations are based on six replicates. Screening of protease activity by zymograms was conducted by Ingrid Munk, Roche Diagnostics GmbH.

The evaluation of tumor lysates originating from different tissues by zymograms indicated high enzymatic activities of MMP-9 and MMP-2 in the pancreatic cancer cell line BxPC-3 and the hepatocellular carcinoma HT1080. Moderate levels of active enzymes were determined in the multiple myeloma cell lines U266B1 and RPMI 8226 with a slight majority of MMP-2 activity. Additionally active proteases were detected in the estrogen receptor, progesterone receptor and Her2 receptor triple negative breast cancer cell lines MDA-MB-468.

Treatment of the multiple myeloma cell line U266B1 with serial dilutions of interferon fusion proteins containing MMP-specific peptide linkers from literature resulted in diverse turn-over rates of the linkers by matrix metalloproteinases depending on the applied sequence. As a negative control a fusion protein with a non-cleavable $(G_4S)_6$ peptide linker was used with an IC50 value of 89.04 pM. The application of a peptide linker derived from Turk *et al.* only slightly improved the IC50 value of the fusion protein to 76.2 pM. Higher linker turn-over rates (31.1 pM) were determined for a fusion protein containing a S-G-K-G-P-R-Q-I-T-A cleavage motif from Kridel *et al.*. A significant shift in the potency of the masking interferon fusion proteins was achieved by the introduction of a MMP-9 specific peptide linker derived from the patent WO 2010/081173A2 with an IC50 value of 13.37 pM. The highest turn-over rate of a peptide linker was determined for the K-G-P-R-S-L-G-K (Fudala *et al.*) amino acid sequence leading to an IC50 value of the interferon fusion protein in the range of 7.46 pM (Fudala et al., 2011; Kridel et al., 2001; Turk, Huang, Piro, & Cantley, 2001).

5.6.2 Peptide linkers selectively cleaved by matriptase

In addition to the previously described screening for peptide linkers cleaved by matrix metalloproteinases another attempt for a tumor specific release of interferon was investigated. The group of LeBeau et al. published data for a tumor specific activity of a membrane bound serine protease called matriptase. Matriptase is distributed all over the body and occurs also in healthy tissues but always in combination with its inhibitor HAI-1. A beneficial characteristic of cancerous tissue is the down-regulation of the enzyme inhibitor HAI-1 in contrast to health tissues (figure 38, b)). Due to this effect, the protease matriptase occurs only in tumors in its enzymatically active form. To utilize this biological effect of tumors, peptide linkers from literature (figure 38, a)) were incorporated in interferon fusion proteins which are specifically cleaved by active matriptase. Generated fusion proteins were tested on the ovarian cancer cell line OVCAR-3 to determine turn-over rates of the applied matriptase specific linker sequences and to select a potential candidate for the bispecific masking interferon fusion protein (figure 38, c)).

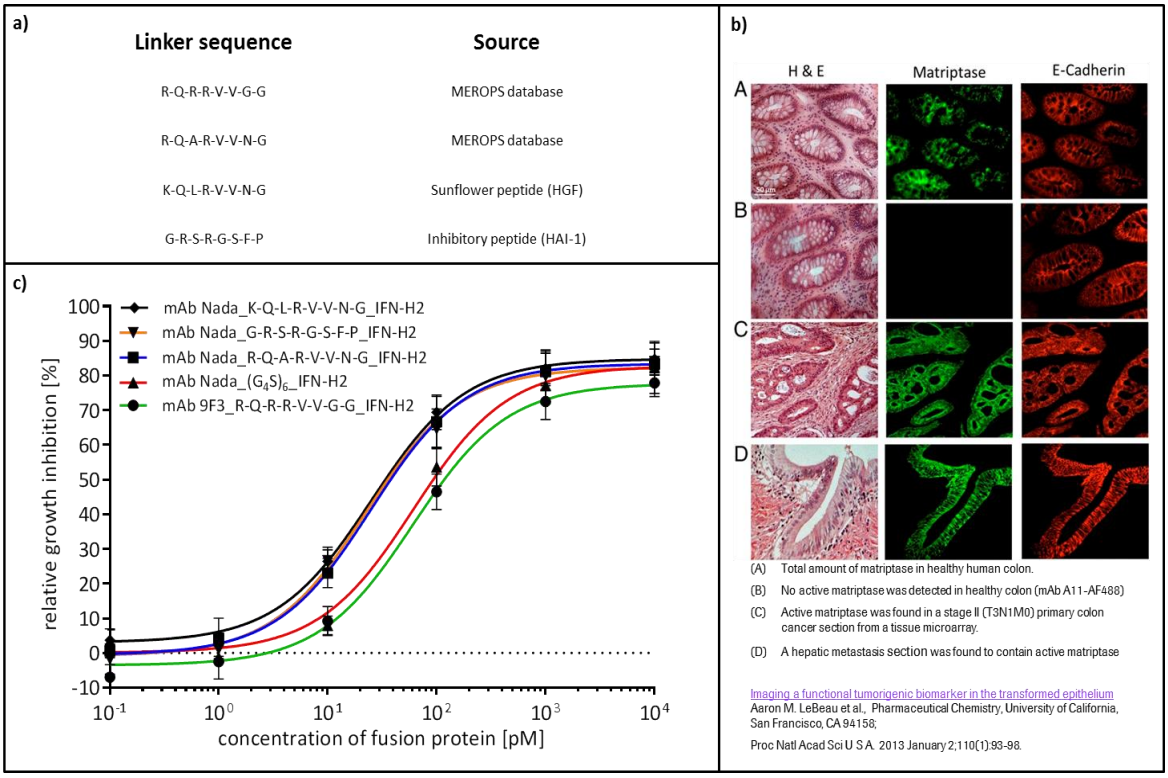


Figure 38: Membrane bound serine-protease cleavable peptide linker

Peptide sequences which contain cleavage motifs for the serine-protease matriptase were gained by literature search and in-house modifications of known matriptase substrates **(a)**. Tumor selective occurrence of enzymatically active matriptase was shown by LeBeau *et al.* **(b)**. Interferon fusion proteins containing matriptase cleavable peptide linkers were tested in serial dilutions [10nM] on the ovarian cancer cell line OVCAR-3 **(c)**. Cell viability and indirect measurement of linker turnover rates was determined via a CellTiter-Glo® luminescence assay. Detected deviations in cell viability compared to untreated cells were blotted and standard deviations calculated on the basis of six replicates.

The matriptase specific peptide linker R-Q-R-R-V-V-G-G originating from the MEROPS data base showed no significant difference compared to a stable $(G_4S)_6$ peptide linker because auf the application of a masking interferon fusion protein (9F3) for the R-Q-R-R-V-V-G-G linker in contrast to non-masking Nada-variants for other matripase linkers. Additional experiments showed that the MEROPS linker was efficiently been cleaved by matriptase (data not shown). An in-house developed point mutation of the MEROPS linker to R-Q-R-R-V-V-N-G significantly improved the turn-over rate of the linker on the ovarian cancer cell line OVCAR-3 to an IC50 value of 25.1 pM. Similar cleavage rates were achieved for fusion proteins containing peptide linkers with sequence similarities to the matriptase inhibitor HAI-1 (22.7 pM) or to the matriptase substrate HGF (25.1 pM).

5.6.3 Dual specific tandem linker

From previously performed screening assays, several suitable peptide linker for a specific cleavage by the tumor associated proteases MMP-9/2 and matriptase were identified. To increase the likelihood of linker cleavages at the tumor site another approach was conducted to combine a matriptase specific sequence with a MMP-9 specific sequence in one peptide linker. The generated "Tandem Linker" combines the amino acid sequences of the matriptase linker derived from the MEROPS database and the sequence for MMP-9 mediated cleavage originating from the patent WO 2010/081173A2. On the N- and C-terminus the tandem linker is flanked by four glycines and one serine as spacers and located between interferon and the CH1 domain of the anti IFN antibody. To determine the cleavage rate of the developed tandem linker, fusion proteins were generated containing a matriptase linker, a MMP-9 linker or the tandem linker with both sequences and tested on the ovarian cancer cell line OVCAR-3 (figure 39).

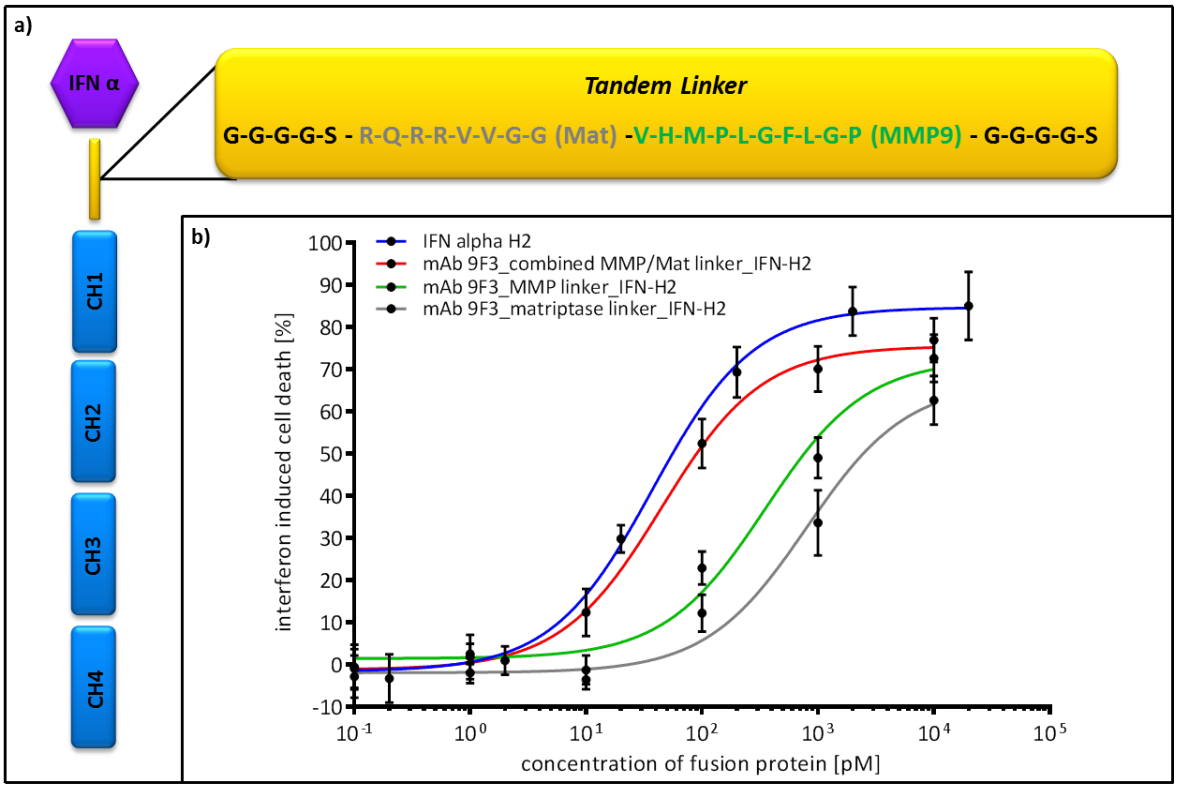


Figure 39: Tandem linker with synergistic turnover rate

The schematic diagram of the antibody heavy chain of the interferon fusion protein visualizes the incorporation site and composition of the used peptide tandem linker **(a)**. Enzymatic turnover of the tandem linker in contrast to monospecific MMP linker and matriptase linker was evaluated on the ovarian cancer cell line OVCAR-3 **(b)**. Cells were treated with serial dilutions of interferon alpha H2 [20nM] or bivalent masking interferon fusion proteins [10nM] which contain previously mentioned linker sequences. Cell viability was determined by using a CellTiter-Glo® luminescence assay after treatment duration of 72 hours. Displayed inhibition curves and standard deviations were calculated on the basis six replicates.

Table 6: IC50 values of fusion proteins containing different peptide linkers

Peptide linker	IC50 [pM]
Interferon alpha H2 (reference)	37.41
Tandem linker	44.71
MMP9/2 linker	350.9
Matriptase linker	797.9

Treatment of OVCAR-3 cells with human interferon alpha H2 resulted in a maximum induction of cell death of 85% with an IC50 value of 37.41 pM. In the presented experiment, unfused interferon alpha H2 was used as reference for 100 % turnover rate of the peptide linker. A masking interferon fusion protein containing a monospecific matriptase cleavable linker achieved a maximum anti-proliferative effect of 62.6 % and an IC50 value of 797.9 pM. A significant improvement of the linker turnover rate was seen by the application of a MMP-9 selective peptide linker in the masking interferon fusion protein with an increased maximum effect of 72.6 % and an IC50 value of 350.9 pM. The combination of both sequences of the MMP-9 linker and the matriptase linker in one tandem linker resulted in a reduction of the IC50 value down to 44.71 pM and a maximum induction of cell death of 76.9 % which indicates a >90 % turnover rate of the tandem linker compared to monospecific peptide linkers.

Used peptide linkers for the interferon fusion protein have to be not only cleaved efficiently by tumor associated proteases, but also show a high grade of stability during circulation in the periphery. To determine the plasma stability of the previously described peptide linkers, fusion proteins with each of the protease specific linker were incubated in murine plasma for 24 hours at 37 °C (figure 40).

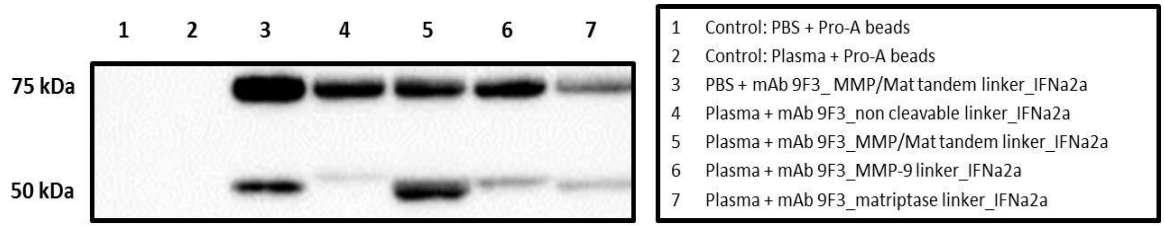


Figure 40: Plasma stability of protease cleavable linkers

Stability of MMP-, matriptase- and tandem linkers in murine plasma was evaluated by Western Blot. Corresponding interferon fusion proteins were incubated for 24 hours in murine plasma at 37 °C and 300 rpm shaking mode. After a recovery step by magnetic protein-A beads, SDS gel electrophoresis was performed and gained protein bands blotted on a PVDF membrane. Antibody heavy chains containing a non-cleaved linker **(75 kDa)** or with a cleaved linker **(50 kDa)** were marked by an overnight incubation with an anti-human IgG1 Fc specific (Fab)2 fragment fused to horse radish peroxidase (HRP). Luminescence signal was gained by the addition of LumiLight™ Western Blot substrate and detected by using a Lumi-Imager™ device.

Control groups of protein-A beads incubated in PBS or mouse plasma did not show any detectable residues from the bead surface on the blot. For the interferon fusion protein containing a tandem protease linker, a small amount of cleaved linkers (~20 %) and a majority of non-cleaved peptide linker (~ 80 %) was detected in samples incubated in PBS. Fusion proteins containing a stable $(G_4S)_6$ peptide linker showed only traces of cleaved constructs and more than 99 % of non-cleaved linkers (75 kDa) after incubation in mouse plasma. Used MMP-9 and matriptase specific linker peptides showed a high degree of plasma stability with similar amounts of cleaved products (lane 6 and lane 7). The comparison of detected antibody heavy chains of lane 3 and lane 5 revealed a moderate plasma stability of the tandem linker. After 24 hours incubation in mouse plasma, half of the tested fusion proteins were unspecifically cleaved by murine proteases in the plasma. Although the presented tandem linker showed significantly improved turnover rates on several cancer cell lines, plasma stability was reduced compared to monospecific peptide linkers.

5.7 Manufacturing and evaluation of the interferon fusion protein lead molecule

Previously described optimizations of each component of the proposed interferon fusion protein were combined in one lead molecule. The interferon fusion protein is composed out of a point mutated interferon alpha H2 which is fused to a heavy chain of the masking anti-IFN antibody by a tandem protease linker. Second half of the bispecific fusion protein is made out of an anti-CD138 targeting moiety which ensures the enrichment of the interferon fusion protein at the target site.

The transmembrane heparin sulfate proteoglycan CD138 (Syndecan-1) is a member of the syndecan protein family which is up-regulated in many multiple myeloma cancer types (figure 19) (O'Connell, Pinkus, & Pinkus, 2004). Due to the tumor selective overexpression of the transmembrane protein, an anti-CD138 targeting moiety was chosen as targeting component of the bispecific interferon fusion protein for clinical indications in multiple myeloma treatment.

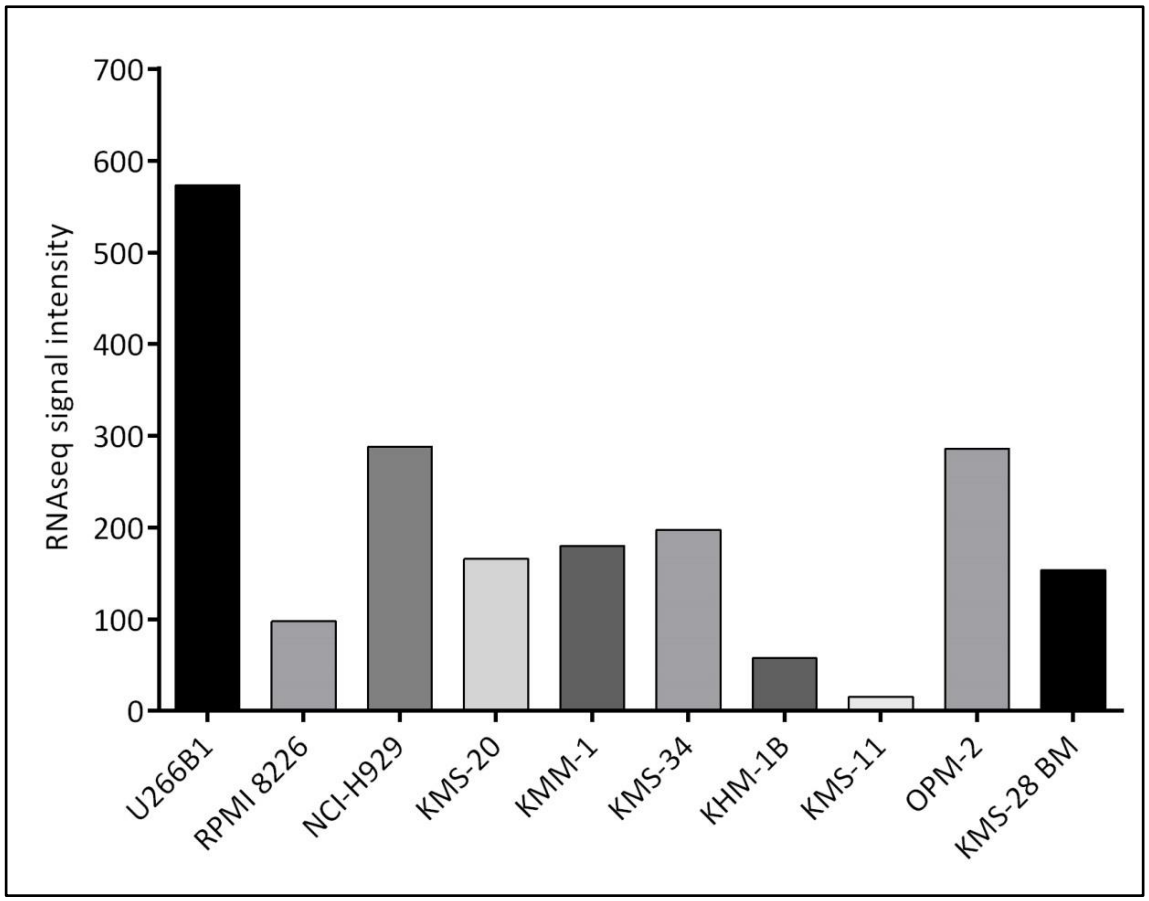


Figure 41: CD138 (Syndecan-1) expression in multiple myeloma cell lines

CD138 expression data were evaluated by RNA sequencing technology originating from Chugai Pharmaceutical Co., Ltd. a member of the Roche Group.

5.7.1 Purification of the bispecific interferon fusion protein

Seven days post transfection, fusion proteins were harvest from the HEK293F culture by centrifugation. Protein A affinity and size exclusion chromatography was used to purify the supernatants and separate aggregates from the monomeric fraction (figure 42).

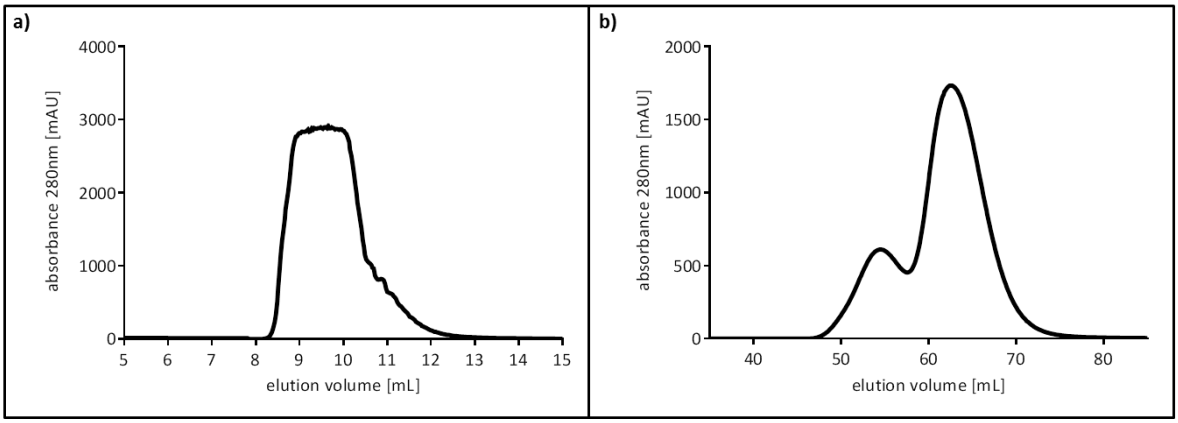


Figure 42: Manufacturing and purification of the bispecific interferon fusion protein

Chromatogram of a protein A affinity chromatography of the bispecific CD138 targeted interferon fusion protein (a). Fusion proteins were trapped by using a HiTrap™ MabSelect Xtra™ (flow rate = 1mL/min) and eluted by a citrate buffer (pH3) induced pH shift. For size exclusion chromatography (b) of the fusion protein, a HiLoad™ 16/600 Superdex™ 200pg column was applied. Gained protein pool from affinity chromatography step was separated by using His-NaCl running buffer (pH6) at a flow rate of 1 mL/min. Protein fractions containing monomeric fusion proteins were collected manually.

The protein A chromatogram from the detected UV absorbance at 280nm displayed a rising peak after eight milliliter elution volume with a maximum height of 3000 mAU. After 10.5 mL elution volume the peak declined and reached the base level after 12.5 mL eluent. The chromatogram from size exclusion chromatography showed a peak from 48 to 58 mL with a height of 611.2 mAU indicating aggregates of the interferon fusion protein. The second peak from 58 to 75 mL elution volume displays the monomeric fraction of the bispecific interferon fusion protein with a maximum height of 1733.1 mAU. Determination of the protein concentration of the collected monomeric fraction revealed a total amount of interferon fusion protein of 12.3 mg of the non-cleavable fusion protein and 16.7 mg of the cleavable fusion protein out of one liter of HEK293F expression culture.

To evaluate the correct composition of the purified bispecific interferon fusion protein out four different antibody chains, a SDS gel electrophoresis was performed and proteins bands visualized by simply blue staining (figure43).

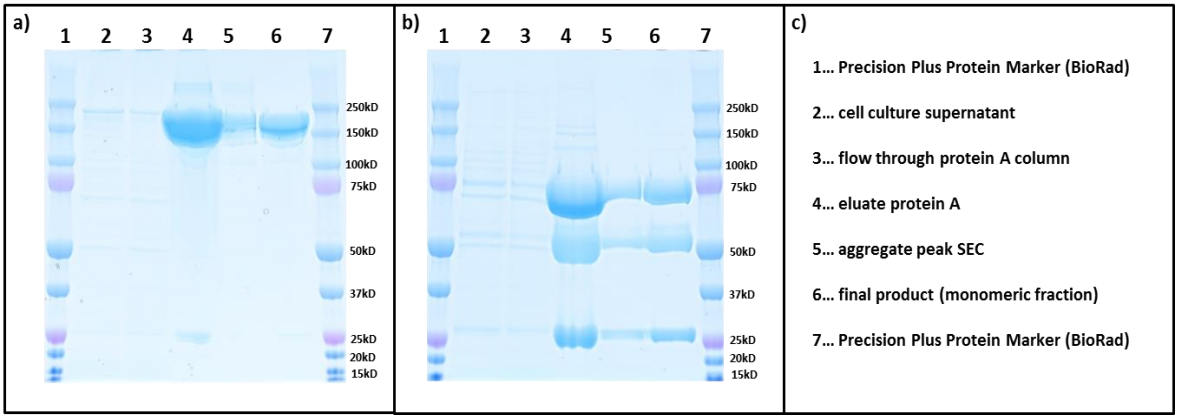


Figure 43: SDS gel electrophoresis of the IFN antibody conjugate

SDS gel electrophoresis to control correct assembly of the bispecific interferon fusion protein was conducted by applying 20 µL of **a)** non-reduced protein pools and **b)** reduced protein pools, on to each lane of NuPage® 4-12% Bis-Tris Gel. **c)** Sample loading scheme for each gel. Electrophoresis was performed at 230 V for 35 minutes. Separated protein bands were visualized by SimpleBlue staining solution. Determination of protein fraction size was determined by comparison with the defined Precision Plus Protein Marker.

On the gel with non-reduced samples from protein purification steps, protein bands could be observed in the eluate from protein A chromatography (lane 4), the aggregate fraction from size exclusion chromatography (lane 5) and the monomeric fraction of the bispecific interferon fusion protein (lane 6) with a molecular weight of 190 kDa which correlates with the calculated molecular weight of the fusion protein. Under reduced conditions (gel b)) protein bands were stained at 75 kDa which indicates interferon fused to one heavy chain of the fusion protein. Additional bands were found at 50 kDa which represent the unfused CD138 targeting arm of the fusion protein. Light chains corresponding to both arms of the interferon fusion proteins were detected at 25 kDa without any separation of both chains due to the identical molecular weight.

5.7.2 Functionality of the CD138 targeting moiety

The transmembrane proteoglycan CD138 was selected as target for the interferon fusion protein due to its overexpression in several multiple myeloma cell lines. To evaluate the functionality of the targeting moiety of the generated bispecific interferon fusion protein, different cell lines were incubated with fluorescence labeled fusion proteins. Live cell imaging of the cultures was performed by confocal microscopy to determine interactions between the interferon fusion protein and tumor cells (figure 44).

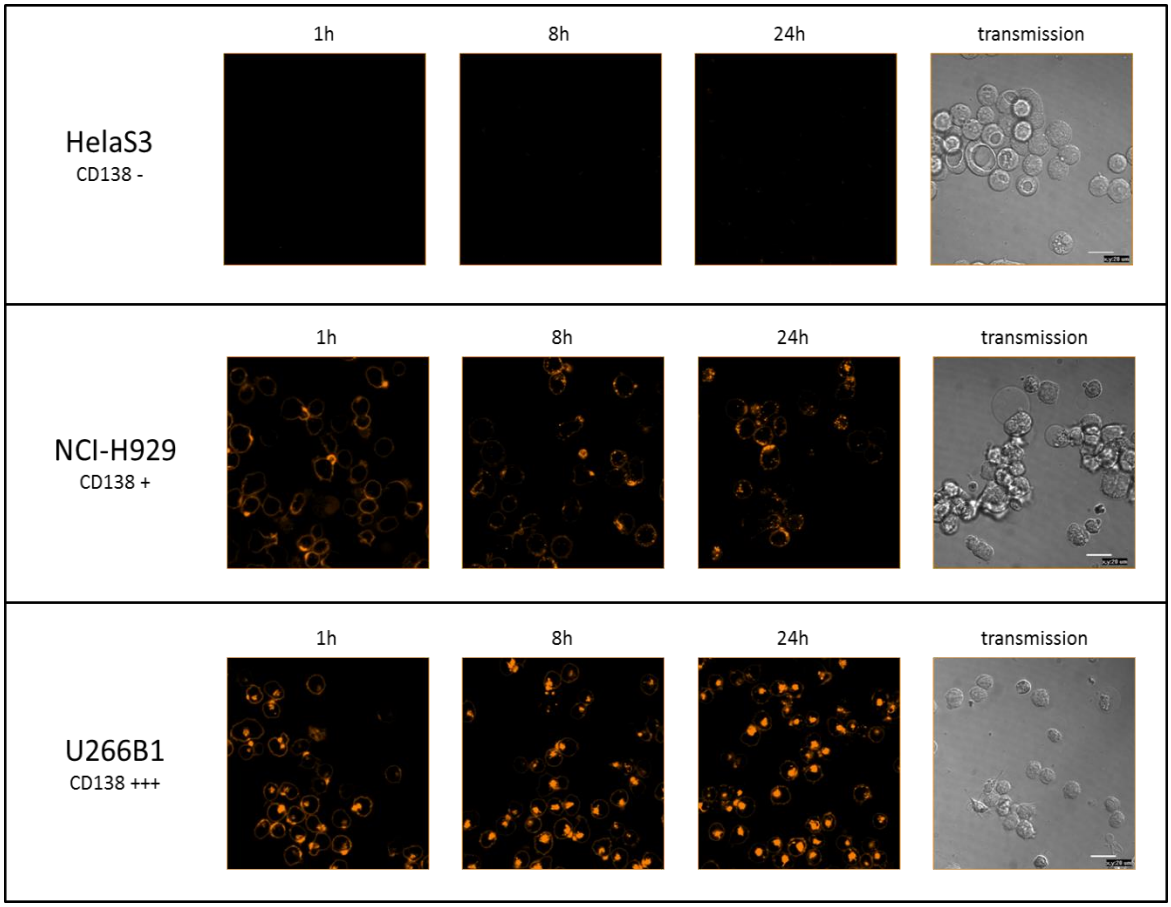


Figure 44: Confocal microscopy of CD138 targeted IFN fusion proteins

Anti CD138 targeted bispecific interferon fusion proteins were labelled by using the Mix-n-Stain™ antibody labeling kit (Biotium). CD138 positive multiple myeloma cell lines U266B1 and NCI-H929 and the CD138 negative cell line Hela-S3 were incubated with labelled fusion protein in the corresponding growth medium. Confocal microscopy was performed by using an Eclipse TE2000-E, D-Eclipse C1si LSM microscope (Nikon) after 1h, 8h and 24 hours. Membrane staining and internalization of the fusion protein was visualized by Nikon EZ-C1 V.3.80 software. Microscopic analysis of prepared samples was performed by Oliver Tonn, Roche Diagnostics GmbH.

The CD138 negative cancer cell line HelaS3 did not show an enrichment of the fluorescence labelled interferon fusion protein on the surface of the cell nor aggregations of internalized fusion proteins in any compartment of the cells. Co-cultivation of CD138 positive NCI-H929 multiple myeloma cells with labelled interferon fusion proteins resulted in strong immobilization of the fusion protein on the surface of the cells already after one hour. Intensity of membrane staining of the NCI-H929 cells increased until next measurement after eight hours. After 24 hours, first internalized aggregates of the interferon fusion protein were determined in cellular compartments which indicates a slow internalization rate of protein at moderate CD138 expression levels. Results from RNA sequencing experiments (figure 41) indicate that the multiple myeloma cell line U266B1 possesses the highest density of CD138 on the cell surface

compared to other tumor cell lines from this indication. Already after one hour, U266B1 cells showed clear membrane staining by the CD138 targeted interferon fusion protein with high levels of internalized fusion proteins. Eight hours post administration, the majority of labelled fusion proteins was internalized and aggregated in the cytosol of the cells. Further incubation until 24 hours showed no difference in membrane staining or the amount of internalized fusion protein compared to images taken after eight hours.

5.7.3 Pharmacologic potency of the interferon fusion protein

Subsequent evaluation of the targeting functionality of the interferon fusion protein, the pharmacologic potency of the fusion protein was investigated. To determine maximum induction of apoptosis and IC50 values of the interferon fusion proteins containing a cleavable tandem linker or a non-cleavable $(G_4S)_6$ linker, cell viability assays were conducted on the multiple myeloma cell line U266B2 over 72 hours (figure 45).

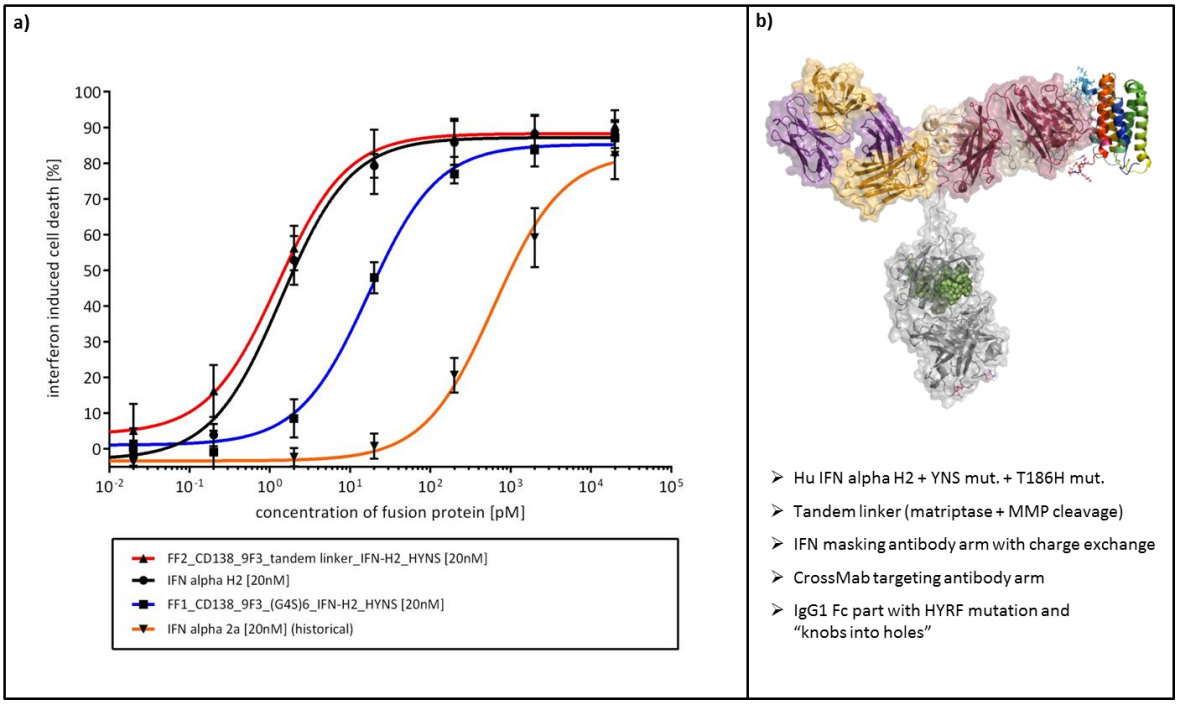


Figure 45: Proof of concept evaluation in multiple myeloma

Cell viability assay of targeted bispecific interferon fusion proteins containing a protease cleavable linker (red curve) or a non-cleavable peptide linker (blue curve) in comparison to human interferon alpha H2 (black curve) and IFN alpha 2a (orange curve) on the multiple myeloma cell line U266B1 **(a)**. Schematic diagram of the tested bispecific interferon fusion protein and listing of applied antibody modifications **(b)**. Cell viability assay was performed by treating 5000 cells/well with serial dilutions of interferon alpha [20nM] or interferon fusion proteins [20nM] for 72 hours. Assay readout was determined by using a CellTiter-Glo® luminescence assay. Blotted values and standard deviations were calculated based on six replicates. 3 D modelling of biomolecules was conducted by Dr. Guy Georges, Roche Diagnostics GmbH.

Table 7: Evaluated IC50 values of proof of concept fusion proteins

Anti-proliferative compound	IC50 [pM]
FF2_CD138_9F3_tandem linker_IFN-H2_HYNS	1.25
Interferon alpha H2	1.38
FF1_CD138_9F3_$(G_4S)_6$_IFN-H2_HYNS	16.84
Interferon alpha 2a	613.2

Treatment of U266B1 cells with serial dilutions of interferon alpha 2a showed a maximum apoptotic effect of 82.9 % and a half maximal inhibitory concentration of 613.2 pM. A decrease of the required IC50 concentration down to 1.38 pM was achieved by the stimulation of the multiple myeloma cells with human interferon alpha H2. Applied dilutions of targeted interferon fusion protein containing a non-cleavable linker induced a maximal growth inhibitory effect of 85.3 % at an IC50 concentration of 16.84 pM. Compared to interferon alpha H2, the masking effect of the fusion proteins triggered an increase in the half maximal inhibitory concentration by more than a factor of 12. In contrast to the masking interferon fusion protein with a non-cleavable linker, fusion proteins with incorporated protease cleavable tandem linkers achieved 88.3 % induction of cell death at a similar IC50 value (1.25 pM) compared to unfused interferon alpha H2 (1.38 pM).

To confirm the previously presented positive pharmacological effects of the bispecific interferon fusion protein to other cell lines and clinical indications, similar cell viability assay were performed to the multiple myeloma cell lines RPMI 8226 and NCI-H929 and the ovarian cancer cell line OVCAR-3 (figure 46).

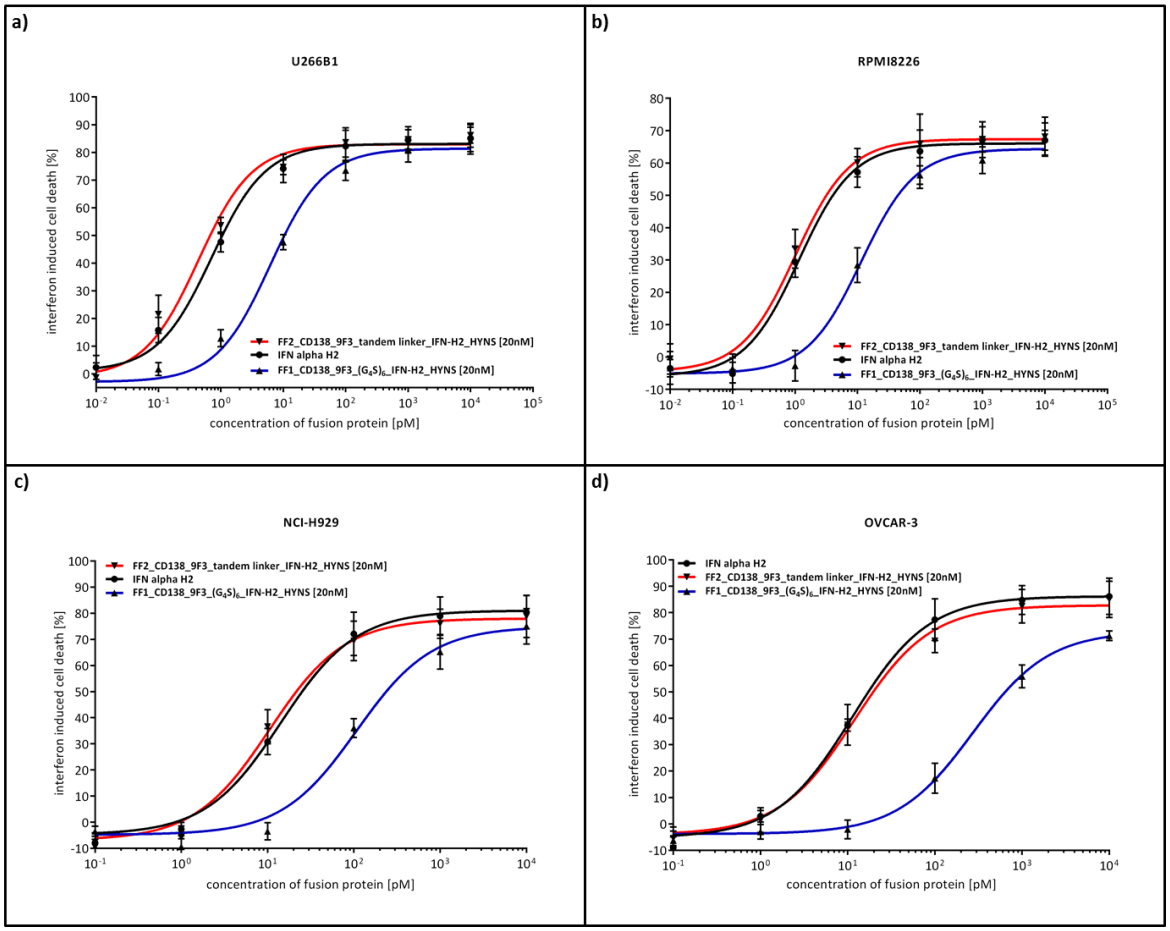


Figure 46: CD138 targeted IFN in several MM cell lines and ovarian cancer

Evaluation of the reproducibility of the previously described efficacy of the targeted interferon fusion protein and application of it on different cancer cell lines. Multiple myeloma cell lines U266B1 **(a)**, RPMI 8226 **(b)**, NCI-H929 **(c)** and the ovarian cancer cell line OVCAR-3 **(d)** were treated with serial dilutions of interferon fusion proteins [20nM] and human interferon alpha H2 [20nM]. Cell viability was determined by CellTiter-Glo® luminescence assay after treatment duration of 72 hours. Blotted inhibition curves and standard deviations were calculated on the basis of six replicates.

Table 8: Similar shifts of IC50 values in different cell lines

	U266B1	RPMI 8226	NCI-H929	OVCAR-3
FF2_CD138_9F3_ tandem linker_IFN-H2_HYNS	0.43	1.01	10.59	11.95
Interferon alpha H2	0.71	1.16	14.26	11.34
FF1_CD138_9F3_$(G_4S)_6$_IFN-H2_HYNS	6.18	11.56	107.7	274.2

In the multiple myeloma cell line U266B1, interferon fusion proteins with a non-cleavable peptide linker (IC50 = 6.18 pM) displayed an increase of the half maximum inhibitory concentration by more than a factor of 14 compared to fusion proteins with an incorporated tandem linker (IC50 =0.43 pM). A comparable difference between masking non-cleavable (IC50 = 11.56 pM) and cleavable (IC50 = 1.01pM) interferon fusion proteins were seen in the RPMI 8226 cell line.

Determined dose response curves in the multiple myeloma cell line NCI-H929 displayed same effects for the evaluated interferon fusion proteins than in other multiple myeloma cell lines, although calculated IC50 values were increased by a factor of 10 due to a decreased interferon sensitivity of NCI-H929. Treatment of the ovarian cancer cell line OVCAR-3 resulted in similar dose response curves of interferon alpha H2 (IC50 = 11.34 pM) and the interferon fusion protein containing a protease cleavable peptide linker (IC50 = 11.95 pM). In contrast to results obtained in multiple myeloma cell lines, the masking effect of the non-cleavable fusion protein triggered an increase in IC50 values between $(G_4S)_6$ and tandem linker by more than a factor of 22. Fusion proteins with a non-cleavable linker showed a half maximal inhibitory concentration of 274.2 pM in contrast to interferon fusion proteins with an incorporated tandem linker (IC50 = 11.95 pM).

Interpretations, possible explanations and consequences of the previously described data are discussed and explained in further detail in the following section of the thesis.

6. Discussion

Since the early discovery of interferon by Isaacs and Lindenmann in 1957, extensive work and scientific approaches to characterize this cytokine were conducted to reveal its anti-viral and anti-proliferative potency. Following first promising treatment results in oncologic indications and extensive promotions of interferon by the pharmaceutical industry in the early 80s, it quickly became clear that the application of this potent cytokine is often accompanied by severe adverse side effects. In many instances, interferon-based therapies in several oncologic indications and infectious diseases significantly interfere with the patient's quality of life. Treatment has to be interrupted due to unspecific responses of interferon in the body which lead to a diverse set of acute and chronic toxicities (Borden et al., 2007; Isaacs & Lindenmann, 1957).

To address these serious issues and possibly broaden the clinical application of interferon in oncology, temporary inhibition of interferon in combination with a tumor selective reactivation of the cytokine holds the potential to reduce or even overcome unwanted effects during interferon-based treatments while the anti-proliferative potential of interferon remains maintained. A possible way to achieve this ambitious goal is described in this thesis, dealing with the fusion of human interferon alpha to a masking bispecific antibody. Due to a temporary inactivation of the cytokine, unspecific interactions during circulation of the fusion protein in the periphery should be significantly reduced with beneficial consequences on the toxicity profile of interferon in oncologic indications. Besides the masking capability of the proposed interferon fusion protein for the improvement of the side effect profile of interferon, tumor specificity of the pharmacologic activity of the cytokine is supported by 1. enrichment of the fusion protein at the target site due to a second targeting moiety of the antibody and 2. selective release and reactivation of interferon in the tumor microenvironment by the cleavage of a connecting peptide linker by tumor-associated proteases. The proposed interferon fusion protein may provide a solution to overcome current obstacles and limitations in interferon-based treatment strategies and enforce an increased clinical application of this potent anti-viral and anti-proliferative cytokine. Combining an improvement of the pharmacodynamical attributes of interferon with a reduced toxicity side effect profile of the cytokine may contribute to more efficient cancer therapies and significant enhancement of patients' quality of life.

Keeping this goal in mind, the following section of the thesis deals with the interpretation of data, gained during the development and optimization of the proposed interferon fusion protein.

6.1 Clinical indications for an interferon fusion protein

Cell viability evaluation after treatment of the cells with IFN alpha 2a, showed diversified anti-proliferative responses varying between cell lines with different tissue origin and between subtypes of the same oncologic indication. Highest response rates to interferon treatment were detected in cell lines derived from multiple myeloma patients which indicates a high therapeutic effect in haematologic tumor types. Results from a meta-analysis of 17 clinical trials using interferon alpha based treatment strategies in multiple myeloma confirm the observed anti-proliferative effect of interferon on multiple myeloma cells (Fritz & Ludwig, 2000). Although the exact mode of action of interferon in multiple myeloma remains unclear, highest treatment efficacies were observed for interferon combination therapies with standard chemotherapeutic agents (Khoo, Vangsted, Joshua, & Gibson, 2011). In-house experiments showed that stimulation of U266B1 and RPMI 8226 multiple myeloma cells with interferon alpha 2a as monotherapy resulted in potent induction of cell death in a range of 65 % to 85 %.

In contrast to the overall potent anti-proliferative effect of IFN on the majority of multiple myeloma cell lines, treatment of OPM-2 and KMS-28 BM cells showed an enforced cell growth. A possible explanation for the varying anti-proliferative effect of Type I interferons on multiple myeloma cell lines was investigated by the group of Taruna Arora (Arora & Jelinek, 1998). Observations of expression levels and activations of cell cycle-associated kinases after stimulation of cell lines with interferon alpha have led to the assumption that interferon mainly interferes with the cell cycle progression of multiple myeloma cells. In the multiple myeloma cell line KAS-6/1, which showed accelerated cell growth in the presence of interferon, an overexpression of cyclin D2 was determined. Cyclin D2 plays a major role in the cell cycle progression from G0/G1 to S phase. In contrast to the growth promoting effect of IFN alpha in KAS-6/1, interferon stimulation of ANBL-6 cells resulted in a potent induction of cell death. It is presumed that the observed anti-proliferative effect of the cytokine is mediated by an overexpression of the kinase inhibitor p19(INK4d) which contributes to cell cycle arrest and induction of apoptosis.

In addition to efficient inhibition of cell growth in multiple myeloma cell lines, interferon treatment showed significant induction of cell death in the triple negative (HER2-, estrogen- and progesterone receptor negative) breast cancer cell lines MDA-MB-468 and MDA-MB-231. According to this observation interferon fusion proteins may be able to be applied in the treatment of triple negative breast cancer which represents an attractive oncologic indication for new potent therapeutics due to the high medical need in this hormone-independent tumor types.

In vitro evaluation of interferon combination therapies on breast cancer cell lines raised the assumption that already small amounts of interferon alpha trigger overexpression of oestrogen receptors which sensitizes cells to hormone receptor inhibitory small molecules like tamoxifen (van den Berg, Leahey, Lynch, Clarke, & Nelson, 1987). Additional investigations of interferon-mediated induction of apoptosis in breast cancer cells assume the involvement of the interferon regulatory factor 1 (IRF-1) in tumor suppression by the activation of cell death-mediating caspases (Bouker et al., 2005).

Regarding approved clinical indications of interferon alpha, additional cell lines originating from malignant melanomas were screened for their responsiveness to interferon based cancer treatment. Gained data showed moderate growth inhibition of cancer cells in the presence of interferon in a range of 18 % to 47 % reduction of viable cells. Although interferon shows just minor effects in the treatment of malignant melanoma, it still remains the only therapeutic agent which leads to a prolongation of the relapse free period and an improvement of overall survival of patients (Hauschild, 2009). Due to the limitation in treatment alternatives, interferon fusion proteins with improved pharmacodynamical attributes and reduced toxicity profiles may be able to contribute to an increased survival of malignant melanoma patients or even reduce the burden of this highly metastatic malady. Summarizing the experimental data gained during the evaluation of a diverse set of cancer cell lines according to interferon-mediated cell death, multiple myeloma seemed to be the most favourable indication for treatment with an interferon antibody fusion protein. According to these findings and the current clinical application of interferon alpha 2a in multiple myeloma treatment, the majority of experiments for the development and optimization of the proposed cytokine fusion protein was performed on multiple myeloma cell lines. To additionally evaluate the interferon fusion protein in solid tumor indications, the ovarian cancer cell line OVCAR-3 was used which showed interferon mediated anti-proliferative effects at similar levels than the most sensitive multiple myeloma cell line U266B1.

To determine suitable oncologic indications for an interferon fusion protein experiments were performed with human interferon alpha 2a due to its approved clinical application and broad knowledge about pharmacokinetic and pharmacodynamic parameters of the cytokine. However, other members of the interferon alpha family may also exhibit potent anti-proliferative activities in oncologic indications and provide suitable candidates for an interferon fusion protein. Treatment of U266B1 cells with all subtypes of the type I interferon family displayed varying degrees of cell death induction in the multiple myeloma cell lines. Other interferon alpha subtypes such as IFNαB2, IFNαH2, IFNαWA and universal IFN alpha showed significantly higher

anti-proliferative response rates compared to the previously applied interferon alpha 2a,. According to the determined differences in the cell death inducing capability of interferon alpha subtypes, further attempts for the improvement of the anti-proliferative potency of interferon alpha were made.

6.2 Improvement of the pharmacological potency of IFN

The comparison of human interferon alpha 2a and interferon alpha H2 in the treatment of U266B1 cells displayed major differences in the anti-proliferative effects of the cytokines. Although both interferon subtypes achieved similar maximum inductions of cell death, interferon alpha H2 showed a reduction in the IC50 concentration by more than a factor of 24 compared IFNα2a. Therefore, this effect was further investigated to possibly explain the differential anti-proliferative activity of interferon subtypes. It is known that all 13 members of the interferon alpha family bind to a common Type I interferon receptor and mediate their biological effects by the activation of a Jak/STAT signalling pathway (Platanias, 2005). To determine differences in the interactions of the cytokines and their receptors, a 3D modelling approach was applied to investigate binding moieties of IFNα2a and IFNαH2 to the human interferon receptor.

Over the past decade several investigations in the field of interferon and its interaction with both receptor units IFNAR1 and IFNAR2 were performed. Interferon-induced anti-proliferative effects are most likely mediated via the interferon receptor subunit IFNAR1 (Eyal Kalie et al., 2007; Piehler et al., 2012). Further experiments to characterize the biological influence of the affinity of interferon to subunits of the IFN receptor made clear that besides the affinity of IFN to each of the IFNAR subunits, the stability of the ternary complex of IFN and both receptor units plays a major role in the potent biological activities of interferon (E. Kalie et al., 2008). Therefore, the binding interaction between interferon alpha 2a and the interferon receptor unit IFNAR1 was analysed by 3D modelling approaches. The anti-proliferative activity of IFNα2a and IFNαH2 was tested by amino acid sequence comparisons of both cytokines. Cristal structure analysis of the IFN alpha 2a / IFNAR1 complex revealed a strong contribution of threonine at position 86 of the interferon peptide to the binding interaction between the cytokine and IFNAR1. The alignment of the amino acid sequences of IFN alpha 2a and IFN alpha H2 showed that IFNαH2 possesses isoleucine instead of threonine at position 86 of the peptide chain. The exchange of threonine to isoleucine possibly explains the more potent anti-proliferative effect of IFN alpha H2 due to an increased hydrophobic interaction of the cytokine with the interferon receptor IFNAR1.

The discovery of the affinity mediating and anti-proliferative effect enhancing amino acid at position 86 of interferon revealed the possibility for an improvement of the pharmacodynamical parameters of the cytokine leading to an enhancement of the anti-proliferative potency of an interferon antibody fusion protein. According to data presented by the company Teva GmbH at the 6th World ADC Summit 2014 in San Diego, efficient treatment of multiple myeloma would require interferon alpha 2a amounts above the maximum tolerable dose (MTD) of the cytokine. In more than half of the treated samples, effective interferon alpha 2a doses were 100 to 1000 times above the MTD. To overcome this limitation of interferon application in multiple myeloma treatment and possibly broaden the therapeutic efficacy of the cytokine, the improvement of pharmacodynamical attributes of interferon is required.

First attempts to improve the efficacy of interferon alpha 2a were conducted by the exchange of threonine at position 86 by leucine, arginine and histidine. Treatment of the multiple myeloma cell line U266B1 with interferon fusion proteins containing point mutated IFN alpha 2a showed that the mutation Thr86His efficiently improved the anti-proliferative potency of the cytokine compared to wild type IFNα2a due to enhanced hydrophobic interactions of the cytokine and its receptor (figure 7). The introduction of arginine instead of threonine at position 86 triggered a loss in the maximum induction of cell death and an increase in the necessary half maximum inhibitory concentration of the interferon fusion protein. Reduced anti-proliferative effects by the Thr86Arg mutation may be explained by sterical hindrances of binding interactions of IFN alpha 2a and IFNAR1 due to the large unfavourable amino acid side chain of arginine.

Besides the discovered Thr86His mutation which leads to an increased affinity of interferon alpha 2a to its receptor, additional work was carried out in the lab of Gideon Schreiber (Eyal Kalie et al., 2007). A phage display library was established to screen for IFN alpha 2a mutants with enforced affinities to IFNAR1 leading to an improvement of the anti-proliferative potency of the cytokine. To reach this goal, interferon alpha 2a was randomly mutated at three positions which seemed to be involved in the binding interaction to IFNAR1. A mutated variant of human IFN alpha 2a was identified which comprised the mutations H57Y, E58N and Q61S (YNS mutations). Further characterizations of the YNS variant showed that binding affinity of IFN (YNS) to IFNAR1 was increased 60-fold compared to wild type interferon alpha 2a. Binding affinity to the interferon receptor unit INFAR2 was not affected. Evaluations of the YNS mutant version of interferon alpha 2a *in vitro* displayed a 150-fold improved anti-proliferative effect compared to wild type interferon on WISH cells. No significant influence of the YNS mutations on the anti-viral potency of the cytokine was determined (Eyal Kalie et al., 2007).

Interferon antibody fusion proteins were generated containing wild type IFNα2a, IFNα2a with an incorporated Thr86His mutation, an IFNα2a YNS mutant version and interferon alpha 2a with both the YNS and Thr86His mutations (HYNS mutation) to evaluate the potency of various IFN mutations in this format. Treatment of the ovarian cancer cell line OVCAR-3 with serial dilutions of interferon fusion proteins containing the point mutations resulted in clear differences regarding the anti-proliferative potency. Compared to wild type interferon alpha 2a, the application of the Thr86His mutation reduced the necessary half maximum inhibitory concentration by more than half. The in-house and interferon fusion proteins with applied YNS mutations showed a reduction in the IC50 value from 613.2 pM of wild type interferon down to 41.3 pM confirming the data by Schreiber et al. (Eyal Kalie et al., 2007).The combination of YNS mutations with the previously in-house discovered Thr86His mutation has triggered an additional shift in the IC50 value of the fusion protein down to 24.2 pM (figure 7).

The 25-fold reduction in the necessary IC50 of the HYNS-mutated interferon fusion protein is explained by exchange of four amino acids mainly responsible for the interaction of the cytokine to IFNAR1. Since affinities to IFNAR2 are not affected by these mutations, published data were confirmed. Following the promising improvements of IFN alpha 2a, Thr86His, YNS and HYNS mutations were additionally applied to the IFN alpha H2. Wild type IFN alpha H2 exhibits a previously described increased anti-proliferative effect compared to wild type IFN alpha 2a. It was hoped to improve even this potent IFN alpha subtype by the application of IFNAR1 affinity-mediating point mutations. Cell viability assays of U266B1 cells revealed a similar potency of fusion protein containing IFN alpha 2a with HYNS mutations or IFN alpha H2 wild type. Introduction of HYNS mutations in IFN alpha H2 just slightly improved its anti-proliferative potency indicating that the affinity of wild type IFN alpha H2 to IFNAR1 is almost maximized. The HYNS mutations of IFNαH2 were able to reduce the IC50 concentration of the fusion protein from 0.9 pM (wild type IFN alpha H2) to 0.6 pM which identified HYNS mutated IFNαH2 as the most potent choice for the proposed masked interferon antibody fusion protein.

To evaluate the potency of interferon fusion proteins with the incorporated HYNS mutation in comparison to standard chemotherapeutic agents, the cell line U266B1 was treated with interferon fusion protein, the standard of care proteasome inhibitor bortezomib (Field-Smith, Morgan, & Davies, 2006), and additional cytotoxic agents. Comparing the IC50 values of the growth inhibition curves of the IFN fusion protein and bortezomib, mutated interferon fusion proteins displayed a 1000-fold improved potency compared to standard of care treatment in multiple myeloma. These promising results supported the hope/expectation for beneficial

application of interferon fusion proteins in this indication to enhance anti-tumor treatment efficacy in patients suffering from multiple myeloma.

Still, the risk of unwanted interactions of interferon in the periphery and aggravation of the side effect profile of the cytokine (Jonasch & Haluska, 2001) was potentially increased. Regarding these concerns and to overcome the dose-limiting toxicity of interferon, an antibody mediated masking approach leading to a temporary inactivation of the cytokine was applied.

6.3 Masking and temporary inhibition of interferon

To overcome in interferon based cancer therapy the limitation by side-effects and possibly enhance the biological availability/activity of the cytokine, temporary inhibition of interferon in interferon antibody fusion proteins by a masking antibody in combination with a selective reactivation of the cytokine in the tumor microenvironment may be able to significantly attenuate toxic side effects.

A related attempt for a temporary inhibition and target selective reactivation of a biomolecule was conducted by the group of Donaldson *et al.* (Donaldson, Kari, Fragoso, Rodeck, & Williams, 2009). To prevent side effects caused by an unspecific binding of therapeutic antibodies to healthy tissue, the group applied a masking approach of the CDR regions of antibodies. Therefore the antigen binding domains of antibodies were blocked by small peptides similar to their natural antigen. The proposed design concept is comprised of two different antibodies which are fused to a blocking peptide, masking the counterpart antibody. As long as the blocking peptides are stably fused to the antibodies, both kinds of antibodies stay in a masked and inactive state. After enrichment of the dual-antibody-construct at the target site, the linker sequence between the antibodies and the masking peptide gets cleaved by target associated proteases. Following the cleavage of the peptide linker and the dissociation of the dual-antibody-construct, each of the antibodies become reactivated and able to induce its pharmacodynamics activity at the target tissue.

The interferon antibody fusion protein presented in this thesis is based on a monoclonal antibody termed 9F3 from the company Genentech Inc. (a member of the Roche Group) and exhibits binding affinity against human interferon alpha 2a. First in-house evaluations of the antibody *in vitro* displayed a strong binding of IFNα2a which has led to the inactivation of the cytokine to a large extend. Due to the masking of interferon by the antibody, most of its biological activity was

lost while it was bound to the antibody. This supports the idea of a temporary inhibition of interferon by an antibody to reduce or even prevent side effects of the cytokine (figure 10).

For the proposed mode of action of the interferon antibody fusion protein release of interferon at the tumor site after cleavage of the peptide linker was required and, at the same time, ensuring an efficient inactivation of the cytokine when bound by the masking antibody . Due to the high affinity of the antibody 9F3 to its antigen interferon alpha 2a in the range of 25 pM, a release of the cytokine from the antibody would have been highly unlikely. To address this issue, several point mutations in the antigen binding domains of the heavy and light chain of the antibody 9F3 were applied to reduce its affinity for IFNα2a. This enables slow release of the cytokine after cleavage of the stabilizing peptide linker. Incorporated point mutations in the variable domain of the light chains caused a tremendous loss of affinity which triggered an insufficient interferon masking capability of the antibody (figure 10). In contrast to that, mutations at position 52 and position 76 in the peptide chain of the variable domain of the heavy chains induced a moderate reduction in the antibody affinity and following this, a suitable binding and masking effect of the modified 9F3 antibody against human interferon alpha 2a (figure 10). Due to the adjustment of the antigen binding affinity of 9F3, the modified antibody was suitable for an interferon antibody fusion protein which exhibits masking and inactivation capability of interferon alpha in combination with possible release of the cytokine at the tumor site.

Antibody affinity modifying effects of the point mutations were additionally confirmed by surface plasmon resonance (SPR) spectroscopy (figure 11). Mutations in the variable heavy domain slightly reduced the affinity of the wild type 9F3 antibody from 25 pM down to 49 pM (Iso52Asp) and 35 pM (Iso76Gly) (figure 11) which resulted in appropriate masking and inhibition of interferon (figure 10). Point mutations in the variable domain of the light chain led to a decrease in affinity of the antibody to 14 nM (Tyr55Ala) and 15 nM (Tyr73Asp) resulting in a quick release of interferon from the antibody (figure 11) and poor masking capability of the antibody (figure 10). It is still unclear why the incorporated mutation at position 119 in the variable domain of the light chain has triggered such significant loss of masking capability of the 9F3 antibody *in vitro*, although SPR measurement displayed only a slight reduction of the affinity down to 41 pM (figure 11). The observed effect might be explained by the change of the binding affinity of the 9F3 antibody at interferon alpha 2a due to the incorporated mutation. Further investigations in the different binding modes of the wild type 9F3 antibody and its point mutated variant to IFNα2a should be conducted by antibody epitope mapping technology which may explain the determined effect on the biological function of the cytokine.

Due to its specificity against a particular member of the interferon alpha family it was unclear to what extend the antibody 9F3 is able to bind and mask other members of the IFN alpha subfamily. To address this issue and determine the masking capability of the 9F3 antibody on other interferon alpha types, cell viability assays with all 13 natural occurring interferon alpha types were performed on the multiple myeloma cell line U266B1 in combination with the 9F3 antibody (figure 12). As expected, strongest binding and cytokine inhibiting effects were observed for the combination of IFN alpha 2a and 9F3 antibodies. According to the innate specificity of the antibody, only moderate or poor masking effects were determined for the majority of IFN alpha family members. The antibody 9F3 displayed a suitable degree of masking in combination with interferon alpha H2, taking into account that reduced affinity supports possible release of the cytokine at the tumor site. As previously shown, interferon alpha H2 has a significantly improved anti-proliferative potency compared to interferon alpha 2a (figure 4) as well being suitably masked by the 9F3 antibody (figure 13) which made this cytokine a potent candidate for the interferon fusion protein.

To characterize the masking and cytokine inactivation capability of the 9F3 antibody compared to a non-masking antibody, a human IgG1 antibody was generated without functional CDR regions (“Nada”-antibody). Therefore, affinity mediating amino acids of the 9F3 antibody were replaced by amino acids (alanine, glycine) with short non-reactive side chains. Due to the exchange of affinity mediating amino acids, the modified “Nada” version of the antibody 9F3 was not capable to bind interferon alpha 2a anymore which was confirmed by SPR measurement (figure 13). Unexpectedly, experiments with bivalent interferon fusion proteins which contained the non-masking “Nada” antibody on the multiple myeloma cell line U266B1 improved the potency of the fusion protein compared to treatment with interferon alpha without antibodies (figure 14). The effect was reproducible in U266B1 cells which fostered the suspicion of enhanced anti-proliferative potency by bivalent non-masked interferon fusion proteins.

Clustering of interferon receptors on the surface of cells by bivalent fusion proteins triggering increased downstream signalling and apoptosis compared to monomeric interferon binding to a single IFN alpha receptor might be a possible explanation for this effect. To further investigate this assumed mode of action of a “Nada” interferon fusion proteins, multiple myeloma cells were treated with a bivalent fusion protein and a monovalent interferon fusion protein with only one cytokine fused to the antibody (figure 14). Goal of the experiment was to even increase the crosslinking of IFN receptors on the cell by pre-incubating interferon fusion proteins with secondary anti-human Fc and anti-human LC antibodies, thus, causing crosslinking of the

interferon carrying antibodies. In case of bivalent fusion proteins, a clear improvement in the anti-proliferative potency of the construct was determined compared to IFN alpha as single agent. However, additional application of crosslinking secondary antibodies did not increase the potency of the bivalent “Nada” fusion proteins (figure 14, a)). A similar experiment with monovalent interferon fusion proteins showed a significantly decrease of the anti-proliferative effect of interferon fusion proteins compared to IFN alpha. Although equimolar amounts of interferon alpha and interferon fusion proteins were applied, no beneficial effect of the monovalent fusion protein was determined even after crosslinking by secondary antibodies (figure 14, b)).

Improved effects of a bivalent “Nada” interferon fusion protein were not able to be confirmed in cell lines from indications other than multiple myeloma which indicates a specific susceptibility of these malignant cells for IFN fusion proteins. Due to the fact that the proposed interferon fusion protein here in this thesis was the first interferon antibody format in a bivalent fashion, no further literature was found which would give hinds for an alternative explanation of the observed effects. Open questions still have to be addressed to reveal the extraordinary mode of action of bivalent non-masking interferon fusion proteins in multiple myeloma derived cell lines.

Summarizing the outcomes and investigations of the masking and temporary cytokine inhibition approach, it was possible to adjust the affinity of the human anti IFN alpha 2a antibody 9F3 to a suitable level for effective masking of interferon in combination with a possible release of the cytokine at the tumor site. According to the specificity of the 9F3 against IFN alpha 2a and its moderate masking of other interferon alpha subtypes, no further affinity adjustments were required for the 9F3 antibody at interferon fusion proteins containing IFN alpha H2. Although efficient masking and inactivation capability of the described fusion protein was shown in several *in vitro* evaluation studies, final proof of concept has to be conducted in immune efficient animal models to determine the consequence of this approach on a desired reduction of unwanted responses and toxic side effects in interferon based cancer treatment. The methodology of temporarily inhibiting a cytokine can also be applied to other types of cytokines with restricted clinical use due to their innate toxicity profile.

A short peptide linker was incorporated in the construct which connects the cytokine to the variable domain of the heavy chain of the masking antibody to achieve a stable binding and masking of interferon during circulation of the fusion protein in the periphery. Due to the close proximity of the cytokine and the antibody, a bound conformation of interferon is favoured in contrast to a linker-free situation with an equilibrium between bound and non-bound cytokine.

Therefore, the selection of a suitable peptide linker between the cytokine and the antibody was of high importance to ensure stable masking and inactivation of interferon in the periphery and selective release of the cytokine at the tumor.

6.4 Protease cleavable peptide linkers

A key feature of the applied peptide linker between interferon and its masking antibody is the incorporated protease cleavage site which provides the possibility for target specific release of the cytokine due to the cleavage of the linker by tumor-associated proteases. Cancerous tissue is well known for the upregulation of certain types of proteases which are produced by the tumor cells for degradation of the extracellular matrix (ECM). The removal or even reduction of components of the ECM contributes to eased tissue invasion of cancer cells and enforces metastasis/colonization. Regarding this essential feature of tumor cells, many therapeutic approaches were conducted with the aim of inhibiting tumor-associated proteases to prevent spreading of cancer cells to healthy tissue (Koblinski, Ahram, & Sloane, 2000).

A subclass of these ECM degrading proteases is made up by the family of matrix metalloproteinases (MMPs). The family of MMPs includes more than 21 human proteases with different substrate specificities, which are able to cleave and degrade nearly every component of the extracellular matrix. According to their structure, MMPs are categorized in five secreted forms and three membrane-bound versions. MMPs are expressed and secreted in an inactive state called zymogens (pro-MMP). Zymogens are converted to their proteolytically active form by a complex mutual crosstalk or by interaction with other subtypes of already activated MMPs. Due to the diverse composition of the MMP family, several functions in the degradation of components of the ECM, cleavage of cell adhesion proteins like cadherins, and the release of the extracellular domain of growth factor receptors (e.g. FGF, HER2/neu and ERBB4) are carried out by matrix metalloproteinases (Egeblad & Werb, 2002).

A common characteristic of most cancer types is the up-regulated expression and activation of MMPs which strongly correlates with tumor progression, enforced tumor cell invasion into healthy tissue, and poor prognostic outcomes in cancer treatment. Although many steps in tumor progression are based on genetic alterations in the genome of cancer cells, the enforced activity of MMPs in human cancer is presumably caused by transcriptional variations and not by alterations of the MMP genes which is probably mediated by oncogene activity or the loss of

tumor suppressor genes (Egeblad & Werb, 2002). Due to a high structural similarity of the catalytic domain of different members of the MMP family, different substrates in the ECM can be cleaved by more than one specific MMP type which explains disappointing clinical results of MMP inhibitors in cancer treatment (Egeblad & Werb, 2002; Kridel et al., 2001).

Despite the inherent similarity of enzymatic active domains of MMPs, several approaches to determine cleavage specificities for distinct subtypes of MMPs were conducted to gain further insight into the complex network of MMPs. The two subtypes MMP-9 and MMP-2 came into the spotlight of scientific research and several approaches to determine substrate specificities and biological functions of both MMPs were investigated. Different methodologies were applied to reveal cleavage motives of MMPs. Studies of the enzymatic activity carried out by phage display libraries (Kridel et al., 2001), screening technologies based on FRET (förster resonance energy transfer) principle (Kridel et al., 2001), and mixture based oriented peptide libraries (Turk et al., 2001) have helped to identify preferred substrates, favoured amino acids within the cleavage motives, and the biological crosstalk of MMPs.

Although direct targeting and inhibition of MMPs did not show to be effective in cancer therapy due to the complex network of proteases with redundant cleavage specificities, tumor-specific up-regulation of this protease family delivers the possibility to utilize this cancer-associated attribute for other therapeutic approaches such as the tumor-selective drug release mediated by the cleavage of linker peptides.

Based on previously described findings concerning specific MMP substrates and their published sequences, several MMP-9- and MMP-2-specific cleavage motives were incorporated into the stabilizing peptide linker between interferon and its masking antibody in the interferon fusion protein. To determine suitable cell lines for the evaluation of cleavage efficacies of the incorporated linkers, several cell lines were screened for their amount of active proteases by zymograms (figure 15, a)). As expected, tumor cell lines from solid indications (pancreatic cancer cell line BxPC3, fibrosarcoma cells HT1080) displayed increased levels of active MMPs compared to tumor cells from hematologic indications. This is due to the engagement of MMPs in degradation of the ECM and metastasis of solid tumors. Besides the requirement of MMPs for solid tumors, cell lines from the hematologic system such as the multiple myeloma cell line U266B1 also displayed proteolytic activity. To determine turnover rates of MMP-specific linker sequences *in vitro*, U266B1 cells were treated with interferon fusion proteins containing cleavable linker peptides found in the literature (figure 15, b) and c)). Fusion proteins containing a peptide linker derived from Turk *et al.* displayed only moderate cleavage (difference in IC 50 values)

compared to a non-cleavable G4S6 peptide linker. Highly efficient cleavage of the incorporated linkers was monitored for interferon fusion proteins with applied peptide sequences from Fudala *et al.* and the patent WO 2010/081173A2. Although the linker sequence V-P-L-S-L-Y-S-G (Fudala *et al.)* displayed highest turnover rates, the MMP-9-specific linker sequence V-H-M-P-L-G-F-L-G-P was selected to be implemented in the final interferon antibody fusion protein. This was done because of the determined instability of the V-P-L-S-L-Y-S-G linker during expression in cell culture and incubation in murine plasma (data not shown). The complexity of the matrix metalloproteinase network and inherent cleavage specificities of members of this family is also reflected by major differences in the amino acid sequence of the MMP-9 specific peptide linkers.

Even though the application of MMP-specific peptide linkers for the interferon fusion protein was successful, more tumor-associated proteases were searched in literature to obtain an alternative strategy for release and reactivation of interferon at the target site. Regarding the functionality of many different kinds of proteases in the degradation of the extracellular matrix, another tumor-associated protease called matriptase was tested. Matriptase, also known by the name MT-SP1, is a representative of the family of type II transmembrane serine proteases and mainly expressed by epithelial cells which are the origin of the most common type of cancer called carcinomas. Up-regulation of matriptase was shown in several human tumors, including lung, ovarian, prostate, and kidney cancer. Until now, the participation of matriptase in cancer progression is not fully understood, although it was shown that this kind of protease mediates the activation of pro-urokinase, vascular endothelial growth factor receptor 2, and pro- hepatocyte growth factor. Due to the previously discovered mode of actions of matriptase, it is assumed that this trypsin-like serine protease mainly contributes to tumor growth and the invasion of tumor cells into the surrounding tissue (Gao et al., 2013; Uhland, 2006).

Activity of matriptase in healthy epithelial tissue is tightly down-regulated by its associated inhibitor hepatocyte growth factor activator inhibitor-1 (HAI-1). Recent studies have shown that in some tumors the natural balance of matriptase and its inhibitor HAI-1 is deregulated by the down-modulation of HAI-1, resulting in high amounts of proteolytically active matriptase (LeBeau et al., 2013). Based on this awareness, the group of LeBeau *et al.* developed a monoclonal antibody which selectively recognizes and binds to the active form of matriptase. Screening with tissue sections derived from human colon cancer xenograftes confirmed the tumor-specific activation of matriptase in 68 % of all tested primary and metastatic colon carcinoma samples (LeBeau et al., 2013). Due to the expression of matriptase on the surface of epithelial cells and the selective activity of the protease in malignant tissue, active matriptase seemed to be a

suitable biomarker in many oncologic indications, thus, delivering an alternative approach for a tumor-selective release of the cytokine from the interferon fusion protein.

First attempts for the incorporation of a matriptase cleavable peptide linker in the interferon fusion protein were based on a common substrate sequence (R-Q-R-R-V-V-G-G) for matriptase derived from the MEROPS protease data bank. Further optimization of the common substrate sequence was performed by an exchange of arginine to alanine at position P2 (developed by Dr. Edgar Voss, Roche Diagnostics GmbH) to enhance the turnover rate of the linker by the protease. Determination of the linker cleavage kinetics on the ovarian cancer cell line OVCAR-3 displayed similar cleavage rates of linker sequence derived from the MEROPS database and sequences which are related to the matriptase inhibitor HAI-1 or its natural substrate HGF (figure 16 c)). The comparison of tested peptide linker sequences has fostered the assumption that arginine at position P1 of the cleavage site mediates an important function to initiate turnover of the linker by matriptase. The addiction of matriptase-mediated cleavage of peptide linkers to arginine at position P1 was also confirmed by several groups who screened for selective substrates or inhibitory compounds for this type II transmembrane serine protease (Beliveau, Desilets, & Leduc, 2009; Colombo et al., 2012).

As described above, several protease cleavable peptide linkers were incorporated into interferon antibody fusion proteins with selectivity for the matrix metalloproteinase 9 (MMP-9) or the membrane bound type II serine protease matriptase. To further increase the likelihood of cleavage of the incorporated peptide linker by tumor-associated proteases, a MMP-9-specific substrate sequence was combined with a matripase-cleavable peptide in one linker. The newly generated “tandem linker” was flanked by a G_4S-spacer at the N-terminus followed by a matriptase cleavable linker, a peptide sequence with selectivity for MMP-9 mediated cleavage and a G_4S-spacer at the C-terminus (figure 17, a)). Following incorporation of the newly developed tandem linker in interferon antibody fusion proteins, cleavage kinetics of the linker were determined using the ovarian cancer cell line OVCAR-3 and compared to fusion proteins containing a monospecific peptide linker (figure 17, b)). Based on the half maximum inhibitory concentrations of constructs with a monospecific matriptase linker (797.9 pM) or MMP-9 specific linker (350.9 pM), a turnover rate of the dual specific tandem linker was expected in a similar range compared to the more potent MMP-9 specific linker. Against all expectations, interferon fusion proteins containing a tandem linker displayed a tremendous improved linker cleavage rate leading to an IC50 value of 44.71 pM which closely corresponds to the anti-proliferative effect of unfused IFN (IC50 = 37.41 pM) measured in the same experiment (figure 17, c)).

Until now it is not fully understood how the combination of two protease specific peptide sequences in one tandem linker is leading to a synergistic effect in the turnover rate of the linker. A possible explanation for the observed effect might be the increased likelihood of the tandem linker to get in close proximity to either one or another of the two proteases which are able to cleave its amino acid sequence. Additionally to the enforced cleavage likelihood, the combination of two different protease cleavable peptides in one linker is leading to a new amino acid sequence in the transition zone between both linkers. Due to this effect, the generated tandem linker may also be cleaved by unknown proteases with specificities to the amino acid sequence in the transition zone of both combined linkers. To investigate the observed synergistic turnover effect of a dual protease-specific tandem in closer detail, further experiments have to be conducted to reveal the exact mode of action of the peptide. During the development of an optimized interferon antibody fusion protein, only one matriptase specific linker was combined with one MMP-9-associated linker in a tandem linker. The combination of alternative substrate sequences for both applied proteases may answer the question whether this effect is restricted to the used amino acid sequences in the performed experiments or whether increased turnover rates can be expected by the combination of two linkers, irrespective of their peptide sequence.

Besides the necessity of a suitable turnover of the peptide linker by tumor-associated proteases, a high grade of stability of the linker during circulation of the fusion protein in the periphery was required. Incubations of fusion proteins containing protease cleavable linkers in murine plasma displayed only low cleavage rates of peptide linkers with single protease specificity (figure 18). In contrast to these findings, western blot analysis of interferon fusion proteins with an incorporated dual specific tandem linker showed a small fraction of cleaved constructs (50 kDa) in samples incubated in PBS and a slight increase of the cleaved fraction over time during incubation in mouse plasma (figure 18). According to these findings, it was assumed that the combination of both protease cleavable peptides in one linker has negatively affected the overall stability of the tandem linker. Decreased stability may correlate with an unspecific cleavage of the new tandem linker by proteases originating from murine plasma. The sequence of the applied matriptase linker contains two arginine residues in a row, which can serve as a substrate for the protease furin leading to an intracellular cleavage of the linker during expression of the fusion protein (Thomas, 2002). Possible ways to overcome this limitation would be to combine other matriptase substrates with a MMP-9 specific linker or to remove possible cleavage sites for unspecific proteases by point mutations of the applied tandem linker. Further combination studies of substrates for tumor-associated proteases in one peptide linker and optimizations of the selective cleavage of them will provide a deeper insight into the synergistic effect of this approach.

Additional findings may also provide a suitable new tandem linker which is able to inhibit increased turnover rates at the tumor site in combination with a high grade of stability during circulation.

Extensive efforts in the optimization of the pharmacodynamic properties of human interferon, the temporary inhibition of the cytokine in the periphery by antibody-mediated masking and the connection of both moieties in one fusion protein by a dual specific protease-cleavable linker makes the generation of a potent interferon fusion protein possible which holds the potential to improve current interferon based treatment strategies in oncology. In addition to the restriction of the anti-proliferative activity of interferon at the tumor site by its local reactivation, a further targeting approach was used to force fast clearance of the fusion protein from healthy tissue by enriching the construct at the tumor. Due to this targeting attempt further decrease the likelihood of unwanted responses of the cytokine in the body are expected which beneficially influence patients` quality of life together with increased levels of the pharmacologically active cytokine at the tumor site.

In spite of the complex composition and assembly of the bispecific antibody, several protein modelling techniques and amino acid modifications were applied to overcome the hurdles of generating this promising fusion protein in high amounts, sufficient for further *in vitro* characterizations and future proof of concept studies in animal models.

6.5 Experiences and findings during manufacturing of a bispecific antibody cytokine fusion protein

Planning and manufacturing of a complex molecule like the proposed interferon antibody fusion protein with a second binding specificity for a tumor-associated antigen required the application of several protein engineering tools to ensure correct pairing of the four different antibody chains of the fusion protein. Dimerization of the antibody heavy chain fused to interferon alpha and the second heavy chain with a binding specificity to CD138 was enforced by the use of the “knobs into holes” technology (Ridgway et al., 1996) which is further described in the method section of the thesis. Although homodimerization of two “knob” – heavy chain was prevented due to steric hindrances, a minor population of “hole”- heavy chain homodimers could still be detected. To circumvent this problem and purify correctly assembled heterodimeric fusion proteins, additional HYRF mutations (Natsume et al., 2008) were introduced in the targeting heavy chain of the fusion protein. Besides these antibody engineering tools, further techniques like the “CrossMab”

technology (Schaefer et al., 2011) and an exchange of charged amino acids in the first constant domains of the antibody were made to control the correct pairing of each antibody light chain to its corresponding heavy chain (described in more detail in the method section of the thesis).

Transient expression of bispecific interferon fusion proteins containing previously mentioned protein modifications resulted in high amounts of captured molecules by protein A chromatography (figure 20, a)). Size exclusion chromatography displayed a moderate fraction of aggregated proteins and a majority of monomeric interferon fusion proteins (figure 20, b)). Despite the complexity of the bispecific fusion protein, it was possible to gain similar protein yields (12 – 17 mg/L) as for unmodified IgG antibodies by transient expression in HEK293F suspension culture. Following purification of the fusion proteins, the correct assembly of the constructs out of four different antibody chains was confirmed by gel electrophoresis (figure 21). Protein bands were detected at 75 kDa corresponding to a heavy chain with fused interferon alpha, at 50 kDa standing for heavy chains containing the targeting moiety of the fusion protein and at 25 kDa displaying the light chains of the construct. Due to the identical size of the interferon masking light chain and the light chain contributing to the targeting moiety of the fusion protein, it was not possible to separate both types of antibody light chains by gel electrophoresis. To further analyse and confirm the correct assembly of the bispecific interferon fusion protein, mass spectroscopy would be an alternative technology to characterize the manufactured molecule in more detail.

With the successful manufacturing and characterization of the targeted interferon fusion protein by simultaneous application of several protein engineering tools, sufficient amounts of the proposed molecule were generated for further analysis of the expected mode of action of the fusion protein and first proof of concept studies *in vitro*.

6.6 Functional characterization of the proposed interferon fusion protein

Based on the screening results of cancer cell lines derived from different oncologic indications, multiple myeloma seemed to be the most promising indication to test the full potential of the optimized bispecific interferon fusion protein (figure 1). Therefore, cancer cell lines derived from multiple myeloma patients were chosen for first proof of concept studies of the fusion protein. A common characteristic of multiple myeloma cells is the overexpression of the transmembrane heparin sulfate proteoglycan CD138 also known as syndecan-1. By up-regulation of CD138 mainly forcing tumor progression it co-localizes with the VEGF receptor 2 (VEGFR-2) leading to neo-

angiogenesis in the tumor mass located in the bone marrow (Lamorte et al., 2012). Besides its important role in angiogenesis, CD138 mediates additional functions in the tumor survival, cell adhesion, and growth of multiple myeloma cells (Dhodapkar et al., 1998). According to other data, overexpression of CD138 in multiple myeloma cell lines was also confirmed by mRNA expression analysis (figure 19) as shown by the Chugay Pharmaceutical company (a member of the Roche group).

For the evaluation of the tumor targeting functionality of the bispecific interferon fusion proteins, cancer cell lines with known CD138 expression levels were selected and treated with fluorescence-labelled fusion proteins. Confocal microscopy revealed an intense membrane staining of the multiple myeloma (MM) cell lines NCI-H929 (CD138 positive) and U266B1 (CD138 highly positive) already after one hour incubation with the fusion proteins (figure 22). Due to the fast enrichment of the labelled interferon fusion proteins on the surface of MM cells and no detectable signals on HelaS3 cells (CD138 negative), targeting of the tumor-associated antigen CD138 was able to be confirmed. In contrast to the CD138 moderate expressing cell line NCI-H929, high amounts of internalized interferon fusion proteins were detected in CD138 overexpressing U266B1 cells. According to this observation, internalization of the transmembrane proteoglycan CD138 seemed to correlate with the level of proteins located on the surface of the cells. Similar data on the internalization of CD138 can be found in literature (Fuki et al., 1997), although the observed correlation between the protein expression level and the degree and speed of internalization of the proteoglycan has not been reported so far.

Successful targeting of the tumor-associated antigen CD138 by one binding moiety of the bispecific interferon fusion protein has further strengthened a proposed suitability of the generated fusion protein for a future clinical application in the indication of multiple myeloma. Besides the proven enrichment of the construct on the surface of the cells, its pharmacologic activity was evaluated by treatment of U266B1 cells with serial dilutions of interferon fusion proteins containing a cleavable or a non-cleavable peptide linker between the cytokine and its masking antibody (figure 23). Results from the performed experiments display an efficient masking and inactivation of the cytokine in case of an applied non-cleavable G_4S_6 peptide linker compared to unfused fully active interferon alpha H2. The increase of the necessary half maximum inhibitory concentration (IC50) of cytokines fused to a masking antibody, clearly demonstrated a significant neutralization of the pharmacologic activity of interferon mediated by the binding of the cytokine by the antibody. In contrast to the strong reduction of the anti-proliferative activity of interferon in case of constructs with an incorporated non-cleavable linker,

interferon fusion proteins with a bispecific protease cleavable tandem linker displayed efficient cell killing effects. Due to the similar IC50 concentrations of unfused human IFN alpha H2 and interferon fusion proteins containing a tandem linker, a 95 - 100 % turnover rate of the protease cleavable linker was reasoned. Gained data verified the proposed mode of action of the targeted interferon fusion protein which deals with a targeted enrichment of a temporarily inactivated cytokine at the tumor site, followed by reactivation of the cytokine after protease-mediated cleavage of the peptide linker between the masking antibody and the cytokine.

To validate the observed pharmacologic potency of the bispecific fusion protein and prove the functionality of the antibody masking approach on a broad basis, additional multiple myeloma cell lines and the ovarian cancer cell line OVCAR-3 were treated with non-cleavable fusion proteins and constructs with an applied protease-cleavable tandem linker (figure 24). The first promising results from cell viability assays in U266B1 cells were could be extended to other multiple myeloma cell lines with similar anti-proliferative activities of the cytokine and antibody masking efficiencies in a comparable range. In multiple myeloma cell lines the masking ability of the applied 9F3 antibody reduced the pharmacologic activity of the cytokine by a factor of 10 - 14 in contrast to the solid tumor cell line OVCAR-3, were a 23-fold decrease in interferon activity was monitored. Based on these findings, it was concluded that cytokine masking and inactivation functions of the interferon fusion protein are increased *in vitro* in adherent cancer cell lines derived from solid tumors. This is likely to correlate with a lower accessibility of the tumor cells compared to multiple myeloma cell lines in suspension culture.

Summarizing the obtained results during the complete elaboration and development of the targeted interferon fusion protein, it was possible to manufacture a new kind of targeted antibody drug conjugated with a presumable application in oncologic personalized health care. Enforced anti-proliferative activity of interferon by selective point mutations in combination with a reduced side-effect profile due to antibody-mediated temporary inhibition of the cytokine might contribute to more efficient cancer therapies and a significant improvement in patients` quality of life in future times.

7 Outlook and perspectives

An inherent genetic instability of the cancer genome and the redundancy in many signalling pathways promoting cell growth and survival, challenge treatment strategies in modern cancer therapies. Due to these characteristics, growth receptor blockage and pathway inhibition approaches often lack clinical efficacy. Recruiting the pleiotropic function of interferon with its direct anti-proliferative and immune-stimulatory mode of action in combination with a tumor-selective activation of the cytokine reveals a new promising attempt in the fight against cancer. This might deliver new possibilities to overcome current limitations in targeted treatment strategies.

Promising results gained from first *in vitro* evaluation studies of the targeted interferon fusion protein support the beneficial value of the proposed antibody cytokine conjugate. Despite the improved potency of interferon, its efficient masking and targeted delivery by the 9F3 antibody, further investigations have to be performed to optimize the plasma stability of the applied protease cleavable tandem linker. Therefore, selective point mutations in the sequence of the tandem linker or the combination of alternative protease cleavable sequences can lead to a reduced turnover of the linker during circulation of the fusion protein in the periphery. Besides the optimization of the applied tandem linker, additional questions have to be addressed *in vitro* before further evaluations of the interferon fusion protein in tumor-bearing animal models.

During the development and optimization of the bispecific interferon fusion protein the main focus was set on the anti-proliferative activity of the cytokine. In addition, further experiments have to be conducted to reveal the influence of immune co-stimulatory effects of interferon in the treatment of cancer. First hints about the function of masked interferon fusion proteins on cells of the immune system can be given by NK - cell and $CD8^+$ - T cell activation assays *in vitro*. Based on the outcome of these investigations, it might be possible to show differences between wild type interferon and masking interferon antibody conjugates in the activation of immune cells. This might foster the argument of a reduced side effect profile of interferon antibody fusion proteins.

Besides the extensive characterization of the interferon fusion protein *in vitro*, a deeper insight into the functionality of the molecule has to be generated by its application in predictive animal models. Therefore, sufficient amounts of the interferon fusion protein have to be provided by the development of human cell lines stably expressing the recombinant protein. Also, optimization of the downstream processing procedure is required to ensure a high grade of purity of the product. First *in vivo* evaluations of the interferon fusion protein in non-tumor bearing mice will help to answer the question whether the expected pharmacokinetic attributes of the fusion protein are similar to normal IgG antibodies due to the applied IgG1 like framework of the fusion protein with a functional Fc part. Based on the determined retention period of the fusion protein in the periphery, a further development of a suitable dosing scheme can be conducted. To determine the anti-tumor activity of the interferon fusion protein *in vivo*, subcutaneous xenograft models of multiple myeloma cells in immune-deficient mice will be a suitable tool to gain a first impression

on the pharmacodynamic property of the molecule. Due to the limitation of a poor cross-reactivity of human interferon alpha to the murine system, investigations of the functionality of the fusion protein *in vivo* have to be performed in humanized mice models. Such models provide a more human-like immune system. Alternatively, surrogate interferon fusion proteins containing the species cross-reactive universal Type I interferon might be used.

Based on the future outcomes of pharmacokinetic and pharmacodynamic studies of the interferon fusion protein in murine animal models, ongoing toxicology studies in cynomolgus monkeys are conceivable. During pre-clinical evaluation of the fusion protein *in vivo*, expectations were raised to overcome current dose-limiting toxicities of interferon while restoring its anti-proliferative effect on cancer cells. The newly developed interferon fusion protein might represent a new potent candidate for clinical studies in the treatment of multiple myeloma. In case of a successful application of interferon fusion proteins in the clinic, the proposed approach of temporary inhibiting a cytokine and its tumor-selective reactivation may serve as a platform technology to reduce unwanted interactions of alternative potent cytokines which are currently limited by severe toxicology profiles. Ongoing pre-clinical and clinical investigations of the interferon fusion protein will hopefully confirm beneficial results gained from first *in vitro* evaluations and contribute to a deeper understanding of the potential and the applicability of this new attempt in modern targeted cancer therapies.

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